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Rapid extraction of DNA suitable for NGS workflows from bacterial cultures using the PDQeX

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ABSTRACT

Background: PDQeX is a novel, single-step DNA extraction method that purifies nucleic acid from sample in under 30 min. **Materials & Methods:** Six bacterial suspensions from species with different cell morphologies and growth optima were made. DNA from half the suspension was purified using PDQeX and the other half using a conventional column purification method. Sequencing and analyses using Ion PGM were performed, blinded to extraction method and species. **Results:** Genomes extracted with either method sequenced successfully. No significant sequence distribution biases were evident between PDQeX and column purification. Surveyed community preference suggested comparable performance between the two extraction methods. **Conclusion:** DNA prepared using the PDQeX performs as well for whole-genome sequencing as DNA purified using a conventional method, albeit much more rapidly.

KEYWORDS:

bacterial DNA preparation • DNA extraction • genome sequencing • Ion PGM • PDQeX • QIAGEN DNeasy • whole-genome sequencing • ZyGEM

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METHOD SUMMARY

We introduce the PDQeX: a new, rapid, single-step method for DNA extraction that is compatible with high-throughput sequencing. Sample is added to the thermoresponsive PDQeX extractor tube with a cocktail of thermostable enzymes. Upon incubation and heating the extracted DNA is expelled into a collection tube ready for use in downstream applications in under 30 min.

INTRODUCTION

The PDQeX system uses a radically different approach to DNA extraction compared with current laboratory methods. It harnesses activities from a range of enzymes extracted from extremophiles coupled with thermoresponsive plastics to extract DNA from samples without the use of centrifugation or harsh solvents. This system has already been demonstrated for PCR, qPCR and STR analysis in human tissue samples [1]; however, it was unknown whether it was compatible with high throughput sequencing. This uncertainty rests on two aspects of the PDQeX system. First, the final extrusion step heats the sample to 95°C; potentially denaturing DNA. Second, buffer components required by the PDQeX process remain in the DNA sample. These buffer components distort 260/230 and 260/280 ratios used as traditional measures of DNA purity.

The PDQeX is a three-component system combining a powerful enzyme-driven extraction chemistry [2] with an innovative extractor cartridge and a temperature control unit [3]. The extraction chemistry consists of a cocktail of thermophilic proteinases and mesophilic cell wall degrading enzymes that systematically lyse cells, destroy nucleases, digest proteins and release nucleic acids. The extractor cartridges, made from thermoresponsive polymers, not only facilitate extraction but also remove enzyme inhibitors from extracts (Figure 1). The temperature-controlled extraction is performed in a chamber at the top of the cartridge. The temperature used to activate the protease also leads to a pressure increase in the closed tube, forcing the extract through a heat-burstable valve and a proprietary purification matrix housed at the bottom of the cartridge. The purification segment removes cell debris, inhibitory polyphenols and polysaccharides. This single-step closed system setup allows for rapid, hands-free preparations of DNA without the danger of cross-contamination.

A side-by-side whole-genome sequencing comparison of DNA prepared using the PDQeX and a standard column method, QIAGEN DNeasy, was proposed. Six thermophilic aerobes with varied cell wall morphologies and growth optima were chosen from the ZyGEM NZ Ltd culture collection. *Meiothermus ruber*, *Thermus* sp. and *Thermus filiformis* are Gram-negative. Two *Geobacillus* sp. and *Alicyclobacillus* sp. are Gram-positive. *Alicyclobacillus* is also an acidophile with optimal growth at pH 3. All other strains grew optimally above pH 7. All sequencing and analyses were performed blind to both extraction method and bacterial species to prevent unconscious bias.

MATERIALS & METHODS

Selection, growth & maintenance of bacterial cultures

Details for each of the bacteria used in this study are given in Table 1. All chemicals were sourced from Sigma Aldrich unless specified otherwise. Strain ZCC225 *Alicyclobacillus* sp. was grown on Brock's acid medium (g/l distilled water), starch (soluble) 2 g, yeast extract 2 g, $(NH4)_2$ SO_4 0.2 g, $MgSO_4.7H_2O$ 0.5 g, $CaCl_2.2H_2O$ 0.25 g, KH_2PO_4 3 g, $FeSO_4.7H_2O$ 0.28 mg, $MnCl_2.4H_2O$ 1.25 mg, $ZnSO_4.7H_2O$ 0.4 mg. Ingredients were dissolved in 900 ml distilled water, pH adjusted to 3 with 1 M H_2SO_4 and autoclaved. Gelrite (Gelzan™ CM) 20 g was sterilized separately in 100 ml distilled water and added to the media once cooled to 50°C to prevent acid hydrolysis. The other strains

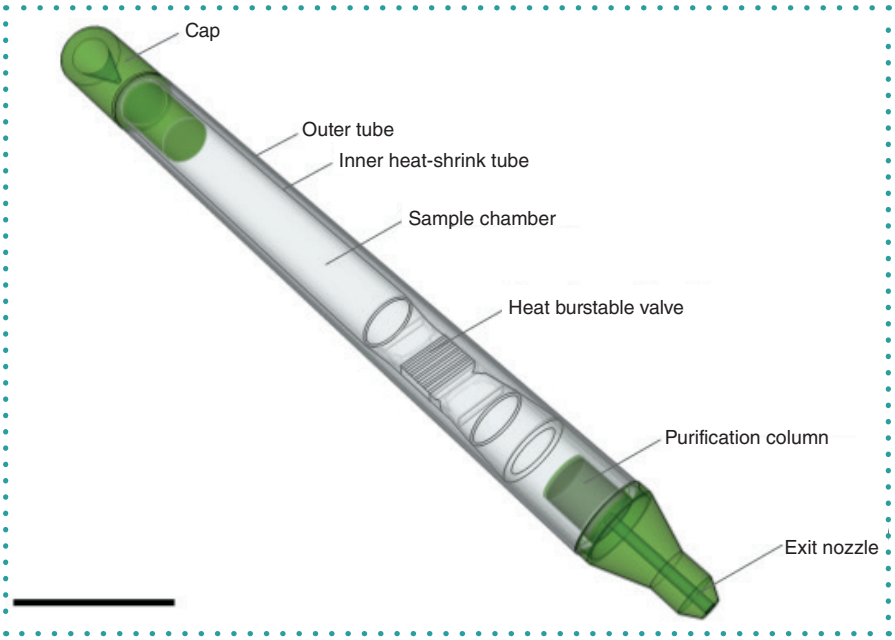

Figure 1. Illustration showing different components of the PDQeX extractor cartridge. The PDQeX extractor cartridge is placed in the separate temperature control unit when in use. Scale bar: ~10 mm.

were grown on Tryptic Soy Broth (Becton, Dickinson and Co., NJ, USA) amended with 2% Gelrite.

Strains were grown from glycerol stocks by plating onto Petri dishes incubated at the respective optimal temperatures (Table 1). Petri dishes were sealed with cling film and placed in zip-lock bags with a moist paper towel to prevent drying. 5-day-old growths were used for DNA extraction because some strains grew slower than others.

DNA EXTRACTION & QUANTIFICATION

Sample preparation

Two sterile inoculation loops (~3 mm^2 each) of plated culture were resuspended in 200 µl of ultrapure water in a 1.5-ml microcentrifuge tube and mixed by pipetting. Half of the bacterial suspension (100 µl) was used for the PDQeX bacterial extraction protocol and the other used for the QIAGEN DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany) bacterial extraction protocol, ensuring that the same amount of starting material was used for both protocols.

Reagents and chemicals described below are proprietary formulations of ZyGEM NZ Ltd and QIAGEN Ltd.

DNA extraction using the PDQeX protocol

The PDQeX Bacteria kit from ZyGEM NZ Ltd was used as per manufacturer's instructions. Briefly, to 100 µl culture, 400 µl of 1x WASH Buffer was added and vortexed vigorously to disperse cells. The WASH buffer reduces polysaccharides and prewashing improves extractions from capsulated bacteria and bacteria producing exopolysaccharides. The cells were centrifuged at 10,000 rcf for 5 minutes and all the supernatant was removed. The pellet was resuspended in extraction mix: 10 µl of 10x GREEN PLUS buffer, 2 µl of *prep*GEM, 2 µl of Lysozyme (10 mg ml^{-1}) made up to 100 µl with ultrapure water. Lysozyme can be omitted for Gram-negatives but was included for all the strains in this study. The extraction mixture was dispensed into PDQeX extractor cartridges and run through the following protocol in the PDQeX 2400 device: 37°C for 5 min, 75°C for 5 min and 95°C for 2 min. At the end of the program, extracts containing purified bacterial DNA were collected in 0.2-ml PCR tubes in the PDQeX collection tray. Extracted DNA was adjusted to 1xTE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) and stored at 4°C.

DNA extraction using the QIAGEN protocol

The bacterial DNA extraction protocol described in the QIAGEN DNeasy Blood and Tissue handbook was followed without deviations. For Gram-positive strains, 100-µl cultures were centrifuged for 10 min at 6000 rcf and the pellet resuspended in 180-µl enzymatic lysis buffer (20 mM Tris-Cl pH 8, 2 mM Na EDTA, 1.2% Triton® X-100, Lysozyme 20 mg/ml). This was incubated for 30 min at 37°C. For Gram-negative strains, the pellet was resuspended in the 180 µl of ATL buffer. 25 ml of Proteinase K and 200 µl of AL buffer were added to the samples, mixed and incubated for 30 min at 56°C. To this was added 200 µl of 100% ethanol. The mix was vortexed and transferred into a DNeasy Mini spin column placed in a collection tube, followed by centrifugation for 1 min at 6800 rcf. The

Table 1. Strain numbers, collection locations, species ID and growth optima of strains used in this study.

Strain #	Collection #	Location	Species	Gram +/-	Optimal pH/temperature (°C)
ZCC225	WP 18 A.1	Waiotapu, New Zealand	*Alicyclobacillus* sp.	+	3/60
ZCC142	Mk 22 A.1	Mokai, New Zealand	*Geobacillus* sp.	+	7.6/70
ZCC17	Ok 4.A1	Orakei Korako, New Zealand	*Thermus filiformis*	-	7.6/70
ZCC16	Fj 3.A1	Savu Savu Beach, Fiji	*Geobacillus* sp.	+	7.6/70
ZCC14	Rt 4.A1	Rotorua, New Zealand	*Thermus* sp.	-	7.6/70
ZCC12	Wai 35.A1	Waimangu, New Zealand	*Meiothermus ruber*	-	7.6/50

column was then washed with 500 µl AW1 and then 500 µl AW2 buffers, centrifuging each time for 1 min at 6800 rcf. The column was placed in a sterile Eppendorf tube, 100 µl AE buffer added and centrifuged for 1 min at 6800 rcf to elute purified bacterial DNA.

DNA quality assessment & quantification

To assess quality of the DNA, electrophoresis of extracts was carried out on 2% Agarose (Bioline Ltd, London, UK) in 1x TBE buffer (Duchefa Biochemie, Haarlem, The Netherlands) (Supplementary Figure 1). Quantification of the extracts was carried out using the fluorometric iQuant™ High Sensitivity dsDNA quantification kit following manufacturer's instructions (GeneCopoeia Inc., MD, USA). Six pairs of extracts were selected based on longest fragment length integrity coupled with highest DNA concentration. Samples were coded to blind the sequencing team to extraction method, species and strain. All samples were sent from Hamilton (North Island, New Zealand) to Dunedin (South Island, New Zealand) as a single shipment. On arrival, samples were re-checked for integrity using 2% agarose E-Gel (Cat#G501802, Invitrogen), and DNA concentrations determined using the Qubit dsDNA HS Assay Kit (Cat# Q32851, Invitrogen).

Ion Xpress Library preparation

DNA was fragmented using the Ion Shear Plus Reagent as per manufacturer's instructions. Sequencing libraries were prepared using the Ion Xpress Plus gDNA Fragment Library protocol with E-gel Size-Select II Agarose Gel 2% size-selection (Cat#G661012, Invitrogen), the Ion Xpress Plus Fragment Library Kit (#4471269, Life Technologies) and Ion Xpress Barcode Adapters (#4471250, Life Technologies). 100 ng starting material was used for all samples except sample 2, which used 84 ng due to its low concentration. The concentration, yield and fragment size distribution of all 12 prepared DNA libraries were checked using a High Sensitivity DNA assay on the Agilent 2100 Bioanalyser (Agilent Technologies, CA, USA).

Three barcodes were used between all 12 sample libraries. Pooled sets of three barcoded libraries were sequenced on four separate runs on the Ion PGM sequencing platform (Life Technologies) using the following kits: ION PGM Hi-Q View OT2 kit (Cat# A29900); ION PGM Hi-Q View sequencing kit (Cat# A30044); Ion 316 chip kit v2 BC (Cat# 4488149). The Ion PGM performed 850 flows for each run.

Data analysis

Sequence from each barcoded library was separated into different data files automatically on the Ion PGM. Sequence read quality was evaluated using FastQC v 0.113 (www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the FASTX-toolkit v 0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/index.html) was used to remove potential adaptor sequences with a read length <50bp, and any low-quality reads containing <40% of bases with a Phred score of ≥25. All reads that remained following quality filtering were *de novo* assembled using Geneious v 9.1.5. Contigs generated from the assemblies were analyzed by BLAST using NCBI (www.ncbi.nlm.nih.gov) to give a list of the most closely related bacterial reference sequences in GenBank. In a second analysis, quality filtered sequence reads were mapped to each bacterial reference sequence. Read length data were statistically analyzed with one-way ANOVA, while number of sequence counts were analyzed with Poisson regression using the software package Stata.

RESULTS

A total of 18 bacterial genomic DNA extractions using both methods were performed simultaneously: six strains extracted in triplicate (Supplementary Figure 1). Using the PDQeX method, the full protocol including prewash and a single transfer step took under 30 mins. The whole extraction was performed in a closed extractor cartridge with no handling of sample post preparation. By contrast, the QIAGEN bacterial extraction method took over 2 h, including two incubations and several pipetting, transfer and centrifugation steps, increasing the possibility of contamination (Figure 2).

Six pairs of DNA samples, one from each extraction method and species, were coded and sent to the sequencing team located in a different city, for Ion PGM sequencing (Table 2). At the time of sequencing and analysis, the sequencing team only knew the sample code number and were blind to bacterial species and extraction method. DNA fragment libraries were constructed from each sample and Ion PGM sequencing performed. Four pools of three barcoded libraries were sequenced on a total of four Ion 316v2 chips. This limited the number of barcodes used across the experiment with equal numbers of libraries constructed from either PDQeX or QIAGEN tagged with each barcode.

Library and sequence run metrics are given in Table 3 (datasets available on request). The mean read length for each library ranged from 234 to 273 bp and the number of quality filtered reads ranged from 423,677 for sample 2 to 2,195,989 for sample 1. Between 95 and 98% of raw reads remained post quality filtering, testament to the raw sequence data quality. Although the sample size was small, a Poisson regression analysis suggested there was a barcode-introduced bias in the number of sequence counts ($p < 0.001$). This suggested bias did not extend to sequence data quality as represented by the lack of statistical difference in sequence read length between libraries and barcodes (Supplementary Table 1).

Geneious *de novo* assembly statistics are given in Table 4. All sequence data files could be assembled except Sample 1. The longest contig of each assembled genome was BLAST searched against the NCBI database and sequence matches used to identify closest bacterial species (Table 2). Samples were paired based on match to the closest reference sequence. Five paired *de novo* assembly statistics were sent to two groups of genome scientists not associated with this project (Group A and B), who were asked to rank each pair of assemblies based on criteria they usually use for their own research projects. A weighted point-scoring system was used to assess which data set was scored as 'best'. QIAGEN-extracted DNA was scored as 'best' for two bacteria and three were scored 'best' for the PDQeX (Table 4). Unweighted preference scores are given in Supplementary Table 2.

Coverage depth and sequence distribution were evaluated by mapping each of the 12 data sets against the six corresponding reference genomes identified in GenBank. As shown in Figure 3, the ▶

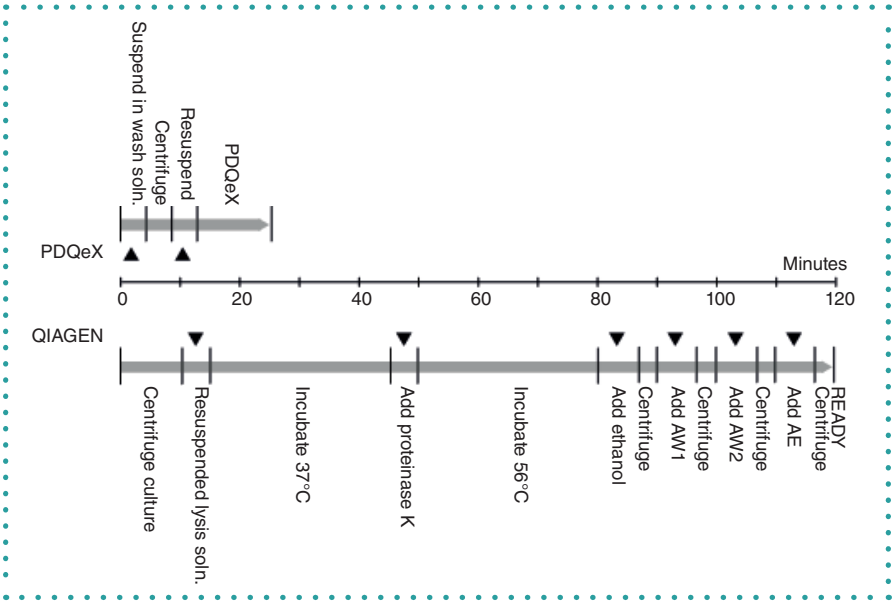

Figure 2. Schematic showing relative processing times and breakdown of the PDQeX and QIAGEN protocols. Black arrows indicate sample transfer or manual addition of reagent.

distribution pattern of mapped reads across each genome was similar for each matched pair of samples indicating that DNA extraction method did not appear to skew sequence distribution. Similarly, the percentage of reads that mapped to its reference was almost identical between each pair of samples. Uniformity of coverage score assigned by the Ion Torrent Coverage Analysis Plug-in was also similar between all sample pairs except for *M. ruber* (PDQeX 77.55% vs Qiagen 91.59%); however, the average depth of coverage was higher for PDQeX (100.5) verses QIAGEN (85.34) for this species.

For DNA pair 1 and 2, approximately 16% of filtered raw reads mapped to the *A. acidocaldarius* reference regardless of DNA extraction method, initially suggesting that this sample may not originate from a pure culture. To test this, 200,000 reads from each dataset were used to search against the NCBI nucleotide database, using BLAST+ (version 2.7.1), and program defaults limited to five target sequences per read (-max_target_seqs 5). The output was imported into MEGAN version 6 [4] to evaluate taxonomic classification. The compare tool (with normalized reads) in MEGAN was used to determine differences between the two samples. The majority of sequences from both sample 1 and 2 mapped to the genus *Alicyclobacillus,* suggesting that these samples are not from a mixed culture but from an organism new to GenBank (Supplementary Figure 2).

DISCUSSION

Isolating nucleic acids from complex matrices continues to be one of the important rate-limiting steps in molecular biology. The adage of garbage-in garbage-out applies well for most downstream analytics. The PDQeX (ZyGEM NZ Ltd) extraction system is a reinvention of DNA purification from sample for use in downstream molecular biology processes. Although rapid, this extraction methodology is effective in both isolating nucleic acids and minimizing enzymatic inhibition as shown in successful qPCR assays [1] [Author, Unpublished Data]. However, as one of the final steps in the PDQeX process involves heating and the 260/280 ratio of extracts deviates from other technologies, the question of whether DNA prepared using this method was suitable for whole-genome sequencing needed to be addressed.

To determine whether PDQeX-extracted DNA was compatible with high-throughput sequencing we designed a whole-genome sequencing experiment using six bacterial species with different growth optima, cell wall morphologies and Gram staining status. All sequencing and sequence metrics comparisons were performed blind to DNA extraction method to protect against unconscious bias. In addition, data were reviewed and called by a team of genome scientists not associated with the project with only limited information on each pair of sequence metrics they were asked to score. On all measures of perfor-

Table 2. Code, concentration of extracted samples and references sequences determined following sequence analysis.

Bacterial strain	PDQeX			QIAGEN			Reference sequence determined post-sequencing
	Code	Qubit ng/μl	Library Barcode	Code	Qubit ng/μl	Library Barcode	
ZCC225	1	12.5	1	2	2.4	2	NC_013205.1 *Alicyclobacillus acidocaldarius*
ZCC142	3	61.8	3	4	61.6	1	CP003125.1 *Geobacillus thermoleovorans*
ZCC17	5	61.8	2	6	41.6	3	NC_006461.1 *Thermus thermophilus*
ZCC16	7	64.2	1	8	39.4	2	CP011832.1 *Geobacillus* sp.
ZCC14	9	52.2	3	10	45.8	1	NC_019386.1 *Thermus oshimai*
ZCC12	11	16.2	2	12	40.8	3	NC_013946.1 *Meiothermus ruber*

Table 3. Library and sequence run metrics.

Sample	Conc. Quant (ng/ul)	Conc. Qubit (ng/ul)	Starting material (ng)	Library pMol	Library av. size (bp)	Barcode	# Raw reads	Mean read length	# Quality filtered reads (Q25 in P40)	% Post filtering
1	5.6	13	100	486	392	1	2,286,908	266	2,195,989	96
2	0.7	2	84	418	386	2	444,169	238	423,677	95
3	14.6	62	100	1702	385	3	850,252	263	820,258	96
4	24.5	62	100	1106	377	1	2,194,140	263	2,122,642	97
5	19.1	62	100	526	375	2	904,779	243	870,119	96
6	15.3	42	100	488	376	3	573,037	243	551,192	96
7	23.5	64	100	1769	375	1	1,396,965	269	1,367,746	98
8	13.0	39	100	4051	380	2	1,537,086	273	1,507,369	98
9	15.3	52	100	526	359	3	629,925	245	612,621	97
10	9.7	46	100	3093	370	1	810,075	250	785,256	97
11	7.6	16	100	1487	349	2	1,391,370	234	1,351,670	97
12	19.6	41	100	3867	365	3	1,073,411	254	1,047,102	98

mance tested in this study, DNA produced from the PDQeX system was shown to be comparable to the QIAGEN DNeasy Blood and Tissue extraction kit.

The simplicity of the PDQeX system led to notable advantages over the QIAGEN extraction kit. First, the PDQeX extraction took significantly less time than QIAGEN: 30 min compared with 2 h. In addition, most of the processing time for the PDQeX was hands-off as, once sample was added to the PDQeX extraction tube, it was simply incubated with no further manipulations required. Second, the reduction in handling and pipetting steps made the PDQeX easier to use and reduced the potential for sample contamination. Whole-genome sequencing is critically dependent on the character of the DNA sample. Anything that reduces the potential for sample cross-contamination enhances the value of the overall outcome.

Only one of the sequence data sets failed to *de novo* assemble due to time-out of the bioinformatics computing system we used. This was Coded Sample 1 from bacteria ZCC225 *A. acidocaldarius*. Sequence data from its pair, Coded Sample 2 could be assembled. It is significant that the sequencing library made from Sample 2 started with less DNA (84 ng compared with 100 ng for all other libraries) and that this was reflected in a lower number of reads

Table 4. *De novo* assembly statistics and blind dataset evaluation.

Code	% Not Incl.	Cont.	Cont. ≥ 1000	Max length (bp)	N50 (bp)	Cont. ≥ N50	Group A†	Group B†	% P or Q
3	0.5	2560	335	108,604	20,594	63	10	16	87% P
4	0.5	9530	507	152,547	993	512	4	0	13% Q
5	0.7	3593	395	34,370	4786	168	N/A	13	81% P
6	0.5	5134	1348	25,383	1749	608	N/A	3	19% Q
7	0.5	2496	236	126,100	22,677	57	10	9	63% P
8	0.4	2132	210	92,375	23,807	57	4	7	37% Q
9	0.4	1897	421	34,728	6052	136	N/A	2	13% P
10	0.2	896	164	75,136	19,503	35	N/A	14	87% Q
11	0.5	5897	517	58,848	2538	255	0	0	0% P
12	0.4	2756	262	136,575	17,187	60	14	16	100% Q
1	–	–	–	–	–	–	–	–	–
2	0.3	800	1726	48,107	4309	20	–	–	–

†Scoring for each response: Preferred = 2; Equal preference = 1; Not preferred = 0.
Cont.: Contig; N/A: Proportion of quality filtered reads not part of the assembly; P = PDQeX method, Q = QIAGEN method.

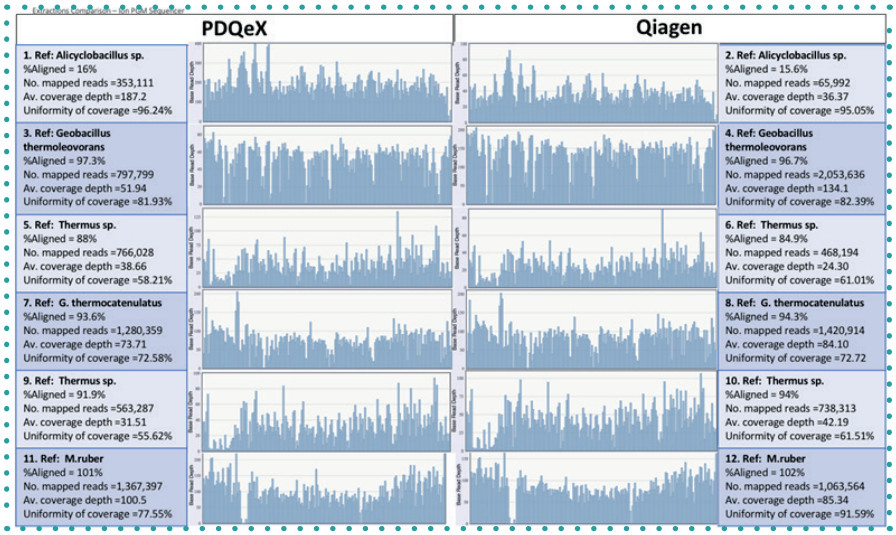

Figure 3. Mapping of bacterial reads to reference genomes using the ion torrent coverage analysis plug-in software tools comparing PDQeX and QIAGEN extraction methods. X-axis corresponds to genome position; Y-axis is base read depth.

following sequencing (423,677 vs 2,195,989). When data sets from Sample 1 and 2 were mapped against a reference, the same low proportion of reads (~16%) mapped to NC_013205.1. We undertook a metagenomic analysis on a subset of Sample 1 and 2 sequences using BLAST and the taxonomic classification tool MEGAN. Almost all sequences grouped with *Alicyclobacillus* indicating a pure culture. These findings suggest that ZCC225 is a new species with no other, more closely related organisms currently represented in GenBank.

Bacteria from the ZyGEM NZ Ltd culture collection had originally been identified based on a combination of physical and metabolic characteristics and closest relatives from 16SrDNA sequences [Author, Unpublished data]. We were able to confirm genera in all cases and further refine species identity from whole-genome data. Culture sample ZCC17 (Coded Samples 5 and 6) was originally identified as *T. filiformis;* however, whole-genome sequencing placed this organism closest to *T. thermophilus* HB8 (NC_006461.1). *M. ruber* (Coded Samples 11 and 12) was confirmed as closest to reference sequence NC_013946.1; however, there is a clear genomic deletion or insertion evident between the reference and ZCC12. Work is ongoing to analyze these genomes further.

DNA extracted using the PDQeX system can be used directly for whole-genome sequencing without further purification. DNA obtained using the PDQeX performed as well as DNA produced by more traditional methods, in this case the QIAGEN column purification system. The simplicity and minimal sample manipulation requirement of the PDQeX system make it an attractive option for obtaining DNA samples for high-throughput DNA sequencing studies.

FUTURE PERSPECTIVE

Sample preparation is key to successful sequencing. Sequencing will be applied to increasingly more biological, commercial and diagnostic problems. The PDQeX is a viable system for preparing quality samples rapidly, cheaply and with minimal chance of sample contamination through reduced operator handling. Its simplicity lends itself to automation and upscaling. In the future, systems like the PDQeX that transform and accelerate sample preparation will become standard in the laboratory. These systems have the capacity to transform fields such as water quality testing, food safety and plant pathology to name a few. In addition, new, more simple and reliable methods for extracting nucleic acids from a sample are required to facilitate molecular testing at the point-of-care and in-field (point-of-need). We see development of new in-lab and point-of-need applications accelerating in the next 5 years.

AUTHOR CONTRIBUTIONS

Jo-Ann L Stanton: Designed the study, polled scientists for data preference, funded and supervised the work, drafted the manuscript. Abishek Muralidhar: Prepared bacterial cultures, optimized and performed DNA extractions, coded samples, edited the manuscript. Christy J Rand: Quantified the DNA, performed IonTorrent sequencing and bioinformatics analysis, edited the manuscript. David J Saul: Supervised preparation of bacterial cultures, invented the PDQeX system, edited the manuscript.

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FINANCIAL & COMPETING INTERESTS DISCLOSURE

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No writing assistance was utilized in the production of this manuscript.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2019-0006

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