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Library preparation protocol to sequence V3-V4 region of 16S rRNA to run in Illumina MiSeq platform

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Protocol status: Working
We use this protocol and it's working

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Abstract

This is an optimised protocol for 16S library preparation of V3-V4 region for sequencing through Illumina MiSeq platform (2 x 300 bp V3 chemistry). This protocol uses Platinum™ SuperFi™ PCR Master Mix instead of 2x KAPA HiFi HotStart ReadyMix given in Illumina 16S protocol. This polymerase master mix has lower error rate than KAPA, making it more suitable for sequencing.

Materials

Equipment

- 96-well Microtiter Plate Magnetic Separation Rack
- 96-well V bottom assay sheath
- 1.5 mL Eppendorf tubes
- Eight 0.2ml PCR strip Tube, Natural, Sterile, 120/Bag
- Sealing Film, Sterile, 50 Sheets
- Multichannel pipette P1-10, P200 and their corresponding tips
- Ice & ice bucket
- Agilent Tapestation 4200
- Qubit
- MiSeq Reagent Kit v3
- Nextera XT Index Kit, 96 indices, 384 samples
- Axygen plate
- Gel tank

Reagent

- Buffer EB
- PCR Water (nuclease free)
- Fresh 80% Ethanol
- Agencourt Ampure XP beads
- High Sensitivity D1000 Reagents
- Qubit 1X dsDNA High Sensitivity Assay Kit
- 2X Platinum™ SuperFi™ PCR Master Mix
- PhiX Control v3
- Agarose

Before start

Th forward and reverse primer along with the highlighted overhang sequence used to amplify the V3-V4 region of 16S rRNA is given below.

Forward Primer: **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGAGGCAGCAG

Reverse Primer: **GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACAAGGGTATCTAATCC

Stage 1 PCR amplification

1

The PCR reaction setup is given below

	Reagents	Volume (µl) for 25µl reaction
	2X Platinum™ SuperFi™ PCR Master Mix	12.5 µL
	Water, nuclease-free	Upto 25 µl
	10 µM forward primer	1.25 µL
	10 µM reverse primer	1.25 µL
	Template DNA	4.6 ng

Add reagents and DNA in a sterilised PCR tube in the order given above

2 PCR cycle conditions:

Initial denaturation at 98°C for 30 sec

25 cycles of :

- 98°C for 10 sec
- 55°C for 15 sec
- 72°C for 30 sec

Final extension at 72°C for 10 min

3 Run 5 µL of PCR product in 1% agarose gel for 20 mins at 100V to check the size of the band (~460 bp)

Stage 1 Clean up

4 Bring the AMPure XP beads to room temperature.

5 Prepare two Axygen plate with each well containing 200 µl of 80% ethanol

6 Using a multichannel pipette set to 20 µl, transfer the entire Amplicon PCR product to the Axygen plate. Change tips between samples.

7 Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed.

8 Add 20 µl of AMPure XP beads (1:1 ratio) to each well on the plate

9 Gently pipette entire volume up and down 10 times using pipette

10 Incubate at room temperature without shaking for 5 minutes.

11 Place a sheath on the coppin device (a device shaped like a 96 well plate with magnets attached at each well similar to 96-well Microtiter Plate Magnetic Separation Rack) and insert into the plate wells containing PCR product

12 Allow beads to bind to the coppin device for 2 mins and use this device to transfer the beads to first ethanol plate.

13 Hold in ethanol for 30 seconds

14 Transfer beads to second ethanol half plate

15 Hold in ethanol for 30 seconds

16 Remove beads from ethanol using coppin device and invert it on the bench so that the beads are facing up.

17 Air dry at room temperature for 10 minutes (still on coppin device)

18 Using a multichannel pipette, add 52.5 µl of 10 mM Tris pH 8.5 (Buffer EB) to each well of the Amplicon PCR plate. Cover when not in use.

19 With air dried bead still on the device, insert sheath into the well containing Tris and remove the coppin device whilst leaving the sheath in the solution

20 Resuspend beads by gently swishing the sheath in the Tris. If beads don't come off, use magnetic base to remove the stuck beads and remove the plate immediately.

21 Incubate at room temperature for 2 minutes without shaking

22 Put fresh sheath on Coppin device and insert into Tris for 2 mins

23 Remove and discard the beads and the sheath.

24 Using a multichannel pipette, remove the cleaned up product from the well and place it into PCR tube

25 Run 5 µL of cleaned-up product in 1% agarose gel for 20 mins at 100V to check the size of the band (~500 bp)

Stage 2 PCR amplification

26

Nextera XT Index Kit (N7XX and S5XX) is used for the following set-up to add barcodes
PCR reaction setup is given below

	Reagents	Volume

		(50 µl)
	Platinum™ SuperFi™ PCR Master Mix	25 µl
	Water, nuclease-free	upto 50 µl
	2µM of index (each)	5 µl
	Cleaned up DNA	5 µl

27 Cycle conditions for the above reavtion volume is

Initial denaturation at 98°C for 30 sec
8 cycles of :

- 98°C for 10 sec
- 55°C for 15 sec
- 72°C for 30 sec

Final extension of 10 min at 72°C.

28 Run 5 µL of PCR product in 1% agarose gel for 20 mins at 100V to check the size of the band (~550 bp)

Stage 2 PCR clean up

29 Follow step 4 to step 25 but use 45 µl of AMPure XP in step 8 and 27.5 µl of 10 mM Tris pH 8.5 in step 18.

30 Run 5 µL of PCR product in 1% agarose gel for 20 mins at 100V to check the size of the band. Run it next to stage 1 clean up product to check whether the product size is increased by addition of barcodes

Quantification of stage 2 cleaned up product

31 Quantify the stage 2 PCR product in Qubit high sensitivity assay

32 Run tapestation on selection of samples to check the length

33 Pool equimolar volume of each sample together to get a final concentration of 4 nM (1.17 ng / µl)

34 Qubit the pooled sample using high sensitivity assay to ensure the concentration is 4 nM (1.17 ng /µl)

Preparation of library for loading

35 Thaw MiSeq reagent cartridge in 25°C water bath

36 Thaw the HT1 reagent and pooled library at room temperature and store it in ice box

37 Prepare a fresh dilution of 0.2N NaOH (800 µl of dH20 + 200 µl of 1N NaOH)

38 Combine 5 µl of pooled library with 5 µl of 0.2N NaOH

Note: Work quickly from this step forward

39 Vortex briefly and centrifuge for 1 min at 300 g.

40 Incubate for 5 mins at room temperature (start as soon as centrifuge stops)

41 Immediately add 990 µl of pre-chilled HT1 to denatured DNA. Place in ice until needed. This gives 20 pM of denatured library

42 Prepare 20 pM dilution of PhiX

43 Dilute denatured DNA to a final concentration of 9.5 pM by taking 285 µl of denatured library and adding 315 µl of pre-chilled HT1 (final volume 600 µl)

44 Invert several times to mix and pulse centrifuge. Place it in ice

45 Dilute 20 pM denatured PhiX to same concentration as final library. Can be performed by adding 285 µl of 20 pM denatured PhiX with 315 µl pre-chilled HT1

46 Invert several times to mix and pulse centrifuge. Place it in ice

47 Combine 60 µl of denatured, diluted PhiX with 540 µl of denatured diluted library

48 Incubate the combined library at 96 °C for 2 min

49 Invert several times to mix and place on ice immediately

50 Invert reagent cartridge and buffer bottle to mix

51 Load sample into reagent cartridge

52 Wash flow cell with water, dry and wash with ethanol

53 Load flow cell, reagent cartridge, buffer bottle, waste bottle into MiSeq. Ensure sample sheet is loaded. Start the run