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Analytical Biochemistry 375 (2008) 391–393
ANALYTICAL BIOCHEMISTRY
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Enhanced solid phase PCR: mechanisms to increase priming by solid support primers

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Received 22 November 2007
Available online 26 January 2008

Abstract

Conventional solid phase amplification regimens such as solid phase PCR (SP–PCR), asymmetric SP–PCR, and bridge PCR are mechanistically limited with respect to amplification efficiency and solid support primer involvement. Here we present enhanced solid phase PCR (ESP–PCR) in which solid support primer priming is facilitated by its nesting and high melting temperature (T_m) relative to the aqueous counterpart. In the study, we demonstrated increased solid support surface loading using ESP–PCR versus standard SP–PCR for three diagnostic targets: *Neisseria gonorrhoeae opa* (9.89-fold), *N. gonorrhoeae pilS* (2.14-fold), and *Chlamydia trachomatis* cryptic plasmid *orf3* (1.41-fold). Furthermore, we applied ESP–PCR to detect five copies of *N. gonorrhoeae* and *C. trachomatis* DNA.

Solid phase amplification approaches in diagnostics offer the prospect of streamlined processing combined with the ability to multiplex analyte detection to relatively high degrees. However, often very low copies of target analytes are present in clinical specimens, requiring optimal assay sensitivity. Conventional symmetric solid phase PCR (SP–PCR)[1] employs balanced aqueous forward and reverse primers and a solid support primer bearing target-specific sequence that matches one of the aqueous primer sequences. Although aqueous amplification proceeds efficiently, solid support priming and loading of amplicon is suboptimal. The higher concentration of aqueous primers and their lack of steric constraint enable the corresponding aqueous primer to outcompete solid support primer for binding to primer binding sites [1]. Asymmetric SP–PCR was designed to overcome the poor solid support loading of SP–PCR [1,2]. In this method, one of the aqueous primers is included at a limiting concentration to lower its competition with solid support primer. However, this approach decreases amplification efficiency and remains suboptimal with respect to solid support amplicon loading [3–5]. Even to this end, asymmetric PCR requires extensive optimization to identify primer ratios and inherently applies selective pressure toward the yield of counterproductive, prematurely abridged products. Bridge PCR uses only solid support primers in the absence of aqueous primers. At each round of amplification, an amplicon bridges over to interact with another solid support primer [6,7]. Although any amplification that occurs contributes to solid support amplicon loading, reported sensitivities are low due to the relatively inefficient reaction kinetics [8,9]. Here we present enhanced solid phase PCR (ESP–PCR), a new mechanism designed to combine the high sensitivity of uncompromised symmetric aqueous PCR with efficient solid support loading. ESP–PCR alters the mechanism by which amplicon is loaded onto solid support by removing competition between aqueous primer and solid support primer to increase solid support primer priming. This is achieved by nesting and raising the melting temperature of solid support primer relative to the aqueous. Aqueous

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[1] *Abbreviations used:* SP–PCR, solid phase PCR; ESP–PCR, enhanced solid phase PCR; *Ng opa, Neisseria gonorrhoeae* opacity gene*; Ng pilS, Neisseria gonorrhoeae pilS; Ct*CP *orf3, Chlamydia trachomatis* cryptic plasmid *orf3;* dNTP, deoxynucleoside triphosphate; dsDNA, double-stranded DNA; RFU, relative fluorescence units; T_m, melting temperature; LATE–PCR, linear-after-the-exponential PCR.

0003-2697/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2008.01.021

primer need not be limited, thereby enabling a more sensitive system. Furthermore, the primer design inherent to ESP–PCR offers the optional potential of applying latter thermal cycles at annealing temperatures permissive exclusively to solid support primer binding.

It should be considered that factors such as the solid support material, solid support size, and surface primer density likely will influence the performance of ESP–PCR [10,11]. However, the experiments detailed in this article were performed using constant solid support material, primer surface density, and linker sequence with the scope and intention of dissecting out the mechanistic benefits of ESP–PCR over SP–PCR.

In this study, we compared ESP–PCR with SP–PCR, employing equally loaded silica microspheres as the solid support, across a range of clinically relevant targets: *Neisseria gonorrhoeae opa* (*Ng opa*), *N. gonorrhoeae pilS* (*Ng pilS*), and *Chlamydia trachomatis* cryptic plasmid *orf3* (*Ct*CP *orf3*) [12,13]. Detailed experimental procedures, including oligonucleotide sequences (Integrated DNA Technologies), can be found in the supplementary material.

Comparative experiments between ESP–PCR and SP–PCR were performed in 20-μl reaction volumes using 1 unit of HotStarTaq (Qiagen). HotStarTaq reaction buffer was supplemented with Mg^{2+} and deoxynucleoside triphosphates (dNTPs, New England Biolabs) to yield reaction concentrations of 2 mM and 200 μM, respectively. Included in reactions were 1 μl of 5 μM aqueous forward primer, 1 μl of 5 μM aqueous reverse primer (Alexa Fluor 647 labeled with a total oligonucleotide/Fluor-labeled oligonucleotide ratio of 2:1), and 1 μl of 1 mg/ml solid support primer-conjugated microsphere suspension. Reactions included 40,000 copies of *Ct*CP *orf3* templates, 400,000 copies of *Ng opa* templates, 40,000 copies of *Ng pilS* templates, or no-template controls (to demonstrate background fluorescent levels) and employed the following thermal profile: 94 °C for 15 min, followed by 30 cycles of 90 °C for 30 s, 44 °C for 1 min, and 72 °C for 1 min, followed by 5 cycles of 90 °C for 30 s, , 44 °C for 2 min, and 72 °C for 2 min. Following solid phase PCR, the bottom 5 μl was transferred to 120 μl of buffer in a 96-well microtiter plate. *.fcs files were generated by FACSArray with the same instrument settings as described previously. Median red fluorescence figures were determined using FCS Express (volume 3).

ESP–PCR resulted in markedly increased solid support amplicon loading versus SP–PCR across all three targets assessed (Table 1), with strong statistical significance: *Ng opa,* 9.89-fold, $P = 0.00000661$; *Ng pilS,* 2.14-fold, $P = 0.001095$; *Ct*CP *orf3,* 1.41-fold, $P = 0.000935$. *P* values relate to the null hypothesis that there is no difference in amplicon loading between ESP–PCR and SP–PCR. Fold increases refer to ESP–PCR relative fluorescence units (RFU)/SP–PCR RFU. Variation in fold increases across targets was not unexpected owing to the inherent variations in hybridization efficiencies between oligonucleotides and the fact that complex competitive hybridization events are involved. Despite this variation, ESP–PCR was universally beneficial across the targets studied. Non-microsphere-bound aqueous amplicons from the same reaction vessels as used for flow cytometry measurements were of equal yield across all targets following ESP–PCR versus SP–PCR as assessed by agarose gel electrophoresis (see supplementary material). Aqueous product yields were also identical with or without microspheres in the reaction mix, indicating that the silica microspheres conjugated to solid support primers did not compromise amplification efficiency. After ESP–PCR, solid support was washed thoroughly via multiple centrifugation and buffer exchange steps and used to template limited cycle reamplification reactions, including previously used aqueous primers and an aqueous version of solid support primer. Single band products of sizes corresponding to those expected of products derived from solid support primer and aqueous reverse primer priming were observed on gel electrophoresis, indicating that ESP–PCR solid support products were specific. Taken together, these data suggest that ESP–PCR was successful in achieving the dual goals of not compromising aqueous phase amplification and increasing solid support surface loading with amplicon relative to standard SP–PCR in a specific manner.

We suggest that these observed benefits are due to loading mechanisms in which, first, the effective concentration of solid support primer is increased by virtue of its relatively high melting temperature (T_m). Linear-after-the-exponential PCR (LATE–PCR) was described recently as improving the generation of single-stranded products by asymmetric PCR [14]. In this approach, a tighter binding, limited concentration primer was employed to increase its effective concentration in the reaction based on the relationship between primer T_m and primer concentration described by the nearest neighbor formula [15]. In ESP–PCR, this principle is applied to the solid support primer. A tighter binding solid support primer has an increased effective concentration and improved kinetics of binding and priming. Second, the solid support primer is nested with respect to its aqueous counterpart. Because the solid

Table 1
Increased loading of silica microspheres with red fluorescence-labeled amplicon following ESP–PCR versus SP–PCR

Target	No-template control SP–PCR (RFU)	SP–PCR (RFU)	No-template control ESP–PCR (RFU)	ESP–PCR (RFU)
Ng opa	37.18	52.98 (1.12)	44.91	523.74 (15.24)
Ng pilS	40.68	74.36 (1.78)	39.60	159.09 (9.92)
*Ct*CP *orf3*	41.79	209.84 (5.10)	38.89	295.39 (8.33)

Note. Standard errors of the mean of triplicate series are in parentheses. RFU, relative fluorescence units.

support primer recognizes a different binding site to the aqueous forward primer, direct competition for amplicon binding is removed. In ESP–PCR, unlike SP–PCR, for solid support priming to be inhibited by blocking of the solid support primer binding site, it is necessary for aqueous forward primer to bind to amplicon template, for polymerase to subsequently find and bind to this substrate, and for polymerization to ensue within a short period of time. In both standard SP–PCR and asymmetric SP–PCR, simply binding of aqueous primer to its primer binding site is sufficient to inhibit solid support priming. In the method of PCR clamping, amplification is specifically blocked by the inclusion of internal (nested) peptide nucleic acid or locked nucleic acid probes [16,17]. Analagous to solid support primer of ESP–PCR, blocking probes of the PCR clamping approach bind to amplicon before primer binding and polymerization have had a chance to occur.

Still applying the ESP–PCR principles, using aqueous primers and thermocycling parameters listed in the supplementary material, we detected five *N. gonorrhoeae* genomes and five *C. trachomatis* genome equivalents per single multiplex ESP–PCR reaction by FACSArray. This approach used solid support primers conjugated to silica microspheres of different diameters (see supplementary material) to facilitate discrimination by flow cytometry. Reactions were templated with *N. gonorrhoeae* genomic DNA and plasmid-containing *C. trachomatis* target region with the inclusion of Jurkat human genomic DNA versus just Jurkat human genomic DNA or water.

Next generation sequencing technologies described recently [18,19] employ asymmetric SP–PCR in an emulsion PCR context. It is possible that the use of ESP–PCR in emulsion PCR instead could facilitate greater sequence reads per run by circumventing the inefficiencies of asymmetric SP–PCR mentioned previously [3–5].

ESP–PCR offers great promise in facilitating streamlined multiplex diagnostics by enabling uncompromised aqueous phase amplification and increased solid support amplicon loading.

Acknowledgment

This work was supported by Genera Biosystems.

Appendix A. Supplementary data

Supplementary material for this article is available in the online version at doi:10.1016/j.ab.2008.01.021.

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