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# Deciphering key parameters enhancing lentiviral vector producer cells yields: Vector components copy number and expression

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**The use of lentiviral vectors (LVs) in gene therapy is expanding, demanding high-quality viral preparations. Producer cell lines for LV production offer robust manufacturing platforms. However, their development is still progressing and more knowledge on the impact of vector components expression levels on vector yields and quality is essential. This work studies the impact of vector cassette expression and stability on vector titer and quality, identifying key parameters in cell line development. Ten heterogeneous LV stable producer clones established through random cassette integration were characterized. The *gag-pol* and *rev* cassettes, expressed under the control of constitutive promoters, showed robust expression generating titers of $10^9$ physical particles (P.P.s)/mL. However, *Pol* and reverse transcriptase expressions were shown to be better indicators of potential functional titers. Envelope and transfer vector expression levels were key to attaining high functional particles yields. The stability analysis of two top clones and their *trans*-complementation with each genetic cassette further supported this conclusion. The producer LV clones expressed constitutively the 4070A envelope, but the overexpression of the VSV-G envelope increased 30-fold the titer supporting the envelope as key determinant in LV quality. This work further elucidates bottlenecks in LV producer cell line development providing insights for their optimization.**

## INTRODUCTION

The most developed and used lentiviral vectors (LVs) in gene therapy are based on HIV-1.[1] LVs have been overtaking gammaretroviral vectors as gene transfer vehicles for therapeutic purposes since safer genotoxicity profiles, due to their genome integration pattern,[2,3] have been reported. Additionally, LVs have the capacity of transducing slowly proliferating and non-proliferating cells by translocating their viral genome (V.G.) across the nuclear membrane.[4] However, in contrast with gammaretroviral vectors, LV manufacture remains suboptimal, generating high-cost bioprocesses. Most LV manufacturing processes rely on transient transfection systems, requiring large amounts of plasmid DNA and transfection reagents, which decrease process reproducibility and increase the downstream complexity.[5] Constitutive and high-yielding LV producer cells enable scalable bioprocesses at lower costs. Such platforms are nevertheless difficult to establish and are characterized by lower productivities than transient transfection systems. Current LV constitutive producer cell lines were established with diverse stable transfection approaches, and expression strategies to cope with LV-associated toxicity of some viral components (viral protease and heterologous *vesicular stomatitis virus G* [VSV-G] envelope).[6–10] The development of standardized procedures to generate robust and high-yielding constitutive producer cell lines is impaired by the lack of knowledge on LV genetic cassettes' optimal design and stoichiometry, and its impact on viral productivity and vector quality. Currently, the most common system to produce this recombinant virus is the third LV generation composed of a four-cassette expression system, each plasmid provided in trans: *rev*, *gag-pro-pol*, *env* (envelope), and transfer vector. However, this split genome approach disregards *Lentivirus* complex and tightly controlled wild-type genome expression, which provides suitable ratios of splicing and translation events for functional viral particle generation.[11] Constitutive producer cells expressing the four LV genetic cassettes lack part of those regulatory mechanisms and only maintain the *gag-pro-pol* genetic cassette ribosomal frameshift translation regulation, providing wild-type ratios between the Gag and Gag-Pro-Pol polyprotein.[12,13] Therefore, addressing LV genetic cassettes ratios is important to further elucidate its impact on vector yields and quality and to assist optimal constitutive LV producer cell line development. This work further elucidates the contribution of each LV genetic cassette expression on vector yields and quality in constitutive producer cells. Previously, we successfully established LentiPro26 cell lines, constitutive LV producer cells (based on third LV packaging generation and using a SIN transfer vector). This was possible by reducing gag-pro-pol and envelope toxicity. These producer cell lines were originated by random integration of the LV genetic cassettes in HEK 293T cell genome. Clone isolation was performed only upon the establishment of a population expressing the four cassettes. The later were transfected and selected stepwise in the sequence: *gag-pol*, *rev*,

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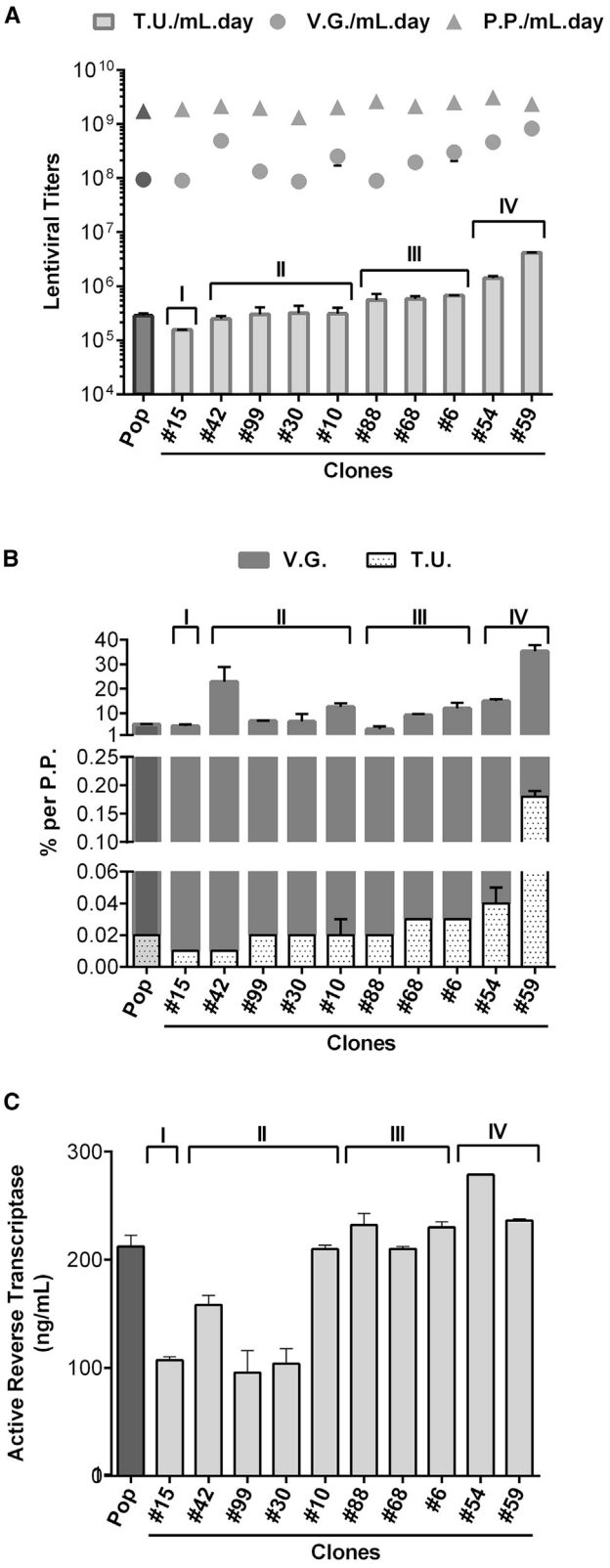


**Figure 1. LV production from LentiPro26 population and clones**

(A) Transducing, genome containing, and P.P.s volumetric titers. (B) Viral vector preparation quality, percentage of particles within total P.P.s. (C) RT activity of LV preparations. Productions were performed in batch mode for 24 h. Data shown represents mean ± SD from biological replicates ($n$ = 2). Pop, population; T.U.s volumetric titer categories: I) ± 1 × $10^5$, II) ± 2 × $10^5$, III) ± 6 × $10^5$, and IV) > 1 × $10^6$.

*env*, and *transfer vector*.[10] As a result, each clone generated is unique. Diverse specific genomic backgrounds and multiple vector cassettes copy number integration and expression stoichiometries were obtained among the cell clones established. LentiPro26 cell lines, therefore, provide an excellent model to study and understand the key factors influencing constitutive LV production. Herein is described the characterization of 10 heterogeneous constitutive LV producer clones regarding viral cassettes genomic copy number, expression level, and their productivity of physical, genome-containing and transducing particles. Moreover, we overexpressed, by *trans*-complementing, each LV genetic cassette to unveil which component impacted mostly LV-transducing units (T.U.s) yields in the top producer clones.

## RESULTS

### Clonal characterization of LV stable producer cells: viral particles yields and quality

LV constitutive producer cell clones were established as described in Tomás et al.[10] The 10 top producer clones, from more than 100 clones isolated, were selected based on their diverse transducing titers productivities as described in Tomás et al.[10] LentiPro26 top 10 clones were first analyzed in terms of LV productivity, namely, physical, genome-containing, and transducing particles volumetric yields, as well as, reverse transcriptase (RT) activity of the LV preparations (Figure 1). The original LentiPro26 cell population was also analyzed providing an average productivity of cells before single cell cloning. Based on the volumetric LV T.U. yields, we distinguished four groups of clones, from very low (I) to high producers (IV), to facilitate the identification of potential limiting or enhancing factors (i.e., expression level of a particular vector component). Groups titers ranged from 1 × $10^5$ T.U./mL/day (I) (below the population T.U. volumetric titer), 2 × $10^5$ T.U./mL/day (II) (within the T.U. volumetric titer of the cell population), 6 × $10^5$ T.U./mL/day (3-fold above the cell population T.U. volumetric titer) (III) to 2 × $10^6$ T.U./mL/day (IV) (10-fold above the cell population T.U. volumetric titer) (Figure 1A). P.P.s and the V.G.s yields of LV preparations were also assessed (Figure 1A). The LV volumetric yields of the P.P.s were found to not limit T.U. titer since were very similar among all clones, 1–3 × $10^9$ P.P./mL/day, whereas of the V.G. varied almost 10-fold from 9 × $10^7$ to 8 × $10^8$ V.G./mL/day. In general, clones with increased V.G. titers presented higher LV transducing volumetric yields (group III and IV). However, exceptions were observed, such as clone #42, which exhibited a high V.G. titer but relatively low T.U. titer.

LV particles volumetric titers were analyzed in terms of viral preparation quality (Figure 1B). Within the total P.P. produced by the clones, 3%–35% contained V.G. Again, in most cases, higher

percentages of V.G. incorporation in the P.P. translated into an increase in the transducing capacity. For example, within the total P.P., clones #54 and #59 (group IV) presented the highest percentage of transducing particles (up to 0.18%) and of V.G. particles content (up to 35%). The exceptions were clone #42 (group II) and clone #88 (group III). Clone #42 showed the second highest percentage of V.G. particles (almost 25% of the P.P.s) but 6.0-fold fewer transducing particles (0.01% of the P.P.s) than top clone #59. However, clone #42 had low *env* expression (see below), impairing particle transducing ability. In contrast, clone #88 presented the lowest V.G. incorporation in P.P. (4%) but had 2.0-fold more transducing particles (0.02% of the P.P.s) than clone #42, which may be explained by its higher *env* expression (see below). Further characterization of LV productivity was performed for RT activity (Figure 1C). Active RT identified in the LV preparations varied from 95 to 280 ng/mL (approximately a 3.0-fold range). High producer clones of groups III and IV showed RT activity values above 200 ng/mL, whereas the low-producing clones of groups I and II showed the lowest RT activity in their LV preparations. These results suggest that active RT is positively linked to LV particle transducing capacity. However, some exceptions were observed, such as in the case of clone #42, which exhibits high RT activity but lower T.U. titers.

### Stable integrated copy number and expression levels of LV genetic cassettes

The genomic copy number and gene expression of the four LV genetic cassettes were assessed for each clone and plotted according to the cell clone transducing particles productivity group (Figure 2). The transfer vector cassette contains two open reading frames: one under the control of a chimeric Rous sarcoma virus (RSV)/HIV-1 LTR, driving the transcription of the full viral RNA genome on the producer cell; and another from an internal human promoter driving the expression of the gene of interest, i.e., GFP to be expressed in the transduced cell (Figure S1). Herein, the transfer vector gene expression and genomic copy number were measured by targeting *mcherry* gene present in the full viral RNA genome only. The transfer vector (Figure 2A) cassette copy number varied among all clones from 2 to 8 copies per cell, whereas the *env* (Figure 2B) (with the exception of clone #59), the *rev* (Figure 2C), and the *gag-pro-pol* (Figure 2D) cassettes presented a more homogeneous copy number distribution. In general, each cassette genomic copy number did not correlate with its mRNA levels, meaning that, for the same number of genomic copies of *rev* and *gag-pro-pol*, mRNA levels were considerably diverse (in the approximately 2–5.0-fold range), but supported similar P.P.s productivities (Figures 1A and 2D). Nevertheless, we found that the clones with higher *gag-pro-pol* mRNA levels (measured targeting a pol sequence) also showed higher RT activities (#54 and #59) (Figures 2D and 1C; Table S1). Since Gag-Pol precursor −1 frameshifting event occurs at a frequency of 5%–10% during translation, *Pol* expression might become limiting despite of the high Gag levels observed of P.P.s. The high producing LV titer clones of group IV (#54 and #59) sustained high mRNA levels of all LV genetic cassettes when compared with others. However, in some cases a relation between titers and LV genetic cassettes expression levels was not observed. For instance, clone #42 (group II) exhibited one of the highest V.G. titers, but was not associated with higher transfer vector expression. However, it exhibited low *rev* and *env* expression. Protein expression levels arising from the transfer vector cassette were assessed by measuring GFP and mCherry fluorescence intensity (Figure S1). Clones with higher transfer vector mRNA levels also presented higher fluorescence intensities.

### LV producer clones' particles productivity stability

LV production stability over time is a determinant factor for the successful commercial and clinical implementation of constitutive LV producer cell lines. Therefore, it is important to assess whether the viral titers remain consistent throughout long culture periods without the presence of antibiotic selection pressure (for each viral cassette) in culture media. This is an important characteristic for a cost-effective, clinical-grade, and large-scale production. Therefore, we conducted a stability study with the top clones from group IV (#54 and #59). The clones were subculture for more than 2 months (68 days) with or without antibiotic selective pressure in the cell culture media. The RT enzyme activity and volumetric productivities of LV transducing, V.G. containing, and P.P. were assessed every week (Figure 3). In the presence of antibiotic selective pressure, both clones maintained volumetric productivities throughout the 2-month culture period. Removal of the antibiotic selective pressure affected the LV production differently for each clone. Clone #54 presented a reduction of 2.0-fold in the transducing titers after 19 days of culture without antibiotics, maintaining $0.8 \times 10^6$ T.U./mL/day production titers until the end of the study. The genome containing and P.P.s production of clone #54 were not affected by the antibiotic removal, ranging between 3 and $5 \times 10^8$ V.G./mL/day and $2–3 \times 10^9$ P.P./mL/day (Figure 3A). Transducing LV titers of clone #59 decreased only 1.2-fold 47 days after antibiotic removal from the culture. From then onward, productivities decreased from $4 \times 10^6$ T.U./mL/day (day 47) to $2.1 \times 10^6$ T.U./mL/day (day 68), a total 2.0-fold reduction. Simultaneously, a gradual decrease in V.G. titers, from $6 \times 10^8$ V.G./mL/day (day 47) to $4 \times 10^8$ V.G./mL/day (day 68) was observed, up to 1.5-fold reduction. Yet, removal of antibiotic selective pressure did not affect physical LV particles yields ($2–3 \times 10^9$ P.P./mL/day). (Figure 3B). Further characterization regarding LV RT activity stability was performed. The lowest values obtained with antibiotic selective pressure, which showed biological variability, were considered the baseline value for the RT activity. Both clones displayed RT variations, with clone #59 exhibiting a consistent gradual decrease in RT activity upon antibiotic removal after day 40 (Figures 3C and 3D).

### LV producer clones' genetic cassettes stability

To determine the genetic stability of the integrated LV expression cassettes, mRNA levels, genomic copy number and protein expression were analyzed throughout the 2-month subculture of clones #54 and #59, with or without antibiotic selective pressure (Figure 4). Day 5 with antibiotic selective pressure was considered the baseline of gene expression. Both clones exhibited gene expression changes within 1.5-fold (higher or lower) from the baseline when cultured

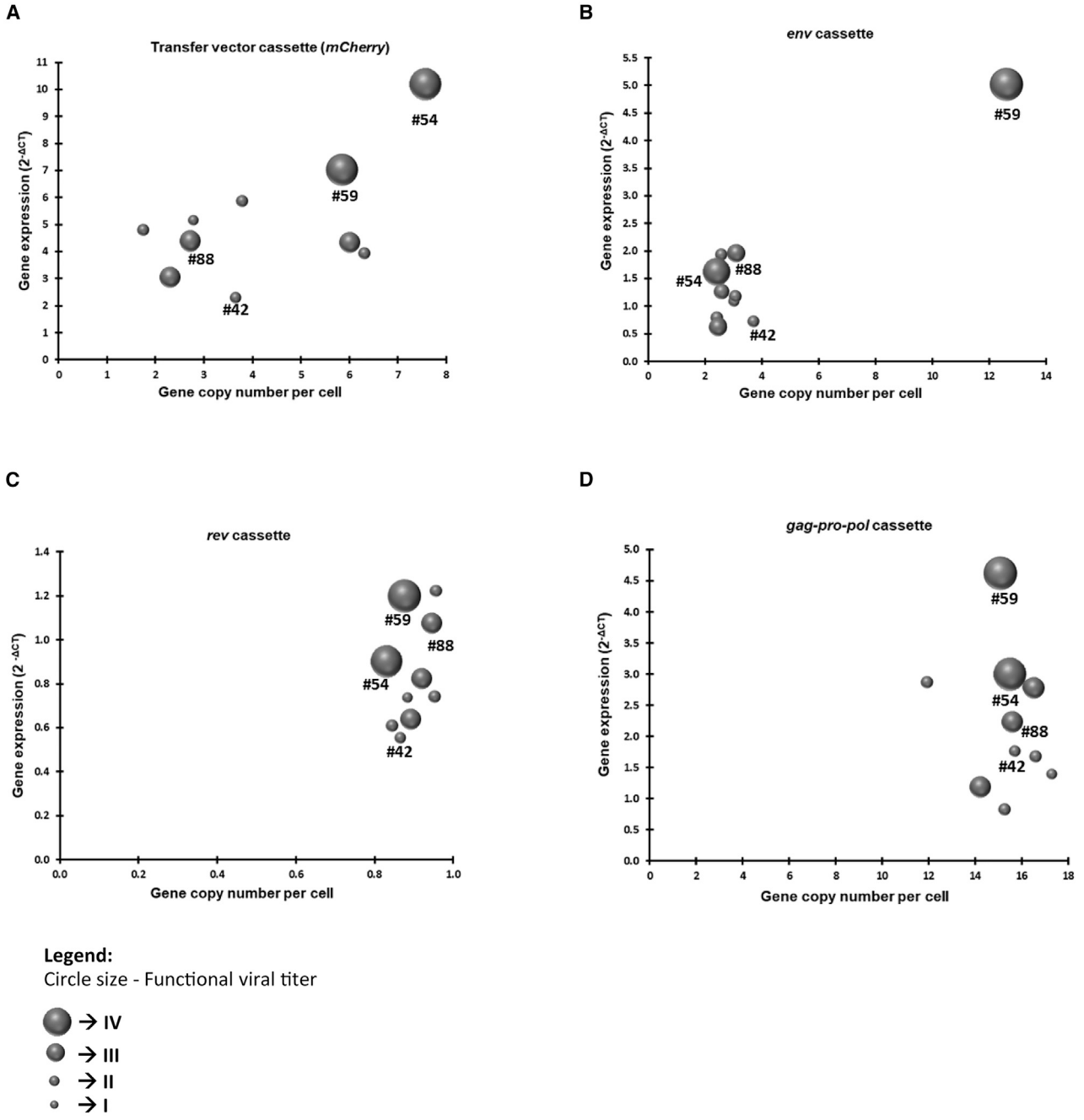


**Figure 2. LV genetic cassettes copy number and normalized gene expression in LentiPro26 clones**

(A) Transfer vector cassette (mcherry). (B) env cassette. (C) rev cassette. (D) gag-pro-pol cassette. Gene expression was normalized using two reference genes (*RPL22* and *UBB*) using the $2^{-\Delta CT}$ method. Data shown represents the mean of biological replicates ($n = 2$). This figure was created using XLSTAT statistical and data analysis solution. See also Figure S1.

in the presence of antibiotic selective pressure throughout the 68 days. These mRNA levels can be considered the interval of biological variability of LV cassettes expression for each clone. In the case of clone #54, upon antibiotics removal from cell culture medium, only *env* mRNA levels decreased below clone expression variability interval (1.7-fold). These results suggest that the loss of transducing LV titers could be a consequence of limited envelope glycoprotein. Clone #59 showed a major decrease in *gag-pro-pol* cassette expression (a 3.3-fold reduction); nevertheless, gene expression absolute values remained higher than clone #54 (data not shown). Over the

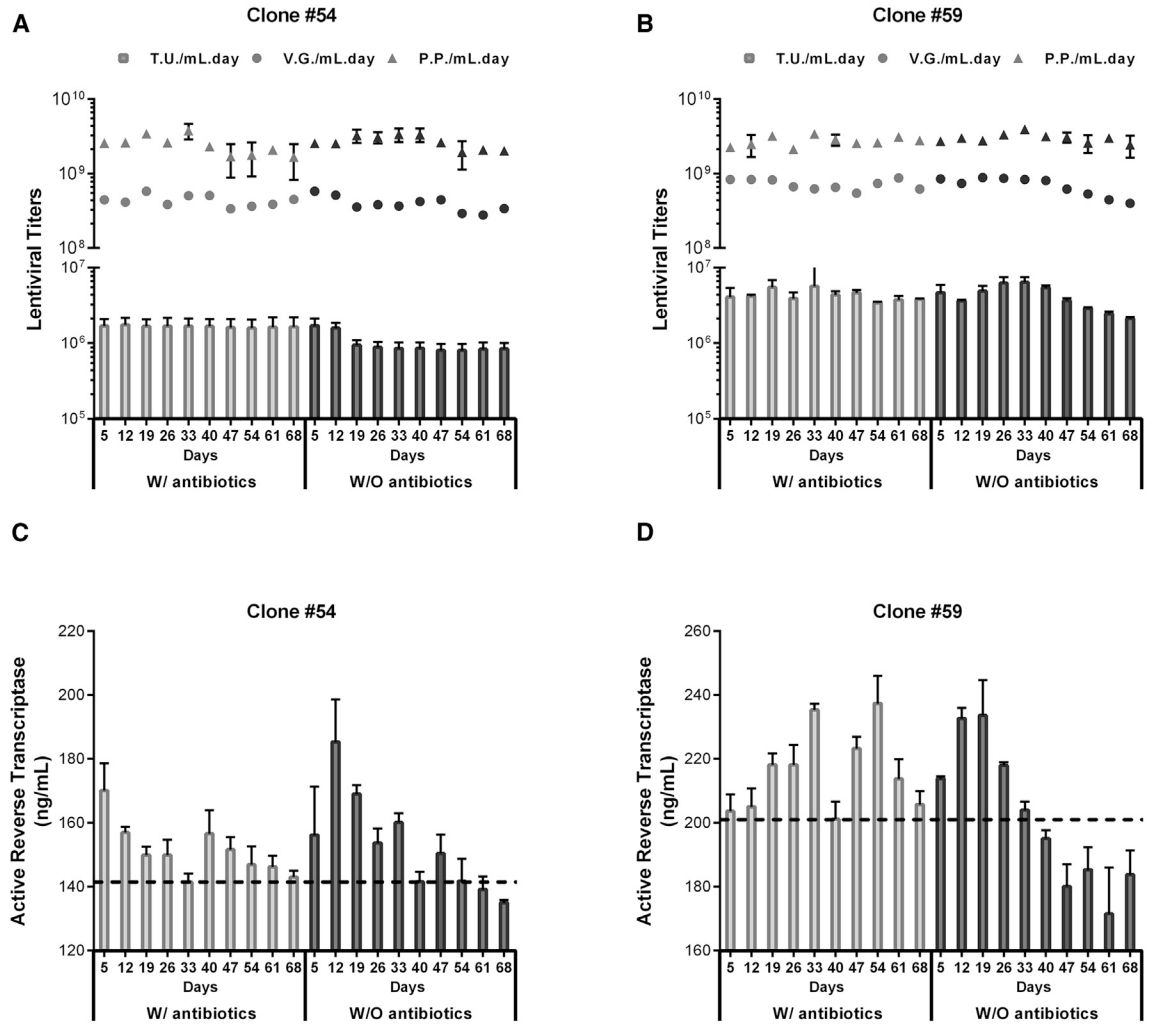


**Figure 3. Stability of clones #54 and #59**

Producer cells were maintained for 2 months (68 days) in culture with (W/) and without (W/O) antibiotic selective pressure. Transducing, genome containing, and P.P.s volumetric titers of clones #54 (A) and #59 (B). RT activity of LV preparations of clones #54 (C) and #59 (D). Volumetric titers of batch (24 h) productions. Data shown are mean ± SD from technical replicates ($n$ = 2).

last week of the study, clone #59 presented a gene expression decrease of 2.0-fold for *env*, 2.0-fold for *rev*, 3.3-fold for *gag-pro-pol*, and 2.4-fold for the transgene (*mcherry*) genetic LV cassettes. The loss of transfer vector expression might be responsible for the decrease in LV genome titers, which thus reduced LV-transducing titers. The genomic stability of both clones was assessed by the number of genomic copies of each LV cassette (Figure S2). The copy number of integrated cassettes remained constant throughout the 68 days, with and without antibiotic selection pressure, meaning that the loss of expression should be due to epigenetic effects (e.g., DNA methylation). Protein expression levels from the transfer vector cassette (Figure S3) support mRNA data. In the absence of antibiotics in cell culture medium, GFP and mCherry fluorescence intensity values of both clones decreased. Still, the absolute fluorescence intensity values of the reporter proteins in clone #54 remained higher than in clone #59.

### LV genetic cassettes overexpression

Further comprehension of how viral components expression might be limiting the transducing viral titers of clones #54 and #59 could provide insights leading to the improvement of LV titer quality through optimization of LV constitutive producer cell line development. To that end, individual *trans*-complementation of the LV genetic cassettes (transfer vector, *rev*, *gag-pro-pol*, *env* [4070A and VSV-G]) was performed in the clones of group IV to assess the overexpression effect on LV T.U.s productivity. Three DNA concentrations (1.5, 2.25, and 3 μg of DNA per million cells) of each of the four viral genetic cassettes were used to transfect transiently each clone. Mock controls

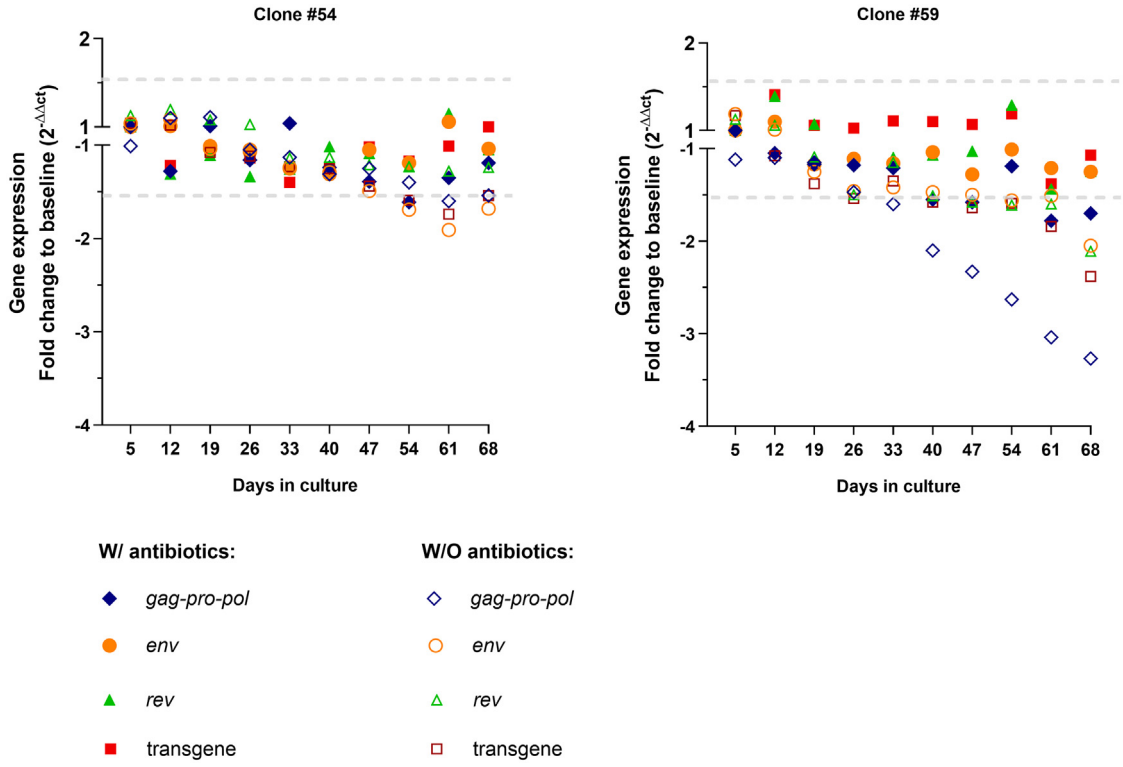


**Figure 4. LV cassettes expression of clones #54 and #59 throughout 68 days in culture**

Producer cells were maintained with (W/) and without (W/O) antibiotic selection pressure in culture medium. Gene expression was normalized using two reference genes (*RPL22* and *UBB*) using the $2^{-\Delta CT}$ method. Data shown are mean of technical replicates ($n$ = 2). See also Figures S2 and S3.

were performed with a non-viral stuffer plasmid. LV genetic cassette mRNA levels of the LV components were also assessed after transfection. The obtained gene expression levels increased according to the amount of transfected viral DNA cassette (Figure S4). The resulting specific transducing LV particles yields were then evaluated (Figure 5). Relative to the non-transfected condition, mock controls of clone #54 and #59 decreased the specific LV titers (data not shown). As such, these controls were used as baseline value to compare the effect of the LV genetic cassette overexpression. Upon the *trans*-complementation of the four LV genetic cassettes, both clones exhibited increased specific LV transducing titers. Clone #54 presented the higher LV transducing titer increase, approximately 7.0-fold, after transfection of *rev* and *env* (4070A) viral cassettes, suggesting that clone #54 LV productivity could be increased by increasing the expressions of these cassettes. In contrast, clone #59 exhibited the higher specific LV-transducing titers increase (6.0-fold) upon transfer vector overexpression. These findings correlate with the data presented in Figure 2, where clone #59 shows lower transfer vector expression (Figure 2A), and clone #54 displays lower env expression (Figure 2B). These results underscore the importance of optimizing the expression of specific components to further enhance LV productivity in these clones. The fusogenic activation of 4070A envelope depends on the cytoplasmatic tail R-peptide recognition and cleavage by the viral protease. We also evaluate the potential of the clones when expressing the G glycoprotein from the rhabdovirus VSV-G envelope which does not require tail R-peptide cleavage for its fusogenic capacity, making it independent of Gag-Pro-Pol expression. Both clones were transfected with the LV genetic cassette encoding VSV-G envelope and presented a substantial increase in specific LV transducing titer of up to 30-fold (clone #54) and 22-fold (clone #59), when overexpressing the VSV-G alternative envelope to 4070A, resulting in a titer of $1.78 \times 10^7$ T.U./mL and $1.97 \times 10^7$ T.U./mL, respectively. These specific LV T.U.s yields surpass the ones obtained for the 4070A overexpression. Results suggest the 4070A envelope limits higher transduction titer productivities in LentiPro26 LV producer cell lines. Furthermore, we simultaneously transfected the four LV genetic cassettes of LentiPro26 (transfer vector, *rev*, *gag-pro-pol*, *env* [4070A] using the 10:1:4:3.6 ratio).[14] Even though, in both clones the specific LV transducing titers increased above the individual LV genetic cassettes trans-complementation (almost 2.0-fold increase), they did not reach the same increased levels as with VSV-G transfection alone.

## DISCUSSION

### Gag-pol and rev genomic copy number and expression

Studying the stoichiometry of vector components expression has historically assisted in the development of numerous constitutive high-producer cells for recombinant retroviruses.[15–18] Although considerable knowledge was generated from these studies, application to LV

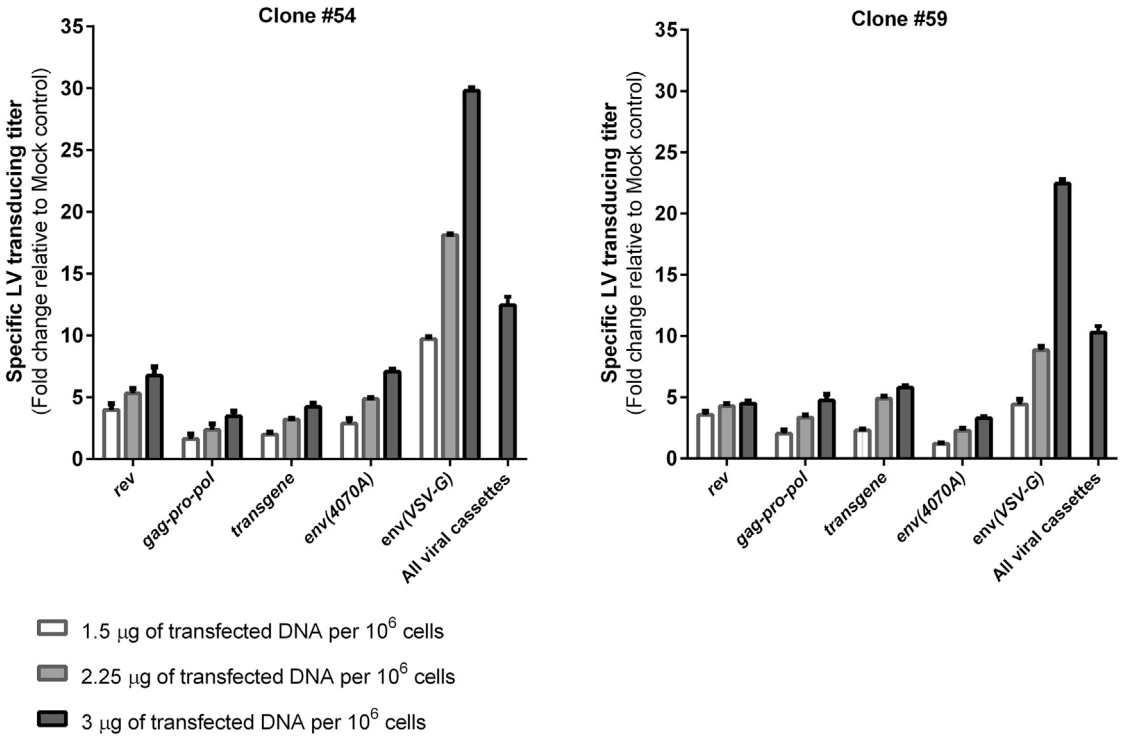


**Figure 5. Specific LV transducing titers after *trans*-complementation of clone #54 and #59**

Viral genetic cassettes were *trans*-complemented at three different DNA amounts (1.5, 2.25, and 3 μg of DNA per million cells). Mock controls were performed with non-viral stuffer plasmid. A mock control was performed transfecting the cells with a non-viral stuffer plasmid and used as baseline value to normalize the specific LV transducing titers. Data shown are mean of technical replicates ($n$ = 2). See also Figure S4.

production systems is restricted. Therefore, comprehending the expression of the cassette and its potential impact on LV yields becomes crucial. To the best of our knowledge, this is the first report to investigate the influence of LV genetic cassette expression levels on productivity and stability of constitutive producer cells. Although high copy number is generally perceived as providing high productivities, herein, the 10 LentiPro26 clones studied showed no correlation between the genomic copy number and the mRNA levels of LV cassettes (Figure 2). This lack of correlation could be a consequence of LentiPro26 development procedure, in which LV cassettes were chemically transfected, thus being randomly integrated into the producer cells genome.[10] Hence, depending on chromosomal integration site (low- or high-expressing *locus*), LV cassette genomic copy number provided different gene expression profiles. Despite that, copy number integration could still provide some insights. It is interesting to observe the *rev* cassette presented very low integrated copies, suggesting it may be the gatekeeping element of LV cytotoxicity. *Rev* enables *gag-pol* full expression.

The *rev* and *gag-pol* cassettes exhibited similar genomic copy number values but up to 5.0-fold expression differences (Figures 2C and 2D). The lower genomic variability of the *gag-pol* and *rev* cassettes may be attributed to various factors. One being the *gag-pol* cytotoxicity (despite the lower cytotoxic profile of T26S viral protease used). Cells exposed to lower cytotoxic levels may have survived and were maintained throughout the antibiotic selection process in the cell line development. Still, only producing clones were analyzed, which may explain the high *gag-pol* cassette copy number.

The expression of *gag-pol* has been pointed as the main challenge limiting LV constitutive producer cell development.[8,9,19] However, in this work, we showed that LentiPro26 clones presented different *rev* and *gag-pol* expression levels (Figures 2C and 2D), enabling similar LV yields of $10^9$ P.P/mL (Figure 1A). Thus, the cell line development process workflow, relying on T26S protease, chemical transfection, and stringent antibiotic selective pressure steps, did not restrict gag-pol expression and LV P.P. production.[10] The decrease in *gag-pol* expression while the copy number remained unaltered in the stability study reinforces the hypothesis of epigenetic silencing mechanisms regulating LV cassette expression stability.[10] The viral cassette genomic site of integration can affect the Gag, Gag-Pol transcription explaining the differences in RT activity observed. Loss in Gag-Pol expression will lead to lower LV titers.

### Envelope and transfer vector genomic copy number and expression

*Env* and transfer vector cassette presented higher variability in integrated genomic copy number in LentiPro26 cell clones. Vector genome

expression levels in the producer cells were previously associated with the resulting genome containing particle titer.[15] In this work, the range of transfer vector expression levels provided yields of $10^7$–$10^8$ V.G./mL/day without a direct correlation (Figures 1A and 2A). Nevertheless, two observations could be made in clones #54 and #59, which exhibited the highest vector genome expression levels (Figure 2A). Clone #54 maintained the V.G. particle titer and vector genome expression throughout 68 days of subculture studies without antibiotics (Figures 3A and 4A). In contrast, clone #59 subcultured in the same conditions showed a decrease of 2.0-fold in V.G. particles titer and 2.4-fold in vector genome expression in a dependent manner (Figures 3B and 4B). Additionally, in the absence of antibiotics, both clones GFP and mCherry fluorescence intensity values decreased (Figure S3). Still, the absolute fluorescence intensity values of the reporter proteins in clone #54 remained higher than clone #59. As such, clone #54 was still able to support V.G. particles titer. These results suggest that lower levels of vector genome availability (in clone #59), could lead to V.G. and T.U. titer loss. LV genetic cassettes trans-complementation results corroborated this observation, since clone #59 exhibited the higher specific LV T.U. titers increase (6.0-fold) when the transfer vector was overexpressed (Figure 5).

Regarding transducing particle yields, the LV genome expression levels of the 10 clones herein analyzed allowed LV titers from $10^5$ to $10^6$ T.U./mL (Figure 1). The one log difference in functional yields could be a result of each clone *env* expression level, as previously shown for gammaretroviral production.[15,17] This LV component is responsible for the first critical step of host recognition; thus, envelope glycoprotein expression and maturation is essential for LV functionalization.[20] When subculturing throughout 68 days without antibiotics, clone #54 functional LV yields decreased 2.0-fold (Figure 3A) and only *env* expression was reduced below clone variability interval (Figure 4A). Additionally, after *trans*-complementation of the *env* (4070A) viral cassette, clone #54 presented a T.U. titer increase of approximately 7.0-fold. This indicates that the productivity of clone #54 might be hindered by low expression levels of the *env* cassette. The *trans*-complementation studies also indicate low efficiency of 4070A envelope processing; when providing VSV-G alternative envelope to both clones, #54 and #59, higher increases in specific transducing titers were observed than when *trans*-complemented with 4070A envelope (Figures 5A and 5B). Inefficient envelope maturation could be due to low protease activity inside the LV particle affecting the envelope fusogenicity state.[11,21]

### Pol expression impact on titer and stability

*Pol* transcribes the PR, RT and the integrase. The RT levels of each clone could further explain the differences between V.G. and T.U. titers, as this enzyme is crucial for the reverse transcription of the viral RNA genome.[22] PR is not only responsible for the Gag-Pol polyprotein processing, as mentioned above, but also responsible for making the 4070A envelope functional. The levels of functionalized particles should be a consequence of both *env* and *pol* expression. This hypothesis is supported by several observations in this work that indicate the need to surpass a threshold of both *env* expression and active RT to attain transducing particles. For example, clone #42 presenting a high V.G. titer was the clone with the lowest T.U. titer, which can be explained by the low *env* expression and/or the low active RT (below 200 ng/mL) (Figure 1C).

The stability study of the LV producer clones confirmed the relevance of *Pol* expression. Both clones were relatively stable, but behaved differently over 2 months of subculture without antibiotic selective pressure. The mild 2.0-fold LV transducing titers decrease of clone #59 seems to be a result of *gag-pol* cassette expression loss (Figures 3B, 3D, and 4). Clone #59 *gag-pol* cassette presented the highest reduction (3.3-fold). The transduction LV particles titer stability started to decrease at 47 days of culture without antibiotic selective pressure, which aligned with P.P yields (Figure 3B) and RT levels (Figure 3D). Nevertheless, gene expression absolute values remained higher than other clones justifying is still high T.U. titer.

Overall, this study advances our understanding of LV stable producer cell lines and highlights critical factors influencing viral production and functionality. The designed *gag-pol* expression cassette successfully generated volumetric titers of $10^9$ P.P. However, the cassette expression resulted in diverse active RT values, which were observed to affect the LV transducing yields. Since *Pol* is expressed in much lower amounts than *Gag*, it may provide a more sensitive parameter for clone screening. If the threshold levels of *Gag-Pol* are successful surpassed, functionalization of particles are then closely linked to balanced mRNA levels of the transfer vector and *envelope*, consistent with findings in constitutive cells producing gammaretrovirus.[17,18] The expression of these components should be optimal. This can be achieved by (1) designing improved genetic cassettes (that provide high expressions and/or high copy number) and (2) protein engineering, both the envelope and the therapeutic protein, such as these have superior stability and functionality. LV specific titers were enhanced by VSV-G envelope *trans*-complementation, underscoring the importance of envelope maturation in viral titer quality. While gene therapy applications are moving for *in vivo* clinical applications, envelope engineering will gain high importance both to attain high tissue specificity and high transducing efficiency.

## MATERIALS AND METHODS

### Plasmids

pGP(T26S)P-blast plasmid, codes for HIV-1 Gag-Pro(T26S)-Pol protein under the control of a cytomegalovirus (CMV) promoter. The expression of a blasticidin resistance marker is coupled to HIV-1 Gag-Pro(T26S)-Pol expression by a spacer region, as described elsewhere.[10]

pREV-hygro-WPRE plasmid codes for HIV-1 Rev protein and the hygromycin resistance marker, both under the control of the RSV U3 promoter, as described elsewhere.[10]

pMONO-zeo-4070A plasmid codes for the murine leukemia amphotropic envelope (4070A) under the control of a CMV promoter. The expression of a zeocin antibiotic resistance marker is linked to the expression of the envelope glycoprotein by an internal ribosome entry site of foot and mouth disease virus, as described elsewhere.[10]

pRRLSIN-mCP-GFP vector transgene plasmid is a SIN LV vector, described elsewhere.[10] The chimeric 5′ LTR-RSV promoter regulates the expression of full-length LV RNA genome, including the expression of the mCherry and GFP reporter genes and the puromycin selection marker. The expression of puromycin resistance marker is coupled to the expression of mCherry by a spacer region. GFP expression is additionally controlled by human phosphoglycerate kinase 1 internal promoter.

pRRLSIN-mCP-GFP-ALB-REV-POL-4070A plasmid has *mCherry*, *gfp*, *ALB*, *rev*, *pol*, and *4070A* amplicon sequences of qPCR primers; thus, it is used as a universal template for calibration curves in gene copy number quantifications. It was generated by cloning a double stranded DNA (gBlock) synthetized by Integrated DNA Technologies (Coralville, Iowa, USA) into the pRRLSIN-mCP-GFP plasmid digested at the XhoI restriction site.

pSELECT-blasti-mcs plasmid (Invivogen, San Diego, CA, USA) was used as a stuffer plasmid for the LV genetic cassettes complementation studies.

### Cell lines and culture conditions

HEK 293T (ATCC CRL-3216), a human embryonic kidney 293-derived cell line expressing SV40 large T antigen, was used for LV T.U.s quantification.

LentiPro26-4070A-mCPGFP population and the top 10 producing clones (#6, #10, #15, #30, #42, #54, #59, #68, #88, and #99),[10] are HEK 293T-derived cell lines constitutively producing HIV-1-based recombinant LV, pseudotyped with 4070A envelope.

Cells were cultured in DMEM (Corning, Corning, NY, USA), supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and maintained at 37°C in a humidified atmosphere containing 8% $CO_2$.

LentiPro26-4070A-mCPGFP were kept under selective pressure supplementing the culture medium with the following antibiotics (Invivogen, San Diego, CA, USA): 20 μg/mL blasticidin, 150 μg/mL hygromycin b gold, 150 μg/mL zeocin, and 0.5 μg/mL puromycin.

Cell concentration and viability were assessed by trypan blue exclusion method.

### Cell growth and LV production studies

Producer cells were seeded at $8 \times 10^4$ cells/cm$^2$ and 0.1 mL/cm$^2$ in the presence or absence of selective pressure. Total RNA, genomic DNA, and whole cell protein extracts were isolated at 48 h after seeding. Medium was exchanged 72 h post-seeding and the supernatant from the following 24-h period was harvested, clarified (using a 0.45-μm cellulose acetate filter), aliquoted, and stored at −80°C until further use. The expression of reporter proteins (GFP and mCherry) in the producer cells was measured by flow cytometry (BD FACSCelesta Flow Cytometer; BD, Franklin Lake, NJ, USA).

### LV genetic cassettes *trans*-complementation

Producer cells were seeded at $2 \times 10^5$ cells/cm$^2$ in tissue culture flasks of 25 cm$^2$. The cells were transfected at seeding using linear 25 kDa polyethyleneimine (PEI; Polysciences Inc, Hirschberg an der Bergstrasse, Germany) at a mass ratio of 1:1.5 (DNA:PEI) and 3 μg of total DNA per million cells. Viral genetic cassettes were transfected at three different DNA amounts (1.5, 2.25, and 3 μg of DNA per million cells) and a non-viral stuffer plasmid was used from 0 μg to 1.5 μg of DNA per million cells to ensure the same transfection conditions. A Mock control was performed transfecting the cells with a non-viral stuffer plasmid. Medium was replaced by 2.5 mL of fresh medium 24 h after transfection. After 48 and 72 h after transfection the medium was exchanged and the supernatant from the following 24-h period was harvested, clarified (using a 0.45-μm cellulose acetate filter), aliquoted, and stored at −80°C until further use. Total RNA was isolated at 48 h after transfection. The cells were counted every day. All the transfection procedures described above were also performed in parallel with a control cell line HEK 293T (ATCC CRL-3216).

### LV particles quantification

Transduction competent LV particles quantification was performed using HEK 293T target cells seeded at $5 \times 10^4$ cells/cm$^2$ in 24-well plates. Transduction was performed 24 h after, in duplicates, by removing the cell supernatant and adding 0.2 mL of viral suspension diluted in fresh DMEM with 10% (v/v) FBS and 8 μg/mL of polybrene (Sigma). Cell concentration was determined at time of transduction. Following spinoculation-centrifugation at 1,200×*g* for 2 h at 25°C, 1 mL of fresh supplemented DMEM was added.[23] Cells were harvested and analyzed 48 h after transduction using BD FACSCelesta Flow Cytometer (BD). The T.U. titer was determined taking into account the percentage GFP positive cells, the cell concentration at the time of transduction and the dilution factor.[10]

The V.G. titer was quantified using a method previously described elsewhere.[24] The protocol was adapted for LV by using primers against the WPRE sequence (Table S1) for cDNA synthesis and qPCR.

For LV total particle quantification, a p24 ELISA was conducted using the Lenti-X p24 Rapid Titer kit (Clontech, Takara, CA, USA), according to the recommendations and manufacturer's instructions.

For RT activity quantification, a colorimetric enzyme immunoassay for the quantitative determination of retroviral RT activity was preformed using the Reverse Transcriptase Assay kit (Roche Applied Science, Penzberg, Germany), according to the recommendations and manufacturer's instructions.

### Genomic DNA and RNA extraction and cDNA synthesis

Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction and stored at −20°C until further use. Total RNA extraction was performed using QIAamp RNeasy Mini Kit (Qiagen) according

to the manufacturer's instructions and stored at −80°C until further use. cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany), in agreement with manufacturer's instructions, using 1 μg of total RNA and anchored-oligo(dT) primers. The cDNA products were aliquoted and stored at −20°C until further use.

### Gene expression and copy number

Relative gene expression was quantified by qPCR and normalized to two reference genes: *Ribosomal Protein* L22 (*RPL22*) and *Ubiquitin B* (*UBB*).[25,26]

Gene copy number was quantified by qPCR, using human *Albumin* (*ALB*) gene as reference gene, assuming two copies per cell (N = 2) in HEK 293T.[27] pRRLSIN-mCP-GFP-ALB-REV-POL-4070A plasmid, harboring all gene target sequences of qPCR primers, was used for the calibration curves. The copy number per cell was calculated based on the following equation[27,28]:

$$\text{Gene copy} \Big/ \text{cell} = \frac{\text{Copy number of target gene}}{\text{Copy number of reference gene } (ALB)} \times \text{genomic ALB copies } (N = 2) \quad \text{(Equation 1)}$$

### Real-time qPCR

qPCR was performed in a thermocycler LightCycler 480 Real-Time PCR System (Roche Applied Science) using a LightCycler 480 SYBR Green I Master (Roche Applied Science) PCR kit. Primers sequences for all reactions are listed in Table S1.

## DATA AVAILABILITY

Data are contained within the article or supplementary material.

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## AUTHOR CONTRIBUTIONS

Conceptualization: A.S.F.O., M.V.F., and A.S.C.; Methodology: A.S.F.O. and M.V.F.; Software: A.S.F.O. and M.V.F.; Validation: A.S.F.O. and M.V.F.; Formal analysis: Investigation: writing original draft preparation, A.S.F.O. and M.V.F.; Writing – review and editing: A.S.F.O., M.V.F., and A.S.C.; Supervision: A.S.F.O. and A.S.C. All authors have read and agreed to the published version of the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## SUPPLEMENTAL INFORMATION

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