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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 × 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

Troubleshooting

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: **TCGT****CGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGAGGCAGCAG

Reverse Primer: **GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GA



Stage 1 PCR amplification

1

The PCR reaction setup is given below

	Reagents	Volume (μl) for 25μl reaction
	2X Platinum TM SuperFi TM 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

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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 μ l 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 μ L 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

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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 TM SuperFi TM 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 reaction 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 μ l of AMPure XP in step 8 and 27.5 μ l of 10 mM Tris pH 8.5 in step 18.
- 30 Run 5 μ L 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 / μ l)
- 34 Qubit the pooled sample using high sensitivity assay to ensure the concentration is 4 nM (1.17 ng / μ l)

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 μ l of dH₂O + 200 μ l of 1N NaOH)
- 38 Combine 5 μ l of pooled library with 5 μ l 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 μ l 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 μ l of denatured library and adding 315 μ l of pre-chilled HT1 (final volume 600 μ l)
- 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 μ l of 20 pM denatured PhiX with 315 μ l pre-chilled HT1
- 46 Invert several times to mix and pulse centrifuge. Place it in ice
- 47 Combine 60 μ l of denatured, diluted PhiX with 540 μ l 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