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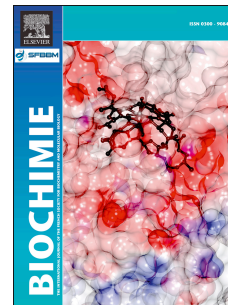
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1 **Amplifying PCR productivity and environmental sustainability through**
2 **shortened cycling protocols**

3

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11

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13

14

15 **Abstract**

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

33

34

35 **1. Introduction**

36

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

43

44 PCR is an important clinical diagnostic tool for genetic microbial detection and
45 identification [5], with specific gene-targeted Real-Time PCR (RT-PCR) and 16S
46 rRNA gene sequencing being the most common methods [6]. RT-PCR is a quick and
47 effective tool for identifying bacterial species or antibiotic resistance genes, but it
48 relies on prior knowledge of target sequences and is limited in the breadth of targets
49 that can be identified in any one test. In contrast, 16S rRNA gene sequencing utilises
50 broad-species-range oligonucleotides that target conserved regions within the gene
51 to allow for simple and cost-effective genus/species level identification without prior
52 knowledge of the disease/infective agent [7,8] and for microbial community analysis
53 [9,10].

54

55 While rapid in comparison to bacterial culture, typical 16S PCR programs can take
56 over 2 hours to complete [11-13], during which time the machine is engaged and
57 cannot be used for other purposes. Advancements in polymerase engineering [14-
58 16] and commercially produced master mixes have increased performance and
59 reduced reaction set up times. However, cycling protocols have largely remained

60 unchanged since the 1990s [11-13,17-19], with extension times of 60-180 seconds
61 still used for amplification during the extension stage. *Taq* polymerases are
62 reportedly capable of incorporating approximately 60 nucleotides per second at 70°C
63 [15, 20], suggesting it may be possible to amplify 1500bp in 25 seconds, translating
64 into a shorter PCR program. While variations in PCR protocols have been published
65 [14,21-23], no study appears to have systemically evaluated the effects of modified
66 PCR cycling parameters on amplicon production. We present a new protocol with
67 shortened cycling times and reduced energy consumption that robustly and
68 reproducibly amplifies a 1466bp 16S rRNA gene fragment across a range of
69 bacterial species with varying genomic GC contents, resulting in increased
70 laboratory productivity and environmental sustainability.

71

72 **2. Materials and Methods**

73 **2.1 Bacteria and DNA extraction**

74 *Bacillus subtilis* (University of Plymouth strain), *Escherichia coli* K12 DH1 (DSM
75 4235) and *Mycobacterium phlei* (DSM 43070), with GC contents of ~36-40%, 50.8%
76 and 73% respectively, were cultured on BHI agar (Merck Life Science UK Limited,
77 Dorset, UK) aerobically at 37°C for 24-48 hours. DNA was extracted from a single
78 colony using the FastDNA™ SPIN Kit for Soil and FastPrep-24 5G Bead Beating
79 Lysis System (MB Biomedical Irvine, CA, USA) according to the manufacturers
80 protocol with the following modification: centrifugation at 12,300xg using a Micro Star
81 12 centrifuge (VWR Lutterworth, Leicestershire, UK). DNA from *E. coli* NCTC 11560,
82 that had previously been amplified, was used as a positive control for PCR. DNA
83 concentration was equalised to 45ng/μL \pm 10% using a NanoDrop™ ND1000

84 spectrophotometer (Thermo Fisher Scientific™ Waltham, MA, USA) and stored at -
85 20°C.

86

87 2.2 PCR Amplification and Energy Monitoring

88 Three commercial PCR master mixes were used: DreamTaq Green PCR Master Mix
89 (Thermo Fisher Scientific™ Waltham, MA, USA), MyTaq™ Red Mix (Meridian
90 Biosciences St. Modiin, Israel), and 2x PCR BIO Ultra Mix (PCR Biosystems London,
91 UK). 50µL reactions were set up comprising 25µL of the respective PCR Master Mix,
92 1µL 27f 5'-AGAGTTTGATCCTGGCTCAG-3' forward primer (10µM) and 1µL 1492r
93 5'-ACGGCTACCTTGTTACGACTT-3' reverse primer (10µM) (Eurofins Genomics,
94 Ebersberg, Germany) [17], 22µL nuclease-free water (Thermo Scientific™), and 1µL
95 of bacterial DNA. Nuclease-free water (Thermo Scientific™ ddH₂O) was used as
96 negative control, while *E. coli* NCTC 11560 DNA was used as the positive control. All
97 reactions were carried out on GeneAmp PCR System 9700 automated thermocycler
98 (Marshall Scientific Hampton, NH, USA) on maximum temperature ramping speed
99 for all cycling programs (Table 1). All PCR amplicons were visualised on a 1%
100 agarose gel in 0.5x tris-acetate-EDTA buffer at pH8, pre-stained with 1x SYBR Safe
101 DNA Gel Stain (Thermo Fisher Scientific™ Waltham, MA, USA), under UV trans-
102 illumination. Power consumption was measured using a Plug-in mains power and
103 energy monitor (Maplin Electronics, Rotherham, UK).

104

105 2.3 PCR Purification, Quantification and Sequencing

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

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

116

117 2.4 Statistics

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

127

128 3. Results

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

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

137

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

155

156 Table 1. PCR programs tested with total cycling duration calculated by PCR System
157 9700 platform and includes a 2-minute initial denaturation and 5-minute final
158 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-cycles 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

	Control Mean ±SEM (ng/μl)	Final Mean ±SEM (ng/μl)	Difference	Mean Loss of Yield (%)	T value	P value
<i>B. subtilis</i>						
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
<i>E. coli</i>						
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
<i>M. phlei</i>						
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**

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

203

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

221 contrast, the manufacture guidelines for MyTaq were shorter than our final protocol
222 (16S V9), however attempts to amplify from the high-GC *M. phlei* DNA proved
223 unsuccessful with a 20 second annealing step, but was possible for the other two
224 bacterial species, suggesting that the high-GC template was problematic at this
225 stage. The manufacturer guidelines for PCR BIO are shorter than the 16S V9
226 programme reported here, and trials with shorter cycling conditions (e.g., 20 seconds
227 for annealing in 16S V4) were still successful, suggesting this master mix could be
228 further optimised. However, this was not explored further here as the aim of this
229 study was to determine a minimum standardised protocol applicable to low-,
230 medium- and high-GC bacteria across multiple manufacturer master mixes.

231

232 Analysis of the amplicon concentration found it to be more consistent and
233 reproducible with the final program (16S V9) across all PCR master mixes compared
234 with the control program (16S V0), with a more consistent product potentially
235 benefitting any downstream analysis. Amplicon concentration varying by as much as
236 ~75ng/ μ L for the control program (16S V0) despite the initial template DNA being
237 equalised to 45ng/ μ L prior to all PCR reactions. The reason for the variability in
238 amplicon concentration using the standard protocol is unknown, though it is possible
239 that the optimised protocol may result in more targeted annealing of the primers and
240 reduced mis-annealing, but this is speculation and requires further study. In
241 summary, the final program (16S V9) reduces 16S PCR completion times by 46%
242 and uses 50% less electricity compared to the control program (16S V0), while
243 producing amplicons at a sufficient concentration for downstream analysis with no
244 discernible detriment. These modifications could allow for the optimisation of
245 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 *MecA* 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 *MecA*. Further optimisation through the examination of
267 oligonucleotide, magnesium and/or GC buffer concentrations may help further
268 reduce overall cycle duration.

269

270 Clinical and biomedical research laboratories are significant energy users [28] and
271 with the UK Government targeting net-zero carbon emission by 2050 [29],
272 sustainability and running costs are increasingly becoming prominent considerations
273 for any laboratory. Green laboratory initiatives including the Enabling
274 Environmentally Sustainable Biomedical Research forum [30], the Laboratory
275 Efficiency Assessment Framework [31] and trials seeking to reduce plastic waste
276 [32] are beginning to address concerns around sustainability and environmental
277 impact within research. Ultra-low temperature freezers have been shown to use 40%
278 less energy when set to -70°C compared with -80°C , with no apparent detriment to
279 their contents [33]. This reduces energy usage and costs while increasing equipment
280 lifespan, leading to a more sustainable working practice. Consumers are also more
281 energy conscious following increasing global energy prices. Indeed, the 41%
282 increase in UK electricity prices in April 2022 was the largest monthly price increase
283 since 1988 [34] making the running of any laboratory equipment more expensive.
284 PCR is a common laboratory method. Shortened cycling protocols would use
285 substantially less electricity, translating into lower energy costs, potentially longer
286 machine shelf life, and improved sustainability, allowing organisations to make
287 cumulative savings towards sustainability initiatives.

288

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

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

301

302 **5. Conclusion**

303 To the authors' knowledge, this is the first study to systematically assess the impact
304 of shortened PCR cycling parameters on the amplification of the 16S rRNA gene in
305 low-, mid- and high-GC template DNA. The final modified program resulted in a 46%
306 reduction in overall run time, with PCR BIO master mix achieving an amplicon
307 concentration comparable to the control program, though there were difficulties when
308 attempting to amplify from high-GC template DNA. For unknown templates, a
309 program with a 5-second denaturation step and 30 seconds for both the annealing
310 and extension steps would allow greater flexibility while only adding 4 minutes and
311 40 seconds to the overall run time, equating to 46% reduction compared to the
312 control program. In contrast, there is evidence programs could be reduced further for
313 known templates comprising $\leq 50\%$ GC. Further research is required to examine the
314 limitations on consistent amplification capability when considering PCR-based
315 productivity and sustainability.

316

317 **Author Contributions**

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

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

323

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328

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480 **Transparency declarations**

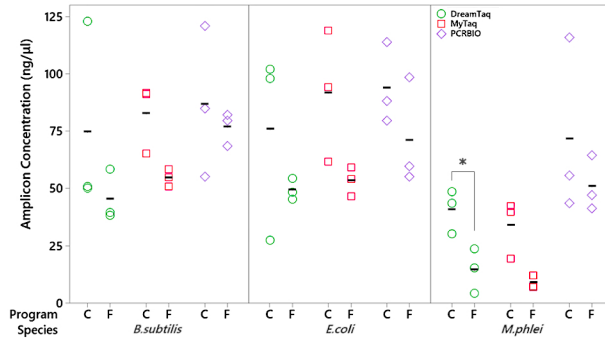
481 None to declare.

482

483

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.



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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

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Declaration of interest/conflict of interest

Authors have none to declare.

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