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Ebola virus sequencing protocol

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ARTIC

Josh Quick

cDNA preparation

1 Mix the following components in an 0.2mL 8-strip tube;

Component	Volume
50µM random hexamers	1 µl
10mM dNTPs mix (10mM each)	1 µl
Template RNA	10 µl
Total	12 µl

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.

2 Gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

3 Incubate the reaction as follows:

65 °C for 00:05:00

Place on ice for 00:01:00

4 Add the following to the annealed template RNA:

Component	Volume
SSIV Buffer	4 µl
100mM DTT	1 µl
RNaseOUT RNase Inhibitor	1 µl
SSIV Reverse Transcriptase	1 µl
Total	20 µl

5 Gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

6 Incubate the reaction as follows:

42 °C **01:30:00**

70 °C **00:10:00**

Hold at **5 °C**

Primer pool preparation

7 If required resuspend lyophilised primers at a concentration of 100µM each

Ebola V2 primers for this protocol were designed using Primal Scheme and generate overlapping 400nt amplicons. Primer names and dilutions are listed in the table below.

8 Generate primer pool stocks by adding **5 µl** of each primer pair to a **1.5 ml** Eppendorf labelled either “Pool 1 (100µM)” or “Pool 2 (100µM)”. Total volume should be **505 µl** for Pool 1 (100µM) and **530 µl** for Pool 2 (100µM). These are your 100µM stocks of each primer pool.

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 until cDNA addition.

9 Dilute this primer pool 1:10 in molecular grade water, to generate 10µM primer stocks. It is recommend that multiple aliquots of each primer pool are made to in case of degradation or 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
		Ebov-10-Pan_17_RIGHT_alt1	GTTTCTGGCACTTCGATACCCA	1	100µM
Ebov-10-Pan_18_LEFT	AAAATCCAAGCAATAATGACTTCACTCC	Ebov-10-Pan_18_RIGHT	TTGATCAATTAAAAGTGTCTCCTCTAATGG	2	100µM
		Ebov-10-Pan_18_RIGHT_alt1	TCGATCAATTTAAAGTATCTCCTCTAATGG	2	100µM
		Ebov-10-Pan_18_RIGHT_alt2	TTGATCAATTAAAAGTATCTCCTCTAATAG	2	100µM
Ebov-10-Pan_19_LEFT	AGATCCAGTTTTATAGAATCTTCTCAGGGA	Ebov-10-Pan_19_RIGHT	AGAAGGGCAATGTCTGTACTTGG	1	100µM
Ebov-10-Pan_19_LEFT_alt1	AGATCCAGTTTTACAGAATCTTCTCAGGGA	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	TCATCCTTGATTCTACAATCATGACAGT	Ebov-10-Pan_28_RIGHT	AGGTGCTGGAGGAACTGTTAATG	2	100µM
Ebov-10-Pan_28_LEFT_alt1	TCATCCTTGATTCTACAATCATAACAGT			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
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	AGCAGAGATGTCAAGATAACTATTGAGT	2	100µM
Ebov-10-Pan_37_LEFT	ACACGAATGCAAAGTTTGATTCTTGA	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	ACATTTGATGTAAAAATTCATTGCCCTG	1	100µM
Ebov-10-Pan_40_LEFT	GTGGGTGCTCAAGAAGACTGTG	Ebov-10-Pan_40_RIGHT	TGAGATTAGAGTTGTGTTGAATCGACA	2	100µM

Ebov-10-Pan_40_LEFT_alt1	GTGGGTGCTCAAGAGGACTGTG	Ebov-10-Pan_40_RIGHT_alt1	TGAGATTAGAGTCGTGTTGAATCGACA	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	GTCAAGGATCTTGGTACAGTGTTACT	Ebov-10-Pan_43_RIGHT	TGAGAAAGAAAAGTTCCGATATTGTGGT	1	100µM
Ebov-10-Pan_43_LEFT_alt1	GCCAAGGGTCTTGGTACAGTGTTACT	Ebov-10-Pan_43_RIGHT_alt1	TGAGAAAGAAAAATTCCGGTATTGTGGT	1	100µM
Ebov-10-Pan_43_LEFT_alt2	GTCAAGGGTCTTGGTACAGTGTTACT	Ebov-10-Pan_43_RIGHT_alt2	TGAGAAAGAAAAATTCCGATATTGTGGT	1	100µM
Ebov-10-Pan_44_LEFT	TTGAGAATGTTCTTTCCTACGCACA	Ebov-10-Pan_44_RIGHT	ACGGTTGCAATATTCTATAAAAGGTGC	2	100µM
Ebov-10-Pan_44_LEFT_alt1	TTGAGAATGTTCTTTCCTACGCGCA	Ebov-10-Pan_44_RIGHT_alt1	ACGGTTGCAATATTCGATAAAAGGTGC	2	100µM
		Ebov-10-Pan_44_RIGHT_alt2	ACGGTTACAATATTCTATAAAAGGTGC	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	TCTCCGAATGATTGAGATGGATGATT	Ebov-10-Pan_48_RIGHT	CTCAGTCTGTCCAAAACCGGTG	2	100µM
Ebov-10-Pan_48_LEFT_alt1	TCTCCGAATGATTGGGATGGATGATT			2	100µM
Ebov-10-Pan_49_LEFT	GATATCTTTTCACGCACGCCGA	Ebov-10-Pan_49_RIGHT	CCACCTGGTTGCTTTGCATTTG	1	100µM
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	AACAGTGACTTGCTAATAAAACCATTTTTG	Ebov-10-Pan_51_RIGHT	AAATACTGAGCTGGTACTTCCCG	1	100µM
Ebov-10-Pan_51_LEFT_alt1	AACAGTGACTTGCTAATAAAGCCATTTTTG			1	100µM
Ebov-10-Pan_51_LEFT_alt2	AACAGTGATTTGCTAATAAAACCATTTTTG			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	ACAATTGTTCCGCGATTAATTATCCAT	1	100µM
Ebov-10-Pan_53_LEFT_alt1	ACAGACCCAATTAGCAGCGGAGA	Ebov-10-Pan_53_RIGHT_alt1	ACAATTGTTCCGCGATTAATTATCCACT	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	ATTGGCTTAATTAAATAACCAGTGGCA	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	AGATTGCAATTGTGAAGAACGTTTCT	Ebov-10-Pan_60_RIGHT	AGAGTGCAGAGTTTATTATGTTGCGT	2	100µM
Ebov-10-Pan_61_LEFT	TCACAATGCAGCATGTGTGACA	Ebov-10-Pan_61_RIGHT	AGGTATTTCTGATTTTACAGTCCTGCC	1	100µM
		Ebov-10-Pan_61_RIGHT_alt1	AGGTATTTATGATTTTACAGTCCTGCC	1	100µM
		Ebov-10-Pan_61_RIGHT_alt2	AGGTATTTCTGATTTTACAGTCATGCC	1	100µM
Ebov-10-Pan_62_LEFT	CCTGTCAGATGGAATAGTGTTTTGGT	Ebov-10-Pan_62_RIGHT	AATTTTTGTGTGCGACCATTTTTCC	2	100µM

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 Pool 1 (10µM) primers per 25µL reaction. Pool 2 has 106 primers the requirement is 4.0µL of Pool 2 (10µM) primers per 25µL reaction. For other schemes, adjust the volume added appropriately.

Multiplex PCR

10 In the mastermix hood set up the multiplex PCR reactions as follows in 0.2mL 8-strip PCR tubes:

Component	Pool 1	Pool 2
NEB Q5 Polymerase 2X MasterMix	12.5 µl	12.5 µl
Primer Pool 1 or 2 (10µM)	3.8 µl	4 µl
Water	6.2 µl	6 µl
Total	22.5 µl	22.5 µl

The **mastermix hood** should prepared by sterilising with UV and treated with MediPal wipes, DNAway and RNAseZap reagents. 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.

This step should be carried out in the mastermix hood and template should not be taken anywhere near the mastermix hood at any stage.

11 In the template hood add 2.5 µl cDNA to each tube and mix well by pipetting.

The **template hood** should prepared by sterilising with UV and treated with MediPal wipes, DNAway and RNAseZap reagents. 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.

12 Pulse centrifuge the tubes to collect the contents at the bottom of the tube.

13 Set-up the following program on the thermal cycler:

Step	Temperature	Time	Cycles
Heat Activation	98 °C	00:00:30	1
Denaturation	98 °C	00:00:15	25-35
Annealing	65 °C	00:05:00	25-35
Hold	4 °C	Indefinite	1

Cycle number should be 25 for Ct 18-21 up to a maximum of 35 cycles for Ct 35

PCR clean-up

14 Combine the entire contents of “Pool 1” and “Pool 2” PCR reactions for each biological sample into to a single 1.5 ml Eppendorf tube.

15 Clean-up the amplicons using the following protocol:

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

15.1 ⚠

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

15.2 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.

15.3 Pulse centrifuge to collect all liquid at the bottom of the tube.

15.4 Incubate for **00:05:00** at room temperature.

15.5 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear.

15.6 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

15.7 Add **200 µl** of room-temperature **[M]70 % volume** ethanol to the pellet.

15.8 Carefully remove and discard ethanol, being careful not to touch the bead pellet.

15.9 **go to step #7** and repeat ethanol wash.

15.10 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.

15.11 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).

15.12 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

15.13 Place on magnet and transfer sample to a clean 1.5mL Eppendorf tube ensuring no beads are transferred into this tube.

15.14 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

Quantification and normalisation

16 Quantify the amplicon pools using the Quantus Fluorometer using the ONE dsDNA assay.

DNA quantification using the Quantus fluorometer
by Josh Quick
PREVIEW RUN

16.1 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

16.2 Set up two **0.5 ml** tubes for the calibration and label them 'Blank' and 'Standard'

16.3 Add **200 µl** ONE dsDNA Dye solution to each tube.

16.4 Mix the Lambda DNA standard 400 ng/µL standard by pipetting then add **1 µl** to one of the standard tube.

16.5 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

16.6 Allow both tubes to incubate at room temperature for **00:02:00** before proceeding.

16.7 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'.

16.8 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

16.9 Label the tubes on the lids, avoid marking the sides of the tube as this could interfere with the sample reading.

16.10 Add **199 µl** ONE dsDNA dye solution to each tube.

16.11 Add 1 µl of each user sample to the appropriate tube.

Use a P2 pipette for highest accuracy.

16.12 Mix each sample vigorously by vortexing for 00:00:05 and pulse centrifuge to collect the liquid.

16.13 Allow all tubes to incubate at room temperature for 00:02:00 before proceeding.

16.14 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.

16.15 On the home screen navigate to 'Sample Volume' and set it to 1 µl then 'Units' and set it to ng/µL.

16.16 Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.

16.17 Repeat step 16 until all samples have been read.

16.18 The value displayed on the screen is the dsDNA concentration in ng/µL, carefully record all results in a spreadsheet or laboratory notebook.

17 Label a 1.5 ml Eppendorf tube for each sample.

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.

18 Normalise the input but adding **10 ng** amplicon pools to each tube and diluting to **10 µl** to have an input concentration of **1 ng/µL**

Quantity of amplicons can vary from 10-50ng depending on the amplicon length, 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.

Native barocoding

19 Barcode the amplicon pools using native barcodes.

One-pot native barcoding of amplicons
by Josh Quick
PREVIEW RUN

19.1 Set up the following reaction for each sample:

Component	Volume
DNA amplicons	**10 µl**
Ultra II End Prep Reaction Buffer	**1.4 µl**
Ultra II End Prep Enzyme Mix	**0.6 µl**
Total	**12 µl**

19.2 Incubate at room temperature for **00:10:00**
Incubate at **65 °C** for **00:05:00**
Incubate on ice for **00:01:00**

19.3 Add the following directly to the previous reactions:

Component	Volume
NBXX barcode	**2.5 µl**
Ultra II Ligation Master Mix	**14.5 µl**
Ligation Enhancer	**0.5 µl**
Total	**29.5 µl**

Use one native barcode from the EXP-NBD104 (1-12) or EXP-NBD114 (13-24) per sample. Use from 6 to 24 barcodes in a library, any fewer and there will be insufficient total material to achieve good yields.

19.4 Incubate at room temperature for 00:20:00

Incubate at 70 °C for 00:10:00

Incubate on ice for 00:01:00

The 70°C incubation is to inactivate the DNA ligase to prevent barcode cross-ligation when reactions are pooled in the next step.

19.5 Pool all barcoded fragments together into a new 1.5 ml Eppendorf tube.

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

19.6 Quantify the barcoded amplicon pools using the Quantus Fluorometer using the ONE dsDNA assay.

DNA quantification using the Quantus fluorometer
by Josh Quick
PREVIEW RUN

19.7 Set up the following AMII adapter ligation reaction:

Component	Volume
Barcoded amplicon pools	30 µl
NEBNext Quick Ligation Reaction Buffer (5X)	10 µl
AMII adapter mix	5 µl
Quick T4 DNA Ligase	5 µl
Total	50 µl

The input of barcoded amplicon pools will depend on the number of barcoded pools and should be between 50 ng (6 barcodes) and 200 ng (24 barcodes).

19.8 Incubate at room temperature for 00:20:00

19.9 Add **50 µl** (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting.

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

19.10 Pulse centrifuge to collect all liquid at the bottom of the tube.

19.11 Incubate for **00:05:00** at room temperature.

19.12 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear.

19.13 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

19.14 Add **200 µl** SFB and resuspend beads completely by pipette mixing.

SFB will remove excess adapter without damaging the adapter-protein complexes. Do not use 70% ethanol as in early clean-ups.

19.15 Pulse centrifuge to collect all liquid at the bottom of the tube.

19.16 Remove supernatant and discard.

19.17 Repeat steps 14-16 to perform a second SFB wash.

19.18 Pulse centrifuge and remove any residual SFB.

You do not need to allow to air dry with SFB washes.

19.19 Add **15 µl** EB and resuspend beads by pipette mixing.

19.20 Incubate at room temperature for **00:02:00** .

19.21 Place on magnetic rack.

19.22 Transfer final library to a new 1.5mL Eppendorf tube.

20 Quantify the final library using the Quantus Fluorometer using the ONE dsDNA assay.

DNA quantification using the Quantus fluorometer PREVIEW RUN
by Josh Quick

Final library can be now be stored in EB at 4°C for up to a week if needed otherwise proceed directly to MinION sequencing.

20.1 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

20.2 Set up two **0.5 ml** tubes for the calibration and label them 'Blank' and 'Standard'

20.3 Add **200 µl** ONE dsDNA Dye solution to each tube.

20.4 Mix the Lambda DNA standard 400 ng/µL standard by pipetting then add **1 µl** to one of the standard tube.

20.5 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

20.6 Allow both tubes to incubate at room temperature for **00:02:00** before proceeding.

20.7 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'.

20.8 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

20.9 Label the tubes on the lids, avoid marking the sides of the tube as this could interfere with the sample reading.

20.10 Add **199 µl** ONE dsDNA dye solution to each tube.

20.11 Add **1 µl** of each user sample to the appropriate tube.

Use a P2 pipette for highest accuracy.

20.12 Mix each sample vigorously by vortexing for **00:00:05** and pulse centrifuge to collect the liquid.

20.13 Allow all tubes to incubate at room temperature for **00:02:00** before proceeding.

20.14 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.

20.15 On the home screen navigate to 'Sample Volume' and set it to **1 µl** then 'Units' and set it to ng/µL.

20.16 Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.

20.17 Repeat step 16 until all samples have been read.

20.18 The value displayed on the screen is the dsDNA concentration in ng/µL, carefully record all results in a spreadsheet or laboratory notebook.

MinION sequencing

21 Prime the flowcell and load **30 ng** sequencing library onto the flowcell.

Priming and loading a MinION flowcell PREVIEW RUN
by Josh Quick

From experience we know 30 ng is optimum loading input for short amplicons.

21.1 Thaw the following reagents at room temperature before placing on ice:

Sequencing buffer (SQB)
Loading beads (LB)
Flush buffer (FLB)
Flush tether (FLT)

21.2 Add **30 µl** FLT to the FLB tube and mix well by vortexing.

21.3 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.

21.4 Rotate the inlet port cover clockwise by 90° so that the priming port is visible.

21.5 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.

21.6 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.

21.7 Wait for **00:05:00** .

21.8 Gently lift the SpotON cover to open the SpotON port.

21.9 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.

21.10 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.

21.11 Mix the prepared library gently by pipetting up and down just prior to loading.

21.12 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.

21.13 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the inlet port and close the MinION lid.

22 Start the sequencing run using MinKNOW.

Starting a MinION sequencing run using MinKNOW PREVIEW RUN
by Josh Quick

22.1 If required plug the MinION into the computer and wait for the MinION and flowcell to ben detected.

22.2 Choose flow cell 'FLO-MIN106' from the drop-down menu.

22.3 Then select the flowcell so a tick appears.

22.4 Click the 'New Experiment' button in the bottom left of the screen.

22.5 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: Select LSK109 as there is no option for native barcoding (NBD104).

Run Options: Set the run length to 6 hours (you can stop the run once sufficient data has been collected as determined using RAMPART).

Basecalling: Leave basecalling turned but select 'fast basecalling'.

Output: The number of files that MinKNOW will write to a single folder. By default this is set to 4000 but can be reduced to make RAMPART update more frequently.

Click 'Start run'.

22.6 Monitor the progress of the run using the MinKNOW interface.