Development and Evaluation of Mock Microbial Community Standards based on DNA Extractability from Bacteria

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Background and significance

The complexities involved in 16S rRNA-based and metagenomics analysis methods pose significant challenges for standardization as bias can be introduced during DNA extraction, amplification, library preparation, sequencing, and bioinformatics analysis. One of the primary challenges in assay standardization is the limited availability of reference materials. To address this issue, we evaluated the use of two mock microbial communities in the form of lyophilized, whole-cell standards as full-process controls.

ATCC® Microbiome Standards

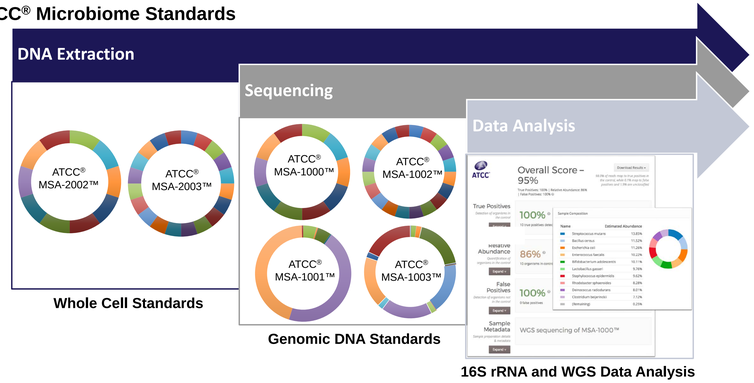
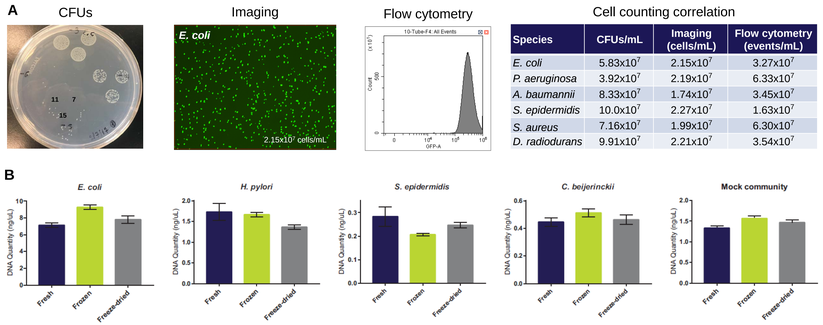


Table 1: Individual bacterial strains within the ATCC® Microbiome Standards

ATCC® No.	Name	Gram Stain	% GC	Genome Size (Mb)	Special Features	Microbiome	16S rRNA Copies	GenBank ID
10987™	*Bacillus cereus*	+	35.2	5.42	Endospores former	Soil	12	NC_003909.8
15703™	*Bifidobacterium adolescentis*	+	59.2	2.09	Anaerobe	Gut	5	NC_008618.1
35702™	*Clostridium beijerinckii*	+	30	6.49	Spores former	Gut/soil	14	NC_009617.1
BAA-816™	*Deinococcus radiodurans*	-	66.7	3.29	Thick cell wall	Gut/environment	7	NC_001263.1
47077™	*Enterococcus faecalis*	+	37.5	3.36	Biofilm producer	Gut	4	NC_017316.1
700926™	*Escherichia coli*	-	50.8	4.64	Facultative anaerobe	Gut	7	NC_000913.3
33323™	*Lactobacillus gasseri*	+	35.3	1.89	Nuclease producer	Vaginal/gut	6	NC_008530.1
17029™	*Rhodobacter sphaeroides*	-	68.8	4.60	Metabolically diverse	Aquatic	3	NZ_AKVW01000001.1
12228™	*Staphylococcus epidermidis*	+	31.9	2.56	Thick cell wall	Skin/mucosa	5	NC_004461.1
700610™	*Streptococcus mutans*	+	36.8	2.03	Facultative anaerobe	Oral	5	NC_004350.2
17978™	*Acinetobacter baumannii*	-	39	4.34	Filaments, capsule	Environment	6	NZ_CP009257.1
17982™	*Actinomyces odontolyticus*	+	65.5	2.39	Type 1 fimbriae	Oral	2	NZ_DS264586.1
8482™	*Bacteroides vulgatus*	-	42.2	5.16	Anaerobe	Gut	7	NC_009614.1
700392™	*Helicobacter pylori*	-	38.9	1.67	Helix Shaped	Stomach/gut	2	NC_000915.1
BAA-335™	*Neisseria meningitidis*	-	51.5	2.27	Diplococcus	Respiratory tract	4	NC_003112.2
33277™	*Porphyromonas gingivalis*	-	48.4	2.35	Anaerobe, collagenase	Oral	4	NC_010729.1
11828™	*Propionibacterium acnes*	+	60	2.56	Aerotolerant anaerobe	Skin	4	NC_006085.1
9027™	*Pseudomonas aeruginosa*	-	66.6	6.26	Facultative anaerobe	Skin	4	NC_009656.1
BAA-1556™	*Staphylococcus aureus*	+	32.8	2.82	Thick cell wall	Skin/respiratory	6	NC_007795.1
BAA-611™	*Streptococcus agalactiae*	+	35.6	2.16	Serogroup B	Vaginal/environment	7	NC_004116.1

Development of whole cell microbiome standards



Cell counting correlation			
Species	CFUs/mL	Imaging (cells/mL)	Flow cytometry (events/mL)
E. coli	$5.83x10^7$	$2.15x10^7$	$3.27x10^7$
P. aeruginosa	$3.92x10^7$	$2.19x10^7$	$6.33x10^7$
A. baumannii	$8.33x10^7$	$1.74x10^7$	$3.45x10^7$
S. epidermidis	$10.0x10^7$	$2.27x10^7$	$1.63x10^7$
S. aureus	$7.16x10^7$	$1.99x10^7$	$6.30x10^7$
D. radiodurans	$9.91x10^7$	$2.21x10^7$	$3.54x10^7$

Figure 1. Development of ATCC® Microbiome Standards. A) To ensure the correct distribution of strains in each of the whole cell standards, we evaluated the quantification of representative bacteria by comparing three methods - colony forming units (CFUs), imaging, and flow cytometry using the SYTO 9 green fluorescent nucleic acid stain. Based on the correlation of the data obtained from these methods, the image cytometry method was selected for the quantification of the whole cell standards. B) Comparison of different preservation methods (live, frozen, and lyophilized whole cells) on DNA extractability. Given the insignificant differences among the methods tested, lyophilization was selected as the method of choice due to the shipping and storage convenience for whole cell microbiome standards.

Evaluation of DNA extraction efficiency from individual bacteria

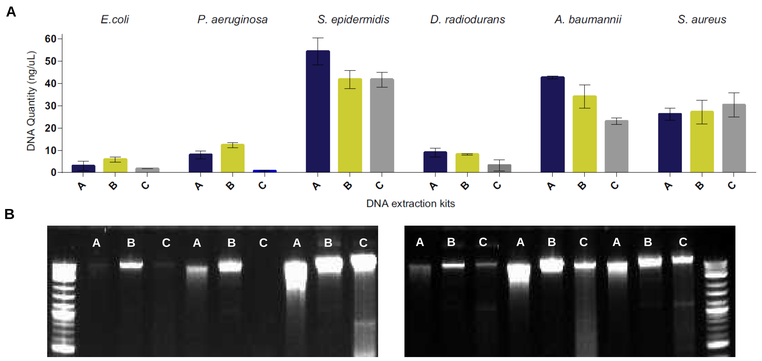


Figure 2. Evaluation of DNA extraction efficiency using individual bacterial strains. A) Quantity of DNA extracted from individual bacterial cultures using three commercial DNA extraction kits commonly used in microbiome research. B) A significant variation in the quantity and quality of DNA recovered was observed among various Gram-negative and Gram-positive strains with the same starting number of bacteria quantified using image cytometry.

Evaluation of DNA extraction efficiency from whole cell mock communities

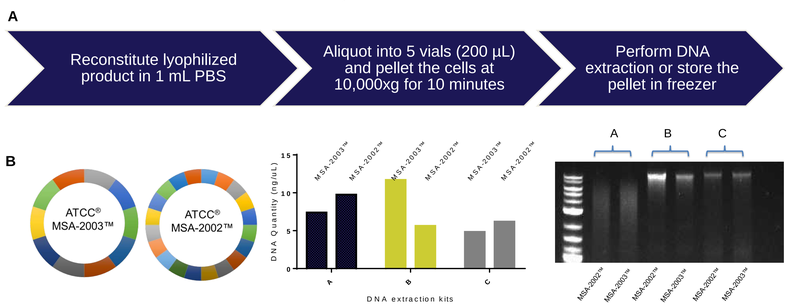
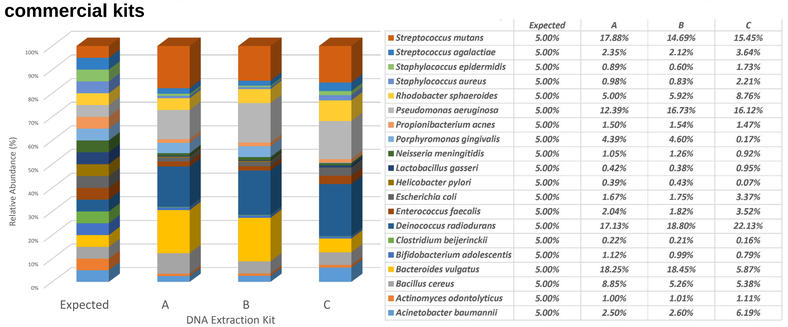


Figure 3. ATCC® Microbiome Standards can help identify bias during DNA extraction. A) Workflow for processing of whole cell mock communities. B) DNA from the whole cell standards (ATCC® MSA-2003™ and ATCC® MSA-2002™) was extracted using three commercial kits and the quality and quantity was assessed.

Community profiling of whole cell mock communities after DNA extraction using three commercial kits



	Expected	A	B	C
Streptococcus mutans	5.00%	17.88%	14.69%	15.45%
Streptococcus agalactiae	5.00%	2.35%	2.12%	3.64%
Staphylococcus epidermidis	5.00%	0.89%	0.60%	1.73%
Staphylococcus aureus	5.00%	0.98%	0.83%	2.21%
Rhodobacter sphaeroides	5.00%	5.00%	5.92%	8.76%
Pseudomonas aeruginosa	5.00%	12.39%	16.73%	16.12%
Propionibacterium acnes	5.00%	1.50%	1.54%	1.47%
Porphyromonas gingivalis	5.00%	4.39%	4.60%	0.17%
Neisseria meningitidis	5.00%	1.05%	1.26%	0.92%
Lactobacillus gasseri	5.00%	0.42%	0.38%	0.95%
Helicobacter pylori	5.00%	0.39%	0.43%	0.07%
Escherichia coli	5.00%	1.67%	1.75%	3.37%
Enterococcus faecalis	5.00%	2.04%	1.82%	3.52%
Deinococcus radiodurans	5.00%	17.13%	18.80%	22.13%
Clostridium beijerinckii	5.00%	0.22%	0.21%	0.16%
Bifidobacterium adolescentis	5.00%	1.12%	0.99%	0.79%
Bacteroides vulgatus	5.00%	18.25%	18.45%	5.87%
Bacillus cereus	5.00%	8.85%	5.26%	5.38%
Actinomyces odontolyticus	5.00%	1.00%	1.01%	1.11%
Acinetobacter baumannii	5.00%	2.50%	2.60%	6.19%

Figure 4. Community profiling of whole cell mock communities. Relative abundance data from shotgun metagenomic sequencing performed on DNA extracted from three different commercial DNA extraction kits. The data was analyzed in One Codex.

Running the control analysis in One Codex

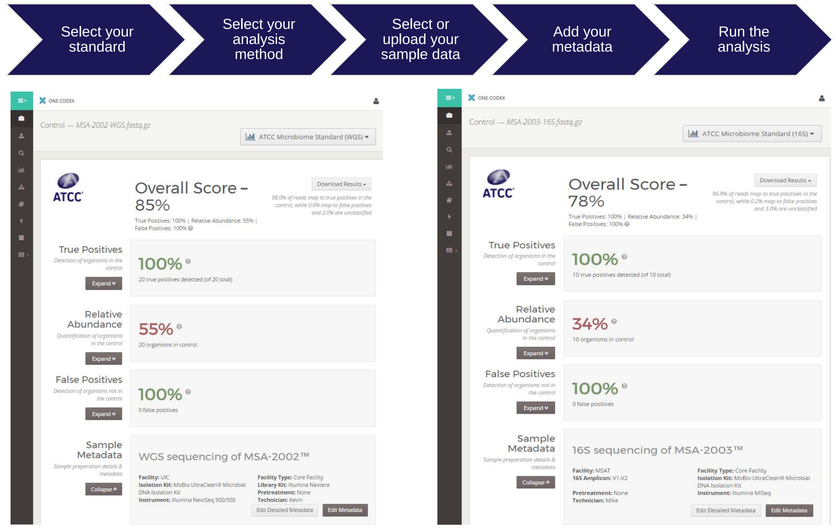


Figure 5. Analyzing data using the One Codex cloud-based web interface. The One Codex platform specifically developed for ATCC® Microbiome Standards can be used to evaluate the number of true-positive, relative abundance, and false-positive scores for 16S rRNA and WGS sequencing methods. The screenshots for shotgun metagenomic sequencing data from ATCC® MSA-2002™ and for 16S data from ATCC® MSA-2003™ are shown. The DNA was extracted using the UltraClean® Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc).

Table 2: Shotgun and 16S Sequencing Data for ATCC whole cell mock communities

ATCC® No.	Analysis type	Laboratory	True positives (Detected/input)	Relative ratio (Correlation coefficient)	False positives (100 – false + penalty)	Overall score (Average of 3 sub-scores)
MSA-2003™ (Lot # 70003364)	Shotgun	Lab A	100%	11%	100%	70%
		Lab B	100%	28%	100%	76%
	16S (V1/V2)	Lab A	100%	34%	100%	78%
		Lab B	100%	29%	99%	76%
MSA-2002™ (Lot # 70003365)	Shotgun	Lab A	100%	30%	100%	77%
		Lab B	100%	55%	100%	85%
	16S (V1/V2)	Lab A	100%	51%	100%	84%
		Lab B	100%	48%	100%	83%

Summary and Conclusions

Given the variability in the DNA extractability from Gram-positive and Gram-negative bacteria, as well as the differences observed between commercial microbiome DNA extraction kits, it is important that reference materials are used to promote standardization. Here, we present the development and evaluation of lyophilized mock microbial communities comprising a diverse bacterial mixture for use as reference materials for DNA extraction, which is the first and foremost step in any microbiome analysis or general molecular diagnostics methodology.

Acknowledgements

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