



Challenge

Accurate and reproducible NGS library preparation for amplicon sequencing of microbial communities

Solution

The Biometra TAdvanced thermal cycler minimizes PCR bias and evaporation for optimal PCR specificity and product yield achieved by high temperature precision and uniformity

Intended audience

Laboratories depending on accurate and reproducible high-quality DNA library preparation for amplicon sequencing

Environmental Microbial Community Analysis Using Next-Generation Sequencing

Introduction

Soil microbial communities are important key drivers for essential biogeochemical functions, soil formation and health, which is directly linked to crop production^[1] as well as one health^[2]. Therefore, the profiling of environmental microbial communities plays a pivotal role for the understanding of their contributions to ecosystem functions.

Molecular analysis of microorganisms from environmental samples is commonly conducted using Next-Generation Sequencing (NGS) techniques. Sequencing of highly variable regions of marker genes represents a standard procedure for microbial community profiling, offering a sensitive method to achieve high sequencing coverage^[3]. This approach provides quick, robust and affordable results, which are highly standardized and comparable using extensive databases.

Those marker gene-based approaches often include a preliminary PCR that is crucial because its quality has

a direct impact on the sequencing results and thus the community profiling. PCR amplification and library preparation workflows depend on thermal cyclers with excellent performance. High temperature accuracy and block uniformity are crucial factors to avoid common PCR bias such as over- and under representation of specific sequences during amplification, especially for amplicons containing GC-rich regions. Thermal cyclers should also be robust to ensure reproducible results across a series of individual runs and should be capable of performing a variety of library preparation protocols in different well formats.

To evaluate the validity of PCR amplified sequencing libraries, standards with a pre-defined community composition (mock communities) can be incorporated as a quality control. Here, we demonstrate a robust amplicon sequencing workflow for 16S rRNA sequencing for the profiling of soil bacterial communities using the Biometra

TAdvanced 96 SG for the first PCR amplification step and evaluate the sequencing results based on a standard microbial community.

Materials and Methods

Samples and reagents

- DNA extracts from bulk soil samples
- Custom PCR mix containing Hot Start Taq DNA Polymerase (New England Biolabs), cat.: M0495S/L
- ZymoBIOMICS® Microbial Community Standard, cat.: D6300

Instrumentation

- Biometra TAdvanced 96 SG thermal cycler, Analytik Jena
- Horizontal agarose gel electrophoresis system, e.g., Biometra Compact Line, Analytik Jena
- Gel documentation system, e.g., UVP GelStudio, Analytik Jena
- Illumina® MiSeq sequencing system

Sample preparation

Bulk soil samples were collected from 0 to 30 cm depth of agroforestry and flower strip systems in Germany. DNA from ten soil samples was extracted using a cetyltrimethylammonium bromide protocol^[4]. As first step of the library preparation, the bacterial 16S rRNA gene from each soil sample was amplified using primers targeting the

V3-V4 region with a specific adapter overhang for p5 and p7 Illumina sequences necessary for library attachment on the flow cell. To assess the quality of the sequencing workflow, the 16S rRNA gene was additionally amplified from a standardized microbial community consisting of eight bacterial strains during library preparation. This allowed evaluation of how accurately the sequencing process could recover the known strain composition. For the first PCR, the DNA extracts were diluted 1:50 while 1 ng of the microbial community standard was processed for constructing the libraries. Three technical replicates at three different annealing temperatures were tested for the PCR of the community standards and one annealing temperature was used for the soil DNA following the PCR protocol shown in Table 1. After amplification, the PCR products were visualized via agarose gel electrophoresis (Figure 1). In a second amplification step, i5 and i7 ligation adapter were added with the index primers, which was performed by LGC Genomics (Berlin, Germany). Paired-end sequencing was conducted on an Illumina MiSeq system (2×300 bp) using V3 chemistry

Table 1: PCR cycler program for amplification of the 16S rRNA gene

Step	Cycle	Profile	Temperature	Holding time	Ramp rate
1	1	Initial denaturation	95 °C	2 min	8 °C/sec
2	24 – 30*	Denaturation	95 °C	20 sec	8 °C/sec
		Annealing	50 – 58 °C**	30 sec	5.5 °C/sec
		Elongation	60 °C	15 sec	8 °C/sec
3	1	Final Elongation	68 °C	5 min	8 °C/sec

* 24 cycles were run for soil samples and 30 cycles for the community standards.

** 3 replicates each at 50 °C, 55 °C and 58 °C

Bioinformatic analysis

After sequencing, the raw reads were demultiplexed and barcodes, adapter and primer sequences as well as short reads < 100 bp were removed using bcl2fast version 2.20 from Illumina. Downstream sequence analysis was performed using DADA2⁵ within the R framework⁶. Single reads were quality filtered, merged and chimeric sequenced were removed. Amplicon Sequencing Variants (ASV) were taxonomically classified against the SILVA ribosomal RNA gene database version 138.1.

Results and Discussion

Reproducible and specific PCR products guarantee an optimal input for NGS library preparation for amplicon sequencing. The first PCR using the Biometra TAdvanced thermal cycler depicted consistent band patterns for specific 16S rRNA PCR products from soil samples and the microbial community standards as validated by electrophoresis on 1.7% (w/v) agarose gels (Figure 1). No visible difference between the PCR products of different annealing temperatures for the standards were observed, depicting the specificity of the PCR setup for these samples.

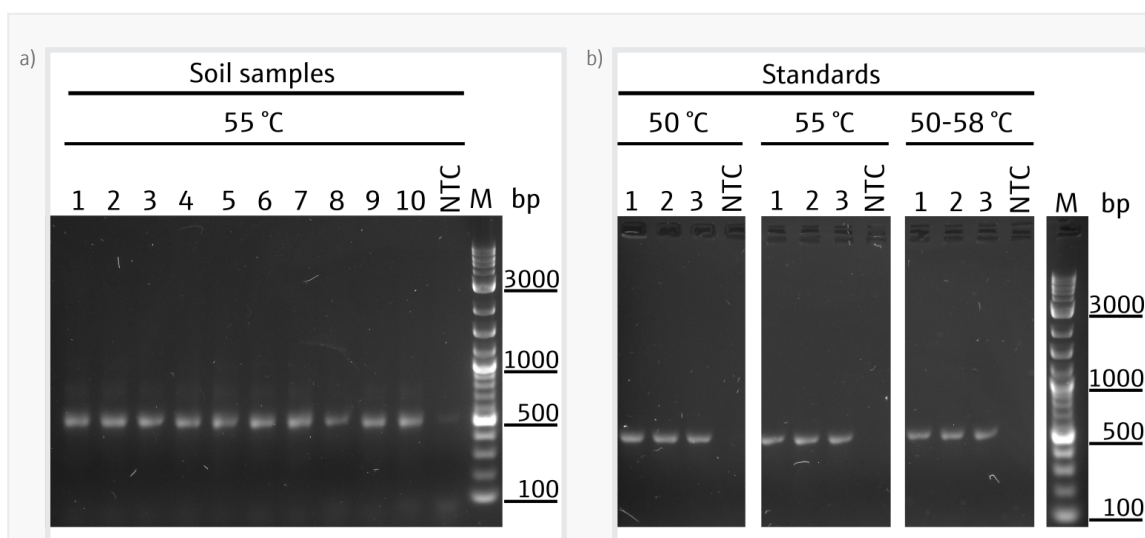


Figure 1: Visualization of fragment size and stability of the first PCR step from a) ten soil samples and b) three replicates for three different annealing temperatures for the ZymoBIOMICS microbial community standard. NTC (no template control) containing 0.5 x TE instead of DNA template. A 1 kb plus DNA Ladder (M) was used for fragment size verification.

The sequencing analysis revealed comparable relative abundance of both gram-positive and gram-negative bacterial strains at the genus level from ten sequenced replicates of the microbial community standard and the theoretical read distribution provided by the manufacturer (Figure 2, Figure 3). In addition, no bias or misclassification of ASV taxonomy was observed. The composition of the soil microbial communities exhibited different dominant bacterial genera and read fractions compared to the standard communities, underpinning the reliability of the sequencing data and the absence of cross-contamination between the standards and soil samples. Given the high diversity of soil microbial communities^[1], species richness and sample heterogeneity was higher than in the standard communities (Figure 4).

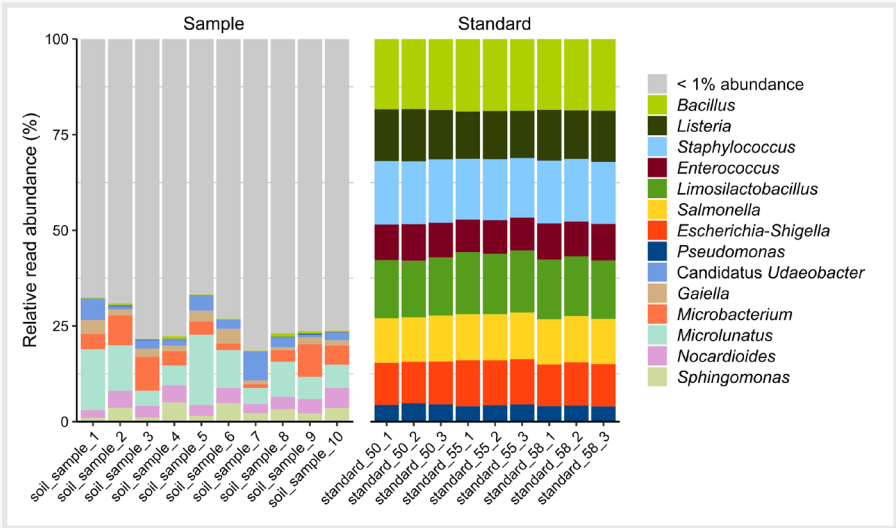


Figure 2: Bacterial community composition of soil samples and microbial community standards. The relative abundance (%) at the genus level is shown for each sequencing library. Taxa with < 1% abundance were grouped together.

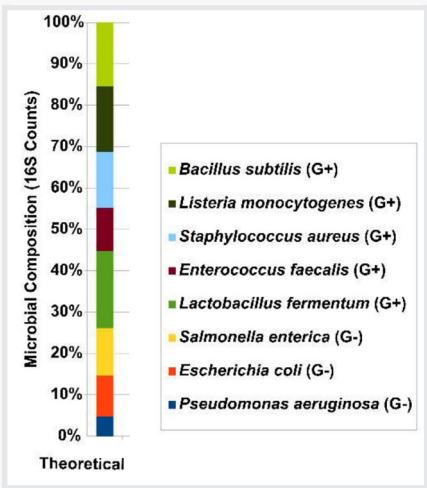


Figure 3: Theoretical size fractions of bacterial strains of the microbial community standard (from ZymoBIOMICS Microbial Community Standard).

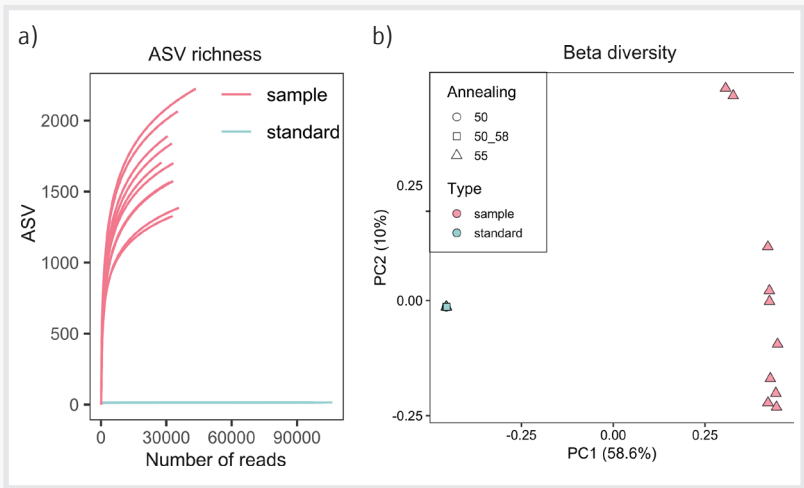


Figure 4: Diversity comparison of bacterial communities between the microbial community standards and soil samples depicted by a) ASV richness expressing the number of reads to ASV, and b) principal component analysis showing the beta diversity across the two sample types.

Summary

Reliable PCR amplification and incubation settings using thermal cyclers are essential for a variety of different library preparation protocols including amplicon sequencing for the acquisition of high-quality sequencing data. Microbial community profiling using the 16S rRNA marker gene represents a reliable, cost-efficient and well-established method in microbial screening in diverse fields of research. Using the high-performance Biometra TAdvanced 96 SG, consistent and reliable PCR products of bacterial communities from soil DNA and a microbial community standard containing a pre-defined mixture of bacterial strains were generated for the subsequent library preparation. All bacterial strains with predefined proportions in the standard community were retrieved at the genus level after sequencing data analysis without unspecific PCR products and ASV classification, confirming the validity of the described amplicon sequencing workflow.



Recommended device configuration

Table 2: Overview of devices

Article	Article number	Description
Biometra TAdvanced 96 SG	846-x-070-241 <i>x = 2 for 230 V, 4 for 115 V, 5 for 100 V</i>	Endpoint PCR thermal cycler with gradient function
Biometra TAdvanced 96 S	846-x-070-251 <i>x = 2 for 230 V, 4 for 115 V, 5 for 100 V</i>	Endpoint PCR thermal cycler

References

[1] Hartmann, M. & Six, J. Soil structure and microbiome functions in agroecosystems. *Nat Rev Earth Environ* 4, 4–18 (2023)

[2] Banerjee, S. & van der Heijden, M. G. A. Soil microbiomes and one health. *Nat Rev Microbiol* 21, 6–20 (2023)

[3] Hugerth, L. W. & Andersson, A. F. Analysing Microbial Community Composition through Amplicon Sequencing: From Sampling to Hypothesis Testing. *Front Microbiol* 8, 1561 (2017)

[4] Beule, L. & Karlovsky, P. Tree rows in temperate agroforestry croplands alter the composition of soil bacterial communities. *PLOS ONE* 16, e0246919 (2021)

[5] Callahan, B. J. et al. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* 13, 581–583 (2016).

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