NEXTFLEX® 16S V3-V4 Amplicon-Seq Kit

(Compatible with Illumina® platforms)

KIT CONTAINS : 48 or 96 BARCODES | 96 or 192 RXNS

USER MANUAL FOR :
#NOVA-4204-03 and -03S
#NOVA-4204-04 and-04S
*Part numbers ending with S are paired with analysis provided by Cosmos-Hub®. Please refer to Appendix B for more information

Contents

This product is for research use only. Not for use in diagnostic procedures.

GENERAL INFORMATION

Product Overview

The NEXTFLEX® 16S V3-V4 Amplicon-Seq Kit is designed to prepare multiplexed amplicon libraries that span the third and fourth hypervariable domain of microbial 16S ribosomal RNA (rRNA) genes. The PCR I primers include random bases up to 10 nucleotides long to increase base diversity and improve sequencing quality. As a result, libraries can be sequenced with little to no phiX. These libraries are only compatible with paired-end sequencing (2x300) on the Illumina® Miseq® or Element® Aviti® platforms.

There are two main steps involved in 16S V3-V4 amplicon processing: an initial PCR amplification using customized PCR primers that target the V3-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.

Kit Overview

The NEXTFLEX® 16S V3-V4 Amplicon-Seq Kit contains enough material to prepare 96 or 192 samples from genomic DNA for Illumina® compatible sequencing.

Note: The 96-reaction kit contains PCR II Primers 1-48, and the 192-reaction kit contains PCR II Primers 1-96.

Contents, Storage and Shelf Life

The shelf life of all reagents is at least 12 months when stored properly. The NEXTFLEX® NGS Cleanup Beads should be stored at 4°C, and all other components should be stored at -20°C.

Kit Contents	Amount (96/192 samples)
GREEN CAP	
NEXTFLEX® PCR Master Mix	(2) 1152 / (4) 1152 µL
ORANGE CAP	
NEXTFLEX® 16S V3-V4 PCR I Primer Mix	192 / 384 µL
PLATE	
NEXTFLEX® PCR II Barcoded Primer Mix	4 µL per well
WHITE CAP BOTTLE	
Resuspension Buffer	6 / 12 mL
Nuclease-free Water	4 / 8 mL
NEXTFLEX® NGS Cleanup Beads	5 /10 mL

Required Materials not Provided.

- 1 ng - 50 ng high-quality genomic DNA in up to 36 µL nuclease-free water
- 96 well PCR Plate Non-skirted (Phenix Research, Cat # MPS-499) or similar
- Adhesive PCR Plate Seal (BioRad, Cat # MSB1001)
- 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

Revvity 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 Revvity at https://www.revvity.com/contact-us/technical-support and choose Next Gen Sequencing as the category.

- Once plate has thawed, spin for one minute before use. This is to ensure all liquid settles to the bottom of the plate.
- The plate seal is intended to be pierced. Do not peel the plate seal from the plate, doing so can easily lead to cross-contamination. Additional thermal heat seals may be applied upon one another to re-seal plate.
- Before use, carefully mix adapters by pipetting up and down several times using a multi- channel pipette with barrier tip. NEVER mix plates by vortexing. Placing a plate on a vortexer to mix samples or barcodes has been proven to result in cross-contamination, even if the plate appears to be securely sealed.
- 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 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 V3-V4 PCR I & PCR II Primer Mixes are used during PCR amplification steps.

Revision History

Version	Date	Description
V23.08	August 2015	Initial Product Launch.
V23.10	October 2023	Rebrand to Revvity.
V24.04	April 2024	Inclusion of “S” parts for Cosmos-Hub.
V24.07	July 2024	PCR II Primer Configuration changed from tubes to plates.
V24.11	November 2024	Inclusion of NEXTFLEX® NGS Cleanup Beads.

NEXTFLEX 16S V3-V4 AMPLICON-SEQ PREPARATION PROTOCOL

NEXTFLEX 16S V3-V4 Amplicon-Seq Sample Preparation Flow Chart

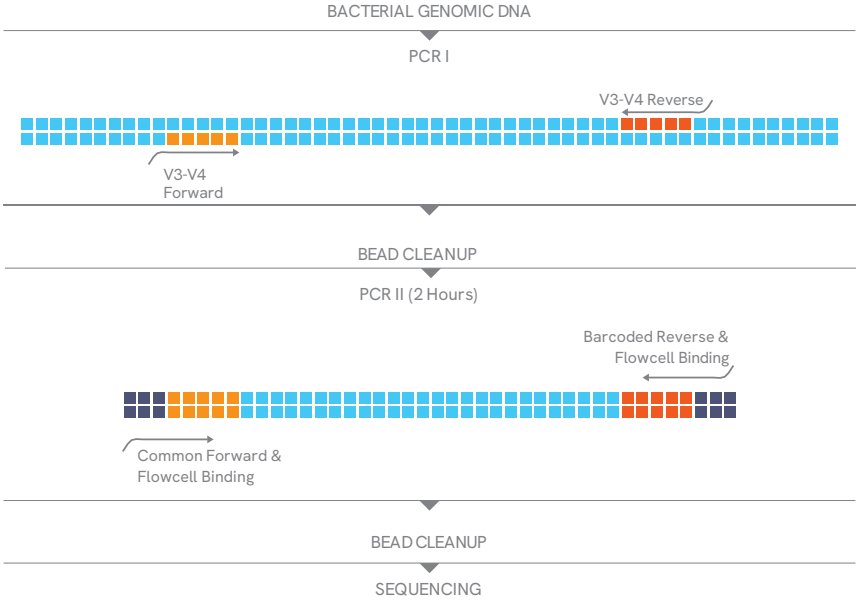


Figure 1: Sample flow chart with approximate times necessary for each step.

Starting Material

The NEXTFLEX 16S V3-V4 Amplicon-Seq Kit 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 NEXTFLEX® NGS Cleanup Beads to come to room temperature and vortex the beads until liquid appears homogenous before every use.
3. Note: Due to the viscosity of certain materials, attempting to prepare more than the stated number of reactions, may result in a shortage of materials. All NEXTFLEX® enzyme components must be centrifuged at 600xg for 5 seconds before opening the tube(s).

STEP A: PCR I Amplification

Materials

Revvity Supplied

GREEN CAP - NEXTFLEX® PCR Master Mix

ORANGE CAP - NEXTFLEX® 16S V3-V4 PCR I Primer Mix

WHITE CAP - Nuclease-Free Water

User Supplied

Thermocycler

96 Well PCR Plate

1 ng - 50 ng High-Quality Genomic DNA (in up to 36 µL Nuclease-free Water)

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

_ µL	High-Quality Genomic DNA (in up to 36 µL Nuclease-free Water)
_ µL	Nuclease-free Water
12 µL	NEXTFLEX® PCR Master Mix
2 µL	16S V3-V4 PCR I Primer Mix
50 µL	TOTAL

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

4 min	95°	
30 sec	95°	Repeat 8 cycles
30 sec	55°	
90 sec	72°	
4 min	72°	

STEP B: PCR I Cleanup

Materials

Revvity Supplied

WHITE CAP - Resuspension Buffer
WHITE CAP - NEXTFLEX® NGS Cleanup Beads

User Supplied

80% Ethanol, freshly prepared (room temperature)

Magnetic Stand

1. Add 50 µL of NEXTFLEX® NGS Cleanup 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 previous step, 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 38 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 36 µL of clear supernatant (purified PCR I product) to new well.

STEP C: PCR II Amplification

Materials

Revvity Supplied

GREEN CAP - NEXTFLEX® PCR Master Mix

PLATE - NEXTFLEX® PCR II Barcoded Primer Mix

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.
   Note: make sure to spin down all reagents prior to opening.

36 µL	Purified PCR I product (from STEP B)
12 µL	NEXTFLEX® 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	24
5	22
10	20
25	18
50	16

STEP D: PCR II Cleanup

Materials

Revvity Supplied

WHITE CAP - Resuspension Buffer
WHITE CAP - NEXTFLEX® NGS Cleanup Beads

User Supplied

80% Ethanol, freshly prepared (room temperature)

Magnetic Stand

1. Add 50 µL of NEXTFLEX® NGS Cleanup 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 previous step, 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 15 µL of clear supernatant to new well.
12. Check the size distribution of the final library by Labchip™ or equivalent and the concentration by Qubit dsDNA HS Assay (Life Technologies).

LIBRARY VALIDATION

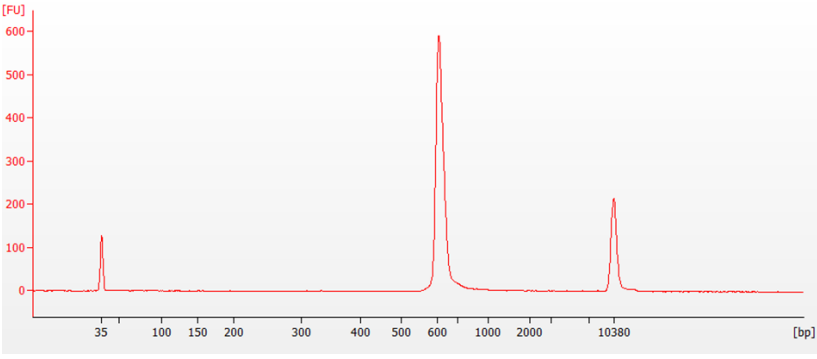


Figure 2. Sample Bioanalyzer HS DNA traces from libraries created from 10 ng of Zymo Research's community DNA standard. The expected fragment size is ~610 bp.*

*Important note – Bacterial hypervariable regions vary in base composition and length.

APPENDIX A

Sequencing

Revvity recommends performing paired-end, single-index sequencing on Illumina® sequencers. Since the amplicon is ~ 610 bp long, sequencing either 2 x 250 or 2 x 300 is required to achieve read overlap and to be able to accurately assign bacterial taxonomic groups. The libraries can be sequenced with or without phiX.

Data Analysis

Since low diversity is a known issue with 16S studies, the kit uses frameshift primers with random sequences of up to 10 nucleotides to combat this issue. These random bases need to be trimmed before any analysis can be done.

A simple command to trim the primer sequences and random bases is below:

*"cutadapt *

*--front CCTACGGGNGGCWGCAG *

*-G GACTACHVGGGTATCTAATCC *

*--output Sample_fastq.R1.trimmed.fastq *

--paired-output Sample_fastq.R2.trimmed.fastq \ Sample_fastq_R1_001.fastq.gz \ Sample_fastq_R2_001.fastq.gz"

Appendix B: Oligonucleotide Sequences

NEXTFLEX® 16S V3-V4 PCR I Primer Mix	
NEXTFLEX®	Sequence 5′ → 3′
16S V3-V4 Forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCT[0-10N]CCTACGGGNGGCWGCAG
16S V3-V4 Reverse	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACTACHVGGGTATCTAATCC

NEXTFLEX® PCR II Barcoded Primer Mix	
NEXTFLEX®	Sequence 5′ → 3′
PCR II Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCT
PCR II Reverse	CAAGCAGAAGACGGCATACGAGAT[XXXXXXXXXXXX][1]GTGACTGGA GTTCAGACGTGTGCTCTTCCGATCT

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

Low Level Multiplexing

Every combination of sequential odd and even numbered barcodes are fully color balanced at all positions of the index. For example, barcodes 5 and 6 are opposite colors at every position, but barcodes 6 and 7 are not.

For a digital copy of indices, please visit our website or this address: https://www.revvity.com/content/nextflex-index-sequences-16s, or contact us at https://www.revvity.com/contact-us/technical-support and choose the “Next Gen Sequencing” category.

Appendix C

Cosmos-Hub Analysis

The NEXTFLEX 16S V3-V4 Amplicon-seq panels can now be bundled with access to Cosmos-Hub; an online software solution that enables fast and easy analysis of complex microbiome data. Cosmos-Hub gives scientists user-friendly access to version-controlled and validated 16S pipelines. The software also enables rapid data interpretation through a comparative analysis software with features such as tables, heatmaps, bar charts, multiple Alpha & Beta Diversity indexes, abundance distribution plots, differential abundance testing, as well as comprehensive statistics between groups.

Customers who purchase parts ending in "S" are entitled to access Cosmos-Hub Analysis Portal to analyze their samples. This machine learning powered software enables rapid and easy interpretation of complex microbiome data. Learn more about Cosmos-HUB.
Please see the steps below on how to access the portal:
Quick Guide:

1. Go to https://cosmos-hub.com/revvity
2. Enter the password: RevvityHub25!
3. Complete the form, including your unique Kit ID (you may enter multiple).
4. A member of the Cosmos-Hub team will reach out to complete your onboarding.
5. Once onboarding is completed, HUB credits will be issued to your account.
6. Upload your 16S data and run your microbiome study for up to 60 days.

Amplicon 16S Profiling Requirements:

- Amplicon 16S profiling workflow accepts *paired-end* sequencing data exclusively.
- Sequencing data files should not exceed a size of 100 MB in fastq.gz format.
- For each sample ID, two paired-end fastq files must be uploaded with "R1_001 " or "R2_001 " followed by the sequencing suffix (e.g., *Sample0123_R1_001.fastq.gz* + *Sample0123_R2_001.fastq.gz*)
- File names should not have any spaces and special characters in them.
- Maintaining an average base quality score >PHRED 20 is expected across all individual reads inside a fastq file.
- A minimum of 10 samples from the same sequencing run must be uploaded for batch analysis through Amplicon 16S profiling workflow, which uses DADA2's denoising and taxonomic classification framework for amplicon 16S analysis.
- The forward and reverse reads must overlap by at least 15 identical bases.

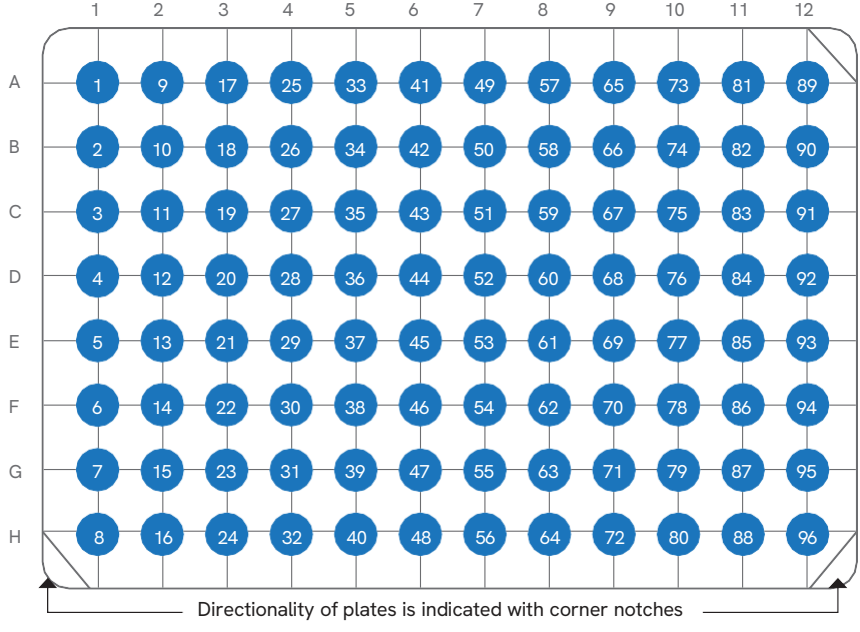
*To receive your Unique Kit ID or want to learn more about this analysis, please reach out at https://www.revvity.com/contact-us/technical-support

APPENDIX D

Plate Format

Plate; 4 µL/well

Representative Plate Orientation:



NOVA-4202-02 & NOVA-4202-02S: Contain only PCR II Primers 1-12 arrayed in columns 1-2

NOVA-4202-03 & NOVA-4202-03S: Contain only PCR II Primers 1-48 arrayed in columns 1-6

NOVA-4202-04 & NOVA-4202-04S: Contain only PCR II Primers 1-96 arrayed in columns 1-12

NOVA-4202-05 & NOVA-4202-05S: PCR II Primers 97-192, arrayed in columns

NOVA-4202-06 & NOVA-4202-06S: PCR II Primers 193-288 arrayed in columns

NOVA-4202-07 & NOVA-4202-07S: PCR II Primers 289-384 arrayed in columns

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