アプタマーによる環境微生物を視覚的に検出する技術の開発

Development of Technique for Detecting Environmental Microorganisms Visually via Aptamers

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1. Introduction

An aptamer is a small, single-stranded oligonucleotide either RNA or single-stranded DNA (ssDNA) that bind to a target molecule with high specificity and affinity. In 1990, Gold and Tuerk founded the method called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) to develop the aptamers. From the basis that polynucleotide or peptide chain will fold with itself to form a stable three-dimensional structure **(Fig. 1)** indicated by stems, loops, bulges, hairpins, pseudoknots, triplexes or quadruplex, aptamers have high affinity for differing molecules ranging from small organic molecules such as metal ions, organic dyes and amino acids up to complex structures of whole cells [1].

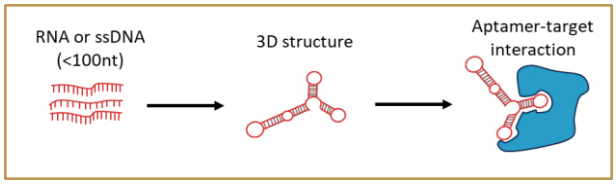

Fig. 1 Representation of aptamer-target interaction [2]

There are more than 25 different SELEX process that has been developed and of them is the whole cell-SELEX. As the name suggests, a chemically synthesized DNA or RNA library is incubated with live bacterial cells which its surface are the targets of the aptamers. Using a simple physical method such as centrifugation, bound aptamers can be separated from the unbound oligonucleotides. The bound aptamers will then be collected and amplified so that it can be used as the aptamer library for the next round of SELEX, commonly required from 8 to 10 rounds. This study aim is to develop aptamers against live bacterial cells grown in suspension through the whole cell-SELEX method [3].

2. Materials and Methods

2.1 Bacterial strains and culture medium

The target cells are 3 types of *Lactobacillus acidophilus*, known as JCM 1132, 5342 and 1021. These bacteria are non-pathogenic and possess a Gram-positive cell wall with a rich variety of potential targets [4]. All bacteria were grown in the MRS broth under anaerobic conditions at 37℃. In addition, the bacteria were harvested in their logarithmic phase of growth. *L. acidophilus* were grown overnight and on the next morning, the cells were subcultured until it reaches OD_{600}=0.3. The cells were then centrifuged at 5000g and 4℃ and washed twice in 1x binding buffer (1xBB). Final resuspension was done with the same buffer. Composition of the 1xBB are 50 mM Tris-HCl (pH 7.4), 5 mM KCl, 100 mM NaCl and 1 mM MgCl2.

2.2 DNA library

Single-stranded DNA (ssDNA) will be used as the initial DNA library for the aptamer selection. The DNA library is made up from 80-nt oligonucleotides consisting of 40-nt randomized region flanked on both sides by 20-nt known primer regions. The sequences of the FAM modified primers are as below:

Forward: 5'-AGCAGCACAGAGGTCAGATG-3'

Reverse: 5'-TTCACGGTAGCACGCATAGG-3'

The making of the initial DNA library starts with the amplification of the DNA template using Platinum™ Taq DNA Polymerase. After analyzing the PCR products and confirming the length of the DNA by using the Agilent 2100 Bioanalyzer, the PCR products were purified using the MinElute PCR purification kit. The double-stranded DNA (dsDNA) library is then heat-denatured to ssDNA at 94℃ for 5 min and cooled at 0℃ for 10 min. The thermocycling parameters were 20 to 40 cycles of 94℃ for 30 s, 57℃ for 30 s and 72℃ for 20 s.

2.3 Aptamer selection

The initial ssDNA library (~10^{13} molecules) was incubated with cells (~10^{8}) in the binding buffer for 45 minutes at room temperature. An excess of tRNA and

BSA were added to the incubation buffer at 10-fold molar for the first round of SELEX. Following incubation, the cells were centrifuged at 12000 rpm at 4℃ for 5 min. The supernatant was then collected as SO fraction. Next, the cells were washed for two times in 250 uL of 1xBB with 0.005% BSA via resuspension and centrifugation in which the supernatants were collected as W1 and W2 fractions. The cells were then resuspended in 100 uL of 1xPCR reaction buffer before heating the cells at 94℃ for 10 min and placed on ice for another 10 min to denature and elute the bound aptamers from the cells. Once again, the cells were centrifuged and the supernatant was collected as CA fraction **(Fig. 2)**.

To eliminate aptamers that bind to the tube wall, re-suspended cell solution was changed to a new microcentrifuge tube in between each incubation, washing and elution step. Negative control (NC) with all medium components but without the ssDNA library was also prepared. All 8 fractions collected, including the NC, were PCR amplified and its products were analyzed. The PCR compositions and thermocycling parameter were the same as above except the addition of 1 μ L of DMSO and increasing the annealing temperature to 69 ℃ to minimize the misamplification during PCR because of the increasing GC contents [3]. The PCR product of CA fraction was used as the DNA library for the next round of SELEX.

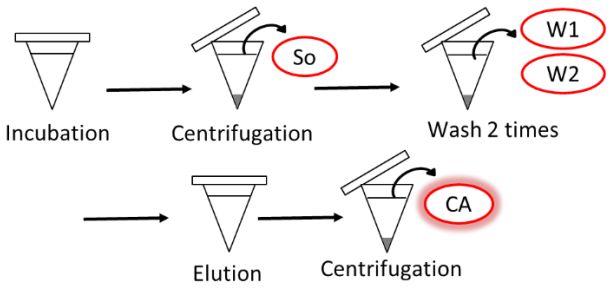

Fig. 2 Collecting each fraction after incubation

3. Results and Discussion

In this study of the whole cell-SELEX, the target bacteria *L. acidophilus* 1132 were investigated first. This is done to improve the techniques of developing aptamers first before furthering with remaining two bacteria. As shown in **Fig. 3**, the PC is the positive control which is the PCR products of the initial ssDNA library. Both the NC SO and NC CA lanes showed several bands which could be because of contamination during the experiment.

At SO lane, no band could be seen while there were bands that can be seen on both W1 and W2 lanes showing that most of the aptamers were bound to the cells but only through further washing that the weakly bound aptamers can be removed from the cells. In the CA lane, there was a single band that is at the same length as the PC, proving that these PCR products were the aptamers that were bound to the cells with high affinity.

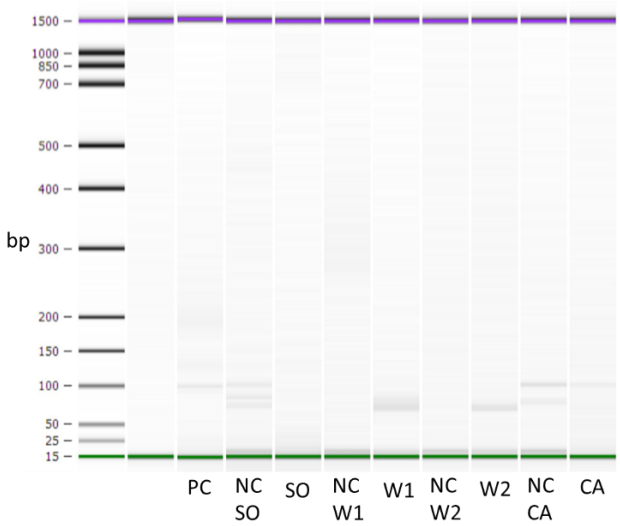

Fig. 3 PCR amplified fractions after the first round of SELEX

4. Conclusions

After several attempts, it is possible through heat-denaturation to develop ssDNA aptamers that can bind to the target bacteria through the whole cell-SELEX method. However, there are still some problems such as contamination and other unknown factors that make it challenging to develop adequate techniques for aptamers development against live bacterial cells.

5. References

[1] Sefah K et al. Development of DNA aptamers using Cell-SELEX. Nat Protoc. 2010 Jun;5(6):1169-85

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[4] Ljungh A et al. Lactic acid bacteria as probiotics. Curr Issues Intest Microbiol. 2006 Sep;7(2):73-89