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Enhanced QIAseq DIRECT SARS-CoV-2 Kit for Illumina MiSeq V.2

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GenomeTrakr Coronavirus Method Development Community

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- This SARS-CoV-2 targeted, a rapid viral RNA library preparation kit compatible with Illumina sequencers, provides an approximately five hour turn-around time from viral RNA sample to sequencing. The kit includes cDNA synthesis, SARS-CoV-2 enrichment, and library amplification and indexing reagents. The Enhanced protocol removes the need for normalization and quantification prior to amplification and indexing. RNA input is 5 µl, regardless of SC2 viral load in the sample extract. Amplification is performed using two primer pools that produce overlapping amplicons that are approximately 250 bp in size; as a result, no fragmentation is necessary prior to sequencing on Illumina platforms. 10 bp unique dual indexes (UDIs) are provided in the kit, and with a cycle read length of 149 for each paired-end read, the sequencing time is just over 28 hours.

DOI

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QIASeq Direct SARS COV-2, Miseq, SARSCOV-2 Enrichment

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Enhanced QIAseq DIRECT SARS-CoV-2 Kit

IMPORTANT: THIS PROTOCOL USES QIAseq DIRECT SARS-CoV-2 Enhancer. To use this protocol, you purchase QIAseq DIRECT SARS-CoV-2 Kit and QIAseq DIRECT SARS-CoV-2 Enhancer.

QIAseq DIRECT SARS-CoV-2 Kit Catalog no.

A (333891), B (333892),
C (333893), D (333894),
E (333886), F (333887),
G (333888), H (333889)

Note: You would need only one index kit with the QIAseq kit. For example if you need QIAseq DIRECT SARS-CoV-2 Kit with index kit A, you will place the order for catalog no: 333891

Components of A (333891), B (333892), C (333893), D (333894), E (333886), F (333887), G (333888), H (333889)

RP Primer, 12 µl 1 tube
EZ Reverse Transcriptase 1 tube
RNase Inhibitor 1 tube
Multimodal RT Buffer, 5x 1 tube
QIAseq 2X HiFi MM 4 tubes (*not used in the Enhanced QIAseq DIRECT SARS-CoV-2 Kit protocol*)
DIRECT SARS-CoV-2 Pool 1 1 tube
DIRECT SARS-CoV-2 Pool 2 1 tube
QIAseq DIRECT UDI Set Variable (A, B, C, D, E, F, G, or H)
Nuclease-Free Water 1 bottle
QIAseq Beads 1 bottle

QIAseq DIRECT SARS-CoV-2 Enhancer Catalog no. 333884

Components

RP Primer, 96 µl 1 tube
UPCR Buffer, 5x 2 tubes
QN Taq Polymerase 1 tube

Note: RP Primer, 12 µl (from QIAseq DIRECT SARS-CoV-2 Kit) and RP Primer, 96 µl (from QIAseq DIRECT SARS-CoV-2 Enhancer) are the same formulation and can be combined/used together.

cDNA Synthesis Procedure

Citation: Padmini Ramachandran, Tamara Walsky, Amanda Windsor, Maria Hoffmann, Chris Grim Enhanced QIAseq DIRECT SARS-CoV-2 Kit for Illumina MiSeq https://dx.doi.org/10.17504/protocols.io.b4htqt6n

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cDNA Synthesis Procedure: Important points before starting

- **Use 5 µl viral RNA input, regardless of viral titer.**
- **Set up cDNA synthesis reaction on ice.**
- **Use a thermal cycler with a heated lid.**

2 Thaw template RNA on ice. Gently yet thoroughly mix, then briefly centrifuge to collect residual liquid from the sides of the tubes and return to ice.

Prepare the reagents required for cDNA synthesis.

- **Thaw RP Primer (random hexamer), Multimodal RT Buffer, and nuclease-free water at room temperature (15–25°C).**
- **Mix thoroughly and then briefly centrifuge to collect residual liquid from the sides of the tubes. Prolonged storage of Multimodal RT Buffer at -20°C can cause white precipitate to form. This is normal. Just ensure to mix until precipitate is fully dissolved.**
- **Thaw RNase Inhibitor and EZ Reverse Transcriptase on ice. Mix by flicking the tubes, briefly centrifuge, and return to ice. After use, immediately return the enzymes to the freezer.**

3 While keeping reagents on ice, prepare the cDNA reaction according to the table below. Briefly centrifuge, “gently yet thoroughly” vortex to mix, and centrifuge briefly again. For multiple reactions, prepare the calculated volume for all reactions, plus 10%.

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Components	Volume/reaction(ul)
EZ Reverse Transcriptase	1
Multimodal RT Buffer, 5x	4
Nuclease-free water	8
RNase Inhibitor	1
RP Primer	1
Template RNA	5
Total volume	**20**

4 **cDNA synthesis incubation.** Incubate as described below.

A	B	C
Step	**Temperature (C)**	**Incubation time (min)**
1	25	10
2	42	50
3	85	5
4	4	Hold

5 Proceed to" SARS-CoV-2 Enrichment Procedure". Alternatively, the samples can be stored at−30 to−15°C in a constant-temperature freeze.

6

SARS-CoV-2 Enrichment Procedure: Important points before starting.

- **Use a thermal cycler with heated lid.**
- **Set up the reactions on ice.**
- **Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color. If a delay in the protocol occurs, simply vortex the beads.**
- **Important: Prepare fresh 80% ethanol daily.**

SARS-CoV-2 Enrichment Procedure

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7 **Prepare the reagents required for target enrichment.**

- **Thaw the DIRECT SARS-CoV-2 Pool 1, DIRECT SARS-CoV-2 Pool 2, and UPCR Buffer, 5x, at room temperature. “Gently yet thoroughly” vortex to mix, and then centrifuge briefly.**
- **Thaw the QN Taq Polymerase on ice. Mix by flicking the tubes, and then centrifuge briefly.**

8 While keeping reagents on ice, set up Pool 1 and Pool 2 reactions for each sample, as described below. Briefly centrifuge, “gently yet thoroughly” vortex to mix, and centrifuge briefly again.

A	B	C
Component	**Pool 1: Volume/reaction (µl)**	**Pool 2: Volume/reaction (µl)**
cDNA from “Protocol: Enhanced QIAseq DIRECT”	8	8
DIRECT SARS-CoV-2 Pool 1	2	—
DIRECT SARS-CoV-2 Pool 2	—	2
UPCR Buffer, 5x	5	5
QN Taq Polymerase	1	1
Nuclease-free water	9	9
Total volume	**25**	**25**

9

Important: For samples with a broad/unknown range of Ct values (i.e., above and below Ct = 32) incubate as described in Table 9.1. For samples with Ct > 32, also incubate as described in Table 9.1. For samples with Ct value < 32, incubate as described in Table 9.2.

9.1 Target enrichment cycling: Samples with broad/unknown range Ct values and samples with Ct > 32

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A	B	C	D
Step	**Time**	**Temperature (°C)**	**Number of cycles**
Heat activation	2 min	98	1
Denaturation	20 s	98	4
Annealing/extension	5 min	63	
Denaturation	20 s	98	29
Annealing/extension	3 min	63	
Final Hold	∞	4	Hold

9.2 Target enrichment cycling: Samples with Ct < 32

A	B	C	D
Step	**Time**	**Temperature (°C)**	**Number of cycles**
Heat activation	2 min	98	1
Denaturation	20 s	98	4
Annealing/extension	5 min	63	
Denaturation	20 s	98	20
Annealing/extension	3 min	63	
Final Hold	∞	4	Hold

10 After amplification, combine the entire contents of “Pool 1” and “Pool 2” PCR reactions for each biological sample into a single well of a plate, giving a volume of 50 µl.

11 Add 50 µl QIAseq Beads to each 50 µl combined sample. Briefly centrifuge ,“gently yet thoroughly” vortex to mix, and centrifuge briefly again.

Note: Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color.

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12 Incubate for 5min at room temperature.

13 Centrifuge the plate until the beads are pelleted (2 min), and then place the plate onto a magnetic rack. After the solution has cleared (2 min or longer), carefully remove and discard the supernatant.

A- Top left: After pooling PCR rxns, add **50 µl room temp QIAseq Beads** to each 50 µl rxn and perform bead clean up.

B: Top right: Example of beads beginning to pellet.

C: Bottom left: Not quite pelleted. **NOTE**: At this point, ~2 min should do it.

D. Fully pelleted beads. **NOTE:** Solution is completely clear.

Important: Do not discard the beads, as they contain the DNA of interest.

Tip: It may be valuable to discard the supernatant twice. The contents settle after the first discard.

14 With the plate still on the magnetic stand, add 200 µl of 80% ethanol. Wait 1 min and carefully remove and discard the wash. Repeat the wash, for a total of 2 ethanol washes. Remove excess ethanol as much as possible.

Important: To, completely remove all traces of the ethanol wash after the second wash, immediately briefly centrifuge and return the tubes to the magnetic stand. Remove the ethanol with a 200 µl pipette first, and then use a 10 µl pipette to remove any residual ethanol. This should be done quickly.

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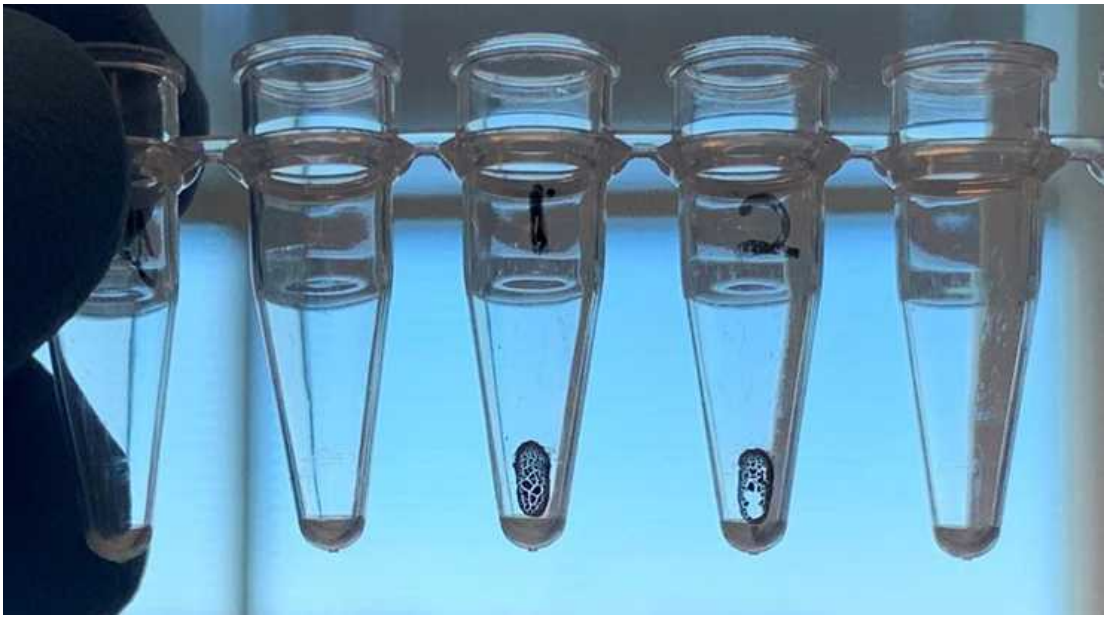
15 With the plate still on the magnetic stand, air-dry at room temperature for 4–8 min (until the beads start to crack and pellet loses its shine).



- **Air dry the beads until ALL EtOH is removed and the beads appear cracked (see image)**
- **To check pellets, turn tube around and look at pellet from back. Holding tubes in front of a sheet of white paper increases ability to visualize cracks.**

16 Remove the plate from the magnetic stand and elute the DNA from the beads by adding 30 µl nuclease-free water. “Gently yet thoroughly” vortex (triturate if necessary) to mix, briefly centrifuge, incubate for 2 min.

17 Centrifuge the plate until the beads are pelleted (2 min), and then return the plate to the magnetic rack until the solution has cleared.

18 Transfer 28 µl to a clean plate. This is now “enriched SARS-CoV-2”.

19 **Important Notes:**

- **We are adding tails ONLY to SARS-CoV-2 sequence in the subsequent Library Amplification and Indexing Procedure, and that will keep all the contaminants at bay when you perform your final amplification.**

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- **So, after you transferred the 28 µl of supernatant from your elution, this is a good time to get a feel for what you have if you want to do a mid-protocol check.**
- **Use a high sensitivity Qubit (for example) and check the concentration of the enrichment reactions after bead cleanup.**
- **With wastewater samples anything that is 1ng/µl and above (even down to 0.5ng/µl with high Ct values) is a success and should definitely move on to final amplification!**

20 Proceed to "Library Amplification and Indexing Procedure". Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

Library Amplification and Indexing Procedure

21

Library Amplification and Indexing Procedure: Important points before starting

- **The QIAseq DIRECT UDI Sets have pierceable foil seals, and the indexes must be pipetted from the plate into separate reaction plates. To prevent cross-contamination, each well is single use.**
- **Set up the reactions on ice.**
- **Use a thermal cycler with a heated lid.**
- **Equilibrate the QIAseq Beads to room temperature (15–25°C) for 20–30 min before use.**
- **Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color. If a delay in the protocol occurs, simply vortex the beads.**
- **Important: Prepare fresh 80% ethanol daily.**

22

Prepare the reagents required for library amplification and indexing.

- **Thaw the QIAseq DIRECT UDI Index Set A, B, C, or D, as well as the UPCR Buffer, 5x, at room temperature. "Gently yet thoroughly" vortex to mix, and**

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then centrifuge briefly.

- **Thaw the QN Taq Polymerase. Mix by flicking the tubes, and then centrifuge briefly.**

23 For samples with a broad/unknown range of Ct values (i.e., above and below Ct = 32) incubate as described in Table 23.1. For samples with Ct > 32, also incubate as described in Table 23.1. For samples with Ct value < 32, incubate as described in Table 23.2.

23.1 Library amplification/indexing cycling conditions: Samples with broad/unknown range Ct values and samples with Ct > 32

A	B	C	D
Step	**Temperature (°C)**	**Time**	**Number of cycles**
Initial denaturation	98	2 min	1
3-step cycling			
Denaturation	98	20 s	
Annealing	60	30 s	11
Extension	72	30 s	
Final extension	72	1 min	1
Final Hold	4	∞	Hold

23.2 Library amplification/indexing cycling conditions: Samples with Ct < 32

A	B	C	D
Step	**Temperature (°C)**	**Time**	**Number of cycles**
Initial denaturation	98	2 min	1
3-step cycling			
Denaturation	98	20 s	
Annealing	60	30 s	14
Extension	72	30 s	
Final extension	72	1 min	1
Hold	4	∞	Hold

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24 On ice, prepare the library amplification and indexing reaction according to Table in Step 25. Briefly centrifuge, “gently yet thoroughly” vortex to mix, and centrifuge briefly again.

Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than what is required for the total number of reactions.

25 **Reaction mix for library amplification and indexing**

A	B
Component	**Volume/reaction**
“Enriched SARS-CoV-2” sample	24 µl
Index from QIAseq DIRECT UDI index plate (A, B, C, or D) Plate	2 µl
Nuclease-free water	12 µl
UPCR Buffer 5x	10 µl
QN Taq Polymerase	2 µl
Total reaction volume	**50 µl**

26 Transfer the plate to the thermal cycler and start the program.

27 Once PCR is complete, add 40 µl of resuspended QIAseq Beads to each 50 µl reaction. Briefly centrifuge, “gently yet thoroughly” vortex to mix, and centrifuge briefly again.

Note: Vortex the QIAseq Beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown color.

28 Incubate the mixture for 5 min at room temperature.

29 Centrifuge the plate until the beads are pelleted (2 min), and then place the plate onto a magnetic rack. After the solution has cleared (2 min or longer), carefully remove and discard

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the supernatant.

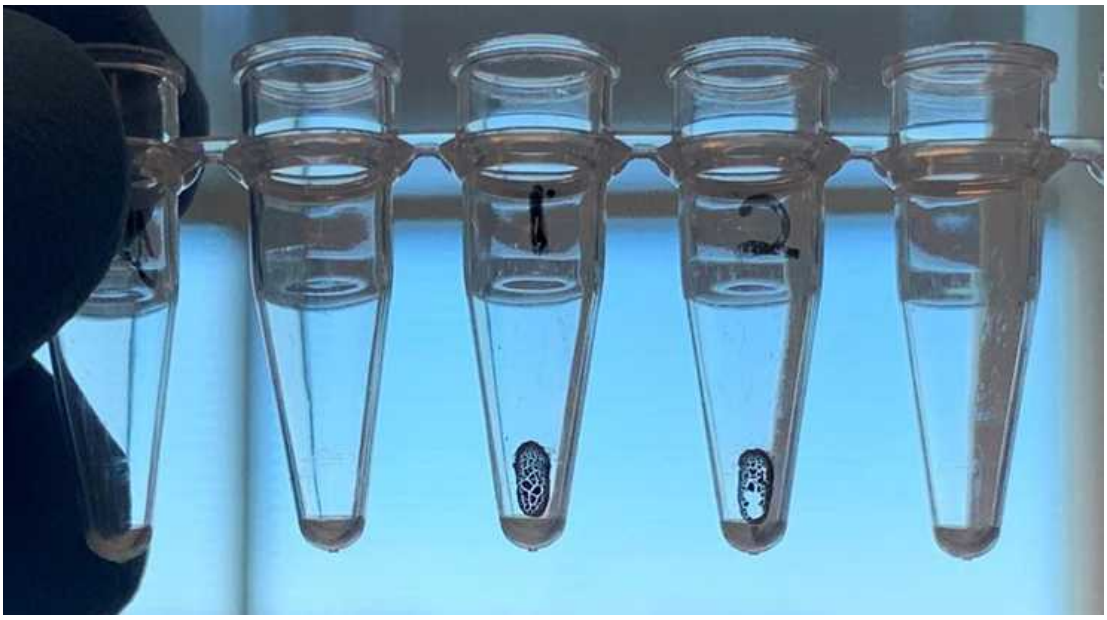
Important: Do not discard the beads as they contain the DNA of interest.

Tip: It may be valuable to discard the supernatant twice. The contents settle after the first discard.

30 With the plate still on the magnetic stand, add 200 µl of 80% ethanol. Wait 1 min and carefully remove and discard the wash. Repeat the wash, for a total of 2 ethanol washes. Remove excess ethanol as much as possible.

Important: To completely remove all traces of the ethanol wash after the second wash, immediately briefly centrifuge and return the plate to the magnetic stand. Remove the ethanol with a 200 µl pipette first, and then use a 10 µl pipette to remove any residual ethanol. This should be done quickly.

31 With the plate still on the magnetic stand, air-dry at room temperature for 4–8 min (until the beads start to crack and pellet loses its shine).



- **Air dry the beads until ALL EtOH is removed and the beads appear cracked (see image).**
- **To check pellets, turn tube around and look at pellet from back. Holding tubes in front of a sheet of white paper increases ability to visualize cracks.**

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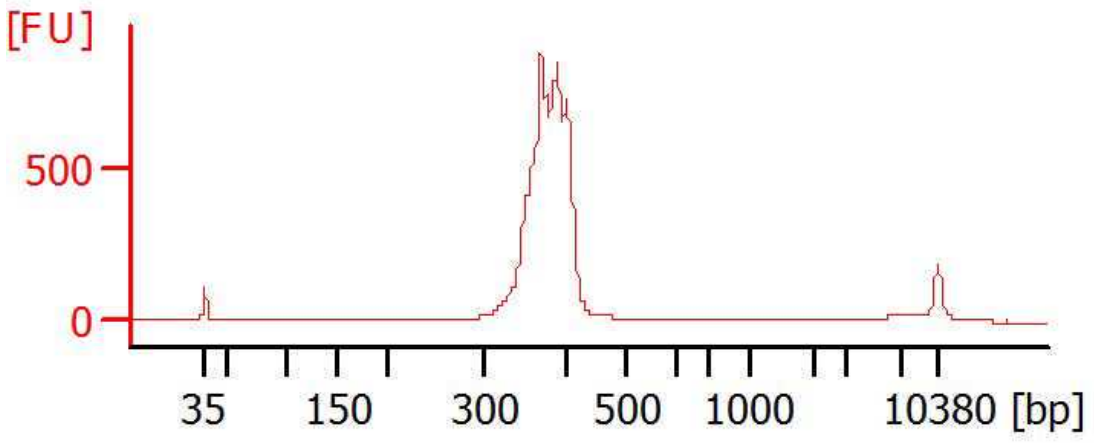
32 Remove the plate from the magnetic stand and elute the DNA from the beads by adding 25 µl nuclease-free water. Vigorously vortex (triturate if necessary) to mix, briefly centrifuge, and incubate for 2 min.

33 Centrifuge the plate until the beads are pelleted (2 min), and then return the plate to the magnetic rack until the solution has cleared (2 min or longer).

34 Transfer 23 µl to a clean plate. This is the “SARS-CoV-2 library”. If not proceeding immediately, the sample can be stored at –30 to –15°C.

35 Quantify the library using Qubit HS assay kit.

36 Assess the quality of the library using a Bioanalyzer, TapeStation (D1000 tape), or Fragment Analyzer. Check for the expected size distribution of library fragments. An example library is shown here.



Quantification, Normalization and Pooling of Libraries

37 Using the attached Post library worksheet, calculate the nM based on the Qubit concentration (ng/ul) and the Tape station (bp) profile. Normalize the “SARS-CoV-2 library”, to 4 nM. Quantify the final pool at the end.

Postlibraryworksheet.xlsx

38 Proceed to loading on to MiSeq (150x 150 cycle) using the attached Sample Sheet.

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Alternatively, the purified “SARS-CoV-2 library” can be safely stored at –30 to –15°C in a constant-temperature freezer until ready to use for sequencing. **SampleSheet.csv**

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