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SWITCHABLE LANTHANIDE FLUORESCENCE PROBES IN HOMOGENOUS MOLECULAR DIAGNOSTICS

by

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To my family

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals (**I–IV**):

I Lehmusvuori, A., Karhunen, U., Tapio, A-H., Lamminmäki, U. & Soukka, T. (2012) High-performance closed-tube PCR based on switchable luminescence probes. *Analytica Chimica Acta* **731:**88–92.

II Lehmusvuori, A., Manninen, J., Huovinen, T., Soukka, T. & Lamminmäki, U. (2012) Homogenous M13 bacteriophage quantification assay using switchable lanthanide fluorescence probes. *Biotechniques* **53:**301–303.

III Lehmusvuori, A., Tapio, A-H., Mäki-Teeri, P., Rantakokko-Jalava, K., Wang, Qi., Takalo, H. & Soukka, T. (2013) Homogenous duplex polymerase chain reaction assay using switchable lanthanide fluorescence probes. *Analytical Biochemistry* **436:**16–21.

IV Lehmusvuori, A., Kiviniemi, M., Ilonen, J. & Soukka, T. (manuscript) Closed-tube HLA-DQA1*05 PCR assay based on switchable lanthanide fluorescence probes and simple dried blood sample punch preparation.

ABBREVIATIONS

ATP	adenosine triphosphate
CMA	competitive reporter monitored amplification
C_t	threshold cycle
Ct	*Chlamydia trachomatis*
DELFIA	dissociation-enhanced lanthanide fluorescent immunoassay
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
EtBr	ethidium bromide
Eu TRF	europium time-resolved fluorescence measurement
f	lanthanide ion ground state
f*	lanthanide ion in excited state
FDA	United States Food and Drug Administration
FP	fluorescence polarization
FRET	fluorescence resonance energy transfer
HEG	hexaethylene glycol
HLA	human leucocyte antigen
IAC	internal amplification control
iC	isocytidine
iG	isoguanosine
LAMP	loop mediated isothermal amplification
LED	light emitting diode
Ln^{III}	lanthanide ion
MRI	magnetic resonance imaging
NASBA	nucleic acid sequence-based amplification
PCR	polymerase chain reaction
PNA	peptide nucleic acid
POC	point-of-care
RNA	ribonucleic acid
SNP	single-nucleotide polymorphism
PP_i	inorganic pyrophosphate
qPCR	quantitative PCR
S/B	signal-to-background ratio
SBE	single base extension
SDA	strand displacement amplification
S_0	singlet ground state
S_1	singlet excited state
ssDNA	single-stranded deoxyribonucleic acid

ssRNA	single-stranded ribonucleic acid
STD	sexually transmitted disease
T_1	triplet state
T1D	type 1 diabetes
Tb TRF	terbium time-resolved fluorescence measurement
TRF	time-resolved fluorescence
TO	thiazole orange
UCP	upconverting phosphor
6-FAM	6-carboxyfluorescein

ABSTRACT

The number of molecular diagnostic assays has increased tremendously in recent years. Nucleic acid diagnostic assays have been developed, especially for the detection of human pathogenic microbes and genetic markers predisposing to certain diseases. Closed-tube methods are preferred because they are usually faster and easier to perform than heterogenous methods and in addition, target nucleic acids are commonly amplified leading to risk of contamination of the following reactions by the amplification product if the reactions are opened.

The present study introduces a new closed-tube switchable complementation probes based PCR assay concept where two non-fluorescent probes form a fluorescent lanthanide chelate complex in the presence of the target DNA. In this dual-probe PCR assay method one oligonucleotide probe carries a non-fluorescent lanthanide chelate and another probe a light absorbing antenna ligand. The fluorescent lanthanide chelate complex is formed only when the non-fluorescent probes are hybridized to adjacent positions into the target DNA bringing the reporter moieties in close proximity. The complex is formed by self-assembled lanthanide chelate complementation where the antenna ligand is coordinated to the lanthanide ion captured in the chelate. The complementation probes based assays with time-resolved fluorescence measurement showed low background signal level and hence, relatively high nucleic acid detection sensitivity (low picomolar target concentration).

Different lanthanide chelate structures were explored and a new cyclic seven dentate lanthanide chelate was found suitable for complementation probe method. It was also found to resist relatively high PCR reaction temperatures, which was essential for the PCR assay applications. A seven-dentate chelate with two unoccupied coordination sites must be used instead of a more stable eight- or nine-dentate chelate because the antenna ligand needs to be coordinated to the free coordination sites of the lanthanide ion. The previously used linear seven-dentate lanthanide chelate was found to be unstable in PCR conditions and hence, the new cyclic chelate was needed.

The complementation probe PCR assay method showed high signal-to-background ratio up to 300 due to a low background fluorescence level and the results (threshold cycles) in real-time PCR were reached approximately 6 amplification cycles earlier compared to the commonly used FRET-based closed-tube PCR method. The suitability of the complementation probe method for different nucleic acid assay applications was studied. 1) A duplex complementation probe *C. trachomatis* PCR assay with a simple 10-minute urine sample preparation was developed to study suitability of the method for clinical diagnostics. The performance of the *C. trachomatis* assay was equal to the commercial *C. trachomatis* nucleic acid amplification assay containing more complex sample preparation based on DNA extraction. 2) A PCR assay for the detection of HLA-DQA1*05 allele, that is used to predict the risk of type 1 diabetes, was developed to study the performance of the method in genotyping. A simple blood sample preparation was used where the nucleic acids were released from dried blood sample

punches using high temperature and alkaline reaction conditions. The complementation probe HLA-DQA1*05 PCR assay showed good genotyping performance correlating 100% with the routinely used heterogenous reference assay. 3) To study the suitability of the complementation probe method for direct measurement of the target organism, e.g., in the culture media, the complementation probes were applied to amplification-free closed-tube bacteriophage quantification by measuring M13 bacteriophage ssDNA. A low picomolar bacteriophage concentration was detected in a rapid 20-minute assay. The assay provides a quick and reliable alternative to the commonly used and relatively unreliable UV-photometry and time-consuming culture based bacteriophage detection methods and indicates that the method could also be used for direct measurement of other micro-organisms.

The complementation probe PCR method has a low background signal level leading to a high signal-to-background ratio and relatively sensitive nucleic acid detection. The method is compatible with simple sample preparation and it was shown to tolerate residues of urine, blood, bacteria and bacterial culture media. The common trend in nucleic acid diagnostics is to create easy-to-use assays suitable for rapid near patient analysis. The complementation probe PCR assays with a brief sample preparation should be relatively easy to automate and hence, would allow the development of high-performance nucleic acid amplification assays with a short overall assay time.

1 INTRODUCTION

Molecular diagnostics (nucleic acid diagnostics) contains a broad range of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) assays. Nucleic acid based tests are used in a wide variety of applications, for example, in forensics to identify individuals [1], evolutionary studies to find phylogenetic relationships among organisms [2] and in environmental microbiology to monitor biodiversity [3]. Clinical nucleic acid testing encompasses, for example, the search of genetic polymorphism related to hereditary diseases and detection of infectious microbial pathogens from various types of human samples [4, 5]. Human pathogens are also searched in drinking and swimming water and in foodstuffs using nucleic acid assays [6, 7]. In addition, molecular diagnostics is used for protein expression studies by measuring messenger RNA [8] and in pharmacogenomics, for instance, to analyze drug response in cancer therapy [9].

Advances in genomics, especially the increased sequence data of different micro-organisms and human genome due to the improvements in sequencing techniques and completion of the Human Genome Project in 2003 have led to the development of a huge number of clinical nucleic acid assays in the past 10 years. Many different technologies and assays have been established, especially for the detection of microbes causing infectious diseases and single-nucleotide polymorphism (SNP) related to genetic diseases. For example, the first United States Food and Drug Administration (FDA) approved nucleic acid based assay, the Legionnaires disease test performed in bacterial culture, was approved in 1986 [10]. The first nucleic acid amplification based test measured directly in a clinical sample, the *Chlamydia trachomatis* assay, was FDA-cleared in 1996 and in 2012 there were approximately 200 FDA-approved or cleared molecular diagnostic tests [11, 12].

Molecular diagnostics of infectious diseases have several potential advantages compared to the traditionally used culture methods. Nucleic acid assays show high clinical specificity because detection is based on microbe-specific stable DNA sequences and high sensitivity is achieved by amplifying the target sequences. Nucleic acid tests enhance the detection of pathogens that are fastidious or uncultivable and this is also the case when the patient is treated with antibiotics before sample collection. The pathogen antibiotic resistance can be identified by detecting antibiotic resistance genes and in some diseases the severity of the condition can be estimated by the quantitative data. Theoretically, one of the main benefits of molecular diagnostics in infectious disease testing is short assay time. Results could be available in a few hours or even faster, whereas cultivation of the microbes requires several hours or even days of incubation. Rapid testing would be valuable especially in critical clinical situations, like in sepsis, where the pathogen identification is needed urgently for the initiation of a specific antimicrobial therapy. [13, 14]

Infectious disease testing represents the majority (70%) of the global molecular diagnostics market [14]. Other commonly used nucleic acid assays are genetic tests

related, for example, to hereditary diseases and different type of cancers and approximately 25% of the FDA approved and cleared nucleic acid assays are human genetic tests such as breast cancer related HER2 tests. Recently, also companion diagnostics have become more common in the therapeutic product development. FDA has lately introduced guidelines that companion diagnostics should be used together with a novel therapeutic product to support the therapeutic product's safety and effective use [15]. Companion diagnostics are used, especially in the development of personalized drugs that have effect only on specific patient population having a particular genotype. The trend of personalized medicine is growing and thus, also the need of companion diagnostics. [9]

Although molecular diagnostic technologies have shown superior features compared to the conventional culture-based methods in infectious disease diagnostics, the traditional phenotypic methodologies such as culture and staining are still the principal methods. Basically, nucleic acid assays are rapid but they often require relatively complex and laborious sample preparation and reagent handling requiring experienced personnel and long hands-on time. There are automated systems, for example, for the detection of infectious diseases [16] but the current sophisticated instrumentation significantly increases the assay costs and the automated systems still usually have several hours turnaround time. In routine clinical diagnostics nucleic acid technologies are nowadays more commonly used in virus diagnostics than for bacteria detection. The virus tests have been developed mainly because culture based virus assays are more complex, laborious and time-consuming to perform than bacteria culturing because a virus requires a host organism for reproduction further complicating the assay protocol. Nowadays, clinical bacterial nucleic acid assays are mainly used to either confirm culture results and or the detection of uncultivable or fastidious bacteria. [13, 17 18]

In the future, molecular diagnostics will be increasingly adapted to routine clinical diagnostics. But before becoming more commonly used, for example, in infectious disease diagnostics, more multiplexed, cost-effective, easy-to-use and even faster assays must be prepared. Multiplexed assays should be able to detect multiple pathogens and resistant markers from one sample in one assay. This would shorten the total analysis time and also decrease the costs. Assays should be automated containing also the sample preparation. Multiplexed nucleic acid amplification assays are already available [4, 19] but one of the main disadvantages of the mainly used multiplexed technologies is that they require post-amplification sample handling increasing the assay time and also causing risk of amplification product cross-contamination. In coming years, the greatest impact of the molecular diagnostics will most likely be in the development of closed-tube point-of-care (POC) infectious disease assays with rapid turnaround time where the results are available in less than one hour or even in 30 minutes. [20-22]

In this thesis, a new high-performance homogenous polymerase chain reaction (PCR) assay concept is described. The introduced system is based on switchable lanthanide fluorescence probe technology where the fluorescence is “turned on” in the presence

of a specific nucleic acid target. Functionality of the system is demonstrated by clinical diagnostic assays and amplification free bacteriophage quantitation assay based on the new technology. The presented technology also opens up possibilities for the development of a highly multiplexed homogenous molecular diagnostic platform.

2 LITERATURE REVIEW

2.1 Fluorescent labels in homogenous nucleic acid assays

Homogenous nucleic acid assays most commonly utilize fluorescence-based detection methods. Fluorescence methods are widely used mainly because of the high sensitivity of the fluorometric techniques and also because fluorescent labels are not as hazardous as traditionally used radioisotope labels [23].

Fluorescence is a particular type of luminescence, more precisely a type of photoluminescence (Table 1). In general, it is a physical effect resulting from excitation of a fluorescent molecule by absorption of a photon followed by de-excitation as emission of a photon. The absorption of a photon by a molecule in the ground state leads to the transition of an electron from its original orbital to an unoccupied orbital and thus, to the excitation of the molecule. The excitation is relaxed by emission of a photon at a higher wavelength (lower energy) than that of the absorbed photon. The change in wavelength occurs because part of the energy is lost in the excited state mainly by the vibrational relaxation. [24]

Table 1. Different luminescence types [24].

Phenomenom	***Mode of excitation***
Photoluminescence (fluorescence, phosphorescence, delayed fluorescence)	Absorption of light (photons)
Radioluminescence	Ionizing radiation (X-rays, *α, β, γ*)
Cathodoluminescence	Cathode rays (electron beams)
Electroluminescence	Electric field
Thermoluminescence	Heating after prior storage of energy (e.g. radioactive irradiation)
Chemiluminescence	Chemical process (e.g. oxidation)
Bioluminescence	Biochemical process
Triboluminescence	Frictional and electrostatic forces
Sonoluminescence	Ultrasounds

A good fluorescent reporter molecule possesses several characteristics. High molar absorptivity and high quantum yield are required for efficient fluorescence signal generation. The quantum yield is the ratio of the number of photons emitted as fluorescence to the total number of absorbed photons and molar absorptivity refers to the ability of the molecule to absorb excitation light at a particular wavelength. A large Stokes shift and narrow excitation and emission spectra are beneficial because overlapping excitation and emission spectra leads to self-quenching of the fluorescence when a fluorophore is used at high concentration. Overlapping spectra with a small Stokes shift may hinder fluorescence measurement because the use of excitation and

emission wavelengths too close to each other can lead to direct measurement of the excitation light. In addition, a large Stokes shift is advantageous for reducing autofluorescence measurement that usually have a small Stokes shift. A long excited-state lifetime usually called as long fluorescence lifetime can be advantageous for reducing the assay's background signal level. The background signal can be decreased by measuring the long-lived sample fluorescence after a short lifetime background signal eliciting mainly from the sample containing autofluorescent material and from the plastic reaction vessel has decayed. A high fluorophore stability is also an important feature as, for example, a fluorophore should be resistant to multiple excitation events without degradation to avoid so called photobleaching and thermostability is needed in a particular type of assays where high reaction temperatures are used. [25]

2.1.1 Organic fluorophores

Synthetic organic fluorophores are the most common reporter molecules in homogenous nucleic acid assays, for example, in closed-tube PCR assays. These fluorescent dyes mainly consist of conjugated cyclic structures, i.e., aromatic groups. Various organic molecules containing aromatic groups are fluorescent but only a fraction of them show high fluorescent intensity suitable for diagnostics. Fluorescent properties of aromatic compounds are difficult to predict and traditionally many of the common fluorophores have been empirically discovered rather than intentionally developed. Although nowadays, at least new derivatives of previously presented fluorophores can be produced by design. [26, 27]

Several fluorophores with different excitation and emission spectra are used in molecular diagnostics. Different fluorescent molecules are based on different conjugated basic structures. For example, fluorescein, one of the oldest fluorophore synthesized in 1871 by Adolf von Baeyer, and its derivate the 6-carboxyfluorescein (6-FAM) are based on the xanthene scaffold as well as the rhodamine B and also rhodamine derivates such as the 5-TAMRA and the Texas red. Another widely used group of fluorophores is based on cyanine structure, such as, the Cy3, Cy5 and Sybr Green I. [27-29] Typical organic fluorophore structures are illustrated in Figure 1.

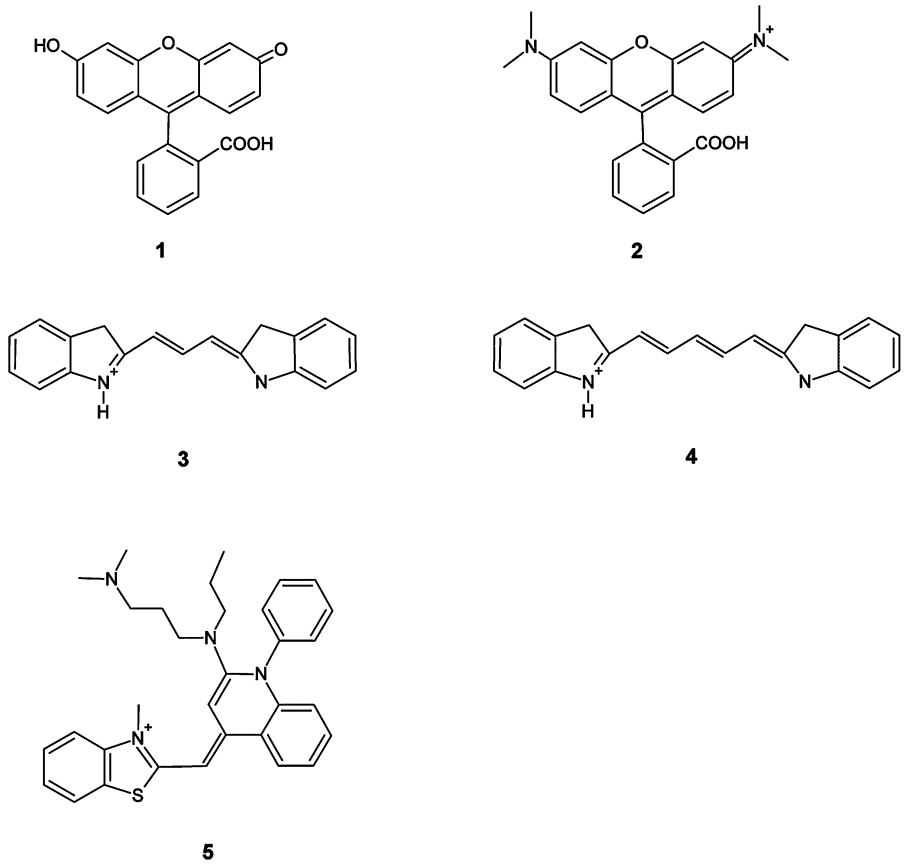

Figure 1. Examples of organic fluorescent molecules used in molecular diagnostics. Molecular structures of **(1)** fluorescein (3',6'-dihydroxyspiro[2-benzofuran-3,9'-xanthene]-1-one); **(2)** rhodamine B ([9-(2-carboxyphenyl)-6-(diethylamino)xanthen-3-ylidene]-diethylazanium); **(3)** Cy3 (2-[(E,3Z)-3-(1,3-dihydroindol-2-ylidene)prop-1-enyl]-3H-indol-1-ium); **(4)** Cy5 (2-[(1E,3E,5Z)-5-(1,3-dihydroindol-2-ylidene)penta-1,3-dienyl]-3H-indol-1-ium); and **(5)** Sybr Green I (N,N-dimethyl-N'-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N'-propylpropane-1,3-diamine).

Absorption of a photon by a fluorophore in the ground state (ground state usually denoted by S_0) leads to the transition of an electron and excitation of the fluorophore to the singlet excited state (S_1). Conjugated structures have delocalized electrons in π-orbitals and the excitation energy mainly promotes π-electron transition to the higher vibrational level usually described as $\pi \rightarrow \pi^*$ transition. Two electrons in the orbital of a molecule in a ground state have opposite spins and excitation to the higher energy state S_1 does not effect the spins. The energy of an electron in the S_1 state is released as emission of a photon (fluorescence) when the electron returns to the S_0 state. [30]

A wide variety of organic fluorophores with different excitation and emission maximum wavelengths suitable for different kind of assay set-ups, for example, multiplexed assays containing several fluorophores in one reaction, are easily

available. Organic fluorophores commonly show strong fluorescence emission with a high quantum yield, even nearly 100% in optimal reaction conditions. [30, 31] Fluorescence lifetimes of organic fluorophores are short, typically, from 1 to 10 ns. Lifetimes are relatively short because fluorophores stay in the excited state only during rapid de-excitation processes like vibrational relaxation of an electron from the higher excited states (e.g. denoted by S_2) to the excited state S_1. [30]

Fluorescence measurements in nucleic acid diagnostics mainly utilize the most common type of fluorescence measurement, the so called steady-state measurement. In steady-state measurement a fluorophore is constantly excited while emission is simultaneously measured and thus, it is a suitable method especially for the measurement of short lifetime fluorescence. This type of measurement can be performed very fast and high fluorescence emission can be detected. However, organic fluorophores usually have a small Stokes shift and thus, the fluorescence detector must be able to precisely detect only the emission signal at a specific wavelength to avoid direct measurement of the excitation light. Organic fluorophores also have wide overlapping excitation and emission spectra and this must be considered when designing multiplexed assays. In addition, autofluorescence from a biological sample material has also nanosecond lifetime and autofluorescence can increase the background signal level and thus, decrease assay sensitivity. [30, 32] Typical emission and excitation spectra of organic fluorescent molecules are shown in Figure 2.

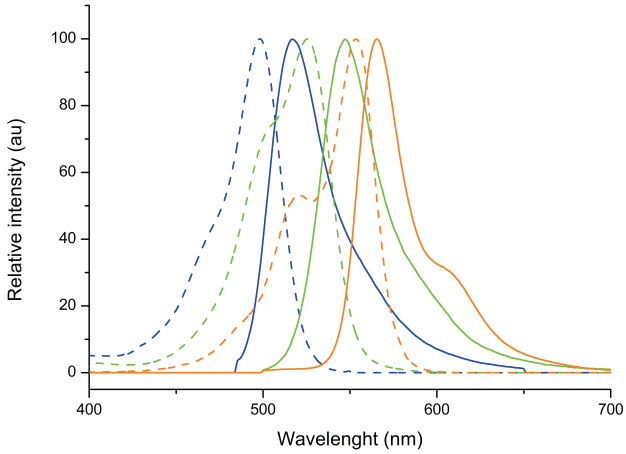

Figure 2. Typical excitation and fluorescence emission spectra of organic fluorophores. Excitation (dashed line) and emission (solid line) spectra of the fluorescein isothiocyanate (blue), rhodamine 6G (green) and cy3 (orange) by Molecular Probes® (Life Technologies Corporation, CA, USA).

2.1.2 Lanthanide chelates

The lanthanides consist of 15 chemical elements belonging to the group of the rare earth metals. Most of the lanthanide ions (Ln^{III}) are luminescent but europium and terbium clearly possess the highest fluorescent intensity and the longest excited-state

lifetime, lasting even up to milliseconds. In addition, also samarium and dysprosium show suitable fluorescent properties (intensity and lifetime) and the chelated Eu^{III}, Tb^{III}, Sm^{III} and Dy^{III} have been used in several fluorescence-based assay applications since the introduction of the lanthanide-based immunoassays by the Finnish researchers in the late seventies. [33, 34]

The fluorescent lanthanide chelates possess unique fluorescent properties such as large Stokes shifts, long fluorescence lifetimes and sharp ion specific emission profiles. The fluorescence properties of the lanthanide elements originate from their exceptional electron configuration that differs from the main group elements. The lanthanides are f-block elements having electrons in the f orbital and the fluorescence occurs mainly from the electron transitions in the 4f orbital, denoted by 4f*→4f transition. The 4f-orbital is shielded by the filled 5s and 5p orbitals resulting in the characteristic narrow emission profiles of the lanthanides. [30, 35-37]

The fluorescent lanthanide ions show unique features valuable for diagnostic assays but because of their low molar absorption coefficients (< 1 M^{-1} cm^{-1}) the f→f* transition by direct excitation is very poor and they require a surrounding organic chromophore structure also called an antenna or a ligand, for the excitation process. In addition, H_2O effectively quenches the fluorescence of the Ln^{III} by the non-radiative relaxation via O–H oscillation of the water molecules coordinated to the lanthanide ion. The organic chromophore chelated to the Ln^{III} absorbs the excitation energy and transfers the energy to the ion, and because the chromophore is coordinated to the Ln^{III} it also efficiently prevents fluorescence quenching caused by the H_2O. [24, 38] Typical excitation and emission spectra of chelated terbium and europium ions [39] are shown in Figure 3.

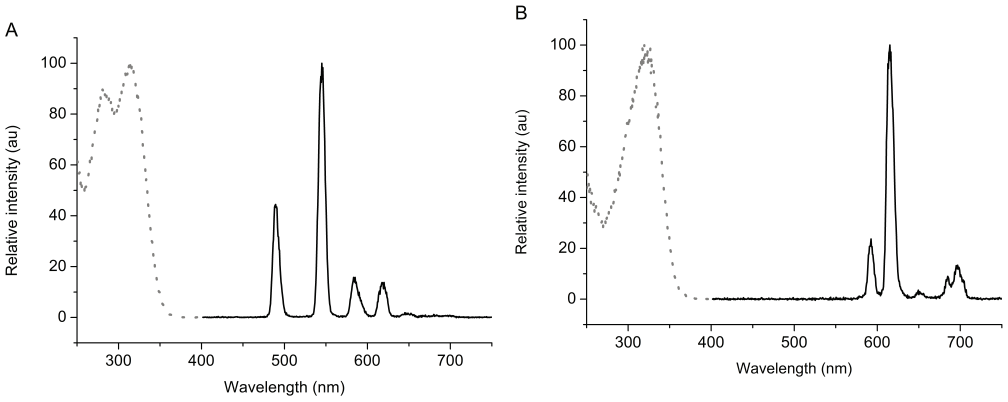

Figure 3. Typical excitation (grey, dashed line) and emission (black) spectra of terbium (A) and europium (B) chelates [39].

The excitation of Ln^{III} by intramolecular energy transfer using an organic chromophore was first reported 1942 by Weissman [40] and since then a large number of different chromophore structures have been synthesized and studied, especially in the past few

decades. Commonly used chromophores usually contains variable numbers of aromatic pyridine groups with substituents such as β-diketones and carboxylic acids [41]. An effective antenna must be able to absorb the excitation energy and effectively transfer the energy to the Ln^{III}. A wide study of the fluorescence properties of 41 different Eu^{III} and Tb^{III} chelates was published in 1997 by Latva *et al.* [42] and recently, a comprehensive review article related to the Ln^{III} chromophores was published by D'Aléo *et al.* [38].

The Ln^{III} excitation process starts with the excitation of the antenna as an electron migrates from the ground state S_0 to the singlet excited state S_1. Then the chromophore undergoes a spin conversion to the triplet state (T_1) in the process called intersystem crossing. Finally, the Ln^{III} is excited by the intramolecular energy transfer where the energy is transferred from the T_1 to the f orbital resonance level of the Ln^{III} ($T_1 \rightarrow f^*$), and the energy can be emitted as lanthanide ion fluorescence ($f^* \rightarrow f$). For successful excitation of the Ln^{III} the chromophore should fill several requirements. The antenna should show minimal radiative and nonradiative transitions in the S_1 and T_1 states and the Ln^{III} antenna complex should not elicit an energy back transfer from excited Ln^{III} to the antenna. [30, 42, 43] One of the key factors is also the triplet state energy level of the antenna because different lanthanide ions have different lowest excited levels and generally it is stated that the highest quantum yield is achieved when energy is transferred from the antenna´s lowest triplet state level to the lowest excited level of the Ln^{III}. However, there are also results showing that when the triplet state level is too close to the lowest excited level of the Ln^{III} the fluorescence is decreased and in fact, more powerful fluorescence is achieved when a chromophore with a slightly higher triplet state energy level is used. [42] For example, the main emission level of the Eu^{III} , i.e., its lowest excited state, is $^5D_0$ but, as described by Latva *et al.* [42], a higher intensity Eu^{III} fluorescence is achieved when an antenna with the triplet state energy level higher than the Eu^{III} resonance level $^5D_1$ or even higher than $^5D_2$ is used. It is also shown that different type of antenna structures give out the largest Eu^{III} quantum yield on different energy levels, for example, for β-diketones this occurs close to $^5D_1$ level and for polyaminocarboxylates near $^5D_2$ level [34].

The Ln^{III} excitation through the chromophore's triplet state ($S_1 \rightarrow T_1 \rightarrow Ln^{III}$) was shown in the early 1960s by Crosby *et al.* [44] and it is a generally and widely accepted theory. However, also other Ln^{III} excitation pathways have been presented and thus, in addition to the antennas triplet state energy level there most likely are also other important factors in the excitation process. One alternative excitation route, a direct energy transfer from S_1 to the Ln^{III}, was first described as early as 1969 by Kleinerman [45] but most of the practical studies have been focused on the triplet state excitation process. However, the singlet excited state pathway has recently raised interest and, for example, Yang *et al.* [46] have discovered a Eu^{III} chelate complex utilizing singlet state excitation route. The antennas using singlet state excitation route can be excited with a light emitting diode (LED)-based light source using wavelengths over 365 nm, whereas Ln^{III} excitation through the triplet state pathway usually requires UV-excitation by xenon or laser light sources. The LED excitation would allow the usage of simpler, smaller and cheaper instruments.

The Ln^{III} excitation through triplet state using higher wavelengths, therefore requiring lower energy, is mentioned to be limited to about 346 nm for Tb^{III} and 385 nm for Eu^{III} by the main fluorescence emission levels of the Tb^{III} ($^5D_4$ 20400 cm^{-1}) and Eu^{III} ($^5D_0$ 17500 cm^{-1}) [47]. The main emission levels set the limit because, for example, the stated theoretical maximum Eu^{III} excitation wavelength of 385 nm is equal to 25974 cm^{-1} and it is assumed that the energy cap between the antenna's singlet and triplet state is at least 5000 cm^{-1} and that the triplet state energy must be at least 3500^{-1} cm higher than the Ln^{III} main emission level [47].

In conclusion, the Ln^{III} excitation pathways are not yet completely understood and thus, the design of efficient Ln^{III} chromophores still requires plenty of experimental work. On the other hand, in the future we can hopefully design new lanthanide chelates with even better fluorescent properties. Excitation energy transfers through the antenna's triplet state and by the direct excitation from S_1 to Ln^{III} are illustrated in Figure 4.

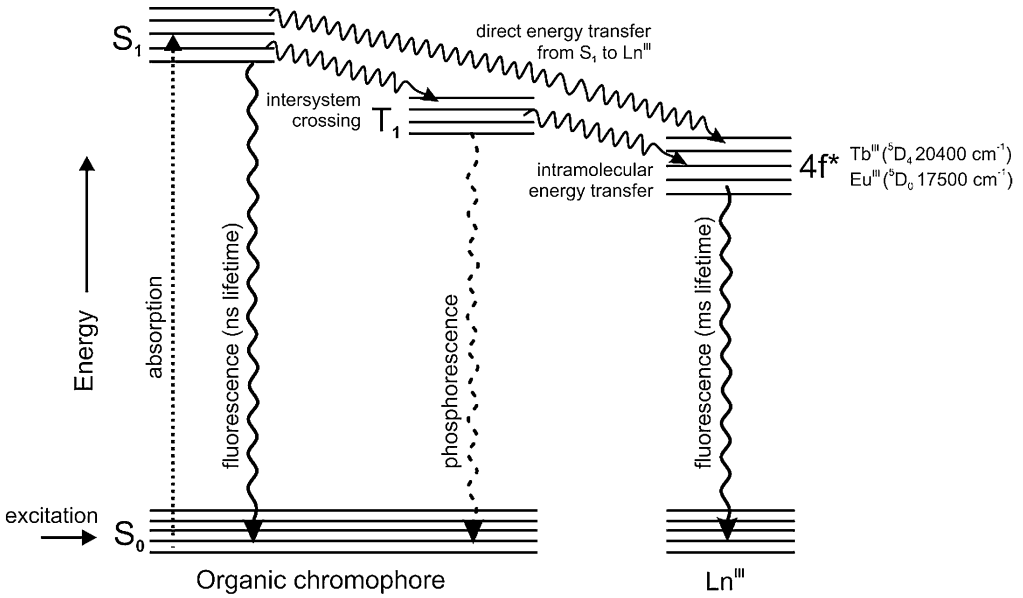

Figure 4. Jablonski diagram illustrating two possible Ln^{III} excitation route: intramolecular energy transfer through the chromophore's triplet state (T_1) and direct energy transfer from the chromophore's singlet state (S_1) to the Ln^{III}. Ln^{III} excited state denoted by 4f* and also Tb^{III} and Eu^{III} main emission levels $^5D_4$ and $^5D_0$ are showed, respectively.

Despite of the unique fluorescence properties, lanthanide chelates have only rarely been used as fluorescent reporters in homogenous nucleic acid assays, such as, closed-tube PCR. One of the main obstacles is that there is no commercially available PCR thermal cycler containing suitable measurement instrument for lanthanides which would be compatible with standard laboratory reaction vessels. However, in the past decade lanthanide chelate-based homogenous PCR assays have been presented, for

example, for the detection of human pathogens [48, 49], quantification of prostate specific mRNA levels in prostate cancer diagnostics [50, 51], and for the detection of single-nucleotide-polymorphism related to genetic disorders [52-54]. In addition, it has been shown that long lifetime lanthanide fluorescence with time-resolved measurement can be advantageous in homogenous PCR assay to the analysis of biological samples containing autofluorescent residues [48, 52].

Assays performed in high temperatures, such as part of the PCR steps, require a stable lanthanide chelate structure. The lanthanide ion can form nine coordination bonds [55] with the nitrogen and oxygen atoms [56]. Lanthanide chelates with maximum number of coordination bonds form stable structures able to keep Ln^{III} chelated even in high temperatures. In addition, Ln^{III} coordination to an organic chelate effectively prevents H_2O coordination to the Ln^{III} and thus, prevents the H_2O quenching effect. Homogenous PCR assays have been reported to base on intrinsically fluorescent nonadentate Eu^{III} or Tb^{III} chelates that elicit quantum yields from 13% to 18% [6, 48, 49, 51, 57]. The structures of the lanthanide chelates used in PCR are illustrated in Figure 5.

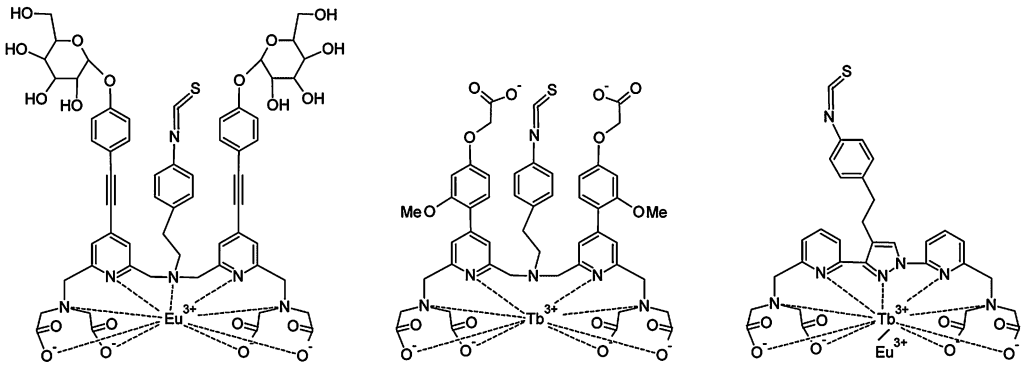

Figure 5. Molecular structures of the intrinsically fluorescent lanthanide chelates used in homogenous PCR. References and systematic names for structure **(1)** {2,2',2",2"'-{ [2-(4-isothiocyanatophenyl)-ethylimino]bis(methylene)bis{4-{[4-(α-galactopyranoxy) phenyl]ethynyl} pyridine-6,2-diyl}bis(methylenenitrilo)}tetrakis(acetato)}europium(III) [58]; **(2)** {2,2',2",2"'-{[2-(4-isothiocyanatophenyl)-ethylimino]bis(methylene)bis{4-[2-methoxy-4-(carboxymethoxy) phenyl]pyridine-6,2-diyl}bis(methylenenitrilo)}tetra kis(acetato)}terbium(III) [59]; and **(3)** {2,2',2",2"'-{{6,6'-{4"-[2-(4-isothiocyanatophenyl)ethyl]pyrazole-1",3"-diyl}bis(pyridine)-2,2'-diyl}bis (methylenenitrilo)}tetrakis (acetato)}terbium/europium(III) [60, 61].

Furthermore, homogenous nucleic acid detection based on sub-micrometer-sized upconverting phosphor (UCP) particles consisting of ytterbium and erbium lanthanides among sodium yttrium tetrafluoride has been introduced [62]. The luminescent properties of the UCPs could be advantageous in nucleic acid diagnostics but nucleic acid amplification assays based on UCPs have not been presented and thus, neither the upconverting phosphor particles nor another fascinating particulate dye, the europium chelate-doped nanoparticles, are introduced in more detailed.

2.1.2.1 Time-resolved measurement

Lanthanide chelates elicit long lifetime fluorescence and thus, time-resolution can be advantageous in fluorescence signal measurement. In time-resolved measurement, pulsed excitation is followed by a delay before the signal counting. During the delay the signal is not measured and autofluorescence, for example, from the plastic reaction vessel and biological sample is decayed. [63] In a typical Eu^{III} time-resolved measurement a delay of 400 µs is used after the excitation and the fluorescence is counted from 400 to 800 µs. This cycle is repeated 1000 times during a total measurement time of approximately 1 s. A fluorometer for time-resolved measurement usually contains conventional fluorometer components and additionally, a measurement unit capable of time-gated measurement. The principle of the time-resolved measurement is illustrated in Figure 6.

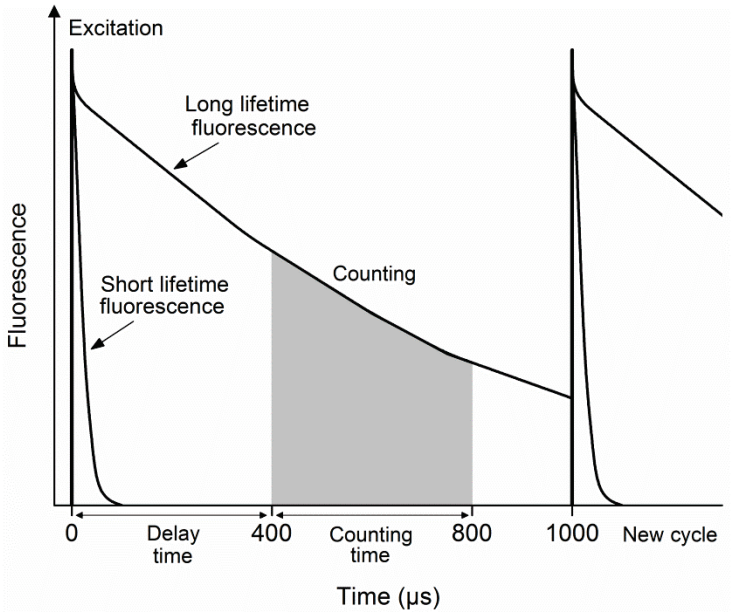

Figure 6. Schematic presentation of fluorescence measurement in time-resolved mode. Modified from the reference [63].

2.2 Nucleic acid amplification methods and sample preparation

Nucleic acid diagnostics are based on the analysis of the nucleic acid, either DNA or RNA. Most of the assays include a nucleic acid amplification step because, for example, human, food and environmental samples rarely contains enough nucleic acids for direct measurement with commonly used techniques. *In vitro* amplification methods used in the molecular diagnostics typically share two basic features: amplification is initiated by the hybridization of a target-specific oligonucleotide called primer, and the target nucleic acid is enzymatically amplified. The amplification of the

target nucleic acid enables the detection of even fewer than 10 target copies in one reaction making the assays highly sensitive [64, 65].

Several amplification techniques have been developed in the past two or three decades. The polymerase chain reaction was introduced in 1985 [66, 67] and it is still the most commonly used nucleic acid amplification technique. PCR is very efficient amplification method resulting in exponential target DNA replication. The principle of the PCR is illustrated in Figure 7. PCR is a widely used method but it requires temperature cycling typically from 50–60 °C to almost up to 100 °C by a specific instrument called PCR thermocycler. Lately, there has been increasing interest for assays based on isothermal nucleic acid amplification. There are several isothermal amplification methods and all of these techniques are performed in a constant temperature, although, a part of these methods requires an initial denaturation step at a high temperature. Isothermal amplification reactions are simpler and cheaper to perform than PCR, and therefore, more suitable especially for point-of-care and lab-on-a-chip type diagnostics. Furthermore, at least three methods (LAMP [68], NASBA [69] and SDA [70]) have shown DNA amplification performance comparable to PCR. [65] The main isothermal amplification methods are introduced in Table 2.

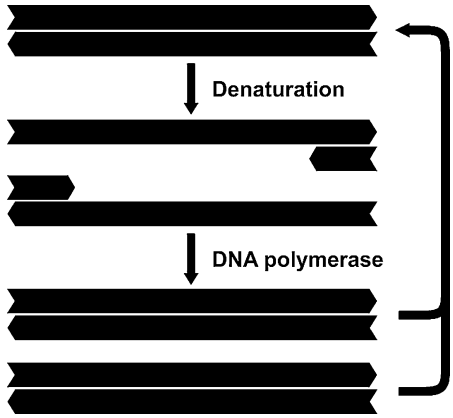

Figure 7. Principle of the polymerase chain reaction (PCR). The target DNA (long thick arrows) is denatured at high temperature and the oligonucleotide primers (short thick arrows) are hybridized to the two target strands when the temperature is decreased (annealing step). DNA polymerase synthesizes new strands by incorporating nucleotides to the primers according to the template strand sequence (extension step). In each amplification cycle the number of target DNA sequences is doubled. The arrowheads indicate the 3' end of the nucleic acids. The flow of the PCR is indicated by the thinner arrows. Modified from the reference [64].

Table 2. Isothermal nucleic acid amplification methods.

Method	Overview	Enzymes	Number of primers / probes	Reaction temperature (°C)	Amplif. power	Amplif. product	Reference
Helicase dependent amplification (HAD)	A helicase enzyme separates the target DNA strands and a single-strand binding protein prevents re-association. DNA polymerase amplifies the target DNA by elongating hybridized oligonucleotide primers.	helicase, ssDNA binding protein, DNA polymerase	2	37–65	+	dsDNA	Vincent 2004 et al., [71] An 2005 [72] Goldmeyer 2007 [73] Jeong 2009 [74]
Loop mediated isothermal amplification (LAMP)	Three pairs of primers and a DNA polymerase with strand displacement activity produce a stem-loop single-stranded DNA (ssDNA) structure. The stem-loop strand functions as a new template and replication continues leading to stem-loop DNA product accumulation.	DNA polymerase	4–6	60–65	+++	Stem-looped DNA	Notomi 2000 [68] Nagamine 2002 [75] Chang 2012 [65]
Rolling circle amplification (RCA) of the padlock probe	Both ends of the padlock probe hybridize to consecutive sequences in the target DNA forming a circle that is closed by ligation. DNA polymerase amplifies the circular padlock probe by elongating a oligonucleotide primer.	DNA ligase, DNA polymerase	2	37	+	ssDNA	1994 Nilsson [76] 1995 Fire [77] 1998 Baner [78]
Strand displacement amplification (SDA)	Two pairs of primers and a DNA polymerase with strand displacement activity produce a DNA product that contains a restriction enzyme recognition site. The restriction endonuclease nicks the DNA and the polymerase extends the 3' end by displacing the downstream strand.	restriction enzyme, DNA polymerase	4	37	++	dsDNA	Walker 1992 [70] Little 1999 [79]
Nucleic acid sequence-based amplification (NASBA)	First, the target sequence (usually ssRNA) is amplified using reverse transcriptase then RNase H and RNA polymerase produce a single-stranded RNA in a cyclic reaction.	reverse transcriptase, RNase H, RNA polymerase	2	37–42	++	mainly ssRNA	Kievits 1991 [69] Compton 1991 [80]

In addition to the isothermal nucleic acid amplification methods replicating the target sequence, there are also isothermal amplification methods that are called signal amplification methods. These methods such as, the branched DNA [81] , beacon assisted detection amplification [82], and Q-beta replicase [83] are generally based on probe amplification initiated by the target nucleic acid.

Enzymatic amplification of the nucleic acids is a relatively sensitive process and can be interfered with the impurities in the analyzed sample. Different type of samples such as, body fluids, foodstuffs and environmental samples contain amplification inhibitors. For example, hemoglobin and urea in blood and urine, respectively, and many organic and phenolic compounds in food samples can inhibit the amplification reaction. Many inhibitors have been indentified but the inhibition mechanisms have not been widely studied and they have mainly remained unclear. To remove the inhibitors sample pretreatment methods are used before the amplification. [84] Although, sample preparation before the amplification is an essential part of the nucleic acid assays, it is only slightly reviewed in this thesis.

Nucleic acid assay sample preparation is commonly based on nucleic acid extraction containing a variable number of different steps such as target (e.g., bacteria or virus) concentration/separation, nucleic acid liberation, and nucleic acid purification steps. Target concentration is needed in assay applications where the sample might contain only few target particles. For example, body fluids, such as blood, rarely contain high concentrations of infectious microbes. It must also be considered that the sample might contain other components that might inhibit the amplification, e.g., red blood cells in blood samples, and these should not be concentrated along with the target particles. Concentration can be performed, for example, by centrifugation, filtration or using antibody-based bacteria capture [48, 85-87]. In some applications bacterial culture prior to the PCR analysis is used [49].

Nucleic acids need to be released from the target organism before the amplification and the simplest method is to lyse the target cells in the PCR by the high temperature of the initial PCR denaturation step. [48, 88] Unfortunately, the heat treatment disrupts only some of the gram-negative bacteria and mammalian cells but the high temperature does not lyse effectively, for example, most of the gram-positive bacteria and yeast cells. Therefore, the disruption of the cells has been achieved by different mechanisms such as mechanical lysis using glass or metal beads together with vortexing, chemical lysis by NaOH, sonication and enzymatic lysis. [89, 90]

After the disruption of the cells the liberated nucleic acids are commonly purified because lysed bacteria residues might interfere with some of the homogenous fluorescence-based detection methods and also amplification. Purification is usually performed using solid phase extraction techniques. The main method is based on silica solid phase that effectively binds nucleic acids in chaotropic salt solution. While bound to the solid phase the nucleic acids can be washed effectively and then eluted in aqueous buffer. [91] Another solid phase method is based on capture probes that specifically hybridize to the target nucleic acid. Nucleic acids can be removed from the

capture probes by denaturing the hybrid in high temperatures, or the nucleic acids can be amplified directly on the solid phase. [92]

Sample preparation commonly contains multiple steps and therefore, it is time-consuming to perform requiring experienced personnel. Automation decreases hands-on-time but current automated systems are usually complex, relatively expensive, and not suitable for miniaturized and rapid near-patient diagnostics. One alternative for complex sample preparation is to create more robust nucleic acid assays that require only minimal sample preparation. The robustness of the assay can be increased, for example, by using DNA polymerase that tolerates inhibitors allowing the use of samples without DNA purification [93]. Additionally, lanthanide chelates and time-resolved fluorescence measurement (TRF) have been used in a PCR assay with simple sample preparation to avoid the measurement of autofluorescence [48, 52].

2.3 Fluorescence-based homogenous nucleic acid detection methods

Different organisms can be identified using nucleic acid assays detecting organism specific genetic material either DNA or RNA. Most of the assays are based on nucleic acid amplification and simultaneous i.e. real-time or post-amplification detection. Nucleic acid detection technologies can be divided, for instance, to heterogenous and homogenous, i.e., closed-tube methods.

In heterogenous assays the target nucleic acids are first amplified. Then the amplified nucleic acids are commonly captured to the solid-phase and detected, for instance, by using fluorescent oligonucleotide probes. Solid-phase capturing allows highly multiplexed detection because different sequences can be detected in one reaction chamber based on spatial differentiation. Assays based on heterogenous detection are widely used but they require post-amplification sample handling that increases assay time, complexity and especially the risk of amplification product cross-contamination. Furthermore, the heterogenous methods are not suitable for quantification because the amount of extensively amplified nucleic acids is not directly proportional to the initial amount.

Assays based on homogenous detection methods are more difficult for multiplexing but, in general, they are easier to use and faster compared to heterogenous methods, and they have low contamination risk. [4, 94] Homogenous methods allow target nucleic acid quantification by measuring the signal in real-time during the amplification, for example, in PCR after every amplification cycle. In real-time quantitative PCR (qPCR) target amount quantification is generally performed by determining the threshold cycle (C_t), also called the quantification cycle (C_q) [95] and calculating the target concentration using a known standard. The threshold cycle is the PCR amplification cycle where the signal exceeds the background signal level, more precisely a threshold signal level that is a statistically significant point above the background level. [95, 96] Homogenous methods can also be used for qualitative measurement; for example, a simple

qualitative so called end-point PCR can be performed by measuring the signal only after the PCR amplification [97, 98].

Several different homogenous nucleic acid detection methods suitable for qualitative and quantitative measurements have been developed during the past two decades. The term fluorophore in the method descriptions below refers to the common organic fluorophores if not otherwise mentioned.

2.3.1 Non-sequence-specific detection methods

DNA intercalative dyes

Some of the organic fluorophores are environmentally sensitive and their fluorescence can be decreased, for example, by an aqueous solvent. Intercalation to the DNA changes the fluorophore's microenvironment leading to an increased fluorescence signal of certain fluorophores. [24, 99] The first method for homogenous detection of amplified nucleic acid was based on DNA intercalative ethidium bromide (EtBr) and published in 1992 [100]. The ethidium bromide shows high DNA binding affinity and when intercalated to the double-stranded DNA (dsDNA) the fluorescence of the EtBr is increased [101]. The fluorescence intensity of the EtBr is proportional to the amount of DNA and therefore, EtBr is suitable for real-time quantitative measurement of the amplified nucleic acids. Later on, the EtBr was replaced with the less PCR-inhibitory and less carcinogenic Sybr Green I that has since become a popular dye in the PCR diagnostics. [102] Intercalative dyes can be used together with any amplification method that produces double-stranded nucleic acids. They are not sequence specific and thus, false amplification products, e.g., primer dimers increase fluorescence signal and can lead to false positive results. However, specificity of the intercalative-dye-based assays can be increased by using a melting curve analysis where different sized amplification products are differentiated. [103]

Multiplexed detection in one reaction solution is not possible using intercalative dyes with different emission spectra, but multiplexed assays based on intercalative dyes have been sucesful when using a melting curve analysis [103, 104]. An automated platform for the detection of 21 respiratory pathogens has recently been published and also FDA-approved (510(k) number, K123620); it is based on homogenous PCR and DNA intercalative dye and also contains integrated sample preparation. The system is based on nested two-step PCR where several different primers are used. The second amplification step is performed in separated reaction chambers where different targets are amplified in specific reaction vessels and after the amplification, detection is performed using melting temperature analysis. [105] The multiplexed homogenous respiratory pathogen assay represents the common trend but also brings out the common problem of the multiplexed assays showing relatively high limit of detection of part of the target microbes.

DNA-assisted excimer formation

The DNA-assisted excimer formation is based on cyclophane interaction with DNA leading to excimer formation. The excimer is a dimer with associated excited

electronic state and the excimer formation can be observed as an emission shift to longer wavelengths compared to the monomer emission. Neelakandan and Ramaih [106] have introduced a cyclophane derivative that does not interact with proteins but binds to the DNA by the weak interaction, e.g., hydrophobic and electrostatic interactions leading to excimer formation and emission maximum wavelength shift from 430 nm to 570 nm and a relatively long fluorescence lifetime of approximately 143 ns. [30, 106, 107]

Bioluminescence-based techniques

Bioluminescence is a form of chemiluminescence where a chemical reaction produces light and thus, it is not fluorescence. However, a recently presented innovative homogenous bioluminescence-based nucleic acid detection method is introduced here. Bioluminescent Assay in Real-Time (BART) is based on isothermal LAMP amplification, ATP sulfurylase and thermostable firefly luciferase enzymes. During the nucleic acid amplification inorganic pyrophosphate (PP_i) is produced and the PP_i is converted to adenosine triphosphate (ATP) by the ATP sulfurylase. The luciferase enzyme produces light in a reaction where the ATP is needed for the oxidation of the luciferin substrate. The BART assay should be a suitable method for low-cost nucleic acid amplification assays because only a simple instrument containing a light detector and producing a constant temperature is needed. [108]

2.3.2 Sequence-specific detection methods

Sequence-specific detection methods are generally based on the fluorescence signal modulation by the oligonucleotide probe hybridization to the target sequence. Nucleic acid amplification assays utilizing sequence-specific methods are highly specific because they are dependent on both the primer(s) and the probe(s) hybridization to the target sequence. Methods based on sequence-specific oligonucleotide probes allow multiplexing, for example, by using probes labeled with different fluorophores [109].

2.3.2.1 FRET-based hybridization methods

The mainly used homogenous sequence-specific detection methods are based on Förster resonance energy transfer (FRET) [110]. The FRET is a nonradiative energy transfer process of the excited-state energy from an excited fluorescent donor molecule to an acceptor molecule leading to the excitation of the acceptor. The energy is transferred through long range dipole-dipole interactions between the donor and acceptor and does not involve emission and reabsorption of a photon. The excited donor is relaxed to the ground state and the acceptors excitation can be observed as fluorescence if the acceptor is a fluorescent molecule. The FRET requires donor and acceptor with similar resonance frequencies, and in practice this means that the donor's emission and acceptor's absorption spectra must overlap. Another requirement is the short distance, from 1 to 10 nm, between the donor and acceptor. [24, 30] In homogenous nucleic acid assays fluorescent and non-fluorescent acceptors have been used. Several sequence specific FRET-based methods are listed in Table 3, and they are described below in more detail.

Table 3. Homogenous sequence specific FRET-based nucleic acid detection methods.

Method	Principle
Hydrolysis probe [111, 112]	5´ nuclease technology
Snake [113]	
Attached universal template probe [114]	
Universal hydrolysis probe [115]	
Universal AEGIS hydrolysis probe [116]	
Molecular beacon [117]	Molecular beacon hairpin method
Amplifluor [118]	
Scorpion [119, 120]	
UniPrimer [121]	
Universal Molecular Beacon (U-MB) [122]	
Mediator probe [123]	Other
FRET probes [102]	
Competitive hybridization [124]	
Competitive hybridization and Hydrolysis probe [57]	
QZyme [125]	
NuPCR,[126]	
MagiProbe [127]	
Invader[128]	
Lion probe [129]	
Single-quantum-dot-based DNA nanosensor [130]	

Hydrolysis probe

The Hydrolysis probe technology was introduced in 1991 and it was the first sequence specific detection method used in homogenous nucleic acid amplification assay and it is also known as the 5´ Nuclease technology and as the TaqMan technology. One oligonucleotide probe is dual labeled with a fluorophore in one end and a quencher in the other. The fluorescence of the intact probe is quenched because the fluorophore and quencher are in close proximity. During the PCR amplification the probe is hybridized to the target sequence and degraded by the *Thermus aquaticus* (Taq) DNA polymerase 5´ nuclease activity while the new strand is amplified. When the probe is degraded the distance between the fluorophore and quencher increases releasing the quenching and increasing the signal. [111, 112, 131] (Figure 8)

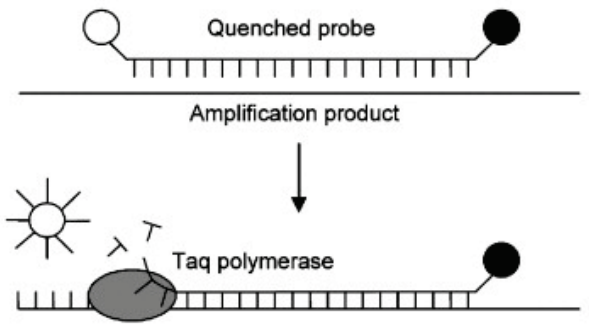

Figure 8. Principle of the Hydrolysis probe technology. The hybridized dual end-labeled Hydrolysis probe is degraded by the DNA polymerase enzyme (grey oval) during the PCR amplification. Increase in fluorescence signal is observed when the distance between the fluorophore (white circle) and quencher (black circle) increases and hence, the quenching efficiency decreases. Modified from the reference [132].

The Hydrolysis probe technology is the most commonly used homogenous nucleic acid detection method, and several modified applications based on the DNA polymerase 5´ nuclease activity have been developed. A Snake method was introduced in 2010 and showed to improve the dual labeled probe degradation and therefore, the fluorescence signal generation [113]. In the Snake system a so called snake primer contains a 5´ overhang sequence (called flap sequence) that does not hybridize to the target sequence but is incorporated into the PCR product when the Snake primer is elongated. Consequently, the complementary strand contains the flap sequence at the 3´ end. After denaturation of the amplified product the 3´ end of the amplified strand self-hybridizes forming a hairpin structure and one unhybridized nucleotide mismatch at the 3´ end. A dual labeled Hydrolysis probe hybridizes next to the self-hybridized sequence. This structure is an optimal substrate for 5´ nuclease activity of the Taq polymerase and results in a more effective 5´ nuclease cleavage of the probe than the original Hydrolysis probe technology.

Preparation of target-specific probes involves relatively laborious and costly research and development work, especially when preparing assays for several different targets. To simplify assay development 5´ nuclease activity based sequence-specific, so-called universal detection methods have been introduced. The universal methods enable usage of a single probe for the detection of several different targets in different reactions. Several methods utilize a primer with a 5´ overhang sequence that incorporates a universal Hydrolysis probe detection sequence into the amplicon. During the PCR amplification a universal dual labeled probe hybridizes to the incorporated sequence and is degraded by the DNA polymerase 5´ nuclease activity [114-116]. In addition, to enhance the 5´ nuclease activity Moser *et al.* [116] incorporated an adjacent isoguanosine (iG) and isocytidine (iC) into the 5´ overhang sequence. The artificial nucleotides are not correctly amplified by the DNA polymerase. Instead, misincorporation occurs opposite to the iG and the amplification is terminated to the adjacent iC resulting in an unpaired 3´ single base flap and, because of the termination also a single-stranded 5´

overhang in the opposite strand. A dual labeled universal probe hybridizes to the overhang next to the 3´ single base flap creating a double-flap gap substrate for the flap endonuclease activity that is mentioned to be part of the DNA polymerase 5´ nuclease [133]. The flap endonuclease cleaves off the fluorophore labeled 5´ flap nucleotide of the probe. Universal detection systems enable the use of the same detection probe in different assays because the detection sequence for the probe is added to the amplicon by the primer elongation and therefore, the target sequence can easily be incorporated into different primers. Unfortunately, universal primer-based systems are prone to false positive results if unspecific primer amplification occurs.

Molecular beacon

The dual-labeled Molecular beacon probe is similar to the hydrolysis probe but it forms a hairpin structure because of short complementary sequences at both ends of the probe. The hybridization of the complementary sequences forces the probe into stem-loop structure and the fluorophore and quencher very close to each other and, therefore, the molecular beacon probes are generally more efficiently quenched than the Hydrolysis probes. The Molecular beacon probes can be used in PCR as Hydrolysis probes and be degraded by the 5´ nuclease activity of the DNA polymerase, but they can also be used without degradation because the hybridization of the probe to the target sequence leads to the linearization of the probe and therefore, increased distance between the fluorophore and quencher leading to increased fluorescence signal. Thus, the Molecular beacon method can be used together with DNA polymerases lacking the 5´ nuclease activity and also for direct single-stranded nucleic acid detection. [117] (Figure 9)

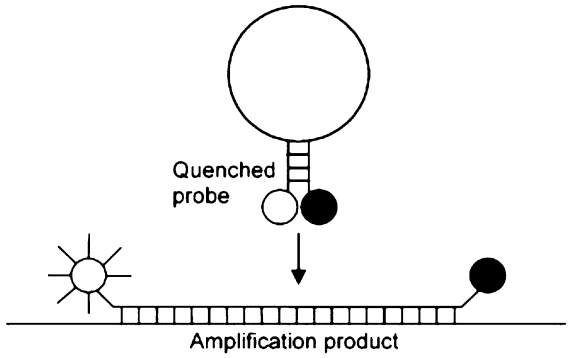

Figure 9. Principle of the Molecular beacon method. The stem-loop structure is linearized by the hybridization of the probe into the target sequence leading to an increased distance between the fluorophore (white circle) and quencher (black circle). The decreased quenching is observed in fluorescence signal increase. Modified from the reference [132].

Numerous different applications utilizing the Molecular beacon hairpin structure have been introduced since introduction of the original method in 1996. The applications are mainly based on a primer containing the Molecular beacon probe at the primer's 5´ end as an overhang tail. The integration of the primer and probe simplifies the assay

because a separate probe oligonucleotide is not needed. The Amplifluor method [118] was the first system joining the Molecular beacon probe and primer. In that system the fluorophore is quenched because the hairpin structure brings the quencher and fluorophore in close proximity just as in common Molecular beacon probe. When a DNA polymerase copies the 5′ tail overhang sequence incorporated to the amplicon the hairpin structure is linearized and the quenching is released. Another method, the Scorpion primer [119, 120] is a similar primer probe fusion as the Amplifluor but it has also a nonamplifiable monomer, for example, hexaethylene glycol (HEG) in the junction of the primer and probe that blocks the DNA polymerase amplification of the primers 5′ tail containing the probe sequence with the fluorophore and quencher. In addition, the scorpion primers 5′ tail loop has a target-recognizing sequence. During PCR amplification the scorpion primer initiates the amplification and is incorporated to the synthesized strand. The following denaturation and annealing steps lead to the self-hybridization of the probe sequence to the target sequence at the 5′ tail into the same strand and therefore, to an increased fluorescence signal by the released quenching.

Molecular beacon-based universal probe systems have also been developed. The UniPrimer system [121] is similar to the Amplifluor method but it involves the usage of additional primer containing a 5′ overhang recognition sequence for the primer probe fusion. The primer with a 5′ recognition site first initiates the target amplification and is incorporated to the amplified target. Then the UniPrimer hybridizes to the amplified strand and is incorporated to the amplicon and the UniPrimer functions as the Amplifluor primer (described in the previous chapter). In another universal system introduced by Li *et al*. [122] a common Molecular beacon probe hybridizes to the primers 5′ overhang sequence and is therefore linearized and unquenched. During the PCR the primers are incorporated to the amplified target and the hybridized molecular beacon probes are displaced by the DNA polymerase enzyme lacking the 5′ nuclease activity. The unhybridized probes form a hairpin structure and are quenched and therefore, a fluorescence signal decrease instead of an increase in the presence of the target is observed.

Universal detection systems may simplify assay development but specificity of the introduced systems is based only on the primers; they are, therefore, prone to false positive results due to unspecific amplification initiated by the primers. Recently, Faltin *et al*. [123] introduced a universal Mediator probe PCR that avoids false signal generation originated from unspecific amplification. The Mediator probe is an oligonucleotide probe that hybridizes to the target sequence between two PCR primers. The probe contains a 5′ overhang that is cut apart from the target recognizing 3′ end by the 5′ nuclease activity of the DNA polymerase during the PCR amplification. The released 5′ tail is called the Mediator and it hybridizes to the universal reporter probe in the reaction solution. The universal reporter probe is a Molecular beacon probe with 3′ overhang sequence complementary to the Mediator and the intact probe is in a hairpin structure and quenched. The Mediator hybridization to the 3′ tail of the reporter creates a free 3′ primer end for the DNA polymerase and the polymerase then amplifies the universal probe sequence. During the amplification the DNA polymerase also degrades the probe's hairpin loop from the 5′ end and/or displaces the hairpin structure leading to released quenching.

Adjacent hybridization probes

The Adjacent hybridization probes, also called the FRET probes, consist of two oligonucleotide probes that hybridize one after another to the target sequence. Both probes are commonly end-labeled (one probe at the 5´ and the other at the 3´ end) with fluorophores in a way that the hybridization of the probes brings the fluorophores in close proximity. The FRET between the fluorophores occurs and the emission of the acceptor can be measured when the probes are hybridized. The Adjacent hybridization probes can be used for the measurement of the amplified (e.g., by PCR) DNA and also for the melting curve analysis. [102] (Figure 10)

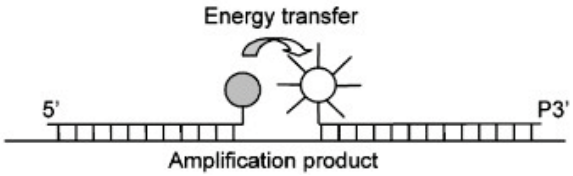

Figure 10. Principle of the Adjacent hybridization probe technology. The hybridization of the probes into adjacent positions brings the donor (gray circle) and acceptor (white circle) fluorophores in close proximity. The acceptor's fluorescence signal increases when the energy transfer occurs. Modified from the reference [132].

Competitive hybridization

The Competitive hybridization method involves the use of two complementary oligonucleotide probes. One probe carries a fluorophore, for example, at the 3´ end and the other a quencher at the 5´ end. In the absence of a target the probes are hybridized and the fluorescence is quenched. In the presence of the target sequence, for example, a PCR amplification product the fluorescent probe hybridizes to the target resulting in an increased fluorescent signal. [124, 134] (Figure 11)

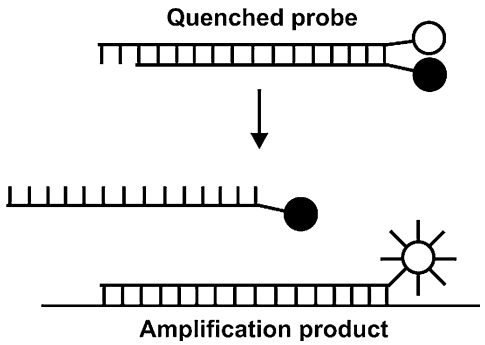

Figure 11. Principle of the Competitive hybridization method. Increased fluorescence signal is observed when the fluorophore (white circle) labeled probe hybridizes to the target sequence leading to increased distance between the fluorophore and quencher (black circle).

Fluorescent lanthanide chelate-labeled probes have been used in homogenous PCR assays based on the Competitive hybridization method and the fluorescent probe hybridization into the target sequence has been increased by using a shorter or only partially complementary quencher probe that decreases the hybridization efficiency of the quencher probe towards the fluorescent probe [48, 135]. Asymmetric PCR has been used to produce a ssDNA target product, that enables more effective fluorescent probe hybridization to the target strand [53]. Another system utilizing fluorescent lanthanide probe and the competitive hybridization method has combined also the 5´ nuclease activity of the DNA polymerase to degrade the fluorescent probe hybridized to the target. The probe degradation leads to the accumulation of the released fluorophore to the reaction solution and, therefore, this system can provide a more efficient fluorescent signal increase [57].

Other methods

The Lion probe method is similar to the Hydrolysis probe technology but it utilizes DNA polymerase 3´ exonuclease activity. A dual end-labeled probe (fluorophore and quencher) contains a mismatch nucleotide at the 3´ end. When the probe is hybridized to the target strand the unpaired labeled 3´ end nucleotide is cut off by DNA polymerase enzyme 3´ nuclease activity. [129]

Another system called QZyme is based on a primer carrying an inactive DNAzyme sequence as a 5´ overhang and a dual end-labeled (fluorophore and quencher) oligonucleotide probe containing a QZyme substrate. During PCR, the primer's 5´ overhang sequence is incorporated to the amplicon and the complementary sequence is a catalytically active DNAzyme. The dual labeled substrate probe hybridizes to the active DNAzyme sequence and is cleaved at the substrate site. [125]

The NuPCR is similar to the Qzyme but it is based on two DNA Partzyme oligonucleotides and a universal dual end-labeled oligonucleotide substrate probe. The DNA Partzyme oligonucleotides hybridize to adjacent positions into the nucleic acid target, for example, to the PCR amplified target sequence, and form a NuZyme nucleic acid enzyme that recognizes the substrate probe and cleaves the probe releasing the fluorophore and quencher molecules in two separate units. [126]

Yamane *et al*. [127] have introduced the MagiProbe method where a single dual labeled oligonucleotide probe contains a fluorescein and a pyrene coupled to adjacent nucleotides in the middle of the probe. The pyrene quenches the signal of the free probe in the solution. When the probe is hybridized to the target sequence the pyrene is intercalated into the DNA complex decreasing the quenching and leading to fluorescence signal increase. The MagiProbe method is suitable also for SNP detection as the fluorescence remains quenched because of an unhybridized pyrene labeled nucleotide.

A fluorescent europium chelate donor and organic fluorophore acceptor based dual end-labeled Decay probe system has been published by Laitala *et al.* [136]. The donor

and acceptor of the free Decay probe in the solution are located very close to each other because of natural conformation of the probe and, therefore, the energy transfer is highly efficient leading to intensive short-lifetime acceptor signal. When the Decay probe is hybridized to the target DNA the donor-acceptor distance is increased leading to less efficient energy transfer and prolonged acceptor fluorescence lifetime.

2.3.2.2 Non-FRET-based hybridization methods

Several homogenous sequence-specific non-FRET fluorescence-based nucleic acid detection methods are listed in Table 4 and introduced below.

Table 4. Homogenous sequence-specific non-FRET nucleic acid detection methods

Method	Principle
Light-up probe [137]	Environmental sensitive fluorophore
Environmental sensitive lanthanide probe [138]	
HyBeacon™ probes [139]	
Binary malachite green aptamer (MGA) [140]	
BODIPY FL primer/probe [141]	Quenching by guanine
Smart probes [142]	
Competitive reporter monitored amplification (CMA) [143]	Other
Excimer-forming two-probe nucleic acid hybridization method [144]	
Fluorescence polarization [145]	
TPX technology [146]	
Diamine catalyzed hemicyanine dye formation through DNA programmed chemistry [147]	
Hybridization directed fluorescent complex formation [148]	

Light-up probe

In the Light-Up probe system a DNA intercalative fluorescent thiazole orange (TO) cyanine dye is coupled to a peptide nucleic acid (PNA) probe. PNA shows increased thermal stability and, in general, PNA-DNA complexes are more stable than DNA duplexes. When the Light-Up probe hybridizes to the target strand the TO dye is forced to bind to the DNA increasing the TO dye fluorescence. Signal intensity increase up to 50-fold due to the probe hybridization has been reported. The Light-Up probe is a simple and promising method for homogenous nucleic acid detection but the functionality has not been demonstrated in homogenous nucleic acid amplification assay. This raises a question of the technology's suitability to real applications where the amplification needs to be performed in the same closed reaction. [137, 149] (Figure 12)

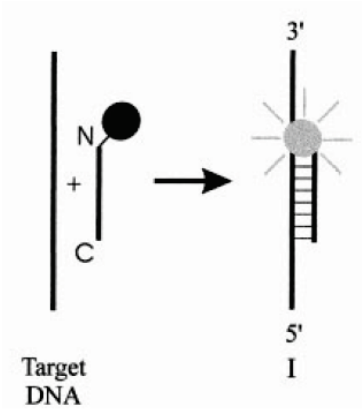

Figure 12. Principle of the Light-Up probe technology. The signal of the fluorophore increases when the probe is hybridized to the target sequence leading to the intercalation of the fluorophore. Modified from the reference [137].

Environmental sensitive lanthanide probe

The method is based on oligonucleotide labeled at its 5´ end with a Tb^{III} chelate sensitive to its immediate chemical environment. The fluorescence of the used Tb^{III} chelate is decreased when it is coupled to the oligonucleotide. The hybridization of the probe to the target sequence in PCR leads to the probe degradation by the DNA polymerase 5´ nuclease activity and to the release of the Tb^{III} chelate to the reaction solution. The detachment of the Tb^{III} chelate increases its fluorescence signal. [138, 150] (Figure 13)

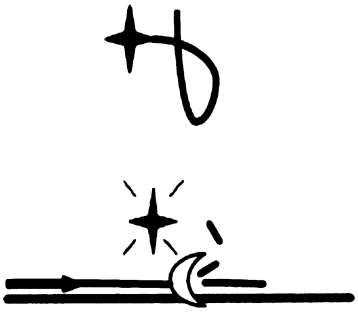

Figure 13. Principle of the Environmental sensitive lanthanide probe technology. The hybridized probe is degraded during the PCR amplification by the DNA polymerase enzyme and the detachment of the lanthanide chelate (star) leads to increased fluorescence signal. Modified from the reference [138].

Competitive reporter monitored amplification (CMA)

The CMA system is based on a capture probe array and fluorophore labeled reporter probes. The reporter probes are complementary to the capture probes and also to the amplified target nucleic acid. The binding of the reporter probes to the capture probes decreases with increasing amplification product concentration, and the fluorescence signal from the capture probe spot decreases during real-time detection of the amplification reaction. Reporter probe-target sequence hybrids in the solution are mechanically removed from the capture spot area by compressing the reaction chamber before the measurement to avoid background signal from the hybrids and from the free fluorescent probes. After measurement the reaction chamber is allowed to uncompress and the reaction solution is allowed to return to the measurement area. [143] (Figure 14)

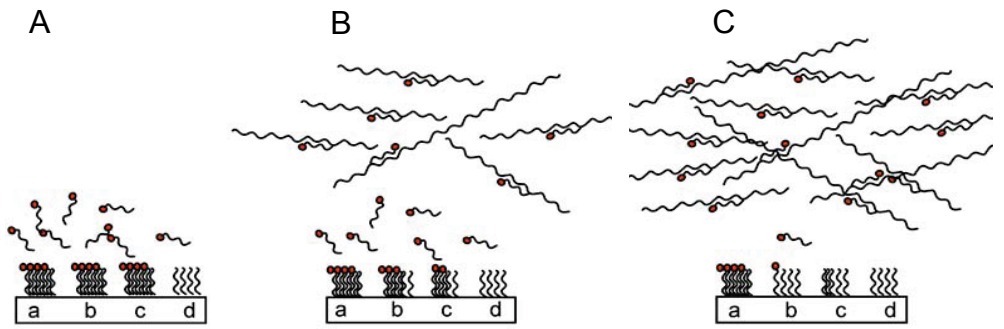

Figure 14. Principle of the Competitive reporter monitored amplification method. Fluorescent reporter probes hybridize to the amplified target sequences (A→C). The target-probe hybrids (and also free reporter probes) are removed from the measurement area by compressing the reaction chamber in the spot area (not illustrated in the figure). Multiplexed detection can be performed by spatial separation using different capture probes (a, b, c and d) and reporter probes in one reaction. Modified from the reference [143].

Excimer-forming two-probe nucleic acid hybridization method

The Excimer-forming method utilizes two oligonucleotide probes, one 5´ and other 3´ pyrene labeled. The hybridization of the probes to the adjacent positions on the target DNA brings the pyrenes in close proximity and leads to excimer formation as well as an emission shift to longer wavelengths (pyrene monomer and excimer emission band maximum wavelengths 378 nm and 495 nm, respectively). The distance of the pyrenes must be 3–4 Å; therefore, the excimer formation requires precise hybridization of the two probes. [144] (Figure 15)

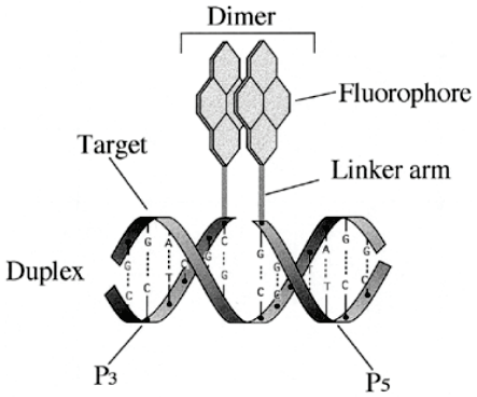

Figure 15. Principle of the Excimer-forming two-probe nucleic acid hybridization method. Two pyrene-labeled probes (P_3 and P_5) hybridize to adjacent positions into the target sequence and form an excimer with a fluorescence signal at a higher wavelength than the pyrene residues. Modified from the reference [151].

Several DNA detection applications based on excimer formation have been published but there are no publications describing nucleic acid amplification and excimer-based detection performed in a homogenous reaction. However, a system combining a signal amplification method called hybridization chain reaction [152] and excimer formation has been introduced [153]. The introduced method utilizes two oligonucleotide hairpin probes that contain pyrene dyes at both ends. The hairpin is formed in a way that the labeled ends are not in close proximity and thus, the excimer is not formed. Target DNA hybridizes to one of the labeled oligonucleotides leading to the opening of the hairpin structure and initiation of hybridization events. This results in a nicked double-helix structure where the 5′ and 3′ pyrenes from different probes are in close proximity forming excimers and, therefore producing a typical emission wavelength shift. Fujimoto *et al.* [154] have presented a Molecular beacon probe type hairpin probe containing pyrene dyes at both ends of the probe. The probe in the hairpin structure emits excimer fluorescence and when the probe is hybridized to the target the pyrenes are separated and excimer signal decreased.

Fluorescence polarization

The fluorescence polarization (FP) method is based on a target-specific oligonucleotide probe that is labeled with a fluorophore. The target's presence can be measured when the polarization of the emission fluorescence changes due to the hybridization of the probe to the target sequence. The fluorophore is excited with polarized light and the rotation of the excited unhybridized probe causes emission fluorescence depolarization. When the probe is bound to the target the fluorescence is more polarized because the larger probe-target hybrid rotates slower than free probe in solution. [24, 145, 155]

Fluorescence polarization has been used in different PCR amplification-based assays. For example, Gibson *et al.* [156] utilized fluorescent probe hybridization and FP measurement together with the amplification refractory mutation system for

genotyping. Another method reported by Latif *et al*. [157] used DNA polymerase 5´ nuclease activity to degrade a fluorescent probe hybridized to the amplified target sequence and FP detection. An intact fluorescent probe has a higher FP value than a cleaved probe and this difference was used for the genotyping of single nucleotide polymorphisms. Zhang *et al*. [158] have developed a target assisted exonuclease III-catalyzed signal amplification fluorescence polarization method where Exo III enzyme's 3´ nuclease activity degrades a hybridized fluorophore-labeled probe. In this system the target DNA is not amplified but the fluorescent probes, one by one, continuously hybridize to the target DNA and are degraded, which leads to a change in the FP value.

ArcDia TPX technology

This technology is based on microparticles and two-photon excitation-based measurement. The microparticles are used as a solid phase for a single-base extension (SBE) reaction to concentrate the nucleic acid analyte and fluorescent dideoxy nucleotides on the surface of the particles. In single-nucleotide polymorphism (SNP) analysis the microparticles are coated with target-specific primers that hybridize next to the polymorphism site. During the SBE reaction the DNA polymerase incorporates a terminating dideoxynucleotide (ddA, ddT, ddC or ddG). One of the four dideoxynucleotides, the dideoxynucleotide complementary to the nucleotide of interest, is labeled with an organic fluorescent dye suitable for two-photon excitation. In the measurement, the optical forces of the near-infra red laser brings the microparticle into diffraction-limited focal point of the laser illumination and only a very small area approximately the volume of 1 femtoliter, is excited. The signal from the microparticles can be distinguished from the background signal because the fluorescent dideoxynucleotides are concentrated on the microparticle's surface and, furthermore, the signal from the free fluorescent dideoxynucleotides in the reaction solution remains low because only a small volume containing individual microparticle is excited. The TPX technology provides an interesting alternative to the FRET-based methods but, for example, the SBE method is based on the incorporation of only one fluorescent dye to one target strand requiring a relatively high analyte concentration and, therefore a pre-amplification of the nucleic acid sample in a separate reaction. [146, 159]

Hybridization directed fluorescent lanthanide complex formation

This technology was first introduced in 1990 by Oser & Valet [148] and it involves the usage of two non-fluorescent oligonucleotide probes, one 3´ labeled with a non-fluorescent lanthanide ion carrier chelate and another 5´ labeled with a light absorbing antenna chromophore. The hybridization of the probes to adjacent positions into the target sequence brings the reporter molecules in close proximity leading to the formation of a fluorescent lanthanide chelate complex. The fluorescent complex is formed by self-assembly of the chromophore and the Ln^{III} carrier chelate. The interaction between the reporter moieties is based on the formation of coordination bonds between the Ln^{III} and a light absorbing chromophore. The intercation is relatively weak and, therefore the free probes in the solution do not form fluorescent lanthanide complex. (Figure 16) The technology has shown high sensitivity (low

picomolar target concentration) in homogenous nucleic acid detection because the background fluorescence signal from unhybridized probes is low and the autofluorescence is avoided by using time-resolved measurement. [160-162]

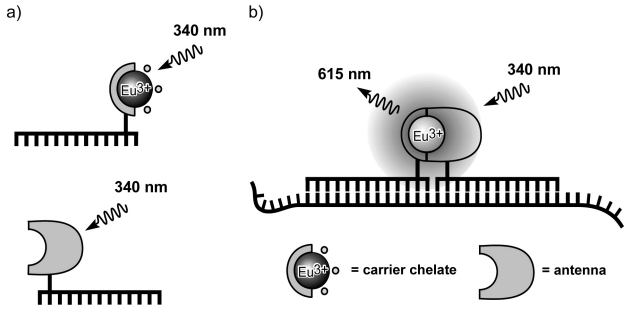

Figure 16. Principle illustrating the Hybridization directed fluorescent lanthanide complex formation technology. The hybridization of two non-fluorescent probes to adjacent positions into the target sequence leads to the self-assembled formation of a fluorescent lanthanide chelate complex. [162]

2.3.3 Conclusions

In conclusion, several different fluorescence-based methods for homogenous nucleic acid detection have been developed during the past 25 years. The FRET-based methods and, especially, the Hydrolysis probe technology are the most commonly used methods in homogenous nucleic acid assays, although some relatively simple non-FRET-based systems have been introduced. The hydrolysis probe method is well-known, has routinely been used for more than a decade and has proven to be a reliable and effective nucleic acid detection method. There is also software available for relatively easy and straightforward Hydrolysis probe design, and several companies provide low-cost syntheses of the probes as a routine service.

However, FRET-based methods have also limitations and drawbacks. In general, background signal levels of the Hydrolysis probe-based assays are relatively high because of imperfect fluorescence signal quenching. In general, FRET-based quenching efficiency decreases with an inceasing distance between the fluorophore and quencher. Hydrolysis probes are commonly dual end-labeled to ensure the separation of the two dyes by the probe degradation but that can lead to a relatively long fluorophore-quencher distance. Furthermore, the secondary structure of the probe might also affect the fluorophore-quencher distance and thus, the quenching efficiency. It has also been reported that different quenchers have different attraction to the fluorophore and this can lead to a difference in quenching efficiency because the attraction can pull some quenchers closer to the fluorophores causing increased quenching [163]. Autofluorescence from the reaction tubes and, especially, from biological sample material increases the background signal level of the assays using common organic fluorophores with steady-state measurement and, therefore effective sample preparation is required for obtaining pure nucleic acid samples. High

background fluorescence results in a low signal-to-background ratio (S/B) that might reduce assay sensitivity. [118, 164, 165] Furthermore, multiplexing using different organic fluorophores is reported to be cumbersome because of the wide overlapping emission spectra of the different fluorophores. In multiplexed assays fluorophores with suitable emission spectra and narrow-band emission filters should be used and, additionally, software for the emission signal processing must be used to discriminate the correct signal from interfering overlapping fluorescence. [166] Multiplexed closed-tube microarrays have been developed by attaching Molecular beacon probes to a solid surface. The hybridization of the target nucleic acid linearizes the hairpin structure of the probe and leads to fluorescence signal increase. However, no closed-tube system containing nucleic acid amplification and molecular beacon probe microarray has been presented and it has been reported that the microarray systems based on the Molecular beacon probes suffer from high background fluorescence signal because of incomplete quenching. [167-169]

3 AIMS OF THE STUDY

The hybridization-directed fluorescent lanthanide complex formation method also called the complementation probe technology has shown high sensitivity in homogenous nucleic acid detection because of low background signal level and high specific fluorescence signal in the presence of the target. The idea of this study was to further improve the complementation probe technology and study the suitability of the method for homogenous PCR based nucleic acid diagnostics. Another aim was to study the performance of the method in different sample materials, for example, urine, blood and bacterial growth medium to be able to design assays using minimal sample preparation. Furthermore, the overall aim was to establish a complementation probe-based homogenous PCR assay concept suitable for rapid and high-performance clinical diagnostics.

More specifically the original publications (titles in bold) had following aims:

I **High-performance closed-tube PCR based on switchable luminescence probes**

To study the suitability and performance of the complementation probe technology in homogenous PCR diagnostics including the design and synthesis of a new thermostable lanthanide (Eu^{III}) chelate.

II **Homogenous M13 bacteriophage quantification assay using switchable lanthanide fluorescence probes**

To explore the potential of the complementation probe method for direct amplification-free target organism measurement by designing a rapid and homogenous bacteriophage quantification method.

III **Homogenous duplex polymerase chain reaction assay using switchable lanthanide fluorescence probes**

To study the applicability of the complementation probe technology for rapid clinical nucleic acid diagnostics by developing a duplex *Chlamydia trachomatis* PCR assay compatible with a simple urine sample preparation. The work also included designing and synthesizing a new light absorbing antenna for Tb^{III}.

IV **Closed-tube HLA-DQA1*05 PCR assay based on switchable lanthanide fluorescence probes and simple dried blood sample punch preparation**

To study the performance of the complementation probe technology in genotyping by applying the method for homogenous HLA DQA1*05 PCR assay compatible with simple blood sample preparation.

4 MATERIALS, METHODS AND BACKGROUND

The key points of the materials and methods of this study are presented here. More detailed descriptions are found in the original publications (**I–IV**). Short introductions and schematic figures are also found in the results and discussion section at the beginning of different chapters.

4.1 Switchable lanthanide chelate complementation probe technology

The principle of the lanthanide chelate complementation probe technology (Figure 16 and Figure 17) based on two oligonucleotide probes, one carrying a non-fluorescent lanthanide chelate and another carrying a light absorbing antenna, was introduced over 20 years ago [148]. The method has been found to be sensitive (low picomolar detection limit) in homogenous nucleic acid detection [160, 162] and also a duplex assay based on Eu^{III} and Tb^{III} complementation probes for the detection of single-nucleotide polymorphism has been introduced [161]. However, a homogenous nucleic acid amplification assay based on the complementation probes is, for the first time, presented in this study.

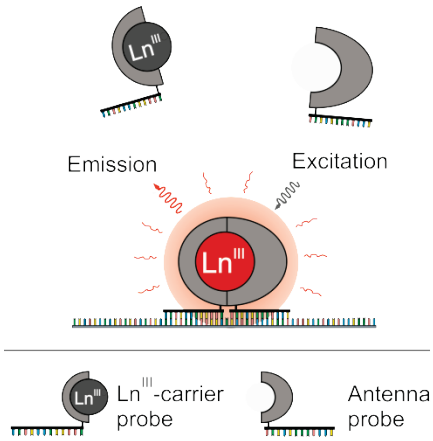

Figure 17. Principle of the Complementation probes-based nucleic acid detection. The non-fluorescent probes hybridize to the target DNA and form a fluorescent lanthanide chelate complex by the self-assembly of the reporter molecules.

4.1.1 Lanthanide ion carrier chelates

The first lanthanide chelate complementation probe assays used DTPA (diethylenetriaminepentaacetic acid) [148] and EDTA (ethylenediaminetetraacetic acid) derivates as lanthanide ion carrier chelates to couple the Ln^{III} to the oligonucleotide probe [160, 161]. Later, also N1-Eu^{III} [N1-(4-isothiocyanatobenzyl)diethylenetriamine-N1,N2,N3,N3-tetrakis(acetato)europium(III)] carrier chelate was used in the

complementation probe system [162]. DTPA and EDTA can form 8 and 6 coordination bonds, respectively, with the lanthanide ion and the N1-Eu^{III} chelates the Ln^{III} by 7 coordination bonds [170]. In contrast, homogenous PCR assays using on inherently fluorescent lanthanide chelates have been based on more stable 9-dentate chelates to ensure the binding of the Ln^{III} in PCR conditions and to prevent H_2O-based quenching [58-61].

In magnetic resonance imaging (MRI) chelated gadolinium ions are injected to the patient and used as a contrast agent. Free Gd^{III} is highly toxic to humans but chelated Gd^{III} is nontoxic and, therefore, stable chelates must be used in MRI. In MRI cyclic chelates based on octadentate DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) structure are commonly used because cyclic chelates are, in general, more stable than linear chelates, e.g., N1-Eu^{III}; therefore, the DOTA based chelates are safer and preferred in MRI. [171, 172]

We hypothesized that the previously used DTPA, EDTA and N1-Eu^{III} would not be stable enough in the PCR reaction conditions and the Ln^{III} would be released from the chelate in the PCR denaturation temperatures of 95–98 °C. Because the complementation probe technology requires the coordination of the light absorbing antenna molecule to the Ln^{III}, stable 9-dentate chelates occupying all the Ln^{III} coordination sites cannot be used.

We designed and synthesized DOTA based cyclic heptadentate europium and terbium carrier chelates 7d-DOTA-Eu^{III} and 7d-DOTA-Tb^{III} [2,20,200-[10-(3-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclo-dodecane-1,4,7-triyl]tri(acetato)Eu^{III}/Tb^{III}], that leave two coordination sites to be occupied by the antenna ligand. The syntheses of 7d-DOTA-Eu^{III} and 7d-DOTA-Tb^{III} were described in the articles published by Karhunen *et al*. 2011 [173] and in the publication **III**, respectively, and they are illustrated in Figure 18.

4.1.2 Light absorbing antenna ligands

The light absorbing antenna ligand needs to form coordination bonds with the Ln^{III} but the interaction with the lanthanide ion carrier chelate should not be too strong to cause a binding of the antenna ligand to the Ln^{III}-carrier chelate in the absence of the target DNA and, therefore, unspecific fluorescence. Previously, a 3-dentate light absorbing antenna ligand for Eu^{III} (Eu^{III} antenna) [4-((isothiocyanatophenyl)ethynyl)pyridine-2,6-dicarboxylicacid] based on the fluorescent 7-dentate europium chelate structure [2,2′,2′′,2′′′-[[4-[4-(-Isothiocyanatophenyl)ethyl]pyridine-2,6-diyl]bis(methylene nitrilo)]tetrakis(acetic acid)], published by Takalo *et al*. 1994 [174] was used in the complementation probe system together with N1-Eu^{III} [162]. That system has shown good sensitivity (22 pM target DNA) with low background signal level and relatively high signal modulation leading to a high signal-to-background ratio of 1400 and a dynamic range of 4 orders of magnitude. [162] We decided to use the same Eu^{III} antenna structure and it is illustrated in Figure 18.

Eu^{III} and Tb^{III} require different light absorbing antenna ligands because they have different lowest excited state levels as discussed earlier in chapter 2.1.2. Tridentate TMP-DPA [4-(2′,4′,6′-trimethoxyphenyl)pyridine-2,6-diyl]dicarboxylic acid)] [41] has been used in PerkinElmers (PerkinElmer Life and Analytical Sciences, MA, USA) heterogenous DELFIA (dissociation-enhanced lanthanide fluorescent immunoassay) technology as a Tb^{III} enhancer. It has shown a millisecond Tb^{III} fluorescence lifetimes and a relatively high quantum yield [41]. A TMP-DPA derivative (Tb^{III} antenna) [4-(3-[2-(4-isothiocyanatophenyl)ethyl]-2,4,6-trimethoxyphenyl)-pyridine-2,6-dicarboxylic acid] containing an isothiocyanate coupling group was synthesized as described in publication **III**. The Tb^{III} antenna is illustrated in Figure 18.

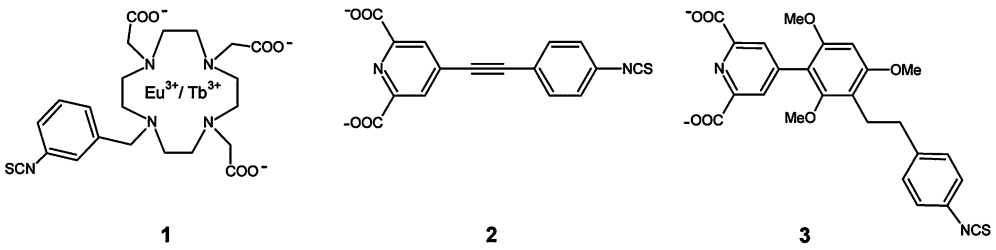

Figure 18. Molecular structures of the complementation probe reporter molecules used in this study. 7d-DOTA-Eu^{III}/Tb^{III} lanthanide ion carrier chelate (1), Eu^{III} antenna (2) and Tb^{III} antenna (3).

4.1.3 Preparation of the complementation probes

The Ln^{III} carrier probes were prepared by coupling the 7d-DOTA-Eu^{III} (Eu^{III} carrier probe) or 7d-DOTA-Tb^{III} (Tb^{III} carrier probe) to the 3′ end of the target-specific oligonucleotides. In addition, N1-Eu^{III} was also used to be able to compare the functionality and thermostability of the 7d-DOTA-Eu^{III} and N1-Eu^{III} chelates.

The antenna probes were prepared by labeling oligonucleotides at the 5′ end with the Eu^{III} antenna (Eu^{III} antenna probe) or with the Tb^{III} antenna (Tb^{III} antenna probe). The Ln^{III} carrier probes and antenna probes were designed to hybridize to adjacent positions (leaving one unhybridized nucleotide between the two probes) into the target sequences bringing the labeled 3′ and 5′ ends in close proximity and hence, allowing the fluorescent lanthanide complex formation by self-assembly of the Ln^{III}-carrier and antenna molecules. The design and preparation of the probes used in different assays are described in publications **I–IV** and the labeling protocol in publication **I**.

4.1.4 The main methodologies and instruments

All the measurements were performed using 50 nM probe concentrations if not otherwise mentioned. Europium and terbium fluorescence signals were measured in time-resolved mode using a 340 nm excitation filter, and 615 nm and 545 nm emission filters for europium and terbium signal measurements, respectively (Eu TRF and Tb

TRF) with 0.4 ms delay and 0.4 ms measurement times. The measurement cycle was repeated 1000 times in a total measurement time of approximately 1 s. The fluorescence signal of the TaqMan probe was measured using a 485 nm excitation filter, 535 nm emission filter and 0.1 s measurement time. The measurements were performed with the PerkinElmers 1420 Victor Multilabel Counter.

The fluorescence emission spectra and lifetimes of the lanthanide chelate complexes formed by the hydridization of the Eu^{III} and Tb^{III} complementation probes to the synthetic oligonucleotide targets (10 nM) were measured with a Varian Cary Eclipse fluorescence spectrophotometer (Varian Scientific Instruments, Australia). The emission spectrum of the Tb^{III} mixed chelate complex (hybridized Tb^{III} carrier and Tb^{III} antenna probes) was measured using 325 nm excitation with 20 nm slit, 450–750 nm emission with 5 nm slit, 0.1 ms delay, and 0.4 ms measurement time; the emission lifetime was measured using 325 nm excitation with 20 nm slit, 545 emission with 20 nm slit, 0.05 ms delay, and 0.05 ms scan gate. The emission spectrum and lifetime of the Eu^{III} mixed chelate complex (hybridized Eu^{III} carrier and Eu^{III} antenna probes) were measured identically to the Tb^{III} complex measurements; an emission spectrum from 550 nm to 750 nm was measured and the emission lifetime was detected at 615 nm, however.

PCR amplification reactions were performed using conventional PCR thermal cycler (PTC-200 DNA engine, MJ Research, MA, USA). Conventional commercial PCR reagent and buffers were used as described in publications **I**, **III** and **IV**. In real-time PCR the reaction plate was removed from the thermal cycler to the multilabel counter for measurement at each measurement point. Three parallel reactions were used in all the measurements, except the clinical samples were tested without parallel reactions.

5 RESULTS AND DISCUSSION

5.1 Properties of the Eu^{III} and Tb^{III} complementation probes

The terms Eu^{III} and Tb^{III} complementation probes refers to the Eu^{III} carrier probe and Eu^{III} antenna probe pair, and to the Tb^{III} carrier probe and Tb^{III} antenna probe pair, respectively.

5.1.1 Fluorescence emission spectra and lifetimes of the Eu^{III} and Tb^{III} complementation probes

The Eu^{III} and Tb^{III} complementation probes formed fluorescent complexes at the presence of the oligonucleotide target and yielded typical Eu^{III} and Tb^{III} emission spectra with the main emission peaks at 615 nm and 545 nm, respectively (Eu^{III} complementation probes emission spectra and lifetime are unpublished data). The emission lifetimes of Eu^{III} and Tb^{III} complementation complexes were 0.9 ms and 1.0 ms, respectively. (Figure 19) The increased fluorescence signal in the presence of the target oligonucleotide indicates a fluorescence Ln^{III} complex formation by coordination bonds and long fluorescence lifetimes enabled the usage of time-resolved measurement in the assay applications.

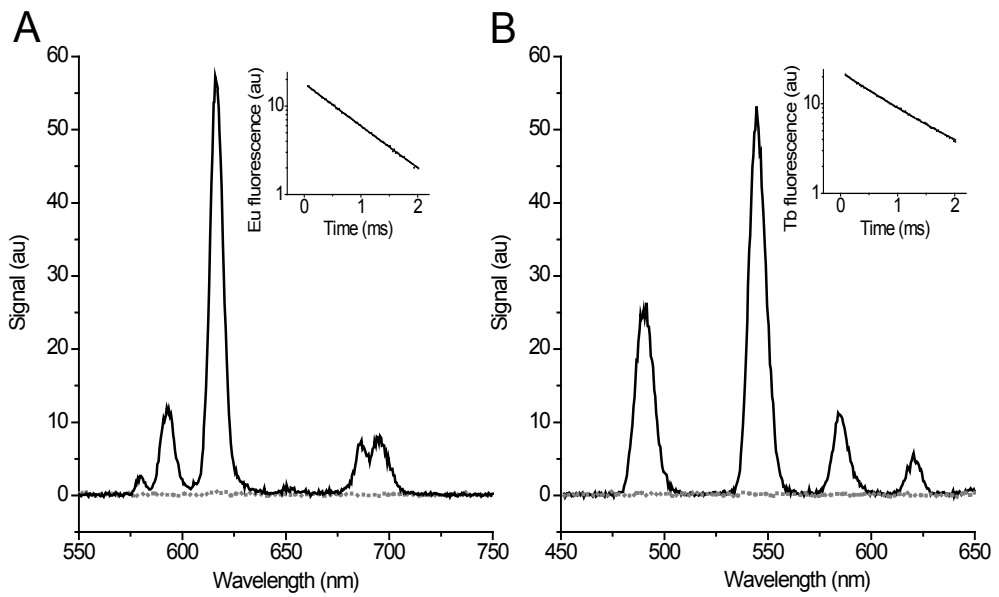

Figure 19. Fluorescence emission spectra and lifetimes of the (A) Eu^{III} and (B) Tb^{III} complexes formed by the hybridization of the Eu^{III} and Tb^{III} complementation probes into the synthetic oligonucleotide target (black line) and in the absence of the target (grey dotted line). Lifetimes of the Eu^{III} and Tb^{III} main emission peaks at 615 nm and 545 nm, respectively, shown in insets.

5.1.2 Performance in closed-tube nucleic acid detection

The nucleic acid detection performances of the Eu^{III} and Tb^{III} complementation probes were studied in homogenous hybridization reactions. The reactions contained either Eu^{III} complementation probes or Tb^{III} complementation probes and different concentrations of complementary synthetic oligonucleotide target. The probes hybridized to the target oligonucleotide and formed a fluorescent complex as illustrated in Figure 17. Fluorescence signal (Eu TRF or Tb TRF) was measured after 5 min incubation at 22 °C. The performance tests are described in more detail in publications **I** (Eu^{III} complementation probes) and **III** (Tb^{III} complementation probes).

Both the Eu^{III} and Tb^{III} complementation probes showed similar nucleic acid detection performances. Detection limits of 15 pM and 10 pM oligonucleotide target concentrations for Eu^{III} and Tb^{III} complementation probes, respectively, were measured. Both assays showed a dynamic range over three orders of magnitude and a high signal-to-background ratio; up to 1000 for Eu^{III} complementation probes and 800 for Tb^{III} complementation probes. Results are illustrated in Figure 20.

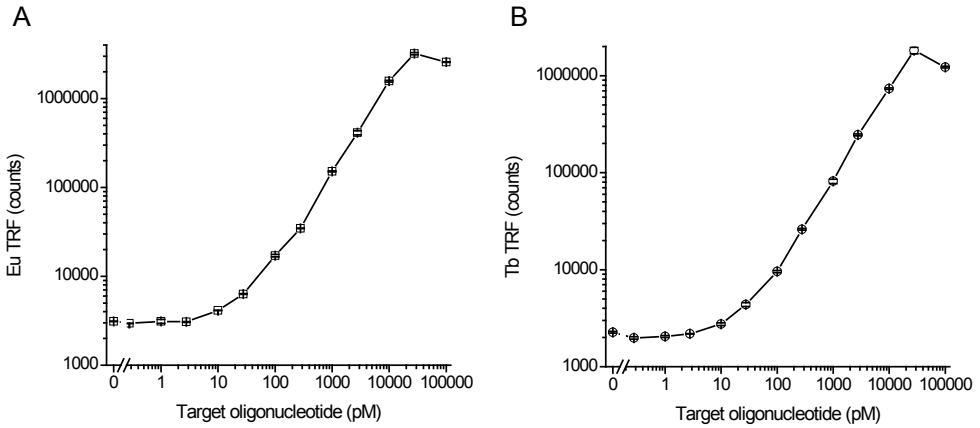

Figure 20. Standard curves of the (A) Eu^{III} and (B) Tb^{III} complementation probes based DNA detection using 50 nM probe concentrations (publications **I** and **III**, respectively).

Other homogenous nucleic acid detection methods, e.g., a FRET-based Adjacent hybridization probes assay (principle of the technology introduced in the chapter 2.3.2.1) with nucleic acid detection sensitivity of 200 pM has been reported using streptavidin containing several biotinylated europium chelates as a fluorescent donor and a Cy5 acceptor [175]. Detection sensitivity of 30 pM has been reported using a similar Adjacent hybridization probes-based assay system with terbium chelates and Cy3 as a donor and acceptor, respectively [176]. In addition, the Competitive hybridization probe system (introduced in chapter 2.3.2.1) with a fluorescein and quencher molecule has shown nucleic acid detection sensitivity of approximately 100 pM with 10 min incubation time and 1 nM probe concentrations [124]. In the same publication, a detection limit of 4 pM was reported using 6 h incubation time and low

concentrations (20 pM) of several probes hybridizing to separate sequences within the same target gene. Low probe concentrations were used to decrease the background signal level limiting the sensitivity but, at the same time the assay kinetics was affected making the system time-consuming and, therefore, impractical.

5.1.3 Performance in homogenous PCR

Thermal stability of the complementation probes

Nucleic acid assays commonly require target amplification to increase the target concentration up to the detection range of the detection methods and, hence, in closed-tube assays the nucleic acid detection method must be compatible with the amplification techniques. Most commonly the target nucleic acids are amplified using the polymerase chain reaction requiring relatively high temperature. Many sensitive nucleic acid detection methods, e.g., the Adjacent hybridization probes assays based on a europium and terbium-labeled streptavidin donor [175, 176], mentioned in the previous chapter, would most likely be dysfunctional in PCR reaction conditions because of the denaturation of the streptavidin in high temperature.

The performance of the complementation probes in PCR reaction conditions, especially the thermostability of the new cyclic 7d-DOTA-Ln^{III} carrier chelate needed to be studied before applying the technology to PCR assay applications. Thermostability was tested by cycling the temperature of the reaction containing Eu^{III} complementation probes and 10 nM synthetic oligonucleotide target concentration between 30 °C and 98 °C for 30 cycles and measuring the Eu TRF at 30 °C after 30 s incubation in every cycle. To compare the effect of the lanthanide chelate structure on the thermostability, the non-cyclic N1-Eu^{III} carrier chelate was also used in this study. The testing process is more precisely described in publication **I**.

In the first cycle of the thermostability testing the reaction temperature was held at 98 °C for 2 min as is usual at the beginning of the PCR during the initial denaturation step. The Eu TRF signal from the Eu^{III} complementation probes noticeably decreased when measured after the first incubation at 98 °C; the following incubations at 98 °C decreased the Eu TRF signal only moderately and, therefore, it was concluded that the Eu^{III} complementation probes were suitable for homogenous PCR. The Eu TRF from the complementation probes using N1-Eu^{III} carrier chelate was dramatically decreased when measured after the first incubation at 98 °C, and during the following 10 temperature cycles the signal was completely decreased to the background fluorescence signal level. The results are shown in Figure 21.

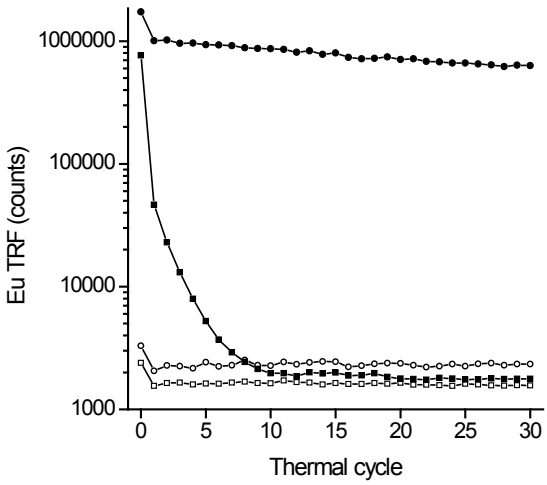

Figure 21. Thermal stability of the Eu^{III} complementation probes. Reactions containing the Eu^{III} antenna probe and Eu^{III} carrier probe labeled with the 7d-DOTA-Eu^{III} carrier chelate (circles) or with N1-Eu^{III} carrier chelate (square) and 10 nM synthetic oligonucleotide target (black symbols) or no target (white symbols). Temperature was cycled between 30 °C and 98 °C and Eu TRF measured in every thermal cycle at 30 °C. (publication **I**)

Homogenous real-time PCR

Various closed-tube PCR assays have been introduced in recent years, especially for the detection of infectious diseases. Both qualitative and quantitative PCR assays are useful, for example, in clinical infectious diseases diagnostics and both assays have been developed. Qualitative assays are sufficient, for example, for the detection of pathogenic bacteria such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae* which are not a part of the normal human bacterial flora and thus, should not be found in the samples of healthy persons. Like the qualitative assays, real-time quantitative PCR assays can also be used for the detection of pathogenic microbes, but they can also be used, for example, to determine disease severity by quantifying the pathogenic microbe load in a clinical sample and to follow the efficiency of drug treatment, e.g., in HIV patients. The qPCR can also be used to differentiate between pathogenicity and commensalism when the target bacterium is a part of the normal human bacterial flora. [18, 177, 178]

The nucleic acid detection performance of the complementation probe technology in homogenous PCR was studied and demonstrated by designing Eu^{III} complementation probes and primers specific to synthetic oligonucleotide target as described in publication **I**. The PCR protocol consisted of 10 amplification cycles without measurement steps followed by 34 amplification cycles with the real-time Eu TRF measurements performed in every second cycle after 30 s incubation at 30 °C. The complementation probes based homogenous PCR assay principle is illustrated in Figure 22. The performance of the assay was compared to the Hydrolysis probe based homogenous PCR assay (method introduced in chapter 2.3.2.1) that is the most

commonly used closed-tube nucleic acid detection method. The Hydrolysis probe was designed to hybridize to the same target DNA as the complementation probes. The dual end-labeled probe contained a 6-carboxyfluorescein and a Black Hole Quencher-1. The same primers were used in both assays, whereas different DNA polymerase enzymes were used because 5´ nuclease activity is needed for probe degradation in the Hydrolysis probe assay, and in the complementation probe assay the degradation would lead to signal loss. Therefore, in the complementation probe PCR a DNA polymerase lacking the 5´ nuclease activity was used. The two assays are described in detail in publication **I**.

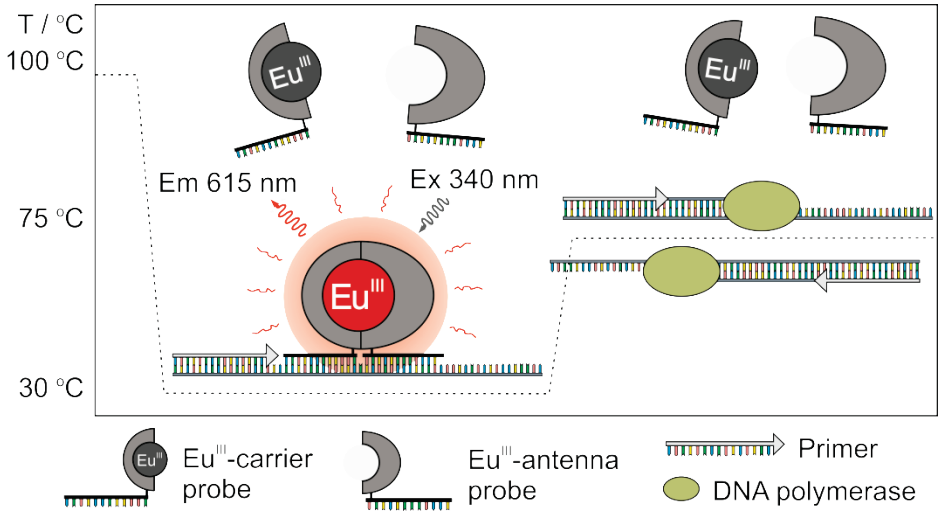

Figure 22. Illustration of the complementation probes based homogenous real-time PCR. After a denaturation step at 98 °C the temperature (dashed line) is decreased to the measurement temperature of 30 °C. The non-fluorescent probes are hybridized to the target and a fluorescent complex is formed. Eu TRF is measured after 30 s of incubation and temperature is then raised to 72 °C for the target DNA extension step. The probes are designed to be unhybridized during the PCR amplification (temperatures 62–98 °C) and they hybridize only at low temperatures.

The Complementation probe PCR assay showed a low background fluorescence signal level of approximately 1500 Eu TRF counts and a high specific signal level maximum of 390000 Eu TRF counts when the target DNA was amplified. The low background and high specific signal levels led to a very high signal-to-background ratio of 190–300. The Hydrolysis probe-based PCR assay signal-to-background ratios were approximately 2 because of a relatively high background signal level approximately 140000 6-FAM fluorescence signal.

The PCR amplification efficiency of both assays was analyzed using standard curves where the base-10 logarithm of the initial template copy numbers and the corresponding threshold cycles were plotted on the x- and y-axis, respectively. The C_t numbers are inversely proportional to the base-10 logarithm of the initial template copy number [179]. According to the standard curves, both assays showed similar and

appropriate amplification efficiency: the complementation probe PCR assay was 97.2% and the Hydrolysis probe based assay efficiency was 93.1%. The efficiency was calculated according to the equation: Efficiency = $10^{(-1/slope)}$ [180, 181]. The threshold cycle levels were detected approximately 6 amplification cycles earlier with the complementation probe method. A linear range of the Complementation probe-based PCR from 100 to 1 x 10^7 DNA template copies/reaction was measured but lower template copy numbers (10 and 1 copies/reaction) were detectable only occasionally. The Hydrolysis probe-based PCR yielded a detectable signal from the reactions containing a minimum of 1000 template copies, whereas reactions containing 100 DNA template copies yielded no signal clearly detectable from the background signal. The real-time PCR results of the both assays are illustrated in Figure 23.

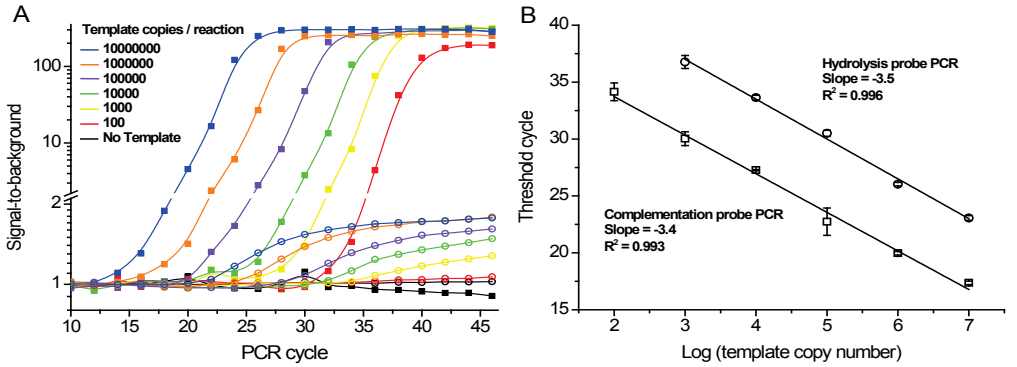

Figure 23. Real-time PCR performance of the Complementation probe-based (square) and the Hydrolysis probe-based (circle) assays. (A) Signal-to-background ratio of real-time PCR using 100–10000000 DNA template copies and (B) standard curve showing linear relationship between the log template copy numbers and threshold cycles. (publication **I**)

The signal-to-background ratios of the Hydrolysis probe assays are commonly of 2–6. The distance between the fluorophore and quencher of the dual end-labeled Hydrolysis probes can be relatively long (20–30 nucleotides) and an increased distance decreases the FRET efficiency and, therefore, the quenching of the intact probe. Low quenching efficiency increases the background signal level and decreases the S/B. Higher signal-to-background ratios of the FRET-based assays has been reported using shorter minor groove binding probes and the Molecular beacon probes. [164, 165, 182, 183] The highest S/B (35) of the FRET-based PCR assays have been reported using the Amplifluor method (introduced in chapter 2.3.2.1) that combines the Molecular beacon probe to the 5' end of the primer. The Molecular beacon method has shown better quenching efficiency than the Hydrolysis probe technology because the stem-loop structure brings the reporter moieties close to each other, but the stem-loop structure competes with the probe-target hybridization and thus, might decrease probe's hybridization efficiency to the target DNA. In the Amplifluor method the Amplifluor primer is linearized when the DNA polymerase replicates the primer sequence and the quenching is effectively released. [118] The Amplifluor method has clearly shown the highest S/B of the

homogenous FRET-based assays, but the ratio still remains almost 10 times lower than in the Complementation probe based PCR assay.

5.2 Homogenous DNA detection assay applications based on the complementation probe technology

Different homogenous nucleic acid assays were designed and developed to study and demonstrate the suitability of the complementation probe technology for PCR-based infectious disease diagnostics (**III**), PCR based genotyping (**IV**) and rapid amplification-free bacteriophage quantification (**II**).

5.2.1 Chlamydia trachomatis duplex PCR assay

Chlamydia infection caused by the bacterium *Chlamydia trachomatis* (*Ct*) is one of the most common sexually transmitted diseases (STD) [184]. More than half of the infected persons are asymptomatic but if untreated the genital *Ct* infection can cause serious complications, e.g., pelvic inflammatory disease and infertility. Many infections remain undetected because asymptomatic persons do not seek to the STD testing and also because part of the tested individuals carrying the infection fail to return to the clinic for results and treatment. [185-187]

Nucleic acid amplification assays have shown higher sensitivity than the traditional culture and immunoassay-type *C. trachomatis* detection methods and the nucleic acid assays are currently considered as the golden standard in *Ct* diagnostics. The present *Ct* nucleic acid assays are mainly performed using urine samples and they have shown good performance with high sensitivity and specificity. However, the *Ct* assays commonly requires a multi-step urine sample preparation containing DNA extraction complicating the *Ct* diagnostics. Automated systems containing sample preparation and nucleic acid amplification with homogenous detection have been developed and are routinely used. The automated *Ct* assays are capable of diagnosing hundreds of assays in a work day but they are relatively expensive and usually located to central laboratories. The overall time from sample collection to treatment initiation is easily delayed by several days because the collected specimen is first transported to the analysis clinic, then analyzed among other samples and, finally, the patient needs to return to the doctor's practice for the prescription. [185, 188, 189]

A rapid, easy-to-use and reliable *Ct* assay providing results while waiting (maximum 1 h) would be valuable for the prevention of *Ct* transmission, especially in resource-poor settings. Recently, an automated *C. trachomatis* nucleic acid amplification assay providing results in 90 min (including *Neisseria gonorrhoeae* analysis in the same reaction) was FDA approved (510(k) number K121710). Although, the system is flexible allowing the initiation of individual sample analyses at different times, the assay time still remains relatively long.

A europium and terbium complementation probes-based homogenous duplex *C. trachomatis* PCR assay (complementation probe *Ct* assay) was developed by designing

primers and Eu^{III} complementation probes specific to the *Ct* cryptic plasmid. *C. trachomatis* contains multiple plasmids, and, plasmid detection can be useful, especially for an early *Ct* infection diagnosis when a urine sample might contain a relatively small number of bacteria and it also allows more flexibility in sample preparation, for example, the use of more diluted samples. Internal amplification control (IAC) was created to confirm PCR functionality when no *Ct*-specific Eu TRF signal is observed (*Ct* negative reaction). Internal amplification control Tb^{III} complementation probes specific to a certain sequence in the IAC target plasmid were designed (IAC plasmid and primers were presented previously [48]) and the IAC target plasmid, IAC primers and IAC specific Tb^{III} complementation probes were included in every reaction. The Eu TRF and Tb TRF signals were measured in every second PCR cycle starting at the cycle 10. Real-time measurement is not required in *Ct* diagnosis but it was performed to follow the functionality of the new complementation probe technology in the urine sample analysis and to determine threshold cycle numbers. The complementation probe *Ct* assay is described in detail in publication **III** and the assay principle is shown in Figure 24.

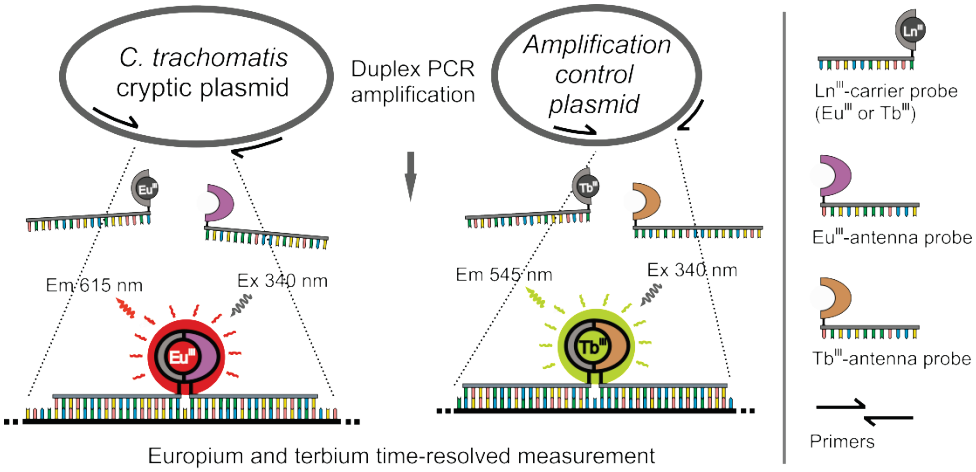

Figure 24. Principle of the complementation probes based *C. trachomatis* duplex PCR assay. (publication **III**)

Urine samples contain variable amounts of different organic and inorganic substances, e.g., ammonium and magnesium crystals that can decrease the PCR performance [190]. Commonly, *C. trachomatis* DNA is extracted and the PCR inhibitors are removed in multistep urine sample preparation procedures. We used a 10 minute simple *C. trachomatis* bacterial concentration and washing procedure based on centrifugation. The *Ct* cells and epithelial cells containing *Ct* cells in the urine sample were concentrated on the bottom of the microcentrifuge tube by centrifugation; this step was followed by suspension (washing) of the cells and subsequent centrifugation. Washed *Ct* bacteria were resuspended in the water and used for PCR analysis. The urine sample preparation is described in publication **III** and previously by Lehmusvuori *et al*. [48].

Total of 100 urine samples were tested with the complementation probe *Ct* assay. As a reference, the same samples were also analyzed with the BD ProbeTec *Ct* ET assay (Becton Dickinson Diagnostic Systems, MD, USA) combined with the automated BUGSn BEADS STI-fast sample preparation (NorDiag, Oslo, Norway) [191, 192]. The complementation probe *Ct* assay correlated 99–100% with the reference method showing good diagnostic accuracy. Both methods detected 50 *Ct* positive samples, while 1 urine sample (*Ct* negative according to the reference method) showed inhibition in the complementation probe *Ct* assay and no Tb TRF signal or Eu TRF signal was detected. In retesting the sample remained inhibitory, but when further diluted 1:1 to H_2O Tb TRF a signal was detected and the sample was diagnosed as *Ct* negative.

The positive urine samples mainly yielded very high *Ct* specific Eu TRF signal-to-background ratios. A maximum S/B ratio of 244 was measured and 80% of the Ct positive samples gave a S/B ratio of over 70 while only 2 positive samples gave a S/B ratio below 6. The *Ct* negative urine samples yielded a maximum Eu TRF signal-to-background ratio of 2. Because of the high S/B ratio difference between the positive and negative samples the complementation probe *Ct* assay result interpretation is clear. In 94% of the tested 50 *Ct* positive samples, the *C. trachomatis*-specific Eu TRF threshold were reached in cycle 26, at the latest, and all the Tb TRF threshold cycles of the Ct negative samples were reached by the cycle 24 (except in the inhibited reaction). The complementation probe *Ct* assay results of the analyzed 100 urine samples are shown in Figure 25.

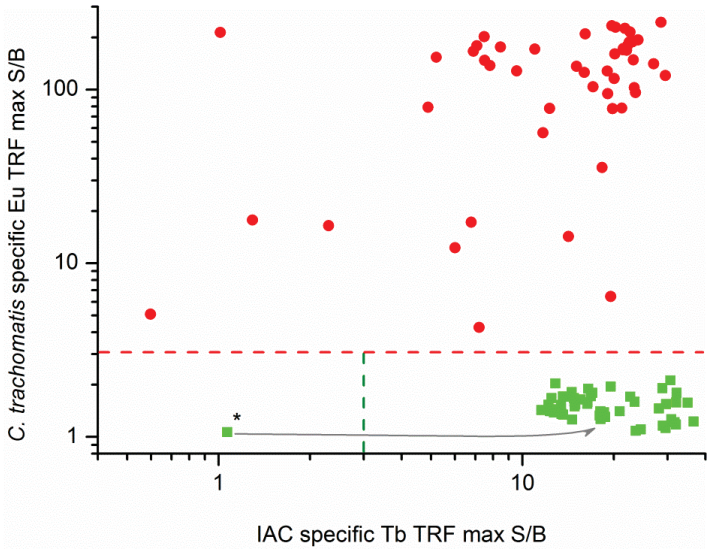

Figure 25. Complementation probe *Ct* assay results of the analyzed 100 urine samples illustrated as the maximum Eu TRF and Tb TRF S/B ratios. *C. trachomatis* positive samples shown in red circles (n = 50) and *Ct* negative samples in green squares (n = 50) according to the reference method. The cut-off level defining the *Ct* status of the urine sample is shown as a dashed red line (sample *Ct* positive if Eu TRF exceeds the cut-off level of 3). Tb TRF cut-off level verifying the functionality of the assay when sample is *Ct* negative is shown as a green dashed line. * One sample was inhibitory, and after additional dilution gave a negative result correlating with the reference method. (publication **III**)

According to 100 analyzed urine samples the complementation probe *Ct* assay performance was better than the performance of the previously presented homogenous rapid dry-reagent *C. trachomatis* PCR assay based on competitive hybridization and intrinsically fluorescent lanthanide chelates [48]. In both assays the same urine sample preparation method was used. More inhibition was reported in the competitive hybridization based *C. trachomatis* assay (3 reactions from a total of 148 urine samples were inhibited, and 2 samples remained inhibitory also after additional dilution). However, only small numbers of urine samples were analyzed in both tests and different DNA polymerase enzymes, devices, and temperature profiles were used. Therefore, it cannot be concluded that the better performance was solely achieved due to the complementation probe technology. However, the complementation probe *Ct* assay seems to have a good performance and the technology allows urine sample analysis after simple sample preparation.

Threshold cycles were reached relative early in the PCR amplification (mainly in cycles 24–26). Short amplification time would be beneficial for rapid nucleic acid diagnostics. The complementation probe *Ct* assay was performed by manually moving the reaction plate between the thermal cycler and measurement device making the assay time-consuming. For rapid *Ct* diagnostics the assay should be performed with automated thermal cycling containing a TRF measurement device, and without real-

time measurement that is not required for qualitative measurement. The reagents should be in a self-contained package, for example, dried in the reaction chamber. Although the centrifugation-based sample preparation was relatively rapid, it requires some expertise as well as a centrifuge, and might be cumbersome especially for point-of-care diagnostics. Instead of the centrifugation, a simpler alternative could be, for example, based on *Ct* bacteria concentration already during the sampling using the FirstBurst urine collector [193]. The collector concentrates the *Ct* cells and, therefore, it might be possible to use a urine sample for analysis directly by only diluting the sample, assuming that the inhibitors are not concentrated during the sample collection.

5.2.2 HLA-DQA1*05 PCR assay

Increased sequence data due to the recent sequencing technology development have led to increased interest for genotyping, for example, using single-nucleotide polymorphism assays [194]. Genotyping can be used to determine risk for certain human diseases, for example, genome-wide association studies have yielded evidence of genetic association to type 1 and 2 diabetes, Crohn´s disease and coronary artery disease [195], and also certain types of cancer have been linked to specific SNPs [196]. Although a large number of SNPs have been identified, so far only a fraction of them have been linked to human diseases. Highly multiplexed whole-genome association studies are usually performed using heterogenous microarray-type chip-based methods [197, 198]. Homogenous FRET-based nucleic acid assays are commonly used to detect cases of a single or a few SNPs related to a known disease, for example, the Factor V Leiden point mutation linked to deep vein thrombosis [199].

Different alleles in human leucocyte antigen (HLA) gene region have been shown to have an impact in about 50% of the cases of type 1 diabetes (T1D), and HLA-DQA1*05 is one of the alleles used in T1D risk calculation. Type 1 diabetes is an autoimmune disease leading to pancreatic β cell destruction and, to a lack of (or insufficient) insulin production [200].

A complementation probe HLA-DQA1*05 end-point PCR assay was developed to study the suitability of the complementation probes to detect single-nucleotide polymorphisms and to convert the routinely used (at the Immunogenetics Laboratory, University of Turku, Finland) heterogenous DELFIA technology-based HLA-DQA1*05 PCR assay [201] to a homogenous format. A Eu^{III} carrier probe and two different antenna probes specific to the HLA-DQA1*05 allele were designed. The antenna probes contained the polymorphism detection site (a minimum of two-nucleotide difference between different alleles) and, therefore, the discrimination of the alleles was mainly based on the antenna probe hybridization. Because it is difficult to design an oligonucleotide probe specific only to the target sequence in a particular temperature using current oligonucleotide designing tools. Two different antenna probes with a two nucleotide difference in the sequence length (Antenna probe 1 with 10 nucleotides and Antenna probe 2 with 12 nucleotides) were prepared as a fixed measurement temperature of 24 °C was used because of the requirements of the measurement device as well as practice limitations. The principle of the

complementation probe HLA-DQA1*05 end-point PCR assay is illustrated in Figure 26.

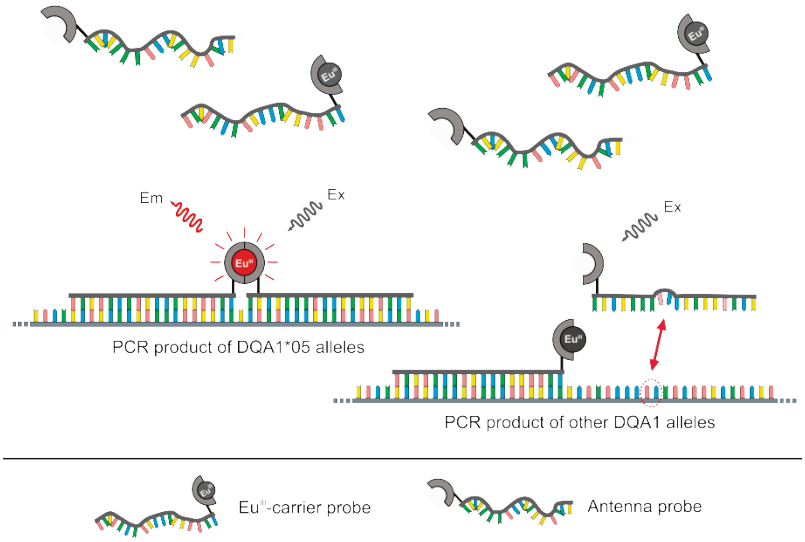

Figure 26. Complementation probe HLA-DQA1*05 PCR assay principle. The probes hybridize to the amplified target DNA at the measurement temperature of 24 °C forming a fluorescent lanthanide chelate complex. The allele discrimination is based on the antenna probe containing the polymorphism detection site. (publication **IV**)

The allele discrimination power of the complementation probes was studied first in the homogenous hybridization assays containing the probes and two different synthetic single-stranded DQA1 allele sequences. The DQA1*05 target was complementary to the probes, while the DQA1*02 target had a two nucleotide difference at the polymorphism site. The probe and synthetic target sequences are shown in Table 5. In the hybridization assay both reactions containing the DQA1*05 target sequence and either the Antenna probe 1 or Antenna probe 2 gave a similar Eu TRF signal, but the longer Antenna probe 1 yielded a high Eu TRF signal also from the hybridization reaction with a non-target allele DQA1*02 (Figure 27).

A melting curve analysis using the Eu^{III} carrier probe, Antenna probe 1 (both 25 nM) and three different synthetic oligonucleotide targets (DQA1*05, DQA1*02 and DQA1*04) was performed to determine a suitable measurement temperature for the Antenna probe 1-based DQA1*05 detection. The melting curve analysis showed clear discrimination between the DQA1*05 and the two other alleles at temperatures between 30 and 39 °C. The results of the melting curve analysis are shown in Figure 27. The assay set-ups are described in detail in publication **IV**.

Table 5. HLA-DQA1*05 PCR assay probe sequences and synthetic oligonucleotide target sequences (complementary target sequences shown in the table) used in the hybridization assay and melting curve analysis.

Oligonucleotide	Sequence 5'—3'
Antenna probe 1	TGAACAGTCTGA
Antenna probe 2	TGAACAGTCT
Eu^{III} carrier probe	CCTAAAACATAAC
Target DQA1*05 allele	GCTGTCCTAAAACATAACTTGAACAGTCTGATTAA
Target DQA1*0201 allele	GCTGTGCTAAAACATAACTTGAACATCCTGATTAA
Target DQA1*04 allele	GCTGTGACAAAACACAACTTGAACATCCTGATTAA

Nucleotides defining the DQA1*05 allele underlined in the Antenna probe sequences and all the divergent nucleotides in the synthetic target sequences marked in gray.
(Complementary target sequences illustrated here for easier visual comparison)

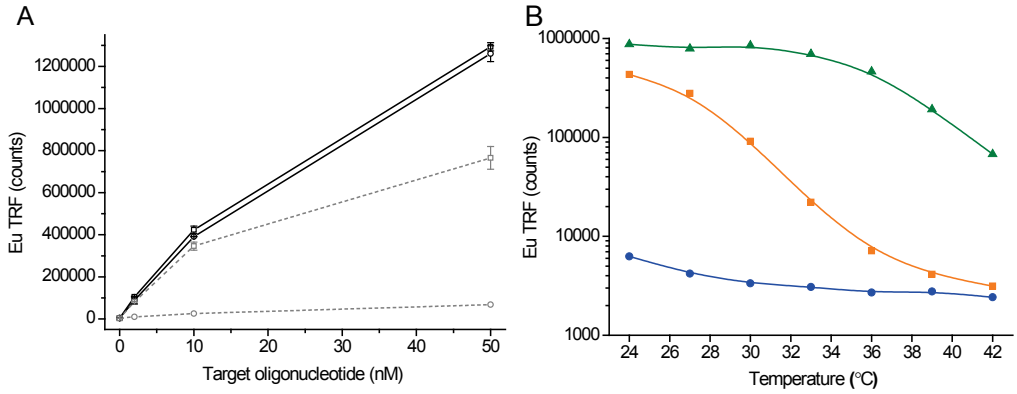

Figure 27. Homogenous hybridization assay (A) and melting curve analysis (B) showing the ability of the HLA DQA1*05 complementation probes to detect the HLA DQA1*05 allele. (A) The Eu^{III} carrier probe and Antenna probe 1 (boxes) or Antenna probe 2 (circles) hybridized to the synthetic DQA1*05 oligonucleotide (black solid line) and to the DQA1*0201 (gray dotted line) at the measurement temperature of 24 °C. (B) The Eu^{III} carrier probe and Antenna probe 1 hybridization to the synthetic oligonucleotide target DQA1*05 (green), DQA1*0201 (orange) and DQA1*04 (blue) in different temperatures. (publication **IV**)

The complementation probe HLA-DQA1*05 end-point PCR assay was evaluated by testing 147 blood samples. The samples were collected on the protein saver cards (Whatman 903 Protein Saver Card, Maidstone, UK) and the DNA was liberated from the dried blood sample punches by rapid cell lysis treatment using high temperature and alkaline reaction conditions. After the lysis treatment the reaction was briefly centrifuged, and the supernatant was analyzed with the complementation probe HLA-DQA1*05 PCR assay. The target DNA was PCR-amplified for 40 amplification cycles followed by Eu TRF measurement at the measurement temperature of 24 °C. The samples were also analyzed with the DELFIA technology based HLA-DQA1*05 PCR assay [201] that was used as a reference assay. The background Eu TRF signal level was

defined as the average signal from the non-HLA-DQA1*05 reactions (defined with the reference assay).

The complementation probe HLA-DQA1*05 PCR assay correlated 100% with the reference assay; 88 samples were genotyped to the DQA1*05 allele. A signal-to-background ratio up to 120, and an average S/B of 54 were measured. The results are illustrated inFigure 28.

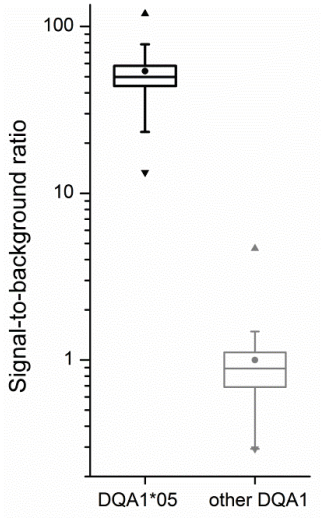

Figure 28. Complementation probe HLA-DQA1*05 end-point PCR assay results. The samples were genotyped as DQA1*05 allele and other DQA1 alleles according to the reference assay, and the S/B of the complementation probe HLA-DQA1*05 PCR assay is shown. A total of 147 blood samples were analyzed, and 88 of them were typed as the HLA-DQA1*05 allele. The box presents the interquartile range (25–75%) and the whiskers determine the 5th and 95th percentiles. The circles and horizontal lines in the boxes denote the average and median, respectively, and the triangles show the minimum and maximum S/B values.

One non-DQA1*05 sample yielded a Eu TRF S/B of 4.5 because one of the two parallel reactions gave a relatively high Eu TRF signal, although the sample was confirmed as non-DQA1*05 with the reference method (and also with other HLA-DQ genotyping assays, data not shown). The evaluation of the complementation probe HLA-DAQ1*05 PCR assay was performed in the laboratory where heterogenous PCR assays and inherently fluorescent europium probes are continuously used and, therefore, it is most likely that the signal of the one reaction was elevated because of an inherently fluorescent europium probe or PCR product contamination.

Definite detection of the HLA-DQA1*05 was achieved with a clear discrimination to other DQA1 alleles. Signal generation in the complementation probe assay is very specific because it requires the hybridization of the two probes to adjacent positions into the target DNA. In addition, genotyping based on the complementation probes is depending only on the hybridization of the probes to the target DNA at adjustable

measurement temperatures (if a measurement device with an adjustable temperature is available). Therefore, probe designing is simple, and very short probes can be used when necessary for optimal discrimination, for example, in genotyping GC rich sequences. Commonly used single probe genotyping methods like, for example, the ones based on the Hydrolysis probe technology require probe degradation by the DNA polymerase enzyme during the PCR amplification. Thus, a relatively long probe able to hybridize in PCR elongation temperatures (typically 60–70 °C) must be used. In general, shorter probes have higher SNP discrimination efficiency than longer probes because one nucleotide difference has a greater effect on the hybridization of a shorter probe. Another commonly used genotyping method, the Molecular beacon, is based on the stem-loop linearization by the hybridization of the probe to the target sequence. The stem-loop formation competes with the probe-target hybridization and can decrease the probe hybridization efficiency compared to the non-competitive methods.

As the sequencing technologies keep developing and, especially, when the costs decrease the whole-genome sequencing might become a routine practice in healthcare. Genotyping with current single-nucleotide polymorphism assays might be replaced by simply sequencing the whole genome. However, in addition to the sequencing cost there are other limitations, for example, the analysis of the huge sequencing data amount with current computers; most likely it will take several years or even decades before the current SNP assays are replaced by sequencing in routine practice. Despite the improvements in sequencing technology, SNP assays will also be used in the future, e.g., to detect somatic mutations related to certain types of cancer. Tumor cells in the bloodstream can be detected because of the cancer specific mutation, and a measurement of the circulating cancer cell level, as well as the measurement of the cancer cell level change in the tumor during the treatment, can be used to evaluate the effect of cancer treatment. To be able to detect mutated cells among the high amount of wild type cells the detection method must be extremely specific and sensitive. The complementation probe technology is highly specific and should be suitable for detecting the tumor cells that represent only a minor proportion of the whole cell count, and this should be studied. [9, 202, 203]

5.2.3 Rapid amplification-free bacteriophage quantification assay

Bacteriophages, also called phages, are bacteria-infecting viruses. In biotechnology they are used, for example, in protein engineering for the screening of large heterologous protein populations by a method called phage display. During a phage display, for example, when screening antibodies from large antibody libraries, the concentration of the bacteriophages needs to be determined. [204, 205]

The current ”gold standard” method for bacteriophage quantification is the plating method where bacterial cells are infected with a diluting series of phages and then plated on a selective agar plate containing antibiotics. After an overnight incubation the colonies are counted and the concentrations determined as colony forming units (cfu). Another commonly used bacteriophage quantification method is based on UV

photometry. Phages consist of protein and DNA and, therefore, light absorption of at 265 nm can be used for phage quantification. The plating method is laborious and time-consuming, and the UV photometry-based method relatively unreliable as any impurities in the sample interfere with the measurement and, therefore, the bacteriophages should be purified using ultracentrifugation requiring a centrifugation time up to 36 h. [206-208]

A rapid bacteriophage quantification assay was created by designing a Eu^{III} carrier and Antenna probes specific to a certain sequence in the M13 bacteriophage single-stranded DNA phagemid. The assay was based on a quantitative detection of the M13 bacteriophage ssDNA phagemid. A simple assay protocol was constructed. First, phages were lysed by incubating at 95 °C for 1 min. After phagemid liberation the temperature was cooled down to 25 °C where the probes were hydridized to the phagemid ssDNA, and the Eu TRF was measured. The assay can be used for the quantification of purified phage preparations and also for direct bacteriophage culture titer determination as illustrated in Figure 29.

A complementation probe bacteriophage assay standard curve was created by first determining the infectivity titer (cfu/mL) of the M13 bacteriophage preparation using the plating method. Then, reactions containing a dilution series of the phages and the complementation probes (10 nM Eu^{III} carrier and 5 nM antenna probe) were prepared, phages were lysed and Eu TRF measured as described previously. The suitability of the assay for direct bacteriophage culture quantification was demonstrated by culturing M13 bacteriophages and measuring the titer every second hour during a 20 h culture. A culture sample was withdrawn in every measurement point, the sample was prepared and Eu TRF measured as described in Figure 29. The Eu TRF signal was converted to cfu/mL by using the standard curve. The assay design and testing is described in more detail in publication **II**.

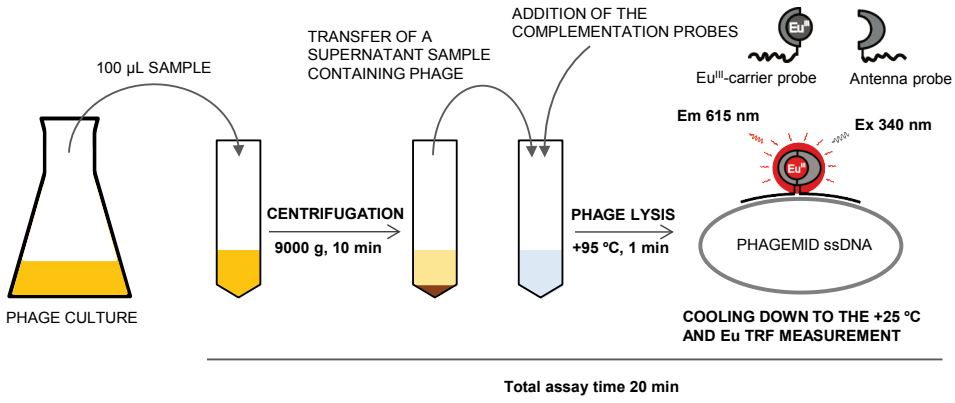

Figure 29. Complementation probe bacteriophage quantification assay principle. In direct phage culture titer determination the bacteria are separated by centrifugation, and a sample from the supernatant containing the phages is 1:7 diluted in the assay buffer containing the

complementation probes. Phages are lysed in high temperature, followed by the hybridization of the probes to the target phagemid and Eu TRF measurement at 25 °C. (publication **II**)

The standard curve showed a bacteriophage detection limit of 1.14 x 10^9 cfu/mL referring to similar low picomolar sensitivity reported previously. Parallel reactions yielded, on average, a 6.4% coefficient of variation showing good repeatability. The bacteriophage production monitoring was successful; in 4 h, the amount of phages had exceeded the assay detection limit, and the maximum phage titer was reached in 8–10 h. (Figure 30) The phage culture quantification was performed in 20 min at each measurement point.

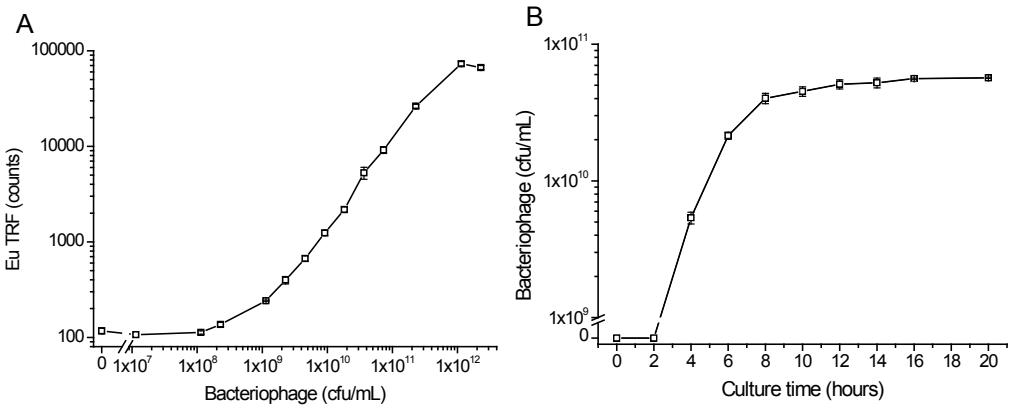

Figure 30. Complementation probe bacteriophage quantification assay (A) standard curve and (B) monitoring of the phage culture during a 20 h culture.

The bacteriophage quantification is based on the measurement of the phage ssDNA and, therefore, the phage size or the protein displayed on the surface of the phage, or impurities in the culture media that could cause variation in the absorbance-based measurement do not interfere with the complementation probes-based method. The same methodology could, most likely, be used for the quantification of any virus. The system could also be useful for the quantification of bacterial cultures when rapid and reliable results are needed and, or when the cultured bacteria tend to grow in clusters that would complicate the plating method-based quantification.

6 CONCLUSIONS

In recent decades, especially in the past 10 years the number of new molecular diagnostic methods and assays has significantly increased. New genotyping methods have been introduced and the interest in microbial diagnostics has turned from traditional phenotypic methodologies towards faster genotypic technologies. The growing sequence databases and the need for faster (and more sensitive) microbial diagnostics have generated this trend. However, in practice the traditional methods relying, for example, on cultures are still the most commonly used procedures in microbe identification.

The nucleic acid diagnostic assays for the detection of microbes causing infectious diseases will keep on developing and will most likely overcome the traditional culture-based methods. But before ruling the microbial diagnostic markets the nucleic acid assays must become more cost-effective and even faster. This could be carried out, for example, by multiplexing the assays so that different pathogens causing similar symptoms could be detected in one reaction. The sample preparation should be rapid and simple to carry out or totally automated. Nowadays, one cause of delay in many healthcare districts is sample transportation from the sampling unit to the place of analysis because sophisticated automated nucleic acid amplification assays are mainly located in central laboratories. Affordable and rapid assays could also be used at small clinics and in resource-poor settings. Rapid and multiplexed high-performance assays would be ideal especially in urgent situations, e.g., in life-threatening bacteremia where an early identification of the microbe as well as antibiotic resistance detection would allow early start of the correct medication. In addition, the diagnostic platform should be flexible allowing individual sample analysis initiation at different times because at the moment samples are usually analyzed in a batch that is another major cause of delay of the analysis.

In this study, the hybridization-directed fluorescence lanthanide complex formation method, here also called the switchable complementation probe technology, was further improved and applied to homogenous PCR diagnostics. Furthermore, theperformance of the complementation probe-based PCR concept for infectious disease diagnostics and for genotyping was evaluated. In addition, amplification free quantitative ssDNA virus detection was demonstrated.

One of the main achievements of this study was the design and preparation of PCR-compatible complementation probes that maintained their performance (fluorescence properties) in the PCR reaction conditions. This gave a basis for the PCR assay applications. The cyclic structure of the designed new heptadentate lanthanide ion carrier chelate created a cavity for the Ln^{III} leading to a higher thermal stability compared to the linear lanthanide chelate. This showed that the lanthanide chelate stability is greatly related to the chelate structure and, therefore, it is possible to prepare stable lanthanide chelates without occupying all the Ln^{III} coordination sites. The heptadentate lanthanide ion carrier chelate structure allowed fluorescent complex

formation by the coordination of the tridentate light absorbing antenna ligand to the Ln^{III} and hence, an excitation energy transfer from the ligand to the lanthanide ion. A light absorbing antenna ligand for terbium was designed and synthesized and it allowed the preparation of duplex PCR assay. Two complementation probe pairs were required for a clinical assay where in addition to the target specific analysis an internal control is needed to monitor the assay functionality. (publications **I** & **III**)

The complementation probes-based closed-tube PCR assays yielded a very high signal-to-background ratio. The high S/B ratio was achieved because of a low background signal level, and there were two main reasons leading to low background fluorescence. First, the long fluorescence lifetime of the lanthanides allowed usage of time-resolved measurement mode and, therefore, the measurement of autofluorescence was avoided. Second, the lanthanides are very poorly excited directly and they require an organic light-harvesting ligand coordinated to the Ln^{III} for efficient excitation. The lanthanide ion carrier chelates and the respective ligand structures were inherently non-fluorescent causing no or only a minimal background signal. In addition, the non-specific binding of the probes or probe degradation do not lead to signal increase. (publications **I** & **III**)

The PCR assays and the bacteriophage quantification method indicated that a simple and rapid sample preparation can be used with complementation probes-based assays. The complementation probe *Ct* assay with the simple 10 min urine sample preparation correlated 99–100% with the reference assay, and the complementation probe HLA-DQA1*05 end-point PCR assay performed after simple DNA liberation from dried blood sample punches correlated 100% with the reference assay. Furthermore, the bacteriophage culture media was only diluted in the assay buffer containing the probes after the bacterial cells were separated from the phages by centrifugation. This leads to the conclusion that complementation probes-based high-performance nucleic acid assays can be developed using a simple sample preparation instead of the commonly used DNA extraction. Simple sample pretreatment should be relatively easy to automate creating a cost-effective system. (publications **II, III & IV**)

Target DNA amplification by PCR using only slightly prepared samples has been proved to be successful previously and was proved to be efficient also in this study [48, 201, 209]. Nowadays, there are also DNA polymerase manufacturers providing so called direct PCR kits and it is stated that DNA can be amplified directly, for example, from several different human specimens, animal tissue and plants without DNA extraction. These different kits could be used together with the complementation probe technology for rapid diagnostics using different types of specimens. Although, the complementation probe technology-based assays showed that urine, blood and bacterial culture media are suitable to be used after a simple processing, the performance of the complementation probes in different sample materials, e.g., stool and sputum needs to be studied to find out what kind of samples can be analyzed directly. Different samples have different composition and, for example, stool samples are very heterogenous and the composition varies depending of the individual's diet. The antenna molecules have carboxylic acid groups that are involved in the

coordination of the antenna ligands to the lanthanide ion carrier chelates. The antenna ligand might be vulnerable to samples containing high amounts of impurities that could be bound to the antenna by weak interactions preventing the fluorescent lanthanide complex formation.

More multiplexed homogenous nucleic acid assays could be performed by applying samarium and dysprosium to the complementation probe technology with suitable antenna ligands. For example, the Eu^{III} and Tb^{III} antenna ligands should be functional with the Sm^{III} and Dy^{III}, respectively, because of similar energy levels. However, samarium and dysprosium have shorter emission lifetimes (Sm^{III} 20–50 µs and Dy^{III} 1–20 µs) and lower fluorescence intensity than europium and terbium, and this would most likely lead to decreased nucleic acid detection performance in homogenous PCR compared to Eu^{III} and Tb^{III} complementation probes. [41, 63] Instead of using different lanthanides, highly multiplexed homogenous PCR assays could be prepared using one of the complementation probes (the lanthanide ion carrier probe or the antenna probe) as a capture probe by coupling it to the solid phase into the PCR reaction chamber. The capture probe and the other probe in the reaction solution would hybridize to the amplified target DNA and form a fluorescent lanthanide complex. A proof-of-principle complementation probe solid-phase array was recently published using synthetic oligonucleotide targets in a room temperature hybridization assay [210]. A complementation probes based homogenous PCR microarray would allow the preparation of rapid high-performance multiplexed PCR assay applications.

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