MULTIPLEX GASTROINTESTINAL PATHOGEN DETECTION WITH A USER FRIENDLY, HIGH-THROUGHPUT SYSTEM

Applied BioCode

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Revised Abstract

Gastroenteritis is the second most common illness after the common cold. Globally, diarrhea accounts for approximately 2 million deaths in children under 5 years old each year, or 19% of total child deaths.[1, 2] High-throughput multiplex assays are desirable for rapid identification of the pathogens that can cause outbreaks of diarrhea and for infection control in healthcare settings.

Using the proprietary barcoded magnetic bead (BMB) technology, Applied BioCode has developed a prototype molecular diagnostic assay for detection of gastrointestinal (GI) pathogens including bacteria, viruses, and parasites. The GI pathogen assay is highly specific and did not show cross-reactivity with organisms tested in the study. Analytically, the assay can detect 10 copies of DNA targets per PCR reaction, and preliminary limit of detection was as low as 10^3 CFU/mL for *C. difficile* and 10^2 PFU/mL for adenovirus 41. Evaluation of 43 clinical specimens previously tested by validated real-time PCR assays showed 100% concordance for *C. difficile* toxin and norovirus. In parallel with the multiplex assay development, we are also developing a prototype automated system in a 96-well format. This system will integrate PCR with post-PCR processing and detection, thereby simplifying the workflow and reducing hands-on time for the users.

In this study, we tested 234 stool specimens with the BioCode GI assay and demonstrated comparable results to a FDA-cleared multiplex test performed in a clinical laboratory.

Methods

Clinical specimens were subjected to vortexing with beads in the easyMAG lysis buffer prior to automated extraction with NucliSENS easyMAG® (bioMerieux).

Following extraction of nucleic acids from stool specimens, DNA and RNA targets were amplified in a one-step RT-PCR in either 9700 or 7500 Fast thermal cycler (Applied Biosystems). PCR products were captured by target-specific probes that were coupled to BMBs with unique barcodes. The presence of captured target sequence on an individual BMB was detected by streptavidin-phycoerythrin (SA-PE) conjugate. Qualitative results for each target were determined by a median fluorescence index (MFI) signal relative to assay cutoffs.

Target specific oligonucleotide probes were synthesized with a 5'-amino modification and a linker. Each probe was conjugated to BMBs with a unique barcode via amide linkage. Probes coupled to BMBs were combined into assay specific probe mix used for capturing biotin-labeled target DNA. Target capture (hybridization) and SA-PE labeling reactions were performed in a Thermo Shaker (PlexBio Co.). Unbound molecules were removed from BMB by washing with a ELx50 magnetic plate washer (BioTek). BMB imaging and fluorescence signal detection was performed on BioCode 1000A reader (Applied BioCode, Inc.).

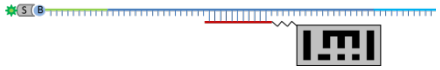


Figure 1. Schematic presentation of a biotinylated target DNA captured by a target-specific probe (red line) coupled to BMB (gray rectangle), and labeled by SA-PE (*S)

BioCode GI Panel Targets

Table 1. Organisms and toxins targeted by BioCode GI Panel

Bacteria
▪ *Clostridium difficile* (toxin A and B)
▪ Campylobacter spp.
▪ Salmonella spp.
▪ Shigella spp./ Enteroinvasive *E. coli*
▪ *E. coli* O157
▪ Enterotoxigenic *E. coli* (LT/ST)
▪ Shiga toxin producing *E. coli* (*stx1/stx2*)
▪ Enteroaggregative *E. coli*
▪ Aeromonas spp.
▪ Vibrio spp. (*V. parahaemolyticus*)
Viruses
▪ Norovirus GI/GII
▪ Adenovirus 40/41
▪ Rotavirus A
Parasites
▪ Cryptosporidium spp.
▪ *Giardia intestinalis*
▪ *Entamoeba histolytica*
Internal Control (MS2)

Barcoded Magnetic Beads (BMB)

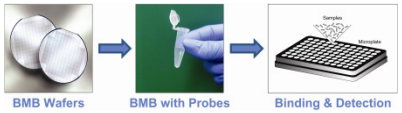


Figure 2. BMBs extracted from wafers are activated in suspension, coupled to probes, and utilized for target capture in microtiter plates.

BioCode-3000 HTS

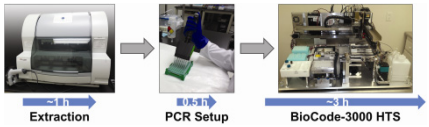
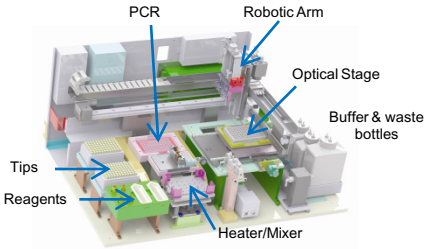


Figure 3. Workflow for BioCode assays includes automated extraction and PCR set up prior to the steps automated by BioCode-3000 HTS (Top). Computer-aided drafting (CAD) image of the prototype instrument (Bottom).



Results

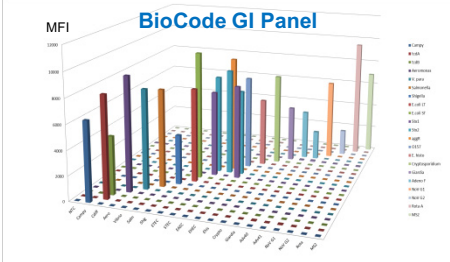


Figure 4. Sample data from the BioCode assay with analytical targets. MFI values (bars) for each target (X-axis) are plotted for each probe.

Table 2. Microorganisms tested for cross reactivity with BioCode assay

Organisms tested for Cross Reactivity		
Acinetobacter baumannii	*Acinetobacter lwoffii*	*Alcaligenes faecalis*
Bacillus cereus	*Citrobacter freundii*	*Enterobacter aerogenes*
Enterobacter cloacae	*Enterococcusfaecalis (vanB)*	*Enterococcusfaecium (vanA)*
Escherichia coli	*Klebsiella oxytoca*	*Klebsiella pneumoniae*
Lactobacillus acidophilus	*Listeria monocytogenes*	*Proteus mirabilis*
Providencia stuartii	*Pseudomonas aeruginosa*	*Serratia marcescens*
Staphylococcus aureus	*Staphylococcus epidermidis*	*Stenotrophomonas maltophilia*
Streptococcus agalactiae	*Vibrio cholerae*	*Yersinia enterocolitica*

No cross-reactivity was observed for the BioCode GI assay with the microorganisms listed above.

Table 3. Preliminary Limit of Detection (LoD) for the BioCode GI assay

Preliminary Limit of Detection		
Target	Preliminary LoD	Average MFI
Adenovirus 41	1x10² PFU/mL	4898
Norovirus GI (recombinant)	7x10³ $TCID_{50}$/mL	6937
Norovirus GII (recombinant)	7x10³ $TCID_{50}$/mL	5672
C. difficile	1x10³ CFU/mL	2624
Salmonella enterica (typhimurium)	7x10³ CFU/mL	4256
Shigella flexneri	7x10³ CFU/mL	5721
E. coli O157	7x10³ CFU/mL	6130
Aeromonas hydrophila	7x10³ CFU/mL	7103
Vibrio parahaemolyticus	7x10³ CFU/mL	7443
Cryptosporidium	7x10³ cells/mL	6736
Giardia intestinalis	1x10² cells/mL	1241

Table 4. Comparison of the BioCode assay with validated real-time PCR for *C. difficile* and noroviruses (GI & GII).

C. difficile		Real-Time PCR		
		Pos	Neg	Total
BioCode GI Panel	Pos	10	0	10
	Neg	0	33	33
	Total	10	33	43

Norovirus (GI/GII)		Real-Time PCR		
		Pos	Neg	Total
BioCode GI Panel	Pos	8	0	8
	Neg	0	35	35
	Total	8	35	43

Results of the BioCode assay showed excellent correlation with validated real-time PCR assays for *C. difficile* and noroviruses.

Results

Table 5. Comparison of the BioCode GI assay with Luminex GPP assay (n =234 stool specimens)

Target Pathogen	Positive results reported by	
	BioCode GI	Luminex GPP
Clostridium difficile	67	65
Salmonella spp.	5	7
Shigella spp./ EIEC	3	4
ETEC	0	0
STEC	0	0
E. coli O157	1	1
EAEC	5	N/A
Campylobacter spp.	1	1
Aeromonas spp.	1	N/A
Vibrio parahaemolyticus	0	N/A
Norovirus (GI & GII)	11	10
Rotavirus A	10	9
Adenovirus F	5	N/A
Cryptosporidium spp.	1	1
Giardia intestinalis	0	0
Entamoeba histolytica	0	N/A
Total positive results	110	98
Invalid results (initial run)	1 (8)	1 (27)*

*Repeat testing for Luminex assay was performed with 1:10 dilution of the sample.

Co-infections: The BioCode assay reported 10 out of 234 samples (4.3%) as positive for more than one pathogen.

Table 6. Comparison of BioCode assay with Luminex GPP for *C. difficile*.

C.difficile		Luminex GPP Results		
		Positive	Negative	Total
BioCode GI Panel	Positive	59	8	67
	Negative	6	161	167
	Total	65	169	234

Positive Agreement	59/65	90.8%
Negative Agreement	161/169	95.3%

Summary & Conclusions

- BioCode GI assay is shown to detect intended targets including bacteria, viruses and parasites.
- BioCode GI assay does not detect other organisms tested for cross-reactivity.
- Preliminary Limit of Detection (LoD) of the assay was determined at 10^3 CFU/mL for *C. difficile* 10^2 PFU/mL for adenovirus 41 and 10^2 cells/mL for Giardia.
- Clinical performance with 234 stool specimens showed comparable results to the Luminex xTAG® GPP assay.
- BioCode-3000 HTS is designed for batch testing of multiplex assays in 96-well format with limited hands-on time and reduced contamination risk.

Acknowledgements

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References

1. Boschi-Pinto C, Velebit L, Shibuya K. Estimating child mortality due to diarrhoea in developing countries. Bull World Health Organ. 2008; 86(9):710–7.
2. Clark B, McKendrick M. A review of viral gastroenteritis. Curr Opin Infect Dis. 2004; 17(5):461–9.

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The assay and platform are currently in development.