# **Chapter 3 - Material and methods dataset**

# **Confirmation of *Cryptosporidium* Occurrence in Irrigation Water, Soil, and Fresh Produce through Spiking Experiments**

The experimental design of this study involved three phases (Figure 3.1) for comparison of DNA extraction methods (Phase 1a) for isolating *C. parvum* DNA using standard oocysts (Waterborne Inc.) of known amounts from water, spinach, and soil with real-time detection (Phase 1b). Phase 2 focused on the optimisation of the droplet digital PCR (ddPCR) assay for the detection of *C.* *parvum* based on positive reference DNA (*C. parvum*). Phase 3 aimed to determine the lowest number of oocysts that could be extracted from artificial contamination trials using the best method with both real-time PCR and ddPCR.

**Figure 3.1:** The experimental design for confirmation of Cryptosporidium occurrence in irrigation water, soil, and fresh produce through spiking experiments.

**Phase 1a: Comparison of DNA extraction methods from PBS**

**Phase 1b: Comparison of DNA extraction method from water, spinach, and soil**

**Phase 2: Optimisation of droplet digital PCR assay for detection of *Cryptosporidium* based on reference DNA**

**Phase 3: Determination of the lowest number of oocysts successfully detected with real-time PCR and ddPCR from the best DNA extraction methods**

* Eleven different DNA extraction methods were tested using real-time PCR.
* Baseline for DNA extraction methods for subsequent testing on water, fresh produce, and soil samples
* PBS (1ml) was spiked with *C. parvum* 1250000, 125000, 12500, 1250, 125, 12.5, and 1.25 oocyst concentrations.
* The best three methods based on phase 1a (DNA extraction methods 9, 10, and 11, Table 3.1) were selected to determine the best method for extraction of DNA from *Cryptosporidium* in water, fresh produce, and soil using real-time PCR.
* Spinach and soil were spiked with known amounts (1250000, 125000, 12500, 1250, 125, 12.5, 1.25) of C. parvum heat-inactivated oocysts.
* To assess the applicability of ddPCR for the detection of *Cryptosporidium* based on reference DNA.
* To assess amplification efficiency and determine the limit of detection of ddPCR.
* Distilled 1L water, soil, and fresh produce (lettuce and spinach) samples in replicates of five and 200ml environmental water in triplicate were spiked with 12500, 1250, 500, 125, 50, 12.5, and 5 *C. parvum* oocysts,
* The Qiagen DNeasy Blood and Tissue kit with a modified lysis step was used for 1L distilled and 200ml environmental water samples, while the Qiagen Powerlyzer DNA isolation kit with an additional lysis step was used for fresh produce and soil samples.
* Extraction efficiency was determined with the nanophotometer and molecular techniques, including real-time PCR and ddPCR.

### **3.2.1 Inactivated *Cryptosporidium* oocysts**

Heat-treated inactivated *C.* *parvum* oocysts (1x107 in 8ml phosphate-buffered saline [PBS]) were purchased from Waterborne Inc. (New Orleans, LA, USA). A tenfold dilution series (1.25–1250000 oocysts per ml and 5–12500 oocysts per ml) was prepared as working solutions in PBS (Oxoid, Hampshire, UK) and subsequently used to determine the best DNA extraction method for *Cryptosporidium* from different matrices and spiking trials for the evaluation of detection sensitivity and limit of detection.

### **3.2.2 Phase 1: Determination of the best DNA extraction method from inactivated oocysts for each sample type**

#### **3.2.2.1 Phase 1a: DNA extraction from PBS**

To determine the recovery efficiency of the purchased oocysts following DNA extraction, inactivated oocysts were suspended in PBS (Oxoid) at concentrations of 1250000, 125000, 12500, 1250, 125, 12.5, and 1.25 per ml and were centrifuged for 5 min at 6000 g (Micro Analytica (Pty) Ltd, Pretoria, South Africa). Subsequently, eleven standard DNA extraction methods, with slight pre-treatment and/or lysis step modifications to lyse oocyst cell walls, were tested to select possible optimal extraction methods for each of the matrices (Table 3.1)*.* These methods included three DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) protocols, a modified Invisorb Spin Tissue Mini Kit (Invitek Molecular, Berlin, Germany) protocol, a standard Genesig easy DNA/RNA extraction kit (PrimerDesign™, [Chandler's Ford](https://www.bing.com/ck/a?!&&p=7b9308865383e7d16331306c186d6d3aeafd467022c181550bcb796d6bfc182bJmltdHM9MTczMTI4MzIwMA&ptn=3&ver=2&hsh=4&fclid=17faa960-e867-6ea8-18a6-bd3ae99a6fff&u=a1L3NlYXJjaD9GT1JNPVNOQVBTVCZxPUNoYW5kbGVyJTI3cytGb3JkJmZpbHRlcnM9c2lkOiI2NWIwYmRmYi1lNWQzLWQ3YjgtY2IwMi02ZTVlZjdkMGQwYTgi&ntb=1), UK) protocol, and six modified DNeasy Blood and Tissue Kit (Qiagen) protocols (Table 3.1).

Following DNA extraction, the concentration was determined using the Qubit dsDNA High Sensitivity assay and a Qubit 2.0 fluorometer (Life Technologies, California, USA). In addition, the purity was determined using the Nanodrop (ThermoFisher Scientific, Massachusetts, USA) according to the manufacturer’s instructions.

#### **3.2.2.2 Phase 1b: DNA extraction from spinach and soil and detection of Cryptosporidium with real-time PCR**

Based on the Qubit HighSensitivity results from Phase 1a, three methods (methods 9, 10, and 11; Table 3.1) were chosen for the evaluation of DNA extraction from spiked spinach and soil samples and to identify the most effective DNA extraction method for Phase 3. Spinach and soil samples were spiked with oocysts covering a clinically relevant range of 1250000, 125000, 12500, 1250, 125, 12.5, and 1.25 per 100 μl.

**Table 3.1:** Summary of eleven different DNA extraction protocols tested for artificially spiked water with Cryptosporidium

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | **DNA Extractions** | | | | | | | | | | |
|  |  | **Method 1** | **Method 2** | **Method 3** | **Method 4** | **Method 5** | **Method 6** | **Method 7** | **Method 8** | **Method 9** | **Method 10** | **Method 11** |
| **Pre-treatment** |  | Freeze-thaw cycle | Freeze-thaw cycle |  | Freeze-thaw cycle |  |  |  |  |  |  |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
| **Extraction procedure** | **Commercial name of kit** | PowerLyzer isolation kit | Invisorb Spin Tissue Mini kit | DNeasy Blood and Tissue kit | PowerLyzer DA isolation kit | DNeasy Blood and Tissue kit | DNeasy Blood and Tissue kit | DNeasy Blood and Tissue kit | DNeasy Blood and Tissue kit | DNeasy Blood and Tissue kit | PowerLyzer DA isolation kit | Genesig easy DNA/RNA extraction kit |
| **Type of kit** | Spin column-based DNA extraction | Spin column-based DNA extraction | Spin column-based DNA extraction | Spin column-based DNA extraction | Spin column-based DNA extraction | Spin column-based DNA extraction | Spin column-based DNA extraction | Spin column-based DNA extraction | Spin column-based DNA extraction | Spin column-based DNA extraction | Magnetic bead-based DNA extraction |
| **Starting step of kit** | Step 2 | Protocol 2 | Step 1 | Step 5 | Step 1 | Step 1 | Step 1 | Step 1 | Step 3 | Step 4 |  |
| **Lysis buffer** | Buffer from kit | Buffer from kit | Buffer from kit | Buffer from kit | Buffer from kit | Buffer from kit | Buffer from kit | Buffer from kit | Modified lysis buffer with proteinase K | Modified lysis buffer with proteinase K | Buffer from kit |
| **Duration of incubation (if modified)** | Manufacturers’ instruction | Modified incubation step:  2 hr @ 52°C | Modified incubation step:  3 hr @ 52°C | Modified incubation step:  1 hr @ 65°C | Modified incubation step:  90 min @ 56°C | Modified incubation step:  5 hr @ 70°C | Modified incubation step:  3 hr @ 70°C | Modified incubation step:  5 hr @ 52°C | Incubation step:  2 hr @ 55°C - 56°C | Incubation step:  2 hr @ 55°C - 56°C | Manufacturers’ instruction |
| **Homogeniser system**  **(company)** | Mechanical: TissueLyzer II (Qiagen) |  |  | Mechanical: TissueLyzer II (Qiagen) |  |  |  |  |  | Mechanical: TissueLyzer II (Qiagen) |  |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
| **Molecular analysis**  (Genesig *Crypto-sporidium* standard kit) |  | Real-time PCR | Real-time PCR | Real-time PCR | Real-time PCR | Real-time PCR | Real-time PCR | Real-time PCR | Real-time PCR | Real-time PCR | Real-time PCR | Real-time PCR |
|  | ddPCR (optimised using ddPCR supermix) | ddPCR (optimised using ddPCR supermix) | ddPCR (optimised using ddPCR supermix) | ddPCR (optimised using ddPCR supermix) | ddPCR (optimised using ddPCR supermix) | ddPCR (optimised using ddPCR supermix) | ddPCR (optimised using ddPCR supermix) | ddPCR (optimised using ddPCR supermix) | ddPCR (optimised using ddPCR supermix) | ddPCR (optimised using ddPCR supermix) | ddPCR (optimised using ddPCR supermix) |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
| **References** |  |  |  | Modified from (Hagos and Molestina, 2022) | Modified from (Farhadkhani et al., 2022) | Modified from (Woolsey et al., 2019) | Modified from (Hagos and Molestina, 2022) | Modified from (Hagos and Molestina, 2022) | Modified from (Hagos and Molestina, 2022) | Modified from  (Mthethwa et al., 2022) | Modified from  (Mthethwa et al., 2022) |  |

**Fresh produce**. Three different DNA extraction protocols (Methods 9, 10, and 11, Table 3.1) were compared for maximum DNA recovery of oocysts from spinach samples. These methods included a DNeasy PowerSoil Pro Kit (Qiagen) protocol, a standard Genesig easy DNA/RNA extraction kit (PrimerDesign™) protocol, and a modified DNeasy Blood and Tissue Kit (Qiagen) protocol. A total number of 21 spinach samples (seven samples for each DNA extraction) were weighed (30g) and placed in weighing boats. For each DNA extraction, samples were contaminated with 100 μl of known amounts of *Cryptosporidium* (1250000, 125000, 12500, 1250, 125, 12.5, 1.25) by spotting across the leaves. After 2hr of drying at room temperature, each of the samples was placed in labelled, sterile polyethylene strainer stomacher bags (Seward Ltd., West Sussex, UK), sealed, and incubated at 4 °C overnight (Razakandrainibe et al., 2020). As a control for natural contamination, non-spiked leaves were incubated in the same way. Contaminated samples were homogenised for 30 sec at 250 rpm in a Homogeniser Laboratory Blender (ThermoFisher Scientific) in 200 ml PBS (Oxoid) supplemented with 0.01% Tween-80 in a 1:4 weight-to-volume ratio (Dixon et al., 2013). Individual vegetable samples were poured into a 50ml Falcon tube and centrifuged at 2500 x g for 10 min (Sigma 1-16KL (Lasec SA (Pty) Ltd, Midrand, South Africa). The supernatant was carefully removed and discarded, and the previous step was repeated until all the biomass was centrifuged. The pellet was resuspended in 1ml distilled water and centrifuged at 10000 rpm for 3 min. After repeating the previous step once more, the pellet was resuspended in 300 µl of PBS (Oxoid) and used for DNA extraction.

**Soil.** Based on the results from the DNA extraction of spinach samples, two different DNA extraction protocols (Methods 9 and 10, Table 3.1) were compared for maximum DNA recovery from oocysts from soil. This included a modified DNeasy Powersoil extraction kit (Qiagen) and a standard Genesig easy DNA/RNA extraction kit protocol (PrimerDesign™). A total number of 14 soil samples (seven samples for each DNA extraction) were weighed (0.25g) and placed into seven Eppendorf tubes. Subsequently, 100 μl of each concentration of the prepared oocyst PBS dilution series (1250000, 125000, 12500, 1250, 125, 12.5, 1.25) was added to the soil and incubated at 4 °C overnight. These samples were directly used for DNA extraction.

### **3.2.3 Detection of *Cryptosporidium* with real-time PCR**

The presence/absence of *Cryptosporidium* was determined using the Genesig Standard Kit for *Cryptosporidium* (Crypto) Genomes (PrimerDesign™) and PrecisionPLUS qRT-PCR Mastermix (PrimerDesign™). The PCR reaction mixture was prepared according to the manufacturer’s instructions to a final volume of 20µl. In addition, a positive control of *Cryptosporidium* DNA and a negative control were also included in the qPCR assay. The thermocycling conditions were as follows: 95°C for 2 min, followed by 50 cycles of 95°C for 10 s and 60°C for 60 s. Data was collected from the green (FAM) channels during each 60°C annealing/extension phase, and the Bio-Rad CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) was used for post-run analysis. The cycle quantification (Cq) value corresponds to the cycle number at which fluorescence surpasses a predetermined threshold, enabling quantification of the target DNA amount.

### **3.2.4 Phase 2: Optimisation of droplet-digital PCR analysis**

Optimisation of the ddPCR amplification protocol and detection limit was first determined using positive reference *C. parvum* DNA (2 × 105 copy number/µl) included in the Genesig Standard Kit for *Cryptosporidium* (Crypto) Genomes (PrimerDesign™). The QX200 Droplet Digital PCR system (Bio-Rad) was used to amplify a tenfold serial dilution of the positive reference DNA sample, ranging from 0.02 to 2x105 copies/µl. Reaction mixtures each contained 13 µl of Bio-Rad supermix for probes (no dUTP) (Bio-Rad), 1.5 µl of *Cryptosporidium* primers/probes included in the Genesig Standard Kit, 4.5 µl of nuclease-free water, and 7 µl of template DNA to make a final volume of 26 µl. A no-template control (NTC) was also included in the reaction. PCR reaction partitioning and droplet generation were performed using the QX200 Droplet Generator (Bio-Rad) with Bio-Rad droplet generation oil for probes. PCR amplification was carried out on a T100 thermal cycler (Bio-Rad), using the conditions: 95°C for 2 min, followed by 50 cycles of denaturation at 95°C for 10 s and annealing at 57°C for 60 s, and a final extension at 72 °C for 10min. The QX200 Droplet Reader and QuantaSoft software (Bio-Rad) were used to read and analyse the reactions. The threshold line was based on the negative and positive droplet separation. The synthetic DNA was further serially diluted using a 1:10 [dilution](https://www.sciencedirect.com/topics/immunology-and-microbiology/dilution) factor to determine the detection limit of the ddPCR protocol.

### **3.2.5 Phase 3: Determination of the lowest number of oocysts detectable with real-time PCR and ddPCR using the best DNA extraction methods per sample matrix**

#### **3.2.5.1 Matrix spiking/artificial contamination trials**

Controlled artificial contamination trials were conducted in the laboratory to establish the lowest number of oocysts that the best method can successfully extract DNA from for water, soil, and fresh produce samples. For each artificial inoculation trial in the respective matrices, a serial dilution of known amounts of oocysts (12500; 1250; 500; 125; 50; 12.5; 5) was prepared, and the assays for determining the limit of detection of real-time PCR and ddPCR were carried out in fivefold. Non-spiked controls were also included and processed in the same manner. The best DNA extraction method determined for each sample type based on the Qubit High Sensitivity measurements of phases 1a and 1b was employed in these trials.

**Water:** Both distilled and environmental water samples were assessed in the artificial contamination trial. Naturally occurring compounds like humic acids and clay in environmental samples can inhibit the amplification process (Fradette et al., 2022). To assess the specificity and robustness of our methodology workflow under more realistic conditions, we additionally spiked environmental water samples, mimicking natural water complexity.

**Artificial inoculation of distilled water:** Using 1L volumes, seven sterile containers were filled with distilled water and spiked with known amounts of *Cryptosporidium* oocysts (12500; 1250; 500; 125; 50; 12.5; 5) (Waterborne Inc). Each spiked sample was filtered through a 0.45 µm nitrocellulose membrane (Sartorius, Johannesburg, SA). Subsequently, the membranes were aseptically cut up and placed into labelled Falcon tubes containing 9 mL of 0.1% BPW (3M, Minnesota, USA). The tubes were vortexed vigorously (ZX3 Advanced vortex mixer, Velp Scientifica, Italy) and centrifuged for 10 mins at maximum speed (Micro Analytica (Pty) Ltd, Pretoria, SA). After discarding the supernatant, the pellet was resuspended in 1ml distilled water and centrifuged at 10000 rpm for three min. After repeating the previous step once more, the pellet was resuspended in 100 µl of PBS (Oxoid) and used for DNA extraction with the modified DNeasy Blood and Tissue Kit (Qiagen) protocol.

**Artificial inoculation of environmental water samples:** A total of eight 1L water samples were collected from water used for irrigation purposes on two small-scale fresh produce (Farm 1 and 2) known to have poor microbiological quality in Gauteng Province. The samples were transported in a cooler box at 4 ⁰C until analysis within 24 hours. In the laboratory, the 8L water samples from each river were split into 200ml samples in sterile containers and spiked directly with known amounts of *Cryptosporidium* oocysts (12500, 1250, 500, 125, 50, 12.5, and 5) (Waterborne Inc.) and filtered through a 0.45 µm nitrocellulose membrane (Sartorius). The water was further processed as described for the distilled water samples.

**Fresh produce:** A total number of 72 samples were included in the fresh produce artificial inoculation study. “Ready-to-eat” baby spinach leaves and lettuce from commercial packages were weighed (30g) in replicates of five and placed in weighing boats and contaminated with 100 μl of known amounts of *Cryptosporidium* (12500; 1250; 500; 125; 50; 12.5; 5) by spotting across the leaves. Sample processing was carried out as described in the artificial contamination trials under Phase 1b.

**Soil:** Commercially available potting soil was used for the inoculation trial. In replicates of five, the soil was weighed (0.25g) and placed into seven Eppendorf tubes. Subsequently, 100 μl of each concentration of the prepared oocyst PBS dilution series was added to the soil and incubated at 4 °C overnight. These samples were directly used for DNA extraction with the DNeasy PowerSoil Pro Kit (Qiagen) protocol.

#### **3.2.5.2 DNA extraction and molecular detection of the lowest number of oocysts from different spiked matrices**

The precision, reproducibility, and limit of detection were determined to establish the lowest concentration of *C. parvum* oocysts that the proposed DNA extraction method from each sample type can effectively extract DNA from. For 1L distilled and 200ml environmental water samples, the Qiagen DNeasy Blood and Tissue kit with a modified lysis step was selected based on the results obtained during the method comparison phase (phase 1a). Meanwhile, the Qiagen PowerLyzer DNA Isolation kit with an additional lysis step was used for spiked fresh produce and soil samples. The protocol was applied in triplicate for environmental water samples and fivefold for 1L distilled water, fresh produce, and soil samples and spiked as previously described. All spiked samples and the negative control (without spiked oocysts) were subjected to the respective DNA extraction protocol, and the extracted DNA was analysed using a nanophotometer and both real-time PCR and ddPCR amplification protocols (Table 3.2). In this study, positive results were defined as detection in at least three out of five replicates.

**Table 3.2:** Design of the study: number of DNA extractions and Cryptosporidium-specific PCR’s according to the parasite concentration for each protocol

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Oocyst Concentration (oocyst/ml)** | **No. of extractions for 1L water** | **No. of extractions for fresh produce** | **No. of extractions for soil** | **Real-time PCR** | | **ddPCR** | |
| Per reaction | Total | Per reaction | Total |
| 12500 | 5 | 5 | 5 | 1 | 15 | 1 | 15 |
| 1250 | 5 | 5 | 5 | 1 | 15 | 1 | 15 |
| 500 | 5 | 5 | 5 | 1 | 15 | 1 | 15 |
| 125 | 5 | 5 | 5 | 1 | 15 | 1 | 15 |
| 50 | 5 | 5 | 5 | 1 | 15 | 1 | 15 |
| 12.5 | 5 | 5 | 5 | 1 | 15 | 1 | 15 |
| 5 | 5 | 5 | 5 | 1 | 15 | 1 | 15 |
| No spike | 1 | 1 | 1 | 1 | 3 | 1 | 3 |

**References**

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