Rapid 16S Metagenomic Library Preparation for Oxford Nanopore Technologies (ONT)® Platform

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ABSTRACT

Metagenomic sequencing is often used to characterize microbial populations in a given sample. Amplifying and sequencing the 16S rRNA gene is a popular method for metagenomic sequencing. Depending on the downstream sequencing method of choice, either a fraction of or the entire 16S rRNA gene is amplified. The quality of the sequencing data and subsequent metagenomic analysis often depends on the choice of the polymerase. In this application note we demonstrate a rapid 16S metagenomic library preparation method for the Oxford Nanopore Technologies (ONT) sequencing platform using repliQa HiFi ToughMix from Quantabio.

INTRODUCTION

The characterization of the composition and diversity of microbial communities has wide-reaching applications in public health, environmental surveillance and medicine. Sequencing of variable regions of the 1500 bp 16S ribosomal rRNA (16S rRNA) gene is an established method of taxonomic identification of bacterial species in a mixed community.[1,2] Analysis of these short regions of the 16S rRNA gene by short read sequencing such as on Illumina® platforms reduces the sequencing burden compared to full gene length analysis, and allows high throughput analysis of microbial communities at reduced cost. However, this short-read methodology is vulnerable to identification bias from potential chimeric sequences produced during library construction and is limited in taxonomic resolution of similar species.[3,4]

More recently, long-read sequencing methods have led to development of full-length 16S rRNA gene sequencing which can reduce biases and improve bacterial classification.[3,4] In particular, the use of Oxford Nanopore Technologies (ONT) MinION® for full-length 16S rRNA gene sequencing can have additional benefits such as low instrument cost, high portability and faster time to result.[5] The ONT 16S barcoding kit provides a convenient and reliable method for 16S library preparation for long read sequencing. However, the protocol is limited by long library preparation times due to the requirement for sample barcoding of full length amplicons using a third party polymerase.

In this application note, we present an optimized workflow to prepare 16S libraries using repliQa HiFi ToughMix (Quantabio #95200) for barcoding PCR with the 16S Barcoding Kit (ONT #SQK-16S024). repliQa HiFi ToughMix is a highly processive, high fidelity PCR master mix. Compared to alternative PCR master mixes used for this library preparation, repliQa HiFi ToughMix reduces PCR cycling time by 50% (Figure 1) and provides the additional benefit of the ToughMix formula, overcoming PCR inhibitors typically found in microbial samples.

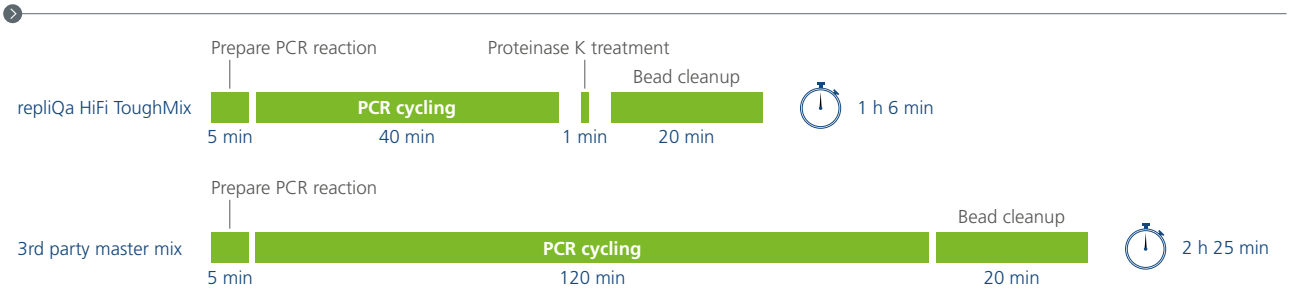

Figure 1 Representation of protocol time for library preparation with the ONT 16S Barcoding Kit using repliQa HiFi ToughMix (and proteinase K treatment) or the 3rd party master mix recommended in the ONT protocol.

METHODS

DNA samples

High molecular weight DNA was extracted from river water samples using DNeasy® PowerWater® Kit (QIAGEN®, #14900-100-NF). Alternatively DNA can be extracted from single species bacterial cultures using Extracta Plus DNA (Quantabio, #95213) or from mixed bacterial communities with additional upstream processing. ZymoBIOMICS™ Microbial Community DNA Standard (Zymo Research #D6305) was used as a mock microbial community for positive control experiments.

16S library preparation

Libraries were prepared using 16S Barcoding Kit 1-24: SQK-16S024 from Oxford Nanopore Technologies following protocol version 16S_9086_v1_revT_14Aug2019,[6] with repliQa HiFi ToughMix in place of the recommended 3rd party master mix. The modified protocol is summarized below.

1. Barcodes were thawed and placed on ice. repliQa HiFi ToughMix was vortexed for 5 s to mix and placed on ice.
2. Each gDNA sample (20 ng) was adjusted to a volume of 10 µl with nuclease-free water. It is recommended to use 10-20 ng high molecular weight input gDNA for this protocol.
3. PCR reactions were prepared in thin-walled PCR tubes according to the table below.

Reagent	Volume (µl)
Nuclease-free Water	5
Input DNA (10-20 ng)	10
repliQa HiFi ToughMix	25
Total	40

4. To each reaction, 10 µl 16S barcoded primers (SQK-16S024) was added, using a different barcode for each sample, to give a total reaction volume of 50 µl.
5. The reaction was mixed thoroughly by pipetting then samples were amplified according to the following cycling protocol.

Step	Temperature	Incubation Time	Cycles
Initial denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	30
Anneal	55°C	5 sec	
Extend	68°C	5 sec	
Final extension	68°C	1 min	1
Hold	4°C	Hold	1
Total Time		40 min	

6. On completion of the PCR cycling, 0.5 µl Proteinase K (QIAGEN #19131) was added, to each reaction, at a final concentration of 0.2 mg/ml and incubated for 1 min at room temperature

   Note: Proteinase K treatment of PCR products is necessary to avoid loss of yield during bead clean up and reduction of reads during sequencing.

7. Cleanup beads (e.g. sparQ PureMag Beads Quantabio, #95196) were equilibrated to room temperature and vortexed thoroughly before use. 30 µl (0.6X) of beads were added to each Proteinase K-treated PCR reaction and mixed well by pipetting.
8. The mixture was incubated at room temperature for 5 min, then placed on a magnetic stand to pellet the beads. The supernatant was discarded.
9. With the tube on the magnetic stand, the beads were washed twice with 200 µl of freshly prepared 80% ethanol, ensuring all of the supernatant was removed after the second wash.
10. The beads were air dried on the magnetic stand for 5 minutes.

    Note: Over-drying beads may result in lower recovery of DNA.

11. The beads were resuspended in 12 µl 10 mM Tris-HCl pH 8.0 with 50 mM NaCl and incubated for 3 min at room temperature.
12. The beads were pelleted on the magnetic stand and 10 µl supernatant carefully collected and transferred to a new tube.

13. Libraries were pooled in equal ratios to a total of 50-100 fmoles in 10 mM Tris-HCl pH 8.0 with 50 mM NaCl.
14. Rapid adapter (1 µL) was added to the barcoded DNA and mixed gently by flicking the tube and spun down.
15. The reaction was incubated for 5 min at room temperature.
16. The prepared library was stored on ice until ready to load.

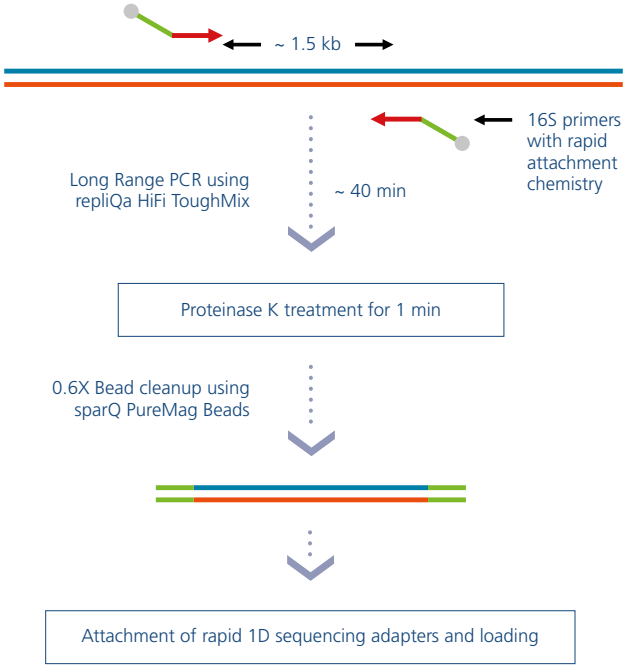

Figure 2 **Summary of library preparation modification using repliQa HiFi ToughMix.**

Priming and loading

Priming and loading the SpotON flow cell was carried out according to the protocol 16S_9086_v1_revT_14Aug2019.[6]

Sequencing

Libraries were sequenced on the MinION platform using R9.4.1 flow cell (ONT #FLO-MIN106D).

Sequencing data was analyzed using MinKnow® software (ONT).

RESULTS

Libraries were prepared from water samples and the ZymoBIOMICS Microbial Community control sample using repliQa HiFi ToughMix with barcoding primers from the ONT 16S Barcoding kit. The PCR amplification time was only 40 min with repliQa HiFi ToughMix compared to approximately 2 h with the standard protocol. It should be noted that library preparation with repliQa HiFi ToughMix requires treatment of the amplification products with proteinase K for 1 min prior to bead clean up, whereas library preparation with the recommended 3rd party master mix does not.

Sequencing demonstrated high numbers of reads from the samples amplified with repliQa HiFi ToughMix, average 210,000 reads/sample for the water samples and 475,000 reads/sample for the mock community control. EPI2Me analysis of sequencing gave 97% classified reads with an average accuracy of 90%, suggesting repliQa HiFi ToughMix is suitable for generating high quality sequencing data using the Oxford Nanopore 16S workflow.

CONCLUSIONS

In this application note we have detailed a protocol for 16S library preparation with repliQa HiFi ToughMix and 16S barcoding kit. In comparison to the standard 16S barcoding kit protocol using a 3rd party master mix for amplification, the presented method with repliQa HiFi ToughMix saves 1 h 20 min in amplification time and requires only an additional 1 min for Proteinase K treatment following PCR. Long-read sequencing of the libraries gave high numbers of reads for each sample demonstrating that repliQa HiFi ToughMix is an exciting alternative master mix for use in preparing 16S libraries for ONT sequencing.

References

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