

Quick-16S™ Plus NGS Library Prep Kit (V3-V4)

Fastest, normalization-free 16S library prep

Highlights

- The most streamlined NGS kit with only 30 minutes of hands-on time for 96 samples.
- 100% automation ready with only a single PCR step and without the need for normalization.
- Real-time PCR enables absolute microbial copy number quantification.

Catalog Numbers:
D6420, D6422



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



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Product Contents

Quick-16S™ Plus NGS Library Prep Kit (V3-V4)	D6420 (96 rxns.)	D6422 (24 rxns.)	Storage Temp.
Equalase™ qPCR Premix	1 ml	400 µl	-20°C
ZymoBIOMICS™ DNase/RNase Free Water	1 ml	1 ml	Room Temp.
ZymoBIOMICS™ Microbial Community DNA Standard (50 ng)	10 µl	10 µl	-20°C
ZymoBIOMICS™ 16S/ITS qPCR Standard	10 µl	10 µl	-20°C
V3-V4 Index Primer Set¹	A ² , 10 µl each (plate)	Z, 20 µl each (tube)	-20°C
PCR Inactivation Solution	100 µl	100 µl	Room Temp.
Select-a-Size MagBead Concentrate²	30 µl	30 µl	4-8°C
Select-a-Size MagBead Buffer²	1 ml	1 ml	4-8°C
DNA Wash Buffer	6 ml	6 ml	Room Temp.
Magnetic Rod	4	4	-
Instruction Manual	1 pc	1 pc	-

1 V3-V4 Index Primer Set A contains the Index Primers V4R ZT701-ZT712 and V3F ZT501-ZT508 already combined together to reach 10 µl total (5 µl of i7 index and 5 µl of i5 index) in a 96-well microplate. V3-V4 Index Primer Set Z contains the Index Primers V4R ZT701-ZT706 and V3F ZT501-ZT504 in tubes. 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 F for additional support and a template for the Illumina MiSeq® sample sheet.

2 Cat# D6420-PSB, D6420-PSC, & D6420-PSD contain primer plates B, C, & D respectively

3 The Select-a-Size MagBead Concentrate and Buffer are shipped at room temperature but should be stored at 4-8°C upon receipt.

Specifications

- **Sample Input** – Purified microbial DNA (≤ 100 ng), free of PCR inhibitors¹.
- **16S V3-V4 Primer Sequences (adapters not included)** – 341f (CCTACGGGCGGCWGCAG, 17 bp) and 806r (GACTACNVGGGTMTCTAATCC, 24 bp). The forward primer 341f is a mixture of the two sequences listed.
- **Primer Coverage**² – bacteria (84.2%), archaea (75.8%), and eukaryota (0.0%).
- **Index Primers** – Dual index (barcodes) to uniquely label samples³.
- **Barcode Sequences** – 10 bp barcodes are available for download by visiting the Documentation section of the D6420/D6422 Product Page at www.zymoresearch.com.
- **Amplicon Size** – The final amplicon size after 1-Step PCR (targeted amplification and barcode addition) is ~606 bp.
- **Sequencing Platform** – Illumina MiSeq® without the need to add custom sequencing primers. We recommend the MiSeq® Reagent Kit v3 (600-cycle). For assistance with sample sheet setup, see Appendix F.
- **Equipment Needed** (user provided) – Microcentrifuge, plate spinner (centrifuge), 96-well real-time quantitative PCR system (SYBR Green compatible, recommended), or standard PCR system, and 96-well real-time PCR plates.

¹ DNA that contains potent PCR inhibitors such as polyphenolics, humic/fulvic acids, tannins, melanin, etc. can be quickly cleaned using the OneStep™ PCR Inhibitor Removal Kit. See Appendix D for additional information.

² Search results from Silva database (March 2020), <https://www.arb-silva.de/search/testprime>.

³ For projects with more than 96 samples, please contact Zymo Research at oemorders@zymoresearch.com for additional indexing solutions.

Product Description

16S rRNA gene sequencing is a routine technique for microbiome composition profiling. Compared to shotgun metagenomics sequencing, 16S rRNA gene sequencing is more cost-effective and more robust; it generally requires less input DNA and is less impacted by the presence of non-microbial DNA. However, 16S rRNA gene 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. Moreover, common 16S library preparation protocols have not been optimized to be cost-effective for large-scale applications.

The **Quick-16S™ Plus NGS Library Prep Kit (V3-V4)** is the fastest and simplest library prep method for high-throughput 16S rRNA sequencing. Distinguishing features of the kit are described below.

Fastest 16S Workflow. The **Quick-16S™ Plus NGS Library Prep Kit (V3-V4)** utilizes a single qPCR/PCR for combined targeted amplification and barcode addition using specially designed primers. After pooling by equal volume, a single clean-up of the final library is performed, rather than multi-well magnetic bead clean-ups. Additional library quantification analysis such as TapeStation® analysis or gel electrophoresis is not necessary. With this workflow, the hands-on time of 16S library preparation is reduced to only 30 minutes (Figure 1).

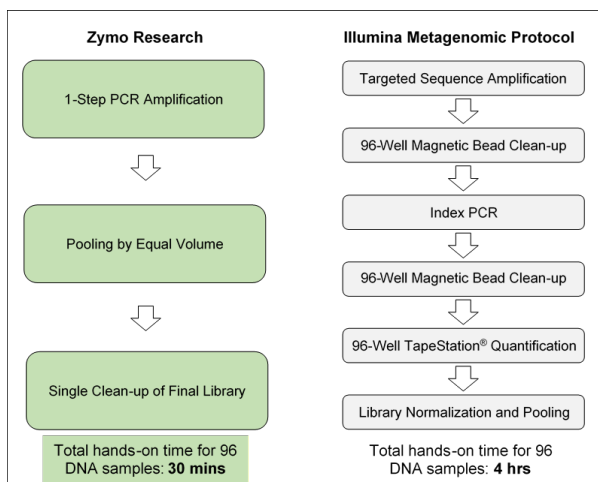


Figure 1. Quick-16S™ Plus NGS Library Prep Kit (V3-V4) workflow versus the Illumina Metagenomic Protocol. Total hands-on time calculations are based on the preparation of 96 DNA samples.

Normalization Free. There is no need to control sample input or PCR cycles. The workflow auto-normalizes and produces similar amounts of sequencing reads across all samples regardless of different DNA inputs (Figure 2). Just pool by equal volumes!

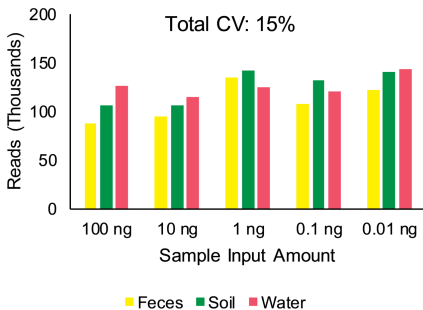


Figure 2. The Quick-16S™ Plus NGS Library Prep Kit (V3-V4) results in similar amounts of reads across different input amounts without normalization. 0.01-100 ng fecal, soil, and water DNA were used as inputs. Libraries were pooled by equal volumes (2 µl each) without further normalization and sequenced using the MiSeq® Reagent Kit v3 (600-cycle). The CV (coefficient of variation) is the ratio of the standard deviation to the mean with lower values corresponding to less dispersion around the mean.

High Quality NGS Library. The workflow has been optimized to minimize amplification bias (Figure 3). PCR chimera formation is maintained below 10% without the need to control PCR cycles (Figure 4).

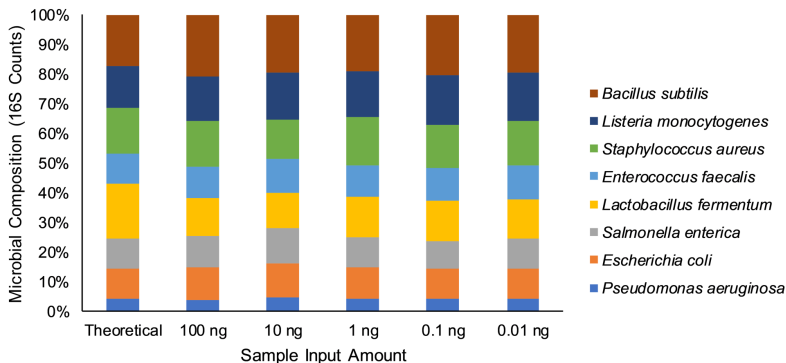


Figure 3. Benchmarked performance with ZymoBIOMICS™ Microbial Community DNA Standard. Bacterial composition profiles are accurate with inputs from 100 ng down to 0.01 ng.

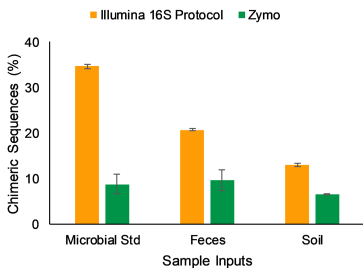


Figure 4. Up to 70% chimera reduction without controlling PCR cycles. Equal amounts of ZymoBIOMICS™ Microbial Community DNA Standard, feces, and soil were used as inputs and amplified for 42 cycles using 1-step PCR and Equalase™ compared to using the Illumina Metagenomic Protocol. Chimeric sequences were predicted with UCHIME (<https://www.drive5.com/uchime>).

Protocol

Before Starting

- ✓ **Sample Quantity Requirement.** To ensure color balance in index sequencing, a minimum of 9 samples per run is recommended using the index primers (ZT501-ZT503, ZT701-ZT703).
- ✓ **Input DNA Guidelines.** All DNA samples should be free of PCR inhibitors¹.
- ✓ **Preparing More than 96 Samples.** Please utilize the Guide for Preparing More than 96 Samples, which is downloadable from the Documentation section of the D6420/D6422 Product Page at www.zymoresearch.com.

Section 1: 1-Step PCR

1. Set up a master mix² according to the table below:

Component	Volume/Reaction
Equalase™ qPCR Premix	10 µl
ZymoBIOMICS™ DNase/RNase Free Water	4 µl
Total	14 µl

2. For each reaction, add 14 µl of the master mix to the appropriate wells of a 96-well real-time PCR plate. A sample of the plate setup can be found on the next page and on the Plate Setup Guide³.
3. From **V3-V4 Index Primer Set**⁴, pierce the foil and add 4 µl of the appropriate **Index Primer V4R ZT7XX** and **Index Primer V3F ZT5XX** combination to the proper wells of the PCR plate as indicated in the diagram on the next page:

(Continued on next page.)

1 DNA that contains potent PCR inhibitors such as polyphenolics, humic/fulvic acids, tannins, melanin, etc. can be quickly cleaned using the OneStep™ PCR Inhibitor Removal Kit. See Appendix D for additional information.

2 Prepare a master mix to include both a positive and negative control.

3 The Plate Setup Guide is available for download by visiting the Documentation section of the D6420/D6422 Product Page at www.zymoresearch.com.

4 If using V3-V4 Index Primer Set Z, add 2 µl of i7 index primer and 2 µl of i5 index primer from the appropriate tubes.

8. Monitor and QC the library preparation when running the reaction on a real-time thermocycler¹.
 - a. For example, a sample that is expected to amplify and shows little or no amplification may indicate an error in the reaction setup (See the Troubleshooting Guide).
 - b. The negative control should not amplify before 35 cycles². Earlier amplification of negative control may indicate process contaminations.
 - c. An example of qPCR amplification with controls is shown in Figure 5 below.

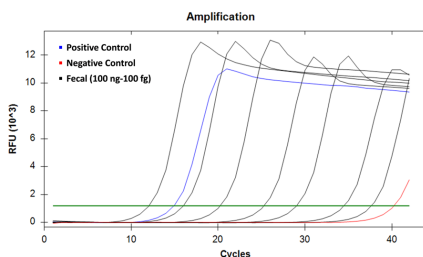


Figure 5. qPCR Amplification Example with Positive and Negative Controls. Serial dilutions of fecal DNA (black) from 100 ng to 100 fg were amplified on a Bio-Rad CFX96™ Real-Time PCR Detection System. The positive (blue) amplified at 14.93 and negative (red) amplified at 40.42. Baseline threshold was set at 1200 RFU.

9. Once the samples have cooled to 4°C, stop the program. Centrifuge plate in a plate spinner to collect condensation in wells and place plate on ice. Proceed to [Section 2](#), or store plate at ≤-20°C for later use.

Section 2: Pooling by Equal Volume

Add 50 µl of **PCR Inactivation Solution** into a new microcentrifuge tube. Pool equal volumes (5 µl³) of PCR products from each well of the plate from [Section 1](#) into the tube⁴ and mix well. Skip the wells of S89-S94 if they are used for qPCR standards. Proceed to [Section 3](#).

1 If real-time PCR was not used, after amplification perform PCR cleanup for a few samples plus positive control. Analyze on a TapeStation® to confirm correct amplicon size (~606 bp).

2 The PCR program runs for 42 cycles, so it is normal to see some amplification from the negative control. The negative control should be sequenced together with other samples. If appropriate for your project, the taxa from the negative control can be subtracted from the analysis.

3 If pooling >96 samples, use 2 µl instead to accommodate all samples.

4 There is no need for additional normalization procedures. To obtain better normalization and adjust pooling volumes, See Appendix B.

Section 3: Final Library Clean-up

1. Equilibrate the **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 **Select-a-Size MagBeads** to the pooled library from [Section 2](#) at a ratio of 0.8x volume. For example, add 400 µl of Select-a-Size MagBeads to 500 µl of the pooled library and PCR Inactivation Solution mixture.
3. Mix thoroughly by pipetting or vortexing until homogenous. Incubate for 5 minutes at room temperature.
4. Place the sample on a magnetic rack¹ 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 supernatant².
6. While the beads are still on the magnetic rack, add 1 ml 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 tube from the magnetic rack and keep the cap open for 3 minutes at room temperature to dry the beads.
9. Add 10-100 µl³ 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 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. Proceed to **Section 4.**

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 10 samples, use 10 µl for elution.

Section 4: Library Quantification

Use a fluorescence-based method (Qubit® dsDNA HS Assay Kit recommended) to quantify the final library. Using a final amplicon size of 606 bp, convert ng/μl to nM using the equation below.

$$\frac{\text{concentration in ng/}\mu\text{l}}{660 \text{ g/mol} \times \text{average library size in bp}} \times 10^6 = \text{concentration in nM}$$

For example: 20 ng/μl DNA of the final library is equivalent to 50.0 nM.

If preferred, a qPCR-based method for quantification may be used¹.

DNA Fragment Analysis (Not Required)

If a fragment analyzer (e.g. TapeStation®) is used to analyze the final library, there may be a lack of a tight band at ~600 bp. Because of the library prep design, some library products have run through additional PCR cycles and might not anneal well. They are perfectly fine for sequencing; which already denatures double-stranded DNA into single strands.

This is your final 16S library.

The ultra-pure pooled library DNA is now ready for use or storage at ≤-20°C. Refer to platform-specific guidelines for preparation for sequencing. No custom sequencing primers are needed.

Illumina MiSeq® Setup:

The MiSeq® Reagent Kit v3 (600-cycle) with 15% PhiX spike-in is recommended. See Appendix F for assistance with sample sheet setup. Remember to set the index size to 10 bp.

¹ For example, KAPA® Library Quantification Kit or similar.

Appendices

Appendix A: Absolute Quantification of Total 16S Copy Number by Real-Time PCR

Absolute quantification is determined with a real-time PCR standard curve using serially diluted amounts of the ZymoBIOMICS™ 16S/ITS qPCR Standard.

1. Dilute the ZymoBIOMICS™ 16S/ITS qPCR Standard (7.5×10^6 copies/ μ l) to 7.5×10^6 copies/ μ l, 7.5×10^4 copies/ μ l and 7.5×10^2 copies/ μ l.
2. Add 2 μ l of the standard and its dilutions to six wells (each in duplicate in positions S89-S94; see plate layout on Page 6) on the same plate that contains unknown samples for library prep.
3. After 1-Step PCR, plot the concentration (16S copies/ μ l) of the standards against the Ct values to produce a standard curve. See Figure 6 and 7 for an example.
4. Calculate the total 16S copy number of unknown samples based on their Ct value with the standard curve accordingly¹.

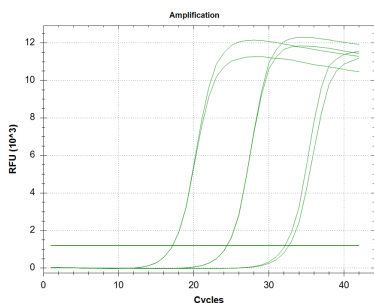


Figure 6. qPCR of Diluted Amounts of 16S/ITS Standard. The standard was amplified on a Bio-Rad CFX96™ Real-Time PCR Detection System. Baseline threshold was set at 1200 RFU.

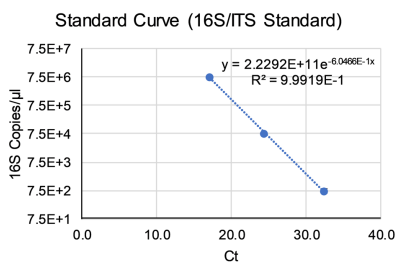


Figure 7. Absolute Quantification using a Standard Curve. Ct can be correlated to the total 16S copies based on the regression.

¹ Excel template for the Absolute Quantification of Total 16S Copy Number is available for download by visiting the Documentation section of the D6420/D6422 Product Page at www.zymoresearch.com.

Appendix B: Improving Read Distribution by Fine-Tuning Pooling Volumes based on qPCR

Typically, pooling samples by equal volumes will result in sufficient normalization and similar number of reads per sample. To obtain even better normalization, the pooling volumes can be adjusted based on Ct values obtained.

First, adjust the fluorescence threshold/cutoff to a certain value so that the positive control¹ has a Ct of 15. Use the following equation to calculate the pooling volume of each well based on its Ct: $\text{Volume } (\mu\text{l}) = 0.000598x^2 - 0.0637x + 3.178$; $x = \text{Ct}$. For example, the pooling volumes for Ct values of 15, 20, and 30 are 2.36 μl , 2.14 μl , and 1.81 μl respectively. This strategy can further reduce the CV (coefficient of variation) of reads assigned to samples by 3-6%.

Appendix C: Index Primer Sets

To accommodate sequencing projects of various sizes, Zymo Research offers primer sets that can uniquely barcode more than 96 samples. The barcodes of each index primer are distinct from one another by at least 5 bp to boost the accuracy of demultiplexing. For projects that require indexes for >96 samples, refer to Cat# D6420-PSB, D6420-PSC, & D6420-PSD that contain primer plates B, C, & D respectively. The Guide for >96 Samples is available for download by visiting the Documentation section of the D6420/D6422 Product Page at www.zymoresearch.com.

Appendix D: Removal of PCR Inhibitors from Starting DNA

The input DNA samples for the *Quick-16S*[™] Plus NGS Library Prep Kit (V3-V4) must be free of PCR inhibitors such as polyphenolics, humic/fulvic acids, tannins, melanin, etc. To further remove PCR inhibitors from purified DNA samples, Zymo Research recommends performing a one-step cleanup with the OneStep[™] PCR Inhibitor Removal Kit. Additional information can be found by visiting the D6030 Product Page at www.zymoresearch.com.

¹ The positive control is the sample that uses 2 μl of the ZymoBIOMICS[™] Microbial Community DNA Standard.

Appendix E: 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.

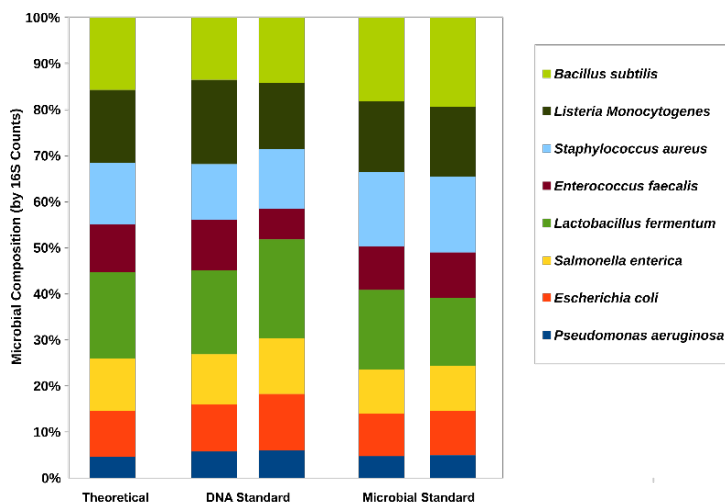


Figure 8. Accurate composition for reliable use to evaluate 16S rRNA sequencing. Characterization of the microbial composition of the two ZymoBIOMICS™ standards by 16S rRNA gene targeted sequencing. The measured composition of the two standards agrees with the theoretical/designed composition. "DNA Standard" 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 F: Illumina MiSeq® Sample Sheet Setup

A template for the Illumina MiSeq® sample sheet is available for download 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®.

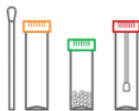
Ordering Information

Product Description	Catalog No.	Size / Format
Quick-16S™ Plus NGS Library Prep Kit (V3-V4)	D6420	96 rxns. / Primer Set A
	D6420-PSB	96 rxns. / Primer Set B
	D6420-PSC	96 rxns. / Primer Set C
	D6420-PSD	96 rxns. / Primer Set D
	D6422	24 rxns. / Primer Set Z

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

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

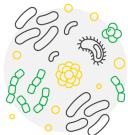
- ✓ Unbiased and inhibitor-free DNA and RNA extraction (high-throughput and automatable) for microbial profiling:



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	<p>Workspace contamination:</p> <ul style="list-style-type: none"> - Clean workspace, microcentrifuge, and pipettes with 10% bleach routinely to avoid contamination. - Use of kit in exposed environment without proper filtration can lead to back ground 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	<p>Adhesive seal:</p> <ul style="list-style-type: none"> - 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. <p>Lid pressure:</p> <ul style="list-style-type: none"> - 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	<p>Sample with high microbial DNA concentration:</p> <ul style="list-style-type: none"> - 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. <p>Sample with low microbial DNA concentration:</p> <ul style="list-style-type: none"> - 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. <p>Abnormal qPCR curves:</p> <ul style="list-style-type: none"> - 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	<p>No single amplicon peak and/or high background:</p> <ul style="list-style-type: none"> - 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

This image shows a single page of white paper with horizontal blue or grey ruling lines. The lines are evenly spaced and run across the width of the page, leaving small margins at the top and bottom. There are no vertical margin lines, and the page is completely blank except for the lines themselves.



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