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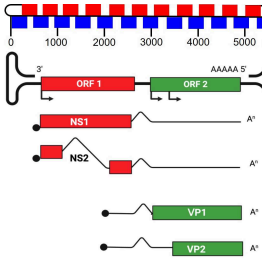
Sequencing of Canine Parvovirus (CPV) from Rectal Swab Samples V.1



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We use this protocol and it's working

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Abstract

Canine parvovirus (CPV) is a highly contagious viral disease that affects dogs, especially puppies. CPV-2 is recognized for its resilience in contaminated environments, ease of transmission among dogs, and pathogenicity for puppies. In this protocol, we have adapted the methodology to allow the recovery of complete CPV-2 genomes directly from clinical samples (dry swabs) from puppies with clinical signals of viral enteritis. A multiplex PCR was designed with primers targeting fragments of 400 to 1,000 base pairs (bp) along the full length of the viral genome. The resulting reads were compared after sequencing with the Nanopore technology. Genome assembly revealed that the smaller fragments generated larger numbers of reads, allowing a more reliable coverage of the genome than those attained with primers targeting larger amplicons. Both new methodologies were efficient in amplification and sequencing.

Materials

Consumables:
Isopropanol
Beta-mercaptoethanol
Ethanol
Nuclease-free water
(primers from Table 1 and 2)
R9.4 Oxford MinION flowcell (FLO-MIN106)(Oxford Nanopore)
Commercial Kits:

* Quick-DNA/RNA™ Viral MagBead (Zymo Research)
* Q5® High-Fidelity 2X Master Mix (New England Biolabs)
* AMPure XP beads (Beckman Coulter)
* Ligation Sequencing kit SQK-LSK-109 (Oxford Nanopore)
* Native Barcoding kits EXP-NBD104 and EXP-NBD114 (Oxford Nanopore)

Equipment:
Thermocycler
Magnetic stand (or automated extractor with magnetic block)
MinION Mk1B device (Oxford Nanopore)

Protocol materials

Quick-DNA/RNA Viral MagBead **Zymo Research** **Catalog #**R2140 / R2141

Q5 High-Fidelity PCR Kit - 200 rxns **New England Biolabs** **Catalog #**E0555L

Ampure XP beads **Beckman Coulter** **Catalog #**A63881

Qubit dsDNA HS Assay kit **Thermo Fisher Scientific** **Catalog #**Q32854

Troubleshooting

Nucleic Acid Extraction using Quick-DNA/RNA Viral MagBead (Zymo Research)

1 This protocol uses Quick-DNA/RNA Viral MagBead **Zymo Research Catalog #**R2140 / R2141 for extraction.

All kit's solutions need to be prepared beforehand, as described by the kit. If you've already prepared the solution, go to step 2. **This is a modified version of the kit's protocol.** The original kit's protocol can be found here.

1.1 **DNA/RNA Buffer:**

Add 500 µL of **beta-mercaptoehtanol** (user supplied) per 100 ml Viral DNA/RNA **Buffer**, (final 0.5% (v/v)

1.2 **DNA/RNA Wash 1:**

Kit R2140 → Add 20 mL of **isopropanol** (2-propanol PA, user supplied) to MagBead DNA/RNA **Wash 1** concentrate

Kit R2141 → Add 80 mL of **isopropanol** (2-propanol PA, user supplied) to MagBead DNA/RNA **Wash 1** concentrate

1.3 **DNA/RNA Wash 2:**

Kit R2140 → Add 30 mL of **isopropanol** (2-propanol PA, user supplied) to MagBead DNA/RNA **Wash 2** concentrate

Kit R2141 → Add 120 mL of **isopropanol** (2-propanol PA, user supplied) to MagBead DNA/RNA **Wash 2** concentrate

1.4 **DNA/RNA Shield™:**

Kit R2140 → Add 25 mL of **nuclease-free water** (user supplied) to 2X DNA/RNA Shield™ concentrate

Kit R2141 → Add 125 mL of **nuclease-free water** (user supplied) to 2X DNA/RNA Shield™ concentrate

1.5 **Proteinase K (20 mg/ml):**

Kit R2140 → Add 1.04 mL of **Proteinase K Storage Buffer** to lyophilized Proteinase K (20 mg)

Kit R2141 → Add 3.12 mL of **Proteinase K Storage Buffer** to lyophilized Proteinase K (60 mg)

Mix by vortexing. Use immediately or store frozen aliquots.

2 Elute each dry Sample into 400 µL of 1X viral DNA/RNA **buffer.**

2.1 Our samples were dry rectal swabs of animals confirmed positive by a rapid test. Samples were collected and stored in a centrifuge tube (without transporting media) at -20 ºC until processing.

3 For each sample, add 10 µL of beads and 4 µL of proteinase K.

Obs: when working with many samples, one can always prepare a mixed solution with both components. Just make to keep the beads suspended while pipetting.

3.1 Transfer the plate (or your tubes) to **magnetic stand** (user supplied). After the beads have pelleted, aspirate and discard the supernatant.

This protocol can be **fast tracked** by the use of an **automated extractor**.
We use EXTRACTA 96 (Loccus).

4 Perform one wash of the pellet (beads) with 250 µL of MagBead DNA/RNA **Wash 1** solution.

5 Perform one wash of the pellet (beads) with 250 µL of MagBead DNA/RNA **Wash 2** solution.

6 Add 250 µL of **80% ethanol** and mix well.
Transfer the sample (liquid and beads) to a new place/tube.

7 Pellet the beads and discard the the supernatant.

8 Add 50 µL of **nuclease-free water** and mix well (this is the elution step).

9 Pellet the beads (magnetically) and transfer the supernatant (containing DNA/RNA) to a new plate/tube.

Multiplex PCR to obtain CPV genome fragments

10 This protocol uses 2 sets of oligos: one set amplifies ~400 bp sequences that overlap about 100 nucleotides with each other (Table 1); the other amplifies ~1000 bp sequences that overlap about 100 nucleotides with each other (Table 2). Primers are listed in the tables below and were design based on Canine parvovirus reference sequence **NC_001539.1** (GenBank).

Primers should be mix into 2 pools for each set. To prepare a pool, add 5 µL of each primer (at [M] 100 micromolar (µM)) as indicated. This will be your 10X (stock) solution. Working solution should be dilute to 1X concentration ([M] 10 micromolar (µM)).

	Name	Pool	Sequence (5′ – 3′)	Size (nt)	%GC	Tm
	CPV-400_0.7_LEFT*	1	ATGTCTGGCAACCAGTATACTG	22	45.50	60.30
	CPV-400_1_LEFT	1	AACCAACTGACCAAGTTCACGT	22	45.45	60.80

	Name	Pool	Sequence (5′ – 3′)	Size (nt)	%GC	Tm
	CPV-400_1_RIGHT	1	GTTCCAGCGAACATCCTTTCCA	22	50.00	61.31
	CPV-400_1.5_LEFT*	1	AGGTGGCGGGCTAATTGTG	19	57.90	59.50
	CPV-400_2_LEFT	2	AAGAAACATGCAGAAAATGAAGCATT	26	30.77	59.73
	CPV-400_2_RIGHT	2	CGTAGCCATTTACCAGTTGCTTG	23	47.83	60.67
	CPV-400_3_LEFT	1	ATGGGGAAAAGATCAAGGCTGG	22	50.00	60.81
	CPV-400_3_RIGHT	1	AGTGTGCTGACAATTTGTCTGTC	23	43.48	59.94
	CPV-400_4_LEFT	2	GGGTGACTATATTAACATACAGACATAAGC	30	36.67	60.59
	CPV-400_4_RIGHT	2	TCCTGGTTGTGCCATCATTTCA	22	45.45	60.68
	CPV-400_5_LEFT	1	ACTTTGCGGGACTTGGTTAGTA	22	45.45	59.81
	CPV-400_5_RIGHT	1	ACAACCAACATTACCCACAGCT	22	45.45	60.61
	CPV-400_6_LEFT	2	CAGTTCTTTTTCATGGACCAGCA	23	43.48	59.93
	CPV-400_6_RIGHT	2	AAACCAAAGTCTCCTGGAAGCT	22	45.45	60.01
	CPV-400_7_LEFT	1	TGGATGTGAAGAAAGACCTGAACA	24	41.67	60.47
	CPV-400_7_RIGHT	1	AACGCCAAGTTGGTTTGATTGT	22	40.91	60.01

	Name	Pool	Sequence (5′ – 3′)	Size (nt)	%GC	Tm
	CPV-400_8_LEFT	2	AGTGGACCTTGCACTGGAAC	20	55.00	60.20
	CPV-400_8_RIGHT	2	GCTTCGTCGTGTTCTTTTGCAG	22	50.00	61.33
	CPV-400_9_LEFT	1	AAATATCTTGGGCCTGGGAACA	22	45.45	59.87
	CPV-400_9_RIGHT	1	ACTGCTCCATCACTCATTGGTG	22	50.00	60.80
	CPV-400_10_LEFT	2	ACCACCTCATATTTTCATCAATCTTGC	27	37.04	60.91
	CPV-400_10_RIGHT	2	TCAACCAATGACCAAGGTGTTACA	24	41.67	60.95
	CPV-400_11_LEFT	1	GTGGTTGTAAATAATATGGATAAAACTGCA	30	30.00	60.00
	CPV-400_11_RIGHT	1	TGTTCTATCCCATTGAAAATAATATCTCCA	30	30.00	59.80
	CPV-400_12_LEFT	2	TGCCATTTACTCCAGCAGCTAT	22	45.45	60.01
	CPV-400_12_RIGHT	2	TCCCATTTGAGTTACACCACGT	22	45.45	60.08
	CPV-400_13_LEFT	1	TTTGCCTCAATCTGAAGGAGCT	22	45.45	60.14
	CPV-400_13_RIGHT	1	ATCATTCGTTACAGGAAGGTTAAAGTT	27	33.33	59.83
	CPV-400_14_LEFT	2	ACACCTGAGAGATTTACATATATAGCACA	29	34.48	60.63
	CPV-400_14_RIGHT	2	ACCTTTCCACCAAAAATCTGAGTAAG	26	38.46	60.18

	Name	Pool	Sequence (5′ – 3′)	Size (nt)	%GC	Tm
	CPV-400_15_LEFT	1	CAAATGGTCAAATTTGGGATAAAGAATTTG	30	30.00	60.15
	CPV-400_15_RIGHT	1	TTCTAGGTGCTAGTTGATATGTAATAAACA	30	30.00	59.56
	CPV-400_16_LEFT	2	TGTTTATTACATATCAACTAGCACCTAGAA	30	30.00	59.56
	CPV-400_16_RIGHT	2	TCTAAGGGCAAACCAACCAACC	22	50.00	61.20
	CPV-400_17_LEFT	1	AGGTTTGTTAGATGGTATACAATAACTGT	29	31.03	59.61
	CPV-400_17_RIGHT	1	AGCTTTAAATACTAATTTACCTTTCCACCA	30	30.00	60.50
	CPV-400_17.5_RIGHT*	1	AAGTATCAATCTGTCTTTAAGGGG	24	37.50	60.10
	CPV-400_18_LEFT*	2	TATAAGGTGAACTAACCTTACCATA	25	32.00	59.20
	CPV-400_18_RIGHT*	2	TTAATATAATTTTCTAGGTGCTAGTTG	27	25.90	59.20

Table 1. Primers targeting 400 bp amplicons

	Name	Pool	Sequence (5'-3')	Size (nt)	%GC	Tm
	CPV-1000_1_LEFT	1	CTGACCAAGTTCACGTACGTATGA	24	45.83	60.93
	CPV-1000_1_RIGHT	1	TGTTCAGTGTAAAGTGTGCTGACA	24	41.67	61.18
	CPV-1000_2_LEFT	2	GTGAATGGGTGACTATATTAACATACAGAC	30	36.67	60.73

	Name	Pool	Sequence (5'-3')	Size (nt)	%GC	Tm
	CPV-1000_2_RIGHT	2	ACCAAACCAAAGTCTCCTGGAAG	23	47.83	60.95
	CPV-1000_3_LEFT	1	AAGCAAATTGAACCAACTCCAGT	23	39.13	59.55
	CPV-1000_3_RIGHT	1	GGTGGTGGTTTACTTCTTTTAGTTGG	26	42.31	60.90
	CPV-1000_4_LEFT	2	CTAAGGACGCTAAAGATTGGGGG	23	52.17	61.00
	CPV-1000_4_RIGHT	2	GTTCCTGTAGCAAATTCATCACCTG	25	44.00	60.77
	CPV-1000_5_LEFT	1	CCATCTCATACTGGAACTAGTGGC	24	50.00	60.82
	CPV-1000_5_RIGHT*	1	TGGATTCCAAGTATGAGAGGCTCT	24	45.83	61.21
	CPV-1000_6_LEFT	2	AACCAAGACTTCATGTAAATGCACC	25	40.00	60.66
	CPV-1000_6_RIGHT*	2	TGGATTCCAAGTATGAGAGGCTCT	24	45.83	61.21

Table 2. Primers targeting 1000 bp amplicons

11 We used

Q5 High-Fidelity PCR Kit - 200 rxns **New England Biolabs Catalog #**E0555L to perform PCR.

Reactions should be set up independent **for each pool**, with a final volume of 25 µL , as bellow:

Reactions for 400 bp products:

* Master Mix 2X - 12.5 µL

* Primer pool 1 **or** 2 - 1.6 µL
* Nuclease-free H20 - 5.9 µL
* DNA - 5.0 µL

Reactions for 1000 bp products:

* Master Mix 2X - 12.5 µL
* Primer pool 1 **or** 2 - 0.4 µL
* Nuclease-free H20 - 7.1 µL
* DNA - 5.0 µL

11.1 The final concentration in a reaction should be [M] 15 nanomolar (nM) per primer.
Therefore, the volume of primers used in a reaction will vary according with the number of primers in the pool. More about this subject can be found in **Quick et al. (2017)**.

12 PCR's run method should be set as: 8m 15s

* 98 °C for 00:03:00
* 35 cycles of (98 °C for 00:00:15 ; 63 °C for 00:05:00)
* Hold at 4 °C

Purifying PCR Products with AMPure XP beads (Beckman Coulter)

13 Mix amplification products of Pool 1 and Pool 2 (final volume 50 µL).

14 We purify the fragments with
Ampure XP beads **Beckman Coulter Catalog #**A63881 , using a modified protocol (original protocol ca be found **here**).

14.1 This is step can also be fast-tracked by the use of an automated system.

15 Add an equal volume of AMPure XP per sample (50 µL).

16 After biding, on a magnetic stand, wash beads once with [M] 80 % (v/v) ethanol.

17 Elute DNA with 20 µL of (sequencing appropriate) buffer.

18 Transfer DNA to a new plate/tube.

19 Quantify your DNA.

19.1 We used Qubit dsDNA HS Assay kit **Thermo Fisher Scientific Catalog #**Q32854

Equipment

Qubit™ 3 Fluorometer	NAME
Fluorometer for nucleic acid quantitation	TYPE
Invitrogen	BRAND
Q33216	SKU
https://www.thermofisher.com/order/catalog/product/br/en/Q33216	LINK
Fluorometer for nucleic acid quantitation	SPECIFICATIONS

Library preparation & sequencing

20 To prepare a library, use the Ligation and Barcoding kits (following manufacturer's instructions).

We used **Ligation Sequencing kit SQK-LSK-109** and **Native Barcoding kits EXP-NBD104 and EXP-NBD114** (Oxford Nanopore)

21 Load library on your flow cell and sequence your sample.

21.1 We used **R9.4 Oxford MinION flow cell** (FLO-MIN106) and **MinION Mk1B** device for sequencing.

Protocol references

Quick, J., Grubaugh, N. D., Pullan, S. T., Claro, I. M., Smith, A. D., Gangavarapu, K., Oliveira, G., Robles-Sikisaka, R., Rogers, T. F., Beutler, N. A., Burton, D. R., Lewis-Ximenez, L. L., de Jesus, J. G., Giovanetti, M., Hill, S. C., Black, A., Bedford, T., Carroll, M. W., Nunes, M., Alcantara, L. C., Jr, ... Loman, N. J. (2017). Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. *Nature protocols*, *12*(6), 1261–1276. https://doi.org/10.1038/nprot.2017.066