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Viral metagenomics using SMART-9n amplification and nanopore sequencing

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

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Abstract

This is a SMART-Seq (Switch Mechanism at the 5′ End of RNA Templates) protocol developed to use random 9n priming and be compatible with ONT RLB rapid, barcoded adapters available from the SQK-RPB004 kit. We have generated cDNA reads in excess of 10 kb from Zika viral reference material with this protocol.

Materials

Reagents required:

Ultrafree-MC Centrifugal Filter (Merck, 10228490)
ZymoBIOMICS DNA Microprep Kit with Lysis Tubes (Zymo, D4301)
Quick-RNA Viral Kit (Zymo, R1034)
Turbo DNase (Thermo Fisher, AM2238)
Clean up kit Zymo Research, (Zymo, R1015)
SuperScript IV (Thermo Fisher, 15307696)
RNase OUT (Thermo Fisher, 10777019)
Ampure XP 60 ml (Beckman Coulter, A63881)
LongAmp Taq 2X Master Mix (NEB, M0287)

Oligos required:

RLB TSO (Order as RNA oligo from IDT):
GCTAATCATTGCTTTTTCGTGCGCCGCTTCAACATrGrGrG
RLB RT 9N:
TTTTTCGTGCGCCGCTTCAACNNNNNNNNN
RLB PCR (not required but useful for testing):
TTTTTCGTGCGCCGCTTCA

Centrifugal filtration

1 Transfer up to 500 µL sample directly onto a Ultrafree-MC Centrifugal Filter column

2 Spin at 5000 x g for 00:01:00

3 Recover filtrate into 1.5 mL Eppendorf tube

4 Remove basket and discard

5 Close lid and place on ice

Viral RNA extraction

6 In a 2 mL Eppendorf tube combine 200 µL sample, 200 µL DNA/RNA Shield (2x concentrate) and mix well by pipetting

7 Add 800 µL Viral RNA Buffer and mix well by pipetting

8 Load 600 µL onto a column in a collection tube and spin at 10000 x g for 00:00:15 discard flow through, place in a new collection tube

9 Add 500 µL Viral wash buffer and spin at 10000 x g for 00:00:15 , discard flow through

10 Add 500 µL 100% ethanol and spin at 10000 x g for 00:01:00 , discard flow through, place in a clean 1.5 mL tube

11 Add 15 µL of DNA/RNA-Free Water and incubate at RT for 00:03:00

12 Spin at 10000 x g for 00:00:15

DNase Treatment

13 Set heat block to 37 °C

14 Set up the following reaction:

Component	Volume
RNA	44 µL
10X TURBO DNase Buffer	5 µL
TURBO DNase	1 µL
Total	50 µL

15 Incubate at 37 °C for 00:30:00

DNase cleanup

16 Add 100 µL RNA Binding Buffer and mix by vortexing 00:00:05 and spin down

17 Add 150 µL 100% ethanol and mix by vortexing for 00:00:15 and spin down

18 Transfer 300 µL to a Zymo-Spin IC column in a 2 mL collection tube and spin at 6000 x g for 00:00:15 discard flow through

19 Add 400 µL RNA Prep Buffer and spin at 6000 x g for 00:00:15 , discard flow through

20 Add 700 µL RNA Wash Buffer and spin at 6000 x g for 00:00:15 , discard flow through and place in a new 1.5 mL Eppendorf tube

21 Add 10 µL DNase/RNase Free Water and incubate at RT for 00:01:00

22 Spin at 6000 x g for 00:00:15

23 Label as 'Viral RNA' and place on ice

Viral DNA extraction

24 Set heat block to 55 °C

25 In a 2 mL Eppendorf tube combine 200 µL sample, 200 µL DNA/RNA Shield (2X concentrate), 20 µL Proteinase K and mix well by pipetting

26 Incubate at 55 °C for 00:30:00

27 Set heat block to 60 °C

28 Add 1.200 mL binding buffer and mix well

29 Load 800 µL onto a Zymo-Spin IIC-Z column in a collection tube and spin at 8000 x g for 00:00:15 , discard the discard flow-through and reload as many times as required

30 Transfer to to a new collection tube add 400 µL DNA Wash Buffer 1 and spin at 8000 x g for 00:00:15 , discard flow-through

31 Add 700 µL DNA Wash Buffer 2 and spin at 8000 x g for 00:00:15 .

32 Add 200 µL DNA Wash Buffer 2 and spin at 8000 x g for 00:01:00 .

33 Transfer to a new 1.5 mL Eppendorf tube and 50 µL DNA/RNA Free Water preheated to 60 °C to the column, incubate at RT for 00:01:00 .

34 Spin at 8000 x g for 00:01:00 .

SMART-9n amplification

35 Combine the following in a 0.2ml 8-strip tube.

Component	Volume
RLB RT 9N (2 uM)	1 µL
dNTPs (10 mM ea.)	1 µL
Template RNA	10 µL
Total	12 µL

36 Mix and spin down.

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

38 Make up the following master mix and add 8 µL to the 12 µL annealed RNA:

Component	Volume
SSIV buffer (5x)	4 µL
DTT (100 mM)	1 µL

RNase OUT	1 µL
SS IV RTase (200 U/ul)	1 µL
RLB TSO (2 uM)	1 µL
Total	20 µL

39 Start the following program on a thermocycler:

42 °C for 01:30:00
70 °C for 00:10:00

40 Set up the following reaction to amplify cDNA in a new 0.2ml 8-strip tube:

Component	Volume
LongAmp Taq 2X master mix	25 µL
RLB (01-12)	0.5 µL
NFW	19.5 µL
cDNA	5 µL
Total	50 µL

41 Start the following program on the thermal cycler:

Step	Temperature	Time	Cycles
Heat Activation	95 °C	00:00:45	1
Denaturation	95 °C	00:00:15	26
Annealing	56 °C	00:00:15	26
Extension	65 °C	00:05:00	26
Final extension	65 °C	00:10:00	1
Hold	4 °C	Indefinite	1

PCR clean-up

42 Clean up products with 1x Ampure XP and elute in 30 ul EB

Protocol

NAME

Amplicon clean-up using SPRI beads

CREATED BY

Josh Quick

PREVIEW

42.1 Vortex SPRI beads thoroughly to ensure they are well resuspended, the solution should be a homogenous brown colour.

Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880

42.2 Add an equal volume (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting. For example add 50 µL SPRI beads to a 50 µL reaction.

42.3 Pulse centrifuge to collect all liquid at the bottom of the tube.

42.4 Incubate for 00:05:00 at room temperature.

42.5 Place on magnetic rack and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear.

42.6 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

42.7 Add 200 µL of room-temperature [M] 70 % volume ethanol to the pellet.

42.8 Carefully remove and discard ethanol, being careful not to touch the bead pellet.

42.9 go to step #42.7 and repeat ethanol wash.

42.10 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette.

42.11 With the tube lid open incubate for 00:01:00 or until the pellet loses it's shine (if the pellet dries completely it will crack and become difficult to resuspend).

42.12 Resuspend pellet in 30 µL Elution Buffer (EB), mix gently by either flicking or pipetting and incubate for 00:02:00 .
Elution Buffer (EB) **Qiagen Catalog #**19086

42.13 Place on magnet and transfer sample to a clean 1.5mL Eppendorf tube ensuring no beads are transferred into this tube.

42.14 Quantify 1 µL product using the Quantus Fluorometer using the ONE dsDNA assay.
QuantiFluor(R) ONE dsDNA System, 100rxn **Promega Catalog #**E4871

Equipment

Quantus	NAME
Fluorometer	TYPE
Promega	BRAND
E6150	SKU
https://www.promega.co.uk/products/microplate-readers-fluorometers-luminometers/fluorometers/quantus-fluorometer	LINK

QC

43 Quantify the PCR products using the Quantus Fluorometer using the ONE dsDNA assay.

Protocol

NAME

DNA quantification using the Quantus fluorometer

CREATED BY

Josh Quick

PREVIEW

43.1 Remove Lambda DNA 400 ng/µL standard from the freezer and leave on ice to thaw. Remove ONE dsDNA dye solution from the fridge and allow to come to room temperature.

QuantiFluor(R) ONE dsDNA System, 500rxn **Promega Catalog #**E4870

43.2 Set up two 0.5 mL tubes for the calibration and label them 'Blank' and 'Standard'

43.3 Add 200 µL ONE dsDNA Dye solution to each tube.

43.4 Mix the Lambda DNA standard 400 ng/µL standard by pipetting then add 1 µL to one of the standard tube.

43.5 Mix each sample vigorously by vortexing for 00:00:05 and pulse centrifuge to collect the liquid.

43.6 Allow both tubes to incubate at room temperature for 00:02:00 before proceeding.

43.7 Selection 'Calibrate' then 'ONE DNA' then place the blank sample in the reader then select 'Read Blank'. Now place the standard in the reader and select 'Read Std'.

43.8 Set up the required number of 0.5 mL tubes for the number of DNA samples to be quantified.

Note

Use only thin-wall, clear, 0.5mL PCR tubes such as Axygen #PCR-05-C

43.9 Label the tubes on the lids, avoid marking the sides of the tube as this could interfere with the sample reading.

43.10 Add 199 µL ONE dsDNA dye solution to each tube.

43.11 Add 1 µL of each user sample to the appropriate tube.

Note

Use a P2 pipette for highest accuracy.

43.12 Mix each sample vigorously by vortexing for 00:00:05 and pulse centrifuge to collect the liquid.

43.13 Allow all tubes to incubate at room temperature for 00:02:00 before proceeding.

43.14 On the Home screen of the Quantus Fluorometer, select `Protocol`, then select `ONE DNA` as the assay type.

Note

If you have already performed a calibration for the selected assay you can continue, there is no need to perform repeat calibrations when using ONE DNA pre diluted dye solution. If you want to use the previous calibration, skip to step 11. Otherwise, continue with step 9.

43.15 On the home screen navigate to 'Sample Volume' and set it to 1 µL then 'Units' and set it to ng/µL.

43.16 Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.

43.17 Repeat step 16 until all samples have been read.

43.18 The value displayed on the screen is the dsDNA concentration in ng/µL, carefully record all results in a spreadsheet or laboratory notebook.

Rapid adapter attachment

44 Pool all barcoded products to a total of [M] 200 Femtomolar (fM) in 10 µL of [M] 10 millimolar (mM) Tris-HCl pH 8.0 with [M] 50 millimolar (mM) NaCl

45 Add 1 µL RAP adapter and mix by pipetting, incubate at RT 00:05:00

MinION sequencing

46 Prime the flowcell and load sequencing library onto the flowcell.

Protocol

NAME

Priming and loading a MinION flowcell

CREATED BY
Josh Quick

PREVIEW

46.1 Thaw the following reagents at room temperature before placing on ice:

Sequencing buffer (SQB)
Loading beads (LB)
Flush buffer (FLB)
Flush tether (FLT)

46.2 Add 30 µL FLT to the FLB tube and mix well by vortexing.

46.3 If required place a new MinION flowcell onto the MinION by flipping open the lip and pushing one end of the flowcell under the clip and pushing down gently.

46.4 Rotate the inlet port cover clockwise by 90° so that the priming port is visible.

46.5 Take a P1000 pipette and tip and set the volume to 800 µL. Place the tip in the inlet port and holding perpendicularly to the plane of the flowell remove any air from the inlet port by turning the volume dial anti-clockwise.

Note

Be careful not to remove so much volume that air is introduced onto the rectangular array via the outlet.

46.6 Load 800 µL of FLB (plus FLT) into the flow cell via the inlet port, dispense slowly and smoothly trying to avoid the introduction of any air bubbles.

46.7 Wait for 00:05:00 .

46.8 Gently lift the SpotON cover to open the SpotON port.

46.9 Load another 200 µL of FLB (plus FLT) into the flow cell via the inlet port, this will initiate a siphon at the SpotON port to allow you to load the library dilution.

46.10 In a new tube prepare the library dilution for sequencing:

Component	Volume
SQB	37.5 µL
LB	25.5 µL
Final library	12 µL
Total	75 µL

Note

Mix LB immediately before use as they settle quickly.

Dilute library in EB if required.

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

46.12 Add the 75 µL library dilution to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop siphons into the port before adding the next.

46.13 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the inlet port and close the MinION lid.