CENTER for PRODUCE SAFETY

CPS 2014 RFP
FINAL PROJECT REPORT

Project Title
Methods for detection of diverse parasites on packaged salads based on (viable) oocysts

Project Period
January 1, 2016 – March 31, 2017 (extended to July 31, 2017)

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Objective

To optimize methods to detect protozoan pathogen (oo)cysts on produce.

Funding for this project provided by the Center for Produce Safety through:
CDFA SCBGP grant# SBC15064

FINAL REPORT

Abstract

Contamination of packaged salads with disease causing microorganisms constitutes a significant health risk for produce consumers. Protozoan pathogens were targeted in this project because they are implicated in produce-borne food outbreaks, optimal methods for their detection in produce have been lacking, and their prevalence in developed countries, such as the United States, is likely to rise due to increasing demands for imported vegetables as well as climate variability projections. Two key challenges to the produce and packaging industry were addressed in the current investigation: 1) development of a rapid, accurate, and affordable test for simultaneous detection of four selected protozoan parasites in leafy greens; and 2) optimization and validation of viability assays that discriminate live from dead parasites on produce. A newly developed qualitative nested multiplex-PCR assay was sensitive (1–10 parasites per reaction) for detection of low levels of parasites and accurately detected 10–50 parasites spiked on 10 grams of spinach, as determined in spiking experiments. For viability assays, reverse transcriptase quantitative PCR (RT-qPCR) was compared with propidium monoazide (PMA) coupled with real-time as well as conventional PCR assays (PMA-qPCR/PCR). The RT-qPCR assays could accurately detect 20 (*Cryptosporidium* and *Toxoplasma*) oocysts or 90 (*Giardia*) cysts spiked on 10 grams of spinach. However, detection of live *Cryptosporidium* and *Giardia* may be underestimated by RT-qPCR when dead (oo)cysts are present in large concentrations. PMA-PCR effectively discriminated live from dead *Giardia* at contamination levels of 220 cysts on 10 grams of spinach, using a relatively simple and affordable approach.

In conclusion, the novel multiplex PCR test developed in this study and the validated viability assays (RT-qPCR/PMA-PCR) are ready for immediate adoption and use by the produce industry to screen leafy greens for the presence of parasites. In addition to the benefit to the leafy green industry, this assay can be optimized for application to other food commodities that are at high risk for contamination with parasites—especially berries and cilantro. The application of viability assays in field surveillance investigations will provide a further advance by discriminating contamination due to live versus dead parasites, and will aid accurate modeling efforts for predicting risk of illness to consumers.

Background

Protozoan contamination of produce is of growing importance due to their current or projected capacity to cause significant illness in consumers of fresh produce. The prevalence of protozoa in developed countries, such as the United States, is likely to rise due to increasing demands for imported produce as well as climate change. However, fresh produce is rarely tested for protozoan pathogens. In addition, current methods used to detect protozoan (oo)cysts (the environmentally robust stage) require time-consuming techniques conducted by specialized laboratories. One critical issue that needs to be addressed for monitoring produce safety of packaged salads is to establish reliable methods for detection of protozoa in this food commodity. With a reliable protozoan detection tool-set, future monitoring will become possible to perform risk assessment in the context of food safety. Thus, the motivation for this project stemmed from a lack of commercially available, user-friendly tests for protozoan pathogens in packaged salads.

The purpose of this study was to develop a new multiplex polymerase chain reaction (PCR) assay that can simultaneously detect and differentiate the presence of four protozoan pathogens in a rapid, accurate and affordable manner. The selected parasites—*Cryptosporidium* spp., *Giardia* spp., *Cyclospora cayetanensis* and *Toxoplasma gondii*—were

specifically targeted in this project due to their current and/or projected capacity to contaminate and cause illness in consumers of leafy greens. The detection limits of the newly developed multiplex PCR assay were assessed in systematic laboratory spiking experiments by using spinach as a model leafy green vegetable. In addition, viability tests including propidium monoazide–quantitative PCR (PMA-(q)PCR), reverse transcriptase qPCR (RT-qPCR), and viability staining were compared to validate and optimize detection of viable protozoan parasites in leafy greens. Combined, the novel tool-set developed in the current study—using multiplex PCR for routine screening, followed by viability tests for further quantification of viable parasites—provides a fundamental advance for the produce industry to identify and manage health risks associated with parasite contamination of leafy green commodities.

Research Methods and Results

Methods

Parasites

Live *Cryptosporidium parvum* (*C. parvum*) oocysts (Iowa isolate) were purchased from Sterling Parasitology Laboratory at University of Arizona (Tucson, AZ, USA). Live *Giardia lamblia* (*G. lamblia*) cysts (H3 isolate) were purchased from Waterborne Inc. (New Orleans, LA, USA). *Toxoplasma gondii* (*T. gondii)* oocysts (Type II) and *Cyclospora cayetanensis* (*C. cayetanensis*) oocysts were generously provided by David Solis at University of California, Davis, and Michael Arrowood at the Centers for Disease Control and Prevention (CDC), respectively. To obtain dead (oo)cysts, desired numbers of live (oo)cysts suspended in 1 ml phosphate buffered saline (PBS) or ultrapure water in microcentrifuge tubes were placed into a heat block at 80ºC for 3 min (*Cryptosporidium* and *Cyclospora*), 70ºC for 15 min (*Giardia*), or 80ºC for 20 min (*Toxoplasma*).

DNA extraction

DNA was extracted from 100-µl (oo)cyst aliquots using the DNeasy Blood and Tissue Kit (Qiagen, CA, USA) with slight modifications. In brief, (oo)cysts were mixed with 180 µl of ATL buffer and subjected to one freeze-thaw cycle (4 min in liquid nitrogen and 4 min in boiling water). Proteinase K (40 µl) was then added and samples were incubated overnight at 56ºC. The nucleic acids were eluted from membrane columns with 50 µl (PCR), or 100 µl (qPCR) of a 1:10 AE buffer and water mixture (see Appendices: SOP – DNA extraction for protozoan (oo)cysts).

Multiplex PCR

A nested PCR assay was developed by designing a new primer set (forward and reverse) to simultaneously amplify a target region of the 18S ribosomal RNA (rRNA) gene of *Cryptosporidium, Toxoplasma,* and *Cyclospora.* The m18S rRNA gene primer set was then multiplexed with a previously established primer set targeting the glutamate dehydrogenase (GDH) gene of *Giardia* (Read et al., 2004) in one external PCR reaction. To discriminate the four parasites via internal nested reaction, three internal primer sets specifically targeting *Cryptosporidium, Toxoplasma,* and *Cyclospora* were designed within the external m18S amplicon (**Table 1**), while nested GDH primers were used for *Giardia* (Read et al., 2004). The specificity and sensitivity of the developed multiplex PCR assay were tested first on individual parasite stocks, followed by testing a mixture of all four protozoan parasites. Amplicons of the expected sizes on agarose gels were validated via sequence analysis. Primary and secondary PCR reactions were carried out in a volume of 50 µl containing 1x PCR buffer 1 with $MgCl_2$ (15 mM), 1.5 U of AmpliTaq® DNA polymerase (Applied Biosystems™, CA, USA), 0.2 µM (primary)

or 0.5 µM (secondary) of each primer, 0.2 mM of deoxynucleotide (dNTP) (Invitrogen, CA, USA), 3.2 µg/µl of bovine serum albumin (BSA) (Calbiochem, CA, USA), and 5 µl of genomic DNA for the external reaction or 2 µl of external amplicon for the internal reaction as DNA template. PCR conditions consisted of an initial denaturation at 94ºC for 3 min followed by 35 cycles at 95ºC for 40 s (denaturation), 58ºC for 40 s (annealing), 72ºC for 90 s (extension), with a final extension step of 72ºC for 4 min. PCR conditions for the internal reaction were similar to those of the external reaction except for the annealing temperature, which was increased to 59ºC for *Giardia, Toxoplasma* and *Cyclospora* and 60ºC for *Cryptosporidium* (see SOP – Multiplex polymerase chain reaction (PCR) with nested PCR approach). The nested amplification products were visualized in a 2% agarose gel containing ethidium bromide, using an ultraviolet (UV) transilluminator.

Restriction fragment length polymorphism (RFLP)

A second approach for simultaneous differentiation of the four selected protozoans was evaluated using a newly developed RFLP assay. The first step of this method follows the same procedure as described above. A single multiplex PCR reaction simultaneously amplifies DNA from all four protozoa using four primers: an m18S primer set co-amplifies *Cryptosporidium, Toxoplasma* and *Cyclospora*, while *Giardia* is amplified using primers targeting the GDH gene. However, instead of following the external PCR with a nested PCR reaction, amplicons are subjected to digestion using the BsaBI restriction enzyme (see SOP – Restriction Fragment Length Polymorphism (RFLP) assay). The resulting patterns allow differentiation of different protozoan parasites, even in the case of multiple parasites present in a single contaminated batch of leafy greens (**Table 2**). Briefly, 5 µl of external amplicon DNA is combined with 0.5 µl BsaBI restriction enzyme, 2 µl 10X CutSmart® Buffer (New England BioLabs Inc., MA, USA) and 12.5 µl sterile PCR grade water. Samples were digested in a 96-well plate at 60ºC for 1 h. Digested DNA products were visualized in a 2% agarose gel containing ethidium bromide, using a UV transilluminator.

Quantitative PCR

Quantitative polymerase chain reaction (qPCR) assays for each parasite were chosen from publicly available literature (Guy et al., 2003; Hill et al., 2007; Opsteegh et al., 2010; Shields et al., 2013). The oligonucleotides of primers and probes of each qPCR assay and their final concentrations in qPCR reaction are indicated in **Table 3**. Each 25-µl qPCR reaction mixture contained 10 µl of template DNA and final concentrations of 1x TaqMan® Environmental Master Mix 2.0 (Applied Biosystems) as well as a specific amount of each primer and probe (Table 3). The thermal cycling conditions included a pre-incubation step of 50ºC for 2 min and 95ºC for 10 min, followed by 40 cycles (45 cycles for *Cyclospora*) of 95ºC for 15 s and 55ºC (*Cryptosporidium*) or 60ºC (all the other protozoa) for 1 min. See SOP – qPCR (quantitative polymerase chain reaction). To prepare standard curves, serial dilutions of (oo)cyst DNA extracts were used and the assay limits of detection (ALODs) were determined using a 99% confidence interval (**Table 4**).

Immunomagnetic separation-direct fluorescent antibody (IMS-DFA) and membrane filtration

IMS was performed using Dynabeads™ GC-Combo (Applied Biosystems) to separate *Cryptosporidium* and *Giardia* (oo)cysts from sample pellets followed by the manufacturer's protocol. The final suspensions of 100 µl were transferred to 3-well SuperStick™ Slides (Waterborne, Inc.) and then air-dried for 24 h followed by DFA staining, including fluorescein isothiocyanate (FITC)-conjugated antibody and DAPI (4'6'-Diamidino-2-Phenylindole, Dihydrochloride) using EasyStain (BTF Precise Microbiology, Inc., PA, USA) per the manufacturer's instructions. The stained (oo)cysts were observed with a Zeiss Axioskop epifluorescence microscope equipped with a FITC filter set.

Toxoplasma and *Cyclospora* were recovered from the supernatant during the IMS procedure after *Cryptosporidium* and *Giardia* (oo)cyst had been separated by magnetic beads as described by previously published protocols (EPA method 1623). Membrane filtration was conducted to concentrate the oocysts on a 25-mm mixed cellulose hydrophilic membrane filter with a 5-µm pore size (Millipore, MA, USA). The entire filter was scanned using a Zeiss Axioskop epifluorescent microscope equipped with a UV emission filter set. The number of sporulated *Toxoplasma* oocysts was enumerated while unsporulated oocysts were recorded as *Cyclospora*. Note that while membrane filtration has been validated as a quantitative detection method for *T. gondii* (Shapiro et al., 2010), this approach has not been validated via independent experiments for *C. cayetanensis* enumeration. Thus, results for this latter protozoan are reported here as tentative observations, with future experiments planned to validate the approach.

Viability assays: RT-qPCR and PMA-qPCR/PCR

To discriminate live from dead protozoa, RT-qPCR assays targeting messenger RNA (mRNA) of heat shock protein 70 (*hsp70*), *β-giardin* and *SporoSAG* genes were performed for *Cryptosporidium, Giardia* and *Toxoplasma*, respectively (Guy et al., 2003; Travaille et al., 2016). The oligonucleotides of primers and probes of each RT-qPCR assay and their final concentrations in qPCR reactions are indicated in **Table 3**. mRNA expression was heat-induced by incubating parasite samples at 45ºC for 20 min before mRNA extraction. mRNA extraction was conducted using the Dynabeads® mRNA DIRECT™ Kit (Invitrogen) following the manufacturer's instructions (see SOP – RNA extraction for protozoan (oo)cysts). The RT-qPCR was performed using a StepOnePlus Real-time PCR System (Applied Biosystems). Each 25-µl mixture contained 12.5 µl Path ID Multiplex One-Step RT-PCR Buffer and 2.5 µl Multiplex Enzyme Mix (Applied Biosystems), a specified amount of each primer and probe (Table 3), and 5 µl of mRNA extract. The cycling conditions were 30 min at 50ºC and 15 min at 95ºC, followed by 40 cycles of 15 s at 95ºC and 1 min at 60ºC (see SOP – RT-qPCR (reverse transcription polymerase chain reaction)). To prepare standard curves, plasmids containing target DNA sequences were constructed for each assay by inserting target PCR amplicons into the PCR™4-TOPO vector of the TA Cloning® Kit for sequencing (Invitrogen). Plasmid DNA was extracted by the QIAprep Spin MiniPrep Kit (Qiagen) and then quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, DE, USA). Serial dilutions of plasmid DNA were used in the range of 10^6–10^1 target gene copies per reaction in six replicates for standard curve analysis. The ALODs for individual assays were determined based on a 99% confidential interval (**Table 4**).

For *Giardia*, PMA (Biotium, Inc., CA, USA) treatment was applied to samples before DNA extraction. The PMA stock dissolved in H_2O was diluted with nuclease-free water and then added into samples to a final concentration of 50 µM. The cyst suspension was incubated with PMA in the dark at room temperature for 5 min and then exposed to light for 15 min using the PMA-Lite™ LED Photolysis Device (Biotium, Inc.) (see SOP – PMA treatment for Giardia). PMA concentration, dark incubation time, and light exposure time were optimized for PMA penetration into cyst walls of dead parasites, as well as DNA binding to chromosomal DNA (leading to inhibition of PCR of nonviable cysts). After DNA extraction, PMA-treated samples were analyzed by the selected qPCR assay for *Giardia* analysis. The previously established nested *Giardia* PCR assay (Read et al., 2004), used in the multiplex PCR assay, was also applied for PMA-PCR analysis and compared with the performance of PMA-qPCR.

Viability staining

Live or heat-inactivated (oo)cysts in 20 µl of PBS or spiking pellets were mixed with 20 µl of DFA in the EasyStain kit (BTF Precise Microbiology, Inc., PA, USA) and 5 µl of 1mg/ml propidium iodide (PI). After vortexing for 20 s, the mixture was incubated in the dark at room

temperature for 20 min. Ten microliters of (oo)cyst mixture stained with fluorescent antibody and PI was then loaded into a 3-well SuperStick™ Slide (Waterborne, Inc.) and observed using an Olympus BH-2 epifluorescence microscope equipped with a FITC and Texas Red multi-band filter set at 200X (see SOP – Viable staining).

Spinach spiking experiments

i) Screening assays

Laboratory screening experiments were conducted using spinach as a model leafy green vegetable, and two treatments were compared for parasite recoveries: i) hand-washing (Wash experiment), and ii) smasher instrument mashing (Mash experiment) (**Fig. 1**). Spinach samples were prepared by placing 10 g of spinach purchased from a local grocery store in individual weighing dishes (Fisher Scientific, CA, USA). Protozoan parasites were heat-inactivated before use, except for *Cyclospora*. Serial dilutions of a parasite mixture, ranging from 10 to 10,000 of individual parasites, were spiked onto spinach (10 grams) by pipetting small droplets (5 µl) onto the surface of leaves. PBS was used as a negative control. Three to five replicates were used for each individual concentration. After 2-h drying at room temperature, samples were placed in Whirl-Pak bags for recovery treatment: i) for the Wash experiment, spinach leaves were placed in an 18-oz Whirl-Pak bag with 100 ml of 0.1% Tween80 and subjected to hand massage for 2 min; ii) for the Mash experiment, spinach leaves were placed in a 24-oz filtered Whirl-Pak bag with 40 ml of 0.1% Tween80 and then agitated mechanically using Smasher™ Blender/Homogenizer (bioMérieux, NC, USA) at FAST mode for 2 min. The eluant solution from each spinach sample was transferred to a Falcon tube and then centrifuged at 3,000 rpm for 15 min at 4^{o}C. In the Mash treatment, an additional 40 ml of 0.1% Tween80 was applied to rinse the filter. After centrifugation, the supernatant was aspirated to reserve approximately 3 ml of pellets for each sample, which was subsequently divided into three microcentrifuge tubes for individual assays (see SOP – Laboratory spiking experiment). Samples were analyzed using 1) the newly developed multiplex nested PCR; 2) optimized qPCR assays; and 3) previously established microscopy-based techniques, including IMS-DFA and membrane filtration. For nucleic acid extraction, the 1-ml (oo)cyst suspensions were further centrifuged at 14,000 rpm for 5 min to retain a 100-µl pellet. The detection limits of mPCR assays on spinach were determined (Table 7), and the probability of detecting spiked protozoan parasites on spinach was calculated by fitting binomial detection data to a logistic regression model (Figs. 4 and 5). Recoveries of target protozoa in the spinach spiking experiments were also estimated using qPCR and microscopy data.

ii) Viability assays

Viability spiking experiments were performed i) to evaluate the sample limits of detection in selected viability assays using serial dilutions of live protozoa mixtures, and ii) to assess the performance of viability assays with different ratios of live to dead (oo)cysts in mixtures (**Fig. 2**). To prepare live and dead mixtures, intact (oo)cysts of each parasite were mixed with heat-inactivated (oo)cysts to obtain the proportions of live/dead (oo)cysts (in %) as 0/100, 25/75, 50/50, 75/25 and 100/0. Every mixture contained a total of 5,000 (oo)cysts of each parasite and was spiked in small droplets onto several spots on the spinach surfaces of each sample. Five replicates were used for each serial dilution or live/dead proportion treatment. Following application of parasites, the spinach samples were air-dried at room temperature for 2 h and placed in an 18-oz Whirl-Pak bag with 100 ml of 0.1% Tween80 for Wash treatment as described under the screening assay experiment above. After the Wash treatment, liquid was recovered and centrifuged at 3,000 rpm for 15 min. The supernatant was discarded, leaving approximately 5 ml of pellets (see SOP – Laboratory spiking experiment). The suspension was aliquoted into five microcentrifuge tubes for further analysis using five individual assays: 1)

PMA-(q)PCR, 2) RT-qPCR, 3) viability staining, 4) qPCR, and 5) multiplex PCR. For molecular tests, the 1-ml suspension was further centrifuged at 14,000 rpm for 5 min to concentrate pellets into a 100-µl pellet.

Quality control

To minimize and monitor cross-contamination during sampling handling and analysis, multiple quality control measures were applied. Blank spikes using PBS without target parasites were used in all spiking experiments to assess potential cross-contamination during parasite spiking and processing. Extraction and reagent negative controls were processed through the entire analytical procedure in molecular analysis. Positive controls consisted of target parasite DNA from concentrated stock solutions.

Data analysis

Statistical analyses were performed using STATA software (StataCorp LLC, TX, USA) to create probability curves for estimating the likelihood of parasite detection in spinach spiking experiments. The probability of detecting spiked protozoan parasites on spinach was determined for each parasite by fitting experimental data (binomial format of presence/absence) to a logistic regression model. Positive and negative results of PCR analyses were directly used in the regression while quantitative data in RT-qPCR and PMA-qPCR were converted to a binomial format (presence/absence) for the application of a logistic regression model. Each regression model was evaluated using the Hosmer-Lemeshow goodness-of-fit test. Regression coefficients were then used to estimate the probability of parasite detection across a range of potential (oo)cyst contamination levels per 10 grams of spinach.

Results

Development of multiplex-PCR

A new multiplex PCR assay for simultaneous detection of *Cryptosporidium* spp., *Giardia* spp., *T. gondii* and *C. cayetanensis* was developed using nested PCR reactions. Several PCR assay conditions including primer concentrations, annealing temperature, cycle numbers and template volume used in the external and internal cycles were evaluated for assay optimization. The optimized condition for nested multiplex PCR assays is specified in the corresponding materials and methods section. The specificity and sensitivity of the developed multiplex PCR assays were tested with the mixture of four protozoan parasites. Multiplex PCR simultaneously amplified all four parasites in an external reaction followed by parasite specific amplification in four individual internal reactions. The assay limits of detection (ALODs) in the parasite mixture were 1 (oo)cyst per reaction for *Cryptosporidium, Toxoplasma* and *Giardia,* and 10 oocysts per reaction for *Cyclospora* (**Table 5**). ALODs appeared to be similar or lower (more sensitive) when each parasite was tested separately. All amplifications from multiplex PCR assays were validated via sequence analysis.

For RFLP analysis, the digestion of the external reaction of multiplex PCR was performed separately on each parasite. Distinct banding patterns were visible, enabling differentiation of all four protozoan parasites (**Fig. 3**). While adequate parasite discrimination was obtained using this approach, further experimentation was not performed with RFLP assays due to an observed ten-fold decrease in parasite detection sensitivity. Because only a single PCR reaction is conducted prior to DNA digestion (external multiplex PCR), the assay proved less sensitive and thus deemed less desirable for the produce industry. Our screening method of choice that was applied in subsequent spinach spiking experiments was therefore the nested multiplex PCR assay.

Optimization and adaptation of viability qPCR

In addition to developing a rapid and sensitive method for routine screening of protozoa, the performance of PMA-(q)PCR and RT-qPCR assays assessing the viability of target parasites was evaluated. PMA treatment was optimized for *Giardia* cysts by adjusting PMA concentration, dark incubation and photolysis time to minimize nucleic acid amplification from heat-inactivated *Giardia* cysts. PMA treatment did not reliably discriminate viable from heat-inactivated *Cryptosporidium* or *Toxoplasma* oocysts due to their robust oocyst walls inhibiting PMA penetration even after death. RT-qPCR methods—quantifying induced mRNA from live cells—did successfully differentiate live from dead parasites for *Cryptosporidium*, *Toxoplasma* and *Giardia.* Therefore, selected viability assays were applied for the subsequent validation through systematic spinach spiking experiments (**Table 6**).

Spiking experiment 1: Screening assays

Multiplex PCR

The first set of spiking experiments was conducted to evaluate the performance of the newly developed assays on salads spiked with the four target protozoan pathogens. To determine the efficiency of different treatment methods in recovering (oo)cysts from spinach, washing of spiked spinach with elution buffer by hand-wash (Wash experiment) was compared with the smasher instrument procedure (Mash experiment). The Wash method provided equivalent or better amplifications of (oo)cysts, as measured by multiplex PCR, compared to the Mash method (**Table 7**). *Cryptosporidium*, *Toxoplasma* and *Cyclospora* could be detected at a low concentration of 10 oocysts per 10 g of spinach, while *Giardia* amplified only as low as 50 and 10,000 cysts per 10 g of spinach using the Wash and Mash techniques, respectively. The Mash treatment resulted in more spinach tissue debris in the elution buffer during treatment, which may have resulted in increased inhibition of DNA amplification during PCR. Also, the fine debris may have adversely affected recovery of the relatively larger *Giardia* cysts (12–15 µm) from the solid mashed leaves to the eluted solution through the filter mesh in the Whirl-Pak filter bags (hole diameter of 330 µm) used in the Mash method to separate liquid and solids by placing spinach in one side and recovering elution buffer from other side. The recovery of (oo)cysts as measured by microscopic and qPCR methods demonstrated lower recovery of *Giardia* with the mash method (**Table 8**) as compared with other pathogens. Additionally, *Giardia* cysts are thought to be less environmentally robust than *T. gondii* and *C. parvum* oocysts; thus, the physical mashing action may have crushed the cyst walls, damaging their physical morphology and/or DNA integrity.

Using the presence/absence detection data from the spinach spiking experiments, logistic regression was applied to create a probability curve for estimating the likelihood of parasite detection under a range of theoretical contamination levels expressed as (oo)cysts per 10 g of spinach. Modeling results suggest that washing spinach leaves yielded better recovery and therefore more sensitive detection of parasites as compared with the Mash approach. Using *Toxoplasma* as an example, oocysts could be accurately detected 90% of the time at contamination levels of 10 or 100 oocysts per 10 g spinach, using the Wash or Mash technique, respectively (**Fig. 4**). When the hand-wash technique was applied, successful detection of parasites would occur at 90% likelihood when contamination levels on 10 g of spinach were 10, 35, 45 and 55 (oo)cysts for *Toxoplasma, Cryptosporidium*, *Giardia*, and *Cyclospora*, respectively (**Fig. 5**).

Spiking experiment 2: Viability assays

The second set of spiking experiments was performed to assess the limits of detection of selected viability assays and their ability to discriminate live from dead (oo)cysts on spinach. In the first experiment, serial dilutions of live *Cryptosporidium, Giardia, Toxoplasma* and

Cyclospora mixtures were prepared to obtain concentrations ranging from 10 to 1,000 (oo)cysts and spiked on spinach in five replicates. Each replicate was divided into five aliquots and analyzed by RT-qPCR, PMA-(q)PCR, viable staining, qPCR and multiplex PCR. RT-qPCR results showed that mRNA amplicons were detected on spinach spiked with as low as 10 *Cryptosporidium* and *Giardia* and 50 *Toxoplasma* (oo)cysts (**Fig. 6**). No targeted mRNA was detected on spinach spiked with PBS as a negative control. Logistic regression of RT-qPCR results indicated that the assays would yield positive detection of parasites 90% of the time when 16, 21 and 93 (oo)cysts for *Cryptosporidium, Toxoplasma* and *Giardia* per 10 g of spinach were present, respectively (**Fig. 7**). PMA-(q)PCR and viable staining were also conducted, but target parasites were only detected in a portion of replicates spiked at the highest concentration of 1,000 (oo)cysts (data not shown).

In the second experiment, spinach was spiked with mixtures of live (intact) and dead (heat-inactivated) parasites to evaluate the effect of different proportions of live (oo)cysts in protozoan contamination, which simulates more realistic environmental conditions. Live and dead (oo)cysts were mixed in defined ratios (0:100, 25:75, 50:50, 75:25 and 100:0) for a total of 5000 (oo)cysts and spiked on spinach in five replicates. The increasing proportions of dead protozoa spiked on spinach may have inhibited the mRNA amplification from live *Cryptosporidium* and *Giardia.* However, detection of live *Toxoplasma* was linearly correlated with increasing proportions of live (oo)cysts with a R^2 value of 0.9961 (**Fig. 8**). This result suggested that the detection of live *Cryptosporidium* and *Giardia* may be underestimated when large concentrations of dead (oo)cysts are present on contaminated salads. Note that false-positive results (RT-qPCR signal amplification) were obtained in three (*Giardia* and *Toxoplasma*) or four (*Cryptosporidium*) out of five replicates when 100% of (oo)cysts present were dead (**Fig. 8**). The concentrations of mRNA amplicons measured in samples containing 100% dead (oo)cysts were equivalent to 0.3–3% of corresponding 100% live (oo)cyst samples. In PMA-PCR analysis, a considerable reduction of DNA amplification with increasing dead (oo)cysts ratios in the mixture was observed (**Table 9**). While *Giardia* DNA was amplified from both 100% live or dead samples without PMA treatment, DNA amplification decreased with increasing proportions of dead (oo)cysts as measured by PMA-PCR. No DNA was amplified on spinach spiked with 100% dead (oo)cysts by PMA-PCR. The probability of detecting viable *Giardia* on spinach was calculated using RT-qPCR, PMA-qPCR and PMA-PCR results from both viability spiking experiments by fitting binomial detection data to a logistic regression model (**Fig. 9**). The quantitative RT-qPCR and PMA-qPCR data were converted to a binomial format of presence/absence data for the application of a logistic regression model. The comparison results suggested that more sensitive *Giardia* detection would occur using PMA-PCR or RT-qPCR analysis (220 or 110 cysts per 10 g of spinach, respectively), as compared with PMA-qPCR. The total costs of PMA-PCR and RT-qPCR analyses in this study were estimated at $17 and $25 per sample, respectively (including the cost for DNA/RNA extraction and for analytical duplicate plus nested PCR reaction in PMA-PCR or for analytical duplicate plus two dilutions in RT-qPCR). Although PMA-PCR may provide relatively lower sensitivity than RT-qPCR, the PMA-PCR assay for the detection of live *Giardia* would be useful, especially when a simpler and more affordable method is preferred in viable *Giardia* monitoring. Microscopic-based viability staining results suggested that discriminating dead from live *Giardia* cysts on spinach was not as clearly visualized as when parasites were suspended in PBS (data not shown); thus, this approach may not be as efficient for *Giardia* viability determination on leafy greens as compared with RT-qPCR or PMA-PCR.

Outcomes and Accomplishments

- A key outcome of the project is the development of a new molecular test that can simultaneously detect and differentiate four important foodborne parasites when present on produce. This assay, a multiplexed, nested conventional PCR test, is simple, rapid (<24 h), and inexpensive. Systematic spiking experiments demonstrated the ease of application of this test on leafy greens, using spinach as a model produce salad. The low detection levels of the assay enable efficient screening of food commodities.
- The optimization and validation of several viability assays for discriminating live from dead parasites on produce presents a fundamental advance in the field of produce safety. Detection of parasite DNA alone is not sufficient for making appropriate management and/or policy decisions that affect public health. Insight on whether positive detection via screening tests is due to the presence of infectious pathogens (as opposed to nonviable organisms) is imperative. Our results demonstrate that RT-qPCR can effectively discriminate viable from dead *Toxoplasma, Cryptosporidium and Giardia* (oo)cysts on spinach; the PMA-PCR assay for the detection of live *Giardia* would be also useful, especially when a simpler and more affordable method is preferred in viable *Giardia* monitoring.
- Currently available commercial methods for protozoan pathogen detection on environmental matrices are laborious, expensive, and require specific personnel training in microscopy (as per EPA method 1623 using IMS DFA for *Cryptosporidium* and *Giardia*), or are completely lacking (for *Toxoplasma* and *Cyclospora*). In comparison, both the screening multiplex PCR and the RT-qPCR as well as PMA-PCR methods used for viability discrimination can be adopted for immediate use by any laboratory with molecular testing capacity.
- The application of user-friendly and cost-effective tests for routine monitoring of produce for parasite contamination will yield field surveillance data essential for investigations aimed at predicting risks of illness to consumers via quantitative microbial risk assessment (QMRA).

Summary of Findings and Recommendations

- The novel multiplex PCR test developed in this project can be immediately adopted by the produce industry to screen leafy greens for the presence of protozoan parasites.
- The multiplex PCR test can be optimized for application to other produce commodities, such as berries and cilantro, which are at high risk for contamination with parasites.
- Applying viability assays in field surveillance investigations is key to estimating contamination due to live versus dead parasites, and will aid accurate modeling efforts to predict the risk of illness to consumers.
- Addressing food safety concerns due to viable *Cyclospora cayetanensis* contamination is especially challenging due to the difficulty encountered in obtaining oocysts for research purposes. Unlike the other three protozoan parasites targeted in this study, *Cyclospora* is a human-specific pathogen and oocysts can only be obtained from diarrheic stool samples of patients. While our research group succeeded in procuring oocysts for development of a screening assay, our collaborator at CDC (Dr. Michael Arrowood) could not verify the viability state of the parasites. A collaborative framework among produce industry partners, academic institutions, state-level public health laboratories, and the CDC is strongly recommended for strategizing surveillance and sample collection approaches that would maximize resources for advancing research on this important emerging infectious disease agent.

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APPENDICES

Publications and Presentations

Stefan Wuertz. Methods for detection of diverse parasites on packaged salads based on (viable) oocysts.

- Oral presentation at CPS Research Symposium, Denver, CO, June 20–21, 2017.
- Oral presentation at CPS Research Symposium, Seattle, WA, June 28–29, 2016.
- Poster presented at CPS Research Symposium, Seattle, WA, June 28-29, 2016.

Publications are currently in preparation.

Budget Summary

Funds from CPS were used to support salaries ($130,129), benefits ($34,848), supplies ($39,318), and travel ($3,027) to support Drs. Wuertz and Kim to attend the annual CPS Research Symposium. Indirect costs totaled $8,248. The total amount spent was $215,573 out of the total award of $223,859.

Suggestions to CPS

- Collaboration with a produce company would **help define future strategies for quantitative microbial risk assessment** by providing **their input on research needs and best approaches for applying effective testing strategies.**

Tables 1–9 and Figures 1–9

Table 1. Primer sets for external and internal PCR in multiplex PCR assays.

	Protozoa	Target gene	Primer	Nucleotide sequences (5' – 3')	Size (bp)	Reference
External reaction	*Cryptosporidium, Toxoplasma, Cyclospora*	18S rRNA	m18S Forward	CGGGTAACGGGGAATTAGGG	751-779	This study
			m18S Reverse	TCAGCCTTGCGACCATACTC		
	Giardia	GDH	Forward	TCAACGTYAAYCGYGGYTTCCGT	455	Read, 2004
			Reverse	GTTRTCCTTGCACATCTCC		
Internal reaction	*Cryptosporidium*	18S rRNA	Forward	TGGAATGAGTTAAGTATAAACCCCT	543	This study
			Reverse	GCTGAAGGAGTAAGGAACAACC		
	Toxoplasma	18S rRNA	Forward	GGTGTGCACTTGGTGAATTCTA	405	This study
			Reverse	TGCAGGAGAAGTCAAGCATGA		
	Cyclospora	18S rRNA	Forward	TCGTGGTCATCCGGCCTT	359	This study
			Reverse	TCGTCTTCAAACCCCCTACTG		
	Giardia	GDH	Forward	CAGTACAACTCYGCTCTCGG	432	Read, 2004
			Reverse	GTTRTCCTTGCACATCTCC		

Table 2. RFLP pattern generated by BsaBI digestion of 18S rRNA gene PCR products amplified by the multiplex external primer set.

Target parasite	PCR assay	Amplicon size (bp)	Digested (Yes/No)	Post-digestion fragment size(s) (bp)
Cryptosporidium	M18s	697	Yes	255 442
Toxoplasma	M18s	715	Yes	559 116
Cyclospora	M18s	723	No	723
Giardia	GDH	455	No	455

Table 3. Primers and probes used in qPCR and RT-qPCR assays and their final concentrations in reaction mixtures.

	Protozoa (target gene)	Label	Oligonucleotide sequence (5'-3')	Final conc (nM)	Reference
qPCR	*Cryptosporidium* (18S rRNA)	Forward	ATGACGGGTAACGGGGAAT	250	Hill et al. 2007
		Reverse	CCAATTACAAAACCAAAAAGTCC	250	
		Probe	FAM-CGCGCCTGCTGCCTTCCTT AGATG-BHQ1	100	
	Giardia (*β-giardin*)	P241-F	CATCCGCGAGGAGGTCAA	600	Guy et al. 2003
		P241-R	GCAGCCATGGTGTCGATCT	600	
		P241-P	FAM-AAGTCCGCCGACAACATGT AC CTAACGA-BHQ1	200	
	Toxoplasma (520-bp repeat element)	Tox-9F	AGGAGAGATATCAGGACTGTA G	700	Opsteegh et al. 2010
		Tox-11R	GCGTCGTCTCGTCTAGATCG	700	
		Tox-TP1	FAM-CCGGCTTGGCTGCTTTTCC TG-BHQ1	100	
	Cyclospora (18S rRNA)	HMPr46	TCGTGATGGGGATAGATTA	1000	Shields et al. 2013
		HMPr43	GCTCTATTTACGCAACTTTC	1000	
		HMPro61	FAM-CTGGTCAGTCCAATGAGTTC ACA-BHQ1	100	
RT-qPCR	*Cryptosporidium* (*hsp70*)	Forward	GGATGCAGGTGCAATTGCT	400	Travaille et al. 2016
		Reverse	CTCTCGCCAGTTCCTTTCTTATCA	400	
		Probe	FAM-ATGTAATGAGAATCATTAAC GAGCCAACTGCAGCT-BHQ1	200	
	Giardia (*β-giardin*)	P241-F	CATCCGCGAGGAGGTCAA	600	Guy et al. 2003
		P241-R	GCAGCCATGGTGTCGATCT	600	
		P241-P	FAM-AAGTCCGCCGACAACATGT ACCTAACGA-BHQ1	200	
	Toxoplasma (*SporoSAG*)	Forward	CGGACAAATGTGGCGTACAC	400	Travaille et al. 2013
		Reverse	GTGATCTTGCGCCGAACAC	400	
		Probe	FAM-TTCTCGTCAAAGCGGCACC ACAGG -BHQ1	200	

Table 4. Assay limits of detection (ALODs) of qPCR and RT-qPCR assays. The ALOD of each assay was determined based on the 99% confidential interval of a standard curve established using serial dilutions of (oo)cyst or plasmid DNA.

	qPCR ((oo)cysts/reaction)	RT-qPCR (gene copies/reaction)
Cryptosporidium	0.1	1
Giardia	12.8	7
Toxoplasma	0.004	1
Cyclospora	0.3	Not applicable

Table 5. Multiplex PCR assay limits of detection (ALODs) tested with individual parasites or a mixture of four target protozoan parasites in phosphate buffered saline.

(Oo)cyst /extract	(Oo)cysts /reaction	Successful amplification / Replicates tested							
		Cryptosporidium		*Toxoplasma*		*Cyclospora*		*Giardia*	
		Single	Mix	Single	Mix	Single	Mix	Single	Mix
1000	100	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
100	10	3/3	3/3	2/3	3/3	3/3	**3/3**	2/3	3/3
10	1	3/3	**1/3**	3/3	**1/3**	**3/3**	0/3	**2/3**	**3/3**
1	0.1	**1/3**	0/3	**3/3**	0/3	0/3	1/3	0/3	0/3
0	0	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3

Table 6. Viability assays tested in viability spiking experiments.

Parasite	Viability assays used in spinach spiking experiments (Yes/No)		
	RT-qPCR	PMA-(q)PCR	Viability staining
Cryptosporidium	Yes	No	Yes
Giardia	Yes	Yes	Yes
Toxoplasma	Yes	No	No
Cyclospora	NA[a]	NA	NA

[a] Not applicable

Table 7. Spiking experiment 1: screening assay. Qualitative detection of pathogens using a multiplex PCR assay in two spinach spiking experiments (Wash vs. Mash). Ten grams of spinach was used per sample.

(Oo)cyst dilution	(Oo)cysts /extract	Successful amplification / replicates tested							
		Cryptosporidium		*Toxoplasma*		*Cyclospora*		*Giardia*	
		Mash	Wash	Mash	Wash	Mash	Wash	Mash	Wash
10000	3333	3/3	NA[a]	3/3	NA	NA	NA	**3/3**	NA
1000	333	3/3	5/5	3/3	5/5	3/3	5/5	0/3	4/5
100	33	3/3	4/5	2/3	5/5	2/3	2/5	0/3	1/5
50	17	3/3	2/5	1/3	4/5	0/3	1/5	0/3	**1/5**
10	3	**1/3**	**1/5**	**3/3**	**3/5**	**1/3**	**1/5**	0/3	0/5
0	0	0/3	0/1	0/3	0/1	0/3	0/1	0/3	0/1

[a] Not applicable. In the wash treatment, 10,000 (oo)cyst dilution was not used. In both spiking tests, the highest oocyst concentration of *Cyclospora* was 1,000 oocysts due to limited oocyst quantity available.

Table 8. Recoveries of 1,000 (oo)cysts spiked on 10 g spinach measured by qPCR and microscopy (IMS-DFA and membrane filtration) in spiking experiments using hand-wash (Wash) or Smasher (Mash) recovery approaches.

	Recovery % ± standard deviation			
	Mash (N = 3)		Wash (N = 5)	
	qPCR	IMS-DFA	qPCR	IMS-DFA
Cryptosporidium	96 ± 11	26 ± 4	79 ± 15	48 ± 4
Giardia	not detected	10 ± 9	39 ± 6	40 ± 6
Toxoplasma	33 ± 31	26 ± 4	34 ± 16	48 ± 7
Cyclospora	22 ± 23	2 ± 2	47 ± 8	30 ± 19

Table 9. Effect of PMA treatment on PCR amplification from different proportions of live and dead *Giardia* cysts mixture spiked on spinach. Genomic DNA from PMA-treated cysts were analyzed using PMA-PCR. For comparison, genomic DNA from non-PMA-treated (oo)cysts were analyzed using multiplex PCR (0% live and 100% live only).

			Successful amplification / Replicates tested				
(Oo)cyst dilution (live:dead %)		(Oo)cysts /extract	PMA-PCR	Multiplex PCR			
			Giardia	*Cryptosporidium*	*Toxoplasma*	*Cyclospora*	*Giardia*
0	: 100	1000	0/5	5/5	5/5	5/5	5/5
25	: 75	1000	3/5	n/a[a]	n/a	n/a	n/a
50	: 50	1000	4/5	n/a	n/a	n/a	n/a
75	: 25	1000	5/5	n/a	n/a	n/a	n/a
100	: 0	1000	5/5	5/5	5/5	5/5	5/5
0		0	0/1	0/1	0/1	0/1	0/1

[a] Not analyzed

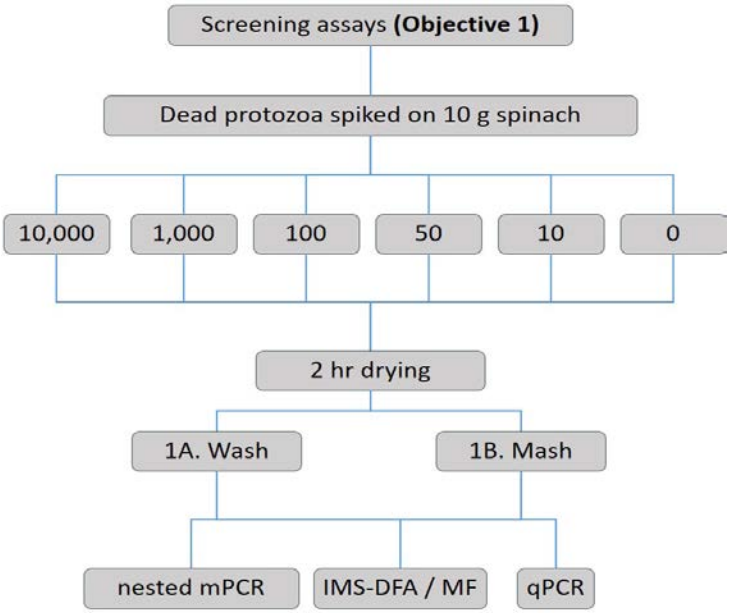

Figure 1. Flow diagram depicting experimental study design for screening assays, in which spinach samples (N = 3 or 5) were spiked with target protozoan parasite mixture at different concentrations.

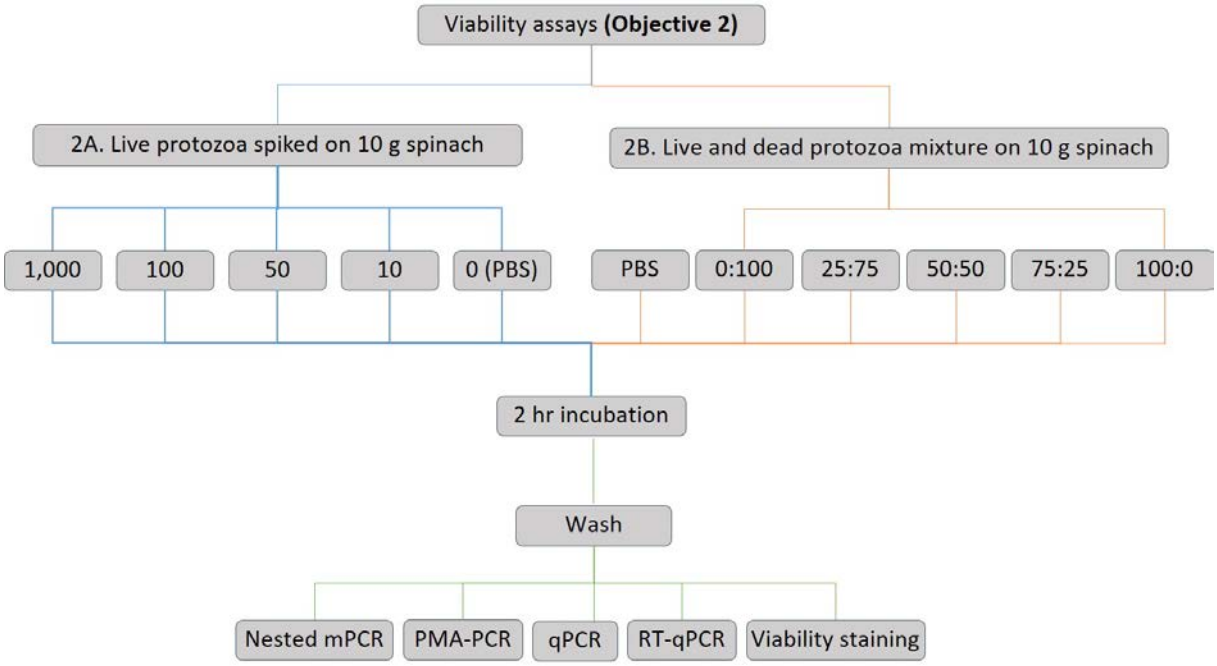

Figure 2. Flow diagram depicting experimental study design for viability assays, in which spinach samples (N = 5) were spiked with serial dilutions of live (oo)cysts or mixtures of different live to dead (oo)cyst ratios.

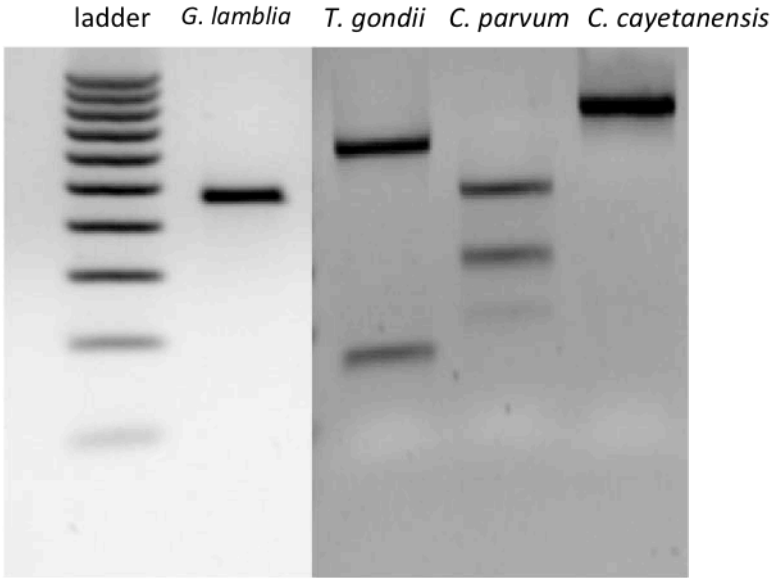

Figure 3. Digestion patterns of external multiplex PCR amplicons of the four selected protozoan parasites obtained via restriction fragment length polymorphism (RFLP) assay.

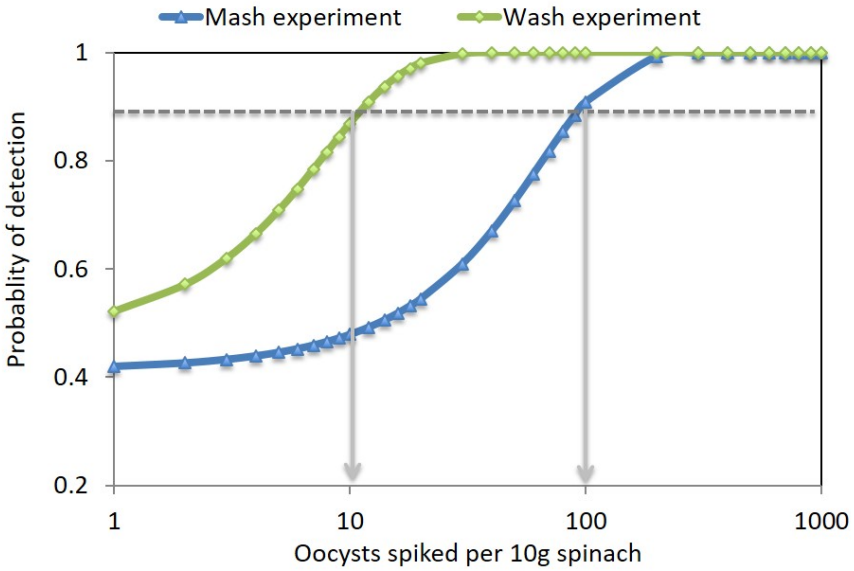

Figure 4. Probability of detecting *T. gondii* oocysts spiked on 10 g of spinach in two screening spiking experiments (Wash vs. Mash). Probability curves were estimated using logistic regression. The dashed line indicates the 90% probability of parasite detection. The arrows depict the estimated parasite concentration detected with 90% likelihood for each target protozoan.

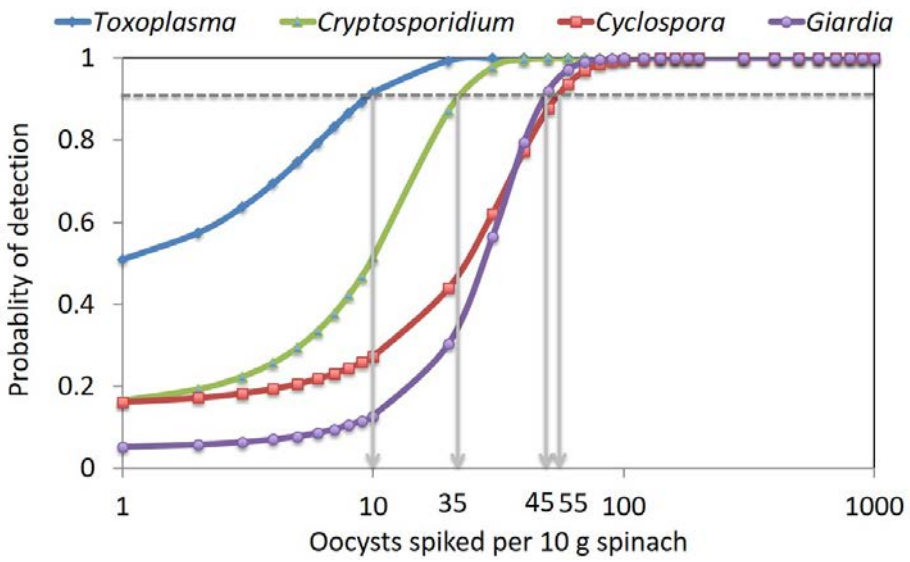

Figure 5. Probability of detecting four target protozoa when recovery of spiked parasites was performed using the hand-wash technique. Probability curves were estimated using logistic regression. The arrows depict the estimated parasite concentrations detected with 90% likelihood for each target protozoan.

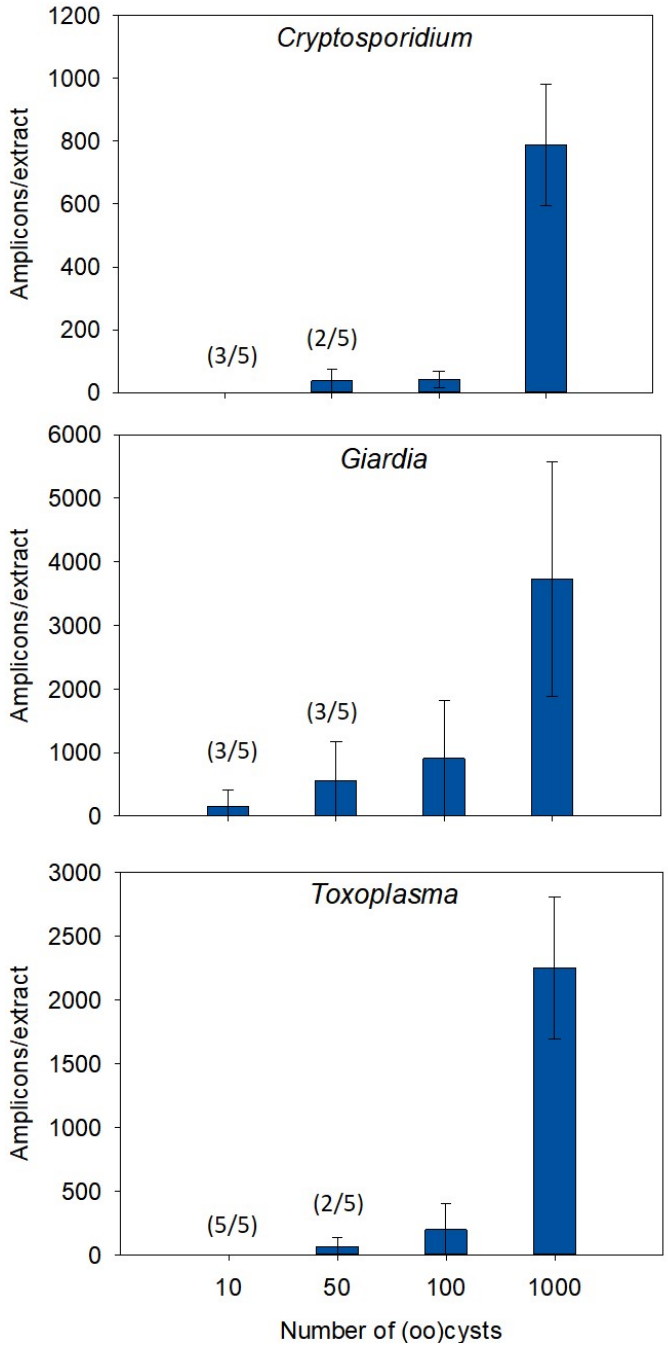

Figure 6. Quantification of mRNA amplicons from serial dilutions of live *Cryptosporidium, Giardia* and *Toxoplasma* spiked on 10 g of spinach measured by RT-qPCR. Non-detect data were assumed to be one-half of the sample limits of detection in graph plotting. The number of non-detects out of five replicates tested is shown in parentheses above each bar, if applicable.

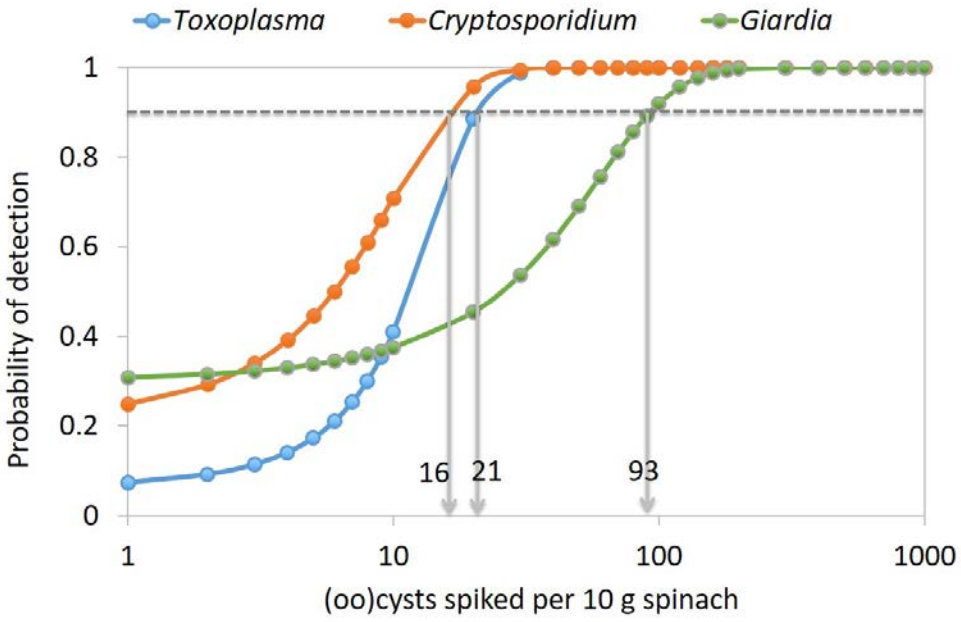

Figure 7. Probability of detecting three viable target protozoan parasites via RT-qPCR assays that discriminate live from dead parasites. Parasites were recovered using the hand-wash technique, and probability curves were estimated using logistic regression. The arrows depict the estimated viable parasite concentrations detected with 90% likelihood for each target protozoan. (Note that *Cyclospora* was not included in viability experiments because the infectivity status of the stocks obtained from CDC could not be verified.)

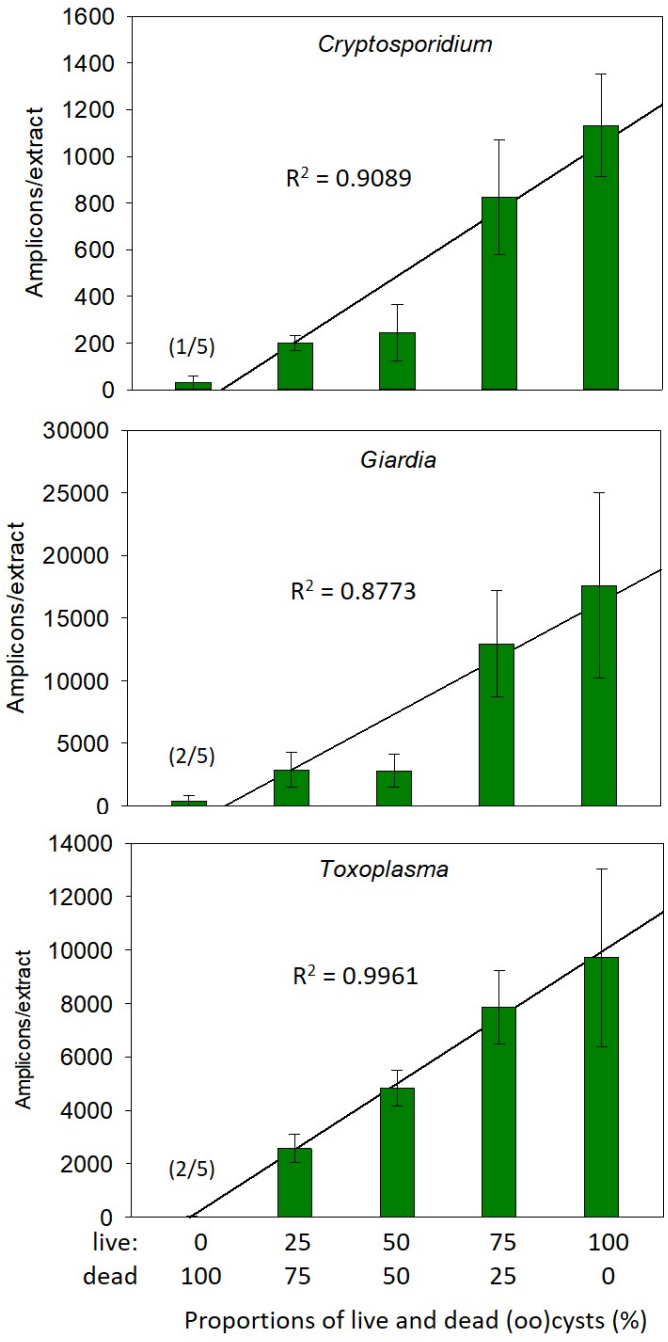

Figure 8. Effect of the proportions of live (oo)cysts on mRNA amplification of protozoa spiked on spinach using RT-qPCR. Each mixture contained 5,000 (oo)cysts of *Cryptosporidium*, *Giardia* and *Toxoplasma* with defined ratios of live to dead (oo)cysts of 0:100, 25:75, 50:50, 75:25 and 100:0. Error bars represent standard deviation of five replicates. Non-detect data were assumed to be one-half of the respective sample limits of detection. The number of non-detects out of five replicates is shown in parentheses, if applicable. Black trend line and R^2 indicate a linear relationship between the proportion of live (oo)cysts and mRNA amplicons obtained via RT-qPCR.

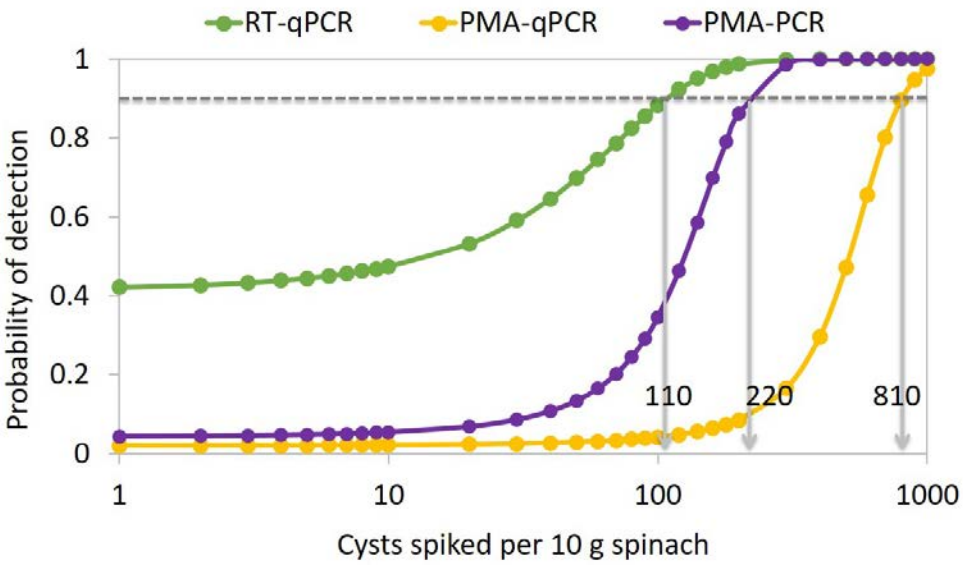

Figure 9. Comparison of probability curves for detection of viable *Giardia* using RT-qPCR, PMA-PCR and PMA-qPCR on spinach in viability spiking experiments. Data from serial dilutions of live (oo)cysts and different ratios of live/dead mixture were combined for the regression. The arrows depict the estimated viable *Giardia* cyst concentrations detected with 90% likelihood using each assay.

Standard Operating Procedure

DNA extraction for protozoan (oo)cysts

1. Aim

DNA extraction from protozoan (oo)cyst suspension based on the DNeasy Blood and Tissue Kit (Qiagen) instruction with modification.

2. Notes

Start with 100 µl (oo)cyst pellets. If sample volume is larger, it should be centrifuged and then the supernatant removed to reserve 100 µl in microcentrifuge tubes before DNA extraction

3. Equipment and Materials

Microcentrifuge
Heat block
Boiling water and liquid nitrogen
DNeasy Blood and Tissue Kit (Qiagen)
Pipette and tips

4. Procedure

1) Prepare a large beaker of boiling water
2) Add 180 µl ATL buffer to sample
3) Prepare liquid nitrogen bucket when samples are ready
4) Perform one freeze/thaw cycle: arrange samples in a floating rack, gently place in liquid nitrogen and let freeze for 4 min. Transfer to boiling water and let boil for 4 min. Remove and let cool for 2-5 min before open the tubes
5) Add 40 µl Proteinase K
6) Mix thoroughly by vortexing
7) Incubate overnight at 56ºC
8) Remove samples from the heat block and increase heat block temperature to 70ºC
9) Add 200 µl AL Buffer, and vortex for 10 s.
10) Put samples in a 70ºC heat block for 10 min
11) Add 200 µl ethanol (96-100%) to samples, and vortex for 10 s
12) Transfer the sample mixture into the DNeasy Mini spin column in 2 ml collection tubes
13) Centrifuge at 9000 rpm for 2 min
14) Discard flow-through and collection tube bottom, and place the spin column in new 2 ml collection tubes
15) Add 500 µl Buffer AW1
16) Centrifuge for 2 min at 9000 rpm
17) Repeat step 14
18) Add 500 µl Buffer AW2
19) Centrifuge for 4 min at 14000 rpm
20) Heat 1:10 AE Buffer and ultrapure water mixture (130 µl AE + 1300 µl water) in microcentrifuge tube to 95ºC in heat block
21) Repeat step 14
22) Centrifuge for 1 min at 14000 rpm, then discard collection tube and place spin columns in clean 1.5 ml microcentrifuge tube pre-labeled with sample ID
23) Add 50 µl (or 100 µl) 95ºC ultrapure water with AE Buffer directly onto the filter column.
24) Incubate at room temperature for 5 min
25) Centrifuge for 2 min at 9000 rpm
26) Check to make sure liquid filtered through to microcentrifuge tubes then remove and discard filter columns

Standard Operating Procedure

Multiplex polymerase chain reaction (PCR) with nested PCR approach

1. Aim
DNA amplification to simultaneously detect and differentiate four protozoan pathogens including *Cryptosporidium, Toxoplasma, Cyclospora* and *Giardia*

2. Notes
The 18S primer set and GDH primer set are multiplexed in the external reaction and simultaneously amplify all four parasites in one tube. In the internal reaction, each parasite is amplified individually from the external reaction PCR amplicon using the pathogen-specific primer set.

3. Equipment and Materials
Thermal cycler
10x PCR buffer
dNTP mix
Forward and reverse primer sets
BSA
Taq Polymerase
Ultrapure quality, PCR-grade water
Template DNA
Pipette and tips
PCR tube strips

4. Procedure
1) Place PCR buffer, dNTP mix, primer sets, BSA and *Taq* Polymerase on ice.
2) Set up an external master mix in a microcentrifuge tube according to the table below. Reaction volume can be scaled up appropriately.

Component	Volume per reaction (µl)	Final concentration
Master mix		
10x PCR buffer	5.0	1x
dNTP mix (10 mM)	1.0	400 µM of each dNTP
m18S forward primer (20 µM)	0.5	0.2 µM
m18S reverse primer (20 µM)	0.5	0.2 µM
GDH forward primer (20 µM)	0.5	0.2 µM
GDH reverse primer (20 µM)	0.5	0.2 µM
BSA (10%)	1.6	3.2 µg/µl
Taq Polymerase (5U/µl)	0.3	1.5U
PCR-grade water	35.1	-
Subtotal	45.0	
Template DNA		
Template DNA, added at step 4	5.0	
Total	50.0	

3) Dispense 45 µl of external master mix into PCR tubes.

4) Add template DNA to the individual PCR tubes. *Note: Preferably this step is done on a different bench space in a PCR hood to minimize cross contamination among samples*.
5) Place the PCR tubes in the thermal cycler and start the cycling program in table below.

Step	Initial activation	3-step cycling (35 cycles)			Final extension	Hold
		Denature	Anneal	Extend		
Temp	94℃	95℃	58℃	72℃	72℃	4℃
Time	3 min	40 sec	40 sec	90 sec	4 min	∞

6) Once the external PCR reaction is done, remove PCR tubes from thermal cycler and put on ice (or store temporarily at 4℃) until use in the internal PCR reactions.
7) For internal reactions, place PCR buffer, dNTP mix, primer sets, BSA and *Taq* Polymerase on ice.
8) Set up an internal master mix in a microcentrifuge tube according to the table below. Reaction volume can be scaled up appropriately.

Component	Volume per reaction (µl)	Final concentration
Master mix		
10x PCR buffer	5.0	1x
dNTP mix (10 mM)	1.0	400 µM of each dNTP
Pathogen specific forward primer (50 µM)	0.5	0.2 µM
Pathogen specific reverse primer (50 µM)	0.5	0.2 µM
BSA (10%)	1.6	3.2 µg/µl
Taq Polymerase (5U/µl)	0.3	1.5U
PCR-grade water	39.1	-
Subtotal	48.0	
Template DNA		
Template DNA, added at step 10	2.0	
Total	50.0	

9) Dispense 48 µl of external master mix into PCR tubes.
10) Add external reaction amplicons to the individual PCR tubes.
11) Place the PCR tubes in the thermal cycler and start the cycling program in table below.

Step	Initial activation	3-step cycling (35 cycles)			Final extension	Hold
		Denature	Anneal	Extend		
Temp	94℃	95℃	59℃*	72℃	72℃	4℃
Time	3 min	40 sec	40 sec	90 sec	4 min	∞

* Use 60℃ for *Cryptosporidium* internal reaction

12) After internal reaction is completed, analyze the internal PCR products on a 2% agarose gel.

Standard Operating Procedure

Restriction Fragment Length Polymorphism (RFLP) assay

1. Aim

DNA digestion of EXTERNAL multiplex (m18s-GDH) PCR amplicon to differentiate *Cyclospora*, *Cryptosporidium*, *Toxoplasma* and *Giardia parasites*.

2. Note

Before digestion, the external amplicon size of *Cyclospora, Cryptosporidium, Toxoplasma* is indistinguishable at ~700 bp, while the Giardia amplicon measures ~ 450 bp. This test provides resolution of amplification product bands to discriminate which parasites are present.

3. Equipment and Materials

Incubator
Gel electrophoresis unit
UV transilluminator
Template DNA
diH_2O (Sterile PCR water)
10x CutSmart® Buffer
Restriction enzyme BsaBI
Pipette and tips

4. Procedure

1) Perform external PCR using m18s-GDH protocol as described elsewhere
2) Prepare Mastermix for digestion as follows:

Reagent	Mastermix per reaction (µL)
diH_2O (Sterile PCR water)	12.5
10x CutSmart® Buffer	2.0
Restriction Enzyme BsaBI	0.5
Subtotal	15.0

3) Calculate total volumes of diH_2O, CutSmart® Buffer, and BsaBI restriction enzyme based on the number of samples and make a stock mastermix solution
4) Pipette 15 µL into each well of a sterile 96-well plate.
5) Add 5 µL DNA sample
6) Incubate the well plate in a sealed Ziploc bag for 1 hour at 60ºC.
7) Increase temperature to 80ºC and incubate an additional 20 min to inactivate the enzyme
8) Prepare a typical agarose gel as you would for running PCR samples.
9) Mix each sample with 4 µL loading dye and load the entire volume (~24 µL) into the appropriate agarose gel well
10) Run gel at 100V for 60 min
11) Obtain an image of the banding patterns on the gel using the geldoc station
12) Analyze results based on expected digestion patterns (or lack of digestion) for each parasite as follows:
    A. *Cyclospora* (723 bp) and *Giardia* (455 bp) are not cleaved
    B. *Cryptosporidium* (697 bp pre-digestion) is cleaved into two fragments measuring 255 bp & 442 bp
    C. *Toxoplasma gondii* (715 bp pre-digestion) is cleaved into two fragments measuring 599 bp & 116 bp

Standard Operating Procedure

qPCR (quantitative polymerase chain reaction)

1. Aim
qPCR quantitatively detect DNA targets of live and dead protozoa (oo)cysts from nucleic acid extracts.

2. Notes
Cycling conditions and final concentrations of primer/probe in the reaction are specific for each parasite. The oligonucleotide sequence, their final concentrations, and assay references can be found in the Table 3 of the final CPS report.

3. Equipment and Materials
StepOnePlus™ Real-time PCR System
Plate spinner
Compression pad
96-well dilution plate and aluminum adhesive film (for dilution plate)
96-well reaction plate and optical adhesive film (for qPCR reaction)
TaqMan® Environmental Master Mix 2.0 (for qPCR)
PathID
Primers and a TaqMan probe set
Nuclease-free water

4. Procedure
1) Place primers and probes used for individual assays on ice.
2) Determine the number of reactions to use based on sample numbers and dilution factors.
3) Prepare a Master mix (note: It contains the Environmental Master Mix 2.0 and TaqMan p/p mix) in a microcentrifuge tube using the reagents listed below. Multiply the volume of each reagent by the number of reactions.

Reagent	Volume (µl) per 25-µl reaction	Final concentration
Master mix		
Environmental Master Mix 2.0 (2x)	12.5	1x
TaqMan p/p mix*	2.5	1x
Subtotal	15.0	-
Template DNA	10.0	
Template DNA, added at step 6		
Total	25.0	

* TaqMan p/p containing forward and reverse primer, TaqMan probe and water for each assay prepared to 10x initial concentration.

Example of TaqMan p/p mix for the *Cryptosporidium* qPCR assay

Reagent	**Volume (µl)**	**Final concentration in qPCR reaction**
TaqMan p/p mix		
Forward primer (100 µM)	2.5	250 nM
Reverse primer (100 µM)	2.5	250 nM
TaqMan probe (100 µM)	1.0	100 nM
Nuclease-free water	94.0	
Sum	100.0	

4) Dispense 15 µl of Master mix to the appropriate well(s) of a 96-well reaction plate.
5) Prepare a 96-well dilution plate with template DNA diluted to the appropriate concentrations. *Note: Preferably this step is done on a different bench space in a PCR hood to minimize cross contamination among samples*.
6) Transfer 10 µl of DNA template into each well.
7) Cover the plate with an optical adhesive film.
8) Centrifuge the plate in a plate spinner to spin down the contents
9) Apply a compression pad to the plate
10) Set up the experimental document in the real-time PCR system software including run mode, sample volume, type of TaqMan dye, and thermal cycling parameters
11) Load the plate into the StepOnePlus instrument
12) Start the run.

Standard Operating Procedure

RNA extraction for protozoan (oo)cysts

1. Aim
mRNA extraction from protozoan (oo)cyst suspension to be used for reverse transcription quantitative polymerase chain reaction (RT-qPCR)

2. Notes
Start with 100 µl (oo)cyst pellets. If sample volume is larger, it should be centrifuged and then the supernatant removed to reserve 100 µl in microcentrifuge tubes before RNA extraction

3. Equipment and Materials
Heat block
Hot water (65°C) and liquid nitrogen
Magnet
Rotator
Microcentrifuge
Dynabeads® mRNA DIRECT™ Kit (Invitrogen)

4. Procedure
1) Prepare a water beaker at 65°C and liquid nitrogen bucket.
2) Turn on heat-block to 45°C and add DI water to wells.
3) Bring Lysis buffer and Washing buffer (A and B) to room temperature prior to start. Elution buffer should be stored on ice.

A. Heat induction

4) Put screw-top sample tubes (containing 100 µl pellets) into wells containing DI water of heat-block to induce mRNA expression at 45°C for 20 min. (In the meantime, perform B. Dynabead Oligo$(dT)_{25}$ Preparation as described below)
5) After heat induction is done, increase heat block temperature to 80°C.
6) Add 200 µl of Lysis/Binding Buffer to a sample tube and mix thoroughly by pipetting to obtain complete lysis.
7) Centrifuge briefly to reduce foam.
8) Perform 6 freeze/thaw cycles by freezing in liquid nitrogen for 1 min and then thawing in 65°C water for 1 min.
9) Remove and let cool for 2 min before open the tube.

B. Dynabeads Oligo$(dT)_{25}$ preparation

10) Resuspend Dynabeads Oligo$(dT)_{25}$ thoroughly before use.
11) Transfer 40 µl from the stock tube to an RNase-free 1.5 ml microcentrifuge tube
12) Place the tube on a magnet (e.g. Dynal MPC-S) for 30 s.
13) Remove the supernatant using a pipette.
14) Remove the tube from the magnet and wash the beads by resuspending in 40 µl of Lysis/Binding Buffer.
15) Proceed to C. Direct mRNA Isolation protocol as described below.

C. Direct mRNA Isolation

16) Remove the Lysis/Binding Buffer from the prewashed Dynabeads Oligo $(dT)_{25}$ by placing on the magnet for 30 s.
17) Remove the tube from the magnet and add 300 µl of sample from Step A.
18) Pipet to resuspend the beads completely in the sample lysate.

19) Incubate with rotating at 15 rpm for 20 min using a rotator at room temperature.
20) Place the tube on the magnet for 2 min and remove supernatant.
21) Wash the beads/mRNA complex with 500 µl of Washing Buffer A at room temperature.
22) Centrifuge briefly to get rid of moist on the rim.
23) Place the tube on a magnet for 30 s.
24) Remove Washing Buffer A.
25) Remove the tube from the magnet and add 300 µl of Washing Buffer B.
26) Place the tube on a magnet for 30 s.
27) Remove Washing Buffer B.
28) Remove the tube from the magnet and add 100 µl of cold Elution Buffer.
29) Incubate at 80°C for 2 min.
30) Immediately place the tube on the magnet.
31) Transfer the supernatant to a new RNase-free microcentrifuge tube.
32) Place this tube on ice for immediate use. Or flash-freeze using liquid nitrogen then keep at -80°C until use.

Standard Operating Procedure

RT-qPCR (reverse transcription polymerase chain reaction)

1. Aim
RT-PCR quantifies mRNA concentrations induced from live but not from dead protozoa.

2. Notes
Cycling conditions and final concentrations of primer/probe in the reaction are specific for each parasite. The oligonucleotide sequence, their final concentrations, and assay references can be found in Table 3 of the CPS Final Report.

3. Equipment and Materials
StepOnePlus™ Real-time PCR System
Plate spinner
Compression pad
96-well dilution plate and aluminum adhesive film (for dilution plate)
96-well reaction plate and optical adhesive film (for qPCR reaction)
Path ID Multiplex One-Step RT-PCR Kit
Primers and a TaqMan probe set
Nuclease-free water

4. Procedure
1) Thaw primers and probes used for individual assays on ice.
2) Determine the number of reactions to use based on sample numbers and dilution factors.
3) Prepare a Master mix (note: It contains the Multiplex RT-PCR Buffer and Enzyme mix as well as TaqMan p/p mix) in a microcentrifuge tube using the reagents listed below. Multiply the volume of each reagent by the number of reactions.

Reagent	Volume (µl) per 25-µl reaction	Final concentration
Master mix		
Multiplex RT-PCR Buffer	12.5	1x
Multiplex Enzyme Mix	2.5	1x
TaqMan p/p mix*	2.5	1x
Nuclease-free water	2.5	-
Subtotal	20.0	-
Template DNA	5.0	
Template DNA, added at step 6		
Total	25.0	

* TaqMan p/p containing forward and reverse primer, TaqMan probe and water for each assay prepared to 10x initial concentration.

Example of TaqMan p/p mix for the *Toxoplasma* RT-qPCR assay

Reagent	**Volume (µl)**	**Final concentration in qPCR reaction**
TaqMan p/p mix		
Forward primer (100 µM)	4.0	400 nM
Reverse primer (100 µM)	4.0	400 nM
TaqMan probe (100 µM)	2.0	200 nM
Nuclease-free water	90.0	
Sum	100.0	

4) Dispense 20 µl of Master mix to the appropriate well(s) of a 96-well reaction plate.
5) Prepare a 96-well dilution plate with template DNA diluted to the appropriate concentrations.
6) Transfer 5 µl of DNA template into each well.
7) Cover the plate with an optical adhesive film.
8) Centrifuge the plate in a plate spinner to spin down the contents
9) Apply a compression pad to the plate
10) Set up the experimental document in the real-time PCR system software including run mode, sample volume, type of TaqMan dye, and thermal cycling parameters
11) Load the plate into the StepOnePlus instrument
12) Start the run.

Standard Operating Procedure

PMA treatment for *Giardia*

1. Aim
PMA treatment is applied prior to DNA extraction to selectively amplify DNA from live *Giardia* cysts only during PCR or qPCR reaction.

2. Notes
Start with 100 µl (oo)cyst pellets. If sample volume is larger, it should be centrifuged and then the supernatant removed to reserve 100 µl in microcentrifuge tubes before PMA treatment.

3. Equipment and Materials
PMA (20 mM in dH_2O)
PMA-Lite photolysis device
Phosphate buffered saline (PBS)
Microcentrifuge

4. Procedure
1) Thaw frozen PMA stock on ice.
2) Take 10 µl and add 90 µl PCR-grade ultrapure water to make 2 mM.
3) Vortex 30 s.
4) Add 2.56 µl of 2mM PMA to the sample tube to make the final concentration of 50 µM in the reaction. Vortex shortly.
5) Incubate for 5 min at room temperature in the dark (e.g. inside a drawer).
6) Place the tube into the PMA-Lite.
7) Photo-activation for 15 min. Shake every 5 min.
8) Add 900 µl of PBS in the tube. Vortex shortly.
9) Centrifuge at 14,000 rpm for 5 min, and remove supernatant to reserve 100 µl of pellets.
10) Proceed to DNA extraction according to the DNA extraction SOP. PMA-PCR can be performed according to the multiplex PCR SOP with slight modification (only *Giardia* external and internal primer set used). PMA-treated DNA extract can be used for *Giardia*-specific qPCR assay without modification (See SOP – qPCR).

Standard Operating Procedure

Viable staining

1. Aim
Microscopy-based observation to differentiate intact and dead *Cryptosporidium* and *Giardia* (oo)cysts

2. Notes

3. Equipment and Materials
Epifluorescence microscope equipped with a FITC and Texas Red filter multi-band filter set
Propidium iodide (PI) (1 mg/ml in 0.1 M PBS, pH 7.2)
EasyStain (direct fluorescent antibody in the EasyStain Kit (BTF Precise Microbiology, Inc.))
3-well slide and cover slip

4. Procedure
1) Transfer 20 µl (oo)cyst pellets in a 1.5 ml tube
2) Add 20 µl of DFA in the EasyStain kit to the tube
3) Add 5 µl PI into the tube
4) Vortex the tubes for 20 s
5) Incubate at room temperature in the dark for 20 min
6) Load 10 µl of stained mixture into a 3-well slide and apply a cover slip Without wash the mixture
7) Place 10 µl aliquots on each well and then apply a cover slip
8) Immediately observe under FITC and Texas Red dual band filter set at 200x.
9) Analyze results as follows:
   A. Liable (oo)cysts appear with green outline and black interior
   B. Dead (oo)cysts have the green outline and pink/reddish interior

Standard Operating Procedure

Laboratory spiking experiment

1. Aim
Systematic spinach spiking experiment to validate the performance of newly developed multiplex PCR assay and selected viability assays

2. Notes
Spinach was used in the present study, however other produce/environmental matrices can be prepared in a similar approach for validation of assays on other substances.

3. Equipment and Materials
Heat-block
Microscope
Hemacytometer
(Micro)centrifuges
Smasher
0.1% Tween80 (filter-sterilized)
18 oz Whirl-Pak bags or 24 oz Whirl-Pak filter bags
15 ml and 50 ml Falcon tubes
Long forceps
Weighing dish

4. Procedure
A. Preparation of spike mixture

1) Count intact (live) protozoan parasite (oo)cyst stocks.
2) If dead parasites are needed, add desired numbers of live (oo)cysts to a microcentrifuge tube suspended in total 1 ml PBS or ultrapure water. Put the tube into a heat block and apply appropriate temperature and time as follows:

Parasite	Heat-inactivation condition
Cryptosporidium	80ºC for 3 min
Giardia	70ºC for 15 min
Toxoplasma	80ºC for 20 min
Cyclospora	-

3) After heat-inactivation is done, recount and double-check the (oo)cyst wall integrity.
4) Calculate the volume to be added to spinach samples.
5) Mix four parasites together to the appropriate concentrations of live and/or dead protozoa (oo)cysts. Proceed to Step B.

B. Inoculation of spike onto spinach

1) Weigh 10 g of baby spinach on disposable weighing dishes.
2) Transfer the samples to a biological safety cabinet.
3) Pipet small droplets (~5 µl) onto the surface of spinach leaves.
4) Air-dry the spinach samples inside the biological safety cabinet with air flow for 2 h.
5) After spike droplets are dried out, proceed to Wash or Mash treatment below. place the spinach samples in 18-oz Whirl-Pak bags (Wash treatment) or 24-oz Whirl-Pak filter bags (mash treatment). Proceed to Step C-1 Wash or C-2 Mash treatment.

C-1. Wash treatment

1) Place the spinach samples in 18-oz Whirl-Pak bags.
2) Add 100 ml of 0.1% Tween80 inside the Whirl-Pak bag. Fold it four times and close it.
3) Remove the excess air out of the bag and fold the openings four times then close it.
4) Rub (massage) by hand for 2 min.
5) Transfer the eluant to two 500-ml Falcon tubes. Proceed to Step D.

C-2. Mash treatment

1) Place the spinach samples in one side of 24-oz Whirl-Pak filter bag.
2) Add 40 ml of 0.1% Tween80 to the Whirl-Pak filter bag.
3) With the opening of Whirl-Pak opened, place the bag inside a bigger zip-loc.
4) Place the double bag into the chamber. Slide the bag along the door to remove the air out of the bag, and avoid leaks when closing the door.
5) Apply FAST blending speed for 120 s.
6) After the cycle is done, take out the eluant from the opposite side of filter bag where sample is located and transfer it to a 50-ml Falcon tube.
7) Wash the filter mesh with additional 40 ml of 0.1% Tween80 by pipetting up and down and transfer it to another 50-ml Falcon tube. Proceed to Step D.

D. Concentration of pellets

1) Centrifuge the Falcon tubes at 3000 rpm for 15 min at 4ºC.
2) Remove supernatant to reserve approximately 5 ml in each tube.
3) Pool the two tubes for each sample together (~10 ml) in a 15-ml Falcon tube.
4) Centrifuge at 3000 rpm for 15 min at 4ºC.
5) Remove supernatant to reserve 1 ml (X assay numbers. For example, reserve ~3 ml for 3 individual assays (mPCR, qPCR, Microscopy) to be used) in the Falcon tubes.
6) Aliquot 1 ml to each microcentrifuge tube for further analysis.
7) Keep the sample in a refrigerator and proceed to further analysis within 48 h.