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Original Article

Asymmetric PCR for good quality ssDNA generation towards DNA aptamer production

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Abstract

Aptamers are ssDNA or RNA that binds to wide variety of target molecules with high affinity and specificity produced by systematic evolution of ligands by exponential enrichment (SELEX). Compared to RNA aptamer, DNA aptamer is much more stable, favourable to be used in many applications. The most critical step in DNA SELEX experiment is the conversion of dsDNA to ssDNA. The purpose of this study was to develop an economic and efficient approach of generating ssDNA by using asymmetric PCR. Our results showed that primer ratio (sense primer:antisense primer) of 20:1 and sense primer amount of 10 to 100 pmol, up to 20 PCR cycles using 20 ng of initial template, in combination with polyacrylamide gel electrophoresis, were the optimal conditions for generating good quality and quantity of ssDNA. The generation of ssDNA via this approach can greatly enhance the success rate of DNA aptamer generation.

Keywords: optimization, asymmetric PCR, single-stranded DNA, Aptamer, SELEX

1. Introduction

Aptamers which are single stranded oligonucleotides that form a variety of three dimensional structures, binding with high affinity and specificity to the target molecules, are generated from SELEX (Mazars and Theillet, 1996). SELEX comprises three basic steps, including the incubation of initial pool of nucleic acid with the target, separation of the target bound and unbound nucleic acid molecules, followed by amplification and regeneration of the bound nucleic acids (Figure 1). Though RNA and DNA aptamer have similar binding affinity towards target molecule, DNA aptamer is more stable owing to the absence of 2'-OH group compared to the former (Wiegand *et al.*, 1996). The generation of DNA aptamer requires the conversion of the dsDNA to ssDNA. Hence, various methods have been devised for the production of ssDNA from dsDNA, which include magnetic separation with streptavidin coated beads (Hultman *et al.*, 1989), lambda exonuclease enzymatic digestion (Higuchi and

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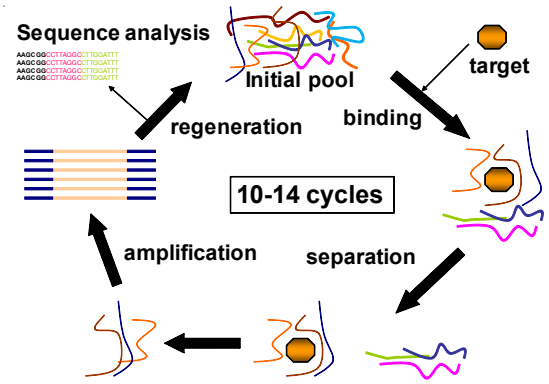

Figure 1. Schematic representation of basic steps of a SELEX experiment

Ochman, 1989) and strand separation of the PCR product containing primer with a terminator and an extension of 20 nucleotides on denaturing urea-polyacrylamide gel (Williams and Bartel, 1985). Another method involves asymmetric PCR, which generates ssDNA due to the unequal concentrations of primers used in the reaction. There are two phases of amplification in asymmetric PCR, which is the dsDNA production followed by ssDNA generation (Gyllensten and Erlich, 1988).

Unfortunately, asymmetric PCR amplification exhibits an overall efficiency of 60-70% in comparison to the efficiency obtained by the conventional PCR, which is 90% or more (McCabe, 1999). Furthermore, asymmetric PCR gives a mixture of ssDNA and dsDNA (Avci-Adali *et al.*, 2001), thus requiring a good purification method to selectively purify ssDNA. However, asymmetric PCR is the most cost effective method for ssDNA production. In this investigation, efforts have been devoted to optimize asymmetric PCR to generate ssDNA, which is very useful for laboratories with low resources. Thus, this study was aimed to demonstrate asymmetric PCR amplification in producing ssDNA and purification of this ssDNA using polyacrylamide gel to produce good quality ssDNA, suitable for generating DNA aptamer.

2. Materials and Methods

2.1 Symmetric PCR

The template used in this experiment was the ssDNA from the elution step in SELEX experiment. This ssDNA is the random ssDNA pool and primers were purchased from Biobasic Inc. (Toronto, Canada). PCR reactions were set in 100 µL of reaction volume with 15-30 ng of template, 5'-AGCTTAGGATCCAACCTGATCT$(N)_{40}$GGTACCAACTGCA TACCGAGCT-3', 5'-AGCTTAGGATCCAACCTGATCT-3' and 5'-AGCTCGGTATGCAGTTGGTACC-3' as the forward and reverse primers, respectively (60 pmol each) in 1 x PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl containing 1.5 mM $MgCl_2$), 200 µM each of four dNTPs (dATP, dGTP, dCTP and dTTP) and 2.5 U Taq DNA polymerase (MBI Fermentas, Lithuania). PCR conditions were as follows: an initial denaturation at 95ºC for 1 min followed by 15 amplification cycles of 45 s of denaturation at 94ºC, 1 min annealing at 55ºC, 1 min elongation at 72ºC and final elongation of 5 min at 72ºC. Nucleospin gel extract II kit (Macherey-Nagel, Duren, Germany) was used to purify the PCR product.

2.2 Asymmetric PCR

The PCR conditions for asymmetric PCR were exactly the same as those of symmetric PCR except for the ratios of primers used. The ratios of primers were varied from 20/2, 20/1, 20/0.2, 20/0.04 to 20/0. In addition, 10, 20, 30, 40, 50, 60, 80 and 100 pmol, which constitutes 10/0, 20/0, 30/0, 40/0, 50/0, 60/0, 80/0 and 100/0 of only sense primer, were also employed. The asymmetric PCR products were analyzed on 3% agarose gel electrophoresis in TAE buffer (40 mM Tris Acetate, 1 mM EDTA, pH 8.0) containing 0.5 µg mL^{-1} of ethidium bromide. 10 µL of the final PCR reaction products were loaded on the gel and visualized by UV transilluminator.

2.3 Southern Blot Analysis

For the purpose of checking for the presence of ssDNA in asymmetric PCR reactions, asymmetric and symmetric PCR reactions were carried out in parallel and subjected to Southern blot analysis. Four sets of PCR reactions in the final volume of 20 µL were carried out (as in Figure Legends). Following the completion of these PCR reactions, all these PCR products were analyzed on 3% agarose gel electrophoresis as explained previously. Next, the gel was subjected to pre-treatment by 0.25 M NaCl and subsequently with (0.4 N NaOH, 0.6 M NaCl). The transfer blot was set-up using 20 x SSC (3 M NaCl, 0.3 M NaOAc), to be blotted onto positively charged nylon membrane (Trans-Blot^{R}Transfer Medium, Biorad). The membrane was subjected to Southern analysis using digoxigenin (DIG) System (Roche Applied Science) according to the manufacturer's specifications. Meanwhile, DIG labeled sense and antisense primers were prepared with the aid of DIG Probe Synthesis Kit (Roche Applied Science). The membrane was hybridized at 42ºC for 4 hrs, washed and detection was carried out with chemiluminescent substrate CDP-Star (Roche Applied Science).

2.5 Gel purification of ssDNA

The asymmetric PCR product was precipitated to a final volume of 20 µL by ethanol precipitation and loaded onto 10% native Polyacrylamide Gel Electrophoresis (PAGE), run in TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3) and stained with 0.5 ug mL^{-1} of ethidium bromide in 1 x TBE buffer for 7 min. The ssDNA was then purified by crush and soak method (Harwood, 1996). The band containing ssDNA was cut using clean sterile razor blade, placed in an eppendorf tube and was crushed thoroughly with a sterile pipette tip followed by addition of 400 µL of elution buffer (1 M NaOAc,

0.01 M EDTA) into the meshed polyacrylamide gel. This was followed by incubation of the mixture at 37ºC for 6-8 hrs with shaking at 900 rpm. The gel particles were then sedimented by centrifugation at maximum speed for 2 min. The supernatant was collected carefully and was ethanol precipitated to a final volume of 20 µL.

3. Results and Discussion

The generation of ssDNA used in SELEX has been made possible by various methods that mostly require higher starting amount of dsDNA. In contrast, our study showed that as low as 20 ng template is adequate to produce a good yield of ssDNA by asymmetric PCR amplification. In order to maximize the yield of ssDNA, optimization of primer concentrations as well as the number of PCR cycles was carried out. The standard $MgCl_2$ concentration of 1.5 mM and the annealing temperature of 55ºC were used, as optimization of annealing temperature is more significant for PCR reactions involving long products or total genomic DNA as the template (Rychlik *et al.*, 1990). Minor effects such as Taq DNA Polymerase concentration can be counterbalanced by adjusting the number of PCR cycles (Kaltenboeck *et al.*, 1992).

3.1 Optimization of both sense and antisense primers

Gyllensten *et al*. (1988) reported on the asymmetric PCR amplification of genomic DNA using a range of concentrations from 50:0.05, 50:0.5, 50:5 to 50:50, and showed that all these primer ratios were able to generate ssDNA. However, Poddar *et al.* (2000) demonstrated that primer ratio of 50:1 and 50:0.5 were the best concentrations for optimal generation of ssDNA in the asymmetric PCR amplification on the conserved region of an adenovirus gene. Furthermore, Kaltenboeck *et al.* (1992) performed a dual PCR approach, in which dsDNA amplified in the first PCR reaction was used directly as an input for the second PCR reaction where the primer concentrations were varied in different proportions (Kaltenboeck and Kousoulas, 1996). However, in the present study, we presume that the direct use of unpurified PCR product as template for the asymmetric PCR will change the primer proportions due to carry-over of the limiting primer from the previous reaction. Therefore, we decided to purify the PCR product by gel extraction method, so that the PCR product of the expected size will be free from excess primers. Among five different ratios tested, the best primer concentration that could lead to the maximum production of ssDNA was the ratio of 20:1 (sense primer: antisense primer) (Figure 2a). To make this observation clear, we have used online ImageJ program (http://rsbweb.nih.gov/ij/) to show the intensities of the amplified bands and a higher yield of ssDNA with the primer ratio 20:1 was seen (Figure 2b). Upon measurement, the intensity of dsDNA was found to be about 30 (arbitrary units) whereas for ssDNA it was 45, showing nearly an increase of 50% in the product. These asymmetric PCR reactions generate both ssDNA and ssDNA with ssDNA migrates faster than dsDNA due to lower molecular weight of ssDNA as compared to dsDNA (Kujau and Wolfl, 1997).

3.2 Optimization of sense primer

PCR reactions with different amounts of only sense primers (10, 20, 30, 40, 50, 60, 80, 100) were also tested (Figure 3a). From the analysis with ImageJ software, the band intensities of ssDNA for all the primer concentrations were

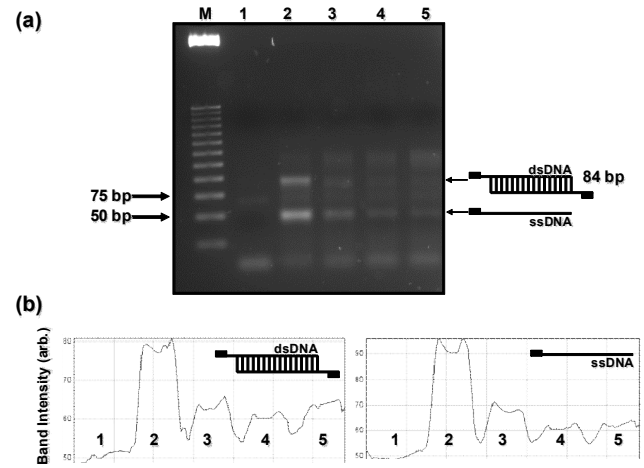

Figure 2. Analyses of PCR products with different ratios of sense to antisense primers. (a) Lane M: 25 bp DNA ladder, Lanes 1-5: Mobility of the PCR products with primer ratios of 20/2, 20/1, 20/0.2, 20/0.04 and 20/0 respectively with 30 PCR cycles on 3% agarose gel (b) Band intensities (peak height) of dsDNA and ssDNA as estimated by ImageJ software.

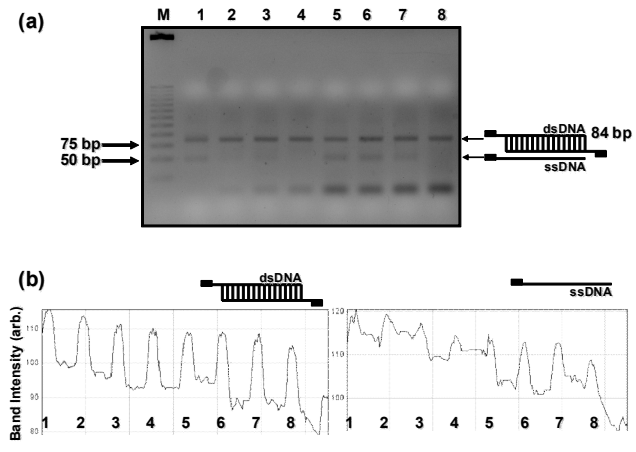

Figure 3. Analyses of PCR products with different amounts of sense primer. (a) Lane M: 25 bp DNA ladder, Lanes 1-8: Mobility of the PCR products with sense primers varied from 10 pmol, 20 pmol, 30 pmol, 40 pmol, 50 pmol, 60 pmol, 80 pmol and 100 pmol with 10 PCR cycles on 3% agarose gel (b) Band intensities of dsDNA and ssDNA as estimated by ImageJ software.

around 5-10 (arbitrary unit) and was found that sense primer alone is also able to generate ssDNA (Figure 3b). Amplification with single primer also generates dsDNA, as the forward primer which is partially complementary (50%) to the sense strand is still able to bind to this secondary, imperfect binding site and serve as a reverse primer (Wang *et al.*, 1991). This priming leads to the production of dsDNA which is produced along with ssDNA when single primer is used in this asymmetric PCR. In contrast, in this study, only dsDNA was produced when the sense and antisense primers were used at the same concentration of 20 pmol as seen in Figure 4. Therefore, the range of 10-100 pmol of only sense primer can also be the optimal range for ssDNA generation.

3.3 PCR cycling variation

Previously, it has been shown that when the PCR cycles were increased to 30 or more, the amount of ssDNA exceeded dsDNA generated from asymmetric PCR of single-copy sequences. However, in contrast to the previous study, we found that the increase in number of PCR cycles will not result in the increase of ssDNA, but instead it will lead to the generation of more background bands, when only sense primer is used (Figure 5a). To further support the agarose gel electrophoresis analysis, band intensity measurement for ssDNA of the three different PCR cycles was carried out. From the ImageJ data, it is known that increasing the PCR cycles from 10 to 20 cycles clearly increases the band intensities which represent the yield of ssDNA, with an improvement of 37.5% (Figure 5b). However, a further increase up to 30 cycles did not result in the increase of ssDNA generation. In fact, the ssDNA generation of PCRs with 10 and 30 cycles was almost similar, as revealed by the band intensity constituted by these two PCR reactions, which was around 25-30 (arbitrary unit). The appearance of these background DNA bands is due to opportunistic mispairing (Tuerk, 1990). This is because the template DNA used in this asymmetric PCR is the amplified random ssDNA pool, as opposed to the template used in the previous study which is genomic DNA (Gyllensten and Erlich, 1988). Due to the fact that random ssDNA comprises multitude of different ssDNA each with a different sequence, these sequences can also pair with other sequences due to complementarity. Moreover, each of these sequences which form base-pair with each other can also serve as a template for PCR amplification. Annealing of 3'-OH of PCR products to the template also leads to higher molecular weight PCR products or smear when the PCR cycles increase (Bell and DeMarini, 1991). Therefore, it is reiterated that this asymmetric PCR amplification can be performed up to 20 cycles for efficient generation of ssDNA. Increasing the PCR cycles up to 30 will lead to the appearance of spurious PCR product if a single primer is used.

On the other hand, upon using 2 primers in different ratios, good amounts of ssDNA can still be ensured concomitant with the increase of the PCR cycles up to 30 despite the presence of some trace amounts of spurious background bands. Although asymmetric PCR reactions containing less amount of ssDNA are preferable over those producing more ssDNA mixed with aberrant products, it is shown that both of these approaches can be used to generate ssDNA based on the suitability of the subsequent purification method and procedure (Saiki *et al.*, 1988).

3.4 Southern blot analysis

Southern Blot analysis was carried out to ensure that asymmetric PCR reactions performed lead to the production of ssDNA. In this investigation, both of the primers were labeled non-radioactively with DIG. As shown in Figure 6a, a clear band was seen in lane 4, which constitutes PCR reaction carried out using the primer ratio of 20:1 (sense to antisense primer ratio) with 20 PCR cycles. On the other hand,

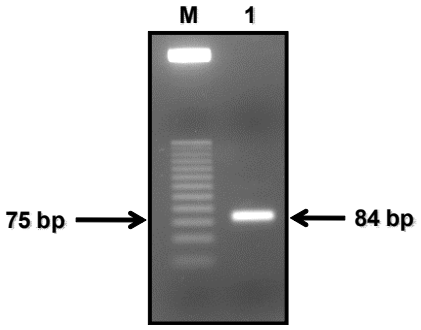

Figure 4. Analyses of symmetric PCR products. Lane M: 25 bp DNA ladder. Lane 1: Mobility of symmetric PCR product with equal amounts of sense and antisense primers (20 pmol) on 3% agarose gel

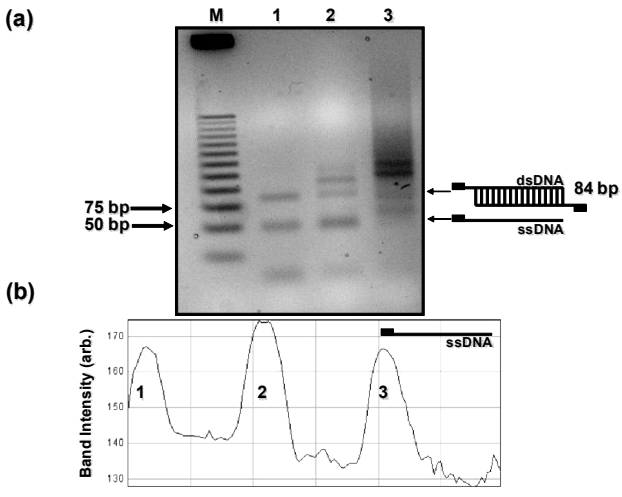

Figure 5. Analyses of PCR products with different PCR cycles (a) Lane M: 25 bp DNA ladder, Lanes 1-3: Mobility of PCR products with PCR cycles 10, 20 and 30 respectively, on 3% agarose gel (b) Band intensities of ssDNA as estimated by ImageJ software.

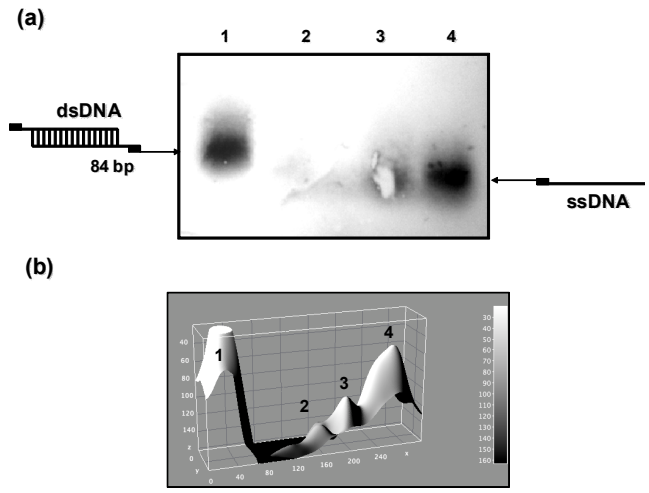

Figure 6. Southern blot analysis of symmetric and asymmetric PCR (a) Lane 1: symmetric PCR with 20 pmol of sense and antisense primer, 20 PCR cycles, Lane 2: 10 pmol sense primer with 10 PCR cycles, Lane 3: 20: 1 ratio of sense to antisense primer with 10 PCR cycles, Lane 4: 20: 1 ratio of sense to antisense primer with 20 PCR cycles (b) 3D plot profile view of Image J software on band intensities, lanes designated similarly as in Figure 7(a)

in lane 3, the same primer ratio employed gave a less intense band than that of in lane 4 as PCR reaction carried out by only 10 PCR cycles. Lane 2 which represents PCR reaction executed using 10 pmol of primer P1 gave a less intense band than the latter and former. All the bands in these lanes are constituted by ssDNA migrating around 50 bp. On the other hand, symmetric PCR reaction carried out using equimolar amounts of sense and antisense primer resulted in the band migrating at 84 bp. The intensity of this band as in lane 1 is higher than that in lane 4, as symmetric PCR product separated into two distinct bands during the pretreatment step (Figure 6b). In this step, HCl treatment resulted in depurination, where glycosidic bond between the purine and sugar component was broken. This was followed by the denaturation of the double-stranded DNA molecules into separate strands by the breakage of the hydrogen bonds (imparted by NaOH), which then bound to two of the DIG-labeled primers (forward and reverse), accounting for the intense band. On the contrary, the PCR reaction carried out with the primer ratio 20:1 which produced only one of the strands, binds to only one of the labeled primers, resulted in band with intensity less than in lane 1 despite the same number of 20 PCR cycles employed.

3.7 Native PAGE based recovery of ssDNA

Upon loading of the asymmetric PCR product onto native polyacrylamide gel, ssDNA appeared to be smeary because ssDNA strands adopt many structural conformations or three dimensional structures. This is due to the presence of many different sequences, aided by intramolecular interactions such as Watson-Crick base-pairings, mismatches, non-Watson-Crick base-pairings, bulges and loops that occur between various parts of ssDNA (Figure 7a). Elution of ssDNA is made possible by cutting the part of polyacrylamide gel constituting the smear and subjected to crush and soak method.

3.8 Scale-up of PCR reaction

In this study, primer amount optimization of asymmetric PCR was carried out in 20 µL of reaction to reduce the consumption of PCR reactants. However, the amount of amplified product in asymmetric PCR is expected to be less than that is obtained by a conventional symmetric PCR (Poddar, 2000). This can be explained by the nearest–neighbour formula which reveals that Tm of limiting primer is several degrees lower than that of excess primer. This accounts for the low amount of ssDNA generation in asymmetric PCR (SantaLucia, 1988). This asymmetric PCR can be carried out in batches of 5-10 tubes of 100 µL of reactions followed by ethanol precipitation to a suitable volume for loading onto native PAGE. Purification of ssDNA from polyacrylamide gel by crush and soak method can result in ssDNA of high quality (Figure 7b). In this study, the amount of ssDNA that could be purified from 100 µL of asymmetric PCR reaction with the starting amount of template as low as 20 ng was 400-500 ng. This is approximately a 20-30 fold increase of yield compared to the initial amount of input DNA (Table 1). In comparison, lambda exonuclease digestion requires starting amount of dsDNA of around 2 µg to produce 400-500 ng of ssDNA, which is much higher than the amount required in asymmetric PCR (Citartan *et al.*, 2011). Asymmetric PCR is the only method of producing ssDNA using minimal amount of dsDNA input.

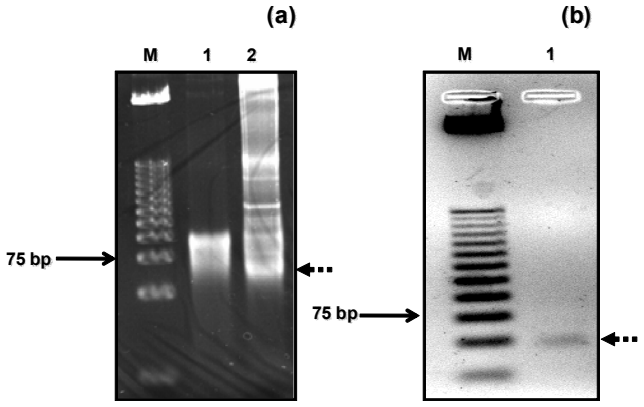

Figure 7. Native Polyacrylamide Gel and agarose gel analysis of asymmetric PCR. (a) Lane 1: 2µL of random ssDNA pool, Lane 2: 20µL of ethanol precipitated asymmetric PCR product on 10% Native polyacrylamide gel (b) Lane 1: 2µL of purified ssDNA from 10% Native PAGE on 3% agarose gel. Dashed arrows indicate the expected band position.

Table 1. Recovery of ssDNA from asymmetric PCR product

Amount of ssDNA recovered following purification from Native PAGE (ng)	Amount of purified symmetric PCR product input for asymmetric PCR (ng)	Fold of increase of output DNA compared to the input DNA (times)
500	20	25.0
550	20	27.5
480	20	24.0
488	20	24.4
400	20	20.0

4. Conclusions

Generation of good yield of ssDNA necessary for aptamer production requires an efficient method, taking into consideration the cost and time of the procedure. Asymmetric PCR reaction provides one of the ways to generate ssDNA. Primer concentration optimization (the amount of sense to antisense primer ratios) and the number of PCR cycles are the main criteria of an asymmetric PCR. We have demonstrated that asymmetric PCR generates ssDNA which can be subsequently purified by native polyacrylamide gel to produce ssDNA with good yield and purity. Therefore coupled with a good purification technique, asymmetric PCR can be universally applied to generate DNA aptamer for other target molecules (where different lengths of random sequence of the initial DNA library pool are used).

Acknowledgements

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