Single-cell dissection of bacterial communities into functional gene carriers with ddPCR

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Abstract

Microbial communities often respond to environmental challenges, such as the presence of antibiotics, as a whole. Dissecting these community-level effects into separate acting entities requires the identification of organisms that carry functional genes for the observed feature. However, unculturable microbes are abundant in various environments, hence making the identification challenging. Moreover, while at present the development and application of single-cell tools for eukaryotic cells are enhancing, the comparable methodologies applicable for prokaryotic cells are still scarce and have not gained broad and solid status as tools for investigating microbial populations. Here, we present a cultivation-free technique that can be utilized to link functional genes with the carrying bacterial species at single-cell resolution. The developed protocol is relatively simple to use, utilizes commercially available droplet microfluidics devices, does not require toxic reagents, and eliminates invalid signals emerging from extracellular DNA. We validate the methodology by studying the conjugative transfer of antibiotic resistance plasmids in an environment challenged by antibiotics. Furthermore, the method can be customized for any given genetic trait to accurately identify its hosting subpopulation from a heterogeneous and potentially uncultivable bacterial community.

Importance

Bacterial systems usually contain numerous different species that may harbor highly similar or identical genes that confer same phenotypic qualities for the community. To decipher the

functions of these systems, we report the development of a novel methodology that enables investigating microbial communities at single-cell level. This user-friendly method utilizes droplet digital PCR (ddPCR) to find and identify carriers of specific genes potentially from various microbial sample types. By pinpointing gene carriers, such as those responsible for antibiotic resistance, this method can provide insights to the behavior of microbial communities and gene transfer therein. By strategically combining the use of common methods (ddPCR and amplicon sequencing), the workflow is highly accessible. Thus, it allows also the researchers without a background in single-cell techniques or access to special equipment to adopt the method for producing single-cell data to serve their own research, enabling new research avenues in microbial genetics and ecology.

Keywords: 16S rRNA gene; droplet digital PCR; droplet microfluidics; functional genes; microbial diversity analysis; single-cell.

Introduction

Microbes are ubiquitous organisms that play important roles in systems shaping life on our planet. Yet, microbes rarely exist in isolation. A single grain of sand contains tens to hundreds of thousands of bacterial species[1], one gram of dry faecal matter has a billion microbial cells and even very slowly growing microbial mats in lava caves have hundreds of distinct taxonomical units in their thick, slimy layers[2]. Within these communities, bacteria form interaction networks where individual cells are often unable to reproduce in the absence of their contemporaries[3]. Ultimately, genetic, metabolic and ecological links between microbes shape the communities and their behaviour[4]. Bacterial systems may, for example, degrade hazardous waste materials, confer resistance to antibiotics and regulate the turnover of limiting nutrients for subsequent levels in trophic cascades. Further, horizontal gene transfer is common in microbial systems and opportunistic genetic characteristics may rapidly switch into more suitable hosts in response to environmental conditions[5]. Disentangling these communities into individual cells that can still be studied with precision and without the influence of potential confounding factors, such as remnant DNA from dead cells, is challenging.

62 Various methodological advances have been achieved in the recent years to help resolve
63 the roles of individual and potentially unculturable microbes within communities. 16S
64 ribosomal RNA sequencing is the current standard for providing an overall picture of the
65 diversity of microbes – living or dead – in the studied sample. However, it fails to associate
66 genetic characteristics to the species. In contrast, total DNA sequencing can cover a lot of the
67 overall genetic variation, but usually without a definitive answer to the question of who acted
68 as the cellular vehicle for any particular gene – or if that gene was indeed actually a part of a
69 living organism at all or merely a remnant from a deceased microbe[6]. Further, (mobile)
70 plasmids in particular, replicate separately from chromosomes in bacterial cells, and hence,
71 metagenomic sequencing fails to identify the host of the potentially crucial genetic element[7].
72 To link genetic traits with particular species, a number of microfluidic techniques have been
73 utilized to identify target traits (such as the ability to cause a disease or carry bacteriophages)
74 from heterogeneous bacterial samples[8,9]. Ottesen and colleagues utilized a chip containing
75 1176 microchambers to divide termite midgut samples to single-cell resolution and used two
76 fluorescent probes to determine the chambers containing 16S rRNA and/or the targeted
77 functional gene[10]. By using a needle to extract the contents of chambers that expressed both
78 fluorescent signals, they were able to associate 16S rRNA sequence with the functional gene.
79 Alternatively, fixed bacterial samples have been used for fluorescent probe hybridization[11].
80 Hybridization methods, however, are relatively laborious and unable to study a large number
81 of cells. In order to study hundreds of thousands of cells from uncharacterized bacterial
82 samples, Spencer and colleagues developed a method to trap individual cells into tiny
83 acrylamide compartments prior to PCR amplification[12]. In this so-called epicPCR, a very
84 localized PCR reaction is utilized to amplify the targeted functional gene and fuse it in
85 concatenation PCR with the 16S ribosomal RNA gene. By sequencing the paired products, the
86 cellular hosts harbouring the studied genetic trait can be identified. While epicPCR is a
87 promising tool for large-scale, single-cell resolution analyses, it is associated with sources of
88 variation that need to be controlled, including the quantification of the template and cross-
89 contamination.

90 Droplet microfluidics utilizes microchannels for the formation of nanolitre-scale
91 droplets. In droplet digital PCR (ddPCR), PCR reagents are enclosed into droplets surrounded
92 and stabilized by oil, enabling thousands of PCR reactions to occur simultaneously. The
93 amplified DNA is detected by using double-stranded DNA-binding fluorescent dye and
94 measured from each droplet one by one. As the sample is randomly distributed across the
95 droplets, both empty (negative) and DNA-containing (positive) droplets are formed. The
96 system is extremely sensitive and capable of detecting even a single copy within a droplet.
97 Finally, the fraction of positive droplets is compared to the total number of droplets and used
98 to calculate the (absolute) quantity of target DNA in the original sample with the help of
99 Poisson statistics[13,14]. DNA can be delivered into droplets also within intact cells instead of
100 pure DNA, allowing ddPCR to be applied for bacterial communities. This principle has been
101 harnessed to trace enterohemorrhagic *Escherichia coli* (EHEC) in food production samples[15,16].
102 In these studies, single bacterial cells were enclosed into droplets. After the cell lysis initiated
103 by the denaturation step of ddPCR, EHEC was detectable via distinct amplification of two
104 virulence genes and their colocalization in the same droplet with fluorescent probes[16].
105 In this study, we present a new adapted technique for investigating bacterial
106 communities at single-cell resolution. The described method utilizes commercially available
107 ddPCR devices (based on droplet microfluidics), is fast to perform, requires only non-toxic
108 reagents, and contains steps to efficiently inactivate extracellular DNA from samples. The
109 method employs paired fusion PCR to link a taxonomic marker to the target gene and allows
110 accurate fluorescence-based verification of single-cell resolution prior to fusion amplicon
111 sequencing. We demonstrate that the method is applicable for precise detection and
112 identification of carriers of a beta-lactamase resistance gene (*bla*CTX-M-14) in the
113 heterogenous bacterial sample, and thus, can be utilized for tracing the movements of mobile
114 genetic elements such as plasmids within the bacterial community. As such, the introduced
115 single-cell ddPCR method provides a convenient approach for different laboratories to identify
116 specific genetic traits from multi-species communities.
117

118

119 **RESULTS**

120 **A microbial single-cell screening from a community**

121 In this study, we developed an easy-to-use microbial single-cell screening method to identify
122 or track hosts of any gene of interest in heterogeneous communities. Our experimental setup
123 focused on tracking an antibiotic resistance gene (ARG) *bla*CTX-M-14 (hereafter referred to
124 as *bla*). A fragment of *bla* is paired with the 16S rRNA gene in fusion PCR to identify
125 taxonomic groups that harbour the ARG. For enabling analysis of individual cells, the bacterial
126 samples are partitioned into compartments by utilizing the QX200™ Droplet Digital PCR
127 System from Bio-Rad. This automated system employs droplet microfluidics for generating
128 equal-sized 1-nanolitre droplets that encapsulate both individual bacterial cells and PCR
129 reagents (Figure 1). During thermal cycling, the bacterial DNA is released from cells via lysis
130 and used as a template for paired amplification of target ARG and 16S rRNA gene. As single-
131 cell PCR-based methods are highly sensitive to any contaminating DNA present in the droplet
132 but outside of the cell, the protocol includes the treatment of the sample prior partitioning for
133 inactivating extracellular DNA. These steps were observed to be crucial for the accurate
134 detection of genetic content that is indeed bound within cellular membranes. By sequencing
135 the paired amplicons, the *bla* carrier population can be identified from the studied bacterial
136 community. Four bacterial species were employed in the development and optimization of the
137 described method: *Escherichia coli* (carrying the target *bla* gene in a conjugative plasmid
138 pEC13), *Pseudomonas fluorescens, Salmonella* Typhimurium and *Klebsiella pneumoniae*.

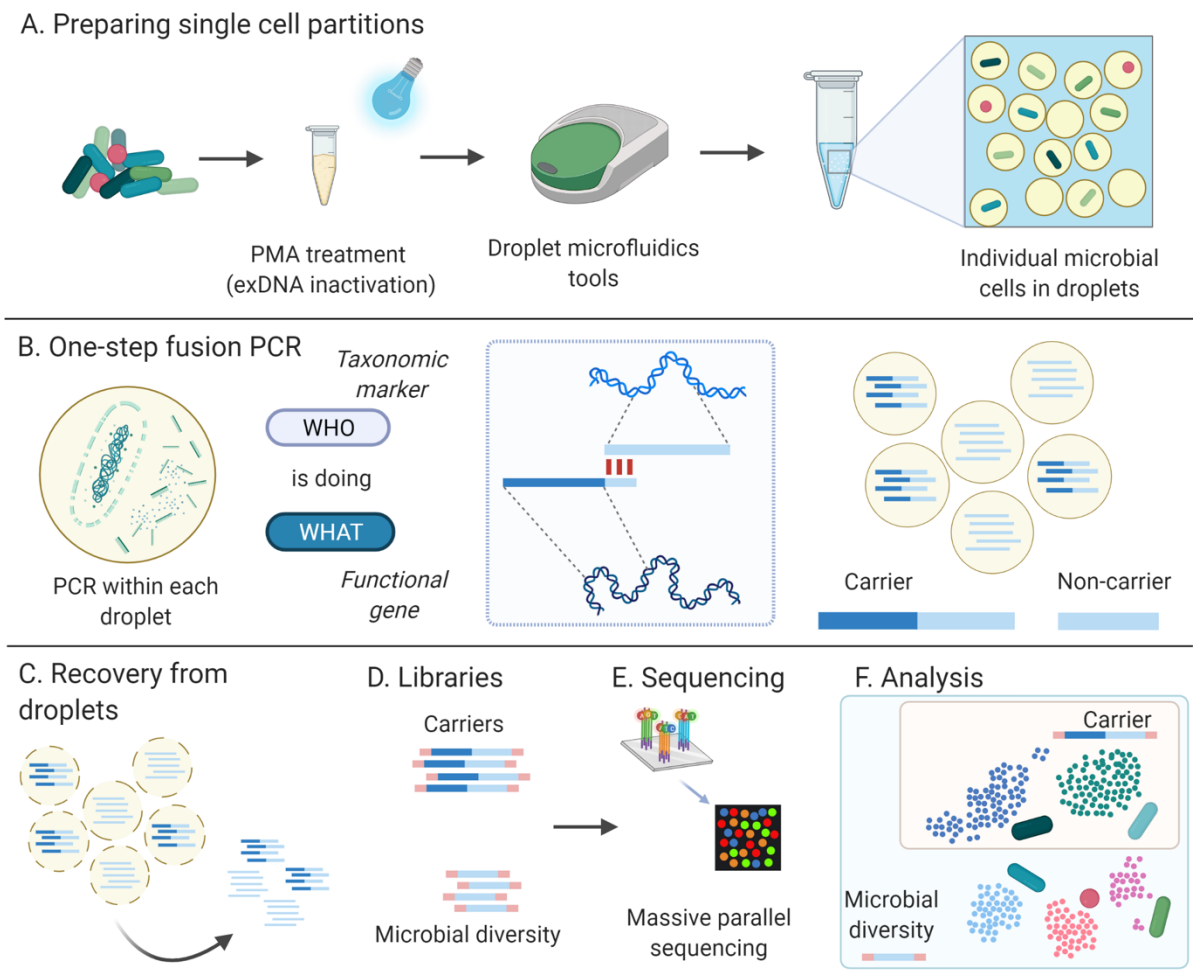
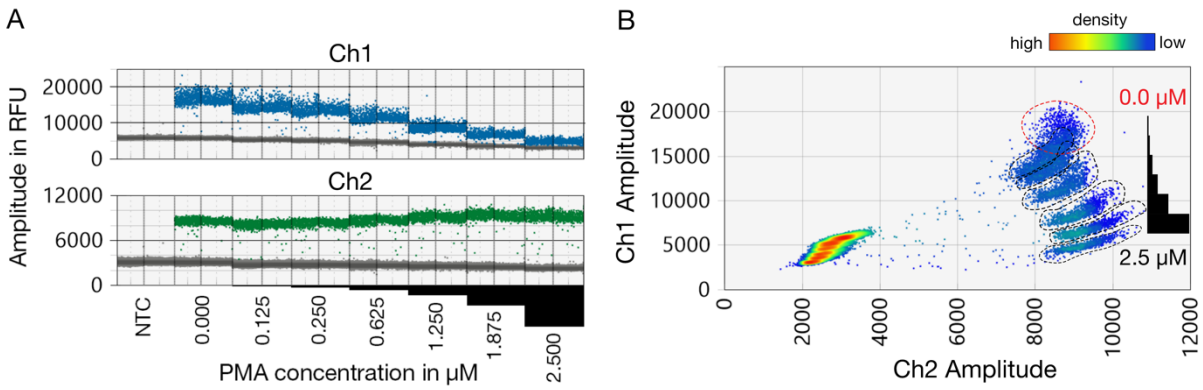


Figure 1. Overview of the single-cell ddPCR method workflow. A) Bacterial samples are prepared and partitioned using droplet microfluidics technology to obtain a single cell per droplet. This step includes treatment with PMA (propidium monoazide) to inactivate exDNA (extracellular DNA), the addition of PCR reagents and, finally, the generation of single-cell -containing droplets with a droplet microfluidics device. B) One-step fusion PCR assay is carried out within each droplet. In droplets containing a carrier cell, *i.e.* a cell that has the functional gene, the taxonomic marker gene and the functional gene are amplified and fused into a concatemer PCR product. The taxonomic marker is the sole product in droplets containing non-carrier bacterial cells. C) The produced amplicons are recovered from droplets by breaking the emulsion using a liquid nitrogen method[17]. D) The recovered amplicons are used to construct sequencing libraries and E) sequenced by massive parallel sequencing. F)

151 The sequence analysis determines the bacterial population carrying the functional gene as well
152 as the overall bacterial diversity in the given sample. Created with BioRender.com.
153
154 **Extracellular DNA (exDNA) inactivation from cell samples**
155 Microfluidic encapsulation traps any content in the sample, including free DNA. Preliminary
156 analysis (not shown) indicated that free DNA (*i.e.* DNA outside of bacterial cells) is abundant
157 in bacterial suspensions even after DNAse treatment and/or repeated washing of the cells. As
158 such, this DNA forms a notable confounding factor for single-cell analyses that aim to reliably
159 identify a specific DNA target that is indeed within the cellular membranes (*i.e.* a single viable
160 cell). We ensured that the process only amplifies DNA originating from a unique cell by
161 treating the samples with propidium monoazide (PMA). PMA is a DNA binding dye that
162 covalently attaches to double stranded DNA (dsDNA) via photoactivation, thus preventing
163 primer annealing and polymerization to occur. Cell membranes are impermeable to PMA,
164 hence only DNA external to intact cells is rendered non-amplifiable. Phosphate buffer saline
165 (PBS) was used for diluting the bacterial cell samples, therefore its maximum non-inhibitory
166 concentration in ddPCR was defined and always kept at safe level (under 0.0625x) (see
167 Supplementary Figure S1).
168 **PMA treatment – fluorescence emission spectral shift**
169 After initial screening, we hypothesized that PMA may influence PCR. At first, we wanted to
170 see if PMA would prevent amplification, therefore we expected the number of positive droplets
171 would reduce with increasing amounts of PMA. As such, the maximum non-inhibitory
172 concentration of PMA in PCR amplification was assessed by adding increasing concentrations
173 of photoactivated PMA to EvaGreen-based ddPCR assays (Figure 2). The reactions targeting
174 the 16S rRNA region (amplified with 27F and 338R primers) of digested *E. coli* DNA were
175 prepared with 0.125 μM up to 2.5 μM of PMA. The Relative Fluorescence Unit (RFU) was

176 obtained from each droplet by the Droplet Reader through fluorescence detection channels 1
177 (Ch1 for FAM) and 2 (Ch2 for HEX/VIC). Analysis of RFU showed that two droplet clusters
178 could be seen: a cluster of negative droplets where amplification had not occurred (low RFU)
179 and a cluster of positive droplets with successful amplification (high RFU). Nonetheless, in
180 Ch1, a pronounced drop in RFU from positive droplet cluster could be seen when increasing
181 concentrations of PMA were used (Figure 2A). The same pattern could not be observed in Ch2
182 data. Here, instead, the positive droplets cluster showed an increase in RFU in higher PMA
183 concentrations. The analysis of Ch1 *versus* Ch2 (Figure 2B) showed a clear distinction between
184 positive and negative droplet clusters. These results suggest that PMA does not inhibit PCR
185 reactions when used in concentrations up to 2.5 µM but affects the fluorescence emission
186 spectrum that disturbs the detection of the DNA binding dye (EvaGreen) from Ch1.

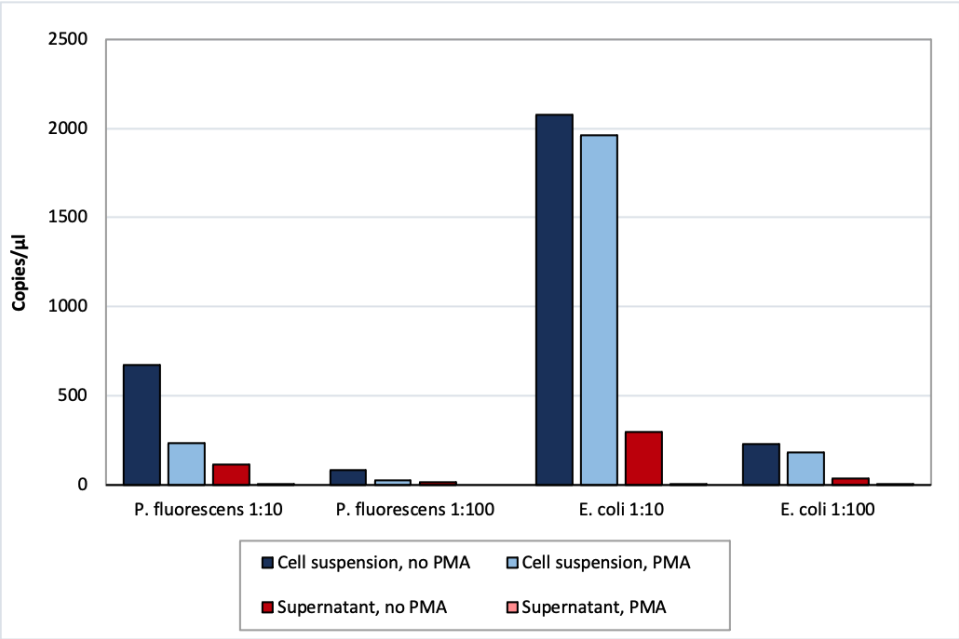


187

188 **Figure 2. Effect of PMA concentration on RFU reading.** Light-inactivated PMA in final
189 concentration ranging from 0.125 to 2.5 µM was added to 16S rRNA ddPCR. After
190 amplification, the fluorescence of each droplet was measured through both detection channels.
191 The increasing PMA concentration resulted in a drop in RFU when measured via channel 1
192 (Ch1 for FAM) and 2 (Ch2 for HEX/VIC).) (A, upper panel), but not with channel 2 (Ch2) (A,
193 lower panel). The positive droplets were identified by two-dimensional plotting of RFU values
194 detected from Ch1 and Ch2 (B). The circles mark roughly the cluster of positive droplets for
195 the different PMA concentration.

196

197 Further, we studied whether PMA inactivates exDNA in samples containing bacterial
198 cells. Cell suspensions of *E. coli* and *P. fluorescens* and their filtrates (suspension filtered
199 through a 0.2 µm filter) were treated with 2 µM PMA and used as a template in ddPCR
200 amplifying a region within the gene for 16S rRNA. For controls, the corresponding samples
201 were treated with sterile water instead of PMA. PMA treatment reduced the obtained 16S rRNA
202 concentration by as much as 71% in *P. fluorescens* and 22% in *E. coli* cell samples (Figure 3).
203 This suggests that exDNA is abundant even in pure bacterial cultures and potentially causes
204 erroneous PCR amplification if the DNA is present inside a droplet together with the trapped
205 bacterial cell. To verify the complete inactivation of exDNA, we filtered out the bacterial cells
206 and exposed the filtrate to PMA. Less than 0.12% of the original contaminating DNA was
207 amplifiable after PMA treatment (Figure 3).



208

209 **Figure 3. Effect of PMA treatment on inactivation of exDNA.** *Pseudomonas fluorescens*
210 and *Escherichia coli* cell dilution (1:10 or 1:100 of an overnight-grown bacterial culture) and
211 their filtrated (0.2 µm) supernatant were treated with 10 µM PMA or sterile water as control.
212 16S rRNA was amplified with single-cell ddPCR and its abundance is presented as copies/µl.

213

214 **One-step fusion PCR**

215 The developed method relies on linking two targeted DNA fragments together by using fusion
216 PCR. To produce the concatemer, we employed one-step fusion PCR with four primers. In this
217 approach, the amplification of both targets occurs within the same reaction. Introduction of a
218 fusion primer into the reaction prompts the attachment of the distinct target fragments into a
219 unified DNA molecule (Figure 4). The fusion primer consists of a priming sequence for a given
220 target sequence added by additional bases at the 5'-end that encode the complementary reverse
221 sequence of the forward primer of the other target. The dynamics of the reaction is driven by a
222 difference in the ratio of forward to reverse primer (within a pair) and the concentration of each
223 primer pair. The outer primers that flank the concatemer sequence are used in higher
224 concentrations than the inner primers. Amplification of the target sequence will eventually
225 diminish due to the depletion of inner primers, thus the matching overhang will prompt the
226 fusion of both target sequences into a paired concatemer. The excess of concatemer flanking
227 primers allows for the amplification of the linked sequence.

228 In our experiments, we used the 16S rRNA universal primers, 27F and 338R, to amplify
229 a variable region that can be used to identify the used bacterial strains. As for ARG target, we
230 designed primers 293F and 320R for amplifying a 101 bp region of the *bla* gene. Furthermore,
231 we modified *bla* reverse primer 320R with a 5'-extension of the reverse complementary
232 sequence of 27F (therefore producing a fragment of 121 bp). The complementary sequence to
233 be produced during PCR was to act as primer and allow annealing of the *bla*-27F overhang to
234 the 16S rRNA fragment, thus fusing both target sequences as a concatemer.

235

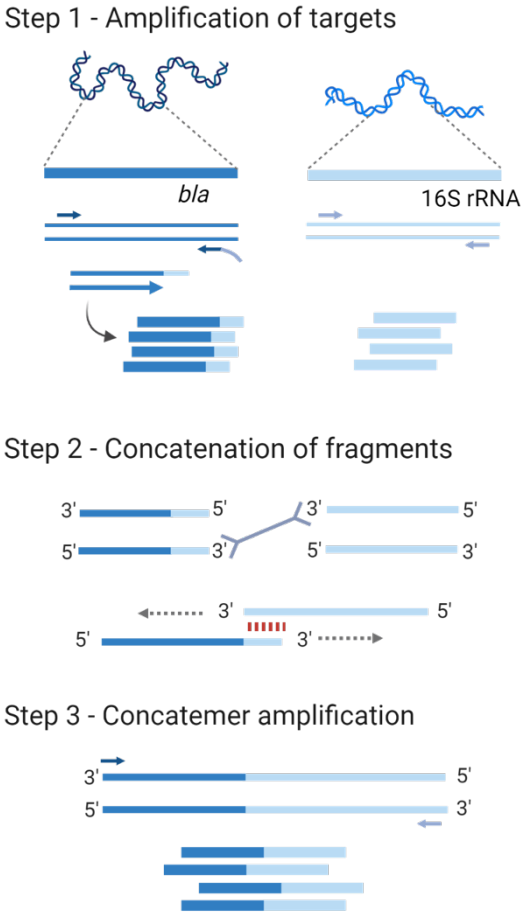
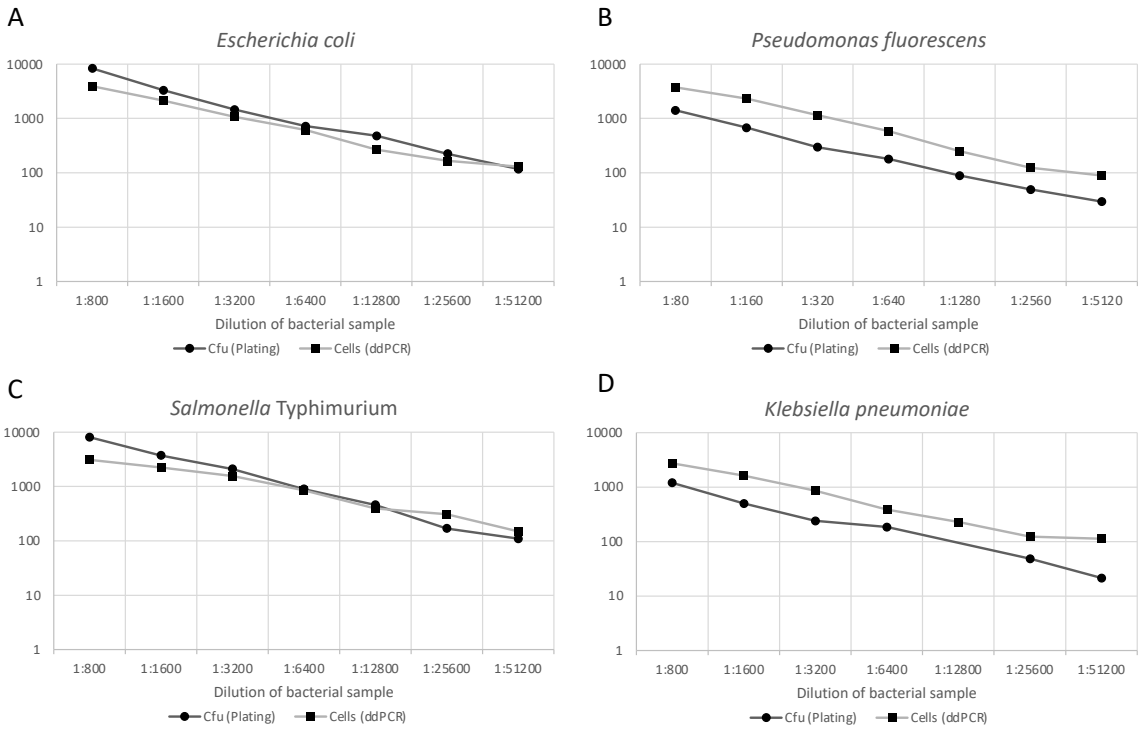


Figure 4. One-step fusion PCR with four primers. The amplification and fusion of 16S rRNA and beta-lactamase gene *bla* are carried out in the same reaction. The fusion process is driven by a fusion primer that is one of the *bla* primers with a 5' -end extension that encodes the reverse complement sequence of the 16S rRNA primer 27F. In step 1, both target regions are simultaneously and independently amplified, generating the intermediate product of the fusion: the *bla* fragment with the tail complementary to 27F. In step 2, the tail will prompt the fusion of 16S and *bla* products. Lower concentration of the inner primers (27F and the modified *bla* primers that bind to the fusion region) facilitates the reaction dynamic to produce the concatemer. After the depletion of the inner primers, in step 3, the amplification of the fusion product is carried out by using the excess of external primers. Created with BioRender.com.

249 **Optimization of primer sets**
250
251 The optimization of one-step fusion PCR was carried out by screening the primer ratio and
252 concentration to assess the efficiency of concatemer production. First, the pairs were tested
253 separately to verify the production of each target in different reaction conditions
254 (Supplementary Figures S2-3). After this, the primers were combined in a single ddPCR
255 reaction to ensure the amplification of the concatemer. The separation of positive and negative
256 clusters was evaluated to define the concentration and ratio to be used in the following assays.
257 Clustering was more efficient at lower ratios and with higher primer concentrations
258 (Supplementary Figure S3).
259 In ddPCR, the amplification of DNA fragments of distinct sizes is indicated by RFU
260 level. Thus, the occurrence and separation of four droplet clusters were sought: a cluster with
261 very low RFU was expected for negative droplets; two clusters with intermediate RFU were
262 expected to result from droplets where a single target was amplified, either a low RFU cluster
263 for *bla* (121 bp product) or higher RFU cluster for 16S rRNA (350 bp product). The cluster
264 with the highest RFU indicated the amplification of the fused concatemer (of 450 bp) (for
265 detailed information, see Supplementary Figure S3). Finally, the conditions for one-step fusion
266 reactions in ddPCR were defined by using 225 nM of 16S rRNA primers in 1:5
267 (forward:reverse) ratio, and 200 nM of *bla* primers in 10:1 (forward:reverse) ratio.
268
269 **Partition of single cells into droplets**
270 In order to verify whether single cells could be delivered to droplets with the ddPCR
271 microfluidic system, we designed an assay where PMA-treated dilutions of *E. coli,*
272 *Pseudomonas fluorescens, Salmonella* Typhimurium and *Klebsiella pneumoniae* cells were
273 used as a template in the ddPCR targeting the 16S rRNA gene. A serial plating assay was
274 prepared in parallel, and the obtained cell density compared to that estimated with ddPCR.

275 With all tested bacterial strains, the bacterial cell density was comparable when measured with
276 serial plating and 16S rRNA single-cell ddPCR; the decreasing trend was detectable between
277 adjacent dilutions. However, in *P. fluorescens* and *K. pneumoniae*, a higher cell concentration
278 was consistently observed with ddPCR in comparison to plating (Figure 5).



279

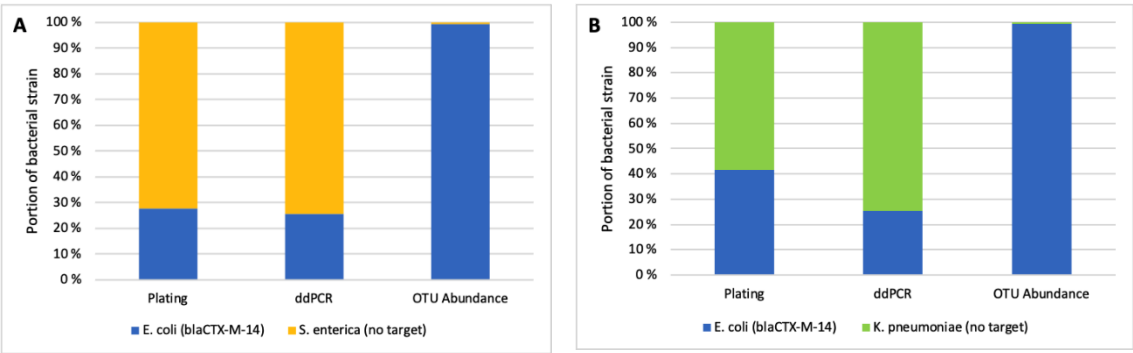
280 **Figure 5. Single cells of *Escherichia coli, Pseudomonas fluorescens, Salmonella***
281 **Typhimurium and *Klebsiella pneumoniae* within droplets.** A dilution series of overnight-
282 grown bacterial cultures was prepared, enclosed into droplets and amplified with 16S rRNA
283 single-cell ddPCR. The cell number per ddPCR sample was determined by 16S rRNA ddPCR
284 and is presented as cells. Parallel serial plating of bacterial cultures on LB agar was performed
285 to measure cfu (colony forming units) per sample.

286

287 **Identifying the ARG carriers by concatemer sequencing**

288 To investigate how effectively the single-cell ddPCR method identifies target gene hosts from
289 a heterogeneous sample, *E. coli* cells carrying *bla*CTX-M-14 were mixed with *S.* Typhimurium

290 and *K. pneumoniae* cells not carrying the gene. Bacterial cell suspensions were first pre-treated
291 with PMA and immediately added to ddPCR reactions to include either a single strain or two
292 strains: *E. coli* with either *S.* Typhimurium or *K. pneumoniae.* The portion of each strain was
293 determined by i) serial plating of each bacterial pure culture, ii) single-cell ddPCR amplifying
294 16S rRNA and iii) OTU abundance (determined via sequencing of bla_16S concatemer
295 produced in single-cell ddPCR) (Figure 6). The portion of *E. coli* cells in the samples ranged
296 from 28-61% when estimated by separate serial plating of the pure cultures. A comparable ratio
297 was obtained with ddPCR from a mix of *E. coli* and S. Typhimurium cells, however from *E.*
298 *coli* and *K. pneumoniae* cell mixes, ddPCR resulted in higher portion of *K. pneumoniae* cells.
299 The mixed cell samples were delivered into single-cell ddPCR in which bla_16S concatemers
300 were produced and proceeded to library preparation and sequencing. The 16S rRNA part of the
301 concatemer was used for identifying the host strain associated with *bla*CTX-M-14 and the
302 abundance of *E. coli*, *S.* Typhimurium or *K. pneumoniae* hosts was determined in each sample.
303 In the mixed samples, the portion of non-carrier (*S.* Typhimurium or *K. pneumoniae*) hosts was
304 0.47-1.15%, suggesting that single-cell ddPCR process effectively diminished the occurrence
305 of false positive host sequences.



307 **Figure 6. Portions of *Escherichia coli, Salmonella* Typhimurium (A) and *Klebsiella***
308 ***pneumoniae* (B) cells in mixed heterogeneous samples determined by serial plating, 16S**
309 **rRNA single-cell ddPCR and bla_16S concatemer single-cell ddPCR and sequencing.** Cell
310 density (colony forming units/ml) from each pure culture was measured separately by serial

311 dilution and plate counting. 16S rRNA single-cell ddPCR was used to measure the cell density
312 of each pure culture separately to determine the portion of each bacterial strain in a mixed
313 sample. Single-cell ddPCR with primers producing bla_16S concatemer followed by fusion
314 amplicon sequencing was utilized to identify and quantify the *bla*CTX-M-14 carriers in mixed
315 samples. The fraction of *bla*CTX-M-14 hosts is presented as OTU abundance.
316
317 **Horizontal transfer of beta-lactamase *bla*CTX-M-14 in synthetic bacterial community**
318 The developed method was applied to track horizontal transfer of a conjugative plasmid within
319 a synthetic microbial community consisting of rifampicin resistant *Klebsiella pneumoniae,*
320 *Comamonas* sp. and *Salmonella* Typhimurium strains. As a plasmid donor, we harnessed
321 rifampicin-sensitive *E. coli* strain with conjugative plasmid pEC13, which renders the host
322 resistance to cephalothin via *bla*CTX-M-14 gene. This plasmid has been shown to be able to
323 rescue antibiotic sensitive cells in its vicinity under lethal antibiotic concentrations[5,18].
324 In bacterial systems, resistance to different antibiotics can exist in separate populations.
325 When these systems are exposed to multiple antibiotics, horizontal transfer of resistance genes
326 may be the only evolutionary pathway for (parts of) the community to survive. As such, our
327 aim was to investigate the transfer of conjugative resistance plasmid to antibiotic susceptible
328 community, if the donor or the recipient, or both were under antibiotic selection. In our
329 experimental setup, the synthetic communities were either sequentially or simultaneously
330 exposed to antibiotics cephalothin and rifampicin (Figure 7A). In practice, *K. pneumoniae,*
331 *Comamonas* sp. and *Salmonella* Typhimurium strains were co-cultured (N=4) either in 1)
332 cephalothin, 2) cephalothin and rifampicin (simultaneous exposure) or 3) rifampicin. After 30
333 minutes, *E. coli* donor carrying pEC13 was introduced into the communities, allowing
334 evolutionary rescue to occur. One hour after the introduction of *E. coli* donor, treatments 1 and
335 3 (i.e. sequential antibiotic exposure treatments), were supplied with rifampicin and

336 cephalothin, respectively. Synthetic communities were cultivated for 3.5 h after which the
337 pEC13 transconjugants were identified via the developed single cell ddPCR method (Figure
338 7A-B). In treatments 1 and 2, where cephalothin was expected to heavily diminish the initial
339 mock community, the proportion of chimeric 16S rRNA sequences was 15.19% (+/- SEM
340 0.56%) and 17.27% (+/- SEM 8.05%), respectively. However, in treatment 3, which allowed
341 the initial rifampicin-resistant community to reproduce prior to the second antibiotic treatment,
342 the proportion of chimeras was only marginal (0.42%, +/- SEM 0.40%). These results suggest
343 that high frequency of non-viable cells may have affected the library preparation via the
344 production of chimeric reads, hence, they were excluded from the sequence analysis.

345 The bacterial community carrying pEC13 was strongly affected by the order in which
346 the antibiotics were introduced into the experimental system (Figure 7B). In treatment 1, in
347 which the community was first exposed to cephalothin, the plasmid carrier population was
348 dominated by the original *E. coli* host. In treatment 3 (first exposed to rifampicin), however,
349 the most abundant plasmid carrier species was *K. pneumoniae*. Simultaneous exposure to both
350 antibiotics led to relatively stochastic behavior of the system, the most abundant plasmid hosts
351 being *E. coli* and *Comamonas* sp. Yet, further pairwise conjugation tests showed that pEC13
352 conjugation between *E. coli* and *Comamonas* sp. was unlikely to occur (data not shown).

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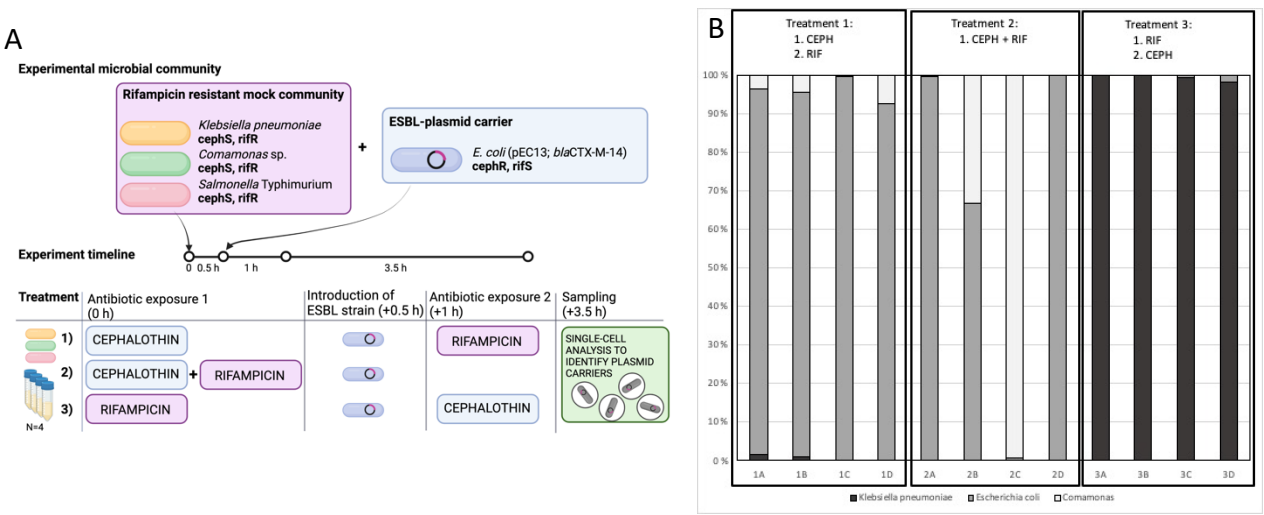

355

356 **Figure 7. Identifying the ESBL-plasmid carrier population (harboring *bla*CTX-M-14**
357 **resistance gene) after sequential or simultaneous antibiotic exposure. A)** Rifampicin
358 resistant mock communities consisting of *Klebsiella pneumoniae, Comamonas* sp. and
359 *Salmonella* Typhimurium were exposed to antibiotics in three different treatments (N=4): 1)
360 cephalothin (CEPH), 2) cephalothin and rifampicin (RIF) or 3) rifampicin. After 30 minutes,
361 *Escherichia coli* (pEC13 with *bla*CTX-M-14) was introduced into the communities. The
362 second antibiotic exposure was given after 1 h with 1) rifampicin and 3) cephalothin.
363 Horizontal transfer of pEC13 within the community was determined with single cell ddPCR
364 (*bla*CTX-M-14 and 16S rRNA fusion) after 3.5 h co-culture and is presented as the frequency
365 of plasmid host species **(B)**.
366
367 **DISCUSSION**
368 Characterizing microbial communities at the resolution of their true operating units, individual
369 cells, is essential for understanding the functions and biological interactions that take place in
370 complex systems. In this study, we provide a stepwise pipeline that utilizes droplet
371 microfluidics for partitioning bacterial samples into single-cell -containing compartments. The
372 method employs fusion PCR that links a 16S rRNA taxonomic marker with the target
373 functional gene (here an antibiotic resistance gene *bla*CTX-M-14), thus enabling the
374 identification of bacteria carrying the target gene after sequencing the paired amplicons.
375 Microbial communities, albeit natural or experimental, are supplemented with exDNA
376 that can prevent accurate analysis of genetic material that solely resides within the boundaries
377 of individual viable cells. exDNA does not only originate from lysed cells, but it is also actively
378 released from bacteria because of different functionalities in the microbial system, such as to
379 aid biofilm formation or mediate horizontal gene transfer (see [19,20] for review). To exemplify,
380 Carini and colleagues demonstrated that extracellularly located relic DNA may significantly

381 bias the observed microbial diversity in soil samples[21]. This bias, however, was shown to be
382 corrected by treating the soil samples with PMA. PMA is a DNA-intercalating dye that only
383 enters dead cells or cells with compromised membranes and hence it has been used in
384 microbiology to identify the viable bacterial population. The covalent binding of PMA to DNA
385 occurs via photoactivation, preventing the DNA from serving as a template for PCR[22]. While
386 exDNA skews the community analyses when conducted on total microbial DNA, it may also
387 disrupt single-cell methodologies that rely on DNA.

388 To investigate the prevalence of exDNA in overnight cultures of *E. coli* and *P.*
389 *fluorescens*, we filtered out the cells and performed 16S rRNA ddPCR for the remaining
390 supernatant. Cell-free supernatant contributed 14-17 % of the total 16S rRNA gene
391 concentration when compared to the corresponding sample where cells were still present. In a
392 preliminary analysis, DNAse treatment was not sufficient in reducing the amplification of
393 exDNA. Hence, the possibility to employ PMA in ddPCR was evaluated. This was done by
394 determining a concentration that did not inhibit the PCR, but effectively blocked the
395 amplification of exDNA in cell-containing and filtrated cell-free samples. The presence of
396 PMA in ddPCR caused a shift in the emitted fluorescence spectrum. However, an accurate
397 categorization of the droplets into positive and negative clusters was still achieved with the 2-
398 dimensional plot of the droplet-emitted fluorescence using two detection channels. As such,
399 potential errors stemming from exDNA can be significantly reduced or eliminated with PMA
400 also in single-cell methods where individual cells and exDNA are trapped within the same
401 compartment.

402 Production of paired concatemer requires a special primer design that allows
403 amplification and fusion of two physically distant genetic regions. The here-employed protocol
404 includes four primers for amplifying both 16S rRNA and target *bla* regions that are then
405 combined with a fusion primer. Four-primer setup changes the reaction dynamics compared to

406 the three-primer protocol utilized in comparable methods such as epicPCR[12]. In the three-
407 primer protocol, the production of fused concatemer is dependent on the presence of the target
408 gene; in case the trapped bacterial cell does not carry it, neither the 16S rRNA gene nor fusion
409 amplicons are produced. The addition of a fourth primer (in this case, 27F) not only enables
410 the production of 16S rRNA gene fragments of noncarrier cells, but also increases the yield of
411 the fusion product (data not shown). Higher outer primer concentration (compared to inner
412 primers) may enhance the production of fusion concatemer[23]. Thus, we tested several primer
413 ratios and total concentrations in order to achieve the most efficient production of *bla*_16S
414 concatemer. The results showed that the best fusion concatemer production and droplet
415 separation was obtained when outer primer concentrations were several times higher compared
416 to that of inner primers (forward to reverse primer ratios 10:1 for *bla* and 1:5 for 16S rRNA
417 primers, where *bla* forward and 16S rRNA reverse primers acted as outer primers). The
418 application of the here-described system for alternative genetic targets may require
419 optimization of primer concentrations and ratios in order to enhance amplification for better
420 DNA yield and for allowing sufficient separation of negative droplets from positive ones in the
421 subsequent fluorescence analysis.

422 In this method, the droplets are generated with a microfluidic device that produces
423 equal-sized droplets with a volume of one nanolitre. Bacterial cells are encapsulated into oil-
424 surrounded droplets during droplet generation. The accuracy of ddPCR to quantify DNA
425 concentrations assumes that targets are randomly distributed into droplets of equal size. In the
426 presented approach, individual cells (instead of gene copies) serve as the quantifiable unit. As
427 such, we first assured the applicability of single-cell ddPCR individually for *E. coli, P.*
428 *fluorescens, K. pneumoniae* and *Salmonella* Typhimurium. To investigate the capability of the
429 method to differentiate *bla*-carriers from non-carriers, *E. coli bla*-carriers were mixed with non-
430 carriers in different ratios and combinations. These mixtures were immediately divided into

431 droplets and the droplets were transferred to a thermal cycler. We employed a prolonged initial
432 denaturation at 95 °C in the ddPCR thermal cycling protocol to ensure cell lysis and
433 accessibility of PCR reagents to the released DNA. As noted above, during PCR, the four
434 primers enable the production of both fusion concatemer from carrier cells and the sole 16S
435 amplicon from non-carriers. As fluorescence is measured after PCR from each droplet one by
436 one, each cell-containing droplet can be detected and hence the ratio of positive to negative
437 droplets can be quantified. The advantage of the QX200 system is that, in addition to its
438 capability to produce uniform droplets, it also inspects the droplet shape and size and excludes
439 abnormal droplets from the analysis. In comparable methods, such as in epicPCR[12] and OIL-
440 PCR[24], partitions of various sizes are formed, hence producing compartments with various
441 ratios of PCR reagents to the target DNA. This, in turn, may result in notably differing amounts
442 of amplified DNA that are produced from a single target cell. As such, the here-described
443 method can provide also a more reliable proxy of the number of positive targets in the initial
444 sample, not only their diversity. Further, Poisson distribution is used to select a suitable number
445 of cells to be loaded into a single ddPCR reaction. In other words, a cell concentration that
446 statistically should enclose only a single cell in a droplet while most droplets remain cell-free
447 is used to seed the droplet generation. We confirmed single-cell resolution by parallel single-
448 cell 16S ddPCR to reveal the potential maximum number of positive droplets produced from
449 the sample and proceeded with only those samples that had a target concentration within the
450 confidence interval.

451 After single-cell ddPCR, the DNA is recovered from all droplets in a single step, thus
452 mixing their contents. At this point, the overall DNA concentration is typically extremely low
453 and needs downstream amplification and barcoding steps to enable amplicon sequencing. The
454 four-primer setup produces single 16S rRNA gene products that can serve as a primer in the
455 subsequent processing and hence may result in false positive host sequences. To prevent cross-

456 binding of fusion amplicons and 16S rRNA products originating from distinct droplets, we
457 used blocking primers in the library preparation steps as described by Spencer and colleagues[12].
458 An excessive concentration of blocking primers binding to both strands of the 27F region was
459 used to prevent the amplification to start from the 16S rRNA region (*i.e.* in the middle of fusion
460 product, single 16S rRNA gene amplicon or genomic DNA), as in this phase, only fused
461 concatemers are to be enriched. Sequencing of full-length fusion amplicons from mixed cell
462 samples revealed that the vast majority of *bla* host sequences belonged to *E. coli*, which was
463 the only true host carrying the resistance gene. To conclude, our results suggest that the steps
464 for eliminating signals that would potentially cause biased host diversity were sufficient to
465 diminish the false host sequences. However, it should be noted that especially in the case of
466 unknown complex communities, the extremely rare hosts of the target functional gene may be
467 shadowed by the background 16S rRNA gene sequences.

468 To demonstrate its applicability, we utilized the single-cell ddPCR method for tracing
469 the horizontal transfer of conjugative resistance plasmid pEC13 within a simple synthetic
470 microbial community consisting of cephalothin resistant (rifampicin sensitive) *E. coli* (carrying
471 pEC13 with *bla*CTX-M-14) and rifampicin resistant (cephalothin sensitive) strains of *P.*
472 *fluorescens, K. pneumoniae* and *Salmonella* Typhimurium. The communities were exposed to
473 three different treatments: 1) cephalothin and after 1 h 30 min to rifampicin, 2) simultaneously
474 to cephalothin and rifampicin or 3) rifampicin and after 1 h 30 min to cephalothin. *E. coli*
475 (pEC13) was introduced 30 min after the first antibiotic exposure. Carriers of *bla*CTX-M-14,
476 and thereby the potential evolutionary rescue via pEC13, were identified with the single-cell
477 ddPCR. When exposed to cephalothin, the susceptible synthetic population was expectedly
478 diminished. Further, after the introduction of *E. coli* with a potential to rescue its accompanying
479 bacteria with the resistance-conferring plasmid, new transconjugant hosts did not seem to
480 appear in the community. In contrast, when rifampicin was administrated as the first antibiotic,

481 the initial resistant population was restored, allowing the spread of rescuing pEC13 plasmid
482 into new hosts before its original host (susceptible to rifampicin) was eliminated from the
483 population. The second antibiotic exposure to cephalothin selects for those bacteria that took
484 in the resistance plasmid and were mainly identified as *K. pneumoniae.* This is in accordance
485 with our previous work where pEC13 was shown to rescue *K. pneumoniae* cells even under
486 lethal cephalothin concentration[18]. The simultaneous exposure to both antibiotics is lethal to
487 all bacterial strains in the community, and therefore assumed not to give enough time for
488 conjugation events. The plasmid host population from this treatment was drastically
489 inconsistent between biological replicates, suggesting that the observed results were likely
490 biased by dead cells dominating the bacterial sample. Moreover, *Comamonas* was not observed
491 to host the pEC13 plasmid, however it was over-represented in some samples, suggesting that
492 certain bacterial behavior, such as secretion of extracellular vesicles, may have significant
493 effects on sequencing results when viable host cells are rare in the community. Nonetheless,
494 these results show that the method can be utilized for identifying the plasmid-carrying sub-
495 community, which itself is shaped by the used antibiotics as well as the resistance phenotypes
496 present in the initial community. Hence, the method has potential for future applications *e.g.,*
497 for studying plasmid-host interactions in more complex microbial communities, such as the gut
498 or soil microbiota – yet it must be acknowledged that different sample types may require further
499 pre-processing steps.
500 In summary, we have outlined a ddPCR-based protocol that enables both counting the
501 number of bacterial cells as well as providing a phylogenetic assignment of functional genes
502 or extrachromosomal elements (plasmids) to their hosts. Available in many molecular biology
503 laboratories, the ddPCR system offers a valuable droplet microfluidic device for high-
504 throughput genomic research of microbial communities.
505

506 **MATERIAL AND METHODS**

507 ***Bacterial strains and cultivation***

508 Bacterial strains with and without the target beta-lactam resistance gene were used in the
509 development of the gene tracking method presented in this study. *Escherichia coli* K-12
510 BL21Gold carrying the conjugative plasmid pEC13 with the target beta-lactamase gene
511 *bla*CTX-M-14[5] served as the antibiotic resistance gene carrier. *Klebsiella pneumoniae*
512 DSM681, *Salmonella enterica* serovar Typhimurium SL5676, *Pseudomonas fluorescens*
513 (ATCC 13525) and *Comamonas* sp. 4B43 (originating from a human fecal sample) were used
514 as noncarriers. The bacteria were cultivated in Luria Bertani Lennox-broth (LB)[25], *E. coli, S.*
515 Typhimurium and *K. pneumoniae* at +37˚C with 120 or 220 rpm shaking, and *P. fluorescens*
516 at room temperature (RT) with 150 rpm. LB-agar (1%) plates were used for quantifying
517 bacterial densities from liquid cultures. *E. coli, S.* Typhimurium and *K. pneumoniae* plates
518 were incubated overnight at +37˚C and *P. fluorescens* 2 days at RT. Media for *E. coli* was
519 supplemented with ampicillin (150 µg/ml) in order to select for pEC13 plasmid.

520 ***ddPCR reactions***

521 The QX200™ Droplet Digital™ PCR system (ddPCR, Bio-Rad Laboratories, Inc.) was used
522 to generate droplets and read the results following the manufacturer's instructions. The
523 reactions were run in C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Inc) with 96
524 Deep-Well Reaction Module. The ddPCR reactions were kept overnight at 12 ˚C before reading
525 the droplets. The results were obtained from channels 1 (FAM) and 2 (HEX/VIC) and analysed
526 with QuantaSoft™ Analysis Pro 1.0 (Bio-Rad Laboratories, Inc). All ddPCR reactions were
527 prepared with QX200™ ddPCR™ EvaGreen® Supermix (2x).

528 ***16S rRNA gene amplification***

529 The amplification of the 16S rRNA gene was done using universal primers 27F and 338R (the
530 primers used in this study are listed in Table 1). After initial optimization of the 16S rRNA

531 primers for ddPCR (see Supplementary Figure S2 for more information), the reactions were
532 prepared using 1x ddPCR supermix and 100 nM of each primer. The amplification conditions
533 consisted of initial incubation at 95 ˚C for 10 minutes, followed by 40 cycles of denaturation
534 at 95 ˚C for 60 seconds, annealing at 55˚C for 60 seconds and elongation at 72 ˚C for 2 minutes,
535 and a final step for fluorescence stabilization with incubation at 4 ˚C for 5 minutes followed
536 by incubation at 90 ˚C for 5 minutes. The ramp rate between steps was 2 ˚C/sec until the last
537 elongation incubation, and 1 ˚C /sec for the fluorescence stabilization. Additionally to the
538 fluorescence analysis by the BioRad droplet reader, a subset of droplets was also imaged under
539 a fluoresence microscope (Zeiss Axio Vert.A1, with Leica MC170HD camera and Zeiss Filter
540 set 09 including Excitation Bandpass Filter 450-490 nm, Emission Longpass Filter 515 nm and
541 Beamsplitter FT 510) to manually assess the difference in fluorescence (Supplementary Figure
542 S4C-D). The produced droplets and the delivery of viable bacterial cells into droplets were
543 inspected under a bright field microscope (Zeiss Axio Vert.A1, with Leica MC170HD camera)
544 (Supplementary Figure S4A-B).

545 ***Propidium monoazide (PMA) for sample treatment***

546 Initially, the maximum non-inhibitory concentration (MNIC) of propidium monoazide (PMA)
547 in ddPCR reaction was tested. PMA (PMAxx™, Biotium) was diluted in PCR grade water to
548 10 µM of final concentration and photoactivated. The treatment consisted of initial incubation
549 in the dark for 10 minutes, followed by incubation for 10 minutes under blue LED light (460
550 nm). The solution was prepared in 500 µl thin-walled polypropylene Qubit™ assay tubes
551 (Invitrogen™) with final volume of 200 µl. Samples were kept on ice layer and mixed
552 occasionally during the treatment by inverting the tube. Lower concentrations were prepared
553 from the treated PMA solution and used in ddPCR, with final concentration ranging from 0.125
554 to 2.5 µM. *E. coli* DNA digested with FastDigest HindIII and NotI (Thermo Fisher Scientific,
555 MA, USA) was used as a template in ddPCR reactions optimized for 16S rRNA. The ddPCR

556 was prepared using 100 nM of each universal primer (27F and 338R) for 16S rRNA
557 amplification. The amplification program was carried out using 5 minutes of initial incubation
558 at 95 ˚C, and 40 cycles of denaturation at 95 ˚C for 30 seconds, annealing at 55 ˚C for 60
559 seconds and elongation at 72 ˚C for 2 minutes. The final fluorescence stabilization and ramp
560 rate were the same as mentioned above.

561 Next, cell samples prepared from *E. coli* and *P. fluorescens* cultures were treated with
562 PMA and used as template in ddPCR. Bacterial suspensions were freshly prepared for every
563 new assay by diluting an overnight culture up to 1000-fold in 1x phosphate buffer solution
564 (PBS). The putative inhibitory effect of PBS (from concentration ranging from 0.015625x to
565 0.25x of PBS) on ddPCR efficiency was determined during the initial optimization of reaction
566 conditions for 16S rRNA primers (Supplementary Figure S1). After determining the maximum
567 non-inhibitory PBS concentration, it was always kept under 0.0625x in the ddPCR reactions.
568 The filtrate was obtained by individually centrifuging 250 µl of each dilution at 5000 × *g* for 3
569 minutes through a 0.2 µm spin filter (Centrifugal Filter, Modified Nylon 0.2 µm, VWR). Both
570 sample types, cell suspension and filtrate, were treated with 10 µM of PMA as described above.
571 After PMA treatment, the cells were diluted in PCR grade water and used as template in ddPCR
572 reaction using 16S rRNA primers. The same reaction conditions used for the MNIC assay were
573 used here. The cell density of the samples was determined by serial dilution and plate
574 cultivation of cell suspensions, before and/or after PMA treatment.

575 ***One-step fusion PCR for concatemer production***

576 Specific primers were designed to detect the pEC13 plasmid and prompt the bla_16S
577 concatemer formation. The plasmid sequence was retrieved from the GeneBank database under
578 accession number KU932024.1. The class A beta-lactamase gene (*bla*CTX-M-14) present in
579 the plasmid was the target sequence chosen and here referred to as *bla*. Primers were designed
580 using the online Primer Designer™ Tool (Thermo Fischer Scientific). To amplify a 101-bp *bla*

581 fragment, 293F was used as a forward primer together with a modified reverse primer rc27F-
582 bla_320R (Table 1). It contains *bla* reverse primer part (320R) and 5'-overhang encoding the
583 reverse complement sequence of 16S rRNA forward primer 27F. A fusion primer enables the
584 production of the 450-bp concatemer via the attachment of the amplified *bla* fragment on its
585 27F-overhang to 16S rRNA fragment (350 bp PCR product amplified by primers 27F and
586 338R) in the same reaction (Figure 2). Initially, both primer pairs (for amplifying *bla* and 16S
587 fragments) were screened separately for forward to reverse ratio and primer pair concentration
588 in ddPCR (Supplementary Figure S3). Reactions were prepared with up to a 100-fold
589 difference between forward and reverse primer molarity, and total concentration ranging from
590 100 to 400 nM. After the initial screening, both primer pairs were used in the same reaction to
591 produce the concatemer. The total concentration of 16S rRNA primers was fixed to 225 nM
592 and the ratio of forward to reverse to 1:5. *bla* primers were used in concentrations ranging from
593 100 to 300 nM and ratios from 10:1 to 30:1 (Supplementary Figure S3C-E). Finally, the primer
594 ratios and concentrations were defined for single-cell experiments. The further reactions for
595 concatemer production were prepared with 225 nM and a ratio of 1:5 (forward to reverse) of
596 16S rRNA primers, and 200 nM and 10:1 (forward:reverse) of *bla* primers.

597 ***Single bacterial cells in droplets***

598 To test whether single bacterial cells are suitable to be used as templates in ddPCR, overnight
599 cultures of *E. coli, P. fluorescens, K. pneumoniae,* and *S.* Typhimurium were diluted to reach
600 a cell density of approximately 1×10^7 cfu/ml (cfu; colony forming units). Bacterial dilutions
601 were PMA-treated as described above with the final PMA concentration of 10 µM. A dilution
602 series of 1:2 was prepared (in PCR grade water) from the PMA-treated cell samples and used
603 as a template in ddPCR. The bacterial cell suspension always constituted ¼ of the total ddPCR
604 reaction volume. The maximum detectable cell number was determined by using 16S rRNA
605 primers 27F and 338R. The corresponding bacterial cell density of the samples was also

606 determined by dilution series and plating on LB plates. The protocol optimized for the
607 amplification of the 16S rRNA gene in ddPCR and specified in the section above was used.

608 ***Detection of beta-lactamase host bacteria from mixed samples with single-cell ddPCR***

609 Overnight cultures of *E. coli, K. pneumoniae,* and *S.* Typhimurium were diluted for PMA-
610 treatment and a 1:2 dilution series was prepared as described above. Diluted bacterial samples
611 containing either one bacterial strain or a mix of two strains: beta-lactamase -carrying *E. coli*
612 and a non-carrier strain *K. pneumoniae* or *S.* Typhimurium was used as template in ddPCR.
613 Both 16S rRNA (amplified with 27F and 338R) and concatemer bla_16S (amplified with four
614 primers; 293F, rc27F-320R, 27F and 338R) were amplified from the same samples in separate
615 reactions. Two replicates were prepared for reading the droplets from the 16S rRNA reaction,
616 and three replicates were prepared for the one-step fusion reaction to be saved and used in the
617 following procedures. The ratio of positive:negative droplets was determined with the Droplet
618 Reader and the samples containing a sufficient number of positive droplets according to 16S
619 rRNA amplification (and therefore verified to contain the cell density required for single cell
620 analysis) were proceeded to DNA recovery. The amplification conditions were the same as in
621 the protocol optimized for 16S rRNA gene amplification in ddPCR (described in the specific
622 section above).

623 ***DNA recovery from droplets and library preparation for sequencing***

624 The amplified *bla*_16S concatemers were recovered from droplets and enriched before
625 preparing the sequencing libraries. The recovery was done by freeze-thawing the ddPCR
626 emulsion using liquid nitrogen (LN2), a method we described recently[17]. After freeze-thawing,
627 phase separation was achieved, and the amplicon-containing phase was collected for further
628 processing. The recovered concatemers were purified with the Agencourt AMPure XP
629 purification system (Beckman Coulter, Brea, CA) using a 0.9x ratio.

630 Sample enrichment was performed before library preparation by reamplifying the
631 recovered *bla*_16S amplicons with primers M13_bla.2_F and 338R (Table 1). The samples
632 were then barcoded with bc_M13 and bc_338R primers, both extended with unique 5'-barcode
633 sequences (bc). Both enrichment and barcoding qPCR reactions were prepared with 1x Maxima
634 SYBR Green/Fluorescein qPCR Master Mix (ThermoScientific™) in a final volume of 25 µl.
635 To prevent the undesired pairing of bla-27F overhang with other 16S rRNA fragments that
636 were present in the sample, the reactions included blocking primers block_27F and block_27R,
637 both in a final concentration of 1 µM. The product of barcoding qPCR was cleaned by gel
638 extraction using NucleoSpin Gel and PCR clean-up kit (MACHEREY-NAGEL, GmbH & Co,
639 KG, Germany), after 1% AGE (agarose gel electrophoresis) run for 1 hour at 100 V. The
640 fragment was further analysed with High Sensitivity D1000 ScreenTape assay in 2200
641 TapeStation System (Agilent Technologies, Inc, CA, USA). PCR conditions for enrichment
642 and barcoding reactions were as follows: the amplification run started with pre-incubation at
643 95 ºC for 5 minutes, followed by 15 cycles of denaturation at 95 ºC for 30 seconds, annealing
644 at 53 ºC for 30 seconds, and elongation at 72 ºC for 30 seconds (with plate read after each
645 cycle). After the run, the product was analysed in a 1.5 % AGE run for 1 hour at 110V. The
646 barcoded and purified *bla*_16S amplicons were proceeded to library preparation with
647 NEBNext® Ultra™ II DNA Library Prep Kit and sequenced with Illumina NovaSeq 6000
648 platform using SP flowcell (PE250). Further sequence analysis of 250-bp paired-end read data
649 was run with CLC Genomics Workbench 12 (Qiagen Bioinformatics). The obtained reads were
650 trimmed with *bla* and 16S rRNA primer sequences bla_293F, bla_320R, 27F and 338R to
651 confirm that they contain both parts of the concatemer. The genetic region between 27F and
652 338R was extracted and used for *de novo* OTU clustering with a 97 % similarity threshold.
653 Rare OTUs (abundance <100 reads) were excluded from the analysis. The reference sequence
654 of each OTU was exposed to BLAST search, using the 16S Ribosomal RNA sequences

655 database. Each OTU was identified according to the BLAST results. Finally, the abundance of
656 identified bacterial hosts carrying *bla*CTX-M-14 present in each sample was determined.

657 ***Horizontal transfer of beta-lactamase under lethal antibiotic exposure***

658 Rifampicin-resistant strains of *K. pneumoniae, S.* Typhimurium and *Comamonas* sp. (bacterial
659 isolate originating from the human fecal sample as part of gut bacterial strain library collected
660 under research permit T300/2020 for Hospital District of Southwest Finland) were used to
661 construct a synthetic bacterial community. 5 µl of each bacterial culture, cultivated overnight
662 in LB medium without antibiotics, were added to 5 ml of fresh LB medium containing 1)
663 cephalothin 2) rifampicin and cephalothin or 3) rifampicin, with four replicates per treatment.
664 The bacterial communities were incubated at +37 ºC for 30 min with 120 rpm agitation, after
665 which *E. coli* harboring conjugative plasmid pEC13 with *bla*CTX-M-14 (conferring resistance
666 to cephalothin) was introduced by adding 5 µl of overnight grown culture into each bacterial
667 community. After 1 h incubation, a second antibiotic pulse was given to treatment 1 with
668 rifampicin and treatment 3 with cephalothin, resulting in the same final antibiotic concentration
669 in all the treatments (50 µg/ml of both cephalothin and rifampicin). The bacterial communities
670 were incubated for 3.5 h after which one cell sample from each community was taken for single
671 cell analysis for tracing the *bla*CTX-M-14 carrier cells.

672 Cell samples from each community were PMA-treated and diluted as described earlier.
673 *bla*_16S concatemers were produced by single-cell ddPCR. Each reaction was run in two
674 replicates. One reaction was read with Droplet Reader to ensure cell density and single-cell
675 resolution. The other replicate was used for DNA recovery with LN2. Recovered fusion
676 amplicons were first purified with QIAquick PCR purification kit (Qiagen) and proceeded to
677 library preparation first with re-amplification PCR (PCR conditions: 95 ºC 5 min, followed by
678 30 cycles of denaturation at 95 ºC for 30 seconds, annealing at 53 ºC for 30 seconds, and
679 elongation at 72 ºC for 30 seconds, with plate read after each cycle) followed by PCR

680 purification (sparQ PureMag Beads, Quantabio) and inspecting the correct amplicon size with
681 AGE (1.5%, 30 min, 120 V). Purified fusion amplicons were barcoded with both 5' and 3'
682 extensions in barcoding PCR (PCR conditions as described above but with 9 cycles). Barcoded
683 amplicons were extracted from agarose gel (1.5%, 1 h, 100 V) by using NucleoSpin® Gel and
684 PCR Cleanup XS kit (Macherey-Nagel) and the amplicon size and DNA concentration were
685 checked with High Sensitivity D1000 ScreenTape assay in 2200 TapeStation System (Agilent
686 Technologies, Inc, CA, USA). Sequencing libraries were prepared with NEBNext® Ultra™ II
687 DNA Library Prep Kit and the samples were sequenced with Illumina NovaSeq 6000 platform
688 using SP flowcell (PE250).

689 Further sequence analysis of 250-bp paired-end read data was run with CLC Genomics
690 Workbench 21 (Qiagen Bioinformatics) and Microbial Genomics module. Paired reads were
691 first merged and trimmed by quality (0.05 as threshold). Reads were then trimmed with forward
692 and reverse primer sequences of *bla*CTX-M-14 to ensure they contain the complete sequence
693 of the target gene, and then with 16S forward primer. Single reads and merged read sequences
694 not covering the complete fusion amplicon length were discarded from the analysis. Finally,
695 the remaining sequence between 16S rRNA forward (27F) and reverse (338R) primer binding
696 sites was used to determine the carriers of the beta-lactamase gene. Only the reads that
697 contained all the primer sequences and that had a length of 300-315 bp (after trimming) were
698 taken to the final analysis. A local 16S database was constructed to represent the synthetic
699 community (consisting of *K. pneumoniae, S.* Typhimurium, *Comamonas* sp. and *E. coli* 16S
700 rRNA gene sequences) and used for reference-based OTU clustering with 99% similarity,
701 allowing the creation of new OTUs (99% taxonomy similarity). OTUs with a total abundance
702 lower than 4000 or recognized as chimeras were left out from the final analysis. The
703 subpopulation carrying *bla*CTX-M-14 was assessed as OTU frequency (OTU abundance/total
704 OTU abundance).

705

706 **Table 1. Primers used in this study.**

Primer name	Sequence 5'-3'	Target
27F[26]	AGAGTTTGATCMTGGCTCAG	16S rRNA forward primer
338R[27]	TGCTGCCTCCCGTAGGAGT	16S rRNA reverse primer
bla_293F	GAAACGTCTCATCGCCGATC	beta-lactamase forward primer
rc27F-bla_320R	CTGAGCCAKGATCAAACTCTGCAGTACAGCGACAATACCG	fusion primer; beta-lactamase reverse primer with 16S rc27F primer
M13_bla.2_F	TGTAAAACGACGGCCAGTCGAGCTGGGCAATCAATTTG	M13 adapter attachment and bla shortening
M13_27F	TGTAAAACGACGGCCAGTAGAGTTTGATCMTGGCTCAG	M13 adapter
27F_block	TTTTTTTTTTAGAGTTTGATCMTGGCTCAG/3SpC3/	blocking primer
rc27F_block	TTTTTTTTTTCTGAGCCAKGATCAAACTCT/3SpC3/	blocking primer
bc_M13F	[bc]TGTAAAACGACGGCCAGT	barcoding primer
bc_338R	[bc]TGCTGCCTCCCGTAGGAGT	barcoding primer

rc = reverse complementary; bc = barcode sequence

707

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712

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714

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794 **Supplementary Data**

795 **Single-cell dissection of bacterial communities into functional gene carriers with ddPCR**

796 **by** Lara Ambrosio Leal Dutra, Matti Jalasvuori, Ole Franz, Kimi Nurminen, Pauliina Salmi,

797 Marja Tiirola and Reetta Penttinen

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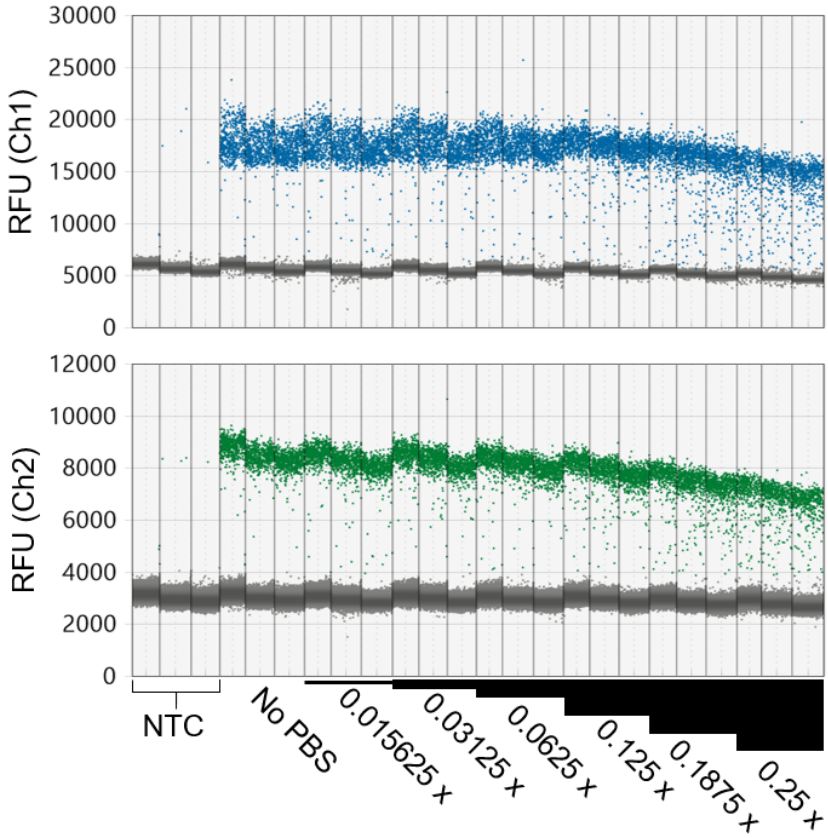


799

800 **Supplementary Figure S1. Inhibitory effect of PBS in ddPCR reaction.** PBS dilutions were

801 added to ddPCR reactions to detect inhibition of amplification. The amplification of 16S rRNA

802 using the ddPCR reaction optimized in this study was used to demonstrate the effect of PBS in

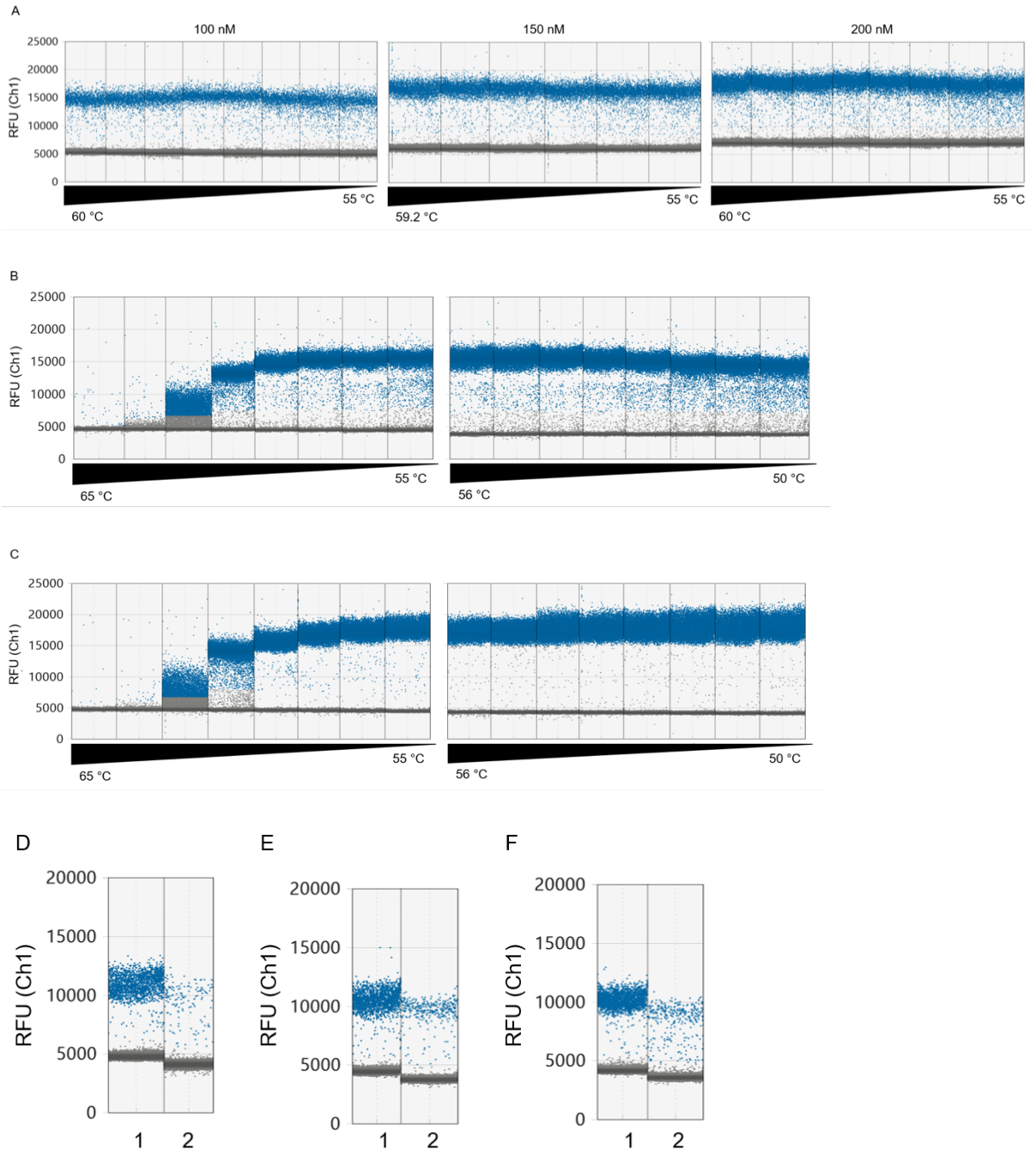
803 ddPCR. *E. coli* DNA was used as a template in the reactions and PBS concentrations ranging

804 from 0.0156x to 0.25x were tested. After the amplification, the RFU (relative fluorescence

805 units) of each droplet was analysed from channels 1 (Ch1; FAM) and 2 (Ch2; HEX/VIC). The

806 amplitude of positive clusters reduced with increasing concentrations of PBS, and more

807 droplets were observed to form the rain effect in reactions with 0.125-0.25x of PBS. These
808 effects could be seen from both channels. PBS concentration of 0.0625x was set as the
809 maximum non-inhibitory concentration for ddPCR reactions.
810



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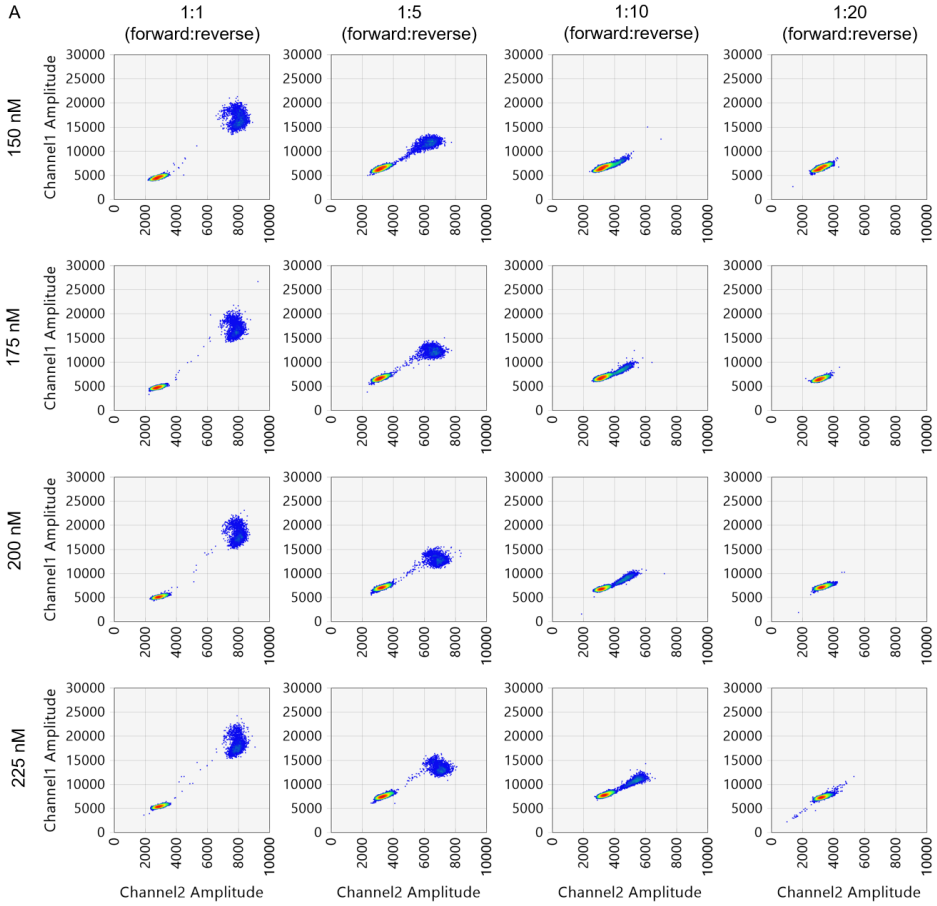
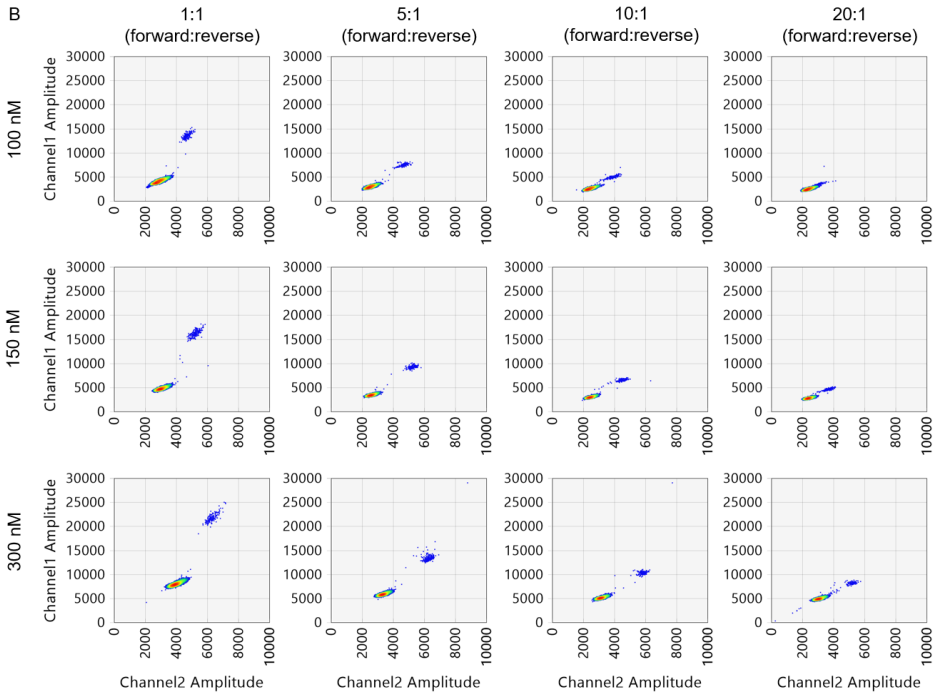
815 **Supplementary Figure S2. Optimization reactions for 16S rRNA amplification in ddPCR**
816 **using universal primers**. The amplification of 16S rRNA using universal primers 27F and

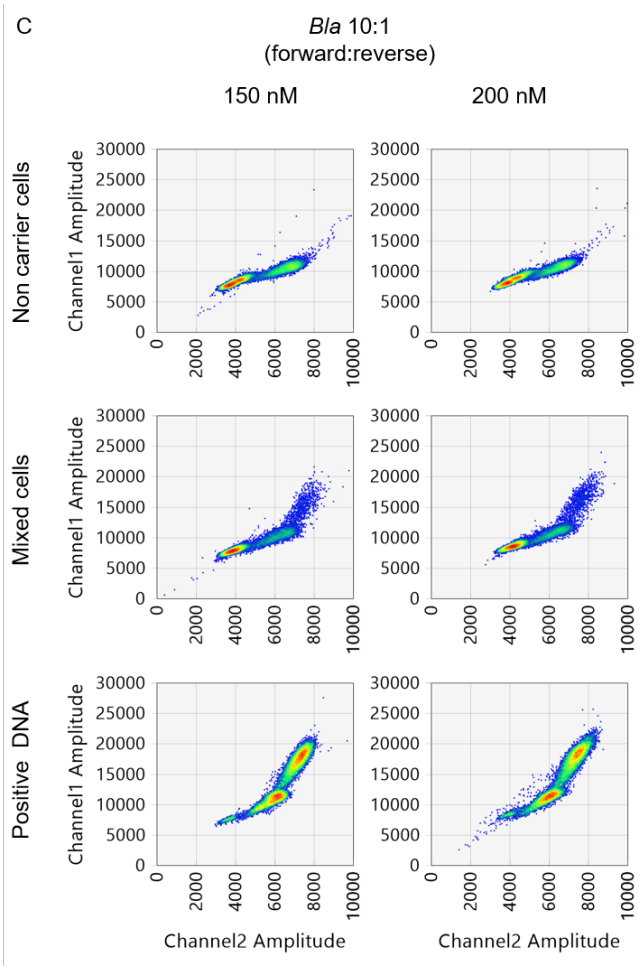
817 338R was optimized for ddPCR by testing primer concentration, annealing temperature,
818 duration of initial incubation and the cycling steps. Initially, three primer concentrations (100
819 nM, 150 nM, 200 nM) were tested in a cycling protocol using an annealing temperature
820 gradient between 60 and 55 °C (A). Heavy "rain effect", *i.e.* droplets' fluorescence not
821 clustering with positive nor negative droplets, was observed in higher primer concentrations
822 and lower annealing temperature. Cycling protocol with two (denaturation and annealing) (B)
823 or three steps (denaturation, annealing, and elongation) (C) were tested *versus* annealing
824 temperature gradients from 65 to 55 °C and 56 to 50 °C. The rain effect was more pronounced
825 in two-step cycles and lower temperatures. Duration of initial denaturation and cyclic
826 denaturation steps were tested using *P. fluorescens* (1) and *E. coli* (2) cell suspensions to
827 optimize the detection of single cells. Initial denaturation at 95 °C for 5 minutes (D), 10 minutes
828 (E) and 95 °C for 5 minutes with a longer cyclic denaturation (1 minute at 95 ° C) (F). The
829 positive droplets formed a narrower cluster in reactions using either longer initial (E) or longer
830 cyclic denaturation (F), when compared with the standard protocol (D). The rain effect
831 decreased in longer initial denaturation. In combination with these results, the following
832 settings were adopted for further assays: long initial incubation (10 minutes at 95 °C); three-
833 step cycling assay with long denaturation (1 minute at 95 °C), annealing at 55 °C (1 minute),
834 and elongation at 72 °C for 2 minutes.
835


A
1:1
(forward:reverse)
1:5
(forward:reverse)
1:10
(forward:reverse)
1:20
(forward:reverse)
150 nM
175 nM
200 nM
225 nM
Channel1 Amplitude
Channel2 Amplitude
0
5000
10000
15000
20000
25000
30000
2000
4000
6000
8000
10000

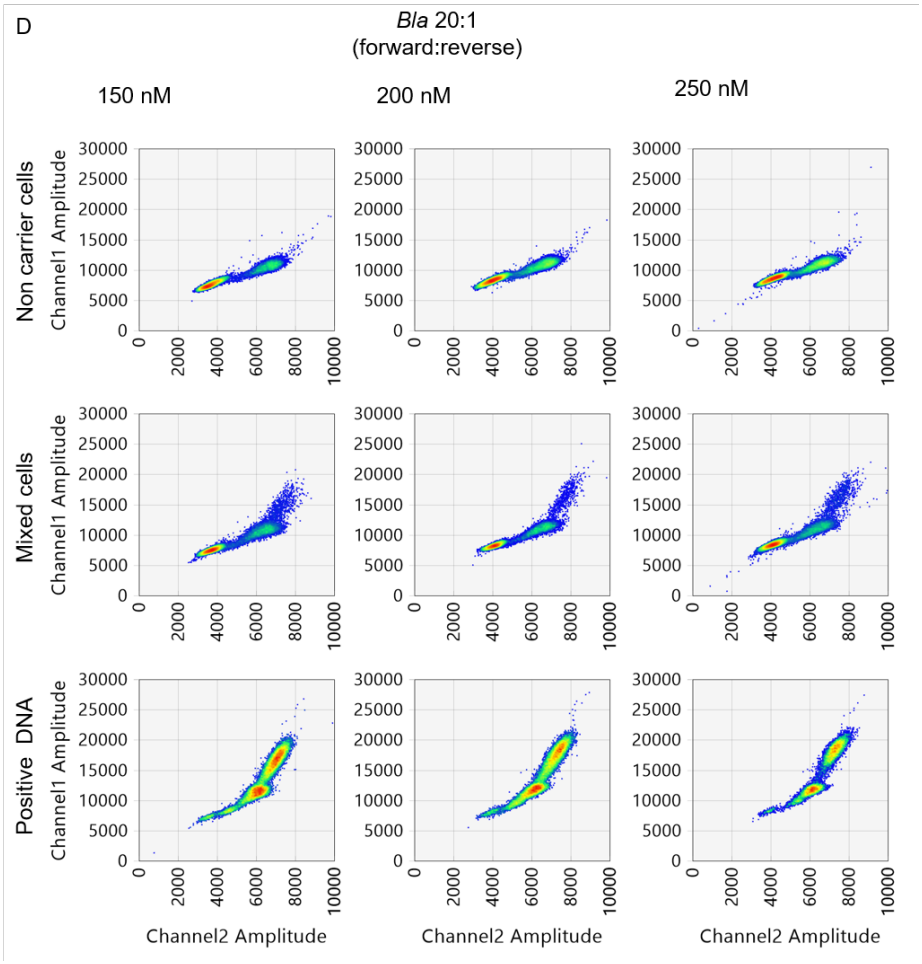
836


B
1:1
(forward:reverse)
5:1
(forward:reverse)
10:1
(forward:reverse)
20:1
(forward:reverse)
100 nM
150 nM
300 nM
Channel1 Amplitude
Channel2 Amplitude
0
5000
10000
15000
20000
25000
30000
2000
4000
6000
8000
10000

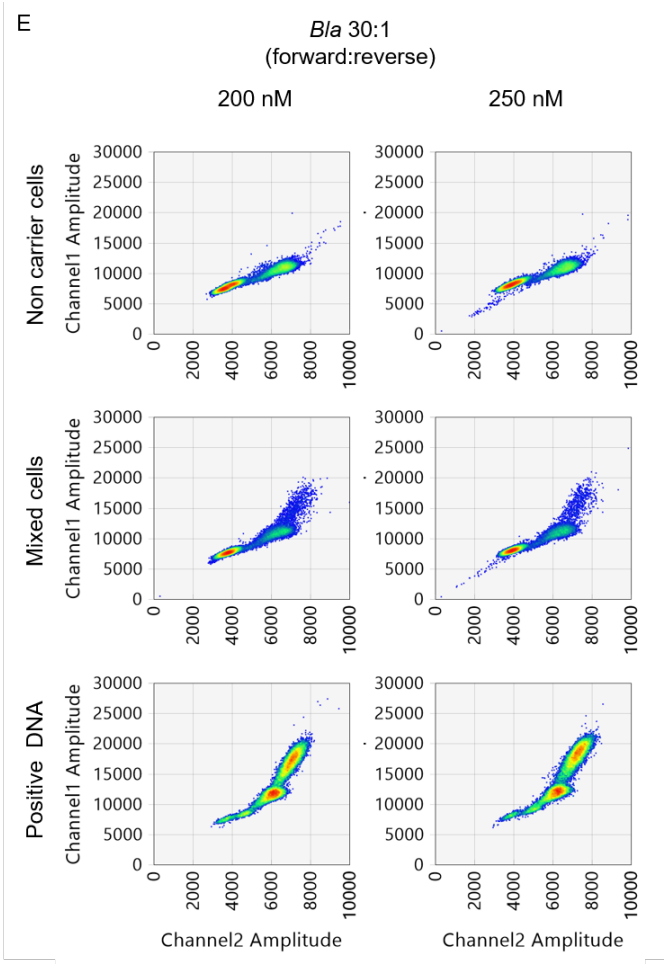
837


C
Bla 10:1
(forward:reverse)
150 nM
200 nM
Non carrier cells
Mixed cells
Positive DNA
Channel1 Amplitude
Channel2 Amplitude
0
5000
10000
15000
20000
25000
30000
0
2000
4000
6000
8000
10000

838


D
Bla 20:1
(forward:reverse)
150 nM
200 nM
250 nM
Non carrier cells
Mixed cells
Positive DNA
Channel1 Amplitude
Channel2 Amplitude
0
5000
10000
15000
20000
25000
30000
2000
4000
6000
8000
10000

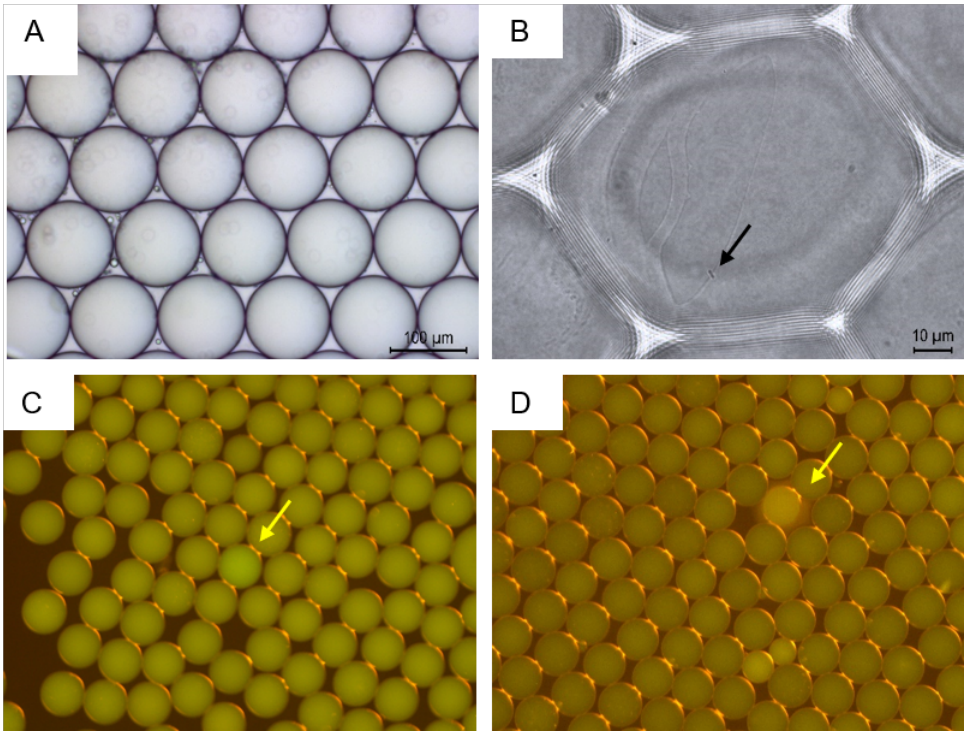
839



840

841 **Supplementary Figure S3. One-step fusion ddPCR optimization.** The fusion reaction to
842 generate the concatemer of 16S rRNA and *bla* was optimized in ddPCR by testing asymmetric
843 molarity of primers in pairs, and varying concentration of each pair. The ratio and concentration
844 were first screened separately for each pair, using *E. coli* DNA as a template. The fluorescence
845 of each droplet was analysed, and the formation of distinct clusters for positive and negative
846 droplets was checked for each sample. 16S rRNA primers were tested with concentrations
847 varying from 150 to 225 nM and ratios 1:1 to 1:20 (forward:reverse) (A). Primers for *bla* were
848 tested with concentrations of 100, 150 and 300 nM and ratio up to 20:1 (forward:reverse) (B).
849 After being tested in separate reactions, both primer pairs were used in the same ddPCR to
850 produce bla_16S concatemer. Bacterial cell suspension of *E. coli* carrying *bla, Pseudomonas*
851 *fluorescens* (non-carrier strain), and DNA was used as template in these reactions. The
852 reactions were prepared with a fixed (total) concentration (225 nM) and ratio of 1:5

853 (forward:reverse) of 16S rRNA primers. Selected concentrations of 150, 200 and 250 nM of
854 *bla* primers were used in one-step fusion ddPCR assay with forward:reverse ratios of 10:1 (C),
855 20:1 (D) and 30:1 (E).

856 

857 **Supplementary Figure S4. Droplet partitions generated for single-cell ddPCR.** Droplets
858 were generated with ddPCR system, single *E. coli* cells were delivered into droplets and imaged
859 using bright field microscopy (Zeiss Axio Vert.A1, with Leica MC170HD camera) (A-B). The
860 black arrow in panel B points to a bacterial cell within a ddPCR partition. Droplets prepared
861 using *E. coli* DNA as a template were subjected to single-cell 16S rRNA ddPCR and imaged
862 using fluorescence microscope (Zeiss Axio Vert.A1, with Leica MC170HD camera and Zeiss
863 Filter set 09 including Excitation Bandpass Filter 450-490 nm, Emission Longpass Filter 515
864 nm and Beamsplitter FT 510) (C-D). The yellow arrows point to droplets with the higher
865 fluorescence emission.

866

867

868