

Chapter 2

Development of Recombinant Canarypox Viruses Expressing Immunogens

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Abstract

Canarypox viruses (CNPV) are excellent candidates to develop recombinant vector vaccines due to both their capability to induce protective immune responses and their incompetence to replicate in mammalian cells (safety profile). In addition, CNPV and the derived recombinants can be manipulated under biosafety level 1 conditions. There is no commercially available system to obtain recombinant CNPV; however, the methodology and tools required to develop recombinant vaccinia virus (VV), prototype of the *Poxviridae* family, can be easily adapted. This chapter provides protocols for the generation, plaque isolation, molecular characterization, amplification and purification of recombinant CNPV.

Key words Canarypox, Transfer vector, Nonessential region, Transfection, Homologous recombination, Visual screening

1 Introduction

Canarypox viruses (CNPV) have been widely used as vectors for vaccine development due to their safety profile and for the protection they induce against infectious diseases [1–3]. Recombinant viruses are based on an attenuated (vaccine) strain of CNPV which can be amplified in the laboratory in avian cell culture such as primary chicken embryo fibroblasts (CEFs) or in several cell lines.

Poxviruses, such as the canarypox virus, have large DNA genomes (175–375 kbp) making it impossible to directly manipulate them genetically to obtain recombinant viruses for expressing foreign antigens. Instead, recombinant viruses are produced inside the cell by homologous recombination between the poxvirus genome and a plasmid vector (named here as “transfer vector,” TV) carrying the desired gene flanked by viral sequences. Afterwards, the viral progeny are a mixed population of recombinant and non-recombinant poxviruses, however only a small percentage

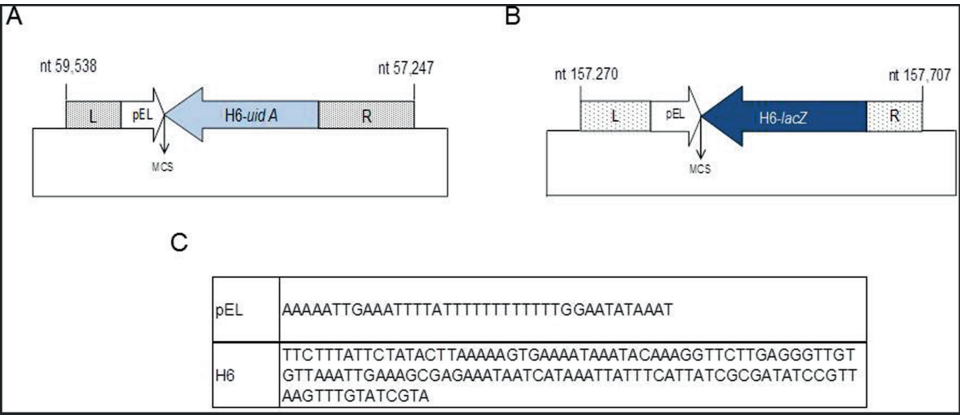


Fig. 1 Scheme of transfer vectors TV-048GUS and TV-134lacZ. TVs have been designed to obtain recombinant CNPV that express a maker enzyme that (a) interrupts the CNPV048 gene or (b) into the intergenic region between the CNPV134 and CNPV135 genes. *pEL* synthetic vaccinia early/late promoter, MCS: multiple cloning site (containing 1 to 5 unique restriction enzyme recognition sites), H6-lacZ: *lac Z* gene (codes for β -galactosidase enzyme) under regulation of vaccinia virus H6 gene promoter, H6-uidA: *uid A* gene (codes for β -glucuronidase) under regulation of vaccinia virus H6 gene promoter, R and L: viral regions which serve as points of recombination with CNPV genome. Genomic nucleotide positions are indicated according to Tulman et al. [14]. (c) DNA sequence of pEL and H6 promoters

(10^{-4} – 10^{-3}) correspond to recombinant virus. Therefore the selection of recombinant viruses, which represent low frequency virus in that mixed progeny, is a vital step for isolation of recombinant CNPVs. The methodology described for the isolation of recombinant canarypox viruses is based on visual screening (through colored lysis plaques) for expression of a marker enzyme (such as β -galactosidase or β -glucuronidase) from the transfected DNA (Fig. 1a, b). This method is not as efficient as those based on direct selection but it is required because no antibiotic/drug resistance gene should be included in the recombinant viral genome that will be used as a vaccine.

2 Materials

2.1 Reagents and Equipment

1. Ultrapure water to prepare solutions.
2. Plasmid purification kits: QIAGEN® Midi, Maxi Kits or Zyppe™ Plasmid Midiprep Kit (Hilden, Germany).
3. Lipofectamine 2000® Reagent (Thermo Fisher Scientific, MA, USA).
4. Phosphate-buffered saline (PBS).
5. 2× Extraction Buffer: 200 mM Tris–HCl pH 8; 20 mM EDTA pH 8; 200 mM NaCl; 2% SDS; 20 mM 2-mercaptoethanol.
6. 5 M potassium acetate.

7. Isopropyl alcohol (99.9%).
8. Ethyl alcohol (70% w/w in distilled water).
9. Cell Scrapers, sterile.
10. NP-40 lysis buffer: 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Nonidet P-40.
11. 6× SDS-Sample Buffer: 375 mM Tris-HCl pH 6.8, 6% SDS, 48% glycerol, 9% 2-mercaptoethanol, 0.03% bromophenol blue.
12. Neutral red (1 mg/mL in water), filter-sterilized.
13. TMN buffer: 0.01 M Tris-HCl pH 7.5, 1.5 mM MgCl₂, 10 mM NaCl.
14. Humidified incubator at 37 °C, 5% CO₂.
15. Water baths (37 and 42 °C).
16. Laminar flow cabinet BSL 2.
17. Inverted microscope.
18. Ultrasonic bath sonicator (e.g., Elmasonic S 30).
19. Ultracentrifuge (e.g., Beckman Coulter), rotor (SW41), and tubes.

2.2 Cells and Virus

1. Cell monolayers of Chicken Embryo Fibroblasts (CEFs) prepared from 11-day-old specific pathogen-free (SPF) embryos [4] or cell lines (e.g., ATCC DF-1, ATCC QM-7, ProBioGen AGE1.CR.pIX®).
2. Canarypox virus: live attenuated vaccine strain (*see Note 1*)

2.3 Cell Culture Media (See Note 2)

1. DMEM: Dulbecco's Modified Eagle Medium, high glucose (4.5 g/mL D-glucose; Gibco®, Thermo Fisher Scientific) supplemented with 3.7 mg/mL sodium bicarbonate, 0.3 mg/mL L-glutamine, 50 µg/mL gentamicin, 66 µg/mL streptomycin, 100 U/mL penicillin.
2. Growth medium: DMEM containing 10% fetal calf serum (FCS).
3. Maintenance medium: DMEM containing 2% FCS.
4. Semisolid overlay medium (first overlay): DMEM containing 2% FCS and 0.7% Low Gelling Temperature (LGT) agarose (SeaPlaque™ Agarose, Lonza, Basel, Switzerland).
5. Semisolid overlay medium with substrate (second overlay): semisolid overlay medium containing enzyme substrate 0.2 mg/mL X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, Inalco S.p. A, Italy) or 0.35 mg/mL Bluo-gal (halogenated indolyl-β-galactoside, Inalco) for β-glucuronidase or β-galactoside, respectively.

2.4 Cell Culture Flasks

1. 25 cm² Tissue Culture Flask (T25).
2. 60 mm cell culture-dish plates (P60).
3. 175 cm² Tissue Culture Flask (T175).

3 Methods

The desired sequence is cloned into the transfer vector (*see Note 3*), under regulation of an early (or early/late) poxvirus promoter (*see Note 4*). The transfer vector is then transfected into CNPV infected CEFs, where the recombination between the viral genome and the TV occurs. Due to the fact that non-recombinant (receptor) CNPV replicate normally, an effective selection/screening method as to be performed to obtain the recombinant CNPV. One strategy involves the screening of the recombinant viruses based on their capability to produced colored (blue) lysis plaques by the expression of a marker enzyme such as β -galactosidase or β -glucuronidase in the presences of a specific chromogenic enzyme substrate. The blue plaques (recombinant virus) are picked and the screening by plaque purification is repeated until a homogenous stock (100% blue plaques) is obtained. Then, the recombinant CNPV is amplified in CEFs to evaluate the presence and expression of the antigen coding sequence. Finally, the recombinant virus is amplified in CEFs and purified through a sucrose cushion.

3.1 Construction and Purification of Transfer Vector

Transfer vectors (TV) carry foreign genes flanked by viral regions which are target sites for recombination with the CNPV genome. In our laboratory two TV have been designed to obtain recombinant CNPV interrupting the CNPV048 gene or the intergenic region between CNPV134 and CNPV135 genes (Fig. 1; [5]). The construction of TV was performed by standard genetic engineering techniques (PCR amplification and cloning). It is also possible to acquire the desired sequences through a service of gene synthesis

1. Subclone the coding sequence of the desired antigen into the CNPV transfer vector downstream of a poxviral promoter. The gene of interest must include its authentic start (ATG) and stop (TAA/TAG/TGA) codons.
2. The correct orientation and identity of the cloned DNA fragment is confirmed by DNA sequencing (*see Note 5*).
3. Prepare a stock of TV plasmid DNA using plasmid purification kits to obtain supercoiled and clean DNA (ultrapure transfection-grade; *see Note 6*)

3.2 Transfection of CNPV-Infected CEFs

Use the following procedure to transfect CEFs monolayer grown on 25 cm² Tissue Culture Flask (T25).

1. One day before transfection seed $3.5\text{--}4.5 \times 10^6$ cells in a T25 to obtain 70–80% confluent the next day (*see Note 7*).

2. Discard the medium, wash the monolayer and add 1 mL of CNPV viral stock to get a multiplicity of infection (moi) of 0.5 (*see Note 8*).
3. Incubate 2 h at 37 °C (with agitation every 20 min) in a humidified CO₂ incubator.
4. Add 5 ml of maintenance medium and incubate 2 h at 37 °C. During this incubation step, prepare complexes for transfection.
5. For each transfection reaction, prepare 5–8 µg DNA (transfer vector) in 20 µl of H₂O and mix with 100 µl DMEM without serum. Mix gently.
6. Mix Lipofectamine 2000® gently before use, then dilute 6.25 µl in 93.75 µl of DMEM (or other medium) without serum. Incubate for 20–30 min at room temperature (without mixing) to stabilize the cationic lipid into cell culture medium.
7. Combine DNA solution with diluted Lipofectamine 2000®. Mix 4–6 times by pipetting and incubate for 15 min at room temperature (complexes are stable for 6 h at room temperature).
8. Remove medium from flask and wash cells two times with DMEM (without serum).
9. For each transfection, add 2 mL of DMEM to the tube containing the complexes DNA/Lipofectamine 2000® and add to each T25 (total volume = 2.24 mL). Mix gently by rocking.
10. Incubate at 37 °C for at least 2 h.
11. To each T25 add 3 mL of growth medium and 40 µl of FCS without removing the transfection mixture. Incubate overnight.
12. Replace medium with fresh maintenance medium.
13. Incubate until cytopathic effect (CPE) is observed (normally between 4 and 5 days).
14. Harvest cells and supernatant, release virus by three freeze–thaw cycles and store at –80 °C before starting the screening of recombinant virus.

3.3 Visual Screening and Plaque Isolation of Recombinant CNPV

Plaque purification of recombinant CNPV will ensure complete removal of the parental virus. Several consecutive rounds (between 8 and 12) of plaque purification have to be performed.

One day before transfection seed 5×10^6 cells in a 60 mm cell culture-dish plates (P60) to obtain 80–90% confluent the next day.

1. Dilute the virus (infection/transfection cell lysate) 1/5 and 1/10 in DMEM (serum free).
2. Add 0.5–1 mL of each dilution to CEFs from which the growth medium has been previously discarded. Infect at least three dish plates per dilution.

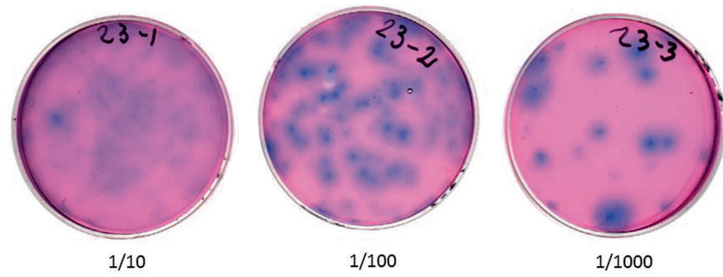


Fig. 2 Screening of recombinant CNPV based on visualization of blue plaques. CEFs grown on 60 mm cell culture-dish plates were infected with recombinant CNPV (diluted 10^{-1} , 10^{-2} , and 10^{-3}) and a second overlay containing X-gluc was added at 4–5 days post-infection

3. Leave the virus on the cells to adsorb for 1 h at 37 °C (tilt dishes every 15–20 min).
4. Remove the inoculum by aspiration or pipetting.
5. Add 3 mL of semisolid overlay medium (first overlay) and incubate for 4–5 days.
Semisolid overlay: prepare DMEM 2× concentrated and add 4% FCS, keep solution at 37 °C. Prepare 1.4% LGT in water, melt using microwave oven (2–3 min at maximum) and keep at 42 °C. Mix equal volume of DMEM/FCS and molten LGT agarose (maintaining the solution at 37 °C), gently add to cell monolayer and leave at room temperature for allowing medium to solidify before introducing into the humidified incubator.
6. CNPV plaques can easily be seen by holding the dish up to the light or using optical microscopy. Add 2.5 mL of semisolid overlay medium with substrate (second overlay).
7. Hopefully the next day it should be possible to see individual blue plaques.
8. Blue plaques are picked with a filtered pipette tip into 500 µL aliquots of DMEM (serum free) in 1.5 mL microfuge tubes.
9. Freeze-thaw × 3 and store at –80 °C.
10. Prepare dilutions (1/5, 1/10 and 1/50 in DMEM) for each picked blue plaque.
11. Repeat steps 2–6.
12. The next day blue plaques have to be seen, picked, freeze-thawed × 3 and stored at –80 °C. This is the first plaque purification step.
13. Prepare dilutions (1/10, 1/100 and 1/1000 in DMEM) for each picked blue plaque.
14. Repeat steps 2–6 until 100% blue plaques are visualized in the greatest dilution (1/1000) (Fig. 2).

15. Amplify recombinant CNPV viral stock by infection with 1 mL of virus (1/10 dilution in DMEM serum free) into a T25 flask of CEFs. Specifically, discard the growth medium, add virus, incubate 45 min, add 4 mL maintenance medium without discarding the inoculum and harvest cells and supernatant when generalized CPE is observed.

3.4 Characterization of Recombinant CNPV Genomes by PCR

Since the screening of recombinant CNPV is based on marker enzyme expression it is important to analyze the presence of the desired gene by PCR. Detection of the foreign gene can be evaluated after four rounds of screening, even though total DNA is obtained from a mixed (wild type, wt) and recombinant) viral population.

Additionally, once the viral progeny produces blue-plaques, it is necessary to confirm the purity of recombinant CNPV stock (absence of wt virus). Thus, a PCR analysis using a combination of three primers in the reaction should be performed. According to the design of those primers, fragments of different length are amplified from wt and recombinant CNPV genomes (*see Note 9* for details of this PCR screen, also *see Fig. 3*).

It is important to keep sterile conditions while performing steps 1–5 (CEFs infection and harvest).

1. Infect CEFs grown in a P60 (80% confluent) with 500 μ L of a 1/10 dilution of recombinant virus (different clones producing blue-plaques), wt CNPV or DMEM (labeled as non-infected cells).
2. Four to five days post infection (dpi) discard medium and wash the monolayer twice with 2–3 mL of sterile PBS (*see Note 10*).
3. Add 1 mL of PBS resuspend the cells by pipetting, and transfer to a microcentrifuge tube.
Remove the cells by pipetting or scrap the cells off in 1 mL of PBS. Transfer the cells to a microcentrifuge tube, spin down by centrifugation (5 min at 200 $\times g$), remove the supernatant, carefully resuspend cells in 1 mL of PBS, centrifuge as before, discard supernatant and resuspend cells in 1 mL of PBS
4. Freeze-thaw three times and store at -80°C .
5. Transfer 350 μ L of each cell lysate to a new microcentrifuge tube. Store the remaining sample at -80°C as a viral stock.
6. Add one volume of 2 \times Extraction Buffer.
7. Mix by vortexing and incubate at 65°C for 10 min.
8. Add 250 μ L of 5 M potassium acetate, mix thoroughly by inverting the tube (and incubate on ice for 20 min).
9. Centrifuge at 10,000 $\times g$, 30 min, 4°C .
10. Transfer 700 μ L the supernatant into a clear microcentrifuge tube.

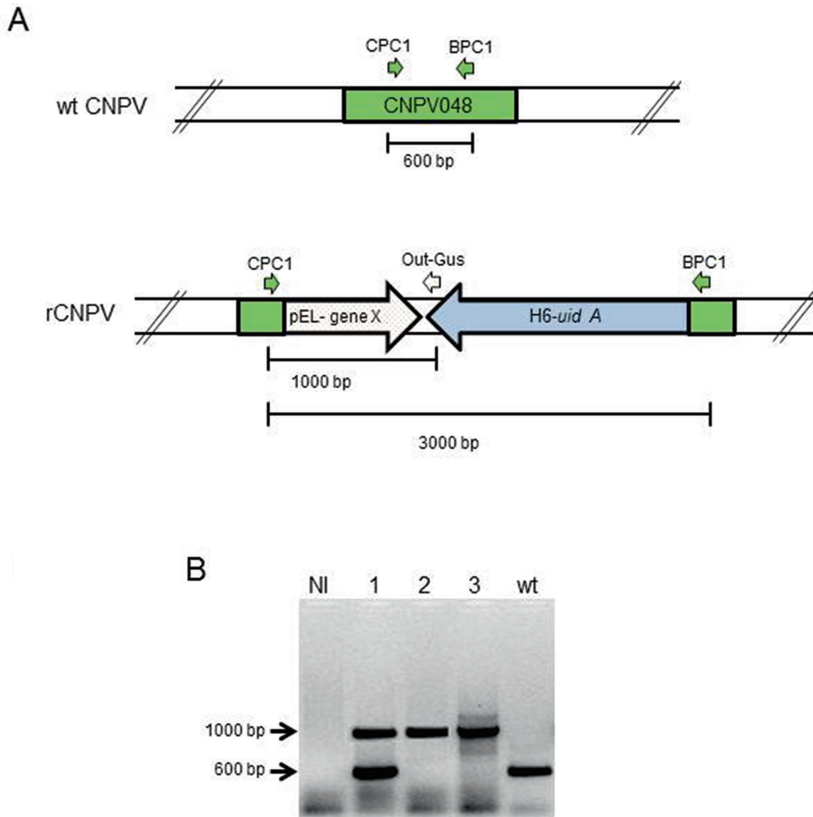


Fig. 3 Determining the purity of recombinant viral stocks by PCR amplification. **(a)** Schematic representation of wild type (wt) and recombinant (r) CNPV genome. Position of the primers used for PCR and expected sizes of amplified products are shown. **(b)** PCR amplification using CPC1 (2× concentrated), BPC1 and Out-Gus primers to analyze rCNPV purity. Total DNA was purified from non-infected cells (NI), non-recombinant CNPV (wt) and different clones (1, 3, to) of recombinant CNPV–infected CEFs. The length of the elongation step in the PCR was not long enough to allow amplification of the 3000 bp fragment on rCNPV genome. Recombinant CNPV clones 2 and 3 are pure (absence of wt CNPV)

11. Add 700 μ l of absolute isopropanol and mix by inverting. Incubate at least 1 h on ice or keep overnight at -20°C .
12. Centrifuge at $10,000 \times g$, 20 min at room temperature.
13. Discard supernatant and wash the pellet twice with 500 μ l of 70% ethanol and centrifuge as before. Discard supernatant by pipetting.
14. Dry the pellet to allow ethanol evaporation.
15. Dissolve the pellet in 50 μ l sterile distilled deionized water and store at -20°C .
16. Use 1.5 μ l of DNA for each PCR reaction to ensure that the desired gene was properly inserted in CNPV genome.

3.5 Total Protein Sample Preparation for Western Blot Analysis

Once the recombinant CNPV (rCNPV) is obtained and the presence of the foreign gene was confirmed by PCR analysis, the expression of the desired antigen must be evaluated by Western blot.

1. Infect CEFs grown in a P60 (80% confluent) with rCNPV (different clones), wt CNPV or DMEM (labeled as non-infected cells). Use a viral dilution to obtain a moi of 1–5.
2. Allow the virus to adsorb for 45 min (mix by rocking every 15 min).
3. Add 3 mL of maintenance medium and incubate 24 h at 37 °C.
4. Discard supernatant, wash the cell monolayer twice with PBS, scrape the cells off in 1 mL of PBS and transfer to a microcentrifuge tube.
5. Centrifuge 5 min at $200 \times g$, 4 °C. Discard the supernatant.
6. Resuspend each pellet in 50 μ L NP-40 lysis buffer by vortexing.
7. Incubate on ice during 45 min and mix by vortexing every 10 min.
8. Centrifuge 5 min at $10,000 \times g$.
9. Recover each supernatant to a new microcentrifuge tube (approximately 40 μ L) and add 8 μ L of 6 \times SDS-Sample Buffer.
10. Boil on hot plate for 10 min and spin samples briefly to bring condensation to bottom.
11. Load 15–20 μ L of each sample into 0.75 mm wells of a polyacrylamide gel.
12. Perform electrophoresis and Western blot as described elsewhere [6].

3.6 Evaluation of Recombinant CNPV Replication by Multiple-Step Growth Curves

The recombinant CNPV is usually obtained by insertion of the foreign gene into a target site identified as nonessential by genomic sequence homology or bioinformatics prediction. However it is important to confirm that this site is nonessential for in vitro replication. This is done by performing multiple-step growth curves in permissive cells.

1. Infect CEF monolayers (grown in P60) with wt CNPV or rCNPV at a moi of 0.01.
2. Allow the virus to adsorb for 45 min (mix by rocking each 15 min), remove the inoculum, and wash the cell monolayers twice with DMEM.
3. Add 3 mL of maintenance medium and incubate at 37 °C.
4. At different times (e.g., 0, 6, 12, 16, 20, 24, 36, 48, and 72 h) post-infection collect separately the cells (which contain the intracellular virus), and the supernatant (which contains the

extracellular virus). Freeze at -80°C and thaw these fractions three times and store at -80°C .

5. Determine the virus titer by performing plaque assays (*see* Subheading 3.7) at each time point. This should be done in duplicate.

3.7 Titration of CNPV: Determining Pfu/ mL

Titration of viral stocks is a critical step before any experimental use of the virus. The plaque assay is one of the most used methods to determine the infective titer of a virus stock. Briefly, cell monolayers are infected with different dilutions of virus suspension and a semisolid agarose overlay is added over the infected cells. As dilution is increasing, sporadic cells become infected. The agarose overlay keeps the cells stable and limits the spread of virus. When the virus lyses the cells, only the immediately adjacent cells become infected. After a few days the viral cytopathic effect can be distinguished as plaques (clear areas) in the cell monolayer. Then, these plaques can be easily visualized by staining with a vital dye (e.g., neutral red) for wt virus or with a second overlay containing the substrate for the expressed marker-enzyme for recombinant CNPV.

1. Thaw virus suspension at room temperature and homogenize by vortexing.
2. Make 10-fold serial dilution (from 10^{-1} to 10^{-8}) of virus in DMEM.
3. Infect cell monolayers (grown in P60, 80% confluent) with 0.5 mL of viral dilution in duplicate (*see* **Note 11**).
4. Allow the virus to adsorb for 1 h (mix by rocking each 20 min), remove the inoculum and wash cell monolayers twice with DMEM.
5. Add 3 mL of semisolid overlay medium (first overlay) and incubate for 4–5 days until plaques can be seen by holding the dish up to the light.
6. To stain wt virus plaques add 2.5 mL of the first overlay containing 50 $\mu\text{g}/\text{mL}$ neutral red: for rCNPV add 2.5 mL of the second overlay containing the appropriate enzyme substrate.
7. Let solidify at room temperature and incubate overnight at 37°C .
8. Count plaques visually (*see* **Note 12**). For wt virus is critical to confirm that the dots are viral lysis plaques by inspection under a microscope.
9. Calculate the titer of the initial viral suspension in pfu/mL using the following formula:

$$\text{pfu} / \text{mL} = \text{No. of plaques} / (D \times V)$$

D = dilution factor.

V = Volume of diluted virus/P60.

3.8 Amplification and Purification of CNPV

This protocol is useful for amplification and purification of recombinant and non-recombinant CNPV. It is recommended to prepare a “master seed” viral stock (e.g., from approximately 15 infected T175) that is used to amplify “working” virus stocks of wt and recombinant CNPV.

1. Infect CEFs monolayers grown on 15–20 T175 by adding 2–4 mL of diluted viral suspension to obtain a moi of 0.1–0.3.
2. Allow virus to adsorb for 1 h at 37 °C, mix by rocking every 15–20 min.
3. Add 30 mL of maintenance medium per flask.
4. Incubate 4–5 days until generalized CPE is observed.
5. Collect cells and supernatants, release virus by three cycles of freeze –80 °C–thaw and store at –80 °C as “primary” viral stock or carry on with following steps for viral purification.
6. Homogenize virus material using an ultrasonic bath sonicator. Fill sonicator with ice-water, place tubes containing viral suspension (25 mL of virus in a 50 mL polypropylene conical tube) and sonicate using sweep frequency mode for 3 min. Repeat three times with 1 min interval between each sonication step.
7. Centrifuge 20 min at $500 \times g$, 4 °C to discard cell debris.
8. Recover supernatant to another tube filtering through sterile gauze.
9. Resuspend the pellet in 10 mL DMEM and repeat **steps 6–8** (*see Note 13*).
10. Collect supernatant. *This is a possible stop point, keeping the supernatant at 4 °C overnight.*
11. Centrifuge 1.5 h at $48,000 \times g$, 4 °C to concentrate viral suspension.
12. Discard supernatant and resuspend pellets in 10–15 mL of TMN buffer by vortexing and pipetting.
Resuspend each pellet in 1–1.5 mL of TMN buffer by vortexing and pipetting (use a filtered tip) to ensure complete resuspension. Then, pool viral suspensions, wash each ultracentrifuge tube with 0.5–1 mL of TMN buffer and pool. Complete with TMN buffer until a final volume of 10–15 mL.
13. Sonicate the viral suspension as before and centrifuge 5 min at $500 \times g$, 4 °C.
14. Prepare sucrose cushions by filling an ultracentrifuge tube (for SW41 rotor) with 4 mL of 25% (w/v) sucrose in TMN buffer.
15. Overlay the viral suspension (supernatant of **step 13**) onto the sucrose cushion (*see Note 14*).
16. Centrifuge 2 h at $160,000 \times g$, 4 °C.

17. Discard supernatant (cell debris and sucrose) and resuspend pelleted viral suspension in TMN buffer using approximately 1 mL/10 T175 (*see* **Note 15**). Separate a 10–20 µl aliquot for viral titration.
18. Store purified viral stock at –80 °C, in aliquots of 0.5–1 mL.

4 Notes

1. Recombinant CNPV vaccines are based on strain ALVAC (Aventis Pasteur) or Abbatista95 (Instituto de Biotecnología, INTA). To our knowledge is quite difficult to find a supplier for a canarypox vaccine. Poximune C (Ceva) or Diftervac (LaDiPreVet, Argentina) are not producing these vaccines anymore. A critical start point is the obtainment of an attenuated strain of canarypox.
2. All media used on cells has to be warmed at 37 °C before use.
3. Unfortunately, there are not commercially available systems to obtain recombinant canarypox virus. So, the platform has to be set up in each laboratory interested in develop it. For example the transfer vectors (TV) have to be designed and constructed and an attenuated strain of CNPV has to be available. Otherwise, collaborative agreements are signed between research groups where one provides the platform and the other evaluates the recombinant CNPV as a vaccine.
4. The first step in designing a transfer vector is the selection of a nonessential target gene. Briefly, selection of a target gene can be done through searching bibliographic databases or by bioinformatics analysis on genome sequences of (avi)poxvirus available on GenBank. The foreign sequences have to be under regulation of poxviral early promoters, which are highly conserved between different members of the *Poxviridae* family [7]. It has been shown that vaccinia virus promoters can efficiently direct the expression of foreign genes in recombinant CNPV [8–10]. Among those are the synthetic vaccinia early/late promoter (pE/L.; [11]), the promoter of 7.5 kDa protein (p7.5; [12]) and vaccinia virus H6 gene promoter (pH 6; [13]). The TV also provides a poxviral transcription stop sequence (TAAATAAATAATTTTAT) downstream of the polylinker where the desired gene is cloned.
5. This is an important check-point to guarantee both that there are no nucleotide mutations (mainly if the desired gene was amplified by PCR) and the initiation of translation occurs from the proper start (ATG) codon.
6. Plasmid DNA for transfection into eukaryotic cells must be clean and free from phenol and sodium chloride as these con-

taminants may kill cells. In addition, salt will interference with formation of lipid complexes, decreasing transfection efficiency.

7. Seed at least 2 T25 flasks with different amount of cells to guarantee that one will be 80% confluence the next day. Viability of primary cell culture can vary a little each time they are prepared.
8. Multiplicity of infection (moi) is the average number of virus particles infecting each cell.

moi = Plaque form ing units (pfu) of virus used for infection/
number of cells.

9. In the example of Fig. 3 the primers used were BPC1 (5' TCCGCTTGATACAGATGGT), CPC1 (5' GATTGAAGATACAGGATTCT) and OutGus (5' CAGCCTCGGGAATTGCTAC). The protocol for a routine Taq PCR of 25 µl reaction contains Taq Reaction Buffer (1×), MgCl₂ (2 mM), dNTPs (200 µM), BPC1 and OutGus primers (50 ng of each), CPC1 primer (100 ng), template DNA from Subheading 3.4 (1.5 µl) and Taq DNA polymerase (0.6 U). For these primers the cycling conditions are:

1 cycle	Initial denaturation	94 °C, 5 min
<i>30 cycles</i>	<i>Denaturation</i>	<i>94 °C, 1 min</i>
	<i>Annealing</i>	<i>50 °C, 1 min</i>
	<i>Extension</i>	<i>72 °C, 1 min</i>
<i>1 cycle</i>	<i>Final extension</i>	<i>72 °C, 10 min</i>

10. CNPV infected CEFs at 4–5 dpi normally show generalized CPE but the monolayer is still fixed to the dish plate. Alternatively, the monolayer can be easily washed by gently adding 1–2 mL of PBS directly to the cell monolayer grown on P60, washing by rocking and discarding the buffer by pipetting. Then, 1 mL of PBS is added to recover cells by pipetting and transferring to a new microcentrifuge tube.
11. Use an appropriate dilution series based on the expected titer. The following expected titers can be used as a guideline: 10⁻⁴ to 10⁻⁸ for sucrose-cushion purified virus, 10⁻¹ to 10⁻⁴ for picked viral plaques, 10⁻³ to 10⁻⁶ for titrating virus in a multiple-step growth curve.
12. Select the virus dilution that produces 20–100 lysis plaques/ P60 to calculate the titer. Neutral red stains healthy cells and the plaques will appear as clear areas. In the case of rCNPV, the

blue lysis plaques are visualized after addition of the second overlay containing X-Gluc or Bluo-gal.

13. During the purification protocol, the greatest loss of virus occurs in the first low speed centrifugation. Repeat **steps 6 to 8** two or three times in order to increase recovery.
14. Complete each tube with TMN buffer until 2 mm from the top to prevent the tube from collapsing.
15. CNPV stocks purified by sucrose cushion are fine for animal work (veterinary vaccines).

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