Rapid Automated Multiplex PCR Diagnostics for Blood Pathogens

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ABSTRACT

Sepsis, the syndrome of infectious illness leading to a systemic inflammatory response, is a leading cause of death in the U.S. Delay in diagnosis and initiation of appropriate therapy is the most common contributor to increased morbidity and mortality. Current microbiological methods used to identify a responsible pathogen are slow and laborious, requiring days for a result. As a large number of pathogens can cause sepsis, broad-spectrum antibiotic therapy must be initiated, and continued until sepsis is ruled out or a pathogen is identified. Recently, molecular tests, such as those using polymerase chain reaction (PCR) have emerged and shown promise for rapid diagnosis of infectious illness. Primarily due to complexity and cost, these have not been widely adopted.

Idaho Technology, Inc. (ITI) has developed the FilmArray™ System (Figure 1), an innovative molecular diagnostics device that streamlines pathogen identification in human samples. The FilmArray System can simultaneously identify up to 32 organisms in ~1 hour, and requires only a minimally trained operator to perform the test. The FilmArray System comprises a uniquely designed "lab-in-a-pouch" and a counter top instrument which together automatically perform all steps of the assay, from nucleic acid extraction to nested multiplex PCR (nmPCR) and data analysis.

ITI's objective is to develop the FilmArray Sepsis System to detect and identify 22 sepsis-causing pathogens from blood culture. Toward this end, we have designed nmPCR assays for both bacterial housekeeping genes and species-specific virulence factors. Assays for the detection and identification of 13 pathogens and the staphylococcal drug resistance gene, *mecA*, have been tested in the FilmArray, and show sensitive and specific detection of pathogen nucleic acid templates. In addition, blood culture samples positive for a variety of pathogens included in the panel were tested. Pathogen identification was concordant between blood culture and FilmArray for all pathogens, including *S. aureus*, *S. pneumoniae* and several enteric species. These results suggest that the FilmArray system is a promising tool for the detection and identification of sepsis causing pathogens in blood culture.

INTRODUCTION

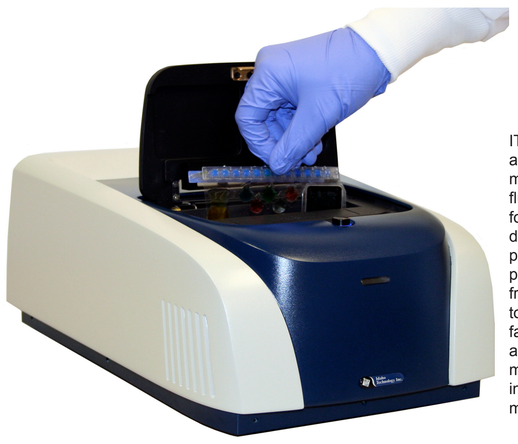
Sepsis is a leading cause of morbidity and mortality in the U.S. and the world. Sepsis in the U.S. has been estimated to be responsible for healthcare costs of $16.7 billion per year, and is the 10th leading cause of death, with as many as 120,000 deaths per year. While sepsis can be effectively treated with antimicrobial agents, rapid response is crucial. For example, after onset of hypotension, survival drops 7.5% per hour over the first six hours. Current methods of pathogen identification are slow, leading to delays in diagnosis. Cultures can take 24-48 hours to become positive, which are followed by Gram staining, then growth of an isolate on solid media, followed by biochemical testing. Overall, it can take up to four days to fully identify a sepsis-causing organism. Molecular diagnostics involving PCR for the detection of multiple pathogens provides more rapid results, however, current systems have not been FDA cleared and are difficult and costly to operate.

In response to the need for a diagnostic system that is rapid, multiplexed and streamlined, ITI is developing the FilmArray Sepsis System. The FilmArray will have several advantages over conventional culture-based detection. First and foremost, the time to detection and identification of pathogens will be significantly decreased, from several days to ~1 hour. Second, the FilmArray provides a low complexity system for the operator, requiring only injection of the blood culture sample into the pouch and starting the instrument. Third, the FilmArray is a closed system that limits contamination.

METHODS

For bacterial detection and identification the FilmArray Sepsis System will employ a nested-multiplex PCR strategy described in Figure. 2. Outer primers are designed to be broad-range, and are based on alignments of housekeeping gene targets (*rpoB*, *gyrB* and *ompA*) from distantly related bacterial species. Gene sequences were obtained through the NCBI database and aligned based on amino acid sequences to identify protein domains conserved across species. Outer primers target these domains, with degenerate nucleotides incorporated to provide cross-species recognition. Inner primers are designed to be species-specific, and are placed in locations where the 3' end includes a characteristic amino acid "signature" conserved among isolates of the same species, but different in other species. Primer 3' mismatches for non-targets help limit cross-amplification. Initial testing of FilmArray Sepsis System primer sets was performed on traditional PCR instruments with clinical bacterial isolates from Primary Children's Medical Center, Salt Lake City, UT. Successful multiplex assays were transferred to the FilmArray System (Figure 1, data in Figures 4, 5 and 6).

Figure 1: The FilmArray Instrument and Pouch



ITI has developed a lab-in-a-pouch system called "FilmArray". It is a medium-scale fluid manipulation system performed in a self-contained, disposable, thin-film plastic pouch. The FilmArray platform processes a single sample, from nucleic acid purification to result, in a fully automated fashion. These system characteristics are ideal for the multiplex testing of pathogens in standard diagnostic sample matrices.

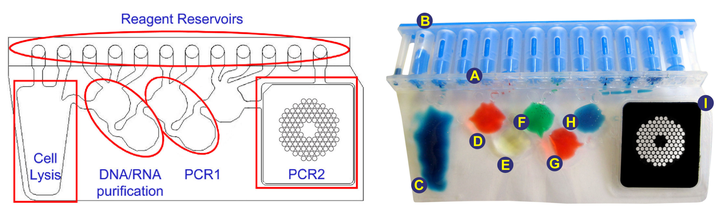
The FilmArray Test System

A FilmArray test is initiated by injecting rehydration solution and a patient blood culture sample into the FilmArray pouch and placing it in the FilmArray instrument. The user enters the sample and pouch type (using a barcode reader) into the software and initiates a run. Results are provided in ~ 1 hour.

The FilmArray pouch has a fitment (see label A) containing all needed freeze-dried reagents. The film portion of the pouch has stations for:

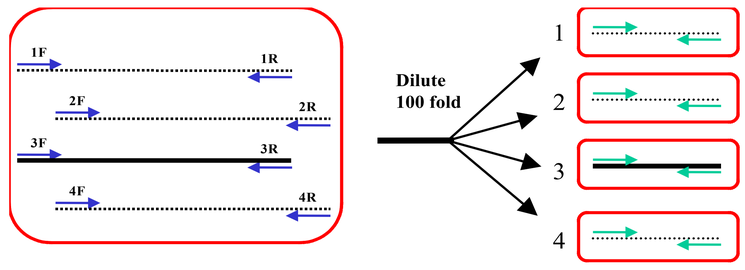
- Cell lysis (Blister C)
- Magnetic-bead based nucleic acid purification (D & E)
- First-stage multiplex PCR (F & G)
- Array of 108, second-stage nested PCRs (I)

PCR primers are dried into the wells of the array and each primer set amplifies a unique product of the first-stage multiplex PCR. The second stage PCR product is detected in real-time using a fluorescent-double-stranded DNA binding dye, LCGreen Plus, developed by ITI and post-PCR product identity is confirmed by high-resolution melt profiling.



A. Fitment with freeze-dried reagents
B. Plungers- deliver reagents to blisters
C. Sample lysis and bead collection
D. Wash station
E. Magnetic bead collection blister
F. Elution Station
G. Multiplex Outer PCR blister
H. Dilution blister
I. Inner Nested PCR array

Figure 2: Schematic of Nested Multiplex PCR



A large volume multiplex PCR (shown here as 4-plex on the left side of figure) is run for a limited number of cycles (20). The reaction is diluted and distributed to individual small PCR reactions that contain primers (green) nested inside the primers (blue) of the first PCR reaction. A template amplified in the first reaction (by the #3 primers) is further amplified in only one of the second reactions.

Figure 3. Proposed Integration of FilmArray into Current Blood Culture Procedures

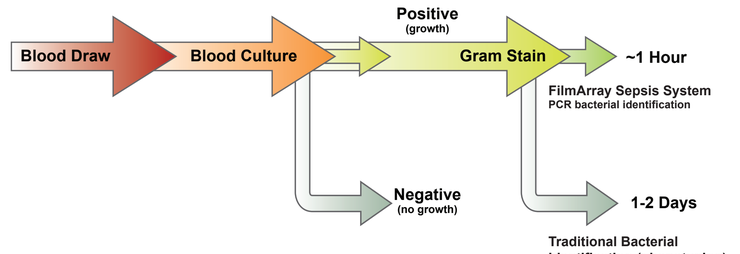
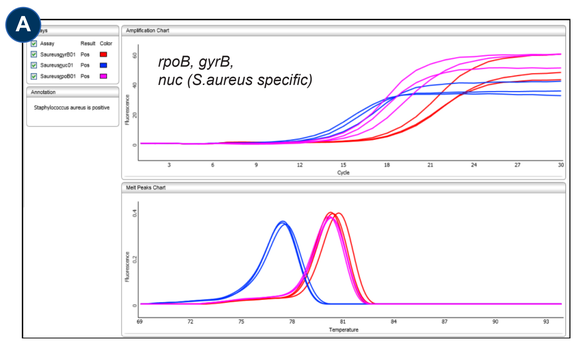


Figure 4. FilmArray Detection of Methicillin-Resistant *Staphylococcus aureus* (MRSA)

A. *S.aureus* organism injected into the FilmArray Sepsis pouch is identified with amplification of the housekeeping genes, *rpoB* (pink), *gyrB* (red), and a virulence target, *nuc* (blue).

B. Detection of the mecA gene from *S. aureus* (same sample shown in panel A). The FilmArray software call is "mecA detected", as *mecA* is sometimes found in some coagulase-negative staphylococci.

C. Raw data showing *nuc* (blue) and *mecA* (orange) with other assays that are negative.



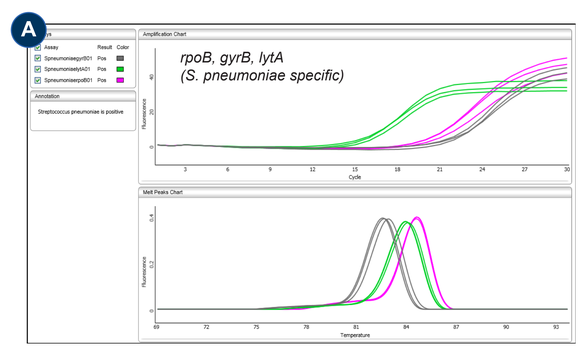
Real time PCR curves (upper panel) are used for assay development purposes only. Melt profiles (lower panel) are used to make the detection/identification calls (organism present or absent)

Table I. Bacteria and Gene Targets Detected in the FilmArray Sepsis System

Gram-positive	Gram-negative
Staphylococcus aureus	*Escherichia coli*
Staphylococcus epidermidis	*Klebsiella oxytoca*
Streptococcus pneumoniae	*Klebsiella pneumoniae*
Streptococcus agalactiae	*Enterobacter cloacae*
Streptococcus pyogenes	*Neisseria meningitidis*
Enterococcus faecalis	*Haemophilus influenzae*
Streptococcus species	*Pseudomonas aeruginosa*
Coagulase negative staphylococcus	Enteric species

Housekeeping genes	Virulence factor genes
RNA polymerase b (*rpoB*)	Pneumolysin (*ply*)
Gyrase B (*gyrB*)	Autolysin (*lytA*)
Outer membrane protein A (*ompA*)	Capsule export protein A (*ctrA*)
	Panton-Valentine leukocidin (*PVL*)
	Nuclease (*nuc*)
	Capsule gene (*bexA*)

Figure 5. FilmArray Detection of *Streptococcus pneumoniae*



A. *S. pneumoniae* organism injected into the FilmArray Sepsis pouch is identified with amplification of three different assays, *rpoB* (pink), *gyrB* (grey), and *lytA* (green). The targets *rpoB* and *gyrB* are housekeeping genes while *lytA* is a virulence gene in *S. pneumoniae*. Outer primers for *rpoB* and *gyrB* capture a broad spectrum of Gram-positive bacteria and inner primers are specific for *S. pneumoniae*. Corresponding melt peaks for the assays are also shown.

B. Amplification of *ply* in *S. pneumoniae* (same sample as shown in panel A). *Pneumolysin* is a virulence factor of *S. pneumoniae*, although it is sometimes present in *viridans streptococci* such as *S. mitis* and *S. oralis*. For this reason it is not used as a sole identifier for *S. pneumoniae* in the FilmArray Sepsis pouch but can have important clinical implications.

Figure 6. FilmArray Identification of *Klebsiella pneumoniae* in a Positive Blood Culture

A. A positive patient blood culture sample shows amplification of *K. pneumoniae ompA* (red) and *gyrB* (blue) assays in the FilmArray Sepsis pouch. The blood culture was diluted 1:1000 with water, mixed with a lysis buffer, and injected into the FilmArray. Microbiological testing of the blood culture sample was concordant with the FilmArray result.

B. The same positive patient blood culture shown in panel A shows amplification of the *Klebsiella rpoB* enteric assay. The assay is designed to detect enteric organisms including but not limited to, *E. coli*, *Klebsiella* species, *Enterobacter* species, *Serratia* species, and *Citrobacter* species.

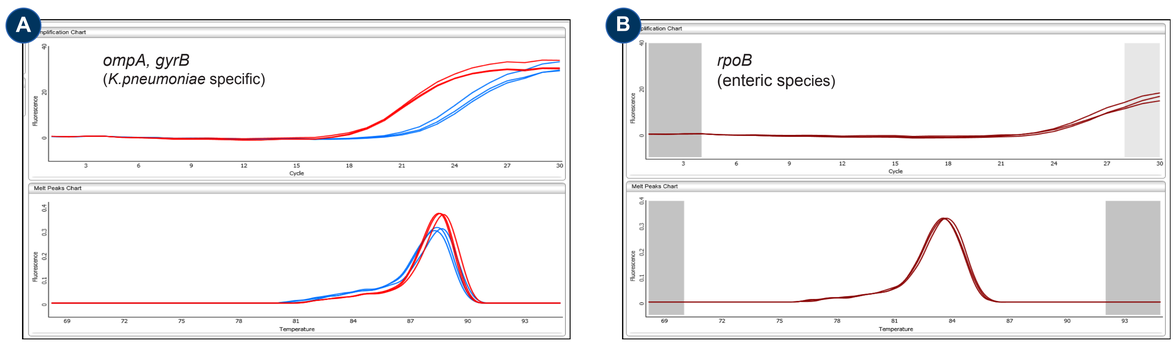


Table II. Blood Culture Pathogen Identification by FilmArray and Traditional Methods is Concordant

Patient No.	FilmArray Result	Blood Culture Result
1	*S. pneumoniae*	*S. pneumoniae*
2	*K. pneumoniae*	*K. pneumoniae*
3	*S. aureus*	*S. aureus*
4	*S. pneumoniae*	*S. pneumoniae*
5	*K. oxytoca*	*K. oxytoca*
6	*S. epidermidis*	*S. epidermidis*

Patient No.	FilmArray Result	Blood Culture Result
7	*S. pyogenes*	*S. pyogenes*
8	*E. cloacae*	*E. cloacae*
9	*E. coli*	*E. coli*
10	*S. aureus*	*S. aureus*
11	Negative	Negative
12	Negative	Negative

Patient No.	FilmArray Result	Blood Culture Result
13	Negative	Negative
14	*E. coli*	*E. coli*
15	*K. pneumoniae*	*K. pneumoniae*
16	*S. aureus*	*S. aureus*

CONCLUSIONS

- These data show proof-of-principal that nmPCR targeting of conserved housekeeping genes and virulence genes can accurately identify bacteria involved in sepsis.
- Broad-range outer primers provide the potential to easily modify the inner targets for different patient populations.
- When fully developed the FilmArray Sepsis System will provide a tool for the rapid and accurate evaluation of bacteria in blood cultures.