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Microfluidics as a tool for rapid biomolecular detection

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Abstract

Here we present a new, fast and sensitive technique for DNA detection using a partial solid phase Circle-to-circle amplification in a microfluidic system. This can hopefully later be integrated into a new portable bio-monitoring system for rapid and efficient detection of hazardous microorganisms.

Keywords

Microfluidics, solid-phase, Circle-to-circle amplification, DNA detection

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Microfluidics as a tool for rapid biomolecular detection

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Sammanfattning

Idag finns det ett stort intresse att utveckla nya metoder som snabbt kan upptäcka mikroorganismer, särskilt de arter som är involverade i mat- och vattenföroreningar, bioterrorism samt infektionssjukdomar som t.ex. fågelinfluensa. En tidig upptäckt av smittor är kritisk, men dagens metoder är långsamma, besvärliga och inte tillräckligt specifika. Att undersöka ett prov efter bakterier, virus eller mikroorganismer och sedan identifiera organismen tar i dag timmar upptill dagar.

Undersökning av förorenade områden skulle underlättas med ett transportabelt instrument som kan utföra en snabb och specifik analys. Detta mobila instrument skulle kunna ge värdefull information för att hitta smittokällan samt kunna hjälpa läkare att snabbt ge den rätta behandlingen.

Här beskrivs en ny metod med potential för snabb och känslig DNA-detektion genom användandet av fastfas i ett mikrofluidiksystem. Detta kan förhoppningsvis sedan bli integrerat i ett nytt transportabelt instrument för snabb och effektiv upptäckt av farliga mikroorganismer. Det här är ett nytt projekt och behöver därför omfattande optimering och nytänkande. Det centrala är utveckling av reagens, detektorsystem, hård- och mjukvara etc. Under mitt examensarbete arbetade jag främst på den molekylära delen med optimering av protokoll och design av DNA-system men även mer tekniska delar, så som design av mikrofluidiksystem och pumpuppställning, berördes.

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Executive Summary

Today there is a large interest in methods for detection of microorganisms especially those species that are involved in air, water and food contamination, infectious diseases e.g. avian influenza, and biological weapons. Early detection is critical, but today's methods are often slow, laborious and not specific enough. Present cultivation-based methods for detection of bacteria, viruses or microbes take days while detection methods using PCR take a few hours.

Analysis of contaminated areas would be greatly facilitated by a transportable instrument that is capable of rapid detection and characterization of a variety of microbial pathogens. This mobile instrument would aid the physicians to give fast and correct medical treatment but it will also give rapid results that can be useful in finding the source of an outbreak.

Here a new, fast and sensitive analysis method for DNA detection is presented. The method combines a solid-phase based capture and amplification with a second step of homogenous amplification in a microfluidic system. Our goal is that the final analysis only will take approximately one hour compared to the current five hours. The molecular method is suitable for integration in a new transportable bio-monitoring system for rapid and efficient detection of hazardous microorganisms currently being constructed in parallel. As this is a novel project the system needs vast optimization and new thinking. The most critical stages in need of optimization/development is the design of the molecular recognition reagents, choices and optimization of the solid phase, choices of detector systems, development of hard - and software etc.

The first investigations focused on determining the characteristics of the solid phase, consisting of streptavidin-coated Sepharose beads. I investigated specificity of the biotin-streptavidin bonds, if the beads were autofluorescent and if the hybridization between the oligonucleotides is selective. The experiments showed low autofluorescence, that the biotin-streptavidin bonds seem to be specific and that the oligonucleotides are selective. I also determined that both ligation and RCA can be performed on the beads. A new oligonucleotide system (System A) was designed especially for this purpose and this system was used exclusively in the rest of the experiments. In the homogeneous phase the temperature and time dependence on the RCA was examined. The results show that the temperature has a great influence, but between 30°C and 37°C the difference is not so prominent. The efficiency of Circle-to-circle amplification (C2CA) was investigated. C2CA is an extended version of RCA where the created RCA products are digested to monomers. These monomers are thereafter religated and amplified a second time using RCA. The results could verify that about a thousand-fold amplification can be achieved within one hour of amplification, proving a high efficiency of the C2CA. To reach the goal of a final analysis in one hour the amplification time has to be reduced. Experiments made show that the time used in both first and second generation of RCA is needed to be addressed, so that the total amplification time is optimized giving a good amplification and sensitivity but still having a high signal to noise enabling detection.

The microfluidic system was designed along with a multichannel pump set-up making it possible to run 8 different samples simultaneously. After initial evaluation we decided to use positive pressure for liquid handling against the more easily handled negative pressure. This was to avoid problems with air leakage, which will lead to lagging of fluids and flow difference which may increase the variability between samples. With the positive pressure setup all the solutions are first loaded in the sample loop of each syringe. Then the column is connected and the solutions are pumped through the column. In the evaluation of the oligonucleotide system designed to "mimic" a realistic detection of a genomic bacterial fragment, the conclusion was made that there is a big variation between samples with identical properties but the fact that the system is working enables further optimizations. The variation can be caused by too high flow in the column, ripping RCA products off the beads, or the retention of free padlock probes and sandwich probes in the tubings and between beads. To further examine the oligonucleotide system two pilot experiments were performed using other analogous techniques. A microarray experiment shows that concentrations, of both padlock and restricted second degree RCA products, down to 1pM could be detected on the array. A qPCR experiment was supposed to measure how much probe that is actually captured in the solid phase, but further experiments need to be performed using new tubing to minimize the background noise. Many more experiments need to be performed before this can be a final method. Especially on the new oligonucleotide system where the variation needs to be dealt with, but also general things like temperature, time, concentration and volume needs to be optimized on-and off-column.

This is a fast evolving area of research and if this technique will work as planned and if it later is incorporated in to a bio-monitoring system that system has the potential to be "State of the art". The system has potential to be robust and reliable, offer a large quantitative dynamic range, distinguish and find trace levels of pathogenic agents against a background of abundant non-pathogenic species and it will offer single-molecule detection (SMD) and thus digital read-out.

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1. Introduction

Today there is a large interest in methods for the detection of microorganisms, especially with those species that are involved in food and water contamination, avian influenza, clinical cases and biological weapons. This interest is now even greater than before as a result of the increased incidents of EHEC, *Salmonella, Clostridium* and *Campylobacter* being found in food and water, high concentrations of *Legionella* in water basins and the use of anthrax and ricin in acts of bioterrorism.

Rapid and early detection are critical to many military and civilian applications, such as food and water safety, defense against biological weapons, and diagnostic microbiology but the detection and identification of microorganisms is slow and difficult today. Present methods for detection of viruses, microbes or their DNA motifs are not specific enough and take from hours up to several days.

Analysis of contaminated areas would be greatly improved by a transportable instrument capable of rapid detection and characterization of a variety of microbial pathogens. This mobile instrument could give results that will aid physicians and veterinarians to give humans and animals fast and correct medical treatment. Fast information can also reduce the time for finding the source of an outbreak.

Here a new, fast and sensitive analysis method for DNA detection is presented. The method will combine the molecular method Circle-to-circle amplification with a solid phase molecular capture in a microfluidic system with a goal that the final analysis only will take one hour compared to the current five hours. This can potentially later be integrated in to a new transportable bio-monitoring system for efficient and specific detection of hazardous microorganisms.

1.1 Background

1.1.1 Microfluidics

Manipulation of fluids in channels with micrometer dimensions is a fairly new field called microfluidics. Microfluidics is at an early stage of development and it is believed to be the next step in evolution of point of care /point of need based diagnostics. It all started in the 1970s at Stanford University with a miniaturized GC device (7), but not until 1990, when the microfluidics research was funded by US Defense Advanced Research Projects Agency (DARPA), did microfluidics receive attention. DARPA wanted to develop portable and easy to use detectors for chemical and biological weapons spurring increased academic interest.

The microfluidic technology is developing very rapidly and is today central in a number of developing miniaturized systems in chemistry, biology and medicine. These miniaturized devices that are capable of performing complete laboratory tasks are called Microscale Total analysis system (μTAS) and this total analysis consists of e.g. sample preparation, amplification and detection units (3). There are many benefits using μTAS compared to conventional sized systems; the reduced consumption of samples and reagents which also reduces the chemical waste and cost, the shorter analysis time, higher sensitivity and resolution, portability and disposability (2). Other advantages using microfluidics is that the micro size minimizes the diffusion distances, closed systems make evaporation impossible, it is easy to automate so no expert operator is needed, and it is easier to have control of concentrations and interactions (4). The disadvantages are among others the difficulties with: interfacing (integration of pumps and such), what material to use, gas bubbles, surface tension, clogging and the lack of monitoring systems (4). So far a few sophisticated μTAS have been created; today the most advanced systems measure DNA and RNA by using PCR for sample amplification and elecrophoretic sizing or real time probes for analysis (5).

Miniaturization affects fluid properties. Reynolds number (Re) is one of the most important numbers in fluid dynamics and aerodynamics. It is dimensionless and describes fluid and gas properties in terms of flow, measuring the ratio of inertial forces to viscous forces. Fluids or gases with Reynolds numbers higher than 3000 have turbulent flow while Reynolds number below 2000 have laminar flow (10). In microfluidic systems the Reynolds number is around 100 making the flow completely laminar, meaning that only diffusion contributes to mixing. This can be a limiting factor, since the reaction rate depends on the diffusion rate. To reduce this problem, several systems that increase the mixing have been designed. Miniaturization also affects the surface-to-volume ratio. The high surface-to-volume and the low masses in microfluidic systems leads to a fast diffusion but also to a high thermal transfer efficiency which contribute to controllable and efficient exothermic or/and high temperature reactions without thermal effects (6).

To build a fully functional microfluidic system there is a need to incorporate different components such as pumps, valves, mixers and of course detectors. The majority of the detectors used today measure changes in fluorescence, since the fluorescent methods are well developed and offers high sensitivity. External microscopes for example EPI-fluorescence- or confocal microscopes are also commonly used for detection, but if the systems should be easy handled or/and transportable built-in detectors, or optofluidics, are necessary. The optofluidic technology combines optic detection with microfluidics making the detection integrated, but it still face several difficulties such as size, cost, sensitivity, requirements for power among others (3, 17)

1.1.1.1 Casting of PDMS

Poly(dimethylsiloxane) or PDMS is a widely used polymer in microfluidics because of its good characteristics. The positive properties of this polymer are that it is inexpensive, non-toxic, biocompatible, optically transparent down to 280nm and that its surface chemistry can be controlled making either reversible or covalent modifications. Furthermore PDMS can be covalently bonded to a wide range of other materials such as itself and glass. It is very hydrophobic but can be converted to a hydrophilic form by plasma treatment. Its elasticity also allows PDMS to be reversibly deformed and an excellent material to make microchannels or other structures in, and it is also easy to incorporate tubings, valves, pumps and such in the PDMS systems (3, 13).

Traditional silicon machining and replica molding are used to fabricate micro channels. A design for the channels is created using computer CAD software, followed by construction of a mould master. This master can be used to form channels by replica molding, casting PDMS against it. To seal the channels the surface of the replica is exposed to air plasma. This creates polar groups on the surface that can irreversibly bond to another air plasma exposed surface, such as a glass or PDMS, forming closed microchannels (13).

1.1.2 Rolling Circle Amplification

The rolling circle amplification (RCA) is a biomolecular detection tool that uses the principles of the rolling circle replication mechanism (RCR). RCR is a process where nucleic acids are replicated rapidly by synthesizing multiple copies of circular molecules of DNA or RNA, such as plasmids, the genomes of bacteriophages, circular RNA genomes of viroids and even some eukaryotic virus genomes(10).

The use of linear oligonucleotides named padlock probes, first described by Nilsson et al. (19), is central in RCA. These padlock probes are designed so that the ends of the linear oligonucleotide base-pair next to each other on a target sequence. If both 5' and 3' end are correctly hybridized to its complementary target sequence the linear padlock probe can be circularized by ligation. This circle then acts as an endless template so that a linear concatemer, consistent of many tandem complementary copies of the circle, is created by that rolling circle replication. The most widely used DNA polymerase in the RCA originates from *Basillus subtilis* phage phi29. It can copy a 100nt circular oligonucleotide into a linear strand with 1000 copies in one hour and it has outstanding strand displacement properties (8), (11).
The RCA product created collapses into a random coil, roughly 1μm in size, when free in solution. Each of the copies have a detection site for a fluorophore-tagged oligonucleotide (detection oligonucleotide, DO), which makes them visible in a fluorescence microscope. The enrichment of fluorophore concentration in the RCA product compared to background enables homogenous detection without prior washes. Therefore making this method suitable for single-molecule detection (SMD) where individual RCA products can be detected and counted(12), see Figure 1. These specific detection oligonucleotides can be manufactured with different fluorophores, making multiplexed pathogen detection possible.

This method can also be used to detect proteins with the use of antibodies, called proximity ligation assay (PLA), Figure 1. A single stranded oligonucleotide is coupled to an antibody as a unique identifier of that antibody, and is then called a proximity probe. Two kinds of proximity probes are added to a sample where each antibody binds to a specific epitope of the same antigen. If both the proximity probes have bound to same antigen, hence are in proximity to each other, the two oligonucleotides can be ligated together by a third oligonucleotide, called a splint, that hybridizes to the ends of the two oligonucleotides. This ligated DNA molecule is restriction digested close to each antibody and the released products can be circularized by an intact restriction oligonucleotide followed by amplification using RCA (14), (16).

The strict requirements needed for enzymatic ligation and the high amplification efficiency contribute to the high sensitivity and specificity of the RCA. Due to the exquisite specificity, prominent detection signals, and low background, it has been demonstrated that individual DNA sequences and single or interacting sets of proteins can be detected in situ using PLA combined with rolling circle replication (20).

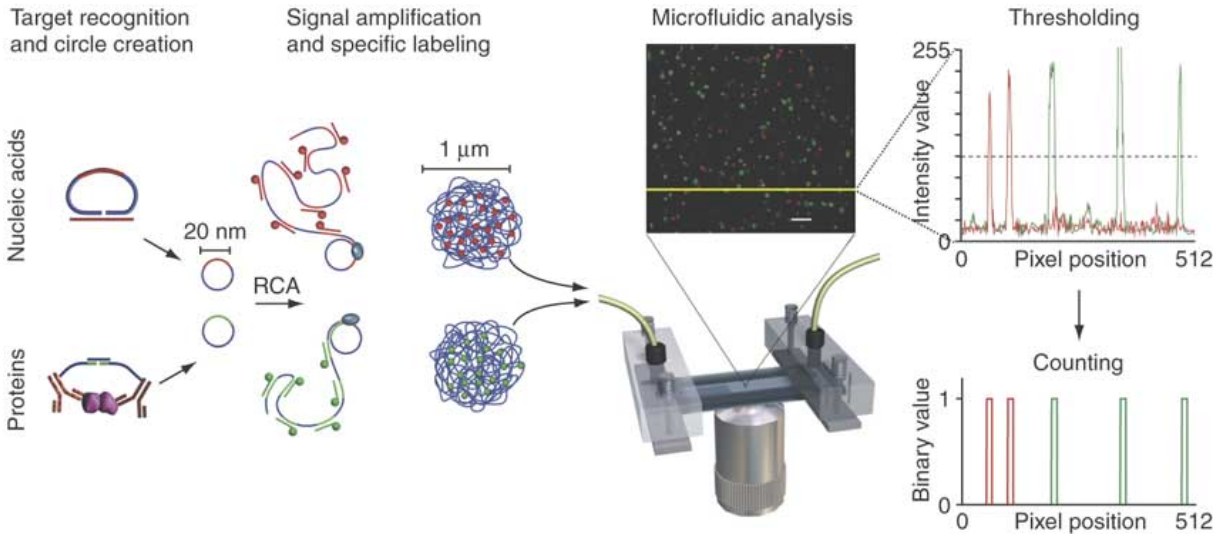


Figure 1. Molecular recognition of a) DNA with padlock probe ligation b) protein with proximity ligation. Formed circles are used as templates in RCA making the linear concatemer, consistent of many tandem complementary copies that will collapse into a coil. Each of the copies has a detection site for a fluorophore-tagged oligonucleotide (DO) which can make them visible in a fluorescence microscope and the fluorophore concentration is therefore going to be much higher in the RCA product coil than the background of free fluorophores. The sample containing RCA products are pumped through a microchannel and a confocal microscope detects the RCA products in line-scanning mode. The raw data is set to a threshold and a binary output file is created that allows the RCA products to be counted. Reprinted by permission from Macmillan Publishers Ltd: NATURE METHODS (Jarvius et al), copyright (2006)(12).

1.1.3 Circle to circle amplification

An efficient way to increase the sensitivity of the RCA even more is to use circle to circle amplification (C2CA) (9). C2CA is an extended version of the RCA, see Figure 2. Here the first generation of RCA product is monomerized by a restriction enzyme. The starting padlock probe has a restriction site and after the first RCA an excess of a restriction oligonucleotide (RO) is added, this RO hybridizes to the site at every repeat. After enzymatic digestion the sample is heated to deactivate the restriction enzyme as well as dissociate the digested fragments. When the temperature is lowered, intact RO hybridizes to the ends of the monomer and since it is energetically preferable to anneal to both ends on same monomer, instead of forming linear concatemers, the monomer is circularized. The intact RO can thereafter serve as a ligation template and a primer in the second generation of RCA (9). This recircularization gives a thousand fold more circles than the starting material, if the first generation RCA lasts for one hour. These circles are now ready for a second generation of RCA, resulting in RCA products with same polarity as the original padlock probe. These second generation RCA products can be detected the same way as the first RCA products but with a DO having a reverse complementary sequence compared to the first generation DO. This process can of course be further repeated to yield even higher amplification and therefore sensitivity.

There are many advantages using C2CA. It enables a high precision strand-specific amplification and since the C2CA is not product inhibited it can yield 100-fold higher monomer concentration than the commonly used PCR. C2CA has also previously been used for multiplexed genotyping of polymorphic loci and for quantitative DNA analysis, with good results (14).

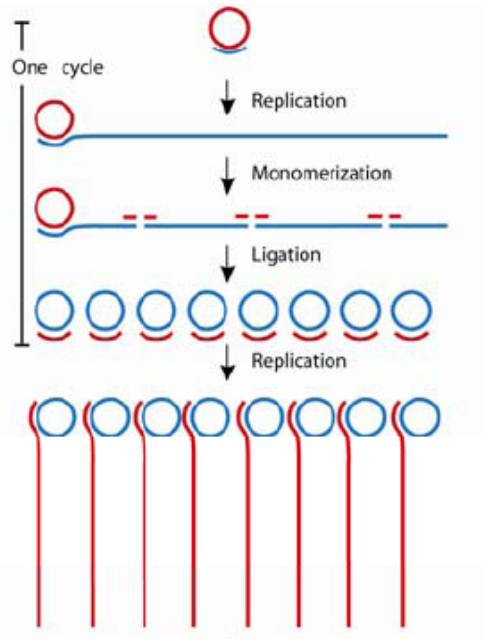


Figure 2 A schematic illustration of the C2CA procedure. After the first RCA the product is monomerized, re-ligated and amplified again. The two polarities of DNA are drawn in red and blue, showing that the second generation RCA product has the same polarity as the padlock. Reprinted by permission from Macmillan Publishers Ltd: NATURE METHODS (Jarvius et al), copyright (2006)(12).

1.1.4 Quantitative polymerase chain reaction

The polymerase chain reaction (PCR) amplifies DNA samples, with a temperature-stable enzyme; DNA polymerase. Theoretically the DNA is exponentially amplified, doubling the number of molecules with each amplification cycle, see Figure 3. The number of cycles and the amount of the PCR end-product can therefore be used to calculate the initial amount of DNA using e.g. an ethidium bromide staining, but several factors complicate this calculation. The PCR reaction is not exponential for the first several cycles, and it will also reach a plateau over time, forcing the measurements of the final amount of DNA to be done while the reaction is in the exponential growth phase. To overcome these difficulties, several different quantitative methods have been developed.

Quantitative polymerase chain reaction (qPCR) is a modified version of the polymerase chain reaction that is used to measure the quantity of DNA or RNA in a sample. The most sensitive quantification methods are done by the real-time polymerase chain reaction, where the amount of DNA is measured with the use of fluorescent markers. These markers attach in double stranded DNA or specific to the single stranded target and the fluorescence can be measured after each cycle of PCR, see Figure 3c.

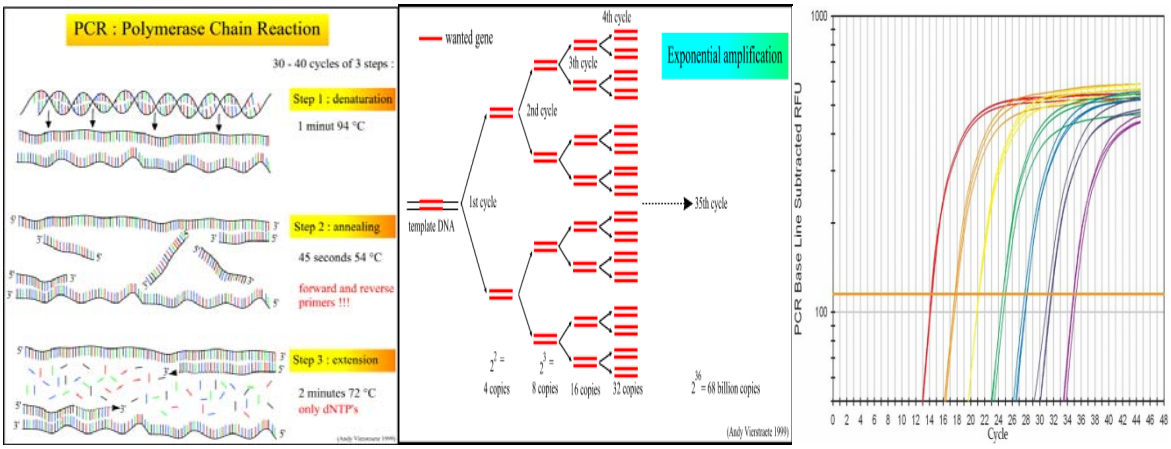


Figure 3 a) Schematic picture of the polymerase chain reaction (PCR). How the double stranded DNA is denatured, how the primers anneal and how two new strands are created from the primers and free dNTPs. b) Schematic picture of the exponential amplification in the PCR. c) Picture showing the results from a typical qPCR run on a dilution series. The DNA signal is exponentially increased each cycle until it reaches a plateau. Pictures is reproduced with permission from Andy Vierstraete and from USB, now a part of Affymetrix, Inc., 2008" (17, 18).

1.1.5 Microarray

Microarray is a commonly used high-throughput tool in molecular biology and in medicine. Two types of microarrays are available; commercial and in-house produced. Microarrays consist of microscopic spots on a solid surface in an array, where each spot contains small amounts of an oligonucleotide with a specific DNA sequence, which is complementary to a DNA or RNA sample, the target. The attached oligonucleotides work as probes which the target hybridize specifically to under high-stringency conditions, see Figure 4. In standard DNA microarrays, the probes are attached to a solid surface, normally glass or a silicon slide, by a covalent bond to a chemical matrix (via epoxy-silane, amino-silane, lysine, polyacrylamide or others). "Spotted microarrays" is a technique used to produce in-house manufactured microarrays. The probes are deposited onto a glass slide with an array of fine pins or needles controlled by a robotic arm that is dipped into wells containing the probes and then depositing each probe at chosen locations on the array surface. This results in a grid of probes ready to be hybridized to complementary ssDNA. The most commonly used probes are synthesized oligonucleotides however cDNA or PCR fragments can also be spotted. These arrays are easily customized for each experiment, choosing the probes and printing locations on the arrays, at a relatively low-cost (10, 21).

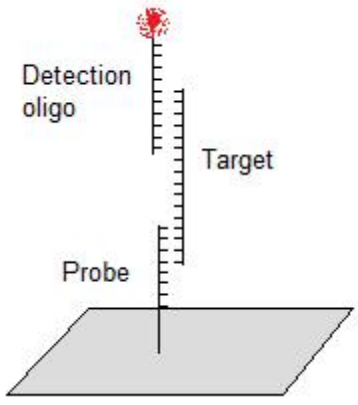


Figure 4. Schematic picture of a microarray. The target hybridizes to the probe and the detection oligonucleotide hybridizes to the target giving a signal.

Hybridization between the probe and its target is usually quantified by fluorescence, where target specific oligonucleotides labeled with fluorophores are used to determine the relative abundance of the target, see Figure 4. Microarrays can also be used to detect single nucleotide polymorphisms (SNPs) or measure changes in expression levels (10, 21).

1.2 Aim

With current methods it is possible to make sensitive and selective detection of bio-molecules, but the analysis is often laborious and time consuming. The overall aim with this project is to create a new, fast and specific analysis method. The method will combine the molecular methods of padlock probe sample recognition coupled to circle-to-circle amplification in a solid phase microfluidic system. The goal is to perform the final analysis within one hour; today this method takes about five hours. The final aim with this project is to develop a combined detection and identification bio-detector for rapid and accurate identification and characterization of hazardous microorganisms which have been released to nature either by accident or on purpose (biological warfare). To reach this final goal the possibilities to simplify the bio-detector to a transportable and rapid unit will be investigated.

This is a novel project and this system needs extensive optimization and innovative thinking. The most critical stages in needs of optimization/development is the design of the molecular recognition reagents, choices and optimization of solid phase, selection of detector systems, development of hard- and software etc. The focus of my work was the molecular part, optimizing protocols of the sample preparation and to design probes etc. Also more technical parts were concerned, like designing the microfluidic system, casting PDMS and the arranging of the pump set-up.

1.2.1 The experimental set-up

The sample preparation consisted of a combined solid-phase and microfluidic system. The protocol started with a series of solid-phase sample processing steps, in which molecular recovery and amplification reactions take place. Microfluidics and solid-phase processing will together minimize the time required for each step and will potentially increase sensitivity due to more efficient target molecule recovery and molecular probing.

Streptavidin Sepharose High Performance (GE, Uppsala) was used as a solid phase. Sepharose is a rigid cross-linked agarose polymer with high chemical stability and these beads were coated with streptavidin. To the beads a biotinylated capture oligonucleotide was bound. The biotin-streptavidin interaction is very strong but not covalent, and can be broken with denaturation conditions or by competition. These beads were packed in a micro-column and attached to a microfluidic system. The fluids in the microfluidic system are driven by a computer controlled 8-channel syringe pump, allowing 8 separate microfluidic systems with columns to be run simultaneously.

Figure 5 gives a schematic view of our experimental set-up. Initially a sandwich probe is pumped through the column, simulating a future target that will be a genomic fragment from a microorganism. It is complementary to a part of the capturing oligonucleotide bound to the solid phase and hybridizes therefore. Then a padlock probe is pumped through together with ligase. When the padlock hybridizes correctly to the sandwich it is ligated to form a circle. This circle works as a never-ending template for RCA. For on-bead amplification a solution containing DNA-polymerase is pumped through the system, generating long ss-DNA amplification products bound to the beads. The RCA product is then digested and released from the beads with a restriction enzyme. The now monomerized RCA products are collected in a tube. To further enhance the signal the monomers are re-ligated to circles with intact restriction oligonucleotides serving as ligation templates and then a new round of RCA is performed, resulting in a partial solid phase C2CA. These second generation RCA products can be labeled with a fluorophore and detected with a confocal-microscope or in the future a simplified portable detector.

The reason of using a solid phase in a microfluidic system is mostly to reduce the mass transport or the diffusion time. By pumping the solutions inside a small microchannel filled with beads the analytes do not have to diffuse so far to find its ligand/target, shortening the total analysis time. High concentrations of padlock probes have been shown, by the Landegren group, to inhibit the RCA. The pumping of fluids in the microfluidic system will wash away the unreacted padlock probes, enabling a usage of high concentration padlock probe to increase the sensitivity without the RCA inhibition. This pumping will also wash away reagents that would interfere later in the experimental chain.

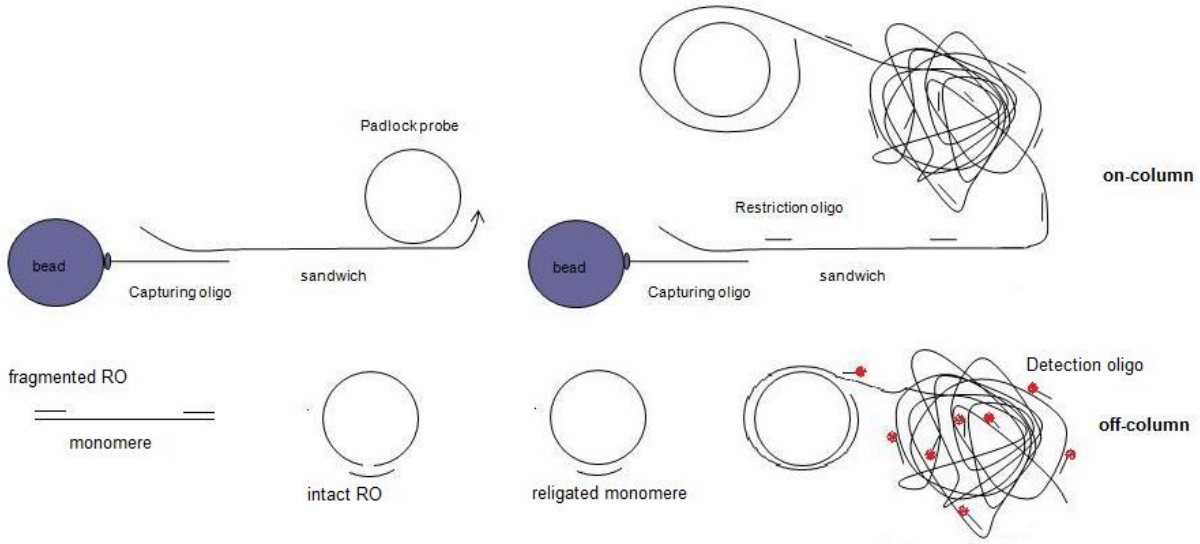


Figure 5. Schematic picture of the sandwich assay on- and off column. First the capturing oligonucleotide is coupled to the beads via a biotin-streptavidin bond, the beads are then packed in columns. Sandwich probes hybridize to capturing oligonucleotides and padlock probes hybridize to the sandwich probes. The padlock probes are ligated to form circles and RCA is performed on the beads. To the RCA products restriction oligonucleotides (RO) hybridize and a restriction enzyme digests the RCA products to single monomers which are released from the beads and pumped out of the columns. The fragmented RO dissociates and intact RO anneals to both ends of the monomers that thereafter is ligated to circles. These circles acts as never ending templates in the second generation RCA, creating approximately 1000 fold more RCA products than in the first generation RCA. The RCA products are labeled with detection oligonucleotides making them ready for detection and counting.

2. Material and methods

2.1 General

2.1.1. Oligonucleotides

The oligonucleotide systems used are described in Appendix 1. System A is new and designed for this purpose while system B was designed earlier. System C was used in the characterization of the beads.

2.1.2 Padlock phosphorylation

Phosphorylation of the 5' end of the padlock probe is necessary for ligation. The padlock probes were therefore phosphorylated in 50mM Tris-HCl (pH 7.6 at 25°C), 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA, 1mM ATP, 10µM Padlock probe and 0.1U/µl T4 PNK (Fermentas, Lithuania) in 37 ºC for 30min followed by inactivation of the enzyme in 65 ºC for 20min.

2.2 Homogeneous phase

2.2.1. Ligation and RCA

Phosphorylated padlock probes were ligated to form circles under following conditions; 33mM Tris-acetate (pH 7.9 at 37°C), 10mM Mg-acetate, 66mM K-acetate, 0.1% (v/v) Tween 20, 1mM DTT, 1mM ATP, 0.1µg/µl BSA st.f., 0.2µM Phosphorylated padlock, 0.6µM Ligation template and 20mU/µl T4 DNA Ligase (Fermentas, Lithuania) in 37 ºC for 15min.

Rolling circle amplification was performed in 33mM Tris-acetate (pH 7.9 at 37°C), 10mM Mg-acetate, 66mM K-acetate, 0.1% (v/v) Tween 20, 1mM DTT, 0.1µg/µl BSA st.f., 100pM ligated circles, 0.1mM dNTP and 0.08U/µl phi29 polymerase (Fermentas, Lithuania) in 37ºC for 60min. The enzyme was inactivated in 65ºC for 5min. To evaluate the results the RCA products were labeled and quantified according to the standard protocol described below.

In the first experiment to study amplification temperature dependence the ligation was performed at 37ºC for 15min and then the RCA reaction was performed at 22 (RT), 30 or 37ºC for 60min. In the ligation temperature studies the reaction temperature of the ligation was set to 22 (RT), 30 or 37ºC for 15min, then the RCA was performed at 37ºC for 60min. Everything else was performed as described above using system B.

2.2.2 C2CA

RCA products were monomerized by incubation at 80°C for 1min followed by 65°C 10min, in a solution containing 33mM Tris-acetate (pH 7.9 at 37°C), 10mM Mg-acetate, 66mM K-acetate, 0.1% (v/v) Tween 20, 1mM DTT and 300nM restriction oligonucleotide (RO) just so the RO's would hybridize to the restriction sites. Then enzymatic digestion was made by adding 0.2U/µl Rsa1 (Fermentas, Lithuania) at 37°C for 30min. The enzyme was heat-inactivated as well as the fragmented RO were dissociated at 80°C for 3min, intact RO hybridizes at 65°C for 5min followed by RT for 10min. The samples was diluted 1000 times and thereafter re-ligated in 33mM Tris-acetate (pH 7.9 at 37°C), 10mM Mg-acetate, 66mM K-acetate, 0.1% (v/v) Tween 20, 1mM DTT, 1mM ATP, 0.1µg/µl BSA st.f., 0.2µM and 20mU/µl T4 DNA Ligase (Fermentas, Lithuania) in 37°C for 15min. The second rolling circle amplification was performed in 33mM Tris-acetate (pH 7.9 at 37°C), 10mM Mg-acetate, 66mM K-acetate, 0.1% (v/v) Tween 20, 1mM DTT, 0.1µg/µl BSA st.f., 100pM re-ligated circles, 0.1mM dNTP and 0.08U/µl phi29 polymerase (Fermentas, Lithuania) at 37°C for 60min. Finally the enzyme was inactivated in 65°C for 5min.

2.2.2.1 Efficiency of the C2CA

RCA was performed as described above on a titration of the first degree ligation; 2/1/0.2/0.02nM of both system A and B. 10% of the sample was removed for detection. The remaining 90% of the samples was digested with restriction enzyme and then diluted thousand times. A C2CA was performed on these diluted samples and another 10% of the sample was removed for detection. The samples was labeled and quantified as described below.

2.2.2.2 Time dependence in the C2CA

Three samples with 20nM ligated circles from system A were amplified for 30min, 18min and 6min respectively. Then a second round of amplification (C2CA) was performed as mentioned above on these samples. All samples were labeled and quantified according to standard protocol described below. To determine the relative concentrations a dilution series and a calibration curve were made with the same system.

2.3 Solid phase

2.3.1 The attachment of oligonucleotides and the packing of the beads

The beads used were Streptavidin Sepharose High Performance (GE, Uppsala). They have a mean diameter of 34µm, low auto-fluorescence and are stable in a wide range of temperatures and pH. Biotinylated oligonucleotides were used to couple to the beads.

The beads need to be washed before a biotinylated oligonucleotide can be coupled and were therefore washed in a binding buffer containing 20mM $NaPO_4$, 0.15M NaCl, pH 7.5. To 600µl of bead slurry an equal amount of binding buffer was added. This was mixed and centrifuged. The supernatant was removed and new binding buffer was added, this washing were repeated 3 times. 75µl of a 1µM biotinylated oligonucleotide was added followed by addition of binding buffer to a final volume of 1ml. This was set on shake for 30min in 37°C and the beads with coupled oligonucleotides were then stored in cold-room.

The "microcolumns" were packed with oligonucleotide coupled beads. These beads are pressed out of a syringe in to a small piece of tubing with a small filter, frit, at the end. The frit keeps the beads in but liquid pressed and later pumped out. The packed column is then washed with 1x binding buffer and kept wet before usage.

2.3.2 Characterization of the beads

Three different experiments were performed to evaluate the characteristics of the beads in terms of autofluorescence and unspecific binding. In all experiments oligonucleotides from System C were used.

In the first experiment 20µl of washed beads and 10µM biotinylated oligonucleotide, labeled with TAMRA, was added in 1X binding buffer and incubated on a shaker for 10 min. Washing was performed using removal of the supernatant followed by addition of new 1X binding buffer. The beads were deposited on a cover slip and analyzed using an EPI microscope.

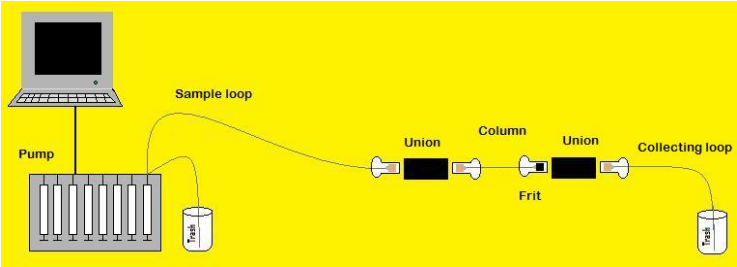
In the second experiment capture oligonucleotide 1 (100nM in 1X binding buffer) was added onto a column. Two detection oligonucleotides (100nM in 1X binding buffer) one Cy3 labeled, specific to the capture oligonucleotide, and one unspecific, Cy5 labeled, were added and then the column was washed with 1X binding buffer. The beads were deposited on a cover slip and analyzed in an EPI microscope.

Two different capture oligonucleotides 1 and 2 (100nM in 1X binding buffer) were coupled to beads in two separate columns. These columns were then pooled together in a tube and then repacked to one column. 100nM of detection oligonucleotides 1 and 2, Cy3- and Alexa 488 labeled, specific to capture oligonucleotides 1 and 2 respectively were added in 1X binding buffer to the column followed by a wash with 1X binding buffer. The beads were deposited on a cover slip and analyzed in an EPI microscope.

2.3.3 The microfluidic design and pump set-up

The microfluidic system was mostly composed of PFA tubings with an inner- resp outer diameter of 0.02" and 1/16" (1512L, Upchurch Scientific). PFA or Perfluoroalkoxy is a good, general replacement for PTFE, commercially known as Teflon. PFA is chemically inert, non-toxic, hydrophilic and it has low friction (10). Compared to PTFE it is more formable, it offers a higher maximum recommended usage temperature and has fewer impurities, making this material ideal to use in the microfluidic system. For liquid handling a Cavro 8 channel syringes pump was used, controlled by an in-house developed software. The 8 channels make it possible to run 8 separate microfluidic systems with separate, but here only 6 of the syringes were used at maximum.

The microfluidic design and pump set-up is described schematically in Figure 6. The sample loop was connected to the pump by a VacuTight™ Standard Nut M6 with ferrule Delrin® for 1/16" Tubing (P-931, Upchurch Scientific). The column was connected to the sample loop via two VacuTight™ Short headless Nuts 10-32 flat PEEK™ with ferrules for 1/16" Tubing (P-844, Upchurch Scientific) and a VacuTight™ Union 10-32 flat PEEK™ (P-845-01, Upchurch Scientific). The column itself was composed of a short piece of tubing connected to a frit in a ferrule™ 0.5µm PEEK™ for 1/16" Tubing with SS ring (P-275, Upchurch Scientific). Two M6 Super Flangeless™ Nuts PEEK™ for 1/16" Tubing (P-219, Upchurch Scientific) and a M6 Super Flangeless™ Union Delrin® (P-602, Upchurch Scientific) connects the column, via the frit in a ferrule™, with the collecting loop (via a Super Flangeless™ Ferrule 1/16" PEEK™ with SS ring (P-250, Upchurch Scientific)). The collecting loop was used to make it easy to collect eluents.



Both a positive and a negative pressure set-up were evaluated. In the negative pressure set-up the different solutions were drawn through the column one at a time. With the positive setup all the solutions were first loaded in the sample loop of each syringe in reverse order. The solutions were separated by air gaps to avoid mixing. Then the column was connected and the solutions were pumped through the column at a speed of approximately 3µl/min.

Figure 6. The microfluidic design and pump set-up. The pump is connected to the column via a sample loop and a union. The column was composed of a short piece of tubing connected to a frit. One more union connects the column with the collecting loop, which was used to make it easy to collect the eluents.

First, different solutions are loaded in the sample loop using negative pressure. The solutions are separated by air gaps to avoid mixing. Then the pre-packed column was connected and with positive pressure the solutions were pumped through the column one by one.

2.3.4 Solid-phase RCA

50nM phosphorylated padlock probes (system A) complementary to the capture oligonucleotides were pumped through the packed column with coupled capture oligonucleotides. The padlock probes were subsequently captured by the capture oligonucleotide, which also serves as a ligation templates. Ligation of the padlock probes to form circles were performed in 33mM Tris-acetate (pH 7.9 at 37°C), 10mM Mg-acetate, 66mM K-acetate, 0.1% (v/v) Tween 20, 1mM DTT, 1mM ATP, 0.1µg/µl BSA st.f. and 20mU/µl T4 DNA Ligase (Fermentas, Lithuania) in room temperature for 5min, see figure 7.

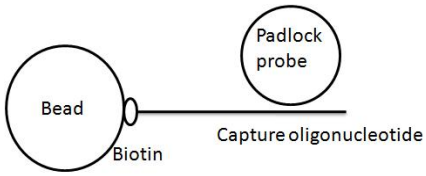


Figure 7. Schematic picture that describes the solid-phase RCA. The padlock probe is hybridized to the capture oligonucleotide, which also serves as a ligation template. The ligated circle can be amplified using RCA with the capturing oligonucleotide working as primer.

Rolling circle amplification was performed in 33mM Tris-acetate (pH 7.9 at 37°C), 10mM Mg-acetate, 66mM K-acetate, 0.1% (v/v) Tween 20, 1mM DTT, 0.1µg/µl BSA st.f., 0.1mM dNTP and 0.08U/µl phi29 polymerase (Fermentas, Lithuania) that was pumped through for 15min at room temperature. Thereafter the column was washed with 1xPhi29 buffer (Fermentas, Lithuania) for 1min. This wash step was later replaced with an air gap. The RCA product was labeled with a detection oligonucleotide in a detection solution that was pumped through the column. The beads were subsequently deposited on a cover slip and analyzed in an EPI microscope.

2.3.5 C2CA

To perform a solid phase C2CA the first generation of amplification was performed on column and the second amplification step was done homogenous. So first a solid-phase RCA is performed as described above without the labeling. To monomerize and release the RCA products from the beads a restriction solution containing 33mM Tris-acetate (pH 7.9 at 37°C), 10 mM Mg-acetate, 66mM K-acetate, 0.1% (v/v) Tween 20, 1mM DTT, 300nM restriction oligonucleotide (RO) and 0.2U/µl Rsa1 (Fermentas, Lithuania) was pumped through the column after the wash step/air gap. This volume was collected in tubes and incubated in 80°C for 3min. This incubation heat-inactivated the enzyme at the same time as it dissociated the fragmented RO. Intact RO hybridized thereafter to the monomers in an incubation step; 65°C for 5min followed by RT for 10min. The samples were thereafter re-ligated and a second generation of RCA was performed as described above in 2.2.2.

2.3.6 Sandwich assay

The sandwich assay is described in Figure 8. A solution containing 33mM Tris-acetate (pH 7.9 at 37°C), 10mM Mg-acetate, 66mM K-acetate, 0.1% (v/v) Tween 20, 1mM DTT, 0.1µg/µl BSA st.f. and 50nM sandwich probe L9995 was flushed through the pre-packed column. This sandwich probe hybridizes to the capture oligonucleotides and then when the padlock probe was flushed through the sandwich probes served as ligation templates. Solid phase RCA and C2CA were then performed as usual.

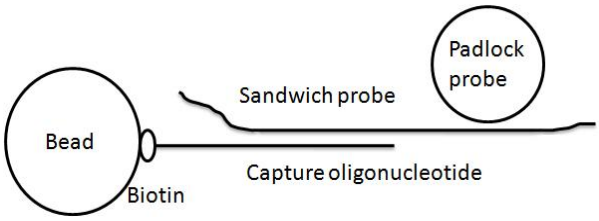


Figure 8. Schematic picture describing the solid-phase sandwich assay. The sandwich hybridizes to the capture oligonucleotide and the padlock probe is hybridized to the sandwich probe, which also serves as a ligation template. The ligated circle can be amplified using RCA with the sandwich probe working as primer.

2.3.6.3 Sandwich capturing

Three solutions containing 33mM Tris-acetate (pH 7.9 at 37°C), 10mM Mg-acetate, 66mM K-acetate, 0.1% (v/v) Tween 20, 1mM DTT, 0.1µg/µl BSA st.f. and 50/5/0,5nM sandwich probe were flushed with different volumes (5/7/10/12/15µl) through pre-packed columns. The different concentrations were flushed through one at a time with new beads for every new concentration starting with the lowest concentration. qPCR was performed as described below with two replicates on each eluate and was then compared with the calibration curve to determine the amount of sandwich probes not captured.

2.3.6.3.1 PCR and qPCR

To optimize the annealing temperature of the primer pair a PCR was made with 10pM Sandwich oligonucleotide (as PCR template), 200nM reverse primer (5'-GCCACAGAGTGTACCGACCTCGTAA-3'), 200nM forward primer (5'-CGTAGAGTAGCCGTGACTAT CCAGT-3'), 200nM dNTP ("U"), 1x PCR buffer (Fermentas, Lithuania), 2.5mM $MgCl_2$ (Fermentas, Lithuania) and 0.02U/µl Polymerase taq platinum (Fermentas, Lithuania). In the negative samples water was added instead of template. The PCR was run for 2min at 95°C, and then 15s at 95°C and 30s at 80-55°C, repeated 30 cycles. The results were then quantified on a 2% agarose gel with ethidium bromide staining.

To make a calibration curve a qPCR was made with 10000/1000/1000/100/10fM of sandwich oligonucleotide (as PCR template), 200nM reverse primer (5'-GCCACAGAGTGTACCGACCTCGTAA-3'), 200nM forward primer (5'-CGTAGAGTAGCCGTGACTAT CCAGT-3'), 200nM dNTP ("U"), 2x SYBR Green, 1x PCR buffer (Fermentas, Lithuania), 2.5mM $MgCl_2$ (Fermentas, Lithuania) and 0.02U/µl Polymerase taq platinum (Fermentas, Lithuania). In the negative samples water was added instead of template. This was run for 2min at 95°C, and then 15s at 95°C and 30s at 56°C, repeated 45 cycles.

2.4 Detection of homogeneous RCA products

2.4.1 Labeling

The RCA products were labeled by hybridization of detection oligonucleotides in 20mM Tris-HCl, 20mM EDTA, 0.5M NaCl, 0,1% Tween, 20nM DO+/DO- Cy3 and 20nM DO FAM. To the C2CA a DO with reversed complementary polarity (DO-) is needed. The FAM DO is not specific to the RCA product but this is used since it makes it possible to discard false positives as they appear double labeled.

2.4.2 Fluidic chip fabrication

A design for the microchannels was created in CAD and a nickel master was then created. A two component silicon rubber elastomer PDMS (Elastosil RT 601, Wacker) was mixed in ratio 10:1 and cast in a mold on the master. This was set to degass in cold-room and thereafter set to solidify in room temperature. When the PDMS had solidified tubing connections were created using a syringe orthogonally against the microchannels, see Figure 9. A corona surface treater (Model BD-20, Electo-Technic Products, Illinois, USA) was used to oxidize this PDMS (30sec) and a thin glass slide(1min). The two surfaces were then bonded together, forming chips with closed microchannels (200x40µm cross section, three per chip), in 65°C for 45min.

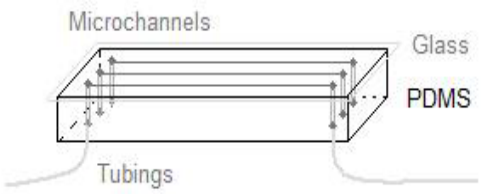


Figure 9. Schematic picture of the fluidic chip. Closed microchannels are formed with the bonding of a glass slide to the pre-cast PDMS. Tubings are connected to the microchannels via the bigger orthogonal channels so that liquids can be pumped through the channel using a syringe pump

2.4.3 Microfluidic quantification and Data analysis

The fluidic chip was connected to a syringe pump PHD-2000 (Harvard instruments) via tubings connected to the microchannels, see figure 8. The labeled RCA products were pumped through the microchannel at a flow rate of 5µl/min. A confocal microscope (LSM 5 META, Zeiss, Germany) was used to detect the RCA products. The focus was set to the center of the microchannel using a Carl Zeiss Fluar 40x/1.3 NA objective, with a pinhole equivalent to a 5µm optical slice. The microscope was operated in line-scanning mode with the scanning path perpendicular to the liquid flow direction. For each sample 30 000 line scans of 512 pixels were recorded, with a pixel time of 1.6 microseconds.

Data from the confocal microscope was stored in a 24-bit rgb-TIF file. A dedicated software written in MATLAB 7.0 (MathWorks, MA, USA) was used to threshold (fluorescent intensity) the data and to create a binary output file used to count the RCA products above the threshold (12). Figure 15 shows the 24-bit RGB-TIFF file where the RCA products appear as red dots and Figure 1 shows the microscopic detection and data analysis.

2.5 Microarray

A homogenous phase C2CA was performed as previously described, with system B. The C2CA products were incubated at 80°C for 1min and then 65°C 10min, in 33mM Tris-acetate (pH 7.9 at 37°C), 10mM Mg-acetate, 66mM K-acetate, 0.1% (v/v) Tween 20, 1mM DTT and 20nM restriction oligonucleotide (RO) so the RO's would hybridize to the restriction sites. Then enzymatic digestion was performed by adding 0.33U/µl Rsa1 (Fermentas, Lithuania) in 37°C for 30min. The enzyme was heat-inactivated at 80°C for 3min.

The spotted microarray was pre-printed with an amino coupled surface capturing oligonucleotide. To the array 500/100/10/1pM 2nd degree monomers/padlock, 10mM Tris-HCl, 10mM EDTA, 0.25M NaCl, and 20nM DO- Cy3 were added and incubated for 2h at 37°C. The slide was then washed in 1X PBS buffer for 5 min, spun dry and then analyzed in a microarray scanner.

3. Results and discussion

Optimal system performance requires every step in the experimental chain to be optimized. The aim was to evaluate and optimize the efficiency of each step in the reaction chain. We started to investigate the homogeneous phase where the temperature and the efficiency could be investigated. The oligonucleotide system design was also evaluated. The foremost goal was to set up and evaluate the solid phase step and the microfluidics. We started to investigate the specificity of the beads and oligonucleotides. The microfluidic system was then designed minimizing flow problems. Then RCA and C2CA was performed on beads both off-column (data not shown) and on-column. The sandwich assay was performed and examined thoroughly. The variation between samples was a concern and to evaluate how much sandwich probe that actually is captured by the capture oligonucleotide a qPCR set up was designed. But many more issues need to be adressed in the future.

3.1 Homogeneous phase

3.1.1 The temperature effect on RCA

One important parameter to evaluate is the temperature. To carry out the reactions at room temperature would be the most effortless way to go, but how much does the temperature affect the reactions? To see the affects of temperature on ligation and the RCA efficiency three reactions were carried out at different temperatures; room temperature (RT~22°C) 30°C and 37°C.

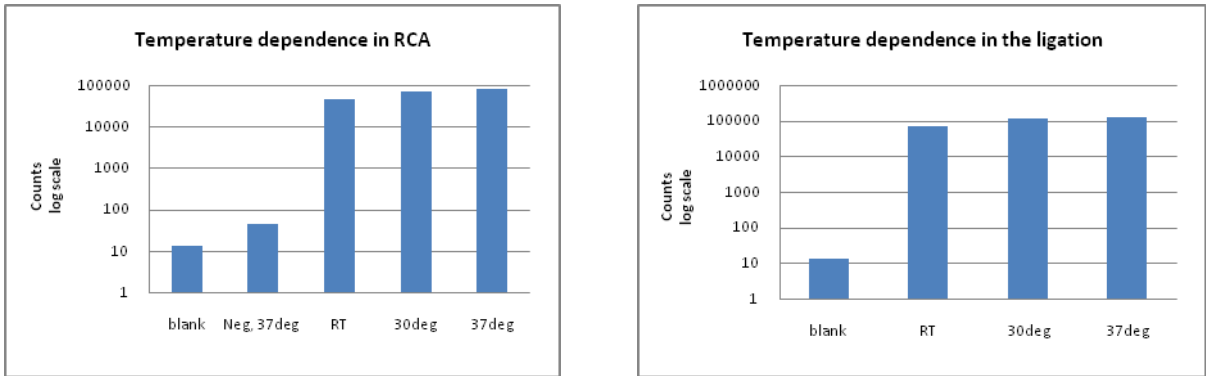


Figure 10. How the temperature affects ligation and RCA reactions on system B. The blank sample is water and the negative sample contains no polymerase at 37 °C.

As one can see in Figure 10, the temperature has a great influence. The performance of the reaction at RT compared to 30°C gives a 40% reduction of counted RCA products in both ligation and RCA, but between 30 and 37°C the difference is not so high (~10%). This means that in the solid phase some kind of heater is needed, and in our case a water bath was used but more sophisticated and smaller systems could be used in the future. The negative controls have a few counts but that is probably contamination from previous samples in the detection system.

3.1.2 C2CA efficiency and time dependence

In this project it is crucial that the C2CA is efficient. To investigate the efficiency of the C2CA a comparison between the first RCA and a thousand fold dilution of the second degree RCA was made. The first RCA copies a 100nt circular oligonucleotide into a linear strand with 1000 copies in one hour. Given complete restriction the second degree RCA products should have about a thousand fold higher concentrations of RCA products than the first one (hence the 1000-fold dilution). The second degree RCA of System A, showed in Figure 11a, has more than a thousand-fold increase of RCA products in all concentrations, which implies that the first RCA must have copied more than thousand copies and that the restriction efficiency must be close to 100 percent. This experiment was made for both system A and B but system B did not perform as well as system A (data not shown) so system A, that was designed for this project, was used exclusively in the rest of the experiments.

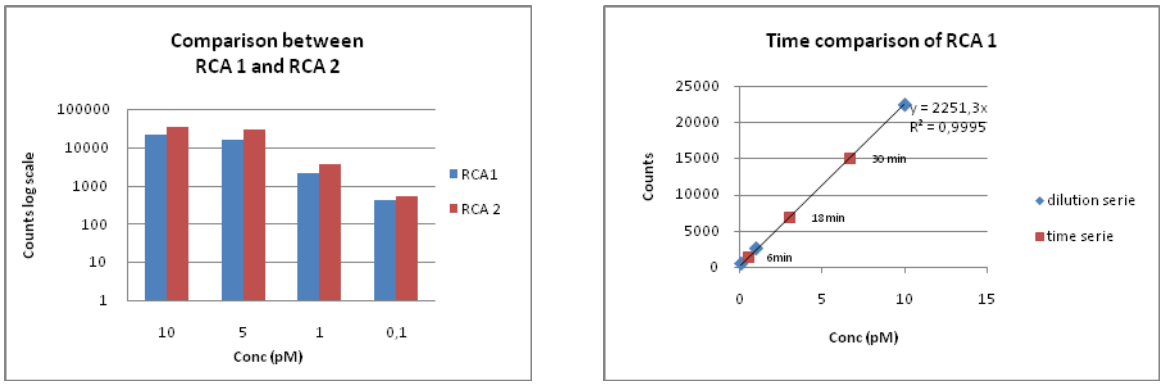


Figure 11 a) Comparison of the first degree RCA and a thousand fold dilution of the second degree RCA in system A. b) Time comparison of the first degree RCA on system A. The derived equation is used to calculate the concentrations of the different time samples.

It is also crucial to reduce the time for the RCA reactions to be able to reach the final goals of one hour. However if the time is reduced too much the amplification is going to be too low. Since there is going to be two amplification steps the relation between them needs to be optimized. The first amplification will increase the number of detectible objects while the second amplification will increase the number of objects above the threshold. Here it is tested how reduction of the first RCA reaction time influences the amplification. To be able to compare the results a dilution series was made. As can be seen in Figure 11b the dilution series is linear. The derived equation can be used to calculate the concentration of the different time samples. The polymerization of DNA is supposed to be linear, meaning that half the time should give the half number of counts and that 10% of the time should give 10% of the number of counts. Figure 7b shows results where half the time gives more than half the expected number of counts, 30% of the time gives 30% of the expected number of counts and a tenth of the time gives less than a ten-fold of expected number of counts. This implies that the polymerization is close to linear and that the differences of counts can be related to sample variations. Still the time in both the first and the second amplification is needed to be optimized, so that the relation between them gives a good amplification and sensitivity but still having a high signal to noise in a short time.

3.2 Solid phase

The used solid phase consisted of Streptavidin Sepharose High Performance (GE, Uppsala) beads. The beads mean diameter is 34µm, they should have a low auto-fluorescence and are stable in a wide range of temperature and pH. Biotinylated oligonucleotides were coupled to the beads. Other solid phases have been evaluated previously but without the same success (data not shown).

3.2.1 Characterization of the beads

It is important to know the characteristics of the beads. Key characteristics investigated were autofluorescence and unspecific binding. This as well as the hybridization of detection oligonucleotides to capture oligonucleotides coupled to beads was examined in the three following experiments.

Firstly a biotinylated oligonucleotide, labeled with TAMRA, was added to beads. This should examine the biotin-streptavidin chemistry, but also the autofluorescence of the beads. The beads were washed and with an EPI-fluorescent microscope the beads were examined. Seeing that only the beads with oligonucleotides were fluorescent and not the ones without (data not shown) it was confirmed that biotin-streptavidin coupling worked but also that the bead autofluorescence was low.

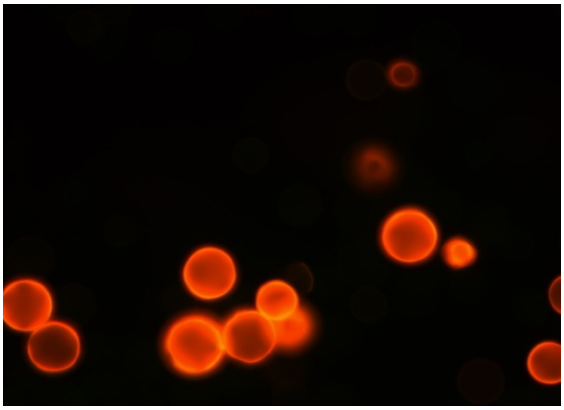


Figure 12. Picture of beads taken on the EPI microscope. Only the specific detection oligonucleotide with Cy3 binds to the beads, red representing Cy3. The unspecific detection oligonucleotide with Cy5 is not bound to the beads and can therefore not be seen.

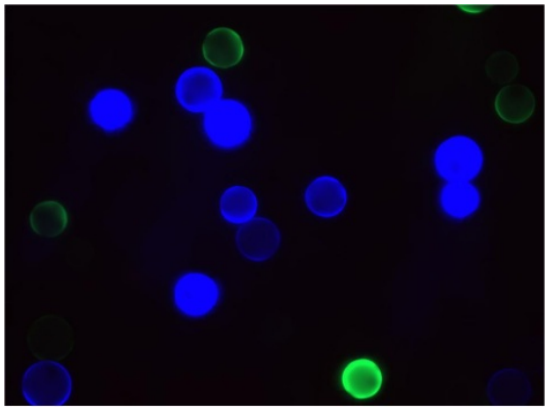


Figure 13. Picture of beads taken on the EPI microscope. The two different detection oligonucleotides hybridize to correspondent capturing oligonucleotide on different beads. Blue represents Cy3 and green represents Alexa 488

The second experiment examines whether oligonucleotides bind unspecifically to beads, and not only through the biotin-streptavidin bond, but also if a detection oligonucleotide binds specifically to a complementary capturing oligonucleotide coupled to the bead. A biotinylated capture oligonucleotide was added to the beads and then a specific and one unspecific detection oligonucleotide was added to the beads. If the binding is specific the specific detection oligonucleotide would be detected on the beads while the unspecific detection oligonucleotide would not be detected. As can be seen in Figure 12 only the specific detection oligonucleotide could be detected, which implies that the binding is specific and that no oligonucleotides without biotin stick to the beads.

To check this further a third experiment was performed. Two different capture oligonucleotides were added onto beads in two separate columns. These columns were then pooled together in a tube and then repacked to one column. Two detection oligonucleotides (Cy3- and Alexa 488 labeled), each different and specific to each of the capture oligonucleotides were added. The beads were deposited on a cover slip and examined in an EPI microscope. The two different detection oligonucleotides were in fact detected on different beads as can be seen in Figure 13. Therefore it could be determined that the oligonucleotides hybridize specifically to the corresponding capture oligonucleotide, that there is no transfer of detection- or capture oligonucleotides between beads ("jumping") and that there is really low unspecific binding to the beads.

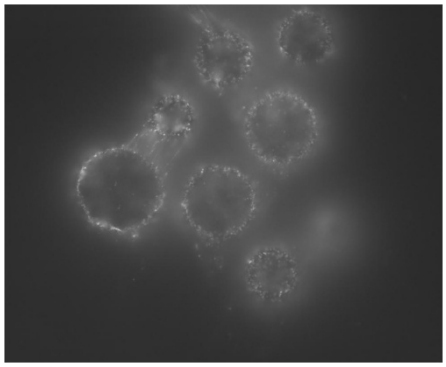
3.2.2 The microfluidic design and pump set-up

The microfluidic system and pump set up is schematically described in Figure 6. The microfluidics was mostly composed of PFA tubings but other parts used were unions and different kinds of nuts and ferrules. For liquid handling an 8-channel syringe pump controlled by a an in-house developed software was used. Only 6 of the 8 syringes were used at maximum. The pump was connected to the column via a sample loop, made of tubings, and a union. The column was composed of a short piece of tubing connected to a frit. The frit worked as a filter making it possible to pack the solid phase, leaving beads inside but liquid pressed out. One more union connected the column with the collecting loop, which was used to make it easy to gather eluents. The two unions make it possible to remove the column from the system, making it easy to pack and unpack the columns but also to load the different solutions in the sample loop.

After several experiments and set-ups with negative pressure, dragging the different solutions up through the column one at a time, a positive pressure setup was used. With this positive pressure setup all the solutions were first loaded in the sample loop of each syringe. The solutions were loaded in reverse order, separated by air gaps to avoid mixing. Then the column was connected and the solutions were slowly pumped through the column. By using positive pressure the problems with leakage, creating air bubbles and flow differences were minimized.

3.2.3 Solid phase RCA and C2CA

To determine the efficiency of RCA on beads a biotinylated capture oligonucleotide was coupled to the beads to capture and serve as a ligation template for a padlock probe. The ligated circle is used as a never ending template for rolling circle amplification using the capture oligonucleotide as primer, see Figure 7. The RCA products were labeled with a fluorophore and analyzed at in an EPI microscope. Figure 14 shows how an EPI-fluorescence picture of RCA performed on beads.



Now that the solid phase RCA worked, the efficiency of the solid phase digestion and the second generation of RCA (C2CA) could be examined. A set up was made where a restriction enzyme was flushed through the column after the RCA digesting the RCA products to monomers. These monomers were gathered by collecting the restriction solution coming out of the column in tubes. In the tubes the monomers were re-ligated, amplified a second time and thereafter labeled and detected in a confocal microscope. This partial solid phase C2CA was successful and the restriction of the first generation RCA products seems to be efficient, data not shown here.

3.2.3.1 The wash step

To get quantifiable data it is important to collect a standardized volume from the restriction containing all the monomers. Different techniques were evaluated to these ends. The wash step between the RCA solution and the restriction solution was dyed with orange G or with Dextran Blue or a big air gap replaced the wash step. The results (not shown here) show that both the dyes inhibit the reaction. The dyes were also retained inside the beads, as a size exclusion chromatography, which could influence later reactions. Currently a big air gap is the most effective and easiest way to go and was therefore used from then on.

Figure 14. A EPI microscope picture of RCA on beads. The RCA products are shown as white and one can see how they are distributed around the beads on the surface. Some RCA products even reach between

3.2.4 Sandwich assay

Our setup was dependent on the sandwich assay to work. The sandwich probe should hybridize to the capturing oligonucleotide on the bead and then work as a ligation template for the padlock probe and thereafter as primer in the RCA, see Figure 8. Here the sandwich probe is synthetic but in the future the sandwich probe will be a genomic fragment from a microorganism e.g. a hazardous bacterium. To test this system the sandwich probe was pumped through the column and then a partial C2CA was performed as described above; including a solid phase RCA, digestion to monomers, re-ligation, a second generation of RCA, labeling and detection in the confocal microscope. Figure 15 shows the data from the confocal microscope. The red dots represent labeled RCA products proving that the sandwich assay works well and that both the capturing of the sandwich probe and the digestion of the RCA products on beads was efficient.

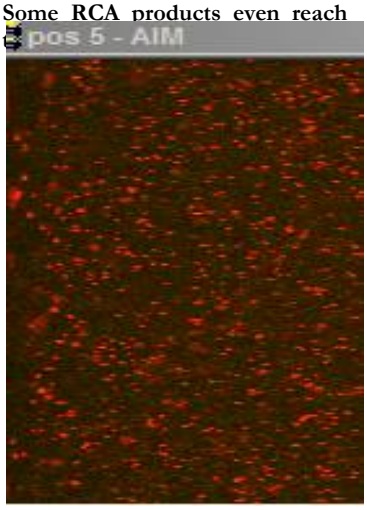


Figure 15. A 24-bit RGB-TIFF file showing that the sandwich assay works. Labeled RCA products appear as red dots, here in a high concentration. This file can later be set to a threshold and the RCA products can be counted.

3.2.4.1 Negative controls

An experiment with multiple negative controls was performed to investigate background noise and also the robustness of the system. The different negative controls were conducted in the solid phase step where one thing crucial for the RCA to work was excluded in each sample; no sandwich, no padlock, no ligase and no polymerase. Two positive samples were also run. As Figure 16 shows the negative samples gives a low signal, confirming that the background noise of the system is low. The fact that the counts are not closer to zero is probably caused by contamination in the detection system were RCA products from previously samples are retained. The two positive samples have as expected high counts but one noticeable thing is the variation between the positive samples, where they differ by 25%. This variation is a problem that needed to be further investigated.

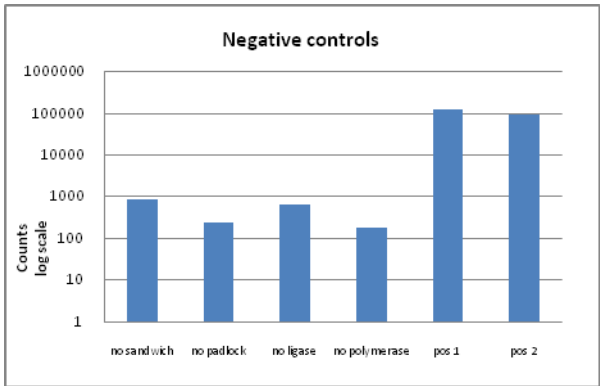


Figure 16. Graph showing the counts of four different negative samples and two positive samples. The negative samples give a very low signal close to zero, while the positives have high but varying counts.

3.2.4.2 Variability between samples

Variability between identical samples should be minimal in this system as well as in all other methods and techniques. As previous data and the data in Figure 17a shows a noticeable difference between identical samples run through different columns. To further investigate this variation, four replicates were made with the same conditions as the column samples in terms of temperatures and times, but in a homogenous phase. The object was to see if the homogeneous phase samples vary as much as the solid phase samples or if it is the beads or the syringes that adds the variability. The data in Figure 17b shows that the variability is high even between the homogeneous samples indicating that the variation could reside from pipetting, analysis or from the enzymatic reactions. It could also be an incomplete digestion or inconsistent polymerization, giving the enzymes too little time or too low temperature. This is most definitely a problem that needs to be managed in the future.

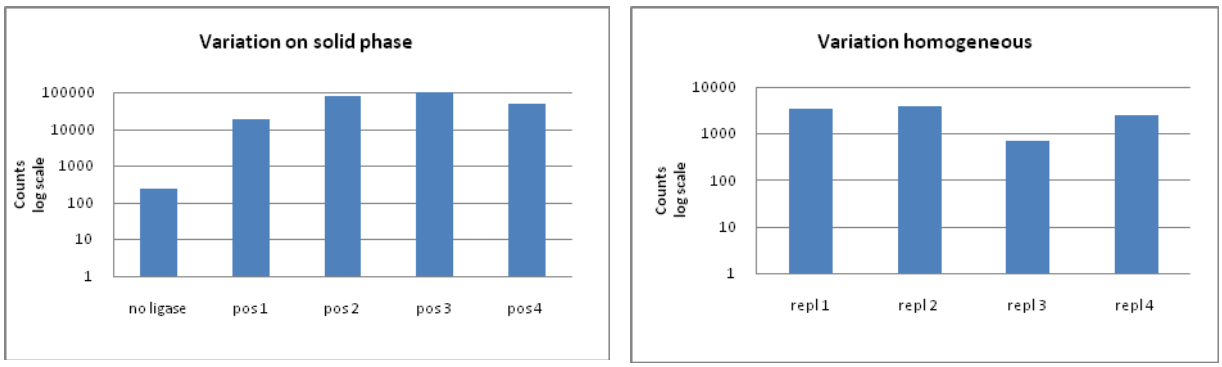


Figure 17 Variability between identical samples. a) The C2CA results from the solid phase b) The C2CA results from a homogeneous phase having same conditions as the solid phase. The variation between samples is high in both cases which might be explained by an insufficient time or temperature for the restriction enzyme or the polymerase.

3.2.4.3 Flow rate

The flow rate of the reactions in the column was approximately 3µl/min. One hypothesis is that a high flow rate may rip RCA products off the beads. To investigate this theory the RCA solution coming out from the column, that should not contain any RCA products, was collected. A restriction solution was both added to the collected RCA solution as well as it was pumped through the column. Then a second round of RCA was performed on all samples, as described before. The analysis of data showed that there are equal amounts of RCA products in the RCA solution samples as the samples collected from the restriction, data not shown here. This indicates that there are RCA products ripping off the beads or that padlock- and sandwich probe are retained between the beads in the column or in the tubings.

3.2.4.4 Evaluating Sandwich probe capturing with qPCR

A qPCR set-up was designed to evaluate how much sandwich probe that is actually captured by the capture oligonucleotide. This can tell us how much sandwich probe we need to use in the continued experiments for efficient capture, how much padlock that needs to be used and also how well the beads works. The sandwich probe mimics a genomic fragment from a microorganism that should be detectable even at very low concentrations. So in the final set-up the sandwich probe is going to be the limiting factor. The solid phase was chosen just because it will increase the sensitivity due to more efficient target molecule recovery. So by using qPCR it can also be tested if low concentrations really bind efficiently to the capturing oligonucleotides.

The sandwich probe is designed with a PCR cassette, using a forward and reverse primer pointing directly at each other. The first qPCR experiments focused on optimizing the annealing temperature of the primers. Optimal annealing temperature was established with a normal PCR using a gradient block. The results were evaluated on a 2%

agarose gel with ethidium bromide staining. Figure 18a shows that the best temperature is 56.8°C, where the band is strongest. The qPCR was therefore run with an annealing temperature of 56°C. A calibration curve was made of the sandwich probe to be able to quantify the qPCR results. An equation was created from the results so that the concentrations of sandwich probe from the capturing experiment could be calculated, see Figure 18b.

The qPCR set up was tested with a pilot experiment that was performed with different concentrations at different volumes of sandwich probe flushed through pre-packed columns. The eluent was collected and run in the qPCR, but the results were not consistent so nothing could be interpreted from this data. This was done on used tubings, and since the qPCR is so sensitive this needs to be remade on new tubings so that no background sandwich probe is present.

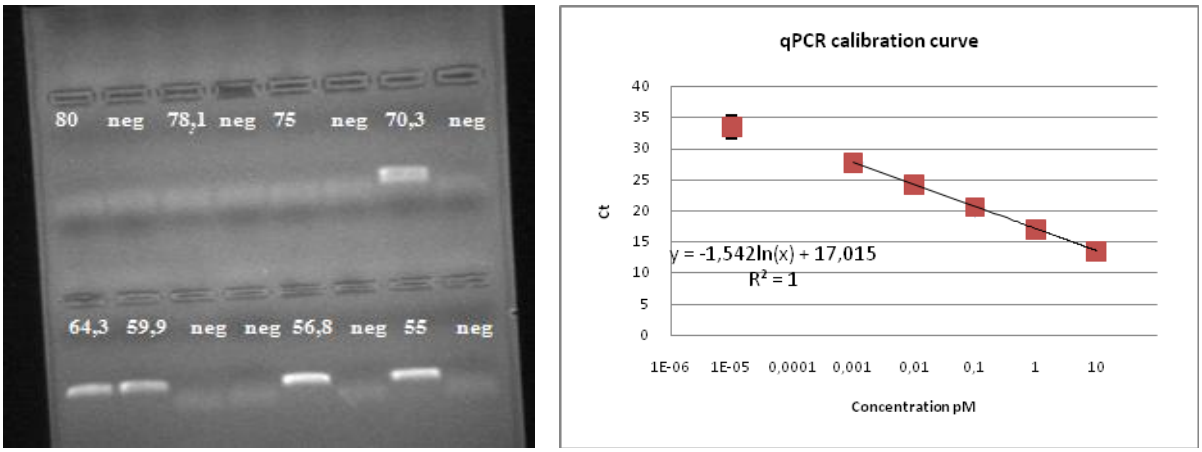


Figure 18 a) Shows a 2% agarose gel with ethidium bromide staining. The samples are PCR products of sandwich oligonucleotide and a corresponding negative (water), with different annealing temperatures. The primers start to anneal at 70.3°C, where the first band is showing, and the optimal temperature to anneal is at 56.8°C, the strongest band. b) Shows a calibration curve for qPCR on the sandwich probe. The dilution series gives a perfect equation that can be used to calculate the concentration of sandwich probe from qPCR data. The first dot is a blank sample containing water and the standard deviations are so low that the error bars are not showing.

3.3 Microarray

If our method is to be used for detection of microorganisms in the future it will be essential to be able to tell what kind of microorganism that is detected. Our microfluidic method has potential for multiplex detection but the ultimate platform for highly multiplexed detection is the microarray platform. A pilot study was performed where both padlock probes and monomerized second generation RCA products, were run on a microarray in a dilution series. This was made with system B, since at that time a surface capturing oligonucleotide for system A did not exist. The samples were hybridized to a detection oligonucleotide and then hybridized to a surface oligonucleotide attached to the array. This pilot study showed that both padlock and digested RCA products with concentrations down to 1pM could be detected on the array (data not shown here), making microarray one more platform that can be used together with our partial solid phase C2CA for multiplexed detection.

4. Conclusions

Today it is more important than ever to have access to analysis instruments that can detect biological agents quickly and correctly. Here we have started to investigate a new, fast and sensitive technique for DNA detection using a partial solid phase C2CA in a microfluidic system. This technique will hopefully later be integrated in to a transportable bio-monitoring system for rapid and efficient detection of hazardous microorganisms.
One benefit of using a solid phase is the possibility to get a more efficient target molecule recovery by increasing the capturing area. The most important benefits for this system are that the microfluidics will minimize the mass transport problems. By pumping the solutions inside a small microchannel filled with beads, or any kind of solid phase, the analytes do not have to diffuse so far to find its ligand/target, shortening the total analysis time. The flowing of solutions in microfluidics will also wash away unreacted padlock probes and sample inhibitors so that high concentrations can be used without inhibiting the RCA reaction. It will also wash away reagents that would interfere in later steps in the experimental chain.

The use of padlock probes and RCA is very beneficial since you get a high specificity due to the padlock and enzymatic ligation procedures and high sensitivity due to the RCA. C2CA will increase the sensitivity even more.

This system is also preferable since one can use it for the detection of DNA, RNA, proteins and other biomolecules. The system also has a great potential for high degrees of multiplexing. Previous analyses have shown that the molecular probing approaches we are taking are less sensitive to interfering substances in complex matrices that inhibit PCR. Microfluidics and a partial solid-phase C2CA will together make an exceptional couple that will minimize the time required for each step and will increase sensitivity due to more efficient target molecule recovery and molecular probing.

Here I have started to investigate this new system and since this is in the beginning of the project the experiments have been wide-ranging, using many different techniques. I have confirmed that the beads are specific to biotin-streptavidin bonds, that the hybridization between the oligonucleotides is specific and that both ligation and RCA can be performed on beads. In the homogeneous phase I have examined the temperature and time dependence on the RCA but also the efficiency of the C2CA. A new oligonucleotide system, which was designed especially for this purpose, was evaluated. The microfluidic system was designed along with the pump set-up making it possible to run 8 different samples simultaneous without problems with leakage, creating air bubbles and flow differences. The sandwich assay was studied and the conclusion made is that there is a big variation between samples with identical properties. This is probably the cause of too high flow in the column or the retaining of free padlock probes and sandwich probes in the tubings, which the flow rate experiment showed. To further examine the sandwich assay two pilot experiments were performed. The qPCR was supposed to measure how much sandwich probe that is actually captured, but this pilot study needs to be repeated using new tubing to minimize the background noise. The microarray experiment shows that concentrations, of both padlock and restricted second degree RCA products, down to 1pM could be detected on the array, making microarray one more platform that can be used together with our partial solid phase C2CA for multiplexed detection.

4.1 Future

Many more experiments need to be done before this can be a final product, especially on the sandwich assay. The first step would be to re-do the qPCR experiment using new tubings. The qPCR could also be used to investigate how low concentrations of sandwich probe will be captured on the column. FACS experiments on the beads can tell how much capturing oligonucleotide that can be bound to the surface of the beads, making the column more efficient. The variability also needs to be addressed. Test with lower flow in the column will be performed and experiments with different rolling times and restriction times will examine whether the restriction isn't complete or if the polymerase is inhibited at room temperature. Other general things to be optimized in the on-column sandwich assay are temperatures, times, concentrations and volumes.

Today the conventional cultivation-based techniques are too slow, as a result have a number of biomarker-based detection strategies have been developed. Commonly real-time PCR is used for genetic assays, but the real-time PCR equipment is sometimes not as stable as preferred. Fast and sensitive mass spectrometry methods such as BAMS (Bio-Aerosol MS) have also been developed for identifying microorganisms. BAMS can distinguish *Bacillus* spores from *Clostridium* spores by analyzing their peptide patterns but it cannot distinguish harmless strains from hazardous ones. Concerning detection of protein markers is it common to use, the enzyme-linked immunosorbent assay (ELISA) but also the immunoassays HandHeldAssays (HHA) and BioVeris (Roche), which are ELISA like bead-based protein detection assays. Unfortunately, reagent kits used for the immunological assays are severely limited in sensitivity and specificity, and cannot discriminate between the many closely related biological agents in the environment.

This is a fast evolving area of research and if this technique will work as planned and if it later is incorporated in to a bio-monitoring system that system holds potential to be "State of the art". The system has potential to be robust and reliable, offer a large quantitative dynamic range, distinguish and find trace levels of pathogenic agents against a background of abundant non-pathogenic species and it will offer single-molecule detection (SMD) and thus digital read-out. If all parts come together it will make this bio-monitoring system the obvious choice of analysis instruments that can detect biological agents rapidly and correctly.

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Appendix 1- Table of oligonucleotides

The different oligonucleotides used during this project

	IDNr	Name	Sequence (5'-3')	Modification
System A				
Capture oligo	L9993	BS_spacer_capture	TACTCGCCCTTGTGGACTGCTCTCTCTCTCTCTCT	3' Biotin
			complement to capture / pcr primer cassette / Recog 1 / Recog 2	
Sandwich probe	L9995	BS_sandwich1	CAGTCCACAAGGGCGAGTA ACGTAGAGTAGCCGTGACTATCCAGTTTACGAGGTCGGTACACTCTGTGGCCGCATAATACCTACGGG TCAAAGAGGGGG	-
			Recog 1 / Restriction / B2_DO (general) / b2_do_40 / Recog 2	
Padlock probe	X00915	B2_P_40	CCCGTAGGTATTATGCG ACTTCT AGAGTGTACCGACCTC AGTA GCCGTGACTATCGACT CTAGTGCTGGATGATCGTCC CCCCCTCTTTGA	-
Ligation template (homogeneous)	X01107	B2_T_40	CGCATAATACCTACGGG TCAAAGAGGGGG	-
Restriction oligo 1st gen	L8764	B2_RO+	AGAGTGTACCGACCTCAGTA	-
Restriction oligo 2nd gen	X01498	B2_FP/RO-	TACTGAGGTCGGTACACTCT	-
Detection oligo 1st gen	X01063	B2_DO	AGTAGCCGTGACTATCGACT	5' Cy3
Detection oligo 2nd gen	L8382	B2_DO-	AGTCGATAGTCACGGCTACT	5' Cy3
unspecific detection oligo 2nd gen	L8592	B2_DO_FAM	TTTAGTAGCCGTGACTATCGACT	5' FITC
System B				
			Recog 1 / Restriction / detection (spec) / detection (general) / Recog 2	
Padlock 1	L9946	BT_P1G-93	CTTCCTCCCTACTGA AGAGTGTACCGACCTC TCGTCGA AGTAGCCGTGACTATCGACT TGCGTCTATTTAGTGGAGCCTATTAACTTAACCAC	
Padlock 2	L8865	BT_P2G	CTTCCTCCCTACTGA AGAGTGTACCGACCTC TCGTCGA TGCGTCTATTTAGTGGAGCCTGCGTCTATTTAGTGGAGCCTATTAACTTAACCAC	
Ligation templat (homogenous)	L8873	BT_T_28	TCAGTAGGGAGGAAGGTGGTTAAGTTAATA	
Capture oligo	L9355	Bio-B2-T-28-yta	TCTCTCTCTCTCTCTCAGTAGGGAGGAAGGTGGTTAAGTTAATA	
Restriction oligo 1st gen	L8871	BT_RG	AGAGTGTACCGACCTC	
Restriction oligo 2nd gen	L8873	BT_T_28	TCAGTAGGGAGGAAGGTGGTTAAGTTAATA	
Surface capturing oligo	L9354	NH2_BT-T-28yta	TCTCTCTCTCTCTCAGTAGGGAGGAAGGTGGTTAAGTTAATA	5' Amin
Detection oligo 1 gen	L8748	B2_DO_27+_Cy3	TGCGTCTATTTAGTGGAGCC	5' Cy3
Detection oligo 2 gen	L9179	B2_DO_27-_Cy3	GGCTCCACTAAATAGACGCA	5' Cy3
System C				
Detection oligo	X749	Inv1216Ar-k TAMRA	TCTCTCTCTCTCTCTCGTGCGACACATGACATCAAC	5' Biotin 3' TAMRA
Capture oligo 1	L8588	B2_DObio	TTTAGTAGCCGTGACTATCGACT	3' Biotin
Detection oligo 1	L8382	B2_DO-	AGTCGATAGTCACGGCTACT	5' Cy3
Unspecific detection oligo	X1163	B-1216-D-Cy5	CCACGCGTTGATGTCATGTGTCGCAC	5' Cy5
Capture oligo2	L9032	B2_DO_20+-biotin	GAGAGGCCGTCGCTATACAT	3' Biotin
Detection oligo 2	L8345	B2_DO_20-ny	ATGTATAGCGACGGCCTCTC	5' Cy3