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Διπλωματική Εργασία με τίτλο: Ανίχνευση παθογόνων βακτηρίων σε τρόφιμα με χρήση ακουστικού βιοαισθητήρα τύπου SAW.

Master Thesis: Detection of pathogen bacteria in food samples using a SAW acoustic biosensor.

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Περίληψη

Η υγιεινή και ασφάλεια τροφίμων είναι ζητήματα που αφορούν όλους μας. Κάθε χρόνο, ανά την υφήλιο παρατηρούνται κρούσματα ασθενειών που οφείλονται σε μολύνσεις τροφίμων και τα οποία μπορεί να οφείλονται σε κακή διαχείρηση αυτών των τροφών κατά την παραγωγή, παρασκευή, συσκευασία ή ακόμα και το μαγείρεμα. Παρόλο που οι έλεγχοι για τις συνθήκες υγιεινής γίνονται όλο και αυστηρότεροι, κρούσματα συνεχίζουν να παρατηρούνται όταν οι συνθήκες για συντήρηση και επεξεργασία τροφίμων δεν τηρούνται ή δεν υφίσταται η απαιτούμενη προσοχή. Τα φαινόμενα αυτά γίνονται πιο έντονα σε χώρες του τρίτου κόσμου όπου οι πόροι και οι δυνατότητες για να εφαρμοστούν τα απαιτούμενα μέτρα είναι περιορισμένοι. Είναι ευκόλως εννοούμενο ότι περιστατικά που οφείλονται σε κακή ποιότητα τροφίμων έχουν πολυδιάστατες συνέπειες. Η πιο σημαντική συνιστώσα είναι η ανθρώπινη και δημόσια υγεία, καθώς μολυσμένα τρόφιμα μπορεί να οδηγήσουν σε λιγότερο ή περισσότερο σοβαρές ασθένειες, ακόμη και στο θάνατο. Επίσης, είναι πιθανή η διάδοση ασθενειών που οφείλονται σε μολυσμένα τρόφιμα και σε άλλες περιοχές του πλανήτη εφόσον αυτά εξάγονται εμπορικά. Το κόστος δεν περιορίζεται μόνο στις ανθρώπινες ζωές, αλλά επεκτείνεται και σε τεράστια έξοδα για ιατροφαρμακευτική περίθαλψη, μειωμένη εμπιστοσύνη του καταναλωτή προς τους παραγωγούς τροφίμων και κατ' επέκταση ισχυρά πλήγματα στην οικονομία του τομέα και στο εμπόριο.

Είναι κοινώς αποδεκτό ότι η συντριπτική πλειοψηφία των ασθενειών που σχετίζονται με μολυσμένα τρόφιμα οφείλονται σε βακτήρια, τα πιο σημαντικά από τα οποία είναι τα *Clostridium perfringens, Listeria monocytogenes, Escherichia coli, Staphylococcus aureus, Bacillus cereus* όπως και μέλη του γένους βακτηρίων *Salmonella.* Αξιοσημείωτο είναι το γεγονός ότι για τις ασθένειες τροφίμων στην πραγματικότητα υπεύθυνα είναι περίπου 20 είδη βακτηρίων, παρά το γεγονός ότι το 90% ή και παραπάνω των ασθενειών αυτών οφείλονται σε βακτήρια.

Κατά συνέπεια, προκειμένου να διασφαλιστεί η υγεία των καταναλωτών και για να αποφευχθούν σημαντικές οικονομικές απώλειες, είναι σημαντικός ο έλεγχος των τροφίμων.

Στην παρούσα εργασία έγινε μια προσπάθεια ανίχνευσης του παθογόνου *Salmonella enterica* οροτύπου Typhimurium, χρησιμοποιώντας δύο διαφορετικές μεθόδους πολλαπλασιασμού DNA, την ευρέως διαδεδομένη PCR (Polymerase Chain Reaction) και την ισόθερμη μέθοδο RCA (Rolling Circle Amplification). Στόχος ήταν ο πολλαπλασιασμός DNA του βακτηρίου εφόσον αυτό υπήρχε, σε δείγματα γάλακτος. Η ανίχνευση πραγματοποιήθηκε με χρήση ακουστικού βιοαισθητήρα τύπου SAW (Surface Acoustic Waves), οι οποίοι εκτός από την ευαισθησία τους και τη δυνατότητα ανίχνευσης χωρίς δείκτες (label-free), έχουν τη δυνατότητα να

συνδυαστούν με συστήματα μικροροής (microfluidic systems), για την κατασκευή φορητών πλατφόρμων Lab-on-Chip (LoC). Αυτές οι πλατφόρμες αναμένεται να προσφέρουν δυνατότητες, όπως ταχύτητα και οικονομία, παράλληλη επεξεργασία πολλαπλών δειγμάτων, μεγάλη ευαισθησία ανίχνευσης, ενώ δεν έχουν την απαίτηση ακριβού εξοπλισμού και χώρου εργασίας, όπως και την παρουσία εξειδικευμένου ανθρώπινου δυναμικού. Ένα ακόμα πλεονέκτημα είναι ότι η προσέγγιση που ακολουθήθηκε δεν απαιτούσε περαιτέρω επεξεργασία των δειγμάτων μετά τη συλλογή τους, όπως καλλιέργεια των βακτηρίων, μειώνοντας έτσι το χρόνο, το κόστος και την τεχνική κατάρτιση που απαιτούν οι συμβατικές μέθοδοι ανίχνευσης μικροοργανισμών.

Αν και στην παρούσα φάση, στην πλειοψηφία των περιπτώσεων, δε χρησιμοποιήθηκαν βακτήρια, αλλά απομονωμένο γενωμικό υλικό από αυτά, υπάρχει η δυνατότητα χρήσης τους απευθείας μετά από μια απλή θέρμανση του δείγματος, ώστε να επέλθει κυτταρική λύση. Στη συνέχεια δοκιμάστηκαν τόσο η PCR όσο και η RCA για τον πολλαπλασιασμό γονιδίου/γονιδίων ενδιαφέροντος και έπειτα ακολούθησε απευθείας ανίχνευση μέσω του βιοαισθητήρα χωρίς επιπλέον επεξεργασία, όπως καθαρισμός. Τα αποτελέσματα ήταν κάτι παραπάνω από υποσχόμενα. Οι δύο μέθοδοι έδωσαν πολύ ικανοποιητικά όρια ανίχνευσης (5 κύτταρα/δείγμα για την PCR και 100 κύτταρα/δείγμα για την RCA έως την παρούσα φάση), συνιστώντας ότι η προσέγγισή μας δεν υπολείπεται σε τίποτα των κλασσικών μεθόδων. Έπειτα, έγινε σύγκριση των δύο μεθόδων ως προς διάφορες παραμέτρους (ευκολία, απαιτήσεις σε υλικά και εξοπλισμό, ταχύτητα, ευαισθησία κλπ) και πρόταση επιλογής μιας εκ των δύο για εφαρμογή σε πλατφόρμα Lab-on-Chip (LoC). Να αναφερθεί ότι στα χρονικά πλαίσια μιας διπλωματικής διατριβής (1 έτος), έγινε κάθε δυνατή προσπάθεια για βελτιστοποίηση των μεθόδων και ειδικά της RCA, για να υπάρξει κάποιο καταληκτικό πρωτόκολλο. Αν και η πρόοδος που έγινε ήταν ικανοποιητική, η όλη διαδικασία επιδέχεται βελτίωσης. Απαιτείται επιπλέον χρόνος για βελτιστοποίηση της μεθόδου, τόσο στην παρασκευή και εφαρμογή της, όσο και στην κατάληξη για την αποτελεσματικότητά της (απαιτούμενος χρόνος, απόδοση σε προϊόν, όριο ανίχνευσης, μείωση κόστους κλπ).

Καθ' όλη την έκταση της παρούσας εργασίας, ευελπιστώ ότι θα καταστεί σαφές ότι η εφαρμογή αναδυόμενων τεχνολογιών, όπως τα συστήματα μικροροής και οι ακουστικοί βιοαισθητήρες συνδυαζόμενες με πιο κλασσικές μεθόδους όπως η PCR ή η RCA για την κατασκευή πλατφόρμων LoC, μπορούν να προσφέρουν πολλά πλεονεκτήματα, όπως αυξημένη ευαισθησία, μείωση του κόστους, παράλληλη και γρήγορη επεξεργασία δειγμάτων σε πολλές εφαρμογές με μεγάλο ενδιαφέρον, όπως η διάγνωση ασθενειών και η ασφάλεια τροφίμων.

Λέξεις κλειδιά: *Salmonella,* PCR, RCA, ακουστικός βιοαισθητήρας τύπου SAW, συστήματα μικροροής, πλατφόρμες LoC, όρια ανίχνευσης

Summary

Food hygiene and safety are matters of significant importance to everyone. Annually, foodborne diseases are reported in a worldwide scale. Sources of the aforementioned diseases may be inappropriate handling during production, preparation, packaging or even food cooking. Although controls become increasingly stricter, cases are still reported due to ineffective food maintenance and handling. These phenomena are more intense to third world countries, where resources and the ability to follow the necessary regulations are not abundant. Apparently, low quality food has numerous consequences. The most important aspect is human and public health, since contaminated food may lead to more or less serious diseases, or even death. Moreover, disease spreading also poses a threat in case the contaminated food is commercially exported to other regions of the planet. The damage is not restricted to human lives though; it expands to overwhelming expenses for medical and clinical treatment, a very negative impact of the consumers' trust towards the producer and can in consequence wreak havoc on the field's economy and trading.

It is widely accepted that the vast majority of foodborne diseases is directly related to bacteria. The most important candidates are *Clostridium perfringens, Listeria monocytogenes, Escherichia coli, Staphylococcus aureus, Bacillus cereus*, as well as members of the *Salmonella* genus. It is noteworthy however, that only about 20 bacteria species are responsible for most foodborne diseases, although that percentage is 90% or even higher.

As a result, to protect consumers' health and avoid significant financial casualties, food safety control is imperative.

In the present work, we tried to detect the pathogen *Salmonella enterica* serovar Typhimurium, applying two different DNA amplification methods, the well-established PCR (Polymerase Chain Reaction), and the isothermal RCA (Rolling Circle Amplification). The aim was bacteria DNA amplification in milk samples, if it indeed existed. Detection was conducted using an acoustic SAW (Surface Acoustic Waves) Biosensor, which apart from its sensitivity and label-free detection potential, gives the option of combination with microfluidic systems to form integrated Lab-on-Chip (LoC) platforms. These platforms are expected to offer many features like speed and reduction in cost, testing of multiple samples in parallel, great detection sensitivity, avoiding the demand of expensive equipment and workspace, as well as the need of specialized and skilled personnel. Another advantage is that our method required no additional sample treatment, like bacteria culture, after the samples have been collected, reducing the time, cost and technical training conventional methods require.

Even though at this stage, in most cases, bacterial genomic DNA was used instead of actual bacteria, it is still possible to use them directly, after heating the sample to inflict thermal lysis. PCR and RCA were both tested for the amplification of a gene(s) of interest followed by direct detection with no further sample treatment like DNA clean-up. The results were promising as both amplification methods offered satisfactory detection limits (5cells/sample for PCR and 100cells/sample for RCA up to the current phase), implying our approach lacks nothing when compared to classic methodologies. The two methods were then compared in terms of simplicity, reagent and equipment requirements, speed, sensitivity etc. and then an effort of suggesting which of the two is more suitable to be used in Lab-on-Chip (LoC) platforms was made. It is important to note that during the time offered for a master diploma thesis (1 year), every possible effort was made to optimize the methods and especially RCA, in order to reach a conclusive protocol. Although the progress was satisfactory, there is still room for improvement. More time is needed for the method optimization, not only for its' preparation and application, but also to conclude about its' effectiveness (required time, product yield, detection limit, cost reduction etc.).

Throughout this thesis, I hope it will be made clear that application of rising technologies, such as microfluidic systems and acoustic biosensors, coupled with more traditional methods like PCR and RCA for the construction of LoC platforms, can offer many advantages, like increased sensitivity, cost-effective methods and parallel and rapid sample treatment for applications of great interest, such as diagnostics and food safety control.

Keywords: *Salmonella,* PCR, RCA, SAW acoustic biosensor, microfluidics systems, LoC platforms, detection limits

Introduction

Salmonella are Gram-, rod-shaped, facultatively anaerobic bacteria of the family Enterobacteriaceae. *Salmonella enterica* serovar Typhimurium poses a major threat for public health, as it is a primary candidate for foodborne disease (Salmonellosis). The annual number of people infected by Salmonellosis was reported to be approximately 1 million [1] in a 2011 report. This fact is not surprising as *Salmonella* can be spread via numerous foods, processed or not, such as raw meat, eggs, dairy products, vegetables, or even water [2-6]. According to Centers of Disease Control (CDC), the Typhimurium serovar has been the most commonly food pathogen isolated for 3 decades (1970-2009), for both human and non-human sources, posing a major health threat, even when mortality is considered [7, 8]. Negative effects do not stop there however. Such cases are a major hit to the economy as well.

It is therefore very important to apply strategies for food quality control. Conventional strategies require sample collection and pre-enrichment before analyses can be applied. This increases the time required by huge amounts, as the detection and confirmation may lead up to several days [9]. In 1996, S. P. NG. *et al* proposed a method that used a T6 Monoclonal Antibody Capture enzyme, reducing the whole process to 30 hours [10]. Other efforts have been made, trying to reduce the total amount of detection, primarily using PCR and Real-Time PCR methods; however, all procedures required several hours of laborious work [11-15]. Two very good publications [16, 17] describe the fact that extensive amounts of time are required for sample pre-enrichment, even when using sensitive methods like Real-Time PCR. Malorny *et al* [16], point in that a pre-enrichment step can sometimes be omitted, but in these cases, the food must either contain a very large number of bacteria, or a method for concentrating the pathogen DNA/food mass ratio has to be included in order to concentrate the DNA of interest vs the total amount of sample taken. Using alternative methods like the Enzyme-Linked Immunoabsorbent Assay (ELISA), has also been applied; to be specific ELISA has been the most commonly antibody assay used for pathogen detection in foods [18, 19], with sandwich ELISA, being commercially available. However, ELISA has a relatively low detection limit and therefore a pre-enrichment step cannot be avoided.

Our approach tries to take a very different path. *Salmonella* genomic DNA is isolated and directly used for a PCR or RCA reaction. After the reaction is over it is directly used to conduct an acoustic experiment using a SAW acoustic device. This method aims to detect the bacterial DNA in a label-free manner without pre-enrichment or post-PCR/RCA steps. If a proof of principle conclusion can be drawn, it is very possible, our method can be applied for an integrated LoC platform, as during the whole procedure, LoC parameters, have always been taken into consideration. More details regarding the whole process will follow in the next sessions.

Polymerase Chain Reaction and Rolling Circle Amplification

The Polymerase Chain Reaction (PCR) [20-22], is an *in vitro* method of DNA amplification. The DNA of interest is exponentially amplified using the enzyme DNA polymerase. For a successful PCR it is important to know the sequences flanking the target DNA. Based on these flanking sequences, oligonucleotide primers are designed, which will hybridize to the 3' and 5' termini of the target sequence, allowing the polymerase to add dNTPs, elongating the primers and producing a copy of the target. Typically, a PCR consists of 3 distinct steps with different temperatures. The first one called the denaturation step is usually conducted at 95^{o}C and serves as a way of breaking the hydrogen bonds of the two strands of DNA, separating them. This is important as the next step which is called the annealing step with temperatures ranging from 55^{o}C to 65^{o}C is the one during which the primers anneal to their complementary sequences and is only achievable once the two strands get separated from each other. The final step called the elongation step is usually carried out at 72^{o}C during which the primers are elongated by the DNA polymerase. The cycle of the three temperatures can be repeated over and over again to achieve an exponential DNA amplification. It is apparent that this method nowadays uses a polymerase that is called Taq and can tolerate high temperatures without being deactivated. It is isolated from *Thermus Aquaticus* [23], a thermophilic bacterium species and can withstand the dramatic temperature changes PCR requires [20, 24, 25]. Figure 1 depicts the classic PCR method.

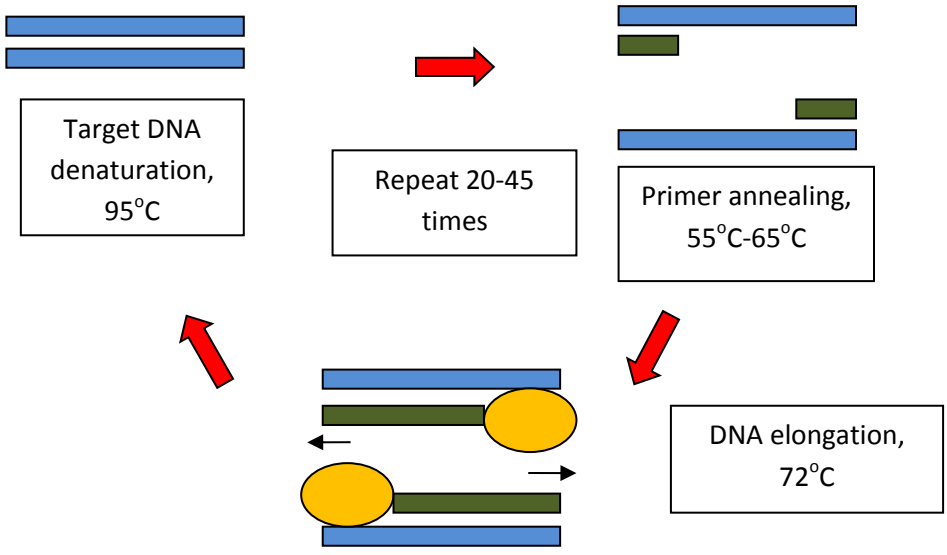


Figure 1. The basic concept of the Polymerase Chain Reaction. First, the DNA target is denaturated at 95^{o}C, so that the two chains get separated. In the second step at 55^{o}C-65^{o}C, the primers anneal to the open strands due to complementarity. The annealing temperature depends on the GC context of the primers. The final step is the elongation of the DNA via DNA polymerase. The arrows indicate the direction of the elongation. The process is repeated several times for high copy yield. Blue indicates the older strands whereas green indicates the newly synthesized ones. Figure not in scale.

PCR is a well-established and widely used tool, and has been used for many purposes including, but not limited to, studying Single Nucleotide Polymorphisms (SNPs) [26, 27], food safety [11-15], diagnostics [28], DNA sequencing and cloning and Southern blotting [29]. Due to its' versatility PCR has been developed and it is used in many variations apart from the basic approach, some of which are Real-Time PCR [30], Reverse Transcription PCR, Asymmetric PCR, Nested PCR, Touchdown PCR and Overlap-Extension PCR [31].

For the purposes of our work, a Multiplex PCR method was used. In a multiplex PCR two or more genes are targeted and amplified at once using two or more sets of primers. In that way, one or more *Salmonella* genes were amplified in the same reaction alongside a human gene which served as an inner control. This approach has been used again in our lab and has been described elsewhere [32]. Also, in many cases, the multiplex PCR has been performed using a Hot-Start PCR kit (Hot-Start PCR). A PCR of this kind aims to eliminate any possible amplification of non-specific primer binding during the early stages of the reaction. Therefore, the enzyme is inhibited either by inhibitors or an antibody and thus inactive at ambient temperatures. When a higher temperature is reached, dissociation of the inhibitors occurs, enabling activation of the enzyme. Alternatively, new approaches use hybrid thermostable polymerases which are inactive, unless the elongation step starts.

Although multiplexing offers PCR a number of advantages, one should be careful; increasing complexity of PCR requires careful primer design, not only in terms of annealing temperatures but also in terms of non-specific hybridization and primer dimer formation (which can occur between one primer and any other primer in the solution including itself). It also requires intense personnel training for learning how to perform multiplex PCR protocols and optimize them, which is costly concerning both time and supplies. Other general PCR problems are ineffectiveness due to inhibition, contamination dangers, as PCR is very prone to them, equipment and workspace requirements and significantly expensive reagents. For example, the Hot-Start and Multiplex Kits used in this work were considerably more expensive than standard PCR reagents.

Rolling Circle Amplification [33, 34], is one of the many isothermal amplification methods that have been developed [35, 36]. The term isothermal suggests that DNA amplification is conducted at one, constant temperature, avoiding the need of thermal cycling like PCR and making a thermocycler a much less relevant piece of equipment, thus reducing the overall cost. RCA utilizes the ability of phi29 polymerase, to produce a concatamer of a circular DNA target by multiples over and over again, taking advantage of the enzyme's strand displacement activity [37, 38]. In brief, a specific primer hybridizes to a circular target and phi29 elongates the product. When the circle is complete the phi29 polymerase displaces the already

formed product and carries on with the reaction, going around of the target for many times, as depicted in figure 2.

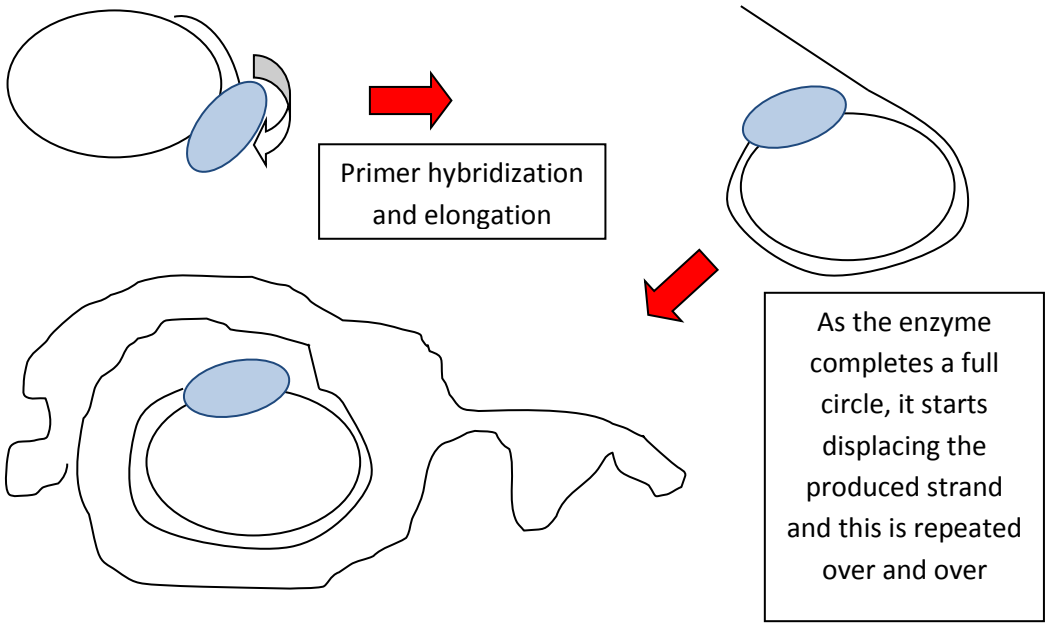


Figure 2. General concept of the Rolling Circle Amplification. By the time the enzyme completes a full circle it starts displacing the produced strand and goes around the template multiple times to produce a very long single-stranded concatamer, which consists of many repeats of the complementary sequence. Figure not in scale.

This makes RCA ideal for plasmid amplification [39, 40], or circular DNA in general [41], however it can also be used to produce RNA instead of DNA products [41]. It should be taken into account that RCA is not limited only to circular targets. In case the target is a linear nucleic acid, a padlock probe which has complementary 5' and 3' termini to the target can be synthesized. Hybridization of the padlock probe to the target creates a local double stranded region which may be accessible to a ligase enzyme (this implies that the 5' terminus of the padlock probe should be phosphorylated for a ligation to occur) [33, 37, 42, 43]. After the ligation is complete, a circular molecule will form that corresponds to the target, and RCA can proceed from that point on. Figure 3 illustrates the way a padlock probe can be circularized for RCA. If a double-stranded DNA molecule is used as the target an additional step of denaturation is crucial so that the two DNA strands get separated from each other and the target site is available to the padlock probe. It is also important to remember that the complementarity between the target sequence and the padlock probe must be absolute, since the ligation will not take place even if a single mismatch is present. This fact makes RCA suitable for SNP mutation detection [44, 45], as well as the possibility to be coupled with other techniques and approaches like magnetic nanobeads [42, 43], gold nanoparticles [46], polymers [47] and biosensors [48-50].

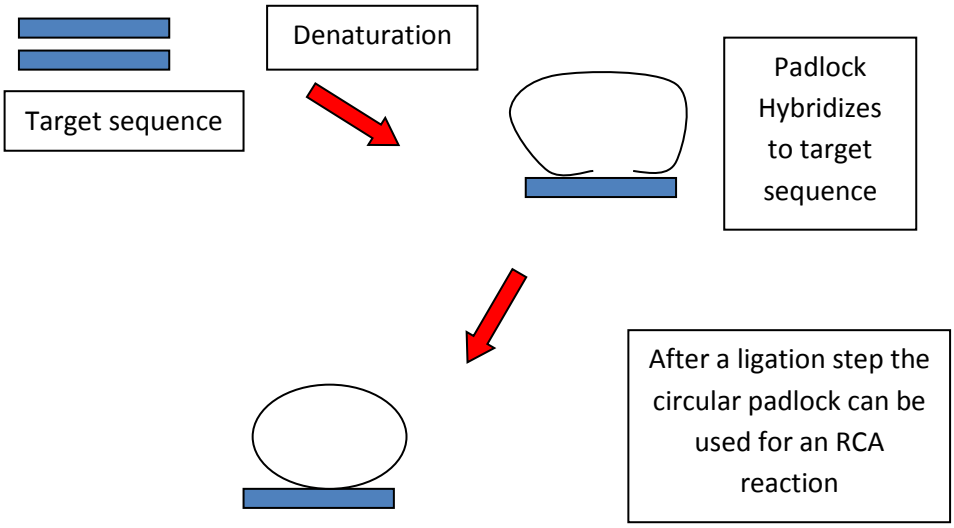


Figure 3. Principle of padlock circularization. The linear molecule binds to the target sequence and after a ligation step it is circularized. Two important conditions have to be met. 1. A single mismatch is not allowed, meaning there has to be strict complementarity between the two sequences, and 2. The 5' terminus of the padlock needs to be phosphorylated. Figure not in scale.

RCA also has alternative forms compared to the classic method. Circle to circle amplification (C2CA) is an RCA alternative where after the first step of RCA is finished the product is digested with a restriction enzyme and the restriction fragments are then used as a target sequence for a new round of padlock ligation and RCA giving the method tremendous amplification capabilities. C2CA is commonly combined with magnetic nanobeads to further increase its' amplification caliber [51]. Multiply-primed RCA is another form where multiple primers are used for the same padlock probe giving the method up to 10,000-fold amplification capability. Another method known as Hyperbranched-RCA, uses a second primer which hybridizes to the first primer's product enabling simultaneous amplification and can offer the benefit of using random hexamers instead of a second primer [52]. All these properties give the RCA method an incredible versatility as it can be used for many applications such as gene expression studies, SNP detection and diagnostics [38].

In this work the standard RCA path was followed. *Salmonella* DNA (either genomic or a synthesized single-stranded target, it will be stated each time) was used to as a target to capture a linear padlock and with the activity of a ligase circularization was achievable. The circular padlock had a primer binding site which was used for primer hybridization and initiation of the reaction. After the RCA was finished the samples were used to conduct acoustic experiments using a Surface Acoustic Waves (SAW) Biosensor without further treatment. More information about Acoustic Biosensors and the Principle of Detection follow in the next session.

Acoustic Biosensors and Principle of Detection

Biosensors are small, analytical devices used for the detection of an analyte combining a biological element with a physicochemical detector [53], without the need of utilizing a biological system directly. A biosensor typically consists of two main parts. The first one is the bioreceptor element, or in a more simple definition the biological sample, which can be a nucleic acid, antibody, protein, whole cell, tissue etc. The second part is the transducer element, which works in a physicochemical way (optical, electrochemical, piezoelectric etc.) and can transduce/convert the biological response of the biological element into an electrical signal. This electrical signal may be further processed and analyzed by other signal processors and/or associated electronics, so that the signal can be displayed in a more user-friendly way via a biosensor reader in a real-time manner. The processed data obtained contain all the analytical information about the biological sample, which may have biochemical, industrial, medical, environmental or other interest. Apparently, biosensors are very versatile systems, as the biological samples that can be used for analysis cover a very wide range, offering them enormous application possibilities.

Depending on the detection principle biosensors can be derived in subgroups. Biosensors can be classified via their biotransducer type. Major categories are electrochemical biosensors, optical biosensors, piezoelectric biosensors, electronic biosensors, gravimetric biosensors etc.

Piezoelectric biosensors in particular, operate based on the piezoelectric effect. That effect describes the electric charge (piezoelectricity) accumulated in solid material due to an applied mechanical stress. Piezoelectricity has been discovered in 1880 by Jacques and Curie [54] and has been reported to be applied to various solid materials such as crystals, DNA and proteins. Surface Acoustic Waves (SAW) biosensors are piezoelectric biosensors, meaning their operating principle utilizes a piezoelectric crystal [54]. The SAW biosensor used in this work generates and detects acoustic waves using interdigital transducers (IDTs) on the surface of the aforementioned piezoelectric crystal [55, 56], enabling the conversion of acoustic waves to electrical signals and vice versa. That means that the acoustic energy is restricted in the range of the acoustic wavelength near the device surface, regardless of the thickness of the substrate [57]. That fact makes the wave very sensitive towards changes on the surface such as conductivity changes, viscoelastic changes and mass deposition. SAW biosensors can operate in a wide range of frequencies which extend from 10KHz to several GHz, depending on the spacing of the IDTs on the device. More detailed information about acoustic biosensors is available in reference [58]. Figure 4 illustrates a SAW biosensor device. The figure was obtained from source [59].

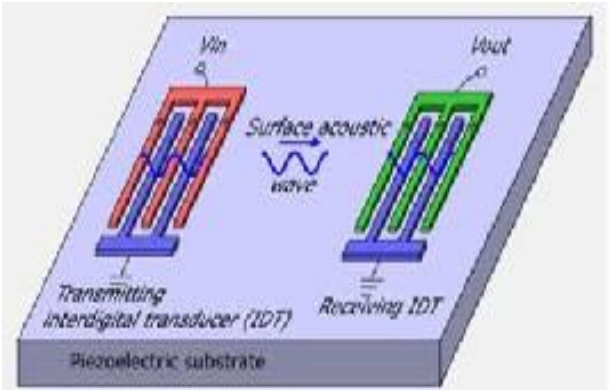


Figure 4. A SAW device. The area between the transmitting and receiving IDT is the detection area, where the biomolecular component will be deposited. Source: Reference [59].

It was previously stated that the SAW biosensor is very sensitive towards changes that take place on the detection area. More specifically, the presence of an analyte on the sensor surface affects the propagation characteristics of an acoustic wave transmitted between the IDTs. It was stated previously that viscoelastic changes and mass deposition on the surface can be detected. Changes in viscoelasticity are monitored as changes in the acoustic wave's amplitude (ΔA), which is an interpretation of the changes of the wave's energy. Similarly, mass deposition affects the velocity of the wave and can be displayed as the change in the wave's phase (Δph). Previous work in our lab showed that the energy change per unit mass (ΔA/Δph), called the acoustic ratio, can be a direct measure of the intrinsic viscosity [η] of a DNA molecule attached to the surface of the sensor [60, 61]. Intrinsic viscosity is directly related to the hydrodynamic volume of the molecules which can be an interpretation of how the mass of the molecule (including surrounding-structural water molecules) is distributed in the volume the molecule occupies. This distribution of mass can be used to deduce the molecule's size and shape [32, 60, 61]. The acoustic ratio is thus an important factor for our approach. Regarding the acoustic ratio of immobilized DNA molecules on an acoustic sensor surface, the following hold true; First of all, it can be used as an acoustic identity for each molecule, as it characteristic of the size and shape of the molecule. This has already been shown using DNA molecules of different sizes [60], same size but different degree of curvature [60], hybridization of double-stranded [62] and triple-stranded [63] DNA molecules, genetic assays for gene expression and mutation screening [32, 64] and characterization of a Holiday nanoswitch junction [65]. Secondly, the acoustic ratio is independent of DNA concentration (provided it is not too extreme) and thirdly, it is independent of previous loading steps [60]. It is therefore possible to test multiple samples of different DNA samples in turn during the same experiment, and thus decreasing the overall experimental time required.

In a 2013 work in our lab [32] we showed that multiplex PCR products can be used at once to conduct an acoustic experiment. A sample containing two PCR products of

different length was used; the ratio of each product has been previously calculated independently and the ratio of the mixture was expected to lie between the two calculated values which indeed happened in an almost linear way. Depending on the mass proportion of the two DNA molecules within the sample, the acoustic ratio was expected to shift towards one or the other end, according to the mass ratio of the short/long DNA, which was indeed observed.

Taking all the above into account, the first part of this work had a similar approach; a multiplex PCR containing a *Salmonella* product and a shorter product derived from a human gene as an inner control was designed. The inner control was kept constant so it could serve as a baseline, whereas the *Salmonella* product was the changing parameter. Changing the initial amount of bacterial DNA was expected to affect the PCR efficiency. The less initial *Salmonella* DNA used, the less PCR product was expected to be amplified. Like in reference [32], when the variable DNA became less and less in the final reaction, the overall ratio used to shift towards the value of the inner control. This approach was used to reduce the initial amount of *Salmonella* template to a minimum where it could be differentiated from the control we used. This control was the same as a multiplex PCR with the exception of bacterial template (note that the bacterial primers were still used to keep every other component of the reaction the same) which was substituted with water. Apparently, only the inner control would be amplified offering a baseline for the acoustic ratio. When a *Salmonella* template dilution could not be discriminated from that baseline it was easily deduced that the previous dilution was our detection limit. Also, to ensure selective detection of the PCR products via the biosensor, one primer of each set used, had a biotin attached to its' 5' terminus. In that way, it could be selectively bound to a sensor surface that was previously saturated with a suitable molecule like Neutravidin (more details in the next sessions). Figure 5 shows the principle of detection when PCR was used as the amplification method.

As far as the RCA is concerned, it was a completely new challenge for our system; there has not been any work previously documenting expected acoustic ratios regarding an RCA product. However, two different approaches were used for the acoustic experiments. The first one was to create circular padlocks and conduct the RCA using a biotinylated primer like in the PCR. Moreover, biotinylated d-UTP was supplemented in the RCA reaction in a minimal amount; this would be a good opportunity to insert random biotins in the product body, creating multiple binding sites for the molecule on a Neutravidin saturated surface. This was done for two main reasons. The first was because the RCA produces gigantic single-stranded molecules which may be up to 70000 bases after 1 hour of reaction. These molecules do not stay linear but hybridize with themselves following strand complementarity rules. It is thus doubtful if that one biotin molecule in the 5' terminus is accessible from the surface-bound Neutravidin. Since biosensors utilize continuous flow-

through protocols, these products would be washed away after a washing step. So, more biotins were needed. The second reason is because each molecule has a different length and conformation, although the general shape is the one of a ''ball of wool''. Since the insertion of biotins is random, each molecule would be bound to the surface in a different way, enabling us to calculate a statistical measurement, which would be impossible otherwise (every molecule is different from the others not only in length but also in conformation). Figure 6 depicts the first RCA approach.

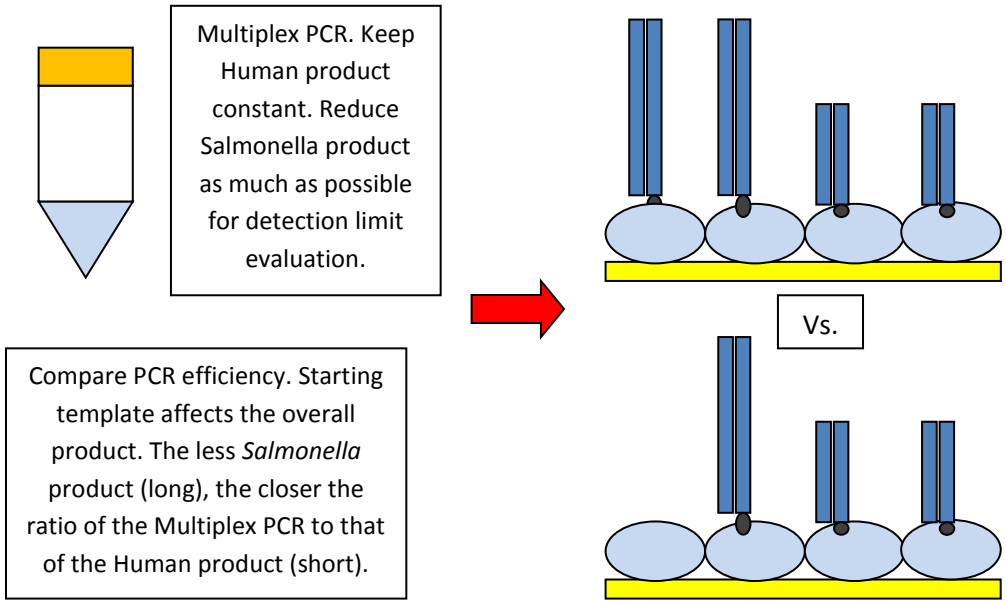


Figure 5. General principle of the detection method when PCR is used for amplification. Two products, one from *Salmonella* (long) and one from Human (short) source are used for a multiplex PCR. The human product serves as an inner control to calculate a baseline and is kept constant. The bacterial product is the variable. The PCR is loaded on a Neutravidin-saturated (light pink) SAW surface (yellow) without further treatment (i.e. purification etc.). Selective binding via a biotin bond (purple) is achievable for calculation of the acoustic ratio of each sample. Based on the proportion of mass of the two molecules a positive sample could or could not be differentiated from the control. The lowest differentiation observed would be the detection limit of the method (directly linked to the initial bacterial concentration). Figure not in scale.

The second RCA approach is shown in figure 7. Since phi29 DNA polymerase has a relatively wide range of activity as far as temperature is concerned (most usually 30^{o}C or 37^{o}C are used, but it can be active from 4^{o}C, up to 37^{o}C), it was assumed that the reaction could be carried out at room temperature (RT). This enables to conduct a reaction directly on the SAW surface, as a SAW device does not have temperature control and consequently operates at RT, and obtain real-time data as the reaction is ongoing and avoid thermocycling after the circularized padlocks are obtained. This approach has been reported previously [48-50], but never using a SAW device and instead using other biosensors. So that approach was simple enough and avoided the need of using multiple biotins, as it ensured that the one biotin of the primer

would be used to bind the product. More specifically, the SAW surface was saturated with Neutravidin. The next step was to use an excess of biotinylated primers to saturate the Neutravidin molecules available, bearing in mind that the ratio of Neutravidin/primer is expected to be 1:1 due to spacing issues [66], although that is not absolute for single-stranded DNA. These primers would serve as a hybridization probe for the circular padlocks and after the RCA mix is injected, the RCA reaction can be initiated obtaining data of the process in a real-time manner, as depicted in figure 7.

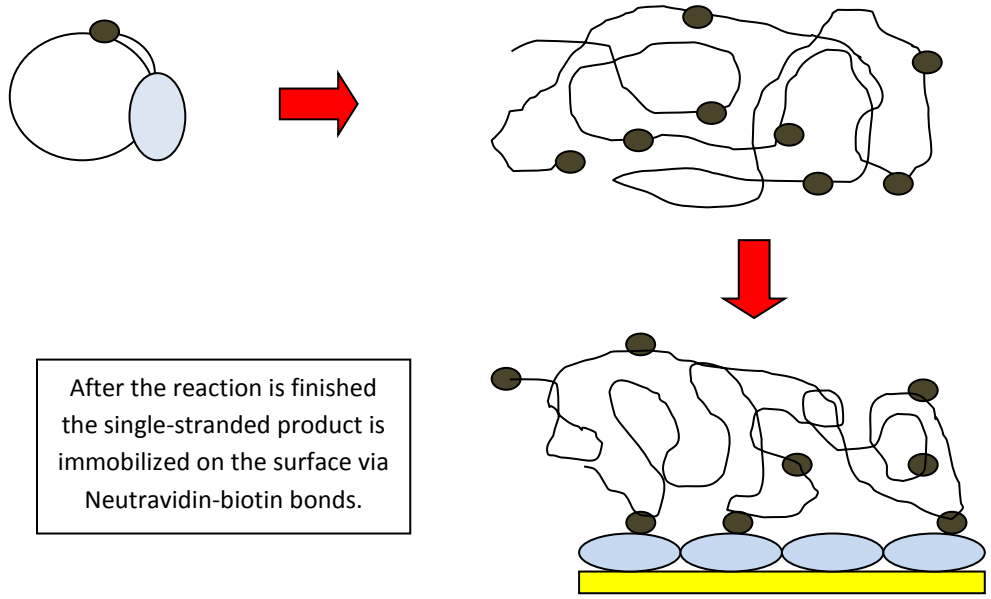


Figure 6. First RCA approach. After completing the RCA reaction, the sample contains single-stranded DNA molecules of various lengths and random biotin insertions. The random biotins will attach each molecule on the surface in a different way. Figure not in scale.

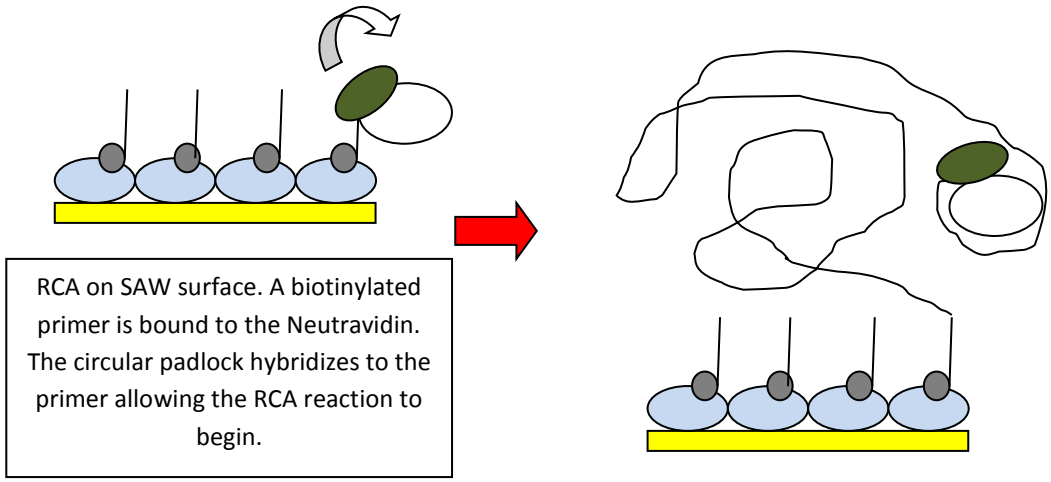


Figure 7. The second approach using RCA utilizes the recognition between the circular padlock and a surface-bound primer to initiate the RCA on the SAW surface. Figure not in scale.

Materials and Methods

Acoustic Devices

The SAW devices used operated at 155MHz using photoresist of an approximate 1μm thickness as a waveguide. Waveguides are structures used to guide the acoustic wave between the IDTs, as well as trapping the acoustic wave energy near the sensor surface [67]. Photoresist is usually used for many applications like patterning and etching of substrates and microelectronics. Other polymers and dielectric materials such as silicon dioxide (SiO_2) and silicon nitride (Si_3N_4) are also used as waveguides. Polymers, although offering increased sensitivity, require cross-linking in order to avoid excessive acoustic loss due to their viscoelasticity [67-69]. Etching (cleaning) of the surface was achieved using a Plasma Cleaner/Sterilizer PDC-002 (Harrick) instrument for 150''. The Network analyzer used was E5061A, ENA Series Network Analyzer (Agilent Technologies), which interpreted the real-time data in a more user-friendly way, using suitable LabVIEW interface software (National Instruments, Austin, TX). The real-time data could be further analyzed using Microsoft Office Excel 2003 (Microsoft Corporation), after the experiments were finished.

All acoustic experiments were carried out in a continuous flow-through manner, where running buffer and samples were exchanging each other in alternate. The running buffer for PCR experiments was Tris 50mM, KCl 10mM, $MgCl_2$ 10mM, pH 7.5, with the exception of experiments using thrA and HemD cases for Multiplex PCR, which was PBS pH 7.4. For RCA experiments the running buffer was PBS, $MgCl_2$ 10mM, pH 7.4. All samples used during an experiment were diluted to the corresponding running buffer. The first step of each experiment was saturation of the sensor surface with Neutravidin (Invitrogen) of a concentration of 100ng/μL-200ng/μL in a final volume of 200μL. After a buffer wash the real-time signal would reach a plateau enabling the run of the next sample. The running flow of all experiments described here was 20μL/min using a peristaltic pump.

Primer sequences for PCR

The Multiplex PCR reactions were carried out using a set of primers targeting the bacterial genomic DNA along with a set for human genomic DNA which served as an inner control and correspond to a region of the BRCA1 gene. The starting amount of the human genomic DNA was kept constant at 1ng/reaction for all PCR reactions to maintain constant background, whereas the initial amount of the bacterial genomic DNA was variable. The bacterial cell equivalents (BCE) used ranged from 100 to 10^6 cells/reaction (or 500fg to 5ng of initial genomic bacterial template, assuming that the genomic DNA of each *Salmonella typhimurium* cell is equivalent to 5fg of DNA)

for the thrA and HemD primer sets and from 10 to 1000 cells/reaction (50fg to 5pg of initial template) for the purE set. Also, negative and positive controls were also used in order to obtain a threshold for our results. In order to conduct the PCR and achieve selective detection with the SAW biosensor, the forward primers of each set were biotinylated at their 5' end, ensuring a selective binding on the sensor surface via a biotin-Neutravidin bond. The primer sequences used for the Multiplex PCR Reactions are summed in Table 1 and were obtained from Integrated DNA Technologies (IDT), with the exception of the purE primer set which was obtained from Metabion International AG. Also, for the experiments concerning the purE set, the human primers HR1FB and HR1R which amplified the inner control fragment were obtained from Metabion International AG as well. The product lengths for the thrA, HemD, purE and HR1 sets were 852bp, 666bp, 510bp and 157bp respectively.

Primer	Sequence
Salmonella typhimurium primers	
thrA forward	5'-Biotin-GTCACGGTGATCGATCCGGT-3'
thrA reverse	5'-CACGATATTGATATTAGCCCG-3'
HemD forward	5'-Biotin-GAAGCGTTAGTGAGCCGTCTGCG-3'
HemD reverse	5'-ATCAGCGACCTTAATATCTTGCCA-3'
purE forward	5'-Biotin-GACACCTCAAAAGCAGCGT-3'
purE reverse	5'-AGACGGCGATACCCAGCGG-3'
Human primers	
HR1FB	5'-Biotin-TCCTGATGGGTTGTGTTTGG-3'
HR1R	5'-TGGTGGGGTGAGATTTTTGTC-3'

Table 1. Primer sequences used for amplification of the *Salmonella* and human genes.

Multiplex and Hot-Start PCR

Regarding the PCR conditions for the thrA and HemD primer sets, the following conditions were used; a final concentration of 1x 2G Multiplex Mix (Kapa Biosystems) was used in a reaction of a final volume of 15μL along with 1ng of human genomic DNA (Clontech) which served as an inner control, 5pmol of each primer (for both the bacterial and human primer set), *Salmonella typhimurium* genomic DNA (generously provided by Dr. Bruno Dupuy, Pasteur Institute, Paris, France) which was equivalent to a desired number of bacteria, ranging from 100 to 10^6 cells per reaction and Nanopure dd-H_2O. The reaction was then conducted with a PeqStar 2x (PeqLab) thermocycler at 95^{o}C for 3', followed by 30 cycles of 95^{o}C for 15'', 55^{o}C for 30'' and 72^{o}C for 30''. The reaction was terminated by a final step at 72^{o}C for 1' and stored forever at 4^{o}C, if necessary. For the purE set the conditions were the same with the following changes: the annealing temperature was 62.5^{o}C instead of 55^{o}C, the denaturation, annealing and elongation steps lasted for 10'' each and the cycles were 35 instead of 30. The product lengths were then detected by using 1/3 of each reaction for agarose gel electrophoresis and ensuring no

contamination was present (agarose purchased from Nippon Genetics). The agarose gel was 2%, 0.5x TBE, the staining was conducted with GelRed (Biotium) and the DNA marker used was the FastGene 100bp DNA Ladder (Takara).

The Hot-Start PCR reaction conditions for the purE primer set (only purE was tested) were different than the conditions for the Multiplex PCR. The reactions contained 1x 2G HotStart Mix (Kapa Biosystems), supplied with 1ng of human genomic DNA (Clontech) which served as an inner control, 2.5pmol of each primer (for both the bacterial and human primer set), *Salmonella typhimurium* genomic DNA which was equivalent to a desired number of bacteria, ranging from 10 to 1000 cells per reaction and Nanopure dd-H_2O. The reaction was conducted with a PeqStar 2x (PeqLab) thermocycler at 95°C for 3', followed by 40 cycles of 95°C for 10'', 62.5°C for 10'' and 72°C for 10''. The reaction was terminated by a final step at 72°C for 1' and stored forever at 4°C, if necessary. Detection of the products was similar to the Multiplex protocol as described above. Tables 2 and 3 summarize the Multiplex and Hot-Start PCR reaction recipes and thermal cycling conditions.

PCR recipe		Thermocycling conditions	
Reagent	Final concentration/quantity	Conditions	
2G Multiplex Mix (2x)	1x	Initial step	95°C for 3'
Human Genomic DNA	1ng	Cycling step (30x or 35x)	
Salmonella forward primer	5pmol or 1/3μM	Denaturation Annealing Elongation	95°C for 15''/10'' 55°C/62.5°C for 30''/10'' 72°C for 30''/10''
Salmonella reverse primer	5pmol or 1/3μM	Final step 72°C for 1'	
Human forward primer	5pmol or 1/3μM		
Human reverse primer	5pmol or 1/3μM	Storage 4°C forever	
Salmonella Bacteria Cell Equivalents *(BCE)*	As desired		
dd-H_2O	Add up to 15μL		

Table 2. Multiplex PCR conditions.

PCR recipe		Thermocycling conditions	
Reagent	Final concentration/quantity	Conditions	
2G Hot-Start Mix (2x)	1x	Initial step	95°C for 3'
Human Genomic DNA	1ng	Cycling step (40x)	
Salmonella for. primer	2.5pmol or 1/6μM	Denaturation Annealing Elongation	95°C for 10'' 62.5°C for 10'' 72°C for 10''
Salmonella rev. primer	2.5pmol or 1/6μM	Final step 72°C for 1'	
Human for. primer	2.5pmol or 1/6μM		
Human rev. primer	2.5pmol or 1/6μM	Storage 4°C forever	
Salmonella Bacteria Cell Equivalents (BCE)	As desired		
dd-H_2O	Add up to 15μL		

Table 3. Hot-Start PCR conditions regarding the purE gene.

RCA reactions

The RCA reactions used for this work include several common pre-RCA steps which primarily aimed the padlock ligation in order to generate a suitable RCA template. These pre-steps were:

Padlock phosphorylation (optional, but highly recommended)

Padlock ligation

It is important to state however, that the steps mentioned could be altered or combined depending on the protocol followed each time. Bearing in mind that additional steps would add cost, experimental time and more importantly different temperatures in the final protocol, it was important to reduce the pre-steps as much as possible. The addition of more temperatures was the most concerning matter, as LoC platforms intend to use as less different temperatures as possible. Increasing and decreasing temperature too many times would not be beneficial as temperature control is a very challenging factor for such platforms, especially when these temperatures are elevated. So, in general, if padlock phosphorylation is not taken into account, the number of temperatures used for the padlock ligation and the RCA reaction would be 3, as it will be explained later. The phosphorylation, ligation and RCA methods are as follow.

1.Padlock probe phosphorylation and ligation

Before setting up and conducting a rolling circle amplification (RCA) reaction, a couple of steps where necessary in order to ensure a circular template for the phi29 DNA polymerase existed. First of all, a linear padlock probe was phosphorylated at its 5' terminus using T4 Polynucleotide Kinase (3' phosphatase minus) (New England Biolabs). The final reaction contained 5 units of T4 Polynucleotide Kinase (3' phosphatase minus), 1x T4 Polynucleotide Kinase Reaction Buffer, 1mM ATP, 5μg BSA and 10μM of linear padlock probe added up to a final reaction volume of 50μL with water ready for injection purchased from a local drug store. The reaction conditions were a simple incubation at 37^{o}C for 30', followed by a step of 65^{o}C for 20' for enzyme inactivation. All reagents were purchased from New England Biolabs with the exception of the linear padlock probe which was purchased from Metabion. The probe sequence was the following: 5'- Pho- GCC TCT ACT CCA TCG TGC AGA TTC TAA G*CC AAA CAC AAC CCA TCA GGA* TCG ATC **GCG CTA AGC GGC GTG GAT A**- 3' and was ordered having the 5' terminus modification (phosphorylation). However, the phosphorylation step was strongly recommended to ensure the modification would be present during the ligation step which followed.

The next step was to conduct a ligation step of the padlock probe in order to produce a circular molecule that would be used for the RCA reaction. For that

purpose, 5μL of the phosphorylated padlock (from the reaction above) were transferred in a new tube supplied with 5 units of AmpLigase (Epicentre), 1x AmpLigase Reaction Buffer (Epicentre), 2.5μg BSA (New England Biolabs), 400nM of the target sequence (Metabion), added up to a final reaction volume of 25μL with water ready for injection. The reaction was conducted at 94^{o}C for 5' (serving as a denaturation step for both cases of single stranded and double stranded DNA samples), followed by 40 cycles of 94^{o}C for 1' and 62.5^{o}C for 2' (for target recognition and padlock probe ligation and circularization). The target sequence corresponds to a region of the purE gene of *Salmonella typhimurium* with the following sequence: 5'- CCA CCG GAA TGC CGC GCG GCA TCT GCA CGA TGG AGT AGA GGC **TAT CCA CGC CGC TTA GCG C**AG CGC TTT GTA CCG GCA CG- 3'. The bold and underlined regions of the target sequence and the padlock probe indicate where the hybridization and the ligation occur. It is important to remember that a strict complementarity is required; otherwise no ligation will occur (even with a single mismatch). Alternatively, ligation was tested in two more additional ways. The first one was that no cycles were performed. Instead, a denaturation step of the target sequence at 94^{o}C for 5'-10', followed by a ligation step of 62.5^{o}C for 30' was used. In that way, temperatures would not be altered too often, giving a more controllable version of the ligation step to apply to LoC platforms. However, the absence of cycling was expected to reduce the amount of padlocks that would be ligated and therefore the overall RCA product levels. The second way was to combine the ligation and RCA steps together using a different ligase that could operate at RT. That would decrease the overall time required down to 60', giving our approach outstanding speed, without necessarily reducing the product performance. Moreover, since RT was applied, a thermocycler was only relevant for padlock phosphorylation, giving our method the option to exclude expensive equipment. And since the padlock was already phosphorylated when ordered and the phosphorylation step is optional, the whole process time could be reduced to 60' and could be achieved just by mixing the reagents for single-stranded targets, or by having a single 5' denaturation step before mixing for double-stranded targets! The latter approach will be described in a following chapter.

2.Thermocycled and on-bench RCA

Once the padlock probe was ligated and circularized, the RCA reaction was ready to be conducted. It is noteworthy that the initial target (the purE gene region) was important only for the padlock ligation and once that was achieved it served no further purpose.

The RCA reaction was performed using 5μL of the ligation reaction (see previous chapter) which were transferred in a new tube supplied with 5 units of phi29 DNA Polymerase (New England Biolabs), 1x phi29 DNA Polymerase Reaction Buffer (New

England Biolabs), 2.5μg BSA (New England Biolabs), 200μM dNTP mix (New England Biolabs), 400nM biotinylated d-UTP (Metabion), 400nM 5'-biotinylated HR1F Human primer (Metabion), added up to a final reaction volume of 25μL with water for injection. The RCA reaction was conducted for 60' either at 30^{o}C using a thermocycler, or at room temperature (on-bench), followed by a step of 65^{o}C for 10' to inactivate the phi29 enzyme. Theoretically, the RCA reaction can produce molecules up to 70kB for 60' of reaction time. The HR1FB Human primer was selected to ensure that hybridization would occur only between the primer and the padlock to avoid non-specific hybridization of the primer to the target sequence (or the *Salmonella typhimurium* genome in general). PCR testing in our lab has shown that the HR1FB primer does not bind to Salmonella genome (data not shown) and production of non-RCA DNA (phi29 also elongates linear sequences, although about 10 times slower than circular ones) would be avoided. The primer sequence can be found in table 1 and binds to the region of the padlock in italics. The 5' terminus biotin would serve as a binding site between the RCA product and Neutravidin used to saturate a biosensor surface during an acoustic experiment. More such binding sites would be introduced randomly throughout the RCA product when phi29 would apply the introduction of biotinylated d-UTP. Note that d-UTP is 500 times less than the regular dNTPs fact that indicates that d-UTP will only be introduced only to a small percentage of the RCA product.

After the reaction was over, 5μL were used along with 1μL of 6x loading dye for gel electrophoresis. The marker used was 100ng of λ DNA-BstEII digest (New England Biolabs) and staining was conducted with GelRed (Biotium). The gel was 0.5x TBE 1% agarose gel (agarose purchased from Nippon Genetics) and was running for at least 2h at 120V-150V.

3.RCA on SAW biosensor surface

An alternative of classic RCA followed by an acoustic experiment is to conduct the RCA reaction directly on the sensor surface. This approach is almost similar to the one mentioned above with small alternations. The steps up to the point and including the ligation are identical. As far as the RCA reaction is concerned the following plan has been followed: a continuous flow-through pattern has been followed alternating between washing buffer and samples. The washing buffer used was PBS, $MgCl_2$ 10mM, pH 7.4. After a first wash, 40μg of Neutravidin (Invitrogen) of concentration of 200ng/μL (in the PBS buffer) was used to saturate the biosensor surface, followed by a second wash. Then, an amount of biotinylated HR1FB Human primer was used which was equivalent to the amount of primer used for 4 RCA reactions as described above (concentration 200nM in the PBS buffer), to ensure that all Neutravidin molecules would be bound with a primer via a Neutravidin-biotin bond. This would create a stable binding site for a circular padlock probe once the

RCA mix would be introduced to the sensor surface enabling the RCA product to be formed on the surface. So, bearing this in mind, after another wash the RCA mix was applied on the surface. The RCA mix was similar to the one described above with two important differences. The first one was that the primer and the biotinylated d-UTP were not used to create the mix. Instead, they were substituted with water. The primer was not used because it was already bound with the Neutravidin from a previous step and the d-UTP was not used because the primer had saturated the Neutravidin molecules making the biotins of the d-UTP irrelevant. The second difference was that the volume of the RCA mix was doubled meaning that it was equivalent to 2 RCA mixes as the ones described above (in fact, 2 RCA reactions were used per experiment instead of one because 25μL was a relatively small volume). Once the reaction mix was deposited on the sensor surface, the flow was stopped so the mix was not washed away and the reaction was left to be carried out on the surface for 60', at RT. Afterwards, a final buffer wash was used so that any unbound molecule was washed away and the acoustic signal was measured. Table 4 summarizes the whole process including the reaction recipes and reaction protocols.

Reaction	Reagent	Final concentration/quantity	Protocol
1.Padlock Phosphorylation	Linear Padlock T4 PNK Reaction Buffer ATP BSA	10μM 5 units 1x 1mM 5μg	37°C for 30' 65°C for 20'
2.Padlock Ligation	Phosporylated Padlock (from step 1) AmpLigase Reaction Buffer BSA Target sequence	5μL 5 units 1x 2.5μg 400nM	92°C or 94°C for 5' (1).40x 92°C or 94°C for 1' 62.5°C for 2'or (2).62.5°C for 30'
3a.Thermocycled or on-bench RCA	Ligation Product (from step 2) Phi29 Polymerase Phi29 Buffer BSA dNTP mix d-UTP HR1FB primer	5μL 5 units 1x 2.5μg 200μM 400nM 400nM	 30°C or RT for 60' 65°C for 10'
3b. RCA on surface (used 2 RCA reactions/experiment), Quantities were doubled	Ligation Product (from step 2) (x2) Phi29 Polymerase (x2) Phi29 Buffer (x2) BSA (x2) dNTP min (x2) Neutravidin (on surface) HR1F primer (on surface) (x4)	10μL 10 units 1x 200μM 400nM 200ng/μL 200nM	 RT for 60'

Table 4. RCA conditions regarding thermocycled, on-bench and on-surface reactions.

4.Combined padlock ligation and RCA

For this approach the following reaction set-up was chosen. 5μL of phosphorylated padlock from the first step (see previous chapters) were transferred in a new tube supplied with 5 units of phi29 DNA polymerase (New England Biolabs), 1x phi29 DNA polymerase Reaction Buffer (New England Biolabs), 2.5μg BSA (New England Biolabs), 200μM dNTP mix (New England Biolabs), 400nM biotinylated d-UTP (Metabion), 400nM 5'-biotinylated HR1F Human primer (Metabion), 400nM of the target sequence (Metabion), 200μM ATP (New England Biolabs), 1500 units of T7 DNA ligase (New England Biolabs), 1x T7 DNA ligase Reaction Buffer (New England Biolabs), added up to a final reaction volume of 25μL with water for injection. The reaction was left at RT for 15', 30', 45' and 60' and was terminated at 65^{o}C for 10'. Then 5μL of the reaction were used along with 1μL of 6x Loading Dye for gel electrophoresis. Table 5 summarizes this approach.

Reagent	Final concentration/quantity	Protocol
Phosphorylated padlock	5μL	
Phi29 DNA polymerase	5 units	
Phi29 buffer	1x	
T7 DNA ligase	1500 units	RT for 15'/30'/45'/60'
T7 buffer	1x	
BSA	2.5μg	65^{o}C for 10'
dNTP mix	200μM	
d-UTP	400nM	
HR1FB primer	400nM	
Target sequence	400nM	
ATP	200μM	

Table 5. The protocol for combining the ligation and the RCA in one step. Several reaction times were examined to detect the product by gel electrophoresis.

5.Other concerns regarding RCA

The RCA approaches mentioned above were the basic ones used for this work. However, there are some matters that need to be mentioned. The first and maybe most important matter is that in all protocols above the target sequence was single-stranded DNA, synthesized by Metabion. This is not the case as far as reality is concerned though. When analyzing a real food sample the scenario where a *Salmonella* double-stranded, genomic DNA is the actual target instead of a synthesized, single-stranded oligonucleotide, is far more likely (this is of course ironic, because the latter case is by no means realistic). It must be understood though that use of the single-stranded molecule was made only for the simplicity of the method and as a proof of principle approach. Double-stranded genomic DNA was actually used as a target. Whenever this is the case it will be mentioned in the Results chapter. Furthermore, for the RCA protocols we only used purE as the target. This choice was not completely random as any from the other two targets were not expected to serve equally well. In some cases, the synthesized target was diluted in

order to match the copy number to an equivalent number of bacteria cells (each *Salmonella* cell has only one copy of the gene). This was of course very crucial, as the synthesized target used to describe the RCA protocols above was equivalent to… $5x10^{16}$ copy numbers, which is of course a very unsatisfactory detection limit. So, serial dilutions were used to reach copy numbers that would be acceptable as a starting target quantity. If this is the case, it will be mentioned accordingly.

Another fact to be mentioned is that due to the ligation step being very long when cycles were used, the protocol was altered for some experiments to reduce the reaction time. More specifically the reaction was down to 20 cycles instead of 40, with 1' for denaturation and 20'' of ligation instead of 1' of denaturation and 2' of ligation that were used previously (temperatures were kept the same). Furthermore, the RCA reaction was reduced to 30', to narrow the whole process time to about 60'-70', which is the approximate time a PCR reaction requires. That would offer the possibility to compare the two methods in terms of speed.

Lastly, due to time limitations, the case were the ligation and RCA were combined in a single step was examined only via gel electrophoresis, meaning there was no chance to conduct acoustic experiments with these samples. Future goals are to use a biosensor surface for these analyses.

Atomic Force Microscopy (AFM) imaging

For further examination of RCA effectiveness samples of thermocycled RCA and its corresponding control were visualized using Atomic Force Microscopy. A thermocycled RCA reaction was prepared as already described along with the corresponding control (which did not contain the target sequence during ligation, leading to failure of circular padlock formation and therefore RCA product). 0.5μL of each the positive and negative samples were transferred in a new tube diluted 10 times and then from these dilutions another 0.5μL of each was diluted 10 times again supplied with 10mM of $MgCl_2$ to a final volume of 5μL (samples RCADPOS and RCADNEG). The remaining volume (24.5μL) of the starting reactions was purified using a phenol/chloroform extraction and ethanol precipitation protocol, as described in the following steps.

1. Mix sample with 1 volume of Tris-saturated phenol and 1 volume of chloroform. Centrifuge at 10000rpm for 5' at RT.
2. Transfer upper aqueous phase in a new tube. Add an equal volume of chloroform and mix gently. Centrifuge at 10000rpm for 5' at RT. Repeat twice.
3. Transfer upper aqueous phase in a new tube. Add 1/10 the volume of Sodium acetate 3M.
4. Add 2.5 volumes of absolute ethanol. Incubate the mixture for 30' at -20^{o}C.

5. Centrifuge for 10’ at 10000rpm at RT. Discard supernatant and rinse the pellet with 100μL of 70% cold ethanol. Air dry the pellet and dissolve in 25μL-50μL of dd-H_2O.

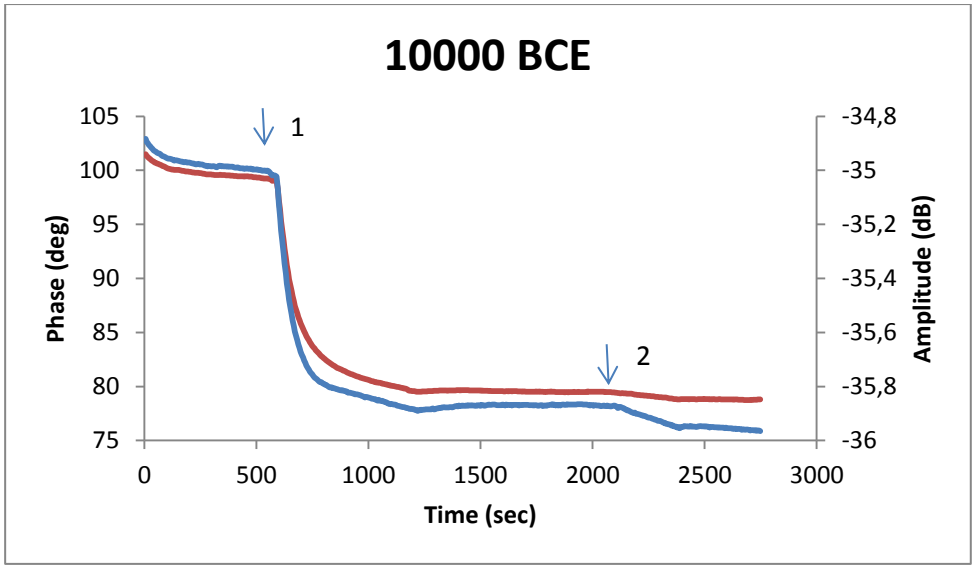
After purification was finished 0.5μL of the samples were diluted 10 times in a final volume of 5μL, 0.5μL of which were diluted another 10 times supplemented with 10mM $MgCl_2$ (samples POSICL and NEGACL). The aim was to compare the AFM images of positive and negative samples before and after DNA purification. The four samples were deposited on freshly cleaved mica (RS Company) and were left there for 30’ at RT. Each mica was rinsed with 1mL of dd-H_2O twice and dried with nitrogen. As reported elsewhere [70], image visualization was carried out in air using the NanoScope IIIa system. The instrument operated in tapping mode and in ambient temperatures using Veeco tips (RTESP model, spring constant 20N/m-80N/m; resonance frequency 267KHZ-298KHZ; tip radius 10nm), and a scan rate of 1Hz-1.5Hz. The scan sizes obtained ranged from 1μm-5μm.

Results

Acoustic Experiments

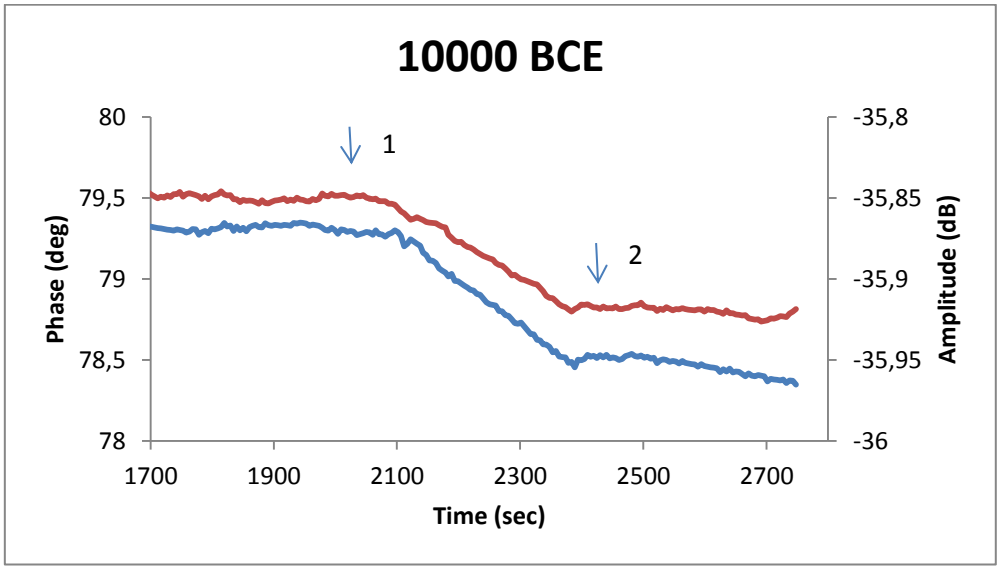
As already stated an acoustic experiment runs in a continuous flow-through manner involving steps of sample loading and buffer washing in an alternate way. Sample deposition on the sensor surface can be monitored as changes in amplitude (measured in dB) and phase (measured in deg) over time in a real-time manner. After each washing step, after the signal reaches a plateau, a sample can be loaded followed by an additional washing step. This process can be repeated several times, the number of which depends on surface saturation. In our experiments the first sample loaded was always Neutravidin for surface saturation and selective binding. After Neutravidin various PCR products were tested without further sample process. For more information regarding the acoustic experiments, refer to the Materials and Methods chapter.

After the experiment is over real-time data can be obtained and further analyzed using a suitable software program. After analyzing the data, calculation of changes in amplitude and phase were obtainable. These changes (ΔA and Δph) would give an acoustic ration for each sample. It is important to note that in order to calculate these values the signal should have reached a plateau. If not, slopes were calculated and correction was needed, so that the signal before and after the change was parallel. In case this was not possible, no measurement could be obtained. A real-time experiment is shown in Graph 1. For convenience issues, the experiment chosen, had only one step of sample loading, although this is not always the case.



Graph 1. Real-time acoustic experiment for 10000 BCE using the thrA primer set along with a human inner control. The changes in amplitude (red) and phase (blue) could be measured giving an acoustic ration for the tested sample. Arrow 1 indicates Neutravidin loading and after a plateau is reached (1500sec-2000sec), the sample was loaded (arrow 2).

It is important to note that the real-time graph depicted above was in a scale that would not be ideal; since the acoustic ratio of the sample was the matter of importance, the scales had to be adjusted to restrict the plot to the part(s) regarding the samples. This would make calculations easier. Graph 2 depicts the sample changes of amplitude and phase of graph 1.



Graph 2. Changes for amplitude and phase for 10000 BCE using the thrA primer set along with a human inner control, as shown in graph 1. Adjustment of the two axes would make calculations much easier. The changes were measured by comparing the values between points 1 and 2.

The graphs depicted above were used to give the reader a general idea of how an acoustic experiment looks like. For spacing issues however, real-time data will not be

included. Instead, tables with the acoustic ratios of all experimental data will be provided. Including all real-time graphs would be extremely exhausting for the reader due to their large number, which is not the intention of this work.

Multiplex PCR

The first Multiplex PCR results that will be presented here are the ones concerning the HemD and thrA primers along with a 157bp region of the BRCA1 gene as an inner control (see Table 2 for more information). PCR samples corresponding to a range of 100 to 10^6 BCE for both sets of primers were prepared. Additionally, negative controls (absence of *Salmonella* DNA) were prepared for both primer sets. An additional sample was prepared for the thrA sets, which included the *Salmonella* product while excluding the human product. Figure 8 demonstrates the PCR reactions for both sets.

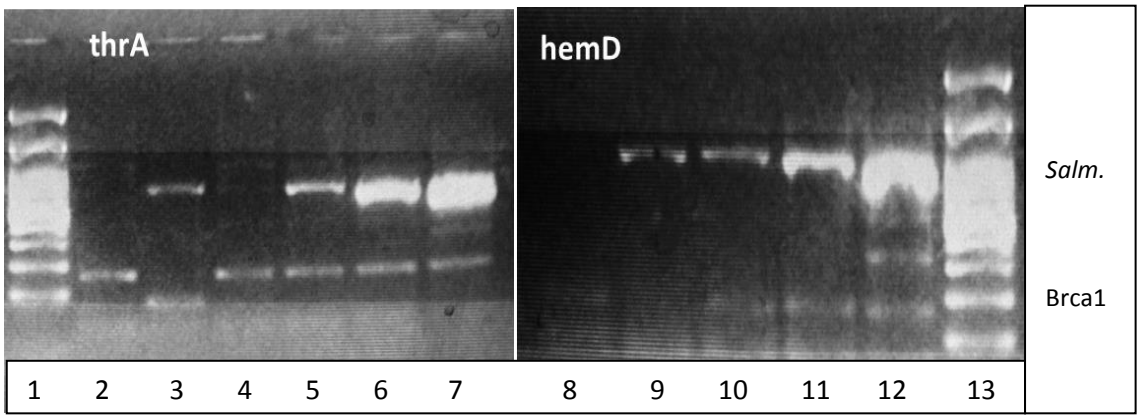


Figure 8. PCR using serial dilutions for thrA and HemD primer sets. Lanes 1 and 13: 100bp DNA Ladder marker. Lanes 2 and 8: negative control, only human product present. Lanes 4 and 9, 5 and 10, 6 and 11, 7 and 12: PCR using serial dilutions of 100, 1000, 10^4 and 10^5 BCE respectively. 10^6 BCE is not shown. Lane 3: *Salmonella* control without the human product. Upper bands: bacterial product. Lower bands: human product. Ladder lowest band: 100bp. Note that the bands of the PCR reactions lower than the 100bp band of the ladder correspond to primer dimers.

Acoustic experiments were performed with all the above PCR reactions and measured. These experiments were not conducted in multiples. The reason is that they were a first approach to test if differentiation between positive and negative samples could be achieved and what the detection limit would be. Tables 6 and 7 summarize all the data obtained from these experiments.

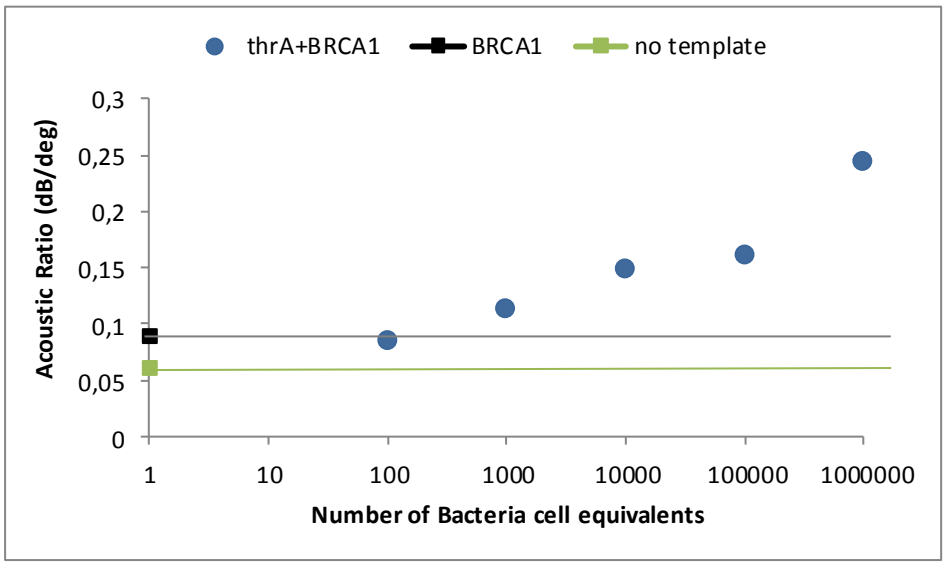
Salmonella Genomic template+human genomic DNA 1ng/reaction, 30 cycles (thrA)			
BCE	Amplitude (dB)	Phase (deg)	Ratio (dB/deg)
0	0.070	0.770	0.0909
100	0.090	1.050	0.0857
1000	0.116	1.018	0.1139
10000	0.092	0.616	0.1494
100000	0.160	0.990	0.1616
1000000	0.180	0.740	0.2432
100/no human DNA	0.030	0.490	0.0612

Table 6. Acoustic data obtained from PCR reactions using the thrA primer set.

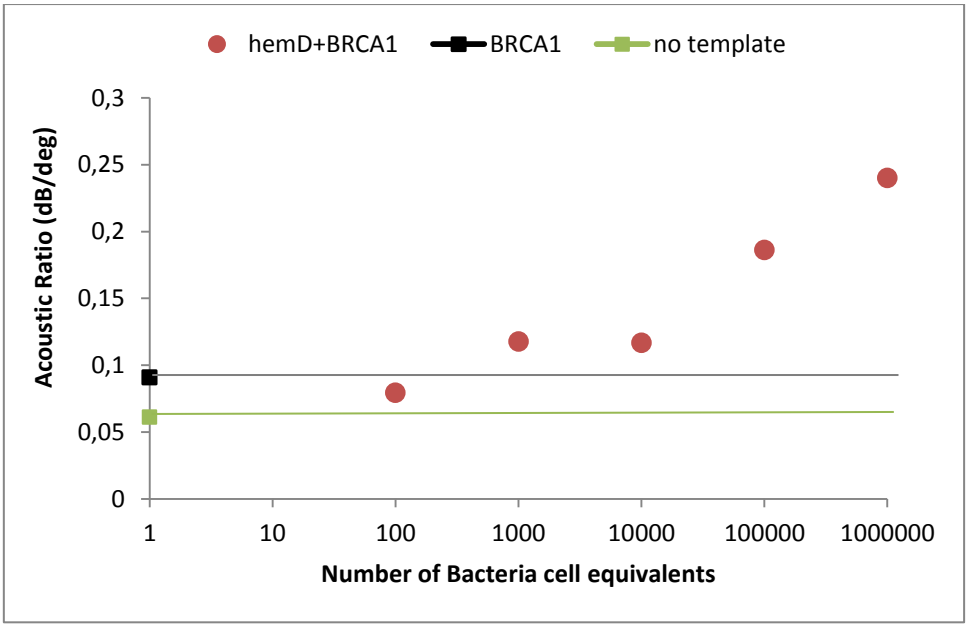
Salmonella Genomic template+human genomic DNA 1ng/reaction, 30 cycles (HemD)			
BCE	Amplitude (dB)	Phase (deg)	Ratio (db/deg)
100	0.050	0.63	0.0793
1000	0.080	0.68	0.1176
10000	0.070	0.60	0.1167
100000	0.132	0.71	0.1862
1000000	0.240	1.00	0.2400

Table 7. Acoustic data obtained from PCR reactions using the HemD primer set.

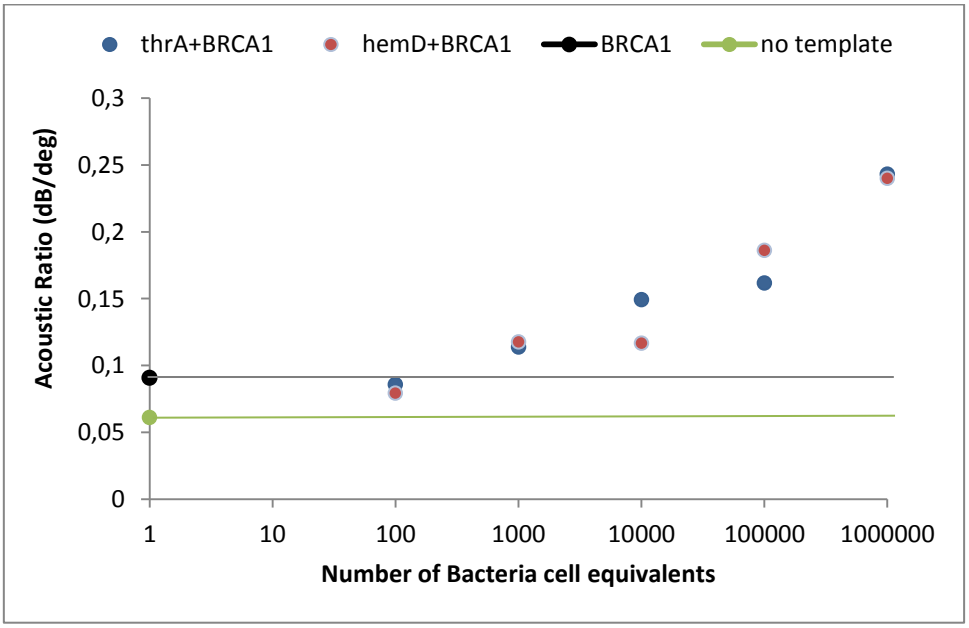
Graphs 3 and 4 plot the acoustic ratios obtained vs the BCE DNA used for each PCR for the thrA and HemD sets respectively, whereas Graph 5 plots both sets of primers vs the BCE DNA used for each PCR. As it immediately can be observed, the two sets of primers showed no big differences as far as the gene targets for the PCR are concerned. Apparently, the detection limit for these sets of experiments was approximately 100 BCE/reaction, which is equivalent to 100 bacterial cells/ reaction before DNA amplification.



Graph 3. Acoustic ration vs BCE plot of multiplex PCR for the thrA *Salmonella* primers. The limit of detection is approximately 100 BCE, which is equivalent to the DNA of 100 bacteria. Horizontal axis is in logarithmic scale.



Graph 4. Acoustic ratio vs BCE plot of multiplex PCR for the HemD *Salmonella* primers. The limit of detection is approximately 100 BCE. Horizontal axis is in logarithmic scale.

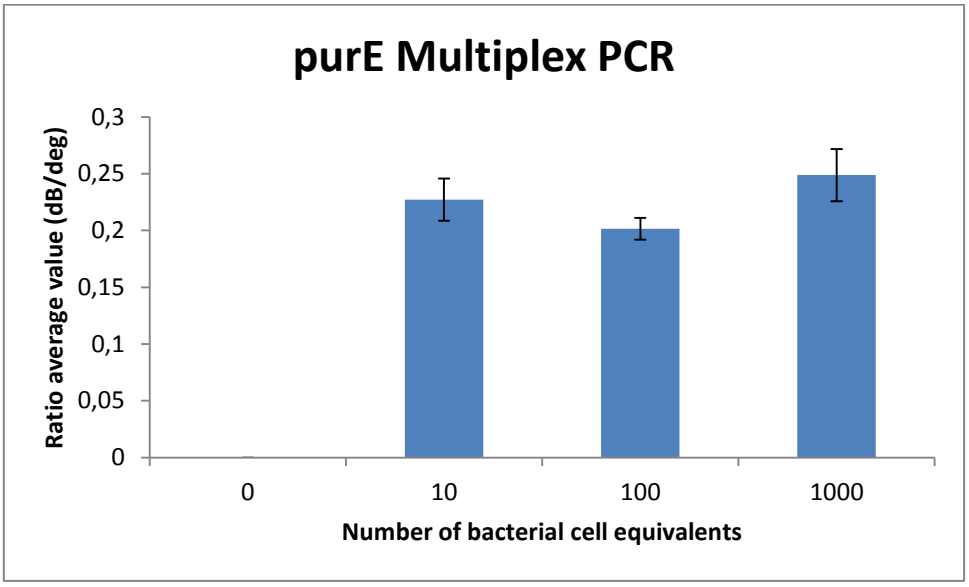


Graph 5. Comparison of thrA (blue) and HemD (red) primer sets. The two scenarios do not exhibit many differences. The detection limit is approximately 100 BCE. Horizontal axis is in logarithmic scale.

The multiplex PCR for purE will be demonstrated below. Dilutions of 0, 10, 100 and 1000 BCE were tested. The reaction conditions were altered for optimization reasons (for example 10'' for the cycling steps are more than enough regarding to the manufacturer and 5 more cycles would allow for a possible reduction to the 100 BCE detection limit). In this case, at least two or three experiments were performed for each dilution. That would enable us to calculate an average value and standard deviation for each dilution enabling to draw safer conclusions and determine a (new) detection limit more accurately.

Salmonella Genomic template+Human Genomic DNA 1ng/reaction, 35cycles (purE)		
BCE	Average Value (dB/deg)	Standard Deviation (dB/deg)
0	0	0
10	0.2271	0.0187
100	0.2016	0.0096
1000	0.2488	0.0230

Table 8. Average values and standard deviations for corresponding BCE dilutions for Multiplex PCR using the purE primer set.



Graph 6. Average value and standard deviation of corresponding BCE dilutions for Multiplex PCR using the purE primer set.

Examining the above information, the first thing that crosses the mind is that for 0 BCE (meaning control with only the Human product present) the average value is 0, fact that contradicts with the previous findings. Although this finding was based on two independent experiments it still makes no sense; a biosensor would detect such a control sample and give measurable changes both in phase and amplitude, giving a specific ratio. However, the change in amplitude in these cases was zero. Even in that case however, the most likely scenario is that there has been some kind of failure during the acoustic experiment and not that the control sample would give a zero change in amplitude (this scenario is highly improbable). Bearing that in mind, we had no choice but to accept the baseline of the thrA and HemD case as the baseline here as well. In fact, since the *Salmonella* product is absent in all cases, the only practical difference between controls of the three primer sets in a PCR mixture are the primers themselves. This difference plays, at least theoretically, no significant role in the measured ratios. If the former baseline (thrA/HemD case) is to be accepted someone could observe that in this case, the 10 BCE used here can be discriminated from the human control. This is very advantageous as the detection limit is lowered significantly, without using pre- or post-PCR treatment. There is one

problem to this whole case, though. The average values of 10 BCE and 100 BCE are almost the same. That means that the two cases cannot be separated when examining real samples. Although 100 cells are an already good detection limit and 10 cells are an even better one, this detection limit cannot be set with absolute certainty. The goal of the project is to go down to a maximum of 100 copies of target (meaning genes, cells or any copy equivalent)/mL of food sample. The detection limits achieved so far are very promising, but there is still room for improvement; these results indicate that even though 10 or 100 cells could not be discriminated as the initial template amount, still, detection can be achieved starting from 10 *Salmonella* cells/mL of food sample.

Hot-Start PCR

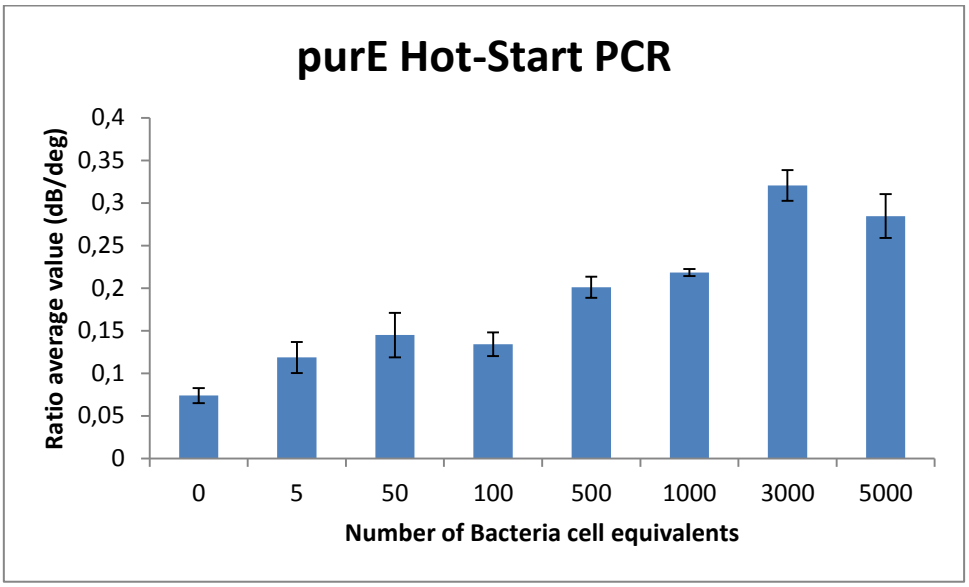
Hot-start PCR was briefly described in the introduction of this thesis and the approach we followed is described in the corresponding chapter of Materials and Methods. Hot-Start PCR has the advantage of utilizing Multiplex PCR protocols as well. PurE primers were used to amplify the corresponding target along with the known inner control. The BCE dilutions used were equivalent to 0, 5, 50, 100, 500, 1000, 3000 and 5000 cells. Each experiment was conducted in triplicates and the results are gathered in Table 9 and Graph 7 below.

Hot-Start PCR, for purE primer set (40 cycles)		
BCE	Average Value (dB/deg)	Standard Deviation (dB/deg)
0	0.0737	0.0089
5	0.1185	0.0183
50	0.1449	0.0261
100	0.1341	0.0138
500	0.2011	0.0124
1000	0.2183	0.0043
3000	0.3205	0.0181
5000	0.2846	0.0257

Table 9. Average values and standard deviations of Hot-Start PCR for the purE primer set.

These data demonstrate that a starting template of 5 BCE can result in amplification detectable by our system, which can also be discriminated from a negative control. This detection limit is the best acquired with PCR in the present work. Still, it is very promising, as this starting material does not undergo pre-enrichment before experimentation. It is obvious that even though the 5 BCE can be detected from a negative sample, they overlap with 50 and 100 BCE, creating the same problem as with Multiplex PCR. However, this is not observed so intensly concerning the 5 BCE; it is more obvious for dilutions that are very near to each other (50 BCE and 100 BCE, 500 BCE and 1000 BCE, 3000 BCE and 5000BCE). This is because these dilutions do differ by less than an order of magnitude, meaning they are very close. Qonsequently, differentiation between them is very hard or maybe impossible, as

these differences are not considered significant and therefore are not detectable. Comparing the 5 BCE with 500 BCE for instance, gives a significant and obvious difference. This happens because these samples have dilutions with difference of some orders of magnitude.



Graph 7. Average value and standard deviation of corresponding BCE dilutions for Hot-Start PCR using the purE primer set.

One can conclude that combining the PCR protocols described here with a SAW acoustic biosensor, offers a great tool for food analysis for pathogen bacteria. This holds true, as detection limits as the ones achieved by this method without further process of the samples are difficult to achieve.

In the next chapters, results of the RCA methods will be presented.

Thermocycled and on-bench RCA

Rolling Circle Amplification (RCA) is an isothermal amplification method which can produce large fragments of single-stranded DNA or RNA molecules (can reach 70Kb in an hour), which contain a target sequence in multiples in a row. These products however, tend to form structures that look like a coil due to the fact that are single-stranded. Running an agarose gel to detect these molecules would require several hours of electrophoresis as the large size would make migration of the molecules very difficult. Another important issue is that RCA does not produce fragments of the same length; the length of the product varies depending on which point the elongation started during the reaction. This factor cannot be controlled and the image collected from an RCA electrophoresis is expected to depict a smear. Regarding the thermocycled and on-bench RCA reactions (which are typically the same apart from the temperature used for elongation), this was found to hold true (Figures 9a and 9b).

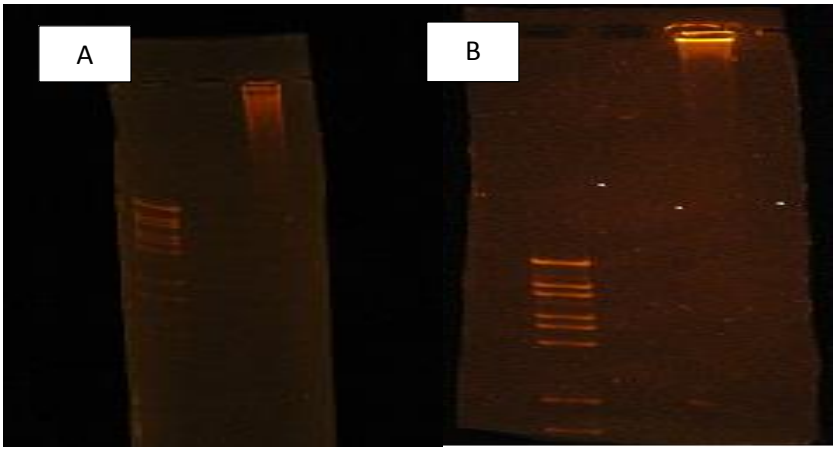


Figure 9. A: Thermocycled RCA (30^{o}C for the RCA reaction). The smear is clearly visible after several hours of electrophoresis. B: On-bench RCA (RT for the RCA reaction). Similarly, the smear is also visible after several hours of electrophoresis. The marker used in both cases was 100ng of λ DNA-BstEII digest (New England Biolabs), upper band of marker 8454bp. The product amplified should be considerably larger. The middle lane of each image is the negative control of the corresponding RCA.

The figure above demonstrates the ability to conduct RCA with or without a thermocycler. This factor could be essential when application of the method would be applied to integrated platforms, as it will simplify the whole attempt considerably. To further clarify the previous findings, 10μL of the total 25μL of the thermocycled RCA reaction were transferred in a new tube for digestion. The principle of this approach lies within the fact that RCA produces concatamers of the padlock probe as a single-stranded DNA molecule. These concatamers include the primer sequence used for elongation in all the repeats of the padlock probe (Figure 2). Incubating the RCA product with an excess of an oligonucleotide complementary to the primer sequence was expected to create local double-stranded regions to the concatamer body. These double-stranded regions were designed to include a unique digestion site when 10 units of BccI restriction enzyme (New England Biolabs) were added for the reaction. Digestion of the product for 30' would lead to 73 bases of single-stranded fragments (same as the padlock size), with small double-stranded regions at each end as shown in figure 10.

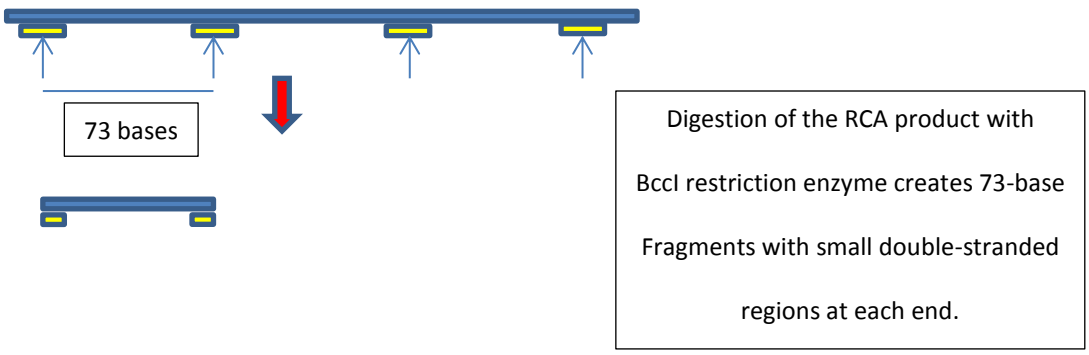


Figure 10. Digestion of RCA products with BccI restriction enzyme. Arrows indicate restriction sites.

This approach was not absolute though; digestion may not have been perfect, leading to formation of fragments with length of multiples of 73 nucleotides. This was not a matter of concern however, as this digestion was conducted solely to enhance the idea that the RCA reactions worked. Gel electrophoresis was conducted to verify this approach. Figure 11 shows the fragments after digestion.

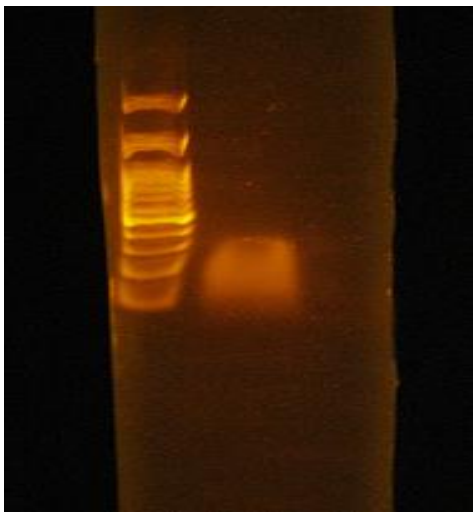


Figure 11. Digested RCA product. The marker used was 100bp DNA Ladder marker (Takara). Bottom band of marker: 100bp and going up for 100bp for each band.

The figure above shows that digestion was nearly perfect as the RCA digestion fragment seems to have been of the expected length. Note that these 73 nucleotides are not exclusively single-stranded. Furthermore, the digestion fragments are so numerous that the band formed is extremely wide. A last thing that should be considered is that digestion was performed only once or twice for clarification issues only. Typically, this step is not part of the whole process and was omitted in almost all cases.

For further evaluation of the success of RCA, thermocycled RCA was also tested using Atomic Force microscopy (AFM). 4 samples, two positives and two negatives were used as described in the corresponding chapter of Materials and Methods. Two RCA samples were purified using a classic phenol/chloroform and ethanol precipitation protocol as already described, and comparison between the samples would be available. In the case of purified vs unpurified RCA positives, we expected that a different image would be obtained since the purified sample was expected to contain only DNA. As depicted in Figure 12 (a-d), it is most likely that this expectation was not met. The images obtained were very similar suggesting the formations observed in the positive samples are coiled single-stranded RCA products. As far as negative samples are concerned, almost nothing was observed on the mica surface. However, this was not considered a false negative result, as mica is a completely smooth surface and on the other hand, according to figure 9a, the negative sample was indeed negative.

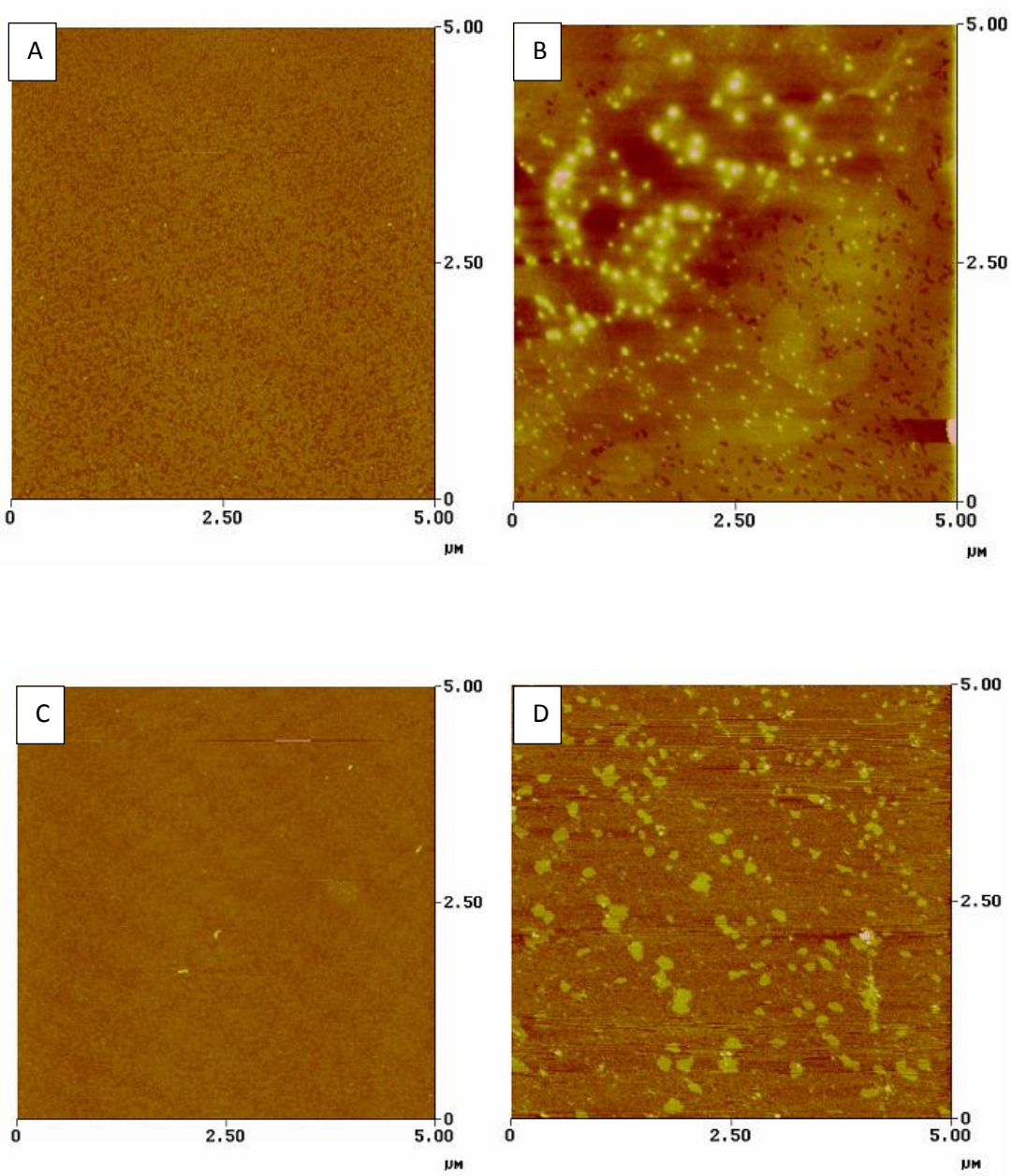


Figure 12. AFM images for RCA samples. A: purified negative control (NEGACL). B: purified positive RCA (POSICL). C: non-purified, diluted negative control (RCADNEG). D: non-purified, diluted positive RCA (RCADPOS). Image scale: 5µm.

The above images indicate that purification did not play a significant role, as comparison between negative and positive samples give no major differences. To ensure that, the same positive samples were analyzed again in a lower scale (2µm) and for the unpurified sample even more (1µm). This can be found in figures 13 (a,b) and 14. Image 12 does not agree with previous reports [39, 46], as to how an RCA visual image should appear. However, when scaling down, the images observed were somehow similar to the ones reported elsewhere [images 3c, 3d and 4 of reference 39]. The differences could have more than one explanation. The first one is that instrumentation was not the same; images of references [39, 46] appear to be

clearer than ours suggesting better equipment. What is more, the approaches used were different, implying that comparison may not be applicable. We assumed that our findings suggest that our products may be surprisingly coiled, leading to very solid structures. Even if these hypotheses prove correct, still images 3c, 3d and 4 of reference [39], bear similarities to ours suggesting that our RCA products are very solidly packed.

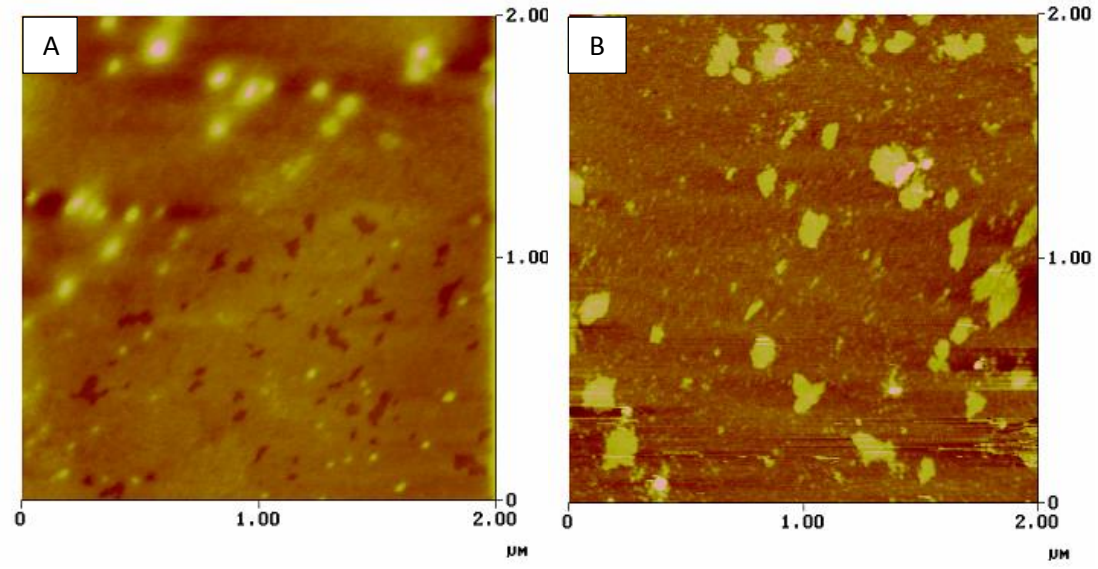


Figure 13. AFM imaging of positive RCA samples on a lower scale (2μm). A: corresponds to POSICL sample from Figure 12b. B: corresponds to RCADPOS sample from Figure 12d.

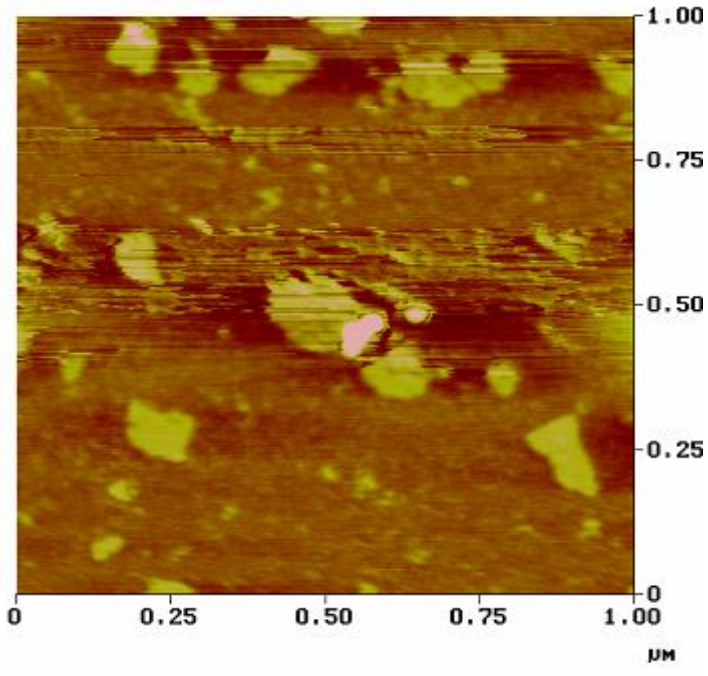
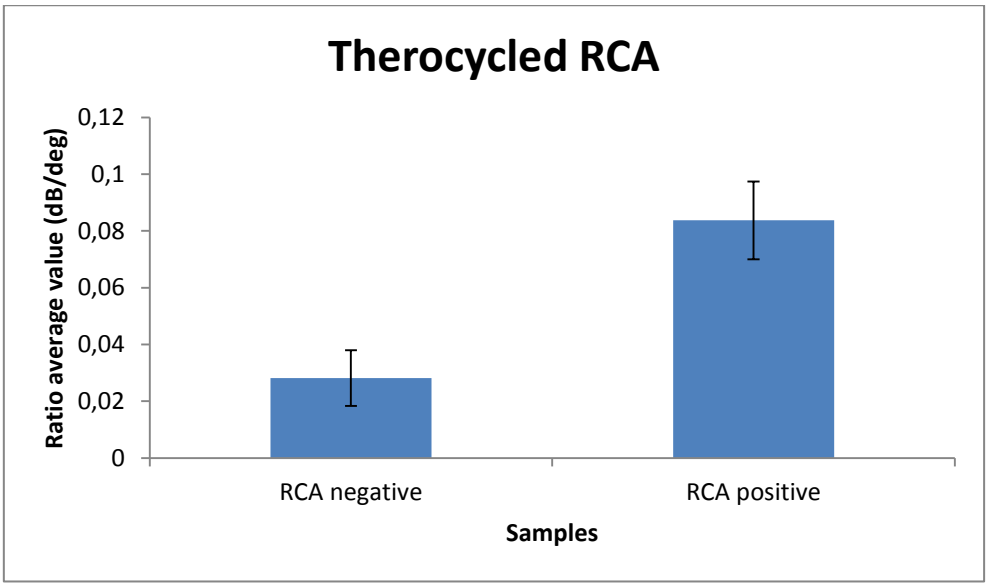


Figure 14. AFM imaging of the RCADPOS sample (corresponds to Figures 12d and 13b). Scale: 1μm.

After ensuring that the RCA reactions gave indeed the desirable product, acoustic experiments were conducted for both the positive and negative samples. For the thermocycled RCA protocol, Table 10 and Graph 8 depict the calculated average values and standard deviations of the acoustic ratios for both positive and negative samples (experiments in quadruplets). Keep in mind that these RCA reactions used the long ligation protocol (40 cycles) and that the initial target sequence corresponded to $5x10^{16}$ bacteria copies of the target! Nevertheless, this was the first approach to apply RCA on a sensor surface and the main goal was to discriminate positive from negative samples before scaling down the initial template.

Thermocycled RCA		
Sample	Average Value (dB/deg)	Standard Deviation (dB/deg)
Negative	0.0281	0.0098
Positive	0.0837	0.0137

Table 10. Acoustic average values and standard deviations of the acoustic ratio for negative and positive thermocycled RCA reactions.



Graph 8. Average values and standard deviations of the acoustic ratio for negative and positive thermocycled RCA.

These results indicate that negative and positive samples could be easily differentiated with our system as far as the thermocycled RCA is regarded. Bearing all above in mind for thermocycled RCA, two more approaches were followed in addition to the experimental data presented above.

The first approach was to dilute the single-stranded target oligonucleotide so that acceptable copy numbers would be used for RCA and acoustic experiments. Serial dilutions were made and RCA was conducted for starting copy numbers of 100, 500 and 1000/reaction. Negative controls were not examined as they are the same with the ones of Table 10 and Graph 8. However, due to limited remaining time, these experiments were conducted only once. The resulting acoustic ratios were 0.0874

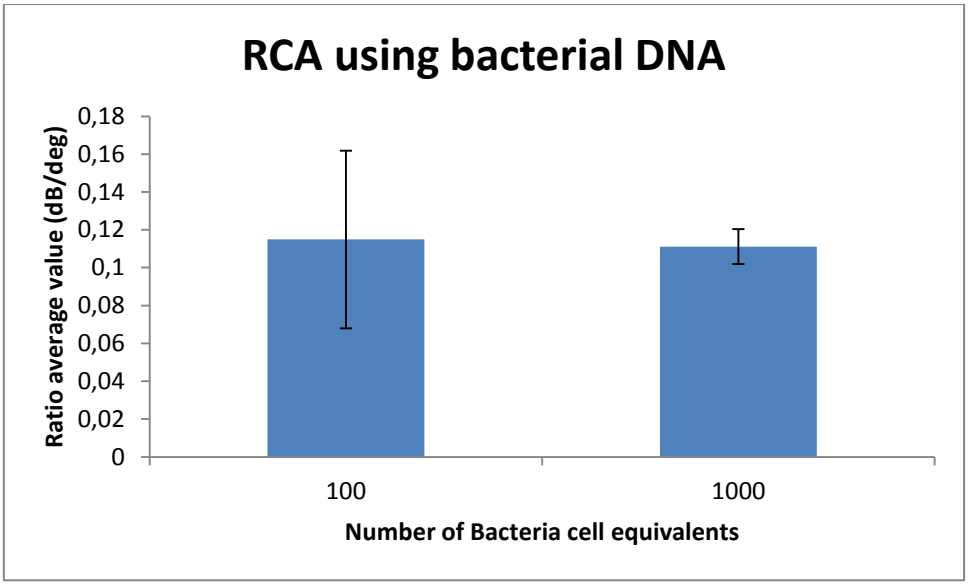
(dB/deg), 0.1060 (dB/deg) and 0.0846 (dB/deg) for 100, 500 and 1000 copy numbers respectively. Although these results are by no means adequate to draw conclusions, it is clear that the calculated values lie in the range of the positive thermocycled RCA presented in Table 10 and Graph 8. It seems that even 100 copy numbers as a starting material give enough product for acoustic detection. If this holds true, then the starting material can be narrowed down to 100 copies or maybe even less, without affecting the detection capabilities of the acoustic experiments and giving very good detection limits. This is important because even though RCA products could not be detected with electrophoresis at this point, the acoustic measurements imply presence of such products. This can be an indirect suggestion of our system's sensitivity and robustness. For further evaluation of these findings more experimentation is required.

The second approach was to use *Salmonella* genomic DNA to conduct RCA and acoustic experiments, as this would be the real case when applying food analysis. For this reason, dilutions of 100 and 1000 BCE genomic DNA were prepared. The two protocols for ligation did not follow the one used up to now; the first one to be tested reduced the number of cycles to 20 and the cycling step durations (refer to the Other concerns regarding RCA chapter). The second one did not include cycling at all. Instead, ligation was performed by heating the sample at 92^{o}C for 5' and then performing ligation at 62.5^{o}C for 30'. In both cases the RCA reaction time was set to 30' instead of 60', so that the whole procedure could be narrowed down to about 1 hour like in the case of PCR offering a comparison possibility in case the acoustic data showed detection. Negative controls were assumed to stay constant from the previous experiments and experiments were performed in triplicates. For the second approach the results are presented below.

Thermocycled RCA using *Salmonella* genomic DNA		
BCE	Average value (dB/deg)	Standard Deviation (dB/deg)
100	0.1149	0.0469
1000	0.1111	0.0092

Table 11. Calculated average value and standard deviation of the acoustic ratio of 100 and 1000 *Salmonella* BCE respectively. This approach followed a fast ligation and fast RCA step limiting the total amount of time for the whole process to 60'-70'.

Obviously, these results suggest that 100 BCE or 1000 BCE give the same result. That could mean that 100 BCE would be adequate for detection, even though differentiation of the two concentrations seems to be improbable. These results are also very close to the previous ones using the synthesized single-stranded target (both the high and low concentration cases), indicating that a starting template equivalent to 100 cells can efficiently provide detection and thus offer a low detection limit.



Graph 9. Calculated average value and standard deviation of the acoustic ratio of 100 and 1000 *Salmonella* BCE respectively. This approach followed a fast ligation and fast RCA step limiting the total amount of time for the whole process to 60'-70'.

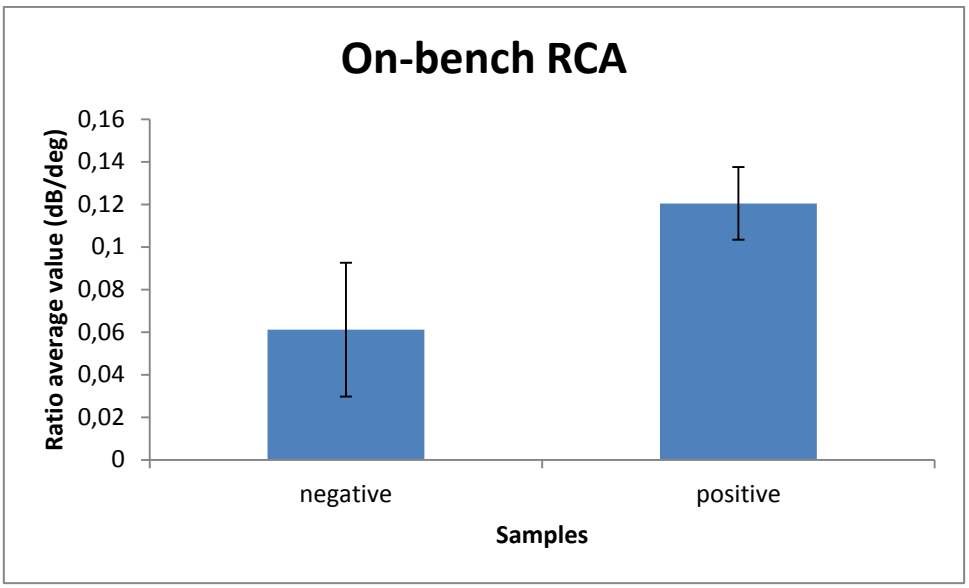
On the other hand, as far as the second approach is regarded, it seems that the method failed. This method resulted in unstable acoustic results which offered no conclusion at all and will not be presented here. It is possible that the padlock probes faced competition from the complementary genomic DNA strand of their target and were removed before ligation could take place. So, ineffective ligation can be held responsible for the inconsistency of these data. It seems cycling may be needed during ligation, so that denaturation of the target can occur often, giving the chance to more and more padlocks to be ligated.

It should be mentioned here that the ligation and RCA conditions were altered only for the experiments concerning the dilutions of the synthesized targets and the experiments concerning the *Salmonella* BCE genomic DNA. The experiments that follow concerning the on-bench RCA used the same ligation and RCA conditions as the thermocycled RCA presented in Table 10 and Graph 8 (40 cycles of ligation and 60' of RCA reaction).

The next step was to examine what the results would be when performing RCA on the bench, at RT. Practically speaking, the thermocycled and on-bench RCA had only one difference, which was the reaction temperature. Consequently, results of them would be comparable with no major differences. Especially for the control samples, it was expected that the results would be almost the same. Regarding the positive samples however, expectations of differences could not be eliminated, as the two samples were not guaranteed to perform equally. On-bench RCA data are presented in Table 12 and Graph 10. Experiments were conducted at least in triplicates.

On-bench RCA		
Sample	Average valure (dB/deg)	Standard Deviation (dB/deg)
Negative	0.0611	0.0314
Positive	0.1205	0.0171

Table 12. Average values and standard deviations of the acoustic ratio for negative and positive on-bench RCA.



Graph 10. Average values and standard deviations of the acoustic ratio for negative and positive on-bench RCA.

It can be immediately observed that even though negative and positive results can be differentiated from each other (even though standard deviations error bars are very close to overlap), the values of both the average value and standard deviation differ from the respective of thermocycled RCA. The results of these sets of experiments are elevated for about 50% for positive samples and almost 100% for the negative ones. The first explanation when observing the positive results could be that RCA had different efficiencies for the different operating temperatures. This however does not explain the great difference between the negative results. It is most likely that different treatment of samples, even negative ones, has a different effect on the ratio of acoustic experiments, indicating that the phenomena near the sensor surface are more complex than a simple DNA binding. This aspect has already been reported in previous work in our lab [64].

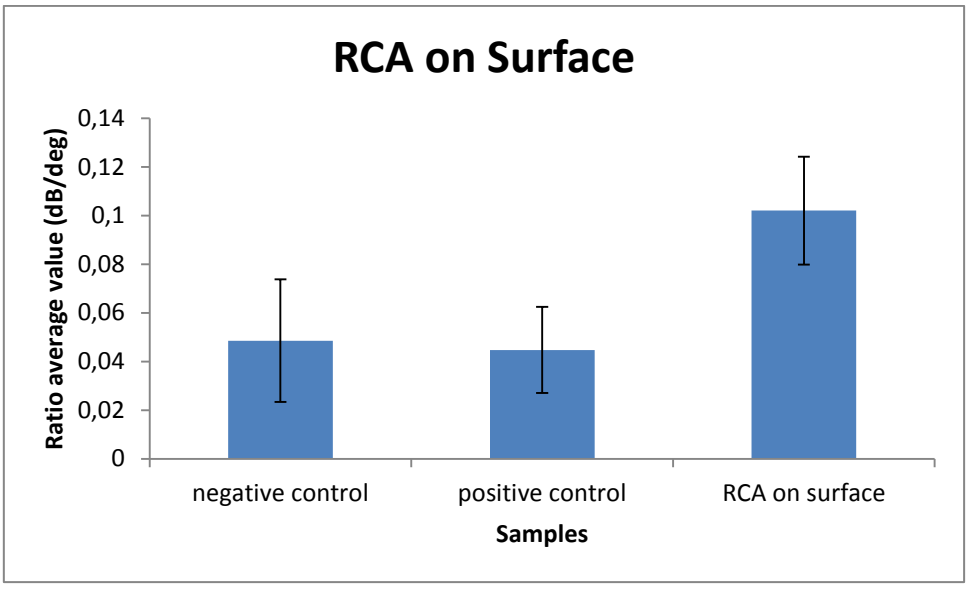
RCA on SAW biosensor surface

Phi29 DNA polymerase can operate at RT, as it has already been shown with experiments conducted on the bench. This led to the challenge of trying to conduct the RCA on the sensor surface itself and monitor its' progress in Real-Time. After a Neutravidin step and wash an excess of HR1FB primers were bound to the Neutravidin molecules via a biotin attached to their 5' terminus. Since the HR1FB

primer has been used so far for the RCA reactions, it would provide an anchor for the circular padlocks. If the whole RCA mix is loaded on the surface and the flow is stopped ligated padlocks were expected to hybridize to the primers. This would enable the phi29 enzyme to initiate RCA on the surface. The conditions of this method are described in the corresponding Materials and methods chapter. RCA was conducted for 60' with this protocol. Negative samples were also tested. These samples were identical to the positive ones with the exception of the target during ligation, which was absent in the control samples. That means that during loading of the RCA sample, circular padlocks would not be present in the negative mix, since ligation was not successful. That would consequently lead to no RCA product formation. Moreover, a positive control was also conducted. The mix would contain normal circular padlocks, but the primer on the surface would be thrA which could not anchor the probe on the surface and therefore RCA would be impossible since there would be no primer available for the enzyme. Experiments were conducted at least 3 times for each sample and are as follow.

RCA on sensor surface		
Sample	Average value (dB/deg)	Standard Deviation (dB/deg)
Negative control	0.0485	0.0252
Positive control	0.0447	0.0177
RCA on surface	0.1020	0.0222

Table 13. Average value and standard deviation of the acoustic ratio of RCA reactions performed directly on the SAW biosensor surface. Negative and positive controls are also included.



Graph 11. Average value and standard deviation of the acoustic ratio of RCA reactions performed directly on the SAW biosensor surface. Negative and positive controls are also included.

The conclusions that can be drawn regarding the results above are that the RCA reaction and the negative control can be differentiated but just barely. This may be a consequence of the fact that during these experiments the samples used where

double compared to the ones used to other experiments. This was done to ensure that the reaction mix would cover the whole surface. This could simultaneously be problematic as well, as the sample was extremely concentrated giving colossal amplitude and phase changes (data not shown). Even after washing, the surface would be so much saturated with molecules from the reaction that evaluating data could be hard. However, even under these slim circumstances, differentiation was achievable, although we were at the limit of differentiation. If further experimentation is to be conducted in the future, the first possible step to gain a clearer picture of the whole case and to be able to differentiate with greater confidence is to dilute the samples to more manageable concentrations. One very good result however is that the negative and positive controls not only overlap, but in fact they are virtually the same. This was expected though; having no circular padlocks with the correct primer, or having circular padlocks with the wrong primer, could only lead to the same result: no RCA products could be amplified. In other words, being able to discriminate the RCA from both positive and negative controls (even barely), was good evidence that RCA can work on the sensor surface directly, at RT, provided that the padlocks are already formed.

Combined padlock ligation and RCA

The basic concept behind this approach was to simplify our method even more and reduce the total process time even down to 25' of total process time. Reactions were prepared and were left on the bench (RT) for 15', 30', 45' and 60' along with a negative control, which had no target template. 10' of heating to 65^{o}C for enzyme inactivation was vital; the RCA has no reason to stop, unless the user deactivates the enzyme or the reaction mix runs out of one or more reagents. Putting the reaction in ice would not help much either as the enzyme can work even to 4^{o}C. Although there was no time to evaluate the procedure further and no acoustic experiments were conducted, some interesting piece of information can be obtained from Figure 15.

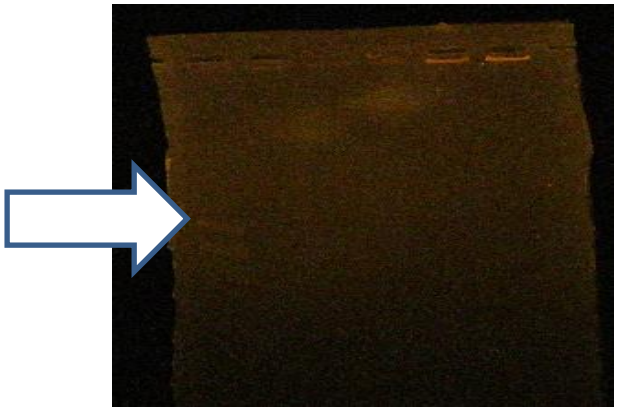


Figure 15. Combination of padlock ligation and RCA at RT. Lane 1: 100ng of λ DNA-BstEII digest (New England Biolabs) marker, arrow: largest band (cannot be seen clearly), Lane 2: negative control. Lanes 3-6: RCA for 15', 30', 45', 60', respectively.

The first thing that comes to mind by seeing Figure 15 is that only the 45' and 60' reactions worked. That is most probably a false conclusion. Being unable to detect the product during electrophoresis does not mean that it does not exist; most probably it is not enough to detect with a semi-quantitative method like electrophoresis. It is most probable that if these reactions are used for AFM imaging and acoustic experiments, then one would gain distinctive results between the reactions and negative controls. Secondly, even though synthetic targets were used, it is still possible to try with genomic DNA or even bacteria cells. The only thing that would be added is a single 5' step of pre-heating for thermal lysis and/or denaturation of the targets along with an excessive amount of padlock probes. Incubation in ice for a short time will decrease the temperature to levels that the enzymes will not be deactivated and then the mix can be added. The excessive amount of padlocks will be the best chance for ligation to occur; once the mix is added there can be no cycling since the phi29 polymerase does not tolerate such temperatures. The most probable scenario is that electrophoresis will give an image similar to figure 15 or even worse. However, examination of the presence of the product via a more sensitive method is the key to evaluate the procedure when using genomic DNA or whole cells. Lastly, it is noteworthy that since ligation and RCA can be combined, there is a good chance the whole process can be achieved directly on a sensors surface. This would be interesting for conducting proof of principle experiments but it is rather difficult to achieve with real samples. This is because this approach would require single-stranded targets; using double-stranded ones would require a denaturation step and unfortunately, temperature control is not applicable on SAW surfaces. Another, problem when using real samples is non-specific binding of the probe to other regions of the genomic DNA, due to low temperature (RT). Although mismatches do not allow ligation, a case of some unpaired bases of the padlock probe, may allow non-consecutive regions of the padlock to bind in other areas of the DNA as well (meaning that one or more bases can be left unpaired forming a single-stranded loop) enabling probe ligation and RCA of a non-specific template. If one decides to combine these steps, several suitable controls should be considered first in order to eliminate this possibility.

Discussion

Biosensors are an emerging field in bioanalytical fields such as diagnostics and pathogen detection [71-76]. Pathogen detection is not limited to foodborne related diseases, though and of course is not limited to the SAW devices, as well. However, foodborne diseases like Salmonellosis affect millions of people globally in an annual basis with severe symptoms that can potentially lead to death. Various detection methods have been applied up to date for *Salmonella* detection, including PCR variants, ELISA and other conventional techniques. The present work attempted to

follow a new strategy. The first step was to amplify *Salmonella* target genes with either the well-established PCR method using a multiplex protocol to include an inner control, or by using the isothermal technique, RCA to produce giant single-stranded amplicons of the target sequence. The amplified samples would then be loaded on a SAW biosensor surface and changes in amplitude and phase (corresponding to viscoelasticity changes and mass deposition on the surface) would be measured to obtain acoustic ratios for evaluation. According to the acoustic ratios, detection limits could be set, as low as the differentiation of controls and positive samples is achievable. Both methods gave good detection limits; PCR went down to 5 BCE/reaction whereas RCA detected 100 BCE/reaction up to the current point, but it is believed to go even lower with further optimization. Both methods exhibited enormous sensitivity when combined with a SAW surface. However, each of them possesses properties that make them more or less applicable for integrated platforms like LoC.

Conventional and multiplex PCR is usually a relatively affordable and fast technique that has high sensitivity. It is usually simple to design and easy to execute. However, as multiplexity is concerned, cost, design difficulty and execution difficulty all increase as multiplexity demands increase; primer design becomes more complicated, cost is increased dramatically, execution may not be as simple as it seems. PCR faces other problems too; very high contamination risks and ineffectiveness due to inhibition, requirement for personnel training which is more demanding for more complicated protocols, requirement for laboratory workspace and equipment (most importantly thermocyclers), high energy demands due to thermocycling and difficulties for portability. Despite these drawbacks, PCR is an all-time classic technique with many advantages which are mentioned below. The capability of multiplexing, the affordable cost (for the standard lab, however this is not always the case), the high sensitivity and the ability to be combined with numerous other molecular techniques, place PCR as maybe the most commonly used technique in the average biology lab. In our case however, the most important drawback of PCR is its' own operating nature. Using three different temperatures in integrated platforms is rather challenging, especially when shifting from one temperature to the other is required to happen within some seconds. This means that when using such systems temperature control is difficult both in terms of temperature shift and stabilization, and timing of the whole process.

RCA on the other hand is a well-established isothermal method utilizing phi29 DNA polymerase to create single-stranded molecules. The cost is comparable to the one of PCR and although at first glance it may look a complicated method, in reality it is rather simple. It has capabilities of multiplexity as well. For example, when designing the padlock probe for this thesis, I also designed padlocks for the thrA and HemD genes as well by alternating the padlock of purE in the termini regions for specific

hybridization to the correct targets, maintaining the rest of the padlock the same (data not shown). In that way multiplexity could be achieved (especially when using genomic DNA) by just using one primer (HR1FB) for the RCA reaction. The reason why the thrA and HemD padlocks were not used is that the purE was expected to hybridize slightly better to its target and of course for timing issues. What is more, RCA operates at a constant temperature enabling operation without expensive equipment. Padlock ligation may be the limiting step however, but with the use of a suitable ligase this is not considered a problem. For example, if denaturation is required, Ampligase is an exceptional ligase due to its incredible thermostability. Ligation and RCA can also be combined as already described to make the method rapid and operate at RT. Comparing PCR with RCA in terms of ease of use, one cannot say that one of them is easier for the user to perform as they both are relatively easy. RCA is not inferior in terms of sensitivity and performance either; results have demonstrated that detection limits achieved by both methods were comparable and with future optimization RCA can be even more sensitive. Other advantages of RCA are that inhibition is always faced adding BSA to all the reaction steps of the procedure, can be combined with other techniques and technologies (see introduction) and is expected to be included in integrated systems easier, offering portability options. Using only one temperature (apart from denaturation steps that may be included) is way easier to control especially concerning that RCA can operate at RT. Additionally, since microfluidic systems utilize sample volume that range from nL to some μL, for sample concentration and increased sensitivity and efficiency, ambient temperatures of RCA do not pose the risk of sample evaporation. This is not always the case for PCR though, as demand for elevated temperatures and cycling can lead to evaporation of samples of such small volumes.

Comparison of PCR and RCA in Table 14 strongly indicates that RCA has advantages that cannot be overlooked when designing LoC platforms. These advantages would place RCA as the suitable amplification method in our design. The most important reason is the ability to use only one temperature for it to work making the whole process more controllable and easier to use. When a user wants to analyze 24 samples simultaneously (''Love-food'' project direction), controlling temperatures for so many samples can be challenging. Use of a single temperature on the other side, adds simplicity without reducing sensitivity and amplifying capability.

Comparison between PCR and RCA		
	PCR	RCA
Ease of use	Moderate[1]	Moderate[1]
Reagent cost	Moderate[1]	Moderate[1]
Specialized equipment	Required	Can be avoided
Workplace	Required	Required
Design	Simple to Difficult[1]	Simple to Difficult[1]
Trained Personnel	Required	Required
Time required/speed	Moderate/Fast	Moderate/Fast
Energy consumption	Moderate to high	Low to moderate[2]
Amplification capacity	Very high	Very high
Number of temperatures	3	1[2]
Thermal cycling	Yes	May be used for ligation
Sensitivity	High	High
Combination with other techniques	Yes	Yes
Use in integrated platforms and portability	Challenging	Easier[2]

Table 14. Comparison of PCR and RCA regarding various parameters. 1: parameter changes with increase of complexity. 2: ligation and denaturation steps that may be needed are not included.

Isothermal amplification technologies have already been established for miniaturization for combination with on-chip techniques like microarrays and microfluidics [36, 77-81]. These advances may be proven crucial for design of LoC platforms as isothermal amplification methods have already been established in analysis for foodborne pathogen detection like *Salmonella* [79, 80, 82-91], as well as other pathogens and agents of health interest [92-95]. It is therefore possible to combine RCA with microfluidic devices and SAW surfaces to create LoC platforms in the near future. RCA is therefore considered more suitable than PCR for this application. This by no means suggests that PCR is inferior to RCA. PCR is well-established and very widely used in all sorts of fields and applications. But for this particular case RCA appears more advantageous.

Throughout this work much effort has been put to RCA condition optimization. The detection limit was 100 BCE/RCA reaction without pre-enrichment. The absence of pre-enrichment is one of the most important aims of this work as experimentation time, resources, working hours and long protocols are avoided. This detection limit is of course satisfactory but it is strongly suggested that optimization continues in the near future, as this detection limit can be possibly lowered even more reaching levels compared to the sensitivity of PCR presented here (5 BCE/PCR reaction). However, even if the data regarding RCA appear promising another question arises: the reason why the acoustic ratios of all positive RCA experiments, regardless of starting template or its' origin, were almost the same. This finding may indicate that actually detection was not achieved by immobilized RCA products on the surface but instead of an immobilized pair of primer and padlock probe. This means that due to

primer and padlock excess, even if RCA products were present, they could not bind on Neutravidin and instead they would be washed away during the washing steps. In order to examine this hypothesis, future work will include experiments that RCA cocktails will be prepared in the same way already presented, but before the reaction initiation enzyme inactivation will take place. This approach leads to preparation of mixtures identical to the ones used for RCA amplification with the important exception that RCA will not actually occur. These mixtures can be then examined for their acoustic ratios and compared to their corresponding findings presented in this work. If the ratios between the new control samples and their respective positive samples differ it can be concluded that the acoustic ratios obtained here depend on the RCA products themselves. If, on the other hand, same results are obtained, the initial hypothesis is expected to hold true, meaning detection was not achieved by RCA products. If this is the case, it is not necessarily a negative finding, as this may be an indication that a low detection limit can be found without actually amplifying a DNA target, but rather comparing negative and positive samples from the ability to produce a circular molecule with ligation. Up to now our data can confirm this detection scenario is possible, based on different conformations of the molecules bound on the sensor surface for negative and positive samples (a primer hybridized to a linear and a circular padlock respectively). Further work is crucial to determine which case holds true; however, if detection was actually achieved by the different conformation of the primer-padlock pair and not from an amplification product, would be a very interesting result. This would increase the overall sensitivity tremendously, as need for DNA amplification is not mandatory. Increased sensitivity is expected by using different SAW surfaces like photoresist surfaces that operate in a higher frequency than 155MHz. In our lab, photoresist surfaces operating approximately at 310MHz are available at the moment. These surfaces exhibit much higher sensitivity than the ones operating at 155MHz indicating more solid and reliable results can be obtained by using them and lower detection limits can be achieved. Use of other acoustic biosensors like the Quartz Crystal Microbalance (QCM-D) can be beneficial as well. Even though QCM-D cannot be applied to the current project, additional results can be very helpful especially for proof of principle matters. Other isothermal amplification techniques can be tried as well; RCA was chosen mainly for its simplicity, sensitivity, ability to work at ambient temperatures including RT and possibility to be coupled with other techniques like magnetic beads, gold nanoparticles and polymers. However, it is possible that another isothermal approach can perform equally better. That can only be learned through personal experience.

All the above can lead to creation of LoC platforms. Such platforms have numerous advantages such as portability, low energy consumption, affordable cost, high sensitivity, high-throughput capabilities, fast analysis, lower fabrication cost and are

user-friendly devices just to name a few. They aim to do many of the analyses a lab can do outside of the lab, thus making the need of workspace, personnel and expensive equipment irrelevant. But these properties are not the only advantages. LoC platforms can have numerous applications as well: diagnostics, food control, disease control, gene screening, plant sciences, molecular biology, chemistry etc. Furthermore, μTAS (Micro Total Analysis Systems) which are a more general approach of integrated platforms aiming to integrate not some but all the processes of a lab are gaining popularity since they aim to minimize a whole lab to a portable device for scientific analysis. Despite all these advantages, LoC platforms have also some disadvantages: they are novel technologies and therefore not completely developed and commercially available, scaling down may sometimes give results opposite than expected, microfabrication can sometimes be inaccurate and some physical and chemical effects like capillary forces, interaction of the construct material with the samples, surface roughness etc. can make processes in LoC platforms more complex than expected theoretically. However, the advantages overcome the disadvantages as LoC platforms will gain increasing popularity in the upcoming years.

Abbreviations

This is a list of the abbreviations used, in order of appearance.

DNA: Deoxyribonucleic Acid

PCR: Polymerase Chain Reaction

RCA: Rolling Circle Amplification

SAW: Surface Acoustic Waves

LoC: Lab-on-(a)-Chip

CDC: Centers of Disease Control

ELISA: Enzyme-Linked Immunoabsorbent Assay

dNTPs: Deoxynucleotide Triphosphates

Taq (polymerase): *Taq* indicates the source organism of the enzyme, *Thermus aquaticus*

SNP: Single Nucleotide Polymorphism

*Phi*29 (DNA polymerase): *Phi29* indicates the source of the enzyme which is *Bacillus subtilis* phage phi29 (Φ29)

RNA: Ribonucleic Acid

C2CA: Circle-to-circle Amplification

IDT: Interdigital Transducer

d-UTP: Deoxyuridine Triphosphate

RT: Room Temperature

PBS: Phosphate-Buffered Saline

Tris: Tris(hydroxymethyl)aminomethane

BCE: Bacteria Cell Equivalents

BP: Base Pairs

TBE: Tris-Borate-EDTA (Ethylenediaminetetraacetic acid)

ATP: Adenosine Triphosphate

BSA: Bovine Serum Albumin

AFM: Atomic Force Microscopy

RPM: Rounds per Minute

QCM-D: Quartz Crystal Microbalance (with Dissipation)

μTAS: Micro Total Analysis Systems

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