



Targeted Whole Genome Sequencing of the Capripoxvirus Genome from Clinical Tissue Samples and Lyophilized Vaccine Batches

Elisabeth Mathijs and Frank Vandenbussche

Abstract

Diseases caused by Capripoxviruses (CaPVs) are of great economic importance in sheep, goats, and cattle. Since CaPV strains are serologically indistinguishable and genetically highly homologous, typing of closely related strains can only be achieved by whole-genome sequencing. In this chapter, we describe a robust, cost-effective, and widely applicable protocol for reconstructing (nearly) complete CaPV genomes directly from clinical samples or commercial vaccine batches in less than a week. Taking advantage of the genetic similarity of CaPVs, a set of pan-CaPVs long-range PCRs was developed that covers the entire genome with only a limited number of tiled amplicons. The resulting amplicons can be sequenced on all currently available high-throughput sequencing platforms. As an example, we have included a detailed protocol for performing nanopore sequencing and a pipeline for assembling the resulting tiled amplicon data.

Key words Capripoxvirus, Targeted sequencing, Whole-genome sequencing, Tiled PCR

1 Introduction

Capripoxviruses (CaPV) are one of the nine currently recognized genera within the subfamily Chordopoxvirinae of the family Poxviridae. The genus Capripoxvirus consists of the Lumpy Skin Disease Virus (LSDV), the Goatpox Virus (GTPV), and the Sheeppox Virus (SPPV). All CaPVs are responsible for economically important diseases in domestic ruminants that threaten the livelihoods of smallholders and poor rural communities in endemic regions [1, 2] and, more recently, farming communities in free regions [3]. Due to their potential to spread rapidly and their significant economic impact, the World Organisation for Animal Health (OIE) has classified all CaPV diseases as notifiable.

Since the different CaPV species cannot be distinguished either morphologically or serologically, CaPVs were originally classified according to the host species from which they were isolated.

However, reliable differentiation between the three CaPV species is only possible at the molecular level. Several real-time PCR (qPCR) tests have been developed for the detection and differentiation of CaPV species. Although these qPCR tests allow the identification of the different CaPV species, they lack the discriminatory power to differentiate closely related strains [4]. Differentiation at the strain level requires much higher resolution, which can only be achieved by comparing whole-genome sequence data.

Unfortunately, CaPVs are difficult to sequence due to their large genome size, skewed nucleotide composition and the presence of multiple homopolymeric and repetitive sequences. Although clinical samples may have a high viral load, viral DNA makes up only a small portion of the total DNA in these samples. To increase the ratio of signal (viral genome) to noise (host genome), most CaPV sequencing protocols require high concentrations and relatively large amounts of viral DNA, which can only be obtained by virus isolation. However, virus isolation is time-consuming and requires appropriate cell culture and biocontainment facilities, which are not available to all laboratories.

In this chapter, we describe a robust, cost-effective, and widely applicable method for obtaining (nearly) complete CaPV genomes directly from clinical samples or commercial vaccine batches. Taking advantage of the genetic similarity of CaPVs, a set of pan-CaPVs long-range PCRs (LR-PCRs) was developed that covers the entire genome with only a limited number of tiled amplicons. The resulting PCR amplicons can be sequenced on all currently available high-throughput sequencing platforms to reconstruct (nearly) complete genomes in less than a week. As an example, we have included a detailed protocol for performing nanopore sequencing and a pipeline for assembling the resulting tiled amplicon data.

2 Materials

Perform procedures with clinical samples in a class II microbiological safety cabinet. Once the DNA is extracted, all subsequent steps can be performed in PCR cabinets to avoid laboratory contamination.

2.1 For All Steps

1. Microfuge tube thermal mixer.
2. Ice bucket or cool elements.
3. Vortex.
4. DNase/RNase free plastics.
5. Benchtop centrifuge.
6. Pipets and filter tips.

2.2 Sample Homogenization and Cellular Debris Sedimentation

1. Scalpel.
2. Petri dishes.
3. Laboratory scale.
4. 1X Phosphate-Buffered Saline, pH 7.4 (PBS).
5. 2 mL sterile plastic tubes.
6. Micro-pestles for microfuge tubes.

2.3 DNase Treatment for Free-DNA Removal

1. 2 U/ μ L DNase enzyme.
2. 10X DNase reaction buffer.

2.4 Viral Nucleic Acid Extraction

1. Waterbaths and/or microfuge tube thermal mixer.
2. 2 mL sterile plastic tubes.
3. Absorbent paper.
4. Gentra Puregene Tissue Kit (Qiagen) including Puregene Cell Lysis Solution, Puregene Proteinase K, Puregene RNase A Solution, Puregene Protein Precipitation Solution, and Puregene DNA Hydration Solution (*see Note 1*).
5. Glycogen solution.
6. Isopropanol.
7. 70% (v:v) ethanol, freshly prepared.

2.5 Real-Time qPCR for DNA Quality Evaluation

1. Real-time PCR instrument.
2. Quantitative PCR SuperMix (2X).
3. 10 μ M Primers: 5'-CATTAGAAGGAGGATATTTTAGATG-3' (forward primer) and 5'-AAACGAGGATTGATGCCAG-3' (reverse primer).
4. 10 μ M Probe: FAM-CACAATGAA-ZEN-GGCCCTCATCTCTACTAATAAGTTCG-BHQ1.
5. PCR-grade water.
6. Multiwell plates or strips for real-time PCR analysis.

2.6 Long-Range PCR Amplification

1. Thermocycler preheated at 98 °C.
2. Microfuge tube thermal mixer preheated at 95 °C.
3. 8-well PCR strips.
4. PCR-grade water.
5. Q5 Hot Start High-Fidelity DNA Polymerase (New England BioLabs).
6. Q5 Reaction Buffer, 5X (New England BioLabs).
7. 5X 5 M Betaine PCR Enhancer.
8. CleanAmp dNTP Mix (TriLink Biotechnologies).

Table 1

Primer sequences for 23 long-range PCR amplicons covering the genome of species comprising the *Capripoxvirus* genus

Primer name	Primer sequence (5'–3')
fp CaPV-F1	AAACCTGTAAATGGATACTTTTTTCATTC
rp CaPV-F1	ATTTGGAAATATAATTGTGTTAACTGTTCT
fp CaPV-F2	TGTGAAAAATTAATCCATTCTTCTAAACAG
rp CaPV-F2	TACATACATTTCAAGTACTAAAAGAGAAGGAA
fp CaPV-F3	ATGTCAACAACATTTTTGCTATTCAATG
rp CaPV-F3	TTTTGGCCAGATATTTACAATGCTATCA
fp CaPV-F4	GATGGACCTAATGGAGTTATTATTGAG
rp CaPV-F4	GAAAAATCAAATGTAAACAAACAGCTGT
fp CaPV-F5	ATTCCATCATTGTTTGGTATTATTCCA
rp CaPV-F5	ACATATCATGTAAATAATAAACGGAAACAAC
fp CaPV-F6	GCTGAAGAATATGAATACAATACGCTAT
rp CaPV-F6	AAAAAACAAAATTTGAAGAACCTAAATCAG
fp CaPV-F7	GTATTGTTCTCCAAGTTTACATCCTT
rp CaPV-F7	CACATTTCTATTTTTTAATAAAATACGATTCCTTTC
fp CaPV-F8	ATCTCCGTTTGCTAAAAAAGATAAAGC
rp CaPV-F8	CATCTATGATAAATCGCACTATGGGTTTTTA
fp CaPV-F9	TGGGACCCAAATTGTTTCAGAATCTAA
rp CaPV-F9	TTTCTAACAAATGGCCAAAAACGTTTATAA
fp CaPV-F10	ATCCCACTTAAGATAATAAGATTTTTTAGAAAC
rp CaPV-F10	TTCCTCAGATTATCCGCTAATTTATTTGA
fp CaPV-F11	CTCTCTAATTTTAGTTATGTTTTTCATCTATCCA
rp CaPV-F11	AGAAAGGAATATTATATGCCCTATAGATATAGA
fp CaPV-F12	CCCAGATAAAGACGCAATAAGTAGAT
rp CaPV-F12	AACGAGTTGTTAGTCATTTGAGATAC
fp CaPV-F13	ACCATTCCAGAATTAAGTTTGATAATAAAC
rp CaPV-F13	GGGGGAAATTATTTTCAGAGTTATTAGAT
fp CaPV-F14	TATTATCATCGTTTTCCAATTAATGAATTAATTAC
rp CaPV-F14	CAACGAAAAACATTGATAAAATCTGATG
fp CaPV-F15	CTTTGATGTTGTTACCACCTTTCC
rp CaPV-F15	TTAAAAGAACAAAGTGGAATGGTAAGATAG
fp CaPV-F16	GAGTAAGATTTGATTTTTGAGATGCTTG

(continued)

Table 1
(continued)

Primer name	Primer sequence (5'–3')
rp CaPV-F16	TTTTCAAACCCGTATTTCATTTTTTACTG
fp CaPV-F17	ACTAAGGTTTGTTTTAGATAAATGGGAT
rp CaPV-F17	GTTTACAATTCTGCATATGATAGTTATATATGG
fp CaPV-F18	GCCATCTAACTCTATTGTAAATCCA
rp CaPV-F18	CCTTTCCTAAATCATCATTTTTTCTC
fp CaPV-F19a	CCRTCTATAGATATTAGAATTGTTAGTAAACC
fp CaPV-F19b	CCGTCTATAGATATTAGAATTATTAGTAAACC
rp CaPV-F19	GTATACATGATATTAGTGCAACATTGTTATG
fp CaPV-F20	GTTTGTATGATGCCAGATTCAGATATTAC
rp CaPV-F20	TAAACATAGACTCTTCTTTCGGTAGAC
fp CaPV-F21	GTTTCGGTTTCATATTTTTTAGCATATTAC
rp CaPV-F21	TGATATAAGTTTCATCCAAAAATCATATGTTG
fp CaPV-F22	GATTTACMCCACTTTTATCTTCTGTATATG
rp CaPV-F22	GACATATTAGATTTTGGAAATATAAGAGGTG
fp CaPV-F23	CCAAAAACGATTTTCATTGTATAAAGAAC
rp CaPV-F23	AAACCTGTAAATGGATACTTTTTTTCATTCAATC

F fragment; *fp*, forward primer, *rp* reverse primer, *CaPV* capripoxvirus

9. 100 μ M CaPV specific primers (Table 1).
10. Equipment and reagents for agarose gel electrophoresis.

2.7 Amplicon Purification and Quantification

1. 1.5 mL low binding microcentrifuge tubes.
2. Magnetic separator for 1.5 mL tubes.
3. SPRI magnetic beads.
4. 80% (v:v) Ethanol, freshly prepared.
5. 5 mM Tris/HCl, pH 8.5 (Elution Buffer).
6. Qubit 4 Fluorometer (ThermoFisher Scientific) (*see Note 2*).
7. Qubit 1X dsDNA HS Assay Kit (ThermoFisher Scientific).
8. Qubit Assay Tubes (ThermoFisher Scientific).

2.8 Amplicon Pooling and DNA Sequencing (See Note 3)

1. 1.5 mL low binding microcentrifuge tubes.
2. 0.2 mL thin-walled PCR tubes.
3. HulaMixer Sample Mixer (ThermoFisher Scientific).

2. MinION Sequencing Device Mk1C (Oxford Nanopore Technologies).
3. Flongle Flow Cell Adapter (ADP-FLG001, Oxford Nanopore Technologies).
4. Ligation Sequencing kit (SQK-LSK109, Oxford Nanopore Technologies).
5. Flongle Flow Cell (FLO-FLG001, Oxford Nanopore Technologies).
6. Flongle Sequencing Expansion (EXP-FSE001, Oxford Nanopore Technologies).
7. NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing (New England BioLabs).
8. SPRI magnetic beads.
9. Magnetic separator for 1.5 mL microtubes.
10. Qubit 4 Fluorometer (ThermoFisher Scientific).
11. Qubit 1X dsDNA HS Assay Kit (ThermoFisher Scientific).
12. DEPC water.

2.9 Sequencing Data Analysis

1. Linux operating computer (*see Note 4*).
2. LILO fork available at <https://github.com/el-mat/LILO-CaPV> (*see Note 5*).

3 Methods

Homogenization and the steps prior to virus inactivation must be carried out in a class II microbiological safety cabinet in a biosafety level 3 laboratory. All disposable materials (e.g., tips, tubes, and pipettes) must be decontaminated before being placed in an appropriate waste container. After virus inactivation (30 min at 65 °C), all manipulations can be performed at biosafety level 2.

3.1 Sample Homogenization and Cellular Debris Sedimentation (See Note 6)

1. Cut a 40 mg piece of clinical tissue and crush it with a pestle in a 2 mL tube containing 300 μ L PBS.
2. Incubate overnight at 56 °C and shake at 1000 rpm.
3. Centrifuge the tubes at 1000 $\times g$ and 4 °C for 15 min and transfer 250 μ L of the supernatant into a clean 2 mL tube.

3.2 DNase Treatment for Free-DNA Removal

1. Add 30 μ L 10X DNase buffer and 20 μ L DNase enzyme to the 250 μ L supernatant. Mix gently by pipetting up and down.
2. Incubate at 37 °C for 15 min and spin down the tubes briefly.

3.3 Viral Nucleic Acid Extraction (See Note 7)

1. In a 2 mL tube, add 500 μL Puregene Cell Lysis Solution to 100 μL of DNase treated sample. Mix gently by pipetting up and down.
2. Incubate for 30 min at 65 $^{\circ}\text{C}$ and spin down the tubes briefly.
3. Add 3 μL Puregene Proteinase K. Mix gently by flicking the tubes.
4. Incubate for 1 h at 55 $^{\circ}\text{C}$ and spin down the tubes briefly.
5. Add 3 μL RNase A and mix gently by inverting the tubes 25 times.
6. Incubate for 15 min at 37 $^{\circ}\text{C}$ and spin down the tubes briefly.
7. Cool the tubes on ice for 1 min.
8. Add 200 μL Puregene Precipitation Solution and vortex at maximum speed for 20 s.
9. Place the tubes on ice for 5 min.
10. Centrifuge the tubes at $13,000 \times g$ and 4 $^{\circ}\text{C}$ for 3 min. The resulting pellets should be firm. Repeat the procedure if necessary.
11. Add 600 μL isopropanol to clean 2 mL tubes and gently pour the supernatants into the tubes.
12. Add 1 μL Glycogen Solution and mix gently by inverting the tubes 50 times.
13. Incubate for 5 min at room temperature.
14. Centrifuge the tubes at $13,000 \times g$ and 4 $^{\circ}\text{C}$ for 5 min.
15. Carefully discard the supernatants and drain the tubes on paper.
16. Add 600 μL of freshly prepared 70% (v:v) ethanol and gently invert the tubes to wash the pellets without disturbing their integrity.
17. Allow the pellets to settle back to the bottom of the tubes (spin down briefly if necessary).
18. Aspirate and discard the supernatants with a pipet without touching the pellets.
19. Allow the pellets to air dry until they are invisible (approximately 5 min).
20. Add 80–100 μL Puregene DNA Hydration Solution and vortex at medium speed for 5 s.
21. Incubate for 1 h at 65 $^{\circ}\text{C}$ to dissolve the DNA pellets.
22. Incubate overnight at room temperature with gentle shaking.
23. Spin down the tubes and store them at -20°C until further analysis.

3.4 Real-Time PCR

Ideally, the crossing point (C_p) value for successful LR-PCR amplification should be between 20 and 27. If the C_p values are below 20, the purified DNA should be diluted at least 1:10 (v:v). At C_p values above 27, the LR-PCRs are less robust, and some amplifications may fail.

1. Prepare the following premix per sample (*see Note 8*): 10 μL Quantitative PCR SuperMix (2X), 1.4 μL forward primer (10 μM), 1.4 μL reverse primer (10 μM), 0.5 μL probe (10 μM), 4.7 μL PCR-grade water, and 2 μL purified DNA.
2. Perform the qPCR reaction using the following program: 50 °C for 2 min, 95 °C for 2 min, 45 cycles of 95 °C for 15 s, and 60 °C for 30 s.

3.5 Long-Range PCR Amplification

PCRs should be performed in batches of no more than eight reactions, as the steps of the protocol should be performed as quickly as possible. Before each amplification, 10 μM primer stocks should be freshly prepared from 100 μM primer stocks.

1. Heat 100 μM primer aliquots for 5 min at 95 °C.
2. Spin down quickly.
3. Prepare forward/reverse 10 μM primer stocks in PCR tubes by mixing: 2 μL forward primer (100 μM), 2 μL reverse primer (100 μM), 16 μL PCR-grade water.
4. Place 10 μM primer stocks on a preheated thermocycler at 98 °C.
5. Prepare the following premix per amplicon: (*see Note 8*): 17.5 μL PCR-grade water, 10 μL Q5 reaction buffer (5X), 10 μL 5 M Betaine (5X) PCR Enhancer, 2 μL CleanAMP dNTP mix, and 0.5 μL Q5 Hot Start High-Fidelity DNA Polymerase.
6. Add 5 μL of DNA template to each mix.
7. Spin down the preheated 10 μM primers.
8. Add 5 μM of preheated 10 μM primers and mix gently by pipetting up and down.
9. Immediately start the LR-PCR using the following program: 98 °C for 3 min, 35 cycles of 98 °C for 10 s, 63 °C for 30 s, 72 °C for 7 min, 72 °C for 2 min, and hold at 4 °C.
10. The amplification products should appear as a single 7.5 kb band when 5 μL of the PCR product is visualized by agarose gel electrophoresis (Fig. 1).
11. The amplification products can be stored at -20 °C prior to further analysis.

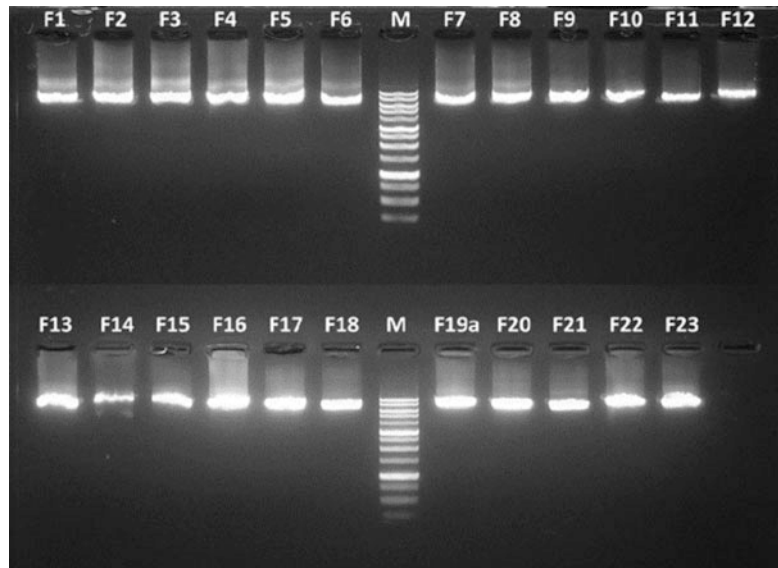


Fig. 1 Agarose gel (1%) electrophoresis analysis of the 23 long-range CaPV PCR amplicons. *M* molecular weight marker range 250–10,000 bp

3.6 Amplicon Purification and Quantification (See Note 9)

1. Transfer 40 μL of the PCR product into a clean 1.5 mL DNA low binding tube.
2. Vortex the SPRI beads until they are completely suspended.
3. Add 72 μL of SPRI beads. Mix by pipetting up and down 10 times.
4. Incubate for 5 min at room temperature.
5. Spin down the tubes and place them on the magnetic separator to separate the beads from the solution. Wait until the solution is clear (up to 3–4 min).
6. Remove the supernatant with a pipet while leaving the tube on the magnetic separator.
7. Wash with 200 μL of freshly prepared 70% (v:v) ethanol without disturbing the beads pellet. Pipette off the ethanol and discard. Repeat this step one more time.
8. Spin down the tube briefly, place it back on the magnetic separator and pipette off any residual ethanol.
9. Let the pellet dry for about 30 s.
10. Remove the tube from the magnetic separator and add 32.5 μL elution buffer. Mix by pipetting up and down until the pellet is completely resuspended.
11. Incubate at room temperature for 2 min.
12. Place the tube on the magnetic separator for 1 min and transfer 30 μL of the supernatant into a new DNA low binding tube.

13. The purified amplicons can be stored at $-20\text{ }^{\circ}\text{C}$ prior to further analysis.
14. Prepare the required number of 0.5 mL Qubit tubes for the two standards and samples.
15. Add 190 μL of the ready-to-use working solution to the standard tubes, and 198 μL to the sample tubes.
16. Add 10 μL of standard 1 or 2 to the respective standard tubes and 2 μL sample to each sample tube.
17. Vortex the tubes and incubate them for 2 min at room temperature protected from light.
18. Read the sample concentrations on the Qubit 4 Fluorometer after performing a new calibration with standards 1 and 2.
19. Each amplicon should have a concentration of at least 20 ng/ μL .

3.7 Amplicon Pooling and DNA Sequencing

1. All 23 amplicons must be pooled equimolarly (e.g., 100 ng of each amplicon) (*see Note 10*).
2. Quantify the pooled amplicons using the Qubit 4 Fluorometer (*see Subheadings 3.8.14–3.8.18*).
3. Dilute the pooled amplicons to 20 ng/ μL in DEPC water in a volume of 50 μL .
4. Add 23.5 μL pooled amplicons, 1.75 μL FFPE DNA Repair Buffer, 1.75 μL Ultra II End-Prep Reaction Buffer, 1 μL FFPE DNA Repair Mix, and 1.5 μL Ultra II End-Prep Enzyme Mix to a 0.2 mL PCR tube. Mix by gently flicking the tube.
5. Incubate for 5 min at $20\text{ }^{\circ}\text{C}$ and for another 5 min at $65\text{ }^{\circ}\text{C}$. Spin down the tubes quickly.
6. Perform a 1:1 (v:v) SPRI bead purification. Transfer the reaction volume to a clean 1.5 mL DNA low binding tube.
7. Add 30 μL of resuspended SPRI beads and mix by flicking the tube.
8. Incubate for 5 min at room temperature on a HulaMixer.
9. Spin down the tube and place it on the magnetic separator to separate the beads from the solution. Wait until the solution is clear (up to 3–4 min).
10. Remove the supernatant with a pipet while leaving the tube on the magnetic separator.
11. Wash the bead pellet with 200 μL of freshly prepared 70% (v:v) ethanol without disturbing the bead pellet. Pipette off the ethanol and discard. Repeat this step one more time.
12. Spin down the tube quickly, replace it on the magnetic separator and pipette off any residual ethanol.
13. Let the pellet dry for about 30 s but avoid cracking the pellet.

14. Remove the tube from the magnetic separator and add 30 μL of elution buffer. Mix by pipetting up and down until the pellet is completely resuspended.
15. Incubate for 2 min at room temperature.
16. Place the tube back on the magnetic separator for 1 min and transfer 30 μL of the supernatant to a new DNA low binding tube.
17. Add to the sample tube: 12.5 μL Ligation Buffer (LNB), 5 μL NEBNext Quick T4 DNA ligase, and 2.5 μL Adapter Mix (AMX) (*see Note 11*).
18. Mix by gently flicking the tube, spin down and incubate the reaction for 10 min at room temperature.
19. Perform a 1:0.4 (v:v) SPRI bead purification. Add 20 μL of resuspended SPRI beads and mix by flicking the tube.
20. Incubate for 5 min at room temperature on a HulaMixer.
21. Spin down the tube and place it on the magnetic separator to separate the beads from the solution. Wait until the solution is clear (up to 3–4 min).
22. Remove the supernatant with a pipet while leaving the tube on the magnetic separator.
23. Wash the bead pellet with 125 μL of Long Fragment Buffer (LFB). Flick the tube to resuspend the beads and then place it back on the magnetic separator. Once the beads are pelleted, discard the supernatant with a pipette. Repeat this step one more time.
24. Spin down the tube quickly, place it back on the magnetic separator and pipette off any residual ethanol.
25. Allow the pellet to dry for about 30 s but avoid cracking the pellet.
26. Remove the tube from the magnetic separator and add 7 μL of elution buffer. Mix by pipetting up and down until the pellet is completely resuspended.
27. Incubate for 10 min at room temperature.
28. Place the tube back on the magnetic separator for 1 min and transfer 7 μL of supernatant to a new DNA low binding tube.
29. Quantify the eluted library using the Qubit 4 Fluorometer (*see Subheadings 3.8.14–3.8.18*).
30. The concentration of the library should be at least 3 ng/ μL to load the required minimum of 3 fmol onto the Flongle flow cell. If the concentration is ≥ 10 ng/ μL , 50 ng of library (in a volume of 5 μL) should be loaded onto the flongle flow cell (*see Note 12*).

31. Load the Flongle flow cell according to the instructions provided by Oxford Nanopore Technologies.
32. Set the sequencing run to 24 h and perform HAC basecalling in the MinKNOW software.
33. Compressed fastq files (*.fastq.gz) containing sequences that have passed quality filtering should be used for genome assembly.

3.8 Assembling the Sequencing Data

1. Clone the GitHub repository (*see Note 5*) and follow the instructions in the README file to install the LILO-CaPV pipeline.
2. In the current working directory, create a folder named “raw/” that contains the compressed fastq files to be analyzed (*see Notes 13 and 14*).
3. Activate the LILO-CaPV environment.
4. Edit the supplied config.file with the correct paths to the CaPV scheme files.
5. Launch the genome assembly as follows: “snakemake -k -s /path/to/LILO-CaPV --configfile /path/to/config.file --cores 8” (*see Note 15*).
6. Check the length of the contig created by LILO-CaPV as follows: “bioawk -c fastx '{ print \$name, length(\$seq) }' sample/polished_trimmed.fa.cap.contigs.” It should be about 150 kbp (*see Note 16*).

4 Notes

1. The protocol was optimized using the Gentra Puregene Tissue Kit from Qiagen. Other extraction kits allowing the purification of high-molecular-weight (100–200 kb) DNA can be used according to the manufacturer’s instructions.
2. For accurate dsDNA quantification, the use of a fluorescent nucleic acid dye is preferable. Alternatives to Invitrogen Qubit are available.
3. Ligation sequencing kits and flow cells for nanopore sequencing should be adapted to the most recent available reagents as the technology keeps evolving.
4. The LILO-CaPV pipeline has been tested successfully on a Windows computer running Windows Subsystem for Linux.
5. The LILO-CaPV pipeline was adapted from the Lilo pipeline <https://github.com/amandawarr/Lilo>.
6. If the starting material is a lyophilized vaccine, resuspend the freeze-dried vaccine pellet in 2 mL PBS and start the protocol immediately at Subheading 3.2, step 1).

7. The extraction should be performed in triplicate to obtain sufficient DNA to perform the downstream PCRs.
8. For multiple samples, we recommend preparing 10% extra premix.
9. Allow the SPRI beads and the Qubit 1X dsDNA HS Assay kit to equilibrate at room temperature for 30 min before use. Keep the Qubit dye away from light.
10. If you want to perform short-read sequencing, amplicons F1 and F23, which contain the inverted terminal repeats (ITR), should not be pooled together. Two separate pools (e.g., pool1 containing F1 to F12 and pool2 containing F12 to F23) must be sequenced separately.
11. Multiple samples can be sequenced simultaneously on the same Flongle flow cell. A barcoding step using the EXP-NBD104 or EXP-NBD114 kit followed by an adapter ligation step using the alternative Adaptor Mix (AMII) must be performed instead.
12. To determine the amount of library that should be loaded according to the required 3–10 fmol, use the “dsDNA: Mass to/from Moles Convertor” from <https://nebiocalculator.neb.com/>.
13. All fastq files from a sample should be combined into a single file. Use the *cat* command to combine the different fastq files.
14. Each sample to be analyzed should have a unique sample name (e.g., sample.fastq.gz). All output files will be written to a folder named after this unique sample file.
15. The number of cores should be adjusted to the number of threads available on your machine.
16. If you have mixed samples or insufficient coverage, you may get a shorter contig or multiple short contigs. To visualize this, the reads can be mapped against a reference genome using minimap2.

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