Advances in Bioresearch
Adv. Biores., Vol 10 (4) July 2019: 40-50

Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.10.4.4050

Advances in Bioresearch

ORIGINAL ARTICLE

PCR amplification of DNA libraries in SELEX rounds: Non –specific amplification and effective intervention approaches

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ABSTRACT
Aptamer generation requires an optimized protocol for generation of the single strand DNA (ssDNA) to be used for target binding and amplification in the SELEX rounds. The randomness of the oligonucleotide library usually results in non-specific products in the SELEX rounds thereby masking the enrichment of specific target bound oligonucleotides. In this study, we report an optimized protocol for effective separation of the ssDNA and workable solutions to overcome non-specific amplifications in aptamer generation using a recombinant protein and a whole cell as target. With the use of 76 and 94 mer oligonucleotide libraries we clearly show the need for optimizing the concentration of alkali (50mM and 10mM respectively) for denaturation and separation of the specific strand of DNA with a recovery percentage of 70% and 29.47% respectively. In the process of aptamer generation to the recombinant protein and the whole cell as target with the 76 mer library, we observed non-specific amplification and attempted six different approaches namely treatment of samples with sodium hydroxide on silica column, purification and PCR amplification without extension step and limiting the number of PCR cycles and their combination to generate specific size amplicons. For non-specific amplification resulting in a higher molecular weight amplicons, excising the specific sized amplicon and gel-purification provides better results. The effectiveness of the solutions attempted to overcome the non-specific amplification and the enrichment observed over the SELEX rounds were confirmed by sequencing the amplicons in Illumina HiSeq 2500 and subsequent data analysis using the FASTAptamer toolkit.
Key words: repetitive amplification; next generation sequencing; SELEX

Received 04.01.2019 Revised 18.03.2019 Accepted 06.05.2019

How to cite this article:
Priyanka M, R. Vignesh, P.S. Vinod, K.G.Tirumurugaan·PCR amplification of DNA libraries in SELEX rounds: Non –specific amplification and effective intervention approaches. Adv. Biores., Vol 10 [4] July 2019.40-50.

INTRODUCTION

DNA well known for storage of the genetic information also mimics the properties of an enzyme in possessing specific molecular recognition and affinity towards the target due to its inherent ability to fold into secondary and tertiary structures in its single strand form [20, 11]. This single stranded DNA or RNA oligonucleotides exhibiting specific target binding properties (may be any small molecule to proteins) are termed as Aptamers [16]. The inherent specificity and greater affinity of aptamers to its selected target had made it applicable to various fields such as pharmaceutical sciences, therapeutics, drug discovery and as delivery and targeting units [7, 12], in the area of diagnostics as sensors [14] or as novel tools in chemical biology [9, 10, 19].

The aptamers are generated by the enrichment process of Systemic Evolution of Ligands by Exponential Enrichment (SELEX) which involves repetitive rounds of incubating an oligonucleotide library with the immobilized target, polymerase chain reaction (PCR) mediated amplification of the bound oligonucleotide, methods to separate specific ssDNA strand from the dsDNA and use of the ssDNA to bind to the targets in the subsequent rounds [13, 5]. There are several methods available for the separation of the specific strand of the ssDNA from the dsDNA amplicons generated during SELEX steps and it is a crucial requirement in aptamer generation. The most widely used approach is the alkali mediated

denaturation and selective separation of the specific strand [21]; while some reports have used lambda exonuclease [3, 8] and asymmetric PCR for this step [4, 17]. However, for application of the alkali mediated denaturation, optimization is required depending on the length of the oligonucleotide library being used.

The common problem that is usually observed during the successive rounds of SELEX is the absence of amplification or the generation of non-specific products due to repetitive amplification that is very difficult to control due to the randomized core of the oligonucleotide library. This hybrid DNA artefact amplification during SELEX rounds might mask the amplification of the specific target bound oligonucleotides and there is loss of enrichment of the interested oligonucleotides [19]. This repetitive amplification (non-specific) might also be due to a chimera formation which is usually observed in the initial rounds of SELEX due to non-specific primer binding to the randomized core (region that enables binding to the target) there by resulting in smaller or larger size amplicons than expected. Under these circumstances, these non-specifically generated amplicons might serve as template and enrichment of those amplicons results in by-product formation [1, 6]. In the process of generating aptamers, the negative SELEX rounds are performed with the cross reacting targets/ the materials used for immobilization to improve the specificity of the aptamers generated. Reports also indicate that this step might contribute to non-specific amplification due to the presence of inhibitor carry over when the unbound material is used for the PCR amplification [2]. Thus in the process of generating aptamers to a target, it is usual to face the several technical obstacles that have been mentioned in individual reports with appropriate solution that worked for the laboratory rather than attempting different approaches. Hence, in this paper, we have concentrated on the two major steps, namely the step that require optimization (the alkali concentration for ssDNA separation) and the other that requires troubleshooting (the several working options and its combination to overcome the repetitive/ non-specific amplification) during the SELEX rounds in aptamer generation. The optimization of the alkali concentration have been demonstrated in our study with the use of different lengths of the oligonucleotide library and we have used immobilized purified protein and a whole cell as target and attempted to provide solutions to the problems of repetitive amplification faced during the SELEX rounds.

MATERIAL AND METHODS

Molecular Biologicals:

The single-stranded DNA (ssDNA) oligonucleotide library and all the primers were obtained HPLC-purified from Eurofins, India. Two oligonucleotide libraries of lengths 76 mer (5'-CGTACGGAATTCGCTAGC-N40-GGATCCGAGCTCCACGTG-3') and 94 mer (5'-TCCATCTCTTCTGTATGTCGAGATCTA-40N-TAGATCTCCTAACCGACTCCGTT ATTT-3') with a central 40 base random nucleotide core flanked by known primer binding sites have been used in this study. The PCR cycles were performed with 5' biotinylated forward and unmodified reverse primers. The other molecular biologicals included Exprime Taq (GenetBio, South Korea), Streptavidin magnetic beads (Thermo Fisher Scientific, India), MinElute PCR and Gel Extraction kits (Qiagen, India).

PCR amplification of the random oligonucleotide library

The optimization steps for PCR amplification of the two random oligonucleotide libraries included different concentrations of both the primer pairs (1, 5 & 10 pmol each); varied concentrations of 5'-biotin tagged forward: unmodified reverse primers (pmol concentrations of 5:5, 5:10, 10:10, 10:5, 1:5, 5:1, 1:10 and 10:1); annealing temperatures (ranging from 50°C to 68°C) and the number of PCR cycles (10, 20, 25 and 30). The PCR conditions for amplification of the random oligonucleotide library included 95 for 5 min, repeated cycles of 95°C for 30 sec; optimized annealing temperature for 30 sec and 72°C for 30 sec with a final extension at 72°C for 5 min.

Generation of single stranded DNA (ssDNA) for use in SELEX

We followed the alkali mediated denaturation coupled with magnetic separation using streptavidin-coated magnetic beads as reported by Wilson (2011) with minor modifications. For this purpose, 250 µg of the streptavidin-coated magnetic beads (Thermo Fischer Scientific, India) were washed with 1X magnetic bead wash and binding buffer (MBWB; 10 mM Tris HCl, 1 mM EDTA and 2 mM Sodium chloride) using a magnetic stand. A concentration of 35 pmol of the purified PCR amplicons were allowed to bind to the beads with different incubation times (15 min – 2 hrs), washed once with MBWB, used different strengths of the alkali solution (5 mM, 10 mM, 25 mM, 50 mM & 75 mM) to the 76 and 94 mer oligonucleotide libraries, incubated at room temperature, magnetically separated the bound 5' biotin labelled anti-sense strand and neutralized the sense-strand free in solution with 10X TE buffer. The neutralized sense strand was purified using the MinElute Gel extraction kit and the concentration determined using a nanodrop spectrophotometer.

SELEX rounds for aptamer generation to different targets

In this study, the aptamer generation were attempted to two different targets (a purified recombinant protein immobilized to solid support & a whole cell target) and the problems encountered during the SELEX rounds are discussed.

Experiment 1-Aptamer generation to a purified recombinant protein immobilized to solid support:

a) ***Immobilization of the purified recombinant protein to polypropylene tubes***: For generation of aptamers, the purified recombinant protein was coated on to polypropylene tubes (Sarstedt, Germany) to perform the SELEX rounds. The purified protein (~ 42 kDa) was coated using 1X carbonate-bicarbonate buffer pH-9.4 and incubated at 4^{o}C overnight. The tubes were washed with phosphate buffered saline (PBS) and the unadsorbed sites blocked with Superblock buffer (Thermo Fischer Scientific, India). The efficiency of protein immobilization was determined with standard concentrations of the purified recombinant protein.

b) ***SELEX steps****:* The optimized concentration of the protein was immobilized to generate DNA aptamers as per the methodology of Scoville *et al.,* [15]. Following overnight coating and blocking as per the protocol mentioned above, we denatured 35 pmol of the 76 mer random oligonucleotide library in 50µl of the DNA binding buffer (25mM Tris, 192 mM glycine, 5mM KH_2PO_4, and 1mM $MgCl_2$ of pH 8.3) at 95^{o}C for 5 minutes, slowly cooled on ice, added to the protein immobilized tubes and incubated for 1 hour at room temperature with intermittent shaking. The tubes were washed with PBS, a 50 µl volume of the PCR mix with 5' biotinylated forward and unmodified reverse primer was added and the PCR performed as per the optimized conditions for 15 cycles. The PCR amplicons of four such tubes were pooled, purified using MinElute PCR purification Kit (Qiagen, Germany), ssDNA separated as per the protocol mentioned above and used in the next round of SELEX. We performed seven rounds of positive SELEX, three rounds of negative SELEX (with superblock buffer and uncoated tubes) and three rounds of competitive SELEX (with decreasing concentration of ssDNA and a fixed concentration of Salmon sperm DNA). We observed a visible smear and non specifically amplified products upon agarose gel electrophoresis in the sixth SELEX round and during the negative SELEX steps. The problems in amplification were overcome by performing the different approaches mentioned below.

Experiment 2-Aptamer generation to a whole cell target:

For generating aptamers to whole cell target, 200 pmol of the synthesized 76 mer DNA library nucleotide library was added to 1 million cells (washed cell line) in 460 µl of cell wash and binding buffer (CWBB; 5 mM Glucose, Phosphate Buffered Saline, pH – 7.4). The mixture was incubated at room temperature for one hour with intermittent shaking. The unbound oligonucleotides were removed by centrifugation, the pelleted cells washed with CWBB for three times and the final cell pellet resuspended in 30µl of Lysis Buffer 1 (LB1; 20 mM DTT, 1.7 µM SDS, 1 mg/ml of Proteinase K), and incubated at 56°C for 1 hour. The mixture was cooled and 15 µl of LB2 (50 mM DTT, 200 mM Potassium hydroxide) was added, incubated at 65°C for 10 minutes, cooled and neutralized with 15 µl of Neutralizing Buffer (NB; 300 mM Potassium chloride, 900 mM Tris Hcl, pH – 8.3). This cell lysate was used as a template for PCR in a volume of 300 µl with the optimized PCR conditions, the amplicons purified using PCR MinElute kit and the ssDNA separated as per the protocol mentioned above and used in the next round of SELEX. We observed a visible smear and lot of non specifically amplified products upon agarose gel electrophoresis in the fourth SELEX round and during the next step of negative SELEX. The problems in amplification were overcome by performing the different approaches mentioned below

Problems in PCR amplification and the technical solutions attempted

Approach 1: To overcome the non specific amplification visible as a smear in the agarose gel upon electrophoresis during the negative SELEX rounds with recombinant protein (the unbound oligonucleotide was processed for the PCR amplification and ssDNA separation in this step), we tried to vary the number of PCR cycles. The unbound supernatant in the negative SELEX round was used as template and amplified for different cycle numbers namely 9, 12, 15, 20 & 25.

Approach 2: To avoid the appearance of smear during the amplification of supernatant from negative round of SELEX with recombinant protein, we used different dilutions of the template 1:5, 1:10, 1: 20, 1:40 & 1:80. In addition, during the PCR we also limited to 20 cycles as well as eliminated the extension step in each PCR cycle.

Approach 3: Following the positive SELEX rounds when the ssDNA was used in the negative SELEX with recombinant protein, we could observe non-specific amplification upon gel electrophoresis. The reason for the presence of nonspecific amplicons could be due to the inhibitor carry over from the binding buffer. To eliminate the effect of inhibitors, we treated the unbound oligonucleotides with sodium hydroxide as reported by Bourke et al. (1999) with some minor modifications. The unbound oligonucleotide sample

was loaded onto the MinElute PCR purification column, briefly centrifuged, washed two times with 0.4N NaOH followed by a 70 % ethanol wash and the product was eluted with 1X Tris EDTA buffer (TE) buffer. The PCR was performed with the eluted sample as the target.

Approach 4: To overcome the above problem we also attempted a combination of alkali treatment of the sample in the MinElute PCR purification column as mentioned above prior to PCR amplification and performed PCR cycles without the extension step.

Approach 5: We also observed a higher molecular weight band (>100 bp) during the SELEX rounds with recombinant protein as target which might be due to mispriming by the unmodified reverse primer. An attempt was made by excising the specific amplicons following agarose electrophoresis and gel extraction using MinElute Gel Extraction kit followed by amplification of the eluted target with varying number of PCR cycles.

Approach 6: During consecutive steps of enrichment of specific target by SELEX with whole cell target, we could not observe any SELEX amplicons upon agarose gel electrophoresis possibly due to inhibitor carry over. We attempted two approaches namely the use of low number of PCR cycles (5, 10, 15 cycles) without extension coupled with dilution of the template.

Sequence enrichment analysis using NGS:

To assess the effectiveness of the methods used in overcoming non specific amplification, we analysed the enrichment of the sequences during subsequent rounds of SELEX by sequencing the PCR amplicons in Illumina HiSeq 2500 platform. The amplicons were ligated with adapters, PCR amplified to generate the libraries, quantified using Qubit and quality checked with the Bioanalyser. Following sequencing the data quality was checked using FastQC and MultiQC software. The adapter sequences were trimmed and the quality passed reads were collapsed and counted. The data was checked for base call quality distribution, % bases above Q20, Q30, %GC, and sequencing adapter contamination. The QC passed reads were analyzed using FASTAptamer. In brief, the reads were collapsed to get unique reads and the number of times it was represented using fastaptamer counts. The enrichment was checked using fastaptamer_ enrichment script. The reads that were represented at least 5 times were used for clustering with Levenshtein edit distance of 4. We retained only top 200 clusters to perform enrichment analysis to generate information on the enriched reads in terms of Reads Per Million (RPM) reads.

RESULTS AND DISCUSSION

This study was initiated during our effort to generate aptamers to a purified recombinant protein and a whole cell target. In generation of aptamers, the randomness of the oligonucleotide library is used as the starting material to generate several structures that specifically bind to the target of interest. The bound structures are enriched through the rounds of SELEX. Thus in the process of generating aptamers by SELEX, the biggest obstacle is the non-specific amplification by product formation. During the SELEX steps we came across some of the common problems that included a smear/non-specific by-product, absence of amplification and an intense high molecular weight band with both the targets at different SELEX rounds. We report here with the workable solutions to overcome the by-product formation in the SELEX rounds to generate aptamers.

In this study we have attempted to develop aptamers to a purified recombinant protein and a whole cell target. For generation of aptamers to the purified recombinant protein, the same was immobilized to polypropylene tubes. Hence, for this purpose we first determined the efficiency of immobilization to solid support as a first step prior to SELEX. The results indicated a binding efficiency 2.5 µg/ml to the polypropylene tubes (Fig. 1). In the SELEX procedure to the purified proteins, the target bound oligonucleotides were amplified in the positive rounds, while the negative SELEX included the cross reacting proteins (the blocking buffer and the tubes). We have used 2 x 10^6 cells in every step for generation of aptamers to the whole cell target. The negative SELEX for the whole cell target included the buffer components and the tubes used for processing the materials.

For this study we had used a 76 and 94 mer long oligonucleotide library for the study and in the process of optimization we could observe efficient amplification of both the libraries at 60° C for 30 cycles with a 10 pmol concentration of both the forward and reverse primers (Fig. 2). Using the above optimized conditions, for the two different libraries we purified the PCR amplicons and optimized the conditions for the alkali mediated denaturation and separation of the ssDNA. As it known that the concentration of the alkali to be used could vary depending on the length and the nucleotide composition, we used different concentrations and exposure time to the alkali solution. We observed that an alkali concentration of 50 mM NaOH & 10 mM NaOH and exposure for a period of 15 min at room temperature resulted in better denaturation and separation of the anti-sense DNA strand using the streptavidin coated magnetic beads with 76 and 94 mer oligo-libraries respectively (Fig. 3 A & C). The sense strand in solution was

neutralized, purified and the concentration determined before use in the subsequent rounds of SELEX. The concentration of the sense strand was 99 ng/µl and 84.94 ng/µl of the sense strand with the 76 and 94 mer libraries respectively. The recovery efficiency of the alkali mediated denaturation and streptavidin mediated separation in our study was 70 % and 29.47 % for the 76 and 94 mer libraries respectively (Fig. 3 B & D). Since the efficiency of the ssDNA recovery was higher with the 76 mer library, the SELEX steps to the purified recombinant protein and the whole cell target used the 76 mer library.

We performed the SELEX rounds with the optimized conditions mentioned above for both the targets (purified recombinant protein and the whole cell) and in each step suitable positive and negative controls were included and the specific amplification was confirmed before the PCR amplicons were processed for ssDNA preparation to be used in the next round of SELEX. We observed a smear like pattern and a higher molecular weight fragment during amplification in the 5th and 6th rounds of SELEX respectively in the approach with recombinant protein (Fig. 4 A), while with whole cell target the appearance of smear was observed in the 5th and subsequent rounds of SELEX (Fig. 4 B). The effect of inhibitors, non-specific binding of the primers to the randomized core of the oligonucleotide library and the presence of targets with diverse and overlapping sequence stretches have been reported to be the major reasons for such problems in SELEX rounds with some of the oligonucleotide libraries [19]. The first appearance of the non-specific amplification in our study was observed in the negative SELEX rounds that used the cross reacting host proteins, blocking buffers and other buffer components. Carry-over of inhibitors from the host cell lysate and other buffers could be potential contributors to the above problem. In such conditions, decreasing the number of PCR cycles have been shown to reduce the non specific amplification due to the presence of inhibitors. Hence, we attempted decreasing the number of PCR cycles as a first attempt however; there was no improvement in the results (Fig. 5 A & B). Inhibitor carry over could be eliminated by diluting the template that is to be used for PCR amplification and improved results have been reported by performing PCR cycles without extension step [19]. Hence as a second approach, we diluted the template (to 5 fold), decreased the number of PCR cycles (10- 15 cycles) and performed PCR cycles with and the without extension step (Fig. 5 A & B).

During the SELEX process with recombinant proteins, inhibitor carryover was first observed during the negative SELEX (also called counter SELEX) rounds that were performed to remove the oligonucleotide structures binding to the cross reacting proteins. The interference of binding buffer components in the unbound supernatant probably results in the appearance of such smear (Fig. 6A). The optimized cycle conditions failed to eliminate the formation of smear (Fig. 6B) and no improvement was observed upon optimization of PCR cycle conditions and using diluted template with elimination of the extension step during PCR amplification (Fig. 6C). As these attempts failed, we followed the approach reported by Bourke et al., 1999, where in the samples were treated with sodium hydroxide, washed and eluted using silica columns (Fig. 6D). This helped us to partially eliminate the smear formation and combining this step along with PCR amplification without extension step helped us in generating specific sized amplicons (Fig. 6E). The report by Tolle et al., 2014, suggests that higher possibility of by-product formation during SELEX was due to the randomness of the library and mispriming. During our SELEX rounds a higher molecular weight product (~100 bp) was also observed (Fig. 6F), when we tried the above approaches (dilution, elimination of extension cycle and alkylation). Hence we excised the specific product from the gel and performed by gel extraction followed by amplification of the product for shorter number of cycles (5-15 cycles) to generate the specific sized amplicons (Fig. 6G).

The solutions attempted in this study for overcoming the repetitive amplification or non-specific amplification was also confirmed for its applicability by next generation sequencing (using Illumina HiSeq 2500 platform) of the amplicons from different rounds of SELEX (round 5, round 6 and round 7 with the recombinant protein target). During the subsequent rounds of SELEX, we observed a higher molecular weight amplicon (100 to 120 bp) in every round of SELEX along with our specific band. The contributors to the non-specific amplification and the successful elimination this problem using the above reported approaches resulted in specific amplification and enrichment of sequences in the subsequent rounds. Following generation of the sequence data and its quality check, the quality passed reads were collapsed to obtain unique reads and using fastaptamer counts. The generated reads were clustered and the top 200 clusters from round 5, round 6 (SELEX round that exhibited repetitive amplification) and round 7 were selected for further analysis. When we compared the round 7 with round 6, we could observe the presence of reverse primer sequence in the core region probably contributing to the mispriming and resulting in by-product formation (Fig. 7). Sequence results of the previous SELEX round (round 5) did not show any non-specific amplification and the solutions reported in this study helped in eliminating the non-specific amplification in the round 7 (Table - 1).

With respect to the whole cell target, we could observe the non specific amplification in the round 5 of SELEX. Following the above solutions, we generated specific amplicons for the rounds 4 and 5. These amplicons were also sequenced in Hiseq 2500 platform and the analysis of the sequences revealed enrichment over the rounds of SELEX. This is evident from the number of sequences in the top 200 clusters as well as the number reads/ RPM in each cluster and the enrichment percent with respect to the previous round of SELEX (Table - 2).

In conclusion, we demonstrate that for generation of aptamers, it is essential to optimize the PCR conditions for amplification of the bound oligonucleotides during the rounds of the SELEX, the concentration of the alkali and the conditions for separation of the ssDNA depending on the length of the oligonucleotide library used. The repeated rounds of PCR for amplification during SELEX experiments can result in the formation of by-products. Enrichment of specifically bound target sequences is barred due to loss of potential binding sequences. We report herewith the different solutions that could potentially tried in the event of non-specific amplification during the process of aptamer generation (applicable to approaches using either purified recombinant protein / whole cell targets). The effective solution arrived at was also confirmed by sequencing the PCR amplicons from the different rounds of SELEX in next generation sequencing platforms.

Table 1: Analysis of the sequence information generated from the Illumina HiSeq 2500 reads to assess the enrichment efficiency (with purified recombinant protein).

Sequence	Length	Round 5 RPM(x)	Round 6 RPM (y)	Round 7 RPM (z)	Enrichment (y/x)	Enrichment (z/x)	Enrichment (z/y)
GGGCGGAACGTGCCAAACACGAAGAGAAAGAAACAACGCC	40	2.16	17.46	8.73	8.08	0.50	4.04
GGGCAACAAAGAAACACGGGAAGCAACAAAACCGAAAGCG	40	5.56	9.84	106.88	1.770	10.862	19.223
GGGCCGACAACAAAGCCAAAAACAAACACAACAGAGCACA	40	6.66	7.84	64.35	1.177	8.208	9.662
GGGCGGAACGTGCCAAACACGAAGAGAAAGAAACAACGCC	40	17.46	8.73	38.99	0.500	4.466	2.233
GGGCGGGGGTGCTGGGGGAATGGAGTGCTGCGTGCGCG	38	5.84	9.62	110.43	1.647	11.479	18.909

Note: Problem was observed in the round 6. The data presented for the round 7 following the implementation of the optimised approach to eliminate non-specific amplification.

Table 2: Analysis of the sequence information generated from the Illumina HiSeq 2500 reads to assess the enrichment efficiency (with whole cell target).

Sequence	Length	Round 4 RPM(x)	Round 5 RPM (y)	Round 8 RPM (z)	Enrichment (y/x)	Enrichment (z/x)	Enrichment (z/y)
GGGACCGACAAACAAAAAACAACAGACATAAACGAAGACA	40	0.07	32.15	42.69	459.3	609.9	1.3
GGGACAGCCCATCAACACAAAAGGAAAAAACGAAGACGCC	40	0.07	5.24	2.51	74.9	35.9	0.5
GAAACAGCAAAGAACCAAACCCAAAAAAGGACCCGCAAGT	40	0.07	3.26	7.53	46.6	107.6	2.3
GGGAGGCGCACCAAAAGAAAACAAACCAGAACCCGACCTA	40	0.07	2.14	2.51	30.6	35.9	1.2
GGAAAAAACAGCACCGGGTCAACCAAACAAACGACAAATA	40	0.07	1.98	5.02	28.3	71.7	2.5

Note: Problem was observed in the round 5. The data presented for the round 8 following the implementation of the optimised approach to eliminate non-specific amplification.

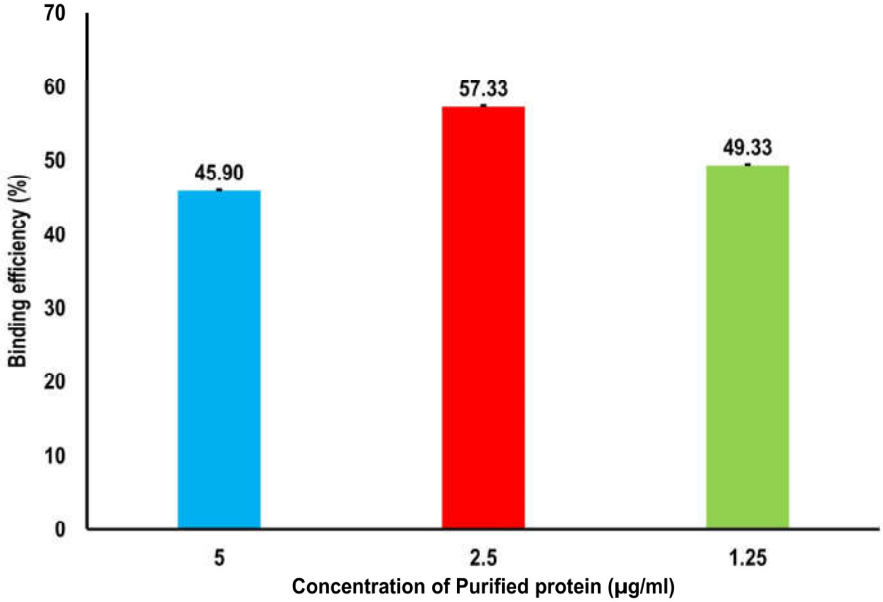

Fig. 1. Efficiency of recombinant protein immobilization to polypropylene tubes: Three different concentrations of the recombinant protein (5, 2.5 & 1.25 µg) were used for immobilization to the polypropylene tubes. *Note: 2.5 µg of the recombinant protein resulted in 57.3% binding to the polypropylene tube.*

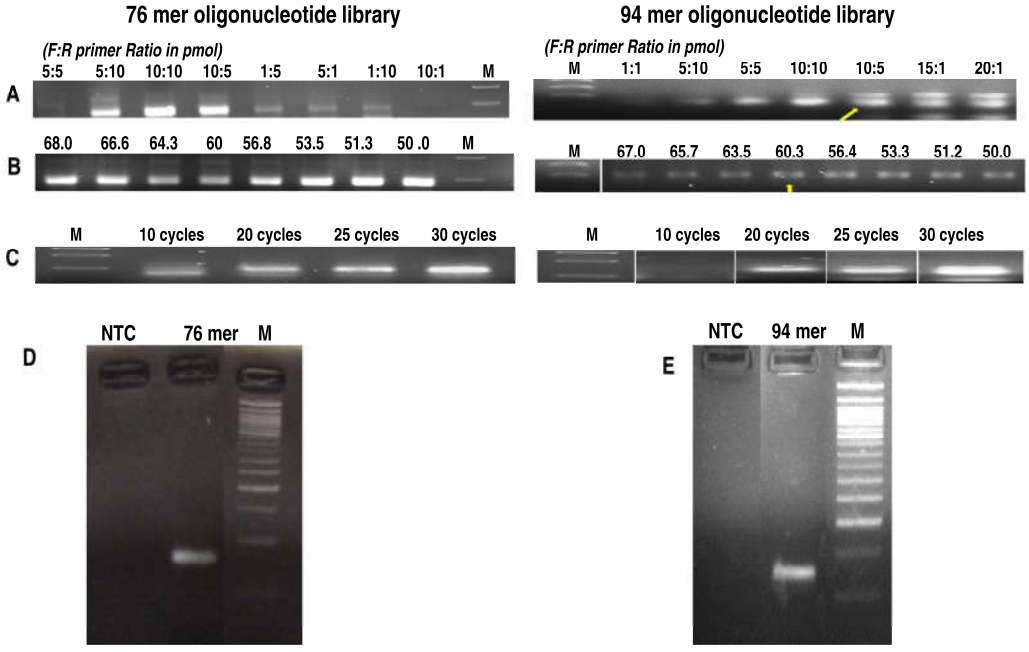

Fig. 2. Optimization of PCR conditions for amplification of 76 mer randomized ssDNA library as template: *A*: *Representative gel image showing analysis of PCR products with different amount of primers.* Lane M: 100 bp DNA ladder, Lanes 1-8: Mobility of the PCR products with varied ratios of biotin modified forward and reverse primer in the ratio as indicated in the image on a 4% agarose gel. ***B:*** *Representative gel image showing effect of different annealing temperature on PCR products.* Lane M -100 bp DNA ladder, Lane 1 - 8 Mobility of the PCR products with different annealing temperatures 50° C to 68° C. ***C:*** *Analyses of PCR amplicons with different amplification cycles.* Lane M – 100 bp DNA ladder, Lane 1 – 4: Mobility of PCR products with PCR cycles 10, 20, 25 and 30 respectively, on 4 % agarose gel. **D & E:** 76 mer (primer ratio 10:10, annealing temp 60°C and 30 cycle) and 94 mer (primer ratio 10:5, annealing temp 60°C and 25 cycle) oligonucleotides library amplicons in the optimized conditions.

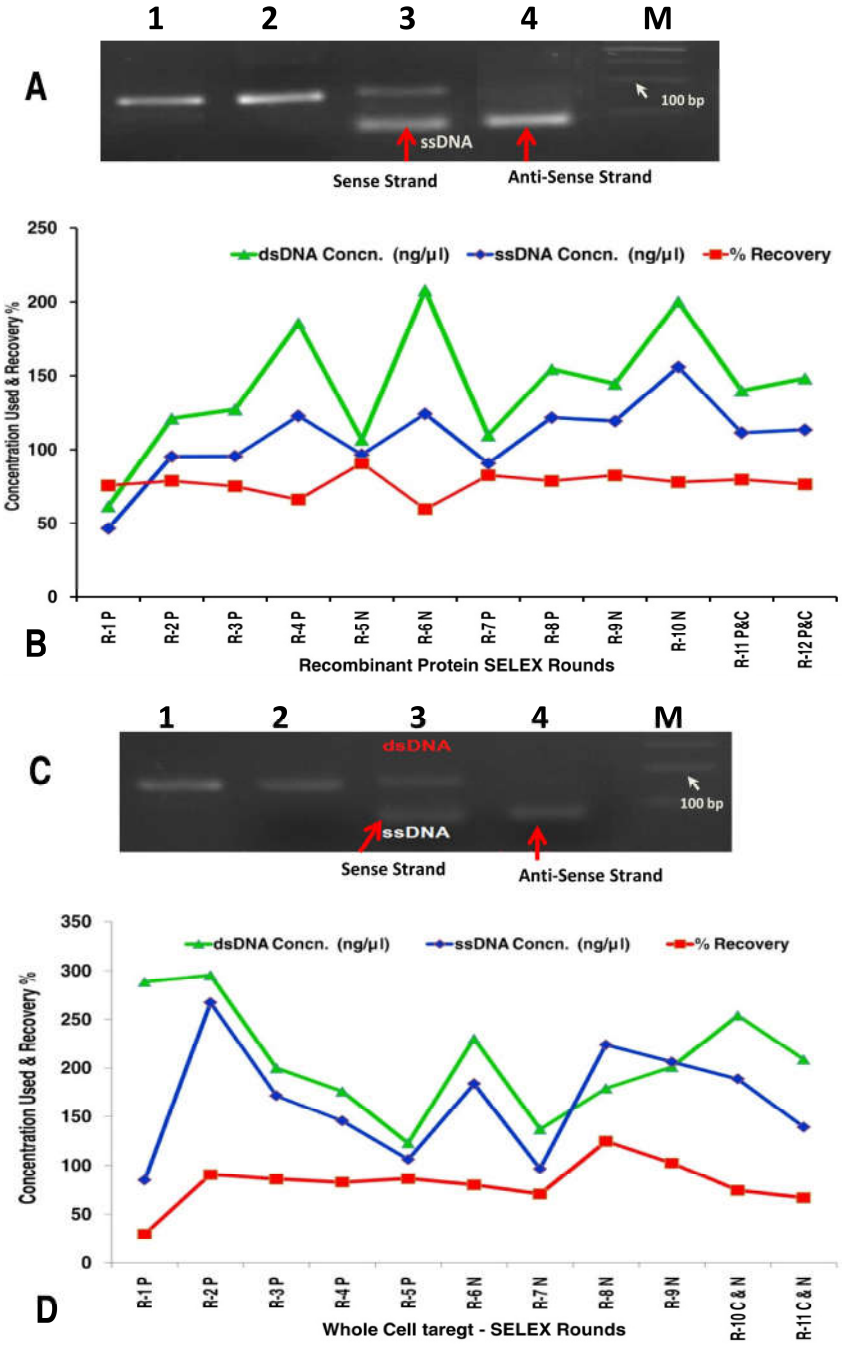

Fig. 3. Analysis of ssDNA obtained from streptavidin mediated alkylation of 76 & 94 mer oligonucleotides library and its recovery percentage. ***A****: Representative gel image of optimized alkali concentration for 76 mer oligo library.* Lane 1 – PCR product of 76 mer oligo library, Lane 2 – PCR purified product of 76 mer oligo library, Lane 3 – separation of ssDNA- sense strand from dsDNA after 50 mM NaoH treatment for 15 min at room temperature, Lane 4 – separation of ssDNA- anti-sense strand after 100 mM NaoH treatment for 15 min at 40° C, Lane M – 50 bp ladder. ***B:*** *Representative gel image of optimized alkali concentration for 76 mer oligo library.* Lane 1 – PCR product of 94 mer oligo library, Lane 2 –PCR purified product of 94 mer oligo library, Lane 3 – separation of ssDNA- sense strand from dsDNA after 10 mM NaoH treatment for 15 min at room temperature, Lane 4 – separation of ssDNA- anti-sense strand after 100 mM NaoH treatment for 15 min at 40° C, Lane M – 50 bp ladder. ***C*:** Recovery percentage of 76 mer oligo library (around 76 %). **D:** Recovery percentage of 94 mer oligo library (around 71%).

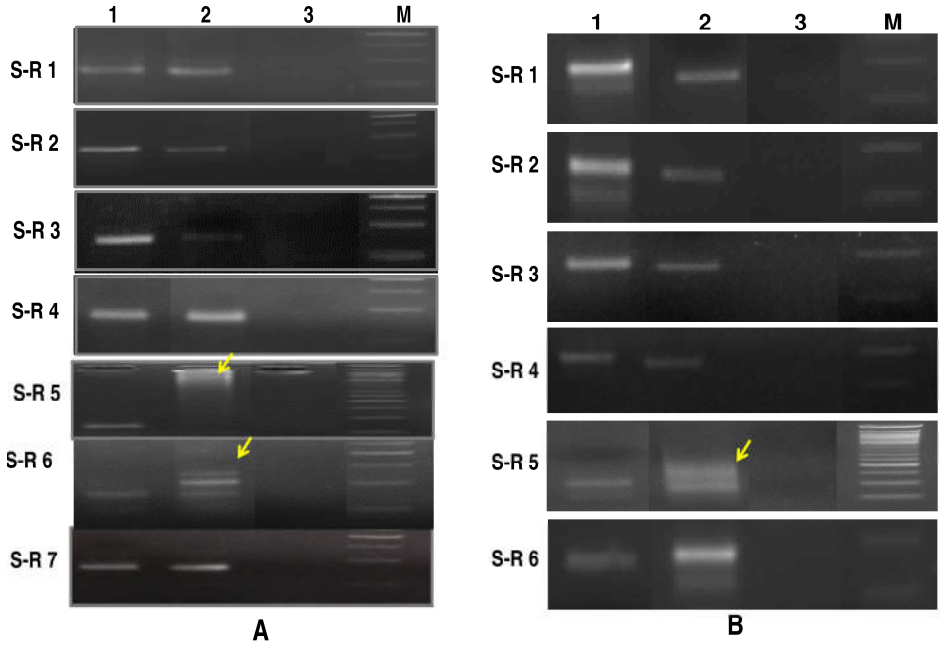

Fig. 4. SELEX Rounds: ***A:*** Representative gel image of SELEX round with purified recombinant protein. **B:** Representative gel image SELEX round with whole cell target. Lane 1 - PCR product of 76 mer oligo library, Lane 2 – PCR product of SELEX; Lane 3 – NTC; Lane M – 50 bp ladder. ***Note****: the nonspecific amplification in SELEX Round -5 (smearing), round 6 (multiple non specific bands) with recombinant protein as target and SELEX Round -5(non specific high molecular product) with whole cell target*

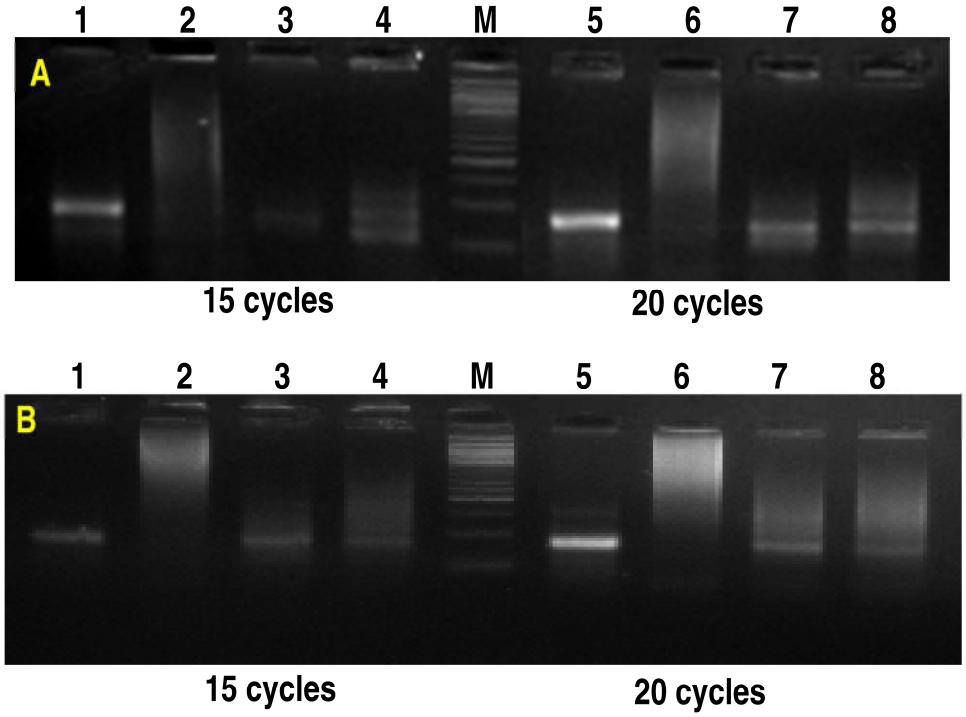

Fig. 5. Approaches attempted for eliminating non–specific amplification in the SELEX rounds with whole cell target: The non-specific amplification was observed in the form of shearing (Lane 2 & 6) in the 5th round of SELEX. The approaches included reducing the number of PCR cycles (Lane 1, 3,4,5,7 & 8) dilution of the template and PCR cycle without extension step (3 & 7). *Note: The specific amplification of the 76 bp amplicon with reduced number of PCR cycles without extension step (Lane 1 & 5)*

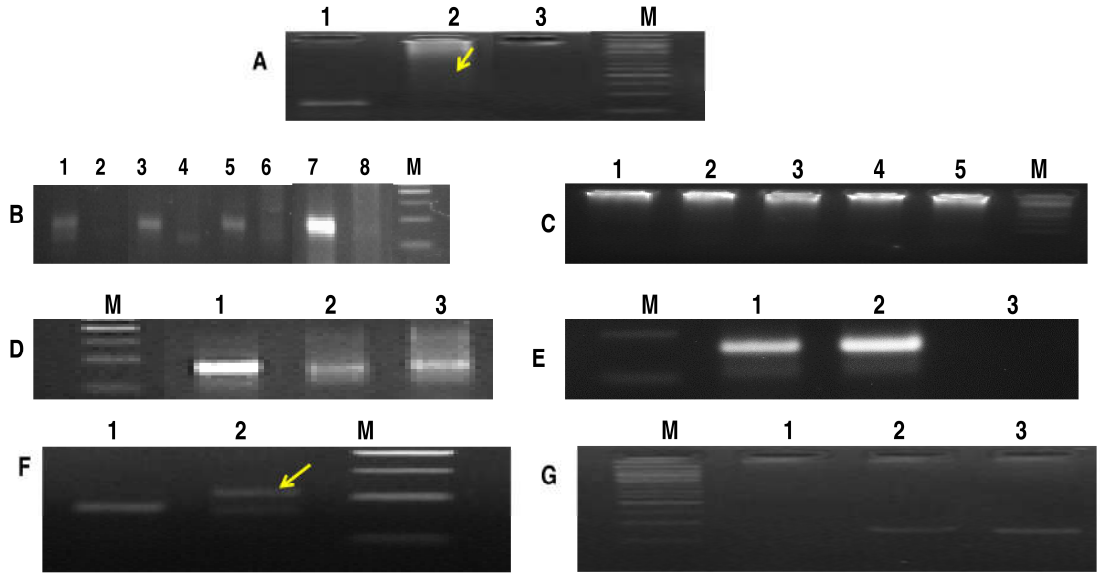

Fig. 6. Solutions attempted for eliminating non–specific amplification in the SELEX rounds with purified recombinant protein as target: The appearance of non-specific amplification in the form of smear in the 5th round of SELEX (A; Lane 2) and the presence of a higher molecular weight amplicon (F; Lane 2); **B**- Varying the number of PCR cycles (9,12,15 & 20 cycles – Positive control in lanes 1,3,5 & 7 and samples in lanes 2,4,6 & 8); **C** - PCR with dilution of the template (2 fold dilutions of the template); **D** - Alkali treatment of the template added on to PCR purification (Lane 2) and gel elution column (Lane 3) respectively, Lane 1 – Positive control; **E** – Combining alkali treatment and elution of the sample followed by PCR cycles without extension (Lane 1 – Positive control, Lane 2 – Sample); **G** – Gel extraction of the specific sized PCR amplicon (Lane2 – Positive control, Lane 3 – Sample); Lane M – 50 bp DNA ladder. *Note: The effectiveness of alkali treatment followed by PCR cycle without extension (E: Lane 2) and gel extraction of the specific amplicon (G)*

SELEX Round - 5
CGTACGGAATTCGCTAGC-GGGCGGGGGTGCTGGGGGAATGGAGTGCTGCGTGCGCG-GGATCCGAGCTCCACGTG
CGTACGGAATTCGCTAGC-GGGCGGGGGTGCTGGGGGAATGGAGTGCTGCGTGTGCG-GGATCCGAGCTCCACGTG
CGTACGGAATTCGCTAGC-GGGCGGGGGTGCTGGGGGAATGGAGTGCTGTGTGCTGCG-GGATCCGAGCTCCACGTG
CGTACGGAATTCGCTAGC-GGGCGGGGGTGCTGGGGGAATGGAGTGCTGCGTGCTGCG-GGATCCGAGCTCCACGTG
CGTACGGAATTCGCTAGC-GGGCGGGGGTGCTGGGGAATGGAGTGCTGCGTGCTGCG-GGATCCGAGCTCCACGTG
SELEX Round - 6
CGTACGGAATTCGCTAGC-GGGTTTGGCTGCGGGACTGGCGGCGTATGGAGCTCGGATCCGGGTTGGGCGGGT-GGATCCGAGCTCCACGTG
SELEX Round - 7
CGTACGGAATTCGCTAGC-GGGCAACAAAGAAACACGGGAAGCAACAAAACCGAAAGCG-GGATCCGAGCTCCACGTG
CGTACGGAATTCGCTAGC-ACGAGCGCAACGCAAAACACGCGGCGCGCACCGACACA-GGATCCGAGCTCCACGTG
CGTACGGAATTCGCTAGC-GGGCCGACAACAAAGCCAAAAACAAACACAACAGAGCACA-GGATCCGAGCTCCACGTG
CGTACGGAATTCGCTAGC-GGGCGGAACGTGCCAAACACGAAGAGAAAGAAACAACGCC-GGATCCGAGCTCCACGTG
CGTACGGAATTCGCTAGC-GGGCGGGGGTGCTGGGGGAATGGAGTGCTGCGTGCTGC-GGATCCGAGCTCCACGTG

Fig. 7. Analysis of the sequence information generated in Illumina HiSeq 2500 platform. The PCR amplicons from the 5, 6 and 7 round of SELEX with the recombinant protein were sequenced in Illumina HiSeq 2500 and the data analysed using FASTAptamer toolkit. Note: the presence of the reverse primer sequence in the SELEX amplicon of round 6 (sequence enriched in the top 3 cluster) probably contributing to the observed non-specific amplification. The sequences enriched in the top 5 clusters in SELEX round 7 does not reveal any primer sequence indicating the efficiency of the solution attempted.

ACKNOWLEDGEMENT

The Funding from DBT a (No.BT/PR17667/AAQ/1/661/2016); DST – Nanomission (SR/NM/NS-1004/2015(G)) & Laboratory facility of TANUVAS are acknowledged herewith.

COMPETING INTERESTS

The Authors have Declared that No Competing Interest Exists.

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