

Sequencing of Ebola Virus Genomes Using Nanopore Technology

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[Abstract] Sequencing of virus genomes during disease outbreaks can provide valuable information for diagnostics, epidemiology, and evaluation of potential countermeasures. However, particularly in remote areas logistical and technical challenges can be significant. Nanopore sequencing provides an alternative to classical Sanger and next-generation sequencing methods, and was successfully used under outbreak conditions (Hoenen *et al.*, 2016; Quick *et al.*, 2016). Here we describe a protocol used for sequencing of Ebola virus under outbreak conditions using Nanopore technology, which we successfully implemented at the CDC/NIH diagnostic laboratory (de Wit *et al.*, 2016) located at the ELWA-3 Ebola virus Treatment Unit in Monrovia, Liberia, during the recent Ebola virus outbreak in West Africa.

[Background] Determining the full-length sequence of virus genomes is an essential procedure in virology. While the classical approach to this involved Sanger sequencing following a primer-walking strategy, newer approaches involve the use of next-generation sequencing methods such as 454, Illumina or Ion Torrent technologies. A common problem with all these technologies, despite their many advantages, is that the required instrumentation is large, expensive, fragile, and therefore difficult to transport. Also, library preparation procedures are often involved. While under usual circumstances these issues are of little consequence, as these machines are run in specialized laboratories with excellent infrastructure, during virus outbreaks in remote areas (for example in case of ebolavirus outbreaks) this can pose significant problems, particularly since the export of samples from affected areas to these specialized laboratories is often politically and logistically challenging. Under these circumstances, the availability of a sequencing technology that can be easily and quickly deployed into remote areas, and allows sequencing to be done directly in an outbreak area, can be invaluable. Therefore, we have tested the MinION sequencing device, which at that time was under development by Oxford Nanopore Technologies (ONT), at the field diagnostic laboratory at the ELWA-3 Ebola virus Treatment Unit in Monrovia (de Wit *et al.*, 2016) during the recent Ebola virus outbreak in West Africa, and developed a protocol for the rapid generation of full-length sequences of Ebola viruses under these conditions. This device employs nanopores, through which nucleotide-strands are transported in a controlled fashion. The nucleotides block and thus modulate an ion-current flowing through those pores, depending on the physical properties of the nucleotides passing through the nanopores, and these current modulations are measured by the device and translated into nucleotide sequences. Results of this test, which indicated that this technology indeed shows great promise as a rapidly deployable and highly usable sequencing platform, are available elsewhere (Hoenen *et al.*, 2016), as are the results of

a similar test using the same sequencing platform performed independently of our own efforts by Quick *et al.* (2016).

Materials and Reagents

Note: This protocol was established and tested during the Ebola virus outbreak in West Africa in January 2015, using materials and reagents available at that time. As the development of the MinION platform progresses rapidly, some modifications might be necessary to adopt the protocol to the materials and reagents available now. Particularly, while at the time the technology was only available to members of the MinION access program, it is now commercially available.

1. 0.2 ml PCR-tubes (ideally in strips of 8) (e.g., Thermo Fisher Scientific, Thermo Scientific™, catalog number: AB0490)
2. 1.5 ml tubes (e.g., Thermo Fisher Scientific, Fisher Scientific, catalog number: S348903)
3. Eppendorf protein LoBind tubes, 1.5 ml, PCR clean (Eppendorf, catalog number: 0030108116)
4. Gloves
5. MinION flowcell, revision 7.3 (Oxford Nanopore Technologies [ONT])
6. RNA freshly purified (or stored at -80 °C) from patient blood samples following appropriate safety protocols
7. SuperScript III First-Strand-Synthesis System (Thermo Fisher Scientific, Invitrogen™, catalog number: 18080051)
8. DEPC-treated nuclease-free water (e.g., Thermo Fisher Scientific, Ambion™, catalog number: AM9906)
9. Primer, 10 µM (see Table 1 for sequences); primer CGGACACACAAAAAGAAAGAAG at a concentration of 2 µM and 10 µM
10. dNTP mix, 10 mM each (e.g., New England Biolabs, catalog number: N0447S)
11. iProof™ high-fidelity polymerase (Bio-Rad Laboratories, catalog number: 1725301)
12. Agencourt AMPure XP beads (Beckman Coulter, catalog number: A63881)
13. His-Tag dynabeads (Thermo Fisher Scientific, Novex™, catalog number: 10103D)
14. Magnetic stand for PCR-purification with Agencourt AMPure XP beads in 1.5 ml tubes (e.g., Beckman Coulter, catalog number: A29182)
15. 70% ethanol
16. Qiagen elution buffer (QIAGEN, catalog number: 19086)
17. 50x TAE buffer (ideally in 10.2 ml aliquots) (e.g., Thermo Fisher Scientific, Thermo Scientific™, catalog number: B49)*
18. Agarose (ideally in pre-weighed aliquots of 0.5 g) (e.g., Thermo Fisher Scientific, Invitrogen™, catalog number: 16500100)*
19. DNA standard (e.g., New England Biolabs, catalog number: N3200S)*
20. 6x gel loading dye (e.g., New England Biolabs, catalog number: B7022S)*
21. Fast Blast DNA stain (Bio-Rad Laboratories, catalog number: 1660420)*

22. NEBNext dA-tailing module (New England Biolabs, catalog number: E6053S)
23. NEBNext end repair module (New England Biolabs, catalog number: E6050S)
24. Blunt/TA ligase master mix (New England Biolabs, catalog number: M0367L)
25. Genomic DNA Sequencing Kit SQK-MAP004 (ONT): contains DNA CS, 2x wash buffer, elution buffer, EP buffer, HP adapter, Adapter mix and Fuel mix

**Note: Optional materials and reagents.*

Equipment

1. MinION sequencing device (ONT)
2. PCR cycler (e.g., Thermo Fisher Scientific, Applied Biosystems™, catalog number: A24811)
3. Vortexer (e.g., Thermo Fisher Scientific, Fisher Scientific, catalog number: S96461A)
4. Mini centrifuges for 1.5 and 0.2 ml tubes (e.g., VWR, catalog number: 93000-204)
5. Pipettes: 10 µl, 20 µl, 100 µl, 1,000 µl, 8 x 20 µl multichannel with filter tips (e.g., Mettler-Toledo, catalog numbers: PR-10, PR-20, PR-100, PR-1000, L8-20XLS+)
6. Racks for 0.2 ml PCR tubes and 1.5 ml tubes (e.g., Thermo Fisher Scientific, Fisher Scientific, catalog numbers: 05-541-85, 05-541-2)
7. PCR cooler rack, 0.2 ml, 4 °C (e.g., Eppendorf, catalog number: 3881000031)
8. Styrofoam box with lid and cold packs (frozen cold pack at bottom, 4 °C cold pack on top, then sample rack on top of that) as cooler box (or alternatively ice box, when ice is available)
9. Electrophoresis chamber with power supply (e.g., Mupid-exU, Takara Clontech)*
10. Microwave oven*
11. Agarose gel casting stand, or alternatively laboratory tape*
12. 250-300 ml glass flask for preparing agarose*
13. Box for agarose gel staining*
14. Additional infrastructure when sequencing under outbreak conditions

Note: This infrastructure is a given in any Western laboratory, but should be considered when establishing the method under field conditions in outbreak areas. While this topic cannot be exhaustively covered in this protocol, the following points should be carefully considered.

- a. Power generator, uninterruptable power supplies, voltage regulators (all depending on the quality of the electricity supply).
- b. Air conditioning if possible, alternatively external heatsink (e.g., a ~30 x 30 cm metal plate) for MinION sequencing device.
- c. Clean water for preparation of 1x TAE, and for DNA staining and destaining - bottled drinking water (ideally in 500 ml bottles) can be used for this purpose if no other source of clean water is available.
- d. Equipment for safe sample inactivation and RNA extraction.
- e. 4 °C fridge, -20 °C freezer, optionally -80 °C freezer for storage of RNA.
- f. Personal protective equipment as deemed necessary.

**Note: Optional equipment.*

Software

1. Laptop running MinKNOW software v 0.48.2.12 (ONT) with internet connection (*e.g.*, via 3G wireless router); for current IT requirements please check the ONT internet site (<https://nanoporetech.com/community/faqs>).

Procedure

A. Reverse transcription (RT) using the SuperScript III First-Strand-Synthesis System

1. Combine 4 µl RNase free water, 1 µl primer (2 µM, CGGACACACAAAAAGAAAGAAG), 1 µl dNTPs, 5 µl RNA, mix by flicking, spin down, incubate for 5 min at 65 °C, put in PCR cooler for > 1 min.
2. Prepare mastermix (6 µl 5x FS buffer, 1.5 µl DTT, 1.5 µl RNase out, 1.5 µl Superscript III. All components are provided as part of the RT kit), add 7 µl to samples, mix by flicking, spin down, incubate for 50 min at 50 °C , 5 min at 85 °C, hold at 4 °C, add 1 µl RNase H, mix by flicking, spin down, incubate for 20 min at 37 °C, hold at 4 °C.

B. PCR using iProof polymerase

1. Set up mastermix for 14 reactions (Table 2; this includes 2 extra reactions that are not used) using the cDNA from step A2 as template, dispense 12 x 44.2 µl into 0.2 ml PCR tube strips on PCR cooler block, add 2.5 µl forward primer (10 µM) and 2.5 µl reverse primer (10 µM) according to table 1, flick, spin down, cycle (Table 3, first PCRs).
2. Set up mastermix for 28 reactions (Table 2; this includes 4 extra reactions that are not used) using the PCR products from step B1 as template, dispense 24 x 44.2 µl into PCR tube strips on PCR cooler block, add 0.8 µl PCR product from step B1 as template (see Table 1 to determine which PCR product from step B1 to use as template), add 2.5 µl forward primer (10 µM) and 2.5 µl reverse primer (10 µM) according to Table 1, flick, spin down, cycle (Table 3, second PCRs). For an overview of the whole procedure see Figure 1.

Table 1. Primer sequences. Each pair of primer sequences (1 forward and 1 reverse primer) correspond to a single reaction.

First PCRs (step B1)	Second PCRs (step B2)	
fwd: GAGTGCGGACAGTTTCCTTC rev: GATGAATGCTGATGACACACTG	rxn 1	fwd: GGCCAAGCATGGAGAGTATG rev: CAAGCTCGGGGAATGTCAC
	rxn 2	fwd: GGGTGGACAACAGAAAAACAG rev: CAAGCTCGGGGAATGTCAC
fwd: CGAAGCCAAACCCGAAGATG rev: GAGAGCATCTTGCAATTGTGTAC	rxn 1	fwd: CCCCTCAATGTGCCCTAATTC rev: GTCGCCTCACAATATCCTTCTAG
	rxn 2	fwd: GCGTAATCTTCATCTCTCTTAG rev: CCATCCTGTCCACCAATTGTC
fwd: CTTGACATCTCTGAGGCAAC rev: GGGTGTGATTTACAGCTAAATGC	rxn 1	fwd: CGAACCACATGATTGGACCAAG rev: CTCATCAGACCTCCGCATTAATC
	rxn 2	fwd: ATATGAGAGAGGACGCCCCC rev: CAGTGAGGATTTATCTGTGGTTAAAC
fwd: GTTAACTTGACATCTCTGCCTTC rev: TGTCGTGAGGATGTACATGATC	rxn 1	fwd: CAAGGCACTATCAGGCAATG rev: CTGCATCAGTCTCTAAGGG
	rxn 2	fwd: CCTCACAATTTCAGTACCAAACG rev: CCGATAGTCCAGCTTATTCG
fwd: CAACCTGGTGGGAAACCATTC rev: TGGACACACAAAAAGAAGAAATAG	rxn 1	fwd: CCGAGAAAATGAATTGATTATGAC rev: AGTTAAATGACTTAGCCAGTATGG
	rxn 2	fwd: CCTCACAATTTCAGTACCAAACG rev: TGGACACACAAAAAGAAGAAATAG
fwd: CGGACACACAAAAAGAAGAAAG rev: CATGGTGAGGTCTCCTGGAG	rxn 1	fwd: CGGACACACAAAAAGAAGAAAG rev: CCTGTTTGCCTTCTTGACTAT
	rxn 2	fwd: CATGGCAATCTGCAACATC rev: GATGAATGCTGATGACACACTG
fwd: CCCCTCAATGTGCCCTAATTC rev: CACACGGTAACTGGAGAGC	rxn 1	fwd: GAGACACTCCATCGAATCCAC rev: GGGTCCTCCGTTGCATTGAC
	rxn 2	fwd: GCGTAATCTTCATCTCTCTTAG rev: CTCGATAATTCTCTCTGGATGATG
fwd: ATATGAGAGAGGACGCCCCC rev: GGGTGTGATTTACAGCTAAATGC	rxn 1	fwd: ATATGAGAGAGGACGCCCCC rev: ATGCAGGGGCAAAGTCATTAG
	rxn 2	fwd: GGTGGAAAGTTTATTGGGCTG rev: GGGTGTGATTTACAGCTAAATGC
fwd: TCTCGAACCATTGTGCTTGG rev: TGTCGTGAGGATGTACATGATC	rxn 1	fwd: CCTCACAATTTCAGTACCAAACG rev: CCGATAGTCCAGCTTATTCG
	rxn 2	fwd: CTGGACAAGTATTTATGTGCTC rev: CAGCTGTTTGCCTTGGAAAAATG
fwd: CGAGAAAATGAATTGATTTATGAC rev: TGGACACACAAAAAGAAGAAATAG	rxn 1	fwd: GAGATCCGTCATTGATACCACAG rev: TGGACACACAAAAAGAAGAAATAG
	rxn 2	fwd: ATGCCACACAAAACCATCTC rev: TGGACACACAAAAAGAAGAAATAG
fwd: CTGGACAAGTATTTATGTGCTC rev: AGTTAAATGACTTAGCCAGTATGG	rxn 1	fwd: CTCCGAATGATTGAGATGGATG rev: TGTCGTGAGGATGTACATGATC
	rxn 2	fwd: CAACCTGGTGGGAAACCATTC rev: GCCGACTTAAATCTCTATTTC
fwd: CAAGGCACTATCAGGCAATG rev: CTGCATCAGTCTCTAAGGG	rxn 1	fwd: CAGTTTTGAAGCTGCACTATG rev: GGGTGTGATTTACAGCTAAATGC
	rxn 2	fwd: CAGTTTTGAAGCTGCACTATG rev: GATATTGTGGTAGTAGATACTCGAG

Table 2. PCR master mix

Component	Volume for 1 reaction (μl)	Volume for 14 reactions (μl) (first PCRs)	Volume for 28 reactions (μl) (second PCRs)
H ₂ O	30.95	433.3	866.6
5x GC	10	140	280
dNTPs	1	14	28
DMSO	1.5	21	42
MgCl ₂	0.5	7	14
iProof	0.25	3.5	7
Template	0.8	11.2	-

Table 3. PCR cycling conditions

First PCRs			Second PCRs		
1 x	30 sec	98 °C	1 x	30 sec	98 °C
10 x	15 sec	98 °C	10 x	15 sec	98 °C
	30 sec	59-54.5 °C (- 0.5 °C/cycle)		30 sec	59-54.5 °C (- 0.5 °C/cycle)
	90 sec	72 °C		60 sec	72 °C
30 x	15 sec	98 °C	30 x	15 sec	98 °C
	30 sec	54 °C		30 sec	54 °C
	90 sec	72 °C		60 sec	72 °C
1 x	3 min	72 °C	1 x	3 min	72 °C

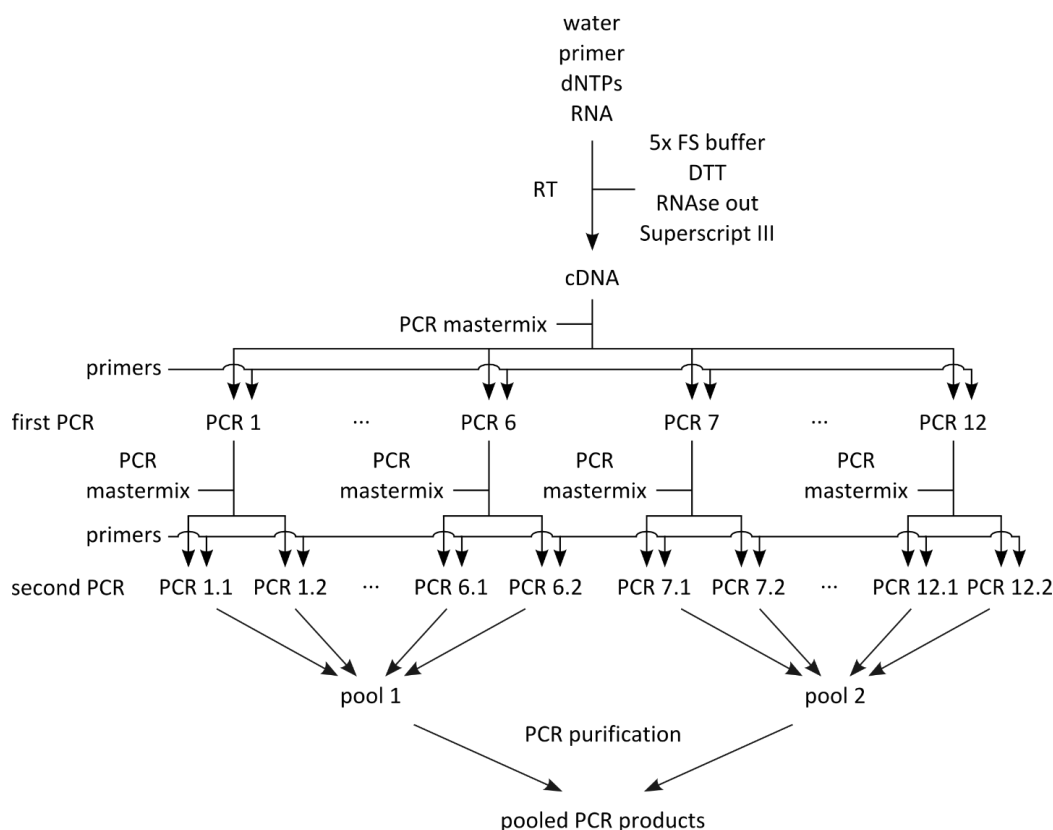


Figure 1. Schematic of PCR amplification prior to library preparation

C. Agencourt PCR-purification

1. Vortex AMPure XP beads for 10 sec.
2. Out of the 24 PCR products, generate two pools of PCR products (Figure 1; the first pool contains PCR products 1.1-6.2, and the second pool PCR products 7.1-12.2), each containing 12 x 40 μ l, for a total of 480 μ l each, and pipet into 1.5 ml Eppendorf tube.
3. Add 720 μ l beads to the pools, mix by pipetting 10 times, incubate for 5 min.
4. Place tube onto magnet, wait 5 min.
5. Take off supernatant completely, being careful not to aspirate any beads, add 1,200 μ l 70% EtOH (do NOT pipet directly onto beads), let sit for at least 30 sec, take off completely with a P1000 pipette.
6. Repeat wash step with 800 μ l 70% EtOH, take off supernatant with a P1000 pipette, spin down for 3 sec, put back on magnet, take off any remaining supernatant with a P10 pipette.
7. Dry beads for about 2 min with open lids, keep lids open from now on.
8. Take tube off magnet, add 60 μ l Qiagen elution buffer, resuspend bead-pellet in elution buffer, incubate for 10 min at room temperature.
9. Place back onto magnet, wait 1 min (until beads have pelleted), take off 50 μ l of eluate. Pool the eluates from the two purified PCR product pools, for a total of 100 μ l containing all PCR products.

D. Optional: Quality control on agarose gel

1. Prepare 1x TAE by adding 10.2 ml 50x TAE into 500 ml water.
2. Combine 50 ml 1x TAE and 0.5 g agarose, bring to boil in microwave.
3. Pour agarose gel, let solidify on a cold pack.
4. Load 15 μ l of DNA ladder and 5 μ l of pooled sample mixed with 1 μ l 6x loading dye onto agarose gel, run for about 20 min at 100 V.
5. Combine 50 ml FastBlast DNA stain and 200 ml water, incubate the agarose gel for 3 min in the diluted DNA stain, then rinse and destain the gel with several changes of water, rocking the gel continuously for 10 to 20 min.
6. Visualize the gel using a white laptop screen as lightbox. Several discrete bands should be visible, corresponding to the various PCR product lengths (strong band at ~2.1 kb, moderate band at ~2.7 kb, weak bands at 1.3, 3.4, and 4.0 kb).

E. Library preparation

1. Combine 80 μ l of pooled, purified PCR products from step C9 and 5 μ l DNA CS, add 10 μ l 10x NEBNext end repair buffer and 5 μ l NEBNext end repair enzyme mix, mix by pipetting, incubate at RT for 30 min.
2. Clean up with 180 μ l Agencourt beads as described in section C, but use 300 μ l EtOH for washing, add 30 μ l elution buffer, take off 25 μ l eluate, put in PCR tube.

3. Add 3 μ l 10x NEBNext dA-tailing buffer and 2 μ l NEBNext dA-tailing enzyme mix, incubate for 30 min at 37 °C.
4. Thaw 2x wash buffer, elution buffer, EP buffer at RT, thaw HP adapter, Adapter mix and Fuel mix in the cooler box.
5. Prepare His-beads:
 - a. Mix 550 μ l 2x wash buffer and 550 μ l nuclease-free water by inverting 10x, briefly spin down.
 - b. Vortex His-Beads for ~30 sec.
 - c. Pipet 10 μ l His-Beads into a LoBind tube, add 250 μ l 1x wash buffer.
 - d. Place on magnet, wait for pellet to form, aspirate off supernatant, add 250 μ l wash buffer, wait 30 sec, aspirate off supernatant.
 - e. Take tube off magnet, resuspend pellet in 100 μ l 2x wash buffer.
6. After 30 min, in a LoBind tube, add (directly to the bottom of the tube) 8 μ l nuclease-free water, 30 μ l DNA from step E3, 10 μ l Adapter mix, 2 μ l HP Adapter, and 50 μ l Blunt/TA ligase master mix, mix by pipetting, if necessary briefly spin down, incubate 10 min at RT.
7. Add 100 μ l washed beads (directly to bottom of tube), carefully mix by pipetting, incubate for 5 min at RT.
8. Place on magnet, wait 2 min for pellet to form, aspirate supernatant, wash 2 x for 30 sec each with 250 μ l 1x wash buffer.
9. With the lid close, briefly centrifuge, put back onto magnet, wait 1 min with lid closed, aspirate off any remaining wash buffer.
10. Take off magnet, resuspend pellet in 25 μ l elution buffer (adding the elution buffer directly to the pellet), wait 10 min with lid closed, put back on magnet, wait 1 min, take off 15 μ l eluate ($\geq$ pre-sequencing mix).

F. Loading of library (Figure 2)

1. Mix 318.5 μ l EP buffer and 6.5 μ l Fuel mix by vortexing, spin down.
2. Prime flow cell two times with 150 μ l of EB/Fuel mix.
3. Mix 6 μ l library, 141 μ l EP, 3 μ l Fuel mix by inverting 10 times, briefly spin down, load, and initiate flow cell run.



Figure 2. Priming/loading of the flowcell

Data analysis

Once the sequencing run is completed on the MinION device, raw data have to be base-called, primer sequences have to be removed, and then all sequences have to be aligned to a reference sequence to build a pile-up, which can then be used for consensus-calling. The exact procedure for doing so, including all used bioinformatics scripts, is published in (Hoenen *et al.*, 2016), which is openly accessible at this link: http://wwwnc.cdc.gov/eid/article/22/2/15-1796_article.

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