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Version 2

Oxford Nanopore Targeted Sequencing Assay for Flongle Flow Cells V.2

 [Pathogens](#)

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We use this protocol and it's working

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Abstract

We adapted a targeted next-generation sequencing (tNGS) panel for wildlife pathogen surveillance to the Oxford Nanopore MinION platform to enable cost-effective detection of SARS-CoV-2 and other pathogens in mesocarnivores. The workflow combines a broad-pathogen tNGS assay with a SARS-CoV-2 whole-genome sequencing panel prior to library preparation, enabling simultaneous pathogen detection and variant identification.

Materials

Materials

Equipment:

Biosafety cabinet

PCR cabinet

Vortex mixer

Microcentrifuge with rotor for 1.5 mL or 2 mL tubes, capable of attaining 17,900 x g

Bench top centrifuge

Magnetic Particle processor (KingFisher Flex, MagMAX-Express 96, Biosprint 96 or equivalent)

Ion ChefTM Instrument

Ion GeneStudioTM S5 System

Thermocycler

Qubit Fluorometer

DynaMag-96 Side Magnet (or similar)/DynaMag-2 (or similar)

Geneious Prime (<https://www.geneious.com/>) or similar for sequence evaluation

Supplies:

- MagMAX Core extraction reagents
- DMEM
- 5.0 mL screw cap tubes
- NGS Reverse Transcription Kit
- Ion AmpliSeq Library Kit Plus (Manual library prep) or Ion AmpliSeqTM Kit for Chef DL8 (Automated library prep)
- WGAG19041_PRD_CFPv01 CFP_with_SARS_2 pool 1 and WGAG19041_PRD_CFPv01 CFP_with_SARS_2 pool 2 (Primer pools are available from ThermoFisher Scientific under number PURDUE_CANINE_FELINE_COMBO2.20211215)
- ARTIC nCoV-2019 panel protocol v5.3.2 pools
- Ion Xpress Barcode Adapters for multiplexing (Manual library prep) or IonCodeTM Barcode Adaptors 1-384 Kit (Automated library prep) (ThermoFisher Scientific)
- Ion 510TM & Ion 520TM & Ion 530TM Kit – Chef (ThermoFisher Scientific)
- Ion S5 sequencing supplies (ThermoFisher Scientific)
- Oxford Nanopore MinION with Flongle Flowcells
- PCR Thermal Cycler
- Library End repair
(Ultra II end prep reaction buffer)
(Ultra II end prep reaction enzyme)
 - Sample Barcoding
(Native barcoding Expansion (Oxford Nanopore EXP-NBD196)
(Ultra II ligation master mix (New England Biolabs cat #E7546L)
(Ultra II ligation Enhancer (New England Biolabs cat #E7546L)



(Short Fragment Buffer (SFB; Oxford Nanopore EXP-SFB001)

(Elution buffer (EB)

- Adapter Ligation

(Adapter Mix II (AMII; Oxford Nanopore EXP-MRT001 or part of EXP-NBD196)

(NEB next quick ligation reaction buffer

(Quick T4 DNA ligase (New England Biolabs cat #E6056L)

(Short Fragment Buffer (SFB; Oxford Nanopore EXP-SFB001)

(Elution buffer (EB; Oxford Nanopore EXP-AUX001)

- AmPure XP reagent (Beckman Coulter)
- Qubit dsDNA HS Assay Kit (ThermoFisher Scientific)
- Gloves
- Lab coat
- Ethanol (70%)
- Low TE
- Microcentrifuge tubes (0.1 mL 8 strip tubes or 0.1 mL or 0.25 mL, 0.5mL and 1.5 mL)
- Pipets and pipet tips



Procedure

1 Nucleic acid extraction

1.1 KingFisher Flex MagMAX Core extraction protocol

In the extraction room, prepare the sample as follows. KingFisher Flex MagMAX Core extraction protocol

In the extraction room, prepare the sample as follows:

Add 2 mL of DMEM to a 5 mL tube.

Swirl the anterior nasal or throat swab and break off the swab tip.

Vortex the sample vigorously for 2 minutes or until it is fully suspended.

Use 200 µL of the supernatant for the downstream process.

1.2 Prepare the Lysis/Binding solution.

In the clean room, combine the following components for the required number of samples.

Component	Volume per sample
MagMAX Core Lysis solution	350µL
MagMAX Core Binding solution	350µL
Total Lysis/Binding solution*	700µL

Vortex at maximum speed for 10 seconds

Store at room temperature for up to 24 hours

2 Prepare the Bead/ Proteinase K mix.

In the clean room, prepare the following to be added to the lysis plate.

2.1 Vortex the MagMAX Core Magnetic Beads thoroughly to ensure the beads are fully resuspended



Component	Volume per sample
MagMAX Core Magnetic Beads	20µL
MagMAX Core Proteinase K	10µL
Total bead mix	30µL

2.2 The bead/ Proteinase K mix can be stored at 4°C for up to one week. Proceed to the next step.

3 Prepare the sample plates.

In the clean room, grab four deep well plates and prepare them as follows.

Plate position	Plate ID	Reagent	Volume per well
1	Lysis Plate	Bead mix	30µL
2	Wash Plate	MagMAX Core Wash Solution 1	500µL
3	Wash Plate 2	MagMAX Core Wash Solution 2	500µL
4	Elution Plate	MagMAX Core Elution Buffer	90µL

3.1 Take the prepared plates to an extraction hood in the extraction room and add Amount 200 µL of each sample to the lysis plate.



- 3.2 Mix the sample with the Bead mix by pipetting up and down several times. Let the mixture incubate for 2 minutes.
- 3.3 Add 700 μ L of Lysis/Binding solution (from step 2) to each sample in the first plate (lysis plate).
- 3.4 Turn the power on to the magnetic particle processor
- 3.5 Using the up and down arrow key, select the program MagMAX Core Flex.
- 3.6 Press the start key. When prompted, load the appropriate plate on the instrument. Properly orient the plates
- 3.7 Press the start key to advance the carousel to the next plate position to load.
- 3.8 When the final plate is loaded, place a deep well comb on the top of the first plate (Lysis Plate) and press the start key. When the program is complete, the instrument will prompt you to remove the elution plate. Remove the elution plate and set it in an extraction hood. Press start to advance the carousel and remove all other plates as prompted. Dispose of all the wash /lysis plates by placing in a biohazard bag.

Place a deep well comb on top of the first plate (Lysis Plate) after loading the final plate, then press the start key.
- 3.9 The eluted product can be used immediately or stored in a labelled 1.5mL tube or sealed elution plate (96 well) at -10°C until use.

4 **Reverse transcription (RT) using the Ion Torrent NGS Reverse Transcription Kit**

For each sample, set up the reaction using the following table:

Component	Volume per Reaction
5X Ion Torrent NGS Buffer	2 μ L
10X Ion Torrent NGS RT Enzyme	1 μ L
RNA Sample	7 μ L
Total Volume	10 μL

Note: You can set up the reaction using a 96-well plate/8-strip tube or individual 0.1-0.25 PCR tubes, depending on the number of samples.

- 4.1 Prepare 4 sets of cDNA per sample.
- 4.2 Mix the reaction mixture well and seal the plate/close the PCR tubes
- 4.3 Execute the following thermocycling conditions on a thermocycler.

Temperature	Time
25°C	10 minutes
50°C	10 minutes
85°C	5 minutes
10°C	Hold

Briefly spin the PCR tube or plate to bring the contents (cDNA) to the bottom of the tube.

- 5 **PCR Assay Preparation using Invitrogen™ Platinum™ Taq DNA Polymerase Kit**
For each sample, prepare **four separate PCR master mixes for the following primer pools:**

- **tNGS pathogens Primer Pool 1:** 5X Pool1: WGAG21047-PRD-CFPV02
- **tNGS pathogens Primer Pool 2:** 5X Pool2: WGAG21047-PRD-CFPV02
- **SARS-CoV-2 Primer Pool 1:** IDT ARTIC nCoV-2019 V5.3.2 Panel
- **SARS-CoV-2 Primer Pool 2:** IDT ARTIC nCoV-2019 V5.3.2 Panel

Each PCR reaction is prepared in a **final volume of 20 μL** , with **15 μL master mix + 5 μL DNA template**.

5.1 **PCR Master Mix Components (per primer pool of the pre-validated multiplex primer pools targeting multiple carnivore pathogens)**

Add 15 μL master mix to each well of the PCR 8-strip well containing cDNA, for a total volume of 25 μL per well sample.

Reagent	Volume Per reaction (μL)
RNase/DNase-free Water	7.15
10X PCR Reaction Buffer	2.5
50 mM MgCl_2	0.75
10 mM dNTP Mix	0.5
tNGS Pathogen Primer pool 1 or 2	5
Platinum [®] Taq DNA Polymerase	0.1

PCR Master Mix Components (per primer pool of the IDT ARTIC nCoV-2019 V5.3.2 Panel)

Add 15 μL master mix to each well of the PCR 8-strip well containing cDNA, for a total volume of 25 μL per well sample.

Reagent	Volume Per reaction (μL)
RNase/DNase-free Water	7.15
10X PCR Reaction Buffer	2.5
50 mM MgCl_2	0.75
10 mM dNTP Mix	0.5
IDT Artic nCoV-2019 Primer pool 1 or 2	4



Platinum [®] Taq DNA Polymerase	0.1

6 Purify PCR reactions—AmPure XP Beads,

6.1 Combine the four PCR assays per sample into a 1.5 mL tube.

6.2 Add 100 μ L beads per sample, mix by flicking the tube, briefly spin down, and incubate at room temperature for 5 min.

6.3 Prepare fresh 80% EtOH at least 10 mL.

6.4 Pellet the beads using a magnetic rack, remove the supernatant with a pipette.

6.5 Add 200 μ L 80% EtOH, avoiding the pellet, remove the EtOH and repeat.

6.6 Briefly spin tubes and remove residual EtOH.

6.7 Resuspend each pellet in 15 μ L nuclease free water, spin down, and incubate at room temp for 2 min.

6.8 Replace tubes on the magnetic rack, remove and keep the supernatant.

7 Measure the concentration using a qubit fluorometer and the 1X dsDNA HS kit

7.1 Use 1 μ L of sample and 199 μ L of working solution.

8 End-Prep (NEBNext Ultra II End Repair/dA-tailing module)



- 8.1 Use approximately 200 fmol per sample for the End-Prep.
- 8.2 Thaw all reagents and mix, do not vortex the enzyme mix.
- 8.3 Use a PCR 8 well strip and use approximately 200 fmol of sample per well.
- 8.4 Add 1.75 μ L reaction buffer NEBNext Ultra II End Repair/dA-tailing kit.
- 8.5 Add 0.75 μ L NEB enzyme mix, keep cooled.
- 8.6 Mix by flicking the tubes and spin down.

- 8.7 Thermocycler Parameters – **“NEB End Repair”**
Use the following program for the end-repair reaction:

	Step	Temperature	Time
	End Repair	20 °C	5 minutes
	Enzyme Inactivation	65 °C	5 minutes
	Final Hold	4 °C	∞ (hold)

- 8.8 Transfer each sample to a 1.5 mL tube and purify with AMPure beads.

9 **Bead purification**

- 9.1 Add 15 μ L of resuspended beads per sample and mix by flicking. Spin down.

- 9.2 Incubate at room temp for 5 min.
- 9.3 Pellet the beads on the magnetic rack until supernatant is clear and remove and discard the supernatant.
- 9.4 Add 200 μ L of fresh 80% EtOH to each tube without disrupting the pellets.
- 9.5 Remove the EtOH and repeat the previous step.
- 9.6 Spin down the tubes and replace on the magnetic rack, remove residual EtOH.
- 9.7 Resuspend in 10 μ L nuclease free water and spin down.
- 9.8 Incubate for 2 min at room temp.
- 9.9 Pellet the beads on the magnetic rack until eluate is clear.
- 9.10 Use 7.5 μ L of this eluate per sample for the next step.

10 **Barcode ligation - NEB Blunt/TA ligase master mix**

- 10.1 Thaw reagent and mix by pipetting and spin down. Place on ice or in refrigerated rack.
- 10.2 Remove appropriate number of barcodes (1 per sample) and thaw at room temp, mix by vortexing and spin down. Place on ice or refrigerated rack.
- 10.3 Thaw EDTA at room temp, vortex and spin down. Place on ice or refrigerated rack.

10.4 **Barcode Ligation Reaction Setup**



Prepare the following in a 1.5 mL microcentrifuge tube for each sample:

Component	Volume per Sample (μL)
End-prepped DNA	7.5
Native Barcode	2.5
Blunt/TA Ligase Master Mix	10.0
Total Volume	20.0

10.5 Mix samples by pipetting and spin down.

10.6 Incubate at room temp for 20 min.

10.7 Add 4 μL EDTA to stop the reaction to each sample.

10.8 Combine all samples into a single 1.5 mL tube.

11 **Bead Purification**

11.1 Add 0.4X beads to the pooled reactions.

11.2 Mix by flicking tube and spin down.

11.3 Incubate at room temp for 10 min.

11.4 Pellet the beads with a magnetic rack until supernatant is clear.

11.5 Keep the tube on the rack and remove the supernatant and discard.



- 11.6 Add 700 μL freshly prepared EtOH without disturbing the pellet. Remove the EtOH with a pipet and discard.
- 11.7 Repeat previous step.
- 11.8 Spin down the tube and place it on the rack, remove the residual EtOH with a pipette and discard.
- 11.9 Remove tube from rack and resuspend in 35 μL nuclease free water by flicking the tube. Spin down.
- 11.10 Incubate at room temp for 10 min, flicking tube approximately every 2 min.
- 11.11 Spin down and place on magnetic rack to pellet beads until eluate is clear.
- 11.12 You can remove the eluate and refrigerate overnight or proceed to the next step.

12 Adapter Ligation (NEB Quick Ligation Module)

- 12.1 Thaw reagents at room temp and mix. Spin tubes and place on ice or refrigerated block.

12.2 Adapter Ligation Reaction Setup

In a 1.5 mL microcentrifuge tube, combine the following:

Component	Volume (μL)
Pooled Barcoded Sample	30.0
Native Adapter	5.0
NEBNext Quick Ligation Buffer	10.0
Quick T4 DNA Ligase	5.0



Component	Volume (μL)
Total Volume	50.0

12.3 Mix by pipetting and spin down.

12.4 Incubate for 20 min at room temp.

13 Final clean-up (AMPure beads)

13.1 Add 20 μL resuspended beads to tube and mix by flicking the tube. Spin down.

13.2 Incubate for 10 min at room temp, flicking tube approximately every 2 min. to mix.

13.3 Spin down and place tube on magnetic rack.

13.4 Pellet beads until supernatant is clear, keep tube on rack and remove supernatant with a pipette and discard.

13.5 Wash beads with 125 μL SFB, flick tube to resuspend the pellet and spin down.

13.6 Replace tube on magnetic bead, pellet beads, and remove the SFB.

13.7 Repeat the previous 2 steps.

13.8 Perform an additional spin, replace tube on the rack and remove residual SFB with a pipette, discard.

13.9 Resuspend pellet with 10 μL elution buffer (EB) from the ONT native barcode kit.

13.10 Incubate at room temp for 10 min, mix by flicking tube approximately every two minutes during the incubation period.

13.11 Spin down and place on the magnet until eluate is clear.

13.12 Remove the eluate and retain. Dispose of the used beads.

14 Determine concentration

14.1 Use 1 μ L of eluate in 199 μ L of 1X dsDNA HS assay and follow the manufacturer's instructions to determine concentration with a Qubit fluorometer. Use approximately 20 fmol per sample for this step.

14.2 Prepare the sample to the correct concentration using the elution buffer from the native barcode kit. The total volume for the library should be 5 μ L.

14.3 Any remaining library can be stored at approximately 4°C for short-term storage (up to 1 month). For long-term storage -80°C is recommended.

15 Sequencing

15.1 Flongle Flow Cell Wash

Component	Volume (μ L)
Flow Cell Flush Buffer (FCF)	117.0
Flow Cell Flush Tether (FCT)	3.0
Total Wash Volume	120.0

15.2 Final Library Loading Mix

Component	Volume (μ L)
Sequencing Buffer (SB)	15.0



Component	Volume (μL)
Loading Beads (LIB)	10.0
Prepared Library	5.0
Total Volume	30.0

- 15.3 The flongle wash is loaded first, followed by the library sample mix. Be sure to not introduce bubbles when loading the flongle because it will mess up the run.

ONT data analysis steps for the tNGS pathogen panel and usage Geneious Prime

16 Reference-based ONT read mapping using FASTA templates and minimap2

Use curated FASTA reference genomes representing clinically relevant bacterial pathogens.

- Upload to **Geneious Prime** [canine_feline_v2_reference.fasta](#)

17 ONT read input preparation

- Use basecalled ONT reads generated from MinION sequencing.
- Retain demultiplexed and adapter-trimmed FASTQ reads with a minimum quality score of $Q \geq 9$.
- Confirm read quality and read count prior to alignment.

18 Read alignment with minimap2

- Align filtered ONT FASTQ reads to the curated FASTA reference template [canine_feline_v2_reference.fasta](#) using **minimap2** using data type Oxford Nanopore settings.

19 Alignment evaluation and validation

- Assess read mapping depth and genome coverage breadth across reference sequences.
- Confirm consistency of aligned reads across multiple genomic regions.
- Evaluate background noise and exclude sporadic or low-complexity alignments.

20 Organism confirmation criteria

- Consider an organism detected when aligned reads show:
- The sequence has to be at least 100 bp

- At least 10 Reads should be detected (across different primer sets detecting the same pathogen)
- Concordance with taxonomic classification results

21 **BLAST analysis**

- Perform sequence similarity searches using **BLAST** against appropriate reference databases (e.g., NCBI nt or curated local databases).
- Use default or optimized BLAST parameters suitable for pathogen comparison.
- Record top hits, percent identity, alignment length, and E-value for each query sequence, at least 99% identity and coverage, and an E-value of at least 0.0001

ONT data analysis steps for the SARS-CoV-2

- 22
- SARS-CoV-2 coverage was determined using CZID: <https://czid.org> using the ONT FastQ files
 - Minimap2 was used to map sequencing reads to the Wuhan SARS-CoV-2 reference sequence (NC_045512.2; MN908947.3) in Geneious Prime.
 - The consensus sequence was used to determine the variant type with Nextclade: <https://clades.nextstrain.org/> SARS-CoV-2 (XBB dataset, updated 7-17-2024).