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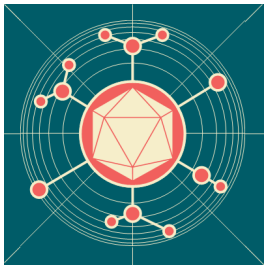
Viral Metagenomics utilising SMART-9N Amplification



Forked from Viral metagenomics using SMART-9n amplification and nanopore sequencing

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We use this protocol and it's working

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Abstract

Following on from the original SMART-9N (Switch Mechanism at the 5′ End of RNA Templates) protocol utilised for viral metagenomics, this protocol offers an optimised approach to improve genome representation in diverse microbial communities and also incorporate DNA viruses.

This protocol is also adapted from the "Rapid metagenomic sequencing for surveillance of bacterial, fungal and viral pathogens using SQK-RPB114.24" protocol published by Oxford Nanopore Technologies (https://nanoporetech.com/document/rapid-sequencing-metagenomics-sqk-rpb114-2).

The protocol utilises a shotgun approach using random 9N priming to reverse transcribe RNA and PCR-amplify RNA present. Furthermore, as the random priming is independent of the poly(A) tail of RNA, DNA is also annealed and amplified (similarly to as described in **https://doi.org/10.1016/j.meegid.2015.03.018)**. Therefore, it is ideal for viral metagenomics to enrich for the viruses present in the sample often obscured by high host backgrounds.

Optimisation Implemented:

- Host depletion has been moved before the total nucleic acid extraction, removing extracellular DNA present in the sample and allowing DNA viruses to be processed through the extraction.
- Total nucleic acid extraction utilising column purification has been replaced by a magnetic bead extraction.
- Primer concentrations have been adjusted to reflect the conditions providing the greatest yield and genome coverage.
- An unmodified PCR primer is utilised in place of the ONT RLB barcoding primers to ensure sufficient amplification for library preparation that the barcoding primers do not provide. We have found that utilising the barcoding primers result in 10x less yield post-PCR in comparison to the unmodified primer.

Materials

Reagents and Consumables:

	Reagent / Consumable	Supplier	Catalogue Number
	HL-SAN Triton Free	ArcticZymes	70911-202
	MagMAX Viral/Pathogen Nucleic Acid Isolation Kit	ThermoFisher Scientific	A42352
	10 mM dNTPs	ThermoFisher Scientific	R0192
	SuperScript™ IV Reverse Transcriptase	ThermoFisher Scientific	18090050
	RNaseOUT™ Recombinant Ribonuclease Inhibitor	ThermoFisher Scientific	10777019
	Q5® High-Fidelity 2X Master Mix	New England Biolabs	M0492S
	AMPure XP Beads for DNA Cleanup	Beckman Coulter	A63880
	Qubit™ dsDNA Quantification Assay	ThermoFisher Scientific	Q32851/Q32854
	Qubit™ Assay Tubes	ThermoFisher Scientific	Q32856
	1.5 mL Eppendorf DNA LoBind tubes	Eppendorf	0030108051
	0.2 mL PCR tubes		
	Nuclease-Free Water		
	Molecular Grade 100% Ethanol		

Equipment:

	Equipment	Supplier	Catalogue Number
	Qubit™ 4 Fluorometer	ThermoFisher Scientific	Q33226
	Vortex		
	Microcentrifuge		
	Thermomixer		
	HulaMixer		
	Magnetic Rack (To fit 1.5 mL Eppendorf tubes)		

	Equipment	Supplier	Catalogue Number
	Thermocycler		

Oligonucleotides:

	Oligonucleotide	Sequence	Type
	RLB RT 9N	TTTTTCGTGCGCCGCTTCAACNNNNNNNNN	DNA
	RLB SSP	GCTAATCATTGCTTTTTCGTGCGCCGCTTCAACATrGrGrG	RNA
	RLB PCR	TTTTTCGTGCGCCGCTTCA	DNA

Troubleshooting

Host Depletion and Extraction

1 Transfer 500 µL of sample into a 1.5 mL Eppendorf tube.

2 Centrifuge at 10000 x g for 00:05:00 .

3 For each sample, transfer 300 µL of supernatant into a separate 1.5 mL Eppendorf tube.

4 To each sample, add 10 µL of HLSAN enzyme and vortex for 00:00:03 .

5 Incubate the reaction on a thermomixer at 37 °C for 00:10:00 at 1000 rpm .

6 Add the following reagents to the reaction and mix by inversion:

	Component	Volume
	Binding Buffer from the MagMAX kit	520 µL
	Magnetic beads from the MagMAX kit	20 µL
	Proteinase K from the MagMAX kit	10 µL
	Total	860 µL

7 Transfer the samples into a thermomixer and incubate at 65 °C for 00:05:00 at 1000 rpm .

8 Transfer the sample tubes to a HulaMixer™ and incubate/mix at room temperature for 00:05:00 using the HulaMixer™.

9 Prepare 2 mL of fresh 80% ethanol per sample in nuclease-free water.

10 Briefly spin down the tubes and pellet on a magnetic rack until the supernatant is clear and colourless for at least 00:05:00 .

11 Keep the tube on the magnetic rack and pipette off the supernatant.

12 Remove the tube from the magnetic rack and add 1 mL of Wash Buffer from the MagMAX kit and gently mix by inverting the tube until fully resuspended.

13 Briefly spin down the tubes and pellet on the magnetic rack until the supernatant is clear and colourless for at least 00:02:00 .

14 Keep the tube on the magnetic rack and pipette off the supernatant.

15 Remove the tube from the magnetic rack and add 1 mL of 80% ethanol and gently mix by inverting the tube until fully resuspended.

16 Briefly spin down the tubes and pellet on the magnetic rack until the supernatant is clear and colourless for at least 00:02:00 .

17 Keep the tube on the magnetic rack and pipette off the supernatant.

18 Remove the tube from the magnetic rack and add 500 µL of 80% ethanol and gently mix by inverting the tube until fully resuspended.

19 Briefly spin down the tubes and pellet on the magnetic rack until the supernatant is clear and colourless for at least 00:02:00 .

20 Keep the tube on the magnetic rack and pipette off the supernatant, allow the pellet to dry for 00:02:00 but do not dry the pellet to the point of cracking.

21 Remove the tube from the magnetic rack and resuspend the pellet by adding 20 µL of nuclease-free water and ensure the pellet is fully resuspended by pipette mixing.

22 To aid with sample elution, transfer the tubes to a thermomixer and incubate at 65 °C for 00:05:00 at 1000 rpm .

23 Pellet the beads on a magnet until the eluate is clear and colourless for at least 00:01:00 .

24 Remove and retain the eluate into a clean 1.5 mL Eppendorf tube.

SMART-9N Annealing, cDNA Synthesis and Amplification

25 Per reaction, combine and mix thoroughly the following in a 0.2 mL PCR tube:

	Component	Volume
	dNTP mix (10 mM)	1 µL
	RLB RT 9N (12 µM)	1 µL
	RNA	10 µL
	Total	12 µL

26 Incubate at 65 °C for 00:05:00 and then snap cool on ice.

27 Per reaction, combine the following to create a master mix:

	Component	Volume
	SSIV Buffer (5X)	4 µL
	DTT (100 µM)	1 µL
	RNase OUT	1 µL
	SSIV RT (200 units/ µL)	1 µL
	RLB SSP (12 µM)	1 µL
	Total	8 µL

28 Add 8 µL of the master mix to 12 µL of the annealed RNA.

29 Incubate on the thermocycler as follows:

	Temperature	Time
	42 °C	90 minutes
	70 °C	10 minutes
	4 °C	Hold

30 Combine the following per reaction to create the amplification master mix:

	Component	Volume
	Q5 High-Fidelity 2X Master Mix	12.5 µL
	RLB PCR (10 µM)	1 µL
	Nuclease-Free Water	9 µL
	cDNA	2.5 µL
	Total	25 µL

31 Incubate the reaction as follows on the thermocycler:

	Temperature	Time	Cycles
	98 °C	45 seconds	1
	98 °C	15 seconds	30
	62 °C	15 seconds	
	65 °C	5 minutes	
	65 °C	10 minutes	1
	4 °C	Hold	1

Quality Control

32 Quantify the PCR products utilising the Qubit™ dsDNA HS (High Sensitivity) Assay Kit on the Qubit Fluorometer.

Protocol

NAME

DNA Quantification using the Qubit Fluorometer

CREATED BY
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Preview

Note

If the Qubit™ fluorometer is not available, alternative fluorometers can be utilised including:

- Promega Quantus™ Fluorometer and the QuantiFluor® dsDNA System (Catalogue Number: E6150 and E2671).
- "DIYNAFLUOR" (DIY Nucleic Acid FLUORometer) - a portable, open-source, <$40 USD Nucleic Acid fluorometer compatible with Qubit™ reagents (**https://doi.org/10.1101/2024.12.16.626200**).

1X Clean-up

33 Add a ratio of 1X AMPure XP beads to the sample and mix gently.

34 Incubate the samples at room temperature on the HulaMixer™ for 00:05:00 .

35 Place the sample on the magnet and allow the beads to pellet before removing the supernatant.

36 On the magnetic rack, wash the beads with 150 µL of 80% Ethanol, being careful not to disturb the pellet. Remove the ethanol and discard.

37 Repeat the previous step.

38 Spin down the pellet and remove any excess ethanol remaining and allow the pellet to lose its shine.

39 Resuspend the pellet in 20 µL of Nuclease-free water by flicking the tube and incubate at room temperature for 00:05:00 .

40 Place the sample on the magnet and transfer the clear supernatant into a fresh 1.5 mL Eppendorf tube.

Sequencing

41 The samples can now be sequenced. We recommend utilising the ONT Native Barcoding Kit (SQK-NBD114.24 / SQK-NBD114.96) for numerous samples or the ONT Ligation Sequencing kit (SQK-LSK114) for a single sample, following the manufacturer's protocol. For metagenomics, it is recommended to utilise the PromethION to generate sufficient data, however, the MinION or GridION can be used for a smaller number of samples or lower input libraries.

Protocol references

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