Identifying the best PCR enzyme for library amplification in NGS

Michael A Quail[1], Craig Corton, James Uphill, Jacqueline Keane and Yong Gu[1]

[*]Correspondence: mq1@sanger.ac.uk

[1]Wellcome Sanger Institute, Hinxton, Cambs., CB10 1SA. United Kingdom.

Michael A Quail: mq1@sanger.ac.uk

Craig Corton: chc@sanger.ac.uk

James Uphill: ju5@sanger.ac.uk

Yong Gu: ygu@sanger.ac.uk

Jacqueline Keane: jacqueline.mcquillan@gmail.com

Abstract

Background

PCR amplification is a necessary step in many next generation sequencing (NGS) library preparation methods[1] [2]. Whilst many PCR enzymes are developed to amplify single targets efficiently, accurately and with specificity, few are developed to meet the challenges imposed by NGS PCR, namely unbiased amplification of a wide range of different sizes and GC content. As a result PCR amplification during NGS library prep often results in bias toward GC neutral and smaller fragments. As NGS has matured, optimised NGS library prep kits and polymerase formulations have emerged and in this study we have tested a wide selection of available enzymes for both short read Illumina library preparation and long fragment amplification ahead of long-read sequencing.

Results

We tested over 20 different Hi-fidelity PCR enzymes/NGS amplification mixes on a range of Illumina library templates of varying GC content and composition, and find that both yield and genome coverage uniformity characteristics of the commercially available enzymes varied dramatically. Three enzymes Quantabio RepliQa Hifi Toughmix, Watchmaker Library Amplification Hot Start Master Mix (2X) “Equinox” and Takara Ex Premier were found to give a consistent performance, over all genomes, that mirrored closely that observed for PCR free datasets. We also test a

range of enzymes for long read sequencing by amplifying size fractionated S. cerevisiae DNA of average size 21.6 and 13.4kb respectively.

Conclusion

The enzymes of choice for short read (Illumina) library fragment amplification are Quantabio RepliQa Hifi Toughmix, Watchmaker Library Amplification Hot Start Master Mix (2X) “Equinox” and Takara Ex Premier, with RepliQa also being the best performing enzyme from the enzymes tested for long fragment amplification prior to long read sequencing.

Keywords: Next-Generation Sequencing, PCR, amplification, polymerase, indexing, barcode, Illumina.

Background

In barely over a decade Next Generation Sequencing has transformed the biological sciences and is now used for a diverse range of applications on a diverse range of species and sample types. From the early days of NGS the concept of bias was recognised. No existing sequencing technology can “read” a genome from start to end so DNA/RNA are fragmented to a size that can be read by the sequencing platform, a library of those fragments is prepared and original sample sequence is reconstituted

following sequencing of the resulting mixture of library fragments. Ideally all of the constituent parts of the genome would be sequenced with equal representation, though in practice this is never the case. During the library prep and sequencing process there are several points that can introduce a bias into the representation, though perhaps the single most-bias introducing step is PCR. Sequencing libraries are comprised of a mixture of fragments of different size, GC and repeat content that together represent the original sample. Smaller, more GC neutral, fragments that don't contain any secondary structure will amplify more efficiently than larger, high GC, high AT fragments and those that are capable of forming secondary structures. Over multiple cycles any bias will be amplified so the number of PCR cycles should be kept to a minimum, indeed for the most even coverage sequencing dataset PCRfree libraries are recommended [3]. Whilst advantageous, PCRfree methods are not always practicable due to the high DNA mass input requirements, necessitating the use of PCR amplification during library prep.

Since its invention in 1986 PCR has proven to be a convenient and effective way to selectively amplify genomic loci of interest and is the most common approach used for targeted selection ahead of both short and long-read sequencing. Whilst the maximal fragment size sequenceable in a contiguous manner using short read sequencing is around 600bp, Pacific Bioscience and Oxford Nanopore Technologies sequencing platforms can generate reads in excess of 100kb. This capability has led to long read sequencing approaches being preferred for *de-novo* sequencing. However, the long-read technologies require significant amounts of input DNA meaning amplification during long read library preparation may be necessary. Here we

compare enzymes for their ability to amplify long DNA fragments and compare yield and sequencing performance.

In 2011 we published a study that identified Kapa HiFi as the best enzyme for Illumina library amplification steps as it gave the most even coverage across a set of microbial genomes of diverse GC content [4]. Whilst most enzymes gave relatively even coverage over the more GC neutral genomes used (*Salmonella Pullorum* and *Staphylococcus aureus*) large differences in coverage representation were observed with libraries generated using different enzymes for the GC rich genome of *Bordetella pertussis* and the AT rich genome of *Plasmodium falciparum*.

Ten years on, we perform a similar study using many currently available and newly developed High Fidelity polymerase mixes/NGS amplification formulations. There are a great many commercially available PCR enzymes. Enzymes were chosen for this evaluation through discussion with individual enzyme manufacturers and suppliers to identify their enzymes that they recommended for NGS, these were mostly hotstart formulations of type II enzymes that have been developed to amplify complex templates and introduce fewer errors than standard Taq polymerases.

Results

Initial evaluation of enzymes for Illumina library amplification

Each enzyme was assessed for its ability to amplify genomic Illumina adapter ligated library fragments of an expected average insert size of approximately 500bp, from a set of four microbial genomes with differing GC content:- *Bordetella pertussis*, 67.7 % GC; *Escherichia coli*, 50.8 % GC; *Clostridioides difficile*, 29.1 % GC; and *Plasmodium falciparum*, 19.3 % GC. For each enzyme tested, 1 ng of pre-PCR library fragments from each genome was amplified using manufacturers recommended denaturation and extension times, with annealing at 60°C for 15 seconds and 14 cycles. Unique dual indexed P7 and P5 amplification primers were used to avoid index hopping [5, 6].
For UDI oligonucleotides used, see **Supplementary Table 1.**

Details of enzymes used and cycling conditions are listed in **Supplementary Table 2**.

After 0.7x Ampure bead cleanup the yield of each library was assessed using fluorimetric measurement (**Supplementary Figure 1**). There were surprising differences in the yields obtained from the enzymes tested with some giving relatively little library. These were repeated with fresh enzyme on a different PCR block, always with the same outcome. Quantabio RepliQa and sparQ, Kapa HiFi, Invitrogen Platinum Superfi II, Thermo Collibri and Phusion U multiplex PCR mastermix, Tools Ultra, Biotool Univerase and Agilent Herculase gave good yields.

Barcoded libraries were pooled in a pseudoequimolar manner according to genome size and run on an Illumina Novaseq 6000 SP or S4 flowcell lane to give > 30x coverage of each genome. To fairly compare results, datasets were randomly trimmed to contain reads representing 30 x coverage. We tabulated the depth of coverage seen at each position of the genome and calculated the fraction of each genome (referred to as Low Coverage Index) that was covered to a depth of less than 15 x i.e. half the mean coverage. Most datasets had <5% low coverage with the GC neutral *E. coli* genome but higher degrees of low coverage were observed for less base balanced genomes with a lot of enzyme, with the extremely AT rich genome of *P falciparum* posing the biggest challenge (**Figure 1**). Quantabio RepliQa, Kapa HiFi, and Collibri had <5% low coverage index with all four genomes.

Further evaluation of enzymes for Illumina library amplification

To test reproducibility further replicate libraries were made from the better performing enzymes, with the addition of Watchmaker Genomics Equinox library amplification mastermix, and Takara Ex Premier (these new formulations had been previously unavailable for testing), under a variety of different cycling conditions; **Supplementary Table 2**. With this selected group, yields were quite high with all templates (**Supplementary Figure 2 and 3**).

Again the low coverage index was calculated for each dataset and enzymes/conditions ranked from low to high LCI for each genome (**Figure 2**).

Whilst some enzymes perform better in certain genomic contexts RepliQa, Watchmaker Equinox, and Takara Ex Premier, give good coverage uniformity with all genomes. To assess the average low coverage index across all genomes we calculated the sum of the low coverage values compared to coverage from PCRfree libraries (**Figure 3**) illustrating that these three enzymes have minimal bias each giving coverage uniformity similar to that seen with PCR free libraries.

The end result of the more even coverage obtained with RepliQa and Equinox relative to other enzymes could be clearly seen in the more challenging GC or AT rich regions where RepliQa, Equinox and PCR free had good coverage in GC rich (locally 100% GC) regions of *B. pertussis* and also in AT rich (locally <4% GC) regions of *P falciparum* (**Figure 4**).

Sequencing data was also obtained for human genome template amplification. Again this showed RepliQa, Watchmaker Equinox and Takara Ex Premier to have the most even genome coverage reflected in the lowest LCI values (**Supplementary Figure 4**).

It has been observed that some enzymes are inhibited with magnetic beads that are commonly used in NGS workflows e.g. SPRI magnetic bead cleanup and size selection, and streptavidin bead capture of biotin labelled DNA fragments. With SPRI cleanup carryover of beads after elution is commonplace and some protocols employ

"with bead" approaches where increased yield is obtained when the beads are not removed after the final elution step [8]. Streptavidin conjugated magnetic beads are also used in NGS protocols for selection of biotin labelled library fragments, most commonly in hybrid capture target enrichment procedures [9], and due to the extremely strong affinity of streptavidin for biotin such methods require PCR amplification of bead bound library fragments. To test if amplification by the enzymes used in the second phase of this study are inhibited by such beads amplification was carried out without beads, with a volume of washed beads in water equivalent to equal sample volume or with the template bound to 50ul of washed streptavidin beads (Dynabeads MyOne Streptavidin T1, Thermo, cat no. 65602). All of these enzymes tested were found to be unaffected by the presence of Ampure or streptavidin magnetic beads, apart from Q5 (**Supplementary Figure 5**).

Accuracy and utility of the human genome sequences obtained with each enzyme were assessed by comparing each dataset with the NA12878 reference genome and variant list (see methods). Both numbers of indels and SNPs detected were slightly greater with 500bp mean inserts compared to 200bp. The three enzymes with the highest sensitivity for SNP and indel detection in the microbial reference genomes were QuantaBio RepliQa, Watchmaker Equinox and Takara Ex Premier (**Table 1**). These enzymes were found to call more SNPs and indels with greater precision, compared to kapa HiFi at rates that are comparable to those seen in the Precision FDA Truth challenge when using 50x PCRfree datasets [10].

There may be times in a high throughput lab or within a clinical sequencing lab when fast turnaround is required when rapid PCR may be desired. Quantabio promote RepliQa for its short extension times. When we tested the enzymes in phase 2 with increasing extension times (5, 15, 30 or 60 seconds) it was observed that Collibri, Q5, RepliQa and Watchmaker Equinox enzymes gave near maximal yield after just 5 seconds of extension whereas Herculase and Kapa HiFi yields increased with extension time (**Supplementary Figure 6**).

The genome of the malaria parasite *Plasmodium falciparum* has an extremely low GC content of 19.3% [11] and has been shown to be one of the most challenging genomes to amplify and sequence [3, 4] [12]. There have been several papers published for this genome detailing methods to minimise the biases introduced by PCR and sequencing including PCR free library approaches [3], [13] and optimised PCR protocols [4, 11] [14, 15]. In this study when using these approaches we find that the fraction of the genome at less than 50% of mean coverage could be decreased even further (**Figure 5**) though the most successful reduction was achieved by using a different approach for different enzymes. The lowest LCI was achieved using RepliQa with denaturation at 94C and extension at 60C as described by Lopez-Barragan, though near similar LCI values were also obtained under these conditions using Collibri and Watchmaker Equinox enzymes. Following on from this we tested RepliQa under a range of denaturation and extension temperature combinations and found that these conditions could not be improved upon (results not shown).

Long range amplification for Long Read sequencing

Long range PCR is a common approach for generation of material for long read sequencing. Many users have found this to be even more challenging with low yield and a bias towards smaller fragments during amplification. To test the suitability of PCR enzymes for this application we prepared size fractionated adapter ligated yeast genome fragments adding Illumina adapters to enable amplification using the same primers as used in the rest of this study.

Sheared *S. cerevisiae* DNA was size fractionated using Sage Sciences ELF or Bluepippin instruments yielding modal fragment sizes of 21.6kb and 13.3kb respectively (**Supplementary Figure 7**). After adapter ligation 1ng of each of these were used as a template for long range PCR with a range of enzymes using manufacturers recommended cycling conditions (**Supplementary Table 2**).

Initially, 12 cycles of PCR was used, but with most enzymes that generated little or no product (data not shown**)** so PCR was repeated for 15 cycles after which time amplicons of the expected size were observed with most enzymes, though yields varied widely (**Supplementary Figure 8**). The long range PCR products were then prepared for Pacific Biosciences HiFi sequencing using manufacturers' recommended amplicon library prep protocol and barcoded adapters. Sequencing yields and coverage obtained are summarised in (**Supplementary Figure 9 and Table 2)**. Due

to extremely low yields after PCR, products from some enzymes gave insufficient yield to obtain significant coverage.

For those amplification product libraries that gave >30x genome coverage low coverage index was calculated. The lowest LCI (indicating more even genome coverage was obtained with RepliQa followed by Terra polymerase (**Supplementary Figure 10**).

Long range PCR can often preferentially amplify smaller templates such that after multiple cycles the amplification reaction can be dominated by such shorter amplicons. Bluepippin size selected templates amplified by RepliQa and terra polymerase gave the longest average subread lengths (library insert size) of approximately 12kb (**Supplementary Figures 11 and 12**). With the larger 21kb ELF fractionated template the majority of reads were obtained from shorter amplification products. RepliQa gave the largest fraction of 20kb subreads (**Supplementary Figure 13**).

By comparing the PacBio HiFi data with the sequence of the *S. cerevisiae* S288C genome reference the error profile of the library generated after amplification with each enzyme could be determined. Terra, LongAmp and Promega Go Long are Taq based polymerase formulations and as a result were observed to give higher rates of particularly mismatch errors compared to the other enzymes that possess proofreading activity. NEB Q5 gave the lowest error rates (**Supplementary Figure 14**).

As might be expected those enzymes that gave the most even genome coverage also gave the best assembly statistics when 30x normalised coverage reads were assembled

in the SMRTlink portal (**Table 2**). Here RepliQa followed by Terra polymerase gave the most contiguous assemblies. The *S. cerevisiae* genome is known to have 16 chromosomes [16] and additional circular chromosomal elements have been reported [17], therefore assemblies from material amplified using these enzymes has given near complete contiguity with the sum of contig lengths matching that expected for the yeast genome and with ELF fractionated fragments amplified for 15 cycles with RepliQa assembling into just 20 contigs.

Discussion

It has been estimated that when sequencing the human genome 30x average genome coverage is required to give local base coverage at >15x so that both homozygous and heterozygous variants can be accurately detected [18] [19]. Indeed for Genomics Englands' clinical human sequencing 95% coverage at >15 x is a key sequence dataset QC metric [20].

Here we compare sequence datasets from different PCR enzymes based on low coverage index, the percentage of the genome that is covered to <15x when using 30x datasets.

We have used 14 cycles PCR with 1ng of template in order to exacerbate any biases so to be able to differentiate between the enzymes used. Even with 14 cycles and a standardised input there was a broad range of yields. With those enzymes that gave a higher yield, fewer PCR cycles could have been used and indeed here we could have overamplified, possibly introducing bias. However, we obtained similar low coverage index values after 10 cycles PCR for these enzymes (results not shown).

The results presented demonstrate that there are distinct differences between PCR enzymes in the yield and evenness of amplification of fragments prior to sequencing. This further confirms that PCR can be a source of bias in genomic data and illustrates that the user should consider which enzyme is used for these applications, particularly for GC biased templates where coverage bias is more pronounced. Some enzymes work well, giving even coverage and low bias, in some situations, but few are capable of unbiased amplification of both GC and AT rich templates. Here, we have demonstrated that RepliQa, Watchmaker Equinox and Takara Ex Premier can amplify extremes of GC content with coverage bias similar to PCR free data and better uniformity than Kapa HiFi that we previously reported to give the best performance [4]. Surprisingly there was considerable variation in yield between enzymes, again illustrating that the user should carefully choose the enzyme used as utilising a low efficiency enzyme may give low yield, requiring the user to employ more cycles of PCR compared to other enzymes and accentuate the bias even more.

The performance of RepliQa, Equinox and also Takara Ex Premier reported here is impressive, with coverage uniformity after 14 cycles of PCR using 1ng template close to that obtained from PCRfree libraries made with 500ng input, even over wide extremes of GC content.

Here the genome of the malaria parasite has been a particular challenge. It is extremely AT rich with long stretches close to 100% AT [11]. Plasmodium causes a severe disease burden especially in sub Saharan Africa, in the 2021 World Malaria report there were 241 million malaria cases and 627 000 malaria deaths worldwide in 2020. As a result its genome is a major focus for researchers who often face challenges with human host DNA contamination and as those infected are mostly young children from which only low volume blood samples can be taken, only low amounts of DNA are often available. Using previously published modifications to cycling conditions we report extremely even genome coverage from just 1ng template using RepliQa, Watchmaker Equinox and Takara Ex Premier.

Sequencing of human genomes, particularly for variant discovery for academic and clinical outcomes, is a major application of NGS. The human genome is much larger and more complex than the microbial genomes used above and so the lead enzymes from this study were also tested for their ability to amplify limiting amounts of human genome template. DNA from the genome in a bottle standard NA12878 was used for this as it is probably the most sequenced human genome and has a good reference and validated list of variants [21]. Here the RepliQa, Watchmaker Equinox and Takara Ex Premier all performed remarkably well giving even coverage (low LCI) as well as

high degrees of SNP and Indel precision all of which are better than the performance seen with Kapa HiFi. There are slight differences in performance with RepliQa and Watchmaker having greater SNP precision (**Table 1**) and RepliQa and Takara Ex Premier slightly better performance on Indels, meaning that from data generated here RepliQa would be the enzyme of choice as it gives superior performance both for Indels and SNPs.

PCR amplification is also used ahead of long read sequencing either as a means of targeted sequencing or for low input template preparation. Standard ONT and PacBio library prep methods are PCR free and require over 1ug input DNA though both sequencing companies have protocols for low input library prep that involves amplification. We have compared a variety of polymerases for their ability to amplify 1ng of adapter ligated long template DNA and found the majority of enzymes to be quite inefficient for amplification of yeast genome fragments of 13.3 and 21kb and highlight a small number of enzymes that are capable of such reactions. The enzymes which performed best resulting in near complete genome coverage were RepliQa and Terra polymerase, though being a derivative of Taq Terra had higher rates of substitution error. Both these enzymes gave superior performance to Takara Primestar GXL reported the by Jia et al., 2014 to be the best enzyme for long template amplification [22], indicating that better enzymes are continuing to be developed.

Having better performing PCR enzymes will enhance the performance of NGS approaches, particularly for long read sequencing and yield the most contiguous

assemblies and most complete analysis of genomic variants. At the time of this report RepliQa appears to give the best outcome for a variety of genomes and applications.

Methods

Genomic DNA

Human NA12878 CEPH/UTAH PEDIGREE 1463 DNA was purchased from Coriell Camden, NJ.

Bordetella pertussis Tohama I (ATCC-BAA-589D-5), *Escherichia coli* MG1655 (ATCC-700926D-5), *Clostridioides difficil*e 630(ATCC-BAA-1382DQ), *Plasmodium falciparum 3D7* (ATCC-PRA-405D) genomic DNA were obtained from ATCC via LGC standards, Teddington, UK.

Saccharomyces cerevisiae S288C genomic DNA (69240-3) was purchased from Merck, Gillingham, UK.

Illumina Library construction

DNA (0.5µg in 100µl of 10mM Tris-HCl, pH8.5) was sheared in an AFA microtube using a Covaris S2 device (Covaris Inc.), with the following settings: for 200 bp fragments (duty cycle 20, intensity 5, 200 cycles/burst, 90 sec) or for 500 bp fragments (duty cycle 20, intensity 5, 200 cycles/burst, 30sec).

Sheared DNA was purified by binding to an equal volume of AMPure XP beads (A63881, Beckman Coulter, Inc. Brea, CA) and eluted in 50µl of 10mM Tris-HCl,

pH8.5. End-repair, A-tailing and short Truseq adapter ligation* were performed using NEBNext UltraII (E7645L, New England Biolabs, Ipswich, MA). After ligation, excess adapters and adapter dimers were removed by Ampure XP clean-up with a 0.9:1 ratio of beads to sample, eluting in 50µl of 10mM Tris-HCl, pH8.5.
Yield of adapter ligated fragments was ascertained by fluorimetric quantification using Qubit high-sensitivity DNA reagents (Q32851, ThermoFisher, Waltham, MA).
*Oligo sequences are supplied in supplementary table S1.

PCR free libraries were prepared using the same approach starting with 500ng of genomic DNA input and ligating “IDT for Illumina” full length unique dual indexed adapters.

PCR

Each PCR was performed using 1ng of template (Illumina truseq adapter ligated sheared DNA), 0.1nmol each of unique dual indexed i7 and i5 barcoding Illumina PCR primers (Supplementary table S1) and 25ul of 2x enzyme premix.

Unless indicated all PCR reaction were performed for 14 cycles on an MJ Tetrad 4 thermocycler that had been validated for accuracy using a Driftcon thermocycler calibration instrument (Cyclertest, Landgraaf, Netherlands).

PCR reaction products were cleaned and size selected using a 0.7:1 ratio of AMPure XP SPRI beads (Beckman Coulter Inc.) to sample, according to the manufacturers' protocol with elution in 30ul of EB buffer.

Illumina sequencing

Prior to sequencing libraries were quantified by real-time PCR, using the SYBR Fast Illumina Library Quantification Kit (Kapa Biosystems cat. no. KK4834).

Libraries were pooled in an equimolar fashion whilst correcting for genome size to facilitate equal sequence coverage from each.

Samples were sequenced on an Illumina Novaseq 6000 instrument with 150 paired end read length and v1.5 chemistry.

Long fragment amplification and Pacific Biosciences Sequencing

Two aliquots of S. cerevisiae DNA S288C were sheared using a Megaruptor 3 (Diagenode, NJ); aliquot 1: 5ug diluted to 150 ul volume with EB buffer was sheared at speed 30 and then 31 to tighten peak, aliquot 2: 10ug diluted to 310 ul volume with EB buffer was sheared at speed 31. Both aliquots of sheared DNA were purified and

concentrated using a 1:1 ratio of PacBio AMPure SPRI beads with elution in 30ul EB. The amount of sheared DNA was measured by fluorimetry using Qubit high sensitivity DNA quantification reagents. Aliquot 1 had 3.1ug, and was size selected on Sage Sciences ELF instrument aiming for a maximum fragment size capture around 20kb. Aliquot 2 had 4.8ug, and 2.4ug was size selected on Sage Sciences Blue pippin instrument with selection set to range 10kb-50kb. Size selected fractions were purified using a 1:1 ratio of PacBio AMPure SPRI beads with elution in 50ul EB. Prior to amplification Illumina adapters were added to the ends of each fragment to enable primer annealing. End-repair, A-tailing and short Truseq adapter ligation were performed using NEBNext UltraII as above. After ligation, excess adapters and adapter dimers were removed by Ampure XP clean-up with a 0.9:1 ratio of beads to sample, eluting in 50µl of 10mM Tris-HCl, pH8.5. Yield of adapter ligated fragments was ascertained by fluorimetric quantification using Qubit high-sensitivity DNA reagents; ELF fractionated DNA was 10.2ng/ul, Blue pippin fractionated DNA was 3.04 ng/ul. Each was diluted to 1ng/ul with EB and 1ul of these dilutions used as template for long range PCR.

After PCR amplified products were concentrated with a 1:1 ratio of PacBio AMPure SPRI beads with elution in 7ul EB, 1ul of which was used for QC and 5ul for PacBio barcoded overhang adapter amplicon library prep with a different barcoded adapter being used for each successful PCR reaction [23].

PacBio libraries were quantified using Qubit high-sensitivity DNA reagents and where possible ligated samples were equimolar pooled, weak samples were pooled in their entirety. Pooled barcoded amplified fragments were sequenced using a Pacbio

Sequel IIe instrument using binding kit v2.2 and sequencing chemistry 2.0, library was loaded at 80pM.

Data processing and analysis

After sequencing, reads were mapped to each genome reference sequence using Minimap2 [24]. SAMtools [25] was then used to generate pileup and coverage information from the mapping output.

The quality of the sequence data was assessed using FastQC v0.11.9.

For human genome sequence data fastq files were automatically aligned to reference GRCh38 and were mapped using bwa version 0.7.17-r1188 and the command bwa mem -t 12 -p -Y -K 100000000 <reference.fa> <read1.fastq.gz> <read2.fastq.gz> and duplicates were marked using biobambam2 version 2.0.79.
The resulting CRAM files were converted to BAM, sorted and indexed with samtools v.1.15.1. The stats and graphs were produced using samtools v.1.15.1.
All bams were subsampled to approx. 33X using samtools v 1.15.1 and seed 42.
Variants were called using GATK HaplotypeCaller v3.5 with special options '-stand_call_conf 2 -stand_emit_conf 2 -A BaseQualityRankSumTest -A ClippingRankSumTest -A Coverage -A FisherStrand -A LowMQ -A RMSMappingQuality -A ReadPosRankSumTest -A StrandOddsRatio -A HomopolymerRun -A TandemRepeatAnnotator'[26].

For each dataset the overlap of variants on chromosomes 1-22 with the GIAB (Genome in a Bottle) v4.2.1 benchmark dataset was calculated using bcftools isec version 1.15.1. The true positive (TP) value was calculated as the number of variant sites that were identified in both the sample and and the GIAB benchmark dataset. The false negative (FN) was calculated as the number of variant sites identified in the GIAB dataset but not identified in the sample. The sensitivity was calculated as TP/(TP+FN) or the number variant sites found in the sample as a percentage of all the variant sites in the giab benchmark dataset [27].

PacBio HiFi read assembly was performed on 30x downsized coverage using IPA [28] assembler within SMRTlink v10.2.

Availability of supporting data

All datasets have been deposited in the ENA read archive. Human genome datasets under accession number ERP141249 and microbial genomes under accession number ERP141224.

Abbreviations

NGS, Next-Generation Sequencing; Tm, Melting Temperature; bp, Base Pairs; PCR, Polymerase Chain Reaction; LCI, Low coverage Index.

Competing interests

MQ is a member of the New England Biolabs panel of Key Opinion Leaders.

Authors' contributions

MQ performed the experiments and performed primary data analysis. MQ designed the experiments. CC and JU performed the PacBio library prep and sequencing work. MQ wrote the manuscript. YGU and JK carried out bioinformatics analysis.

Acknowledgements

The authors thank the Wellcome Trust Sanger Institute core sequencing and informatics teams. This work was supported by the Wellcome Trust [grant number 098051].

Steven Leonard and IDT for design of barcode sequences.

Figure Legends

Figure 1. Low coverage index obtained after 14 cycles of amplification with 1ng of each test microbial genome; BP: *Bordetella pertussis*, EC: *Escherichia coli*, CD:

Clostridioides difficile, and PF: *Plasmodium falciparum*. For each PF was also amplified using a 94C denaturation temperature, “PF 94C”.

Figure 2. Ranked Low Coverage Index values obtained after 14 cycles of amplification with 1ng of each test microbial genome; a) *Bordetella pertussis*, b) *Escherichia coli*, c) *Clostridioides difficile,* and d) *Plasmodium falciparum*.

Figure 3. Multi-genome average low coverage index ranking taking the sum of the LCI values for all 4 genomes compared to that obtained from PCR free data.

Figure 4. Screenshot from Artemis genome browser [7] over regions of a) *B. pertussis* and b) *P. falciparum* genomes. In each the top panel is a GC content plot with the maxima and minima GC content of each region displayed at the far right. The bottom panel is an open read frame plot and the middle panel plots read coverage over each base. The inset panel on each denotes the enzyme used for each coloured line. In a) RepliQa (pink), Watchmaker (blue) and PCRfree data (black) coverage is unaffected whereas with kapa hifi (red) and Collibri (green) coverage drops to near zero in the GC rich region in the region in the centre of the plot. Likewise in b) RepliQa (blue), Watchmaker (black) and PCRfree data (pink) coverage is unaffected whereas with kapa hifi (red) and Collibri (green) coverage drops to near zero in the AT rich region in the region in the centre of the plot.

Figure 5. Low coverage index for amplification of 1ng P falciparum Illumina library template with a range of enzymes using PCR conditions described by Lopez-Barragan [14] (blue), Quail (orange) [4], Oyola [12] (grey) and Aird [15] (yellow).

Tables

Table 1. Indel True Positive, False Negative and Sensitivity values for NA12878 libraries prepared with either approx 200 or 500 bp inserts with different PCR enzymes.

Table 2. Assembly statistics from PacBio HiFI 30 x genome coverage from the various PCR enzyme datasets.

Additional files

Supplementary Table 1. Sequences of unique dual indexing oligonucleotide primers and Illumina sequencing adapter used in this study.

Supplementary Table 2. Enzymes and cycling conditions used in this study.

Supplementary Figure 1: Library yield (ng/ul) obtained after 14 cycles of amplification with 1ng of each test microbial genome; BP: *Bordetella pertussis*, EC: *Escherichia coli*, CD: *Clostridioides difficile*, and PF: *Plasmodium falciparum. For each PF was also amplified using a 94C denaturation temperature, "PF 94C"*.

Supplementary Figure 2. Library yield (ng/ul) obtained after 14 cycles of amplification with 1ng of each test microbial genome; Top: *Bordetella pertussis*, Bottom: *E. coli*

Supplementary Figure 3. *Library yield (ng/ul) obtained after 14 cycles of amplification with 1ng of test microbial genome; Top: C difficile, Bottom: P falciparum*

Supplementary Figure 4*. Low coverage index value for Human NA12878 sequence 25x coverage datasets after amplification with various enzymes.*

Supplementary Figure 5*. Library yields after 14 cycles PCR with 1ng of E.coli Illumina adapter ligated template either without beads present (blue), in the presence of ampure beads (orange) or streptavidin beads (grey).*

Supplementary Figure 6. *Library yields after 14 cycles PCR with 1ng of B. pertussis Illumina adapter ligated template with extension for 5 seconds (blue), 15 seconds (orange), 30 seconds (grey) and 60 seconds (yellow).*

Supplementary Figure 7. *Femtopulse electropherograms of size fractionated yeast DNA from a) ELF and b) Blue pippin.*

Supplementary Figure 8. *a) PCR Yields and b) Agilent Tapestation genomic kit electropherograms from long range PCR with different enzymes after 15 cycles of PCR*

Supplementary Figure 9. *PacBio HiFi Sequel IIe sequence yields and coverage obtained from barcoded long template PCR libraries.*

Supplementary Figure 10. *Low coverage index for sequencing S. cerevisiae 20kb fragments at 30x depth on PacBio Sequel IIe.*
A *denotes ELF fractionation,* ***B*** *Bluepippin.* ***X12*** *samples had 12 cycles PCR,* ***x15*** *had 15 cycles.*

Supplementary Figure 11. *Average subread lengths, ranked by size, after PacBio HiFi sequencing for amplicon libraries that gave >30x genome coverage.*
A *denotes ELF fractionation,* ***B*** *Bluepippin. "****12"*** *samples had 12 cycles PCR,* ***"15"*** *had 15 cycles.*

Supplementary Figure 12. *PacBio HiFi read length heatmap plot from SMRTlink interface for each library sequenced.*

A *denotes ELF fractionation,* ***B*** *Bluepippin.* ***X12*** *samples had 12 cycles PCR,* ***x15*** *had 15 cycles.*

Supplementary Figure 13*. HiFi subread length distributions for ELF size fractionation of a) Terra polymerase and b) repliQa amplified fragments and Bluepippin fractionation of c) Terra polymerase and d) repliQa amplified fragments, after 15 cycles PCR*

Supplementary Figure 14*. Percentage mismatch, insertion and deletion errors in PacBio HiFi reads with datasets that gave >30x genome coverage ranked from lowest to highest rate of mismatch error.*

A *denotes ELF fractionation,* ***B*** *Bluepippin. "****12"*** *samples had 12 cycles PCR,* ***"15"*** *had 15 cycles.*

References

1. Bronner IF, Quail MA: **Best Practices for Illumina Library Preparation**. *Curr Protoc Hum Genet* 2019, **102**(1):e86.

2. Bronner IF, Quail MA, Turner DJ, Swerdlow H: **Improved Protocols for Illumina Sequencing**. *Curr Protoc Hum Genet* 2014, **80**:18 12 11-42.

3. Kozarewa I, Ning Z, Quail MA, Sanders MJ, Berriman M, Turner DJ: **Amplification-free Illumina sequencing-library preparation facilitates improved mapping and assembly of (G+C)-biased genomes**. *Nat Methods* 2009, **6**(4):291-295.

4. Quail MA, Otto TD, Gu Y, Harris SR, Skelly TF, McQuillan JA, Swerdlow HP, Oyola SO: **Optimal enzymes for amplifying sequencing libraries**. *Nat Methods* 2011, **9**(1):10-11.

5. Costello M, Fleharty M, Abreu J, Farjoun Y, Ferriera S, Holmes L, Granger B, Green L, Howd T, Mason T *et al*: **Characterization and remediation of sample index swaps by non-redundant dual indexing on massively parallel sequencing platforms**. *BMC Genomics* 2018, **19**(1):332.

6. Sinha Rea: **Index switching causes 'spreading-of-signal' among multiplexed samples in Illumina HiSeq 4000 DNA sequencing**. *BioRXIV* *https://wwwbiorxivorg/content/101101/125724v1* 2017.

7. Carver T, Harris SR, Berriman M, Parkhill J, McQuillan JA: **Artemis: an integrated platform for visualization and analysis of high-throughput sequence-based experimental data**. *Bioinformatics* 2012, **28**(4):464-469.

8. Fisher S, Barry A, Abreu J, Minie B, Nolan J, Delorey TM, Young G, Fennell TJ, Allen A, Ambrogio L *et al*: **A scalable, fully automated process for construction of sequence-ready human exome targeted capture libraries**. *Genome Biol* 2011, **12**(1):R1.

9. Gnirke A, Melnikov A, Maguire J, Rogov P, LeProust EM, Brockman W, Fennell T, Giannoukos G, Fisher S, Russ C *et al*: **Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing**. *Nat Biotechnol* 2009, **27**(2):182-189.

10. challenge PFT: https://precision.fda.gov/challenges/truth/results.

11. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S *et al*: **Genome sequence of the human malaria parasite Plasmodium falciparum**. *Nature* 2002, **419**(6906):498-511.

12. Oyola SO, Otto TD, Gu Y, Maslen G, Manske M, Campino S, Turner DJ, Macinnis B, Kwiatkowski DP, Swerdlow HP *et al*: **Optimizing Illumina next-generation sequencing library preparation for extremely AT-biased genomes**. *BMC Genomics* 2012, **13**:1.

13. Chappell L, Ross P, Orchard L, Russell TJ, Otto TD, Berriman M, Rayner JC, Llinas M: **Refining the transcriptome of the human malaria parasite Plasmodium falciparum using amplification-free RNA-seq**. *BMC Genomics* 2020, **21**(1):395.

14. Lopez-Barragan MJ, Quinones M, Cui K, Lemieux J, Zhao K, Su XZ: **Effect of PCR extension temperature on high-throughput sequencing**. *Mol Biochem Parasitol* 2011, **176**(1):64-67.

15. Aird D, Ross MG, Chen WS, Danielsson M, Fennell T, Russ C, Jaffe DB, Nusbaum C, Gnirke A: **Analyzing and minimizing PCR amplification bias in Illumina sequencing libraries**. *Genome Biol* 2011, **12**(2):R18.

16. Fisk DG, Ball CA, Dolinski K, Engel SR, Hong EL, Issel-Tarver L, Schwartz K, Sethuraman A, Botstein D, Cherry JM *et al*: **Saccharomyces cerevisiae S288C genome annotation: a working hypothesis**. *Yeast* 2006, **23**(12):857-865.

17. Moller HD, Parsons L, Jorgensen TS, Botstein D, Regenberg B: **Extrachromosomal circular DNA is common in yeast**. *Proc Natl Acad Sci U S A* 2015, **112**(24):E3114-3122.

18. Meynert AM, Bicknell LS, Hurles ME, Jackson AP, Taylor MS: **Quantifying single nucleotide variant detection sensitivity in exome sequencing**. *BMC Bioinformatics* 2013, **14**:195.

19. Meynert AM, Ansari M, FitzPatrick DR, Taylor MS: **Variant detection sensitivity and biases in whole genome and exome sequencing**. *BMC Bioinformatics* 2014, **15**:247.

20. https://research-help.genomicsengland.co.uk/display/GERE/Sample+QC.

21. Zook JM, Chapman B, Wang J, Mittelman D, Hofmann O, Hide W, Salit M: **Integrating human sequence data sets provides a resource of benchmark SNP and indel genotype calls**. *Nat Biotechnol* 2014, **32**(3):246-251.

22. Jia H, Guo Y, Zhao W, Wang K: **Long-range PCR in next-generation sequencing: comparison of six enzymes and evaluation on the MiSeq sequencer**. *Sci Rep* 2014, **4**:5737.

23. Biosciences P: **https://www.pacb.com/wp-content/uploads/Procedure-Checklist-Preparing-SMRTbell-Libraries-using-PacBio-Barcoded-Overhang-Adapters-for-Multiplexing-Amplicons.pdf**.

24. Li H: **Minimap2: pairwise alignment for nucleotide sequences**. *Bioinformatics* 2018, **34**(18):3094-3100.

25. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing S: **The Sequence Alignment/Map format and SAMtools**. *Bioinformatics* 2009, **25**(16):2078-2079.

26. Zook JM, McDaniel J, Olson ND, Wagner J, Parikh H, Heaton H, Irvine SA, Trigg L, Truty R, McLean CY *et al*: **An open resource for accurately benchmarking small variant and reference calls**. *Nat Biotechnol* 2019, **37**(5):561-566.

27. GIAB: **https://ftp-trace.ncbi.nlm.nih.gov/giab/ftp/release/NA12878_HG001/NISTv4.2.1/GRCh38/**.

28. Dunn C, Sovic, I.: **IPA HiFi Genome Assembler**. *GitHub* 2022, **https://github.com/PacificBiosciences/pbipa**.

Figures for Identification of optimal library PCR enzymes

Quail et al.,

Figure 1. Low coverage index obtained after 14 cycles of amplification with 1ng of each test microbial genome; BP: *Bordetella pertussis*, EC: *Escherichia coli*, CD: *Clostridioides difficile*, and PF: *Plasmodium falciparum*. For each PF was also amplified using a 94C denaturation temperature, “PF 94C”.

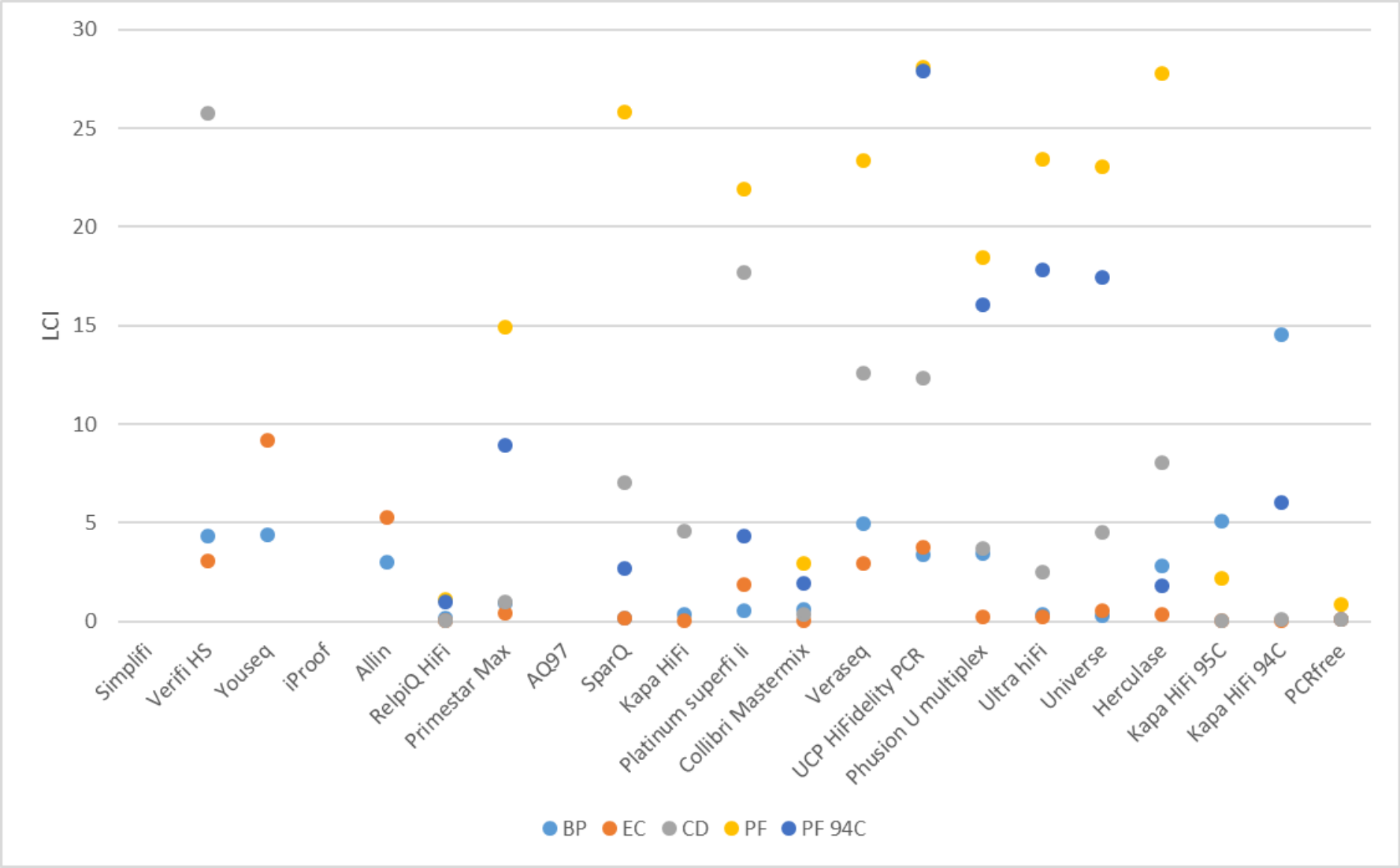


Figure 2. Ranked Low Coverage Index values obtained after 14 cycles of amplification with 1ng of each test microbial genome; a) *Bordetella pertussis*, b) *Escherichia coli*, c) *Clostridioides difficile*, and d) *Plasmodium falciparum*.

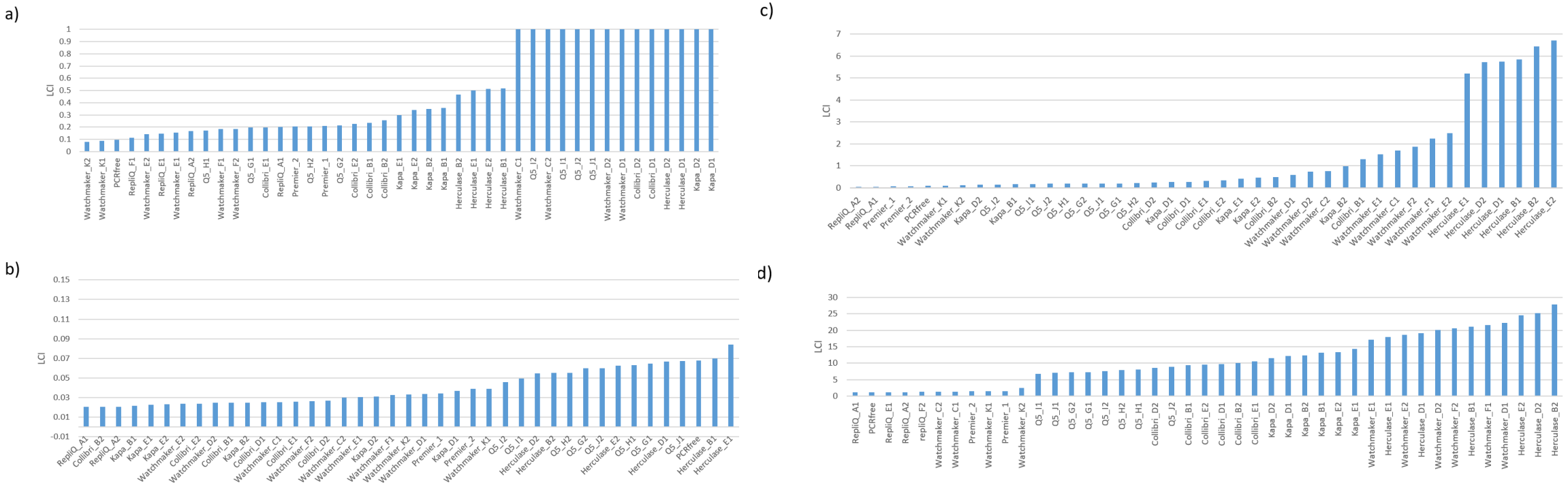


Figure 3. Multi-genome average low coverage index ranking taking the sum of the LCI values for all 4 genomes compared to that obtained from PCR free data.

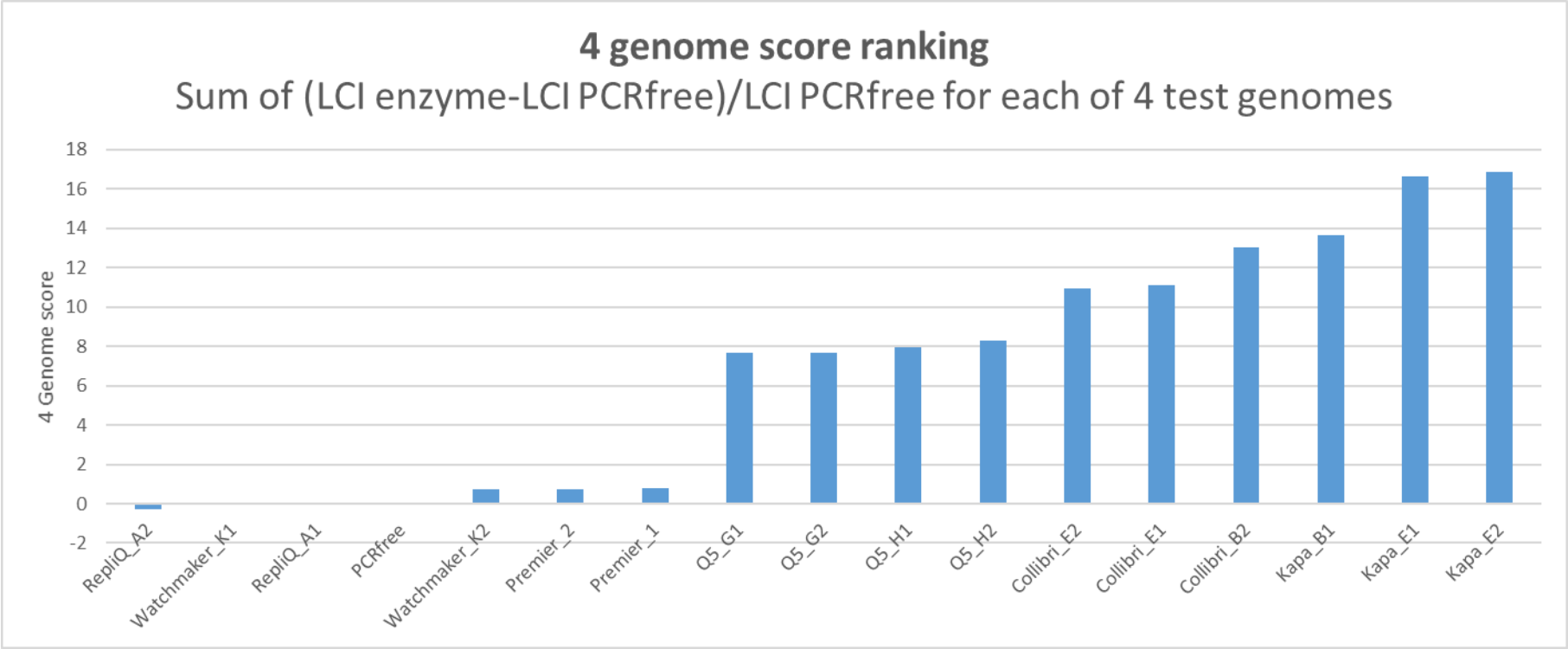
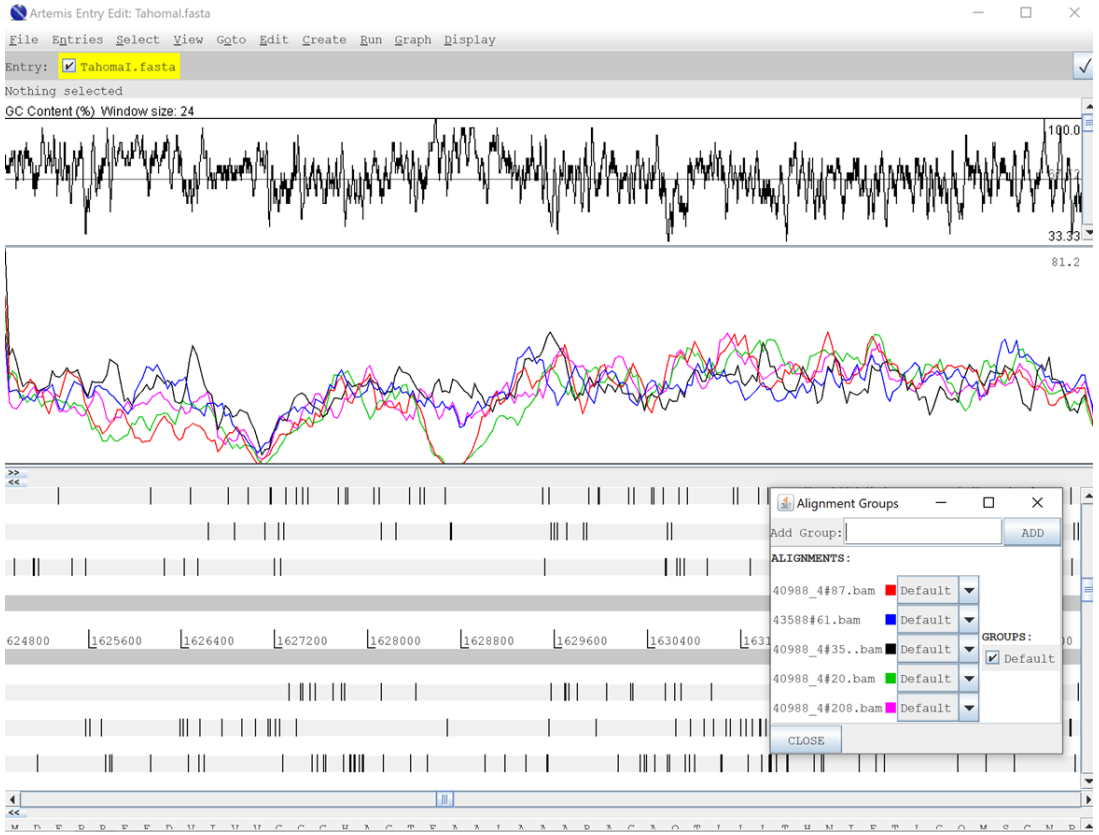


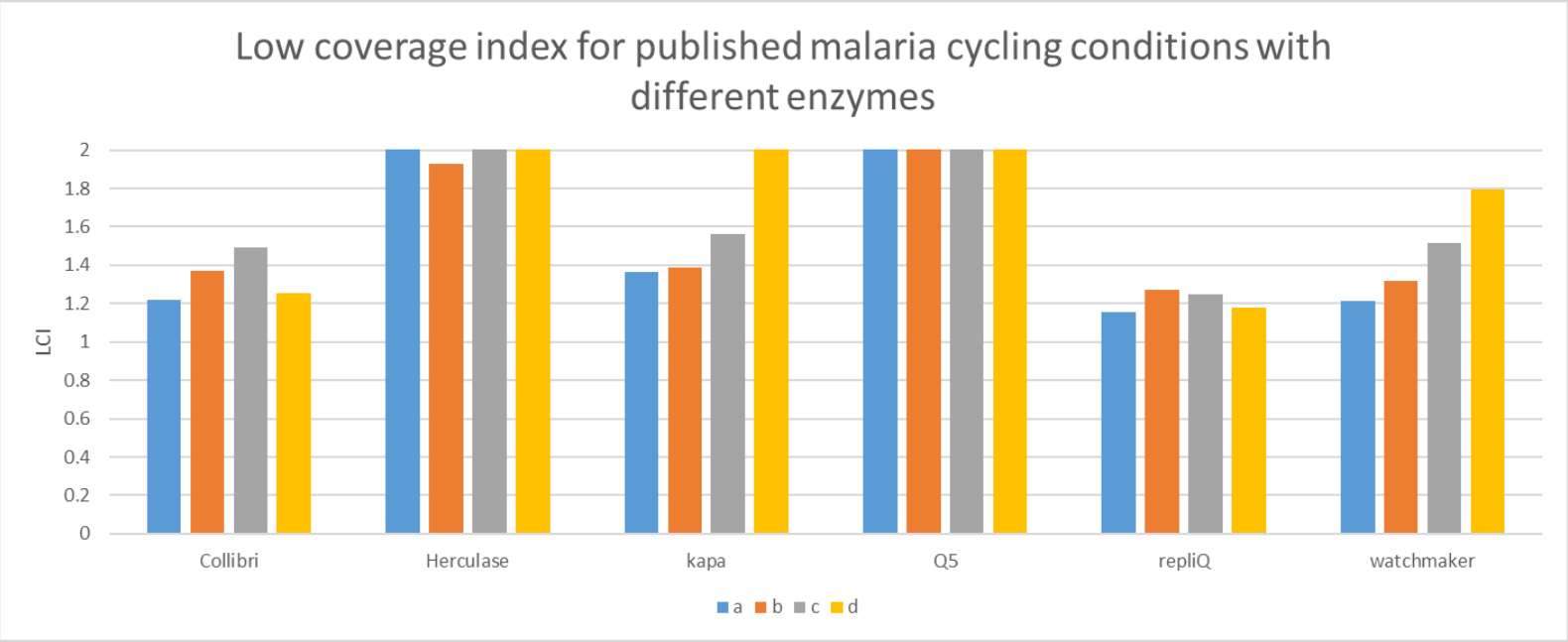
Figure 4. Screenshot from Artemis genome browser [7] over regions of a) *B. pertussis* and b) *P. falciparum* genomes. In each the top panel is a GC content plot with the maxima and minima GC content of each region displayed at the far right. The bottom panel is an open read frame plot and the middle panel plots read coverage over each base. The inset panel on each denotes the enzyme used for each coloured line. In a) RepliQa (pink), Watchmaker (blue) and PCRfree data (black) coverage is unaffected whereas with kapa hifi (red) and Collibri (green) coverage drops to near zero in the GC rich region in the region in the centre of the plot. Likewise in b) RepliQa (blue), Watchmaker (black) and PCRfree data (pink) coverage is unaffected whereas with kapa hifi (red) and Collibri (green) coverage drops to near zero in the AT rich region in the region in the centre of the plot.

a)



b)

Figure 5. Low coverage index for amplification of 1ng P falciparum Illumina library template with a range of enzymes using PCR conditions described by Lopez-Barragan [14] (blue), Quail (orange) [4], Oyola [12] (grey) and Aird [15] (yellow).



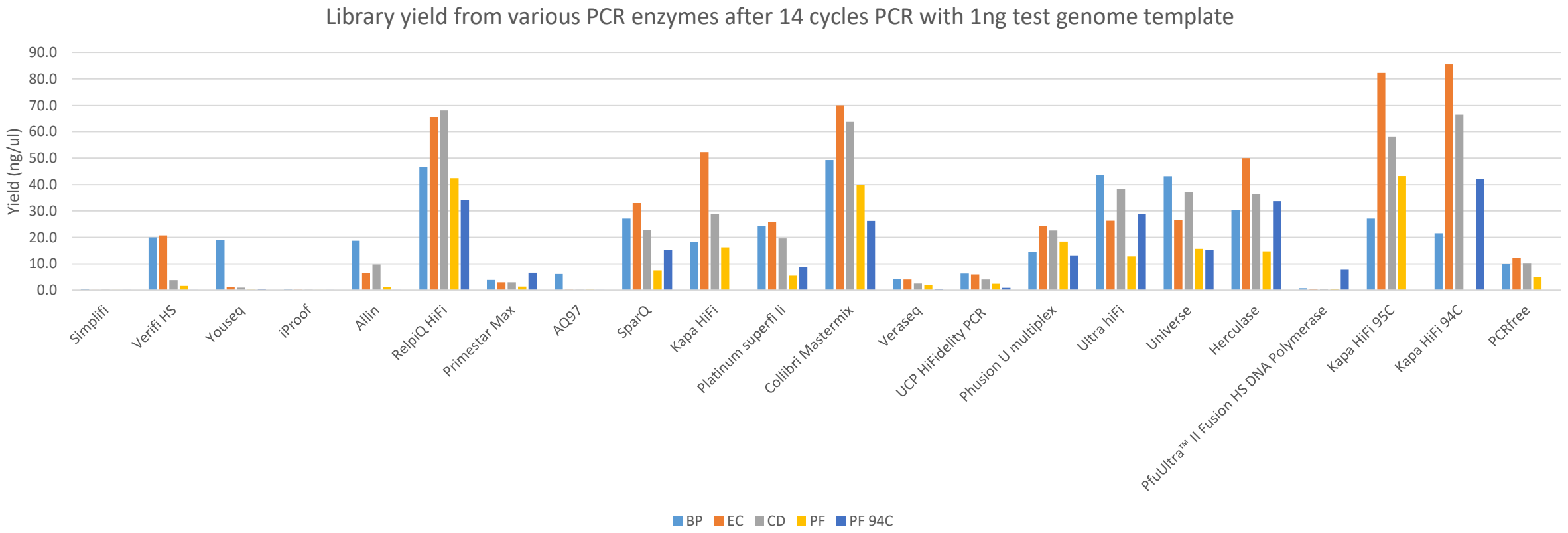
Condition	Publication	Initial extension	Denaturation	Anneal	Extend	Polish
a	Lopez-Barragan	94°C - 2min	94°C - 45 sec	60°C - 30 sec	60°C - 2min	60°C - 3min
b	Quail	98°C - 2min	98°C - 20 sec	60°C - 30 sec	60°C - 2min	60°C - 3min
c	Oyola	94°C - 2min	94°C - 15 sec	60°C - 30 sec	65°C - 2min	65°C - 5min
d	Aird	98°C - 3min	98°C - 80 sec	60°C - 30 sec	60°C - 2min	60°C - 10min

Identification of optimal library PCR enzymes

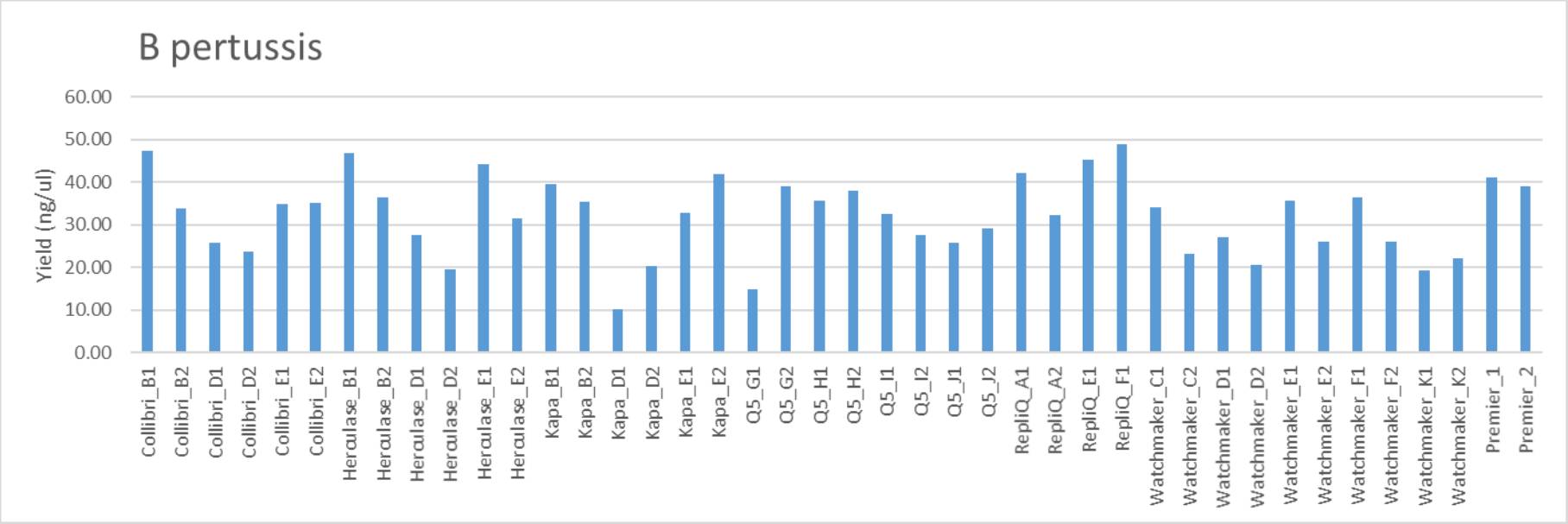
Quail et al.,

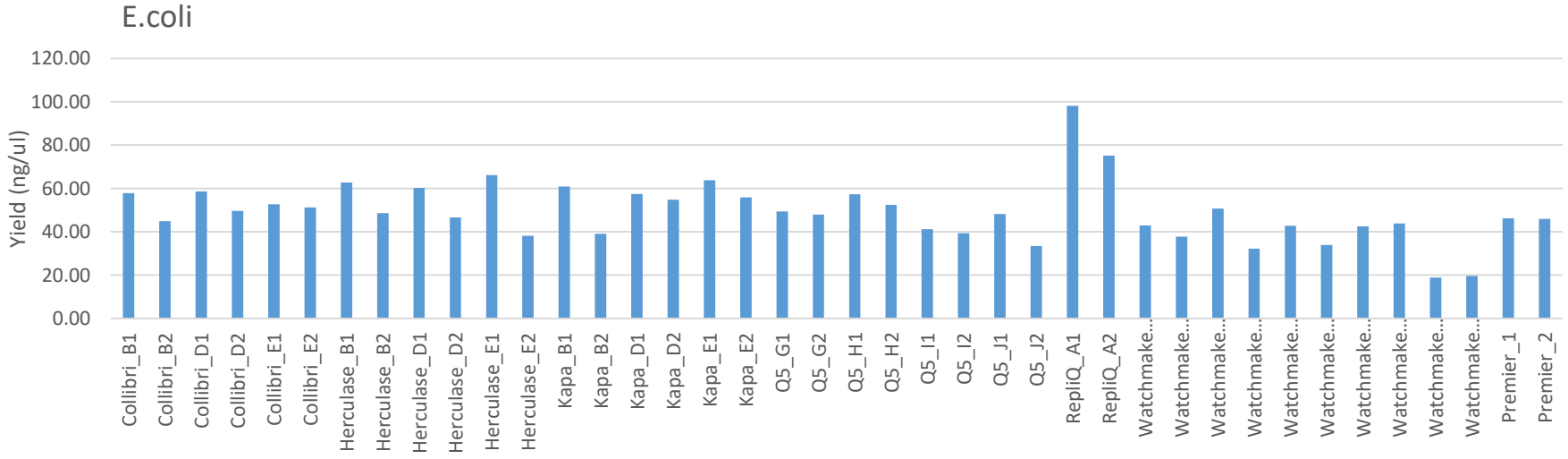
Supplementary Figures

Supplementary Figure 1: Library yield (ng/ul) obtained after 14 cycles of amplification with 1ng of each test microbial genome; BP: *Bordetella pertussis*, EC: *Escherichia coli,* CD: *Clostridioides difficile*, and PF: *Plasmodium falciparum. For each PF was also amplified using a 94C denaturation temperature, "PF 94C".*

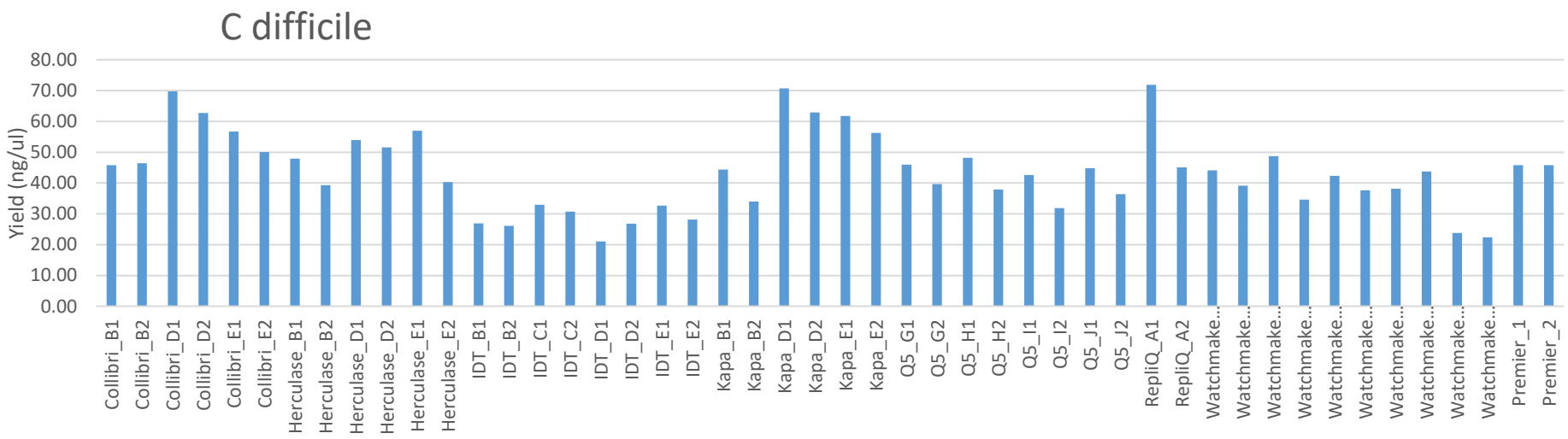


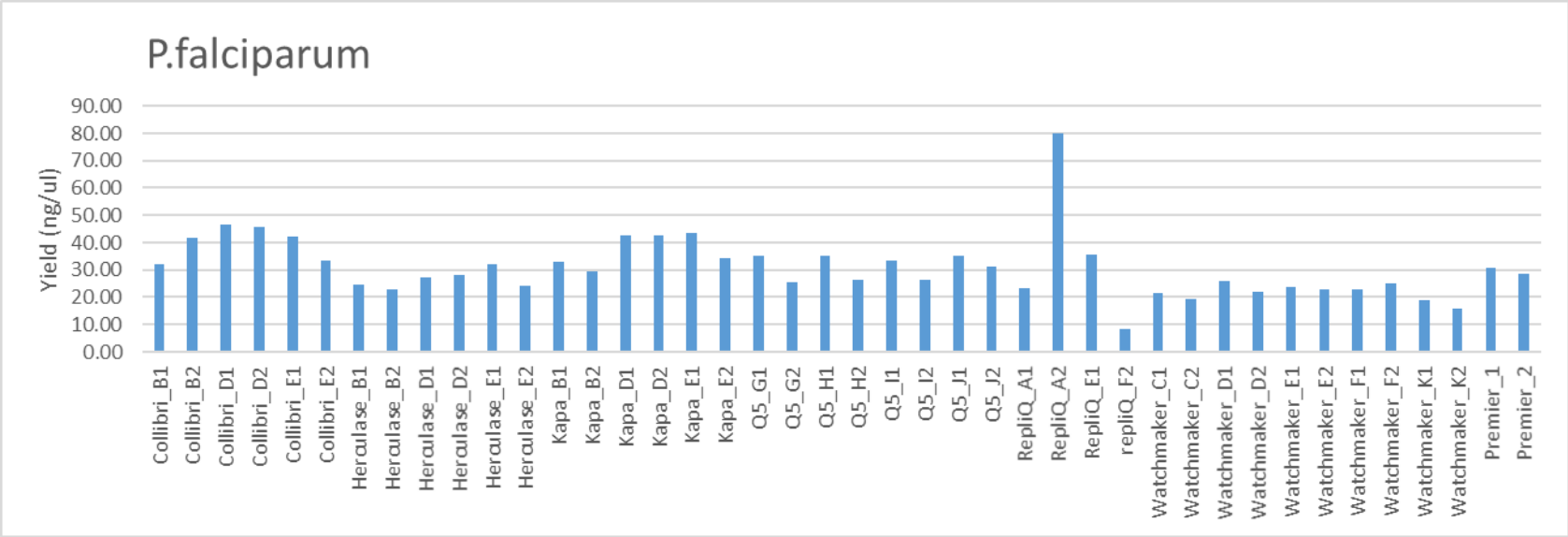
Supplementary Figure 2. Library yield (ng/ul) obtained after 14 cycles of amplification with 1ng of each test microbial genome; Top: *Bordetella pertussis*, Bottom: *E. coli*



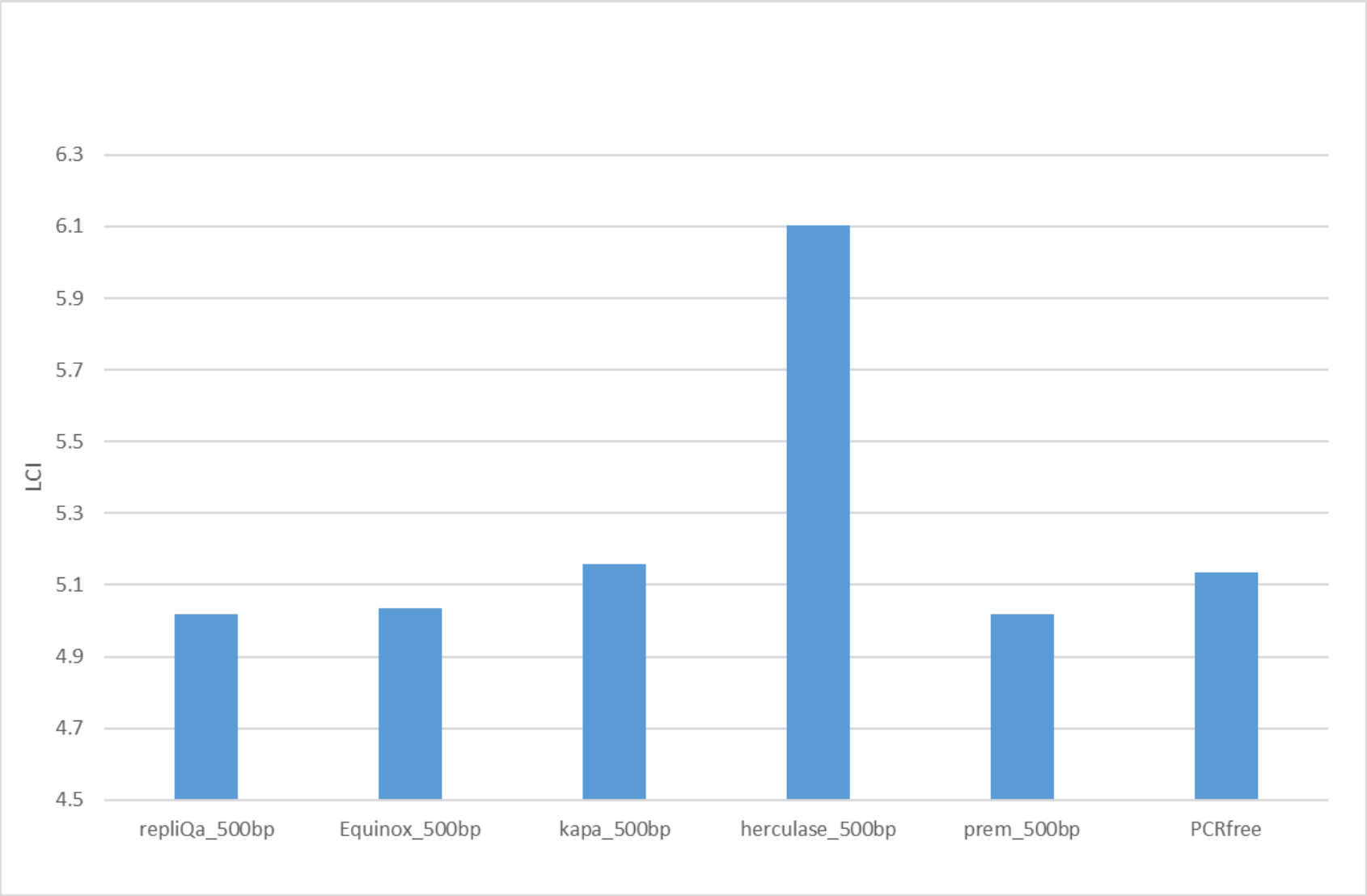


Supplementary Figure 3. Library yield (ng/ul) obtained after 14 cycles of amplification with 1ng of test microbial genome; Top: *C difficile*, Bottom: *P falciparum*

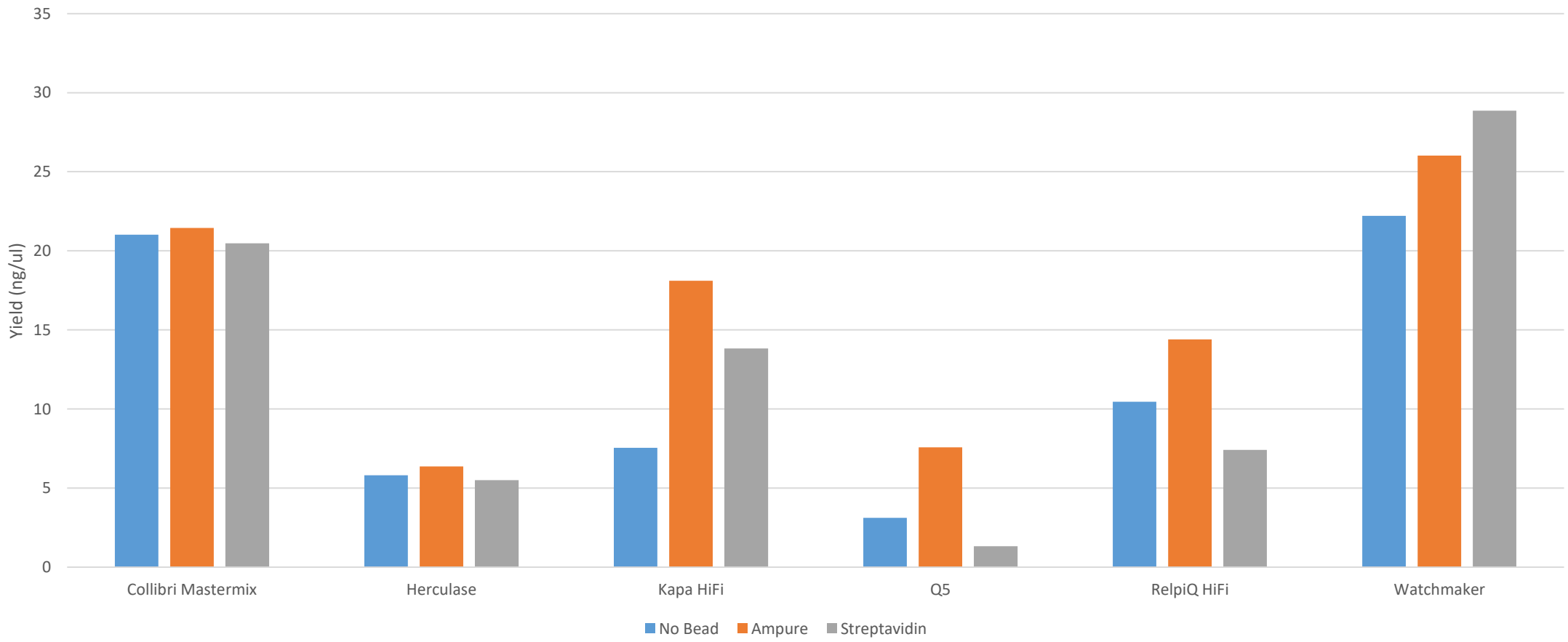




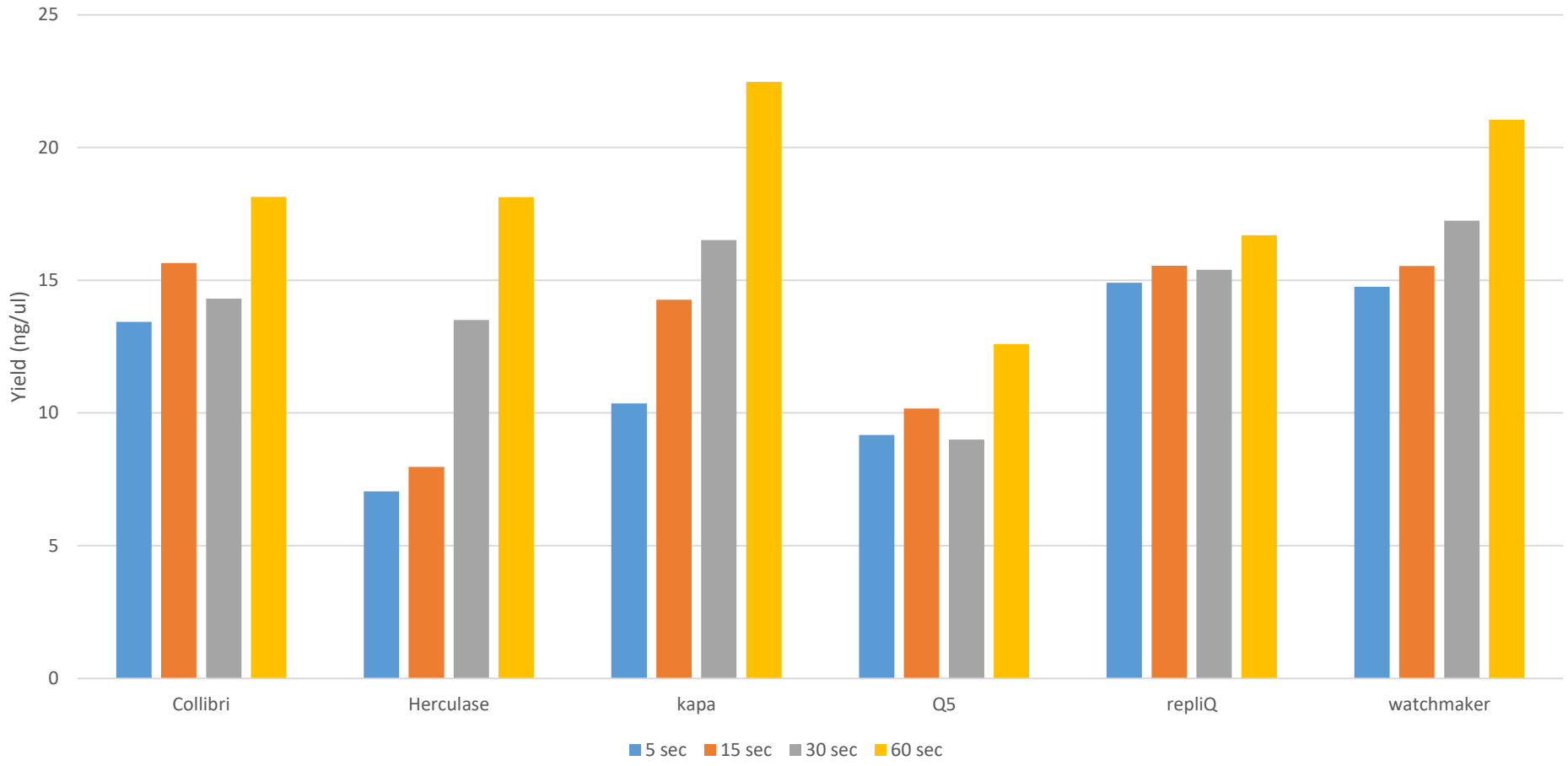
Supplementary Figure 4. Low coverage index value for Human NA12878 sequence 25x coverage datasets after amplification with various enzymes.



Supplementary Figure 5. Library yields after 14 cycles PCR with 1ng of E.coli Illumina adapter ligated template either without beads present (blue), in the presence of ampure beads (orange) or streptavidin beads (grey).

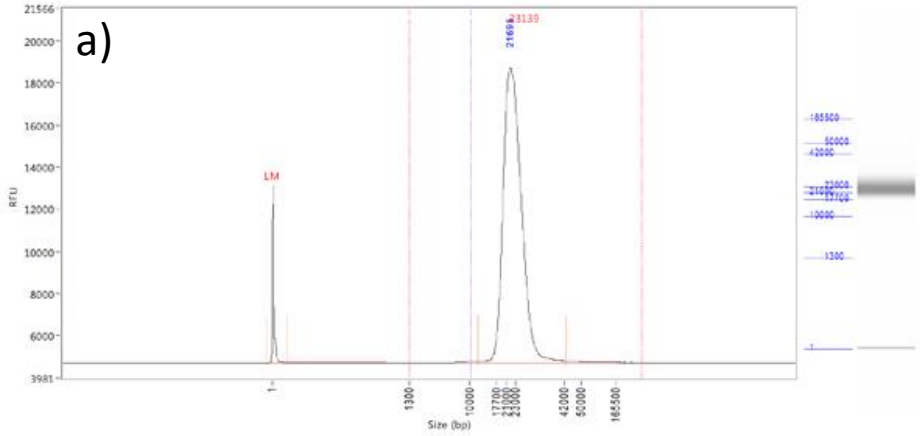


Supplementary Figure 6. Library yields after 14 cycles PCR with 1ng of B. pertussis Illumina adapter ligated template with extension for 5 seconds (blue), 15 seconds (orange), 30 seconds (grey) and 60 seconds (yellow).



Supplementary Figure 7. Femtopulse electropherograms of size fractionated yeast DNA from a) ELF and b) Blue pippin.

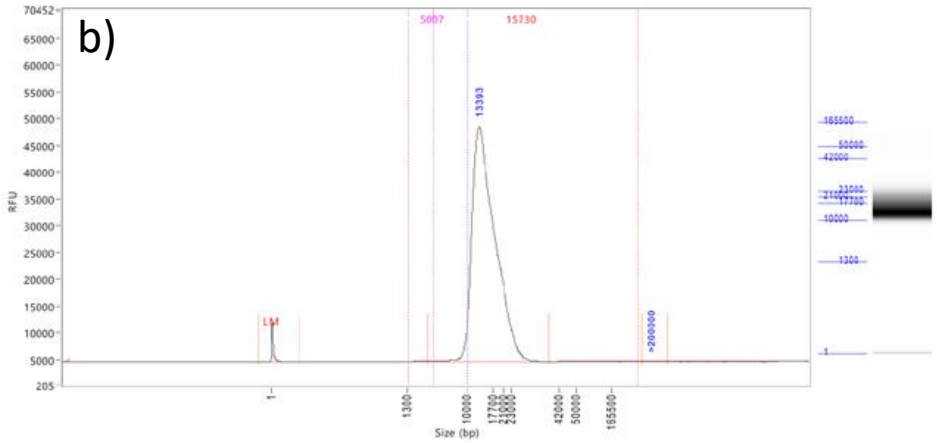
Sample: mq1_yeast_RAD_ELF1
Well Location: F3
Created: 2/11/2022 9:55 AM



Peak	Size (bp)	Conc. (pg/uL)	From (bp)	To (bp)	Avg. Size (bp)	CV%	RFU	Corr. Peak Area
1	1 (LM)	2.0831	0	141	4	298.54	8399	64.374
2	21695	214.1000	12275	42826	22749	13.65	14046	661.652

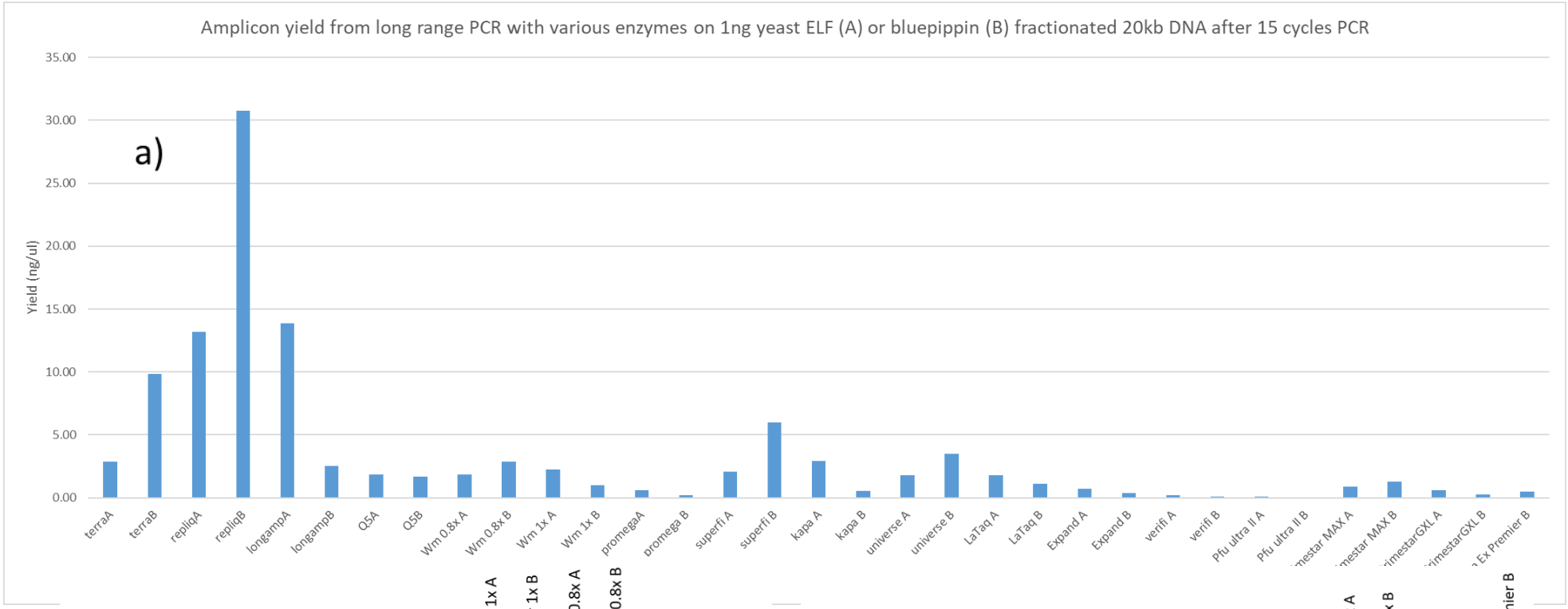
TIC:	214.1000	pg/uL
TIM:	16.2475	pmole/L
Total Conc.:	216.5718	pg/uL

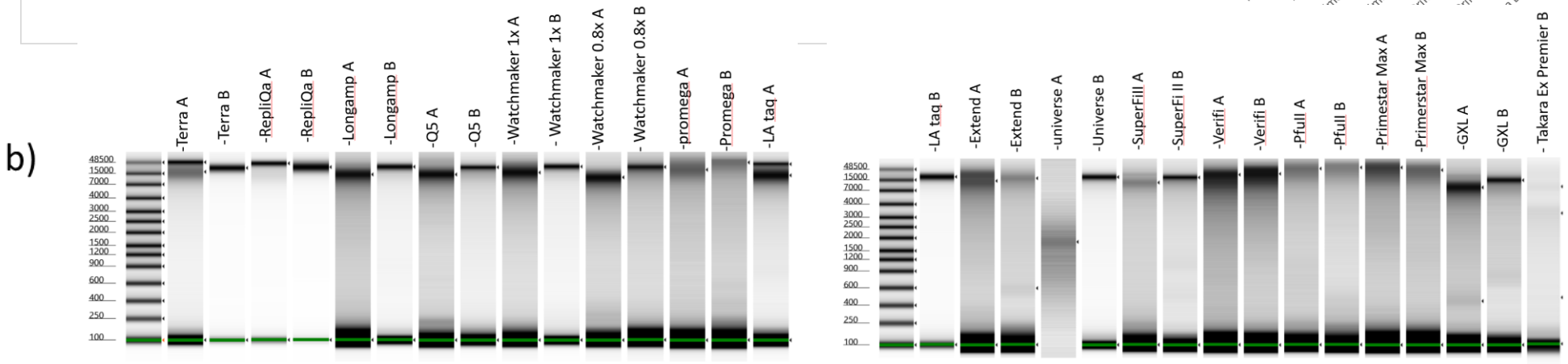
Sample: Yeast #2_12Kb_RAD_PrLIBP
Well location: A2
Created: 2/15/2022 2:21 PM



Peak	Size (bp)	Concentration (ng/uL)	Relative concentration Percent	Molarity (nmole/L)	From (bp)	To (bp)	CV%	RFU
1	1 (LM)	0.0025		0.7748	0	267	360.97	7627
2	13393	1.1130	99.9	0.1183	4158	37888	23.46	43880
3	>200000	0.0010	0.1	0.0000	>200000	>200000	5.63	185

Supplementary Figure 8. a) PCR Yields and b) Agilent Tapestation genomic kit electropherograms from long range PCR with different enzymes after 15 cycles of PCR



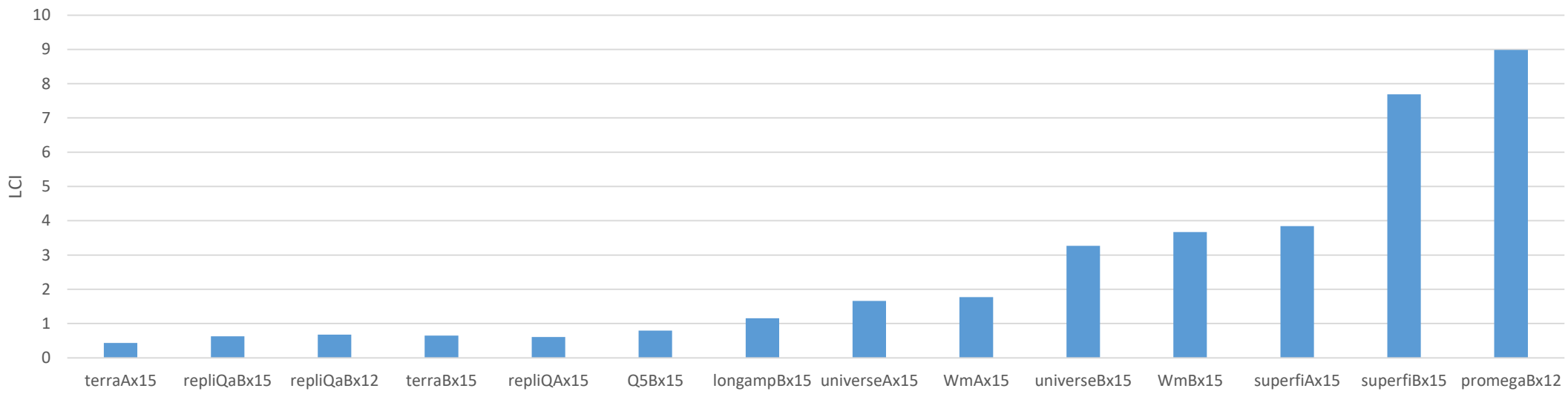


Supplementary Figure 9. PacBio HiFi Sequel IIe sequence yields and coverage obtained from barcoded long template PCR libraries.

Enzyme	Template	PCR cycles	Polymerase Reads	Bases	Estimated Coverage	Sample number	Sample name
Terra	ELF	15	132,515	851,869,210	70.98910083	1	terraELF15
Terra	Bluepippin	15	69,375	811,670,287	67.63919058	2	terrabluepippin15
repliQa	ELF	15	95,403	874,550,481	72.87920675	3	repliqELF15
repliQa	Bluepippin	15	67,874	851,073,366	70.9227805	4	repliqbluepippin15
LongAmp	ELF	15	52,592	256,665,751	21.38881258	5	longampELF15
LongAmp	Bluepippin	15	69,634	626,495,361	52.20794675	6	longamp bluepippin15
Q5	ELF	15	56,635	248,826,686	20.73555717	7	Q5ELF15
Q5	Bluepippin	15	76,603	523,297,797	43.60814975	8	Q5 bluepippin15
Watchmaker Equinox	ELF	15	89,058	419,979,074	34.99825617	9	WmELF15
Watchmaker Equinox	Bluepippin	15	132,438	1,120,679,413	93.38995108	10	Wmbluepippin15
Watchmaker Equinox	ELF	15	63,812	298,235,529	24.85296075	11	WmELF15
Watchmaker Equinox	Bluepippin	15	15,249	119,965,903	9.997158583	12	Wmbluepippin15
Promega Go Taq Long	ELF	15	7,132	35,752,833	2.97940275	13	promegaELF15
Promega Go Taq Long	Bluepippin	15	11	25,388	0.002115667	14	promegabluepippin15
SuperFi II	ELF	15	96,073	536,211,718	44.68430983	15	superfiELF15
SuperFi II	Bluepippin	15	85,578	903,200,782	75.26673183	16	superfibluepippin15
Kapa HiFi	ELF	15	26,974	94,586,892	7.882241	17	kapaELF15
Kapa HiFi	Bluepippin	15	11,265	42,909,095	3.575757917	18	kapabluepippin15
Universe	ELF	15	119,121	599,671,617	49.97263475	19	universeELF15
Universe	Bluepippin	15	51,436	470,686,450	39.22387083	20	universebluepippin15
LaTaq	ELF	15	10,408	53,704,734	4.4753945	21	LaTaqELF15
LaTaq	Bluepippin	15	17,896	163,824,105	13.65200875	22	LaTaq bluepippin15
Expand	ELF	15	12,591	43,700,917	3.641743083	23	ExpandELF15
Expand	Bluepippin	15	10,245	41,390,920	3.449243333	24	Expandbluepippin15
Verifi	ELF	15	20	116,561	0.009713417	25	verifiELF15
Verifi	Bluepippin	15	38	361,609	0.030134083	26	verifibluepippin15
Pfu Ultra II	ELF	15	4	16,767	0.00139725	27	PfuultraIIELF15
Pfu Ultra II	Bluepippin	15	5	18,351	0.00152925	28	PfuultraIIbluepippin15
Primestar Max	ELF	15	28,367	117,920,076	9.826673	29	PrimestarMAXELF15
Primestar Max	Bluepippin	15	28,306	173,936,027	14.49466892	30	PrimestarMAXbluepippin15
Primestar GXL	ELF	15	13	45,198	0.0037665	31	PrimestarGXLELF15
Primestar GXL	Bluepippin	15	20	70,261	0.005855083	32	PrimestarGXLbluepippin15
Terra	ELF	12	20,612	135,188,167	11.26568058	33	TerraF1_12
Terra	Bluepippin	12	24,485	277,871,931	23.15599425	34	TerraB_12
repliQa	ELF	12	32,557	317,548,565	26.46238042	35	repliQaF1_12
repliQa	Bluepippin	12	39,211	508,550,579	42.37921492	36	repliQaB_12
LongAmp	ELF	12	7,294	38,968,104	3.247342	37	LongAmpF1_12
LongAmp	Bluepippin	12	20,402	196,885,031	16.40708592	38	LongAmpB_12
Promega Go Taq Long	ELF	12	11,082	71,125,825	5.927152083	39	promegaF2_12
Promega Go Taq Long	Bluepippin	12	52,834	591,119,208	49.259934	40	promegaB_12

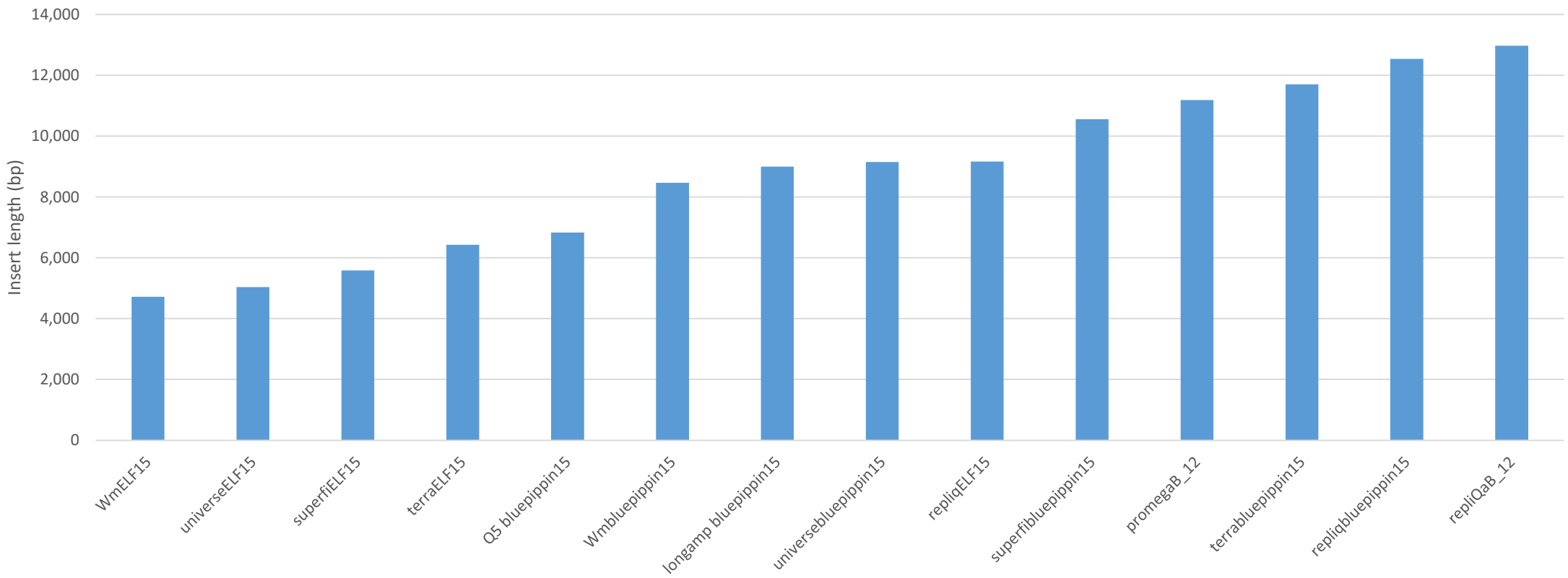
Supplementary Figure 10. Low coverage index for sequencing *S. cerevisiae* 20kb fragments at 30x depth on PacBio Sequel IIe.

A denotes ELF fractionation, **B** Bluepippin. **X12** samples had 12 cycles PCR, **x15** had 15 cycles.



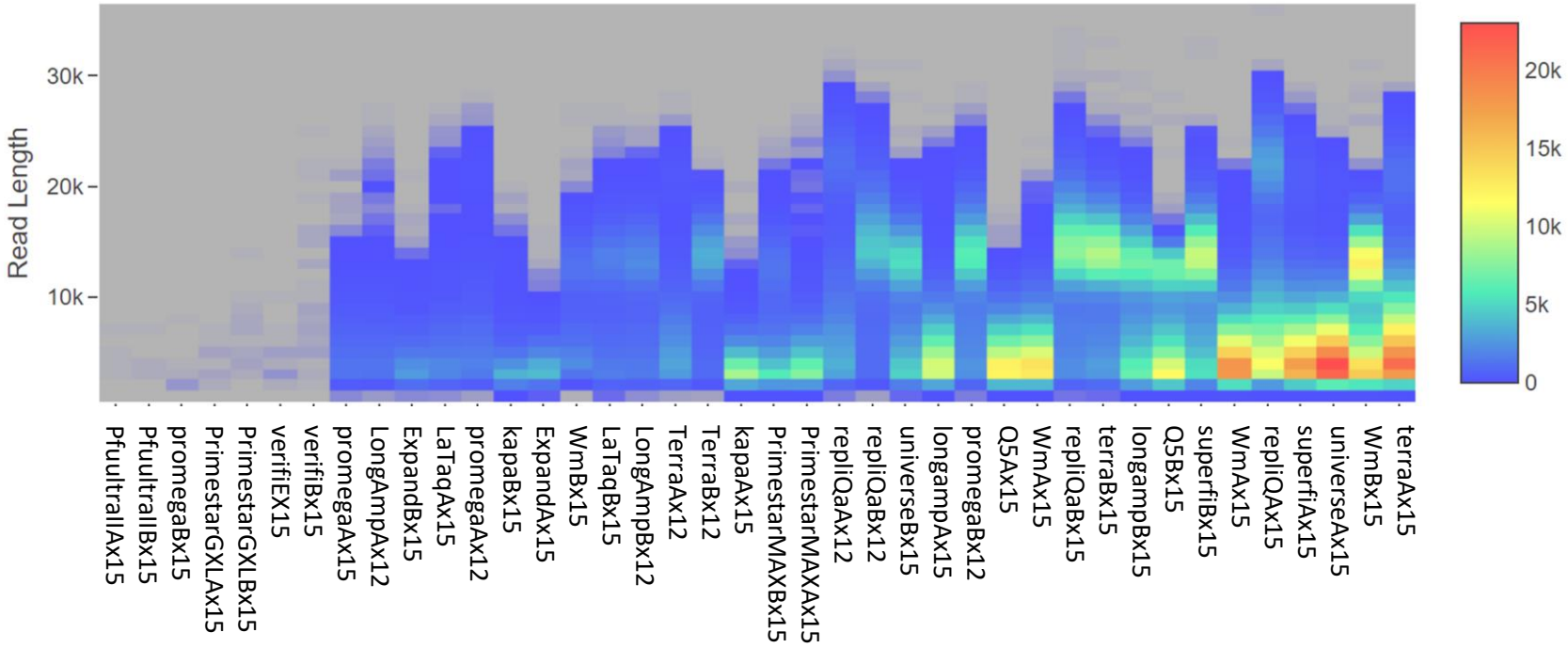
Supplementary Figure 11. Average subread lengths, ranked by size, after PacBio HiFi sequencing for amplicon libraries that gave >30x genome coverage.

A denotes ELF fractionation, **B** Bluepippin. "**12**" samples had 12 cycles PCR, **"15"** had 15 cycles.

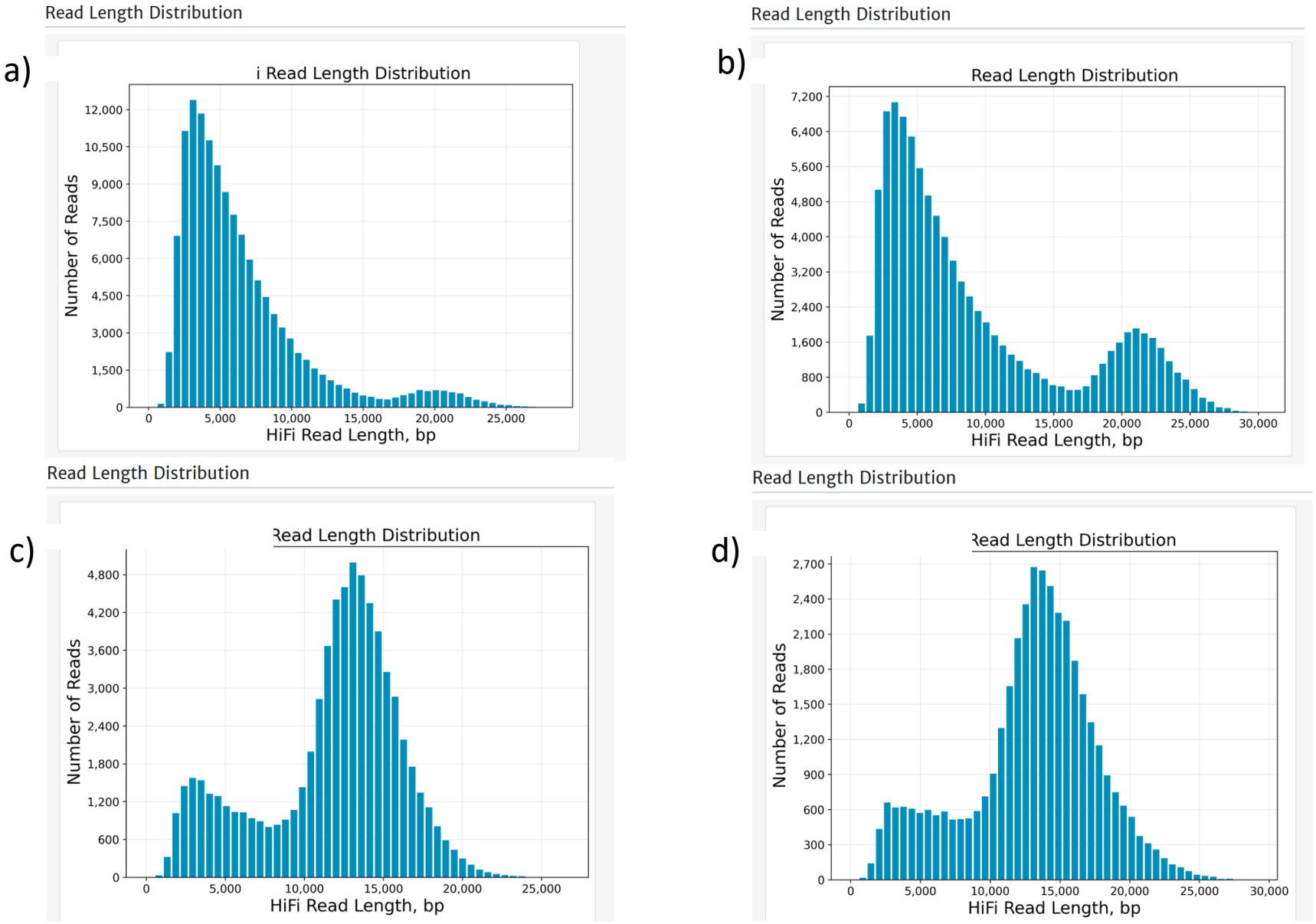


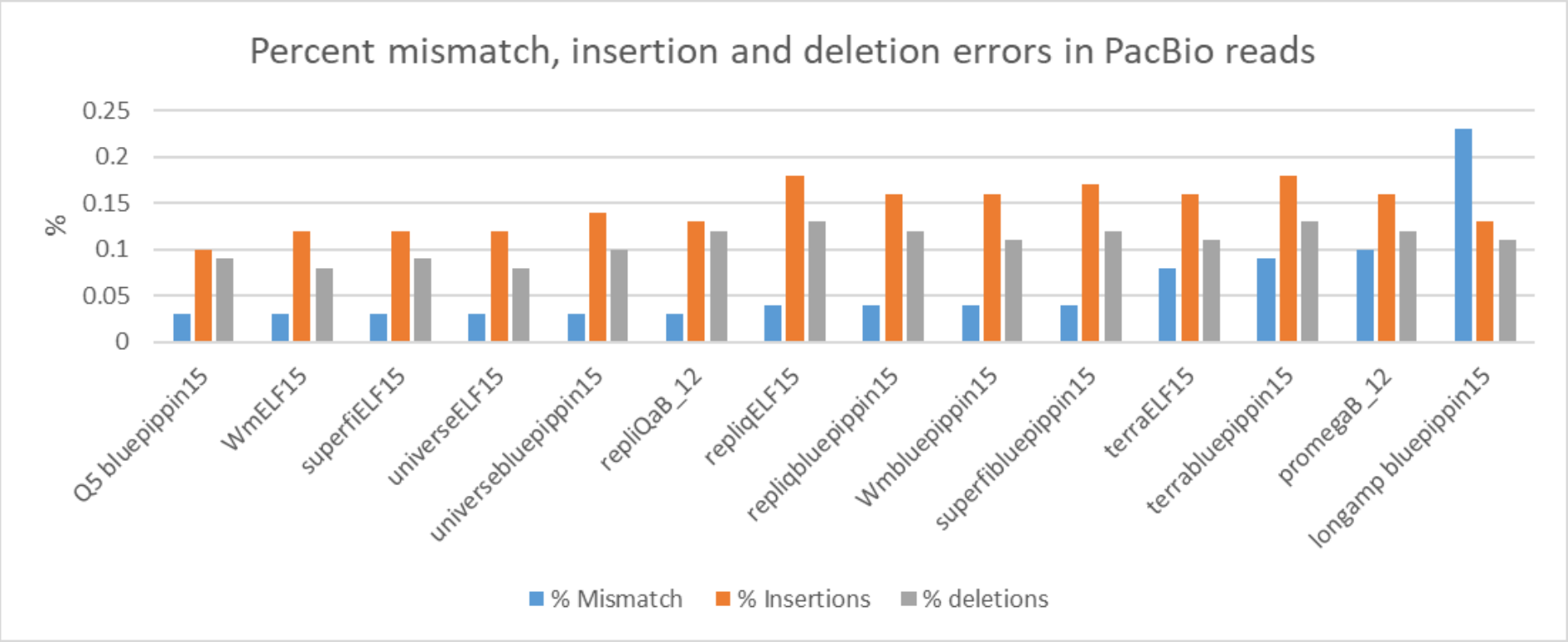
Supplementary Figure 12. PacBio HiFi read length heatmap plot from SMRTlink interface for each library sequenced.

A denotes ELF fractionation, **B** Bluepippin. **X12** samples had 12 cycles PCR, **x15** had 15 cycles.



Supplementary Figure 13. HiFi subread length distributions for ELF size fractionation of a) Terra polymerase and b) repliQa amplified fragments and Bluepippin fractionation of c) Terra polymerase and d) repliQa amplified fragments, after 15 cycles PCR



Supplementary Figure 14. Percentage mismatch, insertion and deletion errors in PacBio HiFi reads with datasets that gave >30x genome coverage ranked from lowest to highest rate of mismatch error.

A denotes ELF fractionation, **B** Bluepippin. "**12**" samples had 12 cycles PCR, **"15"** had 15 cycles.