16S rRNA gene amplicon sequencing library preparation

GENERAL INFORMATION:

- This protocol is based on a semi-automated workflow using a Biomek4000 pipetting robot (Beckman Coulter) to enhance reproducibility and efficacy. It is preceded by a DNA extraction protocol optimized for stool samples (CRC1382-CLA-P04_DNA-extraction). If using samples with low biomass or containing specific microbes that are hard to lyse, adjustments will be necessary (Kennedy et al. 2023).
- For low biomass samples, the number of cycles of the 2nd PCR step can be increased to enhance the chance to obtain sufficient amplicon products; however, do **not** exceed 35 cycles in total; appropriate controls must always be run under the same conditions and provide negative results.
- Amplification may not work for multiple reasons. Increasing dilution of the DNA (e.g., from stool samples) can help diluting potential PCR reaction inhibitors; adding 1 µL of BSA (Sigma Aldrich P5369; 200mg/ml; dilution 1:100.) in the first PCR step and 1.5µL in the second PCR step can help increasing the stringency of reaction, leading to successful amplification.
- For FACS-sorted bacterial samples, DNA extraction with the Mericon Kit (Qiagen DNA Bacteria Plus Kit, Cat. NO: 69534) can be considered.

Do

- Add a blank DNA extraction control for each sample set.
- Add one negative PCR control per PCR (plate).
- Mind potential cross-contamination between samples (e.g., the enclosure of the pipetting robot is equipped with UV-light).
- Prepare low-biomass samples separate from high-biomass samples (e.g., on a separate plate)
- Regularly check if any material/surfaces/reagents you use, is free of potential contamination sources by performing DNA extraction/PCR/agarose gel.

Do <u>NOT</u>:

- Increase the number of PCR cycles above 35
- Pool your samples or DNA extracts prior to the library preparation
- Run Datasets without controls

FURTHER RESOURCES:

Protocols and videos: https://www.crc1382.org/q02/details

- CRC1382-CLA-P04_DNA-extraction

- CRC1382-CLA-V01_Sample-collection
- CRC1382-CLA-V02_DNA-extraction
- CRC1382-CLA-V03_Amplicon-libraries
- CRC1382-CLA-V04_Sequencing-Run-Preparation

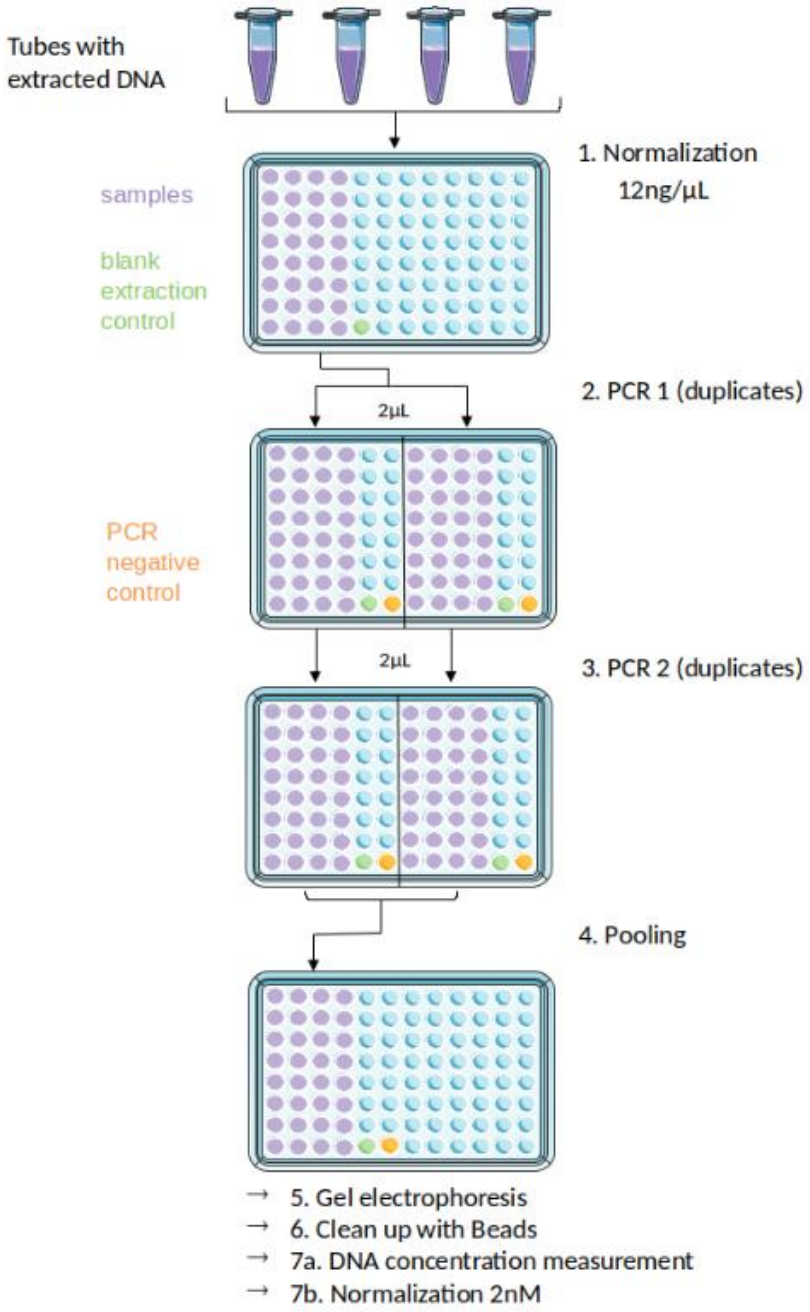

Figure 1: Schematic overview of the amplicon library workflow on 96-well plates
After DNA extraction, the samples (purple tubes and wells) are normalized to 12 ng/µL according to Nanodrop measurements (step 1 below). 2µL is used as template for the first PCR step (step 2 below), which is specific for amplifying the V3-V4 regions of 16S rRNA genes. A blank extraction control (green) and a PCR negative control (orange) are always included. 2µL of PCR1-products are used as template for the second PCR (step 3 below). The primers of the second PCR bind regions in the first primer set, and they contain the sample-specific indices that enable multiplexing and the Illumina adapters for binding to the flow cell. Both PCRs are done in duplicates to reduce primer selection bias. The duplicates are pooled per sample (step 4) before a gel electrophoresis (step 5) is run to check for amplification success. The pooled PCR products are cleaned using AMPureXP beads (step 6). DNA concentrations in the resulting, cleaned sequencing libraries are determined by a fluorometric method (step 7a) before samples are normalized to 2nM (step 7b) and equal volumes are pooled for sequencing.

1. Normalisation of DNA extracts to 12 ng/µL

Dilute each sample to a concentration of 12ng/µL based on previous Nanodrop measurement after metagenomic DNA extraction (see protocol CRC1382-CLA-P04_DNA-Extraction)

2. PCR1 (in duplicates) (15 cycles)

The PCR steps are prepared in duplicates to reduce primer selection bias (Berry et al. 2011). The 16S rRNA gene-targeted primers are 341F-785R (Klindworth et al. 2013).
Negative controls are included in each PCR run (Figure 1, orange well).
Blank DNA extraction samples are also included (Figure 1, green well).

Primer Step 1
341F-ovh
F-overhang / **16S rRNA gene-targeted region**
5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG **CCTACGGGNGGCWGCAG**-3'
+
785r-ovh
R-overhang / **16S rRNA gene-targeted region**
5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG **GACTACHVGGGTATCTAATCC**-3'

Master Mix:

Reagents	**Volume / sample**
Phusion® HF Buffer (without Dye)	4 µL
dNTPs (10mM stock)	0.4 µL
341F-ovh Primer (20 µM stock)	0.125 µL
785r-ovh Primer (20 µM stock)	0.125 µL
Phusion® High-Fidelity DNA Polymerase Hotstart	0.1 µL
DMSO 100%	2.5 µL
DEPC water	10.75 µL

Total volume: 20 µL per sample (18 µL Master Mix + 2 µL DNA suspension)
Template: 2µL from 12ng/µL normalization (=24ng)

PCR program:

98°C	40 s	
98°C	20 s	15 cycles
55°C	40 s	
72°C	40 s	
72°C	2 min	
10°C	∞	

Lid constantly heated to 105°C

3. PCR2 (in duplicates) (10 cycles)

Primer Step 2
341-ovh-HTS-
P5-adapter / 8 index/golay barcode (as in Kozich et al) / overhang
SC501 5'-AATGATACGGCGACCACCGAGATCTACAC ACGACGTG TCGTCGGCAGCGTC-3'
… or any other primer with a different barcode
+
785r-ovh-HTS-
RC-P7-adapter / 8 index/golay barcode (as in Kozich et al) / overhang
SA701 5'-CAAGCAGAAGACGGCATACGAGAT AACTCTCG GTCTCGTGGGCTCGG-3'
… or any other primer with a different barcode

Reagents	**Volume / samples**
Phusion® HF Buffer (without Dye)	10 µL
dNTPs (10mM)	1 µL
e.g. 341-ovh-HTS- SC501 Primer (20µM stock)	0.313 µL
e.g. 785r-ovh-HTS-SA701 Primer (2.5µM stock)*	2.5 µL
Phusion® High-Fidelity DNA Polymerase Hotstart	0.2µL
DMSO 100%	2.5 µL
DEPC water	31.487

*Difference in concentration of fwd. and rev. primer is due to handling by robot

Total volume: 50 µL per sample (48 µL Master Mix + 2 µL Probe)

Template: 2µL, directly from PCR 1 output

98°C	40 s	
98°C	20 s	10 cycles
55°C	40 s	
72°C	40 s	
72°C	2 min	
10°C	∞	

Lid constantly heated to 105°C

Expected product size after PCR 2: ~560 bp

4. Pooling after PCR

Pool (pipette together) the duplicate PCR products for each sample.

5. Gel electrophoresis

Run a 1.5% agarose gel to control for amplification.

Recommendations until protocol is established in your own lab:

1. Run all samples on a gel. Thereafter, 16 randomly selected samples from each plate and the negative controls can be run to check the PCR success.
2. Check some samples on a 1.5% agarose gel for primer dimers **after the clean-up**. If dimers occur (visible at the very bottom of the gel), you can reduce the concentration of primers in your PCR reaction, alter the bead ratio during clean-up, or add another clean up step. **Primer dimers must be removed**, as they bind to the Illumina flow cell, preventing your actual amplicons to bind and be sequenced.

6. Amplicon library clean-up

- Transfer 100 µL of the amplified DNA suspension from Pool (step 5) to wells of a fresh 96-well plate, add 144 µL AMPure beads (1:1.8 ratio), mix well by pipetting up and down at least 10x, and incubate for 5 min at room temperature.
- Place the plate on the magnetic rack; let stand at room temperature for 5 min to separate beads from supernatant.
- With the plate still on the magnetic rack, discard the supernatant by pipetting; be very careful not to touch the beads.
- Still on magnetic rack, add 180 µL 80% ethanol to the beads, and incubate for 30 sec.
- Discard the ethanol by pipetting and repeat previous step once; discard ethanol again.
- Air-dry the beads on the magnetic rack for 5 min (max) to remove the ethanol by evaporation. (**Caution**: Do not over-dry the beads. This may result in lower DNA recovery. When beads start to show cracks, they are too dry).
- Remove the plate from the magnetic rack and elute clean PCR products by pipetting 70 µL of 0.1 x TE buffer into wells; mix well by pipetting up and down at least 10x; incubate at room temperature for 2 min.
- Place the plate back on the magnetic rack, let stand for 5 min and transfer 60 µL* of the suspension containing the eluted DNA to new tube (avoid touching the beads).

*(if not working with an automated platform, transfer volume can be increased to 65 µL)

7. Final steps of library preparation

a) Measure DNA concentrations using a fluorometric method (e.g., picogreen or Qubit)

b) Dilute each library to normalise the concentration to 2 nM according to the following rule:

$$\frac{(\text{concentration in ng/µl})}{(660\ \text{g/mol} \times \text{average library size})} \times 10^6 = \text{concentration in nM}$$

Pool all the samples together (equal volume) to obtain the final, multiplexed DNA library pool; adjust to 13 pM and 25% phiX following the instructions provided in the Illumina protocol:
MiSeq System Denature and Dilute Libraries Guide

See also our own video CRC1382-CLA-V04_Sequencing-run-preparation

Prepare a sample sheet according to the instructions provided by Illumina:
Illumina custom protocol selector
(in our lab: Instrument: MiSeq; software: MCS 4.0; module: Generate FASTQ 3.0.1.)

10. Run sequencer

See our own video CRC1382-CLA-V04_Sequencing-run-preparation

See also Illumina instructions:
Illumina MiSeq system guide

List of material and chemicals used

Normalisation to 12ng/µl		
Name	Company	Ref.
ZymoBIOMICS Microbial Community DNA Standard (2000ng)	Zymo Research	D6306
DNA LoBind® Tubes, DNA LoBind®, 1.5 mL, PCR clean	Eppendorf	30108051
Water for molecular biology, DEPC-treated and sterile filtered	Sigma	7732-18-5
FrameStar® 96 Well Skirted PCR Plate	4titude	4ti-0960
PCR Seal transparent	NeoLab	1209867
PCRs		
Name	Company	Ref.
FrameStar® 96 Well Skirted PCR Plate	4titude	4ti-0960
PCR Foil Seal	NeoLab	1210962
Phusion™ Hot Start II High-Fidelity DNA-Polymerase	Thermo Scientific	F549L
dNTP-Set	Thermo Scientific	R0182
Dimethylsulfoxid (DMSO)	Merck	D8418-50ML
PCR-Cooler 0.2 mL	Eppendorf	22510541
2.0ml Screw Cap Micro Tube PCR-PT	Starstedt	72.694.406
Pooling		
Name	Company	Ref.
1.2ml Low Profile Square Storage Plate	Thermo Scientific	AB1127

Gel		
Name	Company	Ref.
ROTIPHORESE®10x TAE Buffer	Roth	T845.2
Agarose	Sigma	A9539
GelRed® Nucleic Acid Gel Stain	Biotium	41003
Midori Green Advance	Nippon genetics	MG04
GeneRuler 1 kb DNA Ladder	Thermo Scientific	SM0311
Cleanup		
Name	Company	Ref.
Ampure XP	Beckman Coulter	A63881
ROTI®Stock 100x TE	Carl Roth	1052.1
Alpaqua MAGNUM FLX® Enhanced Universal Magnet Plate	NimaGen	A000400
Ethanol absolute ≥99.8%, AnalaR NORMAPUR® ACS, Reag. Ph. Eur. analytical reagent	VWR	20821.321 P
After Cleanup Quantification		
Name	Company	Ref.
Black Assay Plates 96-Well	Brooks Life Sciences	4ti-0263
Quant-iT™ PicoGreen™ dsDNA Assay-Kit	Invitrogen	P11496
Quant-iT™ Qubit™ dsDNA HS Assaykit	Invitrogen	Q32851
Library Prep		
Name	Company	Ref.
Buffer EB	Qiagen	19086
TWEEN® 20 Detergent	EMD Millipore Corp	CAS 9005-64-5
Kimtech Science Trocken-Reinigungstücher	Kimberly Clark	495-1665 or 7552
Phix Control Kit V3	Illumina	FC-110-3001
Miseq® Reagent Kit v3 (600 cycle)	Illumina	MS-102-3003

References

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Kennedy, Katherine M et al. “Questioning the fetal microbiome illustrates pitfalls of low-biomass microbial studies.” *Nature* vol. 613,7945 (2023): 639-649. doi:10.1038/s41586-022-05546-8

Berry D, Ben Mahfoudh K, Wagner M, Loy A. Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. Appl Environ Microbiol 2011;77(21):7846-9.

Klindworth A, Pruesse E, Schweer T, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 2013;41(1):e1.