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FOR REFERENCE PURPOSES

This manual is for Reference Purposes Only. DO NOT use this protocol to run your assays. Periodically, optimizations and revisions are made to the kit and protocol, so it is important to always use the protocol included with the kit.

NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0 - 4

(Illumina Compatible)

Catalog #4203-01 (8 reactions)

innovación tecnológica para laboratorio Rafer

This product is for research use only.

NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0 - 4 - 4203-01

Product Overview

The NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0 is designed to prepare multiplexed amplicon libraries that span the fourth hypervariable domain of microbial 16S ribosomal RNA (rRNA) genes. These libraries are compatible with paired-end sequencing on the Illumina® MiSeq platform.

There are two main steps involved in 16S V4 amplicon processing: an initial PCR amplification using customized PCR primers that target the V4 domain, and a subsequent PCR amplification that integrates relevant flow cell binding domains and unique 12 base pair sample indices. A limited number of cleanup steps ensures maximum recovery of amplicons for downstream sequencing. Custom sequencing read primers are not required with this version of the 16S V4 Amplicon-Seq Kit.

Contents, Storage and Shelf Life

The NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0 contains enough material to prepare eight 16S V4 samples from genomic DNA for Illumina® compatible sequencing. The shelf life of all reagents is 12 months when stored properly. All components can be safely stored at -20°C.

Kit Contents	Amount
GREEN CAP	
NEXTflex™ Amplicon PCR Master Mix	240 µL
ORANGE CAP	
NEXTflex™ 16S V4 PCR I Primer Mix	16 µL
YELLOW CAP	
NEXTflex™ PCR II Barcoded Primer Mix 1 – 4	4 µL
WHITE CAP	
Resuspension Buffer	1 mL
Nuclease-free Water	1.5 mL

Required Materials not Provided

- 1 ng - 50 ng high-quality genomic DNA in up to 33 µL nuclease-free water
- 96 well PCR Plate Non-skirted (Phenix Research, Cat # MPS-499) or similar
- Adhesive PCR Plate Seal (BioRad, Cat # MSB1001)
- Agencourt AMPure XP 5 mL (Beckman Coulter Genomics, Cat # A63880)
- Magnetic Stand -96 (Ambion, Cat # AM10027) or similar
- Thermocycler
- 2, 10, 20, 200 and 1000 µL pipettes / multichannel pipettes
- Nuclease-free barrier pipette tips
- Vortex
- 80% Ethanol, freshly prepared (room temperature)

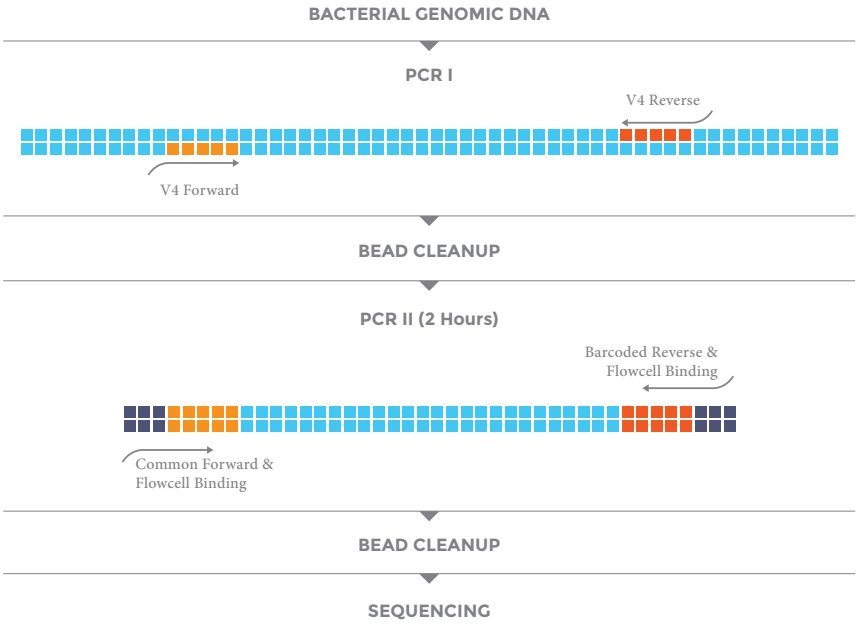
Warnings and Precautions

Bioo Scientific strongly recommends that you read the following warnings and precautions. Periodically, optimizations and revisions are made to the components and manual. Therefore, it is important to follow the protocol included with the kit. If you need further assistance, you may contact your local distributor or Bioo Scientific at nextgen@biooscientific.com.

- Do not use the kit past the expiration date.
- Ensure pipettes are properly calibrated as library preparations are highly sensitive to pipetting error.
- Try to maintain a laboratory temperature of 20°–25°C (68°–77°F).
- Genomic DNA sample quality may vary between preparations. It is the user's responsibility to utilize high quality Genomic DNA. Genomic DNA that is heavily nicked or damaged may cause library preparation failure. Absorbance measurements at 260 nm are commonly used to quantify DNA and 260 nm / 280 nm ratios of 1.8 - 2.0 usually indicate relatively pure DNA. Other quantification methods using fluorescent dyes may also be used. The user should be aware that contaminating RNA, nucleotides and single-stranded DNA may affect the amount of usable DNA in a sample preparation.
- It is required that NEXTflex™ 16S V4 PCR I & PCR II Primer Mixes are used during PCR amplification steps.

NEXTflex™ 16S V4 Amplicon-Seq 2.0 Sample Preparation Flow Chart

Figure 1: Sample flow chart with approximate times necessary for each step.



Starting Material

The NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0 has been optimized and validated using 1 ng - 50 ng of high-quality bacterial genomic DNA.

Reagent Preparation

1. Briefly spin down each component to ensure material has not lodged in the cap or side of tube. Keep on ice and vortex each NEXTflex™ Mix just prior to use.
2. Allow Agencourt AMPure XP Beads to come to room temperature and vortex the beads until liquid appears homogenous before every use.

STEP A: PCR I Amplification

Materials

Bioo Scientific Supplied

GREEN CAP - NEXTflex™ Amplicon PCR Master Mix
ORANGE CAP - NEXTflex™ 16S V4 PCR I Primer Mix
WHITE CAP - Nuclease-Free Water

User Supplied

Thermocycler
96 Well PCR Plate
High-Quality Bacterial Genomic DNA, 1 ng - 50 ng

1. For each sample, combine the following reagents on ice in the PCR plate.

_ µL	High-Quality Bacterial Genomic DNA (1 ng - 50 ng in up to 33 µL)
_ µL	Nuclease-free Water
15 µL	NEXTflex™ Amplicon PCR Master Mix
2 µL	16S V4 PCR I Primer Mix
50 µL	TOTAL

2. Mix reaction well by pipetting.
3. Apply adhesive PCR plate seal and place in thermocycler for the following PCR cycles:

4 min	95°	
30 sec	95°	
30 sec	56°	Repeat 10 cycles
90 sec	72°	
4 min	72°	

STEP B: PCR I Cleanup

Materials

Bioo Scientific Supplied
WHITE CAP - Resuspension Buffer

User Supplied
Agencourt AMPure XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand

1. Add 50 µL of AMPure XP Beads to each sample. Mix thoroughly by pipetting.
2. Incubate at room temperature for 5 minutes.
3. Place the 96 well PCR Plate on the magnetic stand at room temperature until the supernatant appears completely clear.
4. Remove and discard the supernatant. Do not disturb beads. Some liquid may remain in wells.
5. With plate on stand, add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.
6. Repeat step 5, for a total of 2 ethanol washes. Ensure all ethanol has been removed.
7. Remove the plate from the magnetic stand and let dry at room temperature for 3 minutes.
8. Resuspend dried beads with 17 µL of Resuspension Buffer. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.
9. Incubate resuspended beads at room temperature for 2 minutes.
10. Place plate on magnetic stand for 5 minutes or until the sample appears clear.
11. Transfer 16 µL of clear supernatant (purified PCR I product) to new well.

STEP C: PCR II Amplification

Materials

Bioo Scientific Supplied

GREEN CAP - NEXTflex™ Amplicon PCR Master Mix
YELLOW CAP - NEXTflex™ PCR II Barcoded Primer Mix
WHITE CAP - Nuclease-Free Water

User Supplied

Thermocycler
96 Well PCR Plate
Purified PCR I product (from STEP B)

1. For each sample, combine the following reagents on ice in the PCR plate.

16 µL	Purified PCR I product (from STEP B)
17 µL	Nuclease-free Water
15 µL	NEXTflex™ Amplicon PCR Master Mix
2 µL	NEXTflex™ PCR II Barcoded Primer Mix
50 µL	TOTAL

2. Mix well by pipette.
3. Apply adhesive PCR plate seal and place in thermocycler for the following PCR cycles:

4 min	95°
30 sec	95°
30 sec	60°
30 sec	72°
4 min	72°

Repeat cycles as recommended in table below

Input to PCR I (ng)	PCR II Cycles
1	22
5	20
10	18
25	14
50	12

STEP D: PCR II Cleanup

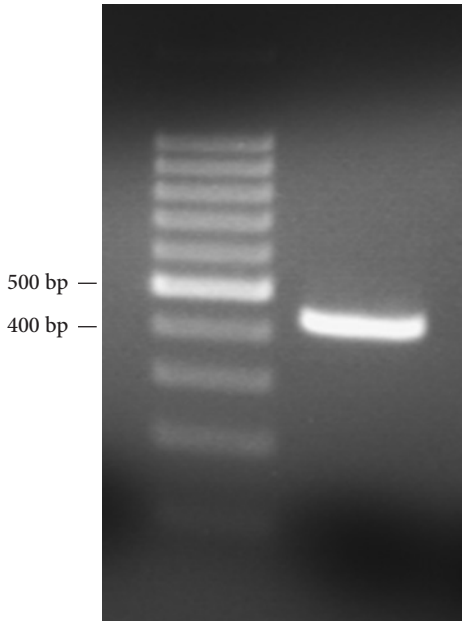
Materials

Bioo Scientific Supplied
WHITE CAP - Resuspension Buffer

User Supplied
Agencourt AMPure XP Magnetic Beads (room temperature)
80% Ethanol, freshly prepared (room temperature)
Magnetic Stand

1. Add 50 µL of AMPure XP Beads to each clear sample. Mix thoroughly by pipetting.
2. Incubate at room temperature for 5 minutes.
3. Place the 96 well PCR Plate on the magnetic stand at room temperature until the supernatant appears completely clear.
4. Remove and discard the supernatant. Do not disturb beads. Some liquid may remain in wells.
5. With plate on stand, add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.
6. Repeat step 5, for a total of 2 ethanol washes. Ensure all ethanol has been removed.
7. Remove the plate from the magnetic stand and let dry at room temperature for 3 minutes.
8. Resuspend dried beads with 17 µL of Resuspension Buffer. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.
9. Incubate resuspended beads at room temperature for 2 minutes.
10. Place plate on magnetic stand for 5 minutes until the sample appears clear.
11. Transfer 16 µL of clear supernatant to new well.
12. To ensure cluster generation, it is recommended that you quantify your library by gel or Agilent Bioanalyzer. To quantify by gel, load 2 µL of 6X Gel Loading Dye and 6-10 µL of PCR Product on a 2% low melt agarose gel + SYBR Gold.
13. Quantitate DNA library templates for optimal cluster density. Libraries generated with this kit are sequenced with standard Illumina sequencing primers.

Figure 2. Gel validation of the NEXTflex™ 16S V4 Amplicon PCR product.



*Important note – Bacterial hypervariable regions vary in base composition and length. For community studies, expect bands that are ~450 bp.

Oligonucleotide Sequences

NEXTflex™ 16S V4 PCR I Primer Mix	
NEXTflex™	**Sequence 5' → 3'**
16S V4 Forward	GACGCTCTTCCGATCTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
16S V4 Reverse	TGTGCTCTTCCGATCTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

NEXTflex™ 16S V4 PCR II Barcoded Primer Mix	
NEXTflex™	**Sequence 5' → 3'**
PCR II Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
PCR II Reverse	CAAGCAGAAGACGGCATACGAGAT[1]XXXXXXXXXXXX[1]GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

[1]XXXXXXXXXXXX denotes the index region of adapter. The index sequences and the respective reverse complement sequences contained in each adapter are listed below.

Reverse Primer Index Sequences and Reverse Complements

Barcoded Primer	Index Sequence (5' → 3')	Reverse Complement
1	GGCCGGCTAGAT	ATCTAGCCGGCC
2	AAGGAAGAGATA	TATCTCTTCCTT
3	GGACGGCATCTA	TAGATGCCGTCC
4	AAGGAAGGAGCG	CGCTCCTTCCTT

For an electronic list of the 16S V4 Primers visit our webpage.

Low Level Multiplexing

Use the following reverse primer combinations for low level multiplexing in this kit:
Pool of 2: (Barcodes 1 & 2) OR (Barcodes 3 & 4)
Pool of 3: (Barcodes 1, 2, & 3), (Barcodes 1, 2, & 4), (Barcodes 1, 3, & 4), OR (Barcodes 2, 3, & 4)

RELATED PRODUCTS

Illumina Compatible RNA NGS Kits and Adapters

- NEXTflex™ Rapid Directional RNA-Seq Kit
- NEXTflex™ RNA-Seq Barcodes
- NEXTflex-96™ RNA-Seq Barcodes

- NEXTflex™ Rapid Directional qRNA-Seq™ Kit

- NEXTflex™ Small RNA Sequencing Kit v2
- NEXTlfex™ Small RNA Barcode Primers

- NEXTflex™ Poly(A) Beads

Illumina Compatible DNA NGS Kits and Adapters

- NEXTflex™ 16S V4 Amplicon-Seq Kit
- NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0
- NEXTflex™ 16S V1-V3 Amplicon-Seq Kit
- NEXTflex™ 18S ITS Amplicon-Seq Kit

- NEXTflex™ Rapid DNA-Seq Kit
- NEXTflex™ Cell Free DNA-Seq Kit
- NEXTflex™ DNA Barcodes
- NEXTflex-96™ DNA Barcodes
- NEXTflex-HT™ Barcodes
- NEXTflex™ Dual-Indexed DNA Barcodes

- NEXTflex™ Bisulfite-Seq Kit
- NEXTflex™ Bisulfite-Seq Barcodes
- NEXTflex™ Methyl-Seq 1 Kit
- NEXTflex™ Msp 1

- NEXTflex™ ChIP-Seq Kit
- NEXTflex™ ChIP-Seq Barcodes
- NEXTflex-96™ ChIP-Seq Barcodes

- NEXTflex™ Pre-Capture Combo Kit
- NEXTflex™ Rapid Pre-Capture Combo Kit
- NEXTflex™ DNA Barcode Blockers

- NEXTflex™ PCR-Free DNA Sequencing Kit
- NEXTflex™ PCR-Free Barcodes

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