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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):
GCTAATCATTGCTTTTTTCGTGCGCCGCTTCAACATrGrGrG
RLB RT 9N:
TTTTTCGTGCGCCGCTTCAACNNNNNNNNNN
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 $^{\circ}\text{C}$ 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 $^{\circ}\text{C}$ 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







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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  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


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PREVIEW


43.1 Remove Lambda DNA 400 ng/ μ L 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 μ L ONE dsDNA Dye solution to each tube.

43.4 Mix the Lambda DNA standard 400 ng/ μ L standard by pipetting then add  1 μ L 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 μ L ONE dsDNA dye solution to each tube.

- 43.11 Add  1 μ L 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.

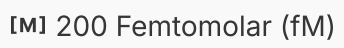

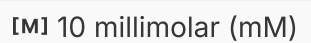
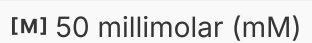




Note

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  200 Femtomolar (fM) in  10 μL of  10 millimolar (mM) Tris-HCl pH 8.0 with  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

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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 flowcell 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.