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Pan-microbial metagenomics protocol v1.2 V.2

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Infectious research

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Protocol status: Working
We use this protocol and it's working

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Disclaimer

This workflow is not accredited.
This mechanical human DNA depletion method is patent pending (PCT/GB2023/051417)

Abstract

Version 1.2 of the pan-microbial metagenomics protocol enhances the depletion of human DNA in clinical samples and improves the detection of microorganisms and resistance genes. The improvements include the double bead-beating step and the use of a more effective endonuclease enzyme. Magnesium is added to optimise the reaction, which is then halted with EDTA.

Another key modification is the processing of BAL samples, which no longer undergo centrifugation prior to bead-beating.

Please remember to cite the original manuscript: Alcolea-Medina *et al*. Unified metagenomic method for rapid detection of microorganisms in clinical samples. Commun Med (Lond). 2024 Jul 7;4(1):135. doi: 10.1038/s43856-024-00554-3. PMID: 38972920

Materials

A	B
Reagent	**Company**
Lysis Matrix D 2mL Tube	MP Biomedicals
MSAN enzyme	ArcticZymes Technologies
EDTA (0.5M)	Thermo Scientific
MgCl2 (1M)	Serva
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	Invitrogen

	A	B
	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	
	Fast SYBR‰ Green Master Mix	Applied Biosystem
	Microbial DNA-Free Water (ID: 338132) Qiagen	ThermoFisher Scientific
	RESPIRATORY SWAB MATRIX NEGATIVE CONTROL	Vircell
	Ethanol	
	Elution buffer	ONT

	A	B
	Equipment	**Company**
	TissueLyser LT	Qiagen
	Thermocycler for the lib prep	VeritiPro Thermal Cycler
	Sorvall Legend X1 / X1R centrifuge	Thermo Scientific
	Real-time Thermocycler	Quantstudio 7
	Eppendorf ThermoMixer	Eppendorf
	Qubit 4 Fluorometer	ThermoFisher Scientific

Preparation of quality controls:

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

2 Mix **300µL of control 1** and **300µL of control 2** in an Eppendorf. Label with date and LOT number.

3 Vortex for 1 minute and Centrifuge at **1,200xg for 5min** immediately prior to each use.

4 Aliquot the volume specified by the manual or automated extraction method and process in parallel with equivalent clinical sample volumes.

5 Store the mixed positive controls at 4°C, it can be used for two extractions.

6 Run a target PCR **(Viasure respiratory panel III)** anytime a new box is opened and record it on the trend analysis spreadsheet.

Negative control: Respiratory Swab Matrix Negative Control (VircellI)

7 Add **500µL** 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
1µL of TMV.

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

8 Reconstitute a new vial of TMV every Monday with **3mL** of Microbial DNA-Free Water (Qiagen). Vortex for 1 min.

9 Aliquot **500µL** of IC into six Eppendorf tubes, labelling each tube with the preparation date and lot number. Store at 4°C.

10 Use one aliquot per day to spike your samples and negative control, vortex for 1 min before spiking

11 For every new vial reconstituted, TMV target PCR should be performed and Ct values should be recorded for trend analysis.
- 200ul of neat was extracted directly from the vial (no depletion performed).
-In our hands the Ct value was 5-6

12 Discard any remaining volume from used aliquot each day and any remaining aliquots by Friday.

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

Spike samples with **1µLof TMV** in the following samples:
-Broncho-alveolar lavage **(BAL)**
-Non-directed Broncho-alveolar lavage **(NBL)**
-Endotracheal tube **(ETT)** aspirates
-Sputum **(SPT)**
-Nasal throat swabs **(NTS)**

Spike with **0.5µL of TMV** the following samples:
-Pleural fluids **(PF)**

Sample preparation:

14 -Respiratory samples validated for this protocol are: **BAL, NBL, ETT, NTS, sputum and PF.**
-Samples processed should be fresh up to two 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.

Sputum and ETT:

15 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 do the following:

16 Add equal volume (2mL) of mucolytic agent Mucolyse to the sample container and Vortex in the safety cabinet for **30 sec**.

17 Leave until liquefication at room temperature (RT).

18 Gently agitate for a further **15 sec** before to proceed to the human DNA depletion

19 If after mucolysing the sample it is still mucoid, pipette **500µL** of the sample and mix with **500µL**of
PBS, vortex well and proceed with the human DNA depletion.

Human DNA depletion:

20 The human DNA depletion is three steps: **centrifugation, bead-beating and endonuclease treatment.**
However, BAL and NBL aspirates are not centrifuged and processed in a different way depending on the appearance of the sample. This is due to the different composition of the sample.

21 **For turbid and mucoid samples:** the human DNA depletion is performed by double bead-beating and endonuclease treatment.

22 **For clear samples with watery appearance even those with some debris:** the human DNA depletion should be avoided and the sample should just be extracted.

1) Centrifugation:

23 Vortex the sample

24 Centrifuge speed **1,200xg for 10 min.**

25 Aliquot **500µL** of the supernatant into an Eppendorf tube
If centrifugation is being removed then vortex sample well and add 500ul of whole sample.

26 Spike **1µL** of TMV to the sample (as indicated above) and **0.5µL** for processing a PF and NTS. Make sure to vortex the TMV aliquot before use.

27 Vortex the sample with the TMV

28 Procced with double bead beating.

2) Bead beating and depletion of free nucleic acid, followed by extraction:

29 Transfer sample from Eppendorf (500ul) to the Matrix **Lysing D tube (MP biomedicals™)** after vortexing for 1 min.

30 Place the matrix tube into the **TissueLyser LT (Qiagen)**. Bead-beat at **50 osc for 3min.** Bead-beat again for 3mins at same settings.

Note: sometimes the sample will become foamy after bead-beating, this is ok.

31 Meanwhile add **10µL of M-SAN** enzyme (ArticZymes Technology) with 1**µL** of MgCl2 (1M) in new Eppendorf tube (1.5 ml) for each sample.

32 Transfer **200µL** of the bead-beaten sample to Eppendorf tubes containing M-SAN, placing the tip at the bottom of the tube to take-up the liquid instead of the foam.

33 Incubate for **10' at 37ºC at 1000rpm** in the Eppendorf ThermoMixer (Eppendorf™).

34 Add **4ul of EDTA** (0.5M) to stop the MSAN reaction.

35 Transfer **200µL to Magnapure 24 (Roche™)** for extraction or proceed using alternative extraction methods validated in your own laboratory

1. Microbial nucleic acid processing and PCR amplification

36 **RT-dsDNA:**

37 Transfer **16µL** of the 50µL extract (from Magnapure) to a PCR tube and add **4µL of LunaScript®** RT SuperMix.

38 Mix by flicking the tube and briefly spinning down in a small bench top Eppendorf centrifuge.

39 Place the tube in the thermocycler and incubate at **25ºC for 2 min, 55ºC for 10 min, 95ºC for 1 min.**

40 Prepare the master mix for the double strand synthesis using the Sequenase Version 2.0 DNA Polymerase using the following volumes:

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		

Table 1: Reagents required for DsDNA master mix.

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

42 Place the tube in the thermocycler. Incubation at **37ºC for 8 min**.

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

44 Add **45µL** of resuspended AMPure XP Beads (AXP) into a new Eppendorf tube for each sample.

45 Add all of the product post dsDNA synthesis (31.2µL) into the Eppendorf tube containing the beads, mix well by flicking the tube and incubate at RT for **5 min**.

46 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.

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

48 Repeat the previous step.

49 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 sec**, but do not dry the pellet to the point of cracking.

50 Remove the tubes from the magnetic rack and resuspend the pellet in **12µL** of Microbial DNA free water. Spin down and incubate for **2 min at RT**.

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

52 Remove and retain **10µL** of eluate for each sample into clean 1.5 mL Eppendorf DNA LoBind tubes, individually.

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

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

54 Add **3µL** of the previous product (dsDNA) to a new PCR tube with **1µL of FRM** (from ONT **Rapid PCR barcoding kit SQK -RPB004**). Mix by flicking the tube and briefly spin down.

55 Place the tube in the thermocycler and incubate at **30ºC for 1 min and then 80ºC for 1 min.**

56 Mix by gently flicking the tube and briefly spin down.

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

58 Mix by gently flicking the tube and briefly spin down.

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

Post PCR:

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

61

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

Table 2: Data collection for qubit concentrations and volume (µL) per sample being added to the pool.

62 **Pool samples as follows:**
Based on the qubit readings, prioritise samples with a qubit reading below 1 ng/µL. For these samples, normalise 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.

63 Clean up 1:1 by adding the same volume of resuspended AMPure XP Beads (AXP) to the same volume of the pooled samples. for example, **50µLof pool = 50µL of beads**

64 Mix well by flicking the tube, incubate for **5 min** in the Hula mixer (rotor mixer) at RT.

65 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.

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

Repeat the previous step.

67 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 sec, but do not dry the pellet to the point of cracking.

68 Remove the tubes from the magnetic rack and resuspend the pellet in **15µL** Microbial DNA-Free Trish buffer. Spin down and incubate for 2 min at RT.

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

70 Remove and retain **10µL** of eluate into clean 1.5 mL Eppendorf DNA LoBind tubes, individually. Make sure no beads are remaining.

71 -Add RAP **1µL**to **10µL** of template, mix gently by flicking the tube, spin down and incubate
at **RT for 5 min**.
- Add **25.5µL** of LB
- Add **34µL** of SQB
- Add **4.5µL** of water
- Mix gently pipetting up and down

Flowcell loading:

72 Thaw FLB and FLT at RT if not thawed from before.

73 Add **30µL** of FLT (vortex and spin before by pipetting) to a FLB (vortex before by pipetting). Mix by vortex.

74 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.

75 Slide the flow cell priming port cover clockwise to open the priming port.

76 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles.

77 Set a P1000 pipette to 200µL and insert the tip into the priming port.

78 Turn the wheel until the dial shows **220-230µL** , to draw back **20-30µL** , 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.

79 Load **800µL** of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for five min.

80 Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

81 Load **200µL** of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

82 Mix the prepared library gently by pipetting up and down just prior to loading.

83 Add **75µL** 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.

84 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.

GridION settings:

85 Select the position of your flow cell on MinKNOW.

86 Type your **experiment name** and **sample ID.**

87 Continue to kit selection and select **Rapid PCR barcoding kit SQK -RPB004.**

88 Continue to run options and change to **24hr.**

89 Continue to analysis and edit options and select **barcode both ends** and **mid read barcode filtering**.

90 Continue to output and unselect fast 5 and edit FastQ to **100 reads per file.**

Bioinformatic pipeline and reporting:

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

92 This is the link for the bioinformatic pipeline analysis **GitHub - GSTT-CIDR/RespiratoryCmg** .
NOTE: A new version will be available soon.

qPCR for the TMV

93 The RT step is performed using the LunaScript master mix explained previously in the step 39.

94 Master-mix calculations as followed:

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

Table 3: Reagents required for TMV qPCR

1094/PHYTO-06-19-0201-FI. Epub 2019 Nov 18. PMID: 31502520.

Primer sequence for TMV:
-TMV_fwd_aps:
Catalog number: 10336022
GGATATGTCTAAGTCTGTTGC
10629186 Nucleotides (25 nmole) 11732013 Desalted.

-TMV_rvr_aps:
Catalog number: 10336022

CAGACAACTCGGGTGCG
10629186 Nucleotides (25 nmole) 11732013 Desalted.

95 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

96 The validation of this method is available on **Unified metagenomic method for rapid detection of microorganisms in clinical samples | Communications Medicine (nature.com)**.