Comparison of Commercial DNA Extraction Kits for Recovery of Bacterial, Protozoan, and Viral Organisms Spiked In Human Fecal Samples

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Abstract:

Background: There is an increasing interest to monitor the gastrointestinal microbiome as it plays an important role in defining human health. Molecular PCR-based approaches are being widely used for detection of normal and pathogenic organisms in human fecal samples. However, there are many "hard to lyse" organisms present in stool that may go undetected if traditional methods of DNA extraction are applied. Efficient extraction of high purity, inhibitor-free DNA or RNA is the main requirement for comprehensive analysis of the gastrointestinal microbiome.

Goal: In this study we evaluated ten DNA extraction kits for efficiency and throughput using organisms (*Cryptosporidium parvum, Clostridium difficile,* Adenovirus 41) that could cause diarrhea and be present in clinical fecal samples.

Materials and Methods: *C. parvum* (10^3-10^6 oocysts/mL), *C. difficile* (10^3-10^6 cfu/mL), and Adenovirus 41 (10-10^4 pfu/mL) were spiked in to stool samples. Ten commercially available DNA extraction kits were evaluated: KingFisher Plant DNA, FastDNA™ Soil, FastDNA®, PowerLyzer™, PowerSoil®, PowerMag™, PowerFecal™, PowerPlant®, a modified Mag-Bind® Stool kit, and a Roche MagNA Pure 96 SV kit. Bead bashing on a FastPrep® 24 was used for all methods. DNA samples were processed using target enrichment multiplex PCR, followed by detection on the Luminex® platform, and extraction efficiency was evaluated based on detection of lowest number of organisms/mL. A phenol/chloroform extraction (TRI® Reagent) was used as an extraction baseline.

Results: We confirmed that bead-bashing is essential for detection of *C. parvum*. Bead matrix (glass, garnet, zirconium, etc.) and bead size have a significant effect on releasing DNA from *C. parvum*. High temperatures or proteinase K digestion demonstrated no benefit to lysis of oocysts. The method using Omega Bio-tek reagents had the lowest limit of detection, detecting *C. parvum* at 10^5 oocysts/mL, *C. difficile* at 10^3 cfu/mL and Adenovirus 41 at 10^5 pfu/mL. Optimal bead bashing occurred with 3 cycles of 40 sec at speed 6.5m/s on the FastPrep 24 using matrix Y plus matrix Z bead tubes. TRI Reagent extraction detected *C. parvum* down to 100 oocysts/mL.

Conclusions: Comparative evaluations of ten commercial DNA extraction methods indicated that a modification of the Omega Bio-tek Mag-Bind Stool kit was the most efficient for recovery of bacterial, protozoan and viral DNA spiked in human stool sample. No high-throughput kit was able to lyse the oocysts as effectively as TRI Reagent. The results of this study can assist diagnostic laboratories with selecting the best extraction method for processing fecal specimens for subsequent detection using molecular tests.

Notification: Limits of detection for the Omega Bio-tek method described in the results were initially reported in the abstract as 10^5 for *C. difficile* and 10 for Adenovirus 41. Further testing indicated that the lowest concentration detected was actually 10^3 for *C. difficile* and 10^2 for Adenovirus 41. The Roche MagNA Pure 96 instrument and another extraction kit were evaluated after abstract submission.

Introduction:

Diarrhea is a leading cause of morbidity and mortality worldwide, particularly in children with 1.5 million children under five years of age dying of diarrhea each year.[1] It can be caused by a host of organisms, including viruses, parasites, fungi, and bacteria. The microbiologic etiology of diarrhea is not clinically obvious, and the detection of enteric pathogens by stool culture is notoriously insensitive compared to molecular methods.

Molecular diagnostic assays on fecal samples have recently emerged for a variety of enteric pathogens, including multiplex PCR and hybridization-based approaches. Efficient DNA extraction from pathogens present in stool is a key requirement for the sensitive performance of molecular tests. However, DNA extraction from fecal samples comes with challenges such as hard-to-lyse organisms and the presence of PCR inhibitors which can mask the identity of the infectious organism.

This study presents evaluation of extraction efficiency and throughput of 10 commercially available DNA extraction kits. The kit performance was evaluated based on the following:

- extraction of good quality amplifiable DNA
- ability to remove the inhibitors inherent with stool samples
- ease of use
- sample throughput

We chose pathogens that represent the diversity of organisms frequently identified in acute and chronic diarrhea:

- *Clostridium difficile*, a spore-forming gram positive bacteria
- *Cryptosporidium parvum*, a protozoan with a tough oocyst wall
- Adenovirus type 41, a DNA virus

Materials & Methods:

Sample Preparation

- Protozoan *Cryptosporidium parvum* oocysts Iowa Isolate (Sterling Laboratory, University of Arizona, Tuscon, AZ)
- Spore forming bacteria *Clostridium difficile* NAP1A 0801620 (Zeptometrix Corporation, Buffalo, NY)
- Adenovirus type 41 0810085CF (Zeptometrix Corporation, Buffalo, NY)

Titered organisms were serially diluted and spiked into a stool matrix of pooled clinical stool specimens submitted for testing on the Diatherix® Gastrointestinal Panel and which no organisms tested for were detected. Each dilution contained all three organisms.

DNA extraction

Name	Vendor	Sample Volume (µL)	Protocol Modification	Bead Bashing
FastDNA™ Spin Kit for Soil	MP Biomedicals	250	No	FastPrep®-24: speed 6, 40 sec
FastDNA® Spin Kit	MP Biomedicals	250	No	FastPrep® 24: speed 6, 40 sec
PowerLyzer™ PowerSoil®	MO BIO	250	No	FastPrep® 24: speed 6, 40 sec
PowerSoil®	MO BIO	250	No	FastPrep® 24: speed 6, 40 sec
PowerFecal™	MO BIO	250	No	FastPrep® 24: speed 6, 40 sec
PowerPlant® Pro	MO BIO	250	No	FastPrep® 24: speed 6, 40 sec
PowerMag™ Soil	MO BIO	250	No	FastPrep® 24: speed 6, 40 sec
KingFisher Plant	Thermo Scientific	600	Yes*	FastPrep® 24: speed 6, 40 sec
DNA and Viral NA Small Volume Kit	Roche	250	Yes**	MixMate® 2000rpm, 5 min
Mag-Bind® Stool DNA	Omega-Biotek	300	Yes***	FastPrep® 24: speed 6.5, 40 sec, 3 cycles
Mag-Bind® Stool DNA	Omega-Biotek	300	Yes****	None
TRI® Reagent	Sigma-Aldrich	1000	No	None

* Followed manufacturer's protocol with a modification to the lysis step. Samples were disrupted with an inhibitor removal agent in MP Biomedicals matrix A tubes.

** Samples were combined with 250µL of Bacterial Lysis Buffer (Roche) in a bead beating tube (Omega Bio-tek) and disrupted on a Eppendorf MixMate. After centrifugation 200µL of supernatant was processed with the Pathogen Universal protocol and eluted into 50µL.

*** All automated wash steps were followed per manufacturer's protocol. Some reagent additions and processes were omitted to increase throughput. Samples were lysed in 300µL of SLX-Mlus buffer (Omega Bio-tek) in a MP Biomedicals lysing matrix tube containing the matrices Y and Z. Eluted with 50µL of provided elution buffer.

**** All automated wash steps were followed per manufacturer's protocol. Samples were lysed by incubating at 65°C for 5 min with 220µL SLX-Mlus buffer, 60µL 20% sarkosyl, and 20µL of 40mg/mL proteinase K (Omega Bio-tek) followed by incubation at 95°C for 5 min. Samples were centrifuged and 275µL of supernatant transferred over.

PCR and Detection

Extracted DNA samples were amplified using the target enriched multiplex PCR (TEM-PCR) Gastrointestinal Panel. TEM-PCR™ is a highly multiplexed, nested, end-point PCR technique covered by US 7,851,148 B2. The Gastrointestinal Panel contains 64 target specific primers which can identify twelve different intestinal pathogens, however only three representative organisms were used in these evaluations. The end-point PCR products generated by TEM-PCR™ were detected on a microsphere-based platform (Luminex, Austin, TX).

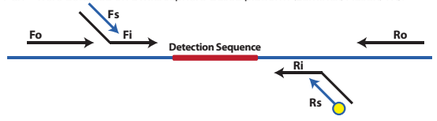
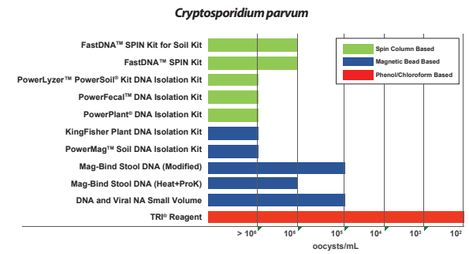


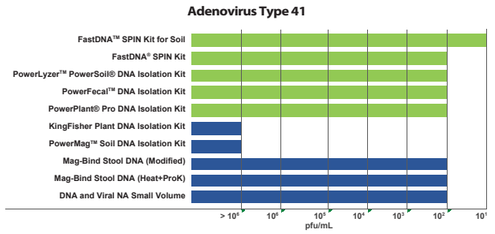
Figure 1. TEM-PCR™ Scheme
Low concentrations of nested gene-specific primers (Fo – forward out; Fi – forward in; Ri – reverse in; and Ro – reverse out) are designed to enrich the genetic targets during the initial PCR cycles. Later in the procedure, a pair of universal SuperPrimers (FS and RS) are used to amplify all targets. The reverse SuperPrimer is labeled with biotin. Biotinylated PCR products are detected with a complimentary detection probe which is covalently coupled to a color-coded microsphere or glass microarray.

Diatherix TEM-PCR™ Gastrointestinal Panel

Clostridium difficile Toxin B gene
Campylobacter jejuni
Escherichia coli strain O157
Listeria monocytogenes
Salmonella enterica
Shigella flexneri
Shigella sonnei
Vibrio parahaemolyticus
Giardia lamblia
Cryptosporidium parvum
Adenovirus 40, 41

Results:





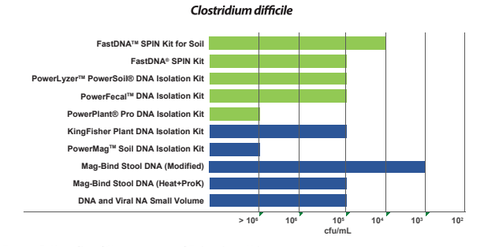


Figure 2. Results of Comparison of Extraction Kits
The lowest concentration of serially diluted organisms detected after PCR is indicated by the bars with the Omega Mag-Bind Stool DNA (Modified) kit yielding the highest overall sensitivity. Although the graphs are organism specific, the three organisms were isolated at the same time from the samples.

Figure 3. Workflow on the MagBind Stool Modified Protocol
The optimal protocol evaluated was a modification of the Omega Biotek MagBind Stool kit. After bead bashing on a FastPrep 24, samples are transferred, a binding solution is added, and all binding, wash, and elution steps are automated thereafter.

Results *(continued)*:

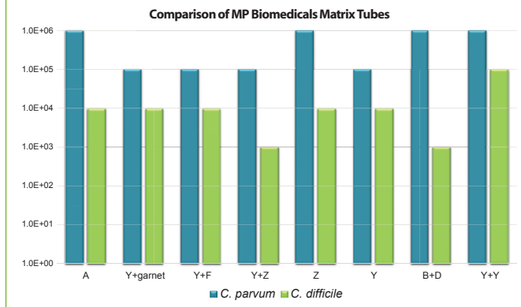


Figure 4. Comparison of bead bashing matrices used with MP Biomedicals FastPrep 24
Bead bashing is required to lyse the tough *C. parvum* oocysts. Several different lysing matrix compositions were tested from MP Biomedicals. Combining matrices to increase the lysis action was also tested. Using two different sizes of a dense ceramic bead (matrix Y + matrix Z) yielded optimal sensitivity.

Table 1. MP Biomedicals bead bashing matrix compositions

Bead Bashing Matrix	Material
A	Garnet & ¼" Ceramic Sphere
B	0.1mm Silica Spheres
D	1.4mm Ceramic Spheres
F	Aluminum oxide & Silicon carbide
Y	0.5mm Zirconium oxide Spheres
Z	2mm Zirconium oxide Spheres

Different sizes, densities, and shapes of lysing matrices were tested to find one that would lyse the *C.parvum* oocysts without damaging the DNA. A combination of two different sizes of a very dense Zirconium oxide ceramic performed the best for DNA extraction from the organisms tested.

Conclusions:

- A modification of the **Omega-Biotek Mag-Bind® Stool DNA** KingFisher™ Flex extraction kit was superior for sensitivity, removal of inhibitors, and ease of use.
- The performance of spin column based kits is comparable to the semi-automated Mag-Bind® protocol but involved more hands-on time.
- Optimization of the Roche MagNA Pure 96 extraction was done with regards to the Roche Small Volume or Large Volume kit and on-board protocols (data not shown). The most sensitive protocol was compared to the results of the other kit evaluations. No significant improvement in sensitivity was seen by using this instrument compared to the Mag-Bind® protocol and MP Biomedicals FastPrep® 24.
- Optimal bead bashing occurs with the FastPrep® 24 using a combination of MP Biomedicals' Matrix Y and Matrix Z beads with 3 cycles of 40 seconds at speed 6.5.
- Heat and proteinase K digestion cannot be used as a substitute for bead bashing as the sensitivity will be lowered.
- Extraction was bench-marked with a TRI® Reagent phenol/chloroform extraction. The caustic nature of the chemicals were able to lyse the oocysts more effectively than any other method tested, however this extraction method is not applicable to a high-throughput laboratory.

1. UNICEF/World Health Organization (2009). *Diarrhoea: Why children are still dying and what can be done.* Retrieved from http//whqlibdoc.who.int/publications/2009/9789241598415_eng.pdf