Love Wave Fully Integrated Lab-on-chip Platform for Food Pathogen Detection - LOVE-FOOD

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Optimized protocol for detection of *S. typhimurium* using the RCA method

Deliverable 7.2

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Executive Summary

One of the main objectives of this work package (WP7) is the development of the most efficient amplification scheme for the detection of *Salmonella Typhimurium* in milk samples. Two different amplification strategies were proposed to be employed and compared in order to achieve the maximum sensitivity without compromising the accuracy of the detection. The first is the standard PCR amplification method, already developed and illustrated in D7.1 (submitted at M12) for two Salmonella gene targets. The second was an alternative strategy employing amplification at one constant temperature and specifically the Rolling Circle Amplification (RCA). D7.2 deals with the design and performance of RCA in two formats: 1. off chip followed by acoustic detection, and, 2. on chip, where amplification and detection occur simultaneously. RCA was found to exhibit a comparable sensitivity to that detected with the PCR, i.e. less than 100 bacteria cell equivalent. The final decision regarding the optimum bioassay to be used in the LOC platform (i.e. microPCR versus RCA) will be determined after the final evaluation of the microPCR device, expected at M28.

Main Text

A. Introduction

The detection method developed within task 7.1 involved the direct immobilization of double stranded PCR products on the sensor surface and subsequent measurement of the acoustic ratio. The method worked efficiently since it was experimentally proven that it can detect less than 10 copies of a DNA target. In general, it showed capability of producing both qualitative and quantitative results. Task 7.2 involves an alternative strategy which was based on isothermal amplification from genomic DNA by the rolling circle amplification (RCA) method. RCA requires the use of padlock probes which are linear oligonucleotides that can be ligated into DNA circles provided that the target sequence is correctly base-paired. After ligation (circularization) a specific primer hybridizes to the circular probe and Phi29 elongates the product. When a circle is complete, the Phi29 polymerase displaces the newly formed DNA strand and carries on with the reaction, going around the target for several times. DNA amplification by RCA was expected to permit simplification of the microchip design as the use of three heating chambers would not be necessary. The isothermal amplification was carried out either in the thermocycler (off-chip) followed by product loading on the device and acoustic detection, or directly on the device (on-chip) for simultaneous amplification and detection. In both cases, the acoustic signal upon immobilization of the RCA product was monitored and evaluated.

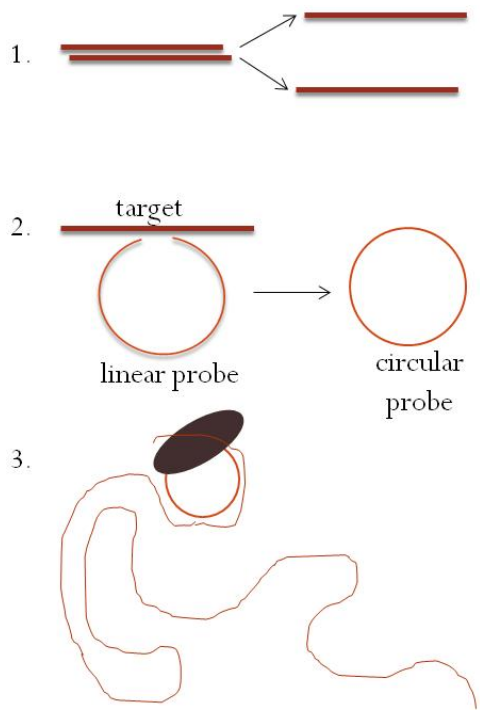

Fig. 1: Schematic depiction of the RCA steps: 1. denaturation and 2. hybridization-ligation and 3, amplification. The black ellipsoid attached to the probe represents the Phi29 enzyme.

B. Experimental part

Acoustic detection of RCA products created off-chip

In a first set of experiments, RCA reactions were performed off-chip and the acoustic ratios of the resulting products were monitored upon subsequent loading on a SAW device operating at 155 MHz. The protocol involved the creation of circular padlocks followed by amplification with Phi29 using a biotinylated primer. RCA produces gigantic single-stranded molecules up to 70000 bases after 1 hour of reaction and each molecule has a different length and conformation with the general shape being the one of a “ball of wool”. To

facilitate immobilization of these products on the sensor surface, biotinylated d-UTPs were supplemented in a minimal amount in the RCA reaction so that multiple biotins would be randomly inserted in the final product body, creating multiple binding sites (fig. 2).

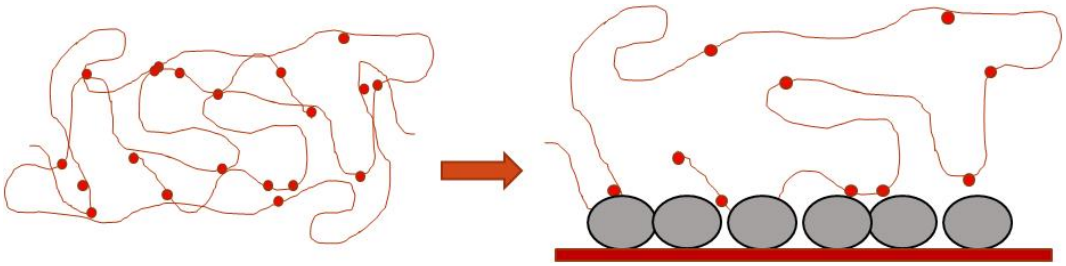

Fig. 2: Acoustic detection of RCA products created off-chip. The RCA products consist of single-stranded DNA molecules of various lengths with random biotin insertions. Each molecule attaches on the surface through biotins in a different way.

The complete assay is summarized as follows: Denaturation and hybridization-ligation steps are performed for 20 to 40 cycles of 1 min at 92^{o}C and 20" up to 2 min at 62.5^{o}C. Amplification mix is then added and the RCA step is performed at 37^{o}C or below for 30 to 60 min. Consequently, the minimum assay time required is approximately 1 hour.

Two types of templates where used for this series of experiments; a synthetic oligo 80 nt long corresponding to the sequence of the Salmonella purE housekeeping gene and isolated Salmonella Typhimurium genomic DNA. **At first,** an excess of the synthetic DNA target template was used (10^{16} copies) in order to produce large amounts of the RCA product for acoustic experiments. The amplification outcome was verified by gel electrophoresis and AFM imaging along with a negative control reaction lacking the target template (fig. 3).

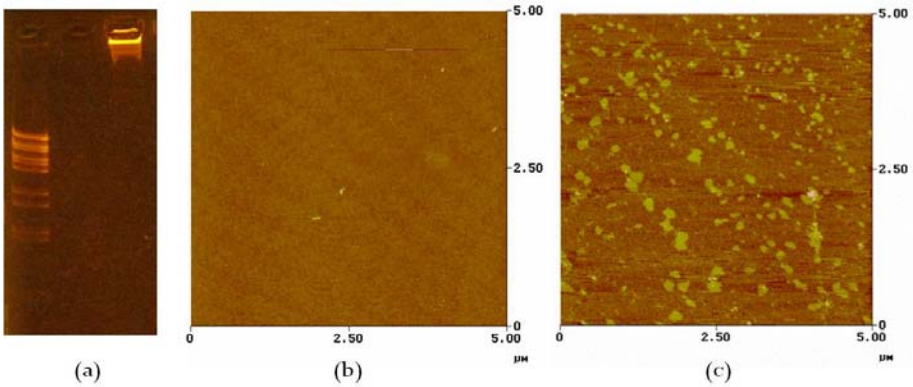

Fig. 3: (a) 1% agarose gel, lane 1: DNA ladder, lane2: negative control reaction, lane 3: RCA reaction. (b) AFM image of the negative control reaction, (c) AFM image of the positive RCA reaction where the coiled single-stranded RCA products are observed as aggregates.

Multiple acoustic measurements (4 repeats) of the control and the RCA reactions were performed and the calculated acoustic ratios showed that the positive amplification reaction

can be clearly distinguished from the negative control (fig. 4). Figure 4 summarizes the acoustic ratios measured for the negative control, the positive reaction with the excess template along with reactions containing as template 100, 500 or 1000 copies of the synthetic target.

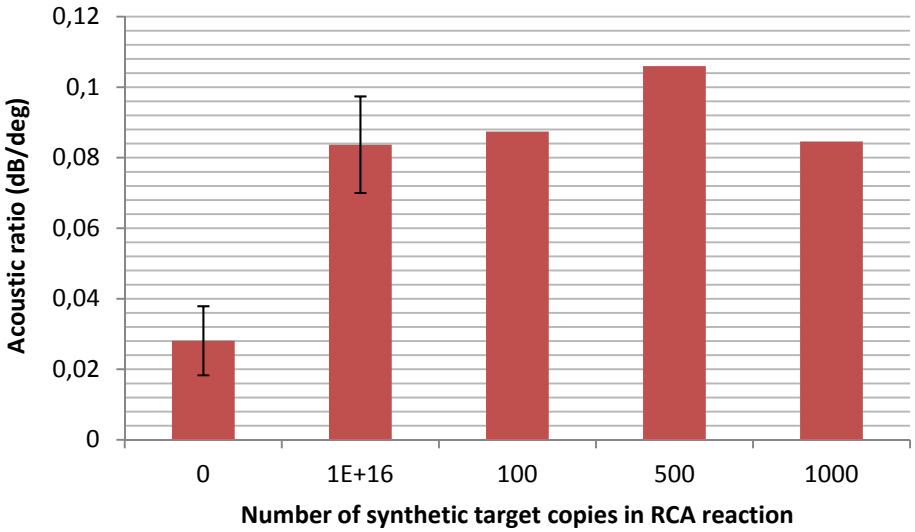

Fig.4: Acoustic detection results of RCA products created off-chip using synthetic DNA template

It seems that even 100 copies as a starting material is sufficient for acoustic detection which is close to the detection limit of the PCR method. It should be noted that the reactions with the low copy number of targets could not be visualized with electrophoresis, although it was detected with the acoustic system.

Secondly, isolated genomic DNA from Salmonella was used as a template in RCA reactions; 100 and 1000 bacterial cell equivalents (corresponding to 100 and 1000 copies of the target sequence, respectively) were used in RCA reactions that were loaded on an acoustic device. The acoustic ratios for these two cases from triplicate experiments were found equal to 0.1149±0.0469 and 0.1111±0.0092 (dB/deg) in respect. These values are in good agreement with those described in fig. 4 indicating that the method can be sensitive to the detection of as low as 100 cells.

Acoustic detection of RCA products created on-chip

In a second set of experiments, amplification of a circular DNA was attempted to be performed on the surface of an acoustic device operating at 155 MHz, taking advantage of the fact that Phi29 DNA polymerase can operate at room temperature, although at slower rates (Soengas et al., J. Mol. Biol., 1995, 253, 517–529). This was also experimentally verified as depicted in fig. 5.

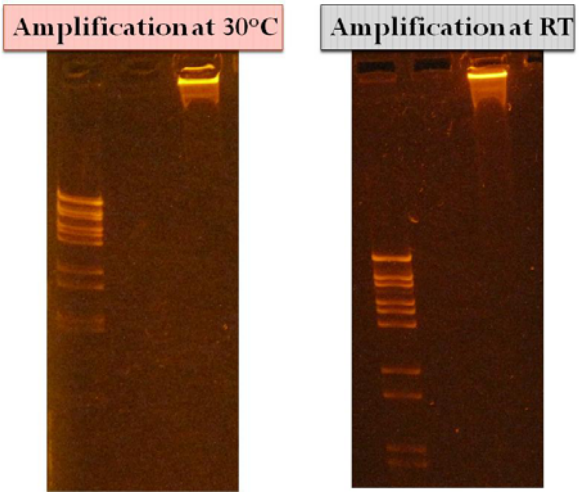

Fig. 5: 1% agarose gel electrophoresis verifying the ability of Phi29 to process polymerization at room temperature compared to 30°C. The first gel image shows the amplification product (lane 3) after 1 hour incubation at 30°C and the second gel image (lane 3) at RT. Lanes 1 and 2 in each gel image show respectively a DNA ladder and a negative control reaction at the same conditions.

The experimental setup involved surface preparation by adsorption of neutravidin protein and subsequent binding of the biotinylated primer used in the previous experiments responsible for initializing rolling circle amplification. Circular padlocks prepared as previously were mixed with Phi29 enzyme and the resulting mix was loaded on the surface under flow. When the surface was completely covered with the amplification mix the flow was stopped for 1 hour during which the circular padlocks were hybridized on the primers and amplification was performed (fig. 6). After 60 minutes, flow was restored and buffer was used to wash any unbound molecules before measuring the acoustic signal.

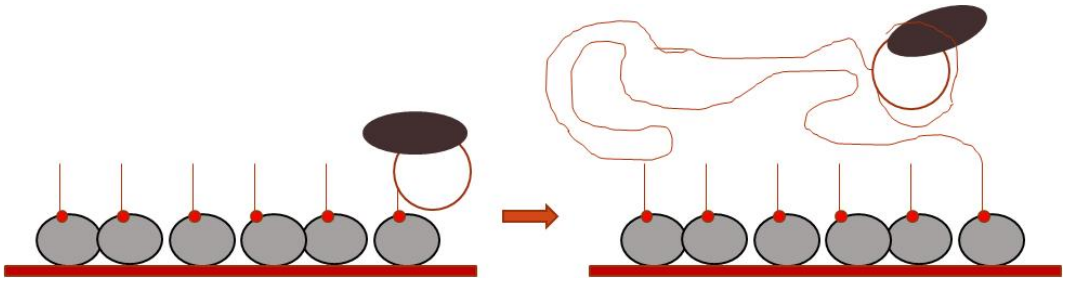

Fig. 6: RCA on-chip. A biotinylated primer is bound to neutravidin. The circular padlock hybridizes to the primer allowing the RCA reaction to begin.

Negative samples were also tested. These samples were identical to the positive ones except for the target which was not included during ligation. As a result, circular padlocks could not be formed which led to no RCA product formation. Moreover, an alternative negative control of specificity was also conducted. The mix contained normal circular padlocks, but the primer on the surface was not complementary to them which would also prohibit initialization of an RCA reaction. Experiments were repeated at least 3 times for each sample and the results are summarized in figure 7.

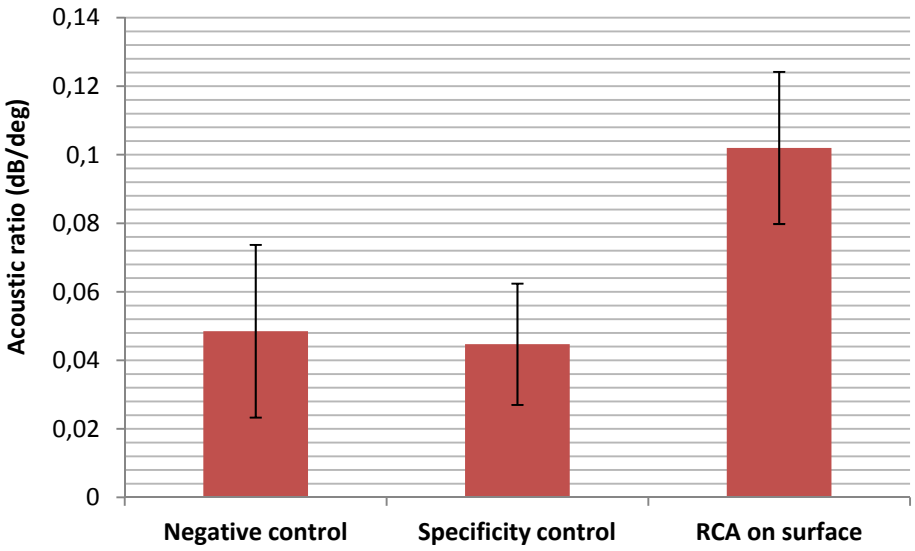

Fig. 7: Acoustic detection results of RCA products created on-chip.

The results show clearly that RCA can be performed and monitored on-chip with specificity when compared to the control reaction with the non-complementary immobilized primer. The acoustic ratio after amplification was found to be 0.102±0.022 while with RCA off-chip was 0.090±0.010, a difference which seems to be within the experimental error. Both negative controls resulted in virtually the same acoustic ratios which can be explained by the fact that in both cases amplification is not initiated either because there are no circular padlocks or because circular padlocks cannot hybridize on the immobilized primer.

Conclusions

During task 7.2 (WP7) the potential of an alternative to PCR amplification strategy for the detection of *Salmonella Typhymurium* in milk samples was investigated. This alternative strategy was based on the rolling circle amplification (RCA) method which requires only one constant temperature for the amplification step and can be used for off-chip as well as on-chip experiments. Experiments were conducted using either synthetic DNA targets or isolated Salmonella genomic DNA and the amplification step was performed either off the device or on the device surface. The results revealed that amplification occurs either off or on-chip since the acoustic ratios are almost identical when both the synthetic and the isolated genomic DNA were used as targets. The detection protocol in total required at least 60 min and this was due to the fact that although amplification requires only one constant temperature, the target recognition step (DNA denaturation, probe hybridization and *circularization) requires two additional ones. In general, the method showed potential as* alternative to PCR displaying similar limits of detection while it can also be performed inside microfluidic chambers similar to those designed for PCR amplification in the LOVE-FOOD platform (fig. 8). However, it should be noted that the 2 extra temperatures required for the denaturation and ligation steps do not lead in a much simpler LOC design than that envisaged for the microPCR.

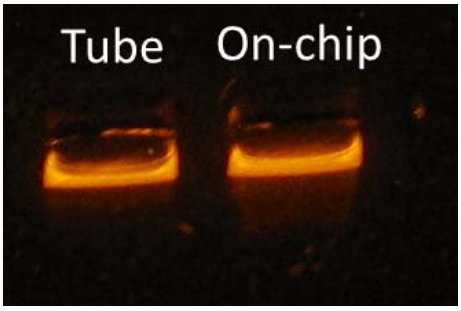

Fig. 8: 1% agarose gel electrophoresis image showing the results of the amplification step with Phi29 polymerase performed either in an eppendorf tube or within a micro-PCR amplification device. The amplification efficiency is similar in both cases.