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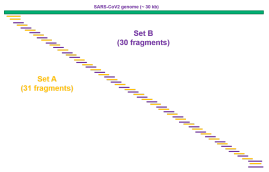
RTPCR Amplification of SARS-CoV2 Whole Genome for Illumina NGS



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Coronavirus Method De...

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Abstract

SUMMARY

This document describes the procedure for performing RTPCR amplification for next-generation sequencing (NGS) of whole genomes from SARS-CoV2 positive clinical samples. This methodology was employed at the Respiratory Virus Unit, Microbiology Services Colindale, Public Health England (RVU-PHE) to sequence the first SARS-CoV2 positive samples.

Reverse transcription (RT) is performed using random hexamer primers. PCR amplification is done using sequence-specific primers which amplify two sets of 30-31 overlapping amplicons (size 1.0 to 1.8 kb), each set independently covering the entire length of the MERS-CoV genome. Once amplicons are obtained, rough equimolar mixtures, clean-up, DNA quantitation and final dilutions are performed to prepare samples for library preparation for Illumina MiSeq next-generation sequencing (NGS).

Primers from set A do not overlap location of primers from set B, except for those binding at the end of the genome (see figure 1). Amplicon mixtures from each set are treated as separate samples throughout the procedure, including assembly. Merging (or comparison) of consensus sequences of both sets is performed at the end.

IMPORTANT: Please note that every amplicon is obtained in a separate PCR reaction and, if using both primer sets, there is a total of ~61 PCR reactions; this protocol is NOT a multiplex approach. We recommend using a 96-well plate per sample.

ADVANTAGES AND LIMITATIONS

The use of both primer sets allows for redundancy in whole genome (WG) coverage, which increases the chances of obtaining full genome sequences when samples have low viral load. Although we haven't formally determined the sensitivity of this assay (or limit of amplification of WG), we have obtained full coverage with samples with CT values of up to 38 (CT value obtained at RVU-PHE with RdRp gene detection within the SARS-CoV2 detection protocol as described by Corman et al. [1]). Variation in storage or transport conditions of samples may affect the integrity of viral nucleic acids.

Another advantage of this protocol is that, as primers from set A and B match different locations on the genome, direct comparison of the sequences obtained with each set allows for curation of primer-induced contamination of the sequences, although we deal with this with a specific step on the bioinformatic assembly pipeline (not described here).

Alternatively, when samples have a relatively good CT (<30) only set A may be used, simplifying the amplification and library steps.

The main limitation of this protocol is its unsuitability for high throughput processing of large batches of samples, although current efforts are being made to adapt the primers to a multiplex approach.

Materials

EQUIPMENT
PCR thermocycler for 96-well plates
Vortex
Pipettes with disposable filter tips
Ice bucket
Microcentrifuge and picofuge
Plate centrifuge
Mother E-base
96-well plates with adhesive seals
1.5ml and 0.2ml polypropylene tubes/strip tubes
Benchtop UV transilluminator (UVP)
Qubit Fluorometer (Life Technologies)
Qubit Assay Tubes (Life Technologies)
FrameStar FastPlate 96 (4titude)
Aluminum StarSeal (PCR) E2796-9792, StarLab

REAGENTS
SuperScript™ VILO™ cDNA Synthesis Kit (ThermoFisher, Invitrogen cat 11754050)
dNTP set 100mM (ThermoFisher, Invitrogen cat 10297018)
Oligonucleotide primers 100pmol/µl stocks (Eurofins) (see Appendix 3)
Platinum Taq DNA polymerase HiFi (ThermoFisher, Invitrogen cat 11304029)
Nuclease-free water (Severn Biotech)
1% or 2% E-gels 48-wells (invitrogen)
10x TBE buffer
DNA 1Kb plus ladder (Invitrogen cat 10787018)
BlueJuice 10X Gel loading buffer (ThermoFisher, Invitrogen cat10816015)
Qubit dsDNA HS Assay Kit (ThermoFisher, Invitrogen Q32854)
QIAquick PCR purification kit (cat 28106)

Safety warnings

Sample inactivation in lysis buffer should be performed at BSL3

1 A total amount of 140 µl of RNA (includes some excess) are needed to set up the necessary volume of RT reactions. RNA should be kept on ice or stored at -80 °C if not immediately used.

2 The RT reaction is as follows (Table 1):

	RT mix	x 1 (µl)	Bulk mix (x 14)
	Water	4.0	56
	5X VILO Reaction mix	4.0	56
	10X Superscript Enzyme mix	2.0	28
	RNA	10.0	140
	Final volume	**20.0**	**280**

Table 1

3 To ensure uniform heat distribution, we aliquot this mix into 7 × 0.2 (strip) tubes.
Cycling program:
25 °C 00:10:00
42 °C 01:00:00
85 °C 00:05:00
Hold at 4 °C
Keep cDNA on ice or store at -80 °C if not immediately used.

4 Combine primers according to Tables 1 and 2 (4.1) and dilute as follows to get a working primer dilution of 5 pmol/µl

Note

Preparation of primer mix pairs (working dilution 5 pmol/µl)
Use different colours of strip tubes, e.g. yellow strip tubes for SET A and purple strip tubes for SET B. Add 180 µl of water + 10µl of each Forward and Reverse primers at 100 pmol/µl, according to Appendix 2. Vortex to mix. Store at -20°C.

Thaw both sets of primer mixes, briefly vortex them and spin down. Using a multichannel pipette (volume 0.5 or 1 to 10 µl) load 4 µl of primer mixes to the corresponding wells of a 96-well PCR plate, according to Figure 2. Keep the PCR plate on a cooler.

Figure 2. SET A (yellow) and SET B (purple)

	1	2	3	4	5	6	7	8	9	10	11	12
A	1A	2A	3A	4A	5A	6A	7A	8A	9A	10A	11A	12A
B	13A	14A	15A	16A	17A	18A	19A	20A	21A	22A	23A	24A
C	25A	26A	27A	28A	29A	30A	31A					
D												
E	1B	2B	3B	4B	5B	6B	7B	8B	9B	10B	11B	12B
F	13B	14B	15B	16B	17B	18B	19B	20B	21B	22B	23B	24B
G	25B	26B	27B	28B	29B	30B						
H												

4.1

The position of primers on the nCOV genome sequence has been based on the sequence of strain hCoV-19/Wuhan/IVDC-HB-01/2019 (GISAID accession EPI_ISL_402119). We gratefully acknowledge the authors (Wenjie Tan et al), originating and submitting laboratory (National Institute for Viral Disease Control and Prevention, China CDC) of this sequence from GISAID's EpiCOV™ Database on which this protocol is based.

	Primer pair	Forward primer	Sequence	Position of 5'end	Reverse primer	Sequence	Position of 5'end	Amplicon size

	1A	ncov-1F	ATTAAAGGTTTATACCTTCCCAGGTAAC	1	ncov-1032R	TCAAAAGGTGTCTGCAATTCAT	1032	1031
	2A	ncov-15F	CCTTCCCAGGTAACAAACCA	15	ncov-1032R	TCAAAAGGTGTCTGCAATTCAT	1032	1017
	3A	ncov-693F	TTGACTTAGGCGACGAGCTT	693	ncov-2030R	CAGATGTGAACATCATAGCATCAA	2030	1337
	4A	ncov-1684F	CGCCATTATTTTGGCATCTT	1684	ncov-3019R	TGAAGCCAATTTAAACTCACCA	3019	1335
	5A	ncov-2665F	TGCCCTTGCACCTAATATGAT	2665	ncov-4010R	GGAACTTAGTTTCTTCCAGAGTTG	4010	1345
	6A	ncov-3686F	CACGAAGTTCTACTTGCACCA	3686	ncov-5015R	CAACTTGCGTGTGGAGGTTA	5015	1329
	7A	ncov-4667F	TCTCTCAAAGTGCCAGCTACAG	4667	ncov-6037R	GCTTGCGTTTGGATATGGTT	6037	1370
	8A	ncov-5687F	TCAGCACCACCTGCTCAGTA	5687	ncov-7021R	AACACCTAAAGCAGCGGTTG	7021	1334
	9A	ncov-6688F	TGCTAAGCCTTTTCTTAACAAAGTT	6688	ncov-8027R	CTGCAACTTCCGCACTATCA	8027	1339
	10A	ncov-7663F	TGATGAAGTTGCGAGAGACTTG	7663	ncov-9019R	TTCAGCAGCCAAAACACAAG	9019	1356
	11A	ncov-8700F	TTGATGGTGGTGTCACTCGT	8700	ncov-10033R	AGAGGTTTGTGGTGGTTGGT	10033	1333
	12A	ncov-9699F	TCATTTGTATTTCCACAAAGCA	9699	ncov-11071R	CAAAGACCATTGAGTACTCTGGA	11071	1372
	13A	ncov-10696F	TGGAGACAGGTGGTTTCTCA	10696	ncov-12030R	TGCATGGAAAGCAAAACAGA	12030	1334
	14A	ncov-11668F	CCGCTACTTTAGACTGACTCTTGG	11668	ncov-13050R	GCAGGCACTTCTGTTGCAT	13050	1382
	15A	ncov-12701F	CCTGTTGCACTACGACAGATG	12701	ncov-14043R	AATACCAGCATTTCGCATGG	14043	1342

	16A	ncov-13663F	CACACTTTCTCTAACTACCAACATGAA	13663	ncov-15028R	ATGCGAAAAGTGCATCTTGA	15028	1365
	17A	ncov-14663F	AAACTGTCAAACCCGGTAATTTT	14663	ncov-16023R	AGACACGAACCGTTCAATCA	16023	1360
	18A	ncov-15686F	TGCGTAAACATTTCTCAATGATG	15686	ncov-17038R	TTGCAACATTGCTAGAAAACTCA	17038	1352
	19A	ncov-16689F	TGCTACTGTACGTGAAGTGCTG	16689	ncov-18034R	AAGTTGCCACATTCCTACGTG	18034	1345
	20A	ncov-17694F	TGCAATTAACAGGCCACAAA	17694	ncov-19031R	TCGTGAAGAACTGGGAATTTG	19031	1337
	21A	ncov-18683F	CATGCTTTTCCACTGCTTCA	18683	ncov-20043R	AACACCATTACGGGCATTTC	20043	1360
	22A	ncov-19664F	TTGATGGACAACAGGGTGAA	19664	ncov-21231R	GTCCACCATGCGAAGTGTC	21231	1567
	23A	ncov-20863F	CATTTTGGTGCTGGTTCTGA	20863	ncov-22226R	CGAAAAACCCTGAGGGAGAT	22226	1363
	24A	ncov-21896F	TTCGAAGACCCAGTCCCTAC	21896	ncov-23214R	CACCTGTGCCTGTTAAACCA	23214	1318
	25A	ncov-22883F	TCTTGATTCTAAGGTTGGTGGT	22883	ncov-24231R	CACCAAAGGTCCAACCAGAA	24231	1348
	26A	ncov-23850F	TTAAACCGTGCTTTAACTGGAATA	23850	ncov-25243R	ATGGCAATCAAGCCAGCTAT	25243	1393
	27A	ncov-24858F	GGCACACACTGGTTTGTAACAC	24858	ncov-26224R	TGCTTACAAAGGCACGCTAGT	26224	1366
	28A	ncov-25886F	TCTTCAATTGTCATTACTTCAGGTG	25886	ncov-27227R	CCTGAAAGTCAACGAGATGAAA	27227	1341
	29A	ncov-26889F	GCCACTCCATGGCACTATTC	26889	ncov-28191R	TTCATAGAACGAACAACGCACT	28191	1302
	30A	ncov-27876F	TTGTCACGCCTAAACGAACA	27876	ncov-29226R	ACATTCCGAAGAACGCTGAA	29226	1350

	31 A	ncov-28871 F	AGGCAGCAGTAG GGGAACTT	288 71	ncov-29848 R	AAAATCACAT GGGGATAGCA	2984 8	977

Table 2. Set A: Primer sequences & combinations

	Prime r pair	Forward primer	Sequence	Position of 5'end	Reverse primer	Sequence	Position of 5'end	Amplicon size
	1B	ncov-193F	CTTACGGTTT CGTCCGTGTT	193	ncov-1541R	GTGGAACCCAAT AGGCACAC	1541	134 8
	2B	ncov-1185F	CGTCACCAAA TGAATGCAAC	1185	ncov-2495R	TTCTGTGGGAAG TGTTTCTCC	2495	1310
	3B	ncov-2193F	GAGACGGTTG GGAAATTGTT	2193	ncov-3523R	TTCAACTTGCAT GGCATTGT	3523	133 0
	4B	ncov-3172F	TCAACCTGAA GAAGAGCAAG AA	3172	ncov-4525R	AGCACCATAATC AACCACACC	4525	135 3
	5B	ncov-4173F	CTAAAAAGGC TGGTGGCACT	4173	ncov-5550R	AGGGTTGTCTGC TGTTGTCC	5550	137 7
	6B	ncov-5192F	CTGGGTAGGT ACATGTCAGC A	5192	ncov-6548R	GATCTGTGTGGC CAACCTCT	6548	135 6
	7B	ncov-6192F	CACCCTCTTT TAAGAAAGGA GCTA	6192	ncov-7537R	TGTACATTCGAC TCTTGTTGCTC	7537	134 5
	8B	ncov-7194F	CCATTTCATC TTTTAAATGG GATT	7194	ncov-8554R	CCACCCTTAAGT GCTATCTTTG	8554	136 0
	9B	ncov-8179F	GCAAGGGTTT GTTGATTCAG A	8179	ncov-9506R	TAAAGGCAACTA CATGACTGTATT CAC	9506	132 7
	10B	ncov-9169F	CCTTGAAGGT TCTGTTAGAG TGG	9169	ncov-10456 R	GAAATTGGGCCT CATAGCAC	1045 6	128 7
	11B	ncov-10080 F	CATCTGGTAA AGTTGAGGGT TGT	1008 0	ncov-11533R	TCTGGCCAAAA ACATGACAG	1153 3	145 3

	12B	ncov-11189F	CCTTCTCTTGCCACTGTAGCTT	11189	ncov-12547R	TGCTGATGCATAAGTAAATGTTG	12547	1358
	13B	ncov-12163F	GGCTGTTGCTAATGGTGATTC	12163	ncov-13547R	TCAAAAGCCCTGTATACGACA	13547	1384
	14B	ncov-13191F	CAGTTACACCGGAAGCCAAT	13191	ncov-14513R	TCCTGATTATGTACAACACCTAGCTC	14513	1322
	15B	ncov-14183F	CCAGGGCTTTAACTGCAGAG	14183	ncov-15544R	CCGTGACAGCTTGACAAATG	15544	1361
	16B	ncov-15167F	TGAAATCAATAGCCGCCACT	15167	ncov-16511R	AAACCAAAAACTTGTCCATTAGC	16511	1344
	17B	ncov-16176F	TTCAAGGTATTGGGAACCTGA	16176	ncov-17551R	CGAGGAACATGTCTGGACCTA	17551	1375
	18B	ncov-17171F	CCGCTGTTGATGCACTATGT	17171	ncov-18533R	TTTATACGCACTACATTCCAAGG	18533	1362
	19B	ncov-18165F	ACCTGGCATACCTAAGGACA	18165	ncov-19542R	CATCATGTTATAAGCATCGAGATACA	19542	1377
	20B	ncov-19191F	TTGGAATTGCAATGTCGATAG	19191	ncov-20478R	ACCTGTTTGCGCATCTGTTA	20478	1287
	21B	ncov-19966F	TGTGCACCACTCACTGTCTT	19966	ncov-21730R	AAGAACAAGTCCTGAGTTGAATG	21730	1764
	22B	ncov-21360F	CAAATCCAATTCAGTTGTCTTCC	21360	ncov-22705R	CCATAACACTTAAAAGTGGAAAATGA	22705	1345
	23B	ncov-22363F	TGGGTTATCTTCAACCTAGGACTT	22363	ncov-23713R	TTTGTGGGTATGGCAATAGAGTT	23713	1350
	24B	ncov-23384F	GGTTGCTGTTCTTTATCAGGATG	23384	ncov-24735R	GAGGTGCTGACTGAGGGAAG	24735	1351
	25B	ncov-24369F	GACTCACTTTCTTCCACAGCAA	24369	ncov-25712R	AGAGAAAAGGGGCTTCAAGG	25712	1343
	26B	ncov-25354F	TGCTCAAAGGAGTCAAATTACATTA	25354	ncov-26734R	AAACAGCAGCAAGCACAAAA	26734	1380

	27 B	ncov-26361 F	TGTGTGCGTACTGCTGCAA	26361	ncov-27712 R	TGCCGCAACAATAAGAAAAA	27712	1351
	28 B	ncov-27361F	TGAAGAGCAACCAATGGAGA	27361	ncov-28719 R	GGGTGCCAATGTGATCTTTT	28719	1358
	29 B	ncov-28397 F	GCCCCAAGGTTTACCCAATA	28397	ncov-29734 R	GGTGAAAATGTGGTGGCTCT	29734	1337
	30 B	ncov-28397 F	GCCCCAAGGTTTACCCAATA	28397	ncov-29871 R	TGATTTTAATAGCTTCTTAGGAGAATGAC	29871	1474

Table 3. Set B: Primer sequences & combinations

5 Prepare a PCR mix following *Table 4*. Thaw and briefly vortex all the required reagents except enzymes. Prepare a mix for ~70 samples (enough for both sets A and B).

Note

Preparation of working dilution of dNTPs (100 mM dNTP Set, Invitrogen, cat 10297018) Mix the four tubes of dNTPs in one bijou tube. Add 1.5 ml of water to reach concentration of 10 mM (Final volume 2.5 ml). Aliquot in 2 ml tubes with screw cap.

	PCR mix	**X 1 (µl)**	**X 70**
	Water	35.8	2,506
	10X High Fidelity Buffer (Invitrogen)	5.0	350
	10 mM dNTPs mix (Invitrogen) See Appendix 1	1.0	70
	50mM MgSO4 (Invitrogen)	2.0	140
	Platinum Taq DNA Polymerase 5 U/µl (Invitrogen)	0.2	14
	Final volume	**44.0**	**3,080**

Table 4

6 Mix with a gentle vortex. Keep on ice. Dispense 44µl of the PCR mix into every well with primers of rows A, B, C, E, F and G.

7 Pull together all 7 cDNA strip tubes into one and load 4 µl of cDNA in each well.

8 Cover the 96-well plate with an adhesive lid or strip caps. Spin briefly the plate.

9 Transfer the 96-well plate to a thermal cycler.
Amplify using the following cycling conditions:
95 °C 00:10:00
followed by 35 cycles of:
94 °C 00:00:30
52 °C 00:00:30
68 °C 00:05:00
Hold at 15 °C

10 **Working in a post-PCR area**

Use 1% or 2% E-gels 48 wells (Invitrogen) for visualisation of the bands, following manufacturer's instructions. For each sample prepare 1× 96-well microtiter plate to mix Loading buffer

11 Prepare diluted Blue Juice loading buffer. Add 10 µl of diluted Blue Juice to every well of rows A, B, C, E, F and G (mirroring the PCR plate) Use multichannel pipettes.

Note

Preparation of Blue Juice diluted 1/25 or 1/50
Mix 10 ml of buffer TBE with 400 µl (1/25) or 200 µl (1/50) µl of undiluted Blue Juice.

12 Add 5 µl of sample from the PCR plate to the microtiter plate. Use multichannel pipettes.

13 Separately, prepare enough mixture of diluted Blue Juice (14µl diluted Blue Juice + 1µl of 1 Kb Plus Ladder) to add to wells marked with M.

14 Transfer 15 µl of samples and ladder dilutions into the E-gels. Load 15 µl of diluted Blue Juice in empty wells (no wells should be empty). Run for 20 minutes and view under shortwave U.V.

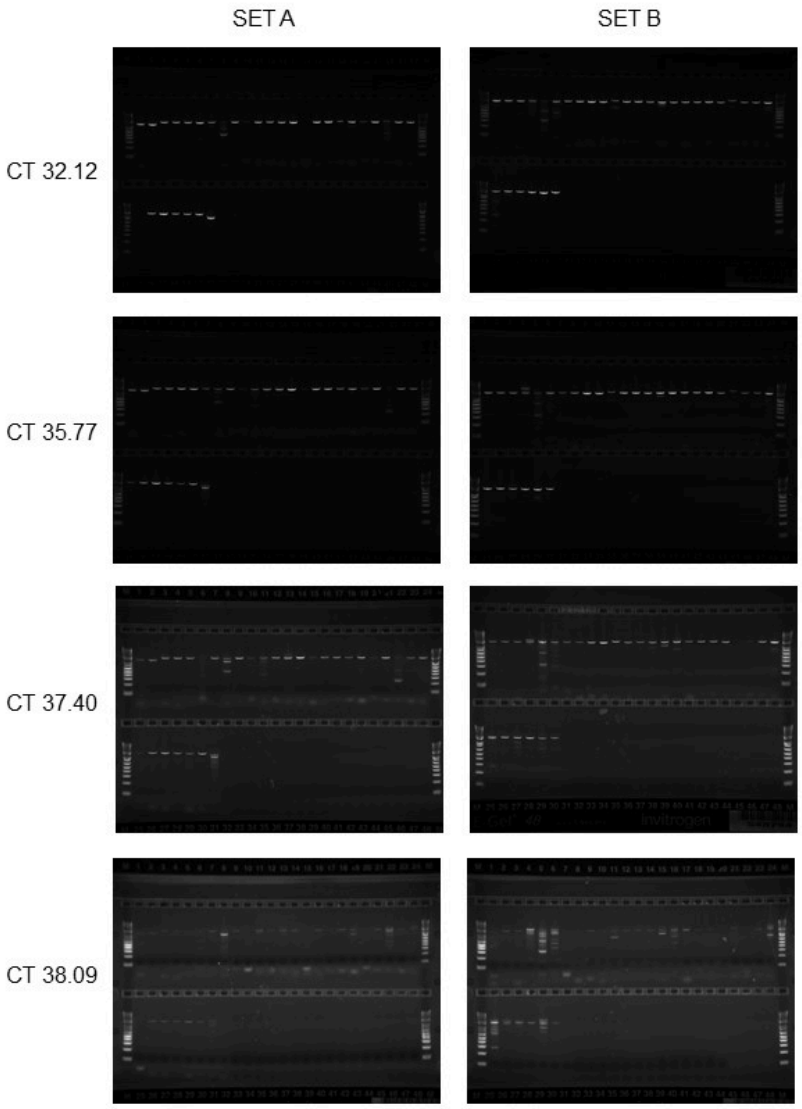
Note

Alternative using multichannel pipette (tips will fit into every other well of 48 wells E-gels): if two samples are run in parallel, load amplicons from sample A in odd wells, and amplicons from sample 2 in even wells. Repeat the procedure with rows B to F. Load 15 µl of the mixture of Blue Juice + Ladder into the wells marked with M at the beginning and end of each gel row. In that way, amplicon 1 of sample A will run next to amplicon 1 of sample B and similarly for each amplicon.

14.1 Some examples of gel pictures are shown here:

Some gel pictures (4 samples)

CT values were obtained with the RdRp gene detection described by Corman et al. (1)



15 To prepare equimolar mixes, ideally PCR reactions should be first cleaned up using e.g. silica columns or magnetic beads and then quantified, but the amount or PCR reactions makes this very impractical, unless using automated methods. Instead, rough equimolar mixes are prepared based on brightness of gel bands: 3 µl of a strong band, 5 µl of a

normal band, and 10 µl of a weak or non-visible band. Mix the appropriate amount of each amplicon from SET A in
an Eppendorf tube (final volume 60 to 300µl). Do not include the negative control. Repeat the procedure for SET B amplicons in a separate tube.

16 Perform PCR product purification using QIAquick PCR purification kit columns, following manufacturer's instructions. Perform repeated additions and spins of the sample until all of it has gone through the column. Elute the purified DNA in 50 µl of molecular grade water.

17 Run quantitation of DNA using the Qubit dsDNA HS reagent (Life Technologies) and the Qubit fluorometer, according to manufacturer's instructions.

18 Dilute the samples to the appropriate concentration for Illumina library preparation using Nextera XT (not described here).