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🌐 Ebola virus sequencing protocol

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Protocol status: Working
 We use this protocol and it's working

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PROTOCOL integer ID:
 28086

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

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.

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

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

Note

[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.

- 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.

Note

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.

- 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	TGTGTGCGAATAAC TATGAGGAAGA	Ebov-10-Pan_1_RIGHT	TTTCCAATGTTTTAC CCCAAGCTTT	1	100μM
		Ebov-10-Pan_1_RIGHT_al t1	TTTCCAATGCTTTAC CCCAAGCTTT	1	100μM
		Ebov-10-Pan_1_RIGHT_al t2	TTTCCAATGTTTTAC CCCAAGTTTT	1	100μM
Ebov-10-Pan_2_LEFT	CAAGCAAGATTGAG AATTAACCTTGGT	Ebov-10-Pan_2_RIGHT	ATCTCCCTGGTACGC ATGATGA	2	100μM
Ebov-10-Pan_2_LEFT _alt1	CAAGCAAGATTGAG AATTAACCTTGAT	Ebov-10-Pan_2_RIGHT_al t1	ATCTCCTTGGTACGC ATGATGA	2	100μM
Ebov-10-Pan_3_LEFT	GGCCTTTGAAGCAG GTGTTGAT	Ebov-10-Pan_3_RIGHT	TCAGTCCTTGCTCTG CATGTAC	1	100μM
Ebov-10-Pan_4_LEFT	CCTTTGCAAGTCTA TTCCTTCCGA	Ebov-10-Pan_4_RIGHT	CTGAGTGCAGCCTTA AAGGAGT	2	100μM
Ebov-10-Pan_4_LEFT _alt1	CTTTTGCAAGTCTA TTCCTTCCGA			2	100μM
Ebov-10-Pan_5_LEFT	AGTTCGTCTCCATC CTCTTGCA	Ebov-10-Pan_5_RIGHT	CTGGAAGCTGATTTT GTTCTTTTTCT	1	100μM
Ebov-10-Pan_6_LEFT	GAGTCTCGCGAACT TGACCATC	Ebov-10-Pan_6_RIGHT	TCCTCGTCGTCCTCG TCTAGAT	2	100μM
Ebov-10-Pan_6_LEFT _alt1	GAATCTCGCGAACT TGACCATC	Ebov-10-Pan_6_RIGHT_al t1	TCCTCATCGTCCTCG TCTAGAT	2	100μM
Ebov-10-Pan_7_LEFT	AGCTACGGCGAATA CCAGAGTT	Ebov-10-Pan_7_RIGHT	GTCCTGTCCTGCTC TTCATCA	1	100μM
		Ebov-10-Pan_7_RIGHT_al t1	GTCCTGTCCTGTTC TTCATCA	1	100μM
		Ebov-10-Pan_7_RIGHT_al t2	GTCCTGTCCTGTTC TTCATCG	1	100μM

Ebov-10-Pan_8_LEFT	TTAACGAAGAGGCA GACCCACT	Ebov-10-Pan_8_RIGHT	TTCCTCTTCAAGGGA GTCTGGA	2	100μ M
Ebov-10-Pan_8_LEFT_alt1	TCAACGAAGAGGCA GACCCACT	Ebov-10-Pan_8_RIGHT_al t1	TTCCTCTTCAAGGGA GTCCGGA	2	100μ M
Ebov-10-Pan_9_LEFT	GTGACAACACCCAG TCAGAACA	Ebov-10-Pan_9_RIGHT	TCTTCCTGTTTTCGT TCCTTGACT	1	100μ M
Ebov-10-Pan_9_LEFT_alt1	GTGACAACACCCAG CCAGAACA	Ebov-10-Pan_9_RIGHT_al t1	TCTTCCTGTTTGCGT TCCTTGACT	1	100μ M
		Ebov-10-Pan_9_RIGHT_al t2	TCTTCCTGTTTGCGT TTCTTGACT	1	100μ M
Ebov-10-Pan_10_LEFT T	ACAATGGGATGATT CAACCGACA	Ebov-10-Pan_10_RIGHT	TCGAGTGCTAGAGA ATTCAATTGACG	2	100μ M
Ebov-10-Pan_10_LEFT T_alt1	ATAATGGGATGATT TAACCGACA			2	100μ M
Ebov-10-Pan_11_LEFT T	ACCTACTAGCCTGC CCAACATT	Ebov-10-Pan_11_RIGHT	AATTGGGTCCGTTTG GGTTTGA	1	100μ M
Ebov-10-Pan_11_LEFT T_alt1	ACCTACTAGCCTAC CCAACATT	Ebov-10-Pan_11_RIGHT_ alt1	AATTGGATCCGTTTG GGTTTGA	1	100μ M
Ebov-10-Pan_12_LEFT T	CCCAAATGCAACAA ACGAAGCC	Ebov-10-Pan_12_RIGHT	TCAATCTTACCCCGA ATCGCAC	2	100μ M
Ebov-10-Pan_12_LEFT T_alt1	CCCAAATGCAACAA ACAAAGCC	Ebov-10-Pan_12_RIGHT_ alt1	TCAATCTTACCCCGA ATTGCAC	2	100μ M
Ebov-10-Pan_13_LEFT T	TATTGGGCCGAACA TGGTCAAC	Ebov-10-Pan_13_RIGHT	TGACAGGTGGAGCA GCATCTTG	1	100μ M

Ebov-10-Pan_13_LEF T_alt1	TATTGGGCTGAACA TGGTCAAC			1	100μM
Ebov-10-Pan_14_LEF T	CATTCATGCTGAGT TCCAGGCC	Ebov-10-Pan_14_RIGHT	GCGAGATATGAACA ATTTTATCTTGGTCG	2	100μM
		Ebov-10-Pan_14_RIGHT_ alt1	GCGAGATAAGGACA ATTTTATCTTGGTCG	2	100μM
		Ebov-10-Pan_14_RIGHT_ alt2	GCGAGATAAGAACA ATTTTATCTTGGTCG	2	100μM
Ebov-10-Pan_15_LEF T	TGAGTATCAGCCCT GGATAATATAAGTC A	Ebov-10-Pan_15_RIGHT	TCGATGGAGTGTCCC CATTGAC	1	100μM
Ebov-10-Pan_15_LEF T_alt1	TGAGTATCAGCCCT AGATAATATAAGTC A	Ebov-10-Pan_15_RIGHT_ alt1	TCGATGGAGTGTCTC CATTGAC	1	100μM
Ebov-10-Pan_16_LEF T	GCAACAGCAATACA GGCTTCCT	Ebov-10-Pan_16_RIGHT	GAAAGCCTGGTTTCC AATTCGC	2	100μM
Ebov-10-Pan_16_LEF T_alt1	GCAACAACAATACA GGCTTCCT	Ebov-10-Pan_16_RIGHT_ alt1	GAAGGCCTGGTTTCC AATTCGC	2	100μM
Ebov-10-Pan_17_LEF T	CCACTTGTCAAGT CAATCGGC	Ebov-10-Pan_17_RIGHT	GTTTCTGGCACTTCG ATTCCCA	1	100μM
		Ebov-10-Pan_17_RIGHT_ alt1	GTTTCTGGCACTTCG ATACCCA	1	100μM
Ebov-10-Pan_18_LEF T	AAAATCCAAGCAAT AATGACTTCACTCC	Ebov-10-Pan_18_RIGHT	TTGATCAATTAAAAG TGTCTCCTCTAATGG	2	100μM
		Ebov-10-Pan_18_RIGHT_ alt1	TCGATCAATTTAAAG TATCTCCTCTAATGG	2	100μM
		Ebov-10-Pan_18_RIGHT_ alt2	TTGATCAATTAAAAG TATCTCCTCTAATAG	2	100μM

Ebov-10-Pan_19_LEF T	AGATCCAGTTTTAT AGAATCTTCTCAGG GA	Ebov-10-Pan_19_RIGHT	AGAAGGGCAATGTC TGTACTTGG	1	100μ M
Ebov-10-Pan_19_LEF T_alt1	AGATCCAGTTTTAC AGAATCTTCTCAGG GA	Ebov-10-Pan_19_RIGHT_ alt1	AGAAGGGCGATGTC TGTGCTTGG	1	100μ M
Ebov-10-Pan_20_LEF T	AGCCAGTGTGACTT GGATTGGA	Ebov-10-Pan_20_RIGHT	AGTTTGTGACATCA CTAACCTGT	2	100μ M
		Ebov-10-Pan_20_RIGHT_ alt1	AGTTTGTGACATCA CTAATTGT	2	100μ M
Ebov-10-Pan_21_LEF T	AGAACATTTTCCAT CCCACTTGA	Ebov-10-Pan_21_RIGHT	AAGCACCTCTTTAT GGAAGGC	1	100μ M
		Ebov-10-Pan_21_RIGHT_ alt1	AAGCACCTCTTTGT GGAAGGC	1	100μ M
Ebov-10-Pan_22_LEF T	TGCCGGTATGTGCA CAAAGTAT	Ebov-10-Pan_22_RIGHT	ATATATTGTCTCATT CAGCTGGAGCA	2	100μ M
Ebov-10-Pan_23_LEF T	CGAGGTTGACAATT TGACCTACGT	Ebov-10-Pan_23_RIGHT	GCAAGGGTTGTTAGA TGCGACA	1	100μ M
		Ebov-10-Pan_23_RIGHT_ alt1	GCAAGGGTTGTCAG ATGCGACA	1	100μ M
Ebov-10-Pan_24_LEF T	TGCAATGGTTCAAG TGCACAGT	Ebov-10-Pan_24_RIGHT	CTGGCACTCTCTTCT CCGGTAT	2	100μ M
Ebov-10-Pan_24_LEF T_alt1	TGCAATGGTTCAAG TGCACAAT			2	100μ M
Ebov-10-Pan_25_LEF T	ACCACAACAAGTCC CCAAAACC	Ebov-10-Pan_25_RIGHT	TAGCTCAGTTGTGGC TCTCAGG	1	100μ M
		Ebov-10-Pan_25_RIGHT_ alt1	TAGCTCGGTTGTGGC TCTCAGG	1	100μ M

Ebov-10-Pan_26_LEF T	ATCTGTGGGTTGAG ACAGCTGG	Ebov-10-Pan_26_RIGHT	GCTTTTCCATGAAGC AATCTGAAGA	2	100μM
Ebov-10-Pan_26_LEF T_alt1	ATCTGTGGATTGAG GCAGCTGG	Ebov-10-Pan_26_RIGHT_alt1	GCTTTGCCATGAAGC AATCTGAAGA	2	100μM
Ebov-10-Pan_26_LEF T_alt2	ATCTGTGGGTTGAG GCAGCTGG			2	100μM
Ebov-10-Pan_27_LEF T	TGGAGTTACAGGCG TTATAATTGCA	Ebov-10-Pan_27_RIGHT	AAAGGCTTCTTTCCC TTGTCAC T	1	100μM
Ebov-10-Pan_28_LEF T	TCATCCTTGATTCT ACAATCATGACAGT	Ebov-10-Pan_28_RIGHT	AGGTGCTGGAGGAA CTGTTAATG	2	100μM
Ebov-10-Pan_28_LEF T_alt1	TCATCCTTGATTCT ACAATCATAACAGT			2	100μM
Ebov-10-Pan_29_LEF T	GAGTACCGTCAATC AAGGAGCG	Ebov-10-Pan_29_RIGHT	CACAGCACATAGAG TCAACAATGC	1	100μM
Ebov-10-Pan_30_LEF T	GATCAAGACGGCAG AACACTGG	Ebov-10-Pan_30_RIGHT	ATCAGACCATGAGC ATGTCCCC	2	100μM
Ebov-10-Pan_31_LEF T	CTGCTGTCGTTGTT TCAGGGTT	Ebov-10-Pan_31_RIGHT	ATGGGATGGATCGTT GCTACCT	1	100μM
		Ebov-10-Pan_31_RIGHT_alt1	ATGGGATGGATCGTT GCTGCCT	1	100μM
		Ebov-10-Pan_31_RIGHT_alt2	ATGAGATGGATCGTT GCTACCT	1	100μM
Ebov-10-Pan_32_LEF T	GCCAAGCATACCTC TTGCACAA	Ebov-10-Pan_32_RIGHT	TGGA CTACCCTGAA ATAGTACTTTGC	2	100μM
Ebov-10-Pan_33_LEF T	TGCGGAGGTCTGAT AAGAATAAACC	Ebov-10-Pan_33_RIGHT	TTCAACCTTGAAACC TTGCGCT	1	100μM

		Ebov-10-Pan_33_RIGHT_alt1	TTCAACTTTGAAACC TTGCGCT	1	100μ M
Ebov-10-Pan_34_LEF T	GCTGAAAAGAAGCT TACCTACAACG	Ebov-10-Pan_34_RIGHT	TCCTTGTCATTGACC ATGCAGG	2	100μ M
Ebov-10-Pan_34_LEF T_alt1	GTTGAAAAAAGGCC TACCTACAACG			2	100μ M
Ebov-10-Pan_34_LEF T_alt2	GCTGAAAAGAAGCC CACCTACAACG			2	100μ M
Ebov-10-Pan_35_LEF T	GTGACTCACAAAGG AATGGCCC	Ebov-10-Pan_35_RIGHT	ACAATCCGTTGTAGT TCACGACA	1	100μ M
		Ebov-10-Pan_35_RIGHT_alt1	ACAACCCGTTGTAGT TCACGACA	1	100μ M
Ebov-10-Pan_36_LEF T	TGCTGTCGTTGATT CGATCCAA	Ebov-10-Pan_36_RIGHT	AGCAGAGATGTCAA GATAACTATTGAGT	2	100μ M
Ebov-10-Pan_37_LEF T	ACACGAATGCAAAG TTTGATTCTTGA	Ebov-10-Pan_37_RIGHT	TGAAACCTAACACA TGTGACCTGC	1	100μ M
		Ebov-10-Pan_37_RIGHT_alt1	TGAAACCTAACACA CGTGACCTGC	1	100μ M
Ebov-10-Pan_38_LEF T	CCCTCAAACAAGAG ATTCCAAGACA	Ebov-10-Pan_38_RIGHT	ACAGTTGCGTAGTTG CGGATTA	2	100μ M
Ebov-10-Pan_38_LEF T_alt1	CCCTCAAATAAGAG ATTCCAAGACA			2	100μ M
Ebov-10-Pan_38_LEF T_alt2	TCCTCAAATAAGAG ATTCCAAGACA			2	100μ M

Ebov-10-Pan_39_LEF T	ACCTAGTCACTAGA GCTTGCGG	Ebov-10-Pan_39_RIGHT	ACATTTGATGTAAAA ATTCATTGCCCTG	1	100μM
Ebov-10-Pan_40_LEF T	GTGGGTGCTCAAGA AGACTGTG	Ebov-10-Pan_40_RIGHT	TGAGATTAGAGTTGT GTTGAATCGACA	2	100μM
Ebov-10-Pan_40_LEF T_alt1	GTGGGTGCTCAAGA GGACTGTG	Ebov-10-Pan_40_RIGHT_alt1	TGAGATTAGAGTCGT GTTGAATCGACA	2	100μM
Ebov-10-Pan_41_LEF T	AAGAAGCGGTTCAA GGGCATAC	Ebov-10-Pan_41_RIGHT	CTATGGAATTCACGG ATCTTTTGAGC	1	100μM
Ebov-10-Pan_41_LEF T_alt1	AAGAAGCAGTTCAA GGGCATAC	Ebov-10-Pan_41_RIGHT_alt1	CTATGGAATTCACGG ATCTTTTGATC	1	100μM
Ebov-10-Pan_42_LEF T	TGCATTTAGCTGTA AATCACACCCT	Ebov-10-Pan_42_RIGHT	AATCATTGGCAACG GAGGGAAT	2	100μM
		Ebov-10-Pan_42_RIGHT_alt1	AATCATTGGCAACG GGGGAAT	2	100μM
Ebov-10-Pan_43_LEF T	GTCAAGGATCTTGG TACAGTGTTACT	Ebov-10-Pan_43_RIGHT	TGAGAAAGAAAAGT TCCGATATTGTGGT	1	100μM
Ebov-10-Pan_43_LEF T_alt1	GCCAAGGGTCTTGG TACAGTGTTACT	Ebov-10-Pan_43_RIGHT_alt1	TGAGAAAGAAAAAT TCCGGTATTGTGGT	1	100μM
Ebov-10-Pan_43_LEF T_alt2	GTCAAGGGTCTTGG TACAGTGTTACT	Ebov-10-Pan_43_RIGHT_alt2	TGAGAAAGAAAAAT TCCGATATTGTGGT	1	100μM
Ebov-10-Pan_44_LEF T	TTGAGAATGTTCTT TCCTACGCACA	Ebov-10-Pan_44_RIGHT	ACGGTTGCAATATTC TATAAAAGGTGC	2	100μM
Ebov-10-Pan_44_LEF T_alt1	TTGAGAATGTTCTT TCCTACGCGCA	Ebov-10-Pan_44_RIGHT_alt1	ACGGTTGCAATATTC GATAAAAGGTGC	2	100μM

		Ebov-10-Pan_44_RIGHT_alt2	ACGGTTACAATATTC TATAAAAGGTGC	2	100μM
Ebov-10-Pan_45_LEF T	CCACAGTTAGAGGG AGTAGCTTTG	Ebov-10-Pan_45_RIGHT	GCTCGTCTGCGTCAG TCTCTAA	1	100μM
Ebov-10-Pan_45_LEF T_alt1	CCACAGTTAGAGGG AGTAGTTTTG			1	100μM
Ebov-10-Pan_46_LEF T	AAGTTACGCTCAGC TGTGATGG	Ebov-10-Pan_46_RIGHT	ATGGAAAGCTGCGG TTATCCTG	2	100μM
Ebov-10-Pan_47_LEF T	TAGGCACTGCTTTT GAGCGATC	Ebov-10-Pan_47_RIGHT	CACAAAGTCAATGG CAGTGCAG	1	100μM
Ebov-10-Pan_47_LEF T_alt1	TAGGCACCGCTTTT GAGCGGTC			1	100μM
Ebov-10-Pan_47_LEF T_alt2	TAGGCACTGCTTTT GAACGATC			1	100μM
Ebov-10-Pan_48_LEF T	TCTCCGAATGATTG AGATGGATGATT	Ebov-10-Pan_48_RIGHT	CTCAGTCTGTCCAAA ACCGGTG	2	100μM
Ebov-10-Pan_48_LEF T_alt1	TCTCCGAATGATTG GGATGGATGATT			2	100μM
Ebov-10-Pan_49_LEF T	GATATCTTTTCACG CACGCCGA	Ebov-10-Pan_49_RIGHT	CCACCTGGTTGCTTT GCATTTG	1	100μM
Ebov-10-Pan_49_LEF T_alt1	GATATCTTTTCACG CACGCCCA	Ebov-10-Pan_49_RIGHT_alt1	CCACCAGGTTGCTTT GCATTTG	1	100μM
Ebov-10-Pan_50_LEF T	TCAAAGTGTTTTGG CTGAAACCCT	Ebov-10-Pan_50_RIGHT	TCCTGAGTAATGTGA AGGGGTCA	2	100μM

Ebov-10-Pan_50_LEF T_alt1	TCAAAGTGGTTTGGCTGAAACCT	Ebov-10-Pan_50_RIGHT_alt1	TCCTGAGTAATGTGAAGGAGTCA	2	100μM
Ebov-10-Pan_51_LEF T	AACAGTGACTTGCTAATAAAACCATTTT TG	Ebov-10-Pan_51_RIGHT	AAATACTGAGCTGGTACTTCCCG	1	100μM
Ebov-10-Pan_51_LEF T_alt1	AACAGTGACTTGCTAATAAGCCATTTT TG			1	100μM
Ebov-10-Pan_51_LEF T_alt2	AACAGTGATTGCTAATAAAACCATTTT TG			1	100μM
Ebov-10-Pan_52_LEF T	AATCGTGCTCACCTTCATCTAACT	Ebov-10-Pan_52_RIGHT	CCCAAACTGTACAGAAGTCCTATCT	2	100μM
Ebov-10-Pan_53_LEF T	ACAGACCCAATTAGCAGTGGAGA	Ebov-10-Pan_53_RIGHT	ACAATTGTTCCGCGATTAAATTATCCAT	1	100μM
Ebov-10-Pan_53_LEF T_alt1	ACAGACCCAATTAGCAGCGGAGA	Ebov-10-Pan_53_RIGHT_alt1	ACAATTGTTCCGCGATTAAATTATCCACT	1	100μM
Ebov-10-Pan_54_LEF T	TCTCAGATGCGGCCAGGTTATT	Ebov-10-Pan_54_RIGHT	TGACCATCACTGTTGTTTGTGCT	2	100μM
Ebov-10-Pan_54_LEF T_alt1	TCTCAGATGCGGCCAGATTATT			2	100μM
Ebov-10-Pan_55_LEF T	TGGAGGAGCAGACACAGAAACA	Ebov-10-Pan_55_RIGHT	ATGACGTTAATTGGCGTGTC	1	100μM
Ebov-10-Pan_55_LEF T_alt1	TGGAGGAGCAGGCACAGAAACA	Ebov-10-Pan_55_RIGHT_alt1	ATGACGTCAATTGGCGTGTC	1	100μM
Ebov-10-Pan_55_LEF T_alt2	TGGAGAAGCAGGCACAGAAACA	Ebov-10-Pan_55_RIGHT_alt2	ATGACGTTAATTGGCGGTCC	1	100μM

Ebov-10-Pan_56_LEF T	CTCACACCGTCTAG TCCTACCT	Ebov-10-Pan_56_RIGHT	TTTGACATAACAGGT AGAAGCATCCT	2	100μ M
Ebov-10-Pan_56_LEF T_alt1	CTCGCACCGTCTAG TCCTACCT			2	100μ M
Ebov-10-Pan_56_LEF T_alt2	CTCACATCGTCTAG TCCTACCT			2	100μ M
Ebov-10-Pan_57_LEF T	ACACGCTAGCTACT GAGTCCAG	Ebov-10-Pan_57_RIGHT	ATTGGCTTAATTAAA TAACCAGTGGCA	1	100μ M
Ebov-10-Pan_58_LEF T	TGAAAGCAGTGGTC CTTAAAGTCT	Ebov-10-Pan_58_RIGHT	TGCTCTAAGATGTGC TAAGTGCTG	2	100μ M
		Ebov-10-Pan_58_RIGHT_alt1	TGCTCTAAGATGTGC CAAGTGCTG	2	100μ M
Ebov-10-Pan_59_LEF T	CGTCGATTCAAAAA GAGGTCCACT	Ebov-10-Pan_59_RIGHT	TCAGAAGCCCTGTC AGCCTTTC	1	100μ M
Ebov-10-Pan_60_LEF T	AGATTGCAATTGTG AAGAACGTTTCT	Ebov-10-Pan_60_RIGHT	AGAGTGCAGAGTTTA TTATGTTGCGT	2	100μ M
Ebov-10-Pan_61_LEF T	TCACAATGCAGCAT GTGTGACA	Ebov-10-Pan_61_RIGHT	AGGTATTTCTGATTT TACAGTCCTGCC	1	100μ M
		Ebov-10-Pan_61_RIGHT_alt1	AGGTATTTATGATTT TACAGTCCTGCC	1	100μ M
		Ebov-10-Pan_61_RIGHT_alt2	AGGTATTTCTGATTT TACAGTCATGCC	1	100μ M
Ebov-10-Pan_62_LEF T	CCTGTCAGATGGAA TAGTGTTTTGGT	Ebov-10-Pan_62_RIGHT	AATTTTTGTGTGCGA CCATTTTTCC	2	100μ M

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 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	4.0 µL
Water	6.2 µL	6.0 µL
Total	22.5 µL	22.5 µL

Note

The **mastermix hood** should be 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.

Note

The **template hood** should be 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

Note

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:

Protocol

NAME

Amplicon clean-up using SPRI beads

CREATED BY

Josh Quick

PREVIEW

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

- 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 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 #15.7 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 μ L Elution Buffer (EB), mix gently by either flicking or pipetting and incubate for 00:02:00 .
Elution Buffer (EB) **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 μ L 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

Quantification and normalisation

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

Protocol

NAME

DNA quantification using the Quantus fluorometer

CREATED BY

Josh Quick

PREVIEW

- 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 **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.

Note

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 μ L ONE dsDNA dye solution to each tube.

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

Note

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.

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.

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.

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.

- 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

Note

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 barcoding

- 19 Barcode the amplicon pools using native barcodes.

Protocol

NAME

One-pot native barcoding of amplicons

CREATED BY

Josh Quick

PREVIEW

- 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

Note

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

Note

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.

Protocol

NAME

Amplicon clean-up using SPRI beads

CREATED BY
Josh Quick

PREVIEW

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

Protocol

NAME

DNA quantification using the Quantus fluorometer

CREATED BY
Josh Quick

PREVIEW

- 19.7 Set up the following AMII adapter ligation reaction:

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

Note

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.

Note

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.

Note

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.

Note

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.

Protocol

NAME
DNA quantification using the Quantus fluorometer

CREATED BY
Josh Quick

PREVIEW

Note

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 **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.
- Note**

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.

Note

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.

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.

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.

Protocol

NAME

Priming and loading a MinION flowcell

CREATED BY

Josh Quick

PREVIEW

Note

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 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.

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

Note

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.

Protocol

NAME

Starting a MinION sequencing run using MinKNOW

CREATED BY

Josh Quick

PREVIEW

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