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High-Titer Production of HIV-Based Lentiviral Vectors in Roller Bottles for Gene and Cell Therapy

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Abstract

Lentiviral vectors are becoming preferred vectors of choice for clinical gene therapy trials due to their safety, efficacy, and the long-term gene expression they provide. Although the efficacy of lentiviral vectors is mainly predetermined by the therapeutic genes they carry, they must be produced at high titers to exert therapeutic benefit for in vivo applications. Thus, there is need for practical, robust, and scalable viral vector production methods applicable to any laboratory setting. Here, we describe a practical lentiviral production technique in roller bottles yielding high-titer third-generation lentiviral vectors useful for in vivo gene transfer applications. $CaPO_4$-mediated transient transfection protocol involving the use of a transfer vector and three different packaging plasmids is employed to generate lentivectors in roller bottles. Following clearance of cellular debris via low-speed centrifugation and filtration, virus is concentrated by high-speed ultracentrifugation over sucrose cushion.

Keywords Gene therapy, Lentivirus, Roller bottles

1 Introduction

Among the numerous viral vectors that have been tested in clinical gene therapy studies so far, HIV-based lentiviral vectors (LVs) stand out particularly due to the long-term transgene expression they provide. LVs have gained widespread use in recent years owing to their favorable features as effective gene transfer vehicles particularly for in vivo applications. The progressively increasing use of LVs in gene transfer studies has demonstrated the necessity to develop methods that will allow high-titer virus production. Scalable, effective, and robust production methods along with high-yield purification steps are critical in this regard. Thus, we aimed to improve the current approaches for high-quality production of LVs in high concentrations in roller bottles, for use in experimental applications.

1.1 Lentiviral Vectors

Lentiviruses are enveloped RNA viruses of the *Retroviridae* (Retrovirus) family. The most studied lentivirus is the human immunodeficiency virus type 1 (HIV-1), which the best characterized lentiviral vectors are derived from. These viruses enter the cell via membrane fusion, after which the positive sense RNA genome is converted into double-stranded DNA by the viral reverse transcriptase to form the proviral DNA. The proviral DNA is then carried into the nucleus to be integrated into the host cell genome by the viral integrase (IN) enzyme [1]. This integration step in the virus' natural life cycle is crucial in providing long-term stable gene expression. Following integration, transcription directed by the LTR (Long Terminal Repeat) regions on the terminal portions of the viral genome takes place and the host cell initiates production of both the lentiviral RNA genome and the lentiviral proteins. Viruses produced in this way are released out of the cell via budding, to infect new host cells [2, 3]. Lentiviruses have the capacity to carry up to 9-kb-long genetic material and can transduce dividing and nondividing cells. Various gene therapy strategies utilize these features of lentiviruses to deliver therapeutic sequences to target cells [4, 5].

Lentiviral genome consists of *cis*- and *trans*-acting components. Important *cis* elements are: Long Terminal Repeats (LTRs), Rev-Responsive Element (RRE), and the packaging signal (Ψ) [1, 3]. LTR sequences reside in the 5′ and 3′ ends of the genome and act as promoters for transcription. Production of the structural and enzymatic proteins occurs under the control of the Rev regulatory protein, which binds to the RRE sequence located within the *env* gene. The posttranscriptional effects provided by Rev include inhibition of viral RNA splicing, stimulation of the nuclear export of the unspliced and incompletely spliced viral mRNAs, and enhancement of the translation of the RRE-containing RNAs [6]. The signal sequence Ψ function in recognition of the viral genome to be inserted into the capsid [7, 8]. The *trans*-acting components of the LV genome include nine open reading frames (ORFs) that code for 15 proteins. Three of these contain conserved genes that encode Gag, Pol, and Env, that are common in all retroviruses as they are required for viral replication. These polyproteins undergo posttranslational cleavage to generate the structural and enzymatic proteins that are essential for the viral life cycle. The outer envelope protein Env is cleaved to yield the surface (SU; gp120) and the transmembrane (TM; gp41) structural proteins. Matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins, on the other hand, are the cleavage products of the Gag polyprotein and form the virion core. Pol also undergoes cleavage to produce three vitally important enzymes: protease (PR), reverse transcriptase (RT), and integrase (IN). These enzymes function in proteolytic processing of the viral precursor polyproteins, conversion of the viral RNA genome into DNA, and integration of this DNA molecule into the host cell genome, respectively [9, 10].

High amounts of full-length transcripts are produced from the integrated proviral genome via the action of the trans-activator protein Tat, which stimulates transcriptional elongation. Tat specifically associates with its cellular cofactor TAK, which is composed of CDK9 and cyclin T, and hyperphosphorylates the carboxyterminal domain of the cellular RNA polymerase II (RNA Pol II) large subunit [11]. This phosphorylation increases RNA Pol II activity and enhances viral transcription to a large extent [12, 13]. The Rev protein, on the other hand, binds to the RRE sequence to provide the nuclear export of the intron-containing viral transcripts into the cytoplasm [14]. This step is essential in the viral life cycle, as unspliced transcripts are needed to be packaged for production of new infectious viral particles. The "accessory proteins" encoded by the remaining genes (Vif, Nef, Vpu, Vpr, and/or Vpx) are not critical for in vitro replication of the virus, as shown in cell culture systems [15].

For LVs to be used as effective gene therapy vectors, the transgene carried by the vector should be integrated into the target cell's genome following infection, and provide transcription and translation of the therapeutic transgene only. Furthermore, biosafety of these vectors should be ensured by rendering them replication-deficient unlike the wild-type virus. Thus, the basic principle for production of biosafe LVs is elimination of the replication competence and prevention of reestablishment of this ability by the virus; so the viral genome should be modified accordingly [16].

Naldini et al. have developed a strategy in 1996 where the lentiviral vector components that are required for viral replication were splitted into three separate plasmids to be expressed during transient co-transfection of producer 293T cells [17]. Vectors produced via this three-plasmid system (packaging, transfer, and envelope) that minimizes the production of replication competent lentiviruses (RCLs) are defined as first-generation LVs [18]. The transfer plasmid contains the transgene, the promoter sequence required for the transcription of the transgene (CMV), the packaging signal (Ψ), and the LTR sequences which function in both conversion of the RNA genome into DNA and integration of the viral genome. All other *trans*-factors required for vector production are included in the packaging plasmid. Accidental packaging of the sequences that would lead to RCL formation is inhibited by deletion of the Ψ sequence. Furthermore, the *env* gene encoding the envelope proteins of the virus is also removed, and a third plasmid coding a heterologous envelope, the vesicular stomatitis virus glycoprotein (VSV-G), is used for pseudotyping the newly generated particles [17]. Use of VSV-G instead of lentiviral gp160 confers high stability to the viral particles and increases resistance to mechanical force. Additionally, VSV-G pseudotyping provides broad tropism over a range of target cells.

Although first-generation LVs contributed a great deal to the overall level of biosafety, recombination risk of the plasmids with each other or with other viruses to generate RCLs could not be eliminated completely. Subsequent studies aimed minimization of the recombination risk associated with the first-generation LV production. Thus, second-generation "multiply attenuated" LVs were produced via removal of the sequences encoding the accessory genes (*vif*, *vpr*, *vpu*, and *nef*) that were included in the packaging plasmids of the first-generation LVs. Products of these genes are essential for the viral life cycle, yet not required for vector production, thus their removal did not impair vector yield or transduction efficiency [19]. Although a minimal risk of RCL formation still existed after these modifications, any such viruses would be devoid of the virulence factors [18]. By removal of the *tat* and *rev* genes that were included in the packaging plasmid used in the second-generation vector production, third-generation LVs were produced, to even further eliminate the RCL formation risk. However, Tat being an essential protein for lentiviral replication, the third-generation vectors had to carry out Tat-independent transcription. This problem was solved by establishment of chimeric LTR regions with Tat-binding sequences removed, through which efficient lentiviral transduction could still be achieved [20]. Furthermore, introducing the essential *rev* gene to the producer cell line on a different plasmid also decreased risk of recombination and RCL formation.

Further modifications to improve vector performance and biosafety in third-generation LVs include a 133-bp deletion introduced into the 3′ LTR region of the viral genome, comprising also the TATA box and the Sp1 and NFkB binding sites. This deletion is naturally transferred to the 5′ LTR site following reverse transcription and results in transcriptional inactivation of the LTR in the integrated provirus, effectively inhibiting viral RNA genome formation. Vectors modified in this way are defined as self-inactivating (SIN) vectors [21, 22]. The transfer plasmid in the third-generation LVs is also designed to contain the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) sequence, which increases the efficiency of transgene expression several folds by a posttranscriptional regulatory effect [23]. Through the above-listed modifications, the idea of using LVs as gene transfer vectors turned into a successful generation of a vector framework that has high efficacy and safety, and also ability to transduce both dividing and nondividing cells.

1.2 Lentiviral Vector Production

Human embryonic kidney (HEK) 293 cells and its derivatives are frequently used in production of the third-generation LVs by co-transfection of four plasmids [17, 20, 24, 25]. Besides a high transfection success in vector production, these human-based packaging cell lines also provide human-type glycosylation patterns on

the Env proteins. This is a significant issue to be considered, especially if the vector is to be used in in vivo applications [26]. In fact, vectors with nonhuman glycosylation patterns are known to be targeted by the human complement system rapidly, within 20 min following application. The most preferred variant of the 293 cells in LV production is the simian virus 40 (SV40) T antigen-expressing HEK293T cells, which are proved to be more efficient cell lines for vector production with increased cell growth and transfection efficiency [25]. SV40 replication origin (SV40ori) in the plasmid backbones is defined as essential for plasmid replication [27]. This context enhances the nuclear import of expression vectors, thus increasing plasmids available for transcription [28].

Besides transient co-transfection of several plasmids to producer cell lines (Fig. 1), an alternative current strategy in LV production involves the use of stable, inducible packaging cell lines that express all lentiviral vector components except for the transfer vector [29]. Among the advantages of the transient gene expression approach compared to the stable packaging cell lines are its flexibility and overall process time [30]. It is an easily applicable method, and various different transient transfection methods have been developed, as it allows modification of different parameters (*see* **Note 1**). The protocol described in this chapter is a third-generation LV vector production method that can be utilized as an intermediate step particularly in transition to large-scale production and summarizes the methodological algorithm to be followed in optimization of LV production, via evaluation of many different parameters suitable for optimization.

2 Materials

- 293T cell line (ATCC CRL-3216)
- FBS (fetal bovine serum) (Biochrom, 50115)
- DMEM (Dulbecco's modified Eagle's medium) (Sigma-Aldrich, D5648)
- IMDM (Iscove's modified Dulbecco's medium), (Sigma Aldrich, I7633)
- Opti-MEM (Gibco, 26600134)
- Chloroquine (Sigma, C6628)
- Petri dishes, 150 mm (CELLSTAR, Greiner)
- Roller bottles (CELLMASTER, Greiner, Ribbed surface)
- pMDLg/pRRE (HIV-1 pGag-Pol, Addgene 12251)
- Rev plasmid (pRSV-Rev, Addgene 12253)
- pMD2.G (pVSV-G, Addgene 12259)
- LV-RFP plasmid (Addgene, 26001)

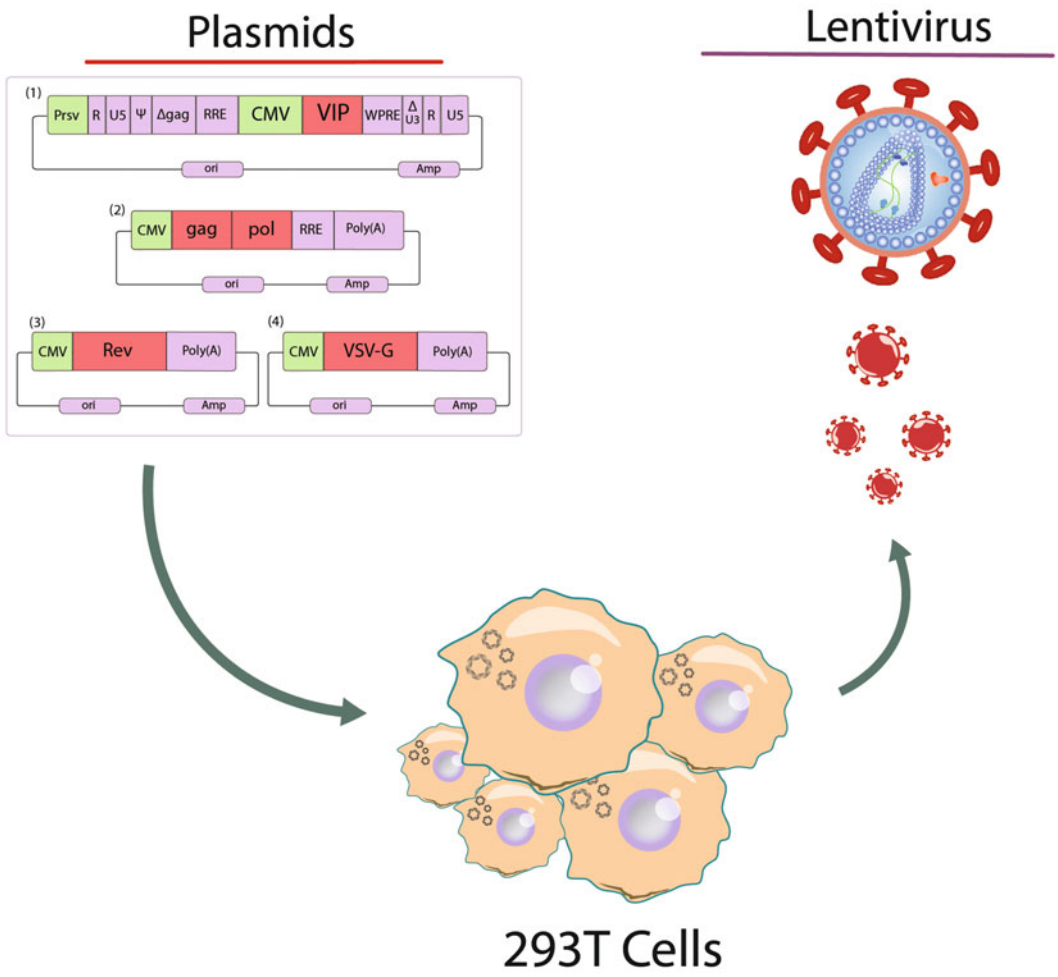

Fig. 1 Transient transfection method for LV vector production. In transient transfection, the transfer vector, packaging, and envelope-coding elements are introduced into the 293T cells via a transfection agent such as $CaPO_4$. Cells produce the vectors in the following few days after transfection. Vectors released from the cells at the end of the process are isolated from the cell supernatant

2.1 Recipes

- 1× PBS (phosphate buffered saline):
  137 mM NaCl
  2.7 mM KCl
  4.3 mM Na_2HPO_4
  1.47 mM KH_2PO_4
  Dissolve the reagents listed above in 800 ml dH_2O
  Adjust the pH to 7.4
  Add distilled water to a total volume of 1 L
  Sterilize solution by autoclaving at 121 °C for 15 min on liquid cycle

Store at +4 °C

- DMEM:

  One bottle of powder DMEM (high glucose), Sigma D5648

  Dissolve in 800 ml dH_2O

  Add 3.7 g $NaHCO_3$

  Add distilled water to a total volume of 1 L dH_2O

  Sterilize solution through a 0.22-μm bottle-top filter

  Add 10% (v/v) FBS, 1% (v/v) Na-pyruvate, and 1% (v/v) pen-strep to 1 L DMEM under aseptic conditions in a Class II Laminar Flow Cabin

  Store at +4 °C

- Opti-MEM:

  13.59 g Opti-MEM Gibco 26600134

  2.4 g $NaHCO_3$

  Dissolve in 800 ml dH_2O

  Adjust the pH to 7.3

  Add distilled water to a total volume of 1 L

  Sterilize solution through a 0.22-μm bottle-top filter

  Store at +4 °C

  Add 10% (v/v) FBS and 1% (v/v) pen-strep to 1 L Opti-MEM under aseptic conditions in a Class II Laminar Flow Cabin prior to use

- IMDM:

  IMDM, powder, Sigma I7633

  Dissolve in 800 ml dH_2O

  Adjust the pH to 7.2

  Add distilled water to a total volume of 1 L

  Sterilize solution through 0.22 μm bottle-top filter

  Store at +4 °C

  Add 10% (v/v) FBS, 1% (v/v) pen-strep, and 25 μM chloroquine to 1 L IMDM under aseptic conditions in a Class II Laminar Flow Cabin prior to use

- 2× HBS (HEPES-buffered solution):

  280 mM NaCl

  10 mM KCl

  1.5 mM Na_2HPO_4 (anhydrous)

  50 mM HEPES

  12 mM Glucose

Dissolve in 800 ml dH_2O
Adjust the pH to 7.05
Add distilled water to a total volume of 1 L
Sterilize solution by 0.22 μm bottle-top filter
Aliquot solution and store at −20 °C

- 2 M $CaCl_2$:
  2 M $CaCl_2$ (anhydrous)
  Dissolve in double distilled water (ddH_2O)
  Sterilize solution through 0.22 μm bottle-top filter
  Aliquot solution and store at −20 °C
- 1× Tris–EDTA (TE):
  10 mM TRIS, pH 8.0
  1 mM EDTA, pH 8.0
  Dissolve in dH_2O
- 0.1× Tris–EDTA (TE): dH_2O solution:
  Dilute 1× TE tenfold to prepare 0.1× TE.
  Mix 2 volumes of 0.1× TE solution and 1 volume of dH_2O.
  Sterilize solution through 0.22 μm bottle-top filter.
  Store at +4 °C.
- HBSS (Hanks' balanced salt solution):
  Buffer 1: Dissolve 8 g NaCl, 0.4 g KCl, and 1 g glucose in 100 ml dH_2O.
  Buffer 2: Dissolve 0.358 g Na_2HPO_4 (anhydrous) and 0.6 g KH_2PO_4, in 100 ml dH_2O.
  Buffer 3: Dissolve 0.73 g $CaCl_2$ in 50 ml dH_2O.
  Buffer 4: Dissolve 1.23 g $MgSO_4{\cdot}7H_2O$ in 50 ml dH_2O.
  Buffer 5: Dissolve 0.35 g $NaHCO_3$ in 10 ml dH_2O.
  PREMIX buffer: Mix 10 ml #1, 1 ml #2, 1 ml #3, 1 ml #4, and 86 ml dH_2O.
  Add 0.1 ml #5 solution to 9.9 ml PREMIX.
  Sterilize solution by 0.22 μm bottle-top filter and store at +4 °C.

2.2 Instruments

- Thermo HeraCell240i CO_2
- Beckman Coulter, Optima L-90K, 365670
- Beckman SW28, 342204
- Multiskan Spectrum Spectrophotometer
- Thermo Class II Laminar Flow Cabin
- Thermo Multi RF Centrifuge

3 Methods

Transfection via calcium phosphate ($CaPO_4$) precipitation is the most widely used method for production of LVs, as a cost-effective, readily applicable, and reproducible method with easily obtained components [31–33]. Nevertheless, the transfection efficiency is directly associated with parameters such as DNA–$CaPO_4$ precipitation, amount of plasmid DNA, calcium and phosphate concentrations, temperature, and duration of the procedure [34]. Thus, various factors should be taken into consideration for successful optimization of the process (*see* **Note 5**).

Precipitate size substantially affects the success of transfection, where small precipitate volumes lead to higher transfection efficiency. As increased incubation times during transfection, on the other hand, will lead to larger precipitates, shorter incubation times should be preferred (*see* **Note 4**) [34]. Another variable affecting the precipitate size is the technique used in preparation of the transfection mixture. Two different methods known as the bubble and vortex techniques were tested to define the more efficient method in our protocol, taking into consideration that the amount of precipitate affects transfection efficiency in both cases. Another factor affecting the transfection yield is the plasmid DNA amounts to be introduced into the producer cell line. Many studies that aimed to define the optimum plasmid amounts for successful transfection in the presence of multiple plasmids conclude that exceeding amounts of transfer plasmid introduced compared to the packaging and VSV-G plasmids result in much higher production yields [27, 35]. Yet the optimum amounts change substantially depending on the structure of the vector used. After extensive literature search, plasmid amounts to be used for transfection into 80–90% confluent cells in a 150-mm petri dish were decided as: 14 μg for Gag/Pol; 6 μg for Rev; 7.5 μg for VSV-G; and 22.5 μg for the transfer vector (RFP) (*see* **Note 2**).

For production of high-titer LVs, it is essential to maintain viability of the cell line and stability of the changing physicochemical conditions during the transfection procedure (*see* **Note 3**). Basic transfection methods are not sufficient particularly for large-scale processes, thus additional agents are required for increased efficiency. Most preferred of these are sodium butyrate, chloroquine, cholesterol, and lipids [35–37]. Chloroquine is an amine that raises endosomal and lysosomal pH levels; the increase in lysosomal pH in turn is believed to prevent degradation of the transfected DNA (*see* **Note 6**) [38, 39]. However, an optimized concentration and duration of exposure should be established to avoid its concentration-, time- and cell type-dependent toxic effects [39]. Two different chloroquine concentrations were used in this study, as 25 and 40 μM [40] (*see* **Note 8**).

Production yield was evaluated by RFP fluorescence. Viral particle numbers following ultracentrifugation were calculated in terms of the integrated copy number via quantitative PCR. Following all optimization studies, 10^{11} transduction units (TU) of lentiviral vector production were established in each roller bottle, and similar concentrations of virus could be obtained from each roller bottle via application of the optimized protocol [41, 42].

3.1 $CaPO_4$ Transfection Method

$CaPO_4$ transfection may be done via the bubble technique or the vortex method:

3.1.1 Bubble Technique

1. Isolate and determine the concentrations of Gag/Pol, Rev, VSV-G, and transfer plasmids (*see* **Note 7**).
2. Calculate the required amounts of plasmids.
3. Add required volumes of plasmids into a Falcon tube.
4. Add 0.1× TE:dH_2O solution into the plasmid mixture to a total volume of 875 μl.
5. Add 125 μl 2 M $CaCl_2$, to obtain a final concentration of 0.25 M in a total volume of 1 ml.
6. Add 1 ml 2× HBS solution in a separate Falcon tube.
7. Create air bubbles in HBS solution by the help of a pipette controller.
8. Meanwhile, add the DNA–$CaCl_2$ mixture dropwise.
9. Incubate the final mixture for 5 min at room temperature.

3.1.2 Vortex Method

1. Isolate and determine the concentrations of Gag/Pol, Rev, VSV-G, and transfer plasmids (*see* **Note 7**).
2. Calculate the required amounts of plasmids.
3. Add required volumes of plasmids into a Falcon tube.
4. Add 0.1× TE:dH_2O solution into the plasmid mixture to a total volume of 875 μl.
5. Add 125 μl 2 M $CaCl_2$, to obtain a final concentration of 0.25 M in a total volume of 1 ml.
6. Add 1 ml 2× HBS solution in a separate Falcon tube.
7. Adjust vortex speed to medium level.
8. Create a constant vortex in 2× HBS solution.
9. Meanwhile, add the DNA–$CaCl_2$ mixture dropwise.
10. Incubate the final mixture for 5 min at room temperature.

3.2 Lentivirus Production

1. Dissolve 293T cells in 10% FBS-containing DMEM.
2. Passage 293T cells 2× prior to transfection.

3. Add transfection media to a 150-cm^2-surface area petri dish at 80–90% of cell confluence ratio.
4. Add transfection cocktails (prepared by either the bubble or the vortex technique) dropwise, following addition of the transfection media.
5. Incubate transfected cells for 4 days at 37 °C in a CO_2 incubator.
6. Observe RFP signals at 24, 48, 72, and 96 h.

3.2.1 Optimization of the Production Conditions

Besides the particle sizes, amount of medium and FBS are also known to affect the transfection yield in LV production. Thus, the optimum production conditions should be set accordingly. In this protocol, different media were used for transfection and collection purposes, and optimization studies were performed under three different conditions (Table 1). RFP plasmid was used as transfer vector in all trials, for the follow-up of the efficiencies of the procedures tested.

At the end of the protocols, use of 10% FBS-containing medium along with the bubble technique appeared as the most efficient method (Fig. 2).

Further optimization studies under eight different conditions were also carried out, where two different chloroquine concentrations (25 and 40 μM) and media with different contents were used, including Opti-MEM, which was reported to enable higher viral titer yields. These conditions are summarized in Table 2.

As evident from the RFP fluorescence signals in Fig. 3, panels a and b, optimization studies enabled determination of the optimum transfection and production media, and detection of the optimum chloroquine concentration [40, 41].

Table 1
Three different conditions for LV production optimization

	Transfection media	Collection media
Exp A	Opti-MEM 2% FBS 1% penicillin/streptomycin	DMEM 2% FBS 1% penicillin/streptomycin
Exp B	Opti-MEM 2% FBS 1% penicillin/streptomycin	DMEM 10% FBS 1% penicillin/streptomycin
Exp C	Opti-MEM 10% FBS 1% penicillin/streptomycin	DMEM 10% FBS 1% penicillin/streptomycin

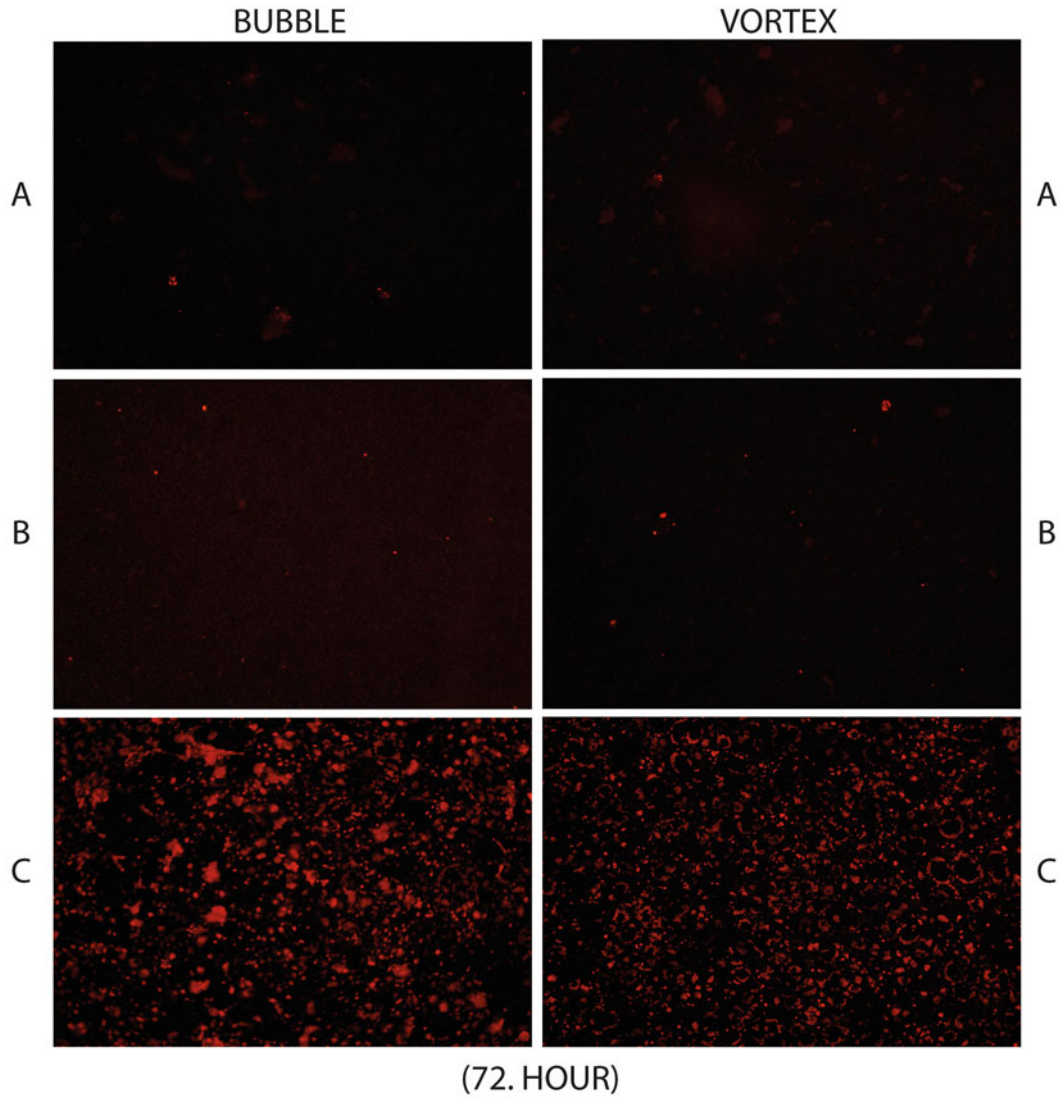

Fig. 2 Comparison of the LV production efficiencies of the bubble and vortex methods 72 h after transfection under three different conditions (**a**, **b**, and **c** panels refer to the different conditions given in Table 1)

3.2.2 Scaling of the Optimized LV Production

Besides optimization of the production conditions, formation of an upscalable protocol is also very significant in LV production. For this purpose, testing of the applicability of the protocol for production in roller bottles is important. A primary task is to define the optimum seeding density and culturing rates for the 293T cells to reach the desired confluency in roller bottles.

Cell Culture in Roller Bottles

The surface area where cells can adhere and grow in roller bottles is approximately 1700 cm^2, whereas the surface area of a 150-mm petri dish is approximately 150 cm^2. Thus, the number of cells

Table 2
Eight different conditions for optimization of LV production in terms of the optimum chloroquine concentration and media combinations

	Transfection media	Collection media
Exp 1	IMDM 25 μM chloroquine 10% FBS 1% penicillin/streptomycin	Opti-MEM 10% FBS 1% penicillin/streptomycin
Exp 2	IMDM 40 μM chloroquine 10% FBS 1% penicillin/streptomycin	Opti-MEM 10% FBS 1% penicillin/streptomycin
Exp 3	Opti-MEM 25 μM chloroquine 10% FBS 1% penicillin/streptomycin	Opti-MEM 10% FBS 1% penicillin/streptomycin
Exp 4	Opti-MEM 25 μM chloroquine 10% FBS 1% penicillin/streptomycin	Opti-MEM 10% FBS 1% penicillin/streptomycin
Exp 5	IMDM 25 μM chloroquine 10% FBS 1% penicillin/streptomycin	OPTIMEM 2% FBS 1% penicillin/streptomycin
Exp 6	IMDM 40 μM chloroquine 10% FBS 1% penicillin/streptomycin	Opti-MEM 2% FBS 1% penicillin/streptomycin
Exp 7	Opti-MEM 25 μM chloroquine 10% FBS 1% penicillin/streptomycin	Opti-MEM 2% FBS 1% penicillin/streptomycin
Exp 8	Opti-MEM 25 μM chloroquine 10% FBS 1% penicillin/streptomycin	Opti-MEM 2% FBS 1% penicillin/streptomycin

grown in a single roller bottle culture system roughly corresponds to that grown in 12 petri dishes.

1. First, a single flask of cells that reach a suitable density for transfection are trypsinized and counted, to define the time it will take 293T cells to reach 80–90% confluency in roller bottles.
2. Cells are seeded into roller bottles with 200 ml DMEM medium and followed for confluency.

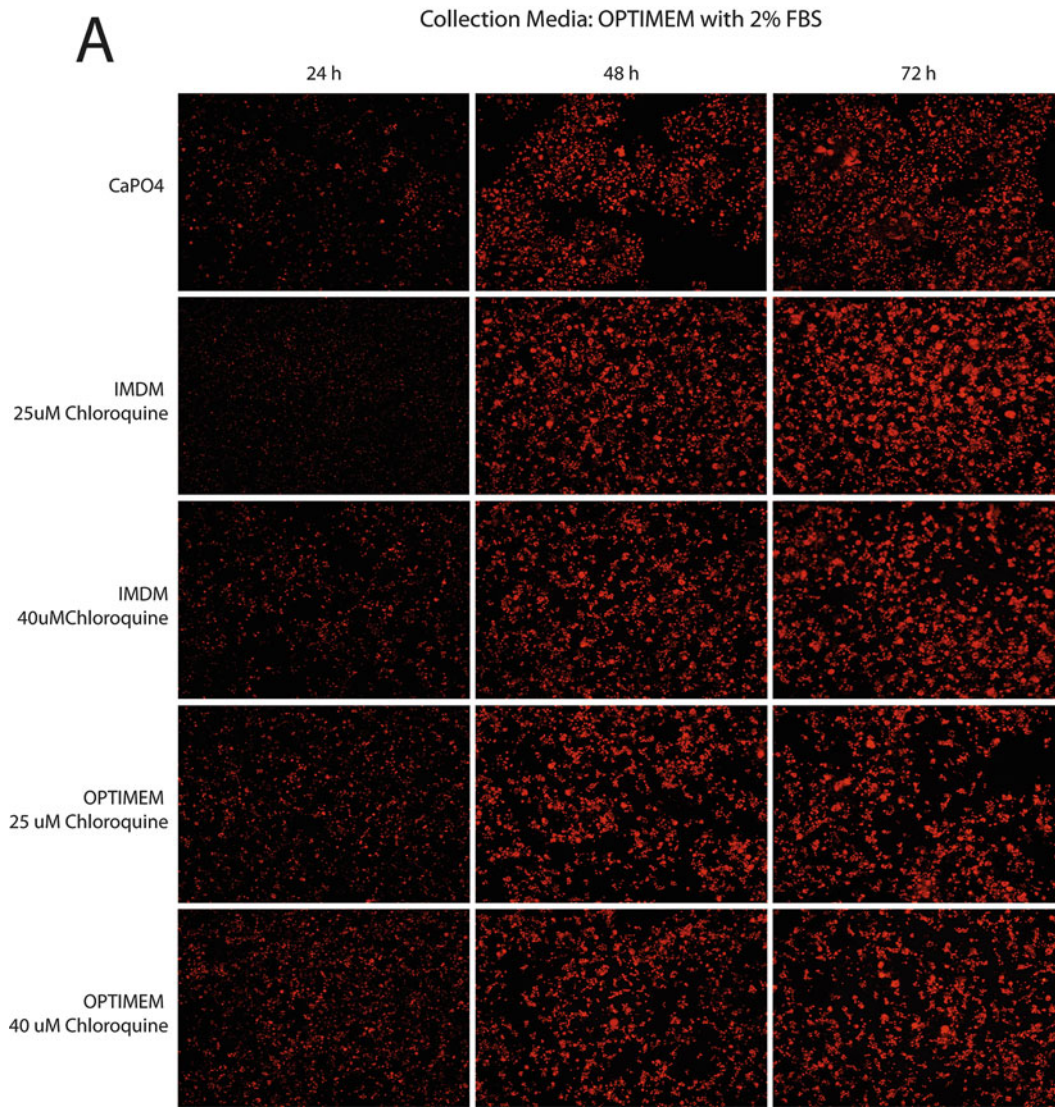

Fig. 3 RFP fluorescence signals at 24, 48, and 72 h of LV production with (**a**) 2% fetal bovine serum (FBS), and (**b**) 10% FBS-containing Opti-MEM used as collection media, along with four different combinations of Iscove's modified Dulbecco's medium (IMDM) and Opti-MEM media and 25 or 40 μM chloroquine at the transfection stage

3. Cells trypsinized from 12 different petri dishes are seeded in roller cell culture bottles, followed by overnight incubation in two different rates as 0.3 and 1 rpm.
4. Following trypsinization the next day, the rate at which 90% of the cells are attached to the surface is detected.
5. After the optimum rate for high attachment is defined, bottles with different densities of cells seeded are subject to incubation at different rates and different durations.

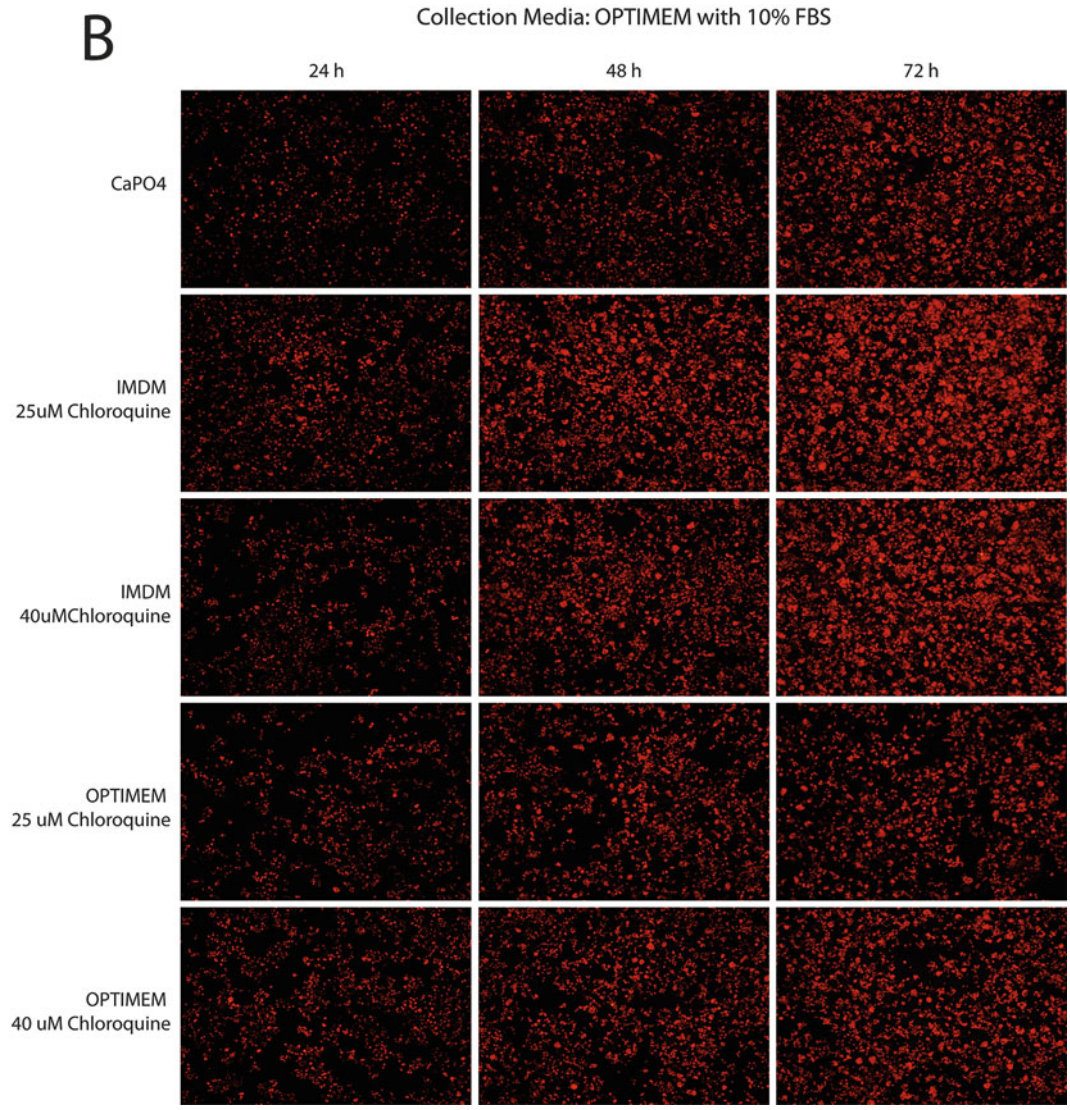

Fig. 3 (continued)

6. Following incubation, cells in the roller bottles are trypsinized and cells counted separately.
7. Optimum parameters for obtaining cell numbers corresponding to 12 petri dishes are defined (Table 3).

According to our results, 293T cells were ready for transfection under the seeding density and revolution time specified in the fifth setup (Fig. 4) [40, 41].

Table 3
Optimization parameters for roller bottle cell culture seeding density, revolution speed, and incubation time

	Seeding density	Speed/time
1. Setup	Two petri dishes (40×10^6 cells)	0.3 rpm/48 h
2. Setup	Three petri dishes (60×10^6 cells)	0.3 rpm/48 h
3. Setup	Four petri dishes (80×10^6 cells)	0.3 rpm/48 h
4. Setup	Four petri dishes (80×10^6 cells)	0.3 rpm/24 h 1 rpm/24 h
5. Setup	Four petri dishes (80×10^6 cells)	0.3 rpm/24 h 1 rpm/16 h

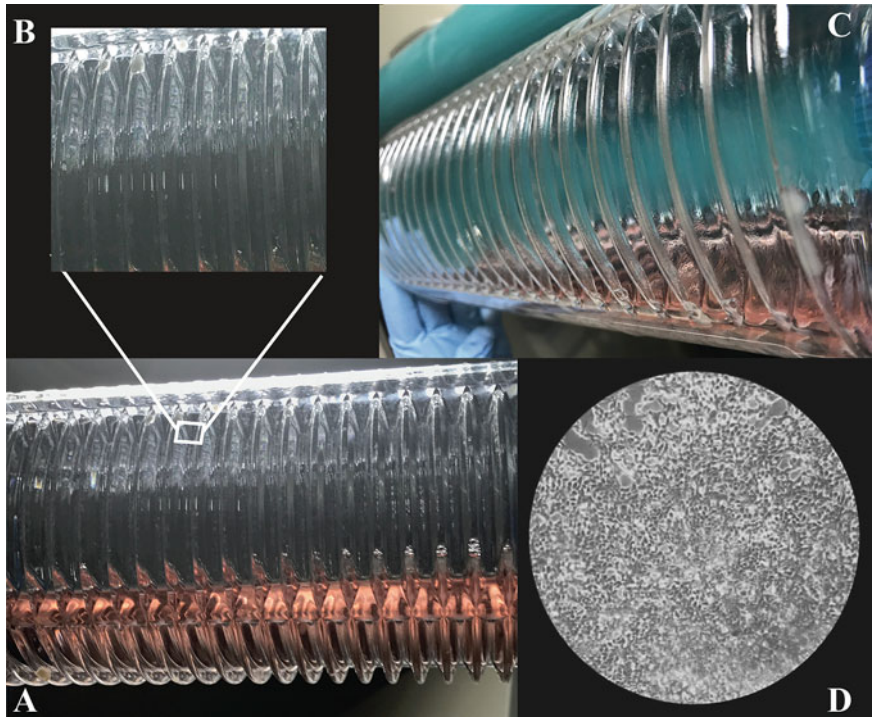

Fig. 4 Cells ready for transfection in roller bottles with ribbed surfaces: (**a**) cells ready for transfection, seeded in roller cell culture bottles, (**b**) closer view of cells attached to the ribbed surface, (**c**) view of a roller cell culture bottle that has a 1700 cm^2 surface area, and (**d**) 293T cells that have reached 100% confluence in 150 mm petri dishes

Table 4
Exemplary calculation of the required plasmid amounts

Plasmid	Required amount	Concentration (μg/μl)	Volume required
Gag/Pol	176	3.02	58
Rev	68	2.9	24
VSV-G	95	5.4	18
RFP (transfer vector)	270	3.1	87

LV Production in Roller Bottles Via $CaPO_4$ Transfection

Cell numbers used for transfection in a single petri dish were adapted to the roller bottles. Accordingly, as the surface area of a single roller bottle corresponds to that of a total of 12 petri dishes, amounts of all solutions were multiplied by 12. Sample volume calculations are given in Table 4.

3.3 Optimized LV Production Protocol in Roller Cell Culture Bottles

HIV-based third-generation LV production via $CaPO_4$ transfection method was optimized in roller cell culture bottles following the steps specified above (Fig. 5) [40, 41].

1. Culture 293T cells in petri dishes that have a 15-cm^2 surface area.
2. Trypsinize four of the petri dishes when cells are 100% confluent.
3. Add trypsinized cells to 200 ml DMEM medium (10% FBS and 1% pen-strep) and transfer to a single roller bottle.
4. Incubate cells in a roller bottle incubator for 24 h at 0.3 rpm speed, allowing them to adhere.
5. Adjust speed to 1 rpm for 16 h for cell expansion (Fig. 6a).
6. Replace the culture media with 180 ml transfection media (IMDM containing 10% FBS and 25 μM chloroquine).
7. Incubate roller bottles for 30 min at a speed of 0.3 rpm.
8. Add required amounts of Gag/Pol, Rev, VSV-G, and transfer plasmids into a Falcon tube.
9. Add 0.1× TE: dH_2O mixture to a total volume of 10,500 μl.
10. Add 1,5 ml 2 M $CaCl_2$, to obtain a final concentration of 0.25 M in a total volume of 12 ml.
11. Add 12 ml 2× HBS in a separate Falcon tube.
12. Add DNA–$CaCl_2$ mixture dropwise using the bubble technique.
13. Incubate the final mixture for 5 min at RT to obtain surface neutralization (Fig. 6b).

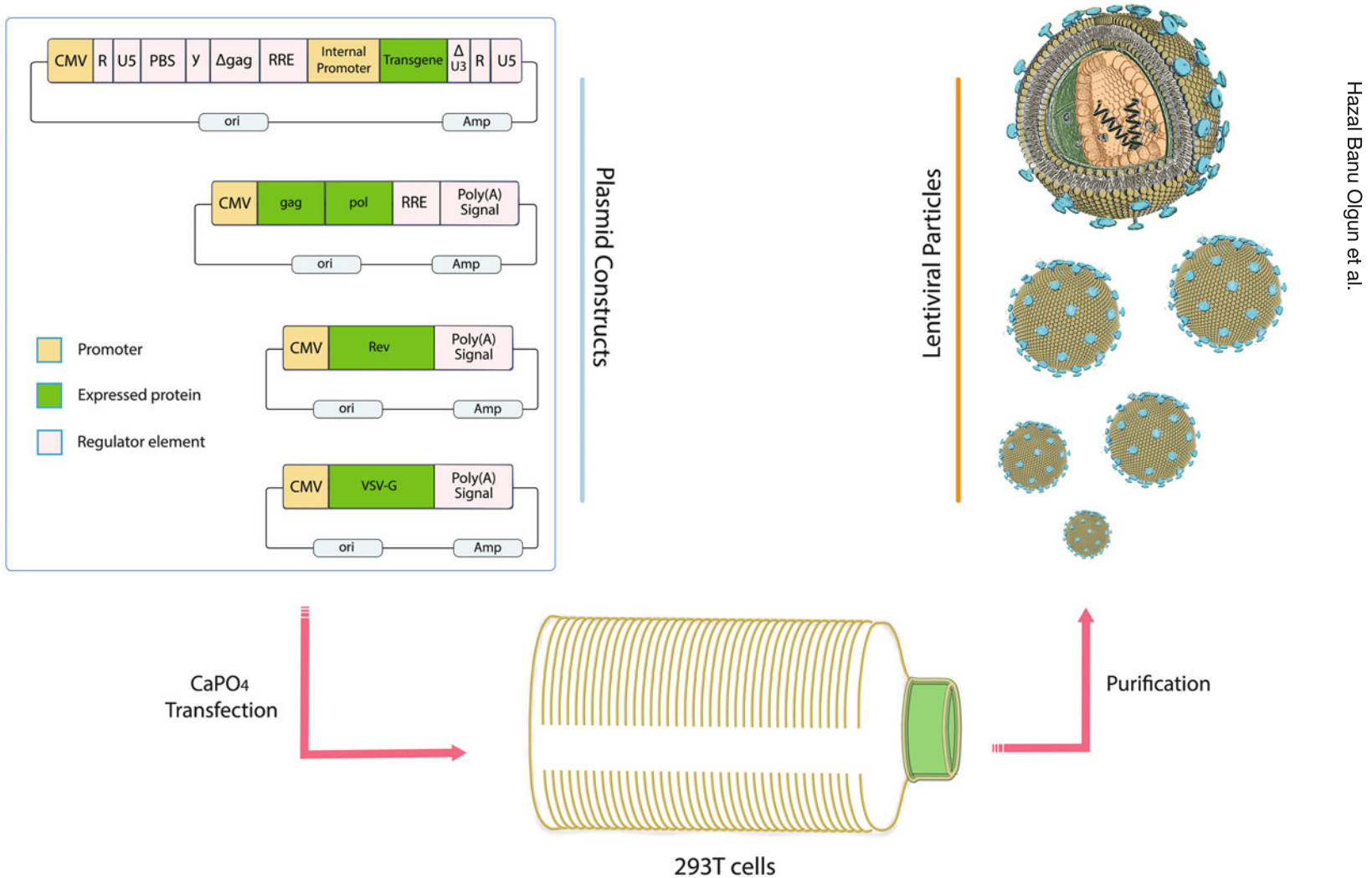

Fig. 5 Figure schematizing production of HIV-based third-generation LVs via $CaPO_4$ transfection method. Image created by Yunus Emre Eksi, MSc

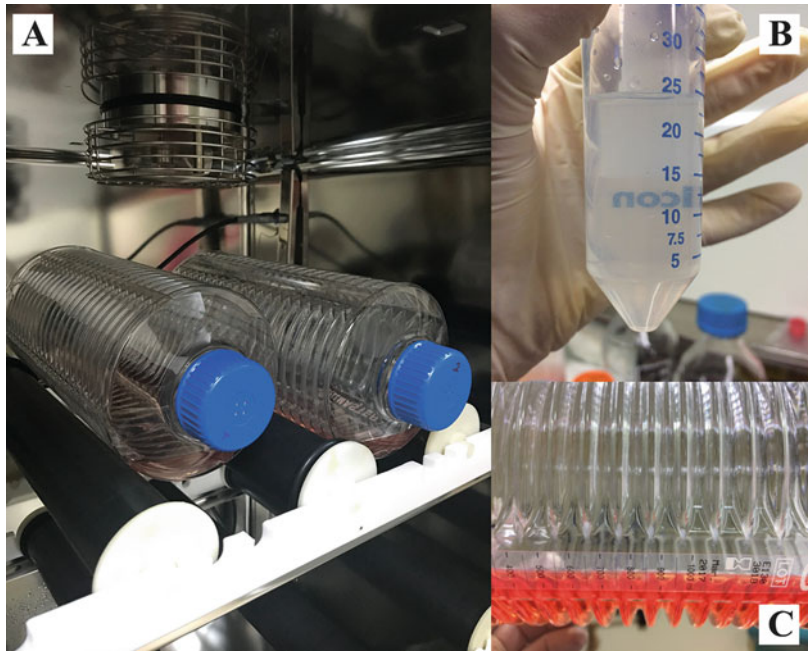

Fig. 6 Figure showing LV production via $CaPO_4$ transfection following cell culture, and transfer into chloroquine-containing medium. (**a**) Incubation of cells ready for transfection, (**b**) preparation of the transfection cocktail, and (**c**) transfer of the transfection cocktail onto the cells in chloroquine-containing IMDM

14. Transfer the prepared transfection cocktail dropwise to the roller bottle containing the transfection media (Fig. 6c).
15. Incubate roller bottles at the speed of 0.3 rpm for 8 h at 37 °C in a CO_2 incubator.
16. Change chloroquine-containing transfection media with 10% FBS-containing Opti-MEM.
17. Incubate roller bottles at 37 °C in a CO_2 incubator up until 72 h following transfection.
18. Harvest the virus-containing supernatants when the incubation time is completed.
19. Centrifuge viral supernatants at 2000 × *g* for 15 min to cleanse the supernatant from cell debris.
20. Filter the viral supernatants through a 0.45-μm vacuum filter.
21. Meanwhile, sterilize the ultracentrifuge tubes by UV irradiation.
22. Dispense viral supernatants as 30 ml per tube.
23. Create a sucrose cushion at the bottom of the tube with 5 ml sucrose solution (10% (v/v)).
24. Concentrate viral supernatants by ultracentrifugation at ~ 82,000 × *g*, 4 °C for 2.5 h (Fig. 7a).

Fig. 7 Ultracentrifugation procedure following the production process. (**a**) Ultracentrifugation instrument, (**b**) viral pellets obtained following ultracentrifugation, and (**c**) schematic representation of the lentivirus. Image created by Yunus Emre Eksi, MSc

25. Discard the supernatants following ultracentrifugation and resuspend the viral pellets in HBSS (Fig. 7b).
26. Collect LVs after resuspension and store aliquoted viral particles at −80 °C, available as ready-to-use.

4 Notes

1. In PEI-based transfections used as an alternative to $CaPO_4$ transfection, parameters such as PEI:DNA ratio, and polyplex amounts per cell differ between experiments that are performed with different media, cell line, plasmid structure, etc. [43, 44]; thus, optimization of the PEI-based techniques should be evaluated accordingly [30, 45].
2. The success of the LV production is directly related to many different parameters such as the cell line used, size of the expression vector, whether a transfection agent is used or not, concentration of the transfection agent, the action mechanism of the transfection agent, and even the nature of the protein expressed from the transgene [28].
3. Although the $CaPO_4$ method provides a high transfection efficiency, it is negatively affected from changes in experimental

parameters such as the pH, precipitation kinetics, and impurities in solutions [27, 32].

4. In formation of the DNA/$CaPO_4$ precipitate, lengthened incubation times cause increased precipitation volumes, thus a decrease in transfection efficiency.
5. Precipitation in $CaPO_4$ transfection is carried out at RT in the presence of HBS at pH 7.05, containing 125 mM Ca^{2+} and 0.75 mM Na_2HPO_4 [46].
6. During $CaPO_4$ transfection in impure solutions, calcium ions in the precipitate may be substituted with ions such as Mg^{2+}, Pb^{2+}, and Zn^{2+}, while phosphate ions may be exchanged with molecules such as carbohydrates in the medium or cellular CO_2. The resulting alterations in CO_2 and CO_3^{+} concentrations lead to pH changes, thus affect the efficiency of the transfection. Buffer solutions such as HEPES should be added into the medium to avoid sudden pH changes [47, 48].
7. The quantity, concentration, and purity of the plasmid DNAs that are introduced into the producer cell line affect the transfection yield. The purity of the plasmids obtained after isolation should be very close to an A260/280 value of 1.8. Quite pure and high concentration yields are obtained in plasmid isolation via the widely used commercial kits, compared to the cesium chloride and ethidium bromide-based isolation protocols. For production of LVs via $CaPO_4$ transfection, the required recombinant DNA amounts are given generally as 1–15 μg/1×10^6 cells [27, 30, 49]. Optimization studies should be carried out for determination of the optimum plasmid amounts to be used in transfections where multiple plasmids are used. Exceeding amounts of transfer plasmid used compared to the other plasmids provide much higher production yields [27, 35].
8. The action mechanisms of the transfection agents to be used in production should be well characterized. If the reagents used are capable of affecting cell viability and transfection, the optimum application method should be defined. If chloroquine is used, cell exposure should not exceed 8–12 h, to avoid toxicity and the resulting decrease in cell viability and viral titers.

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