High-throughput OpenArray solution for vaginal microbiota profiling using TaqMan Assays

In this report, we show that:

- Applied Biosystems™ TaqMan® Assays for vaginal microbiota have been extensively tested to meet rigorous performance criteria
- Applied Biosystems™ OpenArray™ technology allows you to build your own panel of TaqMan Assays that can be used to study a wide range of both pathogenic and commensal microbes at a low cost per sample
- The OpenArray platform, when combined with TaqMan Assays and the Thermo Scientific™ KingFisher™ Flex automated sample preparation system, provides a simple and easy-to-use workflow with minimal hands-on time

Introduction

Traditional culture-based or microscopy-based methods for investigations into vaginal microbiota can lack sensitivity and specificity and also can be subjective, time-consuming, and inaccurate.

Real-time PCR can detect slow-growing, difficult-to-cultivate, or uncultivable 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 bacterial vaginosis, aerobic vaginitis, vaginal candidiasis, and sexually transmitted infections.

We have developed a flexible, low-cost, and high-throughput real-time PCR testing solution for vaginal microbiota profiling. This solution includes a single protocol optimized for DNA isolation from different types of vaginal microbes, a set of validated TaqMan Assays covering 34 pathogenic and commensal microbes, plus control assays for human (RNase P) and bacterial (16S) targets (Table 1).

Table 1. Categorization of microorganisms found in vaginal microbiota.

Organism coverage	Organism name
Bacterial vaginosis	*Atopobium vaginae*
	Bacteroides fragilis
	BVAB2*
	Gardnerella vaginalis
	Prevotella bivia
	*Megasphaera 1**
	*Megasphaera 2**
	Mobiluncus curtisii
	Mobiluncus mulieris
	Mycoplasma hominis
	*Ureaplasma urealyticum***
Aerobic vaginitis	*Staphylococcus aureus*
	Streptococcus agalactiae (group B)
	Enterococcus faecalis
	Escherichia coli
Sexually transmitted infections	*Chlamydia trachomatis*
	Haemophilus ducreyi
	Neisseria gonorrhoeae
	Trichomonas vaginalis
	*Treponema pallidum***
	Herpes simplex virus 1
	Herpes simplex virus 2
	*Mycoplasma genitalium***
Candidiasis	*Candida albicans*
	Candida dubliniensis
	Candida glabrata
	Candida krusei
	Candida lusitaniae
	Candida parapsilosis
	Candida tropicalis
Commensal lactobacilli	*Lactobacillus crispatus*
	Lactobacillus gasseri
	Lactobacillus iners
	Lactobacillus jensenii
Control assays	Pan-bacterial 16S
	Human RNase P

* Bacterial vaginosis–associated bacterium type 2 (BVAB2)—uncultured microbes.
** Hard-to-culture microbes.

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Materials and methods

Optimized DNA isolation from vaginal microbiota using KingFisher Flex Magnetic Particle Processor

A single method for nucleic acid extraction and isolation was developed for different microbes (bacteria, fungi, protozoa, and viruses) using the Applied Biosystems™ MagMAX™ DNA Multi-Sample Ultra Kit. The workflow is compatible with a wide variety of sample collection systems. The DNA yields using the vaginal microbiota protocol from a known amount of spiked-in organisms in different collection media were highly comparable. DNA isolation of 96 samples can be achieved 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 vaginal microbiota detection on the OpenArray platform

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

Purified DNA preparations from vaginal samples were transferred to OpenArray plates using an automated Applied Biosystems™ OpenArray™ AccuFill™ system. The vaginal microbiota workflow on the OpenArray system allows users to analyze up to 192 samples for 34 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 vaginal microbiota (Figure 1).

Please refer to the “Vaginal Microbiota Profiling Experiments Application Guide” (MAN0015669) for detailed workflow information.

Figure 1. Complete solution for profiling vaginal microbiota using Applied Biosystems™ TaqMan® Vaginal Microbiota assays on the OpenArray platform.

Results

Sensitivity and reproducibility of TaqMan Vaginal Microbiota assays

Assessment of the sensitivity, efficiency, and linear dynamic range of TaqMan Vaginal Microbiota assays was conducted by standard curve analysis of the assay runs with the multitarget Applied Biosystems™ TaqMan® Vaginal Microbiota Amplification Control, a plasmid DNA control containing all assay target sequences. The real-time PCR was performed on the amplification control without sample preparation.

The amplification control dilution series, which ranged from 5 x 10^7 to 50 copies/µL, was tested on an OpenArray plate containing all 34 vaginal microbiota target research assays plus control assays, in 4 different subarrays; 8 technical replicates were generated per sample/assay combination.

For most of the assays, we were able to achieve sensitivity down to 1 copy/reaction (50 copies/µL in the PCR) with little variation at lower concentrations (R^2 > 0.98). The sensitivity and linear dynamic range for four different microbe types are demonstrated in Figure 2, with the standard error bars indicating low variation between replicates.

The limit of detection (LOD) was determined for two different processes: PCR reactions on the OpenArray platform, and whole cells from sample preparations. The LOD for PCR was obtained by testing low concentrations of the multitarget amplification control. The LOD for whole-cell analysis was achieved by using the enumerated target microbes that were spiked at various low concentrations into the transport media to imitate vaginal specimen samples, followed by DNA extraction using the vaginal microbiota DNA isolation procedure.

The LOD testing using the amplification control was performed using 418, 266, 152, and 76 copies/µL in the PCR. The LOD testing using the target whole-cell populations was conducted using 600, 1,000, 1,200, 2,000, 3,000, 5,000, and 10,000 enumerated cells spiked per milliliter of vaginal swab transport media, followed by DNA extraction and real-time PCR.

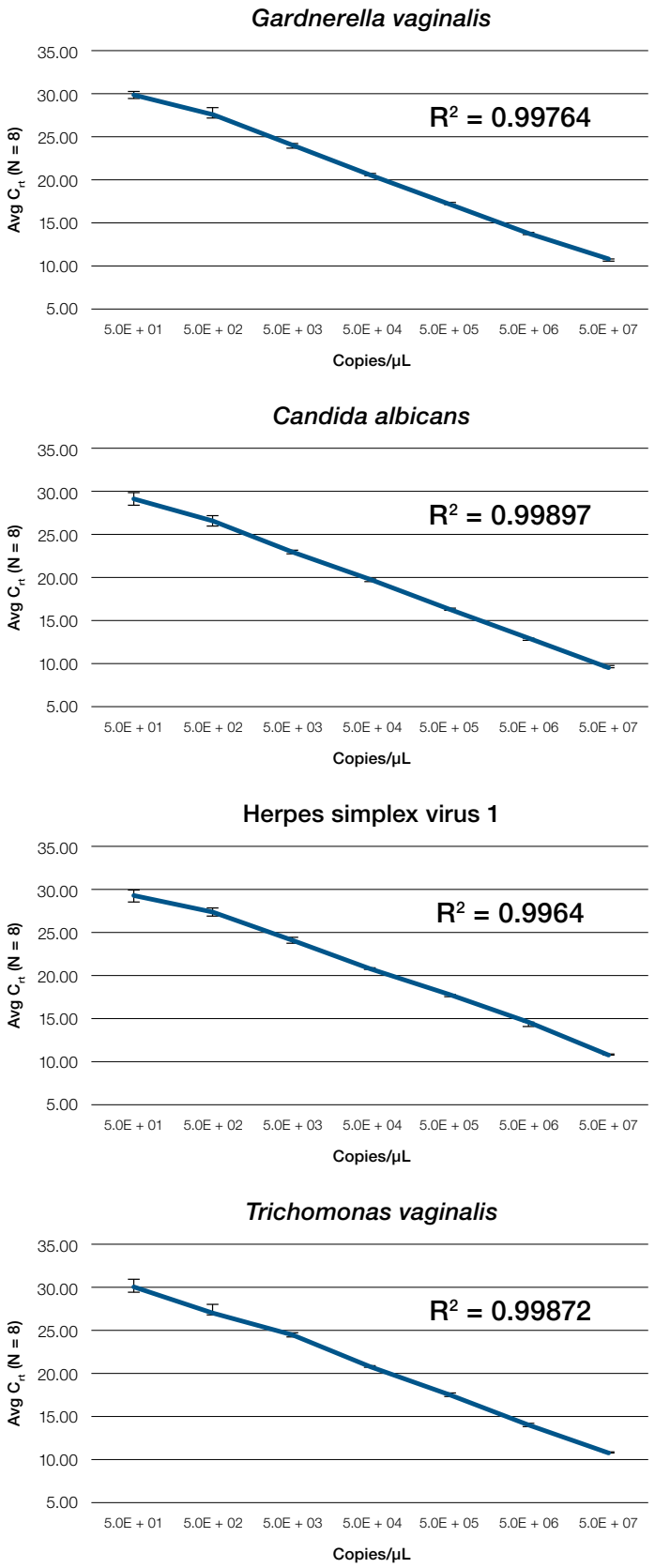

Figure 2. Results for linear dynamic range for a set of 10-fold dilutions of four different types of microbes. The types assayed were bacterium (*G. vaginalis*), fungus (*C. albicans*), virus (herpes simplex virus 1), and protozoan (*T. vaginalis*), ranging from 50 copies/µL (~1 copy/reaction) to 5 x 10^7 copies/µL (825,000 copies/reaction). All four microbe assays show R^2 > 0.99. C_{rt} = relative threshold cycle.

The average LOD for all of our vaginal microbiota assays on the OpenArray platform with >95% confidence is approximately 150 copies/µL (2.5 copies/reaction) for PCR and approximately 5,000 cells/mL for whole cells in swab transport media, prior to extraction (Table 2). Additionally, we were able to achieve reliable sensitivity below this point with high confidence and low variation among the replicates.

Table 2. LOD using amplification control for PCR and measurement of target whole cells.

Microbes	PCR LOD (copies/µL)	Measured whole-cell LOD (cells/mL)
M. hominis	152	10,000
L. jensenii	266	<1,000
S. agalactiae	152	5,000
C. albicans	76	10,000
C. krusei	152	<1,000
N. gonorrhoeae	152	<1,000
C. trachomatis	76	<1,000
T. vaginalis	152	<600
Range	76–266 copies/µL	<600–10,000 cells/sample

The LOD for all our assays was performed without preamplification of the samples. Preamplification is an optional method to potentially increase sensitivity, but given the titer that these organisms are typically observed at in vaginal samples, it appears this option is not required in this particular context.

Optimal specificity of TaqMan Vaginal Microbiota assays

The design of our TaqMan Vaginal Microbiota assays and their target sequences have undergone rigorous bioinformatics selection and analysis to ensure maximum strain coverage while minimizing the potential for off-target cross-reactivity. The TaqMan Vaginal Microbiota assays have been qualified by us—meaning they have been functionally tested against genomic DNAs (gDNAs) and synthetic controls.

Each assay has been tested with on- and off-target synthetic controls, and DNA isolated from target organisms (ATCC) and repository samples have demonstrated specificity, reproducibility, and sensitivity. All 34 vaginal microbiota assays provided highly specific results when screened simultaneously against gDNAs from 28 target microbes on OpenArray plate (Table 3). The overall specificity of the testing of gDNA with the vaginal microbiota assays was 99.7%.

Table 3. Specificity: 28 cultivatable microbial gDNAs at 10^5 copies/µL were screened simultaneously with the 34 target assays. The microbial gDNAs are listed in rows, and the organism assays tested are listed in columns. The vaginal microbiota assays were specific and amplified only their corresponding gDNA samples (real-time PCR relative threshold cycle (C_{rt}) values highlighted in green). The yellow highlighted cell shows off-target amplification in the *Candida dubliniensis* gDNA sample with the *Candida krusei* assay. Orthogonal Sanger sequencing tests confirmed the presence of *Candida krusei* gDNA in the *Candida dubliniensis* sample, demonstrating the robustness and high specificity of the vaginal microbiota assays.

Target gDNA	AV_Ba 04646222_ s1	BF_Ba 04646225_ s1	CA_Fn 04646233_ s1	CD_Fn 04646244_ s1	CG_Fn 04646240_ s1	CK_Fn 04646250_ s1	CL_Fn 04646241_ s1	CP_Fn 04646221_ s1	CT_Fn 04646220_ s1	CHT_Ba 04646249_ s1	EC_Ba 04646242_ s1	EF_Ba 04646247_ s1	GV_Ba 04646236_ s1	HD_Ba 04646228_ s1	HSV1_Vi 04230116_ s1	HSV2_Vi 04646232_ s1	LC_Ba 04646245_ s1	LG_Ba 04646234_ s1	LI_Ba 04646257_ s1	LJ_Ba 04646258_ s1	MC_Ba 04646235_ s1	MH_Ba 04646255_ s1	MM_Ba 04646246_ s1	NG_Ba 04646252_ s1	PB_Ba 04646278_ s1	SA_Ba 04646259_ s1	GBS_Ba 04646276_ s1	TV_Pr 04646256_ s1
Atopobium vaginae	18.77																											
Bacteroides fragilis		23.84																										
Candida albicans			18.60																									
Candida dubliniensis				18.74		22.8																						
Candida glabrata					20.26																							
Candida krusei						12.80																						
Candida lusitaniae							19.70																					
Candida parapsilosis								19.98																				
Candida tropicalis									18.98																			
Chlamydia trachomatis										17.97																		
Escherichia coli											19.75																	
Enterococcus faecalis												19.62																
Gardnerella vaginalis													22.05															
Haemophilus ducreyi														19.49														
HSV1															19.54													
HSV2																18.56												
Lactobacillus crispatus																	18.77											
Lactobacillus gasseri																		20.75										
Lactobacillus iners																			17.41									
Lactobacillus jensenii																				18.67								
Mobiluncus curtsii																					18.85							
Mycoplasma hominis																						18.57						
Mobiluncus mulieris																							19.41					
Neisseria gonorrhoeae																								20.23				
Prevotella bivia																									19.58			
Staphylococcus aureus																										19.33		
Streptococcus agalactiae																											19.82	
Trichomonas vaginalis																												16.18

Complete workflow for accurate and reproducible data

Accurate identification of vaginal microbes using a complete workflow

To further test sensitivity, specificity, and accuracy across different platforms, 34 Aptima™ vaginal swab samples (Hologic), which had been previously characterized for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, were processed using our vaginal microbiota sample preparation solution, and tested in parallel using 384-well and OpenArray plates run on the Applied Biosystems™ QuantStudio™ 12K Real-Time PCR System. Samples were blinded before the testing. The assay results matched well with the decoded identities of the samples (Figure 3).

Reproducibility of data

To demonstrate the reproducibility of the complete vaginal microbiota experimental workflow, each of the 10 vaginal microbiota ThinPrep™ samples (Hologic) was split in half, and each half of the sample was processed and tested at two different geographical locations. When compared, results were highly reproducible with a correlation of >0.99 for most of the samples. Results from real time PCR of two different samples are shown in Figure 4 comparing C_{rt} values for microbial DNAs detected in each sample from different testing sites.

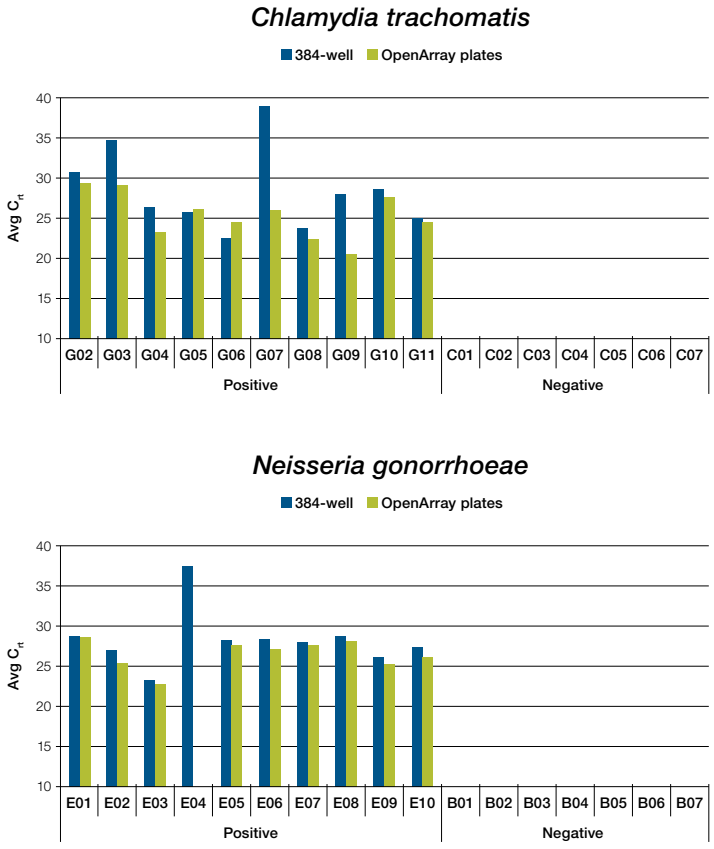

Figure 3. Accurate detection of 34 characterized vaginal swab samples for *C. trachomatis* and *N. gonorrhoeae*. As shown, 100% accuracy, specificity, and sensitivity was observed with 384-well plate testing and 100% of specificity, 99% of accuracy, and 95% (19/20) of sensitivity was observed for *C. trachomatis* and *N. gonorrhoeae* assays on OpenArray plates. The relative difference in sensitivity on the OpenArray platform is primarily due to the low amount of the sample in the 33 nL vs. 384-well plate 10 µL reactions (C_{rt} on 384-well plate is ~37, or less than 1 copy/PCR).

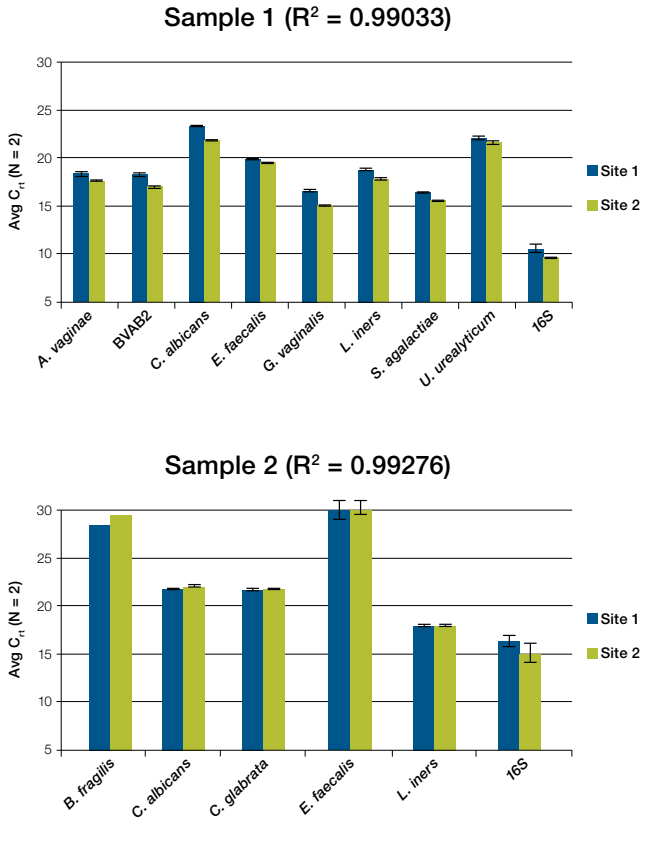

Figure 4. Complete workflow reproducibility. Each sample was split in half and was processed from DNA extraction to testing at two different sites on OpenArray plates. Results were highly reproducible ($R^2 > 0.99$) and concordant with the previous results generated on different platforms, for most of the samples.

Conclusions

- Our real-time PCR solution for vaginal microbiota profiling enables an accurate and reliable molecular profiling workflow, covering a broad range of commensal and pathogenic vaginal microbes
- The TaqMan Vaginal Microbiota assays demonstrated accurate, highly reproducible performance in numerous tests for sensitivity and specificity on different sample types
- The assays performed well with DNA isolated from vaginal research samples from widely used collection media, using optimized protocols for the MagMAX DNA Multi-Sample Ultra Kit
- The qualified TaqMan Vaginal Microbiota assays in combination with the flexible, high-throughput nanofluidic OpenArray format provide an accurate and low-cost solution for investigations to detect and characterize vaginal microbiota

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Ordering information

Product	Quantity	Cat. No.
TaqMan assays and OpenArray plates		
TaqMan OpenArray Real-Time PCR, Format 18	10 pack	4471124
TaqMan OpenArray Real-Time PCR, Format 56	10 pack	4471125
TaqMan OpenArray Real-Time PCR, Format 112	10 pack	4471126
TaqMan OpenArray Vaginal Microbiota Training Plate	1 plate	A32086
QuantStudio 12K Flex OpenArray Vaginal Microbiota Starter Kit	1 kit	A32087
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 Vaginal Microbiota Amplification Control	1 tube (20 µL)	A32040
TaqMan Vaginal Microbiota Amplification Control	1 tube (250 µL)	A32913
TaqMan Vaginal Microbiota Extraction Control	3 pellets	A32039
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

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