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1 # Title

2 Development and on-site evaluation of an easy-to-perform and low-cost food
3 pathogen diagnostic workflow for low-resource communities

4

5 # Short title

6 Simple and low-cost diagnostic platform for low-resource communities

7

8

9

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19 # Abstract

20 Food-borne diseases are a leading cause of illness and death in many developing
21 countries and thus, there is a real need to develop affordable and practical
22 technologies that can help improve food safety in these countries. The ability to
23 efficiently identify food pathogens is essential to allow national regulatory authorities
24 to monitor food quality and implement safety protocols. In this study, we have
25 developed a simple, low-cost ($0.76 (USD)) complete food pathogen diagnostic
26 workflow ideally suited for deployment in low-resource environments that uses a
27 simple four step process (sample enrichment, cell lysis, DNA amplification, and
28 naked-eye readout). The minimal number of steps and equipment involved in our
29 diagnostic workflow, as well as the simplicity of the yes/no flocculation readout,
30 allows non-technical personnel to perform and interpret the assay. To evaluate the
31 system's performance, we tested the entire system on fresh produce samples
32 collected from local farms and markets in Cambodia for the presence of the *E. coli*
33 *O157* O-antigen polymerase, *wzy*. Although this was a proof-of-concept study, our
34 system successfully revealed a clear correlation between the origin and condition of
35 the produce collected and their likelihood of contamination. In conclusion, we believe
36 that our easy-to-perform diagnostic system can have a significant impact on
37 improving food quality and human health if adopted by regulatory authorities in
38 developing countries due to the assay's simplicity, affordability, and adaptability.

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40 # Introduction

41 Over the last 20 years, there has been a significant increase in the incidence of food-
42 borne diseases worldwide [1]. The risk of infection by a food-borne disease is
43 significantly higher in developing countries due to a combination of factors including
44 access to clean water and bathroom facilities, poor hygiene education, inadequate
45 food production and storage practices, and either insufficient food safety legislation
46 or poor implementation of existing legislation [2]. Most food-borne disease infections
47 in these countries result from the consumption of perishable foods sold in informal
48 markets [3] and, as such, food-borne diseases have become a leading cause of
49 illness and death in developing countries [4, 5]. Hence, there is a need for low-cost
50 and simple technologies that can help health authorities to monitor and enforce
51 adequate levels of food safety. In addition to the health benefits, increased food
52 safety standards would likely have economic benefits as a result of increased
53 demand for fresh produce exports [3].
54
55 Shiga toxin-producing *Escherichia coli* (STEC) are a class of *E. coli* responsible for
56 many food-borne outbreaks and sporadic cases of gastrointestinal illness, with a
57 range of symptoms including haemorrhagic colitis (stomach cramping and bloody
58 diarrhoea) and the potentially fatal hemolytic-uremic syndrome (break down of red
59 blood cells, kidney failure, reduction in platelet cells) [6]. In the United States alone,

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60 STEC strains are estimated to cause approximately 176,000 illnesses, 2,400
61 hospitalizations, and 20 deaths each year [7]. Of all the STEC strains, the O157:H7
62 serotype is the most common cause of foodborne illness outbreaks worldwide [8]
63 and is the responsible for all of the deaths by STEC strains in the US [7]. Infection by
64 *E. coli O157:H7* is commonly thought to be associated with the consumption of
65 undercooked meat however, infection can also occur through the consumption of
66 fresh fruit and vegetables [7, 9, 10]. For example, outbreaks of *E. coli O157:H7* in the
67 United States have occurred due consumption of a variety of fresh produce including
68 onions, bagged spinach, lettuce and unpasteurized apple juice [10-12]. Produce and
69 water supplies used for irrigation or consumption can become contaminated by
70 coming in contact with trace amounts of fecal waste from farm animals, which can be
71 carriers of *E. coli O157:H7* but are themselves unaffected by it [6, 13].
72
73 *E. coli O157:H7*'s low infective dose makes it a highly effective and dangerous
74 human pathogen as it can still cause illness even when the initial source of
75 contamination has been significantly diluted [14]. Thus, countries typically adopt a 0
76 CFU limit of *E. coli O157:H7* or other STEC strains in food [15]. The standard
77 method of identifying *E. coli O157:H7* is an involved process that requires a trained
78 microbiologist and takes days to complete [16, 17]. While this method is well
79 established and highly reliable, it is not always practical, especially for developing

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80 countries who have a limited number of trained personnel and testing facilities. DNA
81 amplification-based systems can provide an alternative to detect food borne
82 pathogens as they are low-cost, fast and relatively simple to perform [17].
83 Polymerase chain reaction (PCR) is the conventional DNA amplification-based
84 technique for rapid identification of DNA/RNA biomarkers [18] however, PCR-based
85 methods require a relatively expensive thermocycler and hence are not ideally
86 suitable for low resource environments. Isothermal DNA amplification methods,
87 including Loop-mediated isothermal amplification (LAMP) [19], may potentially
88 overcome equipment costs as they enable DNA amplification to be performed in a
89 low-cost, single temperature heating device.
90
91 We have developed a flocculation-based DNA amplification readout that enables the
92 identification of the presence of amplification even by inexperienced users [20].
93 Thus, in this study, we sought to build upon the advantages of the isothermal DNA
94 amplification and our flocculation readout technique to create a complete food
95 pathogen diagnostic system tailored for developing countries. We have optimized the
96 entire process, from food sampling to pathogen detection, aiming to simplify each
97 step, reduce costs and ensure safety. In a proof-of-concept study, we used our
98 diagnostic workflow to detect *E. coli* O157 in Cambodian fresh produce and

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99 demonstrated that our system is capable of rapidly screening fresh produce for the
100 presence of food pathogens in low-resource environments.
101

102 Results

103 Sample processing and bacterial enrichment

104 In modern food microbiology laboratories, a stomacher machine is typically
105 employed to macerate tissue in growth media prior to microbial enrichment [21]. This
106 equipment is expensive and, in the hands of poorly trained personnel, can be a
107 source of cross-contamination resulting in false positives and misleading results. We
108 therefore developed an equipment free version of a stomacher machine in which
109 samples are collected and placed in individual tubes containing growth media and 3-
110 4 metal ball bearings (S1A Fig). Vigorous shaking of the tubes macerates the tissue
111 and aids the release food pathogens into the media which can then be enriched in
112 an overnight incubation without the need to open the tube, minimizing the risk of
113 cross-contamination.
114

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115 ## Rapid bacterial DNA extraction from fresh produce
116 ## enrichment cultures

117 A critical part of any food pathogen molecular diagnostic is the ability to reliably,
118 cheaply and safely extract amplifiable DNA. As the infective dose of *E. coli O157:H7*
119 is as low as a few hundred cells [7, 14, 22], an initial enrichment step in buffered
120 peptone media for 16-24 hours is required before detection. We reasoned that the
121 cheapest and safest method to extract microbial DNA from enriched samples is to
122 heat the entire culture to 95°C for 30 minutes as this will kill the bacteria in the
123 sample and lyse the *E. coli*, which can be performed without opening the tube. To
124 test this hypothesis, we infected Brussel sprout leaves with *E. coli O157:H7*, and
125 subsequently processed the leaf samples as described above. Tubes containing
126 macerated leaf tissue in buffered peptone water were incubated at 41.5^{o}C for 24
127 hours and then heated at 95°C for 30 minutes. Samples from the boiled culture
128 failed to produce bacterial growth on buffered peptone agar plates, indicating that the
129 heat treatment had killed the bacterial population and the tube was safe to open.
130 Direct addition of 1, 2 or 4 µl of the boiled culture into LAMP reactions did not result
131 in amplification (S1B Fig) indicating that amplification inhibitors are present in the
132 boiled extract. However, a 25-fold dilution of the boiled extract with water prior to
133 addition to the LAMP reaction resulted in strong amplification (S1B Fig).
134

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135 **LAMP primer development**

136 LAMP primers were developed against a number of *E. coli O157:H7* genes (S1
137 Table), including the shiga toxin genes *stx1* and *stx2* [23], the O-antigen polymerase
138 *wzy* [24, 25], the O157-specific biosynthesis gene, *rfbE* [26], and the H7 flagella
139 antigen *fliC* [27]. The primers were used in LAMP reactions to detect the presence of
140 *E. coli O157:H7* in cultures generated from artificially inoculated alfalfa sprouts in
141 three independent experiments. The *stx2-2* primer set was unreliable as it failed to
142 amplify a product in two of the three experiments (Fig 1A) while the *fliC*, *stx2*-2 and
143 *rfbE* primer sets produced amplification products in one of the two non-inoculated
144 control samples indicating that they are prone to false positives (Fig 1B). Based on
145 these results, only the *wzy-1* and *stx2-1* primer sets were found to give reliable
146 amplification of *E. coli O157:H7*.
147

148 **Figure 1. Identification of specific and reliable LAMP primers.** (A) LAMP primer
149 sets designed for *fliC-1*, *wzy-1*, *stx2-1*, *stx2-2*, *rfbE-1* were tested for their ability to
150 detect *E. coli O157:H7* in three artificially inoculated Alfalfa sprout samples. (B) The
151 same sets of LAMP primers were used in two uninoculated Alfalfa sprout control
152 samples.
153

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154 To further test the specificity of the *wzy-1* and *stx2-1* primer sets, we examined their
155 ability to differentiate between *E. coli O157:H7* and *Salmonella enterica*, a
156 genetically similar pathogenic species to *E. coli* [28]. Alfalfa sprouts were inoculated
157 with either 8 or 80 colony forming units (CFU) of *Salmonella enterica* or 1 CFU of *E.*
158 *coli O157:H7* and cultured enriched overnight. LAMP primers designed against the
159 *Salmonella* invasion protein, *invA* (Table S1), were able to specifically identify the
160 *Salmonella* infected alfalfa sprouts while no amplification products were detected in
161 the *E. coli O157:H7* inoculated samples and non-inoculated controls (Fig 2A). The
162 *wzy-1* or the *stx2-1* primer sets produced strong amplifications in the *E. coli O157:H7*
163 infected samples but not in the *Salmonella* infected samples or the non-inoculated
164 controls (Fig 2B).
165
166 **Figure 2. Primers *wzy-1* and *stx2-1* are specific for *E. coli O157:H7*.** (A) LAMP
167 reactions using primers targeting the *Salmonella invA* gene were performed on
168 alfalfa sprout samples inoculated with either 8 or 80 CFU of *S. entrerica serovar*
169 *enteritidis* or 1 CFU of *E. coli O157:H7,* non-inoculated sprouts were used as
170 controls. (B) LAMP reactions using primers *wzy-1* and *stx2-1* were performed on the
171 samples described above.
172
173

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174 ## Stabilization of LAMP reactions to allow room temperature
175 ## transport

176 Procurement of amplification reagents in many developing countries is problematic
177 largely due to erratic power supplies and inadequate cold storage [29, 30]. Freeze
178 drying of reactions could facilitate room temperature transport to, and storage at
179 remote locations. Trehalose is commonly used as a stabilizer of biomolecules during
180 the freeze-drying process [31, 32], however our initial attempts to freeze dry a
181 complete LAMP reaction failed to produce an amplification product upon
182 reconstitution with water either in the presence or absence of trehalose (Fig 3A).
183 Further tests revealed that the presence of betaine, an essential component of the
184 LAMP reaction, negatively affected the activity of the rehydrated reaction (Fig 3A).
185 Therefore, the LAMP reactions were freeze-dried in the absence of betaine and
186 subsequently resuspended in a solution containing betaine at the concentration
187 required for the reaction. To study whether the freeze drying process can stabilize
188 the LAMP reactions for extended periods of time, the dried reactions were left at
189 room temperature (22 - 24°C) for 4, 8 or 21 days before being rehydrated and used
190 to amplify purified *E. coli O157:H7* genomic DNA. Strong amplifications were
191 observed at all three time points with no observable loss of activity over time (Fig
192 3B).

193

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194 **Figure 3. Optimization of freeze-drying conditions.** (A) LAMP reactions
195 containing all necessary components, including primers were freeze-dried overnight
196 in the presence (+) or absence (-) of 5% (w/v) trehalose or 0.8 M betaine. Samples
197 processed without betaine were subsequently rehydrated in betaine solution (0.8 M
198 final concentration) prior to amplification using 2 ng of purified *E. coli O157:H7*
199 genomic DNA as template. (B) Samples freeze-dried with trehalose but without
200 betaine were left at room temperature for 4, 8 and 21 days prior to rehydration with
201 betaine solution and amplification using 2 ng of purified *E. coli O157:H7* DNA as
202 template.
203
204

205 Complete diagnostic workflow and proof of concept 206 testing in Cambodia

207 The individual components developed in this study were combined to create a
208 complete diagnostic workflow suitable for testing fresh produce for *E. coli O157*
209 contamination (Fig 4). To test the full food pathogen diagnostic system in our
210 laboratory, 1 g of Brussel sprout leaves inoculated with *E. coli O157:H7,* were placed
211 in a 50 ml Falcon tube containing three metal ball bearings and 10 ml of buffered
212 peptone. Non-inoculated leaves were used as negative controls. After shaking to
213 disrupt the tissue, tubes were incubated at 41.5^{o}C overnight to allow enrichment and

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214 boiled at 95°C for 30 mins to kill and lyse the bacteria. Freeze dried LAMP reactions,
215 containing *wzy-1* primers, were reconstituted with betaine solution (0.8 M betaine
216 final concentration) and 1 µl of the enriched cultures diluted 15-fold in water added
217 before incubating at 63°C for 50 minutes. The amplification reactions were examined
218 by electrophoresis (Fig 5A) and by the addition of a flocculation solution [20] (Fig
219 5B). No amplification was detected in the water controls or the non-inoculated
220 samples however, a strong amplification was observed in the *E. coli O157:H7*
221 inoculated sample (Fig 5A). The addition of flocculation solution mirrored the results
222 observed on the agarose gel that is, *E. coli* inoculated samples gave a positive
223 flocculation result in which the particles in solution clumped together and rapidly
224 settled to the bottom of the tube and leaving a transparent liquid phase (Fig 5B). In
225 contrast, the water controls and the non-inoculated samples showed a negative
226 flocculation reaction in which the black particles remained suspended.
227
228
229 **Figure 4. Overview of the complete food pathogen detection system.** Sample
230 maceration and enrichment involves shaking fresh produce samples in a tube
231 containing growth media and ball bearings to macerate the tissue and release
232 contaminating pathogens followed by an overnight incubation at 41.5^{o}C. Pathogen
233 cell lysis and DNA release is achieved by incubating the enriched sample at 95°C for

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234 30 minutes. LAMP DNA amplification is performed on a diluted sample of the
235 overnight culture by incubating the reaction in a water bath using a Styrofoam box
236 containing 63°C water. The results of the DNA amplification are visualized by adding
237 flocculation solution. In the absence of amplification the solution will remain black
238 and non-transparent (upper tube), alternatively if the pathogen is detected, the
239 particles in the solution will flocculate and rapidly settle on the bottom of the tube
240 (lower tube).
241
242
243
244
245 **Figure 5. Evaluation of the complete *E. coli O157:H7* diagnostic system.** (A)
246 Freeze dried LAMP reactions containing the *wzy-1* primer set were rehydrated in
247 betaine solution and 1 µl of diluted enriched culture from Brussel sprout leaf samples
248 inoculated with *E. coli O157:H7*. Non-inoculated leaf cultures were used as negative
249 controls as well as water (NTC). (B) 20 µl of flocculation solution was added to the
250 above LAMP reactions and the solutions mixed for 10-15 seconds. Settling of the
251 particles indicates a positive reaction. (C) Fresh produce was collected from three
252 farms and three markets in Cambodia. Samples were assayed for the presence *wzy*
253 using the developed diagnostic workflow. Data shows the percentage of positive

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254 samples (bar graphs) as well as the number of positive and total number of samples
255 from each sampling site (numbers above each bar).
256
257
258 To assess the workflow in field conditions we transported enough reactions to test
259 100 vegetable samples to Cambodia with the *wzy-1* primer set. Cauliflower, radish
260 and bean leaves, as well as bean pods were collected from three different
261 Cambodian farms (S2 Fig) located within an approximate 10km radius of each other.
262 Tissues were processed following the above described workflow (Fig. 4) and positive
263 tests were obtained at all sites. In total, 25%, 39% and 26% of samples tested
264 positive for *wzy* on farms #1, #2 and #3, respectively.
265
266 We also collected samples from three different outlets: a roadside vegetable stall
267 and two large food markets located in a rural area (Figs S3A and S3B) and within the
268 city Phnom Penh (Figs S3C and S3D). The roadside vegetable stall (market #1) was
269 located along a dirt road that was used by local farmers and villagers as well as their
270 farm animals. We found that 56% of the samples obtained from this stall tested
271 positive (Fig 5C, Market #1). Market #2 was located in a small rural village and had a
272 hard packed dirt floor containing numerous puddles of liquid and a low tin roof that
273 protected the produce from direct sun exposure but generated an intense heat with

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274 little air flow (market #2, Figs S3A and S3B). Raw meat products were not
275 refrigerated and were allowed to come in direct contact with fresh produce (Fig S3B)
276 in closely packed stalls where a large number of flies freely moved between the meat
277 and fresh produce. A high proportion (45%) of the samples collected from this
278 market tested positive for *wzy* (Fig 5C). In contrast, the market located within the city
279 (Phnom Penh) (market #3, Figs S3C and S3D) had significantly better ventilation
280 than the rural market and a large proportion of the floor was concreted and appeared
281 clean. The vegetables in this market looked fresh and not wilted like in the rural
282 market. Only one out of 16 samples (6%) from this market tested positive using the
283 *wzy* primer set (Fig 5C, Market #3).
284

285 Discussion

286 The goal of this study was to develop a complete diagnostic workflow for food
287 pathogens tailored to countries with limited resources to enhance food biosecurity
288 capability. Our focus was to create a robust, simple and low-cost system that is safe
289 to perform by people with limited training and equipment. The successful testing of
290 our system in Cambodia on samples collected from the local farms and markets,
291 suggests that we have developed a practical system that meets these requirements
292 and is capable of providing meaningful data that can support food safety initiatives.

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293

294 Culture-based techniques are the most reliable method for food pathogen
295 identification however, these techniques are slow and require highly skilled
296 technicians to perform [33]. The diagnostic workflow presented here is simpler and
297 faster allowing large numbers of samples to be screened in situations where access
298 to suitability trained microbiologists is limited. Our diagnostic workflow takes
299 advantage of the many desirable characteristics of LAMP amplification, including
300 high specificity and the ability to be performed in a simple water bath [34, 35].
301 However, like all DNA amplification reactions, LAMP is not immune to issues of
302 contamination or false-positive amplifications [36]. Thus, it is critically important to
303 optimize sample processing to prevent cross-contamination and develop robust
304 specific primer sets for each target organisms prior to deployment.

305

306 The diagnostic workflow developed here can be easily adapted to detect the
307 presence of target genes from different food pathogens using highly specific LAMP
308 primer sets readily available in the literature [37-39]. Unlike specificity, the sensitivity
309 of the primers is less critical to the success of the assay as the overnight enrichment
310 step significantly increases the pathogen levels in the tested sample. Consistent
311 with this, our assay has successfully detected the presence of 1 CFU of *E. coli O157*
312 on a 1 g sample of Alfalfa sprouts (Fig 2B) emphasizing that the simplicity of the

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313 detection workflow presented here does not limit its capacity to detect trace amounts
314 of pathogens on produce.
315
316 The focus of our investigation in Cambodia was to examine the performance of our
317 workflow rather than perform a comprehensive survey of Cambodian produce. Thus,
318 we purposely biased the sampling by seeking out produce with increased likelihood
319 of *E. coli* contamination such as containing mud splashes or selecting farms with free
320 roaming animals [10, 23, 40, 41]. The data obtained in this study revealed a clear
321 correlation between the origin and condition of the produce and the likelihood of
322 contamination. For example, the location of the food stall (market #1) on a dirt road
323 used by local villagers and their farm animals allowed dirt from the road, potentially
324 carrying zoonotic diseases from animal waste, to blow over the stall increasing the
325 risk of contamination (Fig 5C) [42, 43]. Similarly, the poor drainage, problems with
326 flies and lack of separation between raw meat and fresh produce by some vendors in
327 market #2 also increased the risk of zoonotic disease contamination in fresh
328 produce. Therefore, it is not surprising that these rural locations (market #1 and #2)
329 accounted for 95% of the *wzy*-positive market samples. Consistent with this, other
330 studies that have shown a strong correlation between food-borne disease infections
331 and the selling of produce in markets where the vendors do not follow safe food
332 handling practices or lack access to essential facilities (e.g. clean water, garbage

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333 removal) [3, 42]. Furthermore, a study by the World Health Organization of 'Morning
334 glory' produce harvested from areas surrounding Phnom Penh, found that 100% of
335 samples collected in Cambodian markets were contaminated with *E. coli* species
336 [44]. Collectively, these findings suggest that there is a real need for simple, low-cost
337 diagnostic systems to help the local authorities to improve food safety.
338
339 All the surveyed farms had a similar proportion (25-38%) of produce that tested
340 positive for *wzy* (Fig 5C). As the farms were located within a 10 km radius of each
341 other, these findings suggest that they are exposed to similar levels of pathogenic
342 bacteria through common water supplies [40] or airborne particulates that can move
343 between farms [45]. In this proof-of-concept study, we demonstrate that our simple
344 workflow is capable of obtaining meaningful data on the prevalence of harmful food
345 pathogens in specific locations; such information is critical to improve regional food
346 safety.
347
348 There are many diagnostic systems for food pathogens described in the scientific
349 literature or commercially available. However, our diagnostic workflow has a number
350 of advantages over many of the available systems. Compared to some commercially
351 available diagnostic systems (e.g. lateral flow strips ($6.80 USD each, Romer labs))
352 or systems that involve disposable electronics or custom-made microfluidic parts [46,

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353 47]; our diagnostic system is considerably more affordable; costing $0.76 USD,
354 including all tubes and reagents (S2 Table), which can be further reduced to $0.53
355 USD if the ball bearings and 50ml tubes are carefully decontaminated and reused.
356 Cost is a critical factor for developing countries with very limited budgets devoted to
357 food safety. Lowering the cost of assays will allow increased testing and thereby
358 boosting its efficiency as a biosecurity tool [48].
359
360 The use of LAMP, an isothermal amplification method, allowed us to avoid the
361 expensive thermocyclers needed in conventional DNA amplification techniques such
362 as real-time PCR, substituting it with a simple Styrofoam box. Water was easily
363 adjusted to the desired temperature (63°C) by mixing hot and cold water and using a
364 standard thermometer. A lid was placed on top of the Styrofoam box to keep the
365 water close to the initial temperature for the time needed to perform the reaction.
366 Using this system, the water temperature dropped only 3°C over the 50-minute
367 incubation period. This easily accessible and low-tech approach eliminates the need
368 for expensive scientific equipment for DNA amplification, and with well-designed
369 LAMP primers, can provide accurate detection of pathogens from food samples [21,
370 49].
371

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372 The flocculation-based readout used in our workflow is cheap and does not require
373 any specialized equipment, unlike conventional techniques like agarose gel
374 electrophoresis or modern microfluidic or electronic-based systems. The flocculation
375 solution causes the large DNA amplicons produced in the LAMP reaction to bind to
376 suspended black charcoal particles, forming large clumps that rapidly drop to the
377 bottom of the tube leaving a clear upper phase that is easy to distinguish from the
378 black, non-transparent negative reactions [20]. The simplicity of the flocculation
379 assay enables people with limited scientific training to perform and interpret the
380 assay.
381
382 The ability to perform sample maceration, enrichment and DNA extraction in a single
383 tube without needing to open it between steps makes processing the samples simple
384 and safe and allows the user to handle a large number of samples at once. This
385 process could be made even simpler by using the newly developed 30 second
386 dipstick-based DNA purification technology [50] circumventing the need for any
387 pipetting. Unfortunately, the rapid dipstick purification method was developed after
388 the completion of this study.
389
390 In summary, we have developed a complete food pathogen diagnostic system
391 composed of four key steps: pathogen enrichment, cell lysis/DNA release, LAMP

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392 amplification, and naked eye readout (Fig 4). The minimal number of steps and
393 equipment involved, as well as the simplicity of the presence/absence flocculation
394 readout, allows almost anyone, including those with limited scientific training, to
395 perform the assay. Furthermore, the low cost of the system ($0.76 USD) and broad
396 availability of its reagents, makes the system accessible to countries or institutions
397 with limited resources who might not otherwise be able to afford regular food testing.
398 Every step in the diagnostic workflow has been designed with the World Health
399 Organization's ASSURED philosophy in mind, that is, Affordable, Sensitive, Specific,
400 User-friendly, Rapid, Equipment-free, and Deliverable to those who need it [51]. As
401 the system is based on DNA amplification using specific primers, our diagnostic
402 system can be easily modified to identify a large variety of pathogens including those
403 that infect humans, crops or animals. Therefore, we anticipate that the incorporation
404 of our diagnostic system into food safety programs of developing countries will
405 facilitate improvements to both their food quality and human health.
406

407 Methods

408 Bacterial strains

409 *Escherichia coli O157:H7* str. EDL933 and *Salmonella enterica* serovar *enteritidis*
410 were used to develop and test the diagnostic assay.

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411

412 ## Primer selection.

413 A number of potential *E. coli O157:H7* target genes were selected for primer
414 development including the shiga toxin genes *stx1* and *stx2*, the O-antigen
415 polymerase *wzy*, the O-antigen transporter, and *rfbE*, the H7 flagellar antigen *FliC*
416 (S1 Table). A CLUSTALW alignment was performed for each of each of these genes
417 using *E. coli O157:H7* sequences found on the Genbank database. These
418 alignments were used to design LAMP primer against conserved regions of these
419 target genes using Primer Explorer software V4 (http://primerexplorer.jp/e/).

420

421 ## LAMP DNA amplification

422 Unless otherwise stated, LAMP reactions were performed by in a solution containing
423 20 mM Tris (pH 8.8), 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 0.1% (v/v) Tween-20, 0.8 M
424 betaine, 8 mM $MgSO_4$, 1.2 mM dNTPs, 0.32 U/µl Bst2.0 warm-start (NEB Biolabs,
425 USA), 0.8 µM of FIP and BIP primers and 0.2 µM of F3 and B3 primers. Reactions
426 were incubated at 63°C for 50 minutes followed by a five-minute incubation at 80°C
427 to denature the enzyme.

428

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429 ## Flocculation solution preparation

430 The final, optimized flocculation solution is made from 100-400 mesh activated
431 charcoal (Sigma, St. Louis, USA) and powdered diatomaceous earth that had been
432 ground separately in a coffee grinder for 45 seconds to break up any large particles.
433 400mg of activated charcoal and 600mg of diatomaceous earth were combined in a
434 50 ml solution containing 50 mM Tris (pH 8), 10 mM spermine and 1% (w/v)
435 PEG8000. The flocculation solution can either be stored at 4^oC or -20^oC for at least a
436 year without loss of activity.
437

438 ## Freeze drying method

439 The buffer of Bst 2.0 warm-start DNA polymerase (NEB) was replaced with
440 Isothermal amplification buffer (NEB) by centrifugation of the enzyme in an Amicon
441 Ultracel-30 centrifugal filter device (Merck-Milipore) and subsequently washing in
442 Isothermal amplification buffer twice. Aliquot 3.49 µl of LAMP solution (1.1 U/µl of
443 dialyzed Bst 2.0 warm-start polymerase, 50 mM Tris (pH 8.8), 25 mM $(NH_4)_2SO_4$, 25
444 mM KCl, 20 mM $MgSO_4$, 0.25% (v/v) Triton x100, 3.4 mM dNTPs, 4.6 µM FIP and
445 BIP primers, 0.6 µM B3 and F3 primers, 5% (w/v) Trehalose) into individual 0.2 ml
446 tubes. The solutions were frozen in liquid nitrogen and then immediately transferred
447 to a freeze drier for at least 4 hours under a vacuum of below 150 mTorr.
448

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449 **Vegetable sample collection**

450 In May, 2015, vegetable samples were collected from three Cambodian farms and
451 four markets either in or surrounding Phnom Penh. The markets included a small
452 rural village roadside vegetable stall, two large markets in the outskirts of Phnom
453 Penh and one market within the city. Vegetables collected included cauliflower
454 leaves, beans, sprouts, lettuce and other leafy herbs. Vegetable samples were
455 immediately placed in individual plastic bags that were labelled and stored on ice
456 until they were processed using the complete food pathogen diagnostic system
457 detailed below. Access to farms was arranged by the Cambodian General
458 Directorate of Agriculture (GDA) who obtained verbal approval from the landowners
459 for us to collect plant samples. No protected species were samples in this study.
460

461 **Complete food pathogen diagnostic system**

462 Approximately 1g of vegetable leaf material was placed in a 50 ml tube containing
463 three ball bearings and 10 ml of buffered peptone growth media. The buffered
464 peptone media was pre-aliquoted powder and made up with boiled water
465 immediately prior to use. The tube containing the vegetable sample was sealed with
466 Parafilm M (Bemis, WI, USA) and then shaken vigorously for one minute to aid the
467 release of any food pathogens from the tissue. The samples were incubated
468 overnight at 41.5^{o}C overnight to enrich the bacteria present and then boiled for 30

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469 minutes to both kill any bacteria present and to release their DNA into the media.
470 One microliter of culture that had been diluted 15-fold with water was added to a
471 freeze-dried LAMP reaction that had been rehydrate with 9 µl 0.89M betaine. The
472 reaction was incubated in a lidded Styrofoam box containing 63°C water for 50
473 minutes. 20 µl of flocculation solution (50 mM Tris (pH 8), 10 mM spermine, 1%
474 (w/v) PEG 8000, 0.8% (w/v) powdered activated charcoal, 1.2% (w/v) powdered
475 diatomaceous earth) was then added to each LAMP reaction and the tube gently
476 flicked to encourage mixing of the solutions. Samples in which the particles
477 flocculated and settled on the bottom of the tube within 20 seconds were called
478 positive, whereas those in which the particles remained suspended were negative.
479

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486
487

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655

656

657 Supporting information captions

658

659 **S1 Figure. Optimization of Pathogen DNA extraction.** (A) Image shows tissue
660 that has been macerated after shaking in a 50 mL tube containing 10 ml of buffered
661 peptone water and four ball bearings. (B) LAMP amplification products obtained
662 using *stx2-1* primer set and template consisting of either 1, 2 or 4 µl of undiluted
663 enriched culture or 1 or 2 µl of culture diluted 25-fold in water.

664

665 **S2 Figure. Images of Cambodian farms collection sites.** (A) Collection of
666 cauliflower leaves in individual plastic bags at farm #1. (B) Image of farm #2 which
667 was a small plot in with cauliflower grown in the foreground and beans growing in the
668 background. (C) Image of a cow that freely roamed amongst the cauliflower crop at
669 farm #1.

670

671 **S3 Figure. Images of Cambodian market collection sites.** (A) Image of the
672 cramped conditions in the rural market #2. (B) Image of some of the meat and

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673 produce being sold side by side at market #2. (C) Image of a large city market in
674 Phenom Penh (market #3) with material covered tents and less cramped conditions
675 compared to the rural market. (D) Separation of meat and fresh produce at market
676 #3.
677
678 **S1 Table. LAMP primer sequences used in this study**
679
680 **S2 Table. Costings of individual components of the food pathogen assay.**

C

S2 Figure

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S3 Figure

B

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25x diluted

undiluted

1µl 2µl 1µl 2µl 4µl

Figure 1

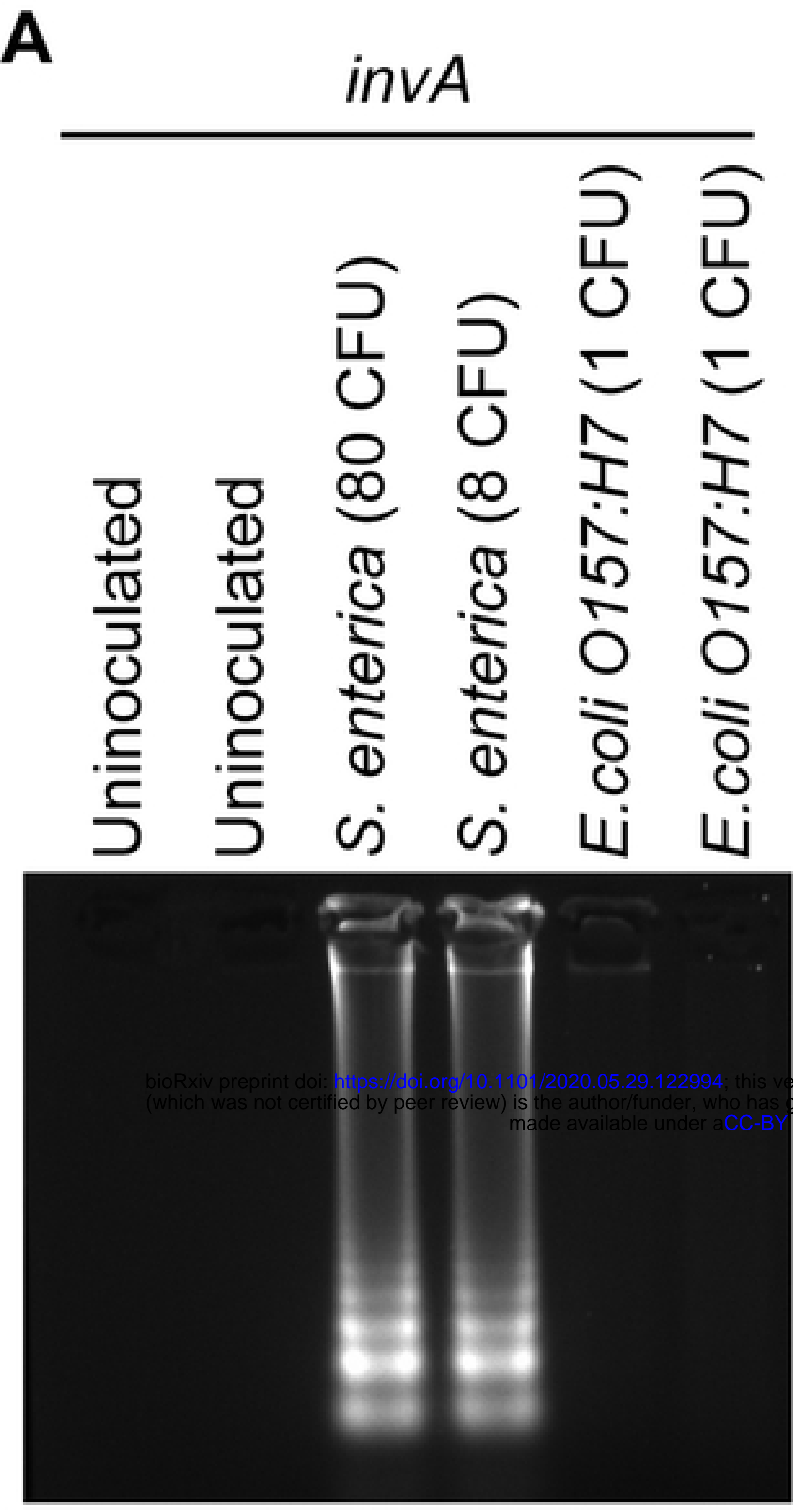

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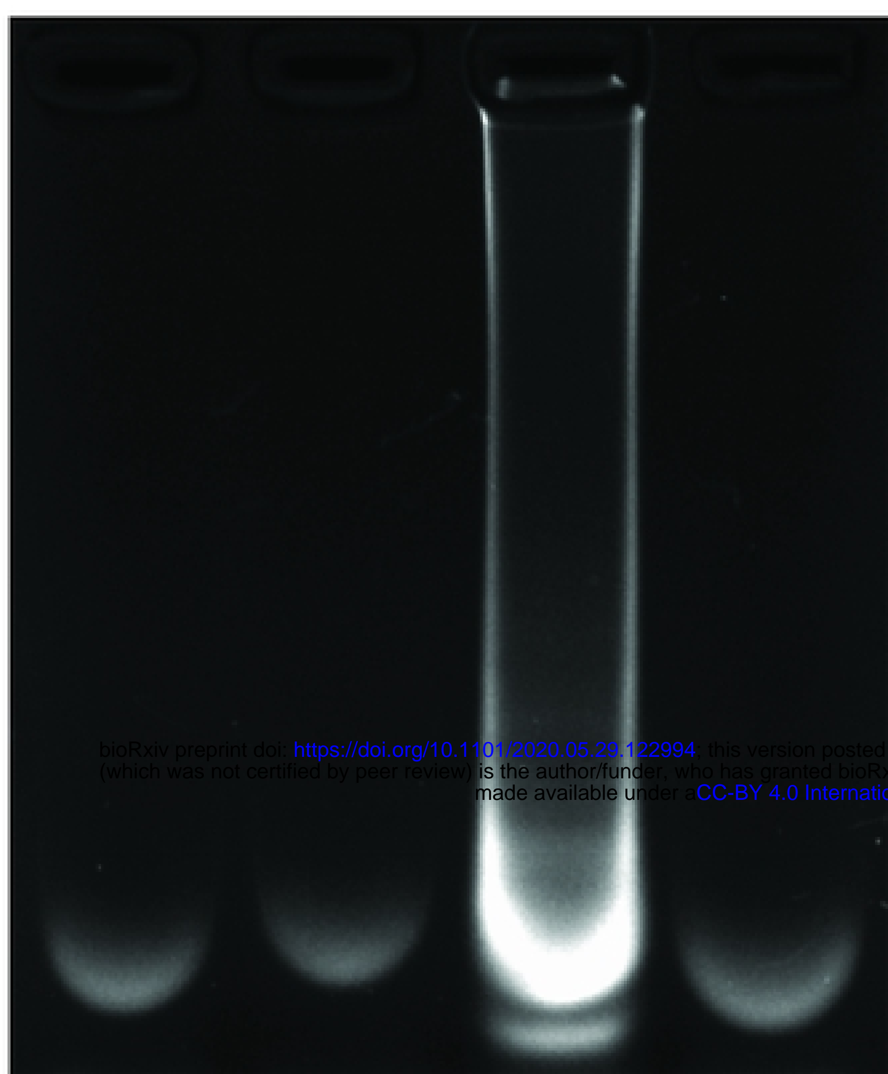
Figure 2

A



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B

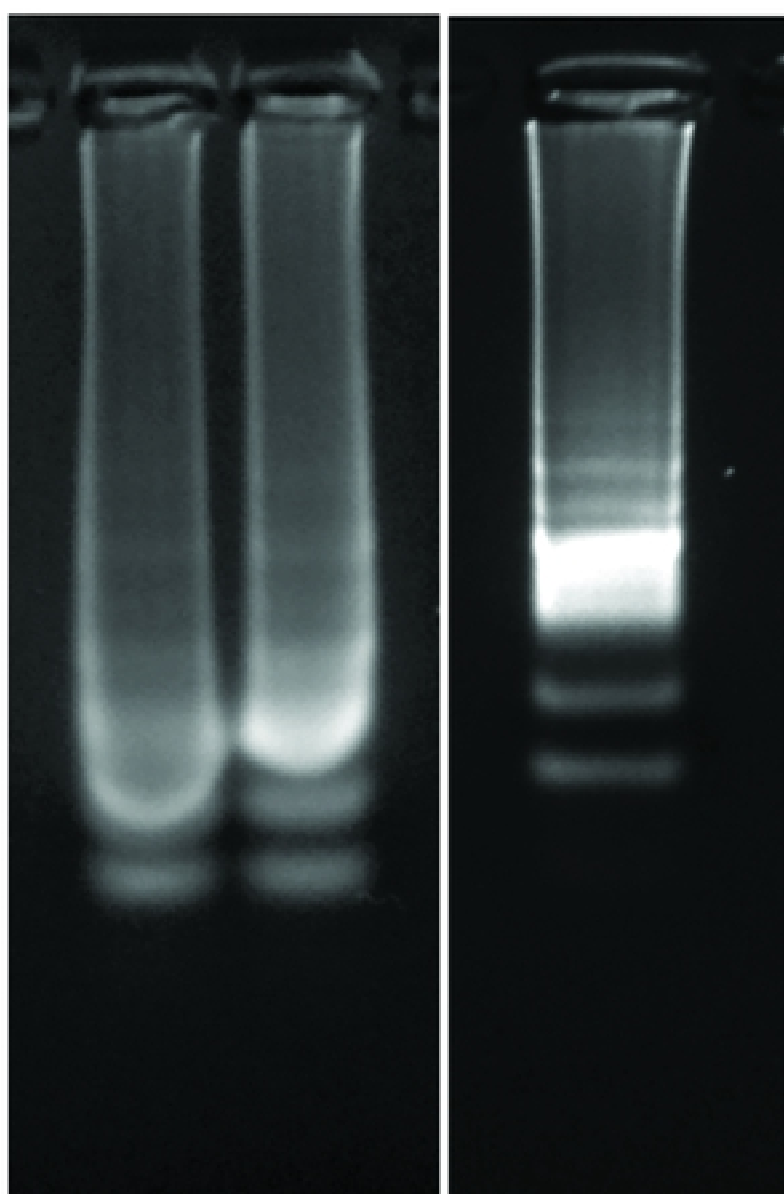
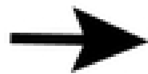
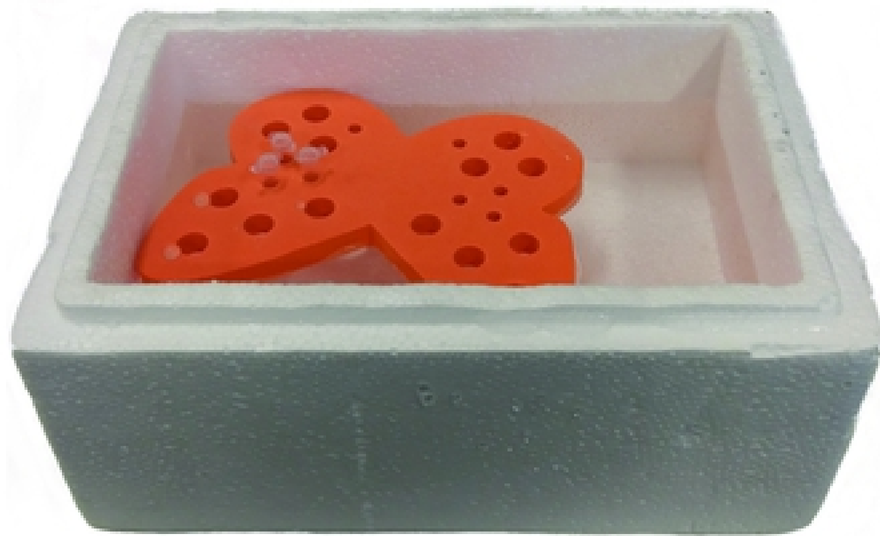


Figure 3





Sample maceration and enrichment
(overnight incubation)

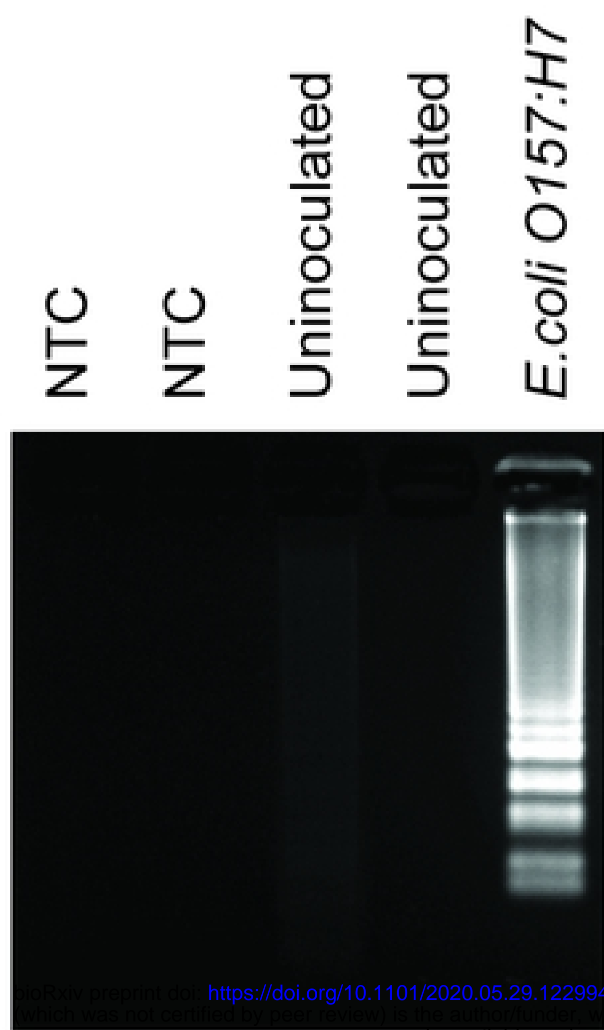
Cell lysis
(30 minutes)

DNA amplification
(50 minutes)

Flocculation readout
(30 seconds)

Figure 4

A



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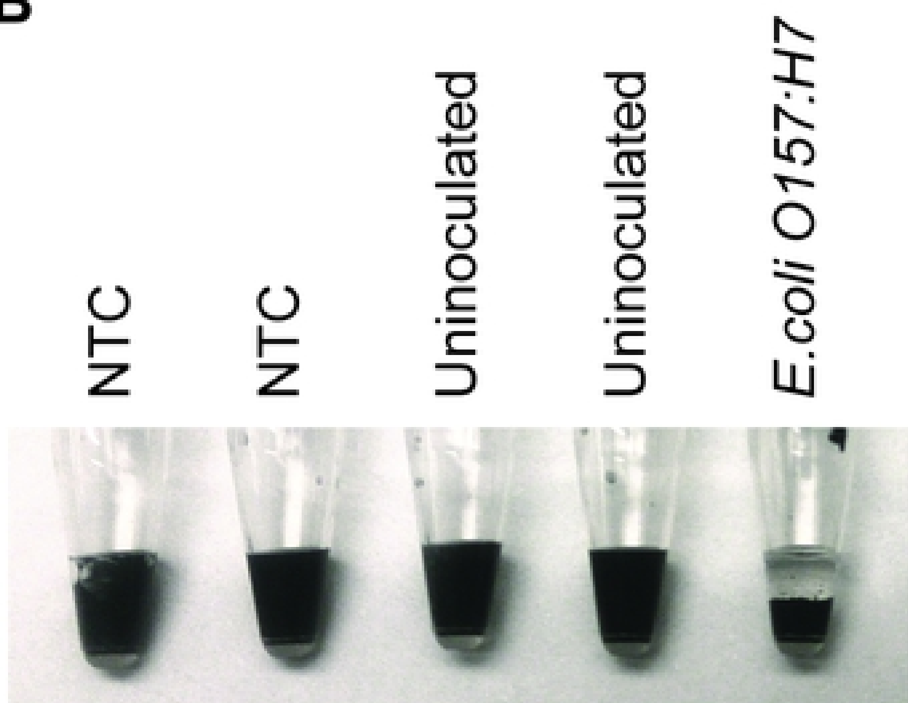
B



C

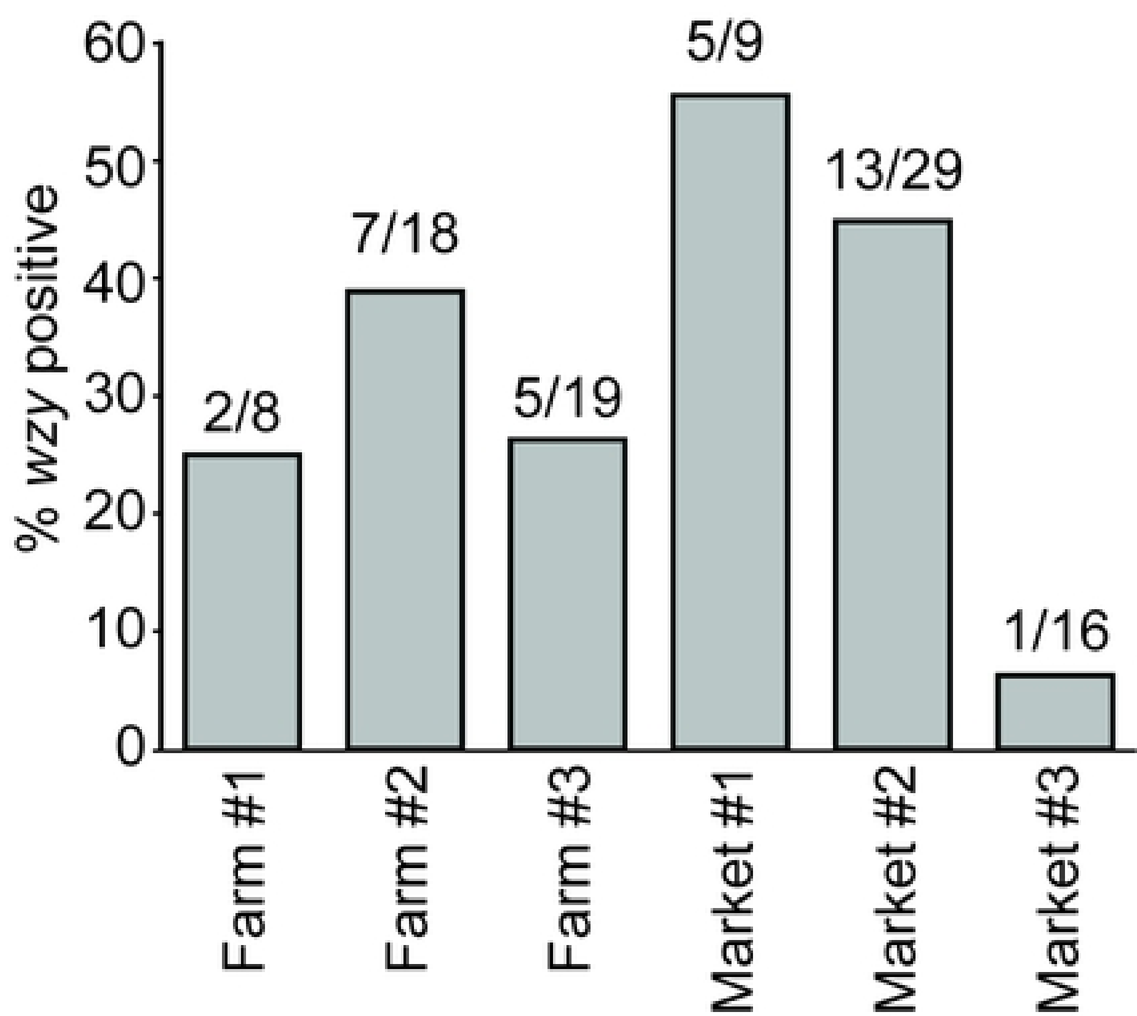


Figure 5

B

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25x diluted undiluted

1µl 2µl 1µl 2µl 4µl

S1 Figure