Essential Strategies to Optimize Asymmetric PCR Conditions as a Reliable Method to Generate Large Amount of ssDNA Aptamers

Running Title: Strategies to Optimize Asymmetric PCR

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Abstract:

Asymmetric PCR, a simple method to generate ssDNA aptamers in SELEX (Systematic Evaluation of Ligand by EXponential enrichments) rounds, is coupled with limitations. We investigated the essential strategies for optimization of conditions to perform a high quality asymmetric PCR. Final concentrations of primers and template, the number of PCR cycles and annealing temperature were selected as optimizing variables. The qualities of visualized PCR products were analyzed by ImageJ software. The highest proportion of interested DNA than unwanted products was considered as optimum conditions. Results revealed that the best values for primers ratio, final template concentration, annealing temperature and PCR cycles were respectively 30:1, 1 ng/µl, 55°C and 20 cycles for the first and 50:1, 2 ng/µl, 59°C and 20 cycles for other rounds. No significant difference was found between optimized asymmetric PCR results in the rounds of 2 to 8 ($P>0.05$). The ssDNA quality in round 10 was significantly better than other rounds ($P<0.05$). Generally, the ssDNA product with less dimers, dsDNA and smear are preferable. The dsDNA contamination is the worst, because it

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can act as antidote and inhibits aptameric performance. Therefore, to choose the best conditions, the lower amount of dsDNA is more important than other unwanted products.

Key Words: Asymmetric PCR, Optimization, ssDNA Aptamer

Introduction:

Normal structure of DNA is typically in a double stranded right-handed helix format in which two strands are posed in opposite directions. DNA is a flexible molecule, which can take various conformations ranging from single-stranded (ssDNA) to triplex or more [1]. In recent years, ssDNA is considered as a functional molecule with interesting utilization due to its essential role in many biological reactions [2]. The ssDNA molecules have been converted to multifunctional tools that can be recruited in some research areas such as allelic diversity studies [3], biophysics and biochemistry, gene expression and genome modification [4] DNA damages [5], DNA binding studies, ssDNA aptamers, etc. [6, 7]. Aptamer is referred to a ligand, which is isolated among a large pool of random sequences during a strict and multi-stage selection procedure called SELEX (systematic evolution of ligands by exponential enrichment). Aptamer ligands are small length nucleic acids (RNA or ssDNA) or peptide that binds specifically with high affinity to a broad range of targets [8]. As mentioned above, the isolation of aptamer is carried out by SELEX procedure. SELEX consists of reiterative cycles of selection, partitioning and amplification. In order to isolate the ssDNA aptamers, at the first round of SELEX, and before the start of each round, an amplified pool of ssDNA should be prepared from recovered target-bound DNA. The production of ssDNA is noted as one of the most critical step to get success in aptamer isolation. Several strategies have been suggested to generate ssDNA such as: 1) The separation of favorite DNA strand using streptavidin-coated magnetic beads, 2) The separation by unequal size primers in PCR procedure [9], 3) Lambda exonuclease digestion [10] 4) T7-based SELEX [11] and 5) Asymmetric PCR. The polymerase chain reaction (PCR) is an enzymatic polymerization of nucleic acids that produces a large amount of double-stranded DNA (dsDNA) [3]. Sometimes just a minor modification in PCR method will lead to major outcomes and constitute a new applicable PCR protocol to achieve a proper aim. Asymmetric PCR is a kind of PCR that produces a large amount of single-stranded DNA by utilizing unequal primer concentrations. In the asymmetric PCR, the primer with lower concentration is involved in the production of dsDNA while the primer with excess concentration is responsible for the generation of ssDNA. The asymmetric PCR followed by a complementary method like gel

purification or enzymatic degradation of the leftover strand can be an efficient method for generating a high purity of ssDNA. The asymmetric PCR, especially used in SELEX procedure for the amplification of short length DNA library, is involved with some problems such as the creation of by-products [12], weak or invisible bands, severe smears and non-separated bands. Offering different protocols by several reports reveal that firstly, the optimization of the asymmetric PCR is difficult, and secondarily the amplification of all ssDNA libraries do not follow a single protocol and unique pattern. In this study, we aimed to introduce the essential strategies and principles that can be used to prepare the optimal conditions for maximum production of ssDNA in the asymmetric PCR.

Materials and Methods:

PCR and Electrophoresis reagents:

General materials for polymerase chain reaction (*Taq* polymerase, enzyme buffer, dNTP, $MgCl_2$) prepared from TOPAZ gene Co., ssDNA random pool (5'-CCT AAC CGA TAT CAC ACT CAC-40N-GTT GGT CGT CAT TGG AGT ATC-3') and its corresponding primers were synthesized by Metabion Co. Agarose powder, Erythro-gel DNA safe stain were prepared from ATP Co.

Library design:

One of the main steps to perform a successful asymmetric PCR is library design. SELEX Library and primer design are linked together, so in order to design a library, the most delicacy should be expended, because in fact the primers are defined by constructing the library. Oligo software (version 7.56) was used to design library construction. The main observed points were equality and proportionality in primer length, melting and annealing temperature, GC content and primer 3′ properties. In this phase it should be tried to avoid primer dimers, cross homology, unequal primers Tm, mismatches and repeats, long run of single nucleotide, restriction enzyme binding sites and every primer secondary structure. Due to the unknown variable region sequences in the library construction, some attributes such as Tm of products and primer specificity are beyond our controls.

Optimization of symmetric PCR:

Symmetric PCR (PCR with equal primers concentration) should be standardized as the prerequisite of asymmetric PCR. The optimized conditions for the first round are different

from other rounds in both symmetric and asymmetric PCR. It is referred to the high purity of template used in the first round. Therefore, the optimization procedure should be done at least for two times. In order to find the best conditions for symmetric PCR, four of the most important factors (the concentrations of primers and template, cycle number and annealing temperature) were considered to be analyzed. One item was changed in each step and others were held fixed. The order of items was adjusted by authors' experiences as follows: A) Primer concentration, B) template concentration, C) the number of PCR cycles, and D) annealing temperature. PCR procedure was performed in a final volume of 25 µl containing 0.5 µl of dNTPs (10 mM), 2.5 µl of PCR buffer (10X), 1.0 µl of $MgCl_2$ (25 mM) and 0.75 units of *Taq* DNA polymerase. Thermo-cycler was programmed to hold 5min at 95°C for initial denaturation and 30s for each three steps (denaturation at 95°C, annealing at variable temperature, extension at 72°C). Other conditions were changed as optimizing items as summarized in Table 1.

Table.1.

Asymmetric PCR Procedure:

Optimization procedure for asymmetric PCR was performed after normalizing symmetric PCR. As mentioned above, because of the high purity of aptamer library used at the first round, the optimized conditions for asymmetric PCR at this round are different from next rounds. So as before, asymmetric PCR also needs to be optimized at least in two steps, first and second (other) rounds. This type of PCR were also performed in a 25 µl reaction volume containing 0.5 µl of 10 mM dNTPs, 2.5 µl of PCR buffer (10X), 1.0 µl of $MgCl_2$ (25 mM) and 0.75 units of *Taq* DNA polymerase. The order of variables for optimization of asymmetric PCR is slightly different from symmetric PCR as follows: unequal primer ratio, DNA template concentration, annealing temperature and the number of PCR cycles. The template concentration and the number of PCR cycles were set as described in the table 1. The annealing temperatures were determined according to the symmetric PCR results. So that the range between +2 to -2°C of optimized temperature in the symmetric PCR with 1°C intervals were checked. Final concentration of the reverse primer was kept constant at 0.04 µM and the effects of different concentrations of forward primer (10, 20, 30, 40, 50, 60 and 70 fold more than reverse primer concentration for the first round and 30, 40, 50, 60, 70, 80 and 90 fold for the second round) were examined. The running program of thermo-cycler was set as pervious.

Gel electrophoresis and ImageJ analysis:

All PCR products (symmetric and asymmetric PCR in both rounds) were analyzed on 2% (w/v) agarose gel electrophoresis. Running of 3 µl of each reaction was performed at room temperature in 1 X TBE buffer (1 mM EDTA, 90 mM boric acid, 90 mM Tris (pH 8.0)) for 20 min at 90 v. To avoid the loss of bands sharpness in gel electrophoresis, the running time of 10-12 min is more suitable. Electrophoresis patterns of PCR products were visualized by erythro-gel safe staining and detected with Bio-Rad Gel-Doc device. ImageJ software was recruited to analyze the electrophoresis gel documented images [13]. So that the intensity of visualized bands (dsDNA and ssDNA), unwanted smears and dimers on agarose gel were compared together by using ImageJ, and the variables with the best scores were selected as the optimal conditions.

Statistical analysis:

The optimum conditions for asymmetric PCR in second round were applied to all next SELEX rounds. These situations were repeated at least 20 times in each round and their ssDNA/dsDNA ratios were determined using ImageJ software. After checking the normality of obtained data of rounds 2, 4, 6, 8 and 10 (as sample rounds) by K-S test, the parametric test of ANOVA followed by Tukey's post-hoc was recruited to find significant differences between results of the rounds. All quantitative results presented in this study are listed as the mean ± SD. All analysis was executed by IBM SPSS Statistics software (version 23). P-values lower than 0.05 were considered as the significant differences between groups.

Results:

Primers design

Oligo software (version 7.56) analysis of equal length forward and reverse primers (21-nt) didn't show any hairpin and false priming neither in forward nor reverse primer. Tm calculated by %GC and 2(A+T)°+4(G+C)° methods were completely the same for both primers. Free energies of duplex and mix duplex primers were more than -4 kcal/mol while the free energies of primers full homology were less than -30 kcal/mol.

PCR optimization

Symmetric PCR was optimized at the first and second rounds in four steps that are summarized in table 1.

The ImageJ scores for gel images were considered as the differentiating scale between the variables. The determining index in the software was the ratio of dsDNA to primer dimers (or

smears) for symmetric PCR. Two other distinctive indexes including the ratio of ssDNA to dsDNA and the ratio of ssDNA to lower (or upper) smears were calculated to find the best conditions for asymmetric PCR. Regarding the noted principles, the best conditions for symmetric PCR at the first round were as follows: 0.2 µM of each primer, 25 PCR cycles and annealing temperature at 57°C. Different template concentrations did not show significant effect according to the software evaluations. Asymmetric PCR at the first round was carried out in an optimized way by observing conditions including; 30:1 of primers ratio (1.2 µM of forward *vs* 0.04 µM of reverse primer), 1 ng/µl of DNA template, 55°C of annealing temperature and 20 PCR cycles (data not illustrated). The optimized conditions for the second round of symmetric PCR, as mentioned in the table 2 and illustrated in the figure 1, were 0.4 µM of each primer, 1 ng/µl of DNA template, 30 PCR cycles and 58°C of annealing temperature. The best conditions for asymmetric PCR at the second round were as follows: 50:1 of primers ratio (2 µM of forward *vs* 0.04 µM of reverse primer), 2 ng/µl of DNA template, 59°C of annealing temperature and 20 PCR cycles (Table 2, Figure2).

Table.2.

Fig.1.

Fig.2.

Asymmetric PCR in other rounds:

Optimized conditions for the asymmetric PCR in the second round was also applied for other rounds of SELEX. The ratio of ssDNA to dsDNA band in the rounds of 2, 4, 6, 8 and 10 (as sample rounds) was analyzed using ImageJ software (Figure 3 and 4). Results for the selected rounds were 1.70 ± 0.163 (CV = 9%), 1.72 ± 0.139 (CV = 8%), 1.82 ± 0.122 (CV = 7%), 1.68 ± 0.131 (CV = 7%) and 2.02 ± 0.135 (CV =6%) for rounds of 2, 4, 6, 8 and 10 respectively. Statistical analysis of data obtained from twenty times repeated PCR reaction in each round using ANOVA, followed by Tukey post-hoc revealed that there is no statistically significant difference between the results of the rounds of 2, 4, 6 and 8 ($P > 0.05$). However, the results obtained from round 10, due to increasing ssDNA/dsDNA ratio showed significant differences with other studied rounds ($P < 0.05$).

Fig.3.

Fig.4.

Discussion:

PCR is a quite effective and useful tool for plenty of molecular biology techniques. One of the important applications of PCR is the generation of large quantities of ssDNA through a process known as asymmetric PCR, in the SELEX procedure to isolate aptamers [14]. Using high purity ssDNA through the SELEX process is one of the secrets to reach success in the production of a high affinity aptamer [15]. This PCR model is associated with some restrictions such as the lack of ssDNA generation, severe production of dsDNA or smears and remaining much primer, which can affect the quality of PCR yield. Limited changes in PCR can lead to improvement of procedure or even the creation of a new method. This study aimed to investigate the optimum conditions to achieve the highest yield of highly pure ssDNA using asymmetric PCR. Carrying out of various studies to optimize asymmetric PCR suggests that this process is somewhat difficult, and also it is not possible to consider a single protocol for all random DNA libraries [16-19]. The first step to get success in asymmetric PCR is the quality of designed library. Perfect design is less mentioned but very important issue in the implementation of a good asymmetric PCR. In SELEX procedure, especially in the final rounds, some problems may occur that are beyond our control. For instance, the creation of rigid secondary structures or the existence of complementary sequences in the library variable region may cause severe by-product and strongly interfere in target amplification [12]. Therefore, the quality of primer design should be high enough to ensure that the PCR problems are only caused by other elements. By well-designed library, the asymmetric PCR does not need to be optimized for each SELEX round. However, if the library is not designed in logical principle, the asymmetric PCR most likely need to be optimized in each round again. In this study, the optimum conditions indicated in second round, were applied in the following rounds and any statistically significant differences did not show between the results of rounds 2 to 8. Even in round 10, the ratio of ssDNA to dsDNA was significantly more than other rounds. Another point is that the optimization procedure of first round is different from other rounds in both PCR types. It is referred to the high purity of ssDNA template used at the first round. It seems that double-stranded DNA remaining in the templates of round 2 and others can play a role similar to reverse primer and prepare the conditions for maximum activity of forward primer [18]. While, the primary library used in the first round is single-stranded and lacks any complementary, so reverse primers should make the template for PCR procedure at early cycles. The main reason for the optimization of symmetric PCR is its regulatory role, meaning that the optimum possible range for a good asymmetric PCR is determined and the need for further experiments will be

reduced. The quality and the purity of ssDNA are more important than its quantity to achieve success in SELEX procedure [20]. Therefore, the PCR product with less dimer and smear is preferable. Double-stranded DNA contamination is worse than smears because it can act as complimentary antidote in SELEX procedure and inhibits aptameric performance [21]. Therefore, in order to select the optimized conditions, the lower amount of double-stranded DNA is more important than lower amount of smear. However, when there is a very little difference between the ratios of ssDNA to dsDNA, then the ratio of ssDNA to smear should be noted. If there is a major difference in the created smear, the condition by which a lower smear is produced is preferable. In the present study, such challenge was occurred in DNA template concentrations and PCR cycles in round 2 of the asymmetric PCR (table 2), where the ratio of ssDNA to dsDNA at 1 ng/µl of template and 10 PCR cycles were a little more than 2 ng/µl and 20 cycles. However, 2 ng/µl of template and 20 PCR cycles were chosen as optimal conditions due to the major difference between the ratios of ssDNA to smear. The optimization of second round of the PCR is more important than the first round, because its conditions are more similar to other rounds and are most likely to determine the optimized conditions until the end of SELEX. Complete removal of dsDNA is not possible in the asymmetric PCR and it is recommended that this technique be performed with a complementary method. For instance, treating the PCR product with low units of lambda exonuclease that digests the remaining dsDNA and leads to a higher purity of ssDNA [22].

Conclusion:

Generating a high yield of pure ssDNA is a vital step to isolate DNA aptamers. Different methods have been suggested to generate ssDNA. Being optimized, the asymmetric PCR reaction is considered as an efficient, rapid and cost effective procedure to provide large amounts of ssDNA. Apart from primer design, our study demonstrated that the experimental design, criteria for choosing the best conditions, ratio of unequal primer concentration, annealing temperature and the number of PCR cycles had the determinative role to reach success in the asymmetric PCR. This method along with other approaches can help to increase product purity.

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Table 1: Program for the optimization of symmetric PCR in the first and second rounds.

	Primers Final Concentration	Templet Final Concentration	PCR Cycles	Annealing Temperature
1	0.2µM 0.4µM 0.6µM 0.8µM	Normal Recommended Concentration (3ng/µl)	Normal recommended cycle (25 cycles)	manufacture recommended annealing temperature (59 °C)
2	Optimized Value	1 ng/µl 2 ng/µl 3 ng/µl 4 ng/µl 5 ng/µl	Normal recommended cycle (25 cycles)	manufacture recommended annealing temperature (59°C)
3	Optimized Value	Optimized Concentration	10 cycles 15 cycles 20 cycles 25 cycles 30 cycles	manufacture recommended annealing temperature (59°C)
4	Optimized Value	Optimized Concentration	Optimized Cycles	8 different temperature with one degree interval from 56 to 63

Table 2: ImageJ analysis of studied variables (table 1) in secondary symmetric and asymmetric PCR. Interested products (dsDNA in symmetric and ssDNA in asymmetric PCR) in each condition were compared with other unwanted products. The variables with higher scores (highlighted rows) were selected as the optimum conditions.

Symmetric PCR		ImageJ analysis	Asymmetric PCR		ImageJ analysis	
Variables and values		dsDNA Band/primers (or smears) ratio	Variables and values		ssDNA/dsDNA (or dsDNA + upper smear)ratio	ssDNA/ upper smears (or primers) ratio
Primers Concentrations (µM)	0.2	2.19	**The ratio between primers** (µM)	30:1 (1.2:0.04)	0.353	0.842
	0.4	2.23		40:1 (1.6:0.04)	1.206	2.702
	0.6	1.2		50:1 (2.0:0.04)	2.949	4.236
	0.8	1.26		60:1 (2.4:0.04)	2.466	3.118
DNA Templet Concentrations (ng/µl)	1	2.81		70:1 (2.8:0.04)	0.987	0.939
	2	2.26		80:1 (3.2:0.04)	1.03	0.738
	3	1.51		90:1 (3.6:0.04)	1.006	1.119
	4	1.56	**DNA Templet Concentrations** (ng/µl)	1	3.523	13.956
	5	1.68		2	3.188	19.167
PCR Cycle	10	2.11		3	1.179	15.828
	15	2		4	1.296	6.365
	20	1.6		5	1.252	2.447
	25	1.7	**Annealing Temperature** (°C)	56	0.92	0.68
	30	2.11		57	0.93	1.37
Annealing Temperature (°C)	56	2.54		58	0.91	2.01
	57	2.64		59	1.12	11.23
	58	3.6		60	0.94	8.7
	59	1.5	**PCR Cycle**	10	1.75	5.64
	60	1.03		15	1.70	7.34
	61	1.12		20	1.73	11.36
	62	1.52		25	1.55	9.25
	63	0.68		30	0.91	0.85

Fig.1. Visualizing the effect of different variables on the second round of symmetric PCR products on 2% agarose gel electrophoresis. The ImageJ graphs show the intensity of dsDNA band and other unwanted products. A) Different concentrations of final primers (µM), B) Different concentrations of DNA template (ng/µl), C) Different cycles of PCR, D) Different temperatures in annealing steps (°C). The number of the lanes are tantamount to different variables in table 2. "M" is referred to 100 bp DNA marker (Thermo Scientific; #SM0623).

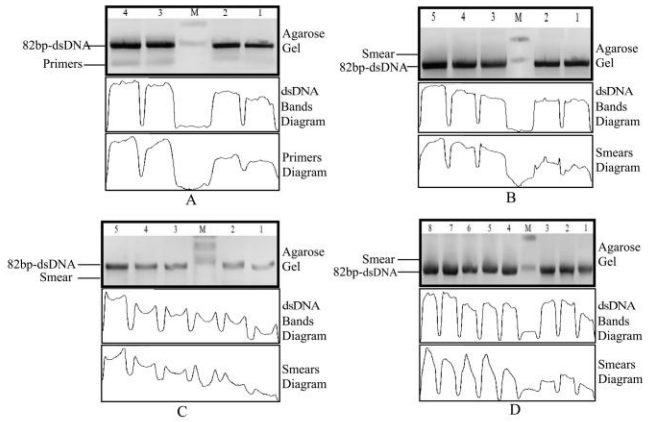

Fig.2. Visualizing the effect of different variables on the second round of asymmetric PCR products on 2% agarose gel electrophoresis. The ImageJ graphs show the intensity of ssDNA band and other unwanted products. A) Different ratios of primers, B) Different concentrations of DNA template (ng/µl), C), Different temperatures in annealing steps (°C), D) Different cycles of PCR. The number of lanes is tantamount to different variables in table 2. "M" is referred to 100 bp DNA marker (Thermo Scientific; #SM0623).

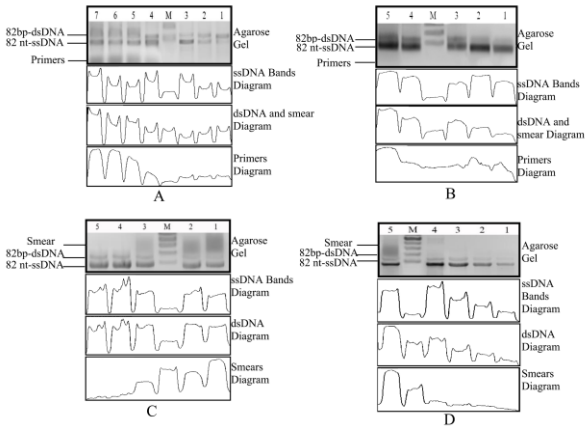

Fig.3. The ratio of ssDNA to dsDNA band in the rounds (R) of 2, 4, 6, 8 and 10 obtained by ImageJ

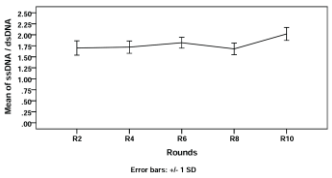

Fig.4. Visualizing the PCR products of different rounds (using the same asymmetric protocol) on 2% agarose gel electrophoresis. The ImageJ graphs show the intensity of ssDNA and unwanted dsDNA band. Lanes 1 to 5 are referred to the rounds of 2, 4, 6, 8 and 10.