Validation and Performance of Sequencing-Based Reference Assays for BioCode GI Panel

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Introduction

Applied BioCode has developed an automated high-throughput molecular diagnostic assay system in a 96-well format. The BioCode GI Pathogen Panel is a 18-plex molecular assay for detection of gastrointestinal pathogens which include bacteria (*Campylobacter*, *C. difficile* toxin A/B, *Salmonella*, Shigella/enteroinvasive *E. coli*, enteroaggregative *E. coli*, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, shiga toxin-producing *E. coli*, *E. coli* O157, *Vibrio*, *Yersinia enterocolitica*), viruses (norovirus group I/II, adenovirus F, rotavirus A), and parasites (*Cryptosporidium*, *Entamoeba histolytica*, *Giardia lamblia*).

To support the clinical trials of the BioCode GI Pathogen Panel, we developed Reference Assays (composite comparator PCR/Sequencing assays) for method comparison for 7 select targets of the Panel.

Methods

Two different SYBR Green PCR/Sequencing assays were validated for each of the 6 targets while ETEC required three assays. NucliSENS® easyMAG™ (bioMérieux) extraction was used for the assays. Targets were amplified with real-time SYBR Green PCR using ABI 7500 system. Presumptive positive results, based on assay-specific Tm ranges, were confirmed by bi-directional sequencing with BigDye Terminator chemistry and ABI 3500 Analyzer. The resulting sequence data was analyzed with ABI Sequence Scanner Software V.2 and SeqMan Pro of the Lasergene 12 Core Suite Software to generate PHRED scores and contig length and ambiguous nucleotides, respectively. NCBI BLAST of each of resulting contigs was performed to generate Identity to Reference, Query Coverage, and Expected Value (E-Value).

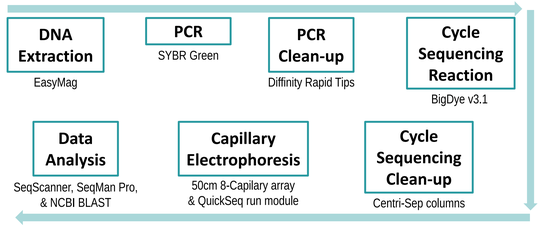

Figure 1. Workflow for Sequencing-Based Reference Assays for the BioCode GI Pathogen Panel. The Reference assays utilize automated nucleic acid extraction (bioMérieux NucliSENS® easyMAG™ System). Target amplification and confirmation of positive result are achieved with real-time SYBR Green PCR using ABI 7500 system followed by Bi-directional Sequencing (BigDye Terminator chemistry and capillary electrophoresis) with ABI 3500 Analyzer.

Limit of Detection (LoD) Study

Table 1. Limit of Detection (LoD) for the Sequencing-Based Reference Assays. The LoD was determined by extracting 20 replicates at the level that was detected 95% of the time.

Bacteria/Parasites/Viruses	Limit of Detection Unpreserved Stool	Limit of Detection Cary Blair Stool
Enteroaggregative *E. coli (EAEC) aggR* + Assays 1 & 2	5.0 E+2 CFU/mL	1.7 E+2 CFU/mL
Enteropathogenic *E. coli* (EPEC) Assay 1	1.0 E+3 CFU/mL	3.3 E+2 CFU/mL
Enteropathogenic *E. coli* (EPEC) Assay 2	5.0 E+3 CFU/mL	1.7 E+2 CFU/mL
Enterotoxigenic *E. coli* (ETEC)- LT & ST1a Assays	1.0 E +4 CFU/mL	3.3 E+3 CFU/mL
Enterotoxigenic *E. coli* (ETEC)- ST1b Assay	1.0 E+5 CFU/mL	3.3 E+4 CFU/mL
Cryptosporidium spp (C. parvum) Assays 1 & 2	5.0 E+4 oocysts/mL	1.7 E+3 oocysts/mL
Giardia lamblia Assay 1	1.0 E+3 oocysts/mL	3.3 E+2 oocysts/mL
Giardia lamblia Assay 2	1.5 E+3 oocysts/mL	5.0 E+2 oocysts/mL
Entamoeba histolytica Assays 1 & 2	5 cysts/mL for	1.7 cysts/mL for
Adenovirus 40	1.0 E+1 $TCID_{50}$/mL	3.3 $TCID_{50}$/mL
Adenovirus 41	1.0 E+1 $TCID_{50}$/mL	3.3 $TCID_{50}$/mL

Analytical Reactivity/ Inclusivity Study

Table 2. Inclusivity Study for the Sequencing-Based Reference Assays. Nucleic acid extraction of all organisms listed using bioMérieux easyMAG was performed in triplicates on contrived stool samples having concentration of 3X LoD predetermined for each of the Reference Assays.

Species/Strain/ Isolate/Toxinotype	Source	Species/Strain/ Isolate/Toxinotype	Source
Enteroaggregative *E. coli* (EAEC) aggR		*Giardia lambia/intestinalis*	waterborne P101
O92:H33 *Escherichia coli* (EAEC)	STEC JM221 TW04440	*Giardia lambia/intestinalis*	BEI NR-9231
E. coli O44:H18	STEC 042 TW04393	*Giardia lambia/intestinalis*	BEI NR-9232
E. coli O111a, 111b:K58:H21	ATCC 29552	*Giardia lambia/intestinalis*	BEI NR-9234
E. coli O104:H4	ATCC BAA-2326	*Giardia lambia/intestinalis*	BEI NR-9235
E. coli, Strain NCDC U14-41	BEI NR-102	*Entamoeba histolytica*	BEI NR-176
Enteropathogenic *E.coli* (EPEC) eae		*Entamoeba histolytica*	BEI NR-177
O127:H6 *Escherichia coli* (EPEC)	STEC E2348/69 TW06375	*Entamoeba histolytica*	BEI NR-178
O111:H2 *Escherichia coli* (EPEC)	STEC DEC12D	*Entamoeba histolytica*	BEI NR-179
O128:H2 *Escherichia coli* (EPEC)	STEC DEC11D	*Entamoeba histolytica*	BEI NR-180
O55:H6 *Escherichia coli* (EPEC)	STEC DEC1E	***Cryptosporidium spp.****	
O86:H34 Escherichia coli (EPEC)	STEC C927-81 TW01273	*Cryptosporidium parvum subtype /IIaA17G1R1*	UKCR UK28
O142:H6 *Escherichia coli* (EPEC)	STEC C765-82 TW01271	*Cryptosporidium parvum subtype /IIaA15G2R1*	UKCR UK29
O114:H2 *Escherichia coli* (EPEC)	STEC 3448-87 TW00148	*Cryptosporidium parvum subtype /IIaA19G1R1*	UKCR UK30
O119:H2 *Escherichia coli* (EPEC)	STEC LT119-80 TW07099	*Cryptosporidium parvum subtype /IIdA22G1*	UKCR UK31
Escherichia coli, Strain CDC	BEI NR 99	*Cryptosporidium parvum subtype /IIdA15G1*	UKCR UK32
Enterotoxigenic E.coli (ETEC) LT/ST		*Cryptosporidium hominis subtype IdA18*	UKCR UKH12
O78:H11 *Escherichia coli* strain H10407 (ETEC)	ATCC 35401	*Cryptosporidium hominis subtype ibA10G2*	UKCR UKH13
O25:K98 *E.coli* (ETEC)	ATCC 43886	*Cryptosporidium hominis subtype IaA14R3*	UKCR UKH14
O78:K80:H12 *E.coli* (ETEC)	ATCC 43896	*Cryptosporidium hominis*	NR2520
O8:K85:K99 *E.coli* (ETEC)	ATCC 31618	*Cryptosporidium meleagridis*	UKMEL10
Adenovirus 40/41			
Sample 3	Clinical Samples	Sample 38	Clinical Samples
Sample 20		Sample 60	
Sample 30		Sample 112	

* Due to lack of titered specimens, *Cryptosporidium* DNA samples obtained from *Cryptosporidium* Reference Unit (Public Health Wales, UK) were tested at ten-fold dilution of each of the *Cryptosporidium* DNA stocks.

Method Comparison Study

Table 3. Method Comparison Study for the Sequencing-Based Reference Assays. Retrospective clinical samples were used for the Method Comparison. A total of 96 specimens (24 EAEC, 52 EPEC, 12 ETEC, 4 adenovirus 40/41, 7 *Giardia*, 9 *Cryptosporidium*, and 3 *E. histolytica*) were tested and compared with results from FDA-cleared BioFire FilmArray® GI Panel and Luminex xTAG® GPP.

Pathogens	Positive Agreement Vs. BioFire FilmArray®		Positive Agreement Vs. Luminex xTAG® GPP	
	TP/(TP+FN)	%	TP/(TP+FN)	%
Enteroaggregative *E. coli*	13/17	76%	7/7	100%
Enteropathogenic *E. coli*	27/28	96%	24/24	100%
Enterotoxigenic *E. coli*	4/5	80%	7/7	100%
Cryptosporidium spp.	5/7	71%	2/2	100%
Entamoeba histolytica	0/0	N/A*	1/3	33%
Giardia lamblia	2/2	100%	0/5	0%
Adenovirus 40/41	1/1	100%	3/3	100%
Total	**52/60**	**87%**	**44/51**	**86%**

* No *Entamoeba histolytica* positives were tested with BioFire FilmArray

Specificity/ Cross Reactivity Study

Table 4. Specificity/Cross Reactivity Study for the Sequencing-Based Reference Assays. Nucleic acid extraction of all organisms using bioMérieux easyMag were performed in triplicates on contrived stool samples having concentration of 10^6 CFU/mL of bacteria or 10^5 Units/mL of viruses or parasites.

Bacteria				
Aeromonas caviae	*Cedecea davisae*	*Enterococcus faecalis*	*Faecalibacterium prausnitzii*	*Megasphaera elsdenii*
Aeromonas hydrophila	*Chlamydia trachomatis*	*Pseudomonas aeruginosa*	*Fusobacterium varium*	*Morganella morganii*
Abiotrophia defectivia	*Citrobacter freundii*	*Shigella boydii (Type 1)*	*Gardnerella vaginalis*	*Peptoniphilus asaccharolyticus*
Acinetobacter baumannii	*Clostridium difficile non-toxigenic*	*Shigella dysenteriae, (Type 1) Newcastle 1934*	*Gemella morbillorum*	*Shigella/EIEC*
Alcaligenes faecalis	*Clostridium difficile toxin A/B*	*Saccharomyces boulardii*	*Haemophilus influenzae*	*Peptostreptococcus anaerobius*
Arcobacter butzleri	*Clostridium histolyticum*	*Salmonella bongori*	*Hafnia alvei*	*Plesiomonas shigelloides*
Bacillus cereus	*Clostridium perfringens*	*Grimontia hollisae (formerly vibrio)*	*Helicobacter pylori*	*Porphyromonas asaccharolytica*
Bacteroides fragilis	*E. coli Non pathogenic strain*	*Serratia mercescens*	*Klebsiella pneumoniae*	*Prevotella melaninogenica*
Leminorella grimontii	*Escherichia coli non pathogenic*	*Shewanella algae*	*Lactobacillus acidophilus*	*Veillonella parvula*
Bifidobacterium breve	Shiga-toxin producing *E. coli (STEC & O157)*	*Staphylococcus aureus*	*Lactococcus lactis*	*Shigella sonnei*
Campylobacter jejuni sub sp .jejuni	*Edwardsiella tarda*	*E. coliO124:HNM (EIEC)*	*Camplyobacter coli*	*Vibrio vulnificus*
Proteus penneri	*Enterobacter cloacae*	*Escherichia hermannii*	*Listeria monocytogenes*	*Streptococcus salivarius*
Provedencia alcalifaciens	*Vibrio mimicus*	*Vibrio alginolyticus*	*Yersinia enterocolitica*	N/A
Shigella flexneri, strain 24570 (Type 2a)	*Vibrio parahaemolyticus*	*Yersinia bercovieri*	N/A	N/A
Viruses			**Parasites**	
Adenovirus 3	Adenovirus 14	Norovirus GI	*Cryptosporidium meleagridis**	*Toxoplasma gondii*
Adenovirus 4	Adenovirus 37	Norovirus GII	*Giardia muris*	*Encephalitozoon intestinalis*
Adenovirus 7a	Cytomegalovirus (CMV)	Rotavirus A	*Encephalitozoon cuniculi*	**Yeasts**
Adeno virus 8	Enterovirus 68	Coxsackie virus	*Toxoplasma gondii*	*Candida albicans*
Rhinovirus 1A	N/A	N/A	N/A	N/A

**C. meleagridis was detected by Cryptosporidium* spp. SYBR Green PCR/Sequencing assays 1 and 2. NBCI BLAST information of forward and reverse primers of *Cryptosporidium* spp. Assays indicated highly significant match of sequence of *C. meleagridis* (7.0 x E^{-04} E-value and 100% Identity to Reference). *C. meleagridis* has been identified in ≤ 1% of persons with diarrhea, and the infectivity and virulence of *C. meleagridis* was similar to that of *C. parvum* or C. *hominis*. It is relevant that in addition to detecting *C. parvum* and C. *hominis, Cryposporidium* spp. SYBR Green PCR/Sequencing assays amplify and detect *C. meleagridis.*

Conclusions

- ❖ The sensitivity of the Sequencing-Based Reference Assays were slightly better for stool in Cary-Blair.
- ❖ The Sequencing-Based Reference Assays detected different strains representing various temporal, geographic, and genetic diversity of targets that each of the assay was designed to amplify, detect and sequence.
- ❖ Positive agreement vs. BioFire FilmArray® for Adenovirus and *Giardia lamblia* targets were 100% while positive agreement for EPEC, ETEC, EAEC, and *Cryptosporidium spp* were 96%, 80%, 76%, and 71%, respectively. Overall positive agreement was 87%.
- ❖ Positive agreement vs. Luminex xTAG® GPP for Adenovirus, *Cryptosporidium spp.,* EAEC, EPEC, *and* ETEC and were 100% while positive agreement for *Entamoeba histolytica* and Giardia lamblia was 33% and 0%, respectively. Overall positive agreement was 86%.
- ❖ The Specificity/Cross Reactivity study shows each of the Sequencing-Based Reference Assays did not cross-react with the organisms tested: bacteria (≥10^6 CFU/mL), viruses or parasites (≥10^5 Units/mL).
- ❖ The Sequencing-Based Reference Assays for select GI pathogens are highly sensitive, specific, and accurate, and can be used as composite comparator PCR/Sequencing assays to rapidly detect and differentiate 7 select targets of the BioCode Gastrointestinal Pathogen Panel.

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Performance of the sequencing-based assays have not been reviewed by the FDA.