Supplemental Information

Performance of human fecal anaerobe-associated PCR-based assays in a multi-laboratory method evaluation study

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1. Standard Operating Procedures (SOPs) used by "core" SIPP laboratories

1.1. Five laboratories involved in planning the SIPP study (SCCWRP, Stanford University, UC Santa Barbara, UCLA, and US EPA/Shanks, collectively known as the "core" labs) agreed to standardize their protocols for DNA extraction, DNA quantification, q/PCR, and data analysis. These SOPs were shared with all participating laboratories, but adherence to these SOPs was optional. All core labs and four "outside" laboratories used the GeneRite DNA-EZ ST kit for DNA extraction. All core labs and eight outside laboratories used a NanoDrop spectrophotometer for DNA quantification. Four core labs also quantified DNA using a fluorescence-based kit (Quant-iT), but only NanoDrop data were used in the analysis for this manuscript. Detailed protocols for q/PCR are found in Table SI-1. Manufacturer and vendor information for q/PCR reagents are found in Table SI-2. The SIPP SOP for using the FlashGel system to visualize conventional PCR products is given in Section 1.2 below. Details of data analysis are described in Ebentier et al. (this issue). Briefly, all core laboratory qPCR data (from both human and general assays) were quantified using lab-specific standard curves. These lab-specific curves were generated by pooling data from standard curves run on each qPCR plate, as well as a "master" standards plate containing triplicates of four separate dilution series. The lower limit of quantification (LLOQ, copies per reaction) was defined as the lowest point on the standard curve for which >50% of the replicates amplified. When samples had amplification in <3 PCR triplicates, a Cq of 40 was substituted for the non-detect replicates and the sample mean of 3 Cq values was used to quantify the copies present in the sample. Outside lab qPCR data were used as-is (as reported by each lab) in this manuscript, with the exception of BLOQ samples. For outside labs that reported BLOQ as quantified copy numbers or non-detects, a LLOQ was determined from their reported standard curves and BLOQ samples were treated as DNQ (see also Section 2 below).

Table SI-1. SOPs for quantitative and conventional PCR conditions used by "core" SIPP laboratories. dNTP = deoxynucleoside triphosphate, rxn = reaction, BSA = bovine serum albumin. See Table SI-2 for vendor information.

Assay	Developer reference	Target gene	oligo names	Reference material (standards)	rxn volume (µl)	template DNA volume (µl per rxn)	Enzyme and buffer (units per rxn)	Mastermix additives (units per rxn)	F primer µM per rxn	R primer µM per rxn	Probe µM per rxn	Thermal cycling conditions	Bio-Rad CFX96 threshold setting	ABI StepOnePlus threshold setting	PCR product visualization
BacH[1]	Reischer et al. (2007)	*Bacteroides* 16S	BacHf, BacH-pT, BacH-pC, BacHr	plasmid	25	2	12.5 µl iQ Supermix, contains hot-start iTaq™ DNA polymerase, dNTPs and buffer	BSA 0.4 µg/µl	0.2	0.2	0.1	95ºC for 3 min, 50 cycles of (95ºC for 15 sec, 61ºC for 15 sec, 72ºC for 45 sec), read at 61ºC anneal	N/A[3]	N/A[3]	N/A
BacHum-UCD	Kildare et al. (2007)	*Bacteroides* 16S	BacHum160f, BacHum193p, BacHum241r	plasmid	25	2	12.5 µl Applied Biosystems Taqman™ Universal Mastermix (cat# 4318157)	BSA 0.05 mg/ml	0.4	0.4	0.08	AB-Uni[2]	100 RFU	0.03	N/A
BsteriF1	Haugland et al. (2010)	*B. stericoris* 16S	BsteriF1DE, BthetP1, BthetR1	plasmid	25	2	12.5 µl Applied Biosystems Taqman™ Universal Mastermix (cat# 4318157)	BSA 0.05 mg/ml	1	1	0.08	AB-Uni	100 RFU	0.08	N/A
BtH[1]	Yampara-Iquise et al. (2008)	*B. thetaiota-omiron* a-1-6 mannanase	BtH-F, BtH-P, BtH-R	genomic *B. thetaiota-omicron* DNA	20	2	10 µl LightCycler Taqman 480 Probe Master Mix (5X)	BSA 0.2 mg/ml	0.2	0.2	0.1	95ºC for 15 min, 45 cycles of (95ºC for 15 sec, 60ºC for 1 min, 72ºC for 5 sec), 40ºC for 30 sec, read during 72ºC step	N/A[4]	N/A[4]	N/A
gyrB[1]	Lee et al. (2010)	*B. fragilis* gyrB	Bf904F, Bf923MGB, Bf958R	genomic *B. fragilis* DNA	25	5	12.5 µl Applied Biosystems Taqman™ Universal Mastermix (cat# 4318157)	N/A	0.5	0.5	0.25	AB-Uni	N/A	automatic threshold determination	N/A
HF183 endpoint	Bernhard & Field (2000)	*Bacteroides* 16S	HF183F, Bac708R	plasmid	25	2	0.625 U TaKarRa ExTaq (cat# RR001AM), 2.5 µl 10X TaKaRa PCR buffer	200 µM each dNTP, 2 mM MgCl2, 0.05 mg/ml BSA	0.2	0.2	N/A	94ºC for 2 min, 35 cycles of (94ºC for 30 sec, 63ºC for 45 sec, 72ºC for 45 sec), 72ºC for 7 min	N/A	N/A	FlashGel, see SOP in Supp Info section 1.2. Correct product is 525 bp
HF183 SYBR	Seurinck et al. (2005)	*Bacteroides* 16S	HF183F, HFsybR	plasmid	25	2	0.625 U Hot GoldStar DNA Polymerase from Eurogentec qPCR core kit for Sybr® Green I (cat #RT-SN10-05), 2.5 µl Eurogentec real-time PCR 10X Buffer (MgCl2-free)	200 µM each dNTP, 2 mM MgCl2, 0.75 µl Sybr® Green I	0.25	0.25	N/A	50ºC for 2 min, 95ºC for 10 min, 40 cycles of (95ºC for 30 sec, 53ºC for 1 min, 60ºC for 1 min)	100 RFU	0.03	melt curve with ramping from 60-94.8ºC at 0.4ºC per 10 sec
HF183 Taqman	Haugland et al. (2010)	*Bacteroides* 16S	HF183F, BthetP1, BthetR1	plasmid	25	2	12.5 µl Applied Biosystems Taqman™ Universal Mastermix (cat# 4318157)	BSA 0.05 mg/ml	1	1	0.08	AB-Uni	100 RFU	0.08	N/A
HumM2	Shanks et al. (2009)	*B. fragilis* hypothetical protein BF3236	HumM2F, HumM2P, HumM2R	plasmid	25	2	12.5 µl Applied Biosystems Taqman™ Universal Mastermix (cat# 4318157)	BSA 0.05 mg/ml	1	1	0.08	AB-Uni	100 RFU	0.08	N/A
Mnif	Johnston et al. (2010)	*Methanobrevi-bacter smithii nifH*	Mnif202F, MnifP, Mnif353R	genomic *M. smithii* DNA	25	2	12.5 µl of 2x Quantitect Mastermix (Qiagen cat# 204343)	N/A	0.5	0.5	0.12	50ºC for 2 min, 95ºC for 15 min, 45 cycles of (95ºC for 1 sec then 50ºC for 1 min)	100 RFU	0.03	N/A

[1]not run by core labs, SOP reported by developer lab

[2]AB-Uni: 50ºC for 2 min, 95ºC for 10 min, 40 cycles of (95ºC for 15 sec then 60ºC for 1 min)

[3]developer lab used Eppendorf Mastercycler® ep realplex (Eppendorf AG, Hamburg, Germany); threshold settings not reported.

[4]developer lab used Roche LightCycler 480 Real-Time PCR System; threshold settings not reported

Table SI-2. Ordering information for (q)PCR reagents used by core SIPP labs.

Item	Manufacturer	Part number	Specifications	Vendor
Primers	Operon	N/A	resuspend in DNase-free water	Operon
Probes	Operon	N/A	resuspend in TE pH 8.0 (from MoBio), put in as many 1000 pmol aliquots as possible	Operon
qPCR MM	Applied Biosystems	4318157	AB Universal MM	Life Technologies
Conventional	Takara	RR001AM	$MgCl_2$ separate	
BSA	GIBCO	15260-037		Invitrogen
DNAse free water	MoBio	100371-020		VWR
qPCR core kit for SYBR® Assay ROX	Eurogentec	RT-SN10-05		Anaspec.com
AE Buffer	Qiagen	19077		Qiagen

1.2. FlashGel System SOP

All conventional PCR products were visualized using Lonza FlashGel 16+1 double tier cassettes (2.2% agarose gel, VWR cat# 95015-624).

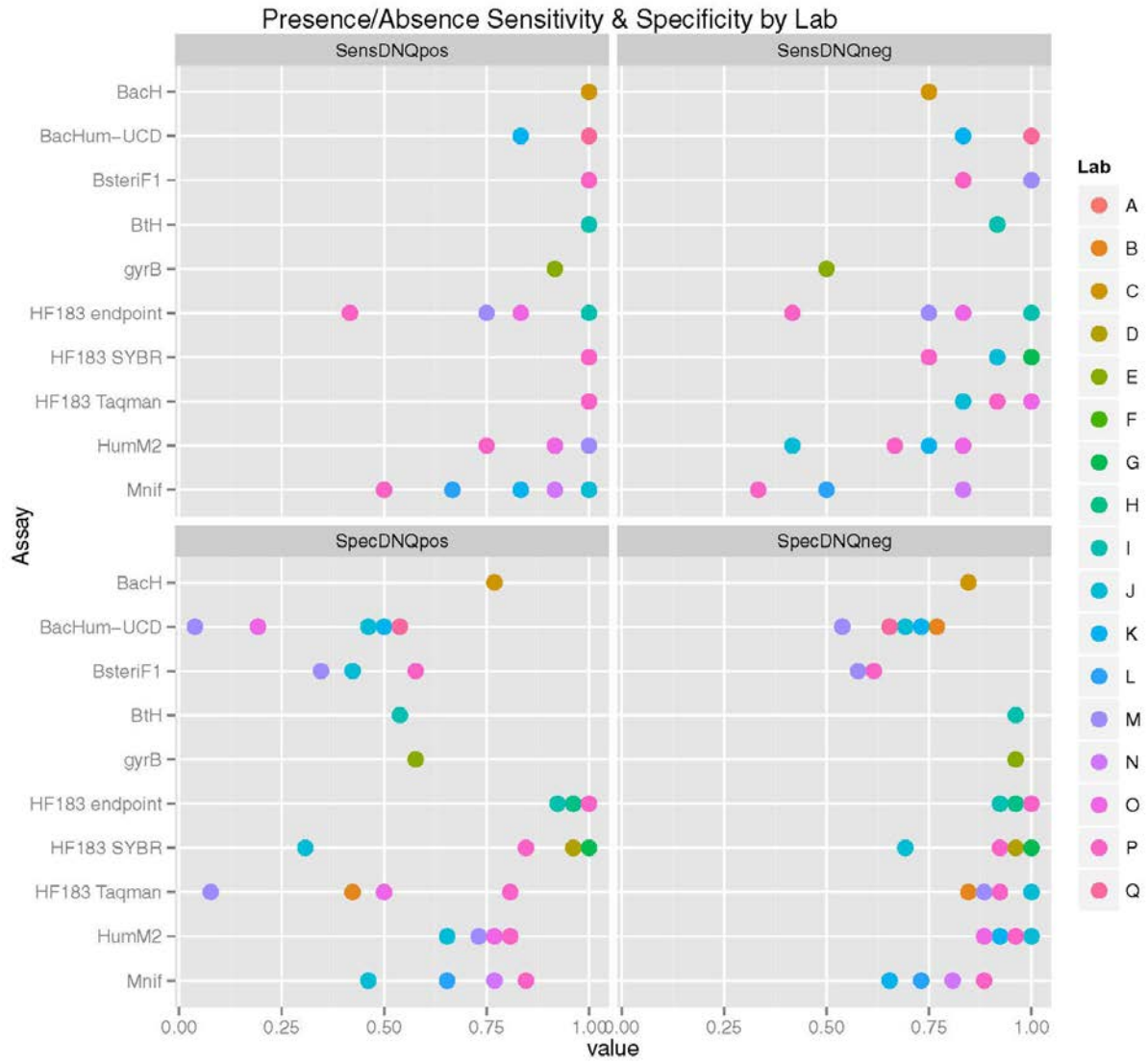
1. Remove the white seals on the gel cassette (leave the vent seals intact).
2. Rinse the gel cassette with dI water (TAE buffer is acceptable as well) and remove any excess liquid from the gel cassette by tilting the cassette and blotting. Do not touch the wells; they should have a bubble of liquid over them.
3. Insert the cassette into the gel dock.
4. Load samples by mixing 2uL sample with 2uL Lonza loading dye (5X loading dye VWR catalog number: 95015-630) and inject into well.
5. Load 4uL of ladder (DNA marker (100bp-4kb) VWR catalog number: 95015-632) into one well (other size ladders can be used (i.e. 1kb); they all worked well in preliminary tests.
6. Set the power source to 275V and allow the gel to run for ~3.5 minutes (Note: Single tier cassettes can run up to 7 min and double tier cassettes up to 5 min).
7. Pictures can be taken anytime during the gel electrophoresis and it is recommended to take one picture every minute since strong UV supply will potentially degrade PCR products.

2. Variable assay performance across laboratories

Table SI-3. Lower limit of quantification (LLOQ, in units of copies per filter) for each lab and assay. The lab "names" are random. BLOQ = below limit of quantification; DNQ = detected, not quantifiable; ND = not detected. Much of the information shown is a summary of data presented in Boehm et al. In press; the LLOQ values calculated in this work and not previously reported are shown in bold.

Assay	Lab	Standard reference material	LLOQ copies/filter	BLOQ reported
BacH	B	Circular plasmid	230	DNQ
BacHum-UCD	A	Circular plasmid	50	DNQ
	G	Circular plasmid	644	DNQ
	H	Circular plasmid	**500**	copy numbers
	J	Circular plasmid	100	DNQ
	L	Circular plasmid	3850	DNQ
	N	Circular plasmid	2404	ND
BsteriF1	A	Linear plasmid	50	DNQ
	G	Linear plasmid	1857	DNQ
	J	Linear plasmid	1000	DNQ
	M	Linear plasmid	957	DNQ
BtH	F	genomic DNA	700	DNQ
gyrB	D	genomic DNA	20000	ND
HF183 SYBR	C	Linear plasmid	250	DNQ
	E	Synthesized oligo	100	DNQ
	G	Linear plasmid	387	DNQ
	M	Linear plasmid	991	DNQ
HF183 Taqman	A	Linear plasmid	50	DNQ
	G	Linear plasmid	2222	DNQ
	J	Linear plasmid	1000	DNQ
	L	Linear plasmid	2948	DNQ
	M	Linear plasmid	1057	DNQ
HumM2	A	Linear plasmid	50	DNQ
	G	Linear plasmid	22486	DNQ
	H	Linear plasmid	**500**	copy numbers
	J	Linear plasmid	1000	DNQ
	L	Linear plasmid	3407	DNQ
	M	Linear plasmid	1113	DNQ
Mnif	G	genomic DNA	2450	DNQ
	H	genomic DNA	**313**	copy numbers
	I	genomic DNA	**1000**	copy numbers
	K	genomic DNA	200	ND
	M	genomic DNA	3282	DNQ

Figure SI-1. Presence/absence sensitivity (top row) and specificity (bottom row) in singleton samples under both DNQ classifications (columns) in each lab. Some data are obscured by multiple points at the same location. The lab "names" are random.



3. Variable DNA extraction yield among laboratories

Figure SI-2a. DNA yield according to DNA extraction kit and quantification method for all labs involved in the method evaluation study (Boehm et al. In press). Note that the two laboratories that used a MoBio kit and a Qubit fluorometer reported lower DNA yield than all other labs.

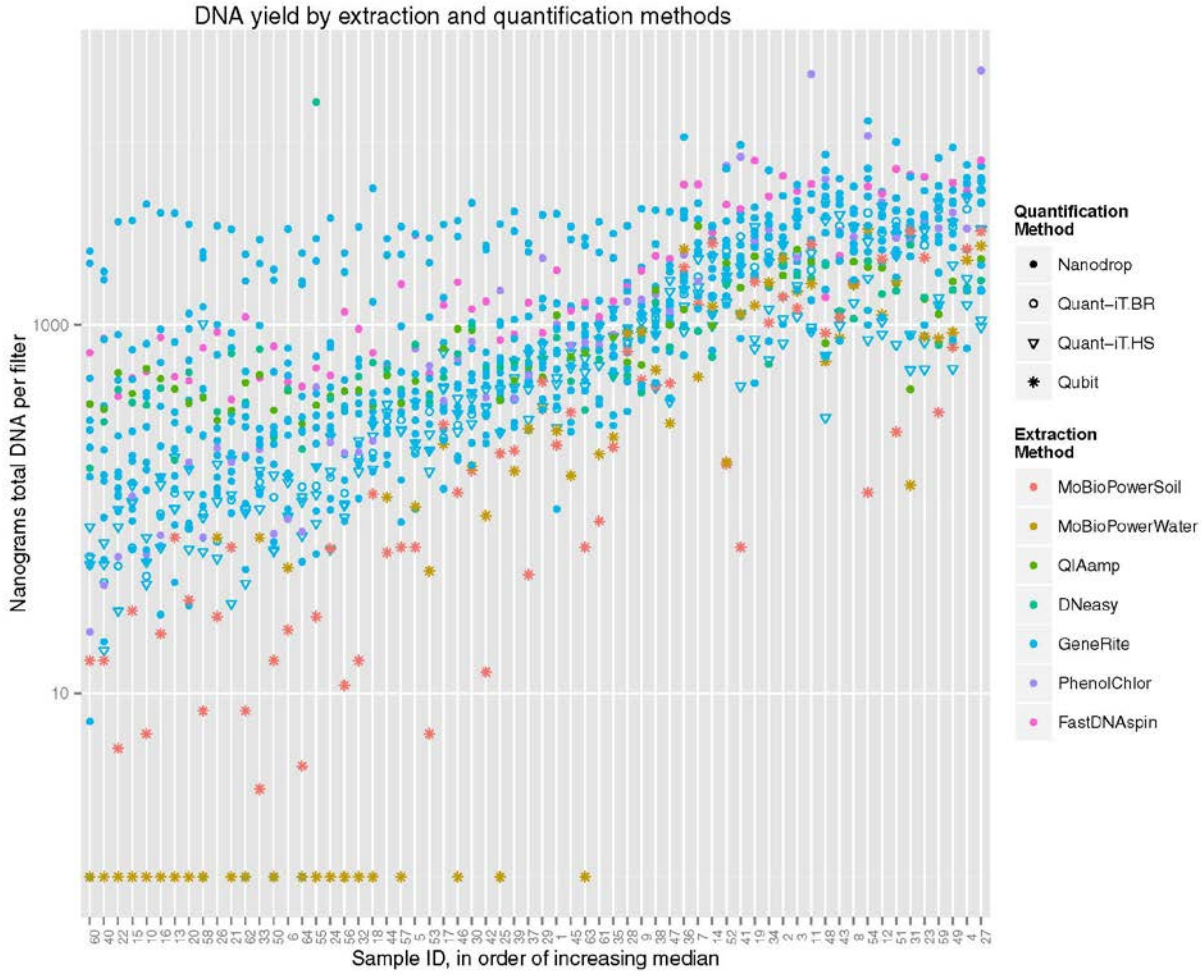
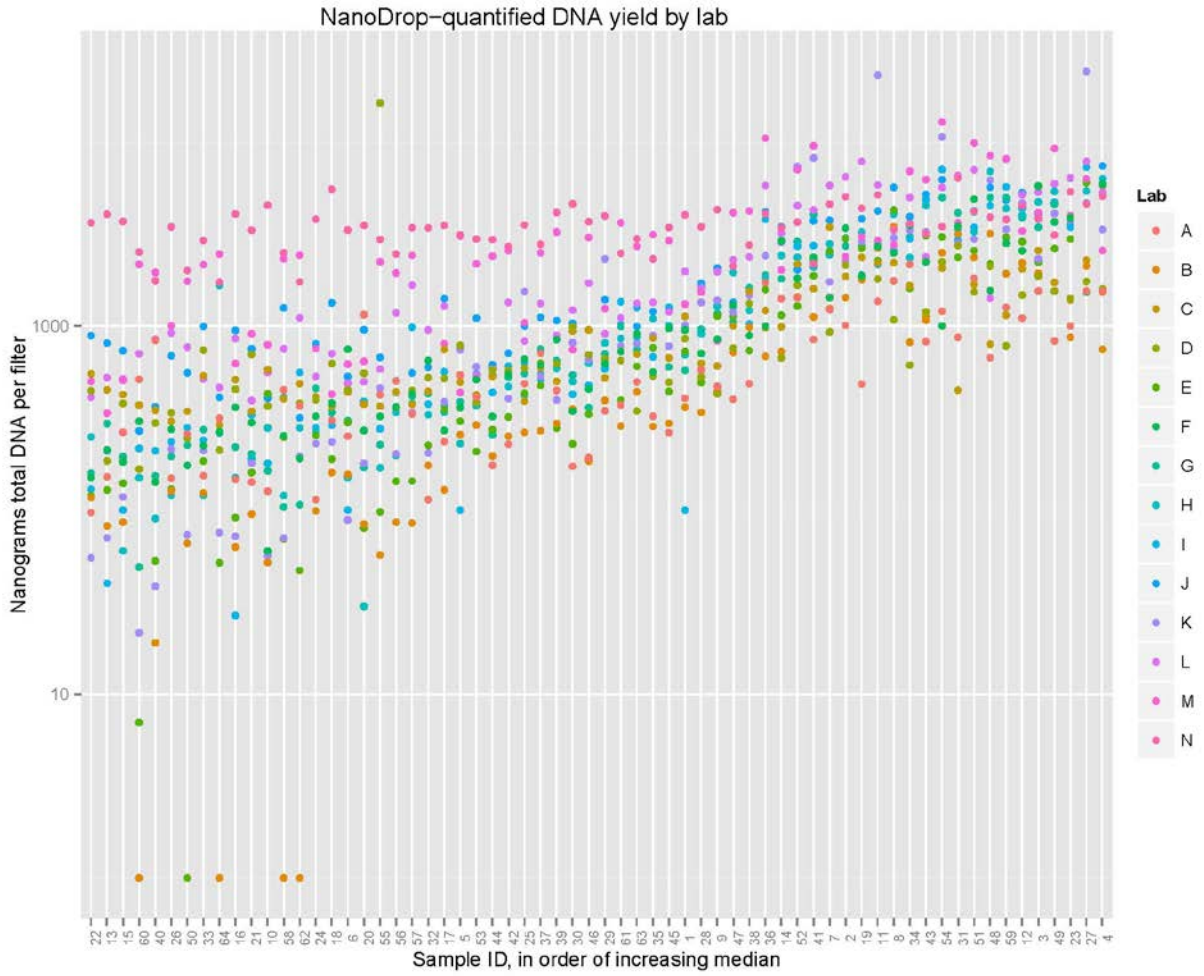


Figure SI-2b. DNA yield among labs measured by NanoDrop. The lab "names" in this figure are random.



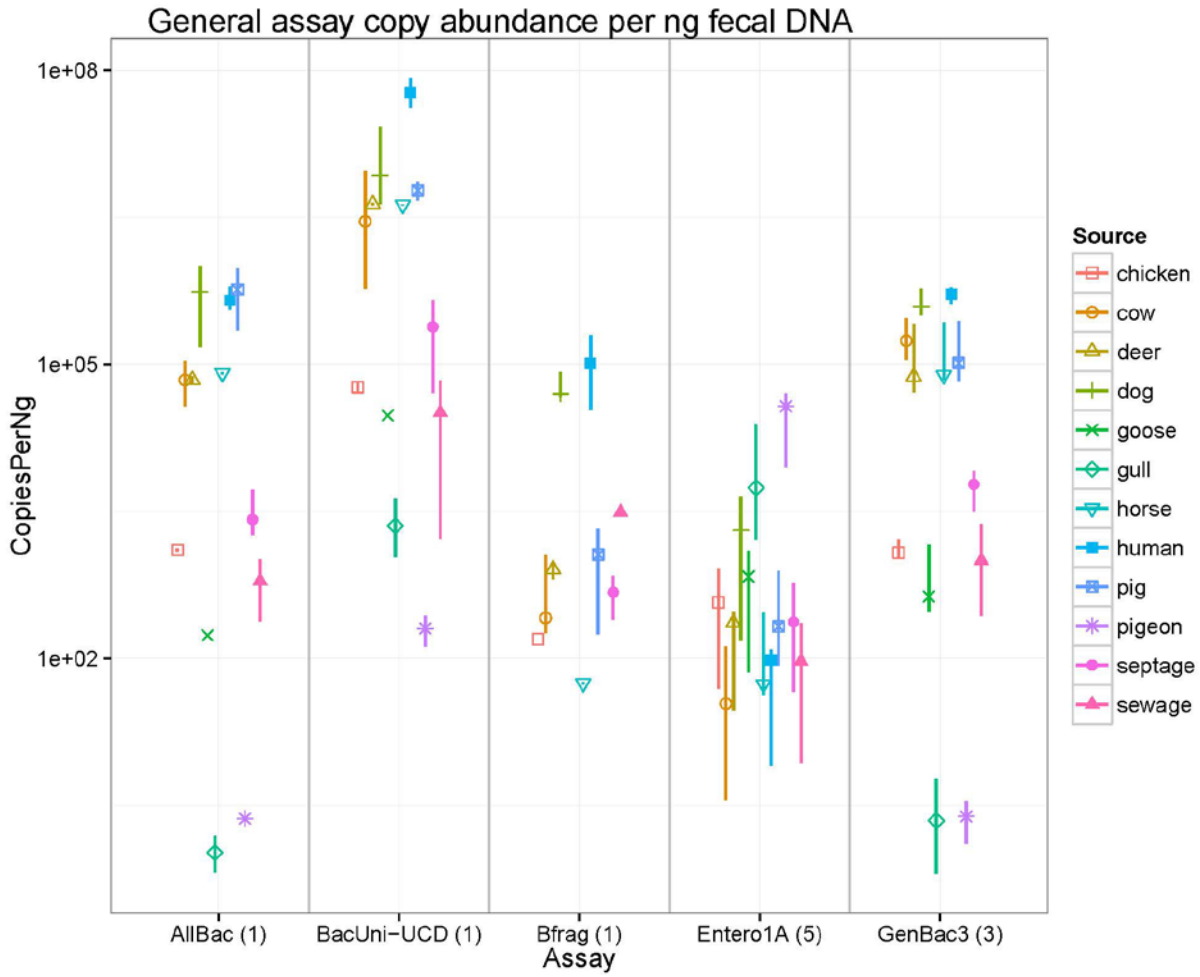
4. Table 4 (main text) recreated with human stool and wastewater (sewage/septage) target sources considered separately

Table SI-4. Human-associated marker abundance under all available fecal source characterizations. Values are median (standard deviation) of log_{10}-transformed copy numbers across all labs. See footnotes to Table 4. The assay with the highest gene copy abundance in a target source (most sensitive) for each unit of measure is shown in bold. The assay with the lowest copy abundance in non-human sources (most specific) is shaded. Note that this definition of "specific" is different than what is used in Table 4.

Assay	Source	*n*	MgWet	DNA	ENT MF	ENT qPCR	*E. coli* MF	*E. coli* qPCR	GenBac	AllBac	BacUni	Bfrag	FecalB
BacH	human stool	4	**7.5 (0.2)**	**5 (0.4)**	**4.8 (0.2)**	–	**1.5 (0.2)**	**1 (0.2)**	–	–	–	–	–
	wastewater	8	–	1.5 (0.9)	1.7 (1)	–	1.3 (1.1)	0.2 (1)	–	–	–	–	–
	non-human	26	2.8 (0.9)	0.9 (0.9)	-1.5 (1.8)	–	-1.5 (1.2)	-2.2 (0.9)	–	–	–	–	–
BacHum-UCD	human stool	24	7.1 (1)	4.8 (1.2)	4.4 (1)	2.3 (0.4)	1.1 (1)	0.6 (1)	**-1 (0.3)**	–	-0.4 (0.1)	–	–
	wastewater	48	–	**2.1 (1)**	**2.5 (0.9)**	-0.7 (0.4)	**2.3 (1)**	**1 (0.9)**	**-1.3 (0.4)**	–	-0.4 (0.2)	–	–
	non-human	156	2.4 (1.7)	0 (1.7)	-0.6 (1.3)	-2.9 (1.3)	-1.3 (1.8)	-2.2 (1.7)	-3.9 (1.9)	–	-3.8 (2.3)	–	–
BsteriF1	human stool	16	6.8 (0.2)	4.6 (0.2)	4.2 (0.2)	2.4 (0.4)	0.9 (0.2)	0.4 (0.2)	-1.2 (0.3)	–	–	–	–
	wastewater	32	–	1.7 (0.5)	2.1 (0.4)	-0.9 (0.5)	1.7 (0.6)	0.6 (0.4)	-1.8 (0.4)	–	–	–	–
	non-human	104	2.9 (2)	0.8 (2)	0.7 (1.4)	-1.8 (2.1)	-1.3 (2)	-2.2 (2.1)	-3.6 (1.8)	–	–	–	–
BtH	human stool	4	5.3 (0.1)	3 (0.2)	2.6 (0.1)	**2.8 (0.5)**	-0.7 (0.1)	-1.2 (0.1)	–	–	–	–	–
	wastewater	8	–	0.6 (0.8)	1.1 (0.5)	**-0.5 (0.8)**	0.8 (0.7)	-0.4 (0.4)	–	–	–	–	–
	non-human	26	0.9 (0.7)	-0.9 (0.4)	-1.1 (1.2)	-2.8 (1.2)	-3.7 (1.6)	-4.3 (1.2)	–	–	–	–	–
gyrB	human stool	4	5.2 (0.3)	3.2 (0.1)	2.6 (0.3)	–	-0.7 (0.3)	-1.2 (0.3)	–	–	–	-1.7 (0.7)	–
	wastewater	8	–	0.9 (0.9)	0.8 (0.5)	–	0.2 (0.4)	-0.5 (0.6)	–	–	–	-2.2 (0.7)	–
	non-human	26	1.2 (0.8)	-0.3 (0.6)	-0.9 (1.5)	–	-2.8 (1.1)	-3.5 (0.8)	–	–	–	-3.4 (1.2)	–
HF183 SYBR	human stool	16	5.9 (1.1)	3.7 (0.8)	3.3 (1.1)	1.4 (1.5)	0 (1.1)	-0.6 (1.1)	-2.5 (1.1)	-2 (0.2)	–	–	-1 (0.5)
	wastewater	32	–	1.3 (0.8)	1.4 (1.2)	-1.2 (0.7)	1 (1.3)	0 (1.2)	-2.1 (0.6)	-2.1 (0.3)	–	–	-1.6 (0.5)
	non-human	104	2.3 (1)	-0.2 (0.8)	-0.9 (1.4)	-3.7 (1.3)	-2.1 (1.4)	-3.2 (1.3)	-5.4 (1.9)	-5.1 (NA)	–	–	NA (NA)
HF183 Taqman	human stool	20	6.9 (0.1)	4.6 (0.2)	4.3 (0.1)	2.5 (0.6)	1 (0.1)	0.5 (0.1)	-1.1 (0.4)	–	–	–	–
	wastewater	40	–	1.7 (0.7)	2.1 (0.5)	-0.6 (0.9)	1.6 (0.6)	0.7 (0.4)	-1.8 (0.6)	–	–	–	–
	non-human	130	1.2 (0.9)	-0.5 (0.8)	-1.1 (1.5)	-4 (1.3)	-2.8 (1.4)	-3.2 (1.1)	-5.1 (2.1)	–	–	–	–
HumM2	human stool	24	5.3 (0.3)	3 (0.5)	2.6 (0.3)	0.9 (0.4)	-0.7 (0.3)	-1.2 (0.3)	-2.7 (0.3)	–	–	–	–
	wastewater	48	–	0.5 (0.7)	0.8 (0.4)	-2.3 (1)	0.4 (0.5)	-0.5 (0.5)	-3 (0.8)	–	–	–	–
	non-human	156	0.8 (0.9)	-1.1 (0.7)	-0.9 (0.8)	-3.7 (1.3)	-2.6 (1.2)	-3.2 (0.7)	-6.2 (1.9)	–	–	–	–
Mnif	human stool	20	5.7 (0.5)	3.3 (0.7)	3.1 (0.5)	0.8 (0.5)	-0.3 (0.5)	-0.8 (0.5)	-2.5 (0.5)	–	–	–	–
	wastewater	40	–	0.1 (0.9)	1.5 (1)	-3.3 (0.6)	1.2 (1.1)	-0.1 (0.9)	-3.5 (0.6)	–	–	–	–
	non-human	130	3.4 (1.2)	0.8 (1.3)	1.6 (2.2)	-1.9 (2.2)	-0.7 (1.9)	-1.3 (1.7)	-4.5 (1.3)	–	–	–	–

5. Abundance of general qPCR gene copies per ng total DNA

Figure SI-3. General assay copy abundance normalized by total DNA mass. Each point is the median value for a given fecal source, and the bars represent the interquartile ranges (25th to 75th percentiles). Fecal sources are indicated by a unique combination of color and shape. The number in parenthesis after the assay name is the number of labs that ran the assay. The fecal *Bacteroides* qPCR assay data are excluded because no filter-matched NanoDrop total DNA measurements were available.



6. *Bacteroides* 16S rRNA gene and oligonucelotide sequence analysis

6.1. Goal: to examine each *Bacteroides* 16S rRNA gene assay's *in silico* specificity by comparing the primer and probe sequences with the human and non-human *Bacteroides* 16S rRNA reference gene sequences available in the National Center for Biotechnology Information (NCBI) database.

6.2. *Bacteroides* primer/probe sequence analysis methods

All data analyses were conducted in Geneious Pro 5.6.3 (Drummond et al. 2012). We used sequence data available from NCBI (the Gene and Nucleotide databases, accessed via Geneious on June 12, 2012) to examine the sequence specificity of the *Bacteroides* 16S rRNA gene assays. The primers and probes were aligned with 1) the reference sequences used to develop the assays, 2) the sequences corresponding to accession numbers reported in the assay developers' papers and 3) 75 other human-associated *Bacteroides* spp. 16S rRNA sequences, including those from fully sequenced genomes and ATCC reference type strains. The database was also searched for non-human fecal *Bacteroides* 16S rRNA sequences corresponding to the fecal sources used in the challenge filter samples. The primers and probes were tested for specificity against the non-human sequences, with a maximum of 2 base mismatches allowed.

6.3. Primer/probe alignment and mismatch results

The alignment of human fecal-associated *Bacteroides* 16S rRNA gene sequences submitted by the assay developers' labs with the related primers and probes reveal a fair amount of overlap in the amplicon regions of these assays (Figure SI-4). For example, the positions of the BacHum-UCD and forward and reverse primers are nearly identical to the HF183 SYBR primers. Indeed, the following primer/probes overlap one another within the same 71-bp region: BacH-pC, BacH-pT, HF183F, BacHum160f, BsteriF1DE, BacHr, and BacHum193p (see Table SI-1 for oligo naming conventions, taken from the original publication of the assays whenever possible). The most unique primers and probes – those with no overlap with any other oligo in the alignment – are BacHf, BthetP1, BthetR1, and Bac708R. While previously the *Bacteroides* species associated with the HF183 marker was unknown (Bernhard and Field, 2000a), it now appears that all of the above assays except BsteriF1 target *B. dorei* (Figure SI-4).

The sequence specificity of each primer and probe was tested against all available non-human fecal-associated *Bacteroides* 16S rRNA gene sequences (n = 645) that corresponded to the fecal hosts in this study. These data included sequences from cow (n=40), chicken (65), dog (74), goose (110), gull (324), horse (19) and pig (13) hosts.

Note that no sequences were found for pigeon or deer. A maximum of two base pair mismatches was allowed in the analysis. The BthetP1, BthetR1, HFsybR, BacHum241r and Bac708R sequences clearly target conserved regions of the 16S gene; these oligos had *in silico* binding – often without any mismatches – in the vast majority of non-human animal sequences (summarized in Table SI-3). Thus it is the forward primers that confer human specificity for these assays (BacHum-UCD, BsteriF1, HF183 Taqman, HF183 SYBR and HF183 endpoint). The HF183F and BacHum160f primers matched only one cow sequence, with two mismatches on the 3' end. The BsteriF1 forward primer matched one cow and one dog sequence, each with one mismatch on the 3' end. The BacH assay had the most specific primer/probe set according to this analysis: the forward primer and both probes matched a few cow (n=2), dog (7) and gull (2) sequences, while the reverse primer had no nonspecific matches at all. Accordingly, BacH also had the least cross-reactivity to the non-human challenge samples. Unfortunately there were not enough fecal-associated sequences in the database to perform similar analyses for the functional gene assays (BtH, gyrB, HumM2, Mnif).

6.4. Primer/probe mismatch discussion

The most distinguishing feature of every PCR-based assay is the DNA sequences of the primers and probes, which play an important role in the host-specificity and sensitivity of the assay. All of the *Bacteroides* 16S rRNA gene-based assays in this study targeted the exact same region of the gene – originally identified by Bernhard & Field (2000b; 2000a) – and several assays shared nearly identical primer and probe regions (Figure SI-4). Given these similarities, one might expect the assays to perform in a similar, if not identical fashion, but this was not quite the case. Performance differences among nearly-identical assays can be due to annealing temperature, salt concentration, mastermix chemistry, thermalcycler platform, and so forth. The finding that *in silico* the probe and reverse primer of HF183 Taqman assay showed binding with hundreds of non-human sequences, yet was one of the most specific assays in the study, indicates that the HF183 forward primer is robustly human-associated. The conserved nature of the reverse primer and probe regions may also contribute to the increased sensitivity that HF183 enjoys.

While there were some parallels between the *in silico* sequence matching and the qPCR results (e.g. BsteriF1 and dog), we expected to see more matches between the oligonucleotides and non-human sequences given the number of hosts that showed cross-reactivity. However, this analysis was limited by the number of non-human sequences available in the NCBI database. For example, we were not able to compare any sequences from deer. This is unfortunate, as deer was a source of false-positive results for every assay, sometimes at levels equivalent to sewage or septage. Some contamination was detected in a small percentage of filter blanks and sample processing controls (see Boehm et al. In press), so it may be that the deer samples were contaminated with a human source during the sample collection or creation process. As such, it would have been ideal to sequence the amplicons from reactions that generated false positive results had we had unlimited resources.

Table SI-5. Summary of nonspecific *in silico* primer binding (≤2 mismatches) in *Bacteroides* 16S rRNA gene-based assays.

Assay	Oligonucleotide	Match
BacH	BacHf	Gull, Cow, Dog
	BacH-pC/BacH-pT	Gull, Dog, Cow
	BacHr	N/A
BacHum-UCD	BacHum160f	Cow
	BacHum193p	Pig, Gull, Goose, Dog, Cow, Chicken
	BacHum241r	Pig, Horse, Gull, Goose, Dog, Cow, Chicken
BsteriF1	BsteriF1DE	Dog, cow
	BthetP1	Pig, Horse, gull goose, dog, cow, chicken
	BthetR1	Pig, Horse, gull goose, dog, cow, chicken
HF183	HF183F	Cow
Taqman	BthetP1	Pig, Horse, gull goose, dog, cow, chicken
	BthetR1	Pig, Horse, gull goose, dog, cow, chicken
HF183 SYBR	HF183F	Cow
	HFsybR	Pig, Horse, gull goose, dog, cow, chicken
HF183	HF183F	Cow
endpoint	Bac708R	Pig, horse, gull, goose, dog, cow, chicken

Figure SI-4. Unique human-associated Bacteroides 16S rRNA gene sequences from NCBI (including assay developers' sequences and reference genomes) aligned with the primers and probes used in this study over the region of the assay amplicons. The sequence that the original HF183 assay was based on (Accession # AF233408) was used as the reference sequence in the alignment. Due to the size of this figure, it is accessible as a pdf using the link below.

ftp://ftp.sccwrp.org/pub/download/DOCUMENTS/AnnualReports/2013AnnualReport/ar13_445_459SI_FigureSI_4.pdf

7. Positive and negative predictive values

In addition to sensitivity and specificity, positive and negative predictive values (PPV and NPV) were calculated under both DNQ classifications each assay (singletons only). PPV and NPV were defined as: PPV = true positives/(true positives + false positives) and NPV = true negatives/(true negatives + false negatives). All three target sources (human stools, sewage and septage) were counted as true positives. These metrics responded similarly to the different classifications of DNQ samples as sensitivity and specificity (Table SI-4). Applying the same 80% criteria as used for sensitivity and specificity, only HF183 endpoint met the benchmark for PPV with DNQ positive. However, with DNQ negative, five assays met the 80% mark for PPV: BtH, gyrB, HF183 SYBR, HF183 Taqman, and HumM2. All assays met or exceeded 80% for NPV under both DNQ classifications. It is important to recognize that the PPV and NPV metrics are valid only for this dataset and cannot be extrapolated to environmental samples, because our challenge filter set does not reflect the prevalence of human fecal contamination found in the environment (Altman and Bland 1994).

Table SI-6. Positive and negative predictive values of the singletons calculated with DNQ samples treated as positive or negative.

Assay	n	Positive predictive value		Negative predictive value	
		DNQ+	DNQ-	DNQ+	DNQ-
BacH	38	67%	69%	100%	88%
BacHum-UCD	228	41%	58%	97%	98%
BsteriF1	152	45%	53%	100%	97%
BtH	38	50%	92%	100%	96%
gyrB	38	50%	86%	94%	81%
HF183 endpoint	266	90%	NA	89%	NA
HF183 SYBR	152	68%	80%	100%	96%
HF183 Taqman	190	46%	84%	100%	98%
HumM2	228	63%	84%	96%	86%
Mnif	190	53%	54%	87%	80%

8. References

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