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We use this protocol and it's working

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16S rRNA gene Library Preparation Protocol

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ABSTRACT

16S rRNA gene library preparation protocol including the following steps: MPure extraction of Bacterial DNA, Manual 16S indexed primer PCR, library clean up with beads, quibit DNA quantification, equimolar mix construction, agarose gel DNA concentration and gel clean up to produce a library ready for Illumina sequencing.

GUIDELINES

Contamination of microbiome samples is a large scale problem, and all steps of this process should be optimised to avoid it. Undertake activity in a freshly cleaned MSC or PCR hood as far as possible. Keep samples closed, use new tips and seal plates well.

MATERIALS

1. DNA Extraction using the MPBio MPure-12

1.1. Equipment

I)Vortex, pipettes, Class II safety cabinet, microcentrifuge.
II)Filter pipette tips, calibrated pipettes, and gloves.
III)MPBio Fast Prep 24
IV)MPBio Mpure 12

1.2. Reagents

V)DNA Extraction Kit = ‘MPure Bacterial DNA Extraction Kit’by MP-Biomedicals (SKU 117022600)
VI)Lysis Tubes: ‘Lysing Matrix E’ (SKU 116914100)

1.3. Material

I)1000ul pipette tips
II)2ml storage tubes

2. 16S rDNA PCR and Clean Up

2.1. Equipment

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I)MSC and/ or PCR hood
II)Vortex, pipettes, Class II safety cabinet, microcentrifuge.
III)Filter pipette tips, calibrated pipettes, and gloves.
IV)Qubit 4.0 Fluorometer (Q32866),
V)96 well PCR plates (8386HM)
VI)Magnetic plate (12027)
VII)Power pack, weighing scale, gel tank (small volume gel tank preferable), UV transilluminator and heat block.
VIII)Thermometer

2.2. Reagents

IX)Q5 High-Fidelity Polymerase Kit from New England Biolabs (M0491S/L)
X)Wizard SV Gel and PCR Clean Up Kit from Promega (A9281)
XI) dsDNA HS Assay Kit (Q32854)
XII)Agencourt AMPure XP beads (A63881)
XIII)10mM Tris ph8 buffer (T1173)
XIV)80% ethanol (make fresh)
XV)Nuclease free water (AM9935)
XVI)DNA Zap (AM9890)
XVII)Agarose
XVIII)1x TBE
XIX)DNA loading dye
XX)100bp DNA ladder (BIO-33056)
XXI)Sybergreen or similar
XXII)See appendices for primer table

2.3. Material

I)10/20/200/1000ul pipette tips
II)2ml tubes (30120094)
III)1.5ml tubes (30123328)
IV)96 well PCR plates (8386HM)
V)Falcon tubes (Corning™ 352070)
VI)QubitTubes (Q32856) by Life Technologies Ltd.
VII)Conical flask 250ml (glass), Measuring cylinder (100ml),
VIII)Gel tray, tank, and comb,
IX)Sterile scalpel
X)Plate Seals

	A	B
	Full length illumina tagged primers	
	Name	Sequence
	V1FW_SD501	AATGATACGGCGACCACCGAGATCTACACAAGCAGCA acactctttccctacacgacgctcttccgatctNNNNAGMGTTYGA TYMTGGCTCAG
	V1FW_SD502	AATGATACGGCGACCACCGAGATCTACACACGCGTGA acactctttccctacacgacgctcttccgatctNNNNAGMGTTYGA TYMTGGCTCAG
	V1FW_SD503	AATGATACGGCGACCACCGAGATCTACACCGATCTACa cactctttccctacacgacgctcttccgatctNNNNAGMGTTYGAT YMTGGCTCAG
	V1FW_SD504	AATGATACGGCGACCACCGAGATCTACACTGCGTCAC acactctttccctacacgacgctcttccgatctNNNNAGMGTTYGA TYMTGGCTCAG
	V1FW_SD505	AATGATACGGCGACCACCGAGATCTACACGTCTAGTGa cactctttccctacacgacgctcttccgatctNNNNAGMGTTYGAT YMTGGCTCAG
	V1FW_SD506	AATGATACGGCGACCACCGAGATCTACACCTAGTATGa cactctttccctacacgacgctcttccgatctNNNNAGMGTTYGAT YMTGGCTCAG
	V1FW_SD507	AATGATACGGCGACCACCGAGATCTACACGATAGCGT acactctttccctacacgacgctcttccgatctNNNNAGMGTTYGA TYMTGGCTCAG
	V1FW_SD508	AATGATACGGCGACCACCGAGATCTACACTCTACACTa cactctttccctacacgacgctcttccgatctNNNNAGMGTTYGAT YMTGGCTCAG
	V2RV_SD701	CAAGCAGAAGACGGCATACGAGATACCTAGTAgtgactg gagttcagacgtgtgctcttccgatctNNNNGCTGCCTCCCGTA GGAGT
	V2RV_SD702	CAAGCAGAAGACGGCATACGAGATACGTACGTgtgactg gagttcagacgtgtgctcttccgatctNNNNGCTGCCTCCCGTA GGAGT
	V2RV_SD703	CAAGCAGAAGACGGCATACGAGATATATCGCGgtgactg gagttcagacgtgtgctcttccgatctNNNNGCTGCCTCCCGTA GGAGT
	V2RV_SD704	CAAGCAGAAGACGGCATACGAGATCACGATAGgtgactg gagttcagacgtgtgctcttccgatctNNNNGCTGCCTCCCGTA GGAGT
	V2RV_SD705	CAAGCAGAAGACGGCATACGAGATCGTATCGCgtgactg gagttcagacgtgtgctcttccgatctNNNNGCTGCCTCCCGTA GGAGT
	V2RV_SD706	CAAGCAGAAGACGGCATACGAGATCTGCGACTgtgactg gagttcagacgtgtgctcttccgatctNNNNGCTGCCTCCCGTA GGAGT
	V2RV_SD707	CAAGCAGAAGACGGCATACGAGATGCTGTAACgtgactg gagttcagacgtgtgctcttccgatctNNNNGCTGCCTCCCGTA GGAGT
	V2RV_SD708	CAAGCAGAAGACGGCATACGAGATGGACGTTAgtgactg gagttcagacgtgtgctcttccgatctNNNNGCTGCCTCCCGTA GGAGT
	V2RV_SD709	CAAGCAGAAGACGGCATACGAGATGGTCGTAGgtgactg gagttcagacgtgtgctcttccgatctNNNNGCTGCCTCCCGTA GGAGT

	A	B
	V2RV_SD710	CAAGCAGAAGACGGCATACGAGATTAAGTCTCgtgactggagttcagacgtgtgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT
	V2RV_SD711	CAAGCAGAAGACGGCATACGAGATTACACAGTgtgactggagttcagacgtgtgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT
	V2RV_SD712	CAAGCAGAAGACGGCATACGAGATTTGACGCAgtgactggagttcagacgtgtgctcttccgatctNNNNGCTGCCTCCCGTAGGAGT

SAFETY WARNINGS

UV - risk assess all work with UV and use appropriate PPE.

Sharps - risk assess all work with sharps, and exclude where possible.

Microbiological Samples - risk assess all work with microbiological samples.

Reagents - read all relevant warnings and paperwork before use.

BEFORE START INSTRUCTIONS

Barcoded primers were reconstituted as instructed by the manufacturer to a stock concentration of 100mM.

DNA Extraction using the MPBio MPure-12:

1 UV the MPure machine before use. 30m

2 Clean MSC with DNAZap. 5m

3 Remove samples to be extracted from the freezer, to thaw in the fridge (4 °C). 10s

Once thawed vortex for 00:00:10 .
Proceed in MSC.

4 A negative control (e.g. nuclease free water), should be done for each kit (approx. every 50 samples) to show the presence of contaminants (particularly important in low biomass samples) and records should be kept to link this to the samples extracted, machine used, and kit information.

5 Pipette mix and remove up to 800 µL from the storage tube and transfer to a labelled 1.5 ml Eppendorf tube 10m

6 Centrifuge tube for 00:10:00 at 13200rpm. 10m

7 Remove and discard all bar 100 µL of the supernatant. Resuspend the bacterial pellet in the remaining liquid. 15m

8 Add 600 µL of BLB2 buffer (MPure Bacterial DNA Extraction Kit', MP Biomedicals)and vortex to further resuspend. 10m

9 Transfer the volume to a labelled lysis tube (Lysing Matrix E, MP Biomedicals). Label the side of the tubes. 10m

10 Place lysis tubes in the 'FastPrep Instrument' and run for 00:00:40 at speed 6000. 40s

11 Centrifuge tube for 00:10:00 at 13500 rpm. 10m

12 Set Up the MPure 12 Reagents and plasticware: 5m

Place the reagent cartridge in the large flat block at the back, it should click into place, and the writing and release flap should be facing toward you.

The clear plastic mixing wells are clipped in front of these.

Then place the black plastic container set into the next slots, into the first of these place a plastic pin, in the third place a tip.

Put an open labelled elution tube in the closest holes in the metal rack (labelled E).

13 Remove the supernatant (450 µL or as close as possible) from each lysis tube to a labelled blue sample tube from MPure bacterial DNA extraction kit. 10m

Place these in the metal rack, in the slots labelled “S” on the machine.

14 Machine instructions: 2m
Select “START”, it will scan the setup from the sheet in the kit.
Select the protocol : Mpure Bacterial DNA extraction kit with barcode: “OP02006”·
Select sample volume 400ul with barcode ”SV0400”·
Select elution volume 50ul with barcode “EV0050”·
Press Enter to start the extraction

The run time is around 1 hour. The machine will stall if an error develops, but will not stall if a tip is lost, or plasticware comes unclipped.

15 Remove elution tubes, checking a reasonable volume is present, and store these at -80 °C in a labelled box. 5m

16S rDNA PCR and Clean Up -Preparation:

16 Clean MSC or PCR hood with DNAZap.

17 Dilute primers to working concentration ([M] 10 millimolar (mM) the stock is kept at 100mM) in NFW, if there are no pre-existing dilutions < 2 weeks old. 20m

18 Aliquot diluted primers into labelled, linked PCR tubes. Freeze upright overnight if necessary. 30m

19 Rack out the samples for the library into 12 x 8 rack according to use, complete plate plan, put on lid, label and store in -80°C. 30m
Remember to include:
- PCR negative control(s) from the relevant DNA extractions
- positive control (mock community or bacterrial DNA)
- negative PCR control (nuclease free water).
- any diluted, or pre-spiked samples.

16S rDNA PCR :

20 Wipe down MSC with Distel/DNAZap, and then 80% ethanol. 10m
Wipe down all equipment with distel and put in the MSC.
Close MSC, and UV.

21 Defrost the reagents on ice. 20m

22 Add 14.25 µL per well nuclease free water to labelled master mix tubes. 5m

23 Add 5 µL per well Q5 buffer (Q5 High-Fidelity Polymerase Kit from New England Biolabs) to the master mix tubes. Mix before use, vortexing if salts have fallen from solution. 5m

24 Add [M] 10 millimolar (mM) dNTP (0.5 µL per well) to the master mix tubes. Mix before use. 5m

25 Pipette mix the master mix, and keep on ice. 2m

26 Record reagent lot numbers on the library plate plan sheet. Try to use the same PCR reagents for all samples, if possible. 1m

27 Add Q5 enzyme (5 µL per well) to individual master mix tubes immediately before use, keep on ice. Pipette mix. 1m

28 Prepare a 96 well plate for use, labelling and adding orientation points as necessary. Use the plate ice block from this point on. 1m

29 Pipette 20 µl of Master mix into each well of the 96 well plate. There is no need to change tips, but the buffer may bubble. 10m

30 1.25 µl of each primer (501-508) can be transferred using a multichannel pipette in the vertical from the set of linked PCR tubes, swap tips if the plate or tubes are touched. Check each tip has taken up the correct volume. 10m

31 1.25 µl of each primer (701-712) can be transferred using a multichannel pipette on the horizontal from a set of linked PCR tubes, tips should be changed after each use. Check each tip has taken up the correct volume. 30m

32 Apply a plate seal and move the plate, and it's chiller unit into a second MSC if available. 1m

33 Retrieve the samples from the fridge, spray the closed rack into the MSC. 1m

34 Carefully remove the plate seal. Change gloves. 2m

35 Add 2.5 µl of each sample DNA template into each well, according to your plate plan. Change tips every time and discard if they have touched anything before use. 45m

36 Return samples to the fridge. 1m

37 Apply a plate seal and use a roller and squeegee to ensure that the wells seal properly. If you find large amounts of loss from outer wells apply autoclave tape around the edges. 1m

38 Run this plate on a PCR machine with a heated lid. 1h 30m

Initially at 98 °C for 2minutes.
32 cycles of 98 °C for 30 seconds, 50 °C for 30 seconds, 72 °C for 1 minute and 30
seconds.
Finally 72 °C for 5mins and hold at 4 °C.

39 Wipe Down MSC and pipettes with DNAZap. 5m

40 Repeat steps 28-40 until the library has been prepared and run in triplicate.

41 These plates can be frozen at -20 °C before clean up.

Magnetic Bead Clean Up:

42 Bring beads to Room temperature, prepare 80% ethanol and TRIS-HCl before beginning.

43 Defrost plates on ice and spin down before opening. Change gloves. 10m

44 Combine the triplicate PCR products into 1 plate using a multichannel pipette. 10m

45 Vortex the beads stock (Agencourt AMPure XP beads) for 30 seconds to ensure even dispersal. Pour into a trough. 1m

46 Add an equal volume of beads per volume of PCR product (probably 75 µL) to each well, gently pipetting up and down x10, changing tips for every column. 10m

47 Incubate at Room temperature for 00:05:00 5m

48 Move the plate to the magnetic stand for 00:02:00 until the supernatant has cleared. 2m

49 Leaving the plate on the magnetic stand use a multichannel pipette to remove and discard the supernatant, changing tips every time. 5m

If you disrupt the beads leave the well until the end to allow them to resettle. The beads should remain attached to the side of the well.

50 Leaving the plate on the magnetic stand wash the beads with 200 µL fresh 80% ethanol, using a multichannel pipette. 5m

51 Incubate on the magnetic plate for 00:00:30. Carefully remove and discard the supernatant into a waste container. 30s

52 Leaving the plate on the magnetic stand wash the beads with fresh 80% ethanol. Using a multichannel pipette add 200 µL of fresh 80% ethanol to each well. 5m

53 Incubate on the magnetic plate for 00:00:30 .Carefully remove and discard the supernatant into a waste container. Use a P20 with fine tips to remove any residual ethanol. 30s

54 Leave the PCR plate on the magnetic plate for 00:10:00 to air dry. 10m

55 Remove the plate from the magnetic stand. Using a multichannel pipette add 27.5 µL of TRIS-HCl pH8 into each well of the plate. 5m

56 Pipette up and down x10 to re-suspend the beads, Some wells may need more flushing than 15m

others, ensure that the beads are fully resuspended.

57 00:02:00 Incubate the plate at room temperature for 00:02:00 4m

58 Put the PCR plate on the magnetic stand for 00:02:00 (or until the supernatant clears). 2m

59 Using a multichannel pipette move 25 µL from each well into a new clean labelled PCR plate. Change tips between samples. Do not disturb the bead pellet . 15m

60 Seal plate thoroughly. If freezing at -20 °C use aluminium foil. Keep at 4 °C overnight. 2m

Quantify PCR products and make Equimolar Mix:

61 Label Qubit tubes with the well names (Sample Tubes). 5m

62 Label two Qubit tubes 'S1' and 'S2' and place 10 µL of Qubit HS Standard 1 into the first and 10µl of Standard 2 into the second RTP.

63 Briefly vortex and spin down the samples. 10m

64 Wrap tape and foil around a 50ml Falcon Tube to ensure no light enters. 1m

65 Into the Falcon Tube, add Qubit HS Buffer (199 µL x (sample no.+2)) and Qubit HS Reagent (1 µL x (sample no.+2)), close and vortex well. This working solution is light reactive; ensure that the duration of light exposure is minimal. 3m

66 Add 190 µL of Working Solution to each of the Standard Tubes, vortex for 00:00:05 and leave for 00:02:00. Ensure there are no bubbles and all liquid is at the bottom of the tube. 2m 5s

67 Select dsDNA HS on the Qubit machine & calibrate using the 2 Standard Tubes. Note down the values for the Standards. 1m

68 Vortex the Working Solution Tube and add 198 µL into a Sample Tube, then add 2 µL of the correct sample, vortex for 00:00:05 and leave for 00:02:00. Ensure there are no bubbles in the Sample Tube and all liquid is at the bottom of the tube. 2m 5s

69 Place Sample Tube into the Qubit machine and record the DNA concentration. 1m

70 Repeat steps 68-69 for the remaining samples. Dilute any samples where the concentration is too high and repeat to get a value.

71 Find the highest and lowest concentration of pooled PCR product & use this to decide the amount of DNA from each sample to be added to equimolar mix (ensure volume of lowest is possible (eg. 20 µL) and volume of largest is possible 1 µL). Remember to scale up any diluted samples.

e.g. Highest = 55.0µg/ml Lowest = 6.2µg/ml
55µg/ml x 3 ul = 165µg 165 / 6.2 = 26.6µl

However, only 20µl any sample, so: 55 x 2 = 110µg 110 / 6.2 = 17.7µl)

72 Once amount of DNA is calculated, use the following formula to work out volume of each sample to be added to equimolar mix: Volume (µl) = DNA amount (µg) / Sample Concentration (µg/ml). If the concentration of a sample is <2ng/µl, add all the sample volume to the equimolar mix to give the best chance of enough reads.

73 Print list of needed volumes in 96 well format.

74 Mix the correct volumes of each pooled PCR product to create the equimolar mix in a labelled, screw cap 2ml eppendorf. Add the largest volumes first. 1h 30m

75 Pipette mix and check that the total volume is correct. 5m

76 Split the equimolar mix volume in half. 2m
Store half of the equimolar mix at -20 °C in a o-ring screwcap tube.

Gel Purification: Removes Primer dimers and concentrates PC... 2h 59m

77 Mix the unfrozen half of the equimolar mix with loading dye as instructed by the dye information leaflet.

78 Make 1% agarose, 1x TBE gel with the nucleic acid gel stain of your choice, load into buffer tank and top up the buffer until it is level with the top of the gel. 20m

79 Load the Equimolar mix into the gel, filling the well(s) as much as possible. 2m

80 Run for 00:10:00 at 90V with the buffer level with the top of the gel. The equimolar mix should have moved into the gel. 10m

81 Add buffer to cover and run for approximately 01:30:00. 1h 30m

82 Use a UV transilluminator to visualize the bands. Cut out the bands with as little excess gel as possible. Wear suitable face and eye covering, and ensure any nearby workers are taking suitable precautions. Dispose of any sharps appropriately. 15m

83 Weigh as many 1.5ml tubes as you have bands, and note their weight. Place the bands in to the 1.5ml tubes. Weigh the tubes to estimate the weight of gel slice. 2m

84 Add 10 µL of Membrane Binding Solution (Wizard SV Gel and PCR Clean Up Kit from Promega) per 10 mg of gel slice and vortex briefly.

Eg. tube + gel slice = 1.48g, tube = 1g, gel slice = 0.48g, add 480µl of Membrane Binding Solution.

85 Place tube into a heat block at 55 °C until gel dissolves completely. You may want to vortex during this incubation to hasten the dissolving. 10m

86 There will only be 1 collection tube used per Equimolar mix. Place a Minicolumn into a Collection Tube and pipette up to 700 µL of dissolved gel into the column. Incubate for 1 minute. 2m

87 Centrifuge the SV Minicolumn at 13200 rpm for 00:01:00. 1m

88 Discard the supernatant into a labelled liquid waste container and reinsert the SV Minicolumn into the Collection Tube. 1m

89 Repeat steps 87-89 until no dissolved gel mixture remains (if > than 700 µL of dissolved gel).

90 Add 700 µL of Membrane Wash Solution (add ethanol before use as instructed by kit). 1m

91 Centrifuge at 13200 rpm for 00:01:00. 1m

92 Discard the supernatant and reinsert the SV Minicolumn into the Collection Tube. 1m

93 Add 500 µL of Membrane Wash Solution. 1m

94 Centrifuge at 13200 rpm for 00:05:00 5m

95 Discard the supernatant. Centrifuge the tube and minicolumn at 13200 rpm for 1 minute.

96 Transfer the SV Minicolumn to a 1.5ml tube and add 100 µL of Nuclease-Free Water directly onto the membrane, incubate for 00:01:00 at RTP. 1m

97 Centrifuge at 13200 rpm for 00:01:00. 1m

98 Check the water has gone through the column and that the membrane is dry. If water remains on the matrix, repeat step 97. 30s

99 Discard SV Minicolumn and split sample, for storage in a labelled screwcap tube with O-ring. Keep half as a spare and submit the other half for sequencing. 5m