Quick-16S™ NGS Library Prep Kit

Catalog No. D6400

Quick Protocol: Low Microbial DNA Samples

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Notice

The optimal DNA concentration for the *Quick*-16S™ NGS Library Prep Kit is 5-20 ng/µl bacterial DNA, excluding host DNA. However, samples with lower microbial DNA concentration than this range may be used as described in this Quick Protocol as well as in the full Instruction Manual.

Protocol

Before Starting

- ✓ **Sample Quantity Requirement.** The PCR conditions and normalization guidelines were designed based on the assumption that there are at least 24 samples in one run; therefore, each run requires a minimum of 24 samples.
- ✓ **Input DNA Guidelines.** All DNA samples should be free of PCR inhibitors.
- ✓ **Choosing a Targeted Primer Set.** Included in the *Quick*-16S™ NGS Library Prep Kit are two 16S primer sets: the *Quick*-16S™ Primer Set V1-V2 and the *Quick*-16S™ Primer Set V3-V4. Use only one primer set for one PCR / well (i.e., do not mix the two sets into one well). For assistance in choosing a targeted primer set, see the table below.

Primer Set	Region Targeted	Features of the Region
Quick-16S™ Primer Set V1-V2	16S V1-V2	• Better species-level resolution for many human-associate microbes when compared to the V3-V4 region. • Excellent coverage for common genera such as *Methanobrevibacter*, *Bifidobacterium, Propionibacterium, Rickettsia, Chlamydia,* and *Treponema.*
Quick-16S™ Primer Set V3-V4	16S V3-V4	• Broader phylogenetic coverage than the V1-V2 region. • Broad coverage for Archaea. • Improved coverage for *Chloroflexi* and *Candidate Phylum Radiation* (CPR).

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Section 1A: Targeted Sequence Amplification

1. Set up a master mix according to the component volumes in the table below:

Component	Volume/Reaction
Quick-16S™ qPCR Premix	10 µl
Quick-16S™ Primer Set V3-V4*	4 µl
ZymoBIOMICS® DNase/RNase Free Water	4 µl
Total	**18 µl**

*Only one *Quick*-16S™ Primer Set should be used during Targeted Sequence Amplification. For more information on the *Quick*-16S™ Primer Sets, please view "Choosing a Targeted Primer Set" on page 1.

2. Add 18 µl of the master mix to each well of a 96-well real-time plate labeled "Targeted Plate." A sample of the plate setup can be found on page 4.
3. Add 2 µl of your DNA samples to individual wells. Include a positive and negative control in the plate. The **ZymoBIOMICS® Microbial Community DNA Standard** (included in this kit) should be used as a positive control.
4. Apply adhesive PCR plate seal. Mix the plate on a plate shaker and centrifuge in a plate spinner.
5. Place "Targeted Plate" in a real-time thermocycler and run the program shown below:

Temperature	Time	
95°C	10 min	
95°C	30 sec	20 cycles
55°C	30 sec	
72°C	3 min	
Plate read	-	
4°C	Hold	

Recommended Program Title: Targeted Sequence Amplification

6. Once the samples have cooled to 4°C, stop the program. Centrifuge "Targeted Plate" in a plate spinner to collect condensation in wells and place plate on ice.
7. To ensure that sufficient amplification has occurred, check if the final fluorescence read of each sample is greater than the fluorescence threshold for the real-time thermocycler, as listed in the table below:

Real-Time Quantitative PCR Instrument	Fluorescence Threshold
BioRad CFX96™ Real-Time PCR Detection System	1,200
Applied Biosystems® 7500 Real-Time PCR System	500,000
Other	Determined by User

8. *Sample Quality Control*: Examine the amplification curve of each sample to confirm that every reaction behaves reasonably.
9. If all samples have had sufficient amplification as determined in Steps 7 and 8, skip to **Section 2**.
10. If any (or all) samples have not had sufficient amplification, proceed to **Section 1B**.

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Section 1B: Additional Targeted Sequence Amplification

The steps below should be performed for low microbial DNA samples that did not achieve sufficient amplification as determined in Steps 7 and 8 of ***Section 1A****.*

1. For samples with final fluorescence reads greater than the fluorescence threshold for the real-time thermocycler, transfer samples to the same well position in a new 96-well real-time PCR plate labeled "Collection Plate." Place "Collection Plate" on ice.
2. To the "Targeted Plate" that contains the remaining samples, apply a new adhesive PCR plate seal. Place back in the real-time thermocycler and run the program shown below:

Temperature	Time	
95°C	1 min	
95°C	30 sec	5 cycles
55°C	30 sec	
72°C	3 min	
Plate read	-	
4°C	Hold	

Recommended Program Name: Additional Targeted Sequence Amplification

3. Once the samples have cooled to 4°C, stop the program. Centrifuge "Targeted Plate" in a plate spinner to collect condensation in wells and place plate on ice.
4. Repeat Steps 1-3 until all samples are collected on the "Collection Plate," or a total of 40 cycles (including the 20 cycles in **Section 1A**) have been run, whichever occurs first. Collect all samples at the end of 40 cycles.
5. Discard the now empty "Targeted Plate." Keep "Collection Plate" on ice and proceed to **Section 2**.

Section 2: Reaction Clean-up

1. Add 1 µl of **Reaction Clean-up Solution** to each reaction well.
2. Apply adhesive PCR plate seal. Mix the plate on a plate shaker and centrifuge in a plate spinner.
3. Place the plate in a real-time thermocycler and run the program shown below:

Temperature	Time
37°C	15 min
95°C	10 min
4°C	Hold

Recommended Program Title: Reaction Clean-up

4. Once the samples have cooled to 4°C, stop the program. Centrifuge the plate in a plate spinner to collect condensation in wells and place plate on ice. Proceed to **Section 3**, or store plate at ≤ -20°C if necessary for later use.

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Section 3: Barcode Addition

1. Set up a master mix according to the component volumes in the table below:

Component	Volume/Reaction
Quick-16S™ qPCR Premix	10 µl
ZymoBIOMICS® DNase/RNase Free Water	4 µl
Total	**14 µl**

2. Add 14 µl of the master mix to each well of a new 96-well real-time PCR plate labeled "Barcoded Plate."
3. From **Index Primer Set A**, add 2 µl of the **Index Primer ZA7xx** and 2 µl of the **Index Primer ZA5xx** to the proper wells as indicated in the diagram below:

Index Primers ZA7xx

Index Primers ZA5xx		ZA701	ZA702	ZA703	ZA704	ZA705	ZA706	ZA707	ZA708	ZA709	ZA710	ZA711	ZA712
		1	2	3	4	5	6	7	8	9	10	11	12
ZA501	A	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73	S81	S89
ZA502	B	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74	S82	S90
ZA503	C	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75	S83	S91
ZA504	D	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76	S84	S92
ZA505	E	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77	S85	S93
ZA506	F	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78	S86	S94
ZA507	G	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79	S87	POS*
ZA508	H	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80	S88	NEG**

* POS: The **ZymoBIOMICS® Microbial Community DNA Standard** should be used as a positive control.
** NEG: A no template control should be used as a negative control.

4. Pipette mix and transfer 2 µl of the DNA samples from the end of Section 2 to the corresponding wells of the "Barcoded Plate."
5. Apply adhesive PCR plate seal. Mix the plate on plate shaker and centrifuge in a plate spinner.
6. Place "Barcoded Plate" in a real-time thermocycler and run the program shown below:

Temperature	Time	
95°C	10 min	
95°C	30 sec	5 cycles
55°C	30 sec	
72°C	3 min	
Plate read	-	
4°C	Hold	

Recommended Program Title: Barcode Addition

7. *Sample Quality Control*: Examine the amplification curve of each sample to confirm that every reaction behaves reasonably.

(Continued on next page.)

8. Keep "Barcoded Plate" in the real-time thermocycler and run the program shown below:

Temperature	Time
72°C	2 min
Plate read	-
4°C	Hold

Recommended Program Title: Plate Read

9. Record and save the fluorescence reading of each reaction from the "Plate Read" program for library quantification in **Section 4**. Proceed to **Section 4**, or store plate at ≤ -20°C if necessary for later use.

Section 4: Library Quantification and Pooling

Steps 1-4 below are for creating a fluorescence standard curve. These measurements for a real-time PCR system should be stable for at least 3 months.

1. To a new 96-well real-time PCR plate labeled "Standards Plate," add 20 µl of each **Fluorescence Standard (1-4)**, in triplicate, to individual wells as indicated below:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C					Fluorescence Standard 1							
D					Fluorescence Standard 2							
E					Fluorescence Standard 3							
F					Fluorescence Standard 4							
G												
H												

2. Apply adhesive PCR plate seal.
3. Place "Standards Plate" in the same real-time thermocycler used for "Barcoded Plate" in **Section 3** and run the program shown below:

Temperature	Time
72°C	2 min
Plate read	-
4°C	1 min

Recommended Program Title: Fluorescence Standards Plate Read

4. Record and save the fluorescence reading of each reaction from the "Fluorescence Standards Plate Read" program for library quantification.

(Continued on next page.)

5. Download the Library Quantification and Pooling Template file by clicking here (USA only), or by visiting the Documentation section of the D6400 Product Page at www.zymoresearch.com.
6. Input the values from the "Fluorescence Standards Plate Read" program (**Section 4**, Step 4) in the appropriate positions in Table 1 of the file.
7. Input the values obtained from the "Plate Read" program (**Section 3**, Step 9) in the appropriate positions in Table 3 of the file.
8. Input the desired amount of product for each sample you would like to use for normalization (e.g. 30 ng) in the appropriate positions in Table 5 of the file. Table 6 will calculate the volume required of each sample for normalization.
9. Place a new microcentrifuge tube on ice. Pool the required volume of each sample from the "Barcoded Plate" into the microcentrifuge tube. Proceed to **Section 5** immediately.

Section 5: Final Library Clean-up

1. Resuspend the magnetic particles of the **Select-a-Size MagBeads** by vigorously shaking until homogenous.
2. Add 0.8 x *volume of pooled library* of the **Select-a-Size MagBeads** to the pooled library from **Section 4**. Use the values found in Table 7 of the Library Quantification and Pooling Template. For example, add 80 µl of Select-a-Size MagBeads to 100 µl pooled library.
3. Mix thoroughly by pipetting or vortexing until homogenous. Incubate for 5 minutes at room temperature.
4. Place the sample on a magnetic rack and incubate for 3-10 minutes at room temperature, or until the magnetic beads have fully separated from solution.
5. Once the beads have cleared from solution, remove and discard the supernatant.
6. While the beads are still on the magnetic rack, add 200 µl of **DNA Wash Buffer**. Remove and discard the supernatant. Repeat this step.
7. While the beads are still on the magnetic rack, aspirate out any residual buffer with a 10 µl pipette tip.
8. Remove sample from the magnetic rack. Incubate for 3 minutes at room temperature to remove all traces of buffer.
9. Add 10-20 µl of **ZymoBIOMICS® DNase/RNase Free Water** to the beads and pipette mix thoroughly. Incubate at room temperature for 2 minutes.
10. Place the sample on a magnetic rack for 1 minute to separate the magnetic beads from eluate.
11. Transfer supernatant to a clean microcentrifuge tube.

The ultra-pure pooled library DNA is now ready for use.