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Development of new methods for multiplexed analysis of biomolecules

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Development of new methods for multiplexed analysis of biomolecules

Abstract

Efficient methods are required to facilitate the analysis and identification of genes and their transcripts. Today, almost all methods for gene analysis involve PCR followed by a sequence analysis method, or alternatively, nucleic acid samples are applied to dense microarrays of hybridization probes. While PCR offers high specificity and sensitivity, microarrays can be used for multiplex analysis, but at the cost of specificity and sensitivity.

In this work a new method with both the ability of performing multiplex analysis and a high specificity and sensitivity is presented. The method is called random array and it can be used for parallel decoding at the single molecule level of products obtained from rolling circle amplification, in this case amplified padlock probes.

Keywords

Random array, Padlock probes, Rolling circle amplification, Multiplex analysis

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Development of new methods for multiplexed analysis of biomolecules

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Populärvetenskaplig sammanfattning

Det behövs nya metoder för att underlätta analys och identifikation av gener. Med de metoder som används idag står man ofta inför valet mellan att få en hög specificitet och känslighet eller möjligheten att kunna utföra många analyser på samma gång.

I det här arbetet presenteras en metod som ger en hög specificitet och känslighet samtidigt som den även har förmågan att kunna utföra många analyser parallellt. Metoden kallas ”Random array” (sv. ”slumpmässig uppställning”) och kan alltså användas till att analysera och identifiera flera gener på samma gång i ett och samma prov.

Om man då vill ta reda på om vissa gener finns i ett prov, tillsätter man först särskilda molekyler som känner igen just de gener man letar efter och binder till dem. För att underlätta detektionen av de gener som hittats förstärker man signalen från dem genom att låta de igenkänningsmolekyler som hittat sin specifika gen kopiera sig själva om och om igen. De kopior som kopierats från samma igenkänningsmolekyl blir sedan till ett litet nystan. Därefter binder man provet till ett objektsglas och tillsätter nya molekyler som binder till nystanen. De nya molekylerna sänder ut ljus i olika färger när man tittar i ett särskilt mikroskop. Varje typ av nystan, som alltså representerar en viss typ av gen, har en viss färg på ljuset och då vet man vilka gener som man hittat i sitt prov.

Examensarbete 20 p
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Uppsala universitet, april 2006

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1. Introduction

Efficient methods are required to facilitate the analysis and identification of genes and their transcripts. Today, almost all methods for gene analysis involves target sequence amplification through polymerase chain reaction (PCR), followed by a sequence analysis method of the target amplified fragment. Alternatively, nucleic acid samples are applied to dense microarrays of hybridization probes.

The most efficient way to reduce the number of reactions needed and the amount of DNA consumed is to analyze several genes in one reaction (multiplexing). This is elegantly achieved in microarray analysis, but the hybridization reactions are limited in sensitivity and specificity, which for example prevents direct analysis of DNA sequences from human genomic DNA samples. Quantitative PCR, on the other hand, has a high specificity and sensitivity, but as the level of multiplexity is increased, i.e. several target sequences are simultaneously amplified, more problems start to add up. This is due to the increased number of primer pairs added to the reaction, which then either can hybridize to each other, forming so-called primer-dimers [1], or primers from different primer pairs can hybridize to a DNA segment, not intended to be amplified, leading to nonspecific amplification [2].

Because of these problems there is a strong demand for new gene analysis methods, preferentially without a PCR amplification step. Currently the possibility of combining methods with a specificity and sensitivity comparable to PCR with a high level of multiplexing is strongly advancing. Several studies on such methods have been presented in the recent years.

One example is the BeadArray technology [3-4], developed by scientists at the company *Illumina Inc*. Samples of DNA are hybridized to an array of beads, randomly sampled from a starting bead population with several different types of beads. Each bead has a gene-specific probe attached to it by a bead identifier. The company has also developed several other assays for genotyping, gene expression and epigenetics [4].

A second example is a method developed by another company, *Affymetrix Inc*. In this method probes of DNA are attached to arrays. These probes consist of one variable sequence and one constant anchor sequence and to this anchor sequence a complementary oligonucleotide is hybridized. The target DNA hybridizes to the variational sequence and can then be ligated with the oligonucleotide bound to the anchor sequence [5].

Another method that should be mentioned is the amplification and sequencing of DNA by PCR in a thin polyacrylamide film on a glass slide [6-9]. The gel keeps each amplification product close to its template. Since the products cannot diffuse in the gel, a number of PCR colonies, called “polonies”, will be formed and one polony will then represent one single template. Due to this localization in the gel, several millions of clones can be amplified and sequenced on one single glass slide.

2. Aim of study

The aim of this master's degree project was to develop a technique for parallel decoding at the single molecule level of products obtained from rolling circle amplification (RCA), in this case amplified padlock probes. The technique is called Random array, in which different RCA-products are immobilized on a surface and then identified by serial hybridization reactions with detection probes, which in this case were oligonucleotides labeled with different fluorophores. With serial hybridizations the method is not dependent on the number of fluorophores available, since the same fluorophore can be used in different hybridization reactions, and the level of multiplexity is thus increased. The project involved optimization of the technique, such as optimizing the hybridization and stripping conditions, but also an investigation of the dynamic range and the possibility to perform multiplex analysis.

3. Theory and background

3.1 Fluorescence based single molecule detection techniques

The quest for new optical methods with the possibility to detect small amounts of biomolecules began in the middle of the 1970's when Hirschfeld demonstrated a method capable of detecting a single antibody molecule tagged with 80-100 fluorophores [10].

Throughout the years, fluorescence based single molecule detection (SMD) techniques have been developed even further and are now a routine in a lot of laboratories. The difference between SMD techniques and classical biochemical analyses is that the SMD enables one to visualize, measure and identify individual molecules, whereas the ensemble techniques only yield information on average properties. This leads to that more information, before hidden by the ensemble techniques, will become available [11].

The techniques are, as the name indicates, based on attaching fluorescent probes to single molecules, like proteins or DNA molecules. The fluorescent probes can for example consist of green fluorescent protein (GFP) which is then fused with the single protein you want to detect [12-14] or they can be fluorescently labeled oligonucleotides which then hybridize to specific DNA segments. Once the fluorescent probes are attached to the single molecules, these molecules can be detected in a fluorescence microscope and give information on for example molecular interactions or just identify that certain molecules are present in the sample and to which extent.

This work will only focus on detection of nucleic acids. Therefore the fluorescent probes used here will be fluorescently labeled oligonucleotides.

3.2 Padlock probes and rolling circle amplification

3.2.1 General

To be able to obtain detectable signals, amplification of the sample is needed. By using something called padlock probes (*see below*) followed by rolling circle amplification, the high sensitivity and specificity accomplished by PCR will remain, but the problems with multiplexing the reaction will be solved.

3.2.2 Padlock probes

Padlock probes are linear oligonucleotides, which are designed to have one target-complementary sequence at each end. The remaining parts of the probes can be designed to include certain tag sequences, which makes it possible to detect and identify the different types of circles. The end sequences are made so that when they hybridize to their target sequences, they will be right next to each other and can then be joined by DNA ligase to form a circle of nucleotides (*Fig. 1*) [15]. The ligases used have been shown to accurately distinguish between matched and mismatched substrates. This means that only absolutely correct matched padlock probes will be ligated and form a circle [16]. Once the ligation step is done, all remaining linear padlock probes can be degraded by adding exonuclease to the

reaction (*Fig. 2*) [15]. One circle will now represent one copy of a specific DNA segment and act as template in the amplification procedure. The padlock probes used in this work were complementary to certain bacterial sequences.

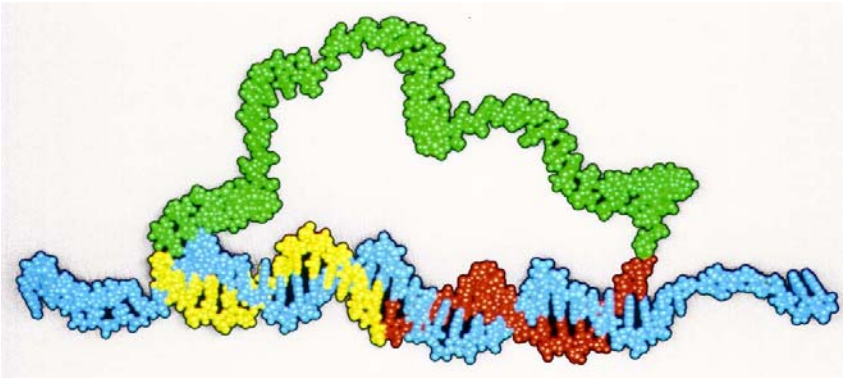

Figure 1. A circularized padlock probe (yellow, green and red) bound to its target (blue). The yellow and the red parts are the target-complementary end sequences, whereas the green part is non-complementary, but consists of the tag sequences needed for detection. Illustration is used with permission from Mats Nilsson.

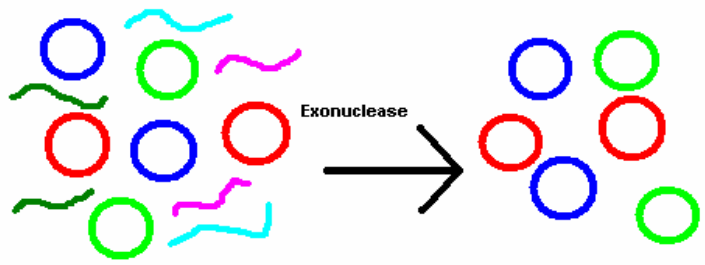

Figure 2. Exonuclease treatment. All linear padlock probes will be degraded by exonuclease, but the circular padlock probes will not be affected.

3.2.3 Rolling circle amplification

The method for amplification used in this thesis is called rolling circle amplification. As mentioned before, the previously formed circles will now act as templates. In comparison with PCR, where you need a primer pair, only one primer is needed here. In fact, the same primer can be used for almost all kinds of circles, since all padlock probes can be designed to have a universal primer sequence. By addition of DNA polymerase, the circles will be amplified, resulting in long linear concatemeric single-stranded DNA molecules. The DNA molecules will have the complementary sequences of the circles repeated ~1000 times depending on the amplification time [17]. These long DNA molecules will then fold and become so called "blobs", discrete objects with a diameter of ~1 µm. As mentioned, different padlock probes can contain different tag sequences for identification of different types of DNA sequences. Since the blobs have repeated sequences complementary to the padlock probes, detection probes with the same sequence as the tag sequence on each padlock probe can hybridize to each of the different types of blobs, ~1000 detection probes per blob. The detection probes are fluorescently labeled with different fluorophores and each type of blob will have a certain fluorophore. This gives an accumulation of fluorophores in each blob, which then makes it possible to detect and identify all the blobs in a fluorescence microscope (*Fig. 3*) [18]

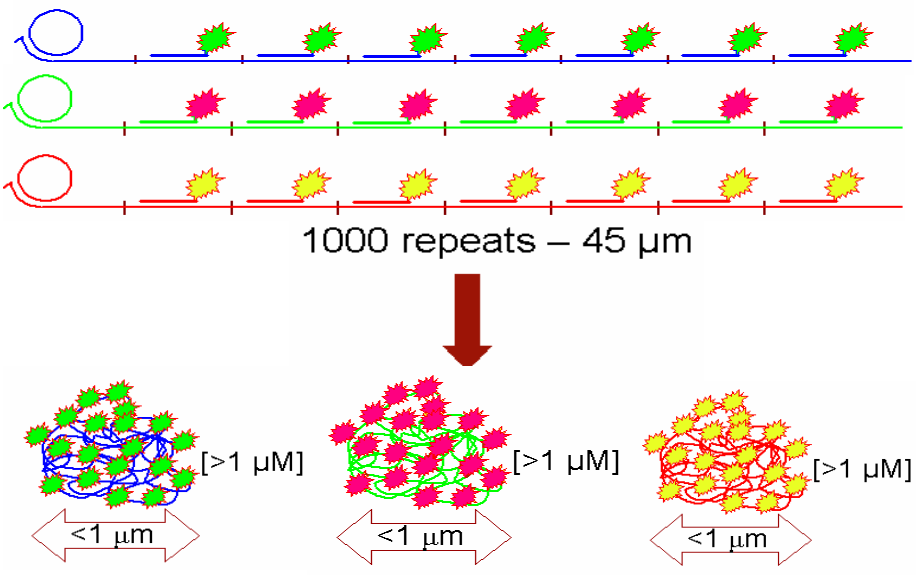

Figure 3. Rolling circle amplification.
Three different types of circles are amplified and blobs are formed. The blobs are then detected with different detection probes specific for each blob.
The illustration (slightly modified) is used with permission from Mats Nilsson.

3.3 Random array

3.3.1 Immobilization of blobs and blocking of surface

Once the padlock probes have been ligated and rolling circle amplification has been performed, the blobs can be immobilized on a glass slide. The glass slides used in this work were coated with poly-L-lysine to create a positively charged surface where the negatively charged DNA molecules can bind. The position of each blob on the glass slide will be at random, hence the name random array (*Fig. 4*). To prevent unspecific binding of fluorophores and fluorophore-connected DNA to the surface, the slide is then blocked with sonicated salmon sperm DNA.

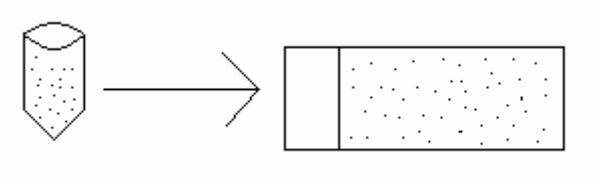

Figure 4. Immobilization of blobs.
Blobs in solution from the rolling circle amplification are immobilized randomly on poly-L-lysine coated glass slides.

3.3.2 Hybridization and stripping

To be able to detect and identify the blobs, a hybridization step is performed. Depending on how many different blobs that are going to be detected, the hybridization is followed by a washing step, called stripping, where the detection probes are washed away and then another hybridization step can be performed using different detection probes. This procedure will then be repeated until all the different blob types have been analyzed.

3.3.3 Image analysis

After each hybridization, the slides are analyzed in a fluorescence microscope with several filters to be able to detect and identify the blobs in that hybridization. Each filter has a different range of excitation and emission wavelength and a certain fluorophore must be

combined with the right filter in order to detect it. A CCD camera is attached to the microscope and the digital images obtained (*Fig. 5*) are then analyzed with certain scripts by using *Matlab*.

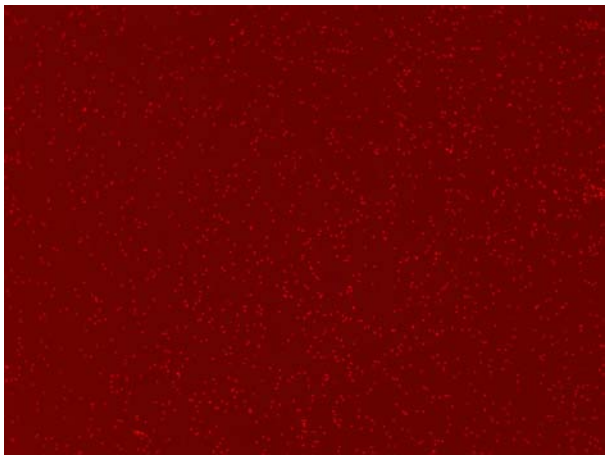

Figure 5. A digital image of blobs on a glass slide. The magnification is 20x.

Two types of scripts were used in this work. The first one gives the number of blobs and their intensities in a certain image. The intensities obtained are mean values of the values of all the peaks reaching above a given threshold and the number of blobs is equal to the number of peaks above the given threshold. The second program makes an alignment of a chosen number of images taken on the same position on a slide after each hybridization in a sequence of hybridizations. All blobs found in the images are given special ID numbers by the script. The purpose of the ID numbers is to be able to identify each type of blob. The ID numbers represent a binary summary of a number of sequential hybridization experiments. The obtained binary codes can then be compared with the expected binary codes of the population of padlock probes that have been used in the experiment. When an unexpected binary code appears, it can be due to dirt or an incomplete experiment. An example of how the numbers are obtained is shown in *Fig. 6*. The script also gives the amount of blobs with the same ID number.

A) **B)** **C)**

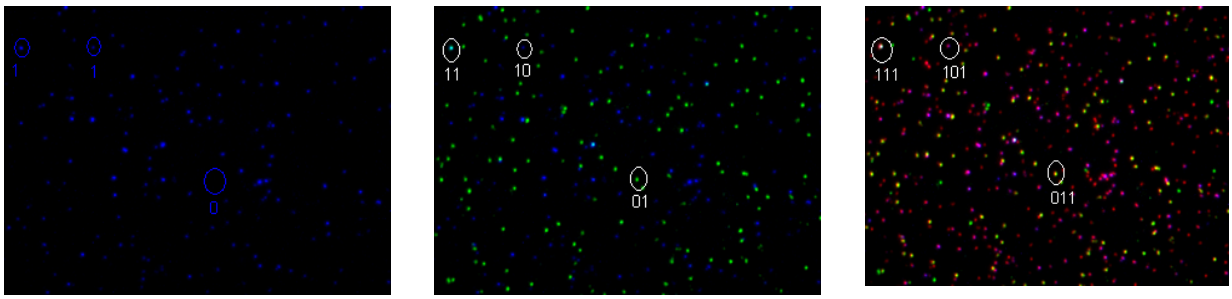

Figure 6. Three images taken on the same spot on a slide. The pictures are either taken in the same hybridization or they are the result of three sequential hybridizations. **A)** All positions where there are blobs with an intensity higher than a given threshold are assigned the value 1, while all positions where the intensity is below the given threshold are assigned 0. Three positions in the image are studied in detail in the following pictures. **B)** The image from A (blue) is aligned with the second image (green). Blobs that occur in both images have a color mixture of green and blue. All positions are again assigned either a 1 or a 0. In the first position there is a blob in both images, which means that the binary code is 11. The second position have a blue blob, but a not a green, i.e. the binary code is 10. In the last position it is the other way around, therefore the binary code is 01. **C)** Here the third image (red) is aligned with the first and second and positions are once again assigned either a 1 or a 0. Each blob has now got an binary code and can be identified according to it. All images are used with permission from Jenny Göransson.

3.4 Hybridization and denaturation kinetics

3.4.1 General

Random array is a technique highly dependent on working hybridization and stripping steps. If these steps do not function as they are supposed to, blobs could either be misclassified or not detected at all.

A lot of studies on hybridization and denaturation of nucleic acids have been done. Common for many of them is the use of formamide and variation in the salt content of the buffer and variations in temperature and pH [19-20, 22-28].

3.4.2 Hybridization kinetics

The hybridization gives the best result when the incubation temperature is between 10 and 30°C below the melting temperature (T_m) of the detection probes [19], where T_m is the temperature at which 50% of the duplexes have their strands separated [20]. In this work the hybridization was performed at a temperature of 37°C and the detection probes used here had a T_m of ~60°C, giving a difference in temperature of 23°C, which is in the suitable range. But since the temperature interval is quite wide, the optimal difference in this case might be smaller. This was investigated by hybridizing in different concentrations of formamide. The reason for using formamide is that studies have shown that formamide may increase the specificity of the hybridization and 1% of formamide lowers the T_m by 0.6-0.7°C [19-20].

Different concentrations of detection probe in the hybridization mixture will also affect the kinetics of the hybridization. If the concentration is too low, the signals from the blobs might be too weak to be detected. A study with different concentrations of detection probes was performed in order to find a concentration that gives a high intensity of the blobs. A higher concentration also increases the on-rate of the detection probes, where the on-rate is the time taken for 50% of the probes to hybridize to their target sequences, for example 0.1 nM has an on-rate of 50 min, 1 nM 5 min and 10 nM 30 s [20].

By adding salt into the hybridization mixture, the hybridization can also be improved, but the higher salt concentration, the higher degree of unspecific hybridization, which then leads to a higher background. This was further studied in this project.

Saline-sodium citrate (SSC) buffer is a buffer containing sodium chloride and sodium citrate and hybridization is often performed in this buffer [22]. Sodium citrate is a chelator, which binds to divalent ions and prevents the ions to interfere with the hybridization. The divalent ions are removed, because it is difficult to find their optimal concentration, since the concentration is relatively sensitive to changes. It is then better to only use monovalent ions instead [20]. In the current protocol, the hybridization mixture contains 1x SSC buffer, i.e. 150 mM NaCl and 15 mM sodium citrate, but increasing the concentration might result in a better hybridization. A small study of this was performed here.

3.4.3 Denaturation kinetics

When it comes to the kinetics of the stripping, i.e. the denaturation of the detection probes, they are quite the same as for hybridization, but reversed.

The temperature of stripping should instead of being lower than the melting temperature, as in the hybridization, be higher. When the temperature reaches T_m or becomes even higher, the hydrogen bonds between the blobs and the detection probes will break and it will be possible to wash away the detection probes [23]. By adding a high amount of formamide to the stripping solution the T_m will be lowered and the stripping temperature will then probably be higher than T_m. To investigate the effect of having formamide in the stripping solution, stripping was performed at different temperatures with several different concentrations of formamide and in some cases with addition of some chemicals, such as detergents and chelators, which might improve the stripping procedure.

Also by increasing the pH of the stripping solution, the detection probes can be denatured and washed away. This is due to the fact that DNA is an acid and when the pH becomes high enough the nucleosides in the DNA will be deprotonated, which results in breaking of the hydrogen bonds between the blobs and the detection probes. The pH was increased by using sodium hydroxide, but too high concentration of NaOH might etch the surface of the glass slides. Some other chemicals were also added to the solutions of high pH and the effect of the variations of the protocol was studied.

3.5 Dynamic range and multiplex analysis

The dynamic range of a method is of great interest. A high dynamic range will lead to easier and faster ways of analyzing samples, since the method will not be so dependent on sample concentration, i.e. a sample with very low concentration of DNA would then give a result as reliable as a sample with high concentration. Furthermore, the larger the dynamic range, the larger the differences in copy numbers in a blob population that can be distinguished.

The ability to analyze several DNA segments in the same reaction is also of great interest, since that too would decrease the experimental time and save resources.

A technique with a combination of a high dynamic range and a high level of multiplexity will certainly be a goal. It would mean that a lot of samples, all with varying DNA concentrations, could be analyzed in the same reaction and the outcome would be just as good as if all samples had been analyzed separately.

In this work a study of the dynamic range of the method random array and its ability to perform multiplex analysis was performed.

For the investigation of the dynamic range, five different samples with three different synthetic templates of various concentrations were mixed with their corresponding padlock probes, which then formed circles that could be amplified with rolling circle amplification. The concentration of the first template was decreased in five samples, whereas the second template concentration was increased. The last template was kept at a constant concentration throughout all of the five different samples and used as an internal reference. This study then showed whether a template of low concentration could be detected even though another template was present at a very high concentration.

The multiplex analysis was performed by having a sample of ten synthetic templates with sequences corresponding to ten different bacteria from the genera *Bacillus* or *Vibrio*. These templates were detected by adding padlock probes specific for each of the ten bacteria. The analysis then showed if all ten types of bacterial sequences could be detected or not, i.e. if the method has the ability of performing multiplex analysis.

This experiment was also performed in order to evaluate the different optimizations made earlier in this project, such as the hybridization and the stripping. If all bacteria are detected and the number of blobs is similar for each type of bacterium, it would indicate that the optimizations of the hybridization and the stripping improved the assay.

4. Materials and methods

4.1 Optimization of hybridization

4.1.1 Synthesis of blobs

4.1.1.1 Oligonucleotides

Synthetic single-stranded oligonucleotides of 30 nt (*DNA Technology*) were used as template. The sequence, corresponding to a region within the *16S* rRNA of the bacterium *Vibrio fischeri*, was designed as followed

5'-CGT GGG AAT ATG CGT TAG TGT GGG GGA TAA-3',

by Magnus Rosenquist at SLU.

From this sequence, the sequence of the padlock probes was designed by Johan Stenberg at the Rudbeck laboratory by using a program called *ProbeMaker*. The sequence was as followed

5'-TAA GGC ATA TTC CCA GGA CTT CAG AGT GTA CCG ACC TCA GTA GCC GTG ACT ATC GAC TTG TCT ATG TTT ACA GCG GGC TTA TCC CCC ACA C-3'

(91 nt, *DNA Technology*).

4.1.1.2 Phosphorylation of padlock probes

Before the ligation step could be performed, phosphorylation of the padlock probes was needed. A mixture of 1x Φ29 buffer (33 mM Tris-acetate (pH 7.9 at 37°C), 10 mM Mg-acetate, 66 mM K-acetate, 0.1% (v/v) Tween 20 and 1 mM DTT, *Fermentas*), 1 mM of ATP (Adenosine triphosphate, *Fermentas*), 1 µM of padlock probes and 0.1 U/µl of T4 PNK (Polynucleotide kinase, *Fermentas*) was incubated first at 37°C for 30 min and then at 65°C for 20 min, in order to inactivate the enzyme.

4.1.1.3 Ligation of padlock probes

For the ligation of the padlock probes, a mixture of 1x Φ29 buffer, 1 mM of ATP, 20 nM of padlock probes (phosphorylated), 60 nM of template and 0.02 U/µl of T4 DNA ligase (*Fermentas*) was incubated at 37°C for 15 min. An excess of template was used, to make sure that all padlock probes were ligated to circles.

4.1.1.4 Rolling circle amplification

To amplify the created circles rolling circle amplification was performed. First, the ligation mixture was diluted with 1x PBS buffer (Phosphate buffered saline, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, sterile filtered) to give a concentration of padlock probes of 200 pM. The reason for diluting with 1x PBS buffer instead of H_2O was that the buffer prevents the template from falling off the padlock. Then a mixture of 20 pM padlock probes, 1x Φ29 buffer, 250 µM of dNTP (*Fermentas*), 0.1 µg/µl of BSA (Bovine serum albumin, sterile filtered, *New England Biolabs Inc*) and 0.4 ng/µl of Φ29 polymerase (*Fermentas*) was first incubated at 37°C for 60 min and then at 65°C for 5 min, in order to inactivate the enzyme. The reason to use BSA in this mix was that Φ29 polymerase is expensive and will attach to the walls of the tubes; BSA prevents this by binding to the tube walls. The filters used for sterile filtering were all Millex™ sterile filters (0.22 µm) from *Millipore*.

4.1.2 Immobilization of blobs

The blobs synthesized through RCA were immobilized on poly-L-lysine coated glass slides, Poly-Prep™ slides from *Sigma-Aldrich*, by pipetting 25 µl of the RCA mixture on each slide, placing a cover slip from *Menzel-Glaser* on top of the slides and then incubating the slides in a humidity chamber at 37°C for 5 min. After 5 min the slides were placed in room temperature in a cuvette containing 1x PBS buffer for 1 min, followed by an ethanol series with washes in 70%, 85% and 99.5% EtOH (*Solveco Chemicals AB*) for 1 min each. The slides were then left to dry.

4.1.3 Blocking of slides

In order to prevent detection probes and fluorophores from unspecific binding to the surface, blocking of the glass slides was necessary. This was done by pipetting 70 µl of sonicated salmon sperm DNA (0.1 µg/µl, sterile filtered, *Amersham Pharmacia Biotech Inc*) on each slide, placing a Lifter Slip (*Erie Scientific Company*) on top, which allows more liquid under than a regular cover slip, and incubating the slides in a humidity chamber at 37°C for 10 min. After 10 min the slides were placed in room temperature in a cuvette containing 1x PBS buffer for 1 min, followed by an ethanol series as in *4.1.2*. The slides were then left to dry.

4.1.4 Hybridization of detection probes

4.1.4.1 General

The detection probe used in the hybridization was 20 nt long (*MWG*) with a sequence equal to a part of the chosen padlock probe,

5'-AGT AGC CGT GAC TAT CGA CT-3'.

The fluorophore attached to the 5' end of the detection probes was Cy 3.

4.1.4.2 Optimization of detection probe concentration

Five different concentrations of detection probe were investigated in a hybridization mixture containing 1x SSC buffer (Saline – sodium citrate, 150 mM NaCl and 15 mM sodium citrate, sterile filtered). The different concentrations were 1 nM, 5 nM, 10 nM, 50 nM and 100 nM.

The hybridization was made by first placing a mask of silicone on top of a glass slide (*Fig. 7*).

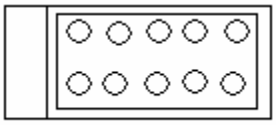

Figure 7. A glass slide with a silicone mask placed on top of it. The mask creates separated wells, in which different solutions can be added without the risk of interfering each other.

The mask divides the slide into several separated areas, where different solutions can be added without interfering with each other. Then the five different hybridization mixtures were added in separate wells (50 µl per well) and the slide was incubated at 37°C with shaking for 30 min. After the incubation, the wells were washed with ~70 µl of 1x PBS buffer twice. The silicone mask was removed and the slide was washed in 1x PBS buffer for 1 min, followed by an ethanol series as in *4.1.2* and then left to dry.

4.1.4.3 Optimization of hybridization time

The incubation time for the hybridization was investigated. The time scale was 2.5 min, 5 min, 10 min, 20 min, 40 min, 80 min and 160 min. The hybridization was done using a silicone mask and a hybridization mixture containing 1x SSC buffer (sterile filtered) and either 10 or 100 nM of detection probe. The incubation temperature was 37°C. After the incubation series, the slides were washed as described in *4.1.4.2*.

4.1.4.4 Optimization of salt concentration

The concentration of salt in the hybridization mixture was optimized by hybridizing in four different wells with four different hybridization mixtures, each containing a different concentration of salt. The hybridization mixtures were made as followed; all four of them contained 100 nM of detection probe, 10 mM of EDTA (sterile filtered) and 20 mM of Tris-HCl (pH 8.0, sterile filtered), together with either 125 mM, 250 mM, 500 mM or 1000 mM NaCl (sterile filtered). There was also a fifth well with the reference hybridization mix (100 nM of detection probe and 1x SSC buffer (sterile filtered)). The slide was then incubated at 37°C for 30 min and washed according to *4.1.4.2*.

4.1.4.5 Optimization of formamide concentration

Five different hybridization mixtures with different concentrations of formamide were added to five different wells on a glass slide. The amounts of formamide were 0%, 10%, 20%, 30% and 40% (sterile filtered, *Merck* or *Sigma-Aldrich*). 100 nM of detection probe and 1x SSC (sterile filtered) was used in all hybridization mixtures. The slide was then incubated at 37°C for 30 min and washed according to *4.1.4.2*.

4.1.4.6 Optimization of SSC buffer concentration

Hybridization was done with two solutions, one with 1x SSC buffer (sterile filtered) and 100 nM detection probe and the other with 2x SSC buffer (sterile filtered) and 100 nM detection probe, at 37°C for 30 min. Washing of the slide was done as in *4.1.4.2*.

4.1.5 Microscopy and image analysis

The blobs on the slides were detected in an epifluorescent microscope (Axioplan 2 imaging, *Zeiss*) equipped with a 100 W mercury lamp, a CCD camera (AxioCam MRm, *Zeiss*) and several excitation and emission filters to be able to visualize different fluorophores. Pictures were taken on three different positions on each slide using a 20x objective (Fluar, *Zeiss*) in a special program, called *AxioVision Release 4.*3. A list of the names of the filters used with each fluorophore can be found in *Table I* in *Appendix A*. The digital images were analyzed by running a script in *Matlab*. The script was written by Carolina Wählby (Dep. of Genetics and Pathology, Uppsala University) and gives the intensity of the blobs. From the received files, mean values of the intensity of the blobs on each slide were calculated.

4.2 Optimization of stripping

4.2.1 Synthesis of blobs

4.2.1.1 Oligonucleotides

Synthetic single-stranded oligonucleotides of 29 nt (*DNA Technology*) were used as template. The oligonucleotides had a sequence corresponding to a region within the *16S* rRNA of the bacterium *Vibrio cholerae*,

5’-CCC TGG GCT CAA AGG AAT CGC ATT TG-3’.

The padlock probes were designed as followed

5’-TAG GTT GAG CCC AGG GAC TTC TAG AGT GTA CCG ACC TCA GTA GCC GTG ACT ATC GAC TTG CGT CTA TTT AGT GGA GCC CAA ATG CGA TTC C-3’

(91nt, *DNA Technology*).

4.2.1.2 Phosphorylation, ligation and rolling circle amplification

Phosphorylation and ligation of the padlock probes were done as in *4.1.1.2* and *4.1.1.3*. The rolling circle amplification was performed according to *4.1.1.4*.

4.2.2 Washing of glass slides

A cuvette containing a solution of 0.1% SDS (sodium dodecyl sulfate) was put in an ultrasonicator. Poly-L-lysine coated glass slides were soaked into the solution and three impulses of ultrasound were run. The slides were then rinsed in MilliQ-H_2O for 1 min, followed by 70% EtOH for another minute and centrifuged to dry for 2 min.

4.2.3 Immobilization of blobs and blocking of slides

The immobilization of blobs on the glass slides and the blocking of the slides were performed according to *4.1.2* and *4.1.3*.

4.2.4 Hybridization of detection probes

The detection probe used was the same as in *4.1.4.1*, which sequence is complementary to a part of both the blobs synthesized here and the ones in the hybridization experiment. The hybridization mixture contained 100 nM of detection probe, 20% formamide (sterile filtered) and 2x SSC buffer (sterile filtered).

The hybridization was either done by using silicone masks as in *4.1.4.2* or by using Lifter Slips, which are a different type of cover slips that allows more liquid (70 µl of hybridization mixture per slide) under than regular cover slips due to the extra bars on the sides of the slips. The incubation time was 30 min at 37°C (in a humidity chamber when using Lifter Slips and with shaking for the masks). After half an hour the slides were washed as in *4.1.4.2*.

4.2.5 Microscopy

The blobs were detected using the same epifluorescent microscope and set up as in *4.1.5*. Lines were drawn with a diamond pen on the back of the glass slide to create crosses, which were then centered in the microscope, to be able to come back to the same position later. Pictures were taken on three different positions on each slide and saved for later analysis.

4.2.6 Stripping of detection probes

4.2.6.1 General

All stripping solutions in the following experiments were sterile filtered through a 0.22 µm sterile filter before use.

The stripping was done in Falcon tubes of 50 ml, the slides were totally soaked in each solution and incubated for 5 min with gentle stirring. After the stripping, the slides were washed in 1xPBS buffer for 1 min, followed by washes in 70%, 85% and 99.5% EtOH for 1 min each and then left to dry.

4.2.6.2 Optimization of formamide concentration

For stripping, six different solutions, divided into two groups, were prepared and tested in different temperatures. The first group consisted of three solutions containing either 30%, 40% or 50% formamide in 20 mM Tris-HCl (pH 8.0) and the temperature tested for these three stripping solutions was 37°C. The second group of stripping solutions were 50%, 60% and 70% formamide and they were all tried out in 37°C and 50°C.

4.2.6.3 Investigation of NaOH concentration

A solution of 10 mM NaOH was tested in room temperature. The same solution together with a second one of 100 mM NaOH were then studied in two temperatures, 37°C and 50°C.

4.2.6.4 Investigation of different stripping conditions in 50% formamide

Three different experiments were set up. All the solutions in the three experiments contained 50% formamide together with different chemicals that might improve the stripping.

In the first experiment, four different solutions containing either 2x SSC buffer, 20 mM EDTA, 0.1% SDS or 20 mM EDTA + 0.1% SDS were tested in 37°C and 50°C. The solutions in the second experiment all contained 20 mM Tris-HCl (pH 8.0) together with either 2x SSC buffer, 300 NaCl, 30 mM EDTA or 300 mM NaCl + 30 mM EDTA and they were only studied in 50°C. A solution containing 2 x SSC buffer was used as a reference this time. In the third experiment, the solutions were 2x SSC buffer, 300mM NaCl, 30 mM sodium citrate and 300 mM NaCl + 30mM EDTA and they were only investigated in 50°C.

4.2.6.5 Investigation of different stripping conditions in 10 mM NaOH

All stripping solutions used here were in 10 mM NaOH.

Four different amounts of formamide (5%, 10%, 20% and 50%) were studied in 37°C. Another experiment with three solutions containing either 20 mM EDTA, 0.1% SDS or 20 mM EDTA + 0.1% SDS was performed in two different temperatures, 37°C and 50°C.

Finally a last experiment was performed, where the best working solutions of 10 mM NaOH were compared with the best solution of formamide at 50°C.

4.2.7 Microscopy and image analysis

After the stripping procedure, the slides were analyzed in the epifluorescent microscope again. The same crosses on the back of the slides as before were centered and pictures were taken. The images were then analyzed together with the ones taken before stripping the slides with the same script as in *4.1.5*.

4.3 Investigation of dynamic range

4.3.1 Synthesis of blobs

4.3.1.1 Oligonucleotides

The investigation of the dynamic range was performed by using three different templates in combination with their padlock probes. The templates were synthetically made (*DNA Technology*) and each template consisted of a specific sequence (*Table II* in *Appendix A*) designed to be found in a region within the *16S* rRNA of one of three different bacteria, *Vibrio cholerae*, *Vibrio fischeri* or *Vibrio vulnificus*. The padlock probes (*DNA Technology*) were designed to specifically bind to one of the three bacteria above (*Table II* in *Appendix A*).

4.3.1.2 Phosphorylation of padlock probes

The phosphorylation of padlock probes was performed according to *4.1.1.2*.

4.3.1.3 Ligation of padlock probes

Five ligation reactions were performed in parallel for 60 min at 55°C and in each of the five reactions all three templates were added in varying concentrations. The concentration of the first template (*V. cholerae*) was decreased 10-fold from 100 pM to 10 fM, whereas the concentration of the second template (*V. fischeri*) was in reversed order, starting at 10 fM and going up to 100 pM. The concentration of the third template (*V. vulnificus*) was kept constant at 1 pM in all five reactions. Each ligation mixture also contained 1x Ampligase buffer (20 mM Tris-HCl pH 8.3, 25 mM KCl, 10 mM $MgCl_2$, 500 µM NAD and 0.01% Triton X-100), 0.2 µg/µl BSA (sterile filtered), 75 mM KCl (sterile filtered), 2 nM of each of the three padlock probes and 0.25 U/µl of Ampligase (*Epicentre® Biotechnologies*).

4.3.1.4 Rolling circle amplification

To amplify the created circles, rolling circle amplification was performed. An RCA mixture of 2x Φ29 buffer, 0.2 µg/µl of BSA (sterile filtered), 375 µM of dNTP (sterile filtered), 3 ng/µl of Φ29 Polymerase and 50 nM of primer with sequence:

5'-TAC TGA GGT CGG TAC ACT CT-3'

(20 nt, *Biomers*) was added to each of the five ligation reactions in ratio of 1:2. The primer used in the RCA mixture hybridizes to all the three types of padlock probes. The reactions were then incubated at 37°C for 60 min, transferred to 65°C and incubated there for 5 min.

4.3.2 Washing of glass slides, immobilization of blobs and blocking of slides

Five glass slides were washed according to *4.2.2* and the five RCA reactions was pipetted on the five washed slides as in *4.1.2*. Blocking of the slides was performed according to *4.1.3*.

4.3.3 Hybridization of detection probes

Three different detection probes with different fluorophores (Cy 3, Cy 5 and Bodipy 493/503) were used to detect all blobs. The three probes were specific for sequences from each of three bacterial species (*Table II* in *Appendix A*).

The hybridization was done using Lifter Slips according to *4.2.2*, but with a hybridization mixture of 100 nM of each detection probe, 20% formamide and 2x SSC buffer (sterile filtered).

4.3.4 Microscopy and image analysis

The same microscope as in *4.1.5* was used to detect the blobs on the five slides. The images taken were analyzed with the same script in *Matlab* as in *4.1.5*.

4.4 Multiplex analysis

4.4.1 Synthesis of blobs

4.4.1.1 Oligonucleotides

Ten different templates with their corresponding padlock probes (*Table III* in *Appendix A*) were used in this multiplex analysis. They were all synthetically made and each template consisted of a specific sequence designed to be found in a region within the *16S* rRNA of one of ten different bacteria (*Table III* in *Appendix A*).

4.4.1.2 Phosphorylation, ligation and rolling circle amplification

Phosphorylation and ligation of the padlock probes were performed separately for each of the ten different padlock probes according to *4.1.1.2* and *4.1.1.3*.

The ligated padlock probes were all mixed and one rolling circle amplification was performed according to *4.1.1.4*, but with a concentration of 2 pM of each type of ligated padlock probe instead of 20 pM.

4.4.2 Washing of glass slides, immobilization of blobs and blocking of slides

A glass slide was washed as in *4.2.2*. The blobs were then immobilized according to *4.1.2* and the slide blocked as in *4.1.3*.

4.4.3 Hybridization, microscopy and stripping of detection probes

In order to detect all ten types of bacterial blobs several sequential hybridizations were performed. For every hybridization a general detection probe, which hybridize to every bacterial blob, and two specific probes were used, giving a total of five hybridizations. The order of the specific detection probes along with their sequences and fluorophores can be found in *Table III* in *Appendix A*.

Each hybridization was performed according to *4.1.4.2*, but with a hybridization mixture of 100 nM of each of the three detection probes (sterile filtered), 20% formamide (sterile filtered) and 2x SSC buffer (sterile filtered). The incubation time for the last three hybridizations was 60 min instead of 30 min.

After each hybridization, the slide was viewed at certain marked positions in the epifluorescent microscope used in *4.1.5* and pictures were taken.

The slide then went through the same stripping procedure as in *4.2.6.1*, with a sterile filtered solution of 50% formamide and 2x SSC buffer. Once the detection probes were washed away another hybridization could be performed.

4.4.4 Image analysis

The images taken were analyzed by running a script, written by Carolina Wählby, in *Matlab*. This script aligns all images taken at the same position on the slide with each other and the outcome is a file with the number of blobs for each blob ID number. Mean values of the number of blobs with the correct ID numbers for the three positions of each bacterium were calculated and compared.

5. Results

5.1 Hybridization

5.1.1 Detection probe concentration

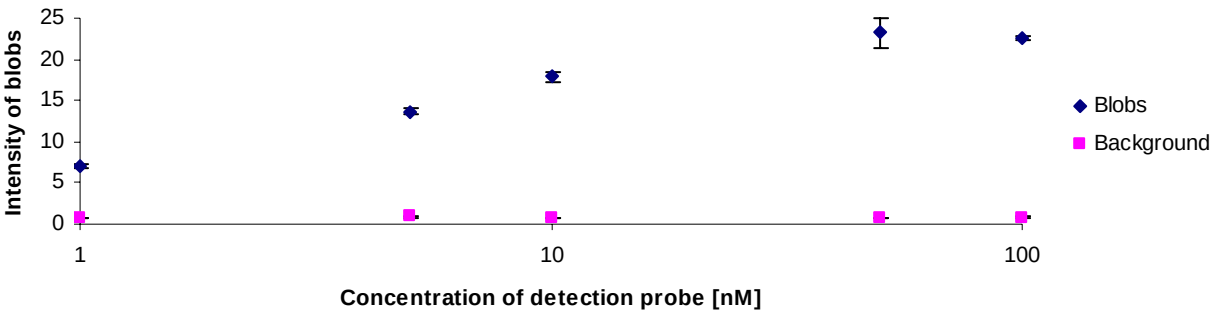

Figure 8. Optimization of concentration of detection probe in a 1xSSC buffer. The hybridization was performed at 37ºC for 30 min. Three pictures for each concentration were taken and analyzed in *Matlab*. The points are mean values of either the intensity of the blobs or the background in the three images for each concentration. The standard deviations were calculated from three images. The different concentrations of detection probe are plotted on a logarithmic scale.

The effect of increasing the concentration of detection probe in the hybridization mixture in order to obtain a higher value of the intensity of the blobs was investigated by hybridizing in five different probe concentrations (1 nM, 5 nM, 10 nM, 50 nM and 100 nM) in a 1x SSC buffer at 37°C for 30 min.

From *Fig. 8* one can see that the intensity of the blobs was dependent on the concentration of detection probe, whereas the background stayed constant even though the concentration of detection probe was increased. The increase of the intensity of the blobs was almost linear from 1 to 10 nM of detection probe, but somewhere before 50 nM of detection probe the intensity leveled off and reached its maximum value.

5.1.2 Hybridization time

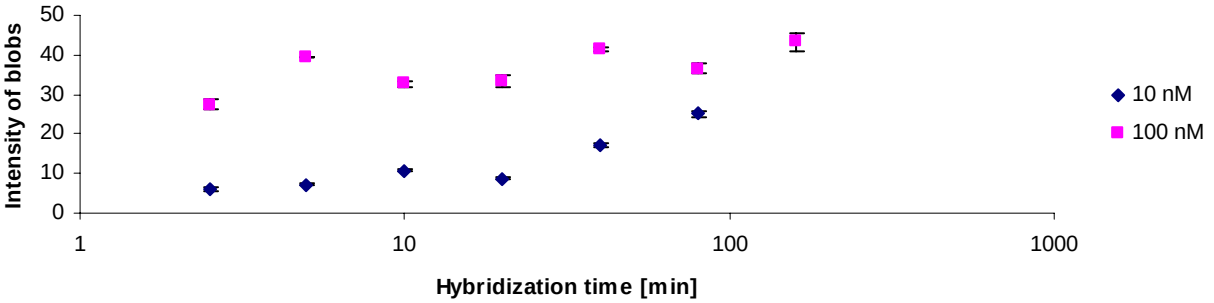

Figure 9. Hybridization with either 10 nM or 100 nM of detection probe in 1x SSC buffer for different incubation times at 37ºC. Each point is a mean value of the intensity of the blobs from three images and the standard deviations were calculated from the three images. Due to experimental problems, it was not possible to look at the last position (160 min) on the slide with 10 nM detection probe. The hybridization times are plotted on a logarithmic scale.

The hybridization time was investigated at 37°C for two different concentrations of detection probe (10 or 100 nM) in 1x SSC buffer and for six different time points. As mentioned earlier, a higher concentration of detection probe increased the on-rate of the probes, i.e. the time taken for 50% of the probes to hybridize to their target sequences.

The result in *Fig. 9* showed that the amount of time needed for the two concentrations of detection probe to reach certain intensity was dependent on the concentration of detection probe. For the 10 nM concentration the intensity of the blobs seemed to increase constantly with increasing hybridization time. The intensity of the blobs for the 100 nM was going somewhat up and down with increasing time, but there might be a slight increase in intensity for increasing time here as well. The last position for the 10 mM solution, which would give the point at 160 min, was not possible to analyze in the microscope, because the position was too far out on the edge of the slide, so the objective could not get close enough.

5.1.3 Salt concentration

By adding salt to a hybridization reaction, the intensity of the blobs can increase, but higher concentrations of salt might give more unspecific hybridization. To determine whether extra salt should be included in the hybridization mixture or not, four different concentrations of sodium chloride (125 mM, 250 mM, 500 mM and 1000 mM) were tried out in four hybridization reactions at 37°C for 30 min.

The only conclusion from this experiment was that the intensity of the background might increase with increasing amount of salt (data not shown)

5.1.4 Formamide concentration

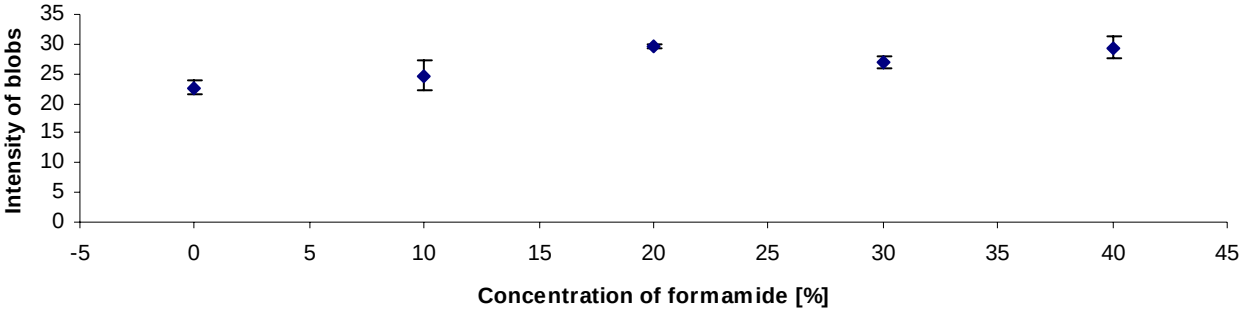

Figure 10. Variation of formamide concentration in a hybridization mixture of 1x SSC buffer. The hybridization was performed at 37°C for 30 min. The values are mean values of the intensities in three images and the standard deviations were calculated from the intensity values of the three images.

The theoretical difference between the melting temperature of the detection probes and the hybridization temperature was 10-30°C. Formamide lowers the melting temperature, so by adding it to a hybridization reaction the hybridization can be improved.

Five different concentrations of formamide (0%, 10%, 20%, 30% and 40%) were investigated at a hybridization temperature of 37°C. The result in *Fig. 10* showed that with increasing concentrations of formamide up to 20%, the intensity of the blobs increased as well. As the concentration reached above 20%, the intensity stayed relatively constant or got even lower than the intensity at 20% formamide.

The background was not affected by the use of formamide in the hybridization mixture (data not shown).

5.1.5 SSC buffer concentration

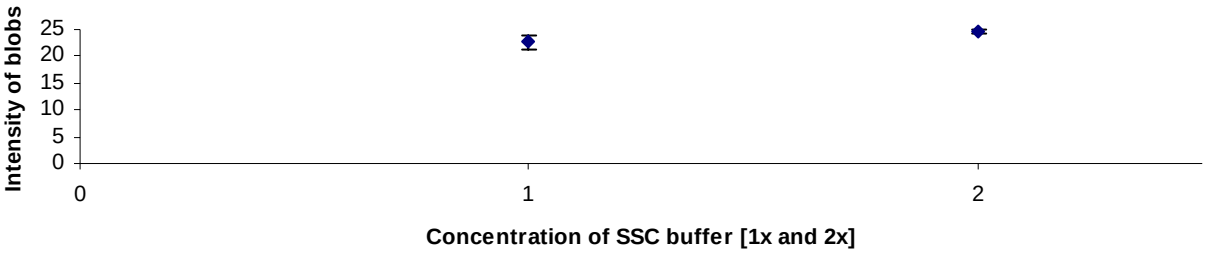

Figure 11. Two different concentrations of SSC buffer in a hybridization mixture of 100 nM detection probe. The hybridization was performed at 37º for 30 min. The points are mean values of the intensities of the blobs in three images for each of the concentrations and the standard deviations were derived from the values of the intensities from the three images.

In the current protocol the amount of SSC buffer in the hybridization mixture was 1x, but a small study, where a hybridization mixture of 1x SSC was compared with one of 2x SSC, was performed. The result showed that the hybridization with the mixture containing 2x SSC buffer gave a slightly higher intensity of the blobs than the one with 1x SSC buffer (*Fig. 11*), without increasing the background (data not shown).

5.1.6 Summary of hybridization

In brief, the results from the optimization of hybridization were that the concentration of detection probe should be at least above 10 nM, formamide should be added to a final concentration of 20% and 2x SSC buffer should be included in the hybridization mixture. The hybridization time could not be defined exactly, but a higher concentration of detection probe required a shorter amount of hybridization time to reach certain intensity.

5.2 Stripping

5.2.1 Formamide concentration

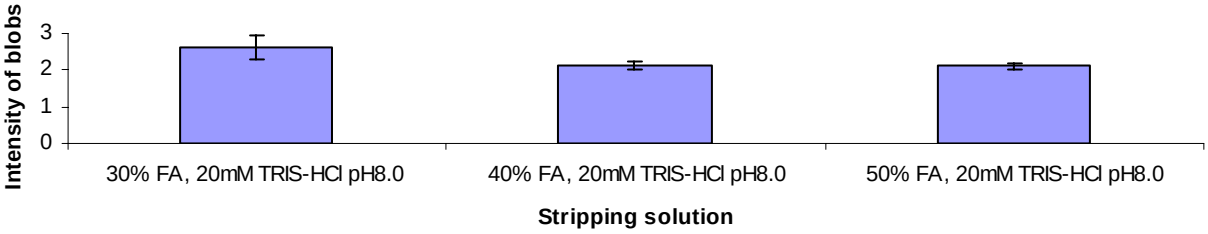

Figure 12. Stripping of glass slides in Tris buffer. Three different concentrations of formamide in 20 mM Tris-HCl was tested as stripping solutions at 37°C for 5 min. The values are mean values of the intensity at three positions on each slide and the standard deviations were determined from the three images.

If the temperature is high enough in comparison to the melting temperature of the detection probes, they will detach from the blobs and new probes can be added. As mentioned, formamide can lower the melting temperature.

Before the stripping procedure, pictures were taken on the blobs and the intensities of the blobs were then calculated using a special script in *Matlab*. Three different concentrations of formamide (30%, 40% and 50%) in 20 mM of Tris-HCl (pH 8.0) were then investigated at 37°C. Pictures were again taken on the same spots and the intensities of the blobs now were then compared with the intensities of the blobs before the stripping. A good result would be a drastic drop in the intensity of the blobs after the stripping compared to the intensity before. It will probably be impossible to decrease the intensity to zero, since there will always be some background signal. The outcome of the experiment can be seen in *Fig. 12*, where the intensity of the blobs was lowest when stripping in 50% formamide. The intensity of the blobs before stripping had a value of ~12 (data not shown).

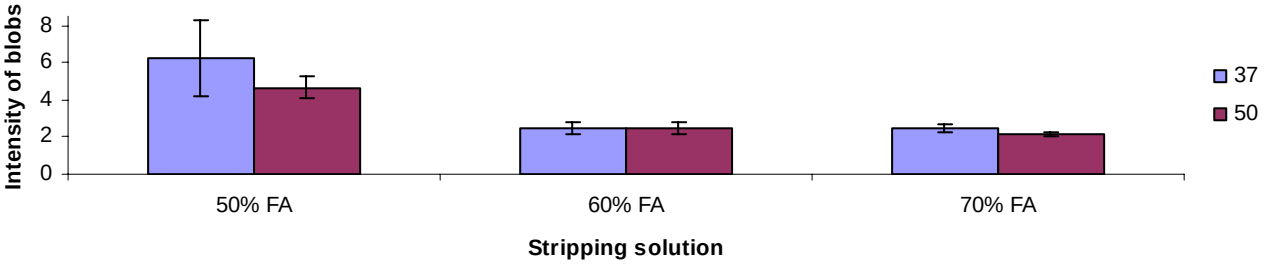

Figure 13. Stripping of glass slides in formamide. Three different concentrations of formamide as stripping solutions in two different temperatures for 5 min were investigated. All values are mean values of the intensity in three images and the standard deviations were calculated from the intensity values of the three images.

Again three concentrations of formamide (50%, 60% and 70%) were tried out, but at two temperatures (37°C and 50°C) and without Tris-HCl this time. The result (*Fig. 13*) showed that the intensity of the blobs decreased with increasing amount of formamide to a certain extent, the differences in intensity between 60% and 70% of formamide were close to zero. Also a high temperature of stripping seemed to be more important with lower concentrations of formamide than higher. Before the stripping the blobs had an intensity of ~23 (data not shown).

By comparing the two diagrams in *Fig. 12-13* one could say that using Tris-HCl (pH 8.0) in the stripping solution seemed to give a better result. Due to some troubles with various exposure times in the microscope for each experiment, which in turn gives different intensities, comparison between different experiments should be done with precaution.

5.2.2 NaOH concentration

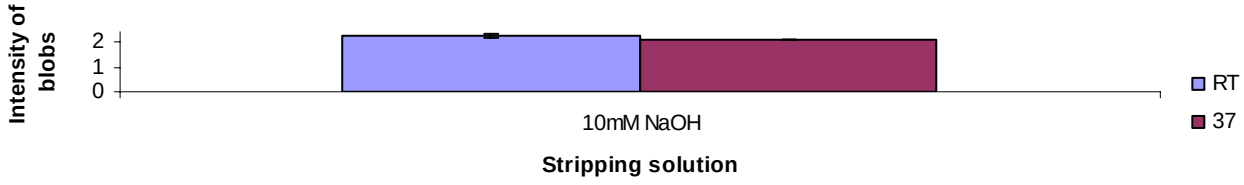

Figure 14. Stripping of glass slides in 10 mM NaOH at room temperature or 37°C for 5 min. Values are mean values of the intensity of three images and the standard deviations were determined from the different images.

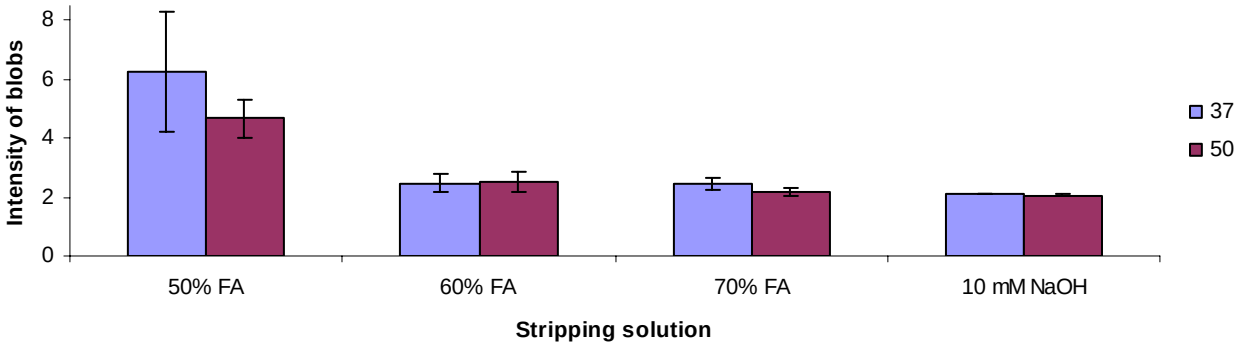

Figure 15. A comparison of stripping conditions: 10 mM NaOH and different concentrations of formamide at two temperatures. The time for stripping was 5 min. Values are mean values of the intensity of three images on each slide and the standard deviations were determined from the different images.

Stripping solutions of different concentrations of sodium hydroxide (10 mM or 100 mM), in order to get a high pH of the stripping solution, in different temperatures (RT, 37°C or 50°C) were investigated.

The diagram (*Fig. 14*) showed that stripping at 37°C resulted in a slightly lower intensity of blobs than stripping at room temperature, and raising the temperature from 37°C to 50°C did not seem to give a large effect either, the intensity after stripping was quite the same (data not shown). Using 100 mM of NaOH instead of 10 mM to reach an even higher pH did not lower the intensity of the blobs after stripping (data not shown).

A comparison of stripping in a solution of only sodium hydroxide or only formamide (*Fig. 15)* was performed and the result showed that it might be slightly better to strip in sodium hydroxide.

5.2.3 Different stripping conditions in 50% formamide

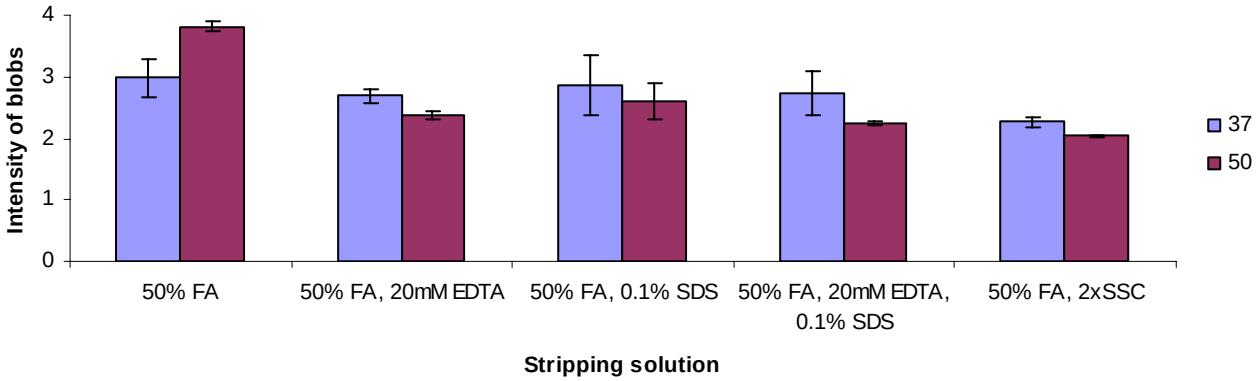

Figure 16. Stripping of glass slides in five different solutions of 50% formamide with different additives for 5 min in two temperatures. The values are mean values of the intensity of blobs from three positions and the standard deviations were calculated from the three positions.

An attempt to improve a stripping solution of 50% formamide was done by adding various additives to the solution and the result can be seen in *Fig. 16*. It showed that the addition of either EDTA, SDS or both gave a decrease in intensity after stripping, but the one that worked

best was the addition of SSC buffer, which is a buffer containing sodium chloride and sodium citrate (2x SSC → 300 mM NaCl + 30 mM sodium citrate). The stripping also seemed to give the best result when it was performed at 50°C. The increase in intensity between 37°C and 50°C for only 50% formamide was probably not correct, it should decrease.

The solution of 50% formamide and 2x SSC was further investigated at 50°C by adding, removing or replacing certain chemicals from the solution. Neither of the chemicals added, removed or replaced improved the outcome of the stripping, the solution of 50% formamide and 2x SSC still gave the lowest intensity of the blobs, i.e. the best result, (data not shown).

5.2.4 Different stripping conditions in 10 mM NaOH

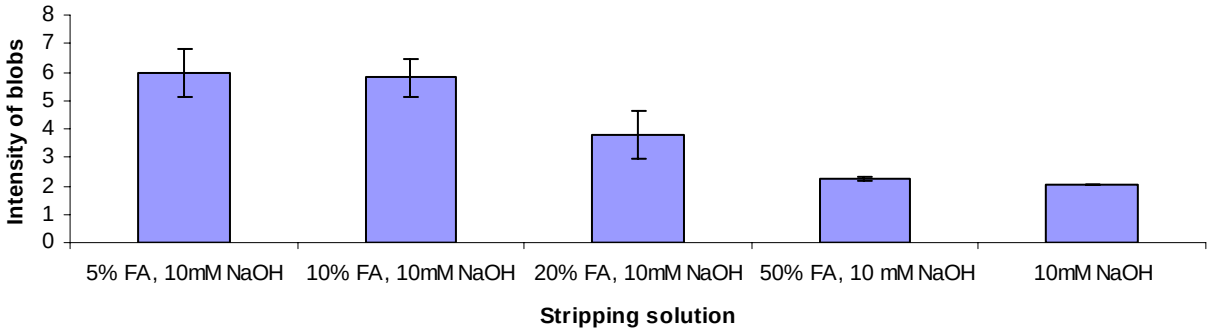

Figure 17. Stripping on glass slides at 37°C in solutions of 10 mM NaOH together with different amounts of formamide for 5 min. The values are mean values of the intensity of blobs from three positions and the standard deviations were calculated from the three positions.

A stripping solution of 10 mM NaOH has been shown to work quite successfully. Experiments have also shown that having formamide in the stripping solution improves the stripping. Here these two chemicals were combined, which gave the result seen in *Fig. 17*, that is when adding formamide to a stripping solution of 10 mM NaOH, the stripping did not work as well as before the addition.

Further possible improvements of the 10 mM solution were studied by adding certain chemicals, such as SDS and EDTA, but none of the solutions tested yielded a better result (data not shown).

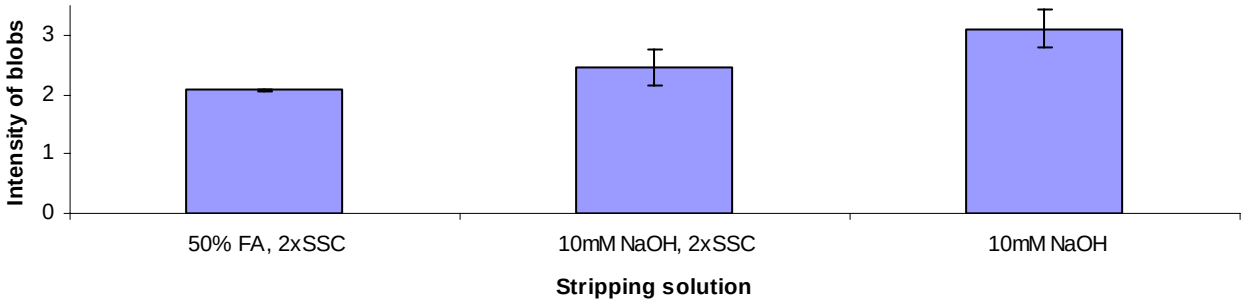

Figure 18. Stripping of glass slides. A comparison of the formamide solution with the best result and the best working solutions of 10 mM NaOH. The stripping temperature was 50°C and the time 5 min. The values are mean values of the intensity of blobs from three positions and the standard deviations were calculated from the three positions.

50% formamide and 2x SSC buffer was the stripping solution of formamide that gave the best result. A comparison was made between this and the two best working solutions of 10 mM NaOH at 50°C. *Fig. 18* shows that of all solutions investigated in this study, the solution consisting of 50% formamide and 2x SSC buffer was the one that removed the detection probes best.

5.2.5 Summary of stripping

In short, the conclusion from the stripping optimization experiments was that the solution used for stripping should consist of 50% formamide and 2x SSC buffer and the temperature for stripping should be 50°C.

5.3 Dynamic range

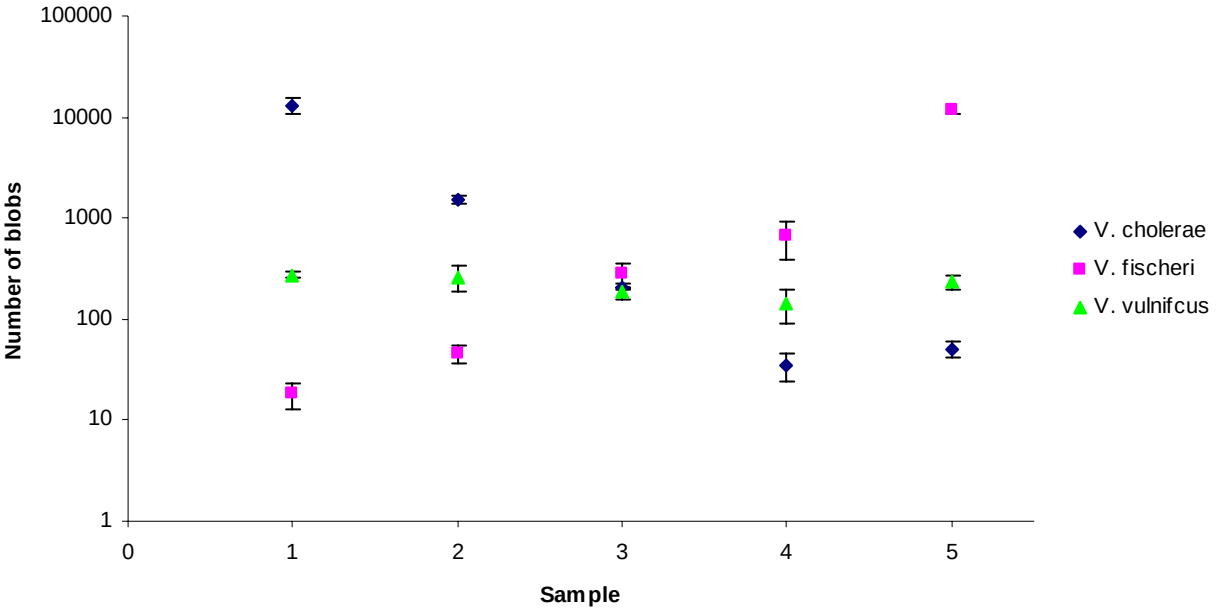

Figure 19. Investigation of the dynamic range. Three synthetic templates with sequences derived from three *Vibrio* bacteria (*V. cholerae*, *V. fischeri* and *V. vulnificus*) were used in different concentrations for each sample, where the template corresponding to *V. vulnificus* was held constant to work as an internal reference. Sample 1: 100 pM *V. cholerae*, 10 fM *V. fischeri* and 1 pM *V. vulnificus*, sample 2: 10 pM *V. cholerae*, 100 fM *V. fischeri* and 1 pM *V. vulnificus*, sample 3: 1 pM *V. cholerae*, 1 pM *V. fischeri* and 1 pM *V. vulnificus*, sample 4: 100 fM *V. cholerae*, 10 pM *V. fischeri* and 1 pM *V. vulnificus* and sample 5: 10 fM *V. cholerae*, 100 pM *V. fischeri* and 1 pM *V. vulnificus*. The mean number of blobs for each template concentration and padlock probe at three positions on each slide are plotted on a logarithmic scale.

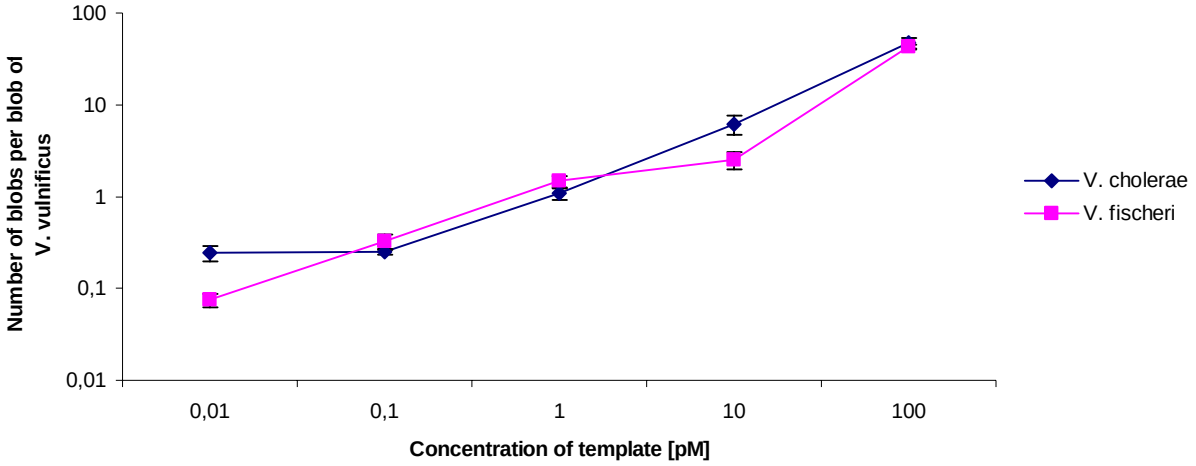

Figure 20. The ratio between the number of blobs for *V. cholerae* and *V. vulnificus* compared with the ratio between the number of blobs for *V. fischeri* and *V. vulnificus*. The template concentration of *V. vulnificus* was kept constant throughout the experiment, whereas the concentration of template for the other two was varying from 10 fM up to 100 pM. The ratios are plotted on a logarithmic scale.

For the investigation of the dynamic range, five samples containing three different templates of different concentrations in each sample where mixed with their corresponding padlock probes. The concentration of one of the templates was kept constant and used as an internal reference, whereas the other two were varied in the different samples (*Fig. 19*). The padlock probes were then ligated and amplified. A hybridization with three specific detection probes were performed and the slide was then analyzed by counting the number of detected blobs in the images taken on the slide.

The result of the study can be seen in *Fig. 19* and *20* and it showed a dynamic range of four orders of magnitude. Each of the three bacteria seemed to follow the expected pattern quite satisfactory, i.e. the number of blobs for *V. cholerae* should decrease 10-fold throughout the experiment, whereas the number of blobs for *V. fischeri* should increase as much as *V. cholerae* decreases, and when it comes to *V. vulnificus*, the number of blobs should be the same in all samples. The only value that did not follow the pattern was the number of blobs for *V. cholerae* in the last sample. Possible explanations to this are further discussed in *6.3*.

Fig. 20 shows the number of blobs of *V. cholerae* and *V. fischeri* per blob of *V. vulnificus*, which was the template with constant concentration throughout the experiment, for each concentration of template. The ratios are following each other in a linear manner, but the increase is not 10-fold as expected.

5.4 Multiplex analysis

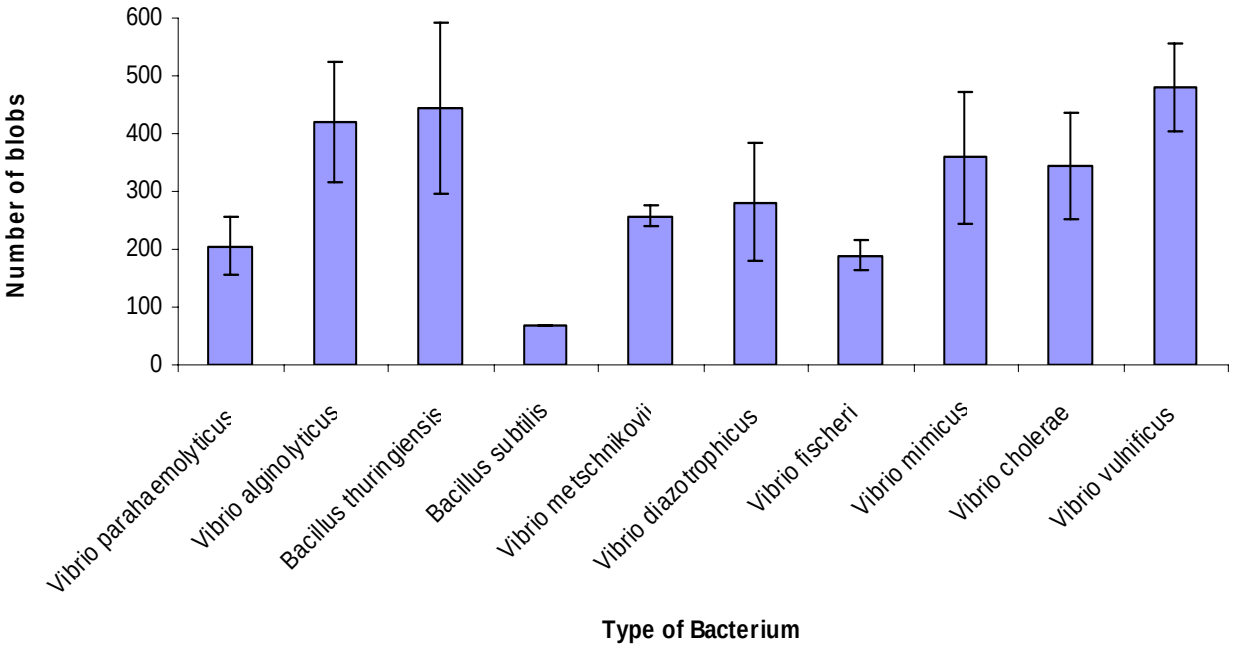

Figure 21. Multiplex analysis. A sample of ten different synthetic templates of the same concentration with specific sequences found in ten different bacteria were analyzed. The detection was made by performing five sequential hybridizations, with one general detection probe and two specific for each hybridization. The first two bacteria in the diagram were detected in the first hybridization, the two next ones in the second and so on. The values are mean values of blobs for three positions on the slide, except for the value of *Bacillus subtilis*, which is the number of blobs only at one position on the slide. The standard deviations were calculated from the three images.

A multiplex analysis, where ten different types of blobs were to be detected, was performed by having a sample containing ten different types of templates with the same concentrations together with their corresponding padlock probes. Five sequential hybridizations were performed in order to detect all ten types of blobs. In each hybridization one general detection probe and two specific ones were used. The images from the hybridizations were then aligned with each other and ID numbers for identification of the different types of blobs were obtained by running a special script in *Matlab*. By checking the different ID numbers obtained from the analysis and comparing them with the expected ID numbers, the number of blobs for each type of bacterium could be found.

The result (*Fig. 21*) showed that all ten types of blobs could be detected, but the number of blobs found for each bacterium differed from each other to a certain degree. When adding the number of blobs for all of the ten oligonucleotides representing the ten bacterial species and comparing that to the total amount of blobs detected at one position, the number for the ten oliognucleotides were approximately only half of all blobs. Except for the blobs detected with all the general probes and only one specific, about 20% of all blobs were only detected by the general probes and not with any of the specific probes. This means that ~30% of all blobs were either not correctly classified or represented some kind of dirt.

This experiment was also performed in order to evaluate the optimizations for hybridization and stripping. An indication of a working stripping procedure in-between each hybridization would be that, when checking all the different types of ID numbers there should not be a lot

of blobs with ID numbers showing that the same blobs are found with two or more different specific probes in different hybridizations.

In this experiment the ID numbers representing a large number of blobs had binary codes indicating that the same blob was detected with the general detection probes, but only with one specific probe (data not shown). This result then showed that the detection probes used in one hybridization were washed away in the stripping procedure performed between the hybridizations, and thus not detected in the next hybridization.

As for the hybridization, the evaluation of the optimizations can be made by checking the number of blobs detected. Since the same concentration of template was used for all ten templates, the same number of blobs should be found for each bacterium. This was not the outcome of this experiment (*Fig. 21*), the number of blobs detected was in the range of 67 for *B. subtilis* to 479 for *V. vulnificus*, but at least all ten different types of probes representing different bacterial species could be detected.

6. Discussion

6.1 Hybridization

The intensity of the blobs after hybridization was found to be dependent on several factors. The hybridization procedure developed in this work was a step towards the optimal conditions of the hybridization for the method random array, but there are more improvements to be done. Now, the hybridization was not stable, because the intensity of the blobs from one experiment varied substantially.

The concentration of detection probe was found to affect the intensity in such a way that increasing the probe concentration resulted in an increase of blob intensity, but only up to a certain concentration, somewhere below 50 nM (*Fig. 8*). After that, the increase of intensity leveled off and the blobs seemed to be saturated. Since it is always interesting to lower the amount of material needed, it might be interesting to do further studies to find out the lowest concentration of detection probe that will still give a high intensity. The studies would probably be performed in the same way as here, but with some more data points in order to make it easier to find the right concentration.

Once the concentration is determined, this may not be the optimal concentration anyway, because the detection probe concentration also affects he hybridization time. Only two concentrations were compared here, 10 nM and 100 nM, but the study showed that the time can probably be reduced if the probe concentration is increased (*Fig. 9*). This result confirmed the fact that a higher concentration of detection probe gives a higher on-rate [20]. Another experiment with more concentrations included should be performed to investigate this further.

The study of the concentration of salt only showed a slight increase of background with increasing concentration of salt. Increasing the concentration of the SSC buffer, increases the salt concentration as well, and this resulted in an increase in blob intensity (*Fig. 11*). One reason for an increased background with increasing amount of salt could be that the salt binds fluorophores, which then causes an increase in background. The concentrations of SSC buffer studied here was only 1x SSC (→ 150 mM NaCl) and 2x SSC (→ 300 mM NaCl), another study where the amount is further increased should be done to see if the blob intensity increases or not at higher concentrations.

Regarding the optimization of formamide in the hybridization mixture, the concentration of formamide found to give the highest intensity was 20% (*Fig. 10*). This concentration is the same as suggested in previously published studies [20, 22].

6.2 Stripping

The results from the optimization of stripping showed that the solution with the best result of all the ones investigated, i.e. the one resulting in the lowest intensity of the blobs, was 50% formamide in 2x SSC buffer in 50°C (*Fig. 18*).

As one can see from the results of stripping, the differences in blob intensity between most of the solutions were relatively small (*Fig. 12-18*). Almost every solution tested in this study resulted in an intensity much lower than the one before stripping and even though there may be some detection probes left, the intensity is probably low enough so that these glass slides

can be analyzed with another threshold value set. But the recommendation is still to use the solution that worked the best, i.e. 50% formamide in 2xSSC buffer.

Stripping in 50% formamide in 2x SSC buffer was also done in 60°C and 70°C, but there were no great differences between the different temperatures (data not shown).

The reason for the improvement of the stripping, when including 2x SSC buffer in the stripping solution, was not clear, but as studies showed removing either NaCl or sodium citrate did not result in a decrease of blob intensity (data not shown).

One might believe that combining NaOH and formamide would give a better result than just having NaOH, but the results showed the opposite (*Fig. 17*). The reason for this was probably because when formamide was added to the sodium hydroxide solution it acted like an acid, and lowered the pH of the solution (data not shown), which then affected the stripping negatively since the use of NaOH was only to get a higher pH.

When pictures of blobs were taken in the epifluorescent microscope after stripping the exposure time was not the same for all experiments. There were also some problems with the settings in the software of microscope, because the exposure time when looking at the same position on a slide had to be set different for different users and different days. Due to these factors the images from different experiments cannot really be compared with each other. This does not affect the total outcome, because some solutions were included in more than one experiment, which then makes it possible to do comparisons between different experiments.

So, in conclusion, the 50% formamide in 2x SSC resulted in the best conditions in the stripping experiments.

6.3 Dynamic range

The dynamic range of this method was found to be of at least four orders of magnitude (*Fig. 19*). This result can be compared with the dynamic range of two other commonly used methods, Q-PCR and microarrays. The dynamic range for a single-plex Q-PCR is approximately 10^8-10^9, but as the level of multiplexity is increased the value is drastically lowered. When using Q-PCR, it is also difficult to detect samples of low concentration in a solution of samples with high concentrations. Microarrays have a dynamic range of approximately 10^3-10^4. This means that the method presented here has a dynamic range that can compete with the dynamic range of both Q-PCR and microarrays, which makes this method interesting for the future. The extra work with having to dilute or concentrate samples, in order to get the right concentrations, will maybe not be totally gone, but at least reduced.

The last value for *Vibrio cholerae* in *Fig. 19* did not follow the expected pattern and one may claim that this decreases the dynamic range to an order of three instead of four. This might be true, but there are other possible explanations.

Since the experiment was not performed in totally sterile environments, it is possible that there was a small contamination of *V. cholerae* either in only the last sample or in all five samples. If the latter was true, the reason why this contamination only affected the outcome of the last sample might be due to that the last sample was the one with the lowest amount of *V. cholerae* template, and a contamination there would then increase the amount of template to a greater extent than a contamination in the other four samples would.

Another explanation could be that the last sample had some kind of dirt that looked like blobs. This would then probably not change the result of the two other bacteria, either because the dirt is only seen in the channel used for detecting *V. cholerae* or because the number of blobs for the other two bacteria is quite high, which means that an addition of a number of blobs will not have such a great effect. But since the expected number of blobs is very low for *V. cholerae* in the last sample, an increase of the calculated number of blobs due to some dirt will definitely affect the result. If the dirt is detected in all three channels, the problem can be solved by analyzing the images with the script used for the multiplex analysis. This script gives the blobs special ID numbers and calculates the number of blobs with the same ID numbers, which then makes it possible to see the number of blobs detected in only one channel.

To be really sure of the dynamic range of this method more experiments should be performed with extra focus on the lower concentrations of template, to be able to find the lowest limit of detection. Templates corresponding to other bacteria than the ones used here should also be studied and compared to the templates in this study.

6.4 Multiplex analysis

From the results in *5.4* one can see that this method definitely has the capability for multiplex analysis, but there are still some problems that need to be solved.

As one can see in *Fig. 21* all ten different types of blobs could be detected, but the number of blobs detected for each type differed.

One reason could be that the ten different templates hybridize to their padlock probes with different efficiencies, which might be due to sequence variations of the templates. This then leads to that some padlock probes will maybe not be ligated and thus no amplification of these padlock probes will be made, resulting in less number of blobs for some templates.

Another reason could be due to some differences in the amplification of the padlock probes. Some padlock probes might not be amplified as well as others, maybe due to sequence variations too. This gives shorter amplification products, which in turn means fewer sites for the detection probes, and the signal from these blobs might then become too weak to be detected.

The different detection probes and their different fluorophores might also affect the number of blobs in a way that some types of fluorophores always gave weaker signals than other, but there were also differences in intensity even if the detection probes had the same type of fluorophore. If the signals get too weak the blobs will not be counted as blobs due to the thresholding. The reason for why some detection probes together with their fluorophores have weaker signals is not clear. It might involve quenching or maybe it could be due to different extinction coefficients, which is a measure of how well a substance absorbs electromagnetic radiation at a particular wavelength.

The problem with the detection probes was probably the main reason for the differences in the number of blobs from probes representing the ten different bacterial species. Other experiments have shown that when hybridizing with a general probe, which always gives a high signal, it was possible to detect blobs not detected or with very weak signals when hybridizing with specific detection probes. This confirms the fact that it was something with the hybridization of the specific detection probes that was causing the difference in the number of blobs.

A positive result though was that the blobs did not seem to be affected by the number of hybridizations and strippings, the number of blobs detected with the general detection probes after each hybridization were about the same as for the first hybridization, ~4500 blobs (data not shown). By adding the number of blobs found with the specific detection probes together, ~3100 blobs, and comparing it to the total number of blobs found by the general probe, there were a lot of blobs not detected by the specific detection probes, but with the general. This further confirmed that the reason for the different number of blobs found for each bacterium was due to the different hybridization efficiencies of the specific detection probes.

6.5 General

We here present a method with a sensitivity and specificity comparable to PCR, but also with the possibility to perform multiplex analysis as in microarrays.

The high specificity and sensitivity is gained through the use of padlock probes and rolling circle amplification. The dual recognition needed for the padlock probes to be circularized and the ability of degrading non-circular DNA by exonuclease, thus allowing only circular DNA to be amplified, are of great importance. Since one RCA product represents one copy of a specific DNA segment, the detection is performed at the single molecule level.

By immobilizing the samples on a surface several sequential hybridizations can be done, where a number of different types of blobs can be detected in each hybridization. This makes it possible to take the level of multiplexing to an even higher degree than before. Now we do not need to detect all the different types of blobs in one hybridization, which we would have needed if the blobs had been in solution. Therefore the method is not dependent on the different types of fluorophores available. The same fluorophore can be used for various amounts of detection probes, as long as they are used in separate hybridizations.

An important fact to mention is that all templates used in this work were synthetically made, so it would be of great interest to investigate how good this method works with genomic DNA, e.g. human or bacterial, and which modifications that are needed to be done.

All the results shown in this report demonstrate that the random array is a method of high potential and with a bright future, but more studies and optimizations of the method are needed before the method can be commercialized.

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Appendix A

Table I. Name of filter to use to a certain type of fluorophore.

Name of filter	Type of fluorophore
FITC	FITC
	Bodipy 493/503
	Bodipy FL
Cy 3	Cy 3
	Bodipy 564/570
Cy 5	Cy 5
	Bodipy 650/665

Table II. Oligonucleotides for the dynamic range. All templates and padlock probes are from *DNA Technology*. The detection probe for *V. cholerae* is from *Biomers* and the other two are from *MWG*.

Vibrio cholerae	
Template (29 nt)	5'-CCC TGG GCT CAA CCT AGG AAT CGC ATT TG-3'
Padlock probe (91 nt)	5'-TAG GTT GAG CCC AGG GAC TTC TAG AGT GTA CCG ACC TCA GTA GCC GTG ACT ATC GAC TTG CGT CTA TTT AGT GGA GCC CAA ATG CGA TTC C-3'
Detection probe (20 nt)	5'-Cy 3-TGC GTC TAT TTA GTG GAG CC-3'
Vibrio fischeri	
Template (30 nt)	5'-CCT GGG AAT ATG CCT TAG TGT GGG GGA TAA-3'
Padlock probe (91 nt)	5'-TAA GGC ATA TTC CCA GGA CTT CAG AGT GTA CCG ACC TCA GTA GCC GTG ACT ATC GAC TTG TCT ATG TTT ACA GCG GGC TTA TCC CCC ACA C-3'
Detection probe (20 nt)	5'-Cy 5-TGT CTA TGT TTA CAG CGG GC-3'
Vibrio vulnificus	
Template (30 nt)	5'-TTG TAA AGC ACT TTC AGT TGT GAG GAA GGT-3'
Padlock probe (91 nt)	5'-ACT GAA AGT GCT TTA CAA CTT CTA GAG TGT ACC GAC CTC AGT AGC CGT GAC TAT CGA CTC TGG ACC TTA ATC GTG TGC GAC CTT CCT CAC A-3'
Detection probe (20 nt)	5'-Bodipy 493/503-CTG GAC CTT AAT CGT GTG CG-3'

Table III. Oligonucleotides for the multiplex analysis. All templates and padlock probes are from *DNA Technology*. The detection probes for the the first general detection, *V. metschnikovii* and *V. diazotrophicus* are from *Biomers* and the remaining are from *MWG*.

General detection probe I (23 nt)	5'-FITC-TTT AGT AGC CGT GAC TAT CGA CT-3'
General detection probe II-V (20 nt)	5'-Cy 3-AGT AGC CGT GAC TAT CGA CT-3'

Hybridization I

Vibrio parahaemolyticus	
Template (27 nt)	5'-GCG GAA ACG AGT TAT CAG AAC CTT CGG-3'
Padlock probe (91 nt)	5'-GAT AAC TCG TTT CCG CCT TCT TCT AGA GTG TAC CGA CCT CAG TAG CCG TGA CTA TCG ACT GGG CCT TAT TCC GGT GCT ATC CGA AGG TTC T-3'
Detection probe (20 nt)	5'-Bodipy 650/665-GGG CCT TAT TCC GGT GCT AT-3'
Vibrio alginolyticus	
Template (24 nt)	5'-AAC GAT AAC GGC GTT GAG CGG CGG-3'
Padlock probe (91 nt)	5'-ACG CCG TTA TCG TTC TTC TTC TTC TAG AGT GTA CCG ACC TCA GTA GCC GTG ACT ATC GAC TGT ACT ACA TTC GTG CGA TGG CCG CCG CTC A-3'
Detection probe (20 nt)	5'-Bodipy 565/570-GTA CTA CAT TCG TGC GAT CG-3'

Hybridization II

Bacillus thuringiensis	
Template (27 nt)	5'-GGC TTC TCC TTC GGG AGC AGA GTG ACA-3'
Padlock probe (91 nt)	5'-CCC GAA GGA GAA GCC ACT TCT TCA GAG TGT ACC GAC CTC AGT AGC CGT GAC TAT CGA CTA AGC CAG ATC GAC CAT CGT ATG TCA CTC TGC T-3'
Detection probe (20 nt)	5'-Bodipy FL-AAG CCA GAT CGA CCA TCG TA-3'
Bacillus subtilis	
Template (26 nt)	5'-ACG TCC CCT TCG GGG GCA GAG TGA CA-3'
Padlock probe (91 nt)	5'-CCC GAA GGG GAC GTA CTT CTT CTA GAG TGT ACC GAC CTC AGT AGC CGT GAC TAT CGA CTG AGA GGC CGT CGC TAT ACA TTG TCA CTC TGC C-3'
Detection probe (20 nt)	5'-Bodipy 650/665-GAG AGG CCG TCG CTA TAC AT-3'

Hybridization III

Vibrio metschnikovii

Template (30 nt)	5’-CTA CTC TTG ACA TCT ACA GGA TCC TGC GGA-3’
Padlock probe (91 nt)	5’-CTG TAG ATG TCA AGA GTA GAC TTC AGA GTG TAC CGA CCT CAG TAG CCG TGA CTA TCG ACT CGT GCG CCT GGT AGC AAA TAT CCG CAG GAT C-3’
Detection probe (20 nt)	5’-Cy 5-CGT GCG CCT GGT AGC AAA TA-3’

Vibrio diazotrophicus

Template (29 nt)	5’-GCA TAA TGT CTT CGG ACG AAA GAG GGG GA-3’
Padlock probe (91 nt)	5’-GTC CGA AGA CAT TAT GCC CTT CTA GAG TGT ACC GAC CTC AGT AGC CGT GAC TAT CGA CTC AAT CTA GTA TCA GTG GCG CTC CCC CTC TTT C-3’
Detection probe (20 nt)	5’-FITC-CAA TCT AGT ATC AGT GGC GC-3’

Hybridization IV

Vibrio fischeri

Template (30 nt)	5’-CCT GGG AAT ATG CCT TAG TGT GGG GGA TAA-3’
Padlock probe (91 nt)	5’-TAA GGC ATA TTC CCA GGA CTT CAG AGT GTA CCG ACC TCA GTA GCC GTG ACT ATC GAC TTG TCT ATG TTT ACA GCG GGC TTA TCC CCC ACA C-3’
Detection probe (20 nt)	5’-Cy 5-TGT CTA TGT TTA CAG CGG GC-3’

Vibrio mimicus

Template (29 nt)	5’-TCT ACT TGG AGG TTG TGA CCT AGA GTC GT-3’
Padlock probe (91 nt)	5’-CAC AAC CTC CAA GTA GAC TTC TTA GAG TGT ACC GAC CTC AGT AGC CGT GAC TAT CGA CTG CGA CAC GTT CCG AGC ATA TAC GAC TCT AGG T-3’
Detection probe (20 nt)	5’-Bodipy 493/503-GCG ACA CGT TCC GAG CAT AT-3’

Hybridization V

Vibrio cholerae

Template (29 nt)	5’-CCC TGG GCT CAA CCT AGG AAT CGC ATT TG-3’
Padlock probe (91 nt)	5’-TAG GTT GAG CCC AGG GAC TTC TAG AGT GTA CCG ACC TCA GTA GCC GTG ACT ATC GAC TTG CGT CTA TTT AGT GGA GCC CAA ATG CGA TTC C-3’
Detection probe (20 nt)	5’-Cy 5-TGC GTC TAT TTA GTG GAG CC-3’

Vibrio vulnificus

Template (30 nt)	5’-TTG TAA AGC ACT TTC AGT TGT GAG GAA GGT-3’
Padlock probe (91 nt)	5’-ACT GAA AGT GCT TTA CAA CTT CTA GAG TGT ACC GAC CTC AGT AGC CGT GAC TAT CGA CTC TGG ACC TTA ATC GTG TGC GAC CTT CCT CAC A-3’
Detection probe (20 nt)	5’-Bodipy 493/503-CTG GAC CTT AAT CGT GTG CG-3’