

Applications

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Rapid and reliable multiplex PCR pathogen detection in whole blood by using the VY00[®] kit on the Mastercycler[®] pro

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Abstract

This Application Note introduces a new protocol for multiplex PCR-based detection (VY00, SIRS-Lab, Jena, Germany) of bacterial and fungal pathogens. Directly from whole blood, sepsis-causing species are identified. Sepsis is one of the most common causes of death in hospitalized patients whereas rapid pathogen detection is a cornerstone in effective therapy.

DNA was isolated from EDTA whole blood samples of intensive care unit (ICU) patients suspected for sepsis. To investigate these DNA isolates for the presence of sepsis-causing pathogens a microbial DNA enrichment was performed followed by a standard multiplex PCR on the Mastercycler pro.

Species- and genus-specific primers for 34 bacterial and 6 fungal targets as well as 5 antibiotic resistances were combined within two primer pools. The amplicons were analyzed by gel-based identification with custom-designed DNA marker ladders. With this protocol, nucleic acid trace detection was successful with an overall sensitivity of 10 to 100 colony forming units (cfu)/mL whole blood.

Introduction

Life-threatening bacterial and fungal infections and their outcomes, sepsis and consecutive organ failure, are frequent complications in hospitalized patients, increasing by 2 % to 7 % worldwide annually. Sepsis results from the host's response to bacterial and fungal infections, whereas malfunction of the defense and repair system seems to be responsible for the development of organ dysfunctions. In Germany, 154,000 patients suffer from severe sepsis each year. With 60,000 deaths, it is one of the most frequent causes of death in the ICUs. About 30 % of the intensive medicine budgets are expended for the treatment of those patients [1]. A prompt and adequate antibiotics, started in the first few hours of infection, assigns the crucial step for an effective therapy [2-4]. Epidemiological data confirm that a doubling of mortality is the consequence of inadequate

therapies [5] and an increase of mortality of more than 7 % per hour is proven in cases of delayed adequate antibiotic treatment [6]. In addition, development and spread of antibiotic resistances are serious health risks, which are mainly contributed to antibiotics overuse [7]. Compared to the so far routinely used culture-based methods, nucleic acid amplification techniques (NAT) allow a more rapid species and resistance detection within several hours. Although their high sensitivity is diminished by factors like high fractions of eukaryotic bulk DNA, salts, and blood ingredients, this can be solved by affinity chromatography sample preparation. This allows for human DNA degradation and decrease of inhibitors which significantly increases overall sensitivity [8]. VY00 (CE-marked 2008) combines such a sample preparation with sensitive multiplex PCR-based pathogen detection and a time-to-result of less than one working day [9, 10].

Materials und Methods

Mechanical Cell Lysis. 5 mL of EDTA whole blood was homogenized and poured into a 15 mL centrifuge tube containing glass-bead matrix, antifoam solution, and *Bacillus subtilis* endospores as internal run control. Mechanical impact was applied using a FastPrep-24 cell lysis device (MP Biomedicals, Solon, OH, USA), set up with 6.5 m/s rotation velocity: 45 s rotation – 5 min intermission – 45 s rotation. Proteolytic digestion of the lysate followed.

Total Genomic DNA Isolation. Digested lysates were poured onto 50 mL spin column matrices. After applying a standard protocol for binding, washing, elution, precipitation, and drying, total DNA was dissolved in an appropriate buffer for subsequent affinity chromatography.

Enrichment of Bacterial and Fungal DNA with LOOXSTER. Total DNA was subjected onto affinity chromatography spin columns. After standard processing, DNA was precipitated, washed, dried and dissolved in 30 μ L water. DNA concentration was determined by the NanoDrop method (Thermo Fisher Scientific, Waltham, MA, USA).

Multiplex PCR Detection of Pathogen-Specific Targets. Standard multiplex PCR was performed within two pools of specific primers. The assay covers the following 34 bacterial and 6 fungal species: *Acinetobacter baumannii*, *Bacteroides fragilis*, *Burkholderia cepacia*, *Aspergillus fumigatus*, *Candida albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. perfringens*, *Enterobacter aerogenes*, *E. cloacae*, *E. faecalis*, *E. faecium*, *Escherichia coli*, *Haemophilus influenzae*, *H. influenzae* capsular type b, *Klebsiella oxytoca*, *K. pneumoniae*, *Morganella morganii*, *Neisseria meningitidis*, *Prevotella buccae*, *P. intermedia*, *P. melaninogenica*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. saprophyticus*, *Stenotrophomonas maltophilia*, *Streptococcus agalactiae*, *S. bovis*, *S. dysgalactiae* subsp. *equisimilis*, *S. mutans*, *S. pneumoniae*, *S. pyogenes*, *S. sanguinis*. In addition, a set of frequent antibiotic resistances (e.g. methicillin, vancomycin, and β -lactamases) are also targeted specifically. *B. subtilis*-specific primers are within each pool as internal run control.

The PCR was set up using the VYOO kit PCR reaction vessels which contain the lyophilized primer mix (final concentration in 25 μ L total reaction volume: 0.1 – 0.4 μ M). In addition, a 25 μ L PCR reaction contained 12.5 μ L of 2x QIAGEN Multiplex PCR Master Mix (QIAGEN, Hilden, Germany) and \leq 1 μ g pathogen enriched DNA filled up with DNA-/DNase-free water. No template controls (NTC) were devoid of DNA. The PCR reaction was performed on the Mastercycler pro (Figure 1). To provide for an optimal performance of this complex multiplex PCR assay it has to be ensured that the device works within its technical specifications. Scheduled maintenance on a regular basis is recommended.

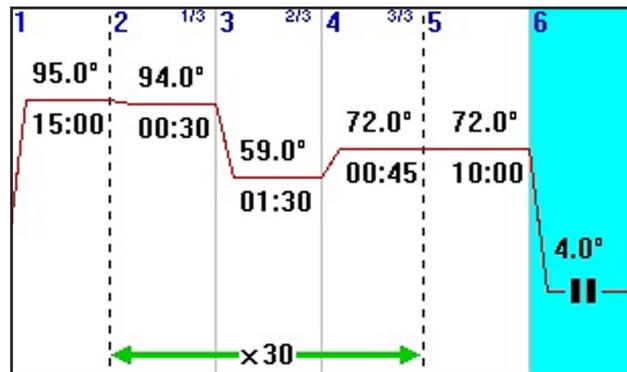


Fig. 1: Temperature profile of the PCR program on the Mastercycler pro.

Multiplex PCR amplicons were analyzed on commercial 3 % TBE agarose gels (ReadyAgarose, Wide-Mini Gel, Bio-Rad GmbH, München, Germany) stained with ethidium bromide and assigned to pathogens and/or antibiotic resistances on the basis of pool-specific length markers.

Results

After preparation of exemplary EDTA whole blood samples from patients suspected for sepsis, agarose gel analysis of PCR products revealed single bands (Figure 2). Amplicons were specific for *Enterococcus faecium* and vancomycin A resistance (pool 2) as compared to species-specific marker bands. Positive control bands (*Bacillus subtilis*) were fully visible and the findings were confirmed by surrounding clinical results. The analytical sensitivity of the assay was tested before to be <10 colony forming units (cfu)/mL. (*E. faecium* and vancomycin A resistance, respectively).

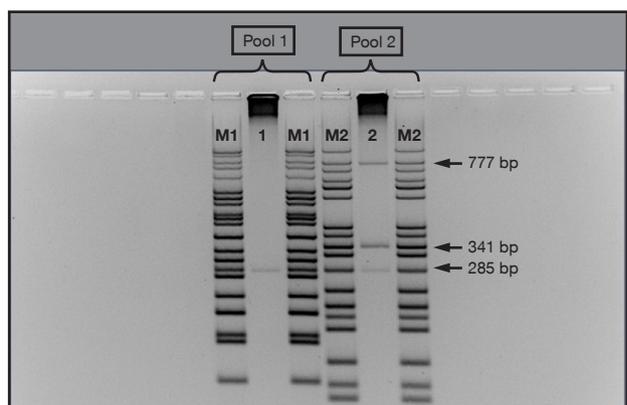


Fig. 2: Proof of *Enterococcus faecium* genomic DNA in EDTA whole blood by VYOO. Samples were taken from ICU patients suspected for sepsis. M1/2: DNA marker pool I/II; 1: sample DNA tested with primer pool I; 2: sample DNA tested with primer pool II; 1: *Bacillus subtilis*-specific band at 285 bp for internal run control; 2: *E. faecium*-specific band at 777 bp, vanA resistance-specific band at 341 bp, *B. subtilis*-specific band at 285 bp (internal run control).

Discussion

The combination of up to 25 primer pairs in a single multiplex PCR reaction is considered to be a challenge regarding sensitivity and specificity. For pathogen detection in body fluids, e.g. sepsis-causative pathogens in whole blood, assays have to cover a capacious panel of targets to yield required clinical utility. While broad-range primers are under ongoing discussion with respect to high numbers of false-positives and the risk of contamination [11], usage of specific primers in combination seems to be an alternative if clinical sensitivity meets the range of diagnostic relevance and if maximum specificity is given.

The presented results of the VYOO PCR detection kit indicate that nucleic acids trace detection is possible applying high plex-grades with desired sensitivity and specificity. The Mastercycler pro fulfills the demanding requirements of such a complex PCR assay: This thermal cycler helps to minimize the technical variance and contributes to a high process reliability in the PCR workflow of such a sensitive and specific assay.

Analysis of whole blood samples by multiplex PCR for bacterial and fungal DNA is a reliable and rapid tool for detection of "low level" infections. It supports and expedites clinical findings and strengthens a directed antibiotic therapy.

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Ordering information

Product	Description	Order no. International	Order no. North America
Mastercycler® pro	(96 well aluminum block), 230 V / 50 - 60 Hz	6321 000.019	-
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	(96 well silver block) 120 V / 50 Hz, with US-plug	6325 000.021	950030020
Mastercycler® pro 384	(384 well aluminum block), 230 V / 50 - 60 Hz	6324 000.010	-
	(384 well aluminum block), 120 V / 50 Hz, with US-plug	6324 000.028	950030030
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