ZYMO RESEARCH

Microbiomics
Made Simple™

Quick-16S™ NGS Library Prep Kit

Fast and flexible 16S sequencing library prep

Highlights

- **Fast:** Only 1.5 hours of hands-on time. No TapeStation® analyses or AMPure® clean-ups required.
- **Accurate:** Utilization of real-time PCR limits PCR chimera formation.
- **Increased Coverage:** Novel primers increase phylogenetic coverage of Bacteria and Archaea and enable species-level resolution for human microbiome profiling.

Catalog Numbers:
D6400

Scan with your smart-phone camera to view the online protocol/video.

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Product Contents

Quick-16S™ NGS Library Prep Kit	D6400 (96 rxns.)	Storage Temp.
Quick-16S™ qPCR Premix	2 x 1 ml	-20°C
Quick-16S™ Primer Set V1-V2	400 µl	-20°C
Quick-16S™ Primer Set V3-V4	400 µl	-20°C
ZymoBIOMICS™ DNase/RNase Free Water	1 ml	Room Temp.
ZymoBIOMICS™ Microbial Community DNA Standard (50 ng)	10 µl	-20°C
Reaction Clean-up Solution	100 µl	-20°C
Index Primer Set[1]	40 µl each	-20°C
Fluorescence Standards Set[2]	60 µl each	-20°C
Select-a-Size MagBeads[3]	1 ml	4-8°C
DNA Wash Buffer	6 ml	Room Temp.
Magnetic Rod	4	-
Instruction Manual	1 pc	-

1 **Index Primer Set A** contains the Index Primers ZA701-ZA712 and ZA501-ZA508. The barcodes of each index primer are distinct from one another by at least 5 bp to boost the accuracy of demultiplexing. Note that the i5 bases remain the same on the sample sheet, while the i7 bases are entered as the reverse complement on the sample sheet. See Appendix I for additional support and a template for the Illumina MiSeq® sample sheet.
2 The Fluorescence Standards Set contains Fluorescence Standards 1-4.
3 The Select-a-Size™ MagBeads are shipped at room temperature but should be stored at 4-8°C upon receipt.

Specifications

- **Sample Input –** Purified microbial DNA ≤ 20 ng/µl, free of PCR inhibitors[1].
- **Index Primers –** Dual index (barcodes) to uniquely label samples[2].
- **Barcode Sequences –** Available for download here (USA Only), or by visiting the Documentation section of the D6400 Product Page at www.zymoresearch.com
- **Amplicon Size –** The size of the 16S V1-V2 region and the 16S V3-V4 region (including primers) is ~350bp and ~460bp, respectively. The final amplicon size after addition of barcoded primers is ~486bp and ~596bp, respectively.
- **Recommended Real-Time PCR Systems[3] –** Bio-Rad CFX96™ Real-Time PCR Detection System (any model), Applied Biosystems® 7500 Real-Time PCR System.
- **Sequencing Platform –** Illumina MiSeq® without the need to add custom sequencing primers. Zymo Research recommends the MiSeq® Reagent Kit v3 (600-cycle) for libraries prepared with the *Quick*-16S™ Primer Set V3-V4 or the MiSeq® Reagent Kit v2 (500-cycle) for libraries prepared with the *Quick*-16S™ Primer Set V1-V2. For assistance with sample sheet setup, see Appendix I.
- **Equipment Needed** (user provided) **–** Microcentrifuge, plate spinner (centrifuge), 96-well real-time quantitative PCR system, and 96-well real-time PCR plates.

1 DNA that contains potent PCR inhibitors can be quickly cleaned using the OneStep™ PCR Inhibitor Removal Kit. See Appendix F for additional information.
2 Up to 384 samples can be supported. For projects with more than 96 samples, please contact Zymo Research at oemorders@zymoresearch.com for additional indexing solutions.
3 Any real-time PCR detection system that can detect and report the SYBR Green fluorophore is also compatible. See Appendix A for additional information.

Product Description

16S rRNA sequencing is a routine technique for microbiome composition profiling. Compared to shotgun metagenomics sequencing, 16S rRNA sequencing is more cost-effective and more robust; it generally requires less input DNA and is less impacted by the presence of host DNA. However, 16S rRNA sequencing has its own challenges. One major challenge is the formation of PCR chimeric sequences, which are artificial sequences resulting from the recombination of two or more PCR templates. Additionally, with common 16S primers, it is difficult to achieve both species-level resolution and broad phylogenetic coverage. Moreover, common 16S library preparation protocols used in the field have not been optimized to be cost-effective for large-scale applications.

The ***Quick*-16S™ NGS Library Prep Kit** aims to standardize the library preparation process for 16S rRNA sequencing. Distinguishing features of the kit are described below.

Rapid 16S rRNA Library Prep. The *Quick*-16S™ NGS Library Prep Kit utilizes real-time (quantitative) PCR (qPCR) rather than endpoint PCR for 16S rRNA amplification, enabling direct quantification of PCR products and eliminating the need for additional library quantification analysis such as TapeStation® analysis or gel electrophoresis. An enzymatic clean-up is introduced between the two PCR steps, saving time and reducing costs as compared to lengthy AMPure® bead-based clean-ups (Figure 1). With these features, the kit dramatically reduces the hands-on time of 16S library preparation (Figure 1).

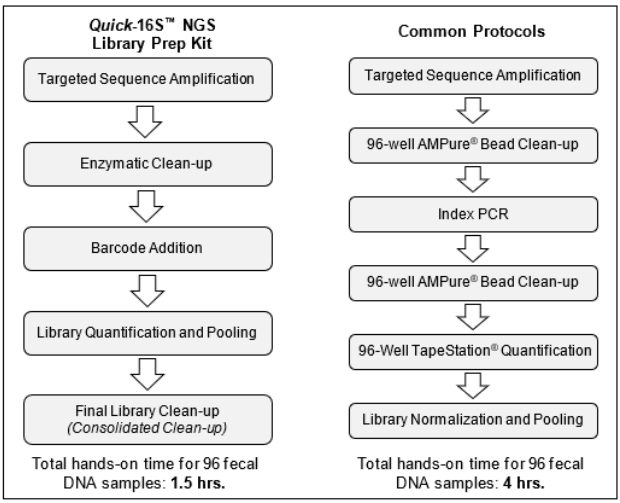

Figure 1. ***The total hands-on time for the Quick-16S™ NGS Library Prep Kit is shorter than that of common library preparation protocols.*** *Total hands-on time calculations are based on the preparation of 96 DNA samples isolated from human stool.*

Simple. The *Quick*-16S™ NGS Library Prep Kit includes all the reagents needed to convert DNA samples to a 16S library. The resulting library is directly compatible with the Illumina MiSeq® without needing additional custom sequencing primers.

Accurate. The utilization of real-time PCR also enables users to control PCR cycles. This limits chimera formation and PCR bias while obtaining enough products for subsequent sequencing. In most cases, the abundance of chimeric sequences is maintained below 2% (Figure 2).

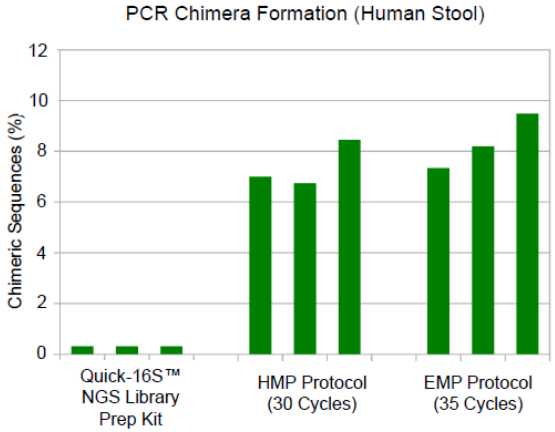

Figure 2. The *Quick*-16S™ NGS Library Prep Kit produces the lowest percentage of chimeric sequences as compared to two common protocols. HMP, Human Microbiome Project 16S sequencing protocol. EMP, Earth Microbiome Project 16S sequencing protocol. Equivalent amounts of the same fecal DNA sample were used as input. Chimeric sequences were predicted with Uchime (https://www.drive5.com/uchime).

Increased Coverage. Due to the rapid expansion of 16S rRNA databases, the insufficient microbial coverage of common 16S primer sets has become evident. Zymo Research has re-designed two common primer sets targeting the 16S V1-V2 and 16S V3-V4 regions based on the most updated 16S reference database and significantly improved their coverage (Figure 3).

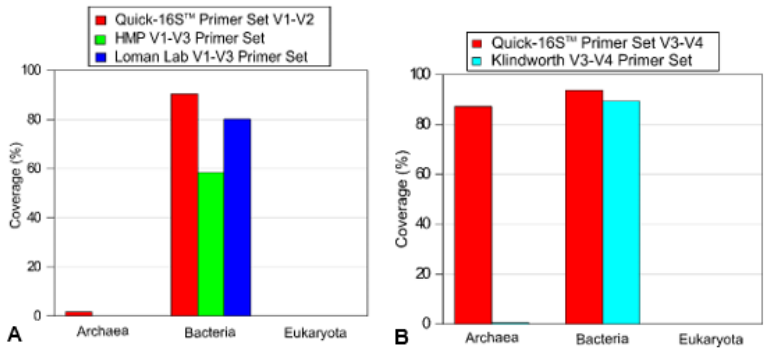

Figure 3. The *Quick*-16S™ Primer Set V1-V2 and the *Quick*-16S™ Primer Set V3-V4 provide better coverage of bacteria and archaea than commonly used primer sets. The coverage over different domains was determined by comparing the primer sequences with the 16S sequences from the Silva database v.1.2.3. A hit was defined as a perfect match between a primer sequence and a 16S sequence in the database. (A) HMP (Human Microbiome Project) V1-V3 primers are 27F (AGAGTTTGATCCTG GCTCAG) and 534R (ATTACCGCGGCTGCTGG). Loman Lab V1-V3 primers1 are 27F (AGAGTTTGAT YMTGGCTCAG) and 519R (GWATTACCGCGGCKGCTG). (B) The Klindworth V3-V4 primers2 chosen for comparison are 341F (CCTACGGGNGGCWGCAG) and 785R (GACTACHVGGGTATCTAATCC).

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[1].
- ✓ **Input DNA Guidelines.** All DNA samples should be free of PCR inhibitors[2]. The optimal range for microbial DNA input is 5-20 ng/µl. Refer to the table below for more detailed input guidelines:

DNA Concentration[3]	Protocol Notes	Quick Protocol Link
High Microbial DNA: 5-20 ng/µl (10 ng/µl)	Follow main protocol, Sections 1-5.	Available for download here (USA Only), or by visiting the Documentation section of the D6400 Product Page at www.zymoresearch.com.
Low Microbial DNA: < 5 ng/µl	1. Follow the main protocol, Section 1. 2. Follow instructions in Additional Protocol for Low Microbial DNA Samples. 3. Return to the main protocol and follow Sections 2-5.	Available for download here (USA Only), or by visiting the Documentation section of the D6400 Product Page at www.zymoresearch.com.

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[1] To evaluate the *Quick*-16S™ NGS Library Prep Kit, use 2 µl of the included ZymoBIOMICS™ Microbial Community DNA Standard as input. In Section 4, skip Step 8 and use entire sample volume to pool in Step 9.
[2] DNA that contains potent PCR inhibitors can be quickly cleaned using the OneStep™ PCR Inhibitor Removal Kit. See Appendix F for additional information.
[3] The concentration only refers to the microbial DNA, not including the host DNA.

✓ **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 per PCR reaction/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[1]	Features[2]
Quick-16S™ Primer Set V1-V2	**16S V1-V2** (27f - 341r)	• Better species-level resolution for many human-associated 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** (341f - 806r)	• Broader phylogenetic coverage than the V1-V2 region. • Broad coverage for Archaea. • Improved coverage for *Chloroflexi* and phyla *Candidate Phylum Radiation* (CPR).

[1] If other targeted regions are preferred, customized primers can be designed and ordered separately. An example is given in Appendix B, presenting the case of the primers of 16S V4 region recommended by the Earth Microbiome Project (EMP, http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/).
[2] Additional information about these primer sets can be found in Appendix C.

Section 1: Targeted Sequence Amplification

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

Component	Volume/Reaction
Quick-16S™ qPCR Premix	10 µl
Quick-16S™ Primer Set (V1-V2 or 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 6.

2. Add 18 µl of the master mix to the appropriate wells of a 96-well real-time PCR plate labeled **"Targeted Plate"**. A sample of the plate setup can be found on page 7, or on the Plate Setup Guide[1].

3. Add 2 µl of your DNA samples to individual wells. Include positive and negative controls in the plate. The **ZymoBIOMICS™ Microbial Community DNA Standard**[2] (included in this kit) is recommended to 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[3].

5. Place **Targeted Plate** in a real-time thermocycler and run the "*Targeted Sequence Amplification*" 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	

(Continued on next page)

[1] The Plate Setup Guide is available for download file here (USA Only), or by visiting the Documentation section of the D6400 Product Page at www.zymoresearch.com.
[2] The composition of the microbial standard can be found in Appendix H.
[3] PCR reactions can be pipette mixed if a plate shaker is not available

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. Examples are listed in the table below.

Real-Time Quantitative PCR Instrument	Fluorescence Threshold
Bio-Rad CFX96™ Real-Time PCR Detection System	1,200
Applied Biosystems® 7500 Real-Time PCR System[1]	500,000
Other[2]	See Appendix A

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

    a. For example, a sample with high microbial DNA concentration that is expected to amplify earlier than 20 cycles and shows little or no amplification may indicate an error in the reaction setup. (See the Troubleshooting Guide on page 18.)

    b. For a sample with low microbial DNA concentration that is not expected to amplify earlier than 20 cycles, it is reasonable that the sample may require further amplification. See **Additional Protocol for Low Microbial DNA Samples** on page 11.

    c. The negative control should not amplify before a total of 35 cycles have been run. (Optional: to ensure that there has not been contamination, transfer the negative control to a new 96-well real-time PCR plate and repeat Step 5 of **Section 1**.)

9. If all samples have had sufficient amplification as determined in Steps 7 and 8, proceed to **Section 2**.

10. If any (or all) samples have not had sufficient amplification as determined in Steps 7 and 8, proceed to **Additional Protocol for Low Microbial DNA Samples** on page 11.

[1] A guide for using the Applied Biosystems® 7500 Real-Time PCR System can be downloaded here (USA Only).
[2] Alternate systems should use the Fluorescence Threshold calculated in Appendix A for this assessment.

Section 2: Reaction Clean-Up

1. Add 1 µl of **Reaction Clean-up Solution** to each reaction well[1].

2. Apply adhesive PCR plate seal. Mix the plate on a plate shaker and centrifuge in a plate spinner[2].

3. Place the plate in a real-time thermocycler and run the "*Reaction Clean-Up*" program shown below:

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

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.

Section 3: Barcode Addition

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

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

2. Add 14 µl of master mix to appropriate wells in a new 96-well real-time PCR plate labeled **"Barcoded Plate"**. The plate setup of the **Barcoded Plate** should match the **Targeted Plate** in **Section 1**.

(Continued on next page)

[1] Tip: Pre-aliquot Reaction Clean-up Solution into PCR strip tubes or dispense into a small volume reservoir and use a multi-channel pipette to distribute to the plate.
[2] PCR reactions can be pipette mixed if a plate shaker is not available.

3. From **Index Primer Set A**[1], 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[2].

Index Primers ZA5xx		Index Primers ZA7xx											
		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** (included in kit) as a positive control.

** NEG: A no template control 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 a plate shaker and centrifuge in a plate spinner[3].

6. Place **Barcoded Plate** in a real-time thermocycler and run the "*Barcode Addition*" 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	

(Continued on next page)

[1] The For other Index Primer Sets, refer to the Plate Setup Guide, available for download here (USA Only), or by visiting the Documentation section of the D6400 Product Page at www.zymoresearch.com.

[2] Tip: Pre-aliquot barcodes into PCR strip tubes and use a multi-channel pipette to distribute to the plate.

[3] PCR reactions can be pipette mixed if a plate shaker is not available

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

    a. If a sample is expected to amplify, the fluorescence read should be greater than the fluorescence threshold of the real-time thermocycler as described on page 5.

    b. For example, if a sample amplifies well during **Section 1: Targeted Sequence Amplification** but has almost no amplification during **Section 3: Barcode Addition**, a mistake may have occurred, e.g. did not add an index to this sample. (See the Troubleshooting Guide on page 18.)

    c. For a sample that did not amplify well during **Section 1: Targeted Sequence Amplification**, such as a negative control, it is reasonable that the sample does not show substantial amplification during **Section 3: Barcode Addition**.

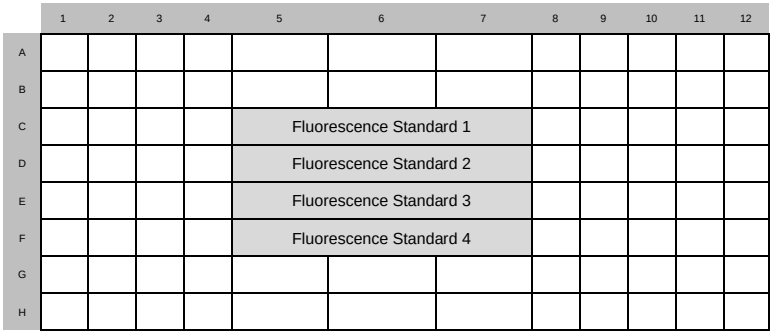
8. Keep **Barcoded Plate** in the real-time thermocycler and run the "*Plate Read*" program shown below.

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

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

Section 4: Library Quantification & Pooling

1. Vortex each Fluorescence Standard (1-4) for ≥ 10 seconds to mix, then centrifuge briefly. In a new 96-well real-time PCR plate labeled **"Standards Plate"**, add 20 µl of each standard, in triplicate, to individual wells (see below):



2. Apply adhesive PCR plate seal.

3. Place **Standards Plate** in the same real-time thermocycler used for **Barcoded Plate**[1] in **Section 3** and run the "*Fluorescence Standards Plate Read*" program shown below:

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

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

5. Download the Library Quantification and Pooling Template file here (USA Only), or by visiting the Documentation section of the D6400 Product Page at www.zymoresearch.com.

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[1] The real-time thermocycler used to create the fluorescence standard curve with this program must be the same real-time thermocycler used in **Section 3: Barcode Addition**.

6. Input the values from the "*Fluorescence Standards Plate Read*" program from **Section 4**, Step 4 in the appropriate positions in Table 1 of the file.

7. Input the values obtained from the "*Plate Read*" program from **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 position 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. Use the required volumes to pool each sample from the **Barcoded Plate** into the tube. Proceed to **Section 5** immediately.

Section 5: Final Library Clean-Up

1. Equilibrate **Select-a-Size MagBead Buffer** to room temperature (15-30°C). Add 30 µl of **Select-a-Size MagBead Concentrate** to the 1 ml **Select-a-Size MagBead Buffer**. Resuspend the magnetic particles by vigorously shaking until homogenous.

2. Add 0.8x volume of **Select-a-Size MagBead Buffer** to the pooled library from **Section 4**, Step 9. Use the values found in Table 7 in the **Library Quantification and Pooling Template** (e.g., add 80 µl of **Select-a-Size MagBead Buffer** 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[1] 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[2].

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[1] Alternatively, the provided Magnetic Rod can be used.
[2] Avoid aspirating any beads when removing the supernatant. To best prevent this, leave 2-5 µl of liquid behind.

6. While the beads are still on the magnetic rack[1], 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[1], aspirate out any residual buffer with a 10 µl pipette tip.

8. Remove tube from the magnetic rack and keep the cap open for 3 minutes at room temperature to dry the beads.

9. Add 10-20 µl[3] 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[1] and incubate for 1 minute at room temperature, or until the magnetic beads have fully separated from eluate.

11. Transfer supernatant to a clean microcentrifuge tube.

This is your final 16S library.

The ultra-pure pooled library DNA is now ready for use or storage at ≤ -20ºC. Please refer to platform-specific guidelines for library quantification and preparation for sequencing.

Recommended Sequencing Reagents:

*For libraries prepared with **Quick-16S™ Primer Set V3-V4**, Zymo Research recommends the **MiSeq® Reagent Kit v3 (600-cycle)**. For libraries prepared with **Quick-16S™ Primer Set V1-V2**, Zymo Research recommends the **MiSeq® Reagent Kit v2 (500-cycle)**. See Appendix I for assistance with sample sheet setup.*

[1] Alternatively, the provided Magnetic Rod can be used.
[2] Avoid aspirating any beads when removing the supernatant. To best prevent this, leave 2-5 µl of liquid behind.
[3] If pooling fewer than 50 samples, use ≥ 10 µl for elution. If pooling more than 50 samples, use 20 µl for elution.

Additional Protocol for Low Microbial DNA Samples

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

1. For samples with final fluorescence reads greater than the fluorescence threshold for the real-time thermocycler (found in the table on page 5), transfer samples to the same well position in a new 96-well real-time PCR plate labeled **"Collection Plate"**. Place the **Collection Plate** on ice.

2. To the **Targeted Plate** that contains the remaining samples, apply a new adhesive PCR plate seal. Place the **Targeted Plate** back in the real-time thermocycler and run the "*Additional Targeted Sequencing Amplification*" 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	

3. Once the samples have cooled to 4°C, stop the program. Centrifuge the **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 1**) have been run, whichever occurs first. Collect all samples at the end of 40 cycles.

5. Discard the now empty **Targeted Plate**. Keep the **Collection Plate** on ice and proceed to Section 2 on page 9.

Appendices

Appendix A: Determination of Fluorescence Threshold for Alternate Systems

Any real-time PCR detection system that can detect and report the SYBR Green fluorophore is compatible with the *Quick*-16S™ NGS Library Prep Kit, though determination of the fluorescence threshold for the system is required for optimal results. This procedure is not necessary for the real-time PCR systems recommended by Zymo Research. To determine the fluorescence threshold for a real-time PCR system, follow the protocol below.

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

Component	Volume
Quick-16S™ qPCR Premix	25 µl
Quick-16S™ Primer Set V3-V4	10 µl
ZymoBIOMICS™ DNase/RNase Free Water	10 µl
ZymoBIOMICS™ Microbial Community DNA Standard (50 ng)	5 µl
Total	**50 µl**

2. Add 20 µl of the master mix to two wells of a new 96-well real-time PCR plate.

3. Apply adhesive PCR plate seal.

4. Place the plate in a real-time thermocycler and run the "*Determination of Fluorescence Threshold*" program shown below:

Temperature	Time	
95°C	10 min	
95°C	30 sec	35 cycles
55°C	30 sec	
72°C	3 min	
Plate Read	---	
4°C	3 min	

5. Record the endpoint fluorescence value of each reaction at the final cycle.

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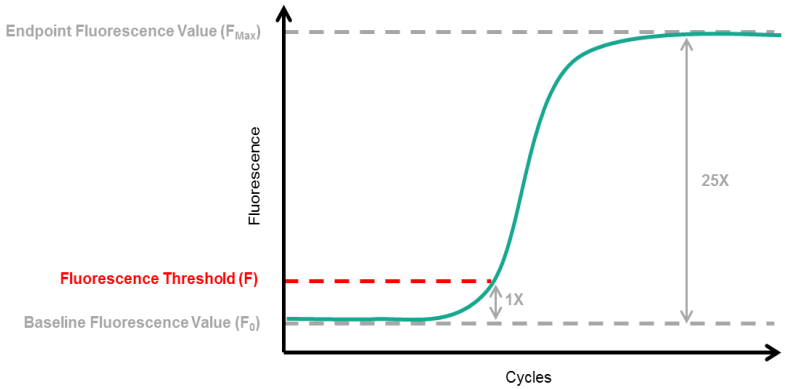
6. Use the average endpoint fluorescence value (F_{Max}) and the baseline fluorescence value of the real-time PCR system (F_0) in the formula below to calibrate the Fluorescence Threshold (F) of the system.

A

$$F = \frac{F_{Max} - F_0}{25} + F_0$$



B

Figure 4. Determination of the Fluorescence Threshold of a real-time quantitative PCR system. (A) The equation for determining the fluorescence threshold, and **(B)** a sample reaction curve graph with fluorescence values indicated. The endpoint fluorescence value of the system is determined by running a reaction to saturation. This value is used with the baseline fluorescence

7. Use the calculated Fluorescence Threshold in **Section 1**, Step 7.

Appendix B: Adapter Sequences for Other Targeted Primers

The *Quick*-16S™ NGS Library Prep Kit can be used with custom primers to target a different region of 16S rRNA gene. To be compatible with this kit, the primers must include the correct adapter sequences as shown below (underlined). See the table below for an example using Earth Microbiome Project (EMP)[1]V4 primers. The starting concentration of each primer should be 10 µM. When added to the reaction in the wells, the final concentration of each primer should be 1 µM.

Primer ID	Primer Sequence Example (5'-3')	Adapter with Primer Sequence (5'-3')
FWD (EMP 515F)	GTGYCAGCM GCCGCGGTAA	TCGTCGGCAGCGTCAGATGTGTATAA GAGACAGGTGYCAGCMGCCGCGGTAA
REV (EMP 806R)	GGACTACNVG GGTWTCTAAT	GTCTCGTGGGCTCGGAGATGTGTATAA GAGACAGGGACTACNVGGGTWTCTAAT

[1] EMP primer sequences were found at http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/.

Appendix C: Targeted Primer Sets

Because different research projects require the analysis of different 16S rRNA gene regions, Zymo Research offers redesigned targeted primer sets for various regions. While some customers prefer to target the V1-V2 region in order to detect human-associated microbes, others prefer the V3-V4 region because of its broader coverage of microbes. Primer sets targeting these regions, the *Quick*-16S™ Primer Set V1-V2 and the *Quick*-16S™ Primer Set V3-V4, are included in the *Quick*-16S™ NGS Library Prep Kit. Information about the *Quick*-16S™ Primer Set V1-V2 can be found in Figure 5 below; information about the *Quick*-16S™ Primer Set V3-V4 can be found in Figure 6 on page 19. For additional assistance in choosing a primer set for your project, please contact Zymo Research at www.zymoresearch.com.

***Quick*-16S™ Primer Set V1-V2.** The V1-V2 region of the 16S rRNA gene has better species-level resolution for many human-associated microbes than other 16S regions. However, common primers targeting this region have poor phylogenetic coverage. For example, the forward primer 27F/8F has poor coverage for *Bifidobacterium*, a common genus found in the human gut, and Propionibacterium, a common genus found on human skin. The *Quick*-16S™ Primer Set V1-V2 dramatically improves the coverage for common human-associated microbes, especially pathogens (Figure 5). It also provides coverage for common methanogenic archaea found in the human gut.

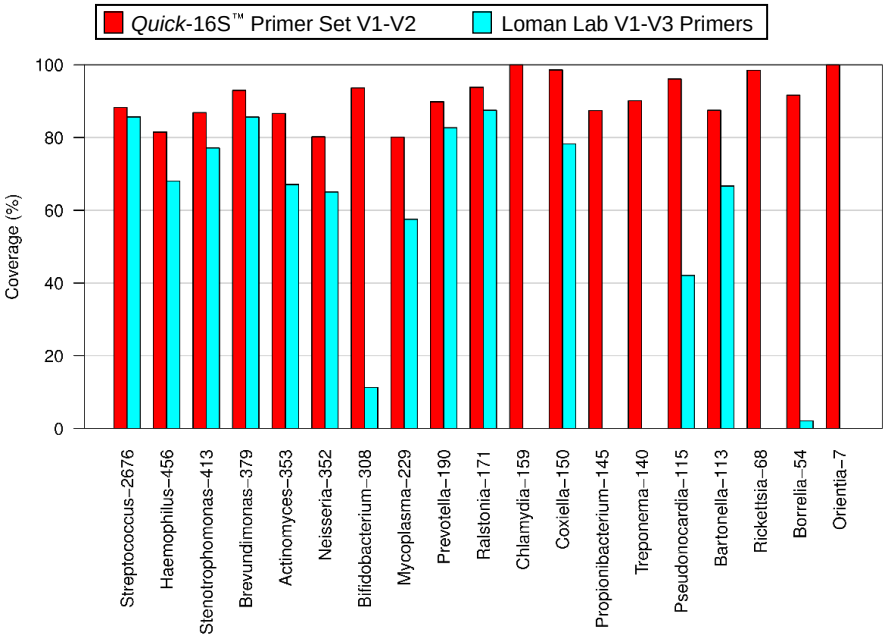


Figure 5. The *Quick*-16S™ Primer Set V1-V2 provides better coverage of microbes than commonly used primer sets. Loman Lab V1-V3 primers were found at: http://joshquick.github.io/metagenomics/2013/11/07/16S-v1-3-on-miseq/. The coverage over selected bacterial genera is shown.

***Quick*-16S™ Primer Set V3-V4.** The V3-V4 region of 16S rRNA gene is a popular target region for 16S sequencing. The primer set containing 341F and 785R proposed by Klindworth et al. (2013)[1] is a popular choice for bacteria profiling. Zymo Research has refined this primer set and built the Quick-16S™ Primer Set V3-V4. The new primer set dramatically improves the coverage of several bacterial phyla and adds broad coverage for the Archaea domain (Figure 6).

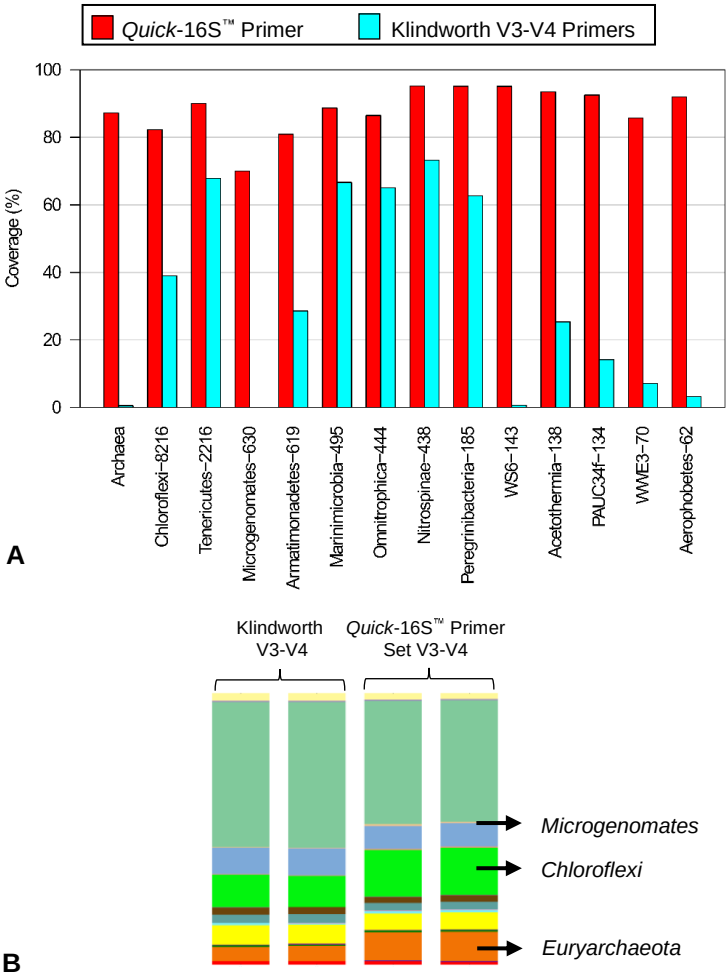


Figure 6. The *Quick*-16S™ Primer Set V3-V4 provides better coverage of archaea and bacteria than commonly used primer sets. The Klindworth[1] V3-V4 primers used for comparison are 341F (CCTACGGGNGGCWGCAG) and 785R (GACTACHVGGGTATCTAATCC). **(A)** Comparing coverage of selected phyla. **(B)** Comparing coverage of phyla in soil with 16S sequencing.

[1] Reference: Klindworth et al. Nucleic Acids Res. 2013 Jan; 41(1): e1.

Appendix D: Index Primer Sets

To accommodate sequencing projects of various sizes, Zymo Research offers various Index Primer Sets that can uniquely barcode up to 384 samples. The barcodes of each index primer are distinct from one another by at least 5 bp to boost the accuracy of demultiplexing. For high-throughput projects that require indexes for more than 96 samples, please contact Zymo Research at oemorders@zymoresearch.comfor a custom solution.

Appendix E: Primer Size Information for Quality Trimming

To perform specific quality trimming following the demultiplexing of libraries prepared with the *Quick*-16S™ NGS Library Prep Kit, the forward and reverse primer sizes of each *Quick*-16S™ Primer Set are listed below:

Primer Set	Forward Primer Length	Reverse Primer Length
Quick-16S™ Primer Set V1-V2	19 bp	16 bp
Quick-16S™ Primer Set V3-V4	16 bp	24 bp

Appendix F: Removal of PCR Inhibitors from Starting DNA

The starting material for the Quick-16S™ NGS Library Prep Kit must be free of PCR inhibitors. For purified DNA that contain contaminants that can inhibit library preparation reactions, Zymo Research recommends performing sample cleanup with the OneStep™ PCR Inhibitor Removal Kit. Additional information can be found here (USA Only), or by visiting the D6030 Product Page at www.zymoresearch.com.

Appendix G: Use with Non-Quantitative PCR Systems

Zymo Research recommends the Quick-16S™ NGS Library Prep Kit be used with a real-time quantitative PCR system. However, it is possible to use a standard thermocycler without quantitative function with this kit, though certain features such as direct quantification and control of PCR chimera formation will be lost. Use of a standard thermocycler with the Quick-16S™ NGS Library Prep Kit is only recommended for DNA samples that have a high concentration of bacterial DNA (e.g. fecal DNA).

To use the Quick-16S™ NGS Library Prep Kit with a non-quantitative PCR system, download the Quick-16S™ NGS Library Prep Kit Quick Protocol: Non-Quantitative PCR here (USA Only), or by visiting the Documentation section of the D6400 Product Page at www.zymoresearch.com.

Appendix H: Composition of ZymoBIOMICS™ Microbial Community DNA Standard (50 ng)

The **ZymoBIOMICS™ Microbial Community DNA Standard (50 ng)** is a mixture of genomic DNA extracted from pure cultures of eight bacterial and two fungal strains. Genomic DNA from each culture is quantified before mixing. The ZymoBIOMICS™ Microbial Community DNA Standard allows for assessment of bias from library preparation, sequencing, and bioinformatics analysis. More information about the standard can be found below.

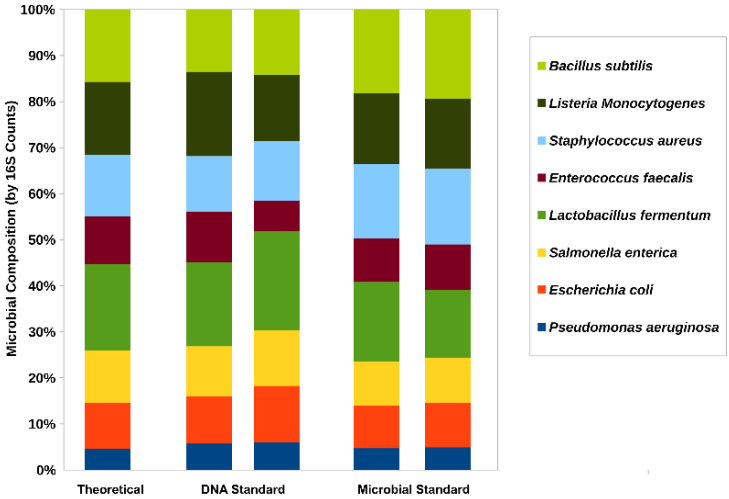


Figure 8. Accurate composition for reliable use to evaluate 16S rRNA sequencing. Characterization of the microbial composition of the two ZymoBIOMICS™ standards by 16S rRNA gene targeted sequencing. The measured composition of the two standards agrees with the theoretical/designed composition. "DNA Standard" represents ZymoBIOMICS™ Microbial Community DNA Standard and "Microbial Standard" represents ZymoBIOMICS™ Microbial Community Standard. 16S composition by 16S rRNA gene targeted sequencing was calculated based on counting the amount of 16S raw reads mapped to each genome.

Appendix I: Illumina MiSeq® Sample Sheet Setup

A template for the Illumina MiSeq® sample sheet is available for download here (USA only), or by visiting the Documentation section of the Product Page at www.zymoresearch.com. Fill in the project and sample information in the highlighted fields, then save the file in comma-separated values (CSV) format for use with the Illumina MiSeq®.

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Ordering Information

Individual Kit Components	Catalog No.	Amount
ZymoBIOMICS™ DNase/RNase Free Water	D4302-5-10	10 ml
ZymoBIOMICS™ Microbial Community DNA Standard (200 ng)	D6305	200 ng
ZymoBIOMICS™ Microbial Community DNA Standard (2000 ng)	D6306	2000 ng

Try the new and improved *Quick*-16S™ Plus NGS Library Prep Kit

Product Description	Catalog No.	Size / Format
***Quick*-16S™ Plus NGS Library Prep Kit (V3-V4)**	D6421-PS1	96 rxns. / Primer Set 1
	D6421-PS2	96 rxns. / Primer Set 2
	D6421-PS3	96 rxns. / Primer Set 3
	D6421-PS4	96 rxns. / Primer Set 4
	D6421-PS5	96 rxns. / Primer Set 5
	D6421-PS6	96 rxns. / Primer Set 6
	D6421-PS7	96 rxns. / Primer Set 7
	D6421-PS8	96 rxns. / Primer Set 8
***Quick*-ITS™ Plus NGS Library Prep Kit (ITS2)**	D6424-PS1	96 rxns. / Primer Set 1
	D6424-PS2	96 rxns. / Primer Set 2
	D6424-PS3	96 rxns. / Primer Set 3
	D6424-PS4	96 rxns. / Primer Set 4
***Quick*-16S™ Plus NGS Library Prep Kit (V4)**	D6430-PS1	96 rxns. / Primer Set 1
	D6430-PS2	96 rxns. / Primer Set 2
	D6430-PS3	96 rxns. / Primer Set 3
	D6430-PS4	96 rxns. / Primer Set 4
***Quick*-16S™ Plus NGS Library Prep Kit (V1-V2)**	D6434-PS1	96 rxns. / Primer Set 1
***Quick*-16S™ Plus NGS Library Prep Kit (V1-V3)**	D6440-PS1	96 rxns. / Primer Set 1

Explore Other Microbiome Products

✓ To collect and transport samples at ambient temperatures:

DNA/RNA Shield™ and Collection Devices	
1X Reagent #R1100	For sample lysis and stabilization of DNA/RNA
2X Concentrate #R1200	Reagent concentrate (2X) for use with liquids at 1:1 ratio
Fecal Collection Tube #R1101	15 mL container (prefilled with 9 mL DNA/RNA Shield™). Direct collection of up to 1g or 1 mL stool
Collection Tube w/ Swab #R1106	12 x 80 mm screwcap container filled with 1 mL DNA/RNA Shield™ and sterile swab for specimen collection

✓ Unbiased and inhibitor-free DNA and RNA extraction (high-throughput and automatable) for microbial profiling:

ZymoBIOMICS™ DNA and RNA Kits	
DNA Miniprep #D4300	Up to 25 µg DNA
DNA Microprep #D4301	Up to 5 µg DNA
MagBead DNA #D4302	Automatable (Tecan, Hamilton, Kingfisher, etc.)
96-Well DNA #D4309	Spin-plate
DNA/RNA Miniprep Kit #R2002	Up to 100 µg DNA/RNA

✓ Microbial standards and references for profiling quality control, benchmarking, positive controls, and to assess performance of entire microbiomic/metagenomic workflows:

ZymoBIOMICS™ Standards and Reference Materials	
Microbial Community Standard #D6300	Contains 8 bacteria and 2 yeasts for QC and method optimization
Microbial Community DNA Standard #D6305	Contains 8 bacteria and 2 yeasts DNA for bioinformatics optimization
Gut Microbiome Standard #D6331	Contains 21 different human gut strains for method benchmarking
Fecal Reference with TruMatrix™ Technology #D6323	Contains real human fecal material for benchmarking and improved data reproducibility

Troubleshooting Guide

Problem	Possible Causes and Suggested Solutions
Background Contamination	Workspace contamination: • Clean workspace, microcentrifuge, and pipettes with 10% bleach routinely to avoid contamination. • Use of kit in exposed environment without proper filtration can lead to background contamination. Check pipettes, pipette tips, microcentrifuge tubes, workspace, etc. for contamination • Make sure all reagent tubes and bottles are properly sealed for storage. Use of these outside a clean room or hood can result in contamination.
Loss of Volume during PCR	Adhesive seal: • A loosened adhesive seal on the PCR plate can lead to sample evaporation. Ensure that the plate seal is secure on every well during targeted sequence amplification. Lid pressure: • Inconsistent lid pressure. Ensure that the lid pressure on the real-time quantitative PCR instrument is consistent over the PCR plate according to the manufacturer's recommendation.
Unexpected or No Amplification of DNA Sample During PCR Program in Section 1	Sample with high microbial DNA concentration: • Reaction setup error. A sample that is expected to amplify but shows little or no amplification during the PCR program in **Section 1** may indicate an error in the reaction setup. Use a new aliquot of the sample and repeat **Section 1**. • Sample may contain high levels of PCR inhibitors. See Appendix D on how to remove these and repeat **Section 1**. Additionally, samples may be diluted to lower concentration (<10 ng/µl) to see if that improves amplification efficiency. Sample with low microbial DNA concentration: • Check negative control. A sample with little microbial DNA may not amplify before the negative control. Either use more concentrated DNA or use more DNA volume during reaction setup. Abnormal qPCR curves: • Proceed as normal. Abnormal qPCR amplification curves may occur, and this is normal performance. This is usually a slight dip in RFU (forming a small "hump").
Diminished Amplicon Bands in Library Analysis	No single amplicon peak and/or high background: • Proceed as normal. There may be a lack of a single band and/or high background if using TapeStation® or similar methods to determine amplicon size. This is normal and is part of the library prep design. Do not use this sizing and quantification data. To properly quantify library, use a fluorescence-based method and calculation in **Section 4**.

For technical assistance, please contact 1-888-882-9682 or email tech@zymoresearch.com

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