Title: High-throughput multi-parallel enteropathogen quantification via nano-liter qPCR

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Abstract

Quantitative molecular diagnostic methods, such as qPCR, can effectively detect pathogen-specific nucleic acid sequences. However, costs associated with multi-pathogen quantitative molecular diagnostics hinder their widespread use. Nano-liter qPCR (nL-qPCR) is a miniaturized tool for quantification of multiple targets in large numbers of samples based on assay parallelization on a single chip, with potentially significant cost-savings due to rapid throughput and reduced reagent volumes. We evaluated a suite of novel and published assays to detect 17 enteric pathogens using a commercially available nL-qPCR technology. Assay efficiencies ranged from 88-98% (mean 91%) and were reproducible across four operators at two separate facilities. When applied to complex fecal material, assays were sensitive and selective (99.8% of DNA amplified were genes from the target organism). Detection limits were 1-2 orders of magnitude higher for nL-qPCR than an existing enteric TaqMan Array Card (TAC), due to nanofluidic volumes. Compared to the TAC, nL-qPCR displayed 97% (95% CI 0.96, 0.98) negative percent agreement and 63% (95% CI 0.60, 0.66) overall positive percent agreement. Positive percent agreement was 90% for target concentrations above the nL-qPCR detection limits. nL-qPCR assays showed an underestimation bias of 0.34 log_{10} copies/gram of stool [IQR -0.41, -0.28] compared with the enteric TAC. Higher detection limits, inherent to nL-qPCR, do not hinder detection of clinically relevant pathogen concentrations. With 12 times higher throughput for a sixth of the per-sample cost of the enteric TAC, the nL-qPCR chip described here is a viable alternative for enteropathogen quantification for studies where other technologies are cost-prohibitive.
Introduction

Quantitative molecular diagnostic methods, such as quantitative polymerase chain reaction (qPCR), can target nucleic acid gene sequences specific to known microbial pathogens. These methods have provided insights in the study of diarrheal disease beyond what can be gained using microbiological cell culture or immunoassays (1–4) and have been applied successfully in the field of pathogen detection for decades (5–9). Over time, molecular diagnostics were developed from single-gene qPCR assays to multiplex reactions (10–14) and to multi-assay, multi-sample arrays that can be operated in parallel on a single chip or card (15–19). Specifically in the field of enteric pathogen detection, a TaqMan Array Card (TAC) was developed by Liu and colleagues (15, 16) and subsequently used in several studies to estimate pathogen-attributable diarrhea burdens (3, 4, 20), as well as the impact of enteric pathogens on child growth (21–23) and vaccine uptake (24, 25). However, despite advances in the throughput of molecular detection of pathogens, costs associated with broad multi-target molecular assays still pose a barrier to their widespread use in epidemiological studies. For instance, the per-sample cost of the enteric TAC is $60-155, not including labor, capital equipment, nor DNA extraction reagents (15).

Compared with TaqMan qPCR arrays, higher-throughput microfluidic qPCR technologies hold potential to decrease per sample costs of multi-target diagnostics. In the case of nano-liter (nL) qPCR, precision robotic dispensing permits smaller reaction volumes, increases throughput, and reduces reagent volumes. While nL-qPCR technologies have been previously applied to pathogen detection, early efforts to develop nL-qPCR pathogen chips were limited by factors such as: (i) high-detection limits associated with small reaction volumes (6-33 nL), (ii)
insufficient assay validation, and (iii) relatively low sample throughput per chip (12-48 samples) (26–28).

In more recent studies, a commercial nL-qPCR technology (SmartChip™ Real-Time PCR, TakaraBio Inc.) was used to design multi-target diagnostics to detect the presence of antibiotic resistance genes in urban wastewater treatment plant effluent, reclaimed water, and environmental samples (29–31) and to evaluate a suite of related dehalogenase genes in complex microbial communities (32). This technology uses 100 nL reaction volumes and allows for flexible configuration of a 5184-well chip that can analyze up to 384 samples (depending on the number of assays included). Using this platform, we developed a nL-qPCR chip with 54 assays (targeting 17 enteric pathogens) across 96 samples in duplicate. Here, we present comprehensive validation of the technology with laboratory standards as well as fecal samples from children in rural Bangladesh. The nL-qPCR enteropathogen chip permits high-throughput, rapid pathogen detection at significantly lower cost per-sample than other methods.

Methods

Assay design

We selected bacterial, protozoan, and helminthic enteropathogens identified as contributing to diarrheal disease in children across twelve countries (33, 34). We computationally designed and screened 175,000 candidate primer pairs to target 16 virulence genes using methods described previously (32). Briefly, amino acid sequences corresponding to all non-redundant members of each target gene’s protein family (Pfam v 27.0) were clustered based on percent pairwise identity using BLASTp all-vs-all search (35). We downloaded
corresponding nucleotide sequences from NCBI, and DNA oligonucleotide primers were
designed to target conserved DNA-level sequence motifs in sequence clusters containing the
target virulence protein for each pathogen. A Python script directed the software primer3 (36)
to develop thousands of candidate primer pairs for each target gene, which were then screened
in silico against other non-target clusters within the same protein family. Up to eight assays per
target gene were selected for laboratory screening. We included an additional 10 published
assays (see Table 1) to assess the suitability for inclusion of previously validated assays
optimized at similar PCR conditions (15, 37–41).

**nL-qPCR procedures and assay pre-screening**

Assays were initially evaluated against 490bp synthesized linear DNA strands (Integrated
DNA Technologies, Inc., Coralville, IA) for each target gene. Oligonucleotide primers (Integrated
DNA Technologies, Inc., Coralville, IA) at a final concentration of 1 µM were added to
LightCycler 480 SYBR Green I Master Mix (Roche Applied Sciences, Indianapolis, IN) and were
robotically dispensed onto nL-qPCR chips using TakaraBio’s SmartChip™ platform. In a separate
plate, samples were added to additional master mix and robotically dispensed onto chips.
Duplicate chips were run, using the standard TakaraBio protocol: 95°C for 3 min, then 40 cycles
of (95°C for 60s, 60°C for 70s). We excluded assays that reproducibly displayed fluorescence in
the negative control (PCR grade water) prior to cycle 28, failed to amplify standards at 100
copies per well, or had PCR efficiencies less than 85%. Final assays were selected based on
optimal performance characteristics as described below. Each chip contained a minimum of
two negative (no-template) controls for each assay.
Analytical performance characteristics

Analytical performance was evaluated in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (42). Assay efficiencies were evaluated with a pool of synthetic DNA standards, described above. Standards were 10-fold serially diluted (10 to $10^6$ copies/reaction). Standard curves were run on a minimum of 15 chips over two instruments at separate facilities (Fremont, CA and East Lansing, MI) and with two different operators at each location. Efficiencies were calculated according to Rutledge and Côté (43); mean efficiency over all runs is reported along with coefficient of variation (CV). Limit of detection (LOD) was determined with pooled synthetic DNA standards spiked into extracted DNA from 10 fecal samples to a final concentration of 10, 100, and 1000 copies/reaction; then each sample was run in duplicate on two separate chips. The mean cycle quantification ($C_q$) value (i.e. the cycle at which sufficient copies of target DNA have been made to produce a fluorescent signal detectable by the instrument) was calculated for duplicate assays on a single chip, and all results under the $C_q$ cutoff of 30 were determined positive. A total of 20 positive samples (10 samples x 2 chips) per target at each concentration were assayed and LOD is defined as the lowest concentration which 95% were positively detected (i.e. where 19 of the 20 were detected).

Inter-assay precision (reproducibility) was assessed across the standard curves used for efficiency calculations measured over 15-20 chips, using different lots of master mix, different batches of oligonucleotide primers, and 4 different operators at 2 separate facilities. We report the mean CV on calculated copy numbers over all points on the standard curve as well as the
range. Intra-assay precision (repeatability) was measured within-chip and between chips.

Within-chip precision was evaluated in three samples in which extracted DNA from fecal samples was mixed with positive controls at high ($10^5$ copies/reaction) and low (100 copies/reaction) concentrations and assayed 20 times on a single chip: we report the CV of calculated copy number across the 20 replicates. Between-chip precision was evaluated in 223 fecal samples collected from a cohort of Bangladeshi children that tested positive for at least one pathogen ($C_q < 30$) plus 18 additional fecal samples into which we added positive controls. Replicates for each sample were run on two chips and the CV of calculated template copies was determined across all four replicates. We report mean CV of calculated template copies over all samples, as well as the number of unique samples included in the calculation of the mean.

Sensitivity and specificity were evaluated using four pools of DNA standards, spiked into extracted DNA from 40 pathogen-free fecal samples. For each pathogen 10 samples contained the target at low concentration (100 copies/reaction), 10 samples at medium concentration (10x the LOD) and 10 samples at high concentration (100x the LOD); an additional 10 samples had no target. Sensitivity and specificity were determined based on positive or negative detection in these 40 samples. In order to further verify assay specificity, we sequenced PCR amplicons obtained from running 96 child fecal samples on the nL-qPCR chip. The Seq-Ready™ TE MultiSample FLEX protocol, PCR clean-up, and DNA quantification prior to sequencing were done in accordance with TakaraBio’s standard procedures, as described previously (Atshemyan, 2017; Firtina, 2017). The resulting paired-end Illumina MiSeq reads were quality filtered and only sequences that were the expected target gene amplicon length (+/-3 bp) were maintained. We verified the intended target (organism and gene) by conducting a nucleotide
BLAST search (35) on each unique sequence. We retained the top hit(s), defined as the highest sequence identity with the lowest E value.

Sample collection

To test the performance of nL-qPCR chip and against the performance of enteric TAC in epidemiology-relevant samples, we utilized 254 fecal samples from children in rural Bangladesh. Children were between 10 and 18 months old and enrolled in the WASH Benefits randomized controlled trial (44–47). Split samples from these children had been previously assayed for pathogens with the TAC technology (manuscript in preparation). Samples were collected by the child’s caregiver into a sterile collection container and placed on cold chain within 165 [IQR 79, 791] minutes, transported to the laboratory and held at -80°C prior to analysis. DNA was extracted according to previously published protocols (16) in the Parasitology lab at the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) and separated into two aliquots: one aliquot was subjected to TAC analysis at icddr,b and the other was shipped on dry ice to Stanford University. Samples were collected after obtaining written, informed consent from the child’s primary caregiver and with approval from human subjects committees at icddr,b (PR-11063), University of California, Berkeley (2011-09-3652), and Stanford University (25863).

Statistical analyses

Data analysis was performed in R statistical software (v3.5.2) and analysis files are available as supplemental files. CV, the standard deviation of replicates divided by the mean,
was used to evaluate precision in accordance with the MIQE guidelines (42). Specifically, CV of calculated copy number, and not $C_q$ value, is reported per Schmittgen and Livak (48) and Hellemans et al. (49). Within-chip, between-chip, and between instrument/operator variances were compared with a pairwise Wilcoxon rank sum test, using the Benjamini-Hochberg procedure to account for multiple comparisons (50). Sensitivity and specificity were calculated using the epi.test function from the epiR package (51). Positive percent agreement and negative percent agreement were calculated in the same manner and are reported with this alternative nomenclature as recommended when no known reference standard is used (52). Exact binomial 95% confidence limits on sensitivity and specificity were calculated according to Collett (53). Unweighted Cohen’s Kappa was calculated using the epi.kappa function with confidence intervals calculated according to Rothman (54). Bias in calculated $\log_{10}$ copy numbers per gram of stool (corrected for extraction and PCR efficiency by normalizing to the positive control PhHV spike-in) was evaluated according to Bland and Altman (55) using the blandr::blandr.statistics function to estimate bias (56); 95% confidence intervals determined per Bland and Altman (57).

**Data Availability and Cost Estimates**

Nucleotide sequences obtained from Bangladeshi child fecal samples for the specificity analysis are deposited in the Sequence Read Archive under BioProject # SUB5519617. Cost estimates for both nL-qPCR and TAC qPCR technologies can be found in Table S1.
Analytical performance

The mean efficiency for each assay, based on the evaluation of standard curves run on 15-20 chips, ranged from 88-98% (mean 91%) with a coefficient of variation of 6.3% [IQR 5.3, 7.3](Table 2). The linearity over all assays on all chips was 0.990 [IQR 0.987, 0.992] and detection limits were between 10-100 copies/100nL reaction, which corresponds to 8x10^5-8x10^6 copies/g of stool (Table 2). Within-chip repeatability was assessed in ten replicates on a single chip: synthetic DNA in high (10^5 copies/reaction) and low (10^2 copies/reaction) concentrations was spiked into DNA extracted from fecal samples. The high concentration displayed a coefficient of variation in calculated copy number of 15% [IQR 8 – 25]); the low concentration had variability of 27% [IQR 18 – 36] (Figure S1).

C_q values across replicate chips were highly repeatable for synthetic DNA standards (R^2 = 0.989, Figure 1a), for synthetic DNA in a complex stool DNA matrix (R^2 = 0.984, Figure 1b) and for DNA extracted from fecal samples collected from children in Bangladesh (R^2 = 0.935, Figure 1c). Fecal samples displayed a median difference in C_q values of 0.39 [IQR 0.15 – 0.81](Figure 1c) across all assays, which corresponds to a coefficient of variation on calculated gene copy number of 28% [IQR 16 – 50] (Table 2, Repeatability). The highest variability was again seen at the lowest concentrations (Figure S2).

Assays were reproducible across two instruments and four operators, again with an inverse relationship noted between variance and concentration (Table S2). At concentrations one or more orders of magnitude above the detection limit, coefficient of variation on calculated copy number ranged from 17 – 44% (Table 2). Coefficient of variation at the limit of detection ranged from 29% to 115% for pathogen virulence and marker genes, the highest of which was
analogous to 17 ± 20 copies detected. The highest variance (319%) observed was for the total bacterial (16S rRNA) assay at the detection limit of 10 copies/reaction. Between-chip variance was similar to variance across two instruments and four operators (p = 0.99) but both were significantly higher than within-chip variance (p < 0.0001, pairwise Wilcoxon rank sum test).

Coefficients of variation of the magnitudes observed are not biologically relevant when analyzing pathogen quantities on the log_{10} scale, as is the normal procedure. Analytical sensitivity ranged from 98-100% and specificity from 90-100% (Table 2) among 60 samples containing combinations of synthetic nucleic acid spiked into DNA extracted from 15 different individuals and assayed in duplicate. To further ensure the specificity of the assays, we sequenced amplicons from 96 fecal samples collected from children in Bangladesh that tested positive for at least one pathogen target. We obtained 1.7M (26,747 unique) sequences with 330 [IQR 142, 1171] unique sequences per assay. Amplicon sequencing showed that the assays were specific. The intended gene target was correctly identified in the top hit(s) (defined as highest identity and lowest E value) for 99.8% of unique sequences. Most (99.7%) of the BLASTn searches returned a database top hit with ≥97% sequence identity. The *Ascaris lumbricoides* assay had highest number of off-target hits: 7/130 of the unique sequences were identified as the same target gene in a closely related species, *Ascaris ovis*.

**Clinical performance**

We analyzed 254 fecal samples collected from children in Bangladesh on both the nL-qPCR chip and the TAC to compare performance. Overall percent agreement was 90% for the >4500 reactions and negative percent agreement was 97% (95% CI 0.96, 0.98)(Cohen’s Kappa =
0.66 (95% CI 0.63 – 0.69)). Positive percent agreement was highly dependent on concentration of the target gene. At concentrations above nL-qPCR detection limits (>10^7 copies/g stool) positive percent agreement was 90%; this dropped to 62% for concentrations near the nL-qPCR detection limits (10^5-10^7 copies/g stool) and fell to 8% for concentrations below 10^5 copies/g stool. In instances where both methods detected the presence of target genes, nL-qPCR assays displayed a median underestimation bias of -0.34 log_{10} copies [IQR -0.41, -0.28] (see Table 3 and Figure S3 for individual assay statistics).

Reactions detected by TAC but not by nL-qPCR were typically below nL-qPCR detection limits, with TAC C_q values approximately 30.9 [IQR 28.6, 33.0] (Figure 2a, black points). The higher detection limits for nL-qPCR assays did not interfere with detection of diarrhea-causing pathogen concentrations, with the exception of the V. cholerae assay which had an etiologic cutoff that was below the nL-qPCR detection limit. The etiologic cutoff (shown as red lines in Figure 2a) indicates the TAC C_q value below which children were highly likely to have diarrhea, i.e. the value at which the odds ratio for diarrhea cases compared to controls was greater than 2 (Liu, 2016, Platts-Mills, 2018). nL-qPCR assays detected all but 8 of the 40 reactions in which TAC assays detected a sample below the etiologic C_q cutoff value (3 of which were for V. cholerae), and typically detected samples well above the cutoff for most assays (Figure 2a). Reactions positive by nL-qPCR but not TAC were also at low concentrations (Figure 2b) and could have been the result of less stringent amplification without the use of probe-based dyes with nL-qPCR.

Contamination may cause false-positive qPCR results, and can occur due to cross-contamination between samples or as a result of free ambient DNA in the laboratory.
environment. Sample cross-contamination occurred rarely with nL-qPCR; amplification of
pathogen virulence or marker genes in no-template controls occurred in <3% of the 4288 no-
template control sample reactions. Moreover, these amplifications resulted in calculated copy
numbers near or below the established limit of detection (median 35 [IQR 28 – 42] calculated
copies). Ambient laboratory contamination was detected more frequently. Amplification of
bacterial 16S rRNA occurred in 46% of no-template controls, and was highly dependent on
operator. In all cases, contamination was near the detection limit with 11 [IQR 7, 25] calculated
copies of bacterial 16S rRNA detected. Cross-contamination, although possible, occurs rarely
and only in low concentration, thereby indicating a low likelihood of false positive results.

Discussion

The nL-qPCR chip evaluated here provides satisfactory analytical performance for
simultaneous analysis of 96 samples against a suite of 17 enteric pathogens for a cost of
<$10/sample. The high-throughput nature of the nL-qPCR chip is particularly advantageous
when large numbers of samples need to be processed in a timely manner, such as in
population-based studies. Above certain thresholds, we found analytical performance to be
comparable to an enteric TAC widely used for investigations of diarrheal disease in diverse
global populations, for roughly a quarter of the per-sample cost ($60; (15)).

The primary difference in performance we observed was that a majority of the nL-qPCR
assays had detection limits 1-2 orders of magnitude higher than TAC. This was due to reactions
that utilize 16 times less sample volume (0.0125 µL compared to 0.2-0.4 µL for TAC;
(15, personal communication with J. Liu, 2019). Furthermore, among 254 fecal samples from
Bangladeshi children 14 months old, most nL-qPCR assays displayed an underestimation bias (i.e. returned a lower estimated number of copies per gram of stool) compared to the enteric TAC. However, these differences do not appear to be limitations in terms of ability to distinguish pathogen loads relevant for diarrheal disease for pathogens with etiological cutoffs established, with the potential exception of infection with *Vibrio cholerae*. Importantly, the TAC and nL-qPCR assays for *V. cholerae* target different virulence genes: hemolysin (*hlyA*) for TAC and toxin-coregulated pilus (*tcpA*) for nL-qPCR. The etiologic cutoffs were established for *hlyA*, which is commonly detected in environmental *V. cholerae* strains that lack both the *tcpA* and cholera toxin genes (58). Thus, discordant detection between the technologies might not represent differences in performance, but rather differential presence of these virulence genes within *V. cholerae* strains. Given that studies have shown low concentrations of *V. cholerae hlyA* gene are observed in feces coincident with diarrheal symptoms in children (3, 4), this might be a superior gene target for *V. cholerae* in pathogen panels. Additional studies should verify the optimal gene target for diarrhea-causing *V. cholerae* species.

In studies where quantitation is required at lower concentrations than were achieved in this study, pre-amplification can be performed as described by Ishii et al. (28). In addition, pre-printing primers directly onto chips, similar to the TAC spotting procedure, can reduce detection limits by nearly 50%. However, a major advantage of the nL-qPCR SmartChip™ is the flexibility of the platform. Therefore, if a research team does opt to pre-print primers onto chips, we suggest also maintaining a stock of unprinted chips on-hand. The current configuration of the chip was designed with large-scale epidemiology studies in mind, thus
increased throughput was prioritized over the inclusion of a higher number of assays. However, researchers wishing to focus on a smaller set of targets can evaluate more samples per plate (further reducing per-sample costs), or the number of samples can be reduced to accommodate an increased number of assay targets. In large-scale studies, replicating analysis for questionable samples is often necessary (e.g. when replicates give discordant results).

Unprinted chips allow for an operator to run a limited suite of sample/assay pairs that need to be reanalyzed: for example, 384 samples with questionable results in the initial run from a large study could be analyzed against a minimal suite of 12 assays on a specially designed chip at the end of the study. This facilitates the resolution of discordant results and minimizes missing values in the final dataset, which will maximize statistical power in the analysis stage.

Unprinted nL-qPCR chips also allow end-users to substitute assays from the ones that we publish here, with appropriate assay validation. This evaluation included 10 pre-published assays that operate at similar PCR conditions, and found they performed well in nL format, suggesting end users have flexibility in re-designing the chip. We further show that seven primer pairs previously validated using TaqMan with probe-based dyes had excellent specificity among 96 fecal samples when utilized with SYBR Green intercalating dye instead. These results suggest the additional reagent costs associated with probes is not necessary to achieve high specificity and is consistent with other findings that have reported equal or superior specificity with SYBR Green compared to TaqMan chemistry (59, 60).
Quantifying nucleic acid targets for large numbers of samples is costly, regardless of the platform used, and recommended best practices are sometimes sacrificed in the face of limited budgets. For example, technical replicates are generally encouraged to facilitate identification of outlier or spurious results, particularly on chip- or card-style platforms, and increase the likelihood of detection near the detection limit where analytical precision is the lowest (15, 61, 62). The nL-qPCR pathogen chip is configured to provide duplicate results for the 24 pathogen-specific virulence and marker genes. This was deliberate as it is impossible to determine *a priori* if a sample will be near the detection limit, particularly in the case of fecal samples where the presence of PCR inhibitors is likely (63, 64). Early versions of the enteric TAC included replicates (15), but those have been replaced by additional pathogen targets in latter versions currently in use for large-scale studies (3, 4). Due to the flexibility in configuration of the nL-qPCR, up to 13 additional pathogen targets could be added without sacrificing duplicate assays, and throughput would still be 8-9 times higher and cost 50% less than the enteric TAC. The lower per-sample cost reduces the temptation to compromise best practices in the face of budgetary constraints.

The nL-qPCR platform has important limitations. First, due to the open chip technology, there is higher likelihood for contamination if not used in a controlled laboratory with minimal ambient contamination and meticulous operators. nL-qPCR does not appear to be well-suited for absolute quantification of total bacteria due to the fact that general bacterial contamination (via 16S rRNA) was detected in almost half of the no-template control samples, albeit at concentrations near the detection limit. To ensure potential low-concentration contamination
is identified, we strongly recommend incorporation of replicates when using this technology or more stringent $C_q$ filtering (e.g. $C_q$ 28 or lower). Another limitation of the current configuration is the omission of viral enteric pathogen targets. The primary aim for this study was to validate the nL-qPCR technology for bacterial and parasitic targets, and we expect that future iterations of the chip will include viral targets, which could be combined with a reverse-transcriptase protocol for the study of RNA as well as DNA viruses.

In conclusion, we found the nL-qPCR pathogen chip to be an acceptable alternative to other methods; particularly for studies with large numbers of samples as savings in both cost and time will be amplified at scale.

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Figure 1. Assay precision across replicate chips for (a) synthetic DNA standards across a 6-fold dilution series, (b) synthetic DNA standards spiked into child fecal samples (n = 60), and (c) child fecal samples (n = 254). Each point represents the replicated results for a single sample-assay reaction run in a specific location on the chip. Points shown with color indicate results from amplified DNA standards with defined input copy number (10-10^6); grey points indicate results from fecal samples with unknown input copy number, absent synthetic standards.
Figure 2. Comparison of nL-qPCR and TAC assays across 254 fecal samples. Samples detected by (a) TAC or (b) nL-qPCR are shown with their respective \( C_q \) values. Blue (top) and purple (bottom) points represent positive detections found in both nL-qPCR and TAC tests. Gray points (top) represent targets undetected by nL-qPCR but detected by TAC, and vise versa (bottom). Red lines represent pathogen TAC etiologic cutoff \( C_q \) values (etiologic cutoffs have not been established for nL-qPCR assays).
Table 1. Assays included on the nL-qPCR pathogen chip. The chip contains each assay in duplicate, except for two of the Quality Control assays (ACAA2 and ESSRA).

| Organism                                      | Gene Target | Sequence (5’ – 3’)                  | Reference       |
|-----------------------------------------------|-------------|-------------------------------------|-----------------|
| **Pathogenic *Escherichia coli***             |             |                                     |                 |
| Enteroaggregative *E. coli* (EAEC)            | *aggR*      | F: CAGCGATACATTAAGACGCCT            | This study      |
|                                                |             | R: TCTTTTGACCAATTTTCGGACA           |                 |
| Enterotoxigenic *E. coli* (ETEC)              | STh (*estA*)| F: TTCACCTTTTCGTCAGGATG             | This study      |
|                                                |             | R: CCCGGTACAAGCAGGATTAC             |                 |
|                                                | STp (*estA*)| F: ACTGAATCACTTGACTCTCTCCAAAA      | This study      |
|                                                |             | R: ACAACAAAAAGTCACAGCAGTAA         |                 |
|                                                | LT (*eltA*) | F: CCTGGATTCATCATGCACCA             | This study      |
|                                                |             | R: TCTGGGTCCTTCATTACAAAGT           |                 |
| Enteropathogenic *E. coli* (EPEC)             | *bfpA*      | F: GCGAAAGGCTACGGTGTTA             | This study      |
|                                                |             | R: GCCTCAGCAGGATATAAGC             |                 |
|                                                | *eaeA*      | F: GGTCAGATTCAGCATAGCGG             | This study      |
|                                                |             | R: CGCGAGCGGTCACCTTTTAA            |                 |
| Shiga toxin-producing *E. coli* (STEC)        | *stx1*      | F: ACAGATGGAAATCTTCAGTCTCTTCT      | This study      |
|                                                |             | R: CTGAATCCCCCTCCATTATGAC           |                 |
|                                                | *stx2*      | F: CGTTAATGCAATGGCGG               | This study      |
|                                                |             | R: TGCAGTAACGGGTTGCGATT            |                 |
| **Other Bacteria**                            |             |                                     |                 |
| *Campylobacter jejuni/coli*                   | *cdtA*      | F: AAAGGATTTGGCGATGCTAGA             | This study      |
|                                                |             | R: CGCCTGTATTGTCATAGG              |                 |
| *Clostridium difficile*                       | *tcdB*      | F: GGTATTACCTAATGCTCAAAATAG        | (15)            |
|                                                |             | R: TTGGTCCATCATTTTTCTAAGC          |                 |
| *Clostridium perfringens*                     | *CPE*       | F: GCTGCTGCTACAGAAAGATTTAAA        | This study      |
|                                                |             | R: AAGCTTTTGTAGTCAAGGGT            |                 |
| *Helicobacter pylori*                         | *ureA*      | F: AAATCGGAACCGTGATAG              | This study      |
|                                                |             | R: TGCCCTCGTGATAGTG                |                 |
| *Salmonella* spp.                             | *invA*      | F: TTGACGGTGCAGATAGTT              | This study      |
|                                                |             | R: CCACCGAAATACCGGCAATT            |                 |
| *Shigella* spp./enteroinvasive *E. coli* (EIEC)| *ipaH*     | F: GTCAGAAGCGTGGAAGAGA             | This study      |
|                                                |             | R: TCAGTAACAGCATGCGCAAGGG          |                 |
| *Vibrio cholerae*                             | *tcpA*      | F: ACAGATAAGAAGGACCGGTCA           | This study      |
|                                                |             | R: GCCCTGGTCTATTTCCTGCA            |                 |
| Organism                                | Gene  | F        | R        | Sequence Length |
|----------------------------------------|-------|----------|----------|-----------------|
| **Yersinia enterocolitica**             | yadA  | F: GCCCAGAAAGATGGAGTAGC | R: CGTGACTAGAGTGCCAATGG | This study |
| **Protozoa & Helminthes**               |       |          |          |                 |
| Cryptosporidium spp.                    | 18S rR| F: GGGTTGTATTTATTAGATAAAAGAACCA | R: AGGCCAATACCCCTACCCTGCT | (15)     |
| Entamoeba histolytica                   | 18S rR| F: ATTGTGCCTGGCATCTAATACCA | R: GCGGACCGTCCCATTATAACA | (36)     |
| Giardia lamblia                         | 18S rR| F: GACGGCTCAGGACAACGGTT | R: TTGCCAGCGGTGCCGG | (36)     |
| Ascaris lumbricoides                    | ITS1  | F: GTAATAGCACGTGGCGGTTTCTT | R: GCCCAAACATGCCACCTATT CC | (37)     |
| Trichuris trichiura                     | 18S rR| F: TGAAACGACTTGCTCATCAACTT | R: CTGATTCTCCGTTAACCGGTTGTC | (15)     |
| **General**                             |       |          |          |                 |
| Total Bacteria                          | 16S rR| F: GTGSTGCAYGGYTGTGCA | R: ACGTCCACCCACCTTCTC | (58)     |
| Total Archaea                           | 16S rR| F: ATTAGATACCCSBGTAGTCC | R: GCCATGCACCWCTTCT | (38)     |
| Total Fungi                             | ITS1  | F: CTGGTCCATTAGAGGAAGTAA | R: GCTCGGTTCCTTCATCGGATGC | (39)     |
| **Quality Control**                     |       |          |          |                 |
| Phocine herpesvirus-1 (PhHV)            | gB    | F: GGCGCAATCACAGATTGAATC | R: GCCGTTCCAACGTACCAA | (40)     |
| Mus musculus                            | ACA2  | F: ACAGATACGCTCGAGTC | R: CTGTTTGCCCTTCTTCTTGCTT | (31)     |
|                                        | B2M   | F: GGTTTCCTGGTGCTTGCTT | R: ACGTAGCAGTTCATGATGTCG | (31)     |
|                                        | ESRRA | F: CCTGCAAAGCCTTCTTCAAG | R: GTCTCCGCTTTGGGATCTC | (31)     |
Table 2. Analytical performance of the nL-qPCR pathogen chip.

| Organism (gene target) | Efficiency % (CVa) | LODb (copies/reaction) | Reproducibility CV above at LODc | Repeatability CV (n)d | Sensitivity | Specificity |
|------------------------|--------------------|------------------------|---------------------------------|-----------------------|-------------|-------------|
| Pathogenic E. coli     |                    |                        |                                 |                       |             |             |
| EAEC (aggR)            | 95 (0.16)          | 8e+05 (10)             | 26,115                          | 19 (75)               | 100         | 93          |
| ST-ETEC (Sth)          | 90 (0.07)          | 8e+06 (100)            | 22,51                           | 44 (8)                | 100         | 100         |
| ST-ETEC (Stp)          | 92 (0.08)          | 8e+06 (100)            | 28,39                           | 28 (13)               | 100         | 100         |
| LT-ETEC (eltA)         | 91 (0.08)          | 8e+06 (100)            | 24,49                           | 18 (25)               | 100         | 100         |
| EPEC (bfpA)            | 89 (0.05)          | 8e+06 (100)            | 26,43                           | 32 (13)               | 100         | 100         |
| EPEC (eaeA)            | 90 (0.05)          | 8e+05 (10)             | 23,55                           | 33 (59)               | 100         | 93          |
| STEC (stxl)            | 89 (0.08)          | 8e+06 (100)            | 18,47                           | 46 (6)                | 100         | 100         |
| STEC (stx2)            | 92 (0.08)          | 8e+06 (100)            | 22,57                           | 62 (16)               | 100         | 100         |
| Other Bacteria         |                    |                        |                                 |                       |             |             |
| Campylobacter jejuni/coli (cdtA) | 92 (0.07) | 8e+06 (100)            | 32,52                           | 29 (35)               | 100         | 100         |
| Clostridium difficile (tcdB) | 90 (0.05) | 8e+06 (100)            | 20,34                           | 25 (8)                | 100         | 100         |
| Clostridium perfringens (CPE) | 88 (0.06) | 8e+05 (10)             | 22,52                           | 24 (2)                | 98          | 100         |
| Helicobacter pylori (ureA) | 91 (0.07) | 8e+06 (100)            | 28,92                           | 42 (2)                | 100         | 100         |
| Salmonella enterica (invA) | 91 (0.07) | 8e+05 (10)             | 21,53                           | 68 (3)                | 100         | 100         |
| Shigella/EIEC (ipaH)   | 90 (0.07)          | 8e+06 (100)            | 20,29                           | 25 (15)               | 100         | 100         |
| Vibrio cholerae ( tcpA) | 89 (0.06)          | 8e+06 (100)            | 20,44                           | 41 (1)                | 100         | 100         |
| Yersinia enterocolitica (yadA) | 92 (0.07) | 8e+06 (100)            | 26,61                           | 64 (2)                | 100         | 100         |
| Protozoa & Helminthes  |                    |                        |                                 |                       |             |             |
| Cryptosporidium (18S)  | 88 (0.07)          | 8e+06 (100)            | 36,103                          | 76 (4)                | 100         | 100         |
| Entamoeba histolytica (18S) | 90 (0.1)    | 8e+06 (100)            | 28,46                           | 54 (2)                | 100         | 100         |
| Giardia (18S)          | 90 (0.07)          | 8e+06 (100)            | 21,31                           | 33 (17)               | 100         | 90          |
| Ascaris lumbricoides (ITS1) | 89 (0.05) | 8e+05 (10)             | 20,53                           | 16 (2)                | 100         | 100         |
| Trichuris trichiura (18S) | 91 (0.07) | 8e+05 (10)             | 17,98                           | 28 (2)                | 100         | 100         |
| General                |                    |                        |                                 |                       |             |             |
| Total Archaea (16S)    | 90 (0.06)          | 8e+06 (100)            | 44,75                           | 70 (2)                | 100         | 93          |
| Total Bacteria (16S)   | 98 (0.09)          | 8e+06 (100)            | 31,319                          | 58 (34)               | 100         | 87          |
| Total Fungi (ITS1)     | 91 (0.1)           | 8e+06 (100)            | 43,54                           | 58 (34)               | 100         | 100         |
| QC                     |                    |                        |                                 |                       |             |             |
| PhHV (gB)              | 92 (0.07)          | 8e+06 (100)            | 22,66                           | 52 (92)               | 100         | 100         |

a Coefficient of variation (CV) of efficiency calculated across 15-20 chips
b Minimum no. of gene copies per g of stool (minimum no. gene copies per 100 nL reaction)
c CV on calculated copy number for all points along the standard curve measured over 15-20 chips, shown separately for concentrations above the LOD and at the LOD
d CV in calculated copy number across 4 replicates as measured in n positive samples, mean CV is reported
Table 3. Bias estimates by assay on calculated $\log_{10}$ copy number per gram of stool for nL-qPCR compared to TAC. The table includes 529 reactions that were concordant for detection on both platforms.

| Pathogen (gene target)                      | n  | Bias (95% CI)         |
|---------------------------------------------|----|-----------------------|
| EAEC (aggR)                                 | 96 | -1 (-1.1, -0.9)       |
| ST-ETEC (STh)                               | 11 | 0.1 (0, 0.2)          |
| ST-ETEC (STp)                               | 25 | -0.5 (-0.9, -0.2)     |
| LT-ETEC (eltA)                              | 40 | -0.8 (-0.9, -0.6)     |
| EPEC (bfpA)                                 | 17 | -0.7 (-0.8, -0.5)     |
| EPEC (eaeA)                                 | 78 | 0 (-0.2, 0.1)         |
| STEC (stx1)                                 | 5  | 0.4 (0, 0.8)          |
| STEC (stx2)                                 | 9  | 0.7 (-0.1, 1.6)       |
| Campylobacter jejuni/coli (cdtA)            | 52 | 0.1 (0, 0.3)          |
| Clostridium difficile (tcdB)                | 9  | -0.8 (-1.5, -0.1)     |
| Helicobacter pylori (ureA)                  | 1  | 1.8 (NaN, NaN)        |
| Salmonella enterica (invA)                  | 1  | -0.1 (NaN, NaN)       |
| Shigella/EIEC (ipaH)                        | 19 | -1 (-1.2, -0.8)       |
| Cryptosporidium (18S)                       | 6  | -1.1 (-2.4, 0.2)      |
| Giardia (18S)                               | 17 | -1.6 (-2.1, -1.1)     |
Figure S1. Within-chip intra-assay precision among samples containing a mixture of synthetic DNA standards and extracted DNA from 3 separate fecal samples. Each sample was assayed 10 times on a single chip. Synthetic DNA was added at either (a) 100 or (b) $10^5$ copies/reaction. Points represent the mean value across the 10 replicates and error bars show the standard deviation. The total bacteria results are not shown as fecal samples contained an unknown quantity of bacterial 16S rRNA which confounds the interpretation.
Figure S2. Calculated copy numbers have the highest variation at the lowest concentrations. Boxplots show median value and inner quartile ranges, points represent the result for a single sample-assay reaction run in quadruplicate (twice each on two chips), colors indicate if the assay targeted the general bacterial 16S rRNA gene or a specific pathogen virulence/marker gene. *** p < 0.001, ** p < 0.01 as determined by Wilcoxon rank sum test.
Figure S3. Bland Altman plots for assays with at least 10 detections in fecal samples in both nL-qPCR and TAC. The blue shaded area represents the mean bias and its 95% confidence interval. The upper (green) and lower (red) limits of agreement and their corresponding 95% confidence intervals are also shown.
Table S1. Cost comparison of consumable supplies for nL-qPCR enteric pathogen chip and enteric TaqMan Array Card (TAC). Nonconsumable costs include a specialized thermocycler instrument (QuantStudio for TAC; SmartChip Cycler for nL-qPCR) and additional instruments for sample handling (centrifuge with TAC-supported bucket adapters for TAC; SmartChip MultiSample Nano Dispenser robotic fluid handling system for nL-qPCR). Cost estimates obtained from Stanford University purchasing system in August 2019.

| Characteristic                              | nL-qPCR pathogen chip | Enteric TAC |
|---------------------------------------------|------------------------|-------------|
| No. reaction wells                          | 5184                   | 384         |
| No. samples (per chip or card)              | 96                     | 8           |
| Cost (per chip or card)                     | $475                   | $600        |
| Mastermix \(^a\) cost (per sample)         | $1.30                  | $78         |
| Primer \(^b\) cost (per sample)            | $0.43                  | NA (spotted on TAC) |
| Consumables-tips, tubes, 96-well plates (per sample) | $3.00 | $1.50 |
| **TOTAL (per sample)**                      | **$9.68**              | **$155**    |

\(^a\) AgPath-ID One-Step RT-PCR Kit (1000 reactions; $1600) for TAC; Roche LightCycler 480 SYBR Green I (103680 reactions; $2500) for nL-qPCR

\(^b\) IDT primer plate with mixed forward and reverse primers at 50uM (good for >6 nL-qPCR chips; $250)
Table S2. Coefficient of variation in target gene copy number across 15-20 chips that were run on two instruments at separate facilities, by two operators at each facility. Synthetic DNA standards were evaluated over a dilution series from 10 to $10^6$ copies/reaction. Coefficient of variation for 10 copies/reaction is not shown when the assay limit of detection was determined to be higher than 10 copies/reaction.

| Organism (target gene) | 10   | $10^2$ | $10^3$ | $10^4$ | $10^5$ | $10^6$ |
|------------------------|------|--------|--------|--------|--------|--------|
| **Pathogenic E. coli**  |      |        |        |        |        |        |
| EAEC (aggR)            | 115.3| 40.9   | 26.1   | 25.0   | 16.3   | 61.8   |
| ST-ETEC (STh)          | 51.1 | 45.0   | 22.7   | 18.4   | 20.5   |        |
| ST-ETEC (STp)          | 39.3 | 38.8   | 25.9   | 22.7   | 29.5   |        |
| LT-ETEC (eltA)         | 48.7 | 39.4   | 26.8   | 19.8   | 22.2   |        |
| EPEC (bfpA)            | 43.3 | 54.2   | 31.8   | 17.2   | 19.8   |        |
| EPEC (eaeA)            | 55.3 | 35.5   | 73.2   | 23.0   | 15.4   | 20.2   |
| STEC (stx1)            | 47.2 | 18.2   | 21.0   | 13.8   | 16.8   |        |
| STEC (stx2)            | 57.3 | 21.8   | 23.5   | 14.6   | 22.8   |        |
| **Other Bacteria**     |      |        |        |        |        |        |
| Campylobacter jejuni/coli (cdtA) | 51.6 | 44.4   | 29.7   | 21.8   | 33.3   |        |
| Clostridium difficile (tcdB) | 34.0 | 19.2   | 27.4   | 15.8   | 21.9   |        |
| Clostridium perfringens (CPE) | 52.1 | 45.8   | 22.4   | 22.8   | 16.0   | 21.7   |
| Helicobacter pylori (ureA) | 92.4 | 25.8   | 35.3   | 13.8   | 31.1   |        |
| Salmonella enterica (invA) | 53.1 | 31.3   | 23.1   | 20.6   | 12.2   | 18.1   |
| Shigella/EIEC (ipaH)    | 29.4 | 19.4   | 20.4   | 13.8   | 21.2   |        |
| Vibrio cholerae (tcpA)  | 44.0 | 22.3   | 25.4   | 15.4   | 18.8   |        |
| Yersinia enterocolitica (yadA) | 60.8 | 101.0  | 29.0   | 16.6   | 21.6   |        |
| **Protozoa & Helminthes** |    |        |        |        |        |        |
| Cryptosporidium (18S)   | 102.9| 112.3  | 40.1   | 31.5   | 19.7   |        |
| Entamoeba histolytica (18S) | 45.9 | 25.6   | 28.6   | 35.6   | 23.7   |        |
| Giardia (18S)           | 31.5 | 19.5   | 21.7   | 12.9   | 27.6   |        |
| Ascaris lumbricoides (ITS1) | 53.2 | 30.6   | 19.1   | 21.6   | 14.7   | 20.2   |
| Trichuris trichiura (18S) | 57.7 | 31.8   | 17.2   | 21.4   | 12.5   | 17.2   |
| **General**             |      |        |        |        |        |        |
| Total Archaea (16S)     | 74.6 | 44.7   | 49.5   | 31.4   | 42.8   |        |
| Total Bacteria (16S)    | 318.9| 78.2   | 91.0   | 29.7   | 16.7   | 30.5   |
| Total Fungi (ITS1)      | 54.5 | 40.1   | 53.5   | 44.8   | 40.5   |        |
| **QC**                  |      |        |        |        |        |        |
| PhHV (gB)               | 66.1 | 25.8   | 22.9   | 15.4   | 21.8   |        |