Panmetagenomics protocol

Reagent	Company	Lot	Expiry date/received date	Extraction day
Lysis Matrix D 2mL Tube	MP Biomedicals			
HL-SAN enzyme	ArcticZymes Technolo			
LunaScript® RT SuperMix Kit (E3010)	New England Biolabs			
Sequenase Version 2.0 DNA Polymerase	ThermoFisher Scientific			
LongAmp® Taq 2X Master Mix	New England Biolabs			
Rapid barcoding Kit SQK-RPB004	Oxford Nanopore Thechnology			
AMPure XP Beads	BeckmanCoulter			
Qubit™ 1X dsDNA High Sensitivity (HS) (Q33230)	ThermoFisher Scientific			
NATtrol™ Respiratory Panel 2.1 (RP2.1) Controls	Zeptometrix			
Viasure respiratory panel III (PCR for PC)	Pro-labs			
Tobacco mosaic virus (PC-0107)	DSMZ			
TMV primers: TMV_fwd_aps -Catalog number: 10336022 GGATATGTCTAAGTCTGTTGC 10629186 Nucleotides (25 nmole) 11732013 Desalted574910 W7149 (E12) TMV_fwd_aps 574910 W7149 (F01) 3 TMV_rvr_aps TMV_rvr_aps -Catalog number: 10336022 CAGACAACTCGGGTGCG 10629186 Nucleotides (25 nmole) 11732013 Desalted	Invitrogen			
Fast SYBR™ Green Master Mix	Applied Biosystem			

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Microbial DNA-Free Water (ID: 338132) Qiagen	ThermoFisher Scientific			
RESPIRATORY SWAB MATRIX NEGATIVE CONTROL	Vircell			
Ethanol				
Elution buffer	ONT			

Equipment	**Company**
TissueLyser LT	Qiagen
Thermocycler for the lib prep	
Real-time PCR for TMV and PC	Quantstudio 7
Eppendorf ThermoMixer	Eppendorf
Qubit 4 Fluorometer	ThermoFisher Scientific

Note: Risk assessment for handling respiratory samples needs to be performed by each laboratory.

Preparation of quality controls:

- **Positive control: NATtrol™ Respiratory Panel 2.1 (RP2.1) Controls (Zeptometrix):**

1. Mix 300ul of control 1 and 300ul of control 2 in an Eppendorf. Label with date and lot number.
2. Vortex for 1 minute Centrifuge at 1,200g for 5min immediately prior to each use.
3. Aliquot the volume specified by the manual or automated extraction method and process in parallel with equivalent clinical sample volumes.
4. Store the mixed positive controls at 4°C, it can be used for two extractions.
5. Run a target PCR (Viasure respiratory panel III) anytime a new box is opened and recorded it on the trend analysis spreadsheet.

- **Negative control: Respiratory Swab Matrix Negative Control (Vircell):**

Add 500ul of Microbial DNA-Free Water to a vial and mix until completely reconstituted as per manufacturer's instructions. Vortex for 30 sec to dissolve and homogenise completely. Spike with 1ul of TMV.

NOTE: run the PC and NC using the same barcodes. We use barcode 11 for the PC and 12 for the NC.

- **Internal control (IC): Tobacco Mosaic Virus (TMV) PC-0107 (DSMZ):**

1. Reconstitute a new vial of TMV every Monday with 3mL of Microbial DNA-Free Water (Qiagen).
2. Vortex for 1 minute.
3. Aliquot 500ul of IC into 6 Eppendorf tubes, labelling each tube with the preparation date and lot number. Store at 4°C.
4. Use one aliquot per day to spike your samples and negative control, vortex for one minute before spiking
5. Do a target PCR on the aliquot used each time a new vial is reconstituted and record the Ct value on the trend analysis spreadsheet (see appendix 1). In our hands this is between 0.5ul and 5ul of TMV to give a final Ct value in the sample of between 5 and 6.
6. Discard any remaining volume from used aliquot each day and any remaining aliquots by Friday.

See Appendix 1 for the TMV targeted PCR.

- **Spiking samples with the internal control (IC):**

- Spiked the sample with 1ul of TMV is the following samples:

  -Broncho-alveolar lavage (BAL),

-Non-directed broncho-alveolar lavage (NBL)

-Endotracheal tube (ETT) aspirates

-Sputum.

- Spike with 0.5ul of TMV the following samples:

  -Pleural fluids

  -Nasal throat swabs

Sample preparation:

-Respiratory samples validated for this protocol are: BAL, NBL, ETT, NTS, sputum and pleural fluids.

-Samples processed should be fresh up to four days old storage in the fridge, older than that it could not be guarantee the RNA virus detection.

- Samples are processed in a different way depending on the sample type.

1. Sputum and ETT:

Mucoid samples such as sputum or ETT should be mucolysed before starting the Human DNA depletion process as follows:

Add approximately 2 mL sputum into a numbered, sterile, plastic universal container or if 2 mL already in original container then use this:

1. Add equal volume (2mL) of mucolytic agent Mucolyse to the sample container.
2. Vortex in the safety cabinet for 30 seconds.
3. Leave until liquefication at RT.
4. Gently agitate for a further 15 seconds before to proceed to the human DNA depletion

If after mucolysing the sample, this is still mucoid, pipette 500ul of the sample and mix with 500ul of PBS, vortex well and proceed with the human DNA depletion.

Human DNA depletion:

The human DNA depletion is three steps: centrifugation, bead-beating and endonuclease treatment. However, BAL, NBL and ETT aspirates are processed in a different way depending on the appearance of the sample. This is due to the different composition of the sample.

1. For turbid and mucoid samples: the human DNA depletion is performed as usual, centrifugation followed by bead-beating and endonuclease treatment.
2. Transparent samples with some mucoid debris: the centrifugation should be spiked and the only bead-beating and endonuclease treatment should be performed.
3. For clear samples with watery appearance: the human DNA depletion should be avoided and the sample should just be extracted.

1) **Centrifugation:**

☐ Vortex the sample

☐Centrifuge 1,200g for 10 min

☐Aliquot 500ul of the supernatant into an Eppendorf tube

☐Vortex the TMV aliquot

☐Spike 1ul of TMV to the sample and 0.5ul if processing a pleural fluid.

☐Vortex the sample with the TMV

☐ Procced with bead beating host-depletion step (2.)

2) **Bead beating and microbial extraction**:

☐Add 500ul of the supernatant post centrifugation or 500ul of whole sample to the Matrix Lysing D tube (MP biomedicals™) after vortexing for 1 minute.

☐ Place the matrix tube into the TissueLyser LT (Qiagen). Bead-beat at 50 osc for 3min. Note: sometimes the sample will become foamy after bead-beating, this is ok.

☐Transfer 200ul of the bead-beaten sample to a new Eppendorf tube, placing the tip at the bottom of the tube to take-up the liquid instead of the foam.

☐Add 10ul of HL-SAN enzyme (ArticZymes Technology).

☐Mix for 10' at 37°C at 1000rpm in the Eppendorf ThermoMixer (Eppendorf™).

NOTE: The HL-SAN is stopped by the Magnapure extraction when the instrument adds proteinase k. However, not all the extraction instruments/kits add proteinase k. If the extraction instrument/kit does not have this step, add the proteinase K manually as followed:

-Add 10ul of proteinase K to 200ul of the samples previously treated with HL-SAN.

-Vortex for 10 seconds.

-Incubate at 56°C for 10 min at 1000rpm.

☐Transfer 200ul to Magnapure 24 (Roche™) for extraction or proceed using alternative extraction methods validated in your own laboratory.

☐ Use total nucleic acid extraction kit 1.1 with pre-set BAL sample parameters and 50ul elution volume. Fast pathogen 200 1.1 is used for processing <8 samples and Pathogen 200

3.2 for >=8 samples. Settings for other extraction robots will need to be established in user laboratories.

Microbial nucleic acid processing and PCR amplification

1. **RT-dsDNA:**

☐ Transfer 16ul of the 50ul extract (from Magnapure) to a PCR tube and add 4ul LunaScript® RT SuperMix. Mix by flicking the tube and briefly spinning down in a small bench top Eppendorf centrifuge

☐ Place the tube in the thermocycler and incubate at 25°C for 2 minutes, 55°C for 10 minutes, 95°C for 1 minute

☐ Prepare the master mix for the double strand synthesis using the Sequenase Version 2.0 DNA Polymerase

dsDNA synthesis	Volume	x _____ samples
5× Sequenase Buffer	2ul	
Microbial DNA-Free Water	7.7ul	
Sequenase Dilution Buffer	0.9ul	
Enzyme	0.6ul	
Total volume		

☐ Add 11.2 ul of the master mix to the 20 ul of product post RT step. Mix by flicking the tube and briefly spin down.

☐ Place the tube in the thermocycler. Incubation at 37°C for 8 minutes.

☐ Prepare stock of 70% (700ul absolute ethanol+ 300ul of Microbial DNA-free water for 1mL) ethanol in Microbial DNA-Free Water.

☐ Add 45ul of resuspended AMPure XP Beads (AXP) into a new Eppendorf tube for each sample.

☐ Add all of the product post dsDNA synthesis (31.2ul) into the Eppendorf tube containing the beads, mix well by flicking the tube and incubate at room temperature for 5 minutes.

☐ Briefly spin down the samples and pellet the beads on a magnet until the eluate is clear and colourless. Keep the tubes on the magnet and discard the supernatant.

☐ Keep the tubes on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

☐ Repeat the previous step.

☐ Briefly spin down and place the tubes back on the magnet. Pipette off any residual ethanol with the 10p pipette. Allow to dry for 30 seconds, but do not dry the pellet to the point of cracking.

☐ Remove the tubes from the magnetic rack and resuspend the pellet in 12ul the Microbial DNA free water. Spin down and incubate for 2 minutes at RT.

☐ Pellet the beads on a magnet until the eluate is clear and colourless.

☐Remove and retain 10 µl of eluate for each sample into clean 1.5 ml Eppendorf DNA LoBind tubes, individually.

NOTE: the sample can be store at -80°C after the RT or dsDNA step

2. <u>Library preparation Rapid PCR Barcoding Kit (SQK-RPB004) Oxford Nanopore:</u>

☐ Add 3 ul of the previous product (dsDNA) to a new PCR tube.

☐ Add 1 ul of FRM (from ONT) into the previous Eppendorf, mix by flicking the tube and briefly spin down.

☐ Place the tube in the thermocycler and incubate at 30º for 1 minute and then 80ºC for 1 minute.

☐ Prepare the master mix for the PCR, adding 20ul of water and 25ul of LongAmp Taq 2X Master Mix per sample and 1ul of the barcode primers to 4 ul of the product obtained after the FRM step.

☐ Mix by flicking the tube and briefly spin down.

☐ Place in the thermocycler. Incubate @ 95º 3', (95º 15'', 56º 15'', 65º 4') x30, 65º 4'

3. <u>Post PCR:</u>

☐ Perform Qubit™ 1X dsDNA High Sensitivity (HS) on the PCR products as the manufacturer's instructions.

Sample number	Barcode	Qbit concentration (ng/ul)	Volume (ul) to be added to the pool

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☐ Pool samples as follows:

Based on the qubit readings, prioritize samples with a qubit reading below 1 ng/µL. For these samples, normalize the qubit concentration of your PCR products to 100 ng/µL.

n=sample

e.g. 100/(n)0.6= 166.67 add the total volume of PCR product.

☐ Clean up 1:1: Add the same volume of resuspended AMPure XP Beads (AXP) as the pool volume to the pool sample

☐ Mix well by flicking the tube, incubate for 5 minutes in the Hula mixer (rotor mixer) at room temperature

☐ Briefly spin down the samples and pellet the beads on a magnet until the eluate is clear and colourless. Keep the tubes on the magnet and discard the supernatant.

☐ Keep the tubes on the magnet and wash the beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

☐ Repeat the previous step.

☐ Briefly spin down and place the tubes back on the magnet. Pipette off any residual ethanol with the 10p pipette. Allow to dry for 30 seconds, but do not dry the pellet to the point of cracking.

☐ Remove the tubes from the magnetic rack and resuspend the pellet in 15 µl Microbial DNA-Free Trish buffer. Spin down and incubate for 2 minutes at RT.

☐ Pellet the beads on a magnet until the eluate is clear and colourless.

☐Remove and retain 10 µl of eluate into clean 1.5 ml Eppendorf DNA LoBind tubes, individually.

☐ Add RAP 1ul to 10ul of template, mix gently by flicking the tube, spin down and incubate at RT for 5 minutes.

☐ Add 25.5ul of LB

☐ Add 34 of SQB

☐ Add 4.5ul water

☐ Mix gently pipetting up and down

4. Flowcell loading:

☐ Thaw FLB and FLT at RT if not thawed from before
☐ Add 30ul of FLT (vortex before by pipetting) to a FLB (vortex before by pipetting).
☐ Mix by vortex.

☐ Open the GridION device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact.
☐ Slide the flow cell priming port cover clockwise to open the priming port.
☐ After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles: 1.
☐ Set a P1000 pipette to 200 µl 2. Insert the tip into the priming port 3.
☐ Turn the wheel until the dial shows 220-230 ul, to draw back 20-30 ul, or until you can see a small volume of buffer entering the pipette tip Note: Visually check that there is continuous buffer from the priming port across the sensor array.
☐ Load 800 µl of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five minutes.
☐ Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
☐ Load 200 µl of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.
☐ Mix the prepared library gently by pipetting up and down just prior to loading.
☐ Add 75 µl of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

☐ Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION or GridION device lid.

5. GridION settings:

1. Select the position of your flow cell on MinKNOW.
2. Type your experiment name and sample ID.
3. Continue to kit selection and select Rapid PCR barcoding kit SQK -RPB004.
4. Continue to run options and change to 24hr.
5. Continue to analysis and edit options and select barcode both ends and mid read barcode filtering.
6. Continue to output and unselect fast 5 and edit FastQ to 100 reads per file.

6. Bioinformatic pipeline and reporting

Curated bioinformatic pipeline will generate automatic reports at 30 minutes (for organism identification) and 2 hours (for AMR determinants). 16-24 hours reports can be generated manually for SNP typing and genomic alignment.

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Bioinformatic pipelines used for this SOP is based on the published pipeline (Charalampous T et al A**m J Resp Crit Care Med**. 2024 209:164-174). Further details available on request.

Appendix 1.

qPCR for the TMV

The RT step is performed using the LunaScript master mix explained previously in the section 3.

-Master mix:

Reagent	Volumen	X___samples
Fast Syber-green	10ul	
Primer Forward (10uM)	1ul	
Primer Reverse (10uM)	1ul	
DNAse-free water	3ul	
Template (cDNA)	5ul	
Total		

1 cycle at 95°C for 20 s, 40 cycles at 95°C for 1 s, 40 cycles at 60°C for 20 s, 1 cycle at 95°C for 15 s, 1 cycle at 60°C for 1 min, and 1 cycle at 95°C for 15 s

Ellis MD, Hoak JM, Ellis BW, Brown JA, Sit TL, Wilkinson CA, Reed TD, Welbaum GE. Quantitative Real-Time PCR Analysis of Individual Flue-Cured Tobacco Seeds and Seedlings Reveals Seed Transmission of Tobacco Mosaic Virus. Phytopathology. 2020 Jan;110(1):194-205. doi: 10.1094/PHYTO-06-19-0201-FI. Epub 2019 Nov 18. PMID: 31502520.

Appendix 2. Sample processing sheet.

Run name	Date

Sample number	Sample lab number	Sample type	McFarland	Centrifugation (Y/N)	Volume of TMV spiked	Barcode