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Development and Evaluation of a Multiplex Suspension Array Protocol for the Detection of Enteric Pathogens from Clinical Specimens

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DEVELOPMENT AND EVALUATION OF A MULTIPLEX SUSPENSION ARRAY PROTOCOL FOR THE DETECTION OF ENTERIC PATHOGENS FROM CLINICAL SPECIMENS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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Abstract

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Foodborne illnesses are a significant public health challenge in the United States, with an estimated 9.4 million illnesses annually attributed to the consumption of contaminated food, of which 59% are estimated to be caused by viruses, 39% by bacteria and 2% by parasites. Timely detection and identification of the pathogens causing foodborne outbreaks is vital for the implementation of outbreak control strategies, allowing public health officials to prevent additional illnesses and maintain confidence in the food supply. Public health laboratories employ a variety of traditional and molecular testing techniques to identify foodborne outbreak etiologic agents. One technology is the Luminex XMap® microsphere system, which is also marketed as the Bio-Plex™ 200. This platform has a multiplexing capability with the potential to simultaneously detect up to 100 targets in one reaction. The studies described here show that

the combination of two Bio-Plex assays with real-time virus assays and one extraction method provides a flexible foodborne outbreak screening algorithm that potentially identifies an outbreak-associated pathogen on the first day of specimen submission and aids in focusing confirmatory laboratory testing. In these studies, two microsphere-based assays were designed for use on the Bio-Plex 200 system as screening assays for the detection of four enteric protozoa (*Giardia intestinalis, Cyclospora cayetanensis, Cryptosporidium parvum, Entamoeba histolytica*) and six virulence determinants of shiga toxin-producing *Escherichia coli* (STEC), enterotoxigenic *Escherichia coli* (ETEC), enteroinvasive *Escherichia coli* (EIEC) and *Shigella* spp. Precision and limits of detections were established for both assays. The sensitivity and specificity of the protozoan assay as compared to reference methods ranged from 81.25% to 100% for most targets, while sensitivity for the *E. histolytica* target was 42.86%. Sensitivity and specificity for the bacterial assay was 100% as compared to reference methods. However, cross-reactivity of the protozoan assay *E. histolytica* target with *E. dispar* and of the bacterial assay *uidA* target with enteropathogenic *E. coli* strains was noted. Additionally, real-time detection of norovirus and rotavirus nucleic acids extracted with the QIAamp DNA Stool Mini Kit was statistically comparable to detection when extracted with the Ambion® MagMAX™-96 Viral RNA Isolation Kit combined with the KingFisher® Magnetic Particle Processor.

CHAPTER ONE INTRODUCTION

Overview of Infectious Gastroenteritis in the United States. It is estimated by scientists at the Centers for Disease Control and Prevention (CDC) that annually in the United States (U.S.), there are 36.4 million domestically-acquired cases of gastrointestinal illness caused by 31 pathogens, with 9.4 million of these cases resulting directly from contaminated food ingestion (100). In addition, over 55,000 hospitalizations and 1,300 deaths are thought to occur due to contamination of food by enteric pathogens (100). The enteric pathogens that cause gastroenteritis include bacteria, viruses, and protozoa, and of the 9.4 million annual foodborne illness cases in the U.S., 59% are estimated to be caused by viruses, 39% by bacteria and 2% by parasites (100). Transmission routes are not limited to contaminated food, as pathogens may also be transferred via waterborne routes or person-to-person via contaminated feces.

Understanding the incidence of foodborne disease, trends associated with foodborne infection and physical characteristics of acute gastroenteritis-causing pathogens, as well as the clinical symptoms of infections caused by these organisms, is vital to the management of outbreak investigations and long-term programs to prevent future foodborne outbreaks. To acquire this type of epidemiologic data, the U.S. utilizes a variety of surveillance systems to detect and prevent foodborne disease. Examples of surveillance systems include, but are not limited to, the national requirement to report "notifiable diseases", an active sentinel laboratory system called FoodNet, a molecular subtyping network called PulseNet, the National

Antimicrobial Resistance Monitoring System (NARMS), and the electronic Foodborne Outbreak Reporting System (eFORS) (32). Epidemiologic data from these surveillance systems are used by public health laboratories for a variety of purposes. For example, prevalence and trend information may lead laboratories to implement new testing assays to detect and/or conduct surveillance for emerging infectious disease strains, or conversely suspend testing due to the low incidence of a particular pathogen. In outbreak investigations, information such as symptoms, incubation periods, geographic locations, and potential modes of transmission are immediately useful in determining what assays or types of testing must be completed first, to facilitate as rapid an identification of the etiologic agent as possible. Discussed below are characteristics, prevalence and trends of enteric pathogens commonly associated with foodborne illnesses and outbreaks, with emphasis on the etiologic agents targeted by the detection assays that are the focus of the project described in this dissertation.

Epidemiology of food- and waterborne intestinal protozoan gastroenteritis. Intestinal protozoa are more commonly associated with prolonged symptoms, particularly in travelers, as opposed to the acute gastroenteritis symptoms seen in bacterial and viral cases (84). Nonetheless, parasites also cause significant acute disease worldwide, especially *Giardia intestinalis*, *Cryptosporidium spp.*, and *Entamoeba histolytica* (91). Although only 2% of domestic foodborne illnesses are estimated to be caused by parasites, the prevalence of *Cryptosporidium* spp., *G. intestinalis,* and *Cyclospora cayetanensis* are significant enough for their inclusion on the CDC's Nationally Notifiable Infectious Conditions list (100).

Infection by enteric protozoa occurs due to the ingestion of oocysts or cysts contaminating food or water sources, and in the cases of *Cryptosporidium* spp. and *Giardia,* as few as 10 oocysts or cysts are required to cause disease (17, 19, 50). Transmission of *Entamoeba,*

Cryptosporidium, and *Giardia* also occurs readily person-to-person, as the organisms are infectious immediately upon excretion. In contrast, *Cyclospora* oocysts must mature in the environment before becoming infectious and therefore person-to-person transmission typically does not occur (24). In the U.S., *Giardia* and *Cryptosporidium* are associated with both waterborne and foodborne outbreaks. These two pathogens alone were linked to over 29,000 cases of illness in 2008, and recently published models estimate that they are responsible for approximately 134,000 cases of foodborne illness in the U.S. annually (17, 19, 100).

The sources of *Cyclospora* spp. infections are variable, ranging from international travel, to foodborne outbreaks, to sporadic cases with no confirmed source (21). Prominent foodborne outbreaks have occurred involving imported foods such as raspberries, basil and snow peas (86). One data source that is used to follow food-related infection trends is the CDC's Foodborne Diseases Active Surveillance Network, or FoodNet, a network that tracks laboratory-confirmed foodborne illness cases across ten states. According to 2009 FoodNet data, the incidence of infection with *Cyclospora* is low in the U.S., with an incidence of 0.07 cases/100,000 population (20). Although only 1,110 laboratory cases were reported during 1997-2008, more recent estimates by the CDC suggest that there is on average over 11,000 domestically-acquired foodborne *Cyclospora* infections annually (21, 100). The low reported numbers versus estimates is most probably attributed to the fact that many laboratories do not test for *Cyclospora* without a specific testing order, and the organism is not readily detected in a routine ova and parasite microscopic examination (21).

Reports of U.S. food- and waterborne infections or outbreaks due to *E. histolytica* are not as abundant in the literature as for other protozoa. However, *E. histolytica* has been implicated in a number of waterborne outbreaks in the U.S. since the 1950s (62). Although infections by *E.*

histolytica are not nationally notifiable, surveillance for *E. histolytica* cases is conducted in the Commonwealth of Virginia, and as of 2009, the five-year average for Virginia cases was 41.4 per year (115). These cases typically represented infections acquired outside the U.S., rather than outbreak-related infections.

Epidemiology of food- and waterborne bacterial gastroenteritis. Based on estimates recently published by the CDC, bacteria are the second-leading cause of foodborne illnesses (39% of annual episodes), with 20 disease-causing strains predominating (100). According to published estimates, the top five bacterial agents implicated in domestically-acquired foodborne illnesses in the U.S. are nontyphoidal *Salmonella* spp., *Clostridium perfringens, Campylobacter* spp., *Staphylococcus aureus,* and *Shigella* spp. (100). In addition, nontyphoidal *Salmonella* spp., *Campylobacter* spp. and *L. monocytogenes* are significant causes of foodborne hospitalizations and/or deaths (100).

The most recent FoodNet report indicates that there were 17,468 laboratory-confirmed cases of foodborne illness due to strains of *Salmonella, Campylobacter,* Shiga toxin-producing *Escherichia coli* (STEC) of both O157 and non-O157 serotypes, *Yersinia, Listeria,* and *Vibrio* reported in 2009 (20). On a positive note, continued declines in cases are occurring for C*ampylobacter*, *Listeria*, *Salmonella,* and *Yersinia.* Significant declines as compared to 2006-2008 levels were exhibited for *Shigella* and shiga toxin-producing *E. coli* (STEC) O157. Unfortunately, cases of illness caused by *Vibrio* spp continue to increase (20).

In addition to food, contaminated recreational and drinking water are also sources of bacterial pathogens that cause acute gastroenteritis, although the case numbers are not as high. According to data from the CDC's Waterborne Disease and Outbreak Surveillance System, 41 gastroenteritis illnesses caused by *Shigella sonnei,* 10 cases of *E. coli* O157:H7 illness and 6

cases of *Campylobacter jejuni* illness resulting from activity in recreational waters were reported in 2005-2006. Lakes and kiddie pools were the sources primarily implicated (14). In 2005, water ingested from a river was the source for an outbreak involving at least 60 individuals and yielded a mix of *E. coli* O157:H7, *C. jejuni,* and *E. coli* O145 (13). *Campylobacter* was additionally implicated in 38 illnesses in 2006 due to contaminated well water (13).

Although not estimated to be one of the top five bacterial agents associated with foodborne illness, *E. coli* strains are of significant public health interest, and are the focus of the Bio-Plex bacterial assay described and evaluated in this paper. *E. coli* organisms have acquired many virulence factors that allow them to expand beyond being commensal intestinal organisms and ultimately lead to infection and disease. These "diarrheagenic" *E. coli* can be characterized based on virulence and pathogenesis into the categories of enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (61). Shiga toxin-producing *E. coli* (STEC) that also contain the invasion gene, intimin, are members of the EHEC grouping.

While STEC O157:H7 is a prominent cause of foodborne infections in the US, several other diarrheagenic *E. coli* strains have been implicated as etiologic agents in outbreaks occurring in the U.S. In a retrospective study of 159 outbreaks occurring from 1971 to 1995, Dalton et al. (33) described an increasing incidence of enterotoxigenic (ETEC) strains causing foodborne outbreaks. During 1975-1989, ETEC was the causative agent of 6% of outbreaks, and between 1990 and1995, the incidence rose to 39%. A similar increase was observed for cruise ship outbreaks, increasing from 6% during 1975-1989 to 36% during 1990-1995. For most outbreak cases, a food item was the implicated source of illness, with seafood being the most

commonly implicated vehicle (33). Based on this study, Dalton recommended that ETEC should be considered as a potential cause of acute gastroenteritis outbreaks exhibiting a 24-48 hour incubation period with a diarrhea to vomiting ratio of greater than or equal to 2.5 and duration of symptoms lasting longer than 60 hours (33). Further investigation into ETEC outbreaks during 1996-2003 revealed that the ETEC serotype O169:H41 had become increasingly prominent (4).

Although reports of foodborne outbreaks due to EIEC and EPEC are not as frequent in the literature, both are considered to be potential causes of foodborne disease (38). In the past several decades, EIEC strains have been attributed to outbreaks due to contaminated French cheese and potato salad (38). EPEC strains are more commonly seen in developing countries, particularly in children. Domestically, food and water may be contaminated by both pathogens and cause illness, although there is a paucity of outbreak reports in the scientific literature, most likely due to the fact that identification of these strains is not routinely included as part of standard foodborne outbreak investigations. The low incidence of EPEC infections was illustrated in a prospective study of diarrheal illnesses in Maryland and Connecticut in which 823 specimens tested with molecular techniques yielded only two EPEC strains (80).

Epidemiology of food- and waterborne viral gastroenteritis. As mentioned previously, viral pathogens are the leading cause of foodborne illness in the U.S, with noroviruses being the most prevalent viral infectious agent (100). Noroviruses are estimated to cause 58% of domestically-acquired gastrointestinal illnesses, equating to an estimated 5.5 million illnesses annually. Of those, 26% are estimated to be due to contaminated food sources (100). Rotaviruses, astroviruses and sapoviruses are estimated to collectively cause slightly over 15,000 illnesses annually, with 1% of those being foodborne (100). In the Commonwealth of Virginia, surveillance is conducted for both noroviruses and rotaviruses, resulting in the detection of

norovirus in over 2,000 specimens and rotavirus in 38 specimens between 2001 and 2009 (45). The initial viral assay design discussed in this paper was intended to detect both noroviruses and rotaviruses.

In addition to the recent CDC estimates, historical data further support the prevalence of norovirus in the U.S. In one study, norovirus was attributed to 93% of non-bacterial outbreaks investigated by the CDC during 1997-2000 (41). In 2006, a significant increase in norovirus cases, including those in foodborne outbreaks, was reported by the CDC (12). The increase was attributed to the emergence of two new co-circulating strains. The rising trend of norovirus cases was experienced across the nation, with food-related venues being the second-most prevalent source of infection in Virginia (45).

Rotavirus has been implicated in 32% of pediatric acute gasteroenteritis (AGE) cases and is the primary pathogen causing AGE in children <5 years old (37, 88). Though mostly associated with infections in children and adults in long-term care facilities, rotavirus has also been linked to consumption of contaminated food and water sources. In 2000, an outbreak among college students was attributed to rotavirus, with deli sandwiches being implicated as the source (11). This outbreak was reported as being unusual since the population was assumed to be naturally immune, and because the suspected source was food. In 1981, rotavirus was the etiologic agent causing a Colorado outbreak due a contaminated community water source (54).

The Role of Pathogen Identification in Outbreak Investigations. Timely detection and identification of the enteric pathogens causing gastrointestinal outbreaks is vital for public health officials to implement strategies for outbreak control and case management, particularly in outbreaks that involve food service workers. Prompt, informed outbreak management allows public health officials in the short-term to stop the outbreak and prevent additional illnesses, thus

maintaining the public's confidence in the food supply (32). For example, rapid identification of the environmentally-hardy norovirus in institutional settings allows swift infection control measures to be instituted, such as staff restrictions, patient isolation, and use of bleach versus quaternary ammonium compounds (22, 65). In addition, the use of pulsed-field gel electrophoresis (PFGE) molecular subtyping by the CDC's PulseNet laboratories has facilitated prompt *Listeria spp., Salmonella spp.* and STEC outbreak identifications and food product recalls (118). To illustrate, PFGE was central to alerting public health officials about a unique cluster of *Salmonella* serotype Typhimurium in 2008, linked to peanut butter products manufactured in a single facility. Control measures culminated in the recall of over 400 commercially-distributed products and the subsequent closure of the manufacturing facility (15).

Pathogen identification also enables the long-term development of prevention strategies and infection control guidelines. For example, establishing the epidemiology of rotavirus infections assisted in the development and subsequent FDA clearance of two rotavirus oral vaccines in the U.S. Continued characterization of this virus is necessary to detect shifts in antigenicity that may compromise or reduce vaccine efficacy (25). Characterizing outbreaks also allows public health officials to identify new pathogens, food vectors, and/or gaps in the food safety system (32). FoodNet surveillance provides a strategic benefit by facilitating an understanding of the foodborne illness burden and an understanding of food practices that may potentially be outbreak-associated (118). For example, a *Listeria monocytogenes* outbreak attributed to turkey deli meat resulted in 54 identified illnesses and the recall of over 30 million pounds of meat products. This outbreak led to intensified USDA regulations for *L. monocytogenes* control, resulting in a 25% reduction in the number of specimens testing positive within one year (48).

Established Methods of Pathogen Identification. Public health laboratories employ a variety of traditional and molecular-based testing techniques to identify etiologic agents responsible for gastroenteritis outbreaks.

Identification of foodborne enteric protozoa. Traditional "gold standard" ova and parasite (O&P) microscopic examinations are typically used to identify the presence of enteric protozoa in clinical stool specimens. However, a known limitation of microscopy is the inability to morphologically distinguish certain strains such as pathogenic *E. histolytica* from non-pathogenic *E. dispar*. *Cryptosporidium* may also be identified using O&P examination, although this organism is usually missed unless a specific request for identification is submitted (55). Identification of *Cryptosporidium* may be facilitated using a modified acid-fast stain for microscopic examination. *Cyclospora* oocysts can also be identified during O&P examination, however, they are quite small. Hot safranin stain may facilitate identification, and presence can also be confirmed by looking for autofluorescence with the use of an excitation filter (86). Several commercial kits are also available for identification of *G. intestinalis* and *C. parvum*, including a lateral-flow immunoassay, a direct fluorescent-antibody (DFA) assay, and microplate enzyme immunoassays. One study compared several of these methods and determined that the DFA method was most effective in detecting both *Giardia* and *Cryptopsoridium* (58). In addition to these techniques, a growing number of singleplex and multiplex molecular assays have been described in the literature for detection and speciation of intestinal parasites, including assays differentiating *E. histolytica* from *E. dispar*. However, these have not yet been widely implemented in public health laboratories for surveillance or outbreak detection purposes.

Identification of foodborne bacterial pathogens. Traditional "gold standard" microbiological methods used for the identification of enteric bacterial pathogens include staining, culture using

selective and differential media, and biochemical testing using manual methods such as the API 20 E® strip (bioMérieux SA, l'Etoile, France) or automated biochemical systems such as the bioMérieux Vitek® and the Seimens MicroScan® instruments. Commercial immunoassay kits have been developed for the detection of shiga toxins produced by *E. coli* in stool and/or culture, although some do not differentiate between Shiga toxin types 1 and 2. In addition, *E. coli* strains may be further characterized using latex agglutination to confirm the O157:H7 serotype. Serological testing of *Salmonella* spp. using antisera specific for somatic and flagellar antigens is conducted in many public health laboratories in order to support national surveillance programs. Due to the increasing availability of genome sequences and simplicity of molecular techniques, information on enteric bacterial virulence factors and their corresponding gene targets is widely available in the scientific literature. This has facilitated an ever-increasing number of singleplex and multiplex PCR assays in the scientific literature for epidemiological use.

Identification of foodborne viruses. Culture methods for enteric viruses are of limited use in public health laboratories. Noroviruses are currently unculturable, and although methods are described in the literature, rotaviruses and astroviruses are not easily cultured (69, 83, 93, 101). Electron microscopy has been considered as the gold standard for virus identification, but this capability is not readily available in most clinical or public health laboratories due to its cost and training requirements. To facilitate rapid identification, commercial enzyme immunoassay (EIA) and latex particle agglutination kits have been developed for norovirus and rotavirus detection, although norovirus diagnostic kits are of limited availability in the U.S. Recently an FDA-cleared norovirus EIA kit became available in the U.S.; however, its use is intended for preliminary screening purposes only with all negative results requiring further confirmation with molecular-based methods (22). According to the World Health Organization and the CDC, the

sensitivity and specificity of several EIA assays for the detection of rotavirus are adequate for surveillance, and these are used in some public health laboratories (16, 117).

As is the case for protozoa and bacteria, many molecular assays for the detection and classification of enteric viruses are described in the literature. Molecular methods are the primary method of detection of noroviruses by laboratories in support of outbreak investigations (22). Use of molecular methods for the detection of rotavirus occurs mainly in research settings and is particularly useful in genotyping virus strains (16).

Investigation of Gastroenteritis Outbreak Cases at DCLS. A primary interest of the Division of Consolidated Laboratory Services (DCLS) and other state public health laboratories is the detection and characterization of common enteric pathogens typically associated with infectious disease outbreaks. DCLS receives clinical specimens for the detection and characterization of enteric pathogens for two main reasons: 1) to confirm and report pathogens on the Virginia Reportable Disease List, as required by law, and 2) to investigate AGE outbreaks within the Commonwealth of Virginia. During 2008-2010, DCLS confirmed over 1,000 bacterial, viral and protozoan pathogens in association with outbreak investigations.

The venues of outbreaks are numerous, ranging from social functions to long-term care facilities to schools and day care centers, and specimen submission to DCLS is triggered by reports of clusters of similar illnesses to the Department of Health. Disease clusters are assigned a formal outbreak number, and a representative number of specimens are submitted for testing to identify an etiologic agent. The initial goal for identifying a pathogen in a cluster investigation is to determine if the cases represent an outbreak, which is defined as two or more cases of a similar illness in unrelated individuals with a common link or exposure (32). Results communicated to epidemiologists can be further used to aid in making decisions regarding future

investigation, additional specimen sampling, food recalls and traceback investigations, isolation and restrictions of ill persons, and infection control. Results are typically not used for diagnostic purposes or for decisions related to patient treatment. In addition to directly supporting outbreak investigations, DCLS also submits representative specimens for selected pathogens to the CDC for further characterization. These results are used to populate data for surveillance programs such as CaliciNet, a national norovirus surveillance program, and a variety of other enteric bacterial surveillance systems for *Salmonella*, STEC, *Shigella* and *Vibrio*.

Current DCLS Enteric Pathogen Detection and Characterization Capabilities. At DCLS, laboratory analyses in response to infectious gastroenteritis outbreaks encompass a variety of methods, including microscopy, culture, EIA and molecular protocols. These methods are performed in separate sections of the laboratory using multiple sample types and a variety of different procedures. Pathogen identification can take as long as six days depending on the pathogen type and the initial testing performed. Figure 1 provides a general overview of the testing capabilities at DCLS, which is reflective of most public health laboratories. The figure also includes a listing of the primary specimen types received, and the testing typically initiated in outbreak situations. The decision regarding which tests to perform is determined by the epidemiological information provided to the laboratory upon submission. In some cases, only minimal information is known (symptoms, time of onset), so the ability to target testing to a specific etiologic agent matching the clinical presentation is not always possible. In other cases, an organism is known due to the fact that the patient was tested elsewhere, and the epidemiologists require additional testing to pinpoint sources and/or follow-up food service employees.

Intestinal protozoan testing at DCLS. Protozoan pathogens have historically been identified at

Figure 1. Overview of typical foodborne outbreak specimen testing algorithm. General overview of the outbreak testing approach at DCLS, the typical specimen types received, and examples of testing typically undertaken in outbreak investigations. The decision regarding which laboratory tests to perform on outbreak-related specimens is determined based on the epidemiological information provided to the laboratory upon specimen submission. Abbreviations : LV-PVA, polyvinyl alcohol; NoV, norovirus; EIA, enzyme immunoassay; RV, rotavirus; RT-PCR, reverse transcriptase polymerase chain reaction; STEC, shiga toxin-producing *E. coli*.

Extraction
(Qiagen DNA Mini Stool Kit)

Assay	Parasite	Bacterium	Virus
Specimen Types (stool)	Unpreserved LV-PVA	Cary-Blair Submitter broth	Unpreserved Cary-Blair
Day 1	Referral laboratory	Plates/broths	NoV real-time RV real-time
Day 2		EIA for toxins Further subculturing	RV real-time RT-PCR *(reflex if not tested Day 1)*
Day 3+		API 20E for enterics Serogrouping STEC real-time	
Time to result	3-6 days	2-6 days	3 hr -2 days

DCLS using fecal concentration and staining techniques. A molecular assay to differentiate *E. histolytica* from *E. dispar* is available, but has been rarely used. Testing for the identification of parasites in clinical specimens has been suspended at DCLS and many other state public health laboratories, resulting in a gap in the ability of laboratories such as DCLS to screen outbreak specimens for protozoan pathogens. Therefore, specimens submitted in association with outbreaks investigations for which an enteric parasite might be implicated must be sent to a private or reference laboratory for testing, greatly delaying the receipt of laboratory results.

Enteric virus testing at DCLS. Enteric virus testing is conducted at DCLS and encompasses real-time and conventional RT-PCR protocols developed by scientists at the CDC for norovirus and rotavirus. In a typical outbreak in which parasites are not suspected, the first testing initiated is typically norovirus real-time RT-PCR, as results are available within just a few hours. Rotavirus real-time RT-PCR testing may also be initiated on the first day, depending on the symptoms of the cases in an outbreak. If symptoms do not prompt the immediate inclusion of rotavirus testing, then testing could be delayed until the next day if all outbreak specimens are negative for norovirus. If the norovirus assay results are positive, rotavirus testing is not typically initiated. If the results are all negative, testing to identify enteric bacteria begins. DCLS also has the capability to further characterize norovirus-positive specimens using sequencing protocols developed by scientists at the CDC. In addition to the genogroup identified by real-time RT-PCR, sequencing provides further genotype information. This information is then entered and tracked using the CaliciNet surveillance program. The typical turnaround time for outbreak results is 3 hours to 2 days, depending on the timing of rotavirus testing.

Enteric bacterial testing at DCLS. Bacterial pathogens are identified by DCLS using microscopy, biochemical testing, and EIA techniques. On the first day of outbreak specimen

submission, plates and broths are prepared and incubated to begin the process of pathogen isolation. On the second day, bacterial identification begins with enzyme immunoassay testing for toxins if the specimens are submitted for reference confirmation. Reference specimens negative for toxins are reflexed for molecular toxin testing. Toxin-positive outbreak specimens continue to be processed using microbiological techniques for organism identification. On day 3 and beyond, final biochemical and confirmatory testing takes place as appropriate for the suspected organism. The approximate time to results is 2-6 days.

Additional STEC characterization is typically performed using real-time PCR molecular methods; however, this testing is mainly used to characterize isolates, and is not performed for outbreak testing purposes. Specific identification of diarrheagenic *E. coli* strains other than STEC O157 is currently not available at DCLS using classical microbiological techniques. A real-time PCR protocol is maintained for ETEC strain detection, although it is typically reserved for outbreaks in which primary testing has not yielded an alternate pathogen identification.

Use of Microsphere-Based Multiplex Screening Assays for Pathogen Identification and Detection. The rapid turn-around time of molecular techniques, combined with increased sensitivity as compared to classical testing methods, has resulted in an explosion of PCR-based testing protocols in the literature. Classical methods such as EIA and microscopy are traditional identification techniques, but are of limited value due to their high detection limits and the inability to differentiate between closely-related pathogens (e.g., *E. histolytica* versus *E. dispar*) (110, 118). For viruses like norovirus and astrovirus, electron microscopy has been a gold standard method. However, sensitivity is hampered due to viral morphology and the accessibility and complexity of this technology (25).

Because molecular techniques can be designed to detect distinct genomic regions, and due to their ability to detect small numbers of a microorganism, they may provide greater sensitivity and specificity versus traditional methods. Another advantage of molecular techniques is that they lend themselves readily to multiplex applications, allowing a number of pathogens to be targeted within one reaction tube. Multiplexing has proven useful in identifying pathogens in clinical specimens, with a variety of protocols described for enteric bacteria, viruses, and parasites (50, 53, 97, 97). However, multiplex assay design is extremely challenging due to the potential for primers to interfere with each other. Careful research and use of design software can facilitate the identification of optimal primer pair combinations. Detection of PCR-amplified products may be accomplished in a variety of ways, including conventional gel electrophoresis and staining or real-time fluorescence detection. Visualization with agarose gel is the most flexible method, as the number of targets that can be visualized at one time is not limited. Real-time detection platforms, however, are limited in the number of fluorophores that may be used for detection at one time.

The Luminex XMap® microsphere technology is an open platform that has the capability for simultaneous detection of up to 100 targets within one reaction. This technology is also marketed by BioRad as the Bio-Plex™ 200 (BioRad Laboratories, Hercules, CA), and its use is the focus of this project. The Bio-Plex 200 system consists of a 96-well microtiter plate reader, a "flow-based" detection system that employs two lasers for detection, and a high throughput fluids module (7). Integral to the system are the 100 different microspheres (sets), each color-coded with a unique mix of two fluorophores. Each microsphere is made specific for a genomic target by chemically oligomerizing amplicon-specific probes to the microspheres, the process of

which is illustrated in Figure 2. This system can detect up to 100 targets in 96 specimens per run, thus providing rapid detection without the real-time fluorophore and batch-size restrictions.

Prior to detection with the Bio-Plex, nucleic acids are extracted from clinical specimens and amplified in multiplex using conventional PCR (Figure 3). One primer of each primer set in the multiplex reaction mix is biotinylated at the 5' end. The amplified specimens are then mixed with a multiplex microsphere "mix" which contains the microsphere sets corresponding to the targets of the assay, and incubated to allow hybridization of biotinylated amplicon corresponding with the target microspheres. After incubation, streptavidin-R-phycoerythrin (SAPE) is added to the sample wells to facilitate detection. The R-phycoerythrin serves as a fluorescent reporter dye and streptavidin binds the dye to the biotinylated amplicon, if present. The plate is then placed on the Bio-Plex reader platform and processed for target detection. Using flow-based fluidics, the Bio-Plex instrument analyzes the microspheres in each sample well individually using lasers to detect specific bead color for identification purposes and the SAPE:biotin-tagged amplicon hybridization. Results are provided by the Bio-Plex 200 platform as a value designated the median fluorescent intensity ("FI" for the Bio-Plex system), which represents the average fluorescence of 100 target-specific beads per specimen well.

Utilization of this technology for the multiplex detection of pathogens has been increasingly described for all pathogen types, in both research and commercial settings, with pathogen detection assays employing the microsphere-based technology of Luminex and/or the Bio-Plex 200 detection platform being described for bacteria, fungi, viruses, and parasites (40, 71, 77, 116). A microsphere-based assay for the detection of intestinal protozoa and parasites was recently described by Taniuchi et al., from which selected pathogen targets are being evaluated in this project (107) To date, only one Luminex-designed nucleic acid assay is

Figure 2. Protocol for coupling pathogen-specific DNA probes to microspheres. This figure provides an overview of the steps required to couple a pathogen-specific probe and a uniquely-numbered microspheres for each target in a Bio-Plex multiplex assay. An image of the purchased microspheres is shown to the top left. The microspheres initially only possess carboxyl groups on their surface. DNA probes are synthesized to contain an amino modifier C12 link at the 5' end. The number of microspheres used to make a batch can vary, but in the studies described herein, five million beads were typically used. Microspheres are pelleted and the storage buffer is removed. A pH 4.5 buffer is added to establish the reaction conditions. Next, the target-specific probe is added followed by addition of EDC {1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride} to react with the carboxyl groups on the microspheres and create a chemical intermediate at each carboxyl site. The intermediate has an affinity for the amines attached to the probes, thus linking the probes to the microspheres. After two washes with buffers, the microspheres are counted using a hemacytometer for accurate addition to the detection plate specimen wells. This process is completed for each target-specific microsphere to be used in the multiplex assay. Prior to a detection reaction on the Bio-Plex, a multiplexed bead mixture is made

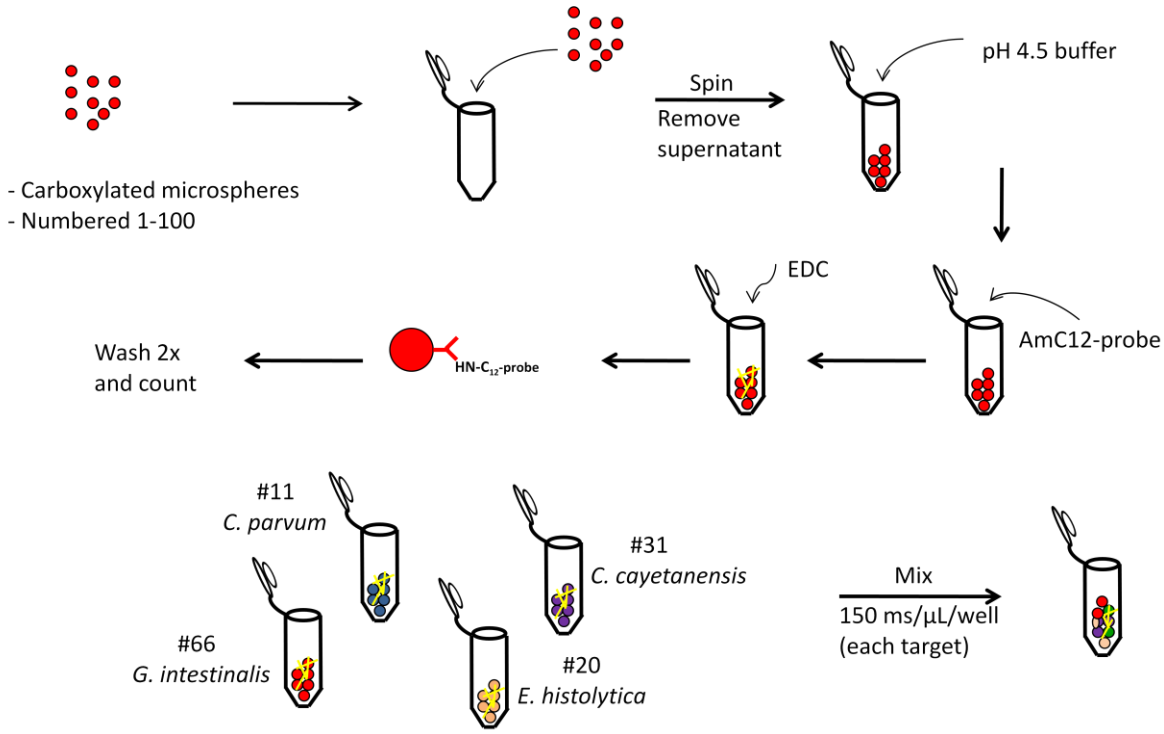
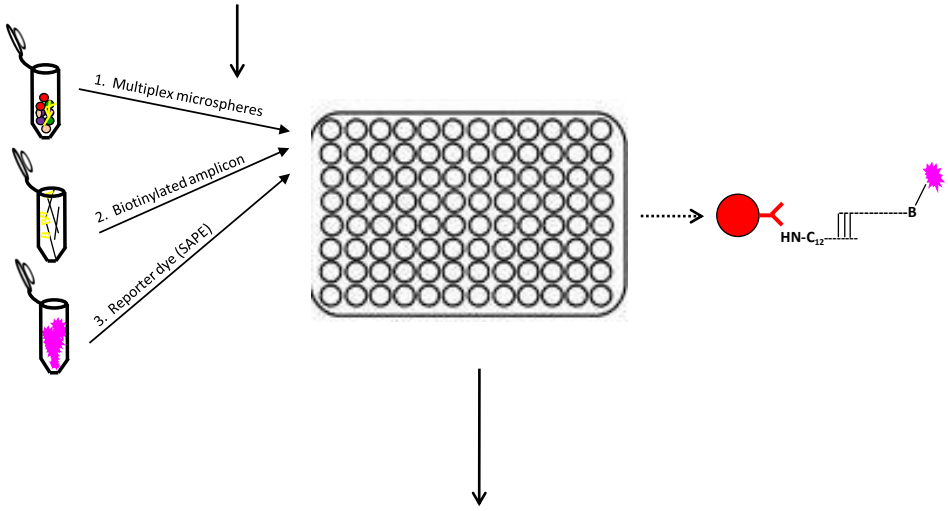

- Carboxylated microspheres
- Numbered 1-100
Spin
Remove
supernatant
pH 4.5 buffer
AmC12-probe
EDC
HN-C12-probe
Wash 2x
and count
#11
C. parvum
#66
G. intestinalis
#31
C. cayetanensis
#20
E. histolytica
Mix
150 ms/µL/well
(each target)

Figure 3. Overview of nucleic acid hybridization and detection methodologies using the Bio-Plex 200. This figure overviews the Bio-Plex 200 detection plate setup and hybridization steps as described in Materials and Methods. Nucleic acids are extracted from clinical specimens and amplified with organism-specific primer sets in which one primer is biotinylated. After amplification, the Bio-Plex plate is prepared. First, organism-specific microspheres are added to the wells of a 96-well plate (1), followed by addition of the biotinylated amplicon (2). This mix is heated to eliminate secondary structures, then incubated to allow hybridization of amplification products to complementary probes. Next, a streptavidin-linked fluorescent reporter is bound to the biotinylated amplicon (3). If the target DNA is present, the amplified DNA will hybridize to the probes on the microspheres, creating a microsphere-amplicon-reporter "sandwich" (middle right). The plate is placed on the Bio-Plex analyzer for fluorescence detection. The Bio-Plex uses two lasers: one to read the spectral address of each bead, thus identifying the organism target, and the second to measure the presence of the reporter dye, thus indicating that a target has hybridized. Histograms in the Bio-Plex software are used to measure bead detection, and final fluorescent intensity results are exported for analysis as qualitative results.

Extract and Amplify
(1 primer/pair biotinylated)

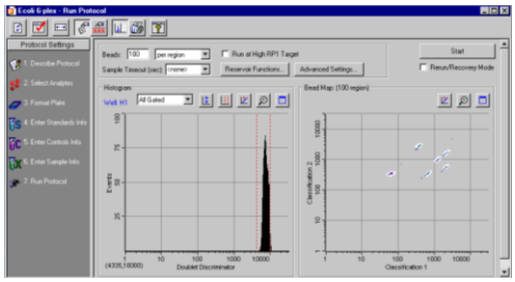
Bio-Plex plate setup



Detection and Results

Positive:

$$(FI_{specimen} - FI_{bkgrd}) \geq 2 \times FI_{bkgrd}$$



available as an FDA-cleared *in vitro* diagnostic kit -- the xTAG™ RVP (Respiratory Viral Panel) kit, which targets 12 respiratory virus targets. Two other bead-based assays are available for research use only: the ResPlex1® assay, which tests for seven respiratory pathogen targets, and the StaphPlex® which tests for 18 targets to allow *Staphylococcus aureus* identification and drug resistance determinations. Luminex Corporation is currently marketing a new xTAG kit targeting 15 intestinal pathogens. However, it is not yet currently available in the U.S.

CHAPTER 2 OVERVIEW OF RESEARCH PROJECT

Introduction to the Research Project. Locally and nationally, there is an urgent need for simple, rapid and cost-effective testing methods for public health laboratories to detect and/or identify pathogens of national health concern (81). Rapid detection is vital in identifying foodborne pathogens, allowing public health officials to quickly become aware of problems with the food supply that otherwise may have gone unnoticed. Once an outbreak is identified, public health officials can control the occurrence of further infections (32). A wide variety of parasites, bacteria and viruses may cause foodborne gastrointestinal infections (100). Therefore, to support the urgency of obtaining outbreak investigation results, simple and rapid testing methods are desired in order to concurrently screen for as many typical foodborne pathogens as possible. As discussed in Chapter 1, there are a wide variety of testing methods available for the detection of foodborne pathogens, ranging from traditional microbiological techniques to multiplex molecular assays. However, each technique is limited in providing a rapid comprehensive foodborne pathogen screening capability, either by the number of pathogens that can be detected concurrently or the time required to obtain a result.

Perhaps the most promising technology to enable comprehensive screening in one testing format for foodborne pathogens is that of multiplex molecular-based assays, many of which are described in the literature. However, many of the assays are developed for real-time (RT)-PCR platforms that may accommodate only a small number of fluorophores, limiting the size of

multiplex reactions and thus the number of pathogens that may be concurrently detected. The potential of the Luminex XMap® microsphere technology incorporated into the Bio-Plex 200 to detect up to 100 molecular targets in one reaction makes the platform an attractive candidate for the development of multiplex assays capable of detecting more targets than currently allowed on real-time multiplex platforms, enabling comprehensive screening of foodborne outbreak clinical specimens on one instrument.

The national emphasis regarding the development of diagnostic techniques for use in public health laboratories, combined with the availability of the advanced multiplex detection capability of the Bio-Plex 200, led to the hypothesis that the integration of multiplex Bio-Plex microsphere protocols with a common extraction method for the simultaneous detection of protozoan, bacterial, and viral organisms would enable prioritization of public health laboratory testing efforts and assist in reducing the timeline for identifying the causative agent of a suspected outbreak.

To address this hypothesis, three specific aims were developed for this research project. The first specific aim was to assess the performance of a Bio-Plex microsphere assay for the detection of four different protozoan pathogens in clinical stool specimens. The second specific aim was to develop and assess the performance of a Bio-Plex microsphere assay for the detection of enteric bacterial pathogens in clinical stool specimens, with an emphasis on diarrheagenic *E. coli* virulence determinants. Finally, the third specific aim was to incorporate a common nucleic acid extraction method to be used in conjunction with the Bio-Plex assays and two standardized real-time RT-PCR viral assays utilized for the detection of norovirus and rotavirus agents.

Rationale for Evaluating a Microsphere-Based Multiplex Testing Platform. The detection of infectious agents associated with enteric outbreaks can be hampered by lack of information,

improper specimen collection, and non-specific symptoms. Outbreak investigations occurring in immigrant populations and at institutions such as schools, day care centers, and long-term care facilities also make information-gathering difficult. In all these cases, the gathering of critical epidemiological data may be hindered by challenges such as language barriers, age and mental status, and may ultimately result in misguided testing decisions and delayed pathogen identification.

Another challenge with respect to outbreak testing is the specimen requirements for individual testing methods. For example, specimens transported in Cary-Blair medium are preferred for traditional bacteriological testing, while formalin and low-viscosity polyvinyl alcohol (LV-PVA) preservation are optimal for traditional parasite microscopy. Depending on the molecular protocols employed by laboratories, some assays may not be validated for certain specimen types. For example, specimens preserved in formalin are typically not acceptable molecular analysis as the formalin may inhibit PCR (94). Should unacceptable specimen types be submitted, the collection of additional specimens may be required, resulting in testing delays. If additional specimens cannot be collected, no further testing can be performed. Additionally, concurrent pathogen testing with multiple methods may require large sample volumes. If sample volume is low, laboratorians must decide which testing can and should be performed first, potentially at the expense of testing for a complete panel of pathogens.

Finally, the characteristics of the pathogens also present obstacles. Most public health laboratories typically characterize outbreaks based on disease onset times and reported symptoms of ill persons. However, many organisms have similar or overlapping symptoms or sources. Listed in Table 1 are a number of pathogens associated with significant numbers of foodborne and waterborne illnesses annually, along with representative incubation times and

typical symptoms (118). The list is arranged in order of incubation period, from shortest to longest. These incubation periods are often used by public health authorities to guide laboratory testing. Of significant note is the overlap in incubation periods and symptoms between many of the pathogens, which can be misleading in trying to initially determine an outbreak etiologic agent and may render diagnostic testing decisions difficult. For example, patients may exhibit similar incubation periods and symptoms when infected with either *Salmonella spp.* or norovirus, particularly early in the illness (118). In another example, misinterpretation of non-specific symptoms was described in one Florida outbreak leading to an initial assessment as norovirus, but further testing revealed the cause to be *Cryptosporidium* (68).

Challenges such as those described above potentially cause a delay in identifying the outbreak pathogen. Using typical laboratory testing protocols, even the preliminary identification of a causative agent may take up to six days post-submission, as previously shown in Figure 1. Designing and employing an integrated screening algorithm that incorporates a common extraction method and multiplex Bio-Plex screening assays to detect enteric pathogens implicated in AGE outbreaks has the potential to allow laboratorians locally, nationally and internationally to test specimens and provide preliminary results for multiple pathogen types on the first day of submission. Therefore, a practical goal for this project was to design a qualitative screening algorithm in which a variety of specimen types are prepared with a single extraction method and tested concurrently using the Bio-Plex platform targeting ten protozoan pathogens and bacterial gene targets in order to decrease the time to preliminary results and to better focus confirmatory testing. Should this initial screening protocol exhibit acceptable performance and meet practical goals, it may serve as a testing backbone to which additional molecular targets may be added to meet future epidemiological testing needs.

Table 1. Symptoms characteristic of infection by common foodborne pathogens. Outbreaks involving infectious agents are typically based on time to disease onset and symptoms. The occurrence of similar or overlapping symptoms or sources have the potential to hinder public health investigation and laboratory testing decisions.

Agent[a]	Incubation Period	Symptoms[b]
Chemical toxins	1 min-3 hrs	Varied
S. aureus enterotoxins	30 min-6hrs	V, D, N, Cr
B. cereus enterotoxins	1-6 hrs or 6-15 hours	V or D, Cr, V
Salmonella spp., nontyphoidal	6 - 72 hours	N, V, D, Cr
C. perfringens enterotoxins	8-24 hours	N, D, Cr
Noroviruses	10-51 hours	V, D, Cr, Hd, N
Shigella spp.	12 – 50 hours	D (bloody), Cr, F, V
Rotaviruses	24-72 hours	V, D (copious)
E. coli O157:H7	3-9 days	Cr, D (bloody), F (low)
Cryptosporidium spp.	2-28 days	V, D, N, F
Giardia intestinalis	7 - 14 days	D, Cr, N, F, Gas
Salmonella typhi	14 – 40 days	F, N, Cr,
Hepatitis A Virus	15-50 days	Jaundice, N

[a] Data from reference 118.
[b] V=vomiting, D=diarrhea, N=nausea, F=fever, Cr=cramps, Hd=headache

Overview of Assays in this Study. Assays targeting enteric protozoa and enteric bacteria were designed and evaluated in this study. In addition, use of the Bio-Plex extraction method was evaluated for use with two standardized real-time RT-PCR assays for virus detection. A general overview of each of the assays that are part of the Bio-Plex screening algorithm is outlined below, with details of the molecular targets provided in specific assay chapters.

Intestinal protozoan assay. A multiplex assay to detect select intestinal protozoa was designed by scientists at the University of Virginia (UVA). An initial design employed two multiplex tests (a two-plex and seven-plex) targeting *G. intestinalis, Enterocytozoon bieneusi, Isospora belli, C. cayetanensis, C. parvum/hominis, Encephalitozoon intestinalis,* and *E. histolytica.* Preliminary testing showed that the assay had potential for successful screening of specimens preserved in LV-PVA (data not shown). However, several pathogens targeted by the assay had no foodborne public health significance in the U.S. Therefore, *E. bieneusi*, *I. belli,* and *E. intestinalis* were removed from the multiplex, and the primers and probes targeting the remaining four pathogens were combined into one multiplex PCR detection assay. Details of the primer and probe sets are provided in Chapter 4. Final assay evaluations were conducted using unpreserved and LV-PVA specimens.

Enteric bacterial assay. An initial six-plex bacterial assay to detect diarrheagenic *E. coli* strains was developed by collaborators at UVA, and included primers and probes specific for EHEC, ETEC and EPEC genome targets. Preliminary testing using stool-inoculated broth specimens (hereafter referred to as "stool broth specimens") submitted for STEC reference confirmation demonstrated the potential of this assay or specimen screening (data not shown). Several changes were made to the multiplex prior to conducting a formal evaluation. Because the incidence of EPEC in foodborne outbreaks is very low in the U.S., primers and probes for two

EPEC molecular targets were removed from the multiplex. Instead, two additions were made, enabling detection of an *E. coli* O157-specific gene target (DCLS addition) and a shared target in enteroinvasive *E. coli* (EIEC) strains and *Shigella* spp. (UVA design). Details of the genomic regions targeted by the primer and probe sets are provided in Chapter 5. Preliminary evaluation of the revised six-plex assay was conducted with stool broth specimens. Because stool broth specimens are not typically submitted for outbreak investigations, final validation of this specimen type was not conducted. Final evaluation of the assay was conducted using stool specimens diluted in Cary-Blair transport medium.

Enteric viral assay. A Bio-Plex viral assay design targeting norovirus genogroups I and II, and Group A rotaviruses was initiated at DCLS. Following preliminary testing, the benefit of adding this assay to complement the other two Bio-Plex assays as part of a multi-pathogen screening algorithm was re-evaluated. The decision to forego a formal evaluation of this assay was made for the following reasons: 1) the norovirus genome targets were identical to standardized real-time RT-PCR protocols currently in use at DCLS, 2) the rotavirus Bio-Plex assay did not provide any additional information than that obtained with standardized real-time RT-PCR protocols currently in use, and 3) the Bio-Plex protocol required twice as much time to complete as the real-time RT-PCR protocols, therefore providing no turnaround time benefit.

The use of real-time RT-PCR assays to test viral agents provides a time advantage over the proposed Bio-Plex assay. However, if outbreak specimens require both Bio-Plex and real-time RT-PCR testing, the existing protocols as designed require two nucleic acid extractions. Therefore, to make the screening algorithm more efficient, use of a common extraction method is optimal. To meet this goal, a study was completed in which nucleic acids were extracted from unpreserved and Cary-Blair specimens using both the Bio-Plex extraction method and the

extraction method currently in use at DCLS for the standardized viral assay. Nucleic acids were then tested using real-time RT-PCR assays to detect norovirus or rotavirus, and results were compared. Achieving comparable results between the two extraction methods would enable laboratories to utilize the Bio-Plex extraction method simultaneously with the real-time RT-PCR assays, thus reducing the need for a second extraction when both Bio-Plex testing and viral testing is required.

Overview of Validation Studies. In order to implement Bio-Plex assays for outbreak specimen screening, specific performance characteristics must be established for each of the assays. Regulations governing validation are provided in the Clinical Laboratory Improvement Amendments (CLIA), section 493.1253, which state that performance characteristics must be established for both qualitative and quantitative assays that are not FDA-cleared or subject to clearance, a category within which the described Bio-Plex assays fall (31). The performance standards to be evaluated include accuracy, precision, analytical sensitivity/specificity, reportable range of results, and reference intervals, as appropriate for the assay (31). To facilitate these studies, the Clinical and Laboratory Standards Institute (CLSI) offers guidance on assessing molecular assay performance characteristics, and a comprehensive review article consolidating validating recommendations is available in the literature (8, 27, 29). Review of these guidelines and publications allowed for the selection of several Bio-Plex assay performance characteristics to be evaluated as part of this project.

Qualitative tests have one of two possible results: detected or not detected (28). The multiplex assays evaluated for this project have three steps: nucleic acid extraction, nucleic acid (template) amplification, and analyte (amplicon) detection. The Bio-Plex instrument is used for the detection step and yields a numerical "median fluorescent intensity" (FI) value that is

translated into a qualitative result of "detected" or "not detected" by the operator. Specific guidance for the validation of qualitative multiplex molecular assays is provided in CLSI documents MM17-A and MM3-A2 and determinations of accuracy, precision, and analytical specificity are required for qualitative molecular assays (27, 29). Because a numerical value is used to generate a qualitative result, assessments of analytical sensitivity, or limits of detection (LoD), are also important as these values potentially impact the positive cutoff point of the assay (30). Therefore, the validation studies performed as part of this project included the performance characteristics of analytical sensitivity (limit of detection, LoD), analytical specificity (cross-reactivity, interference), precision (repeatability, reproducibility), and accuracy (comparison-of-methods).

Reference/Testing materials. The primary specimens used for the validation studies were archived stool specimens, including unpreserved stools and stool specimens diluted in LV-PVA or Cary-Blair transport medium. Bacterial isolates and commercially-purchased protozoan cysts/oocysts were used for both analytical specificity and sensitivity testing studies. Cloned PCR amplification products inserted into vector plasmids were used for selected analytical sensitivity testing. All clinical specimens were anonymized prior to use in the validation testing studies.

Determination of positive cutoff value. All analytical test methods required the establishment of a cutoff value for determining positivity of results. The CLSI guidelines suggest determining the highest FI value that is likely to be observed for a blank or negative sample and using this value to help determine the cutoff (27, 29). However, for the purposes of this project, the FI value for the blank samples was not used as the cutoff for the following reasons: 1) assay cutoff values for selected assays had previously been established by collaborators developing the initial testing

protocols, and 2) preliminary testing in our laboratory showed that negative specimens yielded fluorescent readings lower than the minimum FI regarded to be relevant (<50 FI) by the Luminex Corporation (J. Eveleigh, personal communication). Alternative guidelines suggest that choosing a signal-to-noise ratio between 2:1 and 5:1 allows the nucleic acid targets to be distinguished from background (27). Therefore, the working cutoff for designating positive results was established at two times greater than the background value when background value was subtracted. Precedence for using this cutoff is also provided in the scientific literature (39, 40, 116).

Additionally, a final cutoff value for the Bio-Plex assays under evaluation must be chosen to indicate positivity based on the data gathered during the study. Standardized guidance is not available in the literature; rather, various approaches to assigning a positive value have been described. Typical approaches compare sample and negative background well FI values and calculate cutoffs at either two times background fluorescence, two times background fluorescence with the background fluorescence subtracted, or fluorescent values up to nine times background (39, 40, 71, 116). An alternative approach to establishing cutoff values employs the use of receiver operating characteristic (ROC) plots (71, 107). Data generated for the screening assays described for this project were calculated using a value of two-times background.

Analytical sensitivity or limit of detection (LoD). The LoD, or analytical sensitivity, of an assay describes the lowest amount of analyte that can be detected consistently at a specified level of confidence (8, 27). The LoD can be established using two approaches. First, the LoD can be calculated using the FI data generated with negative specimens (statistical testing) or second, established by testing specimens with known amounts or concentrations of target (empirical

testing) (8). Empirical testing should take place over a series of days to account for procedural variations and the LoD determined for each target in a multiplex and for each matrix type (8, 29).

For this project, empirical testing was used to determine the assay's LoD. Each matrix was spiked with serial dilutions of known concentrations of organism or cloned nucleic acid. It is recommended that 60 data points be collected in LoD studies (8, 30). However, guidelines also acknowledge that availability of resources and cost may impact study design (8, 29). Due to the relatively high cost-per-test, and the intent of these assays to serve as screening assays, not confirmation assays, it was determined that performing a minimum of three replicate experiments per organism or nucleic acid was acceptable. In most cases, five dilutions were tested in each experiment, representing dilutions above, at, and below the anticipated LoD for the target(s) being tested, yielding an average of 15 data points for each assay target per matrix type. The limits of detection were established as a range in which the lower limit represented the lowest dilution yielding one positive result and the upper limit represented the lowest dilution yielding three positive results.

Once the LoD is established for each assay target, it is recommended that data verification be conducted by incorporating additional targets or reaction components that could potentially interfere with the detection of low-concentration targets (29). For example, for the detection of infectious disease agents, it may be useful to add a high-concentration target to a low-concentration target (i.e., pathogen), as this could mimic a true clinical specimen and a high-concentration target could potentially interfere with detection of the low-concentration target. For the purposes of this study, this type of parameter was automatically evaluated in the study since numerous high-concentration nucleic acid targets in combination with low-concentration pathogen assay targets was inherent in the stool specimens, as a wide variety of normal flora

were present in the stool specimens. The question as to whether these assays can detect targeted pathogens in the presence of other organisms is additionally supported by the fact that in most public health outbreak investigations, a single etiologic agent is identified and is often recovered from multiple specimens submitted as part of the outbreak investigation.

Analytical specificity (cross-reactivity/interference). Analytical specificity describes the ability of an assay to detect only the agent that is targeted (29). Assessing analytical specificity may be accomplished in multiple ways: 1) using multiplex data to rule out cross-reactivity or interference of primer and probe sets with non-target nucleic acids, 2) challenging reactions with organisms not specifically targeted by the assay but that may be considered normal flora or causative agents of similar symptoms, 3) spiking specimens with substances that might interfere with the analytical procedure, and/or 4) evaluating sequences for potential cross-reactivity using internet databases and comparison programs (8, 29).

Most of the primer and probe sequences were adapted from published studies for which specificity experiments had been previously described. Therefore, data detailing the non-target organisms against which the primers and probes were tested for cross-reactivity was available. The analytical specificites of the Bio-Plex assays described in this study were further assessed in multiple ways. Novel primer and probe sequences designed for use in this study were tested for potential cross-reactivity by collaborators using the Basic Local Alignment Search Tool (BLAST) maintained by NIH (data not shown). In addition, each target in the multiplex assay was assessed for cross-reactivity to non-complementary nucleic acid templates, mainly using qualitative results obtained during the comparison-of-methods study. Specifically, results for assay targets that were expected to be negative based on reference testing were quantified and compared. If no cross-reactivity exists between non-target primers and probes or with known

non-target organism in the specimen, a negative result should be expected. For example, a *Giardia*-positive specimen is expected to yield negative results when tested using the other three non-*Giardia* primer and probe sets in the protozoan assay multiplex. If all negative results are observed, the data suggests no cross-reactivity. If positive results are detected for any of the targets other than *Giardia*, the data indicates there is potential cross-reactivity between the non-target primers and probes and the nucleic acid template. Finally, additional pathogens were included in order to assess for cross-reactivity to assay primers and probes. For the protozoan assay, the majority of the LV-PVA validation specimens were determined by reference method to contain other intestinal parasites not targeted by the current Bio-Plex assay (e.g., *Blastocytis hominis, Chilomastix mesnili*, etc.). Therefore, the absence of positive results in the presence of non-complementary target can be used to rule out cross-reactivity to these organisms. For the bacterial assay, several sources of non-target pathogens were used to challenge the Bio-Plex assay, including bacterial isolates representing non-targeted *E. coli* strains and serotypes and other enteric bacterial pathogens.

It has been well documented that stool samples contain multiple inhibitors of nucleic acid amplification reactions, including medications and/or their breakdown products, food components, bile salts and normal bacterial flora (111). Directly assessing all the potential interfering substances, known and unknown, in a stool specimen by spiking studies is not practical and was not part of the current validation studies. However, assay interference was indirectly assessed since the specimens tested in the current validation studies were representative of stool specimens typically submitted to DCLS and, therefore, included a range of different consistencies, normal bacterial flora, storage conditions, and preservation methods, and presumably contained a variety of medications and other components reflective of patient

diets, as specimens are submitted from a variety of different patients throughout the Commonwealth of Virginia. To assess interference by these variables, comparison-of-method study data was evaluated for negative results obtained for targets expected to positive when tested with the Bio-Plex system. Specimens yielding false negative results were diluted and tested to determine potential presence of inhibitors.

Precision (repeatability and reproducibility). Precision describes how well replicates of a given measurement agree when tested under defined conditions (8, 29). Precision studies typically incorporate two types of evaluations: repeatability and reproducibility. In repeatability studies ("within-run" studies), specimens of varying concentrations are tested in replicate using the exact same conditions. For the protozoan and bacterial assays, amplicons from specimens representing various concentrations of organism, matrices, and assay targets were tested in ten replicates on a single Bio-Plex run by a single operator.

In reproducibility studies ("between-day studies"), specimens of varying concentrations are tested under a variety of conditions. Ideally, variation should include multiple lots of reagents, multiple operators, various testing times, etc. (29). However, reproducibility studies often employ single lots of reagents and single operators, as was conducted in the current study (8). Reproducibility for the protozoan and bacterial assays was determined by amplifying extracted nucleic acid templates from select specimens and performing the detection step in single wells on the Bio-Plex system, one time per day over five days. Testing occurred only once per day, as opposed to multiple runs, to reflect how the assay would actually be used during an outbreak investigation (8). The reproducibility studies included both replicate detection of the same amplicon over multiple days and replicate amplification combined with Bio-Plex detection over multiple days. Thus, statistics describe both the precision of the detection step and overall

precision of the amplification and detections steps together, taking into account the single variable of time.

Guidelines recommend that precision studies be conducted with specimens of typical clinical analyte concentrations and that represent all specimen matrices to be tested (29). Evaluations of quantitative assays should include specimens not only at the assay cutoff point, but also at other points in the assay measuring range. Evaluations of qualitative assays should emphasize specimens at or near the limit of the assay (8, 29). Because this multiplex assay is a semi-quantitative assay (quantitative results are used to generate a qualitative result), the precision evaluation is based on both recommendations and employs specimens yielding fluorescence results both near the assay cutoff of two times background, and results higher in the measurement range. As recommended, the specimen panels evaluated included the typical specimen matrix type for each assay (29) For both repeatability and reproducibility studies, mean and standard deviation (SD) of the FI minus background results were calculated for both positive and negative multiplex targets. In addition, the coefficient of variation [CV, (SD/mean)x100] was calculated for positive targets. CV was not calculated for negative targets as the resulting percentages are statistically impractical.

Guidance publications also provide varying recommendations for precision study sample size, ranging from 40 replicates for qualitative tests to 120 data points for quantitative tests (8). However, it is acknowledged that achieving these numbers of replicates may not be feasible due to limited specimens and resources (28). Minimizing the panel size, number of replicates, and the scope of the precision study decreases the confidence level of the results and may underestimate the overall assay imprecision. However, because the assay is intended to be used as a screening

assay to rule in suspected pathogens, the precision data was adequately descriptive for this intended use.

The literature contains limited data with respect to the precision of Bio-Plex 200 nucleic acid assays and there are no standards established to determine the acceptability of precision data for molecular assays (8). Therefore, the repeatability and reproducibility data generated by this evaluation were compared to data available in two commercial and scientific studies (75, 77). In order to assess the precision of organism-positive assay targets, the CV data generated in this study were compared to positive target CV data published for the only commercially-available, FDA-cleared, infectious disease microsphere multiplex assay, the xTAG® RVP (Respiratory Viral Panel) (Luminex Molecular Diagnostics, Inc., Toronto). The RVP data differed from the current study data in that the RVP study represented a multiple-laboratory evaluation and measured precision for extraction, amplification and detection steps (75). However, to the author's knowledge it is the only commercial document available in the U.S. describing precision for Luminex-based nucleic acid assays, and therefore was used for comparison purposes.

Fluorescent intensity values less than 50 are below the detection threshold of the Luminex technology, and variation is considered difficult to assess (J. Eveleigh, personal communication). Prior experience with the BioPlex assay has suggested that CV values of negative specimens are often impractically high and potentially misleading; therefore SD values were used to describe the variation for target-negative specimens. To assess statistical acceptability for negative targets, the calculated SD values were compared to those described for an alternate microsphere-based assay described in the literature (77).

Because there are no established standards of precision acceptability for Bio-Plex nucleic acid assays, it is incumbent upon the testing laboratory to determine criteria for acceptability. Standards of precision for chemistry assays provide some basis for analysis, although even these assays demonstrate a 15% to 30% variance (8). Because this technology is relatively new, and well-established precision data is not available, comparisons to that described for the RVP assay and by McNamara et al. (75, 77) were considered acceptable.

Analytical accuracy (comparison-of-methods). Accuracy describes how closely a result agrees with an established reference, or for validation studies, how closely a new method correlates with the reference method (8, 29). Therefore, a comparison-of-methods study may be conducted to evaluate the accuracy of a new method.

Comparison-of-methods studies were conducted to assess the performance of the Bio-Plex protozoan and bacterial assays and to determine their potential for use in outbreak investigations. Previously characterized specimens -- as determined using the reference methods of microscopy, molecular methods, or enzyme immunoassay -- were tested using the appropriate Bio-Plex assay and the results compared to establish the level of agreement between the two methods. Bio-Plex results were designated as either true positive (TP), true negative (TN), false positive (FP), or false negative (FN) (Figure 4). Additionally, the sensitivity (Se) and specificity (Sp) for each assay target were calculated as described in Figure 4. Bio-Plex results that did not correlate with the reference method results were investigated using re-extraction and re-testing, repeating the reference method testing, and/or using alternative molecular assays, as available.

The number of specimens recommended for a comparison-of-methods study varies greatly, ranging from a minimum of 20 specimens to 100 specimens, although typically 40-50

Figure 4. General algorithm to determine correlation between a new test method and the corresponding reference method. To determine correlation (sensitivity and specificity) between a new test method and a reference method, specimens were tested with both methods and the results compared. A grid is generated for each target in the assay. A specimen that is positive when tested with both a reference method and a new method is considered to be a true positive (TP). Similarly, a specimen that tests negative with both methods is considered a true negative (TN). A specimen giving positive results with the reference method and negative results with the new method is a false negative (FN), and likewise, a negative reference method result and a positive result with a new method indicates a false positive (FP). Upon completion of the study, the sensitivity and specificity are then calculated for each target using the equations to the right of the grid.

Reference Method(s)

Bio-Plex

Target	Pos	Neg	Total
Pos	**True Positive (TP)**	**False Positive (FP)**	**TP + FP**
Neg	**False Negative (FN)**	**True Negative (TN)**	**FN + TN**
Total	**TP + FN**	**FP + TN**	**Total**

Sensitivity = [TP/(TP+FN)]x100

High sensitivity:

- Few false negatives
- Negative is really negative (rule-out)

Specificity = [TN/(TN+FP)]x100

High specificity:

- Few false positives
- Positive is really positive (rule-in)

specimens are tested (8). CLSI guidelines for qualitative test comparison studies recommend that 50 positive and 50 negative specimens be tested, although smaller sample sizes may be used with resulting larger confidence intervals (8, 28). The goal for the protozoan and bacterial assays was to test a minimum of 40 specimens. However, for certain targeted pathogens, the number of available specimens was limited; in these cases, all available specimens were tested and the small sample size noted.

The parameters of Se and Sp are important in assessing the appropriateness of assay use and guides result interpretation. The higher the sensitivity, the more confident a user can be that a negative result is truly negative, since there will be few false negatives. Conversely, the higher the specificity, the more confident a user can be that a positive result is truly positive, since there will be few false positives. As discussed previously, the intended use of these Bio-Plex multiplex assays is to screen specimens submitted as part of foodborne or waterborne outbreak investigations. Any positive results would allow for preliminary notification of epidemiologists and a prioritization of confirmatory testing used to characterize the detected pathogen. In most outbreak investigations, up to six specimens are typically accepted for testing. At least two specimens from different patients must be positive with a confirmatory method to assign a causative agent to an outbreak. If all specimens are negative, testing will continue regardless of the screening results as illustrated in Figure 1. Therefore, high specificity is the desired outcome since the decision to continue with testing is based on positive results. A lower sensitivity (higher number of false negatives) is acceptable for the following reasons: 1) all specimens will be confirmed regardless of whether positive or negative, 2) no action decisions will be made based on negative screening results, and 3) multiple outbreak specimens will typically be tested, inherently increasing the overall screening sensitivity for the outbreak. Based on these factors,

the minimum acceptable standards were established to be 80% sensitivity and 95% specificity for the Bio-Plex assays.

CHAPTER 3 MATERIALS AND METHODS

Materials and Methods – Protozoan Assay.

Specimens. Specimens tested in this study were anonymized to fulfill the requirements of the University of Virginia IRB for Health Sciences Research exemption (IRB-HSR #14597). Forty stool specimens preserved with low-viscosity polyvinyl alcohol (LV-PVA) and 30 unpreserved stool specimens were examined. The 40 LV-PVA specimens included specimens positive for the assay targets of *G. intestinalis* (n=8), *E. histolytica/dispar* (n=3), and both *G. intestinalis* and *E. histolytica/dispar* (n=4), as determined by microscopy. The 30 unpreserved stools included specimens positive for *C. cayetanensis* (n=6), *C. parvum* (n=2), *C. hominis* (n=1) and *G. intestinalis* (n=3), as determined by PCR. LV-PVA specimens were stored at room temperature or washed and stored at -80ºC prior to testing. Unpreserved specimens were stored at 4ºC or -80ºC prior to testing. Nucleic acids extracted from the specimens were stored at -80ºC prior to comparative testing. Specimens containing *C. cayetanensis*, *C. parvum,* and *C. hominis* were generously provided by Dr. Alexandre J. da Silva of the Centers for Disease Control and Prevention, National Center for Zoonotic, Vector-Borne, and Enteric Diseases, Division of Parasitic Diseases, Atlanta, GA.

Cyst and oocsyt standards. *G. intestinalis* cysts and *C. parvum* oocysts were purchased as $1x10^6$/4mL concentrations from Waterborne, Inc. (New Orleans, LA). Following vortexing and/or sonication, cysts were ten-fold serially diluted in 1X phosphate-buffered saline. To

establish assay limits of detection, dilutions of 10^4-10^0 cysts (40 µL) were spiked into 200 mg of parasite-negative LV-PVA or unpreserved stool to yield 10^4-10^0 cysts per 200 mg of stool. Spiked stool specimens were vortexed to evenly distribute organisms and stored at 4°C for a minimum of 30 minutes prior to extraction, amplification and detection as described below. Dilution series were tested in triplicate and total numbers of positive and negative qualitative results for each dilution were summarized. The limits of detection were established as a range in which the lower limit represented the lowest dilution yielding one positive result and the upper limit represented the lowest dilution yielding three positive results.

Cloned DNA standards. *C. cayetanensis* and *E. histolytica* DNA was PCR amplified and cloned into the pCR®2.1 TA vector (Invitrogen, Carlsbad, CA) per manufacturer's TOPO TA cloning kit instructions. Screen-positive cells were grown overnight and plasmid DNA isolated using the PureLink™ Quick Plasmid Miniprep Kit per manufacturer's instructions (Invitrogen). The concentration of cloned DNA was determined using a NanoDrop 2000 (Thermo Scientific, Wilmington DE). The cloned nucleic acid stock concentration was used to calculate approximate genome copies/µL for this study as described by Staroscik (106). To establish assay limits of detection, ten-fold serial dilutions were prepared in nuclease-free water and spiked in 10 µL volumes into 200 mg of parasite-negative LV-PVA or unpreserved stool aliquots. LV-PVA specimens were washed twice with 1X PBS prior to spiking with clone. Spiked stool specimens were vortexed to evenly distribute plasmid DNA and stored at 4°C for a minimum of 30 minutes prior to extraction, amplification and detection as described below. Dilution series were tested in triplicate and total numbers of positive and negative qualitative results for each dilution were summarized. The limits of detection were established as a range in which the lower limit represented the lowest dilution yielding one positive result and the upper limit represented the

lowest dilution yielding three positive results. Concentrations expressed as genome copies/µL were converted to reflect approximate organisms/200 mg stool using the following formula: (genome copies/200mg stool) / (genome copies/organism). Copy numbers of the assay gene targets for *Cyclospora* spp. and *E. histolytica* are estimated to be 2-20 copies/oocyst and 200 copies/trophozoite, respectively (5, 112).

Extraction of nucleic acids. Nucleic acid was extracted from stool specimens (200 mg) using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) per manufacturer's instructions, with modification. PVA-preserved stools were washed twice using 1 mL of 1X phosphate-buffered saline per wash and centrifuging at 10,000 rpm for 2 minutes. QIAamp Buffer ASL was added to all specimens, followed by the addition of beads from one UltraClean® Fecal DNA Isolation Kit Dry Bead Tube (Mo Bio Laboratories, Inc., Carlsbad, CA). Beads were not included in the extraction of spiked clone standards in order to preserve the integrity of the cloned DNA. The extraction continued per manufacturer's instructions to the final elution of DNA in 200 µL of AE buffer. Extracted DNA was further purified using a Zymo-Spin™ IV-HRC filtration column (Zymo Research, Orange, CA) per manufacturer's instructions.

Primers and probes. Primers and probes were previously described or designed by collaborators at the University of Virginia (Table 2). Reverse primers were modified at the 5' end with biotin-TEG and probes with the amino modifier C12. Expected amplification products were as follows: *G. intestinalis* (62 bp), *C. cayetanensis* (280 bp), *C. parvum/hominis* (138 bp), and *E. histolytica* (134 bp). Primers and probes were purchased from Integrated DNA Technologies (Coralville, IA) or Eurofins MWG Operon (Huntsville, AL).

Table 2. Oligonucleotide primers and probes for a microsphere suspension array assay for the simultaneous detection of *G. intestinalis, C. cayetanensis, C. parvum/hominis* and *E. histolytica.* Modifications at the 5' end are biotin-TEG (reverse primers) and amino modifier C12 (probes).

Target	Sequence (5'-3')	Ref.
G. intestinalis		(107, 113)
G62F	GACGGCTCAGGACAACGGTT	
G62btn	TTGCCAGCGGTGTCCG	
G62P	CCCGCGGCGGTCCCTGCTAG	
C. cayetanensis		(24, 85)
Cc280F	GTAGCCTTCCGCGCTTCG	
Cc280Rbtn	CGTCTTCAAACCCCCTACTGTCG	
Cc280P	GCATTTGCCAAGGATGTTTT	This study [a]
C. parvum/hominis		(44)
C138F	CGCTTCTCTAGCCTTTCATGA	
C138Rbtn	CTTCACGTGTGTTTGCCAAT	
C138p	CCAATCACAGAATCATCAGAATCGACTGGTATC	
E. histolytica		(98, 107)
Eh134F	AACAGTAATAGTTTCTTTGGTTAGTAAAA	
Eh134Rbtn	CTTAGAATGTCATTTCTCAATTCAT	
Eh134P	ATTAGTACAAAATGGCCAATTCATTCA	

[a] Designed by Dr. Mami Taniuchi, Division of Infectious Diseases and International Health, University of Virginia, Charlottesville, VA (unpublished data)

Coupling of oligonucleotide probes to microspheres. Oligonucleotide probes were coupled to microspheres (Bio-Rad Laboratories, Hercules, CA) using the recommended coupling protocol provided by Luminex Corporation (Austin, TX), with the addition of a 1400 rpm rotation step during the first incubation (74). Coupled microspheres were enumerated using a hemacytometer per the Luminex protocol.

Protozoan assay multiplex PCR. Amplification of extracted nucleic acid was performed in 50 µL reaction volumes using Bio-Rad iQ™ Multiplex Powermix (Bio-Rad). Each reaction contained 25 µL of 2X iQ Multiplex Powermix and 0.3 mmol/L of each primer. Amplification was performed using the 96-well GeneAmp® PCR System 9700 (Applied Biosystems, Carlsbad, CA), and cycling conditions consisted of 3 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 58°C.

Detection of nucleic acid targets using Bio-Plex microsphere assay. Hybridization of amplicons to coupled microspheres was conducted using the recommended hybridization protocol provided by Luminex Corporation, with modifications (73). Briefly, detection reactions were performed in 50 µL volumes in 96-well plates, and consisted of approximately 5000 coupled microspheres for each of the four targets (*G. intestinalis, C. cayetanensis, C. parvum/hominis, E. histolytica*), 33 µL of 1.5X TMAC solution, 12 µL of 1X TE buffer, and 5 µL of amplified DNA. Reaction plates were incubated at 95°C for 3 minutes to denature oligonucleotide secondary structure, then underwent 15 minutes of shaking incubation at 50°C and 800 rpm. After addition of 25 µL of 10 µg/mL streptavidin-R-phycoerythrin reporter dye, plates underwent a second shaking incubation at 50°C and 800 rpm for 10 minutes. Detection of hybridized amplicons was performed using the Bio-Plex® 200 Suspension Array System using a low RP1 target setting. Specimens were tested in single wells, with negative non-template

amplification controls placed at the beginning, middle and end of each detection run. The negative well placed at the end of the run was used as the background well for result calculation. Data output of the Bio-Plex 200 is median fluorescent intensity (FI). In order to determine positivity or negativity, the FI of the background well was subtracted from the FI of the specimen and compared to background. Results greater than or equal to two times the background were considered to be "screen positive".

Trichrome stain. Trichrome staining was performed using Remel Wheatley Trichrome Stain (Remel, Lenexa, KS). Slides were placed into Coplin jars containing 70% alcohol plus iodine for 20 minutes, followed by two washes with 70% alcohol for five minutes each. Slides were then placed into trichrome stain for eight minutes, followed by a rinse with 95% alcohol. Slides were placed into a second jar of 95% alcohol for five minutes, then carbol-xylene for 10 minutes, and xylene for 10 minutes. Slides were mounted with coverslips using Permount and were examined by oil immersion microscopy at 100X.

Singleplex PCR assay for the detection E. histolytica and E. dispar (alternate molecular method). Amplification of nucleic acid was performed using PSP3 and PSP5 primers specific for *E. histolytica* and NSP3 and NPSP5 primers specific for *E. dispar* (26). Singleplex reactions were mixed in 50 µL volumes using ABI AmpliTaq Gold® Master Mix (Applied Biosystems, Carlsbad, CA). Each reaction contained 25 µL of master mix and 0.24 mmol/L of each primer. Template DNA was diluted 1:10 and 10 µL of template added to the reaction volume. Amplification was performed using the 96-well GeneAmp® PCR System 9700 (Applied Biosystems, Carlsbad, CA), and cycling conditions consisted of 5 minutes at 95°C followed by 40 cycles of 15 seconds at 94°C, 15 seconds at 50°C and 90 seconds at 72°C. Amplification was completed with a final extension step at 72°C for 10 minutes. Amplification products were

visualized using 2% agarose gel electrophoresis or Agilent DNA1000 Kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Nucleic acid extracted from *E. dispar* trophozoite cultures was thoughtfully provided by Dr. Ibne Karim Ali, Division of Infectious Diseases and International Health, University of Virginia, Charlottesville, VA.

Precision studies. Precision characteristics of the Bio-Plex 200 assay were determined by testing DNA extracted and amplified from a panel of five specimens that included two LV-PVA stool specimens positive for *G. intestinalis* and *E. histolytica,* respectively, and three unpreserved stool specimens positive for *G. intestinalis, C. cayetanensis,* and *C. hominis,* respectively. The specimens chosen for this panel demonstrated fluorescence results both near the assay cutoff of two times background or results higher in the Bio-Plex measuring range. Repeatability (within-run precision) was determined for select specimens by testing amplicon from a single specimen in ten separate wells of a Bio-Plex detection plate. Results were calculated by subtracting the mean of ten background wells from the Bio-Plex FI output for individual sample wells. The mean and standard deviation (SD) of the FI minus background results for ten individual wells were calculated for both positive and negative multiplex targets. In addition, the coefficient of variation [CV; (SD/mean)x100] was calculated for positive targets. CV was not calculated for negative targets as the resulting percentages are statistically impractical. Reproducibility (between-day precision) was determined for select specimens by amplifying extracted nucleic acids from each specimen and detecting in single wells on the Bio-Plex system one time per day over five days. In order to determine variability in the detection step only, amplicon from the first day of the study was detected in single wells on the Bio-Plex system one time per day for four days. The FI minus background values of each target in the multiplex were calculated for

each specimen by subtracting the FI value of the negative background well at the end of the run from the FI value of each sample well.

Materials and Methods – Bacterial Assay.

Specimens. Specimens tested in this study were anonymized to fulfill the requirements of the University of Virginia IRB for Health Sciences Research exemption (IRB-HSR #14597). Testing of the bacterial Bio-Plex assay was performed using 50 stool broth specimens, which were primarily MacConkey's enrichment broths inoculated with a sterile swab dipped into a small quantity of stool then placed into the broth ("stool broth specimens"). Specimens were stored at -80°C prior to testing. The 50 stool broth specimens included positives for the assay targets of *stx1* (n = 31), *stx2* (n = 21), and *uidA* (n = 4). Specimens were previously characterized by PCR to be positive for one or multiple assay targets. Eight stool broth specimens evaluated were negative for all targets. Validation studies were completed using specimens place into Cary-Blair medium (n = 16), and included stools positive for *E. coli* O157:H7 (n = 2), *E. coli* non-O157 (n = 1), *E. coli* of unknown serotype (n = 3), *Shigella* spp. (n = 4), and *Salmonella* spp. (n = 5). Specimens were stored at -80°C prior to testing. Specimens were previously characterized to be positive for one or multiple assay targets by PCR. One stool specimen in Cary-Blair medium included in the study was determined to contain no pathogenic enteric bacteria by reference methods.

Additional bacterial strains. Additional bacterial isolates used in this study for establishing performance characteristics included ETEC strains (serotypes O153:H45, Ound:H16, Ound:NM, O6:H16, O148:NM) and EPEC strains (serotypes O119:H6, O55:NM, Ound:NM, O115:NM, Orough:H8) graciously provided by Victoria Lappi, Molecular Epidemiology Unit, Public Health Laboratory - Clinical Labs, Minnesota Department of Health, St. Paul, MN. Two EIEC strains

(serotypes O124:NM, O29:NM) were obtained from DCLS archives. Additional EPEC strains (serotypes O111:NM, O55:H6, O111:H2, O128:H1, O86:H34) were thoughtfully provided by Dr. Nancy Strockbine, National Center for Zoonotic, Vector-Borne, and Enteric Diseases, Centers for Disease Control and Prevention, Atlanta, GA.

Bacterial standards. Reference strains for establishing analytical sensitivity included ETEC (ATCC 35401), *E. coli* O157:H7 (EDL 9333), and *Shigella flexneri* (ATCC 12022). Several colonies grown on blood agar plates were inoculated into 0.85% sterile saline to yield a turbidity reading of 0.1, approximating a bacterial count of 10^8 CFU/mL. Ten-fold serial dilutions were performed in 1 mL volumes and 100 µL of the 10^4-10^0 dilutions transferred to blood agar plates. Dilutions were spiked in 10 µL volumes into 200 mg (µL) of Cary-Blair stool negative for enteric bacteria as follows: ETEC (10^8-10^3), O157:H7 (10^8-10^4) and *S. flexneri* (10^6-10^2). Spiked specimens were then extracted, amplified and detected, as described. Inoculated blood plates were incubated overnight, and colonies counted at approximately 24 hours. Dilution series were tested in triplicate and total numbers of positive and negative qualitative results for each dilution were summarized. The limits of detection were established as a range in which the lower limit represented the lowest dilution yielding one positive result and the upper limit represented the lowest dilution yielding three positive results.

Extraction of nucleic acids, isolates and stool broth specimens. Nucleic acid was extracted from isolates or stool broth specimens using a boil preparation method. For isolates, a 1 µL calibrated loop was used to remove bacterial growth from blood agar plates. The bacteria were suspended into 300 µL of nuclease-free water. The sample was vortexed and heated at 100°C for 10 minutes. Following centrifugation at 4,500 rpm for two minutes, the supernatant was removed and used for amplification. For stool broth specimens, 300 µL of specimen was

vortexed, heated and centrifuged, as described. Supernatant was removed and used for amplification.

Extraction of nucleic acids, Cary-Blair specimens. Nucleic acids were extracted from 200 mg of stool diluted in Cary-Blair medium using a QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) per manufacturer's instructions.

Primers and probes. Primers and probes were previously described or designed by collaborators at the University of Virginia (Table 3). Reverse primers were modified at the 5' end with biotin-TEG, and probes with amino modifier C12. Expected amplification product sizes are as follows: *stx1* (132 bp), *stx2* (255 bp), *uidA* (143 bp), *eltA* (62 bp), *estA* (172 bp) and *ipaH* (64 bp). Primers and probes were purchased from Eurofins MWG Operon (Huntsville, AL).

Coupling of oligonucleotide probes to microspheres. Oligonucleotide probes were coupled to BioPlex microspheres (Bio-Rad Laboratories, Hercules, CA) using a standard coupling protocol provided by Luminex Corporation (Austin, TX), with the addition of rotating step at 1400 rpm during the first incubation (74). Coupled microspheres were enumerated using a hemacytometer per the Luminex protocol.

Bacterial assay multiplex PCR. Amplification of extracted nucleic acids was performed in 50 μL reaction volumes using the QIAGEN® Multiplex PCR Kit (QIAGEN, Hilden, Germany). Each reaction contained 25 μL of 2X Multiplex PCR Master Mix, 5 μL of Q-Solution, and 0.2 mmol/L of each primer. Amplification was performed using the 96-well GeneAmp® PCR System 9700 (Applied Biosystems, Carlsbad, CA). Cycling conditions consisted of 15 minutes at 95°C followed by 40 cycles of 30 seconds at 94°C, 30 seconds at 58°C and 60 seconds at 72°C. A final extension step was performed for 10 minutes at 72°C.

Table 3. Oligonucleotide primers and probes for a microsphere suspension array assay for the simultaneous detection of *stx1, stx2, uidA, eltA, estA* and *ipaH*. Modifications at the 5' end are biotin-TEG (reverse primers) and amino modifier C12 (probes).

Target	Sequence (5'-3')	Ref.
stx1		(53)
EH132F	ACTTCTCGACTGCAAAGACGTATG	
EH132Rbtn	ACAAATTATCCCCTGAGCCACTATC	
EH132P3	CTCTGCAATAGGTACTCCA	
stx2		(90)
EH255F	GGCACTGTCTGAAACTGCTCC	
EH255Rbtn	TCGCCAGTTATCTGACATTCTG	
EH255P2	GGGGAGAATATCCTTTAATA	This study[a]
uidA		(119)
uidAF241	CAGTCTGGATCGCGAAAACTG	
uidAR383btn	ACCAGACGTTGCCCACATAATT	
uidAP266[a]	ATTGAGCAGCGTTGG	
eltA (LT)		(53)
ET62F	TTCCCACCGGATCACCAA	
ET62Rbtn	CAACCTTGTGGTGCATGATGA	
ET62P	CTTGGAGAGAAGAACCCT	
estA (ST)		This study[a]
ET172F	TTCACCTTTCGCTCAGGATG	
ET172Rbtn	AGCACCCGGTACAAGCAG	
ET172P	ATTACTGCTGTGAATTGTG	
ipaH		
EI64F	CCTTTTCCGCGTTCCTTGA	This study[a]
EI64Rbtn	CGGAATCCGGAGGTATTGC	
EI64P	CGCCTTTCCGATACCGTCTCTGCA	

[a] Designed by Dr. Mami Taniuchi, Division of Infectious Diseases and International Health, University of Virginia, Charlottesville, VA (unpublished data)

Detection of nucleic acid targets using Bio-Plex microsphere assay.. Hybridization of amplification products to coupled microspheres was conducted using a standard hybridization protocol by Luminex Corporation, with modifications (73). Briefly, detection reactions were performed in 50 µL volumes in 96-well plates, and consisted of approximately 5000 coupled microspheres for each of the six targets (*stx1, stx2, uidA*, LT, ST, *ipaH*), 33 µL of 1.5X TMAC solution, 12 µL of 1X TE buffer, and 5 µL of amplified DNA. Reaction plates were incubated at 95°C for 3 minutes to denature oligonucleotide secondary structure, then underwent 15 minutes of shaking incubation at 50°C and 800 rpm. After addition of 25 µL of 10µg/mL streptavidin-R-phycoerythrin reporter dye, plates underwent a second shaking incubation at 50°C and 800 rpm for 10 minutes. Detection of hybridized amplification products was performed using the Bio-Plex® 200 Suspension Array System using a low RP1 target setting. Specimens were tested in single wells, with negative non-template amplification controls placed at the beginning, middle and end of each detection run. The negative well placed at the end was used as the background well for result calculation. Data output of the Bio-Plex 200 is median fluorescent intensity (FI). In order to determine positivity or negativity, the FI of the background well was subtracted from the FI of the specimen and compared to background. Results greater than or equal to two times the background were considered to be "screen positive".

Real-time PCR for the detection of stx1 and stx2. Amplification of *stx1* and *stx2* targets was performed using a multiplex shiga toxin real-time PCR reaction containing the previously described primers and probes STEC-1, STEC-2, STEC I-HP-1, STEC I-HP-2, STEC II-HP-1, and STEC II-HP-2 (56, 95). Each reaction was prepared as follows using the LightCycler® DNA Master HybProbe kit (Roche Applied Science, Indianapolis, IN), for a total of 19 µL: 2 mM MgCl2, 2 µL of 10X LightCycler DNA Master HybProbe, 0.5 mmol/L of each primer and

0.2 mmol/L of each probe. Each reaction capillary contained 18 µL of the shiga toxin PCR reaction mixture and 2 µL of nucleic acid template. Amplification and detection was performed on the LightCycler® 2.0 Instrument (Roche Diagnostics Corporation, Indianapolis, IN). Cycling conditions consisted of 30 seconds at 95°C followed by 50 cycles of 0 seconds at 95°C, 20 seconds at 50°C and 30 seconds at 72°C. Melting conditions consisted of 0 seconds at 95°C, 10 seconds at 40°C and continuous detection from 40°C to 95°C. The reactions were cooled at 40°C for 2 minutes.

Real-time PCR for the detection of uidA. Amplification of the *uidA* target was performed in singleplex utilizing the previously described primers and probes O157-PT-2, O157-PT-2, O157-HP-1, and O157-HP-2 (10, 53). Each reaction was prepared as follows using the LightCycler® DNA Master HybProbe kit, for a total of 19 µL: 2 mM $MgCl_2$, 2 µL of 10X LightCycler DNA Master HybProbe, 0.5 mmol/L of each primer and 0.2 mmol/L of each probe. Each reaction capillary contained 18 µL of the shiga toxin reaction mixture and 2 µL of nucleic acid template. Amplification and detection was performed on the LightCycler® 2.0 Instrument. Cycling conditions consisted of 30 seconds at 95°C followed by 40 cycles of 0 seconds at 95°C, 10 seconds at 65°C and 20 seconds at 72°C. Melting conditions consisted of 0 seconds at 95°C, 10 seconds at 55°C and continuous detection from 55°C to 95°C. The reactions were cooled at 40°C for 2 minutes.

Real-time PCR for the detection of LT and ST. Amplification of LT and ST gene targets of ETEC was performed utilizing previously described primers and probes (96). To amplify the LT target, each singleplex reaction was prepared as follows using the LightCycler® DNA Master HybProbe kit, for a total of 19 µL: 2 mM MgCl2, 2 µL of 10X LightCycler DNA Master HybProbe, 0.5 mmol/L of each primer and 0.2 mmol/L of each probe. Each reaction capillary

contained 18 µL of the LT reaction mix and 2 µL of nucleic acid template. To amplify ST targets, each multiplex reaction was prepared as follows using the LightCycler® DNA Master HybProbe kit, for a total of 19 µL: 2 mM MgCl2, 2 µL of 10X LightCycler DNA Master HybProbe, 0.5 mmol/L of each primer and 0.2 mmol/L of each probe. Each reaction capillary contained 18 µL of the LT reaction mix and 2 µL of nucleic acid template. Amplification and detection was performed on the LightCycler® 2.0 Instrument. Cycling conditions consisted of 30 seconds at 95°C followed by 50 cycles of 0 seconds at 95°C, 20 seconds at 50°C, and 30 seconds at 72°C. Melting conditions consisted of one cycle of 0 seconds at 95°C, 10 seconds at 40°C, and continuous detection from 40°C to 95°C. The reactions were cooled at 40°C for 2 minutes.

Precision studies. Precision characteristics of the Bio-Plex 200 were determined by testing template DNA extracted and amplified from a panel of four specimens that included Cary-Blair clinical specimens positive for O157:H7 (*stx1, stx2, uidA*) and *S. flexneri* (*ipaH*). A Cary-Blair stool specimen spiked with ETEC (ATCC 35401) was also included (*eltA, estA*). The fourth panel specimen was a Cary-Blair specimen characterized to be negative for pathogenic enteric bacteria. The specimens chosen for this panel represented fluorescence results both near the assay cutoff of two times background (*stx2, uidA, estA*) and higher in the Bio-Plex measuring range (*stx1, eltA, ipaH*). Amplicon for determining repeatability (within-run precision) was obtained by amplifying two 50 µL reactions per specimen and combining the products. Ten separate wells of amplicon from each specimen were then tested on a single Bio-Plex detection plate. Results were calculated by subtracting the mean of ten background wells from the Bio-Plex FI output of individual sample wells. Mean and standard deviation (SD) of the FI-background results for ten individual wells were calculated for both positive and negative

multiplex targets. In addition, the coefficient of variation [CV, (SD/mean)x100] was calculated for positive targets; CV was not calculated for negative targets as the resulting percentages are statistically impractical. Reproducibility (between-day precision) was determined for select specimens by amplifying extracted nucleic acid template from each specimen in single reactions and detecting in single wells on the Bio-Plex system one time per day over five days. In order to determine variability of the detection step only, amplicon from the first day of the study was detected in single wells on the Bio-Plex system one time per day for four days. The FI-background values of each target in the multiplex were calculated for each specimen by subtracting the FI value of the negative background well at the end of the run from the FI value of each sample well.

Materials and Methods – Viral Assay.

Specimens. Specimens were anonymized to fulfill the requirements of the University of Virginia IRB for Health Sciences Research exemption (IRB-HSR #14597). Comparison of extraction methods was performed using unpreserved and Cary-Blair stool specimens. Unpreserved norovirus specimens were stored at 4°C and unpreserved rotavirus specimens were stored at -80°C prior to testing. Cary-Blair specimens were stored at room temperature prior to testing.

Automated extraction of nucleic acids. Nucleic acid was extracted from unpreserved stool specimens and stool specimens diluted in Cary-Blair transport medium (300 mg) using the Ambion® MagMAX™-96 Viral RNA Isolation Kit (Ambion, Inc., Austin, TX) automated on the KingFisher® Magnetic Particle Processor (Thermo Fisher Scientific, Inc., Vantaa, Finland). Extractions were performed according to manufacturers' instructions.

Manual extraction of nucleic acids. Nucleic acids were manually extracted from unpreserved stool specimens and stool specimens diluted in Cary-Blair transport medium (200 mg) using the

QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) per manufacturer's instructions.

Real-time RT-PCR for the detection of norovirus GI and GII. Amplification of norovirus GI and GII targets was performed using two singleplex reactions containing previously described primers and probes (60). Each NoV GI reaction was prepared as follows using the LightCycler® RNA Amplification Kit HybProbe kit (Roche Applied Science, Indianapolis, IN), for a total of 19.8 µL: 3.0 mM MgCl2, 4.0 µL of LightCycler® RT-PCR Reaction Mix HybProbe, 0.4 µL of LightCycler® RT-PCR Enzyme Mix, 0.4 mmol/L of each NoV GI primer and 0.2 mmol/L of each NoV GI probe. NoV GII reactions were prepared as follows, for a total of 19.0 µL per reaction: 2.0 mM MgCl2, 4.0 µL of LightCycler® RT-PCR Reaction Mix HybProbe, 0.4 µL of LightCycler® RT-PCR Enzyme Mix, 0.4 mmol/L of each NoV GII primer and 0.4 mmol/L of NoV GII probe. Reaction capillaries for both GI and GII contained 19 µL of the respective reaction mixture and 1 µL of nucleic acid template. Amplification and detection was performed on the LightCycler® 2.0 Instrument (Roche Diagnostics Corporation, Indianapolis, IN). Cycling conditions began with a 30-minute reverse transcription step at 55°C followed by initial denaturation for 30 seconds at 95°C and 45 cycles of 0 seconds at 95°C, 60 seconds at 58°C and 13 seconds at 72°C. The reactions were cooled at 40°C for 30 seconds.

Real-time RT-PCR for the detection of rotavirus. Amplification of the rotavirus genome target was performed in singleplex utilizing previously described primers and probe (59). Each reaction was prepared as follows using the QuantiTect Probe RT-PCR Kit (QIAGEN, Hilden Germany) for a total of 24 µL: 12.5 µL of 2x QuantiTect Probe RT-PCR Master Mix, 0.25 µL of QuantiTect RT Mix, 0.25 mmol/L of each primer and 0.1 mmol/L of the probe. Sample wells contained 24 µL of reaction mix and 1 µL of RNA template. Prior to addition to the sample well, RNA template was pre-heated to 95°C for 5 minutes, followed by 5 minutes of cooling on

wet ice. Amplification and detection was performed on the ABI 7500 FAST Real-Time PCR System (Applied Biosystems, Foster City, CA). Cycling conditions began with a 30-minute reverse transcription step at 50°C followed by initial denaturation for 15 minutes at 95°C and 45 cycles of 10 seconds at 94°C, 30 seconds at 55°C and 20 seconds at 72°C.

Statistical Methods. Shapiro-Wilk test for normal distribution and Spearman's rho test for correlation were both performed using PASW 18.0 (SPSS Inc., Chicago, IL). Fisher r-to-z transformation to determine correlation coefficient confidence intervals was performed using a VassarStats web-based calculator (72).

CHAPTER 4 EVALUATION OF A PCR-BASED PROTOZOAN BIO-PLEX ASSAY

Overview of Enteric Protozoa and Assay MolecularTargets. Primers and probes targeting select protozoan pathogens were developed for utilization with the Bio-Plex system by collaborators at the University of Virginia. Targeting four enteric protozoa of public health concern in the U.S., the primers and microsphere-coupled probes were combined into one multiplex assay for this project, enabling detection of *Giardia intestinalis, Cryptosporidium parvum, Cyclospora cayetanensis* and *Entamoeba histolytica.*

Giardia intestinalis. *G. intestinalis* (previously called *G. lamblia* or *G. duodenales*) is a flagellate organism and is the most prevalent protozoan in the U.S. (91). Its pathogenesis is attributed to the damage it inflicts on the intestinal mucosa and the resulting immune response (55). Clinical presentation may be asymptomatic, acute and self-limiting after 7-10 days, or chronic (>14 days) (55). Chronic diarrhea due to *Giardia* presents with the distinguishing symptoms of greasy and foul smelling stools that may alternate with constipation or normal stools. Chronic disease presentation may also result in malabsorption and/or lactase deficiency (55).

The genetic target of the primers and probe in this assay is a 62 base-pair region of the small subunit ribosomal RNA gene (SSU rRNA) (113). This region is attractive as a molecular target for two reasons. First, rRNA sequences are typically conserved, accompanied by regions of variability that provide pathogen specificity for molecular assays (102). Second, the rRNA

genes are organized as a cistron that has been reported to be present in repeats numbering from 60 – 132 copies per organism, increasing the potential for detection and overall assay sensitivity (46, 102). The primers and probe were originally designed to be used in a singleplex real-time PCR assay and subsequently were adapted for use in the current BioPlex assay (107, 113).

***Cryptosporidium* spp.** *Cryptosporidium* spp. are intracellular parasites of the phylum Apicomplexa, the sporozoites of which infect intestinal epithelial cells (23). General symptoms of *Cryptosporidium* infection include non-bloody, watery diarrhea which may be accompanied by cramping, nausea and vomiting, which is a result of abnormal absorption and altered secretion by intestinal epithelial cells (23, 91). As is the case for *Giardia*, clinical presentation of *Cryptosporidium* in immunocompetent patients may be asymptomatic, acute, or persistent. In general, symptoms last approximately two weeks (23).

The primers and probe for this assay were previously described for a real-time PCR platform and were designed to amplify and detect a 138 base-pair region that falls within a 450 base-pair sequence described by Laxer et al. (44, 67). No specific gene has been attributed to this region, and the cited Genbank accession identifies the sequence as "*Cryptosporidium parvum* genomic sequence". This region appears to have been chosen for organism specificity and not due to an associated virulence determinant target (44),(67).

***Cyclospora cayetanensis*.** *C. cayetanensis* is a relatively recently-identified intracellular pathogen, also of the phylum Apicomplexa and is currently known to infect only humans (86, 87, 91). The pathogen infects the intestinal epithelium of the small intestine, causing inflammation and villous atrophy. Typical symptoms of infection include diarrhea, nausea, flatulence, low-grade fever, fatigue and weight loss, and may last from a few days to over a month (18, 86).

The genetic target of this primer and probe set is the SSU rRNA gene region. As is typical of rRNA, this region is present in multiple copies, with the total number of copies dependent on the sporulation state of the extracted organisms. Assuming the *Cyclospora* population in stool represents all life cycle stages (sexual and asexual), it is estimated that the copy number is 2-20 per organism (112). The expected amplicon size is 280 base pairs. The primers were previously described for a conventional multiplex PCR assay for the differentiation of *Cyclospora* spp. and *Eimeria* spp. (85). The probe was designed by Dr. Mami Taniuchi of the University of Virginia (M. Taniuchi, unpublished data).

Entamoeba histolytica. *E. histolytica* is characterized by an amoeboid trophozoite stage and an infectious cyst stage (104). Infection may present as asymptomatic or amoebic colitis, and in some cases progress to amoebic liver abscess. Infection in the human host takes place at the colonic epithelium, and is characterized by mucosal thickening and ulceration due to cytolytic activity. The organism can also invade the mucosa, eventually traveling to the liver, causing amoebic liver abscesses (104). Typical colitis cases present with bloody diarrhea and abdominal pain for several weeks, although symptoms may vary in severity between patients (104).

The primers and probe used to detect this pathogen target a 134 base-pair region of the SSU rRNA gene. These were originally designed for use in a singleplex real-time molecular beacon PCR assay for the detection of *E. histolytica*, and were adapted directly to the Bio-Plex system (98, 107). The rRNA genes have been shown to reside on an extrachromosomal circular episome, with an estimated 200 gene copies per organism (6).

Results.

Validation of LV-PVA and unpreserved specimens - precision studies. Repeatability (within-run precision) was determined for a panel of select specimens by testing amplicons from a single

specimen in ten separate wells of a Bio-Plex detection plate, as described in Materials and Methods. To assess repeatability, precision for positive-target specimens was expressed as the CV, which ranged from 5.89% to 30.36% for the five specimens tested in the study (Table 4). Each specimen in the panel also yielded negative results for three additional assay targets. Within-run precision for negative targets was expressed as SD, and ranged from 3.35 – 15.53 (Table 5).

Reproducibility (between-day) precision of the amplification and detection steps combined was determined by amplifying and detecting extracted nucleic acid from select specimens each day for five days, as described in Materials and Methods. Precision was calculated in the same manner as repeatability, with positive-target specimens yielding a CV range of 12.50% to 59.54% (Table 6), and SD values for negative assay targets ranging from 1.72 to 11.51 (Table 7). Precision for the detection step alone was determined by amplifying extracted nucleic acid from each specimen once and detecting each day for four days, as described in Materials and Methods. Evaluation of precision for the detection step alone resulted in a CV value range of 6.20% to 28.71% (Table 8) for positive-target specimens, while negative targets exhibited an SD value range of 3.09-8.96 (Table 9).

Validation of LV-PVA and unpreserved specimens - analytical sensitivity (limits of detection). The analytical sensitivity or limits of detection of the described multiplex assay was evaluated using cultivated *G. intestinalis* cysts and *C. parvum* oocysts. Because quantifiable *C. cayetanensis* and *E. histolytica* organisms were not available, cloned nucleic acid standards were evaluated as a measure of analytical sensitivity for these targets. Table 10 summarizes the limit of detection ranges established for each assay target in both unpreserved and LV-PVA specimen types. Analytical sensitivity of the assays ranged from $10^1 – 10^2$ cysts/200 mg stool for

Table 4. Bio-Plex 200 detection repeatability results for positive targets in LV-PVA and unpreserved stool specimens. Specimen type and positive assay target is indicated for each specimen. Extracted nucleic acid from each specimen was amplified and then detected in ten replicate wells, as described in Materials and Methods. Mean, SD and CV were calculated using result values for each well obtained by subtracting the mean fluorescence of ten background wells from the Bio-Plex FI output of individual sample wells.

Specimen/ Target Organism	Mean (FI) (n = 10 wells)	SD (FI)	CV[a] (%)
LV-PVA *G. intestinalis*	46.30	14.06	30.36
Unpreserved *C. cayetanensis*	81.15	9.85	12.14
Unpreserved *G. intestinalis*	181.65	12.09	6.66
Unpreserved *C. hominis*	2619.90	154.39	5.89
LV-PVA *E. histolytica*	147.55	13.79	9.34

[a] CV: coefficient of variation; (SD / mean) x 100

Table 5. Bio-Plex 200 detection repeatability results for negative targets in LV-PVA and unpreserved stool specimens. Specimen type and positive assay target is indicated for each specimen used in the study. Mean and SD were calculated using result values for each well obtained by subtracting the average of ten background wells from the Bio-Plex FI value. Ranges provided for mean and SD reflect the range of statistics calculated for all specimens of the indicated type (LV-PVA or unpreserved) that were negative for the target listed to the left of the table. The total number of specimens in the panel that were negative for the select target is indicated.

Specimen/Target Organism	Total specimens negative for target	Mean (FI) (n = 10 wells/specimen)	SD (FI)
LV-PVA			
G. intestinalis	1	-3.25	5.61
C. cayetanensis	2	-2.75 - -2.70	4.05 - 4.84
C. parvum/hominis	2	-5.70 – 6.10	3.71 – 4.58
E. histolytica	1	-5.90	3.35
Unpreserved			
G. intestinalis	2	-2.10 – 4.80	4.18 – 6.42
C. cayetanensis	2	-2.05 – 16.30	5.38 – 15.53
C. parvum/hominis	2	-1.70 – 9.95	3.61 – 7.70
E. histolytica	2	-3.95 – 15.30	5.94 – 14.77

Table 6. Amplification and Bio-Plex 200 detection reproducibility results for positive targets in LV-PVA and unpreserved stool specimens. Specimen type and positive assay target is indicated for each specimen used in the study. Extracted nucleic acid from each specimen was amplified and detected each day for five days, as described in Materials and Methods. Mean, SD and CV were calculated by using results representing the specimen FI minus the background well FI for each positive target.

Specimen/ Target Organism	Mean (FI) (n = 5)	SD (FI)	CV[a] (%)
LV-PVA *G. intestinalis*	87.50	32.76	37.44
Unpreserved *C. cayetanensis*	99.00	58.94	59.54
Unpreserved *G. intestinalis*	245.60	30.71	12.50
Unpreserved *C. hominis*	2050.90	258.13	12.59
LV-PVA *E. histolytica*	255.80	50.00	19.55

[a] CV: coefficient of variation; (SD / mean) x 100

Table 7. Amplification and Bio-Plex 200 detection reproducibility results for negative targets in LV-PVA and unpreserved stool specimens. Specimen type and positive assay target is indicated for each specimen used in the study. Nucleic acid extracted from each specimen was amplified and detected each day for five days, as described in Materials and Methods. Mean and SD were calculated using result values for each well obtained by subtracting the mean of one background well from the Bio-Plex FI output of individual sample wells. Ranges for mean and SD reflect the range of statistics calculated for all specimens of the indicated specimen type (LV-PVA or unpreserved) that were negative for the target listed to the left of the table. The total number of specimens in the panel that were negative for the select target is indicated.

Specimen/Target Organism	Total specimens negative for target	Mean (FI) (n = 5/specimen)	SD (FI)
LV-PVA			
G. intestinalis	1	13.90	8.00
C. cayetanensis	2	6.10 – 7.10	6.11 – 7.50
C. parvum/hominis	2	-1.30 – 1.40	9.51 – 11.51
E. histolytica	1	-1.70	6.82
Unpreserved			
G. intestinalis	2	-4.90 – 0.80	1.72 – 7.21
C. cayetanensis	2	0.20 – 6.80	5.89 – 10.04
C. parvum/hominis	2	-4.10 – -1.40	8.15 – 10.57
E. histolytica	2	-6.20 – 4.00	2.58 – 6.28

Table 8. Bio-Plex 200 detection reproducibility results for positive targets in LV-PVA and unpreserved stool specimens. Specimen type and positive assay target is indicated for each specimen used in the study. Extracted nucleic acid from each specimen was amplified once and detected each day for four days, as described in Materials and Methods. Mean, SD and CV were calculated using result values for each replicate well obtained by subtracting the fluorescence of one background well from the Bio-Plex FI output of individual sample wells.

Specimen/ Target Organism	Mean (FI) (n = 4)	SD (FI)	CV[a] (%)
LV-PVA *G. intestinalis*	64.00	18.38	28.71
Unpreserved *C. cayetanensis*	62.00	6.75	10.88
Unpreserved *G. intestinalis*	257.25	19.26	7.49
Unpreserved *C. hominis*	2358.88	148.98	6.32
LV-PVA *E. histolytica*	211.50	13.12	6.20

[a] CV: coefficient of variation; (SD / mean) x 100

Table 9. Bio-Plex 200 detection reproducibility results for negative targets in LV-PVA and unpreserved stool specimens. Specimen type and assay target is indicated. Nucleic acid extracted from each specimen was amplified once and detected each day for four days, as described in Materials and Methods. Mean and SD were calculated using result representing the specimen FI for minus the background well FI for each positive target. Ranges values for each well obtained by subtracting the fluorescent of one background well from the Bio-Plex FI output of individual sample well. Mean and SD ranges reflect the range of statistics calculated for all specimens of the indicated type (LV-PVA or unpreserved) that were negative for the target listed to the left of the table. The total number of specimens in the panel that were negative for the select target is stated.

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Specimen/Target Organism	Total specimens negative for target	Mean (FI) (n = 4/specimen)	SD (FI)
LV-PVA			
G. intestinalis	1	15.38	6.74
C. cayetanensis	2	5.75 – 7.50	6.33 - 7.99
C. parvum/hominis	2	-1.88 – 3.25	5.66 – 6.99
E. histolytica	1	3.38	3.09
Unpreserved			
G. intestinalis	2	1.13 – 4.50	8.04 – 8.96
C. cayetanensis	2	-0.75 – -1.75	7.64 – 7.66
C. parvum/hominis	2	-3.13 – 3.63	4.53 – 4.77
E. histolytica	2	-3.38 – 1.13	4.84 – 5.99

G. intestinalis in unpreserved stools to the maximum estimated range of $3.5x10^2$-$3.5x10^4$ oocysts/200 mg stool for *C. cayetanensis* clone in LV-PVA stools.

Validation of LV-PVA and unpreserved specimens - analytical specificity (cross-reactivity). Many of the LV-PVA specimens tested in this study contained organisms not targeted by the Bio-Plex protozoan assay, including *Blastocystis hominis, Entamoeba coli, Entamoeba hartmanni, Dientamoeba fragilis, Chilomastix mesnili* and/or *Endolimax nana.* No cross-reactivity was observed between these organisms and any of primer and probe sets in the multiplex assay (data not shown). In addition, the specimens positive for *G. intestinalis, Cryptopsoridium* spp., *C. cayetanensis,* and *E. histolytica* tested in the study exhibited no cross-reactivity to non-complementary primers and probes in the multiplex assay.

Validation of LV-PVA and unpreserved specimens - comparison-of-methods. Seventy (70) specimens were tested using the multiplex microsphere assay and results compared to those obtained with the reference methods of either microscopy or PCR. Positive specimens, as characterized by reference methods, included: *G. intestinalis* (n=13), *E. histolytica/dispar* (n=4), *G. intestinalis* and *E. histolytica/dispar* (n=3), *C. cayetanensis* (n=6), *C. parvum* (n=2), and *C. hominis* (n=1). The calculated sensitivity and specificity for each target and corresponding confidence intervals are shown in Table 11. The sensitivity for *G. intestinalis, C. cayetanensis, C. parvum/hominis,* and *E. histolytica* was 81.25%, 83.33%, 100.00% and 42.86%, respectively. Specificity of all targets was at or near 100%.

Discussion. In this study, a multiplex PCR-based Bio-Plex assay targeting intestinal protozoan pathogens was evaluated for precision (repeatability and reproducibility), analytical sensitivity (limits of detection), analytical specificity (cross-reactivity), and performance as compared to reference methods. Results were evaluated for acceptability as a screening method to provide

Table 10. Analytical sensitivity (limits of detection) of the Bio-Plex 200 intestinal protozoan assay. Quantified cysts, oocysts, or cloned amplicon were diluted and spiked into 200 mg stool, as described. Spiked stools were extracted, template amplified, and detected as described. Dilutions were performed in triplicate. Positivity was determined as described for detection of targets in Materials and Methods. The limit of detection range represents the concentration of organism or cloned amplicon in the lowest dilutions yielding a minimum of 1 positive result. Analytical sensitivity was determined for each assay target using both unpreserved and LV-PVA specimens.

Organism	Limit of Detection	
	Unpreserved	LV-PVA
G. intestinalis	$10^1 – 10^2$ (cysts/200 mg stool)	$10^2 – 10^4$ (cysts/200 mg stool)
C. parvum	10^2 - 10^4 (cysts/200 mg stool)	10^3 - 10^4 (cysts/200 mg stool)
C. cayetanensis		
	$7x10^2$-$7x10^3$ (genome copies/200 mg stool)	$7x10^3$-$7x10^4$ (genome copies/200 mg stool)
	$3.5x10^1$-$3.5x10^3$ (oocysts/200 mg stool, estimated)	$3.5x10^2$-$3.5x10^4$ (oocysts/200 mg stool, estimated.)
E. histolytica		
	$4x10^3$ (genome copies/200 mg stool)	$4x10^2$-$4x10^3$ (genome copies/200 mg stool)
	$2x10^2$ (organisms/200 mg stool, estimated)	$2x10^1$-$2x10^2$ (organisms/200 mg stool, estimated)

Table 11. Comparison of multiplex microsphere assay results to reference method results, by assay target. Qualitative results of multiplex PCR testing using the Bio-Plex platform to detect protozoan pathogens in LV-PVA and unpreserved stool specimens were compared to those obtained with the reference methods of microscopy and/or PCR. Sensitivity was calculated using the formula: [TP/(TP+FN)]x100. Specificity was calculated using the formula: TP/(TP+FN)]x100. Confidence intervals were calculated using Wilson's score confidence interval method (28).

	TP (n)	FN (n)	Se (%)	$CI_{Se, 95}$	TN (n)	FP (n)	Sp (%)	$CI_{Sp, 95}$
G. intestinalis	13	3	81.25	L: 56.99 U: 93.41	54	0	100.00	L: 93.36 U: 100.00
C. cayetanensis	5	1	83.33	L: 43.5 U: 97.00	64	0	100.00	L: 94.34 U: 100.00
C. parvum/hominis	3	0	100.00	L: 43.85 U: 100.00	67	0	100.00	L: 94.58 U: 100.00
E. histolytica	3	4	42.86	L: 15.82 U: 74.96	62	1	98.00	L: 91.54 U: 99.72

Abbreviations:
TP, True positive; Bio-Plex 200 positive for an organism identified as positive by reference method;
TN, True negative; Bio-Plex 200 negative for an organism identified as negative by reference method;
FP, False positive; Bio-Plex 200 positive for an organism identified as negative by reference method;
FN, False negative; Bio-Plex 200 negative for an organism identified as positive by reference method;
Se, sensitivity; Sp, specificity; CI. confidence interval; L, lower limit; U, upper limit

preliminary outbreak etiologic agent information to epidemiologists and to focus confirmatory testing decisions in the laboratory.

Precision of the protozoan assay was evaluated by testing a panel of specimens representing stool specimens typically received by public health laboratories in the course of an outbreak investigation. As previously discussed in Chapter 2, the literature contains limited data with respect to the precision of Bio-Plex 200 nucleic acid assays and there are no standards established to specifically determine the acceptability of precision data for molecular assays (8). Therefore, the repeatability and reproducibility data generated by this study were compared to data available in the commercial and scientific literature. In order to assess the precision of organism-positive targets, the CV values calculated in both the repeatability and reproducibility studies were compared to CV values published for the only FDA-cleared infectious disease microsphere multiplex assay on the market, the xTAG® RVP (Respiratory Virus Panel). Coefficient of variation values for the RVP assay were established in a reproducibility study to range from 2.40% to 87.20% (75). This wide range in precision represented testing conducted at multiple laboratories for a variety of targets using single-infection specimens containing a range of virus concentrations (75). As shown in Table 4, the CV value range of 5.89% to 30.36% achieved with the protozoan assay in the repeatability study for all positive-target specimens were comparable to the precision ranges reported for the RVP assay. Similarly, as shown in Tables 6 and 8, the reproducibility study CV values ranging from 12.50% to 59.54% for the amplification and detection steps combined, and 6.20% to 28.71% for the detection step alone, were comparable to those published for the RVP assay. Of note is the marked reduction in the detection step CV values (Table 8) as compared to the CV values for the combined amplification and detection steps (Table 6), suggesting that the amplification step is an important source of

variability in this assay. Although the RVP assay and intestinal protozoan assay precision studies differed in several respects, this comparison suggests that multiplex microsphere-based nucleic acid assays may exhibit a wide range of variability, particularly at the low end of the FI measuring range for positive specimens, and facilitates the conclusion that the Bio-Plex protozoan assay exhibits comparable repeatability and reproducibility for pathogen-positive LV-PVA and unpreserved specimens.

As discussed in Chapter 2, variation of fluorescent intensity values less than 50 are difficult to assess (J. Eveleigh, personal communication). Therefore, standard deviation (SD) was used to assess the variation for target-negative specimens. The SD was compared to similar calculated values described in the literature for a microsphere-based *Plasmodium* spp assay (77). For both *Plasmodium*-negative specimens and negative targets of the malaria assay, SD values ranged from 18.1 to 39.0 based on mean FI values of 81.3 to 121.2 (77). These values were calculated using FI values detected on the Bio-Plex with a high RP1 target setting, which typically results in higher FI values as compared to using the low RP1 target setting. Because the protozoan PCR assay is detected on the Bio-Plex using a low RP1 target setting, the mean FI and SD values for negative specimens in the study described here are lower than those described for the *Plasmodium* assay. However, a relative comparison of the two assays remains informative. As shown in Table 5, the SD range of 3.35-15.53 FI obtained for negative targets in the repeatability study similarly represent the range of variability described for the multiplex microsphere-based assay described by McNamara et al. (77). In addition, the SD value ranges of 2.58 – 11.51 FI and 3.09 – 8.96 FI achieved in the reproducibility study of negative targets for both the amplification/detection and detection-only steps, respectively, are comparable to published data (Tables 7 and 9). This comparison therefore allows us to conclude that the Bio-

Plex protozoan assay exhibits comparable precision for pathogen-negative LV-PVA and unpreserved specimens.

Analytical sensitivity data for microscopic techniques used for the detection and identification of intestinal protozoa is not readily available in the published literature; therefore, comparisons to limits of detection established in this study were not possible. However, it is acknowledged that molecular amplification techniques for the detection of protozoa are typically more sensitive than microscopy (43, 70, 99, 105). Comparing limits of detection for each primer and probe set with published data is informative in understanding how well established primers and probes can be adapted to the Bio-Plex platform. Therefore, the analytical sensitivity (limits of detection) of the Bio-Plex assay primers and probes when used to detect pathogens in unpreserved stools were compared to the published data available for the original testing platforms from which most of the Bio-Plex primers and probes were adapted.

Analytical sensitivity of the *Giardia* primers and probe are previously described for a multiplex real-time PCR format and were estimated to be 10 trophozoites/200 mg stool (50). In addition, Taniuchi et al. (107) recently published a limit of detection of 10^3 *Giardia* cysts/200 mg stool for the Bio-Plex platform. Interestingly, Taniuchi et al. observed a greater limit of detection of 10^3 cysts/200 mg stool when the primers and probe were evaluated using real-time PCR, as described by Haque et al. (107). Therefore, the analytical sensitivity of the *Giardia* primers and probe achieved in this study for unpreserved stool specimens was comparable to that described for the real-time multiplex assay and more sensitive than described for the BioPlex by Taniuchi et al. (Table 10) (50, 107). Differences in Bio-Plex platform analytical sensitivity in this study as compared to Taniuichi et al. may be due to the use of different extraction and/or amplification methods. In contrast, the *C. parvum* target was not as sensitive as that described

by Fontain et al. (44), yielding detection limits 10-fold to 1000-fold higher than the 5 oocysts/PCR reaction described for a singleplex real-time PCR assay (Table 10). Sensitivity of the Bio-Plex assay could be reduced due to the fact that the assay is a multiplex detection assay or due to differences in method biochemistries. The *C. cayetanensis* target limit of detection of $3.5x10^{1}$-$3.5x10^{3}$ oocysts/200 mg stool from this study was comparable only at the lower limit to the sensitivity of 10 oocysts described by Orlandi et al. when the primers were used for multiplex conventional PCR testing (85).

Finally, the *E. histolytica* target exhibited an estimated limit of detection 10 to 100-fold higher than the limit of 1 trophozoite/200 mg stool previously described for a multiplex real-time PCR assay (50). These primers and probe were also tested on the Bio-Plex platform and in a real-time PCR assay developed by project collaborators, and determined to have a limit of detection of 10 trophozoites/200 mg stool in both cases (107). The comparison of assays in this case is not a direct one, as cloned DNA was used to establish the limits of detection of the primers and probe utilized in this study and trophozoites were used in the other two studies (50, 107). Differences in Bio-Plex platform sensitivity may be due to the use of different extraction and/or amplification methods as well as the approach used to establish limit of detection. The limits of detection established using cloned DNA is an estimate, as mathematical formulas are used to calculate both the genome copy values and the organism equivalents. It is also important to note that the limit of detection range for the *E. histolytica* target is a better estimate of trophozoite concentration in stool than that of cyst concentration. This is due to the fact that the conversion factor used to estimate the number of genome copies (Materials and Methods) present in the cloned standards was originally established for trophozoites, and it has been postulated that copy numbers in cysts may be lower (82).

Comparative data describing limits of detection for the protozoan assay targets in LV-PVA specimens is not readily available. When compared to the limits of detection obtained in this study for unpreserved specimens, the limits of detection for the *G. intestinalis, C. parvum/hominis,* and *C. cayetanensis* targets in PVA specimens were approximately 1-2 logs higher. These increased limits of detection are possibly due to the addition of washing steps prior to extraction of *Giardia* and *Cryptosporidium* or due to variation in pipetting clone dilutions of *Cyclospora.* Interestingly, the analytical sensitivity range of the *E. histolytica* target in LV-PVA was lower than that achieved for unpreserved specimens. Again, a plausible source of this discrepancy is pipetting variation when spiking the cloned plasmid. The difference in matrix consistency of the unpreserved and LV-PVA specimens may be considered an alternate source of variation, but is unlikely as the same clone plasmid was used for *Cyclospora* and a similar difference in limit of detection ranges was not observed.

Another practical analysis compares the limits of detection to excreted concentrations of organism available in the literature in order to determine if the assay is sensitive enough to detect organism in clinical specimens. While excretion patterns of protozoa are highly variable, the data are useful in estimating actual performance of the assay on clinical specimens collected as part of an outbreak investigation. For example, a study of asymptomatic pediatric patients infected with *Giardia* reported a variety of cyst excretion patterns, with titers ranging from 10-2000 cysts per milligram of unpreserved stool (34). Converting these titers to $2x10^3$ to $4x10^5$ cysts/200 mg stool enables comparison with the Bio-Plex *G. intestinalis* limits of detection for both unpreserved and LV-PVA specimens. Comparing the approximate *G. intestinalis* target sensitivity range of 10^1 to 10^4 cysts/200 mg stool established in this study for both specimen types suggests that the assay is sensitive enough to detect pathogen in clinical specimens if the

patient is excreting at the time of collection (Table 10). A study in which three methods were used to recover oocysts from unpreserved patient specimens was evaluated and provides a similar means of comparison for the *Cyclospora* target (66). Recovery from suspensions representing 5 mg of stool ranged from one to 7720 oocysts, which can be converted to the average estimate of $4.0x10^1$ to $3.1x10^5$ oocysts/200 mg stool. Comparison of the Bio-Plex *Cyclsopora* limits of detection (Table 10) suggests that this multiplex assay is also capable of detecting physiological concentrations of organism in both unpreserved and LV-PVA stools. Similar comparisons cannot be conducted for the Bio-Plex protozoan assay *C. parvum* and *E. histolytica* targets, as no excretion data is available for either organism.

Specificity of the intestinal protozoa assay primers and probe was evaluated by challenge with non-targeted intestinal parasites in LV-PVA . The assay was challenged with the following organisms: *Blastocystis hominis, Entamoeba coli, Entamoeba hartmanni, Endolimax nana, Dientamoeba fragilis,* and *Chilomastix mesnili.* No cross-reactivity was observed with these organisms, as exhibited by the lack of positive results for any assay targets. In addition, the primers and probes were evaluated for cross-reactivity with other assay targets and similarly no non-specific amplification was observed. Previously-published studies have also demonstrated that the primer and probe sequences do not cross-react with a variety of non-target sequences in other pathogens (44, 113). However, unexpected cross-reactivity of the *E. histolytica* primers and probe to *E. dispar* was identified in one LV-PVA specimen (data not shown). Additional testing using an alternate conventional PCR assay that discriminates between *E. histolytica* and *E. dispar* confirmed cross-reactivity to *E. dispar*. Sequencing of the amplification product identified five mismatched base pairs when compared to the *E. histolytica* probe sequence. The sequence analysis utilizing the nucleotide Basic Alignment Search Tool (BLAST; National

Center for Biotechnology Information, Bethesda, MD) ranked the top three alignments to *E. histolytica* 18S rRNA gene (Genbank AB426549) and two *E. dispar* 18S rRNA genes (Genbank AB282661 and Z49256), with the amplicon sequence aligning most closely to *E. dispar*. Additional testing with DNA extracted from cultures of *E. dispar* trophozoites followed by PCR and Bio-Plex detection also yielded positive results, further supporting the cross reactivity finding (data not shown). It is postulated that despite the observed mismatches between the probe and specimen amplicon, the relatively low stringency of the hybridization conditions in the Bio-Plex assay enables binding of the *E. histolytica*-specific probe to *E. dispar* amplicon sequences. These findings support those of Taniuchi et al. (107), indicating that the primers and probe lack species-specificity on the Bio-Plex platform and therefore may not differentiate between pathogenic and non-pathogenic *Entamoeba* in all specimens.

Finally, overall Bio-Plex assay performance was assessed by comparing the results obtained from testing previously characterized specimens representative of those typically submitted for outbreak investigations with results obtained by performing microscopy and/or PCR. The analytical sensitivity for *G. intestinalis, C. cayetanensis, C. parvum/hominis* and *E. histolytica* was 81.25%, 83.33%, 100.00%, and 42.86%, respectively. A screening assay with high sensitivity is typically desirable as it gives the operator confidence that negative results are true negatives and further testing is not required. However, in most public health laboratories, multiple specimens are submitted in outbreak situations, allowing for increased sampling and increased overall sensitivity. In addition, the proposed overall testing algorithm will require follow-up of testing results with confirmatory methods, particularly in situations in which all specimens submitted yield negative results. Together these approaches allow for some tolerance of lower sensitivity values, as discussed in Chapter 2. Therefore, for the intended use of the Bio-

Plex protozoan as a screening assay, the sensitivity of the *Giardia*, *Cyclospora*, and *Cryptosporidium* targets is acceptable, as the values exceeded the minimum acceptable sensitivity of 80% (Chapter 2). In contrast, the sensitivity of the *E. histolytica* target is markedly low as compared to microscopy, and discrepants could not be resolved upon further troubleshooting with microscopy and an alternate PCR method that discriminates between *E. histolytica* and *E. dispar*.

In order to determine if the presence of inhibitors were the cause of eight false-negative results for the *G. intestinalis, C. cayetanensis,* and *E. histolytica* targets, discrepant specimens were tested using 1:10 and 1:50 dilution of template nucleic acids. Negative results indicated inhibitors were not present (data not shown). Assay limitations that may have contributed to decreased sensitivity include prolonged storage of template DNA used for the study, inability to re-confirm the original reference method results, the presence of amplification products below assay limits of detection and the small number of specimens available for testing. It is acknowledged that the sensitivity of morphologic identification of *E. histolytica* may be as low as 60% target, resulting in false-positive results (105). It is possible that misidentification of morphologic features on microscopy may have occurred for several specimens in this study, contributing to the decreased sensitivity of the *E. histolytica* target.

Rather than ruling out disease, as with traditional diagnostic screening assays, this multiplex assay is intended to be used to screen outbreak specimens to rule in a possible etiologic agent in order to focus further confirmatory testing. Based on this intended use, a high specificity is the priority, as this provides confidence in positive results due to a low incidence of false positive results. The specificity of the four multiplex targets was 98-100%, exceeding the minimum acceptable specificity of 95% established in Chapter 2. The excellent specificity of the assay

facilitates preliminary identification of the etiologic agent leading to the development of effective, data driven public health management practices by epidemiologists.

The evaluations described here have assessed the performance of a microsphere-based multiplex assay for the simultaneous detection of *G. intestinalis, C. cayetanensis, C. parvum/hominis,* and *E. histolytica* in a public health laboratory setting. This assay exhibits acceptable precision for all targets, as compared to data available in the commercial and scientific literature for microsphere-based assays. With the exception of *G. intestinalis,* all targets exhibited higher limits of detection in unpreserved specimens as compared to the real-time and conventional PCR assays for which they were originally designed. These data suggest that the chemistry of Bio-Plex reactions may not facilitate direct adaptation of primers and probes successfully used in other amplification based methods, and instead additional optimization may be required. A practical comparison of the limits of detection with *Giardia* and *Cyclospora* concentrations described for clinical specimens suggests, however, that the protozoan screening assay is capable of detecting the intended targets in typical outbreak specimens. The data also show that analytical sensitivity is somewhat reduced in LV-PVA specimens; therefore, it is recommended that unpreserved specimens be the preferred specimen type for testing. The protozoan assay showed acceptable sensitivity as compared to reference methods for *G. intestinalis, C. cayetanensis,* and *Cryptosporidium* spp. targets based on its intended use as an outbreak screening method. The three targets also exhibited excellent specificity. Because the sensitivity of the assay is less than 100% for several targets, it is recommend that an outbreak investigation algorithm include further testing using alternate protocols when all specimens submitted for an outbreak are negative for assay pathogen targets. In addition, confirmatory testing to verify screening results is recommended. Finally, the

multiplex exhibited no cross-reactivity to a variety of non-target intestinal parasites, with the exception of *E. dispar*. The capability of the *E. histolytica* primers and probe to cross-react with *E. dispar* sequences requires that the primers and probe be re-designed and optimized to achieve improved species specificity. In summary, the data demonstrate that the Bio-Plex multiplex protozoan assay has potential as a screening assay in outbreak investigations, with one target modification, in order to provide preliminary pathogen information to epidemiologists and to better focus confirmatory laboratory testing. In addition, the assay may serve as the backbone to which additional pathogen targets may be added, maximizing the flexibility of the Bio-Plex 200 platform.

CHAPTER 5 EVALUATION OF A PCR-BASED BACTERIAL BIO-PLEX ASSAY

Overview of Enteric Bacterial Pathogens and Assay Molecular Targets. The six-plex bacterial assay developed for and evaluated in this aim of the project targets multiple diarrheagenic *E. coli* strains and *Shigella* spp. The primers and probes include previously published sequences and new unpublished sequences, adapted to or designed for the Bio-Plex platform by collaborators at the University of Virginia and at DCLS. Genetic targets included in the Bio-Plex bacterial assay enable the detection of shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), and enteroinvasive *E. coli* (EIEC), and *Shigella* spp.

STEC. Shiga toxin producing *E. coli* comprise a group of *E. coli* strains that include multiple serotypes, including the most well-characterized foodborne pathogen, *E. coli* O157:H7. Initial symptoms of infection by STEC include watery diarrhea and abdominal pain for 1-2 days, followed by an increase in symptom severity that may include bloody diarrhea for several days (57). Fever is not a prominent symptom. Perhaps of most concern with these strains is the potential for the infection to move beyond hemorrhagic colitis to hemolytic uremic syndrome or thromocytopenic purpura, both of which are life-threatening (57).

STEC strains are characterized by their ability to produce one or both of two shiga-like toxins, the genes of which are designated *stx1* and *stx2*. These toxins were first described in *Shigella dysenteriae* and are composed of one active (A) subunit that interrupts ribosomal RNA to ultimately halt protein synthesis in the infected cell and five binding (B) subunits (61). There

is approximately 55% identity between the *stx1* and *stx2* genes, with the *stx1* sequences' being well-conserved, while the *stx2* sequence exhibits multiple variants. Both *stx1* and *stx2* are encoded on lambdoid lysogenic bacteriophages, while the *stx2e* variant is chromosomally-encoded (79, 109). Bacteria may have single or multiple phage copies (109).

The primers and probe to detect *stx1* were adapted directly from a multiplex real-time PCR assay described by Hidaka et al. (53). The targeted genome sequence is a 132-base-pair region of the gene encoding the A subunit of the *stx1* toxin. The primers for *stx2* detection were adapted from a multiplex conventional PCR assay and yield a 255 base-pair amplification product representing the gene encoding the A subunit of the *stx2* toxin gene (90). The primers are designed to detect all *stx2* variants. The *stx2*-specific probe was designed by Dr. Taniuchi at the University of Virginia (M. Taniuchi, unpublished data).

E. coli O157. Because infections caused by *E. coli* O157:H7 may result in a more severe clinical presentation than those caused by other STEC strains and the fact that *E. coli* O157:H7 strains are more frequently associated with significant foodborne outbreaks, it is desirable to be able to specifically detect O157 serotypes that may be associated with an outbreak (57). Specific detection of O157 strains is possible due to the description of a single base pair mutation in the β-glucuronidase gene (*uidA*) that is conserved in O157 strains versus other STEC serotypes (10, 42). The current assay incorporates primers and a probe described by Yoshitomi et al. that were originally designed for use in a singleplex real-time PCR assay and exhibited optimal Bio-Plex fluorescent data for positive specimens as compared to other primer and probe designs (data not shown) (119). The probe sequence is specific for the O157 base pair mutation.

ETEC. This group of organisms is the primary cause of "traveler's diarrhea" (1). In addition, ETEC strains have been implicated in a variety of outbreaks on cruise ships and within the U.S.,

many cases of which were due to consumption of contaminated food or water (4, 33). The primary symptom of ETEC infection is diarrhea, with varying reports of abdominal cramps, fever, nausea and vomiting. When vomiting is present, diarrhea has generally been reported to occur at least 2.5 times the incidence of vomiting. Duration of symptoms is widely variable among reported patients, with a median duration of >72 hours described for multiple U.S. outbreaks caused by ETEC strains (4, 33).

ETEC strains cause the symptoms of disease through the production of one or both of two toxins that lead to excess intestinal secretion (61). One toxin is termed "heat labile toxin" (LT), and its structure and function are closely related to the cholera toxin of *Vibrio cholerae* (79). There are two serogroups described for LT: LT-I and LT-II. LT-I is produced by ETEC strains that are pathogenic for both humans and animals. In contrast, strains expressing LT-II are found primarily in animals, and have not been associated with human disease (79). The gene for the LT-I toxin is identified as *elt*, and resides extrachromosomally on a plasmid (79). The second toxin that may be produced is a "heat stable toxin" (ST). As is the case for LT, there are several variants of the ST. The toxin variants are small single-peptide toxins of which the STa (ST-1) variant is primarily associated with human disease (61). The *estA* gene that encodes this toxin is located primarily on a plasmid, although the genes have also been found on transposons (79).

The primer and probe set employed in the multiplex for the LT gene was described for use in a multiplex real-time PCR assay, targeting a 62 base-pair region of the A subunit gene (*eltA*) (53). The primer and probe set to detect the *estA* gene (ST) was designed by Dr. Mami Taniuchi of the UVA and produces a 172 base-pair amplification product (M. Taniuchi, unpublished data).

EIEC/Shigella. Enteroinvasive *E. coli* (EIEC) and *Shigella* spp. are highly similar in terms of biochemical identification, genetics and pathogenesis. Separate taxonomies are maintained, however, due to the clinical symptoms of *Shigella* (61). Clinical presentation of *Shigella* infection is that of dysentery: diarrhea with blood and/or mucus, fever, and abdominal cramps (61). In contrast, infection with EIEC manifests similarly to other *E. coli* infections, including watery diarrhea, abdominal cramps, fever and malaise. Progression to dysentery can occur, but is not common (57). Pathogenesis of both organisms is characterized by invasion of intestinal epithelial cells, intracellular replication, invasive movement into adjacent cells, and induction of apoptosis (61).

The genetic region targeted by the Bio-Plex assay for both of these strains is the *ipaH* gene, a multi-copy gene on the large invasion plasmid and chromosomes of both *Shigella* and EIEC strains (9, 52, 89). Investigation of primarily *S. flexneri* strains has shown that *ipaH* gene products are secreted via the organism's invasion plasmid-encoded Type III secretion apparatus (36). Recently the structure and activity of *ipa*H gene products in *Shigella* were elucidated, identifying this family of proteins as a new class of E3 ubiquitin ligases that may be involved in dampening host innate immune responses (103, 121). The primers and probe set employed in this assay was designed by Dr. Mami Taniuchi of UVA, and target a 64-base pair sequence of the *ipaH* gene (M. Taniuchi, unpublished data).

Results.

Preliminary assessment of the multiplex bacterial assay using stool broth specimens. A preliminary evaluation of the bacterial assay 6-plex was performed by testing 50 characterized stool broth specimens using the multiplex Bio-Plex bacterial assay and the qualitative results were compared to those obtained using molecular and shiga toxin enzyme immunoassay

reference methods. The calculated sensitivity and specificity for each target and corresponding intervals are shown in Table 12. The sensitivities for *stx1, stx2,* and *uidA* were 100%, 95% and 100%, respectively. Specificity of all targets was 100%, with the exception of 95% specificity of the *stx1* target.

Validation of Cary-Blair specimens - precision studies. Repeatability (within-run precision) was performed by amplifying nucleic acid from a select panel of specimens using the bacterial assay and detecting in ten replicate wells on the Bio-Plex, as described in the Materials and Methods. The statistics of mean, standard deviation (SD) and coefficient of variation (CV) were calculated, as described. To assess repeatability, the precision for positive-target specimens was expressed as the CV, which ranged from 6.50% to 26.83% for the three specimens tested in the study (Table 13). Each specimen in this panel also yielded negative results for additional assay targets (e.g., O157:H7-positive specimen had negative results for *eltA, estA* and *ipaH*). In addition, the panel included one Cary-Blair specimen known to be negative for enteric bacteria, and therefore yielded negative results for all assay targets. Within-run precision for negative targets was expressed as SD, and ranged from 4.32 – 9.30 (Table 14).

In order to evaluate between-day variation (reproducibility) of the amplification and Bio-Plex steps together, nucleic acid template from the specimen panel used in the repeatability study was amplified and detected for five days using the Bio-Plex bacterial assay, as described in Materials and Methods. The mean, SD and CV were calculated as described. The between-day CV values of the amplification and detection steps combined for positive assay targets ranged from 13.54% to 41.26% (Table15), and SD values (FI) for negative assay targets ranged from 3.14 – 12.76 (Table 16). Evaluation of the precision of the detection step alone resulted in

Table 12. Preliminary assessment of a multiplex Bio-Plex 200 bacterial assay using stool broth specimens. Qualitative results of multiplex testing using the Bio-Plex platform to detect enteric bacterial pathogens in 50 characterized stool broth specimens were compared to those obtained with reference methods. Sensitivity was calculated using the formula: [TP/(TP+FN)]x100. Specificity was calculated using the formula: TP/(TP+FN)]x100. Confidence intervals were calculated using Wilson's score confidence interval method (28).

Target	TP (n)	FN (n)	Se (%)	$CI_{Se, 95}$	TN (n)	FP (n)	Sp (%)	$CI_{Sp, 95}$
stx1	31	0	100.00	L: 88.98 U: 100.00	18	1	95.00	L: 75.36 U: 99.07
stx2	20	1	95.00	L: 77.33 U: 99.16	29	0	100.00	L: 88.31 U: 100.00
uidA	4	0	100.00	L: 51.02 U: 100.01	46	0	100.00	L: 92.29 U: 100.00
eltA (LT)	0	0	N/A	N/A	50	0	100.00	L: 92.87 U: 100.00
estA (ST)	0	0	N/A	N/A	50	0	100.00	L: 92.87 U: 100.00
ipaH	0	0	N/A	N/A	50	0	100.00	L: 92.87 U: 100.00

Abbreviations:
TP, True positive, Bio-Plex 200 positive for an organism identified as positive by reference method;
TN, True negative, Bio-Plex 200 negative for an organism identified as negative by reference method;
FP, False positive, Bio-Plex 200 positive for an organism identified as negative by reference method;
FN, False negative, Bio-Plex 200 negative for an organism identified as positive by reference method;
Se, sensitivity; Sp, specificity; CI. confidence interval; L, lower limit; U, upper limit

Table 13. Bio-Plex 200 detection repeatability results for positive targets in Cary-Blair stool specimens. The known pathogen and bacterial assay positive gene target(s) are indicated for each specimen used in the study. Extracted nucleic acid from each specimen was amplified and then detected in ten replicate wells, as described in Materials and Methods. Mean, SD and CV were calculated using result values for each well obtained by subtracting the mean fluorescence of ten background wells from the Bio-Plex FI output of individual sample wells.

Pathogen/ Gene Target(s)	Mean (FI) (n = 10)	SD (FI)	CV[a] (%)
O157:H7			
stx1	605.50	39.33	6.50
stx2	67.00	9.78	14.60
uidA	74.75	20.06	26.83
ETEC (ATCC 35401)			
eltA (LT)	1140.20	112.76	9.89
estA (ST)	333.30	44.63	13.39
S. flexneri			
ipaH	786.25	81.51	10.37

[a] CV: coefficient of variation; (SD / mean) x 100

Table 14. Bio-Plex 200 detection repeatability results for negative targets in Cary-Blair stool specimens. The bacterial assay gene target and total number of specimens negative for the target are indicated. Extracted nucleic acid from each specimen was amplified and then detected in ten replicate wells, as described in Materials and Methods. Mean and SD were calculated using result values for each well obtained by subtracting the mean fluorescence of ten background wells from the Bio-Plex FI output of individual sample wells. Mean and SD ranges reflect the range of statistics calculated for all panel specimens negative for the respective assay target. The total number of specimens in the panel that were negative for the select target is provided.

Gene Target	Number of specimens	Mean (FI) (n = 10 wells/specimen)	SD (FI)
stx1	3	-1.00 – 3.05	5.47 – 5.84
stx2	3	0.15 – 2.85	5.14 – 5.27
uidA	3	-10.00 – -0.40	4.76 – 5.52
eltA (LT)	3	-3.50 – 2.45	5.33 – 6.51
estA (ST)	3	-3.40 – 0.35	4.32 – 9.30
ipaH	3	-2.70 – 1.70	4.45 – 6.35

Table 15. Amplification and Bio-Plex 200 detection reproducibility results for positive targets in Cary-Blair stool specimens. The known pathogen and bacterial assay-positive gene target(s) are indicated for each specimen used in the study. Extracted nucleic acid from each specimen was amplified and detected each day for five days, as described in Materials and Methods. Mean, SD and CV were calculated by using results representing the specimen FI minus the background well FI for each positive target.

Pathogen/ Gene Target(s)	Mean (FI) (n = 5)	SD (FI)	CV[a] (%)
E. coli O157:H7			
stx1	965.80	309.26	32.02
stx2	104.10	31.91	30.66
uidA	87.50	34.20	39.09
ETEC (ATCC 35401)			
eltA (LT)	993.63	134.56	13.54
estA (ST)	163.00	67.25	41.26
Shigella flexneri			
ipaH	454.10	142.09	31.29

[a] CV: coefficient of variation; (SD / mean) x 100

Table 16. Amplification and Bio-Plex 200 detection reproducibility results for negative targets in Cary-Blair stool specimens. The bacterial assay gene target and total number of specimens negative for the target are indicated. Nucleic acid extracted from each specimen was amplified and detected each day for five days, as described in Materials and Methods. Mean and SD were calculated using result values for each well obtained by subtracting the mean of one background well from the Bio-Plex FI output of individual sample wells. Mean and SD ranges reflect the range of statistics calculated for all panel specimens negative for the respective assay target. The total number of specimens in the panel that were negative for the select target is indicated.

Gene Target	Number of specimens	Mean (FI) (n = 5/specimen)	SD (FI)
stx1	3	2.90 – 9.00	9.97 – 12.76
stx2	3	-6.25 – -3.70	4.70 – 10.29
uidA	3	-6.30 – 3.13	5.23 – 9.56
eltA (LT)	3	-3.60 – -1.00	3.54 – 6.87
estA (ST)	3	-3.20 – 1.50	3.14 – 7.01
ipaH	3	-7.63 – -4.00	3.67 – 7.87

a CV value range of 7.28% to 28.23% (Table 17) for positive-target specimens, while negative targets exhibited an SD value range of 1.65 – 14.26 (Table 18).

Validation of Cary-Blair specimens – analytical sensitivity (limits of detection). The analytical sensitivity, or limits of detection (LoD), of each target in the bacterial assay was evaluated using *E. coli* O157:H7, ETEC (ATCC 35401) and *S. flexneri* (ATCC 12022) isolates spiked into Cary-Blair stool aliquots characterized to be negative for pathogenic enteric bacteria, as described. Dilution series were extracted and tested in triplicate and colony counts on blood agar plates were conducted to determine organism concentrations. Table 19 summarizes the limits of detection established for each assay target in Cary-Blair stool specimens. Analytical sensitivity of the assay ranged from approximately $2.7x10^1$ CFU/200 mg stool to $2.4x10^6$ CFU/200 mg stool. Based on a spiking volume of 10 µL, the LoDs can be converted to a range of $2.7x10^3$ to $2.4x10^8$ CFU/mL (Table 19).

Validation of Cary-Blair specimens – analytical specificity. The organisms present in the Cary-Blair specimens tested in this study exhibited no cross-reactivity to non-complementary primers and probes in the multiplex assay (data not shown). Organisms tested included *E. coli* O157:H7 (n = 2), *E. coli* non-O157 (*stx1*-positive; n = 1), *E. coli* of unknown serotype (*stx1*-positive, n = 3), *Shigella* spp. (n = 4) and *Salmonella* spp. (n = 5). In addition, the assay was challenged with bacterial isolates including ETEC (n = 5; serotypes O153:H45, Ound:H16, Ound:NM, O6:H16, and O148:NM), EPEC (n = 10; serotypes O119:H6, O55:NM, Ound:NM, O115:NM, Orough:H8, O111:NM, O55:H6, O111:H2, O128:H1, and O86:H34), and EIEC (n = 2; serotypes O124:NM and O29:NM) strains. No cross-reactivity was observed with non-complementary

Table 17. Bio-Plex 200 detection reproducibility results for positive targets in Cary-Blair stool specimens. The known pathogen and bacterial assay positive gene target(s) are indicated for each specimen used in the study. Extracted nucleic acid from each specimen was amplified once and detected each day for four days, as described in Materials and Methods. Mean, SD and CV were calculated using result values for each replicate well obtained by subtracting the fluorescence of one background well from the Bio-Plex FI output of individual sample wells.

Pathogen/ Gene Target(s)	Mean (FI) (n = 4)	SD (FI)	CV[a] (%)
E. coli O157:H7			
stx1	818.25	105.02	12.83
stx2	91.50	25.83	28.23
uidA	53.13	4.17	7.85
ETEC (ATCC 35401)			
eltA (LT)	1084.38	78.98	7.28
estA (ST)	63.13	11.51	18.23
Shigella flexneri			
ipaH	635.13	74.33	11.70

[a] CV: coefficient of variation; (SD / mean) x 100

Table 18. Bio-Plex 200 detection reproducibility results for negative targets in Cary-Blair stool specimens. The bacterial assay gene target and total number of specimens negative for the target are indicated. Nucleic acid extracted from each specimen was amplified once and detected each day for four days, as described in Materials and Methods. Mean and SD were calculated using result values for each well obtained by subtracting the fluorescence of one background well from the Bio-Plex FI output of individual sample wells. Mean and SD ranges reflect the range of statistics calculated for all panel specimens negative for the respective assay target.

Gene Target	Number of specimens	Mean (FI) (n =4/specimen)	SD (FI)
stx1	3	-2.75 – 2.38	2.87 – 5.85
stx2	3	-2.13 - 5.75	10.05 - 14.26
uidA	3	-5.88 – 2.38	4.17 – 10.78
eltA (LT)	3	-1.38 – 4.75	2.78 – 5.19
estA (ST)	3	-4.63 - -1.88	1.65 – 5.11
ipaH	3	-2.25 – 8.38	2.87 – 11.14

Table 19. Analytical sensitivity (limits of detection) of the Bio-Plex 200 enteric bacterial assay. Quantified bacterial isolates were diluted and spiked into 200 mg Cary-Blair stool aliquots, as described. Spiked stools were extracted, template amplified, and detected as described. Dilutions were performed in triplicate. Positivity was determined as described for detection of targets in the Materials and Methods section. The limit of detection range represents the lowest concentrations yielding a minimum of 1 positive result. Limit of detection ranges are also converted to CFU/g stool and CFU/mL for analytical comparison..

	stx1	*stx2*	*uidA*	*eltA* (LT)	*estA* (ST)	*ipaH*
LoD, Cary-Blair stool *(CFU/ 200 mg stool)*	$1.0x10^3$-$2.4x10^4$	$3.5x10^1$-$3.5x10^3$	$1.0x10^5$-$2.4x10^6$	$1.5x10^2$-$2.5x10^3$	$1.5x10^4$-$2.5x10^4$	$2.7x10^1$-$2.7x10^2$
LoD, Cary-Blair stool *(CFU/g stool)*	$5.0x10^3$-$1.2x10^5$	$1.7x10^2$-$1.7x10^4$	$5.0x10^5$-$1.2x10^7$	$7.5x10^2$-$1.2x10^4$	$7.5x10^4$-$1.2x10^5$	$1.3x10^2$-$1.3x10^3$
LoD, Cary-Blair stool *(CFU/mL)*	$1.0x10^5$-$2.4x10^6$	$3.5x10^3$-$3.5x10^5$	$1.0x10^7$-$2.4x10^8$	$1.5x10^4$-$2.5x10^5$	$1.5x10^6$-$2.5x10^6$	$2.7x10^3$-$2.7x10^4$

assay targets, with the exception of a repeated positive result exhibited by one EPEC strain of serotype O86:H34 when detected with the *uidA* target.

Validation of Cary-Blair specimens – comparison-of-methods study. Sixteen (16) previously-characterized Cary-Blair stool specimens were tested using the multiplex microsphere assay and results compared to those obtained with molecular and biochemical reference methods. Positive specimens included: *E. coli* O157:H7 (n = 2), *E. coli* non-O157:H7 (n = 1), *E. coli* of unknown serotype (n = 3), *Shigella* spp. (n =4), and *Salmonella* spp. (n = 5). One specimen was negative for pathogenic enteric bacteria. The calculated sensitivity and specificity for each target and corresponding confidence intervals are shown in Table 20. The sensitivity for the *stx1*, *stx2*, *uidA* and *ipaH* targets as compared to reference methods was calculated to be 100.00% for all four targets. No positive specimen data was available to estimate sensitivity of the *eltA* and *estA* targets. Specificity of all targets as compared to reference methods was calculated to be 100%.

Discussion. A comparison-of-methods study was conducted to assess the potential utility of the six-plex enteric bacterial Bio-Plex assay on DCLS platforms. This initial study was completed by testing stool broth specimens typically submitted to state public health laboratories for confirmation of the presence of shiga toxin-producing organisms and characterized to be positive for *stx1*, *stx2*, and/or *uidA* targets. Studies initially focused on these specimen types because a large number of specimens were readily available and the original intent was to validate broth stool matrices for use in outbreak investigations. Results achieved on the Bio-Plex were compared to those obtained with biochemical and molecular reference methods used to characterize the specimens upon submission to DCLS, and sensitivity and specificity were calculated as described. As shown in Table 12, sensitivity for the *stx1*, *stx2*, and *uidA* targets was excellent, exceeding the minimum acceptable sensitivity of 80% established for this assay.

Table 20. Comparison of Bio-Plex 200 bacterial assay results to reference method results, by assay target. Qualitative results of multiplex testing using the Bio-Plex platform to detect bacterial pathogens in Cary-Blair stool specimens were compared to those obtained with molecular and/or biochemical reference methods. Sensitivity was calculated using the formula: [TP/(TP+FN)]x100. Specificity was calculated using the formula: [TN/(TN+FP)]x100. Corresponding 95% confidence intervals were calculated using Wilson's score confidence interval method. (28)

Target	TP (n)	FN (n)	Se (%)	$CI_{Se, 95}$	TN (n)	FP (n)	Sp (%)	$CI_{Sp, 95}$
stx1	6	0	100.00	L: 60.97 U: 100.00	10	0	100.00	L: 72.25 U: 100.00
stx2	2	0	100.00	L: 34.24 U: 100.01	14	0	100.00	L: 78.47 U: 100.00
uidA	2	0	100.00	L: 34.24 U: 100.01	14	0	100.00	L: 78.47 U: 100.00
eltA (LT)	0	0	N/A	N/A	16	0	100.00	L: 80.64 U: 100.00
estA (ST)	0	0	N/A	N/A	16	0	100.00	L: 80.64 U: 100.00
ipaH	4	0	100.00	L: 51.02 U: 100.01	12	0	100.00	L: 75.76 U: 100.00

Abbreviations:
TP, True positive; Bio-Plex 200 positive for an organism identified as positive by reference method;
TN, True negative; Bio-Plex 200 negative for an organism identified as negative by reference method;
FP, False positive; Bio-Plex 200 positive for an organism identified as negative by reference method;
FN, False negative; Bio-Plex 200 negative for an organism identified as positive by reference method;
Se, sensitivity; Sp, specificity; CI. confidence interval; L, lower limit; U, upper limit

Specificity for all targets was also excellent, meeting or exceeding the minimum acceptable specificity of 95% established for this assay.

Several limitations exist for this comparison-of-methods study. First, no stool broth specimens were available containing either ETEC or EIEC/*Shigella* spp., resulting in no sensitivity data for the *eltA*, *estA* and *ipaH* targets. Second, only four *uidA*-positive specimens were available, thus lowering the statistical confidence of the sensitivity data for this target. A limitation in assay performance was also identified, particularly for the *stx2* target. One specimen was negative for the *stx2* target when tested on the Bio-Plex, though it was previously determined to be positive by both the LightCycler reference method and the Bio-Plex a year before (data not shown). This broth specimen was highly pigmented and the boiled preparation yielded equally pigmented supernatant containing template nucleic acid. Testing on both the Bio-Plex and LightCycler as described using 1:10 and 1:50 nucleic acid template dilutions revealed that amplification of the specimen was being inhibited, most likely by the substance causing the pigmentation (data not shown). Such pigmentation is rare in stool broth specimens; therefore, it is recommended that a simple procedure for diluting pigmented stool broth specimens be added to future testing protocols, rather than pursuing a more complicated solution of incorporating an inhibition control.

Following completion of the comparison-of-methods study, further validation was not pursued with stool broth specimens. As mentioned previously, this specimen type is submitted to state reference laboratories in order to enable the isolation and confirmation of shiga toxin-producing *E. coli* bacteria. Since stool-containing broth specimens are not a primary specimen type collected for testing in outbreak investigations, the need for validation of this specimen type with the bacterial assay was not pursued. Instead, validation studies were conducted on the

primary outbreak specimen type submitted during outbreak investigations, which are stools diluted in Cary-Blair medium (referred to here as "Cary-Blair stools" or "Cary-Blair specimens").

Following initial testing of stool broth specimens, the multiplex assay was evaluated using Cary-Blair specimens for the characteristics of precision (repeatability and reproducibility), analytical sensitivity (limits of detection), analytical specificity (cross-reactivity), and performance as compared to reference methods. Validation study results were then evaluated for assay acceptability as a screening method to provide preliminary outbreak etiologic agent information to epidemiologists and to focus confirmatory testing in the laboratory.

As described, precision studies were conducted with a panel of four Cary-Blair specimens representing the type of specimen typically received in the course of an outbreak investigation. As was done in the enteric protozoan study, the CV data generated in the repeatability and reproducibility studies for positive assay targets were compared to positive target CVs published for the xTAG® RVP commercial assay. Coefficient of variation values for the RVP assay were established in a multi-laboratory reproducibility study to range from 2.40% to 87.20% for a range of virus single-infection virus concentrations (75). As shown in Table 13, the within-run (repeatability) CV value range of 6.50% to 26.83% achieved for positive targets with the bacterial assay was comparable to the values reported for the RVP assay. Of some concern was the larger CV exhibited for the *uidA* target as compared to the other two targets near the assay cutoff value (*stx2*, *estA*). The higher variance suggests that low-concentration specimens positive for *uidA* may exhibit a higher incidence of false negative results due to lack of sensitivity. Interestingly, singleplex real-time detection of *uidA* using other established assays

at DCLS have also historically exhibited notable variation in *uidA* detection, suggesting that optimizing the chemistry of detecting the single base pair mutation in O157 strains is inherently difficult and the source of variability.

Similarly, as shown in Tables 15 and 17, the reproducibility study CV value range of 13.54% to 41.26% for the amplification and detection steps combined, and 7.28% to 28.23% for the detection step alone, were comparable to the values published for the RVP assay. It is worth mentioning that on several days of the reproducibility studies the *uidA*-positive specimen yielded negative qualitative results for that target. Based on this performance in both the repeatability and reproducibility studies, it should be noted that low-concentration *uidA* targets may not be detected consistently. As was the case for the protozoan assay, the enteric bacterial assay exhibited a notable reduction in the detection-only CV values as compared to those seen for the combined amplification and detection steps, suggesting that amplification is an important source of variability. Therefore, the comparison of Bio-Plex bacterial assay precision values for positive targets with those of the RVP assay suggests that multiplex microsphere-based nucleic acid assays may exhibit a wide range of variability, particularly at the low end of the FI measuring range for positive specimens, and facilitates the conclusion that the Bio-Plex bacterial assay exhibits comparable precision for Cary-Blair stool specimens.

As performed with the protozoan assay, SD values were used in this study to describe the variation for target-negative specimens and were compared to those described for a microsphere-based assay to detect *Plasmodium* spp. (77). In the malaria assay, SD values ranged from 18.1 to 39.0 for both negative specimens and negative targets, based on mean FI values ranging from 81.3 to 121.2. These values were calculated using FI values detected on the Bio-Plex with a high RP1 target setting, which typically results in higher FI values as compared to using the low RP1

target setting. Because the bacterial assay is detected on the Bio-Plex using a low RP1 target setting, the mean FI and SD values for negative specimens in the study described here are lower than those described for the *Plasmodium* assay. However, a relative comparison of the two assays remains informative. Although not a direct comparison, the SD range of 4.32-9.30 FI achieved in the repeatability study appears to similarly represent the range of variability described for this type of assay (Table 14). In addition, the between-day SD ranges of 3.14 – 12.76 for the combined amplification and detection steps (Table 16) and 1.65 – 14.26 (Table 18) for the detection step only are comparable to published data. Based on these evaluations, the Bio-Plex bacterial assay exhibits comparable precision for pathogen-negative Cary-Blair specimens.

Several primers and probes used in the bacterial multiplex assay were adapted from previously-published real-time or conventional PCR protocols. Therefore, a limited comparison of Bio-Plex assay's analytical sensitivity (limits of detection) to that of previously-described assays was conducted to evaluate how well the described primers and probes were adapted to the Bio-Plex platform. The *stx1* and *eltA* (LT) primers and probes were adapted from a multiplex real-time PCR assay for which the limits of detection were established by diluting organism spiked into enrichment broths, as opposed to the stool aliquots used in this Bio-Plex study (53). The Bio-Plex limits of detection are comparable to or lower than the published values of 2.0-2.7x10^6 CFU/mL for *stx1* and 1.3-1.6x10^6 CFU/mL for *eltA* (Table 19). Reasons for the lower limit of detection of the bacterial assay may be affinity of the probe for the Bio-Plex chemistry, differences in experimental design, or different gene copy numbers in the isolates used.

The *stx2* primers were also adapted, in this case from a published conventional assay (90). The probe was designed in this study for the Bio-Plex assay. Again, comparison of

detection limits is not exact, as the published sensitivity for the primers was established using spiked fecal cultures, not stool, and the probe is a new design. The Bio-Plex limit of detection as determined for a 200 mg stool reaction was slightly lower than the $1x10^3$ CFU/reaction achieved for the conventional assay and may be due to the Bio-Plex chemistry and probe design. In the case of the *uidA* target, both primers and probe from the literature were used in the Bio-Plex assay (119). Comparison of sensitivity data is difficult, however, as the published limit of detection of 1-1000 CFU/g was estimated by spiking food supplemented by enrichment, not a stool matrix. However, the analytical sensitivity of this target as compared to others in the Bio-Plex multiplex indicates that adaptation to the Bio-Plex format reduced the sensitivity of the primer and probe set (Table 19). No comparative data is available for the *estA* (ST) and *ipaH* targets, as they were designed for this study.

A practical comparison of the Bio-Plex assay limits of detection to known excreted concentrations of organism is also useful in determining if the Bio-Plex bacterial assay is sensitive enough to detect organism concentrations typically seen in clinical specimens. As was the case for enteric protozoa, examples of bacterial shedding concentrations are available in the literature. It is important to note that data discussed below for ETEC and *S. flexneri* are from volunteer challenge studies, so the organism concentrations determined to be excreted may not be an exact representation of natural infection. In a study of pediatric patients infected with *E. coli* O157, long-term shedders excreted over 10^6 CFU/g stool, while convalescent shedders were determined to excrete 10^5-10^6 CFU/g stool (63). It was noted, however, that convalescent shedders may excrete lower levels of organism that may not have been detected due to the LoD of the assay used in the Karch et al. study (63). A comparison of the detection limits, as converted to CFU/g stool, for the Bio-Plex *stx1* and *stx2* targets to the study data suggests that

the bacterial assay is capable of detecting physiologic concentrations of organism in clinical specimens (Table 19). Detection of *uidA* may be limited as suggested by the estimated limit of detection and precision data described previously.

An example of ETEC excretion levels is provided in a heat-labile toxin vaccine study, during which volunteers were challenged with $6x10^8$ CFU of organism (76). The median peak excretion was quantified to be $1x10^8$ CFU/g stool, with the implication that excretion levels may be lower. Comparison of the estimated limit of detection ranges for Bio-Plex *eltA* and *estA* targets with these data suggests that the bacterial assay is capable of detecting clinically relevant concentrations of organism (Table 19). In an antibiotic efficacy study, volunteers were challenged with 1500 CFU of *S. flexneri,* and peak shedding in those taking placebo was quantified to be $1x10^3$-$3x10^6$ CFU/g stool (108). Comparison of the estimated Bio-Plex *ipaH* limit of detection with these data indicates the bacterial assay is capable of detecting concentrations of organism that may be expected in clinical specimens (Table 19).

A potential limitation in *estA* (ST) target detection was identified when testing several ETEC isolates previously characterized by the Minnesota Department of Health to contain the *estA1* allele. Confirmation testing upon receiving isolates of serotype Ound:H16 and O148:NM with the Bio-Plex bacterial assay yielded negative results for ST. Subsequent testing using an alternate DCLS ETEC real-time protocol, as described in Materials and Methods, yielded positive ST results (data not shown). Bio-Plex primers and probe sequences were compared with those published for the complete coding sequence for the *estA1* allele of the heat-stable toxin I gene from *E. coli* strain 18D (Genbank accession M58746) (data not shown). Multiple base pair mismatches were identified between the Bio-Plex primers and probe and the published gene sequences, suggesting that detection of this ST allele is hindered in the Bio-Plex assay. Re-

design of the primers and probe or addition of an allele-specific primers and/or probe may be desired.

The Bio-Plex bacterial assay exhibited no cross-reactivity between microsphere targets. In addition, when challenged with ETEC, EIEC, and most EPEC strains, none of the multiplex microspheres yielded positive results. These data complement previously-published studies demonstrating that the primers and/or probe sequences for *stx1, six2* and *eltA* (LT) do not cross-react with non-target sequences in other pathogens (53, 90). Of note is repeated cross-reactivity of the *uidA* probe with one EPEC strain (serotype O86:H34) tested in the study. Similar cross-reactivity with several EPEC strains was also seen with experimental *uidA* probes designed at DCLS (data not shown). As discussed previously, detection of the O157-specific *uidA* sequence is based on a one base-pair mutation as compared to non-O157 strains in the β-glucuronidase gene. Designing reaction conditions to ensure specific hybridization can be a challenge. As described by Yoshitomi et al. (119), even under real-time singleplex conditions very stringent cycling temperature and probe and magnesium concentrations must be established to reduce cross-reactivity to non-O157 strains. Due to the possibility of cross-reactivity with EPEC strains, it is recommended that result interpretation of the *uidA* target be accomplished only when *stx1* and/or *stx2* targets are positive.

Finally, overall performance of the bacterial Bio-Plex assay was assessed by comparing results obtained from testing well-characterized Cary-Blair specimens representative of those typically submitted during outbreak investigations with results obtained by performing the reference methods of biochemical, enzyme immunoassay and/or molecular testing. Based on Bio-Plex and reference method result comparisons, Bio-Plex results were designated as either true positive (TP), true negative (TN), false positive (FP), or false negative (FN), and the

analytical sensitivity and specificity for each assay target were calculated (Table 20). Bio-Plex results that did not correlate with the reference method upon initial testing (FP, FN) were investigated using re-extraction and re-testing with both the Bio-Plex and molecular reference methods, as available. As shown in Table 20, sensitivity for the *stx1, stx2, uidA*, and *ipaH* targets is excellent, exceeding the minimum acceptable sensitivity of 80% established for this assay (Chapter 2). Specificity for all targets is also excellent, exceeding the minimum acceptable specificity of 95% established for this assay (Chapter 2).

Several limitations exist for the comparison-of-methods study. First, no Cary-Blair stool specimens containing ETEC organisms were available, resulting in no comparative data for the *eltA* and *estA* targets. However, successful detection of ETEC strains in Cary-Blair specimens can be anticipated based on the ability to detect low concentrations of organism in the course of the analytical sensitivity (LoD) study (Table 19). Second, the comparison-of-methods study sample size was limited by availability and was thus very small (n = 16). Therefore, the sensitivity confidence limit ranges for *stx1, stx2, uidA* and *ipaH* are fairly wide, illustrating the low statistical confidence provided by the sensitivity calculations. Additional data can be gathered in the course of future outbreak investigations to bolster the statistical confidence. Confidence limit ranges for the calculated specificity of all targets are smaller than those for the sensitivity calculations, providing a higher level of confidence in these data.

An evaluation has been conducted to assess the performance of a microsphere-based multiplex assay for the simultaneous detection of six STEC, ETEC, and EIEC/*Shigella* spp. molecular targets in Cary-Blair stool specimens in a public health laboratory setting. This assay exhibits acceptable precision for all targets, as compared to data available in the commercial and scientific literature (75, 77). A potential limitation is that variability of the *uidA* target in

specimens with organism concentrations near the assay cutoff may lead to inconsistent target detection. The *stx1, stx2,* and *eltA* (LT) targets exhibited similar or lower limits of detection when compared to the previously-published assays from which the primers and/or probes were adapted. In addition, a comparison of the Bio-Plex analytical sensitivity results (CFU/g stool) with those described for clinical and challenge studies suggests that the Bio-Plex assay targets of *stx1, stx2, eltA, estA,* and *ipaH* are capable of detecting physiologic concentrations of organism in clinical specimens. Performance of the *uidA* target in limit of detection studies was not optimal, again suggesting that detection of the *uidA* gene target in Cary-Blair specimens may be problematic. Optimization of the *uidA* primers and probe reaction conditions has been described to be critical to successful O157 strain detection (119). However, conditions for the other primer and probe sets in the multiplex must also be considered, therefore limiting optimization. As discussed earlier in this chapter, testing of alternate primer and probe designs yielded less successful performance, suggesting that the current primers and probe are at this time the best option as long as the operator is aware of the performance limitations. In the case of the *estA* (ST) target, however, re-design of the primers and probe or addition of an allele-specific primer and probe set may be desired in the future.

The assay targets exhibited no cross-reactivity when challenged with a variety of *E. coli, Shigella* spp. and *Salmonella* spp. One notable exception was repeated cross-reactivity of the *uidA* target to a select EPEC strain. Because cross-reactivity to EPEC strains may occur, it is recommended that *uidA* results be considered for interpretation only when *stx1* and/or *stx2* targets are also positive. Finally, comparison of Bio-Plex qualitative results for Cary-Blair specimens with those of reference methods yielded sensitivity and specificity values that exceeded expectations, although confidence of sensitivity data is lowered due to the small

sample size. Despite the small study sample sizes and potential limitations, the Bio-Plex bacterial assay exhibits performance characteristics sufficient for use as a screening assay to provide preliminary etiologic agent information and focus laboratory confirmatory testing in outbreak investigations.

CHAPTER 6 EVALUATION OF A MANUAL METHOD FOR THE EXTRACTION OF VIRAL NUCLEIC ACIDS

Overview of Norovirus and Rotavirus Pathogens and Molecular Assays for Detection of Gene Targets. Noroviruses are the leading cause of acute gastroenteritis in the U.S., and are the leading non-bacterial cause of foodborne illness (22, 100). Rotaviruses are more typically associated with gastroenteritis in pediatric patients, although they have also been implicated in foodborne illnesses (11). An overview of the viruses, their clinical signs and symptoms and testing methods utilized by public health laboratories to detect these viruses is provided below.

Norovirus genogroups I and II. Noroviruses (NoVs) are small round-structured non-enveloped RNA viruses belonging to the family *Caliciviridae* (51). They are currently classified into five genogroups, with GI and GII primarily associated with human infection (3). Genogroups are further subdivided into genotypes based on sequence variations in the capsid genes of the viruses (3, 120). Definitive characterization of NoVs is conducted under the auspices of the CDC's CaliciNet Network, a national NoV electronic surveillance network intended to track NoV strains and outbreak trends through the use of a comprehensive genome sequence database (22). Based on data analyzed through CaliciNet, variants of NoV genotype GII.4 have been the predominant cause of NoV infections in the U.S. since 2001 (22).

Infection with NoV causes acute gastroenteritis, typically after a 12-48 hour incubation period. Symptoms include diarrhea, vomiting, nausea, and/or abdominal cramps. Infrequently patients may experience low-grade fever and/or body aches (22). Infections are usually self-

limiting in immunocompetent people. Prolonged symptoms, including dehydration, can occur in the young, elderly, and hospitalized (22).

Detection and characterization of NoVs are primarily performed using molecular methods, as no culture techniques are available. Only one FDA-approved immunoassay recently became available in the U.S., but is not recommended as a replacement for molecular methods in public health outbreak investigations because negative samples must still be confirmed by a second technique (22). The NoV genome is a single-stranded, positive sense RNA of approximately 7.7kb in length (51). The genome contains three open reading frames (ORFs), of which ORF1 encodes non-structural proteins, ORF2 encodes the major capsid protein VP1, and ORF3 encodes the minor structural protein VP2 (51). Detection of NoV GI and GII is currently performed at state laboratories in collaboration with the CDC using a real-time RT-PCR assay targeting the relatively conserved junction between the ORF1 and ORF2 (60). In addition, conventional RT-PCR methods are performed to target the capsid regions encoded on ORF2 and amplification products sequenced to identify the genotype of the viruses detected in clinical specimens (114).

Group A rotaviruses. Rotaviruses (RVs) are non-enveloped RNA viruses that belong to the family *Reoviridae*, named for their wheel-like shape when visualized using electron microscopy (2). Like NoVs, these viruses are classified based on the characterization of serotypes and/or genotypes of specific viral proteins. Major RV groupings are designated A to G, and are based on antigenicity of epitopes in the most abundant virus structural protein, VP6 (47). Group A rotaviruses are the primary rotavirus type that causes disease in the U.S., and are further characterized based on the serological reactivity of two outer capsid proteins, VP7 and VP4. The VP4 protein determines the P type, and VP7 the G type of the virus (37). Fourteen G types are

known, with G1-G4 being the most common, and it has been shown that serotype directly correlates with genotype. Twenty P types have been identified, with ten known to occur in human RV infections. For the P types, serotype does not correlate directly with genotype (37).

The incubation period for RV infection is usually 24-48 hours. Typical symptoms in both adults and children are fever and vomiting for several days, followed by often profuse diarrhea. However, time to onset of illness and symptoms in adults may be widely variable (2). As is the case for norovirus, dehydration is a concern, particularly in children and the elderly.

The rotavirus genome is made up of 11 segments that are double-stranded. Segments range in size from 660 to 3,300 base pairs (37). Historically at DCLS, as well as other state public health laboratories, a conventional RT-PCR assay targeting the VP6 gene has been used for viral detection (49, 92). Recently, a real-time RT-PCR assay was implemented targeting a structural protein, NSP3, that exhibits a more conserved sequence than that of VP6 (59).

Integration of Enteric Virus Detection into a Foodborne Outbreak Screening Algorithm.

The original intent during the initial stages of this project was to design a multiplex Bio-Plex enteric viral assay, similar to those designed for protozoa and bacteria. Bio-Plex assay design was to complement the other two assays and create a multiple pathogen approach in which three Bio-Plex assays could be utilized to concurrently screen clinical specimens for protozoa, bacteria and viruses of public health significance in foodborne outbreaks. In support of this goal, Bio-Plex primers and probes targeting NoV GI, NoV GII and RV were designed based on sequences previously described for real-time and conventional RT-PCR methods [J. Vinjé, unpublished data; (49, 60, 64)]. While the NoV GII and RV designs adapted readily to the Bio-Plex platform, issues such as lack of sensitivity and cross-reactivity with the NoV GII target were encountered with the NoV GI designs (data not shown). Exhaustive efforts to modify primers, probes and

reaction conditions finally resulted in a set of NoV GI-specific probes that showed promise in multiplex, but still required extensive testing to confirm acceptable performance (data not shown).

As assay development and evaluation progressed, the vision of the integrated screening algorithm was reassessed and it was concluded that continuing to develop a Bio-Plex the virus assay provided no benefit to the overall testing algorithm. In fact, multiple real-time RT-PCR assays for the detection of NoV and RV are readily available at most state public health laboratories and these methods yield confirmatory results faster than the Bio-Plex screening assay. As previously discussed in Chapter 2, additional practical reasons for not pursuing the assay further included the fact that the Bio-Plex norovirus gene targets were the same as those detected using current standardized real-time assays and the Bio-Plex rotavirus gene targets, though different than standardized protocols, provided no additional information than that gained from the current method. Therefore, unlike the parasite and bacterial assays that would be filling a testing void and reducing turnaround time for screening results, a Bio-Plex virus assay would only provide a redundant testing system that would be more costly in terms of supplies and manpower than current confirmatory testing methods.

However, integrating the real-time RT-PCR virus testing procedure into an outbreak screening algorithm which also includes the Bio-Plex protozoan and bacterial assays was required. Harmonization called for the ability to test a single stool specimen with all the assays. Therefore, it was determined that the extraction step needed to be changed to enable an efficient testing algorithm, since the extraction method for the real-time protocols was different than that used for the Bio-Plex assays and a common extraction method was required. The current extraction method utilized for the real-time RT-PCR norovirus and rotavirus methods is the

Ambion® MagMAX™-96 Viral RNA Isolation Kit combined with the automated KingFisher® Magnetic Particle Processor, whereas the manual QIAamp DNA Stool Mini Kit method is used in conjunction with the Bio-Plex assays. Successful extraction of norovirus nucleic acids has been previously described using the Qiagen QIAamp DNA Stool Mini Kit (97). Establishing the Bio-Plex extraction method as the common protocol would allow nucleic acids to be used for testing with not only the Bio-Plex assays, but also the real-time RT-PCR viral assays. Therefore, a parallel study was conducted to compare the results obtained for specimens extracted with both methods and then tested using the NoV and RV real-time RT-PCR assays.

Results.

Comparison of extraction methods. Unpreserved stool specimens and stools in Cary-Blair transport medium known to be positive for NoV or RV were extracted as described using both the Ambion® MagMAX™-96 Viral RNA Isolation Kit combined with the automated KingFisher® Magnetic Particle Processor and the manual QIAamp DNA Stool Mini Kit extraction method, as described (Table 21). Extracted nucleic acids were tested using the standardized real-time RT-PCR assays currently employed at DCLS for NoV or RV, as appropriate. The real-time RT-PCR crossing point values (Ct) obtained upon testing are summarized in Table 22. Pooled study results representing Ct values for Kingfisher-extracted and Qiagen-extracted specimens were tested separately for a normal distribution using the Shapiro-Wilk test (78). Because the p-values for both the Kingfisher and Qiagen data sets were less than 0.05 (α), it can be concluded that the data are not from a normally distributed population (Kingfisher: $W = 0.938$, $p = 0.015$, $n = 47$; Qiagen: $W = 0.922$, $p = 0.004$, $n = 47$). Therefore, correlation between pooled Kingfisher and Qiagen results was performed using the Spearman's rho (rank) correlation, which is appropriate for data that is not normally

Table 21. Summary of the NoV and RV specimens tested in a nucleic acid extraction comparison study. Pathogen, specimen type and total number are summarized for the specimens utilized to compare the automated Kingfisher and manual Qiagen extraction methods.

Virus	Specimen Type	Number (n)
NoV GI	Unpreserved	6
	Cary-Blair	6
NoV GII	Unpreserved	14
	Cary-Blair	7
RV	Unpreserved	7
	Cary-Blair	7

Table 22. Real-time RT-PCR crossing point (Ct) values obtained for specimens positive for NoV GI, NoV GII and RV, using Kingfisher and Qiagen extraction methods. Select specimens positive for NoV and RV were extracted with both the Kingfisher automated extraction method and the manual Qiagen extraction method and tested using real-time RT-PCR assays. (A) The Ct values obtained are for unpreserved and Cary-Blair specimens positive for NoV GI are summarized. (B) The Ct values obtained are for unpreserved and Cary-Blair specimens positive for NoV GI are summarized. (C) The Ct values obtained are for unpreserved and Cary-Blair specimens positive for NoV GI are summarized. Abbreviations: KF, Kingfisher; Q, Qiagen

(A)

Specimen Identification	Unpreserved Specimens		Specimen Identification	Cary-Blair Specimens	
	KF (Ct)	Q (Ct)		KF (Ct)	Q (Ct)
V-S-001	28.19	27.72	V-CB-145	28.70	28.68
V-S-009	36.92	32.90	V-CB-146	37.31	35.74
V-S-118	25.31	23.90	V-CB-147	25.59	22.24
V-S-120	23.76	21.88	V-CB-148	35.87	33.60
V-S-143	34.60	31.46	V-CB-149	36.49	34.57
V-S-144	36.68	23.22	V-CB-150	35.42	33.86

(B)

Specimen Identification	Unpreserved Specimens		Specimen Identification	Cary-Blair Specimens	
	KF (Ct)	Q (Ct)		KF (Ct)	Q (Ct)
V-S-011	22.61	25.71	V-CB-151	29.49	27.79
V-S-013	19.85	17.81	V-CB-152	19.93	17.89
V-S-015	25.13	28.47	V-CB-153	20.91	21.39
V-S-017	28.82	27.73	V-CB-154	16.51	16.58
V-S-021	27.45	26.86	V-CB-157	35.59	33.28
V-S-033	16.73	16.42	V-CB-158	33.9	32.76
V-S-037	20.71	17.52	V-CB-159	28.25	28.05
V-S-038	17.15	16.04			
V-S-047	17.87	17.14			
V-S-055	23.07	22.065			
V-S-056	33.39	37.15			
V-S-059	31.65	28.8			
V-S-063	23.07	18.74			
V-S-066	36.37	32.76			

(C)

Specimen Identification	Unpreserved Specimens		Specimen Identification	Cary-Blair Specimens	
	KF (Ct)	Q (Ct)		KF (Ct)	Q (Ct)
V-S-537	26.14	34.25	V-CB-568	16.46	21.16
V-S-558	14.05	17.01	V-CB-569	16.73	21.08
V-S-559	16.29	23.44	V-CB-570	28.66	32.63
V-S-562	14.27	16.20	V-CB-571	32.28	32.05
V-S-564	17.12	20.13	V-CB-572	31.30	35.39
V-S-565	17.35	18.43	V-CB-574	14.92	19.32
V-S-566	27.05	31.53	V-CB-575	19.17	20.36

distributed (35). The correlation coefficient was calculated to be 0.886, indicating a statistically significant correlation between the Kingfisher and Qiagen results ($p = 0.000$, $\alpha=0.01$). Correlation analysis data output and the corresponding scatterplot are illustrated in Figure 5. Confidence intervals for the correlation coefficient were calculated at the 95% level to be 0.80 – 0.93 using the Fisher *z* transformation as described by Dawson and Trapp (35, 72). The standard deviation of the differences between the Kingfisher and Qiagen Ct values for each specimen was also calculated (S.D. = 3.05), and the 1 S.D. and 2 S.D. ranges are illustrated in Figure 5.

In addition to the statistical analyses, the difference in Ct values of Kingfisher-extracted versus Qiagen-extracted stool specimens (ΔCt) amplified by RT-PCR were calculated and graphed (Figure 6). Delta Ct values greater than 0 (bar above the x-axis) indicate that the Ct value of the Qiagen-extracted specimen is lower. Conversely, delta Ct values less than 0 (bar below the x-axis) indicate that the Ct value of the Kingfisher-extracted specimen is lower. Twenty-seven (27) of 33 norovirus specimens exhibited lower Ct values when extracted with the Qiagen method, and 14 of 14 rotavirus specimens exhibited lower Ct values when extracted with the Kingfisher method.

Discussion. A three-plex viral assay targeting NoV GI, NoV GII and group A RV genome targets was initially designed and evaluated at DCLS. However, when considering the most efficient testing algorithm for screening of outbreak specimens, it was determined that development of a viral Bio-Plex assay was not optimal. Due to the time required to perform the newly developed BioPlex viral assay, it was determined that its use did not improve the timeline for detection of viral agents in association with viral outbreaks. Instead, the focus of integrating an enteric virus testing assay into a multi-agent screening algorithm moved toward establishing a common extraction method. As discussed previously, the Kingfisher extraction method

Figure 5. Spearman rho correlation data output and corresponding scatterplot, comparing Kingfisher and Qiagen extraction methods. Real-time RT-PCR crossing points (Ct) obtained by testing unpreserved and Cary-Blair specimens positive for NoV or RV and extracted with both Kingfisher and Qiagen methods were pooled and assessed for normal distribution. Correlation of Kingfisher and Qiagen results was calculated using the Spearman rho correlation, and 95% confidence intervals determined using the Fisher *z* transformation. Descriptive statistics as determined by analysis using PASW 18.0 are presented in addition to the scatterplot illustrating comparative results. Maroon lines at Ct = 40 indicate positive cutoff value for real-time RT-PCR assays. Dashed blue line: One standard deviation of the difference between Kingfisher and Qiagen Ct values (ΔCt; Kingfisher minus Qiagen). Solid blue line: Two standard deviations of the difference between Kingfisher and Qiagen Ct values (ΔCt; Kingfisher minus Qiagen).

Descriptive Statistic	Output
n	47
Mean, Kingfisher	25.4289
Mean, Qiagen	25.6106
Standard deviation, Kingfisher	7.26226
Standard deviation, Qiagen	6.63405
Spearman's rho	0.886
Significance (2-tailed, $\alpha = 0.01$)	0.000
CI_{95}	0.80 – 0.93

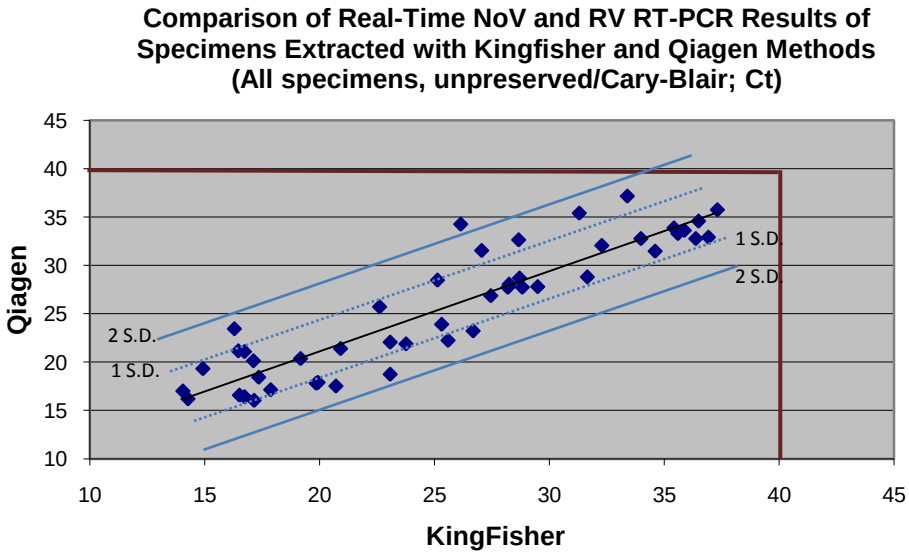
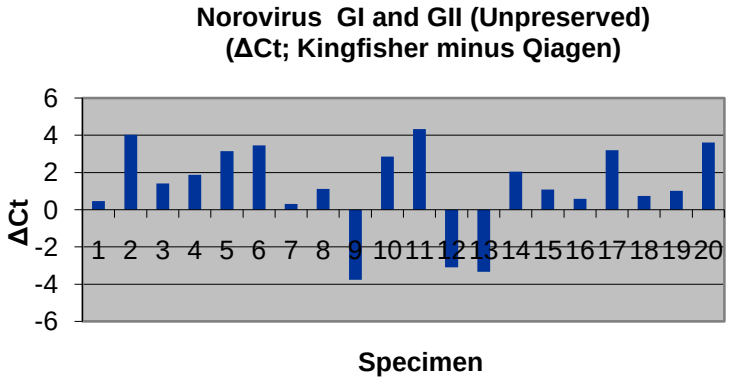
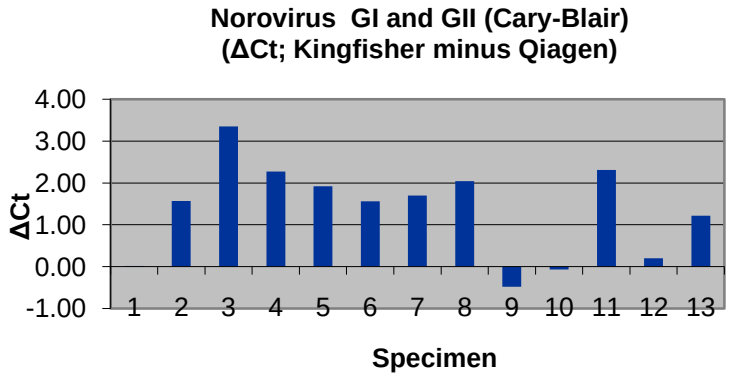


Figure 6. Comparison of real-time RT-PCR crossing points (Ct) for NoV and RV specimens extracted with Kingfisher and Qiagen methods. Crossing points achieved by testing Kingfisher- and Qiagen-extracted NoV and RV positive specimens followed by real-time RT-PCR assays were subtracted to yield a delta Ct (ΔCt; Kingfisher minus Qiagen). Bars above the x-axis indicate a lower Ct achieved for a Qiagen-extracted specimen as compared to Kingfisher; bars below the x-axis indicate a lower Ct achieved for a Kingfisher-extracted specimen as compared to Qiagen. A) Unpreserved specimens positive for NoV GI and GII. B) Cary-Blair specimens positive for NoV GI and GII. C) Unpreserved and Cary-Blair specimens positive for RV.

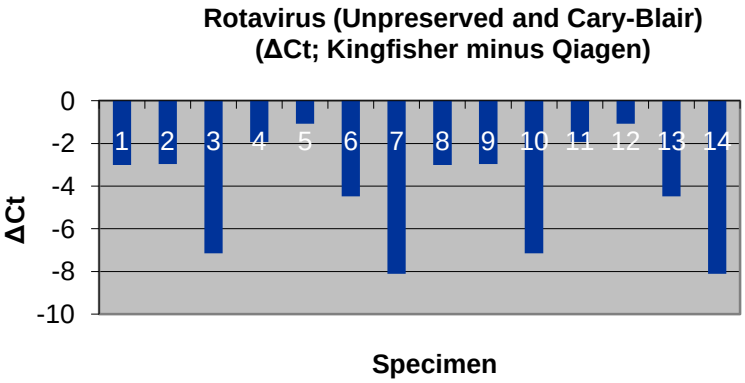
A)


Norovirus GI and GII (Unpreserved)
(ΔCt; Kingfisher minus Qiagen)
ΔCt
6
4
2
0
-2
-4
-6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Specimen

B)


Norovirus GI and GII (Cary-Blair)
(ΔCt; Kingfisher minus Qiagen)
ΔCt
4.00
3.00
2.00
1.00
0.00
-1.00
1 2 3 4 5 6 7 8 9 10 11 12 13
Specimen

C)


Rotavirus (Unpreserved and Cary-Blair)
(ΔCt; Kingfisher minus Qiagen)
ΔCt
0
-2
-4
-6
-8
-10
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Specimen

currently used at DCLS for virus molecular assay testing is different from the Qiagen extraction method used with the protozoan and bacterial Bio-Plex screening assay. Therefore, in order to perform virus testing in conjunction with the Bio-Plex parasite and enteric bacterial screening assays, two extraction methods would be required. The capability of using only one extraction method for both the virus real-time assays and the parasite and bacterial Bio-Plex screening assays was desired. Thus, a comparison was conducted using the current DCLS real-time RT-PCR NoV and RV assays to test positive stool specimens extracted with both the Kingfisher automated method and the Qiagen manual method, as described. The Kingfisher extraction method is currently used to obtain RNA from virus containing stool specimens followed by real-time RT-PCR assay detection. If the Qiagen method yielded comparable results, nucleic acids extracted for Bio-Plex testing could also be used for real-time RT-PCR virus testing, thus reducing the need for a second extraction.

A total of 47 unpreserved and Cary-Blair specimens known to be positive for NoV or RV were extracted with both the Kingfisher and Qiagen methods, tested using real-time RT-PCR protocols, and crossing point (Ct) data pooled and analyzed for statistical correlation (*r*) (35). Correlation between the Kingfisher and Qiagen Ct results were found to be statistically significant and the 95% confidence interval was small (Figure 5). An important practical note is the fact that no specimen Ct values exceeded the positive cutoff values of either assay with either extraction method, providing anecdotal confidence that both extraction methods would yield comparable results. Evaluation of the delta Ct data presented in Figure 6 suggests that the Qiagen extraction method is more efficient for NoV nucleic acid extraction, while the Kingfisher method appears more efficient for RV nucleic acid extraction. However, as noted previously, all specimens yielded positive results in this study as expected regardless of the extraction method.

Preliminary design and testing have led to the development and adaptation of primers and probes targeting NoV GI, NoV GII and RV genome sequences for detection on the Bio-Plex platform. Initial studies suggest that the NoV GII and RV assays may work well in multiplex without the specificity issues encountered during development. Upon conducting a benefit analysis of the three Bio-Plex assays under development, the viral assays were determined to provide redundant qualitative results when compared to current testing capabilities and did not provide any turn-around time advantage at this time.

Although a complete validation of the viral BioPlex assay was not conducted, the primers and probes described are available for future assay development, if desired. The RV primer and probe set has been updated to detect VP6 sequences of recent strains, as recommended by Kerin et al., and could be used on the Bio-Plex or adapted to other detection platforms (64). Sequence-specific probes have been designed to specifically detect predominantly circulating NoV GI genotypes and thus could serve as a model for genotype screening.

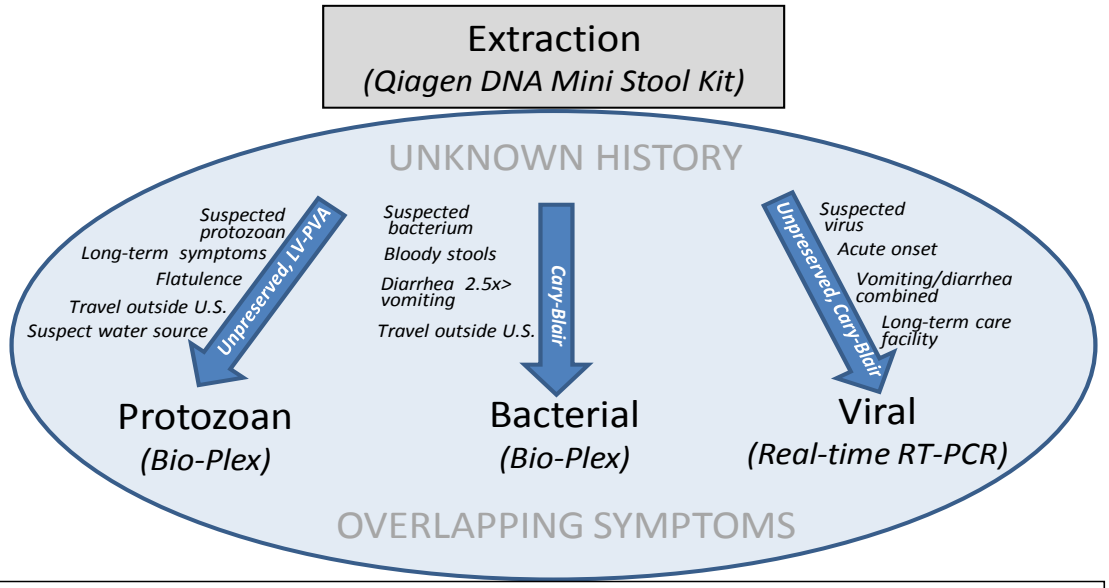
A comparison study has been conducted in order to assess the performance of the Qiagen manual extraction method to the Kingfisher automated extraction method. Comparison of real-time RT-PCR assay results for unpreserved and Cary-Blair specimens positive for NoV GI, NoV GII or RV following RNA extraction with both methods indicates that the positive correlation is statistically significant. Based on this study, it is recommended that specimens to be tested with both Bio-Plex and real-time RT-PCR viral assays be extracted with the Qiagen manual method, eliminating the need for two extractions.

CHAPTER 7 CONCLUSIONS AND OVERALL OBSERVATIONS

Implementation of a Bio-Plex Assay Outbreak Screening Algorithm. Development and implementation of rapid and easy-to-use testing methods to concurrently screen clinical specimens for a variety of foodborne pathogens enables public health laboratories to provide timely preliminary etiologic agent information to epidemiologists or outbreak investigators and to focus subsequent confirmatory testing. Toward this aim, two multiplex assays targeting enteric protozoa and enteric bacteria were developed for the Bio-Plex 200 platform and performance characteristics were established. In addition, detection of viral nucleic acids extracted with two different extraction protocols was compared using a real-time RT-PCR assay currently in use for the detection of viral pathogens. With several limitations, the Bio-Plex assays and manual extraction of viral nucleic acids are suitable for integration into a screening algorithm for use in foodborne outbreak investigations in a public health laboratory setting.

Proposed outbreak screening algorithm. The typical outbreak investigation protocol employed by DCLS is illustrated in Figure 1 (Chapter 1), which summarizes a sometimes lengthy process that incorporates biochemical, enzyme immunoassay and molecular methods, as well as a reliance on reference laboratory testing for detection of intestinal protozoa. Based on the overall vision of this project, and the results of our subsequent studies, we propose the algorithm shown in Figure 7 for screening clinical specimens submitted during foodborne outbreak investigations. The proposed algorithm integrates the enteric protozoan and bacterial multiplex Bio-Plex

Figure 7. Proposed foodborne outbreak investigation testing algorithm with Bio-Plex screening. The proposed algorithm integrates the enteric protozoan and bacterial multiplex Bio-Plex screening assays and real-time RT-PCR viral assays with one nucleic acid extraction method. Examples of possible symptoms and other epidemiologic data that may be provided for use in directing the decision as to whether to use each assay are provided. Confirmatory testing following initial screening with the Bio-Plex assays is recommended below the algorithm, as well as estimated time to achieve preliminary and confirmatory results.



History

Assay

Confirmation:

Some/all positive	*Microscopy, EIA* *Molecular ID* *Reference lab*	*Biochemical ID* *Molecular ID*	*>2 positive confirms*
All negative	*Microscopy, EIA* *Molecular ID* *Reference lab*	*Biochemical ID* *Molecular ID*	*Outbreak neg for NV, RV*
	-----screen with alternate assays if not already performed-----		
Time, preliminary:	*4-6 hrs*	*5-6 hrs*	*N/A**
Time, confirm:	*3-6 days*	*2-6 days*	*3 hr-2 days*

* Virus real-time RT-PCR assay is confirmatory

screening assays and real-time RT-PCR viral assays with one extraction method. These assays, by design, can be used alone or in combination as the situation dictates. Examples of symptoms and other epidemiologic data that may be used to decide which assays to use are illustrated in Figure 7. Because the assays are separate, yet linked with a common extraction method, laboratories can decide to utilize one assay in outbreaks which have detailed epidemiologic data, or all assays in outbreaks for which no case history is provided or symptoms are confusing. Confirmatory testing that is required following initial screening with the Bio-Plex assays is included in the algorithm. The estimated times to achieve preliminary and confirmatory results are also provided.

Patient and/or outbreak history provided by epidemiologists may be useful in identifying which screening (protozoan, bacterial) and/or confirmatory (viral) assay(s) to be performed. In some outbreaks, as in a recent *Giardia* outbreak in Virginia, the identification of the etiologic agent may already be reported for some patients that were diagnosed at local physician offices and/or by laboratory testing conducted at private or commercial laboratories. A decision flow chart is provided in Figure 8 that illustrates how the screening algorithm may be specifically employed in outbreak situations in which information is already known about the etiologic agent. In other outbreaks, public health officials may only provide limited reported symptoms and/or incubation times to help guide testing decision. However, as illustrated in Table 1 (Chapter 2), symptoms for infections may overlap greatly. Figure 9 provides a decision flow chart that may be used to assist in determining assay(s) to perform when information regarding symptoms of outbreak cases is available. In these situations, active involvement of the laboratory's management or scientific team in making testing decisions will be required. However, the proposed decision

Figure 8. Proposed decision chart for outbreaks in which the etiologic agent is known. The proposed decision chart is provided to guide testing decisions for foodborne outbreaks in which an etiologic agent has been previously identified by another laboratory. In these outbreaks, a laboratory may choose to perform only a single assay targeting the previously identified pathogen. The pathogen targets of each of the assays are listed at the top of the decision chart to easily identify the correct assay. If the pathogen information provided by public health officials is not detected by the protozoan, bacterial or viral assays, alternative testing may be required. For the protozoan and bacterial assays, preliminary positive screening results should be communicated to public health officials, prior to obtaining final confirmatory test results. All negative Bio-Plex assay results will require confirmation using standard methodologies employed by the laboratory except for the viral real-time RT-PCR assay results, which are confirmatory.

Foodborne Outbreak Screening Decision Chart -
Pathogen Identification Provided

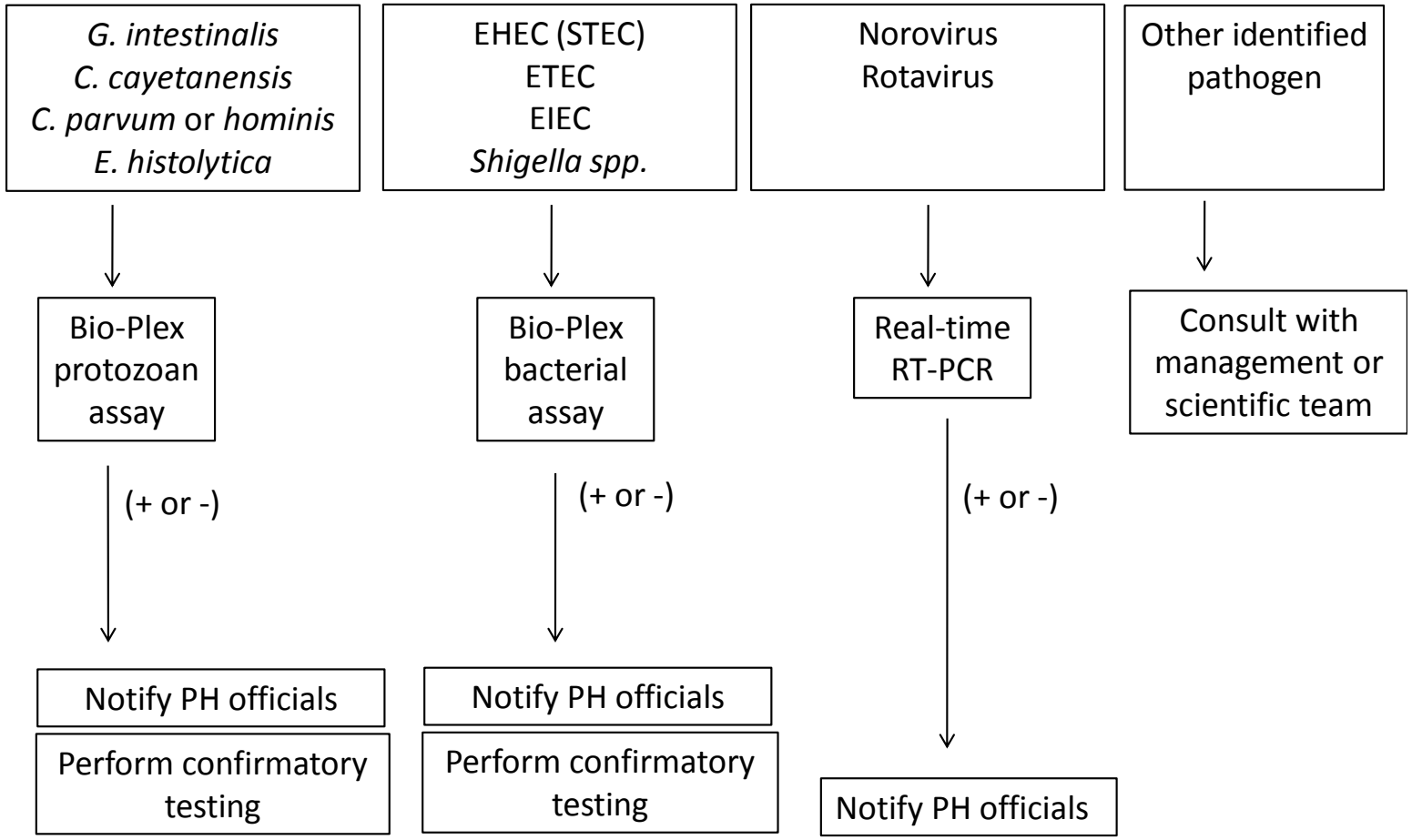
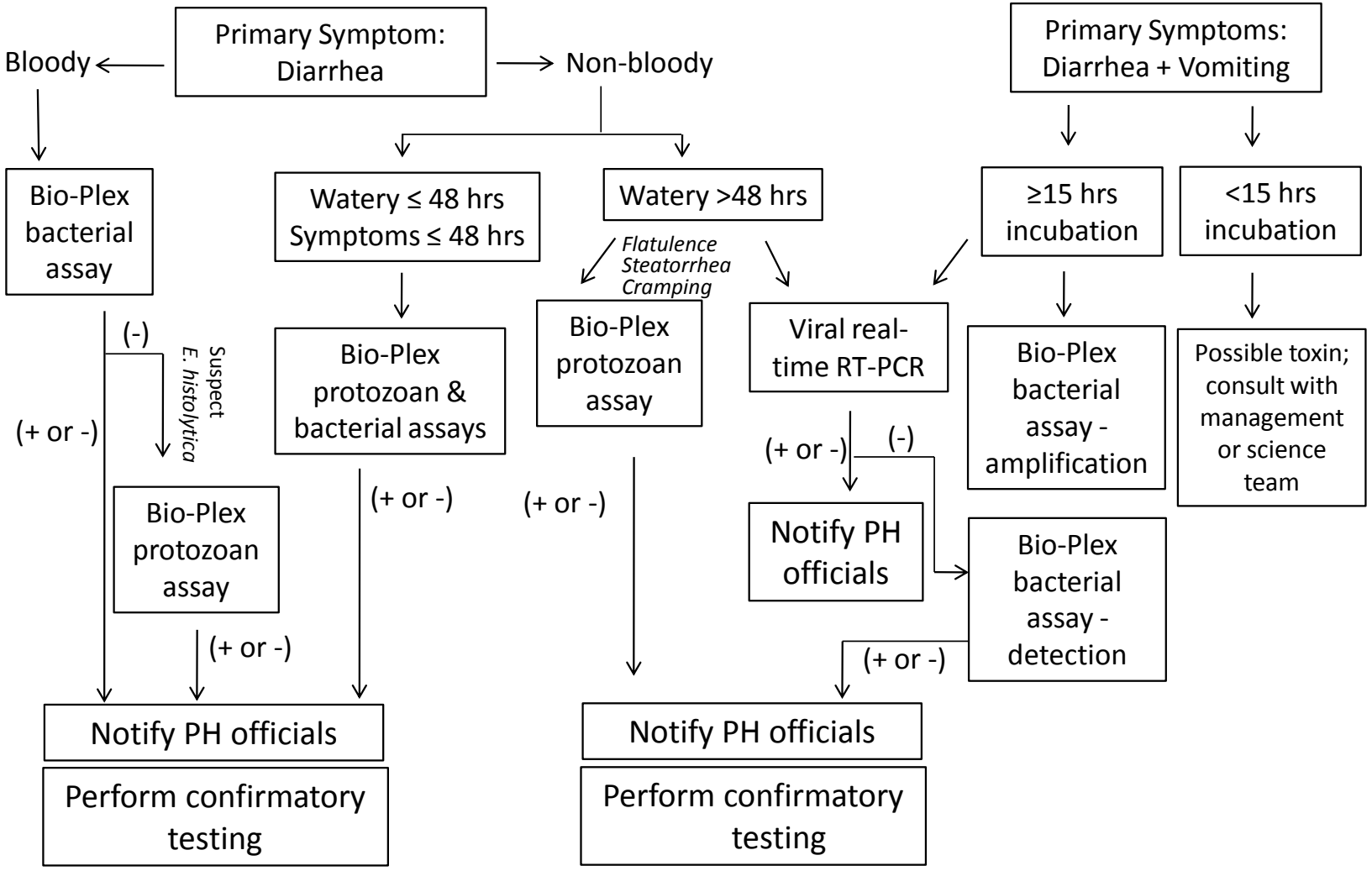


Figure 9. Proposed decision chart for outbreaks in which symptoms are known. The proposed decision chart is provided to assist in guiding testing decisions for foodborne outbreaks in which detailed or sufficient information concerning symptoms and/or incubation periods are provided by public health officials. The chart is based on the symptoms of diarrhea and/or vomiting, and incorporates key incubation periods. Additional symptoms may be experienced by patients, including fever, cramps and/or nausea, depending on the infection, and these data may be further considered in triage decisions at the discretion of laboratory management or scientific staff. For the protozoan and bacterial assays, preliminary positive results should be communicated to public health officials, followed by confirmatory testing. All negative Bio-Plex assay results will require confirmation using standard methodologies employed by the laboratory except for the viral real-time RT-PCR assay results, which are confirmatory.

Foodborne Outbreak Screening Decision Chart - Symptoms Provided by Public Health (PH) Officials

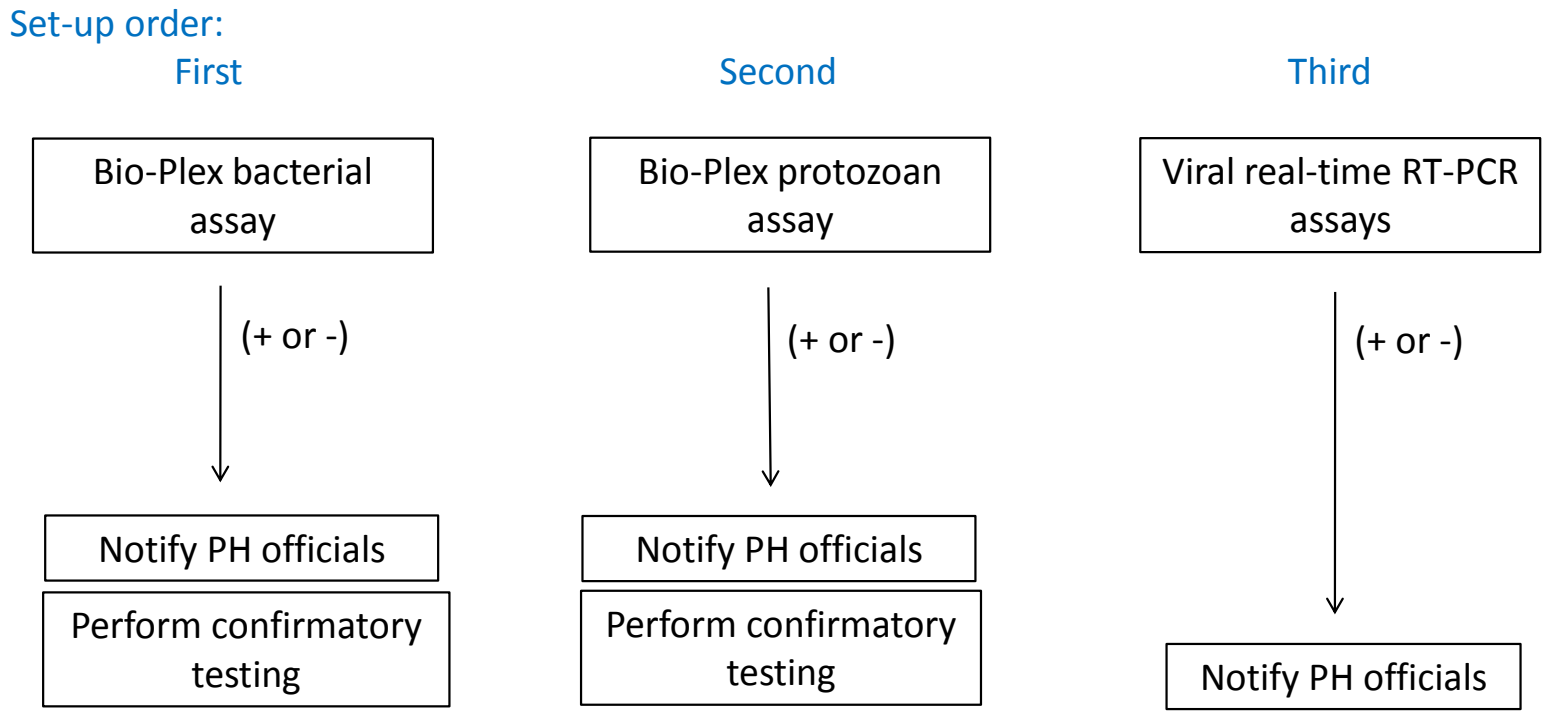


flow chart facilitates screening for the widest variety of pathogens with the least number of screening assays to conserve resources. Finally, in some cases, clinical specimens may be submitted to a public health laboratory for outbreak investigation with sparse or no epidemiologic data available at that time. In these situations, laboratorians have the flexibility to test with all three assays using a single eluted nucleic acid sample from each specimen, as illustrated by the decision chart provided in Figure 10. Examples of non-symptom epidemiologic data, such as travel or location, may be available and may be used to decide which screening assays to perform.

Assuming that all three assays are to be performed concurrently to test specimens submitted in support of an outbreak investigation, it is recommended that conventional amplification (Bio-Plex) and real-time RT-PCR (virus) reactions be set up in a specific order so as to minimize the time to results. The bacterial assay requires the longest amplification period at 2.5 hours, and therefore should be set up first. The protozoan assay should follow, with an amplification of 1.5 hours. Finally, the viral assay may be set up as both amplification and real-time detection only require 1.5 hours. While amplification of the Bio-Plex reactions is occurring, preparations may be performed for the detection steps on the Bio-Plex platform. Once amplification is complete, wells can be prepared on the Bio-Plex detection plate for both the protozoan and bacterial assays. The laboratorian could opt to set up separate detection runs for each assay; however, this would delay preliminary results if only one heated shaker is available as plate setup cannot be accomplished concurrently. Starting with the extraction, estimated time to preliminary Bio-Plex results is 4-6 hours (Figure 7). Time to confirmatory virus results is approximately 3 hours for NV or NV plus RV, and 48 hours if the RV assay is tested as a reflex assay.

Figure 10. Proposed decision chart for outbreaks in which epidemiologic information is limited or unknown. The proposed decision chart is provided to assist in guiding testing decisions for foodborne outbreaks in which the epidemiologic data provided public health officials is limited or unknown. Non-symptom information such as travel, suspected sources, and/or location, if available, may be used in determining which of the assays to perform. In situations in which no epidemiologic data is available, laboratories may choose to perform all three assays following preparation of nucleic acids with the common extraction method. Based on the time required for nucleic acid amplification and detection for each assay, the most efficient set-up order is noted. For the protozoan and bacterial assays, preliminary positive results should be communicated to public health officials, followed by confirmatory testing. All negative Bio-Plex assay results will require confirmation using standard methodologies employed by the laboratory except for the viral real-time RT-PCR assay results, which are confirmatory.

Foodborne Outbreak Screening Decision Chart - Limited or Unknown Epidemiologic Data



Because the Bio-Plex assays are to be used for screening and providing preliminary results to public health officials, follow-up confirmatory testing is recommended (Figure 7). Should some or all of the specimens submitted in support of an outbreak yield positive results for a protozoan target, specimens would be confirmed using microscopy, EIA, and/or molecular methods, if available, or sent to a reference laboratory for confirmation. Additional testing may then be suspended. Positive bacterial assay results may be confirmed with biochemical and/or alternate molecular techniques in order to focus testing on the final identification of the detected organism, as appropriate. Should all specimens be negative for all Bio-Plex assay targets, testing with confirmatory techniques and/or by a reference laboratory would still be required. The time to obtain confirmatory results for protozoan and bacterial testing varies from 2-6 days.

Advantages of the screening algorithm. The recommended foodborne outbreak screening algorithm illustrated in Figure 7 provides several advantages. First, addition of the protozoan and bacterial assays, albeit in a screening capacity, may fill a testing void for a public health laboratory. In the case of DCLS no testing is currently available for the identification of parasites or EIEC. In addition, a real-time PCR assay has been validated for the detection of ETEC, but it is rarely employed. Therefore, the algorithm provides new testing capabilities, and testing with the bacterial multiplex will enable more routine screening for both ETEC and EIEC strains, perhaps revealing a higher prevalence of these strains than is currently understood. Recent reports of at least three outbreaks in the state of New York associated with a restaurant and catered events and affecting over 28 people suggests that the routine addition of ETEC testing in outbreak investigations should be considered (H. Hanson, personal communication). Second, the algorithm reduces the time required to inform public health officials of the possible outbreak etiologic agent, which is a high priority. Third, detection of positive screening targets

allows laboratorians to quickly and efficiently focus confirmatory testing, potentially reducing the unnecessary wasting of resources expended on pathogen identification. A fourth advantage is that of flexibility. The two validated Bio-Plex assays were designed separately, allowing laboratorians to screen with only the assay(s) if desired based on outbreak information. Additional flexibility is provided by the ability of these screening assays to utilize the same extracted nucleic acids for both the Bio-Plex and virus real-time RT-PCR portions, thus eliminating the need to conduct multiple different extractions. Long-term flexibility is afforded by the potential to add primer and probe sets to each multiplex assay to expand the number of pathogens targeted for screening. Finally, since the unpreserved, LV-PVA and Cary-Blair stool specimens tested in the described studies were representative of those specimens typically submitted in an outbreak investigation, and were of the types validated for other confirmatory molecular assays, new specimen preservation methods need not be added to collection protocols.

Disadvantages of the screening algorithm. The recommended screening algorithm and the assays that are incorporated into that algorithm have several disadvantages. The first disadvantage is that of time; the Bio-Plex assays may take up to six hours to complete. Therefore, it is important that specimen processing and testing begin immediately upon specimen arrival in order to gain the maximum benefit of obtaining first-day preliminary results. A second disadvantage is that of programming limitations in the current Bio-Plex software. The version currently in use does not allow for concurrent detection of multiple microsphere mixes (i.e., assays) within the same run on the same plate. Therefore, when a detection plate is set up to include more than one assay, the operator must program the Bio-Plex to detect one multiplex microsphere mix, then quickly re-program to detect a second. A new BioPlex software version eliminates this limitation, and should be considered for future implementation. A third

disadvantage is the low sensitivity of the *E. histolytica* primers, indicating the potential to miss pathogen detection. Despite high specificity for this target, positive results are not readily available for epidemiological use due to potential cross-reactivity with *E. dispar*, thus requiring confirmation with a discriminatory assay. However, the target will remain as part of the validated format at this time. Another disadvantage is that of the potential inability of the bacterial assay to detect *estA1* alleles of the heat stable toxin in ETEC strains. This lack of specificity may result in missed preliminary detection of an outbreak etiologic agent.

Other Considerations for Bio-Plex Protocol Implementation. Although establishing the performance characteristics described in previous chapters is a major element in employing new molecular assays, other practical considerations for deciding to implement Bio-Plex assays include ease of use, personnel qualifications and training, equipment and supply requirements, and cost.

Ease of use of the Bio-Plex assays and system. For a laboratorian trained in molecular techniques, using the Bio-Plex assays to test outbreak specimens is relatively easy. At a minimum, the Bio-Plex assay is no more difficult than performing real-time PCR molecular tests that are routinely performed in a public health molecular laboratory. Chemical solutions required for microsphere-probe coupling and Bio-Plex detection plate setup are easily mixed using readily-available standard protocols and coupling of the target-specific probes to the Bio-Plex microspheres is a straightforward series of adding solutions, centrifuging, and removing supernatant. The manual extraction employs a commercial spin column method that is easy to follow, though it is a bit time-consuming. Setup of the multiplex PCR amplification reactions is equivalent in technique to those used for real-time and conventional molecular PCR methods, and is made easier with the use of commercial master mix kits. Working with the Bio-Plex

microspheres and setting up the detection plate may be initially challenging, as the operator must become comfortable with working swiftly and efficiently so the microspheres do not settle or clump. The most difficult portion of the setup is removing the adhesive film plate cover, which is done twice. Peeling the film off the plate requires firm handling in order to not tear the film and to not cross-contaminate wells. The Bio-Plex system facilitates flexible batch sizes, ranging from 1 well to 96 wells, and use of multi-channel pipettes eases setting up larger batch sizes. Programming of the Bio-Plex is well-described in the software manual, allowing assay parameters to be saved for repeated use and providing flexibility in programming the output order of results without regard for how the wells are set up on the plate. Results may be exported as a spreadsheet for analysis; graph functions are also available, but were not utilized in this project.

Personnel qualifications and training. The Bio-Plex assay and detection plate setup require the same qualifications and training needed to perform other molecular-based assays. Training for an experienced molecular scientist would be minimal, and could realistically be completed following one or two learning and familiarization runs, an observed run, an unobserved run, and a final certification run. Training for an operator with little-to-no experience in molecular testing would, by necessity, be more extensive.

Equipment and supply requirements. In addition to the Bio-Plex system, very little extra equipment is needed to support testing. A heated 96-well plate shaker, sonicator, centrifuges and vortexers are typically standard equipment in a molecular laboratory or can readily be purchased. Facilities for sample extractions, amplification setups and storage are also required. Facility and engineering controls are required for the detection solutions, as they contain chemicals that are respiratory, skin and eye irritants. Solutions should be handled in a fume hood or dead air

cabinet (<1mL only), and although not designated to be carcinogenic, caution should be exercised in allowing pregnant personnel to handle the solutions. There is a significant up-front supply requirement to begin Bio-Plex testing, particularly if microspheres will be coupled in-house. Items to purchase include microspheres, primers, probes, chemicals for coupling and detection solutions, detection plates and covers, Bio-Plex validation and calibration kits and system sheath fluid. Once purchased, the supplies can last several months to a year, particularly if testing is not done daily or in high volume. Other support items such as microcentrifuge tubes, pipettes, pipette tips, deionized water, isopropyl alcohol, plate racks, personal protective equipment, etc. are standard in a molecular laboratory.

Sustainment costs of Bio-Plex assays. Costs for long-term sustainment of the extraction and amplification steps of Bio-Plex assays are similar to those for real-time molecular assays. The pricing for the QIAamp DNA Stool Mini Kit extraction method validated in the described studies lies within the typical range of costs for commercial extraction kits. The costs per test of both master mix kits used for the protozoan and bacterial assays are a bit higher than for other PCR master mix kits, but are less expensive than RT-PCR kits. Pricing for Bio-Plex primers has proven to be less expensive than that for real-time primers and probes, mainly because the Bio-Plex oligonucleotides do not require as high a level of purification following synthesis.

Added costs include the probes, which are inexpensive compared to real-time probes, and the coupling reagents. Most reagents can be purchased relatively inexpensively in bulk. The additional costs of Bio-Plex detection are overcome by the platform's ability to accommodate large multiplex reactions, which is a limitation for real-time platforms. However, if only a two- or three-plex is desired and high throughput is not a goal, the Bio-Plex platform may not be a cost-effective option.

Future Considerations. This paper describes two Bio-Plex assays that have been validated for use as screening tools in foodborne outbreak investigations and a proposed testing algorithm that also maximizes use of extracted nucleic acids for other testing platforms. However, some improvements may be considered to address assay limitations, expand testing capability, and take advantage of instrumentation system upgrades. Due to the low sensitivity of the *E. histolytica* target as compared to reference methods and the potential to cross-react with *E. dispar* gene sequences, it is recommended that a re-designed primer and probe set be incorporated. An alternative approach would be to remove the *E. histolytica* target from the multiplex completely, due to low prevalence of the pathogen in foodborne outbreaks in the U.S. In either case, re-validation would be required. A similar re-design recommendation is made for the *estA* target of the bacterial assay. It is also recommended that performance characteristics of the bacterial assay be established using unpreserved stool specimens, as this specimen type may also be submitted during outbreak investigations in lieu of Cary-Blair specimens. Validating the assay for unpreserved stool specimens provides an additional flexible testing option. As mentioned previously, a new software upgrade is available for the Bio-Plex platform that incorporates concurrent detection of up to twelve multiplex assays on one detection plate. Implementing this software will make the detection step more efficient and require less operator intervention. Finally, additional pathogen targets may be considered for addition to the existing assays or additional multiplex assays implemented for concurrent detection with the protozoan and bacterial assays on the Bio-Plex platform in order to build a more comprehensive screening protocol for foodborne outbreak investigations.

Conclusions. In the studies described here, two multiplex nucleic acid assays for the detection of enteric protozoa and bacteria were designed for the Bio-Plex 200 platform and performance

characteristics evaluated. Both the Bio-Plex protozoan and bacterial assays exhibited precision similar to that described for other microsphere-based nucleic acid methods. Analytical sensitivity of the assay targets was established and comparison to the previously published assays from which most primers and probes were adapted showed that some targets exhibited improved limits of detection on the Bio-Plex platform while others did not. A comparison to organism excretion concentrations in the literature suggests, however, that most assay targets are capable of detecting organism concentrations typically encountered in clinical specimens. Sensitivity and specificity of most targets as compared to reference methods ranged from 81.25% to 100%, which is acceptable for the screening assays. Sensitivity for the *E. histolytica* target in the enteric protozoan assay was 42.86%, another indicator that re-design of this target should be considered. Both the protozoan and bacterial assays exhibited no cross-reactivity to a variety of non-target parasites and bacteria, with the exception of *E. dispar* (*E. histolytica* target in the protozoan assay) and EPEC strains (*uidA* target in the bacterial assay). Additionally, no cross-reactivity between assay targets was noted. Interpretation of results for the *uidA* target only when shiga toxin targets are positive overcomes the cross-reactivity of the *uidA* target with certain EPEC strains. The development of a Bio-Plex multiplex assay to detect viral targets, while promising, was not required due to redundancy with other rapid testing protocols. Real-time detection of NV and RV nucleic acids extracted from clinical specimens with the QIAamp DNA Stool Mini Kit was found to be statistically comparable to detection of nucleic acids extracted with the current viral RNA extraction method on the Kingfisher platform. Therefore, should Bio-Plex testing be desired in addition to viral testing, only one extraction method would be required. In summary, integration of the Bio-Plex and real-time assays with a common extraction method provides a simple, rapid and flexible foodborne outbreak screening algorithm

that can be optimized and tailored based on epidemiological history and preliminary etiologic agent data provided on the day of specimen submission, and may be used to focus subsequent confirmatory laboratory testing.

LIST OF REFERENCES

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