Zika virus whole genome sequencing from clinical samples

Description:

This protocol is for whole genome sequencing of Zika virus from clinical samples. RNA is extracted from using the QIAamp Viral RNA Mini Kit before an additional purification with AmpureXP beads. RT-PCR is performed in two-steps with random hexamers being used to generate cDNA, followed by multiplex PCR to generate overlapping amplicons. After been cleaned-up with AmpureXP, amplicon pools are barcoded using the native barcoding kit from ONT. Up to 12 samples are then run on a single MinION flowcell.

Reagents required:

Random Hexamers (50 µM) – (ThermoFisher cat num. N8080127)
Protoscript II First Strand cDNA Synthesis Kit (NEB cat num. E6560)
Q5 Hot Start High-Fidelity DNA Polymerase (NEB cat num. M0493)
Deoxynucleotide (dNTP) Solution Mix (NEB cat num. N0447)
QIAamp Viral RNA Mini Kit (Qiagen cat num. 52906)
Agencourt AMPure XP (Beckman Coulter cat num. A63881)
Qubit dsDNA HS Assay Kit (Thermo Fisher cat num. Q32854)
SpotON Flow Cell R9 (Oxford Nanopore Technologies)
Nanopore sequencing kit R9 (Oxford Nanopore Technologies)
PCR-grade water
Oligonucleotides (see last page)

1. RNA extraction and purification

Follow procedure in QIAamp Viral RNA Mini Handbook to extract RNA from sample. Freeze thawing of serum and plasma should be avoided as it can cause precipitation of proteins leading to reduced efficiency.

Elute RNA in 50 ul EA buffer. Add 50 ul resuspended SPRI beads (1x ratio) and allow 5 minutes to bind, inverting once a minute. Briefly spin down and place on a magnetic rack, once the beads have pelleted remove the supernatant and discard. With the tube still on the magnetic rack, wash the pellet twice with 80% ethanol. Spin down and remove the residual ethanol then leave to air dry until the pellet loses its shine. To elute, add 30 ul DPEC-treated water and resuspend beads by flicking. Leave for 5 minutes before spinning down and placing on magnetic rack to separate. Transfer the cleaned RNA into an RNase free Eppendorf tube.

2. cDNA generation

NB: The following two steps must be performed in a hood or dedicated pre-PCR area. Wash all surfaces with 1% sodium hypochlorite solution and irradiate labware with UV light for at least 10 minutes.

NB: A water negative control sample must be included in each batch of samples sequenced.

Mix the following in a PCR tube:

7 ul RNA from previous step

1 ul Random Hexamers

Mix by inversion, spin down
Heat in a dry bath at 65°C for 5 minutes then place on a mixture of ice and water
Add the following to each tube

10 ul ProtoScript II Reaction Mix (2X)
2 ul ProtoScript II Enzyme Mix (10X)

Place on the thermocycler and start the following program

25°C for 5 minutes
48°C for 15 minutes
80°C for 5 minutes
Hold at 4°C

3. Multiplex PCR

Make the following mastermixes in 1.5 ml Eppendorf tubes, multiply volumes by the number of samples plus 10%.

	Pool A	Pool B
5X Q5 Reaction Buffer	5 ul	5 ul
10 mM dNTPs	0.5 ul	0.5 ul
Q5 DNA Polymerase	0.25 ul	0.25 ul
Nuclease-free water	15.25 ul	15.25 ul
Primer pool A (10 uM)	1.5 ul	-
Primer pool B (10 uM)	-	1.5 ul

Label 2 PCR tubes per sample (Pools A & B). Mix by inversion and spin down, pipette 22.5 ul of each master mix into the corresponding tube. Add 2.5 ul cDNA from previous step, mix by inversion and spin down. Run the following program on a thermocycler:

Step 1 (Initial denaturation)
98°C 30 seconds
Step 2 (45 cycles)
98°C 15 seconds
65°C 5 minutes
Hold at 4°C

4. Clean-up and quantification

Clean up PCR reactions by adding 25 ul SPRI beads (1x ratio) as previously described. Elute in 30 ul nuclease-free water. Quantify 1 ul of the cleaned amplicons using the Qubit with the high-sensitivity assay as per the manufacturer's instructions.

Optional QC step - Load 2 ul amplicon pools and 1 ul 6x loading dye on a 1% agarose gel to check for specific amplification, a band at 400 bp should be observed.

5. Pooling

To normalize the input to the barcoding library preparation add 125 ng material per pool to a 1.5 ml Eppendorf. Add nuclease-free water to adjust volume to 30 ul.

6. End-prep

Add 4.2 ul Ultra II End-prep buffer and 1.8 ul Ultra II End-prep enzyme mix. Mix by inversion and spin down, incubate at 20°C for 5 minutes and 65°C for 5 minutes.

Clean-up End-prep reactions by adding 36 ul SPRI beads (1x ratio) as previously described. Elute in 15 ul nuclease water.

7. Barcoding

Set up the following ligation reactions to barcode the amplicon pools:

15 ul end prepped DNA
5 ul of one of the barcodes NB01-NB12
20 ul Blunt/TA Ligase Master Mix

Incubate at room temperature for 10 minutes. Incubate at 65°C for 10 minutes to denature ligase. Combine all 12 ligation reactions into a single tube. Clean-up combined barcoded amplicon pools by adding 480 ul SPRI beads (1x ratio) as previously described. Elute in 39 ul nuclease-free water. Quantify 1 ul combined barcoded amplicon pools on the Qubit to confirm 1000 ng DNA has been retained (>25 ng/ul).

8. Adapter ligation

Set up the following ligation reaction, mixing between each addition:

38 ul barcoded amplicon pools
10 ul BAM
2 ul BHP
50 ul Blunt/TA Ligase Master Mix

Mix by inversion and spin down, incubate at room temperature for 10 minutes. Add the following to the reaction:

1 ul HPT

Mix by inversion and spin down, incubate at room temperature for 10 minutes.

9. C1 bead binding

Vortex C1 beads thoroughly, add 50 ul to a 1.5 ml Eppendorf tube. Pellet beads on magnetic rack and remove the supernatant. Wash twice by resuspending in 100 ul BBB, pelleting on a magnetic rack and removing the supernatant. Resuspend beads in 100 ul BBB and add to the ligation reaction from the previous step. Place on a rotator and incubate for 10 minutes. Spin down and place on a magnetic rack to pellet, remove the supernatant. Wash twice by resuspending in 150 ul BBB (it is normal for the beads to adhere to the tube, scrape them off with the pipette tip if necessary), pelleting on a magnetic rack and removing the supernatant. Briefly spin down and remove any residual BBB. Resuspend pellet in 25 ul ELB

and incubate at 37°C for 10 minutes. Pellet on a magnetic rack and transfer the eluate to a new 1.5 ml Eppendorf tube.

10. Quantification and loading

Make up the following priming buffer:

500 ul nuclease-free water
500 ul RBF1

Load 500 ul slowly through the sample loading port using the a P1000 pipette. It is extremely important not to introduce or push and air bubbles into the flowcell. Wait 5 minutes, load another 300 ul priming bugger though the sample loading port. Wait 5 minutes. Flip up the SpotON tab to open the SpotON port, load another 200 ul priming buffer though the sample inlet port. Quantify 1 ul eluted library using the Qubit. Add 100 ng library to a 1.5 ml Eppendorf tube, adjust volume to 37.5 with nuclease-free water. Add the following:

37.5 ul RBF1

Mix by inversion and spin down. Add the 75 ul diluted library to the flowcell by allowing droplets to fall onto the SpotON port using a P100 pipette. Droplets will be drawn into the flowcell by capillary action. If the library is not drawn into the flowcell, perform a further 200 ul prime via the sample inlet port and try loading the library again.

11. Starting the run

Enter the sample ID and start the sequencing run script using MinKNOW.

Primer pools

Pool 1

Name	Sequence	Volume ul
400_1_out_L	GACAGTTCGAGTTTGAAGCGAAAG	9.13
400_1_out_R	AGTATGCACTCCCACGTCTAGT	9.13
400_3_out_L	AGATGACGTCGATTGTTGGTGC	40
400_3_out_R	TACGGTGACACAACCTCCATGT	40
400_5_out_L	AGAACGTTAGTGGACAGAGGCT	8.43
400_5_out_R	TGTGCGTCCTTGAACTCTACCA	8.43
400_7_out_L	TGAAGGGCGTGTCATACTCCTT	3.76
400_7_out_R	CGCCTCCAACTGATCCAAAGTC	3.76
400_9_out_L	GCCTTAGGGGGAGTGTTGATCT	39.87
400_9_out_R	GAGTGGGCATTCCTTCAGTGTG	39.87
400_11_out_L	CAGCCGTTATTGGAACAGCTGT	7.07
400_11_out_R	CCTGGGCCTTATCTCCATTCCA	7.07
400_13_out_L	TGGCAGTGCTGGTAGCTATGAT	5.42
400_13_out_R	AGAGAGAGGAGCATAAACCCCC	5.42
400_15_out_L	CCCTAGCGAAGTACTCACAGCT	7.13
400_15_out_R	TACACTCCATCTGTGGTCTCCC	7.13
400_17_out_L	GTGGTCCATGGAAGCTAGATGC	40

400_17_out_R	CCTCTAAGGGCCTCCTCCATTT	40
400_19_out_L	TATGGATGAGGCCCACTTCACA	12.16
400_19_out_R	GCCATCAAGTATGACCGGCTTT	12.16
400_21_out_L	AGAGACTGACGAAGACCATGCA	12.09
400_21_out_R	CTCCAAAAGCCGCTCCTCTTTT	12.09
400_23_out_L	CGTCTTGATGAGGAACAAGGGC	10.21
400_23_out_R	AAGTGGTCACTGCATGTTGGAC	10.21
400_25_out_L	CCCTGACCCTAATAGTGGCCAT	7.73
400_25_out_R	CCTTCCATTTCTCTCCCAGGGT	7.73
400_27_out_L	AGTGCAAAGCTGAGATGGTTGG	16.65
400_27_out_R	ATGTGTAGAGTTGCGGGAGAGT	16.65
400_29_out_L	AGGATGTGAATCTCGGCTCTGG	2.91
400_29_out_R	ATGCTGCATTGCTACGAACCTT	2.91
400_31_out_L	ACAAGGGGAATTTGGAAAGGCC	8.76
400_31_out_R	CGTAAGTGACAACTTGTCCGCT	8.76
400_33_out_L	CAAACGAATGGCAGTCAGTGGA	3.8
400_33_out_R	ATCCACACTCTGTTCCACACCA	3.8
400_35_out_L	ACCACCTGGGCTGAGAACATTA	40
400_35_out_R	ACCACTAGTCCCTCTTCTGGAG	40
		550.24

Pool 2

Name	Sequence	Volume
400_2_out_L	AAGAAAGATCTGGCTGCCATGC	9.98
400_2_out_R	TGATTCCAACCAGGTTTGCGAC	9.98
400_4_out_L	TCAGGTGCATAGGAGTCAGCAA	10.33
400_4_out_R	GGAGCCATGAACTGACAGCATT	10.33
400_6_out_L	TTGATTGTGAACCGAGGACAGG	16
400_6_out_R	CCATCTGTCCCTGCGTACTGTA	16
400_8_out_L	GGGAGAAGAAGATCACCCACCA	15.24
400_8_out_R	TTGACTGCTGCTGCCAATCTAC	15.24
400_10_out_L	ACGGTCGTTGTGGGATCTGTAA	8.55
400_10_out_R	GTGGGACTTTGGCCATTCACAT	8.55
400_12_out_L	CACTAAGGTCCACGTGGAGGAA	11.57
400_12_out_R	TATCAGCGCCAGATGAGCTACA	11.57
400_14_out_L	CAATGGTTTTGCTTTGGCCTGG	7.13
400_14_out_R	TTTCCCATGTGATGTCACCTGC	7.13
400_16_out_L	GTGGCATGAACCCAATAGCCAT	26.13
400_16_out_R	GCTCCAATGTCCCCATCCTTTG	26.13
400_18_out_L	CTGTTGAGTGCTTCGAGCCTTC	19.37
400_18_out_R	TGGTGAGTTGGAGTCCGGAAAT	19.37
400_20_out_L	GGCTGGAAAACGGGTCATACAG	11.92
400_20_out_R	CCTTTGCTCCGTCCTAAGCTTG	11.92
400_22_out_L	TGGACCAGACACGGAGAGAAAA	40
400_22_out_R	ATTCTGGCTGGCTCAATTTCCG	40
400_24_out_L	TAATGGGAAGGAGAGAGGAGGG	30.8
400_24_out_R	TCTCCACTTGGGGGTCAATTGT	30.8
400_26_out_L	ACTGGAACTCCTCTACAGCCAC	10.52
400_26_out_R	ACCAGGGCCTCCTTTTGTGTAT	10.52

400_28_out_L	GGTGGGGGATTGGCTTGAAAAA	13.64
400_28_out_R	GGGCCTCATAGCTTCCATGGTA	13.64
400_30_out_L	AAAAGTGGACACTAGGGTGCCA	8.29
400_30_out_R	TAATCCCAGCCCTTCAACACCA	8.29
400_32_out_L	AAATGGAAAAAGGGCACAGGGC	7.05
400_32_out_R	TGTCCCATCCAGTTGAGGGTTT	7.05
400_34_out_L	ATTTCCACAGAAGGGACCTCCG	8.5
400_34_out_R	TGACTAGCAGGCCTGACAACAT	8.5
		510.04