Abstract

In this thesis, we tried to improve the initiation of Rolling Circle Amplification (RCA) on genomic DNA using an approach known as Single Primer Extension (SPE). Usually, a synthetic DNA target provides a high signal on RCA after padlock probe hybridisation due to its short 3’ end. However, unfragmented genomic DNA provides low efficiency for RCA initiation due to its long 3’ end.

During SPE, the genomic DNA is used as a template and a biotinylated primer amplifies this target to generate a product of limited length. This amplified product is then captured on to streptavidin-coated magnetic beads. Padlocks specific to the target DNA is then added. They hybridise to the target and closed into circles with the help of a Tth ligase enzyme. Replication of the circularised padlock takes place by a method known as RCA. The Rolling circle products (RCPs) are further amplified with the help of Circle-to-Circle Amplification (C2CA). The RCPS are hybridised with a fluorophore tagged Detection Oligonucleotide (DO) and readout as images containing individual blobs, using a microfluidic system placed on a confocal microscope. The efficiency of SPE was compared with a target which was pre-coupled to the streptavidin-coated magnetic beads prior to padlock hybridisation and ligation. It was seen that a single cycle of RCA was as good as its pre-coupled target control but it was not better than the Standard C2CA genomic DNA control even after changing several factors of SPE. Cycling of SPE always provided a high signal, close to the Std-C2CA synthetic target control. Also a Pre-PCR amplification was tested in this project but did not show any success during the few experiments conducted. Therefore, cycling of SPE could be used as option to test the padlocks on genomic DNA.

Keywords: Rolling Circle Amplification, padlock probes, Circle-to-Circle Amplification, streptavidin-coated magnetic beads, biotinylated primers.

Improvement of RCA initiation on genomic DNA

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Popular Science Summary

Padlock probes are oligonucleotides that have sequence complementary to its target, at both of its ends. They are used as a diagnostic tool to identify pathogens by recognising a specific DNA sequence. Once a padlock probe identifies the target sequence, it is designed such that its ends are adjacent and it can be ligated as a circle with the help of a ligase enzyme. This enzyme provides specificity to use padlock probe as a diagnostic tool.

The ligated padlock probes form circles that may be duplicated to produce many copies of these circles by a process known as Rolling Circle Amplification (RCA). RCA is a common natural method of replication in many circular DNAs present in nature. The products obtained during RCA can be further increased in number by a method known as Circle-to-Circle Amplification (C2CA). During this method, the long concatemer obtained during RCA is cut open into several pieces. These pieces are then joined into many circles with the help of ligase and replicated to produce even more of these circles. At the end of this step, a huge amount of products are obtained. These products can be detected with some flourophore-labelled complementary oligonucleotide, so called detection oligonucleotide, which helps to detect the product by glowing when a laser light is shined on it. By the number of these shiny blobs we can identify the target pathogen.

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Abbreviations

C2CA:	Circle-to-circle amplification
CO:	Capture oligonucleotide
DO:	Detection oligonucleotide
EC:	*Escherichia coli*
PA:	*Pseudomonas aeruginosa*
PDMS:	Poly (dimethyl) siloxane
RCA:	Rolling circle amplification
RCP:	Rolling circle product
RO:	Replication oligonucleotide
SPE:	Single primer extension
TB:	*Mycobacterium tuberculosis*

1. Introduction

Disease diagnosis is an important aspect of medical sciences. Many of the molecular diagnostic tools are designed to target the DNA/RNA sequence of the causative organism. An ideal diagnostic tool is highly specific, sensitive, robust, cost-effective and simple to use. Development of such a tool is a challenging prospect. Many of the tools in use today lack one or many properties of an “ideal” diagnostic tool.

The use of a padlock probe, an oligonucleotide consisting of target-complementary arms, is a promising approach. It is highly specific because the circularisation of the padlock probe on recognition of the target sequence is a ligase dependent step, and requires a perfect match at the ligation site. The circularised probe is amplified using rolling circle amplification (RCA) under isothermal conditions (Nilsson et al., 1996).

Padlock probes can recognise the target sequences simultaneously with great specificity and selectivity. One of the advantages of this method over PCR is that the possibility of cross reactions during PCR can be avoided. (Landegren et al., 1997)

Rolling circle amplification (RCA) is a method used to amplify the circularised padlocks. During this method, the phi29 polymerase starts replicating the circularised padlock probe by displacing the probe-target hybrid at the free 3’ end of the target DNA to produce rolling circle products (RCPs) (Banér et al., 1998). Circle-to-circle amplification (C2CA) is an efficient method used to increase the number of RCPs. During this method, the RCPs obtained from the RCA are monomerised with the help of a restriction enzyme AluI, whose restriction site is made double stranded by Replication oligonucleotides (RO). The RO is also used to prime the second RCA and the cycle continues (Figure 1) (Dahl et al., 2004). These amplified products are detected with the help of a Detection oligonucleotide (DO), which is fluorophore-labelled.

A microfluidic system is used to read out the RCPs obtained from C2CA amplification (Jarvius et al., 2006). Preparation of a microfluidic platform utilises a simple, in-house manufacturing process. A microfluidic system consists of a poly (dimethyl) siloxane (PDMS) chip with channels mounted on them. The surface of the chip with channels etched on them are oxidised by electron beam evaporation. This ensures proper binding of the glass slide onto to the PDMS chip, thereby forming a micro channel. The solution containing RCP is pumped through this channel. The chip is mounted on the confocal microscope, where a laser beam is incident on it. The RCPs tagged with DO appear in the form of individual shiny blobs (Melin et al., 2008). The images are captured and the number of blobs are counted with the help of MATLAB (Figure 2).

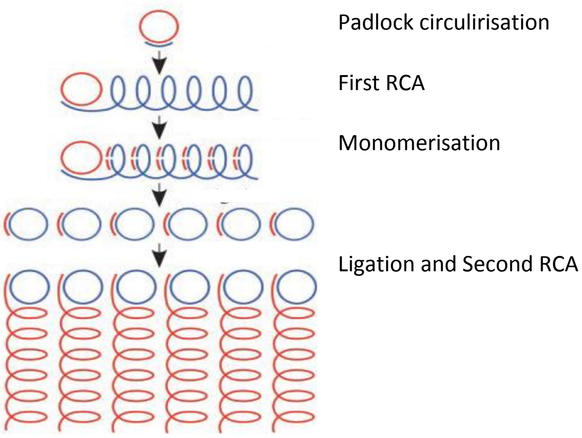
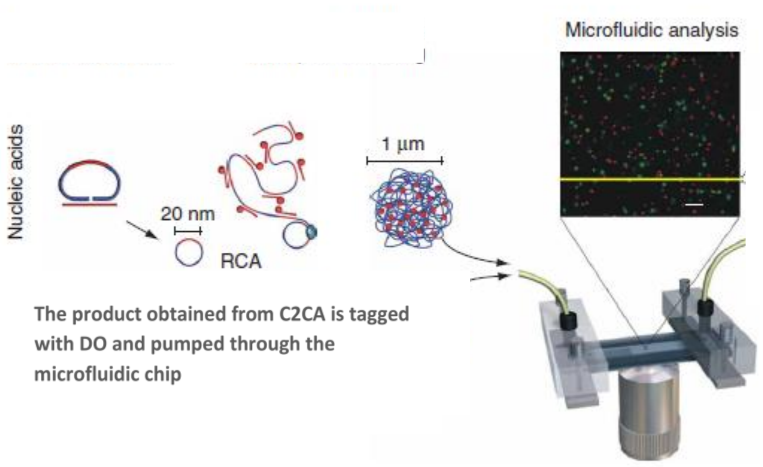


Figure 1: Circle to circle amplification (Dahl et al., 2004).

The padlocks are circularised with the help of ligase, after hybridisation to the target sequence. The circularised padlocks are then replicated by phi29 polymerase enzyme by RCA. After the replication, the newly formed products are cleaved with the help of the restriction enzyme, AluI which is the second step. The restriction enzyme fragmented products are re-annealed with the help of ligase and replicated again using phi29 polymerase enzyme.

RCA on synthetic target provides a good signal because the replication of the padlock can be readily initiated by the phi29 polymerase enzyme due to the availability of the free 3’ end on the target. However, initiation of RCA on genomic DNA has been shown to be less efficient than on synthetic DNA. This is because of the huge mass of the genomic DNA due to which the 3’ end is not readily available for the RCA initiation. Pre-treatment of the genomic DNA becomes necessary for this reason. Several methods of genomic DNA pre-treatment have been carried out routinely, some of which are heat fragmentation at 95°C for different time intervals and restriction enzyme fragmentation.

The problems faced during previous experiments with these pre-treatment methods were that the incubation time optimisation and selection of the restriction enzymes had to be done for each target. In this thesis, a rough optimisation of such pre-treatments were made for different targets such *as Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA) and *Mycobacterium tuberculosis* (TB) in order to compare the efficiency between the two methods.



The product obtained from C2CA is tagged with DO and pumped through the microfluidic chip

Figure 2: Microfluidic read-out system (Melin et al., 2008).

The first part in this figure shows the hybridisation of the padlock probes to its target sequence followed by its circularisation and amplification using the method RCA. The RCPs are then labelled using a fluorophore-tagged DO. The solution containing these RCPs with DO is pumped through the micro-channel of the PDMS chip mounted on the confocal microscope. The image obtained is analysed.

An alternative method called single primer extension (SPE) was extensively tested in this thesis. In this method, a single biotinylated primer is used to replicate the target sequence using the target DNA as its template. This product was then captured on to beads and then hybridised by the padlocks. This was a promising procedure which could possibly eliminate the pre-treatment of genomic DNA. Performing a number of cycles of SPE would always provide a higher signal but the main aim is to improve a single cycle of SPE, which in turn would increase the efficiency of cycling. Also, cycling of SPE is not favourable for Point-of-care diagnostic tools.

Another method which was briefly tested was Pre-PCR. In this method, both the primers are used to obtain an exponential amplification of the target. This increases sensitivity of the padlock recognition.

2. Materials and Methods

2.1. DNA samples

Three systems were tested in the thesis – 1) genomic DNA of *Escherichia coli* (EC, DA5438 strain) courtesy: Camilla Russell, Department of Immunology, Genetics and Pathology, Uppsala University, Sweden, 2) genomic DNA of *Pseudomonas aeruginosa* (PA) courtesy: Pieter Moons, Vaccine and Infectious Disease Institute, University of Antwerp, Belgium and 3) genomic DNA of *Mycobacterium tuberculosis* (TB) courtesy: Anna Engström, Science for Life Laboratory, Stockholm, Sweden. The concentration of the above DNA samples were tested previously using Qubit dsDNA BR kit (Life Technologies, Carlsbad, CA, USA).

It was necessary to denature the genomic DNA at 95°C for fragmentation and effective binding of the padlock to the target sequence. However, the duration of heat fragmentation was tested and optimised for each target.

2.2. Oligonucleotides

The various oligonucleotides used in this project are shown in Table 1. Synthetic targets were designed based on the target sequence on their genomic DNA. The padlocks were tested for efficiency using the synthetic target as positive control. Padlocks were designed such that they consist of ends complementary to the target sequence, binding sites for Replication oligonucleotide (RO) and Detection oligonucleotides (DO).

The design criteria/genes for various synthetic targets were as follows:

Escherichia coli (EC): Synthetic target - L12911, included gene sequence of an antibiotic degrading, glycosyl hydrolases enzyme domain.

Pseudomonas aeruginosa (PA): Synthetic target - L13364, included the gene sequence of *NodT*, an outer membrane factor lipoprotein family which confers drug resistance in PA.

The synthetic target of PA did not serve as a good positive control. Hence, the design was changed later and the padlock was designed accordingly.

Mycobacterium tuberculosis (TB): Synthetic target - L10919, consists sequence of the *rpoB* gene, where the mutations in it can result in resistance towards Rifampicin.

All the padlocks and oligonucleotides were purchased from Integrated DNA Technologies, Inc., Coralville, IA, USA.

Table 1: List of various padlocks, synthetic targets, capture oligonucleotides and detection oligonucleotide used in the project. Abbreviation P in the sequence refers to phosphate and B refers to biotin.

Padlock probes	Target	Sequence (5’-3’)
P5795	EC	P-AAGAGATGCTCGACTGGTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACGCTGCTCGTGGTTAAGCTCTGCATTACCCTTACGCTG
P6018	PA3	P-GGTGGTCGACAGGTCGGTGTATGCAGCTCCTCAGTAATAGTGTCTTACAAGCGTACTCACGACTCATGAAATCGCCGGACTGCC
P4852	TB	P-GGCTCAGCTGGCTGGTGCCGAATGCGGTTCAACAGTCTTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACTTGGTCTGGTCCATGAATT
P6137	PA4	P-GGACAGTTGGCGGCTGTGTATGCAGCTCCTCAGTAATAGTGTCTTACAAGCGTACTCACGACTCATGGCGTCGAACAGGCC
Synthetic target		
L12911	EC	TAAAGCCGACAGCTGCAGTTTCATCAATCACCACGATGCCATGCTCATCTGCCAGTCGAGCATCTCTTCAGCGTAAGGGTAATGC
L13364	PA3	TTCCCGCGGATCGGCGTGGACGGTAGCGGCACCCGCCAGCGTTTGCCGGGCGACCTGTCGACCACCGGCAGTCCGGCGATTT
L10919	TB	ATCACACCGCAGACGTTGATCAACATCCGGCCGGTGGTCGCCGCGATCAAGGAGTTCTTCGGCACCAGCCAGCTGAGCCAATTCATGGACCAGAACAACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCTGGGGCCCGGCGGTCTGTCACGT
L15194	PA4	CGTTCTTCCCGAGCATCAGCCTGACCGCCAACGCCGGCACCATGAGCCGCCAACTGTCCGGCCTGTTCGACGC
Capture oligonucleotide		Sequence (5’-3’)
L12895	EC	B-CTCTCTCTCTCTCTCTCTCTGGCATCGTGGTGATTGATGAAACTGCAGCTGTCGGCTTTA
L13219	PA3	B-CTCTCTCTCTCTCTCTCTCTGCCGCTACCGTCCACGCCGATCCGCGGGAACAGGTCGGCC
L14711	TB	B-CTCTCTCTCTCTCTCTCTCTCGACCACCGGCCGGATGTTGATCAACGTCTGCGGTGTGAT
L15193	PA4	B-CTCTCTCTCTCTCTCTCTCTCCCGGCGTTGGCGGTCAGGCTGATGCTCGGGAAGAACG
Detection oligonucleotide		Sequence (5’-3’)
L14470- TB v2 DO	General	CY3-TTTTTGTAAGACACTATTACTGAGG

2.3. Standard C2CA

The method of amplification used was Circle-to-circle amplification. The target genomic DNA was subjected to heat fragmentation prior to amplification. The duration of heat treatment varied between the different targets. However, a minimum of 2-10 min heat denaturation at 95°C was required. Synthetic target and restriction enzyme digested target did not require this step. One attomole of target was used for the amplification.

2.3.1. Hybridisation and Ligation

A ligation mixture of 10 µl was added to 10 µl of target (1 attomole). The final mixture consisted of 100 nM of padlock probe, 50 nM of CO, 0.2 g/l Bovine Serum Albumin (New England Biolabs, Ipswich, MA, USA), 250 mU/µl of Tth ligase enzyme (Genecraft, Cologne, Germany) along with 1X Tth buffer (Genecraft). After addition of the ligation mixture, the samples were incubated for 5 min at 65°C for effective hybridisation and ligation.

2.3.2. Bead preparation and Coupling

Streptavidin-coated MyOne T1 magnetic Dynabeads (Thermo Fisher Scientific, Waltham, MA, USA) were used in order to provide a solid support for the target to be attached to, with the help of a CO. Five microlitres of the beads were added to each sample after the hybridisation of the padlock to the target. The samples were then incubated for 5 min at room temperature with constant rotation to favour the coupling process. After incubation, the samples were washed once with 100 µl of 1X Wtw buffer (5 mM EDTA, 0.1 M NaCl, 10 mM Tris-HCl pH-7.5 and 0.1% Tween-20 (Sigma Aldrich))

Before addition of the T1 beads, they were washed thrice with 1X Wtw buffer, same volume as that of total volume of beads taken for the experiment. After washing, finally the same volume of the Wtw buffer was added to match the initial total volume of beads taken.

2.3.4. First Rolling Circle Amplification (1RCA)

Twenty microlitres of 1RCA mixture was added to the target which was hybridised with padlock and coupled to the T1 magnetic beads. Contents of the 1RCA mixture included 125 mM dNTP (Thermo Scientific), 0.2 g/l BSA (Thermo Scientific) and 100 mU/µl Φ29 polymerase enzyme (Thermo Scientific) along with 1X Φ29 buffer (Thermo Scientific). The 1RCA mix was added immediately after washing the beads with 1X Wtw buffer and was incubated for 20 min at 37°C and 2 min incubation at 65°C for enzyme inactivation.

2.3.5. Digestion

The products from 1RCA were digested in order to form monomers. This was performed by addition of 5 µl of Digestion mixture which resulted in final constitution of 120 nM of RO P5768, 2 g/l BSA, 120 mU/µl of AluI (New England Biolabs) restriction enzyme and 1X Φ29 buffer. The samples were incubated at 37°C for 2 min and at 65°C for 2 min for enzyme inactivation.

2.3.6. Second Rolling Circle Amplification (2RCA)

After digesting the 1RCA products, the beads were discarded and the solution containing the monomers were retained. To this, 25µl of 2RCA mixture was added. The 2RCA mixture consists of 50 µM dNTP, 0.68 mM ATP, 0.2 g/l BSA, and 14 mU/µl of T4 ligase (Thermo Scientific), 60 mU/µL of Φ29 polymerase enzyme and 1 X Φ29 buffer. 2RCA was performed at 37°C for 20 min and 2 min incubation at 65°C for enzyme inactivation.

2.3.7. Labelling

The products from the 2RCA were labelled with the help of a labelling mixture which consists of a labelling buffer (20 mM EDTA, 20 mM Tris-HCl pH 7.5, 0.1% Tween-20 and 1 M NaCl) and 5 nM of Cy3-conjugated DO L14470. Fifty microlitres of the above labelling mixture was added to each sample tube and incubated for 2 min at 70°C and 15 min at 55°C.

2.4. Pre-coupling of target

Pre-coupling of target which is used as a control for single primer extension involves binding of the target to the streptavidin-coated T1 magnetic beads before hybridisation and ligation of the padlock probes. A pre-coupling mixture consists of 5 µl of 1X B&Wtw buffer (10 mM HCl, 5mM EDTA, 0.1% Tween-20 and 1M NaCl) and 50 nM of CO and 10 µl of 1 amol target.

2.5 Single Primer Extension

SPE was a method performed in order to avoid optimisation of time duration of heat fragmentation. A SPE mixture is similar to that of a PCR mixture, except that a single biotinylated primer is used instead of two primers. Fifteen microlitres of the SPE mix was added to the 10 µl sample (1 amol target DNA). The composition of SPE mix includes final concentration of 200 µM dNTP, 1X PCR buffer, 2 mM $MgCl_2$ (Quanta Biosciences, Gaithersburg, MD, USA), 200 nM biotinylated primer and 200 mU/µl of *Taq* polymerase.

The thermo-cycler was set up based on the steps- Denaturation at 95°C for 5 min, Annealing at 55°C for 30 s and Final denaturation at 95°C for 5 min. After SPE, 5 µl of T1 magnetic beads were added to each sample tube and rotated for 5 min at room temperature. After coupling of the SPE products, the beads were then washed once with 1x Wtw buffer to remove the reagents from the SPE step, specifically to remove the *Taq* polymerase enzyme.

The coupling of the SPE product to the beads was then followed by the hybridisation and ligation step which included addition of 100 nM padlock probe, 250 mU/µl of Tth ligase enzyme along with 1X Tth buffer.

2.6 Pre-PCR

An alternate method, Pre-PCR was tested where both the reverse and forward primers were used to amplify the target. Table 2 shows all the primers used in SPE and Pre-PCR.

2.7 Quantification of the Rolling Circle Products

The rolling circle products were labelled with DO. Once labelled, the samples were pumped through the microfluidic chip mounted on the confocal microscope with an inlet flow of 180 µl/min. The image was captured in several lines (line scan mode) for a period of 29 sec. The captured images were then analysed using MatLab R2014a (MathWorks, MA, USA).

Table 2: List of primers used during SPE & Pre-PCR.

Primer	Target	Sequence
L14723- Biotinylated	EC	TTTTTTTTTTAACGATGCCATGCTCATC
L15200- Biotinylated	PA	TTTTTTTTTTCGAGCATCAGCCTGACC
L14076- Biotinylated	TB	TTTTTTTTTTTCAAGGAGTTCTTCGGCA
L14724	EC	GGCCAACTCCTACCGTA
L15344	PA	TGGCACGCAGGCTGCC

2.8 Genomic DNA isolation

Two methods of genomic DNA extraction from *Escherichia coli* were followed in order to compare the efficiency of both the methods and to test the DNA concentration measurements. Most importantly, to compare the tolerance to different levels of integrity and quality of the DNA of SPE and standard C2CA, respectively.

The first method of extraction was with the help of Thermo Scientific Genomic DNA Purification Kit. The protocol was followed as described in the manual provided in the kit. Lysis Solution, Precipitation solution and NaCl were provided in the kit.

Prior to the DNA isolation, overnight cultures of EC were made by making a starter culture from a single colony of EC. The starter culture was incubated overnight at 37°C shaker incubator. Two hundred microlitres of starter culture was then inoculated into 200 ml of Luria-Bertani (LB) broth and incubated for five to six hours, until the cell reached the late logarithmic phase. This was tested by measuring the OD at 580 nm for every 30 min.

The second method was crude extraction of genomic DNA. Ten millilitres of EC culture was incubated at 95°C for 1 hr. The culture was then spun down at 10,000 rpm for 10 min to separate the debris. The supernatant was collected into a new tube and equal volume of chloroform was added and mixed several times. This was then centrifuged at 12,000 rpm for 10 min. The supernatant containing the genomic DNA was transferred into a new tube and 70% cold ethanol was added to precipitate the DNA. The solution was centrifuged at 12,000 rpm for 5 min to form a pellet. The ethanol supernatant was removed and the precipitate was dried and re-suspended in 50 µl of TE buffer.

3. Results

3.1 Incubation time optimisation for heat fragmentation

The observed optimal incubation times for EC, PA and TB were 20, 5 and 5 min, respectively, as shown in Figure 1.

3.2 Restriction enzyme treatment

Another alternative to heat treatment would be treatment with various restriction enzymes in order to fragment the genomic DNA. The gene sequences were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank). The restriction enzymes were then chosen by running the sequences in NebCutter (http://nc2.neb.com/NEBcutter2). The effect of digestion is shown in the Figure 3.

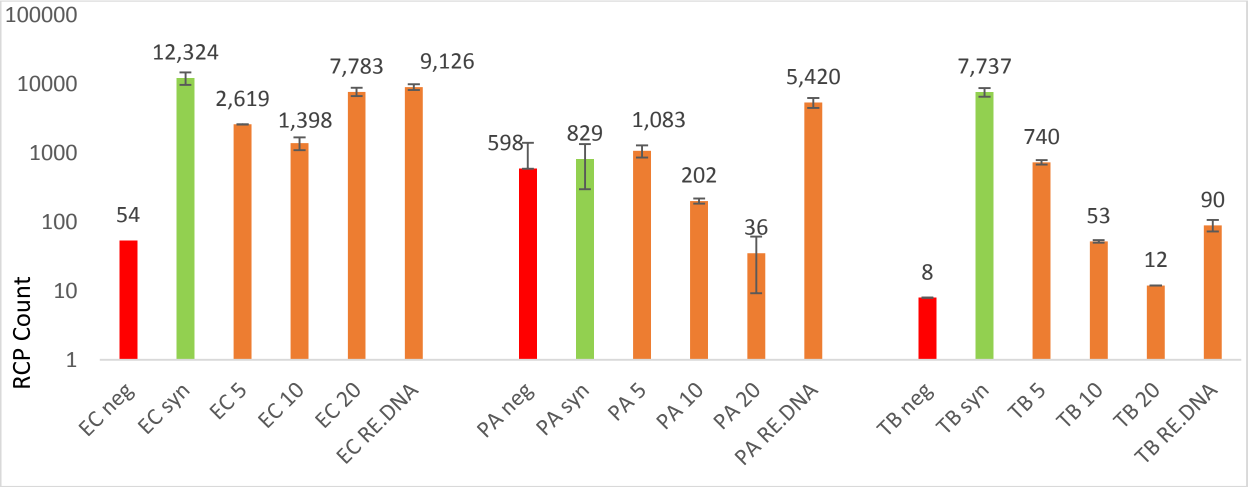


Figure 3: Representation of the effect of different time lengths of heat fragmentation and the effect of restriction enzyme fragmentation on the genomic DNA. The abbreviation syn: synthetic target (positive control), neg: negative control, RE DNA: restriction enzyme treated genomic DNA. The numbers 5, 10 and 20 are in minutes and they indicate the incubation time at 95°C. The error bars indicate standard deviation (n=2).

3.3 Single Primer Extension optimisation

Several factors were tested to improve the single primer extension. Some of them were denaturation time optimisation, annealing time optimisation, extension time optimisation, enzyme concentration, primer concentration and SPE cycle number. Primer concentration and annealing time optimisation was found to be same as studied previously by the group which was verified during this project as well.

3.3.1 SPE with various alterations

To improve the SPE, various factors involved in it were tested. Figure 4 shows the effect of the following changes: replacing Platinum Taq to DreamTaq (T1), including an extension step (T2), extending the final denaturation time (T3), extending coupling time (T4), including an incubation step at 70°C for 5 min (T5) and extending the ligation time to 20 min (T6). Including an extension step increased the efficiency of SPE.

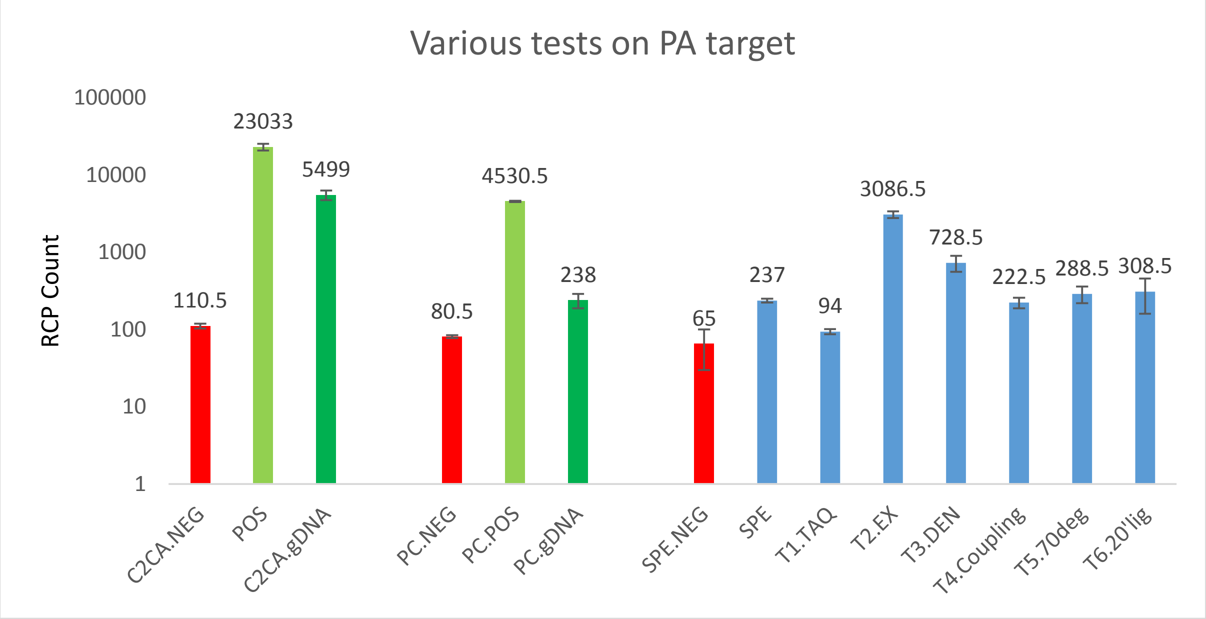


Figure 4: Representation of the effects on SPE due to changes in various factors. SPE refers to Standard Single primer extension protocol followed with no changes. All the conditions were tested against PA pre-coupled genomic DNA. The error bars indicate standard deviation (n=2).

The efficiency of single primer extension was always compared with its pre-coupled target. Also, to make sure that the padlocks were working efficiently, a standard C2CA was always performed simultaneously.

3.3.2 Extension time optimisation

After observing from Figure 4 that including an extension step increased the efficiency of SPE, time optimisation experiment was conducted to obtain the optimal duration for extension. Figure 5 shows that 30 s is the optimal extension time.

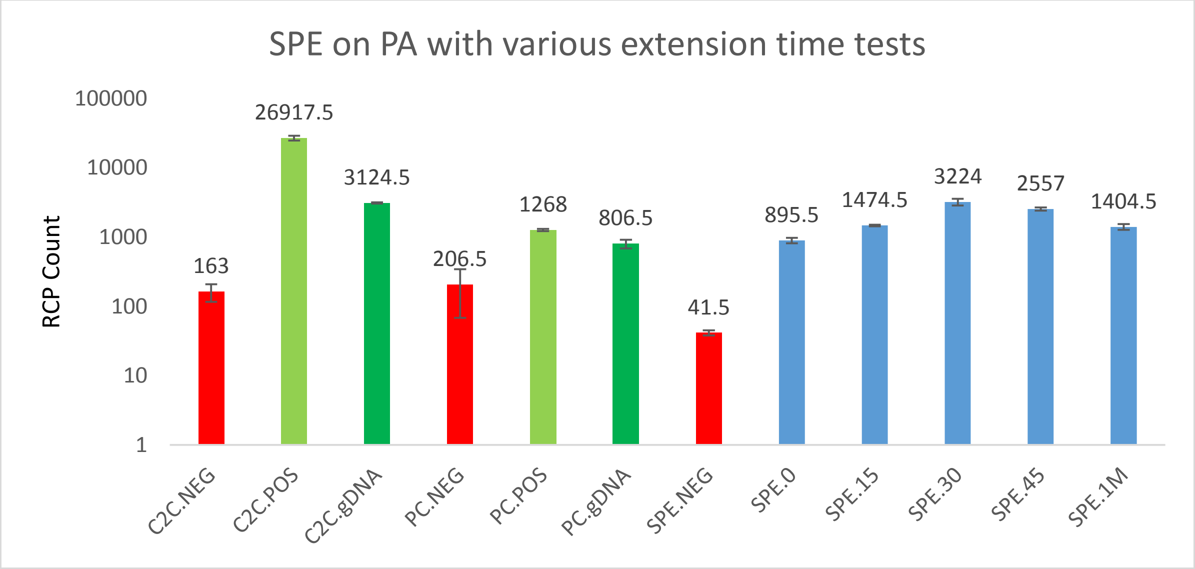


Figure 5: An extension step at 72°C was included in the SPE protocol. Different time duration 0, 15, 30, 45 and 60 seconds extension was tested. All the effects were compared against SPE with no extension step and pre-coupled control. PA genomic DNA was used as control. The error bars indicate standard deviation (n=2).

3.3.3 Annealing time optimisation

Annealing time optimisation was tested previously in the group. However, it was verified in this experiment. Figure 6 shows the effect of different annealing times used. It was observed that 30 s was the optimal annealing time.

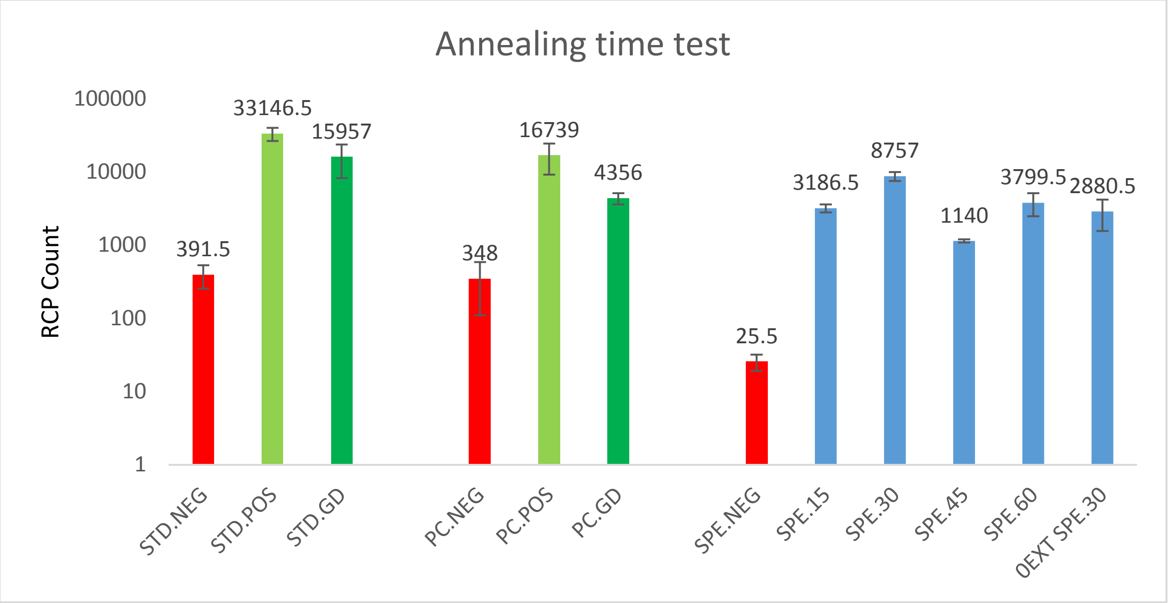


Figure 6: The different annealing times tested were 15, 30, 45 and 60 seconds at 55°C. Also a test with no extension step but with 30 s annealing step was tested, depicted as 0EXT SPE.30 in the graph. PA genomic DNA was used as target. The error bars indicate standard deviation (n=2).

After extension time optimisation, annealing time was tested. It was previously noted that the optimum annealing time was 30 seconds. This was confirmed as seen in Figure 4. The other factor which was confirmed was the optimal primer concentration.

3.3.4 Primer concentration optimisation

Figure 7 represents the effect of different primer concentrations used during SPE. It shows that 200 nM is the optimal primer concentration.

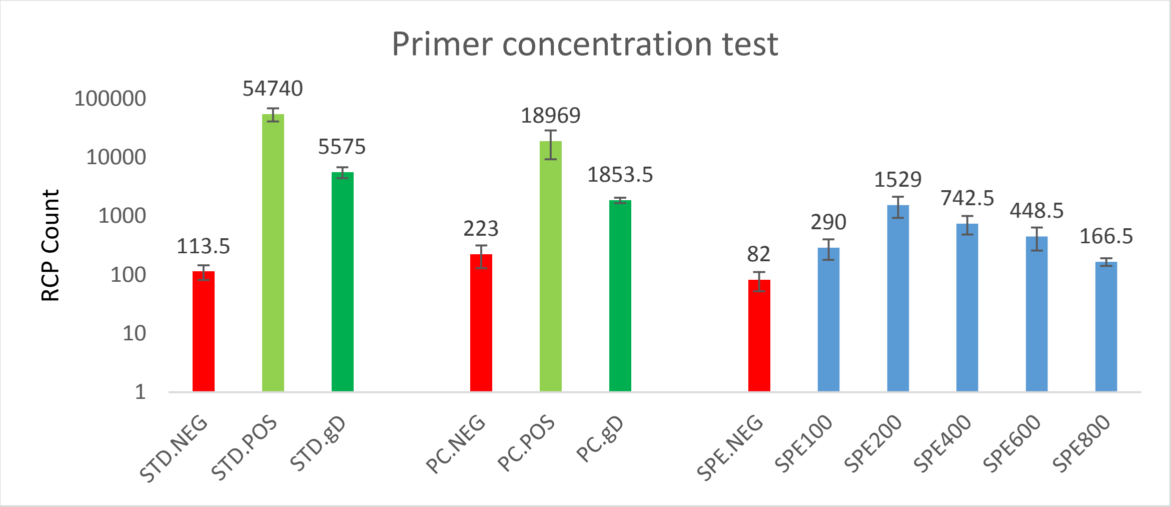


Figure 7: The different primer concentrations used were 100, 200, 400, 600 and 800 nM. PA genomic DNA was used as target. The error bars indicate standard deviation (n=2).

3.3.5 *Taq* polymerase concentration optimisation

Optimisation of the *Taq* polymerase concentration was made by testing its different concentrations. Figure 8 shows the effect of various concentrations on the enzyme.

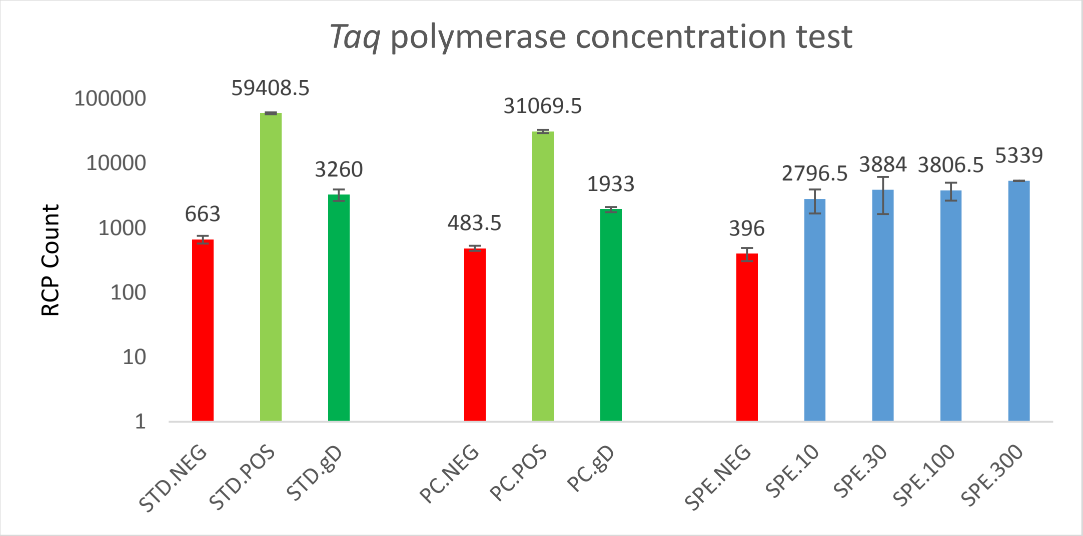


Figure 8: The different concentrations tested were 10, 30, 100 and 300 mU/µl and were used in order to test their effect. All the tests were compared against pre-coupled PA genomic DNA. The error bars indicate standard deviation (n=2).

3.3.6 SPE cycling

Figure 9 shows the effect of performing various cycles of SPE. The different cycles of SPE were compared to the pre-coupled target as well the standard C2CA on synthetic and genomic DNA. It was observed that the highest signal was obtained for 8 cycles.

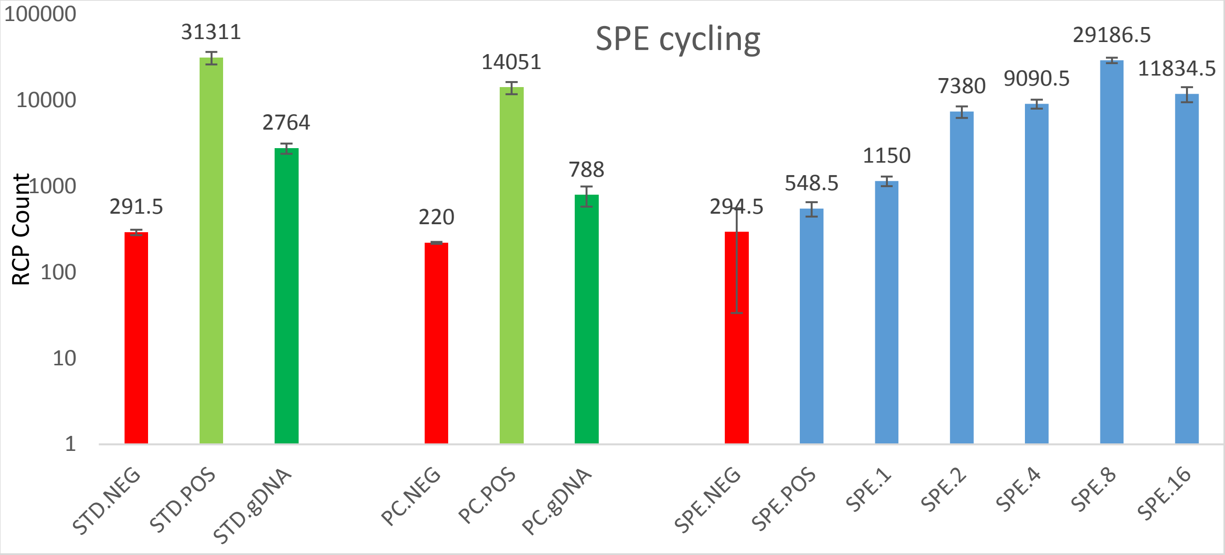


Figure 9: Various number of cycles such as 2, 4, 8 and 16 were tried out. SPE.POS indicates one cycle of SPE without the extension step. However, the other cycles had extension step of 30 s included in them. The error bars indicate standard deviation (n=2).

3.4 Pre-PCR on PA and EC target

Pre-PCR was performed on EC and PA target for 20 cycles. It was then compared with 20 cycles of SPE on both the targets. Both reverse and forward primers were used during pre-PCR in contrast to SPE, where a single primer is used. Figure 10 shows the effect of Pre-PCR on the two targets.

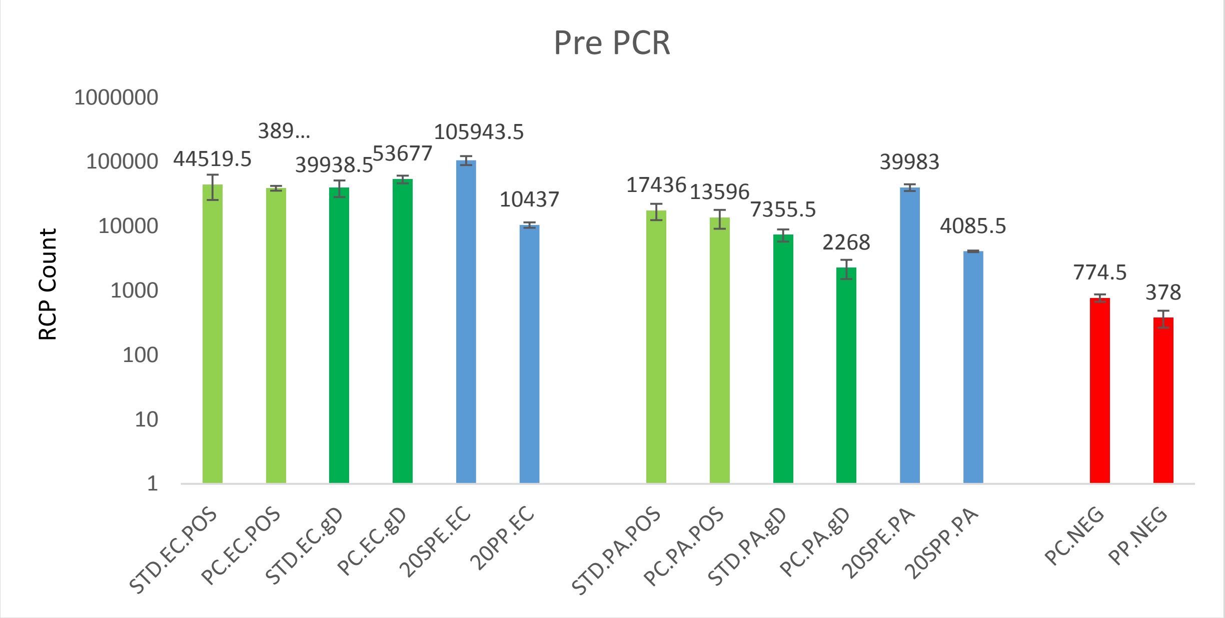


Figure 10: Representation of the effect of Pre-PCR on EC and PA target. 20PP indicates 20 cycles of Pre-PCR and 20SPE indicates 20 cycles of SPE which is used as a control. The error bars indicate standard deviation (n=2).

3.5 Measurement of EC genomic DNA concentration

The isolated genomic DNA from EC was measured using Qubit dsDNA BR Assay kit.

Extraction from kit: 88.5 ng/µl

In-house extraction: 32.5 ng/µl

3.6 Testing of the extracted EC genomic DNA using SPE

The genomic DNA from EC was extracted using two different methods. The genomic DNA from EC was extracted two different methods: in-house method and extraction using a standard kit. SPE was performed on both the DNA to compare the effect of SPE on these two differently extracted DNAs. SPE was performed on both the genomic DNA samples to compare its effect on them.

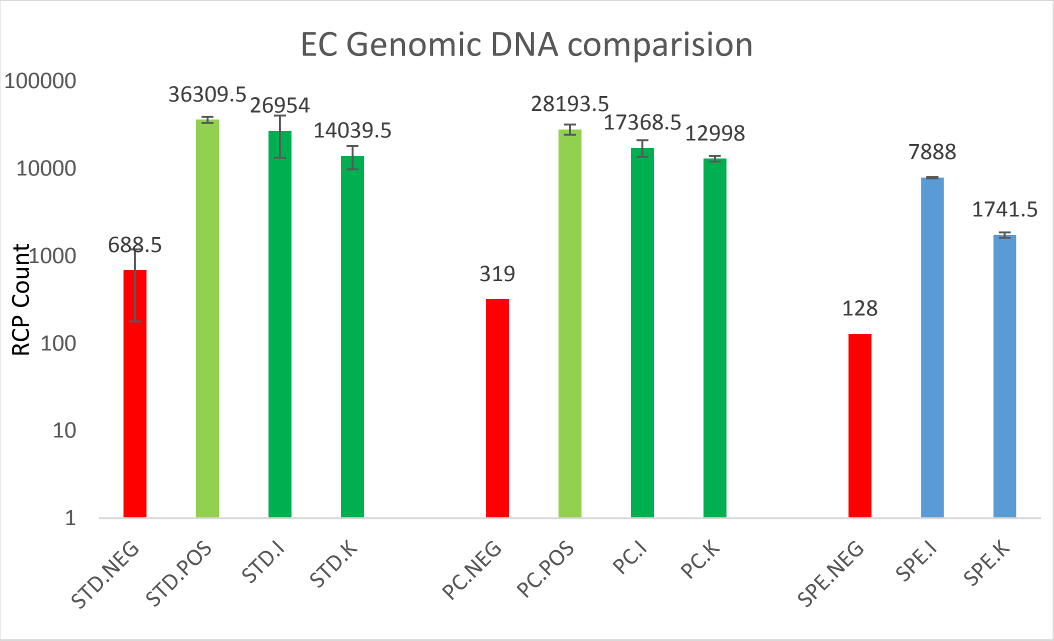


Figure 11: The abbreviation I represents DNA extracted using In-house method and K represents the DNA extracted using the Kit. The error bars indicate standard deviation (n=2).

4. Discussion

Pre-treatment of genomic DNA such as heat fragmentation can be difficult since it varies between the species and different batches of genomic DNA. Other methods like heat fragmentation would be inappropriate for multiplexing since it requires careful selection of enzymes sites which are outside the target sequence. A rough optimisation of incubation time for heat treatment was done and effects of restriction enzyme fragmentation on the genomic targets were studied on three different systems - EC, PA and TB as shown in Figure 3. From this figure, it was observed that EC target showed higher signal with increase in incubation time and showed highest signal for twenty minutes incubation. This was unusual because as previously demonstrated (unpublished data) EC genomic DNA is known to be temperature sensitive. The requirement of longer incubation time could be based on the batch of genomic DNA during extraction.

On the other hand, TB genomic DNA which is known to be heat tolerant (Stewart et al., 2002) showed highest signal with lowest incubation time of five minutes. By running the TB genomic DNA on 1% Agarose gel, it was observed that the DNA was fragmented previously. This would explain the low incubation time requirement for heat fragmentation. However, PA and EC showed better signal with restriction enzyme fragmentation. At this point, it was noted that the synthetic PA DNA, which serves as a positive control for its genomic DNA, showed lower signal than its genomic DNA. It was hence considered to redesign the synthetic DNA and padlocks accordingly.

In order to overcome the disadvantages of the currently used method of pre-treatment of genomic DNA, single primer extension was used. Before testing SPE in all the systems, a series of experiments were performed to choose the most efficient padlock and tested the new synthetic PA target, padlock and capture oligonucleotides.

Single primer extension was extensively tested in this project as it was the primary objective. Initially, a previously roughly optimized SPE protocol was used on the three systems. The efficiency of SPE was always compared with a control where the target was pre-coupled to the beads before the padlock hybridisation and ligation. It was noted that the SPE performed worse than its pre-coupled control. Hence, every step or factor involved in SPE had to be thoroughly tested and re-optimized. Figure 4, shows the effect of various modifications made in the SPE protocol. A significant increase in the signal was seen when an extension step, 72°C for thirty seconds was included after the annealing step. This indicated that the ramping time between annealing step and denaturation step was not enough for the amplification from the target template. Increasing the final denaturation step from two minutes to five minutes increased the signal as well. However, a low signal was observed in the pre-coupled target control. Switching to new batch of Tth ligase improved the signal on the pre-coupled control but it was not better when compared to the standard C2CA control.

The next step was to optimise the extension time. Figure 5 shows the effect of various extension times on SPE. It was noted that the extension time of thirty seconds worked the best for PA target but on repeating this experiment on TB target and EC target, it was observed that the optimum time varied between 30-45 seconds. The optimum time also varied between different thermo cyclers. However, thirty seconds was favourable enough and was retained in the future experiments. Including the extension step improved the SPE performance, but it was only comparable to its pre-coupled target control. The problem with low pre-coupled control signal still persisted. It was later confirmed that low concentration of Tth ligase was used which resulted in the low signal of the pre-coupled target control.

The efficiency of SPE was still low even after changing the various aforementioned factors. *Taq* polymerase concentration effect was tested as well. Usually a concentration of 26 mU/µl of *Taq* polymerase was used during SPE. Figure 8 shows the effect of various concentrations of this enzyme. It can be observed that the concentration used currently is already the optimised one and increase in the concentration further made no significant difference. Though 300 mU/µl of enzyme showed twice the improvement in the signal, it was ten times more enzyme concentration than the current concentration.

Apart from the factors mentioned above, other improvements such as switching from DreamTaq polymerase (Thermo Scientific) to AccuStart (Quanta Biosciences) enzyme also favoured SPE. Increasing the Tth ligase concentration to 625 mU/µl also improved the signal on pre-coupled target. The required improvement was still not observed despite making several changes and optimisations. An alternative solution to this could be by running several cycles of SPE. However, the main goal was to improve SPE for a single cycle. Figure 9 showed that the SPE signal improved dramatically for eight cycles. The signal was comparable to the synthetic target with standard C2CA performed on it. However, a slight dip in the signal was seen for sixteen cycles. This could be due to product re-annealing and also as a result of excessive SPE products competing with the primer and producing even longer products. Therefore, it can be concluded that cycling of SPE for eight cycles could be the possible solution.

From the troubleshooting experiments conducted previously, it was decided to extract EC genomic DNA using two different methods. The in-house method of DNA extraction, where the isolated genomic DNA would be less pure and extraction using a kit, where the isolated genomic DNA would be expected to have less impurities. The two different purities of genomic DNA were used to test the sensitivity of the SPE method towards the quality of the DNA and the presence of impurities.

The concentrations of in-house extracted genomic DNA and kit-extracted genomic DNA were measured using Nanodrop and QuBit dsDNA BR assay kit. The concentrations obtained from the both the methods were compared. It was noted that the Nanodrop had overestimated the concentrations. The theory for this was because the genomic DNA solution had to be homogenous before measuring its concentration. An additional step of incubating at 50°C for ten minutes was necessary to achieve this homogenisation (Nanodrop user manual). Since this step was skipped, there were possibilities that the drop used to measure the concentration contained very high amount of DNA. However, measurement of the concentration using QuBit dsDNA BR assay can be considered more trustworthy since two different dilutions were made and measured and both the readings were consistent. The concentration of the genomic DNA samples were as shown in Table 5.

SPE was carried out on the EC genomic DNA samples. Figure 11 shows the efficiency of SPE on the two types of genomic DNA. The signal on both the DNA samples was lower than its pre-coupled target. In contrast to the original hypothesis that SPE might be tolerant to quality of DNA, it was observed to be sensitive.

Pre-PCR is another promising approach which can be used to overcome the challenges faced during the fragmentation of the target DNA. It was tested on the EC and PA systems. Figure 10 shows the effect of Pre-PCR on the target DNA. Twenty cycles of SPE was also carried out on the target DNA in order to compare them to the Pre-PCR (20 cycles). No signal improvement was seen when Pre-PCR was performed. In fact, the signal dropped in the PA system. During Pre-PCR, exponential amplification takes place and after 20 cycles, the products might re-anneal due to its high concentration. Lower number of cycles can avoid this problem. But further investigation of the problems concerning Pre-PCR could not be carried out.

In conclusion, SPE can be used as an alternative method to pre-treatment of genomic DNA by cycling it to eight cycles. A single cycle of SPE was tried to be improved by looking at every aspect. Making changes such as including an extension step, extending initial and final denaturation time to five minutes and increasing the Tth ligase concentration aided towards the improvement of SPE efficiency. Further improvements can be made by re-designing the primers and avoiding product re-annealing. Multiplexing can also be tried out after SPE.

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