High-throughput Screening of Chemical Compounds to Elucidate Their Effects on Bacterial Persistence
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Corresponding Author:	Mehmet Orman UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	morman@central.uh.edu
Order of Authors:	Prashant Karki
	Mehmet Orman
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UNIVERSITY *of* **HOUSTON**

DEPARTMENT OF CHEMICAL AND BIOMOLECULAR ENGINEERING

Mehmet A Orman
Assistant Professor
4726 Calhoun Rd.
Engineering Build. 1/ S222
Tel: 713-743-6785
Email: morman@central.uh.edu

Dear Dr. Jaydev Upponni,

This is a resubmission of our manuscript (JoVE61597), entitled "High-throughput Screening of Chemical Compounds to Elucidate Their Effects on Bacterial Persistence". The method described here has been developed in our published study (doi: 10.1128/AAC.02393-19) where we showed the effects of osmolytes and environmental pH on *E. coli* persisters. We have proof-read and updated our manuscript to address all the concerns presented by the editor. In addition, we have uploaded a point-by-point description of the changes that have been made to address the editor's concerns.

We would like to thank the editor and reviewers for taking their time to review our manuscript. We look forward to hearing from you.

Sincerely,

Mehmet A Orman, PhD.

1   **TITLE:**
2   High-throughput Screening of Chemical Compounds to Elucidate Their Effects on Bacterial
3   Persistence
4
5   **AUTHORS AND AFFILIATIONS:**
6   Prashant Karki[1], Mehmet A. Orman[1]
7
8   [1]Department of Chemical and Biomolecular Engineering, University of Houston, Houston, TX
9
10 Corresponding Author:
11 Mehmet A. Orman   (morman@central.uh.edu)
12
13 Email Address of Co-Author:
14 Prashant Karki   (pkarki@uh.edu)
15
16 **KEYWORDS:**
17 persisters, high-throughput screening, phenotype microarrays, pH, osmolytes, antibiotics
18
19 **SUMMARY:**
20 In this method paper, we present a high-throughput screening strategy to identify chemical
21 compounds, such as osmolytes, that have a significant impact on bacterial persistence.
22
23 **ABSTRACT:**
24 Bacterial persisters are defined as a small subpopulation of phenotypic variants with the
25 capability of tolerating high concentrations of antibiotics. They are an important health concern
26 as they have been associated with recurrent chronic infections. Although stochastic and
27 deterministic dynamics of stress-related mechanisms are known to play a significant role in
28 persistence, mechanisms underlying the phenotypic switch to/from the persistence state are not
29 completely understood. While persistence factors triggered by environmental signals (e.g.,
30 depletion of carbon, nitrogen and oxygen sources) have been extensively studied, the impacts of
31 osmolytes on persistence are yet to be determined. Using microarrays (i.e., 96 well plates
32 containing various chemicals), we have designed an approach to elucidate the effects of various
33 osmolytes on *Escherichia coli* persistence in a high throughput manner. This approach is
34 transformative as it can be readily adapted for other screening arrays, such as drug panels and
35 gene knockout libraries.
36
37 **INTRODUCTION:**
38 Bacterial cultures contain a small subpopulation of persister cells that are temporarily tolerant to
39 unusually high levels of antibiotics. Persister cells are genetically identical to their antibiotic-
40 sensitive kins, and their survival has been attributed to transient growth inhibition[1]. Persister
41 cells were first discovered by Gladys Hobby[2] but the term was first used by Joseph Bigger when
42 he identified them in penicillin-treated *Staphylococcus pyogenes* cultures[3]. A seminal study
43 published by Balaban et al.[4] discovered two persister types: type I variants that are primarily
44 formed by passage through the stationary phase, and type II variants that are continuously

45 generated during the exponential growth. Persisters are detected by clonogenic survival assays,
46 in which culture samples are taken at various intervals during antibiotic treatments, washed, and
47 plated on a typical growth medium to count the surviving cells that can colonize in the absence
48 of antibiotics. The existence of persisters in a cell culture is assessed by a biphasic kill curve[4,5]
49 where the initial exponential decay indicates the death of antibiotic-sensitive cells. However, the
50 killing trend decreases over time, eventually leading to a plateau region which represents the
51 surviving persister cells.
52
53 Persister cells have been associated with various infectious diseases such as tuberculosis[6], cystic
54 fibrosis[7], candidiasis[8] and urinary tract infections[9]. Almost all microorganisms tested so far were
55 found to generate persister phenotypes, including highly pathogenic *Mycobacterium*
56 *tuberculosis*[6], *Staphylococcus aureus*[10], *Pseudomonas aeruginosa*[7] and *Candida albicans*[8]. Recent
57 studies also provide evidence of the rise of multidrug-resistant mutants from persister
58 subpopulations[11,12]. Substantial efforts in this field have revealed that persistence mechanisms
59 are highly complex and diverse; both stochastic and deterministic factors associated with the SOS
60 response[13,14], reactive oxygen species (ROS)[15], toxin/antitoxin (TA) systems[16], autophagy or self-
61 digestion[17] and ppGpp-related stringent response[18] are known to facilitate persister formation.
62
63 Despite significant progress in understanding the persistence phenotype, the effects of
64 osmolytes on bacterial persistence have not been fully understood. Since the maintenance of
65 optimal osmotic pressure is a necessity for cells' growth, proper functioning and survival, an in-
66 depth study of osmolytes could lead to potential targets for anti-persister strategies. Although
67 laborious, high-throughput screening is a very effective approach for identifying metabolites and
68 other chemicals that play a crucial role in the persistence phenotype[19,20]. In this work, we will
69 discuss our published method[19], where we have used microarrays, i.e., 96 well plates containing
70 various osmolytes (e.g., sodium chloride, urea, sodium nitrite, sodium nitrate, potassium
71 chloride), to identify osmolytes that significantly influence *E. coli* persistence.
72
73 **PROTOCOL:**
74
75 **1.   Preparation of growth medium, ofloxacin solution and *E. coli* cell stocks**
76
77 1.1.   Regular Luria-Bertani (LB) medium: Add 10 g/L of tryptone, 10 g/L of sodium chloride
78 (NaCl) and 5 g/L of yeast extract in deionized (DI) water. Sterilize the medium by autoclaving.
79
80 1.2.   LB agar plates: Add 10 g/L of tryptone, 10 g/L of NaCl, 5 g/L of yeast extract and 15 g/L
81 agar in DI water and sterilize the medium by autoclaving. At the desired temperature (~55 °C),
82 pour ~30 mL of agar medium into square plates (10 x 10 cm). Dry the plates and store at 4 °C.
83
84 1.3.   Modified LB medium: Add 10 g/L of tryptone and 5 g/L of yeast extract in DI water.
85 Sterilize the medium by autoclaving.
86
87 1.4.   Modified LB medium including an osmolyte: Mix 2x modified LB medium with a 2x
88 osmolyte solution in equal volumes. To prepare 2x modified LB medium, add 20 g/L of tryptone

89   and 10 g/L of yeast extract in DI water and sterilize by autoclaving. To prepare the 2x osmolyte
90   solution, dissolve the osmolyte of interest (2x amount) in DI water, and then filter sterilize the
91   solution.
92
93   NOTE: The osmolyte of interest and its concentrations can be determined from the Phenotype
94   Microarray screening assays (see step 4). However, the tested osmolyte and the associated 2x
95   concentration can be adjusted depending on the nature of the research. For example, to study
96   the impact of 1 mM sodium chloride, a 2x osmolyte solution would be a 2 mM sodium chloride
97   solution.
98
99   1.5.   Ofloxacin stock solution (5 mg/mL): Add 5 mg of ofloxacin (OFX) salt in 1 mL of DI water.
100 Add 10 µL of 10 M sodium hydroxide to increase the solubility of OFX in water, and then filter
101 sterilize the solution. Prepare aliquots and store at -20 °C.
102
103 NOTE: Ofloxacin is a quinolone antibiotic that has been widely used for both growing and non-
104 growing bacterial cells[14,21]. The minimum inhibitory concentration (MIC) of OFX for *E. coli*
105 MG1655 cells is within the range of 0.039–0.078 µg/mL[19,20]. Also note that other antibiotics such
106 as ampicillin and kanamycin are commonly used in persister research. The choice of antibiotics is
107 dependent on the nature of the study.
108
109 1.6.   *E. coli* MG1655 cell stocks: Inoculate 2 mL of regular LB medium with a single colony in a
110 14 mL test tube (snap-capped) and culture the cells in an orbital shaker at 250 rpm and 37 °C.
111 When the cells reach the stationary phase, mix 500 µL of the cell culture with 500 µL of 50%
112 glycerol (sterile) in a cryogenic vial and store at -80 °C.
113
114 **2.   Propagation of cells to eliminate pre-existing persisters**
115
116 2.1.   To prepare an overnight culture, scrape a small amount of cells from a frozen cell stock
117 with a sterile pipette tip (do not thaw the glycerol cell stock), and inoculate the cells in 2 mL of
118 modified LB medium in a 14 mL snap-capped test tube. Culture the cells in an orbital shaker at
119 250 rpm and 37 °C for 12 h.
120
121 2.2.   First propagation
122
123 2.2.1. After 12 h, transfer 250 µL of overnight culture to 25 mL of fresh modified LB medium in
124 a 250 mL baffled flask covered with a sterile aluminum foil.
125
126 2.2.2. Grow the cell culture in an orbital shaker at 250 rpm and 37 °C until cells reach the mid-
127 exponential phase ($OD_{600} = 0.5$).
128
129 2.2.3. Measure the optical density at 600 nm (OD_{600}) using a microplate reader every 30 min.
130
131 2.3.   Second propagation
132

133   2.3.1.   At OD_{600} = 0.5, dilute 250 μL of cell culture from the first flask into 25 mL of fresh modified
134   LB medium in a 250 mL baffled flask.

136   2.3.2.   Grow the second cell culture in an orbital shaker at 250 rpm and 37 °C until OD_{600}=0.5.

138   2.3.3.   Measure the optical density every 30 min.

140   NOTE: An overnight culture may have a significant amount of persister cells[4,5,22]. The
141   dilution/growth cycle method[5] described above can be used to eliminate these pre-existing
142   persisters before transferring the cells to microarrays. Their elimination can be validated by
143   quantifying persister levels of overnight cultures with the assay described in step 3. This method
144   has been already validated in a previous study[19].

146   **3.   Validating the elimination of pre-existing persister cells**

148   3.1.   After the second propagation (see step 2.3), dilute 250 μL of the cell culture (OD_{600} = 0.5)
149   in 25 mL of fresh modified LB medium in a 250 mL baffled flask.

151   NOTE: For controls, dilute 250 μL of overnight culture (see step 2.1) in 25 mL of fresh modified
152   LB medium in a 250 mL baffled flask. Before transferring the cells to the flask, the cell density of
153   the overnight culture should be adjusted in fresh modified LB medium to obtain OD_{600} = 0.5.

155   3.2.   Add 25 μL of OFX stock solution (5 mg/mL) into the cell suspension and shake the flask
156   gently to make the assay culture homogeneous. The final concentration of OFX is 5 μg/mL in the
157   assay culture.

159   3.3.   Incubate the assay culture in an orbital shaker at 250 rpm and 37 °C.

161   3.4.   At every hour during the treatment (including 0 h, the time point before adding OFX into
162   the assay culture), transfer 1 mL of the assay culture from the flask to a 1.5 mL microcentrifuge
163   tube.

165   3.5.   Centrifuge the assay culture in the microcentrifuge tube at 17,000 x g for 3 min.

167   3.6.   Carefully remove 950 μL of the supernatant without disturbing the cell pellet.

169   3.7.   Add 950 μL of phosphate-buffered saline (PBS) solution into the microcentrifuge tube.

171   3.8.   Repeat the washing steps 3.5-3.7 for 3x in total until the antibiotic concentration is below
172   the minimum inhibitory concentration (MIC).

174   3.9.   After the final wash, resuspend the cell pellet in 100 μL of PBS solution, resulting in a 10x
175   concentrated sample.

176

177   3.10.   Take 10 µL of the cell suspension and serially dilute six times in 90 µL of PBS solution using
178   a 96 well round bottom plate.

180   3.11.   Spot 10 µL of diluted cell suspensions on antibiotic-free fresh agar plates. To increase the
181   limit of detection, plate the remaining 90 µL of cell suspension on a fresh agar plate.

183   3.12.   Incubate the agar plates at 37 °C for 16 h, and then count the colony-forming units (CFUs).
184   Account for the dilution rates while calculating the total number of CFUs in 1 mL of assay culture.
185   Kill curves are generated by plotting the logarithmic CFU values with respect to the duration of
186   antibiotic treatment.

188   NOTE: A 6 h OFX treatment is sufficient to obtain a biphasic kill curve for *E. coli* cells[19,20]. The
189   persister level of propagated cells (as measured by CFU counts at 6 h) should be significantly less
190   than that of the overnight culture. The procedures described in step 2 and 3 can be performed
191   with regular LB depending on the research design.

193   **4.   Microarray plate screenings**

195   4.1.   Preparing microarray-cell cultures

197   4.1.1   Transfer 250 µL of exponential-phase cells to 25 mL of fresh modified LB medium in a 50
198   mL centrifuge tube. Gently mix the cell suspension to make it homogeneous.

200   NOTE: The exponential-phase cells (OD_{600} = 0.5) are obtained from the second propagation step
201   (see step 2).

203   4.1.2   Transfer the diluted cell suspension into a sterile 50 mL reservoir.

205   4.1.3   Using a multichannel pipette, transfer 150 µL of the cell suspension to each well of a
206   microarray, i.e., a 96 well plate containing various chemicals.

208   NOTE: Wells that do not have chemicals serve as controls.

210   4.1.4   Cover the microarray with a gas-permeable sealing membrane.

212   4.1.5   Incubate the plate in an orbital shaker at 37 °C and 250 rpm for 24 h.

214   NOTE: In these experiments, commercially available plates were used, such as Phenotype
215   Microarrays (PM-9 and PM-10) that include a wide range of osmolytes, pH buffers and other
216   chemicals in a dried state at various concentrations. These microarrays are in half-area 96 well
217   plate formats. Culture volumes should be adjusted depending on the type of plates being used.
218   Microarrays can also be generated manually (see below).

220   4.2   Manual preparation of microarray plates

221

4.2.1 Transfer 75 µL of 2x osmolyte solutions into the wells of a half-area 96 well plate.

4.2.2 Transfer 500 µL of exponential-phase cells to 25 mL of 2x modified LB medium in a 50 mL centrifuge tube. Gently mix the cell suspension to make it homogeneous.

NOTE: The inoculation rate was adjusted to be consistent with the microarray screening protocol described in 4.1.

4.2.3 Add 75 µL of the cell suspension into each well of the half-area 96 well plate containing 2x osmolyte solutions.

4.2.4 Incubate the plate in an orbital shaker at 37 °C and 250 rpm for 24 h.

4.3 Preparing persister assay plates

4.3.1 Prepare 25 mL of modified LB medium containing 5 µg/mL of OFX in a 50 mL centrifuge tube and transfer this medium to a sterile reservoir.

4.3.2 Transfer 190 µL of modified LB medium with OFX from the reservoir into each well of a generic flat-bottom 96 well plate (persister-assay plate) using a multichannel pipette.

4.3.3 Remove the microarray from the shaker (after culturing for 24 h) and transfer 10 µL of cell cultures from the microarray to the wells of the persister-assay plate, containing modified LB medium with OFX.

4.3.4 Take 10 µL of cell suspensions from the persister-assay plate and serially dilute three times in 290 µL of PBS solution using a round-bottom 96 well plate and a multichannel pipette.

4.3.5 Following the serial dilution, spot 10 µL of all serially diluted cell suspensions on antibiotic-free fresh agar plates using a multichannel pipette.

4.3.6 Incubate the persister-assay plate (prepared in step 4.3.3) in an orbital shaker at 37 °C and 250 rpm for 6 h after covering the plate with a gas-permeable sealing membrane.

4.3.7 After 6 h incubation in a shaker, take the persister-assay plate out and repeat the steps 4.3.4-4.3.5.

4.3.8 Incubate the agar plates for 16 h at 37 °C, and then count CFUs. The CFU levels before and 6 h after the antibiotic treatment enable to calculate the persister fraction in each well. The CFU counts before the OFX treatment also help assess the effects of osmolytes and on *E. coli* viability.

5 Validating the identified conditions

265 5.1   Transfer 250 µL of the exponential phase cells from Step 2.3 to 25 mL of fresh modified
266 LB medium containing the osmolyte identified from the microarray screening (see step 4).

268 5.2   Incubate the flask in an orbital shaker at 250 rpm and 37 °C for 24 h.

270 5.3   After 24 h, remove the flask from the shaker and transfer 250 µL of the cell culture to 25
271 mL of fresh modified LB medium in a 250 mL baffled flask.

273 5.4   Add 25 µL of OFX stock solution (5 mg/mL) in the cell suspension and shake the flask
274 gently to make the assay culture homogenous. Incubate the flask in a shaker at 37 °C and 250
275 rpm.

277 5.5   At every hour during the treatment, transfer 1 mL of the assay culture from the flask to a
278 1.5 mL microcentrifuge tube.

280 5.6   Centrifuge the assay culture in the microcentrifuge tube at 17,000 x g for 3 min.

282 5.7   Remove 950 µL of supernatant and add 950 µL of PBS.

284 5.8   Repeat the washing steps 5.6 and 5.7 for 3x.

286 5.9   After the final wash, resuspend the cell pellet in 100 µL of PBS solution.

288 5.10   Take 10 µL of the cell suspension and serially dilute 6x in 90 µL of PBS solution using a 96
289 well round bottom plate.

291 5.11   Spot 10 µL of the diluted cell suspensions on an antibiotic-free fresh agar plate. To
292 increase the limit of detection, plate the remaining 90 µL of cell suspension on a fresh agar plate.

294 5.12   Incubate the agar plate at 37 °C for 16 h, and then count CFUs.

296 **REPRESENTATIVE RESULTS:**
297 **Figure 1** describes our experimental protocol. The dilution/growth cycle experiments (see step
298 2) were adapted from a study conducted by Keren et al.[5] to eliminate the persisters originating
299 from the overnight cultures. **Figure 2A** is a representative image of agar plates used to determine
300 CFU levels of cell cultures before and after OFX treatment. In these experiments, cells were
301 cultured in modified LB medium with osmolytes in half-area 96 well plates as described in step
302 4.2. After incubating the plate in an orbital shaker for 24 h, the persister assay was performed
303 using a generic flat bottom 96 well plate (see step 4.3). The osmolytes and the concentration
304 being tested here were chosen based on our previous study[19], where we performed steps 4.1
305 and 4.3 using the PM-9 plate that includes various osmolytes at different concentrations (sodium
306 chloride, potassium chloride, sodium sulfate, ethylene glycol, sodium formate, urea, sodium
307 lactate, sodium phosphate, sodium benzoate, ammonium sulfate, sodium nitrite and sodium
308 nitrate). The first column in **Figure 2A** shows the CFU counts of the control group. The second

309 column represents a condition where cells were cultured in 100 mM sodium nitrate; this
310 condition was previously found to slightly increase the persister levels[19]. The third column
311 represents a condition where cells were cultured in 60 mM sodium nitrite, and this condition was
312 previously found to significantly decrease the persister levels compared to controls[19]. **Figure 2B**
313 is a graphical representation of the CFU data obtained from the agar plates. **Figure 2C** shows the
314 persister fractions of the cell cultures tested in 96 well plates. To calculate the fractions, persister
315 counts were normalized to the cell counts obtained before the antibiotic treatments. **Figure 3**
316 shows the biphasic kill curves and the persister fractions, respectively, for the assay cultures
317 performed in baffled flasks. In these experiments, cells were first cultured in 25 mL of modified
318 LB medium with the indicated osmolytes in 250 mL baffled flasks for 24 h, and then the cells were
319 transferred to persister-assay flasks for persister enumeration as described in Protocol 5.
320
321 **FIGURE LEGENDS:**
322
323 **Figure 1: The experimental procedure.** Cells from a frozen cell stock were grown overnight (12
324 h) in fresh LB medium. At 12 h, the overnight culture was diluted (1:100) in 25 mL of modified LB
325 medium and grown until OD_{600}=0.5. This propagation step was repeated twice. After the final
326 propagation step, the exponential phase cells (at OD_{600}=0.5) were diluted (1:100) in fresh
327 modified LB medium in a 250 mL baffled flask and a 50 mL centrifuge tube, respectively. The cell
328 suspension in the 250 mL baffled flask was treated with 5 µg/mL of OFX to quantify persisters.
329 The cell suspension in the 50 mL centrifuge tube was transferred to microarrays and incubated
330 for 24 h in an orbital shaker at 250 rpm and 37 °C. The cells from the microarrays were then
331 transferred to persister-assay plates to quantify persisters. This figure was created using
332 biorender.com. This figure has been modified from our previous publication[19].
333
334 **Figure 2: Microarray experiments.** (**A**) Cells were first grown in modified LB medium with or
335 without indicated osmolytes in a half-area 96 well plate for 24 h. The cells were then transferred
336 to a generic flat-bottom 96 well plate and treated with 5 µg/mL of OFX for 6 h. Before and after
337 6 h treatment, cells were serially diluted in PBS, plated on agar plates and incubated at 37 °C for
338 16 h. Each condition has 8 technical replicates. (**B**) CFU measurements were performed to assess
339 the effects of osmolytes on cell viability and persistence, respectively. The straight line indicates
340 the limit of detection (600 CFUs). (**C**) The graph represents the persister fractions of the cell
341 cultures, calculated by taking the ratio of the CFU counts after and before OFX treatment. The
342 persister fraction of the cell culture that has 60 mM sodium nitrite was not calculated as its
343 persister level is below the limit of detection. Each data point was denoted by mean value ±
344 standard deviation, calculated from 8 technical replicates.
345
346 **Figure 3: Validation experiments.** (**A**) Exponential-phase cells were transferred to 25 mL of fresh
347 modified LB medium with indicated osmolytes in 250 mL baffled flasks and cultured for 24 h.
348 Then, the cells were diluted (1:100) in 250 mL baffled flasks containing 25 mL of modified LB
349 medium and treated with 5 µg/mL OFX for 6 h. The CFU counts in the assay cultures were
350 monitored hourly to generate biphasic kill curves. * indicates the condition that significantly
351 affects the OFX persister levels compared to no-osmolyte controls (two-tailed unequal variance
352 t-test, $p<0.05$). (**B**) The graph represents the persister fractions of the cultures. Each data point

353   was denoted by mean value ± standard deviation, calculated from 3 biological replicates. This
354   figure has been modified from our previous publication[19].

356   **DISCUSSION:**
357   The high throughput persister assay described here was developed to elucidate the effects of
358   various chemicals on *E. coli* persistence. In addition to commercial PM plates, microarrays can be
359   constructed manually as described in step 4.2. Moreover, the protocol presented here is flexible
360   and can be used to screen other microarrays, such as drug panels and cell libraries, that are in 96
361   well plate formats. The experimental conditions including the growth phase, inoculation rate and
362   medium can be adjusted to test these libraries. For instance, if one wants to screen a cell library,
363   such as Keio *E. coli* knockout collection[23], they can first transfer the strains from the library to 96
364   well plates that include fresh media, using a multichannel pipette. Once the cells reach the
365   desired growth phase (e.g., exponential or stationary phase), the cells are then transferred to
366   persister-assay plates as described in step 4.3 to enumerate the persister levels of the knockout
367   strains. Similarly, this strategy can be used to screen the *E. coli Promoter* collection[24] (a library of
368   fluorescent reporter strains in 96 well plate formats) to identify promoters that are activated by
369   antibiotics. These promoters can be readily detected by measuring the fluorescence signals of
370   strains in persister-assay plates during the antibiotic treatment.

372   In our experiments, we used a modified LB (lacking NaCl) to avoid any additional effect that could
373   arise from NaCl, considering that the microarrays already have various osmolytes. Although NaCl
374   in regular LB medium is known to be good for preserving the membrane integrity of cells, it has
375   been reported that NaCl at 0-1% range has a minimal effect on cell growth[25]. Moreover, results
376   from propidium iodide (PI) staining and persisters assays in our previous study have shown that
377   the absence of NaCl does not significantly affect the membrane integrity and the persistence of
378   *E. coli* cells[19]. This modification was made specifically to address the concerns in our study and
379   can be changed based on the nature of the research.

381   We have also adapted a method developed by Keren et al.[5] to eliminate pre-existing persisters in
382   our cell cultures prior to inoculation into the microarrays. Type I persisters are known to be
383   generated during the stationary phase; therefore, the direct inoculation of the cells from
384   overnight cultures into microarray plates would transfer a significant number of persisters, which
385   may hinder the effects of osmolytes. With the dilution/growth cycle experiments (see step 2), we
386   were able to significantly reduce these preexisting persisters arising from the overnight
387   cultures[19]. We note that we have cultured the cells in microarray plates for 24 h to be able to
388   count all persisters, including type I variants that are formed during the stationary phase in the
389   presence of osmolytes. However, these adapted techniques can always be modified depending
390   on the nature of the study.

392   We treated the cells in microarrays with 5 µg/mL OFX to enumerate the persisters. Since the
393   washing procedure to remove the antibiotic from 96 samples would be very labor-intensive, cells
394   after the treatment were serially diluted in PBS without washing (see step 4.2). This procedure
395   diluted the OFX concentration more than 30-fold. The diluted cell suspensions were then plated
396   on antibiotic-free agar plates where the OFX was further dispersed. Our preliminary studies

397 where we repeated these experiments with and without washing have verified that this serial-
398 dilution method did not affect the persister levels[19].
399
400 During the multi-channel pipetting in 96 well plates, one should be carefully mixing the cell
401 suspensions to maintain a homogenous cell distribution. To do this, depending on the working
402 volume, it would be beneficial to note the minimum number of pipetting that is needed to make
403 the solution homogeneous. For this purpose, we conducted a simple control experiment where
404 we monitored the pipetting (up and down) by dispersing a dye molecule in PBS and medium
405 under the conditions studied here. Additionally, if working with more complex environments
406 such as pH buffers, we would suggest measuring the pH of the cell culture before and during the
407 incubation in a shaker as the cell culture tends to be very alkaline (pH $\approx$ 8) over time. This can
408 give an idea of the quality as well as the pH range of the buffer used. Additionally, in order to
409 obtain countable CFUs on agar plates from the high-throughput assays, certain conditions, such
410 as the cell inoculation rate, age of cultures, and serial-dilution parameters (PBS volume, dilution
411 rates and dilution times, etc.) should be optimized before testing the microarrays. Finally, the
412 results obtained from the microarrays should be further validated in flasks as the volume of the
413 culture, surface area and aeration in a 96 well plate could have additional effects on the observed
414 results.
415

420
421 **DISCLOSURE:**
422 The authors have nothing to disclose.
423

424 **REFERENCES:**
425 1.   Lewis, K. Persister cells, dormancy and infectious disease. *Nature Reviews Microbiology*.
426 **5** (1), 48–56 (2007).
427 2.   Hobby, G. L., Meyer, K., Chaffee, E. Observations on the Mechanism of Action of
428 Penicillin. *Experimental Biology and Medicine*. **50** (2), 281–285 (1942).
429 3.   Bigger, J. Treatment of staphylococcal infections with penicillin by intermittent
430 sterilisation. *The Lancet*. **244** (6320), 497–500 (1944).
431 4.   Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L., Leibler, S. Bacterial persistence as a
432 phenotypic switch. *Science*. **305** (5690), 1622–1625 (2004).
433 5.   Keren, I., Kaldalu, N., Spoering, A., Wang, Y., Lewis, K. Persister cells and tolerance to
434 antimicrobials. *FEMS Microbiology Letters*. **230** (1), 13–18 (2004).
435 6.   Keren, I., Minami, S., Rubin, E., Lewis, K. Characterization and transcriptome analysis of
436 mycobacterium tuberculosis persisters. *mBio*. **2** (3), (2011).
437 7.   Mulcahy, L. R., Burns, J. L., Lory, S., Lewis, K. Emergence of Pseudomonas aeruginosa
438 Strains Producing High Levels of Persister Cells in Patients with Cystic Fibrosis. *Journal of
439 Bacteriology*. **192** (23), 6191–6199 (2010).
440 8.   LaFleur, M. D., Kumamoto, C. A., Lewis, K. Candida albicans biofilms produce antifungal-

441 tolerant persister cells. *Antimicrobial Agents and Chemotherapy*. **50** (11), 3839–3846 (2006).

442 9.   Allison, K. R., Brynildsen, M. P., Collins, J. J. Metabolite-enabled eradication of bacterial
443 persisters by aminoglycosides. *Nature*. **473** (7346), 216–220 (2011).

444 10.   Lechner, S., Lewis, K., Bertram, R. Staphylococcus aureus persisters tolerant to
445 bactericidal antibiotics. *Journal of Molecular Microbiology and Biotechnology*. **22** (4), 235–244
446 (2012).

447 11.   Barrett, T. C., Mok, W. W. K., Murawski, A. M., Brynildsen, M. P. Enhanced antibiotic
448 resistance development from fluoroquinolone persisters after a single exposure to antibiotic.
449 *Nature Communications*. **10** (1), 1177 (2019).

450 12.   Windels, E. M. et al. Bacterial persistence promotes the evolution of antibiotic
451 resistance by increasing survival and mutation rates. *ISME Journal*. **13** (5), 1239–1251 (2019).

452 13.   Dörr, T., Lewis, K., Vulić, M. SOS response induces persistence to fluoroquinolones in
453 Escherichia coli. *PLoS Genetics*. **5** (12), e1000760 (2009).

454 14.   Völzing, K. G., Brynildsen, M. P. Stationary-phase persisters to ofloxacin sustain DNA
455 damage and require repair systems only during recovery. *mBio*. **6** (5), (2015).

456 15.   Grant, S. S., Kaufmann, B. B., Chand, N. S., Haseley, N., Hung, D. T. Eradication of
457 bacterial persisters with antibiotic-generated hydroxyl radicals. *Proceedings of the National*
458 *Academy of Sciences*. **109** (30), 12147–12152 (2012).

459 16.   Gerdes, K., Maisonneuve, E. Bacterial Persistence and Toxin-Antitoxin Loci. *Annual*
460 *Review of Microbiology*. **66** (1), 103–123 (2012).

461 17.   Orman, M. A., Brynildsen, M. P. Inhibition of stationary phase respiration impairs
462 persister formation in E. coli. *Nature Communications*. **6** (1), 7983 (2015).

463 18.   Korch, S. B., Henderson, T. A., Hill, T. M. Characterization of the hipA7 allele of
464 Escherichia coli and evidence that high persistence is governed by (p)ppGpp synthesis.
465 *Molecular Microbiology*. **50** (4), 1199–1213 (2003).

466 19.   Karki, P., Mohiuddin, S. G., Kavousi, P., Orman, M. A. Investigating the effects of
467 osmolytes and environmental ph on bacterial persisters. *Antimicrobial Agents and*
468 *Chemotherapy*. **64** (5), e02393-19 (2020).

469 20.   Mohiuddin, S. G., Hoang, T., Saba, A., Karki, P., Orman, M. A. Identifying Metabolic
470 Inhibitors to Reduce Bacterial Persistence. *Frontiers in Microbiology*. **11**, 472 (2020).

471 21.   Brooun, A., Liu, S., Lewis, K. A dose-response study of antibiotic resistance in
472 Pseudomonas aeruginosa biofilms. *Antimicrobial Agents and Chemotherapy*. **44** (3), 640–646
473 (2000).

474 22.   Luidalepp, H., Jõers, A., Kaldalu, N., Tenson, T. Age of inoculum strongly influences
475 persister frequency and can mask effects of mutations implicated in altered persistence. *Journal*
476 *of Bacteriology*. **193** (14), 3598–3605 (2011).

477 23.   Baba, T. et al. Construction of Escherichia coli K-12 in-frame, single-gene knockout
478 mutants: The Keio collection. *Molecular Systems Biology*. **2**, 2006.0008 (2006).

479 24.   Zaslaver, A. et al. A comprehensive library of fluorescent transcriptional reporters for
480 Escherichia coli. *Nature Methods*. **3** (8), 623–628 (2006).

481 25.   Hajmeer, M., Ceylan, E., Marsden, J. L., Fung, D. Y. C. Impact of sodium chloride on
482 Escherichia coli O157:H7 and Staphylococcus aureus analysed using transmission electron
483 microscopy. *Food Microbiology*. **23** (5), 446–452 (2006).

484

Figure 1
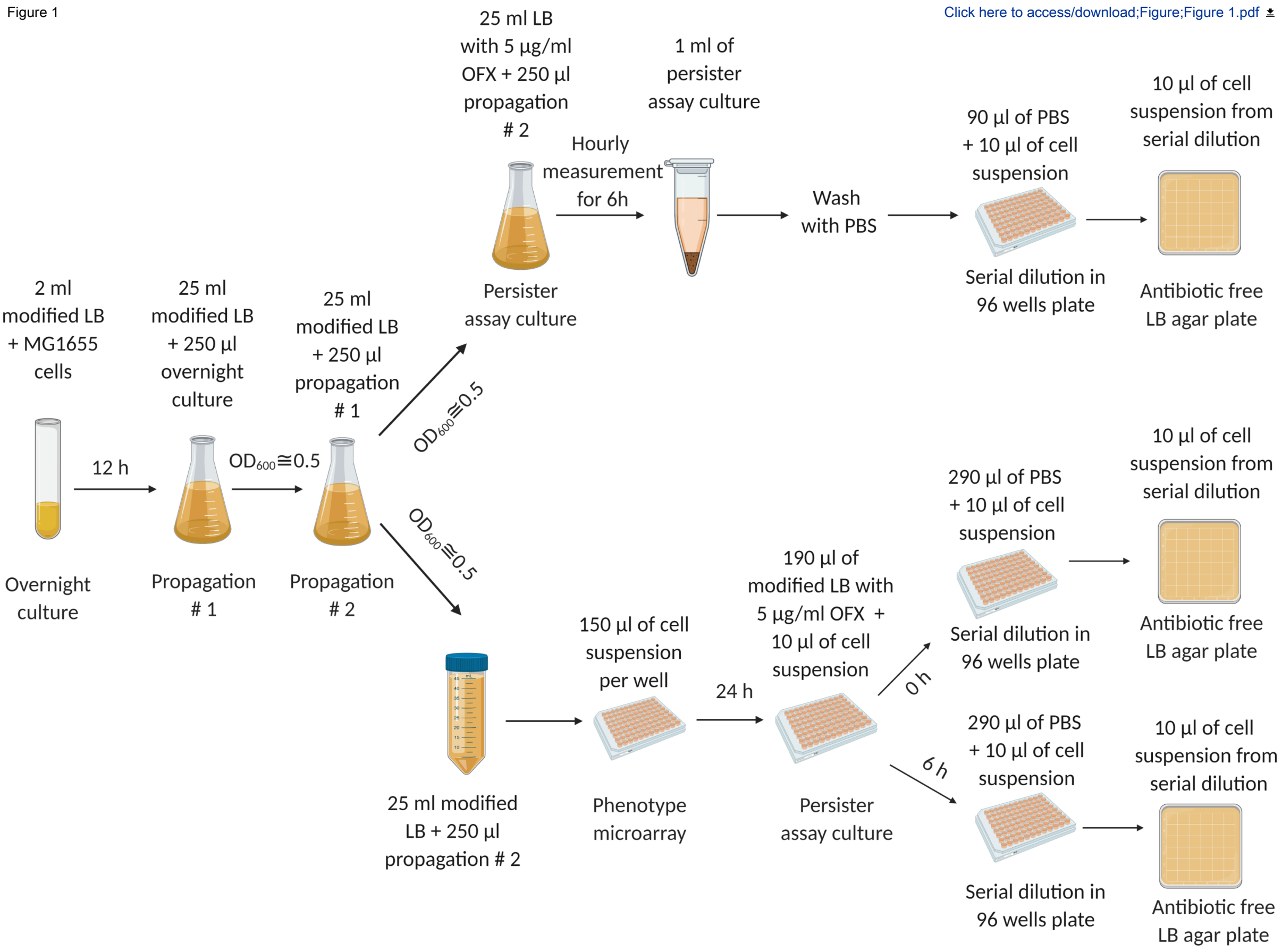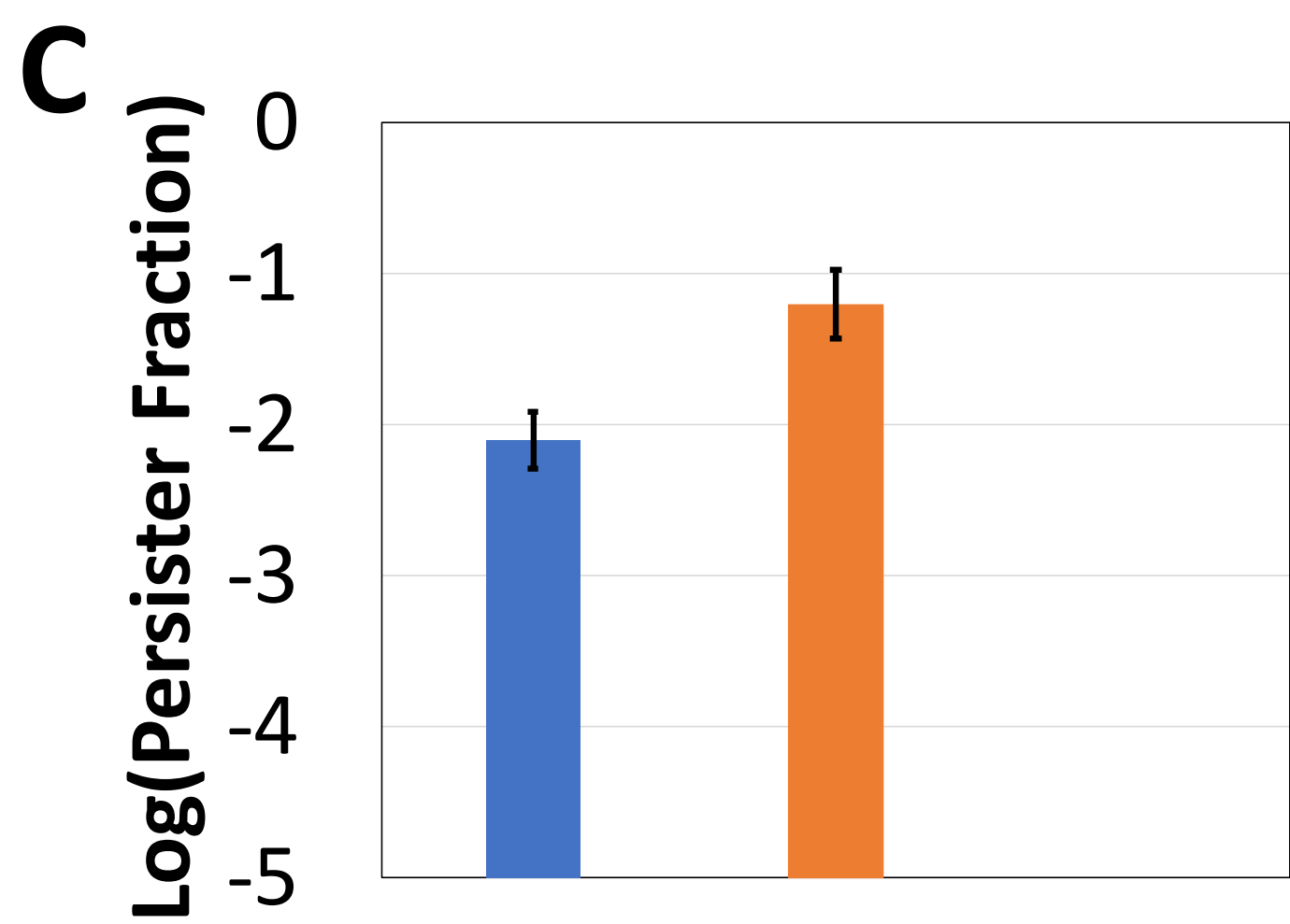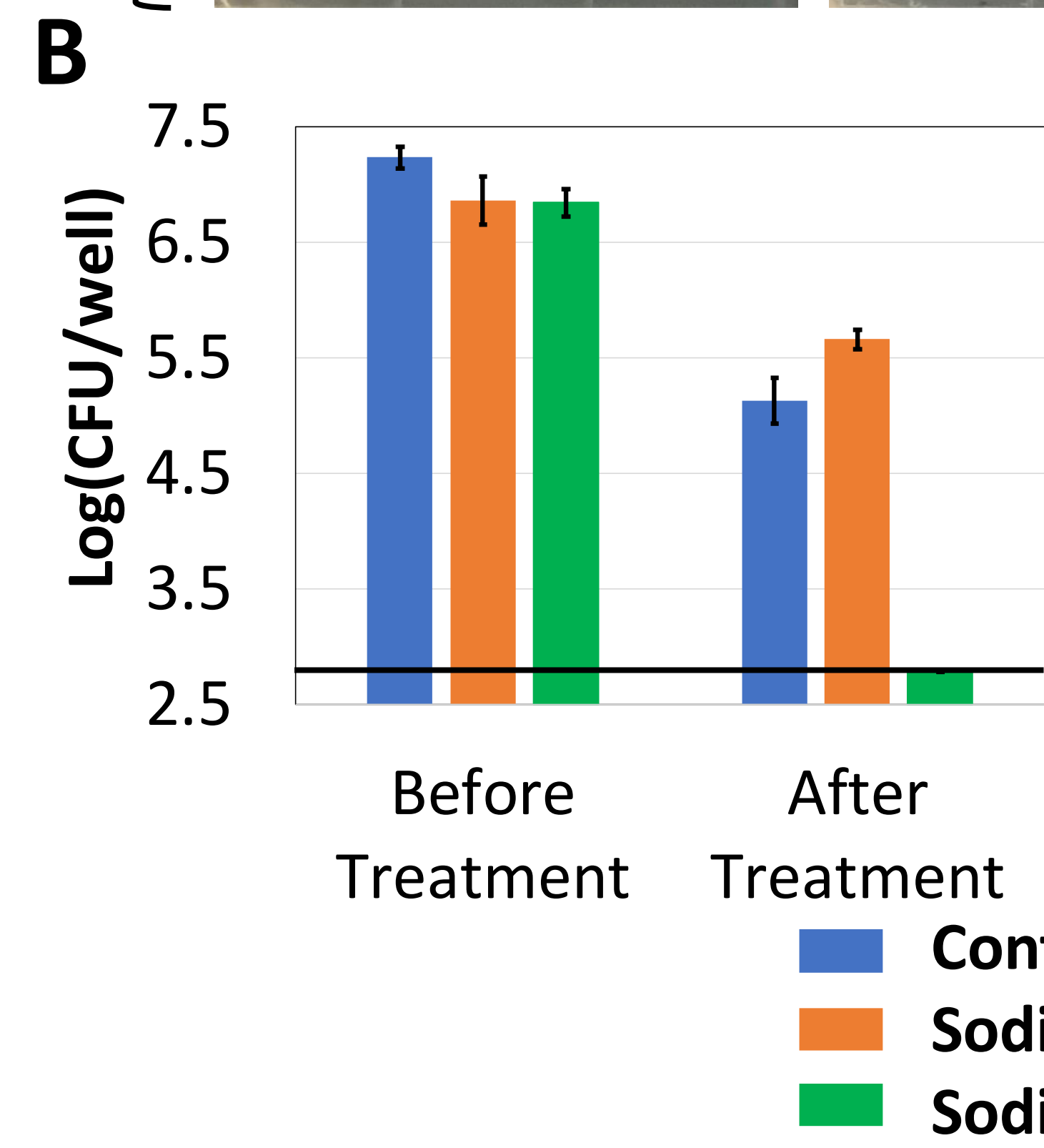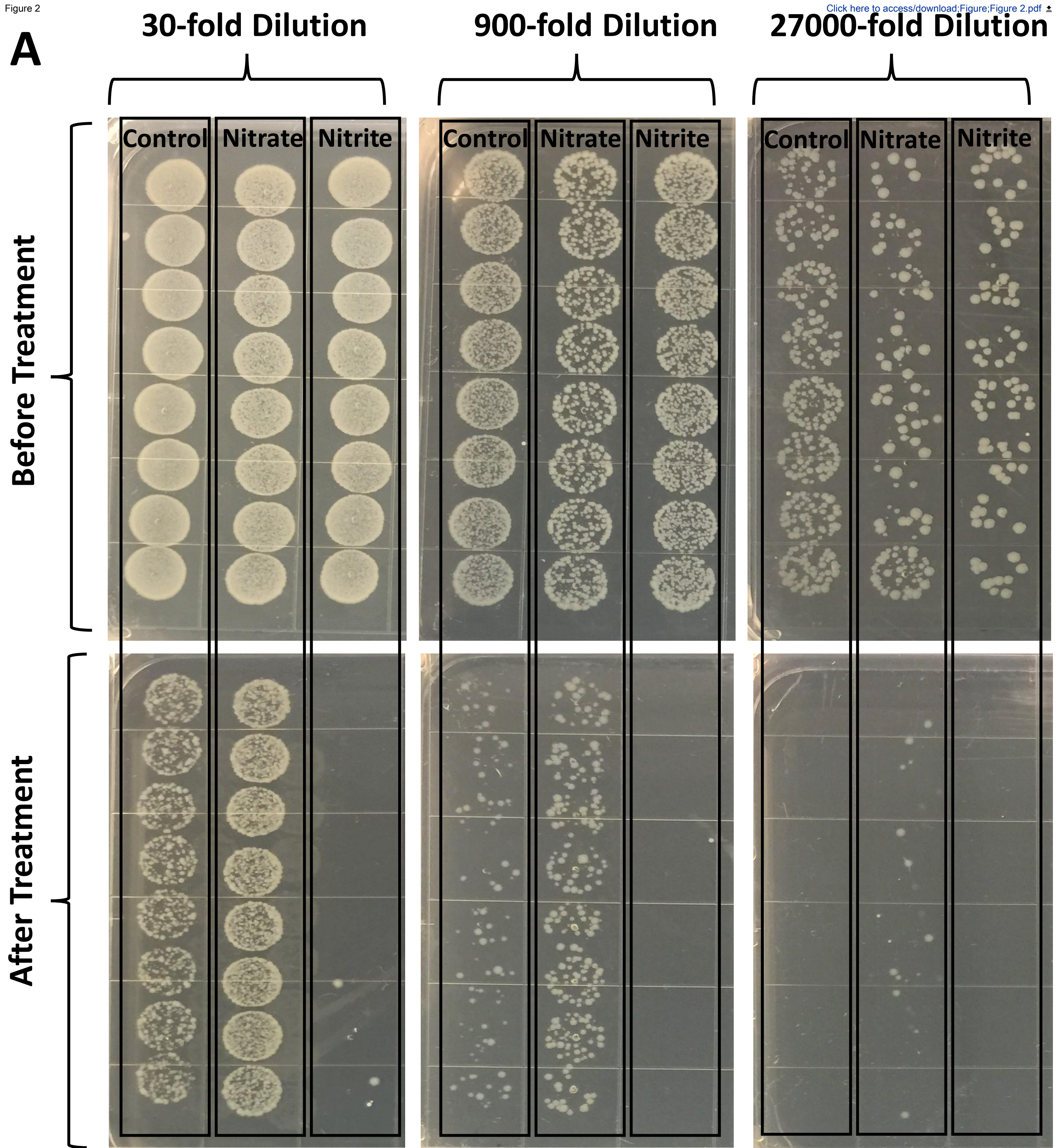
Figure 1

Figure 2

Figure 3
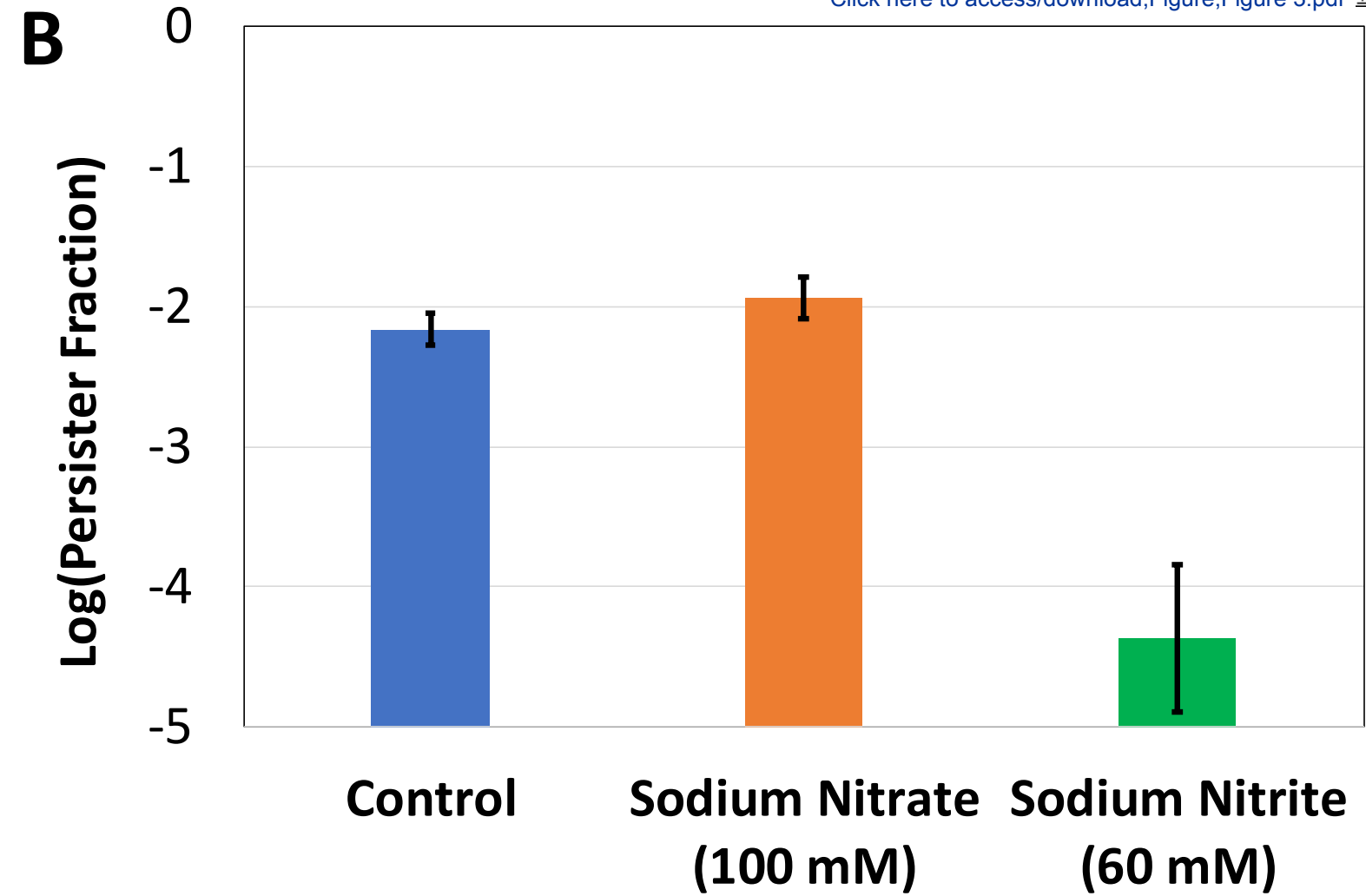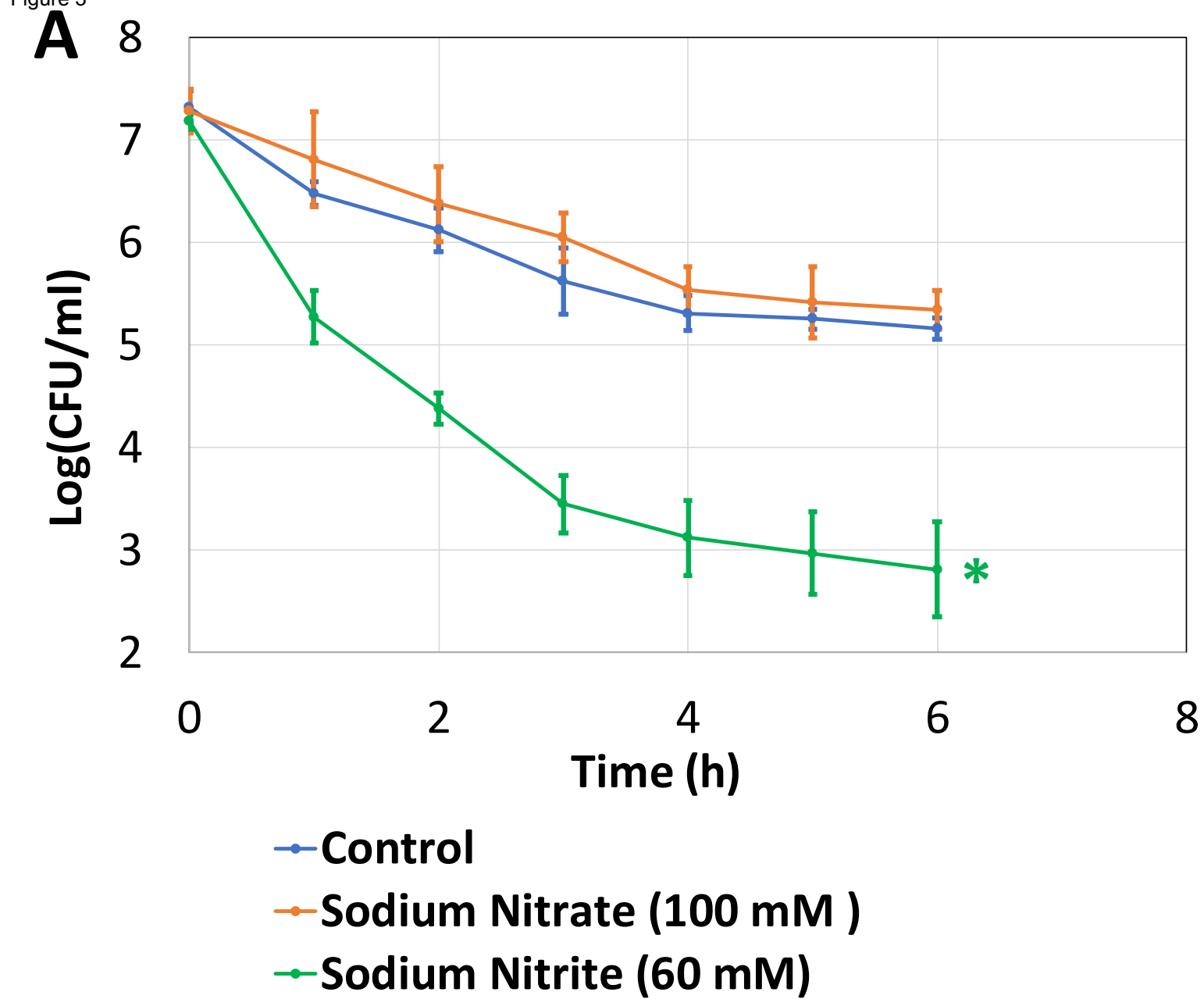
Name of Material/Equipment	Company
14-ml test tube	Fisher Scientific
E. coli strain MG1655	Princeton University
Flat-bottom 96-well plate	USA Scientific
Gas permeable sealing membrane	VWR
Half-area flat-bottom 96-well plate	VWR
LB agar	Fisher Scientific
Ofloxacin salt	VWR
Phenotype microarray (PM-9 and PM-10)	Biolog
Round-bottom 96-well plate	USA Scientific
Sodium chloride	Fisher Scientific
Sodium nitrate	Fisher Scientific
Sodium nitrite	Fisher Scientific
Square petri dish	Fisher Scientific
Tryptone	Fisher Scientific
Varioskan lux multi mode microplate reader	Thermo Fisher Scientific
Yeast extract	Fisher Scientific

Catalog Number

14-959-1B

5665-5161
102097-058
82050-062
BP1425-2
103466-232
N/A
5665-0161
S271-500
AC424345000
AAA186680B
FB0875711A
BP1421-500
VLBL00D0
BP1422-100

Comments/Description

Obtained from Brynildsen lab

Sterilized by gamma irradiation and free of cytotoxins

Molecular genetics grade
HPLC ≥97.5
PM-9 and PM-10 plates contained various osmolytes and buffers respectively

Certified ACS grade
ACS reagent grade
98% purity

Molecular genetics grade
Used for optical density measurement at 600 nm
Molecular genetics grade

Review:

This data (Buffers) is not presented in this manuscript.

- *We have removed the sections related to buffers from the manuscript to make osmolytes our primary focus (see lines 33, 70).*

This needs more clarity…. What are the different chemicals and osmolytes which were screened in the assay.

- *The lists of osmolytes screened in our previous study[1] has been added in the manuscript (see lines 305-307).*

pH conditions not clearly discussed in the protocol section.

- *We have updated the manuscript by removing the statements related to pH conditions from our previous work (see lines 33, 70).*

What are these? how were they obtained? Are these chemicals same as osmolyte? What are the osmolytes which were screened? Why buffer of interest?

- *The chemicals screened in this study are the osmolytes that we had identified in our previous study[1] (see lines 302-307). We have updated the manuscript to clarify this point (see lines 92-96). The list of the osmolytes screened has been mentioned in the current version of manuscript (see lines 305-307). We have removed the terms "buffer of interest" from the manuscript (see lines 89-90).*

Please clearly state the significance of using this antibiotic. So basically you are testing persisters against this one antibiotic?

- *Ofloxacin is a standard antibiotic used for persister assays as it can target both fast and slow growing cells. This has been added as a note in the manuscript. However, the choice of antibiotic depends on the nature of the study (see lines 102-106).*

How do you check this? Will this eliminate persister cells against all the antibiotics? How do you use this to check for the elimination of persister population?

- *The existence of persisters in a cell culture is assessed by a biphasic kill curve where the initial exponential decay indicates the death of antibiotic-sensitive cells.[2] However, the killing trend decreases over time, eventually leading to a plateau region which represents the surviving persister cells. We have added this information in the manuscript (see lines 45-51).*

- *The cell dilution/growth cycle method was developed and verified by Keren and coworkers.[3] We also validated this method in our published study.[1] Since, the dilution/growth cycles were conducted prior to antibiotic treatment it should be applicable for all antibiotics; however, the number of persisters observed can vary depending on the antibiotic used. In addition, we have discussed a control experiment that can be used for further validation (see lines 139-143, 150-152).*

Only one note can follow one step. Please move the notes under the steps which are relevant for the notes.

- ***We have made the changes as directed in the current version of the manuscript.***

Please expand all abbreviations during the first time use.

- ***We have reviewed and addressed this concern in the current version of manuscript.***

After checking for the lack of persisters? O.D.? Cell number?

- ***Cell growth was monitored by OD_{600} measurements. We use exponential phase cells at OD_{600}=0.5. We have added a note to further clarify this concern (see line 199-200).***

With or without osmolyte?

- ***We think the term "modified LB medium without osmolyte" confused the editor. We removed "without osmolyte". It should be "modified LB medium" only (see lines 122-123). Osmolytes were tested in microarray screening and validation experiments.***

Moved here, also notes cannot be filmed. Also as reviewer 1 has suggested, please include steps/discussion to show how one can use 96 well assay plate to perform this study (bringing out different concentrations of osmolytes with different cell numbers or O.D. Also since data presented in figure 2 is generated without these plates it becomes more relevant. Add these as optional steps wherever relevant.

- ***We have added additional steps describing this protocol as suggested (see Protocol 4.2).***

What is the concentration of 2x solution?

- ***The concentration of 2x solution is based on the working solution of the chemical being studied. For example, to study the impact of 1 mM sodium chloride on persistence, the 2x osmolyte solution should be a 2 mM sodium chloride. We have added a note to further explain this concern (see lines 92-96).***

Please make this a step and include the action where actually being done.

- ***These steps have been added in the current version of the manuscript (see lines 219-230).***

Sodium nitrate and sodium nitrite are talked about for the first time here in the representative result. Are these osmolytes? Please bring out clarity on these. Maybe take these as examples in the protocol, include rationale for using these and show these accordingly.

- ***We have updated the representative results section to clarify these concerns (see lines 302-312).***

Why different concentrations of Sodium nitrate and sodium nitrite are used in the assay?

- *These concentrations were selected following the microplate screening data in our previous study.[1] We have mentioned this in the manuscript (see lines 302-303, 308-312).*

What is the limit of detection?

- *The limit of detection for the microarray screening study has been added in the figure legends (see line 340).*

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- *We have added the citations for figures modified from our previous study as directed and have provided pdf of copyright permission for the use of these figures (see lines 332, 353-354).*

As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

- *We have added and discussed the topics listed by the editor in the Discussion and Notes in the updated manuscript.*

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Investigating the Effects of Osmolytes and Environmental pH on Bacterial Persisters

Author: Prashant Karki,Sayed Golam Mohiuddin,Pouria Kavousi,Mehmet A. Orman

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