**Detailed protocol for reverse transcription, PCR and sequencing library preparation.**

Based on the protocol published here (1).

**Prepare the following primer mixes:**

**RPM\_A**: 21 primers. 3 µl of 100 µM stock of the following Reverse primers plus PCR grade water to 200 µl (= 300 pmol x 21 = 6300 pmol/200 µl = 32 pmol/µl. 2 µl per 20 µl RT reaction = 3.2 pmol/µl = 3.2 µM concentration in reaction) .

**Primers in RPM\_A**: nCoV-2019\_1\_LEFT, AR1\_3, AR1\_4, AR3\_11, AR3\_12, AR5\_19, AR5\_20, AR7\_27, AR7\_28, AR9\_35, AR9\_36, AR11\_43, AR11\_44, AR13\_51, AR13\_52, AR15\_59, AR15\_60, AR17\_67, AR17\_68, AR19\_75, AR19\_76.

**RPM\_B**: 21 primers. 3 µl of 100 µM stock of the following Reverse primers plus PCR grade water to 200 µl. (= 300 pmol x 21 = 6300 pmol/200 µl = 32 pmol/µl. 2 µl per 20 µl RT reaction = 3.2 pmol/µl = 3.2 µM concentration in reaction). **Primer Boost:** For Primers BR2\_7, BR2\_8, BR16\_63, BR16\_64 use 9 µl of 100 µM stock.

**Primers in RPM\_B:** BR2\_7, BR2\_8, BR4\_15, BR4\_16, BR6\_23, BR6\_24, BR8\_31, BR8\_32, BR10\_39, BR10\_40, BR12\_47, BR12\_48, BR14\_55, BR14\_56, BR16\_63, BR16\_64, BR18\_71, BR18\_72, BR20\_79, BR20\_80, nCoV-2019\_98\_RIGHT.

**PCR primer mix A (PPM\_A ):** 1.5 µl of each primer (forward and reverse primer) plus 439 µl PCR grade water. = 41 primers (100 pmol/µl) = 4100 pmol in 500 µl water = 8.2 pmol/µl in the PPM. When 2 µl PPM used per 25 µl PCR reaction = 0.66 pmol/µl in reaction (= 660 nM).

**Primers in PPM\_A:** nCoV-2019\_1\_LEFT, AF1\_1, AF1\_2, AR1\_3, AR1\_4, AF3\_9, AF3\_10, AR3\_11, AR3\_12, AF5\_17, AF5\_18, AR5\_19, AR5\_20, AF7\_25, AF7\_26, AR7\_27, AR7\_28, AF9\_33, AF9\_34, AR9\_35, AR9\_36, AF11\_41, AF11\_42, AR11\_43, AR11\_44, AF13\_49, AF13\_50, AR13\_51, AR13\_52, AF15\_57, AF15\_58, AR15\_59, AR15\_60, AF17\_65, AF17\_66, AR17\_67, AR17\_68, AF19\_73, AF19\_74, AR19\_75, AR19\_76 .

**PCR primer mix B (PPM\_B ):** 1.5 µl of each primer (forward and reverse primer) plus 439 µl PCR grade water = 41 primers (100 pmol/µl) = 4100 pmol in 500 µl water = 8.2 pmol/µl in the PPM. When 2 µl PPM used per 25 µl PCR reaction = 0.66 pmol/µl in reaction (= 660 nM). **Primer Boost:** for primers BF2\_5, BF2\_6, BR2\_7, BR2\_8, BF16\_62, BF16\_61, BR16\_63, BR16\_64, use 4.5 µl of 100 µM stock

**Primers in PPM\_B:** BF2\_5, BF2\_6, BR2\_7, BR2\_8, BF4\_13, BF4\_14, BR4\_15, BR4\_16, BF6\_21, BF6\_22, BR6\_23, BR6\_24, BF8\_29, BF8\_30, BR8\_31, BR8\_32, BF10\_37, BF10\_38, BR10\_39, BR10\_40, BF12\_45, BF12\_46, BR12\_47, BR12\_48, BF14\_53, BF14\_54, BR14\_55, BR14\_56, BF16\_61, BF16\_62, BR16\_63, BR16\_64, BF18\_69, BF18\_70, BR18\_71, BR18\_72, BF20\_77, BF20\_78, BR20\_79, BR20\_80, nCoV-2019\_98\_RIGHT.

**1. Set up Reverse Transcription reactions (two reactions per sample).**

Mix the following components in a 0.2mL 8-strip tube, 1 RT\_A and 1 RT-B reaction per sample.

Component Volume (per sample)

**RT\_A\_Reaction**

**Component Volume**

RPM\_A 1 µl

Template RNA 11µl (if using less than 11 µl viral RNA adjust to 11µl with water)  
Total Volume 12µl

**RT\_B\_Reaction**

**Component Volume**

RPM\_B 1 µl

Template RNA 11µl (if using less than 11 µl viral RNA adjust to 11µl with water)  
Total Volume 12µl

Heat at 65 °C for 5:00 mins, and quickly chill in ice/water bath for at least 1:00 min.

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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.

**Reverse Transcription**

Add the following to the 12 µl annealed template RNA sample:

**Component Volume (1 reaction)**

SuperScript III 5X Buffer 4 µl

100mM DTT 1 µl  
RNaseOUT RNase Inhibitor 1 µl

10mM dNTPs mix (10mM each) 1µl

SuperScript III 1 µl

(add 8 µl mix per reaction)

Total Volume 20 μl

**2. Incubate the RT reaction as follows:**

42 °C 50 minutes

70 °C 10 minutes

Hold at 4 °C

**3. Set up Amplicon PCR**

In the Mastermix hood set up the multiplex PCR reactions as follows in 0.2mL 8-strip PCR tubes. Prepare one PPM\_A and one PPM\_B mix per sample.

**Component 1 reaction 8rxns\_ PPM\_A\_Mix 8rxns\_ PPM\_B\_Mix**

5 x Phusion HF buffer 5µl 40 µl 40 µl

10 mM dNTPs 0.5µl 4 µl 4 µl

Phusion DNA Pol 0.5µl 4 µl 4 µl

Primer PPM\_A or PPM\_B

(10μM) 2 µl 16 µl 16 µl

Nuclease-free water 12 µl 96 µl 96 µl  
Total 20 µl per rxn

Aliquot 20 µl per tube PPM\_A\_Mix or PPM\_B\_Mix

In the extraction and sample addition cabinet add **5μl cDNA** to each tube and mix well by pipetting.

**4. Run PCR**

**Step Cycles Temp Time**

Heat Activation 1 98 °C 30sec

Denaturation 35 98 °C 15sec

Annealing 35 63 °C 30sec

Extension 35 72 °C 3 min

Polish 1 72 °C 10 min   
Hold 4 °C infinite

**Table 1. Entebbe Primers**

|  |  |  |  |
| --- | --- | --- | --- |
| **Amplicon\_no** | **Primer\_id** | **Sequence1** | **Position2** |
| 1 | AF1\_1 | GTCCGGGTGTGACCGAAAG | 242 |
| 1 | AF1\_2 | CCTTGTCCCTGGTTTCAACGA | 274 |
| 1 | AR1\_3 | TGCGGGAGAAAATTGATCGTACA | 1894 |
| 1 | AR1\_4 | GCACAGAATTTTGAGCAGTTTCA | 1921 |
| 2 | BF2\_5 | ACGTGCTAGCGCTAACATAGG | 1540 |
| 2 | BF2\_6 | GTTGGAGAAGGTTCCGAAGGT | 1580 |
| 2 | BR2\_7 | TAGCCTTATTTAAGGCTCCTGCAA | 3477 |
| 2 | BR2\_8 | TGCAACACCTCCTCCATGTTTAA | 3459 |
| 3 | AF3\_9 | ACAAACTGTTGGTCAACAAGACG | 3220 |
| 3 | AF3\_10 | CAACAAGACGGCAGTGAGGA | 3233 |
| 3 | AR3\_11 | TTGTGTAGATTGTCCAGAATAGGACC | 4806 |
| 3 | AR3\_12 | TCCATATGTCATTGACATGTCCACA | 5014 |
| 4 | BF4\_13 | TTTGGAAGAAGCTGCTCGGTATAT | 4639 |
| 4 | BF4\_14 | GAAACCATCTCACTTGCTGGTTC | 4772 |
| 4 | BR4\_15 | GGTTTTAGATCTTCGCAGGCAAG | 6380 |
| 4 | BR4\_16 | CCATTAGATCTGTGTGGCCAA | 6534 |
| 5 | AF5\_17 | CACACCCTCTTTTAAGAAAGGAGCTA | 6190 |
| 5 | AF5\_18 | TTGTTTGGCATGTTAACAATGCAAC | 6234 |
| 5 | AR5\_19 | CAGACGCTGATTTTGCAGATGA | 7919 |
| 5 | AR5\_20 | GCACTATCACCAACATCAGACAC | 7994 |
| 6 | BF6\_21 | AGGCTTTTGCAAACTACACAATTG | 7591 |
| 6 | BF6\_22 | TATGCTAATGGAGGTAAAGGCTTTTG | 7574 |
| 6 | BR6\_23 | CGATAGCTACAATACCACCAGCT | 9409 |
| 6 | BR6\_24 | CAATACCACCAGCTACTATAGATGCT | 9397 |
| 7 | AF7\_25 | AAGCTGGTGTTTGTGTATCTACTAGT | 9243 |
| 7 | AF7\_26 | ACCTACCTTGAAGGTTCTGTTAGAG | 9164 |
| 7 | AR7\_27 | CACTACCCAATATGGTACGTCCA | 10885 |
| 7 | AR7\_28 | TGAGTAACAACCAGTGGTGTGT | 11000 |
| 8 | BF8\_29 | CTGGAGTTCATGCTGGCACA | 10560 |
| 8 | BF8\_30 | GTTTTAGCTTGGTTGTACGCTG | 10664 |
| 8 | BR8\_31 | TTTGCCCTCTTGTCCTCAGATCTA | 12313 |
| 8 | BR8\_32 | TGGTATGACAACCATTAGTTTGGCT | 12466 |
| 9 | AF9\_33 | CTCAAGAAGCTTATGAGCAGGCT | 12144 |
| 9 | AF9\_34 | CAAGCTATAGCCTCAGAGTTTAGTTC | 12089 |
| 9 | AR9\_35 | ACGTTGACGTGATATATGTGGTACC | 13770 |
| 9 | AR9\_36 | ACGAGGTCTGCCATTGTGTATT | 13802 |
| 10 | BF10\_37 | GCGGTGTAAGTGCAGCC | 13475 |
| 10 | BF10\_38 | TCGCTTCCAAGAAAAGGACGAA | 13602 |
| 10 | BR10\_39 | CTCAATACTTGAGCACACTCATTAGC | 15406 |
| 10 | BR10\_40 | GTGACAAGCTACAACACGTTGT | 15367 |
| 11 | AF11\_41 | ATTCTATGGTGGTTGGCACAACA | 15219 |
| 11 | AF11\_42 | CAATAGCCGCCACTAGAGGAG | 15173 |
| 11 | AR11\_43 | TAGTGTAGGTGCACTTAATGGCATT | 16932 |
| 11 | AR11\_44 | GGTAAACAACAGCATCACCATAGTC | 16846 |
| 12 | BF12\_45 | AACATGTGACTGGACAAATGCTG | 16566 |
| 12 | BF12\_46 | ACTGACTTTAATGCAATTGCAACATG | 16546 |
| 12 | BR12\_47 | TACAGCAACTAGGTTAACACCTGTAG | 18374 |
| 12 | BR12\_48 | CACCCCTCGACATCGAAGC | 18302 |
| 13 | AF13\_49 | GTGGCAACTTTACAAGCTGAAAATG | 18025 |
| 13 | AF13\_50 | GACATACCTGGCATACCTAAGGAC | 18160 |
| 13 | AR13\_51 | GTTTAATGTTGCGCTTAGCCCAA | 19791 |
| 13 | AR13\_52 | GTAGTCCCAGATCACAGTATTAGCAG | 19859 |
| 14 | BF14\_53 | CGTGTATAACACGTTGCAATTTAGGT | 19454 |
| 14 | BF14\_54 | ACTTTGATGGACAACAGGGTGAA | 19661 |
| 14 | BR14\_55 | CGCGTGGTTTGCCAAGATAATTA | 21285 |
| 14 | BR14\_56 | CATAACCATCTATTTGTTCGCGTGG | 21301 |
| 15 | AF15\_57 | AGCTCATGGGACACTTCGC | 21203 |
| 15 | AF15\_58 | TTGGAGGTTCCGTGGCTATAAAG | 21146 |
| 15 | AR15\_59 | CGATTTGTCTGACTTCATCACCTC | 22770 |
| 15 | AR15\_60 | CTACCGGCCTGATAGATTTCAG | 22971 |
| 16 | BF16\_61 | TGCATCTGTTTATGCTTGGAACAG | 22603 |
| 16 | BF16\_62 | CTCTCTCAGAAACAAAGTGTACGTTG | 22446 |
| 16 | BR16\_63 | CACTTGCTGTGGAAGAAAGTGA | 24371 |
| 16 | BR16\_64 | GTTGACCACATCTTGAAGTTTTCCAA | 24396 |
| 17 | AF17\_65 | GGTGATTGCCTTGGTGATATTGC | 24074 |
| 17 | AF17\_66 | CTGTTTTGCCACCTTTGCTCA | 24138 |
| 17 | AR17\_67 | CCAGCAAAGAAAATAGTTGGCATC | 25816 |
| 17 | AR17\_68 | CGTAACAATTAGTATGCCAGCAAAGA | 25830 |
| 18 | BF18\_69 | TCCCTTTCGGATGGCTTATTGTT | 25514 |
| 18 | BF18\_70 | TGAAATCAAGGATGCTACTCCTTCAG | 25446 |
| 18 | BR18\_71 | GTTGTACCTCTAACACACTCTTGGTA | 27451 |
| 18 | BR18\_72 | CTCACAAGTAGCGAGTGTTATCAG | 27418 |
| 19 | AF19\_73 | ACGCTTTCTTATTACAAATTGGGAGC | 27045 |
| 19 | AF19\_74 | CGCTGTGACATCAAGGACCT | 26994 |
| 19 | AR19\_75 | TGGCAATGTTGTTCCTTGAGGAA | 28755 |
| 19 | AR19\_76 | CAGCCATTCTAGCAGGAGAAGTT | 28885 |
| 20 | BF20\_77 | TAGAGTATCATGACGTTCGTGTTGTT | 28219 |
| 20 | BF20\_78 | ACCCCGCATTACGTTTGGT | 28309 |
| 20 | BR20\_79 | TGGCTCTTTCAAGTCCTCCCT | 29700 |
| 20 | BR20\_80 | GCTCTTCCATATAGGCAGCTCTC | 29779 |
|  | nCoV-2019\_1\_LEFT3 | ACCAACCAACTTTCGATCTCTTGT | 31 |
|  | nCoV-2019\_98\_RIGHT3 | TTCTCCTAAGAAGCTATTAAAATCACATGG | 29866 |

**Footnotes** 1. Sequence listed 5' to 3'.

2. Position in SARS-CoV-2 reference genome GenBank NC\_045512

3. From original ARTIC primer set V.1(2).

**References**

1. Cotten M, Bugembe DL, Kaleebu P, Phan MVT. Alternate primers for whole-genome SARS-CoV-2 sequencing. Virus Evol. 2021 Feb 4;10.1093/ve/veab006.

2. Josh Quick. nCoV-2019 sequencing protocol. 2020; Available from: https://www.protocols.io/view/ncov-2019-sequencing-protocol-bbmuik6w