Video Article

Efficient Recombinant Parvovirus Production with the Help of Adenovirus-derived Systems

Nazim El-Andaloussi*[1,2], Barbara Leuchs*[1], Serena Bonifati[1,2], Jean Rommelaere[1,2], Antonio Marchini[1,2]

[1]Tumour Virology Division F010, German Cancer Research Center (DKFZ)

[2]Inserm Unit 701, German Cancer Research Center (DKFZ)

*These authors contributed equally

Correspondence to: Antonio Marchini at a.marchini@dkfz.de

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Abstract

Rodent parvoviruses (PV) such as rat H-1PV and MVM, are small icosahedral, single stranded, DNA viruses. Their genome includes two promoters P4 and P38 which regulate the expression of non-structural (NS1 and NS2) and capsid proteins (VP1 and VP2) respectively[1]. They attract high interest as anticancer agents for their oncolytic and oncosuppressive abilities while being non-pathogenic for humans[2]. NS1 is the major effector of viral cytotoxicity[3]. In order to further enhance their natural antineoplastic activities, derivatives from these vectors have been generated by replacing the gene encoding for the capsid proteins with a therapeutic transgene (*e.g.* a cytotoxic polypeptide, cytokine, chemokine, tumour suppressor gene etc.)[4]. The recombinant parvoviruses (recPVs) vector retains the NS1/2 coding sequences and the PV genome telomeres which are necessary for viral DNA amplification and packaging. Production of recPVs occurs only in the producer cells (generally HEK293T), by co-transfecting the cells with a second vector (pCMV-VP) expressing the gene encoding for the VP proteins (**Fig. 1**)[4]. The recPV vectors generated in this way are replication defective. Although recPVs proved to possess enhanced oncotoxic activities with respect to the parental viruses from which they have been generated, their production remains a major challenge and strongly hampers the use of these agents in anti-cancer clinical applications.

We found that introduction of an Ad-5 derived vector containing the *E2a, E4(orf6)* and the *VA RNA* genes (*e.g.* pXX6 plasmid) into HEK293T improved the production of recPVs by more than 10 fold in comparison to other protocols in use. Based on this finding, we have constructed a novel Ad-VP-helper that contains the genomic adenoviral elements necessary to enhance recPVs production as well as the parvovirus VP gene unit[5]. The use of Ad-VP-helper, allows production of rec-PVs using a protocol that relies entirely on viral infection steps (as opposed to plasmid transfection), making possible the use of cell lines that are difficult to transfect (*e.g.* NB324K) (**Fig. 2**). We present a method that greatly improves the amount of recombinant virus produced, reducing both the production time and costs, without affecting the quality of the final product[5]. In addition, large scale production of recPV (in suspension cells and bioreactors) is now conceivable.

Video Link

The video component of this article can be found at https://www.jove.com/video/3518/

Protocol

Note that a laboratory with a biosafety level 2 is required for the production of recombinant parvoviruses (recPV).

The protocol is subdivided in two main parts. The first part (production of recPV via transfection) is required to produce the minimal amount of recPVs that serves as inoculum in the second part of the protocol (production of recPVs via infection). Once a small amount of recPVs is produced, the first part of the protocol can be omitted, and the recombinant parvovirus can be amplified only via infection providing the gene encoding for the parvovirus capsid proteins through the adenovirus helper (see below).

1. Production of recPVs via Transfection

1.1 Viral DNA transfection

It is possible to use any established DNA transfection method in this section of the protocol either based on cationic lipids or calcium phosphate. We generally use Fugene HD Transfection Reagent. To control transfection efficiency, we recommend to transfecting additional plate with a plasmid expressing enhanced Green Fluorescent Protein (*e.g.* pEGFP-N1, Clontech). Transfection is considered efficient and optimal for virus production when at least 50% of the cell population results EGFP-positive 24 hours after transfection.

1. 1 day before transfection, seed 2 million HEK293T cells in a 10 cm plate, to obtain 50% cellular confluency at the day of transfection. Grow cells in 10 ml of Dulbecco's Modified Eagle Medium (DMEM) with addition of 10% foetal bovine serum, 2 mM L-glutamine, 100 U per ml Penicillin and 100 µg/ml Streptomycin.
2. Prepare virus-making mixture (1:2:1 molar ratio) in a 1.5 ml tube:
   a. 3 µg of recPV vector harbouring the transgene of interest (e.i. phH-1-GFP[6]).
   b. 6 µg of vector pCMV/VP[5] harbouring the parvovirus capsid gene unit
   c. 8 µg of adenovirus-derived helper plasmid pXX6[7].
3. Dilute the plasmids mix with serum-free medium up to 850 µl (final concentration of 20 ng/µl).
4. Add 42.5 µl Fugene HD (2.5:1 ratio µl Fugene: µg DNA). Do not touch side of tube when adding Fugene to the medium.
5. Vortex gently for 5-10 seconds.
6. Incubate the mixture at room temperature for 30 minutes.
7. Add the transfection mixtures drop-wise to the cells. Shake the plate gently to evenly distribute the mixture throughout.

1.2 Virus production

1. Incubate the plate at 37 °C with 92% humidity and 5% CO_2 for 72 hours before harvest. During this period progeny parvovirus particles are formed from the parvovirus plasmids.

1.3 Virus harvest

1. In a tissue culture hood, scrape the cells within their medium, aspirate and transfer the suspension from the plate into a 15 ml plastic tube.
2. Centrifuge the tube at 250 x g for 5 min at room temperature.
3. Remove 95% of the supernatant.
4. Vortex the tube gently to resuspend the pellet into the remaining supernatant (about 0.5 ml).
5. Freeze the tube at -80 °C. It is possible to stop the production at this stage or to continue with the protocol.
6. Defrost the suspension and bring it at room temperature.
7. Freeze the suspension in a liquid nitrogen bath and thaw in warm water at 37 °C. Vortex the tubes vigorously for 15 sec and repeat the freeze-thaw cycle further two times.
8. Centrifuge the tubes at 16,000x g for 15 min at 4 °C.
9. Collect the supernatant into a fresh tube. The tube at this stage contains the crude virus extract preparation.
10. Proceed with the virus titration (see below). For the recPV carrying the GFP gene, a typical production should give 1-3 x 10^7 viral infectious units corresponding to about 1-3 x 10^{10} viral genome containing particles.

1.4 Virus storage

1. For long-term storage freeze the tube containing the crude virus extract at -80 °C. It is possible to use crude viral extracts as inoculum in the second part of the protocol.

2. Production of recPVs via Infection

Before starting with the recPV production via infection, produce and purify Adenovirus 5 carrying the parvovirus VP gene (Ad-VP helper, described in[5]) according to standard protocol[8]. It is also necessary to have a minimal amount of recPVs carrying the transgene of interest (*e.g.*, produced via transfection as described above).

Below, we describe the recPV production in 175 cm^2 flask (T175) format. Further amplification of the virus stock produced in this way can be conducted in 10 multilayer CellSTACK culture chambers (Corning) with minor adjustments of the protocol proposed.

2.1 Infection

1. 1 day before infection, plate 10 million NB324K cells in a T175 flask to obtain 60-80% cellular confluency at the day of infection. Prepare a second flask containing the same number of cells as a control (non-infected cells). Each T175 should have 20 ml of Minimum Essential Medium (MEM) as a final volume, supplemented with 5% foetal bovine serum, 2 mM L-glutamine, 100U per ml Penicilin and 100 µg/ml Streptomycin.
2. The next morning prepare virus mixture in a 2 ml plastic tube consisting of:
   a. Ad-VP-helper[5] at multiplicity of infection (MOI, infectious units/cell) = 10 (corresponding to 1 x 10^8 infectious units);
   b. recPVs at MOI = 0.5 (corresponding to 5 x 10^6 infectious units). Complete to 1.5 ml with MEM.
3. Apply the entire virus mixture in one of the T175 flask.
4. Incubate the flask for 2 hr at 37 °C, 92% humidity, 5% CO_2 gently shaking every 15 min.
5. Incubate for further 20-24 hr at 37 °C, 92% humidity, 5% CO_2.
6. Replace the cultural medium with fresh medium.

2.2 Virus production

Incubate the flasks at 37 °C with 92% humidity and 5% CO_2 for 48 hours. As indication of efficient viral production, clear signs of cytotoxicity should be observed in the flask containing viral infected cells, starting 36-48 hours post-infection.

2.3 Virus harvest

1. In a tissue culture hood, aspirate and transfer the medium supernatant from the flask into a 50 ml centrifuge tube.

2. Wash the cells twice with 1.5 ml PBS.
3. Trypsinize the cells with 1.5 ml of 0.25 % Trysin-EDTA. Add the cell suspension to the removed medium.
4. Centrifuge the cells and the supernatant at 5,000 x g for 5 min at room temperature.
5. Discard the supernatant.
6. Add 1 ml of TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.7) to the cell pellet and gently vortex the tube to resuspend the cells.
7. Freeze the cell suspension in a liquid nitrogen bath and thaw in warm water at 37 °C. Vortex the tube vigorously for 15 sec and repeat the freeze-thaw cycle three times.
8. Treat the cell suspension with 50 U/ml Benzonase Nuclease for 30 min at 37 °C in order to digest the cell genomic and non-packaged viral DNAs.
9. Centrifuge the tube at 5,000 x g for 20 min at 4 °C.
10. Collect the supernatant into a fresh tube. The supernatant at this stage contains the crude virus extract preparation. Typical yields of recPV-GFP produced following this protocol should be in the range of 1-10 x 10^8 viral infectious units.

2.4 Virus storage

1. For short term storage (maximum 2 months), store virus at 4 °C.
2. For long term storage (longer than 2 months), store virus at -80 °C.

3. RecPV Purification

1. For the purification of recPVs from the crude lysates, we recommend to run an Iodixanol discontinous gradient according to Zolotukhin *et al.* [9]. We recommend using a minimum of 5 ml of crude viral extract (obtained from production in five T175 flasks) for efficient virus purification.

4. Recombinant Parvovirus Titration

1. Perform recPV titration as described in El-Andaloussi *et al.*[5]

5. Quality Controls

1. Use the protocol described in El-Andaloussi *et al.*[5] to check for the presence of undesired replication competent viruses (RCV) carrying out parvovirus plaque assay in NB324K indicator cells.

6. Representative Results

An example of recPVs production via transfection in the presence or absence of adenovirus genomic elements is shown in **Figure 3**. Cells were transfected with pCMV/VP (plasmid carrying the gene encoding for the VP parvovirus capsid proteins) together with phH-1-GFP (a recPV harbouring the *GFP* gene) or phH-1-luciferase (a recPV harbouring the *firefly luciferase* gene), with (+ pXX6) or without (-pXX6) the pXX6 plasmid (carrying the adenovirus E2A, E4(orf6) and VA RNA genes). Equal volume of crude cell extracts were applied to NB324K cells and GFP transduction or luciferase assays were performed as reported in El-Andaloussi *et al.*[5]. A clear increase in the recPVs production was obtained in the presence of pXX6 with production increasing from 0.3 GFP transductional units (TU)/cell obtained according to conventional protocols to approximately 5 TU/cell obtained following our method (**Fig. 3A, B**). A significant increase (about 24-fold) of recPV production was also observed in the case of phH-1-luciferase (**Fig. 3C**).These results indicate that the genetic material contained in pXX6 is able to boost parvovirus production.

A representative example of recPVs production via infection is shown in **Figure 4**. NB324K cells were co-infected with various recPVs (as indicated in the figure) and Ad-VP-helper (harbouring the gene encoding for the PV VP capsid proteins). Ad-VP helper further enhanced recPV production up to >70 TU per seeded cell (**Fig. 4A**) without increasing the occurrence of undesirable replication competent viral particles (**Fig. 4B**).

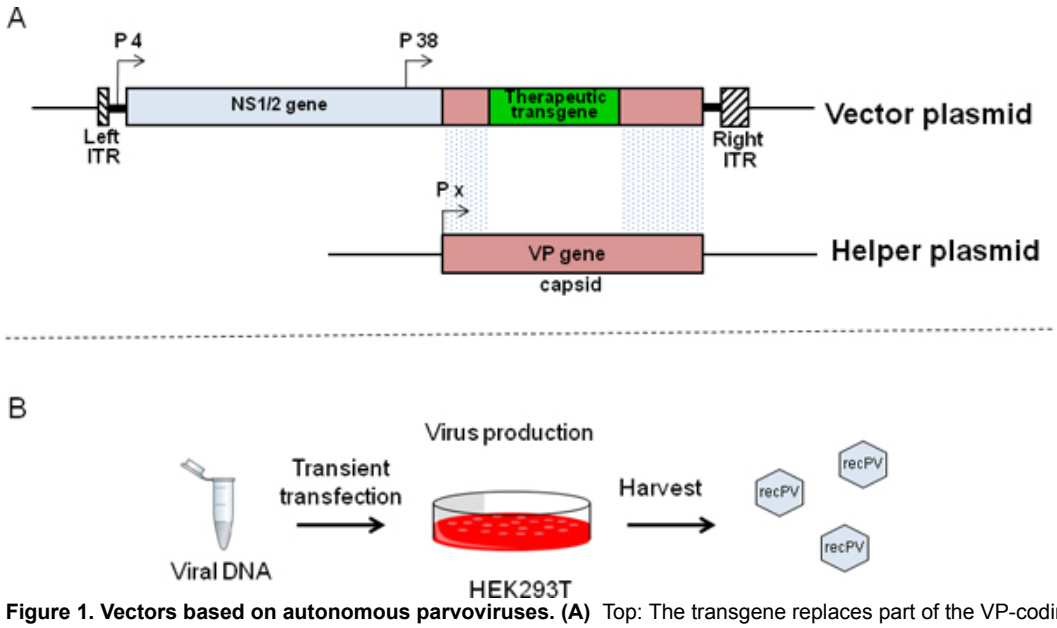

Figure 1. Vectors based on autonomous parvoviruses. (A) Top: The transgene replaces part of the VP-coding genes and is under the control of the viral promoter P38. The genes for NS1/2 are retained and their expression is controlled by the viral promoter P4. The vector genome is flanked by the parvoviral ITRs, which contain *cis*-acting elements that are required for replication and packaging of the recombinant genome. Bottom: A plasmid carrying the VP-gene under either a heterologous (*e.g.* CMV), or autologous (*e.g.* P38) promoter (Px), is supplied *in trans* during recombinant parvovirus production in order to compensate for the disruption of the structural genes in the recombinant genome. ITR, inverted terminal repeat. Figure adapted from [4]. **(B)** Schematic view of the classical protocol used for the production of recPVs. HEK293T cells are transiently transfected with viral DNA (vector and helper plasmids) and after three days, cells are collected and viruses harvested.

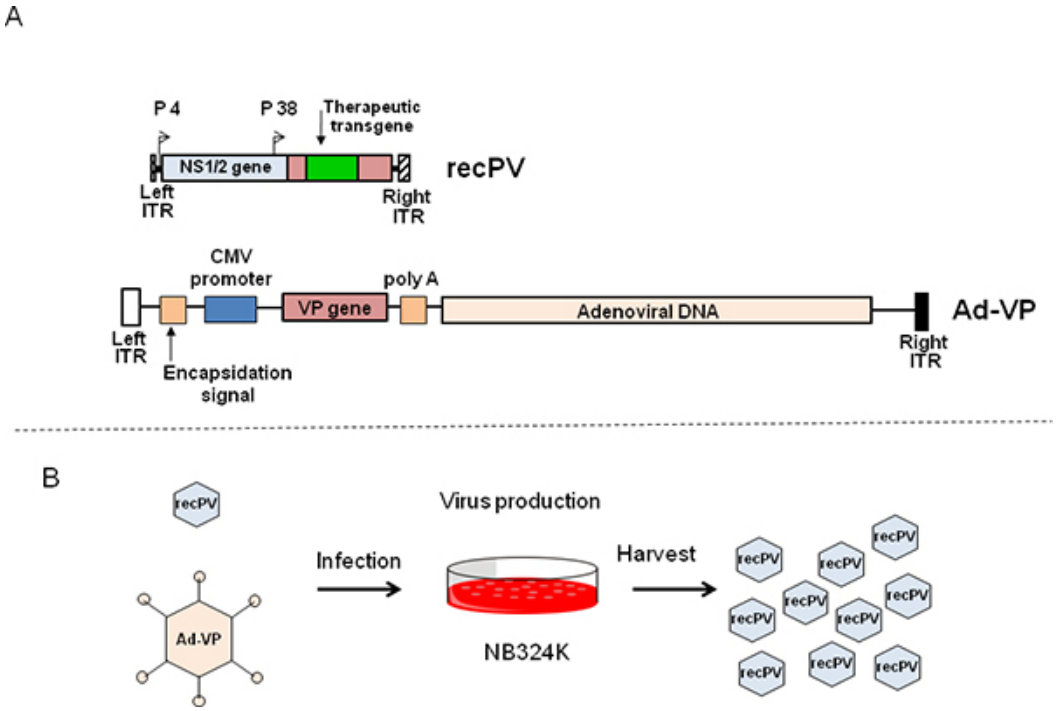

Figure 2. Production of recPVs with the help of the Ad-VP. (A) Schematic maps of recPV and Ad-VP genomes. The recPV contains a heterologous transgene which replaces part of the VP region. The Ad-VP harbors the parvovirus VP gene. **(B)** Schematic view of the protocol described in this manuscript. NB324K cells are co-infected with recPV and Ad-VP viruses. After three days cells are harvested and recPV particles recovered from cell lysate.

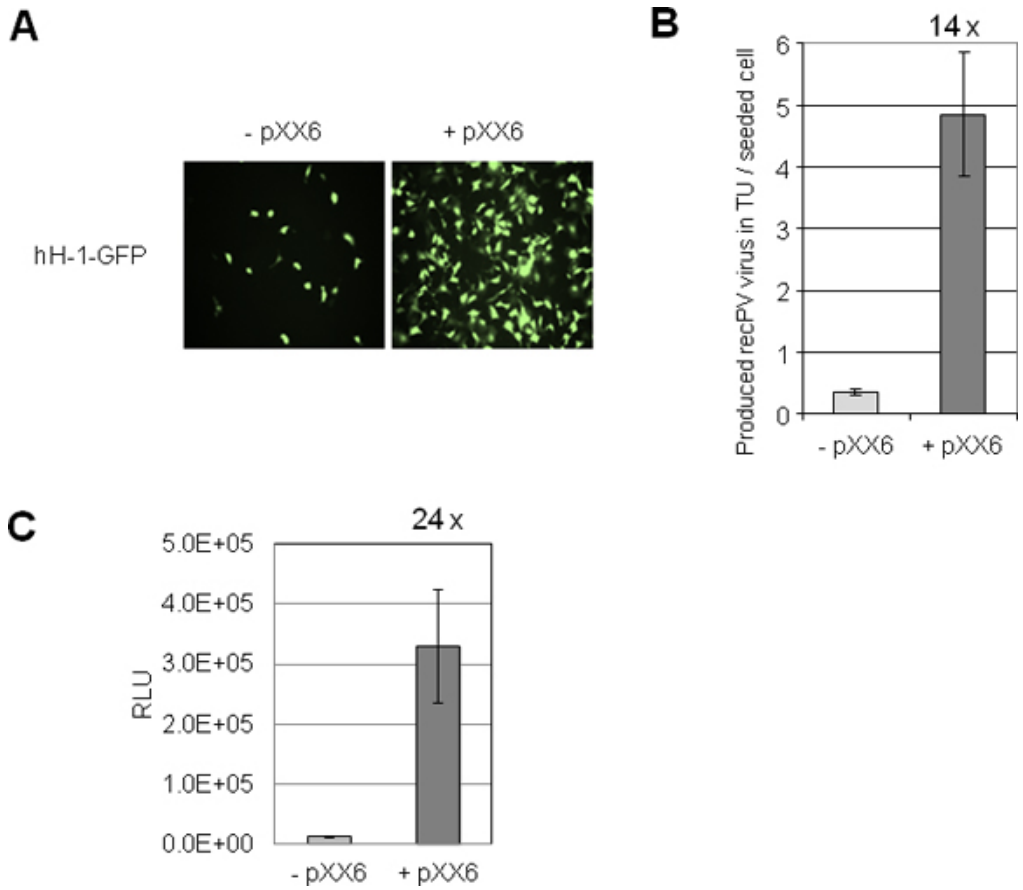

Figure 3. Stimulation of recPV production by adenovirus-based plasmids pXX6 . HEK293T cells, seeded in 10 cm dishes, were transfected with pCMV/VP in combination with phH-1-GFP (A and B) or phH-1-luciferase (C) to produce recPVs. Simultaneously, cells were co-transfected with the adenovirus-derived helper plasmids pXX6 or not, as indicated. Three days post-transfection, cells were harvested and lysed by three freeze and thaw cycles. Equal volumes of crude virus extracts were applied to NBK indicator cells, and transduction assays were carried out. **(A)** Representative micrographs showing GFP positive cells within confluent NB324K monolayers. **(B)** Quantification of the GFP transduction assays expressed in transduction unit (TU) per seeded cell. **(C)** Quantification of the luciferase activity expressed as relative luciferase units (RLU). The luciferase assay was performed as described in El-Andaloussi *et al.*[5]. Columns represent average values from three replicates with standard deviation bars. Number on top of the + pXX6 column, in (B and C), indicates the fold increase in the recPV virus titers, obtained in the presence of pXX6 versus without.

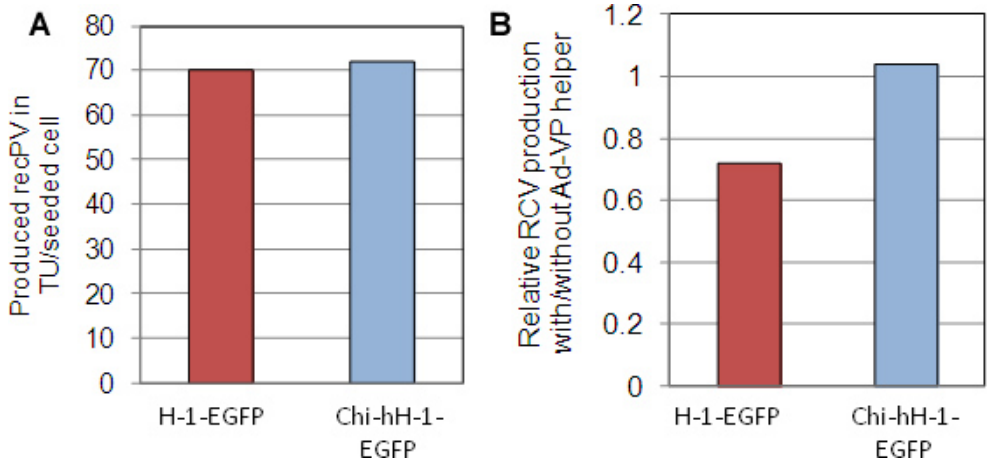

Figure 4. Stimulation of recPV production by means of recombinant Ad-VP helper virus . (A) NB324K cells, were infected with purified Ad-VP helper virus at MOI of 10 (Ad-X unit/cell, titrated with Adeno-X Rapid Titer Kit), and then superinfected with either of the following recombinant parvovirus Chi-hH-1-EGFP[11] (0.1 TU/cell) or H-1-GFP (0.5 TU/cell)[5]. One day post-infection, medium was changed and two days later, cells were collected and lysed via three freeze-and-thaw cycles. Crude cell extracts were used to determine virus titers by transduction assay according to El-Andaloussi *et al.*[5]. TU, transduction unit. **(B)** Viral batches produced in the presence of absence of Ad-VP were analyzed for their content of replication competent viral particles (RCV) by plaque assay on NB324K indicator cells.

Discussion

We have shown that recPV production can be enhanced by the presence of adenoviral genomic elements. We have increased the recPV yields by more than 10 fold (from 0.3 to 5 TU/cell) by providing adenovirus genomic element via transfection and by more than 100 fold co-infecting the cells with Ad-VP-helper in combination with the recPV in comparison to conventional protocols. The protocol described here can be further optimized by determining the most appropriate timing for the delivery of the adenovirus genomic elements and the optimal concentration of Ad-VP helper to be used for the infection.

RecPVs with larger transgenes (more than 1,600 bases) are less efficiently produced. This is probably due to the fact that by inserting the transgene, cis-acting elements necessary for PV proper viral DNA packaging are removed. The optimal size of transgene that can be inserted into the PV genome without affecting its production is up to 700 bases [6]. Also the sort of the transgene inserted may affect recPV production (*e.g.* insertion of genes encoding for cytotoxic proteins may result in lower recPV yields).

Another important aspect to take in consideration during recPVs production is the possible occurrence of replication competent viruses (RCV) that could spontaneously be formed through homologous recombination between the recombinant parvovirus and VP-containing plasmids. The present protocol does not enhance the recombination risk. Nevertheless a RCV quality control should be routinely performed (*e.g.* plaque assay in NB324K indicator cells[5]) at the end of the production in order to assure that viral stocks contain only a negligible amount of RCVs.

The protocol described overcomes previous limitations in the choice of the packaging cell line used, as it renders possible the use of cell lines that are difficult to transfect (and therefore not suitable with protocols based on transfection) but are good producers of PVs. *e.g.* NB324K cells[10] that in our hands were more efficient in producing recPVs than HEK293T (data not shown). A screening of different cell lines is now possible applying the new protocol, and could identify more efficient cells for recPV production. This also paves the way to further exploration of the system for large-scale production of recombinant parvovirus *e.g.* in bioreactors with cells in suspension.

Disclosures

We have nothing to disclose.

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