Chapter 8

Lentiviral Vectors

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Abstract

Lentiviral vectors have evolved over the last decade as powerful, reliable, and safe tools for stable gene transfer in a wide variety of mammalian cells. Contrary to other vectors derived from oncoretroviruses, they allow for stable gene delivery into most nondividing primary cells. In particular, lentivectors (LVs) derived from HIV-1 have gradually evolved to display many desirable features aimed at increasing both their safety and their versatility. This is why lentiviral vectors are becoming the most useful and promising tools for genetic engineering, to generate cells that can be used for research, diagnosis, and therapy.

This chapter describes protocols and guidelines, for production and titration of LVs, which can be implemented in a research laboratory setting, with an emphasis on standardization in order to improve transposability of results between laboratories. We also discuss latest designs in LV technology.

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

1. Introduction

1.1. From Lentiviruses to Lentivectors

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 for 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. 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

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to oncoretroviruses, lentiviruses, such as the human immunodeficiency virus (HIV), are a subfamily of retroviruses that can infect both growth-arrested and dividing cells.

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 post-transcriptional activator called Rev.

The envelope protein (Env) mediates the entry of the vector 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 envelope protein can be substituted by the corresponding protein of another virus. This process, which alters the tropism of the virion, is called pseudotyping. The envelope of the amphotropic strain of MLV was used in some early experiments to pseudotype HIV-derived vectors (1). Its receptor, Pit-2, however, is only present at very low level on hematopoietic stem cells, an important target for gene therapy. Very often, the G protein of vesicular stomatitis virus (VSV-G) is used to pseudotype lentiviral as well as oncoretroviral vector particles, because it is highly stable, allowing for the concentration of the vector by ultracentrifugation, and because its phospholipid receptor is ubiquitously expressed in mammalian cells. Moreover, the association of the VSV-G glycoprotein with viral cores derived from lentiviruses results in vector pseudotypes that can integrate into non-proliferating target cells (2). More selective tropisms were achieved by taking advantage of the natural tropisms of glycoproteins (gps) from other membrane-enveloped viruses (see Table 1).

For instance, the use of surface glycoproteins derived from viruses that cause lung infection and infect via the airway epithelia, like Ebola virus or Influenza virus, may prove useful for gene therapy of the human airway (3). Exclusive transduction of retinal pigmented epithelium could be obtained following subretinal inoculations of some vector pseudotypes in rat eyes (4). Importantly, several viral gps target lentiviral vector to the central nervous system (CNS) such as rabies, mokola, lymphocytic choriomeningitis virus envelope (LCMV) or Ross River viral gps that permit even transduction of specific cell types in the CNS (Table 1). Some other envelope gps have been proven specifically efficient for LV transduction of hepatocytes or skin (Table 1). Likewise, screening of a large panel of pseudotyped vectors established the superiority of the Gibbon Ape Leukemia virus (GALV) and the cat endogenous retroviral glycoproteins (RD114) for

Table 1
Pseudotyping of lentiviral vectors with heterologous envelope glycoproteins relying on the natural tropism of these glycoproteins (after (22))

Glycoprotein	Virus of origin	Targeted cells – tissues	Reference
VSV-G	Vesicular stomatitis virus	Broad tropism (mouse and human cells)	(2)
MLV-10A1 gp	Murine leukemia virus – amphotropic strain	Broad tropism (mouse and human cells)	(5)
MLV-E gp	Murine leukemia virus – ecotropic strain	Broad tropism (mouse cells)	(6)
Rabies gp	Rabies virus	Neurons	(23–26)
Mokola gp	Mokola virus	Neurons Retinal pigment epithelium	(23) (25, 27, 28)
LCMV gp	Lymphocytic choriomeningitis virus	Glioma and neural stem cells	(27, 29, 30)
Ross River gp	Ross River virus	Glial cells	(31)
Ebola gp	Ebola virus	Airway epithelium Skin	(3, 32, 33) (34)
GP64	Baculovirus	Hepatocytes	(35)
HCV gp	Hepatitis C virus	Hepatocytes	(36)
F protein	Sendai virus	Hepatocytes	(37)
RD114 modified gp	Feline endogenous retrovirus	Hematopoietic cells	(7)
GALV modified gp	Gibbon ape leukemia virus	Hematopoietic cells	(5, 7, 38)
HA gp	Hemagglutinin – influenza A virus	Broad tropism – retinal epithelium	(4)
H and F measles gps	Measles virus H (hemagglutinin) and F (fusion protein)	Resting B cells and T cells	(8, 39)

transduction of progenitor and differentiated hematopoietic cells (5–7). Importantly, replacement of the cytoplasmic tail of RD114 and GALV gps with that of MLV-A glycoprotein resulted in strongly increased incorporation of these chimeric gps as well as high titers (5). Measles virus (MV) gps also require a modification of their cytoplasmic tails to allow efficient in corporation onto lentiviral vectors. Interestingly, lentivectors pseudotyped with such modified MV gps can transduce quiescent T and B cells more efficiently than VSV-G pseudotyped LVs (8). Although many different pseudotyped vectors have been generated as described above, pseudotyping with VSV-G gp provides lentiviral vectors with the highest titers and the most robust particles.

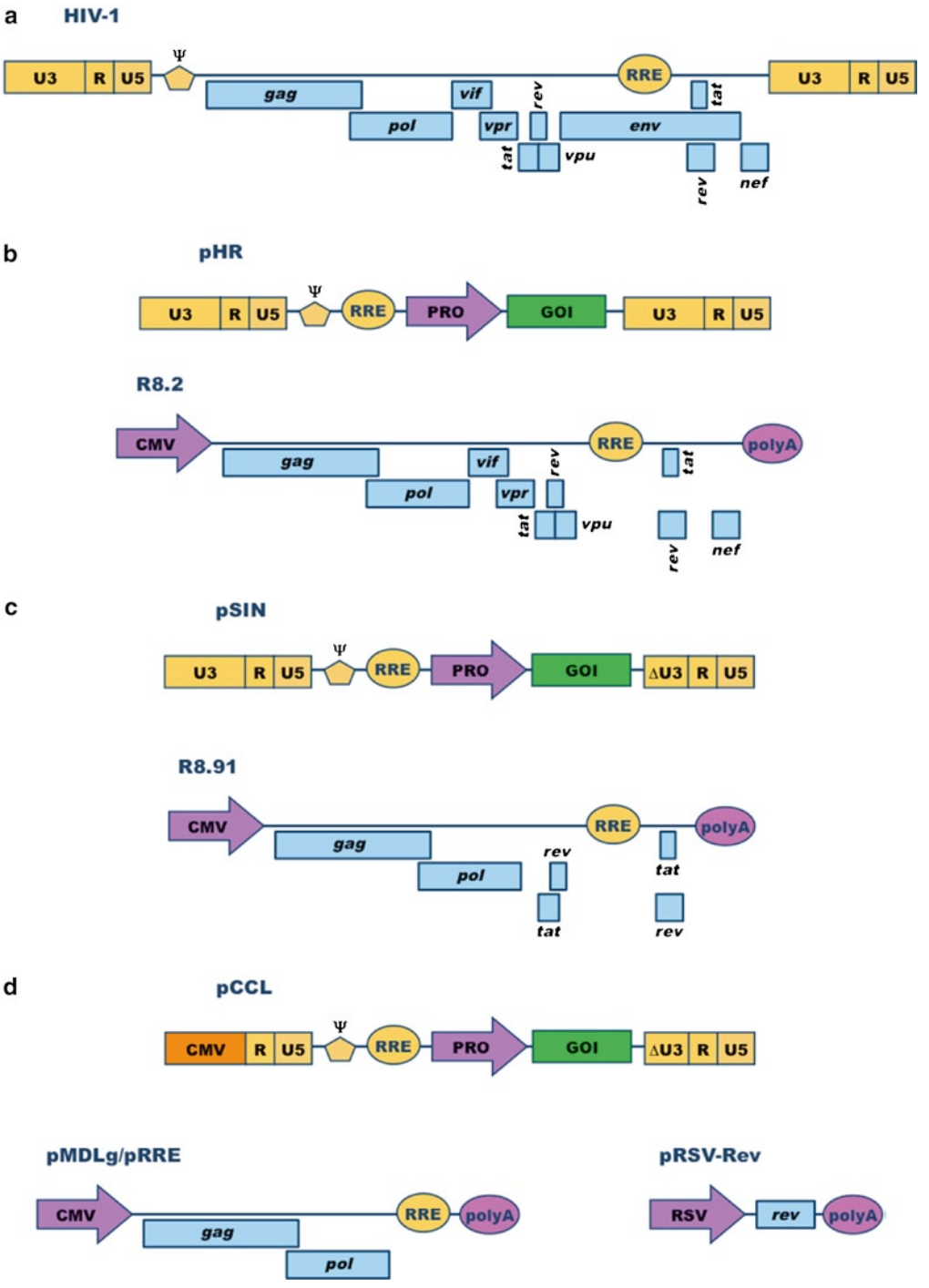
This technique is thus widely and routinely used in basic research as well as in clinical research. Therefore this chapter focuses on production of the VSV-G-pseudotyped vectors.

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 (Fig. 1). 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.

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.

Because lentiviruses can infect both dividing and nondividing cells, vectors were developed from this subgroup of retroviruses with the hope that they would be able to transduce cells that proliferate very little or not at all. 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. 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 HIV1-based vector system

Fig. 1. Evolution in the design of HIV-1 based LV vectors. HIV-1-based LV vectors 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 2 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. *CMV* human cytomegalovirus immediate-early promoter, *RRE* rev-responsive element, *RSV* Rous sarcoma promoter, *polyA* polyadenylation site, *U3-R-U5* HIV-1 LTR, *psi* HIV-1 packaging signal, *PRO* promoter of the internal expression cassette, *GOI* transgene of interest, $\Delta U3$ self-inactivating deletion of the U3 part of the HIV-1 LTR.


a
HIV-1
U3
R
U5
Ψ
gag
pol
vif
vpr
tat
rev
vpu
RRE
env
nef
b
pHR
PRO
GOI
R8.2
CMV
polyA
c
pSIN
ΔU3
R8.91
d
pCCL
pMDLg/pRRE
pRSV-Rev
RSV

(VSV-G pseudotyped LV vectors) because it is presently the most advanced, 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.

1.2. Evolution and Design of Lentivectors

The potential of lentiviral vectors was first revealed in 1996 through the demonstration that they could transduce neurons in vivo (2). Since then, many improvements have been brought to achieve high levels of efficiency and biosafety. The principle, however, remains the same and consists in building replication-defective recombinant chimeric lentiviral particles from three different components, the genomic RNA, the internal structural and enzymatic proteins, and the envelope glycoprotein. The genomic RNA contains all the *cis*-acting sequences, whereas the packaging plasmids contain all the *trans*-acting proteins, necessary for adequate transcription, packaging, reverse transcription, and integration. A diagram of the evolution of HIV1-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 (2). 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 post-transcriptional functions (9). Sensitive tests have so far failed to detect replication-competent-recombinants (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 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 (10). The central polypurine tract of HIV has also been added back in the central portion of the genome of the transgene RNA (11, 12). 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 (13). 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 LV 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) (14). 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 LV 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, however, this third generation is not mandatory, and the second generation system offers a high level of safety for P2 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 provides several desirable features. It contains a gene switch, the TET promoter/rTTA system (15), under the control of the highly and ubiquitously active ubiquitin promoter (16). Transduced cells can also be live-sorted using GFP. The gene of interest can be easily cloned using the Gateway® system, and is expressed in a drug-controlled fashion. When the gene product is toxic, one can thus control its expression in target cells, and also prevent its expression in producer cells, hence avoiding titer drop due to the death of LV-producing cells. The LoxP sequence is duplicated during reverse transcription, and allows the proviral cassette to be excised upon Cre expression (17). Note that, although lentiviral vectors 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 powerful chimeric CAG promoter (CMV enhancer/beta-actin promoter, beta-globin intron) (18) in our hands. Also, the UBI promoter (ubiquitin gene promoter) (16) can be replaced by other ubiquitously active promoters,

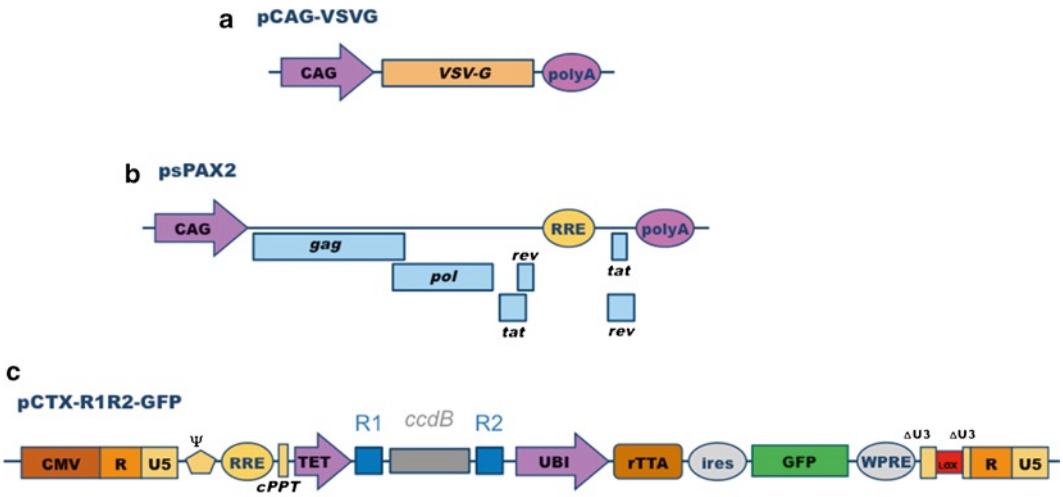


Fig. 2. Example of plasmids used for HIV-1 based LV production. (**a**) The pCAG-VSVG plasmid (courtesy of A. Nienhuis, (6)), providing the envelope of the LV particles is composed of the CAG chimeric promoter, the coding sequence of the Vesicular Stomatitis Virus Envelope protein (VSV-G), and the polyadenylation signal from the rabbit beta-globin gene. (**b**) The second-generation psPAX2 packaging plasmid (P. Salmon, unpublished), providing the structural and enzymatic proteins of the LV particle is composed of the CAG chimeric 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. (**c**) The third generation pCTX-R1R2-GFP vector plasmid, providing the genome of the LV particles is depicted here as an example of the latest development in 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, *R1-ccdB-R2* att-flanked cassette for Gateway® cloning of genes of interest, *UBI* ubiquitin promoter, *rTTA* reverse TET-transactivator, *ires* EMCV internal ribosome entry site, *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.

such as EF1 (19) or EFs (20), or tissue-specific promoters. In that latter case, the gene-switch will be active only in a specific cell type.

Detailed informations (maps, sequences, etc.) as well as other LV backbones are available at our institutional website: http://medweb2.unige.ch/salmon/lentilab/.

1.3. 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 (21).

In general, transduced cells must always be fixed (using formaldehyde or paraformaldehyde as described below) before being taken out of the P2 laboratory. If a live sorting is needed outside of the P2 laboratory, a careful handling and decontamination of the equipment used must be performed afterward.

Given the very broad tropism of VSV-G-pseudotyped lentiviral vectors both in vitro and in vivo, biosafety precautions need to take into account the nature of the transgene. A P2 laboratory, P2 standard equipment, and P2 safety procedures are required. In dependence of the country and the local legislation, procedures using lentiviral vectors must be reviewed and approved by the local biosafety committee of the institution where they are conducted or need authorization from the competent authority. Extra precautions must be taken when working with transgenes that are themselves potential biohazards. For instance, working in a P3 laboratory is recommended for the lentivector-mediated transfer of genes involved in cell proliferation.

2. Materials

All solutions and equipment coming into contact with living cells must be sterile, and aseptic techniques should be used accordingly. All maps and sequences of plasmids described here are available at http://medweb2.unige.ch/salmon/lentilab/. Common plasmids for the generation of HIV1-based lentivectors can be obtained from http://www.Addgene.org. Use ultrapure or double-distilled water in all recipes.

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

1. Producer cells: 293T/17 cells (from ATCC Cat# CRL-11268).
2. D10 medium: Dulbecco's modified Eagle medium (with 4.5 g/l glucose, glutamine, and pyruvate, Invitrogen Cat# 41966052 or equivalent) supplemented with antibiotics and 10% FBS.
3. Serum-free medium: Advanced DMEM (Invitrogen Cat# 12491015) supplemented with 2 mM glutamine.
4. TE buffer: 10 mM Tris–HCl – 1 mM EDTA, 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: pFUGW dissolved at 1 µg/µl in TE buffer.

8. 0.5 M $CaCl_2$: Dissolve 36.75 g of $CaCl_2 \times 2H_2O$ (SigmaUltra Cat# C5080) 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 16.36 g of NaCl SigmaUltra Cat# S7653 (0.28 M final), 11.9 g of HEPES SigmaUltra Cat# H7523 (0.05 M final), and 0.213 g of Na_2HPO_4, anhydrous SigmaUltra Cat# S7907 (1.5 mM final) into 800 ml of H_2O. Adjust pH to 7.00 with 10 M NaOH. Be careful, 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 will be low. Add H_2O to 1000 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 HeBS solution can be kept at +4°C for several weeks without observing significant change in the transfection efficiency.
10. 75% Ethanol in a spray bottle.
11. PBS, pH 7.4.
12. PBS-Ca^{2+},Mg^{2+} at pH 7.4.
13. 20% Sucrose: Dissolve 20 g of Sucrose SigmaUltra in 100 ml of PBS-$Ca^{2+}Mg^{2+}$. Filter sterilize through a 0.22-µm nitrocellulose filter. Store at +4°C.
14. 0.25% Trypsin/EDTA.
15. 13–14% Bleach solution (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 (Cat# 358126, Beckman-Coulter) for ultracentrifuge.
22. Ultracentrifuge (such as Beckman Optima™ L-90K) with SW 28 rotor.

2.2. Titration by FACS

1. Target cells: HT-1080 cells (Cat# CCL-121, ATCC).
2. D10 medium: same as above.
3. Trypsin/EDTA: same as above.
4. MW6 tissue culture plates (Cat# 353224, BD Biosciences).

5. PBS: same as above.
6. 1% Formaldehyde (w/v) in PBS: Mix 1 ml of 37% formaldehyde (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. Titration by qPCR

1. Target cells: HT-1080 cells (same as above).
2. D10 medium: same as above.
3. Trypsin/EDTA: same as above.
4. MW6 tissue culture plates (same as above).
5. PBS: same as above.
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 GmbH, Germany).
8. 2× Reaction buffer (Cat# RT-QP2X-03 Eurogentec, Belgium).
9. 96-well Optical Reaction plate (Cat# 4306737, Applied Biosystems).
10. Optical caps (Cat# N801-0935, Applied Biosystems).
11. Filter tips (1000, 100, and 10 µl).
12. Primers and probe for quantification of HIV sequences (10× GAG set, see Subheading 2.5 below).
13. Primers and probe for quantification of human genomic sequences (10× HB2 set, see Subheading 2.5 below).

2.4. RCR Assay

1. Target cells: HT-1080 cells (same as above).
2. Full HIV-1 genome-containing cells: 8E5 cells (Cat# CRL-8993, ATCC).
3. D10 medium: same as above.
4. Trypsin/EDTA: same as above.
5. MW6 tissue culture plates (same as above).
6. PBS: same as above.
7. Real-time PCR machine (same as above).
8. Genomic DNA extraction kit (same as above).
9. 2× Reaction buffer (same as above).
10. 96-well Optical Reaction plate (same as above).
11. Optical caps (same as above).

12. Filter tips (1000, 100, and 10 µl).
13. Primers and probe for quantification of HIV sequences (10× GAG set, see Subheading 2.5 below).
14. Primers and probe for quantification of human genomic sequences (10× HB2 set, see Subheading 2.5 below).
15. Primers and probe for quantification of HIV packaging sequences (10× PRO set, see Subheading 2.5 below).

2.5. Oligos

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

2.5.1. Human Beta-Actin Taqman® Probe and Primers

1. HB2-P: (probe, sense) 5′-(FAM)-CCTGGCCTCGCTGTC CACCTTCCA-(TAMRA)-3′.
2. HB2-F: (forward primer)5′-TCCGTGTGGATCGGCGGCT CCA-3′.
3. HB2-R: (reverse primer)5′-CTGCTTGCTGATCCACAT CTG-3′.

2.5.2. 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)-ACAGCCTTCTGAT GTTTCTAACAGGCCAGG-(TAMRA)-3′.
2. GAG-F: (forward primer)5′-GGAGCTAGAACGATTCGCA GTTA-3′.
3. GAG-R: (reverse primer)5′-GGTTGTAGCTGTCCCAGTA TTTGTC-3′.

2.5.3. PRO Taqman® Probe and Primers

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

1. PRO-P: (probe, sense) 5′-(FAM)-ACAATGGCAGCAATTT CACCAGT-(TAMRA)-3′.
2. PRO-F: (forward primer) 5′-AGCAGGAAGATGGCCAGT AA-3′.
3. PRO-R: (reverse primer) 5′-AACAGGCGGCCTTAACT GTA-3′.

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

3. Methods

3.1. Production of LV Stocks

1. Maintain 293T cells in D10 medium, 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). Frequent passages and keeping the 293T as individual cells will ensure high transfection efficiency.
2. The day before the transfection, seed 1–10 dishes at 1.5 to 2.5 million cells per dish (10 cm). 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, co-transfect 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	pFUGW	8 µg

4. The vector plasmid (pFUGW 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 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 prewarmed PBS, and gently add 15 ml of fresh

Advanced DMEM, prewarmed 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 LV Stocks

1. For concentration, use 30-ml Beckman conical tubes (Cat# 358126, Beckman-Coulter), in a SW 28 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 3–5 mm dry zone to the top of the tube). Spin at 50,000 g for 120 min at +16°C.
2. Aspirate the medium with a sterile pipette down to the sucrose interface.
3. 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.
4. 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 ~1000-fold concentration.
5. Vortex at half-speed for 2 s.

6. Leave the vector pellet to resuspend for 1–2 h at room temperature or 2–4 h at +4°C.
7. Vortex at half-speed for 2 s.
8. Pipet up and down 20 times and freeze at –80°C in aliquots for long-time storage (see Notes 1–8).

3.3. Titration of LV Stocks

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 productions. 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.
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 a 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 below). We describe here the titration of an Ubiquitin promoter-GFP vector (pFUGW, see above).

1. Add 500 µl of 1% formaldehyde in PBS to the cell pellet obtained at step 7 above. This step will fix the cells and inactivate the vector particles. Samples can thus be taken out of the P2 laboratory.
2. Resuspend the cells thoroughly in the well and transfer them to a 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 (as described in Fig. 3 and Notes 9–11), apply the following formula: Titer (HT-1080-TU/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

When lentivectors contain DNAs coding for genes other than GFP or LacZ, or promoters which are active only in specific primary cells and tissue, FACS titration cannot be used. Therefore,

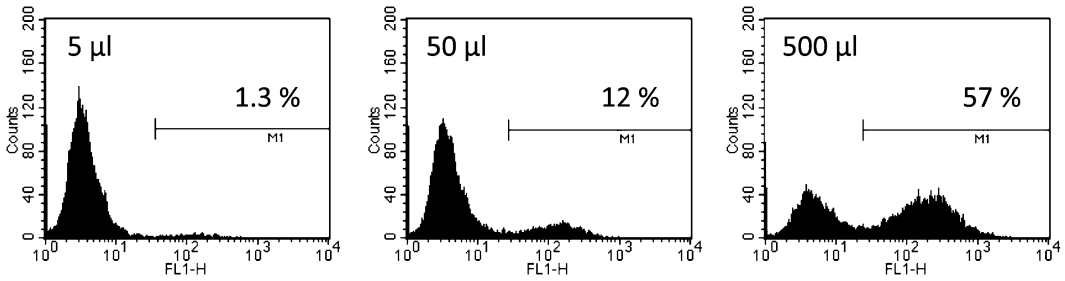


Fig. 3. A representative FACS analysis of HT-1080 cells used for titration of GFP-coding LV. HT-1080 cells (10^5) were incubated with increasing volumes of a supernatant containing a LV expressing GFP under the control of the human Ubiquitin promoter (pFUGW) as described above. After 5 days, cells were 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).

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 above 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 (such as Qiagen DNeasy). 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 a MW6 plate (see general titration procedure above) using the genomic DNA extraction kit, following manufacturer's recommendations. For the DNA elution step, use 100 µl of AE buffer (component of the DNeasy tissue kit) 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):

2× Reaction buffer	7.5 µl
10× Oligo mix (GAG or HB2, see below)	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.

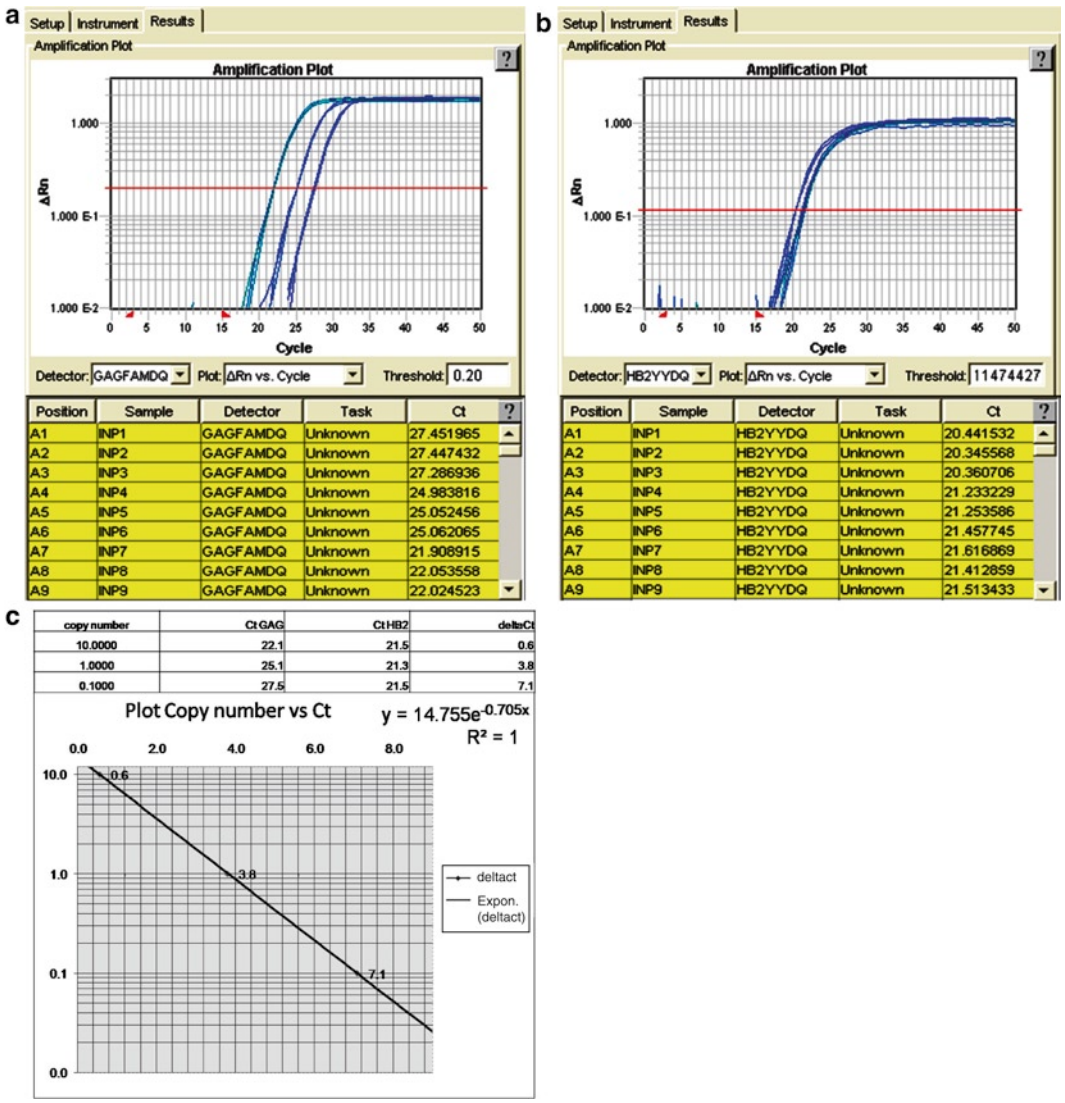


Fig. 4. A representative qPCR analysis used for titration of HIV-1 based LVs. DNA from HT-1080 cells transduced with serial 10-fold dilutions of pFUGW vectors was subjected simultaneously to qPCR titration analysis and FACS analysis as described above. A sample of each dilution was submitted to qPCR 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, panel **A**) or beta-actin sequences (HB2-FAM, panel **B**). Amplification plots were displayed and cycle threshold values (Ct) were set as described in text. 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) (panel **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 (see Note 18).

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 Notes 12–22).
9. Analyze results and calculate titer using the SDS2.2.2 program (Applied Biosystems). An example of amplification profiles of HIV sequences in human DNA is given in Fig. 4.
10. Ask SDS2.2.2 to analyze the amplification reactions.
11. 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-FAM, panel B). These Ct values are the number of cycles required for the amplification curve to cut the absorbance threshold values.
12. Export the results as a Microsoft Excel sheet.
13. Using standards of cells containing 10, 1, and 0.1 copy of LV per cell (see Note 16), ask excel to calculate the ΔCt values (Ct GAG minus Ct HB2).
14. Ask Excel to display an exponential formula giving the copy number as a function of ΔCt.
15. Apply the formula to unknown samples, to calculate their corresponding copy number of HIV sequences.
16. Calculate the titers by applying the following formula: Titer (HT-1080-TU/ml) = 100,000 (target HT-1080 cells) × number of copy per cell of the sample/volume of supernatant (in ml).

3.4. Quantitative PCR Assay of Replication-Competent-Recombinants

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 (21). 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 lentiviral vector (LV) of interest and with standards (see below). This extended growth period allows for dilution of packaging DNA carried over from vector production steps (see Note 23).
2. After ≥3 weeks of cell growth, extract DNA from the transduced cells using a DNeasy 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):

2× Reaction buffer	7.5 µl
10× Oligo mix (GAG, PRO or HB2, see below)	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 (see Notes 12–14).
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 is provided by cells transduced with LV as described above. The second is provided by cells having one copy of full-length HIV genome, such as

8E5 cells (see Note 24 and ATCC website for details about these cells). In the case of 8E5, the DNA will contain 1 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 a 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 1 copy of LV sequence per genome.

3.5. Plasmid Preparation

Plasmids containing retroviral long terminal repeats (LTRs) are prone to undergo deletion in some *Escherichia coli* strains. The Top10 or HB101 strains are strongly recommended for propagating the plasmids used in this section. We also recommend CcdB Survival 2 T1R strain (Invitrogen) for Gateway® clonings. We recommend JetStar Kits (GENOMED GmbH, Germany) 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 co-precipitation, do not precipitate DNA below +20°C.

3.6. Troubleshooting Lentivector Production

Transfection efficiency is the most critical parameter affecting vector titer. 293T/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, but 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.

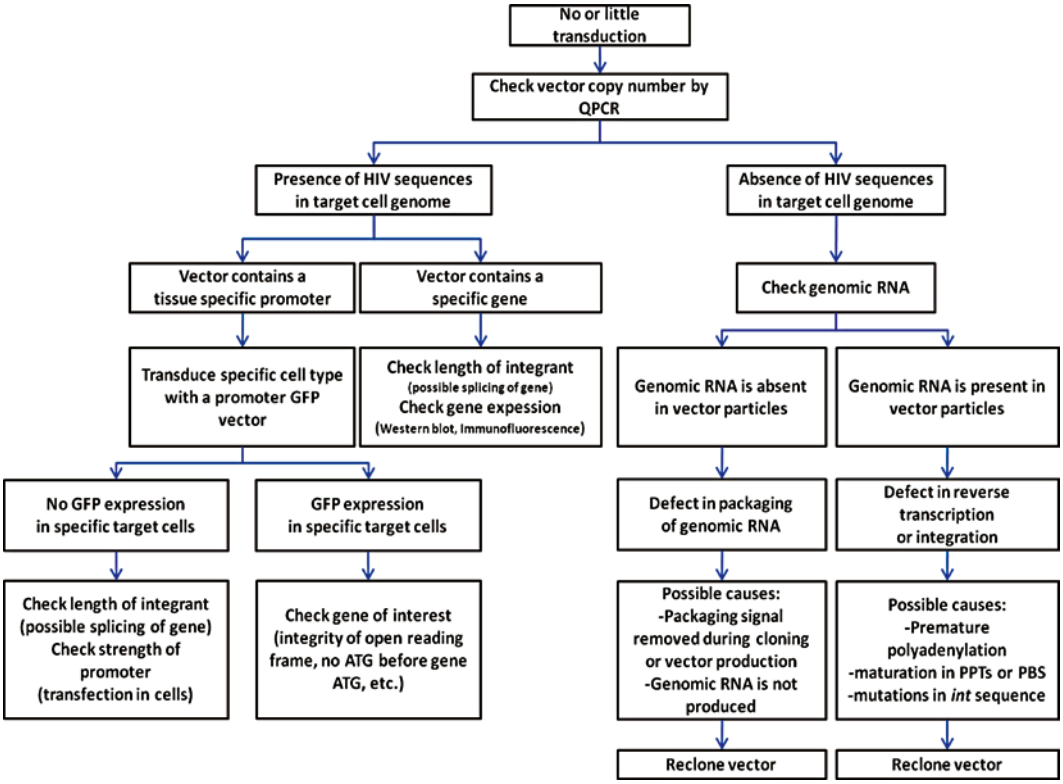


Fig. 5. Troubleshooting diagram for lentiviral vector production and transduction.

In the case of lack of transduction of a specific cell type with a specific lentiviral vector, a synoptic diagram is provided in Fig. 5 to help addressing most of the problems that could account for it.

3.7. Anticipated Results

When applied optimally, the procedure described here yields crude unconcentrated vector titers between 1×10^6 and 1×10^7 TU/ml. 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, are also coated with VSV-G proteins and will co-sediment or copurify 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.

4. Notes

1. P2 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.
2. 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.
3. When full, bags are closed inside the laminar flow hood, then autoclaved.
4. When full, and at least 15 min after neutralization with fresh bleach, the liquid waste bottle can be emptied into a regular sink.
5. In case of a major spill of vector-containing liquid, absorb liquid with paper towels and neutralize with fresh concentrated bleach prior to disposal.
6. In case there is a leak in the SW 28 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.
7. When resuspending the pellets, try to avoid bubbles since it will result in decrease of final volume and hence decrease of yield.
8. Try to avoid repeated freeze-thaw cycles of stored vectors. This may result in drop of titer, although the VSV-G pseudotyped particles are more resistant to this procedure than particles pseudotyped with retrovirus-derived envelopes.
9. A reliable measure of the fraction of GFP+ cells relies on the level of GFP expression. In the example shown in Fig. 3, 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.

10. 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 the cells and inactivate the vectors. Increasing formaldehyde concentration (up to 4% final) will increase the autofluorescence of cells and decrease GFP fluorescence.
11. 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 twice significantly increases, resulting in underestimation of the number of transducing particles.
12. 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.
13. Standard concentrations in 10× oligo sets are 1 μM of probe and 3 μM of each primer in water.
14. Stocks of probes and primers usually come lyophilized and are stored at 100 μM in water.
15. DNA typically comes from 2×10^6 HT-1080 cells (one confluent well of a MW6 plate), extracted and resuspended in 100 μl of Buffer AE (DNeasy Tissue Kit).
16. 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.
17. 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.
18. A prototypic excel worksheet for calculation of qPCR titers can be downloaded from the following link: http://medweb2.unige.ch/salmon/lentilab/QPCRtitration.html
19. 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 1000 or 100 genomes. In this case, consider higher copy numbers for calibration.

20. 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. You must then refer to the Subheading 3.6 to solve this issue.
21. Using careful DNA extraction procedures and standardization as described above, you can expect a reproducibility within a twofold range. Ask your local qPCR expert if you need a more stringent quantification qPCR procedure.
22. Always use pipet tips containing aerosol-barrier filters when preparing solutions, mixes, samples, and plates for qPCR, to prevent cross-contamination.
23. Cells being analyzed for the absence of RCR must be confined cells 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.
24. 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.

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