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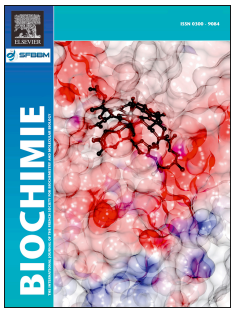

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Amplifying PCR productivity and environmental sustainability through shortened cycling protocols

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Abstract

Since its inception in the 1980s, advancements in PCR technology using improved thermal cyclers, engineered DNA polymerases and commercial master mixes, have led to increased PCR productivity. Despite these advancements, PCR cycling protocols have largely remained unchanged over the same period. This study aimed to systemically evaluate the effect of reduced PCR cycling parameters on amplicon production. The 1466bp fragment from the 16S rRNA gene present in low-, medium- and high-CG bacteria was amplified using three commercially available PCR master mixes. The shortest cycling parameters required to successfully amplify the 16S fragment from all bacteria and master mixes comprised 30-cycles of 5 seconds denaturation, 25 seconds annealing, and 25 seconds extension. While all produced an amplicon with sufficient yield to enable downstream sequence analysis, the PCRBIO Ultra Mix in conjunction with the shortened parameters was found to achieve the highest amplicon yield across low-, medium- and high CG bacteria. Comparing the run times to that of a typical 16S PCR protocol, the shortened cycling parameters reduced the program duration by 46% and consumed 50% less electricity, translating into increased productivity and helping to improve laboratory environmental sustainability.

1. Introduction

Culturing techniques remain the gold standard for bacterial identification in clinical microbiology. However, with some species requiring weeks to culture [1], targeted culture methods potentially missing less common pathogenic species [2], and biochemical testing not always able to discriminate between species [3], this can lead to incomplete or delayed diagnoses that may impact patient care and result in unnecessary antibiotic treatment [4].

PCR is an important clinical diagnostic tool for genetic microbial detection and identification [5], with specific gene-targeted Real-Time PCR (RT-PCR) and 16S rRNA gene sequencing being the most common methods [6]. RT-PCR is a quick and effective tool for identifying bacterial species or antibiotic resistance genes, but it relies on prior knowledge of target sequences and is limited in the breadth of targets that can be identified in any one test. In contrast, 16S rRNA gene sequencing utilises broad-species-range oligonucleotides that target conserved regions within the gene to allow for simple and cost-effective genus/species level identification without prior knowledge of the disease/infective agent [7,8] and for microbial community analysis [9,10].

While rapid in comparison to bacterial culture, typical 16S PCR programs can take over 2 hours to complete [11-13], during which time the machine is engaged and cannot be used for other purposes. Advancements in polymerase engineering [14-16] and commercially produced master mixes have increased performance and reduced reaction set up times. However, cycling protocols have largely remained

unchanged since the 1990s [11-13,17-19], with extension times of 60-180 seconds still used for amplification during the extension stage. *Taq* polymerases are reportedly capable of incorporating approximately 60 nucleotides per second at 70˚C [15, 20], suggesting it may be possible to amplify 1500bp in 25 seconds, translating into a shorter PCR program. While variations in PCR protocols have been published [14,21-23], no study appears to have systemically evaluated the effects of modified PCR cycling parameters on amplicon production. We present a new protocol with shortened cycling times and reduced energy consumption that robustly and reproducibly amplifies a 1466bp 16S rRNA gene fragment across a range of bacterial species with varying genomic GC contents, resulting in increased laboratory productivity and environmental sustainability.

2. Materials and Methods

2.1 Bacteria and DNA extraction

Bacillus subtilis (University of Plymouth strain), *Escherichia coli* K12 DH1 (DSM 4235) and *Mycobacterium phlei* (DSM 43070), with GC contents of ~36-40%, 50.8% and 73% respectively, were cultured on BHI agar (Merck Life Science UK Limited, Dorset, UK) aerobically at 37˚C for 24-48 hours. DNA was extracted from a single colony using the FastDNA™ SPIN Kit for Soil and FastPrep-24 5G Bead Beating Lysis System (MB Biomedical Irvine, CA, USA) according to the manufacturers protocol with the following modification: centrifugation at 12,300xg using a Micro Star 12 centrifuge (VWR Lutterworth, Leicestershire, UK). DNA from *E. coli* NCTC 11560, that had previously been amplified, was used as a positive control for PCR. DNA concentration was equalised to 45ng/µL ±10% using a NanoDrop™ ND1000

spectrophotometer (Thermo Fisher Scientific™ Waltham, MA, USA) and stored at -20˚C.

2.2 PCR Amplification and Energy Monitoring

Three commercial PCR master mixes were used: DreamTaq Green PCR Master Mix (Thermo Fisher Scientific™ Waltham, MA, USA), MyTaq™ Red Mix (Meridian Biosciences St. Modiin, Israel), and 2x PCRBIO Ultra Mix (PCR Biosystems London, UK). 50µL reactions were set up comprising 25µL of the respective PCR Master Mix, 1µL 27f 5'-AGAGTTTGATCCTGGCTCAG-3' forward primer (10µM) and 1µL 1492r 5'-ACGGCTACCTTGTTACGACTT-3' reverse primer (10µM) (Eurofins Genomics, Ebersberg, Germany) [17], 22µL nuclease-free water (Thermo Scientific™), and 1µL of bacterial DNA. Nuclease-free water (Thermo Scientific™ ddH2O) was used as negative control, while *E. coli* NCTC 11560 DNA was used as the positive control. All reactions were carried out on GeneAmp PCR System 9700 automated thermocycler (Marshall Scientific Hampton, NH, USA) on maximum temperature ramping speed for all cycling programs (Table 1). All PCR amplicons were visualised on a 1% agarose gel in 0.5x tris-acetate-EDTA buffer at pH8, pre-stained with 1x SYBR Safe DNA Gel Stain (Thermo Fisher Scientific™ Waltham, MA, USA), under UV trans-illumination. Power consumption was measured using a Plug-in mains power and energy monitor (Maplin Electronics, Rotherham, UK).

2.3 PCR Purification, Quantification and Sequencing

PCR products were purified using the Monarch® PCR & DNA Clean Up Kit (5ng) (New England BioLabs Ipswich, MA, USA) and eluted into 25µL nuclease-free water. Amplicon concentration was quantified using the Qubit 2.0 fluorometer with the

dsDNA BR Assay Kit (Thermo Fisher Scientific™ Waltham, MA, USA). Amplicons were sequenced at the Department of Biochemistry, University of Cambridge, using a 3730XL DNA Analyzer (Thermo Fisher Scientific™ Waltham, MA, USA). DNA sequences were checked using Chromas Lite version 2 (Technelysium Pty Ltd, Australia). Sequence editing and alignments were undertaken using Bioedit v7.7.1 (Tom Hall, Department of Microbiology, North Carolina State University; https://thalljiscience.github.io/).

2.4 Statistics

Amplicon concentration data and PCR program parameters were collated in Microsoft Excel (Version 3202) which was used to calculate the means and differences from triplicate repeats of the Control (V0) and Final (V9) programs (Table 1). Data was analysed using Minitab (Version 19.1.1) to calculate descriptive statistics and general interval charts. A two-sample Welch's t-test was used on each of the nine Master Mix/Species combinations to determine significant difference between Control and Final programs. An Interval Plot was generated using data from all Master Mix/Species combinations to visualise the change in amplicon concentration between control and final programs.

3. Results

The 1466bp fragment of the 16S rRNA gene, amplified by the 27f and 1492r oligonucleotide primers, was selected as a standard target gene to assess the effect of reduced PCR cycling times on amplicon production. PCR master mixes from three different manufacturers, in conjunction with DNA from *Bacillus subtilis*, *Escherichia coli* and *Mycobacterium phlei*, was used to evaluate these changes across species

with low-, medium- and high-CG genomes. All programs tested included the standard 2-minute initial denaturation and 5-minute final extension step as part of the overall run time.

A typical 32-cycle 16S control PCR (16S V0) comprising 30 seconds for denaturation, 30 second for annealing, and 90 seconds for extension, was used as a universal bacterial test program (Table 1). This took 117 minutes to complete and consumed 0.28 kWh of electricity. Changes to the denaturation, annealing and extension times were undertaken independently to ensure successful amplification, with products confirmed by agarose gel electrophoresis. For the extension step, a reduction from 90 to 25 seconds (16S V3) resulted in amplicons of expected size for all PCR master mixes and bacterial species, equating to a 72.2% time saving for this step compared to the control program (16S V0). Attempts with a 20 second extension step (16S V2) resulted in no amplification from *M. phlei* with MyTaq, although amplification was possible with the two other PCR master mixes. For the annealing step, 25 seconds (16S V5) was the minimum time required for successful amplification across all species and master mixes, representing a 16.7% time saving for this step. Tests at 20 seconds (16S V4) were unsuccessful for *M. phlei* with MyTaq. For the denaturation step, amplification was possible across all species and master mixes with 5 seconds (16S V7), reduced from 30 seconds, and representing an 83.5% time saving over that step in the control program.

Table 1. PCR programs tested with total cycling duration calculated by PCR System 9700 platform and includes a 2-minute initial denaturation and 5-minute final extension step, to the nearest 10 seconds.

159

PROGRAM	DENATURATION TIME (94 ˚C)	ANNEALING TIME (54 ˚C)	EXTENSION TIME (72 ˚C)	CYCLE NUMBER	TOTAL PROGRAM DURATION	CALCULATED CYCLE DURATION (EX TEMP RAMPING)
16S V0 (CONTROL)	0:30	0:30	1:30	32	1:57:00	1:27:00
16S V1	0:30	0:30	**0:30**	32	1:25:00	0:55:00
16S V2	0:30	0:30	**0:20**	32	1:19:00	0:49:40
16S V3	0:30	0:30	**0:25**	32	1:23:00	0:52:20
16S V4	0:30	**0:20**	1:30	32	1:52:00	1:21:40
16S V5	0:30	**0:25**	1:30	32	1:55:00	1:24:20
16S V6	**0:10**	0:30	1:30	32	1:46:00	1:16:20
16S V7	**0:05**	0:30	1:30	32	1:44:00	1:13:40
16S V8	**0:05**	**0:25**	**0:25**	**28**	0:59:00	0:32:40
16S V9 (FINAL)	**0:05**	**0:25**	**0:25**	**30**	1:03:00	0:34:30

160

161 Additional time saving was explored through reductions in cycle number. At 28-
162 cycles (16S V8), the amplicon yield with MyTaq and *M. phlei* was 6.34ng/µL based
163 on fluorometric analysis; this is insufficient for downstream analysis such as Sanger
164 sequencing. 30-cyles was found to be the minimum number needed to produce an
165 amplicon concentration sufficient for downstream analysis across the master mixes
166 and species combinations. The final program (16S V9) comprised 30-cycles of
167 denaturation for 5 seconds, annealing for 25 seconds and extension for 25 seconds,
168 with the standard 2-minute initial denaturation and a 5-minute final extension step.
169 This program took 63 minutes to complete and used 0.14kWh of electricity, resulting
170 in a 46.2% reduction in overall run time compared to the control program (16S V0)
171 and required 50% less electricity. Analysis of three independent replicates, for all
172 bacterial species and master mixes, found that mean amplicon concentration was
173 higher from the control program (16S V0) compared to the final program (16S V9)
174 (Figure 1), however, the difference was not significant except with *M. phlei* amplified
175 by DreamTaq ($P = 0.043$) (Table 2). During triplication, amplicon concentration
176 varied by as much as ~75ng/µL (Figure 1), with the larger differences in yield often
177 observed from the control program (16S V0), while the final program (16S V9)

178 typically produced a lower but more consistent product. The reason for such
179 variability is unknown as the template DNA concentration was equilibrated.
180
181 Table 2. Comparison of mean amplicon yield and t-tests for Control and Final
182 program amplicon concentrations, grouped by species/master mix combinations (n =
183 3). *P<0.05 **Mann-Whitney U test performed for the *E. coli*-DreamTaq group due to
184 not having equal variance.
185

B. subtilis	*Control Mean* ±SEM *(ng/µl)*	*Final Mean* ±SEM *(ng/µl)*	*Difference*	Mean Loss of Yield (%)	*T value*	*P value*
DreamTaq	74.67 ±24.20	45.47 ±6.48	29.20	39.1	1.17	0.364
MyTaq	82.70 ±8.70	54.77 ±2.17	27.93	33.8	3.12	0.089
PCRBIO	87.03 ±19.00	76.80 ±4.21	10.23	11.8	0.53	0.651
E. coli						
DreamTaq	75.80 ±24.20	49.40 ±2.70	26.40	34.8	W value** 12.00	0.663
MyTaq	91.63 ±16.60	53.30 ±3.66	38.33	41.8	2.25	0.153
PCRBIO	93.93 ±10.30	71.13 ±13.70	22.80	24.3	1.33	0.277
M. phlei						
DreamTaq	40.73 ±5.46	14.40 ±5.59	26.33	64.6	3.37	0.043*
MyTaq	33.93 ±7.25	8.86 ±1.67	25.07	73.9	3.37	0.078
PCRBIO	71.70 ±22.40	51.00 ±6.99	20.70	28.9	0.88	0.471

186
187 Sanger sequenced amplicons from one control (16S V0) and one final (16S V9) set,
188 across all species and PCR master mixes, using both the 27f and 1492r
189 oligonucleotides, yielded sequence lengths of over 800 bp with no differences.
190 Aligned sequences showed 100% nucleotide identity between reciprocal species for
191 both programs and a corresponding match to those species in the Ribosomal
192 Database Project and NCBI database, indicating that the reduced cycling protocols
193 had no effect on sequence length or quality.
194
195 **4. Discussion**

PCR is now a ubiquitous tool within clinical and research laboratories for the detection of specific genes and species identification. While reagent technology and equipment have improved, cycling protocols have largely remained unchanged for decades. Running machines longer than necessary delays lifesaving information, reduces throughput potential and wastes energy. This study systematically evaluated cycling protocol modifications resulting in shorter run times that have the potential to increase the productivity and sustainability of PCR–based diagnostics.

The 1466bp fragment of the 16S rRNA gene amplified by the 27f and 1492r oligonucleotides [17] was used as a template for this analysis, with programs typically taking around 2 hours to complete depending on published protocols. The final program (16S V9) implemented shortened cycling steps of 5, 25 and 25 seconds for denaturation, annealing and extension respectively, and successfully yielded an amplicon within 63 minutes across high-, mid- and low-GC bacteria using three different PCR master mixes. While a reduction in amplicon concentration was seen, no significant difference was observed compared to the control program (16S V0), except with *M. phlei* amplified using DreamTaq. Of the three master mixes, PCRBIO deliver an average amplicon yield closest to that observed when using the control program (16S V0) and well above the minimum required for downstream analysis, such as Sanger sequencing. The final optimised programme (16S V9) differs from the general guidelines supplied by the various manufacturers, and it is noted that this study specially focused on amplification of the 16S rRNA gene and each manufacturer stipulates that parameters may require optimisation. The final programme (16S V9) was shorter than suggested manufacturer guidelines for DreamTaq for all three cycling stages (denaturation, annealing and extension). In

contrast, the manufacture guidelines for MyTaq were shorter than our final protocol (16S V9), however attempts to amplify from the high-GC *M. phlei* DNA proved unsuccessful with a 20 second annealing step, but was possible for the other two bacterial species, suggesting that the high-GC template was problematic at this stage. The manufacturer guidelines for PCRBIO are shorter than the 16S V9 programme reported here, and trials with shorter cycling conditions (e.g., 20 seconds for annealing in 16S V4) were still successful, suggesting this master mix could be further optimised. However, this was not explored further here as the aim of this study was to determine a minimum standardised protocol applicable to low-, medium- and high-GC bacteria across multiple manufacturer master mixes.

Analysis of the amplicon concentration found it to be more consistent and reproducible with the final program (16S V9) across all PCR master mixes compared with the control program (16S V0), with a more consistent product potentially benefitting any downstream analysis. Amplicon concentration varying by as much as ~75ng/µL for the control program (16S V0) despite the initial template DNA being equalised to 45ng/µL prior to all PCR reactions. The reason for the variability in amplicon concentration using the standard protocol is unknown, though it is possible that the optimised protocol may result in more targeted annealing of the primers and reduced mis-annealing, but this is speculation and requires further study. In summary, the final program (16S V9) reduces 16S PCR completion times by 46% and uses 50% less electricity compared to the control program (16S V0), while producing amplicons at a sufficient concentration for downstream analysis with no discernible detriment. These modifications could allow for the optimisation of laboratory workflows. Automated DNA extraction platforms, such as the EasyMAG®,

246 can complete a run in as little as 40 minutes [24], and when combined with a shorter
247 PCR protocol, could allow for multiple successive runs to be synchronised more
248 effectively and increase sample throughput.
249
250 The findings of this study have the potential for wider impact beyond amplifying the
251 16S gene and could be implemented for the analysis of other genes, leading to much
252 greater productivity, diagnostic potential, and environmental sustainability. For
253 example, Nasution et al [25] outline a protocol for amplifying the 533bp fragment of
254 the *Mec*A gene in methicillin-resistant *S. aureus* (MRSA). This consists of 30 cycles,
255 comprising 180 seconds for denaturation, 30 seconds for annealing and 60 seconds
256 for extension, and is estimated to take 165 minutes (including an estimated 30
257 minutes for temperature ramping). By applying the principles reported here, this
258 program could theoretically be reduced to approximately 57 minutes, equating to a
259 65% reduction in time and electricity usage. Times shorter than those outlined in the
260 final program (16S V9) resulted in amplification failure for *M. phlei*, likely due to its
261 high-GC genome, however they were still capable of amplifying the 16S fragment
262 from *B. subtilis* and *E. coli*. Other studies have also successfully utilised protocols
263 with annealing times as low as 10-15 seconds [10,26-27], suggesting tailored
264 programs with even shorter run times may be possible for known DNA templates,
265 such as species comprising <50% GC content or in the detection of known antibiotic
266 resistance genes such as *Mec*A. Further optimisation through the examination of
267 oligonucleotide, magnesium and/or GC buffer concentrations may help further
268 reduce overall cycle duration.
269

Clinical and biomedical research laboratories are significant energy users [28] and with the UK Government targeting net-zero carbon emission by 2050 [29], sustainability and running costs are increasingly becoming prominent considerations for any laboratory. Green laboratory initiatives including the Enabling Environmentally Sustainable Biomedical Research forum [30], the Laboratory Efficiency Assessment Framework [31] and trials seeking to reduce plastic waste [32] are beginning to address concerns around sustainability and environmental impact within research. Ultra-low temperature freezers have been shown to use 40% less energy when set to -70°C compared with -80°C, with no apparent detriment to their contents [33]. This reduces energy usage and costs while increasing equipment lifespan, leading to a more sustainable working practice. Consumers are also more energy conscious following increasing global energy prices. Indeed, the 41% increase in UK electricity prices in April 2022 was the largest monthly price increase since 1988 [34] making the running of any laboratory equipment more expensive. PCR is a common laboratory method. Shortened cycling protocols would use substantially less electricity, translating into lower energy costs, potentially longer machine shelf life, and improved sustainability, allowing organisations to make cumulative savings towards sustainability initiatives.

PCR is a common technique in teaching on biomedical and biological-based programs. With undergraduate practical sessions typically having a duration of 2-3 hours, performing PCR and its downstream analysis is likely to span multiple sessions to accommodate the long run times. A PCR program of less than 60 minutes could enable the setup, running and gel electrophoresis within a single 3-hour practical session, aiding continuity and improving educational pedagogy.

Furthermore, these principles would also positively impact research and diagnostics undertaken in low- and middle-income countries where limited access to equipment or an uninterrupted/intermittent electricity supply can hamper work. Reduced protocols would enable equipment and available electricity to be used more productively and efficiently, including the possibility to run portable thermal cyclers using battery or solar power for *in situ* analysis of samples in the field.

5. Conclusion

To the authors' knowledge, this is the first study to systematically assess the impact of shortened PCR cycling parameters on the amplification of the 16S rRNA gene in low-, mid- and high-GC template DNA. The final modified program resulted in a 46% reduction in overall run time, with PCRBIO master mix achieving an amplicon concentration comparable to the control program, though there were difficulties when attempting to amplify from high-CG template DNA. For unknown templates, a program with a 5-second denaturation step and 30 seconds for both the annealing and extension steps would allow greater flexibility while only adding 4 minutes and 40 seconds to the overall run time, equating to 46% reduction compared to the control program. In contrast, there is evidence programs could be reduced further for known templates comprising ≤50% GC. Further research is required to examine the limitations on consistent amplification capability when considering PCR-based productivity and sustainability.

Author Contributions

The study was conceptualised, devised, and planned by P.J. Warburton. Data generation, curation, analysis, and visualizations were carried out by all authors.

Writing of the original draft was performed by P.J. Warburton and M. Pedlar, all authors contributed to the reviewing and editing of the manuscript and approve the final article.

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References

[1] G.E. Pfyffer, F. Wittwer, Incubation time of mycobacterial cultures: how long is long enough to issue a final negative report to the clinician? J. Clin. Microbiol. 50 (2012) 4188–4189. https://doi.org/10.1128/jcm.02283-12.

[2] R.F. de Boer, A. Ott, P. Güren, E. van Zanten, A. van Belkum, A.M.D. Kooistra-Smid, Detection of Campylobacter Species and Arcobacter butzleri in Stool Samples by Use of Real-Time Multiplex PCR. J. Clin. Microbiol. 51 (2013) 253–259. https://doi.org/10.1128/jcm.01716-12.

[3] J.C. Lagier, S. Edouard, I. Pagnier, O. Mediannikov, M. Drancourt, D. Raoult, Current and Past Strategies for Bacterial Culture in Clinical Microbiology. Clin. Microbiol. Rev. 28 (2016) 208–236. https://doi.org/10.1128/cmr.00110-14.

[4] A. Akram, M. Maley, I. Gosbell, T. Nguyen, R. Chavada, Utility of 16S rRNA PCR performed on clinical specimens in patient management. Int. J. Infect. Dis. 57 (2017) 144–149. https://doi.org/10.1016/j.ijid.2017.02.006.

[5] D. Aggarwal, T. Kanitkar, M. Narouz, B.S. Azadian, L.S.P. Moore, N. Mughal, Clinical utility and cost-effectiveness of bacterial 16S rRNA and targeted PCR based diagnostic testing in a UK microbiology laboratory network. Sci. Rep. 14 (2020) 10(1):7965. https://doi.org/10.1038/s41598-020-64739-1.

[6] Public Health England, SMI ID 1: introduction to the preliminary identification of medically important bacteria and fungi from culture. https://www.gov.uk/government/publications/smi-id-1-preliminary-identification-of-medically-important-bacteria, 2021 (accessed 15 October 2023)

[7] J.E. Clarridge, Impact of 16S rRNA Gene Sequence Analysis for Identification of Bacteria on Clinical Microbiology and Infectious Diseases. Clin. Microbiol. Rev. 17 (2004) 840–862. https://doi.org/10.1128/cmr.17.4.840-862.2004.

[8] R. Srinivasan, U. Karaoz, M. Volegova, J. MacKichan, M. Kato-Maeda, S. Miller, R. Nadarajan, E.L. Brodie, S.V. Lynch, Use of 16S rRNA Gene for Identification of a Broad Range of Clinically Relevant Bacterial Pathogens. PLoS One. 10 (2015) e0117617. https://doi.org/10.1371/journal.pone.0117617.

[9] J.S. Johnson, D.J. Spakowicz, B-Y. Hong, L.M. Petersen, P. Demkowicz, L. Chen, S.R. Leopold, B.M. Hanson, H.O. Agresta, M. Gerstein, E. Sodergren, G.M.

368 Weinstock, Evaluation of 16S rRNA gene sequencing for species and strain-level
369 microbiome analysis. Nat. Commun. 10 (2019) 1–11. https://doi.org/10.1038/s41467-
370 019-13036-1.
371
372 [10] S. Fujiyoshi, A. Muto-Fujita, F. Maruyama, Evaluation of PCR conditions for
373 characterizing bacterial communities with full-length 16S rRNA genes using a
374 portable nanopore sequencer. Sci. Rep. 10 (2020) 12580.
375 https://doi.org/10.1038/s41598-020-69450-9.
376
377 [11] T.C. Lorenz, Polymerase Chain Reaction: Basic Protocol plus Troubleshooting
378 and Optimization Strategies. Journal of Visualized Experiments. J. Vis. Exp. 63
379 (2012) 3998. https://doi.org/10.3791%2F3998.
380
381 [12] Earth Microbiome Project, 16S Illumina Amplicon Protocol.
382 https://earthmicrobiome.org/protocols-and-standards/16s/, 2018 (accessed 15
383 October 2023).
384
385 [13] H.R.M. Dos Santos, C.S. Argolo, R.C. Argôlo-Filho, L.L. Loguercio, A 16S rDNA
386 PCR-based theoretical to actual delta approach on culturable mock communities
387 revealed severe losses of diversity information. BMC. Microbiol. 19 (2019) 74.
388 https://doi.org/10.1186/s12866-019-1446-2.
389
390 [14] J-Y. Wu, X-T. Jiang, Y-X. Jiang, S-Y. Lu, F. Zou, H-W. Zhou, Effects of
391 polymerase, template dilution and cycle number on PCR based 16 S rRNA diversity

analysis using the deep sequencing method. BMC Microbiol. 10 (2010) 255. https://doi.org/10.1186/1471-2180-10-255

[15] Thermo Fisher Scientific, PCR Setup - Six Critical Components to Consider. https://www.thermofisher.com/uk/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/pcr-education/pcr-reagents-enzymes/pcr-component-considerations.html, 2023 (accessed 15 October 2023).

[16] Z. Ouaray, S.A. Benner, M.M. Georgiadis, N.G.J. Richards, Building better polymerases: Engineering the replication of expanded genetic alphabets. J. Biol. Chem. 295 (2020) 17046-17059. https://doi.org/10.1074/jbc.rev120.013745

[17] W.G. Weisburg, S.M. Barns, D.A. Pelletier, D.J. Lane, 16S ribosomal DNA amplification for phylogenetic study. J. bacterial. 173 (1991) 697–703. https://doi.org/10.1128/jb.173.2.697-703.1991.

[18] Promega, PCR Amplification. https://www.promega.co.uk/resources/guides/nucleic-acid-analysis/pcr-amplification/, 2023 (accessed 15 October 2023).

[19] Merck (2023). Standard PCR Protocol. https://www.sigmaaldrich.com/GB/en/technical-documents/protocol/genomics/pcr/standard-pcr, 2023 (accessed 15 October 2023).

[20] M.A. Innis, PCR protocols: a guide to methods and applications. San Diego, Calif.: Acad. Press. 1994. ISBN: 9780080886718.

[21] R. Sipos, A.J. Székely, M. Palatinszky, S. Révész, K. Márialigeti, M. Nikolausz, Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targetting bacterial community analysis. FEMS. Microbiol. Eco. 60 (2007) 341–350. https://doi.org/10.1111/j.1574-6941.2007.00283.x.

[22] M.A. Sze, P.D. Schloss, The Impact of DNA Polymerase and Number of Rounds of Amplification in PCR on 16S rRNA Gene Sequence Data. mSphere. 4 (2019) e00163-19. https://doi.org/10.1128/msphere.00163-19.

[23] M.C. Witzke, A. Gullic, P. Yang, N.J. Bivens, P.R.F Adkins, A.C. Ericsson, Influence of PCR cycle number on 16S rRNA gene amplicon sequencing of low biomass samples. J. Microbiol. Methods. 176 (2020) 106033. https://doi.org/10.1016/j.mimet.2020.106033

[24] K. Loens, K. Bergs, D. Ursi, H. Goossens, M. Ieven, Evaluation of NucliSens easyMAG for Automated Nucleic Acid Extraction from Various Clinical Specimens. J. Clin. Microbiol. 45 (2007) 421–425. https://doi.org/10.1128/jcm.00894-06.

[25] G.S. Nasution, D. Suryanto, R.L. Kusumawati, Detection of meca gene from methicillin resistant staphylococcus aureus isolates of north sumatera. IOP Conf. Series: Earth. Environ. Sci.130 (2018) 012026. https://doi.org/10.1088/1755-1315/130/1/012026.

[26] R. D'Amore, U.Z. Ijaz, M. Schirmer, J.G. Kenny, R. Gregory, A.C. Darby, M. Shakya, M. Podar, C. Quince, N. Hall, A comprehensive benchmarking study of protocols and sequencing platforms for 16S rRNA community profiling. BMC Genomics. 17 (2016) 55. https://doi.org/10.1186/s12864-015-2194-9.

[27] T.F. Somerville, C.E. Corless, H. Sueke, T. Neal, S.B. Kaye, 16S Ribosomal RNA PCR Versus Conventional Diagnostic Culture in the Investigation of Suspected Bacterial Keratitis. Transl. Vis. Sci. Technol. 9 (2020) 2. https://doi.org/10.1167/tvst.9.13.2.

[28] United States Environmental Protection Agency and U.S. Department of Energy, Laboratories for the 21st Century: An Introduction to Low-Energy Design. https://www.nrel.gov/docs/fy08osti/29413.pdf, 2008 (accessed 15 October 2023).

[29] UK Department for Business, Energy and Industrial Strategy, UK becomes first major economy to pass net zero emissions law. https://www.gov.uk/government/news/uk-becomes-first-major-economy-to-pass-net-zero-emissions-law, 2019 (accessed 15 October 2023).

[30] The Academy of Medical Sciences, Enabling greener biomedical research. https://acmedsci.ac.uk/file-download/47605694, 2023 (accessed 15 October 2023).

[31] UCL, LEAF - Laboratory Efficiency Assessment Framework. https://www.ucl.ac.uk/sustainable/leaf-laboratory-efficiency-assessment-framework, 2023 (accessed 15 October 2023).

[32] D. Kuntin, How to... reduce your lab's plastic waste. The Biologist. 65 (2018) 28-31.

[33] C. Drahl, A Matter of Degree. ACS Cent Sci. 4 (2018) 1294–1297. https://doi.org/10.1021/acscentsci.8b00705.

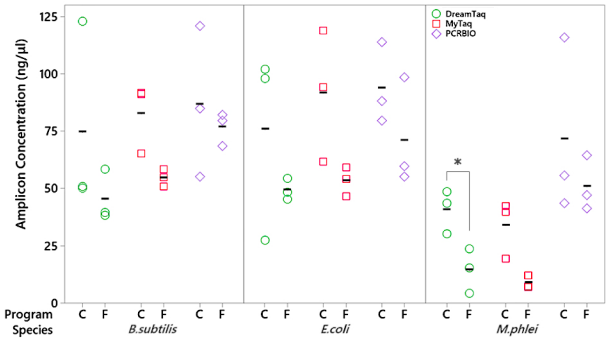
[34] P. Bolton, I. Stewart, Research Briefing: Domestic energy prices. House of Commons Library. Number 9491 https://commonslibrary.parliament.uk/research-briefings/cbp-9491/#:~:text=The%20monthly%20increases%20in%20both,series%20going%20back%20to%201970, 2023 (accessed 15 October 2023).

Transparency declarations

None to declare.

Figure legend

Figure 1. Amplicon concentration of the purified 16S rRNA PCR product amplified from *B. subtilis*, *E. coli* or *M. phlei* using either the 16S V0 control (C) or 16S V9 final (F) PCR program. Three independent replicates undertaken; a green circle represents DreamTaq, a red square represents MyTaq, and a purple diamond represents PCRBIO. The black horizontal dash indicates the mean amplicon concentration (n = 3) and * is $p<0.05$.



Highlights

- Quicker and more environmentally sustainable PCR
- The 16S PCR program duration reduced by 46% compared to typically published protocols
- The 16S PCR program consumes 50% less electricity
- Amplicon produced from low-, mid-, and high-GC bacterial templates

Declaration of interest/conflict of interest

Authors have none to declare.