16S Microbiome + ITS NGS Assay

Library Preparation Kit

REF	Σ
9-131 [Set A]	96 Reactions (Library Preparations)
9-132 [Set B]	96 Reactions (Library Preparations)
9-133 [Set C]	96 Reactions (Library Preparations)
9-131-16	16 Reactions (Library Preparations)

2°C 8°C Magnetic Beads

-30°C -15°C Other Components

RUO

Upon arrival, please store components according to their labels!

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rev 4.0

Instructions for use

Library Preparation Kit for 16S Microbiome + ITS Analysis

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1. Introduction

The kit is intended for preparing NGS libraries for the bacterial 16S rRNA gene- and the fungal nuclear ribosomal ITS region-based characterization of the human microbiome. In the first PCR step, the highly variable bacterial V3-V4 regions and fungal ITS2 region can be amplified simultaneously with locus-specific primers. Primers for bacteria or fungi detection come in separate tubes; hence the addition of fungal ITS2 primers is optional, allowing flexibility to the customer. The second PCR introduces dual index sequences for the assignment of the reads to individual samples during data demultiplexing. The final amplicon structure contains all sequences required to analyze the library pool on Illumina platforms (see Figure 1). This kit has been validated for the Illumina MiSeq instrument and requires at least 2x250bp long reads. It is estimated that fungi account for only 0.01-0.1% of resident gut microbiota. However, despite the very low amount of fungal DNA, it is expected that, on average 2-5% of total sequencing reads are assigned to fungi, with the remaining reads assigned to bacteria.

Graphical Workflow:

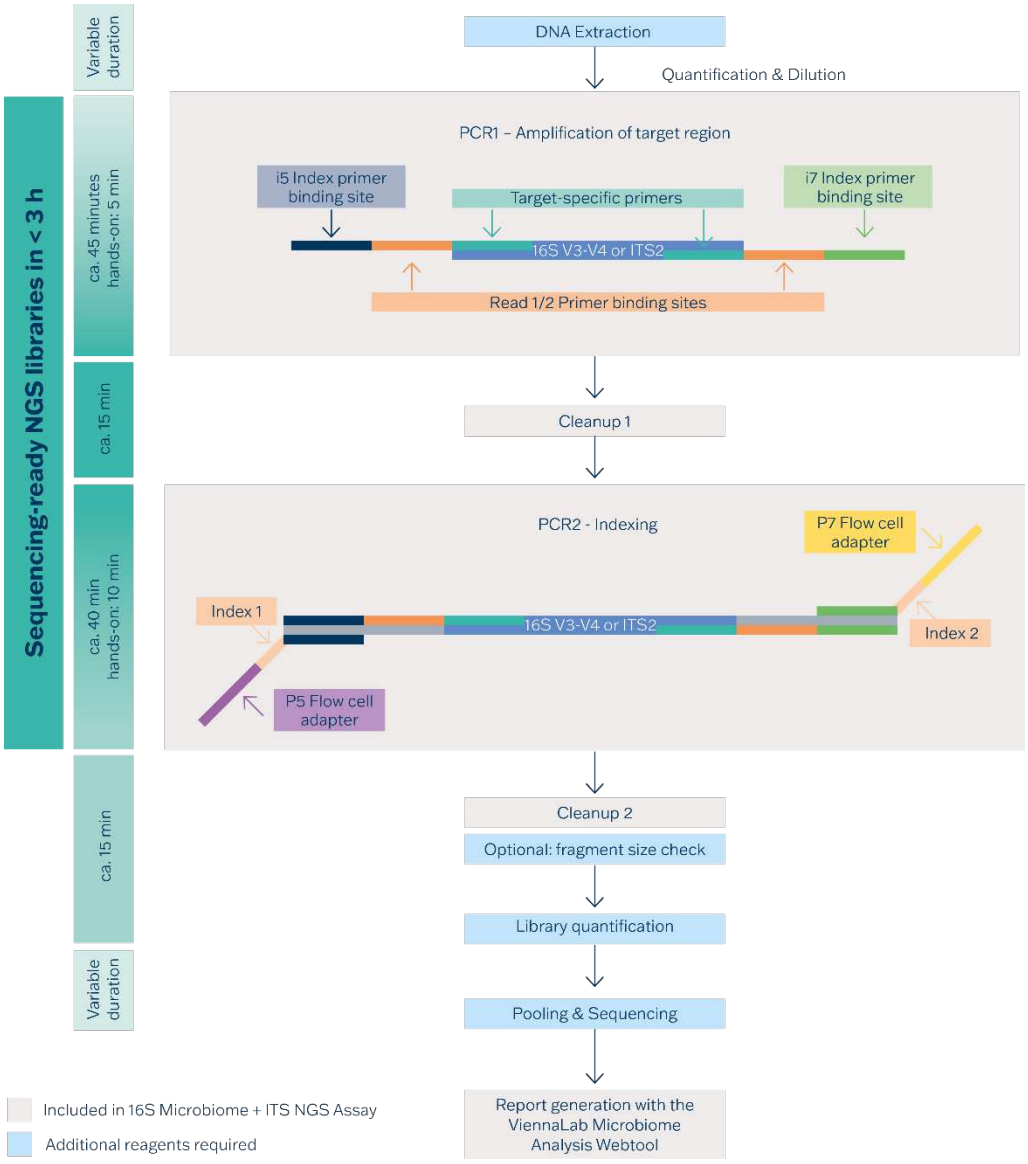

Figure 1 - Graphical Workflow of the library preparation procedure

2. Kit components

[REF 9-131]

Activation code sticker for 100 analyses [attached to the inside of the kit box lid]

Box “Master Mix”	5x 1 ml 16S Master Mix 2x
Box “PCR1 V3-V4 + ITS2 Primer Mixes”	1x 400 µl MIB1 – V3V4 Mix
	1x 400 µl FUN1 – ITS2 Mix
Box “PCR2 Indexing Forward Primers **Set A**”	8x 30 µl MIB2A-F1 – MIB2A-F8 Primer
Box “PCR2 Indexing Reverse Primers 1/2”	8x 30 µl MIB2X-R1 – MIB2X-R8 Primer
Box “PCR2 Indexing Reverse Primers 2/2”	4x 30 µl MIB2X-R9 – MIB2X-R12 Primer
Magnetic Beads	1x 13 ml

[REF 9-132]

Activation code sticker for 100 analyses [attached to the inside of the kit box lid]

Box “Master Mix”	5x 1 ml 16S Master Mix 2x
Box “PCR1 V3-V4 + ITS2 Primer Mixes”	1x 400 µl MIB1 – V3V4 Mix
	1x 400 µl FUN1 – ITS2 Mix
Box “PCR2 Indexing Forward Primers **Set B**”	8x 30 µl MIB2B-F9 – MIB2B-F16 Primer
Box “PCR2 Indexing Reverse Primers 1/2”	8x 30 µl MIB2X-R1 – MIB2X-R8 Primer
Box “PCR2 Indexing Reverse Primers 2/2”	4x 30 µl MIB2X-R9 – MIB2X-R12 Primer
Magnetic Beads	1x 13 ml

[REF 9-133]

Activation code sticker for 100 analyses [attached to the inside of the kit box lid]

Box “Master Mix”	5x 1 ml 16S Master Mix 2x
Box “PCR1 V3-V4 + ITS2 Primer Mixes”	1x 400 µl MIB1 – V3V4 Mix
	1x 400 µl FUN1 – ITS2 Mix
Box “PCR2 Indexing Forward Primers **Set C**”	8x 30 µl MIB2C-F17 – MIB2C-F24 Primer
Box “PCR2 Indexing Reverse Primers 1/2"	8x 30 µl MIB2X-R1 – MIB2X-R8 Primer
Box “PCR2 Indexing Reverse Primers 2/2**”**	4x 30 µl MIB2X-R9 – MIB2X-R12Primer
Magnetic Beads	1x 13 ml

***Note**: REF 9-131, 9-132, and 9-133 differ only in Indexing Primer Sets. If you plan to sequence more than 96 samples on one flow cell, please order different sets (e.g., Set A / REF 9-131 and Set B / REF 9-132). Ensure all individual libraries sequenced in the same pool have a unique indexing primer combination. REF 9-131-16 contains a subset of REF 9-131 Indexing Primers.*

[REF 9-131-16] – *Attention: Indexing Primers overlap with REF 9-131 [Set A]*

Activation code sticker for 16 analyses [attached to the inside of the kit box lid]

Box "Master Mix + PCR1 Primers"	● 1x 1 ml 16S Master Mix 2x
	● 1x 400 µl MIB1 – V3V4 Mix
	● 1x 400 µl FUN1 – ITS2 Mix
Box “PCR2 Indexing Primers”	● 4x 30 µl MIB2A-F1 – MIB2A-F4 Primer
	● 4x 30 µl MIB2X-R1 – MIB2X-R4 Primer
Box "Magnetic Beads"	2x 1.25 ml

3. Equipment and reagents required but not supplied

3.1. Sample preparation

- Recommended for DNA isolation: Stool DNA Isolation Kit (Norgen Biotek).
  Other DNA isolation kits have not been validated and need to be validated by the user.
- Recommended for DNA quantification: fluorometric methods, such as Qubit™.
- DNA input range: 2.5-25 ng (0.5-5 ng/µl) for bacterial-only detection. If simultaneous bacterial and fungal detection is desired, increase the input DNA to 25-50 ng (5-10 ng/µl). The limit of detection for fungal DNA is 10 pg in up to 10 ng of bacterial/human DNA (1000-fold background).

3.2. PCR

- PCR tubes or suitable 96-well plates.
- PCR cycler with lid heating and specified ramp rates.
- Nuclease-free, PCR-grade water (recommended: Illumina wash buffer PW1 or another molecular biology-grade water).

3.3. Sample purification

- 80% ethanol, freshly prepared.
- Magnetic separator for 1.5 ml Eppendorf tubes, PCR strips, or 96-well plates.

3.4. Library Quality Control

- Fragment Analyzer using DNF-473, DNF-474 or DNF-477 kit.

3.5. Library Quantification

- Quantification of the final library pool ready for sequencing is recommended by qPCR, e.g., with JetSeq™ Library Quantification Lo-ROX Kit (meridian Bioscience®) and associated qPCR cycler. Alternatively, a Qubit Fluorometer or a similar device can be used. However, concentrations of final pooled libraries should be compared to qPCR results in the first experiments to establish a normalization factor for further experiments, if needed.

3.6. Sequencing

- Reagents for library denaturation and sequencing: Please order directly from Illumina, Inc. The following sequencing kits can be used: MiSeq Reagent kit v3 (600-cycle), MiSeq Reagent kit v2 (500-cycle), or MiSeq Reagent kit Nano v2 (500-cycle). Optional: PhiX Control v3 (Illumina) spiked in at 5%.

  ***Note**: The use of well-balanced heterogeneity spacers in PCR1 eliminates the need for the addition of PhiX Control v3 (Illumina) in the 16S Microbiome + ITS NGS assay. However, 5% of PhiX can optionally be used for the error rate determination as part of the Illumina sequencing QC procedure.*

4. Laboratory protocol

4.1. First PCR – Amplification of target region(s)

4.1.1. Reaction setup

Use 2.5-25 ng of DNA samples with a concentration of 0.5-5 ng/µl (dilute if needed) for bacterial-only detection. If simultaneous bacterial and fungal detection is desired, dilute the DNA samples to 5-10 ng/µl (25-50 ng total). Set up the PCR reaction as shown in Table 1.
The MIB1 - V3V4 Mix and FUN1 - ITS2 Mix contain different heterogeneity spacers, ensuring sequence heterogeneity in the library pool which is necessary for Illumina sequencing.
Note: *Set up PCR mix on ice.*

Table 1 - Reaction Setup PCR1

V3-V4 – Bacterial detection only

Component	Volume for 1 reaction [µl]	Volume for 10 reactions incl. 5% excess [µl]
16S Master Mix 2x	25.0	262.5
MIB1 - V3V4 Mix	3.2	33.6
DNA template (0.5-5 ng/µl)	5.0	-
Nuclease-free water	16.8	176.4
TOTAL VOLUME	**50.0**	**472.5 (use 45 µl/reaction, add 5 µl DNA template)**

OR

V3-V4 + ITS2 – Simultaneous bacterial and fungal detection

Component	Volume for 1 reaction [µl]	Volume for 10 reactions incl. 5% excess [µl]
16S Master Mix 2x	25.0	262.5
MIB1 – V3V4 Mix	3.2	33.6
FUN1 – ITS2 Mix	3.2	33.6
DNA template (5-10 ng/µl)	5.0	-
Nuclease-free water	13.6	142.8
TOTAL VOLUME	**50.0**	**472.5 (use 45 µl/reaction, add 5µl DNA template)**

4.1.2. PCR program

Create a PCR program according to Table 2. Use a heated lid (>100°C); if applicable, set the heating ramp rate to max. 2.5°C and the cooling ramp rate to max. 1.5°C.

Table 2 - PCR Program PCR1

Temperature	Time	Comment
95°C	03:00	Initial Denaturation
95°C	00:15	20 cycles
55°C	00:15	
72°C	00:30	
72°C	10:00	Final elongation
4°C	Hold	

4.2. Cleanup of PCR1 products

4.2.1. Preparation

- Let the Magnetic Beads equilibrate at room temperature by transferring the bottle to the bench at least 30 min before use.
- Always prepare fresh 80% ethanol.
- Thoroughly resuspend the beads immediately before use.

4.2.2. Cleanup procedure

1. Add 60 µl Magnetic Beads directly to PCR1 products (50 µl) and mix well by pipetting up and down at least 10 times.
2. Incubate for 5 minutes at room temperature.
3. Place the tubes/plate on a magnetic stand to immobilize the beads. Incubate at room temperature until the solution is completely clear (approximately 1-2 minutes).
4. Aspirate and discard the clear supernatant without touching the beads.
5. Keep the tubes/plate on the magnetic stand and add 150 µl 80% ethanol to each tube/well. Do not resuspend the bead pellet.
6. Incubate at room temperature for 30 seconds on the magnetic stand.
7. Aspirate and discard the supernatant without touching the beads.
8. Repeat Steps 5 to 7 one more time for a total of 2 washes.
9. Leave the tubes on the magnetic stand, remove residual ethanol with a pipette and dry the beads for approximately 3 minutes.
   Important: *Residual ethanol may interfere with downstream applications. Do not over-dry the beads, as this will decrease yield. The bead pellet is dry when the appearance of the surface changes from shiny to matte.*
10. Remove the tubes/plate from the magnetic stand.
11. Elute the samples in 25 µl nuclease-free water. Mix well by pipetting up and down 10 times.
12. Incubate at room temperature for 3 minutes.
13. Place the tubes on a magnetic stand and incubate at room temperature until the beads are completely cleared from the solution (approximately 1-2 minutes).
14. Transfer 21.8 µl of the cleared supernatant to a new PCR tube.

4.3. Second PCR – Indexing

4.3.1. Reaction setup

The PCR1 product is used as the template for PCR2. Set up the PCR mix as stated in Table 3. For each sample, select a unique combination of indexing forward and reverse primers. The variation of PCR2 forward and reverse primers allows pooling 96 samples. If a larger number of samples is intended to be pooled, please order kits with different Indexing Primer Sets.

Note: *Set up PCR mix on ice.*
Important: *Make sure that all individual libraries sequenced in the same pool have a unique forward/reverse indexing primer combination and make note of the sample – dual-index combination (see Appendix) for the demultiplexing of the individual samples after sequencing.*
If only a small number of samples is to be sequenced, please use combinations as described in the Appendix for optimal index balancing.

Table 3 - Reaction Setup PCR2

Component	Volume for 1 reaction [µl]
ViennaLab 16S Master Mix 2x	25.0
Indexing Forward primer: MIB2(A/B/C)-F*x*	1.6
Indexing Reverse primer: MIB2X-R*x*	1.6
Product of PCR1	21.8
TOTAL VOLUME	**50.0**

4.3.2. PCR program

Create a PCR program according to Table 4. Use a heated lid (>100°C); if applicable, set the heating ramp rate to max. 2.5°C and the cooling ramp rate to max. 1.5°C.

Table 4 - PCR Program PCR2

Temperature	Time	Comment
95°C	03:00	Initial Denaturation
95°C	00:15	10 cycles
55°C	00:15	
72°C	00:30	
72°C	10:00	Final elongation
4°C	hold	

4.4. Cleanup of PCR2 products

4.4.1. Preparation

- Let the Magnetic Beads equilibrate at room temperature by transferring the bottle to the bench at least 30 min before use.
- Always prepare fresh 80% ethanol.
- Thoroughly resuspend the beads immediately before use.

4.4.2. Cleanup procedure

1. Add 60 µl Magnetic Beads directly to PCR2 products (50 µl) and mix well by pipetting up and down at least 10 times.
2. Incubate for 5 minutes at room temperature.
3. Place the tubes/plate on a magnetic stand to immobilize the beads. Incubate at room temperature until the solution is completely clear (approximately 1-2 minutes).
4. Aspirate and discard the clear supernatant without touching the beads.
5. Keep the tubes/plate on the magnetic stand and add 150 µl 80% ethanol to each tube/well. Do not resuspend the bead pellet.
6. Incubate at room temperature for 30 seconds on the magnetic stand.
7. Aspirate and discard the supernatant without touching the beads.
8. Repeat Steps 5 to 7 one more time for a total of 2 washes.
9. Leave the tubes on the magnetic stand, remove residual ethanol with a pipette and dry the beads for approximately 3 minutes.
   Important: *Residual ethanol may interfere with downstream applications. Do not over-dry the beads as this will decrease yield. The bead pellet is dry when the appearance of the surface changes from shiny to matte.*
10. Remove the tubes/plate from the magnetic stand.
11. Elute the samples in 25 µl nuclease-free water. Mix well by pipetting up and down 10 times.
12. Incubate at room temperature for 3 minutes.
13. Place the tubes on a magnetic stand and incubate at room temperature until the beads are completely cleared from the solution (approximately 1-2 minutes).
14. Transfer 22 µl of the cleared supernatant containing cleaned-up PCR products to a new tube.

4.4.3. (Optional) Check PCR2 product size (e.g. on a Fragment Analyzer)

The expected size of the post-PCR2 library is approximately 600 bp for the bacterial V3-V4 and between 440-540 bp for the fungal ITS2 (See Figure 2).

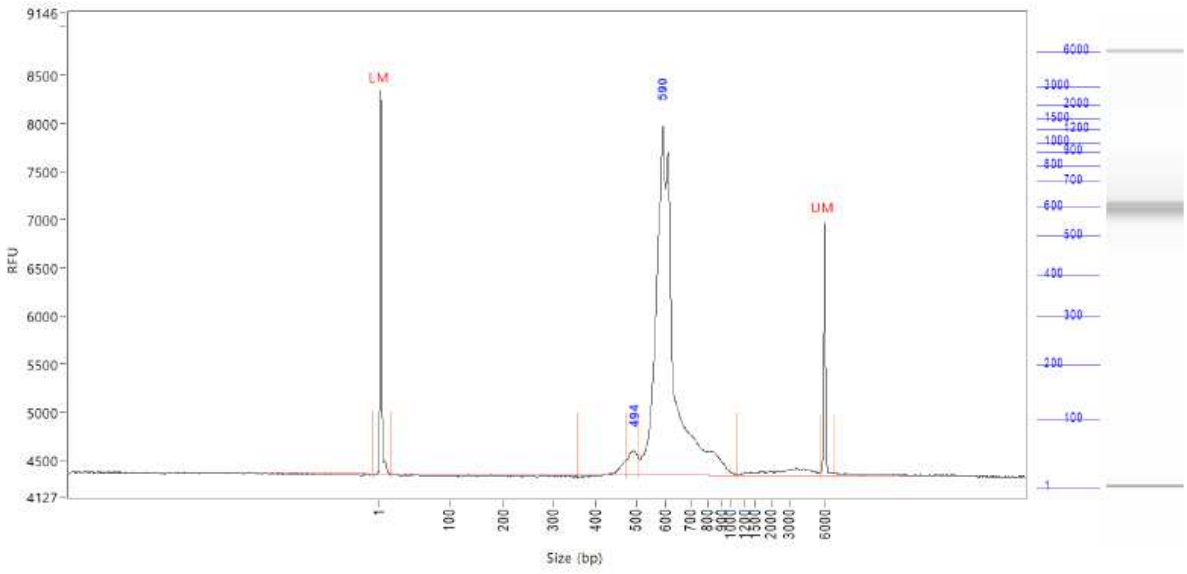

Figure 2 - Fragment Analyzer profile of PCR2 product.

***Note**: It is estimated that fungi account for only 0.01-0.1% of resident gut microbiota. Therefore, in the majority of stool DNA library preparation cases, the V3-V4 peak will be dominant, while a clear fungal ITS2 peak is not always detected. Despite the very low amount of fungal DNA and the absence of ITS2 peak, 2-5% of total reads is usually assigned to fungi after sequencing.*

4.5. Library quantification and pooling

4.5.1. Library quantification

Library quantification by qPCR is recommended, e.g. with JetSeq™ Library Quantification Lo-ROX Kit (meridian Bioscience®) and associated qPCR cycler. Alternatively, a Qubit Fluorometer or a similar device can be used, but concentrations should be compared to qPCR results in the first experiments to establish a normalization factor for further experiments, if needed.

4.5.2. Library dilution and pooling

Dilute all individual libraries to 4 nM and pool for sequencing equimolarly or according to the required amount of data output.
For the assessment of the number of samples that can be sequenced with the used sequencing chemistry, the calculation of the sample concentration and pooling, and the assistance with the sample sheet generation, please refer to the ViennaLab MicrobeCalc™ Excel file that can be downloaded at www.viennalab.com/support/ngs-assays.

5. Sample Sheet and sequencing recommendations

- For library pool denaturation and loading refer to Illumina System Guides.
- A spike-in of 0-5% PhiX is optional (see section 3.6).
- For sample sheet preparation see index sequences listed in Table 5 . For MiSeq the ViennaLab MicrobeCalc™ Excel file can be used (see 4.5.2).
- Sequencing of at least 50000 total reads per sample is recommended for optimal results.

Table 5 - Index sequences for MiSeq, MiSeq i100

i5 Primer	i5 index sequence	i7 Primer	i7 index sequence
MIB2A-F1	TAAGACAC	MIB2X-R1	ACGCTACT
MIB2A-F2	CTGCGTGT	MIB2X-R2	CGAGCGAC
MIB2A-F3	TGTTCTCT	MIB2X-R3	GTCTATGA
MIB2A-F4	CTAATCGA	MIB2X-R4	TATAGCGA
MIB2A-F5	CGTTACTA	MIB2X-R5	CGAGAGTT
MIB2A-F6	ACTATCTG	MIB2X-R6	GACATAGT
MIB2A-F7	TGAACCTT	MIB2X-R7	ACTCACTG
MIB2A-F8	TAAGTTCC	MIB2X-R8	TAGTCTCC
MIB2B-F9	TACGAGAC	MIB2X-R9	TGAGTACG
MIB2B-F10	CGTGAGTG	MIB2X-R10	CTGCGTAG
MIB2B-F11	GACACCGT	MIB2X-R11	ACTACGAC
MIB2B-F12	CTACTATA	MIB2X-R12	GTCTGCTA
MIB2B-F13	GATCGTGT		
MIB2B-F14	GTCAGATA		
MIB2B-F15	AGAGTCAC		
MIB2B-F16	TCATCGAG		
MIB2C-F17	ATCGTACG		
MIB2C-F18	TCGACGAG		
MIB2C-F19	GGATATCT		
MIB2C-F20	TAGACCTA		
MIB2C-F21	ACGTCTCG		
MIB2C-F22	TAGCGAGT		
MIB2C-F23	CTAGAACA		
MIB2C-F24	TGCTAAGT		

6. Quick Guide to sequencing data analysis

6.1. **a)** Upon first use please navigate to **https://microbiome.viennalab.com** and register for the ViennaLab Microbiome Analysis Webtool providing a valid email address and the Activation code you find on the inner side of the kit box lid.
b) If you are an already registered user, please enter the Activation code provided with the kit in your Account profile.

6.2. Upload the raw sequencing data files. Supported file formats include *.fastq and *.fastq.gz and click on Upload .

6.3. You will then be asked to confirm the deduction of 1 sample credit from your balance for each single or two paired sequencing data files by clicking on Upload and Analyze .

***Important**: Do not close the browser window or redirect or reload the page while files are being uploaded, as this would interrupt the upload procedure*.

6.4. When upload and processing are finished sample status will turn from “Processing” to “Ready”.

6.5. You can now review your results and generate reports.

For technical support please contact ViennaLab through the local distributor or directly at **techhelp@viennalab.com**.

APPENDIX

Tables for indexing primer combinations

- If preparing only a small number of samples (e.g. less than 16 samples), use index combinations with consecutive numbers given in the tables below (e.g. use primers F1-F4 and R1-R2 for samples 1-8 as indicated, and F1-F4 and R3-R4 for samples 9-16 and so on for kit [9-131]. For examples see next page.
- Mark which index combinations have already been used and use each primer combination only once.

[9-131]

MIB2-	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12
F1	1	5	9	13	65	69	73	77	33	37	41	45
F2	2	6	10	14	66	70	74	78	34	38	42	46
F3	3	7	11	15	67	71	75	79	35	39	43	47
F4	4	8	12	16	68	72	76	80	36	40	44	48
F5	49	53	57	61	17	21	25	29	81	85	89	93
F6	50	54	58	62	18	22	26	30	82	86	90	94
F7	51	55	59	63	19	23	27	31	83	87	91	95
F8	52	56	60	64	20	24	28	32	84	88	92	96

[9-132]

MIB2-	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12
F9	1	5	9	13	65	69	73	77	33	37	41	45
F10	2	6	10	14	66	70	74	78	34	38	42	46
F11	3	7	11	15	67	71	75	79	35	39	43	47
F12	4	8	12	16	68	72	76	80	36	40	44	48
F13	49	53	57	61	17	21	25	29	81	85	89	93
F14	50	54	58	62	18	22	26	30	82	86	90	94
F15	51	55	59	63	19	23	27	31	83	87	91	95
F16	52	56	60	64	20	24	28	32	84	88	92	96

[9-133]

MIB2-	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12
F17	1	5	9	13	65	69	73	77	33	37	41	45
F18	2	6	10	14	66	70	74	78	34	38	42	46
F19	3	7	11	15	67	71	75	79	35	39	43	47
F20	4	8	12	16	68	72	76	80	36	40	44	48
F21	49	53	57	61	17	21	25	29	81	85	89	93
F22	50	54	58	62	18	22	26	30	82	86	90	94
F23	51	55	59	63	19	23	27	31	83	87	91	95
F24	52	56	60	64	20	24	28	32	84	88	92	96

Examples for indexing primer pipetting scheme for a low sample number

4 samples

Fwd Primer MIB2-	F1	F2	F3	F4
Rev Primer MIB2-	R1			
Sample #	**1**	**2**	**3**	**4**

8 samples

Fwd Primer MIB2-	F1	F2	F3	F4	F1	F2	F3	F4
Rev Primer MIB2-	R1				R2			
Sample #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**

16 samples

Fwd Primer MIB2-	F1	F2	F3	F4	F1	F2	F3	F4
Rev Primer MIB2-	R1				R2			
Sample #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**

Fwd Primer MIB2-	F1	F2	F3	F4	F1	F2	F3	F4
Rev Primer MIB2-	R3				R4			
Sample #	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**

Version	Amendments
rev 3.0	- Update of sample input amount - Change of PCR1 primers from individual primer tubes to one primer pool - Change of PCR1 cycle number - Change of beads clean up ratio and volumes after PCR1 and PCR2 - Change of sample input volume into PCR2 - Change of PCR2 primer sequences - Change of ViennaLab MicrobeCalc™ tool to rev1.4 for demultiplexing
rev 3.1	- Introduction of 16S Microbiome NGS Assay for 16 reactions [REF 9-131-16] - Change of sample - indexing primer combinations - Introduction of examples for indexing primer pipetting scheme for a low sample number (Appendix)
rev 4.0	- Introduction of FUN1 – ITS2 Mix - Adjustment of PCR1 set up for optional FUN1 – ITS2 Mix addition - Deletion of PCR1 product size check paragraph - Change of PCR2 expected sizes - Change of Fragment Analyzer plot of PCR2 product - Added pool quantification - Change of ViennaLab MicrobeCalc™ tool to rev2.0

Notes

Notes

Notes

REF		Σ
9-131	16S Microbiome NGS + ITS Assay [Set A]	96 reactions
9-132	16S Microbiome NGS + ITS Assay [Set B]	96 reactions
9-133	16S Microbiome NGS + ITS Assay [Set C]	96 reactions
9-131-16	16S Microbiome NGS + ITS Assay [16 rxn]	16 reactions

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