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Review Article

Aptamer Selection: Choosing the Appropriate SELEX

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ABSTRACT

Aptamers are oligonucleotides that have an innate ability to bind to various target molecules such as small molecules, toxins, oligonucleotides, proteins and even whole cells with high affinity and specificity. They emerged as strong rivals to the most commonly used biomolecules in the past, antibodies. The aptamer selection process involves evolution of ligands by iterative affinity assays termed as SELEX (Systematic Evolution of Ligands by Exponential Enrichment). This is a tedious task and efforts are being directed to simplify the process and at the same time reduce the turnaround time. Several variants have been designed to overcome the limitations of conventional SELEX process. This review provides sufficient information to a naive researcher for choosing and designing appropriate strategy for selection of aptamers against target of interest.

Keywords: *Aptamers, SELEX, Target, Affinity.*

INTRODUCTION

The ever increasing demand for ligands in point of care tests for clinical diagnosis, food testing, environmental monitoring, drug abuse and in therapeutics has made the search for novel molecules to boom. Aptamer technology has immense potential for providing new tools. Aptamers are short oligonucleotides (ssDNA or RNA) 20-80 nucleotides in length. These bind to their target with high affinity and specificity due to shape complementarity or cumulative effect of non-covalent interactions. Aptamers can be selected for virtually any target (whether immunogenic or not), are stable at transient temperature and do not show batch to batch variation. These characteristics provide an edge to aptamers over antibodies, the molecules currently governing the market. However, researchers world over are showing tremendous interest in aptamers and their potential in varied fields is being tested. The upsurge of interest in aptamer research has led to designing of database (http://aptamer.icmb.utexas.edu/) which provides up-to-date information of the existing aptamers.

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Aptamers are selected by a process termed Systematic Evolution of Ligands by Exponential enrichment (SELEX). It involves selection of a few ssDNA or RNA molecules binding specifically to target with high affinity from a large pool of such molecules. The great deal of work done in this field has led to improvements in the conventional SELEX to either simplify or expedite the process or to suit a particular target or as per the usage of the aptamers. The selection process involves repetition of affinity assays leading to enrichment of pool with high affinity binders with each assay. In this review we intend to provide a detailed description of SELEX and its variants which will allow a naive researcher to choose an appropriate strategy.

2. SELEX

An important requirement for SELEX is an oligonucleotide (ssDNA or RNA) library. The oligonucleotide library consists of ssDNA or RNA molecules about 20-80 nucleotides long. The initial oligonucleotide pool (IOP) which includes 10^{13}-10^{15} different oligonucleotides (each of which has a unique sequence) is often called combinatorial library. SELEX employs the use of combinatorial library for rapid selection of the ligands that have appropriate binding affinity for a given molecular target (Tuerk & Gold, 1990). Aptamers are selected by the process of affinity selection (with the target) carried out iteratively to select high affinity binders over low affinity binders.

2.1 ssDNA aptamer selection

The basic steps of DNA SELEX are outlined in Figure 1. Selection of aptamers start with ssDNA library consisting of 10^{13} to 10^{15} oligonucleotides (James, 2000) each having a unique sequence. Each sequence of the ssDNA library has a random region of 20-80 nucleotides in the middle flanked by constant regions of 18-21 nucleotides long at the ends (Figure 2). The random region defines the affinity for the target whereas the constant regions are meant for primer annealing which allow amplification in the subsequent steps.

Selection is designed such that (few) strong binders are selected from a large random pool of oligonucleotide library. The first step is incubation of oligonucleotide library with the target to allow their interaction. Target could be incubated with the library in free solution (Tuerk & Gold, 1990) or it could be immobilized onto a solid support and then incubated with the library (Majerfeld et al., 2005 & Wallace & Schroeder, 1998).

Oligonucleotides having affinity towards the target bind to it. The next step is partitioning which involves separation of bound oligonucleotides from the unbound ones. This is done by nitrocellulose filter when target is incubated with the library in free solution while unbound oligonucleotides are removed by washing with buffer when the target is bound to solid support. After this step, oligonucleotides bound to target are eluted by heat denaturation (Rhodes et al., 2000) or by use of urea (Low et al., 2009) or both (Stoltenburg, et al., 2005 & Niazi et al., 2008) or by affinity elution with target (Kato et al., 2000). The bound and unbound oligonucleotides are quantified for assessment of SELEX procedure to determine the number of rounds to be conducted to obtain aptamers. Recent protocols describe the use of fluorescent labels (Stoltenburg et al., 2005 & Mann et al., 2005) and radioactive labels (Kiga et al., 1998 & Kwon et al., 2001) to make quantification easy and sensitive while conventional method requires measurement of O.D. at 260 nm (Kato et al., 2000 & Meli et al., 2002). The latter is a simple technique and is still in practice. The oligonucleotides obtained after elution are very few as compared to the initial library. Therefore, pool obtained after selection consisting of both low affinity and high affinity binders is further enriched by more rounds of selection. For this, oligonucleotides are amplified by PCR and dsDNA is obtained. Since ssDNA is required for further selection, the strands of DNA are separated in the subsequent step. This can be carried out by using modified primers that make two strands differ in length and thus allow separation by denaturing PAGE (Figure 3) or by using streptavidin-biotin system. In the former case, the primer for undesired strand is designed to have a poly A lengthener

(hexaethylene glycol) followed by a terminator so as to end the amplification of the desired strand at the terminator while the undesired strand has extra adenine residues thus both the strands differ in length and can be separated on denaturing PAGE gel (terminator lengthener method) described in Figure 3a. Alternatively, ribonucleotide moiety is introduced at 3'end of primer corresponding to undesired strand to allow its cleavage by alkaline hydrolysis and subsequent separation in denaturing PAGE (Navani et al., 2009 & Walder et al., 1993) (Figure 3b). In streptavidin-biotin system, one of the strands is biotinylated and the two strands are separated on streptavidin coated surface upon denaturation (Naimuddin et al., 2007). In addition, asymmetric PCR can be employed wherein the desired strand is enriched by using primer for desired strand in larger amount[6]. Another method for production of ssDNA is using lambda exonuclease which degrades the strand phosphorylated at 5'-end (Citartan et al., 2011) as given in figure 4.

A comparison between modified primer (unequal primers) and streptavidin biotin methods for ssDNA production was experimentally made by Liang et al. 2015 these methods were found to produce almost equivalent recovery of ssDNA but the cost effectiveness of the former method makes it better than the latter. Snap cooling method for obtaining ssDNA from dsDNA is perhaps the simplest method as it requires heating of dsDNA at 85^0C for 15 min. or 95^0C followed by cooling on ice (Chen et al., 2009) prior to incubation with target.

Several rounds of SELEX are used for selecting the aptamers with high affinity. The stringency of the selection is increased in further rounds of SELEX for enrichment of the library with the high affinity binders and this can be done by increasing washings, decreasing incubation time or varying the amount of target or library (Cruz-Aguado & Penner, 2008). Besides this, rounds of counter selection (Niazi et al., 2008) and/or negative selection (Wang et al., 2009) can be introduced. Counter selection is done with structural analogs of target and the binding oligonucleotides are discarded. Negative selection is done with the solid naked support to remove the oligonuleotides that have affinity for the support that forms the background.

2.2 RNA SELEX: RNA SELEX differs from DNA SELEX at several steps (Figure 5). For the selection of RNA aptamers, the random ssDNA library has to be transformed into a RNA library before starting the first round of an RNA SELEX process. For this purpose, either the T7 promoter is added within the constant region or a special sense primer with an extension at the 5' end containing the T7 promoter sequence is used. In the former case, ssDNA library is directly converted to RNA library whereas in latter case, amplification by PCR is done with special sense primer with an extension at the 5' end containing the T7 promoter sequence and an antisense primer to get dsDNA and this dsDNA is then transcribed *in vitro* by T7 RNA polymerase resulting in a randomized RNA library. The RNA pool selected after each round is reverse transcribed and amplified. The new RNA pool for the next SELEX round is then generated by *in vitro* transcription (Tombelli et al., 2005). The remaining steps are similar to DNA SELEX.

2.3 Cloning, sequencing and characterization of enriched aptamer pool

After iteratively executing the rounds of SELEX, a stage arrives where affinity of the target binding candidates cannot be increased further. At this stage, the enriched pool is cloned into an appropriate vector and then sequenced (Stoltenburg et al., 2007). Characterization of aptamers is very important before applying them to therapeutic or diagnostic use. For this, dissociation constants and specificity of the selected aptamers is determined. Aptamers with dissociation constants in low nanomolar range or in picomolar range are desirable. Advent of high throughput sequencing has allowed analysis of several sequences without the need of cloning (Cho et al., 2010 & Schütze et al., 2011). Besides this, it allows assessing enrichment and picking up the sequences that the being

selected preferentially. Thus, this reduces selection time by reducing the selection rounds and resources. But all the laboratories are no equipped with the facility.

Variants of SELEX

Different variants of SELEX have been described in literature either to expedite the selection process or to select aptamers with higher affinity and specificity or to serve a special purpose. Choosing appropriate strategy for selection of aptamers requires consideration of a number of factors including the type of target, available facilities, time, prospective use of the selected aptamers etc. A brief illustration for making a choice among available methods is given in figure 6.

3.1 Toggle SELEX

Species cross-reactivity is desirable for therapeutic applications and efficacy of drugs is checked by conducting trials on experimental animals and then on humans so these drugs (which are aptamers here) must be able to recognize homologous proteins of both the species. This is the reason that most of the potential therapeutic molecules that work efficiently *in vitro* fail to enter clinical trials because these lack efficacy *in vivo*. To overcome this problem a selection strategy that allows the generation of therapeutic agents that are cross-reactive between species has been described by White et al. and is shown in figure 7. RNA aptamers capable of binding to both human and porcine thrombin were generated by conducting selection against human and porcine thrombin in alternating rounds of SELEX. This method provides cross-reactive therapeutic aptamers. In the similar way aptamers capable of recognizing several aminoglycosides were generated by toggling in consecutive rounds of SELEX (Derbyshire et al., 2012). However, it is important to remember that affinity may be compromised while attempting to promote cross-reactivity.

3.2 Capillary electrophoresis SELEX

Tang et al. introduced capillary electrophoresis (CE) as a separation approach for SELEX procedure (Figure 8). The high efficiency of capillary electrophoresis shortens the selection process. In this method, the nucleic acid library is incubated with the target in a vial which is then injected into the capillary and separated by CE, regardless of size or sequence. Oligonucleotides bound to the target travel through the capillary at a rate different from unbound oligonucleotides, allowing separation of bound oligonucleotides from unbound. These are collected as separate fractions thus making partitioning easy. This method requires fewer rounds of selection. A variant of this method termed polymer enhanced capillary transient isotachophoresis has been developed by Saito et al. This group achieved the selection of high affinity aptamers for microbes (*E. coli* and *S. cerevisiae*) in a single round. Initially, the targets for CE-SELEX were only large proteins but later on this was successfully used for small molecules like N-methyl mesoporphyrin (Yang & Bowser, 2013). The limitation of this method is that it requires technical expertise.

3.3 FluMag SELEX

FluMag-SELEX (Figure 9) process as the name suggests uses fluorescent labels for DNA quantification and magnetic beads for target immobilization (Stoltenburg et al., 2005 & Mann et al., 2005). The fluorescent labeling of the selected oligonucleotides makes quantification easier which helps in monitoring the enrichment of the target specific aptamers. Advantage of using magnetic beads is that the quantity of target required for immobilization is small and thus, is method of choice for expensive targets. Further, the magnetic separation technology is a fast and easy method.

3.4 Cell SELEX

Cell SELEX is performed to achieve selection of aptamers for a particular cell type (eukaryotes/prokaryotes) and this is usually done when there is no knowlwdge of cell surface markers. This has been achieved for several bacterial cells (Mozioglu et al., 2015 & Hamula et al., 2016) and cancer cells (Wu et al., 2018 & Graham & Zarbl, 2012). Several variations of this method have been tried viz. Target expressed on cell surface-SELEX or

TECS-SELEX (Ohuchi et al., 2006) Fluorescence activated cell sorting SELEX or FACS-SELEX (Mayer & Ahmed, 2010), Specific target cell SELEX or STACS-SELEX (Meyer et al., 2013) and Sequential Toggle cell SELEX or STC-SELEX (Song et al., 2017). The success rate of cell-SELEX is only 60% but is the method of choice when no information regarding marker is available.

3.5 Genomic SELEX

In genomic SELEX, selection is achieved by conventional method with difference lying only in the library used for selection (Lorenz et al., 2006). Here the variable region of library is derived from the genome of an organism. The sequences displaying highest affinity towards a protein are selected thereafter by SELEX. This method is best suited for DNA interacting proteins.

3.6 GO- SELEX

GO- SELEX, here GO refers to Graphene oxide which is used to increase the stringency of selection. It is added to incubation mixture of library and target or to library before addition of target. The ssDNA molecules absorb to GO by π-π Stacking interaction. The adsorption increases competition and only oligonucleotides with high affinity will interact with target in presence of GO. The increase in stringency of selection helps fasten the selection process. This modification of SELEX was employed for selection of aptamers for okdaic acid (Gu et al., 2016), serpin (Ahmad Raston & Gu, 2015).

3.7 Minimal Primer and Primer free SELEX

ssDNA/RNA molecules fold to form secondry/tertiary structures to interact with the target. The constant regions are therefore also involved in interaction with the target during selection but further truncation to decrease the size of aptamers may lead to unpredictable changes in the affinity and/or specificity of the aptamers. This adds to task of finding the best aptamer after truncation. To overcome this shortcoming, Minimal Primer and Primer free SELEX have been described by Pan et al. In these methods, the starting selection libraries contain a central random sequence but the constant regions may or may not be present. The random region is flanked by only 2 nucleotides on each side in minimal primer method whereas by either a 2- or 0-nucleotides overhang on the 3′end in primer-free method. The advantage of this method is that the selection rounds are performed with DNA oligomers which are generally employed as end product aptamers i.e without the primer regions. The primers are regenerated after selection. The selected oligomers are then amplified and primers are again eliminated before next selection. Disadvantage is that the method requires unique library designing with digestion sites. Steps of hybridization and ligation are added after each selection cycle.

3.8 Fixed region elimination SELEX

This method was devised to avoid interference from the fixed/constant regions by blocking them with complementary sequences before selection. Ouellet et al. explored this method to select aptamers whose function or binding with the target is solely because of the random region. This prevents any loss of the tightly binding molecules because of interference from the constant or fixed regions. The advantage of this method over Minimal Primer and Primer free SELEX is that there is no requirement of special library and the steps of selection remain essentially the same as conventional SELEX except for the additional blocking step (of fixed regions).

3.9 Capture SELEX

Small organic molecules are difficult targets for aptamer selection by SELEX. These cannot be easily immobilized on solid support which is usually required. This SELEX allows selection of aptamers for small targets. A special SELEX library is used which has a docking sequence in the random region meant for hybridization to a complementary oligo fixed on magnetic beads. Oligonucleotides exhibiting high affinity to the target bind to it and are consequently released from the beads during the aptamer selection process. Based on this Capture-SELEX procedure, the successful DNA aptamer selection for the aminoglycoside antibiotic kanamycin A, a

small molecule was described by Stoltenburg et al. and Paniel et al.

3.10 MAI-SELEX (Multivalent aptamer isolation- SELEX)

This variant of SELEX was developed by Gong et al. This selection method is used to generate aptamers with affinity towards different proteins on the same target. It provides aptamer pairs, each member of which recognizes different sites on the target and thus, can be used in sandwich assays. These set of aptamers can greatly enhance the success of sandwich assays for detection of whole cells. This method is described in Figure 10.

3.11 AFM –SELEX (Atomic force microscopy based SELEX)

This method developed by Miyachi et al. exploits the detection of affinity force between sample surface and a cantilever by atomic force microscopy for assessment after each round. Further, the enrichment is attained in few rounds. This method led to selection of aptamers for thrombin in merely three rounds and these exhibited higher affinity than those obtained by conventional SELEX. This method makes aptamer selection fast and efficient but requires equipped laboratory to carry out selection by AFM-SELEX and also trained personnel.

3.12 AEGIS SELEX (artificial expanded genetic information systems)

In this method, unnatural form of DNA was used as starting DNA library. The DNA library had four standard nucleotides A, T, G, C and two nonstandard nucleotides Z and P, the process of SELEX remaining the same. The use of such a library increased sequence diversity and provided more functional groups for interaction with the target. Sefah et al. have successfully tried this method. Special methods are required at different steps i.e. synthesis of library, amplification after each selection and sequencing.

3.13 SOMAmers

SOMAmers (slow off rate modified aptamer) were developed by Somalogic, USA. These are aptamers with modified base which is a dU residues functionalized with t protein-like moieties at the 5-position. Modification of one of the four bases adds to the diversity of library and also resistance to nucleases in *in vivo* applications. These were designed in an attempt to increase the success of selection of aptamers against any difficult protein target against which conventional SELEX has failed.

3.14 Click SELEX

Click SELEX was described by Pfeiffer et al. Like SOMAmers, this variant also employs modified bases. Click Chemistry (copper catalyzed alkyne azide cycloaddition) is used to modify the nucleotides. 5-ethynyl deoxyuridine is incorporated onto the library which can be modified using click chemistry before interaction with target. This modification enhances the interacting capabilities of the oligonucleoties with the target. The modifications are removed during amplification therefore, no specialized amplification methods are required.

3.15 In silico selection of aptamers

In silico approach was tested by Chushak and Stone. They used the in silico approach to reduce the number of sequences in the library for selection of aptamers *in vitro* accelerating the selection process. In this approach, RNA sequences were screened at two levels. Firstly, secondary structures of RNA sequences were generated and sequences were selected based on these structures. The selected sequences were not target specific. At the second level, RNA molecules that could bind to a specific target were identified by computational docking. These selected structures were finally used for selection of aptamers *in vitro.* The computational approach allows selection of sequences from the initial library and leads to a reduction in number of sequences used for final selection and thus the bench work.

3.16 Automated SELEX

Automated SELEX employs use of robotic stations with automated pipetting systems wherein all the steps of SELEX rounds can be conducted without any intervention by the worker. Several rounds can be conducted in continuation. Automated systems can handle multiple targets efficiently since they can be processed in parallel. Eulberg et al. selected

RNA aptamers against the mirror image configuration (D-peptide) of substance P, the so called Spiegelmer, using an automated system. This requires set-up of automated work stations not feasible for all laboratories.

4. Limitations of aptamers

SELEX provides a very promising method of obtaining ligands with wide range of applicability but it has some drawbacks. The cost incurred in selection procedure is high as the reagents and also some targets (required in pure form) used in selection process are expensive. Further, it is a time consuming and a tedious process. Cross reactivity of aptamers is another issue although it can be minimized by counter selection but this sometimes leads to loss of high affinity aptamers. Aptamer selection is a random process and it gives highly variable outcomes even when carried out under identical conditions (Spill et al., 2016).

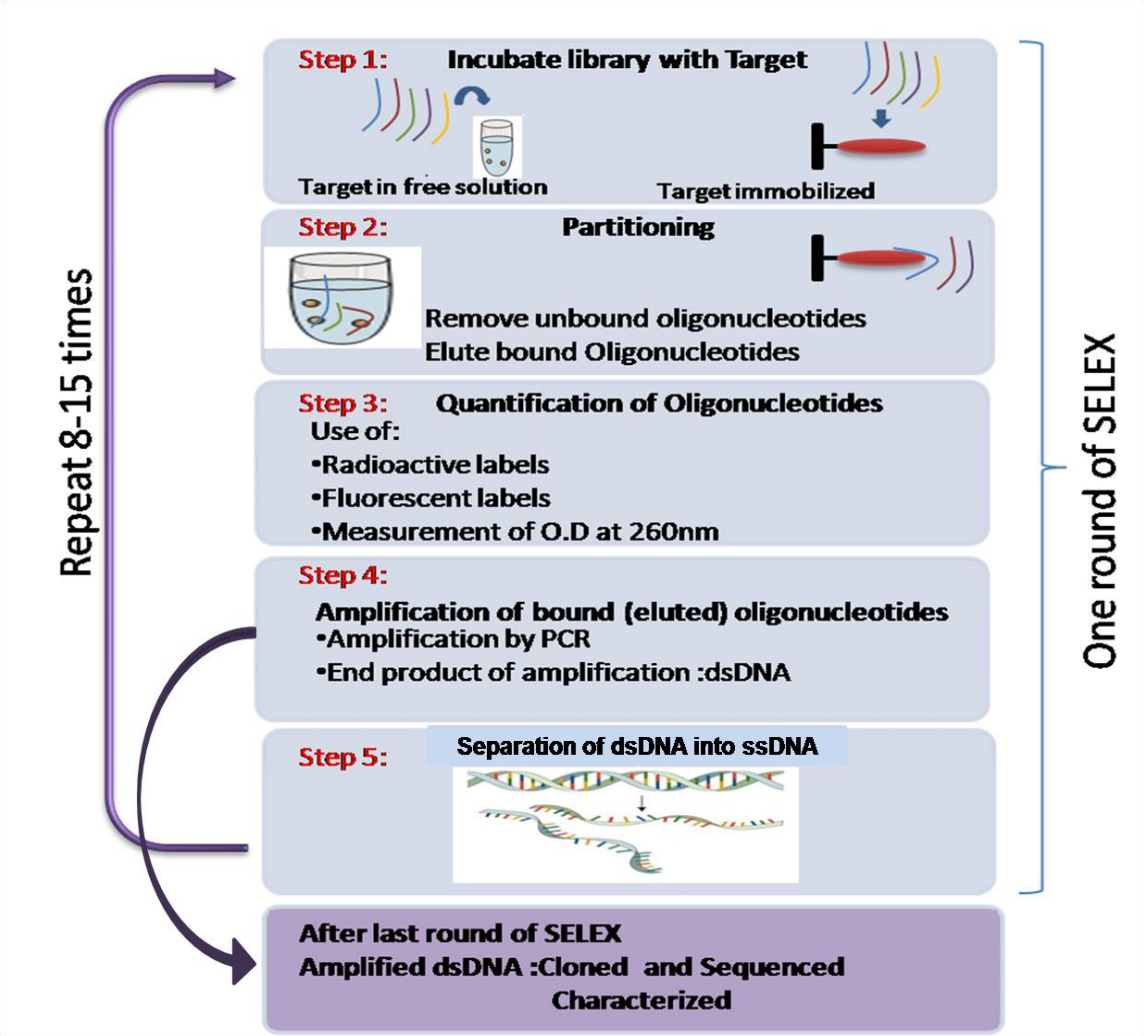

Figure 1: ssDNA SELEX

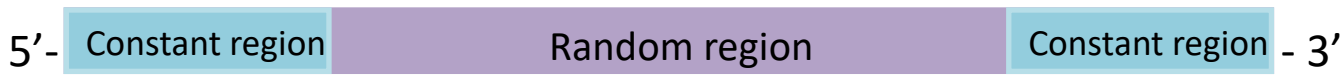

Figure 2: Design of sequences in the ssDNA library

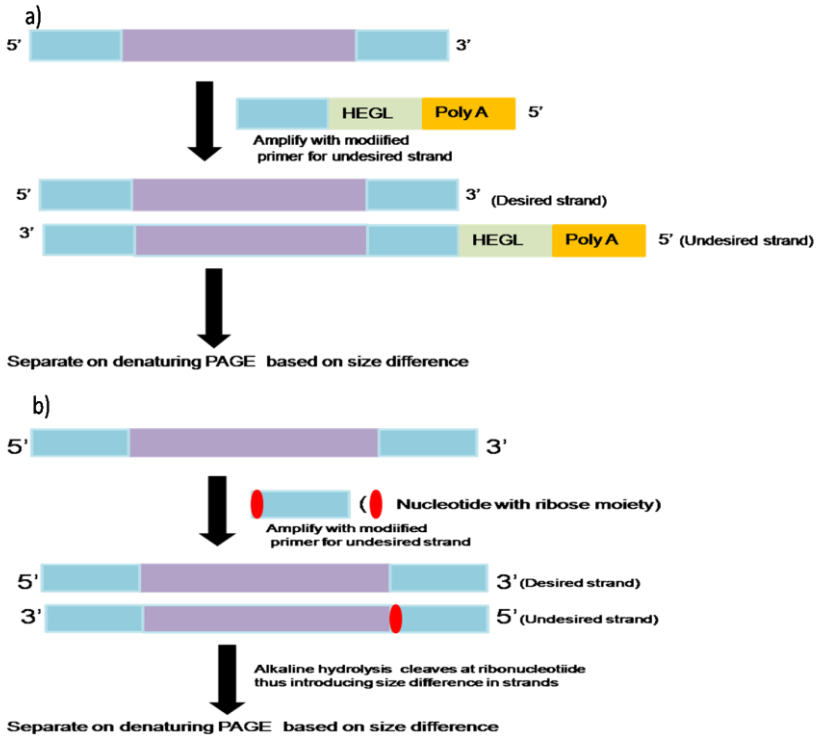

Figure 3: ssDNA separation methods by using modified primers: a) Terminator lengthener method, b) Detachable primer method.

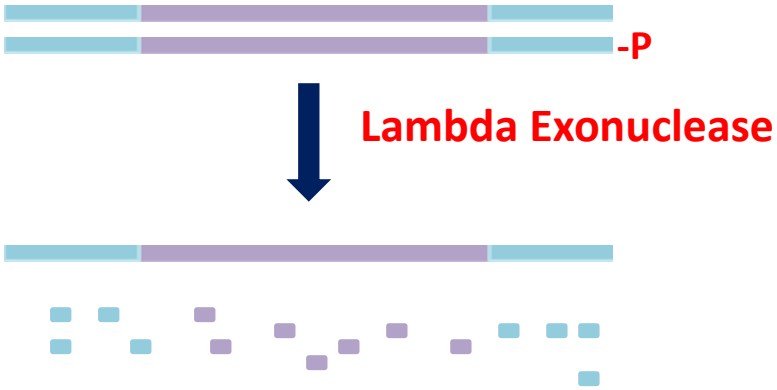

Figure 4: ssDNA production by lambda exonuclease.

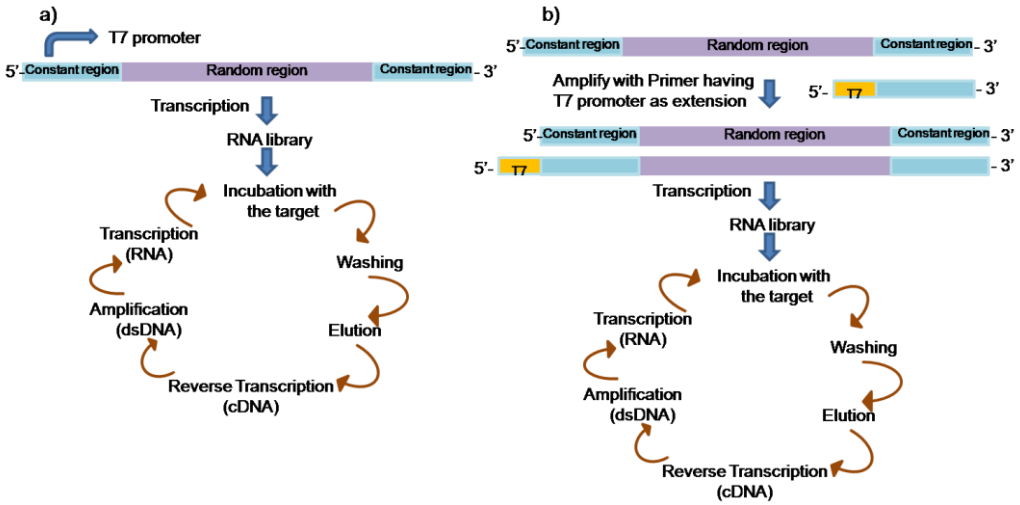

Figure 5: RNA SELEX-a) When T7 promoter is added within the constant region; b) When T7 promoter is added via the primer containing it as an extension

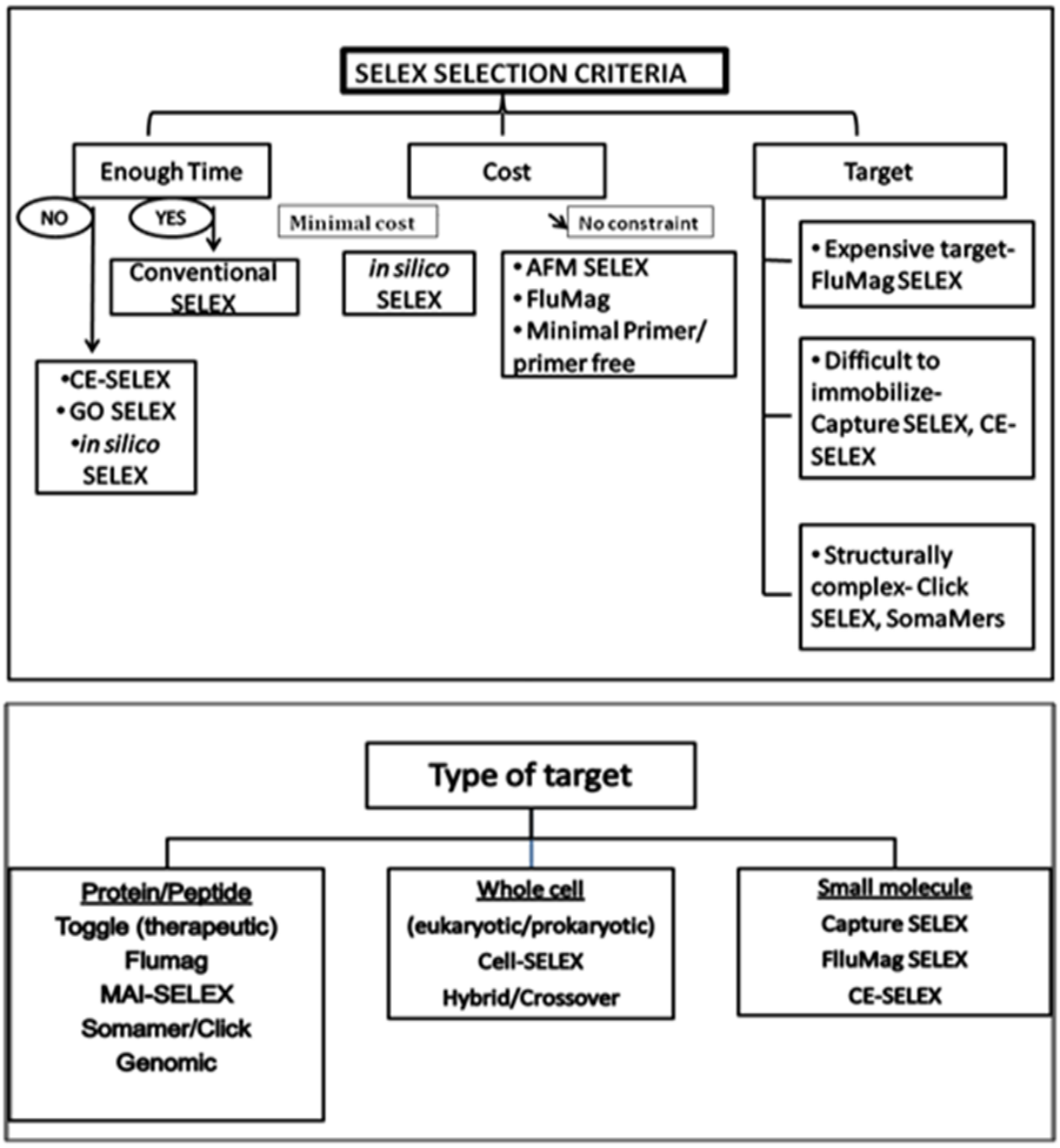

Figure 6: Choosing appropriate SELEX strategy

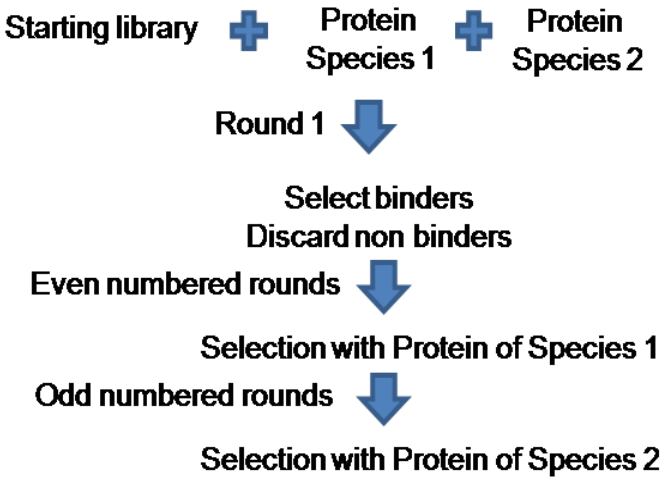

Figure 7: Toggle SELEX

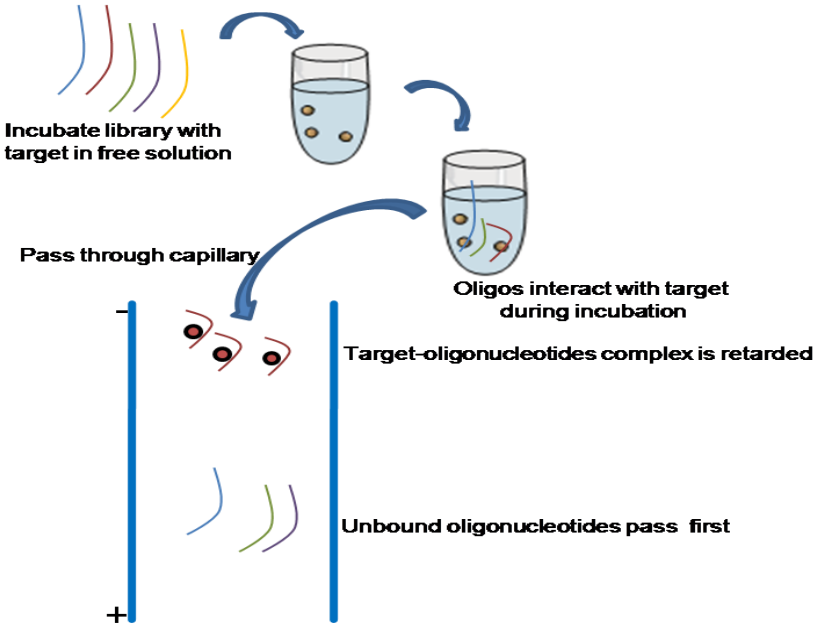

Figure 8: Capillary electrophoresis SELEX

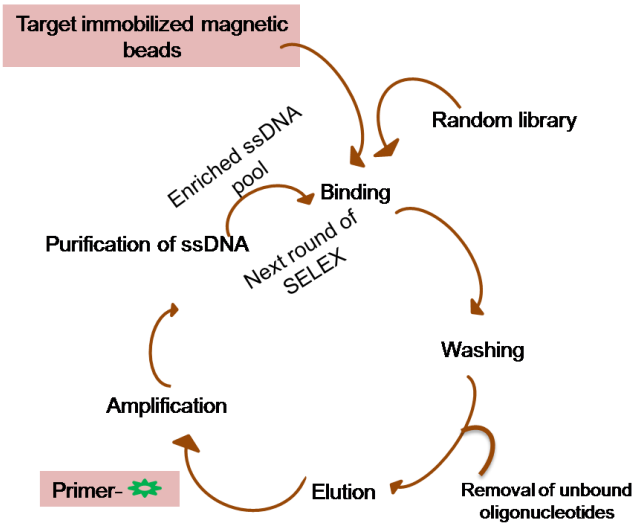

Figure 9: FluMag SELEX

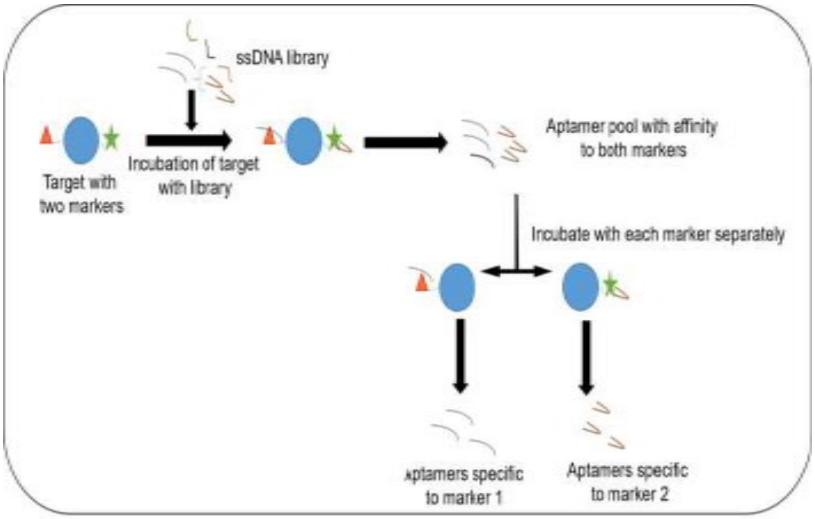

Figure 10: MAI SELEX requires selection in two modules i.e affinity and specificity module.

In affinity module, selection is made against whole target with its two distinct binding sites (markers) wherein aptamers possessing affinity for both sites are generated. In the specificity module, the pool obtained after affinity module is subjected to selection against the two markers separately to sort the molecules with specificity to either of the markers.

5. Summary

There has been an ever increasing interest in research on selection of aptamers against a plethora of targets since the advent of aptamer technology. SELEX technology has evolved and several variants came into existence to achieve successful selection of aptamers.

Despite several attempts to modify the SELEX procedure, the fundamental approach to obtain aptamers remains largely the same involving affinity selection and amplification. The advancements in SELEX however, have made aptamer selection facile and fast. Some variants employing capillary electrophoresis and atomic force microscopy appreciably reduced the number of rounds required to generate aptamers. AFM-SELEX resulted in discovery of even higher affinity aptamers for thrombin than those generated by conventional SELEX. The approaches that augmented the ease of synthesizing aptamers include Flu-Mag SELEX by simpifying partitioning and making quantification sensitive. Several variants reduced the time required for post-selection modification of aptamers by eliminating or reducing the primer regions during SELEX and the end products are aptamers which can be applied unaltered (Minimal primer, primer-free and fixed region elimination SELEX). The aptamers can be produced to possess characterstics rendering them suitable for prospective use viz. selection of aptamers binding to two or more similar targets (toggle SELEX), two or more aptamers binding to different sites of the same target (MAI-SELEX). Conventional SELEX is not an option when the target is structurally complex, SOMAmers and Click SELEX provide alternaive method for aptamer selection.

In silico selection strategy can greatly reduce the bench work for aptamer discovery thereby diminishing the associated cost. Automated workstations for aptamer selection initially require large investment but shorten selection time and reduce labor.

Generating aptamers with potential to be used as therapeutics or diagnostic molecules is a challenge as it requires selection of appropriate strategy suiting the target in hand. Thus, customizing your own strategy with the knowledge of existing variants of SELEX is critical to ensure the success of selection.

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