2017 16S rRNA gene Extraction/PCR/Sequencing Protocol
Version1: May 23, 2017

1. Background

This protocol is derived from a "Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies" (doi:10.1038/nbt.3601). Portions of this protocol have been copied verbatim from the paper and associated protocol (doi:10.1038/protex.2016.030) but adapted to our materials and equipment. For publication purposes, I think it will be sufficient to cite this published protocol protocol.

The purpose of this protocol is to improve accuracy, reduce PCR artifacts such as chimeras, and minimize drop-out of taxa due to mismatches between the amplification or sequencing primers, and to add flexibility in choice of variable region.

This protocol uses a two-step PCR process, where the marker gene of interest is amplified with a pair of adapter-tailed primers in a primary PCR reaction, and sample-specific dual indices and flow cell adapters are added in a subsequent indexing reaction. It could be applied to the following variable regions: V1-V3, V3-V4, V3-V5, V4, V4-V6, and V5-V6 of the 16S ribosomal RNA (rRNA) gene, as well as for variable region V9 of the 18S rRNA gene and the ITS1 and ITS2 regions. The modularity of this design makes it easy to survey new regions of interest, since only a single pair of adapter-tailed locus-targeting primers needs to be synthesized. Primer sets for the primary PCR are listed in **Table 8.1** and the indices in **Table 8.2**.

2. DNA Extraction

2.1. Theory

Ideally we want a high-throughput extraction method that is scalable and would allow for direct sequencing and qPCR analysis that has internal controls to ensure accuracy of microbial profiles. We have been evaluating the ZymoBIOMICS 96-well kits in both the column filtration and magnetic capture implementations. This protocol uses the magnetic capture as the extraction can be scaled any where from ~8 samples to 96 without wasting extra resources thanks to the nature of magnetic capture and the individual strip tubes in the lysing rack. This approach gives good linear recovery of the difficult to lyse *Eggerthella lenta,* down to ~1E4 CFU levels as determined by qPCR. This could be easily replaced with the old Promega-based approach, MoBio PowerSoil, or the column-based varint of this kit.

Using a copy of the 16S_Run_Template.xlsx in buggut.ucsf.edu:/labshare/16S_Runs will help facilitate preparation of the sequencing run sheet and track important details of the experiment.
Note: sample names can not contain "." Instead use "_". THIS IS OPPOSITE TO QIIME!

2.2. Materials

a. ZymoBIOMICS 96 MagBead DNA Kit (Zymo D4302)
b. ZymoBIOMICS Community Standard (Zymo D6300)
c. 1mL Deep well Plates (USA Scientific 1896-1110)
d. TemPlate Semi-Skirt 0.2mL PCR Plate (USA Scientific 1402-9200)
e. Beta Mercaptoethanol (BioRad 1610710)
f. Deep well magnetic capture stand (Sigma Z740158)
g. 96x1200μL Tips (Sartorius 791211)
h. 96x200μL tips (USA Scientific 1120-8710)

i. Multichannel Vacuum manifold (Corning 07200564)
j. 96 well plate vortex (IKA MS 3 360-78005-E1)
k. Biospec Mini-Beadbeater-96 or similar*
**Note: plates will not fit in tissuelyzer or MoBio options!*

2.3. Protocol

Location: Biological Safety Cabinet used for level 2 work (ideally new coy room). Remember that samples, especially those from humans, could contain a wide variety of pathogens and should be treated with universal caution regardless of their origin. After extraction, these should have been deactivated and samples should be protected from outside DNA contamination.

a. Treat extraction area with UV ~15 minutes or 0.5% bleach.
b. Add 750 µL beta-mercaptoethanol to 150mL bottle of MagBinding Buffer
c. Transfer ~50 mg or half fecal pellet, or 200µL liquid sample into lysing plate.
d. Add 650 µL ZymoBIOMICS lysis solution.
e. Disrupt for 5 minutes in Biospec beadbeater (be sure that it is completely tightened).
f. Centrifuge 5 minutes at ~3000g.
g. Transfer 200 µL supernatant to 1 mL deep-well plate.
h. Add 600µL ZymoBIOMICS MagBinding Buffer with BME to each well and mix.
i. Add 25 µL Magnetic Beads to each well and mix.
j. Capture for ~2 minutes or until solution is cleared using magnetic capture stand.
k. Discard supernatant.
l. Add 800µL MagWash1 and mix on vortex for 10 minutes, capture and discard supernatant.
m. Add 800µL MagWash2 and mix on vortex for 10 minutes, capture and discard supernatant.
n. Repeat step m.
o. Using 200µL multichannel, remove any residual ethanol that may be in plate.
p. Using 65˚C heat block for 10 minutes, dry off any residual ethanol (dry bath beside HPLC is currently set up for this).
q. Add 100µL nuclease free H2O and capture again using a spacer to raise plate ~5mm.
r. Transfer 50µL supernatant PCR plate for storage.
s. *Optional: spot check negative controls and real samples with Nanodrop. Successful samples should be >10ng/µL with a 260/280>1.6 and 260/230>1.4. If the 260/230 is <1.4, quantification is not accurate and Qubit should be applied instead. Yield is not an indicator of evenness of extraction.*

3. Primary PCR

3.1. Theory

To prevent against over-amplification, the primary PCR is carried out as a quantitative PCR that provides real-time feed back on amplification. The goal is to get mid-to-late amplification to carry forward for indexing. To get optimal amplification, a 10-fold dilution series is created and the optimal dilution is selected. In PCR, a 10-fold dilution corresponds to a delay of 3.3 cycles, thus 4x10-fold dilutions cover a range of ~13 cycles. In practice, for mouse samples extracted with the above approach, 10-fold dilutions seems to captured nicely at 20 cycles of PCR with lower density samples (such as pure culture gavages) being captured at the 1X concentration. For these types I would recommend running only two dilutions. For more complex or mixed samples types, It is recommended to do a full 4x10-fold dilutions. PCR inhibition can be directly visualized here as 10-fold dilutions amplifying similarly (or better) than its undiluted stock. Remember there should be ~3 cycles between dilutions. Inhibition has not been seen with the Zymo approach, but has been seen with the Promega based extraction. A direct cause of inhibition is carryover of ethanol containing wash buffer from column based approaches.

3.2. Materials

a. 384 Plates for qPCR (Biorad #HSP3865)
b. 96 Well Plates (USA Sci #1402-9200)
c. Optically clear Plate Seals (Biorad Microseal 'B' #MSB1001)
d. DMSO for PCR (Sigma D8418-50mL)
e. SYBR Green I (Sigma S9430) 10x diluted in DMSO to 1000x
f. KAPA HiFi PCR kit (KAPA KK2502)
g. Amplification primers of choice at 100μM (see Table 8.1)
h. Nuclease-free H_2O (Life Tech 0977-023)

Table 3.2.1. Primary PCR Master Mix

Component	1 Rxn (μL)	420 Rxns
Nuclease-free H_2O	5.3955	2606.31
5x KAPA HiFi Buffer	1.8	756
10 mM dNTPs	0.27	113.4
DMSO	0.45	189
1000x SYBR Green	0.0045	1.89
100 μM Forward Primer	0.045	18.9
100 μM Reverse Primer	0.045	18.9
KAPA HiFi polymerase	0.18	75.6
Total	9.0	3780.0

3.3. Protocol

Location: PCR Hood or separate room from other steps

a. Treat PCR area with UV light for ~15 minutes.

b. Add 9μL of PCR master mix (prepared as per **Table 3.2.1**) to all wells of a 384 well plate.

c. Divide 384 plate into 4 quadrants and add 1 μL of template DNA to the first quadrant replicating the plate. This layout is at your discretion.

d. Carry out 3x 10-fold dilutions of each sample into the next 3 quadrants by transferring 1 μL with a multichannel being sure to leave at least 1 no template control. Perhaps by not adding control in the most dilute reaction.

e. Amplify using the BioRad CFX384 according to the parameters of **Table 3.3.1**.

Table 3.3.1. Primary PCR Amplification Parameters

Cycle	Temperature (˚C)	Time
Initial Denaturation	95	5 min
20 cycles[1]:		
Denature	98˚C	20 sec
Anneal	55˚C	15 sec
Extend	72˚C	60 sec
Holding	4˚C	Hold (0 sec)

[1]This should be good for fecal samples, may need more cycles for low abundance, or poorly amplified samples. With V4 primers, primer dimers will occur by cycle 25 and will make success of amplification difficult.

4. Indexing PCR

4.1. Theory

While the primary PCR has created the amplicons for sequencing, there is no identifying information on the DNA to tell the sample of origin. This is the purpose of the indexing PCR. In the former protocol, a single index/barcode was incorporated onto the reverse primer. Here there are two indices/barcodes on both sides of the amplicon. In this way, fewer barcodes can be used as samples are recognized as the combination of left and right barcodes. For example, in our current set up 24 forward, and 16 reverse give 384 combinations. The same amount of sequence capacity would require 384 distinct (and long, and expensive) reverse primers.

4.2. Materials

- **a.** 96 Well Plates (USA Sci #1402-9200)
- **b.** DMSO for PCR (Sigma D8418-50mL)
- **c.** KAPA HiFi PCR kit (KAPA KK2502)
- **d.** Indexing Primer plate (Pick 1 of 4 for each plate to be sequenced without overlapping).
- **e.** Nuclease-free H_2O (Life Tech 0977-023)

Table 4.2.1. Indexing PCR Master Mix (3.3x)

Component	**1 Rxn (μL)**	**110 Rxns**
5x KAPA HiFi Buffer	8	880
10 mM dNTPs	1.2	132
DMSO	2.0	220
KAPA HiFi polymerase	0.8	88
Total	8	880

4.3. Protocol

Location: Anywhere that is not the PCR hood or where final libraries are being prepared. Due to the limited number of cycles, the only risk of contamination here comes from other sequencing libraries.

- **a.** Transfer 2 μL of non-plateaued reaction into a new 96 well plate containing 198 μL of H2O to create a 100-fold dilution of the primary PCR. Label this for long-term storage.
- **b.** In a new 96 well plate, add 6μL master mix to every well (Table 4.2.1).
- **c.** To each well, add 4μL of indexing primer from a stock plate being sure replicate the same layout.
- **d.** Add 10 μL of 100-fold dilution plate being sure to replicate the same layout.
- **e.** Complete amplification as per the protocol in Table 4.3.1.

Table 4.3.1. Indexing Amplification Parameters

Cycle	Temperature (˚C)	Time
Initial Denaturation	95	5 min
10 cycles:		
Denature	98˚C	20 sec
Anneal	55˚C	15 sec
Extend	72˚C	60 sec
Holding	4˚C	Hold (0 sec)

5. Amplicon Pooling

2.1. Theory

While it is absolutely still possible to use PicoGreen pooling of amplicons, we are experimenting with SequalPrep kits. The idea is that the wells of this plate have a positively charged coating such that, under low pH conditions, DNA is bound due to its negative backbone charge. Under neutral to basic conditions the DNA is released. The normalization happens due to saturation of binding and thus it is important that the well be saturated with ~250ng per well. After the protocol, all volumes can be multichanneled into a reservoir and mixed. These must then be concentrated as the concentration will be in the range of 0.2-1ng/μL which while usable, is uncomfortably low. For this I have tested using Qiagen MineEute columns on a vacuum filter, however we should investigate other methods. It is worth noting that after the library has been normalized, there is no concern about cross contamination between samples so the same tips can be used for all wells. **In the future it would be ideal to find a speedvac to concentrate*

2.2. Materials

- **a.** SequalPrep Normalization Plate (Life Tech A10510-01)
- **b.** Qiagen MinElute columns (Qiagen 28004)
- **c.** Qiagen buffer PB (Qiagen 28004)
- **d.** Qiagen buffer PE (Qiagen 28004)
- **e.** Tris-Cl pH 8.5 (Qiagen Buffer EB)
- **f.** Multichannel Vacuum Manifold **optional*
- **g.** Qiagen or MoBio vacuum manifold **optional but saves a lot of time*
- **h.** TempPlate Sealing Foil (USA Scientific 2923-0110)

2.3. Protocol

Location: lab bench

- **a.** Add 15 μL SequalPrep Normalization Binding Buffer to each well of a 96 well plate
- **b.** Transfer 15 μL of PCR production from indexing PCR into each well of a SequalPrep plate
- **c.** Mix by pipetting and incubate at RT for 1h
- **d.** Discard liquid **remember the DNA is bound to plate*
- **e.** Wash with 50 μL SequalPrep Normalization Wash Buffer and pipette up and down to mix
- **f.** Completely remove liquid and discard
- **g.** Tap upside down on kim wipes to remove any residual liquid *using vacuum removal may help here.
- **h.** Add 20 μL SequalPrep Elution Buffer. Seal plate with foil. Mix and centrifuge quickly to pull liquid down.
- **i.** Incubate at RT for 5 minutes.
- **j.** Pool eluted DNA into multichannel reservoir.
- **k.** Transfer eluted DNA into appropriately sized tube.
- **l.** Add 5 volumes of buffer PB to eluted DNA **optional: store some of pooled DNA for backup*
- **m.** 800μL at a time, pass through a MinElute column **Note: using one of the vacuum manifolds here prevents exesive time waiting for centrifuge. Alternatively centrifuge 30s and dispose of filtrate.*
- **n.** Transfer column to 2mL collection tube and add 750 μL buffer PE.
- **o.** Centrifuge for 1 min at ~16,000g and discard flow through.

p. Centrifuge again to ensure complete removal of ethanol.
q. Transfer column to clean 1.5 mL tube and add 12μL of Buffer EB to the center of the column.
r. Incubate at RT for 1 min
s. Centrifuge for 2 min at ~16,000g.
t. Discard column and label tube appropriately.
u. Check concentration using both NanoDrop and QuBit. Record these in your in the LibraryNorm section of your RunSheet.

6. Library QC and Quantification

6.1. Theory

While using the Nanodrop is convienient, it is not accurate for concentrations less than ~10-20ng/μL and lacks the sensitivity required. The QuBit/QuantiT uses a fluorescent dye to specifically quantify dsDNA and is generally much more sensitive and specific than the Nanodrop, and once upon a time was how people normalized sequencing libraries. The most up to date way is via qPCR. This is accomplished by using primers against the Illumina sequencing adapters so in effect, you are only quantifying the portion of DNA that can actually be sequenced for the most sensitive quantification. Accurate quantification is key for getting the correct cluster density on the sequencer **see 7. Sequencing for more information on the importance of cluster density*. This step represents one of the most important steps in your library generation and will directly influence the read depth and quality of your sequencing run. Inaccuracy in this step could completely render your sequencing run useless. *Also while not explicitly required, it is not a bad idea to run a 2% gel and/or a HS DNA bioanalyzer chip to look at size distribution in the library and ensure that size selection is not necessary. If V4 amplicon used, band should be ~427 bp and be free of other bands. If other bands are present, size selection will be required.

6.2. Materials

a. KAPA Library Quantification Kit for Illumina Platforms (KAPA KK4824)
**Standards can be ordered separately as they are limiting reagent of kit (KAPA KK4903)*

b. 384 Plates for qPCR (Biorad #HSP3865)

c. Optically clear Plate Seals (Biorad Microseal 'B' #MSB1001)

6.3. Protocol

Location: lab bench or perhaps tissue culture hood.

a. If first time using kit, add the 10X primer premix to the KAPA SYBRFast qPCR Master Mix (2X)

b. Using whatever layout you choose, 6μL to 30 wells of a 384 well plate ((6 standards x 3 replicates) + (3 dilutions of library x 3 replicates) + 3 NTC))

c. Transfer 4 μL of the 6 standards into triplicate wells.

d. Create a **CAREFULL** dilution series of your library in Tris-Cl pH=8.5 0.05% Tween20 with the following dilutions being sure to mix between:

- **i.** 2 μL of library into 198 buffer (100x)
- **ii.** 2 μL of library into 198 buffer (10,000x)
- **iii.** 20 μL into 180 buffer (100,000x)

e. Transfer 4 μL of these in triplicate to appropriate wells of 384 well plate.

f. Seal plate with optically clear cover and centrifuge briefly.

g. Amplify according to the following parameters on the BioRad CFX384:

Table 4.3.1. Indexing Amplification Parameters

Cycle	Temperature (˚C)	Time
Initial Denaturation	95	5 min
35 cycles:		
Denature	95˚C	30 sec
Anneal/Extend	60˚C	45 sec
Melt Curve	65->95˚C	NA

h. After completion of the run, export the data. From the Cq results: plate view, transfer appropriate values to the Run Log sheet:LibraryNorm into the highlighted boxes. Use the average fragment length appropriate to your amplicon (427 for V4).

i. To be successful, the amplification efficiency should be 90-110% and the R^2 value should be greater than 0.99. Sanity check: Your QuBit/Nanodrop calculations should be in the same ballpark as the qPCR result. If these are extremely different something has gone wrong. Requantify and run bioanalyzer chip to ensure proper library size distribution.

7. Sequencing

7.1. Theory

While many platforms can be applied for sequencing of the amplicons, MiSeq is among the most common due to its relatively longer read lengths and accuracy. While the NextSeq will provide many more reads, the cost of running and shorter read lengths (max 2x150) make it less desirable for some applications. Similarily the HiSeq is also limited to 2x150 currently and provides a level of output not needed for amplicon sequencing. If ultra high-depth community structure is desired, I would propose shotgun sequencing rather than amplicon sequencing. Because we are using a different barcoding structure, it is now also unnecessary to add custom sequencing primers as the built in Illumina primers will suffice. Also, we can now provide a sample sheet such that the sequencer demultiplexes our reads for us. To create a sample sheet, an R script (MakeSampleSheet.R) can be used which will harvest the information in your excel file to create the desired output to upload at sequencing time. The sample sheet can always be changed after the fact, however it is important that the reads are set up for at least 250x8x8x250 to ensure both barcodes are sequenced.

7.2. Materials

a. MiSeq Reagent Kit (Version 3, 600 cycles, $1,377.00 each)
b. Buffer HT1 (Included with MiSeq Kit)
c. Incorperation buffer (Included with MiSeq Kit)

7.3. Protocol

a. Thaw MiSeq reagent kit overnight in fridge (or in room temp water bath)
b. Generate a MiSeq sample sheet using MakeSampleSheet.R
c. Prepare fresh 0.2 M NaOH (10 μL NaOH and 90μL H_2O)
d. In a 1.5 ml microfuge tube, denature 10 µl of 2 nM library by incubating with freshly diluted 0.2 N NaOH for 5 minutes at room temperature.
e. Add 980 µl of Illumina's HT1 buffer to bring the sample to 20 pM.
f. Dilute to 8 pM by mixing 400 µl of 20 pM library and 600 µl of Illumina's HT1 buffer in a clean 1.5 ml microfuge tube.
g. Repeat process to generate 8 pM PhiX library (Start with 2 μL into 8 μL to dilute to 2nM)
h. Remove 150 µl of the 8 pM library and discard. Add 150 µl of 8 pM PhiX to the remaining 850 µl of 8 pM library (15% PhiX spike).
i. ix) Add 600 µl of the 8 pM (15% PhiX) library to the sample well of the MiSeq cartridge and initiate sequencing.

8. Appendices

Table 8.1. Primers for 1˚ PCR.

Primer name	Marker gene	Target region	Sequence
V1_27F_Nextera	16S rRNA	V1-V3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**AGAGTTTGATCMTGGCTCAG**
V3_534R_Nextera	16S rRNA	V1-V3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**ATTACCGCGGCTGCTGG**
V3_357F_Nextera	16S rRNA	V3-V4, V3-V5	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGAGGCAGCAG**
V4_515F_Nextera	16S rRNA	V4, V4-V6	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTGCCAGCMGCCGCGGTAA**
V4_806R_Nextera	16S rRNA	V3-V4, V4	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GGACTACHVGGGTWTCTAAT**
V5F_Nextera	16S rRNA	V5-V6	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**RGGATTAGATACCC**
V5_926R_Nextera	16S rRNA	V3-V5	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CCGTCAATTCMTTTRAGT**
V6R_Nextera	16S rRNA	V5-V6, V4-V6	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CGACRRCCATGCANCACCT**
18S_V9_1391_F_Nextera	18S rRNA	V9	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTACACACCGCCCGTC**
18S_V9_EukBr_R_Nextera	18S rRNA	V9	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TGATCCTTCTGCAGGTTCACCTAC**
ITS1F_Nextera	ITS	ITS1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CTTGGTCATTTAGAGGAAG*TAA**
ITS2_Nextera	ITS	ITS1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GCTGCGTTCTTCATCGA*TGC**
5.8SR_Nextera	ITS	ITS2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**TCGATGAAGAACGCAGCG**
ITS4_Nextera	ITS	ITS2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TCCTCCGCTTATTGATATGC**

Table 8.3. Sequencing Indices

Primer name	Primer sequence	Index set origin	Index name	Index sample sheet	Primer name
F1_MetaIndex	AATGATACGGCGACCACCGAGATCTACACTATAGCCTTCGTCGGCAGCGTC	TruSeq i5	D501	TATAGCCT	F1_MetaIndex
F2_MetaIndex	AATGATACGGCGACCACCGAGATCTACACATAGAGGCTCGTCGGCAGCGTC	TruSeq i5	D502	ATAGAGGC	F2_MetaIndex
F3_MetaIndex	AATGATACGGCGACCACCGAGATCTACACCCTATCCTTCGTCGGCAGCGTC	TruSeq i5	D503	CCTATCCT	F3_MetaIndex
F4_MetaIndex	AATGATACGGCGACCACCGAGATCTACACGGCTCTGATCGTCGGCAGCGTC	TruSeq i5	D504	GGCTCTGA	F4_MetaIndex
F5_MetaIndex	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGTCGTCGGCAGCGTC	TruSeq i5	D505	AGGCGAAG	F5_MetaIndex
F6_MetaIndex	AATGATACGGCGACCACCGAGATCTACACTAATCTTATCGTCGGCAGCGTC	TruSeq i5	D506	TAATCTTA	F6_MetaIndex
F7_MetaIndex	AATGATACGGCGACCACCGAGATCTACACCAGGACGTTCGTCGGCAGCGTC	TruSeq i5	D507	CAGGACGT	F7_MetaIndex
F8_MetaIndex	AATGATACGGCGACCACCGAGATCTACACGTACTGACTCGTCGGCAGCGTC	TruSeq i5	D508	GTACTGAC	F8_MetaIndex
F9_MetaIndex	AATGATACGGCGACCACCGAGATCTACACTGAACCTTTCGTCGGCAGCGTC	TruSeq Amplicon	A501	TGAACCTT	F9_MetaIndex
F10_MetaIndex	AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTC	Nextera i5	N501	TAGATCGC	F10_MetaIndex
F11_MetaIndex	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTC	Nextera i5	N502	CTCTCTAT	F11_MetaIndex
F12_MetaIndex	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTC	Nextera i5	N503	TATCCTCT	F12_MetaIndex
F13_MetaIndex	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGCAGCGTC	Nextera i5	N504	AGAGTAGA	F13_MetaIndex
F14_MetaIndex	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTC	Nextera i5	N505	GTAAGGAG	F14_MetaIndex
F15_MetaIndex	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTC	Nextera i5	N506	ACTGCATA	F15_MetaIndex
F16_MetaIndex	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTC	Nextera i5	N507	AAGGAGTA	F16_MetaIndex
R13_MetaIndex	CAAGCAGAAGACGGCATACGAGATGTCGTGATGTCTCGTGGGCTCGG	TruSeq Amplicon	A701	ATCACGAC	R13_MetaIndex
R14_MetaIndex	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTCTCGTGGGCTCGG	TruSeq i7	D701	ATTACTCG	R14_MetaIndex
R15_MetaIndex	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTCTCGTGGGCTCGG	TruSeq i7	D702	TCCGGAGA	R15_MetaIndex
R16_MetaIndex	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTCTCGTGGGCTCGG	TruSeq i7	D703	CGCTCATT	R16_MetaIndex
R17_MetaIndex	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTCTCGTGGGCTCGG	TruSeq i7	D704	GAGATTCC	R17_MetaIndex
R18_MetaIndex	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTCTCGTGGGCTCGG	TruSeq i7	D705	ATTCAGAA	R18_MetaIndex
R19_MetaIndex	CAAGCAGAAGACGGCATACGAGATACGAATTCGTCTCGTGGGCTCGG	TruSeq i7	D706	GAATTCGT	R19_MetaIndex
R20_MetaIndex	CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTCTCGTGGGCTCGG	TruSeq i7	D707	CTGAAGCT	R20_MetaIndex
R21_MetaIndex	CAAGCAGAAGACGGCATACGAGATGCGCATTAGTCTCGTGGGCTCGG	TruSeq i7	D708	TAATGCGC	R21_MetaIndex
R22_MetaIndex	CAAGCAGAAGACGGCATACGAGATCATAGCCGGTCTCGTGGGCTCGG	TruSeq i7	D709	CGGCTATG	R22_MetaIndex
R23_MetaIndex	CAAGCAGAAGACGGCATACGAGATTTCGCGGAGTCTCGTGGGCTCGG	TruSeq i7	D710	TCCGCGAA	R23_MetaIndex
R24_MetaIndex	CAAGCAGAAGACGGCATACGAGATGCGCGAGAGTCTCGTGGGCTCGG	TruSeq i7	D711	TCTCGCGC	R24_MetaIndex
R1_MetaIndex	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG	Nextera i7	N701	TAAGGCGA	R1_MetaIndex
R2_MetaIndex	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG	Nextera i7	N702	CGTACTAG	R2_MetaIndex
R3_MetaIndex	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG	Nextera i7	N703	AGGCAGAA	R3_MetaIndex
R4_MetaIndex	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG	Nextera i7	N704	TCCTGAGC	R4_MetaIndex
R5_MetaIndex	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGG	Nextera i7	N705	GGACTCCT	R5_MetaIndex
R6_MetaIndex	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGG	Nextera i7	N706	TAGGCATG	R6_MetaIndex
R7_MetaIndex	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGG	Nextera i7	N707	CTCTCTAC	R7_MetaIndex
R8_MetaIndex	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGG	Nextera i7	N708	CAGAGAGG	R8_MetaIndex
R25_MetaIndex	CAAGCAGAAGACGGCATACGAGATCTATCGCTGTCTCGTGGGCTCGG	TruSeq i7	D712	AGCGATAG	R25_MetaIndex
R10_MetaIndex	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGG	Nextera i7	N710	CGAGGCTG	R10_MetaIndex
R11_MetaIndex	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGG	Nextera i7	N711	AAGAGGCA	R11_MetaIndex
R12_MetaIndex	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGG	Nextera i7	N712	GTAGAGGA	R12_MetaIndex

Table 8.2. Amplicon lengths for size selection

Target Gene	Variable Regions	Size
16S	V1-V3	662 bp +/- 20%
16S	V3-V4	550 bp +/- 20%
16S	V3-V5	722 bp +/- 20%
16S	V4	427 bp +/- 20%
16S	V4-V6	685 bp +/- 20%
16S	V5-V6	416 bp +/- 20%
18S	V9	260 +/- 20%
ITS	NA	HIGHLY VARIABLE