

1 Optimization of DNA extraction from human urinary samples for mycobiome community profiling

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19 at the end of this paper.

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24 **Abstract**

25 *Introduction.* Recent data suggest the urinary tract hosts a microbial community of varying composition,
26 even in the absence of infection. Culture-independent methodologies, such as next-generation
27 sequencing of conserved ribosomal DNA sequences, provide an expansive look at these communities,
28 identifying both common commensals and fastidious organisms. A fundamental challenge has been the
29 isolation of DNA representative of the entire resident microbial community, including fungi.

30 *Materials and Methods.* We evaluated multiple modifications of commonly-used DNA extraction
31 procedures using standardized male and female urine samples, comparing resulting overall, fungal and
32 bacterial DNA yields by quantitative PCR. After identifying protocol modifications that increased DNA
33 yields (lyticase/lysozyme digestion, bead beating, boil/freeze cycles, proteinase K treatment, and carrier
34 DNA use), all modifications were combined for systematic confirmation of optimal protocol conditions.
35 This optimized protocol was tested against commercially available methodologies to compare overall and
36 microbial DNA yields, community representation and diversity by next-generation sequencing (NGS).

37 *Results.* Overall and fungal-specific DNA yields from standardized urine samples demonstrated that
38 microbial abundances differed significantly among the eight methods used. Methodologies that included
39 multiple disruption steps, including enzymatic, mechanical, and thermal disruption and proteinase
40 digestion, particularly in combination with small volume processing and pooling steps, provided more
41 comprehensive representation of the range of bacterial and fungal species. Concentration of larger
42 volume urine specimens at low speed centrifugation proved highly effective, increasing resulting DNA
43 levels and providing greater microbial representation and diversity.

44 *Conclusions.* Alterations in the methodology of urine storage, preparation, and DNA processing improve
45 microbial community profiling using culture-independent sequencing methods. Our optimized protocol
46 for DNA extraction from urine samples provided improved fungal community representation. Use of this

47 technique resulted in equivalent representation of the bacterial populations as well, making this a useful

48 technique for the concurrent evaluation of bacterial and fungal populations by NGS.

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52 **Introduction**

53 Multiple organs, such as the gut, oral cavity, and vagina, have long been known to harbor communities of
54 microbes that can protect against or contribute to disease under different circumstances. The urinary
55 tract, however, was widely thought to be sterile until only recently, when extended culture techniques
56 and the detection of microbial DNA definitively demonstrated microbial communities of great diversity
57 within this site.[1-3] Currently, culture-independent microbial characterization using the sequencing of
58 highly conserved DNA regions, such as the ribosomal RNA gene locus (rDNA), is widely-accepted as a
59 useful, sensitive tool to explore microbial populations. These next-generation sequencing (NGS)
60 technologies are particularly useful in characterizing microbes that may be difficult to culture or that are
61 present in low abundance (the “rare biosphere”).[4] Therefore, the composition and diversity of the
62 urinary microbiome has likely been drastically understated, in part, due to dependence on culture
63 methods to identify resident species.

64 With the development of affordable, rapid, and scalable culture-independent methods for the study of
65 bacterial communities, the last decade has seen a massive expansion in studies aimed at profiling
66 commensal communities in a multitude of organs not included in the large-scale Human Microbiome
67 Project (HMP), such as the urinary tract. Using NGS methods, multiple studies have demonstrated that
68 perturbations in the urinary microbiota appear to correlate with Lower Urinary Tract Symptoms (LUTS).[5-
69 13] The clinical significance and utility of these alterations, however, remain unclear, primarily due to
70 challenges that persist for the characterization of microbes from low biomass specimens, such as urine.

71 Due to these limitations, we still lack vital information about the content of normal urine and its
72 relationship to dysbiosis and/or disease. Studies examining the urinary microbiome thus far demonstrate
73 wide variation in their ability to consistently detect microbial species. In many studies, approximately half
74 of patient samples do not have bacterial sequences of sufficient quality for analysis[2, 6, 14]; in other
75 studies, this efficiency could be improved with the use of multiple amplification steps[11], but this may

76 introduce new biases that could skew results. This low sequencing efficiency is likely due to the
77 combination of low biomass and the unique qualities of urine, which include high variability in
78 osmolality/salt content, high abundance of PCR inhibitors, and fluctuating levels of cellular material, all in
79 all making urine a challenging biological fluid to study. The question remains as to whether these
80 sequence-negative samples are truly negative for microbes or whether our detection methods are
81 inadequate to fully characterize these specimens. Until this question can be answered, it remains a very
82 real possibility that the subset of samples analyzed, the “sequence-positive” group, may represent a
83 unique subgroup within the analyzed population with higher microbial loads, whose findings cannot be
84 generalized to the larger sample population.

85 Even less is known about the composition of non-bacterial populations, such as fungi, viruses, archaea,
86 and protozoa, in the genitourinary tract and other human organs, primarily from a lack of well-researched
87 tools for their analysis. Despite these challenges, alterations in the fungal microbiota (the “mycobiome”)
88 in the absence of frank infection have been demonstrated in multiple human diseases, such as hepatitis
89 [15], atopic dermatitis [16], inflammatory bowel disease [17-19], cystic fibrosis [20], allergy/atopy [21],
90 asthma [22], and psoriasis [23, 24]. As yet, only a few analyses have examined aspects of the urinary
91 mycobiome. *Candida spp.* have been detectable in urinary samples by culture,[5-8] demonstrating their
92 viability. Fungi were also detectable in urine from patients with urological chronic pelvic pain syndromes
93 (UCPPS) using the targeted Ibis T-5000 Universal Biosensor system.[25] Interestingly, fungi were detected
94 more frequently in UCPPS patients during symptomatic flares, while no significant differences in the
95 bacterial microbiota could be identified, implicating fungi as important players in lower urinary tract
96 symptomatology. Even in this culture-independent study, however, fungi were detected in less than 10%
97 of patients overall. Again, it is unclear if this low number is representative of the absence of fungi in the
98 majority of subjects or represents severe limitations in our current technologies.

99 Further progress in identifying consistent microbial markers or understanding the pathophysiology of
100 microbial interactions in the urinary tract requires methodologies that adequately and reliably
101 characterize these populations, and which include fungi and other microbes in addition to bacteria. In this
102 study, we sought to identify the most effective strategies for extracting and identifying microbial DNA
103 from urine, with a focus on enhancing the detection of fungi. Using an iterative approach, we optimized
104 urine sample processing at multiple steps to increase DNA yields and population representation to
105 generate more consistent data from sequencing-based microbial population analyses.

106

107 **Materials and Methods**

108 This study was approved by the Cedars-Sinai Institutional Review Board (Pro00033267) and written
109 consent was obtained from all subjects.

110 *DNA yield assessment*

111 Overall DNA yields and quality (assessed by OD₂₆₀/OD₂₈₀ ratios) were measured on the NanoDrop 2000
112 Spectrophotometer (Thermo Scientific). Fungal DNA levels were assessed in duplicate by quantitative
113 Real-Time Polymerase Chain Reaction (qRT-PCR) analyses on a Mastercycler Realplex2 (Eppendorf) using
114 the SYBR Green PCR kit as instructed by the manufacturer (Applied Biosystems). Fungal levels were
115 assessed using the Fungiquant primers (forward: 5'-GGRAAACTCACCAGGTCCAG-3'; reverse: 5'-
116 GSWCTATCCCCAKCACGA-3')[26] that recognize a highly-conserved segment of the fungal 18S rDNA
117 region, while bacterial levels were assessed using 16S rDNA primers (forward: 5'-
118 ACTCCTACGGGAGGCAGCAGT-3'; reverse: 5'-ATTACCGCGGCTGCTGGC-3'), a universal primer with broad
119 specificity for bacteria. The qRT-PCR protocol employed an initial denaturation at 94°C for 10 min,
120 followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C
121 for 2 min, followed by an elongation step at 72°C for 30 min. Relative quantity of bacterial and fungal DNA
122 yields, consistent from experiment to experiment, was calculated by the comparative CT method ($2^{-\Delta\Delta C_T}$
123 method)[27] and normalized to a DNA standard curve derived from a mixed bacterial and fungal culture
124 that remained constant over all tests. Samples with greater than 3% variance between duplicates were
125 reanalyzed in duplicate. An aliquot of 1 µl of the PCR product was evaluated by 2% agarose gel
126 electrophoresis.

127 *Evaluation of Individual protocol enhancements*

128 For the initial, iterative analyses, specimens were obtained from mid-stream urine collections from
129 multiple male and female subjects, all of whom denied any urinary symptoms, after preparation of the
130 external urethral meatus with chlorhexidine gluconate wipes. Urine specimens were mixed well, then

131 divided into 1 ml samples and centrifuged at 5000 relative centrifugal force (rcf) to pellet cellular material
132 prior to parallel processing to test the individual protocol variations described below.

133 *Enzymatic disruption.* Sample pellets were initially resuspended in 500 μ l enzyme buffer (0.5 M Tris, 1mM
134 EDTA, and 0.2% 2-mercaptoethanol, pH 7.5). We added 200 U/ml Lyticase (Sigma Aldrich), 20 mg/ml
135 Lysozyme (Thermo Scientific), both enzymes, or buffer alone without enzyme. Samples were then
136 incubated for 30 min. at 30°C, with inversion of the tubes every 5-10 min. Subsequently, samples were
137 centrifuged at 1500 rcf for 5 min, the supernatant removed, and the pellet resuspended in 800 μ l of Stool
138 DNA Stabilizer (Stratec Biomedical).

139 *Mechanical disruption.* Physical disruption of cell walls was accomplished with bead beating. The 800 μ l
140 post-enzymatic digestion cell suspension was transferred to a 2 ml centrifuge tube containing 100 μ l 0.1
141 mm and 300 μ l 0.5 mm silica beads (Biospec Products, Inc.). Samples were agitated twice for 1 min each
142 on a standard Vortex mixer using a Vortex Adapter for bead beating (MO BIO Laboratories Inc.). Samples
143 were centrifuged for 15 s at 17000 rcf between bead beating periods.

144 *Thermal disruption.* Samples were heated to 95°C for 10 min, with a brief vortex to ensure adequate
145 mixing 5 min into the incubation. After a second, brief vortexing step, samples were incubated on ice (0°C)
146 for 5 min, then centrifuged for one min. at 17000 rcf after each boil/freeze cycle.

147 *Proteinase digestion.* After cell wall disruption, cell lysates were transferred to new tubes containing an
148 equal volume of buffer AL (Qiagen) containing varying concentrations of Proteinase K (0, 12, 24, 48, 72,
149 96, 120, or 144 mAU/ml)(Qiagen), then incubated at 70°C for 10 min.

150 *Addition of carrier DNA.* When specified, polyadenylic acid carrier DNA (PolyA) (Roche Diagnostics) was
151 added to the cell lysates at the time of proteinase K digestion.

152 *Column DNA extraction.* Following the specified disruption and digestion steps, 250 μ l 100% Ethanol was
153 added and briefly mixed by vortexing, prior to applying the cell lysates to Qiagen Mini DNA Spin columns
154 (Qiagen). The columns were washed twice with a column volume of buffer AW (Qiagen) by centrifugation
155 at 17000 rcf for 1 min and residual alcohol removed with a third spin without wash buffer. DNA was then
156 eluted from the column in 60 μ l warm Tris-EDTA (TE) buffer.

157 *Confirmation of protocol components in aggregate analysis*

158 For 4 subjects (2 male and 2 female), >60 ml of urine were obtained and divided into 1 ml samples within
159 1 h of sample collection. Twenty unique conditions were analyzed following centrifugation at one of three
160 centrifugation conditions: 1) 1500 rcf for 15 min., 2) 5000 rcf for 20 min., or 3) 16000 rcf for 10 min. The
161 resulting pellets were frozen and stored at -80°C. 20 unique combinations of the conditions explored in
162 initial, iterative analysis were performed, with inclusion or exclusion of the individual enzymatic
163 treatments, mechanical and thermal disruption steps, and proteinase digestion in almost all
164 combinations. For this panel of conditions, carrier DNA was included in all samples to provide better
165 discrimination of differences in these low volume samples.

166 Relative DNA yields for each condition were determined by fungal-specific qPCR as specified above; for
167 each sample, yields were scaled to equal variance for all samples to allow plotting of the median yields
168 for each condition as a heat map.

169 *Determination of optimal sample volume*

170 Large volume urine samples (>100 ml) from 3 male and 3 female subjects were mixed well and subdivided
171 into 1, 2.5, 5, 10, 25, and 50 ml aliquots. Each sample was centrifuged at 1500 rcf and the supernatant
172 decanted. After pelleting, all samples were identically processed using the optimized protocol detailed
173 above. All aliquots for an individual subject were processed in batches to minimize batch-to-batch

174 variation. The resulting fungal DNA concentrations were then quantitated by qRT-PCR. Taxal diversity was
175 also examined by 2% agarose gel electrophoresis.

176

177 *Sample subdivision and pooling*

178 To evaluate if processing lysates in smaller volumes provided increased DNA yields, samples were
179 subdivided into smaller aliquots after mechanical disruption. Seven identical urine specimens were
180 pelleted, digested with lysozyme and lyticase, then subjected to bead beating with a mixture of silica
181 beads as detailed above. Sample quantities ranging from 100 μ l to 400 μ l (of an approximately 500 μ l total
182 lysate volume) at 50 μ l intervals were aspirated off of the beads and subjected to thermal disruption,
183 proteinase K digestion and DNA-column binding and elution.

184 To examine if total DNA yields could be increased by pooling these smaller aliquots, sample lysates were
185 subdivided into two 250 μ l aliquots after mechanical disruption, then subjected to thermal disruption and
186 proteinase digestion separately. These two samples were then applied to either a single DNA-binding
187 column in succession or to two separate columns, eluted and pooled after elution. Overall and fungal-
188 specific DNA yields were then measured using NanoDrop DNA quantitation and fungiquant qRT-PCR.

189 *Light microscopy*

190 After centrifugation, cellular pellets from urine were resuspended in 5 ml PBS and mixed well with a
191 pipette. A 10 μ l aliquot was transferred to a 75 \times 26-mm glass slide and covered with an 18 \times 18-mm
192 coverslip, ensuring that the sediment was uniformly distributed but not escaping from the edges of the
193 coverslip. Using an inverted IX51 microscope (Olympus), images without staining were captured at \times 400
194 (objective lens 40 \times in combination with wide field 10 \times eyepiece) to generate a field area of 0.196 mm².

195 *Comparison with commercial methods*

196 We compared our optimized approach to three, commonly used commercial kits for DNA extraction: PSP®
197 Spin Stool DNA Plus Kit (Stratec Biomedical), PureLink™ Microbiome DNA Purification Kit (ThermoFisher
198 Scientific), and QIAamp DNA Stool Mini Kit (Qiagen). Large volume urine specimens (>120 ml) from 9
199 subjects were divided into four 30 ml specimens and pelleted by centrifugation at 1500 rcf. Mid-vaginal
200 swabs were obtained from female subjects using FloQSwabs (Copan Diagnostics). Swabs were gently
201 agitated for 30 min in 500 µl enzyme buffer (0.5 M Tris, 1mM EDTA, and 0.2% 2-mercaptoethanol, pH 7.5),
202 before removing the swab; the resulting cell suspension was then processed as for urine specimens. The
203 identical urine samples and vaginal swabs were processed according to the manufacturers' protocols for
204 each kit or using our optimized protocol. Fungal and bacterial DNA yields in the eluents were then
205 assessed by qPCR as specified above.

206 *Microbial Sequencing Analysis.*

207 *Library Generation.* DNA was isolated from urine using the specified protocols as described above. Fungal
208 ITS1 and bacterial 16S regions amplicons were generated by PCR using the primers below modified to
209 include Nextera XT v2 barcoded primers (Illumina) to uniquely index each sample. PCR reactions utilized
210 Platinum SuperFi DNA Polymerase (Invitrogen) according to the following protocol: initial denaturation at
211 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 30 s, and
212 elongation at 72°C for 2 min., followed by an elongation step at 72°C for 30 min.

Amplicon	Forward	Reverse
ITS1	5'-CTTGGTCATTTAGAGGAAGTAA-3'	5'- GCTGCGTTCTTCATCGATGC-3'
16S (8F&R357)	5'-AGAGTTTGATCMTGGCTCAG-3'	5'-CTGCTGCCTYCCGTA-3'

213 *Next-generation sequencing.* Amplicons generated above were sequenced at 2x300 paired-end
214 sequencing on the Illumina MiSeq sequencer, according to manufacturer's instructions. Raw data
215 processing and de-multiplexing was performed using on-instrument MiSeq Reporter Software v2.6 as per
216 manufacture recommendations. Demultiplexed 16S sequence data were processed and analyzed as

217 previously described including OTU assignment by alignment to the GreenGenes reference database (May
218 2013 release) at 97% identity.[28] For analysis of ITS1 sequence data, raw FASTQ data were filtered to
219 enrich for high quality reads including removing the adapter sequence by Cutadapt v1.4.1,[29] truncating
220 reads with average quality scores less than 20 over a 3-base pair sliding window and removing reads that
221 do not contain the proximal primer sequence or that contain a single unknown base. Filtered pair-end
222 reads were then merged with overlap into single reads using SeqPrep v1.1 wrapped by QIIME v1.9.1.[30]
223 Processed high-quality reads were then aligned to previously observed host sequences (including rRNA
224 and uncharacterized genes in human) to deplete potential contamination. Operational taxonomic units
225 (OTU) were identified by alignment of filtered reads to the Targeted Host Fungi (THF) custom fungal ITS
226 database (version 1.6), [31] using BLAST v2.2.22 in the QIIME v1.9.1 wrapper with an identity percentage
227 $\geq 97\%$.

228 *Diversity analysis*

229 We performed rarefaction analysis. The original OTU table was randomly subsampled (rarefied) to create
230 a series of subsampled OTU tables. Alpha diversity was calculated on each sample using the OTU table
231 and a variety of metrics (chao1, observed species, etc.). The results of the alpha diversity were collated
232 into a single file and the number of species identified for each sample versus the depth of subsampling
233 was plotted. Shannon diversity indices were selected to show composite readout of microbial population
234 evenness and richness.

235 *Statistical analysis*

236 Differences in DNA yields between groups were compared using a two-tailed, paired Student's *t* test with
237 a 95% confidence interval. Data are presented as means \pm SEM, unless otherwise stated. Statistical
238 analyses were performed using Microsoft Excel 2016 (version 1803) or RStudio version 3 as appropriate.

239

240 **Results**

241 *Sequential optimization of fungal DNA extraction*

242 To optimize the procedure of isolating urinary microbial DNA, we began with a protocol described in the
243 initial isolation of bacteria from urine specimens,[5, 32]. Small volumes of urine were initially centrifuged
244 to concentrate cells and microorganisms, then subjected to DNA extraction using a standardized kit
245 involving DNA binding and elution from an affinity column.

246 To concentrate the rare cellular material present in urine, samples were centrifuged under three
247 conditions previously described for the isolation of fungi from low biomass fluids.[32-34] Samples
248 prepared with an initial centrifugation speed of 1500 relative centrifugal force (rcf) for 20 min. yielded
249 fungal DNA levels at least 1.5-fold higher than those prepared at 5000 rcf for 10 min., while yields from
250 those centrifuged at 16000 rcf for 10 min. were substantially lower (Figure 1A).

251 **Figure 1. Optimization of Microbial DNA Extraction Requires Multiple Disruption Steps.** (A) Eight variations
252 in the protocol (at left) were noted to increase yields as determined by quantitative PCR. Relative fungal
253 DNA yields were calculated from quantitative PCR using the Fungiquant pan-fungal PCR primer pair and
254 normalized to a mixed fungal DNA standard. The negative control samples were processed in parallel, but
255 did not have any input cellular material. Multiple protocol variations, such as enzymatic pre-digestion (B)
256 or carrier DNA use during DNA column binding (C), were tested individually in triplicate for multiple
257 subjects (minimum n=4), both male and female, before incorporating.

258 Fungi and some bacteria have cell walls, which can be resistant to digestion, leading to their absence or
259 underrepresentation in culture-independent analyses. To optimize the isolation of organisms with robust
260 cell walls, we examined the utility of an initial enzymatic digestion step to aid in cell wall dissolution.[35]
261 Lysozyme, a glycolytic hydrolase that catalyzes the breakdown of peptidoglycan in gram-positive bacterial
262 cell walls, is known to enhance gram-positive bacterial detection.[36] Lyticase, which hydrolyzes the poly-
263 $\beta(1\rightarrow3)$ -glucose present in yeast cell wall glycans, has been widely used in yeast DNA extraction, including
264 PCR-based clinical assays.[34, 37] These enzymes were tested alone and in combination in comparison to
265 omission of this step. Consistently, the combination of the two enzymes resulted in improved yields of
266 both total DNA (data not shown) and relative fungal DNA levels calculated by qPCR (Figure 1B).

267 Particularly for fungi, physical disruption techniques, such as the thermal and mechanical steps described
268 above, significantly improve fungal DNA purification,[38-40] again by further breaking down tough cell
269 walls. Bead beating, which we performed using multiple sizes of silica beads, can be particularly useful in
270 isolation of fungi such as *Aspergillus*, which is known to play a role in multiple human diseases.[41, 42] An
271 additional thermal disruption step, with two freeze-boil (0°C/95°C) cycles, was also evaluated. Both
272 methods used in isolation enhanced DNA extraction efficiency 2-3-fold over baseline (Figure 1A).

273 These disruption steps were followed by an additional digestion step with Proteinase K, a broad-spectrum
274 serine protease, to remove any protein contamination and inactivate any remaining DNAase activity prior
275 to cell and nuclear lysis. We tested a range of proteinase concentrations; while inclusion of the enzyme
276 was important in enhancing DNA extraction efficiency, varying the proteinase concentration had much
277 less effect. While a concentration of 24 mAU/ml (0.8 µg/ml) tended to provide the best results, a range
278 of concentrations from 12-144 mAU/ml (4.8 µg/ml) did not differ significantly in their enhancement of
279 DNA recovery (data not shown).

280 To maximize DNA recovery, we also evaluated the addition of carrier DNA. Because naturally occurring
281 carriers, such as salmon sperm DNA, contain rDNA sequences with partial homology to other eukaryotic
282 DNA, we chose a synthetic carrier, polyadenylic acid, which has shown efficacy in enhancing recovery of
283 low abundance DNA from human biological samples.[43] Supplementation of carrier DNA increased both
284 overall (data not shown) and fungal-specific DNA yields 2-4 fold across all samples (Figure 1C). The best
285 combination of all techniques tested resulted in an almost 14-fold increase in fungal DNA yields,
286 comprising an optimal protocol utilizing low-speed centrifugation, enzymatic, mechanical, and thermal
287 cell wall disruption, inclusion of carrier DNA, and proteinase K digestion in combination.

288 *Confirmation of protocol on standardized samples*

289 Each of the individual conditions noted to increase DNA yields were tested in aggregate on a panel of
290 urine specimens from both male and female patients. Large volume (>75 ml) urine specimens from 4
291 subjects (2 male and 2 female) were divided into small equal aliquots (1 ml), and then processed in parallel
292 to confirm the enhancement of DNA purification with the modifications observed in the individual
293 experiments detailed above. This larger-scale optimization panel assessed the variations in cell wall
294 disruption methods (thermal and mechanical), enzymatic pre-treatment methods (lysozyme and lyticase),
295 proteinase K digestion, and centrifugation speed in almost all combinations (Figure 2). Calculation of the
296 relative fungal DNA yields from these 60 variations in isolation methodology revealed a clear pattern, with
297 improved yields resulting from the optimized protocol defined above with multiple disruption methods,
298 combined enzymatic digestion, and lower centrifugation speeds.

299 **Figure 2. Large-Scale Confirmation of Optimization of Microbial DNA purification.** The individual
300 conditions noted to increase yields were tested in aggregate in a larger-scale optimization panel. DNA was
301 concurrently isolated from 60 identical 1 ml urine samples from each of 4 subjects with variations in cell
302 wall disruption methods (as indicated at the top), enzymatic pre-treatment methods (bottom), and
303 centrifugation speeds (rows indicated at right adjacent to heat map). Fungal DNA yields from these 60
304 variations in isolation methodology were calculated from Fungiquant qPCR as described in Figure 1, then
305 scaled across all samples. Values are expressed as a heat map, with bright red signifying the highest yields
306 and black the lowest yields across all samples.

307 *Effect of sample volume on community profiling*

308 Specimen volume is thought to influence the representation of microbial complexity determined by NGS,
309 particularly in low biomass specimens, such as urine[44], which was also suggested by our preliminary
310 results (Figure 1C). To determine the magnitude of the effect of sample volume on microbial yields and
311 community depth and diversity, we examined microbial profiles across a range of urine sample volumes.
312 Large volume urine specimens from individual subjects were divided into 1, 2.5, 5, 10, 25, and 50 ml
313 aliquots and processed in parallel according to our optimized protocol described above. Fungal yields
314 (Figure 3A) were greatest with larger urine volumes. However, the optimal volume of initial urine was 25

315 ml, with 10 and 25 ml samples yielding substantially greater DNA concentrations than smaller or larger
316 amounts. Average yields across all specimens *decreased* in 50 ml samples.

317 **Figure 3. Fungal community representation is influenced by specimen volume.** (A) DNA was isolated from
318 a range of urinary volumes in male and female subjects (n=3 each) and assessed by qPCR for fungal DNA.
319 Calculated fungal DNA concentrations were calculated by normalization to a fungal standard. The optimal
320 concentrations were achieved using 25 ml urine specimens. *: $P < 0.05$ in comparison to 1 mL yields. (B)
321 Following fungal DNA amplification by qPCR using broad-spectrum fungal primers, products from 25, 5
322 and 1 ml samples were assessed by 2% agarose gel electrophoresis. Standards indicating the PCR product
323 size are shown on the left. Each band represents unique taxa within the urinary fungal population. NPC:
324 no primer control.

325 We also assessed community complexity by gel electrophoresis following PCR-based amplification of the
326 fungal ITS1 rDNA region in which different sized products represent unique fungal taxa (Figure 3B). In
327 comparison to sample sizes of 5 ml or less, 25 ml provided a more comprehensive representation of the
328 range of fungal species with an increased number of bands of varying sizes representing unique taxa for
329 larger initial sample sizes. Across all volumes, urine from male subjects consistently demonstrated lower
330 yields. Only at the 25 ml volume were fungal DNA yields consistently above quality control thresholds.

331

332

333 *Effect of urine storage and centrifugation conditions on DNA extraction efficiency*

334 In handling urine, we sporadically observed after centrifugation a substantial, sand-like pellet of varying
335 colors. The appearance of this non-cellular pellet material was observed with refrigeration (>2 hours) of
336 urine samples prior to processing and with high-speed centrifugation (16,000 rcf). Post-centrifugation
337 pellets from larger urine volumes (>50 ml) also would frequently contain this material, even when pelleted
338 at lower speeds (1500-5000 rcf) and processed at room temperature. Microscopic examination of these
339 samples revealed a range of crystalline forms, typically amorphous urates or phosphates, depending on
340 urinary pH. When these microcrystal salts appeared, DNA quality, as assessed by OD_{260}/OD_{280} ratios, was

341 significantly lower. Relative DNA yields were also consistently lower, suggesting that larger crystal burden
342 interfered with DNA purification. One such post-centrifugation specimen (shown in Figure 4A)
343 demonstrates a red-orange, sandy pellet, the “brick-layer’s dust” characteristic of amorphous urates.
344 Confirmation of crystal composition was supported by microscopic analysis (Figure 4B) as well as chemical
345 properties; these pellets could be dissolved by either heating to a temperature >60°C or adding sodium
346 hydroxide. In a smaller subset of alkaline urine specimens, refrigeration or high-speed prolonged
347 centrifugation resulted in a light-colored sandy pellet, which could be identified as amorphous phosphates
348 by microscopy (Figure 4C). Chemical composition was confirmed by solubility in glacial acetic acid and
349 resistance to dissolution with heating[45]. Other crystal forms were occasionally noted, such as the
350 “envelope”-type crystals characteristic of calcium oxalate (Figure 4D inset), but these did not typically
351 constitute any sizable portion of the crystalline material. We were able to minimize the appearance of
352 crystalline salts through a combination of expedient processing (within 4 hours of sample acquisition), the
353 avoidance of refrigeration, and optimization of sample size and centrifugation speed.

354 **Figure 4. Urine storage and centrifugation conditions impact DNA extraction efficiency.** In a subset of urine
355 samples, both refrigeration and high-speed centrifugation were associated with precipitation of varying
356 crystals that interfered with DNA purification. (A) A single urine specimen before and after refrigeration
357 and centrifugation at 5000 rcf. In the post-centrifugation specimen, a red-orange, sandy pellet was
358 observed after centrifugation consistent with the “brick-layer’s dust” characteristic of amorphous urates.
359 (B) The pellet seen in A was examined by light microscopy (x400 magnification), revealing disorganized
360 amorphous urate crystals. (C) Amorphous phosphates from alkaline urine. (D) The “envelope”-type
361 crystals characteristic of calcium oxalate could also be identified in urine (magnified in the inset picture),
362 but did not constitute the majority of the crystalline material.

363 As amorphous urates and phosphates can inhibit individual steps in DNA purification and PCR
364 amplification, we next sought to determine if varying processing volumes could minimize any impact of
365 these salt contaminants on DNA purification and subsequent PCR amplification. In addition, we
366 hypothesized that smaller sample volumes might be more effectively heated for thermal disruption. After
367 combined enzymatic treatment and mechanical cell wall disruption, we subdivided samples into varying
368 aliquot sizes for the two boil/freeze cycles, proteinase K digestion, and DNA isolation using a DNA-binding

369 column. Volumes ranging from 25% to 80% (100-400 μ l in 50 μ l increments) of the total sample lysate
370 were applied to the spin columns before washing and DNA elution. Small sequential increases in DNA
371 yields were seen up to 250 μ l, but then plateaued, without additional increase in DNA yields with larger
372 volumes (Supplemental Figure 1A).

373 **Supplemental Figure 1. Smaller volume sample processing and pooling increases DNA purification yields.**
374 Standardized urine samples from 4 subjects were pelleted and processed using the optimized purification
375 protocol for enzymatic treatment and cell wall disruption. (A) Prior to the addition of proteinase K, varying
376 quantities of the total sample lysate were transferred to new tubes for digestion and DNA column binding.
377 Lysate quantities \geq 250 μ l provided equivalent yields. (B) Prior to the addition of proteinase K, sample
378 lysates were divided into 250 μ l aliquots. Processing of a single 250 μ l aliquot (No pooling) was compared
379 to the results if the lysate was split into two aliquots and processed in parallel, then later pooled on either
380 a single DNA-binding column and eluted as a single sample (1 column) or purified separately on two
381 columns, eluted independently and then pooled (2 columns). Control samples were processed in parallel
382 and did not have any input cellular material.

383 These data suggested that a portion of the DNA in our samples was not either effectively digested or
384 binding to the extraction column. We therefore attempted pooling of subdivided samples; sample lysates
385 were divided into equal halves (\sim 250 μ l) and processed in parallel before column binding. Lysates were
386 then either pooled onto a single column in two subsequent binding steps and eluted in a single elution or
387 bound and eluted from separate columns and pooled after elution. Pooling of two 200-250 μ l aliquots on
388 a single DNA column provided the best DNA yields (Supplemental Figure 1B).

389 *Our optimized method outperforms previously described and commercial DNA isolation methods.*

390 We then evaluated our method in comparison to several commercial DNA kits commonly used for
391 microbial analysis. This optimized protocol yielded higher concentrations of DNA and greater species
392 diversity for fungal DNA than identical samples processed with the PSP[®] Spin Stool DNA Plus Kit,
393 PureLink[™] Microbiome DNA Purification Kit, and QIAamp DNA Stool Mini Kit (Figure 4). As the ideal goal
394 of this method would be the simultaneous examination of both fungal and bacterial populations, we also
395 assessed the utility of the optimized protocol in the isolation of bacterial DNA. Our protocol consistently

396 outperformed commercial methods for the purification of fungal as well as bacterial DNA (Figure 5A). To
397 assess the applicability of this protocol to other human commensal microbial communities, we analyzed
398 a panel of vaginal swabs as well. Our protocol enhanced fungal and bacterial recovery from vaginal swabs
399 significantly. While the method previously described for urine samples[5, 32] using Qiagen DNA isolation
400 kits (Qiagen) was already better than the commercial kits tested, the optimized protocol increased the
401 yield of fungal DNA approximately 200% ($p<0.001$) for vaginal swabs and 130% ($p<0.005$) for 30 ml urine
402 samples. Bacterial yields differed even more profoundly, increasing yields approximately 240% for vaginal
403 swabs and 200% for urine over levels seen with the best of previously described methods ($p<0.001$).

404 **Figure 5.** *Our optimized DNA extraction method outperforms commercial methods.* We compared fungal
405 (left) and bacterial (right) extraction and characterization after our optimized protocol in comparison to
406 three commercial DNA preparation kits using standardized urine and vaginal swab samples. (A) Individual
407 urine specimens were divided into equal aliquots of 30 ml each. DNA was isolated from each aliquot using
408 the specified methods; this process was repeated in quadruplicate. Samples were assessed by qPCR for
409 fungal (left) and bacterial (right) DNA. Calculated DNA concentrations were determined by normalization
410 to a mixed fungal and bacterial standard with a known DNA concentration. *: $P<0.001$, **: $P<0.005$. (B,C)
411 Samples were sequenced in quadruplicate by next generation sequencing for the ITS1 (left) and 16S (right)
412 primers. (B) The stacked bar plots represent the mean relative abundances for the fungal (left) and
413 bacterial (right) populations in individual sequencing runs. (C) Shannon diversity indices were calculated
414 from the microbial populations resulting from NGS for each purification method.

415 The improved yields translated to an improved representation of urinary microbial community diversity
416 as assessed by NGS. Qualitatively, a community of greater richness and evenness, as measured using the
417 Shannon Diversity Index (Figure 5C), was seen; multiple taxa were absent or underrepresented in other
418 purification methods (Figure 5B). The optimized method consistently resulted in the highest diversity of
419 all methods. While these differences were not statistically significant, our optimized technique provides
420 equivalent or improved bacterial and fungal community representation across multiple biological sample
421 types.

422

423 **Discussion**

424 Extraction of DNA from fungal cells in urine has proven challenging for multiple reasons. Fungi are thought
425 to be low abundance in most body sites and are structurally more robust and difficult to lyse. Multiple
426 challenges in the identification and characterization of fungal species, such as incomplete annotation in
427 common databases, inconsistent taxonomic classification, and variable conservation of the ribosomal
428 locus across divisions of the fungal kingdom[46], complicate studies of fungi in any biologic niche. The
429 combination of these problems with the technical difficulties of working with urine specimens has left
430 previous explorations of the urinary fungal microbiota inadequate to examine anything more than a few,
431 well-characterized species.[25] Given the experiences of others attempting fungal isolation in low biomass
432 specimens, such as blood, we anticipated that substantial modifications of typical protocols used for the
433 isolation of bacteria from urine would be necessary to assess adequately the fungal populations present.
434 As optimal depth of sequencing requires the highest concentrations of DNA possible, we hypothesized
435 that successful fungal DNA extraction for sequencing would require concentration of cellular material
436 from larger volumes of urine, multiple disruption steps to break down fungal cell walls, and inactivation
437 of the abundant PCR inhibitors present in urine. An iterative approach to the optimization of fungal DNA
438 extraction confirmed these suspicions, with multiple modifications from commonly-used standard DNA
439 extraction methods needed to provide consistent, good quality fungal DNA for sequencing-based
440 assessments.

441 Sample size was very important. Approximately 40% of low volume specimens (e.g. 1 ml urine) did not
442 provide adequate sequencing depth for analysis (<1000 reads per sample), while samples >10 ml
443 consistently provided excellent depth of coverage in ~95% of samples. While this volume threshold had
444 previously been suggested[44], our data provides objective confirmation that such a threshold is
445 important for microbial analyses. Unexpected was the discovery that the best results were not associated
446 with the largest, initial sample size, with an optimal samples size of 25-30 ml. Interestingly, centrifugation

447 speed made a substantial difference, with slower speeds yielding better results. While both these results
448 seem counter-intuitive, our data suggest that this decrease in fungal DNA seen with higher centrifugation
449 speeds and larger sample volumes is due to the accumulation of amorphous crystals common in urine
450 that interfere with DNA extraction and amplification by PCR. While it is possible that other methodologic
451 variations, such as filter-based concentration methods or magnetic bead separations, could provide
452 improved results, our initial attempts using these methods did not appear promising.

453 As anticipated, multiple cell wall disruption methods (thermal, mechanical, and enzymatic) provided much
454 improved fungal DNA yields. An additional digestion with proteinase K was helpful at improving DNA
455 quality as well, although the precise amount of enzyme was less important. The use of carrier DNA to
456 enhance DNA column binding efficiency was crucial. Parallel processing of cell lysates in smaller batches
457 with serial application of these samples to a single DNA binding column also improved yields. The
458 individual improvements in fungal DNA extraction for each of these steps justified their inclusion into the
459 optimized protocol.

460 We have also attempted multiple other variations on our protocol that are not described in this paper,
461 such as preheating the DNA elution buffer to 37°C or performing a second elution from the DNA-binding
462 column in a small volume, as none of these possibilities made significant differences in the resulting DNA
463 concentrations. Our results without these additional steps were sufficient for genomic sequencing.

464 One drawback to these additional steps is that this protocol takes significantly more time than the
465 available commercial kits, 150-180 min in contrast to 75-90 min. The substantial improvement in the
466 quality and quantity of isolated microbial DNA, however, is clear, consistently providing reliable DNA for
467 NGS analyses of microbial populations.

468 The samples utilized as test specimens throughout this paper were voided. Contamination from nearby
469 sites, such as skin, urethra, and vagina (in women), can contribute heavily to the microbial content of

470 voided samples[44]. When compared directly (Figure 3), fungal levels in samples from women were 2-3
471 fold higher than those seen for men. While this could reflect a difference in the urinary fungal content
472 between genders, it may also merely reflect differences in contamination from nearby urogenital sites. As
473 a result, this paper does not seek to make conclusions about the composition of the urinary mycobiome,
474 but instead sought to explore the solutions needed to characterize microbial content from urine
475 specimens. Larger scale studies, which are currently underway, using a multitude of samples will be
476 needed to explore the urinary mycobiome. However, while the samples used in this study were voided in
477 origin, we have since confirmed that this enhanced protocol is successful at producing sufficient quality
478 fungal DNA to obtain good depth of sequencing from a limited number of catheterized urine samples and
479 those obtained by suprapubic aspirate.

480 For microbial populations of low abundance, as presumed for the urinary tract, maximizing the quantity
481 of template DNA for analysis is extremely important. When DNA quantities are barely in the range of
482 detection, small variations in sample quantity or quality or even minor fluctuations in physiologic
483 conditions may result in large misleading population shifts. If certain benign urologic conditions are
484 associated with changes in the overall abundance of fungi in urine, as has been suggested for UCPPS,[25]
485 then methods that fail to adequately represent the population at the lower, baseline levels will
486 underrepresent the populations present in these circumstances. It is likely in that situation that culture-
487 independent microbial analyses will incorrectly identify the upregulation or novel appearance of particular
488 taxa, providing misleading conclusions about disease pathophysiology. These problems are compounded
489 by the fact that urine composition and concentration is highly variable, even within a single individual.
490 Certain disease conditions are associated with systematically smaller void volumes, which might also
491 significantly bias such results. The increased DNA concentration and quality achieved using this optimized
492 approach seek to minimize these biases and provide the most accurate results in the use of sequencing-
493 based methods to define the urinary mycobiome.

494 It has been widely recognized for bacterial DNA extraction that different sample preparation and DNA
495 extraction protocols can produce dramatically different results.[47-50] Protocols utilizing mechanical and
496 enzymatic disruption steps have consistently given the best representations of bacterial community
497 structure, but in no case have the obtained results provided completely accurate representations of
498 standardized samples.[50] In fungal studies,[51] optimal conditions vary for individual fungal species;
499 therefore, while standardized methods are generally useful for fungal and bacterial DNA extraction from
500 biologic specimens, every method will have some bias in extraction efficiency. No single extraction
501 method is reliable and optimal for all species in all specimens. While our results from a range of subjects
502 and specimens confirmed the efficacy of this optimized protocol in aggregate, there were individual
503 variations in fungal community patterns. Our optimized protocol as defined was not always the most
504 effective for every subject assessed. The greatest variations occurred with centrifugation conditions; it is
505 likely that for subjects for whom there is a lower urinary salt content there would be improved results
506 with higher centrifugation speeds. Such biases are inevitable for all stages in the process of culture-
507 independent sequencing-based identification of microorganisms. It remains important to keep these
508 biases in mind when interpreting results, as well as to confirm results through multiple methodologies.

509 In conclusion, we present a method for microbial DNA isolation that results in a better representation of
510 the overall fungal and bacterial populations, both in terms of the population diversity as well as
511 identification of low abundance taxa that are lost with less sensitive methods. All of these benefits appear
512 to occur without a significant loss in bacterial community representation, making this the best available
513 method for microbial analyses of urine samples.

514

515 **Conclusion**

516 Studies examining urinary fungal populations have been limited by the inability to consistently isolate the
517 microbial DNA from low biomass urinary samples. This report describes an optimized protocol for the
518 analysis of urinary fungi that is also highly effective for the concurrent analysis of urinary bacterial
519 populations. The simultaneous and efficient extraction of fungal and bacterial DNA from urine for use in
520 culture-independent microbial analyses is thus possible with this refined technique, providing more
521 reliable methods for the detection and exploration of multiple microbial kingdoms from a single specimen.

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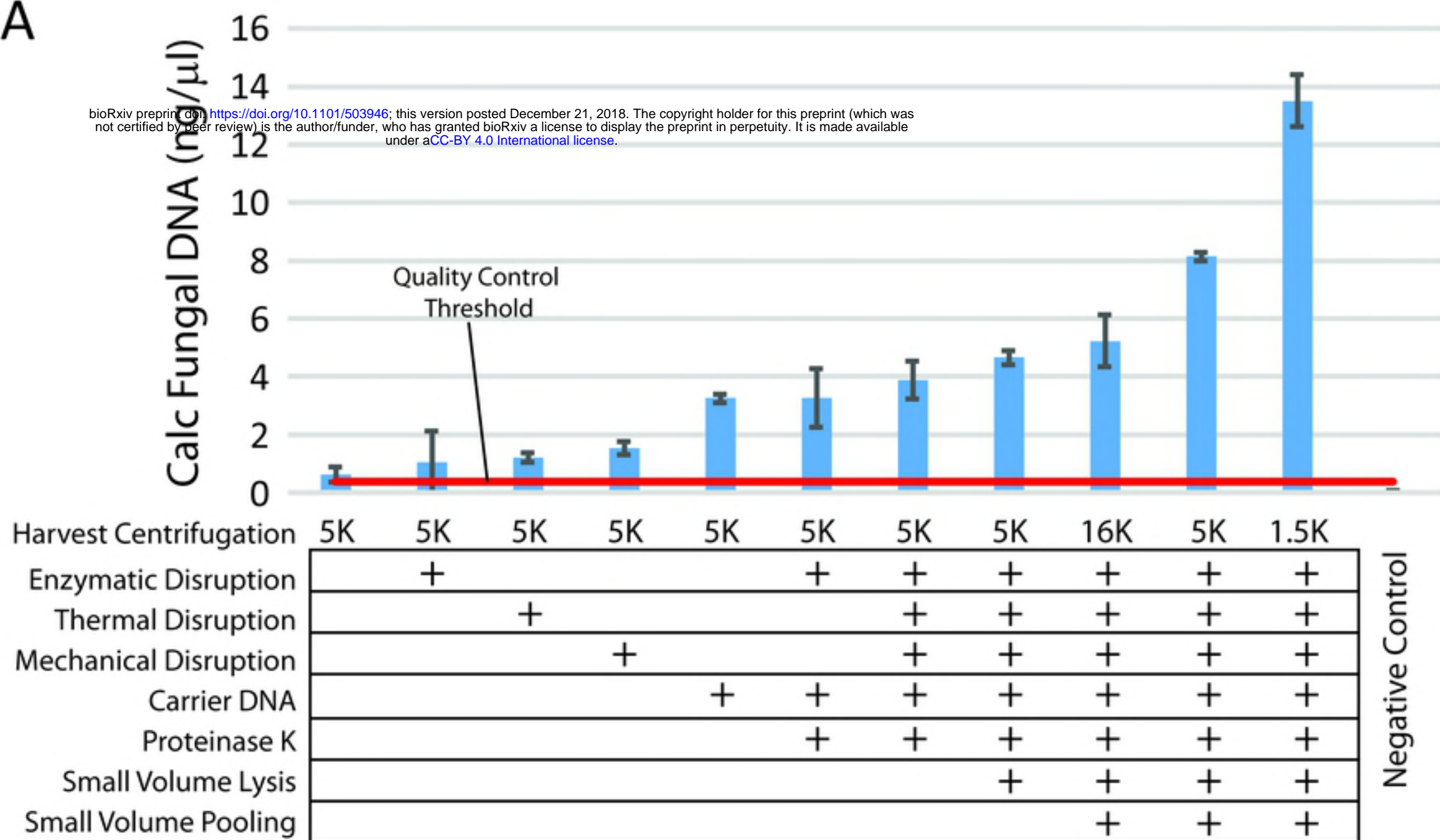
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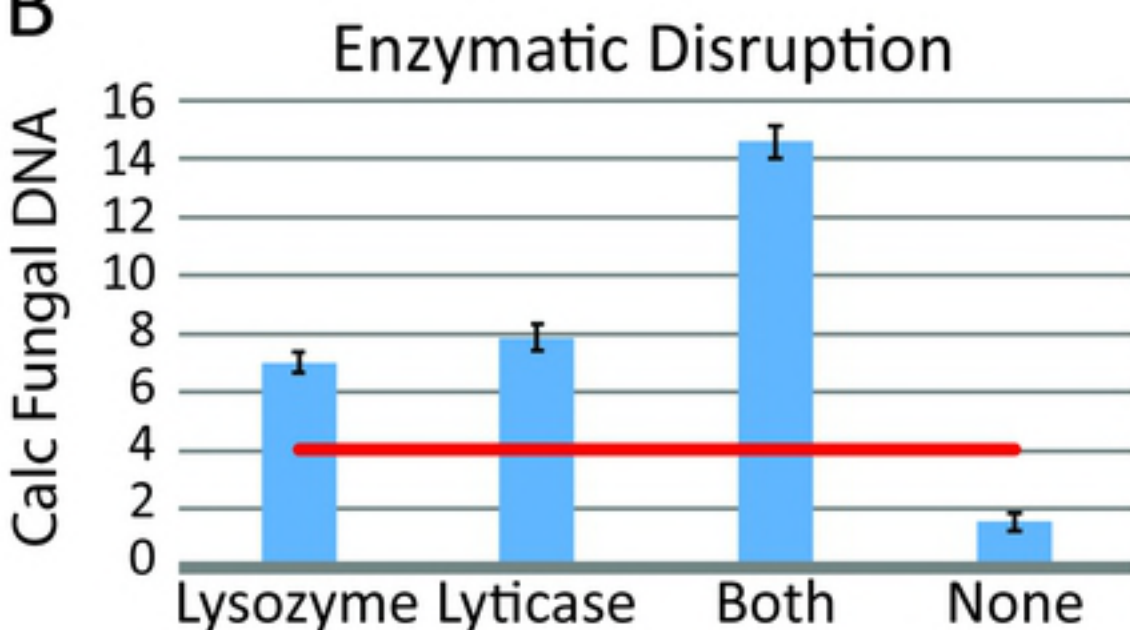
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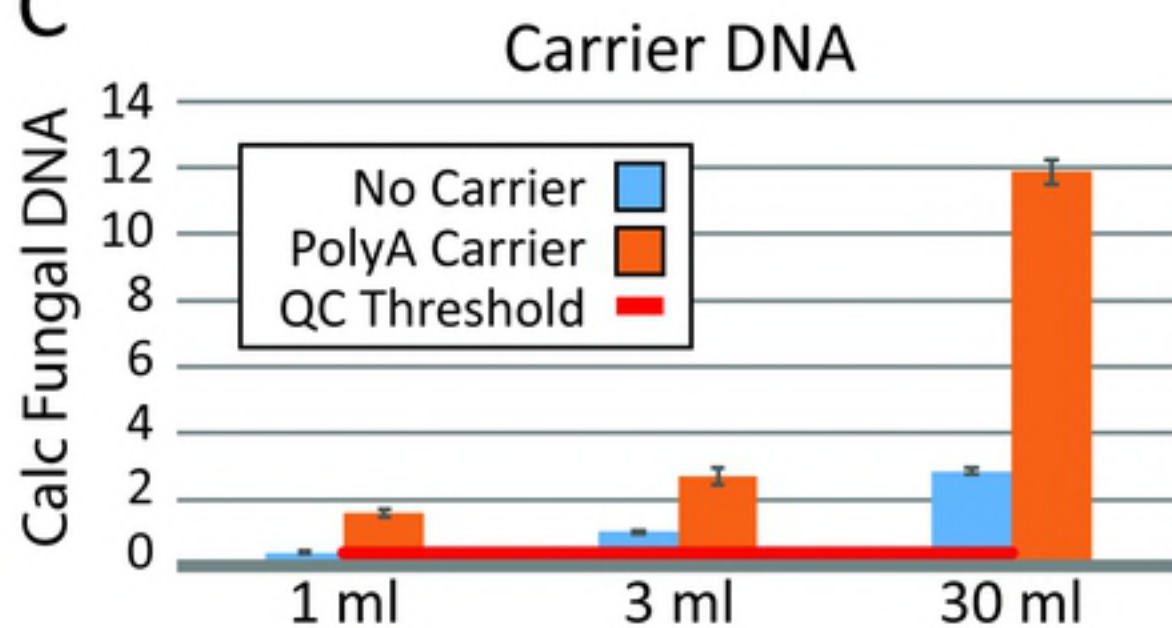


Figure One

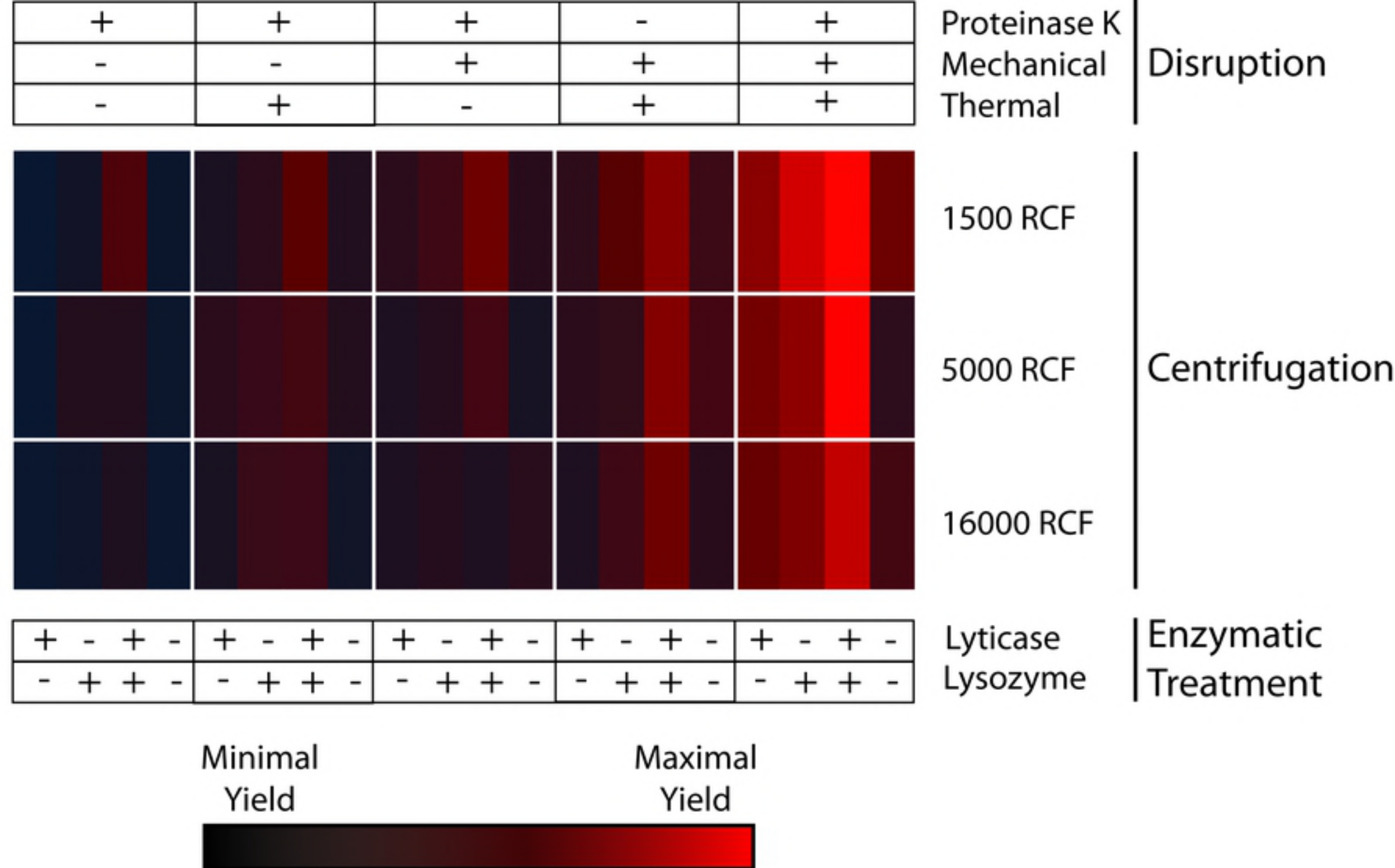
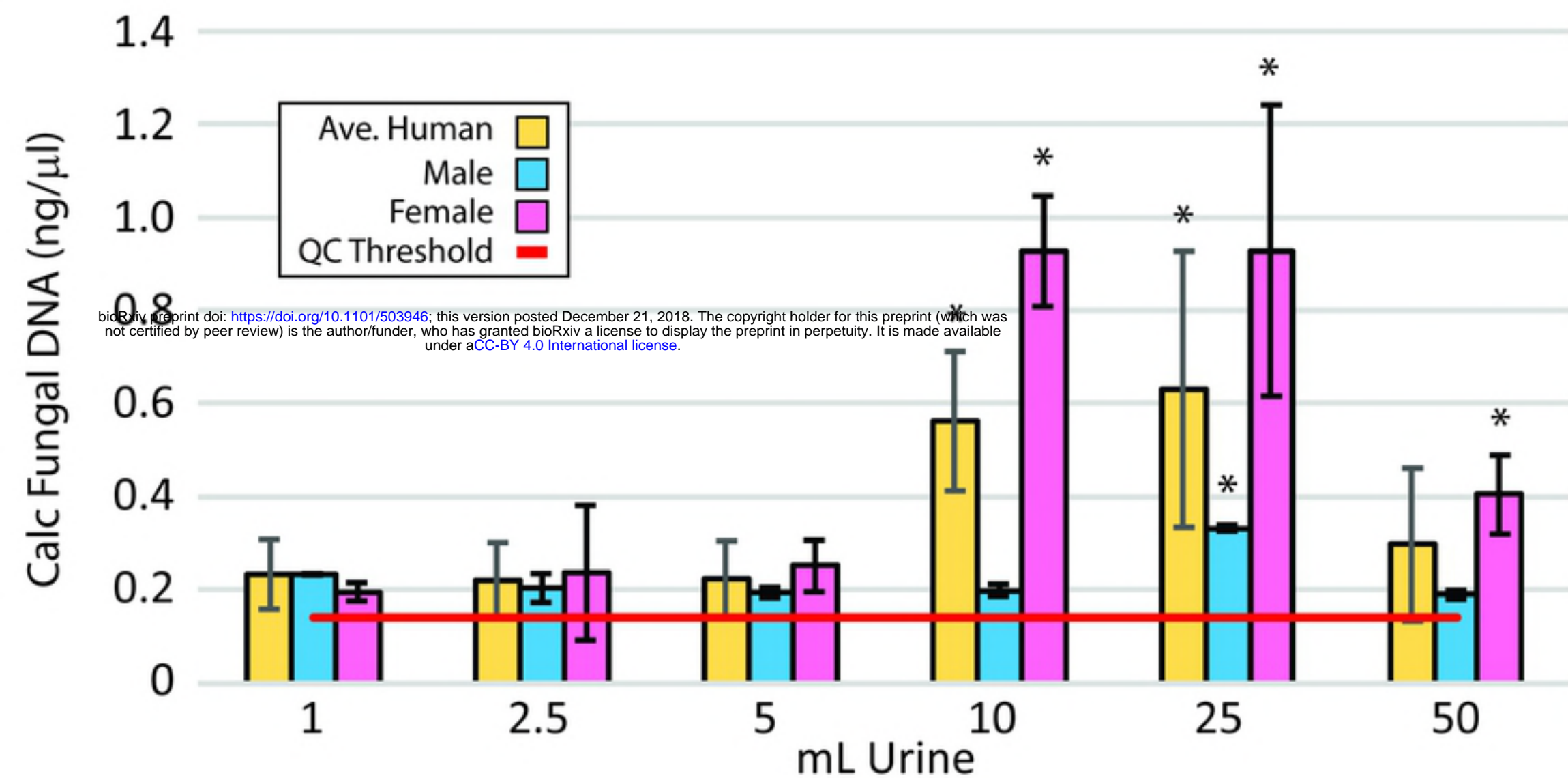


Figure Two

A



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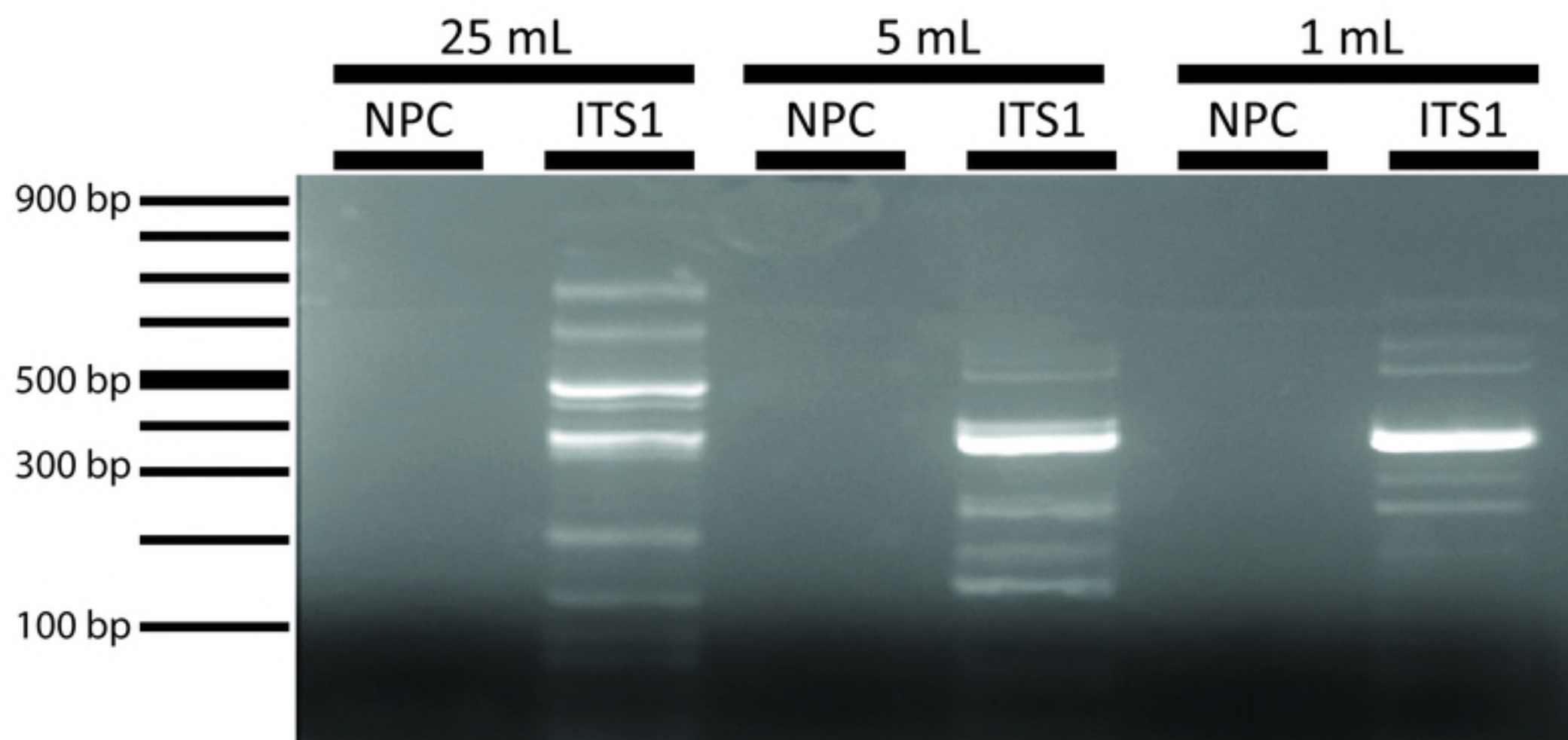


Figure Three

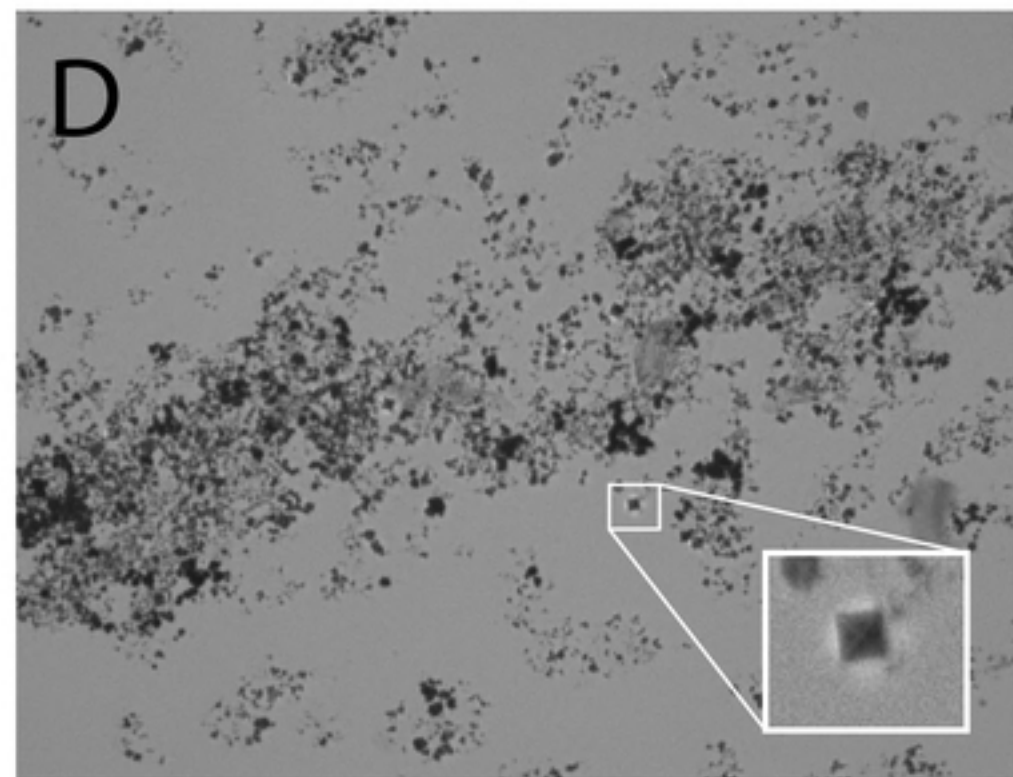
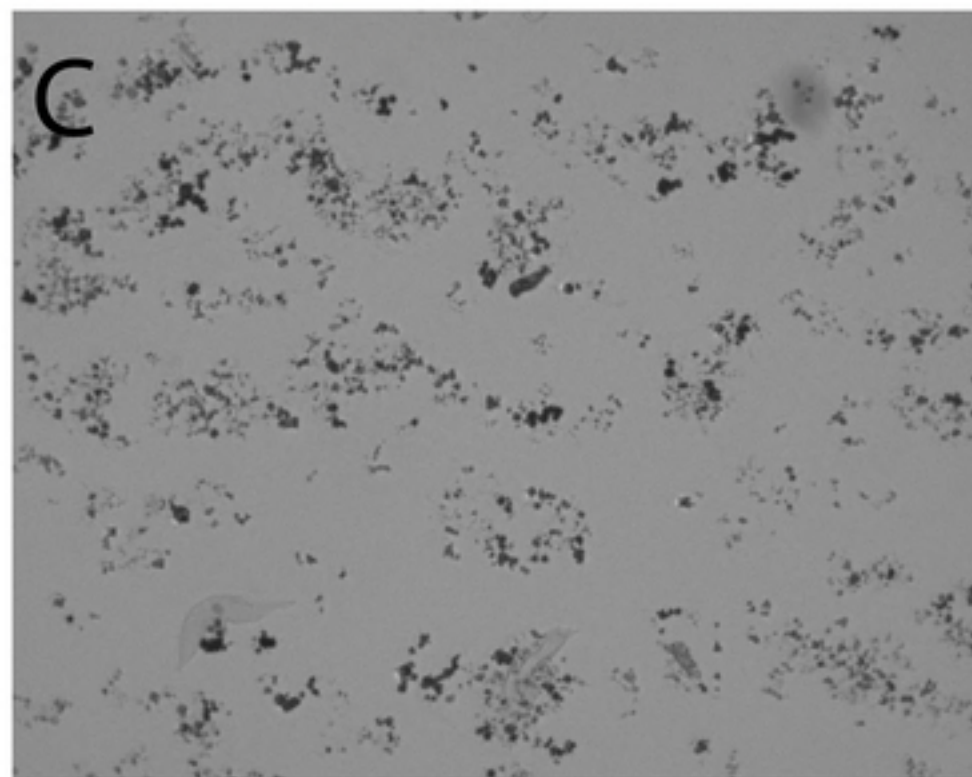
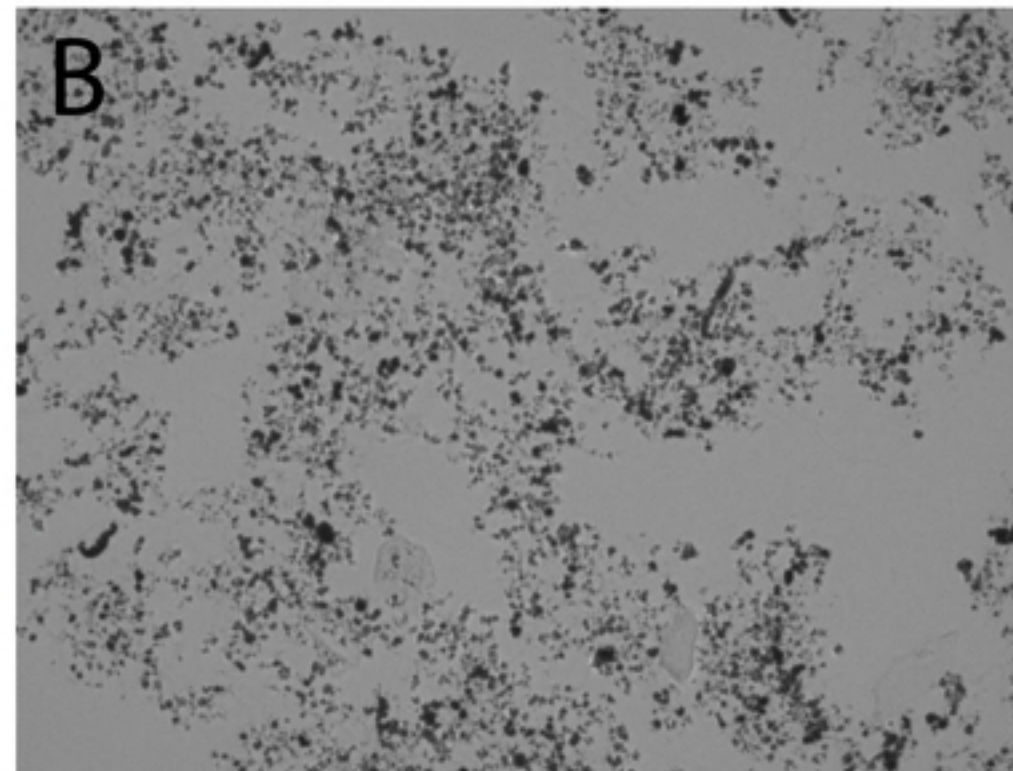
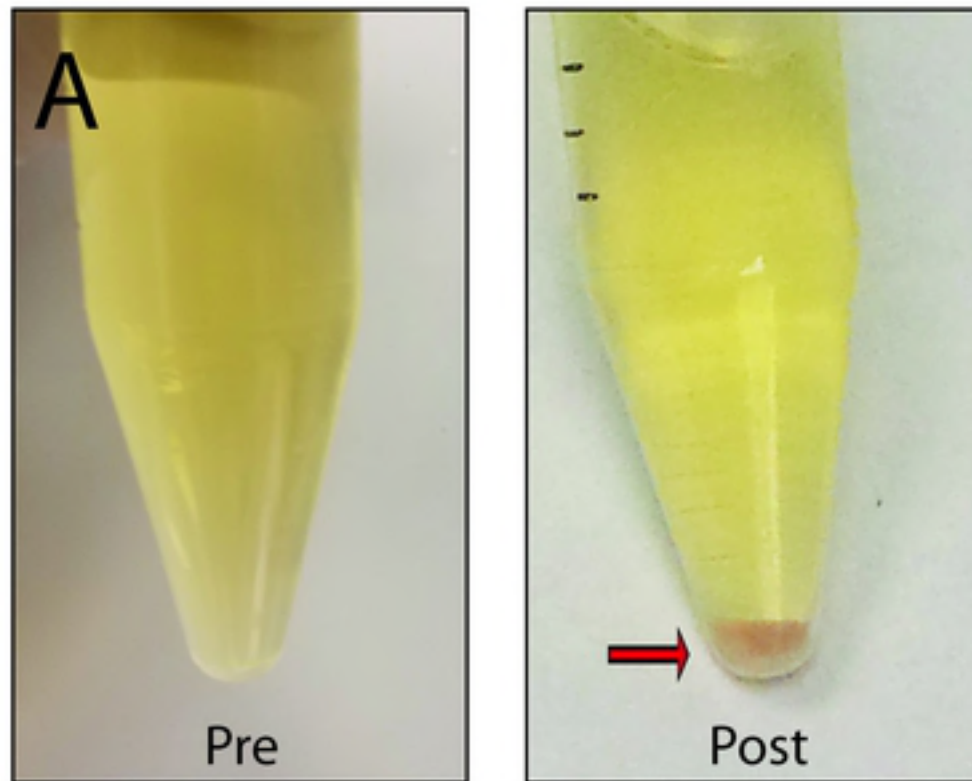


Figure Four

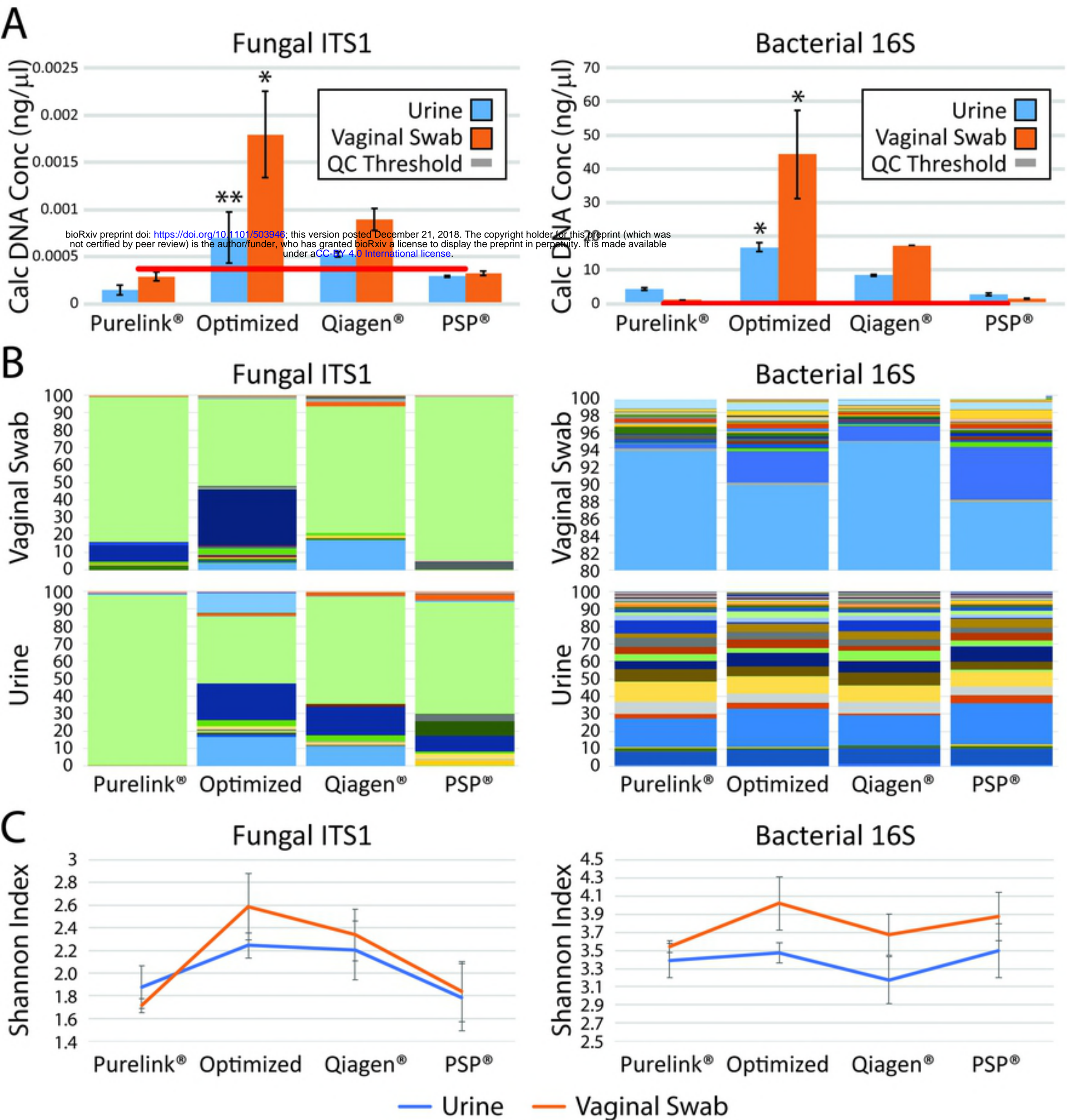


Figure Five