High-throughput profiling of urinary tract microbiota using TaqMan Assays

In this report, we show that:

- Applied Biosystems™ TaqMan® Assays for urinary tract microbiota have been extensively tested to meet rigorous performance criteria while maintaining a low cost per sample
- Applied Biosystems™ OpenArray™ technology can be used to simultaneously investigate a wide range of pathogenic microbes involved in urinary tract infections that can be missed or hard to grow using traditional methods
- The OpenArray platform, when combined with TaqMan Assays and the Thermo Scientific™ KingFisher™ Flex automated sample preparation system, provides a simple and straightforward workflow with minimal hands-on time

Introduction

Each year, around 150 million people are affected by symptomatic or asymptomatic urinary tract infections (UTIs), which could present serious health issues. Currently, UTIs are diagnosed by clinical symptoms and urine analysis (bacterial culture and the presence of white blood cells), and are often treated with antibiotics. However, the urinary tract hosts a diverse and complex microbial community, and some of those organisms can be difficult to culture. Emerging evidence shows that urinary tract microbiota may exert profound effects on urinary tract health, both positive and negative. Traditional culture-based or microscopy-based methods for investigation of urinary tract microbiota can lack sensitivity and specificity; they can also be subjective, time-consuming, and inaccurate.

Real-time PCR can detect slow-growing or difficult-to-cultivate microorganisms, and can be used when traditional microbiological techniques lead to ambiguous results. Utilizing real-time PCR techniques along with microorganism-specific TaqMan Assays enables rapid and accurate detection and categorization of microorganisms involved in urinary tract microbiome composition and dynamics. Housing many of these TaqMan Assays on a single panel provides a molecular approach to detecting the presence of specific species and indicating the overall status of urinary tract microbiota. With this knowledge, appropriate courses of action can be designed.

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We have developed a flexible, low-cost, high-throughput real-time PCR testing solution for urinary tract microbiota profiling. This solution includes a single protocol optimized for DNA isolation from different types of urinary tract microbes, a set of verified TaqMan Assays covering 17 pathogenic and commensal microbes, plus control assays for human RNase P and xeno DNA targets (Table 1).

Materials and methods

Optimized DNA isolation from urinary tract microbiota

A single method for nucleic acid extraction from multiple microbes (gram-positive and gram-negative bacteria, and fungi) was developed using the Applied Biosystems™ MagMAX™ DNA Multi-Sample Ultra Kit. The workflow is compatible with BD Vacutainer™ urine collection cups and tubes. DNA can be isolated from 96 samples using this optimized workflow on the Thermo Scientific™ KingFisher™ Flex Magnetic Particle Processor or the Applied Biosystems™ MagMAX™ Express-96 Magnetic Particle Processor, within 2 hours, with 30 minutes of hands-on time.

High-throughput detection of urinary tract microbiota on the OpenArray platform

OpenArray technology is a high-throughput, flexible-format, nanofluidic real-time PCR system that utilizes a microscopic-scale stainless-steel plate with 3,072 wells for individual 33 nL reactions where TaqMan Assays are spotted according to customer specifications.

Purified DNA preparations from urinary tract samples were transferred to OpenArray plates using the automated Applied Biosystems™ OpenArray™ AccuFill™ system.

The urinary tract microbiota workflow on the OpenArray system allows for analysis of up to 192 samples for 17 distinct microbial species in a single 2-hour qPCR run with 30 minutes of hands-on time. Users can go from sample to data in less than 6 hours using the complete workflow solution for detection and characterization of urinary tract microbiota (Figure 1).

Please refer to the Urinary Tract Microbiota Profiling Experiments Application Guide (Pub. No. MAN0017750) for detailed workflow information.

Table 1. Targets of the TaqMan OpenArray urinary tract microbiota assay panel.

Classification	Organism name
Bacteria	*Acinetobacter baumannii*
	Citrobacter freundii
	Enterobacter aerogenes
	Enterobacter cloacae
	Enterococcus faecalis
	Enterococcus faecium
	Escherichia coli
	Klebsiella oxytoca
	Klebsiella pneumoniae
	Morganella morganii
	Proteus mirabilis
	Proteus vulgaris
	Providencia stuartii
	Pseudomonas aeruginosa
	Staphylococcus saprophyticus
	Streptococcus agalactiae (group B)
Yeast	*Candida albicans, RPPH1* gene
Control assays	Human RNase P, *RPPH1* gene
	Xeno DNA

Figure 1. Complete solution for profiling urinary tract microbiota using TaqMan urinary tract microbiota assays on the OpenArray platform.

Results

Sensitivity and linear dynamic range of TaqMan urinary tract microbiota assays

Assessment of the sensitivity, efficiency, and linear dynamic range of TaqMan urinary tract microbiota assays was conducted by serial dilution analysis of the multitarget Applied Biosystems™ TaqMan® Urinary Tract Microbiota Amplification Control, a plasmid DNA control containing all assay target sequences. This control ensures the stoichiometric equivalence of all of the target DNA sequences. Real-time PCR was performed on the amplification control without sample preparation.

The amplification control dilution series, which ranged from 10^7 copies/µL to 10^2 copies/µL, was tested on an OpenArray plate containing research assays for all 17 urinary tract microbiota targets plus control assays; 3 technical replicates were generated per sample–assay combination.

For all of the assays, sensitivity was achieved down to 100 copies/µL with little variation at lower concentrations (R^2 >0.99). The sensitivity and 5-log linear dynamic range for 9 representative assays are displayed in Figure 2.

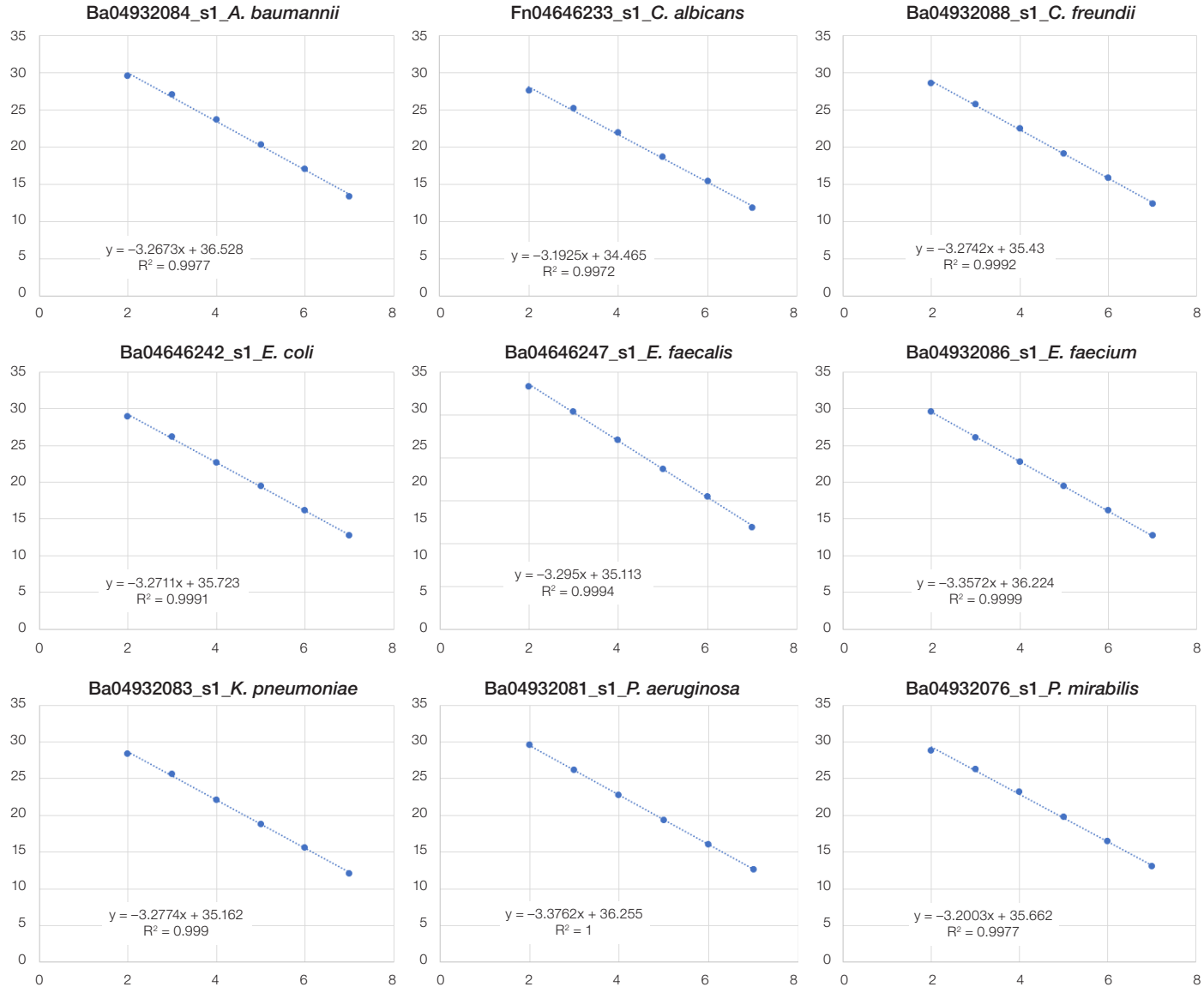


Figure 2. Dynamic range of TaqMan urinary tract microbiota assays on an OpenArray platform. Ten-fold serial dilutions were prepared with the TaqMan Urinary Tract Microbiota Amplification Control, spanning 5 orders of magnitude from 10^7 to 10^2 copies/µL. PCR reactions were prepared by mixing 2.5 µL of diluted plasmid with 2.5 µL of Applied Biosystems™ TaqMan® OpenArray™ Real-Time PCR Master Mix for each subarray. Each subarray was spotted with 3 replicates of all 17 assays. Data from 9 representative assays are shown in scatter plots, where the x-axis is $\log_{10}$ of plasmid concentration (copies/µL) and the y-axis shows the real-time PCR's mean relative threshold cycle (C_{rt}) values obtained at each concentration. All assays were able to detect 100 copies/µL, with at least 5 orders of magnitude of linearity where R^2 is greater than 0.99.

The LOD was determined for PCR reactions on the OpenArray platform by testing low concentrations of a pool of genomic DNA (ATCC) from all targets. LOD testing was performed using 10-fold serial dilutions from 10^5 copies/µL down to 10^2 copies/µL in the PCR reactions. The average LOD of all of our urinary tract microbiota assays on the OpenArray platform is at least 100 copies/µL as shown in Figure 3. The LOD of all of our assays was determined without preamplification of the samples. Preamplification is an optional step that potentially helps to increase the sensitivity of the assay, but it is not required given that these organisms are typically observed at sufficient titer in urine samples.

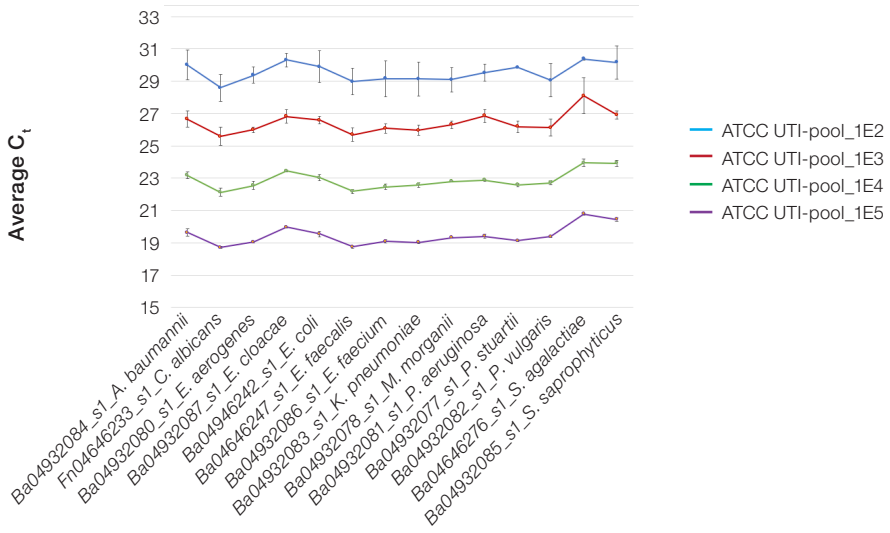


Figure 3. LOD for genomic DNA. Serial dilutions of 10^5 copies/µL down to 10^2 copies/µL were prepared using a pool of genomic DNA samples (ATCC). The pool contained genomic DNA from targets. For each concentration, 2.5 µL of the genomic DNA pool was mixed with 2.5 µL of OpenArray Real-Time PCR Master Mix, and loaded onto each subarray of an OpenArray plate on which all the urinary tract microbiota assays were spotted. A total of 4 replicates were run on an OpenArray plate for each concentration. All 4 replicates were able to detect approximately 100 copies/µL, although with a higher standard deviation of replicates than at higher target concentration.

Optimal specificity of TaqMan urinary tract microbiota assays

The TaqMan urinary tract microbiota assays have undergone rigorous bioinformatic analysis to help ensure maximum strain coverage while minimizing the potential for off-target cross-reactivity.

Each assay has been tested with on- and off-target DNA isolated from target organisms in our inclusivity panel (ATCC) (Table 2) to evaluate accuracy, specificity, reproducibility, and sensitivity. All 17 urinary tract microbiota assays provided highly specific results when screened simultaneously against gDNA from all 17 target microbes on an OpenArray plate (Table 3).

Table 2. Panels of ATCC genomic DNA (gDNA) used for inclusivity and exclusivity testing. gDNA isolated from 17 target and 17 nontarget strains was used to test the 17 urinary tract microbiota assays for specificity—**(A)** inclusivity panel of target organisms and **(B)** exclusivity panel of nontarget organisms.

A.	Target name	ATCC ID	B.	Target name	ATCC ID
1	*Acinetobacter baumannii*	BAA-1710D-5	1	*Acinetobacter bereziniae*	17924_D2
2	*Citrobacter freundii*	8090D	2	*Citrobacter koseri*	BAA-895_D2
3	*Klebsiella oxytoca*	700324D	3	*Raoultella planticola*	33531_D2
4	*Klebsiella pneumoniae*	700603D-5	4	*Proteus hauseri*	13315_D2
5	*Morganella morganii*	35200D	5	*Proteus penneri*	35198_D2
6	*Proteus mirabilis*	12453D	6	*Moellerella wisconsensis*	35621_D2
7	*Proteus vulgaris*	6380-D2	7	*Brenneria salicis*	15712_D1
8	*Providencia stuartii*	33672D	8	*Pseudomonas denitrificans*	13867_D1
9	*Pseudomonas aeruginosa*	27853D-5	9	*Pseudomonas syringae*	11528_D1, BAA-978D, BAA-871D-5
10	*Staphylococcus saprophyticus*	15305D-5	10	*Pantoea agglomerans*	27155_D1
11	*Enterobacter cloacae* ATCC13047	13047D-5	11	*Corynebacterium glucuronolyticum*	51862_D2
12	*Enterococcus faecium* MMC4	51559D-5	12	*Enterococcus hirae*	10541D-5
13	*Enterobacter aerogenes*	13048_D1	13	*Hafnia alvei*	51873D-5
14	*Escherichia coli*	BAA-460D-5	14	*Staphylococcus epidermidis*	12228D-5
15	*Enterococcus faecalis*	700802D-5	15	*Streptococcus mitis*	49456
16	*Candida albicans*	110066D-5	16	*Candida dubliniensis*	MYA-646
17	*Streptococcus agalactiae*	BAA-1138D-5	17	*Salmonella enterica* subsp.	700720

Testing of nontarget organisms showed that the TaqMan urinary tract microbiota assays also demonstrated low cross-reactivity with closely related species in our exclusivity panel (Table 2B).

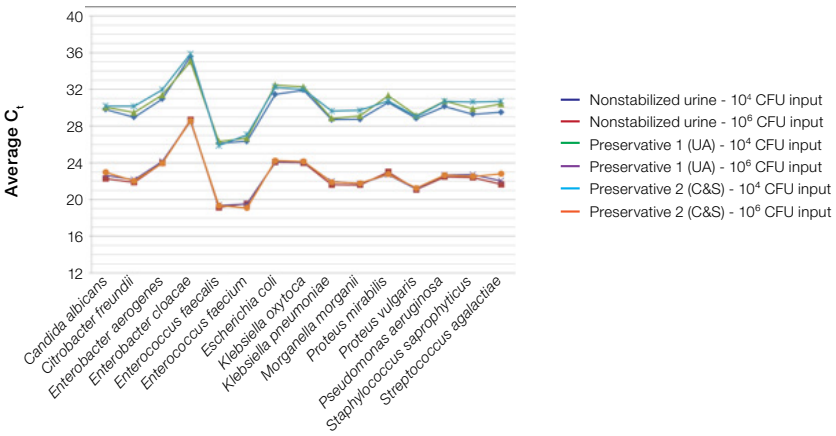


Figure 4. Detection of titered organisms added to urine samples with and without preservative. 1 mL of urine with or without a preservative was spiked with titered organisms. Results were highly reproducible when 10^6 or 10^4 CFU of each organism were added to 1 mL of sample used in the extraction.

Table 3. Specificity (inclusivity) results. gDNAs at 10^5 copies/µL from a total of 17 cultivatable microbes were screened simultaneously with the 17 target assays. The microbial gDNAs are listed in columns, and the assays tested are listed in rows. The urinary tract microbiota assays were specific and amplified only their corresponding gDNA samples (C_{rt} values from 4 replicates, in shaded cells). The data were filtered according to our recommended criteria for identification of positive amplification, namely where C_{rt} < 31, AmpScore > 1.2, and C_q confidence > 0.8 for >50% of replicates. The result for *C. freundii* in the unshaded cell shows off-target amplification in individual replicates that were not found in other replicates; the result could be caused by potential cross-reactivity or by impurities in the gDNA sample.

Assay \ Genomic DNA	*A. baum.*	*C. albi.*	*C. freu.*	*E. aero.*	*E. cloa.*	*E. coli*	*E. faeca.*	*E. faeci.*	*K. oxyt.*	*K. pneu.*	*M. morg.*	*P. aeru.*	*P. mira.*	*P. stua.*	*P. vulg.*	*S. agal.*	*S. sapr.*
Ba04932084_ s1_ A. baumannii	20.01																
Fn04646233_s1_ C. albicans		18.80															
Ba04932088_ s1_ C. freundii			19.95														
Ba04932080_ s1_ E. aerogenes				19.53													
Ba04932087_s1_ E. cloacae					19.80												
Ba04646242_ s1_ E. coli						19.84											
Ba04646247_s1_ E. faecalis							18.87										
Ba04932086_ s1_ E. faecium								19.49									
Ba04932079_ s1_ K. oxytoca									20.96								
Ba04932083_ s1_ K. pneumoniae										19.13							
Ba04932078_ s1_ M. morganii											19.50						
Ba04932081_s1_ P. aeruginosa												20.60					
Ba04932076_ s1_ P. mirabilis			29.66 (3)										20.23				
Ba04932077_s1_ P. stuartii														19.42			
Ba04932082_ s1_ P. vulgaris															18.80		
Ba04646276_ s1_ S. agalactiae																20.29	
Ba04932085_ s1_ S. saprophyticus																	20.72

There was no performance difference between the nontarget organisms tested individually and in pools (data not shown). Occasionally, sporadic signals were observed, but these results did not meet criteria for positive amplification for minimum C_{rt} value or number of replicates showing amplification. Furthermore, these background C_{rt} values are not significant due to the large differences in C_{rt} values ($\Delta C_{rt} > 10$) between target and nontarget microbes.

Detection of titered organisms in urine samples

All targets were tested using urine samples spiked with 14 titered control organisms. Results were highly reproducible using 10^6 or 10^4 colony-forming units (CFU) of input into 1 mL of sample used in the extraction, as shown in Figure 4. No significant difference was observed for urine with or without preservative.

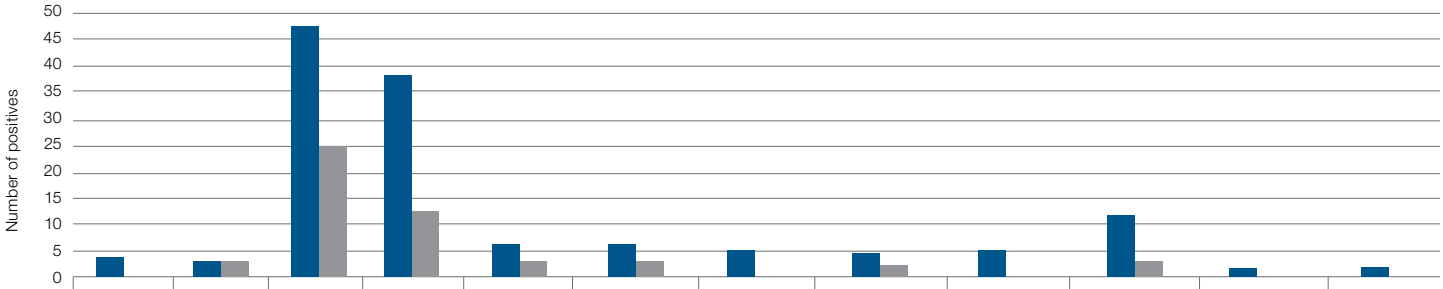
Accurate identification of urinary tract microbes using the complete workflow

To further test sensitivity, specificity, and accuracy, cultured samples consisting of 40 positives and 30 negatives (i.e., no-growth culture results), were tested using the TaqMan urinary tract microbiota panel workflow. The gDNA was extracted using the MagMAX DNA Multi-Sample Ultra Kit on the KingFisher Flex system, then analyzed by qPCR on the OpenArray plates containing the TaqMan urinary tract microbiota panel assays. Organisms detected in the 40 positive samples using culture methods were also detected using the OpenArray panel (Figure 5).

The TaqMan urinary tract microbiota assays combined with the OpenArray panel were able to detect additional targets that did not grow in the culture.

For example, mixed flora of >100,000 CFU/mL was reported for samples where individual organisms could not be identified. However, the species present were readily identified within mixed-flora samples using the TaqMan urinary tract microbiota assays with the OpenArray panel (Table 4).

A subset of 7 samples from the pool were selected for Sanger sequencing to provide additional support for the presence of targets detected by qPCR but not in culture. Sequencing data were 100% concordant with OpenArray urinary tract microbiota panel results. This indicates that the OpenArray panel is more sensitive than culture-based detection (Table 5). OpenArray results agreed with 28 of 30 negatives, or samples showing no growth. The two discrepant no-growth samples (samples 18 and 20 in Table 5) were subjected to Sanger sequencing, which confirmed targets detected by qPCR.



	C. albicans	*C. freundii*	*E. coli*	*E. faecalis*	*K. oxytoca*	*K. pneumoniae*	*M. morganii*	*P. aeruginosa*	*P. mirabilis*	*S. agalactiae*	*C. parapsilosis*	*C. tropicalis*
qPCR	3	1	48	38	7	7	5	4	5	12	1	1
Culture	—	1	25	13	2	2	—	1	—	3	—	—

Figure 5. Detection of organisms in urine samples using the complete workflow. Samples that were analyzed in culture were also analyzed using the TaqMan urinary tract microbiota panel workflow. DNA was isolated from 1 mL of urine and analyzed using the TaqMan urinary tract microbiota assays with the OpenArray panel. All targets detected in culture (gray) were also detected using the TaqMan urinary tract microbiota panel and qPCR (blue). Additional organisms were detected by qPCR that were either not above the detection limits in culture, or where mixed-flora results interfered with species identification, resulting in the greater target counts obtained by qPCR vs. culture.

Table 4. Multiplex screening of urinary tract microbiota on the OpenArray platform using clinical research samples. Each sample was tested at two different geographical sites on two different OpenArray panels built with the same assays. The results from the two sites were identical for all samples: the subset of samples and pathogens detected at both sites are highlighted in green, with "+" indicating presence of the uropathogen. Blank cells indicate absence of detection of the pathogen in that sample. Both sites were able to detect multiple pathogens, including fungal and bacterial targets that are difficult to culture.

		OpenArray panel result									
Research sample number	**Culture result (CFU/mL)**	***C. albicans***	***C. freundii***	***E. coli***	***E. faecalis***	***K. oxytoca***	***K. pneumoniae***	***M. morganii***	***P. mirabilis***	***S. agalactiae***	***S. aureus***
1	*E. coli* >100,000			+							
2	*E. coli* >100,000 *E. faecalis* 50,000–100,000			+	+	+					
3	*E. faecalis* >100,000				+		+				
4	*E. coli* >100,000 *E. faecalis* 10,000–50,000			+	+		+		+		
5	*K. pneumoniae* >100,000						+				
6	*K. oxytoca* >100,000					+	+				
7	Coagulase-negative staph >100,000 *P. pentosaceus* >100,000								+	+	
8	*E. coli* >100,000			+				+		+	
9	*E. coli* 10,000–50,000		+	+							
10	No significant growth <10,000			+						+	
11	Mixed flora >100,000			+						+	
12	Mixed flora >100,000			+	+				+	+	
13	Mixed flora >100,000			+	+		+	+	+		
14	No data	+			+						
15	No data			+	+	+					
16	Mixed flora >100,000			+	+					+	
17	No growth (negative)										
18	No growth									+	
19	Mixed flora >100,000			+	+						
20	No growth										+

Table 5. Sanger sequencing of culture-tested samples to evaluate OpenArray results. Sequencing was performed on extracted DNA that was obtained using the OpenArray sample preparation method for 7 samples with no growth, mixed flora, or no data from culture tests. (In the case of a mixed-flora result, the culture method cannot distinguish between different organisms.) Sequencing data confirming targets detected by qPCR indicate that the OpenArray panel can be used to accurately identify the correct pathogens with greater sensitivity than that obtained by the culture methods.

Research sample number	Culture result (CFU/mL)	Target analyzed by Sanger sequencing	Detected by qPCR	Confirmed by Sanger sequencing
13	Mixed flora >100,000	*K. pneumoniae*	Yes	Yes
		P. mirabilis	Yes	Yes
		E. faecalis	Yes	Yes
14	No data	*E. faecium*	Yes	Yes
		C. albicans	Yes	Yes
15	No data	*K. oxytoca*	Yes	Yes
		K. pneumoniae	No	Yes (not detected)
		E. coli	Yes	Yes
16	Mixed flora >100,000	*E. faecalis*	Yes	Yes
		S. agalactiae	Yes	Yes
18	No growth	*S. agalactiae*	Yes	Yes
19	Mixed flora >100,000	*E. coli*	Yes	Yes
		E. faecalis	Yes	Yes
20	No growth	*S. aureus*	Yes	Yes

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Conclusions

- Our real-time PCR solution for urinary tract microbiota profiling enables an accurate and reliable molecular profiling workflow, covering a broad range of commensal and pathogenic urinary tract microbes.
- The TaqMan urinary tract microbiota assays demonstrate accurate, highly reproducible performance in numerous tests for sensitivity and specificity in the presence or absence of preservative.
- The assays performed well with DNA isolated from urinary tract research samples in a widely used collection medium, using optimized protocols for the MagMAX DNA Multi-Sample Ultra Kit.
- The qualified TaqMan urinary tract microbiota assays in combination with the flexible, high-throughput nanofluidic OpenArray format provide an accurate and low-cost solution for detecting and characterizing urinary tract microbiota.

Ordering information

Product	Quantity	Cat. No.
TaqMan assays and OpenArray plates		
TaqMan OpenArray Urinary Tract Microbiota Comprehensive Plate	1 plate	A39900
Controls and master mixes		
TaqMan OpenArray Real-Time PCR Master Mix	1 x 1.5 mL	4462159
TaqMan OpenArray Real-Time PCR Master Mix	1 x 5 mL	4462164
TaqMan Urinary Tract Microbiota Amplification Control	1 tube (5 x 50 µL)	A39174
TaqMan Universal DNA Spike-In Control	1,000 µL	A39175
Instrumentation and sample preparation		
QuantStudio 12K Flex Real-Time PCR System, with OpenArray block	1 system	4472380
QuantStudio 12K Flex AccuFill System	1 system	4471021
KingFisher Flex Purification System	1 system	5400620
MagMAX DNA, Multi-Sample Ultra Kit	500 preps	A25597
MagMAX Viral/Pathogen Ultra Nucleic Acid Extraction Kit	100 preps	A42356

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