January 2022

QIAseq® DIRECT SARS-CoV-2 Handbook

Targeted whole viral genome library preparation direct from purified RNA

Sample to Insight

QIAGEN

Contents

Kit Contents

QIAseq DIRECT SARS-CoV-2 Kit	
Catalog no.	**A (333891), B (333892), C (333893), D (333894), E (333886), F (333887), G (333888), H (333889)**
No. of reactions	**96**
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
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

* RP Primer, 12 µl, and RP Primer, 96 µl, are the same formulation and can be combined/used together, both in "Protocol: Enhanced QIAseq DIRECT" and "Appendix B: Legacy QIAseq DIRECT Protocol".

† QIAseq 2X HiFi MM is used in "Appendix B: Legacy QIAseq DIRECT Protocol".

QIAseq DIRECT SARS-CoV-2 HT Catalog no. Number of reactions	A–D (333898), E-H (333899) 384
RP Primer*	4 tubes
EZ Reverse Transcriptase	4 tubes
RNase Inhibitor	4 tubes
Multimodal RT Buffer, 5x	4 tubes
QIAseq 2X HiFi MM†	16 tubes
DIRECT SARS-CoV-2 Pool 1	4 tubes
DIRECT SARS-CoV-2 Pool 2	4 tubes
QIAseq DIRECT UDI Set A–D or E–H	A–D (A, B, C, and D) or E–H (E, F, G, and H)
Nuclease-Free Water	4 bottles
QIAseq Beads	4 bottles

* RP Primer, 12 µl, and RP Primer, 96 µl, are the same formulation and can be combined/used together, both in "Protocol: Enhanced QIAseq DIRECT" and "Appendix B: Legacy QIAseq DIRECT Protocol".

† QIAseq 2X HiFi MM is used in "Appendix B: Legacy QIAseq DIRECT Protocol".

QIAseq DIRECT SARS-CoV-2 Enhancer* Catalog no. Number of reactions	333884 96 Reactions
RP Primer, 96 µl†	1 tube
UPCR Buffer, 5x‡	2 tubes
QN Taq Polymerase‡	1 tube

* The QIAseq DIRECT SARS-CoV-2 Enhancer is purchased separately from the QIAseq DIRECT SARS-CoV-2 Library Kit.

† RP Primer, 12 µl, and RP Primer, 96 µl are the same formulation and can be combined/used together, both in "Protocol: Enhanced QIAseq DIRECT" and "Appendix B: Legacy QIAseq DIRECT Protocol".

‡ UPCR Buffer, 5x, and QN Taq Polymerase are used in "Protocol: Enhanced QIAseq DIRECT".

The QIAseq DIRECT SARS-CoV-2 Enhancer is purchased separately from the QIAseq DIRECT SARS-CoV-2 Library Kit.

Shipping and Storage

The QIAseq DIRECT SARS-CoV-2 Kit is shipped in two boxes. Box 1 (enzymes, reagents, and UDI plate) are shipped on dry ice or blue ice and should be stored immediately upon receipt at –30 to –15°C. Box 2 (QIAseq Beads) is shipped on cold packs and should be stored immediately upon receipt at 2 to 8°C.

The QIAseq DIRECT SARS-CoV-2 Enhancer is shipped on dry ice or blue ice and should be stored immediately upon receipt at –30 to –15°C.

Intended Use

The QIAseq DIRECT SARS-CoV-2 Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the QIAseq DIRECT SARS-CoV-2 Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

This kit is specially designed to aid in the research of the SARS-CoV-2 virus, which is the causative agent of coronavirus disease 2019 (COVID-19). Viruses consist of nucleic acid (viral genome) and a limited number of proteins that aid with entry into the host cells, replication of the genome, and production of virions. While viral genomes can be comprised of RNA or DNA, SARS-CoV-2 is encoded by RNA. The size of the entire SARS-CoV-2 virus genome is under 30 kb and can be mixed with host RNA when isolating from a human sample, making it challenging to reconstruct the whole genome of the virus.

While next-generation sequencing (NGS) has become a vital tool, streamlined library preparation solutions remain elusive for SARS-CoV-2 assessment. The QIAseq DIRECT SARS-CoV-2 Kit represents a rapid library prep, enabling high-throughput SARS-CoV-2 mutation surveillance on Illumina® instruments.

Principle and procedure

The enhanced QIAseq DIRECT SARS-CoV-2 protocol utilizes a streamlined, four-hour workflow for enrichment and library prep of the SARS-CoV-2 virus genome (Figure 1). Compared to the legacy QIAseq DIRECT SARS-CoV-2, the enhanced workflow improves robustness, uniformity of coverage, and removes quantification/normalization prior to library amplification and indexing.

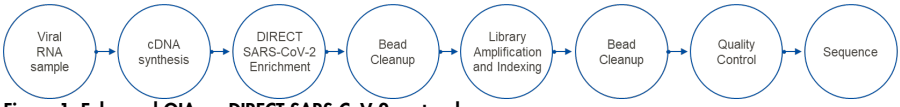

Figure 1. Enhanced QIAseq DIRECT SARS-CoV-2 protocol.

cDNA synthesis and SARS-CoV-2 enrichment

The QIAseq DIRECT SARS-CoV-2 workflow begins with random-primed cDNA synthesis (no rRNA depletion or poly-A selection is required). This reaction is flexible with regard to input RNA; 5 µl viral RNA input is recommended as a starting point, regardless of viral titer.

Following cDNA synthesis, multiplexed primer pools are used to prepare two pools of approximately 225–275 bp QIAseq DIRECT SARS-CoV-2 overlapping amplicons. The two enriched pools per sample are then pooled into a single tube and purified using a QIAseq Bead cleanup.

Library amplification and sample indexing

SARS-CoV-2 enriched samples are amplified and sample-indexed. During this reaction, unique dual indexes (UDIs) are added to the samples. UDIs effectively mitigate the risk of read misassignment due to index hopping. This is enabled by filtering misassigned reads during the demultiplexing of individual samples, thus generating highly accurate output data. For more information on QIAseq UDIs, please refer to Appendix A: QIAseq DIRECT Unique Dual Indexes.

Next-generation sequencing

The QIAseq DIRECT SARS-CoV-2 libraries are compatible with Illumina NGS platforms including iSeq® 100, MiniSeq®, MiSeq®, NextSeq® 500/550, NextSeq 1000/2000, HiSeq® 2500, HiSeq 3000/4000, and NovaSeq™ 6000. Dual 10-bp indexes and 149-bp paired-end reads are required. Table 1 describes the number of clusters/flow cell per Illumina Instrument. When the enhanced QIAseq DIRECT protocol is used for nasopharyngeal (NP) swab samples, our starting recommendation is 125,000 clusters/sample; depending on the application, viral titer, sample type, RNA preparation method, and other potential variables, increasing the cluster allocation per sample may prove to be beneficial.

Table 1. Illumina sequencers and clusters/flow cell

Instrument	Version	Clusters/flow cell (M)
iSeq 100	i1 Reagents	4
MiniSeq	Mid Output	8
MiniSeq	High Output	25
MiSeq	v2 Reagents	15
MiSeq	v3 Reagents	25
NextSeq 500/550	Mid Output	130
NextSeq 500/550	High Output	400
NextSeq 1000/2000	P1	100
NextSeq 1000/2000	P2	400
NextSeq 1000/2000	P3	1200
HiSeq 2500	Rapid Run v2 with cBot	150 (per lane)
HiSeq 3000/4000		312.5 (per lane)
NovaSeq 6000	SP	800
NovaSeq 6000	S1	1600

Data analysis

Downstream NGS data can be analyzed with the QIAGEN CLC Genomics Workbench.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Consumables and reagents

- Nuclease-free pipette tips and tubes
- PCR tubes (0.2 ml individual tubes or 8-well tube strips) (VWR®, cat. no. 20170-012 or 93001-118) or plates
- 1.5 ml LoBind® tubes (Eppendorf®, cat. no. 022431021)
- 100% ethanol (ACS grade)
- Nuclease-free water
- Ice

Laboratory equipment

- Single-channel pipette
- Multichannel pipette
- Magnetic racks for magnetic beads separation: DynaMag™-96 Side Magnet (Thermo Fisher Scientific, cat. no. 12331D)
- Microcentrifuge
- Thermal cycler
- Vortexer
- Library QC: Bioanalyzer®, TapeStation, Fragment Analyzer (Agilent®), or similar.

Important Notes

General precautions

- Observe good laboratory practices to minimize cross-contamination of nucleic acid products.
- Always use PCR tubes, microcentrifuge tubes, and pipette tips that are certified sterile and DNase- and RNase-free.
- Before starting, wipe down work area and pipettes with an RNase and DNA cleaning product such as RNase Away® (Thermo Fisher Scientific) or LookOut® DNA Erase (Sigma Aldrich).
- For consistent genome amplification and library construction and amplification, ensure that the thermal cycler used in this protocol is in good working order and has been calibrated according to the manufacturer's specifications.
- Please read the entire protocol before beginning. Take note of required products, notes, recommendations, and stopping points.

Protocol: Enhanced QIAseq DIRECT

Important: This protocol uses QN Taq Polymerase and UPCR Buffer, 5x. It does not use the QIAseq 2X HiFi MM. The Enhanced QIAseq DIRECT protocol is described in Figure 1.

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.

Procedure

1. 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.

2. Prepare the reagents required for cDNA synthesis.
   - 2a. Thaw RP Primer (random hexamer), Multimodal RT Buffer, and nuclease-free water at room temperature (15–25°C).
   - 2b. 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.
   - 2c. 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. On ice, prepare the cDNA synthesis reaction according to Table 2. Briefly centrifuge, "gently yet thoroughly" vortex to mix, and centrifuge briefly again.

   Note: Do not dilute the RP Primer.

   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.

Table 2. Setup of cDNA synthesis reaction

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

4. Incubate as described in Table 3.

Table 3. cDNA synthesis incubation

Step	Temperature (°C)	Incubation time
1	25	10 min
2	42	50 min
3	85	5 min
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 freezer.

SARS-CoV-2 Enrichment Procedure

Important points before starting

- 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.

Procedure

6. Prepare the reagents required for target enrichment.
   - 6a. 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.
   - 6b. Thaw the QN Taq Polymerase on ice. Mix by flicking the tubes, and then centrifuge briefly.

7. On ice, prepare two target enrichment reactions per sample according to Table 4. 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.

Table 4. Setup of SARS-Cov-2 enrichment reactions

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**

8. For samples with a broad/unknown range of Ct values (i.e., above and below Ct = 32) incubate as described in Table 5a. For samples with Ct > 32, also incubate as described in Table 5a. For samples with Ct value < 32, incubate as described in Table 5b.

Table 5a. Target enrichment cycling: Samples with broad/unknown range Ct values and samples with Ct > 32

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	
Hold	∞	4	Hold

Table 5b. Target enrichment cycling: Samples with Ct < 32

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	
Hold	∞	4	Hold

9. 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.

10. 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.

11. Incubate for 5 min at room temperature.

12. 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.

    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.

13. 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.

14. 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).

15. 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.

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

17. Transfer 28 µl to a clean plate. This is now "enriched SARS-CoV-2".

18. 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

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.

Procedure

19. Prepare the reagents required for library amplification and indexing.
    19a. 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 then centrifuge briefly.
    19b. Thaw the QN Taq Polymerase. Mix by flicking the tubes, and then centrifuge briefly.

20. For samples with a broad/unknown range of Ct values (i.e., above and below Ct = 32) incubate as described in Table 6a. For samples with Ct > 32, also incubate as described in Table 6a. For samples with Ct value < 32, incubate as described in Table 6b.

Table 6a. Library amplification/indexing cycling conditions: Samples with broad/unknown range Ct values and samples with Ct > 32

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

Table 6b. Library amplification/indexing cycling conditions: Samples with Ct < 32

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

21. On ice, prepare the library amplification and indexing reaction according to Table 7. 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.

Table 7. Reaction mix for library amplification and indexing

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**

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

23. Once PCR is complete, add 45 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.

24. Incubate the mixture for 5 min at room temperature.

25. 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.

    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.

26. 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.

27. 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).

28. 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.

29. 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).

30. 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.

31. Assess the quality of the library using a Bioanalyzer, TapeStation, or Fragment Analyzer. Check for the expected size distribution of library fragments. An example library is shown in Figure 2:

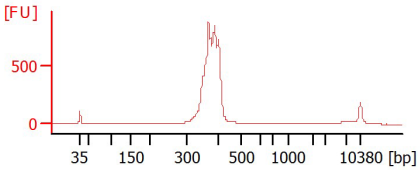

Figure 2. Example QIAseq DIRECT SARS-CoV-2 library.

32. Quantify and normalize the "SARS-CoV-2 library", and proceed to "Protocol: Sequencing Setup on an Illumina Instrument". 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.

Protocol: Sequencing Setup on an Illumina Instrument

"SARS-CoV-2 libraries" are compatible with Illumina NGS platforms, including iSeq100, MiniSeq, MiSeq, NextSeq 500/550, NextSeq 1000/2000, HiSeq 2500, HiSeq 3000/4000, and NovaSeq 6000.

Important points before starting

- **Important**: To make sequencing preparation convenient, download Illumina compatible sample sheets for different sequencing instruments on **www.qiagen.com**, and refer to "Appendix A: QIAseq DIRECT Unique Dual Indexes".
- **Important**: 149 bp paired-end sequencing with dual 10 bp indexes should be used.
- For complete instructions on how to denature sequencing libraries and set up a sequencing run, please refer to the system-specific Illumina documents.

Sample dilution, pooling, sequencing, and data analysis

- Dilute the individual "SARS-CoV-2 libraries" to a concentration of 4 nM, then combine libraries with different sample indexes in equimolar amounts. The recommended starting final loading concentration of the pooled "SARS-CoV-2 libraries" to load onto a MiSeq is 10 pM, or 1.6 pM on a MiniSeq, or 1.6 pM on a NextSeq instrument.
- Dilute the individual "SARS-CoV-2 libraries" to a concentration of 10 nM, then combine libraries with different sample indexes in equimolar amounts. The recommended final loading concentration of the pooled "SARS-CoV-2 libraries" to load onto a NovaSeq instrument is between 175 and 265 pM.

Protocol: Data Analysis

- Downstream NGS data can be analyzed with QIAGEN CLC Genomics Workbench. Desktop or server versions are available.
- QIAGEN CLC Genomics Workbench is a comprehensive analysis package for the analysis and visualization of data from all major NGS platforms. The workbench supports and seamlessly integrates into a typical NGS workflow, and is available for Windows, Mac OS X, and Linux platforms. Incorporating cutting-edge technology and algorithms, QIAGEN CLC Genomics Workbench supports key NGS features within genomics, transcriptomics, and epigenomics research fields. Additionally, it includes all the classical analysis tools of QIAGEN CLC Main Workbench.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page in our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information or protocols in this handbook (for contact information, visit **support.qiagen.com**).

		Comments and suggestions
Low library yields		
a)	Suboptimal reaction conditions due to low RNA quality	Make sure to use high-quality RNA to ensure optimal activity of the library enzymes.
b)	Residual ethanol after QIAseq Bead washes	Ensure that (1) all ethanol has been removed and (2) QIAseq Beads have been thoroughly dried, according to the protocols listed in the handbook.
Dimers observed in final library traces		
a)	Residual ethanol after QIAseq Bead washes	Residual ethanol during QIAseq Bead washes can also result in library dimers. Ensure that (1) all ethanol has been removed and (2) QIAseq Beads have been thoroughly dried, according to the protocols listed in the handbook.
b)	Ultra-low viral titer	With ultra-low viral titers (20 copies and under), there is the possibility of dimers. As ultra-low viral titer samples conceptually do not require as many sequencing reads as higher viral titer samples, this should not present an issue during next-generation sequencing.

Appendix A: QIAseq DIRECT Unique Dual Indexes

Generation of sample sheets for Illumina instruments

Index sequences for QIAseq DIRECT Unique Dual Indexes are available for download at **www.qiagen.com**. Sequencing on the NextSeq, HiSeq X™, or HiSeq 3000/4000 system follows a different dual-indexing workflow than other Illumina systems. If you are manually creating sample sheets for these instruments, enter the reverse complement of the i5 index adapter sequence. If using Illumina Experiment Manager, BaseSpace®, or Local Run Manager for run planning, the software will automatically reverse complement index sequences when necessary.

Ready-to-use sample sheets containing all QIAseq DIRECT Unique Dual Indexes are available for MiSeq, NextSeq, MiniSeq, HiSeq, and NovaSeq instruments. These can be conveniently downloaded from **www.qiagen.com**. These can be imported and edited using the Illumina Experiment Manager Software, Illumina Local Run Manager, or any text editor. Make sure to download the appropriate sample sheet for NextSeq, HiSeq X, or HiSeq 3000/4000 systems depending on whether you are using Local Run Manager or manually configuring the sequencing run.

QIAseq DIRECT UDI Layouts

The layouts of the single-use QIAseq DIRECT UDI plates is shown in Figure 3. The index motives used in the QIAseq Unique Dual Index Kits are listed at **www.qiagen.com**. To make sequencing preparation more convenient, you can download Illumina-compatible sample sheets for different sequencing instruments on **www.qiagen.com**.

QIAseq DIRECT UDI Set A: RUDI-96AA

	1	2	3	4	5	6	7	8	9	10	11	12
A	i7RUDI-001 i5RUDI-001	i7RUDI-009 i5RUDI-009	i7RUDI-017 i5RUDI-017	i7RUDI-025 i5RUDI-025	i7RUDI-033 i5RUDI-033	i7RUDI-041 i5RUDI-041	i7RUDI-049 i5RUDI-049	i7RUDI-057 i5RUDI-057	i7RUDI-065 i5RUDI-065	i7RUDI-073 i5RUDI-073	i7RUDI-081 i5RUDI-081	i7RUDI-089 i5RUDI-089
B	i7RUDI-002 i5RUDI-002	i7RUDI-010 i5RUDI-010	i7RUDI-018 i5RUDI-018	i7RUDI-026 i5RUDI-026	i7RUDI-034 i5RUDI-034	i7RUDI-042 i5RUDI-042	i7RUDI-050 i5RUDI-050	i7RUDI-058 i5RUDI-058	i7RUDI-066 i5RUDI-066	i7RUDI-074 i5RUDI-074	i7RUDI-082 i5RUDI-082	i7RUDI-090 i5RUDI-090
C	i7RUDI-003 i5RUDI-003	i7RUDI-011 i5RUDI-011	i7RUDI-019 i5RUDI-019	i7RUDI-027 i5RUDI-027	i7RUDI-035 i5RUDI-035	i7RUDI-043 i5RUDI-043	i7RUDI-051 i5RUDI-051	i7RUDI-059 i5RUDI-059	i7RUDI-067 i5RUDI-067	i7RUDI-075 i5RUDI-075	i7RUDI-083 i5RUDI-083	i7RUDI-091 i5RUDI-091
D	i7RUDI-004 i5RUDI-004	i7RUDI-012 i5RUDI-012	i7RUDI-020 i5RUDI-020	i7RUDI-028 i5RUDI-028	i7RUDI-036 i5RUDI-036	i7RUDI-044 i5RUDI-044	i7RUDI-052 i5RUDI-052	i7RUDI-060 i5RUDI-060	i7RUDI-068 i5RUDI-068	i7RUDI-076 i5RUDI-076	i7RUDI-084 i5RUDI-084	i7RUDI-092 i5RUDI-092
E	i7RUDI-005 i5RUDI-005	i7RUDI-013 i5RUDI-013	i7RUDI-021 i5RUDI-021	i7RUDI-029 i5RUDI-029	i7RUDI-037 i5RUDI-037	i7RUDI-045 i5RUDI-045	i7RUDI-053 i5RUDI-053	i7RUDI-061 i5RUDI-061	i7RUDI-069 i5RUDI-069	i7RUDI-077 i5RUDI-077	i7RUDI-085 i5RUDI-085	i7RUDI-093 i5RUDI-093
F	i7RUDI-006 i5RUDI-006	i7RUDI-014 i5RUDI-014	i7RUDI-022 i5RUDI-022	i7RUDI-030 i5RUDI-030	i7RUDI-038 i5RUDI-038	i7RUDI-046 i5RUDI-046	i7RUDI-054 i5RUDI-054	i7RUDI-062 i5RUDI-062	i7RUDI-070 i5RUDI-070	i7RUDI-078 i5RUDI-078	i7RUDI-086 i5RUDI-086	i7RUDI-094 i5RUDI-094
G	i7RUDI-007 i5RUDI-007	i7RUDI-015 i5RUDI-015	i7RUDI-023 i5RUDI-023	i7RUDI-031 i5RUDI-031	i7RUDI-039 i5RUDI-039	i7RUDI-047 i5RUDI-047	i7RUDI-055 i5RUDI-055	i7RUDI-063 i5RUDI-063	i7RUDI-071 i5RUDI-071	i7RUDI-079 i5RUDI-079	i7RUDI-087 i5RUDI-087	i7RUDI-095 i5RUDI-095
H	i7RUDI-008 i5RUDI-008	i7RUDI-016 i5RUDI-016	i7RUDI-024 i5RUDI-024	i7RUDI-032 i5RUDI-032	i7RUDI-040 i5RUDI-040	i7RUDI-048 i5RUDI-048	i7RUDI-056 i5RUDI-056	i7RUDI-064 i5RUDI-064	i7RUDI-072 i5RUDI-072	i7RUDI-080 i5RUDI-080	i7RUDI-088 i5RUDI-088	i7RUDI-096 i5RUDI-096

QIAseq DIRECT UDI Set B: RUDI-96BA

	1	2	3	4	5	6	7	8	9	10	11	12
A	i7RUDI-097 i5RUDI-097	i7RUDI-105 i5RUDI-105	i7RUDI-113 i5RUDI-113	i7RUDI-121 i5RUDI-121	i7RUDI-129 i5RUDI-129	i7RUDI-137 i5RUDI-137	i7RUDI-145 i5RUDI-145	i7RUDI-153 i5RUDI-153	i7RUDI-161 i5RUDI-161	i7RUDI-169 i5RUDI-169	i7RUDI-177 i5RUDI-177	i7RUDI-185 i5RUDI-185
B	i7RUDI-098 i5RUDI-098	i7RUDI-106 i5RUDI-106	i7RUDI-114 i5RUDI-114	i7RUDI-122 i5RUDI-122	i7RUDI-130 i5RUDI-130	i7RUDI-138 i5RUDI-138	i7RUDI-146 i5RUDI-146	i7RUDI-154 i5RUDI-154	i7RUDI-162 i5RUDI-162	i7RUDI-170 i5RUDI-170	i7RUDI-178 i5RUDI-178	i7RUDI-186 i5RUDI-186
C	i7RUDI-099 i5RUDI-099	i7RUDI-107 i5RUDI-107	i7RUDI-115 i5RUDI-115	i7RUDI-123 i5RUDI-123	i7RUDI-131 i5RUDI-131	i7RUDI-139 i5RUDI-139	i7RUDI-147 i5RUDI-147	i7RUDI-155 i5RUDI-155	i7RUDI-163 i5RUDI-163	i7RUDI-171 i5RUDI-171	i7RUDI-179 i5RUDI-179	i7RUDI-187 i5RUDI-187
D	i7RUDI-100 i5RUDI-100	i7RUDI-108 i5RUDI-108	i7RUDI-116 i5RUDI-116	i7RUDI-124 i5RUDI-124	i7RUDI-132 i5RUDI-132	i7RUDI-140 i5RUDI-140	i7RUDI-148 i5RUDI-148	i7RUDI-156 i5RUDI-156	i7RUDI-164 i5RUDI-164	i7RUDI-172 i5RUDI-172	i7RUDI-180 i5RUDI-180	i7RUDI-188 i5RUDI-188
E	i7RUDI-101 i5RUDI-101	i7RUDI-109 i5RUDI-109	i7RUDI-117 i5RUDI-117	i7RUDI-125 i5RUDI-125	i7RUDI-133 i5RUDI-133	i7RUDI-141 i5RUDI-141	i7RUDI-149 i5RUDI-149	i7RUDI-157 i5RUDI-157	i7RUDI-165 i5RUDI-165	i7RUDI-173 i5RUDI-173	i7RUDI-181 i5RUDI-181	i7RUDI-189 i5RUDI-189
F	i7RUDI-102 i5RUDI-102	i7RUDI-110 i5RUDI-110	i7RUDI-118 i5RUDI-118	i7RUDI-126 i5RUDI-126	i7RUDI-134 i5RUDI-134	i7RUDI-142 i5RUDI-142	i7RUDI-150 i5RUDI-150	i7RUDI-158 i5RUDI-158	i7RUDI-166 i5RUDI-166	i7RUDI-174 i5RUDI-174	i7RUDI-182 i5RUDI-182	i7RUDI-190 i5RUDI-190
G	i7RUDI-103 i5RUDI-103	i7RUDI-111 i5RUDI-111	i7RUDI-119 i5RUDI-119	i7RUDI-127 i5RUDI-127	i7RUDI-135 i5RUDI-135	i7RUDI-143 i5RUDI-143	i7RUDI-151 i5RUDI-151	i7RUDI-159 i5RUDI-159	i7RUDI-167 i5RUDI-167	i7RUDI-175 i5RUDI-175	i7RUDI-183 i5RUDI-183	i7RUDI-191 i5RUDI-191
H	i7RUDI-104 i5RUDI-104	i7RUDI-112 i5RUDI-112	i7RUDI-120 i5RUDI-120	i7RUDI-128 i5RUDI-128	i7RUDI-136 i5RUDI-136	i7RUDI-144 i5RUDI-144	i7RUDI-152 i5RUDI-152	i7RUDI-160 i5RUDI-160	i7RUDI-168 i5RUDI-168	i7RUDI-176 i5RUDI-176	i7RUDI-184 i5RUDI-184	i7RUDI-192 i5RUDI-192

QIAseq DIRECT UDI Set C: RUDI-96CA

	1	2	3	4	5	6	7	8	9	10	11	12
A	i7RUDI-193 i5RUDI-193	i7RUDI-201 i5RUDI-201	i7RUDI-209 i5RUDI-209	i7RUDI-217 i5RUDI-217	i7RUDI-225 i5RUDI-225	i7RUDI-233 i5RUDI-233	i7RUDI-241 i5RUDI-241	i7RUDI-249 i5RUDI-249	i7RUDI-257 i5RUDI-257	i7RUDI-265 i5RUDI-265	i7RUDI-273 i5RUDI-273	i7RUDI-281 i5RUDI-281
B	i7RUDI-194 i5RUDI-194	i7RUDI-202 i5RUDI-202	i7RUDI-210 i5RUDI-210	i7RUDI-218 i5RUDI-218	i7RUDI-226 i5RUDI-226	i7RUDI-234 i5RUDI-234	i7RUDI-242 i5RUDI-242	i7RUDI-250 i5RUDI-250	i7RUDI-258 i5RUDI-258	i7RUDI-266 i5RUDI-266	i7RUDI-274 i5RUDI-274	i7RUDI-282 i5RUDI-282
C	i7RUDI-195 i5RUDI-195	i7RUDI-203 i5RUDI-203	i7RUDI-211 i5RUDI-211	i7RUDI-219 i5RUDI-219	i7RUDI-227 i5RUDI-227	i7RUDI-235 i5RUDI-235	i7RUDI-243 i5RUDI-243	i7RUDI-251 i5RUDI-251	i7RUDI-259 i5RUDI-259	i7RUDI-267 i5RUDI-267	i7RUDI-275 i5RUDI-275	i7RUDI-283 i5RUDI-283
D	i7RUDI-196 i5RUDI-196	i7RUDI-204 i5RUDI-204	i7RUDI-212 i5RUDI-212	i7RUDI-220 i5RUDI-220	i7RUDI-228 i5RUDI-228	i7RUDI-236 i5RUDI-236	i7RUDI-244 i5RUDI-244	i7RUDI-252 i5RUDI-252	i7RUDI-260 i5RUDI-260	i7RUDI-268 i5RUDI-268	i7RUDI-276 i5RUDI-276	i7RUDI-284 i5RUDI-284
E	i7RUDI-197 i5RUDI-197	i7RUDI-205 i5RUDI-205	i7RUDI-213 i5RUDI-213	i7RUDI-221 i5RUDI-221	i7RUDI-229 i5RUDI-229	i7RUDI-237 i5RUDI-237	i7RUDI-245 i5RUDI-245	i7RUDI-253 i5RUDI-253	i7RUDI-261 i5RUDI-261	i7RUDI-269 i5RUDI-269	i7RUDI-277 i5RUDI-277	i7RUDI-285 i5RUDI-285
F	i7RUDI-198 i5RUDI-198	i7RUDI-206 i5RUDI-206	i7RUDI-214 i5RUDI-214	i7RUDI-222 i5RUDI-222	i7RUDI-230 i5RUDI-230	i7RUDI-238 i5RUDI-238	i7RUDI-246 i5RUDI-246	i7RUDI-254 i5RUDI-254	i7RUDI-262 i5RUDI-262	i7RUDI-270 i5RUDI-270	i7RUDI-278 i5RUDI-278	i7RUDI-286 i5RUDI-286
G	i7RUDI-199 i5RUDI-199	i7RUDI-207 i5RUDI-207	i7RUDI-215 i5RUDI-215	i7RUDI-223 i5RUDI-223	i7RUDI-231 i5RUDI-231	i7RUDI-239 i5RUDI-239	i7RUDI-247 i5RUDI-247	i7RUDI-255 i5RUDI-255	i7RUDI-263 i5RUDI-263	i7RUDI-271 i5RUDI-271	i7RUDI-279 i5RUDI-279	i7RUDI-287 i5RUDI-287
H	i7RUDI-200 i5RUDI-200	i7RUDI-208 i5RUDI-208	i7RUDI-216 i5RUDI-216	i7RUDI-224 i5RUDI-224	i7RUDI-232 i5RUDI-232	i7RUDI-240 i5RUDI-240	i7RUDI-248 i5RUDI-248	i7RUDI-256 i5RUDI-256	i7RUDI-264 i5RUDI-264	i7RUDI-272 i5RUDI-272	i7RUDI-280 i5RUDI-280	i7RUDI-288 i5RUDI-288

QIAseq DIRECT UDI Set D: RUDI-96DA

	1	2	3	4	5	6	7	8	9	10	11	12
A	i7RUDI-289 i5RUDI-289	i7RUDI-297 i5RUDI-297	i7RUDI-305 i5RUDI-305	i7RUDI-313 i5RUDI-313	i7RUDI-321 i5RUDI-321	i7RUDI-329 i5RUDI-329	i7RUDI-337 i5RUDI-337	i7RUDI-345 i5RUDI-345	i7RUDI-353 i5RUDI-353	i7RUDI-361 i5RUDI-361	i7RUDI-369 i5RUDI-369	i7RUDI-377 i5RUDI-377
B	i7RUDI-290 i5RUDI-290	i7RUDI-298 i5RUDI-298	i7RUDI-306 i5RUDI-306	i7RUDI-314 i5RUDI-314	i7RUDI-322 i5RUDI-322	i7RUDI-330 i5RUDI-330	i7RUDI-338 i5RUDI-338	i7RUDI-346 i5RUDI-346	i7RUDI-354 i5RUDI-354	i7RUDI-362 i5RUDI-362	i7RUDI-370 i5RUDI-370	i7RUDI-378 i5RUDI-378
C	i7RUDI-291 i5RUDI-291	i7RUDI-299 i5RUDI-299	i7RUDI-307 i5RUDI-307	i7RUDI-315 i5RUDI-315	i7RUDI-323 i5RUDI-323	i7RUDI-331 i5RUDI-331	i7RUDI-339 i5RUDI-339	i7RUDI-347 i5RUDI-347	i7RUDI-355 i5RUDI-355	i7RUDI-363 i5RUDI-363	i7RUDI-371 i5RUDI-371	i7RUDI-379 i5RUDI-379
D	i7RUDI-292 i5RUDI-292	i7RUDI-300 i5RUDI-300	i7RUDI-308 i5RUDI-308	i7RUDI-316 i5RUDI-316	i7RUDI-324 i5RUDI-324	i7RUDI-332 i5RUDI-332	i7RUDI-340 i5RUDI-340	i7RUDI-348 i5RUDI-348	i7RUDI-356 i5RUDI-356	i7RUDI-364 i5RUDI-364	i7RUDI-372 i5RUDI-372	i7RUDI-380 i5RUDI-380
E	i7RUDI-293 i5RUDI-293	i7RUDI-301 i5RUDI-301	i7RUDI-309 i5RUDI-309	i7RUDI-317 i5RUDI-317	i7RUDI-325 i5RUDI-325	i7RUDI-333 i5RUDI-333	i7RUDI-341 i5RUDI-341	i7RUDI-349 i5RUDI-349	i7RUDI-357 i5RUDI-357	i7RUDI-365 i5RUDI-365	i7RUDI-373 i5RUDI-373	i7RUDI-381 i5RUDI-381
F	i7RUDI-294 i5RUDI-294	i7RUDI-302 i5RUDI-302	i7RUDI-310 i5RUDI-310	i7RUDI-318 i5RUDI-318	i7RUDI-326 i5RUDI-326	i7RUDI-334 i5RUDI-334	i7RUDI-342 i5RUDI-342	i7RUDI-350 i5RUDI-350	i7RUDI-358 i5RUDI-358	i7RUDI-366 i5RUDI-366	i7RUDI-374 i5RUDI-374	i7RUDI-382 i5RUDI-382
G	i7RUDI-295 i5RUDI-295	i7RUDI-303 i5RUDI-303	i7RUDI-311 i5RUDI-311	i7RUDI-319 i5RUDI-319	i7RUDI-327 i5RUDI-327	i7RUDI-335 i5RUDI-335	i7RUDI-343 i5RUDI-343	i7RUDI-351 i5RUDI-351	i7RUDI-359 i5RUDI-359	i7RUDI-367 i5RUDI-367	i7RUDI-375 i5RUDI-375	i7RUDI-383 i5RUDI-383
H	i7RUDI-296 i5RUDI-296	i7RUDI-304 i5RUDI-304	i7RUDI-312 i5RUDI-312	i7RUDI-320 i5RUDI-320	i7RUDI-328 i5RUDI-328	i7RUDI-336 i5RUDI-336	i7RUDI-344 i5RUDI-344	i7RUDI-352 i5RUDI-352	i7RUDI-360 i5RUDI-360	i7RUDI-368 i5RUDI-368	i7RUDI-376 i5RUDI-376	i7RUDI-384 i5RUDI-384

Figure 3. QIAseq DIRECT UDI sets: Set A layout (UDI 1–96), Set B layout (UDI 97–192), Set C layout (UDI 193-288), and Set D layout (UDI 289–384).

Appendix B: Legacy QIAseq DIRECT Protocol

Important: This protocol uses QIAseq 2X HiFi MM. This protocol does not use QN Taq Polymerase or UPCR Buffer, 5x. The legacy QIAseq DIRECT protocol is described in Figure 4.

Figure 4. Legacy QIAseq DIRECT SARS-CoV-2 workflow.

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.

Procedure

1. 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.

2. Prepare the reagents required for cDNA synthesis.
   - 2a. Thaw RP Primer (random hexamer), Multimodal RT Buffer, and nuclease-free water at room temperature (15–25°C).
   - 2b. Mix thoroughly and then briefly centrifuge to collect residual liquid from the sides of the tubes. Prolonged storage of Multimodal RT Buffer at –30 to –15°C can cause white precipitate to form. This is normal. Just ensure to mix until precipitate is fully dissolved.
   - 2c. 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. **Important**: Dilute the RP Primer 11-fold. As an example, add 2 µl of RP Primer to 20 µl of nuclease-free water to create 22 µl of RP-Primer (11-fold diluted). Briefly centrifuge and vortex to mix.
   On ice, prepare the cDNA synthesis reaction according to Table 8. 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.

Table 8. Setup of cDNA synthesis reaction

Component	Volume/reaction (µl)
Template RNA	5
RP Primer (11-fold diluted)	1
Multimodal RT Buffer, 5x	4
Nuclease-free water	8
RNase Inhibitor	1
EZ Reverse Transcriptase	1
Total volume	**20**

4. Incubate as described in Table 9.

Table 9. cDNA synthesis incubation

Step	Temperature (°C)	Incubation time
1	25	10 min
2	42	50 min
3	85	5 min
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 freezer.

SARS-CoV-2 Enrichment Procedure

Important points before starting

- 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.

Procedure

6. Prepare the reagents required for target enrichment.

   6a. Thaw the DIRECT SARS-CoV-2 Pool 1 and DIRECT SARS-CoV-2 Pool 2 at room temperature. "Gently yet thoroughly" vortex to mix, and then centrifuge briefly.

   6b. Thaw the QIAseq 2X HiFi MM on ice. Mix by flicking the tubes, and then centrifuge briefly.

   Note: If a precipitate is present, bring to room temperature for 5 min, and dissolve the precipitate by mixing with a pipettor and gentle vortexing.

7. On ice, prepare two target enrichment reactions per sample according to Table 10. 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.

Table 10. Setup of SARS-Cov-2 enrichment reactions

Component	Pool 1: Volume/reaction (µl)	Pool 2: Volume/reaction (µl)
cDNA from "Appendix B: Legacy QIAseq DIRECT Protocol"	5	5
DIRECT SARS-CoV-2 Pool 1	2	–
DIRECT SARS-CoV-2 Pool 2	–	2
QIAseq 2X HiFi MM	12.5	12.5
Nuclease-free water	5.5	5.5
Total volume	**25**	**25**

Table 11. Target enrichment cycling conditions

Step	Time	Temperature (°C)	Number of cycles
Heat activation	2 min	98	1
Denaturation	20 s	98	35
Annealing/extension	3 min*	63	
Hold	∞	4	Hold

* For low quality samples, increasing annealing/extension to 5 min can improve results.

8. Incubate as described in Table 11.

9. 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.

10. 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.

11. Incubate for 5 min at room temperature.

12. 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.

    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.

13. 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.

14. 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).

15. 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, and incubate for 2 min.

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

17. Transfer 28 µl to a clean plate. This is now "enriched SARS-CoV-2".

18. Proceed to "SARS-CoV-2 Enrichment Quantification and Normalization Procedure". Alternatively, the samples can be stored at –30 to –15°C in a constant-temperature freezer.

SARS-CoV-2 Enrichment Quantification and Normalization Procedure

Potential options for concentration readings:

- Qubit® Fluorometer (Thermo Fisher Scientific)
  Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific cat. no. Q32851 or Q32854)
  Qubit Assay Tubes (Thermo Fisher Scientific cat. no. Q32856)
- NanoDrop™ Spectrophotometer (Thermo Fisher Scientific)
- QIAxpert® System (QIAGEN)

Procedure

19. Using a small portion (approx. 1–2 µl) of the sample, quantify the enrichment reaction using a Qubit, NanoDrop, QIAxpert, or similar instrument. To do this, follow the respective user manual for your chosen instrument.

    Note: Ensure that the concentration reading is on the linear scale. If the concentration is not on the linear scale, dilute a small portion of the target enriched sample, and requantify.

20. Once concentrations have been determined, dilute an aliquot of each "enriched SARS-CoV-2" to a common concentration using nuclease-free water, in a volume of 23 µl or less. The diluted samples should be pipetted into a clean, empty PCR plate.

    Note: The goal is to add 100 ng of "enriched SARS-CoV-2" during library amplification and indexing; nonetheless, if 100 ng is not achievable, less can be used.

21. Proceed to "Library Amplification and Indexing Procedure".

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.
- **Important:** Depending on the sample type and viral titer, diluting the DIRECT UDI indexes 8-fold in nuclease-free water may improve performance by reducing dimers.
- 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.

Procedure

22. Prepare the reagents required for library amplification and indexing.
    - 22a. Thaw the QIAseq DIRECT UDI Index Set A, B, C, or D at room temperature. Mix by gently vortexing the plate and then centrifuge briefly.
    - 22b. Thaw the QIAseq 2X HiFi MM on ice.
      Note: If a precipitate is present, bring to room temperature for 5 min, and dissolve the precipitate by mixing with pipettor and gentle vortexing.
    - 22c. Mix by flicking the tubes, and then centrifuge briefly.

23. Program a thermal cycler with a heated lid according to Table 12.

Table 12. Library amplification and indexing cycling conditions

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	98	2 min	1
3-step cycling			
Denaturation	98	20 s	
Annealing	60	30 s	7*
Extension	72	30 s	
Final extension	72	1 min	1
Hold	4	∞	Hold

* Based on input of 100 ng of "enriched SARS-CoV-2". If 50 ng is added, perform 8 cycles. If 10 ng is added, perform 10 cycles.

24. **Important:** Depending on the sample type and viral titer, diluting the DIRECT UDI indexes 8-fold in nuclease-free water may improve performance by reducing dimers. For the QIAseq DIRECT UDI plates, pierce the foil seal associated with each well that will be used, and transfer 2 µl (each well contains a forward primer and a reverse primer, each with a unique index) to the diluted "enriched SARS-CoV-2" sample plate prepared in "SARS-CoV-2 Enrichment Quantification and Normalization Procedure".

    Important: Only one UDI pair should be used per amplification reaction.

    Important: The QIAseq DIRECT UDI index plates are stable for a maximum of 10 freeze–thaw cycles. If all 96-wells have not been used at one time, cover used wells with foil and return to the freezer. Do not reuse wells from the QIAseq DIRECT UDI index plates once the foil seals have been pierced. This would risk significant cross-contamination.

25. On ice, prepare the library amplification and indexing reaction according to Table 13. 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.

Table 13. Reaction mix for library amplification and indexing

Component	Volume/reaction
Diluted "enriched SARS-CoV-2" sample	Variable (100 ng*)
Index from QIAseq DIRECT UDI index plate (A, B, C, or D) Plate	2 µl
QIAseq 2X HiFi MM	25 µl
Nuclease-free water	Variable
Total reaction volume	**50 µl**

* Based on input of 100 ng of "enriched SARS-CoV-2". 50 ng or 10 ng can also be added. If lower amounts have been added, adjust the number of cycles accordingly in Table 12.

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

27. Once PCR is complete, add 45 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 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).

32. Remove the plate from the magnetic stand and elute the DNA from the beads by adding 25 µl nuclease-free water. "Gently yet thoroughly" 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.

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. Assess the quality of the library using a Bioanalyzer, TapeStation, or Fragment Analyzer. Check for the expected size distribution of library fragments. An example library is shown in Figure 2.

36. Quantify and normalize the "SARS-CoV-2 library", and proceed to "Protocol: Sequencing Setup on an Illumina Instrument". 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.

Ordering Information

Product	Contents	Cat. no.
QIAseq DIRECT SARS-CoV-2 Kit A, B, C, D, E, F, G, H	For 96 reactions: Contains all buffers and reagents for cDNA synthesis, high-fidelity SARS-CoV-2 amplicon enrichment, and high-fidelity library amplification; includes index set A with UDIs 1-96 (pierceable foil seal allowing usage of defined parts of plate)	333891 333892 333893 333894 333886 333887 333888 333889
QIAseq DIRECT SARS-CoV-2 HT (A–D), (E–H)	For 384 reactions: Contains all buffers and reagents for cDNA synthesis, high-fidelity SARS-CoV-2 amplicon enrichment, and high-fidelity library amplification; includes index sets A, B, C and D with UDIs 1-384 (pierceable foil seal allowing usage of defined parts of plates)	333898 333899
QIAseq DIRECT SARS-CoV-2 Enhancer	For 96 reactions: Contains buffers and reagents to enhance the performance of the QIAseq DIRECT SARS-CoV-2 Kits	333884

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Document Revision History

Date	Changes
04/2021	Initial revision
01/2022	Updated the "Kit Contents" section. Deleted the "Important: Additional Required Products" section. Updated the "Shipping and Storage" section to provide storage instructions for the DIRECT SARS-CoV-2 Enhancer. Updated the Principle and procedure section to describe the enhanced workflow. Added NextSeq 1000/2000 (Versions P1, P2, and P3) in Table 1. Reformatted writing of protocols and procedures. Updated the main protocol or procedures as an enhanced version of the workflow, which is now the new, recommended protocol. In the enhanced version, Tables 5 and 6 were revised into two parts. In the enhanced protocol, the "Protocol: SARS-CoV-2 Enrichment Quantification and Normalization" section was removed. Updated the components and volume information of the reaction mix for library amplification and indexing in Table 7. In the enhanced protocol, the bead cleanup after library amplification/indexing is a 0.9X bead to sample ratio. Added the Appendix B (Legacy QIAseq DIRECT Protocol). In the legacy protocol, the recommended SARS-CoV-2 target enrichment cycles was increased to 35. In the legacy protocol, the bead cleanup after library amplification/indexing was changed to a 0.9X bead to sample ratio. Throughout both protocols, mixing by pipetting was updated to gently yet thoroughly" vortex to mix.

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