# *Cryptosporidium* Genotyping Method for Regulatory Microscope Slides [Project #4099]

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**OBJECTIVES:**
The overall project objective was to develop a simple, reliable, and cost-effective method for the genotyping of *Cryptosporidium* from 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.

**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.

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. 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. Three different DNA purification methods were compared. 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 that 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 that have a significant adverse effect on the PCR. The 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 following were the specific conclusions from this project:

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 that 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 U.K. 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 included the following:

1. Hsp70 false negatives may occasionally occur. Therefore, when using conventional PCR, samples that 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.

**RESEARCH PARTNER:**

U.S. Environmental Protection Agency

**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.