"Development of a Localized Circle-to-Circle Amplification Protocol for Sensitive DNA Analysis"

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SUMMARY

There is a very famous quote by Black Elk that says – “The power of the world always works in circles”. Researchers applied it into the field of life science and there came the discovery of the versatile new technology called the Rolling Circle Amplification (RCA). The invention of this new technique is really an important advantage to the various sectors like molecular diagnostics, laboratory research and also to pharmacogenomics. The technology is very reliable while taking into account the various important parameters like sensitivity, specificity, selectivity, detection efficiency, robustness and reproducibility. RCA is a generation of linear polymers, by making use of a small, single stranded, circular DNA probe called padlock probe (≈ 100 nucleotides in length), in a number from hundreds to billions in an isothermal invitro method all because of the hybridization-sparked enzymatic reactions.

RCA has now come up with its extension called Circle to Circle Amplification (C2CA). The basic point here is to subject the first RCA products to second round of rolling circle amplification. This is done by monomerizing the first RCA product that will then serve as padlock probes for the capture probes of second generation RCA. This technology so far has proved to be excellent when performed in-solution.

So the aim of my project was to transfer the C2CA assay from solution to a solid phase so that the RCA product can be localized on the solid phase. The method could be applied in DNA sequencing for easy library preparation and also for rapid diagnostic techniques coupled to padlock detection. To fulfill the target three designs were framed termed as Restriction Design, Nicking Design 1 and Nicking Design 2 based on the results obtained. The general setup for the whole project consists of a solid phase (magnetic beads) to which the two capture probes are coupled by streptavidin-biotin binding. 1RCA and 2RCA are then generated on these S1 and S2 probes, respectively. In all the three plans the difference was in either the architecture of the S1 and S2 probes or the sequence of the steps taken to carry out the whole reaction.

Till now we have been successful in optimizing various reaction steps that include hybridization and ligation of the padlock to S1, nicking of the S1 probe and finally the first round of rolling circle amplification for the 1RCA. The difficulty was after the 1RCA while moving towards the 2RCA. For the restriction design the digestion with MboI after 1RCA for getting the monomers that will work as templates for 2RCA worked fine but the ligation was not acceptable. So the nicking design 1 was adopted assuming that the ligation of a single strand will be easier along with using low Tm. The monomerization was again satisfying but this time also a very little ligation was observed and therefore nicking design 2 was planned. The main scheme here was the addition of a ligation oligo for better ligation results. Finally together with the nicking we also got acceptable ligation. This is where we are now and the future plan includes the optimization of this ligation step for the start of 2RCA together with the further optimization of incubation time and temperature.
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1. INTRODUCTION

1.1. From Cells To DNA

The planet Earth on which we reside basically consists of two types of entities—the living things and the non-living things. Carbon, hydrogen, oxygen and nitrogen are the four main chemical elements that comprise both of them. The way the molecules made by these elements organize themselves into cells distinguishes the living things from all other matter. The Cell is the smallest functional building unit of life. All living objects are made of one or more cells whether it is a tiny bacterium or the largest whale. So, a cell can be considered to be that precious gem without which the life will be impossible. On the basis of the cell theory, cells contain hereditary information that is transferred to other cells during the process of cell division. This genetic information abbreviated as DNA (deoxyribonucleic acid) is required by an organism to develop, survive and reproduce. Almost all cells in a multicellular organism consist of the same DNA. The information of the DNA is converted into proteins which are responsible for performing most of the tasks in the human body. This happens in basically two steps. Firstly the signal in the DNA is transcribed into mRNA and then in the second step this mRNA is translated into amino acids which are the building blocks of proteins. DNA can be defined as a garland of chemical components called nucleotides consisting of a phosphate group and a sugar group which are joined by ester bonds and one of the four types of nitrogen bases namely adenine (A), thymine (T), guanine (G) and cytosine (C) as was discovered by Watson and Crick in 1953. The two strands of DNA are antiparallel to each other. "A" always base pair with "T" by two hydrogen bonds and "G" always base pairs with "C" with three hydrogen bonds between them. Almost 99.9% of these bases are similar in all people.

1.2. Molecular Methodologies For Sensitive DNA Analysis

Today because of the two main projects called the Human Genome Project and the ENCODE (The Encyclopedia of DNA Elements) project a vast and huge amount of knowledge and data is available to all researchers globally, which they can use and apply to further and future medical discoveries and developments. One main focus of research labs worldwide is to find cures for different diseases and for this DNA information is a must. As the human code has become more public, the molecular tool laboratories all over the world are putting in their best efforts to come up with new and advanced techniques for DNA sequence analysis. Isolation, amplification and detection of a particular DNA sequence is the first most need of a molecular biology experiment. To fulfill this requirement many methods/techniques have been developed and many are in the first or the final stage of establishment. All these molecular amplification methods are a must to laboratory research, molecular diagnostics and also to pharmacogenomics.
These methods are basically classified into two types—either target amplification or signal amplification. Sensitivity, specificity, selectivity, detection efficiency, robustness and reproducibility are the various important parameters which makes any particular assay reliable. Sensitivity refers to the smallest amount of the sample that the assay can easily measure. Specificity is the detection of the correct analyte whereas selectivity is to distinguish the molecule of interest from other suchlike molecules. Detection efficiency is a symbol of detecting the probability of whether the assay has taken place or not. If an assay manages to remain unaffected by the small changes in the procedure it is referred to as robustness of the assay. Finally reproducibility means that the result should be the same whether it is performed in different laboratories or between experiments. Polymerase Chain Reaction (PCR), Strand Displacement Amplification (SDA), Ligase Chain Reaction (LCR) and Nucleic Acid Sequence Based Amplification (NASBA) fall in the category of target amplification methods. On the other hand methods like branched DNA (bDNA), hybrid capture and DNA cleavage-based signal amplification are the list of signal amplification methods. PCR is the most widely used method for DNA amplification. TaqMan, molecular beacons, amplifluors, and scorpions are the latest advancements in PCR for real time fluorescence detection. LCR is a method in which two very closely touching oligonucleotides are firstly ligated by DNA ligase upon their hybridization to the specific DNA target and are then amplified. In SDA a single stranded probe containing two different fluoros is incorporated into the amplification product thus eliminating fluorescence quenching. RNA and DNA can both be amplified by another method called NASBA by making use of reverse transcriptase, RNaseH, T7 RNA polymerase, and two primers. While coming to signal amplification methods, bDNA is a technique that gives amplification by attaching many alkaline phosphatase molecules to DNA dendrimers. Then by using a proper substrate against alkaline phosphatase, chemiluminescence can be produced. The method of Hybrid Capture involves the formation of RNA-DNA hybrid on the solid phase coated with specific antibodies. Here also the use of particular substrate provides chemiluminescence. Invader is a cleavage based technique. In this method two partially overlapping probes are cleaved by endonuclease upon binding to the target DNA and then these cleaved products bind to a second probe having a fluorophore and quencher which are further cleaved by endonucleases thus generating fluorescence. So this was the brief introduction of some of the different target and signal amplification methods that are used in various research labs worldwide.

An advantage to the molecular lab world was a method that can perform both the target and signal amplification. And this benefit is available with a versatile new technology discovered in the mid 1990’s named Rolling Circle Amplification (RCA). This technique is unique in a way when compared to other DNA amplification methods as it can be used to detect proteins on a microarray and in cells. RCA can be done in-solution as well as on a solid phase. Loop mediated isothermal amplification of DNA (LAMP) and SMart Amplification Process (SMAP) are two other isothermal DNA amplification technologies that can be summed up with RCA for reliable quantification, higher sensitivity and faster analysis. RCA was developed the first time
in the lab of Prof. Ulf Landegren. As every technique advances with time so do RCA. This lab has already developed the extension of RCA called Circle to Circle amplification. The approach behind this method is to recircularize the first generation rolling circle products (tandem repeated complements of the original DNA circles) after their monomerization for the start of the second generation rolling circle products and so on the process can be further repeated\textsuperscript{22}. I have worked on the extension of RCA i.e. C2CA but before describing that I would like to elaborate the process of RCA.

**Table 1  Properties of various nucleic acid amplification technologies\textsuperscript{10}**

<table>
<thead>
<tr>
<th>Property</th>
<th>PCR</th>
<th>LCR</th>
<th>SDA</th>
<th>NASBA</th>
<th>bDNA</th>
<th>Invader</th>
<th>RCA</th>
</tr>
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<tr>
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<td>√</td>
<td>√</td>
<td>√</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>√</td>
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<tr>
<td>RNA Target Amplification</td>
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<td>×</td>
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<tr>
<td>Protein Signal Amplification</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>√</td>
</tr>
<tr>
<td>Multiplexing</td>
<td>Little</td>
<td>×</td>
<td>Little</td>
<td>Little</td>
<td>×</td>
<td>×</td>
<td>√</td>
</tr>
<tr>
<td>Mesothermal</td>
<td>×</td>
<td>×</td>
<td>√</td>
<td>√</td>
<td>√</td>
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<td>√</td>
</tr>
<tr>
<td>Amplification within cells</td>
<td>√</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>×</td>
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<tr>
<td>Amplification on microarrays</td>
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<td>√</td>
<td>×</td>
<td>×</td>
<td>×</td>
<td>√</td>
</tr>
<tr>
<td>Sensitivity(copies)</td>
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<td>500</td>
<td>100</td>
<td>500</td>
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</tr>
<tr>
<td>Range (logs)</td>
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<td>3</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Specificity (allele discrimination factor)</td>
<td>50</td>
<td>5000</td>
<td>50</td>
<td>50</td>
<td>10</td>
<td>3000</td>
<td>100,000</td>
</tr>
</tbody>
</table>
(✓=Can Perform, ×=Cannot Perform).

1.3. Rolling Circle Amplification (RCA)

Generation of linear polymers that make use of a small, single stranded, circular DNA probe (∼100 nucleotides in length) in a number from hundreds to billions in an isothermal in vitro method is referred to as Rolling Circle Amplification (RCA)\(^{23}\) (Fig.1). All this happens because of the hybridization-sparked enzymatic reactions. Basically the process consists of a primer which is a single stranded DNA either in solution or immobilized on to a solid phase through its 5’ end. A padlock probe which is a few (∼100) nucleotides of DNA in length is hybridized to this primer due to complementarity. Then the padlock probe can be ligated to the primer followed by the process of polymerization carried out by the DNA polymerase. So, in this way the padlock is copied. The process can generate a billion-fold amplification in a total time of 1-2 hours\(^{23}\). It is shown that when a 100 nucleotide DNA minicircle undergoes RCA reaction it results in the formation of 1000 copies of the same DNA minicircle within 1 hour\(^{24-25}\), resulting in a 1000-fold increase. RCA is a miracle to the diagnostic world for the direct or indirect detection of DNA/RNA, protein and other biomarkers\(^{23}\). The process of RCA can be single primed as well as double primed. Single primed refers to what is described above but in the case of double primed one primer hybridizes to the DNA minicircle as in single primed whereas the other primer is complementary to the repeated single stranded DNA formed in the first RCA product\(^{24-26}\). Fluorescence\(^{27}\), UV absorbance, radio labeling\(^{28}\) and gel electrophoresis\(^{24,29}\) are various methods that can be used for the detection of the RCA products.

1.3.1 Pros & Cons of RCA

RCA has a lot of advantages compared to other amplification methods. The first important point here is that it is a very simple technology\(^{20}\). It is an isothermal process that does not require special instrumentation for maintaining or changing the temperature. Also, it can use a wide range of DNA polymerases and is not limited to just the thermostable enzymes, as is the case with PCR. It’s also true to say that the choice is limited when the focus mainly remains on efficient amplification\(^{23}\). Also, contrary to PCR, the rolling circle amplification is a linear process, and uses the original circular template during the whole amplification. For this reason incorporation of mistakes by the polymerase will remain isolated events and not be exponentially amplified as in PCR. The technique is inexpensive, highly sensitive and provides a high range and high multiplexity for diagnostic methods\(^{23}\) (compared to PCR). The RCA based assays are also less complicated when compared with other signal and target amplification techniques described above\(^{23}\).

But there is a famous quote that says that nothing can be perfect and that is the reason that pencils have erasers. So I just want to add that the process of RCA also has some disadvantages. Special care should be taken to avoid false positives or possible contamination that can be caused by either the removal of the non-specific DNA strands to the target or by the release of the
probes by the hybridized targets. The use of endo and exonucleases in the experiment can solve this problem\textsuperscript{28,30}. “The power of the world always works in circles”-this is a famous quote by Black Elk and this is completely proven right by the power of Rolling Circle Amplification.

1.3.2 Clinical Applications Of RCA On Small Scale

RCA has added a lot of stars to itself by showing victory in clinical applications. This method has been used for scoring of simple nucleotide polymorphisms for nanogram sensitivity in human genomic DNA samples\textsuperscript{31,32}. In one study RCA has been compared with PCR and LCR for pathogen diagnosis for the detection of \textit{Chlamydia trachomatis}\textsuperscript{33}. The results showed that RCA was very efficient in detecting \textit{C. trachomatis} bodies in a number as few as 10 in less than 2 hours thus confirming it to be an alternative method to PCR and LCR because of its simplicity and isothermal amplification. RCA was also successful in detection of hotspot somatic mutations that were present in very low amount with high sensitivity that enabled universal microarrays\textsuperscript{34}. Immuno-RCA has proved to be a great advantage for analysis of allergen specific immunoglobulins\textsuperscript{23}. RCA has also become a great success for proteomic surveys\textsuperscript{35}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{rca_diagram.png}
\caption{Pictorial view of RCA – a DNA amplification method for sensitive DNA analysis. The figure shows a DNA minicircle with a complementary primer attached to it. Next step is the entry of DNA polymerase that goes all through the circle to make copies of the primer in the form of a long polymer\textsuperscript{35A}.}
\end{figure}

1.4. Circle To Circle Amplification

As already mentioned before, my project is on the extension of RCA, termed as Circle to Circle Amplification (C2CA). In this process the first RCA products are further subjected to a second round of RCA by their monomerization that will serve them as padlock probes for the primers
(capture probes) of the second generation RCA. In this way the process can be possibly repeated for further generations (Fig.2).

**Figure 2.** The mechanism of Circle to Circle Amplification (C2CA) method. Padlock probe with (+) polarity (in red) shows complementarity to the replication oligonucleotide [RO (-)] (in blue). The priming of these two results in the repeated copies of the padlock (blue) thus amplifying the RO (-). This product is restriction digested to form monomers and then these monomers are circularized and serve as templates for the second round of RCA. For this the primer is of opposite polarity [RO (+) in red] complementary to RO (-). The RCA product from this second round is again monomerized and circularized. This RCA product is the twin of the original padlock and can be further subjected to more rounds of C2CA

1.4.1 Advantages and Applications

C2CA in-solution protocol has shown good results with 10 ng aliquots of genomic DNA and proving itself to be a very powerful and sensitive technique for amplification and analysis of sets of padlock probes. Single stranded linear concatemers with a desired polarity are the end results of this technique. This method can yield a 100- fold higher concentration of monomer products compared to PCR. Also, the products do not cause hindrance to the amplification reaction. The process is really precise for quantification as the amplification of each generation goes in a linear fashion. Multiplexed genotyping of polymorphic loci and quantitative DNA analysis are the two major areas of application described for C2CA insolution.
2. Aim Of The Project

The aim of my project was to transfer the Circle to Circle Amplification (C2CA) assay from solution to a solid phase (magnetic beads) in order to increase sensitivity and to simplify detection and also to localize the RCA product on the beads. To achieve the target various steps were taken. The first one was the conjugation of the RCA primers to the solid phase. Then optimization of the different padlock concentrations, hybridization, ligation and nicking times. The project also included the optimization of the conditions for the monomerization and recircularization reactions for second RCA. Last but not the least evaluation of the sensitivity of the optimized assay was done.

2.1. Application of the method

- In next generation sequencing as an easy way for library preparation.
- For rapid diagnostic techniques coupled to padlock detection.

2.2. The Approach To The Project

In the whole project basically three designs were used for optimizing the first round of amplification and for heading towards the second round of amplification. These three designs were named Restriction Design, Nicking Design 1 and Nicking Design 2. Initially when the project was started only the restriction design was imagined for getting the first and second RCA and also at that time it was thought that if everything will run smoothly then even the third RCA can be started. But as this is research where everything can unexpectedly change it did for us too. Because of this, two more plans were designed for first and second RCA respectively based on the results obtained. The detailed description of all the three plans along with their pictorial representation is given one by one below (Figs 3-6).

2.2.1. Restriction Design (RD)

This roadway to the second round of amplification is based on the restriction sites present on the S2 probe for the restriction enzyme MboI. These restriction sites are also present on the S1 probe because as said earlier there was a thought of starting the third round of amplification also, after the 2RCA. The S1 probe to which the padlock probe (in this case an oligo of 94 nucleotides) will hybridize and be ligated is protected at the 3” end by 2-O-methyl U. S2 probes are also protected by 2-O-methyl U at the 3” end. The idea behind the protection on the S1 probe is to avoid the digestion of the S1 probes, to which the padlock probes are not hybridized, by the 3”-5” exonucleolitic activity of the φ29DNA polymerase. In this way these S1 probes will be protected for the third round of amplification and obviously S2 probes for second round of amplification. But there is a need to remove this 3” protection from the S1 probes to which the padlock probes have been hybridized. This is because the amplification process by the φ29 DNA polymerase will only start when the protected end from the S1 probe is removed. For this the nicking enzyme
*NtBsmAI* is used. Nicking enzymes cut only one DNA strand unlike restriction enzyme that cut both strands. The first rolling circle product (1RCP) generated will now hybridize to the S2 probe because of complementarity, creating a restriction site for the endonuclease *MboI*. As *MboI* is a restriction digestion enzyme it will cut two DNA strands that will include the digestion of the 1RCP to form monomers and at the same time cut the S2 probe to remove the protected end present in the 3” end enabling the start of 2RCA. But before the start of the 2RCA the monomers of the 1RCP will hybridize and circularize onto the S2 probes as per the scheme are to be ligated because only then the circles will be formed that will serve as templates for the second round of rolling circle amplification. This ligation step is aided by a helper probe. Finally the reaction will continue with 2RCA after addition of the polymerase and dNTPs. Figures 3 and 4 give complete description of the restriction design.

**Figure 3.** Restriction design for the first rolling circle amplification product. The first picture shows the binding of the magnetic bead with the two capture probes S1 and S2 on which the 1RCA and 2RCA will take place respectively. Followed by the hybridization and ligation of the padlock probe to the S1 probe and then by nicking of the S1 probe for the start of the first round of rolling circle amplification on the S1 probe.
Figure 4. Pathway to 2RCA by restriction design using the first rolling circle product. The road to 2RCA involves first the digestion of the 1RCA product and the protected 3” end of the S2 probe by the restriction enzyme MboI continued with the hybridization, circularization and ligation (aided by helper probe) of these monomerized fragments to the S2 probe with the start of 2RCA on S2 probe.

2.2.2. Nicking Design 1 (ND1)

For this approach the S1 probe used did not have a protection at its 3” end. Because of this there is no need of a nicking step here. Therefore the padlocks will be hybridized, ligated and then subjected directly to rolling circular amplification to get the 1RCP. After the hybridization of the 1RCP onto the S2 probe it is monomerized by the nicking enzyme NtBsmAI. Only the 1RCP will be nicked whereas the S2 probe remains intact with the protection at its 3” end. One uracil nucleotide is incorporated into the S2 probe, this time together with its protection at the 3” end. This is done for the efficient removal of the protection at the 3” end of the S2 probe. The nicking site is localized in the 3” end of the S2 probe; hence, the 3” end of the nick on the 1RCP will only have 8 nucleotides of complementarity with the S2 probe while the other end of the nick on the 1RCP will be stably hybridized to the S2 probe with 30 nucleotides. In this way it is thought that the low Tm of one end of the nick on the 1RCP will allow the circularization between both the ends of the 1RCP monomers that will then detach and hybridize to the adjacent S2 probes. After the ligation step followed by the removal of the protection on the S2 probe the second round of
amplification will start. FPG-UDG (FPG is formamidopyrimidine (fapy)-DNA glycosylase and UDG is Uracil-DNA glycosylase) treatment will be used for the removal of the protection on S2 probe which will work by digesting the uracil nucleotide present just below the protection. Thus, this will result in not only removing the uracil but also the protection on the 3” end of the S2 probe to make a path for 2RCA. The main idea was that ligation of the single strand could be easier than the ligation of the double strand that was seen in RD. ND1 is pictured nicely in the figure 5.

**Figure 5.** Pictorial view of the Nicking Design 1. The 1RCA will start directly on S1 after hybridization and ligation of the padlock to it as the S1 probe is not protected. Then *NtBsmAI* will nick the 1RCP followed by hybridization of the nicked 1RCP onto S2. Hybridized and circularized 1RCP monomers will be ligated continued with FPG-UDG treatment for removing the 3” end protection and the uracil nucleotide on S2. Finally the 2RCA will start.
2.2.3. Nicking Design 2 (ND2)

The step for reaching till the 1RCA is the same as for ND1. This is because the S1 probe is unprotected thus omitting the need for the nicking step. After the hybridization and ligation of the padlock probe to the S1 probe the first round of rolling circle amplification will start directly. But the design for the S2 probe is different from the ND1. In this case instead of relying on the thermal instability of the hybridization of the nicked 1RCP to the 3” end of the S2 probe three uracil nucleotides are included in the S2 probe at its 3” end below the protection. This is done to digest this part of the S2 probe that is with the protection and 3 uracil sequence effectively, thus allowing the addition of a ligation oligo in the next step. Digestion will be carried out by FPG/UDG enzymes. The ligation oligo together with the ligase will stably ligate the nicked 1RCP thus forming a circle. This is because the ligation oligo here will utilize the sequence of the 1RCP monomer that will be made available after the uracil digestion. This sequence previously in the nicking design 1 was hybridized to the probe but now it is supposed to be in use after the addition of the ligation oligo. The sequence of events in this design will look as follows: - hybridization, ligation and 1RCA; nicking of the 1RCP hybridized to the S2 probes, partial digestion of the S2 probe by FPG/UDG (this step will also release the protection of the S2 probes); circularization of the nicked 1RCP with the addition of the ligation oligo and ligase followed by the second round of rolling circle amplification. The scheme is drawn below for an easy understanding (Fig.6).
Figure 6. Nicking Design 2. The S1 probe is not protected so the padlock after hybridization and ligation to S1 will directly roll. The 2nd picture shows the nicking of the 1RCP that is hybridized to the S2 probes. 3rd drawing is the circularization of the 1RCP monomers onto the S2 probe. After this is the FPG/UDG treatment of the S2 probe followed by ligation aided by ligation oligo. Finally the last picture shows the start of the 2RCA on S2.

There is no “circle to circle amplification protocol to be carried out on a solid phase” reported to date to our knowledge although the research for this is may be carried out in different molecular research labs all over the world.
3. Materials and Methods

The basic protocol for the whole project was the same. The difference lied in the use of different capture and padlock probes, different concentrations for the padlock probe. The list also comprised of optimizing the different times for ligation and nicking reactions together with the use of different elution methods for the rolling circle amplification products. Finally for quantifying the amplified product qPCR was used along with radioactivity techniques. Sometimes when there occurred a failure in the experiment the reactions on beads were instead tried in-solution for confirming the results. The destination was a second round of amplification starting with the first RCA product. But sometimes the second RCA was not so fruitful on beads so it was done in-solution or many times the first RCA product from in-solution was added to the beads for the initiation of the second RCA. But the protocol for both in-solution and on beads is the same, the only difference of being the environment in which the reaction was set up. In-solution experiments were only carried out to cross check the results that were obtained by performing the set up on beads. The project basically involved the standardization of the protocol for circle to circle amplification on beads.

3.1. Coupling Of The Magnetic Beads To The Capture Probes

The first main step of the experiment was the preparation of the solid phase for the amplification process. The solid phase used were either the Dynabeads® MyOne™ Streptavidin C1 magnetic beads or the Dynabeads® MyOne™ Streptavidin T1 beads. Both the C1 and T1 beads can be used for the binding of DNA although T1 beads are specific for both proteins and DNA but on the other hand C1 beads are specifically designed for nucleic acids. But the comparison of these two magnetic beads in one experiment done by us showed that both are specific to DNA in an equal manner and also there was no background seen. So we used T1 magnetic beads in initial experiments and later for some experiments we shifted to C1 beads. These beads are a matrix to which Streptavidin is covalently coupled for the efficient and easy binding of the Biotinylated compounds\(^ {37} \). These Biotinylated compounds in our case are the capture probes named as S1 and S2 capture probes. The beads are small in size and very well suited for automated applications. The S1 and S2 capture probes that were used are basically oligonucleotides of different length depending on the design used (ranging from 30 to 40 nucleotides). The specific biotinylation was done to the 5’ end of these oligonucleotide primers so that the 3’ end remained free for polymerization. In both the probes a 10 nucleotide poly-T long spacer arm is present that is used basically to get rid off stearic hindrance. The sequence for the S1 capture probe used in the Restriction Design was Bio-TTTTTTACATAATGATAGCAGGTCAGTCTACGTGUUUU (All the U”s at the 3’-end are 2-0-methyl RNA) and that for the S2 capture probe for the same design was Bio-TTTTTTTUUUCTGCGAATTTATACTGTCTTGATCTGUUUU (All the U”s at the 3’-end are 2-0-methyl RNA. Also there are 3 normal U”s used after the poly-T spacer, in case one wants to elute using FPG/UDG instead of the water elution method). The S1 probe used in the Nicking Design was
Bio-TTTTTTTTTTTAAACGCTGTCATGTCGTGGCAGTTCTCACAGATCAAG. The two different S2 probes used in the Nicking Design were ND2 digestable and ND2 intact. Bio-TTTTTTTTTTTAAACGCTGCGACATGAATGAGAGTCAATCAGTGUCACGUACGAGACCTUUUU corresponds to the sequence of ND2 digestable (The last 3 U’s are 2-0-methyl-RNA in order to have protection from the exonuclease activity of the phi29 polymerase. The U before these 3 U’s is a normal U that can be removed by the FPG/UDG treatment). The sequence for the ND2 intact was Bio-TTTTTTTTTTTAAACGCTGCGACATGAATGAGAGTCAATCAGTGTCACGTAGACGAGACCTUUUU (The last 3 U’s are 2-0-metyl-RNA for protection against the exonuclease activity of the phi29 polymerase. The U before these 3 U’s is a normal U that can be removed by the FPG/UDG treatment).

To fit nicely the Streptavidin coupled magnetic beads together with the Biotinylated compounds which are the S1 and S2 capture probes the direct method was used. The first step included the preparation of the S1/S2 mix. For this, one eppendorf tube with 10 µM S1 probe and 10 µM of S2 probe, 32 µl each giving 1:1 ratio were mixed making the final volume to 320 µl with MQ water. The same ratio of S1 and S2 probes (1:1) is used till date in all the experiments. The total concentration of the two probes in the mixture was 640 pmols. In one experiment different ratios of the two probes were used. In one tube 3:1 was used and in the other 5:1. Different capture probes were tried in the whole project depending on the results obtained in order to finalize the correct probe for the protocol that can be used together with the correct ratio to be used for the two capture probes. The eppendorf tube prepared with the S1/S2 probes was kept on ice and in another tube 160 µl of the C1/T1 magnetic beads was added. Using a magnet the buffer was removed from the beads in which they were initially suspended. Then the beads were given two times washing with 400 µl of 2X binding and wash buffer to provide the environment for binding of S1/S2 probes to the magnetic beads. The beads were finally resuspended in 320 µl of the same buffer. To these 320 µl magnetic beads 320 µl of the S1/S2 mix kept on ice was added. This 640 µl of total suspension was then incubated at room temperature (RT) for 30 minutes on a spinner. On the completion of the incubation time the magnetic beads that are coupled with the S1/S2 capture probes were given three times washings with 200 µl of 1X binding and wash buffer in order to get rid off the probes that are not bound to the magnetic beads. The beads and the S1/S2 mixture was then after these washings resuspended in 640 µl of 1XTE and was stored at 4°C in the fridge for future use. The final concentration of the beads in this mixture was now 1x10^6 beads/µl. The solution containing S1/S2 mix together with magnetic beads was finally resuspended in 1XTE because TE is a buffer that protects DNA from degradation.

3.2. Hybridization And Ligation Of The Padlock Probe on The S1 Capture Probe

The hybridization reaction involves the circularization of the padlock probe to the S1 capture probe. The padlock sequence used in the Restriction Design was GTGCTATCATTAGTCTGCGAATTATATCAGTGAGAATCTGAGAGACGACGACAT

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GAATGAGAGTCAATCAGTGTCACGTAGACGAGACCT and that used for the Nicking Design was
CCACGACATGAATGAGAGTCAATCAGTGTCACGTAGACGAGACCTGTGCTATCATTA
GTCTGCGAATTATATCTGTCTTGATCTGTGAGAACTG. After the 5” and 3” end of the padlock forms a circle having a nick though, against the complementary S1 probe, ligase is used to seal the gap. Depending on the design hybridization of 10 fM padlock probe (94 nucleotides in length) was done either in 40 µl 1× buffer (NEB) or in 1×φ29 buffer (fermentas), supplemented with 0.25 mg/ml final BSA. The hybridization was allowed to go on for 20 minutes at 37°C before the addition of T4 ligase (fermentas) to a final concentration of 0.04 U/µl and ATP to a final concentration of 1 mM. After an additional 30” (in one experiment 5, 15 and 60” were also tested) at 37°C the reaction was stopped by washing twice in 200 µl of the buffer to be used in the next step (nicking or RCA, according to the design).

Table 2. The complete reaction set up for the hybridization and ligation reaction.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Stock Concentration</th>
<th>Final Concentration in the reaction</th>
<th>1 reaction set up (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific density beads</td>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>BSA</td>
<td>10 mg/ml</td>
<td>0.25 mg/ml</td>
<td>1</td>
</tr>
<tr>
<td>Padlock</td>
<td>100 fM</td>
<td>10 fM</td>
<td>4</td>
</tr>
<tr>
<td>φ29 buffer</td>
<td>10X</td>
<td>1X</td>
<td>3</td>
</tr>
<tr>
<td>ATP</td>
<td>10 mM</td>
<td>1 mM</td>
<td>4</td>
</tr>
<tr>
<td>Ligase</td>
<td>1U/µl</td>
<td>0.04U/µl</td>
<td>1.6</td>
</tr>
<tr>
<td>Water</td>
<td>MQ</td>
<td></td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total reaction volume =</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The ingredients for the in solution in context to hybridization- ligation reaction are also the same. The difference is that firstly only the S1 capture probe is used along with other constituents that include padlock probe, ATP, T4 ligase, φ29 buffer and MQ water all in one tube, for the reaction to take place. This is due to that there is no use of beads here. Also, in-solution reactions require the inactivation of the enzymes which was not done in case of the solid phase set up since everything can be taken away from the beads. So after incubating the ligation reaction for 20 minutes at 37°C it was inactivated at 65°C for 10 minutes to stop the activity of the T4 ligase enzyme.

### 3.3. Nicking Reactions

Nicking is done to remove the protected end i.e. the methylated RNA from the S1 capture probe for the start of the 1RCA cycle in the RD system, and to monomerize the first RCP in the ND1 and ND2 designs. The nicking enzyme \textit{NtBsmA1} on a double stranded DNA substrate cleaves only one strand of the DNA. The reaction used a final concentration of 0.15 U/µl of \textit{NtBsmA1} (NEB) supplemented with 0.25 mg/ml of BSA in 1x Buffer 4 (NEB). The samples were incubated for 30” at 37°C and the reaction was stopped by washing twice with the buffer for the next step.

**Table 3.** Reaction set up for the nicking reaction.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Stock Concentration</th>
<th>Final Concentration in the reaction</th>
<th>1 reaction set up (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>10 µg/µl</td>
<td>0.25 µg/µl</td>
<td>1</td>
</tr>
<tr>
<td>NEB4 buffer</td>
<td>10X</td>
<td>1X</td>
<td>4</td>
</tr>
<tr>
<td>\textit{NtBsmA1} enzyme</td>
<td>5U/µl</td>
<td>0.15 U/µl</td>
<td>1.2</td>
</tr>
<tr>
<td>Water</td>
<td>MQ</td>
<td>33.8</td>
<td></td>
</tr>
</tbody>
</table>

Total reaction volume=40
Nicking reaction was also done at a wide range of time differences. 0, 5, 15, 30 and 60 minutes. All these different nicking time tubes were given two times washings with 200 μl of 1Xφ29 buffer which is the buffer that will be used for the 1RCA reaction later. A no amplification control was also included here for the no RCA reaction.

In the case of in-solution experiments after the inactivation of the ligation reaction 0.5 μl of \textit{NtBsmAI} –the nicking enzyme was added directly into the solution followed by incubation at 37°C for 30 minutes. The enzyme was inactivated by heating at 65°C for 20 minutes.

### 3.4. RCA Reactions

First and second RCA reactions were performed in 40 μl of 1Xφ29 buffer (fermentas) supplemented with 0.25 mg/ml BSA and in the presence of 0.250 mM dNTPs and 0.2 U/μl of φ29 polymerase (fermentas). The reaction was incubated for 60" at 37°C and stopped by washing twice in 200 μl of the buffer to be used in next step. The ingredients required for this reaction with their stock concentrations, final concentrations and their amount required for one reaction set up is all described in table 4 for both the first and second RCA and the control.

**Table 4.** Complete reaction plan for the 1\textsuperscript{st} and 2\textsuperscript{nd} RCA reaction together with the control.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Stock Concentration</th>
<th>Final Concentration in the reaction</th>
<th>1 reaction set up (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>10 µg/µl</td>
<td>0.25µg/µl</td>
<td>1</td>
</tr>
<tr>
<td>dNTPs</td>
<td>2.5mM</td>
<td>0.25mM</td>
<td>4</td>
</tr>
<tr>
<td>φ29 buffer</td>
<td>10X</td>
<td>1X</td>
<td>4</td>
</tr>
<tr>
<td>φ29 polymerase</td>
<td>10U/µl</td>
<td>0.2U/µl</td>
<td>0.8 for 1RCA and 0 for Control</td>
</tr>
<tr>
<td>Water</td>
<td>MQ</td>
<td></td>
<td>30.2 for 1RCA and 31 for control</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Total reaction volume =</strong></td>
<td>40</td>
</tr>
</tbody>
</table>

| 1 reaction set up (µl) | 40                    |
3.5. Quantification Of The 1RCA Product Using qPCR And Polyacrylamide Gel

3.5.1. qPCR Analysis

For the qPCR analysis the first step was the release of the 1RCA product from the magnetic beads i.e. the elution of the 1RCA product. The second step involved the digestion of this 1RCA product which was actually in a form of a long linear polymer. The digestion was done to get monomers. These monomers were then amplified using the appropriate forward and reverse primers with the qPCR machine.

Elution Methods

Two kinds of methods were used for eluting the 1RCA product from the magnetic beads. This elution was only done to later amplify the RCP by qPCR. In initial experiments elution was done using MQ water. This elution method was used when the S1 probe that was used had a sequence of „T” (thymine) as the spacer arm before the biotin. For this the 1RCA product was given three times washings with 50 µl of 1XTE. Then finally the sample was eluted in 30 µl of water (MQ) followed by heating at 75°C for 20 minutes. After 20 minutes the supernatant that contained the 1RCA was taken in fresh tubes, using the magnet. The beads were not thrown rather were also kept to check whether there was RCA product also left on them.

Before analysis by qPCR the eluted 1RCA product was monomerized using the restriction enzyme XapI together with a restriction probe (Apo Helper). For the enzyme to recognize the XapI sites on this long fragment a double strand is needed. For this Apo Helper 2(+) was used. This digestion was kept at -20°C in the freezer till the start of the qPCR. XapI is used for digestion as there are XapI sites on the long linear RCA product.

In later experiments the S1 probe that was used contained the sequence of „U” (uracil) as the spacer arm. When this „U” sequence is released the whole long linear RCA fragment will detach from the magnetic beads. So for this pathway FPG-UDG elution was done. FPG is formamidopyrimidine (fapy)-DNA glycosylase and UDG is Uracil-DNA glycosylase. Both these enzymes cleave uracil. When the uracil sequence was cleaved this resulted in the release of the 1RCA product. The long linear fragment was again monomerized using XapI and Apo Helper 2(+) probe. For this roadway after the 1RCA reaction the beads were given two times washes with 200 µl of 1XNEB4 and then all the above mentioned enzymes were added along with the appropriate buffers as per the table 5.
Table 5. Reagents to be used for the FPG-UDG elution method with their respective quantities for one reaction set up.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Stock Concentration</th>
<th>Amount required for 1 reaction (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEB4</td>
<td>10X</td>
<td>4</td>
</tr>
<tr>
<td>FPG</td>
<td>8U/µl</td>
<td>3</td>
</tr>
<tr>
<td>UDG</td>
<td>1U/µl</td>
<td>1.5</td>
</tr>
<tr>
<td>XapI</td>
<td>500U</td>
<td>1.5</td>
</tr>
<tr>
<td>Apo Helper 2(+)</td>
<td>1µM</td>
<td>3</td>
</tr>
<tr>
<td>Water</td>
<td>MQ</td>
<td>27 Total volume = 40</td>
</tr>
</tbody>
</table>

This reaction was incubated at 37°C for 1 hour. After the incubation time was over the supernatant was transferred to fresh eppendorf tubes. The supernatant contained the monomers of the RCA product. Beads were also kept after resuspending them in the same reaction buffer for later qPCR analysis together with the supernatant.

When the experiments were done in-solution after 1RCA reaction, 1.5 µl of XapI was added together with 0.5 µl of 10 µM Apo Helper 2(+) probe, 0.5 µl of 10XΦ29 buffer, for a total reaction volume of 5 µl made up with MQ water. This was also incubated at 37°C for only 30 minutes and then the enzyme was inactivated by heating at 80°C for 20 minutes.

Real Time Quantitative PCR

The main advantage of qPCR over traditional PCR is that it allows the measurement of the PCR product concentration after each round of amplification. The main concept here is that a fluorescent dye with the help of a labeled hybridizing probe either directly or indirectly binds to the lot of DNA that is produced and each cycle of amplification gives the fluorescence values that can be recorded. The DNA concentration is directly proportional to the fluorescent signal over a broad range. The template that was present at the beginning of the reaction can be calculated by a linear correlation between the fluorescent intensity and the PCR product. Ct value or the threshold cycle is the main important parameter while describing qPCR. It is the point at which the statistical significance of fluorescence above the baseline/background can be detected. The dye used in the qPCR analysis was SYBR Green I which is an intercalating dye allowing DNA detection and analysis without using sequence–specific probes. This dye upon binding to
double stranded DNA emits a fluorescent signal that can be measured. 494 nm and 521 nm are the excitation and emission maxima respectively for the dye used\textsuperscript{39}. The table 6 gives the information for the qPCR master mix preparation.

**Table 6.** Reaction plan for qPCR run.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Working Concentration</th>
<th>1 reaction set up (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Forward primer</td>
<td>10µM</td>
<td>0.75</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>10µM</td>
<td>0.75</td>
</tr>
<tr>
<td>PCR mix</td>
<td>2X</td>
<td>12.5</td>
</tr>
<tr>
<td>Water</td>
<td>MQ</td>
<td>8</td>
</tr>
</tbody>
</table>

Along with the unknowns in the qPCR 96-well plate was run the standard curve and the No Template Control. No Template Control was run to check for reagent contamination or primer-dimers. After the 96-well plate was prepared it was given a short spin at 2100 rpm for 1 minute in a centrifuge (eppendorf centrifuge 5804 R) to settle down everything. Afterwards the plate was put into the qPCR machine (Stratagene Mx3000P\textsuperscript{TM}). The 2X PCR mix contains Maxima\textsuperscript{®} Hot Start Taq DNA Polymerase, Maxima\textsuperscript{®} SYBR Green qPCR buffer, SYBR\textsuperscript{®} Green I and dUTP. The polymerase in the mix contains heat-labile blocking groups added to the amino acid residues. As the enzyme is inactive at room temperature it provides higher specificity for DNA amplification. The qPCR buffer in the mix contains KCl and (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} that results in high specificity of primer annealing. The buffer composition is such that it also gives a wide range of MgCl\textsubscript{2} concentration. In order to prevent carryover contamination between reactions the mix includes dUTP that partially replaces dTTP in the PCR product that is being accumulated.

**qPCR Program**

95°C 10 minutes (Initial Denaturation)
45 cycles 95°C 30 seconds (Denaturation)
56°C 45 seconds (Annealing)
72°C 45 seconds (Extension)

The initial denaturation is to get rid of the antibodies attached to the polymerase (hot start) so that it can start to polymerize. Then the double stranded DNA is denatured at 95°C for 30 seconds followed by annealing at 56°C for 45 seconds and extension of the fragments at 72°C for 45 seconds. Total cycles used by the program were 45.
Dissociation curve was also run for which the program is given below:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>55°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>95°C</td>
<td>30 seconds</td>
</tr>
</tbody>
</table>

Melting curve was run to check the melting temperatures of the amplification products. The theory is that as the double-stranded DNA starts melting to single-stranded DNA there occurs a drop in fluorescence because the dye dissociates.

### 3.5.2. Radioactive Labeling Of The RCA Reactions & Analysis By Polyacrylamide Gel

The RCA reactions were the same as previously described with the difference that 20 uCi [alpha-32P] dGTP were included in the dNTP mix.

After the 1RCA reaction was done by using radioactively labeled dGTP, the sample was run on denaturing TBE-urea 6% polyacrylamide gel ( invitrogen). This gel was used because it has urea that denatures DNA. So in the gel only single-stranded DNA was seen. The readymade gel was opened and then wiped with a tissue. The white strip from the bottom of the gel was removed so that the gel comes in contact with the electrode in the gel apparatus. The samples were eluted in 8 µl of 2X dye. The samples were denatured at 80°C for 3 minutes. This is because loading dye and buffer contain urea that will denature the DNA. The gel was pre run at 70V for compensation of different salt concentration. Afterward the samples were loaded. The gel was run at 170V for 50”. After the run of the gel, it was exposed to a phosphoimager screen overnight by keeping it in a cassette. Next day the screen was scanned in a phosphoimager.

### 3.6. Ligation Reactions On The S2 Probes

Basically all ligation reactions on the S2 probes were performed in 40 µl 1x buffer 4 or 1x phi29 buffer, using a final concentration of T4 ligase of 0.04 U/µl supplemented with 0.25 mg/ml BSA.

In the case of ND2 design the ligation is aided by the addition of 400 nM ligation oligo that templates the reaction after the partial digestion of the S2 probe by FPG/UDG. The sequence of the ligation oligo used was CACGTAGACGAGACCTGTGCTATCAT (Used for ligation when using the ND2 digestable probe).
3.7. FPG-UDG Digestion

Table 7. Complete recipe for the reaction.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Stock Concentration</th>
<th>Amount required for 1 reaction(µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEB4</td>
<td>10X</td>
<td>3</td>
</tr>
<tr>
<td>UDG</td>
<td>1U/µl</td>
<td>1.5</td>
</tr>
<tr>
<td>FPG</td>
<td>8U/µl</td>
<td>1.5</td>
</tr>
<tr>
<td>Water</td>
<td>MQ</td>
<td>24</td>
</tr>
</tbody>
</table>

Total Volume = 30

3.8. Detection Of First And Second RCPs With HRP Labeled Probes

40 µl of the first and second RCPs were detected using hrp-labeled probes complementary to the same region on the first or second RCP respectively. As a substrate the quantablu fluorogenic substrate was used (thermo scientific) and the peroxidase reaction was done according to instructions in 150 µl volume of the provided washing solution for 30” before adding the sop solution. The fluorescence was read at 420 nanometers in a Saphire microplate reader.
4. Results

As already mentioned in the „aim of the project” section we used three different designs for the optimization of this project. I will discuss the results for all three plans one by one.

4.1. Results For Restriction Design

For this design the 1RCA results were kind of satisfactory but the 2RCA was found to be in very low amount. For the 1RCA the method used for quantification was qPCR as is described in the previous section. After the 1RCA the elution of the product was done either by FPG/UDG treatment or by water depending on the spacer arm used. After this the 1RCP was digested by XapI in the aid of the specific helper probe to form the monomers. These monomers as discussed earlier were then amplified in the qPCR machine using the appropriate primers and PCR mix. The amplification plots and the standard curve for this design is given below (Fig.7).

Figure 7. The picture on the left shows the amplification plot for the 1RCA together with the control and the standard curve. The image on the right side is the graphical view of the standard curve. From left to right ( for the amplification plot picture on the left): Blue-Standard 1, Light Blue-1RCA, Red-Standard 2, Orange-Beads, Green-Standard 3, Grey-Standard4, Light Green-Control, Violet-No Template Control, Blue-Water.
The control includes everything in it except for the φ29 DNA polymerase. The standard curve shows a nice linear plot. The padlock concentration used was 10 fM. The standard curve ranges from 1 pM to 1 fM. Also the amplification plots were fine. As can be seen in the picture above there was no amplification plot for the no template control so it can be concluded that there was no contamination or primer-dimer formation. The following figure displays the graphical result of the above given pictures (Fig.8).

**Figure 8.** The picture shows the fold amplification of the material eluted from the beads after 1 cycle of rolling amplification compared to the RPC still present on the beads and compared to the same reaction performed in solution.
520 times fold enrichment was seen here for the 1RCA in RD system. So this showed that most of the 1RCA product was eluted from the beads. The method used here for the elution of the 1RCP from the beads was FPG-UDG treatment.

But when water was used in the same experiment for elution the following result was obtained (Fig. 9).

**Figure 9.** qPCR results for 1RCP and control when water was used for elution.

Only 280 times fold enrichment was seen this time. Therefore no good elution was obtained. The elution efficiency reduced here because the method used for elution of the 1RCP was with water. The results showed that more than 1/3 of the products were left on the beads thus depicting low elution efficiency. The starting padlock concentration used was 10 fM again.
As already mentioned in the material methods section for the 1RCA different ligation and nicking times were tested. The figure below shows the qPCR results for those experiments approached by the restriction design (Fig.10).

**Figure 10.** qPCR results for different ligation and nicking times.

Basically four times were checked for ligation and nicking (Fig.10). These included 5, 15, 30 and 60 minutes. The control was taken for only one time i.e. 60 minutes. The first set of peak is for 5'' of ligation and nicking time followed by the peaks for 15, 30 and 60 minutes of ligation and nicking time in the restriction design together with comparison to what is left on the beads and with the same reaction performed in solution. 5 minutes time for ligation and nicking shows a fold enrichment of 520 with 15 minutes showing that of 580, 30 minutes of 570 and finally 60 minutes of 630. The starting padlock concentration was again 10 fM. The amplification of the 60 minutes is similar to the 30'' just a bit higher. In this step 30 minutes of ligation and nicking time was finally standardized for the project.

Moving on to check the results for 2RCA done by this approach, the RCPs from two different starting concentrations (1 pM and 10 fM) of padlock were detected using HRP labeled probes complementary either to the first RCP (from the 1 pM concentration) or to the second RCP (from the starting concentration of 10 fM) (Fig.11). The substrate used was molecular probes quantablue and the fluorescence was read in a microplate reader (saphire II). The comparison of a dilution
from the first RCP of the highest concentration with the undiluted second RCP from the lower concentrations showed that there was a very poor second rolling amplification (Fig.11).

**Figure 11.** In this picture a dilution of a first RCA cycle obtained starting from 1 pM (and diluted to 500, 100 and 10 fM) is compared to 2 RCA cycles made from a starting concentration of 10 fM. The picture shows that the two RCA cycles do not even reach the fluorescence intensity of the 100 fM dilution of the single cycle amplification. This means that there is not more than around 10 fold amplification in the second RCA.

So to confirm what actually went wrong the 1RCA product was radioactively labeled using $\alpha$-P$^{32}$-dGTP in the NTP mix. Then this 1RCA product was run on an acryl amide gel, the result of whose are shown below (Fig.12).
Figure 12. From left to right:-Lane 1 seems to show some ligation product (the band just on top of the last monomer band); lane 2 shows that the RCP has been cut by MboI into mainly monomers and dimers. Lane 3 shows the intact RCP, while lane 4 shows that the ligated material do not survive the exonuclease (exonuclease 1 and 3) treatment. This means that even if there is some ligation this has not produced the circular templates required for the second RCA.

The gel picture therefore concludes that the digestion with MboI has worked in a very good manner but the ligation reaction seemed to be very inefficient. The exonuclease treatment will only be survived by the circles formed by the ligation reaction on the monomers of 1RCP. This is
clearly visible in the gel. This is an indication that ligation might have worked but for forming linear ligation products and not circles. As these are linear ligation products so, on the treatment of exonuclease enzyme they were digested completely and therefore nothing is visible in the gel.

4.2. Results For Nicking Design 1

Circles are the necessary need for the start of 2RCA as these circles that will be formed from the monomers of 1RCP will only serve as padlock for second round of rolling circle amplification. The ligation was found to be a major problem in the restriction design. So for this reason the plan was changed and nicking design 1 was adopted. The qPCR results for the first round of rolling circle amplification for this design are elaborated below (Fig.13).

Figure 13. Amplification obtained after one cycle of RCA in the ND1 design was satisfactory, showing amplification over 600 folds.
To confirm the monomerization and ligation another gel was run after radioactively labeling the RCP (Fig. 14).

<table>
<thead>
<tr>
<th>Lane</th>
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<td>S1/S2 ratio</td>
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**Figure 14.** From left to right: Lane 1 shows the intact RCP, lane 2 shows that the RCP has been nicked by NtBsmAI and has produced mainly monomers and dimmers. Lane 3 shows that there is some ligation mainly the dimer band, but lane 4 shows that the ligated sample do not survive the exonuclease treatment so whatever was ligated it was ligated in a linear form and no circles were produced.

The gel picture confirms that the monomerization is really good again, but even when the nicking design 1 is used but once again very little ligation was seen.
4.3. Results for Nicking Design 2

To improve the efficiency of ligation next design was framed named as nicking design 2. In this design the first steps are the same as for the ND1 design, with also here an unprotected S1 probe. The design of the S2 probe is different. The sequence of events in this design will look as follows: hybridization, ligation and first RCA; nicking of the 1RCP hybridized to the S2 probes, partial digestion of the S2 probe by UNG/FPG (this step also releases the protection on the S2 probes); circularization of the nicked RCP with the addition of ligase and a ligation oligo followed by the second RCA. Therefore a better ligation was expected this time. The figure 15 show the qPCR result obtained by this design.

![Graph showing amplification results](image)

**Figure 15.** In the ND2 system the amplification is again around 600 folds.

To test the efficiency of the nicking and ligation reactions the RCP was again radioactively labeled. This time a good result for ligation could be seen. This is visible in the following given gel picture (Fig.16).
Figure 16. From left to right: Lane one shows the intact RCP. Lane 2 shows that the RCP has been nicked by NtBsmAI mainly into monomers and to a smaller degree into dimers (some of the RCP is uncut and still left in the well). Lane 3 shows that the nicked RCP gets completely digested by the exonuclease treatment (as expected as it is linear). Lane 4 shows the products of ligation. It seems that both the dimers and monomers get ligated into their circular forms, but also it can be seen that a consistent proportion of the material is ligated into higher molecular weight forms. Lane 5 shows that some bands survive the exonuclease treatment and can be identified as circular forms. Lane 6, 7 and lane 8, 9 are respectively ligated material treated and not treated with exonuclease but processed on beads with different S1/S2 ratios as indicated in the picture.
5. Discussion

5.1. Restriction Design

The method used here for quantification of the 1RCA products was qPCR. For this the 1RCA was eluted from the beads in two different ways- either by FPG/UDG treatment or by using water depending on the architecture of the S1 probe. FPG/UDG elution was more efficient than the elution with water as almost the entire 1RCA product was eluted from the beads and only a very less amount of the product was left on the beads. The reason for low efficiency when water was used for elution can be high salt concentration, still present in the eluted product because of the buffers that were used in the previous steps of experiment as the elution was done in small volumes.

Different ligation and nicking times were also checked for the 1RCA product obtained by the restriction design. For this 30 minutes of ligation and nicking time was standardized for the experiment out of the different times checked. 30 minutes of ligation and nicking was found to be optimum for the whole experiment as this was also the requirement of the procedure to have a shorter period of time for different steps in the experiment.

The results for the 2RCA were analyzed by the use of HRP labeled probes complementary to the 2RCA product as already mentioned in the results section. Then by the use of the appropriate substrate fluorescence was produced which was afterwards compared with the fluorescence which was obtained for the 1RCA products when respective complementary HRP labeled probes were used against them. The results showed very poor second rolling circle amplification in comparison to the first round of rolling circle amplification. There was supposed to be basically two reasons for this less satisfactory result of 2RCA obtained by the restriction design. The first problem could be that the restriction digestion done by *MboI* was not successful and the second reason can be that the ligation did not work fine. To confirm for the restriction digestion and for the ligation acryl amide gel was run for the radioactively labeled 1RCA product. The result confirmed that the ligation did not work fine for the restriction design as the little ligation product that was seen in the gel did not survive the exonuclease treatment. This proved that the ligation product was not circular but instead was linear that’s the reason that it got chewed up by the exonuclease. Therefore as there is practically no ligation (or at least very less) then there is no circularization and there is no template for the second RCA. We also tried other enzymes earlier, including *AccI* and *KpnI* but got similar results as we obtained with *MboI*. The reason is difficult to tell, most probably it can be due to physical constrains of the molecules that are not free in solution but linked to the physical supports as the beads are. So nicking design 1 was framed.

5.2. Nicking Design 1

In the nicking design 1 the S1 probe is not protected and because of this reason nicking is not required. Therefore the padlock can directly hybridize, ligate and start to roll for the formation of 1RCP. After the 1RCP the step chosen here for getting the monomers was nicking with *NtBsmAI* enzyme and not restriction digestion as was done in restriction design. This was preferred...
because it was thought that, on a solid phase, the ligation of a single strand on a template could be easier than the ligation of double stranded ends. Concerning the protection of the S2 probe it was removed after ligation step by FPG/UDG treatment as the S2 probe has one uracil nucleotide this time below the protection as mentioned before. Also it was assumed that the low Tm of only 8 nucleotides at the 3” end of the S2 probe after the nicking will favor the detaching of the monomer of the 1RCP from the S2 probe where it was initially hybridized and will let it go to circularize on an adjacent S2 probe.

qPCR results were again satisfactory for the 1RCA. The nicking and ligation results were checked again by running the gel. Though the nicking worked fine but again the ligation was not able to survive the exonuclease treatment and so nicking design 2 was framed. While doing the ND1 we were also doing the ND2 in parallel, so as the ND2 showed much better ligation we choose that one instead.

5.3. Nicking Design 2

In this case instead of relying on the thermal instability of the hybridization of the nicked RCP to the 3”-end of the S2 probe, we included in the probe 3 uracils. This will allow basically digesting, part of the S2 probe and help the next step by adding a ligation oligo. The ligation oligo will stably template the nicked RPC, now utilizing also the sequence that has become available after the uracil digestion and that previously hybridized to the probe. Hence a better and positive result for ligation was imagined this time.

qPCR results for the 1RCA gave good fold enrichment for the solid phase as well as for the in-solution experiments. Very less product was left on the beads. This time as expected the ligation worked fine together with the nicking. This is because the gel results for the nicking design 2 showed the survival of the ligation from the exonuclease treatment. Therefore it proved that the circular ligated product was obtained here thus providing the DNA circles for the start of the second round of rolling circle amplification on the S2 probe.
6. Future Work

This is where we are now. The next step in this project is to optimize this ligation step for the start of the 2RCA. For this the scheme is to change the ratio of the S1 and S2 probes. This is because when the density of the S2 probes will be lowered then this can possibly decrease the chance of producing linear products instead of circles by the adjacent S2 probes. But the future work also includes the further optimization of time and temperature.

One main problem with the ND2 system could be the amount of linear ligation products that is produced along with the circularized ones. This will disturb the second RCA as the small fragments that are left from the S2 probe after the UNG treatment could start firing the amplification from linear templates, reducing considerably the rolling of the correct circular templates. It would therefore be important to titrate a 5’-3’ exonuclease treatment that will allow to eliminate the presence of linear products but at the same time leave the correct circular templates hybridized to the S2 probes.

More efforts will be made also on trying to get the ligation work in the ND1 system. In this case in fact, the problem of very long linear products would be reduced as this design does not contemplate the addition of an external ligation template and more importantly it ensures that the amplification reaction can only start from the S2 probes.
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