April 2019

QIAseq® 16S/ITS Panel Handbook

For high-throughput targeted next-generation sequencing of metagenomic samples

Sample to Insight

QIAGEN

Contents

Kit Contents

QIAseq 16S/ITS Screening Panel	**(24)**	**(96)**
Catalog no.	**333812**	**333815**
Number of reactions	**24**	**96**
Screening Panel Pool	3 pools	3 pools
UCP Master Mix	2 x 500 µl tubes	5 x 500 µl tubes
UCP PCR Water	6 x 1.9 ml tubes	18 x 1.9 ml tubes
QIAseq Beads	1 bottle	1 bottle
Quick-Start Protocol	1	1

QIAseq 16S/ITS Region Panel	**(24)**	**(96)**
Catalog no.	**333842**	**333845**
Number of reactions	**24**	**96**
16S/ITS Region Panel	1–3 pools	1–3 pools
UCP Master Mix	Up to 2 x 500 µl tubes	Up to 5 x 500 µl tubes
UCP PCR Water	Up to 7 x 1.9 ml tubes	Up to 22 x 1.9 ml tubes
QIAseq Beads	1 bottle	1 bottle
Quick-Start Protocol	1	1

QIAseq 16S/ITS Smart Control	**(10)**
Catalog no.	**333832**
Number of reactions	**10**
Smart Control	1 tube
UCP PCR Water	1.9 ml tube

QIAseq 16S/ITS 24-index I (for Illumina®)	**24 indices**
Catalog no.	**333822**
Number of samples	**96**
RS-D# (4 µM)	36 µl (2 tubes each)
FS-D# (4 µM)	48 µl (1 tube each)
QIAseq Read1 Primer I (100 µM)	24 µl
QIAseq 16S/ITS Read2 Primer (100 µM)	24 µl

QIAseq 16S/ITS 96-index I (for Illumina)	**96 indices**
Catalog no.	**333825**
Number of samples	**384**
Box contains QIAseq Read1 Primer I, QIAseq 16S/ITS Read2 Primer (100 µM, 24 µl) and arrays. Each array well contains one universal PCR primer pair that is printed for PCR amplification and sample indexing – enough for a total of 384 samples (for indexing up to 96 samples per run) for 16S/ITS sequencing on Illumina MiSeq® platforms.	

QIAseq 16S/ITS 384-index	**384 indices**
Catalog no.	**333827**
Number of samples	**384**
Box contains QIAseq Read1 Primer I, QIAseq 16S/ITS Read2 Primer (100 µM, 24 µl) and 4 sets of arrays (set A, set B, set C, set D). Each array well contains one universal PCR primer pair that is printed for PCR amplification and sample indexing. Each kit is enough for a total of 384 samples (for indexing up to 384 samples per run) for 16S/ITS sequencing on Illumina MiSeq platforms.	

Storage

QIAseq 16S/ITS Panels are shipped in 2 boxes. Box 1 is shipped on dry ice or blue ice, and Box 2 is shipped at room temperature (15–25°C). Upon receipt, all of the components in Box 1 should be stored immediately at –30 to –15°C in a constant-temperature freezer. All of the components in Box 2 should be stored immediately at 2–8°C.

The QIAseq 16S/ITS Index Kits and Smart Control are shipped on dry ice or blue ice and should be stored at –30 to –15°C upon arrival.

Intended Use

The QIAseq 16S/ITS Panels are intended for molecular biology applications. These products are 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 QIAseq 16S/ITS Panels, QIAseq 16S/ITS Index Kits, and QIAseq 16S/ITS Smart Control is tested against predetermined specifications to ensure consistent product quality.

Introduction

Microbial life is present in almost every environment on earth. This microbial life is partly composed of archaea/bacterial and fungal cells, and the composition of each environment or community (i.e., sample type) can be highly unique.

All species of archaea/bacteria contain the ribosomal 16S gene in their genome; likewise, all fungal species contain the ITS gene in their genome. The 16S gene has regions of conserved sequences interspersed with regions of high variability. The highly variable regions of 16S are commonly used to taxonomically classify bacterial species that reside in a sample. The surrounding conserved regions can serve as targets for 16S primers that will amplify all archaea/bacteria species in a sample. Classification of bacterial species in any sample type can be performed by first amplifying regions of the 16S gene and then performing next-generation sequencing (NGS) on the variable regions. Furthermore, this is applicable to ITS classification of fungal species. Shifts in archaea/bacterial/fungal communities can be observed by comparing different samples.

When performing 16S NGS experiments, the targeted region of interest depends on the desired classification power. To aid in the selection of the region(s) with the highest discrimination power, the QIAseq 16S/ITS Screening Panel can be used. The QIAseq 16S/ITS Screening Panel separates 6 amplicons into 3 pools that, together, cover the entire ribosomal 16S gene. In addition, the QIAseq 16S/ITS Screening Panel contains primers for ITS to amplify fungal DNA. The contents of each pool are as follows:

- Pool 1: V1V2, V4V5, and ITS
- Pool 2: V2V3 and V5V7
- Pool 3: V3V4 and V7V9

Therefore, in one library construction, all regions of the bacterial 16S gene and the fungal ITS gene are targeted and sequenced. If desired, specific regions can then be chosen for further studies, or the screening panel can also be used.

A prominent concern with existing 16S PCR (polymerase chain reaction) protocols is that PCR master mixes and lab water are contaminated, and this will negatively impact the community profile. This is especially important when looking at taxa that may occur at low abundances. To address this problem, the QIAseq 16S/ITS Panels contain a UCP (ultra-clean production) Master Mix and UCP PCR Water that have been produced in an ultra-clean environment and quality controlled to prevent contamination. This produces 16S sequencing reads with a minimal number of contaminating reads and higher confidence of sequencing results.

16S NGS is also challenging because the single amplicon design results in low-base diversity. This results in reduced clusters passing filter and reduced sequence quality (Q30 scores). Ultimately, the consequence is fewer reads that can be used for operational taxonomic units (OTU) analysis. To overcome this issue, the QIAseq 16S/ITS Panels incorporate phased primers that add 0–11 additional bases before the 16S primer. This technology increases the base diversity, which is especially important within the first 11 cycles of sequencing and ultimately leads to higher Q30 scores and usable reads. At the same time, it eliminates the need to add PhiX, increasing the available reads.

Principle and procedures

The QIAseq 16S/ITS Panels utilize a 2-stage PCR workflow for targeted enrichment of 16S and ITS genes (Figure 1). The first PCR step incorporates a phased primer pool to enrich for conserved regions of the 16S gene and ITS gene. Following a reaction cleanup with QIAseq Beads, library amplification then introduces sample indices and ensures that sufficient target is present for NGS. Following a final cleanup, the libraries are quality controlled (Bioanalyzer® or TapeStation®) and quantified using a the QIAseq Library Quant system. The number of multiplexed samples depends on the number of interrogated variable regions. This is outlined in Table 1.

The QIAseq 16S/ITS Panels offer a 16S/ITS processing control (QIAseq 16S/ITS Smart Control) that monitors both proper library construction and contamination introduced by the environment or user. The process control is used as a sample during library preparation. After sequencing, any environmental contamination introduced can be identified after bacterial/fungal classification.

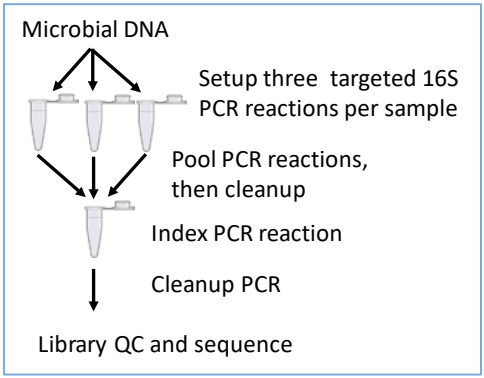

Figure 1. Schematic of QIAseq 16S/ITS Panel workflow. The number of targeted 16S PCR reactions depends on the number of required primer pools. The QIAseq 16S/ITS Screening Panel requires 3 targeted 16S PCR reactions, because 3 primer pools are required.

Table 1. MiSeq Sequencing Kit compatibility and sample multiplexing guideline

Panel	MiSeq Reagent Kit*	No. of samples†	Library concentration loaded on MiSeq
Screening Kit	v3 (600-cycle kit)	12–24	10 pM
V1V2	v2 (500-cycle kit)/ v3 (600-cycle kit)	24–96 (up to 384‡)	v2: 6 pM, v3: 10 pM
V2V3	v3 (600-cycle kit)	24–96 (up to 384‡)	10 pM
V3V4	v3 (600-cycle kit)	24–96 (up to 384‡)	10 pM
V4V5	v2 (500-cycle kit)/ v3 (600-cycle kit)	24–96 (up to 384‡)	v2: 6 pM, v3: 10 pM
V5V7	v2 (500-cycle kit)/ v3 (600-cycle kit)	24–96 (up to 384‡)	v2: 6 pM, v3: 10 pM
V7V9	v2 (500-cycle kit)/ v3 (600-cycle kit)	24–96 (up to 384‡)	v2: 6 pM, v3: 10 pM
ITS	v2 (500-cycle kit)/ v3 (600-cycle kit)	24–96 (up to 384‡)	v2: 6 pM, v3: 10 pM

* If using MiSeq v3 Reagent Kit, setup with paired end read program = 276 x 2. If using MiSeq v2 Reagent Kit, setup with paired end program = 251 x 2.

† Number of samples multiplexed on each MiSeq sequencing run depends on sample type and complexity. This needs to be experimentally determined.

‡ If multiplexing more than 96 samples, use v3 (600 cycle) MiSeq Reagent Kit.

Data analysis

Sequencing is performed on an Illumina MiSeq NGS system using a v2 kit with 251 x 2 paired end run or a v3 kit with 276 x 2 paired end run. It is highly recommended to perform sequencing data analysis with the CLC Genomics Workbench with Microbial Genomics Module and QIAseq 16S Panel Analysis. Please refer to “Protocol: Data Analysis Using CLC Microbial Genomics Module”, page 38. Alternatively, the generated FASTQ files can be analyzed with open-source 16S data analysis software. Importantly, if the QIAseq 16S/ITS Screening Panel or multiple region panels are used, the individual amplicons must be demultiplexed before bacterial/fungal classification. This can be performed within the CLC Microbial Genomics Module.

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.

- High-quality nuclease-free water
- Nuclease-free pipette tips and tubes
- LoBind® microfuge tubes (1.5 ml)
- PCR tubes (0.2 ml individual tubes or tube strips) (VWR, cat. no. 20170-012 or 93001-118)
- Ice
- Microcentrifuge
- Thermal cycler
- MagneSphere® Technology Magnetic Separation Stand (Promega, cat. no. Z5342) or DynaMag™-2 Magnet (Thermo Fisher Scientific, cat. no. 12321D)

  Library QC:

  Agilent® 2100 Bioanalyzer: High Sensitivity DNA Kit (Agilent, cat. no. 5067-4626) or 4200 TapeStation: High Sensitivity D1000 ScreenTape® (Agilent, cat. No. 5067-5584)

  Preferred library quantification method:

  qPCR instrument and QIAseq Library Quant System: QIAseq Library Quant Array Kit (QIAGEN, cat. no. 333304) or QIAseq Library Quant Assay Kit (QIAGEN, cat. no. 333314)

Important Notes

DNA preparation and quality control

High-quality DNA is essential for obtaining optimal sequencing results. The most important prerequisite for DNA sequence analysis is consistent, high-quality DNA from every experimental sample. Therefore, sample handling and DNA isolation procedures are critical to the success of the experiment. Residual traces of proteins, salts, or other contaminants may either degrade the DNA or decrease the efficiency of – if not block completely – the enzyme activities necessary for optimal targeted DNA amplification.

DNA quantification and quality control

For best results, all DNA samples should also demonstrate consistent quality according to the following criteria:

- Concentration and purity determined by UV spectrophotometry

  The concentration and purity of DNA should be determined by measuring absorbance using a spectrophotometer such as a Nanodrop®. Prepare dilutions and measure absorbance in 10 mM Tris·Cl, pH 8.0. The spectral properties of nucleic acids are highly dependent on pH.

- *A*260:*A*280 ratio should be greater than 1.8.

  If QIAseq 16S/ITS Screening Panel is to be used, then follow "Protocol: QIAseq 16S/ITS Screening Panel", page 17. If a single 16S/ITS region or multiple regions are chosen, then follow "Protocol: QIAseq 16S/ITS Region Panels", page 24.

96-sample indexing for QIAseq 16S/ITS Panels on Illumina MiSeq Platform

The indices RS-D501 to RS-D508 (for p5-RS2-ID#) are adapted from the TruSeq® HT/TruSeq CD index D501–D508. The indices FS-D701 to FS-D712 (for p7-FS2-ID#) are adapted from the TruSeq HT indices D701-D712.

For QIAseq 16S/ITS 96-Index I (HT Array), the sample index PCR reaction primer pairs (single p5-RS2-ID# and single p7-FS2-ID# primer) are dried down in each well. Refer to Table 2 for unique dual index layout.

Table 2. Illumina dual indexed HT array layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	D701-D501	D702-D501	D703-D501	D704-D501	D705-D501	D706-D501	D707-D501	D708-D501	D709-D501	D710-D501	D711-D501	D712-D501
B	D701-D502	D702-D502	D703-D502	D704-D502	D705-D502	D706-D502	D707-D502	D708-D502	D709-D502	D710-D502	D711-D502	D712-D502
C	D701-D503	D702-D503	D703-D503	D704-D503	D705-D503	D706-D503	D707-D503	D708-D503	D709-D503	D710-D503	D711-DD503	D712-D503
D	D701-D504	D702-D504	D703-D504	D704-D504	D705-D504	D706-D504	D707-D504	D708-D504	D709-D504	D710-D504	D711-D504	D712-D504
E	D701-D505	D702-D505	D703-D505	D704-D505	D705-D505	D706-D505	D707-D505	D708-D505	D709-D505	D710-D505	D711-D505	D712-D505
F	D701-D506	D702-D506	D703-D506	D704-D506	D705-D506	D706-D506	D707-D506	D708-D506	D709-D506	D710-D506	D711-D506	D712-D506
G	D701-D507	D702-D507	D703-D507	D704-D507	D705-D507	D706-D507	D707-D507	D708-D507	D709-D507	D710-D507	D711-D507	D712-D507
H	D701-D508	D702-D508	D703-D508	D704-D508	D705-D508	D706-D508	D707-D508	D708-D508	D709-D508	D710-D508	D711-D508	D712-D508

384-sample indexing for QIAseq 16S/ITS Panels on Illumina MiSeq Platform

For QIAseq 16S/ITS 384-Index I (HT Array), the sample index PCR reaction primer pairs (single S50X and single N7XX primer) are dried down in each well. The indices S502–S522 and N701–N729 are adapted from Nextera®XT v2 indices. Refer to tables 3–6 for unique dual index layout for each plate set.

Table 3. Unique dual index layout for plate set A

	1	2	3	4	5	6	7	8	9	10	11	12
A	N701-S502	N702-S502	N703-S502	N704-S502	N705-S502	N706-S502	N707-S502	N710-S502	N711-S502	N712-S502	N714-S502	N715-S502
B	N701-S503	N702-S503	N703-S503	N704-S503	N705-S503	N706-S503	N707-S503	N710-S503	N711-S503	N712-S503	N714-S503	N715-S503
C	N701-S505	N702-S505	N703-S505	N704-S505	N705-S505	N706-S505	N707-S505	N710-S505	N711-S505	N712-S505	N714-S505	N715-S505
D	N701-S506	N702-S506	N703-S506	N704-S506	N705-S506	N706-S506	N707-S506	N710-S506	N711-S506	N712-S506	N714-S506	N715-S506
E	N701-S507	N702-S507	N703-S507	N704-S507	N705-S507	N706-S507	N707-S507	N710-S507	N711-S507	N712-S507	N714-S507	N715-S507
F	N701-S508	N702-S508	N703-S508	N704-S508	N705-S508	N706-S508	N707-S508	N710-S508	N711-S508	N712-S508	N714-S508	N715-S508
G	N701-S510	N702-S510	N703-S510	N704-S510	N705-S510	N706-S510	N707-S510	N710-S510	N711-S510	N712-S510	N714-S510	N715-S510
H	N701-S511	N702-S511	N703-S511	N704-S511	N705-S511	N706-S511	N707-S511	N710-S511	N711-S511	N712-S511	N714-S511	N715-S511

Table 4. Unique dual index layout for plate set B

	1	2	3	4	5	6	7	8	9	10	11	12
A	N716-S502	N718-S502	N719-S502	N720-S502	N721-S502	N722-S502	N723-S502	N724-S502	N726-S502	N727-S502	N728-S502	N729-S502
B	N716-S503	N718-S503	N719-S503	N720-S503	N721-S503	N722-S503	N723-S503	N724-S503	N726-S503	N727-S503	N728-S503	N729-S503
C	N716-S505	N718-S505	N719-S505	N720-S505	N721-S505	N722-S505	N723-S505	N724-S505	N726-S505	N727-S505	N728-S505	N729-S505
D	N716-S506	N718-S506	N719-S506	N720-S506	N721-S506	N722-S506	N723-S506	N724-S506	N726-S506	N727-S506	N728-S506	N729-S506
E	N716-S507	N718-S507	N719-S507	N720-S507	N721-S507	N722-S507	N723-S507	N724-S507	N726-S507	N727-S507	N728-S507	N729-S507
F	N716-S508	N718-S508	N719-S508	N720-S508	N721-S508	N722-S508	N723-S508	N724-S508	N726-S508	N727-S508	N728-S508	N729-S508
G	N716-S510	N718-S510	N719-S510	N720-S510	N721-S510	N722-S510	N723-S510	N724-S510	N726-S510	N727-S510	N728-S510	N729-S510
H	N716-S511	N718-S511	N719-S511	N720-S511	N721-S511	N722-S511	N723-S511	N724-S511	N726-S511	N727-S511	N728-S511	N729-S511

Table 5. Unique dual index layout for plate set C

	1	2	3	4	5	6	7	8	9	10	11	12
A	N701-S513	N702-S513	N703-S513	N704-S513	N705-S513	N706-S513	N707-S513	N710-S513	N711-S513	N712-S513	N714-S513	N715-S513
B	N701-S515	N702-S515	N703-S515	N704-S515	N705-S515	N706-S515	N707-S515	N710-S515	N711-S515	N712-S515	N714-S515	N715-S515
C	N701-S516	N702-S516	N703-S516	N704-S516	N705-S516	N706-S516	N707-S516	N710-S516	N711-S516	N712-S516	N714-S516	N715-S516
D	N701-S517	N702-S517	N703-S517	N704-S517	N705-S517	N706-S517	N707-S517	N710-S517	N711-S517	N712-S517	N714-S517	N715-S517
E	N701-S518	N702-S518	N703-S518	N704-S518	N705-S518	N706-S518	N707-S518	N710-S518	N711-S518	N712-S518	N714-S518	N715-S518
F	N701-S520	N702-S520	N703-S520	N704-S520	N705-S520	N706-S520	N707-S520	N710-S520	N711-S520	N712-S520	N714-S520	N715-S520
G	N701-S521	N702-S521	N703-S521	N704-S521	N705-S521	N706-S521	N707-S521	N710-S521	N711-S521	N712-S521	N714-S521	N715-S521
H	N701-S522	N702-S522	N703-S522	N704-S522	N705-S522	N706-S522	N707-S522	N710-S522	N711-S522	N712-S522	N714-S522	N715-S522

Table 6. Unique dual index layout for plate set D

	1	2	3	4	5	6	7	8	9	10	11	12
A	N716-S513	N718-S513	N719-S513	N720-S513	N721-S513	N722-S513	N723-S513	N724-S513	N726-S513	N727-S513	N728-S513	N729-S513
B	N716-S515	N718-S515	N719-S515	N720-S515	N721-S515	N722-S515	N723-S515	N724-S515	N726-S515	N727-S515	N728-S515	N729-S515
C	N716-S516	N718-S516	N719-S516	N720-S516	N721-S516	N722-S516	N723-S516	N724-S516	N726-S516	N727-S516	N728-S516	N729-S516
D	N716-S517	N718-S517	N719-S517	N720-S517	N721-S517	N722-S517	N723-S517	N724-S517	N726-S517	N727-S517	N728-S517	N729-S517
E	N716-S518	N718-S518	N719-S518	N720-S518	N721-S518	N722-S518	N723-S518	N724-S518	N726-S518	N727-S518	N728-S518	N729-S518
F	N716-S520	N718-S520	N719-S520	N720-S520	N721-S520	N722-S520	N723-S520	N724-S520	N726-S520	N727-S520	N728-S520	N729-S520
G	N716-S521	N718-S521	N719-S521	N720-S521	N721-S521	N722-S521	N723-S521	N724-S521	N726-S521	N727-S521	N728-S521	N729-S521
H	N716-S522	N718-S522	N719-S522	N720-S522	N721-S522	N722-S522	N723-S522	N724-S522	N726-S522	N727-S522	N728-S522	N729-S522

Custom sequencing primers for Illumina platform

For QIAseq 16S/ITS panels, custom sequencing primers are required for both R1 and R2 to start the reads at the phased primers. Refer to “Protocol: Sequencing Setup on Illumina MiSeq”, page 34.

For further information, please refer to the Illumina website and search for “miseq-system-custom-primers-guide-15041638-01” for a guide on loading custom primers on a MiSeq.

Protocol: QIAseq 16S/ITS Screening Panel

This protocol is designed for the amplification of all 16S/ITS rRNA variable regions.

Important points before starting

- Use high quality DNA.
- If DNA concentration is >1 ng/µl, then dilute to 1 ng/µl. If DNA concentration is ≤1 ng/µl, then use 1 µl of sample per PCR reaction.
- Ensure reactions are thoroughly mixed, as well as prepared and incubated at the recommended temperatures.
- 80% ethanol should be freshly prepared using nuclease-free water and mixed thoroughly by vortexing.
- QIAseq Beads need to be homogenous. Thoroughly resuspend the beads immediately before use and process the beads quickly. If a delay in the protocol occurs, simply vortex the beads again.

Procedure

1. Thaw on ice the QIAseq 16S/ITS Screening Panel Pool 1, QIAseq 16S/ITS Screening Panel Pool 2, QIAseq Screening 16S/ITS Panel Pool 3, UCP Multiplex Master Mix, UCP PCR Water, and samples.
2. On ice, prepare 3 PCR reactions per gDNA sample by following Table 7. Briefly centrifuge, mix by pipetting up and down 10 times, and briefly centrifuge again.

Table 7. Preparation of QIAseq 16S/ITS Screening Panel PCR

Component	Panel pool 1	Panel pool 2	Panel pool 3
Microbial DNA sample*	1 µl	1 µl	1 µl
UCP Multiplex Master Mix	2.5 µl	2.5 µl	2.5 µl
Panel pool 1	1 µl	–	–
Panel pool 2	–	1 µl	–
Panel pool 3	–	–	1 µl
UCP PCR Water	5.5 µl	5.5 µl	5.5 µl
Total volume	**10 µl**	**10 µl**	**10 µl**

* The QIAseq 16S/ITS Smart Control can be added instead of a microbial DNA sample. Refer to Appendix A: Preparation and Use of QIAseq 16S/ITS Smart Control.

3. Incubate the reactions in a thermal cycler as described in Table 8.

Table 8. Setup of QIAseq 16S/ITS PCR reaction

Step	Time	Temperature
Hold	2 min	95°C
3-step cycling		
Denaturation	30 s	95°C
Annealing	30 s	50°C
Extension	2 min	72°C
12 cycles*		
Final extension (1 cycle)	7 min	72°C
Hold	∞	4°C

* If samples contain low bacterial/fungal content, use 20 cycles of 16S PCR.

4. Remove the tubes/plate from the thermal cycler and briefly centrifuge.
5. Add 20 µl of UCP PCR Water to each of the PCR reactions.

6. Pool the PCR reactions from the same microbial DNA sample into a single LoBind tube or the well of a 96-well PCR plate. The total volume for each microbial DNA sample should be 90 µl.
7. Add 1.1x volume (99 µl) of QIAseq Beads to each combined sample from step 5. Mix well by pipetting up and down 12 times and then briefly centrifuge.
8. Incubate for 5 min at room temperature.
9. Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.
10. Centrifuge briefly and carefully remove any residual liquid. Add 55 µl of nuclease-free water. Mix by pipetting up and down 12 times until the beads are fully resuspended. Incubate for 2–5 min at room temperature.

    Note: UCP PCR Water is not needed at this step.
11. Return the tubes/plate to the magnetic rack until the solution has cleared, and then carefully transfer 50 µl of the supernatant that contains the 16S/ITS PCR product to new tubes or plate.
12. Add 1.1x volume (55 µl) of QIAseq Beads to each sample. Mix well by pipetting up and down 12 times and briefly centrifuge.
13. Incubate for 5 min at room temperature.
14. Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.

    Note: Keep the beads on the magnetic stand while handling the supernatant and during the washing steps.

    Important: Do not discard the beads, because they contain the DNA of interest.
15. Add 200 µl of 80% ethanol while it is on the magnetic rack/plate. Rotate the tube (3 times) or move the plate side to side in the 2 positions of the magnet to wash the beads. Carefully remove and discard the wash.

16. Repeat the ethanol wash as in step 15.

    Important: Completely remove all traces of the ethanol wash after the second wash. To do this, briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol first with a 200 µl pipette, and then use a 10 µl pipette to remove any residual ethanol.

17. With the tubes/plate still on the magnet, air-dry at room temperature for 5 min (up to 15 min when using a plate).

    Note: Visually inspect that the pellet is completely dry. When completely dry, the beads should have a "cracked" appearance.

18. Remove the tubes/plate from the magnetic stand and elute the DNA from the beads by adding 35 µl UCP PCR Water. Mix well by pipetting. Incubate for 2–5 min at room temperature.

19. Return the tubes/plate to the magnetic rack until the solution has cleared.

20. Transfer 32.5 µl of the supernatant to clean tubes/plate.

21. Proceed to "Protocol: Preparation of QIAseq 16S/ITS Screening Panel Sample Index PCR Reaction", page 21. Alternatively, the cleaned-up products can be stored at –20°C in a constant-temperature freezer.

Protocol: Preparation of QIAseq 16S/ITS Screening Panel Sample Index PCR Reaction

This protocol uses the product from “Protocol: QIAseq 16S/ITS Screening Panel”, page 17.

In this protocol, sample indices and sequencing adaptors are added.

Important points before starting

- Set up the QIAseq 16S/ITS Screening Panel Sample Index PCR Reactions on ice.
- Ensure reactions are thoroughly mixed, as well as prepared and incubated at the recommended temperatures.
- 80% ethanol should be freshly prepared using nuclease-free water and mixed thoroughly by vortexing.
- QIAseq Beads need to be homogenous. Thoroughly resuspend the beads immediately before use and process the beads quickly. If a delay in the protocol occurs, simply vortex the beads again.

Procedure

1. Thaw on ice the required QIAseq 16S/ITS Indices, UCP Multiplex Master Mix, and UCP PCR Water.
2. Place on ice the tubes/plate containing the 16S PCR product.
3. Add the components to the tubes/plate containing the 16S PCR product according to Table 9. If using the HT array sample index format, add sample and UCP Multiplex Master Mix to the corresponding well of the index plate. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

   Important: To prevent contamination of UCP Multiplex Master Mix, aliquot a sufficient volume for all samples to a sterile tube and then transfer the required amount to each sample.

Table 9. Preparation of QIAseq 16S/ITS Sample Index PCR Reaction

Component	Per sample	HT array (96- or 384-index kit)
16S PCR product	32.5 µl	32.5 µl
UCP Multiplex Master Mix	12.5 µl	12.5 µl
p5-RS2-ID# (4 µM)*	2.5 µl	-
p7-FS2-ID# (4 µM)*	2.5 µl	-
UCP Water	-	5 µl
Total volume	**50 µl**	**50 µl**

* Use a unique p5-RS2-ID# + p7-FS2-ID# combination for each microbial DNA sample.

4. Incubate the reactions in a thermal cycler according to Table 10.

Table 10. Setup of QIAseq 16S/ITS Sample Index PCR Reaction

Step	Time	Temperature
Hold	2 min	95°C
3-step cycling		
Denaturation	30 s	95°C
Annealing	30 s	60°C
Extension	2 min	72°C
14 cycles		
Final extension (1 cycle)	7 min	72°C
Hold	∞	4°C

5. Remove the tubes/plate from the thermal cycler and briefly centrifuge.
6. Add 0.9X volume (45 µl) of QIAseq Beads to each sample from step 5. Mix well by pipetting up and down 12 times and briefly centrifuge.
7. Incubate for 5 min at room temperature.

8. Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.

   Note: Keep the beads on the magnetic stand while handling the supernatant and during the washing steps.

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

9. Add 200 µl of 80% ethanol while it is on the magnetic rack/plate. Rotate the tube (3 times) or move the plate side to side in the 2 positions of the magnet to wash the beads. Carefully remove and discard the wash.

10. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol wash after the second wash. To do this, briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol first with a 200 µl pipette, and then use a 10 µl pipette to remove any residual ethanol.

11. With the tubes/plate still on the magnet, air-dry at room temperature for 5 min (up to 15 min when using a plate).

    Note: Visually inspect that the pellet is completely dry. When completely dry, the beads should have a "cracked" appearance.

12. Remove the tubes/plate from the magnetic stand, and elute the DNA from the beads by adding 30 µl nuclease-free water. Mix well by pipetting. Incubate for 2–5 min at room temperature.

    Note: UCP PCR Water is not needed at this step.

13. Return the tubes/plate to the magnetic rack until the solution has cleared.

14. Transfer 25 µl of the supernatant to clean tubes/plate. This is the final Microbial 16S/ITS Screening Panel Sequencing Library.

15. Proceed to "Protocol: Library QC and Quantification", page 31. Alternatively, the completed QIAseq 16S/ITS Screening Panel Sequencing Library can be stored at –20°C in a constant-temperature freezer.

Protocol: QIAseq 16S/ITS Region Panels

This protocol is designed for the amplification of specific 16S/ITS rRNA regions.

Important points before starting

- Use high quality DNA.
- If DNA concentration is >1 ng/µl, then dilute to 1 ng/µl. If DNA concentration is <1 ng/µl, then use 1 µl of sample per PCR reaction.
- Depending on how many and which 16S/ITS regions, the number of 16S/ITS Region PCR reactions ranges from 1 to 3 per sample. Check Table 11 to determine the number of PCR reactions per samples to set up.
- Ensure reactions are thoroughly mixed, as well as prepared and incubated at the recommended temperatures.
- 80% ethanol should be freshly prepared using nuclease-free water and mixed thoroughly by vortexing.
- QIAseq Beads need to be homogenous. Thoroughly resuspend the beads immediately before use, and process the beads quickly. If a delay in the protocol occurs, simply vortex the beads again.

Procedure

1. If more than one 16S region is to be sequenced, use Table 11 to determine the number of PCR reactions per sample. At the same time, only certain regions can be multiplexed together in the same PCR reaction.

Table 11. QIAseq 16S/ITS Region Multiplex Guideline

	V1V2	V2V3	V3V4	V4V5	V5V7	V7V9	ITS
V1V2	–	X	X	Yes	Yes	Yes	Yes
V2V3	X	–	X	X	Yes	Yes	Yes
V3V4	X	X	–	X	X	Yes	Yes
V4V5	Yes	X	X	–	X	X	Yes
V5V7	Yes	Yes	X	X	–	X	Yes
V7V9	Yes	Yes	Yes	X	X	–	Yes
ITS	Yes	Yes	Yes	Yes	Yes	Yes	–

Important: Choose one relevant 16S region from the vertical column and then choose one 16S region from the horizontal row. If marked with "Yes", then the primers for the 2 regions can be used in the same 16S PCR reaction (multiplexed). If marked with "X", then they must be in separate 16S PCR reactions. Count the number of required 16S PCR reactions and continue to step 2.
Note: ITS can be mixed with any 16S region.

2. Thaw on ice the QIAseq 16S/ITS Region Panel(s), UCP Multiplex Master Mix, UCP PCR Water, and samples.

   On ice, prepare the required number of PCR reactions per gDNA sample by following Table 12. Briefly centrifuge, mix by pipetting up and down 10 times, and briefly centrifuge again.

Table 12. Preparation of QIAseq 16S/ITS Region PCR

	Per sample		
Component	**Panel pool 1**	**Panel pool 2 (optional)**	**Panel pool 3 (optional)**
Microbial DNA sample*	1 µl	1 µl	1 µl
UCP Master Mix	2.5 µl	2.5 µl	2.5 µl
Region Panel 1	1 µl	1 µl	1 µl
Region Panel 2	Optional (1 µl)	Optional (1 µl)	Optional (1 µl)
Region Panel 3	Optional (1 µl)	Optional (1 µl)	Optional (1 µl)
UCP PCR Water	Varies	Varies	Varies
Total volume	**10 µl**	**10 µl**	**10 µl**

*The QIAseq 16S/ITS Smart Control can be added instead of a microbial DNA sample. Refer to Appendix A: Preparation and Use of Smart Control.

3. Incubate the reactions in a thermal cycler as described in Table 13.

Table 13. Setup of QIAseq16S/ITS Region PCR Reaction

Step	Time	Temperature
Hold	2 min	95°C
3-step cycling		
Denaturation	30 s	95°C
Annealing	30 s	50°C
Extension	2 min	72°C
12 cycles*		
Final extension (1 cycle)	7 min	72°C
Hold	∞	4°C

*If samples contain low bacterial/fungal content, use 20 cycles of 16S PCR.

4. Remove the tubes/plate from the thermal cycler and briefly centrifuge.
5. If the sample requires multiple PCR reactions, add 20 µl UCP PCR Water to each PCR reaction, and pool PCR reactions from same sample into a single LoBind tube or the well of a PCR plate. If there is only one PCR reaction per sample, add 40 µl UCP PCR Water.
6. According to the number of PCR reactions per sample, add 1.1x volume of QIAseq Beads to each sample. Mix well by pipetting up and down 12 times.
   - 1 PCR reaction per sample = add 55 µl of QIAseq Beads per tube/well
   - 2 PCR reactions per sample = add 66 µl of QIAseq Beads per tube/well
   - 3 PCR reactions per sample = add 99 µl of QIAseq Beads per tube/well
7. Incubate for 5 min at room temperature.
8. Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.
9. Centrifuge briefly and carefully remove any residual liquid.
10. Add 55 µl of nuclease-free water. Mix by pipetting up and down 12 times until the beads are fully resuspended. Incubate for 2–5 min at room temperature.

    Note: UCP PCR Water is not needed at this step.

11. Return tubes/plate to magnetic rack until solution has cleared, and then carefully transfer 50 µl of the supernatant that contains the 16S/ITS PCR product to new tubes or plate.
12. Add 1.1x volume (55 µl) of QIAseq Beads to each sample. Mix well by pipetting up and down 12 times.
13. Incubate for 5 min at room temperature.
14. Place the tube/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.

    Note: Keep the beads on the magnetic stand while handling the supernatant and during the washing steps.

    Important: Do not discard the beads, because they contain the DNA of interest.
15. Add 200 µl of 80% ethanol while it is on the magnetic rack/plate. Rotate the tube (3 times) or move the plate side to side in the 2 positions of the magnet to wash the beads. Carefully remove and discard the wash.
16. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol wash after the second wash. To do this, briefly centrifuge and then return the tubes or plate to the magnetic stand. Remove the ethanol first with a 200 µl pipette, and then use a 10 µl pipette to remove any residual ethanol.
17. With the tubes/plate still on the magnet, air-dry at room temperature for 5 min (up to 15 min when using a plate).

    Note: Visually inspect that the pellet is completely dry. When completely dry, the beads should have a "cracked" appearance.
18. Remove tubes/plate from magnetic stand, and elute DNA from beads by adding 35 µl UCP PCR Water. Mix well by pipetting. Incubate for 2–5 min at room temperature.
19. Return tubes/plate to magnetic rack until the solution has cleared.
20. Transfer 32.5 µl of the supernatant to clean tubes/plate.
21. Proceed to "Protocol: Preparation of QIAseq 16S/ITS Region Panel Sample Index PCR Reaction", page 28. Alternatively, the cleaned-up PCR products can be stored at –20°C in a constant-temperature freezer.

Protocol: Preparation of QIAseq 16S/ITS Region Panel Sample Index PCR Reaction

This protocol uses the product from “Protocol: QIAseq 16S/ITS Region Panels”, page 24.

In this protocol, sample indices and sequencing adaptors are added.

Important points before starting

- Set up the QIAseq 16S/ITS Region Panel Sample Index PCR Reactions on ice.
- Ensure reactions are thoroughly mixed, as well as prepared and incubated at the recommended temperatures.
- 80% ethanol should be freshly prepared using nuclease-free water.
- QIAseq Beads need to be homogenous. Thoroughly resuspend the beads immediately before use, and process the beads quickly. If a delay in the protocol occurs, simply vortex the beads again.

Procedure

1. Thaw the required QIAseq 16S/ITS Indices, UCP Multiplex Master Mix and UCP PCR Water.
2. Place on ice the tubes/plate containing the QIAseq 16S/ITS Region Panel PCR product.
3. Add the components to the tubes/plate containing the 16S PCR product according to Table 14. If using the HT array sample index format, add sample and UCP Multiplex Master Mix to the corresponding well of the index plate. Briefly centrifuge, mix by pipetting up and down 12 times, and centrifuge briefly again.

   Important: To prevent contamination of the low bioburden Master Mix, aliquot a sufficient volume for all samples to a sterile tube, and then transfer the required amount to each sample.

Table 14. Preparation of QIAseq 16S/ITS Region Panel Sample Index PCR Reaction

Step	Per sample	HT array (96- or 384-index kit)
16S/ITS Region Panel PCR Product	32.5 µl	32.5 µl
UCP Master Mix	12.5 µl	12.5 µl
p5-RS2-ID# (4 µM)*	2.5 µl	–
p7-FS2-ID# (4 µM)*	2.5 µl	–
UCP Water	–	5 µl
Total volume	**50 µl**	**50 µl**

* Use a unique p5-RS2-ID# + p7-FS2-ID# combination for each microbial DNA sample.

4. Incubate the reactions in a thermal cycler according to Table 15.

Table 15. Preparation of QIAseq 16S/ITS Region Panel Sample Index PCR Reaction

Step	Time	Temperature
Hold	2 min	95°C
3-step cycling		
Denaturation	30 s	95°C
Annealing	30 s	60°C
Extension	2 min	72°C
19 cycles		
Final extension (1 cycle)	7 min	72°C
Hold	∞	4°C

5. Remove the tubes/plate from the thermal cycler and briefly centrifuge.
6. Add 0.9x volume (45 µl) of QIAseq Beads to each sample from step 5. Mix well by pipetting up and down 12 times.
7. Incubate for 5 min at room temperature.

8. Place the tubes/plate on a magnetic rack for 5 min. After the solution has cleared, carefully remove and discard the supernatant.

   Note: Keep the beads on the magnetic stand while handling the supernatant and during the washing steps.

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

9. Add 200 µl of 80% ethanol while it is on the magnetic rack/plate. Rotate the tube (3 times) or move the plate side to side in the 2 positions of the magnet to wash the beads. Carefully remove and discard the wash.

10. Repeat the ethanol wash.

    Important: Completely remove all traces of the ethanol wash after the second wash. To do this, briefly centrifuge and return the tubes or plate to the magnetic stand. Remove the ethanol first with a 200 µl pipette, and then a 10 µl pipette to remove any residual ethanol.

11. With the tubes/plate still on the magnet, air-dry at room temperature for 5 min (up to 15 min when using a plate).

    Note: Visually inspect that the pellet is completely dry. When completely dry, the beads should have a "cracked" appearance.

12. Remove the tubes/plate from the magnetic stand, and elute the DNA from the beads by adding 30 µl nuclease-free water. Mix well by pipetting. Incubate for 2–5 min at room temperature.

    Note: UCP PCR Water is not needed at this step.

13. Return the tubes/plate to the magnetic rack until the solution has cleared.

14. Transfer 25 µl of the supernatant to clean tubes/plate. This is the final 16S/ITS Region Panel Sequencing Library.

15. Proceed to "Protocol: Library QC and Quantification", page 31. Alternatively, the completed QIAseq 16S/ITS Region Panel Sequencing Library can be stored at –20°C in a constant-temperature freezer.

Protocol: Library QC and Quantification

This protocol determines the quality and quantity of each QIAseq 16S/ITS library.

Important points before starting

- A portion of either the QIAseq 16S/ITS Screening Panel or QIAseq 16S/ITS Region Panel, 25 µl total volume of sequencing library, is the starting material for the library QC and quantification. When not in use, the QIAseq 16S/ITS Screening or Region Panel sequencing library should be stored on ice.
- Library QC involves the use of an Agilent 2100 Bioanalyzer or TapeStation.
- Library quantification involves the use of QIAGEN's QIAseq Library Quant System: QIAseq Library Quant Array Kit (cat. no. 333304) or QIAseq Library Quant Assay Kit (cat. no. 333314).

Library QC (Agilent 2100 Bioanalyzer or TapeStation)

1. Analyze 1 µl of the QIAseq 16S/ITS Screening Panel or QIAseq 16S/ITS Region Panel sequencing library on an Agilent Bioanalyzer or TapeStation using a high-sensitivity DNA chip according to the manufacturer's instructions. A typical QIAseq 16S/ITS Screening Panel or QIAseq 16S/ITS Region Panel sequencing library is shown in Figure 2. Figure 3 shows a library that has been constructed from the QIAseq 16S/ITS Smart Control. These libraries have an extra ~440 base pair peak present as this represents the ITS amplicon, which is at equal abundance to the other 16S amplicons.

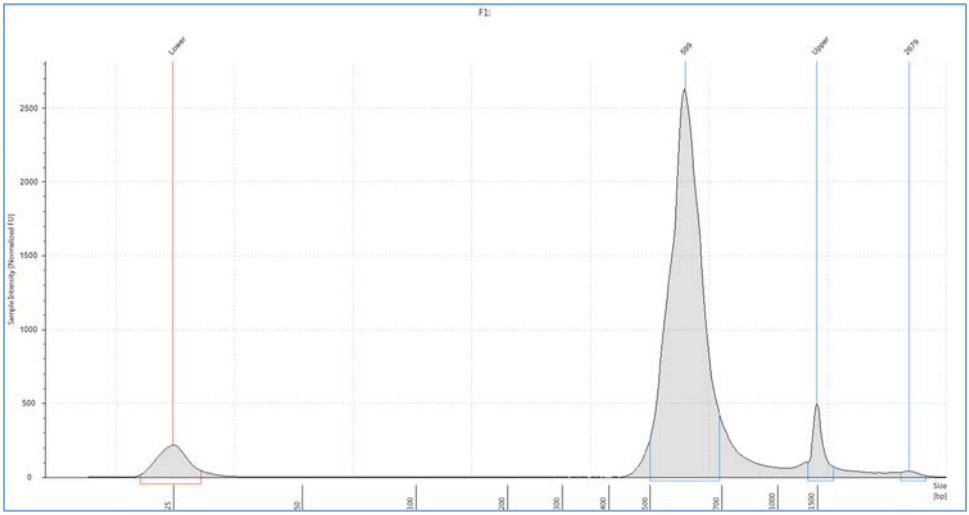

Figure 2. Bioanalyzer trace of library prepared with QIAseq 16S/ITS Screening Panel or QIAseq 16S/ITS Region Panel.

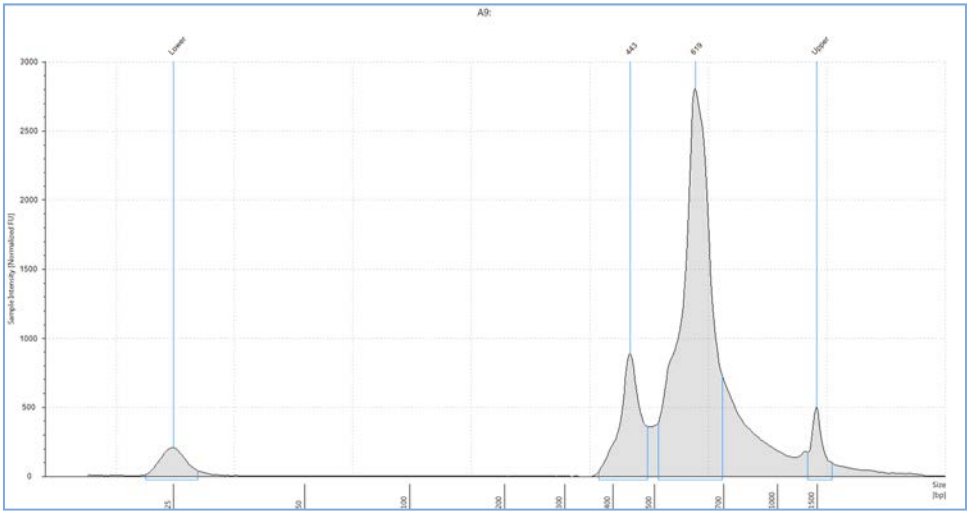

Figure 3. Bioanalyzer trace of library prepared with QIAseq 16S/ITS Smart Control (as DNA sample) and the QIAseq 16S/ITS Screening Panel.

2. Proceed to “Library Quantification” on the next page.

Library Quantification

3. The library yield measurements of the Bioanalyzer or TapeStation system use fluorescent dyes that intercalate into DNA or RNA and cannot discriminate between DNA with or without adapter sequences. Real-time PCR-based methods provide an accurate quantification of complete QIAseq 16S/ITS Screening Panel or QIAseq 16S/ITS Region Panel sequencing libraries with full adapter sequences. Therefore, QIAGEN's QIAseq Library Quant Array Kit or Assay Kit, which contains laboratory-verified forward and reverse primers, together with a DNA standard, is highly recommended for accurate quantification of the prepared library.

   2 nM of QIAseq 16S/ITS Screening Panel or QIAseq 16S/ITS Region Panel libraries should be used as input for the denaturation procedure to load the MiSeq sequencing instrument.

4. Proceed with "Protocol: Sequencing Setup on Illumina MiSeq", page 34.

Protocol: Sequencing Setup on Illumina MiSeq

Important points before starting

- Recommendations for library dilution concentrations and library loading concentrations are based on QIAGEN's QIAseq Library Quant System (see "Protocol: Library QC and Quantification", page 31).
- **Important**: QIAseq Read1 Primer (Custom Read1 Sequencing Primer) and QIAseq 16S/ITS Read 2 Primer (Custom Read2 Sequencing Primer) must be used when performing sequencing on an Illumina platform.
- Custom Read Primers go into the following specific MiSeq reagent cartridge positions:
  - QIAseq Read1 Primer: MiSeq Position #18
  - QIAseq 16S/ITS Read 2 Primer: MiSeq Position #20
- Paired-end sequencing must be used for the QIAseq 16S/ITS Screening Panel or QIAseq 16S/ITS Region Panel on an Illumina platform.
- For complete instructions on how to denature sequencing libraries, prepare custom index primers, and set up a sequencing run, please refer to the system-specific Illumina documents.

Sequencing preparations for MiSeq

1. Sample sheet setup:

   Set up a sample sheet with Custom Sequencing Read 1 Primer and Custom Sequencing Read 2 Primer using Illumina Experiment Manager v1.2.

   Note: If using Illumina Experiment Manager v1.4 or later, see Appendix B: Setting Up with Updated Version of Illumina Experiment Manager.

   The QIAseq 16S/ITS Screening Panel or QIAseq 16S/ITS Region Panel Sample Indices are compatible with Illumina's TruSeq HT adapter sample index system.

Select and check the parameters as follows (Figures 4a and 4b):

- Category: **Other**
- Select Application: **FASTQ Only**
- Library Prep Kit: **TruSeq HT** (384-index kit use **Nextera XT v2**)
- Index Reads: **2**
- Read Type: **Paired End**
- Cycles for Read 1: **276** (**251** if using MiSeq v2 500 cycle kit)
- Cycles for Read 2: **276** (**251** if using MiSeq v2 500 cycle kit)
- **Important**: Check "Custom Primer for Read 1"
- **Important**: Check "Custom Primer for Read 2"
- **Important**: Check "Use Adapter Trimming"
- **Important**: Check "Use Adapter Trimming Read 2"

Figure 4a. Sample Sheet Wizard using Illumina Experiment Manager. MiSeq Application Selection.

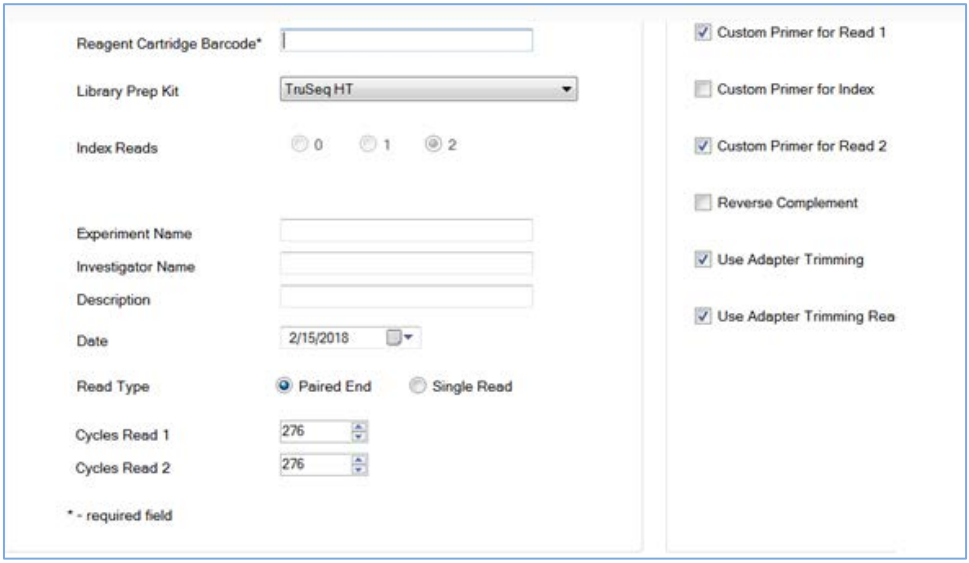

Figure 4b. Sample Sheet Wizard using Illumina Experiment Manager. Workflow parameters.

2. Sample dilution and pooling:

   Dilute the final libraries to 2 nM for the MiSeq.

   Then, combine libraries with different sample indices in equimolar amounts if similar sequencing depth is needed for each library.

   Note: Recommendations for the library dilution concentrations are based on the QIAseq Library Quant System.

3. Library preparation and loading:

   Prepare and load the library on a MiSeq according to the *MiSeq System Denature and Dilute Libraries Guide* (**sapac.support.illumina.com/downloads/prepare_libraries_for_sequencing_miseq_15039740.html**).

   The final denatured library concentration is 10 pM on a MiSeq (v3 kit) or 4 pM (v2 kit).

   Note: Recommendations for the library loading are based on the QIAseq Library Quant System.

4. Custom Sequencing Primer for Read 1 and Read 2 preparation and loading:

   Use 597 µl HT1 (Hybridization Buffer) to dilute 3 µl of QIAseq Read1 Primer to obtain a final concentration of 0.5 µM.

   Use 597 µl HT1 to dilute 3 µl of QIAseq Read2 Primer to obtain a final concentration of 0.5 µM.

   Load 600 µl of the diluted QIAseq Read1 Primer to Position #18, and load 600 µl of the diluted QIAseq Read2 Primer to Position #20 of the MiSeq reagent cartridge.

   For more details, refer to Illumina's *MiSeq System: Custom Primers Guide* (**sapac.support.illumina.com/downloads/miseq-system-custom-primers-guide-15041638.html**). Refer to Figure 5 for guidance on loading the primers into positions on the MiSeq Reagent Cartridge.

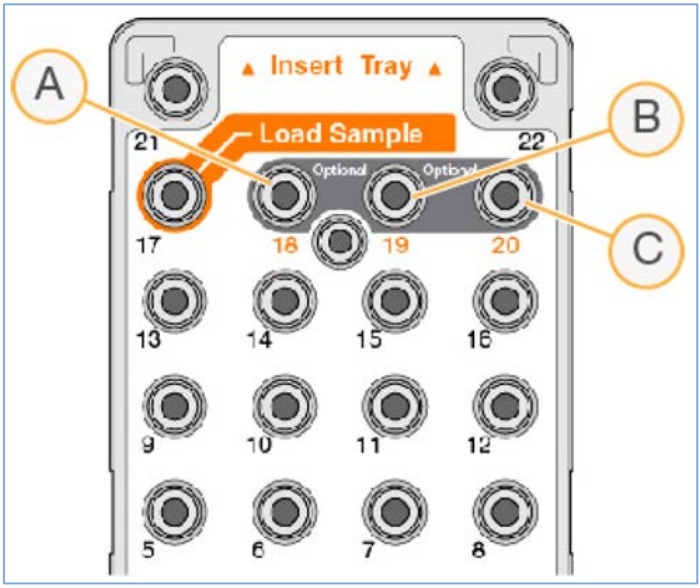

Figure 5. Loading the Primers into Positions on the MiSeq Reagent Cartridge. (A) Position 18 for Read1 Custom Primer. **(B)** Not used. **(C)** Position 20 for Read2 Custom Primer.

5. Upon completion of the sequencing run, proceed to "Protocol: Data Analysis Using CLC Microbial Genomics Module", page 38.

Protocol: Data Analysis Using CLC Microbial Genomics Module

Important points before starting

- If using the CLC Microbial Genomics Module, refer to the *CLC Microbial Genomics Module* User Manual (**resources.qiagenbioinformatics.com/manuals/clcmgm/current/User_Manual.pdf**).

Bacterial and Fungal Classification

1. Import FASTQ files.

   Import → **Illumina** → check **Paired reads** → select FASTQ files

2. Demultiplex amplicons (for use with Screening Panel or multiple Region Panels).

   Toolbox → **QIAGEN 16S Panel Analysis** → **Demultiplex Reads by Barcode** → If demultiplexing more than one sample, check **Batch** → Select FASTQ files → In Metadata table, select **barcodes all** file; in Barcode, select **Barcode** → check **Save**

3. Bacterial Classification

   Toolbox → **Microbial Genomics Module** → **Metagenomics** → **Amplicon Based OTU Clustering** → **Workflows** → **Data QC and OTU Clustering** → If Screening Panel or multiple Region Panels were used, select same region for all samples (i.e., Samples 1–24: V1V2) → Quality limit = 0.05 (default) → OTU picking = Reference based OTU → Select **OTU database** → Check **Save**

   If applicable, repeat for each region.

   After OTU clustering analysis, OTU tables can be merged into one table, if desired.

   Toolbox → **Microbial Genomics Module** → **Metagenomics** → **Abundance Analysis** → **Merge Abundance Tables**

4. Fungal Classification

   Toolbox → **Microbial Genomics Module** → **Metagenomics** → **Amplicon Based OTU Clustering** → **Workflows** → **Data QC and OTU Clustering** → Select demultiplexed FASTQ files. Select ITS region for each sample (i.e., Samples 1–24, ITS region) → Quality limit = 0.05 (default) → OTU picking = Reference based OTU → Select **UNITE** → Check **Save**

5. Comparing 16S variable regions – to compare performance of different 16S variable regions in terms of diversity.

   To determine which region contains the most OTU sequences and, therefore, potentially has the highest diversity, check the "Total predicted OTUs" in the OTU report for each region analysis.

   Alternatively, using the merged abundance table from step 3 (in Table view), select **Region** under "Aggregate sample." Then select the region that contains the highest number of features with greater than 5-10 reads at target taxonomy level (ie, family, genus or species). Also, alpha diversity can be measured to determine which region exhibits the highest diversity.

6. For more in-depth analysis, please refer to the *CLC Microbial Genomics Module* User Manual.

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 at 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 and/or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

		Comments and suggestions
Low library yield		
a)	DNA contains PCR inhibitors carried over from sample preparation	If samples contain high concentration of PCR inhibitors (such as stool or soil), use appropriate sample DNA extraction kit.
b)	Improper reaction setup	Ensure reactions are thoroughly mixed, prepared, and incubated at recommended temperatures.
c)	Excess ethanol not removed during bead cleanup steps	After each second ethanol wash, ensure that excess ethanol is removed. Briefly centrifuge and return the tubes or plate to the magnetic stand. Remove with a 10 µl pipette to remove any residual ethanol. In addition, allow beads to dry for the appropriate amount of time.
d)	Insufficient starting material	If starting material is significantly less than 1 ng bacterial or fungal DNA, up to 4 µl of microbial DNA sample can be added per QIAseq 16S/ITS reaction. Adjust UCP PCR Water so that total 16S/ITS PCR reaction volume equals 10 µl. Also, use 20 cycles at the 16S/ITS PCR step. If library yields are still low, then additional cycles (3–5) can be added at the sample index PCR step.
Primer-dimers observed		
a)	Excess ethanol not removed during bead cleanup steps	After each second ethanol wash, ensure that excess ethanol is removed. Briefly centrifuge and return the tubes or plate to the magnetic stand. Remove with a 10 µl pipette to remove any residual ethanol. In addition, allow beads to dry for the appropriate amount of time.
b)	Improper mixing of QIAseq beads and PCR reaction	After adding QIAseq beads, mix by pipetting up and down 12 times.
c)	Input DNA amount too low	If input DNA is equivalent to less than 1 pg, then primer-dimers may be formed. If starting material is significantly less than 1 ng, up to 4 µl of microbial DNA sample can be added per QIAseq 16S/ITS reaction. Adjust UCP PCR Water so that total 16S/ITS PCR reaction volume equals 10 µl. Also, use 20 cycles of 16S/ITS PCR.

		Comments and suggestions
Sequencing Issues		
a)	Too low or too high cluster density	Accurate library quantification is the key for optimal cluster density on any sequencing instrument. PCR-based quantification method is recommended. Other methods may lead to the incorrect quantification of the library especially when there is overamplification.
b)	Very low clusters passing filter	Make sure the library is accurately quantified and that the correct amount is loaded onto the sequencing instrument. **Important**: The QIAseq Read1 Primer I and the QIAseq 16S/ITS Read 2 custom sequencing primers *must* be used when sequencing on Illumina MiSeq platform.

Appendix A: Preparation and Use of QIAseq 16S/ITS Smart Control

QIAseq 16S/ITS Smart Control is a synthetic DNA construct that contains target sequences for all QIAseq 16S/ITS primers interspersed with nonbacterial/nonfungal sequences. When the Smart Control is included during library preparation (as a separate sample), it can monitor both the library construction process and contamination introduced by the environment or user. Any contaminating reads detected by the Smart Control can then be subtracted for all samples that were prepared.

Preparation and use

When working with high bacterial content samples, simply add 1 µl of the QIAseq 16S/ITS Smart Control to each primer pool PCR reaction as described in the protocol.

When working with low bacterial content or low biomass samples, dilute the QIAseq 16S/ITS Smart Control immediately before use. Prepare dilution by adding 2 µl of stock Smart Control + 198 µl of UCP PCR Water. Discard after use. Do not store.

Appendix B: Setting Up with the Updated Version of Illumina Experiment Manager

Illumina Experiment Manager v1.4 or later

The QIAseq 16S/ITS Screening Panel or QIAseq 16S/ITS Region Panel Sample indices are compatible with Illumina's TruSeq or Nextera XT v2.

Select and check the parameters as follows:

- Category: **Other**
- Select Application: **FASTQ Only**
- Library Prep Workflow:
  - QIAseq 24- or 96-index kit: **TruSeq Nano DNA**
  - QIAseq 384-index kit: **Nextera XT**
- Index Adapters
  - QIAseq 24- or 96-index kit: **TruSeq DNA CD Indexes** (96 indexes)
  - QIAseq 384-index kit: **Nextera XT v2 Index Kit**
- Index Reads: **2** (Dual)
- Read Type: **Paired End**
- Cycles for Read 1: **276** (**251** if using MiSeq v2 500-cycle kit)
- Cycles for Read 2: **276** (**251** if using MiSeq v2 500-cycle kit)

- **Important**: Check "Custom Primer for Read 1"
- **Important**: Check "Custom Primer for Read 2"
- **Important**: Check "Use Adapter Trimming"
- **Important**: Check "Use Adapter Trimming Read 2"

Ordering Information

Product	Contents	Cat. no.
QIAseq 16S/ITS Screening Panel (24)	For targeted enrichment of 16S/ITS from 24 microbial DNA samples	333812
QIAseq 16S/ITS Screening Panel (96)	For targeted enrichment of 16S/ITS from 96 microbial DNA samples	333815
QIAseq 16S/ITS Region Panel (24)	For targeted enrichment of single or multiple 16S/ITS from 24 microbial DNA samples	333842
QIAseq 16S/ITS Region Panel (96)	For targeted enrichment of single or multiple 16S/ITS from 96 microbial DNA samples	333845
QIAseq 16S/ITS Smart Control (10)	Process control for monitoring library construction and contamination with the QIAseq 16S/ITS Panels	333832
QIAseq 16S/ITS 24-index I (96)	Twenty-four sample indices, and custom read primers compatible with Illumina platforms; enough to process a total of 96 samples	333822
QIAseq 16S/ITS 96-index I (384)	Ninety-six sample indices, and custom read primers compatible with Illumina platforms. Enough to process a total of 384 samples	333825

Product	Contents	Cat. no.
Related Products		
QIAseq Library Quant Array Kit	Reagents for quantification of libraries prepared for Illumina or Ion Torrent® platforms; array format	333304
QIAseq Library Quant Assay Kit	Reagents for quantification of libraries prepared for Illumina or Ion Torrent platforms; assay format	333314
DNeasy PowerSoil Kit (100)	For the isolation of microbial genomic DNA from all soil types	12888-100
QIAamp PowerFecal DNA Kit (50)	For the isolation of DNA from stool, gut material, and biosolids	12830-50
QIAamp DNA Microbiome Kit (50)	For the isolation of bacterial microbiome DNA from mixed samples	19091

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
December 2018	Updated procedure in amplification of all 16S/ITS rRNA variable regions. Added sample indexing for QIAseq 16S/ITS Panels on Illumina MiSeq Platform in "Important Notes" section.
April 2019	Added 384-index product information in Kit Contents, Table 1, and "Important Notes". Added other details to Table 1. Specified which amplicons are contained in the 3 QIAseq 16S/ITS Screening Panel pools. Corrected typographical errors in Table 2. Added Appendix B: Setting Up with the Updated Version of Illumina Experiment Manager. Specified number of tubes for RS-D# and FS-D# in QIAseq 16S/ITS 24-index I (for Illumina) kit contents. Removed reference to GeneGlobe Data Analysis portal in "Data analysis" section. Adjusted some recommended volumes, times, etc. in protocols.

Notes

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