J.Natn.Sci.Foundation Sri Lanka 2024 52 (4): 493 - 500
DOI: http://dx.doi.org/10.4038/jnsfsr.v52i4.12070

RESEARCH ARTICLE

Biotechnology

Establishment and evaluation of real time PCR based SELEX platform for the identification of protein binding aptamers: A pilot study in Sri Lanka

AI Abeykoon[1*], KMN Kumarasinghe[1], NV Chandrasekharan[2] and PMTB Wickramasinghe[3]
[1] *Department of Pre-Clinical Sciences, Faculty of Medicine, General Sir John Kotelawala Defence University, Ratmalana, Sri Lanka.*
[2] *Department of Chemistry, Faculty of Science, University of Colombo, Colombo, Sri Lanka.*
[3] *Department of Biochemistry and Clinical Chemistry, Faculty of Medicine, University of Kelaniya, Kelaniya, Sri Lanka.*

Submitted: 01 March 2024; Revised: 26 October 2024; Accepted: 12 December 2024

Abstract: Aptamers are single-stranded DNA (ssDNA) affinity reagents capable of substituting conventional antibodies in processes of molecular recognition. Their higher affinities, lower costs of production and longer shelf lives of aptamers are making them increasingly popular and replacing conventional antibodies in the fields of diagnostics and therapeutics. Systematic evolution of ligands by exponential enrichment (SELEX) is a well-established and efficient technology for generation of aptamers with high affinity against various targets including whole cells, isolated proteins, and small molecules. This study is the first to report efforts in exploring SELEX for isolating protein-binding aptamers using a quantitative PCR and NGS-based approach in a local context. A low-cost selection platform was developed by coating microwell plates with human serum albumin, the target protein of interest, for quantitative and qualitative solid phase immunoassays. Changes in affinity and diversity were monitored through analysis of amplification plots, melt curves, remelt curves and high-resolution melt curve (HRM). Following eight selection cycles, the enriched DNA was subjected to high-throughput sequencing, and the two most abundant sequences were identified. The sequences were evaluated through in-silico binding assays, which resulted in comparable binding affinities expected for aptamers. Among the monitoring techniques, the amplification curve analysis was a valuable tool in understanding changes in pool affinity. Although the melt curve initially lacked sufficient resolution in the early stages of SELEX, the re-melt curve and HRM analysis accurately reflected pool diversity during this time. Thus, we demonstrate that it is feasible to use locally available technology for the successful development of aptamers. This highlights the potential to produce affinity reagents locally on a commercial scale in the future.

Keywords: PCR, protein bindiny aptamers, SELEX platform.

INTRODUCTION

Aptamers are single-stranded deoxyribonucleic acids (ssDNA) or ribonucleic acids (ssRNA) that can bind target molecules with high affinity and specificity. Depending on the sequence of nucleotides, aptamers can fold into unique and distinct three-dimensional conformations, which can bind to target molecules with high affinity and selectivity. Systematic evolution of ligands by exponential enrichment (SELEX) is a well-established technology for generating synthetic DNA and/or RNA molecules with high affinity against various targets, including whole cells, isolated proteins, and small molecules. These designer DNA or RNA molecules are alternatives to antibodies in developing theranostics in biomedical research (Ellington & Szostak, 1990; Tuerk & Gold, 1990).

Aptamers offer significant advantages over antibodies in many aspects. They can be developed against almost any target. Moreover, their high thermal stability, relatively high resistance to both biological and chemical degradation, and low cost of production are just a few

* Corresponding author (isuruabeykoon@kdu.ac.lk; https://orcid.org/0000-0002-2189-1004)

of the many superior qualities, which make aptamers preferable to antibodies (Famuko *et al*., 2007).

Aptamer research is a fast-developing field with an annual revenue valued at USD 151 million in 2021 and projected to reach USD 342 million by 2026 (Markets & Markets, 2022). Despite this significant global trend, Sri Lanka is lagging behind in aptamer research even though the country possesses adequate technology to engage in SELEX and aptamers. This paper discusses the initial efforts and challenges in the establishment of the SELEX platform for the first time in Sri Lanka, paving the way for the future development of a wide array of aptamers with applications in both diagnostics and therapeutics.

MATERIALS AND METHODS

Design and preparation of ssDNA library

The initial library design was based on the work published by Percze *et al*. in 2017. A 50 pmol sample of the library was dissolved in 100 µL of selection buffer and the solution incubated at 95 °C for 5 min in a heat block. The heated library was then immediately cooled by placing it on ice for 15 min. Prior to binding, the library was allowed to equilibrate to room temperature.

Optimization of PCR for amplification of ssDNA library

All PCR reactions were conducted in a volume of 20 µl. An initial denaturing step at 95 °C for 5 min was followed by twenty cycles of the standard PCR protocol, with a denaturing step at 95 °C for 10 s, an annealing step at 63 °C for 10 s, and an extension step at 72 °C for 10 s. A final extension step was performed at 72 °C for 1 min.

The optimized conventional PCR cycling parameters and reagent concentrations/volume were used as a baseline for the optimization of quantitative PCR. Template concentrations ranging from 1µM to 10^{-8}µM were tested.

Optimization of biotin/streptavidin-based ssDNA generation

Following this procedure, streptavidin-coated magnetic Dyna beads (MagSi-STA by Magtivio) were used for the generation of single-stranded DNA.

A 100 µl sample of the PCR product was added to prepared beads and pulse vortexed for 10 s and then incubated at room temperature for 30 min. The solution was then briefly centrifuged, and the supernatant discarded. Next, 250 µl of water was added, pulse vortexed, briefly centrifuged, and the supernatant discarded to remove unbound sequences. The beads were then resuspended in 5 µl of 0.15M NaOH and incubated for 3 min. The supernatant was carefully separated and neutralized with 2.5 µl of 0.3M HCl. The solution was then stored at 4 ºC until further use.

Generation of ssDNA was confirmed through 4% agarose -gel electrophoresis (50V for 60 min).

Selection procedure/target binding assay

A 50 pmol (50 µl of 10 µM) sample of the library was dissolved in 100 µl of binding buffer. The library solution was heat-denatured and snap-cooled in ice for 15 min. Before binding, the library was allowed to equilibrate to room temperature.

The prepared library was added to human serum albumin (HSA) (Sigma Aldrich) coated microtiter plate wells and incubated for 1 h. The supernatant was then removed, and the wells were washed thoroughly three times with 200 µl of PCR grade water to remove any unbound sequences.

Elution of bound sequences was conducted using a Qiagen DNA mini kit with slight modifications described below.

To a sample well, 100 µl of buffer ATL was added and incubated at room temperature for 30 min with occasional vortexing. Next, 100 µl of buffer AL was added and thoroughly mixed by vortexing for 15 s. The mixture was then incubated for 10 min at room temperature. A 200 µl volume of absolute ethanol was added to the well and vortexed for 15 s. The mixture was then placed in a minicolumn provided by the manufacturer and centrifuged at 6000 x g for 1 min. The flow-through was discarded and the column was placed in a new collecting tube. A volume of 500 µl of buffer AW1 was added and centrifuged at 6000 x g for 1 min. The flow-through was discarded and the column was placed in a new collecting tube. Next, 500 µl of buffer AW2 was added and the tube was centrifuged at 20,000 x g for 3 min. The flow-through was discarded and the column was placed in a new collecting tube. Finally, 100 µl of PCR grade water was added, incubated for 1 min and centrifuged at 6000 x g for 1 min. The flow-through was carefully separated and stored at 4 ºC until further use.

Table 1: Stringency changes across SELEX cycles

SELEX Cycle number	Num. of washes	Incubation time (minutes)	Incubating temperature (ºC)	Use of shaking platform
I	1	60	25	No
II	2	45	25	No
III	3	45	45	No
IV	4	30	45	No
V	5	15	45	Yes
VI	6	15	45	Yes
VII	6	10	45	Yes
VIII	6	10	55	Yes

Conducting SELEX cycles

A SELEX cycle involves incubating the prepared library with the target, extracting the bound fraction, PCR amplifying the bound fraction, and generating ssDNA. The stringency of each cycle of SELEX was gradually increased to isolate stronger binders for the target. Stringency improvement was achieved through two main aspects: (i) changes in selection conditions, and (ii) inclusion of negative selection cycles.

Changes in time, temperature parameters, and the number of washes were the main approaches to improving stringency during selection (see Table 1). In addition, as higher cycle numbers were reached, incubation was done on a shaking platform with the frequency gradually increasing with each cycle.

Monitoring SELEX process

Quantification of the bound fraction

A real-time PCR assay was developed to quantify target-bound oligonucleotides from each SELEX cycle. Quantitative PCR was performed on the Rotor Gene Q PCR system with a reaction volume of 20 µl. The reaction mixture consisted of 10 µl of x2 SYBR Green PCR Master mix, 2 µl of 10 µM unlabeled forward and reverse primers, and 2 µl of DNA template (target-bound oligonucleotides washed after each cycle). Two dilutions of 10^4-fold and 10^5-fold per sample were analyzed, each in duplicates for SELEX cycles II-IV. For SELEX cycles 5 and 6, 10^5-fold and 10^6-fold dilutions were used, respectively. The cycling conditions were pre-incubation at 95 ºC for 5 minutes, initial denaturation at 95 ºC for 10 s, annealing at 63 ºC for 10 s, and extension at 72 ºC for 10 s concluded by a final elongation at 72 ºC for 1 min.

Melt curve analysis

PCR-amplified products from each SELEX cycle were subjected to gradual melting in the quantitative PCR system. For analysis, the sample temperature was gradually increased from 50 ºC to 95 ºC at a step rate of 0.3 ºC/min. Changes in fluorescence intensity were monitored continuously, and melting peaks were calculated with the cycler software (Rotor-Gene Q software).

NGS sequencing

The aptamer pools were amplified by PCR and NGS libraries were prepared by nick repair and barcoding using IonXpress™ barcode adapters (ThermoFisher Scientific, USA). The NGS templates of the libraries were prepared by clonal amplification of barcoded libraries using the Ion OneTouch™ 2 System. The amplified libraries were enriched using the Ion One Touch ES enrichment system, and the template positive ion sphere particles were loaded onto an Ion 540 chip and sequenced using the Ion Genestudio S5 system at Genelabs Medical (Pvt) Ltd., Sri Lanka. The Ion Torrent Suite™ software was used for data analysis including the generation of the Fastq file, which was used for further downstream analysis.

In silico target binding assay

3D structures of the first two aptamer sequences derived from the sequencing results were generated using the Builder GUI menu in PyMOL 2.5.4 (http://www.pymol.org). The sense DNA strands were folded using sculpt models and the generated files were exported as PDB files. The plasma-derived human serum albumin was selected as the target protein and its PDB file was downloaded from the protein data bank (5z0b; https://www.rcsb.

org/ structure/5z0b). The sequence TCGTCTGCTCCG TCCAATACCCCCGGCTTTGGTTTAGAGGTAGTT GCTCATTACTTGTACGCTCCGGATGTTTGGTG TGAGGTCGTGC-3' was used as the reference sequence. The ZDOCK server1 was used to identify aptamer-protein binding. The PDB files of the aptamers and target proteins were submitted to the ZDOCK server. All amino acids in the target protein were selected for the analysis, and the probable binding complexes were determined with the ZDOCK score. Further, the likelihood of the sequences being aptamers was measured using the PPAI web server2 and prediction scores were calculated using the optimal threshold value of 0.44 to predict true protein-aptamer pairs.

Comparative wet lab binding assay

A selection platform for ssDNA aptamers was prepared by immobilizing HSA in microtiter plate wells (Nunc-Immuno™ Microtiter™ 96 well solid plates – Thermo Scientific).

An unselected DNA aptamer library was used as the negative control and the HSA binding ssDNA sequence, reported in the literature (referred to as aptamer 1), was used as the positive control.

A 2 µl sample of a 1000 pmol/µl library was dissolved in 98 µl of binding buffer to prepare a 100 µl solution of 20 pmol/µl. The diluted library was then incubated at 95 ºC for 5 min in a heat block and immediately cooled by placing it on ice for 15 min. The library was allowed to equilibrate to room temperature, before binding.

A 200 pmol (20µl of 100 pmol/µl) sample of aptamers 1 (positive control) and 2 (test sequence) were dissolved separately in 80 µl of binding buffer to prepare a 100 µl solution of 20 pmol/µl. The aptamer solutions were then incubated at 95 ºC for 5 min in a heat block and then immediately cooled by placing it on ice for 15 min. Before binding, the aptamer solutions were allowed to equilibrate to room temperature.

The controls and test samples were incubated in the albumin-coated plates on a shaking incubator for 30 min at room temperature.

A 50 µl sample was aspirated from each well and stored at 4 °C until further use. The already optimized real-time PCR assay was used to quantify ssDNA before and after binding.

RESULTS AND DISCUSSION

Optimization of PCR for amplification of ssDNA library

A template concentration of $1x10^{-4}$ µM was identified as the ideal concentration for obtaining a well-defined band on the agarose gel. An annealing temperature of 63 °C produced clear bands after twenty rounds of standard PCR cycles (Figure 1).

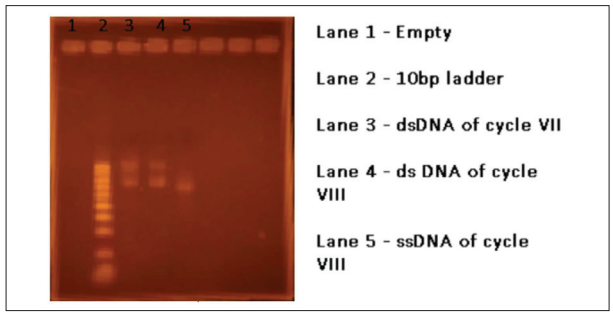

Figure 1: Gel electrophoresis in 4% agarose. Lane 1: empty lane, Lane 2: 10 bp ladder, Lane 3: dsDNA of cycle VII, Lane 4: dsDNA of cycle 8, and Lane 5: ssDNA of cycle 8.

Optimization of quantitative PCR for amplification of ssDNA library

The optimized conventional PCR cycling parameters and reagent concentrations/volume provided efficient amplification in the quantitative PCR setup at template concentrations of $1x10^{-4}$µM (Figure 2).

Melt curve analysis

The melt curve analysis of PCR amplified products from each SELEX cycle (I to VIII) demonstrated a narrowing of peaks and a shift in peak towards higher temperatures indicating a reduction in pool diversity (Figure 3).

In silico target binding assay

Both sequences selected through NGS data analysis yielded comparable ZDOCK scores (Table 2) and interactions with HSA protein (Figure 6). The PPAI server analysis for the two sequences yielded a score of 0.47 predicting the sequences as probable binders for the defined target.

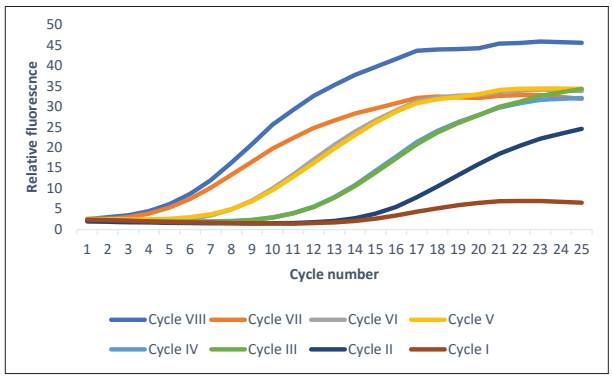

Figure 2: Amplification curves for dilutions of ssDNA library

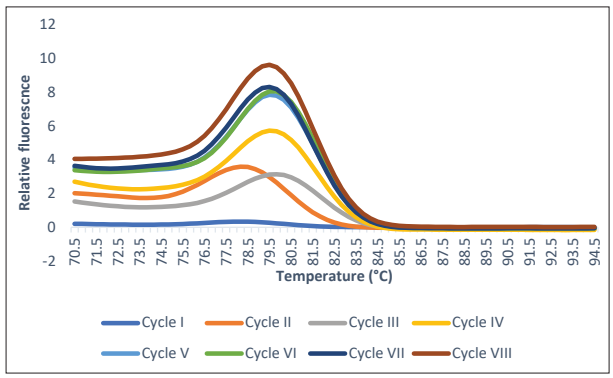

Figure 4: Re-melt curve analysis of SELEX cycles I-VIII

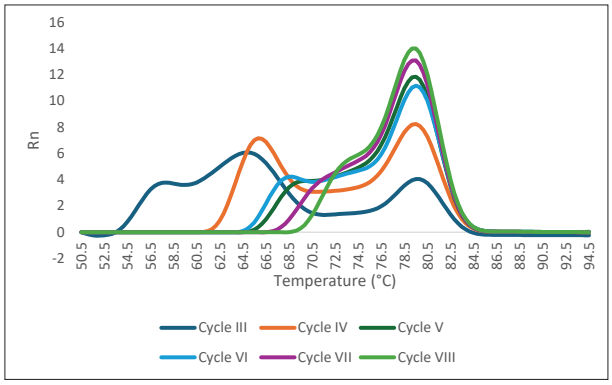

Figure 3: Melt curve analysis of SELEX cycles III to VIII

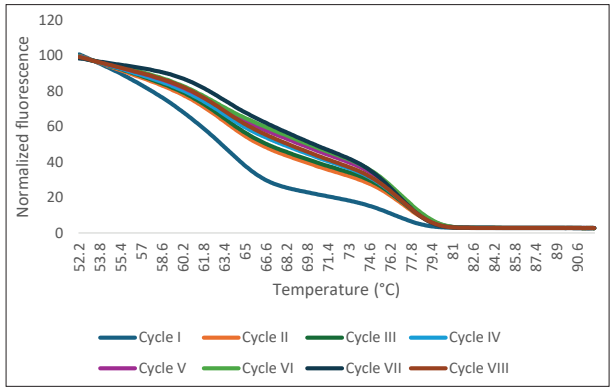

Figure 5: Normalized HRM analysis of SELEX cycles I-VIII

Table 2: Sequences of selected aptamers, their frequencies, ZDOCK, PPAI results

Sequence	Count	ZDOCK score	PPAI results	
			Prediction result [a]	Prediction score [b]
Seq 1 AGAGGAAAGCGGAGGCGTAGTGGTT	123	1067.844	Yes	0.47
Seq 2 CCCATAGAGAGGAAAGCGGAGGCGTAGTGGTT	85	1190.363	Yes	0.47

[a] Prediction result ('yes' represents that it is an aptamer, and 'no' represents that it is not an aptamer)

[b] Prediction score (the probability that the sequence is an aptamer)

Wet lab analysis

The respective Ct values obtained for original and post incubation ssDNA are presented in Table 3.

The DNA concentrations were calculated using a standard curve (Supplementary Figure 1) generated for serial dilutions of the ssDNA library. The threshold cycle numbers from three independent experiments were averaged, and the standard deviation was calculated for each concentration analyzed (Table 4).

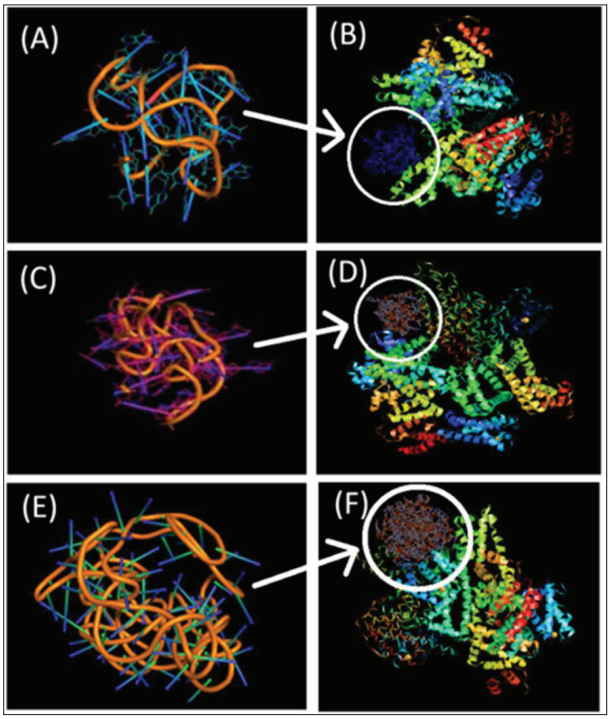

Figure 6: 3-D modelling and ZDOCK interaction of selected DNA sequences with target protein HSA.
(A) scrambled unrestricted structure of aptamer sequence 01
(B) ZDOCK interaction of sequence 01 with albumin
(C) scrambled unrestricted structure of aptamer sequence 02
(D) ZDOCK interaction of sequence 02 with albumin
(E) scrambled unrestricted structure of control sequence
(F) ZDOCK interaction of control sequence with albumin.

Table 3: DNA concentration and Ct values for samples at pre-incubation and post-incubation

Series number	Content	Ct	ΔCt
1	Positive control/Aptamer 1 (Pre incubation)	22.531	~3
2	Positive control/Aptamer 1 (Post incubation)	25.421	
3	Test sequence/Aptamer2 (Pre incubation)	18.737	~1
4	Test sequence/Aptamer 2 (Post incubation)	19.884	
5	Negative control/Library (Pre incubation)	15.711	~0
6	Negative control/Library (Post incubation)	15.593	

Table 4: Average threshold cycles and standard deviation for ssDNA library dilutions

DNA concentration (μM)	Negative logarithmic derivative of concentration	Average Ct'	SD
$1x10^{-8}$	8	19.40	6.6
$1x10^{-7}$	7	14.27	1.1
$1x10^{-6}$	6	12.20	2.9
$1x10^{-5}$	5	11.10	4.0
$1x10^{-4}$	4	10.34	3.8

Monitoring SELEX is crucial to ensure the isolation of aptamer sequences with high affinity and specificity towards a defined target. In this experiment, an aptamer library was enriched towards human serum albumin through eight cycles of SELEX. The process utilized real-time PCR-based approaches for both amplification and monitoring. Amplification plots were considered in affinity assessments of aptamer pools from cycles I to VIII. The first reported use of quantitative PCR in SELEX monitoring was by Adali *et al*. (2013) where the selected fraction of aptamers in every cycle of SELEX was quantified. The optimized qPCR allowed for the detection of aptamers ranging from 5 pg to 0.02 pg with a 98.7% efficiency (Adali *et al*., 2013). In contrast, comparison studies by Mencin *et al* (2014) using non-saturating SYBR Green based quantification revealed some significant drawbacks of the assay. The higher affinity of SYBR Green towards AT compared to GC resulted in a low copy number when selection was directed towards GC-rich motifs enrichment. This effect was even more prominent in the latter cycles of SELEX giving a fourfold difference compared to the fluorescence-based assay results. This study highlights the usefulness of a saturation dye for ssDNA quantification in SELEX experiments (Mencin *et al*., 2014). Our experiment targeting HSA used saturating SYBR green (Promega) for the generation of amplification signals since the final sequence was not known at the start of the experiment. The initial cycle of SELEX is known to yield minimal sequences demonstrating a drastic reduction in DNA quantity (Mencin *et al*., 2014; Song *et al*., 2017). Our findings were in agreement with this, demonstrating extremely low bound fractions in SELEX cycle I. However, from cycles II to VIII, a gradual increase in the bound fraction was observed, reflecting a gradual increase in the pool's affinity towards the target.

Dynamics of diversity changes across the SELEX process were evaluated through melt curve, re-melt curve, and HRM analysis. Melt curve analysis is frequently used to assess RT-PCR amplicon length and product purity in dye-based RT-PCR assays. The melt profile and dissociation kinetic properties of double-stranded DNA depend on factors such as sequence composition, length and concentration of products. This approach has been explored as a monitoring tool for SELEX to determine reductions in diversity across advancing selection cycles. Theoretically, a near Gaussian peak is expected for the melting profile of the library, where narrowing of the peak is expected with advancing cycles due to the reduction of diversity. In a comparison study of monitoring approaches, Mencin *et al*. (2014) observed this phenomenon starting with a library of a normal distribution, with a gradual reduction of the peak width occurring up to cycle four. In subsequent cycles, well defined melting peaks were observed and the peaks of cycles seven and nine were almost super imposed, reflecting the convergence of library diversity towards a specific sequence. Our experiment targeting HAS used melt curve analysis, which revealed a gradual convergence of melt profile peaks towards a specific temperature. A bimodal distribution was observed in SELEX cycles III-VIII as exemplified in many previous reports of melt curve analysis (Mencin *et al*., 2014; Luo *et al*., 2017; Song *et al*., 2017). A gradual increase in peak temperature was observed across SELEX cycles confirming a reduction in pool diversity.

Vanbraband *et al*. (2014) reported the use of melting curve analysis after a short re-annealing step known as remelt curve analysis (rMCA) as a useful tool for diversity analysis in enrichment monitoring. Conventional melt curves of the amplified products were obtained by increasing the temperature from 60 °C to 95 °C. Reannealing was performed at 70 °C for 1 minute followed by a second melting analysis (remelting) from 70 °C to 95 °C at 0.5 °C/s. During this stringent re-annealing phase, both hetero- and homoduplexes were formed with their proportions depending on the sequence diversity. As the diversity of SELEX pools decreased, more homoduplexes were observed in the reannealing phase shifting the obtained rMCA melting temperatures and providing crucial information on enrichment in terms of diversity (Vanbraband *et al*., 2014). In contrast to Vanbraband's findings our study in the temperature range of 70 °C to 95 °C revealed a single peak. However, across SELEX cycles a clear shift in peak temperature was observed reflecting pool enrichment. These changes were evident in the initial stages of SELEX highlighting the importance of remelt curve analysis as a monitoring tool over conventional melt curve analysis.

HRM analysis is an advanced version of conventional melt curve analysis where sequence-related melting profiles revealing single nucleotide changes are generated (Farrar *et al*., 2017). Unlike the classical melt curve, HRM analysis monitors melting differences in temperature gaps of less than 0.5 °C, generating a comparatively higher density of data points per 1 °C. Such enriched data can provide information on subtle differences in sequences. The use of HRM analysis and the availability of HRM data on the library behavior across SELEX cycles are sparse. This experiment presents early reports on using HRM analysis as a monitoring tool. Melting profile changes were observed even in the early stages of SELEX with HRM, whereas conventional amplification plots or melt curve analysis failed to detect such early changes. A gradual shift in the melting profile further reflected the gradual enrichment of the library pool.

The affinity was monitored based on unbound fraction determination. This method of affinity determination was employed to avoid practical limitations associated with determinations of the bound fraction of aptamers. Such bound fraction quantifications require the separation of the aptamer-target complex from the binding platform and/or isolation of DNA from the target, both of which carry risks of yielding lower quantities of aptamer, thereby affecting the final affinity calculations. In contrast, determination of original and unbound aptamer concentrations could be conducted by directly sampling the original and unbound solutions, thereby allowing for a more accurate measure of affinity.

The ssDNA library used as the negative control produced similar Ct values for both initial incubating and unbound fractions implying that negligible binding has occurred and that the concentration of the initial incubating DNA and final unbound fractions remained consistent. The positive control in this experiment had a Ct of 22.5 (rounded to the first decimal) for the initial incubation concentration. The Ct value for the post-incubation eluted sample was 25.4, resulting in a Ct difference of nearly 3, suggesting that a significant amount of the initial incubating DNA had bound to the coated well. The Ct difference for the test sequence used was 1.2 (Table 3) indicating a significant binding of the test sequence compared to the negative control.

The first round of NGS on the aptamer library selected from the final SELEX cycle identified 540 potential sequences of aptamers. Among these, there were 432 unique sequences each appearing once, 36 unique sequences appearing twice, and approximately 40 sequences appearing three or more times (see Supplementary Table 1). This is consistent with previous

literature on NGS results from final SELEX cycles targeting various protein targets where over ≈100 different sequences have been identified as potential binders (Schütze *et al*., 2011; Stoltenburg & Strehlitz, 2018; Cho *et al*., 2010). Two sequences with the highest frequencies were selected for further in silico testing.

Both sequences yielded comparable ZDOCK scores, with the probability of the selected sequences being an aptamer identified as 0.44 for each. The PPAI server predicted the two sequences as probable.

CONCLUSION

The PCR system allows for both pool amplification and monitoring of SELEX, simultaneously. Among the monitoring techniques, the amplification plot analysis has revealed a valuable tool in understanding pool affinity changes. Additionally, melt curve, re-melt curve and HRM analysis have provided insights into pool diversity across SELEX. Although the melt curve lacked sufficient resolution during the initial stages of SELEX, the re-melt curve and HRM analysis accurately reflected pool diversity changes during these early stages. Sequencing results from NGS analysis reflected this enrichment of the aptamer library, resulting in a binding sequence with comparable binding affinity.

Therefore, the authors suggest the possibility of using local resources to identify protein binding DNA aptamers. They further recommend improvements to the SELEX procedure by utilizing NGS analysis during the early cycles to achieve better selection. Finally, they suggest further characterization of selected sequences through association/dissociation kinetic assessments.

REFERENCES

Aiswarya, P. U., Gopika, R., Jinju, J., Malavika, M. K., Franklin, J., Jinu, G. (2023). Aptamers: Features, synthesis and applications. *Chemistry and Biodiversity, 20*(10). https://doi.org/10.1002/cbdv.202301008

Avci-Adali, M., Wilhelm, N., Perle, N., Stoll, H., Schlensak, C., & Wendel, H.P. (2013). Absolute quantification of cell-bound DNA aptamers during SELEX. *Nucleic Acid Therapeutics, 23*(2), 125–30. https://doi.org/10.1089/nat.2012.0406

Cho, M., Xiao, Y., Nie, J., Stewart, R., Csordas, A.T., Oh, S.S. (2010). Quantitative selection of DNA aptamers through microfluidic selection and high-throughput sequencing. *Proceedings of the National Academy of Sciences of the United States of America, 107*(35), 15373–8. https://doi.org/10.1073/pnas.1009331107

Ellington, A.D., Szostak, J.W. (1990). In vitro selection of RNA molecules that bind specific ligands. *Nature*, *346*(6287), 818–22. https://doi.org/10.1038/346818a0

Famulok, M., Hartig, J.S., Mayer, G. (2007). Functional aptamers and aptazymes in biotechnology, diagnostics, and therapy. *Chemical Reviews*, *107*, 3715–43. https://doi.org/10.1021/cr0306743

Farrar, J. S. & Wittwer, C. T. (2017). High-resolution melting curve analysis for molecular diagnostics. In G. P. Patrinos (Ed.), *Molecular Diagnostics* (3rd ed., pp. 79-102). Academic Press.

Luo, Z., He, L., Wang, J., Fang, X., Zhang, L. (2017). Developing a combined strategy for monitoring the progress of aptamer selection. *Analyst*, *142*(17), 3136-3139. https://doi.org/10.1039/c7an01131h

Markets and Markets. (2022). Aptamers market revenue trends and growth drivers. https://www.marketsandmarkets.com/Market-Reports/aptamers-technology-market-1167.html

Mencin, N., Šmuc, T., Vraničar, M., Mavri, J., Hren, M., Galeša, K. (2014). Optimization of SELEX: Comparison of different methods for monitoring the progress of in vitro selection of aptamers. *Journal of Pharmaceutical and Biomedical Analysis*, *91*, 151–159. https://doi.org/10.1016/j.jpba.2013.12.031

Schütze, T., Wilhelm, B., Greiner, N., Braun, H., Peter, F., Mörl, M. (2011). Probing the SELEX process with next-generation sequencing. *PLoS One*, *6*(12), 1–10. https://doi.org/10.1371/journal.pone.0029604

Song, M.S., Sekhon, S.S., Shin, W.R., Kim, H.C., Min, J., Ahn, J.Y., Kim, Y.H. (2017). Detecting and discriminating shigella sonnei using an aptamer-based fluorescent biosensor platform. *Molecules*, *22*(825). https://doi.org/10.3390/molecules22050825

Stoltenburg, R., Strehlitz, B. (2018). Refining the results of a classical SELEX experiment by expanding the sequence data set of an aptamer pool selected for protein A. *international journal of molecular science*, *19*(2), 642. https://doi.org/10.3390/ijms19020642

Tuerk, C., Gold, L. (1990). Systematic evolution of ligands by exponential enrichment. RNA ligands to bacteriophage T4 DNA polymerase. *Science*, *249*(4968), 505–510. https://doi.org/10.1126/science.2200121

Vanbrabant, J., Leirs, K., Vanschoenbeek, K., Lammertyn, J., Michiels, L. (2014). reMelting curve analysis as a tool for enrichment monitoring in the SELEX process. *Analyst, 139*(3):589-595. https://doi.org/10.1039/c3an01884a