Amplification of bacterial full-length 16S gene with barcoded primers

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Procedure & checklist

This procedure describes the PCR amplification of bacterial full-length 16S rRNA genes (V1—V9 regions) using up to 192 dual indices. See Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 for a detailed library preparation procedure.

Overview	
Samples	1—192
Metagenomic DNA input for PCR	1-2 ng
16S rRNA degenerate forward primer sequence*	5'GCATC/barcode/AGRGTTYGATYMTGGCTCAG3'
16S rRNA degenerate reverse primer sequence*	5'GCATC/barcode/RGYTACCTTGTTACGACTT3'

*Full sequence with barcode shown in the appendix

Before you begin

Order the 32 barcoded primers, as listed in the appendix, from your preferred oligo vendor. Upon receipt, the primers should be resuspended at a 100 μM concentration in 1XTE or elution buffer and stored at -20°C. Avoid repeated freeze-thaw cycles. The Oligo Order Sheet and FASTA file for demultiplexing are available on our **Multiplexing page**.

Prior to setting up the PCR, make a working dilution of each barcoded primer stock at 2.5 μM in elution buffer.

Measure the DNA concentration of each sample using a Nanodrop Spectrophotometer or Qubit fluorometer with the 1X dsDNA HS assay.

Normalize DNA samples to a concentration of 500 pg/μL in elution buffer or nuclease-free water.

Thaw KAPA HiFi HotStart 2x ReadyMix PCR Reagent on ice and mix well before use.

General best practices

Keep all KAPA HiFi HotStart reagents and reactions on ice until PCR; the high proofreading activity of the enzyme will rapidly degrade primers at room temperature. This is generally true for all high-fidelity polymerases.

Use high-quality DNA and work in a PCR-clean environment to avoid contamination.

Use non-template control (NTC) to check for contamination.

Normalize DNA input amounts so that ~ 2ng of DNA is used per PCR well. Generating similar amounts of 16S rRNA gene amplification per well will help balance the amplicon pool for SMRTbell® library preparation.

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Metagenomic samples with high amounts of host or non-bacterial DNA may require higher amounts of input DNA into PCR. Scale DNA input concentration as appropriate to achieve the necessary amount of amplification.

Use the following barcoded primer design for up to 192 dual indices. Do not repeat any barcode combinations in a single experiment.

Add the forward 16S rRNA barcoded primer across the rows of the 96-well PCR plate as shown in

BARCODED FORWARD PRIMER MASTER MIX TO ADD	Row
>16S_For_bc1005 Master Mix (17 µL per well)	A
>16S_For_bc1007 Master Mix (17 µL per well)	B
>16S_For_bc1008 Master Mix (17 µL per well)	C
>16S_For_bc1012 Master Mix (17 µL per well)	D
>16S_For_bc1015 Master Mix (17 µL per well)	E
>16S_For_bc1020 Master Mix (17 µL per well)	F
>16S_For_bc1022 Master Mix (17 µL per well)	G
>16S_For_bc1024 Master Mix (17 µL per well)	H

Figure 1. Barcoded forward primer mix added across the rows A—H.

Add the reverse 16S rRNA barcoded primer down the column of the 96-well PCR plate as shown in figure 2. For the 1st 96-well plate use the first 12 reverse primers, and for the 2nd 96-well plate use the last 12 reverse primers listed in the appendix.

BARCODED REVERSE PRIMER TO ADD (3 µL PER WELL)											
>16S_Rev_bc1033	>16S_Rev_bc1035	>16S_Rev_bc1044	>16S_Rev_bc1045	>16S_Rev_bc1054	>16S_Rev_bc1056	>16S_Rev_bc1057	>16S_Rev_bc1059	>16S_Rev_bc1060	>16S_Rev_bc1062	>16S_Rev_bc1065	>16S_Rev_bc1075
1	2	3	4	5	6	7	8	9	10	11	12

Figure 2. Barcoded reverse primers to add down each column on the 96-well PCR plate.

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Spot check PCR prior to pooling samples to ensure amplification was successful.

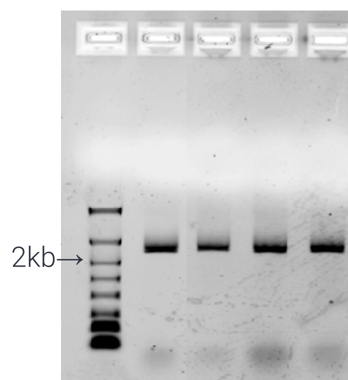

Figure 3. Example agarose gel spot-check of individual PCR reactions. 1 µL from each of four independent PCR reactions was analyzed per lane of a 1.2% agarose Lonza DNA Flash Gel according to the manufacturer's recommendations. The PCR products were of the expected size (~1.5 kb) and of comparable quantity as determined by visual inspection of their band intensities.

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Materials and kits needed

Item	Part number
16S amplification	
KAPA HiFi HotStart ReadyMix PCR kit	Roche KK2600 (or KK2601 or KK2602)
Barcoded 16S primers	Any Oligo vendor
96-well block PCR thermocycler	Any major lab supplier (MLS)
Nuclease-free PCR grade water	Any MLS
Single channel pipettes (P2, P10, P20, P200, and P1000)	Any MLS
96-well PCR plate	Any MLS
QC tools	
Qubit Fluorometer	Thermo-Fisher Q33238
Qubit 1X dsDNA High Sensitivity Kit	Thermo-Fisher Q33231
NanoDrop Technologies ND-2000 UV/Vis Spectrophotometer or equivalent	Thermo-Fisher ND-2000
BioAnalyzer or 4200 TapeStation	Agilent technologies G2939BA or G2991BA
General lab supplies and equipment	
DNA LoBind tubes, 2.0 mL	Eppendorf 022431048
DNA LoBind tubes, 5.0 mL	Eppendorf EP0030108310

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PCR amplification of bacterial 16S rRNA

✔	Step	Instructions
	1.1	Add the components listed below into a 2.0- or 5.0-mL DNA LoBind tube. Increase component volumes for the number of samples (N) being amplified plus a 25% overage for pipetting margin.

Component	Volume per N (µL)	N = ___	For 96-plex*	For 192-plex*
PCR-grade water	1.5 x N x 1.25	___ µL	180.0 µL	360.0 µL
2X KAPA HiFi HotStart ReadyMix	12.5 x N x 1.25	___ µL	1500.0 µL	3000.0 µL
Total volume	**14.0 x N x 1.25**	**____ µL**	**1680.0 µL**	**3360.0 µL**

1.2 Label 8 microfuge tubes A, B, C, D, E, F, G, and H to assign a specific barcoded forward primer to each tube (see table below).

Tube	Barcoded Forward Primer
A	16S_For_**bc1005**
B	16S_For_**bc1007**
C	16S_For_**bc1008**
D	16S_For_**bc1012**
E	16S_For_**bc1015**
F	16S_For_**bc1020**
G	16S_For_**bc1022**
H	16S_For_**bc1024**

1.3 Add the appropriate volume of PCR master mix into each of the eight tubes (A—H). Use the table below for calculating the volume of PCR master mix to use per tube.

Component	N wells per row (µL)	96-plex mix (µL)	192-plex mix (µL)
PCR master mix (from 1.1)	14.0 x N x 1.2	201.6	403.2

1.4 Add the appropriate volume of barcoded forward primer (2.5 µM) to each respective tube (A—H). Use the table below for calculating the volume of forward primer to use per tube. Mix by pipetting 10 times.

Component	N wells per row (µL)	96-plex Mix (µL)	192-plex Mix (µL)
barcoded forward primer (2.5 µM)	3.0 x N x 1.2	43.2	86.4
Total tube volume	**17.0 x N x 1.2**	**244.8**	**489.6**

1.5 Dispense **17 µL** of the PCR master mix + barcoded forward primer (tubes A-H) to each respective well across rows A-H of a 96-well plate. For a 192-plex experiment design, use two 96-well plates.

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1.6	Add **3 µL** of the respective barcoded reverse primer (2.5 µM) to each column (1–12). For a 96-plex, use the first 12 reverse primers, and for a 192-plex, use all 24 reverse primers listed in the Appendix. The total volume in each well is **20 µL**.
1.7	Add **5 µL** (1-2 ng) of each diluted gDNA sample to a single well of the 96-well PCR plate(s) for a total reaction volume of **25 µL**.
1.8	Mix well by pipetting. Seal the plates tightly with adhesive seal to prevent evaporation during PCR. Briefly spin the plate in a refrigerated centrifuge (4°C) to ensure that the entire sample volume is at the bottom of each well.
1.9	Place PCR plate, or plates, on thermocycler and run the following program: (see table below)

	Step	Temperature	Time
1	Initial Denature	95 °C	3 minutes
2	Denature	95 °C	30 seconds
3	Anneal*	57 °C	30 seconds
4	Extend	72 °C	60 seconds
		Repeat steps 2 to 4 for a total of 25 cycles	

*Refer to manufacturer's recommendations for your thermocycler instrument to set the ramp rate for the annealing step to ≤ 3°C per second.

1.10	Spot-check amplification results by directly loading 1 µL of one or more PCR reactions onto an agarose gel. A typical result is shown in Figure 3 above. The expected target amplicon size is ~1500 bp, and the amount of amplicon material generated from each sample should be comparable as assessed by analyzing the relative intensity of the ~1500 bp PCR product on an agarose electrophoresis gel. If available, you may also use an Agilent Bioanalyzer System or TapeStation System to spot-check PCR product size and quantity.
1.11	Pool an **equal volume** of each barcoded amplicon together in a 2.0 mL DNA LoBind tube. Do not exceed a total volume of 800 µL. • 96 samples use 8 µL per sample (for a total of approximately 800 µL) • 192 samples use 4 µL per sample (for a total of approximately 800 µL)
1.12	Proceed to Preparing multiplexed amplicon libraries using SMRTbell prep kit 3.0 or freeze pooled sample at -20°C until ready to proceed.

PROTOCOL COMPLETE

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Appendix

The list includes sequences for thirty-two primers (8 forward and 24 reverse) that can used for up to 192 dual indices. Each primer contains a 5' spacer sequence (GCATC), a 16-base barcode (in bold), and the degenerate gene-specific sequence for amplifying the bacterial 16S rRNA gene (V1—V9 regions). Degenerate base identities are: R = A,G; Y = C,T; M = A,C.
Avoid repeated freeze-thaw cycles.

Barcoded forward and reverse16S gene-specific primers recommended for use with this procedure (PacBio barcode sequences are **bolded**).

Barcoded forward primer	
>16S_For_bc1005	/5Phos/GCATC**CACTCGACTCTCGCGT**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1007	/5Phos/GCATC**TCTGTATCTCTATGTG**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1008	/5Phos/GCATC**ACAGTCGAGCGCTGCG**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1012	/5Phos/GCATC**ACACTAGATCGCGTGT**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1015	/5Phos/GCATC**CGCATGACACGTGTGT**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1020	/5Phos/GCATC**CACGACACGACGATGT**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1022	/5Phos/GCATC**CACTCACGTGTGATAT**AGRGTTYGATYMTGGCTCAG
>16S_For_bc1024	/5Phos/GCATC**CATGTAGAGCAGAGAG**AGRGTTYGATYMTGGCTCAG
Barcoded reverse primer	
>16S_Rev_bc1033	/5Phos/GCATC**AGAGACTGCGACGAGA**RGYTACCTTGTTACGACTT
>16S_Rev_bc1035	/5Phos/GCATC**CAGAGAGTGCGCGCGC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1044	/5Phos/GCATC**CGCGCGTCGTCTCAGC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1045	/5Phos/GCATC**AGAGAGTACGATATGT**RGYTACCTTGTTACGACTT
>16S_Rev_bc1054	/5Phos/GCATC**TCTGTAGTGCGTGCGC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1056	/5Phos/GCATC**ATGTGCGTGTGTGTCT**RGYTACCTTGTTACGACTT
>16S_Rev_bc1057	/5Phos/GCATC**CTCTCAGACGCTCGTC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1059	/5Phos/GCATC**TATCTCAGTGCGTGTG**RGYTACCTTGTTACGACTT
>16S_Rev_bc1060	/5Phos/GCATC**TGTGTCTATACTCATC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1062	/5Phos/GCATC**TATAGACTATCTGAGA**RGYTACCTTGTTACGACTT
>16S_Rev_bc1065	/5Phos/GCATC**GTATGTGAGAGAGCGC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1075	/5Phos/GCATC**CACGCGACGCTCTCTA**RGYTACCTTGTTACGACTT
>16S_Rev_bc1076	/5Phos/GCATC**GAGAGCGCGAGTGCAC**RGYTACCTTGTTACGACTT

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>16S_Rev_bc1082	/5Phos/GCATC**GTGCTCTGTGTGTCAC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1083	/5Phos/GCATC**TGCGTGTATGTCATAT**RGYTACCTTGTTACGACTT
>16S_Rev_bc1089	/5Phos/GCATC**ACGAGATACTCGCGCG**RGYTACCTTGTTACGACTT
>16S_Rev_bc1096	/5Phos/GCATC**CTGTGTAGAGAGCACA**RGYTACCTTGTTACGACTT
>16S_Rev_bc1098	/5Phos/GCATC**TGATGTGACACTGCGC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1100	/5Phos/GCATC**ACTACTGAGACATAGA**RGYTACCTTGTTACGACTT
>16S_Rev_bc1101	/5Phos/GCATC**TATATCGCGTCGCTAT**RGYTACCTTGTTACGACTT
>16S_Rev_bc1105	/5Phos/GCATC**GCGTACTGCGACTGTG**RGYTACCTTGTTACGACTT
>16S_Rev_bc1107	/5Phos/GCATC**ATATATGCACGCTCTA**RGYTACCTTGTTACGACTT
>16S_Rev_bc1110	/5Phos/GCATC**CGCTGTATACACGCTC**RGYTACCTTGTTACGACTT
>16S_Rev_bc1112	/5Phos/GCATC**AGAGACTGTAGCGCAC**RGYTACCTTGTTACGACTT

Revision history (description)	Version	Date
Initial release	01	June 2018
Updates throughout based on KAPA HiFi HotStart ReadyMix PCR Kit.	02	June 2019
Updated to provide the sequences (and ordering information) for 8 barcoded forward, and 12 barcoded reverse, 16S-specific primers that can be combined for multiplexed analysis for up to 96 samples using an asymmetric barcoding strategy. Clarify preparation of PCR master mixes for the amplification of 96 samples.	03	February 2020
Added 12 more Reverse Primers for a total of 24, allowing multiplexing of up to 192 samples.	04	January 2021
Removed Express template prep kit 2.0 steps for SMRTbell library preparation. Protocol covers only the 16S rRNA gene amplification steps. Added overview table, and Before you begin and General best practices sections. Title changed to reflect that the protocol describes only the amplification steps for bacterial 16S rRNA gene amplification.	05	May 2022

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