Fully Automated Lysis and Purification of Multiple Gastrointestinal Pathogen Nucleic Acid for Simultaneous Detection from a Single Stool Sample

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J. Green, A. Clark, T. Hayes, K. Rasband, J. Killpack, D. Jones, M. Rogatcheva, R. J. Crisp, S. Thatcher; BioFire Diagnostics, LLC, Salt Lake City, UT.

INTRODUCTION/BACKGROUND

Several methods are currently used to identify pathogens that cause diarrhea, including: culture for bacteria, microscopy for parasites, nucleic acid preparation and PCR for some bacterial and viral organisms, as well as antibody-based methods for some viral targets. These methods lack sensitivity, are not comprehensive in nature, and have relatively long processing times which significantly impact diagnosis. A rapid, fully automated, integrated sample preparation and pathogen detection system, the FilmArray™ Gastrointestinal (GI) Panel, was developed to detect multiple pathogens, including protozoan cysts and bacterial spores, from a single stool sample. The semi-solid nature of stool poses challenges to sample preparation in the FilmArray system, which employs automated fluidic processing. An in-line stool filtration system (100mm pore size allows targeted parasites to pass) was integrated to eliminate stool particulates.

DEVELOPMENT OF SAMPLE PURIFICATION FOR FILMARRAY GI

The Sample Purification of the existing FilmArray system was originally developed with multiple sample matrix types in mind, presenting an already effective platform for the development of the FilmArray GI Panel. However, the specific characteristics of stool, as well as a more difficult-to-lyse panel of organisms did present challenges that had to be overcome. Development of a novel filtration device for introduction of sample into the system allowed for the processing of stool samples. Additional optimization of the FilmArray's bead-beating based mechanical lysis resulted in increased sensitivity for difficult-to-lyse organisms in a complex sample matrix. The Sample Purification of the FilmArray GI Panel is a fundamental component for allowing effective detection of this very diverse group of GI Pathogens.

THE FILMARRAY GI PANEL

Simultaneous detection of 22 targets:

Bacteria

- *Campylobacter (jejuni, coli* and *upsaliensis)*
- *Clostridium difficile*
- *Plesiomonas shigelloides*
- *Salmonella*
- *Vibrio (parahaemolyticus, vulnificus* and *cholerae)*
- *Vibrio cholerae*
- *Yersinia enterocolitica*

Diarrheagenic *E. coli/Shigella*

- Enterotoxigenic *E. coli* (ETEC) *lt/st*
- Enteropathogenic *E. coli* (EPEC)
- Shiga-like toxin-producing *E. coli* (STEC)*stx1/stx2*
- *Shigella*/Enteroinvasive *E. coli* (EIEC)
- Enteroaggregative *E. coli* (EAEC)
- *E. coli* O157

Viruses

- Adenovirus F40/41
- Human Astrovirus
- Norovirus GI/GII
- Rotavirus A
- Sapovirus (I, II, IV, and V)

Parasites

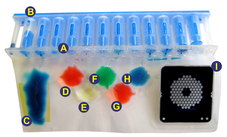
- *Cryptosporidium*
- *Cyclospora cayetanensis*
- *Entamoeba histolytica*
- *Giardia lamblia*

The FilmArray System

The FilmArray is a lab-in-a-pouch, medium-scale fluid manipulation system performed in a self-contained, disposable, thin-film plastic pouch. The FilmArray platform processes a single sample, from nucleic acid purification to result, in a fully automated fashion.

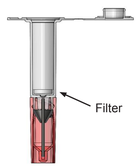
The FilmArray GI pouch has a fitment (B) containing all needed freeze-dried reagents and plungers that plunge liquids to the film portion of the pouch. This portion consists of stations for cell lysis (C), magnetic-bead-based nucleic acid purification (D & E), first-stage multiplex PCR (F & G) and an array of 102, second-stage nested PCRs (I).

PCR primers are dried into the wells of the array, and each primer set amplifies a unique product of the first-stage multiplex PCR. The second-stage PCR product is detected in a melting analysis using a fluorescent-double-stranded DNA binding dye, LCGreen™.



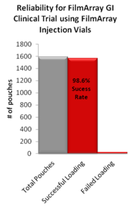
A. Fitment with freeze-dried reagents
B. Plungers-deliver reagents to blisters
C. Sample lysis and bead collection
D. Wash station
E. Magnetic bead collection blister
F. Elution station
G. Multiplex outer PCR blister
H. Dilution blister
I. Inner nested PCR array

Sample Injection Vial



The physical properties of stool, specifically the presence of variable amounts of particulates, presented a unique challenge for sample processing and movement through the FilmArray's closed fluidic system.

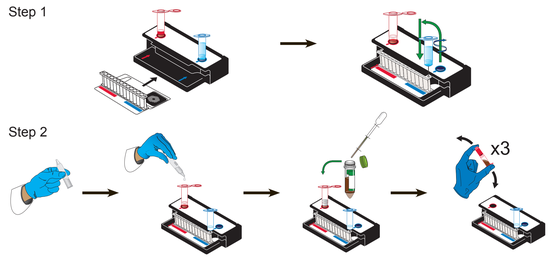
A novel filtration system was developed which mitigates the negative physical properties of stool, while allowing all target pathogens to be detected.



This system is applicable for other solid sample matrices as well as liquid matrices.

During the FilmArray GI Clinical Trial the system success rate was almost 99%, with no samples lost. This rate is inclusive of the entire system including both Hydration and Sample Injection Vials as well as the pouch itself. This failure rate is comparable to the syringe loading method used by other FilmArray Panels, and also results in an improved workflow when compared to the syringe method.

Sample Processing and Pouch Loading Instruction



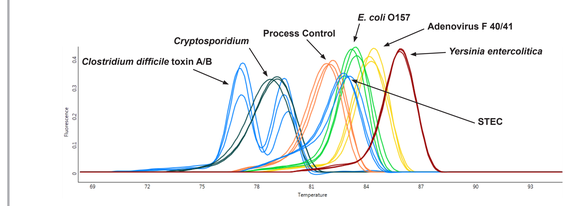
Testing requires minimal pre-processing of specimens. The stool is diluted in Cary Blair medium (1:4), filtered by vacuum through the FilmArray Injection Vial, and loaded into the FilmArray GI pouch (see Instruction guide). The user enters the sample and pouch type (using a barcode reader) into the software and initiates a run.

MATERIALS AND METHODS

- 10 bacteria, 3 parasites, and 5 viral pathogens known to cause gastrointestinal illness were tested in this Study.
- For all bacterial pathogens tested, fresh cultures were grown and enumerated before being incorporated into the testing.
- For all parasitic pathogens tested, fresh enumerated stocks were purchased and used for testing.
- Testing of viral targets was done using enumerated stocks for Adenovirus and Astrovirus.
- Additional testing of viral targets was done using clinical samples of Norovirus, Rotavirus, and Sapovirus due to a lack of available quantified organism.
- Organisms were divided into 3 pools of up to 6 organisms per pool for testing in FilmArray GI Panels. Pools were organized in a way to separate similar organisms, as well as test all organism types (bacterial, viral, parasitic) in each pool.
- Each pool was serially diluted and spiked into 5 unique stool samples (20 total) to determine whether multiple pathogens can be detected within a single sample, as well as to characterize the effects of multiple backgrounds.
- Over 700 GI Panels were processed using the FilmArray system to generate the results summarized here.

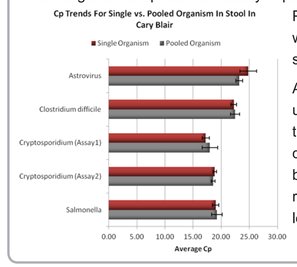
Pool A	Pool B	Pool C
Cryptosporidium parvum (Waterborn P102)	*E. coli* lt/st (O78-PS 2.1507)	Campylobacter jejuni (ATCC BA-1234)
Clostridium difficile (ATCC 17857)	*Shigella sonnei* - EIEC (ATCC 29930)	E. coli pAA plasmid (EAEC 92.0141)
EDL933 (ATCC 43895)	*Vibrio cholerae* (ATCC 14035)	*Plesiomonas shigelloides* (ATCC 14029)
Yersinia entercolitica (CDPH-4277)	Rotavirus A (PCMC-027)	*Giardia lamblia* (Waterborne P101)
Adenovirus (Type 40 - Dugan Strain)	*Entoamoeba histolytica* (ATCC 30459)	*Sapovirus* (PCMC-035)
Norovirus (PCMC-025)		Astrovirus (NCPV #936 Strain ERE IID 2868)

Multiple Detections from a Single Sample



The lysis and purification processes that have been developed and optimized for the FilmArray GI Panel allow for the detection of multiple pathogens from the same patient sample. This includes difficult-to-lyse organisms such as *Cryptosporidium* oocysts or *Clostridium difficile* spores, as well as bacterial and viral targets.

Additional experiments were performed to illustrate that there is no impact on sensitivity when multiple analytes are present. Note that there is no significant difference in Cp's when organism is spiked individually or pooled; highlighting the fact that the FilmArray GI Panel can not only detect multiple pathogens within the same sample, but do so in a very sensitive manner.



All data presented here has been generated using stool in Cary Blair as background. With this in mind, it is important to realize that multiple detections and a high level of sensitivity has been achieved even in a sample matrix that is rich with non-pathogenic organisms and high levels of nucleic acids.

RESULTS

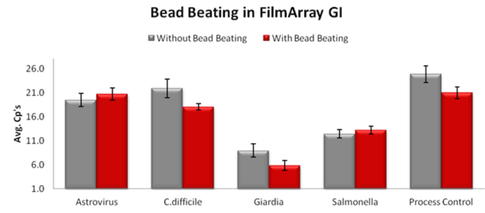
All-Organism Study Positivity				
	Organism	Organism/mL		
		10^2	10^3	10^4
Bacteria	*Campylobacter (jejuni, coli, &upsaliensis)*	0%	81%	100%
	Clostridium difficile Toxin A/B	0%	17%	100%
	Plesiomonas shigelloides	73%	100%	100%
	Salmonella	93%	100%	100%
	Vibrio cholerae	0%	6%	53%*
	Yersinia entercolitica	87%	100%	100%
Diarrheagenic *E. coli/Shigella*	Enterotoxigenic *E. coli* (ETEC) lt/st	87%	100%	100%
	Enteropathogenic *E. coli* (EPEC)	100%	100%	100%
	Shiga-like toxin-producing *E. coli* (STEC) stx1/stx2	100%	100%	100%
	Shigella/Enteroinvasive *E. coli* (EIEC)	93%	100%	100%
	Enteroaggregative *E. coli* (EAEC)	80%	97%	100%
	E. coli O157	87%	100%	100%
Viruses	Adenovirus F 40/41	100%	100%	100%
	Astrovirus	100%	100%	100%
	Norovirus GI/GII***	7%	30%	73%
	Sapovirus***	93%	90%	100%
	Rotavirus A***	7%	97%	100%
Parasites	*Cryptosporidium*	60%	72%	96%**
	Entamoeba histolytica	13%	79%	100%
	Giarda lamblia	93%	100%	100%
	Cyclospora cayetenensis	Not Tested		

*100% Positivity was observed at $5x10^4$
**100% Positivity was observed upon retesting
***Enumerated organism unavailable; 10-fold dilutions of a known clinical sample

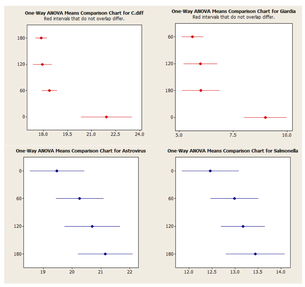
- All organisms on the FilmArray GI Panel were detected.
- Using the Sample Injection Vials for sample loading allowed 100% of samples to be tested in the FilmArray GI Panel.
- Both DNA and RNA targets were effectively isolated from stool for downstream detection in the FilmArray GI Panel.
- 100% detection of pooled pathogens was observed for up to 6 pathogens from a single sample.
- Minimal inhibition was observed as a result of effective sample purification which incorporates nested multiplex PCR.
- Reliable detection was observed in the range of at 10^2-10^4 organisms/mL for all organisms tested.
- Detection of difficult-to-lyse organisms, such as *Cryptosporidium* and *Clostridium difficile*, was aided by enhanced mechanical lysis in the FilmArray.
- Positivity can vary due to the highly variable and complex nature of the stool background itself, as well the difficulty of effectively mixing spiked organism into stool samples.

Mechanical Lysis

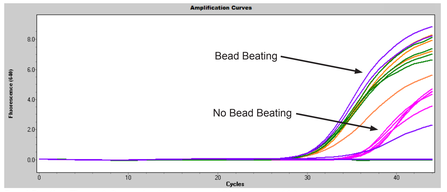
The FilmArray GI Panel utilizes a bead beating protocol using ceramic beads that has been specifically optimized for GI pathogens in stool in Cary Blair. This includes notoriously difficult-to-lyse organisms such as *Cryptosporidium*, *Clostridium difficile*, and *Giardia*, in addition to the many other bacterial, parasitic, and viral pathogens that are known to cause GI illness.



Optimized bead beating in the FilmArray GI Panel allows for more sensitive detection of organisms known to populate the GI tract in the form of spores, cysts, and oocysts.



- Analysis by 1-way ANOVA confirms the significant advantage of optimized bead beating for organisms such as *Clostridium difficile* and *Giardia* while also showing no significant effect on other "easy-to-lyse" targets.
- 3.8 cycle improvement in Cp observed for *Clostridium difficile*.
- 3.1 cycle improvement in Cp observed for *Giardia*.
- No significant shift observed for Astrovirus and *Salmonella*.



Bead Beating Time: 0 Seconds, 60 seconds, 120 seconds, 180 seconds

Additional experiments illustrate a 4.1 cycle improvement in Cp's for *Cryptosporidium parvum* ($1x10^6$ oocysts/ml in stool in Cary Blair) with the incorporation of mechanical lysis that has been optimized for the FilmArray GI.

CONCLUSION

Extraction, purification, and detection of this very diverse set of gastrointestinal organisms in a complex stool matrix in about an hour can be achieved using a modified sample-to-result automated system.

The FilmArray GI Panel has been optimized to quickly and efficiently lyse, extract, purify, and detect nucleic acid from 22 different known GI pathogens. By incorporation of a novel filtration method for introducing sample as well as optimizing the existing bead beating, the sample purification of if the FilmArray GI Panel has proven to be an efficient and sensitive method for detection of GI pathogens.

The ability to quickly detect this comprehensive collection of pathogens from a single sample has the potential to revolutionize the way gastrointestinal illness is diagnosed, and greatly improve patient care.

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CONTACT INFORMATION
Jeremy Green
BioFire Diagnostics
Jeremy.Green@BioFireDX.com