

# Multi-diagnostic qPCR assay for rapid detection of the most frequent human pathogens associated with Sepsis.

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## Introduction

Sepsis is a syndrome commonly called "blood stream infection" defined by the presence of bacteria (bacteremia) or other infectious organisms and/or their toxins in the blood (septicemia). Sepsis is commonly associated with clinical symptoms of systemic illness characterized by a generalized inflammatory response due to the above mentioned bloodstream infection, and is one of leading killers in general intensive care unit population. For the treatment of septic patients, it is important to perform a rapid and accurate identification of the causative microorganisms of the syndrome, in order to use the appropriate medicine. Growth in liquid media is the conventional method for detecting microorganisms associated with this syndrome. However, the blood culture method requires several days to detect and identify the bacteria and to run a later test for susceptibility to antibiotics. Therefore, in the absence of an appropriate diagnostic test, the sepsis-treatment involves broad-spectrum antibiotics in advance of the test, which are seriously increasing resistance to antibiotics as well as the costs related to its treatment. In order to achieve this goal, we have used **LionProbes®**, owned by Biotools B&M Labs., S.A., to detect amplified PCR products belonging to specific bacterial pathogens. Polymorphisms in 16S rDNA gene have been selected to design genus-specific probes for the detection of *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae*, the main microorganisms associated with sepsis under common hospital conditions. We have adapted the qPCR assay to a gelified 96-well plate format to increase the throughput and the use of the **Gelification®** technology (Biotools B&M Labs. S.A.) in order to stabilize the reaction mixture, and to minimize the risk of contamination and non correct manipulation, storage and transportation.

## Materials and methods

We have included in the analysis DNA from clinical isolates of *E. coli*, *K. pneumoniae*, *S. pneumoniae* and *S. aureus* provided by the Hospital Universitario La Paz, Madrid. The choice intends to allocate in a single tube-strip mayor taxonomic groups for nosocomial infection (gram positive and gram negative). The SeptiGap PCR system uses the bacterial 16S rDNA region as the target for amplification and detection. Polymorphisms in this region have been used for the design and validation of the **LionProbes®** (Table 1). We have designed one primer and one **LionProbe®** labeled with 5'-FAM and 3'-TAMRA for each pathogen. This detection combines the use of a DNA polymerase (*Pfu*) with 3'-5' proofreading activity and oligonucleotide labeled with a fluorophore and a quencher. The design of this probe incorporates two mismatched positions at 3' that doesn't hybridize with the template DNA when the rest of the primer is hybridized. Then *Pfu* repairs the error, releasing the quencher at 3', yielding the emission of fluorescence. (Figure 2). PCR reactions were performed using total genomic DNA of the four pathogens as a template. Analysis of PCR results was performed using the Applied Biosystems 7500® Instrument and with a manual interpretation of the amplification curves and melting-point curves. Results were confirmed in Corbett Rotorgene 6000 instrument.

Figure 1: Schematic representation of specific probes designed from ribosomal sequences.

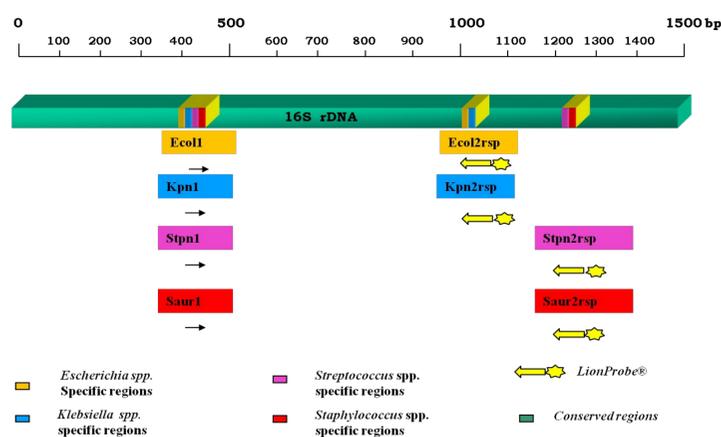


Table 1: Microorganisms and primers used in this study. Real-time PCR results: (1) positive amplification, (0) no results.

Genus	Ecol1/Ecol2rsp	Kpn1/Kp2rsp	Stpn1/Stpn2rsp	Saur1/Saur2rsp
<i>Escherichia coli</i>	1	0	0	0
<i>Klebsiella pneumoniae</i>	0	1	0	0
<i>Streptococcus pneumoniae</i>	0	0	1	0
<i>Staphylococcus aureus</i>	0	0	0	1

Figure 2: Schematic representation of Lionprobes® Real-time PCR detection

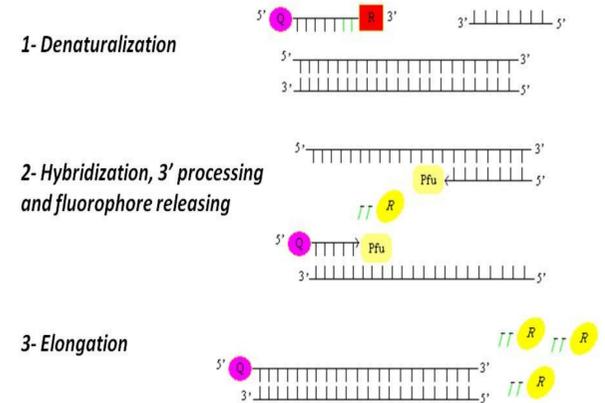
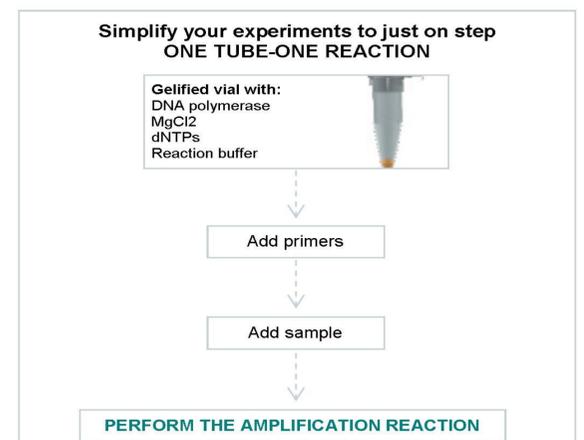


Figure 3: Schematic representation of Gelification® technology



## Results and discussion

We have optimized the PCR reactions for the specific amplification of the four pathogens. All the results are summarized in table 1. Specific real-time amplification was obtained for *E. coli*, *K. pneumoniae*, *S. pneumoniae* and *S. aureus* using the pair of primer-probe Ecol1/Ecol2rsp, Kpn1/Kpn2rsp, Stpn1/Stpn2rsp and Saur1/Saur2rsp respectively (Figures 1, 4). We have compared the  $C_T$  values among different pathogens using an initial concentration of DNA template, detecting  $C_T$  of 18 for *E. coli* and *S. pneumoniae*,  $C_T$  of 19 for *K. pneumoniae* and  $C_T$  of 22 for *S. aureus*. The detection limits of the pairs of probes were established in standard curves representing the threshold cycles ( $C_T$ ) versus the log of 10-fold serial dilutions (1 to  $10^{-6}$  ng/ $\mu$ l) of DNA template of the four pathogens. The lowest estimated amounts of DNA detected by PCR are in the four pathogens in the range of  $10^{-5}$  ng/ $\mu$ l (Figure 5). Rapid diagnosis within species identification will allow the clinician rapid evaluation of septic patient, adjusting the antimicrobial chemotherapy. The advantage of PCR-based detection system is that the microorganism causing sepsis does not have to be viable at the time of sampling. Rapid bacterial identification reduces costs by decreasing the length of hospitalization and conserving hospital resources, narrowing chemotherapeutic treatments.

Figure 4: Specific amplifications of the four pathogens using primer-probes: (A) Ecol1/Ecol2rsp, (B) Kpn1/Kpn2rsp, (C) Stpn1/Stpn2rsp and (D) Saur1/Saur2rsp

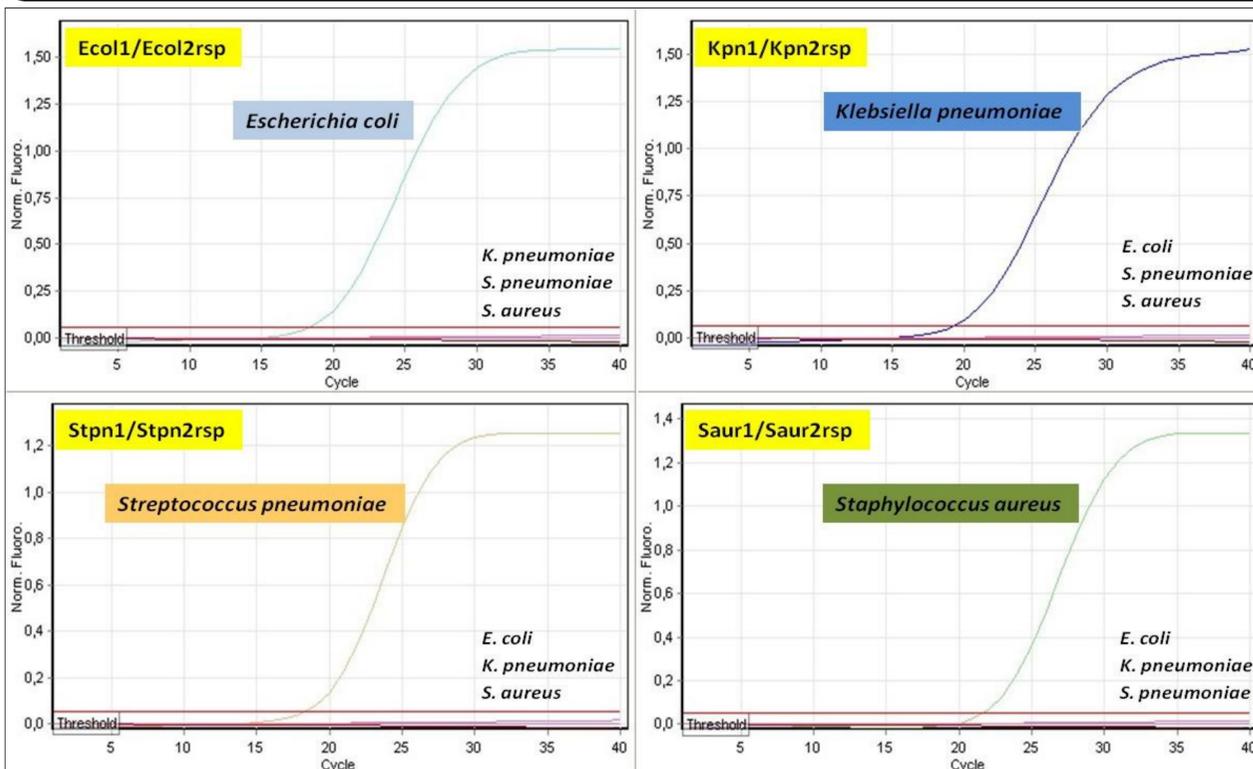
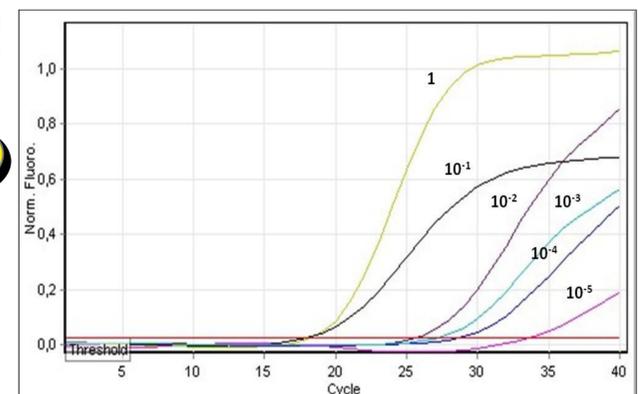


Figure 5: Serial dilutions of genomic DNA (ng/ $\mu$ l) of *E. coli* using Ecol1/Ecol2rsp in RT-PCR



## Conclusions

•We have designed and developed four specific **LionProbes®** for the molecular identification with Real-time PCR of the four main pathogens causing of sepsis: *E. coli*, *K. pneumoniae*, *S. pneumoniae* and *S. aureus* on the basis of the sequence polymorphism of the 16S rDNA region .

•The use of these probes at same PCR conditions allows the specific detection of the four septic nosocomial pathogens.

•Real-time PCR shows that the minimal concentration four causing-sepsis pathogens DNA that can be detected with *these specific LionProbes®* is  $10^{-5}$  ng/ $\mu$ l.