The Influence of High Fidelity DNA Polymerase on 16S rRNA Gene Sequencing

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Abstract

Background. An increasing body of research has found that various methodological steps can have an impact on the observed microbial community when using 16S rRNA gene surveys. These components include, but are not limited to, preservation media, extraction kit, bead beating time, and primers. Both cycle number and high fidelity (HiFi) DNA polymerase are sometimes overlooked when sources of bias are being considered. Here we critically examine both cycle number and HiFi DNA polymerase for biases that may influence downstream diversity measures of 16S rRNA gene surveys.

Methods. DNA from Fecal samples (n = 4) were extracted using a single PowerMag DNA extraction kit with a 10 minute bead beating step and amplified at 15, 20, 25, 30, and 35 cycles using Accuprime, Kappa, Phusion, Platinum, or Q5 HiFi DNA polymerase. Mock communities (technical replicates n = 4) consisting of previously isolated whole genomes of 8 different bacteria were also amplified using the same PCR amplification approach. First, the number of OTUs (Operational Taxonomic Units) was examined for both fecal samples and mock communities. Next, Bray-Curtis index, the error rate, sequence error prevalence, and chimera prevalence were assessed based on cycle number and HiFi DNA polymerase. Finally, the chimera prevalence correlation with number of OTUs was assessed for both cycle number and HiFi DNA polymerase dependent differences.

Results. At 35 cycles there were significant differences between HiFi DNA polymerase for fecal samples (P-value < 0.0001). These HiFi dependent differences in the number of OTUs could be identified as early as 20 cycles in the mock communities (P-value = 0.002). Chimera prevalence varied by HiFi DNA polymerase and these differences were still observed after chimera removal using VSEARCH. Additionally, the chimera prevalence had a strong positive correlation with the number of OTUs observed in a sample and this association was not changed by chimera removal with VSEARCH.

Conclusions. Due to the impact of HiFi DNA polymerase on the number of OTUs, common diversity metrics that incorporate this value could give artificially inflated numbers due to higher undetected chimeras. When designing 16S rRNA gene survey studies it is important to consider both the cycle number and the type of HiFi DNA polymerase that will be used since it can increase or decrease the number of OTUs that are observed.

Introduction

Recently there has been an increasing focus on standardizing methodological approaches in microbiota research (Kim et al., 2017; Hugerth & Andersson, 2017). In particular, investigating ways that 16S rRNA gene surveys can be made more reproducible has been a predominant driver of this standardization push (Lauber et al., 2010; Salter et al., 2014; Song et al., 2016; Gohl et al., 2016). Although 16S rRNA gene sequencing has been much maligned for introduced biases, many of these same considerations also affect metagenomic sequencing (Nayfach & Pollard, 2016; Costea et al., 2017). Between the two approaches similar bias considerations include preservation media, storage conditions, DNA extraction kit, PCR, and sequence library preparation. Thus, for these overlapping considerations biases identified for 16S rRNA gene sequencing will also likely influence metagenomic sequencing results.

Currently, preservation media used, storage conditions, and DNA extraction kits chosen are the most commonly studied biases. The study of these specific biases has become so large, aggregating them all together has become a difficult task and some researchers provide resources to actively track new findings (e.g. Microbiome Digest - https://microbiomedigest.com/microbiome-papers-collection/microbiome-techniques/sample-storage/). Within the literature, DNA extraction kits have consistently been shown to add bias to downstream analysis (Salter et al., 2014; Costea et al., 2017). However, the current literature on preservation media and storage conditions has been more mixed, with some studies showing biases while others do not (Lauber et al., 2010; Dominianni et al., 2014; Sinha et al., 2015; Song et al., 2016; Luo et al., 2016; Bassis et al., 2017). Although these are important sources of bias they are not the only sources that should be critically examined. The type of DNA polymerase chosen could have a wide ranging affect on downstream results due to error rates and chimeras that may not be easily resolved using bioinformatic approaches.

A recent study in *Nature Biotechnology* showed that there were clear differences between normal and high fidelity (HiFi) DNA polymerase and that you could reduce error and chimera generation by optimizing the PCR protocol (Gohl et al., 2016). Another important component of this study was that the number of OTUs generated were not easily removed using the authors chosen bioinformatic pipeline regardless of DNA polymerase used (Gohl et al., 2016). Although it is probably not surprising that normal DNA polymerase performed worse than HiFi DNA polymerase, it is natural to extend this line of inquiry and ask whether different HiFi DNA polymerase contribute different biases to downstream sequencing results. There is some reason to think that this may be the case since many of these HiFi DNA polymerase come from different families (e.g. *Taq* belongs to the family A polymerases) and may intrinsically have different error rates that cannot be completely removed with modifications (Ishino & Ishino, 2014). In this study we critically examine if any of five different HiFi DNA polymerases introduce significant biases into 16S rRNA gene surveys, if this is a cycle dependent phenomenon, and whether they can be removed using a standard bioinformatic pipeline.

We amplified the V4 region of the 16S rRNA gene in both fecal and mock community samples using either Accuprime, Kappa, Q5, Phusion, or Platinum HiFi DNA polymerase. First, we tested if we could identify differences in the number of OTUs between the different HiFi DNA polymerase for both the fecal and mock samples. Next, since there were differences based on HiFi DNA polymerase, we examined if a community measure such as the Bray-Curtis index would be affected. After assessing these community measures we then examined if the different HiFi DNA polymerase had different per base error rates as well as chimera prevalence. Finally, since differences in chimera prevalence, based on HiFi DNA polymerase, could not be completely removed using bioinformatic approaches we tested whether the chimera prevalence correlated well with total number of observed OTUs.

Materials & Methods

Human and Mock Samples: A single fecal sample was obtained from 4 individuals who were part of the Enterics Research Investigational Network (ERIN). The processing and storage of these samples have been published previously (Seekatz et al., 2016). Clinical data and other types of meta data were not utilized or accessed for this study. All samples were extracted using the MOBIO™ PowerMag Microbiome RNA/DNA extraction kit (now Qiagen, MD, USA). The ZymoBIOMICS™ Microbial Community DNA Standard (Zymo, CA, USA) was used in this study and is made up of *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica*, *Lactobacillus fermentum*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus subtilis* at equal genomic DNA abundance (http://www.zymoresearch.com/microbiomics/microbial-standards/zymobiomics-microbial-community-standards).

PCR Protocol: The five different high fidelity (HiFi) DNA polymerase that were tested were AccuPrime™ (ThermoFisher, MA, USA), KAPA HIFI (Roche, IN, USA), Phusion (ThermoFisher, MA, USA), Platinum (ThermoFisher, MA, USA), and Q5 (New England Biolabs, MA, USA). The PCR cycle conditions for Platinum and Accuprime followed a previously published protocol (Kozich et al., 2013) (https://github.com/SchlossLab/MiSeq_WetLab_SOP/blob/master/MiSeq_WetLab_SOP_v4.md). The HiFi DNA polymerase activation time was 2 minutes, unless a different activation was specified. For Kappa and Q5, the protocol previously published by Gohl and colleagues was used (Gohl et al., 2016). For Phusion, the company defined conditions were used except for extension time, where the Accuprime and Platinum settings were used.

Both fecal and mock samples cycle conditions started at 15 and increased by 5 up to 35 cycles with amplicons used at each 5-step increase for sequencing. The fecal PCR consisted of all 4 samples at 15, 20, 25, 30, and 35 cycles for each Taq (total samples =

100). Although, the mock communities also had 4 replicates for 15, 20, 25, and 35 cycles and 10 replicates for 30 cycles for all HiFi DNA polymerase (total samples = 130). No mock community sample had enough PCR product at 15 cycles for adequate 16S rRNA gene sequencing.

Sequence Processing: The mothur software program was utilized for all sequence processing steps (Schloss et al., 2009). Generally, the protocol followed what has been previously published (Kozich et al., 2013) (https://www.mothur.org/wiki/MiSeq_SOP). Two major differences from the stated protocol were the use of VSEARCH instead of UCHIME for chimera detection and the use of the OptiClust algorithm instead of average neighbor for Operational Taxonomic Unit (OTU) generation at 97% similarity (Edgar et al., 2011; Rognes et al., 2016; Westcott & Schloss, 2017). Sequence error was determined using the 'seq.error' command on mock samples after chimera removal and classification to the RDP to remove non-bacterial sequences (Schloss et al., 2009; Cole et al., 2013; Rognes et al., 2016).

Statistical Analysis: All analysis was done with the R (v 3.4.3) software package (R Core Team, 2017). Data transformation and graphing was completed using the tidyverse package (v 1.2.1) and colors selected using the viridis package (v 0.4.1) (Garnier, 2017; Wickham, 2017). Differences in the total number of OTUs were analyzed using an ANOVA with a tukey post-hoc test. For the fecal samples the data was normalized to each individual by cycle number to account for the biological variation between people. Bray-Curtis matrices were generated using mothur after 100 sub-samplings at 1000, 5000, 10000, and 15000 sequence depth. The distance matrix data was analyzed using PERMANOVA with the vegan package (v 2.4.5) (Oksanen et al., 2017) and Kruskal-Wallis tests within R. For both error and chimera analysis, samples were tested using Kruskal-Wallis with a Dunns post-hoc test. Where applicable correction for multiple comparison utilized the Benjamini-Hochberg method (Benjamini & Hochberg, 1995).

Analysis Workflow: The total number of OTUs after sub-sampling was analyzed for both the fecal and mock community samples. Cycle dependent affects on Bray-Curtis indices were next assessed for the fecal samples looking at both overall cycle differences and within individual differences for the previous cycle (e.g. 20 versus 25, 25 versus 30, etc.). Based on these observations we wanted to next analyze potential reasons for these differences. First, analysis of general sequence error rate, number of sequences with an error, and base substitution were assessed in the mock community for each DNA polymerase. After assessing these errors, the total number of chimeras was determined after sequence processing. For the community based measures, the fecal samples were analyzed at 4 different sub-sampling sequence depths (1000, 5000, 10000, and 15000) while the mock community samples were analysed at 3 levels (1000, 5000, 10000).

Reproducible Methods: The code and analysis can be found here https://github.com/SchlossLab/Sze_PCRSeqEffects_XXXX_2017. The raw sequences can be found in the SRA at the following accession number **need to upload still**.

Results

The Number of OTUs is Dependent on the HiFi DNA Polymerase Used: In order to compare the number of OTUs across individuals a Z-score normalization by individual, by cycle number, was employed on the number of OTUs data. After normalization, we identified that there was a HiFi DNA polymerase dependent difference that was consistent across sub-sampling [Figure 1]. Lower cycle numbers (15-20) tended to result in less differences between HiFI DNA polymerase while cycle numbers of 25, 30, and 35 tended to have more distinct differences [Figure 1]. All sub-sampling levels were significantly different only at 35 cycles (P-value < 0.0001) [Table S1]. There were differences in HiFi DNA polymerase at 25 and 30 cycles but the sub-sampling depth had to be 5000 or higher (P-value < 0.05) [Table S1]. Platinum HiFi DNA polymerase was the main driver of the differences observed across all sub-sampling depths at 35 cycles, based on a Tukey post-hoc test (P-value < 0.05) [Table S2].

This HiFi DNA polymerase dependent difference in the number of OTUs was also observed in the mock community samples with the same DNA polymerases having high (Platinum) and low (Accuprime) number of OTUs [Figure 2 & Table S3]. Conversely, differences between HiFi DNA polymerase were observed as early as 20 cycles and a sub-sampling depth of 1000 sequences (P-value = 0.002) [Table S3]. Using a Tukey post-hoc test differences between Platinum and the other HiFi DNA polymerases was the major driver of the differences seen at different cycle numbers and sub-sampling depths [Table S4]. Both fecal and mock samples consistently showed that across sub-sampling depth and cycle number the lowest number of OTUs identified was from Accuprime™ while the highest was from Platinum for both fecal and mock samples [Figure 1 & 2].

Minimal Bray-Curtis Differences are Detected by Cycle Number: Overall, there was little difference between each respective 5-cycle increment at higher cycle numbers, for

both fecal and mock samples, and this was consistent across the different sub-samplings [Figure 3]. There were only two differences between 5-cycle increments that were identified. First, there were differences for the same fecal sample between 20 versus 25 cycles that was independent of HiFi DNA polymerase but dependent on sub-sampling depth (sub-sampled to 1000 = 0.51 (0.4 - 0.79) (median (25% - 75% quantile)), sub-sampled to 5000 = 0.43 (0.33 - 0.63), sub-sampled to 10000 = 0.4 (0.24 - 0.43)) [Figure 3A]. Second, for the mocks, where data is available, there were larger difference between 20 and 25 cycles (sub-sampled to 1000 = 0.88 (0.42 - 0.91)) [Figure 3B]. However, these differences between the next 5-cycle increment do not persist once 25 cycles are reached [Figure 3].

Using PERMANOVA to test for differences within HiFi DNA polymerase groups based on cycle number, only Phusion had cycle dependent differences at 1000 and 5000 sub-sampling depth (P-value = 0.03 and 0.01, respectively). Phusion was one of only two HiFi DNA polymerases that managed to have samples for the 1000 sub-sampling depth at 15 cycles. Next, we assessed whether there were any major differences between 5-cycle increments within each sample. We found that there was no detectable difference in Bray-Curtis index when comparing to the previous 5-cycle increment (P-value > 0.05). However, Phusion at 1000 sub-sampling depth had a P-value = 0.02 before multiple comparison correction. It should be noted that at higher sub-sampling depths these differences in Bray-Curtis indices disappear for both differences in cycle number and within 5-cycle increments within an individual.

Sequence Error is Dependent on both HiFi DNA Polymerase and Cycle Number: Differences by HiFi DNA polymerase in the median average per base error varied without a clear pattern across sub-sampling depth [Table S5]. Generally, the highest per base median average error rates were for the Kappa HiFi DNA polymerase [Figure 4]. This error rate was minimally affected by both the 'pre.cluster' step and chimera removal by VSEARCH [Figure 4]. There were small differences in the per base error rate between

the various HiFi DNA polymerase at lower cycle numbers and larger differences at higher cycle number with Platinum having the largest differences of all the HiFi DNA polymerase [Figure 4B-C and Table S6].

The total sequences with at least one error had multiple differences at different cycle numbers and was mostly alleviated by the use of the 'pre.cluster' step [Figure S1]. Major differences before this 'pre.cluster' step were driven by large differences in Accuprime™ and Platinum versus the other HiFi DNA polymerase tested [Figure S1 & Table S7 & S8]. Although Accuprime™ had the lowest per base error rate it had the largest number of sequences with at least one error, regardless of cycle number or sub-sampling depth [Figure S1]. However, this increased number of sequences with an error can be drastically lowered with existing bioinformatic approaches [Figure S1]. Investigation of whether there were HiFi DNA polymerase dependent effects on base substitution found that there were generally no biases in the types of substitution made [Figure S2].

Chimeric Sequences are HiFi DNA Polymerase Dependent and Correlate with Number of OTUs: After chimera removal using VSEARCH and removal of sequences that did not classify as bacteria, we assessed the percentage of sequences that were still chimeric within our mock community. At all levels of sub-sampling and cycle number there were significant differences between the HiFi DNA polymerase used (P-value < 0.05) [Table S9]. Differences between Platinum and all other HiFi DNA polymerases accounted for the vast majority of these differences independent of cycle number and sub-sampling depth when using a Dunn's post-hoc test [Table S10]. Generally, across sub-sampling depth and cycle number Accuprime™ had the lowest chimera prevalence of all the HiFi DNA Polymerases regardless of whether 'pre.cluster' or chimera removal with VSEARCH had been used [Figure 5].

For all DNA polymerase, a positive correlation was observed between chimeric sequences and number of OTUs, with this correlation being strongest for Accuprime, Platinum and

Phusion HiFi DNA Polymerase [Figure 6]. The R^2 value between the number of OTUs and chimeric sequences did not change from the use of 'pre.cluster' and the removal of chimeras with VSEARCH [Figure 6]. Taken together, this data suggests that a strong correlation exists between the number of OTUs and the prevalence of chimeric sequences.

Discussion

Our observations build upon previous studies (Gohl et al., 2016) by showing that even different HiFi DNA polymerases have significant differences in the number of OTUs and that changes in total OTUs correlate with chimeras not removed after sequence processing [Figure 1-2 & 5]. This is important since many diversity metrics rely on the total number of OTUs as part of their calculations and changes to the total number of OTUs could drastically change the results, as well as the findings. Our observations show that HiFi DNA polymerase can have a noticeable affect on the OTUs generated and these differences are consistent across sub-sampling depth and PCR cycle number [Figure 2-4]. These differences were observed in high biomass samples, where biases introduced by such components like kit contamination may have less of an effect, suggesting that these differences may be exacerbated in low biomass samples.

Although we did not observe strong differences in the Bray-Curtis index, based on cycle number, the data suggests that there may be differences between 15 and 20 cycles versus higher cycle numbers, such as 30x, that are commonly used. Additionally, there was no difference within individuals between corresponding 5 cycle increments (e.g. 15 to 20, 20 to 25, etc.). Conversely, we may not have enough samples to adequately judge the true magnitude difference between 20x and 25x communities and higher cycle numbers analyzed, even though there is a clear trend to suggest that 20x is very different then 25x [Figure 3]. This finding, in conjunction with the PERMANOVA results, suggest that cycle number can change bacterial community calculations but that these differences are minimal once 25 cycles are reached. Increasing the sub-sampling depth, for some DNA polymerase, may reduce some of these observed community differences at lower cycle numbers.

Increasing the cycle number also exacerbated chimera prevalence differences between

the different HiFi DNA polymerases [Figure 5]. The chimera prevalence was strongly correlated with the number of OTUs which is relied upon heavily for many community metric calculations. However, Bray-Curtis analysis with PERMANOVA showed few differences based on DNA polymerase. It is possible that many of the increased number of OTUs are not highly abundant allowing the Bray-Curtis index to be able to successfully down-weight these respective OTUs (Minchin, 1987). The choice of downstream diversity metric could be an important consideration in helping to mitigate these observed changes due to high chimera prevalence in specific HiFi DNA polymerases (e.g. Platinum).

Our observations suggest that there are clear HiFi DNA polymerase dependent differences in both per base error rate and chimeras that cannot be removed using bioinformatic approaches [Figure 4 & 5]. Although it may be a natural assumption that the variation may be due to the DNA polymerase family, the highest chimera rate, from Platinum, was a family A polymerase while the lowest, from Accuprime, was also an A polymerase (Ishino & Ishino, 2014). In fact, from the material safety data sheet (MSDS), the differences between the two mixes is not immediately apparent. Both Accuprime and Platinum contain a recombinant *Taq* DNA polymerase, a *Pycrococcus* spp GB-D polymerase and a platinum *Taq* antibody. It is possible that differences in how the recombinant *Taq* was generated could be the main reason for the differences in chimera rate.

Conclusion

Our findings show that measures that rely on number of OTUs will be specific for a particular study and may not be easily generalized to other studies investigating a similar area. Care should be taken when choosing a HiFi DNA polymerase for 16S rRNA gene surveys since intrinsic differences can change the number of OTUs observed as well as potentially influence diversity based metrics that do not down weight rare observations.

Acknowledgements

The authors would like to thank all the study participants ERIN whose samples were utilized. We would also like to thank Judy Opp and April Cockburn for their effort in sequencing the samples as part of the Microbiome Core Facility at the University of Michigan. Salary support for Marc Sze came from the Canadian Institute of Health Research and the Michigan Institute for Clinical and Health Research Postdoctoral Translational Scholar Program.

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Figure 1: Normalized Fecal Number of OTUs. The x-axis represents the different sub-sampling depths used and the y-axis is the normalized within individual number of OTUs. The red line represents the overall mean Z-score normalized number of OTUs for each respective HiFi DNA polymerase. The dashed black line represents the overall Z-score normalized mean number of OTUs.

Figure 2: Mock Sample Variability in Number of OTUs based on HiFi DNA Polymerase. A) Sub-sampled to 1000 reads. B) Sub-sampled to 5000 reads. C) Sub-sampled to 10000 reads. The dotted line represents the number of OTUs generated when the mock reference sequences are run through the pipeline.

Figure 3: Community Differences by Five-Cycle Intervals and Sub-sampling Depth. A) Fecal samples within person difference based on the next 5-cycle PCR interval. B) Mock samples within replicate difference based on the next 5-cycle PCR interval.

Figure 4: HiFi DNA Polymerase Per Base Error Rate in Mock Samples. A) Error rate before the merger of sequences with pre.cluster and the removal of chimeras with VSEARCH. B) Error rate before the removal of chimeras with VSEARCH. C) Full pipeline. The error bars represent the 75% interquartile range of the median.

Figure 5: HiFi DNA Polymerase Chimera Prevalence in Mock Samples. A) Chimera sequence percentage before the merger of sequences with pre.cluster and the removal of chimeras with VSEARCH. B) Chimera sequence percentage before the removal of chimeras with VSEARCH. C) Full pipeline. The error bars represent the 75% interquartile range of the median.

Figure 6: The Correlation between Number of OTUs and Chimeras. A) Correlation before the merger of sequences with pre.cluster and the removal of chimeras with VSEARCH. B) Correlation before the removal of chimeras with VSEARCH. C) Correlation with full pipeline.

Figure S1: HiFi DNA Polymerase Sequence Error Prevalence in Mock Samples. A) Sequence error prevalence before the merger of sequences with pre.cluster and the removal of chimeras with VSEARCH. B) Sequence error prevalence before the removal of chimeras with VSEARCH. C) Full pipeline. The error bars represent the 75% interquartile range of the median.

Figure S2: HiFi DNA Polymerase Nucleotide Subsitutions in Mock Samples.