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Viral metagenomics using SMART-9n amplification and nanopore sequencing

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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 TEXT

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 1.2 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

Amplicon clean-up using SPRI beads
by Josh Quick
PREVIEW RUN

42.1 ⚠

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

Agencourt AMPure XP
by 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 #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)
by 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
by Promega
Catalog #: E4871

Quantus
Fluorometer
Promega E6150

QC

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

DNA quantification using the Quantus fluorometer PREVIEW RUN
by Josh Quick

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
by 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.

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.

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.

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 **200 Femtomolar (fM)** in **10 µl** of **10 Milimolar (mM)** Tris-HCl pH 8.0 with **50 Milimolar (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.

Priming and loading a MinION flowcell
by Josh Quick
PREVIEW RUN

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.

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

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.