"Investigations of a bio-synthetic approach to access large padlock as a part of a new concept for detecting DNA on a microarray"

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Dedicated to my Parents,

Ashok Kotenkar &Sabita Kotenkar

Dankeschön..... Thank you……

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SUMMARY

Ever since the discovery of deoxyribonucleic acids, DNA analytics has been a field which has gained immense popularity. As the demand increases, more and more sophisticated technologies and methods have been invented and implemented, to analyze this basic structure of life. The main task for the analysis of DNA, is to design probes that select only a particular sequence of DNA, and help in their identification. It would be highly beneficial for medical diagnostics, food industry and for other industrial applications, if these selected sequences could be detected with higher specificity on microarrays as so fare. A couple of methods and probes like quantitative PCR, molecular beacons, Tagman probes, to name only a few have been developed for this purpose but there are still disadvantages in using DNA hybridization analyses on microarrays for organism identification if a mixture of biological samples are to be analyzed.

One of the solutions to increase specificity and sensitivity that might be an answer to improve signal quality and reliability is the use of "Padlock Probes".

Padlocks are single stranded oligonucleotides, which are at least 100 bases long or more, and have their 5' and 3' ends complementary to the target molecule. In the presence of a complementary target, the ends of the padlock probes place themselves adjacent to each other and are ligated. Detection of the circularized probe then depends on whether primer binding sites, signal oligonucleotide binding sites, haptens, etc. are integrated in the non-target part of the padlock. These probes are extremely sensitive, owing to the two control steps, hybridization of both the ends and their ligation. A single base pair mismatch leads to complete failure in their functioning.

So far the main hurdle connected to the padlock approach, is to overcome the difficulties in synthesizing them, because the common route of DNA synthesis is exhausted at a length of about <100 bases, and the quality, concentration and the phosphorylation of the padlocks is extremely poor.

The idea of this study was to develop a protocol based on the principle of gene synthesis by ligation with the following new approaches: First, generation of padlocks much longer than 100 bases to enable incorporation of new sequence functions, secondly simple and cost-effective protocol, thirdly high yield and fourth opportunity to incorporate repetitive and other functional sequences using module-like building blocks. First, ligation steps and polymerase reactions were used which facilitate the construction of a 120 bases padlock. The sequences were confirmed using Southern blot and sequencing. In order to increase the yield of the constructed probes, rolling circle amplification method and restriction enzymes were used to generate multiple copies of single stranded and phosphorylated 120 bases padlock with precise sequence.

To make the padlocks popular and be able to use them in newer approaches, padlocks longer than 120 bases will be required. Hence, after successfully finishing this first step repeated sequences were introduced to realize the use of padlocks as a new DNA amplification system. Repetitive sequences are generally difficult to be synthesized. This experience was confirmed since many modifications of the protocols mentioned above, did not lead to the desired result. But finally, hybridization and ligation reactions were used, to construct a 205 bases long padlock with a threefold repetitive sequence inserted in to the non-target specific part of the single stranded DNA. Although this achievement is a good step forward, this protocol is not efficient in producing longer padlocks with higher repetitive sequences. Quite a few methods were tested but proved unsuitable for this task.

The bio-synthesized padlock containing a threefold repetitive sequence has been used for elaborating the new DNA amplification system, which is part of another PhD thesis. The main part of the discussion is devoted to further padlock applications which are very promising in research as well as for commercial biotechnology.

ZUSAMMENFASSUNG

Seit der Entdeckung der Desoxyribonukleinsäure hat der Bereich der DNA-Analytik erheblich an Bedeutung gewonnen. Mit steigender Nachfrage sind immer mehr ausgeklügelte Technologien und Methoden erfunden und am Markt etabliert worden, um diese Grundbausteine des Lebens zu analysieren. Die Hauptaufgabe bei der Analyse von DNA ist die Entwicklung von Sonden, die nur an bestimmte DNA Abschnitte binden und somit bei deren Identifikation helfen. Es wäre für medizinische Diagnostik, Lebensmittelindustrie und andere industrielle Anwendungen in hohem Grade vorteilhaft, die Spezifität des Nachweises der ausgewählten Sonden auf dem Microarray zu steigern. Eine Reihe Methoden und Sonden wie z. B. Quantitative PCR, Molecular Beacons oder Tagman Sonden sind zu diesem Zweck entwickelt worden. Es gibt aber weiterhin Schwierigkeiten, wenn man das Prinzip der DNA-Hybridisierung auf Microarrays zur Detektion von Organismen verwendet, besonders bei der Analyse von Gemischen biologischer Proben.

Einer der Ansätze, um die Spezifität und die Sensitivität zu erhöhen und dadurch die Verlässlichkeit der Methodik zu verbessern, ist der Gebrauch von „Padlocks-Sonden“.

Padlocks sind einzelsträngige, mindestens 100 Basen lange Oligonukleotide, die am 5' und 3' Ende komplementär zum Zielmolekül sind. In dessen Gegenwart lagern sie sich mit diesem derartig zusammen, dass die beiden Enden jeweils an benachbarte Nukleotide binden. In dieser Form kann ein Padlock durch eine Ligase ringförmig geschlossen werden. Der Nachweis der zirkularisierten Probe erfolgt dann über Primer Bindestellen, Bindestellen für kleine Signaloligonukleotide, Haptene o.ä., die sich in dem verbindenden Abschnitt zwischen den beiden endständigen Zielsequenzen befinden. Derartige Sonden sind infolge von zwei sequenzabhängigen Schritten (Hybridisierung beider Enden und deren Ligation)

extrem spezifisch. Eine einzelne Paarfehlpaarung kann somit nachgewiesen werden.

Bis jetzt war die größte Schwierigkeit beim Einsatz des Padlock Ansatzes, dass die gängigen Methoden der DNA Synthese nur Oligonukleotide bis ca. 100 Basen in zufriedenstellender Qualität herstellen können. Bei längeren Padlocks ist die Genauigkeit der Synthese, die Ausbeute und der Phosphorylierungsgrad sehr schlecht.

Die Idee dieser Arbeit ist, ein Protokoll nach dem Vorbild der Gensynthese durch Ligation zu entwickeln, das folgende Kriterien erfüllt: Erstens die erzeugten Padlocks sollen deutlich länger als 100 Basen sein, damit in die Nicht-Ziel-Sequenz Abschnitte eingebunden werden können, die weitere Funktionen erfüllen können. Zweitens muss die Synthese einfach und kostengünstig sein. Drittens soll eine hohe Produktausbeute erzielbar sein. Und schließlich soll viertens die Verwendung von repetitiven Sequenzen und anderen funktionellen Abschnitten nach dem Baukastensystem möglich sein.

Zunächst wurden Ligase- und Polymerasereaktionen verwendet, die die Synthese einer 120 Basen langen Padlock-Sonde ermöglichten. Die synthetisierte Gesamtsequenz wurde mit Hilfe von Southern Blot und Sequenzierung verifiziert. Um die Ausbeute der konstruierten Proben zu erhöhen, wurde Rolling-Circle Amplifikation in Kombination mit Restriktionsenzymen verwendet. Hierdurch wurden einzelsträngige, phosphorylierte Proben von 120 Basen Länge mit der korrekten Sequenz in großer Anzahl hergestellt.

Um eine breitere Anwendung von Padlock-Sonden auch im Hinblick auf neue Einsatzmöglichkeiten zu ermöglichen, sind Oligonukleotide länger als 120 Basen erforderlich. Daher wurden nach erfolgreichem Abschluss dieses ersten Schritts repetitive Sequenzen mit in die Konstruktion einbezogen, um die Anwendung von Padlocks in einem neuen DNA Detektionssystem zu ermöglichen. Repetitive Sequenzen sind grundsätzlich schwierig zu synthetisieren. Dieser Sachverhalt wurde bestätigt, da trotz einer Vielzahl von Änderungen des oben erwähnten

Protokolls zunächst kein erwünschtes Ergebnis erzielt werden konnte. Aber schließlich gelang es mit Hilfe von Hybridisierungs- und Ligationsreaktionen ein 205 Basen langes Padlock mit einer dreifachen repetitiven Sequenz im Nicht-Ziel-Abschnitt des Einzelstrangs herzustellen. Obwohl dies ein guter Schritt vorwärts ist, ist dieses Protokoll nicht effizient genug, um längere Padlocks mit einer höheren Anzahl an repetitiven Sequenzen zu produzieren. Für diese weitere Erhöhung wurden eine Reihe von Methoden untersucht, erwiesen sich aber als unzulänglich für diese Aufgabe.

Die enzymatisch synthetisierte Padlock mit der dreifachen Sequenzwiederholung wurde innerhalb einer anderen Dissertation zur Ausarbeitung eines neuen DNA-Nachweisverfahrens genutzt. Der Hauptteil der Diskussion ist weiteren Padlock Anwendungen gewidmet, die sowohl für die Forschung als auch für die biotechnologische Anwendung großes Potential haben.

TABLE OF CONTENTS

1 INTRODUCTION

1.1 Background

Deoxyribonucleic acid (DNA) is the main information carrier, where instructions for the functioning of the cells and transferring it from one generation to the next are stored. With the revelation of its structure in 1953, by James Watson and Francis Crick and Rosalind Franklin, the biggest mysteries of how the genetic material was transferred from one generation to the next was deciphered. But about 50 years since the structure of the unit of life was first deduced, there are still so many unanswered questions. The quest to get these answers is ongoing.

DNA molecule which is basically made up of sugar (deoxy- ribose) phosphate backbone, where the 3′ Carbon of one sugar molecule is attached to the 5′ Carbon of the next by the phosphate groups, with bases attached to it, is extremely flexible owing to the hydrogen bonds present. The double helical structure formed by the anti-parallel arrangement of the DNA strand and hydrogen bonds (between the bases) facilitates replication and bestows other functionality to the DNA (Figure 1.1). The bases form two hydrogen bonds (Adenine (A) (purine) always pairing with Thymine (T) (pyrimidine) or three hydrogen bonds (Guanine (G)

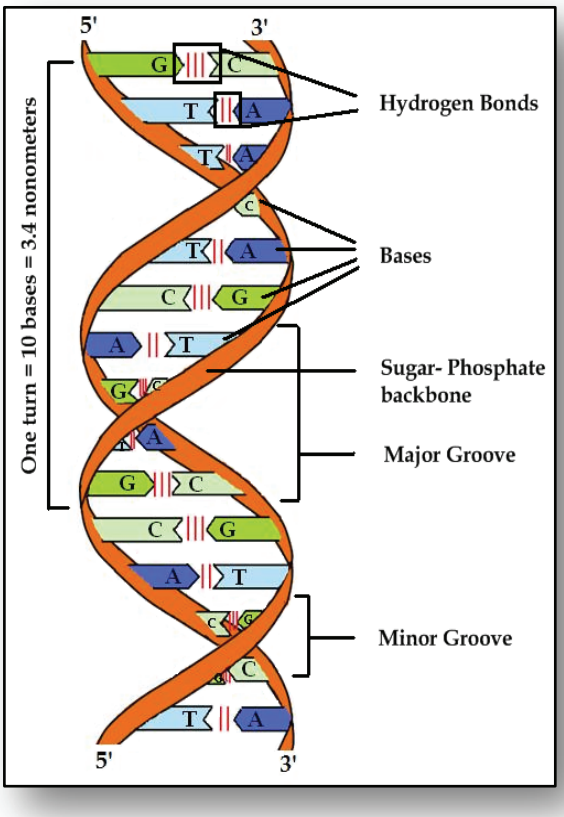


Figure 1.1 Basic structure of the double helix (this figure has been modified from the original figure source: http://tigger.uic.edu/classes/phys/phys461/phys450/ANJUM04/)

(purine) pairing with Cytosine (C) (pyrimidine) where the GC bonding is stronger than the AT bonding.

Discoveries and inventions were at a boom, once the structure of double helix was put forth. The next step was to understand the various mechanisms that the DNA undertakes to sustain life in the living beings. The inquisitiveness to gain more in-depth knowledge about the different life forms including the humans, gave rise to the field of DNA analytics. There was a sluggish growth rate in the number of new inventions, as there weren't enough sensitive techniques which would be reliable and would permit the comprehensive exploration of life. Creating sensitive methods for detection and analysis was the key highlight of the research then.

The list of how DNA analysis would help is very long. A few examples of it would be: giving a detailed insight of the mechanisms of diseases, or how a particular gene responds to drugs, or how a manipulation in a particular strain of microorganism would increase the yield of a product and also introduce many more functionalities in the organisms. By studying the DNA sequence of the living beings, one can also track down their ancestors and create an evolutionary tree. As there are a vast number of living organisms which are still to be discovered, having a library of the one's already discovered, can save a lot of time and resources. Portals such as *National Center for Biotechnology Information* (*NCBI*) or *European Molecular Biology Laboratory (EMBL)* to name a few, are the most commonly used to submit sequenced data. Dr. Paul Hebert in 2003 introduced the concept of having a small varying fragment like a "Signature sequence" for each individual, which can efficiently distinguish the different species in the same region, and the term "DNA Barcoding" was coined (Hebert et al., 2003). Sequencing the "Signature sequences" assist the classification of the individuals, by helping create phylogenetic trees. But the critical point here also, is to design and standardize a reliable method that helps in acquiring the barcode of each and every individual, without generating any reservations. Hence even today, although there are a range of methods available for

sensitive DNA analysis, there is still an extensive demand to develop more sensitive and robust DNA detection methods.

Analysis of DNA can give plenty of information which can be extremely useful for understanding mechanisms, or in manipulating it to get it to function in a desired fashion. One opportunity that was introduced in 1977 by Frederick Sanger, was to decipher the sequence of the DNA strand, using the chain termination method which utilizes the chain terminating nucleotide, di-deoxynucleotide, for sequencing (Sanger, Nicklen et al. 1977). But recent development of the new generation of sequencers, as e.g. developed by 454 Life Sciences (http://www.454.com/) enables high throughput sequencing of individuals at a low cost and shorter time (Gharizadeh, et al. 2006). A new "pyrosequencing" method was developed by Mostafa Ronaghi (Ronaghi et al. 1996, 1998, 2001) and since then, there are more than 700 papers published on this method. The basic principle is shown in Figure 1.2. Although there are some disadvantages in comparison to the Sanger's

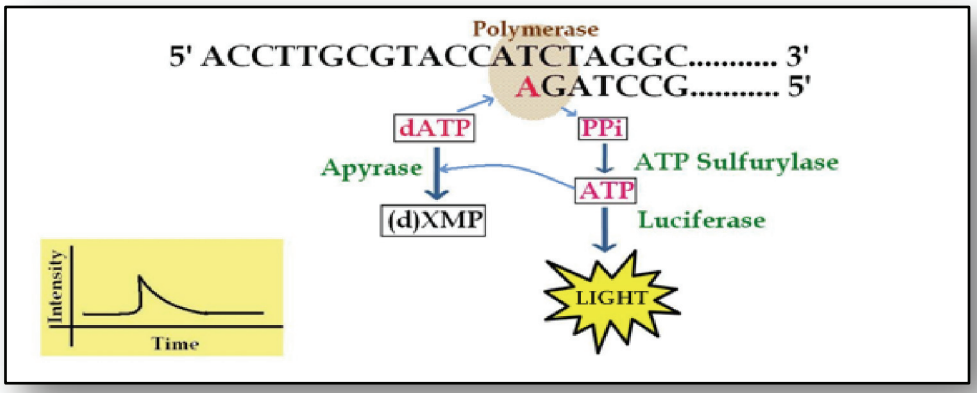


Figure 1.2 Schematic representation of the principle of Pyrosequencing method. This picture has been re- drawn completely from the site: http://med.stanford.edu/sgtc/technology/high-throughput.html

Here, nucleotide, dATP, is added to form the complementary strand of the single-stranded template, to which a sequencing primer has already been annealed (top right of the diagram). This reaction is carried out in the presence of polymerase, ATP-sulfurylase, luciferase and apyrase enzymes. One molecule of pyrophosphate (PPi) is released for every nucleotide incorporated into the growing strand by the DNA polymerase, and is converted to ATP by sulfurylase. Visible light is produced from luciferin in a luciferase-catalyzed reaction that utilizes the ATP produced above, and unincorporated nucleotides are degraded by apyrase between each cycle. The table on the left shows the intensity of the light produced.

sequencing method, like the read length possible so far ranges between 200- 500 bases in the laboratory (Green et al., 2006; Gharizadeh, B., et al. 2002) which is far less than possible by Sanger's method (Marziali, A., 2001; Franca et al. 2002) and the inefficiency in the presence of homopolymeric stretch in the sequence, especially if the stretch consists of more 3 Thymine bases (Gharizadeh1, Herman, et al; 2006). Pyrosequencing method saves time, labour and is easy to use while analyzing smaller sequences and the sequencing reaction starts just after the primer, unlike the Sanger's method where a gap of few bases is witnessed. While the recently developed sequencing methods are extremely sensitive, they can simultaneously detect many samples, can easily distinguish different species or Single nucleotide polymorphism (SNPs) in the DNA by comparing the signal outputs (Ahmadian et al. 2000), these methods fail when there is a mixture of sample to be analyzed.

The use of hybridization based assays, opened a new path for sequence specific detection of multiple samples from a mixture. The first method dealing with hybridization technique as a means for DNA identification was Southern blotting, which basically involved immobilization of the target DNA on a nitrocellulose membrane and hybridizing it with the labeled oligonucleotide for detection (Southern, 1977). Although this method rendered sensitive sequence specific detection and the stringency of the experiments could be modulated, analyzing a large number of samples was extremely tedious and parallel analysis of lots of samples is not feasible. Recently, based on this simple principle of hybridization of the target DNA with small oligonucleotide probes for detection, there are a lot of methods developed to detect allele specific variations. Most of them use florescence based detection due to which there is a possibility of detecting simultaneously multiple samples (Kosman D, et al. 2004). To name a few would be the molecular beacons based detection (Tyagi S. et al., 1996), or the TaqMan® assay (Lee LG, et al., 1993).

For parallel DNA detection and analysis of multiple and mixed samples the, "DNA Chip", which was introduced (Saiki et al, 1989) is currently the most exploited

method. The DNA Chip method, commonly known as "Microarray", differs in many ways in comparison to the Southern blotting method. Here the small oligonucleotide is immobilized on a solid surface to which the labeled DNA could bind and be detected, contrary to that done in Southern blot technique. Also with the current development, it is possible to scan 40,000 small oligonucleotides on a Chip. Testing multiple samples in a single experiment and very reliable usage for the analysis of long-range PCR products for discovering single sequence variations across the whole genome in known genes and for genotyping (Hinds et al., 2005, Cargill et al., 1999, Hacia et al., 1996) are the chief advantages of the microarray technique. Also as the probes are bound on a solid surface, the unbound targets can easily be washed away, reducing the background, and also the knowledge of the exact position of the probe on the slide facilitates genotyping of the target sample (Wang et al. 1998). But the main problem is of cross hybridizations when two closely related DNA are evaluated on the same scanning area. Also the cost for designing and developing it is quite high and lastly due to the intermediate step of PCR being involved for labeling and amplifying the target, the target sequence must be known and should be amplified correctly.

The Polymerase Chain Reaction (PCR) method which was invented by Kary Mullis (Mullis et al., 1987; Saiki, et al., 1989) is an amplification method, which is one of the

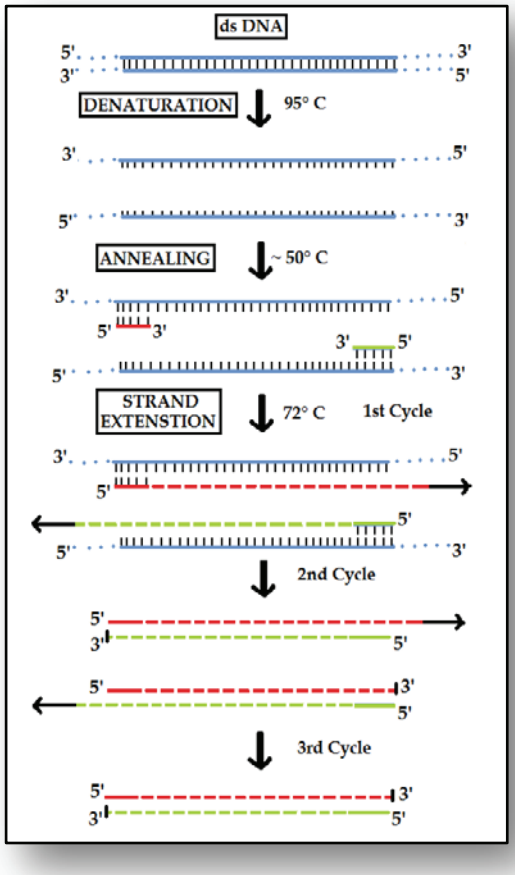


Figure 1.3 Schematic representation of a PCR amplification reaction.

Each of the cycling steps of PCR consists of initially denaturation of the DNA, annealing of the primer and strand extension using polymerase.

standard methods for most of the DNA amplification. The basic principle is as shown in Figure 1.3 Though PCR has been used very commonly and is a robust method to amplify DNA and for genotyping among many other applications, there are flaws too. Due to the exponential amplification of PCR, it is quite sensitive, and even a small contamination can be amplified and inhibit the target amplification. Furthermore, when two or more template strands are amplified simultaneously for multiplexing, due to the presence of multiple primer pairs sometimes leads only to the superfluous "primer- dimer" artifacts or selective amplification of a particular target, as the sequence of the target also plays a role to define the efficiency of PCR. Although microarrays have the capacity to carry on multiplexing reaction, the intermediate PCR steps currently used is limiting the exploitation of this technology to the fullest.

Rolling circle amplification (RCA) method originated from the rolling circle replication carried out by strand displacing DNA polymerases in certain bacteria and viruses (Fire et al., 1995; Liu et al., 1996) in the last decade has rapidly gained significance in the past few years as multiple copies of the DNA can be generated from a single circularized DNA. It being a very robust and isothermal amplification method the number of copies generated in the form of concatemers exceeds 10^9 which is much more than the number of copies generated by the classical amplification methods (Kuhn, Demidov et al. 2002). The main advantage of RCA over PCR is that: 1) the reaction is isothermal; 2) the original template is always the source for generating products unlike PCR, where the product of one cycle is the template for the next cycle, thus increasing the probability of error and, 3) lastly single stranded products can easily be generated using RCA whereas the asymmetric PCR used for this purpose fall short. There are variations of rolling circle amplification such as RAM (Ramification Amplification) (Zang et al, 1998; 2001; 2006) and HRAM (Hyperbranched Rolling Circle Amplification) (Lizardi et al., 1998), which additionally enhance the production of multiple copies by adding primers which continuously further amplify the product generated by rolling circle.

Other isothermal methods of amplification also have been used for the purpose of signal amplification. Like in the invasive cleavage assay (Lyamichev et al. 1999, 2000) where about 10^6 to 10^7 cleaved fluorescently labeled probes are easily detected for each target per hour without the need of amplification of the target using PCR or the Helicase dependent amplification (HAD) (Vincent et al., 2004; An et al., 2005), Exponential nucleic acid amplification reaction (EXPAR) (Ness et al., 2003), Multiple displacement amplification assay (MDA) (Dean et al., 2002), Loop Mediated Isothermal Amplification assay (LAMP) (Notomi et al., 2000; Nagamine et al., 2001), Transcription Mediated Amplification assay (TMA) (Wood et al., 1996; Hill et al., 2001), Strand Displacement Amplification assay (SDA) (Walker et al., 1992), Nucleic acid Sequence based Amplification assay (NASBA) (Compton etal., 1991) to name a few.

Each of the above method is good, but none of them meets all the requirements desired. The predicament is finding a probe that is (a) extremely sequence specific and can detect even small amounts of DNA without the need of an intermediate amplification step and; (b) with which multiplexing is feasible. The search for a method that can provide sensitivity, specificity and high throughput is on.

The types of probes that fulfill the requirement of sequence specificity, are the ligation based probes. DNA ligase is known for its property of ligating nicks, which is exploited in these assays. Even a single base pair mismatch near the ligation site leads to the failure in ligation. Based on this principle, Ligation Chain Reaction (LCR) has two oligonucleotides hybridized to each of the double stranded DNA, such that on each strand the two oligonucleotide probes lie adjacent to each other.

When there is a perfect match the probes are ligated (Figure 1.4). There is a cycling process involved so that the number of the ligated probes is enlarged and hence avert the need for amplifying the target (Barany, 1991a; Barany and Gelfand, 1991). Though these probes have two checkpoints for ensuring sequence specific detection, the first being hybridization and the second being the ligation reaction, the probes themselves tend to form “probe- dimers”.

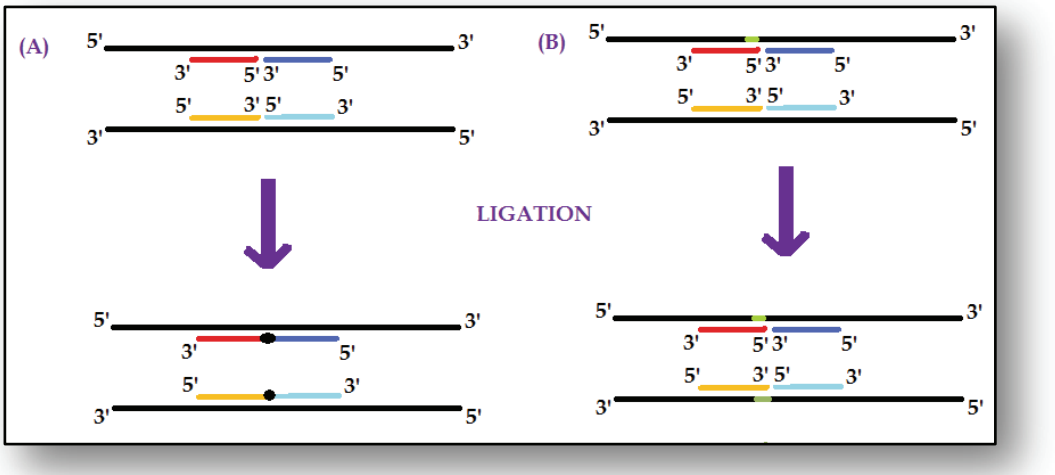


Figure 1.4 The Ligase chain reaction.

In the first part of the figure (A), a part of the DNA is separated and hybridized with 4 oligonucleotide probes. Since there is a perfect match between the probes and the target, after ligation the oligonucleotide probes are ligated. In the figure (B), the DNA strand has a single base pair mutation at the joining part of the oligonucleotides. Therefore even after the ligation reaction no ligation is observed.

1.2 Padlocks as extremely potent analytical tools

A modification to this method which could be the key to these hitches might be the "Padlock probes" introduced for the first time in *SCIENCE* in 1994 (Nilsson, et al. 1994). Since then they have been used for varied DNA and RNA applications. Basically, Padlock probes are single stranded 5′ phosphorylated oligonucleotides which have their 5′ end and their 3′ end complimentary to the target sequence and joining the 5′ and the 3′ arms of the probes is the "linker segment" (Figure 1.6). Also various functionalities can fit into the linker segment of the probes, like primer binding site (Zang et al., 1998), zip or tag sequence to bind to the chip or other solid surfaces (Szemes et al., 2005), modified bases (Baner et al., 2001), flurophores (Nilsson et al., 2000) to name a few.

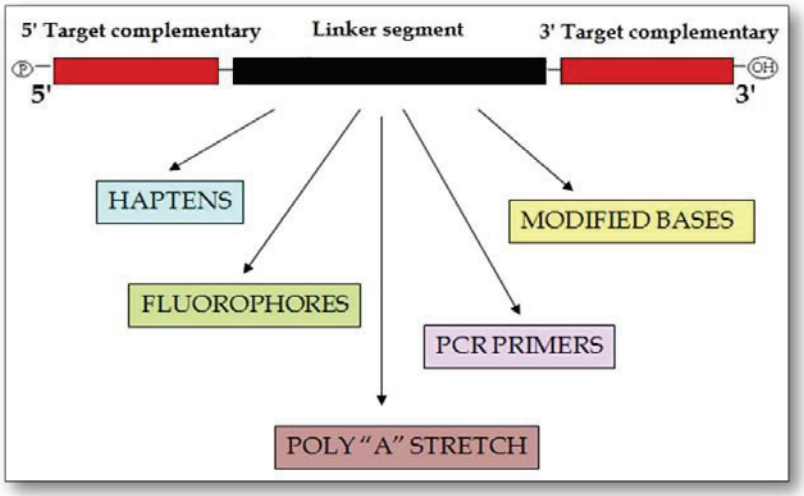


Figure 1.5 Basic structure of Padlock probes with some of the possible functionalities that can be introduced in the linker segment.

When the probe comes in contact with the target sequence, it wraps its 5′ and 3′ arms around the target in such a way that the 5′ phosphate and the 3′ -OH lie adjacent to each other. The probe can then be circularized using ligase enzyme.

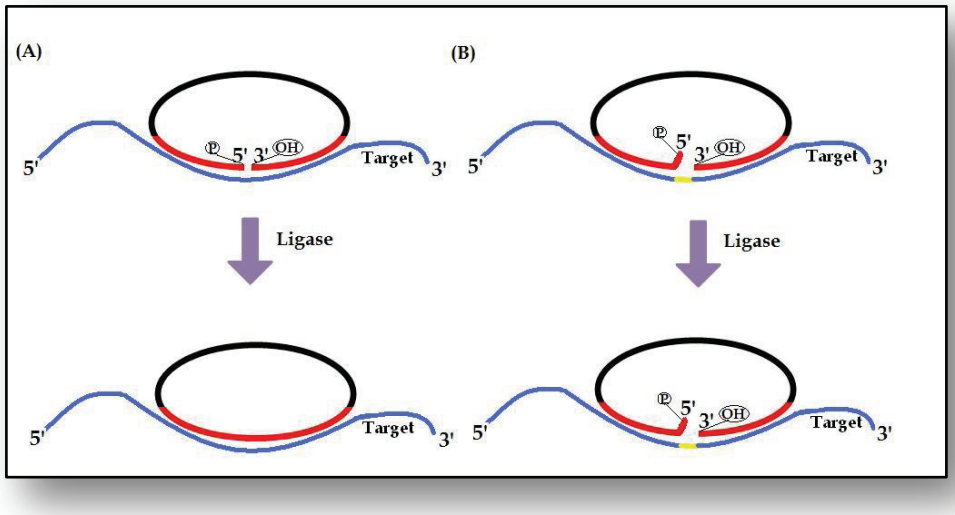


Figure 1.6 Functioning of a Padlock probe. (A) Here when the padlock probe hybridizes on the target sequence, there is an exact match and hence when ligase is added there is a circularized probe generated. (B) Unlike figure (A) here there is a base pair mismatch at the 5′ end and hence even after the addition of ligase no circularization reaction is observed.

The circularization reaction fails, if the arms of the probe are not exactly complimentary to the target. Even a single base pair mismatch leads to the failure of circularization reaction (Figure 1.5). Due to the lack of hydrogen bonds, the circularized probes are resistant to denaturation washes and also to exonuclease digest to get rid of unwanted products. Padlock probes were described to be highly specific and were shown to reduce the background (Nilsson, Malmgren et al. 1994). Though they are referred to with different names such as Molecular Padlock Probes (MPP) (Erik L. McCarthy and Teressa J. Egeler 2006), CLIPS (Zhang and Liu 2003), Molecular Inversion probes (Paul Hardenbol, Johan Baner, et al 2003), Connecter Inversion probe (CIPer) (Akhras et al., 2007), etc. their job remains the same.

The unsurpassed benefit is that these probes are sequence specific and after the hybridization of the probes and ligation, the target molecule along with the un-reacted probes, and all the other artifacts can be digested using exonucleases, thus making system less complex. These probes exploit the functionalities in the linker segment to detect the as low as 0.6 fmol (McCarthy et al., 2006) or even lower amount of DNA. The circular nature of the padlock probes, after the reaction with the desired target, makes them a suitable reaction for rolling circle amplification (Baner et al., 1998; Zang et al., 1998, 2001)

The combination of rolling circle amplification and padlock probes has definitively more advantages over other amplification techniques. The main advantage is that the reactions are isothermal and hence there is no need for a cycler and therefore this method can be used easily in for field research. Also with the grouping of rolling circle amplification and padlock probes, the signal generated can be localized, hence enabling analysis on a Chip. During multiplexing, different probes amplify different target making multiplexing achievable compared to the PCR (Zang et al., 1998; Hsuih et al., 1996).

Few of the applications where padlock probes have already been used are Identification of microorganisms based on multiple motifs, indicator organisms for

soil health status, and pathogens dependent on cultivated crop; Detection of expanded T-cell clones for assistance in diagnosis and guide therapy in infectious, inflamatory, autoimmune conditions and in tumour immunotherapy (Baner et al, 2005); Nucleotide sequence distinction; Target copy no. Measurements; Parallel gene analysis (Baner et al, 2003); In situ genotyping individual DNA molecules (Larsson et al, 2004); Detection of Alu sequences and mtDNA in comets using padlock probes (Shaposhnikov et al, 2006); Determination of the plus and minus strand of HIV-I (D. Thomas et al, 2007); Detection of foot and mouth disease, vesicular stomatitis and swine vesicular disease viruses (Baner et al, 2007); Sequencing.

Though these probes are incredibly proficient, their use is comparatively limited. One of the reasons for that are the current methods used for constructing of these probes. The main requirement for the probes is that,

(1) The 5′ end of the probe should be phosphorylated;

(2) The 5′ and the 3′ end of the probes have to have the correct sequence, as the inability to achieve this might render the probes unusable and

(3) The concentration of probes must be high. The other parts of the padlock reaction that need work are: improvising the ligation efficiency; designing effective method to use padlock probes for quantification and miniaturization of the system. To overcome these hurdles, the following methods have been worked on:

1.2.1 Synthesis of Padlock probes

- **[A] Chemical synthesis of probes:** The construction of these probes is generally carried out by the chemical synthesis method (Caruthers et al., 1983) using the main four steps; (1) Deprotection; (2) Coupling; (3) Capping and; (4) Oxidation. Here phosphoramidite monomers are used. The construction proceeds in the 3′ to 5′ direction. The 3′ carbon of the nucleotide is bound to the solid support. During the construction reaction, the protective trityl group at the 5′ carbon of the sugar molecule of the nucleotide bound to the solid surface is removed using

trichloroacetic acid (TCA) and the phosphoramidite nucleoside is introduced. The coupling reaction between the phosphoramidite nucleoside and terazol creates and intermediate tetrazole phosphoramidite. The intermediate reacts with the hydroxyl group at the 5′ end of the receiving nucleotide creating a linkage. The tetrazole is reconstituted and the reaction continues further. The use of tetrazole enabled the construction of longer probes (Berner et al. 1989) (Figure 1.7). A modification to the above method was pioneered to overcome the limitations of having truncated 5′ ends of the oligonucleotides by Marek Kwiatkowski in 1996. Here the main difference was the use of commercially available 1,3-dichloro1,1,3,3-tetraisopropyl disiloxane as support for construction and the usage of Tetrabutylammonium fluoride to cleave the linker segment. These steps made the construction reaction quite accurate.

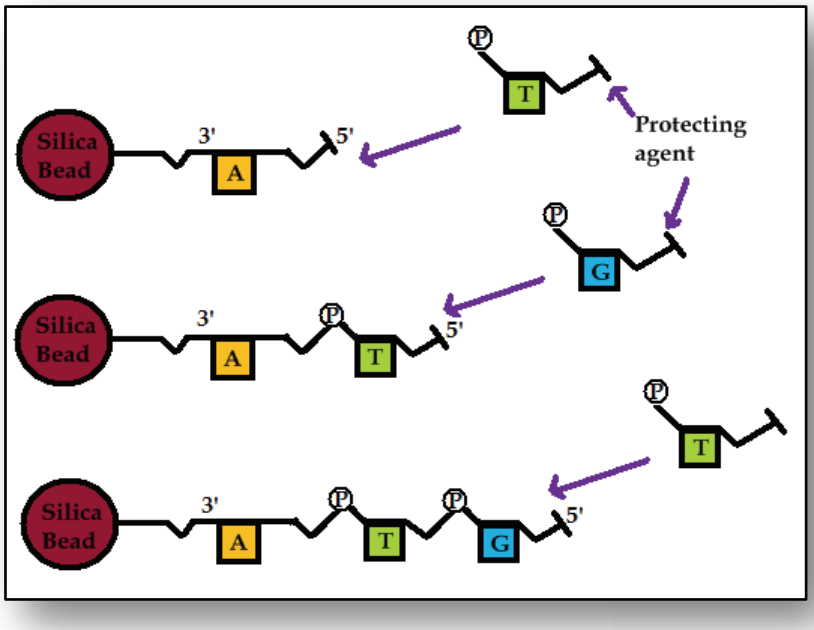


Figure 1.7 Schematic representation of Chemical synthesis of probes

- **[B] PCR generated Padlock probes**: This method was introduced by Antson et al. in 2000, to trounce the chemical synthesis method due to its shortcomings. Here basically the target specific regions were used as PCR primers. The PCR reaction was carried out on a λ DNA using biotinilated target specific primers.

Also fluorescently labeled nucleotides were used to insert labeled molecules in the linker segment of the probe (Figure 1.8).Using this method probes of the size 50, 100, 150, 200, 400 and 800 were created.

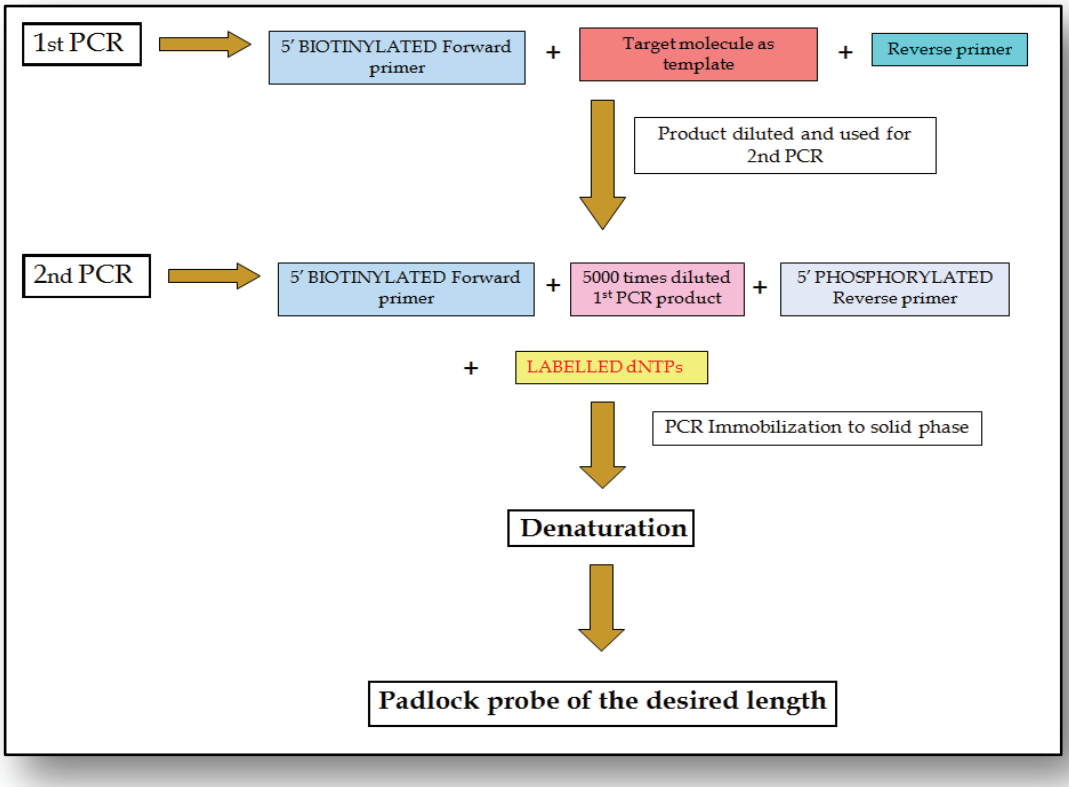


Figure 1.8 Schematic representation of the steps involved in the construction of padlock probes using the PCR based method.

- **[C] Ligation-based synthesis of probes**: This method was based on the principle of gene synthesis and used the enzyme ligase during construction. Here basically pre- synthesized short oligonucleotides were used for construction of longer probes (Borodina et al., 2003). Here three fragments, common primer, left locus primer and the right locus primer were hybridized in the ratio 1:1.1:1.1 respectively with the help of right locus adaptor and left locus adaptor in the ratio 1.2:1.2. The left locus primer and the right locus primer were then ligated to the common primer in the center. The probe was then phosphorylated and PAGE purified (Figure 1.9)

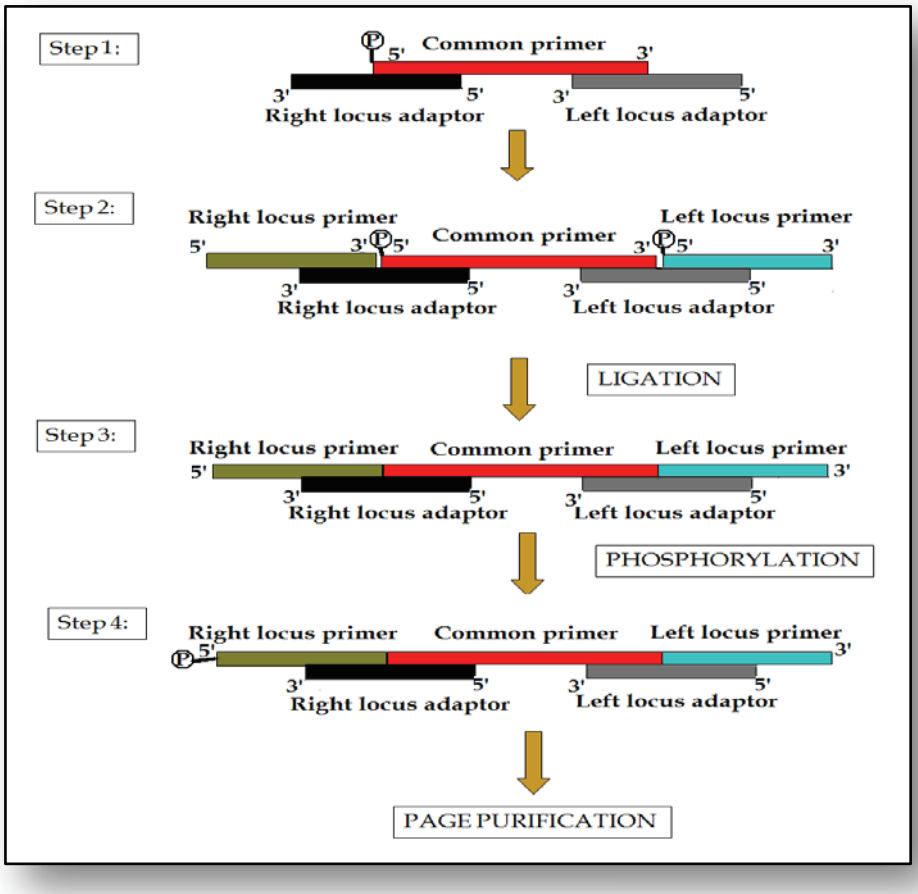


Figure 1.9 Schematic representation of construction of padlock probes using ligation based assay

The above methods of construction do have advantages, but each of them has a set of disadvantage too. The list of advantages and disadvantages of the above methods is given in **Table 1.1**.

Table 1.1 Advantages and disadvantages of the different methods used for construction of padlock probes. The advantages and disadvantages are only based in view of construction of padlock probes.

No.	METHOD	ADVANTAGES	DISADVANTAGES
1.	**Standard automated solid-phase oligonucleotide Probe synthesis (Chemical synthesis)** (Caruthers et al., 1983)	-	a) As the length of the padlock probe to be synthesized usually exceeds 90 nt, the specificity is greatly reduced and many types of imperfect molecules accumulate during synthesis. b) The functionality of the padlock probe can be vulnerable if a deletion has occurred due to inefficient capping. c) As the required concentration increases, the cost also increases decreasing the reliability of obtaining the exact sequence. d) Phosphorylated oligonucleotides of more than 110bps are not reliable and are very difficult or even impossible to construct.
2.	**PCR-generated padlock probes** (Antson et al., 2000)	a) Longer and significantly labeled probes can be generated. b) Dense labeling enhances detection.	a) The specificity of the PCR – synthesized Padlock probe to circularize is reduced to only 60- 70% from ~90% efficiency of the chemically synthesized probes. b) The efficiency has been tested only for the pilot scale generation of Padlock probes. c) It is impossible to insert modified bases into a defined position during PCR.
3.	**Ligation based synthesis of padlock probes with block structures** (Borodina et al., 2003)	a) Modifications can be introduced into the sequence. b) The synthesis scale can be manipulated as required. c) No expensive instruments required.	a) PAGE purification is tedious and time consuming. b) The amount of probes generated is dependent on the amount of oligonucleotide added in the initial stage.

1.2.2 *Criteria required for popular usage of Padlock probes*

In order to use these padlock probes to their fullest potential, there is a desperate need of a method that can construct high concentrated functional padlock probes with target specific ends and phosphorylation. Also from the Table 1.1 it is comprehensible that all the currently available methods for the construction of Padlock probes have many disadvantages compared to the advantages they offer. So the main task for the success of these probes is the construction of these probes. Then new method must be efficient and must fulfill the following criteria:

- Inexpensive construction
- High concentration
- Construction of longer probes
- Construction of functional probes
- Construction of repetitive sequences

1.2.3 *Proposed strategy*

The proposed approach would be using the gene construction method which utilizes the enzyme ligase to join pre- synthesized oligonucleotides, to create the desired long sequence. Construction of short oligonucleotides by phosphoramidite chemistry is usually error free and they can be used for constructing longer molecules. The overlapping segments for hybridization can be set to 10- 12 bp (Engels et al., 1987) in order to get stable hybridization and at the same time avoid most of the secondary structures.To reduce the costs of short oligonucleotides, a polymerase reaction can be introduced to create the remaining part of the molecule as described by Joachim for the synthesis of gene (Engels et al. 1989; Jayaraman et al. 1991; Prodromou et al. 1992; Majumder 1992; Stemmer et al. 1995; Holowachuk et al. 1995; Singh et al. 1996; Au 1998; Tsuge 2003; Young 2004; Rouillard 2004;). The fill-in method followed by the ligation reaction would be the distinctive difference between Joachim's PCR based gene synthesis method and the method designed for this study, 'Ligation and polymerization based assay'.

Also the partial double stranded construct can be converted to single stranded form by exploiting the Exonuclease I and III's inability to cleave phosphorothioate modification (Rogers et al., 1980). Using both these enzymes in conjunction can get rid of the other artifacts formed during hybridization, thus reducing the complexity of the reaction. After construction the probe concentration won't be as high as desired, hence an amplification method could be introduced.

As the construct is single stranded, it can easily be circularized and amplified using rolling circle replication. After a restriction digestion reaction, the product consists of a 5' phosphorylated and 3' hydroxyl group, it could be used to cleave the rolling circle amplified padlock probe to generate high concentration of functional probes. The schematic representation of an attempt to make functional, phosphorylated and highly concentrated probes is given in Figure 1.10 and Figure 1.11 respectively.

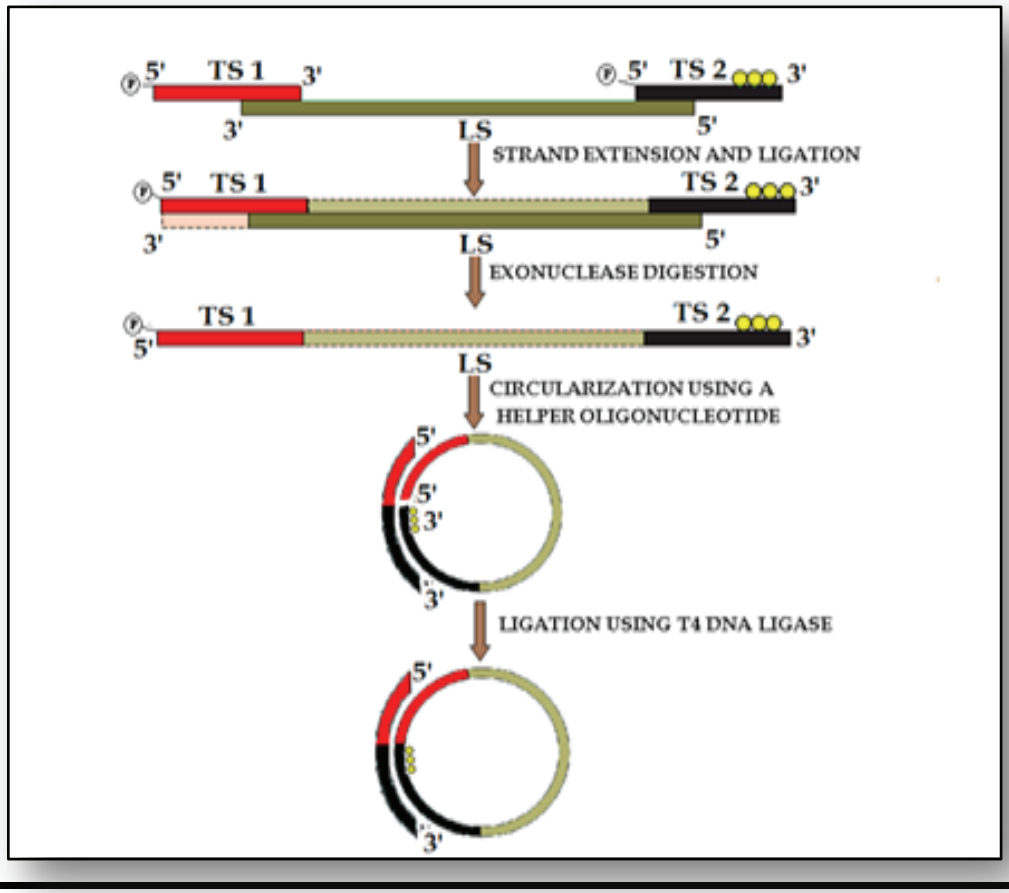


Figure 1.10 Schematic representation of the method designed for construction, of padlock probes

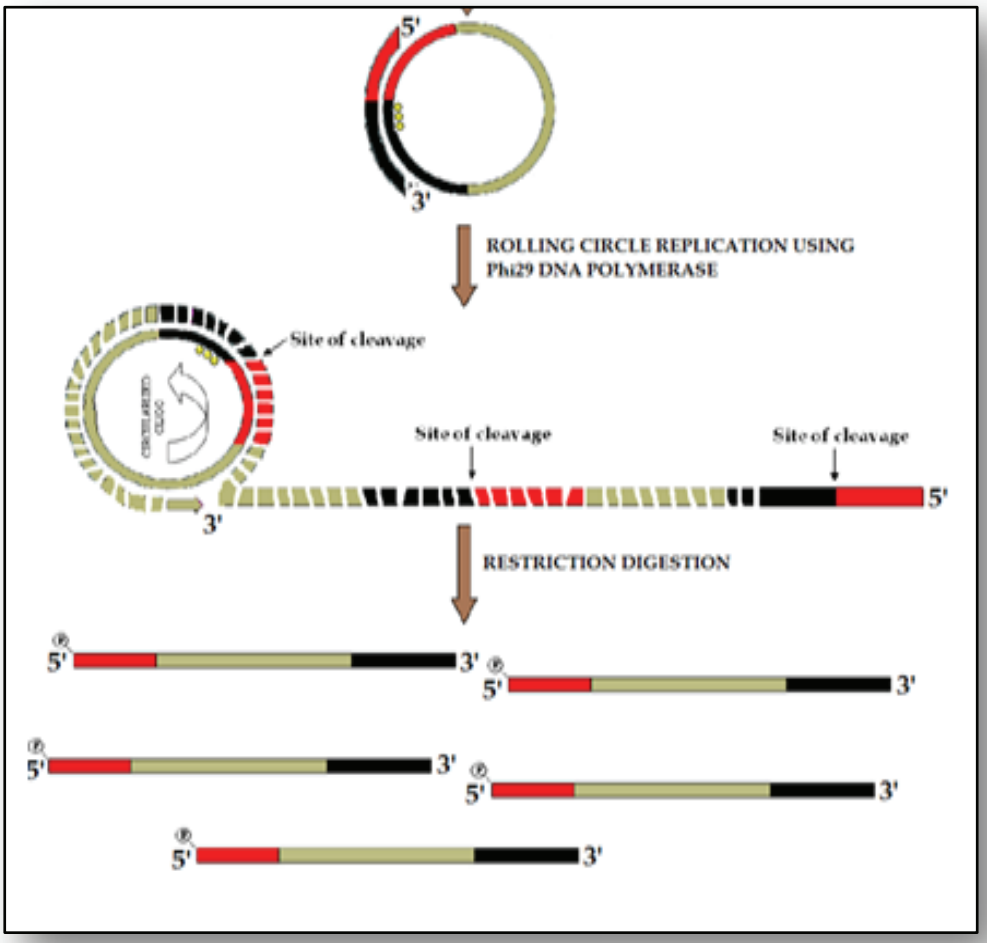


Figure 1.11 Schematic representation of the method designed for amplification and generation of functional padlock probes form the construct

As a part of the EU project GenSensor Nanoparts, a new sensitive approach is aimed to detect and quantitate the amount of DNA left behind in the solution after fishing with the nanobeads, and thus validate the working of the whole system. The Kotenkar-Rühle-Kracht approach to fulfill this aspiration combined three aspects. Firstly, to construct cheap longer probes with repetitive sequences introduced in its linker segment (scope of this work). These repetitive linker segments can act as target for the next level of padlock probes which can act as a method for signal amplification (PhD work of Frank Rühle). Also the introduction of a poly 'A' stretch can enable bioluminescence detection of the amplified probes (PhD work of Björn Kracht) and hence an extremely sensitive detection and quantification method can

be put forth. As the probes are amplified with the addition of each layer, there is only signal amplification and not target amplification as in PCR reaction hence facilitating quantification. The schematic representation of this approach is shown in Figure 1.12 respectively.

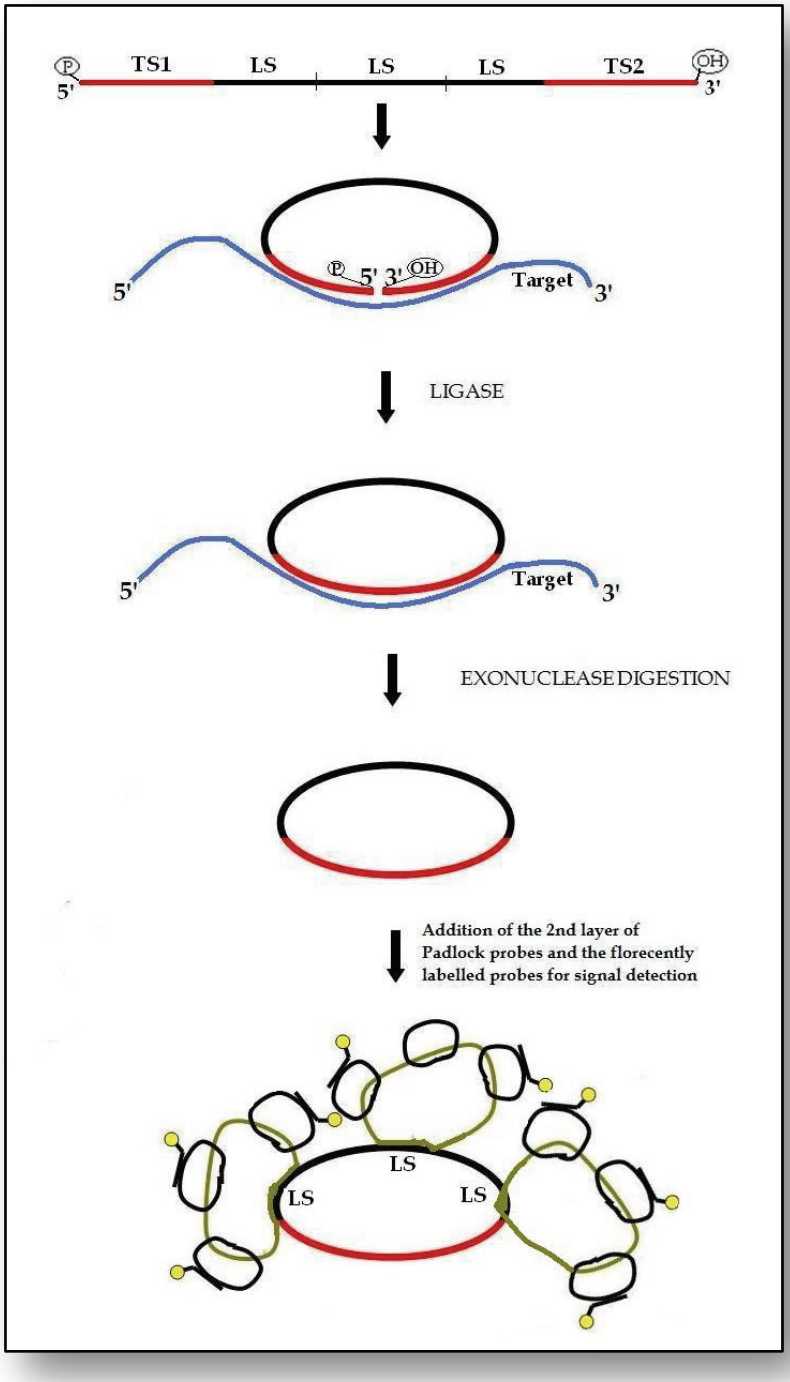


Figure 1.12 Functioning of the longer padlock probes with multiple linker segments (Kotenkar-Rühle-Kracht-Approach)

1.3 Aim of this study

In the scope of this study, the goals that have been attempted to be achieved are divided into two parts:

1. Firstly, to design a standardized protocol to construct high concentration of padlock probes with phosphorylated 5′ end and exact sequences at the 5′ and the 3′ end of the probe at a low cost.
2. Secondly to create and standardize a protocol for constructing high concentration of long and phosphorylated padlock probes with as many as possible repetitive sequences incorporated in it for or the Kotenkar-Rühle-Kracht approach.

An attempt has been made to fulfill all the above criteria and develop a method for the production of padlock probes. The task has been tackled in three major steps:

1. Construction of padlock probes
2. Amplification of the constructed padlock probes
3. Generation of multiple single stranded functional padlock probes

2 MATERIAL AND METHODS

2.1 Materials

2.1.1 Oligonucleotides

All the oligonucleotides that were used as primers, probes, "circs" or other elements including the starting material for the construction of padlocks were synthesized by the company Thermo Fisher Scientific, Ulm, who are using the phosphoramidite chemistry. The oligonucleotides in all cases were HPLC purified. They were obtained in desiccated form and were diluted to a final concentration of 100 pmol/µl, using double distilled water. Aliquots were made from each of the oligonucleotides which were further diluted as per need. 321 bases padlock was ordered from Shingene, China and a vector with the padlock sequence inserted in it was ordered from Microsynth, Switzerland.

2.1.1.1 Construction and Amplification

The target sequence for which the padlocks were designed for, was selected from *S. epidermidis* Domain 5 as a part of the EU project "GenSensor Nanoparts". For the construction of the probe five different approaches were worked on. For two of the approaches, ligation and polymerization based system and continuous ligation based system, the design was created using Visual OMP and Mfold such that no secondary structure or conformations of the oligonucleotides used for the construction of padlocks, inhibit its assembly and creation and also such that the functionality of the created padlock is not affected by its secondary conformations. For the other three methods namely, stepwise based system, bead based system and pre-ligation based system, Mr. Hannes Weber designed a set of possible fragments which had the desired functionality required for the final padlock and

which showed minimal secondary structures. The final sequence of the desired padlock was put together by Frank Rühle.

In each of the construction, the oligonucleotides were annotated depending on their role during the construction and amplification of the padlocks. The detailed list of the abbreviations used and what they stand for is mentioned in Table 2.1. In the first two methods of construction, ligation and polymerization based system and continuous ligation based system, a fill in reaction was introduced and hence the helper oligonucleotide was not completely complementary, but partially complementary at the both the ends to the target specific oligonucleotides. The constructed padlocks were then amplified using rolling circle amplification using the circ oligonucleotide as a primer. Below in the Table 2.2 and Table 2.3 the details of the sequences of the oligonucleotides used for construction of padlocks and their respective modifications are mentioned.

Table 2.1 List of abbreviations used for labelling the oligonucleotides used for the various purposes during construction, amplification and digestion.

ABBREVIATIONS	MEANING OF THE ABBRIVIATIONS
He "Name of Oligonucleotide"	Stands for helper oligonucleotide which aids the ligation reaction during construction. It is followed by the name of the oligonucleotide to which it is complementary
rC" Name of Oligonucleotide"	Stands for the reverse complementary sequence of the expected final product
TS	Stands for Target specific region of the padlock
TS1	Stands for target specific region near the 5' end of the padlock
TS2	Stands for target specific region near the 3' end of the padlock
LS	Stands for linker site where different functionalities can be introduced
BS	Stands for binding sites for the next generation of probes
Circ	Stands for the oligonucleotide that facilitates the circularization of the constructed padlocks for further acts as a primer for rolling circle amplification.

Table 2.2 Sequences of oligonucleotides that were used for construction using ligation and polymerization based system and continuous ligation based system.

LABEL	OLIGONUCLEOTIDE SEQUENCE (5′ – 3′)	MODIFICATION	Tm
rC R1 DV TS1	TTTGATTGTTAGAACGTATGCATCGCCTATATGTAATTGTGCCT	5' Phosphate	67
rC R1 DV PTO TS2	GCGTATAGAGAAAGACCTTTAGATGATTCTTCGAAACGGTAAACT	5' Phosphate, last three bases of 3' end PTO modified	67
R1 DV He LS	CTAAAGGTCTTTCTCTATACGCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGGCACAATTACATATAGGCGA	5' Phosphate	77
rCR2 DV BS	GCGTATAGAGAAAGACCTATATGTAATTGTGCCT	5' Phosphate	62
R2 DV LS	GTCTTTCTCTATACGCTCTCTCTCTCAGGCACAATTACATATAG	-	68
R1/2 DV Circ	TTCTAACAATCAAAAGTTTACCGTTTC	-	54
R1 DV syn Template	TTTGATTGTTAGAACGTATGCATCGCCTGTATGTTAGAGAAATTGACCTTAAGCAACGCCTATAGTCCATCGAAACGGTAAACT	5' Phosphate	78

Table 2.3 Sequences of oligonucleotides that were used for stepwise based system, bead based system and pre-ligation based system.

LABEL	OLIGONUCLEOTIDE SEQUENCE	MODIFICATION	Tm
rC R3 DV TS2	CAGTCGAGTTCATCGCACCATCAAACGCTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTG	5' Phosphate	69
rC R3 DV TS1	ACTATCTGCATACCTATAGCTCCC	5' Phosphate, last three bases of 3' end PTO modified	55
rC R3 DV BS	ATATGCGTCTGTGAAATGTGCTCGGTGGTAAAGGCAAGG	5' Phosphate	69
rC R3 DV HeTS2-BS	AGACGCATATCACAAAAAAAAA	-	49
R3 DV HeTS1-BS	TATGCAGATAGTCCTTGCCTTT	-	53
R3 DV HeBS-BS	CAGACGCATATCCTTGCCTTT	-	54

R3 DV HeBS-BS lng	GCACATTTCACAGACGCATATCCTTGCCTTTACCACCGA	-	69
R3 DV Bio HeBS-BS	AACAATCAAAAGTTTACCGTTTCGA	5' Biotin	53
R3 DV Circ new	ATGAACTCGACTGGGGAGCTATAG	-	58
rC R4 DV TS2	TTGATTGTTACATCGCACTACAACCTAACGTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTG	5' Phosphate	63
rC R4 DV TS1	TAGATTCTTCGAAACGGTAAACTT	5' Phosphate, last three bases of 3' end PTO modified	51
rC R4 DV BS	ATATGAACTCGACTGGGGAGCTATAGGTATGCAGATACA	5' Phosphate	66
rC R4 DV HeTS2-BS	CGAGTTCATATCACAAAAAAAAA		46
R4 DV HeTS1-BS	CGAAGAATCTATGTATCTGCA	-	49
R4 DV HeBS-BS	CGAGTTCATATTGTATCTGCAT	-	49
R4 DV Target	GCACATTTCACAGACGCATATCCTTGCCTTTACCACCGA	-	68
R4 DV Circ	TAACAATCAAAAGTTTACCGTTTCGAAGAATC	-	57

2.1.1.2 Restriction Digestion

In order to get functional padlocks from the rolling circle product, the rolling circle product was subjected to enzymatic digestion. Since most the restriction enzymes need a double stranded region for cutting and as the rolling circle product is single stranded, oligonucleotides were added to create partial double strand to aid cutting reaction. Also an oligonucleotide having restriction enzyme recognition site and a stem-loop secondary structure near its 5′ end was used for digesting the rolling circle product with an aim of avoiding the limitation of having a restriction site near the 5′ and the 3′ ends of the constructed padlocks, hence provide flexibility while constructing the padlocks. The sequences of the oligonucleotides that assisted the restriction digestion reaction are listed in

Table 2.4 in the 5′ to 3′ direction.

Table 2.4 Sequences of oligonucleotides that were used for all cleaving the rolling circle product for all the construction systems

LABEL	OLIGONUCLEOTIDE SEQUENCE	MODIFICATION	Tm
rC R1 DV RF new	ATTGACCTTAAGCAACGCCT	-	50
rC R1 DV RFelong	TATGTTAGAGAAATTGACCTTAAGCAACGCCTATAGTCCATC	-	66
rCR1 DV RF BtgZI	TTTGATTGTTAGAACGTATGCATCGCCTATATGTAA	-	63
rCR1 DV RF Stem-loop	CATCGCCTCCAGTTTTCTGGAGGCGATGTTGTGCCTGAGAGAG	-	74
rCR1 DV RF GsuI	CGAAGTGCCGGGGCAGGATCTCCAGCGGATA	-	70
rCR1 DV deoxy	ATTGACCTGGGCCGTTGCTTTGCAACAC	-	64

2.1.1.3 PCR Amplification

During construction of the padlocks, the oligonucleotides hybridized and formed altered combinations than the expected padlocks and the concentration of these products varied. In order to distinguish and select the right product for further amplification, PCR step was introduced. The sequences are listed in Table 2.5.

Table 2.5 Sequences of oligonucleotides that were used for PCR and sequencing reactions

LABEL	OLIGONUCLEOTIDE SEQUENCE	MODIFICATION	Tm
rCR1 DV Seq Primer	CGAAACGGTAAACTTTTGATTGTT	-	52
R1 DV Seq FP Primer	AGAACGTATGCATCGCCTATA	-	53
rCR1 DV Seq RP Primer	AACAATCAAAAGTTTACCGTTTCGA	-	53
R3 DV LPrimer	CAGTCGAGTTCATCGCACCA	5' Phosphate	55
rCR3 DV Rprimer	GGGAGCTATAGGTATGCAGATAGTC	-	56
Se_DV 2	CACCGACACTGAATAACATCG	-	59
Se_DV 2+	CGTTTAGAAGAGGCAGGCTTA	-	59

2.1.1.4 Detection system

For detecting whether the constructs have the required fragments, a sensitive method other than the gel based system was desired. Southern blotting was the

method of choice. The probes that were required for the same are listed below in Table 2.6.

Table 2.6 Sequences of oligonucleotides that were used as probes for Southern blotting

LABEL	OLIGONUCLEOTIDE SEQUENCE	MODIFICATION	Tm
R3DVDIG TS1	GGGAGCTATAGGTATGCAGATAG	5′ Deoxygenin	50.1
R3DVDIG TS2	TTTGATGGTGCGATGAACTCGACT	5′ Deoxygenin	61.9
R3DVDIG BS	ACAGACGCATATCCTTGCCTTTAC	5′ Deoxygenin	58.6
R3DVDIG rca	ACTATCTGCATACCTATAGCTCCC	5′ Deoxygenin	55
R3DVDIG rca	CAGTCGAGTTCATCGCACCATCAA	5′ Deoxygenin	59

2.1.2 Solutions and reagents

2.1.2.1 Buffers and Reagents

[A] Construction and Amplification

1. **1X NEBuffer 2 (T4 DNA Polymerase Buffer) (New England Biolabs)**
   50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT
   pH 7.9 at 25°C
2. **1X T4 DNA Ligase Reaction Buffer (New England Biolabs)**
   50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/ml BSA
   pH 7.5 at 25°C
3. **1X Ampligase® Reaction Buffer (Epicentre® Biotechnologies)**
   20 mM Tris-HCl (pH 8.3 at 25° C), 25 mM Potassium chloride(KCl), 10 mM $MgCl_2$, 0.5 mM NAD, 0.1 % Triton® X-100
4. **1X Reaction Buffer (Exonuclease III) (Fermentas)**
   66 mM Tris-HCl (pH 8.0 at 30° C), 0.66 mM $MgCl_2$
5. **1X Phi DNA Polymerase Buffer (Fermentas)**
   33 mM Tris-acetate (pH 7.9 at 37° C), 10 mM Magnesium acetate, 66 mM Potassium acetate, 0.1 % Tween 20, 1 mM DTT

6. **PCR Mix**

   20 mM dNTP (Molzym), 100 mM $MgCl_2$ (Molzym), 10X PCR buffer (Molzym)

[B] Restriction Digestion

1. **1X NEBuffer 4 (New England Biolabs) (For BtgZI enzyme)**

   20 mM Tris-acetate, 10 mM Magnesium acetate, 50 mM Potassium acetate, 1 mM DTT

   pH 7.9 at 25° C

2. **1X Buffer B (Fermentas) (For GsuI enzyme)**

   10mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 0.1mg/ml BSA

3. **Deoxyribozyme reaction mix**

   HEPES (Serva), NaCl (Fluka), $CuCl_2$ (Serva)

4. **1X Buffer O (Fermentas)(For BspTI enzyme)**

   50 mM Tris-HCl (pH 7.5 at 37°C), 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA

[C] Detection System

I] Gel based detection

1. **1X TBE Buffer**

   89 mM of Tris-Base (pH 8.2) (Sigma), 89 mM of boric acid (Roth), 1 mM EDTA (Roth)

   pH- 7.2

2. **1X TAE Buffer**

   40 mM Tris-acetate (Sigma), 1 mM EDTA (Roth).

3. **Agarose** (Peqlab)

   peqGOLD Universal Agarose

4. **Polyacrylamide gel with urea**

   10 X TBE (500 µl), 40% Acrylamide (Serva) (2500 µl), 2% BAA (Bio- Rad) (425 µl), Urea (Sigma) (2.4 g), TEMED (Gibco BRL) (50 µl), 10% APS (Sigma) (5 µl)

5. **Loading dye with urea**

   Loading dye + (8 M Urea, 20 mM EDTA, 5 mM Tris-HCl (pH 7.5))

6. **Polyacrylamide gel without urea (12%)**

   10 x TBE (500 µl), Water (3000 µl), 40%Acrylamide: bis -acrylamide (Sigma- Aldrich) (1500 µl), TEMED (5.7 µl), 10 % APS (41.7 µl)

7. **Loading Dye** (Fermentas) (6 X)

8. **Ladders** (Fermentas)

   Gene ruler® Ultra low ladder, Gene ruler® 100 bp Ladder, Gene ruler® 1 Kb Ladder, Gene ruler®1 Kb plus ladder

9. **SYBR Green I/ II solution** (Molecular Probes® Invitrogen)

   10,000 X concentrate in 1 XTBE

10. **Ethidium bromide solution** (Serva)

    0.5 µg/ml in 1 X TBE

11. **Gel Purification Kits and Reagents**

    PCR- Kombi Kit (Seqlab), **FTA elute (classic)** (Whatman), **High Pure DNA extraction Micro Kit** (Roche), Sodium acetate (3 M) (Merck), 70 % Ethanol , Parafilm (Pechiney)

II] Southern blot based detection

1. **Denaturating Buffer**

   500 mM NaOH (Applichem), 1.5 M NaCl (Fisher Scientific)

2. **Neutralizing Buffer**

   0.5 M Tris, 1.5 M NaCl, 1 mM EDTA

   pH 7.0

3. **10 x SET**

   1.5 M NaCl, 0.3 M Tris, 20 mM EDTA

   pH 8.3

4. **DIG P1**

   0.1 M Maleic acid (Merck- Schuchardt), 0.15 M NaCl

   pH 7.5

5. **DIG P2**

   5% w/v Milk powder (Oxoid) in DIG P1

6. **DIG P3**

   0.1 M Tris, 0.1M NaCl, 50 mM $MgCl_2$ (Riedel- de Haën)

   pH 9.5

7. **Premixed BCIP/NBT Solution** (Sigma-Aldrich)

   0.48 mM NBT (nitro blue tetrazolium), 0.56 mM BCIP (5- bromo-4-chloro-3-indolyl phosphate), 10 mM Tris-HCl (pH 9.2), 59.3 mM $MgCl_2$

8. **Anti- Digoxigenin- AP, Fab fragments** (Roche)
9. **Biodyne® B Membrane** (Blot Membrane) (Pall Corporation) (0.45µM)
10. **Whatman® Chromatograph paper 17 CHR** (Whatman)

[III] Sequencing reaction

1. BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems)

2.1.2.2 Enzymes

1. **T4 DNA Polymerase enzyme** (New England Biolabs) (3 U/µl)
2. **T4 DNA ligase enzyme** (New England Biolabs) (400 U/µl)
3. **Exonuclease III enzyme** (Fermentas) (200 U/µl)
4. **Exonuclease I enzyme** (Fermentas) (20 U/µl)
5. **Ampligase® DNA ligase enzyme** (Epicentre® Biotechnologies) (5 U/µl)
6. **Phi 29 DNA Polymerase enzyme** (Fermentas) (10 U/µl)
7. **Taq Polymerase** (Molzym) (5 U/µl)
8. **BtgZI restriction enzyme** (New England Biolabs) (2 U/µl)
9. **GsuI restriction enzyme** (Fermentas) (5 U/µl)
10. **BspTI restriction enzyme** (Fermentas) (10 U/µl)

2.1.2.3 Miscellaneous

1. 20 mM dNTP (Molzym)
2. 10 mg/ml Dynabeads (Dynal Biotech; Invitrogen)
3. 1 M DTT (Fermentas)

2.1.3 Equipments

- Cycler: Eppendorf Mastercycler® ep gradient, Biometra Trio-Thermoblock
- Vortex: Merck® eurolab
- Mixer: Eppendorf Thermomixer compact
- Centrifuge: Eppendorf 5415D
- Incubator: Merck® eurolab - Binder
- Shaker: MR-1 Mini-rocker shaker, IKA® MTS 4 schüttler
- Spectrophotometer: NanoDrop ND- 1000
- UV light table: Camag Reprostar II
- Camera: Cannon Powershot A520
- Scanner: Cannon CanoScan FB630U
- Microwave: AEG Micromat
- Sequencer: Perkin Elmer ABI - Prism™ 310
- Crosslinker: Vilber Lourmat Bio-Link (254nM)
- Hybridization oven : MWG- Biotech Mini 10
- Polyacrylamide gel- electrophoresis apparatus: Bio- Rad Mini Protean II™
- Agarose gel- electrophoresis apparatus: Biometra Agagel
- Voltage supply for Agarose and Polyacrylamide gels: Bio-Rad Power n Pac 300
- Pipette: Eppendorf Micro litre pipettes

2.1.4 Software

2.1.4.1 Secondary structure analysis and calculating the properties of oligonucleotides and DNA

- VisualOMP version 3.2.7
- Oligo Calc: Oligonucleotide Properties Calculator http://www.basic.northwestern.edu/biotools/oligocalc.html

- The Rensselaer bioinformatics web server
  http://mfold.bioinfo.rpi.edu/
- IDT Scitools
  http://eu.idtdna.com/SciTools/SciTools.aspx?c=EU

2.1.4.2 Mapping restriction sites and choosing restriction enzymes

- REBASER- The Restriction Enzyme Database
  http://rebase.neb.com/rebase/rebase.html
- Restriction mapper version 3
  http://www.restrictionmapper.org/

2.1.4.3 Sequencing

- Chromas Freeware Version 1.45
- SeqMAN II (DNA Star Inc. 2000), Version 4.05
- Genedoc Version 2.6.002

2.1.4.4 Miscellaneous

- Microsoft Visio 2007
- Microsoft Excel 2007
- Paint.NET v3.31
- Irfanview version 3.98
- Microsoft word 2007
- Endnote XI

2.2 Methods

2.2.1 Bioinformatics

All the probes were targeted to ***S.epidermidis*** Domain 5. As this PhD work was a part of EU project, "Gensensor Nanoparts", the target was selected in the scope of this project. For the 120 bases padlocks the linker segments that was chosen was based on the PhD work of Dr. Sascha Todt, which gave maximum hybridization. Rest of the molecules were then designed using M-fold (from the DNA mfold server: 1996-2008, Michael Zuker, Rensselaer Polytechnic Institute; http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi) and meticulous work with Visual OMP DNA software. The occurrences and avoidance of secondary structure were tested using these software, and a padlock sequence which fits into the most acceptable category was selected. The required assets of the desired padlock were as follows:

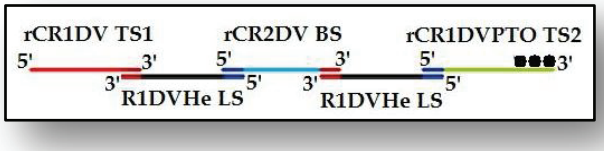


Figure 2.1 Schematic representation of the hybridization of the oligonucleotides used for constructing padlocks with repetitive sequences by modifying the ligation and polymerization assay.

- While construction using fragment sequences, no secondary structures especially near the hybridizing parts at the given reaction temperature, should be observed.
- Also no cross hybridization between the different fragments during construction should occur.
- The binding site should be different and unique in each probe.
- The difference in the Tm of the fragments used for construction should not be more than 15° C.
- Primer binding site should be incorporated in the linker segment.

- Poly A stretch was incorporated for detection using bioluminescence reaction.
- Restriction enzyme recognition site should be near the target recognition fragment.

With Visual OMP, the following criterions were considered

- Reaction buffer (Monovalent ions were set to 108 mM)
- Reaction temperature (37° C, 45° C and 60° C)

Note that when the fragments were designed for the construction of padlocks, they were reverse complementary to the sequence of the desired probe. As a rolling circle amplification reaction was a part of the construction method, the desired sequence of the probe would then be obtained after amplification.

For the longer probes a set of sequences were designed by Mr. Hannes Weber (Universität Bremen FB 3, Mathematik und Informatik) using bioinformatics tools and optimized in terms of the secondary structure formation and the Tm of the hybridizing oligonucleotides during construction,by Mr. Frank Rühle using the visual OMP software.

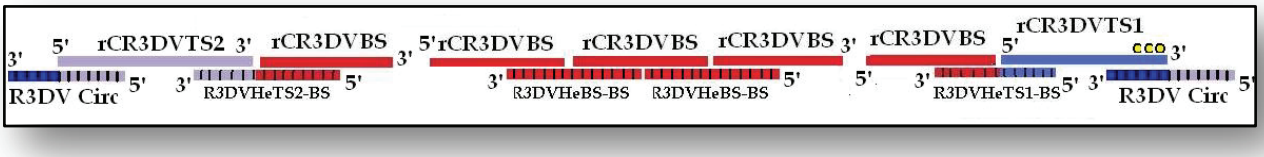


Figure 2.2 Schematic representation of the hybridization of the oligonucleotides used for constructing long padlocks with repetitive sequences.

2.2.2 Construction assays

There were five construction methods tried and standardized in order to get satisfactory results. Due to limitations in each of the methods the next was tried. The protocols followed and schematic representations of each of the protocol are shown below. The schematic representation consists of only those reactions which are expected to generate the desired product. Other reaction steps and possibilities are not shown, but they do occur in the system to an unknown extent.

2.2.2.1 Ligation and polymerization based system

This assay combines two enzymatic steps, polymerization and ligation. The construction fragments were hybridized together by heating and cooling, and then subjected to polymerization reaction and finally the fragments were joined together to get the desired product using ligation reaction. The unwanted strand and the un-reacted oligonucleotides in the solution were digested using a combination of exonuclease I and III exonucleases. This step was introduced to reduce the complexities of the system and to increase the yield of the desired product. This protocol was later modified to suit the construction of longer padlocks. Different ratios were tried to get the desired product. Both the detailed construction protocols pursued is given below followed by their respective schematic representation in Figure 2.1 and Figure 2.2.

PROTOCOL:

1. **Hybridization of rCR1DVTS1, rCR1DVPTOTS2 and R1DVHeLS**
   [R1DVTS1 (5 µM), rCR1DVPTOTS2 (5 µM) and R1DVHeLS (2.5 µM)] or [R1DVPTOTS2 (2.5 µM), rCR1DVTS1 (2.5 µM), R1DVLS (5 µM) and rCR2DVBS (3.75 µM)] were mixed with water and 1X T4 ligase buffer (New England Biolabs) to make the final volume to 50 µl. The mixture was then kept at 37° C for 10 min, heated to 90° C for 10 min and then slowly cooled to RT on a thermomixer (Eppendorf)(for more details see Appendix-A1.1).
2. **New strand synthesis using polymerase**
   Mixture from step 1 (30 µl) was mixed with 1 mM dNTP, 1X T4 Polymerase buffer (New England Biolabs), 0.1 U/µl T4 Polymerase (New England Biolabs), and the system volume was adjusted to 50 µl using distilled water. The mixture was then incubated in the thermo cycler (Eppendorf) at 12 ° C for 6 hrs and the enzyme was inactivated at 75° C for 20 min. For the synthesis of the new strand incubation at 12° C for 12 hrs was tested too.
3. **Ligation of the extended strand**

Mixture from step 2 (39.9 µl) was mixed with 1X T4 Ligase buffer (New England Biolabs), 8 U/µl T4 ligase (New England Biolabs) The system volume was adjusted to 50 µl using distilled water and incubated at 16 ° C for 6 hrs. The enzyme was inactivated at 65° C for 15 min in the thermo cycler (Eppendorf)

4. **Exonuclease digestion of the unwanted strand**

Mixture from step 3 (40 µl) was mixed with 1X exonuclease III buffer (Fermentas) and 2.5 U/µl exonuclease III (Fermentas). The mixture was then incubated at 37 ° C for 3 hrs and then at 65° C for 15 min.

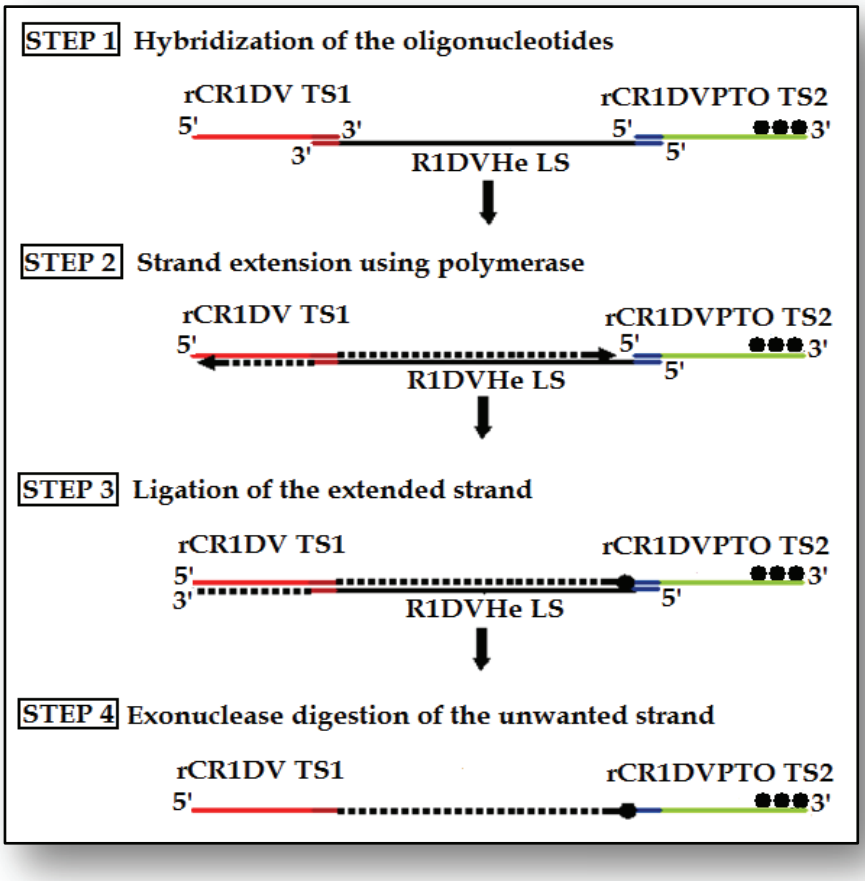


Figure 2.3 Schematic representation of construction using ligation and polymerization based system

2.2.2.2 Continuous ligation based system

This protocol in principle was just a modification of the ligation and polymerization based system. Here the two steps of polymerization and ligation were combined. Merging these two steps gave two advantages. The first one being the reduced time, and the second one was the circumvention of repeated heating and cooling steps. The modified protocol is as given below. Many ratios and different reaction time and conditions were altered before getting the final protocol.

PROTOCOL:

1. **Hybridization of rCR1DVTS1, rCR1DVPTOTS2 and R1DVHeLS**
   R1DVPTOTS2 (6 µM), rCR1DVTS1 (6 µM), R1DVLS (9 µM) and rCR2DVBS (7.2 µM) were mixed with water and 1X N2 polymerase buffer (New England Biolabs) to make the final volume to 100 µl and incubated at 37° C for 10 min, 90° C for 5 min, cooled to 40° C,40° C for 1 min. It was re-heated to 80° C for 1 min and finally cooled slowly to 30°C (for more details see Appendix- A1.2).
2. **New strand synthesis and ligation using polymerase and ligase**
   Mixture from step 1 (32 µl) was mixed with 1 mM dNTP, 1 X T4 ligase buffer (New England Biolabs), 16 U/µl T4 ligase (New England Biolabs), 1 X N2 polymerase buffer (New England Biolabs), 0.12 U/µl T4 Polymerase (New England Biolabs), and the system volume was adjusted to 50 µl using distilled water. The mixture was then incubated at 12° C for 6 hrs, 16° C for 8 hrs and then at 75°C for 20 min (for more details see Appendix- A1.2)
3. **Exonuclease digestion of the unwanted strand**
   Mixture from step 2 (20 µl) was mixed with 1X exonuclease III buffer (Fermentas) and 2.5 U/µl exonuclease III (Fermentas) and 0.5 U/µl exonuclease I (Fermentas) and incubated at 37° C for 4 hrs and heat inactivated at 65° C for 15 min.

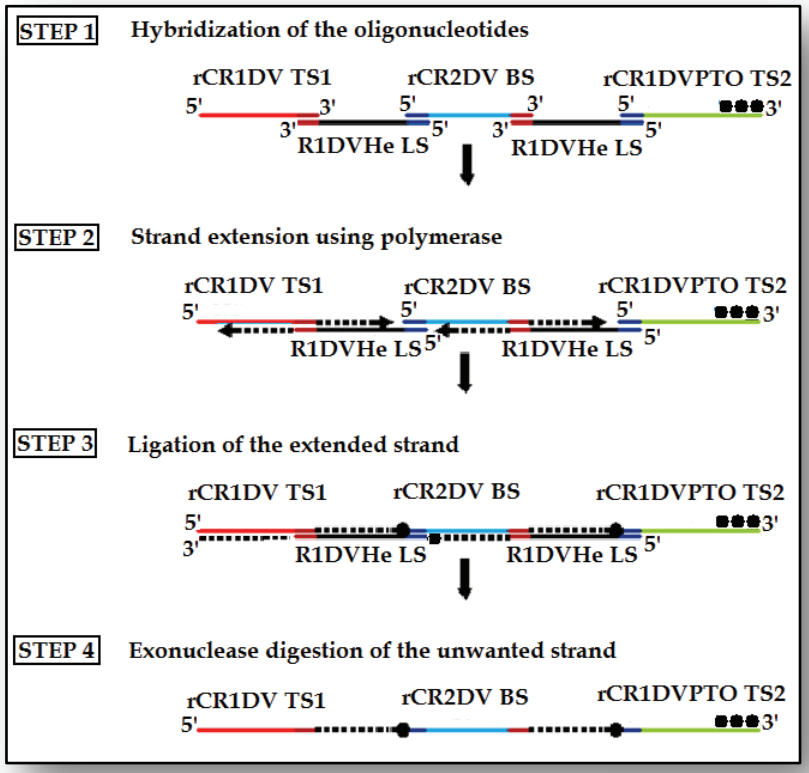


Figure 2.4 Schematic representation of construction using continuous ligation based system for longer probes

2.2.2.3 Stepwise based system

This protocol was followed, as the reactions could be partially controlled using varying concentrations of the different constructing oligonucleotides added in each step. This protocol reduced the complexity of the system to great extent. Also a major variation compared to the previous two methods was the design of the fragments. Here onwards the fragments were engineered such that the use of polymerase was eluded, thus, further minimizing the complexity of the system. Here two identical protocols with varying starting material, one in which constructions began with rCR3DVTS1 as the starting fragment and the other with rCR3DVTS2 as the first fragment were used. The main difference in the protocol where rCR3DVTS1 was the starting material was that the step no 1 and step no. 5

are interchanged in the protocol given below. Schematic representation of both the methods follows after the protocol in Figure 2.4 and Figure 2.5

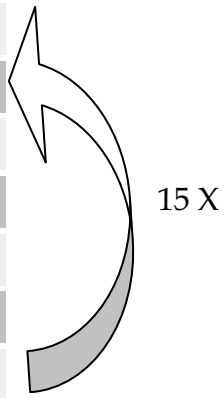
PROTOCOL:

1. **Annealing and Ligation of rCR3DVTS1, rCR3DVBS and R3DVHETS1-BS**

   rCR3DVTS1 (1 µM), rCR3DVBS (1 µM), and rCR3DVHeTS1-BS (1 µM) were mixed with 1X Ampligase buffer (Epicentre® Biotechnologies), 0.2 U/µl Ampligase (Epicentre® Biotechnologies) and water to make the final volume to 50 µl and incubated as shown in Table 2.7.

   Table 2.7 Temperature profile that was used for hybridization for the Stepwise based assay.

TEMPERATURE	TIME
90° C	4 min
90° C	30 sec
40° C	3 min
45° C	3 min
50° C	3 min
55° C	3 min
60° C	3 min



2. **Annealing of R3DVHEBS-BS**

   Mixture from step 1 (50 µl) was mixed with rCR3DVHeBS-BS (0.5 µM) vortexed, centrifuged and incubated at 65° C for 10 min and then slowly cooled to 30 ° C.

3. **Annealing and Ligation of rCR3DVBS**

   Mixture from step 2 (43 µl) was mixed with rCR3DVBS (375 nM) and incubated at 37° C for 60 min. 0.1 U/µl T4 ligase (New England Biolabs) and 1X T4 ligase buffer (New England Biolabs) was added to 37 µl of the annealed mixture and incubated at 37° C for 60 min and at 65° C for 10 min.

4. **Annealing of R3DVHEBS-BS_lng**

   Mixture from step 3 (33 µl) was mixed with R3DVHEBS-BS_lng (250 nM) and incubated at 45° C for 60 min.

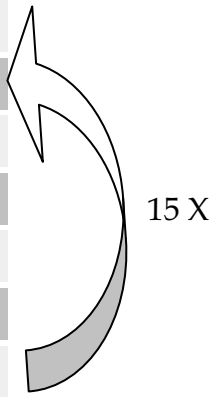
5. **Annealing and Ligation of rCR3DVTS2, rCR3DVBS and R3DVHETS2-BS**

rCR3DVTS2 (1 µM), rCR3DVBS (1 µM), and rCR3DVHeTS2-BS (1 µM) were mixed with 1X Ampligase buffer, 0.2 U/µl Ampligase and water to make the final volume to 50 µl. The mixture was treated as shown in

Table 2.8:

Table 2.8 Temperature profile used for annealing and ligating rCR3DVTS2, rCR3DVBS and R3DVHETS2-BS in the stepwise based method

TEMPERATURE	TIME
90° C	4 min
90° C	30 sec
40° C	3 min
45° C	3 min
50° C	3 min
55° C	3 min
60° C	3 min



6. **Annealing and ligation of STEP 4 products and STEP 5 products**

   0.1 U/µl T4 ligase and 1X T4 ligase buffer was added to the mixture from step 4 (26 µl) and mixture from step 5 (3.25 µl) and incubated at 37° C for 60 min and then at 65° C for 10 min.

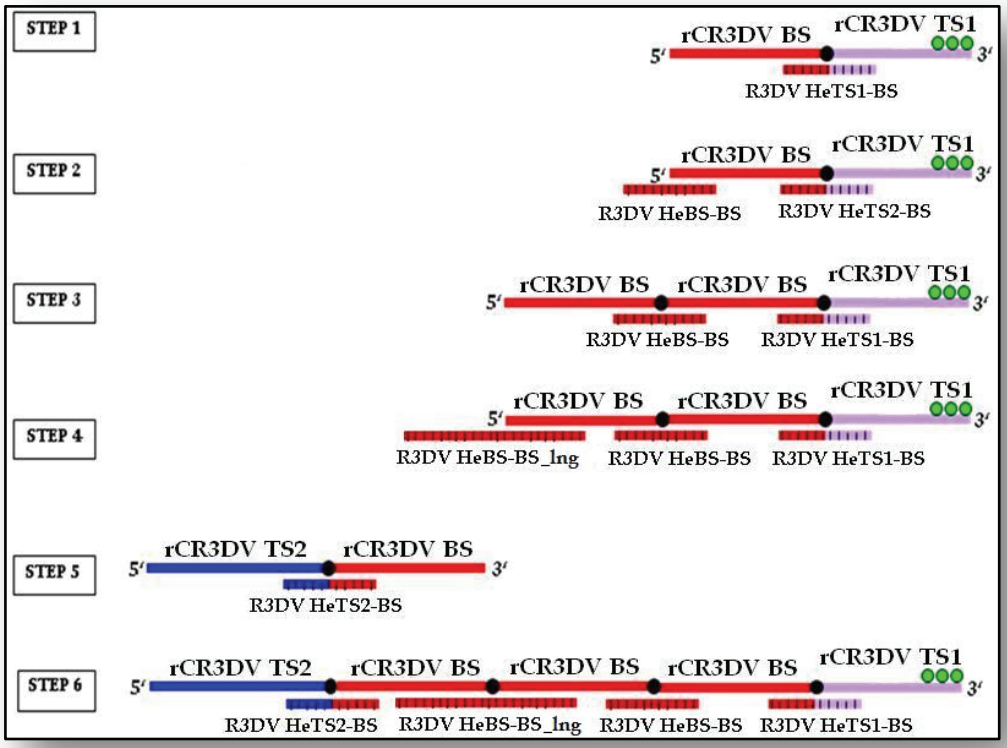


Figure 2.5 Schematic representation of construction using stepwise based system and rCR3DV TS1 as the starting oligonucleotide.

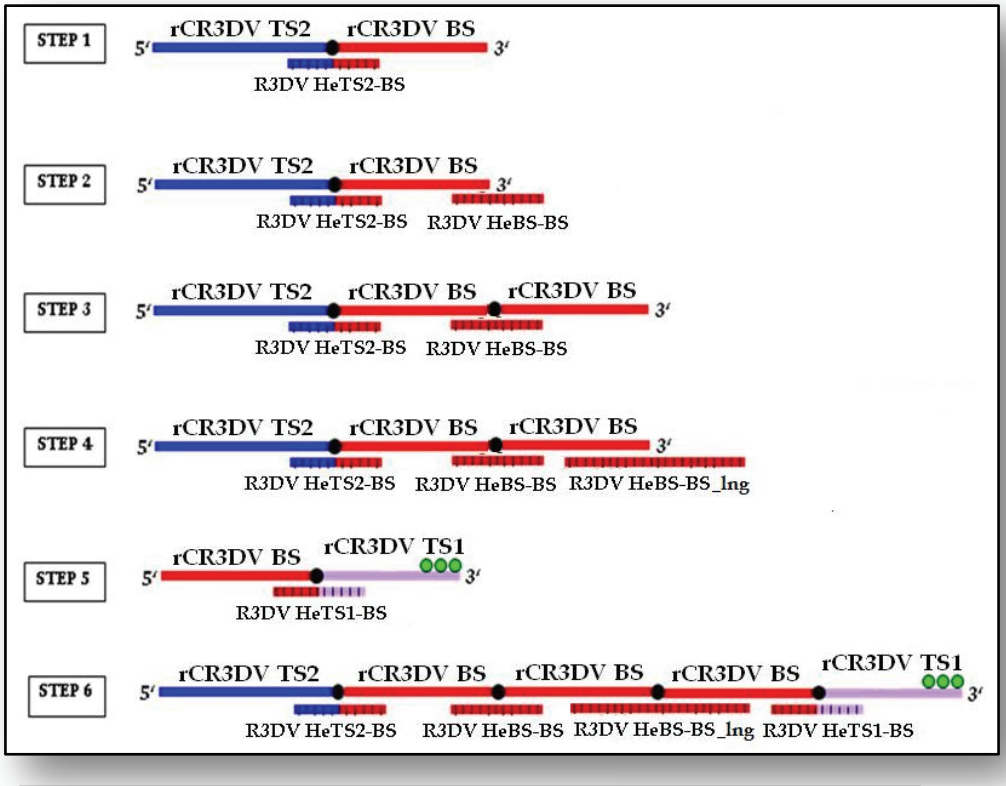


Figure 2.6 Schematic representation of construction using stepwise based system and rCR3DV TS2 as the starting oligonucleotide.

2.2.2.4 Bead based system

The solid support (Streptavidine beads) was used so that the washing steps could be introduced to get rid of the not hybridized and not ligated oligonucleotides. The pre-ligated molecules were constructed using the same protocol as given in section 1.2.1.3 step 1.

PROTOCOL:

1. R3DVBioHeBS-BS (1.5 µM) and 1X T4 Ligase buffer (New England Biolabs) was added to the Dynabeads that were washed with 1X T4 ligase buffer (New England Biolabs) and incubated on a shaker at 37° C for 30 min.
2. The unbound R3DVBioHeBS-BS was washed using a magnet and an additional 1X T4 ligase buffer wash.
3. The beads were re-suspended in 1X T4 ligase buffer, pre- ligated (rCR3DVBS + R3DV HeBS-BS) (1 µM) and 0.2 U/µl T4 DNA ligase and incubate on the shaker at 37° C for 120 min.
4. The excess molecules were again washed on the magnet using 1X T4 ligase buffer and re-suspended in pre-ligated (rCR3DVTS2+ rCR3DVBS + R3 DV HeTS2-BS) (1 µM), 0.2 U/µl T4 DNA ligase and 1x T4 ligase buffer and incubated at 37° C for 120 min on the shaker.
5. The washing steps were again repeated same as above and the beads were then suspended in R3DVCirc new (1.5 µM) and incubated on the shaker at 37° C for 60 min.
6. The cup was incubated on the shaker at 37° C for 60 min after the washing steps and re-suspension in pre ligated (rCR3DVTS1+ rCR3DVBS + R3 DV HeTS1-BS) (1 µM) and 0.2 U/µl T4 DNA ligase.
7. After a final wash with 1X T4 ligase buffer the beads were suspended in 20 µl double distilled water.
8. The cup was heated at 95° C for 5 min and was immediately cooled on ice and the supernatant was collected and stored at 4° C for further use.

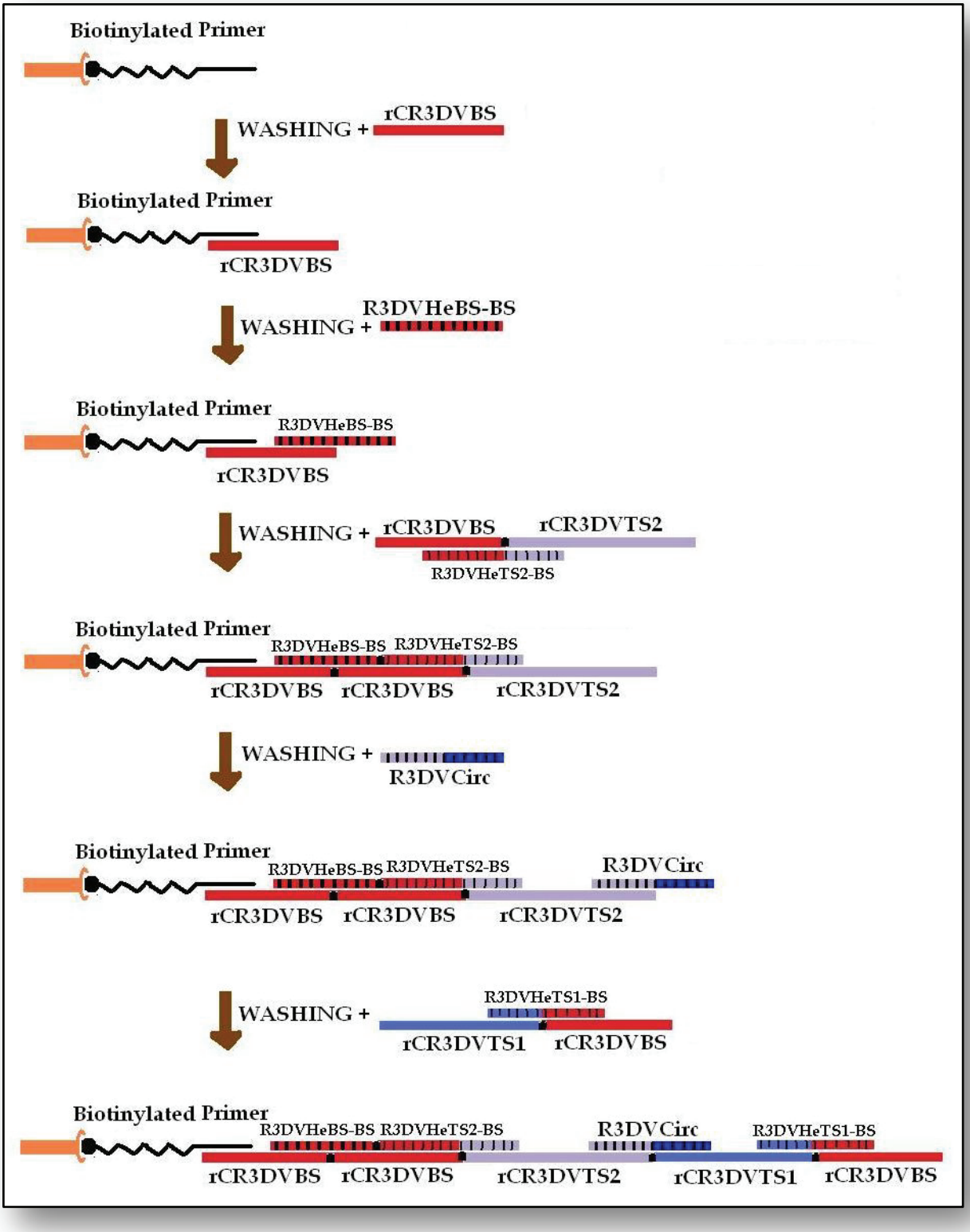


Figure 2.7 Schematic representation of the bead based method

2.2.2.5 Pre-ligation based system

This method was used to construct longer molecules. First all the molecules were added together and different variation in the conditions was done (for more details see Appendix- A1.3)

PROTOCOL:

The protocol that was finally followed and standardized was: The pre- ligated molecules were constructed as given in section 1.2.1.3 step 1. Pre-ligated (rCR3DVTS1+ rCR3DVBS + R3 DV HeTS1-BS) (1 µM), (rCR3DVTS2+ rCR3DVBS + R3 DV HeTS2-BS) (1 µM) and (rCR3DVBS + R3 DV HeBS-BS) (1 µM) were mixed either in the ratio 1:1:2, 1:1:1.2 and 1:1:1 with 1X T4 ligase buffer (New England Biolabs) and 0.2 U/µl T4 ligase (New England Biolabs) and incubated at 37° C for 2 hrs and followed by enzyme inactivation at 65° C for 10 min or in the ratio 1:1:2 with 1 X Ampligase buffer (Epicentre® Biotechnologies) and 0.2 U/µl Ampligase (Epicentre® Biotechnologies) incubated as shown in Table 2.9

Table 2.9 Temperature profile for construction using Pre- ligated method with Ampligase

TEMPERATURE	TIME	
90° C	4 min	
90° C	30 sec	9 X
40° C	3 min	
45° C	3 min	
50° C	3 min	
55° C	3 min	
60° C	3 min	

2.2.3 Amplification assays

In order to have increased concentration different amplification assays were followed. The methods that were followed are listed below with detailed protocol.

2.2.3.1 Rolling Circle amplification (RCA)

The rolling circle amplification is divided into two parts; firstly, the circularization of the target molecule and secondly, the continuous amplification of the target (Fire et al., 1995).

2.2.3.1.1Circularization of the target

A helper oligonucleotide was added in the molar excess of 1:1.5 compared to the starting template to be circularized. The amount of ligase needed of the circularization reaction, and the time required for the complete circularization reaction, was the crucial point in this reaction. So a series of tests were performed with. First the general protocol pursued is mentioned followed by the variations that were introduced for the standardization of the reaction.

PROTOCOL:

1. **Standard Method:**
   a. Target molecule (70 nM), Circularization helper molecule (R3DVCirc new or R1/2DVCirc) (125 nM), DTT (10 mM) (Fermentas), 1X T4 DNA ligase reaction buffer, and water were mixed to give a final system volume of 100 µl and heated to 65° C for 10 min and slowly cooled to 30° C.
   b. 0.5 U/µl of T4 DNA ligase was then added to the mixture and incubated at 16° C for 12 hrs and heat inactivated at 65° C for 10 min.
2. **Varying concentration of ligase in a reaction for a definite time at a definite temperature:**
   Five varying concentrations of ligase (0.1 U/µl- 2 U/µl) were used for the reaction. Rest of the protocol was as in the standard method.
3. **Different incubation temperatures with constant ligase concentration and constant incubation time:**

The reaction was carried out at three different temperatures i.e. at 16˚ C, R.T., and at 37˚ C. for 12 hours and the concentration of ligase was kept constant at 0.5 U/µl. Rest of the protocol was as in the standard method.

4. **Different time intervals of the reaction with a constant concentration of ligase and a definite temperature:**

   Here the ligase concentration was kept constant at 0.5 U/µl and an aliquot was collected after an interval of every 3 hours, for 12 hours. Rest of the protocol was as in the standard method.

2.2.3.1.2 Amplification of the circularized target:

PROTOCOL:

1. **Standard Method:**
   a. The circularized target (47.14 ng/µl) was mixed with 1X Phi29 DNA polymerase buffer (Fermentas) and water. The cup was heated to 80° C for 3 min and then slowly cooled to 30° C.
   b. 10 mM DTT (Fermentas), 5 mM dNTP (Molzym), and 0.4 U/µl of Phi29 DNA polymerase (Fermentas) were added to the cup and the system volume was adjusted to 100 µl and incubated at 37° C for 5 hrs and 65° C for 15 min.
2. **Varying the time of the reaction:**

   Here the reaction was carried out at 37^0 C using 0.4 U/µl enzyme concentration and aliquots were removed at an interval of 1 hour for 12 hours. Rest of the protocol was as in the standard method.
3. **Varying the temperature of the reaction:**

   Two different incubation temperatures, 16^0 C and 37^0 C, were tested at 0.4 U/µl enzyme concentration for 5 hours. Rest of the protocol was as in the standard method.

2.2.3.2 Polymerase chain reaction (PCR)

Polymerase chain reaction was used as an intermediate step to amplify the constructed molecules. The cycles which were selected were based on the Yap

McGee Method (Yap et al., 1991). The protocol followed varied a bit depending on its purpose and are mentioned in the protocols below:

PRIMERS:

1. Primer 1 (R3DV LPrimer)
2. Primer2 (rCR3DV Rprimer)

PCR MIX:

Table 2.10 PCR reaction profile

CONTENTS	CONCENTRATIONS	
	GIVEN	REQUIRED
PCR Buffer	10 x	1 x
dNTP's	10 mM	0,4 mM
Primer 1	100 µM	0,4 µM
Primer 2	100 µM	0,4 µM
$MgCl_2$	100 mM	1 mM
Taq Polymerase (Molzym)	5 U/µl	0,02 U/µl
H_2O	Adjusted so that the final system volume was 50 µl	
Sample	5 ng/µl	0.2ng/µl
For PCR amplification of the constructed molecule 0.5µl of the sample was used.		

PCR CYCLE:

Table 2.11 PCR temperature profile for Target template amplification

STEP NO.	TEMPERATURE	TIME
1	94.0° C	1 min
2	94.0° C	1 min
3	53.9° C	30 sec
4	72.0° C	28 sec
5	Go to 2, 29 times	
6	72.0° C	10 min

Table 2.12 PCR temperature profile for amplifying the constructed molecule

STEP NO.	TEMPERATURE	TIME
1	94.0° C	1 min
2	94.0° C	1 min
3	53.2° C	30 sec
4	72.0° C	11 sec
5	Go to 2, 9 times	
6	88.0° C	1 min
7	53.2° C	30 sec
8	72.0° C	11 sec
9	Go to 6, 29 times	
10	72.0° C	10 min

Annealing temperatures varied from 53.2° C to 55° C amplifying the constructed molecule

2.2.3.2.1 Purification of Polymerase chain reaction products (PCR)

Qiagen QIAquick® PCR Purification Kit was used to purify the PCR products. The protocol given by the supplier was followed exactly. The purified product was eluted in 30µl of double distilled water.

2.2.4 Restriction digestion assays

2.2.4.1 BtgZI digestion

The rolling circle products were cleaved using BtgZI enzyme. The BtgZI enzyme, first isolated by New England Biolabs (United States Patent 7029900, issued on April 2006) is known to efficiently cleave double stranded DNA asymmetrically on the same side after 10 bases of the 3′ end and after 14 bases of the 5′ end. Different helper molecules were used to facilitate cleavage. The design of the stem-loop helper was based on the design published by Podhajska (Podhajska et al., 1985)

where the stem-loop helper was used for digesting double stranded DNA with FokI enzyme. The protocols of the reaction using each of the probes are given below.

PROTOCOL:

1. **Using helper oligonucleotide**

   In a 20 µl reaction mix, 1 X NEBuffer 4 (New England Biolabs), rolling circle product (8 µl), and helper oligonucleotide (rCR1DVRF BtgZI) (2 µM) and distilled water were mixed. The cup was heated to 90^0 C for 3 min and then slowly cooled down to R.T. at the rate of 1^0 C/minute. The cups were briefly mixed after the addition of BtgZI enzyme (0.1 U/µl) (New England Biolabs) and were incubated for 120 min at 60^0 C. was and the enzyme was inactivated at 80^0C for 20 min. The samples were cooled to 4^0 C and observed on agarose gel.

2. **Using stem-loop helper oligonucleotide**

   In 20 µl reaction mix 1 X NEBuffer 4 with rolling circle product (8 µl), and Stem loop helper (rCR1 DV RF Stem-loop) (2 µM) and distilled water were mixed. Rest of the incubation profile was similar to the one followed above in **Using helper oligonucleotide**

3. **Without helper molecule**

   1 X NEBuffer 4 along with rolling circle product (10µl) and BtgZI enzyme (0.08 U/µl) was mixed and the system volume was adjusted to 25 µl with distilled water. The mixture was incubated at 60° C for either 4 hrs or 12 hrs and the enzyme was heat inactivated at 80° C for 20 min.

4. **Alternate protocol**

   Alternatively, the cup with the reaction mixture, 1 X NEBuffer 4 with rolling circle product (10 µl), BtgZI enzyme (0.08 U/µl) and the system volume adjusted to 25 µl with distilled water, was heated to 68^0 C for 1 min and then slowly cooled down to R.T. at the rate of 1^0 C/minute. The cups were incubated for 12 hrs at 60^0 C and at 80^0 C for 20 min.

2.2.4.2 GsuI digestion

The GsuI enzyme was also used to cleave rolling circle product and compare its activity with BtgZI enzyme. GsuI enzyme (Fermentas 2000-2001 Catalog, Product No. ER0461/ER0462) is known to efficiently cleave double stranded DNA asymmetrically on the same side after 16 bases of the 3′ end and after 14 bases of the 5′ end.

PROTOCOL:

1. **Using helper oligonucleotide**

   1 X Buffer B (Fermentas), rolling circle product (2 µl) and helper oligonucleotide (rCR1 DV RF GsuI) (4 µM) were mixed and the system volume was adjusted to 20 µl with distilled water. After heating the cup to 90^0 C for 3 min and then slowly cooling to RT, GsuI enzyme (0.25 U/µl) (Fermentas) was added and was incubated for 12 hrs at 30^0 C. The enzyme was inactivated at 65^0 C for 20 min.

2. **Using stem-loop helper oligonucleotide**

   1 X Buffer B, rolling circle product (8 µl) and Stem loop helper (rCR1 DV RF Stem-loop) (4 µM) were mixed and the system volume was adjusted to 20 µl with distilled water. The temperature profile was similar to the one above in 'Using helper oligonucleotide with GsuI enzyme'.

3. **Without helper molecule**

   In 25 µl system volume, 1 X Buffer B, rolling circle product (10 µl), GsuI enzyme (0.20 U/µl) and distilled water were mixed. The mixture was incubated at 30° C for 12 hrs and at 65° C for 20 min.

2.2.4.3 Deoxyribozyme digestion

The protocol for digestion using deoxyribozymes was based on the protocol by (Nir Carmi. Et al., 1996)

PROTOCOL:

According to the protocol described by Nir Carmi 1996 the deoyribozymes were incubated with the RCA products and analysed on a gel.

HEPES (0.05 M), NaCl (0.3 M) and Deoxyribozyme (250 nM) were added and the volume was adjusted with water to 100 µl. After the reaction mix was heated to 90° C for 1 min, $CuCl_2$ (0.01 mM), RCA product (1:10 diluted) were immediately added and incubated at 23^0 C for 20 min. The reaction was then immediately stopped and run on an agarose gel.

2.2.4.4 BspTI enzyme

PROTOCOL:

1. **Using short and long helper oligonucleotide**

   For facilitating cleavage a partial double strand was created by the addition of a short and long helper molecule. 1:10 diluted rolling circle product (37.5 µl), 1 X Buffer O (Fermentas), and short helper oligonucleotide (rCR1DV RF new) (5 µM) or long helper oligonucleotide (rCR1DV RFelong) (5 µM) were heated to 90° C for 3 minutes and cooled to 30° C. 1 U/µl of BspTI enzyme (Fermentas) was added and incubated at 37° C for 2 hrs and the enzyme was inactivated at 65° C for 20 min.

2.2.5 Detection systems

2.2.5.1 Gel based detection

2.2.5.1.1 Agarose gel electrophoresis

PROTOCOL:

Agarose gel was the key detecting method used for analysing the constructed products, amplified products and digested products based on their molecular weight. 2 µl of loading dye with generally 6 µl or 8µl (for products of BtgZI digestion) of the samples were loaded on the gel. For rolling circle products, 1 µl loading dye with 4 µl sample was loaded on the gel. 1 µl of required ladder along with 1µl loading dye (6 X) and 4 µl water were mixed and added to the gel for predicting the approximate run of the test samples. Either 2 % agarose

gel in 1 X TBE buffer (most of the gels), 3 % of agarose gel 1 X TBE for better resolution (separation) of the fragments (Southern blotting), or 1 % agarose gel in TAE buffer (some gel extraction protocols) were placed in a chamber filled either with 1 X TBE or 1 X TAE buffer depending on the gel being used and was run for 60 – 70 min (approximately 3/4th its running length) at 100 volts and stained in either Ethidium bromide (Franklin and Locker, 1981) (more specific for double stranded DNA), or SYBR Green I (Zipper, H. *et al.*, 2004) (more specific for double stranded DNA and more sensitive than Ethidium bromide) or SYBR Green II (Schmidt et al., 1997) (more specific for single stranded DNA) depending on the product being analysed for 45 min and then viewed under UV light. A picture of the gel was taken using the Canon Powershot camera attached to the gel documentation system.

2.2.5.1.2 Polyacrylamide gel electrophoresis

PROTOCOL:

For high resolution of some products polyacrylamide gels with and without 0.8 M urea were used as described in Table 2.12. Before loading with special care the urea containing gels according to Table 2.13 and 2.14, samples were heated at 80°C for 10 min. on a shaker to prevent secondary structures. Gels running with urea at 220 V for 25 min. and without at 100 V and 60 min respectively, were stained in 1:10000 X diluted SYBR Green I or SYBR Green II solutions for 45 min.

Table 2.13 Composition of the polyacrylamide gels with and without urea

REAGENTS	I (with urea)	II (without urea)
system volume (µl)	**10**	**10**
Acrylamide (40%) (µl)	5000	5000
bis- acrylamide (2%) (µl)	850	850
TBE (10X) (µl)	1000	1000
Urea (g)	4.8	–
APS (10 %) (µl)	100	100
TEMED (µl)	10	10

Table 2.14 Loading profile for PAGE without urea

PAGE without urea	1	2
Sample	Ultra Low ladder	Sample
Amount (µl)	2	8
Loading Dye (µl)	5	5

Table 2.15 Loading profile for PAGE with urea

PAGE with urea	1	2
Sample	Ultra Low ladder	Sample
Amount (µl)	2	8
T.E. (µl)	8	2
Loading Dye with urea (µl)	5	5

2.2.5.1.3 Gel extraction

PROTOCOL:

For extraction of the desired band from agarose gel, seven different kits were tested. The protocols followed were exactly according to the manual given by the manufacturer. Another gel extraction method that was followed for agarose gel extraction was the "Freeze Squeeze" method (Clare L Mckay, 2006). The protocol is as given below:

1. Agarose gel was run and the desired band was excised and frozen for 24hrs.
2. The frozen gel piece was then placed in a parafilm, whose two sides were folded. The gel piece was trapped in the closed right angle of the pocket and gently squished.
3. The resulting liquid was collected and sodium acetate ($1/10^{th}$ the volume of extracted liquid) and 70 % ethanol (twice the volume of the extracted liquid were added and centrifuged at 14000 rpm for 5 minutes.
4. The supernatant was carefully discarded and the pellet was air dried and resuspended in 20 µl ddH_2O.

Alternatively for polyacrylamide gels the following protocol was followed:

1. 50 µl of double distilled water was added to the piece of gel that was cut and placed on a shaker at 25° C for 10 min.
2. The cup was centrifuged at 13,200 rpm for two minutes and the supernatant was carefully removed, collected and stored at 4° C till further use.

2 µl of each sample was used for the measurement on the Nanodrop (spectrophotometer) to check the concentration after gel extraction. Blank was set using 2 µl of ddH_2O.

2.2.5.2 Southern blot

PROTOCOL:

A. Gel:

3 % TBE Gel was run and stained using SYBR Green II and was cut to get the required size of 5, 5 * 8 cm and denatured in denaturation buffer for 20 min at RT and then in neutralization buffer for 20 min at RT.

B. Blotting:

On the glass trough filled with 1 Litre 20 x SSC, was placed a big glass slide. Whatman paper was laid on the glass slide and both its front and back ends (lengthwise) were dipped into the SSC buffer in the glass trough. On it was placed a small set of wet Whatman paper, wet in SSC buffer. The denatured and neutralized gel was inverted on it and the membrane was carefully placed on it, followed by the bigger set of wet Whatman paper, a bunch of cut „Green tissues“ and glass slides to add weight for facilitating diffusion. This whole set up was placed at 4° C overnight.

C. Crosslinking:

The Membrane was carefully recovered and dried for a few minutes, before the controls were loaded on the very bottom and the top of the Membrane. 1 µl of rCRDV TS1, rCRDV TS2, and rCRDV BS were added as control from the 1 µM given stock added to the membrane at the very bottom and the three DIG labelled probes (10 µM) were added to the membrane at the very top along with 2 µl of denaturating solution

on each of the controls and dried for sometime, before treating it with 400 mJ/m^2 UV Light (254 nm) in the UV chamber.

D. Hybridization of the Probe:

The Membrane was then soaked in 4 x SET+10 x Denhardt's Solution for 1 hour on the shaker at 50° C for pre-hybridization, before being cut to its respective size before hybridization. To the three different three side sealed cover each containing a cut membrane, 4 x SET+ 10 x Denhardt's Solution with 10 pmol /ml of respective Digoxigenin labelled probe (1 µl probe in 10 ml solution) was added, and the fourth side of the covers were sealed, and the covers were incubated on the shaker 50 °C for 3 hours for hybridization.

E. Washing after Hybridization:

The cut membranes were washed separately 4 times each in the 3 x SET+ 0.1 % SDS solution for 5 minutes (each wash) on a shaker followed by a short washing step at RT in DIG P1 buffer Using new DIG P1 buffer the membranes were re-washed at RT for 15 min and then transferred to a new box and incubated with DIG P2 for 30 minutes at RT for blocking.

F. Binding of the Antigen (Anti-Digoxigenin-AP- Conjugate):

6 µl of the supernatant of the centrifuged Anti-Digoxigenin-AP-Conjugate (1:5000 dilution) (centrifuged at highest speed for 5minutes) was mixed with 30 ml of DIG P2 buffer before adding 10 µl of it to each of the cut membrane in a sealed cover and incubating it on a shaker at RT for 30 min.

G. Washing after Antigen binding:

Membrane was then washed two times in DIG P1 solution for 15 minutes each, at RT on a shaker and then equilibrated in DIG P3 solution for 2 minutes at RT on a shaker in a new box.

H. Colour Reaction:

The Membrane was sealed in the covers along with 5/8 ml of BCIP/NBT and was incubated at RT on the shaker till the generation of colour, and lastly it was rinsed in ddH_2O and dried and before being scanned.

2.2.5.3 Sequencing and Purification

2.2.5.3.1 Sequencing

PROTOCOL:

Sequencing reaction was carried out based on the Sanger's method of sequencing (Sanger et al. 1977), on the ABI Prism310 (Applied Biosystems, Foster City, USA) sequencer. The sequencing reaction was carried out by the manufacturer's protocol (Rosenblum et al. 1997).

Since the material to be sequenced was single stranded padlocks, it was very difficult to get sequences, hence rolling circle products were used as starting material for sequencing. As the rolling circle products were extremely concentrated, it was diluted 1:10 and 2 µl of the diluted product was used as template in contrast to the 6 µl of the restricted digested rolling circle product when used as template. The program of the cycler was set for 25 cycles to 95^0 C for 15 sec, 50^0 C for 15 sec and 60^0 C for 240 sec. The samples were then purified using the sodium acetate precipitation method before adding the samples into the sequencer.

2.2.5.3.2 Purification

PROTOCOL:

Purification was carried out using the sodium acetate precipitation method (Shapiro, 1981).

1. A mixture of Sodium acetate (3 M) (4 µl) (Merck), absolute ethanol (100 µl) (Riedel-de- Haen) and water (30µl) was added to each of the samples and centrifuged for 20 min at RT at 13500 rpm.
2. After discarding the supernatant by suction, 1000 µl of 70% ethanol (freshly prepared) was added to each of the cups and centrifuged for 10 min at RT at 13500 rpm.
3. The supernatant was discarded by suction, and the pellets were air dried and hydrated with 30 µl distilled water and sequenced.

2.2.5.4 Sequence editing

The sequence chromatograms that were obtained were checked on Chromas (McCarthy, 1998) and SeqMAN II (DNA Star Inc. 2000). The sequences were then aligned with the expected sequence using Genedoc software (Nicolas, 1997).

2.2.6 Exonuclease digestions

To get rid of the unwanted strand and reduce the complexity of the system, Exonuclease I and Exonuclease III enzymes were used. Both these enzymes were used in conjunction.

PROTOCOL:

Exonuclease digestion was generally done after the construction protocols to reduce the complexity of the unwanted products formed, before proceeding to the next steps. The reaction mixture contained 1 X exonuclease III buffer (Fermentas), 0.5 U/µl exonuclease I (Fermentas), 2.5 U/µl exonuclease III (Fermentas) and the samples were incubated at 37° C for 2 hrs and the enzymes were denatured at 85° C for 25 min.

3 RESULTS

3.1 Construction of 120 bases padlocks

The aim was to develop a method for constructing high concentration of phosphorylated 120 bases padlocks, having the required sequences especially at the 5′ and 3′ ends, overcoming existing limitations in the currently available methods. The main approach was based on the gene synthesis method for getting padlocks with the precise sequence. To get higher concentration of the phosphorylated padlocks, rolling circle amplification and restriction digestion was used. Rolling circle amplification and restriction digestion were also a proof of the desired padlocks being constructed. The sequences were designed in such a way, that only when both the target specific sequences were present, circularization reaction and hence rolling circle amplification would occur. Restriction digestion was also another checkpoint, as only those amplified constructed padlocks would be cleaved to give functional padlocks, which had both the target specific regions incorporated in them.

3.1.1 Construction methods

3.1.1.1 Ligation and polymerization based system

The construction of 120 bases product was done using a fill- in reaction. Different conditions were varied to avoid the occurrence of undesirable products. Aliquots of the different stages of the construction were run on a 1.8 % agarose gel as shown in Figure 3.1. The gel picture below is of the standardized protocol for the construction of 120 bases. In between each step there were not much differences seen, except in the lane 4 and lane 5, where it was clearly seen that most of the material was digested, hinting towards the amount of undesired products that get formed. Due to the presence of PTO modification (Stein et al., 1988) incorporated in

the padlock, during construction; it was resistant to exonuclease digestion. As the products formed after each step of construction were partially double stranded, their running behaviour cannot be directly compared to the available double stranded marker. Probably the upper band observed below the 200 bp mark of the ladder might be the desired 120 bases of padlock in the intermediate steps, and the lower band observed below the 75 bp mark of the 1 kb ladder might be the excess oligonucleotides that did not hybridize or ligate to give the desired product.

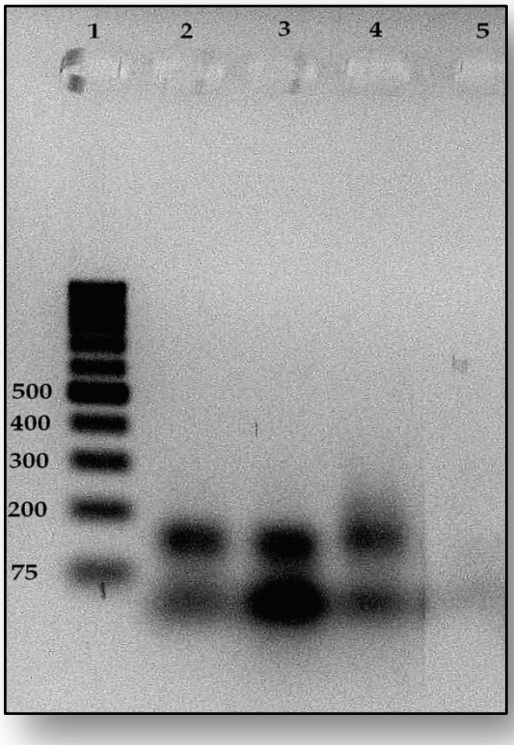
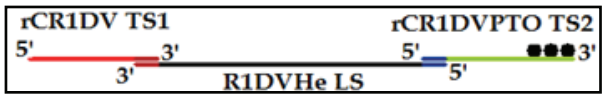


Figure 3.1 Different stages during the construction of 120 bases padlocks.

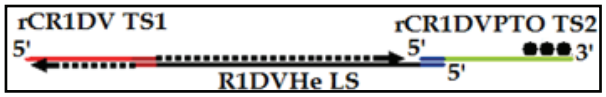
KEY:

1. 1 kb plus ladder

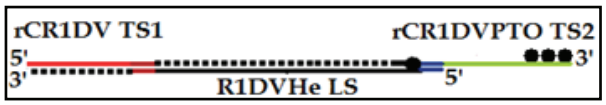
2. rCR1DV TS1 + rCR1DV PTO TS2 + R1DVHe LS hybridized together. (STEP 1)



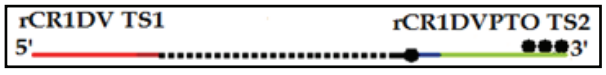
3. Strand extension using T4 DNA polymerase. (STEP2)



4. Ligation of the extended strand using T4 ligase. (STEP 3)



5. Exonuclease digestion of the second strand. (STEP 4)



3.1.2 Amplification

In order to select and increase the concentration of the desired probes, amplification of the constructed padlocks was carried out. Before the amplification process, the

probes were circularized using that standardized conditions (see material and methods; section 2.2.3.1.1- Standard protocol, page 45). Since a diluted concentration of the constructed probe was needed for the circularization reaction, the circularized product when checked on agarose gel showed no band. When this circularized product was concentrated, it could be easily viewed on the agarose gel. For most of the experiments the circularization reaction was not confirmed on the agarose gel and was directly proceeded by rolling circle amplification reaction. Rolling circle amplification was also carried out using the standardized protocol (see materials and methods; section 2.2.3.1.2- Standard protocol page 45). The protocol was extremely robust and hence even extremely diluted circular molecules gave products much longer than 10000 bases. The product was in such a large concentration even after 5 hrs of incubation, that the product hardly came out of the well as observed in the lane 2 in Figure 3.2.

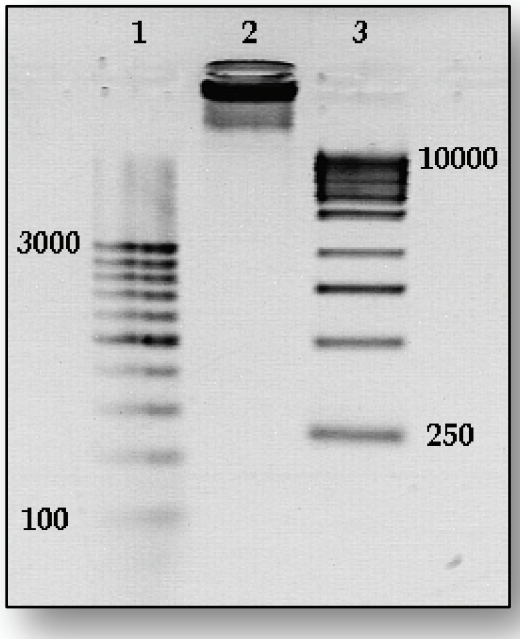


Figure 3.2 Rolling circle product of the constructed probe as a starting material.

KEY: 1- 100 base pair ladder; 2- Rolling circle product of the circularized constructed 120 bases padlock; 3- 1 kb ladder

3.1.3 Generation of functional probes

Different methods for cleaving the rolling circle product were worked with, in order to get the finest method for getting functional probes, without complicated procedures. The results below are of the procedures that were only tried for 120 bases padlocks.

3.1.3.1 Using a helper molecule for assisting digestion with restriction enzymes

Two different helper molecules, one short (rCR1DVRFnew) and the other longer (rCR1DVRFelong), were tested, as the literature about the enzyme BspTI gave no information about the number of base pairs of double strand required for the enzyme to stick and cleave (Janulaitis., unpublished observations). Restriction enzyme BspTI was used for digesting the RCA product, as this was the only enzyme that had its recognition site in the target specific region of the padlock which was being constructed. As the restriction digestion helper molecules were added in excess to achieve complete digestion of the rolling circle product, after digestion the excess un-reacted helper molecules were seen below the 80 bp mark of the 100 bp ladder on the agarose gel. The lane in which the longer helper

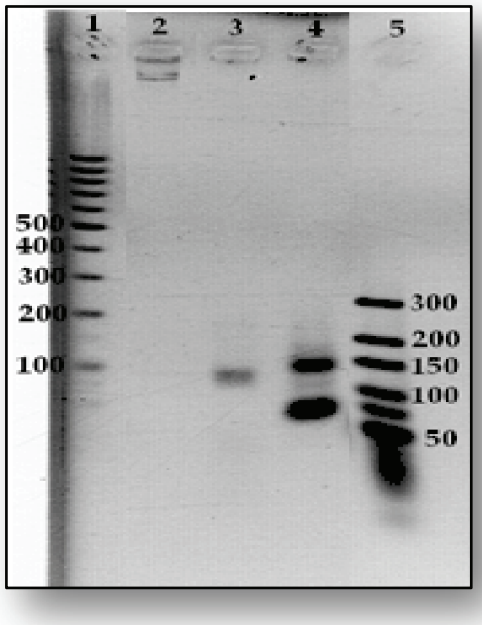


Figure 3.3 Restriction digestion of the rolling circle product using BspTI enzyme

KEY: 1- 100bp Ladder; 2- 1:10 diluted RCA product; 3- Restriction digestion with the aid of 20 bp restriction fragment, rCR1DVRFnew; 4- Restriction digestion with the aid of 40 bp restriction fragment, rCR1DVRFelong; 5- Ultra low ladder

molecules were added to the rolling circle product, stronger bands were seen below 80 bp mark of the ladder as seen in lane 4 of Figure 3.3. Also in Figure 3.3, the expected band (of 120 bases) was observed in lane 3 and 4 just above the 100 bp mark of the ladder, indicating the presence of the desired product. The main disadvantage of using the BspTI was that the recognition site of this enzyme needs

to be at the ends of the desired constructed padlocks, so that during construction, the rolling circle product can be cleaved to give functional padlocks. This also restricts the choice of the target sequence.

As restriction enzymes BtgZI and GsuI do not have the recognition and the cleavage site at the same point, but instead cleavage few bases away from its recognition site, these enzymes could be used for cleaving the rolling circle product to give the desired padlock. The rolling circle product was cleaved with BtgZI enzyme using a short helper restriction fragment (rCR1DVRFBtgZI) (material and methods section 2.2.4.1- using helper oligonucleotide, page- 48). The Figure 3.4 shows that, when only the rolling circle product along with the helper restriction fragment were incubated in the absence of enzyme and run on the gel in the lanes 4, 7 and 10, no cleavage was observed indicating that the enzyme was required and

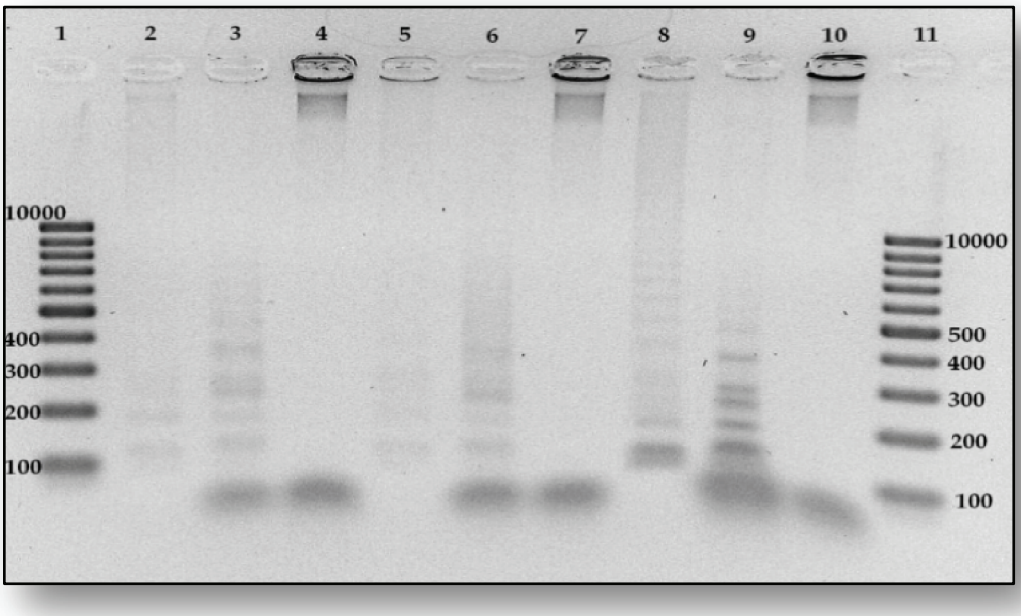


Figure 3.4 Restriction digestion of the rolling circle product along with the helper molecule for varying time intervals between 30 min - 90 min.

KEY: 1 –100 bp ladder; 2 – RCA product + BtgZI after 30 min digestion ; 3 - RCA product + BtgZI + Restriction fragment (rCR1DVRFBtgZI) after 30 min digestion ; 4 - RCA product + Restriction fragment (rCR1DVRFBtgZI) after 30 min digestion ; 5 - RCA product + BtgZI after 60 min digestion ; 6 – RCA product + BtgZI + Restriction fragment (rCR1DVRFBtgZI) after 60 min digestion; 7 - RCA product + Restriction fragment (rCR1DVRFBtgZI) after 60 min digestion ; 8 – RCA product + BtgZI after 90 min digestion; 9 – RCA product + BtgZI + Restriction fragment (rCR1DVRFBtgZI) after 90 min digestion; 10 - RCA product + Restriction fragment (rCR1DVRFBtgZI) after 90 min digestion; 11 – 100 bp ladder

no other contents of the reaction could cleave the rolling circle product. The lanes 3, 6, and 9, which contained the products after 30 min, 60 min and 90 min of incubation respectively with the restriction fragment (rCR1DVRFBtgZI), the RCA product and enzyme, showed the desired products along with a laddered structure, indicating incomplete digestion. But in the lanes 2, 5 and 8, where the products of the reaction containing only the rolling circle product and the enzyme were loaded, incomplete digestion was observed too, even after 90 min of incubation.

In Figure 3.5 the gel picture of the reaction after 120 min of digestion, showed completely digested rolling circle in both, lane 2 and lane 3, in the presence and in the absence of restriction fragment (rCR1DVRFBtgZI).

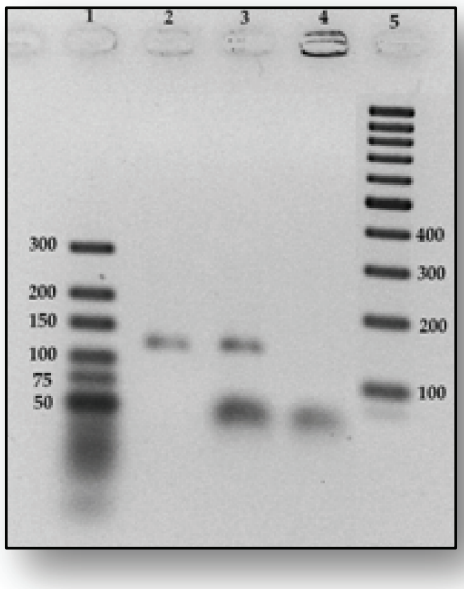


Figure 3.5 Restriction digestion of the rolling circle product along with the helper molecule for 120 min.

KEY: 1 –Ultra low ladder; 2 – RCA product + BtgZI after 120 min digestion; 3 - RCA product + BtgZI + Restriction fragment (rCR1DVRFBtgZI) after 120 min digestion; 4 - RCA product + Restriction fragment (rCR1DVRFBtgZI) after 120 min digestion; 5- 100 bp ladder

When GsuI enzyme was used with the helper molecule (rCR1DVRFGsuI) to cleave the rolling circle product, no cleaved product was observed on the gel even though the reaction was carried out for 12 hrs at 30° C. In contrast to the results observed when the rolling circle product was digested using only BtgZI enzyme and run on the gel, where the desired cleaved product was observed.

Stem-loop helper molecule was used in the view of avoiding the presence of

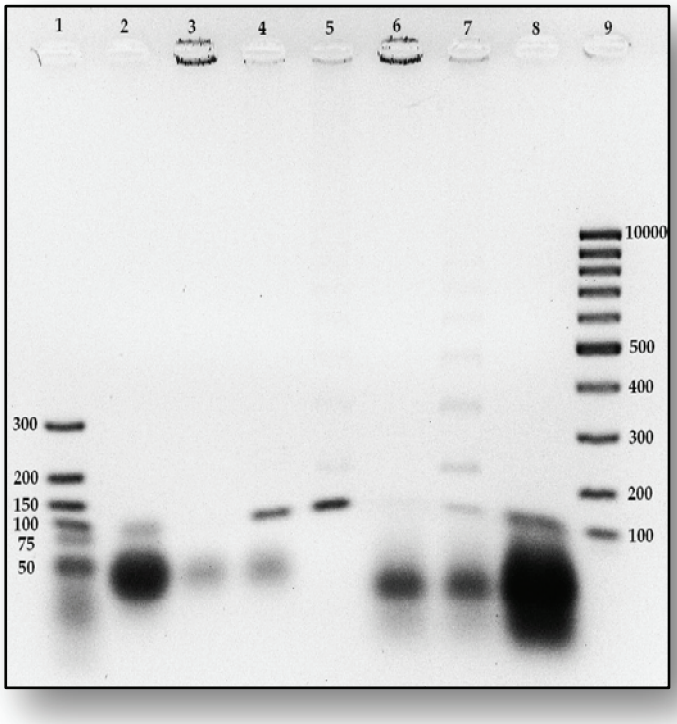


Figure 3.6 Cleaving rolling circle product with the aid of restriction fragment or step-loop restriction fragment.

KEY: 1- Ultra low ladder; 2- Restriction fragment (rCR1DVRFBtgZI); 3- RCA product+ restriction fragment (rCR1DVRFBtgZI); 4- RCA product+ restriction fragment (rCR1DVRFBtgZI) + BtgZI; 5- RCA product+ BtgZI; 6- RCA product+ stem-loop restriction fragment (rCR1DVRFstemloop); 7- RCA product+ stem-loop restriction fragment (rCR1DVRFstemloop) + GsuI; 8- Stem loop restriction fragment (rCR1DVRFstemloop); 9- 100 bp ladder

restriction site anywhere near the target specific regions of the probes for the convenience for construction. GsuI restriction enzyme was used to check the working of the stem-loop restriction fragment. The functionality of GsuI enzyme was checked previously on a double stranded DNA fragment which possessed the recognition site for the enzyme, where complete digestion was observed, and hence the same conditions were used for all the digestion reactions. When the stem-loop restriction fragment (rCR1DVRFstemloop) was used for cleaving the rolling circle product using the GsuI enzyme, as observed in lane 7 in Figure 3.6, a laddered structure was seen indicating incomplete digestion, as these bands match the length of products expected from incomplete digestion. Even though more amount of enzyme was later added and the incubation time was increased, no change in the laddered structure was observed. It also suggests that the GsuI enzyme requires a stretch of double stranded region in the recognition site, as well as in the cleavage site. The digestion of the rolling circle product with the BtgZI enzyme always

showed a distinct desired band on the gels, in presence and also in the absence of the restriction fragment (rCR1DVRFBtgZI). But when the restriction fragments were used for digesting the rolling circle product with BtgZI enzyme, there were two distinct bands observed. The lower band, near the 50 bp mark of the ladder was the excess restriction fragment, added for the reaction. The upper band just above the 100 bp mark of the ladder was of the desired padlocks.

3.1.3.2 Using deoxyribozyme for assisting digestion

A short oligonucleotide which functioned as a deoxyribozyme was used to cleave the 1:10 diluted rolling circle product of the constructed probe. The design of the deoxyribozyme was based on the paper from Nir Crami (Carmi et al., 1998) who for the first time demonstrated the capability of Class II deoxyribozymes to cleave single stranded DNA sequence specifically. Since the concentration was adjusted in each of the control and test samples, such that they have the same dilution factor before running the gel, the intensities of the band can be directly compared. In the Figure 3.7, lane 4 shows slightly and lane 5 show no trace of the rolling circle when compared to lanes 2 and 3 respectively near the top of the lane, indicating that the

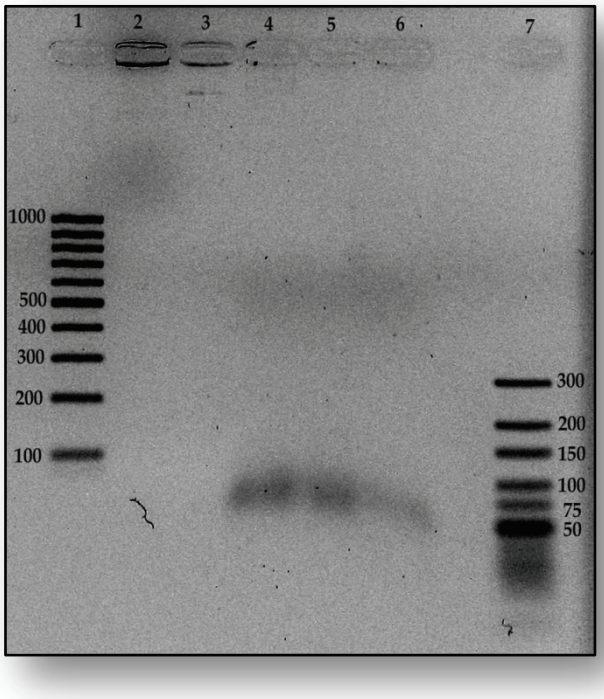


Figure 3.7 Deoxyribozyme cleavage reaction for the cleavage of rolling circle product.

KEY: 1- 100bp ladder; 2- undiluted rolling circle product; 3- 1:10 diluted rolling circle product; 4- Deoxyribozyme with undiluted rolling circle product; 5- deoxyribozyme with 1:10 diluted rolling circle product; 6- only deoxyribozyme (negative control); 7- ultra low ladder

rolling circle product was digested. But at the same time when the lanes 4 and 5 at the bottom were compared with lane 6, which contained only the deoxyribozyme with the buffer, the bands were almost at the same level. The difference observed was the change in the intensity of the bands. Also the cleaved bands were lower than its expected size which should be slightly above the 100 base pair mark of the ladder. But this could be due to various structures formed by the single stranded molecule. In addition, the deoxyribozyme remains attached to the cleaved probe influencing the running behaviour of the cleaved padlocks on agarose gel. The fragments could not be identified as the sequencing reaction failed for these molecules. Also the functionality tests were negative using these probes. These might be due to the deoxyribozymes still attached to probes, prohibiting its functionality by blocking its ends.

3.2 Construction of Longer Probes

The design that was used for constructing 120 bases padlocks was modified and repetitive sequences (binding sites for the next generation of padlocks) were added in the linker segment, so that these padlocks could be used for the Kotenkar-Rühle-Kracht approach, where different layers of padlocks were added, facilitating signal amplification. The Ligation and polymerization based assay and the Continuous ligation based assay were first experimented with. A lot of unpredictable products were generated which could not be interpreted. Also using two enzymes, ligase and polymerase, further complicated the system and made the analysis of the results difficult. To reduce the complexity of the system for easy interpretation of results and to facilitate the production of the desired padlocks, a new system was designed which required the use of only ligase enzyme. In the new design also, binding sites for the next generation of padlocks were incorporated. Since the number of binding sites incorporated during construction in the padlocks was directly proportional to the signal amplification factor, the incorporation of a higher number of binding sites in the constructed padlocks would be desirable. Four different methods,

namely Block based approach, Stepwise based approach, Bead based approach and Pre-ligation based approach were worked on. The different parameters experimented on the block based approach were done with Frank Rühle, some of which gave definite clue about malfunction of this approach. The major hurdle was the formation of distinct circular product of rCR3DVBS- rCR3DVBS using R3DV HeBS-BS during construction, without the incorporation of the target specific oligonucleotides (rCR3DVTS1 and rCR3DVTS2). This product was not digested even after prolonged incubation with exonucleases and gave a rolling circle product which was not cut (as seen in Figure 3.8) (for more details refer appendix A2). Hence the other three methods, Stepwise based approach, Bead based approach and Pre-ligation based approach, were tested simultaneously, to check whether these methods were able to produce the desired padlock with more number of binding sites.

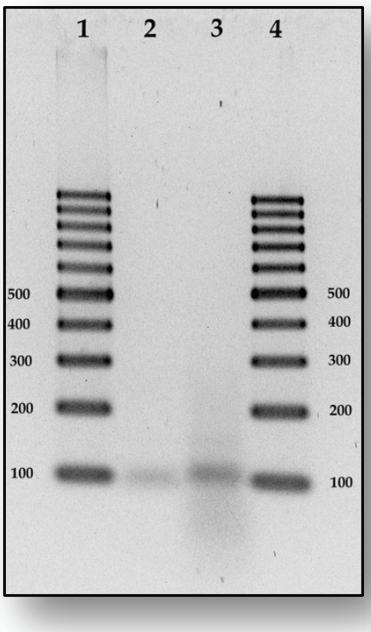


Figure 3.8 Construction of padlocks using the block based method

KEY: 1- 100bp Ladder; 2- Construction using 500nM of 1:1:1:1:1:1 ratio of (rCR3DVTS1+ rCR3DVTS2+ rCR3DVBS+ R3DVHeTS1BS+ R3DVHeTS2BS+ R3DVHeBSBS)+ **with Ampligase(0.01U/µl)** and annealing temp **45^{0}C** treated **with Exonuclease I and III; 3-** Construction using 500nM of 1:1:1:1:1:1 ratio of (rCR3DVTS1+ rCR3DVTS2+ rCR3DVBS+ R3DVHeTS1BS+ R3DVHeTS2BS+ R3DVHeBSBS)+ **with Ampligase(0.01U/µl)** and annealing temp **45^{0}C**; 4- 100bp Ladder

3.2.1 Construction Methods, amplification and restriction digestions

3.2.1.1 Continuous ligation based system

The design for constructing 120 bases probes was slightly modified, as shown in Figure 2.1 for constructing longer probes. Although exactly the same protocol was followed as used for constructing 120 bases probes, the results were not the same. An additional PCR step was introduced to enhance the intensity of the required bands to improve further analysis.

Different ratios of the different fragments rCR1DVTS1, rCR1DVPTOTS2, rCR2DVBS, and R2DVLS were tested to get maximum incorporation of binding sites, and hence longer probes. The gel picture in Figure 3.9 shows the PCR product

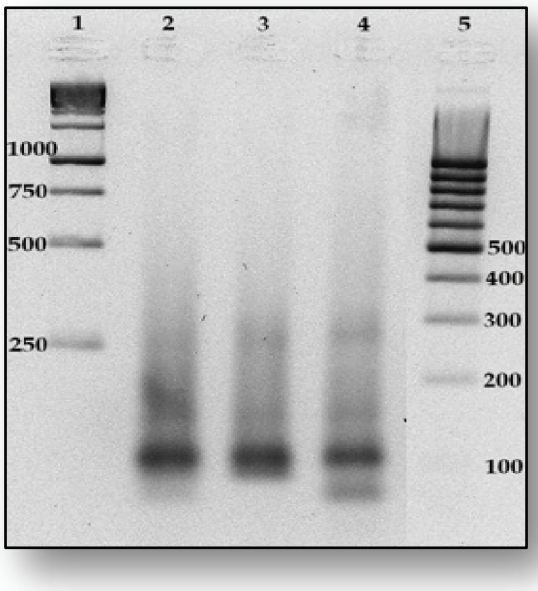


Figure 3.9 Construction of longer probes using varying ratio of the fragments used for construction.

KEY: 1- 1 kb ladder; 2- PCR product of the constructed probe with **1:1:1.5:2** (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio; 3- PCR product of the constructed probe with **1:1:7.5:10** (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio; 4- PCR product of the constructed probe with **3:3:1.5:2** (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio; 5- 100 bp ladder

of constructed products. The second lane consists of PCR product of the construction product carried out with 1:1:1.5:2 (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio, the third lane with 1:1:7.5:10 (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio and the fourth lane with 3:3:1.5:2

(rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio. Each of the lanes showed a slight smear. Amid the smear, there were also few distinct bands observed in the lanes. One band which was common to all the lanes was the one slightly above the 100 bp mark of the ladder.

When the construction was carried out with two different ratios **1:1:1.2:1.5** and **1:1:1:1.2** (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) and three different hybridization conditions were used such as: (a) 37°C 15min, 90°C 3min, 90°C slowly cool to 40°C, 40°C 1min, 80°C 1min, cool to 30°C, (b) 37°C 15min, 90°C 3min, 90°C-20°C in thermocycler 5min/°C and (c) 37°C 15min, 90°C 3min, 90°C-75°C in thermocycler 2min/°C, 75°C-30°C in thermocycler 30min/°C, 30°C-15°C in thermocycler 2min/°C. The gel picture below (Figure 3.10) shows that distinct bands were obtained after PCR. Also the band near the 100 bp mark of the ladder as in the previous in Figure 3.9 was extremely strong.

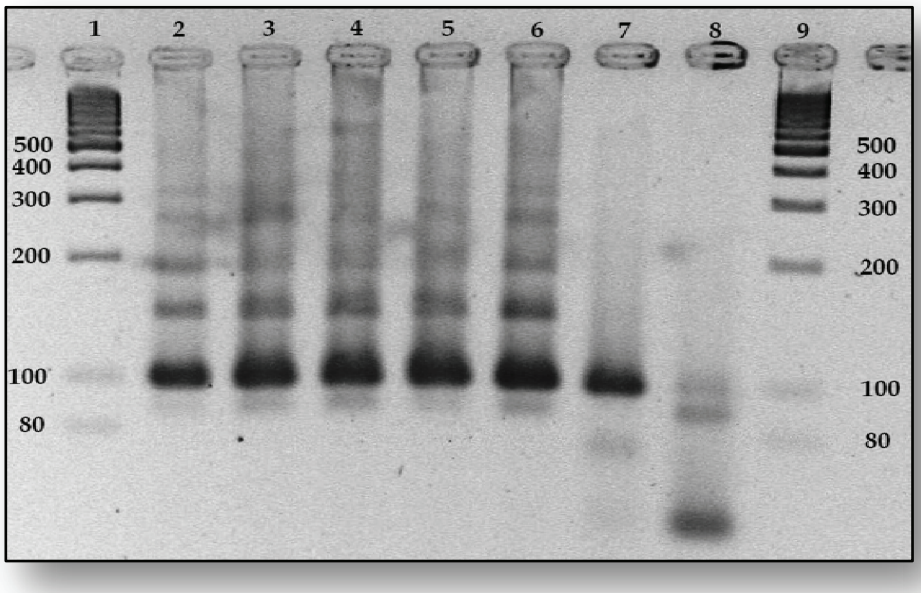


Figure 3.10 Construction of longer probes using varying hybridization conditions and varying ratio of the fragments used for construction.

KEY: 1- 100 bp ladder; 2- PCR product of the constructed probe with **1:1:1.2:1.5** (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio and (a) hybridization condition; 3- PCR product of the constructed probe with **1:1:1.2:1.5** (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio and (b) hybridization condition; 4- PCR product of the constructed probe with **1:1:1.2:1.5** (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio and (c) hybridization condition; 5- PCR product of the constructed probe with **1:1:1:1.2** (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio and (b) hybridization condition; 6- PCR product of the constructed probe with **1:1:1:1.2** (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio and (c) hybridization condition ; 7- all the construction fragments; 8- PCR negative (PCR reaction with water as a template); 9- 100 bp ladder

When these PCR products were analyzed on the agarose gel using two different stains, such as SYBR green I (which preferably stains double stranded DNA) and SYBR green II (which preferably stains single stranded DNA) (as seen in Figure 3.11) to compare the intensities of the bands, the gel pictures clearly showed the presence of single stranded molecules, thus indicating the occurrence of an asymmetric reaction.

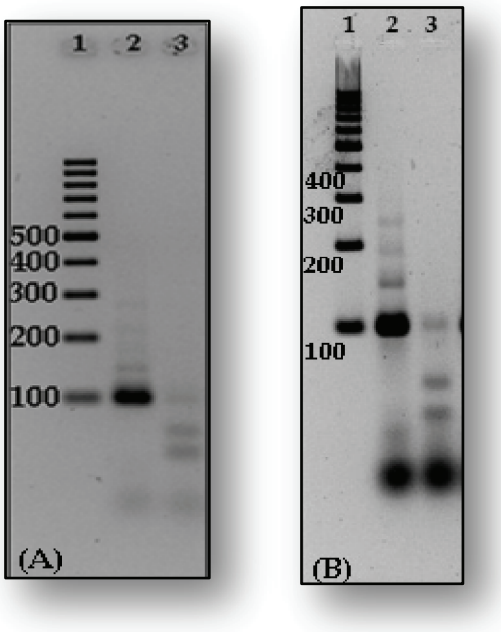


Figure 3.11 Staining of PCR products with more double strand specific SYBR Green I (A) and more single strand specific SYBR Green II (B).

Here just after construction a PCR was run with 2µM concentration of each of the primers and the PCR products were stained using two different stains. (A) Here the SYBR Green I stain was used to stain the PCR products; (B) Here SYBR Green II

Another test was done to prove that asymmetric reaction was going on and that there were more undigested oligonucleotides in the reaction mix after exonucleases digestion of the constructs, which acted as primers along with the two primers added for the PCR amplification to generate single stranded product, was positive. Here the property of exonuclease I to cleave only single stranded DNA from the 3' to 5' end was exploited. After the PCR, the product was digested with exonuclease I and to confirm that there were very few products left, the exonuclease digest was further used as the template for PCR. Disappearance of most of the bands after the exonuclease I digestion of both, the PCR product and the PCR product of the exonuclease I digest, confirmed that there were a lot of single stranded products generated. Due to undigested oligonucleotides from the construction reaction, which acted as primers, these false products were formed (Figure 3.12).

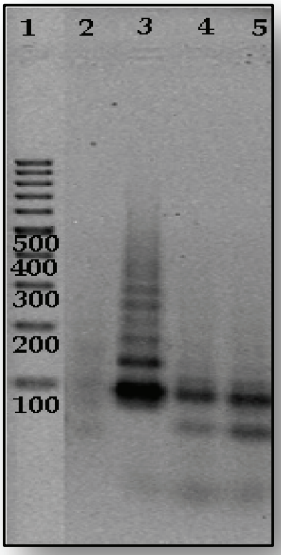


Figure 3.12 PCR conformation test for the formation of false products after construction using continuous ligation method

KEY: 1- 100bp Ladder; 2- Construct after exonuclease III digestion; 3- PCR of the digested constructed padlock; 4- Exonuclease I digestion of the PCR product; 5- PCR product of the exonuclease I digested product.

Drastically varying ratios were used to check the difference in the banding pattern. PCR products of 1:1:20:21 (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio and 20:20:1:21 (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio of constructed probes were viewed on the agarose gel. As seen in Figure 3.13 the smear was not as much as observed in the previous experiments. Also there were distinct and more bands observed.

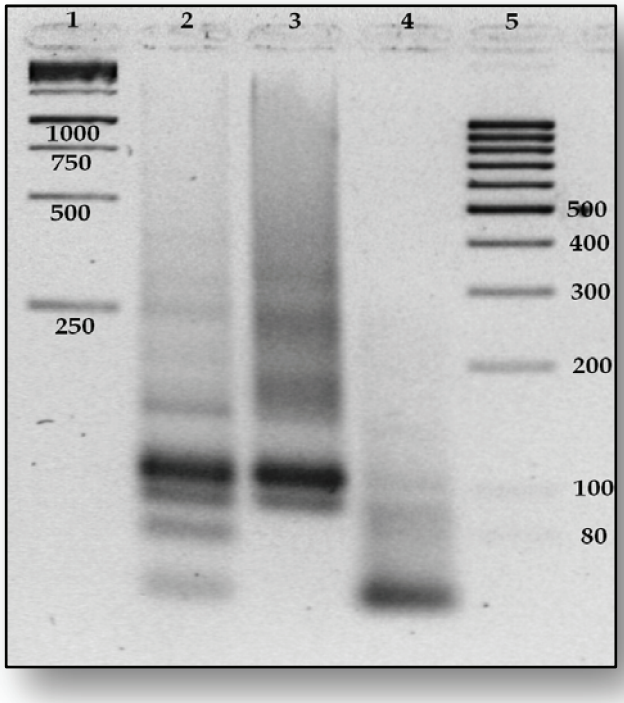


Figure 3.13 Construction of longer probes using drastically varying ratio of the fragments used for construction.

KEY: 1- 1 kb ladder; 2- PCR product of the constructed probe with **1:1:20:21** (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio; 3- PCR product of the constructed probe with **20:20:1:21** (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio; 4- PCR negative; 5- 100 bp ladder

In order to get pure product of a particular size, the bands having a length of more than 100 bp compared to the marker band were cut from lane 2, were extracted using Qiagen gel extraction kit and a re-PCR was carried out. As shown in Figure 3.14 the re-PCR gave only two distinct bands, irrespective of the position from which the band was cut. The negative control also showed the presence of those bands, but the intensity was extremely low compared to the wells which had the sample present in it.

The gel extracts were circularized and amplified using rolling circle amplification using the standardized protocol. But there was no rolling circle product observed and the gel was totally blank. The control reaction showed a product indicating that

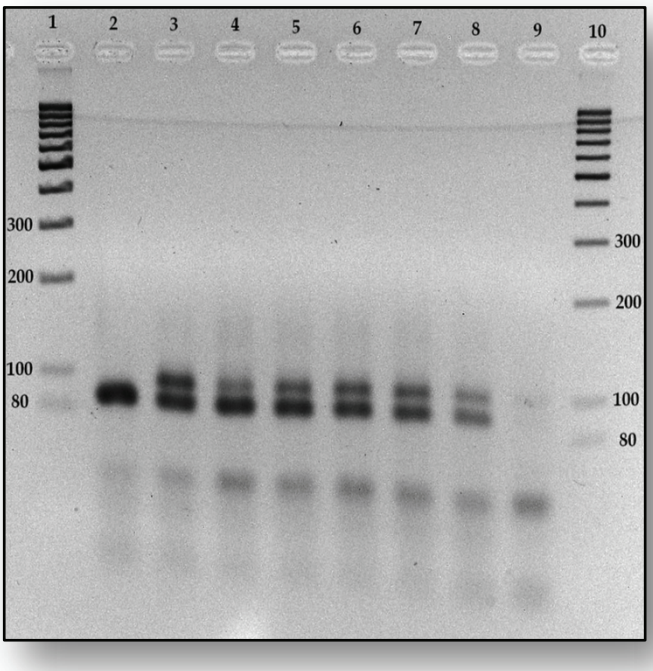


Figure 3.14 re-PCR of the bands extracted from the PCR of the constructed probes carried out using 1:1:20:21 ratio of the different fragments.

KEY: 1- 100 bp ladder; 2- re-PCR product of the first band above 100 bp of the PCR product of constructed probe; 3- re-PCR product of the second band above 100 bp of the PCR product of constructed probe; 4- re-PCR product of the third band above 100 bp of the PCR product of constructed probe; 5- re-PCR product of the fourth band above 100 bp of the PCR product of constructed probe; 6- re-PCR product of the fifth band above 100 bp of the PCR product of constructed probe; 7- re-PCR product of the sixth band above 100 bp of the PCR product of constructed probe; 8- re-PCR product of the seventh band above 100 bp of the PCR product of constructed probe; 9- PCR negative; 10- 100 bp ladder

the reagents were functioning, and there was a problem with the gel extracts, thus confirming the re-PCR results.

Instead of the ambiguous PCR step, higher amount of starting material was used to create longer probes. The ratios of the fragments were same as the previous experiment, **1:1:1.2:1.5** (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS), and

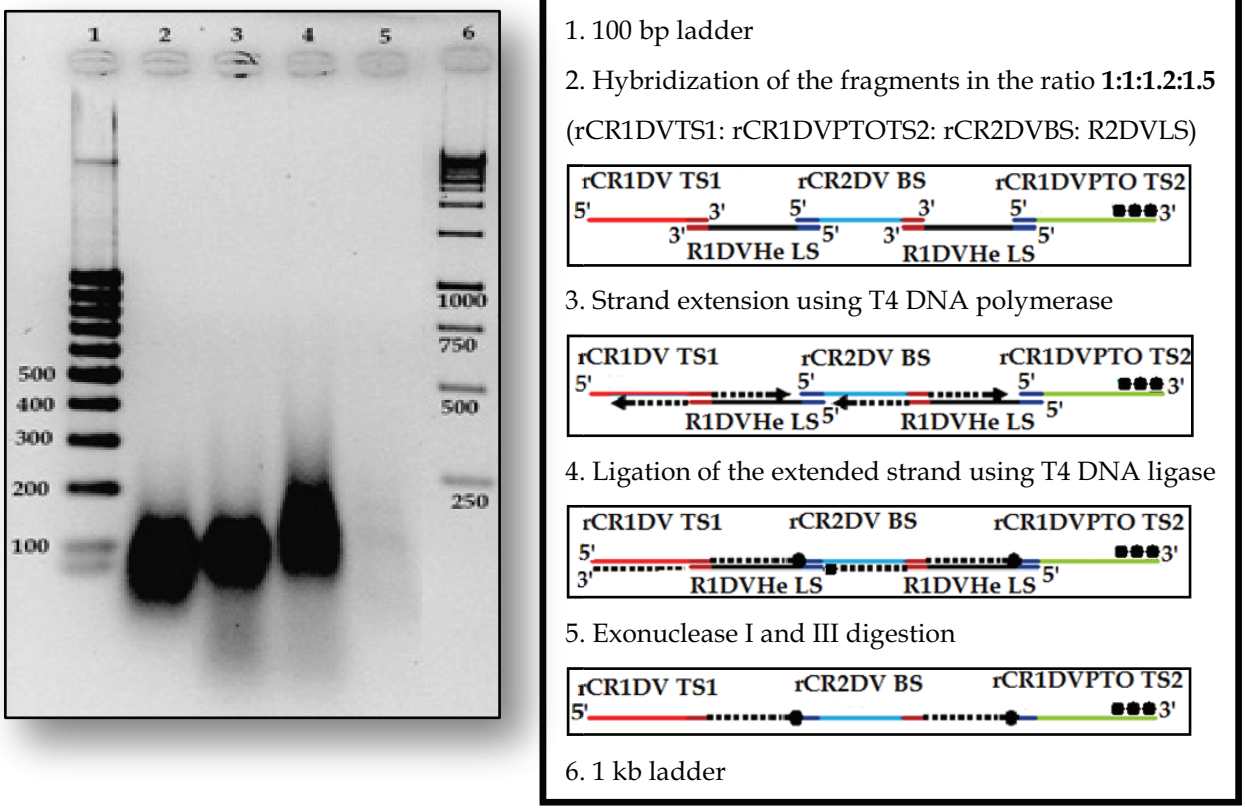


Figure 3.15 Construction of long padlock probe using 1μM final concentration and 1:1:1.2:1.5 (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio of the fragments.

the reaction conditions were identical. The difference between lane 4 and the lane 5 in Figure 3.15 was a clear indication of the unwanted products being generated. After exonuclease digestion only those molecules containing the PTO modified rCR1CVPTO TS2, were left back in the reaction mix, drastically reducing the complexity of the reaction. Two very faint but distinct bands, one at the 200 bp mark of the ladder and the other between the 200 bp mark and the 100 bp mark of the ladder, were observed in lane 5, indicating the presence of padlocks with three and two binding sites respectively. The smear below the 100 bp mark of the ladder in the same lane consists of all the other sequences which contain the PTO modification and were resistant to the exonuclease digestion.

A variation to the protocol is shown in Figure 3.16, where the polymerization and ligation steps were combined in one step, reducing the number of steps. The bands

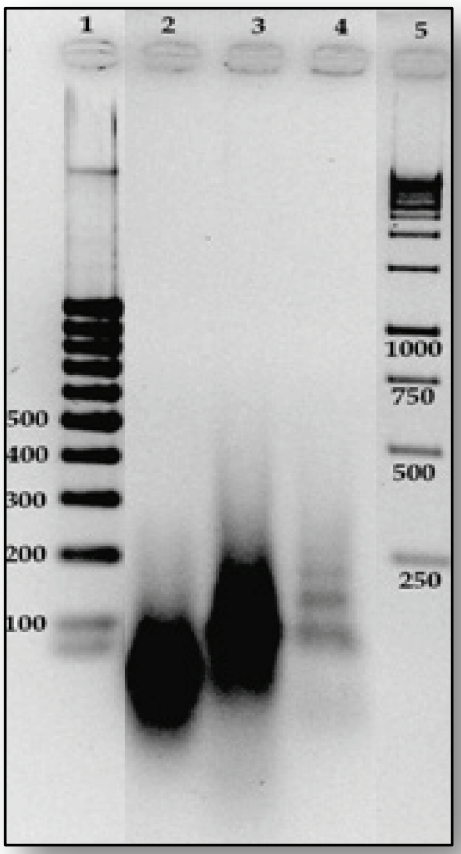
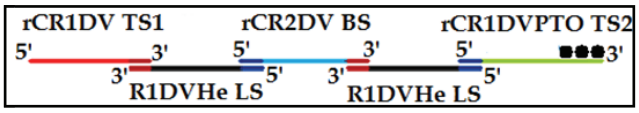


Figure 3.16 Construction of long padlock probe using 1μM final concentration and 1:1:1.2:1.5 (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio of the fragments and combining the polymerization and ligation steps.

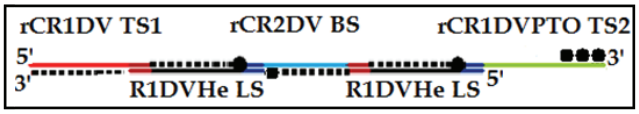
KEY:

1. 100 bp ladder

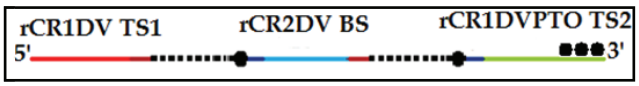
2. Hybridization of the fragments in the ratio **1:1:1.2:1.5** (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS)



3. Strand extension using T4 DNA polymerase and ligation of the extended strand using T4 DNA ligase



4. Exonuclease I and III digestion



5. 1 kb ladder

observed in the lane 4 were much clearer and showed much higher intensity, compared to the band observed in the lane 5 in the Figure 3.15. Also the number of bands that were observed was more. The approximate heights of the bands matched the expected band length of padlocks containing one, two, three and four binding sites (from bottom to the top), when compared to the ladder. The final constructed product was re-run on another gel and the desired bands of padlocks with one- three binding sites incorporated were extracted, using the Qiagen gel extraction kit, for further processing.

Though the gel extracts showed successful extraction for two of the samples, when checked on the gel after extraction, the PCR results and rolling circle replication contradicted those results. The PCR of the gel extracts showed a distinct band near the 100 bp mark of the ladder in all the lanes, and a faint band at the same level in the negative control, which could not be interpreted (Figure 3.17). In the lanes 3 and

4 in Figure 3.17 other than the band near the 100 bp mark of the ladder, one faint additional band, padlock with one binding site incorporated (expected size= 153

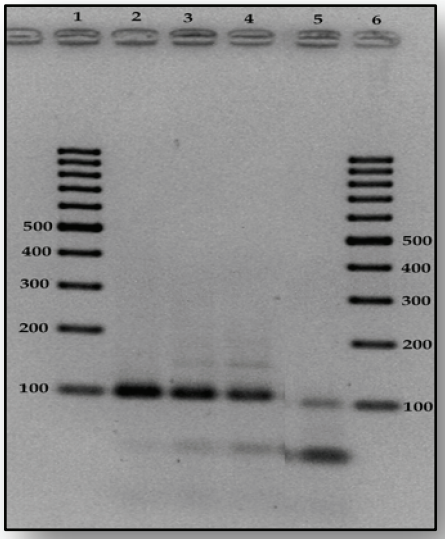


Figure 3.17 PCR products using the gel extracts as the starting material.

KEY: 1- 100 bp ladder; 2- PCR product of the smallest band extracted from the gel; 3- PCR product of the second band extracted from the gel; 4- PCR product of the largest band extracted from the gel; 5- PCR negative (no sample present); 6- 100 bp ladder

bases), was observed in both the lanes. In addition to the two bands, in lane 4 a faint band padlock with two binding sites incorporated (expected size= 197 bases) was observed. When the same gel extracts were circularized and amplified using

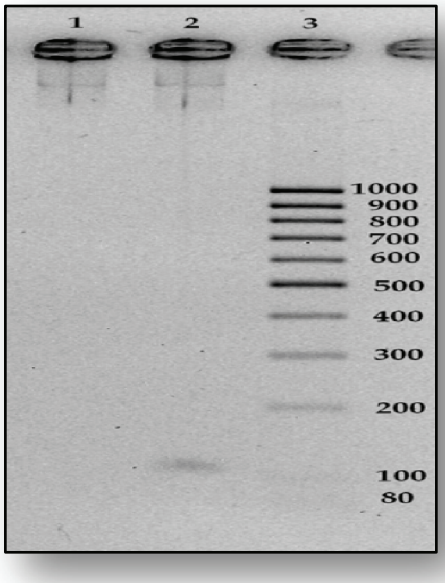


Figure 3.18 Rolling circle product of the whole construction carried out using 1µM starting material and 1:1:1.2:1.5 (rCR1DVTS1: rCR1DVPTOTS2: rCR2DVBS: R2DVLS) ratio of the fragments.

KEY: 1- Rolling circle product of the constructed probe; 2- BtgZI digestion of the rolling circle product; 3- 100 bp ladder

rolling circle amplification method, no rolling circle product was observed for any of the gel extracts. But when the constructed material was amplified using rolling

circle amplification, before gel extraction, a distinct rolling circle product was observed (Figure 3.18). Also when this rolling circle product was cleaved using the standardized conditions for BtgZI digestion, a distinct band higher than 100 bp mark to the ladder was observed, which fits to the expected size of 143 bp of the constructed padlock with only one binding site (rCR2DVBS) incorporated. The results show that, it was not possible to incorporate more than one binding site using the Continuous ligation assay. The PCR reaction created products that could not be interpreted. As, in addition to the primers added for amplification reaction after construction, there were the undigested PTO modified oligonucleotides used for construction, which also could act as primers and generate a lot of undesired false products.

Different conditions for hybridization were tested, but functional longer probes could not be constructed using this method. Although after combining the polymerization and ligation steps during construction, bands above 300 bp mark of the ladder could be seen (as shown in Figure 3.19), i.e. padlocks with about six binding sites incorporated (expected size= 373 bases). But these bands when cut from the gel and circularized, did not produce any rolling circle product. Also these gel extracted fragments gave unusual bands after PCR as seen in the pictures before.

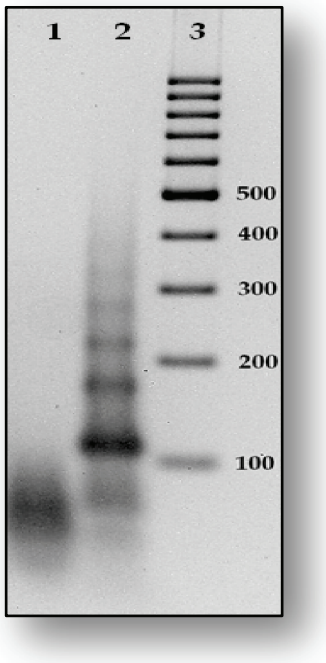


Figure 3.19 Construction of longer probes by first hybridizing rCR2DVBS and R2DVLS in the ratio 1.2:1.5 **and then the addition of rCR1DVTS1 and rCR1DVPTOTS2 in the ratio 1:1, and secondly strand extension and ligation.**

KEY: 1- Hybridization of the fragments, first rCR2DVBS and R2DVLS and then addition of rCR1DVTS1 and rCR1DVPTOTS2; 2- Polymerization and ligation reaction on the hybridized molecules; 3- 100 bp ladder

3.2.1.2 *Stepwise based system*

In the view of controlling the unwanted products formed, the stepwise construction method was used. The concentration control during the addition of each of the fragment restricted to some extent,

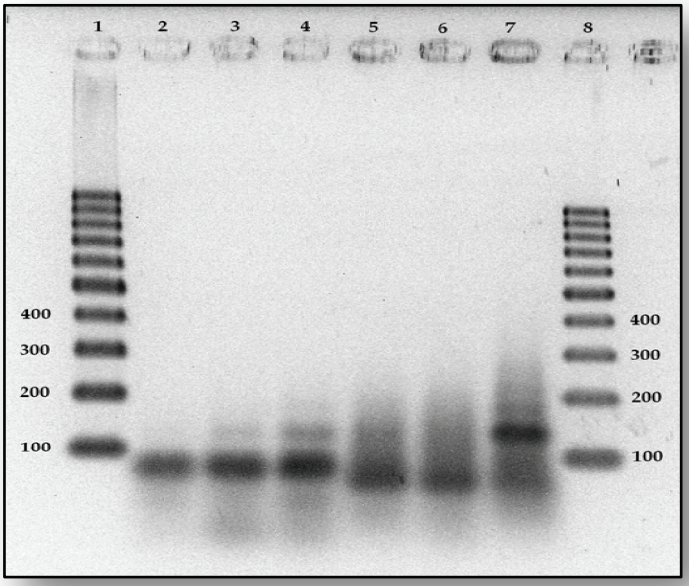
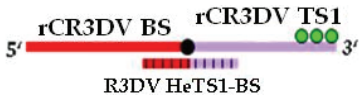


Figure 3.20 Construction of longer probes using the stepwise based method.

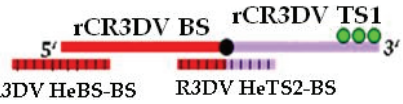
KEY:

1- 100 bp ladder;

2- Hybridization and ligation of (rCRDV TS1 + rCR3DV BS + rCR3DV HeTS1-BS) (1:1:1ratio);

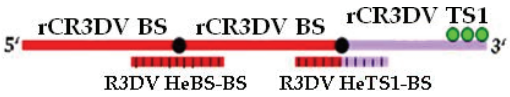


3- Addition of R3DV HeBS-BS and hybridization to step no. 2;

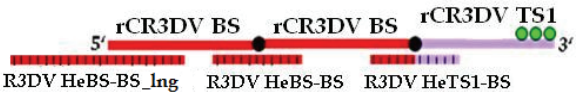


4- Addition of 375nM rCR3DV BS to step no. 3;

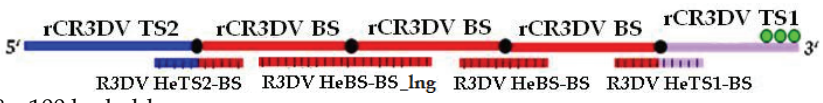
5- Ligation of the hybridized molecules;



6- Addition of 250nM R3DVHeBS-BS lng to step no. 5;



7- Addition and ligation of pre-ligated (rCRDV TS2 + rCR3DV BS + rCR3DV HeTS2-BS)(1:1:1ratio) and product of step no. 6;



8- 100 bp ladder

the formation of unwanted products. In Figure 3.20 different steps during the stepwise construction method are seen. In the lane 7 one distinct band around 150 bp mark of the ladder was seen, which was the padlock with two binding sites inserted in it (expected size= 166 bases) and about two faint bands along with a smear were seen above it, which were the padlocks with three (expected size= 205 bases) and four binding sites incorporated (expected size= 244 bases). As each fragment was added, there was also a gradual increase in the bands and also in the smear. , This might be due to the creation of unwanted products or due to the different running behaviour of the single stranded constructs of varied length. The construct and the exonuclease digest of the construct were run on a polyacrylamide gel to get distinct bands for gel extraction. In Figure 3.21, the bands that were labelled were the bands that were extracted. From the gel picture, it can clearly be observed that padlocks containing not more than three binding sites (expected size= 205 bases) were created, as the last band observed in lane 3 of Figure 3.21 was just above the 200 bp mark of the ladder.

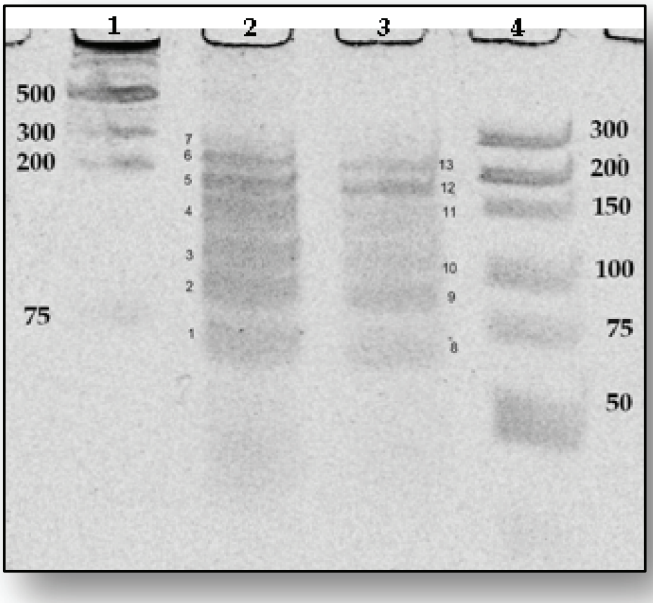
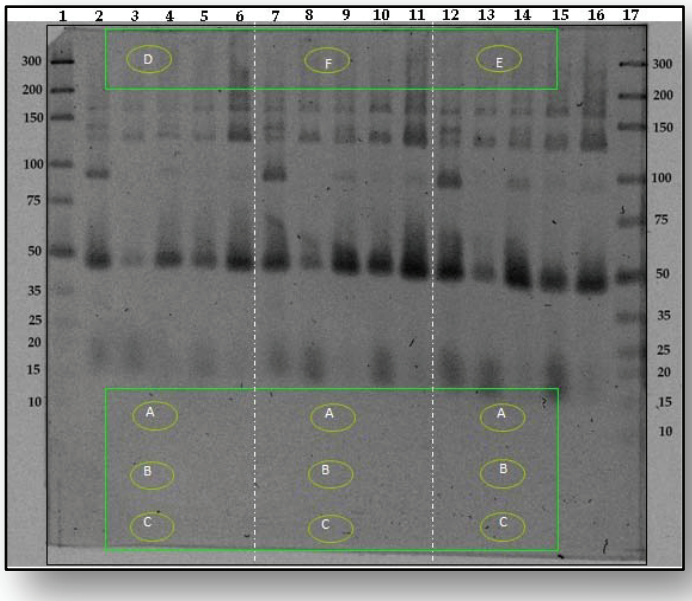


Figure 3.21 Polyacrylamide gel of the construct and the exonuclease digest of the construct constructed using stepwise based assay

KEY: 1- 1 kb plus ladder; 2- Final product after construction using stepwise based assay; 3- Exonuclease I and III digestion of the final product after construction using stepwise based assay; 4- Ultra low ladder

A PCR reaction was run to amplify the gel extracted fragments. But each band, of the gel extracts gave multiple bands which were not interpretable. To confirm whether these bands were unwanted products or whether they were the desired padlocks with both the target

specific regions included, Southern blotting was done for the PCR product of few of the extracted bands. In Figure 3.22, the dotted line shows where the membrane was cut after blotting for further processing.



A	Controls	rCRDV TS1
B		rCRDV TS2
C		rCR3DV BS
D	DIG- labelled probe	R3DVDIG TS1
E		R3DVDIG TS2
F		R3DVDIG BS

Figure 3.22 Gel picture of the PCR products from the gel extracts of the constructed molecule along with the markings used for Southern blotting

3% agarose gel was run to get distinct and separated bands for easy interpretation. The bands cannot be compared to the ladder as the gel percentage is higher and the running behaviour of the bands change with the percentage of agarose used. The membrane was divided into three parts after blotting to keep

KEY: (for both Figure 3.22 **and** Figure 3.23**)**

Lane	Name
1, 17	GeneRuler Ultra Low
2, 7, 12	PCR product of PAGE extract of band no. **1** from Figure 3.21
3, 8, 13	PCR product of PAGE extract of band no. **9** from Figure 3.21
4, 9, 14	PCR product of PAGE extract of band no. **10** from Figure 3.21
5, 10,	PCR product of PAGE extract of band no. **3** from Figure 3.21
6, 11,	PCR product of PAGE extract of band no. **4** from Figure 3.21

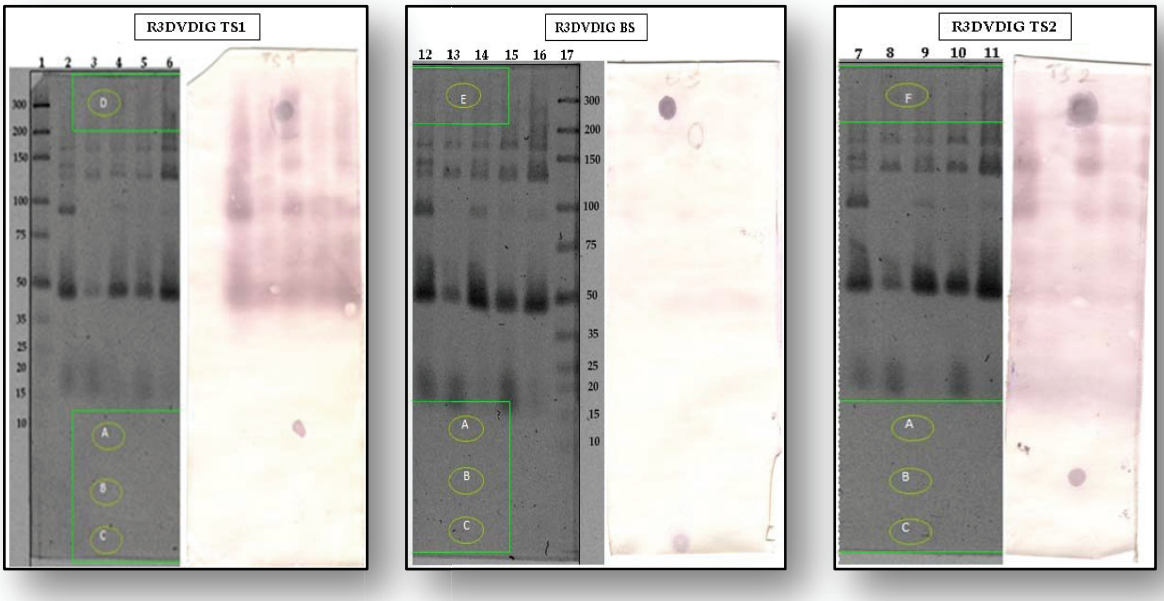


Figure 3.23 Scanned pictures of the membrane after Southern blotting of the PCR products. The extreme left figure of the membrane was treated with only DIG- labeled TS1 fragment (R3DVDIGTS1), the middle figure of the membrane was treated with only DIG- labeled BS fragment (R3DVDIGBS), and the extreme right figure of the membrane was treated with only DIG- labeled TS2 fragment (R3DVDIGTS2)

The membrane stained with DIG labelled BS fragment (R3DVDIGBS), was not stained enough. There was very faint banding observed. Although from the other two membranes in Figure 3.23, it could be concluded that there were constructed fragments which contain rCRDVTS1 and rCRDVTS2 fragments, the Southern blot failed to wrap up whether these constructed structure was of one single padlock containing at both the ends rCRDVTS1 and rCRDVTS2 or two different constructs running at the same position having rCRDVTS1 or rCRDVTS2 incorporated in it. Hence Figure 3.23 just gave a hint about which bands have which fragments incorporated within them.

The circularization and BtgZI digestion steps were exploited to confirm whether the desired padlocks were constructed. Only if both the target specific fragments were present, they could be circularized and amplified using rolling circle amplification. After the sequence specific cleavage of the rolling circle product, obtaining a cleaved product of the desired length would substantiate the presence of both the target specific sites.

The gel extracts of the constructed padlocks did not give a rolling circle product. When the whole padlock construct without gel extraction and its exonuclease digested products were used for rolling circle amplification, a strong rolling circle product was observed as seen in Figure 3.24. After exonuclease digestion, lane 3

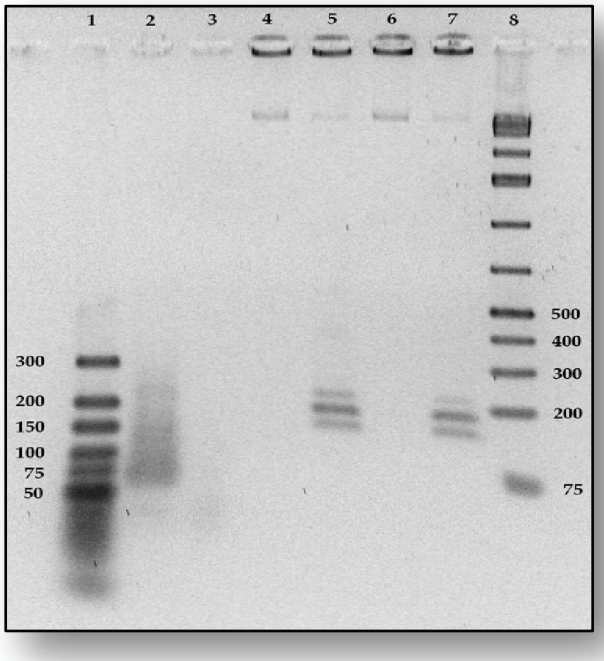


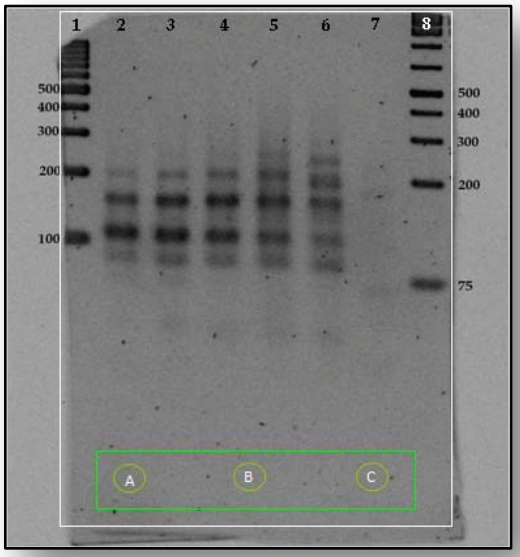
Figure 3.24 Rolling circle amplification and BtgZI cleavage of the constructed probes constructed using stepwise based method.

KEY: 1- Ultra low ladder; 2- Product of stepwise construction method; 3- Exonuclease I and III digestion of the product of stepwise construction method; 4- Rolling circle product of step no. 2; 5- BtgZI digestion of the rolling circle product from step no. 4; 6- Rolling circle product of the exonuclease digested construct; 7- BtgZI digestion of the rolling circle product from step no. 6; 8- 1 kb plus ladder

seemed empty, although when the contents of the same well were used for rolling circle amplification it gave a product, confirming the limitation of the detection limit of agarose gel and the sensitivity of rolling circle amplification. Also in the lanes 5 and 7 three distinct bands were observed and those bands match the expected size of the constructed padlocks with two, three and four repetitive binding sites incorporated. The gel extraction of these bands and sequencing of the gel extracts did not give conclusive results.

A variation of this stepwise method was also done, with an aim to reduce the unwanted and undigested PTO modified constructs in the final product, after construction. The protocol followed remained almost the same, but the starting material changed. Instead of using pre-ligated (rCRDV TS1 + rCR3DV BS + rCR3DV HeTS1-BS) as in the previous approach, pre-ligated (rCRDV TS2 +

rCR3DV BS + rCR3DV HeTS2-BS) were used as starting material to build the required molecule. With this variation the PTO modified oligonucleotide (rCRDV TS1) was added in extremely reduced concentration only the final step of construction. This prevented the accumulation of unwanted and indigestible products as observed in lane 7 in Figure 3.25. Three gels (Figure 3.25) of each step of the construction were blotted on three membranes, so that the presence of all the three fragments (rCRDVTS1, rCRDVTS2, and rCR3DVBS) could be confirmed on each of the membrane.



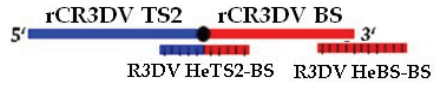
A	Controls	rCRDV TS1
B		rCRDV TS2
C		rCR3DV BS

Figure 3.25 One of the three gel pictures of the different steps of construction using rCRDV TS2 as the starting material along with the markings used for Southern blotting.

KEY:

1- 100 bp ladder;

2- Addition and hybridization of R3DV HeBS-BS to ligated (rCRDV TS2 + rCR3DV BS + rCR3DV HeTS2-BS) (1:1:1ratio);



3- Addition of 375nM rCR3DV BS to step no. 2;

4- Ligation of the hybridized molecules;

5- Addition of 250nM R3DVHeBS-BS lng to step no. 4;

6- Addition and ligation of pre-ligated (rCRDV TS2 + rCR3DV BS + rCR3DV HeTS2-BS)(1:1:1ratio) and product of step no. 5;

7- Exonuclease I and III digestion of the construct from step no. 6

8- 1 kb plus ladder

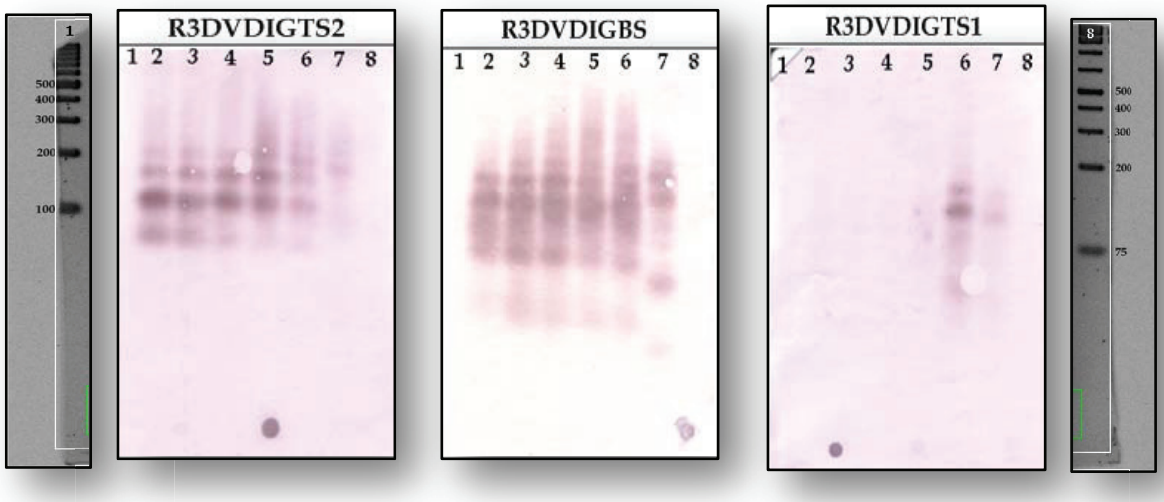


Figure 3.26 Scanned picture of the membranes after Southern blotting of the different steps of probe construction using the stepwise based assay with rCRDV TS2 as the starting material.

The extreme left membrane was treated with only DIG- labeled TS2 fragment (R3DVDIGTS2), the middle membrane was treated with only DIG- labeled BS fragment (R3DVDIGBS), and the extreme right figure of the membrane was treated with only DIG- labeled TS1 fragment (R3DVDIGTS1)

The membranes in Figure 3.26 clearly show the different steps of construction. According to the protocol, rCRDVTS1 was added only in the fifth step as clearly evident in lane 6, of all the three membranes. In the extreme right membrane lanes 2 to 5 were empty, as no rCRDVTS1 was present in the system. Even though the lane 7 was practically empty on the agarose gel stained with SYBR green II, Southern blotting gave distinct bands. The last lane in all the three blots was the most important part, as it was the final product of construction. It can be clearly seen that there were two bands which correspond to the padlocks containing two (expected size= 166 bases) and three (expected size= 205 bases) binding sites incorporated in them. These bands show the presence of all the three fragments i.e. both the target specific regions as well as the binding sites. Starting with the pre-ligated rCRDVTS2 instead of pre-ligated rCRDVTS1 definitely reduces the artefacts and the complexity of the system.

3.2.1.3 Bead based system

Bead-based method was followed as an alternative method and its efficiency was compared to the stepwise method. The fragments that were used were the same as used for the stepwise based approach. As seen in Figure 3.27, lane 2 showed no signs of DNA being present; it can be concluded that the separation of the constructed padlocks from the beads was complete. In the lane 3, a distinct band between the 150 bp and 200 bp mark of the ladder was seen amid the smear corresponding to the padlock with two binding sites incorporated (expected size= 166 bases). No padlocks longer than the 200 bp mark of the

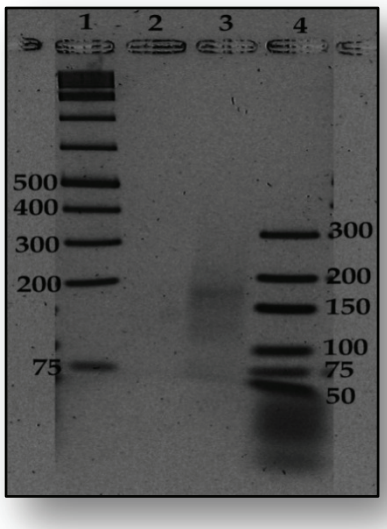


Figure 3.27 Gel picture of the final product of construction using the bead based assay

KEY: 1- 1 kb plus ladder; 2- Only beads re-dissolved in water after the separation of the required strand; 3- Final product of construction using bead based assay after separation from the beads; 4- Ultra low ladder

ladder were observed, though a faint smear was seen.

When this sample was circularized, amplified using rolling circle replication and cleaved with BtgZI enzyme, it generated two padlocks with two and three binding sites incorporated in them. One of the bands that were obtained lay near the 200 bp mark of the ladder corresponding to the padlock with three binding sites incorporated (expected size= 205 bases) and the other was between the 150 bp mark and the 200 bp mark of the ladder corresponding to the padlock with two binding sites (expected size= 166 bases) as seen in Figure 3.28. The rolling circle was carried out for two hours only, instead of five hrs that was usually done. The smear below the 75 bp mark was of the oligonucleotides that were used in excess for the circularization reaction. These oligonucleotides in no way affect or influence the BtgZI cleavage reaction.

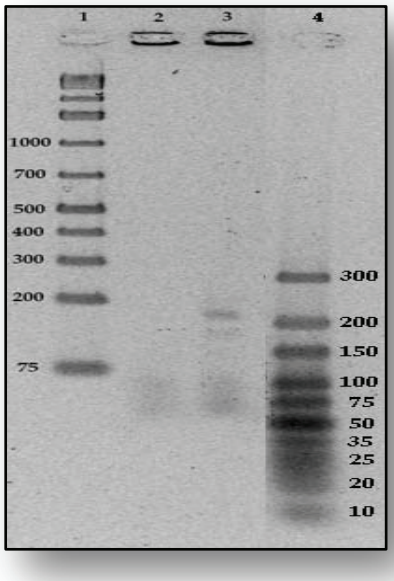


Figure 3.28 Rolling circle amplification of the bead based constructed probe and the cleavage to the rolling circle product using BtgZI enzyme

KEY: 1- 1 kb plus ladder; 2- Rolling circle product of the constructed probe using bead based assay; 3- BtgZI digestion of the rolling circle product; 4- Ultra low ladder

3.2.1.4 Pre-ligation based system

In this method pre-ligated constructs were put together with the aim of achieving longer padlock probes. Three different variations were tried and in all the three cases the ratio of the pre-ligated fragment was 1:1:1 [(rCRDVTS1 + rCR3DVBS + rCR3DVHeTS1-BS) : (rCRDVTS2 + rCR3DVBS + rCR3DVHeTS2-BS) : (rCR3DVBS +

rCR3DVHeBS-BS)]. Figure 3.29 shows the final product of all the three variations. In all the three lanes, not much of a difference in the bands was seen. A distinct difference was the absence of bands below 75 bp mark of the ladder in the lane 2, which signified the complete utilization of the pre- ligated (rCRDVTS1 + rCR3DVBS + rCR3DVHeTS1-BS) (expected size= 63 bases). Also the band in the

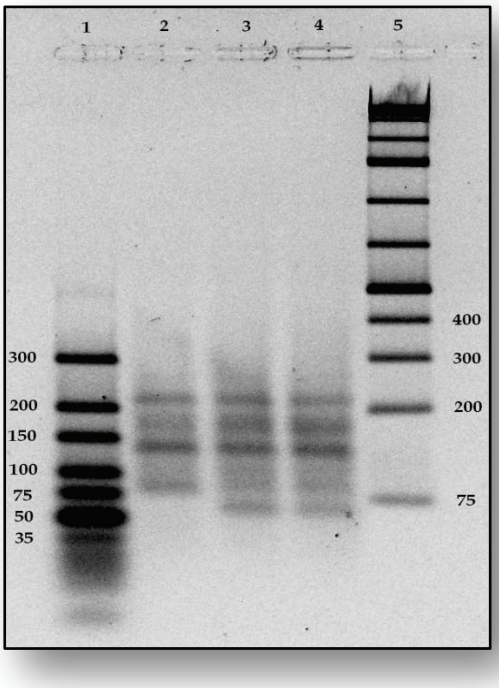


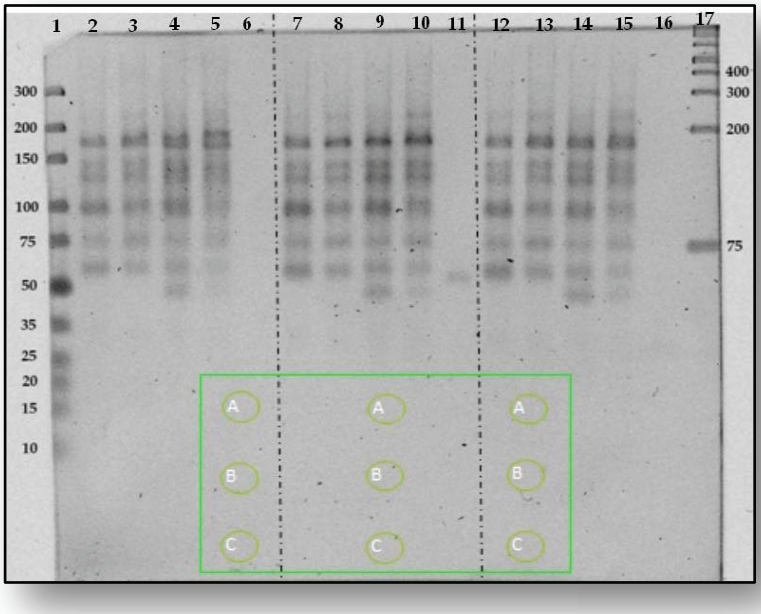
Figure 3.29 Construction using the pre-ligation based assay and three different variations during construction

KEY: 1- Ultra low ladder; 2- Construction using pre-ligation based system with Ampligase enzyme for ligation; 3- Construction using pre-ligation based system with T4 ligase enzyme for ligation; 4- Construction using pre-ligation based system with first hybridization reaction and then ligation with T4 ligase enzyme; 5- 1 kb plus ladder

lane 2 near the 75 bp mark of the ladder was much stronger than in lane 3 and 4 which corresponds to (rCR3DVBS + rCR3DVHeBS-BS) circles (expected size= 78 bases). These bands were resistant to exonuclease I and III digestion (Figure 3.32), even though they did not contain any PTO modified oligonucleotides. . The three prominent bands observed in all the three lanes, matched the expected size of the padlocks with one (expected size= 127 bases), two (expected size= 166 bases) and three (expected size= 205 bases) binding sites incorporated. Southern blotting of all the three methods confirmed the presence of all the fragments, hence assuring the existence of padlocks with one, two and three binding sites incorporated in them.

The following slight variation in the above method was done. Instead of taking the ratio of the pre- ligated molecules to be 1:1:1, a ratio of 1:1:1.2 [(rCRDVTS1 +

rCR3DVBS + rCR3DVHeTS1-BS): (rCRDVTS2 +rCR3DVBS +rCR3DVHeTS2-BS): (rCR3DVBS + rCR3DVHeBS-BS)] was used. An additional band corresponding to the padlock with four (expected size= 244 bases) binding sites incorporated in it was observed when the ratio was changed. Basically no significant difference was seen when Ampligase or T4 ligase was used for the ligation reaction in the desired product formed. Southern blotting of that 3 % gel with variation was done and the results are as shown in Figure 3.30 and Figure 3.31.



A	Controls	rCRDV TS1
B		rCRDV TS2
C		rCR3DV BS

Figure 3.30 Gel picture of the final products obtained after construction using pre- ligated material and the four different variations along with the markings used for Southern blotting

KEY: (for both Figure 3.30 **and** Figure 3.31 **)**

Lane No.	Name
1, 17	Gene Ruler Ultra Low and 1Kb plus ladder
2, 7, 12	[(rCRDVTS1+rCR3DVBS):(rCRDVTS2+rCR3DVBS):(rCR3DVBS + rCR3DVBS)]= 1:1:1; Ampligase
3, 8, 13	(rCRDVTS1+rCR3DVBS):(rCRDVTS2+rCR3DVBS):(rCR3DVBS + rCR3DVBS)]= 1:1:1.2; Ampligase
4, 9, 14	(rCRDVTS1+rCR3DVBS):(rCRDVTS2+rCR3DVBS):(rCR3DVBS + rCR3DVBS)]= 1:1:1; T4 ligase
5, 10, 15	(rCRDVTS1+rCR3DVBS):(rCRDVTS2+rCR3DVBS):(rCR3DVBS + rCR3DVBS)]= 1:1:1.2; T4 ligase
6	rCRDVTS1 (10nM)
11	rCRDVTS2 (10nM)
16	rCRDVBS (10nM)

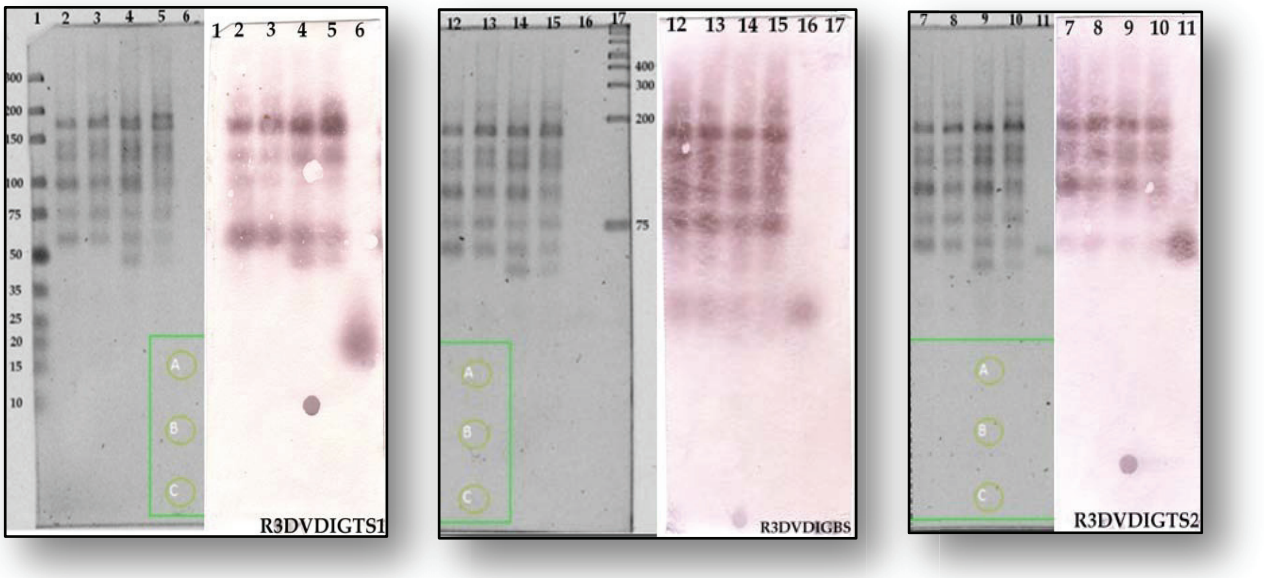


Figure 3.31 Scanned pictures of Southern blot of the final product of the construction using pre-ligated material

The extreme left membrane was treated with only DIG- labeled TS1 fragment (R3DVDIGTS1), the middle membrane was treated with only DIG- labeled BS fragment (R3DVDIGBS), and the extreme right figure of the membrane was treated with only DIG-labeled TS2 fragment (R3DVDIGTS2)

The blots clearly show that the bands formed by the padlocks containing one, two and three binding sites have all the three fragments (rCRDVTS1, rCRDVTS2 and rCRDVBS) incorporated. The change in the ratio that was used during construction, produced an additional band which corresponds to the padlock containing four binding sites. It showed the presence of all the three fragments in it. But even when the previous ratio was used, faint presence of padlocks with four binding sites could be observed on the developed bolts.

Although the Southern blot, just after construction, showed the presence of the desired padlocks, when the constructs were treated with exonucleases to reduce the complexity of the reaction, most of the bands were missing. In Figure 3.32, lanes 3, and 7 showed the presence of mainly only padlocks with two and three binding site. Lane 5 showed the presence of padlocks with two and three binding sites along with very faint presence of padlock with four binding site. In the lane 9, padlock

with three binding sites was distinctly observed, with faint bands of padlock with two and four binding sites. This might be due to the low detection limit of agarose gel compared to Southern blot. But nevertheless, there was a definite alteration observed in the constructed padlocks, with the change in the ratios of the fragments.

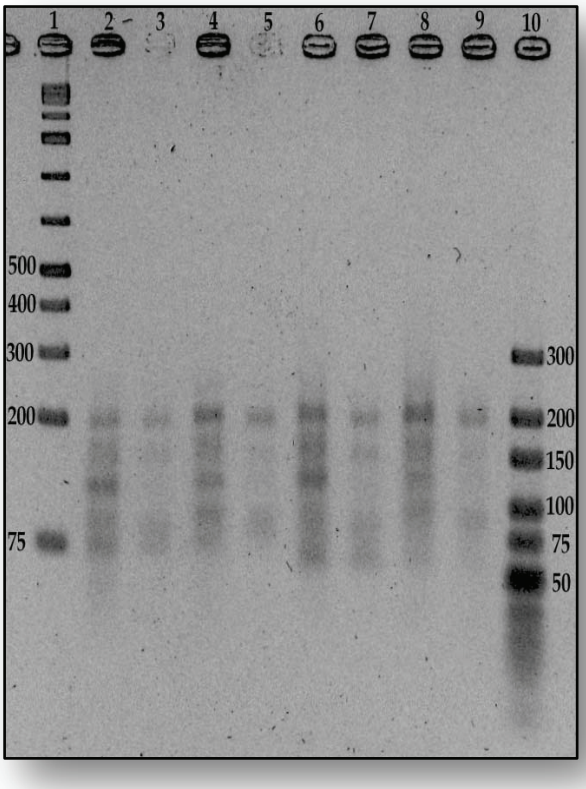


Figure 3.32 Gel picture of the Exonuclease digest of the constructed probes using the pre- ligation method.

KEY: 1- 1 Kb plus ladder; 2- Ligated [(rCRDVTS1+rCR3DVBS):(rCRDVTS2+rCR3DVBS):(rCR3DVBS + rCR3DVBS)] in the ratio 1:1:1 with Ampligase; 3- Exonuclease I and III digest of the step 2; 4- Ligated [(rCRDVTS1+rCR3DVBS):(rCRDVTS2+rCR3DVBS):(rCR3DVBS + rCR3DVBS)] in the ratio 1:1:1.2 with Ampligase; 5- Exonuclease I and III digest of the step 4; 6- Ligated [(rCRDVTS1+rCR3DVBS):(rCRDVTS2+rCR3DVBS):(rCR3DVBS + rCR3DVBS)] in the ratio 1:1:1 with T4 ligase; 7- Exonuclease I and III digest of the step 6; 8- Ligated [(rCRDVTS1+rCR3DVBS):(rCRDVTS2+rCR3DVBS):(rCR3DVBS + rCR3DVBS)] in the ratio 1:1:1.2 with T4 ligase; 9- Exonuclease I and III digest of the step 8; 10- Ultra low ladder

But when the exonuclease digest of the constructs were used for rolling circle amplification and digested with BtgZI enzyme, the product of lane 3, 5 and 7 from Figure 3.32 gave two clear bands, indicating the presence of two and three binding sites in the probes. The sample from lane 9 from Figure 3.32 gave only one single band after BtgZI digestion of its rolling circle product as seen in Figure 3.33. None of the digests generated the padlocks with four binding sites. This might be due to the competition occurring for amplification during RCA, because of the presence of varied multiple targets. The abundantly available targets were easily amplified, compared to the targets in extremely low concentration.

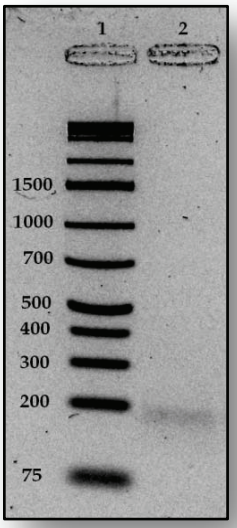


Figure 3.33 BtgZI digest of the rolling circle product of the exonuclease digested probe made from the fragments in the ratio 1:1:1.2 and T4 ligase.

KEY: 1- 1 kb plus ladder; 2- BtgZI digest of the rolling circle product of Exonuclease I and III digest of ligated [(rCRDVTS1+rCR3DVBS):(rCRDVTS2+rCR3DVBS):(rCR3DVBS + rCR3DVBS)] in the ratio 1:1:1.2 with T4 ligase.

The single band observed in Figure 3.33 was extracted and sequenced. But no sequence was obtained, due to the inefficiency of gel extraction kits for small single stranded DNA.

When the ratio of the pre- ligated molecules were further altered to 1:1:2 [(rCRDVTS1 + rCR3DVBS + rCR3DVHeTS1-BS) : (rCRDVTS2 + rCR3DVBS + rCR3DVHeTS2-BS) : (rCR3DVBS + rCR3DVHeBS-BS)] and 0.2 U/µl of T4 ligase was used, a single band (padlock with three binding sites) was observed with a smear below near the 200 bp mark of the ladder as seen in Figure 3.34 Final product of the probes constructed using the pre-ligation method with T4 ligase and 1:1:1, 1:1:2 and 1:1:1.3 ratios of the different fragments. in lane 2. As the ratio was changed to

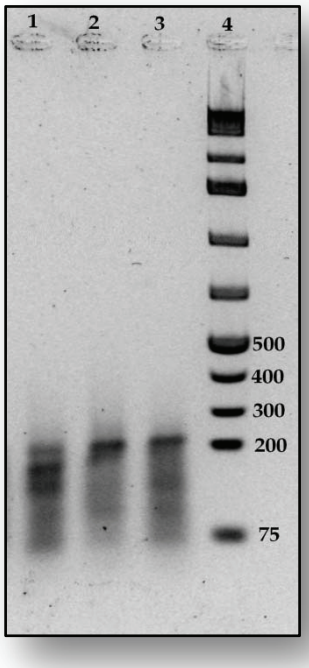


Figure 3.34 Final product of the probes constructed using the pre-ligation method with T4 ligase and 1:1:1, 1:1:2 and 1:1:1.3 ratios of the different fragments.

KEY: 1- Ligated [(rCRDVTS1+rCR3DVBS):(rCRDVTS2+rCR3DVBS):(rCR3DVBS + rCR3DVBS)] in the ratio 1:1:1 with 0.1 U/µl T4 ligase; 2- Ligated [(rCRDVTS1+rCR3DVBS):(rCRDVTS2+rCR3DVBS):(rCR3DVBS + rCR3DVBS)] in the ratio 1:1:2 with 0.2 U/µl T4 ligase; 3- Ligated [(rCRDVTS1+rCR3DVBS):(rCRDVTS2+rCR3DVBS):(rCR3DVBS + rCR3DVBS)] in the ratio 1:1:1.3 with 0.2 U/µl T4 ligase; 4- 1 kb plus ladder

1:1:1.3 [(rCRDVTS1 + rCR3DVBS) : (rCRDVTS2 + rCR3DVBS) : (rCR3DVBS + rCR3DVBS)] additional bands lower than the 200 bp mark of the ladder was observed in lane 3. Further when the ratio of the fragments was 1:1:1 [(rCRDVTS1+rCR3DVBS): (rCRDVTS2+rCR3DVBS): (rCR3DVBS+rCR3DVBS)] stronger lower bands were observed, indicating the effect of the varying ratios on

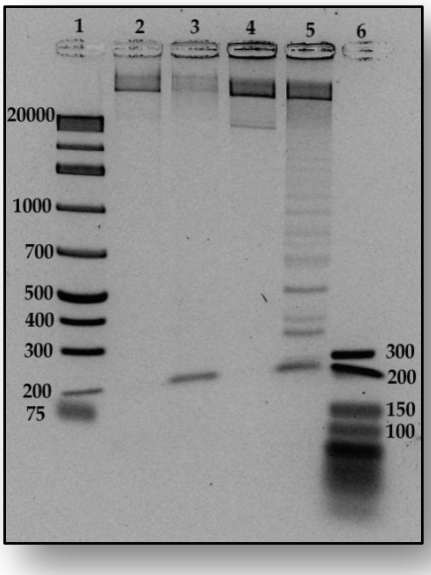


Figure 3.35 BtgZI digestion of purified and not purified rolling circle product.

KEY: 1- 1 kb plus ladder; 2- diluted rolling circle product before purification; 3- BtgZI digestion of the diluted rolling circle product; 3- rolling circle product after purification; 4- BtgZI digestion of the purified rolling circle product; 6- Ultra low ladder

the padlocks and the artefacts formed. Rolling circle product of lane 2 gave a single band after BtgZI digestion.

The attempt to extract the single band from the gel that was achieved after BtgZI digestion of the rolling circle product, was proved futile. As the construct after rolling circle amplification and after complete digestion with BtgZI enzyme gave only one distinct band, the whole rolling circle product was purified using Sodium acetate precipitation method. The purified product was then cleaved with BtgZI enzyme again. This was done to get rid of the excess primers and dNTPs. As seen in Figure 3.35, when the rolling circle product was cleaved before purification, a single band was observed as in lane 3. But when the same rolling circle product was purified, there was a laddered structure observed after cleavage. The bands corresponded to the lengths of more than two padlocks bound together, indicating that the cleavage was insufficient to get the desired single band of padlock

containing three binding sites. More amount of enzyme was added to the mixture and it was re-incubated, but there was very slight difference observed in band pattern. But when the purified rolling circle product was heated at 90° C for 5 min and incubated at 37° C for 12 hrs, just after dissolving the purified pellet in double distilled water after purification, it showed a complete digestion and generated single stranded padlocks with three binding sites. As there was complete digestion of the purified rolling circle product, these padlocks could be directly used as functional probes without the need of further purification. After purification as no additional oligonucleotides or any other components like dNTPs were added for BtgZI digestion. They could be directly used as probes, as the hindrances caused for the efficient functioning of the padlocks could be avoided. Also as no purification of the padlocks was needed, the concentration was much higher and the loss of final products during the purification was avoided.

3.3 Gel extraction of the probes

Nine different protocols were tested for the extraction of bands from agarose gel and polyacrylamide gels. But in none of the samples a peak at 260 nm was observed while measuring the samples on the Nanodrop. In general the extraction kits failed to extract the padlocks, after construction or after the digestion of rolling circle product with BtgZI enzyme. There was a significant loss of sample observed after extraction. Although FTA elute (classic) (Whatman) gave as high as 640 nM concentration after extraction of single stranded construct, no rolling circle product was observed when this extract was used as a starting material for the reaction. Table 3.1 gives a list of the methods used and the final concentration of the bands after extraction.

Table 3.1 Spectrophotometric results of gel extraction methods for PCR products, constructs, exonuclease digest of constructs and BtgZI digested products.

No.	Sample ID	ng / µL	Length (nt /bp)	DNA [nM]
1	Ultrafree-DA (Millipore) (ds DNA)	6.5	283	34.80
2	Ultrafree-DA (Millipore) (ds DNA)	5.78	205	42.72
3	Nucleospin Extract II (Macherey- Nagel) (ds DNA)	7.84	283	41.97
4	Nucleospin Extract II (Macherey- Nagel) (ds DNA)	6.96	205	51.44
5	Mini- elute Reaction clean up kit (Qiagen) (ds DNA)	1.34	283	7.17
6	Mini- elute Reaction clean up kit (Qiagen) (ds DNA)	2.29	205	16.93
7	PCR- Kombi Kit (Seqlab) (ds DNA)	5.44	283	29.13
8	PCR- Kombi Kit (Seqlab) (ds DNA)	5.37	205	39.69
9	Squeeze and crush method (ds DNA)	48.23	205	356.47
10	Squeeze and crush method (ds DNA)	44.99	283	240.87
11	QIAquick® Gel Extraction Kit (Qiagen) (ds DNA)	131.16	205	969.40
12	High Pure DNA extraction Micro Kit (Roche) (ss DNA)	0.72	127	17.18
13	High Pure DNA extraction Micro Kit (Roche) (ss DNA)	2.43	166	44.36
14	High Pure DNA extraction Micro Kit (Roche) (ss DNA)	1	205	14.78
15	High Pure DNA extraction Micro Kit (Roche) (ss DNA)	0.91	119	23.17
16	High Pure DNA extraction Micro Kit (Roche) (ds DNA)	32.17	427	114.15
17	PAGE extraction protocol (ss DNA)	1.78	166	32.49
18	PAGE extraction protocol (ss DNA)	0.6	205	8.87
19	FTA elute (classic) (Whatman) (after construction) (partial ds)	41.25	205	304.88
20	FTA elute (classic) (Whatman) (after exonuclease digestion of the construct) (ss DNA)	43.31	205	640.21
21	Freeze and squeeze (BtgZI digested product) (ss DNA)	7.92	205	117.07

3.4 Sequencing

The gel extracts of the BtgZI digestion products were sequenced. As no sequence chromatogram was obtained, rolling circle products which gave only one single product on BtgZI digestion were used as templates. The 205 bases padlock with three binding sites incorporated, 166 bases padlock with two binding sites incorporated and the 120 bases constructed padlock were sequenced. The chromatograms were edited using Chromas and seqMAN software. The edited sequence was aligned with the expected sequence in GeneDoc, to compare whether the probe contained the exact sequence as desired. For 205 bases probe there was a few bases mismatch observed in the middle of the probe, probably a result of sequencing errors. But for 120 bases and 166 bases probes, the sequences obtained were an exact match to the expected sequence. (Appendix- A3)

3.5 Confirming the functionality of probes

3.5.1 120 bases Probes

To check the functionality of the 120 bases probes, the probes were reacted with an oligonucleotide having the same sequence as the product. Also the functionality of the probes was tested with a 472 bp PCR amplified fragment from the S. *epidermidis*

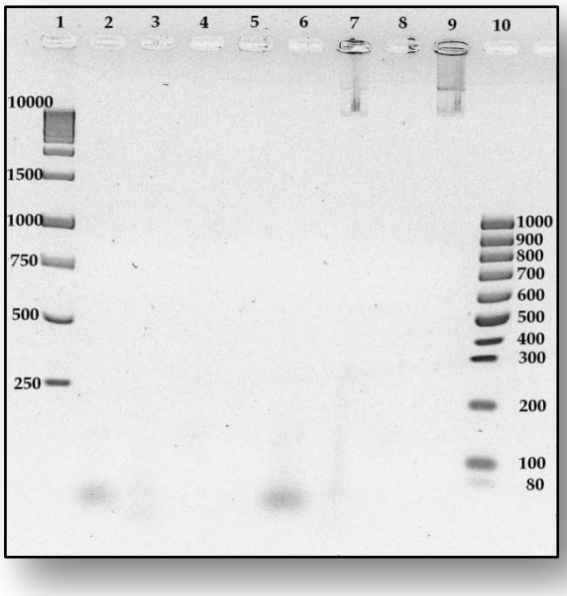


Figure 3.36 Testing the functionality of 120 bp probes with target oligonucleotide

KEY: 1- 1 kb ladder; 2- Target oligonucleotide + 120 bp probe + Ampligase buffer; 3- Rolling circle amplification of step 2; 4- Exonuclease digestion of step 2; 5- Rolling circle amplification of step 4; 6- Target oligonucleotide + 120 bp probe + Ampligase buffer+ 0.2 U/µl Ampligase; 7- Rolling circle amplification of step 6; 8- Exonuclease digestion of step 6; 9- Rolling circle amplification of step 8; 10- 100 bp ladder.

Domain V. The functionality test was based on the ability of the padlock to react with the target and form circular products, proving the presence of right sequence at the 5′ and 3′ end of the padlock and the presence of phosphate group at the 5′ end. This test was just qualitative, as rolling circle amplification of the padlock was involved. Quantification and the ratio of functional probes to non-functional probes could not be determined. As seen in Figure 3.36, only the reaction containing ligase gave a rolling circle product in lane 7 compared to lane 3 where no ligase was present, and hence no circularization occurred. Even when the circularized product was digested using exonucleases, a rolling circle product was formed (lane 9). Similarly in Figure 3.37, in lanes 1 and 2 the 472 bp PCR product of the target and the 120 bases probe can be easily visualized. After exonuclease digestion in lane 4 and 5 only the circularized product should remain. Since the concentration of the circularized product was not within the range of detection by SYBR Green I stain, the lanes seemed empty. A rolling circle product in the lane 6 and no rolling circle product in lane 7 was a clear indication that the constructed padlocks were phosphorylated with the right bases at the 5′ and the 3′ end. Also there were no factors during the reaction that could contribute to give a false positive result.

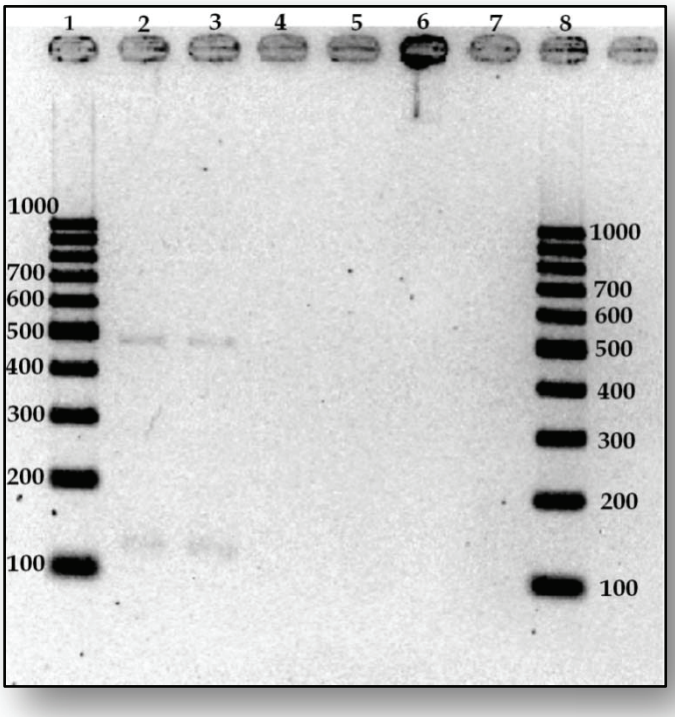


Figure 3.37 Testing the functionality of 120 bp probes with the 472 bp PCR product from target

KEY: 1- 100 bp ladder; 2- 472 bp PCR product from the target + 120 bp probe + T4 ligase buffer + T4 ligase (0.2 U/µl); 3- 472 bp PCR product from the target + 120 bp probe + T4 ligase buffer; 4- Exonuclease digestion of step 2; 5- Exonuclease digestion of step 3; 6- Rolling circle amplification of step 4; 7- Rolling circle amplification of step 5; 8- 100 bp ladder.

3.5.2 Longer Probes

As the long probes were a part of the Kotenkar-Rühle-Kracht approach of construction, amplification and bioluminescence detection, the constructed padlock was tested accordingly, and the experiments were done in close collaboration with B. Kracht and F. Rühle. The functionality of the constructed padlocks with three binding sites incorporated, was compared with the synthetically order padlock, with six binding sites incorporated. The two padlocks, constructed RK (205 bases) and synthetically ordered SG (321 bp) had identical sequence but the number of binding sites varied. After construction of 205 bases of long padlock (RK) using pre-ligation method, the concentration of the probes was checked on the Nanodrop to be 77.23 ng/µl (1.15 µM) and for the SG probes the concentration was 77.67 ng/µl (0.73 µM). Both the probes after hybridization to the target (472 bp PCR product from the target) were treated with exonuclease. The signals were measured by adding a primer binding just before the poly 'A' stretch. Below in Figure 3.38, all the results obtained from the experiment are summarized (for details refer Appendix- A4). Note that this was just an initial experiment and the exonuclease digestion did not seem working. As observed in Figure 3.38, for the RK padlocks the signal strength was reduced when the padlocks were reacted with the target in comparison to when the padlocks were measured separately. Although the well with ligase and the hybridized padlock and target did show a decrease in signal after exonuclease digestion, as the well containing only the padlock and the target was not digested as expected, the results cannot be substantiated. But when the constructed padlocks ligated using the target were amplified using rolling circle, a distinct rolling circle product was observed in comparison to the constructed padlocks with the target and no ligase. Thus confirming the functionality of the constructed padlock and the errors occurred for the bioluminescence reactions, during the exonuclease digestion. The Shine Gene probes (321 bases), on the other hand did not give high output signals. One reason for this might be due to the

restrictions caused by its length (personal correspondence with Dr. Landegren), which can lead to secondary structure formation and result in low or no signals (Antson et al., 2000). To substantiate the quality of the ShineGene padlocks further tests must be done.

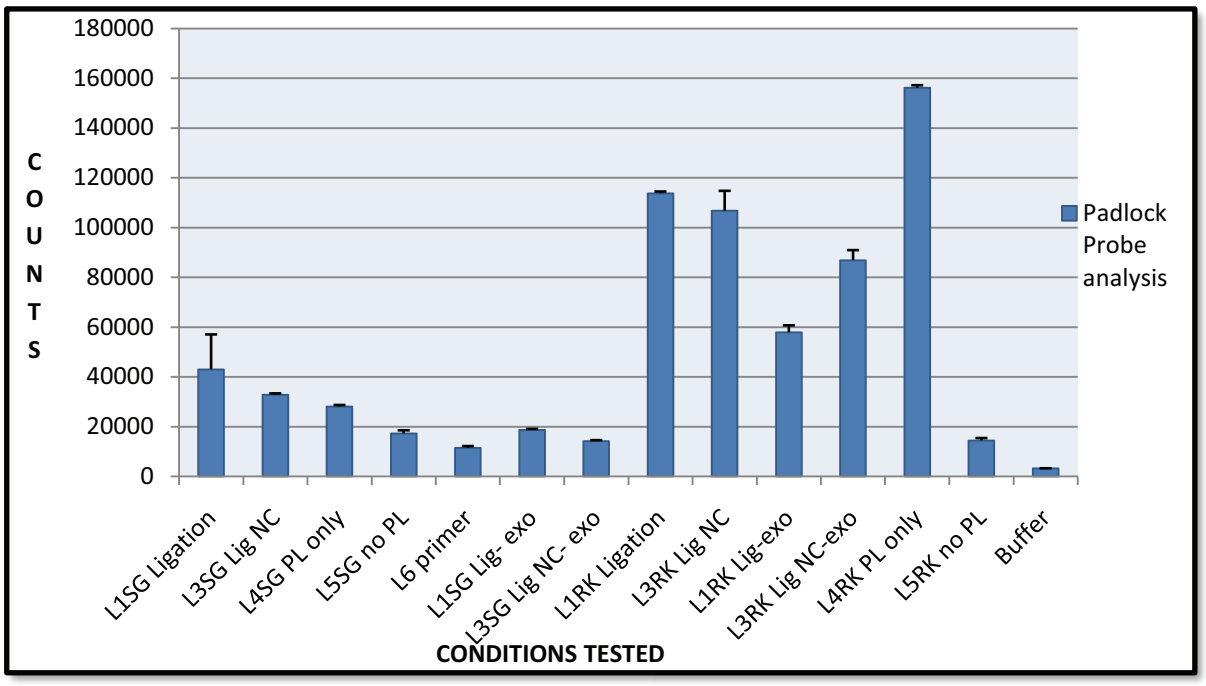


Figure 3.38 Analysis of two long padlock probe, one constructed synthetically and the other enzymatically, using bioluminescence reaction.

Abbreviations used	Reaction conditions
L1SG Ligation	SG padlock with target (472 bp PCR product) and ligase
L3SG Lig NC	SG padlock with the target (472 bp PCR product) and without ligase (negative control)
L4SG PL only	Only SG padlock
L5SG no PL	No SG padlock
L6 primer	Only primer used for the bioluminescence reaction
L1SG Lig- exo	Exonuclease I and III digestion of L1SG ligation reaction (ligated SG padlock)
L3SG Lig NC- exo	Exonuclease I and III digestion of L1SG Lig NC reaction (SG padlock+ target-ligase)
L1RK Ligation	RK padlock with target (472 bp PCR product) and ligase
L3RK Lig NC	RK padlock with the target (472 bp PCR product) and without ligase (negative control)
L1RK Lig- exo	Exonuclease I and III digestion of L1RK ligation reaction (ligated SG padlock)
L3SG Lig NC- exo	Exonuclease I and III digestion of L1SG Lig NC reaction (SG padlock+ target-ligase)
L4RK PL only	Only RK padlock
L5RK no PL	No RK padlock
Buffer	Buffer used for the bioluminescence reactions

4 DISCUSSION

Generally the pre-requisite of having the length of the padlock at least between 70-100 bases (Nilsson et al., 1994), is critical in deciding the method of choice for constructing padlocks. The need for a robust method for the construction of padlocks was generated due to the shortcomings of the currently available methods for construction as shown in Table 1.1. The results obtained in this study, do not completely overcome the pre-existing difficulties in the construction of padlocks. But nevertheless the methods used in this study allow variations in the gene synthesis method specifically, for constructing functional single stranded 120 bases padlock probes. Also the combination of rolling circle amplification and the novel BtgZI enzyme, gives the opportunity to have high concentration of phosphorylated padlocks with at least three repetitive sequences incorporated in them. This can be a a major contribution for developing new padlock application methods.

4.1 Construction of Padlocks

Since the first gene synthesis method pioneered by Khorana (Khorana et al., 1968; Agarwal et al., 1970) was introduced till date where 582,970-base pair Mycoplasma genitalium genome was reconstructed by the J. Craig Venter Institute (Gibson et al., 2008), the field of gene synthesis has been rapidly growing. The basic principle of hybridizing short oligonucleotides and ligating them, remains common in most of the gene synthesis method still. But as more as the number of oligonucleotides to be ligated increases, as higher the probability is of getting the relative amount of the desired product reduced (Nassal et al., 1987). While long overlapping fragments results in stable hybridization reactions, it also results in the formation of secondary structure formation.

Generally for constructing single stranded DNA, the unwanted strand of the double stranded DNA, constructed using PCR or gene synthesis method, is got rid off by modifying the wanted strand with biotin and selectively washing off the

unwanted strand (Antson et al., 2000; Myer et al., 2001). Alternatively, the single stranded DNA has been constructed using just hybridization and ligation method of gene synthesis and a denaturating gel separation (Borodina et al., 2003). This study was also based on the basic principles of gene synthesis methods, but instead of having a biotinylated modification or using denaturating gels, PTO modification was introduced which facilitated the exonuclease digestion of all the unwanted byproducts of the construction reaction. Also this modification does not interfere with the further reactions like ligation (technical information offered by Thermo scientific and Fermentas). The 'Stepwise based assay' described in this study, was a new approach tried to prevent the accumulation of unwanted byproducts during the reaction and thus avoid the intermediate washing steps. The use of 'Ligation and polymerization based assay', introduced in this study for constructing single stranded DNA, reduces the number of oligonucleotides required compared to what is required during the construction of genes. The fill-in reaction followed by the ligation reaction was the distinctive difference between Joachim's PCR based gene synthesis method and the method designed for this study, 'Ligation and polymerization based assay'. Few companies do offer padlocks as long as 120 bases, but the cost is extremely high and also the amount of phosphorylation at the 5' end of the padlock is poor (observed by F. Rühle). As one of the aims of the study was to construct 120 bases padlocks at a low cost, below in Table 4.1 and

Table 4.2 a cost comparison of concentration of 125 pmol of the synthetically produced padlocks and the constructed padlocks using the standardized 'Ligation and polymerization based assay' is reported. While calculating the yield and costs for construction of padlocks, the amplification factor is not considered. Only the final concentration after construction is considered for the constructed padlocks. When

the amplification factor will be taken into account the cost of the reaction will go down further.

Table 4.1 Cost of padlock probes constructed using the phosphoramidite chemistry without phosphorylation

REQUIREMENTS	AMOUNT SUPPLIED	PRICE (per cup ordered) (tax included) (Euros)	PRICE FOR 125 pmol (Euros)
Synthetic padlock (120 bases)	1.3 nmol	135.84	16.98

Table 4.2 Cost report for construction of 120 bp probes using the Ligation and polymerization based assay

REQUIREMENTS	AMOUNT SUPPLIED	PRICE (per cup ordered) (tax included) (Euros)	AMOUNT REQUIRED FOR ONE REACTION	PRICE FOR ONE REACTION (Euros)
Oligo 1 (45 bp)	5.9 nmol	68.3	0.25 nmol	2.89
Oligo 2 (44 bp)	24.6 nmol	20.24	0.25 nmol	0.21
Linker segment (74 bp)	18.8 nmol	54.76	0.125 nmol	0.36
Phi 29 DNA Polymerase (10 U/µl)	250 U	64.96	10 U	2.6
Helper Oligo(27 bp)	44.6 nmol	14.41	0.1 nmol	0.032
DTT	5 g	40.6	4 µmol	0.21
BtgZ I (2 U/µl)	50 U	76.56	2 U	3.06
T4 DNA polymerase (3 U/µl)	150 U	75.4	2.5 U	1.26
dNTP (10 mM)	0.2 ml	18.79	4 µM	0.94
T4 DNA Ligase (400 U/µl)	20,000 U	81.2		0.97
Exonuclease III (200 U/ µl)	4000 U	23.78	25 U	0.15
			TOTAL	**= 12.682**

The next focus was on constructing padlocks with repetitive sequences incorporated in them, as all the gene synthesis papers published from 1970 till date (Engels et al., 1989; Gibson et al., 2008), there is always a special mention about the uniqueness of the overlapping sequences. All the protocols published do mention the additional care that was taken while designing these oligonucleotides for constructing the genes, so that there are no repetitive sequences at least near the overlapping areas as that would give to severe secondary structures and lower the concentration of the desired product drastically. Although gene synthesis is

commercially a routine procedure, but due to the incorporation of repetitive sequences in the padlocks, it was denied. But the incorporation of repetitive sequences in the padlock is critical for the Kotenkar-Rühle-Kracht approach. Out of the eight gene synthesis companies contacted for this purpose only two companies agreed to deliver a long padlock with six repetitive sequences incorporated. But only ShineGene, China, who at the end of 2007 have started the synthesis of gene, agreed to deliver it finally at a very low price. But the first tests using the ShineGene padlocks did not give usable results (Figure 3.38), further analysis of this probe is necessary to confirm its functionality.

To enable construction with the repetitive sequences, the two enzyme system was changed to one enzyme system, to reduce complexity of the reaction. The basic design format for gene synthesis by Khorana (Khorana et al., 1970) was followed, with the difference of using the repetitive sequences instead of unique sequences and changes in the experimental conditions. When these new sets of oligonucleotides were used for construction using varying ratios and conditions, it just gave rise mainly to the formation of a circular product with the repetitive sequences of rCR3DVBS- rCR3DVBS (Figure 3.8). Since the repetitive sequences are abundant, the possibility of finding maximum hybridizing reactions among the repetitive sequence cannot be avoided (Engels et al., 1989). When only the repetitive sequences were hybridized the band patterns observed were similar to when all the oligonucleotides were added for hybridization, indicating that the products formed contain predominantly the repetitive sequences. After both the constructs were digested, the strong band observed in the both the cases was left untouched, signifying the presence of circular products which cannot be digested. This corresponds to the results published by Bang in Nature methods (Bang et al., 2008).

To control the circular product formation, stepwise construction method and bead based construction method were endeavored. In the bead method stringent washing conditions to get rid of the unbound oligonucleotides was possible and in the Stepwise based method the concentration regulation limited the formation of

unwanted products. Also in both these methods, the chances for the formation of truncated products are not restricted and cannot be avoided. But the circularization reaction acts like the first filter, where only the molecules having the both target specific sequences can be circularized. Even if low activity of single stranded blunt end ligation is observed or circularization of the repetitive sequences which cannot be avoided, they either won't be amplified. Even if they are amplified, they won't be cleaved if both the target specific fragments are not incorporated (second filter), thus delivering the desired padlocks. The Stepwise method produced padlock with three repetitive sequences incorporated.

Another, less time consuming, easy to handle and robust method was worked on in the scope of this study. Here separate pre-ligation reactions were carried out and later these pre-ligated products were mixed together in specific ratios. Manipulating the varying ratios proved that one single desired product could be obtained (Figure 3.29). Using this method aspiration for obtaining one single product containing three binding sites was fulfilled. Sequencing of this padlock gave the expected sequence. Thus padlocks with three repetitive sequences could be constructed.

One of the problems before sequencing was of deciphering whether the product obtained after the construction reaction was the anticipated one or not. As the gel based methods were used for detection of the products based on their sizes, it was extremely challenging to clarify whether the bands obtained are the desired ones. The main reason for this was the unavailability of single stranded markers. Basically the running behaviour of single stranded DNA or oligonucleotides on an agarose gel is dependent on their sequences and the secondary structures formed. Denaturing polyacrylamide gels were used, but the bands obtained could not be compared to the ladder, as the ladder was double stranded. Most of the products obtained were partially double stranded (basically single stranded with some parts being double stranded) after construction, hence interpreting was tedious. Use of Southern blotting and polyacrylamide gels with urea helped in the understanding

of the bands. Later, obtaining the sequences confirmed the interpretation to be right and hence in this case it can be concluded that the single stranded DNA on 2 % agarose ran almost similarly as a double stranded DNA would run. Also for most of the detection on the gel, ethidium bromide was replaced by SYBR Green I as SYBR Green stains are less carcinogenic (Singer et al., 1999), they are at least 5 times more sensitive (Invitrogen product manual) and are extremely light sensitive thus facilitating usage of the gel extracted products without much contamination.

4.2 Amplification of the padlocks

Rolling circle amplification was the method of choice for amplifying the constructed single stranded padlocks. During rolling circle amplification the original template is always used as the source, unlike that in PCR, where the products generated in one cycle become the templates for the next cycle, thus increasing the error during construction. Also the phi 29 DNA polymerase is extremely efficient for rolling circle amplification as it exhibits high processivity (> 70 kb) and is easily able to displace the double strand during amplification by its strand displacing activity without the need of processivity factors and helicases (Blanco et al., 1989; Blanco & Salas, 1996). More than ~ 1000 monomers of the desired product can be generated within one hour. Also longer incubation with the enzymes results in more product unlike PCR where the increase in the number of cycles generates false product. From the experiments it was observed that rolling circle replication is extremely sensitive and robust. It can also easily generate product if there only 100 circularized starting templates (Pe´rez-Arnaiz et al., 2006). The critical part was the circularization reaction. Inefficiency of circularization reaction led to the failure ingenerating a rolling circle product. If the template concentration of the molecules to be circularized is very high, then circularization reaction fails as more linear ligations occur. The other way round when the template to be circularized is extremely low, it is difficult for both the ends of the template to come together and ligate and hence results in failure of circularization

reaction (Zimmerman et al., 1983). Rolling circle amplification step was introduced for construction, to increase the concentration of the functional padlocks. Rolling circle was previously used for amplifying oligonucleotides upto 80 bases (Kool et al., 1996). But only recently Lohmann published using rolling circle amplification for amplifying the constructed padlocks (Lohmann et al., 2007). But the padlock probes constructed were not more than 100 bases due to the restrictions introduced by the chemical synthesis of longer oligonucleotides.

4.3 Generation of functional padlocks

Generation of functional padlocks from the rolling circle amplified product was initially done as described by Dahl (Dahl et al., 2005), by adding helper oligonucleotides for creating double stranded regions and cleavage using Class II restriction enzymes. The restriction digestion reaction generates 5′ phosphate and hence additional phosphorylation reaction was not required. Although using this method functional padlock was generated, there was a need of purifying the cleaved product from the cut helper oligonucleotide which is a tedious task. Also a hindrance was crafted of having a restriction site near the target binding regions of the padlock. This restricts the flexibility of choosing the padlock binding region on the target DNA. Also for SNP detection, not always there is a single nucleotide base change near the restriction sites. Therefore this method is not practical for facilitating the wide usage of padlocks. Alternative would be the usage of deoxyribozymes (Nir Carmi et al., 1998) for cleaving the rolling circle product. Though the deoxyribozyme binding design is not restricted to a specific binding position like in the case of Class II restriction enzymes, it requires a stretch of four purines in a row for binding and cleavage. Additional to the tedious designing, the use of deoxyribozymes also requires an additional purification step, thus increasing the costs and decreasing the concentration of the desired padlocks.

The use of Class IIS restriction enzymes overcame the problems faced by the above methods. The property of Class IIS enzymes of cleaving few bases away from their

recognition site (Takanami et al., 1974), removed the prerequisite of restriction enzymes having their site near on the target specific regions of the padlock probes. The criteria for selecting the Class IIS restriction enzyme was the number of bases it cuts away from its recognition site. BtgZI and GsuI enzymes were selected over the other 17 enzymes that fit into the selecting filter as these two enzymes had the least number of technical difficulties listed by the supplier. When using a helper molecule and both these enzymes, cleavage reaction was done, the required product was generated. Also to avoid having any restrictions of having the recognition site for these enzymes on the target specific regions, stem-loop helper oligonucleotides with recognition sites on the stem- loop were used but it was found in efficient to cleave the rolling circle product completely. BtgZI enzyme was chosen over GsuI enzyme for its ability to cleave single stranded DNA without the use of any helper oligonucleotides (data to be published), thus avoiding the need for purification.

As the rolling circle product contains leftover dNTPs and primers for initiating the rolling circle reaction, using the product directly after cleavage would give wrong readings of the actual concentration. Also these residual artefacts may hinder in the further reactions. Purifying the rolling circle product using the sodium acetate ethanol precipitation and cleaving the concatemer with BtgZI enzyme helped generate functional padlock probes with as relatively high concentration as 1.15 µM (ShineGene, China, delivered 0.77 µM).

The main problem arises when after construction there are two or more padlocks additional to the desired padlock. Gel extraction after the BtgZI digestion failed as most of the gel extraction kits are specific for double stranded DNA. Standardizing a protocol for gel extraction of the single stranded DNA could not be covered in the scope of this work. But it was found, that HPLC separation could easily separate the desired padlock from the rest, resulting in the method of choice.

The work done in the scope of this project suggests new methods that can be used for the construction of padlocks overcoming some of the shortcomings of the

methods used so far. Also, as these methods do not require any expensive machinery or complicated methodology it can be easily used routinely. If the desired length of padlocks can be extracted directly after construction and then amplified, the concentration of the padlocks would increase further. The yield calculation clearly states that higher concentration of functional padlocks can be generated using the above methods. Here basically lab scale synthesis was worked on and one has to consider the possibilities of large scale synthesis of these padlocks. Also, for the incorporation of more number of repetitive sequences more work needs to be done. The attempt to design new methods, which would facilitate construction of padlocks, irrespective of their size or sequence was not fulfilled completely.

120 bases padlock was constructed in high concentration with phosphorylated 5' ends and the sequencing reactions confirmed the sequence as desired fulfilling the first part of the aim. The 'Ligation and polymerization method' used for the construction of 120 bases padlock is a new method for constructing single stranded probes. But this method fails when repetitive sequences are involved during construction.

The second part of the aim was not accomplished completely as a protocol for the incorporation of only three repetitive sequences in the padlock could be standardized. The other factors such as high concentration, exact sequences at the 3' and the 5' end of the padlock and phosphorylation of the padlock were fulfilled nevertheless. For the Kotenkar-Rühle-Kracht approach, it would be desirable to have more repetitive sequence incorporated. All the three methods used, Stepwise based assay, Bead based assay, and Pre-ligation based assay, did give padlocks with three repetitive sequence incorporated in them. But using the 'Pre-ligation based assay', a pure construct containing only padlocks with three repetitive sequences was achieved unlike the other two methods, where a mixture of one, two and three repetitive sequences incorporation was observed.

Three papers based on the results of this study are in preparation. The first paper deals with the alternative method of constructing functional padlocks using the Ligation based assay. As the construction with repetitive sequences has not been attempted before, the second paper deals with difficulties involved during construction using repetitive sequence and a possible method for incorporation of at least three repetitive sequences in the padlock. The last paper deals with the novel activity of the BtgZI enzyme to cleave single stranded DNA, which has not been published before.

5 OUTLOOK

The use 'Ligation and polymerization method', in combination with rolling circle amplification and BtgZI digestion can be the basis of a new method for constructing error free functional padlocks in high concentration, as was aimed in the first part of this study. This method fulfills all the prerequisites necessary for the popular use of these probes. But using this method would just provide an alternative efficient method for construction than the pre-existing once. The real challenging part of constructing padlocks with incorporation of the desired number of repetitive sequence was not completely fulfilled. The methods used in the scope of this study enabled the possibility of having up to three repetitive sequences incorporated in the padlock.

The challange of constructing the padlocks with desired and variable number of repetitive sequences in its linker segment requires to overcome the following challenges:

1. The occurrence of circles of the repetitive sequences: Though in this study the occurrences of the repetitive sequences were limited by the use of stringent washes and controlled concentration of the oligonucleotides used during construction, but could not be avoided totally.
2. Formation of truncated products: The construct also consisted of truncated molecules that can affect the identification of the desired products, as the truncated molecules consisting of the oligonucleotide with PTO modification are resistant to digestion.

Overcoming these difficulties will definitely give a solution for constructing large probes or DNA consisting of repetitive sequences.

ABREVIATIONS

µl	microliter
µM	micromolar
A	Adenine
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
BAA	Bisacrylamide (N,N'-Methylenebisacrylamide)
bp	base pair (s)
BS	Binding segment
BSA	Bovine Serum Albumin
C	Cytosine
$CuCl_2$	Copper (II) chloride
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dNTPs	deoxinucleotidetriphosphates
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
G	Guanine
hrs	hours
ddH_2O	double distilled water
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kb	kilo bases
LS	Linker segment
M	Molar
min	minutes
$MgCl_2$	Magnesium chloride
m^2	Metre square
ml	milliliter
mJ	millijoule

mM	millimolar
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
nM	nanomolar
nm	nanometre
nmol	nanomol
nt	nucleotide (s)
pmol	picomol
pM	picomolar
PCR	Polymerase Chain Reaction
PTO	Phosphorothioate
RCA	Rolling circle replication
rC	reverse complementary
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulfate
sec	seconds
SNP	Single Nucleotide Polymorphism
SSC	sodium chloride sodium citrate
T	Thymine
TAE	Tris-acetate- EDTA containing buffer
TBE	Tris-borate- EDTA containing buffer
TE	Tris-HCl- EDTA containing buffer
TEMED	N, N, N', N'-tetramethylethylenediamine
Tm	melting temperature of complementary nucleic acids
Tris-HCl	2-amino-2-(hydroxymethyl) propane-1,3-diol hydrochloride
TS	Target specific binding regions
U	Units
V	Volt

TABLE OF FIGURES

TABLE OF TABLES

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APPENDIX

A1. Different variation of conditions used for the different construction methods

A1.1 Ligation and polymerization based system

1. Hybridization of rCR1DVTS1 (TS1), rCR1DVPTOTS2 (TS2) and R1DVHeLS (LS)

a. TS1:TS2: LS= **1:1:2**

- 4°C 30 min, 68°C cool to 47°C, 47°C 10 min, 90°C 10 min, cool to RT
- 37°C 10 min, 90°C 10 min, cool to 10°C
- 4°C -60 min, 68°C- 47°C, 47°C 10 min, 90°C 10 min, cool to RT
- 37°C 15 min, 90°C 3 min, cool to 40°C, 80°C 1 min, cool to 30°C

b. TS1:TS2: LS= **1:1:1.5**

- 37°C 10 min, 90°C 10 min, cool to 10°C
- 37°C 15 min, 90°C 3 min, cool to 40°C, 80°C 1 min, cool to 30°C

c. TS1:TS2:LS= **1:1:7.5**

- 4°C 30 min, 68°C- 47°C, 47°C 10 min, 90°C 10 min, cool to RT

d. TS1:TS2:LS= **3:3:1.5**

- 4°C -30 min, 68°C- 47°C, 47°C 10 min, 90°C 10 min, cool to RT

e. TS1:TS2:LS= **1:1:20**

- 37°C 15 min, 90°C 3 min, cool to 40°C, 80°C 1 min, cool to 30°C

f. TS1:TS2:LS= **20:20:1**

- 37°C 15 min, 90°C 3 min, cool to 40°C, 80°C 1 min, cool to 30°C

g. TS1:TS2: LS= **1:1:1.2**

- 37°C 15 min, 90°C 3 min, 90°C-40°C, 40°C 1 min, 80°C 1 min, cool to 30°C
- 37°C 15 min, 90°C 3 min, 90°C-20°C in thermocycler 5 min/°C
- 37°C 15 min, 90°C 3 min, 90°C-75°C in thermocycler 2 min/°C, 75°C-30°C in thermocycler 30 min/°C, 30°C-15°C in thermocycler 2 min/°C

A1.2 Continuous ligation based system

Following ratios were tested before fixing on the final ratio. Also various hybridization conditions were tested as listed below:

1. **Hybridization of rCR2DVTS1(TS1), rCR2DVPTOTS2 (TS2) and R2DVHeLS (LS), rCR2DVBS(BS),**

a. TS1:TS2:BS:LS= **1:1:1.2:1.5**

- 37°C 15 min, 90°C 3 min, 90°C-40°C, 40°C 1 min, 80°C 1 min, cool to 30°C
- 37°C 15 min, 90°C 5 min, 90°C-40°C, 40°C 1 min, 80°C 1 min, cool to 30°C

b. CS:LS= **1.2:1.5** heat and cool and add TS1:TS2:= **1:1**

- 37°C 15 min, 90°C 3 min, 90°C-40°C in thermomixer, 40°C 1 min, 80°C 1 min, 80°C-30°C, 30°C 120 min

c. TS1:TS2: LS= **1:1:1.5**

- 37°C 15 min, 90°C 5 min, 90°C-40°C, 40°C 1 min, 80°C 1 min, cool to 30°C

d. TS2:BS: LS= **1:1.2:1.5**

- 37°C 15min, 90°C 5min, 90°C-40°C, 40°C 1min, 80°C 1min, cool to 30°C

e. TS1:BS: LS= **1:1.2:1.5**

- 37°C 15min, 90°C 5min, 90°C-40°C, 40°C 1min, 80°C 1min, cool to 30°C

f. BS: LS= **1.2:1.5**

- 37°C 15min, 90°C 5min, 90°C-40°C, 40°C 1min, 80°C 1min, cool to 30°C

g. BS: LS= **1.2:1.5**

- 37°C 15min, 90°C 5min, 90°C-40°C, 40°C 1min, 80°C 1min, cool to 30°C
  Add to the above mixture TS1:TS2= **1:1,** 30°C 30min.
- 95°C 5min, 95°C 30sec, 80°C 1min, 70°C 2min, 60°C 3min, 50°C 4min, 40°C 5min, 35°C 5min, 30°C 5min, 25°C 5min, 20°C 30min
  Add to the above mixture TS1:TS2= **1:1,** 30°C 60min.

h. BS: LS= **1:1**

- 95°C 5min, 95°C 30sec, 80°C 1min, 70°C 2min, 60°C 3min, 50°C 4min, 40°C 5min, 35°C 5min, 30°C 5min, 25°C 5min, 20°C 30min
  Add to the above mixture TS1:TS2= **0.5:0.5,** 30°C 60min.
- 95°C 5min, 95°C 30sec, 80°C 1min, 70°C 2min, 60°C 3min, 50°C 4min, 40°C 5min, 35°C 5min, 30°C 5min, 25°C 5min, 20°C 30min

Add to the above mixture TS1:TS2: LS= **0.7:0.7:0.2,** 30°C 60min.

- 37°C 45min, 90°C 5min, 95°C – 40°C, 40°C 15min, 80°C 1min, 80°C – 30°C, 30°C 30min

2. New strand synthesis and ligation using polymerase and ligase

a. 12°C 2 hrs, 16°C 2 hrs, 75°C 20 min

b. 12°C 12 hrs, 16°C 12 hrs, 75°C 20 min

c. 12°C 12 hrs, 75°C 20 min

d. 12°C 6 hrs, +Ligase, 16°C 6 hrs, 75°C 20 min

e. 2°C 2 hrs, +Ligase, 16°C 2 hrs, 75°C 20 min

f. 12°C 12 hrs,+ Ligase, 16°C 12 hrs, 75°C 20 min

g. 12°C 6 hrs, +Ligase, 12°C 6 hrs, 75°C 20 min

A1.3 Pre-ligation based system

1. Hybridization of rCR3DVTS1 (TS1), rCR3DVPTOTS2 (TS2), rCR3DVBS (BS) and R3DVHeBS-BS (HeBS-BS)

a. TS1:TS2:BS:Circ= **1:1:1:1**

- (+/- Ampligase) (94°C 4min, (94°C 30sec, 45°C 5min)X 20, 45°C 60 min)

b. TS1:TS2:Circ:BS=**1:1:1:3**

- (+/- Ampligase) (94°C 4min, (94°C 30sec, 45°C 5min)X 20, 45°C 60 min)

c. TS1:TS2:Circ:BS=**3:3:3:1**

- (+/- Ampligase) (94°C 4min, (94°C 30sec, 45°C 5min)X 20, 45°C 60 min)

d. BS: HeBS-BS= **1.2:1**

- (+/- Ampligase) (94°C 4min, (94°C 30sec, 45°C 5min)X 20, 45°C 60 min)
- (+/- T4 Ligase) (94°C 5min, 16°C 12hrs, 85°C 15 min)

e. Pre ligated (TS1:BS)(1:1) + BS: HeBS-BS= **1:1**

- (+ Ampligase) (94°C 4min, (94°C 30sec, 45°C 5min)X 20, 45°C 60 min)

- (+ /-Ampligase, +/-40% PEG) (94°C 4min, (94°C 30sec, 45°C 5min)X 20, 45°C 60 min)

f. Pre ligated (TS1:BS)(1:1) + BS: HeBS-BS = **1:10**

(+/- Ampligase) (94°C 4min, (94°C 30sec, 45°C 5min)X 20, 45°C 60 min)

g. BS: HeBS-BS = **1:1**

(Concentrations tested: 40µM, 20µM, 10µM, 5µM, 1µM, 700nM, 500nM and 250nM)

- (95°C 5min, 30°C 1min) X2, Phi 29polymerase, 37°C 30min, 65°C 15min, +T4 Ligase, 16°C 12hrs, 65°C 10min.
- (+T4 Ligase, +11%PEG) (20°C 60min, 65°C 10min)
- (+/-T4 Ligase, 10%PEG, 150mM NaCl) (37°C 60min, 65°C 10min)
- (+ Ampligase (0.1U/µl, 0.05U/µl, and 0.01U/µl) (94°C 4min, (94°C -30sec, 45°C 5min)X 25, 45°C 120 min)
- (+ Ampligase (0.1U/µl)) (94°C 4min, (94°C 30sec, 30°C 5min)X 25, 30°C 120 min)
- (+ Ampligase (0.1U/µl)) (94°C 4min, (94°C 30sec, 50°C 5min)X 25, 30°C 120 min)
- (+ Ampligase (0.1U/µl)) (94°C 4min, (94°C 30sec, 60°C 5min)X 25, 30°C 120 min)
- (+ Ampligase (0.5U/µl) (94°C 4min, (94°C 30sec, 50°C 5min)X 10, 50°C 60 min)
- (+ Ampligase (0.5U/µl) (94°C 4min, (94°C 30sec, 50°C 5min)X 4, 50°C 60 min)
- (+ /-Ampligase (0.5U/µl) (94°C 4min, (94°C 30sec, 50°C 5min)X 1, 50°C 60 min)
- (+/-Ampligase (0.2U/µl)) (94°C 4min, (94°C 30sec, 50°C 5min)X 10, 50°C 60 min)
- (+/-Ampligase (0.2U/µl)) (90°C 4min, (90°C 30sec, 40°C 3min, 45°C 3min, 50°C 3min, 55°C 3min, 60°C 3min)X 15)
- (+Ampligase (0.1U/µl, 0.2U/µl)) (90°C 4min, (90°C 30sec, 40°C 3min, 45°C 3min, 50°C 3min, 55°C 3min, 60°C 3min)X 15)

h. BS: HeBS-BS_ lng= **1:1**

(Concentrations tested: 10µM, 100nM)

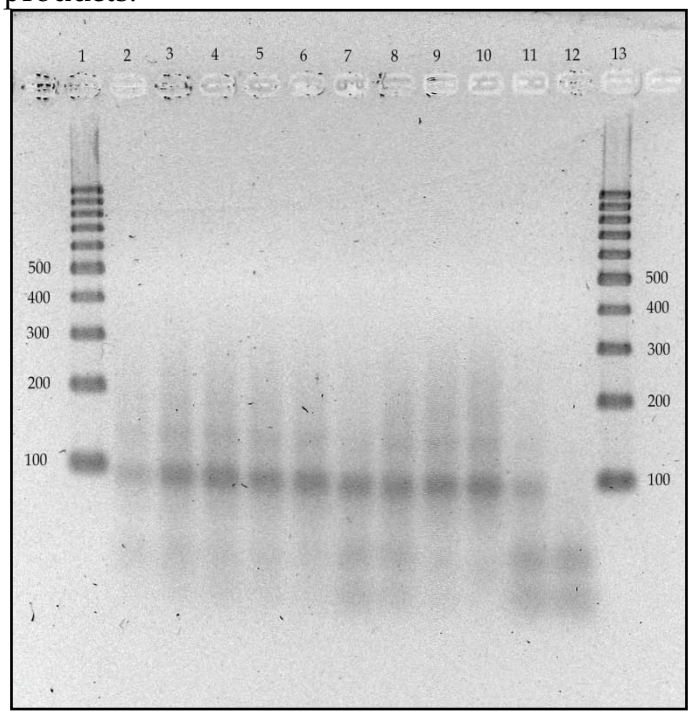
- (+/-Ampligase (0.2U/µl) (94°C 4min, (94°C 30sec, 50°C 5min)X 10, 50°C 60 min)
- (+/-Ampligase (0.2U/µl) (90°C 4min, (90°C 30sec, 40°C 3min, 45°C 3min, 50°C 3min, 55°C 3min, 60°C 3min)X 15)

A2. Results of the block based method

As the block based method gave uninterruptable results only all the fragments were used for construction. Hence only two fragments (rCR3DV BS and R3DVHeBS-BS) were used to construct multimeric structure, to which later the target specific sequences could be added. The previous constructions using rCR3DV BS and R3DVHeBS-BS were used as a starting material for the next reactions in a hope that longer products would be formed.

Also drastic ratios of the fragments were taken to observe the changes in the length of the product formed. PEG was also used to see its influence on the product. Different cycle nos., enzyme amounts, concentration of fragments and annealing temperatures were also varied. Although these variations gave different patterns on the gel, none of them gave the desired multimeric structure. Most of the tests just gave a smear without any bands, other than the circularized product, being formed. The main drawback was the formation of circular products which would be predominant in the system. Though other multimeric structures might be present, their concentration is extremely low. Hence this method proved futile and alternative methods were worked on to achieve the goal of constructing padlocks with repetitive sequences incorporated in them. The figure below shows some of the results obtained by varying the enzyme concentration and template concentration. As seen in the figure a distinct band is observed near the 100 bp

mark of the ladder. This band was proved to be BS-BS circles after exonuclease digestion of the products.



Construction of padlocks using the Block based method by varying the enzyme concentration and template concentration

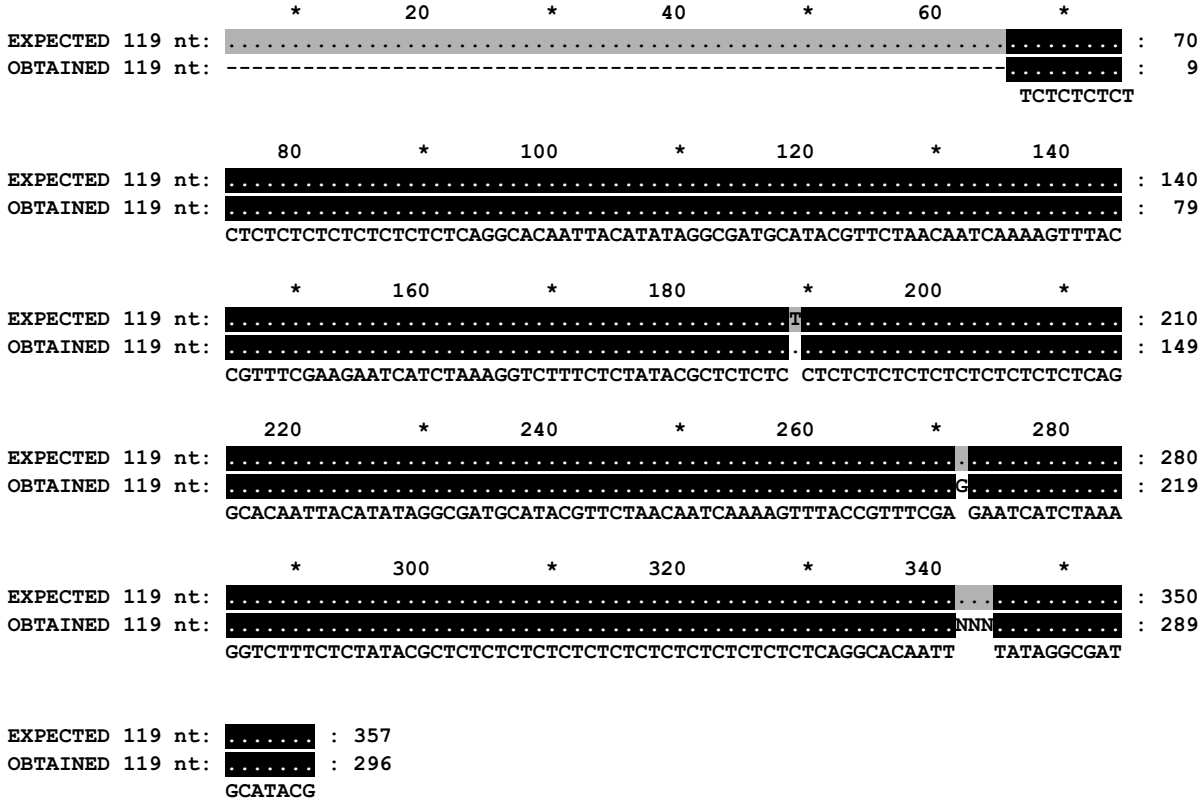
KEY:

1. 100bp Ladder
2. Pl construction with BS: HeBS_BS =**1:1 (250nM) with Ampligase (0.1U/µl)** and annealing temp **45^{0}C**
3. Pl construction with BS: HeBS_BS =**1:1 (500nM) with Ampligase(0.1U/µl)** and annealing temp **45^{0}C**
4. Pl construction with BS: HeBS_BS =**1:1 (700nM) with Ampligase(0.1U/µl)** and annealing temp **45^{0}C**
5. Pl construction with BS: HeBS_BS =**1:1 (500nM) with Ampligase(0.1U/µl)** and annealing temp **45^{0}C**
6. Pl construction with BS: HeBS_BS =**1:1 (500nM) with Ampligase(0.05U/µl)** and annealing temp **45^{0}C**
7. Pl construction with BS: HeBS_BS =**1:1 (500nM) with Ampligase(0.01U/µl)** and annealing temp **45^{0}C**
8. Pl construction with BS: HeBS_BS =**1:1 (500nM) with Ampligase(0.1U/µl)** and annealing temp **30^{0}C**
9. Pl construction with BS: HeBS_BS =**1:1 (500nM) with Ampligase(0.1U/µl)** and annealing temp **45^{0}C**
10. Pl construction with BS: HeBS_BS =**1:1 (500nM) with Ampligase(0.1U/µl)** and annealing temp **50^{0}C**
11. Pl construction with BS: HeBS_BS =**1:1 (500nM) with Ampligase(0.1U/µl)** and annealing temp **60^{0}C**
12. Pl construction with BS: HeBS_BS =**1:1 (500nM) without Ampligase** and annealing temp **45^{0}C**
13. 100bp Ladder

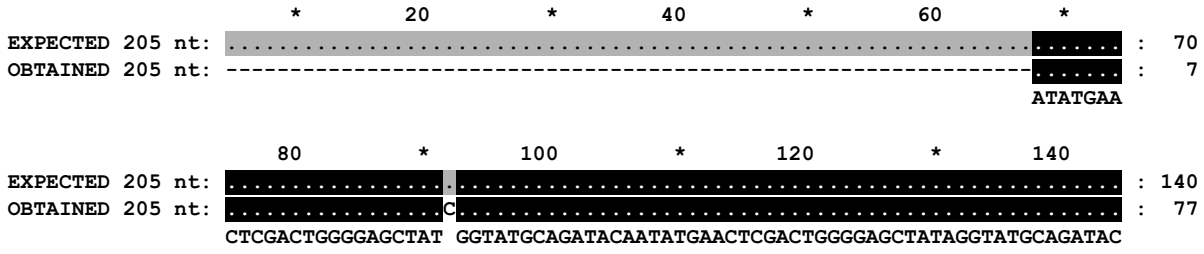
A3. Sequencing of the constructed padlocks

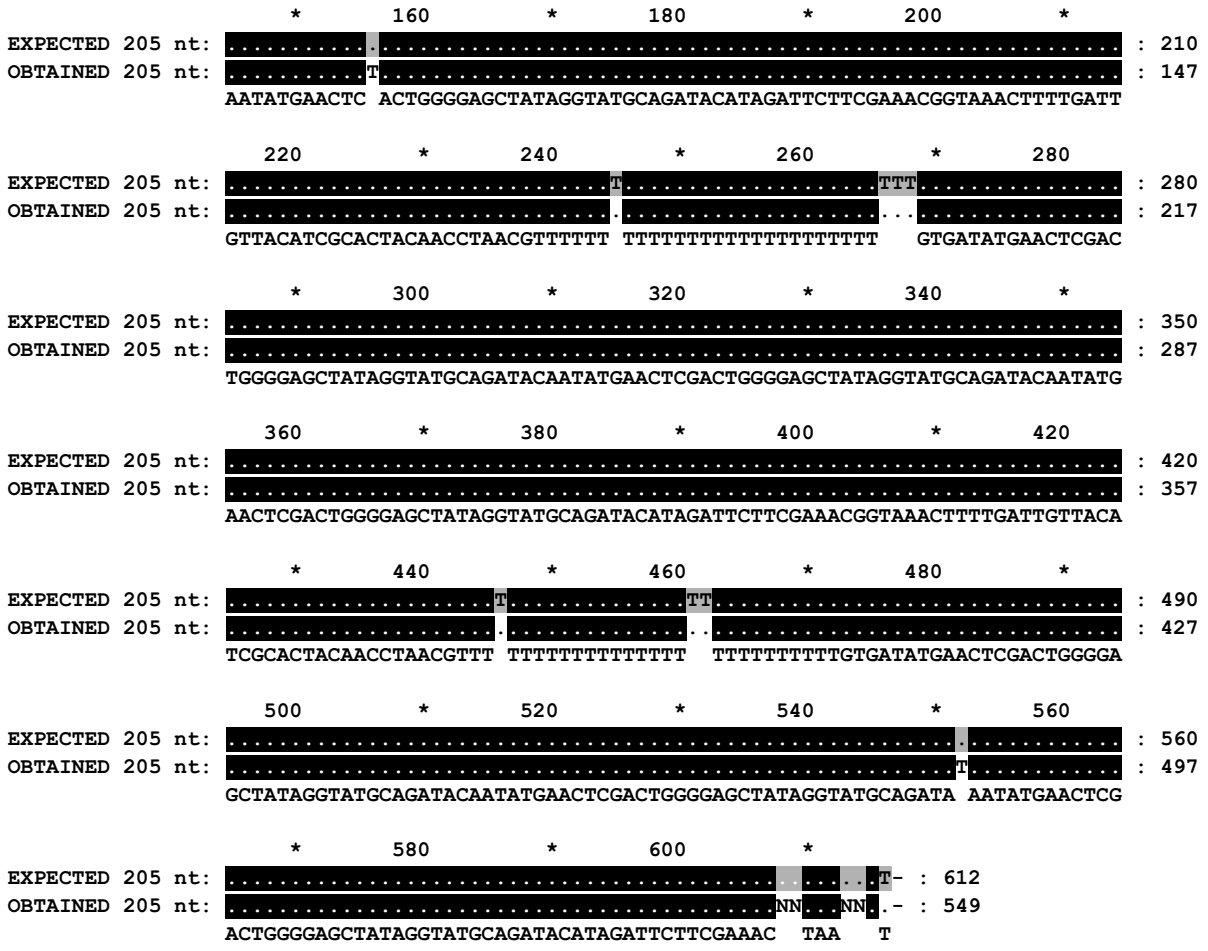
The rolling circle products of the constructed padlocks were sequenced. Below is the comparison of the expected sequences to the sequence obtained of the rolling circle product after construction.The doted parts and the parts highligted in black indicate homology between the expected and obtained sequences of the rolling circle products of the constructed probes.

1. Sequenced rolling circle product of the 120 bases padlock probe



2. Sequenced rolling circle product of the 205 bases padlock probes




* 160 * 180 * 200 *
EXPECTED 205 nt: : 210
OBTAINED 205 nt: : 147
AATATGAACTC ACTGGGGAGCTATAGGTATGCAGATACATAGATTCTTCGAAACGGTAAACTTTTGATT
220 * 240 * 260 * 280
EXPECTED 205 nt: : 280
OBTAINED 205 nt: : 217
GTTACATCGCACTACAACCTAACGTTTTTT TTTTTTTTTTTTTTTTTTTT GTGATATGAACTCGAC
* 300 * 320 * 340 *
EXPECTED 205 nt: : 350
OBTAINED 205 nt: : 287
TGGGGAGCTATAGGTATGCAGATACAATATGAACTCGACTGGGGAGCTATAGGTATGCAGATACAATATG
360 * 380 * 400 * 420
EXPECTED 205 nt: : 420
OBTAINED 205 nt: : 357
AACTCGACTGGGGAGCTATAGGTATGCAGATACATAGATTCTTCGAAACGGTAAACTTTTGATTGTTACA
* 440 * 460 * 480 *
EXPECTED 205 nt: : 490
OBTAINED 205 nt: : 427
TCGCACTACAACCTAACGTTT TTTTTTTTTTTTTT TTTTTTTTTTGTGATATGAACTCGACTGGGGA
500 * 520 * 540 * 560
EXPECTED 205 nt: : 560
OBTAINED 205 nt: : 497
GCTATAGGTATGCAGATACAATATGAACTCGACTGGGGAGCTATAGGTATGCAGATA AATATGAACTCG
* 580 * 600 *
EXPECTED 205 nt: : 612
OBTAINED 205 nt: : 549
ACTGGGGAGCTATAGGTATGCAGATACATAGATTCTTCGAAAC TAA T

A4. Bioluninescence reaction data

The values obtained for the bioluminescence reaction is given below. This reaction was used to compare the functionality of constructed probes and synthetically ordered ones. There were three replicates done of each reaction.

	Replikates					
Daten bei 19,95 sec	R1	R2	R3	Mean	Stabw	Stabw %
L1SG Ligation	58790	38233	32043	43022	14001.8353	32.5457565
L3SG Lig NC	32600	33390	32576	32855	463.190386	1.40978751
L4SG PL only	27471	28019	28667	28052	598.696362	2.13421235
L5SG no PL	18424	17324	15890	17213	1270.66334	7.38214112
L6 primer	11943	11690	10562	11398	735.249844	6.45050309
L1SG Lig- exo	19081	18362	18610	18684	365.2182	1.95467611
L3SG Lig NC- exo	13800	14386	14362	14183	331.616244	2.33817978
L1RK Ligation	114524	113133	113638	113765	704.142741	0.61894497
L3RK Lig NC	97633	110324	112362	106773	7980.79326	7.47454249
L1RK Lig-exo	56762	55952	61067	57927	2749.31355	4.7461694
L3RK Lig NC-exo	82276	88943	89481	86900	4013.52626	4.61855726
L4RK PL only	156871	156643	154962	156159	1042.595	0.66765106
L5RK no PL	14976	14986	13405	14456	909.917762	6.29454029
Buffer	3138	3124	3219	3160	51.2867754	1.62282804