High quality single amplicon sequencing method for illumina platforms using 'N' (0-10) spacer primer pool without PhiX spik-in

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Abstract

Illumina sequencing platform requires base diversity in initial 11 cycles for efficient cluster identification and color matrix estimation. This limitation yields low quality data for amplicon libraries having homogeneous base composition. Spike-in of PhiX library ensures base diversity but overall reduces the number of sequencing reads for data analysis. To overcome such low diversity issues during amplicon sequencing on illumina platforms we developed high throughput single amplicon sequencing method by introducing 'N' spacers in target gene amplification primers that are pooled for simple handling. We evaluated the efficiency of 'N' spacer primers by targeting bacterial 16S V3-V4 region, demonstrating heterogonous base library construction. Addition of 'N' spacer causes sequencing frame shift at every base that leads to base diversity and produces heterogenous high quality reads within single amplicon library. We have written a python script "MetReTrim" to trim the heterogenous 'N' spacers from the pre-processed reads. This method terminates the need for PhiX spike-in and allows for multiplexing of multiple samples, greatly reducing the overall cost and yields improved sequence quality.

Introduction

Amplicon sequencing is an important and widely used tool for inferring the presence of taxonomic groups in microbial communities, detecting genetic variation embedded in complex and genetic backgrounds, and is far more cost-effective than untargeted sequencing when large amounts of undesired genetic material is present (Lundberg et al., 2013; Callahan et al., 2019). Illumina HiSeq and MiSeq sequencing platforms are extensively used for performing paired-end sequencing to generate millions of reads for amplified fragments of

the 16S rRNA gene, the internal transcribed spacer (ITS) region and different marker genes (Holm et al., 2019). Illumina's sequencing-by-synthesis technology uses fluorescently labelled reversible terminator-bound dNTPs. Red laser illuminates A and C and green laser illuminates G and T fluorophores. Different filters are employed to image and identify the four different nucleotides. The similar emission spectra of the fluorophores (A and C as well as G and T) and limitations of the filters increases chances of low base call quality and mismatch rate in homogeneous sequence libraries (Schirmer et al., 2015). Therefore, for effective template generation and accurate base-calling on Illumina platforms it is required to have nucleotide diversity (equal proportions of A, C, G, and T nucleotides) at each base position in a sequencing library (Muinck et al., 2017; illumina 2014). Libraries of low sequence diversity like 16s rRNA gene are highly homogenous and commonly spiked with high-diversity library such as PhiX, to alleviate the problem of homogenous signals generated across the entire flow cell. However, it reduces the overall sequence read throughput and multiplexing options because of it being a non-target (PhiX) library (Holm et al., 2019; Muinck et al., 2017). The base diversity in first few cycles, particularly in the first 11 bases of the amplicon, are crucial for identification of the sequencing clusters on the flow cell and color matrix estimation (Jensen et al., 2019; Holm et al., 2019). Even though the research field has progressed in successful sequencing of 16S rRNA with Illumina V3–V4 region primers, the problems of drop in read quality and inherent error rate still remain unresolved (Jensen et al., 2019). Another approach to deal with this issue is by sequencing libraries tagged with heterogeneity spacers at the 5' end of the target gene amplicon during library preparation. The heterogeneity spacers are short sequences linked to index adaptors or to the gene specific amplification primers in the form of 0-7 bases and minimizes the need for PhiX spike-in to 10 % by introducing base complexity at the start of sequencing reads yielding high quality sequencing and increased multiplexing capacity (Muinck et al., 2017; Holm et al., 2019; Herbold et al., 2015; Kozich et al., 2013). However, designing primers or index adaptors consisting varying length of heterogeneity spacer with unique sequences for different types of amplicon libraries is a complex process due to the fact that every base sequenced at given time should contribute to diversity (A ~25%, T ~25%, G ~25%, C ~25%) during the sequencing run and also requires PhiX spike-in. The PhiX spike-in hinders the use of Miseq and Hiseq platforms to great levels. Another drawback is handling more number of heterogeneity primer pairs instead of a single gene specific primer pair. Amplification of target gene with 0-7, 1-6, 2-5 and so on combinations of primer pairs makes the experimental setup tedious and requires minimum 8 samples to be pooled for confirming base complexity.

To resolve the technical limitations of single amplicon sequencing on Illumina platforms and challenges encountered during heterogeneity spacer primer designing, we added 'N' nucleotides to the 5' end of the gene specific primers for amplifying the gene. The 'N' nucleotide bases are added in 0-10 fashion in forward and reverse gene specific primers. Pool of 'N' (0-10) spacers-linked gene specific primers are used for amplification and library synthesis incorporating diversity within single library. In addition, the pool design reduces the number of primer combinations to single set compared to previous studies (Liyou et al., 2015; Jensen et al., 2019). This contributes to increased base diversity at each sequencing cycle in all the libraries that are multiplexed during a sequencing run on Illumina platform. Our 'N' (0-10) spacer primers form libraries with improved base diversity leading to a higher quality data. Since our method is devoid of PhiX spike-ins, this allows for sequencing of more number of samples and a reduction in the overall cost. Further, this strategy of using 'N' (0-10) spacer design can be simply adopted for generating high quality single locus amplicon sequencing in a high throughput manner on any illumina platform.

Methods

1. Reagents

Product	Supplier	Catalog Number
Nuclease-free water	Ambion	AM993
Ethanol	Merck kGaA	64-17-5
AMPure-XP Magnetic Beads	Beckman Coulter	A63881
Ultrapure 1 MTris-HCL pH 8.0	Invitrogen	15568-025
DNA High Sensitivity Qubit Reagents	Invitrogen	Q32854
2x KAPA HiFiHotStart Ready Mix	KapaBiosystems	KK2602
Agilent 2100 DNA 1000 Bioanalyzer Kit	Agilent	5067-1504
Nextera XT Index kit V2	Illumina	15032350
NaOH	Sigma-Aldrich	1091361000
MiSeq V3 reagent cartridge and Flow cell	Illumina	MS-102-3003
pH Test Strips (7.0-14.0)	Sigma	P-4411
16S Amplicon PCR Forward 'N' spacer Primers	Sigma	Sigma
16S Amplicon PCR Forward 'N' spacer Primers	Sigma	Sigma

1.1. *Buffer*

1.2.1 1mM Tris-HCL: In falcon tube add 500 µl of 1 M Tris-HCL pH 8.0 to 49.5 mL Nuclease free water. Prepare fresh and keep at RT.

1.2.2 0.1N NaOH: Add 50ul of 2N NaOH to 950ul of Nuclease free water. Check the pH using pH test strips. The pH should be >12.5.0.1 N NaOH should be freshly prepared for denaturing libraries.

2. Equipment

Product	Supplier	Catalog Number
Multichannel Pipette0.5 – 10 µL	Eppendorf	4922000021
Multichannel Pipette 10 – 100 µL	Eppendorf	4922000048
Multichannel Pipette 30 – 300 µL	Eppendorf	4922000064
Plate centrifuge	Eppendorf	5430R
PCR machine	BIO-RAD	C1000 touch
Thermomixer	ThermoFisher	ThermomixerC96
well plate	ThermoFisher	SP-0446
Sealing mat	Axygen	AM-96-PCR-RD
Magnetic stand 96	Invitrogen	AM10027
TruSeq Index Plate Fixture	Illumina	(FC-130-1005)
Qubitfluorometer	Invitrogen	Q33238
Miseq Platform	Illumina	SY-410-1003

3. Experimental design

3.1. *Primer Design-*

Bacterial 16S V3-V4 region was targeted to study the efficiency of 'N' spacer primer. The primer contains Illumina adapter overhang sequence (blue), 'N' spacer region (red) and Target Gene specific primer (green). (Fig.1, Table 1 and Table 2). The primers were ordered as standard desalted PCR primers.

Illumina adapter overhang Sequence:

Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG

Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

Forward and Reverse primer stocks were diluted to 5uM and equal volumes of each forward and reverse primer were pooled together.

Critical Step: For freshly ordered primers it is recommended to check efficiency of each primer before pooling them. Perform PCR using control template and any combination of forward and reverse primer in total 11 PCR reaction setup.

3.2. *Starting Material*- Genomic DNA 12.5 ng

4. Detailed Protocol

4.1.*First round PCR-Amplifying target gene*

Assemble the following components per reaction

Reagents	Per well
Microbial Genomic DNA (12.5 ng)	2.5µL
Forward Primer Pool (5uM)	1µL
Reverse Primer Pool (5uM)	1µL
2x KAPA HiFi HotStart ReadyMix	12.5µL
Nuclease Free Water	8µL

Use 96 well plate for more samples.

Carry out the following PCR program:

(i) 95°C for 3 min,

(ii) 95°C for 30s, 55°C for 30s, 72°C for 30s for 25 cycles

(iii) 72°C for 5 min, Hold at 10°C

[Optional] Check for PCR amplification by estimating the concentration of few amplicons on Qubit using DNA High Sensitivity Qubit Reagent.

4.2.*Amplicon Cleanup:*

4.2.1. Centrifuge the Amplicon PCR plate at 1,000 × g at 20°C for 1 minute.

4.2.2. Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly distributed

4.2.3. Add 0.8X (20 µl) of AMPure XP beads to each well using multichannel pipette, mix the beads by gently pipetting entire volume 10-15 times. Seal plate and shake at 1800 rpm for 2 minutes.

4.2.4. Incubate the plate for 5 minutes at RT

4.2.5. Keep the plate on Magnetic stand for 5 minutes or until the supernatant is clear.

4.2.6. Without disturbing the plate kept on Magnetic stand, discard the supernatant using Multichannel Pipette.

4.2.7. With the Amplicon PCR plate on the magnetic stand, wash the beads by adding 150ul of freshly prepared 80% ethanol to each sample well. Incubate for 30 seconds. Carefully remove and discard the supernatant.

4.2.8. Repeat the above step for total two washes. Using multichannel pipette carefully remove the leftover ethanol from each well.

4.2.9. With the Amplicon PCR plate on the magnetic stand, air-dry the beads for 2 mins. Do not over dry the beads

4.2.10. Remove the Amplicon PCR plate from magnetic stand, using multichannel pipette resuspend the beads in 25 µl of 10 mMTris pH 8.0 in each well.

4.2.11. Mix the beads by gently pipetting entire volume 10-15 times to ensure proper resuspension of beads. Seal plate and shake at 1800 rpm for 2 minutes.

4.2.12. Incubate for 2 minutes at RT

4.2.13. Place the plate on the magnetic stand for 5 minutes or until the supernatant is clear.

4.2.14. Using a multichannel pipette, carefully transfer 22.5 µl of the supernatant to new 96 well PCR plate.

4.2.15. Quantify the amplicons using Qubit DNA HS reagent kit.

[Optional] Verify the amplicon size on Bioanalyzer using DNA 1000 chip. For V3-V4 region expected size after PCR is ~550-560 bp.

4.3.*Second round PCR- Nextera XT Index Barcoding*

This step adds Index 1 (i7) and Index 2 (i5) sequences to generate uniquely tagged libraries by amplifying the target gene amplicons using illumina Nextera XT Index Kit V2. The forward primer contains the P5 adapter end that binds to the flow cell, unique 8 nt index and the Illumina read 1 primer binding site. The reverse primers

consist of the P7 adapter end that bind to the flow cell,unique 8 nt index and the Illumina read 2 primer binding site. Refer to Illumina's Index Adapters Pooling Guide for selection of compatible primer combinations.

In TruSeq Index Plate Fixture arrange the Nextera XT Index 1 primer (i7) tubes horizontally from 1-12 fashion and Nextera XT Index 2 primer (i5) tubes vertically in 1-8 fashion. Transfer 1ul of purified product to a new 96 well PCR plate and place it on TruSeq Index Plate Fixture.

Set up the following reaction using Multichannel pipette for adding Index primer 1 and primer2:

Reagents	Per well
Amplified Product	1 µl
Nextera XT Index Primer 1	5 µl
Nextera XT Index Primer 2	5 µl
2x KAPA HiFi HotStart ReadyMix	25 µl
Nuclease Free water	14 µl

Gently pipette up and down 10-15 times and seal the plate with septa. Centrifuge the plate at 1,000 × g at 20°C for 1 minute.

Carry out the following PCR program:

(i) 95°C for 3 min,

(ii) 8 cycles of 95°C for 30s, 55°C for 30s, 72°C for 30s

(iii) 72°C for 5 min, Hold at 6°C

4.4.*Index Amplicon Cleanup:*

4.4.1. Centrifuge the Amplicon PCR plate at 1,000 × g at 20°C for 1 minute.

4.4.2. Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly distributed

4.4.3. Add 1X (50 µl) of AMPure XP beads to each well using multichannel pipette, mix the beads by gently pipetting entire volume 10-15 times. Seal plate and shake at 1800 rpm for 2 minutes.

4.4.4. Incubate the plate for 5 minutes at RT

4.4.5. Keep the plate on Magnetic stand for 5 minutes or until the supernatant is clear.

4.4.6. Without disturbing the plate kept on Magnetic stand, discard the supernatant using Multichannel Pipette.

4.4.7. With the Amplicon PCR plate on the magnetic stand, wash the beads by adding 150ul of freshly prepared 80% ethanol to each sample well. Incubate for 30 seconds. Carefully remove and discard the supernatant.

4.4.8. Repeat the above step for total two washes. Using multichannel pipette carefully remove the leftover ethanol from each well.

4.4.9. With the Amplicon PCR plate on the magnetic stand, air-dry the beads for 2 mins. Do not over dry the beads.

4.4.10. Remove the Amplicon PCR plate from magnetic stand, using multichannel pipette resuspend the beads in 37.5 µl of 10 mMTris pH 8.0 in each well.

4.4.11. Mix the beads by gently pipetting entire volume 10-15 times to ensure proper resuspension of beads. Seal plate and shake at 1800 rpm for 2 minutes.

4.4.12. Incubate for 2 minutes at RT

4.4.13. Place the plate on the magnetic stand for 5 minutes or until the supernatant is clear.

4.4.14. Using a multichannel pipette, carefully transfer 35 µl of the supernatant to new 96 well PCR plate.

4.4.15. **Quantify the libraries using Qubit DNA HS reagent kit.**

4.4.16. **Check the final library on a Bioanalyzer DNA 1000 chip to verify the size. Expected size for V3-V4 Region is ~630-640bp.**

4.4.17. **Library Normalization and Pooling**

Calculate DNA concentration in nM, based on the size and concentration of library

$$\frac{(\text{Concentration in ng/µl})}{(660\ \text{g/mol} \times \text{average library size})} \times 10^6 = \text{concentration in nM}$$

Dilute the final library using 10 mMTris pH 8.0 to 4 nM. Aliquot 5 µl from each diluted library and mix aliquots for pooling libraries with unique indices. Depending on coverage needs, 96 or more than 96 libraries can be pooled for one MiSeq run. Check the concentration of pooled library and calculate the nM considering the average size of all libraries. It should be ~4nM

5. Library Denaturing and MiSeq Loading

5.1.1. Keep the Miseq Reagent for thawing at RT

5.1.2. Freshly prepare 0.2N NaOH

5.1.3. Keep the thawed HT1 buffer in Ice

5.1.4. Denature the Library

5.2. Combine the following in a 1.5 ml tube:

4 nM pooled library (5 µl)

0.2 N NaOH (5 µl)

5.3. Mix with pipette and vortex briefly

5.4. Centrifuge the sample solution at 280 × g at 20°C for 1 minute

5.5. Incubate for 5 minutes at room temperature to denature the DNA into single strands.

5.6. Add 990 µlPrechilled HT1 buffer. Adding HT1 buffer results in a 20 pM denatured library in 1 mMNaOH.

5.7. Dilute the denatured DNA to the desired concentration using the following example:

Final Library Concentration	6 pM	7 pM	8 pM	9 pM	10 pM
20 pM denatured Library	180 µl	210 µl	240 µl	270 µl	300 µl
Pre-chilled HT1 buffer	420 µl	390 µl	360 µl	330 µl	300 µl

5.8. Invert several times to mix and then pulse centrifuge the DNA solution.

5.9. Place the denatured and diluted DNA on ice.

5.10. Library sequencing- Load the Denatured Library in Miseq V3 Reagent Cartridge and start the sequencing run for 2 X 300 paired end read.

6. Heterogeneity 'N' spacer Trimming:

In-house python script "MetReTrim" was written to trim the heterogeneity 'N' spacers from the 5' end of the reads.. Please visit the following link for details on how to download the software and other usage related information: https://github.com/Mohak91/MetReTrim. The algorithm looked for the given unique primer sequence(s) in each read and allowed upto 3 mismatches during the search. Once

the primer sequence was completed, all the bases before the start of the primer sequence were trimmed. The primer sequence was retained in the processed reads. Two files were generated in the output directory- 1) fastq file containing the processed reads and 2) fastq file containing unprocessed reads. The unprocessed reads were a result of primer sequences in the reads having more than 3 mismatches or insertions and deletions. The software was run as a command line using the following syntax:

For paired-end reads,

MetReTrim -i <path to fastq files folder> -o <desired path to trimmed output> -f <primer sequence for forward read> -r <primer sequence for reverse read>

Results

The experiment was designed to obtain libraries using 'N' spacer primers from Metagenomic DNA and *E. coli* DNA (Fig. 1). The libraries were prepared from 5 set of N spacer primer pool combinations. The 'N' spacer primer pool combinations were made by equimolar pooling of forward and reverse primer (Table 3) Equimolar pool of barcoded libraries prepared using 5 set of 'N' spacer primer pool combinations were denatured and spiked in Hiseq 2500 Rapid-V2 2X100bp run to check base distribution at each sequencing cycle. Extracting fastq files from raw data, data de-multiplexing and Illumina adapters trimming was done using Bcl2fastq conversion software. The fastq files generated for Metagenomic DNA and *E.coli* DNA were analysed using in-house python script to check for the diversity at each base position in read 1 and read 2 sequence reads. The read 1 sequence with 6N spacer and 7N spacer (Fig. 2A, 2B and 3A and 3B) exhibited base diversity in the first ten nucleotides, allowing for better identification of clusters in the first few cycles, however at 11^{th} and 12^{th} base position, the contribution of A and C nucleotide reduced to ~15% this lead to poor base quality scores. Also the base diversity pattern is similar for Metagenomic and *E.coli* DNA. This confirms that 'N' spacer primer pool is able to generate base diversity in amplicon libraries from pure culture as well. Analysis of Read1 sequence for 8N and 9N primer pool (Fig. 2C, 2D and 3C and 3D) comparatively showed more promising base diversity but nucleotide distribution at position 15^{th}-16^{th} showed bias towards Green Laser Registry. Distribution of nucleotides for Green and Red laser registry plays a critical role in obtaining good quality reads, therefore Fastq results were analysed for 10N primer pool (Fig. 2E and 3E). We found that the 10N primer pool combination although showed significant increase in G nucleotide beyond 12^{th} base position the effect was balanced by elevated percentage of A and C nucleotide responsible for Red laser registry.

To evaluate the result obtained we applied our approach to prepare 16S V3-V4 amplicon library for DNA extracted from pure *E. coli* culture using 10N primer pool (Fig. 3). The library was denatured with 0.2N NaOH. Paired end sequencing was performed on illumina MiSeq platform using 2x300 V3 sequencing kit producing 290bp reads per end with loading concentration 11pM. The average quality scores (Q30) were ~92% without PhiX spike-in. Per base sequence quality of libraries prepared using our method ('N' spacer V3-V4 primers) without PhiX Spike-in (Fig. 4B) showed increased sequence quality compared to libraries prepared using standard illumina V3-V4 primers with 10% PhiX spike-in (Fig 4A).

Comparison with the previous published amplicon sequencing protocols

Liyou et al., 2015 tried to shift the sequencing frame of amplicons by using spacers of 0–7 bases, however in our studies we observed that spacers consisting 7 bases are not sufficient to resolve the issue of unbalanced base distribution and requires higher percent of PhiX spike-in. Jensen et al., 2019 designed heterogeneity primers by adding specific nucleotide bases to 16SV3-V4 primers to create 10 oligonucleotide sets and carried out Miseq run with 10% PhiX. This approach requires preparation of minimum 10 libraries to be pooled to generate base diversity. Adopting this strategy to other amplicon library preparation needs a careful designing of heterogeneity spacers to ensure base diversity by considering the sequence of primer used to amplify the gene of interest. In our study we designed primers by adding 'N' spacers (0-10) and pooled them together that results in base variability within individual library leading to effective laser registry without need for PhiX spike-in. The maximum amplicon sequence length compromises for the base sacrifice by sequencing spacers. Pooling the primers for library preparation simplifies the experimental setup and designing modified primers by effortless addition of 'N' spacers (0-10) upstream of the target gene binding region. Jensen et al., 2019 also reported slower drop in quality scores at 185–189 position in sequence reads in contrast our method shows higher quality Q scores upto 230-240 base position and a slower drop thereafter (Fig 4B). Holm et al., 2019 presented extremely high-throughput and high-quality 300 bp paired-end reads from 1,568 amplicon libraries per lane on a HiSeq 2500 instrument with 5% PhiX spike-in. 0-7 base heterogeneity spacer strategy was used for library preparation. In our study we presented high quality paired end 2x290bp sequencing run on Miseq without PhiX spike-in and also proved that a minimum of 0-10 base heterogeneity spacer is needed to resolve the issue of base diversity.

Conclusion

In this study, we designed heterogeneity primers by adding (0-10) 'N' spacers to 16S V3-V4 specific forward and reverse primer to generate amplicon with complex base diversity due to the frameshift effect. Addition of 'N' nucleotide to primers and pooling them together makes the experimental setup faster and simple. The use of 'N' spacer primers generates nucleotide distinctiveness within individual libraries at each base resulting in better identification of clusters during library sequencing run and enhance confidence in nucleotide base calls. This allows for multiplexing of more number of samples with no requirement of PhiX spike-in. This greatly reduces the overall cost and attains improved data quality. The 'N' spacer strategy can be used to amplify and sequence other amplicon regions. We have developed pipeline to specifically remove the heterogeneity spacers in sequenced reads.

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Author contributions: AP conceived and designed the study. TN performed all the experiments under the guidance of AP. MS performed the bioinformatics analysis. TN, MS and AP wrote the paper.

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Table 1: Forward 'N' spacer primers required for the First round PCR

Primer Name	Forward Primer Sequence (5'-3')
16S_F_10N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNNNNCCTACGGGNGGCWGCAG
16S_F_9N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNNNCCTACGGGNGGCWGCAG
16S_F_8N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNNCCTACGGGNGGCWGCAG
16S_F_7N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNNCCTACGGGNGGCWGCAG
16S_F_6N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNCCTACGGGNGGCWGCAG
16S_F_5N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNCCTACGGGNGGCWGCAG
16S_F_4N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNCCTACGGGNGGCWGCAG
16S_F_3N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNCCTACGGGNGGCWGCAG
16S_F_2N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNCCTACGGGNGGCWGCAG
16S_F_1N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG
16S_F_0N	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG

Table 2: Reverse 'N' spacer primers required for the First round PCR

Primer Name	Reverse Primer Sequence (5'-3')
16S_R_0N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC
16S_R_1N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNGACTACHVGGGTATCTAATCC
16S_R_2N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNGACTACHVGGGTATCTAATCC
16S_R_3N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNGACTACHVGGGTATCTAATCC
16S_R_4N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNGACTACHVGGGTATCTAATCC
16S_R_5N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNGACTACHVGGGTATCTAATCC
16S_R_6N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNGACTACHVGGGTATCTAATCC
16S_R_7N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNNGACTACHVGGGTATCTAATCC
16S_R_8N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNNNGACTACHVGGGTATCTAATCC
16S_R_9N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNNNNGACTACHVGGGTATCTAATCC
16S_R_10N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNNNNNNGACTACHVGGGTATCTAATCC

Table 3: 'N' spacer primer pool combination

'N' spacer primer pool combinations	Forward Primer Pool	Reverse Primer Pool
6 N Spacer primer pool	6N, 5N, 4N, 3N, 2N, 1N, 0N	6N, 5N, 4N, 3N, 2N, 1N, 0N
7 N Spacer primer pool	7N, 6N, 5N, 4N, 3N, 2N, 1N, 0N	7N, 6N, 5N, 4N, 3N, 2N, 1N, 0N
8 N Spacer primer pool	8N, 7N, 6N, 5N, 4N, 3N, 2N, 1N, 0N	8N, 7N, 6N, 5N, 4N, 3N, 2N, 1N, 0N
9 N Spacer primer pool	9N, 8N, 7N, 6N, 5N, 4N, 3N, 2N, 1N, 0N	9N, 8N, 7N, 6N, 5N, 4N, 3N, 2N, 1N, 0N
10 N Spacer primer pool	10N, 8N, 7N, 6N, 5N, 4N, 3N, 2N, 1N, 0N	10N, 8N, 7N, 6N, 5N, 4N, 3N, 2N, 1N, 0N

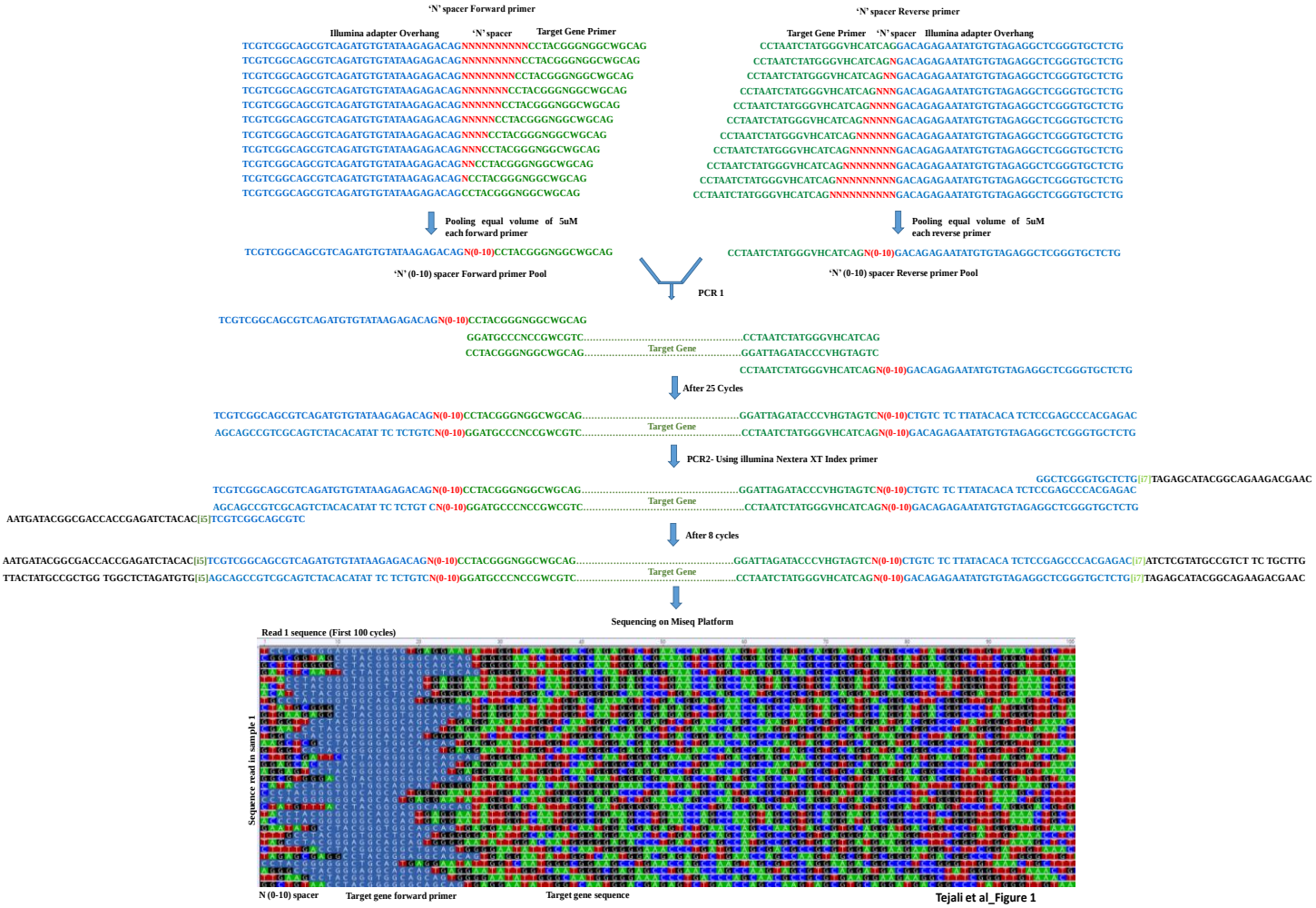
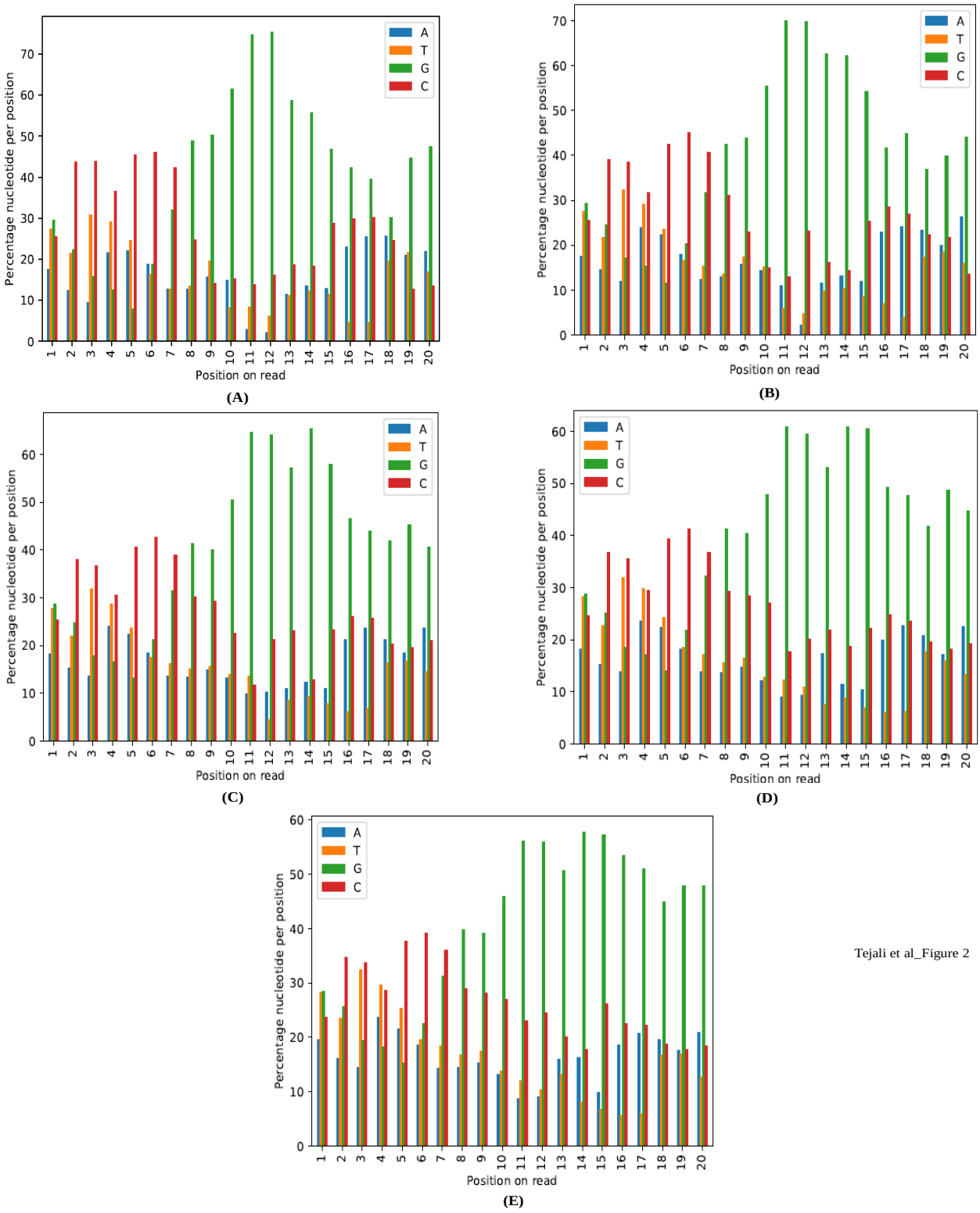


Fig. 1. **Schematic representation of 16S amplicon sequencing workflow**. *E.coil* DNA was amplified for 16S V3–V4 regions using gene specific 'N' spacer primers with overhang adapters. The amplicons were subjected to second PCR using Nextera XT index primers. Illumina MiSeq platform was used for sequencing the final libraries. The first 100 sequencing cycles illustrates the base diversity generated within a sample amplified using 'N' spacer primers.



Tejali et al_Figure 2

Fig. 2. Graphical representation of base diversity in Metagenomic 16S V3-V4 amplicon sequencing using **(A)** 6N **(B)** 7N **(C)** 8N **(D)** 9N **(E)** 10N spacer primer pool sets.

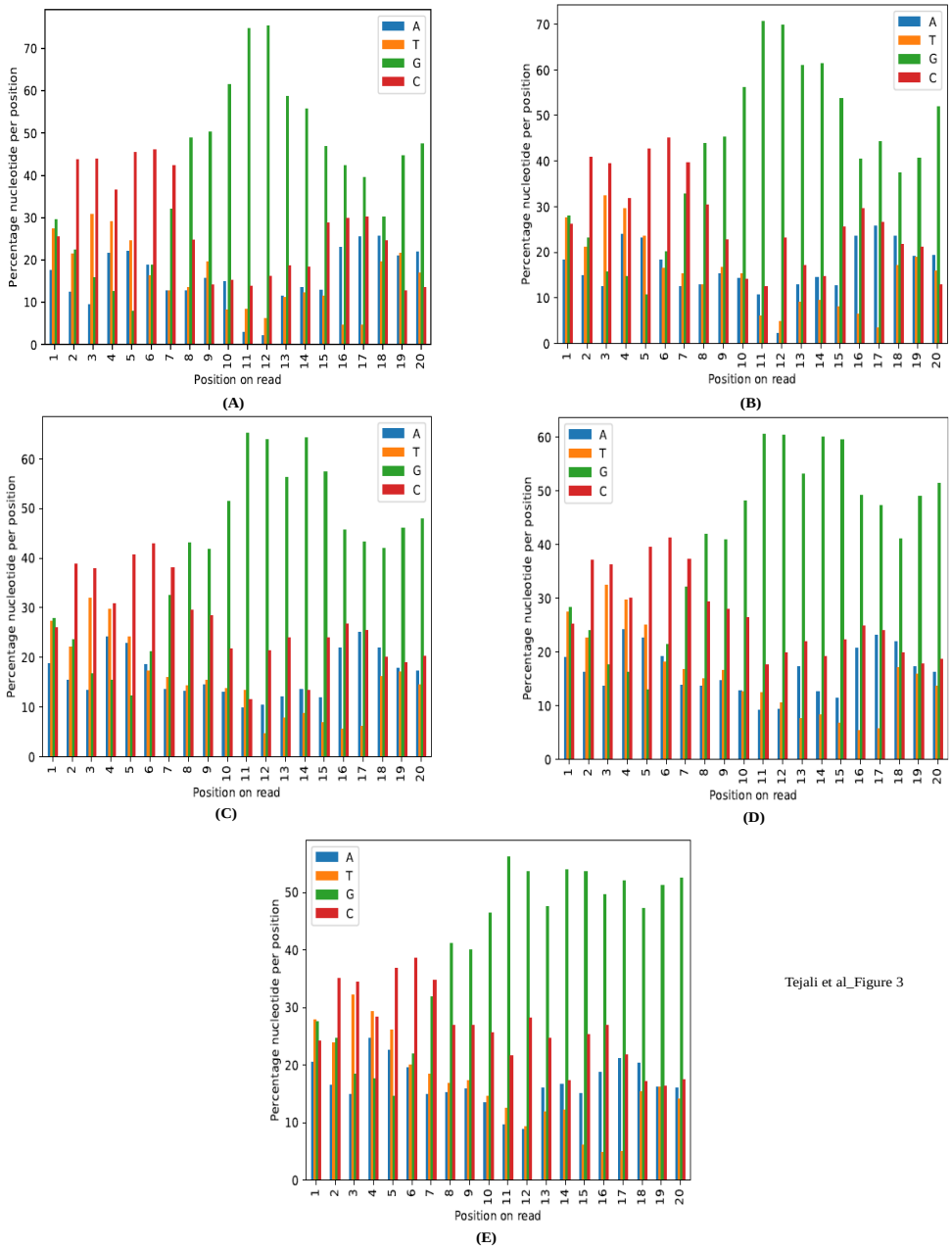


Fig. 3. Graphical representation of base diversity in *E. coli* 16S V3-V4 amplicon sequencing using **(A)** 6N **(B)** 7N **(C)** 8N **(D)** 9N **(E)** 10N spacer primer pool sets

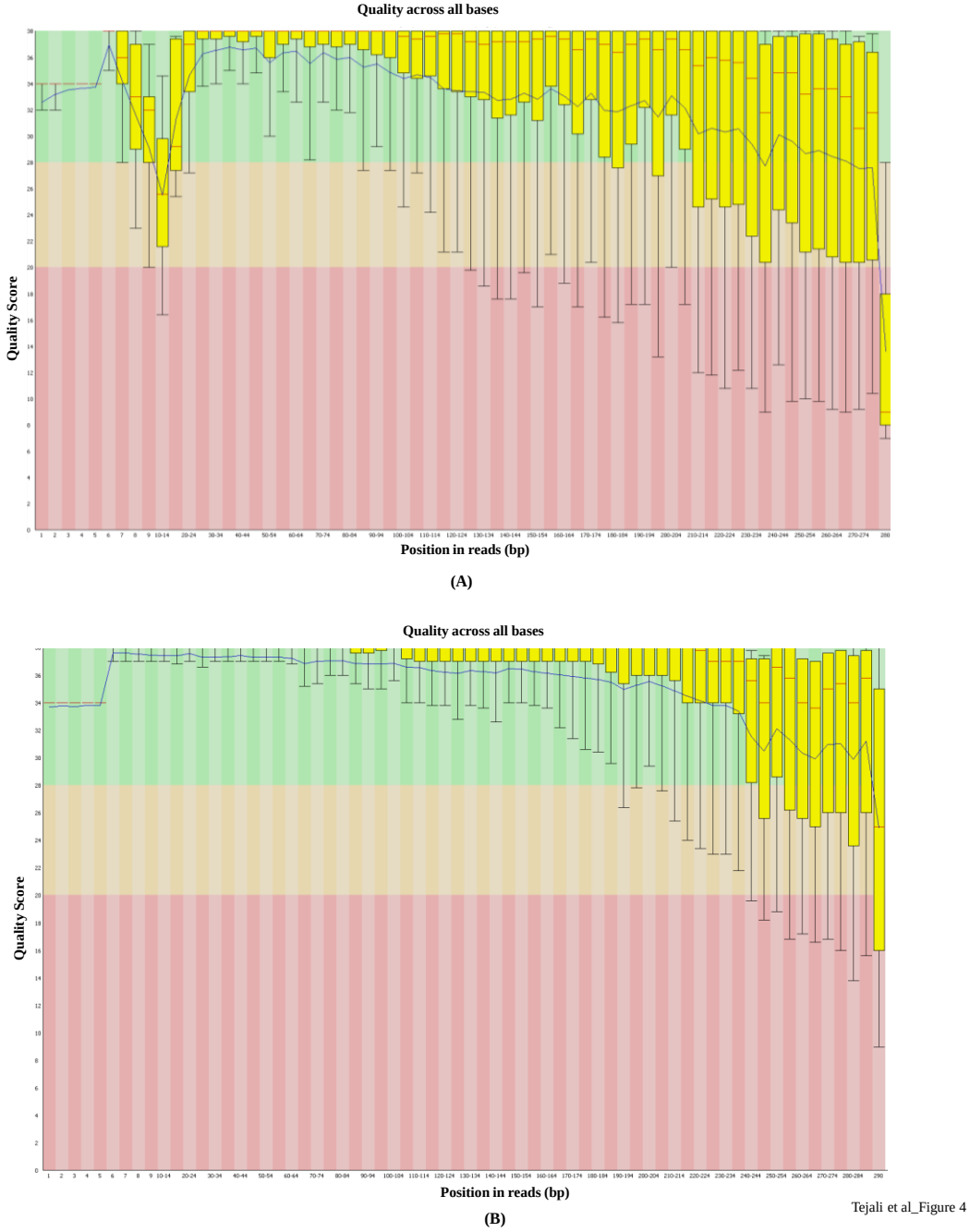


Fig. 4 **(A)** Per base sequence quality of libraries prepared using Standard illumina V3-V4 primers with 10% PhiX spike-in. **(B)** Per base sequence quality of libraries prepared using 'N' spacer V3-V4 primers without PhiX Spike-in. The quality scores showed increased base sequence quality compared to libraries prepared using Standard illumina V3-V4 primers with 10% PhiX spike-in