Chapter 25

Generation of Human Cell Lines Using Lentiviral-Mediated Genetic Engineering

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Abstract

Even now, most human cell lines used in research are derived from tumor cells. They are still widely used because they grow well in vitro and so far have helped answering several basic biological questions. However, as modern biology moves into more sophisticated areas, scientists now need human cell lines closer to normal primary cells and further from transformed cancerous cells. The recent identification of cellular genes involved in cell cycling and senescence, together with the development of molecular tools capable of cleanly integrating transgenes into the genome of target cells, have moved the frontier of genetic engineering. In this chapter, we present a detailed hands-on protocol, based on lentivirus-derived vectors and a combination of two native cellular genes that has proven very efficient in generating immortal cell lines from several human primary cells, while preserving most of their original properties.

Key words: Lentivirus, Vector, Lentivector, Gene transfer, Genetic engineering, Cell engineering, Cell line

1. Introduction

1.1. A Brief History of Human Cell Lines

The first deposited cell line (ATCC number CCL-1) was described in 1943 from a chemically induced mouse mammary tumor (1). The second deposited cell line (ATCC number CCL-2) is the famous HeLa cell line that was isolated in 1952 from a cervix carcinoma (2). Since then, several human cell lines have been deposited, most of them after in vitro expansion of human tumor cells. These naturally occurring tumor cells have arisen after unknown or partially known transformation events that have conferred them with the desirable features for a cancerous cell, i.e., autonomous proliferation, lack of senescence, lack of contact inhibition, anchorage-independent growth, angiogenesis, decrease or lack of MHC antigen expression, etc. The sum of these successive selection processes in vivo results in the "transformation" of a normal cell into a

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cancerous cell. From the biologist standpoint, the main advantage is the robust growth in vitro. Indeed, these cell lines have helped define the composition of culture media, and thus they have blazed the trail of modern cell culture. The inconvenience is that, in most cases, we do not know what molecular event (or events) has occurred to render these cells tumorigenic. In almost all cases, they display an abnormal and unstable karyotype, have lost several features of their tissue of origin, and have lost the ability to differentiate. Of note, some widely used human cell lines, such as the HT-1080 cells (ATCC number CCL-121) display a near normal karyotype. This may be due to the fact that their transformation event was the activation of the Ras oncogene (3).

One of the first "genetic engineering" of human cells was the transformation of human embryonic kidney cells by transfection of DNA from Adenovirus type 5 (4). The resulting cell line is the famous 293 cell line (ATCC number CRL-1573). Although 293 cells are polyploid and tumorigenic, they are relatively stable and are used for many biological studies as well as further genetic engineering by the introduction of other transgenes. Although the procedure leading to the generation of the 293 cell line can be considered as "crude" by present standards, this type of experiment paved the way to modern genetic engineering.

The first description of "viral genetic engineering" was the transformation of rodent cells by transduction of SV40 large T antigen using a retroviral vector (5). It was rapidly followed by retroviral transduction of human cells of various origins with combinations of oncogenes, including SV40 large T, Ras and/or HPV16 E6 and E7 genes. The use of these genes generated countless cell lines from various tissues and animals, but there always seemed to be an issue of genomic or phenotypic stability. HPV16 E6 and E7 genes immortalized and induced genomic instability in human bronchial epithelial cells (6), human mesenchymal cells (7), and pancreatic duct epithelial cells (8). Since it has been shown that HeLa cells are expressing HPV16 E6 and E7 genes, this is the likely reason why they are instable and have a chromosome number ranging from 70 to 164. This genomic instability is best illustrated by SV40 large T antigen, that, when used to induce or enhance cell proliferation, invariably generated genomic instability and dedifferentiation (9–11). The immortalizing capacity of SV40 large T antigen is generally attributed to its inhibitory association with p53, which relieves the cell cycle G1 checkpoint (12). However, p53 also controls apoptosis following DNA damage (13). This could explain why introduction of SV40 large T antigen is invariably followed by genomic instability and chromosomal aberrations.

This issue of immortalization versus genomic stability and maintenance of a differentiated phenotype is crucial and is best summarized by the paradigm of human pancreatic beta-cells. Unlimited supplies of human pancreatic beta-cells would represent

a major breakthrough towards the cure of diabetes. However, even though some rodent beta-cells are available, originating either from X-ray-induced tumors (INS-1) (14) or from transgenic expression of SV40 large T antigen such as MIN6 cells (15) or βTC-tet cells (16), no human beta-cell line is available yet. In early experiments, while trying to generate a human beta-cell line, Wang et al. described the isolation of an epithelial cell line from human fetal pancreas, using retroviral transduction of SV40 large T and activated Ras (17). Using the same approach, neither fetal nor adult human pancreatic cells could generate long-term insulin-producing cells. Insulin expression was gradually lost and never recovered, a likely consequence of genomic instability or dedifferentiation induced by the strong proliferative signals provided by these two oncogenes (18).

In the 1990s, the issue of cell senescence was elucidated by the discovery that the telomerase enzyme could prolong life-span of cells by delaying telomere shortening, hence "resetting" the aging clock of the cells (19, 20). Moreover, it was shown soon after that ectopic expression of telomerase alone was not associated with transformation or tumorigenicity (21, 22). Prevention of telomere shortening was, however, not sufficient for in vitro expansion of most human cells. One needed to deliver another signal to force cells to enter cell cycle or to keep them cycling.

A paradigm of "mild" cell cycle promoter was provided by the study of Jacobs et al., who showed that Bmi-1, a transcriptional repressor belonging to the Polycomb group, and downregulating expression of the ink4a locus genes p16 and p19Arf, could immortalize fibroblasts (23). Following this report, several immortal human cell lines have been created using the combination of Bmi-1 and telomerase, amongst those, vascular endothelial cells (24), myoblasts (10), cementoblast progenitors (25), mesenchymal cells (26, 27), skin, mammary, and lung epithelial cells (28, 29), skin fibroblasts (30), and glial cells (31), all with preserved and stable phenotype, and no or minimal genomic alterations, even after extended culture periods. Interestingly, Bmi-1 has also been shown to correlate with stem cell maintenance of normal and cancer cells, and its expression is linked to poor prognosis in human tumors (32–34). From a cell engineer standpoint, this latter feature has three implications. First, on one hand, it may prove useful if successful immortalization of a given cell type requires a certain level of "stemminess." Second, it may be detrimental if "stemminess" prevents full terminal differentiation of the desired cells. And last but not least, it implies that viral vectors carrying Bmi-1 must be handled with special precautions.

Taken together, it is now obvious that the generation of all these cell lines using molecular tools to introduce this specific combination of Bmi-1 and telomerase has paved the way to "molecular immortalization" of many other human cell types.

1.2. HIV-1-Based Lentiviral Vectors as Molecular Tools for Genetic Engineering

Retroviral vectors have three characteristics of a highly attractive gene delivery system. First, they integrate their genetic cargo into the chromosome of the target cell, a likely prerequisite for long-term expression. Second, they have a relatively large capacity, close to 10 kb, allowing the delivery of most cDNAs. Finally, they do not transfer sequences that encode for proteins derived from the packaging virus, thus minimizing the risk that vector-transduced cells will be attacked by virus-specific cytotoxic T lymphocytes.

An infectious retroviral particle comprises an RNA genome that carries *cis*-acting sequences necessary for packaging, reverse transcription, nuclear translocation, and integration, as well as structural proteins encoded by the gag and env genes, and the enzymatic products of the pol gene. The assembly of these components leads to the budding of the virion at the plasma membrane of the producer cell. In lentiviruses, the efficient expression of Gag and Pol requires a virally encoded posttranscriptional activator called Rev.

Conventional retroviral vectors, however, are of limited usefulness for many applications because they are derived from oncoretroviruses such as the mouse leukemia virus (MLV), and, as a consequence, cannot transduce nondividing cells. In contrast to oncoretroviruses, lentiviruses, such as the human immunodeficiency virus (HIV), are a subfamily of retroviruses that can infect both growth-arrested and dividing cells, a feature that can be essential when trying to immortalize slow or nondividing primary cells. The proof-of-principle of this concept was first provided with vectors derived from HIV-1, using the adult rat brain as an in vivo paradigm (35). Since then, gene delivery systems based on animal lentiviruses such as the simian and feline immunodeficiency viruses (SIV and FIV) and the equine infectious anemia virus (EIAV) have been described. This chapter presents exclusively the HIV-1-based vector system because it is presently the most advanced, efficient and versatile, and because, in its latest version, it offers a level of biosafety that matches, if not exceeds, that of the MLV-derived vectors currently used in the clinic.

The envelope protein (Env) mediates the entry of the viral particle into its target. HIV-1 Env specifically recognizes CD4, a molecule present on the surface of helper T cells, macrophages, and some glial cells. Fortunately, as with all retroviruses, the HIV-1 Env can be substituted by the corresponding protein of another virus. This process, which alters the tropism of the virion, is called pseudotyping. More selective tropisms were achieved by taking advantage of the natural tropisms of glycoproteins from other membrane-enveloped viruses. Although many different pseudotyped vectors have been generated using glycoproteins from other membrane-enveloped viruses, the G protein of vesicular stomatitis virus glycoprotein (VSV-G) is the most widely used envelope to pseudotype lentivectors for three reasons. First, VSV-G provides

the highest vector titers. Second, it provides the highest vector stability, allowing for the concentration of vector particles by ultracentrifugation. And third, with its phospholipid receptor being ubiquitously expressed in mammalian cells, lentivectors pseudotyped with VSV-G can be used to transduce virtually all cell types. This technique is thus widely and routinely used in basic research as well as in clinical research and this chapter focuses on production of the VSV-G-pseudotyped vectors.

HIV is a human pathogen. However, its pathogenic potential stems from the presence of nine genes that all encode for important virulence factors. Fortunately, six of these genes (namely Env, Vif, Vpr, Vpu, Nef, and Tat, see Fig. 1) can be deleted from the HIV-derived vector system without altering its gene-transfer ability. The resulting multiply-attenuated design of HIV vectors ensures that the parental virus cannot be reconstituted. In addition, other improvements have been brought to achieve high levels of efficiency and biosafety.

When producing vector stocks, it is mandatory to avoid the emergence of replication-competent recombinants (RCRs). In the retroviral genome, a single RNA molecule that also contains critical *cis*-acting elements carries all the coding sequences. Biosafety of a vector production system is therefore best achieved by distributing the sequences encoding its various components over as many independent units as possible to maximize the number of recombination events that would be required to recreate a replication-competent virus. In the lentiviral vector systems described here, vector particles are generated from three or four separate plasmids, each providing either the genomic RNA containing the transgene and all the *cis*-acting sequences, or the internal structural and enzymatic proteins necessary for adequate transcription, packaging, reverse transcription and integration, or the envelope glycoprotein necessary for vector entry into the target cell. This ensures that only replication-defective viruses are produced, because the plasmids would have to undergo multiple and complex recombination events to regenerate a replication-competent entity. A diagram of the evolution of HIV-1-based systems is depicted in Fig. 1.

The first generation of lentiviral vectors was manufactured using a packaging system that comprised all HIV genes but the envelope (35). In a so-called second generation system, five of the nine HIV-1 genes were eliminated, leaving the gag and pol reading frames, which encode for the structural and enzymatic components of the virion, respectively, and the tat and rev genes, fulfilling transcriptional and posttranscriptional functions (36). Sensitive tests have so far failed to detect RCRs when this system is used. This good safety record, combined with its high efficiency and ease of use, explains why the second generation lentiviral vector packaging system is utilized for most experimental purposes. In a third generation system, geared up towards clinical applications, only gag, pol, and rev genes

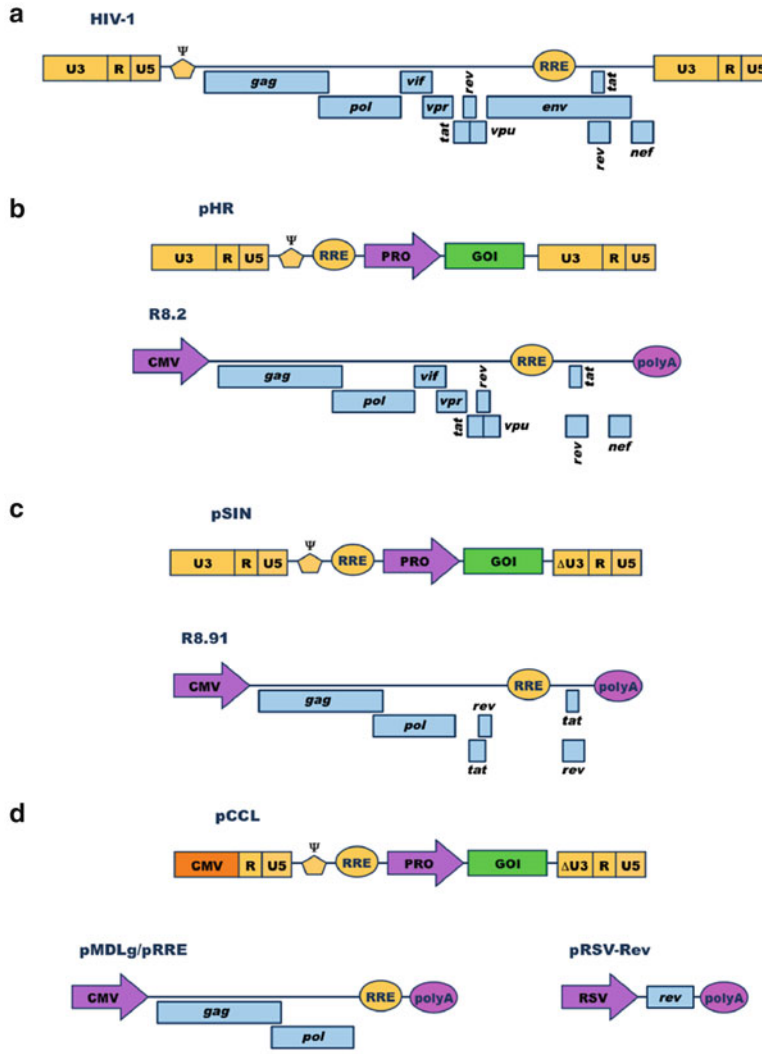

Fig. 1. Evolution in the design of HIV-1-based lentivirus vectors (LVs). HIV-1-based LVs are derived from wild-type HIV-1 (**a**) by dissociation of the *trans*-acting components (*blue boxes*) coding for structural and accessory proteins (gag, pol, env, tat, rev, vif, vpr, vpu, nef) and the *cis*-acting sequences required for packaging and reverse transcription of the genomic RNA (LTR U3-R-U5, psi, RRE) (*yellow boxes*). (**b**) First generation system. The pHR vector genome has intact 5′ LTR and 3′ LTR. The R8.2 packaging plasmid expresses all HIV-1 proteins except Env. (**c**) Second generation system. The pSIN vector genome has a self-inactivating (SIN) deletion in the U3 sequence of the 3′ LTR. The R8.91 packaging plasmid expresses only the structural and regulatory proteins of HIV-1. (**d**) Third generation system. The pCCL vector genome has a chimeric 5′ LTR that is independent of the Tat protein. The packaging system is composed of two plasmids, pMDLg/pRRE coding of the structural proteins of HIV-1 and pRSV-Rev providing the Rev protein. Note that all vector systems need the presence of complementary plasmid providing the env gene. *LTR* long-terminal repeat, *CMV* human cytomegalovirus immediate-early promoter, *RRE* rev-responsive element, *RSV* Rous sarcoma virus promoter, *polyA* polyadenylation site, *U3-R-U5* HIV-1 LTR, *psi* HIV-1 packaging signal, *PRO* virus promoter of the internal expression cassette, *GOI* transgene of interest, *deltaU3* self-inactivating deletion of the U3 part of the HIV-1 LTR.

are still present, using a chimeric 5′ LTR (long-terminal repeat) to ensure transcription in the absence of Tat.

The genetic information contained in the vector genome is the only one transferred to the target cells. Early genomic vectors were composed of the following components. The 5′ LTR, the major splice donor, the packaging signal (encompassing the 5′ part of the gag gene), the Rev-responsive element (RRE), the envelope splice acceptor, the internal expression cassette containing the transgene, and the 3′ LTR. In the latest generations, several improvements have been introduced. The Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) has been added to increase the overall levels of transcripts both in producer and target cells, hence increasing titers and transgene expression (37). The central polypurine tract of HIV has also been added back in the central portion of the genome of the transgene RNA (38, 39). This increases titers at least in some targets. The U3 region of the 3′ LTR is essential for the replication of a wild-type retrovirus, since it contains the viral promoter in its RNA genome. It is dispensable for a replication-defective vector and has been deleted to remove all transcriptionally active sequences, creating the so-called self-inactivating (SIN) LTR (40). SIN vectors are thus unable to reconstitute their promoter and are safer than their counterparts with full-length LTRs. Finally, chimeric 5′ LTRs have been constructed, in order to render the lentiviral promoter Tat-independent. This has been achieved by replacing the U3 region of the 5′ LTR with either the CMV enhancer (CCL LTR) or the corresponding Rous sarcoma virus (RSV) U3 sequence (RRL LTR) (41). Vectors containing such promoters can be produced at high titers in the absence of the Tat HIV transactivator. However, the Rev-dependence of these third generation lentiviral vectors (lentivectors, LVs) has been maintained, in order to maximize the number of recombination events that would be necessary to generate an RCR. This latest generation represents the system of choice for future therapeutic projects. In the laboratory and in vitro genetic engineering, however, this third generation is not mandatory, and the second generation system offers a high level of safety for BSL-2 conditions. For most research applications, it is thus easier to use only three plasmids, i.e. an envelope plasmid, a second generation plasmid providing Gag, Pol, Tat, and Rev proteins, and any vector genome plasmid (second generation with native 5′ LTR or third generation with chimeric 5′ LTR) since the presence of Tat is required for optimal activity of the native LTR and does not affect the activity of the chimeric LTRs. Thus, for in vitro and vivo research, we advise to use an all-purpose packaging plasmid, such as the psPAX2 which encodes for the HIV-1 Gag, Gag/Pol, Tat, and Rev proteins.

The vector plasmid represented in Fig. 2 carries a LoxP recombination site. This LoxP sequence is duplicated during reverse

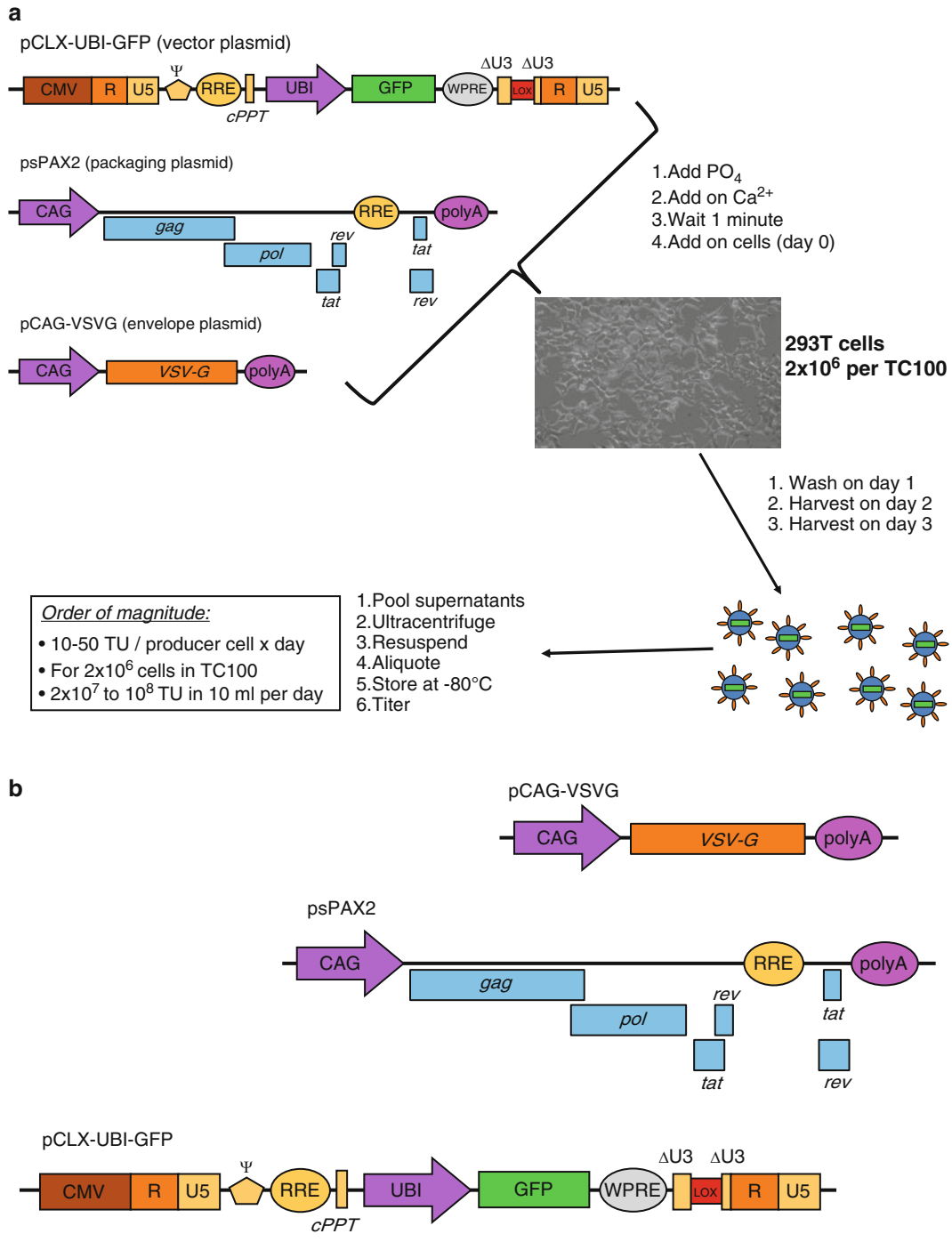

Fig. 2. Schematic diagram of lentivector production. This diagram summarizes the steps described in Subheading 3 of this chapter, to produce LVs. (**a**) The three plasmids encoding for the vector genome, the structural and accessory genes, and the envelope are mixed and introduced by transfection into 293T cells. The vectors particles are then harvested, concentrated, and titered as described in Subheading 3. The expected quantities of vectors are described in Subheading 3.8. (**b**) The pCAG-VSVG plasmid (courtesy of A. Nienhuis, (45)), providing the envelope of the LV particles is composed of the CAG compound promoter, the coding sequence of the Vesicular Stomatitis Virus Envelope protein (VSV-G), and the polyadenylation signal from the rabbit beta-globin gene. The second-generation psPAX2 packaging plasmid (P. Salmon, unpublished),

transcription and allows the integrated proviral cassette to be excised upon expression of the Cre bacterial recombinase (24). Note that, although LVs can theoretically accommodate up to 9 kb of transgenic sequence, some inserts can induce a rapid and important titer drop. This is the case, for example, for the CAG promoter in our hands. Also, the UBI promoter can be replaced by other ubiquitously active promoters, such as EF1 or EFs, or tissue-specific promoters.

Detailed information (such as maps, sequences, etc.) are available at our institutional website (http://medweb2.unige.ch/salmon/lentilab).

1.3. Current Applications and Developments

In a little more than a decade, it has become obvious that "historical" cell lines derived from tumors must be confined to "basic cell culture tasks" and that primary cells are essential to address biologically relevant questions. This is why more and more companies now propose human primary cells of various tissue origins. However, the tissue sources are limited, the life span is finite and the price can be an issue especially when up-scaling is required. Also, it is still difficult for some cell types to standardize all parameters from one preparation to another.

As a consequence, immortalization is becoming a viable alternative to provide researchers with unlimited, reproducible, and standardized human cell sources. As described above, the use of "strong oncogenes" such as SV40 large T, E6, or E7 is limited since they induce transformation and genomic instability. The final in vitro outcome is most often similar to in vivo tumorigenesis, with cells accumulating unknown molecular events, resulting in unstable and dedifferentiated cells. On the contrary, the use of "mild immortaligenes" such as Bmi-1 and telomerase, seems to preserve original phenotypes and genomic stability. Although other genes has been described and are being tested for a similar mild immortalization, such as CBX7 (42), CDK4 (43), CDK6, CyclinD1, or members of the E2F family, the Bmi-1/telomerase combination currently offers the best compromise. If the target primary cells grow well in culture, telomerase alone can suffice by providing infinite lifespan. Considering the efficiency, safety and versatility of lentivectors on one hand, and the growing list of immortalized human cell lines created using the Bmi-1/telomerase gene combination on the other hand, we propose this "molecular

Fig. 2. (continued) providing the structural and enzymatic proteins of the LV particle is composed of the CAG compound promoter, the gag, pol, tat, and rev genes, the Rev-responsive element of HIV-1 (RRE), and the polyadenylation signal from the rabbit beta-globin gene. The third-generation pCLX-UBI-GFP vector plasmid, providing the genome of the LV particles is depicted here as an example of a classical LV design. The 5′ LTR is composed of the CMV promoter and the R and U5 regions of HIV-1. This renders it tat-independent. *psi* HIV-1 packaging signal, *RRE,* rev-responsive element, *cPPT* central polypurine tract, *UBI* ubiquitin promoter, *GFP* green fluorescent protein, *WPRE* Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element, Δ*U3* self-inactivating deletion of the U3 part of the HIV-1 LTR, *lox* Cre recombinase LoxP target sequence.

toolbox" as a start point for the immortalization of human primary cells, as well as a means for further genetic tailoring of such cells, by introduction of additional genes (30).

In this chapter, we thus describe the two lentivectors composing our "star cocktail," i.e. pLOX-CW-Bmi1 and pLOX-hTERT-iresTK, together with standard GFP vectors. Once integrated in the genome of the target cells, these lentivectors can be excised using the Cre bacterial recombinase. This can be required if removal of Bmi-1 is needed for terminal differentiation. Finally, pLOX-hTERT-iresTK encodes for the thymidine kinase of HSV-1 and thus provides a safety feature consisting in the conditional ablation of unexcised growing cells using Acyclovir or its analogs (24).

1.4. Safety Issues

The system presented here contains numerous safeguards as compared to the first-generation HIV vectors, in which genes encoding all HIV-1 proteins, except for Env, were present. A second generation was characterized by the exclusion of four accessory genes (vif, vpr, vpu, and nef). These deletions improved considerably the safety of the vector because they excluded major determinants of HIV-1 virulence. In the third-generation system, described in this unit, Gag, Pol, and Rev are the only HIV-1 proteins still present. Vectors with self-inactivating (SIN) LTR and produced with the third generation packaging system have been tested for RCR. Thus far, no RCR have been detected amongst a total of 1.4×10^{10} transducing units (44).

Since VSV-G-pseudotyped LVs have a broad tropism, both in vitro and in vivo, biosafety precautions need to take into account the nature of the transgene. LVs containing non-hazardous genes (such as GFP) should be handled in a biosafety level (BSL) laboratory as prescribed by the appropriate Institutional Biosafety Committee. We handle LVs containing non-hazardous genes (such as GFP) in a BSL-2 laboratory. We handle LVs containing genes that are themselves potentially biohazardous (such as Bmi1, hTERT, or other genes involved in cell proliferation described in this chapter) in a BSL-3 laboratory. Details on BSL-2 and BSL-3 laboratory, standard equipment and P2 safety procedures can be found at our institutional website (http://medweb2.unige.ch/salmon/lentilab).

In general, transduced cells can be fixed (using formaldehyde or paraformaldehyde as described in Subheading 3) before being taken out of the BSL-2 or BSL-3 laboratory. If a live-sorting of transduced cells is needed outside of the BSL-2 laboratory, a careful handling and decontamination of the equipment used should be performed afterwards. However, once they have cleared the RCR assay (see Subheading 3.5.4), LV-transduced cells, including LV-immortalized cells can be considered as any other human cell line.

2. Materials

All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly. All maps and sequences of plasmids described here are available at our institutional website (http://medweb2.unige.ch/salmon/lentilab). Common plasmids for the generation of HIV-1-based lentivectors can be obtained from www.Addgene.org. Use ultrapure or double-distilled water in all recipes.

2.1. HIV-1-Based Lentiviral Vectors

1. pCLX-CW-GFP (CMV promoter, GFP gene, excisable).
2. pCLX-UBI-GFP (Ubiquitin promoter, GFP gene, excisable).
3. pLOX-CW-Bmi1 (CMV promoter, mBmi-1 gene, excisable).
4. pLOX-TERT-iresTK (CMV promoter, hTERT gene, HSV-1 thymidine kinase gene, excisable).
5. pLOX-Cre (CMV promoter, Cre recombinase gene, excisable).

2.2. Production of HIV-1 Based Lentiviral Vectors by Transient Transfection of 293T Cells

1. Producer cells HEK293T/17 cells (ATCC, see Note 1).
2. Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, glutamine, pyruvate, antibiotics, and 10% FBS (abbreviated D10).
3. Serum-free Advanced DMEM (Life Technologies™) supplemented with 2 mM glutamine.
4. TE buffer: Tris–HCl 10 mM–EDTA 1 mM, pH 8.0 used to redissolve all plasmids.
5. Envelope plasmid pCAG-VSVG: dissolved at 1 μg/μL in TE buffer.
6. Packaging plasmid psPAX2 (encoding HIV-1 Gag, Pol, Tat, and Rev proteins): dissolved at 1 μg/μL in TE buffer.
7. Vector plasmid (such as pCLX-CW-GFP): dissolved at 1 μg/μL in TE buffer.
8. $CaCl_2$ Solution: 0.5 M Dissolve $CaCl_2$ (Sigma-Aldrich®, see Note 2) into 500 mL of H_2O. Filter sterilize through a 0.22-μm nitrocellulose filter. Store at −70°C in 50 mL aliquots. Once thawed, the $CaCl_2$ solution can be kept at +4°C for several weeks without observing significant change in the transfection efficiency.
9. 2× HeBS (HEPES-buffered saline): Dissolve NaCl (0.28 M final), HEPES (0.05 M final), and Na_2HPO_4, (1.5 mM final) into 800 mL of H_2O. Adjust pH to 7.0 with 10 M NaOH (see Notes 3 and 4). Add H_2O to 1,000 mL and make the final pH adjustment. Filter sterilize through a 0.22-μm nitrocellulose filter. Store at −70°C in 50 mL aliquots. Once thawed, the

2× HeBS solution can be kept at +4°C for several weeks without observing significant change in the transfection efficiency.

10. Ethanol 75% in a spray bottle.
11. PBS, pH 7.4.
12. PBS–$Ca^{2+}Mg^{2+}$, pH 7.4.
13. Sucrose 20%: Dissolve 20 g of sucrose (see Note 5) in 100 mL of PBS–$Ca^{2+}Mg^{2+}$. Filter sterilize through a 0.22-µm nitrocellulose filter. Store at +4°C.
14. Trypsin 0.25%/EDTA (Life Technologies™).
15. Bleach solution 13–14% (w/v).
16. 10-cm Tissue culture dishes.
17. 37°C Humidified incubators, 5% CO_2.
18. 1.5-mL Microcentrifuge tubes, sterile, disposable.
19. 15- and 50-mL Conical centrifuge tubes, sterile.
20. 50 mL Syringes and 0.45-µm pore size PVDF filters.
21. 30-mL Beckman Konical tubes for ultracentrifuge.
22. Ultracentrifuge (such as Beckman Optima™ L-90 K) with SW28 rotor.

2.3. Titration of Lentivectors

2.3.1. Titration by FACS

1. Target cells HT-1080 cells (ATCC).
2. D10 medium (see Subheading 2.2, item 2).
3. Trypsin/EDTA (see Subheading 2.2, item 14).
4. MW6 tissue culture plates.
5. PBS.
6. Formaldehyde 1% (w/v) in PBS: Mix 1 mL of formaldehyde (37% w/v) in 36 mL of PBS. Store at +4°C.
7. Fluorescence-activated cell sorter (FACS; Becton Dickinson with 488 nm excitation laser and green filter) and appropriate tubes.

2.3.2. Titration by qPCR

1. Target cells HT-1080 cells (ATCC).
2. D10 medium (see Subheading 2.2, item 2).
3. Trypsin/EDTA (see Subheading 2.2, item 14).
4. MW6 tissue culture plates.
5. PBS.
6. Real-time PCR machine (ABI PRISM® 7900HT Real Time PCR System, Applied Biosystems or equivalent, with a dedicated analysis program, SDS2.2.2, Applied Biosystems or equivalent).
7. Genomic DNA extraction kit (DNeasy Blood & Tissue Kit, Qiagen).
8. qPCR Master Mix (Eurogentec).

9. 96-well Optical Reaction plate (Applied Biosystems).
10. Optical caps (Applied Biosystems).
11. Filter tips (1,000, 100, 10 μL).
12. Primers and probe for quantification of human genomic sequences (10× HB2 set, see Subheading 2.6.1 and Notes 6–8).
13. Primers and probe for quantification of HIV sequences (10× GAG set) (see Subheading 2.6.2 and Notes 6–8).

2.4. Immortalization of Human Primary Cells

IMPORTANT: Immortalization of human primary cells using lentivectors pseusotyped with the VSV-G envelope and containing Bmi-1 or telomerase, as well as production, concentration, and titration of immortalizing lentivectors must be performed as per local biosafety requirements. We use BSL-3 (see Note 9).

We downgrade LV-immortalized cells (i.e., BSL3 to BSL-2 conditions) only after they have cleared the RCR assay (see Subheading 3.5.4 and Note 10).

1. Polystyrene 24 well-multidish.
2. Culture medium appropriate for the primary cells to immortalize.
3. Aerosol-barrier filter tips (1,000, 100, 10 μL).

2.5. Genetic Analysis of Lentimmortalized Cells

2.5.1. PCR Detection of Immortalizing Lentivectors

1. Standard molecular biology equipment (see Notes 11 and 12).
2. Genomic DNA extraction kit (DNeasy Blood & Tissue Kit, Qiagen).
3. REDTaq® ReadyMix™ PCR Reaction Mix (Sigma-Aldrich®).
4. 0.2 mL PCR tubes.
5. Filter tips (1,000, 100, 10 μL).
6. Primers for amplification of pLOX-CW-Bmi1 and pLOX-TERT-iresTK sequences (see Subheading 2.6 and Notes 6–8).

2.5.2. Quantification of Lentivector Copy Numbers by qPCR

1. Real-time PCR machine (ABI PRISM® 7900HT Real Time PCR System, Applied Biosystems or equivalent, with a dedicated analysis program, SDS2.2.2, Applied Biosystems or equivalent).
2. Genomic DNA extraction kit (DNeasy Blood & Tissue Kit, Qiagen).
3. qPCR Master Mix (Eurogentec).
4. 96-well Optical Reaction plate (Applied Biosystems).
5. Optical caps (Applied Biosystems).
6. Filter tips (1,000, 100, 10 μL).
7. Primers and probe for quantification of HIV sequences (10× GAG set, see Subheading 2.6 and Notes 6–8).
8. Primers and probe for quantification of human genomic sequences (10× HB2 set, see Subheading 2.6 and Notes 6–8).

2.5.3. Replication-Competent Recombinant Assay

1. All materials described in Subheading 2.5.2.
2. Genomic DNA from full HIV-1 genome-containing cells, such as 8E5 cells (ATCC) or full-length HIV-1-containing plasmid (see Notes 13 and 14).
3. Primers and probe for quantification of HIV packaging sequences (10× PRO set, see Subheading 2.6 and Notes 6–8).

2.6. Oligos

Oligos can be ordered on-line from several companies such as Eurogentec or Sigma-Aldrich®. FAM fluorescent dye can be replaced by other equivalent molecule, and TAMRA can be replaced by other quenchers.

2.6.1. Human Beta-Actin Taqman® Probe and Primers

These oligos are used to normalize for the amount of genomic DNA and are specific for the human beta-actin gene.

1. HB2-P (probe, sense) 5′-(FAM)-CCTGGCCTCGCTGTCCACCTTCCA-(TAMRA)-3′.
2. HB2-F (forward primer) 5′-TCCGTGTGGATCGGCGGCTCCA-3′.
3. HB2-R (reverse primer) 5′-CTGCTTGCTGATCCACATCTG-3′.

2.6.2. GAG Taqman® Probe and Primers

These oligos are used for amplification of HIV-1-derived vector sequences and are specific for the 5′ end of the gag gene (GAG). This sequence is present in all HIV-1 vectors for it is part of the extended packaging signal.

1. GAG-P (probe, antisense) 5′-(FAM)-ACAGCCTTCTGATGTTTCTAACAGGCCAGG-(TAMRA)-3′.
2. GAG-F (forward primer) 5′-GGAGCTAGAACGATTCGCAGTTA-3′.
3. GAG-R (reverse primer) 5′-GGTTGTAGCTGTCCCAGTATTTGTC-3′.

2.6.3. PRO Taqman® Probe and Primers

These oligos are used for amplification of sequences present in RCRs and are specific for the region of the pol gene coding for the HIV-1 protease (PRO).

1. PRO-P (probe, sense) 5′-(FAM)-ACAATGGCAGCAATTTCACCAGT-(TAMRA)-3′.
2. PRO-F (forward primer) 5′-AGCAGGAAGATGGCCAGTAA-3′.
3. PRO-R (reverse primer) 5′-AACAGGCGGCCTTAACTGTA-3′.

2.6.4. CMV-Bmi Primers

These primers are for PCR detection of the pLOX-CW-Bmi1 integrated cassette in the genomic DNA of transduced cells. To avoid amplification from the endogenous Bmi-1 gene, these primers are designed to amplify a sequence comprised between the CMV

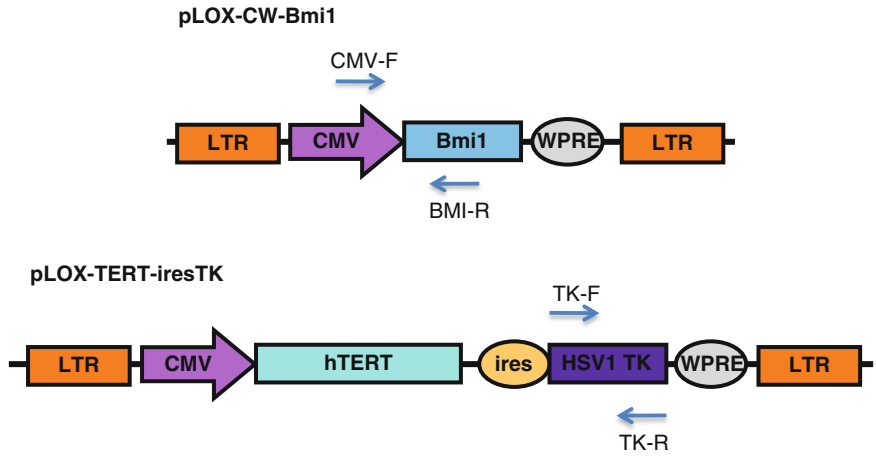

Fig. 3. Schematic diagram of PCR detection of immortalizing lentivectors. Schematic diagrams of pLOX-CW-Bmi1 and pLOX-TERT-iresTK are shown. *Arrows* indicate orientations and approximate locations of binding sites of primers described in Subheading 2.6.

promoter and the Bmi-1 gene. The corresponding amplified region is depicted in Fig. 3.

1. CMV-F (forward primer) 5′-GCAGAGCTCGTTTAG TGAACCGTC-3′.
2. BMI-R (reverse primer) 5′-CCAGACCACTCCTGAACA TAAGGTC-3′.
3. The size of the amplicon is 350 bp.

2.6.5. HSV1 TK Primers

These primers are for PCR detection of pLOX-TERT-iresTK integrated cassette in the genomic DNA of transduced cells. We could not generate PCR amplicons from CMV and hTERT sequences. Hence, detection of pLOX-TERT-iresTK is performed by specific amplification of the HSV1 thymidine kianse. The corresponding amplified region is depicted in Fig. 3.

1. TK-F (forward primer) 5′-GATGACTTACTGGCGGGTGT-3′.
2. TK-R (reverse primer) 5′-GGCCCGAAACAGGGTAAATA-3′.
3. The size of the amplicon is 620 bp.

3. Methods

3.1. Production of Lentiviral Vectors

IMPORTANT: Production, concentration, and titration of immortalizing lentivectors, must be performed as per local biosafety requirements. We use BSL-3 (see Notes 9 and 15–19).

1. Maintain 293T cells in D10 medium (see Subheading 2.2, item 2) in 10 cm tissue culture dish in a 37°C humidified incubator

with a 5% CO_2 atmosphere, and split them at ratio 1:10 using Trypsin/EDTA, three times per week (e.g., every Monday, Wednesday, and Friday).

2. The day before the transfection, seed 1–10 dishes at 2.5 million cells per dish. Cells must be approximately 1/2 to 2/3 confluent on the day of transfection. Incubate overnight in a 37°C humidified incubator with a 5% CO_2 atmosphere. On the following day, cotransfect the cells according to the following recipes.
3. For one plate of 10 cm, mix in a sterile 1.5 mL microcentrifuge tube.

Envelope plasmid	pCAG-VSVG	4 μg
Packaging plasmid	psPAX2	8 μg
Vector plasmid	pCLX-CW-GFP	8 μg

4. The vector plasmid (pCLX-CW-GFP given as example above) can be second or third generation since the psPAX2 plasmid provides Tat protein.
5. Adjust to 250 μL with sterile buffered water and mix well by pipetting.
6. Add 500 μL of 2× HeBS and mix well by pipetting.
7. Put 250 μL of 0.5 M $CaCl_2$ in a 15-mL sterile conical tube.
8. To each 15 mL tube containing the $CaCl_2$ solution, slowly transfer, dropwise, the 750 μL of DNA/HeBS mixture, while vigorously vortexing. Vigorous vortexing will ensure the formation of a fine precipitate that can be taken up efficiently by cells.
9. Leave the precipitates (1 mL final volume per tube) at room temperature for 5–30 min.
10. Add the 1 mL of precipitate dropwise to the cells in 10 mL of medium in one culture dish prepared as above. Mix by gentle swirling until the medium has recovered a uniformly red color.
11. Place the dish overnight in a 37°C humidified incubator with a 5% CO_2 atmosphere.
12. Early the next morning, aspirate the medium, wash with 10 mL of pre-warmed PBS and gently add 15 mL of fresh Advanced DMEM (see Subheading 2.2, item 3) pre-warmed to 37°C. Incubate for 24 h. If 293T cells adhere poorly, washing with PBS can be omitted.
13. Transfer the supernatant from each plate to one 50 mL centrifuge tube. Close the tubes, and spray them with 70% ethanol before taking them out of the hood. Store the supernatant at +4°C. Add another 15 mL of fresh Advanced DMEM, prewarmed to 37°C. Incubate for another 24 h with the cell monolayer.

14. Pool the supernatants of day 1 and 2 and centrifuge for 5 min at 500 × *g*, at 4°C, to pellet detached cells and debris.
15. Filter the 30 mL of pooled supernatant (total harvest from 2 days: 30 mL/dish) with a 50 mL syringe connected to a 0.45 μm PVDF disk filter.

The LV stocks can be stored at +4°C for 1–4 days without significant titer loss, before they are used for transduction of target cells or further processing such as concentration. For longer storage, LV stocks must be kept at −80°C.

The transfection can be started late in the afternoon and the medium changed early the next morning. If you notice cell toxicity, you can transfect early in the morning and change the medium late in the afternoon the same day. The transfection procedure can be scaled up to ten culture dishes of 10 cm, or other cell culture systems with equivalent or larger surface.

3.2. Concentration of Lentiviral Vectors

1. For concentration, use 30 mL Beckman conical tubes, in a SW28 rotor in an ultracentrifuge. Put 4 mL of 20% sucrose on the bottom of the tube. Very slowly pour the supernatant on the surface of the sucrose cushion until the tube is full (allow a 2 mm dry zone to the top of the tube). Spin at 50,000 × *g* for 120 min at +16°C.
2. Remove conical tube from SW28 bucket (see Note 20).
3. Aspirate the medium with a sterile pipette down to the sucrose interface.
4. Aspirate the sucrose until you have 1–2 mL of colorless sucrose solution and then invert the tube while aspirating the remaining sucrose. Never touch the bottom of the tube where the vector pellet is.
5. Place the conical tube in a 50 mL Falcon tube and quickly add 30–100 μL of PBS–$Ca^{2+}Mg^{2+}$ on the pellet (not always visible). Do not leave the pellet dry for more than 5 min or it may result in significant titer decrease. Close the Falcon tube. You can resuspend the vector pellet of one tube in a minimal volume of 30 μL. In this case, you will achieve a ~1,000-fold concentration.
6. Vortex at half-speed for 2 s.
7. Leave the vector pellet to resuspend for 1–2 h at room temperature or 2–4 h at +4°C.
8. Vortex at half-speed for 2 s,
9. Pipet up and down 20 times and freeze at −80°C in aliquots for long time storage (see Notes 21 and 22).

3.3. Titration of Lentiviral Vectors

Titers of viruses in general and lentivectors in particular critically depend on the methods and cells used for titration. The quantification of vector particles capable of achieving every step from cell binding to expression of the transgene depends on both vector and cell

characteristics. First, the cell used as target must be readily permissive to all steps from viral entry to integration of the vector genetic cargo. Second, the expression of the foreign gene must be easily monitored and rapidly reach levels sufficient for reliable quantification. Early vectors had the lacZ bacterial gene as reporter, under the control of the CMV promoter. Current vectors now have the green fluorescent protein (GFP) gene as a reporter, under the control of promoters that are active in most primary cells.

Measured titers can also vary with the conditions used for titration, i.e., volume of sample during vector-cell incubation, time of vector-cell incubation, number of cells used, etc. For several years now, numerous laboratories have been using HeLa cells as target cells for LVs. Although these cells are easy to grow and 100% susceptible to transduction by VSV-G-pseudotyped LVs, they are very unstable in terms of morphology and karyotype. For this reason, we are now using HT-1080 cells, which are stable, of human origin and give titers identical to HeLa cells.

Physical titration based on the quantification of HIV-1 capsid p24 antigen is not used anymore in our lab. Instead, our current standard procedure relies on determination of infectious titers by transduction of HT-1080 target cells. Also, we always produce a test batch of a standard GFP lentivector alongside all LV production. This test batch is used to monitor the overall efficiency of the procedure and detects any anomaly in producer cells or reagents that will result in titer drop.

Here we described a procedure that is used on a weekly basis in our lab for several years and that has been standardized in order to compare titers from one batch to another one or from one lab to another one. Changes in this procedure can be made, but one must keep in mind that, for example, reducing the cell culture surface or increasing the number of target cells will result in an increase of the final calculated titer, from the exact same vector batch.

3.3.1. General Procedure

1. On day 0, seed HT-1080 cells at 50,000 cells per well in MW6 plate in D10. Make sure that HT-1080 cells are well separated and uniformly distributed in the well.
2. On day 1, put into three independent wells 500, 50, or 5 μL of the vector suspension (either pure from unconcentrated supernatants or diluted in complete medium if it comes from a concentrated stock, i.e. 1/100 if the vector is concentrated 100-fold).
3. Polybrene can be omitted for transduction with VSV-G pseudotyped vectors since this compound does not influence permissivity of cells to VSV-G pseudotyped vectors.
4. On day 2, remove the supernatant and replace by 2 mL of fresh D10.
5. On day 5, wash the cells with 2 mL of PBS; detach them with 250 μL of Trypsin/EDTA for 1 min at 37°C.

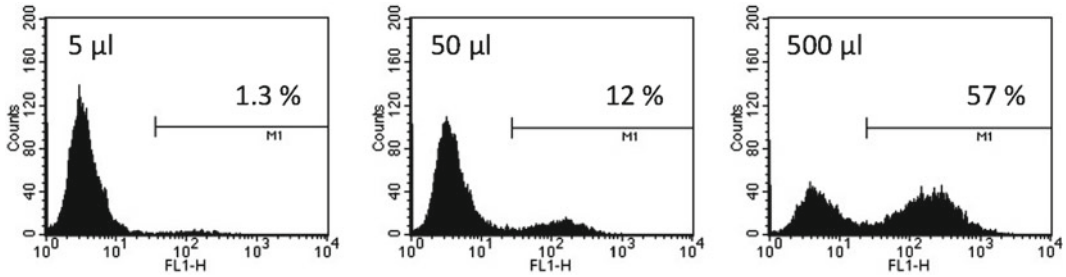

Fig. 4. A representative FACS analysis of HT-1080 cells used for titration of GFP-coding LV. HT-1080 cells (10^5) were incubated with increasing volumes of a supernatant containing an LV expressing GFP under the control of the human Ubiquitin promoter (pCLX-UBI-GFP) as described in Subheading 2.1. After 5 days, cells were detached, fixed, and analyzed by FACS for GFP fluorescence (*x* axis, 4-decade log scale, FL1) versus number of cells (*y* axis, linear scale). The percentage of GFP-expressing cells was measured by placing a marker discriminating between GFP-negative (mean of fluorescence intensity 3–4) and GFP-positive cells (mean of fluorescence intensity 200).

6. Add 250 µL of D10 and mix well to resuspend the cells. This step inactivates the trypsin and EDTA.
7. Spin cells in a microcentrifuge for 2 min at 200×*g*. Note that if you need to run an FACS analysis and a qPCR analysis on the same sample, you must split your cells in two separate microcentrifuge tubes.

3.3.2. Titration of Lentivectors by FACS

This method can only be used to titer stocks of vectors that carry a transgene that is easily monitored by FACS (such as GFP, or any living colors, or any membrane protein that can be detected by flow cytometry), and whose expression is governed by a promoter that is active in HT-1080 cells (tissue-specific promoter-containing vector must be functionally assayed in specific cells, and titered by qPCR in HT-1080 cells (see Subheading 3.3.3).

We describe here the titration of an Ubiquitin promoter-GFP vector (pCLX-UBI-GFP, see Subheading 2.1).

1. Add 500 µL of 1% formaldehyde in PBS (see Subheading 2.3.1, item 6) to the cell pellet. This step will fix the cells and inactivate the vector particles. Samples can thus be taken out of the BSL-2 laboratory.
2. Resuspend the cells thoroughly in the well and transfer them to an FACS tube.
3. Analyze the cells in a flow cytometer. If you are not familiar with flow cytometry, you must seek help from your institutional FACS specialist.
4. Once chosen the appropriate dilution (see Fig. 4 and Notes 23–25), apply the following formula: Titer (HT-1080-transducing units/mL) = 100,000 (target HT-1080 cells) × (% of GFP-positive cells/100)/volume of supernatant (in mL).

3.3.3. Titration of Lentivectors by Quantitative PCR (qPCR)

When lentivectors contain DNAs coding for genes other than GFP or LacZ and promoter which are active only in specific primary cells and tissue, FACS titration cannot be used. Therefore, most new LVs will need an alternative method to measure the number of copies of LV stably integrated in HT-1080 target cells, after transduction as described in Subheading 3.3.2 for GFP vectors. This assay, however, only measures the number of LV copies integrated in the target cell genome. The overall functionality of the vector must be tested at least once in cells in which the promoter is active and/or with appropriate techniques to detect the expression of the transgene product. The qPCR assay proceeds as follows, using a real-time PCR machine. HT-1080 cells are transduced as for FACS analysis. Then, one half can be used if FACS analysis is performed in parallel, or target cells can be lysed directly in the plate and the DNA is extracted using a genomic DNA extraction kit and following general procedures for qPCR analysis (see Notes 26–29). Then, a fraction of the total DNA is analyzed for copy number of HIV sequences using the following real-time PCR protocol.

1. Extract target cell DNA from each individual well of an MW6 plate (see Subheading 3.3.1) using the genomic DNA extraction kit, following manufacturer recommendations. For the DNA elution step, use 100 µL of AE buffer instead of 200 µL.
2. Perform qPCR or store DNA at –20°C until use.
3. Prepare a mix containing everything but the sample DNA for the number of wells needed for the qPCR analysis, including all samples and standards in duplicates or triplicates, according to the following recipe (for one well):

qPCR Master Mix	7.5 µL
10× oligo mix (GAG or HB2, see Subheading 2.6 and Notes 6–8)	1.5 µL
DNA sample	1 µL
H_2O	5 µL

4. Distribute 14 µL of this mix into the wells of a 96-well Optical Reaction plate.
5. Add sample DNAs.
6. Close with optical caps.
7. Centrifuge the plate at 200 × *g* for 1 min to bring all liquid on the bottom of the wells.
8. Place the 96-well Optical Reaction plate in the real-time PCR machine and run the appropriate program depending on the fluorochromes and quenchers used in your Taqman probes (see Note 30).

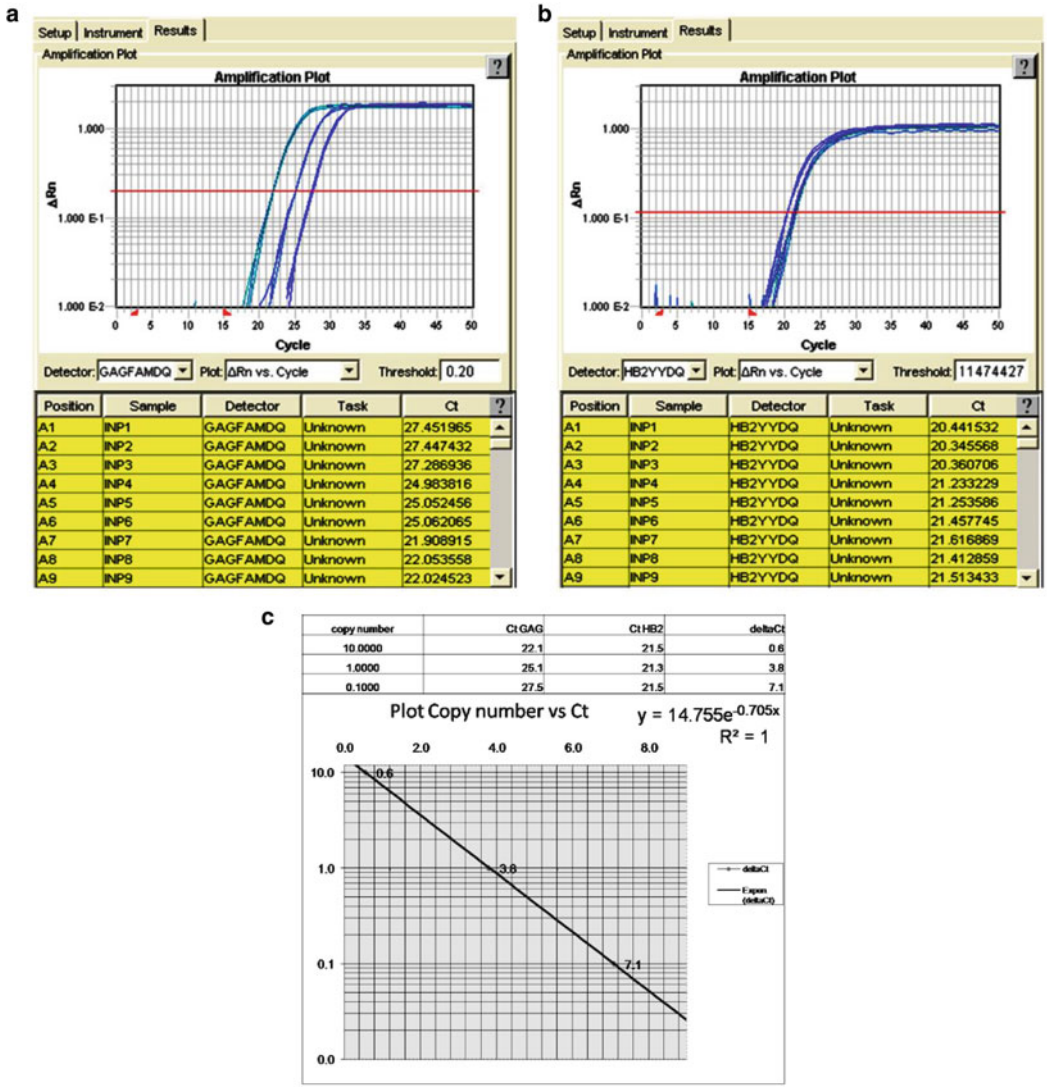

Fig. 5. A representative qPCR analysis used for titration of HIV-1-based LVs. DNA from HT-1080 cells transduced with serial tenfold dilutions of pCLX-UBI-GFP vectors was subjected simultaneously to qPCR titration analysis and FACS analysis as described in Subheading 3. A sample of each dilution was submitted to qPCR amplification and monitoring using an ABI PRISM® 7900HT Real Time PCR System (Applied Biosystems), and sets of primers and probes specific for HIV gag sequences (GAG-FAM, (**a**) or beta-actin sequences (HB2-YY, (**b**) Amplification plots were displayed and cycle threshold values (Ct) were set as described in Subheading 3.3.3. Values of GAG Ct and HB2 Ct were exported in an Excel worksheet to calculate ΔCt values (*x* axis, linear scale) and plot them against copy number values (*y* axis, log scale) (**c**) A sample giving 10% of GFP-positive cells was set as cells containing 0.1 copy of HIV sequences per cell. The regression curve can then be used to calculate GAG copy numbers (*Y* value) of unknown samples by applying the formula to ΔCt values (*X* values) of the sample.

9. Analyze results and calculate titer using the SDS 2.2.2 program. An example of amplification profiles of HIV sequences in human DNA is given in Fig. 5.
10. Set the threshold values (Ct) where the amplification curve is the steepest, both for the gene of interest (GAG-FAM, panel

A) and for the internal control (HB2-YY, panel B). These Ct values are the number of cycles required for the amplification curve to cut the absorbance threshold values.

11. Export the results as a Microsoft Excel sheet (see Note 31).
12. Using standards of cells containing 10, 1, and 0.1 copy of LV per cell (see Note 28), ask excel to calculate the ΔCt values (Ct GAG minus Ct HB2).
13. Ask Excel to display an exponential formula giving the copy number as a function of ΔCt.
14. Apply the formula to unknown samples, to calculate their corresponding copy number of HIV sequences.
15. Calculate the titers by applying the following formula: Titer (HT-1080-transducing units/mL) = 100,000 (target HT-1080 cells) × number of copy per cell of the sample/volume of supernatant (in mL) (see Notes 32–34).

3.4. Immortalization of Human Primary Cells

IMPORTANT: Immortalization of human primary cells using lentivectors pseudotyped with the VSV-G envelope and containing Bmi-1 or telomerase, as well as production, concentration, and titration of immortalizing lentivectors, must be performed in a BSL-3 laboratory (see Notes 9 and 15–19). Immortalized cells can be downgraded to BSL-2 conditions once they have cleared the RCR assay (see Subheading 3.5.4 and Note 10).

1. On day 0, seed primary cells to immortalizing in triplicate in an MW24 cluster (see Subheading 2.4) at cells densities of 1,000, 3,000, 10,000, 30,000 or 100,000 cells per well per 500 μL of their dedicated medium.
2. On day 1, wash the cells and add 500 μL of fresh medium.
3. Choose for transduction the well triplicate where the cell density is between 1/8 and 1/4 confluence. Depending on the cell size, this should amount to approximately 10,000 cells. You may add one extra well of each seeding density to detach and count cells in order to have a more precise estimation of lentivector quantities to add in further steps.
4. Add pLOX-CW-Bmi1 lentivector at an MOI of 1 (i.e. 10^4 TU) in one well and at an MOI of 10 (i.e. or 10^5 TU) in another well. The total volume of added lentivector suspension should not exceed 10% of the initial volume (500 μL of culture medium + 50 μL of vector). Keep one well as untransduced control.
5. On day 2, wash cells and add 1 mL of fresh medium.
6. Then, change medium and split cells as usual until you have enough cells to freeze, perform a CMV-BMI PCR detection and a copy number qPCR assay (see Subheadings 3.5.2 and 3.5.3) or move directly to telomerase transduction step. This should be between 1 and 2 weeks minimum, especially if a

qPCR assay of copy number is performed. Indeed, cells need to go through 3–5 cell divisions to dilute and lose unintegrated copies of lentivector DNA. Note that at this step, you may already observe a change in growth rates between transduced and untransduced cells.

7. Prepare cells in MW24 clusters and transduce them with pLOX-TERT-iresTK lentivector as described above for Bmi-1 lentivector.
8. Wash and expand cells as described above until you have enough cells to freeze and perform a TK PCR detection and a copy number qPCR assay (see Subheadings 3.5.2 and 3.5.3). Note that at this step, untransduced cells may already have ceased to grow and/or died of senescence.

3.5. Genetic Analysis of Lentimmortalized Cells

These genetic analysis assays are designed to assess for the presence of pLOX-CW-Bmi1 and pLOX-TERT-iresTK lentivectors and calculate their copy numbers. They also ensure for the absence of replication-competent recombinants in the final immortalized cells, and thus provide a biosafety label for these cells as the production, even at low levels, of infectious immortalizing vectors may represent a serious biohazard outside of BSL–3 environment. These assays should be performed at least 3 weeks after the last transduction, especially if a qPCR assay of copy number is performed. Indeed, cells need to go through 3–5 cell divisions to dilute and lose unintegrated copies of lentivector DNA.

3.5.1. Sample Preparation

1. Extract DNA from approximately 10^6 cells using the genomic DNA extraction kit, following manufacturer recommendations. For the DNA elution step, use 100 µL of AE buffer instead of 200 µL.
2. Perform PCR or store DNA at –20°C until use.

3.5.2. PCR Detection of Immortalizing Lentivectors

1. In a 200 µL PCR tube, prepare the following mix:

RedTaq reaction buffer 2×	10 µL
10× oligo mix (CMV-BMI or TK, see Subheading 2.6 and Notes 6–8)	2 µL
DNA sample	1 µL
H_2O	7 µL

2. Use DNA from Bmi + Tert immortalized cells as positive control. In case you do not have such DNA, you can prepare a stock solution at 10 pg/mL of the corresponding lentivector plasmid DNAs, diluted in control DNA from untransduced cells at 5 µg/mL (approximately equivalent to 10^6 cells/mL).
3. Use DNA from primary cells as negative control.

4. Run the PCR for 42 cycles with the following parameters:

94°C	20 s
55°C	1 min
72°C	1 min

5. Run 10 μL of PCR products in a 2% agarose gel.
6. Positive band for CMV-BMI PCR has a size of 350 bp.
7. Positive band for TK PCR reaction has a size of 620 bp.

3.5.3. Quantification of Lentivector Copy Numbers by qPCR

This assay is basically run as the qPCR titration assay described above. The specificity of this assay is that it is run with genomic DNA from immortalized cells instead of HT-1080 cells and that the copy number is directly calculated from the standard curve obtained from HT-1080 cells containing various copy numbers of LV-GFP. The normalization of LV copy numbers per genomic DNA (hence per cell) is more relevant when using standard DNA from HT-1080 cells rather than from HeLa cells. Indeed, HT-1080 cells have a close to normal diploid DNA content, whereas HeLa cells have a chromosome count ranging from 70 to 164.

3.5.4. Replication-Competent Recombinant Assay

The absence of Replication-Competent Recombinants (RCRs) is essential to downgrade the biohazard level of cells that have been transduced by retroviral vectors, including LVs. We propose here a test based on the detection (or absence of detection) in the chromosomal DNA of transduced cells of HIV sequences that are absent in the vector plasmid (vector genome) but are present in the packaging plasmid and are essential for HIV (or RCR) replication. The target sequence chosen in our assay is located in the sequence coding for the viral protease that is present in the packaging plasmid, essential for virus replication and absent in the vector genome. Although the assay described here is performed on a small number of cells, at least 3 weeks after initial transduction, it can be scaled up to meet requirements for the detection of RCR in preclinical vector batches. Other RCR tests have been described in the literature. One earlier paper describes a true RCR assay which failed to detect any RCR in vector batches produced from third generation packaging systems (44). Several other tests have been described, but they detect biological entities that need *trans*-complementation to replicate. Although these assays can measure the level of recombination during the production of lentivectors, they are not suitable to detect genuine RCR that may represent a biological hazard due to potential dissemination within primary human cells.

1. At least 3 weeks prior to assay, transduce HT-1080 cells with LV of interest and with standards (see below). This extended growth period allows for dilution of packaging DNA carried over from

vector production steps. In contrast to qPCR titration described above, two types of standards are needed in this protocol. One standard corresponds to cells containing vector sequences only (LV standard, target for GAG oligo set), and one corresponds to cells containing all HIV sequences (HIV standard, target for GAG and PRO oligo sets). The first standard is provided by cells transduced with LV as described above. The second standard is provided by cells having one copy of full-length HIV genome, such as 8E5 cells, or by full-length HIV-1-containing plasmid (see Notes 13 and 14).

2. After ≥3 weeks of cell growth, extract DNA from the transduced cells using a DNAeasy kit according to the manufacturer's instructions. Store DNA at –20°C until use. The number of cells and final volume should be such that 1 µL of the final DNA solution corresponds to 10^4 cells.
3. For each sample or standard, prepare three independent mixes containing everything but the sample DNA for the number of wells needed for the qPCR reaction, including all samples and standards in duplicates, according to the following recipe (for one well):

qPCR Master Mix	7.5 µL
10× oligo mix (GAG, PRO or HB2, see Subheading 2.6 and Notes 6–8)	1.5 µL
DNA sample	1 µL
H_2O	5 µL

4. Distribute 14 µL of this mix into the wells of a 96-well Optical Reaction plate.
5. Add sample DNAs.
6. Close with optical caps.
7. Centrifuge the plate at 200 × *g* for 1 min to bring all liquid on the bottom of the wells.
8. Place the MW96 in the real-time PCR machine and run the appropriate program depending on the fluorochromes and quenchers used in your Taqman probes.
9. Analyze as described in the qPCR titration section. In this case, however, two types of standards are used. One standard corresponds to cells containing vector sequences only (LV standard, target for GAG oligo set), and one corresponds to cells containing all HIV sequences (HIV standard, target for GAG and PRO oligo sets). The first standard is provided by cells transduced with LV as described above. The second standard is provided by cells having one copy of full-length HIV genome, such as 8E5 cells. In the case of 8E5, the DNA will contain one

copy of HIV per genome. Serial tenfold dilutions of 8E5 DNA into human DNA (up to 10^{-3} copy per genome) can be performed to provide an HIV DNA standard curve. A negative control both for LV sequences and HIV sequences will be provided by HT-1080 cells.

10. Results are expressed as Ct values for each oligo set, i.e., GAG-HB2ΔCt and PRO-HB2ΔCt. The sample DNA will be considered negative for PRO sequences and hence negative for RCR if its PRO-HB2ΔCt value is similar to the PRO-HB2ΔCt value of HT-1080 cells, with a GAG-HB2ΔCt value above the range corresponding to one copy of LV sequence per genome.

3.6. Plasmid Preparation

Plasmids containing retroviral LTRs are prone to undergo deletion in some *E. coli* strains. The Top10 strain is strongly recommended for propagating the plasmids used in this section. We recommend JetStar Kits to prepare DNAs for transfection. The last step of the DNA prep should be an additional precipitation with ethanol and resuspension in TE. Do not treat DNA with phenol/chloroform as it may result in chemical alterations. Also to avoid salt coprecipitation, do not precipitate DNA below +20°C.

3.7. Troubleshooting Lentivector Production

Transfection efficiency is the most critical parameter affecting vector titer. HEK293T/17 cells are highly transfectable using a variety of protocols. When establishing vector production procedures, it is highly recommended that the transfection protocol be optimized using a plasmid encoding GFP. Transfection efficiency should not be assessed solely on the basis of the percentage of GFP-positive cells and also on the mean fluorescence intensity, which reflects the number of plasmid copies taken up by the cells. This makes FACS analysis of the transfected cells mandatory. FACS can be done as soon as 15 h after the transfection, allowing many variables to be tested rapidly. The factors most likely to impact on the transfection efficiency are the pH of the 2× HeBS solution, the quality of the batch of fetal bovine serum used, the cell density, the total amount of DNA per plate, and the quality of DNA. A coarse precipitate will give poor transfection, whereas a fine precipitate (barely visible after application on cells) will give good transfection. As a rule of thumb, the precipitate will be coarser as the pH of 2× HeBS increases, the DNA quantity decreases, the temperature or the incubation time for precipitate formation increases.

In the case of lack of transduction of a specific cell type with a specific LV, a synoptic diagram is provided at the following URL to help addressing most of the problems that could account for it.

http://medweb2.unige.ch/salmon/lentilab/troubleshootingdiagram.pdf

3.8. Anticipated Results

When applied optimally, the procedure described here yields crude unconcentrated vector titers between 1×10^6 and 1×10^7 TU/mL. Note that pLOX-TERT-iresTK gives unconcentrated titers approximately ten times lower. After centrifugation, a yield of at least 50% is expected. A similar 50% yield is also expected after one freeze/thaw cycle. The cells produce equally during the 48 h post-transfection. You maximize the total yield by harvesting twice.

Note that there is no current procedure for purification per se, of infectious particles. The only methods available (ion exchange, centrifugation, etc.) will only concentrate the vector particles and/or wash soluble material. One must keep in mind that all other particulates generated by the producer cells, such as defective vector particles and exosomes, have a similar density to infectious vector particles and are also coated with VSV-G proteins, and will thus co-sediment or co-purify with infectious vector particles. This implies that there is no current way to enrich in infectious particles a vector stock displaying a poor infectivity index. Defective particles will be enriched alongside causing an increase in cell toxicity.

Most human cells transduced with lentivectors at MOIs ranging from 1 to 10 will end up with 1–5 copies of viral DNA in their genome. One should test transduction sensitivity of each cell type to immortalize. A sensitive cell may need only an MOI of 2. Some resistant cells may need an MOI of 10 or more to achieve one or two copies of integrated transgene. A few days to a few weeks after transduction with Bmi-1 and telomerase, immortalized cells will show sustained proliferation while untransduced control cells will undergo cell cycle arrest and senescence. PCR detection of immortaligenes lentivectors as well as qPCR quantification of their copy number will confirm molecular immortalization.

4. Notes

1. HEK293T/17 cells are highly transfectable using the $CaPO_4$ technique. Choosing other cells or other variants of 293T cells may result in lower titers. We recommend using ATCC Cat. # CRL-11268. Also, frequent passages and keeping the 293T as individual cells will ensure high transfection efficiency, hence high titers. A stock of HEK293T/17 should be frozen at early passages. When 293T producer cells in culture start showing repeated low titers of control LVs, the cells must be discarded and replaced by a fresh batch from the frozen stock.
2. For the preparation of $CaPO_4$ transfection solutions, we recommend high-quality chemicals such as SigmaUltra Cat. # C5080. Less pure chemicals may contain small amounts of contaminants that can affect transfection and viability of 293T producer cells.

3. For the preparation of 2× HeBS, we recommend high-quality chemicals such as NaCl, SigmaUltra Cat. # S7653, HEPES, SigmaUltra Cat. # H7523, and Na_2HPO_4, SigmaUltra Cat. # S7907.
4. For the 2× HeBS solution, obtaining a proper pH is very important. Below 6.95, the precipitate will not form, above 7.05, the precipitate will be coarse and transfection efficiency low.
5. For the preparation of sucrose 20% solution, we recommend high-quality chemicals such as sucrose, SigmaUltra, Cat. # S7903.
6. Stocks of probes and primers usually come lyophilized and are stored at 100 μM in water.
7. Standard concentrations in 10× oligo sets for qPCR are 1 μM of probe and 3 μM of each primer in water.
8. Standard concentrations of 10× oligo sets for classical end-point PCR are 2 μM of each primer in water.
9. BSL-3 procedures are described in our institutional website (see http://medweb2.unige.ch/salmon/lentilab). Please consult this site or your institutional biosafety officer for details about local BSL-3 procedures. In particular, personnel handling "immortaligene-containing" lentivectors must work in Class-2 laminar flows wear a double pair of gloves, a safety gown and protective glasses.
10. Cells being analyzed for the absence of RCR must be confined in a culture flask with vented cap until result of RCR analysis. If the result is negative, the biohazard level of the cells can be downgraded; after spraying the flask with 75% ethanol, it can be transferred outside of the culture laboratory.
11. These procedures require standard molecular biology equipment such as an agarose gel apparatus and a generator, a gel imager and a classical end-point PCR machine.
12. For analytical and preparative DNA visualization, we now use SYBR® Safe DNA gel stain (Cat. # S33102, Life Technologies™) together with a Safe Imager™ 2.0 Blue-Light Transilluminator (Cat. # G6600EU, Life Technologies™). This staining system related to SYBR Green dye is not carcinogenic. It is thus less biohazardous and gives higher cloning efficiencies than ethidium bromide.
13. ATCC recommends that 8E5 cells be handled in a P3 laboratory. Indeed, although they contain a full copy of noninfectious HIV, they can form syncytia with uninfected CD4+ cells.
14. A plasmid containing full-length HIV-1 DNA can be obtained from the AIDS Repository (https://www.aidsreagent.org/reagentdetail.cfm?t=molecular_clones&id=55).

15. P2 and P3 practices require that open tubes always be handled in the laminar flow hood. Tubes can be taken out of the laminar flow only when they are closed, and sprayed with 75% ethanol.
16. All solid waste and plasticware must be discarded in a trash bin in the laminar flow hood and all liquids must be aspirated into a liquid waste bottle containing fresh concentrated bleach. Refill the liquid waste bottle with fresh bleach when the color of the liquid is no longer yellow.
17. When full, bags are closed inside the laminar flow hood, then autoclaved.
18. When full, and at least 15 min after neutralization with fresh bleach, the liquid waste bottle can be emptied into a regular sink.
19. In case of a major spill of vector-containing liquid, absorb liquid with paper towels and neutralize with fresh concentrated bleach prior to disposal.
20. In case there is a leak in the SW28 buckets, remove the tubes in the hood, fill the buckets with 75% ethanol, and invert them several times. Leave under the hood for ≥20 min. Discard the 75% ethanol and remove the conical adapters under the hood. Spray the adapters with 75% ethanol and leave them under the hood for >20 min.
21. When resuspending the pellets, try to avoid bubbles since it will result in decrease of final volume and decrease of yield.
22. Try to avoid repeated freeze-thaw cycles of stored vectors. This may results in drop of titer, although the VSV-G pseudotyped particles are more resistant to this procedure than particles pseudotyped with retrovirus-derived envelopes.
23. A reliable measure of the fraction of GFP+ cells relies on the level of GFP expression. In the example shown in Fig. 4, GFP-positive and GFP-negative cells can be readily discriminated when GFP is expressed from a human Ubiquitin promoter and allowed to accumulate in cells for 4–5 days. A marker can then be set to measure the fraction of transduced versus total cells.
24. Cells fixed with formaldehyde can be stored in the dark at +4°C for several hours. A final 0.5% formaldehyde concentration is enough to fix cells and inactivate vectors. Increasing formaldehyde concentration (up to 4% final) will increase the autofluorescence of cells and decrease GFP fluorescence.
25. In a typical titration experiment, only dilutions yielding to 1–20% of GFP-positive should be considered for titer calculations. Below 1%, the FACS may not be accurate enough to reliably determine the number of GFP-positive cells. Above 20%, the chance for each GFP-positive target cell to be transduced more than once significantly increases, resulting in underestimation of the number of transducing particles.

26. Always use pipet tips containing aerosol-barrier filters when preparing solutions, mixes, samples, and plates for qPCR, to prevent cross-contamination.
27. It is advisory to run a dual titration (FACS plus qPCR) using one GFP vector alongside the other vectors, for each experiment of qPCR titration. This will help comparing the FACS titration with the qPCR titration.
28. Standards of HT-1080 cells containing 10, 1, and 0.1 copy of LV per cell can be prepared from HT-1080 cells transduced with a GFP vector, using serial tenfold dilutions. The 0.1 copy per cell standard will be provided by the sample displaying 10% of GFP-positive cells.
29. DNA typically comes from 2×10^6 HT-1080 cells (one confluent well of an MW6 plate) extracted and resuspended in 100 µL of Buffer AE (DNAeasy Tissue Kit).
30. The precise settings of a qPCR protocol depend on the real-time PCR machine used. This aspect is beyond the scope of this protocol. If you are not familiar with qPCR techniques, you should seek advice from your local qPCR expert or from the technical assistance of your real-time PCR machine.
31. A prototypic excel worksheet for calculation of qPCR titers can be downloaded from the following link: http://medweb2.unige.ch/salmon/lentilab/QPCRtitration.html.
32. Using standard DNA extraction procedures in a laboratory context where HIV sequences are often handled, you can expect a level of background contamination with HIV sequences corresponding to cells containing 1 copy per 1,000 or 100 genomes. In this case, consider higher copy numbers for calibration.
33. Vector stocks failing to give higher than 0.01 copy per genome in a qPCR assay, using the highest titration dose, must have experienced one or several problems during their design, packaging, and/or production and cannot be used. You must then refer to the troubleshooting in Subheading 3.7 to solve this issue.
34. Using careful DNA extraction procedures and standardization as described above, you can expect reproducibility within a twofold range. Ask your local qPCR expert if you need a more stringent quantification qPCR procedure.

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