

1 Quantifying and understanding well-to-well contamination in microbiome research

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43 **Abstract:**

44 Microbial sequences inferred as belonging to one sample may not have originated from
45 that sample. Such contamination may arise from laboratory or reagent sources or from
46 physical exchange between samples. This study seeks to rigorously assess the
47 behavior of this often-neglected between-sample contamination. Using unique bacteria
48 each assigned a particular well in a plate, we assess the frequency at which sequences
49 from each source appears in other wells. We evaluate the effects of different DNA
50 extraction methods performed in two labs using a consistent plate layout including
51 blanks, low biomass, and high biomass samples. Well-to-well contamination occurred
52 primarily during DNA extraction, and to a lesser extent in library preparation, while
53 barcode leakage was negligible. Labs differed in the levels of contamination. DNA
54 extraction methods differed in their occurrences and levels of well-to-well contamination,
55 with robotic methods having more well-to-well contamination while manual methods
56 having higher background contaminants. Well-to-well contamination was observed to
57 occur primarily in neighboring samples, with rare events up to 10 wells apart. The effect
58 of well-to-well was greatest in samples with lower biomass, and negatively impacted
59 metrics of alpha and beta diversity. Our work emphasizes that sample contamination is
60 a combination of crosstalk from nearby wells and background contaminants. To reduce
61 well-to-well effects, samples should be randomized across plates, and samples of
62 similar biomass processed together. Researchers should evaluate well-to-well
63 contamination in study design and avoid removal of taxa or OTUs appearing in negative
64 controls, as many will be microbes from other samples rather than reagent
65 contaminants.

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68

69 **Importance:**

70 Microbiome research has uncovered magnificent biological and chemical stories across
71 nearly all areas of life science, at times creating controversy when findings reveal
72 fantastic descriptions of microbes living and even thriving in once thought to be sterile
73 environments. Scientists have refuted many of these claims because of contamination,
74 which has led to robust requirements including use of controls for validating accurate
75 portrayals of microbial communities. In this study, we describe a previously
76 undocumented form of contamination, well-to-well contamination and show that
77 contamination primarily occurs during DNA extraction rather than PCR, is highest in
78 plate-based methods as compared to single tube extraction, and occurs in higher
79 frequency in low biomass samples. This finding has profound importance on the field as
80 many current techniques to 'decontaminate' a dataset simply relies on an assumption
81 that microbial reads found in blanks are contaminants from 'outside' namely the
82 reagents or consumables.

83

84 Keywords: microbiome, contamination, 16S rRNA gene, metagenomics, built-
85 environment, genomics, low-biomass

86 **Introduction:**

87

88 Massively high-throughput sequencing has enabled fundamental changes to the study
89 of microbial ecology. Increased throughput and sequencing depth has empowered
90 researchers to utilize multiplexing to increase sample sizes to thousands per study [1–
91 6]. However, new ways of knowing require new understanding of potential flaws and
92 confounds. Many studies have addressed computational and statistical challenges
93 associated with analyzing 16S rRNA gene sequence data, including the impacts of
94 sequence similarity clustering [7], diversity estimation, and data compositionality [8], to
95 name just a few. There has also been substantial effort to reduce confounding
96 experimental effects via standardization of microbiome sample processing methods,
97 including sample collection, preservation [9], DNA extraction [10–12], library preparation
98 [6,13–17] and sequencing [5]. Together, these approaches have facilitated large-scale
99 metaanalyses such as the Earth Microbiome Project ‘EMP’ (earthmicrobiome.org) [2].
100 Despite these efforts, a significant amount of experimental noise remains in any given
101 microbiome study.

102
103 Contamination, or the observation of sequence reads in a sample coming from
104 microbes that weren’t originally part of that sample, remains one of the most pernicious
105 types of experimental noise. Microbial rRNA gene copies can be found even in ‘sterile’
106 reagents, leading to that presence of background signal derived from DNA extraction
107 kits [18], PCR mastermix [19], and other consumables [20]. It is now widely understood
108 that such contaminants must be considered in microbiome analyses, especially when
109 dealing with low-biomass samples where contaminant rRNA gene copies make up a
110 larger fraction of the community [7,21–25]. Various engineering strategies have been
111 proposed and are utilized to minimize contamination including physical separation of
112 rooms used for DNA extractions and PCR, wearing additional PPE [26] to cover skin to
113 prevent technician-induced contaminants, UV sterilization of plastic consumables or
114 reagents, or ethidium oxide treatment of consumables [20].

115
116 Beyond physically limiting contamination, the use of positive and negative controls is
117 increasingly being used to assess and quantify contamination in a study, allowing for
118 the potential of contaminant removal in silico [27]. Methods such as Katharoseq [11],
119 utilize the ratio of read counts and composition of positive and negative controls, to
120 determine criteria for sample inclusion. Others have emphasized the importance of
121 including negative controls to understand background contamination [28]. Based on the
122 idea that contaminants are primarily derived from external sources, some have
123 proposed the strategy of simply identifying this ‘contaminome’ profile and then removing
124 them from the dataset [29]. This, however fails to contend with the potential that
125 contaminants may arise from other samples within a study itself. Such between-sample
126 contamination has been observed as a product of ‘barcode-swapping’ between samples
127 as a byproduct of Illumina ex-Amp sequencing reactions, and has also been suggested
128 to arise from improper assignment of barcodes to neighboring clusters in image
129 processing [30]. Anecdotally, we have also observed instances that appear to arise from
130 physical cross-contamination of samples. Since most DNA extractions and PCR
131 reactions are performed on multiple samples at once, often times in 96-well format, we
132 reasoned it would be important to take into consideration that nearby samples could in
133 fact contribute to contamination of negative controls.

134
135 To evaluate this hidden factor of contamination, we designed an experiment to
136 empirically characterize the frequency and nature of well-to-well contamination using
137 different DNA extraction and sample handling protocols. By placing 16 unique bacterial
138 “source” isolates at high biomass in individual wells across plates of alternating low-
139 biomass “sink” bacteria and no-template blank wells, we were able to observe and
140 quantify well-to-well transfer events under different scenarios, including automated
141 plate-based extraction and manual tube-based extraction protocols. We further included
142 libraries from an additional, unique, isolate that were extracted and amplified separately
143 to account for potential instrument-based cross-contamination mechanisms such as
144 barcode-swapping or miss-assignment. To further validate results, we processed an
145 additional two 96-well plates at another microbiome facility.

146
147 **Results:**

148
149 We designed a 96-well plate layout containing 16 unique source bacteria (~10,000,000
150 cells per well, corresponding to 10^8 cells ml^{-1}), 24 sink wells (containing *V. fischeri* at
151 ~100,000 cells per well, 10^6 cells ml^{-1}) and 48 blank wells (Figure 1a). At UCSD, a total
152 of three replicate sample plates were DNA extracted: two using the Epmotion5075 with
153 magnetic bead cleanups on Kingfisher robots (Plate 1, Plate 2) and one manually with
154 column cleanups (Tube). All three plates were then processed each with two unique
155 PCR plates in triplicate as outlined (Figure 1). In addition, 16 gDNA replicates of a
156 *Clostridium* isolate were processed on its own 96-well plate and amplified in a separate
157 PCR reaction, to allow for detection of instrument-based barcode miss-assignment. A
158 mock community comprised of all source isolates and the sink isolate was created and
159 then serially diluted and processed as well to validate sample amplification. Details on
160 the actual plate map patterns can be found in Additional file 1 for all eight PCR plates. A
161 total of 3,756,064 reads from 713 samples resulted in 6305 features. A summary table
162 was generated to describe well-to-well and background contamination occurrences
163 across the samples (Additional Table 1). One of the 16 source microbes was highly
164 contaminated with background contaminants and did not produce the expected
165 sequence results, but was included in the analysis as we did not want to bias our
166 results.

167
168 Well-to well contamination events were analyzed by counting fraction of reads from a
169 given source well appearing in other source wells, low biomass sink wells, or blanks. In
170 our setup, well-to-well contamination was visualized to occur in all six PCR replicate
171 plates in both labs. Based on the visualized plate patterns, the pattern of well-to-well
172 contamination was observed to be higher in plate extractions compared to tube
173 extractions, and was more prominent in wells directly surrounding the source well,
174 suggesting a physical mechanism for well-to-well contamination (Figure 2). We
175 quantified the distance by measuring contamination counts as a function of the
176 Pythagorean distance from the source well, and determined that the highest rates of
177 contamination occurred in the immediate proximate wells for both plate and tube
178 extractions, but with a stronger distance-decay relationship for the plate vs. the tube

179 extractions (Figure 3). The supplementation of Antifoam-A to wells during DNA
180 extraction did not reduce well-to-well contamination (Additional file 2).

181
182 Another possible contributing source of inter-sample contamination is barcode leakage,
183 i.e. reads originating from a given sample being identified as originating from a different
184 sample due to read errors in the barcode. Such “barcode-hopping” behavior has been
185 observed in labs using 8 bp barcodes in the Microbiome Quality Control project [31]. In
186 order to quantify the contribution of such events in our 12 bp barcode design, we
187 designed another plate containing 16 replicate wells of a single *Clostridium* isolate.
188 Since these samples were sequenced together with the PCR replicate plates, barcode
189 leakage would be expected to result in *Clostridium* reads appearing in the PCR
190 replicate plates samples. Barcode leakage was quantified by counting the number of
191 reads originating from barcodes not present in the plate, and no such reads were
192 observed, indicating that for the 12 bp Golay error correcting barcodes sequenced in
193 these conditions, this is a very rare event (less than 1 in 3.75E6 reads), and is not a
194 contributing factor to inter-sample contamination.

195
196 To further quantify the total effect of well-to-well contamination, we compared the
197 proportion of microbial community source for each sample across the three DNA
198 extraction plates (plate 1, plate 2, and tube) and for each of the two PCR replicate
199 plates (PCRA and PCRB) from each extraction (Figure 4). Contamination frequency and
200 relative abundance was highest in plate 1 followed by plate 2 and lowest in the tube
201 plate (Additional file 3). NTCs were composed of primarily background contaminants in
202 the tube extractions for both PCR replicates (median fraction of well-to-well reads 0).
203 However, in some plate extraction NTCs, the majority of reads originated from well-to-
204 well reads (median fraction of well-to-well reads of 0.78, 0.9, 0.44 and 0.77 for plate 1:
205 PCRA, PCRB; plate 2: PCRA, PCRB respectively) (Additional file 4). Sink wells were
206 also partially contaminated with source microbes, particularly in the Plate 1 replicate.
207 The total occurrence (prevalence) of well-to-well contamination across the various
208 sample types and extraction methods along with summarizing compositional effects of
209 well-to-well contaminants on samples (mean, median and max) is detailed in Additional
210 Table 2. For NTCs, 47.5% of blanks from tubes and 95.7% of blanks from plate
211 extractions had well-to-well contamination. For low biomass samples, 15.0% of sink
212 wells from tubes and 67.4% of sink wells from plate extractions had well-to-well
213 contamination (Table 1).

214
215 To determine if DNA extraction method (tube vs plate) had an impact on well-to-well
216 contamination, we compared relative abundances of well-to-well contaminants for
217 NTCs, sink, and source samples independently (Figure 5a). Well-to-well contamination
218 was affected by extraction method, and was generally higher in plate-based extractions
219 compared to manual single tube extractions (Kruskal-Wallis $P < 0.0001$, Figure 5a).
220 Further, the proportion of well-to-well contamination was greater in samples with lower
221 starting biomass (NTCs, 0-100 cells and sinks, approximately 100,000 cells) than in
222 source wells, which had higher starting biomass (approximately 10,000,000 cells) while
223 controlling for extraction method (Figure 5b). Well-to-well contamination was greatest in
224 samples with lower microbial biomass.

225
226 In order to validate these results in an independent lab, in addition to the samples
227 processed at UCSD, we sent away bacterial samples to be processed at an outside
228 facility using the manual single tube extraction and plate extraction (although due to
229 available facilities both utilized a column cleanup step rather than magnetic beads). All
230 results for replicate PCR plates and robot extraction replication were summarized for
231 overall comparison purposes (Table 1). While controlling for site (UCSD only), the total
232 fraction of reads from samples (mean, median, and max out of 100%) caused by well-
233 to-well contamination was highest in NTCs followed by sink and lastly source microbes
234 for both the tube (NTC: 4.57%, 0%, 56.0%; sink: 0.05%, 0.0%, 2.78%; source: 0.13%,
235 0.01%, 2.99%) and plate (NTC: 58.26%, 65.79%, 100.0%; sink: 6.9%, 0.078%, 15.61%;
236 source: 0.94%, 0.04%, 50.67%) extraction methods (Table 1 and Figure 4). The NTCs
237 of samples processed outside of UCSD had well-to-well contamination consistent with
238 the other tube methods while the sink samples had higher well-to-well contamination
239 and overall background contamination than both tube and plate processed samples at
240 UCSD (Table 1).

241
242 Since well-to-well contamination can introduce additional bacteria to samples, it has the
243 potential to inflate alpha and decrease resolution in beta diversity metrics, especially for
244 binary metrics (such as number of observed species, Jaccard dissimilarity, or
245 unweighted UniFrac distance). While all of our source and sink control samples should
246 have only had one unique sOTU, richness was typically much higher than this due to
247 contamination including background kit contaminants along with well-to-well
248 contaminants. We calculated the total richness per sample, which should have been
249 one, and determined the percentage of that richness which was due to well-to-well
250 contamination. Both well-to-well contaminants and background kit contaminants
251 contribute to this inflated richness. Controlling for site (UCSD only), we determined that
252 well-to-well contamination inflated richness estimates for both tube and plate extracted
253 samples by contributing to on average (0.96%, 12.7%; tube, plate) of sink sample
254 richness and (6.51%, 13.76%; tube, plate) of source sample richness.

255
256 We next assessed the impact of well-to-well contamination on beta-diversity
257 measurements of the communities. Specifically, for each unique DNA extraction plate,
258 we performed pairwise well_ID comparisons of the PCR replicates for each of the three
259 sample types including NTCs, sink, and source microbes. Because well-to-well
260 contamination generally only made up a small proportion of the total reads of each
261 sample, binary metrics (which tend to emphasize the impact of rare taxa) were more
262 affected than abundance-weighted metrics. (Additional file 5).

263
264 To further elaborate on this observation and quantify where well-to-well contamination
265 was coming from (PCR process only or DNA extraction), we compared replicate plates
266 which were processed using the robot. This included two separate DNA extraction
267 plates and then two PCR plates for each extraction plate. For each PCR replicate plate,
268 96 pairwise distances were computed and categorized by sample type for each of the
269 two DNA extraction plates (light red shade Additional file 5b). In addition, the pairwise
270 distances from each of the 96 wells of the two replicate DNA extraction plates

271 processed on the robots were also compared for the PCR replicate plate PCRA only.
272 We found much less between-PCR than between-extraction variance, indicating that the
273 combination of stochastic effects plus well-to-well contamination for DNA extraction is
274 greater than for stochastic effects plus well-to-well for PCR (Additional file 5b).

275

276 **Discussion:**

277

278 Understanding experimental biases or noise in microbiome research is critical to
279 drawing accurate inferences of the microbial world. Since microbes are everywhere [2],
280 it is extremely important to limit and ideally eliminate false positives in sample
281 signatures. Contamination is a combination of background contaminants (DNA
282 extraction kits, PCR mastermixes, and enzymes), processing contaminants (equipment,
283 air, technicians), and plate contaminants (well-to-well contamination). In this study, we
284 showed that well-to-well contamination can play a major role in microbiome studies,
285 especially when using plate-based DNA extraction methods and for samples with low
286 starting biomass. This type of contamination is difficult to detect and relatively
287 infrequently discussed, but should be considered when designing and evaluating
288 research. The majority of research to date has focused on identifying microbial
289 contaminants in reagents and consumables [12,18,21] and subsequently using
290 bioinformatics techniques to simply subtract out these contaminant taxa [22,27,32].
291 Existing tools to remove contaminant taxa or OTUs (operational-taxonomic units) from a
292 dataset largely focus on these background contaminants, and don't yet consider the
293 case of contamination from proximal wells [27]. We show in this study that a large
294 fraction of reads in the blank (NTC) samples originate from neighboring wells. In this
295 study, we observed that contamination between samples can account for a significant
296 fraction of the overall observed diversity in a sample, especially for no-template control
297 blanks that are physically adjacent to relatively high-biomass samples. Given this, the
298 simple approach of removing any taxa found in blanks is likely to remove the most
299 prominent "real" taxa in a dataset. More sophisticated methods using additional
300 information (such as the 'decontam' package [27]) are absolutely necessary in the face
301 of well-to-well contamination, even for addressing the problem of reagent contaminants.

302

303 Identifying and removing well-to-well contamination *in silico* is challenging, as
304 contamination events between wells are largely independent, and thus cannot be
305 statistically identified and removed across a study in the same way that reagent
306 contaminants are. However, several observations from this experiment should help
307 researchers in planning experiments to minimize its effects. First, plate-based DNA
308 extractions are much more susceptible to well-to-well contamination than the more
309 painstaking tube-based extractions; for critical experiments, automated plate-based
310 extractions should be carefully reconsidered. Second, even for tube-based extractions,
311 well-to-well contamination was greatest in wells immediately adjacent to the source.
312 Thus, sample location on plates should be explicitly considered in experimental design.
313 When plating samples for extraction, it is important to block and/or randomize
314 treatments across 96-well plates. Third, well-to-well contamination has the greatest
315 impact in low-biomass samples, especially when they are processed adjacent to high-

316 biomass samples that can act as sources. Because of this, it is important to have an
317 awareness of the absolute concentration of microbial cells in samples, and to ensure
318 that only samples of similar biomass are processed together. Lastly, when analyzing
319 datasets, it is important to be aware that different methods will have different
320 sensitivities to well-to-well contamination. For example, alpha-diversity estimates can be
321 highly inflated by well-to-well contamination in samples with low starting diversity; and
322 for beta-diversity estimates, binary metrics such as Jaccard or unweighted UniFrac are
323 more likely to be affected than abundance-weighted metrics. Other experimental
324 approaches to reduce the impacts of well-to-well contamination bear further
325 investigation. These might include use of higher-fidelity liquid handling approaches [33],
326 or broader adoption of unique-per-sample positive control spike-ins to allow the direct
327 observation and statistical disambiguation of cross-contamination [34]. Methods which
328 rely on identifying and subtracting putative contaminants from datasets need to be used
329 with extreme caution, particularly if the identified sequence variants are present in
330 primary samples.

331
332 Understanding experimental noise is extremely important for improving and guiding
333 microbiome research best practices [23,24]. Specifically, addressing ‘hot’ negative
334 controls is one of the great challenges to genomics based research. Since well-to-well
335 contamination is an important component of this, we emphasize that for any given
336 experiment, it is critical to identify any kit-specific background contaminants in a lot to
337 best accurately remove contaminant taxa. While we have good power to estimate
338 frequency of well-to-well contamination in our assays, extrapolating the frequency of
339 well-to-well contamination in assays from other labs and methods is still a challenge.
340 This suggests that while we can generalize to well-to-well contamination being a
341 widespread problem, we can’t generalize the quantities or specifics. Further, this argues
342 for other labs spending the effort to do similar in-house tests to evaluate their own
343 pipelines. To identify these background contaminants, we recommend using a variety of
344 positive controls titrations both at the DNA extraction stage and PCR stage [11].
345 Companies which manufacture high-throughput DNA extraction will need to invest in
346 research and development to reduce well-to-well contamination. Lastly, measuring and
347 accounting for well-to-well contamination identification and reduction will be critical for
348 diagnostic research going forward [35–40].

349
350 Conclusions:

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352 Contamination is a serious impediment to reproducibility in any genomics study,
353 particularly microbiome research. As emerging diagnostic tests for environmental health
354 and human health become more mainstream, it will be crucial for these tests to address
355 variability in microbiome signal due to well-to-well contamination. Our study identified
356 and quantified a previously undetected source of contamination in microbiome studies.
357 We show that intensity of well-to-well contamination varies per extraction method with
358 plate-based methods and lower biomass samples having higher rates of contamination.

359 Our findings demonstrate the importance for the community to accept standards to best
360 monitor and quantify these sources of noise in a given study.

361

362 **Methods:**

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364 **Sample collection and processing**

365 A total of 17 bacterial isolates including *Brevibacterium sp*, *Corynebacterium stationis*,
366 *Brachybacterium sp*, *Arthrobacter sp*, *Propionibacterium acnes*, *Bacillus sp*,
367 *Staphylococcus equorum*, *Staphylococcus succinus*, *Streptococcus angiosis*,
368 *Desulfovibrio sulfodismutans*, *Serratia sp*, *Halomonas sp*, *Psychrobacter sp*,
369 *Pseudomonas fragi*, *Vibrio rumo*, *Eschericia coli*, and *Vibrio fischeri* were collected and
370 stored in PBS solution. The optical density, OD600, was measured for all isolates and
371 the corresponding cell density estimated. Sixteen of these microbes (all except *V.*
372 *fischeri*) were diluted to a final density of 1e8 cells per ml in a single 50 ml conical vial
373 and were designated as 'source' organisms. The *V. fischeri* isolate was diluted to 1e6
374 cells per ml, designated as the 'sink' microbe, and stored in a single 50 ml conical. Both
375 source and sink microbes were stored in a -80 °C freezer until making aliquots for
376 extractions. In addition, a mock community was created using these isolates by
377 combining equal volume of all samples which also served as a reference for accounting
378 for processing biases. An additional isolate of *Clostridium sp*. was measured and
379 aliquoted into 16 different 2 ml tubes to be used for barcode testing. For DNA extraction
380 at UCSD, 100 ul of 'source' and 'sink' samples were aliquoted into 2 96-well DNA
381 extraction robot plates and 96 2-ml bead beating extraction tubes as indicated in the
382 diagram (Additional file 1, Figure 1a). Following the Earth Microbiome Project protocol
383 [2], the Qiagen PowerMag kit (Qiagen, Cat# 27500-4-EP) was used for robot extractions
384 while the Qiagen DNeasy PowerSoil kit (Qiagen, Cat# 12888-100) was used for
385 'manual, single-tube' extractions. To test the effect of antifoam on reducing well-to-well
386 contamination, we added 2 ul of antifoam-A concentrate (Sigma-Aldrich, Cat#A5633-
387 25G) to half of each of the robot plates (Figure 1b-c). In addition to processing samples
388 at UCSD, an additional 192 samples were plated (96) in a 96-well plate and 96
389 individual 2-ml bead beating tubes and sent to Argonne National lab in the same
390 platemap scheme. The manual tube samples were processed using the Qiagen DNeasy
391 PowerSoil kit (Qiagen, Cat# 12888-100) while the manual plate samples were
392 processed using the Qiagen DNeasy PowerSoil HTP 96 kit (Qiagen, Cat# 12955-4).

393

394 **Amplicon sequencing**

395 To distinguish between well-to-well contamination derived from DNA extraction versus
396 PCR setup, each UCSD processed DNA extraction plate (2 robot plates and 1 manual
397 plate) were subjected to two separate triplicate PCR reactions (Figure 1b-d). The mock
398 community dilution plate and barcode testing plate were processed with a single
399 triplicate PCR reaction each. The EMP 16S rRNA V4 primers 515f/806rB were used to
400 amplify the samples. Equal concentrations of amplicons from each sample from all 8
401 plates were pooled and sequenced using a MiSeq [5,13,14]. The 192 samples DNA
402 extracted at Argonne were processed using the same EMP primers and method but on
403 a separate MiSeq run. Amplicon data was uploaded to Qiita [41] and processed with

404 Qiime 1.9.1 [42]. Exact sequence tags from the first read were generated using the
405 deblur pipeline under default parameters as described in the publication [43].

406

407 **Statistical analysis**

408 Sequences processed with deblur were positively filtered against the reference
409 database as part of the default workflow in deblur. In addition, singleton sequences
410 were omitted from the dataset. The dataset was not rarified in order to best quantify
411 well-to-well contamination for all samples processed. The sequence tags were identified
412 for all of the positive controls used in this study and included in supplement (Additional
413 Table 2). Sequences which did not have 100% match to those original controls were
414 considered 'background contaminants' whereas the *Vibrio fischeri* deemed as 'sink
415 microbes' and the 16 unique isolates deemed collectively as 'source microbes'. For
416 each of the 16 source microbes, 1 sink microbe, and 1 barcode leakage microbe, a
417 custom script was used to generate 96-well plate maps to visualize well-to-well
418 contamination. The distances of microbial dispersal 'jumping' was then calculated for
419 each individual isolate using a custom script. Summary statistics of read counts,
420 richness, and contamination metrics are summarized (Additional Table 3). To determine
421 if well-to-well contamination was higher in robot compared to manual extractions, the
422 composition of well-to-well contaminants was compared within NTCs, sink, and source
423 independently, using the Kruskal-Wallis test. Further, to determine if well-to-well
424 contamination was associated or more frequent with lower biomass samples, well-to-
425 well composition was compared across the NTCs, sink, and source within each
426 extraction method independently using Kruskal-Wallis test.

427

428 To determine the impact of well-to-well contamination on beta-diversity microbiome
429 analyses, we calculated distance metrics of both Bray-Curtis [44,45] and Jaccard [46]
430 and compared within categories. The three different extraction plates each had two
431 separate PCR plates processed. The pairwise distances of unique Well_ID was
432 calculated using both metrics for each of the two PCR plates belonging to each of the
433 three DNA extraction plates. Sample types were grouped into NTCs (non-template
434 control or blank), sink, or source. Within each group, the distances were compared
435 using the Mann-Whitney test. To calculate effects for the entire pipeline which includes
436 both PCR and DNA extraction, we combined the pairwise distances of the Well_IDs for
437 each of the three DNA extraction plates (robot 1, robot 2, and manual) and grouped by
438 sample type (NTC, sink, or source). Again, we compared the total dissimilarities of Bray-
439 Curtis vs. Jaccard for each sample type using Mann-Whitney test.

440

441 **List of abbreviations**

442 EMP: Earth Microbiome Project

443 NTC: non-template control (sterile water blanks)

444 sOTU: sub-operational taxonomic unit

445 sink: lower biomass microbial isolate used in experiment (approximately 100,000 cells)

446 source: higher biomass microbial isolate (1 of 16) (approximately 10,000,000 cells)

447 Well_ID: refers to the well position (in a 96-well plate: A1-A12 → H1-H12) from which
448 the sample was processed

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JJM, AA, JS, GH, RK designed experiment

JJM, GH, JG processed samples

JJM, AA, JS analyzed and interpreted results

AA, JS developed custom scripts for various platemap analyses and distance-k

JJM, AA, JS, RK helped to write the manuscript

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Additional files

Supplemental Table S1 (full characterization of w2w and metadata)

Supplemental Table S2. Summary statistics on well-to-well contamination across DNA

Supplemental Figure S1: Platemap descriptions of experimental design

Supplemental Figure S 2: The use of antifoam (antifoam = 1) does not reduce well-to-well contamination

Supplemental Figure S 3: Sources of contamination (well-to-well and background contaminants) across manual and robot extraction plate and PCR replicate plates. Summary of compositionality of NTCs (n=48) vs. sink (n=32) vs. source microbes (n=16) processed in two facilities across five DNA extraction plates (a) UCSD tube extraction, (b) UCSD plate extraction 1, (c) UCSD plate extraction 2, (d) Argonne tube extraction, (e) Argonne plate extraction. UCSD DNA extractions were processed each twice thus had two PCR per plate (PCR A, PCR B).

Supplemental Figure S 4: Summary composition of reads (median, inter-quartile range) of specific sample types: NTCs, sink, or source microbes.

Supplemental Figure S 5. Determining the origin of well-to-well contamination and its impact on distance metrics from 96 unique WellIDs across three DNA extraction plates and six PCR plates. (a) Summary comparison of use of compositional (Bray-Curtis) or presence-absence (binary Jaccard) to describe microbial communities from NTCs (red), sink microbes (lower biomass), or source microbes (higher biomass). (b) Determining the effects of well-to-well contamination from PCR processing only (PCR replicates) compared to the entire process of DNA extraction and PCR (DNA extraction replicates). The statistical tests are performed on dark colors only while lightly shaded bars indicate the replicates for robot extraction plates.

542 **Figure Legends:**

543

544 Fig 1: Plate design, experimental design. (a) ntc 'white', sink 'purple', and source 'green'
545 samples are distributed in a checkboard pattern across the plate. Antifoam A is added
546 to last half (b) and first half (c) of the 96-well plates processed with the robot. The
547 manual samples did not get antifoam A. Each unique DNA extraction plate is processed
548 in duplicate PCR plates.

549

550 Fig 2: Example of plates with cross contamination; Each panel depicts a 96-well plate
551 with source, sink and blank wells denoted by "X", "O" empty squares respectively.
552 Colors indicate the number of reads from a specific bacteria (*Psychrobacter spp.*,
553 present in well E5). Panels a,b c,d and e,f correspond to two PCR replicates of robotic
554 extraction 1,2 and manual extraction respectively.

555

556 Fig 3: Distance decay relationship

557

558 Fig 4: Summary statistics of sample fraction composition of well-to-well contaminants
559 compared across extraction types (blanks - pink, sink - blue, source - purple) and
560 across extraction methods (tube vs. plate). Samples processed at UCSD in circles with
561 no outline and samples processed at Argonne as circles with dark border. All samples
562 with 0 well-to-well contamination occurrences are given a count of 0.00001 to enable
563 visualization on graph (labeled 0 counts). Median and interquartile range are displayed
564 in black lines over the data points.

565

566 Fig 5: Well-to-well effect size. Proportion of sample containing well-to-well contaminants
567 as organized by (a) sample type (ntc, sink, source) and (b) extraction method. Statistical
568 analysis within bars performed using Kruskal-Wallis non-parametric testing.

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Table 1. Summary of contamination (well-to-well and background) impact on NTCs, low biomass, and high biomass sample types

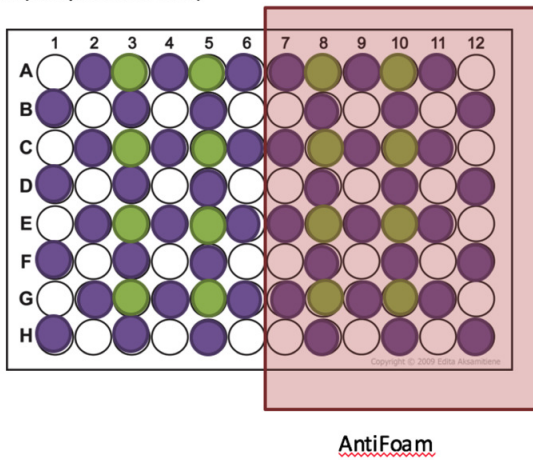
Type	Location	Extract	(Well-to-well)						(background-kits)		
			Prev mean	Richness Total	Richness W2W	Composition (mean, median, max)			Composition (mean, median, max)		
ntc(n)											
61	UCSD	m_tube	0.4754	20	0.041	0.046	0	0.56	0.9382	1	1
32	Argonne	m_tube	0.5313	165	0.016	0.009	0.0003	0.0823	0.9915	0.9997	1
28	Argonne	m_plate	0.1071	8	0.042	0.031	0	0.7517	0.9686	1	1
116	UCSD	r_plate	0.9569	15	0.278	0.583	0.6579	1	0.3307	0.2025	1
sink(n)											
93	UCSD	m_tube	0.1505	20	0.01	5E-04	0	0.0278	0.0335	0.0168	0.9873
48	Argonne	m_tube	0.5	189	0.017	0.023	0	0.5934	0.7808	0.8382	0.9878
46	Argonne	m_plate	0.3261	16	0.066	0.14	0	0.9871	0.5846	0.6267	1
187	UCSD	r_plate	0.6738	15	0.127	0.007	0.0008	0.1561	0.0091	0.0023	0.4051
source(n)											
31	UCSD	m_tube	0.6129	18	0.065	0.001	0.0001	0.0299	0.083	0.0029	1
16	Argonne	m_tube	0.875	21	0.138	2E-04	0.0002	0.0007	0.1154	0.0041	0.9998
16	Argonne	m_plate	0.8125	17	0.168	0.024	0.0001	0.364	0.1313	0.0032	0.9999
64	UCSD	r_plate	0.7031	12	0.138	0.009	0.0004	0.5067	0.0732	0.0016	1
Prev-prevalence (number of samples with any well-to-well contamination / total number of samples)											
Extract: DNA extraction method used											
m-manual (non-robotic based extraction)											
r-robot based DNA cleanup											

a

● Vibrio sink (1/100)

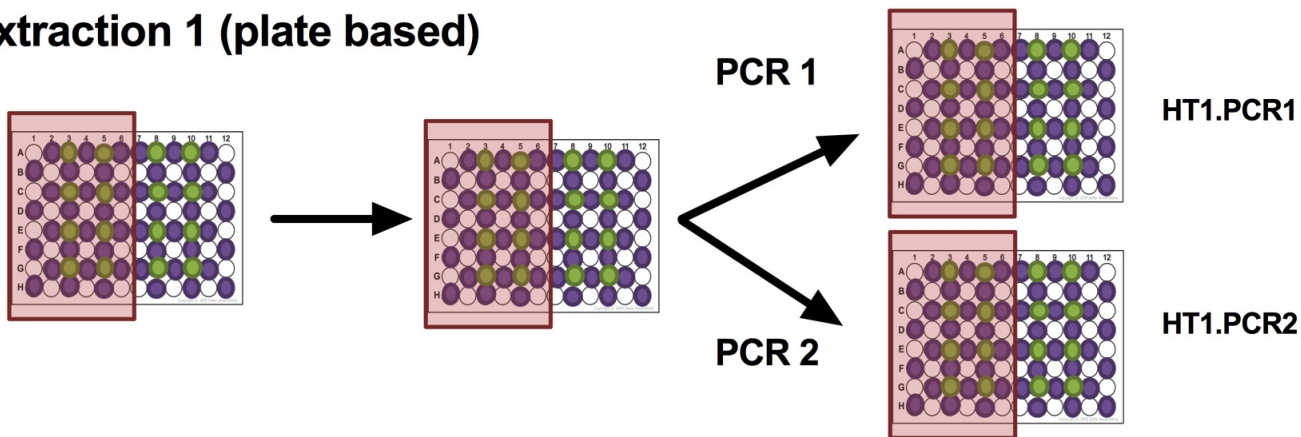
● Source bacteria (unique each well)

○ Blank NTC



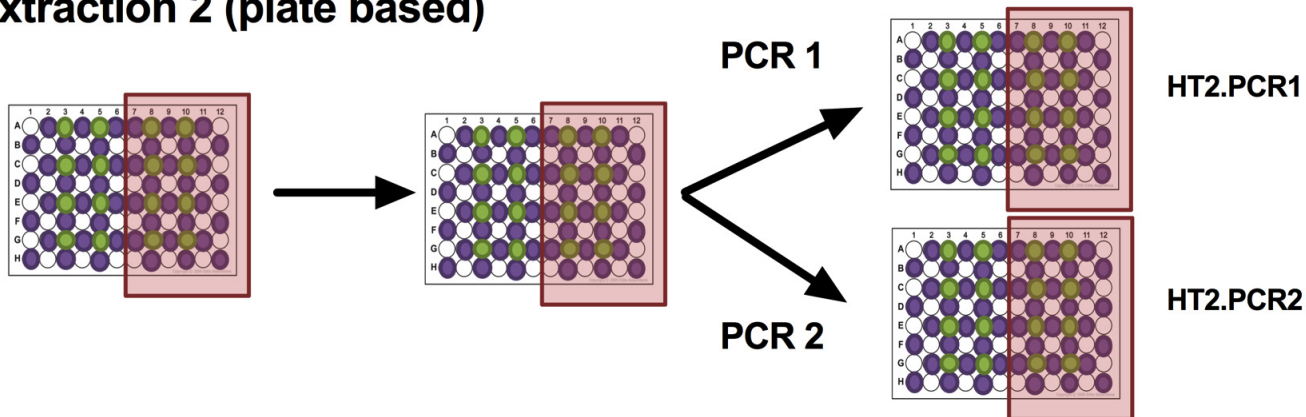
b

DNA Extraction 1 (plate based)



c

DNA Extraction 2 (plate based)



d

DNA Extraction 3 (individual tube)

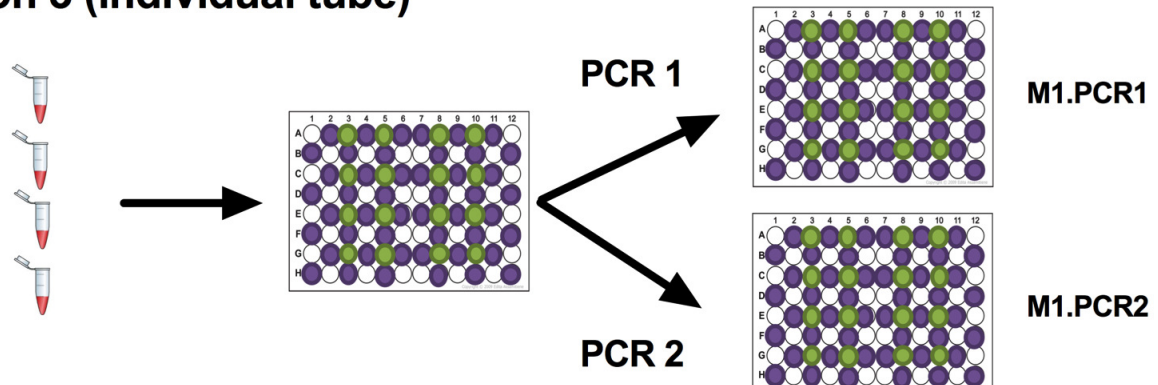


Figure 1

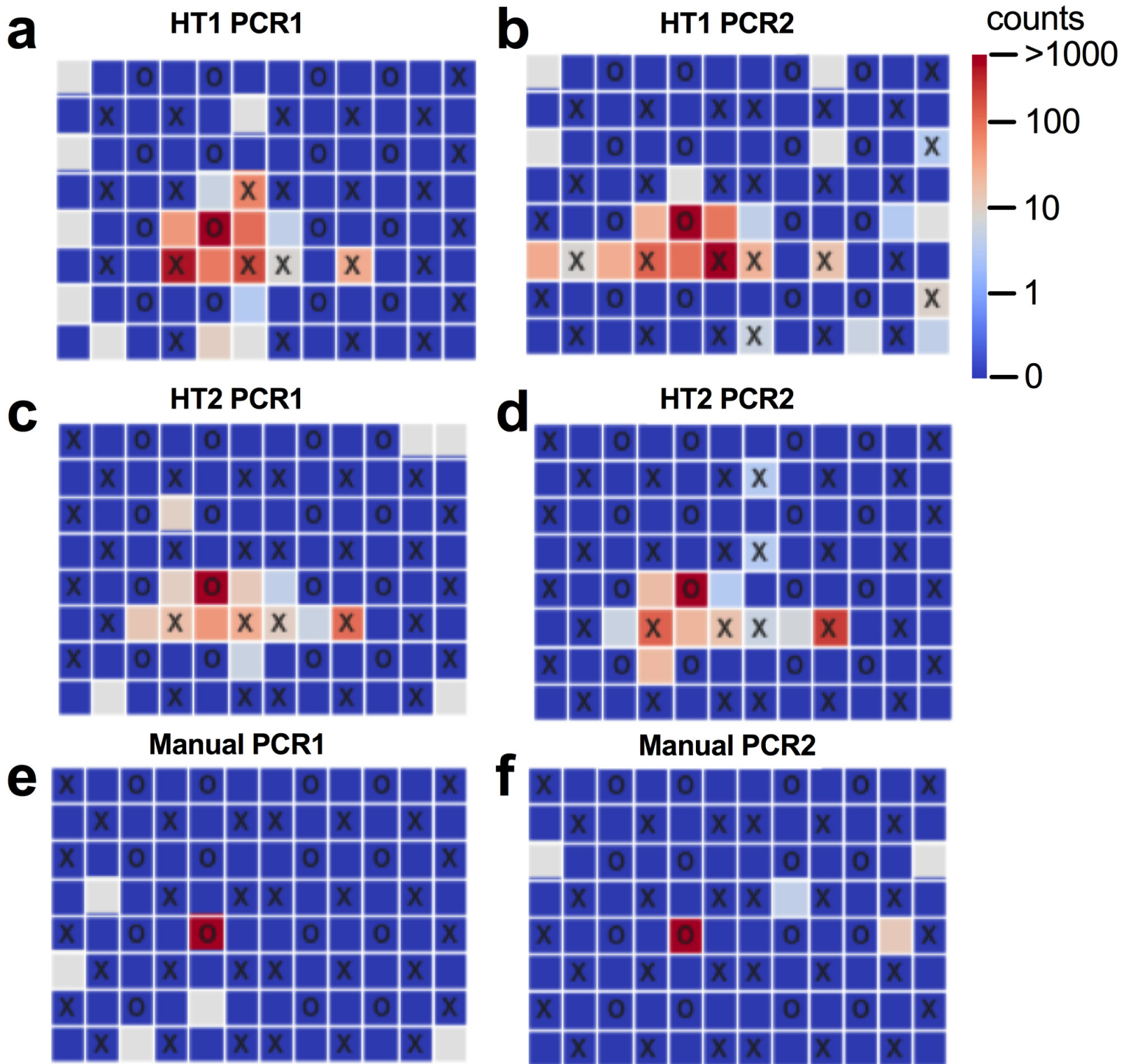
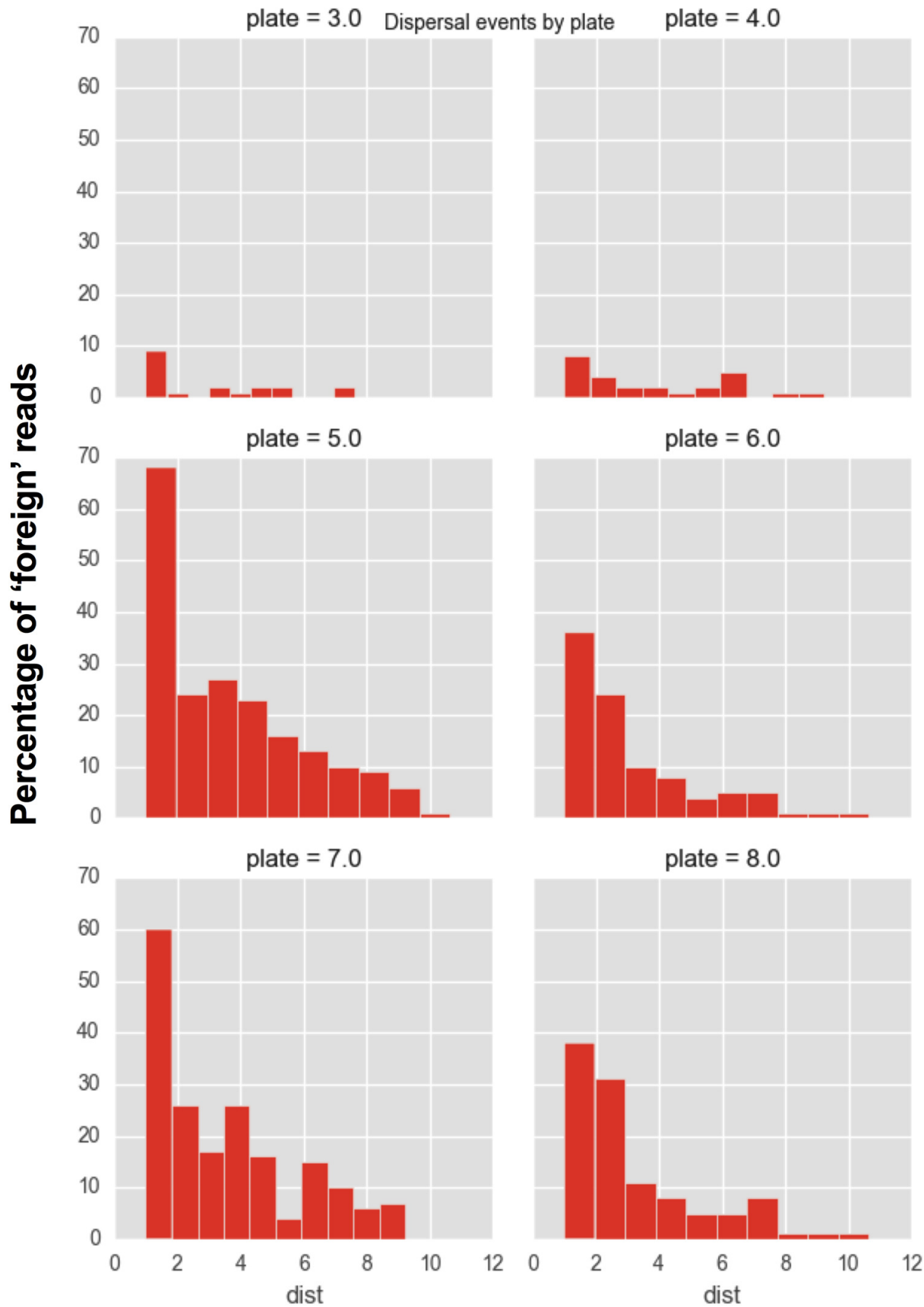


Figure 2

a

Tube



Distance (number of wells) away from source spike in microbe

Figure 3

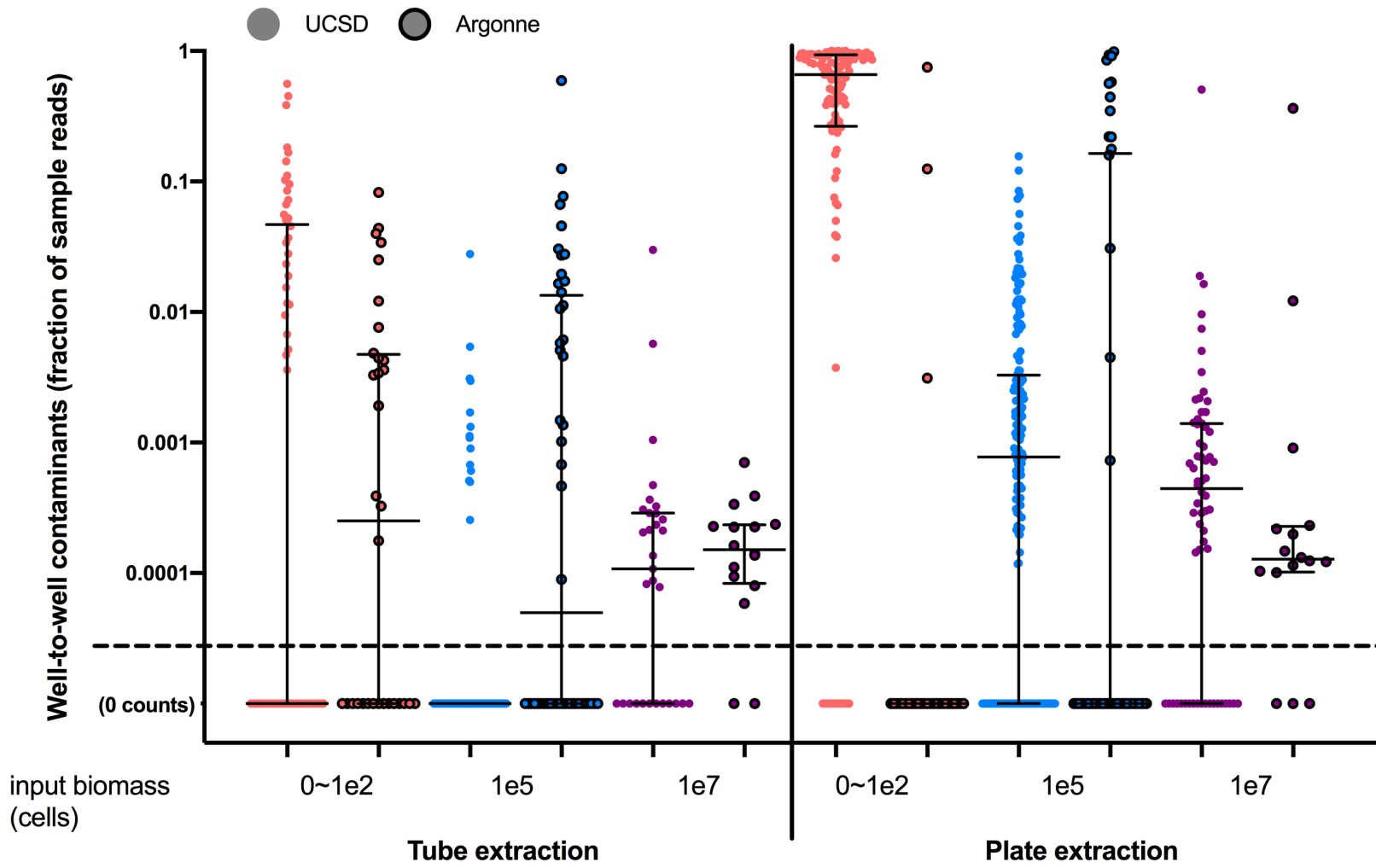


Figure 4

