

# *Cryptosporidium* Genotyping Method for Regulatory Microscope Slides

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# *Cryptosporidium* Genotyping Method for Regulatory Microscope Slides

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## CONTENTS

LIST OF TABLES .....	vii
LIST OF FIGURES .....	ix
FOREWORD .....	xi
ACKNOWLEDGMENTS .....	xiii
EXECUTIVE SUMMARY .....	xv
CHAPTER 1: INTRODUCTION .....	1
Molecular Epidemiology of Cryptosporidiosis.....	1
Regulatory Monitoring.....	1
Previous <i>Cryptosporidium</i> Slide Genotyping Research .....	2
Research Needs .....	3
Research Approach .....	4
CHAPTER 2: MATERIALS AND METHODS .....	5
<i>Cryptosporidium</i> spp. Oocysts and DNA .....	5
Oocyst DNA Extraction and Purification .....	5
PCR Primers and Conditions .....	6
Slides.....	7
CHAPTER 3: RESULTS AND DISCUSSION.....	9
Oocyst Retention and Removal From Slides .....	9
Methanol Fixation and Retention of Oocysts on Slides.....	9
Recovery of Oocysts from Slides .....	10
Hsp70 and 18S Multiplex PCR .....	11
Optimization of dUTP Concentration for Sensitive Amplification While Maintaining PCR Product Carryover Protection .....	11
Initial Hsp70 and 18S Multiplex PCR Trials.....	11
PCR Technical Challenges .....	14
Specificity of the Hsp70 and 18S Multiplex PCR .....	17
High Resolution Melt (HRM) Genotyping of <i>Cryptosporidium</i> .....	17
Extraction and Purification of Oocyst DNA.....	20
Effect of ChargeSwitch Kit Elution Buffer on PCR .....	20
Initial Evaluation of Chelex/TE Freeze-Thaw Lysis With and Without ChargeSwitch Lysis Buffer and ChargeSwitch DNA Purification.....	21
Effect of TE Buffer from Chelex/TE on PCR .....	22
Comparison of Oocyst Lysis Using Chelex/MGW or MGW Only .....	22
Comparison of Chelex/MGW Oocyst Lysis With and Without Further ChargeSwitch DNA Purification .....	24
Comparison of Oocyst Lysis Using Chelex/MGW With Different Freeze-Thaw Conditions .....	25

Comparison of Oocyst DNA Preparation Using Chelex/MGW With and Without Subsequent DNA Purification Using the ChargeSwitch Kit or Qiagen Kit With Carrier DNA.....	28
Evaluation of Matrix Effect on PCR Using Pooled Scrapings From <i>Cryptosporidium</i> -Negative Method 1622 Field Slides .....	29
Spin Filtration Removal of Chelex From DNA Extracts.....	32
Method Evaluation Using Matrix-Free and Seeded Field Slides.....	33
Removal of Mounting Medium from Slides.....	33
Adverse Effects of Slide Mounting Media Containing Formalin on PCR Amplification .....	33
Method Evaluation Using Seeded Method 1623 Field Slides .....	35
Method Evaluation Using Seeded UK DWI Field Slides.....	36
<b>CHAPTER 4: SUMMARY AND CONCLUSIONS.....</b>	<b>39</b>
Conclusions.....	39
Recommendations.....	40
<b>APPENDIX A: LIST OF MATERIALS AND SUPPLIES.....</b>	<b>41</b>
<b>APPENDIX B: REAGENT PREPARATION.....</b>	<b>43</b>
<b>APPENDIX C: DETAILED SAMPLE PROCESSING PROTOCOL.....</b>	<b>47</b>
<b>REFERENCES .....</b>	<b>51</b>
<b>ABBREVIATIONS .....</b>	<b>55</b>

## TABLES

2.1	<i>Cryptosporidium</i> species and genotypes used in this study.....	6
3.1	Comparison of oocyst lysis using Chelex/TE freeze-thawing or Chelex/TE/lysis buffer freeze-thawing plus ChargeSwitch DNA purification .....	21
3.2	Effect of Carryover TE Buffer from the Chelex/TE lysis method on PCR .....	22
3.3	Comparison of oocyst freeze-thaw lysis with Chelex/MGW or MGW only.....	23
3.4	Comparison of oocyst freeze-thaw lysis with Chelex/MGW with and without further ChargeSwitch DNA purification .....	24
3.5	Effect of slide mounting media on PCR detection of matrix-free slides seeded with single flow-sorted <i>C. parvum</i> oocysts.....	34
3.6	Method evaluation using <i>Cryptosporidium</i> -negative Method 1623 field slides seeded with single flow-sorted oocysts .....	36
3.7	Method evaluation using <i>Cryptosporidium</i> -negative UK DWI field slides seeded with single flow-sorted <i>C. parvum</i> oocysts.....	38



## FIGURES

3.1	Photomicrographs of slide wells after removing oocysts by scraping with a bacteriological loop or closed cell foam swab .....	10
3.2	Amplification plot for <i>C. parvum</i> and <i>C. hominis</i> simplex and multiplex hsp70 and 18S PCR.....	12
3.3	SYBR Green 1°C step melt curves for <i>C. parvum</i> and <i>C. hominis</i> simplex and multiplex hsp70 and 18S PCR.....	12
3.4	SYBR Green 0.1°C step high resolution melt curves for <i>C. parvum</i> and <i>C. hominis</i> multiplex hsp70 and 18S PCR.....	13
3.5	Gel electrophoresis detection of <i>C. parvum</i> and <i>C. hominis</i> simplex and multiplex hsp70 and 18S PCR amplicons .....	13
3.6	Lot-to-lot variability of Applied Biosystems magnesium chloride PCR reagent .....	14
3.7	Lot-to-lot variability in fluorescence observed for Applied Biosystems SYBR Green PCR buffers .....	15
3.8	Differences in the resolution of real-time PCR melt curves and quality of PCR products due to the use of different PCR platforms.....	16
3.9	Specificity of the hsp70 and 18S multiplex PCR for different human-pathogenic and animal-associated <i>Cryptosporidium</i> genotypes.....	17
3.10	Differentiation of <i>Cryptosporidium</i> genotypes within the same melt peak bin using HRM genotyping.....	18
3.11	Flow diagram for interpretation of RotorGene HRM genotyping data .....	19
3.12	Simplex 18S PCR amplification plot for <i>C. parvum</i> Iowa, <i>C. parvum</i> Iowa (CpAZ), and <i>C. muris</i> oocysts .....	25
3.13	High resolution melt curves of simplex 18S PCR amplicons from <i>C. parvum</i> Iowa, <i>C. parvum</i> Iowa (CpAZ), and <i>C. muris</i> oocysts.....	26
3.14	Simplex 18S PCR amplification plot for <i>C. parvum</i> Iowa and <i>C. hominis</i> oocysts .....	27
3.15	High resolution melt curve of simplex 18S PCR amplicons from <i>C. parvum</i> Iowa and <i>C. hominis</i> oocysts.....	27

3.16	Direct use of Chelex/MGW oocyst DNA as PCR template compared to additional DNA purification using the ChargeSwitch kit or Qiagen kit with carrier DNA .....	29
3.17	Pooled scrapings/washes from <i>Cryptosporidium</i> -negative Method 1622 field slides used to evaluate matrix effects on PCR and the need for DNA purification .....	30
3.18	Effects of pooled scrapings/washes from <i>Cryptosporidium</i> -negative Method 1622 field slides on PCR .....	31
3.19	Gel electrophoresis analysis of simplex 18S PCR products from Chelex/MGW oocyst lysate samples with and without pooled Method 1622 field matrix.....	31
3.20	Spin filter removal of Chelex resin used in the oocyst DNA extraction procedure and foam used to scrape slides.....	32
3.21	Gel electrophoresis analysis of 18S and hsp70 PCR products from <i>C. parvum</i> seeded microscope slides stained with or without BTF Fixing Buffer containing formalin.....	34

## **FOREWORD**

The Water Research Foundation (Foundation) is a nonprofit corporation that is dedicated to the implementation of a research effort to help utilities respond to regulatory requirements and traditional high-priority concerns of the industry. The research agenda is developed through a process of consultation with subscribers and drinking water professionals. Under the umbrella of a Strategic Research Plan, the Research Advisory Council prioritizes the suggested projects based upon current and future needs, applicability, and past work; the recommendations are forwarded to the Board of Trustees for final selection. The Foundation also sponsors research projects through the unsolicited proposal process; the Collaborative Research, Research Applications, and Tailored Collaboration programs; and various joint research efforts with organizations such as the U.S. Environmental Protection Agency, the U.S. Bureau of Reclamation, and the Association of California Water Agencies.

This publication is a result of one of these sponsored studies, and it is hoped that its findings will be applied in communities throughout the world. The following report serves not only as a means of communicating the results of the water industry's centralized research program but also as a tool to enlist the further support of the nonmember utilities and individuals.

Projects are managed closely from their inception to the final report by the Foundation's staff and large cadre of volunteers who willingly contribute their time and expertise. The Foundation serves a planning and management function and awards contracts to other institutions such as water utilities, universities, and engineering firms. The funding for this research effort comes primarily from the Subscription Program, through which water utilities subscribe to the research program and make an annual payment proportionate to the volume of water they deliver and consultants and manufacturers subscribe based on their annual billings. The program offers a cost-effective and fair method for funding research in the public interest.

A broad spectrum of water supply issues is addressed by the Foundation's research agenda: resources, treatment and operations, distribution and storage, water quality and analysis, toxicology, economics, and management. The ultimate purpose of the coordinated effort is to assist water suppliers to provide the highest possible quality of water economically and reliably. The true benefits are realized when the results are implemented at the utility level. The Foundation's trustees are pleased to offer this publication as a contribution toward that end.

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## EXECUTIVE SUMMARY

### OBJECTIVES

The overall project objective was to develop a simple, reliable, and cost-effective method for the genotyping of *Cryptosporidium* from US Environmental Protection Agency (USEPA) Method 1622/23 and United Kingdom Drinking Water Inspectorate (UK DWI) slides that can be readily adopted by the typical utility and water quality testing laboratory that has little or no molecular biology experience. Specific objectives were to: optimize the removal of *Cryptosporidium* oocysts from slides; optimize DNA extraction and purification from low numbers of oocysts; develop and evaluate a single-round multiplex PCR targeting the *Cryptosporidium* genes for 18S ribosomal RNA (18S) and heat shock protein 70 (hsp70) for differentiating human-pathogenic and animal-associated *Cryptosporidium*; perform preliminary method evaluation using matrix-free and field matrix slides seeded with single flow cytometry sorted oocysts; and develop a protocol and demonstration DVD for technology transfer to water quality laboratories.

### BACKGROUND

*Cryptosporidiosis* is a significant diarrheal illness in both healthy and immunocompromised humans, and waterborne outbreaks of cryptosporidiosis continue to occur worldwide. The genus *Cryptosporidium* has at least 20 different recognized species and approximately 50 genotypes based on oocyst morphology (size and shape) and infection site (i.e. intestines or stomach), preferential host and genetic/genome analysis (Feng, Dearen et al. 2009). Research studies and regulatory monitoring programs have determined that *Cryptosporidium* oocysts are found in most surface waters, and may also be found in finished drinking water. Oocysts found in water may originate from a variety of sources, including human sewage and wastewater treatment plant effluent, livestock, and wild animals.

Research using polymerase chain reaction (PCR) techniques has greatly enhanced our knowledge of the species and genotypes of *Cryptosporidium* oocysts found in surface waters. These studies have shown that oocysts of wildlife- and livestock-associated *Cryptosporidium* species and genotypes are frequently found in surface water. Subsequently, important issues have been raised with regard to *Cryptosporidium* risk assessment and the development of watershed management strategies. Of major concern for *Cryptosporidium* risk assessment is the fact that only *C. hominis*, *C. parvum*, and *C. meleagridis* appear to readily infect immunocompetent healthy humans, and animal-related *Cryptosporidium* may pose little or no human health risk. From a watershed management perspective, different strategies are needed to control human, wildlife, and livestock sources of oocysts.

The current Method 1622/23 and the UK DWI regulatory methods are microscopy-based. While these methods are capable of enumerating low levels of oocysts, they do not determine the species or genotypes of the *Cryptosporidium* detected. Researchers have attempted to address this shortcoming by developing PCR methods for genotyping oocysts directly recovered from water samples or from microscope slides. While valuable information can be obtained by directly genotyping oocysts from water samples, simultaneous and accurate quantitation of

oocysts is not possible with the current PCR methods. Therefore, methods focused on genotyping oocysts recovered from regulatory slides are the most useful since the overriding regulatory requirement of oocyst quantitation is satisfied.

Several research groups have developed methods for genotyping *Cryptosporidium* oocysts recovered from regulatory slides. However, most of these methods are not practical for water utility and water quality laboratories since they rely on complicated and cumbersome multi-step nested PCR restriction fragment length polymorphism (RFLP) protocols. At present, there are no regulatory PCR-based water quality tests, and consequently the majority of utility and water quality laboratories have limited or no molecular experience. Nested PCR is prone to intra-lab contamination with PCR product, and the extensive manipulation of PCR products for RFLP analysis increases this risk. Therefore, the current project was undertaken to develop a slide genotyping method that can readily be used by water quality labs to distinguish human-pathogenic (i.e. *C. hominis*, *C. parvum*, and *C. meleagridis*) from animal-associated *Cryptosporidium* oocysts.

## APPROACH

Research addressed the strengths and weaknesses of previously developed slide genotyping methods to develop a user-friendly method for routine use by water quality laboratories. It is also important to note that research focused on genotyping single oocysts rather than mixtures of different genotypes since the majority of positive regulatory field slides have only 1 to 3 oocysts present. Flow cytometry was used to precisely sort oocysts directly into microcentrifuge tubes or onto slides, with most experiments using single oocysts. The majority of trials were performed using human-pathogenic *C. parvum* and animal-associated *C. muris* oocysts.

Bacteriological loops and closed cell foam swabs were compared for the removal of oocysts from slides. Microscopic examination was used to verify oocyst removal and evaluate uniformity of scraping. Several oocyst DNA extraction methods were compared, including freeze-thaw lysis of oocysts in different buffers, with or without additional DNA purification. Lysis buffers evaluated included: Chelex 100 resin in Tris EDTA (Chelex/TE) buffer; Chelex 100 resin in molecular grade water (Chelex/MGW); and Chelex/TE combined with a commercial lysis buffer. Three different DNA purification methods were compared, including the direct use of Chelex/MGW freeze-thaw oocyst DNA alone or with additional purification using the Invitrogen ChargeSwitch forensic DNA kit or Qiagen DNA kit purification. Development and evaluation of the single-round 18S and hsp70 multiplex PCR method for differentiating human-pathogenic and animal-associated *Cryptosporidium* included: incorporation of PCR product carryover prevention reagents to prevent false positives (critical for laboratories new to molecular biology and PCR); specificity testing using different *Cryptosporidium* species; and standard and real-time PCR with high resolution melt (HRM) analysis. Preliminary method evaluation was performed using matrix-free and Method 1623 and UK DWI field matrix slides seeded with single flow cytometry sorted oocysts.

## RESULTS AND CONCLUSIONS

Both bacteriological loops and closed cell foam swabs were very effective (95 - 100%) in removing oocysts from slides. However, microscopic examination of slides scraped with loops

showed areas which were not scraped, while slides scraped with closed cell foam swabs showed uniform and complete scraping. Therefore, the use of closed cell foam swabs for scraping slides was adopted. A water wash of the slide well was incorporated into the slide processing protocol for the removal of residual mounting medium that may interfere with the PCR. It was also discovered that some brands of mounting media contain formalin which have a significant adverse effect on the PCR. Our standard Chelex/MGW freeze-thaw procedure was shown to be superior to the other lysis and DNA extraction methods based on qPCR analysis of flow-sorted oocyst samples with and without pooled Method 1623 field matrix.

The use of PCR product carryover prevention chemistry was successfully incorporated into the PCR protocol. Multiplex PCR experiments with conventional gel electrophoresis detection and real-time PCR with high resolution melt (HRM) analysis indicated that human-pathogenic *C. parvum*, *C. hominis* and *C. meleagridis* could readily be distinguished from animal associated *Cryptosporidium* spp. based on the presence of the hsp70 amplicon and/or melt analysis of the 18S amplicon. Further, most of the individual species could be resolved using HRM analysis. However, only the 18S target amplified for some *C. parvum* samples, and these samples were therefore falsely negative for the hsp70 target (approx. 15% of samples). Hsp70 false negatives were not an issue for real-time PCR analysis, since the melt curves of the 18S products for these samples correctly identified each as *C. parvum*.

Results from multiple experiments using single flow-sorted *C. parvum* or *C. muris* oocysts seeded onto matrix-free slides indicated the method had an approximate 70% positive detection rate. Preliminary method performance was also evaluated using Method 1623 and UK DWI field slides from diverse water sources seeded directly with single flow-sorted *C. parvum* or *C. muris* oocysts. For Method 1623 field slides, 9 of 10 and 15 of 20 slides seeded with a single *C. parvum* or *C. muris* oocyst, respectively, tested positive by PCR. Method performance with UK DWI field slides was similar, with 9 of 10 slides seeded with single flow-sorted *C. parvum* oocysts testing positive by PCR. Therefore, an overall 83% positive detection rate was obtained for Method 1623 and UK DWI field slides seeded with single oocysts. The specific conclusions from this project were:

1. A streamlined method for the genotyping of single *Cryptosporidium* oocysts from USEPA Method 1622/23 and UK DWI slides was developed. The method protocol is included as an appendix to this report.
2. Some slide mounting media contain formalin which has a significant adverse effect on PCR. Therefore, only mounting media that do not contain formalin (e.g. Method 1622/23, Waterborne without formalin, and Cellabs media) are recommended if slide genotyping will be performed.
3. The single-round multiplex hsp70 and 18S PCR protocol allows the genotype differentiation of human-pathogenic *C. hominis*, *C. parvum*, and *C. meleagridis* from animal-associated *Cryptosporidium* genotypes (i.e. identification as human-pathogenic or animal-associated).
4. The developed method may be used with conventional and real-time PCR instruments with high resolution melt (HRM) analysis capabilities, allowing for flexibility in infrastructure investment.
5. HRM analysis allows further discrimination of species and genotypes.

## **APPLICATIONS AND RECOMMENDATIONS**

The developed method represents a critical first step for water utility and water quality laboratories wishing to gain added value from *Cryptosporidium* regulatory monitoring. Under the current USEPA Long-Term 2 Enhanced Surface Water Treatment Rule (LT2), public water suppliers serving populations over 10,000 are required to monitor for *Cryptosporidium* in their source water using USEPA Method 1622/23. The first round of LT2 monitoring was completed in 2008, and another round of monitoring is required to begin in 2015. There is no requirement for archival of LT2 slides, although many utilities and commercial water quality labs have saved their slides from the first round of monitoring. Current UK regulatory efforts mostly focus on monitoring finished drinking water, but some source waters are also being monitored. In the UK *Cryptosporidium*-positive slides must be archived for one year. Therefore, PCR analysis of archived slides and slides from upcoming monitoring present excellent opportunities to obtain valuable information on the occurrence of *Cryptosporidium* genotypes in waters with only a modest amount of additional effort and cost. Identifying *Cryptosporidium* genotypes present in water will allow a more accurate assessment of the public health significance of waterborne *Cryptosporidium*, provide data for sound treatment plant infrastructure decisions, and aid the development of effective watershed management plans. Recommendations include the following:

1. Hsp70 false negatives may occasionally occur. Therefore, when using conventional PCR, samples which yield only the 18S PCR product should be confirmed using high resolution melt (HRM) analysis. Post-PCR HRM analysis of products generated by conventional PCR is straightforward and may be performed by contract laboratories. This is important in accurate determination of the presence or absence of human-pathogenic *Cryptosporidium*.
2. In this study, preliminary results using Method 1623 and DWI field slides were promising. However, additional evaluation of the method is needed, including technology transfer to end-users. Further method evaluation is currently being performed under Water Research Foundation Project 4284, which includes a technology transfer workshop and international round robin.
3. Development of additional PCR genotyping methods should continue so that a toolbox of analytical options is available to meet the specific needs of the water industry and regulatory agencies. Additional research in this area is currently being performed under Water Research Foundation Project 4179.

## **MULTIMEDIA**

In addition to the method protocol included as an appendix, this report includes a method demonstration DVD. The DVD includes demonstration of oocyst removal from slides, DNA extraction, and pre-PCR setup.

## CHAPTER 1: INTRODUCTION

### MOLECULAR EPIDEMIOLOGY OF CRYPTOSPORIDIOSIS

Cryptosporidiosis continues to be a significant diarrheal illness in both healthy and immunocompromised humans, as well as agriculturally important livestock species worldwide. The dollar amount spent on lost wages, sick time, medical treatments, in addition to investments in infrastructure to prevent transmission of the highly environmental resistant oocysts is estimated in the tens of millions each year. *Cryptosporidium* species infect a diverse group of vertebrate hosts including amphibians, birds, fish, mammals, and reptiles. Currently, at least 20 different *Cryptosporidium* species and approximately 50 genotypes are recognized based on oocyst morphology (size and shape) and infection site (i.e. intestines or stomach), preferential host, and genetic/genome analysis (Feng, Dearen et al. 2009). This includes some exotic species, such as *C. wrairi* found in guinea pigs (Vetterling, Jervis et al. 1971), and *C. scophthalmi* in turbot (Alvarez-Pellitero, Quiroga et al. 2004).

It appears that only *C. hominis*, *C. parvum*, and *C. meleagridis* readily infect immunocompetent humans in all age groups (McLauchlin, Amar et al. 2000; Leoni, Amar et al. 2006). Leoni et al. (Leoni, Amar et al. 2006) genotyped 2414 human clinical specimens obtained between 1985 and 2000 and found *C. parvum* and *C. hominis* in 99% of samples. The remaining samples were identified as containing *C. meleagridis* (0.9 %), *C. felis* (0.2 %), *C. andersoni* (0.1 %), *C. canis* (0.04 %), *C. suis* (0.04 %) and the *Cryptosporidium* cervine genotype (0.04 %). Similarly, Chalmers et al. (Chalmers, Elwin et al. 2009) genotyped 7,758 human clinical specimens obtained between 2000 and 2003, and 96% were identified as *C. parvum* and/or *C. hominis*. The remaining 1% of typeable samples was identified as *C. meleagridis* (0.7%), *C. felis* (0.05%), cervine genotype (0.05%), *C. canis* (0.01%), horse genotype (0.01%), and skunk genotype (0.01%). There have also been two documented cases of cryptosporidiosis due to the W17 chipmunk genotype (Feltus, Giddings et al. 2006). With one recent exception, only *C. parvum* and *C. hominis* have been identified as causes of waterborne and foodborne outbreaks of cryptosporidiosis (Xiao and Ryan 2008). The only exception is the 2008 outbreak in Northamptonshire, England, which was caused by the rabbit genotype (Chalmers, Robinson et al. 2009). It is noteworthy that multilocus genotyping of the rabbit genotype indicated that it is very closely related to *C. hominis*, as opposed to typical animal-associated genotypes (Xiao, Sulaiman et al. 2002).

With this knowledge, it is reasonable that detection methodologies that differentiate the three *Cryptosporidium* species (*C. hominis*, *C. parvum* and *C. meleagridis*) causing the vast majority of cryptosporidiosis in immunocompetent humans from those typically animal-related genotypes may meet the needs for evaluating health risk of waterborne *Cryptosporidium* and for watershed management.

### REGULATORY MONITORING

Under the current USEPA Long-Term 2 Enhanced Surface Water Treatment Rule (LT2), public water suppliers serving populations over 10,000 are required to monitor for

*Cryptosporidium* in their source water using USEPA Method 1622/23. The first round of LT2 monitoring was completed in 2008, and another round of monitoring is required to begin in 2015. There is no requirement for archival of LT2 slides, although many utilities and commercial water quality labs have saved their slides from the first round of monitoring. Current *Cryptosporidium* regulatory efforts in the UK mostly focus on monitoring finished drinking water, but high risk source waters are also being monitored. In the UK, *Cryptosporidium*-positive slides must be archived from three months (England and Wales) to one year (Scotland).

USEPA Method 1622/23 for the detection of *Cryptosporidium* in water (USEPA 2005) has greater recovery efficiency and precision compared to its predecessor, the ICR Method. Nevertheless, Method 1622/23 still relies on immunofluorescent assay microscopy to detect oocysts, and therefore has significant shortcomings. In particular, Method 1622/23 is not capable of determining the species or genotype of the *Cryptosporidium* detected. The UK DWI method (UK Drinking Water Inspectorate 2005) is very similar to Method 1622/23, and therefore shares this weakness. This is a significant limitation when gauging the public health risk posed by waterborne *Cryptosporidium*. However, a strength of these microscopy-based methods is that they are capable of enumerating the typically low levels (1 to 3) of oocysts found in water samples.

## PREVIOUS CRYPTOSPORIDIUM SLIDE GENOTYPING RESEARCH

Researchers have attempted to address the shortcoming of microscopy-based detection of *Cryptosporidium* by developing PCR methods for genotyping oocysts directly recovered from water samples or from microscope slides. While valuable information can be obtained by directly genotyping oocysts from water samples, simultaneous and accurate quantitation of oocysts is not possible with the current PCR methods. Therefore, methods focused on genotyping oocysts recovered from regulatory slides are the most useful since the overriding regulatory requirement of oocyst quantitation is satisfied.

Several groups of investigators have described PCR protocols for the speciation and genotyping of *Cryptosporidium* oocysts from clinical and water quality monitoring slides. Amar et al. (Amar, Pedraza-Diaz et al. 2001) described the *Cryptosporidium* oocyst wall protein gene (COWP) PCR restriction fragment length polymorphism (RFLP) genotyping of *Cryptosporidium* oocysts from human clinical specimen slides. While the method was able to correctly identify the genotype of *Cryptosporidium* present in 85% of the samples tested, the authors reported that it had poor sensitivity. The sensitivity of this protocol is not likely sufficient for application to environmental water samples which typically contain very low numbers of oocysts.

PCR-RFLP genotyping of *Cryptosporidium* oocysts on water quality slides has also been investigated by a number of groups (Ruecker, Bounsbath et al. 2005; Nichols, Campbell et al. 2006; Sunnotel, Snelling et al. 2006; Ruecker, Braithwaite et al. 2007). Sunnotel et al. (Sunnotel, Snelling et al. 2006) described a strategy for the removal of oocysts from USEPA Method 1622/23 slides and nested PCR-RFLP genotyping. In their approach they used laser capture microscopy to “catapult” oocysts from slide surfaces into microcentrifuge tubes held above the slides. Although their approach was interesting, the capital equipment cost is almost \$200,000 and the protocol was not tested on any environmental water samples.

Ruecker et al. (Ruecker, Bounsbath et al. 2005) described a nested 18S ribosomal RNA gene (18S) PCR-RFLP protocol that included the removal of oocysts from slides and multiple PCR and RFLP analyses from each sample to verify results. They tested their protocol

on three Method 1623 microscope slides containing 17 to 27 oocysts each. For all three samples they found mixes of animal-related *Cryptosporidium* species and did not detect any *Cryptosporidium* of human health significance. The authors noted false negatives due to PCR inhibitors and inter-replicate variability for the same DNA sample, leading to replicate-dependent results. Their recent work included the analysis of a much larger number of samples, with genotype analysis successful for 31 of 92 slides which tested positive for *Cryptosporidium* by microscopy (Ruecker, Braithwaite et al. 2007). The authors noted that 57 of the 92 slides had three or fewer oocysts, and that it was possible their method might not have been sensitive enough to detect these low numbers of oocysts. Most recently, they reported a 45% detection rate for field slides having 1 to 2 naturally occurring oocysts (Ruecker, Matsune et al. 2009). More importantly, their results confirm their earlier findings that human-pathogenic *Cryptosporidium* rarely occur in the watershed they studied.

Nichols et al. (Nichols, Campbell et al. 2006) described nested 18S PCR-RFLP genotyping of *Cryptosporidium* oocysts recovered from UK Drinking Water Inspectorate (DWI) method slides from various water samples. The authors compared the performance of their PCR primers with the primers of Xiao et al. (Xiao, Alderisio et al. 2000) which were also used in the Sunnotel et al. and Ruecker et al. studies described above. The authors found that their PCR was more sensitive and reproducible than that of Xiao et al. based on the use of both purified oocysts and environmental samples. The authors analyzed 33 *Cryptosporidium*-positive DWI method slides which contained between 1 and 130 oocysts each, with most containing over 3 oocysts. Mixtures of animal-related *C. andersoni* and *C. muris* oocysts were found on most slides, and some slides were also found to contain *C. parvum* and/or *C. hominis*. It should be noted that most sample results were listed as “mixed” genotypes present, for example “*C. muris* or *C. andersoni* and *C. parvum* or *C. hominis*”.

## RESEARCH NEEDS

*Cryptosporidium* species capable of infecting both humans and animals are frequently detected in environmental water samples. This is an important finding from a regulatory and utility perspective, since source waters contaminated with animal-related *Cryptosporidium* may pose little or no health risk to humans. Identifying *Cryptosporidium* genotypes present in water will allow a more accurate assessment of the public health significance of waterborne *Cryptosporidium*, provide data for sound treatment plant infrastructure decisions, and aid the development of effective watershed management plans. PCR analysis of archived slides and slides from upcoming monitoring represent excellent opportunities to obtain valuable information on the occurrence of waterborne *Cryptosporidium* genotypes with only a modest amount of additional effort and cost.

The previously described methods of *Cryptosporidium* slide genotyping have limited practical use by water utilities and water quality laboratories for a variety of reasons, including: complicated and cumbersome multi-step nested PCR-RFLP protocols which are prone to contamination and poor reproducibility, especially for novice laboratories; uncertain detection sensitivity with one to three oocysts; uncertainties regarding the human health significance of the findings, and; impractical capital laboratory equipment cost. In addition, most of the published protocols have not been thoroughly evaluated using environmental water samples. For these reasons, a simple and reliable method for the speciation and genotyping of *Cryptosporidium*

from Method 1622/23 and UK DWI slides that typical utility and water quality laboratories can readily adopt is needed.

## RESEARCH APPROACH

Based on our knowledge of human cryptosporidiosis, it is reasonable that a slide genotyping method that can differentiate the *Cryptosporidium* species which cause the vast majority of cryptosporidiosis in immunocompetent humans (*C. hominis*, *C. parvum* and *C. meleagridis*) from typically animal-related genotypes may meet the needs of the water industry and regulatory communities. However, the method should also provide analytical end-products which are amenable to more detailed analysis if greater discrimination of genotypes is desired.

Specific considerations were incorporated into the research approach to aid technology transfer to water quality laboratories having little or no molecular experience. These considerations included: the use of single-round PCR instead of nested PCR with RFLP analysis to help reduce the risk of intralab contamination with PCR product and subsequent false positives; the incorporation of PCR product carryover prevention reagents to help prevent false positives; PCR platform flexibility, with the option of using either standard PCR or real-time PCR instruments to accommodate different levels of infrastructure investment; analytical end-products which are amenable to more detailed analysis if greater discrimination of genotypes is desired. It is also important to note that research focused on genotyping single oocysts rather than mixtures of different genotypes since the majority of positive regulatory field slides have only 1 to 3 oocysts present. Flow cytometry was used to precisely sort oocysts directly into microcentrifuge tubes or onto slides, with most experiments using single oocysts. The majority of trials were performed using human-pathogenic *C. parvum* and animal-associated *C. muris* oocysts, since oocysts of these two species are commercially available.

Specific objectives of this project were to:

1. Optimize the removal of *Cryptosporidium* oocysts from microscope slides by comparing the previously described use of bacteriological loops to the use of closed cell foam swabs.
2. Evaluate the use of different DNA extraction and purification using low numbers of flow cytometry enumerated *Cryptosporidium* oocysts.
3. Develop a heat shock protein 70 (hsp70) and 18S ribosomal RNA (18S) gene multiplex PCR that can differentiate human-pathogenic from animal-associated *Cryptosporidium*, and that can be performed using both conventional PCR and real-time PCR with high resolution melt (HRM) analysis.
4. Evaluate the specificity of the developed PCR protocol using different *Cryptosporidium* species.
5. Evaluate method sensitivity using single flow cytometry enumerated oocysts sorted directly into microcentrifuge tubes or onto matrix-free microscope slides.
6. Perform preliminary testing of the protocol using Method 1623 and UK DWI field slides seeded with single flow-sorted *Cryptosporidium* oocysts.
7. Development of a protocol and demonstration DVD to aid technology transfer to water quality laboratories.

## CHAPTER 2: MATERIALS AND METHODS

Since this was a method development project, many different variations of sample processing and analysis were performed. Therefore, general information on materials and methods is provided below, while specific experimental variations are described in their respective section of Chapter 3. Most importantly, detailed information is included in the appendices: a list of materials and supplies (Appendix A); reagent preparation (Appendix B); and detailed sample processing protocol (Appendix C).

### ***CRYPTOSPORIDIUM* spp. OOCYSTS AND DNA**

Only *C. parvum* and *C. muris* oocysts are commercially available. *C. hominis* is also propagated by a research laboratory and is available to other researchers but is costly due to the expensive propagation methods used. Due to lack of commercial availability, it is generally difficult to obtain oocysts or DNA of other *Cryptosporidium* species. Despite these challenges, we were able to attain a variety of *Cryptosporidium* oocysts or DNA to test in this project (Table 2.1). Mouse-propagated *C. parvum* Iowa isolate and *C. muris* RN66 oocysts were obtained from Waterborne, Inc. (New Orleans, Louisiana). Bovine-propagated *C. parvum* Iowa (CpAZ) isolate oocysts were obtained from the University of Arizona (Tucson, Arizona). Flow cytometry sorting of oocysts was performed by WSLH, and oocyst stocks were typically 30 days or less post-shedding in age.

### **OOCYST DNA EXTRACTION AND PURIFICATION**

Our standard Chelex 100/1X Tris EDTA (Chelex/TE) freeze-thaw method (Di Giovanni and LeChevallier 2005) was performed by eight cycles of freezing in liquid nitrogen and thawing at 94°C to 98°C for approximately 1 min each cycle. Modifications of the Chelex/TE freeze-thaw method were performed, including the use of ChargeSwitch magnetic bead DNA purification kit (Invitrogen; Carlsbad, California) lysis buffer during the Chelex freeze-thawing step; the addition of the ChargeSwitch lysis buffer to the sample lysate after Chelex freeze-thawing; and the use of lower concentrations of TE buffer (e.g. 0.5X) or molecular grade water (MGW) for preparation of the Chelex slurry. The final method protocol uses Chelex 100 prepared in molecular grade water (Chelex/MGW). Oocyst freeze-thaw lysates (DNA) were either used directly as template in PCR or further purified using the ChargeSwitch DNA purification kit or Qiagen DNeasy tissue kit (Qiagen; Valencia, California) with carrier DNA following the protocol of Ruecker et al. (Ruecker, Bounsombath et al. 2005).

**Table 2.1**  
***Cryptosporidium* species and genotypes used in this study**

Species/genotype	Typical Host	Significant Pathogen for Healthy Humans	Source
<i>C. parvum</i> Iowa	Cattle, Humans	Yes	Waterborne, Inc.
<i>C. parvum</i> Iowa (CpAZ)	Cattle, Humans	Yes	University of Arizona
<i>C. hominis</i> Y049, TU502, and Ch4	Humans	Yes	CHD, Tufts University, and NPHS Microbiology Swansea
<i>C. meleagridis</i> TU1867	Birds	Yes	Tufts University
<i>C. muris</i> RN66	Rats/mice	No	Waterborne, Inc.
<i>C. andersoni</i>	Cattle	No	CHD and University of Calgary
Cervine genotype	Deer/sheep	No	NPHS Microbiology Swansea
<i>C. baileyi</i>	Chickens	No	CHD
<i>C. serpentis</i>	Snakes	No	CHD

## PCR PRIMERS AND CONDITIONS

Our previously described CPHSPT2 primers targeting the *Cryptosporidium* heat shock protein 70 gene (hsp70) were used (LeChevallier, Di Giovanni et al. 2003; Aboytes, Di Giovanni et al. 2004; Di Giovanni and LeChevallier 2005), which result in a 346 bp product. The primers have the following sequence: CPHSPT2F 5' TCCTCTGCCGTACAGGATCTCTTA 3' and CPHSPT2R 5' TGCTGCTCTTACCAAGTACTCTTATCA 3'. The hsp70 primers were used for the detection of human-pathogenic *Cryptosporidium*, and will amplify DNA from *C. parvum*, *C. hominis* and *C. meleagridis*. The primers will also amplify DNA from *C. wrairi*, a species very closely related to *C. parvum* (Xiao, Fayer et al. 2004). *C. wrairi* is not considered a significant human pathogen. However, since *C. wrairi* is typically found in guinea pigs and is extremely rare in the environment, it is not considered an issue with regards to hsp70 primer specificity. For the 18S ribosomal RNA (18S) gene locus (18S), the CPB DIAG PCR primers of Johnson et al. (Johnson, Pieniazek et al. 1995) were used, resulting in a 435 bp product. These primers are

*Cryptosporidium* genus specific, and will detect all *Cryptosporidium* species, and as such were used as the diagnostic amplicon for animal-associated *Cryptosporidium*.

The results of the hsp70 and 18S multiplex PCR were interpreted as follows. Human-pathogenic oocysts (i.e. *C. parvum*, *C. hominis* and *C. meleagridis*) have either both the 18S and hsp70 or the hsp70 only amplicon bands detectable; while animal-associated oocysts have only the 18S amplicon band. The only exception is the case of hsp70 false-negatives in which only the 18S amplicon is obtained for human-pathogenic oocysts, and which high resolution melt (HRM) analysis must be used for correct identification.

The composition of the PCR mastermix varied by experiment, but the final method mastermix is as follows. Each 50 µl reaction mixture contained 1X GeneAmp Gold buffer (Applied Biosystems; Foster City, California); 3.0 mM MgCl<sub>2</sub>; 200 µM of each dATP, dCTP, and dGTP; and 80 µM dUTP and 120 µM dTTP (GE Healthcare Life Sciences; Piscataway, New Jersey); 200 nM of each CPHSPT2 and CPB DIAG primer; 0.75 µg µl<sup>-1</sup> bovine serum albumin (BSA; Sigma Chemical Co.; St. Louis, Missouri); 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) or Cheetah HotStart Taq DNA polymerase (Biotium; Hayward, California); 1X EvaGreen (Biotium); and 1.0 U AmpErase uracil N-glycosylase (UNG; Applied Biosystems).

Real-time PCR amplification was performed using either an Applied Biosystems 7000 thermal cycler or Corbett Rotor Gene 6000 high HRM thermal cycler (Corbett Life Science; Sydney, Australia; now marketed as the Qiagen RotorGene Q 5plex HRM instrument). Final method PCR conditions were as follows: UNG digestion at 50°C for 10 min; initial denaturation at 95°C for 10 min; 55 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 30 s; followed by a final extension at 72°C for 10 min. To perform melt analysis using the Applied Biosystems 7000 instrument, the final extension step was followed by pre-melt conditioning at 50°C for 30 s and default dissociation (melt) with 1°C (fixed by system software) melt steps from 60°C to 95°C. To perform high resolution melt analysis (HRM) on the RotorGene 6000 HRM instrument (the preferred cycler for the final method) the final extension step was followed by pre-melt conditioning at 50°C for 30 s and melt analysis with 0.1°C melt steps from 80°C to 86°C. PCR products were confirmed by gel electrophoresis. Aliquots (10 µl) of each completed PCR were analyzed on 2% agarose gels containing ethidium bromide, and visualized by ultraviolet light illumination.

During each cycle of the PCR and as amplicons are generated, increases in SYBR Green or EvaGreen fluorescence was detected by the thermal cycler. The fluorescence signal increases in direct proportion to the concentration of the PCR amplicon being formed. The threshold cycle ( $C_T$ ) is the fractional PCR cycle number at which a significant increase in signal fluorescence above baseline is first detected for a sample. Therefore the lower the  $C_T$  value, the greater initial concentration of target in the sample or more efficient/sensitive PCR obtained.

## SLIDES

The majority of slides used in this study were Dynal Spot-On/IDEXX SingleSpot slides (IDEXX; Westbrook, Maine). Both matrix-free (no water sample debris present) and *Cryptosporidium*-negative Method 1623 and UK DWI field method slides were used. *Cryptosporidium*-negative Method 1623 field slides were stained with EasyStain anti-*Cryptosporidium* and anti-*Giardia* fluorescein isothiocyanate (FITC) labeled antibodies (BTI; North Ryde, Australia); while UK DWI slides were stained with Cellabs (Brookvale, New South

Wales, Australia) anti-*Cryptosporidium* FITC labeled antibody. Field matrix slides represented raw and finished drinking water samples from various locations in the US and Scotland, were stored at 4°C, and were greater than 1 year old when used for genotyping experiments. Flow-sorted oocysts were stained in solution prior to sorting with Waterborne Crypt-a-Glo anti-*Cryptosporidium* FITC labeled antibody and 4', 6'-diamidino-2-phenylindole (DAPI). Field slides seeded with flow-sorted oocysts were not stained again after being seeded.

## CHAPTER 3: RESULTS AND DISCUSSION

### OOCYST RETENTION AND REMOVAL FROM SLIDES

Experiments were performed to address several issues related to oocyst retention and removal from slides. First, we planned to use slides seeded directly with flow-sorted oocysts, and there was some concern over the retention of oocysts on the slides. For successful detection and accurate evaluation of the developed method, it was important for oocysts to remain on the slides after removal of coverslips. In an early meeting with Dr. Huw Smith and his staff at the Scottish Parasite Diagnostic Laboratory (SPDL), methanol fixation of slides was discussed. In the UK DWI method, methanol fixation of slides (after the sample has dried) is required, whereas it is optional for Method 1622/23. It was hypothesized that methanol fixation was important for oocysts retention on the slides. Similarly, there was some concern that oocysts flow-sorted directly onto slides with field matrix may not be retained well, since the oocysts would not be imbedded in the matrix.

With regards to oocyst removal from slides, prior research of Nichols et al. (Nichols, Campbell et al. 2006) reported the successful use of bacteriological loops to scrape slides and remove oocysts. While the investigators reported efficient removal of oocysts using loops, we had some concern that oocysts may occasionally be missed when using loops. Therefore, experiments were performed to evaluate the use of closed cell foam swabs for scraping slides and removing oocysts. It was hypothesized that foam would make better contact with the slide surface and provide for thorough and consistent scraping of the slide surface. Closed cell foam was used to help avoid entrapment of oocysts in the foam and subsequent interference with DNA extraction.

#### Methanol Fixation and Retention of Oocysts on Slides

Ten *C. parvum* Iowa isolate oocysts were flow-sorted onto matrix-free IDEXX SingleSpot slides by WSLH. Forty replicate slides were prepared, with twenty of these fixed with methanol and allowed to air dry prior to sealing. Ten replicate methanol-fixed and unfixed slides were shipped to CHD for analysis, with the remaining slides analyzed at WSLH. Slides were examined by immunofluorescent microscopy to confirm oocysts counts. Coverslips were carefully removed and the slides recounted. Both laboratories reported that none of the oocysts for both the unfixed and methanol-fixed slides were lost during cover-slip removal.

Similarly, there was some concern over oocyst retention on field slides seeded directly using flow cytometry. The concern was that seeded oocysts may come off the slides when the processing laboratory removed the coverslips since the oocysts were not embedded into the field matrix. To address this concern, WSLH removed coverslips from ten *Cryptosporidium*-negative Method 1622/23 field slides (provided by CHD). Residual mounting medium was removed using two warm PBS rinses and two water rinses of the slides, being careful not to disturb the environmental debris on the slides. Ten pre-stained oocysts were flow-sorted onto each slide, the slides dried at 37° C, methanol-fixed, and mounted with coverslips. Oocysts on the slides were enumerated by IFA microscopy. Coverslips were removed the next day and the slides recounted. All oocysts were retained on each of the slides. This is a particularly important finding since the

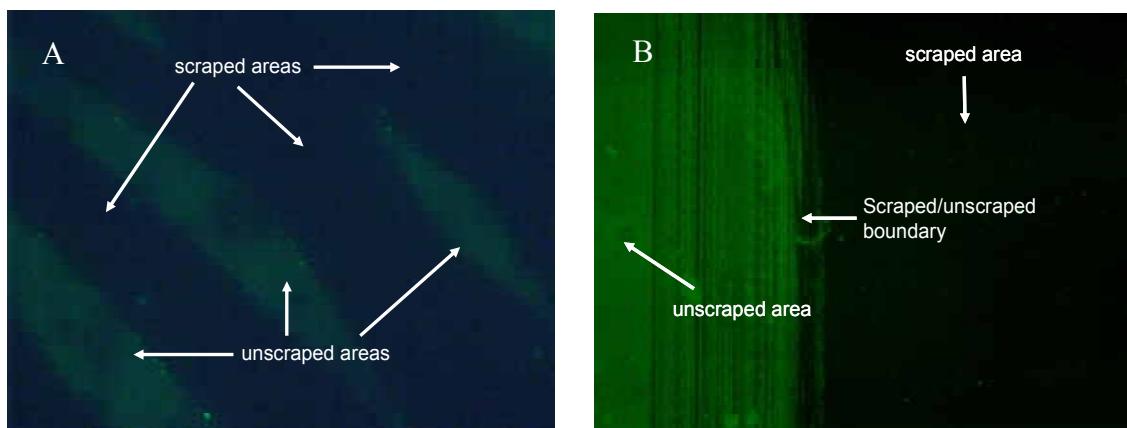
WaterRF Project 4284 round-robin method evaluation will make extensive use of field slides seeded with flow-sorted oocysts.

Overall, results were inconclusive as to the beneficial effect of methanol fixation for oocyst retention. However, since all oocysts on methanol fixed slides were retained and methanol fixation is required for the UK DWI method, we decided to methanol fix slides in all subsequent experiments.

### Recovery of Oocysts From Slides

A critical step in the slide processing protocol is the removal of oocysts from slides. Preliminary experiments were performed using bacteriological loops for the removal of oocysts from microscope slides using the technique described by Nichols et al. (Nichols, Campbell et al. 2006). Ten replicate matrix-free slides seeded with a single flow-sorted oocyst were examined by microscopy at AgriLife Research before and after oocyst removal using loops. The loop oocyst removal procedure was found to be effective for all replicates. Further, while the project team was at the SPDL, the project team and SPDL staff scraped 28 slides using loops, and only one slide had incomplete removal of oocysts.

Although the loop removal of oocysts was efficient, gaps in the scraping pattern were observed upon microscopic examination of the slides (Figure 3.1A). Therefore, closed cell foam was also evaluated in an attempt to improve scraping of the slides. The increased contact area of the foam with the slide was anticipated to allow more complete and uniform scraping of the slide. Closed cell foam sheets were cut it into small pieces using flame sterilized scissors, and foam pieces were gripped with a flame sterilized fine tip hemostat. After scraping the slide, the tip of the hemostat was placed over the microcentrifuge tube containing the slide washes, the hemostat unlocked, and the foam dropped into the tube. Microscopic observation of slides scraped with the closed cell foam swabs revealed complete scraping of the slide surface without the gaps typically observed with the bacteriological loops (Figure 3.1B). A subsequent experiment using closed cell foam swabs to scrape ten matrix-free slides seeded with single flow-sorted oocysts indicated that the procedure was effective for all replicates.



**Figure 3.1** Photomicrographs of slide wells after removing oocysts by scraping with a bacteriological loop (A) or closed cell foam swab (B). The unscraped areas appear light green due to residual immunofluorescent staining background. Note that in the slide scraped with closed cell foam there are no gaps in the scrape pattern as seen with the bacteriological loops. Magnification approximately 250X.

## HSP70 AND 18S MULTIPLEX PCR

### Optimization of dUTP Concentration for Sensitive Amplification While Maintaining PCR Product Carryover Protection

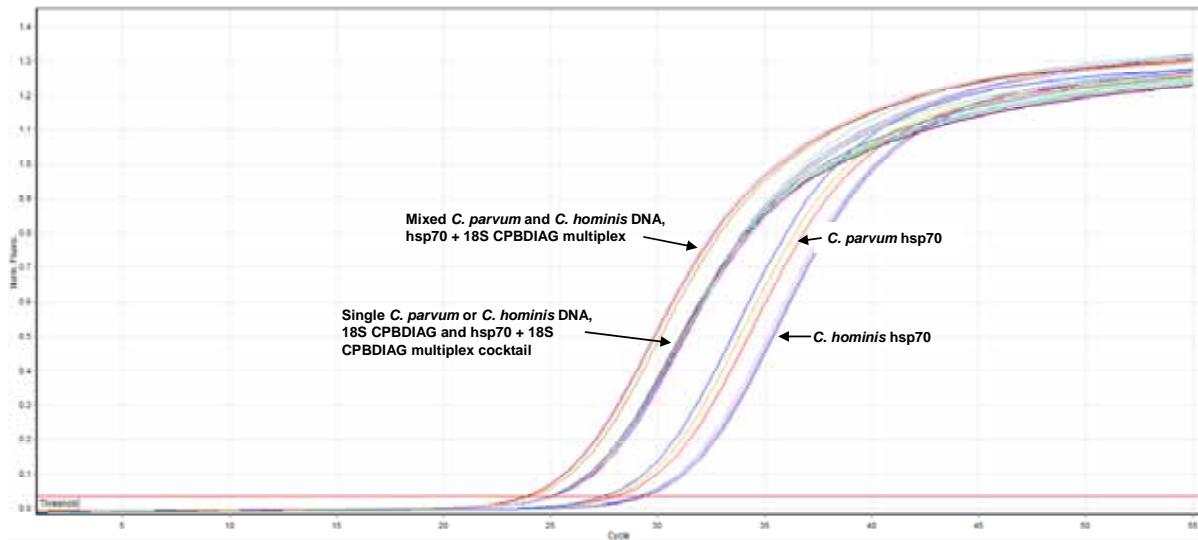
We confirmed the findings of Xiao, Alderisio and Singh (Xiao, Alderisio et al. 2006) that a 4:6 ratio of dUTP/dTTP used in the PCR provides greater sensitivity over the use of only dUTP and still provides PCR product carryover prevention. PCR mastermixes were prepared using either the conventional 200  $\mu$ M concentration of dTTP, 800  $\mu$ M dUTP or with 80  $\mu$ M dUTP and 120  $\mu$ M dTTP (a 4:6 dUTP/dTTP ratio). Real-time hsp70 and 18S PCR was performed using replicate 10 oocyst DNA templates and the  $C_T$  values compared. There was no significant difference between the use of 200  $\mu$ M dTTP or a 4:6 dUTP/dTTP ratio ( $P > 0.05$ ) for the hsp70 PCR. However, the two treatments did differ for the 18S PCR ( $P = 0.003$ ), although the differences in mean  $C_T$  values were quite small:  $24.24 \pm 0.12$  and  $24.72 \pm 0.06$  for 200  $\mu$ M dTTP and 4:6 dUTP/dTTP, respectively. This 0.5 cycle difference in mean  $C_T$  values translates to only about a 15% difference in amplification. In comparison, mean  $C_T$  values for reactions containing 800  $\mu$ M dUTP (our previous conditions) were greater than 2 cycles higher than reactions containing 200  $\mu$ M dTTP, indicating the use of only dUTP reduced amplification.

For evaluation of PCR product carryover prevention, amplicons from 4:6 dUTP/dTTP reactions were diluted and used as template in new reactions with or without the addition of uracil-N-glycosylase (UNG). The UNG enzyme degrades PCR products containing uracil, therefore preventing the amplification of trace amounts of contaminating PCR product for the prevention of false positives due to intra-laboratory contamination. In our trials, the reactions with the addition of UNG still amplified, likely due to overloading the reactions with PCR product template (not “trace” amounts). However, the  $C_T$  values of the reactions with UNG were approximately 12 cycles higher (about 10,000-fold lower amplification) than the reactions without the addition of UNG for both the hsp70 and 18S amplicons. Collectively, these results indicate that the use of 4:6 dUTP/dTTP provided good amplification sensitivity while still providing an acceptable level of PCR product carryover prevention, and was therefore incorporated into the protocol.

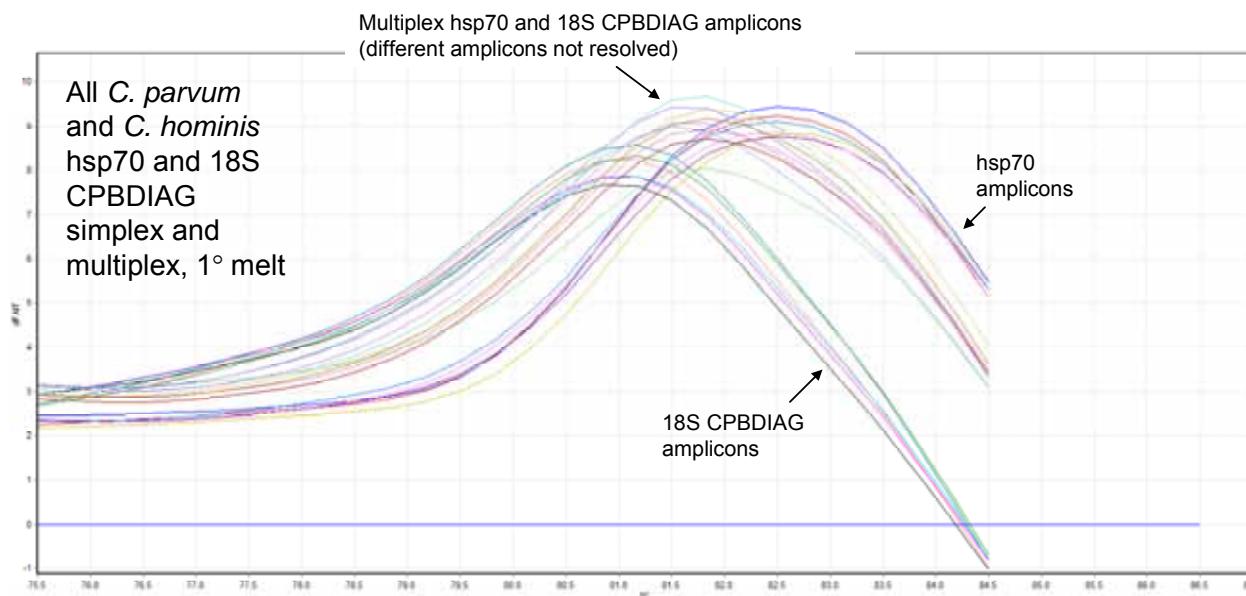
### Initial Hsp70 and 18S Multiplex PCR Trials

Initial experiments evaluating single round hsp70 and 18S CPB-DIAG multiplex PCR were performed using *C. parvum* Iowa and *C. hominis* TU502. Oocyst DNA samples (approx. 5 oocyst equivalents) of each isolate were analyzed separately using both simplex and multiplex hsp70 and 18S PCR. Further, *C. parvum* and *C. hominis* DNA were mixed together and analyzed using multiplex hsp70 and 18S CPB-DIAG. SYBR Green melt curves were generated using both 1°C and 0.1°C melt step increments. All PCR for these trials was performed using the RotorGene 6000 HRM thermal cycler.

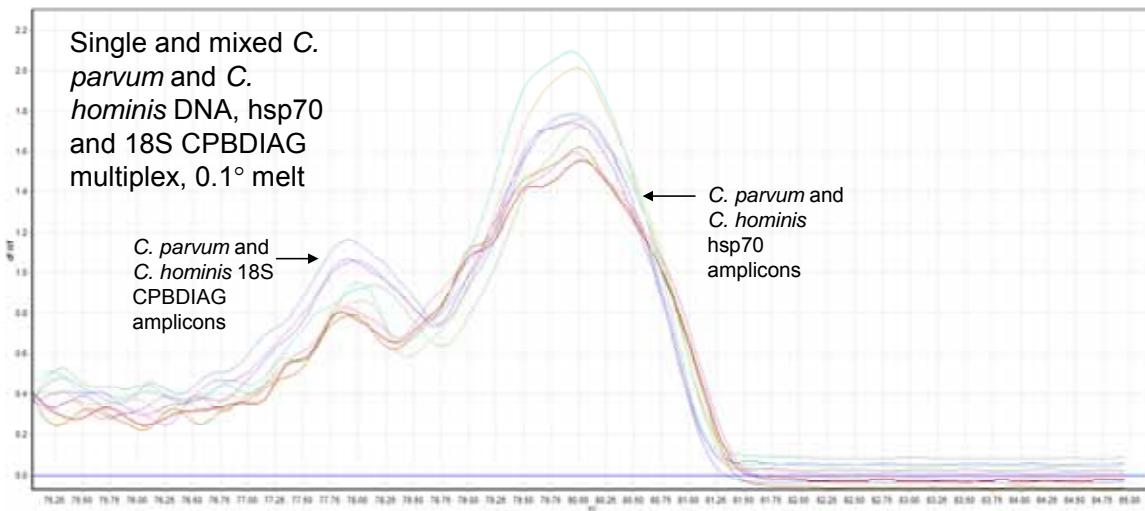
The amplification plot for the samples is included as [Figure 3.2](#). The 18S and multiplex hsp70 and 18S reactions amplified earlier than the simplex hsp70 reactions. This is likely due to the higher copy number of 18S target compared to hsp70 (20 copies and 4 copies per oocyst, respectively). SYBR Green melt curves with 1°C and 0.1°C melt step increments are presented in [Figures 3.3](#) and [3.4](#), respectively. Note that for the 1°C step melt curve that the hsp70 and 18S amplicons form one large peak for the multiplex reactions, and the two different PCR amplicons cannot be resolved ([Figure 3.3](#)).



**Figure 3.2** Amplification plot for *C. parvum* and *C. hominis* simplex and multiplex hsp70 and 18S PCR. Reactions were seeded with DNA equivalent to approximately five oocysts.



**Figure 3.3** SYBR Green 1°C step melt curves for *C. parvum* and *C. hominis* simplex and multiplex hsp70 and 18S PCR.



**Figure 3.4 SYBR Green 0.1°C step high resolution melt curves for *C. parvum* and *C. hominis* multiplex hsp70 and 18S PCR.**

In contrast, the hsp70 and 18S products can be resolved for the multiplex reactions by using 0.1°C step high resolution melt analysis (Figure 3.4). This indicated that high resolution melt analysis is capable of determining whether only the 18S amplicon is present, or if both hsp70 and 18S amplicons are present. The 18S amplicon melt peak appeared smaller likely due to background fluorescence from the hsp70 amplicon which melts at a higher temperature. Gel electrophoresis analysis of PCR products was also performed (Figure 3.5). The hsp70 and 18S amplicons are readily distinguishable based on molecular weight size (346 bp and 435 bp, respectively) in both the simplex and multiplex reactions.



**Figure 3.5 Gel electrophoresis detection of *C. parvum* and *C. hominis* simplex and multiplex hsp70 and 18S PCR amplicons.**

## PCR Technical Challenges

### *Lot-to-Lot Variability of PCR Reagents*

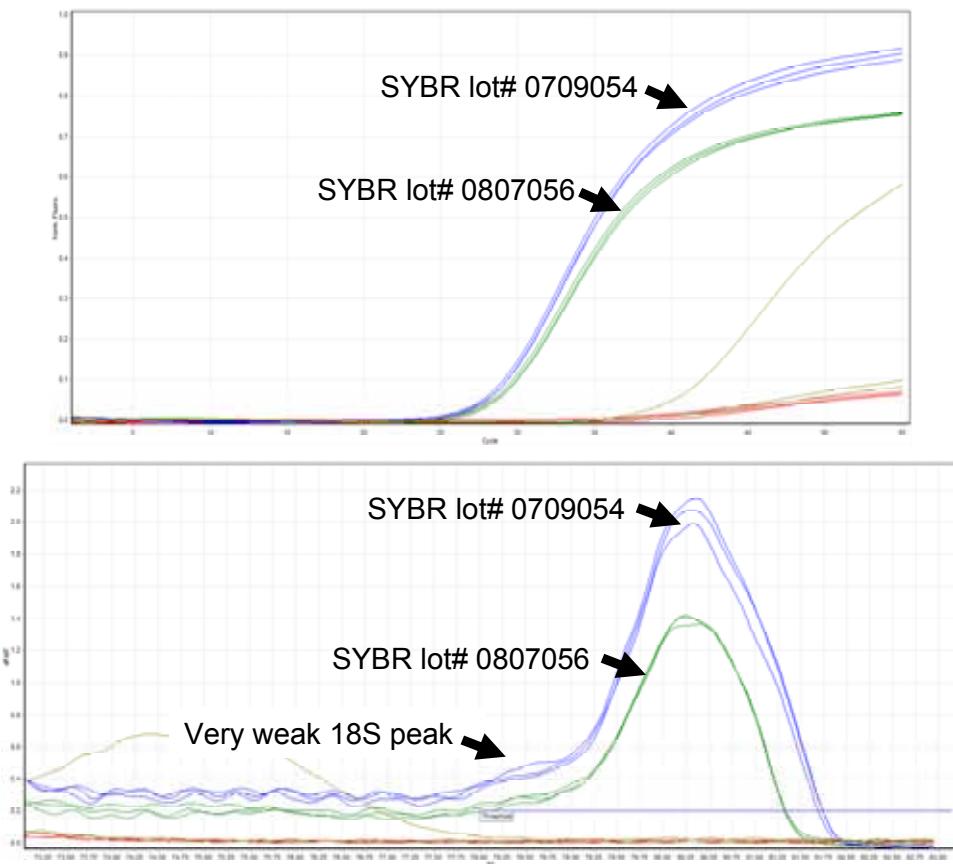
Over the course of the study, a few PCR reagent lot-to-lot variability issues came to light. The first issue was with magnesium chloride ( $MgCl_2$ ) purchased from Applied Biosystems and supplied with their AmpliTaq Gold DNA polymerase and PCR buffers. At  $MgCl_2$  levels of 3.0 mM and higher, as used in the PCR for this project, a BSA/magnesium precipitate sometimes occurred during thermal cycling of PCR samples. This precipitate did not appear to have an adverse effect on PCR detection or real-time PCR fluorescence readings, but may cause difficulties in taking sample aliquots for gel electrophoresis. To investigate this issue, four different lots of  $MgCl_2$  were used to prepare different PCR mastermixes, with the same lot of BSA used for each mastermix. The reactions were monitored during the PCR cycling and it was discovered that the BSA/magnesium precipitate formed during the first few cycles of PCR after the samples had been heated to 95° C and cooled to the 60° C annealing temperature. Results of the experiment indicated that samples prepared with  $MgCl_2$  Lot C had a significant amount of precipitate, while Lots A and B had minor amounts, and Lot D had no visible precipitate at all (Figure 3.6). Applied Biosystems technical support was not able to help us resolve this issue, and it unfortunately continues to occur.



**Figure 3.6 Lot-to-lot variability of Applied Biosystems magnesium chloride PCR reagent. Variation in the amount of BSA/magnesium precipitate formation in PCR samples after thermal cycling was observed.**

A lot-to-lot variability issue was also found with the Applied Biosystems SYBR Green PCR buffer. We found that different lots of SYBR Green PCR buffer resulted in PCR products with significant differences in their fluorescence as revealed by real-time PCR amplification plots and melt curves (Figure 3.7). Further, only the hsp70 product melt peak was readily visible, making it difficult to identify the presence of the 18S product melt peak for *C. parvum*, *C. hominis*, and *C. meleagridis* samples (compare to the RotorGene EvaGreen melt curve in Figure 3.8). Applied Biosystems technical support informed us that they frequently see variability in their SYBR Green PCR buffers and recommended the use of their Power SYBR

Green buffer. We had previously evaluated the Power SYBR Green buffer and were not pleased with its performance. Therefore, we decided to pursue the use of EvaGreen, a new dye which is recommended for high-resolution melt (HRM) analysis. We were previously aware of this dye but had not pursued using it for this project because there are currently no commercially available EvaGreen PCR buffers designed specifically for our application. EvaGreen has several advantages over SYBR Green including higher fluorescence, greater thermal stability, and less PCR inhibition at high concentrations. EvaGreen is manufactured by Biotium, and is available as a standalone reagent that can be added to the PCR mastermix. Biotium technical support informed us that most PCR buffers are not optimized for use with EvaGreen, particularly the ABI GeneAmp Gold buffer used with the AmpliTaq Gold DNA polymerase which we were using in our protocol. Despite this potential issue, we found the use of EvaGreen and GeneAmp Gold buffer resulted in superior real-time PCR amplification plots and melt curves compared to ABI SYBR Green PCR buffer. Consequently, we incorporated the use of EvaGreen into our final protocol.

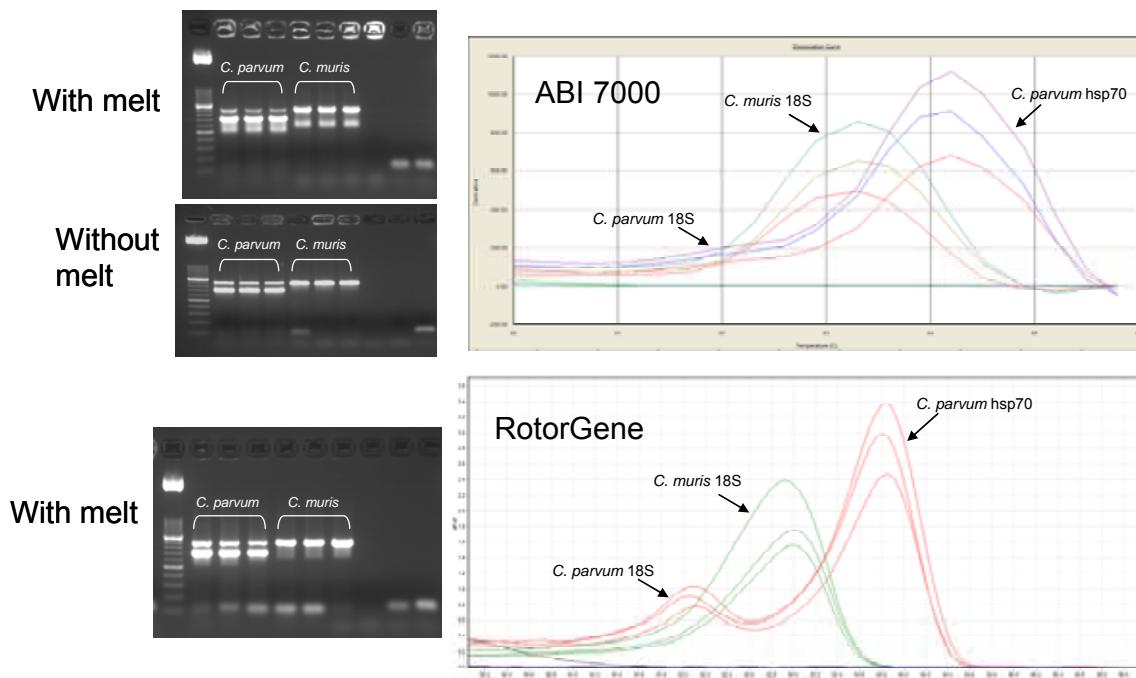


**Figure 3.7** Lot-to-lot variability in fluorescence observed for Applied Biosystems SYBR Green PCR buffers. Real-time PCR amplification plot (top) and DNA melt curves (bottom) for reactions prepared with two different lots of SYBR Green PCR buffer. Reactions were seeded with *C. parvum* DNA equivalent to approximately 10 oocysts.

### Differences Related to PCR Platforms

In preparation for the Project 4284 Technology Transfer Workshop, quality control samples were run on the RotorGene and Applied Biosystems ABI 7000 real-time PCR cyclers. As expected, the ABI 7000 instrument melt curves were inferior to the melt curves generated on the RotorGene. The ABI instrument is not capable of precision temperature steps (fixed by the software at 1°C) for the melt curve analysis, which causes loss of peak resolution ([Figure 3.8](#)). Unexpectedly, the gel electrophoresis analysis of PCR products generated with the ABI 7000 instrument also revealed a significant amount of smearing and noise ([Figure 3.8](#)). Upon further investigation it was determined that this was due to artifact generated during the melt analysis on the ABI instrument. In addition to the differences seen between the RotorGene and ABI instrument, we have also seen melt analysis performance issues with the Roche LightCycler, LightCycler II, and LightCycler 480 instruments.

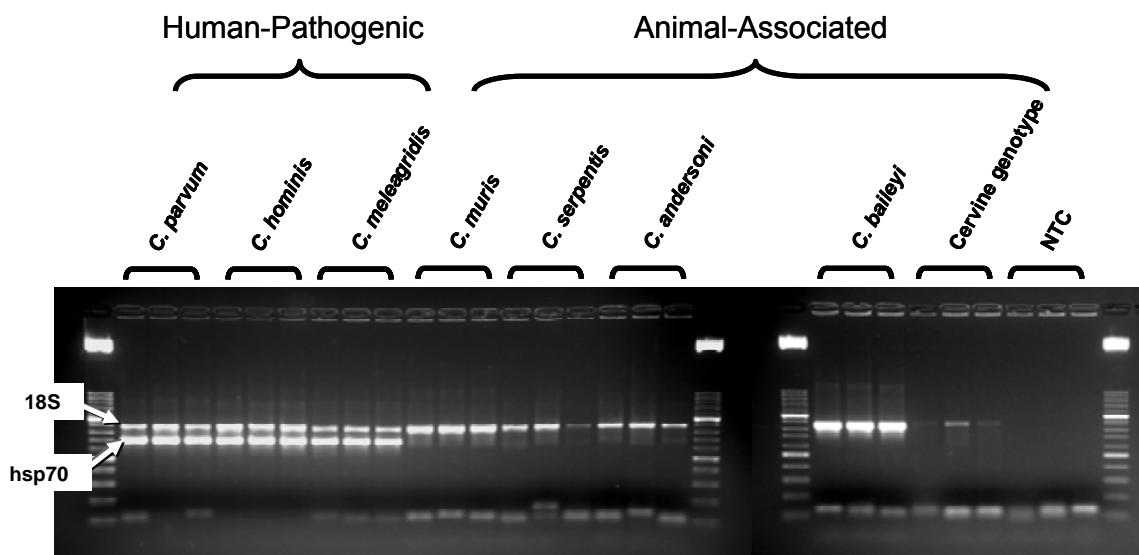
Unfortunately, effects of different PCR platforms on method performance will occur. This will be one of the issues addressed in Project 4284, since the participating laboratories will be using different conventional and real-time PCR thermal cyclers. Platform differences are particularly an issue for high resolution melt analysis, as some instruments do not have these capabilities (as demonstrated in [Fig. 3.8](#)). However, there may also be differences in PCR amplification due to different instruments. Since we are providing PCR reagents and control DNA to the Project 4284 labs, we will be able to investigate this issue further and will then be able to make specific recommendations on how to validate method performance using different PCR platforms.



**Figure 3.8 Differences in the resolution of real-time PCR melt curves (right panels) and quality of PCR products (left panels) due to the use of different PCR platforms. Mastermixes were prepared using EvaGreen dye and reactions were seeded with DNA equivalent to approximately 10 *C. parvum* or *C. muris* oocysts.**

## Specificity of the Hsp70 and 18S Multiplex PCR

The specificity of the hsp70 and 18S multiplex PCR was tested using oocyst DNA of the following different *Cryptosporidium* genotypes: *C. parvum*; *C. hominis*; *C. meleagridis*; *C. muris*; *C. serpentis*; *C. andersoni*; *C. baileyi*; and the cervine genotype. As expected, only the human-pathogenic *C. parvum*, *C. hominis*, and *C. meleagridis* had both the hsp70 and 18S amplicons; while the animal-associated genotypes had only the 18S amplicon (Figure 3.9). For the analysis of a slide with a single oocyst, the presence of only an 18S amplicon would indicate the oocyst was an animal-associated *Cryptosporidium* genotype. Similarly, the presence of both hsp70 and 18S amplicons (or only the hsp70 amplicon) would indicate that the oocyst was human-pathogenic (i.e. *C. hominis*, *C. parvum*, or *C. meleagridis*). Furthermore, results indicated that conventional gel electrophoresis of hsp70 and 18S multiplex PCR products can differentiate human-pathogenic from animal-associated oocysts. The use of a conventional PCR thermal cycler is therefore an option for the developed method, providing flexibility in infrastructure investment for water quality laboratories. Suitable conventional PCR instruments cost as little as \$5,000, whereas high resolution melt analysis PCR instruments cost approximately \$30,000 to \$50,000.



**Figure 3.9 Specificity of the hsp70 and 18S multiplex PCR for different human-pathogenic and animal-associated *Cryptosporidium* genotypes. NTC, no template negative control.**

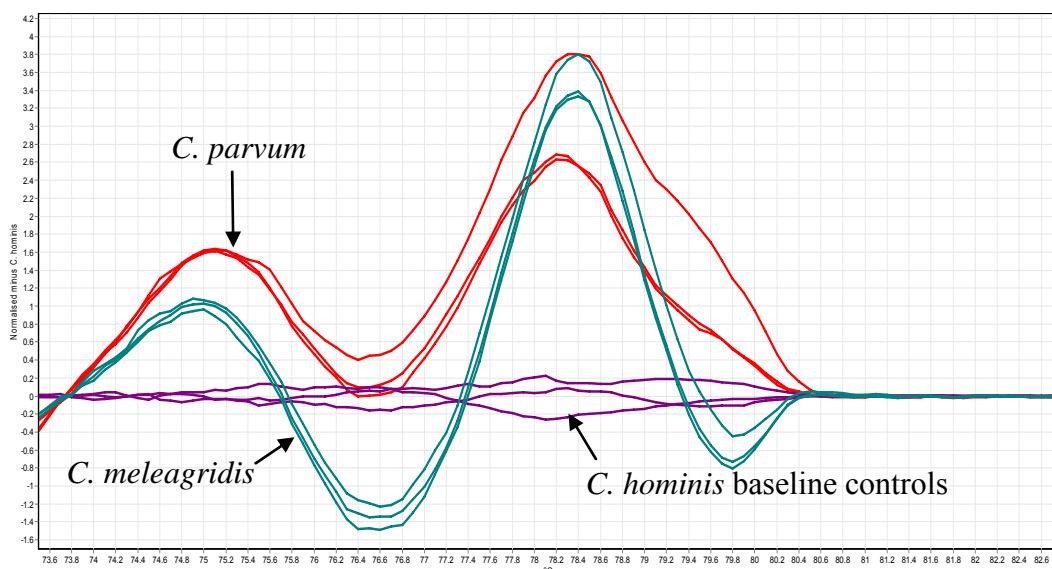
## High-Resolution Melt (HRM) Genotyping of *Cryptosporidium*

As shown previously, analysis of high resolution melt curves can determine if the hsp70 and 18S melt peaks are present, allowing the simple differentiation of human-pathogenic and animal-associated *Cryptosporidium* genotypes based on the presence of the hsp70 product. In addition, HRM-based genotyping of samples is possible. HRM genotyping is best performed on samples which have only one PCR product present. Therefore, the ability of HRM genotyping to

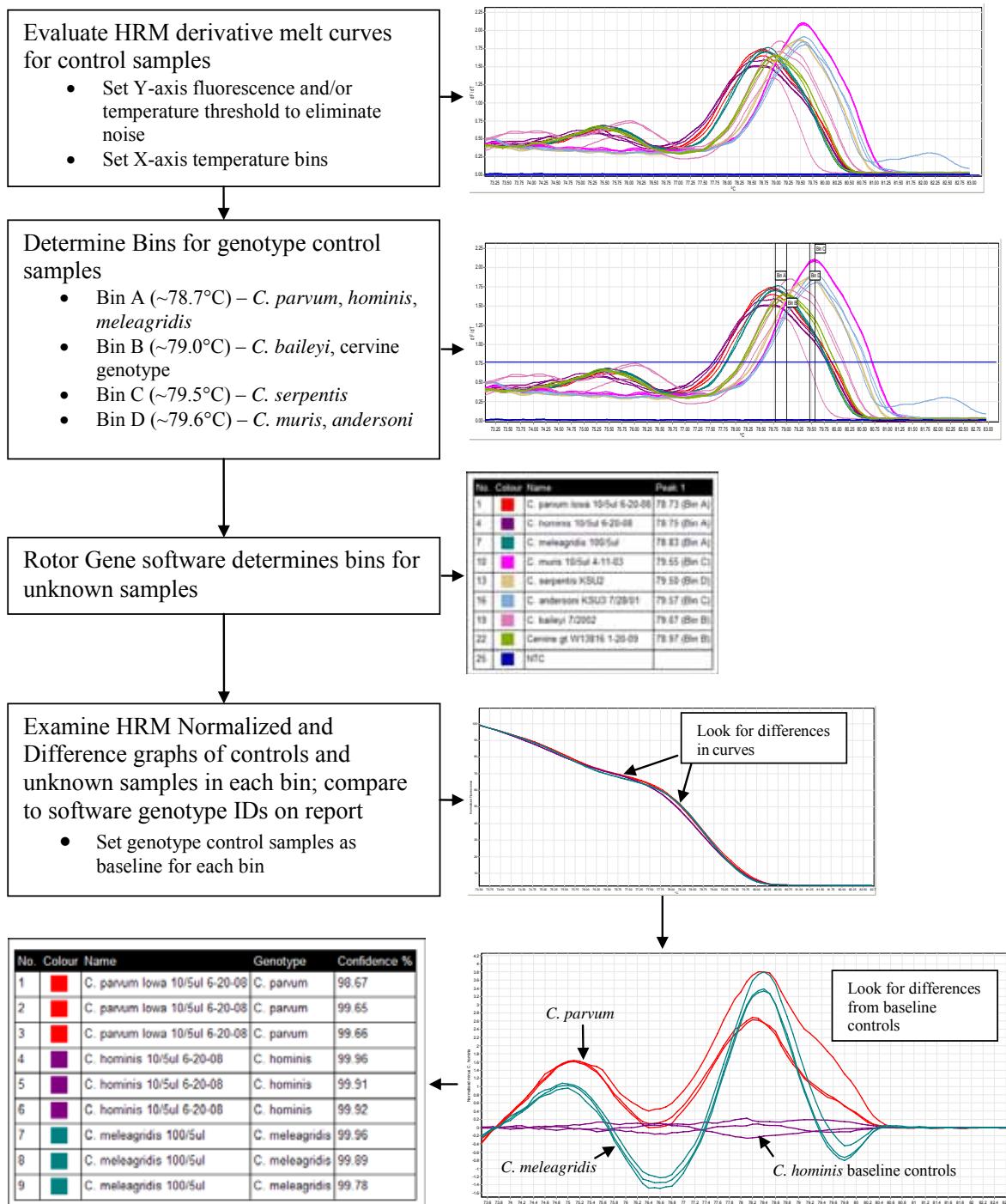
differentiate *C. parvum*, *C. hominis*, *C. meleagridis*, *C. muris*, *C. baileyi*, *C. andersoni*, *C. serpentis* and the cervine genotype was evaluated using simplex 18S PCR and the RotorGene 6000 HRM thermal cycler. It should also be noted that these evaluations were performed using SYBR Green and therefore melt temperatures in these examples are lower than will be obtained using the final method with EvaGreen. Further, it should be noted that there are many different factors which affect high-resolution melt curves, including dUTP and magnesium chloride concentrations and the use of different PCR buffers. Deviation from the final protocol included in this report will result in different HRM results.

Results indicated that the *Cryptosporidium* genotypes could be grouped into four melt peak bins, differentiating human-pathogenic genotypes (*C. parvum*, *C. hominis*, and *C. meleagridis*) from animal-associated genotypes. The bins contained the following species: *C. parvum*, *C. hominis*, and *C. meleagridis* (Bin A); *C. baileyi* and the cervine genotype (Bin B); *C. serpentis* (Bin C), and; *C. muris* and *C. andersoni* (Bin D). HRM genotyping indicated that *Cryptosporidium* genotypes within the same melt peak bin could also be differentiated (e.g. *C. parvum*, *C. hominis*, and *C. meleagridis*; [Figure 3.10](#)). To interpret the HRM genotyping data, a multi-step process was followed. This process included the evaluation of melt peaks, followed by further HRM analysis of samples within the same bin using the system software. A flow diagram for HRM genotyping data interpretation is included as [Figure 3.11](#). In addition to the subjective interpretation of the HRM results, the system software may be used to automatically identify the genotypes (lower left panel of [Figure 3.11](#)). The default confidence level threshold for automated genotype identification is 90%, but can be modified by the user. Importantly, appropriate *Cryptosporidium* genotype controls should be included in the PCR run for accurate HRM analysis of unknown samples.

Since hsp70 false negatives may occasionally occur for human-pathogenic oocysts, HRM genotyping is not only useful for further discrimination of *Cryptosporidium* genotypes but also confirmation of 18S products. Therefore, when using conventional PCR, samples which yield only the 18S PCR product should be confirmed using HRM analysis (post-PCR HRM analysis) or DNA sequencing to avoid incorrectly identifying the sample as animal-associated *Cryptosporidium*.



**Figure 3.10 Differentiation of *Cryptosporidium* genotypes within the same melt peak bin using HRM genotyping.** This example is an HRM difference graph of 18S PCR products from Bin A human-pathogenic *C. parvum*, *C. hominis*, and *C. meleagridis*.



**Figure 3.11 Flow diagram for interpretation of RotorGene HRM genotyping data. HRM data was obtained for different *Cryptosporidium* genotypes using simplex 18S PCR. Note that these data were obtained using SYBR Green and therefore melt temperatures are lower than will be obtained using the final method with EvaGreen.**

## **EXTRACTION AND PURIFICATION OF OOCYST DNA**

Efficient extraction and purification of DNA from single oocysts was a critical component for development of a successful slide genotyping method. The use of Chelex 100 resin for the extraction of DNA has been reported by *Cryptosporidium* researchers and others. Chelex resin chelates divalent cations, and although the exact mechanism of action is uncertain, it is thought to protect DNA during the freezing and thawing cycles commonly used to lyse *Cryptosporidium* oocysts. Chelex has been shown to be as efficient as proteinase K and phenol-chloroform extraction (Walsh, Metzger et al. 1991). We have used Chelex in our *Cryptosporidium* and *Cyclospora* oocyst DNA extraction procedures for several years (e.g. (Sturbaum, Ortega et al. 1998; Di Giovanni, Hashemi et al. 1999; LeChevallier, Di Giovanni et al. 2003; Di Giovanni, Betancourt et al. 2006). For this project, modifications of our Chelex 100/Tris EDTA (Chelex/TE) oocyst freeze-thaw lysis method (Di Giovanni and LeChevallier 2005) were evaluated.

While oocyst DNA may be used directly for the PCR, additional purification may be necessary to remove PCR inhibitors present in environmental matrices. Two kit-based DNA purification methods were evaluated: the Invitrogen ChargeSwitch kit and the Qiagen DNA kit. The ChargeSwitch kit was designed for the purification of DNA from very small samples, such as forensic samples. The ChargeSwitch DNA kit was successfully used in WaterRF Project 3021 for the purification of *Cryptosporidium* DNA from single clusters of cell culture infection. The oocyst DNA purification protocol of Ruecker et al. (Ruecker, Bounsombath et al. 2005) using the Qiagen kit with carrier DNA was also evaluated.

Our standard Chelex/TE freeze-thaw method (Di Giovanni and LeChevallier 2005) was performed using eight cycles of freezing in liquid nitrogen and thawing at 94°C to 98°C for approximately 1 min each cycle. Chelex/TE freeze-thaw method modifications were evaluated, including: the use of ChargeSwitch kit lysis buffer during the Chelex freeze-thawing step; the addition of the ChargeSwitch lysis buffer to the sample lysate after Chelex freeze-thawing; and the use of lower concentrations of TE buffer (e.g. 0.5X) or molecular grade water (MGW) for preparation of the Chelex slurry. Chelex freeze-thaw lysates were then either used directly as template in PCR or further purified using the ChargeSwitch or Qiagen DNA purification kits.

### **Effect of ChargeSwitch Kit Elution Buffer on PCR**

One of the first issues to be evaluated was the effect of the ChargeSwitch kit elution buffer on PCR. The kit uses a special buffer optimized for elution of DNA from the magnetic beads used in the kit. Typically this solution buffer would not have any effect on downstream PCR analysis of the samples. However, due to the very low concentration of DNA in our application, we use half of the purified sample in a single PCR. This could potentially lead to adverse effects on the PCR due to the composition of the kit elution buffer. To evaluate this possibility, reaction mixes were prepared using either molecular grade water (MGW) or up to 15 µl of ChargeSwitch kit elution buffer. All reactions ( $n = 3$ ) were seeded with oocyst DNA equivalent to approximately 10 oocysts. Reactions prepared with MGW had a mean  $C_T$  of  $29.7 \pm 0.3$ , while reactions prepared with elution buffer had a mean  $C_T$  of  $29.5 \pm 0.1$ . Therefore, results indicated that the ChargeSwitch kit elution buffer did not have any adverse effect on PCR amplification.

### Initial Evaluation of Chelex/TE Freeze-Thaw Lysis With and Without ChargeSwitch Lysis Buffer and ChargeSwitch DNA Purification

Several experiments were performed to compare our standard Chelex/TE freeze-thaw oocyst lysis method to the use of Chelex/TE/ChargeSwitch lysis buffer (Table 3.1). The lysates were used directly as template in the PCR for the standard Chelex/TE freeze-thaw lysis method. For Chelex/TE/lysis buffer freeze-thawed samples, oocyst DNA was further purified using the ChargeSwitch kit. Flow-sorted *C. parvum* oocysts in microcentrifuge tubes were used for these experiments.

**Table 3.1**  
**Comparison of oocyst lysis using Chelex/TE freeze-thawing or Chelex/TE/lysis buffer freeze-thawing plus ChargeSwitch DNA purification**

Number of flow-sorted oocysts	Chelex/TE freeze-thawing		Chelex/TE/lysis buffer freeze-thawing plus ChargeSwitch DNA purification	
	Number of positive PCR/number of replicates	Mean ± SD C <sub>T</sub> Value	Number of positive PCR/number of replicates	Mean ± SD C <sub>T</sub> Value
1	0/6	NA*	0/13	NA
3	0/6	NA	9/15	36.8 ± 1.6
5	0/5	NA	8/15	35.6 ± 1.9

\*NA, not applicable.

These results were somewhat unexpected, since none of the standard Chelex/TE freeze-thaw samples tested positive. We routinely use this method to prepare oocyst DNA PCR positive controls. However, we typically use 5 µl or less of the oocyst lysate per PCR, while for this project we used the entire sample lysate (20 µl) in a single PCR. Therefore, we suspected PCR inhibition due to carryover of too much TE buffer into the PCR. Additional experiments were designed to address this issue.

### Effect of TE Buffer From Chelex/TE on PCR

Several experiments were performed to address the potential PCR inhibition caused by TE buffer in the Chelex/TE slurry. Reaction mixes were either prepared with molecular grade water (MGW) or the supernatant from Chelex/1X TE or Chelex/0.5X TE sample blanks. Reactions were seeded with *C. parvum* oocyst DNA equivalent to approximately 2 or 10 oocysts prepared using the standard Chelex/TE freeze-thaw method (our standard PCR positive control method). Results are presented in [Table 3.2](#).

**Table 3.2**  
**Effect of carryover TE buffer from the Chelex/TE lysis method on PCR**

DNA concentration (approx. no. oocysts)	MGW*		Chelex/0.5X TE		Chelex/1X TE	
	Number of positive PCR/number of replicates	Mean ± SD C <sub>T</sub> Value	Number of positive PCR/number of replicates	Mean ± SD C <sub>T</sub> Value	Number of positive PCR/number of replicates	Mean ± SD C <sub>T</sub> Value
2	6/6	33.3 ± 0.7	3/3	35.7 ± 0.8	3/3	42.2 ± 3.6
10	6/6	30.5 ± 0.7	3/3	35.6 ± 2.3	0/6	NA**

\*MGW, molecular grade water.

\*\*NA, not applicable.

The data clearly indicated increasing PCR inhibition with increasing TE buffer carryover/concentration. Magnesium in the PCR mix is needed for *Taq* DNA polymerase activity and EDTA in the TE buffer can chelate magnesium and cause PCR inhibition, although the PCR was unexpectedly sensitive to even small amounts of TE buffer. Even reaction mixes prepared with only MGW showed an adverse effect of carryover TE from the Chelex/TE oocyst DNA lysates used to seed the reactions, with the C<sub>T</sub> values of reactions seeded with 10 oocyst DNA (5 µl of lysate) being higher than reactions seeded with 2 oocyst DNA (1 µl of lysate). We therefore investigated the use of Chelex slurry prepared with MGW instead of TE buffer.

### Comparison of Oocyst Lysis Using Chelex/MGW or MGW Only

Due to the PCR inhibition problems caused by TE buffer in our standard Chelex/TE lysis method, we evaluated the preparation of Chelex slurry in molecular grade water (Chelex/MGW). We had some concerns that Chelex/MGW might not provide the same amount of DNA

protection during the freeze thaw lysis as the standard Chelex/TE. This is because TE adds pH buffering to the Chelex slurry, and also due to the metal chelating property of EDTA that may function synergistically with the divalent cation chelating property of Chelex resin.

We performed several experiments to evaluate whether the use of Chelex/MGW for freeze-thaw lysis provided a benefit over freeze-thaw lysis in only MGW. IFA-enumerated *C. parvum* oocyst stocks were used for these experiments. Oocysts were freeze-thaw lysed in either Chelex/MGW or in MGW only, and sample volumes adjusted so that the Chelex/MGW supernatants (the slurry is half resin by volume) and the MGW only samples had equivalent DNA concentrations.

**Table 3.3**  
**Comparison of oocyst freeze-thaw lysis with Chelex/MGW or MGW only**

Approx. number of oocysts	Chelex/MGW*			MGW**	
	Number of positive PCR/number of replicates	Mean ± SD $C_T$ Value	Number of positive PCR/number of replicates	Mean ± SD $C_T$ Value	
10	10/10	46.9 ± 1.7	0/10	NA***	
100	5/5	30.6 ± 0.4	2/5	49.6 ± 2.6	

\*Chelex/MGW, Chelex/molecular grade water slurry.

\*\*MGW, molecular grade water.

\*\*\*NA, not applicable.

Results indicated that samples lysed with Chelex/MGW amplified better than samples lysed with MGW only (Table 3.3). Although all replicates of Chelex/MGW freeze-thawed samples amplified,  $C_T$  values were unexpectedly high for the number of oocysts compared to experiments with flow-sorted oocysts (*C. parvum* Iowa (CpAZ)). There were two lots of *C. parvum* Iowa (Waterborne) oocysts used for these experiments. The same lot of oocysts was used for two trials at the 10 oocyst level and was 19 days post-shedding in age. A different lot of oocysts was used for the 100 oocyst level experiment and was 7 days post-shedding in age. There are several possible explanations for these findings, including errors in oocyst levels due to IFA enumeration and dilution and/or differences in lysis efficiency or oocyst integrity between the Waterborne and CpAZ Iowa isolates. Previous research by Nichols and Smith (Nichols and Smith 2004) reported differences in the efficiency of freeze-thaw lysis for different *C. parvum* isolates. This latter possible explanation was investigated through side-by-side lysis comparison using flow-sorted *C. parvum*, *C. hominis* and *C. muris* oocysts (see “Comparison of Oocyst Lysis Using Chelex/MGW With Different Freeze-thaw Conditions”).

### Comparison of Chelex/MGW Oocyst Lysis With and Without Further ChargeSwitch DNA Purification

Since the use of Chelex/MGW appeared useful for oocyst lysis, additional trials were performed evaluating its use with and without further DNA purification using the ChargeSwitch kit. Flow-sorted *C. parvum* Iowa (CpAZ) oocysts in microcentrifuge tubes were used for these experiments. Chelex/MGW freeze-thaw lysis was performed, then the lysates were used either directly as template in PCR or further purified using the ChargeSwitch kit.

**Table 3.4**  
**Comparison of oocyst freeze-thaw lysis with Chelex/MGW with and without further ChargeSwitch DNA purification**

Number of flow-sorted oocysts	Chelex/MGW*		Chelex/MGW + ChargeSwitch DNA purification	
	Number of positive PCR/number of replicates	Mean ± SD C <sub>T</sub> Value	Number of positive PCR/number of replicates	Mean ± SD C <sub>T</sub> Value
1	3/5	35.4 ± 0.4	1/5	37.4
3	5/5	34.4 ± 0.6	3/5	41.1 ± 8.3

\*Chelex/MGW, Chelex/molecular grade water slurry.

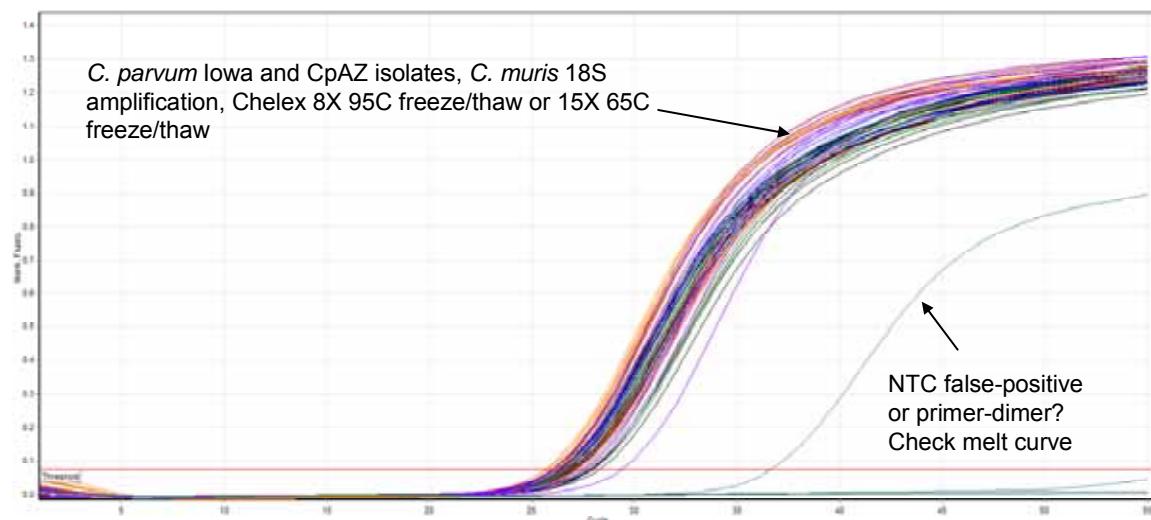
The use of Chelex/MGW without additional ChargeSwitch DNA purification was the most successful, with 3 out of 5 replicates for a single oocyst and 5 out of 5 replicates at the 3 oocyst level testing positive with a single round of hsp70 PCR (Table 3.4). Fewer positives for samples further purified with the ChargeSwitch DNA kit were likely due to loss of DNA during purification. This explanation is supported by the higher C<sub>T</sub> values observed for those samples that did amplify.

Based on these results, ChargeSwitch DNA purification did not appear as successful as using Chelex/MGW oocyst lysates directly in the PCR. However, the benefits of DNA purity and removal of PCR inhibitors versus DNA concentration needed to be weighed for the analysis of environmental samples. Subsequently, an experiment was performed using pooled environmental water matrix to investigate the need for DNA purification (see “Evaluation of Matrix Effect on PCR Using Pooled Scrapings From *Cryptosporidium*-Negative Method 1622 Field Slides”).

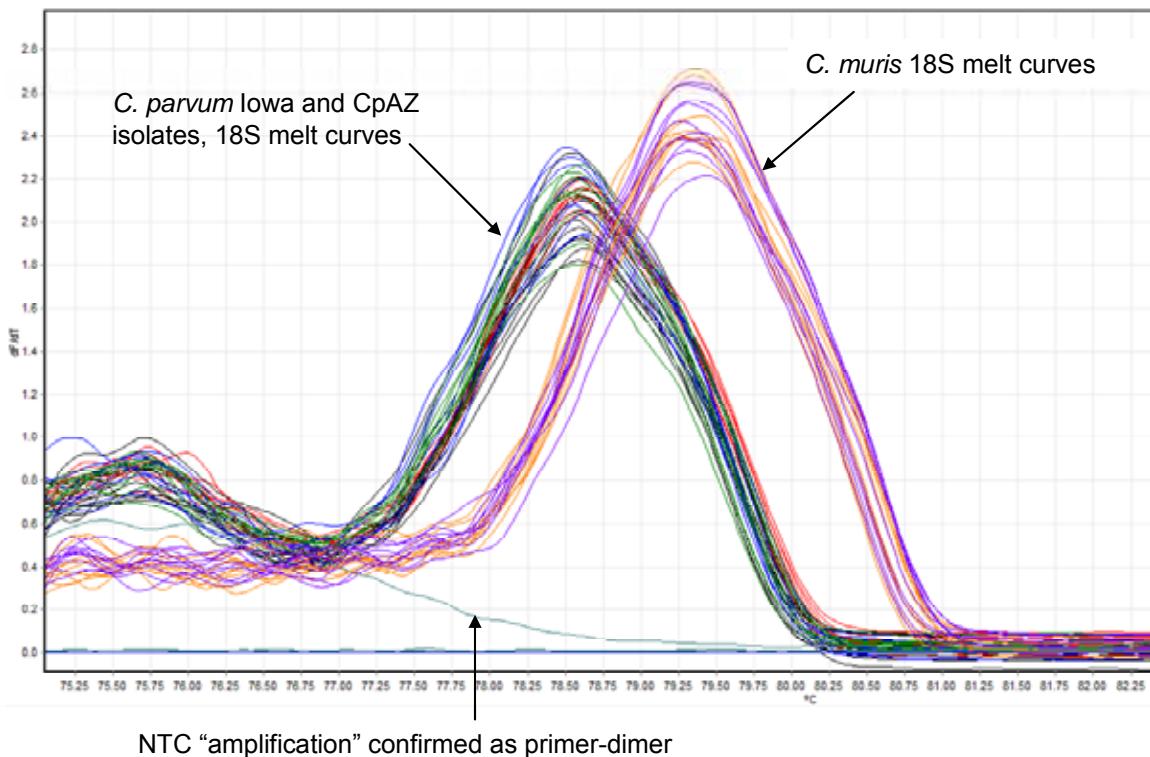
## Comparison of Oocyst Lysis Using Chelex/MGW With Different Freeze-Thaw Conditions

Two sets of experiments were performed to evaluate the lysis efficiency of 8 cycles of Chelex/MGW freeze-thawing with liquid nitrogen and 95°C (our protocol) compared to 15 cycles of freeze-thawing with liquid nitrogen and 65°C (SPDL protocol). The first experiment included the use of *C. parvum* Iowa, *C. parvum* Iowa (CpAZ), and *C. muris* oocysts. Ten oocysts of each isolate were flow-sorted into microcentrifuge tubes, with twenty replicates prepared per isolate. Chelex/MGW was added to each sample and ten replicates of each isolate were subjected to each of the freeze-thaw conditions. The entire lysate of each sample was analyzed using real-time simplex 18S PCR. Results indicated the samples all had similar amplification profiles (Figure 3.12). Statistical analysis of  $C_T$  values using ANOVA indicated that there were no significant differences between the two freeze-thaw methods ( $P > 0.05$ ), with the exception of *C. muris*. For *C. muris* the eight cycles of freeze-thawing in liquid nitrogen and 95°C provided slightly better amplification ( $P = 0.009$ ), with a mean  $C_T$  value of  $25.99 \pm 0.23$  as compared to  $27.02 \pm 1.09$  ( $n = 10$ ) for the SPDL freeze-thaw cycling.

In this experiment, one no template negative control (NTC) sample appeared to have late amplification (Figure 3.12). Upon examination of the high resolution melt curve, the “amplification” was confirmed to have been caused by primer-dimer formation (Figure 3.13). This conclusion was confirmed by gel electrophoresis of the samples, and a faint primer-dimer band was observed for the sample (data not shown).

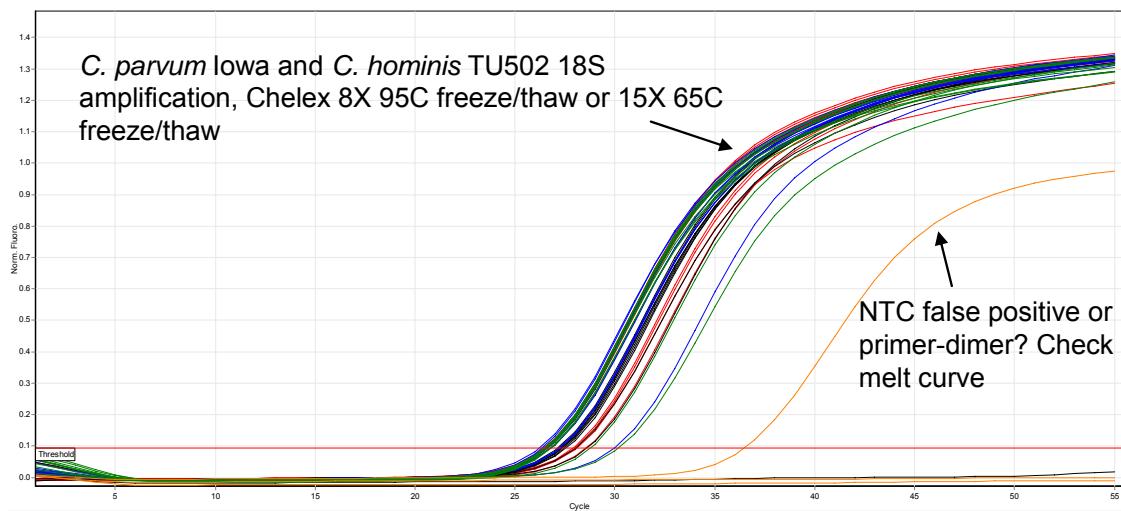


**Figure 3.12** Simplex 18S PCR amplification plot for *C. parvum* Iowa, *C. parvum* Iowa (CpAZ), and *C. muris* oocysts. Each sample contained ten flow-sorted oocysts lysed using Chelex/MGW and either 8 cycles of freeze-thawing with liquid nitrogen and 95°C or 15 cycles of freeze-thawing with liquid nitrogen and 65°C.

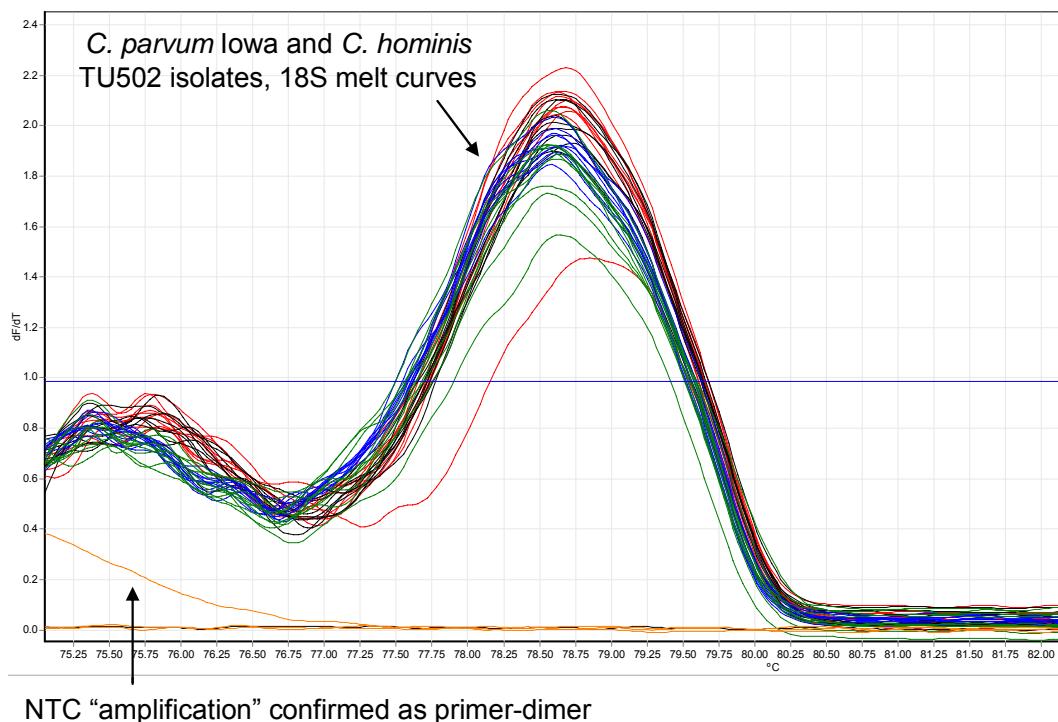


**Figure 3.13** High resolution melt curves of simplex 18S PCR amplicons from *C. parvum* Iowa, *C. parvum* Iowa (CpAZ), and *C. muris* oocysts.

For the second lysis efficiency experiment, *C. parvum* Iowa and *C. hominis* TU502 oocysts were evaluated in the same manner as in the first lysis experiment. The *C. parvum* Iowa oocysts used for this experiment came from a different lot of oocysts than the first experiment to see if there were any lot-to-lot variations in lysis efficiency. As observed in the first experiment, the amplification plots for the oocysts lysed with the two different freeze-thaw conditions were similar (Figure 3.14), and statistical analysis of  $C_T$  values using ANOVA indicated that there were no significant differences ( $P > 0.05$ ). Similar to the first experiment, a single no template negative control (NTC) sample appeared to have late amplification (Figure 3.14). Upon examination of the high resolution melt curve, the amplification was also confirmed to have been caused by primer-dimer formation (Figure 3.15). As opposed to the melt curve differences observed between *C. parvum* and *C. muris* (Figure 3.13), the *C. parvum* and *C. hominis* 18S amplicons could not be distinguished simply on the appearance of their melt curves. This was anticipated due to their close phylogenetic relatedness. However, subsequent experiments determined that HRM genotyping could successfully differentiate these closely related species (see Figure 3.10).



**Figure 3.14** Simplex 18S PCR amplification plot for *C. parvum* Iowa and *C. hominis* oocysts. Each sample contained ten flow-sorted oocysts lysed using Chelex/MGW and either 8 cycles of freeze-thawing with liquid nitrogen and 95°C or 15 cycles of freeze-thawing with liquid nitrogen and 65°C.



**Figure 3.15** High resolution melt curve of simplex 18S PCR amplicons from *C. parvum* Iowa and *C. hominis* oocysts.

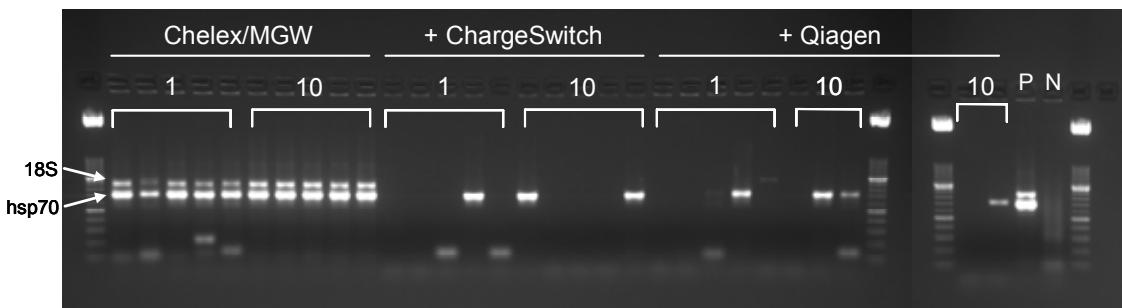
### Comparison of Oocyst DNA Preparation Using Chelex/MGW With and Without Subsequent DNA Purification Using the ChargeSwitch Kit or Qiagen Kit With Carrier DNA

As described earlier, preliminary experiments indicated that using Chelex/MGW oocyst lysates directly in the PCR might be a better approach than attempting to further purify the DNA. However, there was still some concern that DNA purification would be required to remove PCR inhibitors from environmental samples. However, reduced DNA recovery with increased sample manipulation is a common issue, and the benefits of DNA purity and removal of PCR inhibitors need to be balanced with DNA yield to optimize detection.

To address this issue a series of experiments were performed to evaluate the direct use of oocyst lysates in the PCR compared to samples receiving additional purification using the ChargeSwitch or Qiagen DNA kits with the use of carrier DNA. The use of carrier DNA is a critical component of the Qiagen DNA purification protocol used by Norma Ruecker in Norm Neumann's laboratory (University of Calgary and Alberta Provincial Public Health Lab) for the purification of oocyst DNA from Method 1622/23 slides (Ruecker, Bounsombath et al. 2005). Norma kindly provided us with a detailed copy of her current protocol, which we followed with only two modifications: omission of the heat lysis step (not needed since oocysts were already lysed), and elution of DNA with 50  $\mu$ l of elution buffer (twice with the same volume) instead of elution with 100  $\mu$ l. We also added the use of carrier DNA to the ChargeSwitch protocol in an attempt to increase DNA yields. Five replicates of 1 and 10 flow-sorted *C. parvum* oocysts in microcentrifuge tubes were evaluated using each of the DNA preparation methods.

Direct use of the Chelex/MGW oocyst lysates performed significantly better than samples receiving additional DNA purification with either the ChargeSwitch or Qiagen kits. All five replicates of 1 and 10 oocyst Chelex/MGW lysates amplified, with mean  $C_T$  values of  $27.98 \pm 0.27$  and  $25.52 \pm 0.94$ , respectively. In contrast, the ChargeSwitch purified samples had only one replicate of a single oocyst that amplified ( $C_T = 34.70$ ), and only two of the 10 oocyst samples amplified ( $C_T$  values of 35.47 and 38.28). The Qiagen purified samples amplified poorly as well, and had only one replicate of a single oocyst that amplified ( $C_T = 33.95$ ), and only three of the 10 oocyst samples amplified ( $C_T$  values of 29.83, 33.09, and 42.60).

Gel electrophoresis analysis of PCR products revealed some interesting findings (Figure 3.16). As observed in earlier multiplex PCR trials with higher numbers of oocysts, the hsp70 amplicon was amplified more efficiently than the 18S amplicon despite there being three times as many copies of 18S target compared to the hsp70 target. The preferential amplification of the hsp70 amplicon is likely due to several factors, including the slightly smaller size of the hsp70 product (346 bp vs. 435 bp), and that the annealing temperature is slightly higher than optimum for the 18S primer set. It is therefore likely that both hsp70 and 18S targets were present for the ChargeSwitch and Qiagen samples, but at very low template levels, and the hsp70 target was preferentially amplified.



**Figure 3.16 Direct use of Chelex/MGW oocyst DNA as PCR template compared to additional DNA purification using the ChargeSwitch kit or Qiagen kit with carrier DNA. One (1) and ten (10) flow-sorted *C. parvum* oocysts were used, and all samples were analyzed using hsp70 and 18S multiplex PCR. P, *C. parvum* positive control; N, negative control.**

#### Evaluation of Matrix Effect on PCR Using Pooled Scrapings From *Cryptosporidium*-Negative Method 1622 Field Slides

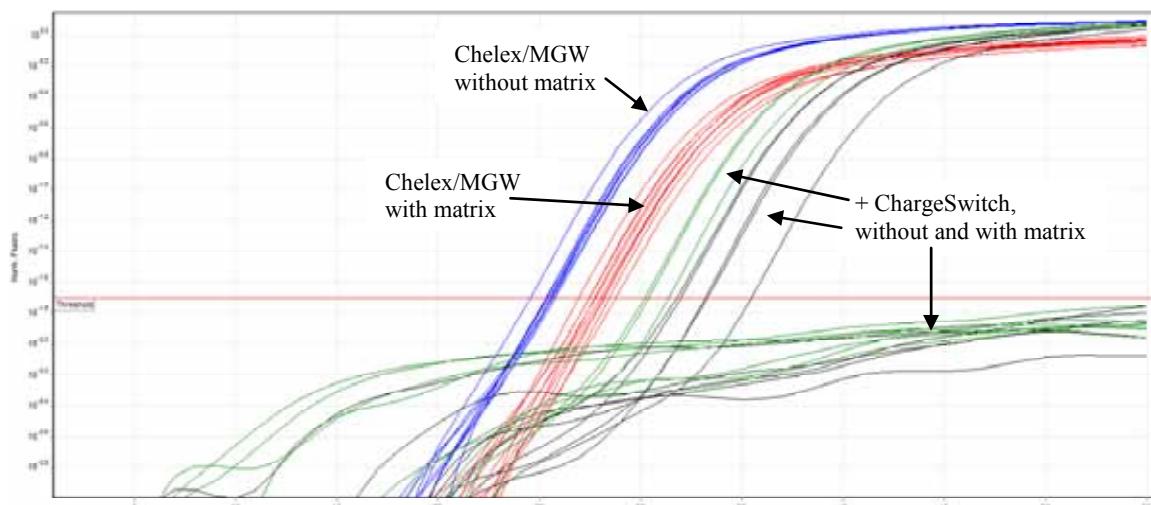
From previous experiments it was shown that the Chelex/MGW method provided the highest oocyst DNA yield, while the ChargeSwitch and Qiagen kits provided lower DNA yield but likely greater DNA purity and removal of PCR inhibitors. Despite these findings, it was still possible that environmental samples would require DNA purification for the removal of PCR inhibitors. The balance between higher template concentration and DNA purity still needed to be evaluated, and therefore an experiment was performed to evaluate the effect of Method 1622 field matrices on PCR. Twenty two *Cryptosporidium*-negative Method 1622 field slides were scraped using closed cell foam swabs and the scrapings/washes pooled. The pooled slide scrapings/washes was very dirty and contained a significant amount of debris (Figure 3.17), providing a good challenge to PCR. The use of a pooled sample provided a homogeneous environmental matrix for the comparison of the use of Chelex/MGW oocyst lysates in the PCR with and without further DNA purification using the ChargeSwitch kit.



**Figure 3.17 Pooled scrapings/washes from *Cryptosporidium*-negative Method 1622 field slides used to evaluate matrix effects on PCR and the need for DNA purification.**

Twenty microliters of the pooled slide scrapings/washes were aliquoted into twenty microcentrifuge tubes and sent to the WSLH. Flow cytometry was used to seed each tube with ten *C. parvum* oocysts and to prepare ten oocyst matrix-free controls. Oocyst DNA was prepared using the Chelex/MGW freeze-thaw procedure, with half of the replicates receiving further DNA purification with the ChargeSwitch kit. Two PCR runs were performed, the first using 5 µl of each 20 µl DNA sample as template, and the second using the remaining 15 µl of each sample. These conditions allowed us to better evaluate the effects of environmental matrices on PCR.

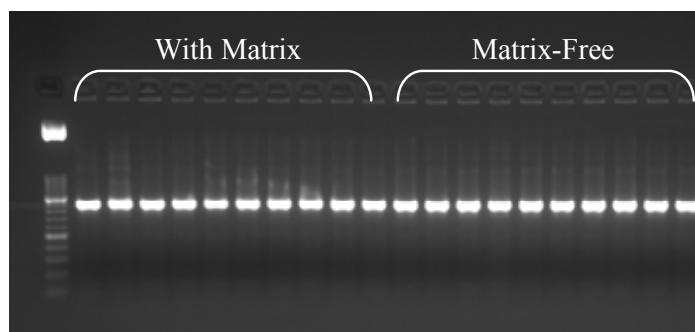
As observed previously, the use of the Chelex/MGW lysates directly in the PCR performed much better than samples receiving additional DNA purification. For the PCR run using 5 µl of DNA template, all ten replicates of Chelex/MGW samples with and without environmental matrix amplified. Results for these samples indicated that there was only a minor effect of the matrix on PCR, with matrix-free control samples having a mean  $C_T$  of  $26.01 \pm 0.19$ , while samples with matrix had a mean  $C_T$  of  $26.69 \pm 0.50$ . In contrast, the ChargeSwitch purified samples amplified poorly and only six of ten matrix-free control replicates (mean  $C_T$  of 40.40), and only four of ten replicates with matrix had amplification (mean  $C_T$  of 40.10).



**Figure 3.18 Effects of pooled scrapings/washes from *Cryptosporidium*-negative Method 1622 field slides on PCR.** Samples were seeded with 10 flow-sorted *C. parvum* oocysts and lysed using the Chelex/MGW freeze-thaw procedure, with and without subsequent ChargeSwitch DNA purification, and analyzed using simplex 18S PCR.

Similar results were obtained for the PCR run using 15  $\mu$ l of each DNA sample as template, with the exception that the matrix effect was more pronounced for the Chelex/MGW samples, although all ten replicates with matrix still amplified (Figure 3.18). Chelex/MGW matrix-free control samples had a mean  $C_T$  of  $25.44 \pm 0.34$ , while samples with matrix had a mean  $C_T$  of  $27.91 \pm 0.53$ . Despite this reduction in amplification, gel electrophoresis revealed no differences in amplicon intensities for samples with and without matrix (Figure 3.19). Similar to the previous experiment, the ChargeSwitch purified samples amplified poorly, and only three of ten matrix-free control replicates and four of ten replicates with matrix had amplification.

In summary, the most successful approach was the direct use of the Chelex/MGW lysates as template in the PCR, without further purification of DNA. It is noteworthy that this approach allowed much of the environmental debris to be carried over into the PCR, with only the larger debris separated by the brief centrifugation used. These data indicated that the hsp70 and 18S multiplex PCR was robust and moderately resistant to matrix effects.

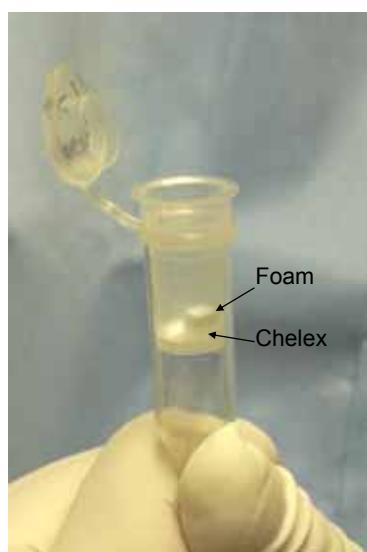


**Figure 3.19 Gel electrophoresis analysis of simplex 18S PCR products from Chelex/MGW oocyst lysate samples with and without pooled Method 1622 field matrix.**

## Spin Filtration Removal of Chelex From DNA Extracts

Chelex resin used in the DNA extraction procedure chelates divalent cations, including magnesium, which is a metal cofactor required for *Taq* DNA polymerase activity. PCR inhibition can be caused by Chelex resin carried over into the PCR cocktails, with small amounts causing reduction in amplification and shifted melt curves, while large amounts will completely inhibit the PCR. Chelex carryover is not an issue when using our Chelex oocyst DNA PCR positive controls since it is easy to avoid the Chelex resin at the bottom of the tube when withdrawing the supernatant for use as PCR template. In contrast, it can be difficult to avoid Chelex resin when working with samples from slides, since the entire sample supernatant will be used as PCR template.

Since we wanted the final protocol to be user-friendly and robust, we investigated the use of microcentrifuge spin filters for the removal of Chelex resin from samples. The spin filters have a 0.45 µm cellulose acetate membrane that filters out the Chelex resin and the foam used to scrape slides ([Figure 3.20](#)). In addition, spin filtration also removes environmental debris (e.g. clay, silt, and algae) and IMS beads co-recovered with oocysts from slides, potentially preventing interference with the PCR. Despite these advantages, there was still the possibility that some oocyst DNA would be lost when using the spin filters, so we investigated the possibility. Aliquots of supernatant from a Chelex oocyst DNA PCR positive control were transferred to new microcentrifuge tubes and additional molecular grade water (MGW) and Chelex/MGW slurry added to each. The samples, including the Chelex resin, were then transferred to spin filters and centrifuged. Tubes were rinsed with a small volume of MGW, the rinses transferred to the spin filters, and the centrifugation repeated. Analysis of real-time PCR  $C_T$  values indicated that the spin filtration did not cause any measurable loss of DNA ( $P = 0.31$ ), and melt curves for both types of samples were similar (data not shown). Excellent results were obtained in subsequent experiments with single oocyst tube controls, matrix-free slides, and seeded UK DWI field matrix slides processed with the protocol incorporating spin filtration (see [Table 3.7](#)).



**Figure 3.20 Spin filter removal of Chelex resin used in the oocyst DNA extraction procedure and foam used to scrape slides. The filter insert is removed from the microcentrifuge tube and the Chelex-free sample filtrate at the bottom of the tube is used as PCR template.**

## METHOD EVALUATION USING MATRIX-FREE AND SEEDED FIELD SLIDES

### Removal of Residual Mounting Medium From Slides

PCR experiments performed during the method optimization phase of this project used oocysts sorted into microcentrifuge tubes rather than onto slides, and typically 7/10 to 10/10 of the single oocyst tube controls tested positive. In addition, method optimization experiments performed early on in the project indicated that steps for the removal of oocysts from slides and DNA extraction were efficient. We then began experiments using seeded slides to evaluate the draft method protocol. To our surprise, poor results were obtained for initial experiments with matrix-free slides seeded with single oocysts, and only 1/10 to 3/10 samples tested positive by PCR.

One possible explanation for the poor performance was interference by residual mounting medium on the slides. Mounting media can contain pH buffers and glycerol which may interfere with the PCR. A wash of the slide well was therefore incorporated into the protocol to remove traces of mounting media. An experiment was performed to verify retention of oocysts on the slides following this additional wash. Matrix-free slides (20 replicates) were seeded with pre-stained single flow-sorted *C. parvum* oocysts and mounted with coverslips. Coverslips were removed the following day and residual mounting medium was removed using a 50 µL water rinse of the slide well. Washed slides were examined using IFA microscopy and no oocyst loss was observed.

### Adverse Effects of Slide Mounting Media Containing Formalin on PCR Amplification

Incorporation of the water wash to remove residual mounting medium resulted in only a minor improvement in PCR detection. It was subsequently hypothesized that there was a lasting adverse effect of a reagent used in slide preparation which was interfering with the assay. We then realized that some mounting media may also contain formalin. Formalin may cause DNA fragmentation and fixation of proteins to DNA, both of which interfere with the PCR. Upon further investigation it was found that the Waterborne, Inc. mounting medium that WSLH had been using for slide preparation contained 2% formalin. Waterborne prepared a formalin-free version of their mounting medium for our evaluation.

The Waterborne mounting media, as well as the Method 1623, Meridian Merifluor (Meridian Bioscience, Inc.; Cincinnati, Ohio), and BTF mounting media were evaluated for their effects on PCR detection. Results clearly indicated that formalin-containing mounting media adversely affected the PCR detection of oocysts ([Table 3.5](#)). Collectively, 37 of 40 slides prepared with mounting media not containing formalin tested positive by PCR compared to only 6 of 30 slides prepared with mounting media containing formalin. Therefore, the use of mounting media containing formalin is not recommended when slide genotyping will be performed.

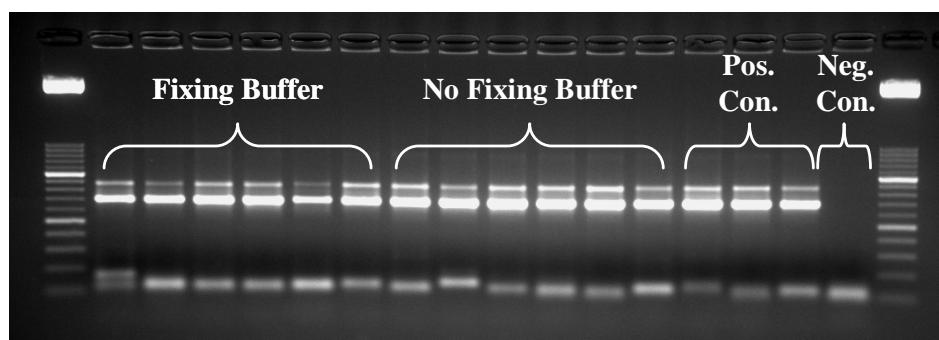
The BTF mounting medium does not contain formalin, however, the BTF Fixing Buffer used in slide staining does. In a follow-up experiment, samples processed with BTF Fixing Buffer exhibited a slight reduction in amplification and greater variability based on real-time PCR results and gel electrophoresis analysis of products ([Figure 3.21](#)). Although the BTF mounting medium does not contain formalin and does not appear to affect PCR detection, the use of the BTF Fixing Buffer is not recommended. The Cellabs mounting medium, which does not contain formalin, was tested in a subsequent trial and found to be compatible with the slide

genotyping method (see “Method Evaluation Using Seeded UK DWI Field Slides” and Table 3.7). Method 1623 and Cellabs mounting media were used for subsequent method evaluation trials.

**Table 3.5**  
**Effect of slide mounting media on PCR detection of matrix-free slides seeded with single flow-sorted *C. parvum* oocysts**

Mounting medium	Contains formalin	Number of PCR-positive replicates	
		Experiment 1	Experiment 2
Waterborne, Inc. with formalin	Yes	1/10	2/10
Waterborne, Inc. without formalin	No	9/10	ND*
Method 1623	No	10/10	9/10
Meridian	Yes	3/10	ND
BTF (without the use of Fixing Buffer)	No	9/10	ND

\*ND, not done.



**Figure 3.21** Gel electrophoresis analysis of 18S and hsp70 PCR products from *C. parvum* seeded microscope slides stained with or without BTF Fixing Buffer containing formalin.

## Method Evaluation Using Seeded Method 1623 Field Slides

After resolving the mounting medium issue, the final method protocol was ready to be evaluated using seeded field slides. Method 1623 field slides from various source waters which previously tested negative for *Cryptosporidium* using IFA microscopy (*Cryptosporidium*-negative) were provided by CHD and were sent to WSLH for seeding with oocysts. WSLH removed the slide coverglasses; removed residual mounting medium using warm PBS while being careful not to disturb the environmental debris; then flow sorted either a single *C. parvum* or *C. muris* oocyst directly onto each slide. Slides were remounted using Method 1623 mounting medium and sent to the AgriLife Research laboratory for processing. Microcentrifuge tubes containing single flow-sorted oocysts (tube controls) and unseeded *Cryptosporidium*-negative Method 1623 field slides were also processed as controls.

Nine of ten *Cryptosporidium*-negative Method 1623 field slides seeded with single *C. parvum* oocysts tested positive by PCR (Table 3.6). One of these nine positive samples was falsely negative for the hsp70 target, although HRM analysis of the 18S amplicon correctly identified the sample as *C. parvum*. For this experiment, results for the slide samples were actually better than the tube controls, for which 6 of 10 tested positive (with no hsp70 false negatives). Similar results were obtained for *C. muris*, with 15 of 20 Method 1623 field slides seeded with single oocysts testing positive by PCR. Results for seeded slides were similar to those obtained for the tube controls in this experiment, with 8 of 10 controls testing positive by PCR. All unseeded *Cryptosporidium*-negative Method 1623 field slides tested negative.

**Table 3.6**  
**Method evaluation using *Cryptosporidium*-negative Method 1623 field slides seeded with single flow-sorted oocysts**

Sample	Real-time PCR positives/reps	Gel electrophoresis positives/reps	HRM genotype ID <i>C. parvum/C. muris</i>	Gel electrophoresis hsp70 false negatives (false negatives for human infectious <i>Cryptosporidium</i> )
Method 1623 field matrix slides seeded with single <i>C. parvum</i> oocysts	9/10	9/10	9/0	1/9
<i>C. parvum</i> tube controls	6/10	6/10	6/0	0/6
Method 1623 field matrix slides seeded with single <i>C. muris</i> oocysts	15/20	15/20	0/15	NA*
<i>C. muris</i> tube controls	8/10	8/10	0/8	NA
Unseeded <i>Cryptosporidium</i> -negative field slide controls	0/10	0/10	NA	NA

\*NA, not applicable

### Method Evaluation Using Seeded UK DWI Field Slides

A similar evaluation of the final method was also performed using *Cryptosporidium*-negative UK DWI field slides spiked directly with *C. parvum* oocysts using flow cytometry. In addition to testing the final method on UK DWI field slides, the use of the Cellabs antibody stain

and mounting medium was also being evaluated. This was an important issue since Scottish Water uses the Cellabs stain and mounting medium and we planned on using many of their slides for the multi-laboratory method evaluation in Project 4284. The Cellabs antibody stain used by Scottish Water contained the counterstain Evans Blue which may potentially interfere with the PCR (specifically the fluorescence readings taken during real-time PCR). The Cellabs mounting medium does not contain formalin and was therefore not expected to adversely affect the PCR.

*Cryptosporidium*-negative DWI field slides were sent from the Scottish Water lab to WSLH for seeding. WSLH removed the slide coverglasses; removed residual mounting medium using warm PBS while being careful not to disturb the environmental debris; then flow sorted a single *C. parvum* oocyst directly onto each slide. The slides were remounted using Cellabs mounting medium, sealed, and sent to the AgriLife Research laboratory for processing. Microcentrifuge tubes containing single flow-sorted oocysts (tube controls) and seeded matrix-free UK DWI (with Cellabs stain and mounting medium) and Method 1623 control slides were included. Samples were processed following the final method protocol, including spin filter removal of Chelex resin.

Nine of ten *Cryptosporidium*-negative UK DWI field slides seeded with single *C. parvum* oocysts tested positive by PCR ([Table 3.7](#)). One of these nine positive samples was falsely negative for the hsp70 target, although HRM analysis of the 18S amplicon correctly identified the sample as *C. parvum*. Similar detection rates were observed for UK DWI and Method 1623 matrix-free control slides and single oocyst tube controls ([Table 3.7](#)). Overall, experiments using seeded matrix-free and field matrix slides indicated the developed method had very good performance.

**Table 3.7**  
**Method evaluation using *Cryptosporidium*-negative UK DWI field slides  
seeded with single flow-sorted *C. parvum* oocysts**

Sample	Real-time PCR positives/reps	Gel electrophoresis positives/reps	HRM <i>C. parvum</i> genotype ID	Gel electrophoresis hsp70 false negatives (false negatives for human infectious <i>Cryptosporidium</i> )
UK DWI field matrix slides seeded with single <i>C. parvum</i> oocysts	9/10	9/10	9/9	1/9
UK DWI matrix-free slides seeded with single <i>C. parvum</i> oocysts	8/10	8/10	8/8	2/8
Method 1623 matrix-free slides seeded with single <i>C. parvum</i> oocysts	10/10	10/10	10/10	1/10
Single <i>C. parvum</i> oocyst tube controls	9/10	9/10	9/9	1/9

## CHAPTER 4: SUMMARY AND CONCLUSIONS

Currently, at least 20 different *Cryptosporidium* species and approximately 50 genotypes are recognized based on oocyst morphology (size and shape) and infection site (i.e. intestines or stomach), preferential host, and genetic/genome analysis. However, *C. hominis*, *C. parvum*, and *C. meleagridis* are responsible for approximately 98% of the cryptosporidiosis in immunocompetent humans. *Cryptosporidium* species capable of infecting both humans and animals are frequently detected in environmental water samples. This is an important finding from a regulatory and utility perspective, since source waters contaminated with animal-related *Cryptosporidium* may pose little or no health risk to humans.

The overall objective of this project was to develop a simple, reliable, and cost-effective method for genotyping single *Cryptosporidium* oocysts present on USEPA Method 1622/23 and UK DWI slides. Specifically, we aimed to develop a method which was user-friendly, compatible with conventional PCR or real-time PCR using high resolution melt (HRM) analysis, and capable of distinguishing human-pathogenic (i.e. *C. hominis*, *C. parvum*, and *C. meleagridis*) from animal associated *Cryptosporidium*. Research tasks for this project focused on removal of oocysts from slides; DNA extraction and purification from single oocysts; development of a single-round hsp70 and 18S multiplex PCR; and preliminary method evaluation using matrix-free and field matrix slides seeded with single flow cytometry sorted oocysts.

### CONCLUSIONS

1. A streamlined method for the genotyping of single *Cryptosporidium* oocysts from USEPA Method 1622/23 and UK DWI slides was developed. A detailed method protocol and demonstration DVD are included in this report to facilitate technology transfer to water quality laboratories.
2. The single-round multiplex hsp70 and 18S PCR protocol allows the genotype differentiation of human-pathogenic *C. hominis*, *C. parvum*, and *C. meleagridis* from animal-associated *Cryptosporidium* genotypes (i.e. identification as human-pathogenic or animal-associated).
3. The developed method may be used with conventional and real-time PCR instruments with high resolution melt (HRM) analysis capabilities, allowing for flexibility in infrastructure investment.
4. HRM analysis allows further discrimination of species and genotypes.
5. Some slide mounting media contain formalin which has a significant adverse effect on PCR. Therefore, only mounting media that do not contain formalin (e.g. Method 1622/23, Waterborne without formalin, and Cellabs media) are recommended if slide genotyping will be performed.
6. An overall detection rate of 90% (18/20) was obtained using matrix-free Method 1623 and UK DWI slides seeded with single *Cryptosporidium* oocysts.
7. An overall detection rate of 83% (33/40) was obtained using Method 1623 and UK DWI field matrix slides seeded with single *Cryptosporidium* oocysts.

8. An hsp70 false negative rate of 14% (5 of 36 positive samples) was observed for Method 1623 and UK DWI slides seeded with single *C. parvum* oocysts. However, HRM analysis of the 18S PCR products correctly identified each sample as *C. parvum*.

## RECOMMENDATIONS

The developed method represents a critical first step for water utility and water quality laboratories wishing to gain added value from *Cryptosporidium* regulatory monitoring. PCR analysis of archived slides and slides from upcoming monitoring present excellent opportunities to obtain valuable information on the occurrence of *Cryptosporidium* genotypes in waters with only a modest amount of additional effort and cost. Identifying *Cryptosporidium* genotypes present in water will allow a more accurate assessment of the public health significance of waterborne *Cryptosporidium*, provide data for sound treatment plant infrastructure decisions, and aid the development of effective watershed management plans. Recommendations include the following:

1. Hsp70 false negatives may occasionally occur. Therefore, when using conventional PCR, samples which yield only the 18S PCR product should be confirmed using high resolution melt (HRM) analysis. Post-PCR HRM analysis of products generated by conventional PCR is straightforward and may be performed by contract laboratories. This is important in accurate determination of the presence or absence of human-pathogenic *Cryptosporidium*.
2. In this study, preliminary results using Method 1623 and DWI field slides were very promising. However, additional evaluation of the method is needed, including technology transfer to end-users. Further method evaluation is currently being performed under Water Research Foundation Project 4284, which includes a technology transfer workshop and international round robin.
3. Development of additional PCR genotyping methods should continue so that a toolbox of analytical options is available to meet the specific needs of the water industry and regulatory agencies. Additional research in this area is currently being performed under Water Research Foundation Project 4179.

## APPENDIX A: LIST OF MATERIALS AND SUPPLIES

<b>Item</b>	<b>Company</b>	<b>Catalog #</b>
<b>Sample Processing</b>		
Alcohol wipes	VWR	18889-002
Kimwipes or paper towels		
5% commercial bleach		
Cotton swabs	VWR	10806-001
Non-acetone nail polish remover		
Sterile scalpel #10	Bard-Parker, VWR	371110, BD371110
Scalpel handles	VWR	BD-371030
Molecular grade water	Lonza, VWR	51200, 12001- 380
Aerosol/filter barrier micropipette tips (20 and 200 $\mu\text{L}$ sizes)	VWR	e.g. 14217-726 and 14217-728
Closed cell foam (2 mm thick and approx. 3 x 3 mm square)	Wrightway Sports, Sandy Utah; Kip Hirrlinger, <a href="mailto:kip@wrightwaysports.com">kip@wrightwaysports.com</a> , 801-699-9311	001-100; 12 x 18" sheet, 2 mm thickness, cut into 3 mm wide strips
Halsted-mosquito type hemostat	VWR	25607-302
Safe Seal microcentrifuge tubes (0.65 mL)	VWR	53550-970 (Sorenson # 16090)
<b>DNA Extraction</b>		
Dry heat bath and blocks	VWR	52434-204, 13467-447
5 ml tube; Graduated, Flat-Base for Chelex/MGW	Qiagen	990552
Chelex 100 resin	Bio-Rad	143-2832
Large bore genomic micropipette tips	VWR	46620-642
Square floating microtube racks	VWR	60986-094
Liquid nitrogen		
Spin-X Centrifuge Tubes Filters, Corning (0.45 $\mu\text{m}$ , sterile)	Corning, VWR	Corning #8162, VWR # 29442-756
Aerosol/filter barrier micropipette tips (20 and 200 $\mu\text{L}$ sizes)	VWR	e.g. 14217-726 and 14217-728
<b>PCR</b>		
Microcentrifuge tube opener	BelArt, VWR	199250001, 47743-758

Aerosol/filter barrier micropipette tips (10 to 1000 $\mu$ L sizes)	VWR	e.g. 14217-730
Forceps		
1.5 mL microcentrifuge tubes		
GeneAmp® 10X PCR Gold Buffer & MgCl2, 1 Set (1.5 mL)	Applied Biosystems	4306894 or 4306898
dNTP set 100 mM (see reagent preparation App. B)	GE Healthcare Life Sciences	28-4065-51
dUTP 100 mM (see reagent preparation App. B)	GE Healthcare Life Sciences	28-4065-41
HPLC purified <i>Cryptosporidium</i> ( <i>C. hominis</i> , <i>parvum</i> , <i>meleagridis</i> ) hsp70 primers Di Giovanni and LeChevallier, 2005 CPHSPT2F primer 5' TCCTCTGCCGTACAGGATCTCTTA 3' and CPHSPT2R primer 5' TGCTGCTCTTACCACTACTCTTATCA 3' (see reagent preparation App. B)		
HPLC purified <i>Cryptosporidium</i> genus 18S primers Johnson et al. 1995 CPB-DIAGF 5'- AAGCTCGTAGTTGGATTCTG-3' and CPB-DIAGR 5' TAAGGTGCTGAAGG AGTAAGG-3' (see reagent preparation App. B)		
EvaGreen Dye 20X in water	Biotium	31000
Bovine Serum Albumin (BSA, Elisa Grade) (see reagent preparation App. B)	Sigma	A7030
Cheetah Hot Start Taq DNA Polymerase	Biotium	29050
AmpErase Uracil N-glycosylase (UNG)	Applied Biosystems	N8080096

### Gel Electrophoresis

Agarose I	Amresco	0710-500G
Loading buffer (see reagent preparation App. B)		
DNA Molecular Weight Marker XIII	Roche	1721925
Ethidium bromide	VWR	EM-4410
1X TBE Buffer	VWR	EM8830

## APPENDIX B: REAGENT PREPARATION

### Chelex/MGW

The Chelex/MGW protocol is a slight modification (water used instead of TE buffer) of the method reported in: Di Giovanni, G.D., LeChevallier, M.W., 2005. Quantitative PCR assessment of *Cryptosporidium parvum* cell culture infection. Appl Environ Microbiol. 71, 1495-1500.

***Please note that current batches of Chelex produced by Bio-Rad are at pH 10, and if the pH is not adjusted very poor results will be obtained.***

**All volume manipulations should be performed using sterile serological pipettes.**

1. Add 10g of Chelex 100 resin (BioRad Cat# 143-2832) pH 10 to a 400 to 500 mL sterile beaker with sterile stir bar.
2. Add 30 mL of molecular grade water (MGW), carefully rinsing the resin from the beaker wall. This mixture will be roughly a 1:1 volume-to-volume suspension of Chelex resin and MGW.
3. Add 3.5 mL of 1.0 N HCl (standard solution, e.g. VWR Cat# VW3202-1) with stirring. Rinse any resin from the beaker wall with 5 mL of MGW and continue to stir for 1 hour.
4. Let settle by standing for 2 min, aspirate the supernatant, being careful not to remove the resin, to a total volume of approximately 25 mL of Chelex/MGW.
5. To remove the salts formed during pH adjustment, add 200 mL of MGW, stir for 10 minutes, let settle, then pipette off the supernatant leaving approximately 30 mL Chelex/MGW.
6. Add another 200 mL of MGW, stir for 10 minutes, let settle for 2 min, then pipette off the supernatant leaving approximately 1:1 Chelex/MGW volume-to-volume ratio.
7. Let sit covered overnight.
8. Stir briefly and let settle for 2 minutes. Transfer a small volume of supernatant to a microcentrifuge tube. Using a pH meter and microelectrode (e.g. Thermo Orion 2 Star meter kit # 1111001 and Thermo Micro Combination pH Electrode # 9802BN) check the pH. The pH should be approximately between 7.6 and 7.8, but a range from 7.0 to 8.0 is acceptable.
9. While stirring, transfer 4 mL aliquots to graduated 5 mL flat bottom tubes (Qiagen Cat# 990552). Let tubes sit upright overnight then adjust the molecular grade water volume as necessary to achieve a 1:1 Chelex/MGW volume-to-volume ratio.
10. Chelex/MGW can be stored at room temperature for up to 1 year. Ensure there is a 1:1 volume-to-volume ratio of resin and MGW prior to each use and adjust the MGW if necessary.

### PCR Reagents

Avoid multiple freeze/thaws of nucleotides by preparing single use aliquots.

33mM dGCATPs (GE Healthcare Life Sciences catalog # 28-4065-51, 100 mM)

Thaw tubes only once. Vortex briefly and quick spin. Mix 250 µL of each dGTP, dCTP and dATP together, vortex briefly and quick spin. Aliquot 25 µL volumes into 0.65 mL tubes ( $\pm$  30 tubes)

10mM dTTP (dTTP tube from GE Healthcare Life Sciences catalog # 28-4065-51 or #28-4065-31, 100 mM)

Thaw tube only once. Vortex briefly and quick spin. Transfer aliquots of 120 µL into two 2 mL tubes. Add 1080 µL of molecular grade water to each = 1200 µL of 10 mM dTTP. Vortex briefly and quick spin. Aliquot 50 µL volumes into 0.65 mL tubes ( $\pm$  45 tubes)

10mM dUTP (GE Healthcare Life Sciences catalog # 28-4065-41, 100 mM)

Thaw tube only once. Vortex briefly and quick spin. Transfer aliquots of 120 µL into two 2 mL tubes. Add 1080 µL of molecular grade water to each = 1200 µL of 10 mM dUTP. Vortex briefly and quick spin. Aliquot 50 µL volumes into 0.65 mL tubes ( $\pm$  45 tubes)

Primers - HPLC purified. We purchase ours from Invitrogen. Good for 1 year after rehydration.

CPB-DIAG *Cryptosporidium* genus 18S primers of Johnson, D. W., N. J. Pieniazek, et al. (1995). "Development of a PCR protocol for sensitive detection of *Cryptosporidium* oocysts in water samples." Appl Environ Microbiol **61**(11): 3849-3855.

CPB-DIAGF 5'-AAGCTCGTAGTTGGATTCTG-3'  
CPB-DIAGR 5' TAAGGTGCTGAAGGAGTAAGG-3'  
435 bp product

CPHSPT2 Human-infectious *Cryptosporidium* (*C. hominis*, *parvum*, *meleagridis*) hsp70 primers of Di Giovanni, G. D. and M. W. LeChevallier (2005). "Quantitative PCR assessment of *Cryptosporidium parvum* cell culture infection." Appl Environ Microbiol **71**(3): 1495-1500.

CPHSPT2F primer 5' TCCTCTGCCGTACAGGATCTCTTA 3'  
CPHSPT2R primer 5' TGCTGCTTACCAAGTACTCTTATCA 3'  
346 bp product

Primers are rehydrated using molecular grade water added directly to the tubes received from the supplier. First, 200 pmol/µL stocks of each primer are prepared. Then 4 pmol/µL primer mixes are prepared.

*200 pmol/µL primer stocks*

Even though freeze-dried, briefly centrifuge to make sure all material is at the bottom of the tube. Determine the amount of primer (in nmol or pmol) provided by supplier. This is either located on the primer tube itself or on the paperwork which accompanied the shipment. Calculate the amount of molecular grade water to add to the tube that will result in a 200 pmol/µL concentration. For example:

130.8 nmol (130,800 pmol) + 654 µL Molecular Grade Water (MGW) = 200 pmol/µL

Add the appropriate amount of MGW, cap and briefly vortex making sure to rinse the entire inside of the tube, including under the cap, then quick spin. Repeat for each primer.

*4 pmol/µL primer mix*

Briefly vortex and quick spin the 200 pmol/µL primer stocks. Prepare primer mixes in 0.65 mL tubes.

CPB-DIAG primer mix – Add 10 µL of each CPB-DIAGF and CPB-DIAGR 200 pmol/µL primer stock to 480 µL Molecular Grade Water (MGW) = 500 µL of 4 pmol/µL CPB-DIAG primer mix

CPHSPT2 primer mix – Add 10 µL of each CPHSPT2F and CPHSPT2R 200 pmol/µL primer stock to 480 µL Molecular Grade Water (MGW) = 500 µL of 4 pmol/µL CPHSPT2 primer mix

Bovine serum albumin (BSA; 30 mg/ml)

Use ELISA grade BSA (Sigma Cat #A7030). Prepare in a sterile 50 ml conical tube.

1. Add 1.2 g BSA slowly to 38.4 ml of molecular grade water. Swirl intermittently during addition of BSA to help it dissolve. It may also need to be heated at 65°C.
2. Heat at 65°C for 6 hours to inactivate DNases.
3. Expose to ultraviolet light (bacteriological hood) for 1 hour to render endogenous DNA non-amplifiable.
4. Add 40 µl of 100X Tris-EDTA (TE, pH 8.0; catalog no. T9285; Sigma) buffer for a final concentration of 0.1X TE.
5. Prepare 500 µl aliquots and freeze at -20°C. Can be stored for up to one year.

Preparation of *C. parvum* and *C. muris* 10 oocyst DNA PCR Positive Controls

Contact Rebecca Hoffman at the Wisconsin State Laboratory of Hygiene for flow cytometry sorting of oocysts [beckyh@mail.slh.wisc.edu](mailto:beckyh@mail.slh.wisc.edu). Request 300 *C. parvum* or *C. muris* oocysts sorted into 0.65 ml microcentrifuge tubes containing 100 µl molecular grade water.

1. To each tube containing 300 oocysts, add 100 µl of Chelex/MGW (see reagent preparation protocol) using a large bore P200 filter tip (e.g. VWR Cat# [-642](#)). Make sure the Chelex/MGW stock is vortexed in between drawing each aliquot, with the goal of maintaining a 1:1 resin/water ratio. The final density of the DNA extract will be equivalent to approximately 10 oocysts in 5 µl (tube labeled as “10/5λ”).
2. Lyse the oocysts using 8 cycles, approx. 1 min each, of freezing in liquid nitrogen and thawing at 95°C using a dry heat bath (make sure samples are completely frozen and thawed each cycle). Shake samples down after each thaw step.
3. Store the oocyst lysates (without removing Chelex) at -20 °C for up to six weeks or -80 °C for several months.
4. Just prior to use, vortex on high speed, quick spin a few seconds, and use 5 µl of

supernatant as template (equivalent to DNA from 10 oocysts). Be careful not to transfer the Chelex resin to the PCR mastermix, as it will inhibit the PCR.

## Gel Electrophoresis

### Maniatis Type 1 6X Gel Electrophoresis Sample Loading Buffer

0.01 g bromphenol blue (0.10%)

4.0 g sucrose (40%)

Add 1X TE buffer (approx. pH 8) up to 10 mL, divide into 1 mL aliquots store at room temperature. Use 2  $\mu$ L buffer per 10  $\mu$ L of PCR product.

### DNA Molecular Weight Marker XIII – 50 bp ladder (Roche 1721925)

Add reagents below to a full tube of marker:

80  $\mu$ L of loading buffer

149  $\mu$ L of MGW

48  $\mu$ L of 10X GeneAmp Gold buffer (Applied Biosystems; Foster City, California)

Use 3  $\mu$ L per gel lane

Store at - 20°C.

### Running the Gel

Products and reagents used:

Agarose – Amresco Agarose I 500g (VWR catalog # 100513789)

TBE Buffer 1x (stock is bought as 10x TBE Buffer OmniPur, VWR catalog # EM8830).

Gel Electrophoresis Sample Loading Buffer

DNA Molecular weight marker (50 bp ladder)

30 tooth comb, 1.5 mm thickness

Ethidium bromide at a final concentration of 0.5  $\mu$ g per mL of agarose solution (stock is bought as 10 mg/mL ethidium bromide solution, OmniPur, VWR catalog #EM-4410)

Prepare a 2% agarose gel in 1x TBE buffer by completely dissolving the agarose using heat (e.g. with a microwave). Add the appropriate amount of ethidium bromide to the slightly cooled molten agarose, then pour the agarose into the casting tray. Let the agarose solidify, then transfer the tray containing the solidified gel to the electrophoresis tank containing 1x TBE buffer.

Mix 10  $\mu$ L of each PCR product with 2  $\mu$ L of gel electrophoresis sample loading buffer. Load samples into the agarose gel wells, always loading molecular weight marker into the first and last wells used.

Run the gel for 60 minutes at room temperature and at a constant 5 V per cm of gel length (e.g. a 20 cm gel would be run at a constant 100 V).

## APPENDIX C: DETAILED SAMPLE PROCESSING PROTOCOL

Revision 3-29-10

### Removal of Oocysts From Regulatory Slides and DNA Preparation Using Chelex Freeze-Thaw Lysis

Note: The removal of oocysts from slides is a modification of the procedure described by Nichols, Campbell and Smith (2006; Applied Environ Microbiol). The Chelex freeze-thaw procedure follows Di Giovanni and LeChevallier (2005; Applied Environ Microbiol) with the exception that molecular grade water is used to prepare the Chelex instead of 1X TE.

#### For flow sorted oocysts in microfuge tube controls

1. Sample volume should be 25 µl in molecular grade water (MGW).
2. Proceed to Step 1 of the Chelex Freeze-Thaw Lysis procedure.

#### Removal of oocysts/cysts from slides

1. Place slides on a Kimwipe or paper towel.
2. Wipe slides with a Kimwipe moistened with 10% bleach, followed by an alcohol wipe. Try not to touch the coverslip area of the slide after this point.
3. Moisten a cotton swab with non-acetone nail polish remover and swab the nail polish around the coverslip.
4. Use a sterile scalpel to cut around the coverslip, then lever a corner of the cover slip from the slide surface. *Be careful not to cut yourself!*
5. If a dried film is present, try to keep it in place over the slide well using a sterile pipet tip.
6. Place the coverslip inverted onto a clean Kimwipe.
7. Wash residual mounting medium off by adding 50 µl of molecular grade water (MGW) gently to the edge of the slide well. Holding the slide with both hands, tilt and rotate the slide to roll droplet around well. Tilt slide and aspirate wash using a cotton tip swab placed at the edge of the well. Do not touch the well with the swab. Approximately 3 to 5 µl of water will remain in the well.
8. Add 15 µl of MGW to the center of the slide well.
9. Scrape the entire well surface using a piece of closed cell foam (2 mm thick and approx. 3 x 3 mm square) held in a mosquito-type hemostat (VWR # 25607-302). Use the hemostat to pinch and grasp the center of the foam piece, rather than picking it up from the edges. Scrape around the edge of the well first, followed by scraping the entire well using up-and-down strokes while moving across the width of the well for a total of four passes across the well. Try to minimize sample spread/splattering outside of the well. See accompanying video for a demonstration of the technique.
10. Rotate the slide 90° and scrape again, then place the hemostat on a rest so that it does not touch any other surface. *Do not discard the foam.*
11. Aspirate the MGW from the bottom of the foam piece and transfer to a 0.65 ml microcentrifuge tube, then transfer the MGW from the well to the tube. *Keep the same pipet tip for the next wash of the slide.*
12. If no film from over the slide well area came off with the coverslip, add 15 µl of MGW to the slide well and proceed to the next step. If part of the film came off with the coverslip,

- add the 15 µl of MGW to the coverslip, rehydrate and scrape film, then transfer the wash to the slide well.
13. Rotate the slide 90° and scrape the entire well again.
  14. Place the tip of hemostat in the sample tube, unlock, and add the foam scraper to the tube. Use the pipet tip used for sample transfer to knock the scraper into the tube if needed.
  15. Aspirate the wash from the well using the same pipet tip used for the first slide wash transfer and pool with first wash in the 0.65 ml tube – total volume should be approx. 30 µl.
  16. If desired, keep the slide for examination by microscopy to verify oocysts were removed.
  17. Centrifuge samples briefly to bring droplets down to the bottom of the tube.

#### Chelex Freeze-Thaw Lysis

1. Add 20 µl of 1:1 Chelex/MGW using a large bore P200 tip (VWR # 46620-642) to each sample.

*Make sure the Chelex/MGW stock is vortexed and inverted immediately before drawing each aliquot to prevent settling of the resin and to maintain a 1:1 volume-to-volume ratio of resin and MGW.*
2. Lyse the oocysts using 8 cycles, approx. 1 min each, of freezing in liquid nitrogen and thawing at 95°C (make sure samples are completely frozen and thawed each cycle). Shake samples down after each thaw step.
3. Quick spin sample, then transfer, including Chelex and foam, to a 0.45 µm cellulose acetate microfuge spin filter (Corning Spin-X #8162, VWR # 29442-756). Use a 20 µl tip to transfer the foam piece to the spin filter. Next use a large bore pipet to resuspend the sample and Chelex and transfer the sample to the spin filter. *Keep the 0.65 ml sample tube.*
4. With the spin filter hinge in the 12 o'clock position, centrifuge the sample at high speed for 30 seconds.
5. Add a 5 µl MGW rinse to the 0.65 ml sample tube, briefly vortex, then quick spin in the microcentrifuge. Transfer this rinse to the spin filter using a 20 µl tip.
6. With the spin filter hinge in the 6 o'clock position, centrifuge the sample at high speed for 30 seconds.
7. Discard the spin filter insert containing the Chelex resin and foam, then cap the sample tube. The sample volume should be 40 to 45 µl and duplicate 20 µl volumes will be used as template in the PCR.
8. Proceed immediately to PCR or store at -80 °C.

## PCR Mastermix Calculation Spreadsheet (included on Method Demonstration DVD)

Di Giovanni <i>Cryptosporidium</i> hsp70 and 18S multiplex EvaGreen PCR			Date:	
			3/29/2010	
<b>Experiment Name/Label on PCR Plate</b>				
<b>Analyst</b>				
	Rxn Info	in uL		
	# of Rxns	1		
	Sample Volume	20	Note: Use duplicate 20 uL template volumes for slide and tube samples. Use triplicate 5 uL template volumes for <i>C. parvum</i> and <i>C. muris</i> 10 oocysts/5 uL PCR controls (avoid Chelex and be sure to add 15 uL water to PCR tube/well to bring up reaction volume). Use triplicate 20 uL water for no template PCR controls (NTCs).	
	Final Rxn Vol	50		
	Rxn Conc		Stock	Stock Units
10X Gold PCR Buffer w/o Mg (ABI)	1	10	X	
MgCl <sub>2</sub> (25 mM) (ABI)	3	25	mM	
each dGTP, dCTP, dATP (33 mM mix) (GE)	200	33	nM each	
dUTP (10 mM) (GE)	80	10	mM	
dTTP (10 mM) (GE)	120	10	mM	
CPHSPT2 Primer Mix	200	4	pmoles/uL (nM)	
CPB-DIAG Primer Mix	200	4		
EvaGreen stock (Biotium)	1	20	X	
BSA (30 mg/mL)(in house)	0.75	30	mg/mL (ug/uL)	
Cheetah Taq (Biotoium) or AmpliTaqGold (ABI) (Units)	2.5	5	U/uL (U)	
AmpErase UNG (ABI) (UNG; 1U/rxn)	1.0	1	U/uL (U)	
<b>Date Prep or Exp.</b>	<b>MASTER MIX</b>	Amt (uL)	<u>Added</u> <u>(check)</u>	Final Calc
	Molecular Grade Water	7.4		Final Units
	10X Gold PCR Buffer w/o Mg (ABI)	5	1	X
	MgCl <sub>2</sub> (25 mM) (ABI)	6	3.0	mM
	each dGTP, dCTP, dATP (33 mM mix) (GE)	0.3	200	uM each
	dUTP (10 mM) (GE)	0.4	80	uM
	dTTP (10 mM) (GE)	0.6	120	uM
	CPHSPT2 Primer Mix	2.5	200	nM each
	CPB-DIAG Primer Mix	2.5	200	nM each
	EvaGreen stock (Biotium)	2.5	1	X
	BSA (30 mg/mL)(in house)	1.25	0.75	ug/uL
	Cheetah Taq (Biotoium) or AmpliTaqGold (ABI) (Units)	0.5	2.5	Units/rxn
	AmpErase UNG (ABI) (UNG; 1U/rxn)	1	1.0	Units/rxn
Total MaterMix Volume =	30			
<b>Add 30 uL MasterMix to each PCR tube/well</b>				
Amplification conditions are as follows: UDG digestion at 50°C for 10 min; initial denaturation at 95°C for 10 min; 55 cycles of denaturation at 95°C for 30 sec and annealing at 60°C for 1 min, extension at 72°C for 30 sec; followed by a final extension at 72°C for 10 min. Add the following after the final extension step for high resolution melt analysis (RotorGene 6000 HRM, LightCycler, etc.): 50°C for 30 sec and 0.1°C melt steps from 80°C to 86°C.				
Di Giovanni CPHSPT2F primer 5' TCCTCTGCCGTACAGGATCTTCA 3' and CPHSPT2R primer 5' TGCTGCTTACCAAGTACTCTTATCA 3'				
Di Giovanni, G. D. and M. W. LeChevallier (2005) Appl Environ Microbiol 71(3): 1495-1500				
346 bp product from heat shock protein 70 gene (Human pathogenic Cryptosporidium - <i>C. parvum/hominis/meleagridis</i> )				
High resolution melt or sequence analysis will discriminate <i>C. parvum/hominis/meleagridis</i>				
<i>Cryptosporidium</i> genus 18S primers CPB-DIAGF 5'-AAGCTCGTAGTTGGATTCTG-3' and CPB-DIAGR 5' TAAGGTGCTGAAGGAGTAAGG-3'				
435 bp product from 18S rRNA gene				
Johnson, D. W., N. J. Pieniazek, et al. (1995) Appl Environ Microbiol 61(11): 3849-3855.				
High resolution melt or sequence analysis will discriminate genotypes				



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## ABBREVIATIONS

18S	18S ribosomal RNA
AgriLife El Paso	Texas AgriLife Research Center at El Paso
ANOVA	Analysis of variance
BSA	Bovine serum albumin
°C	Degree Celsius
CHD	CH Diagnostic and Consulting
Chelex/TE	Chelex 100/Tris EDTA
Chelex/MGW	Chelex 100/molecular grade water
C <sub>T</sub>	Threshold cycle (for real-time qPCR)
DAPI	4', 6'-diamidino-2-phenylindole
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	Deoxyribonucleic Acid
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
DWI	Drinking Water Inspectorate
EDTA	Ethylenediaminetetraacetic acid
F	Forward (when used in conjunction with a primer name)
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HRM	High resolution melt
hsp70	Heat shock protein gene (70 kDa)
IFA	Immunofluorescent assay
IMS	Immunomagnetic separation
kDa	Kilodalton
L	Liter
LT2	Long Term 2 Enhanced Surface Water Treatment Rule
MgCl <sub>2</sub>	Magnesium Chloride
MGW	Molecular grade water
µg/L	Micrograms per liter
µg/mL	Micrograms per milliliter

$\mu\text{l}$	Microliter
$\mu\text{m}$	Micrometer
$\mu\text{M}$	Micromolar
mg/mL	Milligrams per milliliter
min	Minute
mL	Milliliter
mM	Millimolar
n	Number
NA	Not applicable
ND	Not done
NTC	No template negative control
P	Probability
PAC	Project Advisory Committee
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
qPCR	Quantitative polymerase chain reaction
R	Reverse (when used in conjunction with a primer name)
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
s	Second
SPDL	Scottish Parasite Diagnostic Laboratory
spp.	Species
TBE	Tris-Borate-EDTA buffer
TE	Tris-EDTA
UK	United Kingdom
US	United States
UNG	Uracil N-glycosylase
USEPA	United States Environmental Protection Agency
V	Volts
WaterRF	Water Research Foundation
WSLH	Wisconsin State Laboratory of Hygiene





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