bioRxiv preprint doi: https://doi.org/10.1101/244517; this version posted July 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

NanoAmpli-Seq: A workflow for amplicon sequencing for mixed microbial communities on the nanopore sequencing platform.

Szymon T Calus[1], Umer Z Ijaz[1*], Ameet J Pinto[2*]

Affiliations:

[1]School of Engineering, University of Glasgow, Glasgow, G12 8QQ, United Kingdom

[2]Department of Civil & Environmental Engineering, Northeastern University, 269 SN, 360 Huntington Avenue, Boston, MA, USA

* Correspondence to:

Ameet Pinto, Department of Civil & Environmental Engineering, Northeastern University. a.pinto@northeastern.edu

Umer Ijaz, School of Engineering, University of Glasgow, UK. umer.ijaz@glasgow.ac.uk

bioRxiv preprint doi: https://doi.org/10.1101/244517; this version posted July 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Abstract

Background: Amplicon sequencing on Illumina sequencing platforms leverages their deep sequencing and multiplexing capacity, but is limited in genetic resolution due to short read lengths. While Oxford Nanopore or Pacific Biosciences platforms overcome this limitation, their application has been limited due to higher error rates or smaller data output.

Results: In this study, we introduce an amplicon sequencing workflow, i.e., NanoAmpli-Seq, that builds on Intramolecular-ligated Nanopore Consensus Sequencing (INC-Seq) approach and demonstrate its application for full-length 16S rRNA gene sequencing. NanoAmpli-Seq includes vital improvements to the aforementioned protocol that reduces sample-processing time while significantly improving sequence accuracy. The developed protocol includes chopSeq software for fragmentation and read orientation correction of INC-Seq consensus reads while nanoClust algorithm was designed for read partitioning-based *de novo* clustering and within cluster consensus calling to obtain full-length 16S rRNA gene sequences.

Conclusions: NanoAmpli-Seq accurately estimates the diversity of tested mock communities with average sequence accuracy of 99.5% for 2D and 1D2 sequencing on the nanopore sequencing platform. Nearly all residual errors in NanoAmpli-Seq sequences originate from deletions in homopolymer regions, indicating that homopolymer aware basecalling or error correction may allow for sequencing accuracy comparable to short-read sequencing platforms.

Keywords: amplicon sequencing, nanopore sequencing, *de novo* analyses, sequencing accuracy

bioRxiv preprint doi: https://doi.org/10.1101/244517; this version posted July 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

Background

Amplicon sequencing, particularly sequencing of the small subunit rRNA (SSU rRNA) gene and internal transcribed spacer (ITS) regions, is widely used for profiling of microbial community structure and membership [1-4]. The wide-scale application of amplicon sequencing has been driven mainly by the ability to multiplex 100's of samples on a single sequencing run and obtain millions of sequences of target communities on high-throughput sequencing platforms [1, 4]. The primary limitation of these commonly used technologies (i.e., Illumina's MiSeq, Ion Torrent PGM, etc.) is that their read lengths are short, ranging from 150-400 bp [5]. While excellent at bulk profiling of microbial communities through multiplexed deep sequencing, short read lengths are limited in the taxonomic resolution of sequenced reads and more so, are not amenable to robust phylogenetic analyses to assess the relationship between sequences originating from unknown microbes with those in publicly available databases. An important effect of the proliferation in short read sequencing applications has been a decrease in the rate at which long higher quality sequences, particularly of SSU rRNA genes, are being deposited in public databases. This effect is to some extent being mitigated through assembly and curation of near full-length SSU rRNA genes from metagenomic datasets [6-9] and will continue to be mitigated with novel approaches for SSU rRNA sequencing using synthetic long read approaches [10].

The introduction of long-read single molecule sequencing platforms, such as PacBio's single-molecule real-time sequencing (SMRT) and single molecule sensing technologies on the Oxford Nanopore Technologies (ONT) MinION™ platform, has opened the possibility of obtaining ultra-long reads [5, 11]. While sequencing throughput and raw data quality of long read single molecule sequencing approaches are yet to rival that of short read platforms, the ability to obtain ultra-long

bioRxiv preprint doi: https://doi.org/10.1101/244517; this version posted July 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

reads can overcome several limitations of the latter [12]. For instance, long-read sequencing combined with various error correction approaches [13, 14] has been used to obtain high-quality single contig microbial genomes [14] or increase assembly quality of previously sequenced but fragmented eukaryotic genomes assemblies [15, 16], which was not feasible using short-read approaches. Long-read sequencing capabilities have also been recently leveraged to sequence near full-length SSU rRNA genes (e.g., 16S rRNA) [17-21] or even the entire *rrn* operon [20, 22].

A majority of the studies utilizing either the SMRT or nanopore sequencing platforms have limited data their analyses efforts to sequence classification, due to the fact that widely used sequence classifiers are tolerant of high sequencing error rates [23, 24]. However, these classification-only approaches are limited in their ability to differentiate between closely related sequences, risks false detections (i.e., read incorrectly classified at the family or genus levels due to high error rates), and are unable to identify organisms that are not represented in the reference databases. In contrast, some studies have gone beyond sequence classification by using consensus sequence construction to improve overall sequence accuracy. The consensus sequence creation efforts thus far can be categorized into two approaches. The first approach involves mapping raw, noisy reads to custom or publicly available reference databases (i.e., SILVA) [25]. Subsequently, reads mapping to the same reference sequence are then used for the semi-automated or manual construction of a consensus sequence using overlapping alignments [20, 22]. While this approach does result in improved accuracy of the consensus sequence, clustering of reads based on mapping of noisy reads to reference databases has significant limitations. First, incorrect read mapping to a reference is prevalent due to high error rates of raw reads. Second, the reliance on a reference database ensures that reads originating from organisms not represented in the reference database are ignored. The

bioRxiv preprint doi: https://doi.org/10.1101/244517; this version posted July 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

more robust alternative towards high accuracy consensus sequence generation would be a completely *de novo* approach, i.e., generation of a consensus sequence without the use of any reference database.

To our knowledge, there are three reports of *de novo* data processing to reduce error rate from long read sequencing of amplicons from mixed microbial communities [17, 21, 26]. Both Singer et al [26] and Schloss et al [17] utilized the circular consensus sequencing approach of SMRT sequencing coupled with a range of quality filtering (i.e., mismatches to primer, quality scores) and sequence clustering (i.e., pre-cluster) to generate consensus sequences from reads clustered into operational taxonomic units (OTUs) and achieved error rates of 0.5% [26] and 0.027% [17] for full-length 16S rRNA gene sequencing libraries. For the later effort [17], the number of operational taxonomic units (OTUs) of the processed data were also highly similar to the theoretical number of OTUs in tested mock communities, indicating that the application of this protocol for naturally derived mixed microbial communities is likely to result in robust diversity estimates. Li et al. [21] developed Intramolecular-ligated Nanopore Consensus Sequencing (INC-Seq) protocol for consensus-based error correction of nanopore sequencing reads with a median accuracy of 97-98%. The INC-Seq workflow involves amplicon concatermization to link multiple identical copies of the same amplicon on a single DNA molecule, sequencing of the concatamerized molecules using 2D sequencing chemistry on the nanopore sequencing platform, followed by consensus-based error correction after aligning the physically linked concatemers on each sequenced DNA strand. By using this approach, Li et al [21] were able to increase the median sequence accuracy of processed reads to 97-98%. While this significant improvement allowed for

bioRxiv preprint doi: https://doi.org/10.1101/244517; this version posted July 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

taxonomic classification of sequences to the species level, it did not allow for sequence clustering for diversity estimation due to residual median error rates of approximately 2-3%.

In this study, we leverage and expand on the INC-Seq protocol developed by Li et al [21] to provide a complete workflow for amplicon sequencing and *de novo* data processing, NanoAmpli-Seq, applied to near full-length 16S rRNA gene of mock communities that results in high-quality sequences with a mean sequence accuracy of 99.5±0.08%. The current version of NanoAmpli-Seq includes modifications to the library preparation protocol for INC-Seq and fixes a key issue with INC-Seq consensus sequences while adding a novel read partitioning-based sequence clustering approach which results in an accurate estimation of diversity of mixed microbial communities and results in higher sequence accuracy by allowing within OTU sequence alignment and consensus calling. Further, we demonstrate that NanoAmpli-Seq works equally well on the (now obsolete) 2D sequencing chemistry and the recently released 1D2 sequencing chemistry on the MinION™ device. While important limitations such as suboptimal re-construction of community structure and error rate of ~0.5% remain, the proposed approach may be used for sequencing of long amplicons from complex microbial communities to assess community membership with cautious utilization of sequences from low abundance OTUs due to likely lower sequence accuracy ranging from 99-99.5% accuracy.

Results

Experimental design and workflow. The NanoAmpli-Seq protocol was developed and validated using amplicon pools consisting of near full-length 16S rRNA gene of a single organism (*Listeria monocytogens*) or an equimolar amplicon pool of near full-length 16S rRNA genes from 10

bioRxiv preprint doi: https://doi.org/10.1101/244517; this version posted July 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

organisms (Supplementary Table S1). The amplicon pools were generated by PCR amplifying near full-length 16S rRNA genes from genomic DNA of the target organism(s) using primers and PCR reaction conditions as described in the materials and methods section. The respective amplicon pools were subsequently prepared for sequencing using the INC-Seq workflow as outlined in Figure 1, with a few significant modifications. Briefly, the amplicon pools were self-ligated to form plasmid-like structures which was followed by digestion with plasmid-safe DNAse to remove the remaining non-ligated linear amplicons. The DNA pool consisting of plasmid-like structures was subject to rolling circle amplification (RCA) using random hexamer-free protocol using a combination of primase/polymerase (PrimPol) and hi-fidelity Phi29 DNA polymerase [27]. The RCA product was subject to two rounds of T7 endonuclease I debranching and g-TUBE fragmentation followed by gap filling and DNA damage repair. Description of the protocol including reagent volumes and incubation conditions is provided in the materials and methods and a step-by-step protocol is provided in the supplementary text. The prepared amplicon pools for both single organism and 10 organism mock community samples were then subject to library preparation using the standard 2D (SQK-LSK208) (Runs 1 and 2) and 1D2 (SQK-LSK308) (Runs 3 and 4) kits using ONT specifications and sequenced on the MinION™ MK1b device followed by basecalling using Albacore 1.2.4.

bioRxiv preprint doi: https://doi.org/10.1101/244517; this version posted July 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

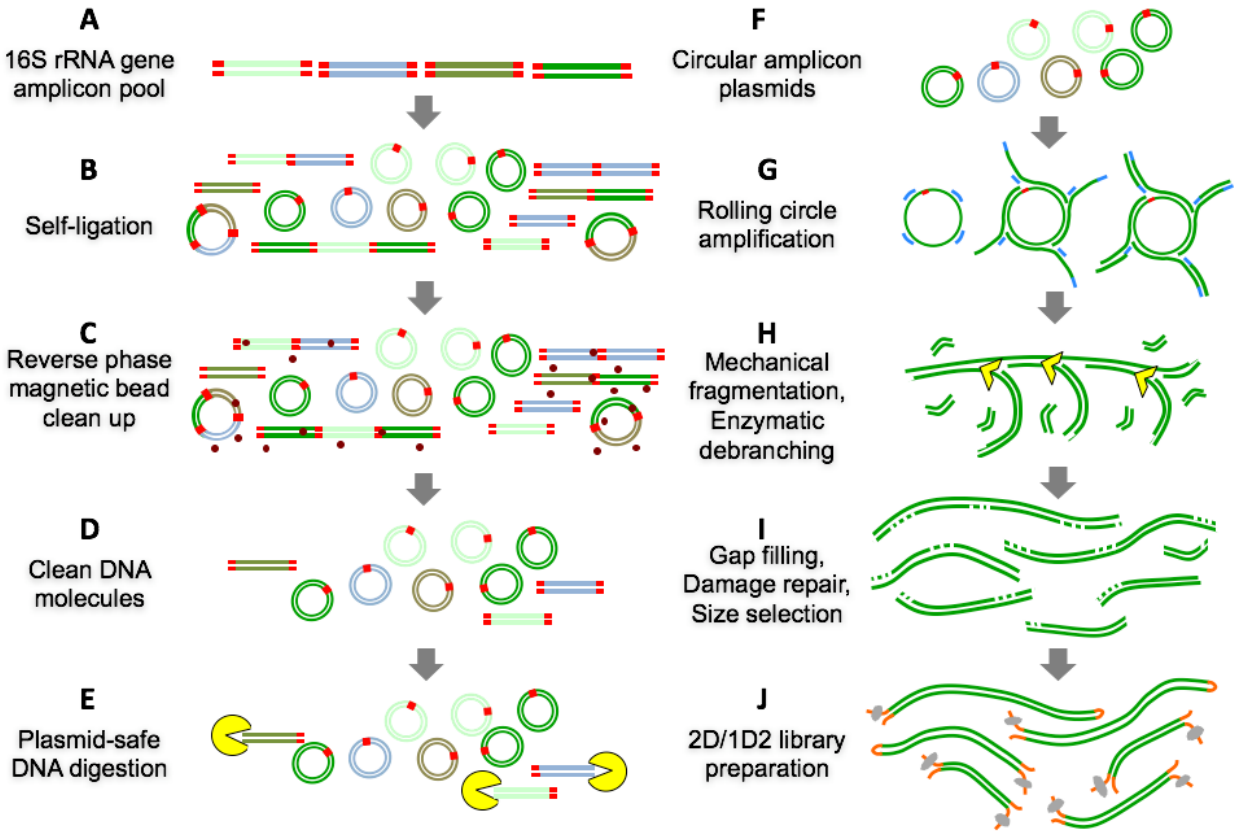


Figure 1: Overview of the sample preparation protocol for 16S rRNA gene pool preparation, plasmid-like structure preparation, enzymatic debranching and mechanical fragmentation, and 2D and 1D2 library preparation including intermediate clean-up steps.

Each resulting read consisted of multiple concatamerized physically linked amplicons from the one original 16S rRNA gene amplicon. The long concamtermized amplicon reads were subject to INC-Seq's anchor based alignment and consensus error correction using three different alignment options (i.e., blastn, Graphmap, and partial order alignment (POA)) and followed by iteratively running PBDAGCON on the consensus for error correction (INC-Seq flag "iterative"). Reads with irregular segment length, unmappable anchors, and potentially chimeric molecules (i.e., concatemers from more than one original 16S rRNA gene amplicon) were removed during the generation of the INC-Seq consensus read. Manual inspection of INC-Seq consensus reads revealed that a vast majority had an incorrect orientation of primers (Figure 2A). Specifically, the

bioRxiv preprint doi: https://doi.org/10.1101/244517; this version posted July 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

forward and reverse primers did not occur at the ends of the INC-Seq consensus reads but rather were co-located at varying positions along the length of each read. Efforts to manually split INC-Seq reads and re-orient the forward and reverse splits based on primer orientation revealed the presence of tandem repeats of nearly identical sequences, which affected efforts to merge the forward and reverse read splits (Figure 2B, 2C).

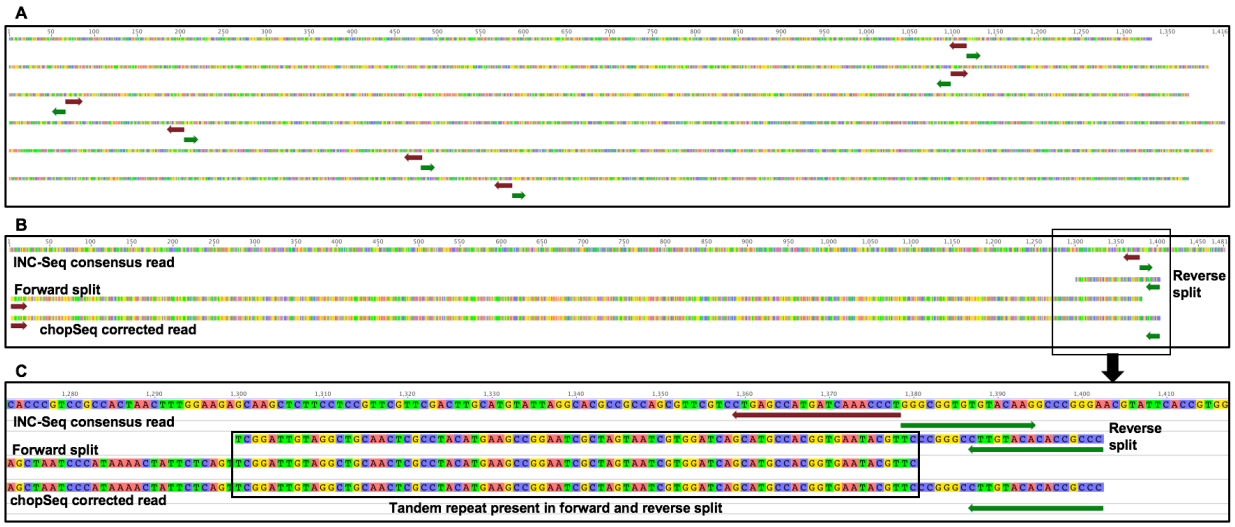


Figure 2: **(A)** Example of INC-Seq consensus reads showing the improper orientation with forward (maroon) and reverse (green) primers co-located and incorrectly oriented. **(B)** Manual splitting and re-orientation of the reads revealed the presence of tandem repeats in the forward and reverse splits which were identified and removed using chopSeq. **(C)** An expanded view of tandem repeat region in Figure 2B.

To this end, we developed the chopSeq algorithm as part of the NanoAmpli-Seq workflow. The chopSeq algorithm uses pairwise2 open source library from Biopython package to identify user provided primers (forward "–f", reverse "–r") sequences including degenerate bases in the INC-Seq consensus reads. Primer detection is carried out in different orientations and primer match scores for each orientation are generated. Subsequently, primer sequences in the INC-Seq consensus read with the highest mean score are re-oriented and any overhang is removed. Re-orienting reads using primer orientation resulted in the identification of insertions consisting of repeated sequence patterns, i.e., tandem repeats. These tandem repeats were identified using

bioRxiv preprint doi: https://doi.org/10.1101/244517; this version posted July 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

etandem algorithm from EMBOS open source software package (http://emboss.bioinformatics.nl/cgi-bin/emboss/etandem) and various features of these repeats were delineated, i.e. tandem minimum repeat, tandem maximum repeat, and mismatch rate. The percent identify between tandem repeat is estimated iteratively measuring the sequence similarity between co-occurring segments using window size ranging from 10 bp to 350 bp with diminishing sequence similarity threshold with increasing window size. The sequence similarity threshold with increasing window size was applied as longer tandem repeats tend to have lower similarity to each other compared to shorter. After completing re-orientation of reads in the removal of tandem repeats, the forward and reverse splits are merged into a single read, and any read that does not match prescribed length threshold (i.e., 1300-1450 bp) is discarded. This process of primer identification and tandem repeat removal can also be visualized by turning on verbosity mode (flag = -v) and the results can be exported in fasta format.

To enable fully reference-free analyses, we developed the nanoClust algorithm which takes the fasta file of chopSeq corrected reads as input and then performs read-partitioning based *de novo* clustering using VSEARCH [28] to delineate OTUs at a user-specified sequence similarity threshold (i.e., 97% in this study) followed by within OTU read alignment and consensus calling for each OTU. The nanoClust algorithm is written in python and requires Biopython packages such as Seq, SeqIO, AlignIO, aligninfo and pairwise2. This algorithm was explicitly designed for *de novo* clustering because standard *de novo* clustering approaches such as VSEARCH [28] and the clustering approaches available in mothur [29, 30] vastly overestimated the richness of the mock community when using chopSeq corrected reads (see details below). The nanoClust algorithm takes chopSeq corrected reads in fasta format, splits the reads into partitions based on

bioRxiv preprint doi: https://doi.org/10.1101/244517; this version posted July 4, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

user-defined partition size, implements VSEARCH [28] for dereplication, chimera detection and removal in each partition, and clustering for each partition to identify the partition category with optimal (i.e., maximum) number of OTUs (not counting singleton OTUs) and (3) discards singletons. Following this, nanoClust extracts read IDs for each OTU bin from the best performing partition, the extracted read IDs for each OTU bin are then used to obtain full-length chopSeq corrected reads, a subset of reads that fall within 10% of the average full-length read distribution within each OTU bin are aligned using Multiple Alignment using Fast Fourier Transform (MAFFT) [31, 32] with G-INS-i option, followed by consensus calling to obtain full-length representative sequence for each OTU. The entire data processing workflow is shown in Figure 3.

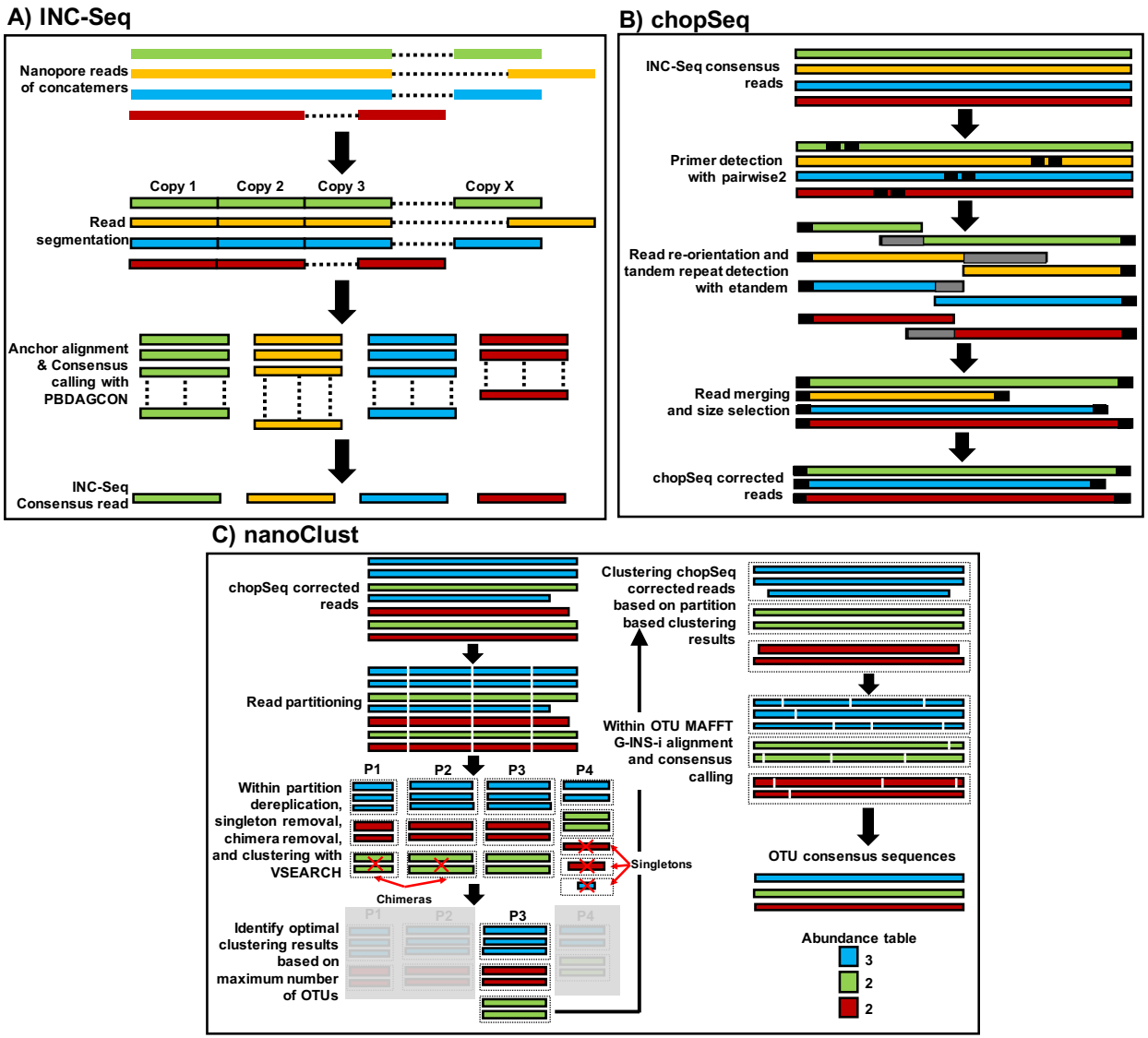


Figure 3: **(A)** Overview of the INC-Seq based anchor alignment and iterative consensus calling using PBDAGCON. **(B)** INC-Seq consensus reads were subject to chopSeq based read reorientation followed by tandem repeat removal and size selection to retain reads between 1300-1450bp. **(C)** chopSeq corrected reads are subject to partitioning followed by VSEARCH based binning to identify optimal binning results using partition that generates maximum number of OTUs (without singletons). MAFTT-G-INS-i was then used for sequence alignment of a subset of full length reads from each OTU bin for the best performing partition and the alignment was used to create the OTU consensus read.

228 **Modifications to the original INC-Seq protocol significantly reduces time required for**
229 **amplicon concatemer pool preparation.** While the proposed DNA preparation protocol is based
230 on the previously developed INC-Seq approach [21], it contains multiple improvements that allow
231 for faster and more efficient library preparation. These modifications include reduced incubation
232 times for self-ligation step and plasmid-safe DNAse digestion process. More importantly, the
233 current protocol utilizes Tth PrimPol [27] and Phi29 DNA polymerase enzymes for RCA which
234 minimizes the formation of unspecific products that may occur when using random hexamers.
235 Similarly, the NanoAmpli-Seq protocol utilizes T7 endonuclease I enzyme for enzymatic
236 debranching of RCA product combined with mechanical fragmentation step involving use of the
237 g-TUBE. Thus, while our protocol increases the number of intermediate steps for sample
238 preparation, by optimizing each step, it reduces the overall time required for sample DNA
239 preparation to 6 hours (approximately 70% reduction compared to the original INC-Seq protocol).
240 These improvements not only result in analyses of near full-length 16S rRNA gene (i.e., twice the
241 amplicon size of the previously developed INC-Seq approach), but the combination of the
242 improved protocol with appropriate data processing modifications resulted in significant increase
243 in high-quality data post-processing.
244
245 **NanoAmpli-Seq data yield for 2D and 1D2 experiments.** Runs 1, 2, 3, and 4 resulted in 29420,
246 59490, 142233, and 301432 raw records with post basecalling read lengths ranging from 5bp to
247 43kbp and 5bp to 234kbp for 2D and 1D2 sequencing protocols (Supplementary Figure S1). The
248 pass reads to total raw reads ratio ranged from 28% for 2D and 7-9% for the 1D2 experiments
249 (Table 1). It is unclear if the low yield of pass reads, particularly for the 1D2 experiments, were
250 due to the concatemerization process, DNA damage during enzymatic debranching and

mechanical fragmentation that was unrepaired in the subsequent steps, or due to basecalling issues. All pass reads were subjected to INC-Seq processing to allow for consensus-based error correction using reads with a minimum of three concatemers per read (i.e., reads with less than three concatemers were excluded for any subsequent analyses) as compared to six concatemer threshold used by Li et al [21]. The number of concatemers per read passing INC-Seq threshold ranged from 3 to 21 and 3 to 42 for 2D and 1D2 data (Supplementary Figure S1). The total number of reads passing the three concatemer threshold ranged from 36-75% of the base called reads depending on the experiment, sequencing protocol, and alignment approach during INC-Seq processing (Table 1). This was significantly higher than those reported by Li et al [21], primarily due to the use of three compared to six concatemer threshold recommended previously.

Table 1: Summary of the total number of reads and median read lengths at each step of the data processing workflow for all experiments.

	Number of Reads				Median Read Length			
Protocol	**2D**	**1D2**	**2D**	**1D2**	**2D**	**1D2**	**2D**	**1D2**
Experiment	**One organism**	**One organism**	**Ten organism**	**Ten organism**	**One organism**	**One organism**	**Ten organism**	**Ten organism**
Run	1	4	2	3	1	4	2	3
Raw records	29420	301432	59490	142233	-	-	-	-
Pass reads	8108	20888	16403	12011	4868	7305	5401	6418
INC-Seq aligner	**Blastn**							
INC-Seq	3911	7618	7011	9081	1394	1433	1396	1413
chopSeq	3911	7618	7011	9081	1383	1387	1377	1374
chopSeq-size select	3748	7288	5186	7265	1384	1388	1377	1375
nanoClust	2997	6153	3677	5465	1396	1398	1384	1386
INC-Seq aligner	**Graphmap**							
INC-Seq	3902	7631	7004	9169	1400	1439	1401	1420
chopSeq	3902	7631	7004	9169	1384	1388	1378	1377
chopSeq-size select	3765	7399	5190	7496	1384	1389	1377	1377
nanoClust	2981	6179	4141	5490	1397	1396	1384	1386
INC-Seq aligner	**POA**							
INC-Seq	3913	7643	7025	9191	1414	1457	1415	1443
chopSeq	3913	7643	7025	9191	1394	1396	1386	1387
chopSeq-size select	3779	7088	5076	6954	1394	1396	1385	1386
nanoClust	3184	5993	3916	5622	1398	1389	1384	1386

264 **INC-Seq processed reads demonstrated incorrect read orientation and presence of tandem**
265 **repeats.** While the median read lengths for post INC-Seq were generally in the expected range
266 (i.e., 1350-1450 bp) (Table 1) and similar for all three alignment methods used (Supplementary
267 Figure 2), manual inspection of the reads revealed several instances of incorrect read orientation
268 (Figure 2). The amplicon pool preparation protocol relies on RCA of plasmid-like structures
269 constructed through self-ligation of linear amplicons followed by a combination of enzymatic
270 debranching and mechanical fragmentation to generate linear molecules with multiple
271 concatemers. Considering the fragmentation and debranching steps are not driven by sequence
272 specificity, it would be reasonable to assume that the resulting linear amplicon is unlikely to have
273 the correct orientation, i.e. 16S rRNA gene-specific forward and reverse primers do not flank the
274 entire amplified region. Indeed, we found a vast majority of the 2D and 1D2 INC-Seq consensus
275 reads were incorrectly oriented for the single organism sequencing runs, with forward and reverse
276 primers not located at the ends of the reads. As a result, the reads were chopped at the primer sites
277 and re-oriented to allow for the forward and reverse primers to be correctly oriented. However,
278 during the process of read re-orientation, we also discovered the presence of inserts in the form of
279 tandem repeats. Additional inspection of these inserts revealed that they were composed of
280 multiple repetitive sequences, with the length of these inserts ranging from 10 bp to in excess of
281 1500 bp (for rare cases) with median tandem repeat size ranging from 12 to 62 bp. The proportion
282 of INC-Seq processed reads with tandem repeats varied from 60-75% but did not reveal any
283 significant effect of type of aligner used during INC-Seq consensus calling or the sequencing
284 chemistry itself. Interestingly, however, the length distribution for the tandem repeats was strongly
285 associated with the sequencing chemistry (Figure 4).

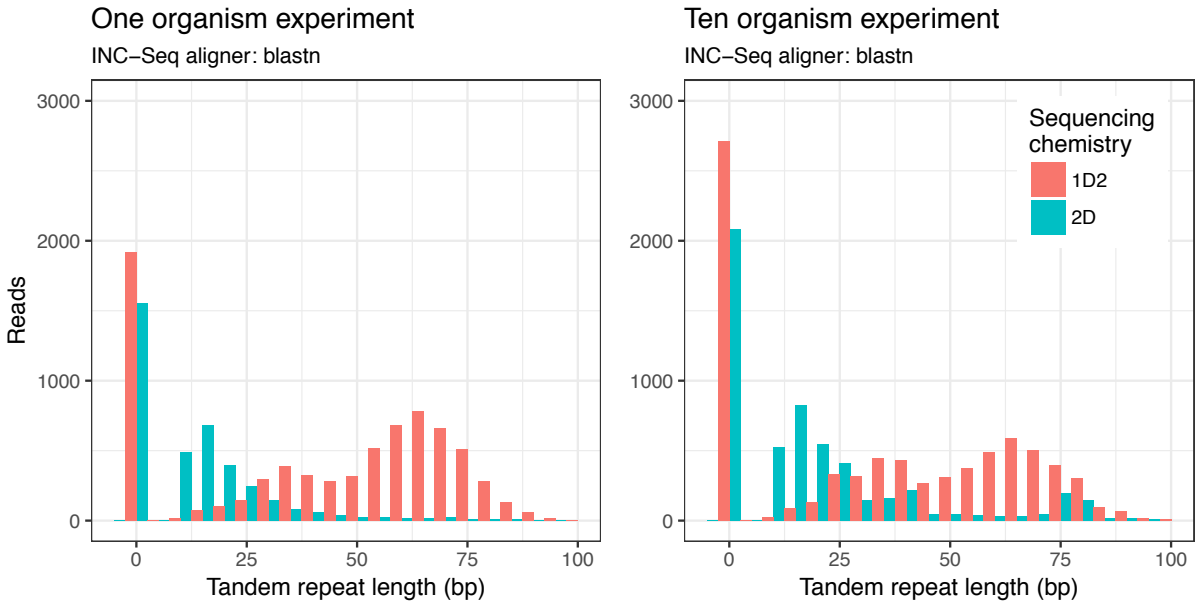


Figure 4: Histogram of tandem repeat length distribution of the INC-Seq processed reads did not show any effect of the aligner used during the INC-Seq process, but rather a marked effect of the sequencing chemistry. Results are only shown for INC-Seq consensus reads generated using blastn aligner.

Specifically, the 1D2 reads had longer tandem repeats as compared to the 2D reads and demonstrated a bimodal distribution of tandem repeat lengths as compared to the 2D data which showed a unimodal tandem repeat length distribution (Figure 4). While the template and complements in the 2D sequencing chemistry are physically linked by a hairpin adapter, they are not physically linked in the 1D2 sequencing chemistry; this could likely be the cause of differences in tandem repeat length distribution between 2D and 1D2 experiments.

Read re-orientation and tandem repeat removal significantly improves sequence quality. BLASTn analyses of INC-Seq reads against reference database composed of 16S rRNA gene sequences of one (Run1 and Run4), or ten organisms (Run 2 and 3) revealed that a combination of incorrect read orientation and presence of tandem repeats significantly affected overall sequence quality. While the average sequence similarity between INC-Seq consensus reads and the reference

303 sequence was 97±0.37%, the portion of the INC-Seq consensus read demonstrating a contiguous
304 alignment to the reference sequence varied significantly (Figure 5); the remaining section of the
305 read typically resulted in shorter secondary alignments with similar sequence similarity to that of
306 the primary alignment. However, post chopSeq the average proportion of the read aligning to the
307 reference sequences increased to 96±2.3% with additional step of discarding reads less than 1300
308 bp and greater than 1450 bp increasing the average proportion of the read aligned to 98.4±0.7%
309 while the sequence similarity between chopSeq (97.5±0.42%) and chopSeq followed by size
310 selection (chopSeq_SS) (98±0.23%) remained similar to or slightly better than INC-Seq processed
311 read. This demonstrates that read reorientation and tandem repeat removal resulted in
312 reconstruction of reads with a high level of similarity to the reference sequences (Figures 5).

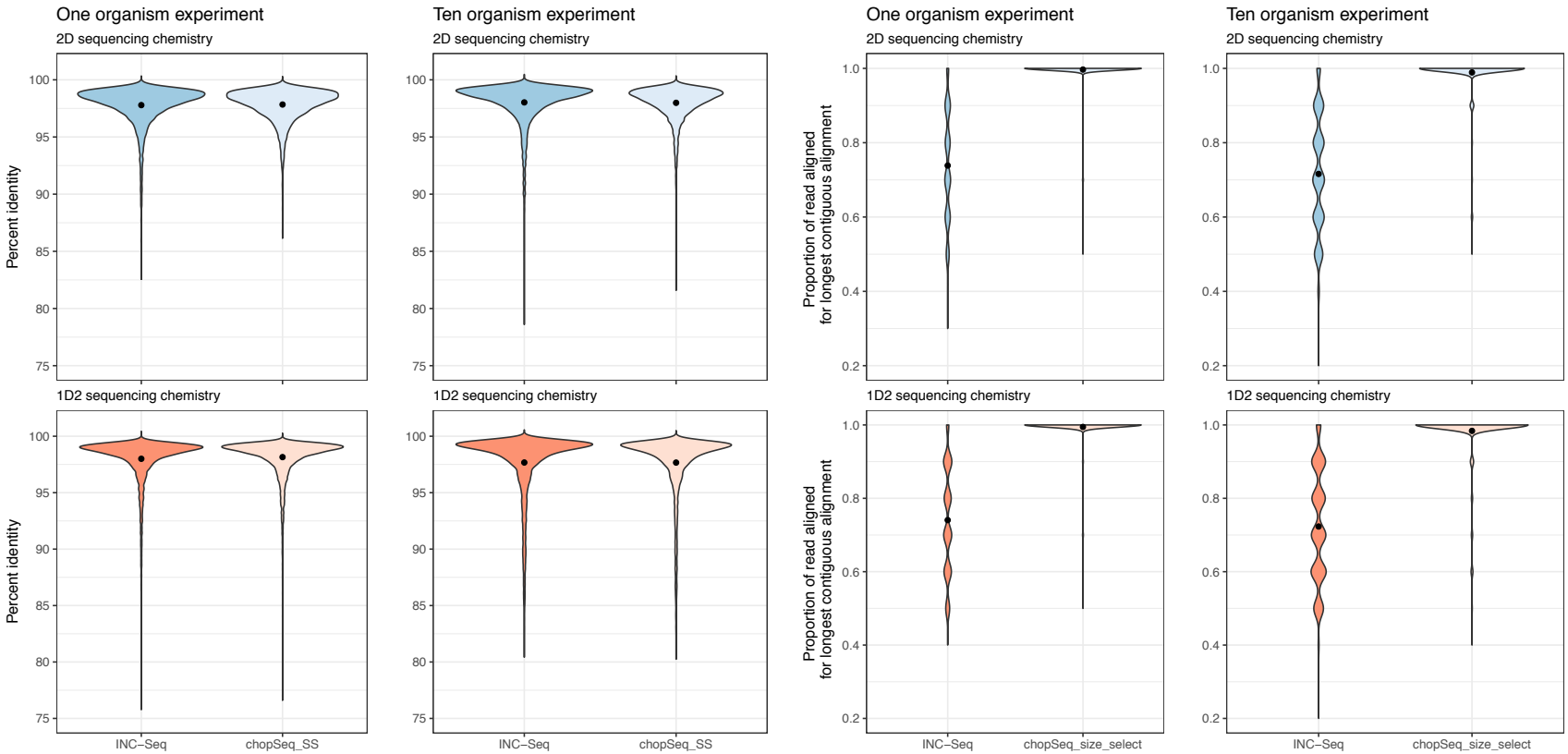


Figure 5: While the distribution of percent identities of INC-Seq and chopSeq processed reads (chopSeq_SS) to reference sequences was on average 97-98%, variable lengths of the INC-Seq processed reads aligned to the reference sequences. In contrast, nearly the entire length of the chopSeq processed reads aligned to the reference sequence without affecting overall sequence similarity. Results are only shown for INC-Seq reads generated using blastn aligner.

Inspections of the read to reference alignment length ratio indicated that the primary source of sequence error for both INC-Seq and chopSeq corrected reads originated from deletions; i.e., the majority of reads had a read to reference alignment ratio lower than 1. While deletions in reads were also strongly associated with sequence accuracy for post chopSeq and size selected reads, a small proportion of chopSeq corrected reads showed read to reference alignment ratios of greater than 1 (Figure 6), suggesting that insertions were less prominent than deletions.

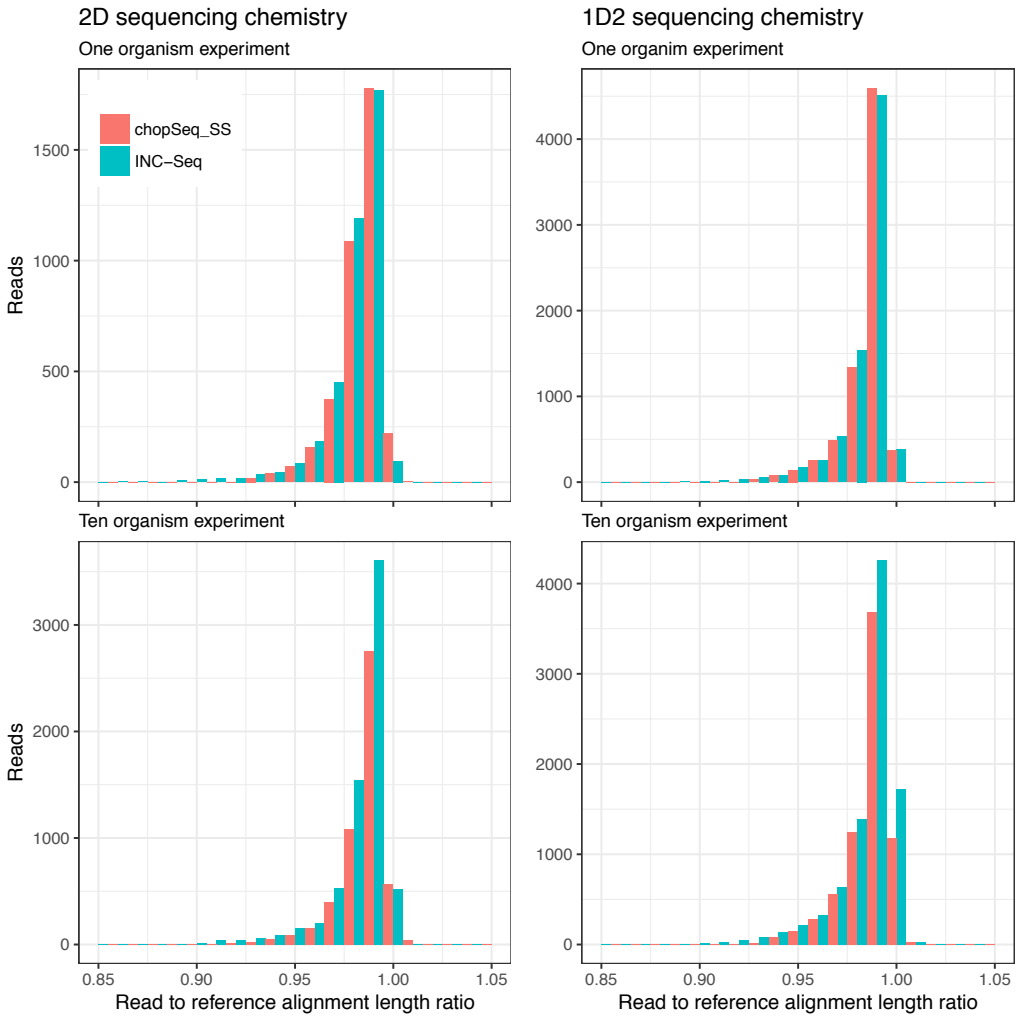


Figure 6: The ratio of the alignment length of INC-Seq and chopSeq corrected reads to that of the corresponding reference sequences was consistently lower than 1, suggesting that deletions in the INC-Seq and chopSeq corrected reads were the primary cause of dissimilarity with the reference sequences. Results are only shown for INC-Seq reads generated using blastn aligner.

329 ***De novo* clustering of chopSeq corrected sequences followed by within cluster consensus**
330 **calling significantly enhances sequence accuracy.** The overall sequence accuracy increased to
331 an average of 97.9±0.23% following chopSeq read correction and size selection with 98±0.23%
332 of the read aligning to the reference (Figure 5). However, approximately 5 and 10% of reads for
333 the 2D and 1D2 runs, respectively, exhibited sequence accuracy of less than 95% with some
334 sequences aligning over less than 50% of the read length even after chopSeq correction and size
335 selection. These poor-quality reads could not be selectively filtered out based on any commonly
336 used quality filtering criteria (e.g., maximum homopolymers length, primer mismatches, etc.) and
337 significantly affected clustering of reads into OTUs. For instance, VSEARCH based clustering of
338 full length post chopSeq and size selected reads (INC-Seq aligner: blastn) at a 97% sequence
339 similarity threshold resulted in 817 (with 777 singletons) and 1301 (with 1238 singletons) for 2D
340 and 1D2 data for single organism experiment and 2122 (with 1742 singletons) and 2725 (with
341 2447 singletons) for 2D and 1D2 data for 10 organism experiments. We hypothesized that accrual
342 of residual errors over the entire read length hampered the accuracy of the OTU clustering and that
343 accurate clustering was more likely over shorter regions of the reads due to fewer absolute errors.
344 To this end, we developed nanoClust which utilizes partitioning of reads in user-defined lengths,
345 followed by application of VSEARCH within each partition for dereplication, singleton removal,
346 chimera detection and removal, clustering at user-defined sequence similarity threshold (i.e., 97%
347 sequence similarity in this study), followed by within cluster sequence clustering and consensus
348 calling.
349
350 We tested the effect of the choice of partition length on the estimation of the richness of the mock
351 communities (i.e., number of observed OTUs) and overall sequence accuracy post within-OTU

352 MAFFT-G-INS-i alignment and consensus sequence construction. To this effect, we varied the
353 number of partitions from one (i.e., partition length of 1300 bp) to seven (i.e., partition length 180
354 bp). With increasing number of partitions (i.e., decreasing partition length), the number of OTUs
355 being detected were significantly inflated above the theoretical threshold while at the same time
356 the average sequence accuracy decreased (Figure 7).

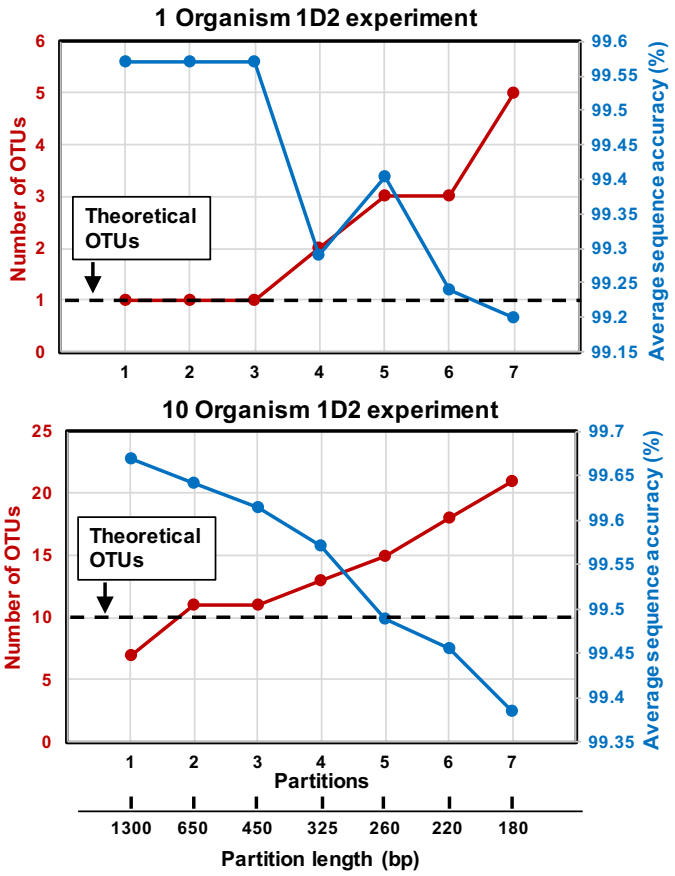


Figure 7: Increasing the number of partitions (and decreasing partition length) during nanoClust processing results in inflation in the number of OTUs observed and a decrease in overall sequence accuracy.

357

358 This was consistent for both the one organism and 10 organism experiments for both 1D2 and 2D
359 experiments (Figure 7). As the partition size decreased the number of OTUs decreased and the
360 overall sequence accuracy increased. The highest average sequence was observed with a single
361 partition, but the number of OTUs was lower than theoretical for the 10-organism experiment.

362 Further, using a single partition approach also resulted in discarding a significant number of
363 sequences that were deemed singletons prior to OTU clustering. Specifically, while three less
364 OTUs were detected in the single partition approach the total number of sequences retained post
365 clustering were 20% lower as compared to when two or three partition approach was used.
366 Considering the tradeoff between sequencing depth (and the resultant impact on detection of lower
367 abundance OTUs), the extent of deviation from the theoretical number of OTUs and overall
368 sequence accuracy, we recommend using either the two or three partition approach which result in
369 similar outcomes on all three metrics.

370

371 **Table 2**: Number of OTUs detected and consensus sequence accuracy for all experiments using the
372 nanoClust for OTU clustering and consensus calling approach.

	Number of OTUs				Average Consensus Accuracy (%)			
Protocol	**2D**	**1D2**	**2D**	**1D2**	**2D**	**1D2**	**2D**	**1D2**
Experiment	**One organism**	**One organism**	**Ten organism**	**Ten organism**	**One organism**	**One organism**	**Ten organism**	**Ten organism**
Run	1	4	2	3	1	4	2	3
Theoretical	1	1	10	10				
INC-Seq aligner	**Blastn**							
OTUs detected	1	1	11	11	99.36	99.5	99.47	99.61
Spurious OTUs	0	0	1	1	-	-	99.22	99.37
Non-Detect	0	0	0	0	-	-	-	-
INC-Seq aligner	**Graphmap**							
OTUs detected	1	1	11	11	99.43	99.43	99.44	99.61
Spurious OTUs	0	0	1	1	-	-	99.29	99.5
Non-Detect	0	0	0	0	-	-	-	-
INC-Seq aligner	**POA**							
OTUs detected	1	2	10	12	99.5	99.61	99.60	99.52
Spurious OTUs	0	1	0	2	-	99.57	-	98.67
Non-Detect	0	0	0	0	-	-	-	-

373

374 The nanoClust approach with three partitions was far superior to the direct clustering of full-length
375 reads and resulted in an accurate determination of the number of OTUs, with one-two spurious
376 OTUs (when using 3 concatemers threshold for INC-Seq) and no false negatives (Table 2)
377 depending on the type of experiment and INC-Seq aligner used. MAFFT-G-INS-i alignment of

378 50 reads from each OTU resulted in consensus reads with the entire read aligned to the reference,
379 and average consensus read of 99.5% and accuracy values for individual OTUs ranging from 99.2
380 to 100%. Nearly all errors in the nanoClust consensus reads originate from single base pair
381 deletions in a few homopolymers regions (homopolymers > 4 bp) and no detectable insertions,
382 with one-two mismatches associated with the spurious OTUs (Figure 8). While accurate OTU
383 estimation allowed for single OTUs to be detected in the one organism experiment, the overall
384 community structure deviated from the theoretical community structure for the ten organism
385 experiments (Figure S4) and thus additional protocol optimization is essential to ensure levels of
386 deviation from theoretical community structure does not exceed what may be seen from PCR
387 biases [33]. Phylogenetic analyses of the consensus sequences demonstrated close placement of
388 the OTU consensus sequences with their corresponding references, with excellent pairwise
389 alignment between the two (Supplementary Figure S3).

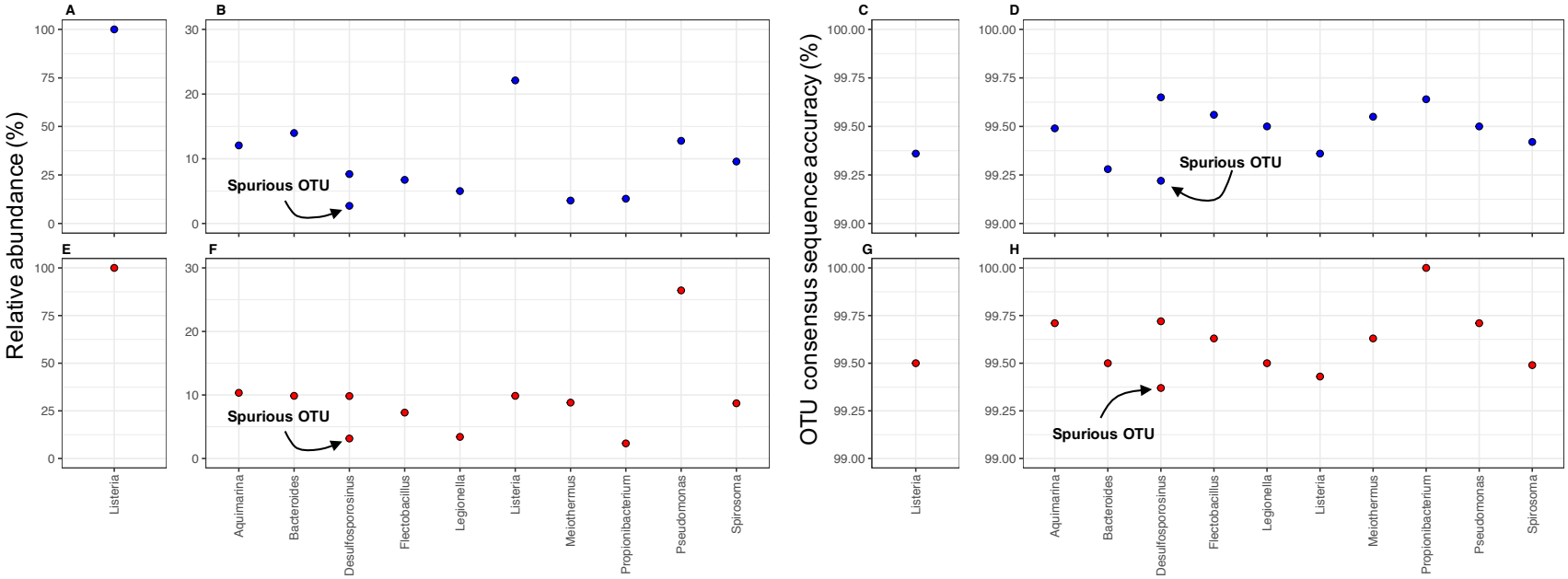
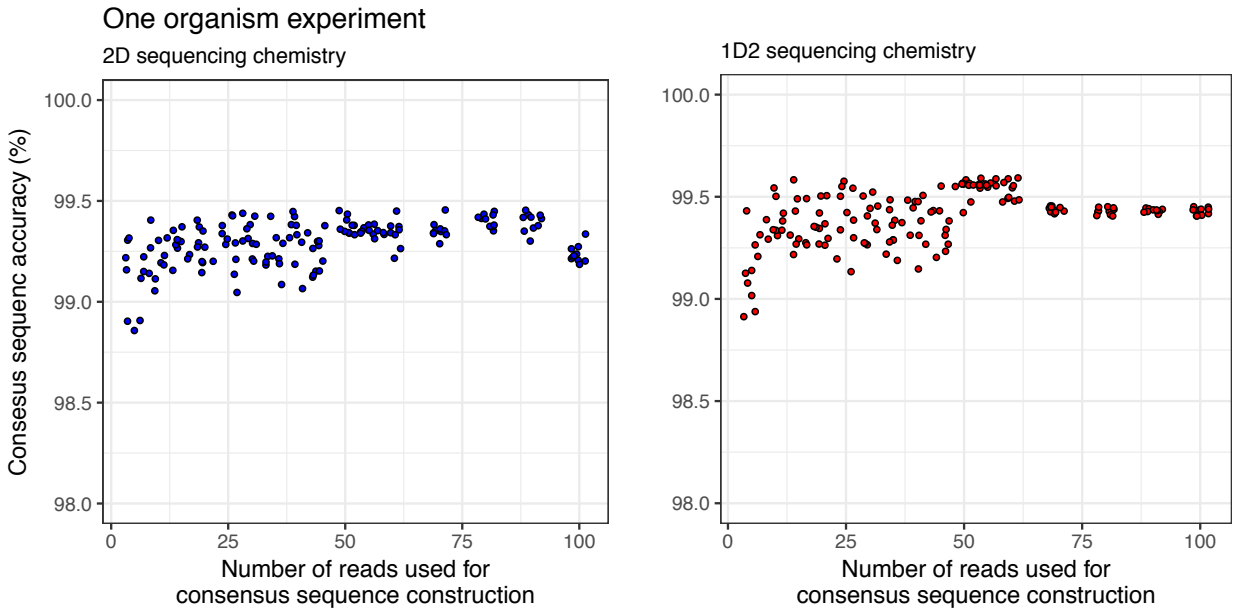


Figure 8: Relative abundance of OTUs for one (A, E) and ten organism experiments (B, F) for 2D (blue data points) and 1D2 (red data points) experiments post nanoClust when using blastn algorithm during INC-Seq. nanoClust clustering and consensus sequence generation resulted in few spurious OTUs and average similarity to the reference sequence of ~ 99.5%. Results are shown for one (C, G) and ten organism experiments (D, H) for 2D (blue data points) and 1D2 (red data points) experiments with the use of blastn during INC-Seq. The results were similar for Graphmap and POA alignment methods used during INC-Seq.

395 The nanoClust implementation in this study included a specified threshold of a maximum of 50
396 reads per OTU to generate OTU consensus sequences. This was feasible because our study
397 focusses on a single organism and even mock community of ten organisms. Thus, the process of
398 consensus construction was not limited by the number of reads that could be recruited. However,
399 it would be critical to determine the potential for poor quality consensus sequence due to fewer
400 reads with an OTU in naturally derived mixed microbial communities. To this end, we varied the
401 number of reads used for consensus sequence construction from 5 to 100 for 2D and 1D2 data
402 from the one organism experiment (Figure 9).



403

404 **Figure 9**: Consensus sequence accuracy plateaus with the use of 10-15 reads for MAFFT-G-INS-i alignment and consensus
405 calling. However, with increasing number of reads used for consensus calling the variability in consensus sequence accuracy from
406 repeated sampling of data diminishes significantly for both 2D and 1D2 sequencing chemistry. Data is shown for one organism
407 experiment where the blastn aligner was used during INC-Seq.

408 The consensus sequence accuracy surpasses 99% with the use of more than five reads for
409 consensus sequence construction and plateaus at approximately 10-15 reads. However, the
410 variability in accuracy with repeated random sampling of data was much more pronounced when
411 fewer than 50 reads were used for both 2D and 1D2 data. This suggests that consensus sequence

412 accuracy is reliably high only for OTUs where a minimum of 50 reads are available for use in
413 constructing the consensus sequence. This would have an impact on sequence quality of low
414 abundance OTUs.
415
416 **NanoAmpli-Seq based improvements in sequence accuracy are not primarily associated with**
417 **changes in nanopore sequencing chemistry.** The INC-Seq study [21] utilized data generated
418 from flow cells with R7 pores, while the present study used R9.4 and 9.5 pores with reported
419 higher sequencing accuracy. Improvements in sequencing chemistry and basecalling allowed us
420 to reduce the concatemer threshold for INC-Seq from six to three, which significantly increased
421 the amount of data used for analysis. The second significant improvement of the updated
422 sequencing chemistry is the much higher sequencing output. However, neither of these
423 improvements result in improved data quality post INC-Seq processing alone (Figure 5). Thus, the
424 chopSeq and nanoClust algorithms are critical for obtaining 99.5% sequence accuracy.
425
426 To demonstrate this, we re-processed the "Ladder replicate" data made available through the
427 original INC-Seq study [21] using the NanoAmpli-Seq workflow. While re-analyzing R7
428 chemistry generated data from Li et al [21], we detected tandem repeats and incorrect primer
429 orientation issue highlighted in this study. chopSeq was successfully able to remove tandem
430 repeats and re-orient reads such that nearly the entire length of the read was now correctly aligned
431 to the reference sequence for most of the reads. Thus, while INC-Seq reaches a median sequence
432 accuracy of 97-98% (as described in the Li et al. [21]), post-processing by chopSeq improves read
433 quality through read re-orientation and tandem repeat removal (Figure 10).

434

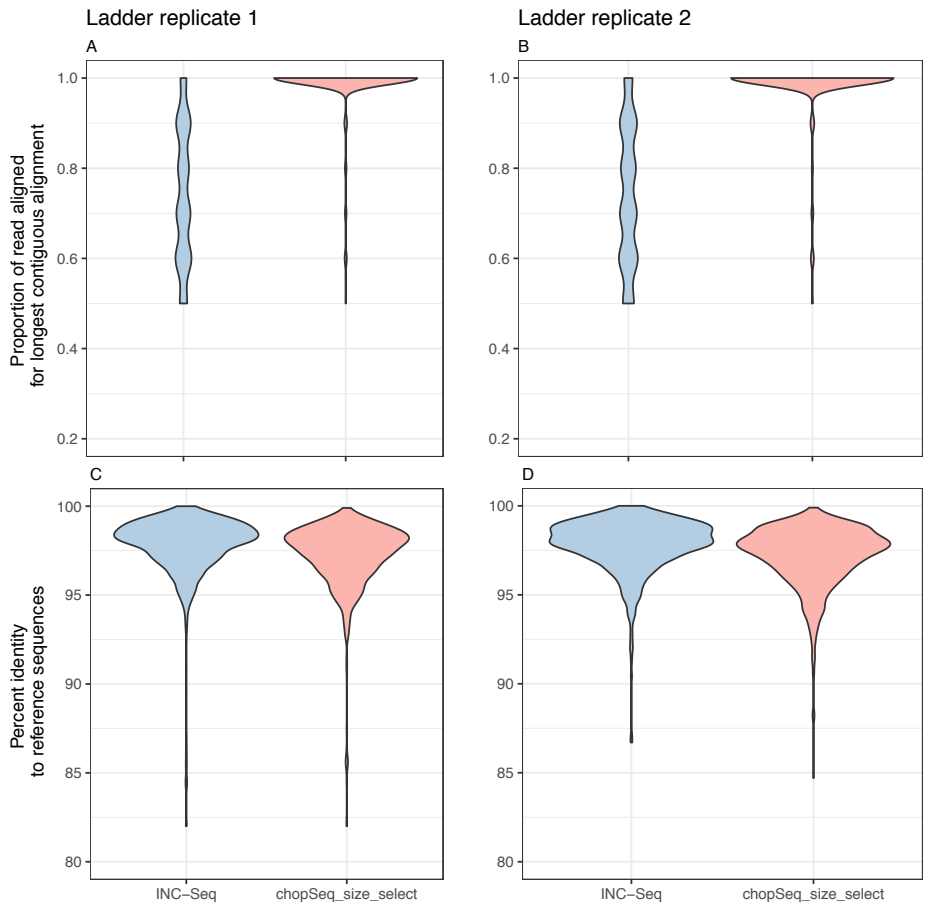


Figure 10: chopSeq based read re-orientation and tandem repeat removal allowed for nearly the entire length of the read to be aligned to the reference sequences (A, B) while maintaining the median sequence accuracy of INC-Seq consensus reads to ~97-98%.

450 Furthermore, nanoClust based clustering and consensus calling results in an average sequence
451 accuracy of 99.5% for the data generated by Li et al [21]. The data generated by Li et al. [21]
452 included the V3-V6 region of the 16S rRNA gene from 10-organism mock community with a
453 staggered community structure including closely related organisms. This resulted in a theoretical
454 number of 8 OTUs at 97% sequence similarity. Specifically, *Staphylococcus aureus* and
455 *Staphylococcus epidermis* clustered into a single OTU at 97% sequence similarity (their 16S rRNA
456 gene V3-V6 hypervariable regions are 98.8% similar to each other) and *Klebsiella pneumoniae*

457 and *Salmonella typhimurium* clustered into a single OTU at 97% sequence similarity (their 16S
458 rRNA gene V3-V6 hypervariable regions are 97.6 % similar to each other). Further, the Li et al.
459 [21] only generated 2100 INC-Seq consensus reads combined for the two replicate sequencing
460 runs. As a result, two of the low abundance OTUs with a relative abundance of 0.2% (*Neisseria*),
461 and 0.1% (*Faecalibacterium*) were not detected after processing with chopSeq and nanoClust. This
462 non-detection of low abundance OTUs is primarily a function of low sequencing depth rather than
463 of the NanoAmpli-Seq workflow. Further, the sequence accuracy of most of the detected OTUs
464 was in excess of 99.5%, while that of a single OTU (i.e., *Fusobacterium*) was 98.75% (Figure 11).
465 Thus, we conclude that incorporating chopSeq correction of INC-Seq consensus reads followed
466 by nanoClust based clustering and consensus calling was vital for improved sequence accuracy,
467 irrespective of the changes in the sequencing chemistry.

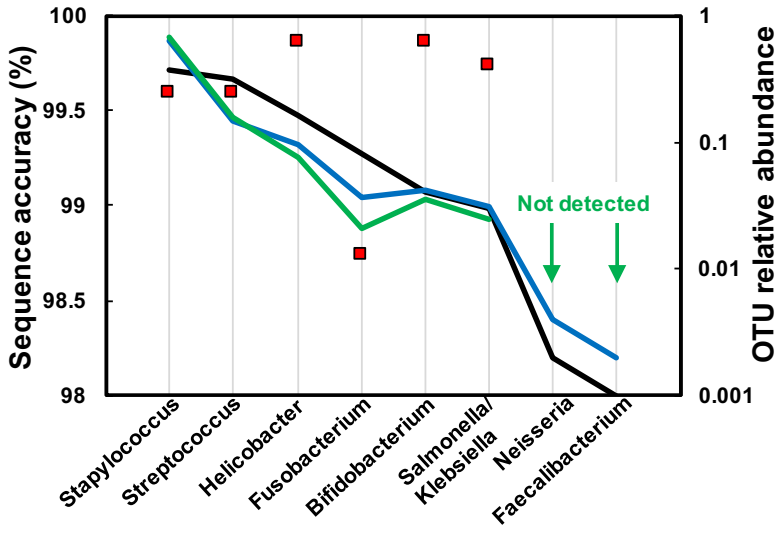


Figure 11: Sequence accuracy of detected OTUs post nanoClust for combined ladder replicate was ~99.5% (red squares). Two OTUs were not detected post nanoClust processing (green line) due to their low abundance (<0.5%) in the constructed mock community (black line). The blue line shows the relative abundance as reported by Li et al.

468

469

470

471 **Discussion and conclusions**

472 The current study uses mock communities to develop and validate the NanoAmpli-Seq workflow
473 for long amplicon sequencing on the nanopore sequencing platform. While this study focusses on
474 the near full-length 16S rRNA gene, in principle the approach outlined by the NanoAmpli-Seq
475 workflow should be amenable to amplicons generated from PCR amplification of any target gene
476 irrespective of target gene length. While leveraging the previously described INC-Seq protocol,
477 NanoAmpli-Seq adds several novel components which significantly enhances the amplicon
478 sequencing workflow for the nanopore platform. The improvements over the previously described
479 INC-Seq protocol involve modifications to both library preparation (i.e. PrimPol based primer
480 synthesis for RCA, debranching and fragmentation, shorter protocol length) and the data analyses.
481 Specifically, we identify and fix the issues associated with incorrect read-orientation and presence
482 of tandem repeats in INC-Seq consensus reads, thus allowing for nearly the entire length of the
483 chopSeq corrected reads to be aligned to the reference with accuracies (97-98%) similar to those
484 described by Li et al [21]. While the original INC-Seq protocol prescribed a concatemer threshold
485 of six, we halved the concatemer threshold to three. Thus, more than doubling the number of INC-
486 Seq consensus reads available as a proportion of the base called reads. We could use a lower INC-
487 Seq concatemer threshold due to both enhances in basecalling and sequencing accuracy of the
488 nanopore platform [34]and the ability to perform another round of alignment and consensus calling
489 during the nanoClust step.

490

491 The correction of INC-Seq consensus reads using chopSeq did not allow for sequences with high
492 enough quality for direct OTU clustering using VSEARCH. However, the read partitioning-based
493 sequence clustering allowed for accurate determination of the number of OTUs in the mock

494 community. Further, *de novo* sequence clustering using nanoClust provided the opportunity to
495 significantly increase the number of sequences used for consensus calling. In this study, we used
496 50 reads for consensus calling (i.e. 150x coverage considering three concatemer threshold set for
497 INC-Seq) which resulted in average sequence accuracy of 99.5%. The use of more than 50 reads
498 for consensus calling in nanoClust did not improve sequence accuracy while reducing the number
499 of reads resulted in reduced precision. This threshold of 50 reads for both the 2D and 1D2
500 sequencing data suggests that the OTUs with fewer than 50 reads are likely to have sequence
501 quality lower than those OTUs with greater than 50 reads. It should however be noted that using a
502 10 read threshold (i.e., 30x coverage when including three concatemer threshold for INC-Seq)
503 consistently allowed for sequence accuracy consistently higher than 99% and thus sequence
504 classification to species level using any of the current sequence classification approaches [35, 36]
505 (i.e., RDP classifier) would be reliable even for lower abundance OTUs.
506
507 While the NanoAmpli-Seq workflow represents a significant improvement in amplicon
508 sequencing on the nanopore platform, some fundamental limitations remain. For instance, the
509 NanoAmpli-Seq sequence accuracy is still lower than those reported for short amplicons [3] or
510 those generated from the assembly of SSU rRNA from metagenomic sequencing on the Illumina
511 Platform [7, 9], and the full-length 16S rRNA sequencing on the PacBio platform using the
512 approach described previously [17]. Our analysis shows that the sequence accuracy does not
513 improve with more than 50 sequences used in the nanoClust based consensus calling process.
514 Nearly all the errors in the OTU consensus sequences originate from single deletions at
515 homopolymers regions, specifically for homopolymers greater than 4 bp. This homopolymer error
516 issue on the nanopore platform is well known [14, 37] and is likely to best resolved during the base

517 calling process rather than subsequent data processing or by processing signal data (rather than
518 base called data) all the way through clustering, followed by base calling as the final step. The
519 second limitation of our approach is the low data yield at the base calling step, i.e. base called
520 reads represents only a small portion (i.e., 7-9% for 1D2 data) of the raw records. This data loss is
521 significant and could potentially deter the widespread use of the nanopore platform for amplicon
522 sequencing. While the precise cause of the low yield of pass reads post base calling is unclear, the
523 proportion of pass reads in our study are not significantly different from those reported elsewhere.
524 One current option would be to directly work with 1D rather than 1D2 data. However, the
525 maximum sequence accuracy of 1D reads post INC-Seq consensus construction was only 94% and
526 unsuitable for processing with chopSeq and nanoClust. The NanoAmpli-Seq workflow includes a
527 *de novo* clustering step, and as long as the sequence accuracy post chopSeq is ~97% (3-10
528 concatemers required), the binning process should provide for sufficient coverage for consensus-
529 based sequence correction to accuracies in excess of 99%. The final limitation of our approach is
530 that the nanoClust relies on generating consensus sequences from multiple DNA sequences and
531 thus there is the likelihood of clustering and generating a multi-species consensus from closely
532 related species, i.e. those within 97% sequence similarity to each other. While we did not find
533 evidence for this "multispecies consensus sequence" while analyzing data from Li et al. [21] which
534 included closely related organisms, this possibility cannot be ignored. And thus, we recommend
535 that researchers refrain from depositing NanoAmpli-Seq processed sequences in publicly available
536 references databases, but utilize this approach for rapid screening of mixed microbial communities
537 and limit the use of NanoAmpli-Seq processed data for within-study sample microbial community
538 comparisons. Future improvement to avoid the likelihood of "multispecies consensus sequence"
539 would be to utilize primers with barcodes consisting of random N bases (i.e., unique molecular

540 tags), similar to that used by Karst et al. This could allow clustering of reads originating from the
541 same original sequence using the unique molecular tags.
542
543 **Methods**
544 Mock community description and preparation
545 Two different mock communities were constructed for the experiments outlined in this study. First,
546 a single organism mock community was constructed by amplifying the near full-length of the 16S
547 rRNA gene from genomic DNA of *Listeria monocytogens* using primers sets 8F (5'-
548 AGRGTTTGATCMTGGCTCAG-3') and 1387R (5'-GGGCGGWGTGTACAAG-3'), both with
549 5' phosphorylated primers (Eurofins Genomics) [26]. Phosphorylated ends are essential for the
550 subsequent self-ligation step. PCR reaction mix was prepared in 25µl volumes with use of 12.5µl
551 of Q5® High-Fidelity 2X Master Mix (New England BioLabs Inc., M0492L), 0.8µl of 10pmol of
552 each primer, 9.9µl of nuclease-free water, (Roche Ltd.) and 1ng of bacterial DNA in total followed
553 by PCR amplification as described previously[26]. PCR amplicons from replicate PCR reactions
554 were combined and purified with use of HighPrep™ PCR magnetic beads (MagBio, AC-60050)
555 at 0.45x ratio. The ten organism mock community was constructed from purified near full-length
556 16S rRNA amplicons of 10 organisms. Briefly, genomic DNA from 10 bacteria were obtained
557 from DSMZ, Germany (Supplementary Table S1) and the aforementioned primers, PCR reaction
558 mix and thermocycling conditions were used to independently PCR amplify the near full-length
559 16S rRNA gene, followed by purification using HighPrep™ PCR magnetic beads as detailed
560 above. The purified amplicons from each organism were quantified on the Qubit using dsDNA HS
561 kit, normalized to 4ng/µl, and combined to generate an amplicon pool consisting of an equimolar
562 proportion of the 16S rRNA gene amplicons of the 10 organisms.

563 DNA sequencing library preparation.

564 To circularize the linear amplicons into plasmid-like structures, 5µl of Blunt/TA Ligase Master
565 Mix (New England Biolabs, M0367L) was added to 55 µl of amplicon pool at a concentration of
566 1ng/µl and incubated for 10min at 15°C then 10min at room temperature to (total time = 20
567 minutes). Not all linear amplicons self-ligate into plasmid-like structures, but some are likely to
568 cause long chimeric linear amplicons. These long chimeric structures were removed using
569 magnetic bead-based purification with the following modifications. HighPrep™ PCR magnetic
570 beads were homogenized by vortexing followed by aliquoting 50µl into a sterile 2ml tube and
571 placed on a magnetic rack for 3min. A total of 25µl of the supernatant was carefully removed using
572 a sterile pipette to concentrate the beads to 2x its original concentration. The tube was removed
573 from the magnetic rack and vortexed vigorously to resuspend the beads. This concentrated bead
574 solution was used at a ratio of 0.35x to remove any amplicons greater than 2000 bp in the post-
575 ligation reaction mix. Briefly, the post-ligation product was mixed with concentrated bead solution
576 at the 0.35x ratio by vortexing followed by incubation for three minutes at room temperature. The
577 tube was placed on the magnetic rack to separate the beads from solution, followed by transferring
578 of clear liquid containing DNA structures less than 2000 bp into new sterile tubes. Sample
579 containing short self-ligated molecules was subject to another round of concentration using
580 standard magnetic beads at 0.5x ratios according to manufacturer instructions and eluted in 15µl
581 of warm nuclease-free water. Concentrated and cleaned DNA pool consisting of plasmid-like
582 structures and remaining linear amplicons was then processed with Plasmid-Safe™ ATP-
583 Dependent DNase (Epicentre, E3101K) reagents to digest linear amplicons using the mini-prep
584 protocol according to manufacturer instructions and was followed by another round of cleanup

585 with magnetic beads at 0.45x ratio as described before and then eluted in 15µl of warm nuclease-
586 free water.
587
588 The pool containing plasmid-like structures was subject to RCA with use of TruPrime^{TM} RCA Kit
589 (Sygnis, 390100) random hexamer-free protocol. Samples were prepared in triplicate and
590 processed according to manufacturer protocol with all incubations performed in triplicate for 120-
591 150 min depending on the assay efficiency. The progress of RCA was monitored by measuring the
592 concentration of DNA using Qubit® 2.0 Fluorometer at 90, 120 or 150 min time points. The
593 negative control sample, consisting of reagents without any circularized plasmid-like amplicons,
594 were processed and analyzed concomitantly with the samples. The final concentration of the RCA
595 product after 150 minutes of incubation was typically 70 ng/µl when using a starting DNA
596 concentration of 0.35-0.4 ng/µl with no detectable unspecific product formation in the negative
597 control.
598
599 Replicates RCA products were combined (~4.5µg of DNA in total) and subject to de-branching
600 and fragmentation of post-RCA molecules to remove hyperbranching structures generated during
601 RCA. The RCA product was first treated with T7 endonuclease I enzyme (New England BioLabs,
602 M0302S) by adding 2µl of the reagent to the 65µl of RCA product followed by vortexing and
603 incubation as recommended by the manufacturer. Subsequently, the reaction mix was transferred
604 into a g-TUBE (Covaris, 520079) and centrifuged at 1800 rpm for 4min or until the entire reaction
605 mix passed through the fragmentation hole. The g-TUBE was reversed, and centrifugation process
606 was repeated. Post debranching and fragmentation, short fragments were removed using the
607 modified bead-based cleanup step using concentrated bead solution (see above for concentration

608 procedure). Concentrated beads were mixed with fragmented RCA product at a 0.35x ratio,
609 vortexed for 15sec, and incubated at room temperature for 3min then placed on a magnetic rack
610 until the beads separated and the supernatant was removed. The beads were subsequently washed
611 with 70% freshly prepared ethanol according to manufacturer protocols. Size-selected amplicons
612 bound to the beads were eluted in 41µl of warm nuclease-free water. Preliminary experiments
613 indicated that one round of de-branching did not completely resolve the hyperbranching structure,
614 which was inferred based on poor sequencing yield likely caused due to pore blocking by
615 hyperbranched DNA. As a result, a second round of enzymatic de-branching using T7
616 endonuclease I was added and the de-branched product was cleaned a second time using the bead-
617 based clean-up step. Figure S4 shows an example BioAnalyzer traces of the RCA product post-
618 debranching/fragmentation and post-cleanup using magnetic bead-based protocol.
619
620 Finally, the de-branched RCA product was treated with NEBNext® FFPE DNA Repair Mix (New
621 England BioLabs, M6630S) for gap filling and DNA damage repair caused during g-TUBE
622 fragmentation and T7 endonuclease I enzyme. All reagent components were combined with de-
623 branched RCA product according to manufacturer recommendations and incubated at 12°C for
624 10min then at 20°C for another 10min. Post incubation, the repaired RCA product was cleaned
625 using standard magnetic beads at 0.5x ratio, washed with 70% ethanol, and eluted in 46µl of warm
626 nuclease-free water. The concentration of the DNA product was measured using Qubit and was
627 approximately 20-25 ng/µl with a total yield of ~1000 ng of DNA with product size typically
628 ranging from 1500bp to 20,000 bp. A total of 45µl DNA pool of concatamerized amplicons was
629 prepared for sequencing using the standard 2D and 1D2 library preparation protocol by Oxford
630 Nanopore Technologies (SQK-LSK208, SQK-LSK308) according to manufacturer specifications

631 to obtain pre-sequencing mix. Moreover, the final concentration for prepared libraries was
632 determined using dsDNA HS kit on the Qubit instrument. A detailed step-by-step protocol is
633 provided in the supplementary text.
634
635 DNA sequencing.
636 The MinION MkIB was connected to Windows personal computer compatible with Oxford
637 Nanopore Technologies requirements. R9.4 (FLO-MIN106) and R9.5 (FLO-MIN107) flow cells
638 were placed onto the MinION Mk1B (Oxford Nanopore Technologies). Platform quality control
639 was performed using MinKNOW software (v1.4.2 for 2D and v1.6.11 for 1D2 libraries). Only
640 flow cells containing above 1100 active pores were used in this study. Each flow cell was primed
641 twice according to ONT specifications using priming buffer consisting of equal parts of running
642 buffer (RBF1) and nuclease-free water with 10 min breaks between subsequent primes. The
643 loading mix was prepared with a 12µL pre-sequencing mix, 75µL of RBF1, and 63µL of nuclease-
644 free water. Loading mix was sequenced with MinKNOW settings appropriate for 2D or 1D2
645 options and standard 48h processing time for every run. Albacore 1.2.4 was used to convert raw
646 signals into HDF5 file format using switch options FLO-MIN106 and SQK-LSK208 for 2D data
647 and FLO-MIN107 and SQK-LSK308 for 1D2 data.
648
649 Data processing
650 HDF5 raw signals from each sequencing run were converted to FASTQ format using Fast5-to-
651 Fastq (https://github.com/rrwick/Fast5-to-Fastq) and then from FASTQ to FASTA with seqtk
652 (https://github.com/lh3/seqtk). The resultant data was subject to INC-Seq
653 (https://github.com/CSB5/INC-Seq) processing using blastn, Graphmap, and POA aligners with

654 concatemer threshold of 3 with the iterative flag for consensus error correction using
655 PBDAGCON. INC-Seq consensus reads were subject to chopSeq by specifying forward and
656 reverse primer sequences and upper (1450 bp) and lower (1300 bp) read size thresholds for size
657 selection (chopSeq_SS). The chopSeq processed reads were subsequently processed using
658 nanoClust with partition size limits (flag "-s") of 0,450,451,900,901,1300 which splits the reads
659 into three partitions of 450, 450, and 400 bp respectively prior to further processing. VSEARCH
660 was used for chimera removal (using uchime) followed by clustering of reads in each partition at
661 a hardcoded sequence similarity threshold of 97%. For the optimal binning results, nanoClust then
662 outputs consensus sequence for each OTU based on MAFFT-G-INS-i pairwise alignment as
663 described previously and outputs an OTU table with reads corresponding to each OTU after
664 discarding singletons.
665
666 Fasta files from all stages (i.e. raw, INC-Seq, chopSeq, chopSeq_SS, and OTU consensus
667 sequence) were analyzed for read lengths in R [38]. The number of concatemers on each raw read
668 was estimated by dividing the length of each raw read with the length of its corresponding INC-
669 Seq consensus read. At each stage of processing, the reads were aligned to reference dataset using
670 blastn, and only the match with the highest bitscore was considered. The ratio of read to reference
671 alignment at appropriate points (as discussed above) was estimated based on blastn results. The
672 percent identity from the blastn results were used to measure consensus sequence accuracy for the
673 nanoClust output. All figures for the manuscript were generated in R using packages "ggplot2",
674 "gridExtra", and "cowplot" (https://github.com/wilkelab/cowplot), as appropriate. Neighbor-
675 Joining tree construction (Figure S3 and S4) was performed after muscle alignment [39] (default

676 parameters) and using Jukes-Cantor model was constructed in Geneious (version 8) using 100
677 bootstraps.
678
679 **Availability and requirements**
680 Project name: NanoAmpli-Seq
681 Project home page: https://github.com/umerijaz/nanopore
682 Operating system: Linux
683 Programming language: Python
684
685 **Availability of supporting data**
686 All data is available on European Nucleotide Archive (ENA) under primary accession number:
687 PRJEB21005.
688
689 **Abbreviations:**
690 ONT, Oxford Nanopore Technologies; RCA, Rolling circle amplification; SMRT, single
691 molecular real-time; INC-Seq, Intramolecular-ligated Nanopore Consensus Sequencing
692
693 **Declarations**
694 **Acknowledgements**: This research was supported by EPSRC award no: EP/M016811/1. STC is
695 supported by the EPSRC Doctoral Training Center at University of Glasgow. UZI is supported by
696 a NERC Fellowship (NE/L011956/1).
697

698 **Author contribution:** STC, UZI, and AJP designed and developed the experiments. STC
699 performed the experiments. UZI wrote the code for chopSeq and nanoClust. STC, UZI, and AJP
700 analyzed the data and contributed equally to writing this manuscript.

701

702 **References**

703 1. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J and Fierer N. Ultra-
704 high-throughput microbial community analysis on the Illumina HiSeq and MiSeq
705 platforms. ISME J. 2012;6 doi:10.1038/ismej.2012.8.
706 2. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et
707 al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample.
708 Proceedings of the National Academy of Sciences. 2011;108 Supplement 1:4516-22.
709 doi:10.1073/pnas.1000080107.
710 3. Kozich JJ, Westcott SL, Baxter NT, Highlander SK and Schloss PD. Development of a
711 dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence
712 data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol. 2013;79
713 doi:10.1128/aem.01043-13.
714 4. Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, et al. Nuclear
715 ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker
716 for Fungi. Proceedings of the National Academy of Sciences. 2012;109 16:6241-6.
717 doi:10.1073/pnas.1117018109.
718 5. Goodwin S, McPherson JD and McCombie WR. Coming of age: ten years of next-
719 generation sequencing technologies. Nat Rev Genet. 2016;17 6:333-51.
720 doi:10.1038/nrg.2016.49.
721 6. Yuan C, Lei J, Cole J and Sun Y. Reconstructing 16S rRNA genes in metagenomic data.
722 1367-4811 (Electronic) doi:10.1093/bioinformatics/btv231.
723 7. Miller C, Baker B, Thomas B, Singer S and Banfield J. EMIRGE: reconstruction of full-
724 length ribosomal genes from microbial community short read sequencing data. Genome
725 Biology. 2011;12 5:R44.
726 8. Burke CA-O and Darling AE. A method for high precision sequencing of near full-length
727 16S rRNA genes on an Illumina MiSeq. 2167-8359 (Print).
728 9. Miller CS, Handley KM, Wrighton KC, Frischkorn KR, Thomas BC and Banfield JF.
729 Short-Read Assembly of Full-Length 16S Amplicons Reveals Bacterial Diversity in
730 Subsurface Sediments. PLOS ONE. 2013;8 2:e56018. doi:10.1371/journal.pone.0056018.
731 10. Karst SM, Dueholm MS, McIlroy SJ, Kirkegaard RH, Nielsen PH and Albertsen M.
732 Retrieval of a million high-quality, full-length microbial 16S and 18S rRNA gene
733 sequences without primer bias. Nature Biotechnology. 2018;36:190.
734 doi:10.1038/nbt.4045.
735 11. Mardis ER. DNA sequencing technologies: 2006–2016. Nature Protocols. 2017;12:213.
736 doi:10.1038/nprot.2016.182.
737 12. Wommack KE, Bhavsar J and Ravel J. Metagenomics: Read Length Matters. Applied
738 and Environmental Microbiology. 2008;74 5:1453-63. doi:10.1128/aem.02181-07.

739 13. Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH and Phillippy AM. Canu:
740 scalable and accurate long-read assembly via adaptive k-mer weighting and repeat
741 separation. Genome Research. 2017;27 5:722-36. doi:10.1101/gr.215087.116.
742 14. Loman NJ, Quick J and Simpson JT. A complete bacterial genome assembled de novo
743 using only nanopore sequencing data. Nat Methods. 2015;12 doi:10.1038/nmeth.3444.
744 15. Chin C-S, Peluso P, Sedlazeck FJ, Nattestad M, Concepcion GT, Clum A, et al. Phased
745 diploid genome assembly with single-molecule real-time sequencing. Nature Methods.
746 2016;13:1050. doi:10.1038/nmeth.4035.
747 16. Michael TP, Jupe F, Bemm F, Motley ST, Sandoval JP, Loudet O, et al. High contiguity
748 Arabidopsis thaliana genome assembly with a single nanopore flow cell. bioRxiv. 2017;
749 doi:10.1101/149997.
750 17. Schloss PD, Jenior ML, Koumpouras CC, Westcott SL and Highlander SK. Sequencing
751 16S rRNA gene fragments using the PacBio SMRT DNA sequencing system. PeerJ.
752 2016;4:e1869. doi:10.7717/peerj.1869.
753 18. Benítez-Páez A, Portune KJ and Sanz Y. Species-level resolution of 16S rRNA gene
754 amplicons sequenced through the MinION™ portable nanopore sequencer. GigaScience.
755 2016;5 1:4. doi:10.1186/s13742-016-0111-z.
756 19. Cusco A, Vines J, D'Andreano S, Riva F, Casellas J, Sanchez A, et al. Using MinION to
757 characterize dog skin microbiota through full-length 16S rRNA gene sequencing
758 approach. bioRxiv. 2017; doi:10.1101/167015.
759 20. Kerkhof LJ, Dillon KP, Häggblom MM and McGuinness LR. Profiling bacterial
760 communities by MinION sequencing of ribosomal operons. Microbiome. 2017;5 1:116.
761 doi:10.1186/s40168-017-0336-9.
762 21. Li C, Chng KR, Boey EJ, Ng AH, Wilm A and Nagarajan N. INC-Seq: accurate single
763 molecule reads using nanopore sequencing. GigaScience. 2016;5:34. doi:D - NLM:
764 PMC4970289 OTO - NOTNLM.
765 22. Benitez-Paez A and Sanz Y. Multi-locus and long amplicon sequencing approach to
766 study microbial diversity at species level using the MinION portable nanopore sequencer.
767 Gigascience. 2017;6 7:1-12.
768 23. Shin J, Lee S, Go M-J, Lee SY, Kim SC, Lee C-H, et al. Analysis of the mouse gut
769 microbiome using full-length 16S rRNA amplicon sequencing. Scientific Reports.
770 2016;6:29681. doi:10.1038/srep29681.
771 24. Mitsuhashi S, Kryukov K, Nakagawa S, Takeuchi JS, Shiraishi Y, Asano K, et al. A
772 portable system for rapid bacterial composition analysis using a nanopore-based
773 sequencer and laptop computer. Scientific Reports. 2017;7 1:5657. doi:10.1038/s41598-
774 017-05772-5.
775 25. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA
776 ribosomal RNA gene database project: Improved data processing and web-based tools.
777 Nucleic Acids Research. 2013;41:590-6. doi:10.1093/nar/gks1219.
778 26. Singer E, Bushnell B, Coleman-Derr D, Bowman B, Bowers RM, Levy A, et al. High-
779 resolution phylogenetic microbial community profiling. The Isme Journal. 2016;10:2020.
780 doi:10.1038/ismej.2015.249.
781 27. Picher ÁJ, Budeus B, Wafzig O, Krüger C, García-Gómez S, Martínez-Jiménez MI, et al.
782 TruePrime is a novel method for whole-genome amplification from single cells based on
783 TthPrimPol. Nature Communications. 2016;7:13296. doi:10.1038/ncomms13296.

784 28. Rognes T, Flouri T, Nichols B, Quince C and Mahe F. Vsearch: a versatile open source
785 tool for metagenomics. PeerJ. 2016;4 doi:10.7717/peerj.2584.
786 29. Schloss P, Westcott S, Ryabin T, Hall J, Hartmann M, Hollister E, et al. Introducing
787 mothur: Open Source, Platform-independent, Community-supported Software for
788 Describing and Comparing Microbial Communities. Appl Environ Microbiol. 2009;
789 doi:10.1128/AEM.01541-09.
790 30. Westcott SL and Schloss PD. De novo clustering methods outperform reference-based
791 methods for assigning 16S rRNA gene sequences to operational taxonomic units. PeerJ.
792 2015;3:e1487. doi:10.7717/peerj.1487.
793 31. Katoh K, Misawa K, Kuma K-i and Miyata T. MAFFT: a novel method for rapid
794 multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research.
795 2002;30 14:3059-66.
796 32. Katoh K and Toh H. Recent developments in the MAFFT multiple sequence alignment
797 program. Briefings in Bioinformatics. 2008;9 4:286-98. doi:10.1093/bib/bbn013.
798 33. Pinto AJ and Raskin L. PCR Biases Distort Bacterial and Archaeal Community Structure
799 in Pyrosequencing Datasets. PLoS ONE. 2012;7 8:e43093.
800 doi:10.1371/journal.pone.0043093.
801 34. Jain M, Olsen HE, Paten B and Akeson M. The Oxford Nanopore MinION: delivery of
802 nanopore sequencing to the genomics community. Genome Biology. 2016;17 1:239.
803 doi:10.1186/s13059-016-1103-0.
804 35. Wang Q, Garrity G, Tiedje J and Cole J. Naive Bayesian classifier for rapid assignment
805 of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol. 2007;73
806 16:5261-7. doi:10.1128/AEM.00062-07.
807 36. Werner JJ, Koren O, Hugenholtz P, DeSantis TZ, Walters WA, Caporaso JG, et al.
808 Impact of training sets on classification of high-throughput bacterial 16s rRNA gene
809 surveys. ISME J. 2012;6 1:94-103. doi:10.1038/ismej.2011.82.
810 37. Feng W, Zhao S, Xue D, Song F, Li Z, Chen D, et al. Improving alignment accuracy on
811 homopolymer regions for semiconductor-based sequencing technologies. BMC
812 Genomics. 2016;17 7:521. doi:10.1186/s12864-016-2894-9.
813 38. RCoreTeam. R: A Language and Environment for Statistical Computing R Foundation
814 for Statistical Computing, Vienna, Austria, 2014.
815 39. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high
816 throughput. Nucleic Acids Research. 2004;32 5:1792-7. doi:10.1093/nar/gkh340.

NanoAmpli-Seq: A workflow for amplicon sequencing for mixed microbial communities on the nanopore sequencing platform.

Szymon T Calus, Umer Z Ijaz, Ameet J Pinto

Protocol for sample preparation for NanoAmpli-Seq workflow.

Consumables required:

1. Sterile Filtered pipette tips from any vendor
2. 0.2 ml PCR grade tubes from any vendor
3. Wide Bore Filtered pipette tips from any vendor
4. Qubit dsDNA HS Assay kit. Vendor: ThermoFisher Scientific. Catalog number: Q32851.
5. Primers for PCR amplification of 16S rRNA gene with phosphorylated 5' ends can be ordered from any provider:
    a. Forward primer: 8F: [PHO] AGRGTTTGATCMTGGCTCAG
    b. Reverse primer: 1387R: [PHO] GGGCGGWGTGTACAAGRC
6. The master mix for PCR amplification: Q5® High-Fidelity 2X Master Mix. New England Biolabs, Inc. Catalog number: M0492S
7. Nuclease-free Water. Vendor: New England Biolabs, Inc. Catalog number: B1500S
8. HighPrep™ PCR paramagnetic bead solution. Vendor: MAGBIO. Catalog number: AC-60050
9. 70% ethanol (prepared from denatured ethanol).
10. Magnetic stand for 1.5 ml tubes. MagStrip Magnet Stand 10. Vendor: MAGBIO. Catalog number: MBMS-10
11. Blunt/TA Ligase Master Mix. Vendor: New England Biolabs, Inc. Catalog number: M0367S
12. DNA LoBind Tubes, 1.5 ml. Vendor: Eppendorf. Catalog number: 003018078
13. Plasmid-Safe™ ATP-Dependent DNAse. Vendor: Epicentre. Catalog number: E3101K.
14. TruePrime™ RCA kit. Vendor: Expedeon. Catalog number: 390100
15. T7 endonuclease I. Vendor: New England Biolabs, Inc. Catalog number: M0302S.
16. g-TUBE. Vendor: Covaris. Catalog number: 010145
17. NEBNext® FFPE DNA Repair Mix. Vendor: New England Biolabs Inc. Catalog number: M6630S
18. Ligation Sequencing Kit 1D. Catalog number: SQK-LSK108 and/or Ligation Sequencing Kit 1D2. Catalog number: SQK-LSK308. Vendor: Oxford Nanopore Technologies
19. Flow Cell (R9.4) and/or Flow Cell (R9.5). Vendor: Oxford Nanopore Technologies

Equipment required:

1. PCR thermocycler from any vendor
2. PCR hood
3. Thermal mixer with appropriate blocks from any vendor
4. Pipettes from varying volumes range from any vendor
5. Centrifuge for 2 ml and 0.2 ml tubes from any vendor
6. MinION™ Mk1b device and compatible personal computer

Step 1: PCR amplification of 16S rRNA gene.

1. Combine the following components using volumes below or as appropriate for your experiment in a PCR tube.
    a. Q5® High-Fidelity 2X Master Mix: 12.5 µl
    b. Forward primer (10 pmol): 0.8 µl
    c. Reverse primer: (10 pmol): 0.8 µl
    d. Template DNA: 1 µl
    e. Nuclease-free water: 9.9 µl.

2. Amplify PCR reaction mix at the following PCR conditions:

Segment	Temp (°C)	Time (sec)	Cycles
Initial denaturation	98	00:30	1
Denaturation	98	00:05	20
Annealing	59	00:10	
Extension	72	00:35	
Final extension	72	02:00	1
Hold	8	00:00	1

Step 2: PCR product clean up.

1. Follow vendor instructions (MAGBIO) to clean PCR product at 0.45x bead ratio as described at the following URL: http://www.magbiogenomics.com/image/data/Literature/Protocols/HighPrep%20PCR%20Protocol.pdf

Step 3: PCR product concentration estimation.

1. Follow vendor instructions (ThermoFisher) to determine the concentration of cleaned PCR product using Qubit™ dsDNA HS Assay kit as described at the following URL: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Qubit_dsDNA_HS_Assay_UG.pdf

Step 4: Self-ligation for the formation of plasmid-like structure.

1. Dilute PCR product from step 2 to 2-3 ng/µl using nuclease-free water
2. Mix 55 µl of diluted PCR product with 5 µl of Blunt/TA Ligase Master Mix in 0.2 ml PCR grade tubes
3. Gently mix by flicking the tube a few times
4. Centrifuge tube for 10 seconds
5. Incubate for 10 min at 15°C in a PCR thermocycler
6. Gently mix by flicking the tube a few times
7. Centrifuge tube for 10 seconds
8. Incubate for another 10 min at room temperature.

Step 5: Reverse phase cleanup.

1. Vortex HighPrep™ PCR paramagnetic bead solution
2. Transfer 50 µl of bead solution into clean DNA LoBind 1.5 ml tube

3. Place the tube on a magnetic rack until beads separate from the liquid
4. While the tube is on the magnetic rack, remove 25 µl of liquid from the tube using sterile pipette tip, taking care not to disturb the beads
5. Remove the tube from magnetic rack and gently vortex to resuspend the beads.
6. Add the self-ligation mix from step 4 to concentrated beads at a ratio of 0.35x bead ratio.
7. Incubate the mixture for 3 minutes at room temperature.
8. Place tube on a magnetic rack
9. Allow the beads to separate from the liquid. The beads contain long linear amplicons (potentially chimeric amplicons) while the liquid contains short linear amplicon and plasmid-like structures. Carefully remove the clear liquid from the tube and move it to step 6.

Step 6: Plasmid and short amplicon clean-up.

1. Follow vendor instructions (MAGBIO) to clean plasmid and short amplicon mix at 0.45x bead ratio as described at the following URL: http://www.magbiogenomics.com/image/data/Literature/Protocols/HighPrep%20PCR%20Protocol.pdf

Step 7: Removal of linear molecules from plasmid mix.

1. Combine 42 µl of clean product from step 6 (eluted in nuclease-free water) with 2 µl of 25 mM ATP solution, 5 µl of 10X reaction buffer and 1 µl of Plasmid-Safe DNASe in 0.2 ml PCR grade tube. The last three ingredients are provided with the Plasmid-Safe™ ATP-Dependent DNAse kit.
2. Incubate at 37°C for 15 minutes in a PCR thermocycler
3. Clean product as described in Step 2
4. Determine the concentration of DNA in the cleaned product as described in Step 3.

Step 8: Rolling Circle Amplification (RCA).

Perform RCA in triplicate for each sample and include negative controls using nuclease-free water instead of cleaned product from step 7. Below are reaction conditions and volumes for a single RCA reaction:

1. Combine 2.5 µl of cleaned product from step 7 with 2.5 µl of Buffer D (provided with TruePrime™ RCA kit) in 0.2 ml PCR grade tube and incubate at room temperature for 3-5 minutes
2. While the sample is being incubated, prepare the amplification mix consisting of 9.3 µl of nuclease-free water, 2.5 µl of reaction buffer, 2.5 µl of dNTPs, 2.5 µl of Enzyme 1 and 2.5 µl of Enzyme 2. All ingredients are included in the TruePrime™ RCA kit
3. After 10 minutes, add 2.5 µl of Buffer N (provided with TruePrime™ RCA kit) and prepared amplification mix to the tube
4. Mix by pipetting and incubate tube at 30°C for 150 minutes.
5. Follow Step 3 to determine the concentration of DNA in samples and negative controls.

Step 9: Enzymatic de-branching.

1. Combine triplicate reactions from step 8 into a single 0.2 ml PCR grade tube
2. Mix thoroughly to prepare single RCA product per sample

3. Combine 65 µl of RCA product with 2 µl of T7 endonuclease I and incubate at room temperature for 5 minutes.

Step 10: Mechanical fragmentation.

1. Transfer enzymatically de-branched RCA product into g-TUBE using wide bore pipette tips
2. Centrifuge at 1800 rpm for 4 minutes or until entire reaction mix passes through the fragmentation hole
3. Reverse the g-TUBE and centrifuge again at 1800 rpm for 4 minutes or until entire reaction mix passes through the fragmentation hole
4. Clean product with as described in Step 5, with the exception that the clear liquid after bead separation is discarded and bead-bound DNA is eluted according to vendor outlined protocol
5. Repeat step 9 (i.e. Enzymatic de-branching) on the g-TUBE fragmented product.
6. Clean product as described in Step 5, with the exception that the clear liquid after bead separation is discarded and bead-bound DNA is eluted according to vendor outlined protocol.

Step 11: DNA Damage repair.

1. Combine 53.5 µl of product from Step 10 with 6.5 µl of FFPE DNA Repair Buffer and 2 µl of NEBNext FFPE Repair mix in a 0.2 ml tube
2. Incubate at 20°C for 15 minutes
3. Clean product as described in Step 2
4. Quantify DNA concentration as described in Step 3.

Step 12: **DNA library preparation and nanopore sequencing.**

1. Prepare 2D or 1D2 libraries for nanopore sequencing according to the protocols described for the SQK-LSK108 or SQK-LSK308 kits by Oxford Nanopore Technologies.
2. Primer appropriate flow cell using protocols outlined by Oxford Nanopore Technologies.
3. Finally, sequence the prepare libraries on appropriate flow cells as outlined by Oxford Nanopore Technologies.

Table S1: Names and DSM catalog numbers of bacteria used to construct mock communities for the single organism and ten organism experiments and their corresponding accession numbers are shown below. The 16S rRNA genes were extracted from genome assemblies using RNAmmer[1] for bacteria for use in estimation of sequencing accuracy. Where genome assemblies were unavailable, the 16S rRNA gene sequence in Genbank was utilized.

Organism name	DSM catalog number	Genbank assembly accession number	16S rRNA gene accession number
One organism experiment			
Listeria monocytogenes	19094	HE999705.1	
Ten organism experiment			
Aquimarina intermedia	17527	-	AM113977
Bacteroides vulgatus	1447	CP000139	-
Desulfosporosinus orientis	765	CP003108	-
Flectobacillus major	103	ATXY00000000	-
Legionella pneumophila	7513	AE017354	-
Listeria monocytogenes	19094	HE999705.1	-
Meiothermus ruber	1279	CP001743	-
Propionibacterium acnes	16379	AE017283	-
Pseudomonas aeruginosa	1128	NC_009656	-
Spirosoma linguale	74	CP001769	-

1 Lagesen, K. *et al.* RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Research* **35**, 3100-3108, (2007).

Figure S1: Violin plots showing the read length distribution of raw data (i.e., post base calling with Albacore 1.2.4), and the number of concatemers on each base called read estimated using read lengths from INC-Seq processing using the "blastn" alignment approach for all four experiments involving both 2D and 1D2 chemistry.

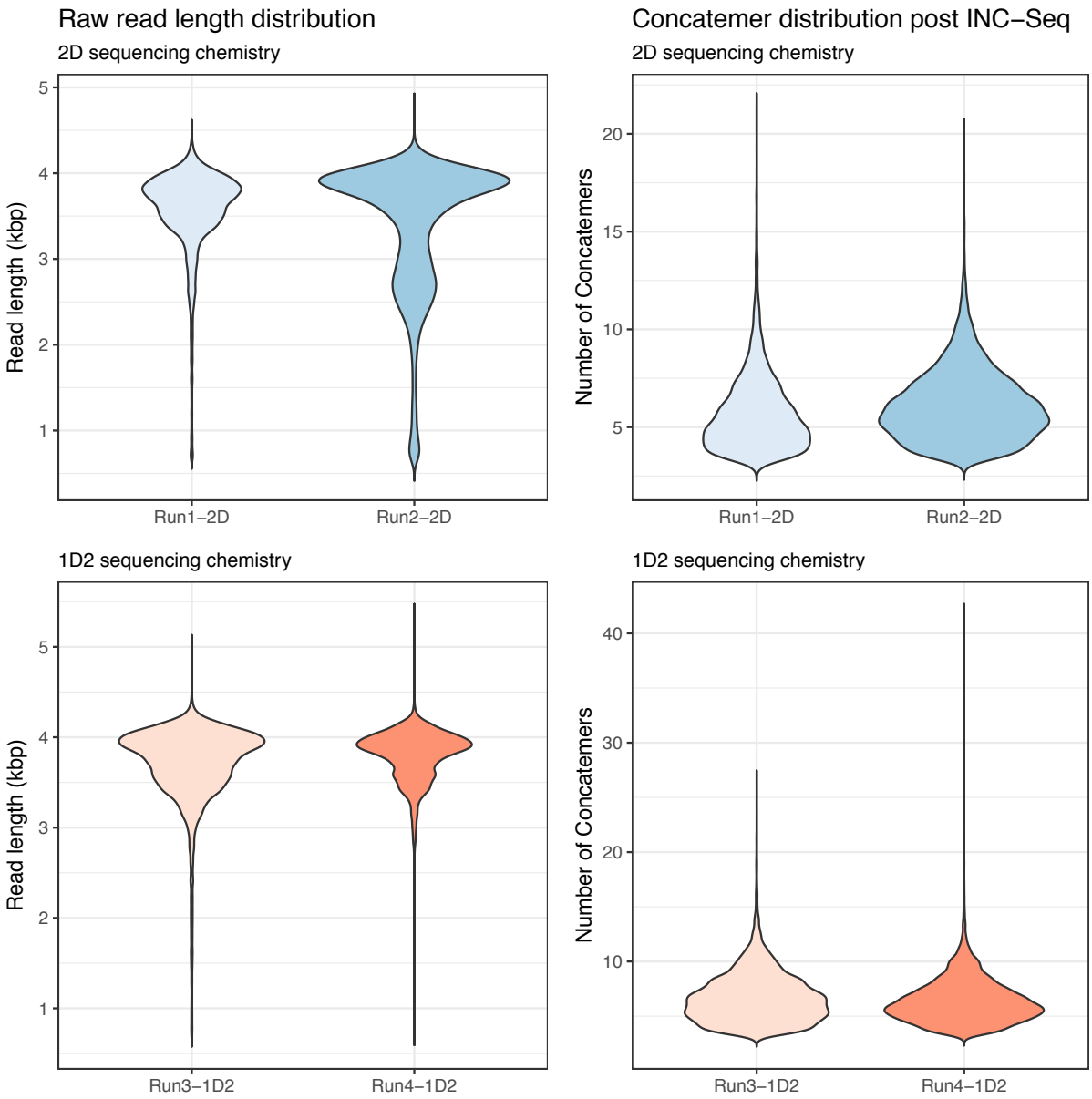


Figure S2: Violin plot of read length distributions for INC-Seq processed reads, chopSeq corrected reads after tandem repeat removal, and post-size-selection of chopSeq corrected reads and size selected reads (chopSeq_SS) for one organism experiments using both 2D and 1D2 sequencing chemistry.

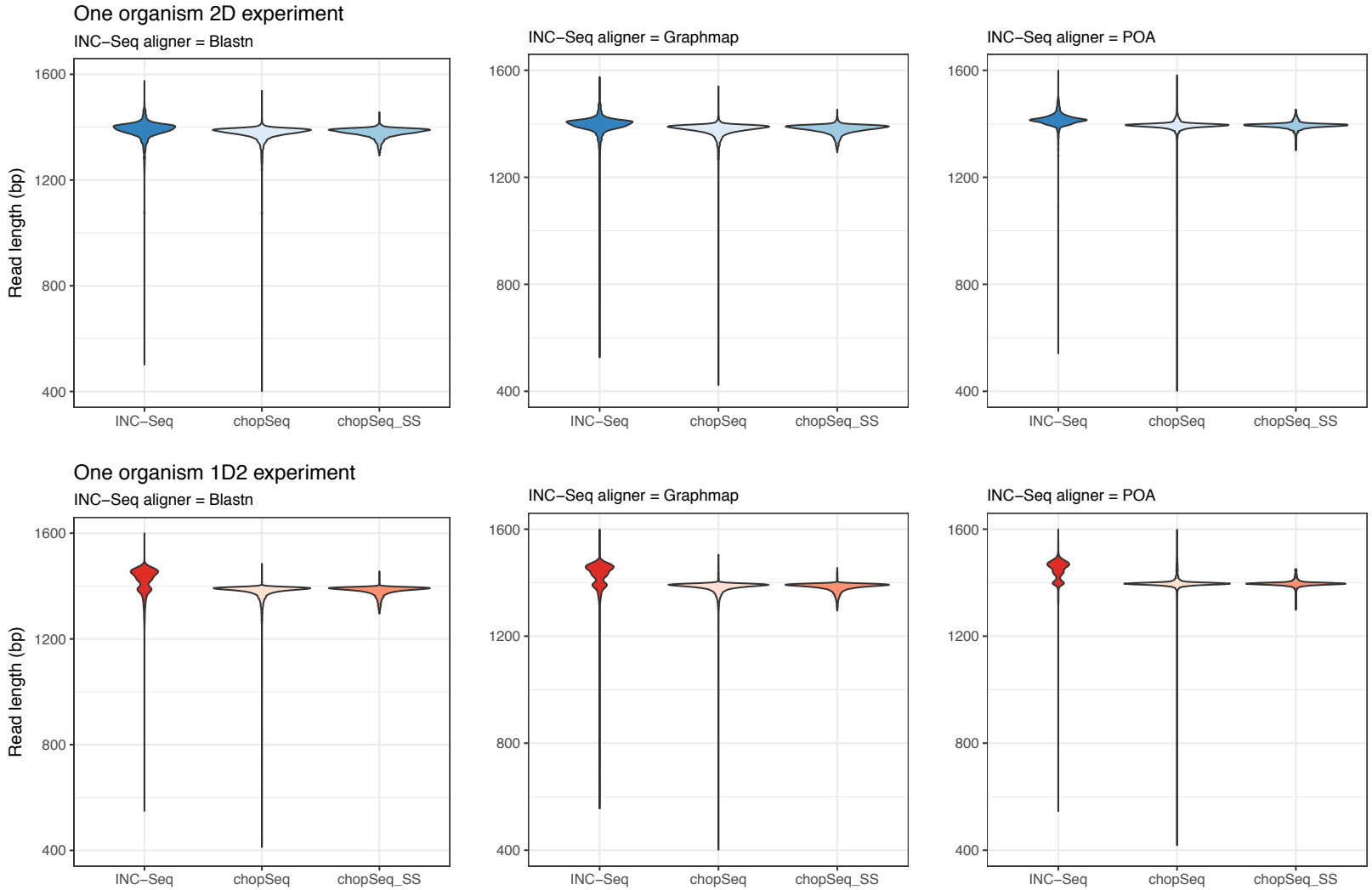


Figure S3: Consensus sequences from OTUs from Run 3 (1D2 sequencing chemistry, INC-Seq aligner: blastn) were combined with reference sequences and aligned using muscle (default parameters) and Neighbor-Joining tree using Jukes-Cantor model was constructed in Geneious (version 8) using 100 bootstraps. Reference sequences are labelled in green, and OTUs are labelled in red.

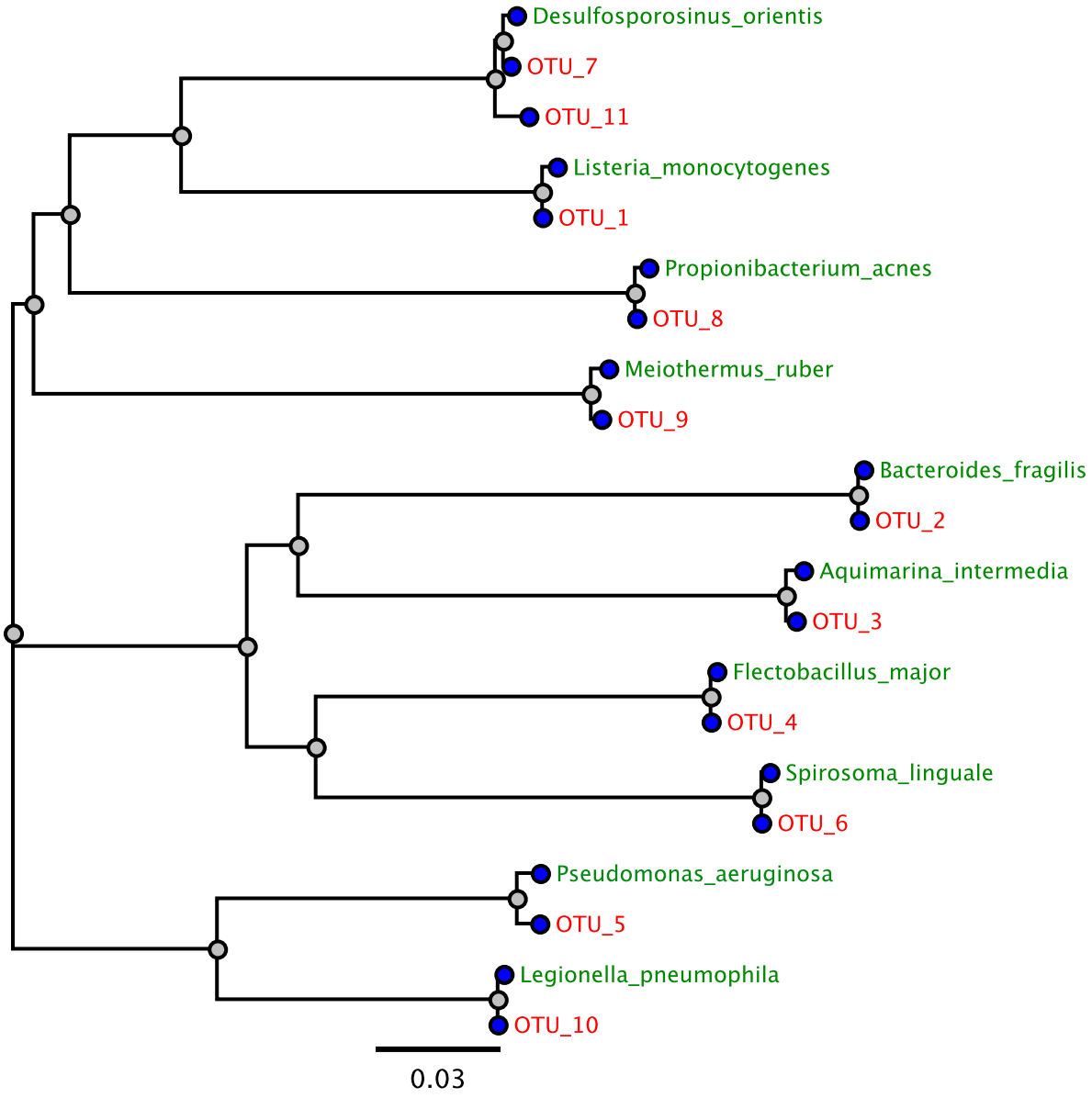
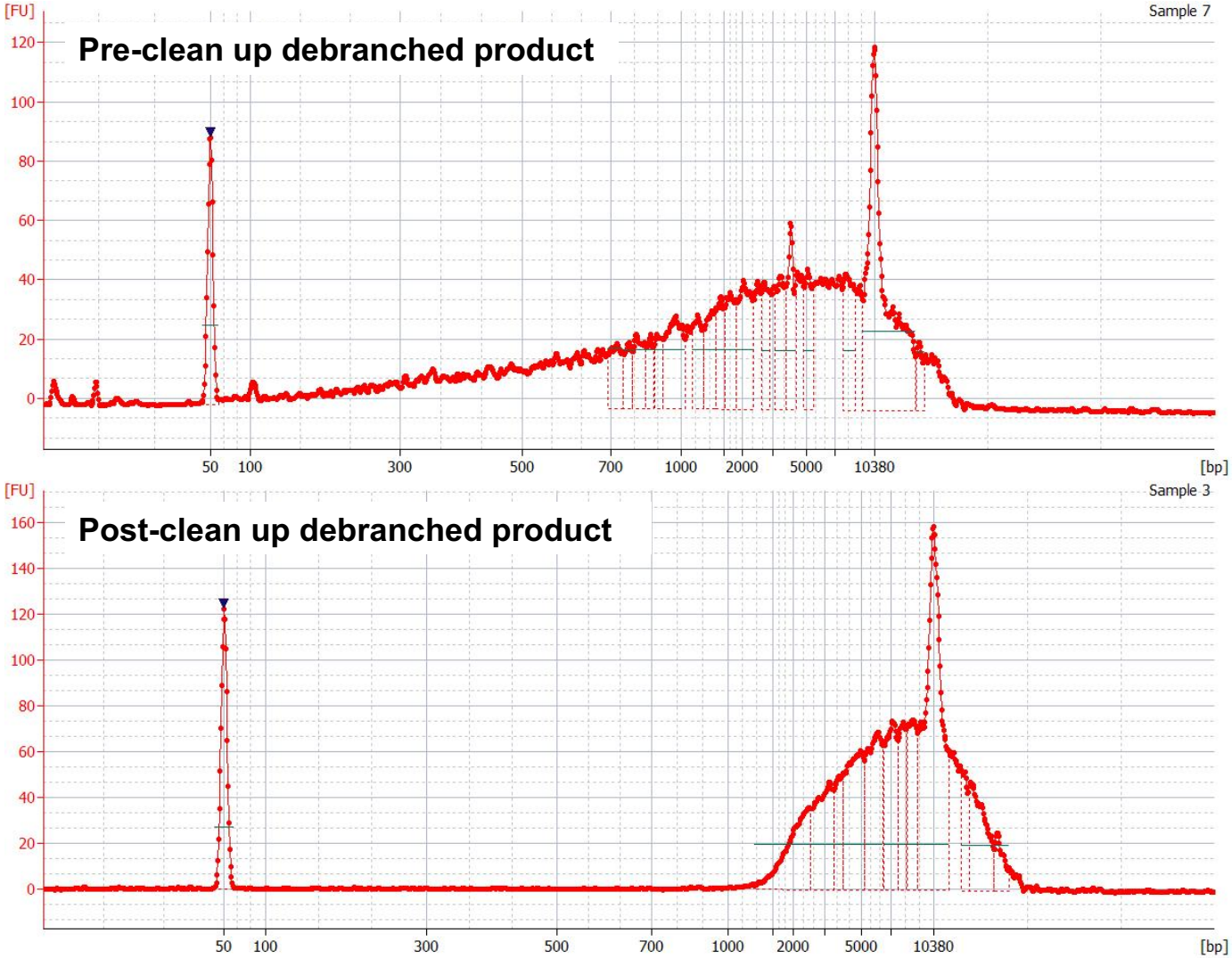



Figure S4: Example Bioanalyzer traces of de-branched product pre- and post-cleanup. The post-cleanup product was then treated for gap filling and DNA damage repair prior to being used for library preparation of sequencing according to ONT protocol for 2D and 1D2 chemistries.