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Evaluation of TaqMan real-time PCR for the detection of viable Cryptosporidium parvum oocysts in environmental water samples

Melissa A. Cameron
University of South Florida

Evaluation of TaqMan Real-Time PCR for the Detection of Viable *Cryptosporidium parvum* Oocysts in Environmental Water Samples

by

Melissa A. Cameron

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Department of Global Health
College of Public Health
University of South Florida

Co-Major Professor: Boo H. Kwa, Ph.D.
Co-Major Professor: Lillian M. Stark, Ph.D.
Donna J. Haiduven, Ph.D.

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Dedication

To my mother

who always told me I had the ability

to achieve any goal and continuously

encouraged and motivated

me to pursue my dreams.

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List of Symbols and Abbreviations

Symbol and Abbreviations	Description
%	Percent
'	Prime
º	Degrees
°C	Degrees Centigrade
ABI	Applied Biosystems Inc.
bp	Base pairs
CDC	Centers for Disease Control and Prevention
C. parvum	*Cryptosporidium parvum*
Ct	Cycle threshold
DAPI	4',6-diamidino-2-phenylindole
DIC	Differential Interference Contrast
DNA	Deoxyribonucleic Acid
EPA	Environmental Protection Agency
g	Gravitational acceleration
HCl	Hydrochloric Acid
hsp	Heat Shock Protein
ID_{50}	Infective Dose, 50%
IFA	Immunofluorescence assay
IMS	Immunomagnetic Separation
ml	milliliter
N	Normal
NaOH	Sodium hydroxide
nm	nanometers
NPV	Negative Predictive Value
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PPV	Positive Predictive Value
µl	microliter
µm	micrometer
µM	micromolar
U.S.	United States
UV	Ultraviolet

Definitions

Cyst: a phase or form of an organism characterized by a thick and environmentally resistant cell wall. It is produced either in response to environmental conditions or as a normal part of the life cycle of the organism

Effluent: the outflow of water usually from a waste water facility that has been treated in order to be released back into the environment. It may be further treated for use as reclaimed water

Fomites: an object (such as an article of clothing) that may be contaminated with infectious organisms and serve in their transmission

ID_{50}: used for the dose of an infectious organism required to produce infection in 50 percent of the experimental subjects

Inhibition: something that forbids, debars, or restricts

Oocyst: an encysted zygote of certain sporozoans, e.g. *Cryptosporidium*, characterized by a thick and environmentally resistant cell wall. It is a phase or form of an organism produced either in response to environmental conditions or as a normal part of the life cycle of the organism

Raw water: water taken from the environment (ground and surface) that is subsequently treated or purified to produce potable water in a water purification works

Reclaimed water: wastewater (sewage) that has been treated and purified for reuse, rather than discharged into a body of water. It is frequently used to irrigate golf courses and parks and to fill decorative fountains

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ABSTRACT

Cryptosporidium parvum is of growing public health concern due to its ability to survive typical water treatment processes. In order to protect the public from infection, the Environmental Protection Agency developed Method 1623 for the detection of *Cryptosporidium* oocysts in environmental water samples. Execution of this method is time consuming, and the results do not provide an accurate estimation of viability. Therefore, current research is focused on creating a real-time PCR method for the accurate detection of viable *Cryptosporidium parvum* in environmental water samples.

This thesis presents the development of a real-time PCR method, and the results obtained in its use on field samples. The assay was standardized using multiple dilution series in addition to positive and negative controls. Environmental water samples were tested using this method and Method 1623 for comparison. The results were compared statistically to determine the degree of correlation between methods. The data show that the real-time PCR method correlates well to Method 1623. In addition, the assay was determined to be more cost effective and less labor intensive than Method 1623. Although these early findings are promising, additional research and development are needed before the proposed assay can be used in industry.

Introduction

Cryptosporidium

Cryptosporidium parvum, commonly known as "crypto," originally thought to only cause disease in animals, has become a major public health concern. Over recent decades, *Cryptosporidium* has been linked to most waterborne outbreaks in the United States (Leav et al., 2003). It resists chlorination and is difficult to remove by filtration due to its small size. Hence, *Cryptosporidium* has become a major threat in United States' water supplies (Guerrant, 1997). Subsequently, it has become the most common cause of human waterborne disease in the United States (CDC, 2005). The disease caused by *Cryptosporidium*, known as Cryptosporidiosis, is an enteric illness in humans and animals and has become recognized as a significant cause of diarrhea in humans. Though the disease is self-limiting in those with healthy immune systems, it is potentially life threatening in the growing number of individuals with compromised immune systems (Guerrant, 1997). For this reason, water sources must be closely monitored to assure the health of the public.

Discovery

Cryptosporidium was first described as an intracellular organism in the mucosa of mice by E.E. Tyzzer in 1907 (Hannahs, 2007). It is a minute coccidean parasite and the only genus in the family of Cryptosporidiidae. *Cryptosporidium* was originally known as an intracellular parasite and the cause of enterocolitis in many animal species including

mammals, birds, and fish (Markell et al., 1999). This ability to infect a vast variety of hosts sets it apart from other coccideans. Nevertheless, it was not until 1976 that *Cryptosporidium* was first discovered to cause disease in humans (Leav et al., 2003). The first reported case involved a 3 year old girl from Tennessee who developed a severe yet self-limiting enterocolitis (Markell et al., 1999). An intestinal biopsy was performed and examination of the intestinal mucosa showed the causative organism to be *Cryptosporidium parvum*. There are approximately 20 different species of *Cryptosporidium*, with the primary cause of illness in humans and most mammals attributed to *Cryptosporidium parvum* (Roberts & Janovy, 2000).

Epidemiology

Cryptosporidiosis may be acquired from domestic animals as a zoonosis with mainly bovine and human reservoirs (Leav et al., 2003). It is a common cause of short-term diarrhea (Roberts & Janovy, 2000). Humans acquire the parasite by ingesting it in its oocyst form after it is excreted in the stool of infected animals or people (Leav et al., 2003). Though distributed world wide and endemic in developing countries, Cryptosporidiosis is only seen in developed countries in sporadic outbreaks mainly affecting children and people who are immunocompromised (Leav et al., 2003).

Cryptosporidiosis is often significantly under diagnosed due to its self-limiting nature. Among diagnosed cases, a vast majority have been linked to the ingestion of water contaminated with *Cryptosporidium* oocysts. There have been several waterborne outbreaks of cryptosporidiosis in the U.S. (Markell et al., 2000). An environmental study found between 67% and 95% of the surface water throughout the U.S. is contaminated

with *Cryptosporidium* oocysts (Markell et al., 1999). Due to their small size, 4 to 5µm in diameter, *Cryptosporidium* oocysts are difficult to filter from water supplies. They are also resistant to chlorination. This has lead to numerous outbreaks throughout the U.S despite water treatment efforts.

The first reported outbreak occurred in 1984 and was due to the fecal contamination of an artesian well in Texas. Another highly publicized outbreak occurred in Milwaukee, Wisconsin in 1993 (Leav et al., 2003). The outbreak affected approximately 403,000 people and was the largest waterborne outbreak in the U.S. (Guerrant, 1997). Of these, many became severely ill and several of those who were immunocompromised died.

Drinking water has been identified as source of infection. Most outbreaks have occurred in communities where the local water utilities were meeting state and federal guidelines. Other sources of infection have been linked to public wave pools and to consumption of unchlorinated well water (Markell et al., 1999). The vast possibilities for infection and outbreaks illustrate the importance of routine monitoring of water supplies.

Transmission

The transmission of *Cryptosporidium parvum* is via the fecal-oral route. Infected individuals and animals shed the parasite in their feces in the form of oocysts as few as five days after initial infection and for up to five weeks after the diarrheal illness ends (Roberts & Janovy, 2000). A single bowel movement from an infected individual or animal may release millions of oocysts (CDC, 2005). Oocysts can contaminate water, soil and food. *Cryptosporidium* oocysts have a thick wall which allows them to survive

well in the environment and withstand chlorination (Guerrant, 1997). Once the oocysts are shed into the environment there are multiple means of transmission. For example, from animal to person: a person comes in contact with an infected animal, picks up the oocysts through contact and accidentally ingests the parasite. This means of transmission has been seen on dairy farms where the pathogen is present in 50% of the calves on 90% of dairy farms; however, it is rare in other environments (Hannahs, 2007).

Another mode of transmission is person to person contact. This occurs predominately in child daycare centers, nursing homes, and hospitals where the occupants need supervision and assistance with personal hygiene. Infection may spread quickly if special attention is not given to cleanliness when changing diapers or handling contaminated fomites of infected patients. Like animal to human transmission, person to person transmission is rare (Hannahs, 2007).

The most common means of transmission for *Cryptosporidium parvum* is via contaminated food or water. Most outbreaks world wide have been transmitted through contaminated drinking water and recreational water parks (Leav et al., 2003). Source water is easily contaminated by water runoff from farms and grazing areas. Once oocysts are in the water supply, they are very difficult to remove. Upon ingestion of the contaminated water, the individual becomes infected, begins excreting oocysts and the cycle continues.

Clinical Features

Cryptosporidium parvum is the etiologic agent of cryptosporidiosis. Based on human studies *C. parvum* has an infective dose (ID_{50}) of 132 oocysts. However,

infection may occur upon ingestion of as few as 30 oocysts by healthy individuals and a single oocyst in those immunocompromised (Guerrant, 1997). Once ingested, sporozoites are released and parasitize the brush lining of the epithelial cells located in the gastrointestinal or respiratory tract. This may cause a variety of symptoms two to ten days post infection depending upon the age and immune status of the individual (Leav et al., 2003).

In individuals with healthy immune systems, the disease may be asymptomatic but generally causes a watery or mucous-like diarrhea that may or may not be accompanied with abdominal pain. Other symptoms may include varying degrees of nausea and vomiting accompanied with dehydration, low grade fever and weight loss (Markell et al., 1999). Symptoms in healthy individuals may cycle, causing a period of a few days during which the individual seems to be improving before the symptoms return (CDC, 2005). The symptoms are usually self-limiting and mild lasting one to two weeks. However, children and pregnant women should be closely monitored due to their increased sensitivity to dehydration (Hannahs, 2007).

Individuals with a compromised or deficient immune system, such as people with HIV/AIDS, those who have undergone a transplant or chemotherapy, and individuals with inherited immune disease, are much more likely to suffer from more severe symptoms (CDC, 2005). Since the early 1980's, Cryptosporidiosis has become an important contributory factor in the death of AIDS patients (Guerrant, 1997). The infected individual may experience cholera-like watery diarrhea with as many as 6 to 25 bowel movements per day, with a stool fluid loss of up to 20 liters per day. They may

also experience severe abdominal pain, nausea and vomiting. Complications may occur due to prolonged diarrhea, malabsorbtion and dehydration. In immunocompromised and deficient individuals, infection may occur in multiple areas of the body in addition to the intestines by penetration of the luminal surface (Hannahs, 2007).

Cryptosporidium has been found in the sputum, lung biopsy materials and the biliary tract. Symptoms may subside, but often become chronic and life-threatening. There have been many deaths attributed to Cryptosporidiosis in immunocompromised individuals (CDC, 2005).

Treatment and Prevention

Cryptosporidiosis is diagnosed by identification of the organism in biopsy material or detection of oocysts in stool samples (Guerrant, 1997). The disease may not require any treatment in healthy individuals due to its self-limiting nature however some may be treated with nitazoxanide (CDC, 2005). Treatment for individuals with poor health or weakened immune systems is more difficult. The effectiveness of drugs such as nitazoxanide is unclear in the immunocompromised (CDC, 2005). The illness is not usually curable in these individuals and, as the immune status worsens, the symptoms may recur and worsen producing a chronic infection (CDC, 2005).

There are few measures that may be taken to prevent possible infection by *Cryptosporidium parvum*. The best way to prevent illness is to abstain from drinking water or consuming food that may be contaminated with the parasite. One must also practice good hygiene and use caution when traveling; especially abroad, i.e. proper hand

washing to avoid fecal-oral contamination, drinking bottled water and consuming only cooked foods (CDC, 2005).

Water Treatment & Detection Methods

Public health and municipal water authorities have taken action in an attempt to assure the safety of public drinking water. The U.S. Safe Drinking Water Act (1974) requires drinking water utilities to meet stringent standards for maximum levels of microbiological and chemical contaminants (Viessman & Hammer, 1998). The treatment process used at each facility is determined by the type of raw water source and the quality of finished water desired. To successfully remove a protozoan like *C. parvum*, there must be effective chemical treatment and filtration (Viessman & Hammer, 1998). It is difficult and very costly to remove all *Cryptosporidium* from water supplies because of the parasite's resistance to chlorination and small oocyst size (Guerrant, 1997). Minor problems in the treatment process may go unnoticed and allow *C. parvum* to enter the water supply. Therefore, it is very important that municipal water supplies are treated and monitored regularly to prevent the public from becoming ill. In order to do this, improved and faster methods of detection are necessary.

Water Testing (Environmental Protection Agency Methods 1622 & 1623)

The U.S. Environmental Protection Agency (EPA) created Methods 1622 and 1623 as a means of routine monitoring of water sources to prevent the occurrence of outbreaks like that witnessed in Milwaukee. Method 1622 is specific for the detection and enumeration of *Cryptosporidium* oocysts and Method 1623 is specific for the detection and enumeration of both *Cryptosporidium* oocysts and *Giardia* cysts in

environmental water samples (EPA, 2001). The methods are performed by filtration of the water to be assayed, elution of the organisms from the filter, immunomagnetic separation (IMS) of the oocysts from the matrix and immunofluorescence assay (IFA) microscopy and differential interference contrast (DIC) microscopy (EPA, 2001).

Analysis

The primary method for identifying and enumerating the number of cysts and oocysts in environmental water samples, per EPA Methods 1622 and 1623, is by immunofluorescent staining and microscopic examination. Two types of stain are used. The first stain used is an immunofluorescent stain, for example EasyStain C&G (BTF, Cat. #ESTAIN80) which is designed to bind specifically to the protein coat of *Cryptosporidium* and *Giardia* using monoclonal antibodies that cause the oocysts and cysts in the sample to fluoresce a bright apple green color. This allows for quick identification of potentially viable oocysts and cysts.

Giardia and *Cryptosporidium* may also be assayed with a secondary stain, 4',6-diamidino-2-phenylindole, commonly known as DAPI. DAPI is a fluorescent dye that binds to A-T rich double stranded DNA, producing a sky blue color when viewed with a UV filter block (excitation 550nm, emission 600nm) (Polysciences, INC., 1999). Positive DAPI staining is an indicator of potential viability, whereas DAPI negative oocysts are considered nonviable since they lack intact DNA (EPA, 2001). The slides are also examined with differential interference contrast (DIC) microscopy for internal structures characteristic of *Giardia* cysts and *Cryptosporidium* oocysts.

These staining methods are capable of providing a rough estimate of the number of potentially viable oocysts in a water sample; however, there are problems with these methods. Because they do not differentiate between different *Cryptosporidium* species, oocysts counted in this process may be of a species that is not known to cause harm to the general public. In addition, DAPI has been shown to overestimate the number of viable oocysts in a sample by studies in which mice were infected with DAPI positive samples yet never produced infection (Jenkins et al., 2000).

In addition to the non-species specific nature of these stains, they are also labor intensive to perform and examine, taking hours to complete. Examination of the slides must be performed by experienced laboratorians trained in the science. However, a great deal of the interpretation of the results is left to the discretion of the examiner. Although both of these stains allow us to assess the possibility of contaminants in water samples, there needs to be a less subjective, more specific and efficient means of detecting viable *Cryptosporidium* in environmental water samples in order to significantly reduce the possibility of misinterpretation.

Real-Time PCR

In attempts to find a more specific and timely method for the detection of *Cryptosporidium* in environmental water samples, scientists are looking toward real-time polymerase chain reaction (PCR). It is a method that allows for the logarithmic amplification of short strands of DNA and detection in "real-time" by the reporting of fluorescent probes. Theoretically, a single copy of a particular sequence can be amplified and detected, through the use of appropriate primers and probes, with a direct relationship

between the starting target and the amount of product at a given cycle (Ambion, 2007). This is accomplished by cycling the sample through various thermal cycles, usually ranging in number from 40 to 50, during which the DNA is replicated (Ambion, 2007). As the DNA replicates, the probe searches for a specific target nucleic acid sequence. The probe attaches to the target DNA and cleaves, creating a fluorescence that is detected by the real-time PCR instrument after each thermal cycle is completed.

This method is commonly used for detection and quantification of various viruses and parasites in numerous sample mediums. The ability to detect a specific sequence and the fast result time (2 to 3 hours) makes real-time PCR a good candidate for future *Cryptosporidium* testing and water monitoring. Many studies have been done in attempts to create a method that detects viable *Cryptosporidium* in water samples; however, few have been done using environmental samples as opposed to spiked laboratory samples.

In 1995, Wagner-Wiening and Kimmig detected viable *Cryptosporidium parvum* oocysts using traditional PCR. The study used oocysts that were placed in media and excysted to assure viability. The PCR generated a product 873 base pairs (bp) in length encoding an oocyst protein. The procedure was successful in detecting viable oocysts; however, the results were not easily replicated or predictable.

Kaucner and Stinear (1997) detected viable *C. parvum* oocysts in large volumes, 20 to 1,500 liters, of spiked creek and river water. They devised a method for detecting a smaller segment of DNA measuring only 307 bp in length, from a heat shock protein found in *C. parvum*. The method was not tested on environmental samples. These two

studies did not permit enumeration of oocysts but they did set the course for further research in the area.

A Most-Probable-Number assay was developed by Slifko, Huffman, and Rose (1999) that enumerated infectious *C. parvum* oocysts in cell culture systems. They also were able to determine from this study that the age of the oocysts affected its infectivity. This discovery illustrated the ability of oocysts enumeration and a need for a more precise method for determining the viability of oocysts.

Gobet and Toze (2001) conducted a study to determine the relevance of heat shock protein (hsp) 70 messenger RNA and DNA to determine the viability of *Cryptosporidium parvum* oocysts. The study compared an assay using this protein with methods utilizing mouse infectivity and immunofluorescent dyes. The poor specificity and sensitivity of immunofluorescent dyes and the unreliability of infectivity assays lead to the determination that DNA encoding for hsp70 was the best indicator of viability. They also noted that the amount of DNA detectable in the oocysts decreased quickly after they became nonviable allowing for more precise detection of only viable oocysts.

In 2003, Fontaine and Guillot developed a method for an immunomagnetic separation real-time PCR for the quantification of *C. parvum* in water samples. The method followed the previously used EPA Method 1622 for the detection of *Cryptosporidium* species in water samples. They were able to detect as few as 5 oocysts in spiked samples. Though this method was successful with laboratory spiked samples, it did not use hsp70 as the target and was not designed to detect only viable oocysts.

During the same period of time, LeChevallier, et al. (2003) were comparing EPA Method 1623 with a cell culture PCR method. The two methods produced similar results. Their study showed the usefulness of hsp70 for detection of viable *C. parvum* in water samples. Though the method was successful and able to detect low quantities of viable oocysts, it utilized traditional gel based PCR and was labor intensive to perform.

The results of both 2003 studies just described were used in the development of the present research presented herein. The method developed for this study used immunomagnetic separation followed by real time PCR with hsp70 as the target in order to detect only viable oocysts in spiked water samples and environmental samples received at the Florida Department of Health, Bureau of Laboratories in Tampa.

Objectives

Many methods have been used to assess the viability of *Cryptosporidium parvum* oocysts. For example, one method consists of growing cell cultures and inoculating the cells with oocysts. This is done to determine if the oocysts are viable (capable of causing infection). This method is time-consuming, taking weeks to perform, and underestimates of the number of viable oocysts. Method 1623, developed by the US Environmental Protection Agency, is the current test used by most laboratories. Although this method is widely used, it is also a time-consuming method requiring at least one day for processing. The results depend on the interpretation of the technologist and may lead to an overestimate of the number of viable oocysts in the sample.

The inaccuracy and inefficiency of current testing methods necessitates finding a more precise and practical method for laboratories to determine oocyst viability in public water sources. Current research utilizing the real-time PCR shows promise. Primers and probes have been reported in the literature for testing the viability of *Cryptosporidium parvum* oocysts, but the tests are not standardized and are not currently in routine use, nor have they been evaluated to determine their efficiency in a public health laboratory.

The hypothesis of this study is that a protocol can be developed for real-time PCR based assay for testing of *Cryptosporidium parvum* in environmental water samples that is as sensitive as the current testing methods. This method is anticipated to give a more accurate estimate of oocyst viability due to the specific nature of the test and the

elimination of examiner interpretation. This new method will decrease the total time needed to complete the assay and allow for quicker reporting of results. This will, in turn, aid in the prevention of illness and allow control measures to be implemented in a more timely manner should an outbreak occur.

This study evaluates the potential use of real-time PCR to determine *Cryptosporidium parvum* oocyst viability by comparing the viability of *C. parvum* oocysts estimated by EPA Method 1623 with TaqMan real-time PCR.

The study has four specific aims:

1) to standardize and validate a protocol for using Real Time PCR detection on viable *Cryptosporidium parvum* oocysts in various types of water samples;
2) to determine the sensitivity and statistical relationship of real-time PCR method as compared to IFA/DAPI staining methods;
3) to determine if the statistical relationship varies by water sample types, i.e. raw, treated waste water (effluent), reclaimed water, or drinking water (potable); and,
4) to determine the best testing method for viable *Cryptosporidium* oocysts by analyzing cost and time efficiency.

The overall goal of this study is to enhance public health by improving current *Cryptosporidium* detection methods in environmental water samples, and to aid in the prevention and control of infection.

Materials and Methods

Water Sample Submission and Processing

Water samples are received weekly at the Florida Department of Health Bureau of Laboratories in Tampa, to be tested for *Cryptosporidium* and *Giardia*. The samples are submitted from various water and waste water utilities throughout the state of Florida. Sample types received vary depending on the facility requesting the testing which include raw (ground or surface), effluent (treated waste water), reclaimed (re-use), and drinking (potable) water. The raw water used in this study is from ground or surface water sources. Raw water is treated and purified to be used as potable or drinking water. Effluent is the outflow of water usually from a waste water facility that has been treated in order to be released back into the environment. Effluent can be further treated and purified, known as reclaimed water, for use in irrigation systems and outdoor fountains.

On average the Tampa Laboratory receives approximately 130 samples a year; however this number is steadily increasing. Upon submission, samples are logged, numbered and stored at 4ºC until testing. All samples are tested within 96 hours of collection per EPA Method 1623 requirements.

Sample Size and Selection

A sample size of 32 was needed in this study to assure statistically significant results. The sample size was determined using the sample size calculator developed by Cameron and Baldock based upon a population of 130 (the average number of samples

collected per year), a sensitivity and specificity of 80%, a prevalence of 45% (based on an average of 59 positive samples per year), a level of significance of α=.05, and a power of 95% (AUSVET, 1998). Due to the limited number of samples collected each year, every sample received between September and December of 2006 was assayed in this study. This resulted in a final sample size of 40 for this study.

Water Sample Analysis

EPA Method 1623

All water samples received are tested for *Cryptosporidium* and *Giardia* protozoa using EPA Method 1623. This method will be summarized here. A complete protocol is available at http://epa.gov/waterscience/methods/1623.pdf.

In the field, water sources are passed through filters (IDDEX, Cat. # FMC10601) which are designed to trap *Cryptosporidium*, *Gardia* and extraneous material. The filters are sent on ice to the Department of Health for processing and testing. Upon arrival at the laboratory, the samples are numbered and held between 0 and 8ºC until tested.

Elution

The Filta-Max system (IDDEX Cat. #FMC 10102, FMC 10301, FMC 12001) is used to elute the material off of the submitted filter. The apparatus consists of an elution tube and plunger containing a membrane designed to concentrate the oocysts and cysts (Figure 1). The filter is placed in the elution tube along with any remaining liquid from the filter carriage. The filter is processed using a series of washes to assure all protozoa are concentrated on or above the membrane at the base of the tube. The membrane, with protozoa attached, is removed from the device and washed to remove the oocysts and

cysts from the membrane. The membrane is discarded. The oocyst suspension is placed in 50 ml sterile polypropylene conical tubes and centrifuged at 1,500g for 15 minutes. This results in the formation of a pellet, consisting of oocysts, and the supernatant.

The supernatant is removed to leave a volume of approximately 5ml above the pellet. At this point, the pellet size is measured and recorded. The pellet is divided in two with one portion continuing on to the immunomagnetic separation step, the other preserved with 10% neutral buffered formalin and archived. For the purpose of this study, the second portion of the pellet continued through the IMS step and was processed for real-time PCR testing (Figure 2).

Immunomagnetic Separation

Dynal flat-sided tubes (Dynal Inc., Cat. #740.03) are labeled and 1ml each of buffer A and buffer B from the Dynal IMS kit (Dynal Inc., Cat. #730.02) is added to each tube. Both pellets are resuspended by vortexing for 2 minutes. The sample pellets are removed with a pre-wetted pipette and added to the appropriate Dynal tube. The conical tubes are rinsed twice with the appropriate amount of sterile water in order to produce a total volume of 12ml in the Dynal tube. Magnetic beads with antibodies specific for *Giardia* and *Cryptosporidium* are added. The Dynabeads Giardia-Combo and Crypto-Combo vials (Dynal Inc., Cat. #730.02) are vortexed and 100µl from each vial are added to each tube. The Dynal flat-sided tubes are then placed on a rotation device (Dynal Inc., Cat. #947.01) and rotated at a speed of 18 rotations per minute for one hour (Figure 3).

After the samples complete the rotation, the *Cryptosporidium* oocysts and *Giardia* cysts present in the samples should be attached to the magnetic beads by an antigen-

antibody reaction. The Dynal flat-sided tubes are removed from the rotator and placed in a magnetic holder with the flat portion of the tube facing the magnet (Figure 4). The tubes are rotated in the holder 90º end to end for two minutes at one tilt per second causing the magnetic beads to adhere to the flat portion of the Dynal tube. The supernatant is immediately decanted; the tubes are removed from the magnetic holder and placed in a test tube rack. The tube containing the magnetic beads is rinsed three rinses with a 1X buffer A solution to assure removal of the magnetic beads from the Dynal tubes: 0.5ml of the 1X buffer A solution is added to the tube. The tube is caped and gently rocked to rinse the beads from the flat side of the tube. The solution is removed using a pre-wetted pipette and added to a microfuge tube. The process is repeated with an additional wash using 0.5ml and a final wash of 0.4ml. The microfuge tube is capped, placed in a holder and a magnetic strip is added to the holder (Figure 5). The tubes are gently rocked back and forth through 180º for one minute at one rock per second allowing the magnetic beads to adhere to the side of the tube. The supernatant is removed leaving the magnetic beads with any attached protozoa. At this point, the two samples separated at the beginning of the IMS proceed to different steps. One microfuge tube continues to the IMS disassociation step, staining with DAPI and EasyStain, and microscopic examination while the other proceeds to the experimental nucleic acid extraction, real-time PCR and analysis.

Disassociation

The magnetic bar is removed from the microfuge tube holder and 50µl N HCl is added to the tube. The tube is vortexed for 50 seconds, placed back into the tube holder

without the magnetic strip and let stand for 10 minutes. This causes the oocysts to disassociate from the beads. The tube is vortexed for 30 seconds and the tube is tapped to assure any drops in the cap return to the base of the tube. The magnetic strip is placed back into the holder. The holder is placed at a slant and let stand for 10 seconds. A well slide is labeled and 10µl of 1.0 N NaOH is added to the well. Without removing the magnetic strip the supernatant is removed from the microfuge tube and added to the well slide. The disassociation step is repeated and the supernatant is added to the slide. The slide is now ready for staining.

Staining

During the staining process a positive and negative slide are also prepared for controls. The slides containing the sample and the controls are placed on a slide warmer set to 37ºC and allowed to air dry. Each slide is treated with 50µl of absolute methanol and allowed to air dry. The slide is removed from the warmer and 50µl of DAPI staining solution (Sigma, Cat. #D9542-1MG) is added to each slide and allowed to stand for 2 minutes. The solution is removed from the slide by tilting it on a paper towel. A volume of 50µl of sterile water is added to the well and the slide stands for one minute. The water is removed as before by tilting the slide on a paper towel. 50µl of EasyStain, consisting of an immunofluorescence reagent designed for use on *Cryptosporidium* oocysts and *Giardia* cysts in water samples, (BTF, Cat. #ESTAIN80) is added to the slide and the slide is placed in a humid, dark chamber at room temperature for 30 minutes. The stain is removed from the slide by tilting it on a paper towel. At this time, 300µl of Fixing Buffer from the EasyStain Kit (BTF, Cat. #ESTAIN80) is added to the slide. The

slide stands for 2 minutes and the buffer is removed by placing the slide tilted on a paper towel. 10µl of mounting buffer is added to the well and a cover slip is put in place. The cover slip is sealed in place using two coats of clear nail polish around its edges. Once dried, the slide is ready for microscopic analysis.

Microscopy

A Zeiss epifluorescent microscope (Zeiss, model #AXIOSKOP2) is used to scan the entire well at 200X or 400X for an apple-green fluorescence which indicates the possible presence of cysts and oocysts. When bright apple-green fluorescing ovoid or spherical objects, ranging in size from 4 to 6µm in diameter, are observed with highlighted edges they are counted and recorded (Figure 6). A UV filter block (excitation 550nm, emission 600nm) is put in place for DAPI examination.

The oocysts may exhibit one or more of the following characteristics: a light blue internal staining and no distinctive nuclei, an intense blue internal staining, or no more than four distinct sky blue nuclei (Figure 7). The results are recorded for the first ten organisms examined. If the organism exhibits a green rim without internal structures, it is characterized as DAPI negative. Organisms are recorded as DAPI positive if there is a strong blue internal stain or distinctly stained nuclei are present. The UV filter is removed; magnification is increased to 1000X (oil emersion) for DIC examination of possible internal structures.

Real-Time PCR

Extraction

Samples that do not undergo the IMS disassociation step mentioned above continue to the experimental real-time PCR method. 1ml of 1X phosphate buffered saline (PBS) added to each microfuge tube containing the magnetic beads to wash away any residual Buffer A solution. The samples are vortexed, returned to the magnetic holder, and rocked 180º for 2 minutes allowing the beads to attach to the side of the tube containing the magnet. A solution is prepared at a 25% weight-to-volume concentration of Chelex 100 resin (BioRad, Cat. #143-2832) and sterile reagent grade water to be used in the extraction. The wash supernatant is removed from the microfuge tube containing the magnetic beads and 200µl of Chelex 100 resin solution is added to the tube.

The DNA is extracted using a simple freeze-thaw method. The tubes are submerged into liquid nitrogen for 2 minutes. Upon removal, they are immediately placed into a water bath at 95ºC for 2 minutes. This freeze-thaw process is repeated for four more cycles. After the final cycle, the sample is placed in a microcentrifuge and spun at 10,000g for 10 minutes to separate the beads and Chelex 100 resin from the supernatant containing the DNA. The sample lysate is now ready for PCR testing and is stored in a -20ºC freezer until real-time PCR is performed.

Positive and Negative Controls

Positive and negative controls are used to assure accuracy in the PCR testing. Viable *Cryptosporidium parvum* oocysts in 1X PBS were obtained from Waterborne Inc. (New Orleans, LA) with a guaranteed number of viable oocysts at 1 x 10^6 per 4ml. A

volume of 500µl is removed from the vial and placed in a screw top microfuge tube marked positive. An additional 500µl is removed from the vial, boiled for one minute in order to render the *Cryptosporidium* oocyst nonviable and is placed in a screw capped microfuge tube marked negative. Both tubes undergo the same freeze thaw extraction method used on the samples.

Standardization of PCR Assay

The PCR assay that was developed for this study was based on methods used by LeChevallier et al. for the detection of viable *Cryptosporidium* and Fontaine et al. for IMS detection of *Cryptosporidium parvum*. The methods were combined to allow for testing of viable oocysts in environmental water samples.

Primers and probes specific for *Cryptosporidium parvum* hsp70 DNA were obtained from Operon (Huntsville, AL). Primer and probe sequences to be used are as follows:

forward primer: 5' TCCTCTGCCGTACAGGATCTCTTA 3';

reverse primer: 5' TGCTGCTCTTACCAGTACTCTTATCA 3';

TaqMan probe: 5' 6-carboxyfluorescein

TGTTGCTCCATTATCACTCGGTTTAGA 6-

carboxytetramethylrhodamine 3'.

Based on experimental results the optimal concentration for the primers and probe in this study are 100µM and 25µM respectively and are subsequently used to test all collected samples.

Real-Time PCR Protocol

Real-time PCR is performed with the Applied Biosystems (ABI) 7500 Fast Real-Time PCR System (Applied Biosystems, CA) and TaqMan One Step RT-PCR Master Mix Kit (Applied Biosystems, Cat. #4309169). A template is created for calculating the quantities of each master mix ingredient per sample being tested; an example is seen in Figure 8. For each sample to be assayed, a master mixture is made containing: Dnase/Rnase free water, 6.35µl; TaqMan Universal 2X PE Master Mix, 12.5µl; forward primer, 0.25µl; reverse primer, 0.25µl; probe 0.15µl; enzyme, 0.50µl. These ingredients are combined in a SafeLock 1.5 microfuge tube (Eppendorf, Cat. #0540334B), vortexed, and briefly pulsed to remove any drops from the lid.

Samples are tested on a MicroAmp Fast Optical 96 well reaction plate (Applied Biosystems, Cat. #4346906) along with positive and negative controls, a positive control dilution series for quantification and spiked samples to determine if the is any inhibition of the PCR run. The template sheet also illustrates the well location of each sample, spiked samples, controls, and dilution series (Figure 8). Each plate is loaded with 15µl of master mix per well. A volume of 10µl of DNA template from the extracted sample is added to the appropriate well creating a total reaction volume of 25µl (Figure 9). The mixture is mixed by pipetting up and down carefully so as to not create bubbles or aerosols. Spiked samples are loaded with 15µl of master mix, 8µl of sample and 2µl of a known positive control. A well is also loaded with the same volumes of master mix, sterile water and positive control for comparison against spiked samples. An optical

adhesive cover (Applied Biosystems, Cat. #4311971) is placed on the plate to seal the wells.

The sample plate is loaded in the ABI 7500 (Figure 10). The detectors, FAM and TAMRA, are selected and applied to the entire plate. The optimum cycling times are programmed. These include an initial denaturing step of 95ºC for 10 minutes and an amplification step of 50 cycles of 95ºC for 30 seconds followed by 60ºC for 1 minute. The sample volume is set to 25µl and a run mode of 9600 emulation with detection occurring at the 60ºC stage. The run takes approximately 3 hours to complete.

TaqMan Analysis

Once the run is completed, the manual Ct and manual baseline settings are selected. Start and end cycles are set to 13 and 25 respectively, based on experimental results. The threshold is moved to the mid point of the exponential portion of the curves (Figure 11). The data is analyzed and cycle threshold (Ct) values are displayed following the instrument instructions. The Ct values displayed for each sample allow for enumeration and determination of run validity (Figure 12).

Enumeration and Inhibition

Based on the Ct values for the positive and negative controls one can assess if the run is valid. Positive controls should have a Ct value of less than 45 and the negative control should be listed as undetected. The Ct value gives a reference value for the enumeration of the amount of viable *Cryptosporidium* in the sample. For example, if a known number of 250 oocysts are placed in the positive control well, the Ct values of

each sample can be compared to the Ct of the positive control, estimating the number of oocysts in each sample.

Some field samples may inhibit the PCR reaction. In order to account for this, the spiked samples are examined. The Ct value for the well containing the water spike (8µl water and 2µl positive control) is compared to the Ct value of each spiked sample. If the Ct value is less than two standard deviations from the spiked control Ct, the sample is not considered to be inhibitory. Ct values on spiked samples that are 2 to 4 standard deviations greater than the control spike are reported with minor inhibition. Samples that are 4 to 6 standard deviations above the control are reported with major inhibition. Samples 6 or more standard deviations above the control and Ct's listed as undetected are reported with major or complete inhibition, respectively.

Figure 1 Iddex elution device used in EPA Method 1623 for the elution of *Cryptosporidium* oocysts and *Giardia* cysts

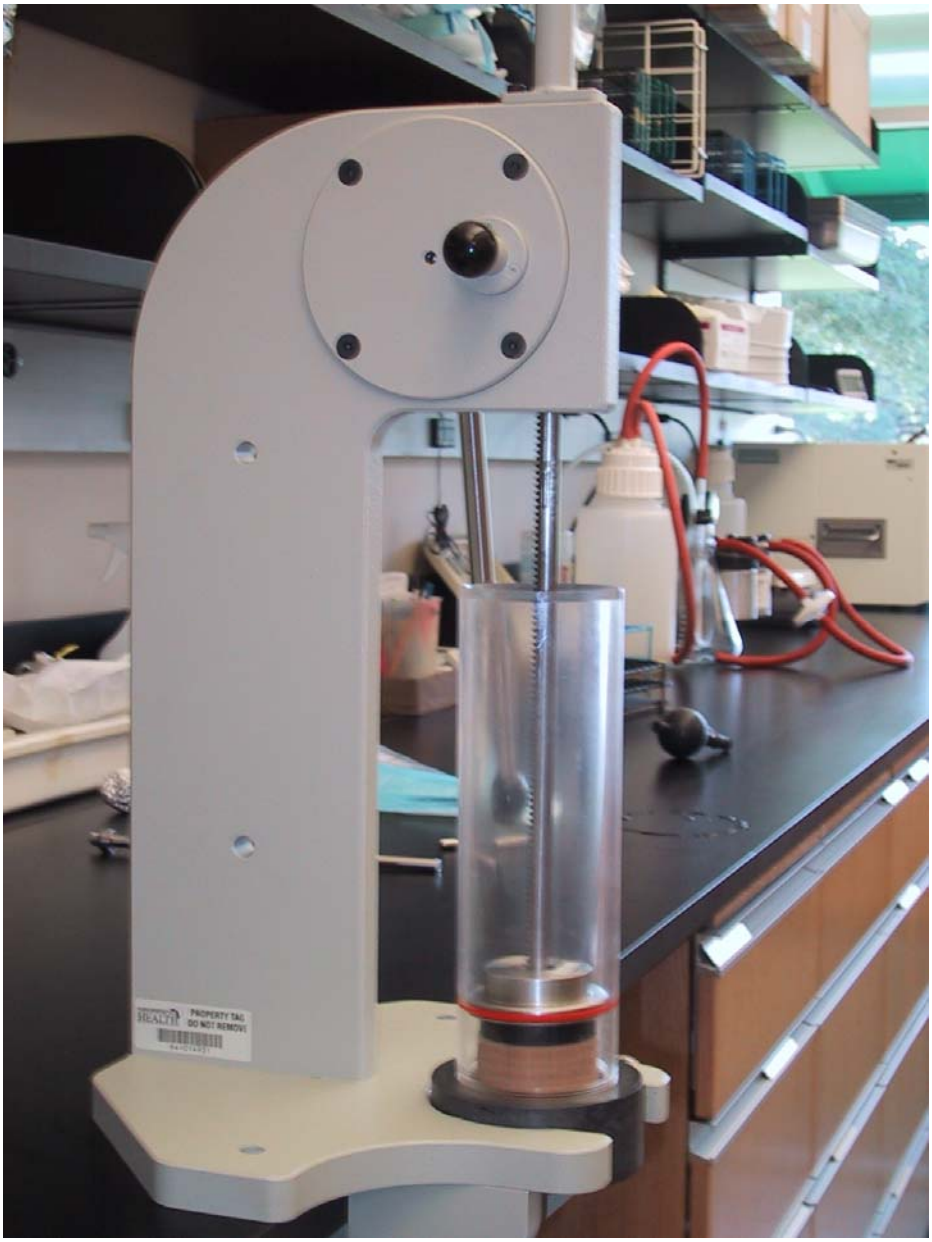

Figure 2 Flow chart of sample processing and time requirements for processing 8 samples using Method 1623 vs. real-time PCR

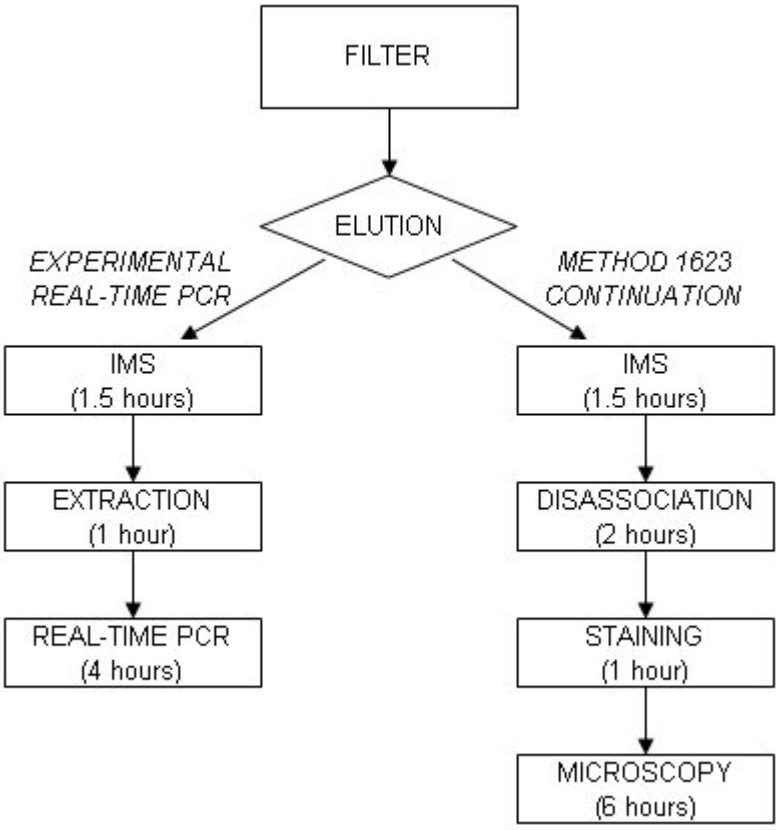

Figure 3 Immunomagnetic separation procedure: Dynal rotation instrument with flat sided tubes mounted

Figure 4 Immunomagnetic separation procedure: Dynal flat sided tubes in magnetic holder for the removal of the supernatant

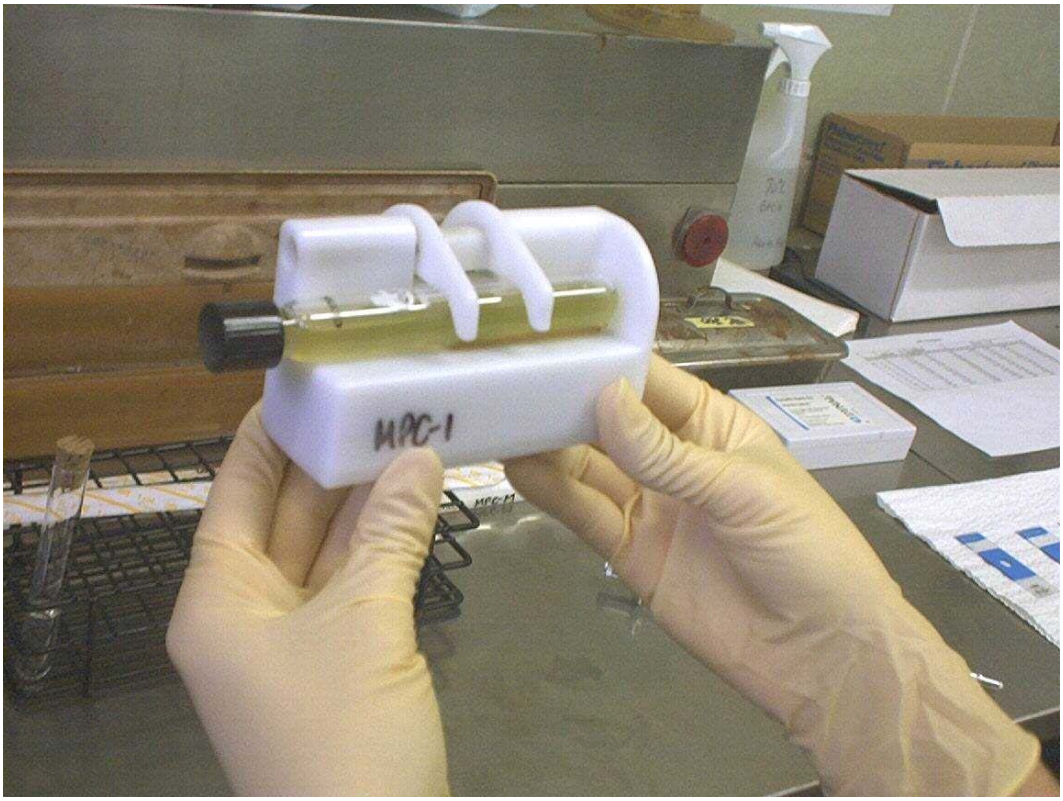

Figure 5 Immunomagnetic separation procedure: Washed beads are added to microfuge tubes in a magnetic holder for additional washes

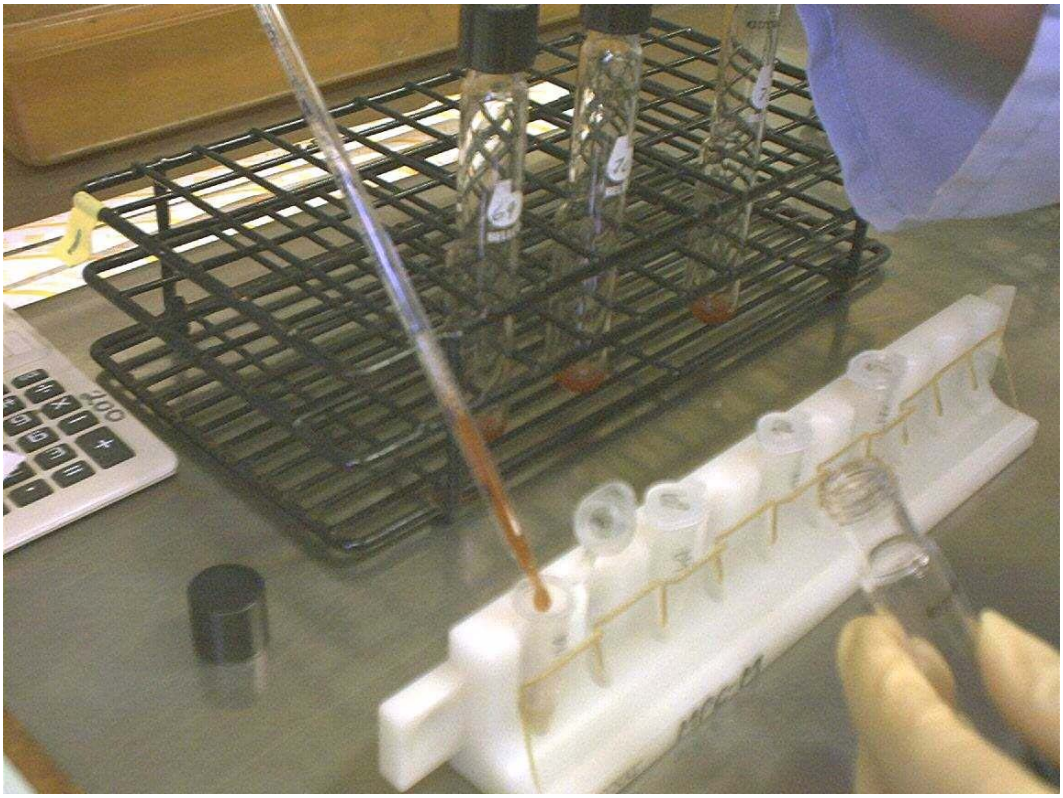

Figure 6 *Cryptosporidium* producing apple-green fluorescence with IFA (obtained from www.griffin.uga.edu)

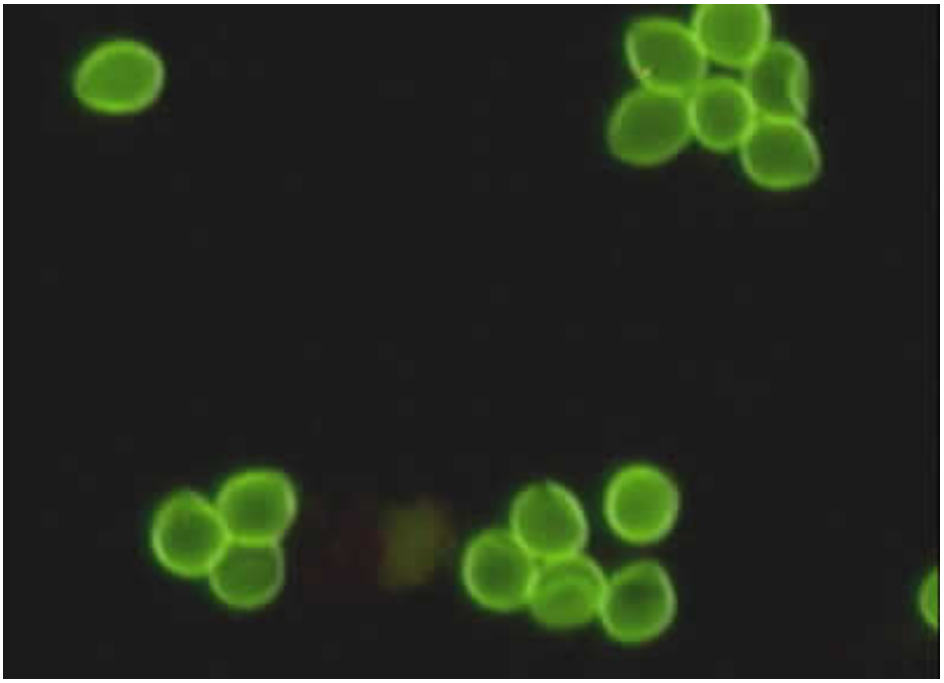

Figure 7 *Cryptosporidium* producing bright blue fluorescence with DAPI staining (obtained from www.griffin.uga.edu)

Figure 8 TaqMan template for master mix calculations and well placement of samples, spikes, controls and dilution series

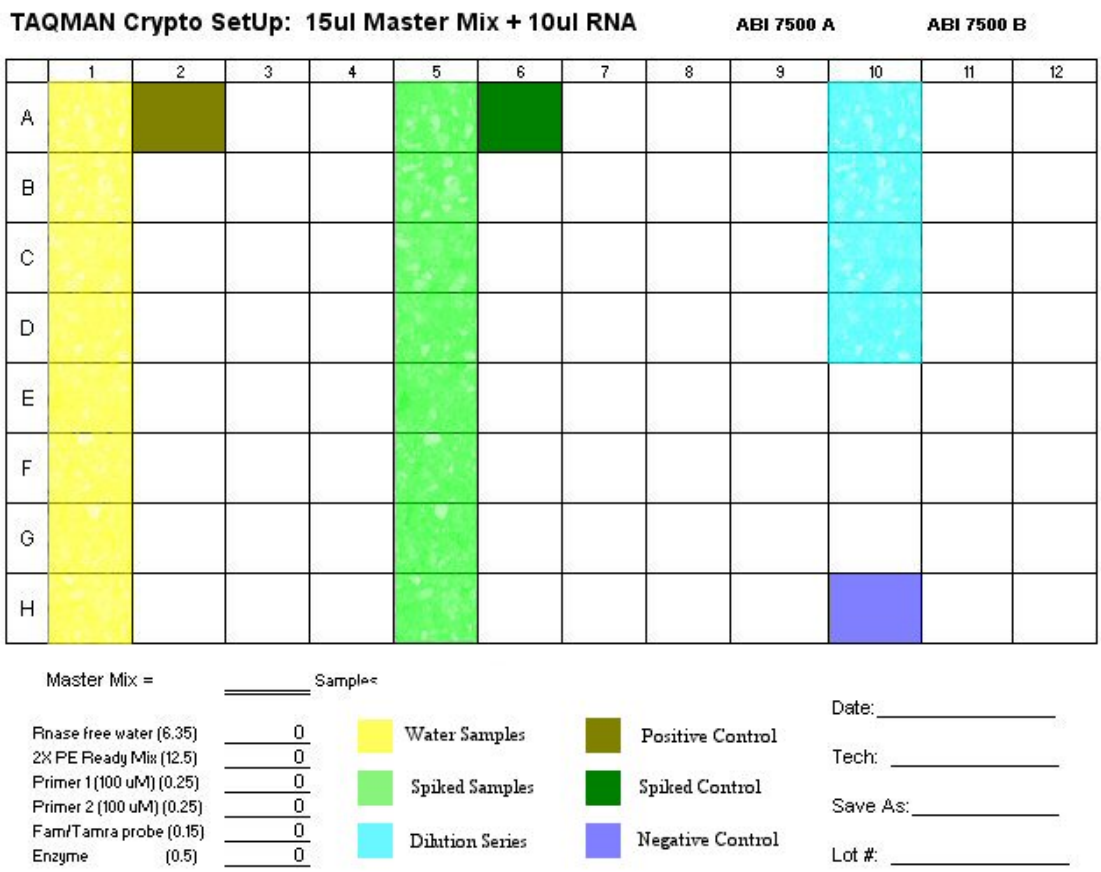

Figure 9 Real-time PCR procedure: Loading of sample into 96-well MicroAmp plate for analysis

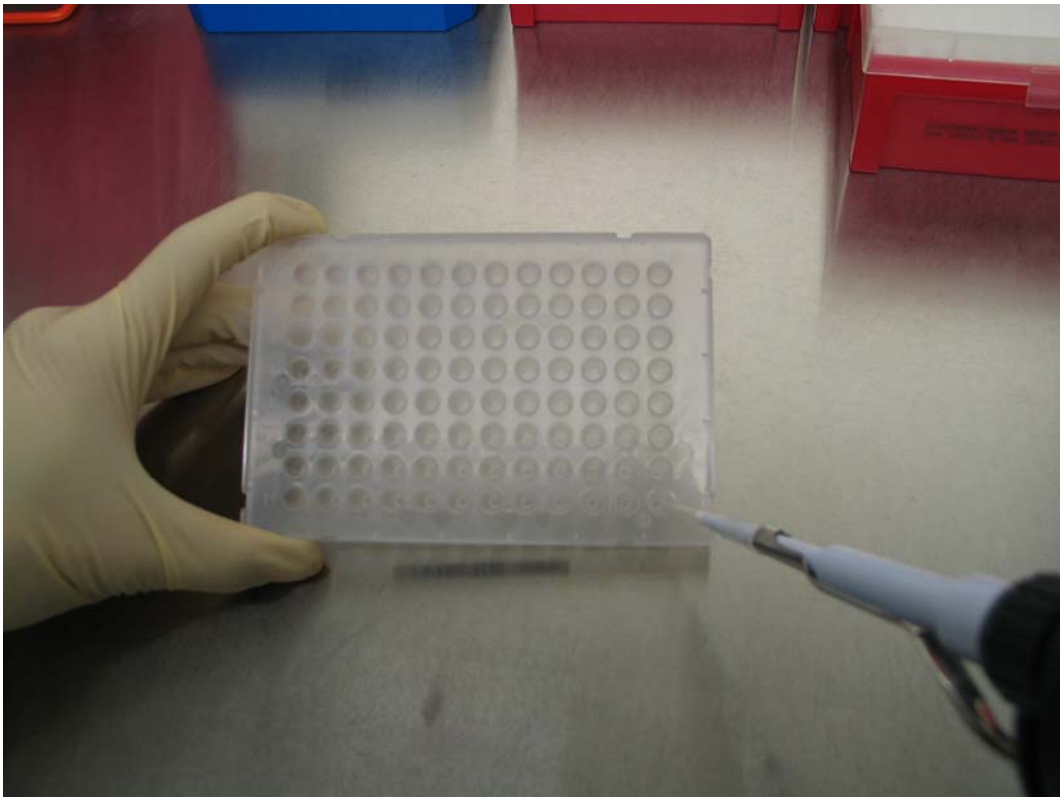

Figure 10 Real-time PCR procedure: MicroAmp 96-well plate loaded on the ABI 7500

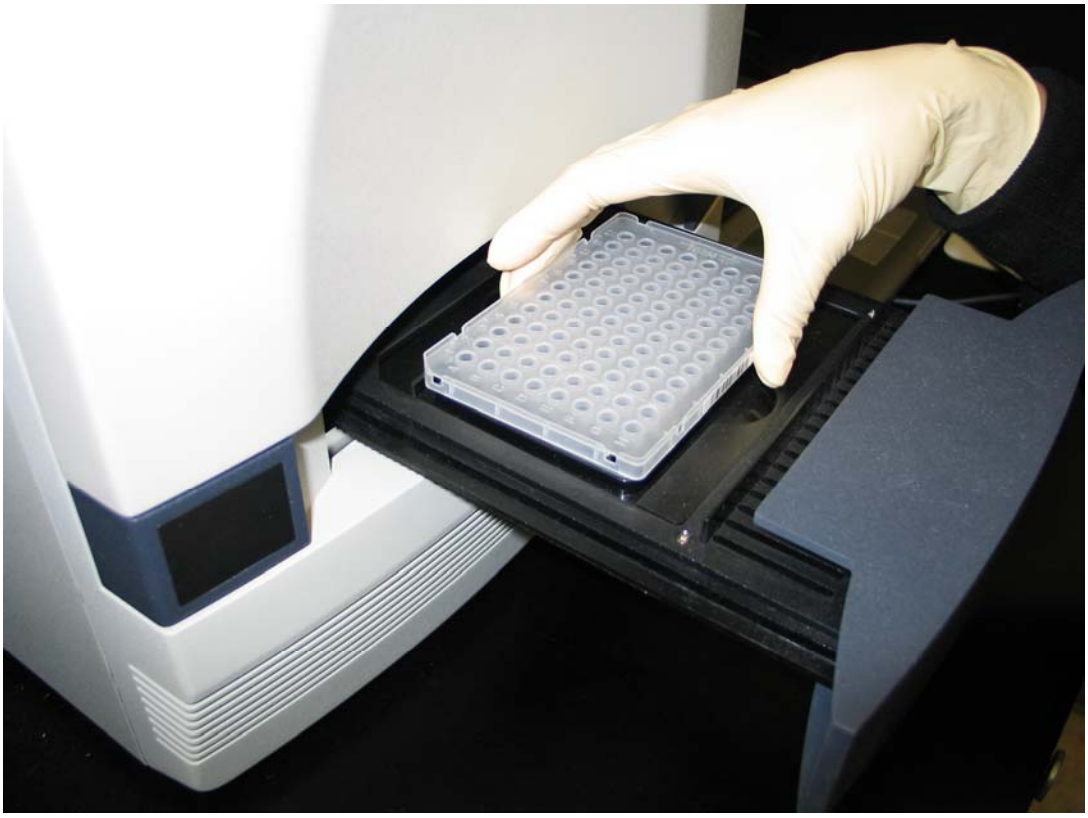

Figure 11 TaqMan analysis: Placement of the threshold in the center of the exponential phase of the curve for determination of Ct values

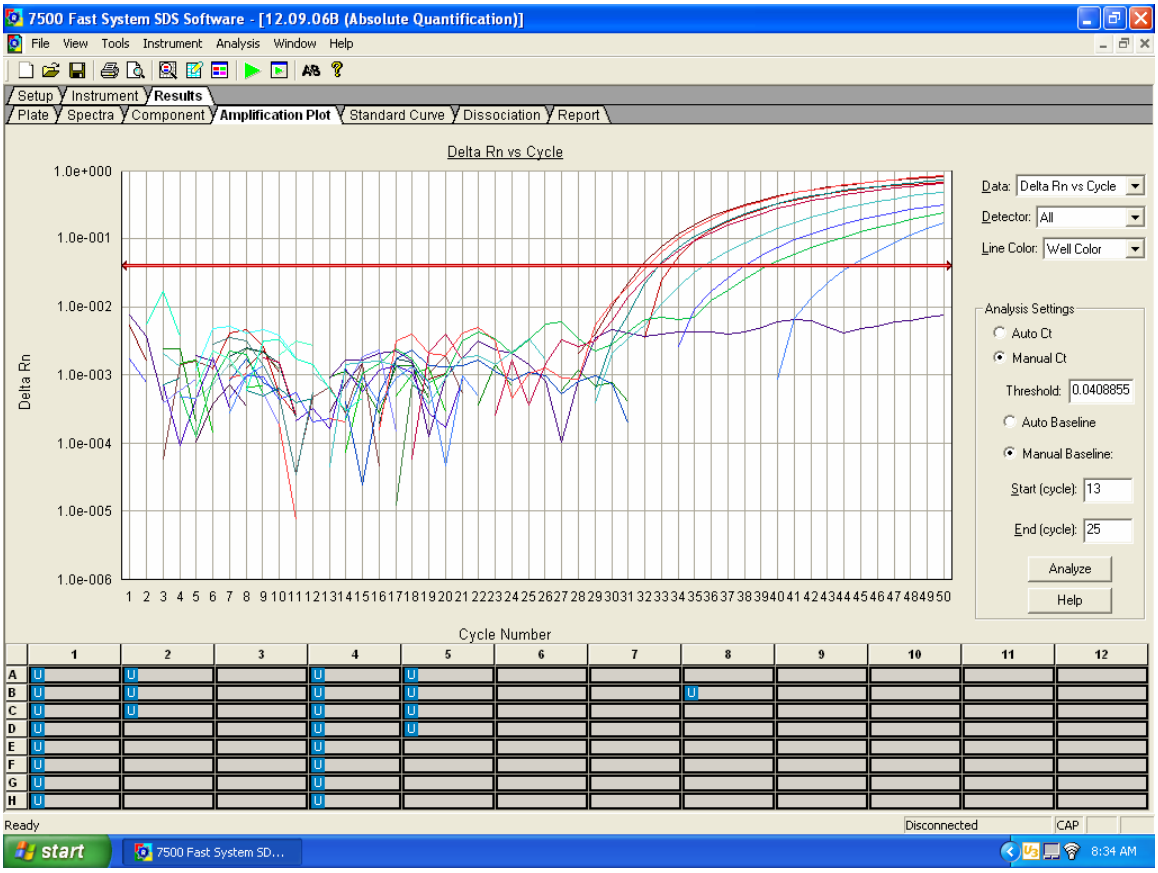

Figure 12 TaqMan analysis: Displayed Ct values after analysis of PCR results

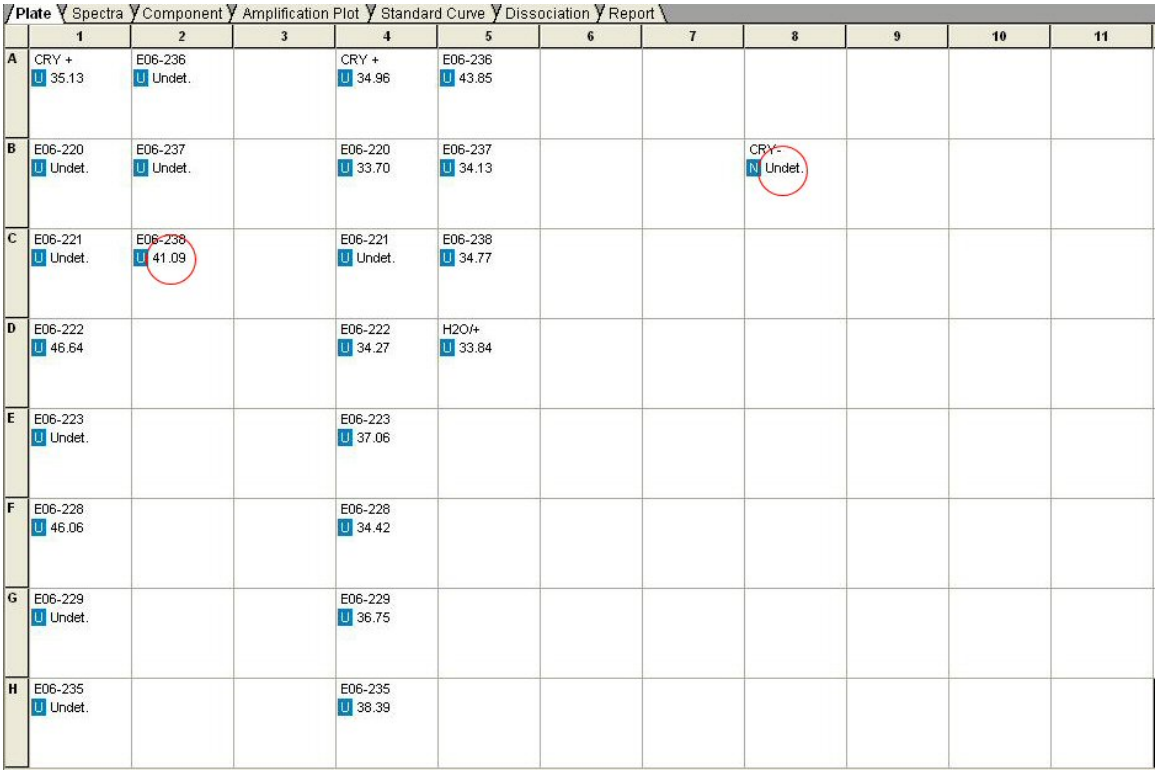

Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | Report

	1	2	3	4	5	6	7	8	9	10	11
A	CRY + U 35.13	E06-236 U Undet.		CRY + U 34.96	E06-236 U 43.85						
B	E06-220 U Undet.	E06-237 U Undet.		E06-220 U 33.70	E06-237 U 34.13			CRY- N Undet.			
C	E06-221 U Undet.	E06-238 U 41.09		E06-221 U Undet.	E06-238 U 34.77						
D	E06-222 U 46.64			E06-222 U 34.27	H2O/+ U 33.84						
E	E06-223 U Undet.			E06-223 U 37.06							
F	E06-228 U 46.06			E06-228 U 34.42							
G	E06-229 U Undet.			E06-229 U 36.75							
H	E06-235 U Undet.			E06-235 U 38.39							

Results

Real-Time PCR Standardization

A required minimum sample size of 32 was determined by use of the Cameron and Baldock sample size calculator. Due to the unpredictable schedule of sample submission by water and waste water utilities, every sample received between September and December 2006 was used in this study instead of a random sampling. This resulted in a final sample size of 40. All field samples were processed and tested for viable *Cryptosporidium parvum* oocysts using the method developed for this study.

Positive and negative controls were tested against various primer and probe concentrations ranging from 30 to 300µM for primers and 10 to 200µM for probes. Using the 3 primer and probe concentrations (60/25, 30/10, 100/25) that accurately detected positive and negative controls, multiple dilution series of positive controls were assessed to determine the best concentrations to be used in the real-time PCR protocol. This was determined by finding the concentrations of primers and probes that resulted in a correlation coefficient closest to one to illustrate perfect unity. Based on this data, a concentration of 100µM for the primers and 25µM for the probe was selected for use in this study (Figure 13).

Three ratios of master mix to DNA template were tested to determine the best ratio to use for the assay. The first test utilized a master mix volume of 20µl with 5µl of template. The second used a 10µl master mix volume and a 15µl template volume.

Amplification curves produced from these two tests were jagged and did not have a distinctive exponential phase. The third mixture tested included 15µl of master mix and 10µl of DNA template. This run generated amplification curves that were smooth with a distinctive exponential phase, indicating the 15:10 ratio of master mix and DNA template to be most suitable for use in this PCR protocol.

Initially, 45 cycles of DNA amplification were used in the PCR protocol. Results from this first run produced nicely shaped curves but the curves did not complete the exponential phase prior to the run termination. Therefore, the real-time PCR runs were assessed at 50 and 55 cycles. The 50 cycle run resulted in smooth and clearly defined exponential curves at 50 cycles, while the 55 cycle run produced results similar to those of the 45 cycle trial. Once cycling times were determined, the linear graph of the dilution series was examined to find the cycle number at which all fluorescent curves were at zero and converge. This cycle was identified as 13, and the start cycle was set to this value (Figure 14). Initial amplification occurred at 30 cycles. The end cycle which determines the end of the background noise, was set to 5 cycles before this point, or 25 (Figure 15).

Sample Classification and Determination of Inhibition

Water samples were categorized into 4 groups based on the type of water noted on the submission paperwork. Of the 40 samples collected, 21 were reclaimed water, 10 were raw (ground or surface) water, 5 were effluent, and 4 were potable water samples. These samples were analyzed using real-time PCR and further classified.

Real-time PCR assays utilizing positive control dilution series were analyzed and a standard deviation from the mean of 0.699 was calculated. The inhibition of PCR by

the sample matrix was determined in increments of 2 standard deviations. Ct values less than two standard deviations above the spiked control Ct were reported as having no inhibition. Ct values 2 to 4 standard deviations above the spiked control were reported with minor inhibition. Samples 6 or more standard deviations from the control and Ct's listed as undetected were reported with major and complete inhibition, respectively. Inhibition was detected in all water types in varying degrees except in the potable water samples (Figure 16). Effluent water samples illustrated the highest degree of inhibition at 80%, with 20% of the samples showing minor inhibition and 60% displaying complete inhibition (Figure 17). Similar results were observed in the raw water samples (70%); complete inhibition was observed in 60% of the samples and 10% produced minor inhibition (Figure 18). Reclaimed water samples produced an overall inhibition of 52%, where 28% of the samples produced complete inhibition, 5% produced major inhibition, and 19% showed minor inhibition (Figure 19). Samples tested from potable water sources were the only samples that produced no inhibition.

Statistical Analysis of Assay Results

A statistical analysis was performed on the results obtained from each real-time PCR on field samples, with triplicate runs used to account for variability. Using two-by-two tables comparing real-time PCR and DAPI results from the split IMS pellet, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of this PCR assay were determined. The real-time PCR assay had a specificity of 100% and a sensitivity of 56%, a PPV of 100% and a NPV of 73% (Table 1).

In addition to the aforementioned tests, statistical tests which study equivalency and measure the association between variables with dichotomous outcomes were performed on the data obtained from this project. Additional tests included the Kappa measure of agreement, Pearson's Correlation, and Youden's J. These were performed using diagnostic effectiveness software (Simple Interactive Statistical Analysis, 2007).

The Kappa measure of agreement determines the degree of agreement between compared tests. A value of zero is produced if there is no agreement between tests and a value of one results if the tests are in perfect agreement (i.e., they correctly predict the outcome). Values that fall between zero and one are classified by degrees of agreement (Szklo & Nieto, 2000). The Kappa value for this test was determined to be 0.59, indicating a substantial agreement between DAPI and real-time PCR.

Pearson's correlation indicates the amount of correlation between the expected Ct, based on percent DAPI positive oocysts, and mean Ct values. A value close to one indicates a good linear correlation between the values whereas values of zero indicate there is no correlation between the two values. This assay produced a Pearson's Correlation value of 0.64, indicating a positive correlation between the two tests.

The final statistical test performed on the data set was Youden's J, which determines if the results are in agreement or produced solely be chance. A value of one indicates the tests are in perfect agreement. A value of zero indicates the results of the test occurred due to chance alone (Szklo & Nieto, 2000). The real-time PCR assay had a Youden's J of 0.56 (Table 2).

Due to the high level of inhibition, a statistical analysis was repeated on all samples that did not produce inhibition to determine the overall performance of the real-time PCR assay in the absence of inhibition. The samples that produced inhibition were removed from the data set and counted as failed runs. The additional statistical analysis performed on the revised data set increased the sensitivity of the test to 89%. It also resulted in an increase in the NPV and Pearson's correlation to 90% and 0.89, respectively. The Kappa value also increased to 0.89, showing a better correlation between the two testing methods (Tables 3 & 4).

Figure 13 Comparison of correlation coefficients for positive control dilution series vs. Ct values for determination of optimum primer and probe concentrations

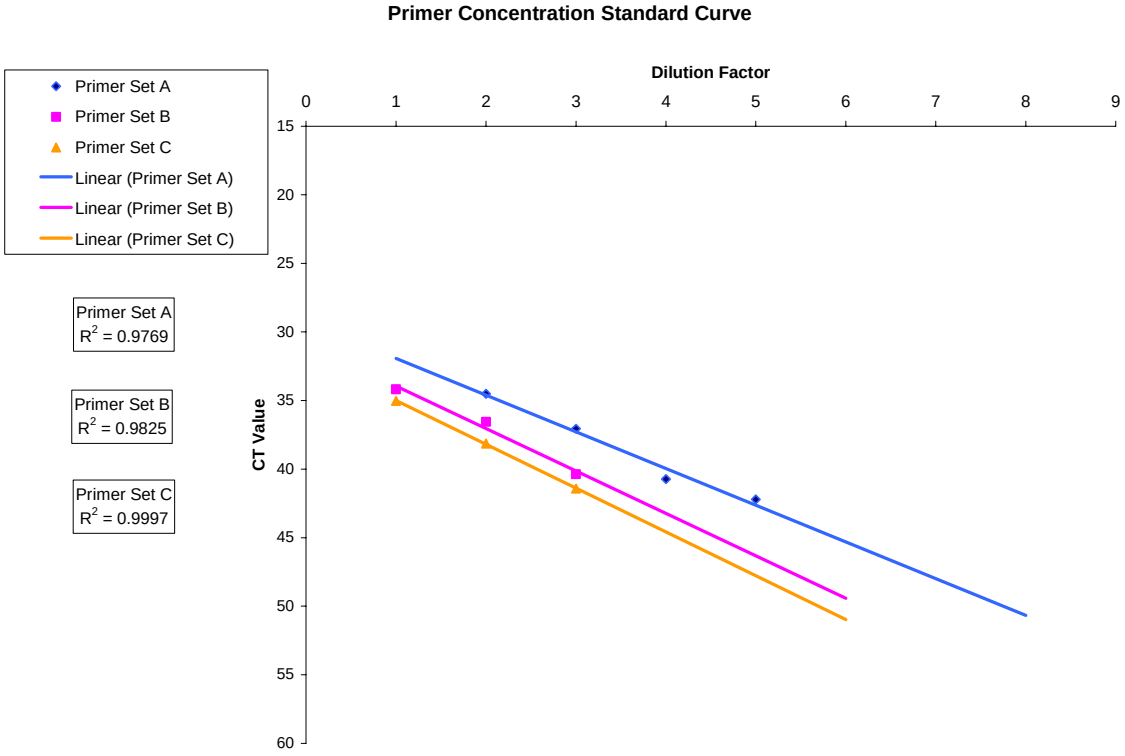

Figure 14 Screen capture illustrating the linear plot of fluorescence with all samples converging at zero for determination of the start cycle setting

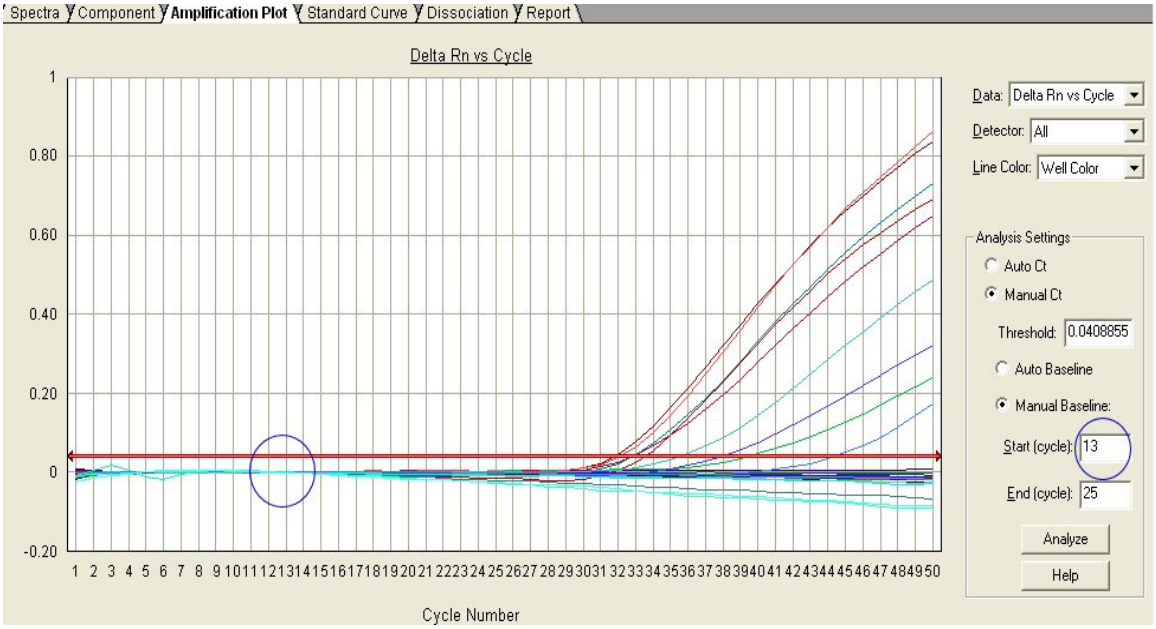

Figure 15 Screen capture illustrating the cycle at which amplification begins for determination of the end cycle setting

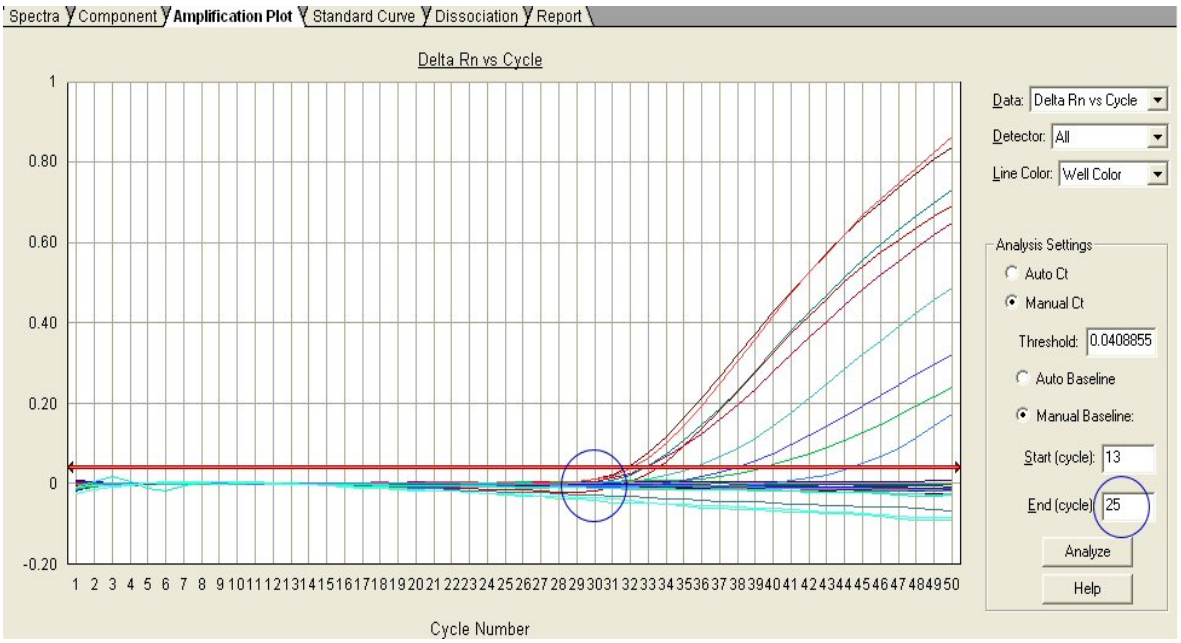

Figure 16 Percentage of samples inhibiting the real-time PCR process by matrix type

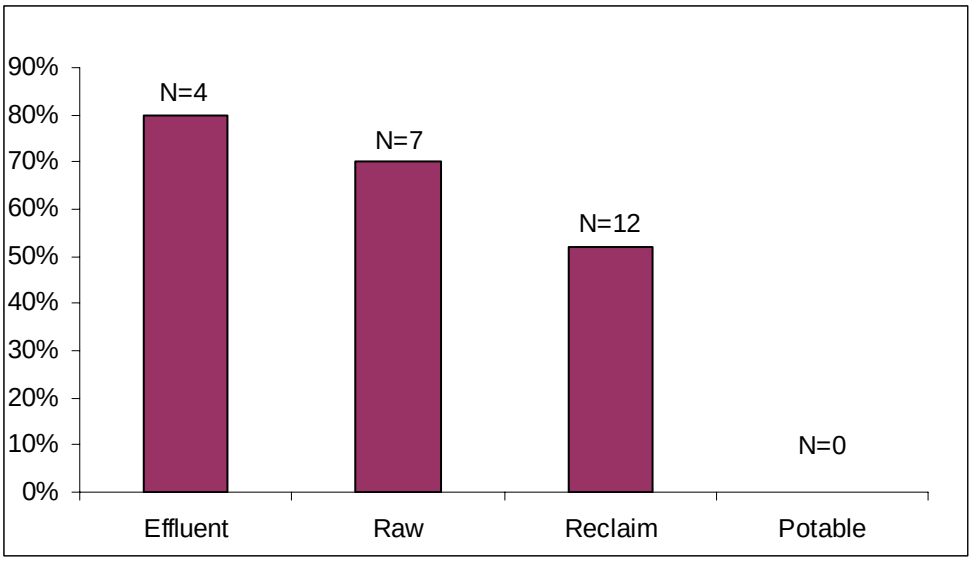

Figure 17 Percentage of effluent water samples inhibiting the real-time PCR process by degrees of inhibition

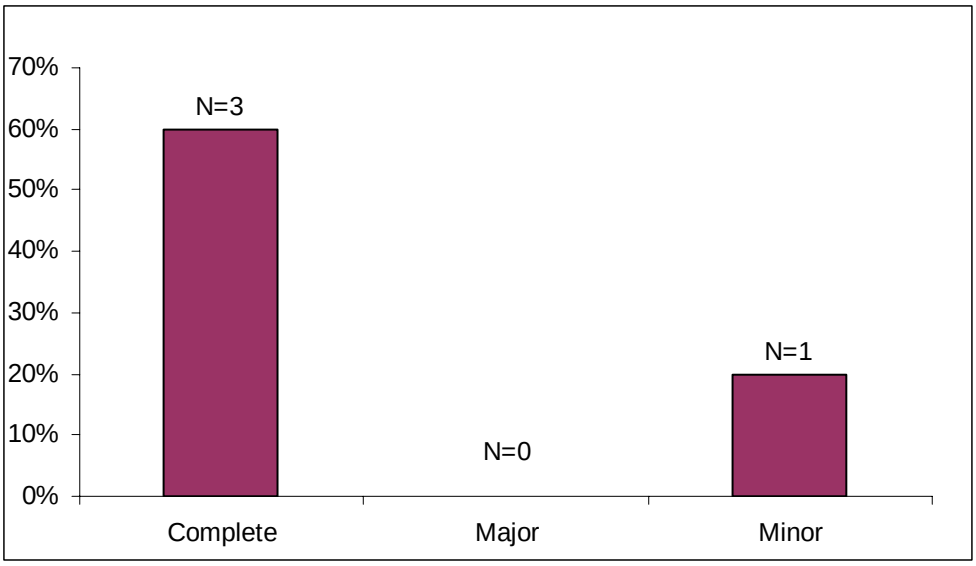

Figure 18 Percentage of raw water samples inhibiting the real-time PCR process by degrees of inhibition

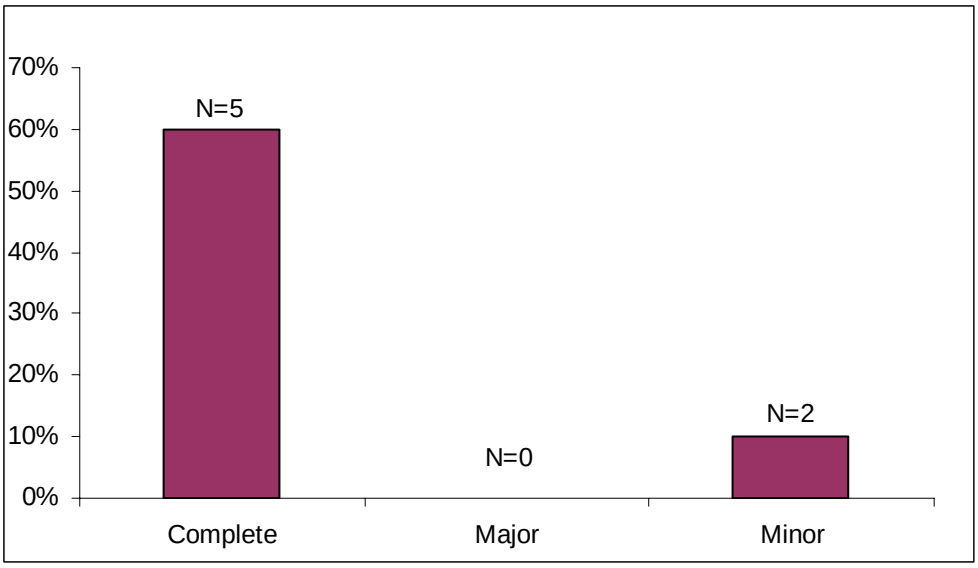

Figure 19 Percentage of reclaimed water samples inhibiting the real-time PCR process by degrees of inhibition

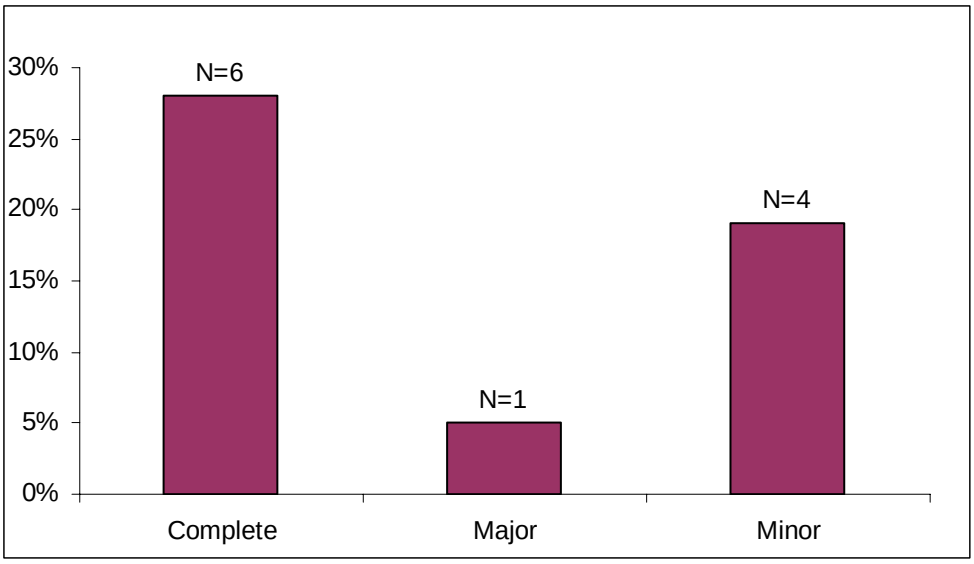

Table 1 Comparison of DAPI and Real-time PCR results for field sample data

Real-time PCR	DAPI Stain Results			
	DAPI +	**DAPI -**	**Totals**	
PCR +	9	0	9	**Sensitivity** 56% **Specificity** 100% **PPV** 100% **NPV** 73%
PCR -	9	22	31	
Totals	18	22	40	

Table 2 Correlation analysis of field sample data set comparing real-time PCR vs. DAPI

Correlation Statistical Analyses			
Assay	**Pearson's**	**Youden's J**	**Kappa**
PCR	0.64 p=0.0000	0.56	0.59

Table 3 Comparison of DAPI and Real-time PCR results for the adjusted field sample data

Real-time PCR	DAPI Stain Results			
	DAPI +	**DAPI -**	**Totals**	
PCR +	8	0	8	**Sensitivity** 89% **Specificity** 100% **PPV** 100% **NPV** 90%
PCR -	1	9	10	
Totals	9	9	18	

Table 4 Correlation analysis of adjusted field sample data set comparing real-time PCR vs. DAPI

Correlation Statistical Analyses			
Assay	**Pearson's**	**Youden's J**	**Kappa**
PCR	0.89 p=0.0001	0.89	0.89

Discussion

This study describes the development of a real-time PCR protocol for the detection of *Cryptosporidium parvum* in environmental water samples. Due to *C. parvum's* low infective dose and its ability to evade conventional water treatment, it is important to devise an assay that has the ability to reliably detect viable oocysts in a variety of water types. Testing of municipal water systems was established by the EPA to ensure the safety of the public, and Method 1623 has been successful in the detection of *Cryptosporidium* and *Giardia* in environmental water supplies. However, the method is very labor intensive and tedious to perform. Also, Method 1623 does not have the ability to distinguish between different species of *Cryptosporidium* or *Giardia*, and it does not have the ability to give an accurate estimate of viability.

A real-time PCR assay for the detection of *C. parvum* would be beneficial to facilities that regularly perform water testing. Real-time PCR has the ability to dramatically decrease the time required to obtain and report results (Figure 2). This type of assay has the ability to narrow the range of species of *Cryptosporidium* detected to *C. parvum* specifically. Therefore, it will only detect those species that are of human concern. It also has the ability to accurately estimate oocysts viability due to the use of the hsp70 DNA as a target, since hsp70 quickly degrades once the oocyst become nonviable.

Real-time PCR was shown to accurately detect *C. parvum* oocysts in cell cultures in a study by Fontaine et al. (2003). It has also been demonstrated by LeChevallier et al.

that viable *C. parvum* oocysts are detectable with high specificity in samples when using hsp70 gene as a target (2003). These two methods brought the option of real-time PCR for the detection of viable *C. parvum* oocysts to the forefront. This study built upon the results of these two studies to produce a protocol for a real-time PCR assay that detected viable *C. parvum* in environmental water samples.

The testing of various primer and probe concentrations yielded a final concentration of 100µM for the primers and 25µM for the probe used in this assay. These concentrations produced a correlation coefficient of 0.9997 illustrating an almost perfect relationship between the number of viable oocysts and the Ct values. As the number of oocysts increased in the sample the Ct values accurately and predictably decreased linearly.

The assay was further evaluated to determine the optimum master mix to DNA template ratio. The amplification plots for each ratio were compared and a 15:10 (master mix: template) ratio was determined to be best suited for this assay. Whereas other concentrations produced jagged curves and minor exponential phases that were difficult to decipher, the amplification plot for this mixture was smooth and free of excess background noise. It also produced a clear exponential phase with a distinct plateau, allowing for data analysis.

Amplification plots were also assessed to determine the number of cycles that should be used in each PCR run. Initially 45 cycles were used, resulting in plots that had a very small exponential phase and never reached a plateau. The small exponential phase did not allow for accurate analysis of the run, due to the difficulty of aligning the

threshold in the center of the phase. The number of cycles were reset to 50 resulting in a much larger exponential phase and plateau in the final cycles. This adjusted the placement of the threshold and allowed for the accurate reporting of Ct values.

The assay showed great potential for detecting viable oocysts with the successful run of multiple dilution series. The results of these assays accurately detected the presence of viable oocysts and did not report oocysts that were rendered nonviable. Assays of dilution series of viable oocysts indicated the method was able to detect low numbers of oocysts ranging from 1 to 4, making real-time PCR a useful tool for testing potable water samples, as these are required to be oocyst free.

The data generated by the 2x2 tables was used to determine the specificity, sensitivity, PPV and NPV of the PCR assay. The assay had a specificity of 100% and a NPV of 73% with field samples. This was an indication that the assay was successful at correctly identifying negative field samples and had a low tendency to produce false negative results. In other words, samples reported as negative were true negatives and did not contain viable *C. parvum* oocysts. A sensitivity of 56% and PPV of 100% were determined for the PCR assay. Though the assay was not capable of correctly identifying all samples positive for viable oocysts, the samples that were reported were true positive samples containing viable oocysts.

Correlation studies were also performed comparing the real-time PCR assay to the DAPI stained portion of the pellet. These tests included the Kappa measure of agreement, Youden's J, and Pearson's correlation. The Kappa and Youden's J values of 0.59 and 0.56, respectively, indicate there is agreement between the two testing methods

and the results are not due to chance alone. A value of 0.64 was obtained for the Pearson's correlation. This indicates the assay does show moderate correlation with the DAPI results. Though there is agreement between the testing methods and a moderate correlation in the results, the assay didn't perform well enough to be used on all water matrices.

The statistical analysis may have been influenced by the high number of samples that inhibited the real-time PCR runs. A high percentage (80%) of waste water effluent samples displayed inhibition to PCR. This caused many true positive samples to report as negative because the runs failed. This was also observed with the raw water and reclaimed water samples, having 70% and 52% inhibition, respectively. The levels of inhibition produced an interesting correlation to current water treatment. Raw water showed a level of inhibition of 70%, suggesting the inhibition was not caused by the treatment but by a impurity already in the water supply. Effluent may show a high level of inhibition due to the high level of chemical and biological agents it contains. Since effluent may be further treated and purified to produce reclaimed water, it is logical for reclaimed water to have a lower inhibition that effluent. However, reclaimed water treatment is not as rigorous as potable water treatment, since the water is not used for human consumption. This may give insight as to why reclaimed water produced an inhibition level of 52%. Since potable water has to endure a more rigorous treatment procedure, the contaminants that were in the original raw water sample have been removed producing an inhibition of 0%.

Due to the high level of inhibition, it was necessary to determine the true value of the PCR assay in the absence of inhibition. Samples that produced inhibition were removed from the original data set, and the runs were listed as failed. The total number of successful PCR runs was 18. The data was then analyzed again using only values from the runs that produced a PCR result. This produced a noticeable difference in the performance ratings of the assay. The specificity and PPV were unaffected and remained at 100%, however, the sensitivity increased to 89% and the NPV to 90% indicating a lower probability of reporting false negative results. The Pearson's correlation and Kappa values both increased to 0.89 indicating the results were not due to chance and there is a more substantial correlation between the two tests. This analysis shows the inhibition of the assay by matrix factors not removed in sample processing lead to the low proficiency of identifying positive samples and was not caused by a flaw in the PCR assay design.

Focusing in particular on the necessary materials and the "hands-on" time required for each method, that is, the amount of time a sample must be handled in some manner by the person executing the protocol, a cost comparison of Method 1623 to real-time PCR was completed. There was a marked decrease in the cost of performing real-time PCR on 8 samples as opposed to completing Method 1623. The cost associated with completion of Method 1623 on 8 samples is approximately $750.00. The EasySeed, used for positive control, and EasyStain, the fluorescent antibody stain, used in Method 1623 add substantially to the cost of Method 1623. In addition, on average a hands-on time of

9 hours is needed to complete the disassociation step, staining, to examine the slides and report the results.

In contrast, the real-time PCR assay would cost approximately $145.00 to test the same 8 samples. The major cost associated with this assay is attributed to the cost of the 96 well plates and covers; however it is offset by minimal hands-on labor of only 2 hours. The result is that the real-time PCR assay is much more cost effective than Method 1623 (Table 5).

These results further show the potential for a real-time PCR assay in the absence of inhibition. The varying levels of inhibition may give some insight into the underlying cause, however further research should be performed to determine the inhibiting factors and methods for their elimination before performing the real-time PCR assay. The assay works well with potable water samples, producing no inhibition and accurate detection of viable *C. parvum* oocysts. In addition, the ability to test multiple samples in a single run paired with lower total cost make the assay more efficient to perform.

Another important consideration in implementing a new assay is the amount of time needed to execute the test and report the results. The current Method 1623 takes approximately 9 hours to test 8 samples, from disassociation of the beads to reading the slides and reporting the results. The hands-on time required to prepare 8 samples for real-time PCR is 2 hours. Since the PCR run is completed in 3 hours, a total of 5 hours is needed to complete the assay. This cuts the time needed for reporting results almost in half. Another benefit of the PCR assay is its ability to run multiple samples without

drastically increasing the turn around time. Nevertheless, for both methods, processing of the water filter through IMS may require 4 to 6 hours to perform.

Conclusion

The monitoring of environmental water sources is important in the implementation of control measures to prevent outbreaks and protect the population from possible infection. EPA Method 1623 is a practical and effective method for the detection of *Cryptosporidium* in water samples; however it is costly to perform and requires substantial time to report results. It also does not have the ability to distinguish between various species of *Cryptosporidium* or give an accurate determination of viability.

In summary, the objectives of this study were achieved. The real-time PCR assay developed was validated with the use of positive and negative controls. The results of this assay are comparable to the DAPI results obtained using EPA Method 1623. The study determined that this real-time PCR assay may be capable of providing detection of viable *C. parvum* oocysts in potable water samples in a more cost effective manner than Method 1623. The real-time PCR assay developed in this study has the potential to be used on other types of water samples once the problem of inhibition is solved.

The rapid detection of viable C. parvum in environmental water samples by real-time PCR would allow for a more accurate determination of the risk to public health. It would allow proper authorities to issue boil water warnings and potentially decrease the risk of infection. This method may potentially be used to assess treatment methods for

use in reclaimed water. The increased use of reclaimed water in residential and recreational areas raises the possible risk of individuals to become infected with C. parvum. The use of real-time PCR may more accurately assess this risk reclaimed water imposes on the public and prevent infection. This real-time PCR assay has the potential to allow for faster detection of viable C. parvum in environmental water samples which may aid in the prevention and control of future infections.

Table 5 Cost comparison of the continuation of Method 1623 vs. the experimental real-time PCR assay for 8 sample

Continuation of Method 1623		Experimental Real-time PCR	
Item	**Cost**	**Item**	**Cost**
1.5 Microfuge Tubes	$0.50	1.5 SafeLock Tubes	$2.80
Cover slips	$2.00	1.5 Microfuge Tubes	$0.50
DAPI	$0.20	0.6 Microfuge Tubes	$0.60
Easy Seed	$440.00	96 Well MicroAmp Plate	$30.00
EasyStain	$74.40	Chelex 100 Resin	$0.40
Well Slides	$8.00	Liquid Nitrogen	$2.00
		Optical covers	$14.00
		One Step PCR Master Mix	$41.60
		Primers	$0.60
		Probe	$2.00
		C. parvum oocysts	$0.80
Labor Hours @ $25.00/Hr	$225.00	Labor Hours @ $25.00/Hr	$50.00
Total:	$750.10	Total:	$145.30

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