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Solid Phase PCR for rapid multiplex detection of *Salmonella* spp. at sub species level, with amplification efficiency comparable to conventional PCR

Wai Hoe Chin[1], Yi Sun [2*], Jonas Høgberg[1], Tran Quang Hung[1], Anders Wolff[2] and Dang Duong Bang[1]

[1] National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, Søborg DK-2860, Denmark

[2] Department of Micro- and Nanotechnology, Technical University of Denmark, Lyngby Kgs-2800, Denmark

[*] To whom correspondence should be addressed. Tel: +45 45256319; E-mail: Sun.Yi@nanotech.dtu.dk

Corresponding author. Mailing address: Associate Professor Yi Sun, Department of Micro- and Nanotechnology, Technical University of Denmark, Kgs. Lyngby Dk-2800, Denmark

Abstract

Solid-phase PCR (SP-PCR) has attracted considerable interest in different research fields since it allows parallel DNA amplification on surface of a solid substrate. However, the applications of SP-PCR have been hampered by the low efficiency of the solid phase amplification. In order to increase the yield of the solid-phase amplification, we studied various parameters including the length, the density as well as the annealing position of the solid support primer. A dramatic increase in signal to noise ratio (S/N) was observed when increasing the length of solid support primers from 45 to 80 bp. The density of primer on the surface was found to be important for the S/N ratio of the SP-PCR, and the optimal S/N was obtained with density of 1.49×10^{11} molecules/mm². In addition, the use of solid support primers with a short overhang at the 5' end would help to improve the S/N ratio of the SP-PCR. With the optimized conditions, the SP-PCR can achieve amplification efficiency comparable to a conventional PCR, with a limit of detection (LOD) of 1.5 copies/µl (37.5 copies/reaction). These improvements will pave the way for wider applications of the SP-PCR in various fields such as clinical diagnosis, high-throughput DNA sequencing and single-nucleotide polymorphism analysis.

Keywords: Solid phase PCR, high efficiency, rapid diagnostics, multiplex detection

Introduction

Solid-phase PCR (SP-PCR) is a technique that amplifies target nucleic acids on a solid support with one or both primers immobilized on the surface. Spatial separation of the primers minimizes significantly undesirable primer interactions, thereby preventing the formation of primer dimers and allowing higher multiplexing amplification. This circumvents the limitations of conventional PCR in high-throughput and multiplex applications and holds significant promise in *e.g.*, multi-analyte diagnostics [1], DNA sequencing [2], and large-scale single-nucleotide polymorphism analysis [3]. Since the first solid-phase PCR was successfully performed [4], numerous studies have been further developed in this field [5-7] . In addition, solid-phase PCR in a picowell array for immobilizing and arraying 100,000 PCR products was demonstrated [8]. Despite of the apparent advantages, SP-PCR has not been widely applied as it should. The major concern of SP-PCR is the low amplification efficiency (referred to yield of the SP-PCR amplification), which can cause significant negative implications in nucleic acid-based analysis. One widely used SP-PCR strategy is called bridge amplification, where both forward and reverse primers are physically attached onto a solid support, and at each round of the amplification, an amplicon bridges over to interact with another solid support primer [9]. It was generally

accepted that the lack of diffusion in SP-PCR would cause relatively inefficient reaction kinetics, and consequently the method has poor limits of detection [10].

One of methods for enhancing the diffusion in SP-PCR is to supplement the reaction mixture with unbound primers, so that the reaction proceeds simultaneously in both the liquid phase and the solid phase on the surface. For example, symmetric SP-PCR employs balanced aqueous forward and reverse primers as well as a solid support primer with the same sequence as one of the aqueous primers [6]. In this approach, initial amplification in the liquid phase efficiently increases the copy numbers of the starting template, thereby improving the probability template that can interact with the solid support primer. Later another asymmetric SP-PCR was proposed in which the aqueous primer that corresponds to the solid support primer is included at a limit concentration [1]. This approach reduces the competition between the aqueous and the solid primers during annealing and extension steps, which results in efficient loading of amplicons onto the solid support. To further increase solid support priming, Khan *et. al.,* [11] enhanced the SP-PCR technique by using a nested solid support primer for the aqueous PCR product. The nested primer bears a higher melting temperature, therefore at later stage of the PCR cycling, the annealing temperature is increased so that the priming competition is more favoured to the solid support primer. The use of the above mentioned approaches successfully increased the diffusion of DNA amplicons during the SP-PCR reaction, and demonstrated a 10-fold improvement in amplicon yield as compared to a normal SP-PCR [11]. However, the amount of the DNA template required for positive PCR in that study was 2 orders of magnitude higher than that required in solution PCR [11].

To explain the low amplification efficiency of the SP-PCR, the mechanisms of the SP-PCR were investigated by several groups. Palanisamy *et. al.,* [10] showed that only 36% of the immobilized primer were extended during a SP-PCR, which was substantially lower than that of in the solution PCR where 90-100% of primers are involved in the reaction. In addition, it was observed that the amplified signals of the SP-PCR in that study were hampered in the later stage of the PCR. These facts suggested that the SP-PCR is not exclusively limited by diffusion, but more likely by the surface amplification itself. Thus, other studies devote to investigate the mechanisms of the surface amplification. Different hypotheses were underlined in order to explain why the surface amplification could not be occurred by an exponential growth as in the case for solution PCR. The possible reasons include:

1) Masking effect (Fig. 1a). Since the length of PCR amplicons are usually much longer than that of the primers, they could "mask" adjacent primers (approximately one gyration radius of the amplicon) so that these primers are unable to participate in the next round of the SP-PCR amplification [10];

2) Molecular crowding effect (Fig. 1b). Molecules separated by less than their radius of gyration will interact sterically and repulse each other. When surrounded by high density amount of immobilized surface primers, the free-floating template tends to move away from the surface. Those molecules have less chance to anneal to the solid support primer [12];

3) Neighbouring DNA interactions (Fig. 1c). As SP-PCR progresses, the immobilized amplicon density increases and the DNA template may likely hybridize to an adjacent amplicon in preference to the primer. This could prevents the primer extension and limits the SP-DNA amplification [10].

2

Within this theoretical framework, we have hypothetically identified some factors that may influence the yield of DNA during the solid-phase amplification, such as 1) the length of the surface support primer, 2) the density of the surface support primer and 3) the annealing position of the solid support primer within the liquid phase stage of the SP-PCR. The use of a longer surface support primer may reduce the masking effect, and at the same time may facilitate the hybridization of the primer to the target. A low density of the solid support primer may be insufficient for the SP-PCR to amplify while too high density of the solid support primers will increase the molecular crowding effect; therefore optimization of solid support primer density will be very important. In addition, the position where the solid support primer anneals to the PCR amplicon generated from the liquid phase PCR amplification may also play a significant role in enhancing the SP-PCR efficiency, as a shorter 5' overhang of the template may reduce the interaction with the neighbouring amplicons and lead to a better annealing efficiency. Although all these factors have been investigated for the hybridization in DNA microarray, no systematic study has been done for the SP-PCR. In this paper, the theoretical considerations were addressed and tested experimentally. A SP-PCR method was developed and optimized for multiplex detection of *Salmonella* at subspecies level. The results revealed that much higher surface primer involvement (up to 77.2%) was achieved. We demonstrated for the first time, that the newly develop SP-PCR can achieve a sensitivity similar to conventional PCR.

Material and methods

***Salmonella* DNA extraction**

Salmonella Enteritidis reference strain (CCUG 92243) and *Salmonella* Typhimurium a chicken isolate (DVI-Jeo 3979 Jgt.110) were used in this study. Bacterial cultures were grown at 37°C for 18 h in Buffered Peptone Water (BPW). One mL of the BPW was collected and centrifuged at 15,000 × g for 5 min at 5°C. The supernatant was discarded and the bacterial pellet was washed with 1 mL phosphate buffered saline (PBS; Oxoid). Bacterial chromosomal DNA was extracted from the pellet using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration was determined using the Nano drop instrument (Thermo Scientific, USA). The DNA preparation was stored at -20°C until use. By considering the genome size of *Salmonella* of approximately 4.8 Mb [13], the genome equivalence can be calculated using the equation (1) (see below). The calculated number of the genome equivalent was used when determining the limit of detection (LOD) for the SP-PCR.

Genome equivalent $= \frac{AB}{CDE}$ (**1**)

A = Amount of DNA in nanogram.
B = Avogadro's number which is 6.022×10^{23}.
C = Genome size of *Salmonella* which is 4.8 Mb.
D = Multiply by 1×10^9 to convert to nanograms
E = Assumption that the average weight of a base pair (bp) is 650 Daltons in double stranded DNA.

Primer design

PCR primers were designed for specific detection of *Salmonella* spp. at subtype level. A Nucleotide-BLAST from National Center for Biotechnology Information (NCBI) was used to test for homology within the database, and Primer-BLAST was used to design the primers [14]. Four different primer sets targeting 4 different genes: the *hil*A, *sdf, sef*A and *fli*C of *Salmonella* spp. were designed to specifically identify different *Salmonella* genus. The *hil*A gene is specific for *Salmonella* genus, the *sdf* gene is specific for serotypes *Salmonella* Enteritidis; the *sef*A gene is specific for serotypes *Salmonella* Dublin and *Salmonella* Gallinarum; and the *fli*C gene is specific for *Salmonella* Typhimurium and *Salmonella* Kentucky. One primer set, namely Universal *Campylobacter*, (UC), targeting the *16S* rRNA gene of *Campylobacter spp.* was used as a negative control [15]. For each gene, one pair of a forward and a Cyanine-5 (Cy5) labelled reverse primer were designed for the liquid phase amplification and a nested probe was designed as the solid support primer. Fluorophore Cy5 was chosen in order to reduce the quenching effect caused by the interaction with nucleotides. In this study, the self-quenching effect was not considered in the analysis. To enhance the amplification efficiency of the SP-PCR, the reverse primers were designed to have a melting temperature (T_m) 3.2 - 9°C higher than those of the forward primers (Table 1). All the primers were synthesized and purchased by DNA technology (Aarhus, Denmark). The primers sequences are shown in Table 1.

Immobilization of solid support primers on cyclic olefin copolymer (COC) polymer slides

A simple Ultraviolet (UV) cross-linking method for attaching TC-tagged DNA oligonucleotides on various substrates described previously [16] was used. The technique has been showed to have not only high versatility but also high thermal stability comparable to other tedious and expensive covalent bonding methods [17]. In this study, this method was used to immobilize the solid support primers on COC polymer. COC microscope slides (Microfluidic ChipShop, Germany) was cleaned in 70% ethanol using sonication for 30 minutes, then rinsed with Milli-Q water (Millipore Corporation, USA), and air dried. The five oligonucleotide solid support primers with poly(T)10–poly(C)10 tails (Table 1) were diluted in 5 × saline sodium citrate (SSC) buffer (Promega, WI, USA) with 0.004% Triton X-100 (Sigma-Aldrich, USA). The solid support primers solutions were spotted on the cleaned COC slides using a non-contact array nano-plotter 2.1 (GeSim, Dresden, Germany) with Picoliter pin that deposited 0.06 nL/spot and average 100 µm diameter of spot. Each solid support primer was spotted in six consecutive spots. After drying, the slides were exposed to UV irradiation at default setting with 10 minutes (254 nm with energy of 0.3 J/cm^2) in an UV Crosslinker (Stratalinker 2400, Stratagene, CA, USA) to immobilize the primers onto surface of the substrate. Then the slide was washed in 0.1 × SSC solutions for 5 min, rinsed in deionized water and dried at 25°C to let water evaporate naturally. The slides were treated with bovine serum albumin (BSA) (2.5 mg/mL) for 30 minutes and rinsed with Milli-Q water and air dried before use for the SP-PCR. The average of immobilization efficiency was 50% (Fig. S4).

Solid phase PCR reaction

A 25 µL of SP-PCR reaction mixture was prepared. The SP-PCR mixture consists of 1 × Phusion® Human Specimen PCR Buffer (Thermo Fisher Scientific), 400 nM of *hil*A forward and 1600 nM *hil*A reverse primers, 200 nM of *sdf* forward and 800 nM *sdf* reverse primers, 200 nM of *sef*A forward and 800 nM *sef*A reverse

primer, 600 nM of *fli*C forward and 2400 nM *fli*C reverse primer, 400 ng BSA (Sigma- Aldrich St. Louis, USA) and 0.05 U/µL Phusion Hot Start II High-Fidelity DNA polymerase (Thermo Fisher Scientific). A Gene Frame (Thermo Fisher Scientific) was used to create a 25 µL reaction chamber surrounding the solid support primer array. The PCR master mix was loaded by pipette into the gene frame and sealed with a cover slip. The SP-PCR was conducted in a flat-bed PCR thermocycler (MJ research, Canada), where a piece of 1 cm thick polystyrene insulation foam (Silvan, Copenhagen Denmark) was used to separate the slides from the lid of the PCR thermocycler. The SP-PCR conditions were: 94˚C 5 minutes follow by 30 cycles of 94˚C for 10 seconds, 60˚C for 20 seconds, 72˚C for 20 seconds, then another 15 PCR cycles of 94˚C for 10 seconds, 65˚C for 20 seconds, 72˚C for 20 seconds. A higher annealing temperature was used in the later 15 PCR cycles to enhance the SP-PCR (see primer design). After the SP- PCR, the chamber was washed with $0.1 \times$ SSC and 0.1% of Sodium dodecyl sulphate (SDS) (Promega, WI, USA) for 5 minutes then rinsed with deionized water and dried at room temperature. The slide was ready for scanning.

Data analysis

After the SP-PCR, the slides were scanned using a BioAnalyzer 4F/4S scanner with 5000ms shutter time (LaVision BioTec GmbH, Bielefeld, Germany). Microarray image was analysed using GenePix pro 7.0 software (Molecular devices). A circle was drawn and adjusted to the size of the spot and the mean light intensity value was determined as signal. A square surrounding the circle was drawn and the mean intensity was used as the background. The standard deviations of background were calculated based on background from six spots. The signal to noise ratio (S/N) in this study was defined as the mean of signal of the 6 spots on the array, subtracting the mean background, and divided by the standard deviations of the background. All the experiments were performed in triplicate and the *UC* solid support primers were used as negative control (Table 1).

Optimization of the solid-phase PCR

The effect of solid support primer length

Different lengths of the *hil*A solid support primers ranging from 45 bp to 80 bp were designed (Table S1). To test the effect of the solid support primer length, 60 µM of each solid support primer was spotted on COC substrates with volume of 0.06 nL/spot and the average spot size of 100 µm in diameter. The slides were then subjected to the SP-PCR reaction as described.

The effect of solid support primer density

Different concentrations of the *hil*A solid support primers (with primer length 70 bp) ranging from 5 µM to 100 µM were spotted on COC slides. The volume of the droplet deposited by Nano plotter was approximately 0.06 nL. The immobilization efficiency of UV-crosslinking method is around 50%, so the actual solid support primer density is from $1.24x\ 10^{10}$ to 2.48×10^{11} molecules/mm^2. The SP-PCR reaction was carried out with the same condition as described.

The effect of solid support primer annealing position

To define the effect of the annealing position of the solid support primer on the efficiency of the SP-PCR, two types of solid support primers: the end type and the central type, were designed for the *hil*A*, *sdf*, *fli*C and *sef*A genes to target different positions of the liquid phase PCR amplicons (Table S2). All the solid support primers had the same length of 80 bp, but varied in the distance to the 5' end of the liquid-phase PCR amplicons. All these solid support primers were spotted at the same concentration of 60 µM for testing the SP-PCR.

Specificity and sensitivity of SP-PCR

After optimization of all the parameters such as the length of the solid support primers, the concentration and the annealing position of the solid support primer, four solid support primers targeting the *hil*A, *sdf, sef*A *and fli*C genes were spotted on the COC slides for testing the specificity and the sensitivity of the multiplex SP-PCR reaction. Genomic DNA of *S.* Enteritidis and *S.* Typhimurium were used as templates to investigate the specificity of the developed SP-PCR. A series of 10-fold dilution ranging from 1.5×10^5 copies/µl to 1.5×10^{-1} copies/µl were employed for sensitivity test.

For comparison, a conventional liquid-phase PCR was performed with the same PCR condition including 45 PCR cycles as the SP-PCR and used as reference. The PCR amplified products were quantified on Agilent 2100 BioAnalyzer (Palo Alto, CA, USA) using DNA 12000 chips.

Involvement of immobilised solid support primers in SP-PCR

The percentage of the immobilised solid support primers involved in the SP-PCR was determined by using a standard curve. Serial dilutions of control solid support primer with Cy5 label ranging from 3.75 µM to 80 µM were spotted on COC slides. The S/N ratio was determined and the data was used to make a standard curve (Fig. S5 and S6). In theory, the S/N ratio at surface density of 1.49×10^{11} molecules/µm^2 (corresponding to the 60 µM spot) should represent the case when 100% of the immobilized solid support primers are extended during SP-PCR. A "Trend" function in Microsoft Excel (Microsoft Office Excel 2010) was used to fit the S/N ratio of the solid support primers targeting the *hil*A, *sdf*, *sef*A*, and *fli*C genes, respectively, obtained after SP-PCR to the standard curve. The percentage of the involvement of solid support primers in SP-PCR was calculated by the equation (2). This calculation excluded the effect of quenching effect of fluorophores.

$$\frac{a}{1.49 \times 10^{11}} \times 100\% = b \qquad\qquad (2)$$

a = Surface density (molecules/µm^2) of the solid support primer obtained by fitting S/N to the standard curve.
b = Percentage of solid support primers extended during SP-PCR.
1.49×10^{11} corresponds to the surface density (molecules/µm^2) of the 60 µM spot (assuming 50% loss during washing)

Results and discussion

Effect of the length of the solid support primer on the efficiency of SP-PCR

It has been shown that a longer solid support primer increased the intensity of the signal in microarray hybridization [18]. However, to our knowledge no work has been done to study the effect of the length of the

solid support primer on the efficiency of the SP-PCR. In this study, we designed the solid support primer for *hil*A gene with different lengths and spotted on COC slide at concentration of 60 µM. As shown in Fig. 2, the signal to noise ratio (S/N) was increased when the solid support primer length was increased from 45 bp to 80 bp. The experimental results indicated that increase of the length of the solid support primer will increase the SP-PCR amplification signals.

In SP-PCR, the long solid support primer facilitates the hybridization of the solution PCR products. As the primer length increases, more complementary binding sites are available for the target [19]. Thus, the longer solid support primer has a higher probability to interact with the target than the shorter one. This implies that longer solid support primer can provide higher sensitivity. Moreover, increasing the solid support primer length may also address a number of limits in solid phase amplification. One of the possibilities is that a longer solid support primer can alleviate the masking effect. After immobilizing the primer to the solid surface, the solid support primers are expected to extend further away from the immobilized surface. This will make them more accessible to DNA targets and DNA polymerase for more efficient annealing and extension.

Effect of the density of the surface solid support primer on the efficiency of SP-PCR

To investigate the effect of the density of the solid support primer on SP-PCR efficiency, the SP-PCR was carried out on COC slides containing 70 bp *hil*A solid support primer with concentrations ranging from 5 µM to 100 µM (corresponding to the surface density from 1.24×10^{10} to 2.48×10^{11} primers/mm^2). The correlation between the S/N ratio and the density of the solid support primer is shown in Fig. 3. Less signals was observed when the solid support primer density was below 4.96×10^{10} molecules/mm^2 (20 µM). The fluorescence intensity increased significantly when the density exceeded 9.92×10^{10} molecules /mm^2 (40 µM) and the maximum S/N ratio was obtained at density of 1.49×10^{11} molecules /mm^2 (60 µM).

The results suggested that within a certain range of the density of the solid support primers, the SP-PCR amplification may be limited by insufficient primers. Since higher surface density may result in higher concentration of the solid support primers involved in the SP-PCR and therefore enhanced the SP-PCR amplification efficiency. However, above a certain limit, the high amount of the solid support primers tends to inhibit the SP-PCR amplification. The soundest explanation is that the molecular crowding effect plays an important role in the case. When the solid support primers are separated by less the radius of gyration, the steric effect from neighbouring solid support primers will result in a repulsive force on the targets. That raises the difficulty for the liquid phase PCR products to anneal to the solid support primers. In addition, the high-density negatively charged DNA could also create charge repulsion that repulse the target. In theory when the length of the solid support primers after extension reaching 112 bp the SP-PCR extension product will has a theoretical gyration radius of 3 nm. In order to keep the solid support primers with a distance larger than the gyration radius, the density of solid support primers should approximately be 1.1×10^{11} molecules/mm^2. This theoretical number fits well to the 1.49×10^{11} molecules/mm^2 (60 µM) as determined by experiment. The optimized surface density of 1.49×10^{11} molecules/mm^2 (60 µM) was employed for further experiments to achieve an efficient SP-PCR amplification on the surface.

Effect of the annealing position of the solid support primer

In order to examine the effect of the annealing position of the solid support primers on the liquid phase amplicons, two types of solid support primers, the end-type and the center-type for the *hil*A, *fli*C and *sef*A genes were designed (Fig. 4). In Fig. 4, the red strand and blue strand represent the immobilized solid support primers and the two primers from liquid phase PCR, respectively. The center-type of the solid support primer targets the central part of the liquid phase PCR amplicons, whereas the end-type of the solid support primer is located closer to the 5' end of the target, resulting in a shorter 5' end overhang. The results showed that the intensity of the fluorescence signal increased from 3 to 19 fold for all the three genes when using the end-type solid support primers (Fig. 5, Fig. S1 and S2). Therefore, the extension of the annealed solid support primer and the DNA target is highest when the solid support primer is located closer to the 5' end of the target.

Peytavi *et al.,* [20] observed a similar effect in microarray hybridization. This phenomenon could be explained by the free energy related to the hybridization reaction between the solid support primer and target. In SP-PCR, in the case of the 80 bp solid support primer, the number of nucleotides bound to the target is the same for both types solid support primers: the end and the center types, the enthalpy is equal in all the annealing reactions. Hence the free energy is affected only by the entropy term. However, for the end-type solid support primer, the loss in configurational entropy upon the annealing is less than in the case of the center-type solid support primer, thus it has a higher opportunity for annealing all its sequences to the target and therefore result in a better SP-PCR efficiency.

In addition, in the end-type solid support primer, the effect of the neighboring DNA interactions is limited, since, the target anneals to the end-type solid support primer. The shortened 5 'end overhang makes it more difficult for the target to interfere with an adjacent amplicon. Therefore, the inhibitory effect due to neighboring DNA interactions is less likely to occur, which leads to a higher yield of the surface amplicons in the end-type solid support primer.

Sensitivity and efficiency of the multiplexed SP-PCR

With the optimized conditions, a multiplexed SP-PCR was performed to detect *Salmonella* spp. as well as identify the serotypes of *Salmonella*. Four 80 bp end-type solid support primers for the *hil*A, *sdf*, *sef*A and *fli*C genes were spotted on COC slides with a density of 1.49×10^{11} primers/mm^2. Fig. 6 showed the layout of the array. The *hil*A, *sdf* and *sef*A genes were used to detect S. Enteritidis while the *fli*C gene was used to differentiate *S.* Typhimurium. The Chromosomal DNA of *S.* Enteritidis and *S.* Typhimurium were used as templates in the SP-PCR. As shown in Fig. 6b, the two *Salmonella* strains were identified accurately by the distinct patterns of PCR amplicons. No fluorescence signal was observed for the *Campylobacter* UC negative control solid support primer, as well as no cross amplification was observed (Fig. S3). The results showed that the multiplex SP-PCR was highly specific for detection of *Salmonella* at sub-species.

The sensitivity of the multiplex SP-PCR was determined and compared to a conventional PCR (Fig. 7 and Fig. 8). The fluorescence signals of the SP-PCR array increased with an increase of template concentrations, suggesting that more amplification occurred on the surface at higher template concentration. It was also noticed that there is a high variation in the S/N ratio among different genes. As suggested by Khan *et al*., [11] this phenomenon was owing to the inherent difference in hybridization efficiencies between oligonucleotides. In addition, the Cy5 quenching efficiencies of different nucleotides were: adenosine (15%), cytidine (15%),

guanosine (19%) and thymidine (10%) [21]. The quenching efficiencies might contribute to the variations of S/N ratio among different genes. In this study, the quenching effect of different nucleotides was not considered in the analysis. Despite of the variations, remarkably high percentage of solid support primers involved in the SP-PCR was achieved for all the four genes as summarized in Table 2. The lowest DNA concentration that is able to be detected by the SP-PCR on COC slide was 1.5 copies/µl (37.5 copies/reaction) for both *S.* Enteritidis and *S.* Typhimurium, which was comparable to the result of the conventional PCR. The percentage of solid support primers involved in the SP-PCR amplification was calculated and listed in Table 2. Approximately 44.9% - 77.2% of the solid support primers were extended during the solid phase amplification (the effect of quenching effect of fluorophores excluded), which was significantly higher than the 36% as reported previously [10]. The results showed that by addressing all the issues that affect the yield of the surface amplification, the SP-PCR efficiency can be improved enormously.

Conclusion

In this study, we have strategically improved and characterized different parameters such as the length of the immobilized solid support primer, the surface density of solid support primer and the annealing position of the solid support primer in the targets that theoretical influence the efficiency of the SP-PCR. The theoretical hypotheses were confirmed experimentally by performing a multiplex SP-PCR on COC slides to detection and identification of *Salmonella* spp. at sub species. The average S/N ratio of *hil*A gene of 310.7 was obtained with the end-type 80 bp solid support primers at surface density of 1.49×10^{11} molecules/mm^2. It increased 19 fold as compared to 45 bp center type which showed an average S/N ratio of 16.1 at surface density of 1.24×10^{10} molecules/mm^2. With all the optimized conditions, the detection limit of 1.5 copies/µl (37.5 copies/reaction) was obtained and up to 77% of surface solid support primers were involved in the surface amplification. To our knowledge, this study presents the first evidence that the SP-PCR amplification can achieve a high sensitivity that is comparable to conventional PCR. This work provided guidelines for developing efficient DNA amplification on a solid surface, which would greatly facilitate the applications of SP-PCR amplification in different fields such as multiplexed diagnostics, next generation sequencing and high-throughput screening.

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Conflict of Interest,

The authors declare that they have no competing interests.

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