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DIPLOMARBEIT

Titel der Diplomarbeit

Establishment of an internal control for a DNA microarray-based assay aiming at the detection of bloodstream infection relevant pathogens

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1. Abstract

Severe sepsis and septic shock are major healthcare problems and, although there has been extensive research, remain the major causes for mortality in intensive care units. For a faster detection of sepsis causing pathogens a DNA microarray-based assay was established which allows the identification of 30 different bloodstream infection relevant pathogens from whole blood samples.

During this work an internal control was implemented in order to monitor each working step of the microarray protocol, including DNA extraction, PCR and labeling reactions as well as hybridization. Microarray data allow a semi-quantitative analysis of DNA concentrations. However, internal control sequences are needed for normalization and to control obtained data, as signal intensities underlie considerable fluctuations. In order to validate the results of microarray quantification, a real-time PCR protocol was established.

Most PCR based analyses have the shortcoming to only generate information about the presence of bacteria but not about the physiological activity of those bacteria. This problem can be solved by detecting RNA instead of DNA since most RNA is quickly degraded and is only detectable in actively metabolizing cells.

Considering these facts a screening of the viability status of diverse pathogens, based on microarray technology was realized. Therefore the protocol for the identification of pathogens causing bloodstream infections applying a DNA chip was optimized to allow the detection of RNA. Several RNA amplification techniques were tested, among them a method called NASBA (nucleic acid sequence-based amplification). Besides this an mRNA detection protocol for diverse housekeeping genes was established to evaluate the possibility for quantification of mRNA.

Zusammenfassung

Schwere Sepsis und septischer Schock verursachen gravierende Probleme in Spitälern und Ambulanzen. Trotz intensiver Forschung auf dem Gebiet, bleibt Sepsis eine der häufigsten Todesursachen in der Intensivmedizin. Für die rasche Identifizierung von Sepsis verursachenden Pathogenen, wurde ein DNA Microarray entwickelt. Dieser ermöglicht die Identifikation von 30 verschiedenen Erregern von Blutstrominfektionen direkt aus Vollblut.

In dieser Arbeit wurde eine interne Kontrolle etabliert um die Effizienz eines jeden Teilschrittes des Microarray Protokolls zu kontrollieren. Dies inkludiert die DNA Extraktion aus Vollblut, PCR- und Labeling-Reaktionen sowie die Hybridisierung. Darüber hinaus ermöglichen diese Ergebnisse eine semiquantitiative Analyse von DNA Konzentrationen. Die Signalintensitäten der internen Kontrolle werden für eine Normalisierung und Überwachung der Daten von pathogen-spezifischen Sonden benötigt, da diese erheblichen Schwankungen unterliegen können. Ein real-time PCR Protokoll wurde etabliert, um die Ergebnisse der Microarray basierten Quantifizierung zu evaluieren.

PCR basierte Methoden geben nur Ergebnisse über ein Vorhandensein von bakterieller DNA, jedoch nicht über die physiologische Aktivität der Erreger. Anhand der Detektion von mRNA anstelle von DNA kann dieses Problem gelöst werden, da RNA sehr schnell abgebaut wird und nur in Zellen mit aktivem Metabolismus nachweisbar ist.

Aus diesem Grund wurde ein Screeningverfahren basierend auf Microarray Technologie getestet, welches der Bestimmung des Viabilitätsstatus verschiedener Pathogene mittels mRNA Detektion dient. Um eine RNA Detektion zu ermöglichen, wurde das Protokoll für den Identifikations-Chip für Sepsiserreger adaptiert. Hierfür wurden verschiedene RNA Amplifikationstechniken getestet, inklusive einer Methode namens NASBA (nucleic acid sequence-based amplification). Eine Quantifizierung der mRNA verschiedener Haushaltsgene wurde ebenfalls etabliert.

2. Introduction

2.1 Medical Microbiology

Severe sepsis and septic shock are prominent healthcare problems which affect millions of people worldwide each year [Dellinger *et al.,* 2008]. Despite an extensive research for about two decades, sepsis remains a major cause of mortality in intensive care units, with mortality rates ranging from 30 to 70% [Riedemann *et al.,* 2003b]. Levy *et al.* stated that severe sepsis is accounting for 150.000 deaths per year in Europe, thus making it the most common cause of death in noncoronary critical care units. Treatment costs for severe sepsis amount up to €7.6 billion in the EU and sepsis incidences are rising at a rate of 1.5 % per year due to population aging and an increase in immunecompromised people [Hunter, 2006].

The first signs of sepsis are unspecific and often unclear. Within hours patients with no obvious signs of disease can develop severe symptoms that, left untreated, are ultimately leading to death. By definition a patient with sepsis suffers an actual or assumed infection going along with symptoms indicating a SIRS (systemic inflammatory response syndrome) [Rivers *et al.,* 2005]. SIRS criteria, defined in the 1992 statement from the ACCP/SCCM Consensus Conference, are listed in table 1. SIRS is considered to be present when a patient shows at least two of the in 1992 stated criteria [Levy *et al.,* 2003]. However, SIRS being a host response can be caused by an infection but also by non-infectious conditions and therefore does not specifically indicate sepsis [Levy *et al.,* 2003]. Non-infectious conditions stimulating a SIRS are for example trauma, malignancy and surgeries such as coronary artery bypass grafting [Kerbaul *et al.,* 2004; Hunter, 2006].

SIRS criteria, as defined by the ACCP/SCCM Consensus Conference	
Body temperature:	higher than 38°C or lower than 36°C
Heart rate:	over 90 beats per minute
Respiratory rate:	higher than 20 breaths per minute or a $PaCO_2$ lower than 32 mmHg
Leukocyte count:	higher than 12,000 cells/µl or lower than 4,000 cells/µl

Table 1: Clinical findings indicating the presence of a SIRS (systemic inflammatory response syndrome) as defined by the ACCP/SCCM Consensus Conference in 1992 [Levy *et al.,* 2003; Rivers *et al.,* 2005].

Severe sepsis is defined as sepsis accompanied by a dysfunction of an organ (e.g. the hematologic system, the pulmonary system, the renal system) [Levy *et al.,* 2003].

Patients suffering from septic shock experience a state of falling blood pressures (arterial hypotension) and require vasopressor support [Rivers *et al.,* 2005; Levy *et al.,* 2003]. Hypotension is identified by a systolic blood pressure lower than 90 mmHg or a mean arterial pressure lower than 65 mmHg [Rivers *et al.,* 2005].

Until recently sepsis was considered an over the top immune response [Hotchkiss and Karl, 2003]. Inflammation normally helps to eliminate microorganisms from an origin of infection. However, in sepsis patients inflammation becomes a systemic syndrome showing numerous symptoms (see SIRS definition) [Decker *et al.*, 2004]. Accounting to these assumptions, clinical trials conducted during the past 40 years were mainly focused on anti-inflammatory agents [Riedemann *et al*., 2003a]. However, none of these studies showed significant improvement in survival of septic patients [Hotchkiss and Karl, 2003; Riedemann *et al*., 2003a].

One applied target for therapy was tumor necrosis factor alpha (TNF-α). It was shown that the administration of high amounts of endotoxins or bacteria in animal models, lead to high levels of circulating cytokines like TNF-α [Hotchkiss and Karl, 2003]. Additionally injection of TNF-α into animals evoked symptoms comparable to those of sepsis [Riedemann *et al*., 2003a]. These animals died from an endotoxic shock and passive immunization against TNF-α increased survival rates [Hotchkiss and Karl, 2003; Riedemann *et al*., 2003a]. However, because therapy approaches in humans failed, it was considered that these animal models do not mirror the disease pattern of sepsis patients [Hotchkiss and Karl, 2003].

Fast detection of pathogens is critical for survival of patients with bloodstream infections and septicaemia. A large scale study of Kumar *et al.*, including 2.731 patients suffering from septic shock, showed that over the first 6 hrs after the onset of recurrent or

persistent hypotension, a critical marker of septic shock, mean survival rates decreased by 7.6 % with each hour passed without initiation of effective antimicrobial therapy. The time span before effective treatment and associated survival rates are illustrated in figure 1. While 82.7 % of patients survived if the time span between first evidence of hypotension and administration of effective antimicrobials was 30 minutes, the survival rate was only 42.0 % if it took more than five hours for patients to receive the appropriate treatment [Kumar *et al.,* 2006].

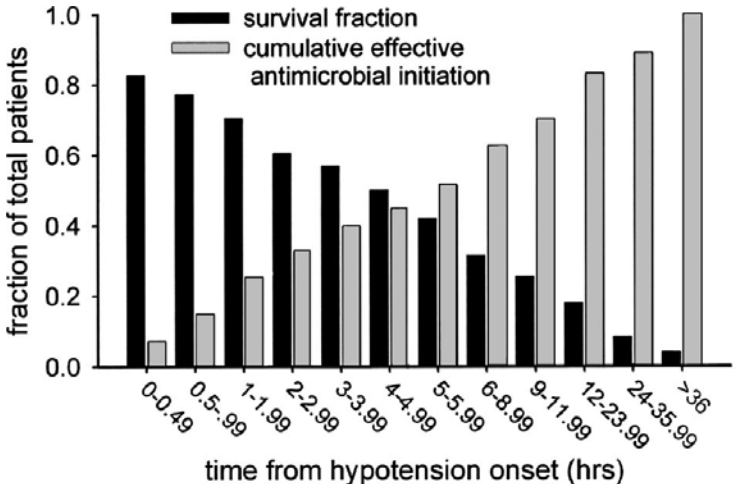


Figure 1: Survival rates of patients who received effective antimicrobial treatment after 0 to over 36 hours after showing first signs of hypotension (a critical marker of septic shock) are illustrated in this figure. The y-axis represents the fraction of total patients while the x-axis represents the time passed after hypotension onset. Black bars indicate survival rates while grey bars represent the fraction of patients receiving effective treatment at a given time point [Kumar *et al.*, 2006].

2.2 Clinical Diagnostic: Traditional microbiological techniques

Blood cultures are currently the standard technique for the detection and identification of bloodstream infection inducing pathogens in patients with fever [Peters *et al*., 2004; Wiesinger *et al*., 2007]. Since Libman in 1906 first published guidelines on the realization of over 700 blood cultures, the techniques have evolved leading to fully automated systems for the detection of microbial growth [Peters *et al*., 2004; Libman, 1906]. Today automated systems, such as BacT/Alert (Biomérieux, Lyon Area, France) and BACTEC9240 (BD, Franklin Lakes, USA) are the standard cultivation procedures in clinical diagnostics [Wiesinger *et al*., 2007].

Both systems detect an increase in CO_2 levels, an indicator for microbial growth, using continuously monitored fluorescent (BACTEC9240) or colorimetric (BacT/Alert) sensors [Shigei *et al*., 1995; Nolte *et al.,* 1993; Kocoglu *et al.,* 2005].

Automated blood culture systems have considerably reduced the time needed to detect positive blood cultures [Shigei *et al.*, 1995]. However, as blood culture systems are dependent on microbial growth rates, results are obtained after a minimum of 24 hours [Wiesinger *et al.*, 2007]. Other limitations of blood cultures are the often poor sensitivity for slow growing and fastidious pathogens and the reduction in sensitivity when blood samples are taken after the onset of antimicrobial therapy [Peters *et al.*, 2004].

Therefore, nucleic acid-based molecular techniques for the identification of microorganisms become more and more influential [Peters *et al.*, 2004].

2.3 Clinical diagnostic: Nucleic acid-based methods

Molecular biology was revolutionized by the invention of the polymerase chain reaction (PCR) by Mullis in 1984 [Mullis *et al.*, 1986]. In the following years detection of nearly every clinically relevant bacterial pathogen was allowed via new PCR-based assays [Mothershed and Whitney, 2006]. Also, in the late 1980s other nucleic acid amplification methods were developed, including ligation-mediated amplification [Wu and Wallace, 1989] and transcription-based amplification [Kwoh *et al.,* 1989] [Monis and Giglio, 2006]. However, these methods never gained equal acceptation for diagnostic applications than PCR methods. This might arise from the superiority of PCR applications regarding simplicity and cost-effectiveness [Monis and Giglio, 2006].

The main advantage of nucleic acid-based assays is their shorter time to results compared to conventional blood culturing methods, since they are not dependent on bacterial growth [Mothershed and Whitney, 2006]. Also, lower detection limits and the detection of specific microorganisms, including non-cultivable or fastidious microorganisms, can be accomplished by the use of nucleic acid-based techniques. However, there are some disadvantages such as false positive signals due to contamination or the lack

of viability information due to the solely detection of pathogen specific nucleic acids [Mothershed and Whitney, 2006].

Several nucleic acid based methods applied in clinical diagnostics are briefly presented in the following chapters and further detailed information is given on methods used in this work.

2.3.1 Nested PCR

Nested PCR is a conventional PCR method with initial target amplification with an outer primer pair followed by a second amplification with an inner primer pair. Because of the second amplification nested PCR shows an improved sensitivity [Mothershed and Whitney, 2006; Ratcliff *et al.*, 2002].

2.3.2 Ligase chain reaction (LCR)

LCR is based on the ligation of two adjacent oligonucleotide primers that hybridize to the target DNA [Wiedmann *et al.,* 1994]. It is based on a thermostabile ligase, which will only ligate the two probes if they exactly match the template DNA, making LCR a candidate method for the detection of single nucleotide polymorphisms (SNPs) [Wiedmann *et al.*, 1994; Monis and Giglio, 2006]. The two primers are positioned in a way that the SNP overlaps with the 3' end nucleotide of the upstream primer. If the sequences match, the ligase will join the two primers. In order to exponentially amplify the product of the ligation, a second pair of primers is used. In a cycling reaction the ligation products can serve as a template for the next ligation reaction, which leads to doubling of the ligation product in each cycle, similar to PCR amplification [Wiedmann *et al.*, 1994]. LCR is a promising technology especially for SNP detection and for use in microarrays, despite some recently reported problems with reproducibility [Monis and Giglio, 2006]. A commercially available test for *Clamydia* detection based on LCR was taken off the market in 2003 because of problems concerning negative controls and non-repeating positives [Mothershed and Whitney, 2006; Monis and Giglio, 2006].

2.3.3 Strand displacement amplification (SDA)

SDA is an isothermal amplification technique for DNA [Walker *et al.,* 1992]. It is rather complicated as it requires two sets of primers (which bind adjacent to each other), two enzymes and a modified deoxynucleotide (dATPS) [Monis and Giglio, 2006]. The initial steps of the reaction include the primers binding in positions flanking the target sequence. The inner primer pair (closest to the target sequence) have restriction enzyme recognition sites on their 5' ends. The four primers are then extended by an exonuclease deficient DNA polymerase (Klenow fragment). Products of the outer primer pair displace the products of the inner primer pair, which then serve as template for further extensions. Extension and displacement reactions finally produce hemiphosphorothioate dsDNA with restriction recognition sites on one or on both ends, respectively (see figure 2) [Walker *et al.*, 1992]. These hemiphosphorothioate restriction recognition sites are cut by the restriction enzyme, generating a single stranded nick. The nick is then repaired by DNA polymerase, generating a new strand of DNA which displaces the strand downstream to the nick. The process is exponential because the displaced strand serves as target for new primers (of the inner primer pair) promoting further extensions [Monis and Giglio, 2006; Walker *et al.,* 1992]. SDA allows the production of 10^9 copies of target DNA per reaction and is the basis for some commercially available detection tests, for example BDProbeTec (Becton Dickinson) [Mothershed and Whitney, 2006].

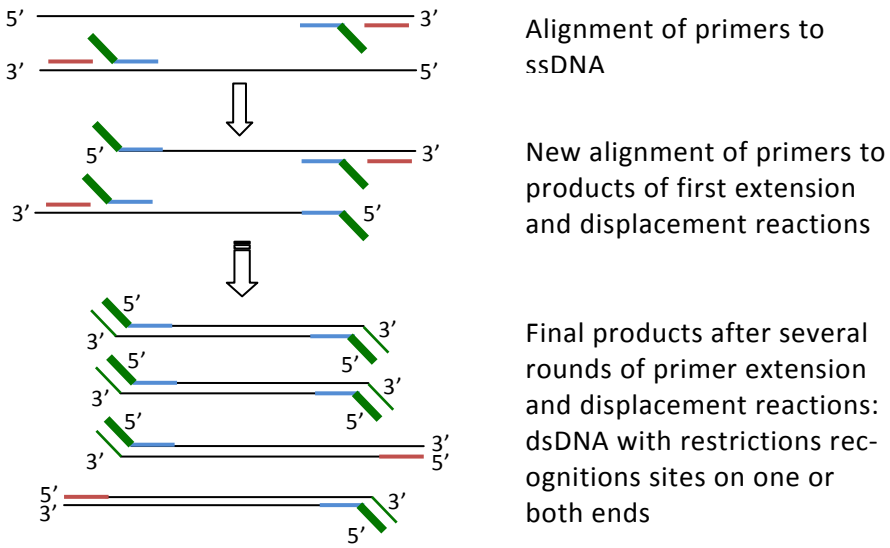


Figure 2: Extension and displacement reactions in SDA generating hemiphosphorothioate dsDNA with restriction recognitions sites on one of both ends, respectively. **Red lines**: outer primer pair; **blue and green lines**: inner primer pair with restriction recognition site (bold green lines).

2.3.4 Hybrid capture tests

The Hybrid Capture (HC) assays from Digene (Gaithersburg, MD), aim for signal amplification instead of target amplification. The HC1 assay introduced in 1995 aimed to detect 14 human papilloma virus (HPV) types, and was improved in a second generation of hybrid capture assays (HC2) [Vernick and Steigman, 2003]. The concept is the hybridization of pathogenic DNA to a specific RNA probe to form a RNA/DNA hybrid. This hybrid is then captured by specific antibodies that bind it to a solid substrate, and is subsequently detected by a second antibody carrying the alkaline phosphatase enzyme. Alkaline phosphatase cleaves the phosphate moiety from a specific luminescent substrate which then emits light. Signal amplification is achieved because many antibodies bind to one RNA/DNA hybrid [Mothershed and Whitney, 2006].

2.3.5 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization applying fluorescently labeled rRNA probes can detect and identify microorganisms in complex samples such as blood culture and tissue within a few hours. Even multiple species can be detected in one assay when the specific probes are labeled with different dyes. The procedure involves fixing of the microbial cells with suitable chemicals, permeabilizing the cells and finally hybridizing the sample on a glass slide or in solution with the 15-25 nucleotide long probes. After a washing step fluorescence detection is realized by fluorescence microscopy or flow-cytometry [Wagner *et al.,* 2003; Mothershed and Whitney, 2006]. Specific FISH probes were developed for the identification of many bacteria species, including *Pseudomonas aeruginosa* [Jansen *et al.,* 2000], *Staphylococcus spp.* [Kempf *et al.,* 2000], *Streptococcus spp.* [Trebesius *et al.,* 2001] and several others.

2.3.6 Mass spectrometry

Mass spectrometry is an analytical technique that ionizes target molecules by firing electrons at them. The mass/charge ratio of the generated charged molecules is then analyzed and a molecular signature is generated [Mothershed and Whitney, 2006]. Important information about biomolecules such as their molecular composition and structure

can be obtained by mass spectrometry. However, at the beginning, analysis of nucleic acids was limited to oligonucleotides shorter than ~20 bp, due to inefficient generation of gas-phase ions of larger samples [Meng *et al.,* 2004; Gut, 2004]. New ionization techniques like electrospray ionization (ESI) and matrixassisted laser desorption/ionization (MALDI) solved that problem and revolutionized analysis of nucleic acids by mass spectrometry [Meng *et al.,* 2004]. MALDI is fast and in combination with time-of-flight analysis (MALDI-TOF) especially suited for analysis of complex molecules [Gut, 2004]. Therefore mass spectrometry with MALDI-TOF ionizations is considered a valuable tool for bacterial detection in clinical diagnostics [Mothershed and Whitney, 2006].

2.3.7 Real-time PCR and RT-PCR

During real-time PCR the DNA amplification and detection is carried out simultaneously. The advantages to conventional PCR based amplification are lower duration and a reduction of the risk for contamination, since both steps are performed in a closed system without the need for an agarose gel electrophoresis [Peters *et al.,* 2004; Rosey *et al.,* 2007; Tobler *et al.,* 2006]. Real-time PCR methods are based on the generation of a fluorescent signal in each cycle which can be measured directly and simultaneously [Peters *et al.,* 2004]. Detection chemistries can be divided into two groups: intercalating dyes which bind to dsDNA (eg SYBR® Green, ethidium bromide, SYTO9) and probe based detection systems (eg TaqMan® probes, 3' MGB probes, molecular beacons) [Monis and Giglio, 2006]. The detection methods applying TaqMan® probes and SYBR Green® will be explained in more detail, because these two methods are among the most important ones.

2.3.7.1 SYBR® Green Real-time PCR:

SYBR® green is a molecule which fluoresces when bound to dsDNA. With each PCR cycle more dsDNA is generated, leading to an increase in fluorescence by SYBR® green. Because no probes are needed, SYBR® green is a less expensive method to detect DNA amplification [Mackay *et al.,* 2002]. Amongst its disadvantages is that SYBR® green detects any dsDNA, including primer-dimers and non-specific amplification products. However, melting curve analysis allows the discrimination of specific product from nonspecific products or primer-dimers [Mothershed and Whitney, 2006].

2.3.7.2 TaqMan® Real-time PCR:

This detection method relies upon fluorescence resonance energy transfer (FRET), between a fluorophore and a non-fluorescent quencher [Mackay *et al.,* 2002]. The TaqMan® probe is a 20-30 bp long oligonucleotide, which is specific to a certain sequence within the desired amplicon. This probe carries the reporter fluorescent dye (e.g. TAM) at the 5' end and the quencher (e.g. TAMRA) at the 3' end [Peters *et al.,* 2004; *Gibson et al.,* 1996]. On the complete TaqMan® probe the fluorescent dye and the quencher molecules lie in close proximity, therefore fluorescent emission of the dye is extinguished by the quencher [Mackey *et al.,* 1992; Gibson *et al.,* 1996]. During PCR the Taq Polymerase extends the new DNA strand and displaces and hydrolyses the probe via its 5'-3' exonuclease activity. This releases the reporter dye from the quencher, generating a fluorescent signal [Peters *et al.,* 2004; Mackay *et al.,* 2002]. In TaqMan® assays no melting curve analysis can be performed to validate the amplification of a specific product, therefore it is especially important to test the specificity of primer/probe combinations [Monis and Giglio, 2006].

The kinetics of PCR ought to be known to analyze real-time PCR results. When fluorescence intensity is plotted against the cycle number in an ideal real-time PCR reaction, a sigmoid growth curve is generated, which can be seen in figure 3 [Mackay *et al.,* 2002]. PCR amplification can be divided into four stages. It starts with a pre-log phase where amplification is not yet fully efficient. This phase is followed by the logarithmic phase, in which the DNA copy number ideally doubles every cycle [Higuchi *et al*., 1993]. Then the reaction slows due to product accumulation and limitation of primers and enzyme. This phase is called the transition phase, which finally leads to the plateau phase in which little or no further amplification of DNA takes place [Mackay *et al.*, 2002].

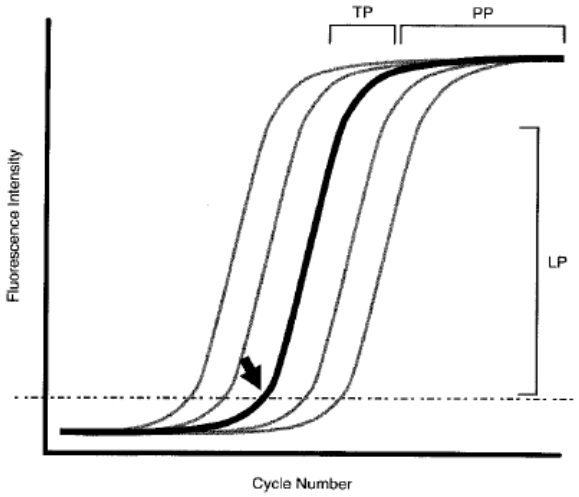


Figure 3: Cycle numbers were plotted against fluorescence intensity in real-time PCR. Logarithmic (LP), transition (TP) and plateau phases (PP) are indicated. At the dash dotted line the measured fluorescence intensity goes beyond a background level and is now clearly detectable. This cycle number (black arrow) is called the threshold cycle, which is an important value in real-time PCR. The threshold line is set by the user and should lie within the logarithmic phase of the PCR reaction.
The threshold cycle number depends on the initial DNA copy number, thus making real-time PCR an effective method to quantify DNA. Different starting amounts of DNA produce a shift in the sigmoidal growth curve either to the left (less starting DNA) or to the right (more starting DNA) leading to a change in threshold cycles.

Early cycles of amplification are not detectable, because the specific fluorescence cannot be distinguished from background fluorescence. The cycle at which the specific fluorescence first reaches significant levels, is called the threshold cycle and is dependent on the starting copy number of DNA [Mackay *et al.*, 2002]. Samples containing more DNA will reach significant fluorescence intensity at an earlier cycle than samples containing only small amounts of DNA. Quantification is possible by relating the threshold cycles of unknown samples to the values of a standard curve [Peters *et al.*, 2004].

Therefore the DNA amplification of real-time PCR needs to be highly efficient. Reliable quantification can only be achieved when the target sequence is logarithmically amplified, which is a prerequisite for the comparison of different samples. Logarithmic amplification is achieved when the amount of DNA doubles every cycle. Therefore after n cycles the amount of DNA is the 2^n fold of the starting amount. To calculate the efficiency of the PCR a dilution series is amplified. In a ½-dilution series the single steps should

differ by 1 cycle and 1/10-dilutions should differ by ~3.3 cycles (because $2^{3.3}$=10). The final copy number can be calculated applying the following formula:

$$final\ copy\ number = initial\ copy\ number \times 2^n$$

with n representing the number of thermal cycles.

2.3.8 Microarray technology

DNA microarray technology allows the parallel and high throughput analysis of thousands of genes, or the simultaneous identification of many different bacteria and viruses in a single assay [Borel *et al.,* 2008; Loy and Bodrossy, 2006].

Only 10 years ago analysis of gene expression was a slow, low throughput process, where only one gene at a time was investigated typically by northern blot analysis [Hardiman, 2004]. The post-genomic era, with publicly available sequences of whole genomes, drastically changed science and scientific methods. Microarrays, which allow a highly parallel analysis of gene expression, perfectly represent that post-genomic era of high throughput science [Hardiman, 2004].

2.3.8.1 What is it and how does it work?

A microarray is a solid substrate (e.g. a glass slide) to which the capture DNA, either in form of oligonucleotides or the cDNA of whole genes, are immobilized by covalent linkage [Bryant *et al.,* 2004]. Protein microarrays, which apply proteins instead of DNA as capture molecules, usually consist of immobilized antibodies or antigens. DNA is transferred onto the array surface in form of microscopic spots, each consisting of thousands of oligonucleotide copies. Nucleic acids in the test sample (the target DNA) are labeled (e.g. via fluorescence dyes) and then hybridized to the capture DNA on the chip. After hybridization of the target DNA, spots with complementary probes will emit fluorescence, which can be detected by specific scanners.

In a two color analysis the samples are taken under different circumstances and are labeled with two different fluorescent dyes, which allow sample discrimination on the chip (e.g. one testing sample and one comparison or standard sample). Two different fluorophores are widely used for labeling of DNA during microarray experiments. They

are named Cy3 and Cy5 and emit light at 570 nm (green part of the spectrum) and 670 nm (red part of the spectrum), respectively. When sample DNA is labeled with both dyes and hybridized to one spot it appears yellow, meaning that respective sequence is present in both samples. Up and down regulated genes in the samples can be identified with this system based on the color of the spots [Hardiman, 2004]. Figure 4 shows a DNA microarray hybridized to fluorophore-labeled *E. coli* DNA (green spots).

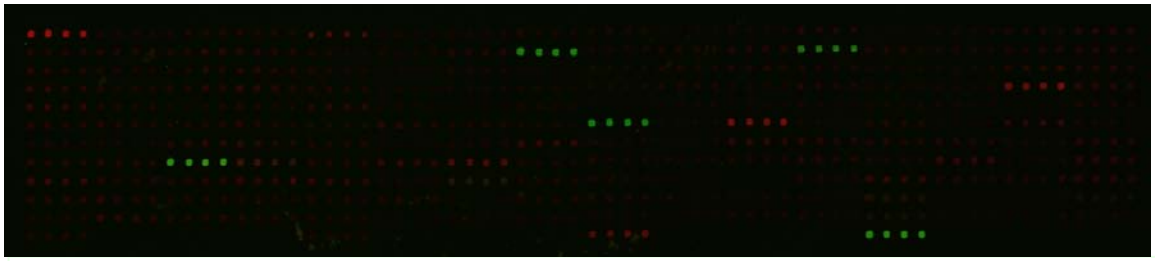


Figure 4: The microarray results of a hybridization with labeled *E. coli* DNA are shown in this figure. **Green spots**: *E. coli* DNA labeled with the dye Atto532, hybridized to chip probes. **Red spots**: hybridization control DNA labeled with Cy5.

2.3.8.2 Fabrication of microarrays

Different types of microarrays can be distinguished by their spotting method. There are contact (e.g. pins) and non-contact (piezoelectric and photolithographic) techniques, which differ in their mode of probe deposition. Contact printing is widely used and includes methods such as contact pin printing and microstamping. There are several non-contact printing methods and most of them originated from the printing industry. Ink-jetting methods are thermal ink jet, microsolenoids, piezoelectric, and acoustic ink printing [Hsieh *et al.,* 2004]. Other non-contact printing methods include photochemistry-based methods (e.g. photolithographic spotting), laser writing and electrospray deposition [Barbulovic-Nad *et al.,* 2006]. Contact pin printing, piezoelectric printing and photolithographic spotting will be discussed in detail.

Contact pin printing: Microarrays are produced by printing presynthesized ssDNA (or dsDNA) onto glass slides [Kato-Maeda *et al.,* 2001]. Especially full-length cDNAs but also oligonucleotide probes are used for this platform. A robot arm which carries a set of pins (see figure 5), first dips the pins into the probe suspension and then prints the probes onto specific locations on the glass slides. Glass slides with different surfaces (amino-propylsilane reactive groups, aldehyde reactive groups, epoxy reactive groups)

are available and the chemistry by which the DNA is linked to the slide depends on the surface. Aldehyde slides have aldehyde groups as reactive group on the glass surface. DNA probes are 5' terminally amino modified and bind to the aldehyde groups by forming a Schiff base, thus covalently linking the DNA to the glass slide. Spotted DNA microarrays can be produced "in house" also by research institutions.

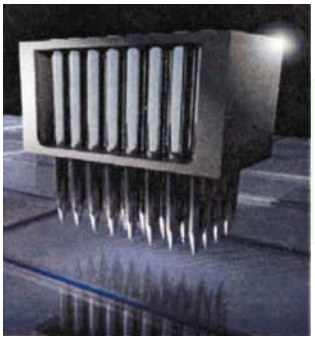


Figure 5: A picture of a microarray spotter head with pins [Kato-Maeda *et al.,* 2001].

Piezoelectric printing: Most inkjet printers produce heat to vaporize the ink and create a bubble. By expanding this bubble a tiny amount of ink is pushed out of the nozzle and onto the paper. This technique, however, cannot be used for biological material as the heat would damage proteins and DNA [Hsieh *et al.,* 2004]. In piezoelectric systems a small piezoelectric element is located near each nozzle. When a small electric charge is applied to the piezo ceramics it causes a change in shape [Hsieh *et al.,* 2004] or generates an acoustic wave [Nagaraj *et al.,* 2008] which leads to displacement of a small droplet of sample from the nozzle. The advantages of this method are the variable and small droplet size (tens to hundreds of picoliters) and the high-ejection rate [Hsieh *et al.*, 2004; Nagaraj *et al.,* 2008].

Photolithographic spotting: By photolithography production, arrays with extremely high information content can be produced. They are constructed by directly synthesizing 25 bp (Affimetrix) to 60 bp (Agilent) long oligonucleotides *in situ* onto the solid substrate [Hardiman, 2004]. A schematic representation of the process is shown in figure 6. A solid support is covered with light-sensitive masking agents. Upon illumination through a photolithographic mask, selected areas are deprotected and reactive hydroxyl groups are exposed. 5'-hydroxyl protected nucleotides then couple to the deprotected areas,

followed by capping and oxidation steps. The process is repeated with a different mask to deprotect other areas and gradually "build" a sequence one nucleotide after the other [Lipshutz *et al.,* 1999; Pease *et al.,* 1994]. Such high density oligonucleotide arrays, however, cannot be produced in house and are only available from commercial suppliers [Kato-Maeda *et al.,* 2001]. Especially noteworthy are GeneChips from Affimetrix. The company pioneered the field of microarray technology and their GeneChips are quite popular due to their high number of probes, a good level of reproducibility and because they are delivered ready for use [Hardiman, 2004]. Other vendors of microarray platforms are Agilent and Amersham Biosciences.

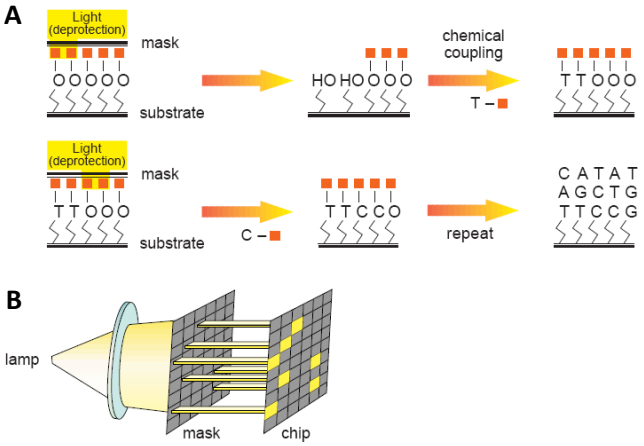


Figure 6: Representation of the photolithographic spotting technique. **A**: graphical representation of the spotting process; the orange boxes represent protections groups; for further information about the spotting process see text; **B**: schematic of the lamp, mask and array in photolithographic spotting [Lipshutz *et al.,* 1999]

2.3.8.3 Analysis of microarray data

Microarray experiments produce an enormous amount of data. **Normalization** of microarray data is a crucial step in making them comparable. Normalization generally requires a set of probes that are expected to give constant signals (e.g. housekeeping genes) [Bryant *et al.,* 2004]. However, if such probes are not available a quantile normalization approach can be chosen [Wiesinger *et al.,* 2007]. Normalization of other factors, such as background fluorescence, variation in DNA amount in different spots, differences between slides, is done by specialized software [Bryant *et al.,* 2004].

Clustering of data allows the classification and grouping of genes based on similarities and differences in their expression patterns, which is represented by different signal intensities after hybridization. All different clustering algorithms have in common the assumption that genes with similar expression pattern are likely to engage in related processes [Ferea and Brown, 1999]. There are a variety of clustering methods, each having its strengths and weaknesses, which can be divided into the following two groups: designated supervised and unsupervised clustering. Supervised clustering uses reference vectors (based on external biological or clinical knowledge) while in unsupervised clustering no such vectors are applied [Eisen *et al.,* 1998].

The patterns created by the clustering software can be represented in **expression or heat maps**, that allow the data to be observed in an intuitive manner [Ferea and Brown, 1999; Eisen *et al.,* 1998]. A heat map is a two-dimensional graphical representation of the data, in which for example probes and hybridizations correspond to columns and rows, respectively. Also colors are used to illustrate values quantitatively and qualitatively [Eisen *et al.,* 1998; Wiesinger *et al.,* 2007]. An example of a heat map is shown in figure 7.

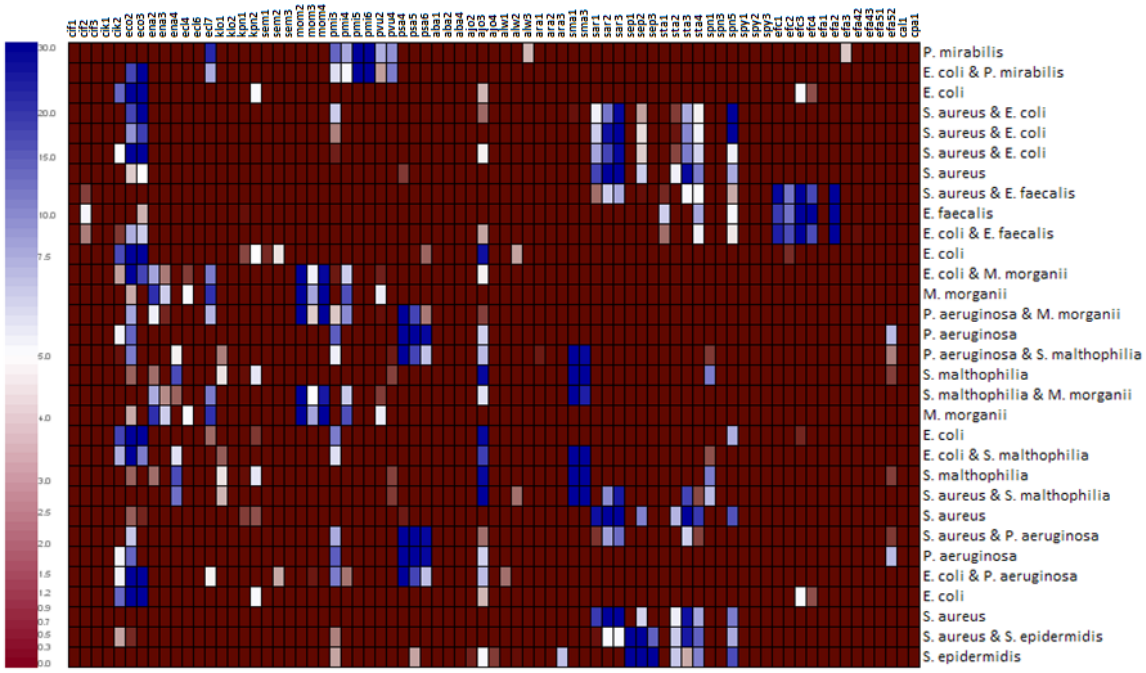


Figure 7: Example of a heat map, drawn after a hierarchical clustering. Data were obtained by a parallel identification of different pathogens using microarray technology. Probes are printed in columns and hybridizations are printed in rows. The color corresponds to signal intensities where the color blue represents high signal values and the color red represents low or no signal values. Image by Wiesinger *et al.,* 2007.

2.3.8.4 Applications

One of the most important applications for microarrays is gene expression monitoring. In order to understand gene function, its expression is investigated in manner of time and reason [Lipshutz *et al.,* 1999]. To address these questions, the expression of large numbers of genes needs to be observed in different tissues at several times and under different circumstances. Especially oligonucleotide arrays with millions of probes are well suited for this task [Lipshutz *et al.,* 1999].

Since many pathogenic genomes are sequenced and the human genome project is completed, new methods for the identification of pathogen host interactions are available [Bryant *et al.,* 2004]. DNA Microarrays are ideal to study those interactions because they allow the parallel, high-throughput detection of thousands of genes [Bodrossy and Sessitsch, 2004; Bryant *et al.,* 2004].

Microarrays also have big potential in many other areas of microbiology including the field of clinical diagnostics. By a careful selection of probe sets, several thousand microbial strains and also species, genera and families can be detected and distinguished in a single assay [Bodrossy and Sessitsch, 2004]. For infection diagnostic applications the main advantage of new microarray techniques are speed and accuracy. Reliable results are obtained after maximal 6 hours with further potential for reduction of total analysis time.

An ideal target gene for probe design is the 16S rRNA gene. It is present in all bacteria and encodes the same product in all bacteria. The 16S rRNA gene consists of many different domains. Some of them are highly conserved with almost identical sequences in different species and others have variable sequences. These inspired Woese in 1987 to consider rRNA the “ultimate molecular chronometer” [Barken *et al.,* 2007]. Figure 8 shows a schematic representation of the 16Sr RNA. Probes against highly conserved regions of the 16S rRNA gene will detect almost any bacterial species, while probes against variable regions of the gene are specific for a few or even only one bacterial species. Conserved regions are also the optimal target for universal primers in order to amplify DNA from unknown species.

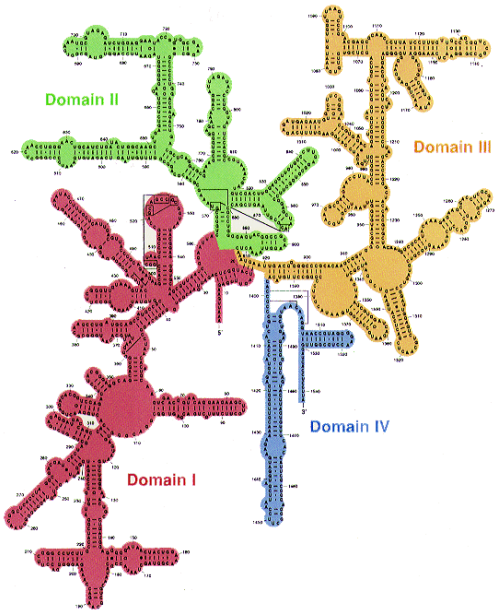


Figure 8: A schematic representation of the secondary structure of the 16S rRNA of *E. coli.* The colors represent the different domains of the rRNA. Image by Garrett und Grisham, 1995

On this concept Wiesinger *et al., 2007* established a DNA microarray-based assay which allows the identification of 30 different bloodstream infection relevant pathogens (bacteria and fungi) from whole blood samples. On the DNA chip 96 probes specific for sequences within constant and variable regions of the 16S rDNA gene (for fungi the 18S rDNA gene) were spotted, allowing species-specific identification of human pathogens. This concept is considered to allow fast detection of sepsis or septicemia causing microbes also in clinical practice.

2.4 Assessment of bacterial viability

Traditionally used culture methods may be inferior to PCR based methods in terms of handling-time and sensitivity but they only detect viable organisms [Birch *et al.,* 2001]. The new generation of PCR based methods detect bacteria at higher sensitivity. However, they do not provide any information about the viability status of the cells. Inactive bacteria, such as dormant cells and also dead cells with their DNA not yet degraded, are all detected in the same way as active cells [Bodrossy *et al.,* 2006].

It was believed that DNA would be degraded more rapidly than any other cellular components in dead cells, making intact DNA a good indicator for the viability of bacteria. Later it was shown that the correlation between cell viability and detection of DNA is

actually very poor [Keer and Birch, 2003]. Masters *et al.* found that in actively killed cells, DNA can be detected even after long periods of time, when plate counts have long declined to zero [Masters *et al.,* 1994].

Messenger RNA (mRNA) is a very labile molecule with a half-life of seconds to minutes and was proposed to be a better target for analyzing bacterial viability. Ribosomal RNA (rRNA), which has a longer half-life than mRNA was also tested as a marker for viability. In these test a good correlation between detection of rRNA and viability of the cells was found. However, mRNA was found to be a better indicator for bacterial viability than rRNA [Keer and Birch, 2003].

Both the rRNA per cell and the RNA:DNA ratio strongly correlate with the cells' growth rate. This positive correlation has been shown for a number of bacteria including *E. coli*, *A. aerogenes*, *S. typhimurium* and some resin acid-degrading bacteria [Muttray and Mohn, 1999; Kemp *et al.*, 1993]. The chemical composition of the growth medium only influences DNA and RNA quantities of a cell by affecting its growth rate [Muttray and Mohn, 1999].

In the following two methods for the amplification of RNA are presented:

2.4.1 One step RT-PCR

RNA can be amplified by reverse transcription of the RNA into cDNA followed by a PCR reaction. This approach, however, has several disadvantages. Due to two separate reactions the risk of a contamination of the sample is increased, as well as time consumption and labor.

In one step RT-PCR (reverse transcription-PCR), both enzymes, the reverse transcriptase and the DNA polymerase, are together in one reaction mix, allowing reverse transcription and DNA amplification to take place in a one reaction step. First an incubation at approximately 37-42°C is carried out for generation of cDNA from the RNA template. This step is followed by normal PCR temperature cycles, starting with an incubation step at 95°C. Because reverse transcriptase is inactivated at high temperatures and the appli-

cation of a hot-start DNA polymerase, reverse transcription and DNA amplification reactions do not occur simultaneously but sequentially in a single tube.

One step RT-PCR kits are distributed by many vendors including Qiagen (OneStep RT-PCR Kit), Invitrogen (SuperScript™ III One-Step RT-PCR System), Clontech (TITANIUM™ One-Step RT-PCR Kit) and Applied Biosystems (TaqMan® Gold RT-PCR Kit).

2.4.2 Nucleic acid sequence-based amplification (NASBA)

In order to realize reverse transcription of RNA to cDNA and simultaneous amplification of cDNA a method called NASBA (nucleic acid sequence-based amplification) was introduced.

NASBA is an isothermal RNA amplification method utilizing the concurrent activity of three enzymes: avian myeloblastosis virus reverse transcriptase (AMV-RT), RNase H, and T7 RNA polymerase [Churruca *et al.,* 2007; Morre *et al.,* 1996]. The method was first described by Kievits *et al.* in 1991 and has since then been applied for the detection of many pathogens, including human immunodeficiency virus type 1 (HIV-1) RNA [Bruisten *et al.,* 1993; van Gemen *et al.,* 1994], *Campylobacter jejuni* and C. *coli* in chicken meat [Churruca *et al.,* 2007], *Escherichia coli* [Keer and Birch, 2003] and *Mycoplasma pneumonia* [Loens *et al.,* 2003].

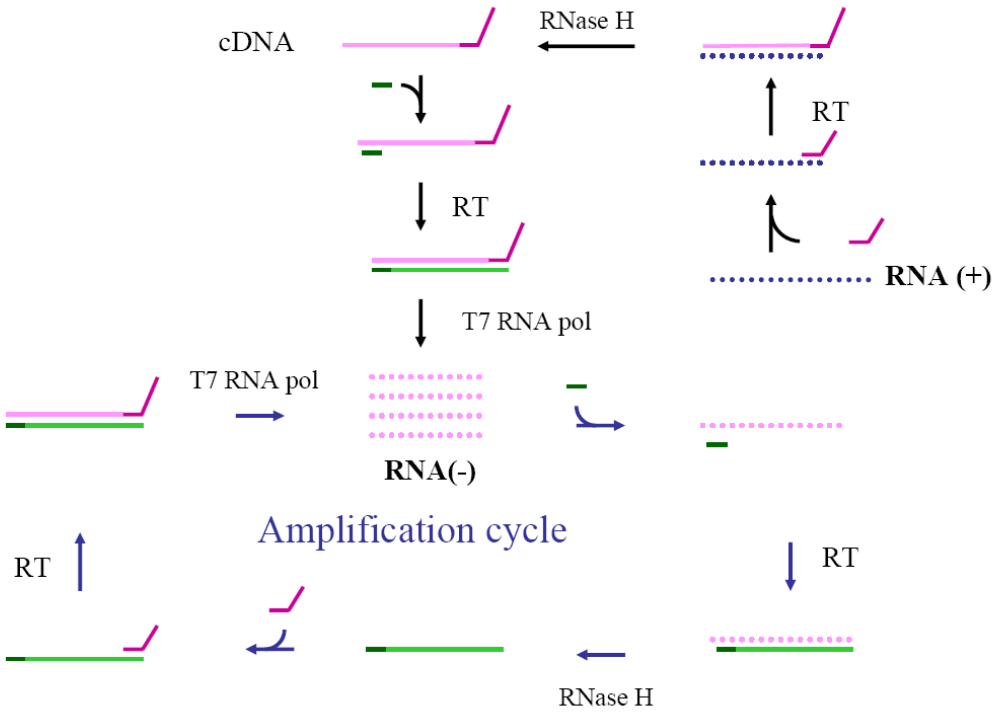


Figure 9: Overview over the nucleic acid sequence-based amplification (NASBA). It amplificates RNA applying three enzymes: RT (reverse transcriptase), T7 RNA pol (T7 RNA polymerase) and RNase H. RNA is plotted as dotted lines (with color blue and pink for RNA(+) and RNA(-), respectively), while cDNA is drawn in continuous lines (color green). Primers are short lines (with colors purple and dark green for first and second primers, respectively). For detailed information about the NASBA reaction see text. Graphic presented by Alain Houde at Nucleic Acid based Technologies, Baltimore 2007

Besides the three already mentioned enzymes, the NASBA reaction also needs oligonucleotide primers, one containing a recognition sequence for T7 RNA polymerase, dNTPs and NTPs. Figure 9 shows an overview of the NASBA reaction. It starts with first primer annealing (which contains the T7 recognition sequence on the 5'end) to the RNA. The reverse transcriptase then synthesizes a complementary strand of cDNA. RNase H digests the RNA from the RNA/cDNA hybrid, allowing the second primer to align to the cDNA. The reverse transcriptase then synthesizes the second strand of cDNA. This double stranded DNA product is a copy of the original RNA and carries the recognition sequence for T7 polymerase. This represents the starting point of the amplification cycle in which T7 polymerase *in vitro* transcribes RNA from the template DNA. These newly produced RNAs then serve as template for repeated cycles of amplification. The whole reaction is performed at a single temperature and achieves an approximately 10^6 to 10^9-

fold amplification of the target, with the main reaction product being ssRNA [Gracias and Mc Killip, 2007; Cook, 2003].

2.5 Objectives for this work

This work is based on the "sepsis chip" developed at the ARC (Austria Research Centers) by Herbert Wiesinger-Mayr (see Microarray section in the introduction for further information) and includes the integration of an internal control (spike-in) to the protocol.

An internal control is a DNA sequence that is not present in the targeted sample, but is then spiked into the sample (just before cell lysis) and passes through most working steps of the protocol, including DNA extraction from whole blood, DNA amplification and hybridization. Therefore the internal control sequences serve as a control for the correct functioning of each single step within the whole protocol, including DNA extraction, PCR and labeling reactions as well as hybridization.

During this work different plant sequences (from the potato) and 16S rRNA gene sequences of methanotrophic bacteria were analyzed for utilization as internal control. Sequences of those species were easy to obtain in the laboratory and are naturally not present in human blood. In order to use these sequences as internal control, they were amplified and the optimal spike-in concentration was determined. In order to detect the internal control new probes were designed and incorporated into the microarray chips.

In combination with internal control the microarray data allow a semiquantitative analysis of samples. An objective of this work was the establishment of a quantification approach for bacteria applying the sepsis chip. However, quantification applying microarray technology is not exact, since signal intensities underlie considerable fluctuations between assays. Therefore, internal control probes and diverse "housekeeping" genes are important to control microarray data and quantification results. In order to validate the results of microarray quantification, a real-time PCR was established.

Another research topic was amplification of RNA, since RNA is a better target to assess bacterial viability than DNA. Several amplification methods for RNA were tested, including one step RT-PCR and a method called nucleic acid sequence based amplification

(NASBA). Necessary quantification was realized by real-time PCR and microarray analysis (semi-quantitative). Future perspectives are the establishment of a protocol which allows the parallel detection of DNA and RNA with the advantage that the viability status of bacteria could be evaluated directly on the chip.

3. Material & Methods

3.1 Sepsis Identification chip

3.1.1 Probe design

Most species specific probes were already in use and tested in previous chip layouts and were published by Wiesinger *et al*., 2006. All new probes for the "Sepsis Identification" chip are listed in table 10 in appendix 1.

The sequences of the plant DNA were obtained by sequencing and the sequence information of the 16S rDNA of methanotrophic bacteria and of the housekeeping genes were downloaded from the NCBI database [http://www.ncbi.nlm.nih.gov]. After a database of all sequences was established, probes for the microarray were designed. In order to control probe sequences, the arb-silva database SSURef_0207_1_4_tree_silva_opt.arb was downloaded and newly designed probes were analyzed using arb software.

Probes were ordered from Microsynth (Balgach, Switzerland) and diluted to a concentration of 100 µM by adding the amount of water indicated in the Quality Certificate of Microsynth. All probes carried 5' oligo thymidine residues as spacer molecules and were 5' amino-modified for covalent linkage to the chip surface.

3.1.2 Spotting

The spotting mix consisted of spotting buffer (6x SSC, 3 M Betain) plus hybridization control probe BSrev of a concentration of 3% of the final probe concentration (that were 15µl of BSrev in 1 ml of spotting mix). Spotting mixtures were prepared in 396 well plates by adding 17 µl spotting buffer and 17 µl of the probe solution (100 µM, Microsynth). The plate was closed using a gluing metal-foil, centrifuged at 900 g for 1 min and stored at -20°C until spotting.

Spotting was performed by using the contact arrayer Omnigrid from GeneMachines (Genomic Solutions, Ann Arbor, USA) on CSS-100 Silylated glass slides (Cel Associates, Pearland, USA). The new chips were tested by hybridizing *E. coli* and *S. aureus* DNA.

3.2 Preparation of internal control sequences

3.2.1 Methanotrophic 16S rDNA gene

The DNA samples of four methanotrophic species were obtained by Levente Bodrossy. Details of the methanotrophic species are listed below (see table 2).

Species name	Abbreviation	Date of recovery
Methylocaldum szegediense	msz	23.01.1996
Methylococus capsulatus BL5	mca	17.03.1994
Methylococus capsulatus BL13	mca	16.03.1994
Methylosinus trichosporium	mtr	14.02.2001
Methylomicrobium agile	mag	14.07.2003

Table 2: Methanotrophic species, used abbreviations and the date of recovery are listed.

The 16S rRNA genes of the obtained sequences were amplified by PCR (details see PCR reaction with MolTaq 16S Kit, Molzym).

3.2.2 Plant DNA

The genomic potato DNA and the specific primers to amplify sequences of ~1,300 bp, were obtained from Frederieke Trognitz. Sequence details are listed in table 3.

Name of potato DNA fragment	Primers
15010	15010F, 15010R
25760	25760F, 25760R
38025	38025R, 38025R

Table 3: Names and primers of potato DNA. F and R in primer names represent forward and reverse primers, respectively.

Because the potato is tetraploid, there are four homologous sets of chromosomes with different alleles of genes. Thus PCR amplification of the genomic potato DNA with one specific primer pair yields up to four slightly different products. However, for the use as internal control a product with 100% sequence identity was needed. This was realized by cloning PCR products of desired sequences into *E.coli* hosts followed by the isolation of the DNA insert of one particular clone. The detailed protocol is described below.

3.2.2.1 Cloning protocol

The cloning of the potato DNA sequences was carried out, using the StrataClone™ PCR Cloning Kit (Stratagene, La Jolla, USA) according to the manufacturer's instructions.

3.2.2.2 Preparation of the sequences

The potato genomic DNA was amplified by PCR using the target specific primers (see table 3). The PCR was carried out as described in chapter 3.6.3. Aliquots of the PCR products were analyzed on an agarose gel to verify amplification of the desired fragment.

3.2.2.3 Ligation of the insert

3 µl StrataClone™ Cloning Buffer and 1 µl of StrataClone™ Vector Mix were added to 2 µl of the PCR product. The components were mixed by repeated pipetting and then incubated at room temperature for 5 min. Afterwards the reaction was placed on ice.

3.2.2.4 Transformation of the Competent Cells

StrataClone SoloPack competent cells were thawed on ice. 1 µl of the cloning reaction mixture was added to the competent cells. The transformation mixture was incubated on ice for 20 min followed by a heat-shock at 42°C for 45 sec. The cells were immediately placed on ice for 2 min. 250 µl of SOC medium was added to the transformation reaction and cells were allowed to recover for 1 h at 37°C with agitation. 50 µl and 100 µl of the transformation mixture were plated on separate LB-ampicillin-X-gal plates. Plates were incubated at 37°C for 24 h followed by storage at 4°C. White clones were successfully transformed with potato DNA sequences.

3.2.2.5 LB-ampicillin-X-gal plates

500 ml of plating agar contained 5 g of trypton (Fluka Biochemika, Buchs, Switzerland), 2.5 g of yeast extract (Fluka Biochemika), 5 g of NaCl (Merck, Whitehouse Station, USA), 7.5 g of Agar (Fluka Biochemika) and 300 µl of ampicillin (conc.: 100mg/ml). Plates were finally mixed with 40 µl of 2% X-gal and spread into Petri dishes.

3.2.2.6 PCR amplification of vector inserts

PCR amplification of the inserts was performed using primers M13 forward (GTAAAAC-GACGGCCAG) and reverse (CAGGAAACAGCTATGAC) (primer sequences against vector sites flanking the insert) in a concentration of 0.25 µM, respectively. 20 µl of the PCR mixture contained 2 µl 10x buffer (Invitrogen, Carlsbad, USA), 0.8 µl of $MgCl_2$ (50 mM, Invitrogen), 2 µl dNTP mix (2.5 mM of dATP, dCTP, dTTP and dGTP each, Roche, Mannheim, Germany) and 0.2 µl Taq-Polymerase (5 U/µl, Invitrogen). White clones were picked with an inoculating loop. The burden of bacteria was streaked on LB-ampicillin-X-gal plates. The remaining cells on the inoculating loop were dipped into the PCR tubes containing 20 µl of PCR mix for resuspension of cells. PCR cycling included an initial denaturation step at 95°C for 10 min, followed by 35 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min using a Biometra 7300 Thermocycler (Goettingen, Germany). Temperature cycles were terminated at 72°C for 10 min. PCR products were stored at -20°C upon sending to sequencing.

In order to be able to amplify the plant sequences along with the 16S rRNA gene of pathogens, it was necessary to ligate the standard pathogen primer sequence to the plant amplicons. This was realized by PCR amplifying the plant sequences with specific adapter primers. These adapter primers consisted of sequences specific to the plant DNA plus the sequences of the standard 16S rDNA primers (sequences see table 12). Amplification with adapter primers was carried out applying the MolTaq 16S PCR Kit (Molzym, Bremen, Germany) as described in chapter 3.6.3.

PCR products were all diluted accordingly (1/10,000) before adding them to another PCR. This dilution is very important, as PCR reacts very sensitive to vast amounts of DNA and satisfactory amplification was only achieved with decent amounts of inserted DNA.

3.3 Quantification of internal control DNA

Internal control DNA amplified by PCR was purified using the MSB Spin PCRapace Kit (Invitek, Berlin, Germany). The protocol is described here briefly: 250 µl of Binding Buffer (Invitek) were added to the PCR sample, mixed well and transferred onto a provided Spin Filter column. Columns were centrifuged at 12,000 rpm for 3 min. To elute the purified PCR products, the Spin Filter column was transferred to a 1.5 ml receiver tube and 50 µl of Elution Buffer (Invitek) were added to the surface of the column membrane. An incubation period for 1 min at room temperature was followed by a centrifugation of the columns at 10,000 rpm for 1 min.

Then the purified methanotrophic and plant DNA was quantified via NanoDrop® measurements giving the DNA concentration in ng/µl. The corresponding DNA sequences were copied into BioEdit v.7.0.9 and the program calculated the length, nucleotide composition and also the molecular weight in Dalton of one copy of dsDNA. The conversion factor of Dalton to kilogram is $1.660538782\times10^{-27}$. Therefore, the molecular weight in Dalton multiplied by the conversion factor gives the weight in kilogram per DNA copy, which can be easily converted to nanogram per DNA copy. The result of the NanoDrop® measurement in ng/µl is then divided by the nanogram per DNA copy, giving the result of copy number per µl.

3.4 DNA isolation from bacterial culture

3.4.1 Bacterial culture

All reference strains tested in this study were obtained from the American type culture collection (ATCC) or the “Deutsche Sammlung für Mikroorganismen und Zellkultur” (DSMZ). For long term storage all bacterial strains were kept as 30% glycerol stocks at -80°C. 1 ml of an overnight culture of the bacterial strain (in Caso-Bouillon, incubated at 37°C, 160 rpm) was transferred into an eppendorf tube. The cells were centrifuged at 5,000 rpm for 5 min, supernatants were discarded and pellets were resuspended in 1 ml 1x PBS and again centrifuged at 5,000 rpm for 5 min. This washing step was repeated once. Finally the pellet was taken up in 1 ml 1x PBS. The suspension was diluted 1/10

and the optical density at 625 nm was determined. The cell concentration was adjusted to 10^8 cells per ml by comparing to Mc Farland standard # 0.5. The calculated volume was transferred into a new eppendorf tube and TE buffer (for lysis applying the Epicentre kit; 10 mM Tris-HCl [pH 8.0], 1 mM EDTA, Epicentre) or ddH_2O (for other lysis methods) was added to a final volume of 100 µl (final concentration = 10^8 cells/100 µl). This suspension was the starting material for different lysis experiments.

3.4.2 Lysis of the bacterial cells

Several methods of bacterial cell lysis were tested and compared during this work.

3.4.2.1 Cell lysis applying a commercial kit

The Master Pure Gram Positive DNA Purification Kit (Epicentre, Madison, USA) was used for cell lysis. The protocol was adapted to our requirements. 1 µl of Ready Lyse Lysozym was added to the cell suspension followed by incubation for 30 min at 37°C. 1 µl of proteinase K (50 µg/µl) was diluted into 100 µl of Gram Positive Lysis Solution and added to the cell suspension. The cells were then incubated at 65°C for 15 min followed by a heating step at 95°C for 15 min. Before proceeding with DNA and RNA extraction, 500 µl of buffer RLT Plus + 1 % β-mercaptoethanol (RNeasy Plus lysis buffer, Qiagen) were added to the sample.

3.4.2.2 Boiling protocol

The cell suspension was heated to 95°C for 15 min briefly vortexed and centrifuged (15,000 g, 10 min). The supernatant (contained the bacterial DNA) was transferred to a new tube. In order to separate DNA and RNA with the Qiagen kit (see chapter 3.4.3. “DNA and RNA purification”) 600 µl of buffer RLT Plus were added.

3.4.2.3 Bead beating

600 µl of buffer RLT Plus + 1 % β-mercaptoethanol (Qiagen, Venlo, Netherlands) were added to the cell suspension before putting the mixture onto 0.1 mm silica spheres (MP Biomedicals, Solon, USA). Then, the sample was blended three times in a bead beater (Fast Prep FP120, MP Biomedicals) for 30 sec at maximal setting. The suspension was then centrifuged at 15,000 g for 15 min. The supernatant was proceeded for DNA and RNA extraction applying the Qiagen kit.

3.4.2.4 Lysis applying the RLTPlus buffer (Qiagen)

RLT Plus buffer + 1 % β-mercaptoethanol (Qiagen) was added to the cells at a certain step of each lysis method. However, for this protocol the cells were solely lysed by addition of 600 µl buffer RLT Plus.

The extracted DNA was purified using the AllPrep DNA/RNA Mini Kit (Qiagen), in order to obtain DNA and RNA ready for molecular methods.

3.4.3 DNA and RNA purification

DNA and RNA purification was carried out applying the AllPrep DNA/RNA Mini Kit (Qiagen). After addition of buffer RLT Plus (step 3 in the AllPrep DNA/RNA Mini handbook, 11/2005 protocol) all steps were realized according to the protocol. Homogenization of the cells, as described in step 3 of the protocol, was not necessary since we were not working on cell tissue but bacterial cell culture.

The lysed cells were transferred onto an AllPrep DNA spin column and centrifuged for 30 sec at 10,000 rpm. RNA flows through while the DNA binds to the column and is kept for later processing.

3.4.3.1 Total RNA Purification

The flow-through, which contains the RNA, was mixed with one volume of 70 % ethanol by pipetting. Up to 700 µl of the sample were transferred to an AllPrep RNA spin column and centrifuged at 10,000 rpm for 15 sec. The flow-through was discarded.[1] Then 700 µl of buffer RW1 were added to the spin column and again centrifuged at 10,000 rpm for 15 sec. The flow-through was discarded. The same procedure was done with 500 µl of buffer RPE to wash the spin column membrane. This washing step was repeated once by addition of 500 µl of buffer RPE and centrifuged at 10,000 rpm for 2 min. After the flow-through was discarded the column was again centrifuged at full speed for 1 min to remove any traces of the washing buffer. The column was then placed in a DNA-free collection tube and 50 µl of RNase-free water were added. A last

[1] starting point for optional On-Column DNase Digestion see chapter 3.4.3.3

centrifugation at 10,000 rpm for 1 min was carried out to elute the RNA. All RNA was stored at -20°C until use.

3.4.3.2 Genomic DNA purification

500 µl of buffer AW1 were added to the AllPrep DNA spin column and centrifuged at 10,000 rpm for 15 sec. After the flow-through was discarded, 500 µl of buffer AW2 were added to the column and centrifuged at full speed for 2 min, to wash the membrane. The column was then placed in a collection tube and 100 µl of elution buffer EB were added to the membrane. After an incubation time of 1 min the DNA was eluted by centrifugation at 10,000 rpm for 1 min and stored at -20°C.

3.4.3.3 Optional On-Column DNase Digestion Using the RNase-Free DNase Set

First the DNase I stock solution was prepared by injecting 550 µl RNase-free water into the vial containing DNase I, using a needle and syringe. DNase I stock solution was then stored in single-use aliquots at -20°C. For DNase digestion the RNA purification protocol was followed until the washing steps with buffer RW1. The step in the Total RNA Purification protocol, at which optional DNase Digestion starts, is indicated by a footnote in the text. 350 µl of buffer RW1 were added to the AllPrep RNA spin column and centrifuged for 15 sec at 10,000 rpm to wash the membrane. 10 µl of DNase I stock solution were gently mixed with 70 µl of Buffer RDD. The resulting DNase I incubation mix was added to the column membrane and incubated at room temperature for 15 min. After incubation 350 µl of buffer RW1 were added to the membrane and centrifuged for 15 sec at 10,000 rpm. After discarding the flow-through the Total RNA Purification protocol was continued starting with the first addition of buffer RPE.

3.5 Pathogen DNA Isolation from whole blood

Various methods for the isolation of bacterial DNA from whole blood were tested by Herbert Wiesinger-Mayr. Good results combined with ease of use were obtained by the MolYsis Kit from Molzym. Therefore, this protocol was used to isolate pathogenic DNA from whole blood and also for experiments with internal control DNA addition just before cell lysis.

3.5.1 Preparation of bacterial culture

1 ml overnight (o/n) culture of a bacterial strain (in 6 ml Caso-Bouillon, incubated at 37°C, 160 rpm) was transferred into an eppendorf tube. The cells were centrifuged at 5,000 rpm for 5 min, supernatants were discarded and pellets were resuspended in 1 ml 1x PBS. This washing step was repeated once. Suspensions with three different cell concentrations were prepared. A bacterial suspension with a concentration of 10^8 cells per ml was prepared applying the McFarland standard #0.5 (see chapter 3.4.1 "bacterial culture"). The 10^6 and 10^5 cells/ml suspensions were made by diluting the 10^8 cells/ml cell suspension accordingly.

3.5.2 Spiking of whole blood

70 µl bacterial cell suspension were added to 7 ml of whole blood. Therefore, the final pathogen concentration in whole blood was 10^3 to 10^6 cells per ml. 1 ml of the spiked blood was then aliquoted into new eppendorf tubes. The internal control DNA was added later in the protocol, just before bacterial cell lysis.

3.5.3 Bacterial DNA isolation applying MolYsis Kit

DNA isolation was performed according to manufacturer's instructions (Molzym).

3.5.3.1 Human cell lysis

250 µl of buffer CM were added to 1 ml of whole blood and vortexed at full speed for approximately 20 sec. Then 250 µl of buffer DB1 and 10 µl of MolDNAseB were added and vortexing for appr. 20 sec was repeated, followed by an incubation step at room temperature (RT) for 15 min. The bacterial cells were harvested by centrifugation at 13,000 rpm for 10 min and decantation of the supernatant.

3.5.3.2 Bacterial cell lysis

Cells were washed by addition of 1 ml of buffer RS and vortexed for 10 sec. Cells were again harvested by centrifugation at 13,000 rpm for 5 min and decantation of the supernatant. The bacterial pellet was resuspended in 80 µl of buffer RL by pipetting and

vortexing for 10 sec. After addition of 20 µl of BugLysis solution the cells were again vortexed for 10 sec, followed by addition of internal control DNA. An incubation step at 37°C and 1,000 rpm for 30 min was carried out prior to transferring 150 µl of buffer RP and 20 µl of Proteinase K to the cells and vortexing for 10 sec. Subsequent to an incubation step at 56°C and 1,000 rpm for 10 min, 250 µl of buffer CS were added and vortexed for 10 sec.

3.5.3.3 DNA purification

For DNA purification 250 µl of binding buffer were added to the cell lysate and vortexed for 10 sec. The suspension was then transferred to a spin column and centrifuged at 13,000 rpm for 30 sec. The flow-through was discarded and the spin column was replaced to the collection tube. 400 µl of buffer WB were added to the column and the column was centrifuged at 13,000 rpm for 30 sec. The flow-through was discarded and the column was washed with 400 µl of DNA-free 70% ethanol. Subsequent to this washing step the column was centrifuged at 13,000 rpm for 3 min. Then the column was transferred to a 1.5 ml elution tube and 100 µl of DNA-free deionized water (preheated to 70°C) were added and the column was incubated for 1 min. In order to elute the DNA the column was centrifuged at 13,000 rpm for 1 min. Eluted DNA was stored at -20°C until further usage.

3.6 Amplification of bacterial 16S rDNA

Several PCR master mixes were tested in order to increase efficiency and minimize risk of contamination.

3.6.1 PCR applying Taq DNA Polymerase

Recombinant Taq DNA Polymerase (Invitrogen) was used as standard protocol. PCR amplification of 16S rRNA gene was realized applying the forward primer 45 and the reverse primer 1391 (0.6 µM) (Microsynth). Primer sequences are listed in Appendix 1. A 25 µl PCR mixture contained 2.5 µl 10x PCR buffer (Invitrogen), 1 µl $MgCl_2$ (50 mM, Invitrogen), 2 µl dNTP mix (2.5 mM of dATP, dCTP, dTTP and dGTP each, Roche, Mannheim,

Germany), 0.25 µl Taq-Polymerase (5 U/µl, Invitrogen) and 1 µl of bacterial DNA extraction.

3.6.2 PCR applying the ImmoMix Red Kit

The ImmoMix Red kit (Bioline, London, UK) is a ready-to use PCR mixture. 25 µl of PCR mix contained 12.5 µl of 2x PCR buffer (Bioline), 0.5 µl of primers forward and reverse, each (15 µM) (Microsynth) and 1 µl of target DNA. The Immomix Red buffer contains a red dye, making the addition of loading buffer for gel electrophoresis, obsolete.

3.6.3 PCR applying the Molzym 16S basic mix

Molzym 16S basic mix is a guaranteed DNA-free PCR mix (Molzym, Bremen, Germany). 25 µl of PCR mix contained 0.5 µl of primers forward and reverse, each (15 µM) (Microsynth), 10 µl of 2.5x PCR mix, 0.8 µl of MolTaq polymerase and 1 µl of target DNA.

3.6.4 Cycling conditions

PCR cycling conditions were the same for all PCR mixes and included an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min using a Biometra 7300 Thermocycler (Goettingen, Germany). Temperature cycles were terminated at 72°C for 10 min to complete partial amplicons, followed by storage at 4°C until further usage.

Successful amplification was confirmed by transferring 9 µl of the PCR products plus 1 µl of 10x loading buffer) on a 1.5 % agarose gel (SeaKem; Biozym, Oldendorf, Germany) in TBE buffer (0.1 M Tris, 90 mM boric acid, 1 mM EDTA) (Invitrogen) supplemented with ethidium bromide. The run was carried out at 170 mV for 45 min.

3.7 Primer Extension

The primer extension method was used for labeling of 16S rDNA PCR products. 6 µl of the PCR product was added to the primer extension mix containing 1.5 µl of forward

primer 45 (15 µM), 2.5 µl of 10x ThermoPol buffer (New England BioLabs Inc., Ipswich, Massachusetts), 0.5 µl of dNTP mix (10 mM of dGTP, dATP and dTTP, 5 mM of dCTP, Roche), 0.5 µl of Atto 532 labeled dCTP (5 mM, Mo Bi Tec, Göttingen, Germany), 0.25 µl of $MgSO_4$ (100 mM, New England BioLabs Inc.), and 0.8 µl of Vent (exo-)polymerase (2 U/µl, New England BioLabs Inc.) for a 25 µl reaction volume. Cycling program started with a denaturation step at 95°C for 3 min, followed by 25 cycles of 95°C for 20 seconds, 60°C for 20 sec and 72°C for 20 sec. Primer extension was completed at 72°C for 3 min followed by storage at 4°C until further usage.

3.8 Hybridization with glass cover slip

Before hybridization, slide surfaces were blocked in urea buffer (3 M urea, 0.1% SDS) for 30 minutes, shortly washed in WashII (0.1 x SSC, 0.2 % SDS) and dried by centrifugation (900 rpm for 2 min in a Multifuge 3 S-R from Heraeus). A hybridization mix containing 6.25 µl of 20x SSC, 0.3 µl of 10% SCS and 0.5 µl of a hybridization control (10 nM, BSrev: 5'end Cy5-labeled oligonucleotide sequence: AAG CTC ACT GGC CGT CGT TTT AAA) was added to 25 µl of the labeled primer extension product. The mixture was heated to 95°C for 3 min for denaturation of DNA strands. To start the hybridization reaction 24 µl of this mixture were transferred on a cover slip (22 x 22 mm) and subsequently applied to the microarray surface. Hybridization was realized at 65°C in a vapor saturated pre-heated chamber for 1 hour.

3.9 Hybridization with Agilent Slides

The applied protocol was similar to the hybridization using glass cover slips except for processing Agilent slides an increased hybridization volume of 100µl was needed. Therefore the Primer Extension reaction was carried out in a volume of 50µl. The primer extension products were then diluted in dH_2O to a volume of 75µl. The volumes of the hybridization mix were changed to 21.4 µl of 20x SSC, 1.1 µl of 10% SDS and 1.7 µl of hybridization control. After denaturation (95°C for 3 min), 100 µl of the mixture were transferred onto the Agilent Slides (Agilent Technologies, Santa Clara, USA). Then the microarray was applied (see figure 10a). The Agilent hybridization chamber (see figure

10b), was assembled following the manufacturer's instructions. Hybridization was realized at 65°C for 1 hour at 10 rpm.

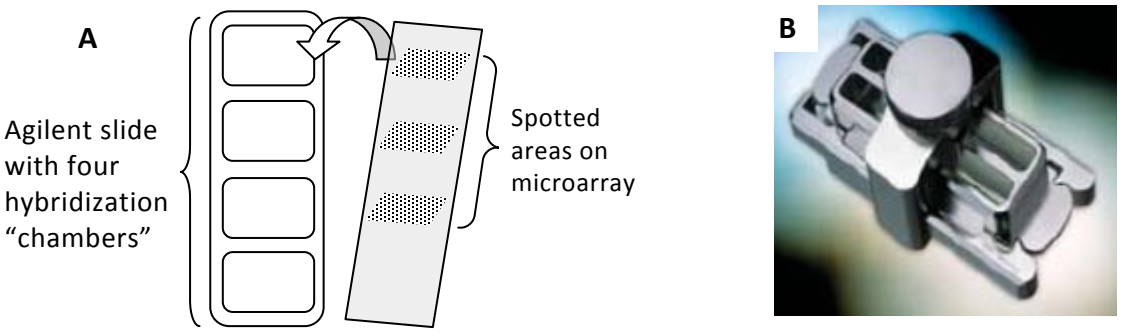


Figure 10: Agilent hybridization technology; **A**: first steps in assembly of Agilent hybridization chamber; **B:** Agilent hybridization chamber, picture by Agilent Technologies

3.10 Washing procedure after hybridization

After hybridization slides were washed first in 2x SSC, 0.1 % SDS for 5 min, then in 0.2x SSC for 2 min and finally in 0.1x SSC solutions for 1 min. Afterwards slides were again dried by centrifugation (900 rpm, 2 min).

3.11 Signal detection and analysis

Slides were scanned at a resolution of 10 µm with an Axon Genepix 4000A microarray scanner (Axon, Union City, California) at two different sensitivity levels of the photomultiplier for each slide. Those were 500 pmt (photo multiplier) and 600 pmt each for red and green channels in two-color analysis, respectively. Obtained images were analyzed using the Genepix software. A grid specifying the name of each probe and the position on the microarray was created by Omnigrid software (Genomic Solutions, Ann Arbor, USA). This grid was imported into Genepix software and placed across the microarray image either manually or automatically by the software. The software then performed background correction by applying the following formula:

$$mean\,absolute\,signal\,intensity - mean\,background = background\,corrected\;\;probe\,signal$$

Figure 11 illustrates signal intensity values of probes and background signal intensities.

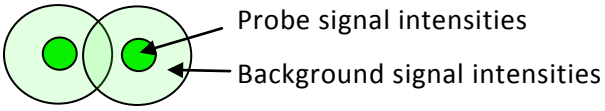


Figure 11: Illustration of probe and background signal intensities. A grid specifying the exact position and name of each probe was applied either manually or automatically by the Genepix software. The mean of absolute signal intensities within the defined area of the probe was calculated, as well as the mean of signal intensities in the area of three times the diameter of the spot around each probe (background signal).

Each probe was spotted on the array fourfold (quadricates). The arithmetic mean of these four spots was also calculated by the GenePix software. All results were exported as gpr (GenePix results) files. Graphs were generated in Excel software by drawing the background corrected arithmetic mean signal values of quadricate probes (y-axis) against the probe name (x-axis).

3.12 Real-Time PCR

Two different kits for real-time PCR were applied: the SYBR® Green PCR Master Mix and the TaqMan® Universal PCR Master Mix (each from Applied Biosystems, Foster City, USA).

3.12.1 SYBR® Green PCR Master Mix

PCR amplification of 16S rRNA gene was realized using the primer pair 521F and 673R (900 nM, Microsynth). The 20 µl PCR mixtures contained 10 µl 2x PCR buffer (Applied Biosystems), and 2 µl template DNA.

PCR cycling included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min using the GeneAmp® 5700 Sequence Detection System from Applied Biosystems. After realization of the temperature cycles a melting curve analysis was carried out, by heating the sample from 60°C to 95°C.

3.12.2 TaqMan® Universal PCR Master Mix

Primers 331F and 797R were added to real-time PCR for a final concentration of 900 nM. The 20 µl PCR mix further contained 10 µl of 2x TaqMan PCR Master Mix (Applied Biosystems), 2 µl of the TaqMan probe (2.5 µM, Microsynth) and 1 µl of the template DNA.

Cycling conditions were equal to SYBR® Green PCR conditions, except the opportunity to carry out melting curve analysis.

3.12.3 Data analysis

Data analysis was the same for SYBR® Green real-time PCR and for TaqMan® Universal PCR.

The raw data was the fluorescence intensity measured after each thermal cycle. The raw data of real-time PCR analysis was plotted on a logarithmic scale with fluorescence drawn on the y-axis and cycle number drawn on the x-axis.

The threshold cycle was defined as cycle number, at which specific signals of the samples clearly increased and were distinguishable from background signals. At that point amplification has reached the logarithmic phase, making the increase in fluorescence linear on a logarithmic scale. This cycle number is calculated by the GeneAmp 5700 SDS software.

For DNA quantification a standard curve was generated. It is a dilution series of DNA with known concentration. In a 1/2 dilution series threshold cycles between single dilution steps should differ by one cycle. In order to determine the efficiency of the PCR as well as suitability for quantification, a line chart was produced, with the dilutions steps on the x-axis and the threshold cycles on the y-axis. A trendline was plotted, with the equation and the R-squared value displayed in the chart. The equation is important for quantification and will be discussed in detail later. The R-squared value represents the efficiency of the PCR reaction and should ideally be above 0.9. An R-squared value of 1 is achieved when the reaction is 100% efficient, meaning that the amount of DNA doubles in every cycle.

The equation in the line chart with two variables x and y is as follows: $y = m * x + b$
The two constants m and b define the slope of the graph and the crossing point of the

graph with the y-axis, respectively. By inserting the threshold cycle of the unknown sample in the equation as y value the variable x can be calculated. The variable x represents the dilution step of the standard curve which equals the unknown sample in respect to the DNA concentration. If the concentration of the unknown sample is exactly the same as one dilution step of the standard curve, then x is an integer. Normally the value x is a rational number and therefore positioned somewhere between two steps of the dilutions series. To determine the concentration of the unknown sample a second equation is necessary. For a 2 fold dilution series with the highest concentration being 100,000 copies per reaction this equation is:

$$y = 200{,}000\, e^{-0.69x}$$

By inserting the already calculated variable x as x in this second equation and then calculating the y value, the exact concentration of the unknown sample is determined.

3.12.3.1 Dissociation curve analysis in SYBR® Green real-time PCR assays

In order to proof specific product amplification a dissociation curve analysis was carried out. Unspecific amplification as well as primer dimers were visualized as further peaks in the dissociation curve. Dissociation curve analysis takes place after thermocycling and is realized by slowly increasing the sample temperature from 60°C to 95°C. When the temperature reaches the melting point of a specific PCR product, the two strands of dsDNA separate, leading to a decrease of fluorescence.

3.13 RNA based techniques

RNA from bacterial culture was obtained as described earlier (see M&M Testing of different lysis methods for bacteria) and dilution series of these bacterial RNA suspensions were prepared (dilutions 1x, $^1/_5$, $^1/_{25}$).

3.13.1 Omniscript Reverse Transcription Kit (Qiagen)

Reverse primer 1391 (1.1 µM, Microsynth, Balgach, Switzerland) was used for reverse transcription of RNA into cDNA. No forward primer was needed for this reaction. 2 µl 10x buffer RT, 2 µl dNTP mix (5 mM each dNTP, Roche), 1 µl RNase Inhibitor (10 U/µl,

Qiagen), 1 µl Reverse Transcriptase (4 U/µl) and 10 µl of diluted RNA were mixed and diluted with H_2O to a final volume of 20 µl as recommended in the Omniscript Reverse Transcription Handbook 05/2004. The reverse transcription reaction was carried out at 37°C for 60 min.

3.13.2 Superscript Reverse Transcription Kit (Invitrogen)

0.5 µl of forward primer 45 (15 µM, Microsynth), 0.5 µl of reverse primer 1391 (15 µM, Microsynth) and 1 µl of dNTP mix (10mM, Roche) were added to 10 µl of diluted RNA and then heated to 65°C for 5 min, followed by an incubation on ice for at least 1 min. Then 4 µl of 5x first-strand buffer (Invitrogen), 1 µl of 0.1 M DTT (Qiagen), 1 µl of RNase Inhibitor (10 U/µl, Qiagen), 1 µl of H_2O and 1 µl of Superscript III Reverse Transcriptase (200 U/µl, Invitrogen) were added to a final volume of 20 µl. Reaction conditions were 52°C for 60 min followed by 70°C for 15 min.

3.13.3 Avian Myeloblastosis Virus (AMV) Reverse Transcriptase (Invitrogen)

0.5 µl of forward primer 45 (15 µM, Microsynth), 0.5 µl of reverse primer 1391 (15 µM, Microsynth) and 2 µl of dNTP mix (10 mM, Roche) were added to 10 µl of template RNA and then heated to 65°C for 5 min, followed by an incubation on ice for 1 min. Then 4 µl of 5x cDNA synthesis buffer (Invitrogen), 1 µl of 0.1 M DTT (Qiagen), 1 µl of RNase Inhibitor (10 U/µl, Qiagen) and 1 µl of Cloned AMV Reverse Transcriptase (15 U/µl, Invitrogen) were added to a final volume of 20 µl. Reaction conditions were 45°C for 60 min followed by 85°C for 15 min.

2 µl of cDNA were amplified in a following PCR. PCR and gel analysis of products were performed as described above.

3.13.4 One-Step RT-PCR Kit (Qiagen)

Primers 45F and 1391R (sequences see table 11 in Appendix 1) were used at a final concentration of 0.6 µM. 25 µl reaction mixture contained 5 µl 5x OneStep RT-PCR buffer, 10 µl H_2O (RNase free), 1 µl dNTP mix (10 mM of each dNTP, Qiagen), 1 µl of RNase Inhi-

bitor (10 U/µl, Qiagen), 1 µl of OneStep RT-PCR Enzyme Mix (Qiagen) and 5 µl of template RNA. For reverse transcription reaction the samples were incubated at 50°C for 30 min. An initial PCR activation step at 95°C for 15 min was followed by 40 temperature cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min using a Biometra 7300 Thermocycler (Goettingen, Germany). Temperature cycles were terminated at 72°C for 10 min, followed by storage at 4°C until further usage.

3.13.5 Home-made NASBA

Reverse primers (internal name: 317R_T7, 673R_T7 and 1391R_T7) consisted of the T7 promotor sequence plus a sequence specific for the 16S rRNA. The standard 45 forward primer (also used for standard PCR amplifications) was used for NASBA reactions. NASBA reaction was realized as previously described by Song *et al.,* 2000. Reactions were carried out in a volume of 25 µl, consisting of 5 µl RNA dilution, 3.8 µl enzyme mix and 15.4 µl amplification mix (40 mM Tris-HCl pH 8.4, 2 mM each NTP (Roche), 1 mM each dNTP (Roche), 10 mM DTT, 12 mM $MgCl_2$, 90 mM KCl, 0.18 µM of each primer and 15% DMSO). Enzyme mix containing 40 U T7 polymerase (Roche), 7.5 U AMV-RT (Invitrogen), 0.2 U RNase H (New England Biolabs Inc.), 10 U RNase Inhibitor (Qiagen) and 400 µg/ml BSA (Sigma Aldrich, St. Louis, Missouri) were added to the reaction after denaturing target RNA at 65°C for 5 min and incubation at 41°C for 5 min. Reactions were incubated for 90 min at 41°C. Products were loaded onto 1.5% agarose gels for analysis.

3.13.6 NASBA applying the NucliSENS EasyQ® Basic Kit v2 (Biomérieux)

All steps were performed according to the NucliSENS EasyQ® Basic Kit v2 Guidance document (Biomérieux, Lyon Area, France). Typically the NASBA mix was prepared for eight samples, since the lyophilized enzyme and reagent spheres were aliquoted for this sample number. First the enzyme solution was prepared by adding 45 µl enzyme diluent to the lyophilized enzyme sphere. The solution was placed at room temperature for 5 min before gently mixing by tapping the tube. The target RNA specific primer mix was prepared by diluting 10 µl of each primer (15 µM) with 10 µl of water (included in the kit). The KCl solution (80 mM) was prepared by mixing 16 µl KCl stock solution with 14 µl

water. 64 µl reagent sphere diluent, 24 µl KCl solution and 8 µl primer mix were transferred to a new tube and briefly vortexed. Finally the reagent sphere was added and vortexed immediately until a clear solution was obtained. Each PCR tube was supplemented with 5 µl of target RNA and 10 µl primer reagent mix. Subsequent to vortexing the tubes were placed in the thermocycler. Incubation was carried out at 65°C for 2 min followed by 41°C for 2 min. Then 5 µl of the enzyme solution were added to each tube and the samples were incubated at 41°C for 90 min, followed by storage of the products at 4°C.

4. Results & Discussion

4.1 Probe design for the "Sepsis Identification" chip

A database for arb software package [Ludwig *et al.,* 2004] was established, which was the basis for specific probe and primer design. 16S rRNA gene sequences from methanotrophic bacteria and DNA sequences from housekeeping genes were obtained from the ncbi database [http://www.ncbi.nlm.nih.gov] and subsequently loaded into arb database. Amplified plant DNA was sequenced and the results were loaded into the arb database. Data analysis was carried out using the probe design function of the arb software package and as result the DNA sequences for primers and probes were obtained. The sequences are listed in table 10 in Appendix 1.

The phylogenetic tree based on 16S rDNA analysis shows the relation between the pathogens represented on the "Sepsis Identification" chip. An illustration can be seen in figure 12.

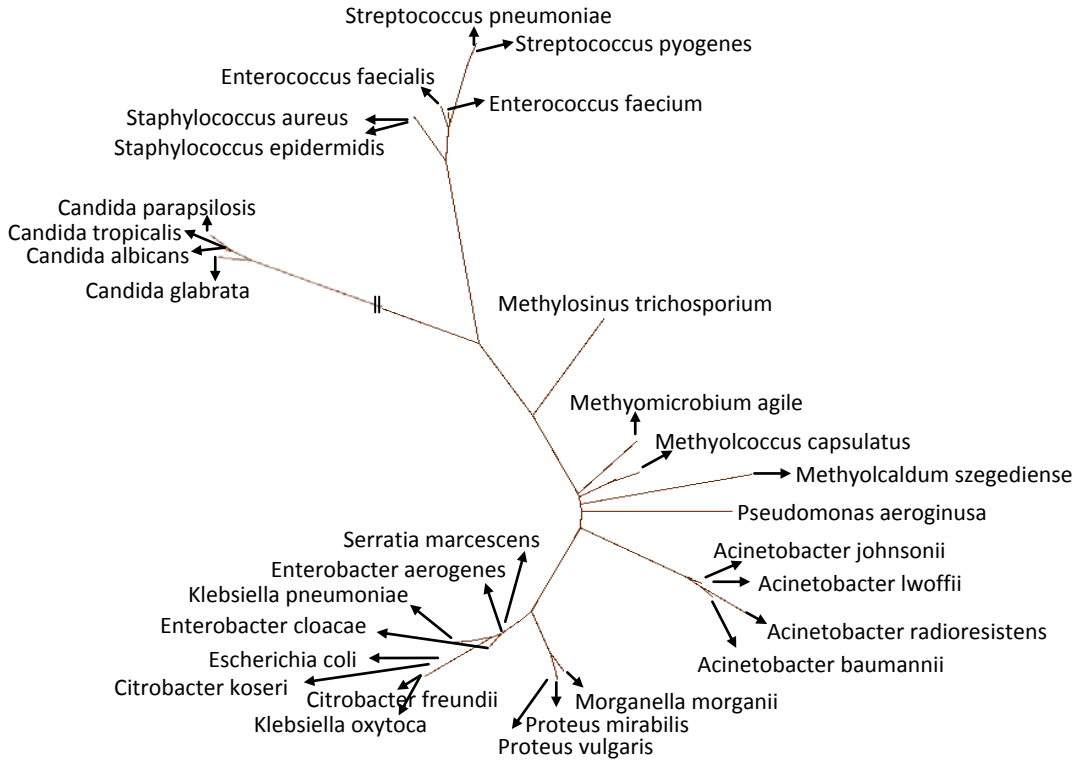


Figure 12: Phylogenetic tree based on 16S rDNA analysis showing the relations between pathogenic species represented on the sepsis identification chip.

4.1.1 Hybridization control BSrev

The spotting mix consisted of spotting buffer plus hybridization control probe BSrev at a final concentration of 3 % of the total probe concentration. The target for BSrev was 5' labeled with the dye Cy5 (emits red light), therefore it was distinguishable from the amplified pathogen sequences which were Atto 532 (emits green light) labeled. As BSrev was added to the spotting buffer, all probes contained BSrev and gave weak Cy5 signals at 635 nm. The BSrev specific signal intensities between different probes showed big differences, although the spiked-in BSrev concentration was the same. Additionally, BSrev specific signals were continuously decreasing the longer the spotted slides were stored before hybridization (see figure 13).

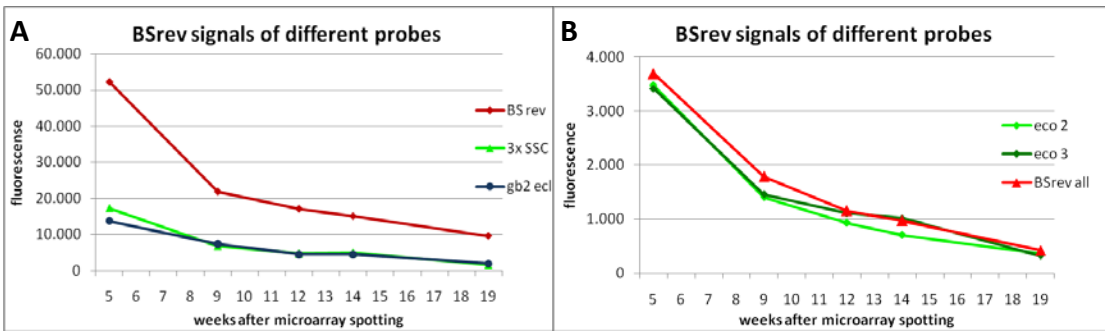


Figure 13: Signals of spiked-in BSrev of selected probes over 19 weeks. Signal intensities are plotted on the y-axis while the weeks after spotting are plotted on the x-axis. Probe BSrev (100 % BSrev), negative control 3x SSC (buffer control plus 3 % BSrev) and probe gb2 ecl (A) showed high BSrev specific signals while probes eco2 and eco3 (B) generated relatively low signals. BSrev all shows the mean values of BSrev specific signals of all probes of a microarray.

Four weeks after spotting of the slides probes gb2 ecl and eco2 generated BSrev specific signals of 13,800 and 3,400, respectively. Signal intensities for these probes dropped to 2,000 and 350, when hybridization was performed four months after spotting. Therefore, those two spots demonstrated the differences in BSrev signal intensities as well as their decrease over time very well. The decrease in signals intensities was observed for all spots. Probe BSrev (100 % BSrev spotted) showed the highest signal of 52,300 shortly after spotting and dropped considerably over the next four months to 10,000. This effect was also observed with the mean values of BSrev specific signals of all probes, where the signals decreased from 3,600 to 400 over the course of 19 weeks. This de-

crease in signals was only observed for BSrev specific signals and not for pathogen specific signals.

As BSrev was added to the pathogen specific probes, each spot would emit signals of both wave lengths as BSrev (Cy5 labeled) and the pathogenic DNA (labeled with Atto 532) bind to the probes in these spots. The effects of pathogen specific signals on BSrev signals were determined by the example of *E. coli* specific probe eco2 and eco3. High and low pathogen specific signals were compared to respective BSrev signals (see figure 14). No correlation could be detected. Fluctuations in BSrev signal intensities do not correspond to the signal intensities of pathogen specific probes but are dependent on the age of the chips.

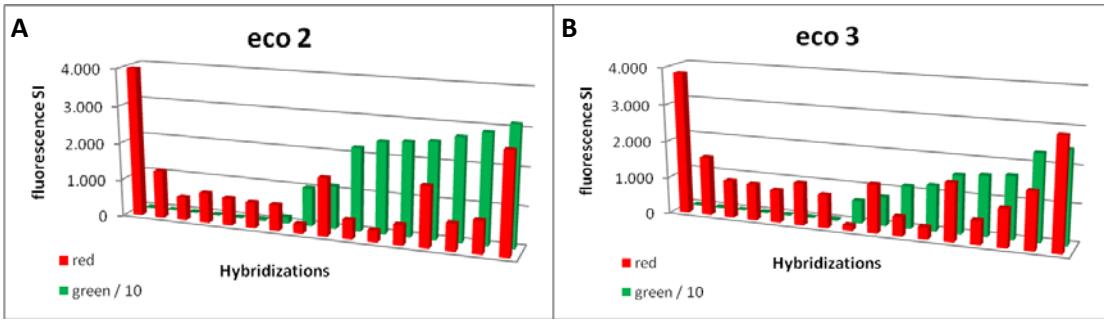


Figure 14: *E. coli* specific and BSrev signals of probes eco2 (**A**) and eco3 (**B**) in different experiments. Fluorescence signal intensities (SI) were plotted on the y-axis while hybridizations where plotted on the x-axis. Values were sorted by *E. coli* specific signal intensities (green bars) from smallest to largest. Red bars indicate BSrev signal intensities. In order to plot both (green and red) signals on one axis, the green signals (Atto 532) were divided by 10.

4.2 Establishment of internal control

The internal control was added for proof of each working step of the microarray protocol. The signals of the internal control monitor the efficiency of DNA isolation, PCR amplification, labeling reactions and hybridization. During each working step the internal control sequence must not interfere with the analysis of pathogenic DNA from the sample.

4.2.1 Preparation of internal control DNA

The following chapters will describe the results of the establishment of an internal control into a pathogen identification protocol. Further the internal control supports semi-quantitative analysis based on chip technology.

4.2.1.1 Potato sequences

Potato DNA was thankfully received from Frederieke Trognitz. PCR was realized with three different primer pairs (15010F and R; 25760F and R; 38025R and R) targeting different fragments, each amplifying sequences of ~1,400 bp. PCR products were loaded onto 1.5 % agarose gels, purified and the DNA concentration was measured using NanoDrop.

The potato is a tetraploid plant and therefore has four homologous sets of chromosomes. For the usage as internal control, however, it is important that only one completely homologous sequence is prevalent in one sample. Thus the amplified sequences were cloned into *E. coli* and enriched by cultivation. PCR amplification of the insert of one single clone ensured that only one allele was amplified in one sample.

Cloning was achieved, using the StrataClone™ PCR cloning kit. The LB-plates carrying the transformed clones are shown in figure 15. White clones were successfully transformed, while blue clones did not carry any plant sequences.

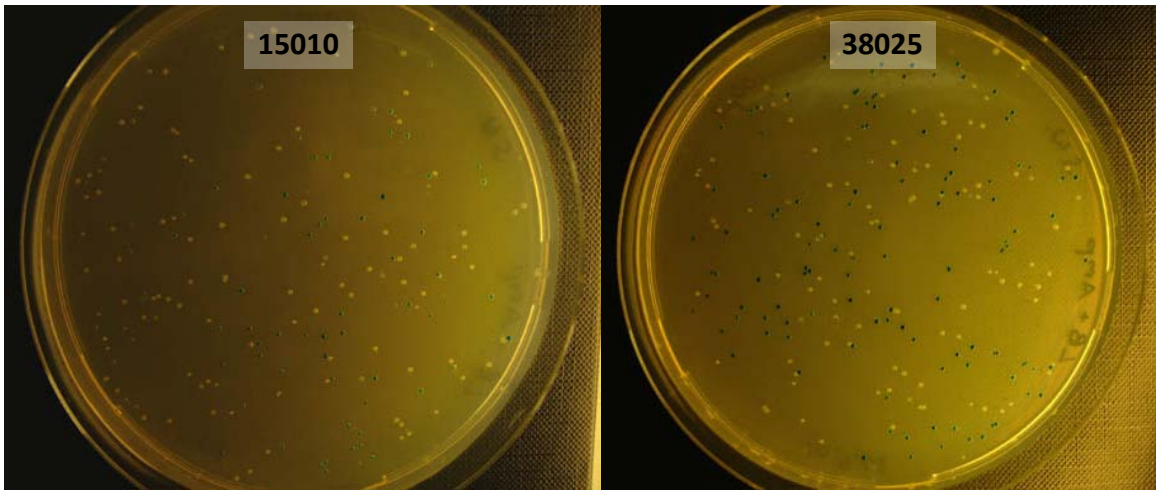


Figure 15: Pictures of transformed *E. coli* clones plated onto LB-ampicillin-X-gal plates. 15010 and 38025 correspond to the plant sequences, used for cell transformation. **White colonies**: successfully transformed clones; **Blue colonies**: did not uptake any plasmid, thus they did not carry any plant sequences

Four white clones of each plate were selected and the extracted plasmids were used as template for PCR amplification. Figure 16 shows the PCR products of the four isolations per DNA sequence. Amplified potato DNA samples were then prepared for sequencing.

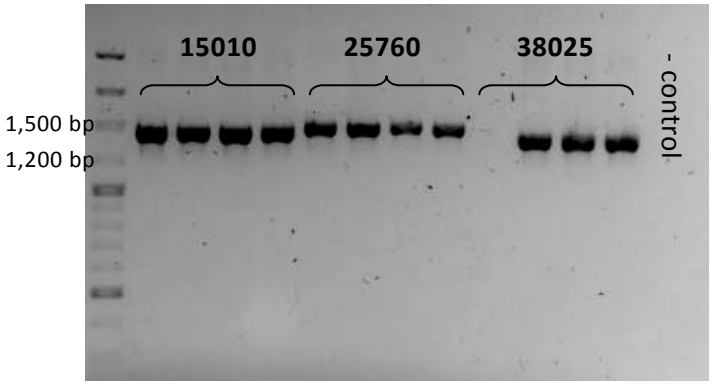


Figure 16: 1.5% agarose gel showing PCR products of each clone insert. Four clones of each target DNA sequence were picked and amplified. The length of the DNA products was approximately 1,400 bp, which corresponds to the length of the potato sequences. Amplification of the inserted plant sequences worked for all clones, except for the first clone picked from the 38025 harboring plate.

In most cases multiplex PCR (uses more than one primer pair) is less efficient than singleplex. In order to obtain best sensitivity only one pair of primers should be added to an assay. Therefore plant DNA had to be amplifiable applying bacterial primers. Thus adapter primers were introduced which contained the plant primers flanked by bacterial primers. The internal control sequences were amplified using these adapter primers (see figure 17). The products of this amplification are the plant sequences ligated with the sequences for primer 45F and primer 1391R.

Amplification of the plant DNA with the adapter primers did not work as expected. More than one band was observed on the gel. Therefore, a second amplification was performed using the bacterial 16S rRNA specific primers, to support solely amplification of already successfully ligated PCR products. After this amplification only a few samples showed bands. Further investigation revealed that the bacterial reverse primer sequence was linked to the 3' end of the plant reverse primer. Figure 17 shows the correct orientation of each primer sequence. Both, the forward and the reverse primer, require the bacterial 16S rDNA specific sequence on the 5' end of the primer.

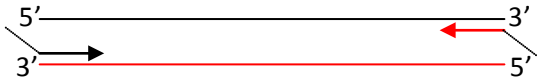


Figure 17: Design of adapter primers. The forward primer (homologous to the template strand) is drawn in black while the reverse primer (homologous to the coding strand) is drawn in red. The arrow indicates the direction of the primer (5' to 3') and the dotted line aligned to each primer represents the 16S rDNA specific primer sequence.

In order to control integrity of the plant sequences after several amplification steps, they were reamplified using the potato specific primers. This reamplification resulted in more than one band on the agarose gel for the 15010 sequence, as shown in figure 18.

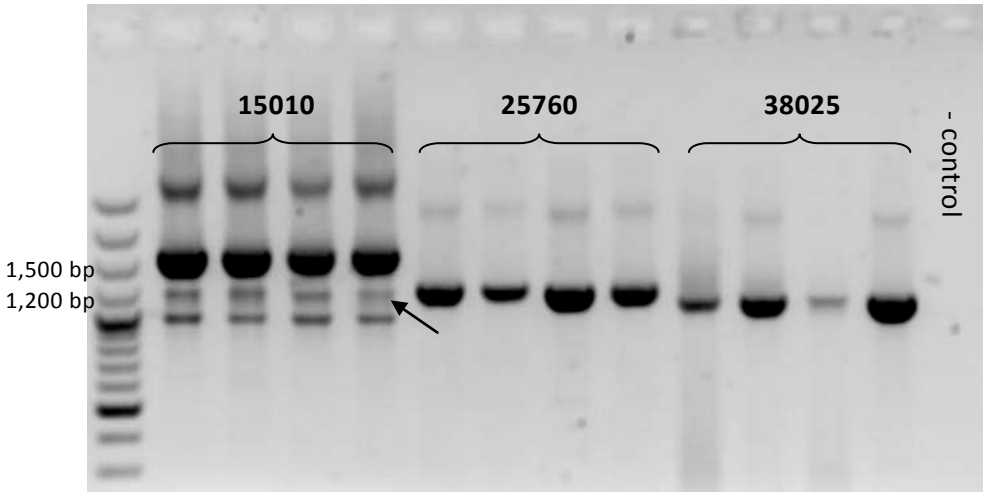


Figure 18: PCR products of amplified plant sequences loaded onto a 1.5% agarose gel. Plant sequences were first amplified using the adapter primers and then a second amplification was performed using the bacterial primers 45F and 1391R. This product was applied for re-amplification with plant primers in order to examine product integrity. 15010, 25760 and 38025, are the different plant sequences. The 15010 sequence gave four distinct bands on the gel. Only the second band from the bottom (indicated with an arrow) contained the desired sequence.

It is very important that the PCR products used as internal control are not a mixture of specific and unspecific amplification products. Therefore a DNA stock containing more than the specific sequence could not be used for internal control. Thus, a gel extraction was carried out with the targeted band cut from the gel and used for PCR product extraction. Subsequently the extracted DNA was initially amplified with the new adapter primers and in a following amplification with the bacterial 16S rDNA specific primer pair 45F and 1391R. Figure 19 shows amplification with the bacterial specific primer pair did now work resulting in clearly visible single bands per lane. This indicates a successful ligation of the bacterial primer sequences to the plant DNA.

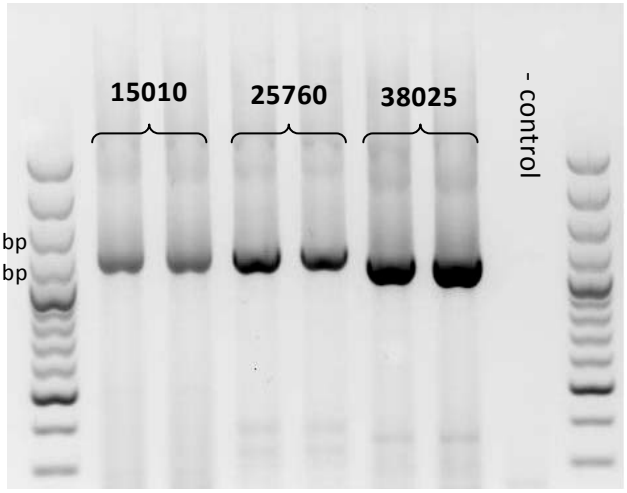


Figure 19: Agarose gel showing PCR products of amplified potato sequences applying primers 45F and 1391R. Designations 15010, 25760 and 38025 correspond to respective plant sequences.

Primary amplification with the primers 45F and 1391R worked perfectly, however, a subsequent amplification step of the first PCR product using the same primer pair only gave very little or no product. A repetition of the PCR did not lead to a solution of the problem. Therefore the initial PCR product of the amplification using the adapter primers was applied as internal control and was spiked into the sample at different working steps.

4.2.1.2 16S rDNA of methanotrophic bacteria

Genomic DNA from different methanotrophic bacteria were thankfully obtained by Levente Bodrossy. 16S rDNA was amplified using the standard primer pair 45F and 1391R. Figure 20 shows the PCR products after initial amplification of all sequences.

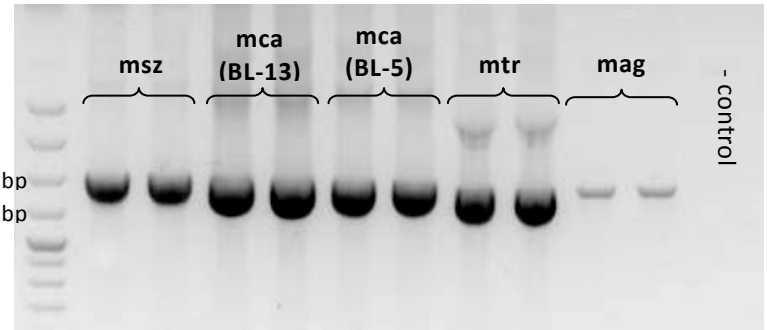


Figure 20: Agarose gel showing PCR products of the amplification of methanotrophic bacterial DNA. **Abbr.:** msz: *Methylocaldum szegediense*; mca: *Methylococcus capsulatus* (BL-13, BL-5 subgroup, respectively); mtr: *Methylosinus trichosporium*; mag: *Methylomicrobium agile*

The DNA from the methanotrophic bacteria did not need to be sequenced, because 16S rRNA sequences of the corresponding species were available in our arb database.

4.2.2 Quantification of internal control DNA

The ready to use sequences of methanotrophic bacteria and potato were quantified using NanoDrop®. The ng/µl (result of NanoDrop) was converted into copy number per µl; the formula is noted in the Material & Methods part. NanoDrop® results and calculated copy numbers per µl are listed in table 4.

sequence	ng/µl (mean of several measurements)	260nm/280nm (mean)	molecular weight of dsDNA sequence	copy number per µl
msz	111	1.85	827,685 Da	$8.1*10^{10}$
mca	116	1.86	821,056 Da	$8.5*10^{10}$
mtr	100	1.85	793,198 Da	$7.6*10^{10}$
mag	101	1.87	824,263 Da	$7.4*10^{10}$
15010	18	2.01	753,441 Da	$1.4*10^{10}$
25760	20	1.84	732,597 Da	$1.7*10^{10}$
38025	20	1.71	727,388 Da	$1.7*10^{10}$

Table 4: Results of NanoDrop® measurements and calculated copy number per µl of each internal control sequence. Each sequence was measured several times and means are listed. The molecular weight of each sequence (dsDNA) in Dalton (Da) was calculated using BioEdit software.

Dilution series were generated for all internal control sequences, as various concentrations of internal control would be needed in subsequent experiments. Methanotrophic DNA was diluted 1/8 to a concentration of circa 10^{10} copies per µl preceding several 1/10 dilution steps starting with 10^9 copies per µl and ending with 10^1 copies per µl as highest dilution. The PCR products of plant DNA already had a concentration of approximately 10^{10} copies per µl. A 1/10 dilution series was generated for plant PCR products starting with approximately 10^9 copies per µl and ending with 10^1 copies per µl.

4.2.3 Determination of optimal copy number of internal control

A certain amount of internal control DNA has to be added to each sample. In case too little control DNA is added, the internal control could not be detected on the microarray,

if too much DNA is put into the sample, it could hamper the amplification of the pathogen DNA.

4.2.3.1 Potato DNA

In order to determine the ideal concentration of internal control per assay a dilution series of the internal control originated from potato sequences was again amplified by PCR. In some assays a mixture of internal control and pathogen DNA was provided, to identify possible obstructive influence of the plant DNA on the amplification of pathogen DNA. The results of this experiment are shown in figure 21.

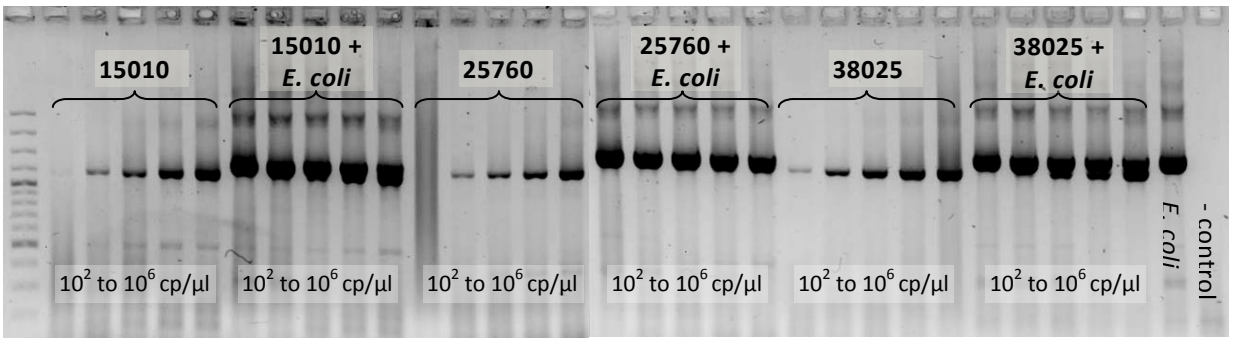


Figure 21: Agarose gel showing plant sequences with and without addition of *E. coli* DNA. 15010, 25760 and 38025: designations of the plant sequences; **10^2 to 10^6 cp/µl**: concentration of initially spiked-in internal control in copy number per reaction. The concentration of *E. coli* DNA always was 10^7 cells per ml giving a final concentration of 10^4 copies per assay.

The addition of the internal control originated from plant sequences to the pathogenic DNA did not hamper the amplification of any sequence. The detection limit of the plant DNA was identified as approximately 100 copies per reaction. In the row of sequence 38025 a clear band is visible at this concentration, while the band for 15010 at this concentration is barely visible. Amplification of 100 copies per µl DNA from the 25760 sequence did not work due to unknown problems. However, when comparing the bands generated by the PCR products with an initial 10^3 copies per reaction, they have a similar intensity for 15010 and 25760 sequences, suggesting that the detection limit for DNA from 25760 also is 100 copies per reaction (see figure 21). On the gel internal control and *E. coli* sequences generate two distinct bands, as the amplified plant DNA is slightly shorter than the amplified 16S rRNA gene fragment of bacteria. The two bands are especially visible for coamplified *E. coli* plus 38025 DNA. For *E. coli* plus 15010 DNA a slight

shift towards a higher electrophoretic mobility is observed with increasing concentrations of 15010 DNA. Two bands or an increasing electrophoretic mobility were not observed for *E. coli* plus 25760 DNA. However, analysis was difficult as 25760 PCR products alone and products of coamplified *E. coli* plus 25760 DNA were loaded onto two gels and only presented side by side for better visualization.

PCR products were subsequently labeled and hybridized onto the "Sepsis identification" chip. Average signal intensities of the bacterial probes did not decline, in samples where 10^3 copies of internal control DNA were added (see figure 22). Further *E. coli* specific signal intensities even increased at higher numbers of added plant DNA (e.g. probe eco2 gave a signal of 32,800 when 10^4 copies per µl of 15010 DNA were spiked-in while the signal was 31,100 when no plant DNA was added). The addition of plant DNA clearly did not hamper amplification or hybridization of *E. coli* DNA.

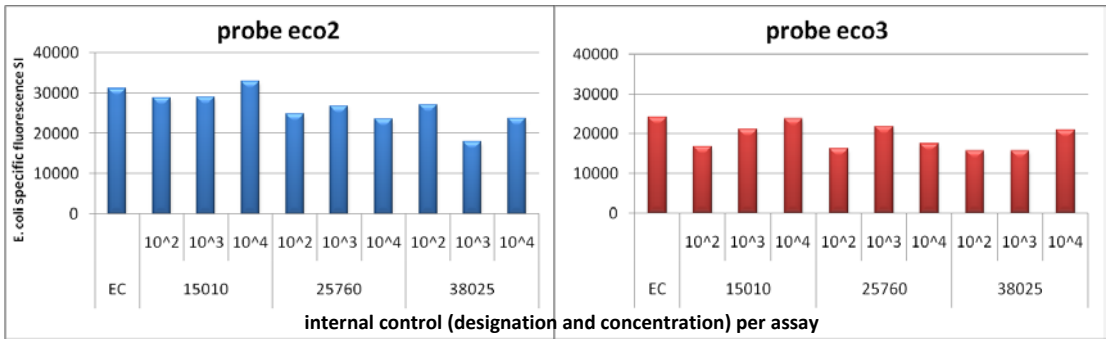


Figure 22: Influence of different concentrations of plant DNA on signal intensities of *E. coli* specific probes: left graph eco2 and right graph eco3. Signal intensities of eco2 and eco3 probes do not decrease with increasing concentrations of internal control in the sample. The concentration of *E. coli* DNA in each sample was 10^7 cells per ml. Signal intensities (SI) of *E. coli* specific probes are plotted on the y-axis. The concentration of plant DNA added to each sample is indicated on the x-axis. In the EC designated sample no plant DNA was added.

Table 5 lists signal intensities of plant specific probes at different spike-in concentrations. Except for 25760 a concentration of 10^2 copies per assay would give satisfactory probe signals when only plant DNA was amplified. However, addition of pathogenic DNA at a concentrations of 10^5 copies per assay hampered amplification of plant DNA resulting in lower plant specific signals by approximately 50 %. In detail probe iKc1-l gave a high signal of 15,000 when 10 cells per assay *E. coli* were added (to an internal control concentration of 10^3 copies per assay), which dropped to 8,000 when the concentration of *E. coli* DNA is 10^7 cells per assay. Signal decreases were not that consistent for all

probes (probe iKb3-l shows the highest signal when 10^5 cells per ml *E. coli* DNA were added). However, there was a clear tendency that plant specific probe signals decrease with increasing concentrations of pathogenic DNA. Based on these results and on the results of the agarose gel, it was decided to work with a concentration of 10^3 copies / assay of plant DNA in further experiments.

15010						
***E. coli* conc.**	**-**			**10^1**	**10^2**	**10^5**
Plant conc.	**10^2**	**10^3**	**10^4**	**10^3**		
iKb1-k	-	9000	16000	3500	1500	2000
iKb1-l	3000	13000	19000	2500	5500	2000
iKb2-k	-	3000	4000	3000	3000	-
iKb2-l	2000	9000	17000	2000	4500	1500
iKb3-k	3000	10000	12000	3000	3000	2000
iKb3-l	7500	15000	20000	7000	7000	9000
25760						
***E. coli* conc.**	**-**			**10^1**	**10^2**	**10^5**
Plant conc.	**10^2**	**10^3**	**10^4**	**10^3**		
iKa1-k	-	4500	6000	2000	-	3000
iKa1-l	-	9500	14000	4000	5000	6000
iKa2-k	-	7000	10500	7000	5000	2000
iKa2-l	-	8500	15000	3500	2500	2000
iKa3-k	-	-	-	-	-	-
iKa3-l	-	9000	15000	14000	7500	5000
38025						
***E. coli* conc.**	**-**			**10^1**	**10^2**	**10^5**
Plant conc.	**10^2**	**10^3**	**10^4**	**10^3**		
iKc1-k	-	800	1500	-	-	-
iKc1-l	16000	17000	20000	15000	11000	8000
iKc2-k	4500	7500	9500	6500	2000	-
iKc2-l	8000	11000	14000	8000	3000	1000
iKc3-k	2000	5000	6500	2500	-	-
iKc3-l	4000	9000	10000	5000	1000	-

Table 5: Comparison of internal control probe signals at varying concentrations of spiked-in plant PCR product (copies per assay) and pathogenic DNA (corresponding to cells per assay). **Plant probes**: iKa1-k, iKa1-l, iKb1-k etc. **Color code**: green: fluorescence signal intensity of 0-5,000; blue: 5,000-10,000; red: >10,000

4.2.3.2 16S rDNA of methanotrophic bacteria

The dilution series of the quantified PCR products of methanotrophic 16S rDNA was hybridized, to investigate the ideal concentration for detection on the microarray. The detection limit was shown to be approximately 500 to 1,000 copies of DNA per PCR reaction. Subsequently different methanotrophic DNA concentrations were added to PCR

assays containing pathogenic DNA. After amplification the products were loaded onto an agarose gel (figure 23).

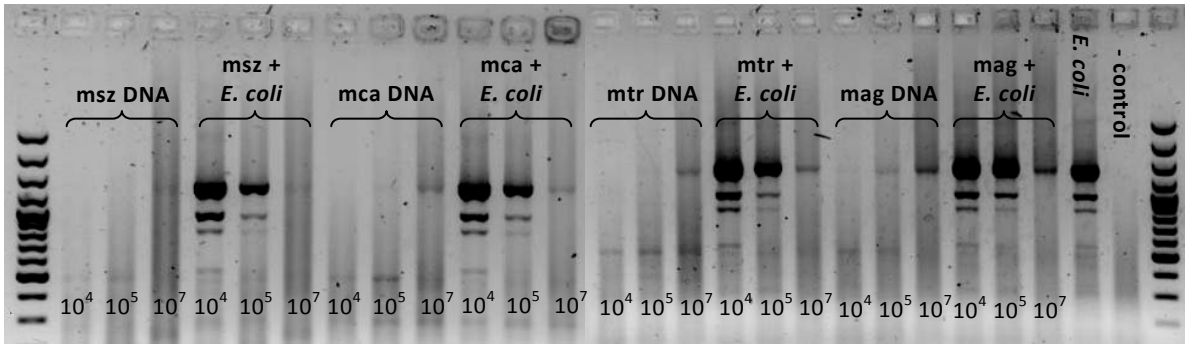


Figure 23: Comparison of amplification efficiency of solely methanotrophic DNA and in combination with *E. coli* DNA. Products were loaded onto an agarose gel. All concentrations (10^4, 10^5, 10^7) are copy numbers per reaction and refer to PCR products of methanotrophic DNA only. The concentration of *E. coli* DNA was 10^5 cells per assay.

Regarding these results the addition of diluted PCR products of methanotrophic DNA probably interferes with the amplification of the pathogenic DNA. The band intensity of *E. coli* DNA decreased successively with the addition of higher concentrations of internal control DNA. When 10^7 copies methanotrophic DNA were added to the *E. coli* DNA, only a very small band was observed. In order to confirm specific amplification the PCR products were hybridized onto the microarray.

These results confirmed that only very little *E. coli* DNA was amplified, when 10^7 copies methanotrophic DNA were added. In these samples the pathogen specific signals dropped significantly to 13,000 for probe eco2 and 11,000 for eco3. Whereas the sample containing 10^5 copies of internal control plus 10^5 cells / assay *E. coli* DNA, showed high *E. coli* specific probe signals on the microarray (21,000 for probe eco2 and 16,000 for eco3). Therefore, the concentration of methanotrophic DNA was kept as low as possible. A concentration of 10^3 copies per reaction of methanotrophic DNA gave only unsatisfactory signals on the microarray (probe mtr1-L signal intensities ranged from zero to 4,500). Therefore, 10^4 copies per assay of internal control were added for further experiments.

It remains unclear why pathogen specific amplification is stronger inhibited by the addition of methanotrophic 16S rDNA that by the addition of internal control originated from plant DNA. An explanation could be that the primers in use work better for the

methanotrophic sequences than for *E. coli* DNA. This, however, only partially explains the drastic decrease of PCR product in samples where 10^7 copies of methanotrophic DNA were added.

4.2.4 Internal control probe evaluation

Specific probes for detection of each tested internal control were designed using arb software package. These probes were assessed for sensitivity as well as specificity to the targeted internal control.

4.2.4.1 Internal control derived from potato DNA

Several hybridizations confirmed the high specificity of the plant probes. However, some probes gave stronger signals than others.

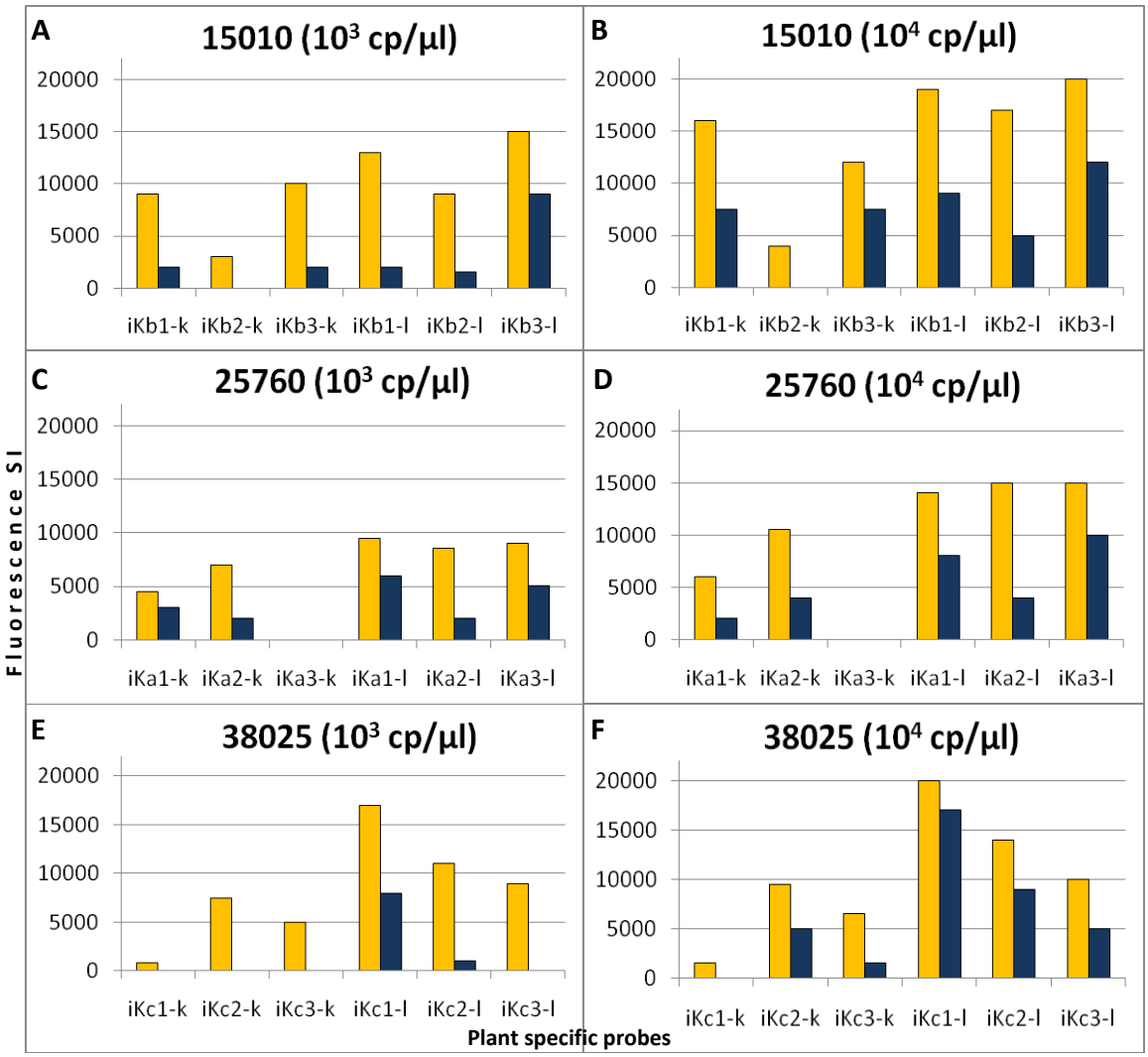


Figure 24: Evaluation of probes specific for internal control derived from plant DNA. The average signal intensities of diverse hybridizations are plotted in column charts. Sequence names and concentrations are indicated in the chart title (cp/µl: copy number per µl). Signal intensities are plotted on the y-axis while each column on the x-axis represents one specific probe. **Yellow columns** represent samples containing only copies of the internal control DNA. **Dark blue columns** represent samples of internal control DNA parallel amplified with *E. coli* DNA (c=10^7 cells/assay).

Figure 24 shows the results of hybridizations with different concentrations of internal control originated from plant DNA. Best signal intensities were obtained by longer probes (iKa1-l, iKa2-l, iKa3-l, iKb1-l …) which generated signals up to 20,000 when amplified without addition of pathogen DNA. Most short probes (iKa1-k, iKa2-k, iKa3-k, ikb1-K …) did not give strong signal intensities, especially when spiked to pathogenic DNA at high concentrations. Long probes such as iKb3-l, iKa1-l and iKc1-l showed specific signals of 9000, 6000 and 8000 when spiked to 10^5 cells per assay *E. coli* DNA, respectively. Best

signals from short probes were obtained by iKb3-k and iKa1-k, generating signals of 2000 and 3000, respectively. Short probes specific for 38025 gave no signals at all, when spiked to high concentrations of *E. coli* DNA. It was expected for longer probes to generate higher signals, as annealing efficiency correlates with the length of the complementary sequences. However, this makes longer probes also more susceptible to generating unspecific signals. Therefore, both longer and shorter probes were designed. All internal controls deriving from plant sequences were targeted by at least one highly specific probe which also generated high signal intensities. These were probes iKb3-l for 15010, iKa1-l and iKa3-l for 25760 and iKc1-l for 38025. Therefore, all three plant sequences were qualified as internal control and were utilized in further experiments.

4.2.4.2 Internal control derived from methanotrophic bacteria

Several hybridizations were accomplished, in order to select probes specific for the DNA of targeted methanotrophic bacteria. The results of these hybridizations are listed in table 6.

Species	name of probe	specific	unspecific	no signal
Methylocaldum szegediense / 16S rDNA	msz1			❖
	msz2			❖
	msz3			❖
	msz1-L			❖
	msz2-L			❖
	msz3-L			❖
Methylococcus capsulatus / 16S rDNA	mca1		✓	
	mca2			❖
	mca3	+		
	mca1-L		✓	
	mca2-L		✓	
Methylosinus trichosporium / 16S rDNA	mtr1		✓	
	mtr2			❖
	mtr3	+		
	mtr1-L	+++		
	mtr2-L			❖
	mtr3-L			❖
Methylomicrobium agile / 16S rDNA	mag1			❖
	mag2			❖
	mag3			❖
	mag1-L		✓	
	mag2-L			❖
	mag3-L		✓	

Table 6: Results of different hybridizations for the assessment of methanotrophic probe specificity. Quantified PCR products (dilution series) of methanotrophic 16S rDNA were PCR amplified, labeled and hybridized to the "Sepsis identification" chip. The products of the PCR amplification were also loaded onto an agarose gel (figure 23). Some probes (such as mca1 or mtr1) gave unspecific signals and are indicated in column unspecific. Column no signal highlights probes that did not give any signals. "+" indicates low specific signals, "+++" indicates high and specific signals.

As indicated in table 6 most probes gave no or unspecific signals. High specific signals were only obtained by probe mtr1-L. Some probes did not give any signals. This might derive from low PCR efficiency for these sequences, although the primers match well with the targeted sequence in the methanotrophic 16S rDNA gene. The PCR products loaded onto an agarose gel (figure 23) showed low amplification efficiency for *M. szegediense*, whose specific probes did not generate any signals. *M. agile* specific probes did give unspecific or even no signals. However, the agarose gel revealed good performance of sequence amplification. The bad performance of most methanotrophic specific probes might be a combination of low PCR efficiency (especially for *M. szegediense*) maybe due to bad working primers or other PCR related amplification problems and problems with the probes itself.

Figure 25 shows the results of the microarray analysis of methanotrophic specific probes. This figure demonstrates high numbers of cross-hybridization of many methanotrophic specific probes. Even the negative control sample (figure 25 D) generated unspecific signals from five methanotrophic probes. These probes gave strong signals independently of DNA present in the sample, making them not applicable as internal control probes. Other probes gave signals when methanotrophic bacteria DNA was in the sample, independent of the species (e.g. mag3-L).

Only three probes generated specific signals. Probes mtr3 and mca3 showed relatively low specific signals of approximately 9,300 each. Only probe mtr1-L generated a high specific signal (32,800). Addition of internal control to the samples at a concentration of 10^4 copies / assay did only slightly hamper amplification of pathogenic DNA (see chapter 4.2.3.2: 16S rDNA of methanotrophic bacteria). Based on hybridization results the internal control deriving from *M. trichosporium* seemed to be best suited and was therefore applied for further experiments.

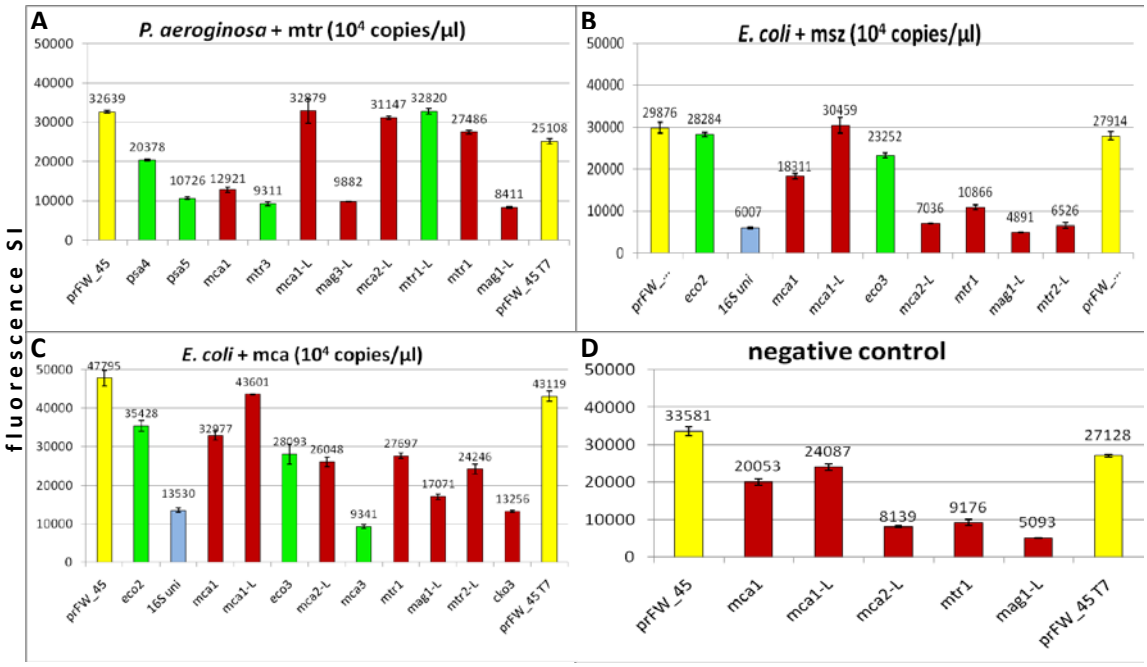


Figure 25: Results of microarray analysis. The DNA of different pathogens was amplified together with the methanotrophic PCR product, followed by a hybridization on the "Sepsis Pathogen Identification" chip. **A:** *M. trichosporium* DNA was spiked to *P. aeruginosa* DNA; **B:** *M. szegediense* was spiked to *E. coli* DNA; **C:** *M. capsulatus* internal control DNA was spiked to *E. coli* DNA; **D:** negative control hybridized to the chip. Signal intensities (SI) are drawn on the vertical axis, while probes are plotted on the horizontal axis**.** Methanotrophic species and corresponding probe names are listed in table 6. **Yellow bars** are signals generated from specific probes, used as control probes. **Green bars** are specific probe signals. **Red bars** represent unspecific signal values.

4.3 Comparability of internal control probe signals

In order to investigate the probe specificity and reproducibility of internal control probes, several hybridizations of pathogenic DNA coamplified with internal control DNA in one sample were carried out. The mean values from two different hybridizations are plotted in figure 26.

It is difficult to compare the results of different hybridizations as the signal intensities are subject to considerable fluctuations. It was the aim to make these values comparable by normalizing them with the internal control and thus enabling semiquantitative analysis.

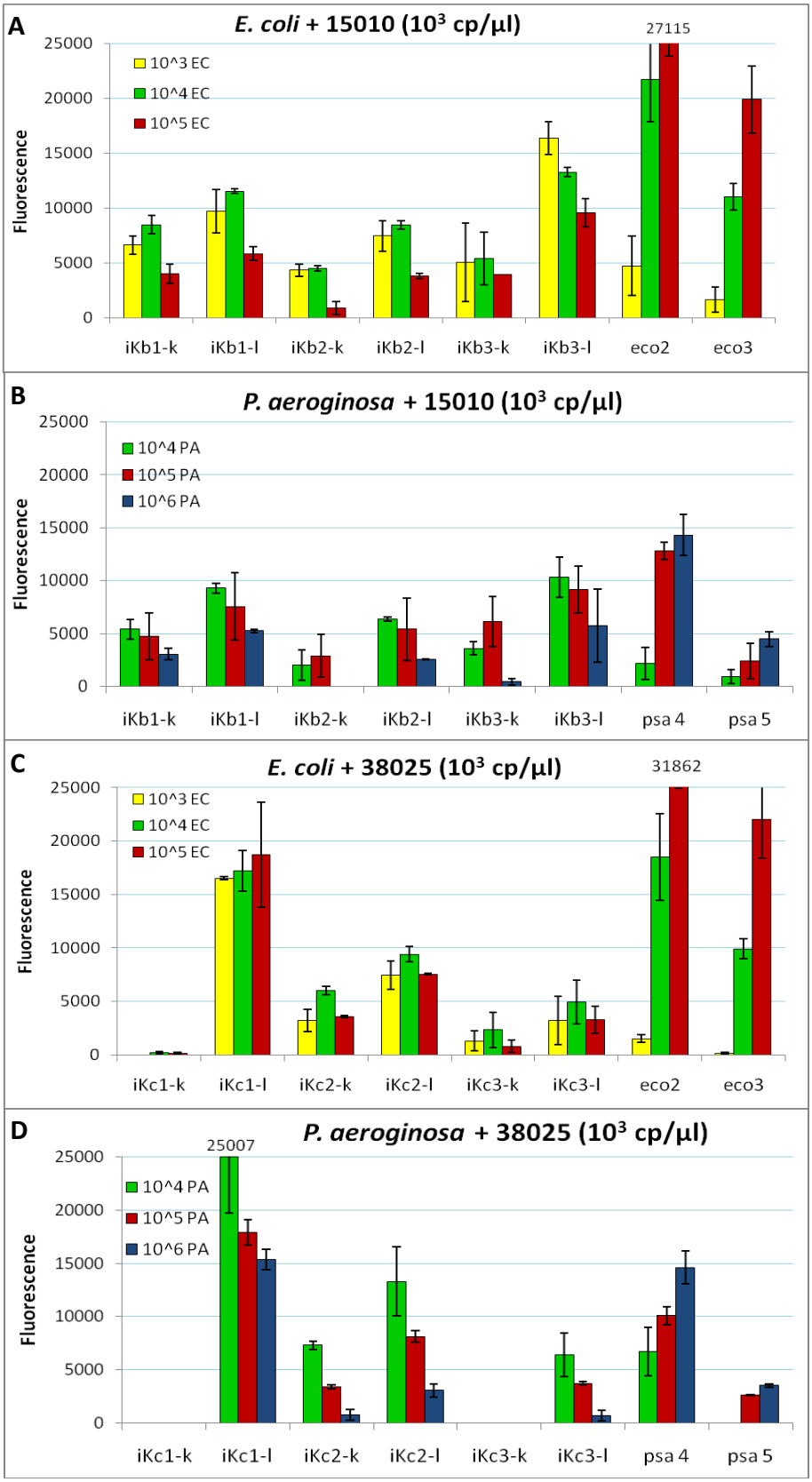


Figure 26: Evaluation of internal control probes of different hybridizations. A dilution series of *E. coli* (EC) and *P. aeruginosa* (PA) was spiked with internal control DNA, PCR amplified and hybridized onto the "Sepsis Identification" chip. The ideal concentration of control DNA was previously determined (10^3 copies per µl and assay). Different colors represent different pathogen DNA concentrations as indicated in the chart legend.

Figure 26 A and B show internal control 15010 spiked to dilutions of *E. coli* and *P. aeruginosa* DNA, respectively. Plant specific signal intensities decreased with increasing concentrations of pathogenic DNA present in the sample. For example a sample containing 10^3 cells per ml *E. coli* DNA additionally to the plant sequence (10^3 copies per assay), generated an iKb2-l signal of ~16,400 while in the sample containing 10^5 cells per ml *E. coli* DNA the iKb2-l signal was only 9,500. However, the decrease of plant specific signal intensities with increasing concentrations of pathogen DNA was less distinct for samples containing *E. coli* DNA than it was for *P. aeruginosa* samples.

While amplification of both species worked equally well (both species still showed bands on the gel when 10^4 cells / ml were amplified, but none when 10^3 cells / ml were amplified), the probes specific for *E. coli* generated much higher signals than the *P. aeruginosa* specific probes. Therefore, the detection limit is higher for *E. coli* than for *P. aeruginosa* DNA, allowing the detection of only 10^3 cells / ml *E. coli*.

15010 specific probe signals were a little less when the internal control was spiked to *P. aeruginosa* DNA than to *E. coli* DNA. The values for probe iKb1-l were 9,300 when spiked to 10^4 cells / ml *P. aeruginosa* but 11,600 when spiked to the same concentration of *E. coli* DNA. It was the other way round with 38025 specific probes. These probes generated surprisingly strong signals when the internal control was spiked to 10^4 cells / ml *P. aeruginosa* DNA. Signals of probes iKc1-l and iKc2-l were 25,000 and 13,300 when spiked to *P. aeruginosa* and 17,200 and 9,400 when spiked to the same amount of *E. coli* DNA.

Probes with small standard deviation values are better comparable and more reliable than probes with a high standard deviation. The ideal internal control probe generates relatively high, specific signals and shows fluctuations comparable to those from pathogen specific probes. These features are important for an internal control to enable semiquantitative analysis in microarray experiments.

Generally it was shown that standard deviation of mean probe signal intensities over several hybridizations relative to the signal intensity was lowest for long internal control probes. Especially probes iKb1-l, iKb2-l, iKc1-l and iKc2-l showed low average values of 31 %. Considerably higher were fluctuations of pathogen specific probes (49 % in aver-

age) and short internal control probes (esp. iKb3-k, iKc2-k) generated the highest standard deviation values relative to their mean signal intensities (55% in average).

For the 15010 sequence, probe iKb1-l was most suitable as internal control, followed by probe iKb3-l. For the 38025 sequence, probe iKc1-l worked best, followed by probe iKc2-l. This choice was confirmed by the mean signal intensities of all hybridizations (independent of pathogen species and concentrations). Based on all results, probe iKc1-l seemed to be the best internal control probe, as it provides high specific fluorescence (mean of all hybridizations is >18,000) and, compared to other probes, showed rather low fluctuations (standard deviation relative to signal intensity <20 %).

4.4 DNA Isolation from whole blood

Proper bacterial DNA Isolation from whole blood is one of the most important steps for the detection of pathogens with sufficient sensitivity and specificity. Several methods were tested by Herbert Wiesinger-Mayr. One of the best working assays, although relatively work intensive, was the MolYsis Kit from Molzym. Therefore, this kit was tested for the recovery of bacterial DNA as well as internal control DNA from whole blood samples. Bacterial cells were spiked to fresh whole blood, while the internal control DNA was added just before pathogen cell lysis. Bacterial DNA isolation worked well in comparison to isolations from pure culture, when the protocol was carefully followed. After PCR amplification a relatively small concentration of bacterial DNA could be detected on the gel (figure 27).

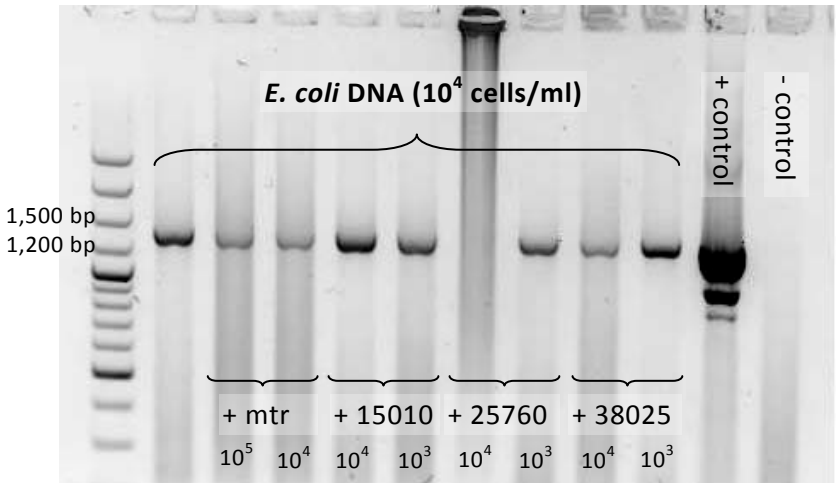


Figure 27: Agarose gel after electrophoresis of PCR products obtained by amplification of bacterial and internal control DNA. *E. coli* cells were spiked to whole blood in a final concentration of 10^4 cells per ml blood. Plant derived internal control DNA was added in concentrations of 10^3 and 10^4 copies per ml blood while mtr internal control was added in concentrations of 10^4 and 10^5 copies per ml blood. **Abbr**.: **mtr:** *M. trichosporium*; **15010**, **25760** and **38025** indicate the different plant internal control sequences; **positive control** sample: *E. coli* DNA (c=10^7 cells per µl) isolated from pure culture.

After microarray analysis of the DNA isolates, high *E. coli* specific signals were detected. However, the specific signals of internal control derived from plant DNA were relatively low. This might be caused by a partial loss of the DNA during the isolation process from whole blood or by an interference of the high background of human DNA in subsequent PCR and labeling reactions. However, low specific signals can partially be compensated by increase of pmt (photomultiplier transmission) during the scanning process. Figure 28 shows the microarray analysis results of different samples.

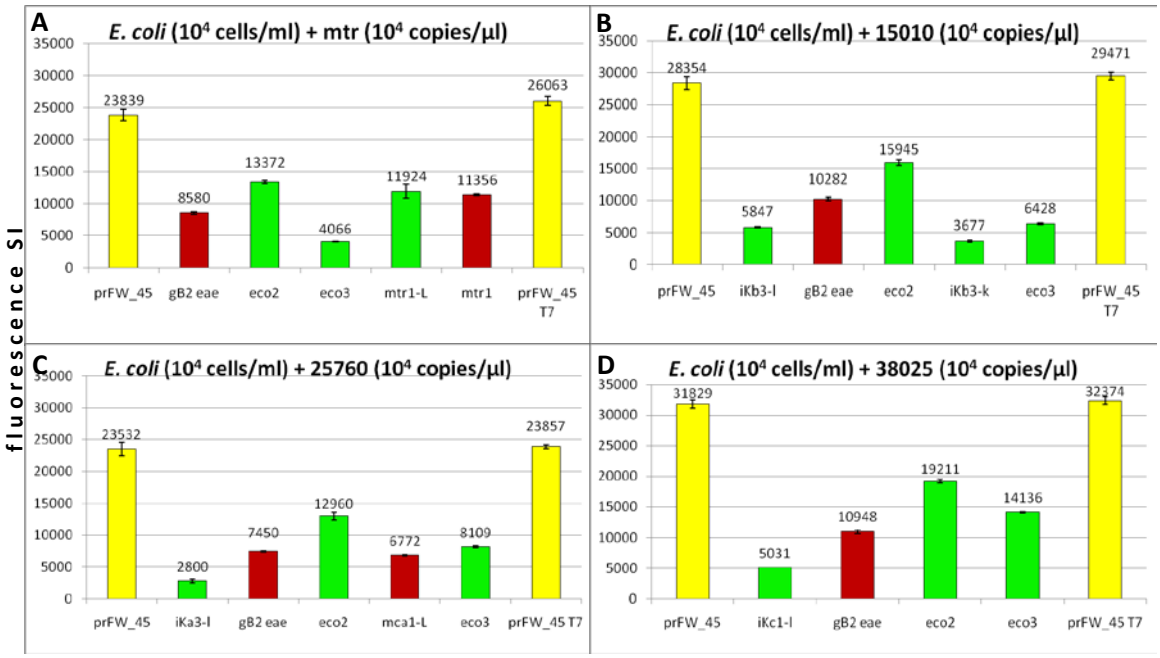


Figure 28: Results of microarray analysis. Whole blood was spiked with *E. coli,* and internal control DNA was added just before bacterial cell lysis. Then the pathogenic and internal control DNA was isolated from blood, PCR amplified and analyzed with the "Sepsis Identification" chip. **A:** *M. trichosporium* 16S rDNA was spiked to whole blood; **B:** 15010 internal control *DNA* was spiked to blood; **C:** 25760 *DNA* was spiked to and recovered from blood; **D:** 38025 internal control DNA was spiked to whole blood. On the **vertical axis** the signal intensities (SI) of the single probes obtained by microarray analysis are plotted. The **horizontal axis** shows the probe names. **Color code: yellow:** control probes for successful protocol realization; **green:** pathogen specific probes; **red:** unspecific signals

Figure 28 shows that the microarray signals for *E. coli* specific probes were rather high. Interestingly, *M. trichosporium* internal control probe signals were high as well (12,000), although the probe signals for all three plant sequences (iKa1-3, iKb1-3 and iKc1-3) were quite low. By spiking 10^3 copies of plant internal control DNA to 10^4 cells per ml *E. coli* DNA isolated from pure culture and subsequent PCR amplification, high specific signals were obtained after microarray analysis. Important plant specific probes iKb1-l, iKb3-l and iKc1-l generated signals of 11600, 13300 and 17200, respectively (see figure 26). However, when 10^4 copies internal control DNA were spiked to whole blood along with *E. coli* cells (c=10^4 cells per ml blood) only little plant specific signals could be detected on the microarray. Specific signal values were zero from probe iKb1-l (decline is 11600), 5800 from iKb3-l (decline of 7500) and 5000 from iKc1-l (decline of 15200). This clearly indicated that a huge amount of internal control DNA got lost during isolation from whole blood. No big differences were observed for pathogen specific signal intensities

between DNA isolation from pure culture (signal of probe eco2 was 20,100) or from whole blood (mean signal intensity was 15,400 for eco2), however. The loss of internal control during DNA isolation from whole blood might be caused by the column purification of DNA included in the MolYsis kit. These columns are specialized for the isolation of bacterial genomes which are typically well above 5 million bases in length. The internal control sequences, however, are only approximately 1,400 bp long and therefore, might not attach to the column as efficiently as longer sequences.

4.5 Quantification by Real-time PCR

In order to confirm the semiquantitative data obtained by microarray analysis a real-time PCR protocol was established. Several considerations have to be made for introducing a well working protocol, which allows the quantification of unknown samples. The primer pair should amplify a target fragment shorter than 150 bases and should not show runs of identical nucleotides. Furthermore, melting temperature (Tm) values of the primers should lie between 58 and 60°C and complementarities within or between primers should be avoided [Applied Biosystems, Real-Time Tour 2008]. Primer sequences used in this work are listed in table 11 in Appendix 1. The ideal primer concentration was determined to be 900 nM. Finally a reliable and distinct standard curve needs to be established for a successful quantification of unknown targets. The standard curve ideally amplificates with high efficiency and covers a wide range of concentrations.

4.5.1 Selection of primer pair

Different primer pair combinations were tested in order to select a good working primer pair for use in real-time PCR. All primer pairs were specific for the 16S rRNA gene of bacteria.

Real-time PCR efficiency is optimal when the amplified sequence is shorter than 200 bp (base pairs) because the ability of PCR product doubling increases with decreasing amplification length. Therefore a good working primer pair needs to be selected.

Different primer pair combinations were tested in a normal PCR and the products analyzed by agarose gel electrophoresis. Selected results are listed in table 7.

forward primer	reverse primer	amplicon length	PCR efficiency
8F	1491R	1,483 bp	++
45F	1391R	1,346 bp	+++
45F	154R	109 bp	+
45F	316R	271 bp	++
347F	514R	167 bp	+
347F	673R	326 bp	+++
521F	673R	152 bp	+
783F	926R	143 bp	+-
916F	1391R	475 bp	+++

Table 7: List of primer pairs with their respective amplicon lengths and amplification efficiencies. "+++": excellent amplification; "++": good amplification; "+" acceptable amplification; "+-": bad amplification

In our experiments the amplification of larger fragments, usually worked better, than the amplification of smaller sequences. Figure 29 shows that amplification of *E. coli* and *S. aureus* DNA with the primer pair 347F and 673R (amplicon length is 326 bp) worked better than with the primer pair 347F and 514R (amplicon length is 167 bp). However, a few primer pairs amplifying smaller fragments were selected for application in real-time PCR.

Figure 29: Agarose gel showing PCR products amplified with different primers. A DNA dilution series of 10^7 cells/ml to 10^3 cells/ml of *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) DNA were amplified using Taq Polymerase mix (Invitrogen) and primer pairs 347F and 514R, 347F and 673R, respectively. + control: *Enterococcus faecium* DNA (10^8 cells/ml); - control: water control

Some negative control samples showed bands in the gel (figure 29). To overcome this problem, several PCR mixes (Taq Polymerase from Invitrogen, ImmoMix Red from Bioline and Molzym 16S basic from Molzym) were tested. Amplification with the ImmoMix resulted in huge amounts of PCR product as visualized by gel electrophoresis (data not shown). However, severe problems concerning contamination of negative control samples were encountered. PCR applying the Taq Polymerase Mix and the Molzym mix showed an equal efficiency of amplification. As seen in figure 29, amplification with the Taq Polymerase mix also showed slight bands in negative control samples. As no problems concerning the negative control were observed with the Molzym mix, this PCR mix was used for amplification of DNA in further experiments.

4.5.2 SYBR® Green PCR Master Mix

SYBR® Green real-time PCR was tested by applying the primer pair 521F and 673R at two different concentrations (500 nM and 900 nM). *E. coli* DNA corresponding to 10^5 cells was added per real-time PCR assay.

Figure 30 shows threshold cycles of a DNA dilution series. Ct values were lower when primers were added at a concentration of 900 nM compared to 500 nM. Average threshold cycle values in samples containing undiluted template DNA were 15.3 for a primer concentration of 500 nM and 15.2 for primer concentrations of 900 nM. Bigger differences were observed in 1/100 diluted samples of the template DNA. The values were 20.2 and 19.0 for primer concentrations of 500 nM and 900 nM, respectively. Therefore a concentration of 900 nM was chosen for further experiments. However, threshold cycle differences between single dilution steps of *E. coli* DNA did not correlate exactly. A threshold cycle difference of 1 (or slightly over 1, when PCR efficiency is not 100 %), is expected from single ½ dilution steps, as in PCR the DNA amount doubles with every cycle. However in some assays the threshold cycle of a ½ dilution was lower than the threshold cycle of the original DNA (see figure 30). This indicates that inhibitors of the PCR were present in the samples. These results were confirmed in further experiments. Threshold cycles between assays were subject to considerable fluctuations. While mostly the threshold cycle differences between single ½ dilution steps were zero to 1.8, in

extreme cases differences of up to minus 6 cycles were observed between undiluted samples and ½ dilutions.

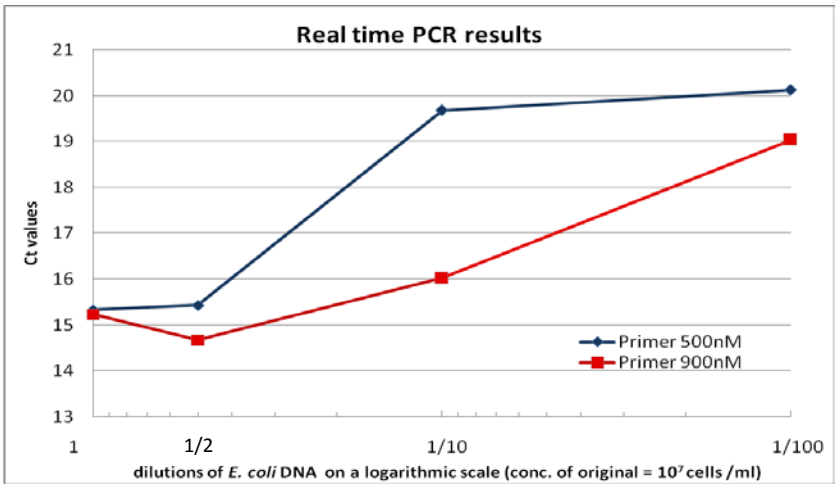


Figure 30: Results of real time PCR experiment with primer pair 521F and 673R at concentrations 500 nM and 900 nM. The x-axis shows applied dilutions of the original DNA (indicated as 1) on a logarithmic scale. Threshold cycles (Ct) are drawn on the y-axis.

Dissociation curve analysis available for SYBR® Green PCR provided a good indication that only the desired fragment was amplified. Unspecific amplification or generation of primer dimers is visualized in the dissociation curve by a second peak in the chart. Figure 31 shows the dissociation curve for real-time PCR applying SYBR® Green and the primer pair 521F and 673R in concentrations of 500 nM and 900 nM. Only one peak can be seen in this dissociation curve, thus revealing specific amplification of primer pair 521F and 673R.

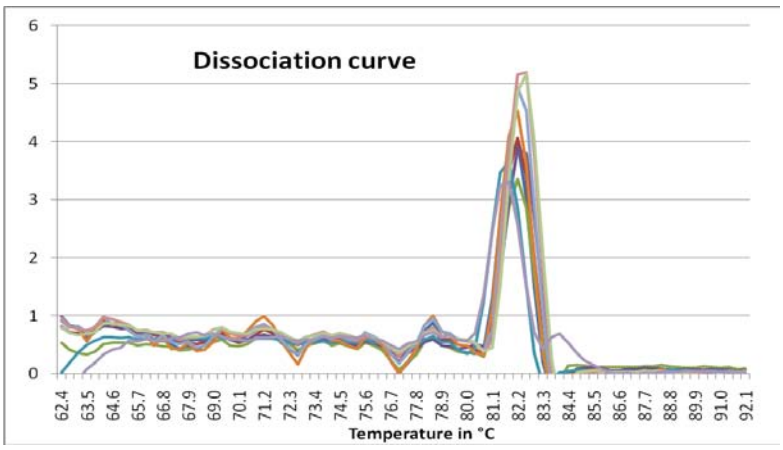


Figure 31: Graph of SYBR® Green real-time PCR dissociation curve. Increasing temperatures are drawn on the x-axis, while the derivative of detected fluorescence intensity is drawn on the y-axis.

4.5.3 TaqMan® Universal PCR Master Mix

Real-time PCR using TaqMan® probes was compared to SYBR® Green assays. However, as the results were not better than in real-time PCR applying SYBR® Green, it was settled on the SYBR® Green PCR Master Mix (Applied Biosystems) for further experiments. A dilutions series of *S. aureus* DNA (starting concentration corresponding to 10^5 cells) was generated and amplified using both real-time PCR assays. Threshold cycles were reached 7 cycles later with the TaqMan® mix on average. For example the 1/20 dilution of the template DNA had a threshold cycle of 20 when amplified with SYBR® Green but of 27 when amplified with the TaqMan® mix. This might be due to the fact that only one TaqMan probe (only one fluorophore) is present per strand of dsDNA, while multiple copies of SYBR® Green bind. Therefore, later threshold cycles in TaqMan® PCR do not necessarily mean that it did not work as well as SYBR® Green real-time PCR.

4.5.4 Generation of a standard curve for quantification

By relating threshold cycles of unknown samples to a standard curve, quantification is possible. A good standard curve was obtained by establishing a ½ dilution series of *M. trichosporium* PCR products (see figure 32) and amplification with the SYBR® Green real-time PCR mix. In these high diluted samples, no PCR inhibitory substances were present, which enabled efficient amplification of target DNA. However, the 16S rRNA sequences of methanotrophic bacteria are slightly different from human pathogens like *E. coli* and *S. aureus*. The previously applied primer pair 521F and 673R, therefore did not work with DNA from *M. trichosporium*. Primer pair 521F and 764R was selected for real-time PCR experiments amplifying DNA from methanotrophic bacteria.

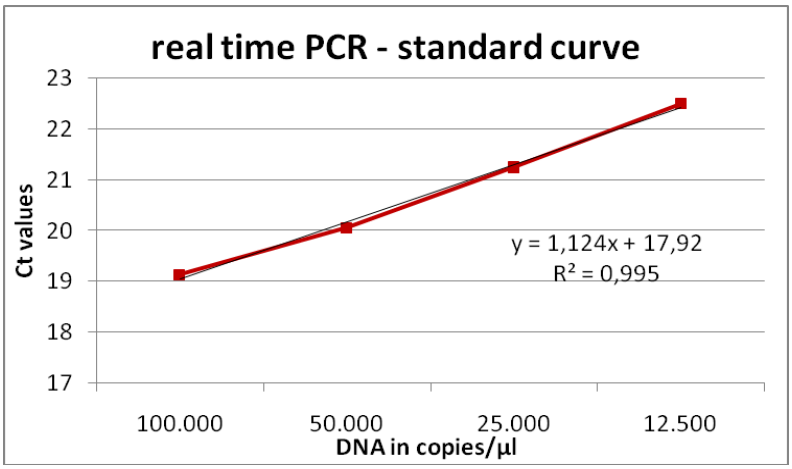


Figure 32: Standard curve obtained by the amplification of a ½ dilution series applying the primer pair 521F and 764R at a concentration of 900 nM. **Template DNA:** PCR product of *M. trichosporium* DNA quantified by NanoDrop®, diluted to concentrations from 100,000 to 12,500 copies per µl. One µl of DNA suspension was added per assay. A trendline was applied to the standard curve in order to determine the slope and R^2 value of the curve

The standard curve of figure 32 can be used for quantification. The distance between Ct values of single dilution steps is 1.124 (see formula in figure 32), which means that the DNA is doubled every 1.124 cycles (corresponding to an efficiency of 95.5 %). A factor of 1 would mean that the amplification was 100 % effiecient, which is almost impossible to reach in real-time PCR [Applied Biosystems, Real-Time Tour 2008]. For quantification the R squared value should be above 0.9. In out experiments this value was 0,995.

Although the qualitiy of the standard curve was sufficient (confirmed by the R squared value), a quantification of target DNA was not possible. The sample DNA was obtained by lysis of bacterial cells from pure culture or from whole blood followed by purfication. There were still PCR inhibitory substances in these samples which prevented a reliable amplification and therefore a quantification.

4.6 Assessment of bacterial viability

DNA cannot be used as target for the assessment of bacterial viability, since in actively killed cells the DNA persists and can be detected for long periods of time [Masters *et al.*, 1994]. RNA, especially mRNA is the better target for analyzing bacterial viability [Keer and Birch, 2003]. Several methods for the amplification of 16S rRNA and different mRNA

targets were tested. The best method would be integrated into the sepsis chip protocol, to be able to directly detect RNA on the chip. The calculations of ratios between DNA and RNA would allow the discrimination between alive and dead bacteria.

4.6.1 Superscript III Reverse Transcriptase and AMV Reverse Transcriptase

Superscript reverse III transcriptase (Invitrogen) did only work when high amounts of target RNA were used, as visualized by gel electrophoresis. Similar results were obtained by Avian Myeloblastosis Virus reverse transcriptase (AMV-RT). In both cases, the products of the reverse transcription reaction were added to a PCR to amplify the previously generated cDNA. Figure 33 shows the results of Superscript III and AMV-RT amplification of RNA. On the gel it can be seen that Superscript reverse transcription worked slightly better (stronger band in the 1/50 dilution sample) than AMV-RT.

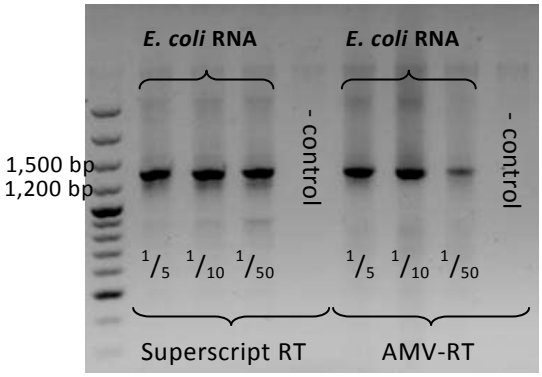


Figure 33: Products of Superscript III RT and AMV-RT followed by PCR loaded onto 1.5% agarose gel. 1/5, 1/10 and 1/50: dilutions of *E. coli* RNA (initial conc. corresponding to 10^5 cells).

4.6.2 Omniscript Reverse Transcription (Qiagen)

Omniscript reverse transcription followed by PCR, also worked with low amounts of template RNA. Purified RNA equivalent to 10^5 cells was added per Omniscript reverse transcription assay. After the subsequent PCR the products were loaded onto a gel leading to thick bands (figure 36). Also after hybridization high pathogen specific signals were detected on the microarray. Therefore Omniscript reverse transcriptase (Qiagen) was considered to work better than Superscript reverse transcriptase (Invitrogen) and AMV-RT (Invitrogen) in terms of sensitivity. Omniscript reverse transcription was then further applied for positive control samples for the experiments based on nucleic acid sequence-based amplification (NASBA).

4.6.3 Nucleic acid sequence-based amplification (NASBA)

4.6.3.1 Home-made NASBA

NASBA was carried out following the protocol by Song *et al.,* 2000. Subsequent to the NASBA reaction no PCR was needed, as in NASBA an amplification of the target RNA is accomplished. However, the NASBA reaction did not perform with sufficient efficiency.

Because of the necessary activity of three enzymes in NASBA, there are several factors that can inhibit or hamper the reaction. A lot of parameters were changed during troubleshooting. First the functionality of two out of the three enzymes was tested using the primers T7-8F and 1391R. The AMV-RT (Invitrogen) was tested by reverse transcribing a RNA template followed by a PCR reaction to amplify the cDNA (the results can be seen in part 4.6.1 of the results section). Then the T7 polymerase (Roche) was tested by realizing an *in vitro* transcription reaction. The template DNA needed to harbor the T7 promoter sequence. Therefore a PCR was performed applying the primer pair T7-8F and 1391R, which ligated the T7 sequence to the amplicon. The diluted PCR product was then added as template DNA to the *in vitro* transcription reaction. The results of this *in vitro* transcription can be seen in figure 34.

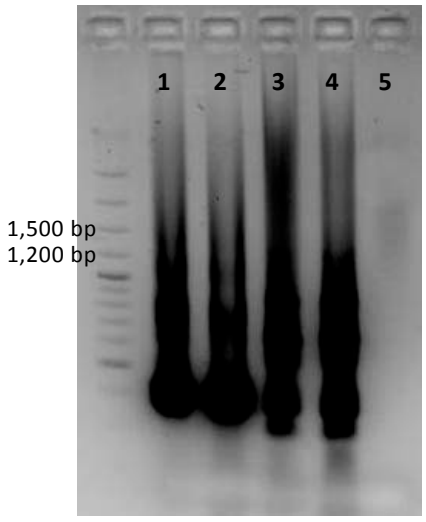


Figure 34: T7 in vitro transcription products loaded onto a 1.5 % agarose gel. **Template DNA**: *E. coli* RNA reverse transcribed into cDNA and subsequently PCR amplified applying primers T7-8F and 1391R. **Lane 1 and 3**: 1 µl of template DNA was added into the reaction; **lane 2 and 4**: 5 µl of template DNA were used; **lane 5**: negative control sample

Figure 34 shows that the *in vitro* transcription worked. However, the products do not form one distinct band, but a smear, indicating that the RNA was degraded either before or during the running of the gel. The activity of the RNase H (New England Biolabes Inc.) was not tested individually.

During the next step the efficiency of the NASBA buffer was determined. An Omniscript reverse transcription reaction was carried out using the buffer prepared for the NASBA reaction instead of the typically used buffer RT (Qiagen). The reverse transcription applying the NASBA buffer did work, although not as efficiently as with the buffer delivered by Qiagen for reverse transcription. Distributed buffers have to pass extensive performance tests before they are sold. Therefore, it was expected that purchased ready-to-use buffers work better than home-made ones. Plus, the NASBA buffer does not only have to provide the optimal environment for reverse transcription but also for *in vitro* transcription and RNase H mediated reactions.

4.6.3.2 NASBA applying the NucliSENS EasyQ® Basic Kit v2 (Biomérieux)

The home-made NASBA mixture was compared to the efficiency of the commercially available NASBA kit from Biomérieux. When the NASBA products were directly loaded onto the gel no bands were observed. However, after a subsequent PCR bands on the gel were visible. Therefore, it was considered that the reverse transcription reaction in NASBA did work, but at a very low sensitivity. One possible explanation was that the T7 promoter sequence was not or wrongly aligned to the target sequence.

The NASBA reaction applying a new set of primers together with the Biomérieux kit gave bands on the gel (see figure 35). Although the bands appear to be blurry, they did migrate with the correct electrophoretic mobility. The products of the primer pairs 45F and 317R_T7, 45F and 673R_T7, 45F and 1391R_T7 were expected to be 272 bp, 628 bp and 1346 bp in length, respectively (see figure 35 A). After a subsequent PCR the samples gave strong and clear bands.

In figure 35 B a dilution series of *S. aureus* RNA was amplified by NASBA. Also during this experiment blurry bands were obtained. The single dilution steps are identifiable, except the 1/1,000 dilution gave a stronger band, than the 1/100 dilution step. Also the sequences were migrating with the correct electrophoretic mobility, as expected for a product of the primer pair 347F and 673R_T7, which is 326 bp in length.

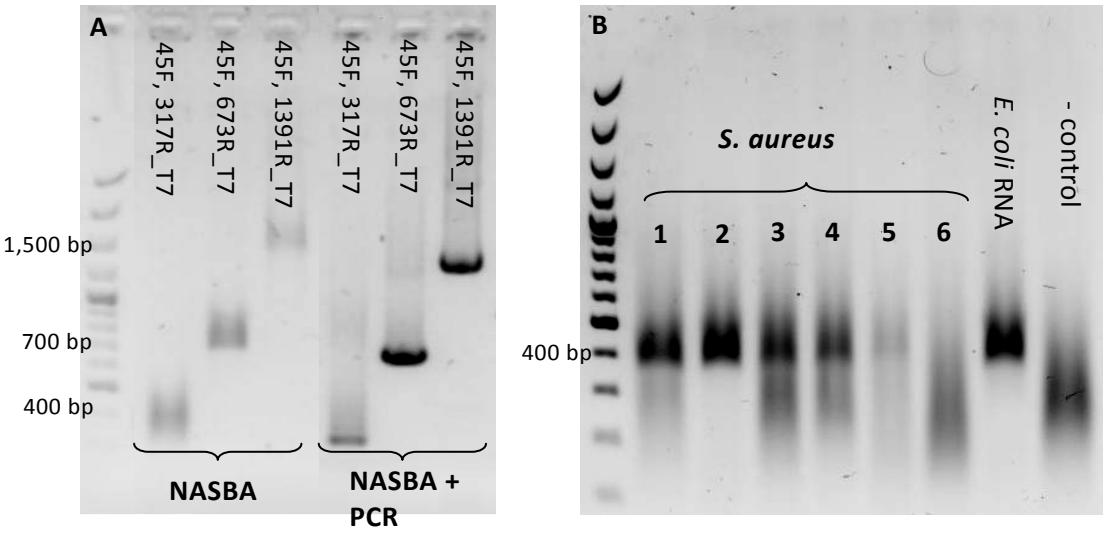


Figure 35: NASBA products applying the NucliSENS EasyQ® Basic Kit v2 were loaded onto 1.5% agarose gels. **A:** new reverse primers 317R_T7, 673R_T7 and 1391R_T7 were tested. Products were loaded directly (left) and after a subsequent PCR (right) onto the gel; **B:** NASBA of a dilution series of *S. aureus* RNA (original concentration corresponds to approximately $5*10^7$ cells per assay) applying primers 347F and 673R_T7. Numbers 1-6 correspond to dilution steps of a 10 fold dilutions series (10^{-2} to 10^{-7}, respectively). Undiluted *E. coli* RNA (concentration corresponds to $5*10^7$ cells per assay) was added to a positive control sample. The negative control sample showed a band. However, this band was extremely blurry and did show an increased electrophoretic mobility compared to the other samples. The same effect was observed in the $1/10^{-7}$ diluted sample and also, less distinct in lane 1, 3 and 4.

Also in subsequent experiments the products of the NASBA reaction gave always blurry bands. NASBA applying the NucliSENS EasyQ® Basic Kit v2 did work, however, RNA amplification applying other methods such as reverse transcription followed by PCR gave better results.

4.6.4 OneStep RT-PCR (Qiagen)

Another method for the amplification of RNA is Reverse transcription-PCR (RT-PCR). It combines reverse transcription of RNA and amplification of cDNA in one assay, with the advantages of speed and a reduced risk of contamination. OneStep RT-PCR from Qiagen gave the best results for the amplification of RNA. Also, when only small amounts of template RNA were added to the reaction, a lot of product could be detected by gel electrophoresis.

OneStep RT-PCR was compared to Omniscript reverse transcription followed by PCR realized as a two-step assay. The results are shown in figure 36. In order to prevent possible DNA amplification the RNA template was DNAse digested prior to RT-PCR.

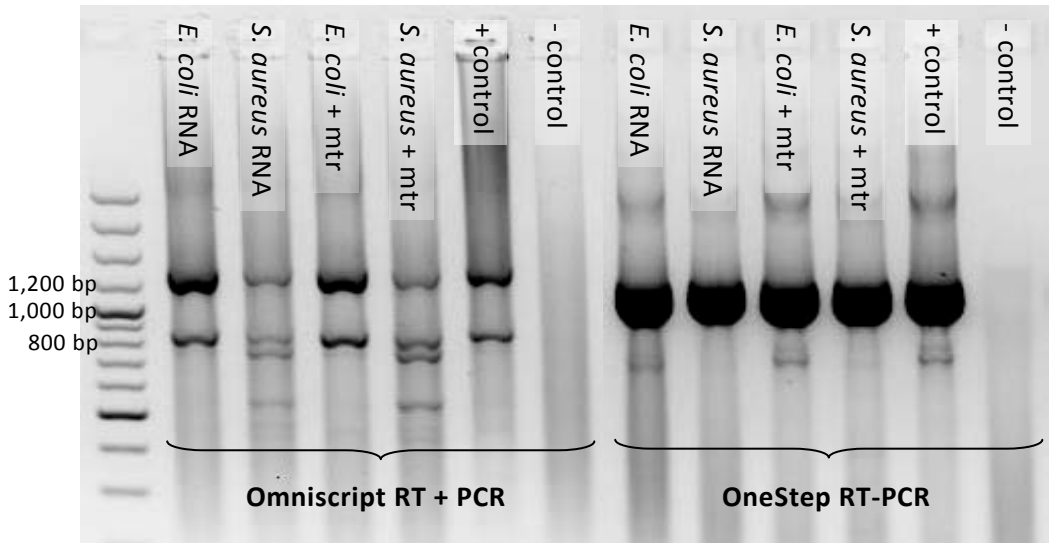


Figure 36: Comparison of Omniscript reverse transcription followed by PCR and One-Step RT-PCR. *E. coli* and *S. aureus* RNA was added at a final concentration of 10^5 cells per assay. *M. trichosporium* (mtr) DNA was added in a concentration of 10^4 copies per assay. **+ control:** Highly concentrated *E. coli* RNA

OneStep RT-PCR worked considerably better than Omniscript reverse transcription followed by PCR (see figure 36). The detection limit for OneStep RT-PCR was 10^3 cells per assay for *E. coli* RNA and 10^2 cells per assay for *S. aureus* RNA, as determined in following experiments (data not shown). After hybridization of the samples high pathogen specific signals were obtained on the microarray (figure 37). *E. coli* specific probes eco2 and eco3 generated signals of 13000 and 6600, respectively and *S. aureus* specific probes sar2 and sar3 showed signal intensities of 4200 and 6100, respectively. Therefore, OneStep RT-PCR was the method of choice for RNA amplification.

M. trichosporium was added to the sample to evaluate signal intensities of pathogen specific probes. The efficiency of every microarray assay can be determined by analyzing the signal intensities of the internal control. By normalizing pathogen specific signals with the internal control signals, fluctuations between assays are compensated, which makes semiquantification possible. However, no signals were obtained on the microarray for mtr specific probes (figure 37).

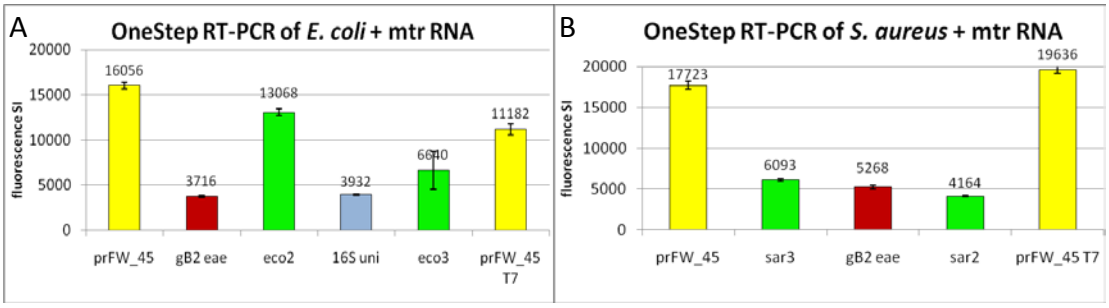


Figure 37: OneStep RT-PCR amplified *E. coli* (A) and *S. aureus* (B) RNA hybridized to the "Sepsis Identification" Chip. *M. trichosporium* RNA was added in a concentration of 10^4 copies per assay. On the **vertical axis** signal intensities obtained by microarray analysis are plotted. The **horizontal axis** shows the probe names. **Color code: yellow:** specific probes for control of successful protocol realization; **green:** pathogen specific probes; **red:** unspecific signals

4.6.5 Housekeeping genes

Since mRNA is a better target for the assessment of bacterial viability than rRNA [Keer and Birch, 2003], several primers and probes were designed for diverse housekeeping genes. For assessment of a quantification method it is necessary to determine the amount of RNA of cultures in different growth phases and calculate the ratio of DNA. Housekeeping genes are essential for the survival of the cell and therefore are constantly expressed. That makes the mRNA of housekeeping genes especially interesting for the viability status testing of bacteria.

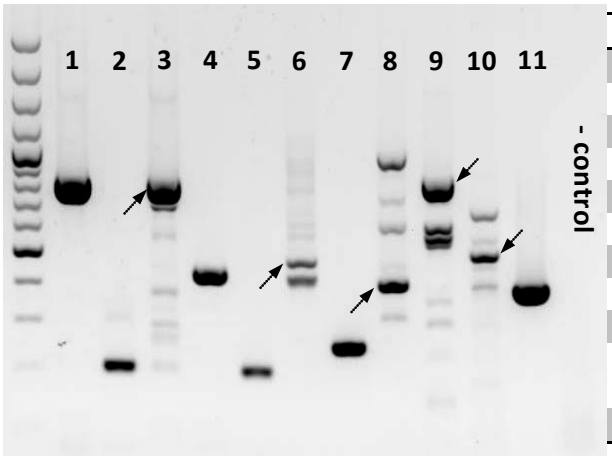
However, before bacterial quantification applying the mRNA of housekeeping genes is possible, several considerations must be kept in mind. Many housekeeping genes do not harbor highly conserved regions, making it difficult to identify sequences within these genes specific for a wide range of bacteria. This makes the design of probes and primers for housekeeping genes exceptionally difficult. An arb database was established, which was the basis for specific probe and primer design. Sequences were obtained from the NCBI database [http://www.ncbi.nlm.nih.gov] and subsequently loaded into arb. As a result of these data analysis different primers and probes were designed. The sequences are listed in table 13 in Appendix 1.

Table 8 lists selected housekeeping genes and names the proteins they encode.

Gene name	Encoded protein
fusA	Translation elongation factor G
leuS	Leucyl-tRNA synthetase
pyrG	CTP synthase
recG	ATP-dependent DNA helicase
glpK	Putative glycerol kinase
gyrB	DNA gyrase subunit B

Table 8: Housekeeping genes and names of encoded proteins.

The DNA of housekeeping genes was amplified to test for specificity of primers and probes. Figure 38 shows the products of this amplification loaded onto a 1.5 % agarose gel.



Nr	Species	Pr Fw	Pr Rev	Length
1	*E. coli*	fusA3f	fusA4r	~800 bp
2	*E. coli*	fusA3f	fusA5r	~170 bp
3	*E. coli*	leuS3f	leuS4r	~780 bp
4	*E. coli*	pyrG3f	pyrG4r	~400 bp
5	*E. coli*	pyrG3f	pyrG5r	~150 bp
6	*E. coli*	recG3f	recG4r	~400 bp
7	*S. aureus*	glpK1f	glpK2r	~250 bp
8	*E. coli*	gyrB1f	gyrB1r	~370 bp
9	*E. coli*	gyrB2f	gyrB3r	~775 bp
10	*P. aerugi-nosa*	gyrB3f	gyrB3r	~440 bp
11	*S. aureus*	gyrB4f	gyrB4r	~340 bp

Figure 38: PCR products of diverse housekeeping genes. The table on the right side of the figure lists the corresponding genes, species and primers to the numbers shown in the gel. The expected length of the amplified sequence was compared to the position of the band on the gel, to make sure that the primers were specific and only the desired sequence was amplified (specific bands are indicated by an arrow). The names of the primers were composed of the abbreviations for the amplified gene, a number and an f for forward primers or an r for reverse primers.

Most primers for housekeeping genes were specific for one product and thereby only showed one band on the gel (figure 38). Although a few primer pairs generated several bands, the band with the correct electrophoretic mobility is always the most prominent one (in figure 38, indicated by an arrow). Best working primer pairs were specific for the genes fusA, pyrG, glpK and gyrB and did only show a single band per lane. Therefore these genes with corresponding primers were considered best suited for quantification

experiments. Not all primer and probes were specific for all bloodstream relevant pathogens. The specificity is indicated in table 13 in Appendix 1.

To amplify housekeeping genes of a wide variety of pathogens, a multiplex PCR with more than one primer pair would be necessary. In order to amplify *gyrase B* genes from *E. coli*, *S. aureus* and *P. aeruginosa* in one sample, three primer pairs were added. Multiplex amplification worked almost as well as singleplex PCR. Furthermore, only one specific band was observed on the gel (figure 39).

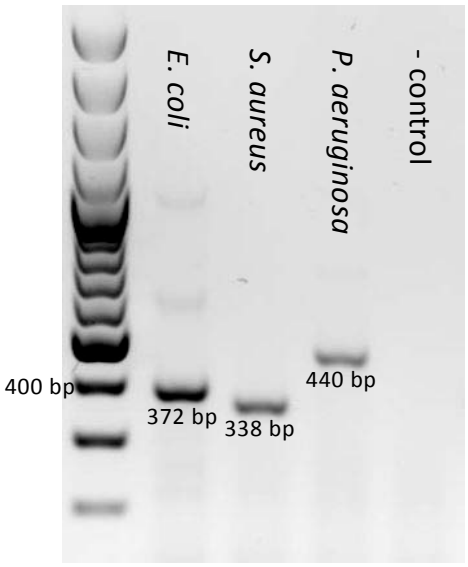


Figure 39: Multiplex PCR amplification of gyrase B genes from *E. coli*, *S. aureus* and *P. aeruginosa* applying primers gyrB1f and gyrB1r, gyrB3f and gyrB3r, gyrB4f and gyrB4r. The lanes in this gel correspond to lanes 8, 11 and 10 in figure 38.

To test for the specificity of the designed probes, hybridization with the amplified **gyrase G** gene was performed. With a setup like that the parallel detection of housekeeping RNA for possible viability determination combined with species identification would be possible. The results are summarized in table 9.

Species specificity	Name of probe	Specific	Unspecific	No signal
E. coli	gB1 eco			❖
	gB2 eco		✓	
	gB3 eco	**+**		
S. aureus	gb1 sau	++		
	gb2 sau	**++**		
	gb3 sau	**+++**		
P. aeruginosa	gB1 pae	++		
	gB2 pae	**+**		
	gB3 pae			❖

Table 9: Results of the hybridization of amplified gyrase G gene. Probes specific for the gyr G gens of *E. coli*, *S. aureus* and *P. aeruginosa* were tested. Signal intensities of specific probes were indicated with "+" (specific signal), "++" (good signal intensity) and "+++" (high specific signals). Probes generating unspecific signals or no signals at all were indicated in corresponding columns.

Most probes for the gyrase G gene were specific. However, they did not deliver very high fluorescence signals on the microarray at the adjusted pmt level of 500.

A real-time PCR was performed amplifying housekeeping genes. In a first approach only the DNA was amplified. This experiment only evaluated the quality of the primers but had no significance for quantification approaches and determination of viability status, as this would require the amplification of RNA. Figure 40 shows that the amplification of fusA, pyrG and leuS genes worked fine as the threshold fluorescence was reached at cycles 20.0, 20.7 and 26.7, respectively. The primers for glpK did not work, however. A threshold cycle of 40 means that the threshold fluorescence was not reached.

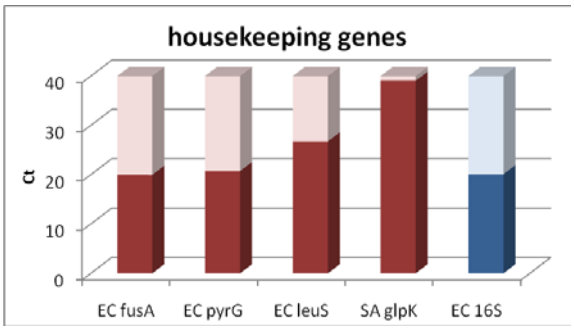


Figure 40: Results of real time PCR amplification of housekeeping genes. Threshold cycles are plotted on the y-axis. The lower the threshold cycle, the better did the amplification work. To highlight this, cycles from the threshold cycle to the maximal cycle number of 40, are also drawn but in a lighter color. 16S rDNA of *E. coli* (blue) was plotted as a control, while the housekeeping genes fusA, pyrG, leuS and glpK are of red color. **EC**: *E. coli*, **SA**: *S. aureus*.

For the use of housekeeping genes in quantification and viability determination, protocols need to be established for microarray analysis of RNA and for real-time PCR amplification of RNA.

5. Conclusion

5.1 Establishment of internal control

Internal control sequences were implemented in the sepsis chip protocol. Some difficulties had to be overcome during the process. Problems with sequencing and amplification of the plant DNA could be solved. Another challenge was caused by the methanotrophic 16S rDNA sequences, because of poor specificity of the probes, which prevented most of them from being used as internal control. In order to use these sequences as internal control new probes should be designed with sufficient differences in sequences to be specific. It might be more promising, however, to focus on the further improvement of the plant internal control.

After establishment of the internal control, the spike-in of the control sequences just before pathogen lysis led to satisfying results after microarray hybridization. Internal control sequences are needed for normalization and to control obtained pathogen specific data, which is the base for semiquantitative analysis of initial DNA concentrations. Therefore, signal fluctuations of internal control probe signals are acceptable or even desired, if they correspond to fluctuations of pathogen specific probes.

It proved difficult to normalize the pathogen specific probe signals using the internal control. High fluctuations of signals between different experiments were observed for both pathogen specific and internal control probes. However, fluctuations in internal control signals were not correlated with fluctuations in pathogen specific signals.

To improve the correlation of different probe signals of experiments, the protocol (especially DNA isolation, PCR and hybridization reactions) needs to be optimized. Also a normalization method should be established, as comparing of probe signals was previously slow and inefficient. A good normalization method would allow the analysis of more data and would therefore produce better results.

5.2 Quantification via Real-time PCR

A real-time PCR protocol was established to confirm the semiquantitative results we were planning to obtain by microarray analysis. Initially, some problems were encountered concerning the reliability and accuracy of the results.

Some real-time PCR results suggested that with water diluted samples would contain more DNA than nondiluted samples, which cannot be correct. A possible explanation would be a contamination of the water with bacteria or their DNA, which is unlikely. Further testing strengthened the assumption that PCR inhibitory substances in the sample DNA were the cause of these discrepancies. In diluted samples less inhibitors were present, leading to a better PCR amplification.

Real-time PCR experiments were repeated with highly diluted samples generated from PCR products. Due to the high dilution, in these samples very little or even no inhibitory effect was observed. Real-time PCR amplification worked more accurate with these samples. In order to establish a standard curve, which is necessary for quantification, NanoDrop® measurements were used to quantify PCR products. They were subsequently diluted to 10^5 copies per µl. Dilution series of these samples were then amplified by real-time PCR, giving a reliable standard curve.

However, quantification of DNA samples isolated from bacterial culture or even from whole blood proved to be difficult, due to PCR inhibitory substances. Real-time PCR related problems were excluded as amplification of highly diluted samples worked well. Further DNA isolation and purification protocols need to be tested, in order to eliminate PCR inhibitors from the sample DNA and to allow proper quantification.

5.3 Viability status of bacteria

Due to antimicrobial treatment the viability status of disease causing pathogens may vary, especially in patients suffering from multiple infections. In order to determine the viability status the focus was concentrated on RNA instead of DNA. RNA is a short-lived molecule and is mostly degraded in dead bacteria, while DNA can be detected even after long periods of time in actively killed cells [Masters *et al.,* 1994].

5.3.1 RNA amplification techniques

Several RNA amplification techniques were tested in order to find the most efficient one. Also a method called nucleic acid sequence-based amplification (NASBA) was introduced, which allows for the isothermal amplification of RNA via the combined actions of three enzymes.

Some problems were solved before establishment of a working NASBA protocol. First, all the reagents and enzymes were purchased individually and buffers were self prepared. RNA amplification did not work properly with this setting. Therefore, a ready to use NASBA Kit from Biomérieux was tested. At the beginning amplification did not work due to wrong designed primers. After redesign of the T7 promotor carrying primers, first results were observed. However, amplification was still poor and did not reach the sensitivity of PCR. Results could not be improved by optimization during following experiments.

Other RNA amplification techniques worked better than NASBA. Especially Omniscript (Qiagen) reverse transcription followed by PCR and the OneStep RT-PCR Kit (Qiagen) led to a strong amplification of target RNA. The OneStep RT-PCR Kit from Qiagen was the method of choice for RNA amplification, because amplification worked best and reverse transcription and PCR reactions occur subsequently in one tube, which saves time and decreases the risk of contamination.

5.3.2 Housekeeping Genes

Several probes for housekeeping genes were added to the sepsis chip. The RNA of these genes is constantly expressed because their products are crucial for cell functioning. This makes the mRNA of housekeeping genes especially interesting for quantification and determination of the viability status of bacteria. Several primers specific for these genes were designed and tested. Also protocols for RNA amplification and multiplex PCR were established, making on-chip analysis of housekeeping genes possible.

However, for quantification RNA amplification in real-time PCR is necessary. Furthermore, products of reverse transcription could be used as template for real-time PCR. Also many commercial kits for reverse transcription real-time PCR are available, but were not tested in this work. Such a protocol needs to be established and problems with PCR inhibitory substances (see above) need to be overcome in order to allow reliable quantification of RNA.

6. Acknowledgements

Especially I want to thank my family for the great support and my supervisor Herbert Wiesinger-Mayr for motivating me with his drive and passion for the subject and for giving me great ideas.

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I have to thank Frederieke Trognitz for providing plant sequences and helping me with the cloning protocol and Levente Bodrossy for providing 16S rDNA sequences of methanotrophic bacteria.

Finally I thank Werner Lubitz for being my supervisor at the university.

7. References

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8. Appendix 1 – sequences of primers and probes

8.1 New probes designed for the "Sepsis Identification" Chip

Specific for methanotrophic bacteria		
Spezies / gene	**Name of probe**	**Sequence [5'-3']**
Methylocaldum szegediense / 16S rDNA	msz1	TTTTTACTCGCCACCCACCGGTTA
	msz2	TTTTTCCGCCTTTCTTCCCCGCTT
	msz3	TTTTTGGTTCCCCAGATGTCAAGACCAG
	msz1-L	TTTTTACACTCAAGCTCGCCAGTATCAACTGCCATTCCCAGGTT
	msz2-L	TTTTTATCTCTCAAGGGTTCCCCAGATGTCAAGACCAGGTAA
	msz3-L	TTTTTTAGAGTTCCCGTCTTTACGCTGGCAACTAGGGACAA
Methylococcus capsulatus / 16S rDNA	mca1	TTTTTCACAACAAGGCAGATTCCTACGCATTAC
	mca2	TTTTTCACACTCGAGCCTGACAGTATCCAAT
	mca3	TTTTTCCATGCAGCACCTGTGTCTTGG
	mca1-L	TTTTTAGAGTTATCCCCCACAACAAGGCAGATTCCTACGCATTA
	mca2-L	TTTTTACCAACTAGCTAATCCGACGTAGGCTCATCTTATTGCGCGA
Methylosinus trichosporium / 16S rDNA	mtr1	TTTTTACTCACCCGTCTGCCACTGAC
	mtr2	TTTTTCCTCTCCCGGACTCTAGATCGCC
	mtr3	TTTTTGAGATTTGCTCCGGGTCACCC
	mtr1-L	TTTTTTCAAGTTTCCCTGAGTTATTCCGAACCGAAAGGTACGTT
	mtr2-L	TTTTTAGGTTGAGCCTCGGGATTTCACCCCTGACTTAACAAT
	mtr3-L	TTTTTATCTCTGACGACCATACCGGACATGTCAAAAGCTGGTAA
Methylomicrobium agile / 16S rDNA	mag1	TTTTTATCCCACGCAGGCTCATCTCATA
	mag2	TTTTTTCTATAGGTAATGTCAAGTCTGCCGGGT
	mag3	TTTTTAGAGTTCCCAGCATCACGTGTTGG
	mag1-L	TTTTTTATTAGCTTGAGTTTCCCCAAGTTATCCCCCACTACTA
	mag2-L	TTTTTTGCTTCTTCTATAGGTAATGTCAAGTCTGCCGGGTATTA
	mag3-L	TTTTTGAAAACCTTTATAAAGACCAACGGCTAGTTGACATCGT
Specific for potato DNA		
15010	iKc1-k	TTTTTTTCTCAGAGTTCCCTCGTGATTGATGG
	iKc2-k	TTTTTATGAACGCGGATGACATACTGTACCA
	iKc3-k	TTTTTATGGTCCTTTAAGGTGTGGCCACA
	iKc1-l	TTTTTAAGAATTTAGAGCACGTTCCATAATCTGTTGATAAAGAGATAAATA-GATT
	iKc2-l	TTTTTTCTTGACTACTACAAAGTAAATAGCGAGAACCATTAATATATGTAA-CATAT
	iKc3-l	TTTTTTTAGTTTTA-TATTCTTACTCTTGTTAGTTTCATCTTTCCTCGAATTTGCAT
25760	iKa1-k	TTTTTTTCCACTAGTGTCTTGCGGCACT

	iKa2-k	TTTTTTGATGAATCCACTGAGTGCTGACCTAG
	iKa3-k	TTTTTAGAGCAAAGACACAGATTTGGCGTGT
	iKa1-l	TTTTTAACAGTGTTTCGTTGTAATTAACTTTGCTCAATAAGTTTATATAAA-GAGA
	iKa2-l	TTTTTAAACAACTAATCTCAAACTTAATCAGTTATAA-CAAAAAAAGCTCATCTCA
	iKa3-l	TTTTTAGAGAGATTAGTAAGTAACAACTCATTTTTTGATTAGTGAGTAA-CAATTA
38025	iKb1-k	TTTTTGGAGAACCCATTTGTCAAGCATAACTCAAGT
	iKb2-k	TTTTTTGAGGTACACACAAGCTGACCCAGA
	iKb3-k	TTTTTGGATTCATCCCAACTGCTTTCCATTCA
	iKb1-l	TTTTTACAAAATACACTTGGTATATAGAAATTTTATTCTAGGGGAGAACC-CATTT
	iKb2-l	TTTTTTGTTAGTCTAATGCACGTTGATTTAAGGATATTCTCCAAAAGTTA-TAATA
	iKb3-l	TTTTTAATATAGTCATCCAAACATGAACTCTTTTCCAAATAACTTATTTTTA-GAA
Specific for housekeeping genes		
Enterobacteriaceae/ fusA	fA1 Gen	TTTTTCATGGTTGCAGCGCCGTCATG
Enterobacteriaceae/ fusA	fA2 Gen	TTTTTGAACACCACCAACTGCGCAGT
Enterobacteriaceae/ pyrG	pG1 Gen	TTTTTGCAGGGATTCGATATCACCTACAGTACCG
Enterobacteriaceae/ pyrG	pG2 Gen	TTTTTATTGAGGCCACGGGCTTCAAGA
S. aureus/glpK	gK1 sau	TTTTTTGCATCATGTTCAACCCAACCTGA
Enterobacteriaceae/ gyrB	gB1 Gen	TTTTTTTCACCTCRGARGARACCAGYTTGT
E. coli/gyrB	gB1 eco	TTTTTGCAGACGAATGGAAACGCCGG
E. coli/gyrB	gB2 eco	TTTTTGGAAACGACCGCAATCAGGCCT
E. coli/gyrB	gB3 eco	TTTTTCTGATTTCACCTCAGAAGAAACCAGTTTGTT
K. pneumonia/ gyrB	gB1 kpn	TTTTTATTTCGCTTTTTTGCTATAGCCCTCTTTGTCC
K. pneumonia/ gyrB	gB2 kpn	TTTTTAGTTCGTTCATCTGCTGCTCCACC
K. pneumonia/ gyrB	gB3 kpn	TTTTTCGAGTCATCTCACGAGCGCGAC
K. oxytoca/ gyrB	gB1 kox	TTTTTACTTCGCTTTTTTGCTGTAACCCTCTTTATCC
K. oxytoca/ gyrB	gB2 kox	TTTTTAGTTCGTTCATCTGCTGCTCTACCG
K. oxytoca/ gyrB	gB2 kox	TTTTTGGGTCATTTCACGCGCACGAC
E. cloacae/ gyrB	gB1 ecl	TTTTTTTGGTCTGTGAGGAGAACTTCGGATCC
E. cloacae/ gyrB	gB2 ecl	TTTTTGCCCTGCTTCGCAGAACCG
E. cloacae/ gyrB	gB3 ecl	TTTTTTTTTACCCACCACGATTTTCGCGTC
E. aerogenes/ gyrB	gB1 eae	TTTTTTTAGTCTGAGAGGAGAACTTCGGATCCG
E. aerogenes/ gyrB	gB2 eae	TTTTTGCCCTGTTTCGCAGAGCCG
E. aerogenes/ gyrB	gB3 eae	TTTTTTCGATAATTTTGCCGACCACGATTTTCGCAT
C. freundii/ gyrB	gB1 cfr	TTTTTAAACCGCCCGCTGAGTCC

C. freundii/ gyrB	gB2 cfr	TTTTTCATCTTGTCGAAACGCGCCTTCTC
C. freundii/ gyrB	gB3 cfr	TTTTTTTCGCAAAACCGCCCGCTGA
P. aeruginosa/ gyrB	gB1 pae	TTTTTGCGAAGTACTTGCCCATCTCCTGT
P. aeruginosa/ gyrB	gB2 pae	TTTTTAGTCACCCTCCACGATGTACAGTTC
P. aeruginosa/ gyrB	gB3 pae	TTTTTGCGATTGCGGCCCTGCTT
S. pneumoniae/ gyrB	gB1 spn	TTTTTGACCACTTCGCTATTTCCCAATTTGGTCTT
S. pneumoniae/ gyrB	gB2 spn	TTTTTTTCACTGAAGAGGCGATTGGTAATCTTGAC
S. aureus/ gyrB	gb1 sau	TTTTTATCTACAACTTGACGCACTTCAGAATTACCT
S. aureus/ gyrB	gb2 sau	TTTTTTCAACCACTGTACGTGCGACTTGTG
S. aureus/ gyrB	gb3 sau	TTTTTTCTTCAGGACTTTKACTAGAGCAATCGGCT
Bacteria/ 16S rDNA	16S uni	TTTTTAACGCTTGCACCCTCCGTATT

Table 10 lists all probe sequences designed during this work and added to the previously published "Sepsis ID" chip. Probes already in use with the preceding chip are listed in Wiesinger *et al.*, 2007.

8.2 Bacterial 16S rDNA specific primers

Name	Sequence [5'-3']	length
8F	GAGTTTGATCCTGGCTCAG	19
45F	GCCTAWCACATGCAAGTCGA	20
321F	TCCTACGGGAGGCAGCAGT	19
347F	GAGGCAGCAGTRGGGAA	17
521F	GCAGCCGCGGTAATACG	17
783F	AGGATTAGATACCCTGGTAGTCCA	24
916F	GAATTGACGGGGGCCC	16
154R	GTATTAGCTACCGTTTCCAGT	21
316R	GCCGTGTCTCAGTYCCA	17
514R	GCGGCTGCTGGCAC	14
673R	CACCGCTACACVTGGAATTC	20
764R	TGTTTGCTCCCCAYGCTTT	19
797R	GGACTACCAGGGTATCTAATCCTGTT	26
926R	GCTTGTGCGGGCCC	14
1391R	ACGGGCGGTGTGTAC	15
1491R	TACGGYTACCTTGTTACGACTT	22
T7_8F	TAATACGACTCACTATAGAGAGTTTGATCCTGGCTCAG	39
317R_T7	GCCGTGTCTCAGTYCCATAATACGACTCACTATAG	35
673R_T7	CACCGCTACACVTGGAATTCTAATACGACTCACTATAG	38
1391R_T7	ACGGGCGGTGTGTACTAATACGACTCACTATAG	33

Table 11: List of 16S rDNA specific primers used in this work. Primers ending with an F are forward primers, while primers ending with an R are reverse primers. Primer names including T7 do have the T7 promoter sequence aligned to the 16S rDNA sequence.

8.3 Adapter primers

Plant sequence	Adapter primer	Sequence [5'-3']	Length
15010	P45F-15010F-iKb	GCCTAWCACATGCAAGTCGAA-CATCCCAATTTGGTTACTGCCCTG	45
	P15010R-1391R-iKb	AGGGGACAATGGACCAACTTCTTCATCACGGGCGGTGTGTAC	44
25760	P45F-25760F-iKa	GCCTAWCACATGCAAGTCGATCCTTAT-GATGGTGGAGTTTTCCAG	45
	P25760R-1391R-iKa	AAAGCAATTATAGCTCGACAAACAGACGGGCGGTGTGTAC	41
38025	P45F-38025F-iKc	GCCTAWCACATGCAAGTCGAAtGGGCGCTGCATGTTTCGTG	41
	P38025R-1391R-iKc	ACACCTTTGTTGAAaGCCATCCCACGGGCGGTGTGTAC	40

Table 12: List of adapter primers which were used for the ligation of bacterial primers to plant DNA in order to amplify pathogenic and plant DNA together in one assay with only one primer pair.

8.4 Primers specific for housekeeping genes

Target	Specificity	Name	Sequence [5' to 3']	Length
fusA	*Enterobacteriaceae; Plesiomonas shigelloides*	fusA3f	CATCGGTATCAGTGCKCACATCGA	24
		fusA4r	CAGCATCGCCTGAACRCCTTTGTT	24
	Enterobacteriaceae	fusA5r	TAGCCATACCAGACCAGAATGC	22
leuS	*Enterobacteriaceae; Plesiomonas shigelloides*	leuS3f	CAGACCGTGCTGGCCAACGARCARGT	26
		leuS4r	CGGCGCGCCCCARTARCGCT	20
pyrG	*Enterobacteriaceae; Plesiomonas shigelloides*	pyrG3f	GGGGTCGTATCCTCTCTGGGTAAAGG	26
		pyrG4r	GGAACGGCAGGGATTCGATATCNCCKA	27
	Enterobacteriaceae;	pyrG5r	TGCCCCAGGTCCAGGTCGGTTT	22
recG	*Enterobacteriaceae; Plesiomonas shigelloides*	recG3f	GGCGACGTTGGCTCMGGKAAAAC	23
		recG4r	GGGTGCGGGGGATSGGSGTKGC	17
glpK	*S. aureus*	glpK1f	AATTGCAGGGGTAGCACAACGTG	23
		glpK2r	TGACGTGATTGCCAAACAATTGCGTGA	27
gyrB	*Enterobacteriaceae;*	gyrB1f	GCCTGATTGCBGTBGTHTCC	20
		gyrB1r	TACCCTTCAGCGGCAGRATCG	21
		gyrB2f	TTYGARTAYGAMATYTGGCGAAACG	25
	P. aeruginosa	gyrB3r	GATYTTRCCCTTSAGCGGCAG	21
		gyrB3f	ACCTGAACAACTACATCGARGMCGAA	26
	S. aureus	gyrB4r	TGCGTTCTAGAGTCACGACCAGATTTT	27
		gyrB4f	ACAGCAATTATATCTATCAAA-CATGGTGACCTCA	34

Table13: List of primers specific for different housekeeping genes. Pathogen specificity is also indicated in this table. Most housekeeping genes are not as highly conserved as some regions of the 16S rRNA genes, therefore the design of primers specific for a wide variety of pathogens was difficult and not always successful. Primers fusA3f, fusA4r, leuS3f, leuS4r, pyrG3f, pyrG3r, recG3f and recG4r were designed by Salerno *et al.,* 2007.

Curriculum vitae

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Work experience:

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Aug. 2004:	arsenal research office occupation
Aug. 2001:	Kromberg & Schubert GmbH Co. KG office occupation

Additional skills:

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- Programming skills in java and php
- Microsoft office skills
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