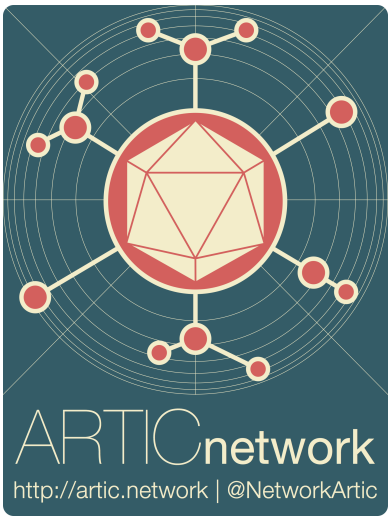

Ebola virus sequencing protocol

Nanopore | amplicon | native barcoding

Document: ARTIC-EBOV-seqSOP-v2.0.0
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Forked from: doi:10.1038/nprot.2017.066
Author: Luke Meredith, Josh Quick

Overview: The following protocol is adapted from the methods of Quick et al. (2017) *Nature Protocols* **12:** 1261–1276 doi:10.1038/nprot.2017.066 and covers primers, amplicon preparation and clean-up, then uses a single-tube protocol to barcode and adaptor ligate the library, before running minION.

This document is part of the Ebola virus Nanopore sequencing protocol package:

http://artic.network/ebov/

Related documents:

Ebola primer scheme:

https://github.com/artic-network/primer-schemes/tree/master/ZaireEbola/V3

Ebola virus Nanopore sequencing protocol:

http://artic.network/ebov/ebov-seq-sop.html

Ebola virus Nanopore sequencing kit-list:

http://artic.network/ebov/ebov-seq-kit.html

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Preparation

Equipment required:

2 Portable nucleic acid preparation hood or equivalent
1 12V vortex
1 Sprout portable centrifuge
1 P1000 Eppendorf pipette
1 P100 Eppendorf pipette
1 P10 Eppendorf pipette
1 1.5mL/0.6mL convertible tube rack
1 Quantus Fluorometer
1 miniPCR machine.
1 Heat block
1 Magnetic rack

Consumables required:

SuperScript IV Reverse Transcriptase
Q5 Hot Start High-Fidelity 2X Master Mix
Ebola Zaire Primers V3
NEBNext Ultra II End Repair/dA-Tailing Module
Blunt/TA Ligase Master Mix
Aline PCRCLEAN DX 50ml
Nanopore Ligation Sequencing Kit 1D
Nanopore Native Barcoding Expansion Kit
Nanopore R9.4.1 Flow cell
1.5mL Eppendorf Tubes
0.2mL 8-strip Tubes
50mL Falcon Tubes
0.5ml PCR Tubes
QuantiFluor ONE dsDNA System
Nuclease-free water
70% Ethanol
P1000 pipette tips
P100 pipette tips
P10 long-reach pipette tips
Paper towelling
Clinical waste sharps containers

Safety, containment and contamination recommendations

Back-tie hydrophobic lab gown
Gloves
UV light sterilizers
MediPal Decontamination wipes
DNAway and RNAse Zap reagent

Protocol

Part 1: cDNA synthesis with Superscript IV reverse transcriptase

NOTE ON HOOD PREPARATION: To prevent cross contamination of both the sample and other reagents, this should be carried out in the SAMPLE PREPARATION HOOD, which is pre-sterilised with UV and treated with MediPal wipes, DNAway and RNAseZap reagent. Wipe down the hood with each sequentially, allowing 5 minutes for drying between each. Pipettes should also be treated in the same way, and UV treated for 30 mins between library preparations.

1. Set up the following reaction:

50µM random hexamers	1µL
10mM dNTPs mix (10mM each)	1µL
Template RNA	11µL
TOTAL	12µL

NOTE: Viral RNA input from a clinical sample should be between Ct 18-35. If Ct is between 12-15, then dilute the sample 100-fold in water, if between 15-18 then dilute 10-fold in water. This will reduce the likelihood of PCR-inhibition.

1. Gently mix (avoid vortexing) then pulse spin the tube to ensure maximum contact with the thermal cycler.
2. Incubate the reaction as follows:

Denaturation	65°C	5 mins
Primer annealing	Ice	1 mins

3. Add the following to the annealed template RNA:

SSIV Buffer	4µL
100mM DTT	1µL
RNaseOUT RNase Inhibitor	1µL
SSIV Reverse Transcriptase	1µL
TOTAL	20µL

4. Gently mix (avoid vortexing) then pulse spin the tube to ensure maximum contact with the thermal cycler.
5. Incubate the reaction as follows:

Extension	42°C	90 mins
Inactivation	70°C	10 mins

6. cDNA is now ready for amplicon generation.

Part 2: Ebola Amplicon Preparation

NOTE ON HOOD PREPARATION: To prevent cross contamination of both the sample and other reagents, this should be carried out in the MASTERMIX HOOD, which is pre-sterilised with UV and treated with MediPal wipes, DNAway and RNAseZap reagent. Wipe down the hood with each sequentially, allowing 5 minutes for drying between each. Pipettes should also be treated in the same way, and UV treated for 30 mins between library preparations.

Primer dilution and preparation

1. Ebola primers for this protocol were designed using Primal Scheme and generate overlapping 400nt amplicons. Primer names and dilutions are listed in the table below.
2. Primers should be prepped and aliquoted PRIOR TO DEPARTURE in a STERILE PCR CABINET. At NO stage should primers or PCR reagents be anywhere near the template or amplicons until use.
3. Resuspend lyophilised primers at a concentration of 100µM each
4. Generate primer pool stocks by adding 5µL of each primer pair to a 1.5mL Eppendorf labelled “Pool 1, 100µM” or “Pool 2, 100µM”. Total volume should be 505µL of Pool 1 and 530µL of Pool 2. This is a 10x stock of each primer pool.
5. Dilute this primer pool 1:10 in molecular grade water, to generate 10µM primer stocks. Recommend that multiple aliquots of each primer pool are made to account for any risks of degradation of contamination.

Name	Sequence	Name	Sequence	Pool	[Stock]
Ebov-10-Pan_1_LEFT	TGTGTGCGAATAACTATGAGGAAGA	Ebov-10-Pan_1_RIGHT	TTTCCAATGTTTTACCCCAAGCTTT	1	100µM
		Ebov-10-Pan_1_RIGHT_alt1	TTTCCAATGCTTTACCCCAAGCTTT	1	100µM
		Ebov-10-Pan_1_RIGHT_alt2	TTTCCAATGTTTTACCCCAAGTTTT	1	100µM
Ebov-10-Pan_2_LEFT	CAAGCAAGATTGAGAATTAACCTTGGT	Ebov-10-Pan_2_RIGHT	ATCTCCCTGGTACGCATGATGA	2	100µM
Ebov-10-Pan_2_LEFT_alt1	CAAGCAAGATTGAGAATTAACCTTGAT	Ebov-10-Pan_2_RIGHT_alt1	ATCTCCTTGGTACGCATGATGA	2	100µM
Ebov-10-Pan_3_LEFT	GGCCTTTGAAGCAGGTGTTGAT	Ebov-10-Pan_3_RIGHT	TCAGTCCTTGCTCTGCATGTAC	1	100µM
Ebov-10-Pan_4_LEFT	CCTTTGCAAGTCTATTCCTTCCGA	Ebov-10-Pan_4_RIGHT	CTGAGTGCAGCCTTAAAGGAGT	2	100µM
Ebov-10-Pan_4_LEFT_alt1	CTTTTGCAAGTCTATTCCTTCCGA			2	100µM
Ebov-10-Pan_5_LEFT	AGTTCGTCTCCATCCTCTTGCA	Ebov-10-Pan_5_RIGHT	CTGGAAGCTGATTTCGTTCTTTTTCT	1	100µM
Ebov-10-Pan_6_LEFT	GAGTCTCGCGAACTTGACCATC	Ebov-10-Pan_6_RIGHT	TCCTCGTCGTCCTCGTCTAGAT	2	100µM
Ebov-10-Pan_6_LEFT_alt1	GAATCTCGCGAACTTGACCATC	Ebov-10-Pan_6_RIGHT_alt1	TCCTCATCGTCCTCGTCTAGAT	2	100µM
Ebov-10-Pan_7_LEFT	AGCTACGGCGAATACCAGAGTT	Ebov-10-Pan_7_RIGHT	GTCCCTGTCCTGCTCTTCATCA	1	100µM
		Ebov-10-Pan_7_RIGHT_alt1	GTCCCTGTCCTGTTCTTCATCA	1	100µM
		Ebov-10-Pan_7_RIGHT_alt2	GTCCCTGTCCTGTTCTTCATCG	1	100µM
Ebov-10-Pan_8_LEFT	TTAACGAAGAGGCAGACCCACT	Ebov-10-Pan_8_RIGHT	TTCCTCTTCAAGGGAGTCTGGA	2	100µM
Ebov-10-Pan_8_LEFT_alt1	TCAACGAAGAGGCAGACCCACT	Ebov-10-Pan_8_RIGHT_alt1	TTCCTCTTCAAGGGAGTCCGGA	2	100µM
Ebov-10-Pan_9_LEFT	GTGACAACACCCAGTCAGAACA	Ebov-10-Pan_9_RIGHT	TCTTCCTGTTTTCGTTCCTTGACT	1	100µM
Ebov-10-Pan_9_LEFT_alt1	GTGACAACACCCAGCCAGAACA	Ebov-10-Pan_9_RIGHT_alt1	TCTTCCTGTTTGCGTTCCTTGACT	1	100µM
		Ebov-10-Pan_9_RIGHT_alt2	TCTTCCTGTTTGCGTTTCTTGACT	1	100µM
Ebov-10-Pan_10_LEFT	ACAATGGGATGATTCAACCGACA	Ebov-10-Pan_10_RIGHT	TCGAGTGCTAGAGAATTCAATTGACG	2	100µM
Ebov-10-Pan_10_LEFT_alt1	ATAATGGGATGATTTAACCGACA			2	100µM
Ebov-10-Pan_11_LEFT	ACCTACTAGCCTGCCCAACATT	Ebov-10-Pan_11_RIGHT	AATTGGGTCCGTTTGGGTTTGA	1	100µM
Ebov-10-Pan_11_LEFT_alt1	ACCTACTAGCCTACCCAACATT	Ebov-10-Pan_11_RIGHT_alt1	AATTGGATCCGTTTGGGTTTGA	1	100µM
Ebov-10-Pan_12_LEFT	CCCAAATGCAACAAACGAAGCC	Ebov-10-Pan_12_RIGHT	TCAATCTTACCCCGAATCGCAC	2	100µM
Ebov-10-Pan_12_LEFT_alt1	CCCAAATGCAACAAACAAAGCC	Ebov-10-Pan_12_RIGHT_alt1	TCAATCTTACCCCGAATTGCAC	2	100µM
Ebov-10-Pan_13_LEFT	TATTGGGCCGAACATGGTCAAC	Ebov-10-Pan_13_RIGHT	TGACAGGTGGAGCAGCATCTTG	1	100µM
Ebov-10-Pan_13_LEFT_alt1	TATTGGGCTGAACATGGTCAAC			1	100µM
Ebov-10-Pan_14_LEFT	CATTCATGCTGAGTTCCAGGCC	Ebov-10-Pan_14_RIGHT	GCGAGATATGAACAATTTTATCTTGGTCG	2	100µM
		Ebov-10-Pan_14_RIGHT_alt1	GCGAGATAAGGACAATTTTATCTTGGTCG	2	100µM
		Ebov-10-Pan_14_RIGHT_alt2	GCGAGATAAGAACAATTTTATCTTGGTCG	2	100µM
Ebov-10-Pan_15_LEFT	TGAGTATCAGCCCTGGATAATATAAGTCA	Ebov-10-Pan_15_RIGHT	TCGATGGAGTGTCCCCATTGAC	1	100µM
Ebov-10-Pan_15_LEFT_alt1	TGAGTATCAGCCCTAGATAATATAAGTCA	Ebov-10-Pan_15_RIGHT_alt1	TCGATGGAGTGTCTCCATTGAC	1	100µM
Ebov-10-Pan_16_LEFT	GCAACAGCAATACAGGCTTCCT	Ebov-10-Pan_16_RIGHT	GAAAGCCTGGTTTCCAATTCGC	2	100µM
Ebov-10-Pan_16_LEFT_alt1	GCAACAACAATACAGGCTTCCT	Ebov-10-Pan_16_RIGHT_alt1	GAAGGCCTGGTTTCCAATTCGC	2	100µM
Ebov-10-Pan_17_LEFT	CCACTTGTCAGAGTCAATCGGC	Ebov-10-Pan_17_RIGHT	GTTTCTGGCACTTCGATTCCCA	1	100µM

Name	Sequence	Name	Sequence	Pool	[Stock]
		Ebov-10-Pan_17_RIGHT_alt1	GTTTCTGGCACTTCGATACCCA	1	100µM
Ebov-10-Pan_18_LEFT	AAAATCCAAGCAATAAT-GACTTCACTCC	Ebov-10-Pan_18_RIGHT	TTGATCAATTAAAAGT-GTCTCCTCTAATGG	2	100µM
		Ebov-10-Pan_18_RIGHT_alt1	TCGAT-CAATTTAAAGTATCTCCTCTAATGG	2	100µM
		Ebov-10-Pan_18_RIGHT_alt2	TTGATCAAT-TAAAAGTATCTCCTCTAATAG	2	100µM
Ebov-10-Pan_19_LEFT	AGATCCAGTTTTATAGAATCTTCT-CAGGGA	Ebov-10-Pan_19_RIGHT	AGAAGGGCAATGTCTGTACTTGG	1	100µM
Ebov-10-Pan_19_LEFT_alt1	AGATCCAGTTTTACAGAATCTTCT-CAGGGA	Ebov-10-Pan_19_RIGHT_alt1	AGAAGGGCGATGTCTGTGCTTGG	1	100µM
Ebov-10-Pan_20_LEFT	AGCCAGTGTGACTTGGATTGGA	Ebov-10-Pan_20_RIGHT	AGTTTGTCGACATCACTAACCTGT	2	100µM
		Ebov-10-Pan_20_RIGHT_alt1	AGTTTGTCGACATCACTAACTTGT	2	100µM
Ebov-10-Pan_21_LEFT	AGAACATTTTCCATCCCACTTGGA	Ebov-10-Pan_21_RIGHT	AAGCACCCTCTTTATGGAAGGC	1	100µM
		Ebov-10-Pan_21_RIGHT_alt1	AAGCACCCTCTTTGTGGAAGGC	1	100µM
Ebov-10-Pan_22_LEFT	TGCCGGTATGTGCACAAAGTAT	Ebov-10-Pan_22_RIGHT	ATATATTGTCTCATTCAGCTGGAGCA	2	100µM
Ebov-10-Pan_23_LEFT	CGAGGTTGACAATTTGACCTACGT	Ebov-10-Pan_23_RIGHT	GCAAGGGTTGTTAGATGCGACA	1	100µM
		Ebov-10-Pan_23_RIGHT_alt1	GCAAGGGTTGTCAGATGCGACA	1	100µM
Ebov-10-Pan_24_LEFT	TGCAATGGTTCAAGTGCACAGT	Ebov-10-Pan_24_RIGHT	CTGGCACTCTCTTCTCCGGTAT	2	100µM
Ebov-10-Pan_24_LEFT_alt1	TGCAATGGTTCAAGTGCACAAT			2	100µM
Ebov-10-Pan_25_LEFT	ACCACAACAAGTCCCCAAAACC	Ebov-10-Pan_25_RIGHT	TAGCTCAGTTGTGGCTCTCAGG	1	100µM
		Ebov-10-Pan_25_RIGHT_alt1	TAGCTCGGTTGTGGCTCTCAGG	1	100µM
Ebov-10-Pan_26_LEFT	ATCTGTGGGTTGAGACAGCTGG	Ebov-10-Pan_26_RIGHT	GCTTTTCCATGAAGCAATCTGAAGA	2	100µM
Ebov-10-Pan_26_LEFT_alt1	ATCTGTGGATTGAGGCAGCTGG	Ebov-10-Pan_26_RIGHT_alt1	GCTTTGCCATGAAGCAATCTGAAGA	2	100µM
Ebov-10-Pan_26_LEFT_alt2	ATCTGTGGGTTGAGGCAGCTGG			2	100µM
Ebov-10-Pan_27_LEFT	TGGAGTTACAGGCGTTATAATTGCA	Ebov-10-Pan_27_RIGHT	AAAGGCTTCTTTCCCTTGTCACT	1	100µM
Ebov-10-Pan_28_LEFT	TCATCCTTGATTCTACAATCAT-GACAGT	Ebov-10-Pan_28_RIGHT	AGGTGCTGGAGGAACTGTTAATG	2	100µM
Ebov-10-Pan_28_LEFT_alt1	TCATCCTTGATTCTACAAT-CATAACAGT			2	100µM
Ebov-10-Pan_29_LEFT	GAGTACCGTCAATCAAGGAGCG	Ebov-10-Pan_29_RIGHT	CACAGCACATAGAGTCAACAATGC	1	100µM
Ebov-10-Pan_30_LEFT	GATCAAGACGGCAGAACACTGG	Ebov-10-Pan_30_RIGHT	ATCAGACCATGAGCATGTCCCC	2	100µM
Ebov-10-Pan_31_LEFT	CTGCTGTCGTTGTTTCAGGGTT	Ebov-10-Pan_31_RIGHT	ATGGGATGGATCGTTGCTACCT	1	100µM
		Ebov-10-Pan_31_RIGHT_alt1	ATGGGATGGATCGTTGCTGCCT	1	100µM
		Ebov-10-Pan_31_RIGHT_alt2	ATGAGATGGATCGTTGCTACCT	1	100µM
Ebov-10-Pan_32_LEFT	GCCAAGCATACCTCTTGCACAA	Ebov-10-Pan_32_RIGHT	TGGACTACCCTGAAATAGTACTTTGC	2	100µM
Ebov-10-Pan_33_LEFT	TGCGGAGGTCTGATAAGAATAAACC	Ebov-10-Pan_33_RIGHT	TTCAACCTTGAAACCTTGCGCT	1	100µM
		Ebov-10-Pan_33_RIGHT_alt1	TTCAACTTTGAAACCTTGCGCT	1	100µM
Ebov-10-Pan_34_LEFT	GCTGAAAAGAAGCTTACCTACAACG	Ebov-10-Pan_34_RIGHT	TCCTTGTCATTGACCATGCAGG	2	100µM

Name	Sequence	Name	Sequence	Pool	[Stock]
Ebov-10-Pan_34_LEFT_alt1	GTTGAAAAAAGGCCTACCTACAACG			2	100µM
Ebov-10-Pan_34_LEFT_alt2	GCTGAAAAGAAGCCCACCTACAACG			2	100µM
Ebov-10-Pan_35_LEFT	GTGACTCACAAAGGAATGGCCC	Ebov-10-Pan_35_RIGHT	ACAATCCGTTGTAGTTCACGACA	1	100µM
		Ebov-10-Pan_35_RIGHT_alt1	ACAACCCGTTGTAGTTCACGACA	1	100µM
Ebov-10-Pan_36_LEFT	TGCTGTCGTTGATTCGATCCAA	Ebov-10-Pan_36_RIGHT	AGCAGAGATGTCAAGATAACTAT-TGAGT	2	100µM
Ebov-10-Pan_37_LEFT	ACACGAATGCAAAGTTTGATTCT-TGA	Ebov-10-Pan_37_RIGHT	TGAAACCTAACACATGTGACCTGC	1	100µM
		Ebov-10-Pan_37_RIGHT_alt1	TGAAACCTAACACACGTGACCTGC	1	100µM
Ebov-10-Pan_38_LEFT	CCCTCAAACAAGAGATTCCAAGACA	Ebov-10-Pan_38_RIGHT	ACAGTTGCGTAGTTGCGGATTA	2	100µM
Ebov-10-Pan_38_LEFT_alt1	CCCTCAAATAAGAGATTCCAAGACA			2	100µM
Ebov-10-Pan_38_LEFT_alt2	TCCTCAAATAAGAGATTCCAAGACA			2	100µM
Ebov-10-Pan_39_LEFT	ACCTAGTCACTAGAGCTTGCGG	Ebov-10-Pan_39_RIGHT	ACATTTGATGTAAAAATTCATTGC-CCTG	1	100µM
Ebov-10-Pan_40_LEFT	GTGGGTGCTCAAGAAGACTGTG	Ebov-10-Pan_40_RIGHT	TGAGATTAGAGTTGTGT-TGAATCGACA	2	100µM
Ebov-10-Pan_40_LEFT_alt1	GTGGGTGCTCAAGAGGACTGTG	Ebov-10-Pan_40_RIGHT_alt1	TGAGATTAGAGTCGTGT-TGAATCGACA	2	100µM
Ebov-10-Pan_41_LEFT	AAGAAGCGGTTCAAGGGCATAC	Ebov-10-Pan_41_RIGHT	CTATGGAATTCACGGATCTTTTGAGC	1	100µM
Ebov-10-Pan_41_LEFT_alt1	AAGAAGCAGTTCAAGGGCATAC	Ebov-10-Pan_41_RIGHT_alt1	CTATGGAATTCACGGATCTTTTGATC	1	100µM
Ebov-10-Pan_42_LEFT	TGCATTTAGCTGTAAATCACACCCT	Ebov-10-Pan_42_RIGHT	AATCATTGGCAACGGAGGGAAT	2	100µM
		Ebov-10-Pan_42_RIGHT_alt1	AATCATTGGCAACGGGGGGAAT	2	100µM
Ebov-10-Pan_43_LEFT	GTCAAGGATCTTGGTACAGTGT-TACT	Ebov-10-Pan_43_RIGHT	TGAGAAAGAAAAGTTCCGATATTGT-GGT	1	100µM
Ebov-10-Pan_43_LEFT_alt1	GCCAAGGGTCTTGGTACAGTGT-TACT	Ebov-10-Pan_43_RIGHT_alt1	TGAGAAAGAAAAATTCCGGTATTGT-GGT	1	100µM
Ebov-10-Pan_43_LEFT_alt2	GTCAAGGGTCTTGGTACAGTGT-TACT	Ebov-10-Pan_43_RIGHT_alt2	TGAGAAAGAAAAATTCCGATATTGT-GGT	1	100µM
Ebov-10-Pan_44_LEFT	TTGAGAATGTTCTTTCCTACGCACA	Ebov-10-Pan_44_RIGHT	ACGGTTGCAATATTCTATAAAAGGT-GC	2	100µM
Ebov-10-Pan_44_LEFT_alt1	TTGAGAATGTTCTTTCCTACGCGCA	Ebov-10-Pan_44_RIGHT_alt1	ACGGTTGCAATATTCGATAAAAGGT-GC	2	100µM
		Ebov-10-Pan_44_RIGHT_alt2	ACGGTTACAATATTCTATAAAAGGT-GC	2	100µM
Ebov-10-Pan_45_LEFT	CCACAGTTAGAGGGAGTAGCTTTG	Ebov-10-Pan_45_RIGHT	GCTCGTCTGCGTCAGTCTCTAA	1	100µM
Ebov-10-Pan_45_LEFT_alt1	CCACAGTTAGAGGGAGTAGTTTTG			1	100µM
Ebov-10-Pan_46_LEFT	AAGTTACGCTCAGCTGTGATGG	Ebov-10-Pan_46_RIGHT	ATGGAAAGCTGCGGTTATCCTG	2	100µM
Ebov-10-Pan_47_LEFT	TAGGCACTGCTTTTGAGCGATC	Ebov-10-Pan_47_RIGHT	CACAAAGTCAATGGCAGTGCAG	1	100µM
Ebov-10-Pan_47_LEFT_alt1	TAGGCACCGCTTTTGAGCGGTC			1	100µM
Ebov-10-Pan_47_LEFT_alt2	TAGGCACTGCTTTTGAACGATC			1	100µM
Ebov-10-Pan_48_LEFT	TCTCCGAATGATTGAGATGGAT-GATT	Ebov-10-Pan_48_RIGHT	CTCAGTCTGTCCAAAACCGGTG	2	100µM
Ebov-10-Pan_48_LEFT_alt1	TCTCCGAATGATTGGGATGGAT-GATT			2	100µM
Ebov-10-Pan_49_LEFT	GATATCTTTTCACGCACGCCGA	Ebov-10-Pan_49_RIGHT	CCACCTGGTTGCTTTGCATTTG	1	100µM

Name	Sequence	Name	Sequence	Pool	[Stock]
Ebov-10-Pan_49_LEFT_alt1	GATATCTTTTCACGCACGCCCA	Ebov-10-Pan_49_RIGHT_alt1	CCACCAGGTTGCTTTGCATTTG	1	100µM
Ebov-10-Pan_50_LEFT	TCAAAGTGTTTTGGCTGAAACCCT	Ebov-10-Pan_50_RIGHT	TCCTGAGTAATGTGAAGGGGTCA	2	100µM
Ebov-10-Pan_50_LEFT_alt1	TCAAAGTGGTTTGGCTGAAACCCT	Ebov-10-Pan_50_RIGHT_alt1	TCCTGAGTAATGTGAAGGAGTCA	2	100µM
Ebov-10-Pan_51_LEFT	AACAGTGACTTGCTAATAAAAC-CATTTTTG	Ebov-10-Pan_51_RIGHT	AAATACTGAGCTGGTACTTCCCG	1	100µM
Ebov-10-Pan_51_LEFT_alt1	AACAGTGACTTGCTAATAAAGC-CATTTTTG			1	100µM
Ebov-10-Pan_51_LEFT_alt2	AACAGTGATTTGCTAATAAAAC-CATTTTTG			1	100µM
Ebov-10-Pan_52_LEFT	AATCGTGCTCACCTTCATCTAACT	Ebov-10-Pan_52_RIGHT	CCCAAAACTGTACAGAAGTCCTATCT	2	100µM
Ebov-10-Pan_53_LEFT	ACAGACCCAATTAGCAGTGGAGA	Ebov-10-Pan_53_RIGHT	ACAATTGTTCCGCGATTAAT-TATCCAT	1	100µM
Ebov-10-Pan_53_LEFT_alt1	ACAGACCCAATTAGCAGCGGAGA	Ebov-10-Pan_53_RIGHT_alt1	ACAATTGTTCCGCGATTAAT-TATCCACT	1	100µM
Ebov-10-Pan_54_LEFT	TCTCAGATGCGGCCAGGTTATT	Ebov-10-Pan_54_RIGHT	TGACCATCACTGTTGTTTGTGCT	2	100µM
Ebov-10-Pan_54_LEFT_alt1	TCTCAGATGCGGCCAGATTATT			2	100µM
Ebov-10-Pan_55_LEFT	TGGAGGAGCAGACACAGAAACA	Ebov-10-Pan_55_RIGHT	ATGACGTTAATTGGCGTGTCCC	1	100µM
Ebov-10-Pan_55_LEFT_alt1	TGGAGGAGCAGGCACAGAAACA	Ebov-10-Pan_55_RIGHT_alt1	ATGACGTCAATTGGCGTGTCCC	1	100µM
Ebov-10-Pan_55_LEFT_alt2	TGGAGAAGCAGGCACAGAAACA	Ebov-10-Pan_55_RIGHT_alt2	ATGACGTTAATTGGCGCGTCCC	1	100µM
Ebov-10-Pan_56_LEFT	CTCACACCGTCTAGTCCTACCT	Ebov-10-Pan_56_RIGHT	TTTGACATAACAGGTAGAAGCATCCT	2	100µM
Ebov-10-Pan_56_LEFT_alt1	CTCGCACCGTCTAGTCCTACCT			2	100µM
Ebov-10-Pan_56_LEFT_alt2	CTCACATCGTCTAGTCCTACCT			2	100µM
Ebov-10-Pan_57_LEFT	ACACGCTAGCTACTGAGTCCAG	Ebov-10-Pan_57_RIGHT	ATTGGCTTAATTAAATAACCAGTG-GCA	1	100µM
Ebov-10-Pan_58_LEFT	TGAAAGCAGTGGTCCTTAAAGTCT	Ebov-10-Pan_58_RIGHT	TGCTCTAAGATGTGCTAAGTGCTG	2	100µM
		Ebov-10-Pan_58_RIGHT_alt1	TGCTCTAAGATGTGCCAAGTGCTG	2	100µM
Ebov-10-Pan_59_LEFT	CGTCGATTCAAAAAGAGGTCCACT	Ebov-10-Pan_59_RIGHT	TCAGAAGCCCTGTCAGCCTTTC	1	100µM
Ebov-10-Pan_60_LEFT	AGATTGCAATTGT-GAAGAACGTTTCT	Ebov-10-Pan_60_RIGHT	AGAGTGCAGAGTTTATTATGTTGCGT	2	100µM
Ebov-10-Pan_61_LEFT	TCACAATGCAGCATGTGTGACA	Ebov-10-Pan_61_RIGHT	AGGTATTTCTGATTTTACAGTCCT-GCC	1	100µM
		Ebov-10-Pan_61_RIGHT_alt1	AGGTATTTATGATTTTACAGTCCT-GCC	1	100µM
		Ebov-10-Pan_61_RIGHT_alt2	AGGTATTTCTGATTTTACAGTCAT-GCC	1	100µM
Ebov-10-Pan_62_LEFT	CCTGTCAGATGGAATAGTGTTTTG-GT	Ebov-10-Pan_62_RIGHT	AATTTTTGTGTGCGACCATTTTTCC	2	100µM

NOTE: Primers need to be used at a final concentration of 0.015µM per primer. In this case, Pool 1 has 101 primers in it so the requirement is 3.8µL of 10µM primers Pool 1 per 25µL reaction. Pool 2 has 106 primers so needs 4.0µL of 10µM primers Pool 2 per 25µL reaction. For other schemes, adjust the volume added appropriately.

1. Set up the amplicon PCR reactions as follows in 0.5mL thin-walled PCR or strip-tubes:

Reagent	Pool 1	Pool 2
NEB Q5 Polymerase 2X MasterMix	12.5µL	12.5µL

Reagent	Pool 1	Pool 2
Primer Pool 1 or 2 (10µM)	3.8µL	4.0µL
Water	6.2µL	6.0µL
TOTAL	22.5µL	22.5µL

NOTE: This should be carried out in the mastermix hood and cDNA should not be taken anywhere near the mastermix hood at any stage.

1. In the TEMPLATE HOOD add 2.5µL of cDNA to each Pool1 and Pool2 reaction mix and mix well.
2. Pulse centrifuge the tubes to remove any contents from the lid.
3. Set up the cycling conditions as follows:

Step	Temperature	Time	Cycles
Heat Activation	98°C	30 seconds	1
Denaturation	98°C	15 seconds	25-35
Annealing	65°C	300 seconds	25-35
Hold	4°C	Indefinite	1

NOTE: Cycle number should be 25 for Ct18-21 up to a maximum of 35 cycles for Ct 35

1. Clean-up the amplicons using the following protocol in the TEMPLATE HOOD:
    a. Combine the entire contents of “Pool1” and “Pool2” PCR reactions for each biological sample into to a single 1.5mL Eppendorf tube.
    b. Mix sample gently, avoid vortexing.
    c. Ensure Aline beads are well resuspended by thoroughly mixing prior to addition to the sample. Mixture should be a homogenous brown colour.
    d. Add an equal volume of Aline beads to the tube and mix gently by either flicking or pipetting. This should be approximately 50µL, so add 50µL of beads.
    e. Pulse centrifuge the tubes to remove any beads or solution from the lid or side of the tube.
    f. Incubate for 5 mins at RT.
    g. Place on magnetic rack and incubate for 2 mins or until the beads have pelleted against the magnet and the solution is completely clear.
    h. Carefully remove and discard the solution, being careful not to displace the bead pellet.
    i. Add 200µL of room-temperature 70% ethanol to the pellet.
    j. Carefully remove and discard ethanol, being careful not to displace the bead pellet.
    k. Repeat steps `i` to `j` to wash the pellet again.
    l. Briefly pulse centrifuge the pellet and carefully remove as much ethanol as possible using a 10µL tip.
    m. Allow the pellet to dry for 1 mins, being careful not to overdry (if the pellet starts to crack then it is too dry).
    n. Resuspend pellet in 30µL of water, and incubate for 2 mins.
    o. Place on magnet and CAREFULLY remove water and transfer to a clean 1.5mL Eppendorf tube. MAKE SURE that no beads are transferred into this tube. In some cases a pulse centrifugation can be used to pellet residual beads.

p. Quantify the amplicons pools using the Quantus Fluorometer following ONE dsDNA protocol.

Part 3: Quantus Quantification of Amplicon Pools

1. Set up the required number of 0.5mL tubes samples.

> **NOTE:** Use only thin-wall, clear, 0.5mL PCR tubes.

1. Label the tube lids. Do not label the side of the tube as this could interfere with the sample reading.
2. Add 199µL ONE dsDNA dye solution to each tube.
3. Add 1µL of each user sample to the appropriate tube.

> **NOTE:** Use a P2 pipette for highest accuracy.

1. Mix each sample vigorously by vortexing for 3–5 seconds.
2. Allow all tubes to incubate at room temperature for 2 minutes before proceeding.
3. 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.

1. Add 200µL ONE dsDNA Dye solution to two 0.5mL tubes.
2. Add 1µL Lambda DNA standard 400 ng/µL provided in the kit to one of the tube. These two tubes are the blank sample and standard required to perform the single point calibration procedure.
3. 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’.
4. On the home screen navigate to ‘Sample Volume’ and set it to 1 ul then ‘Units’ and set it to ng/µL.
5. Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.
6. Repeat step 12 until all samples have been read.
7. The value displayed on the screen is the dsDNA concentration, carefully **record all results** in a spreadsheet or laboratory notebook.

Part 4: Barocoding and adaptor ligation: One-pot protocol.

NOTE: This is a 'one-pot ligation' protocol for native barcoded ligation libraries. We have seen no reduction in performance compared to standard libraries, and is made faster by using the Ultra II® ligation module which is compatible with the Ultra II® end repair/dA-tailing module removing a clean-up step. If you have the time I would recommend using the double incubation times in blue, if you are in a hurry the times in red are a good compromise between speed and efficiency.

1. Set up the following end-prep reaction for each biological sample:

DNA (20 ng)	16.7µL
Ultra II End Prep Reaction Buffer	2.3µL
Ultra II End Prep Enzyme Mix	1µL
Total	20µL

NOTE: Quantity of amplicons can vary from 10-50ng, any more than this and the molarity of DNA ends will be too high for efficient barcoding. You need to have 6 samples per native barcoded library to have sufficient material at the end.

1. Incubate at RT for 20 mins then 65°C for 10 mins
2. Place on ice for 30 secs
3. Add the following directly to the previous reactions:

NBXX barcode	2.5µL
Ultra II Ligation Master Mix	22.5µL
Ligation Enhancer	0.7µL
Total	45.7µL

NOTE: Use a SINGLE barcode per biological sample.

1. Incubate at RT for 30 mins, 70°C for 10 mins then place on ice.

NOTE: This is to inactivate the DNA ligase to prevent barcode crossover.

1. Pool all barcoded fragments together into a clean 1.5 ml Eppendorf tube
2. Add 45.7µL Aline beads per sample.
3. Incubate for 5 mins.
4. Place on a magnet rack for 2 mins or until clear.
5. Remove solution.
6. Add 200µL 70% ethanol to the tube still on the magnetic rack.
7. Remove and discard ethanol without disturbing the pellet.
8. Repeat steps 11 and 12.
9. Spin down and remove residual 70% ethanol and air dry for 1 min.
10. Resuspend in 31µL EB.

11. Incubate off the magnetic rack for 2 mins.
12. Replace on magnetic rack.
13. Wait until clear and then carefully remove solution and transfer to a clean 1.5mL Eppendorf tube.
14. Remove 1µL and assess concentration by Quantus as described in previous section.
15. Set up the following adapter ligation reaction:

Cleaned-up barcoded amplicon pools (~60ng)	30µL
NEBNext Quick Ligation Reaction Buffer (5X)	10µL
AMII adapter mix	5µL
Quick T4 DNA Ligase	5µL
Total volume	50µL

16. Incubate at RT for 30 mins.
17. Add 50µL Aline beads
18. Incubate for 5 mins
19. Place on a magnetic rack until clear
20. Remove supernatant
21. Add 200µL SFB and resuspend by flicking

CAUTION: do not use 80% ethanol

1. Place on magnetic rack until clear
2. Remove supernatant
3. Repeat SFB wash
4. Spin down and remove residual SFB
5. Add 15µL EB and resuspend by flicking
6. Incubate at RT for 2 mins.
7. Place on magnetic rack.
8. Carefully transfer solution to a clean 1.5mL Eppendorf tube.
9. Remove 1µL and assess concentration by Quantus (wait until beads have settled before measuring).

NOTE: Library can be now be stored at 4°C if required, but for best results it would be best to proceed immediately to sequencing.

Part 5: Priming and loading the SpotON flow cell

1. Thaw the following at RT before placing on ice:
    - Sequencing buffer (SQB)
    - Loading beads (LB)
    - Flush buffer (FLB)
    - Flush tether (FLT)
2. Add 30 ul FLT to the tube of FLB and mix well.
3. Flip back the MinION lid and slide the priming port cover clockwise so that the priming port is visible.

IMPORTANT: Care must be taken when drawing back buffer from the flow cell. The array of pores must be covered by buffer at all times. Removing more than 20-30µL risks damaging the pores in the array.

1. After opening the priming port, check for small bubble under the cover. Draw back a small volume to remove any bubble (a fewµLs):
    - Set a P1000 pipette to 200µL
    - Insert the tip into the priming port
    - Turn the wheel until the dial shows 220-230µL, or until you can see a small volume of buffer entering the pipette tip.
2. Load 800µL of FLB plus FLT into the flow cell via the priming port, using the dial-down method described in step 5, avoiding the introduction of air bubbles.
3. Wait for 5 minutes.
4. In a new tube prepare the library dilution for sequencing:

Reagent	Volume
SQB	37.5µL
LB	25.5µL
Library (~30ng)	12µL
Total	75µL

5. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
6. Load 200µL of the priming mix into the flow cell via the priming port (**NOT** the SpotON sample port), avoiding the introduction of air bubbles.
7. Mix the prepared library gently by pipetting up and down just prior to loading.
8. Add 75µL of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop siphons into the port before adding the next.
9. Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.
10. Double–click the MinKNOW icon located on the desktop to open the MinKNOW GUI.
11. If your MinION was disconnected from the computer, plug it back in.
12. Choose the following flow cell type from the selector box:
    - `FLO-MIN106` : R9.4.1 flowcell
13. Then mark the flow cell as `Selected` .
14. Click the `New Experiment` button at the bottom left of the GUI.
15. On the New experiment popup screen, select the running parameters for your experiment from the individual tabs:
    - **`Experiment`**

Name the run in the experiment field, leave the sample field blank.

- **Kit**

  Selection LSK109 as there is no option for native barcoding (NBD104)

- **Run Options**

  Set the run length, usually 1-2 hours.

- **Basecalling**

  Leave basecalling turned on and check the HAC (high accuracy model) is selected

- **Output**

  The number of files that MinKNOW will write to a single folder. By default this is set to 4000

16. Click `Start run` .
17. Allow the script to run to completion.
18. The MinKNOW Experiment page will indicate the progression of the script; this can be accessed through the `Experiment` tab that will appear at the top right of the screen
19. Monitor the Message panel on the right hand side for errors.