DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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**OTT SCHELER**

# The application of tmRNA as a marker molecule in bacterial diagnostics using microarray and biosensor technology

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Ref.II Scheler O, Kaplinski L, Glynn B, Palta P, Parkel S, Toome K, Maher M, Barry T, Remm M, Kurg A (2011). Detection of NASBA amplified bacterial tmRNA molecules on SLICSel designed microarray probes. BMC Biotechnology 11:17

Ref. III Scheler O, Kindt JT, Qavi AJ, Kaplinski L, Glynn B, Barry T, Kurg A, Bailey RC (2012). Label-free, multiplexed detection of bacterial tmRNA using silicon photonic microring resonators. Biosensors and Bioelectronics, *in press*

**Author's contributions:**

Ref. I The author conducted and designed the study, performed most of the experiments (NASBA amplification and microarray hybridization), analyzed the data, was responsible for drafting the paper.

Ref. II The author participated in experiment design, performed most of the experiments (*in vitro* transcription, NASBA amplification and microarray hybridization), participated in data analysis, was responsible for drafting the paper.

Ref. III The author conducted and designed the study, performed most of the experiments (*in vitro* transcription, biosensor platform set-up, hybridization experiments), participated in data analysis, was responsible for drafting the paper.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| CFU | colony forming unit |
| FISH | fluorescence in situ hybridization |
| ISR | intergenic spacer region |
| ITS | internal transcribed spacer |
| NASBA | nucleic acid sequence based amplification |
| RT | reverse transcriptase |
| SPR | surface plasmon resonance |
| T7 | bacteriophage T7 |
| tmRNA | transfer-messenger RNA |

# INTRODUCTION

There is a growing need for quick and reliable approaches for microorganism detection and identification worldwide. Although traditional culture-based technologies provide very trustworthy and accurate approach for bacterial detection and identification, they are also time- and labor-consuming and can be applied only to analyze a small fraction of bacteria that can be cultured. Those weaknesses have caused a necessity for alternative technologies that are capable for faster and more precise analysis of bacterial composition in medical, food or environmental samples. Most common tactics nowadays is to analyze the nucleic acid component of analyte solution and determine the bacterial composition according to specific nucleic acid profiles that are detected and identified. Theoretically every bacterial species and strain contain unique characteristic target regions that can be used for their specific identification.

In the first part of current thesis a literature overview is given about the different technologies that are used for nucleic acid-based bacterial detection. Main focus is on nucleic acid amplification and hybridization-based detection methods with emphasis on microarray and biosensor technologies, and their practical application in bacterial diagnostics. In second part of the literature overview, a description of different DNA and RNA molecules that have been targeted for bacterial detection and identification is reviewed. Longer explanation is given about the trans-translation mediating RNA molecule called tmRNA that is used as a target marker molecule in the current thesis.

The research section describes two different methods that apply tmRNA for bacterial detection and identification. Firstly, a microarray-based technology is described where target tmRNA molecules are amplified using Nucleic Acid Sequence Based Amplification (NASBA) and labeled fluorescently prior the hybridization experiment. The developed method was applied for tmRNA detection from bacterial total RNA samples. In second part of the research tmRNA molecules are specifically targeted using real-time label-free biosensing platform that is based on the optical microring resonator technology. Potential quantitative nature and sensitivity of the biosensor is demonstrated using *in vitro* synthesized tmRNA molecules.

# 1. REVIEW OF THE LITERATURE

## 1.1. Bacterial diagnostics

There are many bacteria causing poisoning or severe infections on human beings that can lead to serious health issues and even death. Precise understanding of processes in microflora and identification of pathogenic agent is needed both in clinical diagnostics and in food safety monitoring in order to treat and/or minimize the effect caused by potentially harmful bacteria. The current "gold standard" approach for detection and identification of such pathogenic bacteria is usually based on traditional culture-based methods. The roots of those methods can be traced back to the early works of the founders of modern clinical microbiology Pasteur and Koch more than a century ago. In brief, these methods typically involve isolation of bacteria by their cultivation on specialized microbiological media, followed by morphological or biochemical analysis. While being very reliable and accurate, the traditional methods may require several days and even weeks to get final results. In addition there are also several important pathogens that are difficult or even impossible to cultivate and can therefore remain undetected by conventional culture-based methods. Such time- and labor-consuming nature combined with lack of cultivation methods for certain bacterial groups are serious weaknesses that set a limit to traditional methods in microbial diagnostics, where fast and precise analysis of potentially dangerous situations is often desirable. The development of technologies in molecular biology over the last couple of decades enables direct and specific molecular analysis of different bacterial components, offering potentially faster and more conclusive identification of bacterial species, while addressing the shortfalls of traditional techniques (Amann et al., 1995; Barken et al., 2007; Lazcka et al., 2007; Kostic et al., 2008; Jasson et al., 2010; Velusamy et al., 2010).

One option is to use any of several immunoassay formats that have been developed to investigate unique antigens or antigen-related antibody component of samples in order to look for the possible pathogenic bacteria (Andreotti et al., 2003; Banada and Bhunia, 2008). Another common possibility is to examine the nucleic acid sequences of different bacteria and look for the characteristic target regions of each species that can be exploited for their detection and identification in clinical, environmental or food samples. The advantages of nucleic acid-based detection over culture-based and immunological methods include: rapidness, less demanding handling procedures, and often also higher specificity and sensitivity. Both DNA and RNA can be used as a target molecule for bacterial diagnostics, depending on the experiment setup and technological requirements of used approach (Barken et al., 2007; Ludwig, 2007; O'Connor and Glynn, 2010). While DNA is very stable molecule that can be easily isolated from different biological samples, RNA on the other hand is more labile and is easily degraded, especially when the microorganism is killed.

Although more demanding from handling perspective, the presence of RNA may sometimes give better insight then DNA into viability of the bacteria under investigation (Birch et al., 2001; Keer and Birch, 2003; O'Connor and Glynn, 2010).

### 1.1.1. Nucleic acid-based bacterial diagnostics

The use of nucleic acid sequences for diagnostic purposes has followed closely the key technological advances in molecular biology over the last three-four decades. The detection and characterization methods of DNA and RNA molecules were pushed forward by several major inventions that included isolation of nucleic acid restriction and amplification enzymes and development of different hybridization techniques. Each improvement and their combinations were soon applied correspondingly to improve bacterial diagnostics.

A common principle for direct detection of nucleic acid sequences from environmental or clinical samples is use of short specific oligonucleotide probes that hybridize to complementary target sequences. One widely used technology for such direct analysis is Fluorescence In Situ Hybridization (FISH) that was developed in the end of 1980s. In FISH, fixed and intact bacteria are permeable for short fluorescently labeled oligonucleotides that enter the cell and hybridize to complementary target rRNA regions. Specific identification and quantification of single microbial cells is then achieved by visualizing labeled duplex regions using either fluorescence microscopy (DeLong et al., 1989; Amann et al., 1990) or flow cytometry (Wallner et al., 1997). Over time, FISH has been applied for identification, visualization and localization of various bacteria in many fields of analytical microbiology; most commonly in environmental research (Amann et al., 2001; Daims et al., 2001; Pernthaler and Amann, 2004), but also in diagnostics in clinical microbiology (Kempf et al., 2000; Peters et al., 2006) and food safety analysis (Schmid et al., 2005). FISH and other FISH-based technologies that have been established can in addition to rRNA recognition be similarly used to detect and identify other nucleic acid target molecules like mRNA (Pernthaler and Amann, 2004), tmRNA (see section 1.2.5.) (Schönhuber et al., 2001) and DNA sequences (Pratscher et al., 2009).

Another important field where rapid progress has occurred over the past few decades is nucleic acid amplification technologies. Most recognized and used of those technologies is "polymerase chain reaction" (PCR) that was developed in the middle of 1980s. In PCR, DNA region of interest is enzymatically amplified by DNA polymerase in exponential manner using a pair of specific DNA primers and controlled thermal cycling (Saiki et al., 1985). PCR was soon implemented in microbial diagnostics in combination with already existing nucleic acid analysis mechanisms such as dot-blot ( Steffan and Atlas, 1988; Persing et al., 1990), Southern blot (Greisen et al., 1994; Wilson et al., 1990), restriction fragment length polymorphism typing (Deng et al., 1992; Persing et al., 1990) and sequencing (Yamamoto and Harayama, 1995). Many different

PCR-based methods have emerged since then and have found use in bacterial diagnostics. For example, multiplex-PCR is application in which many different targets are amplified in the same reaction and subsequently analyzed by gel electrophoresis (Chattopadhyay et al., 2004; Keto et al., 2001; Strålin et al., 2005). Another key development of traditional PCR was a technology that enabled not only the detection but also the quantification of the DNA product during the amplification reaction (Higuchi et al., 1993). Real-time PCR, as the method is called, has since become a widely used tool in microbial diagnostics (Espy et al., 2006; Postollec et al., 2011) with applications ranging from simple and quick detection of a single certain bacterium (Uhl et al., 2003) to more complex multiplex real-time PCR analysis targeted for several pathogens (Thurman et al., 2011). In order to implement PCR to amplify RNA target molecules, a reverse transcriptase-mediated synthesis of complementary DNA strand has to precede conventional polymerase chain reaction (Kawasaki et al., 1988). Such reverse-transcriptase PCR (RT-PCR) can be used in bacterial diagnostics combined with endpoint detection of amplified nucleic acid (Klein and Juneja, 1997), or in real-time format (Fey et al., 2004). Single-cell or one colony forming unit (CFU) detection sensitivity can be achieved using many different PCR-based methods: for example in combination with dot-blot (Steffan and Atlas, 1988), as well as in more modern real-time format (Lucas et al., 2008). While PCR has been by far the most common nucleic acid amplification technology, there are also many alternatives that have successfully been applied in microbial diagnostics. Most well-known of those alternative amplification technologies are isothermal enzymatic methods that do not require active thermo-cycling as PCR does. The isothermal nature of those methods offers distinct advantages over PCR method with regard to the cost and simplicity of instrumentation needed for the nucleic acid amplification process. Methods like “strand displacement amplification” (SDA) where 5’–3’ exonuclease-deficient DNA polymerase amplifies primer-bound DNA strands (Walker et al., 1992) and “nucleic acid sequence-based amplification” (NASBA) (Compton, 1991) have all been later applied in bacterial diagnostics (Ge et al., 2002).

### 1.1.2. NASBA

In NASBA (figure 1), target RNA molecules are amplified at constant temperature around 40 °C by using a specific set of oligonucleotide primers and sequential activity of three different enzymes: reverse transcriptase (RT), RNaseH and T7 RNA polymerase.

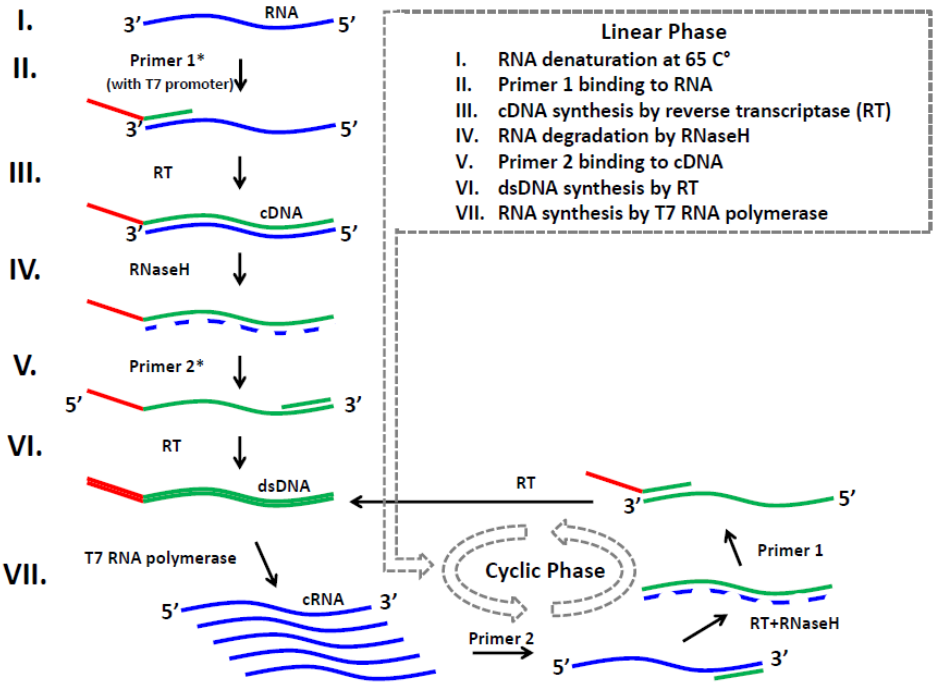


**Figure 1.** A NASBA amplification schematic. The linear phase of NASBA is described briefly in text box and thoroughly in text according to the amplification steps presented in roman numerals. The asterisk* denotes that cRNA amplification products will be synthesized using currently pictured primer setup with T7 promoter attached to the Primer 1. Alternatively, promoter sequence can be added to primer 2 that would yield in inverse-NASBA amplification and production of positive strands of initial target RNA.

First, a target RNA molecule is quickly denatured for 5 minutes at 65 °C (figure 1–I.), followed by forward primer binding (figure 1–II.) to the complementary region of RNA target molecule (primer also includes 5' sequence corresponding to T7 RNA polymerase promoter). Complementary DNA strand is then synthesized by RT (figure 1-III.), making the initial RNA substrate instantly available for degradation by DNA/RNA duplex specific RNaseH (figure 1-IV.). This enables binding of the second primer oligonucleotide to new cDNA strand (figure 1-V.) trailed by second strand DNA synthesis by RT that also possesses DNA-directed DNA polymerase activity (VI.). New dsDNA with its T7 promoter sequence is hence ready for T7 RNA polymerase to start producing new complementary RNA (cRNA) molecules (figure 1-VII.). After these initial linear steps, a cyclic phase of NASBA proceeds where all newly synthesized RNA molecules act as substrates for primer binding, DNA synthesis, DNA/RNA duplex-specific RNA degradation, and again RNA synthesis by T7 RNA polymerase (Compton, 1991). The T7 promoter sequence can alternatively

be included in the 5' end of the second primer to achieve production of positive strand of initial target RNA in a process called inverse-NASBA (Tauriainen et al., 2006). NASBA is highly specific for RNA amplification and unintentional DNA amplification can only occasionally be triggered by the absence of target RNA or at extreme excess of proper target DNA (Deiman et al., 2002). High RNA specificity makes NASBA also less sensitive to genomic DNA contamination and therefore more suitable for applications where microbial viability can be assessed by target RNA detection, giving it another important advantage over PCR-based methods (Keer and Birch, 2003; Mader et al., 2010). For diagnostic purposes, NASBA has been successfully combined with different detection platforms like Northern blot, enzyme-linked gel assay (ELGA) (van der Vliet et al., 1993), enzyme-linked immunosorbent assay (ELISA) (Gill et al., 2006) and also in real-time detection format with molecular beacons (van Beckhoven et al., 2002; O'Grady et al., 2009; Rodríguez-Lázaro et al., 2004). Detection sensitivity of 1 CFU by using NASBA amplification has been described (Loens et al., 2006; O'Grady et al., 2009) previously. In principle, NASBA is similar to two other closely related RNA amplification methods called "transcription-based amplification system" (TAS) where only two enzymes (RT and T7 polymerase) are used (Kwoh et al., 1989) and "self-sustained sequence replication" (3SR) where T7 promoter sequence is present in both primers resulting in production of both RNA strands (Guatelli, 1990). TAS is actively being used in microbial diagnostics by licensed technology holder Gen-Probe Inc. under the name Transcription Mediated Amplification (TMA) (http://www.gen-probe.com/science/).

### 1.1.3. Nucleic Acid Microarrays

Microarray technology offers the capability to carry out quick and highly parallel hybridization analysis of complex nucleic acid mixtures in a single assay. A typical microarray consists of a high number of different capture probes attached to the solid surface in precisely ordered arrangement, where each position corresponds to specific target nucleic acid molecule. DNA microarray probes (either oligonucleotides or longer DNA fragments) capture and identify the presence of labeled complementary target molecules from analyzed solution. The event of probe-target hybridization is usually detected and quantified by fluorescence-based methods. The number of different targets that can simultaneously be analyzed have steadily grown from 48 in the first published microarray paper (Schena et al., 1995) up to several million on a single microarray chip with current state-of-the-art platforms developed by Illumina (www.illumina.com) or Affymetrix (www.affymetrix.com). Such high capability for miniaturized multiplexing, the ability to detect and identify more than one target molecule simultaneously from the same small specimen in molecular diagnostics, is the key advantage that microarrays have over otherwise highly specific and sensitive technologies like culture- and PCR-based

technologies (Kostic et al., 2008; Severgnini et al., 2010). While microarray technology was originally developed for gene expression analysis (Schena et al., 1995) and has since maintained its importance in genetics- and genomics-related research, it has also found many applications in microbiology and infectious disease diagnostics that contribute steadily around 8–9% of all microarray-related publications (Miller and Tang, 2009). In first publication that described potential use of microarrays for determinative and environmental studies in microbiology, Mirzabekov and colleagues used an array of probes complementary to 16s rRNA of different bacteria. Five different preparation types of target nucleic acid were used in experiments: total RNA, enriched ribosome solution, *in vitro* transcribed rRNA and PCR synthesized double-stranded and single-stranded ribosomal DNA (rDNA). All of them provided detectable hybridization signals that enabled discrimination between compared microorganisms (Guschin et al., 1997).

Nucleic acids can be analyzed in microarray experiments without any prior amplification by simple, direct hybridization approach by targeting marker molecules with high concentration in order to get sensitive signal. Many different methods have been developed for direct detection of 16S rRNA that already is naturally amplified at the average level of about 10000–20000 copies per cell. Such direct detection method is useful in circumstances where possible amplification-based bias during the detection experiments has to be avoided (Leski et al., 2010). Chandler and colleagues applied microarrays for direct detection of 16S rRNA from soil bacteria using helper DNA oligonucleotides (chaperones) to increase target binding efficiency and obtained the detection sensitivity equivalent to $7.5x10^6$ cells (Chandler et al., 2003; Small et al., 2001). Another possible option for direct nucleic acid analysis is to use samples that already contain high titers of examined bacteria and have therefore plenty of target material for hybridization experiments. A microarray containing 120 probes for different protein encoding genes in three different bacteria was used to identify and characterize bacterial pathogens that cause bloodstream infections (Cleven et al., 2006).

Although direct detection methods are easy to implement and do not require additional time-consuming steps that add complexity, target nucleic acids are generally amplified prior the hybridization step in microarray technology in order to gain more sensitivity and sometimes also specificity (Leski et al., 2010). PCR-based methods that are massively used in many fields of molecular biology (also in bacterial diagnostics as described previously), have also been favorite choices for target nucleic acid amplification related to microarray hybridization experiments. Universal PCR primers were used to amplify a variable region of bacterial 23S rDNA from range of different bacterial cultures. Amplification products were subsequently identified on custom-made nylon chip microarray consisting of 30 probes corresponding to 24 different bacterial species. Described system was capable of specific detection and identification of both pure and mixed cultures of different bacteremia causing pathogens

(Anthony et al., 2000). In another work, six bacterial virulence factor genes were amplified in one-tube multiplex PCR, followed by linear amplification step with the presence of only one primer in order to produce single-stranded labeled DNA product. Microarray-based hybridization detection of those gene markers was used to identify and distinguish six different foodborne illness causing bacteria from the *Listeria* genus (Volokhov et al., 2002). From food safety monitoring perspective, combining multiplex PCR and culture pre-enrichment with following microarray-based detection, Bej and colleagues could detect as low as 1 CFU of pathogenic bacteria from shellfish (Panicker et al., 2004). RT-PCR with universal primers was used to amplify intergenic spacer regions (ISR) in precursor-rRNA in the work published by Antranikian and colleagues. Using RT-PCR in combination with microarray-based detection enabled them to detect and identify seven different bacterial taxa associated with beer spoiling. More information about the growth status of these species was also obtained by investigating ISR regions that usually degraded quickly in growing cells (Weber et al., 2008).

All previously described methods in this section are based on low- to medium-density microarrays. Such arrays are usually custom-made and have at maximum few hundred or thousand pre-synthesized probes spotted and immobilized onto the chip surface. Low- to medium-density microarrays are suitable for routine clinical diagnostics of infectious diseases because the slides are easily prepared, inexpensive and require only the most basic equipment for printing, hybridization and scanning. Modifications like adding new probes or redesigning the whole chip layout can be introduced relatively easily and quickly (Mikhailovich et al., 2008).

In alternative approach, high-density microarrays can be manufactured by in-situ synthesizing oligonucleotide probes directly onto the surface of the microarray chip. Although being rather expensive platform that requires sophisticated data investigation software, the major advantages of these types of microarrays are the reproducibility of the manufacturing process, and standardization of reagents, instrumentation and data analysis (Dalma-Weiszhausz et al., 2006). A Multi-Pathogen Identification (MPID) high-density microarray with 53 660 probes was developed for identification of 18 pathogenic prokaryotes, eukaryotes and viruses. Multiplex-PCR was used to amplify specific DNA sequences of pathogenic bacteria that contained pathogenicity and virulence genes, and previously uncharacterized regions. Each organism was represented by three to ten different diagnostic regions on MPID with several overlapping probes. The simultaneous identification of multiple diagnostic regions allowed an accurate identification of each pathogen with a resolution limit at species level using spiked environmental samples (Wilson et al., 2002). In a more recent and ambitious attempt, a high-density microarray for pathogen detection was developed that contained target probes for all bacteria and viruses for which full genome sequences were available at that time. In that work, microarray probes were designed to cover both already known organisms and also be

suitable for not yet sequenced species with homology to sequenced organisms. While precise strain or subtype identification was not initial goal of probe design, the combined information of multiple probes during the data analysis made it possible nevertheless. Described array enabled detection and characterization of multiple viruses, phages, and bacteria in a sample up to the family and species level in clinical fecal, serum and respiratory samples (Gardner et al., 2010). Using high-density microarrays in combination with whole-genome amplification technology, Kennedy and colleagues could detect as little as one genome copy of pathogenic bacteria (Berthet et al., 2008).

### 1.1.4. Biosensors

According to the Biosensors World Congress (www.biosensors-congress.elsevier.com): ”Biosensors (figure2) are defined as analytical devices incorporating a biological material (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, natural products etc.), a biologically derived material (e.g. recombinant antibodies, engineered proteins, aptamers etc) or a biomimic (e.g. synthetic receptors, biomimetic catalysts, combinatorial ligands, imprinted polymers etc) (figure 2-II.) intimately associated with or integrated within a physicochemical transducer or transducing microsystem (figure 2-III.), which may be optical, electrochemical, thermometric, piezoelectric, magnetic or micromechanical. Biosensors usually yield a digital electronic signal (figure 2-IV.) which is proportional to the concentration of a specific analyte or group of analytes (figure 2-I.). While the signal may in principle be continuous, devices can be configured to yield single measurements to meet specific market requirements.”

The potential advantages of biosensors over other previously described analytical technologies are considered being shorter experiment time, lower cost and also easier handling. In microbial diagnostics, three main transduction principles that have been most used for nucleic acid-based biosensing technology are: mass-sensing, electrochemical sensing and optical sensing (Lazcka et al., 2007).

In mass-sensitive DNA/RNA biosensors, the event of probe-target hybridization is monitored and transduced into a readable signal by detecting small changes of mass on the sensor surface. One such type of mass-sensitive biosensors is quartz crystal microbalance (QCM) which allows monitoring of hybridization events, using an oscillating piezoelectric crystal with the specific nucleic acid probe molecules immobilized on its surface. The increased mass that is associated with the hybridization reaction, results in a proportional decrease of the oscillating frequency that can be monitored in real-time (O’Sullivan, 1999). Several methods have been published over time that describe QCM-based detection of PCR amplified gene targets from bacteria for diagnostic purposes (Mo et al., 2002; Wu et al., 2007). In another approach, small cantilever beams can be used for mass-sensitive detection of biological

binding events. Cantilevers can be used similarly to QCM as piezoelectric sensor where added mass causes detectable shift in resonance frequency (Hwang et al., 2007) or, alternatively, the mass-induced bending of the cantilever can be monitored by optical means (McKendry et al., 2002).

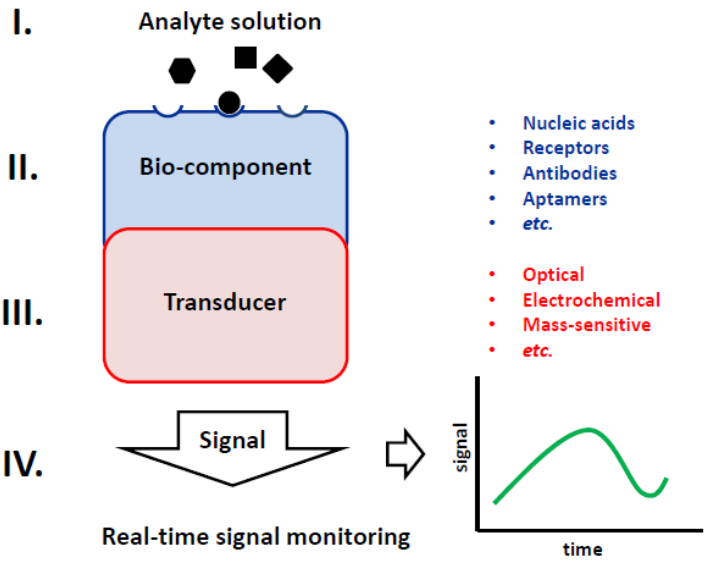


**Figure 2.** Working principle of a biosensor. A specific event of analyte binding (I.) to the bio-component (II.) of the biosensor is converted via transducer (III.) into a detectable signal that can be monitored in real time (IV.). A more thorough explanation is given in the main text according to roman numerals on the figure, respectively.

Electrochemical biosensors are usually based on the detection of changes in current or potential, caused by interactions occurring at the sensor-sample matrix interface. The techniques are generally classified according to the observed parameter: current (amperometric), potential (potentiometric) or impedance/conductance (impedimetric/conductometric) (Lazcka et al., 2007). In nucleic acids-based bacterial diagnostics: amperometric biosensors have been described for detection of PCR amplified bacterial toxin genes (Palchetti and Mascini, 2008), potentiometric sensors for direct detection of pathogenic bacteria by targeting the 16S rRNA molecules (Wu et al., 2009), and impedance-based biosensing has been applied for the antibiotic resistance detection by targeting short DNA oligonucleotides (Kaatz et al., 2012) and longer PCR-amplified DNA fragments (Corrigan et al., 2012).

Third widely used and described transducing principle for biosensors in microbial diagnostics is optics. There are many different sub-classes for optical biosensors that are based on different optical transducing technologies (Lazcka et al., 2007; Velusamy et al., 2010). Probably the most popular principle of optical biosensing that can be used for label-free detection and identification of

nucleic acid targets is surface plasmon resonance (SPR). It is a phenomenon that describes a condition when a certain amount of light energy is transferred to the groups of electrons on the metal surface, and as a result, the intensity of the reflected light from the surface is reduced. That, in turn, is dependent on the refractive indices of the media at both sides of the metal surface (sensor chip). Minor changes that occur in the refractive index of the thin metal film sensor chip when nucleic acid targets bind to the surface bound probe molecules can be measured by monitoring shifts in either the reflection angle or the wavelength of the light that is being beamed onto the metal senor chip (Cooper, 2003; Tudos and Schasfoort, 2008). In bacterial diagnostics the use of SPR for detecting nucleic acid targets has been described for example recently in the work by Liao and colleagues. They amplified the regions of 16S rDNA of four different pathogenic bacteria using linear- after-the-exponential (LATE)-PCR protocol that yielded in ssDNA target molecules. Four-channel SPR setup enabled real-time detection and quantification of amplified target DNA molecules at concentration levels down to 0.01 nM (Wang et al., 2011). Variation of the SPR technique called SPR imaging (SPRi) is a technology in which multiple adsorption interactions can under identical conditions be monitored in a single microarray format. Direct and specific detection of full-length 16S rRNA at 2nM concentration has been described by using SPRi (Nelson et al., 2001).

### 1.1.5. Microring resonators

One new emerging class of sensitive label-free optical sensors is called microring resonators. Their working principle is based on the refractive index sensitivity of optical microcavity structures that are supported by adjacent waveguide arrangements. Light coupled to the adjacent linear waveguide is localized around the circumference of the microring under precise conditions of optical resonance (figure 3.-I.), defined by the geometry of the cavity (micro-ring) and the refractive-index of the surrounding environment. The resonance condition is described by:

$$m\,\lambda = 2\pi r n_{eff}$$

where $m$ is an integer, $\lambda$ is wavelength, $r$ is the radius of the resonator, and $n_{eff}$ is the effective refractive index of the environment near the microring. The wavelength at which the resonance occurs is extremely narrow and is observed as a sharp dip in transmission spectrum (figure3.-II.). Target nucleic acid hybridization onto probe-modified microring surface induces change in local refractive index that, in turn, is detected as a shift in resonant wavelength (figure 3-III.).

Several possible diagnostic applications have been described that use microring resonator arrays for bioanalysis. For example, an array of microrings that was modified with either nucleic acid or antibody probes was capable of successful detection of viral nucleic acids, whole bacteria and quantification of

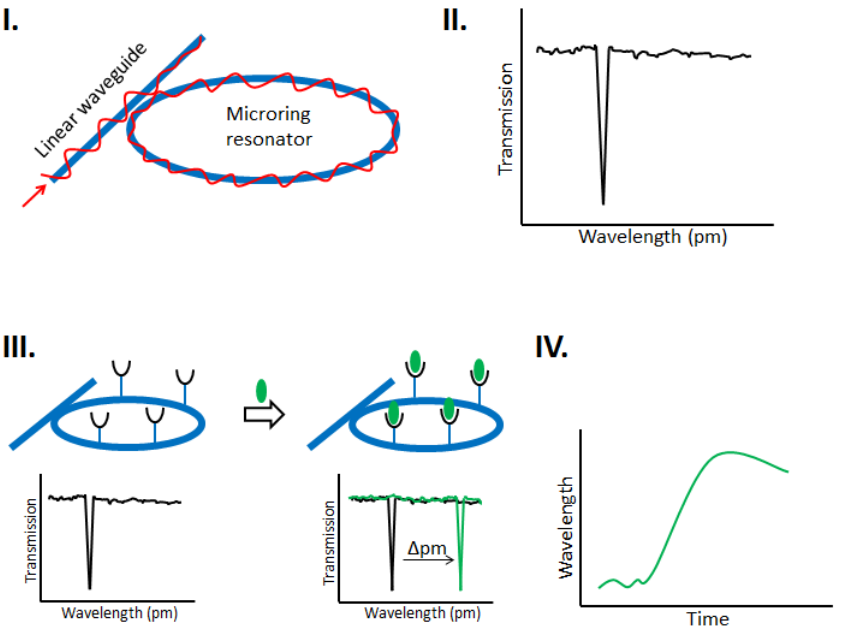


**Figure 3.** The working principle of a biosensor based on microring resonator. Light from the linear waveguide is coupled into the microring (I.) under precise optical resonance conditions described by formula $m\lambda=2\pi r n_{eff}$ (further explained in text). Resonance wavelength is observed as a sharp dip in transmission spectrum (II.). Changes in local refractive index caused by specific probe-target interaction induce shift in resonance wavelength (III.) that can be monitored in real time on a corresponding sensorgram (IV.)

target proteins (Ramachandran et al., 2008). Microring resonators can also easily be implemented for more complex analysis of nucleic acid component of the analyte solution. For example, Qavi and Bailey described a simultaneous multiplexed detection and quantification of four different clinically relevant human miRNA targets with additional microarray sensor chip regeneration and reuse possibilities (Qavi and Bailey, 2010). Since then they have upgraded the sensitivity of described system (Qavi et al., 2011a) and also demonstrated the possible use of their platform for discriminating between single mismatched target molecules (Qavi et al., 2011b). Microring resonators are considered being highly reproducible, scalable and cost effective alternatives to other traditional optical sensing technologies due to their relatively cheap and easy fabrication process via commercially widely available semiconductor processing method. Furthermore, microring sensors require, for functioning and operating, optical technology that is already commonly available in telecommunications, reducing the need for expensive additional hardware development (Bailey et al., 2009). Current state-of-the-art technology enables simultaneous monitoring and analysis of 128 different positions in a single microring resonator chip assay (www.genalyte.com)

## 1.2. Marker molecules in bacterial diagnostics

In theory, within every bacterial species and strains there are unique DNA or RNA sequences that can be used to detect and identify those bacteria. Nucleic acid-based detection technologies in bacterial diagnostics rely on the analysis of those specific marker regions that indicate the presence and abundance of certain bacteria in samples under investigation. Such biomarker should ideally be present in bacteria at a relatively high copy number for easier extraction and detection, while also being sufficiently heterogeneous at the sequence level to allow differentiation of the pathogen at strain or species levels (Glynn et al., 2006; O'Connor and Glynn, 2010). Unfortunately such "ideal" marker molecule does not exist or at least has not been identified yet; therefore several different molecules are being used for bacterial detection and identification. The selection of a suitable marker molecule (or combination of several different) is usually determined by the experiment setup and depending on particular bacterial species or taxa that are investigated. Popular marker molecules in microbial diagnostics include (but are not limited to) ribosomal RNA (and DNA), genes involved in cellular metabolism processes, and also more specific virulence factors and pathogenicity related genes.

### 1.2.1. Ribosomal RNA and DNA

Based on the previously described criteria for a feasible biomarker in bacterial diagnostics, rRNA molecules are considered highly suitable. They are universally present in all bacteria with a very high copy number, reaching from few hundred to even hundred thousand copies per cell (averaging around 10000). Evolutionary conservation of different regions of the rRNA sequence is highly variable, enabling targeting of both large taxonomic groups (such as phyla) and also allowing more specific detection of bacteria at lower level using the same RNA molecule (Amann and Fuchs, 2008). The origins of using rRNA for the analysis of bacteria can be traced back to the pioneering works of Woese and colleagues who applied 16S rRNA sequence data to divide the living world into three domains: eukaryotes, bacteria and archaea (Woese and Fox, 1977). Since then, the 16S rRNA has been used extensively for bacterial diagnostics and has achieved a "gold standard" status among other bacterial marker molecules (O'Connor and Glynn, 2010). In previous sections of this thesis, direct targeting of 16S rRNA in analytical microbiology has already been described by using several different technological approaches like FISH (1.1.1.), microarrays (Guschin et al., 1997; Small et al., 2001; Chandler et al., 2003) (1.1.2.), and also biosensors (Nelson et al., 2001; Wu et al., 2009) (1.1.3.). In combination with different amplification strategies, both rRNA (Fey et al., 2004) and also its corresponding genomic rDNA sequence can be applied for diagnostics either in simple Southern blot assay (Greisen et al., 1994) (1.1.1.) or in more complex microarray format (Guschin et al., 1997) (1.1.2.).

Additional advantage of 16S rRNA, as a target for bacterial identification, is that respective sequences have already been described for almost all of the known bacteria allowing for quick and relatively easy probe design for various detection platforms (Amann and Fuchs, 2008). That has enabled the development of high-density microarrays that contain probes covering most of the described bacterial taxa. Andersen and colleagues designed a "PhyloChip" microarray that contained roughly 300000 probes complementary to different regions of 16S rRNA from nearly 9000 different operational taxonomic units (OTUs). OTU is defined as a cluster of 16S rRNA sequences that are all complementary to a set of specific probes (on average 24 per cluster). The taxonomic belonging of each OTU was assigned according to the affiliation of its member organisms in Bergey's manual of systematic bacteriology 2001 issue. The requirement of a sequence-specific interaction of multiple unique probes in a single set was implemented to increase the confidence of specific detection of each OTU. Described microarray was used for monitoring bacterial populations in different environments in combination with universal PCR amplification of rDNA sequences (Brodie et al., 2006; DeSantis et al., 2007), or alternatively by direct hybridization of rRNA or double-stranded cDNA (dscDNA) (Deangelis et al., 2011).

Still, several studies have shown that 16S rRNA (or its gene sequence) is not the ultimate marker molecule in bacterial diagnostics that fits every possible circumstance as in many this biomarker does not allow to differentiate between closely related species. For example, it has been reported, that in case of the *Streptococcus* genus, the 16S rRNA gene sequence is not variable enough to allow identification of closely related species or subspecies. Instead, sequence comparison of several other protein-encoding genes turned out as more informative allowing better discrimination between the members of *Streptococcus* genus (Glazunova et al., 2009). Similar situations have been described for other bacterial taxa (Yamamoto and Harayama, 1995; Mollet et al., 1997; Schönhuber et al., 2001; Martens et al., 2007; Weng et al., 2009). Alternative marker molecules in these studies will be discussed later in following sections. Another drawback, that has emerged regarding the use of 16S rRNA genes, is the presence of multiple copies of non-identical sequences of that gene are often present in some bacterial genome. Such intragenomic heterogeneity may further invalidate the use of this target for precise diagnostic applications in some cases (Case et al., 2007; Martens et al., 2007; Kilian et al., 2008). Low differentiation power of this marker molecule at species level combined with copy number heterogeneity has driven analytical scientists to look for alternative marker molecules that can be used for more accurate bacterial diagnostics.

One possible option, to overcome low discriminating power of highly conserved 16S rRNA or rDNA, is to use 23S rRNA or its gene sequence. While possessing all characteristics of a good marker molecule described also for 16S rRNA, the 23S rRNA molecule is considered having more variation between the species, including those of medical and food safety importance. This

property enabled detection and identification of specific PCR-amplified 23S rRNA genes on a low-density microarray platform for identification of a foodborne infection (Hong et al., 2004) or in previously described bacteremia analysis in section 1.1.2. (Anthony et al., 2000). Another possible choice for a diagnostics marker in bacteria are the intergenic spacer regions (ISR, also known as internal transcribed spacer- ITS) between the ribosome genes in the ribosomal operon (*rrn*) (Gürtler, 1999; Milyutina et al., 2004). From a diagnostics perspective, the intergenic region between 16S and 23S rRNA (ITS1) has found most consideration for bacterial analysis as it contains hyper-variable regions that should theoretically allow differentiation even at strain level (García-Martínez et al., 2001). As an example of ISR based diagnostics, the detection of beer spoilage bacteria was described (1.1.2.) using ISR amplification by RT-PCR in combination with microarray-based detection and identification. More information on viability of the bacteria under investigation was also achieved by targeting ISR that is usually degraded quickly in growing cells (Weber et al., 2008). On the other hand, the ribosomal operon is usually found in multiple copies within most bacterial genomes, and as previously described also with 16S rDNA, the variation of ISR sequences between these copies has also been described (Gürtler, 1999; Milyutina et al., 2004). Unless such intragenomic heterogeneity of ribosomal genes is carefully considered and analyzed while developing the diagnostics assay, correct typing of many bacterial species or strains remains somewhat questionable (Lenz et al., 2010).

### 1.2.2. Universal protein-coding marker molecules

There are many protein-coding genes that participate in universal metabolic processes that are present basically in all bacteria (also referred to as housekeeping genes). Their ubiquitous presence combined with sequence heterogeneity between different species and higher taxa has been found suitable for identification and classification of bacteria in environmental and diagnostics studies (Santos and Ochman, 2004).

One such candidate for a good marker molecule in bacterial diagnostics is a RNA polymerase β subunit coding gene *rpoB*. Many studies demonstrate the superiority of *rpoB* over 16S rRNA in sequence divergence and thus species differentiation capability, for example among species of *Enterobacteriaceae* family (Mollet et al., 1997), *Bacillus* (Ki et al., 2009), *Geobacillus* (Weng et al., 2009), *Streptococcus* (Glazunova et al., 2009), and *Bartonella* (Renesto et al., 2001) genera. As a practical proof-of-principle for diagnostic purposes, DelVecchio and colleagues utilized *rpoB* gene as a specific chromosomal marker for real-time PCR detection and identification of *Bacillus anthracis* that is a causal agent of anthrax, a serious infection among both livestock and humans, making it a potent biological warfare agent (Qi et al., 2001).

The "DNA gyrase subunit B"-coding *gyrB* gene is another popular housekeeping gene that has been recommended for bacterial detection and

identification due to its good species differentiation capability ( Yamamoto and Harayama, 1995; Glazunova et al., 2009). A common pair of specific primers was used to PCR-amplify *gyrB* gene sequences from 14 different *Mycobacterium* species. Single-stranded RNA products were derived from amplified dsDNA templates for following hybridization-based detection on microarray. Precise species identification was obtained according to unique hybridization patterns for each species of mycobacteria, and the described method could differentiate even between closely related *Mycobacterium* species (Fukushima et al., 2003). In a more ambitious attempt a *gyrB*-based diagnostic microarray was described for detection of the 24 most relevant food- and water-borne pathogens and indicator organisms at species and/or genus level. Applicability of this microarray system for the detection and identification of food-borne pathogens was validated by using artificially and naturally contaminated food samples (Kostic et al., 2010). In order to achieve a wider range of specificity encom passing many different bacteria taxa, several marker molecules can be used simultaneously in a single microarray platform. Cao and colleagues designed a microarray that contained specific probes for ITS1 and *gyrB* of most prevalent and devastating waterborne pathogenic agents (Zhou et al., 2011).

The gene for heat shock protein 60 (*hsp60*, also known as *groEL*) has also actively been investigated as a potential alternative marker molecule for bacterial identification (Goh et al., 1996). Within the *Streptococcus* genus, the *hsp60*gene sequence is even considered as the most suitable marker molecule for species- and subspecies-level identification, as well as for phylogenetic analysis in comparison with other previously mentioned marker genes like 16S rRNA, *rpoB*, and *gyrB* (Glazunova et al., 2009). In another work, RT-PCR amplified *hsp60* mRNA molecules were used as targets to detect and differentiate between viable pathogenic food-borne illness-causing *Campylobacter* species on an electronic reverse-microarray platform. Heat-denatured biotinylated RT-PCR amplified target DNA molecules were electronically delivered onto a microarray chip where they were bound onto the testing sites through the biotin-streptavidin interaction. Detection and identification of bacteria was achieved by reverse hybridization of fluorescently labeled species-specific reporter probes onto surface-bound target molecules (Zhang et al., 2006).

The list of universal protein-coding marker molecules does not end here. Other possible alternative target biomarkers that have been investigated in microbial diagnostics include (but the following list is certainly not limited to): glutamate-6-phosphate dehydrogenase gene *gdh* (Hoshino et al., 2005; Nielsen et al., 2009), heat shock protein 70 gene *hsp70* (Straub et al., 2002), bacterial recombinase gene *recA* (Mahenthiralingam et al., 2000; Thompson et al., 2004; Weng et al., 2009; Zbinden et al., 2011) housekeeping gene *rpsA* (Martens et al., 2007) and many more.

### 1.2.3. Functional gene markers

In addition to exact bacterial composition of investigated environment or biological sample, it is often also important to know what kind of processes those bacteria can perform and/or how dangerous they are. Analysis of functional gene markers that either participate in specific metabolic processes in certain bacteria or determine their virulence, should give better understanding of microbial communities and their responses/ adaptations to surrounding environment.

A high-density GeoChip microarray has been developed for analyzing microbial community composition, structure and functional activity. The microarray consists of ~28 000 probes covering approximately 57 000 gene variants from 292 functional gene families involved in carbon, nitrogen, phosphorus and sulfur cycles, energy metabolism, antibiotic resistance, metal resistance and organic contaminants degradation. For the analysis of bacterial taxonomic composition, a probe set for targeting universal marker molecule *gyrB* in different taxa was also included. GeoChip 3.0 analysis of soil microbial communities in a multifactor grassland ecosystem showed that the structure, composition and potential activity of soil microbial communities significantly correlated with the plant species diversity (He et al., 2010). In more specific environmental studies; *nirK, nirS* and *amoA* genes have been applied for nitrogen cycle analysis, and methane mono-oxygenase gene *pmoA* for methane cycle monitoring using either FISH (Pratscher et al., 2009) or microarray format ( Wu et al., 2001; Stralis-Pavese et al., 2011).

From an analytical perspective, functional genes can be targeted in order to get information about the possible pathogenicity of investigated bacterial population and its susceptibility for treatment. Narayanan and colleagues developed a diagnostic microarray for human and animal bacterial diseases, and also for their virulence and antimicrobial resistance genes. Designed microarray was able to detect 40 different bacterial pathogens of medical, veterinary and zoonotic importance (Peterson et al., 2010). Combining multiplex-PCR with microarray-based hybridization detection, Witte and colleagues analyzed different clinical isolates of *Staphylococcus aureus* for the presence of 10 clinically and therapeutically relevant antibiotic resistance genes (Strommenger et al., 2007). In another work, a similar antibiotic resistance analysis of a single target gene was described by applying impedimetric biosensor platform for the detection of respective PCR-amplified gene sequence (Corrigan et al., 2012).

Interestingly, there are some nucleic acid sequences that can be used both as universal markers for bacterial taxa analysis as well but also allow more precise functional analysis of certain populations. For example, *rpoB* gene that was described in previous section has also been linked in several studies with rifampicin (antibiotic) resistance. Drug-resistant strains of *Mycobacterium tuberculosis* strains have been detected and identified by analyzing the mutations in the *rpoB* gene that cause the resistance. Described methods include both microarray-based analysis of *rpoB* (Gingeras et al., 1998; Troesch et al.,

1999; Mikhailovich et al., 2001) as well as SPR biosensor-based detection (Rachkov et al., 2011).

### 1.2.4. Non-annotated marker regions

One possible option in bacterial diagnostics is to use probe sequences that do not target certain gene or RNA molecules, but some unique nucleotide patterns that are present in bacterial genome. While designing such probes, the annotation and the biological background of a target region is usually irrelevant. Such approach typically uses whole genome sequence data for specific probe design. In previous section 1.1.2., one such microarray was described that was designed for the detection of all known viruses and bacteria with complete genome sequence available by that time. Described high-density microarray was able to detect and characterize multiple viruses, phages, and bacteria up to the family and species level in clinical fecal, serum, and respiratory samples (Gardner et al., 2010). In another work, a comparative genomic approach was used for probe design in developing a microarray with specific probes for 11 major food-borne pathogens (Kim et al., 2008).

Ultimately, the whole genome of the bacterium can act as a marker sequence, providing highest possible level of information about its metabolism as well as clinical relevance regarding tolerance for antibiotics, virulence and toxicity. Next-generation sequencing technologies already enable increasingly cheaper and quicker production of bacterial genome sequences, accompanied by vast enlargement of corresponding databases for storage and analysis of the corresponding data. It has been predicted that in near future a routine diagnostics scenario will include direct sequencing of whole genomes of a bacterial population and comparison of obtained results against databases containing well-annotated data giving necessary information for future treatment of patients and other actions (Joseph and Read, 2010). While such scenario is not yet common in everyday medicine or environmental analysis, first steps towards such future have been made. For example, in environmental studies, the direct metagenomic analysis has been conducted to study bacterial community structure and phosphorus-removing potential in wastewater (Albertsen et al., 2011). As an example in medical studies, a whole genome analysis has been demonstrated for high-resolution analysis of pathogenic species and their antibiotic resistance (Berthet et al., 2008).

In addition, there is also an interesting option to design microarrays that contain probes without any prior knowledge about the target bacterium or its genome sequence. A high-density "Universal Bio-Signature Detection Array" (UBDA) was developed that contained probes complementary to every possible 9-mer oligonucleotide sequence ($4^9 = 262144$ probes). Each genome hybridized onto the probes on this array has a unique pattern of signal intensities. That data can theoretically be used to affiliate investigated samples into known phylogenomic relationships even with mixed analytical samples. The utility of a

UBDA microarray as a possible tool in diagnostics was demonstrated by comparing and distinguishing between genetic signatures from closely related *Brucella* species. The data gathered from the UBDA microarray experiments could also be used for analyzing phylogenetic relationships between different organisms (Shallom et al., 2011).

### 1.2.5. tmRNA

Transfer-messenger RNA (tmRNA) molecule was first described as a component belonging to a previously unknown 10S RNA fraction of small and stable RNA molecules (Lee et al., 1978; Jain et al., 1982). The name tmRNA derives from the fact that it resembles both transfer and messenger RNA (figure 4). tmRNA is encoded by *ssrA* gene in bacterial genomes (Chauhan and Apirion, 1989) and it participates in a process called trans-translation where stalled ribosomes get recycled and faulty truncated peptides are addressed for degradation. Using its tRNA-like domain tmRNA enters the ribosome that has stucked during the protein synthesis, followed by addition of proteolytic tag to the nascent faulty protein according to the small reading frame that is present in its mRNA-like domain. After that the ribosomal components are released and the tagged protein will be degraded (Felden et al., 1997; Keiler et al., 2000; Keiler et al., 1996; Withey and Friedman, 2002).

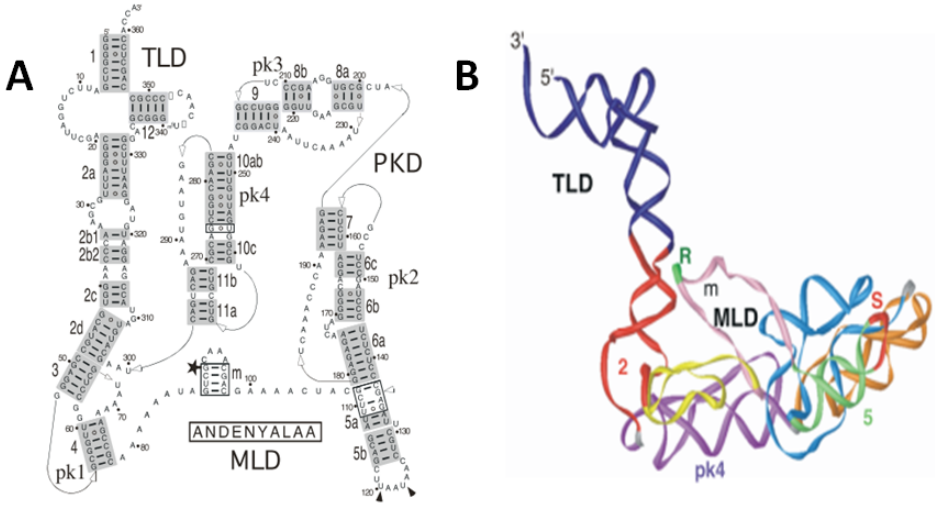


**Figure 4.** A) Secondary structure of *Escherichia coli* tmRNA molecule. Helices are highlighted in gray and numbered from 1 to 12. Three domains are distinguished: the tRNA-like domain (TLD), the mRNA-like domain (MLD) with proteolysis tag coding-sequence, and the structural pseudoknot domain (PKD). B) 3-D model of corresponding Escherichia coli tmRNA molecule. Both figures adapted from (Burks et al., 2005).

In 2001 it was first proposed that tmRNA molecules can be used as alternative biomarkers for bacterial identification. The sequence analysis of tmRNA molecules from several different bacterial taxa showed that this molecule can also be used for phylogenetic assignment and also for diagnostic analysis using FISH as an example. tmRNA also allowed for more precise differentiation among certain species when compared to 16S rRNA (Schönhuber et al., 2001). Another important characteristics that make tmRNA molecules attractive as diagnostic markers are their relatively high number in the cell averaging around 1000 copies (Glynn, 2007), and likely presence of their respective gene (*ssrA*) in all bacteria (Keiler et al., 1996; Keiler et al., 2000).

Real-time PCR assays for specific *ssrA* gene detection have been described for monitoring presence of pathogenic *Listeria* and *Salmonella* species in culture-enriched food samples (O'Grady et al., 2008; McGuinness et al., 2009) and also for clinical analysis of pathogenic Group B streptococci (Wernecke et al., 2009). Approximately 1000-order gain in sensitivity was obtained for detection of *Salmonella* when tmRNA transcript was targeted instead of its gene sequence in a real-time RT-PCR assay, proving again the advantages of naturally amplified target marker molecule (McGuinness et al., 2010). Another RNA amplification technology NASBA (section 1.1.1.1.) has also been used for tmRNA-based detection and identification of bacteria. Respective applications include endpoint detection of amplification products on SPR biosensor platform (Glynn et al., 2008), real-time detection and quantification analysis of bacteria for food safety analysis (O'Grady et al., 2009) and also a proof-of-principle concept of Integrated microfluidic tmRNA purification and real-time NASBA lab-on-a-chip device for molecular diagnostics (Dimov et al., 2008).

# 2. AIMS OF STUDY

The overall goal of current thesis was to develop new analytical methods for bacterial detection and identification using tmRNA as a target marker molecule. The experimental part consists of two sections with following objectives:

I. Selection of suitable tmRNA-specific oligonucleotide capture probes for differentiation between selected bacterial species. Development of a labeling protocol for NASBA amplification products that is suitable for further microarray-based detection. Combining of selected microarray probes with NASBA amplification technology and testing the sensitivity of the system for bacterial detection (Ref. I and II).

II. Use of tmRNA-specific probes from previous section in combination with optical microring resonator technology to develop a concept of a biosensor capable of quick real-time detection, identification and quantification of tmRNA targets in a direct hybridization assay (Ref. III).

# 3. RESULTS AND DISCUSSION

## 3.1. Bacterial strains and tmRNA genes used in current study

In current study six different bacterial test strains were addressed to develop concepts of new diagnostics methods for bacterial detection and identification: *Streptococcus pneumoniae* ATCC 33400 (*S.pneumoniae*), *Streptococcus pyogenes* ATCC 12344 (*S.pyogenes*), *Klebsiella pneumoniae* ATCC 13883 (*K.pneumoniae*), *Moraxella catarrhalis* ATCC 25238 (*M.catarrhalis*), *Streptococcus agalactiae* (*S.agalactiae*) and Group C/G streptococcus (GrC/G). *SsrA* genes from those bacteria were also inserted into the pCR® II-TOPO vector (under the transcriptional control of either T7 or SP6 promoter sequence for *in vitro* transcription of tmRNA molecules (group C and G *ssrA* sequences used in this work were identical and were thus represented by a shared gene vector). The choice of bacterial strains and their tmRNA molecules originates from tmRNA panel used in EU FP6 SLIC project #–513771 that this PhD thesis is partially based on (Ref. I and II). Main goal of that project was to develop new technologies that enable fast and precise detection of bacteria that can cause severe infections in human respiratory system. The aforementioned list of bacteria contains some of the major agents that cause those infections. Main test bacteria in current work as well as in SLIC project was *Streptococcus pneumoniae*, an important human pathogen related to several diseases, mostly associated with pneumonia.

## 3.2. NASBA-microarray technology (Ref. I and II)

### 3.2.1. Specificity of tmRNA-specific probes

Series of hybridization experiments were conducted to test the specificity of designed oligonucleotide probes and their suitability for the use in development of diagnostic technology. Probes were designed using SLICSel 1.0 software (http://bioinfo.ut.ee/slicsel) that is based on nearest-neighbor thermodynamic modeling. In total 97 oligonucleotide probes were designed complementary to the different regions of *S. pneumoniae'*s tmRNA (the main target molecule). Negative control tmRNA molecules were prepared from five other bacteria: *S. pyogenes*, *S.agalactiae*, GrC/G streptococcus, *K.pneumoniae* and *M.catarrhalis*. All tmRNA sequences were synthesized *in vitro* and then hybridized individually to the panel of *S.pneumoniae* tmRNA specific probes on microarray. Ref. II Figure 1 shows a scatter plot of relative signal intensities of control tmRNA hybridizations onto microarray probes according to their binding energy difference $\Delta\Delta G$ between target and control RNA. From a total of 463 hybridization events only 20 (~4.3%) gave relative signal intensities higher than

preset 10% false positive signal threshold condition. For the remaining 443 hybridizations (95.7%) the control signals remained under the threshold level. Designing probes with higher binding energy difference (ΔΔG) decreased the possibility of a false positive signal. For example, choosing the probes with the minimum ΔG difference of 4 kcal/mol was sufficient to avoid all the false-positive bindings over the threshold while in the case of ΔG difference 2 kcal/mol 6 signals remained over the 10% signal threshold (~1.5% of hybridizations). The average hybridization signal intensities of target and control tmRNAs (all five together and individually) are shown on a bar chart and complementary table in Ref II, Figure 2. Nearly fivefold increase of the probe specificity was achieved with ΔΔG condition 4 kcal/mol as the average false-positive control tmRNA signal intensity dropped from 2.46% to 0.55%. All of the average false-positive hybridization signals of individual tmRNAs were lower at higher minimum ΔΔG criteria. In general, reference tmRNAs from bacteria belonging to the *Streptococcus* genus showed stronger than/or near average false-positive hybridization signals while signals of more distant *K.pneumoniae* and *M.catarrhalis* remained under the overall average. *K.pneumoniae* tmRNA produced lowest average false-positive signals in all three different minimum ΔΔG conditions and had no signals over the 10% threshold. All of the false-positive signals higher than 10% were contributed by 10 individual microarray probes.

When results were analyzed without these cross-hybridizing probes the average hybridization signal intensities were under 1% for all of the used control tmRNAs. In general, the hybridization experiments with *in vitro* synthesized target and control tmRNA molecules proved that SLICSel-designed tmRNA-specific probes can be used for bacterial identification, and differentiation between species. By implementing stringent binding energy difference criteria during the probe design, SLICSel can minimize the possibility of designing probes resulting in false-positive signals. In our validation experiment, the hybridization binding energy difference ΔΔG 4 kcal/mol between the control and target tmRNA was sufficient to eliminate all the false-positive control signals over the needed threshold level (Ref II, Figure 1). We achieved an almost fivefold increase in average probe specificity by using stringent ΔΔG criteria 4 kcal/mol (Ref II, Figure 2). Although, the specificity of average SLICSel-designed probe is high, there is no 100% guaranteed approach for the *in silico* oligonucleotide probe design for hybridization-based experiments with surface-immobilized probes. Additional probe specificity evaluation *in vitro* and low quality probe removal still remain as necessary steps in any microarray experiment (Pozhitkov et al., 2006). In our case, the removal of 10 probes was needed to assure that hybridization signals with control tmRNAs remain safely under the established 10% threshold level.

### 3.2.2. NASBA product labeling for microarray experiments

The NASBA protocol was modified to include aminoallyl-UTP (aaUTP) molecules that were incorporated into nascent RNA during the NASBA reaction. Post-amplification labeling with fluorescent dye was carried out and tmRNA hybridization signal intensities were measured using microarray technology.

Two different aaUTP salts (aaUTP sodium salt and aaUTP lithium salt) were evaluated and optimum final concentrations were identified for both.

Addition of aaUTP to NASBA mix resulted in a concentration-dependent effect on the reaction performance. Concentrations of up to 0,5 mM for sodium salt and 1 mM for lithium salt did not influence NASBA efficiency as seen from the amount of RNA produced (Ref I, Figure 1A), whereas higher concentrations did inhibit the amplification efficiency. The exact ratio of aaUTP to rUTP (and to all other nucleotides correspondingly) and its influence on NASBA reactions cannot be determined precisely as the manufacturer's protocol does not provide information about the composition of Reagent sphere (component of bioMerieux NASBA kit containing NTP and dNTP molecules). Ref I, Figure 1A shows the average amount of RNA product generated with amplification reactions comparing the effect of two different aaUTP salts that were used. Specific amplification of *S.pneumoniae* tmRNA molecules was verified by observing only one peak of predicted size (307 nucleotides) nucleic acid on RNA 6000 chip electropherogram (Agilent Bioanalyzer). Corresponding microarray signal intensities of labeled NASBA products are shown on Ref I, Figure 1B. Data for hybridization signals with aaUTP lithium salt concentrations from 0,125 mM to 8 mM and for aaUTP sodium salt concentrations from 0,125 mM to 2 mM are given, respectively. Increased microarray signal intensity was observed in parallel with increasing aminoallyl-UTP concentration in NASBA reaction up to 1 mM for sodium and 2 mM for lithium salt. For aaUTP lithium salt, the final concentrations within the range of 1 mM and 2 mM resulted in the highest average microarray signals; while highest average signals with using aaUTP sodium salt were obtained between it 0,5 mM and 1 mM concentration, respectively. The final 2 mM concentration of aaUTP Li-salt in NASBA reaction resulted in highest microarray signals overall, being twice as high as the strongest signals observed with using 1 mM aaUTP Na-salt.

To our knowledge, this is the first time that the widely used NASBA technology has been combined with microarray-based RNA detection using a one-step NASBA product labeling method. Previous methods described for NASBA amplicon detection have used additional enzymatic steps after the amplification and extra labeled probes (Gill et al., 2006; Morisset et al., 2008). In our case, the NASBA protocol was modified by addition of aminoallyl-UTP molecules allowing labeling of the reaction product with aminoreactive fluorescent dye. Indirect labeling of RNA for microarray purposes via incorporation of aaUTPs was preferred over direct incorporation of fluorescently labeled nucleotides, as it has been proved to be more efficient with T7 RNA polymerase-based amplification technologies ('t Hoen et al., 2003). Two

different aaUTP salts and their effect to hybridization were compared, and both enabled sufficient fluorophore incorporation providing easily detectable microarray signals. Considering that aminoallyl-UTPs tested in the current report are produced as different salts, this may contribute to the difference in RNA quantity in NASBA reaction (Ref I, Figure 1A) causing decrease in microarray signal intensities (Ref I, Figure 1B). The aaUTP Na-salt may have more impact than the Li-salt aaUTP on the co-operation of the NASBA enzymes. Different monovalent cations have previously been shown to have unequal impact on similar enzymatic reactions (Taube et al., 1998). However, as the manufacturers do not provide exact composition of aaUTP storage solution, other unknown components may also contribute to the observed difference in behavior of used aminoallyl-UTPs.

### 3.2.3. Sensitivity of NASBA-microarray

To test specific tmRNA probes for their potential use in microbial diagnostics; a new microarray was designed that consisted of the 25 best-performing probes out of 97 according to their specificity and sensitivity in the validation experiments. For control purposes, oligonucleotide probes specific to *S.pyogenes*, *S.agalactiae*, *K.pneumoniae* and *M.catarrhalis* were also added to the microarray. tmRNA molecules of *S. pneumoniae* were amplified from three different total RNA dilutions (equivalent to teh RNA content of 0.1, 1 and 10 CFU, respectively) and labeled for microarray hybridization. Microarray signals were obtained with all three total RNA dilutions in each three parallel experiments including 10 fg of total RNA (equivalent to 0.1 CFU). Microarray signal intensity increased with higher input RNA concentration, 0.1 CFU being the lowest and 10 CFU the highest in three replicate experiments (Ref II, Figure 3). Hybridization experiments with NASBA-amplified negative control solution provided no significant signals over the background level on microarray. NASBA control experiments with excess amounts of total RNA mix from four control species (*S.pyogenes*, *S.agalactiae*, *K.pneumoniae* and *M.catarrhalis*) were performed to verify the specificity of the NASBA-microarray-based detection method. 10 pg of total RNA from each control species were added, making the background RNA ratio to target RNA 4 × 10:1, 4 × 100:1 and 4 × 1000:1, respectively. Addition of control total RNA-s to NASBA reaction did not cause any changes in microarray signal intensities; all *S.pneumoniae* target dilutions were amplified and detected on the microarray while the negative control remained blank. The capability of the described NASBA-microarray method to detect tmRNA from low amounts of bacteria was also confirmed experimentally when the total RNA was prepared from dilutions of *S.pneumoniae* cultures (0.1 to 10 CFU) instead of using total RNA dilutions, making the experiment setup closer to real-world diagnostic situations where only small amounts of target bacteria may be present.

A key advantage of the NASBA-microarray technology relevant to microbial diagnostics is that the detection and the identification of the correct target can be optimized at two different steps in the experimental protocol. The selection of oligonucleotide primers determines the specificity of the NASBA amplification phase while a second specificity checkpoint is provided by used microarray probes. Specific amplification of a single RNA molecule or wider selection of various RNAs in case of multiplex-NASBA is possible. Certain rules have been described for the NASBA primer pair design (Deiman et al., 2002), but as no convenient software has yet been developed it still remains a trial-and-error approach. In our case the primer set was designed according to the aforementioned rules to amplify a near full length tmRNA molecule from *S.pneumoniae*. We included additional control probes specific to *S.pyogenes*, *S.agalactiae*, *K.pneumoniae* and *M.catarrhalis* in the microarray to evaluate the specificity of NASBA amplification step conducted in the presence of a non-*S.pneumoniae* total RNA background. The composition of capture probes on the microarray depends on overall goal of the experiment. In our case the objective was to specifically detect tmRNA molecules from *S.pneumoniae* total RNA and test the sensitivity of the method. Our intention was to investigate whether the method is capable of detecting 1 CFU by using tmRNA as a target molecule. Previous works have shown that detection of 1 CFU by using NASBA amplification of rRNA (Loens et al., 2006) or tmRNA (O'Grady et al., 2009) is possible. The addition of highly parallel microarray-based hybridization detection to this amplification technology could represent a significant advance in microbial diagnostics; particularly in situations where high number of different bacterial species may be present (*e.g.* in environmental samples) or in clinical settings where it is necessary to identify one particular infection causing species from a large panel of potential pathogens. We successfully detected and identified *S.pneumoniae* tmRNA molecules from all three different dilutions of total RNA used in the experiments (Ref II, Figure 3). Our experiments proved that 0.1 CFU equivalent total RNA was sufficient to produce strong reproducible hybridization signals on our microarray. The addition of background total RNAs to the NASBA reaction mix provided no signals on respective control probes on microarray, confirming the high specificity of NASBA-microarray technology and also its components: NASBA primers and microarray probes. In case of the specific tmRNA detection from 0.1 CFU equivalent of *S.pneumoniae* total RNA, the amount of non-specific RNA exceeded the target in 4000 to 1 ratio. The high level of specificity and sensitivity that was achieved demonstrates the potential and suitability of NASBA-microarray technology for pathogen detection in microbial diagnostics or in more complex analysis of microbial taxa in the environment.

## 3.3. tmRNA detection using microring resonators (Ref.III)

In this part of the thesis, the quantitative detection of specific tmRNA molecules for bacterial biosensing is demonstrated using arrays of silicon photonic microring resonators functionalized with tmRNA-specific oligonucleotides. *S.pneumoniae* tmRNA was used as a main target RNA molecule while tmRNA molecules from three other pathogens (*K.pneumoniae, E. faecium* and *S.agalactiae*) were used for comparison purposes. Microring resonator array contained specific probes for both *S.pneumoniae* and *S.agalactiae* that were selected out of wider set based on their specificity and sensitivity in fluorescent microarray hybridization experiments described in sections 3.2.1 and 3.2.3 of current thesis. A schematic of the tmRNA hybridization assay is shown in Ref III, Figure 1. In this assay, a DNA probe complementary to the target tmRNA of interest is covalently attached to the microring surface, after which a solution containing the target tmRNA is flowed across the sensor. The hybridization of tmRNA onto the probe-modified sensor surface results in a change in the wavelength of the light that is resonantly coupled into the microring, resulting in an easily measured shift.

### 3.3.1. RNA preparation for the biosensor experiment

Unlike small nucleic acids such as siRNAs and miRNAs, tmRNAs frequently possess significant secondary and tertiary structures (Burks et al., 2005) that can complicate simple hybridization-based detection. This is of particular concern when making measurements at near room temperature (Kaplinski et al., 2010), that is convenient from a sensor operation perspective. Our early results highlighted these challenges, as evidenced by relatively slow binding of tmRNA molecules where the capture probe saturation was achieved in hours instead of minutes in case with short complementary probes. To address this challenge, three additional RNA preparation methods were investigated to determine the optimal conditions for tmRNA detection at room temperature. These methods included: (i) chemical fragmentation of the tmRNAs using $ZnCl_2$, (ii) denaturation of the target tmRNAs by heating at 95$^{o}$C before cooling back to room temperature, and (iii) thermal denaturation of the targets in the presence of chaperone oligonucleotides designed to assist in unfolding the tmRNA. The chaperone sequences were previously designed in our group and demonstrated to bind to predicted secondary structure regions in *S.pneumoniae* tmRNA, prevent refolding of tmRNA after denaturation and therefore enhance tmRNA hybridization (Kaplinski et al., 2010). As shown in Ref III, Figure 2, fragmentation of the tmRNA was the most effective method in order to enhance both the binding kinetics and overall net response magnitude. We attribute this primarily to the reduced secondary structure present in the shorter (80–120

nucleotides) tmRNA fragments. Our results agree with the previous report by Wu and co-workers in which RNA fragmentation was also found as the most effective strategy to improve hybridization efficiency and sensitivity in a fluorescent microarray analysis (Liu et al., 2007). Consequently, tmRNA molecules were fragmented in all subsequent experiments to improve the sensor performance. Once fragmentation was established as the most effective pre-treatment for tmRNA samples, we sought to optimize fragmentation time. As shown in Ref III, Figure 3, the time in which the sample was exposed to $ZnCl_2$ fragmentation solution was systematically varied from zero to 60 minutes, and the resulting hybridization responses were measured using identically prepared sensors. These experiments indicated that 10 min of treatment was sufficient for optimal sensor performance. Interestingly, we did not observe any significant change in the non-specific sensor response as a function of fragmentation time.

### 3.3.2. Sensitivity and dynamic range of biosensor

In order for a microbial diagnostic technology to be useful, it must respond quantitatively and specifically to low levels of target bacterial marker molecules on a relatively high background of non-target material. This is due to the diversity of bacterial species potetially present in clinical or other types of samples. Addressing specificity first, we functionalized a single sensor array with ssDNA capture probes targeting bacterial tmRNAs from *S.pneumoniae* and *S.agalactiae*. We subsequently introduced a series of tmRNAs from four bacterial species (*K.pneumoniae*, *E. faecium*, *S.pneumoniae*, and *S.agalactiae*) sequentially across the sensor surface. Each tmRNA solution contained 1.66 pmoles of the target. As seen in Ref III, Figure 4, *K.pneumoniae* and *E.faecium* tmRNA did not elicit a response while subsequent hybridization steps with both *S.agalactiae* and *S.pneumoniae* tmRNA demonstrated strong and specific responses from the microrings modified with complementary capture probes. Different response magnitudes from *S.pneumoniae*- and *S.agalactiae*-specific microrings can be attributed to differences in the probe length and hybridization properties (melting temperatures, binding affinity, nucleotide composition and positioning) of the different DNA capture probe-RNA target pairs. Additionally, targeting of complementary regions in fragmented tmRNA molecules can still be unevenly hindered by any remaining secondary structure. While these probe sequences can be further designed to reduce these differences, our results already clearly demonstrate the potential of microring resonator platform to directly detect bacterial tmRNAs and also discriminate between different bacterial strains on the basis of differential hybridization.

Having demonstrated the specificity of the method, we then focused on establishing the quantitative utility of the platform towards tmRNA detection. Probe-functionalized microring sensors were exposed to different quantities of *S.pneumoniae* tmRNA, ranging from 52.4 fmol to 16.6 pmol. A cocktail of control tmRNAs from three other bacteria (1.66 pmol each from: *K.pneumoniae*, *E.faecium* and *S.agalactiae*) were also added to the hybridization

mixture as a background to mimic the complex matrix in which tmRNA analytes could be found naturally. The concentration-dependent responses, shown in Ref III, Figure 5, provided 52.4 fmol (or 100 µl of 524 pM tmRNA solution) as a limit of detection. This value roughly corresponds to 3.16 x $10^{10}$ tmRNA molecules or 3.16 x $10^{7}$ CFU of *S.pneumoniae*, with a dynamic range of nearly three orders of magnitude. The limit of detection reported herein surpasses previous reports on the direct label-free detection of microRNAs and DNA using the same measurement technology (Qavi and Bailey, 2010; Qavi et al., 2011b). This increased sensitivity may be due to the larger size of the tmRNA targets. Even after fragmentation, the detectable targets are still 80–120 nucleotides in length, compared to the previously investigated 22 nucleotide microRNA sequences. Furthermore, this detection limit is comparable to that achieved by a well-known and widely used optical biosensing technology-surface plasmon resonance imaging (SPRi), in which the detection of 2nM of *Escherichia coli* 16S rRNA was reported in a similar direct hybridization assay (Nelson et al., 2001).

Extrapolating beyond this initial report, the potential multiplexing capability of this silicon photonic platform is attractive for more informative bacterial diagnostics, whereby the presence of a larger number of targeted bacteria can be simultaneously probed using a relatively simple and rapid assay protocol. Although the obtained level of sensitivity is far from that achieved with regular culture-based approach or with regular molecular methods, the incorporation of signal and/or target amplification technologies should provide sensitivity levels comparable to real-time NASBA or real-time RT-PCR-based detection of tmRNA molecules, albeit with additional sample and/or assay manipulation steps. Recently, an antibody specific to DNA-RNA duplexes was incorporated onto this microring resonator platform and a ~3 order of magnitude improvement in limit of detection was achieved for miRNA analysis (Qavi et al., 2011a). Also, larger refractive index tags can be introduced to dramatically increase limits of detection, as compared to solely label-free analyses (Luchansky et al., 2011). Importantly, both these additional amplification steps can be implemented in two hours or less, and thus the assay retains its quick time-to-result mode as compared to traditional microbiology-based analysis. Aside from the sensor platform itself, increases in sensitivity can also be accomplished by applying either culture enrichment methods (McGuinness et al., 2009; O'Grady et al., 2008) or tmRNA amplification using previously described NASBA technology prior to hybridization. While the biosensor experiments described here demonstrate early stage studies designed to show detection platform applicability to *in vitro* synthesized target tmRNA molecules, this platform has already been applied to significantly more complex RNA-containing samples (Qavi and Bailey, 2010; Qavi et al., 2011a) and thus the assays described herein should be applicable to more complex samples with only minor modifications to hybridization conditions or with minor additional pre-analysis sample preparation.

# CONCLUSIONS

In current thesis tmRNA molecule was used as a target marker molecule to develop new concepts of diagnostic methods to detect and identify bacteria. Two different methods are described that are based on either microarray or biosensor technology, correspondingly.

I. A panel of tmRNA specific probes was designed and tested for specificity in microarray hybridization experiments using *in vitro* synthesized tmRNA molecules from *Streptococcus pneumoniae* and five control species. Subsequently, a labeling protocol was developed for NASBA amplified tmRNA molecules from bacterial total RNA samples. The technology is based on addition of aminoallyl-UTP molecules to the NASBA reaction mix that can later be labeled using fluorescent dye molecules. Described NASBA amplification technology in combination with designed capture probes in microarray format enabled specific detection of as low as 0.1 CFU equivalent of bacterial tmRNA molecules from series of total RNA mixture dilutions.

II. A set of selected tmRNA-specific probes from previous section were combined with emerging optical microring resonator-based label-free biosensing technology for diagnostics. A chemical fragmentation of the tmRNAs using $ZnCl_2$ was established as the most effective pre-treatment technology that yielded in quickest hybridization signal with biosensor. The sensitivity and the dynamic range of the biosensor were then determined using *in vitro* synthesized tmRNA molecules. The limit of detection for the designed biosensor was 52.4 fmol that roughly corresponds to $3.16 \times 10^{10}$ tmRNA molecules or $3.16 \times 10^{7}$ CFU of *S.pneumoniae*, with a dynamic range of nearly three orders of magnitude.

Both described technological concepts can be adapted for further use in testing different patient samples, food products or environmental material in a highly parallel manner suitable for quick detection of multiple bacterial species.

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# SUMMARY IN ESTONIAN

## tmRNA kasutamine markermolekulina bakterite tuvastamisel mikrokiibi ja biosensor tehnoloogia kaudu

Käesolevas doktoritöös antakse kirjanduse põhjal ülevaade erinevatest tehnoloogiatest, mida kasutatakse nukleiinahappe põhises bakteriaalses diagnostikas. Põhirõhk on erinevatel nukleiinhapete paljundamise meetoditel ning hübridisatsiooni-põhistel detektsiooni tehnoloogiatel. Käsitletud on erinevate mikrokiibi ja biosensor tehnoloogiate põhimõtteid ning nende võimalikke kasutusviise bakterite tuvastamisel. Kirjanduse ülevaate teises osas antakse ülevaade DNA ja RNA järjestustest, mida saab kasutada markerjärjestusena erinevate bakterite tuvastamisel ja üksteisest eristamisel. Pikemalt tutvustatakse tmRNA molekule, mida kasutatakse markerjärjestusena käesoleva doktoritöö raames välja töötatud diagnostiliste meetodite puhul. tmRNA on kõikides bakterites leiduv, keskmiselt 300–400 nukleotiidi pikkune spetsiifiline RNA molekul, mis abistab rakus valgusünteesi mehhanismi. Pooleli jäänud või vigase translatsiooni korral võimaldab tmRNA koos abivalkudega ribosoomi vabastamise teistest valgusünteesi komponentidest ning suunab pooliku valgu lagundamisele.

Töö praktilises osas kirjeldatakse kahte erinevat meetodit, kus tmRNA detektsiooni kaudu tuvastatakse lahustest erinevaid baktereid. Esimese puhul paljundatakse lahuses olevad tmRNA molekulid kõigepealt NASBA tehnoloogiat kasutades. NASBA on on isotermiline meetod, kus RNA paljundatakse kolme ensüümi: pöördtranskriptaasi, RNaseH ja T7 RNA polümeraasi koostöös. Seejärel toimub paljundatud ning märgistatud tmRNA molekulide tuvastatamine ja täpne identifitseerimine mikrokiibi tehnoloogiat kasutades. Kirjeldatud NASBA-mikrokiip tehnoloogiat rakendati spetsiifiliste tmRNA-de tuvastamiseks erinevate bakterite totaalsest RNA lahusest. Praktilise osa teises pooles kirjeldatakse tmRNA-de detektsiooni märkevaba reaal-ajas toimiva biosensor süsteemi abil, mis põhineb optilisel mikroring resonaator tehnoloogial. Optilised mikroring resonaatorid on sellised sensorid, mis tunnevad ära oma pinna lähedal toimuvaid bioloogilisi reaktsioone nende poolt tingitud keskkonna optilise murdumisnäitaja muudu kaudu. Kirjeldatud biosensori spetsiifilisust, tundlikkust ning kvantiseerimisvõimet demonstreeritakse *in vitro* sünteesitud tmRNA molekulide abil. Kuigi mõlema meetodi puhul kasutati testsüsteemina erinevaid hingamisteede haigusi põhjustavaid baktereid ning nende vastavaid liigispetsiifilisi tmRNA molekule, on kirjeldatud tehnoloogiad lihtsasti kohandatavad ka teiste RNA järjestuste ning erinevate bakteri-liikide korral.

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# PUBLICATIONS

# CURRICULUM VITAE

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### Education

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| 1989–2001 | Rapla Ühisgümnaasium |
| 2001–2005 | BSc Gene Technology (Molecular Diagnostics), Department of Biotechnology, IMCB, University of Tartu |
| 2005–2007 | MSc Gene Technology (Molecular Diagnostics), Department of Biotechnology, IMCB, University of Tartu |
| 2007–... | PhD student in Molecular and Cell Biology (Biotechnology), Department of Biotechnology, IMCB, University of Tartu |
| 2011 | DoRa PhD Student semester abroad in University of Illinois at Urbana-Champaign, USA |

### Professional employment

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| 2008–2009 | researcher, Estonian Biocentre, |
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### Scientific work

The research carried out during my graduate studies can be summarized as developing new methods for specific detection and identification of different bacteria by targeting specific nucleic-acid sequences. The backbone of my studies has been trans-translation mediating RNA molecule called tmRNA that can be used as species- or other taxon-specific marker sequence for bacterial diagnostics. Researched methods include NASBA-microarray technology and optical microring resonator-based biosensor array.

### Publications


1. Scheler O, Glynn B, Parkel S, Palta P, Toome K, Kaplinski L, Remm M, Maher M, Kurg A (2009). Fluorescent labeling of NASBA amplified tmRNA molecules for microarray applications. BMC Biotechnology, 15(9), 45–50.

2. Kaplinski L, Scheler O, Parkel S, Palta P, Toome K, Kurg A, Remm M (2010). Detection of tmRNA molecules on microarrays at low temperatures using helper oligonucleotides. BMC Biotechnology, 10(34), 1–10.
3. Scheler O, Kaplinski L, Glynn B, Palta P, Parkel S, Toome, K, Maher M, Barry T, Remm M, Kurg A (2011). Detection of NASBA amplified bacterial tmRNA molecules on SLICSel designed microarray probes. BMC Biotechnology, 11(1), 11–17.
4. Vlassov S, Scheler O, Plaado M, Lõhmus R, Kurg A, Saal K, Kink I (2012). Integrated carbon nanotube fibre–quartz tuning fork biosensor. Proceedings of the Estonian Academy of Sciences, 61(1), 48–51.
5. Scheler O, Kindt JT, Qavi AJ, Kaplinski L, Glynn B, Barry T, Kurg A, Bailey RC. Label-free, multiplexed detection of bacterial tmRNA using silicon photonic microring resonators. Biosensors and Bioelectronics, *in press*

# ELULOOKIRJELDUS

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| 2005–2007 | MSc Geenitehnoloogia (molekulaardiagnostika),, Biotehnoloogia õppetool TÜMRI, Tartu Ülikool |
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### Erialane teenistuskäik

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| 2008–2009 | Teadur, Eesti Biokeskus, |
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### Teadustegevus

Minu kraadiõpingute jooksul teostatud teadustöö saab kokkuvõtvalt sõnastada kui uute meetodite välja töötamine bakterite tuvastamiseks ja identifitseerimiseks spetsiifiliste nukleiinhappe järjestuste kaudu. Kesksel kohal nendes uuringutes kasutati trans-translatsioonil osalevat RNA molekuli nimega tmRNA, mille järjestust siis saab kasutada liigi- või kõrgema taksoni-spetsiifilise markermolekulina bakteriaalses diagnostikas. Arendatud meetoditeks on NASBA-mikrokiip ning optilistel mikroring resonaatoritel põhinevad biosensor-kiibid.

### Publikatsioonid

1. Scheler O, Glynn B, Parkel S, Palta P, Toome K, Kaplinski L, Remm M, Maher M, Kurg A (2009). Fluorescent labeling of NASBA amplified tmRNA molecules for microarray applications. BMC Biotechnology, 15(9), 45–50.

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4. Vlassov S, Scheler O, Plaado M, Lõhmus R, Kurg A, Saal K, Kink I (2012). Integrated carbon nanotube fibre–quartz tuning fork biosensor. Proceedings of the Estonian Academy of Sciences, 61(1), 48–51.
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