# Inducible Packaging Cells for Large-scale Production of Lentiviral Vectors in Serum-free Suspension Culture

Sophie Broussau[1,2], Nadine Jabbour[1], Guillaume Lachapelle[1], Yves Durocher[3], Rosanne Tom[3], Julia Transfiguracion[3], Rénald Gilbert[1,4] and Bernard Massie[1,3,5]

*[1]Groupe de Vecteurs de Génomique et Thérapie Génique, Institut de Recherche en Biotechnologie, Conseil National de Recherches Canada, Montréal, Quebec, Canada; [2]Département de Microbiologie et Immunologie, Faculté de Médecine, Université de Montréal, Quebec, Canada; [3]Groupe de Technologie des Cellules Animales, Institut de Recherche en Biotechnologie, Conseil National de Recherches Canada, Montréal, QC, Canada; [4]Neuromuscular Research Group, Montreal Neurological Institute, Montreal, Quebec, Canada; [5]Institut National de la Recherche Scientifique–Institut Armand-Frappier, Université du Québec, Laval, Quebec, Canada*

We have developed new packaging cell lines (293SF-PacLV) that can produce lentiviral vectors (LVs) in serum-free suspension cultures. A cell line derived from 293SF cells, expressing the repressor (CymR) of the cumate switch and the reverse transactivator (rtTA2$^S$-M2) of the tetracycline (Tet) switch, was established first. We next generated clones stably expressing the Gag/Pol and Rev genes of human immunodeficiency virus-1, and the glycoprotein of vesicular stomatitis virus (VSV-G). Expression of *Rev* and VSV-G was tightly regulated by the cumate and Tet switches. Our best packaging cells produced up to $2.6 \times 10^7$ transducing units (TU)/ml after transfection with the transfer vector. Up to $3.4 \times 10^7$ TU/ml were obtained using stable producers generated by transducing the packaging cells with conditional-SIN-LV. The 293SF-PacLV was stable, as shown by the fact that some producers maintained high-level LV production for 18 weeks without selective pressure. The utility of the 293SF-PacLV for scaling up production in serum-free medium was demonstrated in suspension cultures and in a 3.5-L bioreactor. In shake flasks, the best packaging cells produced between 3.0 and $8.0 \times 10^6$ TU/ml/day for 3 days, and the best producer cells, between 1.0 and $3.4 \times 10^7$ TU/ml/day for 5 days. In the bioreactor, 2.8 liters containing $2.0 \times 10^6$ TU/ml was obtained after 3 days of batch culture following the transfection of packaging cells. In summary, the 293SF-PacLV possesses all the attributes necessary to become a valuable tool for scaling up LV production for preclinical and clinical applications.

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## INTRODUCTION

Lentiviral vectors (LVs) are becoming widely popular as gene transfer vehicles because of their relatively large transport capacity (~8 kilobase) and their ability to integrate their genomes into the chromosomes of dividing and non-dividing cells for stable transgene expression (reviewed in refs. 1,2). Since the first reports in the mid 1990s describing the possibility of using viral vectors derived from human immunodeficiency virus-1 as a way to deliver transgenes to a wide variety of cells including post-mitotic cells,[3–6] several modifications have been made to improve their efficacy and safety. A common procedure to generate LVs is by transfecting mammalian cells (most often 293 cells) with several plasmids encoding the necessary components for the synthesis and assembly of the virion. Although relatively efficient (because titers in the range of $10^7$ transducing units (TU)/ml can be obtained by transient transfection) this method is cumbersome and difficult to scale-up. For this reason, several laboratories have generated stable packaging cell lines that produce all the components necessary for the assembly and functioning of LVs.[7–15] Production of LVs using packaging cell lines is advantageous, because there is requirement only for a single DNA that carries the gene of interest flanked by the two human immunodeficiency virus long terminal repeats (the transfer vector). Moreover, packaging cell lines open the possibility of generating cell lines that can produce a particular LV without further modifications (producer cells), provided they are stably transfected with the transfer vector or stably transduced with an LV.

An ideal packaging cell line should be stable, should be capable of producing large quantities of LVs, and should grow in suspension culture for scale-up and in serum-free medium for safety reasons. Because of the reported cytotoxicity of the LV protease, and also of the glycoprotein of vesicular stomatitis virus (VSV-G) that is often utilized to pseudotype LV,[16,17] it has been difficult to generate good packaging cell lines. This problem of cytotoxicity can be solved by regulating the expression of VSV-G and of some LV components using an inducible transcription system that is activated at the time of production. The tetracycline (Tet)–dependent regulatory system[7,9,12,14,15,18] and the inducible ecdysome system[11] have be successfully employed for producing LV packaging cell lines. When the best of these cell lines are used, the titers ($2–3 \times 10^7$ TU/ml) reported [12,14] are

***Correspondence:*** *Bernard Massie, Institut de recherche en biotechnologie, Conseil national de recherches Canada, 6100, Royalmount Avenue, Montréal, Quebec, Canada H4P 2R2. E-mail: bernard.massie@cnrc-nrc.gc.ca*

comparable with the titers obtained by transient transfection. In these studies, induction (and therefore LV production) occurs after the removal of the Tet inhibitor or its analogue doxycycline (Dox) from the culture medium (Tet-Off system). The LV packaging cell lines described to date were not adapted to serum-free culture and to suspension culture, which are two important requirements for facilitating large-scale production of LV in a clinical setting. In addition, poor stability of the packaging cells (caused by leaky expression of VSV-G) was documented when using the Tet-Off system.[12] Finally, because induction does not occur immediately after the removal of an inhibitor from the culture medium, there may be a significant delay before production of LV begins. For large-scale production, it is also more practical to add an inducer to the growth medium rather than removing an inhibitor. In this study, we generated stable packaging cell lines (293SF-PacLV) with the capacity to produce high-titer (up to $3.4 \times 10^7$ TU/ml) of LVs in suspension culture and in serum-free medium. The expression of VSV-G and *Rev* was tightly regulated at the transcription level, using the optimized Tet-On switch[19] in conjunction with our newly developed inducible transcription system (cumate switch) derived from the *p-cym* operon of *Pseudomonas putida*.[20] Induction is promoted by the addition of two inducers (Dox and cumate) to the growth medium. We also demonstrate the usefulness of the 293SF-PacLV for producing LVs in shake flasks as well as in a 3.5-l bioreactor.

## RESULTS

### Development of packaging cell lines: Two-step approach

Several intermediates were required to produce the LV packaging cells that we call 293SF-PacLV. The strategies used in generating these cells are described below and are summarized in **Table 1**. A description of the plasmids used for producing the cells is presented in **Figure 1**. We used two approaches to generate the packaging cell lines. In the first approach (Two-step), a stable clone expressing the *Gag/Pol* and *Rev* genes is first generated and characterized before producing a clone expressing VSV-G and additional Rev. In the second approach (One-shot), all the LV components (Gag/Pol, rev, and VSV-G) are added simultaneously through a single transfection event. Many retroviruses and LVs are pseudotyped with VSV-G, because this glycoprotein confers a broad tropism and increases the virion stability.[17] Given the considerable cytotoxicity of VSV-G, we decided to tightly regulate its expression using the Tet-On system[19] and the repressor system of the cumate switch.[20] Induction is promoted by adding two inducers, Dox and cumate, to the growth medium. Dox promotes binding of the reverse tetracycline transactivator (rtTa2$^s$-m2) to the TR5 promoter, and cumate promotes the release of the cumate repressor (CymR) from the CuO operator (**Figure 1a**). We first generated a cell line derived from a clone of 293 cells (293SF)[21] adapted to grow in suspension and in serum-free medium that expresses CymR (**Table 1**). 293SF were transfected with pMPG-BFP/CMV5-CymR/tk-neo (**Figure 1b**) and a resistant pool of cells was isolated in the presence of neomycin. Clones were then isolated by limiting dilution of the pool. The clones were maintained for 6 weeks in the absence of selective pressure in order to test their stability. The CymR function was analyzed using an adenoviral vector (AdV) expressing $\beta$-galactosidase ($\beta$-gal) regulated by the CMV5-CuO promoter. The best $\beta$-gal On/Off ratio in the presence and absence of cumate was used in order to select the clone producing the optimal quantity of CymR. Clone G, which showed an On/Off ratio of 14, was selected as a recipient for the rtTA2$^S$-M2 transactivator. 293SF-CymR-G cells were transfected with plasmid pUDHrtTA2$^S$-M2.hygro (**Figure 1b**). A pool of hygromycin-resistant cells was generated, and clones were obtained by limiting dilution of the pool in the absence of selection. The functioning of CymR and rtTA2$^S$-M2 produced by the clones was tested using an AdV encoding the green fluorescent protein (GFP) regulated by the TR5 and CuO promoters. The clone with the best On/Off ratio was chosen for the development of the packaging cell lines. This was done by transfecting it with pMPG-RSV-Rev/CMV-Gag/polRRE, a plasmid that encodes the *Rev*, *Gag*, and *pol* genes of human immunodeficiency virus-1, as well as the resistance for phleomycin as a fusion protein with GFP. Phleomycin-resistant clones were isolated in 96-well plates. The clones with the best GFP expression level were analyzed for LV production by transient transfection with pCSII-CMV5-GFPq (**Figure 1c**) and VSV-G. The titers obtained with the best clones were 10- to 55-fold lower than those obtained when the same clones were transfected with plasmids encoding Rev regulated by rous sarcoma virus (RSV) (pRSV-Rev).[22] Two clones (#19 and #64) were then subcloned by limiting dilution and analyzed for LV production by transient transfection. As observed for the parent clones, the LV titer obtained was significantly higher in the presence of additional Rev (**Supplementary Figure S1a**).

Because the previous results indicated that the quantity of Rev produced by the 293SF-Rev-Gag-Pol cells was not optimal, we co-transfected two of the best subclones (#19-17 and #64-8)

**Table 1 Steps used for generating the 293SF-PacLV**

| Name | 293SF-CymR | 293SF-CymR-rtTA2$^s$-M2 | 293SF-Rev-Gag-pol | PacLV (Two-step) | PacLV (One-shot) | Stable producer #16-22-22[a] | Stable producer #29-6-14[b] |
|---|---|---|---|---|---|---|---|
| Parent cell lines used | 293SF | 293SF-CymR | 293SF-CymR-rtTA2$^s$-M2 | 293SF-Rev-Gag-pol | 293SF-CymR-rtTA2$^s$-M2 | 293SF-PacLV #16-22 | 293SF-PacLV #29-6 |
| Plasmid[c] used for transfection | 1 | 2 | 3 | 5, 7 pPuro | 4, 6, 7 pPuro | LVR2-GFP[d] | LVR2-GFP[d] |
| Stability tested (weeks) | 6 | 8 | Not tested | 18 | 18 | 18 | 18 |
| Best clone | G | #1 | # 19-17 | # 16-22 | # 29-6 | # 16-22-22 | # 29-6-14 |

*Abbreviation*: pPuro, plasmids encoding the puromycin resistance.
[a]293SF-PacLV generated using the Two-step strategy; [b]293SF-PacLV generated using the One-shot strategy; [c]the number used in **Figure 1b** to identify the particular plasmid; dcell line obtained by lentiviral (LV) transduction.

with plasmids encoding the puromycin resistance (pPuro), Rev (pkCMV5-CuO-Rev) and VSV-G (pTR5-CuO-VSVg-IRES-GFPq) to generate a cell line expressing VSV-G and more Rev. VSV-G was regulated by the cumate switch and the Tet switch, whereas Rev was regulated by the cumate switch only. The puromycin-resistant clones were first screened for VSV-G expression by measuring GFP (through the IRES-GFP) after induction with Dox and cumate. The LV production was then analyzed by transient transfection with pCSII-CMV5-GFPq. Co-transfection of the best clones with pRSV-Rev increased the LV titer by only twofold to fourfold (data not shown), which was lower than the earlier level. The three best clones were subcloned in the absence of selective pressure. LV production was analyzed by transient transfection as described earlier. The production from the six best subclones could be improved by only twofold to threefold after the addition of Rev, thereby indicating that the quantity of Rev was nearly optimal (**Supplementary Figure S1b**). The efficacy of the double switch was investigated by measuring the increase of GFP expression (from the stably integrated VSV-G-IRES-GFPq cassette) after induction of clone #16-22 (**Figure 2**). An induction factor over 2,500 was observed in the presence of cumate and Dox. GFP expression in the presence of the two inducers was much higher than when each inducer was used separately. In the presence of cumate only, the GFP expression level increased by a factor of 4.5, thereby indicating that the cumate switch improves the tightness of the Tet switch by this factor.

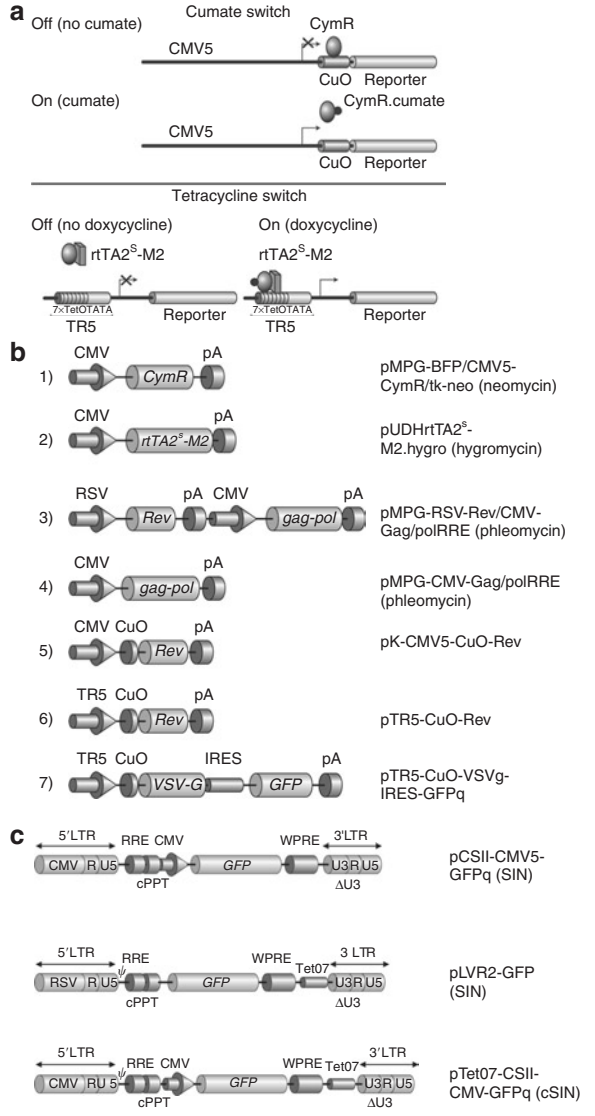


**Figure 1 Inducible promoter systems and plasmids used in the present study.** (**a**) The cumate and Tet inducible systems. In the absence of cumate, the CymR binds to the CMV5-CuO promoter and prevents transcription. Cumate inhibits binding of CymR to CMV5-CuO, allowing transcription. Doxycycline (Dox) induces binding of the rtTA2$^S$-M2 transactivator to the TR5 promoter, which triggers transcription. No binding of rtTA2$^S$-M2 occurs in the absence of Dox. (**b**) Description of the plasmids used for generating the PacLV cell lines. The genes and regulatory elements carried by each plasmid are indicated. The antibiotic resistance is also indicated in brackets. (**c**) Structure of the transfer vectors used in this study. CMV, immediate early enhancer and promoter of cytomegalovirus; CymR, cumate-regulated repressor; RSV, rous sarcoma virus promoter; TR5, tetracycline-regulated promoter; CuO, binding site for the cumate-regulated repressor; IRES, internal ribosome entry site; LTR, long terminal repeat; U3, U5, and R, unique 3′, unique 5′, and repeat regions of the LTR; Ψ, packaging signal; ΔU3, deletion of the U3 region that removes its promoter activity; RRE, rev-responsive element; WPRE, woodchuck hepatitis virus post-transcriptional regulatory elements. GFP, green fluorescent protein; SIN, self-inactivating vector; cPPT, central polypurine tract; VSV-G, glycoprotein of vesicular stomatitis virus.

## Development of packaging cell lines: One-shot approach

Because Rev is required for the efficient expression of the protease from the *Gag* gene, which has been reported to be cytotoxic,[16] even a relatively small amount of Rev could be detrimental to the cells. For this reason, we decided to generate a packaging cell line expressing Rev under very tight regulation. We therefore constructed pTR5-CuO-Rev, which contains the Rev coding sequence doubly regulated by the Tet switch and the cumate switch (**Figure 1b**). The 293SF-CymR-rtTA2$^S$-M2 cell line was co-transfected with plasmids encoding *Rev*, *Gag*, *Pol*, VSV-G, and the resistance for puromycin (**Table 1**, PacLV, One-shot). Stable clones were selected in the presence of puromycin. Clones having the highest GFP induction factor after addition of Dox and cumate were analyzed for LV production by transfection with transfer vector pCSII-CMV5-GFPq. The two best clones

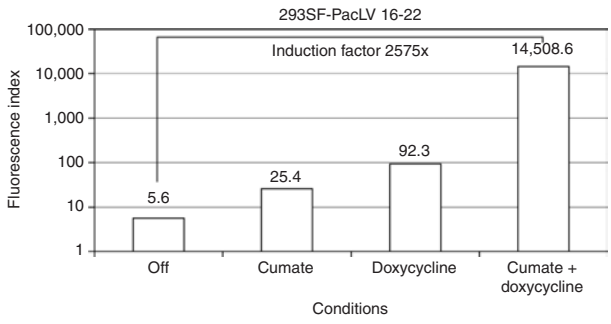


**Figure 2 Efficacy of the doubly regulated switch.** Packaging cell line #16-22 was induced by addition of cumate, doxycycline (Dox) or both. Forty eight hours later, the fluorescence index [(percentage of green fluorescent protein (GFP)-positive cells) × (mean fluorescence intensity)] of the cell population was measured by flow cytometry. The expression of GFP is caused by the VSVG-IRES-GFP transcript that is doubly regulated by the Tet and CuO promoter. Off, fluorescence index in the absence of cumate and Dox. IRES, internal ribosome entry site; VSVG, glycoprotein of vesicular stomatitis virus.

were then subcloned without selection and analyzed for LV production. The transfection of the best subclones with pCSII-CMV5-GFPq and pRSV-Rev did not increase the amount of LVs produced, thereby suggesting that the quantity of Rev produced by the clones was not limiting (**Supplementary Figure S1**). We then compared the LV production levels obtained in the best clones when using the Two-step (#16-22) and One-shot (#29-6) approaches (**Figure 3**). Titers of $8.6 \times 10^6$ and $2.6 \times 10^7$ TU/ml were obtained for #16-22 and #29-6, respectively. The amount of p24 produced after induction was also evaluated by enzyme-linked immunosorbent assay (ELISA). The amounts of p24 produced were 39 and 571 ng/ml by #16-22 and #29-6, respectively. The fact that the specific activity (TU/ng of p24) was 219,000 for #16-22 and 45,000 for #29-6 indicates that the latter clone produced more defective or empty virions.

## Production of LVs in suspension culture by the packaging cells

We tested clones #16-22 and #29-6 for LV production in suspension culture in shake flasks, using serum-free media. The cells were transfected with pCSII-CMV5-GFPq, induced by addition of Dox and cumate, and incubated with constant agitation for 7 days, with the culture medium either being replaced on a daily basis or not replaced. The amount of LVs produced was determined every day to find the timing of maximum production. As shown in **Figure 4**, the behavior of the two clones was quite similar. In cultures in which the medium was replaced daily, the production of LVs was higher on days 2–3, and decreased at later time points. By contrast, when the medium was not changed, the quantity of functional LVs reached a maximum on days 2–3 and remained to that level during the whole experiment. The highest titer ($7.7 \times 10^6$ TU/ml) was obtained on day 3 for clone #16-22. This titer was 3.5 times lower for clone #29-6 under the same conditions. For this experiment, the cells were transfected using a mixture of DNA and polyethylenimine (PEI). The production from #16-22 was higher than for #29-6 because the transfection in the former clone was more efficient when this method was used (data not shown). Because of its higher yield in suspension culture (**Figure 4**), we chose clone #16-22 to test the production in a bioreactor. A volume of 2.8 l of cells was transfected with pCSII-CMV5-GFPq and incubated in batch culture in a bioreactor for 3 days. This time point was chosen because it was the time point of maximum production in shake flasks (**Figure 4**). At the end of the production, the titer obtained was $2 \times 10^6$ TU/ml. After a 100-fold concentration, the titer was $1.2 \times 10^8$ TU/ml which corresponds to a 60% recovery.

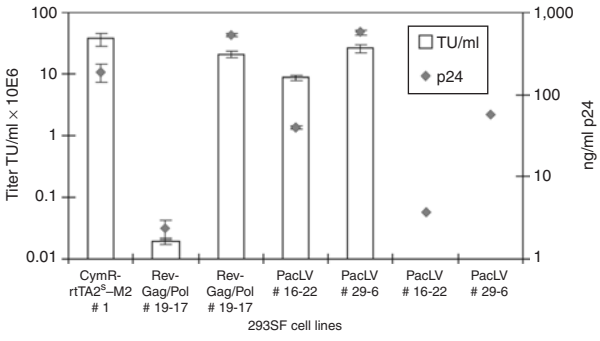


**Figure 3 Production of lentiviral vectors (LVs) by transient transfection of the 293SF-PacLV.** LVs were produced by transfecting the indicated cells (see **Table 1** for details). After the 293 cells were infected, the amount of LVs produced was determined by flow cytometry and by measuring the amount of p24 in the supernatant. The cells were transfected with the following plasmids (from left to right): Bar 1: the four packaging plasmids: pCSII-CMV5-GFPq, pRSV-Rev, pMDLg/pRRE#54, and pTR5-CuO-VSVg-IRES-GFPq; bar 2: pCSII-CMV5-GFPq and pTR5-CuO-VSVg-IRES-GFPq; bar 3: pCSII-CMV5-GFPq, pTR5-CuO-VSVg-IRES-GFPq, and pRSV-Rev; bars 4 and 5: pCSII-CMV5-GFPq; bars 6 and 7: untransfected cells. The experiment was performed in sets of three. All the mean values ± SD (TU/ml) were significantly different from one another ($P < 0.01$). TU, transducing unit.

## LV production using stable producers

Neither the preparation of large quantities of plasmid nor the transfection procedure would be necessary if a cell line (a producer) that synthetizes all the essential viral functions including the viral RNA were available. Using such a cell line, LV production could be initiated solely by adding the inducers, such as Dox and cumate. We therefore evaluated the ability of our packaging cells to generate stable producers. Clones #29-6 and #16-22 were transduced with a conditional-SIN-LV, produced by transfecting packaging cells with the transfer vector pLVR2-GFP[23] (**Figure 1c**). Clones were isolated by limiting dilution of the pool of transduced cells. The clones having the highest GFP expression following induction with Dox were expended. We then tested the production in serum-free suspension culture of the best clones from parent cells #16-22 (16-22-22) and #29-6 (#29-6-14). The medium was changed on a daily basis and the number of infectious particles was determined by flow cytometry. The amount of p24 produced was also analyzed by ELISA (**Figure 5**). The behavior of the two clones was quite similar, except for the absolute amount of LVs produced, which was two to three times higher for clones #29-6-14. The number of infectious particles produced increased every day until it reached a maximum at day 4. It then decreased progressively. The highest titer ($3.4 \times 10^7$ TU/ml) was obtained at day 4 when #29-6-14 was

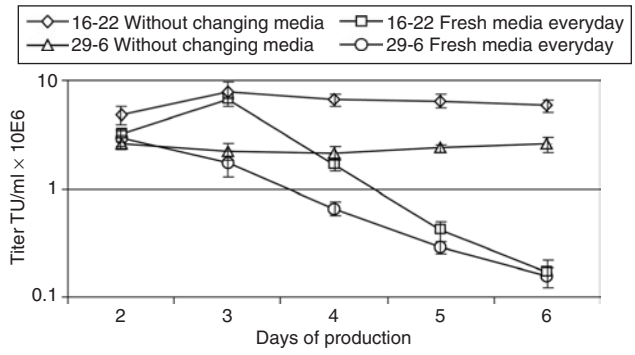


**Figure 4 Lentiviral vector (LV) production in shake flasks using packaging cells.** Twenty five milliliter suspension cultures of clones #16-22 (Two–step) and #29-6 (One-shot) containing $2.5 \times 10^7$ cells were transfected with pCSII-CMV5-GFPq using polyethylenimine. The amount of LVs in the growth medium was titrated on 293A cells by flow cytometry each day. The production achieved with the medium being replaced with a fresh medium on a daily basis was compared with the production when the medium was left unchanged. The data shown are the mean values ± SD ($N = 2$). TU, transducing unit.

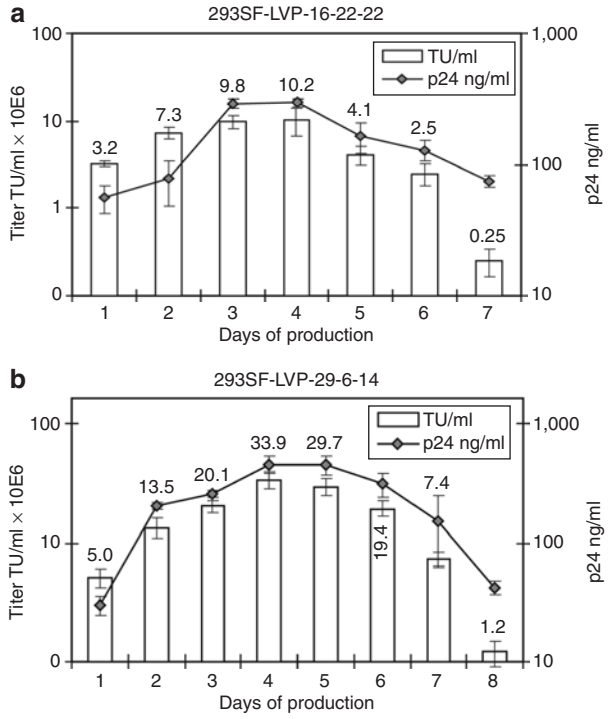


**Figure 5 Lentiviral vector (LV) production in shake flasks using stable producers.** Twenty milliliter suspension cultures of clones 16-22-22 (Two-step) and 29-6-14 (One-shot) containing $3.0 \times 10^7$ cells were induced by addition of doxycycline and cumate. The amount of LVs in the medium, which was changed every day, was titrated on 293A cells by flow cytometry. The quantity of p24 in the medium was also determined by enzyme-linked immunosorbent assay. The data shown are the mean values ± SD ($N = 3$).

used. The quantity of p24 produced by the cells at different time points followed the same pattern. On day 1, the specific activity of the viral prepration (TU/ng of p24) was 54,000 and 166,000 for #16-22-22 and #29-6-14, respectively, and decreased progressively. At the end of the production (day 6 or 7) the specific activity was lower by a factor of 3 to 4, thereby indicating that more defective particles were produced at later time points.

The relative stability of four different producer clones derived from packaging cells #29-6 was evaluated. The LV production was compared after 4 weeks and 18 weeks of culture in the absence of selective pressure. As shown in **Figure 6**, the titers obtained with these four clones did not decrease significantly after 18 weeks of culture. These data indicate that, using the packaging cells described in this study, it is possible to obtain producers whose long-term stability is more than adequate for a large-scale production process. In addition, tests to detect the presence of replication-competent lentiviruses were carried out by infecting 293 cells using concentrated stock of LVs as described.[24] No replication-competent lentiviruses were detected by ELISA analysis for P24, and no VSV-G was detected by polymerase chain reaction (PCR).

## LV production using a pool of stable producers

Because subcloning and analysis of individual clones is a time-consuming process, we investigated whether high levels of LVs could be obtained using a pool of cells. For this experiment,

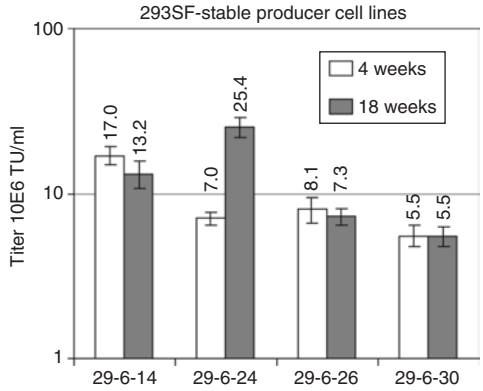


**Figure 6 Stability of producer cells.** Four different producer clones were maintained in culture without selective pressure and without induction (no doxycycline (Dox) and no cumate) for 4 and 18 weeks. The production of lentiviral vectors (LVs) was then induced by addition of Dox and cumate. The amount of LVs produced at 48 hours after induction was analyzed using flow cytometry. The data shown are the mean values ± SD ($N = 2$).

packaging cells #29-6 were transduced with a conditional-SIN-LV produced by transfecting packaging cells with pTet07-CSII-CMV-GFPq (**Figure 1c**). The production efficiency of the pool of producers was then tested in serum-free suspension culture in shake flasks. The medium was changed on a daily basis and the number of infectious particles was determined by flow cytometry (**Supplementary Figure S2**). The production pattern of the pool was very similar to that of the two clones for which the data are shown in **Figure 5**, except for the fact that the highest titer ($1.9 \times 10^7$ TU/ml) was obtained at day 3 instead of day 4, and that the duration of useful production was one day shorter.

## DISCUSSION

In this study, we employed two strategies (Two-step and One-shot) to create several cell lines capable of producing high-titers of LVs in serum-free suspension culture. We also demonstrated the feasibility of generating producers that remain stable for at least 18 weeks in the absence of selective pressure. The LV production in serum-free culture medium was validated in shake flasks for the packaging cells as well as the producer cells (clones and pool), and in a 3.5-l bioreactor for the packaging cells. In shake flasks, the best titer ($3.4 \times 10^7$/ml) was obtained with the use of a stable clone. This was five times higher than the titer obtained by transient transfection of our best packaging cells under similar conditions. In addition to making more LVs, the stable producers secreted the LVs for a longer period of time (7 days) as compared to the transfected packaging cells (4 days). The transfection efficiency of the packaging cells being lower than 100%, and the fact the transfer vector DNA can be degraded or lost after transfection, could explain these differences. For *in vivo* applications, stable producers possess the advantage of being able to produce LVs that are not contaminated by tubulovesicular structures containing plasmid DNA that stimulates dendritic cells.[25] We also demonstrated that the isolation of individual clones from a pool of producers is not essential for obtaining elevated titers of LVs. However, because a pool consists of a poorly characterized heterogeneous cell population, it should not be used for clinical application. LV production

was more than three times lower in the bioreactor compared to the shake flask. One possible reason for this is that the transfection efficiency is somewhat variable, and is usually lower when larger culture volumes are transfected. Despite this, the titer we obtained with the bioreactor was comparable to the titer obtained recently by Segura *et al*.,[26] who produced LVs in a similar bioreactor by transfecting 293 cells with plasmids encoding all the components necessary for LV production. It should be noted, however, that the use of packaging cells simplifies the production process, because only one plasmid (the transfer vector), as opposed to four plasmids, is required for transfection.

The quantity of LVs produced by our best clones (packaging and producers) under optimal conditions is comparable to the quantity produced by the best packaging cells reported in the literature.[9,12–15] An important characteristic of our packaging cell lines is their ability to produce high levels of LVs in suspension culture and in serum-free medium; this is a notable advantage for scale-up, safety, and regulatory requirements. To our knowledge, no other reports have described efficient LV packaging cells possessing these two important attributes. The excellent stability of our packaging cells and the elevated LV titers produced by them are most likely a consequence of the gene-switch systems we employed to control the On/Off expression of some packaging elements. Induction is performed by adding two compounds (cumate and Dox). This is far more convenient then removing a compound (Dox) as is done for the other packaging cell lines available. One disadvantage of using the inducers cumate and Dox is that they remain in the supernatant, and thus contaminate the preparation of LV. However, these two compounds can be partly removed with one or two cycles of ultracentrifugation. In addition, the vector can be purified more extensively using size-exclusion chromatography.[27] In the Two-step approach, we first generated a clone stably expressing *Gag/Pol* and *Rev*, and then inserted the gene for VSV-G. In our first attempt to generate a packaging cell line, Rev was constitutively expressed using the RSV promoter. We demonstrated that the production of LVs could be increased by providing additional Rev, suggesting that our best clones did not synthesize sufficient amount of this protein. Constitutive expression of Rev is probably cytotoxic in the presence of the *Gag/Pol* gene, because Rev is required for efficient processing and export of the *Gag*/*Pol* transcript and therefore for the synthesis of the protease,[28] which has been reported to be cytotoxic.[16] For this reason, during the second step which consisted of inserting the envelope gene (VSV-G), we introduced *Rev* again, but this time regulated by the cumate switch. The LV titers obtained by the resultant clones were comparable to the titer obtained after transfecting all the packaging functions. The amount of LVs was not significantly increased in the presence of additional Rev, indicating that it was not a limiting component anymore. A similar strategy was used by Klages *et al*.[9] to generate LV packaging cells lines by indirectly controlling the expression of the *Gag/Pol* gene using an inducible Rev expression cassette.

In the second strategy (One-shot), the packaging functions and VSV-G were introduced simultaneously into the cell line. *Rev* and VSV-G were both doubly regulated by the cumate and Tet switches. The main advantage of this strategy is its rapidity, with the cells being transfected only once. Flow cytometry and p24-ELISA were used for evaluating the amount of LVs produced by the cells. Flow cytometry measures functional virions (TU/ml), whereas p24 is an estimate of the number of physical particles and does not distinguish between functional and inactive virions. The infectivity (TU/ng of p24) can be used for estimating the integrity of the preparation of LVs. The infectivity values of our various LV preparations, which varied between 45,000 and 200,000, was in the range of infectivity described by others.[14,29,30] Because our stable producers are amenable to perfusion culture in a bioreactor (a mode of production that has been shown to significantly improve retroviral vector production),[31] we expect that an approximately tenfold increase in LV productivity can be achieved if this technology is used for production.

In summary, we have generated efficient and stable LV packaging cell lines that can by used for generating stable producers. The 293SF-PacLV allows scaling-up production in shake flasks and in bioreactors in serum-free culture medium. These properties render these cells very amenable for use in producing large batches of LVs.

## MATERIALS AND METHODS

***Plasmid construction.*** Plasmids were constructed according to standard procedures and purified by chromatography (Qiagen, Valencia, CA). The construction of the plasmid pMPG-BFP/CMV5-CymR/tk-neo has been described previously.[20] In order to construct pUHDrtTA2$^S$-M2-hygro, the hygromycin-resistant gene was removed from pSL-hyg[32] by *Sal*I/*Xho*I digestion and ligated into pUHDrtTA2$^S$-M2 (ref. 19) digested with *Ssp*I/*Xho*I. The plasmid pMPG-CMV-Gag/polRRE was constructed by removing the CMV-g/pRRE fragment from pMDLg/pRRE#54 (ref. 22) by *Hae*II/*Snab*I digestion. The fragment was ligated into pMPGB43 (ref. 33) in which the hygromycin-resistant gene was replaced with the phleomycin/GFP fusion gene. Before ligation, the B43 heavy and light chains were removed by digesting pMPGB43 with *Pac*I and *Snab*I. The phleomycin/GFP gene was generated using pUT531 (ref. 34) carrying the *Sh-Gal*fusion gene conferring phleomycin resistance and $\beta$-gal activity.[35] GFP was amplified using PCR and cloned in pUT531 at the *Pvu*II site to remove $\beta$-gal. Plasmid pK-RSV-Rev was generated with PCR, using primers 5′-CGCGGACCGATGTACGGGCCAGATATA-3′ and 5′-CCAAGCTTTG ATCACTATTCTTTAGCTCCTGA-3′ and pRSV-Rev[22] as template. The PCR product was digested with *Rsr*II/*Hind*III and inserted in front of the rabbit poly-A signal.[33] The plasmid pMPG-RSV-Rev/CMV-Gag/polRRE was produced by inserting the RSV-Rev-polyA expression cassette of pK-RSV-Rev into the *Asc*I site of pMPG-CMV-Gag/polRRE by digesting both DNA with *Asc*I. In order to produce pK-CMV5-CuO-Rev, the RSV promoter of pK-RSV-Rev was removed by digestion with *Xho*I and replaced with the *Xba*I/*Eco*RV fragment containing the CMV5-CuO promoter of pAdCMV5-CuO-mcs-IRES-GFP-MLP-Ps (Qbiogene, Carlsbad, CA). The plasmid pTR5-CuO-VSVg-IRES-GFP was constructed by first isolating the VSV-G coding sequence with PCR, using primers 5′-CATAGATCT GCCATGAAGTGCCTTTTGTACTTAGCC-3′ and 5′-CATAGATCTG AGTTACTTTCCAAGTCGG and pSVCMVinVSVg (kindly provided by Jacques Galipeau, Jewish General Hospital, Montreal, Canada). The PCR product was introduced into the *Bgl*II site of pAd-TR5-CuO-mcs-ires-GFP.[36] The plasmid pTR5-Cuo-Rev was generated by replacing the CMV5-CuO promoter from pK-CMV5-CuO-Rev with the TR5-CuO promoter from pTR5-CuO-VSVg-IRES-GFP by *Afl*II/*Blp*I digestion. The plasmid pCSII-CMV-GFPq was constructed by inserting the GFP coding sequence of pAdCMV5-GFP[37] into pCSII-CMV-mcs[38] by digesting both DNAs with *BamH*I. The plasmid pCSII-CMV5-GFPq was generated by replacing the *Snab*I-*BstB*I fragment containing the cytomegalovirus (CMV) promoter of pCSII-CMV-GFPq with the CMV5 promoter of pAdCMV5-GFP[37] isolated with the same enzymes. The plasmid pTet07-CSII-CMV-GFPq was generated by introducing the Tet07 promoter into the 3′–long terminal repeat of pCSII-CMV-GFPq. The Tet07 promoter was removed from pLVR2-GFP[23]

by *Bsm*I/*Xho*I digestion and ligated into pCSII-CMV-GFPq previously digested with *Pme*I and *BspE*I.

***Cell lines and AdVs.*** 293SF[21] and its derivatives were grown in low-calcium-serum-free medium (Invitrogen, Grand Island, NY). For suspension culture, the cells were grown in shake flasks at 120 rpm. The 293A (American Type Culture Collection) and 293rtTA (see later text) were grown in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT) supplemented with 5% fetal bovine serum. All the cell lines were maintained at 37 °C in a 5% $CO_2$ humidified atmosphere. The switch efficacy was evaluated using AdVs produced and tiltered with standard procedures.[37] An AdV expressing GFP regulated by a Tet promoter (AdTR5-GFP)[37] was used for testing the efficacy of the 293rtTA and 293SF-CymR-rtTA2$^S$-M2 with and without 1 µg/ml of Dox (Sigma). An AdV expressing $\beta$-gal controlled by the CMV5-CuO promoter AdCMV5-CuO-LacZ[20] was used for testing the efficacy of the 293SF-CymR. An AdV carrying GFP regulated by TR5-CuO promoter (GFP/Pab13; provided by Y. Langelier; Université de Montréal, Canada) was used for evaluating the double switch of 293SF-CymR-rtTA2s-M2 with and without 1 µg/ml Dox (Sigma-Aldrich, St. Louis, MO) and 10 µg/ml cumate (Sigma-Aldrich, St. Louis, MO). At 48 hours after infection, the cells were analyzed by flow cytometry (GFP expression) or by measuring the conversion of ortho-nitrophenyl-$\beta$-D-galactopyranoside at 420 mm ($\beta$-gal expression).

***LV production by transient transfection.*** Cells were transfected using PEI (PEI 25-kd linear; Polysciences, Warrington, PA) as described[39] or using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Four to six hours later, the medium was replaced with fresh medium supplemented with 1 µg/ml Dox and 10 µg/ml cumate. The supernatant containing the LVs was collected at 48 and 72 hours after transfection by low-speed centrifugation and filtered through 0.45-µm pore size–HT Tuffryn membrane (Pall Life Science, Ann Arbor, MI) The supernatant was used fresh, or frozen at −80 °C, or concentrated by centrifugation on a 20% sucrose cushion.[27]

***Generation of cell lines.*** The cell lines were produced by transfecting 80% confluent 10-cm plates using PEI. The cell line expressing the transactivator rtTA2$^S$-M2 (293rtTA) was produced by transfecting 293A cells with pUDHrtTA2$^S$-M2.hygro. Hygromycin B (150 µg/ml; Invitrogen, Carlsbad, CA) was added to the growth medium 24 hours later to produce a pool of hygromycin-resistant cells. The pool was cloned by limiting dilution in 96-well plates. Other cell lines: 293SF-CymR, 293SF-CymR-rtTA2$^S$-M2, 293SF-Rev-Gag/pol, and 293SF-PacLV were generated in a similar manner, using the plasmids and parent cell lines described in **Table 1**. At 48 hours after transfection, some cells were directly plated into 96-well plates in the presence of the selection (see Results for details). G418 (Wisent, St. Bruno, Canada), Hygromycin B, Phleomycin (Sigma-Aldrich, St. Louis, MO) and puromycin (Sigma-Aldrich, St. Louis, MO) were used at 100, 30, 5, and 0.3 µg/ml respectively.

***Transduction and titration of LVs.*** Cells were seeded 24 hours prior to the experiment. The LVs were diluted in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 8 µg/ml of polybrene (Hexadimethrine Bromide; Sigma) and incubated for 30 minutes at 37 °C. The cell culture medium was removed and the LV/polybrene mix was applied directly onto the cells, which were then incubated overnight at 37 °C. The medium was removed and replaced with fresh culture medium. The cells were analyzed for GFP expression 72 hours later using a Coulter EPICS XI flow cytometer (Beckman-Coulter, Fullerton, CA). Quantification of p24 protein in the LV preparation was determined by ELISA.[40] Hybridomas expressing anti-p24 antibodies (183 H12-5C and 31-90-25) were provided by Michel Tremblay (Centre Hospitalier Université Laval, Québec, Canada).

***Production of LVs in suspension.*** For the packaging cells, flasks of 125 ml were seeded in triplicate with 25 ml of LC-SFM supplemented with 0.1% (vol/vol) of lipid mixture (Sigma-Aldrich, St. Louis, MO) and 0.5% (wt/vol) of Gelatin Peptone N3 (Organotechnie, La Courneuve, France) containing $3 \times 10^5$ cells/ml. Forty eight hours later, when the cell density reached $1 \times 10^6$ cells/ml, they were transfected with PEI. At 4 hours after transfection, the cells were induced by adding 1 µg/ml Dox and 10 µg/ml cumate. The LV titer was determined on a daily basis by flow cytometry as described earlier. For the producers, subclones #16-22-22 and #29-6-14 were seeded in triplicate in 125-ml flasks at $1.5 \times 10^6$ cells/ml in 20 ml LC-SFM supplemented with 1 µg/ml Dox and 10 µg/ml cumate. The LVs were collected daily, clarified using low-speed centrifugation, and kept frozen at −80 °C until titration. The cells were resuspended into fresh medium supplemented with 1 µg/ml of Dox and 10 µg/ml of cumate. The LVs were titrated by flow cytometry and by ELISA as described earlier.

***Production of LVs in a bioreactor.*** A 3.5-l bioreactor (Chemap CF-3000; Mannedorf, Switzerland) was retrofitted with three surface baffles to improve aeration. The operation parameters were as described earlier.[41,42] The bioreactor was seeded with 293SF-PacLV #16-22 in LC-SFM supplemented with 0.1% (vol/vol) lipid mixture and 0.5% (wt/vol) of Gelatin Peptone N3 to an initial density of $2.6 \times 10^5$ cells/ml. When the density level reached $0.83 \times 10^6$ cells/ml, the cells were transfected with 2.8 mg of pCSII-CMV5-GFPq in 43.5 ml DNA-PEI polyplex mixture in LC-SFM, as described previously without 10 mmol/l HEPES.[43] The DNA:PEI ratio used was 1:3 and the final DNA concentration was 1 mg/L. Thirty minutes after transfection 1 µg/ml of Dox and 10 µg/ml of cumate were added. LV production proceeded for 72 hours and was titered by flow cytometry. The cells were removed by clarification, and the LV was concentrated by ultracentrifugation at 37,000*g* for 3 hours at 4 °C on a 20% sucrose cushion. The pellet was resuspended in 5% sucrose, 20 mmol/l Tris–Cl, pH7.5, filtered through a 0.45 µm filter, and titered by flow cytometry.

***Detection of replication-competent lentivirus.*** Tests to detect the presence of replication-competent lentivirus were carried out as described earlier.[24] Briefly, 293 cells were transduced with concentrated stocks of LVs (multiplicity of infection >150 TU/cell). After 3 weeks of culture, the culture supernatant was collected and used for infecting 293 cells (indicator culture). The amount of p24 in the supernatant was determined using ELISA. One week after infection of the indicator culture, the presence of VSV-G was tested for, using PCR of the genomic DNA. Neither p24 nor VSV-G DNA was detected.

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## SUPPLEMENTARY MATERIAL

**Figure S1.** Effects of Rev on LV production by the packaging cells.
**Figure S2.** Production of LVs in shake flasks using a pool of producer cells.

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