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 CosmosID-Hub®. Please refer to Appendix B for more information

NEXTFLEX® 16S V3-V4 Amplicon-Seq Kit

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 CosmosID-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

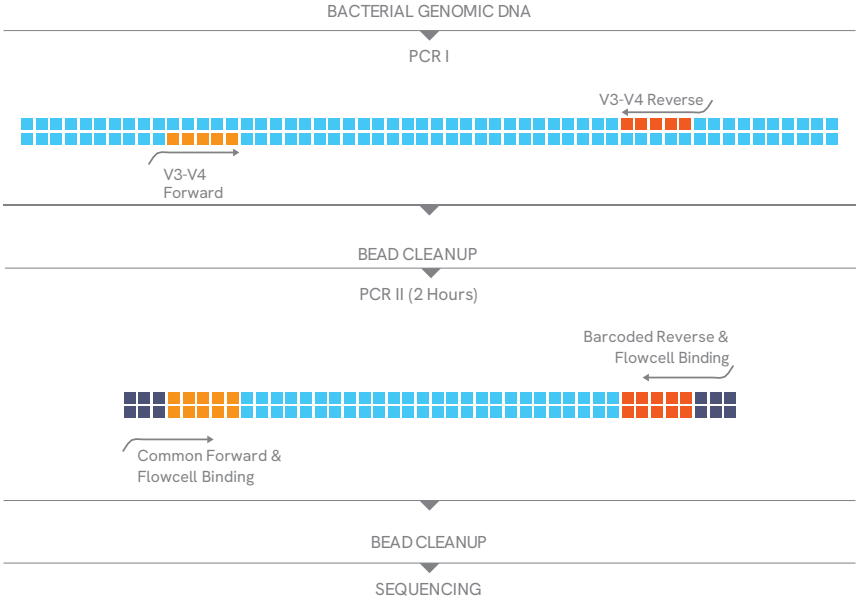


Figure 1: Sample flow chart with approximate times necessary for each step.

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°	*Repeat cycles as recommended in table below*
30 sec	60°	
30 sec	72°	
4 min	72°	

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

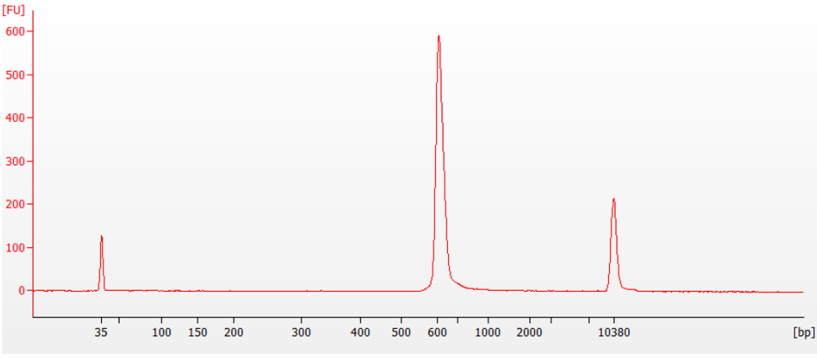


Figure 2. Sample Bioanalyzer HS DNA traces from libraries created from 10 ng of Zymo Research's community DNA standard. The expected fragment size is ~610 bp.*

*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"

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<u>XXXXXXXXXXXX</u>[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.

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
5	GGACGGCGCTCG	CGAGCGCCGTCC
6	CCGGACTCTCGA	TCGAGAGTCCGG
7	GGCCGGCCGAGC	GCTCGGCCGGCC
8	CCGGACTGAGCT	AGCTCAGTCCGG
9	GGACGCGGCAGT	ACTGCCGCGTCC
10	CCGGAGAAGTAA	TTACTTCTCCGG
11	GGCCGCGCGTCA	TGACGCGCGGCC
12	CCGGAGATCATT	AATGATCTCCGG
13	GGACGTACGCTT	AAGCGTACGTCC
14	AAGGACTGATAA	TTATCAGTCCTT
15	GGACGCGATGAC	GTCATCGCGTCC
16	CCGGAGAGACGG	CCGTCTCTCCGG
17	GGACGTAGCGAA	TTCGCTACGTCC
18	CCGGAAGAGCGT	ACGCTCTTCCGG
19	GGCCGCGTACTG	CAGTACGCGGCC
20	AAGGATCAGTAC	GTACTGATCCTT
21	GGCCGTATATCC	GGATATACGGCC
22	CCGGAAGCTATG	CATAGCTTCCGG
23	GGCCGATGCCTC	GAGGCATCGGCC
24	CCGGATCCTTAT	ATAAGGATCCGG
25	GGACGATCGGAG	CTCCGATCGTCC
26	CCGGATCGAATA	TATTCGATCCGG
27	GGACGATTAAGA	TCTTAATCGTCC
28	CCGGATCAGGCG	CGCCTGATCCGG
29	GGACGATATTCT	AGAATATCGTCC
30	CCGGATCTCCGC	GCGGAGATCCGG
31	GGACCGGCCATG	CATGGCCGGTCC
32	AAGGTACGTGAC	GTCACGTACCTT
33	GGACCGGTTGCA	TGCAACCGGTCC
34	CCGGTCAACAGG	CCTGTTGACCGG
35	GGACCTTGGGCT	AGCCCAAGGTCC
36	CCGGTACCAAGC	GCTTGGTACCGG
37	GGACCTTCCCGA	TCGGGAAGGTCC
38	CCGGTACGTTCG	CGAACGTACCGG
39	GGCCCTTAAATC	GATTTAAGGGCC
40	AAGGTCAGTTCT	AGAACTGACCTT
41	GGACCAAGGCGG	CCGCCTTGGTCC
42	CCGGTTGCATCA	TGATGCAACCGG
43	GGCCCAACCGCC	GGCGGTTGGGCC
44	CCGGTTGGTAGT	ACTACCAACCGG
45	GGACCAATTATT	AATAATTGGTCC

46	CCGGTTGACGAC	GTCGTCAACCGG
47	GGCCTGAGATTT	AAATCTCAGGCC
48	CCGGCCGCGCAC	GTGCGCGGCCGG
49	GGACTGACTAAA	TTTAGTCAGTCC
50	CCGGCCGGCGTG	CACGCCGGCCGG
51	GGACTGATCGGG	CCCGATCAGTCC
52	CCGGCCGATACA	TGTATCGGCCGG
53	GGACTCTGAAAG	CTTTCAGAGTCC
54	CCGGCGCCGGTA	TACCGGCGCCGG
55	GGACTCTCTTTC	GAAAGAGAGTCC
56	AAGGCTAGCCAG	CTGGCTAGCCTT
57	GGCCTCTTCCCT	AGGGAAGAGGCC
58	AAGGCTACGGTC	GACCGTAGCCTT
59	GGACTCTAGGGA	TCCCTAGAGTCC
60	AAGGCTATAACT	AGTTATAGCCTT
61	GGACTTCGAGGC	GCCTCGAAGTCC
62	AAGGCCGCGACG	CGTCGCGGCCTT
63	GGCCTTCCTCCG	CGGAGGAAGGCC
64	AAGGCCGGCTGC	GCAGCCGGCCTT
65	GGACTTCTCTTA	TAAGAGAAGTCC
66	AAGGCCGATCAT	ATGATCGGCCTT
67	GGACTTCAGAAT	ATTCTGAAGTCC
68	AAGGCCGTAGTA	TACTACGGCCTT
69	GGACTAGGACCA	TGGTCCTAGTCC
70	CCGGCTAATGTT	AACATTAGCCGG
71	GGACTAGCTGGT	ACCAGCTAGTCC
72	CCGGCTATACAA	TTGTATAGCCGG
73	GGACTAGTCAAC	GTTGACTAGTCC
74	CCGGCTACGTGG	CCACGTAGCCGG
75	GGACTAGAGTTG	CAACTCTAGTCC
76	AAGGCGCGCACA	TGTGCGCGCCTT
77	GGCCACAGTACC	GGTACTGTGGCC
78	AAGGGTTAATTT	AAATTAACCCTT
79	GGCCACATGCAA	TTGCATGTGGCC
80	AAGGGTTCCGGG	CCCGGAACCCTT
81	GGACACAACGTT	AACGTTGTGTCC
82	AAGGGTTGGCCC	GGGCCAACCCTT
83	GGACATGGTGTG	CACACCATGTCC
84	CCGGGAACCAAA	TTTGGTTCCCGG
85	GGACATGCACAC	GTGTGCATGTCC
86	CCGGGAATTGGG	CCCAATTCCCGG
87	GGACATGACACA	TGTGTCATGTCC
88	CCGGGAAGGTTT	AAACCTTCCCGG
89	GGACAACGTCAT	ATGACGTTGTCC
90	CCGGGTTAAGGA	TCCTTAACCCGG
91	GGACAACTGACG	CGTCAGTTGTCC
92	CCGGGTTCCTTC	GAAGGAACCCGG
93	GGCCAACACTGC	GCAGTGTTGGCC
94	CCGGGTTGGAAG	CTTCCAACCCGG

95	GGCTGGTCATAC	GTATGACCAGCC
96	CCGAACCTTAGG	CCTAAGGTTCGG
97	GGATGGTACGCA	TGCGTACCATCC
98	CCGAACCGGCTT	AAGCCGGTTCGG
99	GGATGCAGTTAT	ATAACTGCATCC
100	CCGAAGGCCCTC	GAGGGCCTTCGG
101	GGCTGCACAATA	TATTGTGCAGCC
102	CCGAAGGTTTCT	AGAAACCTTCGG
103	GGATGCATGGCG	CGCCATGCATCC
104	CCGAAGGAAAGA	TCTTTCCTTCGG
105	GGATGCAACCGC	GCGGTTGCATCC
106	AAGAATTGGGAT	ATCCCAATTCTT
107	GGCTGTGGTCGA	TCGACCACAGCC
108	AAGAACCAAGAG	CTCTTGGTTCTT
109	GGCTGTGCAGCT	AGCTGCACAGCC
110	AAGAACCGGAGA	TCTCCGGTTCTT
111	GGCTGTGACTAG	CTAGTCACAGCC
112	AAGAACCTTCTC	GAGAAGGTTCTT
113	GGATGACCACGG	CCGTGGTCATCC
114	CCGAATTGGTCA	TGACCAATTCGG
115	GGATGACTGTAA	TTACAGTCATCC
116	CCGAATTAACTG	CAGTTAATTCGG
117	GGCTGACACATT	AATGTGTCAGCC
118	AAGAAGGTTGAA	TTCAACCTTCTT
119	GGATCGAGAAGC	GCTTCTCGATCC
120	AAGATATATTAT	ATAATATATCTT
121	GGATCGACTTCG	CGAAGTCGATCC
122	CCGATCGGCCGA	TCGGCCGATCGG
123	GGATCGATCCTA	TAGGATCGATCC
124	CCGATCGATTAG	CTAATCGATCGG
125	GGATCGAAGGAT	ATCCTTCGATCC
126	CCGATCGTAATC	GATTACGATCGG
127	GGCTCCTGATCA	TGATCAGGAGCC
128	CCGATGCCGCGG	CCGCGGCATCGG
129	GGATCCTCTAGT	ACTAGAGGATCC
130	AAGATTATATAC	GTATATAATCTT
131	GGCTCCTTCGAC	GTCGAAGGAGCC
132	CCGATGCATATT	AATATGCATCGG
133	GGATCCTAGCTG	CAGCTAGGATCC
134	AAGATTAGCGCA	TGCGCTAATCTT
135	GGCTCTCCTGAA	TTCAGGAGAGCC
136	CCGATATTACGT	ACGTAATATCGG
137	GGATCTCTCAGG	CCTGAGAGATCC
138	AAGATCGCGTAA	TTACGCGATCTT
139	GGATCTCAGTCC	GGACTGAGATCC
140	CCGATATGCATG	CATGCATATCGG
141	GGATCAGGAGAG	CTCTCCTGATCC
142	AAGATGCCGATC	GATCGGCATCTT
143	GGCTCAGCTCTC	GAGAGCTGAGCC

144	CCGATTAGCTAT	ATAGCTAATCGG
145	GGATCAGTCTCT	AGAGACTGATCC
146	AAGATGCATCGA	TCGATGCATCTT
147	GGCTCAGAGAGA	TCTCTCTGAGCC
148	CCGATTATAGCG	CGCTATAATCGG
149	GGCTTGGCCTGA	TCAGGCCAAGCC
150	CCGACCAGTCCG	CGGACTGGTCGG
151	GGATTGGTTCAG	CTGAACCAATCC
152	CCGACCACAGGC	GCCTGTGGTCGG
153	GGCTTGGAAGTC	GACTTCCAAGCC
154	AAGACACTGAAG	CTTCAGTGTCTT
155	GGATTCCGGTGG	CCACCGGAATCC
156	CCGACGTCACCA	TGGTGACGTCGG
157	GGCTTCCTTGTT	AACAAGGAAGCC
158	CCGACGTACAAC	GTTGTACGTCGG
159	GGATTCCAACAA	TTGTTGGAATCC
160	AAGACTGTGTTT	AAACACAGTCTT
161	GGATTAACCCAT	ATGGGTTAATCC
162	CCGACTGGTTTC	GAAACCAGTCGG
163	GGGTTAATTTGC	GCAAATTAACCC
164	CCCACTGCAAAG	CTTTGCAGTGGG
165	GGATAGCGCAAA	TTTGCGCTATCC
166	AAGAGAGAGTGG	CCACTCTCTCTT
167	GGATAGCCGTTT	AAACGGCTATCC
168	CCGAGCTTCACA	TGTGAAGCTCGG
169	GGATAGCTACCC	GGGTAGCTATCC
170	AAGAGAGCTGTT	AACAGCTCTCTT
171	GGCTAGCATGGG	CCCATGCTAGCC
172	AAGAGAGGACAA	TTGTCCTCTCTT
173	GGATACGGCTTC	GAAGCCGTATCC
174	AAGAGTCCTCAG	CTGAGGACTCTT
175	GGCTACGCGAAG	CTTCGCGTAGCC
176	AAGAGTCGAGTC	GACTCGACTCTT
177	GGATACGTAGGA	TCCTACGTATCC
178	AAGAGTCAGACT	AGTCTGACTCTT
179	CCAGCGCGCCAT	ATGGCGCGCTGG
180	TTGCTAGAGGGC	GCCCTCTAGCAA
181	CCCGCGCTAACG	CGTTAGCGCGGG
182	TTGCTAGCTTTA	TAAAGCTAGCAA
183	CCAGCGCATTGC	GCAATGCGCTGG
184	TTGCTAGGAAAT	ATTTCCTAGCAA
185	CCAGCTAGCACC	GGTGCTAGCTGG
186	TTGCTCTCTGGG	CCCAGAGAGCAA
187	CCAGCATGCTGA	TCAGCATGCTGG
188	TTGCTGACTCCT	AGGAGTCAGCAA
189	CCAGCATCGACT	AGTCGATGCTGG
190	TTGCTGATCTTC	GAAGATCAGCAA
191	CCAGCATTAGTC	GACTAATGCTGG
192	TTGCTGAAGAAG	CTTCTTCAGCAA

193	CCAGCATATCAG	CTGATATGCTGG
194	TTGCTGAGAGGA	TCCTCTCAGCAA
195	CCCGTGTGTCTC	GAGACACACGGG
196	TTGCCAACCTAG	CTAGGTTGGCAA
197	CCAGTGTCAGAG	CTCTGACACTGG
198	TTGCCAATTCGA	TCGAATTGGCAA
199	CCAGTGTACTCT	AGAGTACACTGG

200	TTGCCAAGGATC	GATCCTTGGCAA
201	CCCGTCAGTGAA	TTCACTGACGGG
202	TTGCCTTAACGG	CCGTTAAGGCAA
203	CCAGTCACACTT	AAGTGTGACTGG
204	TTGCCTTGGTAA	TTACCAAGGCAA
205	CCCGTACTGGAT	ATCCAGTACGGG
206	TTGCCGGAAATA	TATTTCCGGCAA
207	CCGGTACACCTA	TAGGTGTACCGG
208	TTACCGGTTTAT	ATAAACCGGTAA
209	CCGGACTTCTAG	CTAGAAGTCCGG
210	TTACGTAATCTC	GAGATTACGTAA
211	CCGGACTAGATC	GATCTAGTCCGG
212	TTCCGTAGCTCT	AGAGCTACGGAA
213	CCACATGGTCAA	TTGACCATGTGG
214	TTGGGCCAAGGG	CCCTTGGCCCAA
215	CCACAGTCATGC	GCATGACTGTGG
216	TTGGGAATTAAT	ATTAATTCCCAA
217	CCACAGTGTACG	CGTACACTGTGG
218	TTGGGAACCGGC	GCCGGTTCCCAA
219	CCACTAGAGAAA	TTTCTCTAGTGG
220	TTGGCGCGCTGG	CCAGCGCGCCAA
221	CCACTAGTCTTT	AAAGACTAGTGG
222	TTGGCGCATCAA	TTGATGCGCCAA
223	CCACTTCAGTTC	GAACTGAAGTGG
224	TTGGCCGGCACT	AGTGCCGGCCAA
225	CCACTTCTCAAG	CTTGAGAAGTGG
226	TTGGCCGCGTGA	TCACGCGGCCAA
227	CCACTTCCTGGA	TCCAGGAAGTGG
228	TTGGCCGTACAG	CTGTACGGCCAA
229	CCACTTCGACCT	AGGTCGAAGTGG
230	TTGGCCGATGTC	GACATCGGCCAA
231	CCACTCTAGCCG	CGGCTAGAGTGG
232	TTGGCTAGCGTA	TACGCTAGCCAA
233	CCACTCTTCGGC	GCCGAAGAGTGG
234	TTGGCTAATACG	CGTATTAGCCAA
235	CCACTCTCTAAT	ATTAGAGAGTGG
236	TTGGCTATATGC	GCATATAGCCAA
237	CCACTCTGATTA	TAATCAGAGTGG
238	TTGGCTACGCAT	ATGCGTAGCCAA
239	CCACTGAAGGGT	ACCCTTCAGTGG
240	TTGGCATGCCAC	GTGGCATGCCAA
241	CCACTGATCCCA	TGGGATCAGTGG

242	TTGGCATCGGTG	CACCGATGCCAA
243	CCACTGACTTTG	CAAAGTCAGTGG
244	TTGGCATTAACA	TGTTAATGCCAA
245	CCACTGAGAAAC	GTTTCTCAGTGG
246	TTGGCATATTGT	ACAATATGCCAA
247	CCACCAATTTAC	GTAAATTGGTGG
248	TTGGTGTCAAGT	ACTTGACACCAA
249	CCACCAACCCGT	ACGGGTTGGTGG
250	TTGGTGTGTTCA	TGAACACACCAA
251	CCACCAAGGGCA	TGCCCTTGGTGG
252	TTGGTGTACCTG	CAGGTACACCAA
253	CCACCTTAATAT	ATATTAAGGTGG
254	TTGGTCAGTAGC	GCTACTGACCAA
255	CCACCTTCCGCG	CGCGGAAGGTGG
256	TTGGTCATGCTA	TAGCATGACCAA
257	CCACCTTGGCGC	GCGCCAAGGTGG
258	TTGGTCACATCG	CGATGTGACCAA
259	CCACCGGAAGCC	GGCTTCCGGTGG
260	TTGGTACTGAGG	CCTCAGTACCAA
261	CCACCGGCCTAA	TTAGGCCGGTGG
262	TTGGTACGTCTT	AAGACGTACCAA
263	CCACGATATAGC	GCTATATCGTGG
264	TTGGAGAGATAT	ATATCTCTCCAA
265	CCACGATTATCG	CGATAATCGTGG
266	TTGGAGACTATA	TATAGTCTCCAA
267	CCACGATCGCTA	TAGCGATCGTGG
268	TTGGAGATCGCG	CGCGATCTCCAA
269	CCACGATGCGAT	ATCGCATCGTGG
270	TTGGAGAAGCGC	GCGCTTCTCCAA
271	CCGCGTAATTCA	TGAATTACGCGG
272	TTAGACTGAATG	CATTCAGTCTAA
273	CCGTCCTCTTCC	GGAAGAGGACGG
274	GGAATGCGCCGT	ACGGCGCATTCC
275	CCCTCCTGAAGG	CCTTCAGGAGGG
276	GGGATGCATTAC	GTAATGCATCCC
277	CCGTCGAAGCTC	GAGCTTCGACGG
278	TTAATATTATAG	CTATAATATTAA
279	CCGTCGATCGAG	CTCGATCGACGG
280	GGCATCGATATA	TATATCGATGCC
281	CCATCGACTAGA	TCTAGTCGATGG
282	GGGATCGGCGCG	CGCGCCGATCCC
283	CCGTCGAGATCT	AGATCTCGACGG
284	GGCATCGCGCGC	GCGCGCGATGCC
285	CCATGACACTAC	GTAGTGTCATGG
286	GGGAATTGGAGG	CCTCCAATTCCC
287	CCCTGACTGATG	CATCAGTCAGGG
288	TTGAAGGAAGAC	GTCTTCCTTCAA
289	CCGTGACGTCGT	ACGACGTCACGG
290	GGCAATTAAGAA	TTCTTAATTGCC

291	CCATGTGACATA	TATGTCACATGG
292	TTGAACCTTGAT	ATCAAGGTTCAA
293	CCATGTGTGTAT	ATACACACATGG
294	TTGAACCAACTA	TAGTTGGTTCAA
295	CCGTGCAACGCT	AGCGTTGCACGG
296	TTCAATTGGCTC	GAGCCAATTGAA
297	CCATGCATGCGA	TCGCATGCATGG
298	TTGAATTCCGAG	CTCGGAATTCAA
299	CCGTGCACATAG	CTATGTGCACGG
300	GGAAAGGTTAGC	GCTAACCTTTCC
301	CCGTGCAGTATC	GATACTGCACGG

302	GGAAAGGCCGAT	ATCGGCCTTTCC
303	CCATGGTACCGG	CCGGTACCATGG
304	GGGAACCGGGAC	GTCCCGGTTCCC
305	CCGTGGTCAATT	AATTGACCACGG
306	GGAAACCTTTCA	TGAAAGGTTTCC
307	CCGCATGACTGG	CCAGTCATGCGG
308	TTCGGCCGGAAA	TTTCCGGCCGAA
309	TTGGGAAGGCCG	CGGCCTTCCCAA
310	AAACATGACGTC	GACGTCATGTTT
311	GGACATGTGTGT	ACACACATGTCC
312	AAGGGCCAACCA	TGGTTGGCCCTT
313	TTCCAGATTAGC	GCTAATCTGGAA
314	AATGGCGCATAG	CTATGCGCCATT
315	TTTCAGAAATCG	CGATTTCTGAAA
316	CCCGGATTGCGC	GCGCAATCCGGG
317	TTCCACTCCCTG	CAGGGAGTGGAA
318	CCTGGTATGGCA	TGCCATACCAGG
319	TTCCATCTTCTT	AAGAAGATGGAA
320	AATGGATACTAC	GTAGTATCCATT
321	TTTCATCAAGAA	TTCTTGATGAAA
322	AACGGATGTCGT	ACGACATCCGTT
323	TTTCAAGGGTCT	AGACCCTTGAAA
324	AACGGTAACATA	TATGTTACCGTT
325	TTTCAAGCCAGA	TCTGGCTTGAAA
326	AACGGTAGTGCG	CGCACTACCGTT
327	TTCCAAGAACTC	GAGTTCTTGGAA
328	AATGGTATGTAT	ATACATACCATT
329	TTCTGCTGGCCT	AGGCCAGCAGAA
330	AATAAGCACGTA	TACGTGCTTATT
331	TTCTGCTCCGGA	TCCGGAGCAGAA
332	AATAAGCTGCAT	ATGCAGCTTATT
333	TTCTGCTAATTC	GAATTAGCAGAA
334	AATAAGCGTACG	CGTACGCTTATT
335	TTATGTCGGTTA	TAACCGACATAA
336	AATAAATACACT	AGTGTATTTATT
337	TTCTGTCCCAAT	ATTGGGACAGAA
338	AATAAATTGTGA	TCACAATTTATT
339	TTCTGTCTTGGC	GCCAAGACAGAA

340	AATAAATCACAG	CTGTGATTTATT
341	TTTCACCGCAAT	ATTGCGGTGAAA
342	AAAGGGTCTGTC	GACAGACCCTTT
343	AAATTAGATATC	GATATCTAATTT
344	TTTACTATCGAT	ATCGATAGTAAA
345	AATTAGTCCTCA	TGAGGACTAATT
346	TTAAGCCTGATT	AATCAGGCTTAA
347	AAATAGTTTCTG	CAGAAACTATTT
348	GGTAGAACAGCA	TGCTGTTCTACC
349	AAATAGTAAGAC	GTCTTACTATTT
350	GGTAGAATGATG	CATCATTCTACC
351	AAATACAGGTCG	CGACCTGTATTT
352	GGGAGTTCACGC	GCGTGAACTCCC

353	AAATACACCAGC	GCTGGTGTATTT
354	TTTAGGGTGTAG	CTACACCCTAAA
355	AAATACATTGAT	ATCAATGTATTT
356	GGTAGTTACATA	TATGTAACTACC
357	AAATACAAACTA	TAGTTTGTATTT
358	GGGAGTTTGTAT	ATACAAACTCCC
359	AATTATGGGCTC	GAGCCCATAATT
360	TTAAGAAACGCG	CGCGTTTCTTAA
361	GGACGTAATAGG	CCTATTACGTCC
362	AAGGACTTCGCC	GGCGAAGTCCTT
363	GGACCGGAACGT	ACGTTCCGGTCC
364	CCGGTCATGTCC	GGACATGACCGG
365	GGCCTGAAGCCC	GGGCTTCAGGCC
366	CCGGCCGTATGT	ACATACGGCCGG
367	GGATGGTGTATG	CATACACCATCC
368	CCGAACCAATCC	GGATTGGTTCGG
369	GGCTGACGTGCC	GGCACGTCAGCC
370	CCGAATTCCAGT	ACTGGAATTCGG
371	GGCTCTCGACTT	AAGTCGAGAGCC
372	AAGATCGATGCC	GGCATCGATCTT
373	CCAGAGACTGCC	GGCAGTCTCTGG
374	TTGCGATGCAGG	CCTGCATCGCAA
375	CCACTAGCTCCC	GGGAGCTAGTGG
376	TTGGCGCTAGTT	AACTAGCGCCAA
377	CCACTAGGAGGG	CCCTCCTAGTGG
378	TTGGCGCCGACC	GGTCGGCGCCAA
379	CCACCGGTTCGG	CCGAACCGGTGG
380	TTGGTACACTCC	GGAGTGTACCAA
381	CCGTGGTTGGCC	GGCCAACCACGG
382	GGCAACCAAAGT	ACTTTGGTTGCC
383	CCGCATGCAGTT	AACTGCATGCGG
384	TTAGGCCTTCCC	GGGAAGGCCTAA

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.

Appendix B

CosmosID-HUB Analysis

The NEXTFLEX 16S V3-V4 Amplicon-seq panels can now be bundled with access to CosmosID-HUB; an online software solution that enables fast and easy analysis of complex microbiome data. CosmosID-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 CosmosID-Hub Analysis Portal to analyze their samples. This machine learning powered software enables rapid and easy interpretation of complex microbiome data. Learn more about CosmosID-HUB.

Please see the below steps on how to access the portal:

Quick Guide:

1. Go to https://cosmosidhub.com/revvity/
2. Complete the form, including your unique Kit ID (you may enter multiple).
3. A member of the CosmosID-HUB team will reach out to complete your onboarding.
4. Once onboarding is completed, HUB credits will be issued to your account.
5. 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-

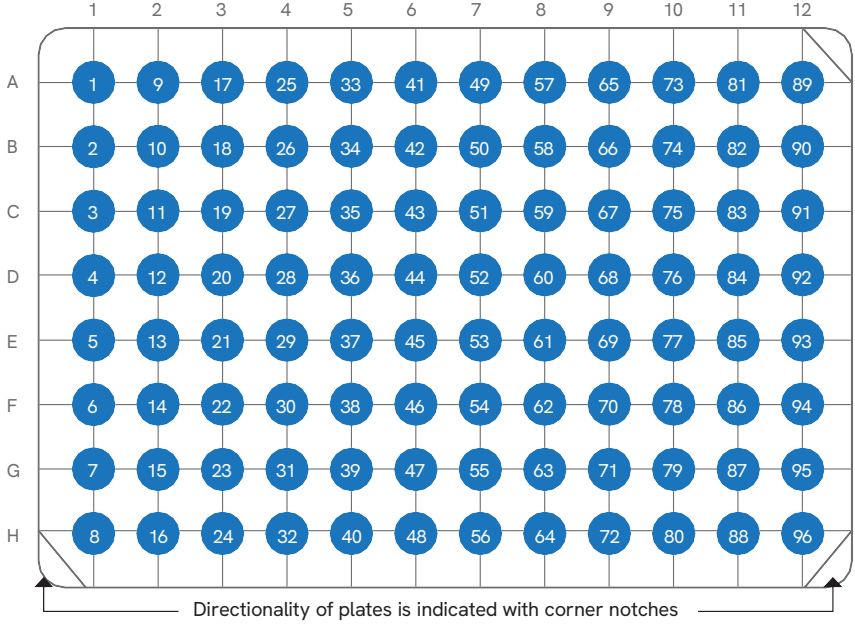
us/technical-support

APPENDIX C

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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