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Development of the iCubate Molecular Diagnostic Platform Utilizing Amplicon Rescue Multiplex Polymerase Chain Reaction

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We utilized Amplicon-Rescue Multiplex PCR (ARM-PCR) and microarray hybridization to develop and validate the iC-GPC Assay, a multiplexed, *in vitro* diagnostic test that identifies five of the most common gram positive bacteria and three clinically relevant resistance markers associated with bloodstream infections (BSI). The iC-GPC Assay is designed for use with the iC-System™, which automates sample preparation, ARM-PCR, and microarray detection within a closed cassette. Herein, we determined the limit of detection for each of the iC-GPC Assay targets to be between 3.0×10^5–1.7×10^7 CFU/mL, well below clinically relevant bacterial levels for positive blood cultures. Additionally, we tested 106 strains for assay inclusivity and observed a target performance of 99.4%. 95 of 96 non-target organisms tested negative for cross-reactivity, thereby assuring a high level of assay specificity. Overall performance above 99% was observed for iC-GPC Assay reproducibility studies across multiple sites, operators and cassette lots. In conclusion, the iC-GPC Assay is capable of accurately and rapidly identifying bacterial species and resistance determinants present in blood cultures containing gram positive bacteria. Utilizing molecular diagnostics like the iC-GPC Assay will decrease time to treatment, healthcare costs, and BSI-related mortality.

KEYWORDS: *Molecular Diagnosis, ARM-PCR, Microarray, In Vitro Diagnostic (IVD), Infectious Disease Detection, Sepsis, Bloodstream Infection.*

INTRODUCTION

Molecular diagnostic assays are growing in popularity and clinical application given their ability to decrease time to diagnosis, while improving sensitivity and specificity. Molecular technologies play an important role and provide detection methods in sequencing,[1] microarrays,[2–4] fluorescent *in situ* hybridization (FISH),[5–6] Real-Time PCR,[7] and many others.[8] However, most published laboratory-based molecular tests can only detect one or a few microorganisms and are impractical for the diagnosis of many infectious diseases. For example, FISH is limited to the detection of one or a few specific targets. Real-Time PCR or qPCR offers great sensitivity and speed with the integration of PCR amplification and detection; however, it is difficult to develop multipex assays for qPCR due to inherent technology and fluorophore limitations. In recent years, multiplex PCR technologies have overcome the limitations of qPCR, allowing for the detection of multiple specific gene products from a single experiment.[9–10] Therefore, multiplex technologies are ideally suited for improving the diagnostic capacity of molecular assays.[11–12]

Bloodstream infections (BSI) and subsequent sepsis are now the 10th leading cause of death in the US.[13–14] The most important aspect of BSI treatment is rapid diagnosis and administration of the appropriate treatment, which directly correlates to patient outcomes, hospitalization length, and treatment cost.[15–17] A number of studies confirm the urgency of rapid identification of bloodstream

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pathogens and antimicrobial resistance markers and the subsequent benefit to the effective targeting of antimicrobial therapy and thus patient survival.[18–19] Conventional bacterial identification methods used in the clinical setting are based on culture; however, identification of microbes through these techniques is limited and time consuming. With the advent of Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS),[20–24] the time to identification of positive blood cultures can be shortened to approximately 5 hours after the organism is isolated in culture; however, conventional culture-based antimicrobial susceptibility testing is still required, which may take an additional 12–24 hours.[25] These factors necessitate the need for more rapid and accurate diagnostics to decrease time to treatment and reduce mortality rates.[26]

One of the most pressing areas in BSI diagnostics has been the development of assays that detect gram positive pathogens, which are responsible for 52% to 77% of all BSI.[15–16] Herein, we developed and validated a multiplexed, *in vitro* molecular diagnostic assay, the iCubate Gram Positive Cocci Assay (iC-GPC Assay), which is performed on the iC-System. The iC-System automates sample preparation, Amplicon-Rescue Multiplex PCR (ARM-PCR), and microarray detection within a closed cassette. The iC-GPC Assay identifies the following targets from positive blood cultures: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Enterococcus faecalis*, *Enterococcus faecium*, as well as *mecA*, *vanA* and *vanB* resistance genes. Development and validation of this assay will provide clinicians with a valuable tool to rapidly detect and identify the causative organisms and resistance markers of BSI and thereby improve patient outcomes.

MATERIALS AND METHODS

Source of Samples

The 19 reference strains used in the Limit of Detection (LoD) study were received from American Type Culture Collection (ATCC, Manassas, VA) and JMI Laboratories (North Liberty, IA). A total of 106 inclusivity strains were received from BEI Resources (Manassas, VA), ATCC, Medical College of Wisconsin (MCW, Milwaukee, WI), and Zeptometrix (Buffalo, NY). A total of 96 strains used for exclusivity testing were titered and received from ATCC or Zeptometrix. The positive control strain, Bacillus thuringiensis Z096, was obtained from Zeptometrix. The human whole blood (K2 EDTA) used in this study was obtained from BioreclamationIVT. Culturing was performed under aerobic or anaerobic conditions depending on the bacterial species and according to the manufacturer's instructions. Reference strains were grown on TSA 5% sheep blood agar plates and confirmed by Gram stain to be gram positive cocci.

Study Design

The iC-System is a sample-to-answer, automated system, which consists of an iC-Processor, an iC-Reader, and an iMac computer (Fig. 1). Each pre-assembled iC-GPC Cassette contains the reagents needed to perform the assay as well as a printed universal microarray for the detection of target analytes.

To conduct an iC-GPC Assay, an aliquot of blood culture media containing gram positive bacteria is pipetted into the sample well of the iC-Cassette. The iC-Cassette is then inserted into the iC-Processor, which performs nucleic acid extraction, ARM-PCR, and hybridization onto a microarray following a predefined software-driven script. Nucleic acid extraction is performed using heat (~102 °C) to lyse the bacterial sample. Following lysis, the released DNA is used for subsequent ARM-PCR. During hybridization, the amplified target binds to both a complimentary spot on the microarray and to a gene-specific fluorescent probe for target detection. Once processing is complete, the iC-GPC Cassette is transferred to the iC-Reader for fluorescence-based detection and data processing. The iC-Reader, using a laser and photomultiplier tube (PMT) optics system, scans the microarray located in the cassette and forms a 2D digital array image, which is then sent to the iC-Report software for data analysis. A final report is displayed on an iMac indicating the presence of target species and associated antibiotic resistance markers.

Amplicon-Rescued Multiplex PCR

ARM-PCR (US Patent 07999092) involves a two-step amplification process. During the first step of amplification, nested gene-specific primers (Forward-out, Fo; Forward-in, Fi; Reverse-out, Ro; and Reverse-in, Ri) are used to enrich assay targets. The inside primers (Fi and Ri) contain built in priming sites for the second stage PCR as well as index sequences that direct array hybridization after amplification. Once these tagged sequences are incorporated into the first stage PCR (PCR1) products the second stage PCR (PCR2) for exponential amplification is performed using a pair of communal primers specific to the incorporated tags. The communal primers are included at a high concentration at an asymmetric ratio, which enriches single-stranded DNA for microarray hybridization (Fig. 2). For PCR1, 50 μL Alpha Master Mix containing nested primers and PCR enzymes is transferred into the sample well. ARM-PCR occurs in the sample well using three heaters within the iC-Processor controlled at distinct temperatures to initiate thermal cycling. After PCR1 amplification is complete, the mixture is diluted and mixed with PCR Master Mix containing communal primers for PCR2.

Microarray Hybridization

In this study, a universal microarray was designed to detect up to 30 targets in a single experiment. The probes printed on the microarray can be used as tags for the identification

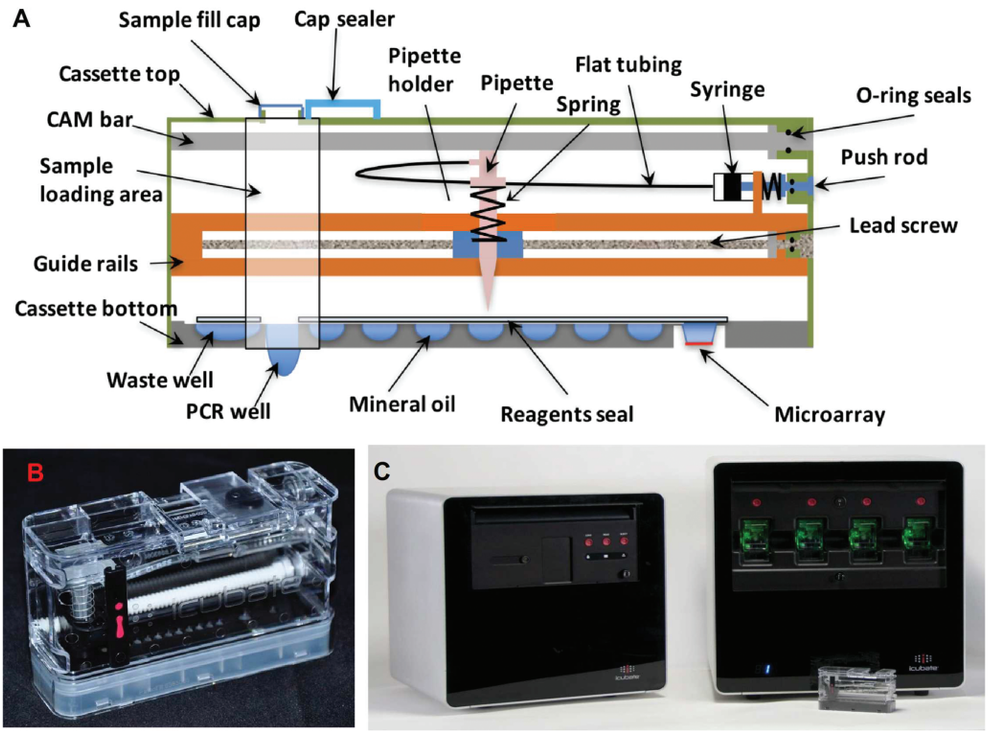

Figure 1. **Diagram of the iC-system and iC-cassette. (A) X-ray view of the iC-cassette with a closed design; (B) iC-cassette; (C) The iC-system including the iC-reader (left) and iC-processor (right).**

of targets for various assays. The universal microarray is mounted and secured in a defined compartment within the iC-Cassette. The probes are arranged on the microarray in a two-dimensional grid pattern and include 30 different target probes in replicates of four, four replicates each of a positive control and negative control probe, and 8 fluorescent orientation spots used for locating and orienting the microarray pattern during scanning and data processing. For the iC-GPC Assay, the single stranded DNA generated by PCR2 is captured by the corresponding index detection probe on the microarray, and a gene-specific fluorescent-labeled detector probe is used for signal detection (See Fig. 2(3)). During hybridization, 45 μL nucleic acid target is mixed with 195 μL hybridization buffer containing an optimal concentration of fluorescent-labeled gene-specific detection probes of 100 pmol/μL. The mixture is then transferred onto the universal microarray for hybridization. The array is incubated using a heater in the iC-Processor at 55 °C for 45 minutes. Following hybridization, unbound fluorescent-labeled detectors are removed by two washes with wash buffer I (1X Saline-Sodium Citrate, SSC plus 0.1% Sodium Dodecyl Sulfate, SDS) followed by four washes with wash buffer II (0.1X SSC plus 0.1% SDS). After washing is complete, the iC-Cassette is ejected from the iC-Processor.

Data Analysis

After processing is complete, the iC-Cassette is scanned in the iC-Reader. Light scatter from the fluorescent-labeled probes is imaged and intensities from the microarray spots are used to make decisions regarding the presence (Detected) or absence (Not Detected) of the iC-GPC targets. A spot on the microarray is read as positive only if the mean intensity value is greater than or equal to the cut-off. The default value of the cut-off is determined to be the background median value *BG(md)* of the microarray plus three times the standard deviation of the background, *BG(sd)*. Five representative strains (Table I) were evaluated to verify the cut-off for each iC-GPC target. The iC-System software calculates the target spot detection threshold for a given iC-Cassette based on a histogram of the scanned image data. The "Balanced Threshold Method" of image analysis is applied to the digitized image data set to determine which target spots are positive or negative. A minimum of three of the four replicate spots per target must be positive for a given target to be labeled as detected.

Limit of Detection

Limit of Detection (LoD) studies were performed by diluting targets in a matrix comprised of blood culture bottle media spiked with human blood. Preliminary tests were conducted using an 11 step dilution series and testing

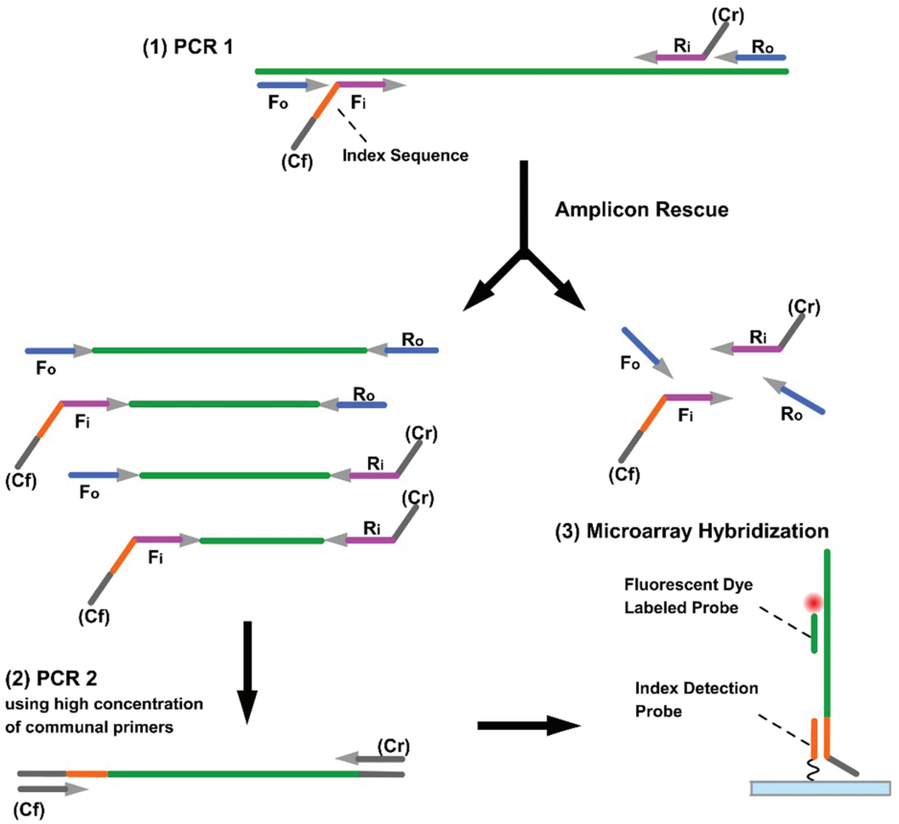

Figure 2. **Illustration of ARM-PCR and microarray hybridization. (1) PCR1: Nested gene-specific primers (Forward-out, Fo; Forward-in, Fi; Reverse-in, Ri and Reverse-out, Ro) are used to enrich assay targets; Fi and Ri contain built in priming sites (Cf, Forward communal; Cr, Reverse communal) for the second stage PCR as well as index sequences that direct array hybridization. (2) PCR2: Using PCR1 amplicon as template, an asymmetric amplification is performed in PCR2 using high concentrations of communal primers to amplify all the targets. (3) Microarray hybridization: In the "Sandwich" hybridization, the single-strand DNA generated by PCR2 will be captured by the corresponding index detection probe on the array, and a gene-specific fluorescent labeled probe is used for signal detection.**

each concentration in 10 cassettes. Initial concentrations were approximated to 1×10^8 CFU/mL for each organism using a Sensititre™ Nephelometer (Waltham, MA) and then diluted to the target concentration in BCB matrix. Final concentrations were confirmed by plating and colony counts. Target performance was used to approximate a 95% performance concentration, which was confirmed by testing 60 cassettes spread across three unique cassette lots. The target LoD was defined as the concentration at which $\geq$95% but $<$100% of targets were successfully detected.

Analytical Reactivity (Inclusivity)

106 strains of target organisms were tested for analytical reactivity. A 1×10^8 CFU/mL stock solution for each organism was made and diluted in BCB matrix to concentrations of approximately 2–3 $\times$ LoD. Each organism was tested in triplicate. In the event of a false negative result, the organism was grown to initial bottle positivity in BCB matrix and repeated in replicates of ten. Plating and colony counts were used to confirm purity and final concentration.

Analytical Specificity (Exclusivity)

Exclusivity strains included organisms phylogenetically related to iC-GPC targets, as well as common blood culture contaminants. A total of 96 organisms were purchased at high titers and tested at the highest concentrations possible, considered 1×10^8–1×10^9 CFU/mL for bacteria and fungi and 10^6 to 10^7 copies/mL for viruses. Bacteria and fungi were tested in BCB media with human blood added and viral organisms were tested directly in transport media. Each organism was tested in triplicate.

Reproducibility

To test the reproducibility of the iC-GPC Assay and iC-System, five representative target organisms and one negative control organism, *Corynebacterium striatum* (MCW), were evaluated at two concentrations: initial bottle positivity and eight hours beyond initial bottle positivity.

Table I. iC-GPC assay 95% target LoDs, evaluated using 19 reference strains.

Target	Organism	Reference strain #	95% LoD (CFU/mL)	Defined target LoD (CFU/mL)
gseA	*S. epidermidis*[a]	ATCC 700566	2.36×10^6	1.6×10^6–1.7×10^7
	S. epidermidis	ATCC 35984	8.27×10^6	
	S. epidermidis	ATCC 12228	1.70×10^7	
	S. epidermidis	ATCC 49134	1.64×10^6	
nuc	*S. aureus*[a]	ATCC 700699	2.30×10^6	1.7×10^6–4.4×10^6
	S. aureus	ATCC BAA1768	4.38×10^6	
	S. aureus	ATCC BAA977	1.66×10^6	
	S. aureus	ATCC 25923	3.68×10^6	
mecA	*S. epidermidis*	ATCC 700566	7.38×10^5	7.4×10^5–9.5×10^6
	S. epidermidis	ATCC 35984	7.28×10^6	
	S. aureus	ATCC 700699	9.36×10^6	
	S. aureus	ATCC BAA1768	9.45×10^6	
lytA	*S. pneumoniae*[a]	ATCC 6301	6.00×10^6	1.3×10^6–6.0×10^6
	S. pneumoniae	ATCC 700673	1.30×10^6	
ddl(EFLS)	*E. faecalis*[a]	ATCC 51299	5.76×10^6	3.0×10^5–5.8×10^6
	E. faecalis	ATCC 700802	2.16×10^6	
	E. faecalis	JMI 12536	5.28×10^6	
	E. faecalis	ATCC BAA2128	3.03×10^5	
	E. faecalis	ATCC 29212	4.44×10^5	
ddl(EFCM)	*E. faecium*[a]	ATCC 700221	4.88×10^6	4.9×10^6–7.9×10^6
	E. faecium	ATCC 51559	7.85×10^6	
	E. faecium	ATCC 35667	6.16×10^6	
	E. faecium	ATCC BAA2127	5.56×10^6	
vanA	*E. faecium*	ATCC 700221	7.20×10^5	7.2×10^5–1.1×10^7
	E. faecium	ATCC 51559	1.05×10^7	
	E. faecalis	JMI 12536	1.21×10^6	
vanB	*E. faecalis*	ATCC 51299	5.76×10^6	3.9×10^6–5.8×10^6
	E. faecalis	ATCC 700802	3.88×10^6	

Note: [a]Five representative strains were used for cut-off validation, optimization, and reproducibility test.

A 1×10^8 CFU/mL stock solution for each organism was made and then diluted to 1×10^3 CFU/mL in saline. 300 μL was inoculated into BD BACTEC Plus Aerobic/F bottles with human blood and bottles were allowed to incubate on the BD BACTEC 9050 System (Franklin Lakes, NJ). Testing was performed by two independent operators at each of three sites, two external and one internal. The six organism panel was tested in triplicate at each concentration across five, non-consecutive days. Testing was evaluated across three unique cassette lots and four iC-Systems.

RESULTS AND DISCUSSION

Optimizing ARM-PCR

Multiplex PCR is notoriously complex and requires extensive optimization to balance sensitivity and specificity. To maximize the sensitivity and specificity of each assay target, multiple primer concentrations and PCR cycling conditions from 5 representative strains (Table I) were tested and optimized. To prevent the generation of non-specific products, the cycle number for PCR1 reaction was set at 25. After multiplex amplification in PCR1, product is carried through a second amplification stage using communal primers. However, carryover of primers from PCR1 to PCR2 can inhibit communal primer activity. In order to optimize the first stage dilution ratio for PCR2, several dilution series tests were performed. PCR1 amplicon from *S. pneumoniae* ATCC 6301 strain targeting the *lytA* gene was used to optimize the amplicon rescue reaction (Fig. 3). Using this product, asymmetric PCR2 and microarray hybridization were performed as described (see Materials and Methods); both gel electrophoresis and fluorescence intensities were used to determine assay performance and the optimal dilution ratio. As shown in Figure 3, amplicon rescue performed using a 10 to 50-fold dilution can minimize the carryover of first stage primers while providing sufficient amplicon for use in the second amplification. Dilution by less than 5-fold resulted in carryover of PCR1 primers, which interfered with PCR2, and caused a significant decrease in assay sensitivity. By incorporating this dilution scheme, ARM-PCR is capable of achieving the sensitivity and specificity needed for molecular diagnostic assays.

Multiplex PCR technologies are poised to provide a huge advantage for molecular-based methods by delivering organism identification and drug resistance information concurrently. In traditional multiplex PCR, each target

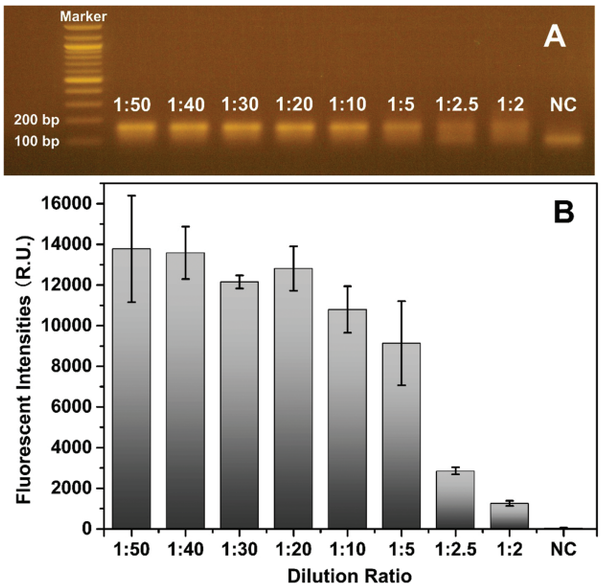

Figure 3. Amplicon rescue optimization. Amplicon rescue optimization of *lytA* gene performed by different dilution ratios ranging from 1:50 to 1:2, NC (negative control, no PCR1 amplicon added to PCR2). (A): 2% Gel electrophoresis results. (B): Signal intensity plots of hybridization of the asymmetric second stage PCR amplicon to the detection microarray. Target *lytA* was spotted in four replicates on the microarray, the fluorescent signal of each spot was determined by subtraction of local background from the mean intensity of each spot. The signal intensity bars represent an average of mean net intensities obtained from the 4 replicate spots for target *lytA* at each dilution ratio. The error bars represent the standard deviation of the mean net intensity of 4 replicate spots.

requires its own optimal reaction conditions, so increasing the number of targets requires that the reaction conditions for each individual target are less than optimal. Furthermore, crowded primers generate primer-dimers and reduce the amplification efficiency by consuming assay reagents, leading to significant discrepancies in amplicon yields among targets. The potential uneven amplification makes it difficult to accurately identify the pathogens and drug resistance marker simultaneously in a single reaction. In this study, we introduced ARM-PCR technology utilizing target-specific, nested primers to solve the loci incompatibility problem. In ARM-PCR, target-specific amplification is performed using very low primer concentrations in the first step and a pair of communal primers at high primer concentrations in the second amplification to enrich all targets. The only primers used for exponential amplification are a pair of high concentration communal primers; consequently, all the co-amplified targets have similar amplification efficiencies. Increased compatibility among multiple targets allows the assay to be reorganized or remixed in accordance with specific requirements. In addition, new targets can be added without significantly reducing the sensitivity of the assay. This feature of ARM-PCR has also been used to amplify the immune repertoire for high throughput sequencing.[27]

Optimizing iC-GPC Assay

The iC-Cassette contains a printed universal microarray and gene-specific fluorescent detector probes for detection of each iC-GPC Assay target. The optimal concentration of detection probes during hybridization was determined to be 100 pmol/μL. The optimal hybridization temperature was determined to be 55 °C. Following hybridization, unbound fluorescent-labeled detectors were removed by two washes at room temperature with wash buffer I followed by four washes with wash buffer II (see Materials and Methods). For software analysis of detected targets, the targets cut-offs were initially set at a default value: Cutoff (Default) = $BG(md) + 3BG(sd)$. This cutoff value indicates that a target must have a signal at three times the background level to be considered positive. During optimization, the cut-off values of *gseA* and *ddl* were increased to $BG(md) + 4BG(sd)$, and the *mecA* cut-off value was increased to $BG(md) + 5BG(sd)$ to minimize occasional false positive signals seen with these targets.

Incorporating ARM-PCR into an automated cassette system provides numerous advantages in both development and the clinical setting. The first strength of the iC-GPC Assay is its design around a universal microarray. Using a microarray that contains no gene specific sequence, but instead relies on index sequences incorporated into ARM-PCR primer design, makes development of new assays quick and efficient. A second benefit of the iC-GPC Assay is the closed nature of the iC-Cassette that limits the chances of contamination and exposure of lab personnel. Another advantage to the automated ARM-PCR system is the ease of use for the lab technician. Loading the cassette is accomplished through simply pipetting the sample into the cassette with no additional sample processing. This along with the open access of the iC-System allow for an expedited laboratory workflow that requires minimal technician and system down time.

In order to ensure assay performance, a positive control is built into the iC-GPC Assay. The positive control is an intact, inactivated strain of *Bacillus thuringiensis* that is added to the sample and undergoes all processing steps with the sample. The positive control was constructed by designing primers specific to the *cry* gene of *Bacillus thuringiensis* strain Z096.[28] Primer and *Bacillus thuringiensis* concentrations were optimized for the detection of 1×10^4 CFU/mL in the assay in order to balance target sensitivity with positive control detection.

Limit of Detection

A crucial component of any molecular test is to be able to detect organisms at clinically relevant concentrations. As such, we sought to determine the LoD for the iC-GPC Assay defined as the lowest concentration of organism that

can be detected 95% of the time. Using 19 strains, a minimum of two for each target, we conducted preliminary LoD testing at 11 concentrations ranging from 5×10^7 to 1×10^5 CFU/mL. After initial testing narrowed the performance range, further dilutions were performed around the target concentration until a 95% detection point was achieved. The target concentration and performance were confirmed by testing 60 cassettes, 20 from each of three different lots. By this method, we determined the LoD for each of the targets in the iC-GPC Assay to be in the range of 3.0×10^5 CFU/mL to 1.7×10^7 CFU/mL (Table I). These concentrations are well below the concentrations where blood culture bottle positivity is indicated, generally $>1 \times 10^8$ CFU/mL.

Analytical Reactivity (Inclusivity)

Molecular assays must demonstrate they are capable of covering the diversity of isolates seen under clinical settings in order to be practically applicable. In order to confirm the inclusivity of the iC-GPC Assay, 106 inclusivity panel members were chosen to represent temporal, geographic, and genetic diversity (Fig. 4). To ensure no loss in sensitivity between isolates, all strains were tested at 2–3 times the target LoD in blood culture bottle/blood media. Each strain was initially tested in triplicate, and a 99.37% (316/318) detection rate was observed. The two strains displaying only 2 of 3 true positive results were repeated in replicates of 10 with a 100% success rate for a final performance of 99.40% (336/338). A BLAST *in silico* analysis was performed to supplement cassette testing for strains that were difficult to obtain. This study suggests the iC-GPC Assay is sufficiently inclusive and will encompass any minor sequence variations in gene targets present across diverse strain backgrounds.

Analytical Specificity (Exclusivity)

While the targets in the iC-GPC Assay comprise the majority of gram positive cocci associated with BSI, non-target organisms may either cause BSI or be introduced into the blood culture bottle media during the blood draw. Therefore, it is important for any molecular assay to display no cross-reactivity with common contaminants or other causative organisms. A panel of non-target gram positive bacteria, gram negative bacteria, viruses,

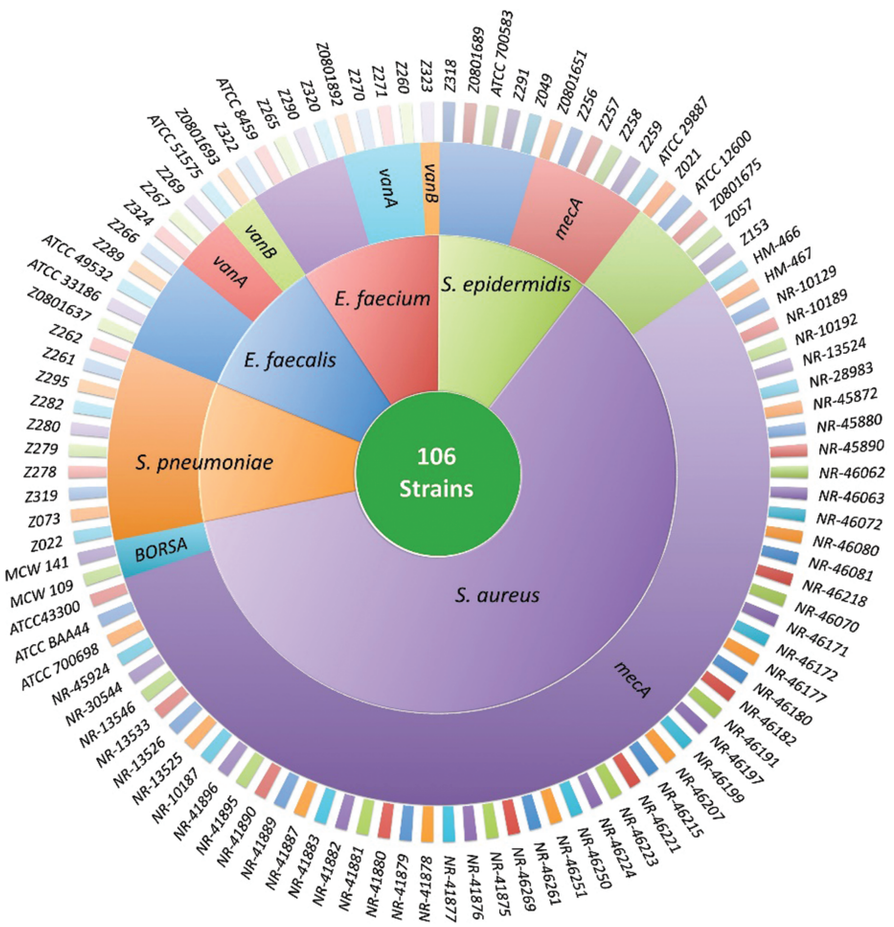

Figure 4. **iC-GPC assay inclusivity. Diagram depicts the 106 bacterial strains utilized for inclusivity studies.**

Table II. **iC-GPC assay analytical specificity (exclusivity).**

Abiotrophia defectiva *A. defectiva*	***Candida Species*** *C. albicans* *C. catenulate* *C. dubliniensis* *C. glabrata* *C. guilliermondii* *C. krusei* *C. parapsilosis* *C. tropicalis*	***Coxsackievirus*** Coxsackievirus	***Enterovirus*** Enterovirus (*Type* 71)	***Micrococcus luteus*** *M. luteus*	***Salmonella spp*** *S. spp* (*typhimurium*)
Acinetobacter Species *A. baumannii* *A. lwoffi*	***Citrobacter Species*** *C. amalonaticus* *C. freundii* *C. koseri* *C. sedlakii*	***Cryptococcus neoformans*** *C. neoformans*	***Escherichia Species*** *E. coli* *E. hermannii*	***Oerskovia enterophila*** *O. enterophila*	***Staphylococcus Species*** *S. capitis* *S. delphini* *S. haemolyticus* *S. hominis* *S. intermedius* *S. lugdunensis* *S. lutrae* *S. pettenkoferi* *S. schleiferi* *S. schleiferi subsp. coagulans* *S. warner*
Aerococcus viridans *A. viridans*	***Clostridium Species*** *C. difficile* (*NAP-1 toxigenic*) *C. difficile* (*non-toxigenic*) *C. oedematiens* (*novyi*)	***Cytomegalovirus*** Cytomegalovirus	***Fusobacterium varium*** Fusobacterium varium	***Pediococcus pentosaceus*** *P. pentosaceus*	
Aeromonas hydrophila *A. hydrophila*	***Collinsella aerofaciens*** *C. aerofaciens*	***Echovirus*** Echovirus	***Klebsiella Species*** *K. oxytoca* *K. pneumoniae*	***Planococcus citreus*** *P. citreus*	
Alcaligenes faecalis *A. faecalis*	***Corynebacterium Species*** *C. amycolatum* *C. genitalium* *C. jeikeium*	***Edwardsiella tarda*** *E. tarda*	***Kocuria kristinae*** *K. kristinae*	***Propionibacterium acnes*** *P. acnes*	
Anaerococcus tetradius *A. tetradius*		***Eggerthella lenta*** *E. lenta*	***Kytococcus schroeteri*** *K. schroeteri*	***Proteus Species*** *P. mirabilis* *P. penneri* *P. vulgaris*	
Aspergillus niger *A. niger*		***Enterobacter Species*** *E. aerogenes* *E. cloacae*	***Lactobacillus Species*** *L. acidophilus* *L. plantarum subsp. plantarum* *L. reuteri*	***Providencia Species*** *P. alcalifaciens* *P. arettgeri* *P. stuartii*	
Bacillus cereus *B. cereus*		***Enterococcus Species*** *E. avium* *E. casseliflavus* *E. cecorum* *E. dispar* *E. gallinarum* *E. hirae* *E. raffinosus*	***Leminorella grimontii*** *L. grimontii*	***Pseudomonas Species*** *P. aeruginosa* *P. putida*	
Bacteroides fragilis *B. fragilis*			***Leuconostoc mesenteroides*** *L. mesenteroides*	***Rothia mucilaginosus*** *R. mucilaginosus*	
Campylobacter Species *C. coli* *C. jejuni*					

Note: [b] *Streptococcus bovis* was detected as E. faecalis, none of the remaining 95 organisms demonstrated any detectable cross-reactivity.

Table III. iC-GPC assay reproducibility performance by target and concentration.

Organism/ Gene target/ Concentration	Overall performance	Overall performance % [95% CI]	False negatives	False positives	Positive controls check failures	System failures
S. epidermidis (gseA) bottle ring	89/89	100.0 [95.86–100.0]	0/89 (0.00%)	1/976 (0.10%)	0/90 (0.00%)	1/90 (1.11%)
S. epidermidis (gseA) bottle ring + 8 hours	90/90	100.0 [95.91–100.0]	0/90 (0.00%)	0/975 (0.00%)	0/90 (0.00%)	0/90 (0.00%)
S. aureus (nuc) bottle ring	89/89	100.0 [95.86–100.0]	0/89 (0.00%)	0/976 (0.00%)	1/90 (1.11%)	0/90 (0.00%)
S. aureus (nuc) bottle ring + 8 hours	89/90	98.9 [93.97–99.80]	1/90 (1.12%)	0/975 (0.00%)	0/90 (0.00%)	0/90 (0.00%)
S. pneumoniae (lytA) bottle ring	88/88	100.0 [95.82–100.0]	0/88 (0.00%)	0/977 (0.00%)	1/90 (1.11%)	1/90 (1.11%)
S. pneumoniae (lytA) bottle ring + 8 hours	89/89	100.0 [95.86–100.0]	0/89 (0.00%)	0/976 (0.00%)	0/90 (0.00%)	1/90 (1.11%)
E. faecalis (ddl) bottle ring	89/89	100.0 [95.86–100.0]	0/89 (0.00%)	2/976 (0.20%)	0/90 (0.00%)	1/90 (1.11%)
E. faecalis (ddl) bottle ring + 8 hours	89/89	100.0 [95.86, 100.0]	0/89 (0.00%)	3/976 (0.31%)	1/90 (1.11%)	0/90 (0.00%)
E. faecium (fcm) bottle ring	90/90	100.0 [95.91–100.0]	0/90 (0.00%)	0/975 (0.00%)	0/90 (0.00%)	0/90 (0.00%)
E. faecium (fcm) bottle ring + 8 hours	89/89	100.0 [95.86–100.0]	0/89 (0.00%)	0/976 (0.00%)	1/90 (1.11%)	0/90 (0.00%)
mecA bottle ring	177/178	99.4 [96.89–99.90]	1/178 (0.56%)	1/887 (0.11%)	1/180 (0.56%)	1/180 (0.56%)
mecA bottle ring + 8 hours	180/180	100.0 [97.91–100.0]	0/180 (0.00%)	0/885 (0.00%)	0/180 (0.00%)	0/180 (0.00%)
vanA bottle ring	90/90	100.0 [95.91–100.0]	0/90 (0.00%)	0/975 (0.00%)	0/90 (0.00%)	0/90 (0.00%)
vanA bottle ring + 8 hours	89/89	100.0 [95.86–100.0]	0/89 (0.00%)	0/976 (0.00%)	1/90 (1.11%)	0/90 (0.00%)
vanB bottle ring	89/89	100.0 [95.86–100.0]	0/89 (0.00%)	0/976 (0.00%)	0/90 (0.00%)	1/90 (1.11%)
vanB bottle ring + 8 hours	89/89	100.0 [95.86, 100.0]	0/89 (0.00%)	0/976 (0.00%)	1/90 (1.11%)	0/90 (0.00%)

and fungi that may be present in positive blood cultures were tested at high concentrations to evaluate iC-GPC Assay exclusivity. Of the 96 organisms tested, only one potential cross-reactant, *Streptococcus bovis*, was observed with the *Enterococcus faecalis* (*ddl*) target (Table II). None of the remaining 95 organisms tested demonstrated any detectable cross-reactivity, thereby assuring a high level of assay specificity. Additionally, no potential cross-reactivity was indicated from an *in silico* exclusivity analysis.

Reproducibility

Assays intended for commercial use must be usable by any trained operator in any lab setting to be a successful product. Reproducibility of the iC-GPC Assay was evaluated across three sites, six operators, four systems, and

Table IV. iC-GPC assay reproducibility performance by operator.

Operator	Overall performance	Overall percentage [95% CI]	Positive controls check failures	System failures
Site 1, Operator A	177/178	99.4% [0.9689, 0.9990]	2/180 1.11%	0/180 0.00%
Site 1, Operator B	175/178	98.3% [0.9516, 0.9943]	2/180 1.11%	0/180 0.00%
Site 2, Operator C	174/175	99.4% [0.9683, 0.9990]	2/180 1.11%	3/180 1.67%
Site 2, Operator D	176/178	98.9% [0.9600, 0.9969]	0/180 0.00%	2/180 1.11%
Site 3, Operator E	176/177	99.4% [0.9687, 0.9990]	2/180 1.11%	1/180 0.56%
Site 3, Operator F	179/179	100.0% [0.9790, 1.0000]	0/180 0.00%	1/180 0.56%

Table V. iC-GPC assay reproducibility performance by cassette lot.

Lot	Overall performance	Overall percentage [95% CI]	Positive controls check failures	System failures
Lot A	352/353	99.7% [0.9841, 0.9995]	4/360 1.11%	3/360 0.83%
Lot B	352/354	99.4% [0.9796, 0.9984]	4/360 1.11%	2/360 0.56%
Lot C	353/358	98.6% [0.9677, 0.9940]	0/360 0.00	2/360 0.56%

three unique cassette lots. Five target and one non-target organism were tested at two clinically relevant concentrations, initial bottle positivity and eight hours beyond initial positivity. iC-GPC Assay performance stratified by target and concentration is provided in Table III. Overall performance based on target detection was 99.3%. The operator-to-operator reproducibility performance was between 98.3% and 100% (Table IV). The lot-to-lot reproducibility performance was between 98.6% and 99.7% (Table V). No differences in performance were observed across sites, operators, systems, or cassette lots, demonstrating that these variables have no effect on instrument or assay performance.

CONCLUSION

Rapid and accurate identification of a broad range of microbial pathogens is key for the successful management of patients with BSI.[29] In this study, the analytical performance of the iC-GPC Assay was validated on the iC-System to identify gram positive organisms and resistance markers within 4.5 hours. In addition to analytical validation, the iC-GPC Assay has undergone extensive clinical trials and received FDA approval. Clinical performance was comparable to current molecular diagnostics, as well as traditional culture techniques.[30] These studies demonstrate the assay can accurately and rapidly identify bacterial species and their resistance determinants simultaneously in blood cultures containing gram positive bacteria. Utilizing molecular diagnostics like the iC-GPC Assay will improve time to treatment, decrease healthcare costs, and decrease BSI related mortality.

Conflict of Interests

This study was supported by iCubate, Inc. Jian Han is the founder and CSO of iCubate, Inc. All other authors are employees or contract employees of iCubate, Inc.

Author Contributions

Conceived and designed the experiment: Hongna Liu Kathryn Heflin Jian Han Song Li. Performed the experiments: Hongna Liu Matt Conover Leslie Wagner Song Li. Analyzed the data: Hongna Liu Kathryn Heflin Song Li. Contributed reagents/materials/analysis tools: Jeff Bertrand Phillip Ewing Stanley Lu Scott Clemmons John Watts. Wrote the manuscript: Hongna Liu Kathryn Heflin Jian Han Matt Conover Song Li.

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