1. SCOPE

This is a suggested protocol for the generation of 16S rRNA amplicons from mixed DNA samples. It assumes the extraction was carried out using the MoBio 96 well Power Soil kit. This SOP is derived from the illumina_sop.txt file created by Greg Gloor. It covers the amplification part of preparing samples, quantification and pooling will be carried out at the LRGC.

2. PRINCIPLE

Microbiome profiling is used to identify and enumerate the organisms in samples from diverse sources such as soil, clinical samples and oceanic environments. This profiling is an important first step in determining the important bacterial and protist organisms in a biome and how they interact with and influence their environment. Microbiome profiling is usually achieved by sequencing PCR- amplified variable regions of the bacterial 16S and of the protistan small subunit ribosomal RNA genes [4,5]. Other sequences, such as the GroEL genes may also be targeted for independent validation [6]. The microbial profile of a sample may be determined by traditional Sanger sequencing, by terminal restriction length polymorphism analysis or by denaturing gradient gel electrophoresis (reviewed in [7]). The recent introduction of massively parallel 454 pyrosequencing has resulted in a radical increase in the popularity of microbiome profiling because a large number of PCR amplicons can be sequenced for a few cents per read [4,8]. However, while constituting a tremendous improvement over previous methods, pyrosequencing is constrained by cost limitations and a relatively high per-read error rate. The high error rate has led to some discussion in the literature about the existence and importance of the ‘rare microbiome’ [9]. New methods for analyzing pyrosequencing output suggest that much of the rare microbiome is composed of errors introduced by the sequencing method [10].

Until recently, the Illumina sequencing-by-synthesis method of parallel DNA sequencing was thought to be unsuitable for microbiome profiling because the sequencing reads were too short to traverse any of the 16S rRNA variable regions. This can be partially circumvented by identifying maximally informative sites for specific groups of organisms (eg. [11]). A recent report demonstrated that short sequences derived from Illumina sequences could be used for robust reconstruction of bacterial communities. This group used Illumina sequencing to determine the partial paired-end sequence of the V4 16S rRNA region in a variety of samples using single-end sequence tagged PCR primers [12]. Now using the longer reads of the next generation of Illumina sequencers we can cover other regions including the V4 to V5 region documented in this protocol.

The overlapping paired-end reads will give us complete coverage of the variable regions. The combination of sequence tags at each end of the overlapped reads allow us to use a small number of primers to uniquely tag a large number of samples. The Illumina sequencing method is an approximate order of magnitude cheaper than the per-read cost of pyrosequencing. The cheaper per-read costs allows economical experiments on large numbers of samples at very large sequencing depths. These methods can be applied to paired-end sequencing of the microbial V3, V5, V6 and the

eukaryotic V9 rRNA regions. Choice of variable region will have an effect on the ability to delineate particular organisms, for example, *Lactobacillus* are best differentiated in the V6 region while for stool samples V3 or V4/5 are likely to be more informative. **Modified from Gloor et al. 2010*

3. MATERIAL REQUIREMENTS

3.1 Primer Plates

Three primer plates of the V4/V5 primers have been prepared, 2 full plates of primer combinations, and a third with 64 primer combinations. *Note: Primers are ordered from left to right, top to bottom*. When amplifications are to be performed, the plate should be as complete as possible as each plate contains primers with balanced nucleotide composition in the first 12 positions which is important for calibrating base calling (ie, if the primer combinations aren't used properly the sequencing data will be suffer from poor quality).

Premade primer plates have been created and are stored in the -20 chest freezer in F0-100 (Burton Lab). The primer sequences and layout of primers are listed in Appendix 1 and also in the dropbox file primerlayouts_template.xlsx.

Due to the length of the primer, freeze thaw should be avoided so each plate is intended for one time use. It is imperative when picking which primer plates to use that sample number is considered to maximize plate usage as well as for sharing, each sample can only be present with one primer combination per run.

Ensure before starting any experiment that you have planned the layout of samples and if a run is to be shared between projects that no overlapping barcodes are used.

3.2 DNA Samples

Samples should be arrayed in 96 well plates with minimum 50 µL per well. Ideally the first and last wells (A1 and H12) were used as extraction blanks and can be pooled into A1. This leaves H12 on the primer plate to be used as a NTC (no template control) for PCR. DNA yield from extraction is a poor estimator of PCR product yield due to a number of factors including PCR inhibitors and eukaryotic DNA contamination. Should test amplifications need to be carried out, a batch of test primer (V4L1 and V5R1) has been prepared and is available from Jordan.

!!!!!!!!BEFORE RUNNING YOUR PCR Fill out the template and save as a new file. It will automatically generate the samples.txt file for downstream analysis UPDATED_primerlayouts_template.xlsx!!!!!!!!!

3.3 Biomek 3000 Laboratory Automation Workstation

Four programs have been created in the Biomek Software:

- V4V5MiSeq-AXYGEN-Plates1and2
- V4V5MiSeq-AXYGEN-Plate3
- V4V5MiSeq-MOBIO-Plates1and2
- V4V5MiSeq-MOBIO-Plate3

The difference in these programs reflects if the plates of DNA are in AXYGEN PCR Microplates or MoBio plates and has the tip go to different depths for removing template. Programs for Plates 1 and 2 are for the full plates while Plate 3 is for partial plates.

3.4 Promega GoTaq 2x Hot Start Colorless MasterMix

GoTaq® DNA Polymerase is supplied in 2X Colorless GoTaq® Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 4mM MgCl2.
Cat # M5132
2000 µL is required per full plate

3.5 Disposables

Axygen PCR Microplates: PCR-96M2-HS-C
Biomek AP96 P20 Tips, Pre-sterile with Barrier (case of 10 racks): 717256
Biomek AP96 P250 Tips, Pre-sterile with Barrier (Case of 10 racks): 717253
Biomek quarter reservoirs: 372790
Biomek reservoir holder
Foil PCR 96-well plate covers
Generic flatbottom 96 well plates (serve as holder for Axygen plates)

4. SETTING UP THE BIOMEK 3000

The first step in using the Biomek 3000 is to turn on the power, the toggle switch is located on the back left of the unit near on the power unit which runs to the wall. On the accompanying computer, open “Biomek Software”.

Ensure that there is no pipetting head on the Biomek. If there is, manually release it by pushing the black release switch on the moving pod, and place it back in the tool holder. Click Instrument>Home Axis to reset the machine and prepare for the run. Next click open protocol and pick the appropriate protocol from section 3.3 to run.

The arrangement of tools, tips and plates has been manually programmed in and should not be changed. If you click on instrument setup a diagram will be shown. An example image is included in Appendix 1.

The following should be the layout:

1. PM1 should contain the tool holder with the pipette heads in the 3 middle slots arrange from left to right: p200>mp200>p20.
2. PM2 should contain 200ul barrier tips
3. PM3 should contain 20ul barrier tips

4. P3 should contain the quarter reservoir in the further right holder, containing 2000µL master mix/ full plate reaction (1400µL per partial).
5. P5 should contain the primer plate and is the site where master mix and template will be added to each well. The primer plate should be sitting in one of the black 96 well plates acting as a holder.
6. P7 should contain the plate of extracted DNA conforming to the standards outlined in section 3.2. If the template is in a semi-skirted Axygen plate, this should also be positioned in one of the black 96 well plate holders.

5. EXECUTING PROTOCOL

Once you are confident in the setup, you are ready to execute the protocol. Click Run protocol, and enter to verify deck layout. The rest should run automatically. In the event of an error about verifying position of tips mid run, hit retry to continue, this will not affect your run. The final reaction contains: 20 µL primer mix (10µL left, 10 µL right at 3.2 pmol/ul), 20 µL GoTaq Hot Start Mastermix, 2 µL template DNA.

5. MACHINE SHUTDOWN

After running the protocol, the probe should have returned to its home position and all pipetting heads in their starting positions. At this point the machine can be powered down. Pool the remaining master mix and refreeze being clear to mark the date of original use. Using a foil PCR plate cover, firmly seal the plate by pushing the cover down over each well, and sealing the corners with the end of a Sharpie to trace the wells. Before PCR, briefly centrifuge to bring all components to the bottom of the well.

6. PCR

A PCR protocol called V4V5Miseq has been programmed in to the Eppendorf thermocycler in F3-127. The cycle parameters are listed in Appendix 3. **To avoid problems with evaporation, start the cycle before loading the plate and let the heated lid reach >100˚C before progressing**. Put the plate in the machine, spin the knob to lower the heated lid as far down as it will go and press enter to progress to the program. At completion, freeze the plate and transfer on ice to David Carter at Robart's for normalization, cleanup and subsequent adapter elongation. **Tell David you will need a 600 cycle kit but that you want it to be run at 2x220.**

In the event of samples that are highly likely to have not amplified well (ex. urine, tissue) it may be warranted to spot check some samples. In this case run on a 1.5% agarose gel in TBE (Tris Borate EDTA) buffer to verify no amplicon in the NTC or extraction blank. The product of successful amplifications should be ~380 bp. An example gel is included in Appendix 4. It is imperative that once the amplification has taken place that the products never be brought back into extraction/Biomek room.

Appendix 1: PRIMERS AND LAYOUT

These primers amplify from 515-806bp
see: http://www.earthmicrobiome.org/emp-standard-protocols/16s/

V4L1	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNccaaggttGTGCCAGCMGCCGCGGTAA
V4L2	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNaaggttccGTGCCAGCMGCCGCGGTAA
V4L3	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNggttccaaGTGCCAGCMGCCGCGGTAA
V4L4	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNttccaaggGTGCCAGCMGCCGCGGTAA
V4L5	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNccttggaaGTGCCAGCMGCCGCGGTAA
V4L6	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNttggaaccGTGCCAGCMGCCGCGGTAA
V4L7	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNggaaccttGTGCCAGCMGCCGCGGTAA
V4L8	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNaaccttggGTGCCAGCMGCCGCGGTAA
V4L9	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNtccgttcgGTGCCAGCMGCCGCGGTAA
V4L10	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNgaatccatGTGCCAGCMGCCGCGGTAA
V4L11	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNcttaggtcGTGCCAGCMGCCGCGGTAA
V4L12	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNaggcaagaGTGCCAGCMGCCGCGGTAA
V4L13	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNtggcttcgGTGCCAGCMGCCGCGGTAA
V4L14	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNcaatggatGTGCCAGCMGCCGCGGTAA
V4L15	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNgttacctgGTGCCAGCMGCCGCGGTAA
V4L16	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNaccgaacaGTGCCAGCMGCCGCGGTAA
V5R1	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNccaaggttGGACTACHVGGGTWTCTAAT
V5R2	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNaaggttccGGACTACHVGGGTWTCTAAT
V5R3	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNggttccaaGGACTACHVGGGTWTCTAAT
V5R4	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNttccaaggGGACTACHVGGGTWTCTAAT
V5R5	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNccttggaaGGACTACHVGGGTWTCTAAT
V5R6	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNttggaaccGGACTACHVGGGTWTCTAAT
V5R7	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNggaaccttGGACTACHVGGGTWTCTAAT
V5R8	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNaaccttggGGACTACHVGGGTWTCTAAT
V5R9	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNtccgttcgGGACTACHVGGGTWTCTAAT
V5R10	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNgaatccatGGACTACHVGGGTWTCTAAT
V5R11	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNcttaggtcGGACTACHVGGGTWTCTAAT
V5R12	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNaggcaagaGGACTACHVGGGTWTCTAAT
V5R13	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNtggcttcgGGACTACHVGGGTWTCTAAT
V5R14	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNcaatggatGGACTACHVGGGTWTCTAAT
V5R15	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNgttacctgGGACTACHVGGGTWTCTAAT
V5R16	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTNNNNaccgaacaGGACTACHVGGGTWTCTAAT

MiSeq V4 V5 Primer Plate 1 Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	V4L1_V5R1_A1	V4L1_V5R2_A2	V4L1_V5R3_A3	V4L1_V5R4_A4	V4L1_V5R5_A5	V4L1_V5R6_A6	V4L1_V5R7_A7	V4L1_V5R8_A8	V4L1_V5R9_A9	V4L1_V5R10_A10	V4L1_V5R11_A11	V4L1_V5R12_A12
B	V4L1_V5R13_B1	V4L1_V5R14_B2	V4L1_V5R15_B3	V4L1_V5R16_B4	V4L2_V5R1_B5	V4L2_V5R2_B6	V4L2_V5R3_B7	V4L2_V5R4_B8	V4L2_V5R5_B9	V4L2_V5R6_B10	V4L2_V5R7_B11	V4L2_V5R8_B12
C	V4L2_V5R9_C1	V4L2_V5R10_C2	V4L2_V5R11_C3	V4L2_V5R12_C4	V4L2_V5R13_C5	V4L2_V5R14_C6	V4L2_V5R15_C7	V4L2_V5R16_C8	V4L3_V5R1_C9	V4L3_V5R2_C10	V4L3_V5R3_C11	V4L3_V5R4_C12
D	V4L3_V5R5_D1	V4L3_V5R6_D2	V4L3_V5R7_D3	V4L3_V5R8_D4	V4L3_V5R9_D5	V4L3_V5R10_D6	V4L3_V5R11_D7	V4L3_V5R12_D8	V4L3_V5R13_D9	V4L3_V5R14_D10	V4L3_V5R15_D11	V4L3_V5R16_D12
E	V4L4_V5R1_E1	V4L4_V5R2_E2	V4L4_V5R3_E3	V4L4_V5R4_E4	V4L4_V5R5_E5	V4L4_V5R6_E6	V4L4_V5R7_E7	V4L4_V5R8_E8	V4L4_V5R9_E9	V4L4_V5R10_E10	V4L4_V5R11_E11	V4L4_V5R12_E12
F	V4L4_V5R13_F1	V4L4_V5R14_F2	V4L4_V5R15_F3	V4L4_V5R16_F4	V413_V5R1_F5	V413_V5R2_F6	V413_V5R3_F7	V413_V5R4_F8	V413_V5R5_F9	V413_V5R6_F10	V413_V5R7_F11	V413_V5R8_F12
G	V414_V5R1_G1	V414_V5R2_G2	V414_V5R3_G3	V414_V5R4_G4	V414_V5R5_G5	V414_V5R6_G6	V414_V5R7_G7	V414_V5R8_G8	V415_V5R1_G9	V415_V5R2_G10	V415_V5R3_G11	V415_V5R4_G12
H	V415_V5R5_H1	V415_V5R6_H2	V415_V5R7_H3	V415_V5R8_H4	V416_V5R1_H5	V416_V5R2_H6	V416_V5R3_H7	V416_V5R4_H8	V416_V5R5_H9	V416_V5R6_H10	V416_V5R7_H11	V416_V5R8_H12

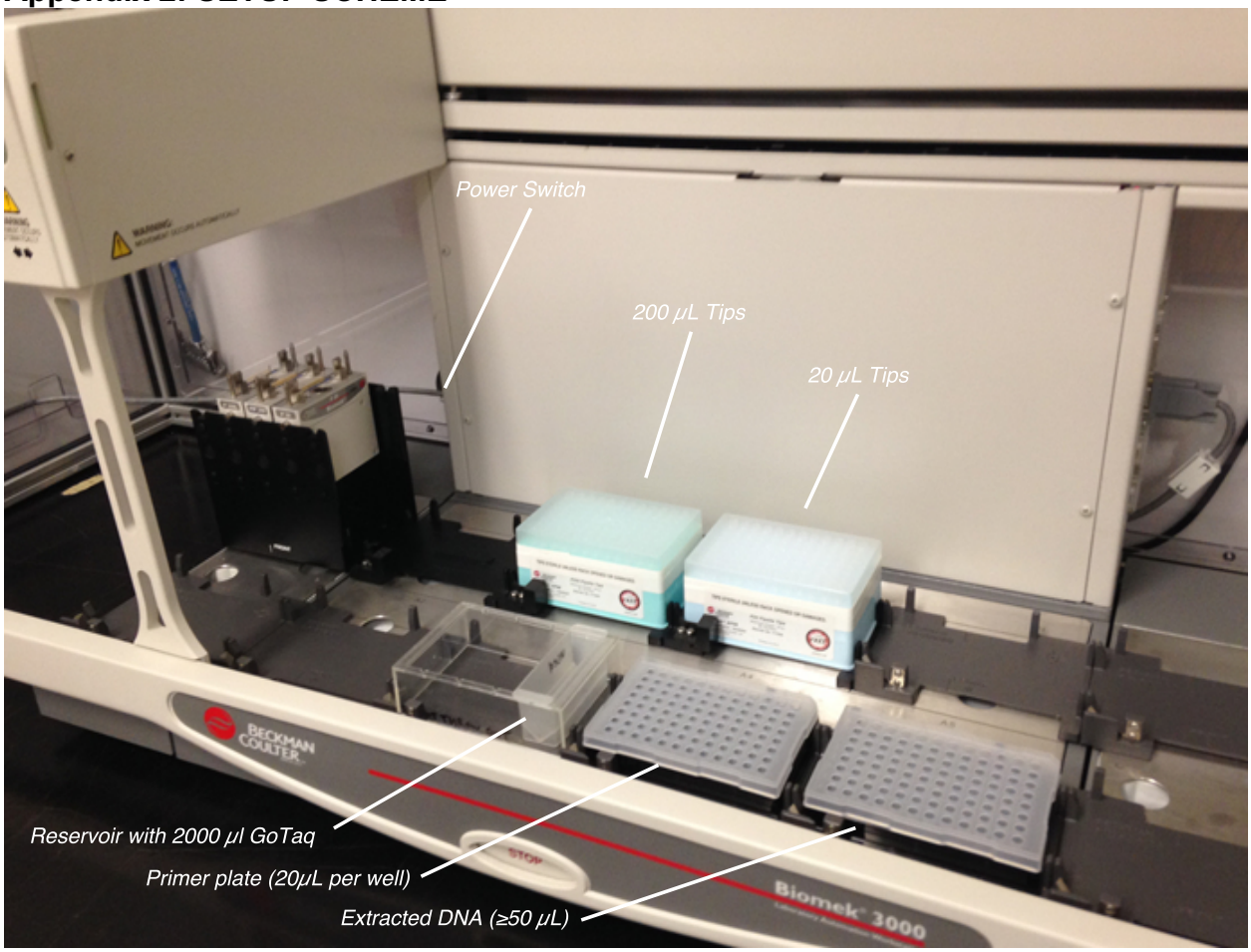
MiSeq V4 V5 Primer Plate 2 Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	V4L5_V5R1_A1	V4L5_V5R2_A2	V4L5_V5R3_A3	V4L5_V5R4_A4	V4L5_V5R5_A5	V4L5_V5R6_A6	V4L5_V5R7_A7	V4L5_V5R8_A8	V4L5_V5R9_A9	V4L5_V5R10_A10	V4L5_V5R11_A11	V4L5_V5R12_A12
B	V4L5_V5R13_B1	V4L5_V5R14_B2	V4L5_V5R15_B3	V4L5_V5R16_B4	V4L6_V5R1_B5	V4L6_V5R2_B6	V4L6_V5R3_B7	V4L6_V5R4_B8	V4L6_V5R5_B9	V4L6_V5R6_B10	V4L6_V5R7_B11	V4L6_V5R8_B12
C	V4L6_V5R9_C1	V4L6_V5R10_C2	V4L6_V5R11_C3	V4L6_V5R12_C4	V4L6_V5R13_C5	V4L6_V5R14_C6	V4L6_V5R15_C7	V4L6_V5R16_C8	V4L7_V5R1_C9	V4L7_V5R2_C10	V4L7_V5R3_C11	V4L7_V5R4_C12
D	V4L7_V5R5_D1	V4L7_V5R6_D2	V4L7_V5R7_D3	V4L7_V5R8_D4	V4L7_V5R9_D5	V4L7_V5R10_D6	V4L7_V5R11_D7	V4L7_V5R12_D8	V4L7_V5R13_D9	V4L7_V5R14_D10	V4L7_V5R15_D11	V4L7_V5R16_D12
E	V4L8_V5R1_E1	V4L8_V5R2_E2	V4L8_V5R3_E3	V4L8_V5R4_E4	V4L8_V5R5_E5	V4L8_V5R6_E6	V4L8_V5R7_E7	V4L8_V5R8_E8	V4L8_V5R9_E9	V4L8_V5R10_E10	V4L8_V5R11_E11	V4L8_V5R12_E12
F	V4L8_V5R13_F1	V4L8_V5R14_F2	V4L8_V5R15_F3	V4L8_V5R16_F4	V413_V5R9_F5	V413_V5R10_F6	V413_V5R11_F7	V413_V5R12_F8	V413_V5R13_F9	V413_V5R14_F10	V413_V5R15_F11	V413_V5R16_F12
G	V414_V5R9_G1	V414_V5R10_G2	V414_V5R11_G3	V414_V5R12_G4	V414_V5R13_G5	V414_V5R14_G6	V414_V5R15_G7	V414_V5R16_G8	V415_V5R9_G9	V415_V5R10_G10	V415_V5R11_G11	V415_V5R12_G12
H	V415_V5R13_H1	V415_V5R14_H2	V415_V5R15_H3	V415_V5R16_H4	V416_V5R9_H5	V416_V5R10_H6	V416_V5R11_H7	V416_V5R12_H8	V416_V5R13_H9	V416_V5R14_H10	V416_V5R15_H11	V416_V5R16_H12

MiSeq V4 V5 Primer Plate 3 Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	V4L9_V5R1_A1	V4L9_V5R2_A2	V4L9_V5R3_A3	V4L9_V5R4_A4	V4L9_V5R5_A5	V4L9_V5R6_A6	V4L9_V5R7_A7	V4L9_V5R8_A8	V4L9_V5R9_A9	V4L9_V5R10_A10	V4L9_V5R11_A11	V4L9_V5R12_A12
B	V4L9_V5R13_B1	V4L9_V5R14_B2	V4L9_V5R15_B3	V4L9_V5R16_B4	V4L10_V5R1_B5	V4L10_V5R2_B6	V4L10_V5R3_B7	V4L10_V5R4_B8	V4L10_V5R5_B9	V4L10_V5R6_B10	V4L10_V5R7_B11	V4L10_V5R8_B12
C	V4L10_V5R9_C1	V4L10_V5R10_C2	V4L10_V5R11_C3	V4L10_V5R12_C4	V4L10_V5R13_C5	V4L10_V5R14_C6	V4L10_V5R15_C7	V4L10_V5R16_C8	V4L11_V5R1_C9	V4L11_V5R2_C10	V4L11_V5R3_C11	V4L11_V5R4_C12
D	V4L11_V5R5_D1	V4L11_V5R6_D2	V4L11_V5R7_D3	V4L11_V5R8_D4	V4L11_V5R9_D5	V4L11_V5R10_D6	V4L11_V5R11_D7	V4L11_V5R12_D8	V4L11_V5R13_D9	V4L11_V5R14_D10	V4L11_V5R15_D11	V4L11_V5R16_D12
E	V4L12_V5R1_E1	V4L12_V5R2_E2	V4L12_V5R3_E3	V4L12_V5R4_E4	V4L12_V5R5_E5	V4L12_V5R6_E6	V4L12_V5R7_E7	V4L12_V5R8_E8	V4L12_V5R9_E9	V4L12_V5R10_E10	V4L12_V5R11_E11	V4L12_V5R12_E12
F	V4L12_V5R13_F1	V4L12_V5R14_F2	V4L12_V5R15_F3	V4L12_V5R16_F4								
G												
H												

Appendix 2: SETUP SCHEME



Appendix 3: PCR CYCLE PARAMETERS

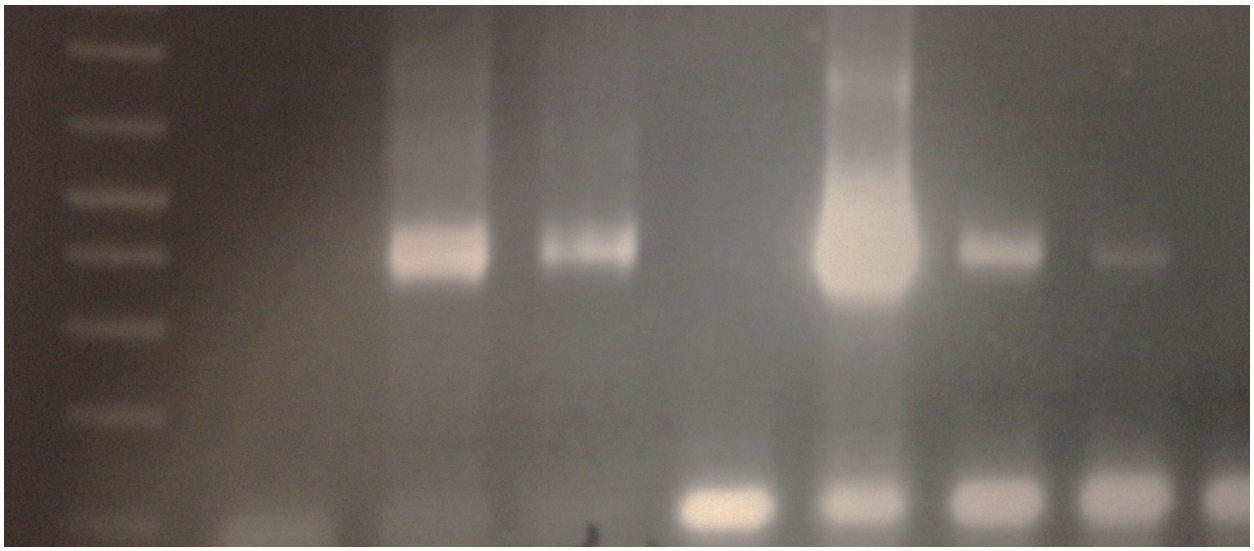
Lid: 105˚C
HOLD until press enter @ 95˚C (This makes sure the lid has heated to prevent evaporation)
2 min @ 95˚C (to activate the GoTaq which is meant for hot start applications)
25 cycles of:

- 1 min @ 95˚C
- 1 min @ 50˚C (recommended annealing based on EMP protocols http://www.earthmicrobiome.org/emp-standard-protocols/16s/)
- 1 min @ 72˚C

NO FINAL ELONGATION

**cycles should not be raised much higher than 25 cycles to reduce likely hood of PCR errors or amplifying things that weren't really there. Could be raised to 30 cycles but be cautious.*

Appendix 4: GEL ELECTROPHORESIS CHECKING FOR SUCCESSFUL AMPLIFICATION



PCR products run on a 1.5% agarose gel in TAE at 120 V for 1h. Lanes (1) 100bp ladder (2) NTC (3) 4 µL Fecal extraction PCR (4) 4 µL Fecal extraction PCR (5-9) 20 µL Urine extraction PCR products. Note the product should be a hair less than 400bp. Note that no successful visible product was obtained in lanes 5 and 9.