CENTER for PRODUCE SAFETY

CPS 2021 RFP
FINAL PROJECT REPORT

Project Title

Cyclospora cayetanensis monitoring in agricultural water

Project Period

January 1, 2022 – December 31, 2023 (extended to March 31, 2024)

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Objectives

1. *Design and understand the parameters for the C. cayetanensis aptamer synthesis via systematic evolution of ligands by exponential enrichment (SELEX).*
2. *Design and test a colorimetric microfluidic biosensor platform for detection of Cyclospora cayetanensis intact oocysts spiked in agricultural water, and measure parameters critical for achieving biosensing functionality.*

Funding for this project was provided partly through the CPS Campaign for Research

FINAL REPORT

Abstract

Currently, there is a lack of standard detection and identification technologies for *Cyclospora cayetanensis*. There are several obstacles in the way of advancement towards technologies for early detection and mitigation of this parasite in agricultural water and produce. Among these, a lack of commercially available biorecognition elements, such as antibodies, is notable. Here, we report on the discovery of five unique aptamers, which specifically recognize and bind to the surface antigen TA4 of *C. cayetanensis*.

Background

There are currently no standardized easy-to-use methods for the early detection of *Cyclospora cayetanensis*, and the infections with this organism are understudied in the developed world. Microscopy methods, immunofluorescence microscopy, and polymerase chain reaction (PCR) are the only currently available detection methods for this parasite [1-7]. While these can offer good to excellent sensitivity, they also require trained laboratory personnel, high cost, high turnaround times, and are challenged by the presence of PCR inhibitors. On the last point, with nucleic acid detection methods, it is difficult to detect oocysts from produce washes or feces, which contain PCR inhibitors; this is why detection of intact oocysts, as the one proposed by our team, would present with a solution that will offer a much more reliable detection than the methods currently available. Typically, detection of intact pathogens requires targeting of surface antigens with antibodies, which are available in many cases but not for *C. cayetanensis*. Thus, the first step towards making a quantum leap towards field deployable *C. cayetanensis* detection in agricultural water would be the development of such antigen targeting ligands, such as DNA aptamers.

Research Methods and Results

Objective 1

1. **Cloning, expression, and purification of *Cyclospora cayetanensis* specific proteins.** Obtaining *Cyclospora cayetanensis* samples to perform the SELEX round is very difficult in the U.S. Hence, *C. cayetanensis* oocyst protein biomarkers were cloned and expressed for purification to be used in the subsequent experiments. The identified proteins are known to be found in the *Cyclospora* spp. oocysts, *viz.* a) sporulated oocyst TA4 (TA4), b) Heat Shock Protein (HSP70), and c) oocyst wall protein 2.

    1.1. TA4 gene cloning, overexpression, and purification. To clone and express the *C. cayetanensis* TA4 in *E. coli,* the sequences for the above-mentioned genes were obtained from the NCBI database. The sequences were optimized and cloned into the pET-22b(+) or pHIS-2 vector. Each plasmid was used for transformation into various BL21 strains (**Fig. 1**) and evaluated for expression (**Fig. 2**). Based on the results of the colony screening, pilot-scale (10 mL) experiments were performed to determine the optimum conditions for the expression of the target proteins. The optimized protocol was used going forward for the large-scale (5 L) expression and purification of the target proteins. Briefly, the *E. coli* BL21 was grown in TB broth and induced with IPTG for 12 h. After the induction, the bacterial cells were harvested and homogenized with a French press at 15-

20 psi pressure. The prepared mix was loaded onto a HisTRAP® column for chromatographic separation. The fractions from the HisTRAP® chromatography were collected and analyzed (**Fig. 3**). SDS-PAGE was performed to identify the chromatography fractions with the target protein (**Fig. 4**). The selected fractions were pooled and again loaded onto a Superdex® 200 chromatography column for further purification (**Fig. 5**). The obtained fractions were analyzed with SDS-PAGE and Western Blotting (**Fig. 6**) to determine the quality of the target proteins. The obtained fractions were aliquoted and stored at -80°C for future use in SELEX rounds.

1.2. Cloning, overexpression, and purification of HSP70 and wall protein 2. To clone and express the *C. cayetanensis* protein HSP70 and wall protein 2 in *E. coli,* the sequences for the above-mentioned genes were obtained from the NCBI database. The sequences were optimized and cloned into the pHIS-2 vector. Each plasmid was used for transformation into various BL21 strains and evaluated for expression. Based on the results of the colony screening, pilot-scale experiments were performed to determine the optimum conditions for the expression of the target proteins. The optimized protocol was used going forward for the large-scale (5 L) expression and purification of the target proteins. Briefly, the *E. coli* BL21 was grown in TB broth and induced with IPTG for 12 h. After the induction, the bacterial cells were harvested and homogenized with a French press at 15-20 psi pressure. The prepared mix was loaded onto a HisTRAP® column for chromatographic separation. The fractions from the HisTRAP® chromatography were collected and analyzed (**Fig. 7**).

2. **Conjugation of TA4 antigen with the functional magnetic beads.** Dynabeads M-280 beads, (Invitrogen) have tosyl groups on their surface. The TA4 antigen possesses an amino group at its N-terminus. When these two components are combined in the presence of a borate buffer at pH 7.8, a nucleophilic substitution reaction takes place. During this reaction, the amino group of the TA4 antigen displaces the tosyl group on the magnetic beads, resulting in the formation of a strong covalent bond between the bead and the TA4 antigen.

3. **Binding of amplified ssDNA library on magnetic beads and its elution.** TA4 conjugated beads were prepared by suspending and washing them in a sterile binding buffer (20 mM Tris-HCl, 100 mM NaCl, 2 mM MgCl2, 5 mM KCl, 1 mM CaCl2, pH 7.6). The single-stranded DNA (ssDNA) library was first amplified using polymerase chain reaction (PCR) in 20 parallel reactions. Each 50 µL reaction mixture contained 100 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.75 mM $MgCl_2$, 0.2 mM of each dNTP, 100 ng of ssDNA library, and 0.2 µM of both forward and reverse primers. The amplified product was then purified using a PCR product purification kit and resuspended in the binding buffer. To convert double-stranded DNA (dsDNA) into ssDNA, the purified dsDNA was heated at 90°C for 5 minutes and then quickly cooled on ice. The ssDNA was then allowed to bind to the beads in the binding buffer at 20°C for 30 min. Unbound oligonucleotides were removed by washing the beads six times with 100 µL of binding buffer each time. Following this, the beads were treated with an elution buffer (40 mM Tris-HCl, 0.02% Tween-20, 10 mM EDTA, 3.5 M Urea; pH 8.0) at 80°C for 10 minutes, using 100 µL of elution buffer each time (**Fig. 8**). The final eluate obtained after each SELEX round was amplified, and the PCR product was purified using gel electrophoresis (**Figs. 9-11, 13, 14** and **16-23**) before being used in the subsequent SELEX round.

4. **Positive, negative, and counter selection rounds.** To date, we have performed 13 SELEX rounds. During these rounds, the ssDNA library or the last eluent from the previous SELEX round was preincubated with pristine magnetic beads for negative selection (**Fig. 12**), or with *Eimeria tenella or Eimeria maxima* oocysts for counter selection rounds (**Fig. 15**). For positive selection rounds, the ssDNA library or last eluent from the previous SELEX round was incubated with TA4-conjugated magnetic beads (**Fig. 8**). To facilitate binding with the TA4-conjugated magnetic beads, the mixture was incubated for 30 minutes, with intermittent flickering every 5 minutes. The unbound oligonucleotides and bound oligonucleotides were then collected through sequential elution (100 µL each) using the elution buffer. The washing and elution obtained with each buffer were collected in separate tubes. For the assessment and quantification of SELEX efficiency, 1 µL of each elution was used for the amplification of aptamers through PCR. The resulting PCR products were then analyzed using agarose gel electrophoresis to determine the success of the selection process. Following the initial incubation of the library with TA4 conjugates in each SELEX round, a preparative PCR of the eluted binders was carried out. This step is critical in the SELEX process, as random PCR of libraries can easily generate nonspecific products that are often smaller or multiple times larger than the library. These nonspecific PCR products, called molecular parasites, can remain present throughout the SELEX process and ultimately compromise the aptamer selection outcome. They can go unnoticed in the early rounds but increase exponentially in number and become more apparent in later rounds. The positive selection rounds were carried out in rounds 1, 2, 3, 5, 7, 9, 11, 12, and 13. Round 4 involved negative selection, and counter selection was performed in round 6, 8 and 10.

5. **Determination of the aptamer sequences.** To determine the sequence of the aptamers from the last elute after 13th round of SELEX, PCR amplification was performed, followed by ligation with the pGEM-T vector. The prepared plasmids were transformed into the DH5α *E. coli* competent cells. The transformed cells were plated and after 24 h individual bacterial colonies were grown in LB-ampicillin broth for about 12 h. Plasmids were isolated with a commercial plasmid isolation kit. The presence of DNA insert was confirmed by PCR and sent for sequencing. The obtained sequences were analyzed, and the aptamer sequences were identified (**Table 1**). The secondary structure of the obtained sequences was determined with nupack web server (**Fig. 24**).

6. **The binding affinity constant (K_d) determination.** To determine K_d, 1x10^7 TA4-coated magnetic beads were incubated with 100 µL of 10-800 nM aptamer (labeled at the 5' end with 6-carboxyfluorescein) for 1 h at room temperature. Unbound aptamers were collected by five quick washings with binding buffer; bound aptamers were collected after incubating the beads with eluting buffer at 90^{o}C for 10 min with mild shaking. The fluorescence of unbound and bound aptamers was measured at the excitation wavelength (494 nm) and the emission wavelength (520 nm) of 6-carboxyfluorescein. The binding affinity constant of the respective five aptamers is listed in **Table 1**. The K_d ranges between ~91–198 nM.

Objective 2

1. **PURE-SCAN** (Purdue's Enhanced Sensing System for Cyclospora Analysis in Agricultural Water using Nanomaterials) **system for onsite analysis of paper-based µPAD sensing devices.** PURE-SCAN, was designed for onsite color analysis of microfluidic paper-based analytical devices (µPADs). The primary objective of the PURE-SCAN device is to record and analyze the colorimetric sensor response in the presence and absence of *Cyclospora cayetanensis* in the test sample. The device has been prototyped using PLA material through 3D printing, and it incorporates a high-quality camera system with LED lights and a Raspberry Pi 4B (as depicted in **Fig. 26**). The colorimetric sensors utilized in the system undergo a color change from red/reddish pink to blue/blueish gray in the presence of Cyclospora cayetanensis, which can be correlated with the concentration of contaminants in the test sample. The developed PURE-SCAN system optimally illuminates the paper-based µPAD colorimetric sensors and captures an image, which is subsequently processed on the device to generate a calibration curve. This calibration curve allows for the quantification of contaminating pathogen concentration in the test samples. The processed image and data can also be uploaded to cloud-based storage for further processing and data storage. The image analysis program is written in Python and is accessible through a graphical user interface (GUI). It contains two main functions that can be initiated through GUI buttons. The first function captures an image of the developed sensor placed below the camera in the imaging device. The image is then cropped to a square ratio of 400x400 pixels, and two samples and one reference site are cropped to isolate each region of interest (ROI). The second function calculates the pixel values of each region, utilizing OpenCV to determine the values of each pixel for every sample site in both the HSV and RGB color spaces. The mean (±SD) pixel values are then determined, as well as the standard deviation, for each sample site. A background subtraction is performed, and the subtracted values are reported via a popup text box, which can be easily read by the operator.

2. **Synthesis of polystyrene-gold nanoparticles (PS-AuNP).** The PS-AuNP particle was synthesized to develop a color-based diagnostic test for TA4. Briefly, 100 µL of polystyrene beads (PS) was mixed with 4 mL of DI water, and sonicated for 5 minutes. Then, we added 8 mL of DI water, along with 63.4 µL of 254 mM gold (III) chloride trihydrate, in DI water solution and stirred for 5 minutes at room temperature. Next, we added 20 mL of DI water and 82.86 mg of trisodium citrate dihydrate, before adding 13.84 mL of DI water to achieve a final concentration of 0.35 mM for gold (III) chloride trihydrate and 6.125 mM for trisodium citrate dihydrate. The solution was then stirred for 30 minutes at room temperature, followed by heat treatment in a water bath at 95°C for 20 minutes with stirring. Transmission electron microscopy shows the size and distribution of gold nanoparticles on the polystyrene core (**Fig. 27**). The synthesized particles have a plasmon peak at 523 nm (**Fig. 28A**). A salt aggregation assay was performed to determine the red-to-blue/purple color change (**Fig. 28C**). We are still working on using these particles in the building and optimizing the microfluidic paper-based analytical devices.

Outcomes and Accomplishments

1. *Cyclospora cayetanensis* detection required the identification of its biomarkers. Obtaining *C. cayetanensis* oocysts in the US for research is difficult. We identified, cloned, and purified the *C. cayetanensis* protein biomarker known as sporulated oocyst TA4 antigen.
2. To identify the ligand aptamers that will specifically bind to the TA4, SELEX rounds were performed. The obtained DNA aptamers were sequenced. We have identified 5 unique aptamer sequences to date.
3. The binding affinity constant of the obtained sequences was determined to identify the aptamer with the strongest binding affinity to TA4.
4. The PURE-SCAN (Purdue's Enhanced Sensing System for Cyclospora Analysis in Agricultural Water using Nanotechnology) system for onsite analysis of paper-based analytical devices (μPADs) was developed. The device performance parameters still need to be validated.

Summary of Findings and Recommendations

Cyclospora cayetanensis research in the US is limited due the unavailability of *Cyclospora* spp. oocyst samples. To date, attempts to grow *C. cayetanensis* have not succeeded[8]. Despite such limitations, we have cloned, overexpressed, and purified the *C. cayetanensis* sporulated oocyst TA4 antigen that is a potential biomarker. The prepared protein has been used for the discovery of aptamers. We have, at present, identified 5 unique aptamer sequences. These aptamers were characterized to determine the binding affinity to the TA4 protein. The binding affinity of the respective aptamers is 92.1–198.2 nM.

APPENDICES

Publications and Presentations

Presentations

A. Barui and L. Stanciu. *Cyclospora cayetanensis* aptasensor development for monitoring in agricultural water. Cyclospora Task Force Webinar. Sept 2023.

A. Barui and L. Stanciu. Aptamers as New and Emerging Methods for Detection and Characterization of Parasites in the Environment. IAFP 2023, Toronto, Canada.

Budget Summary

This project was awarded $159,032 in research funds, and all funds were spent.

Figures 1–29 and Table 1 (see Annexure 1 below)

References cited

1 Resendiz-Nava, C. N. *et al.* A Molecular Tool for Rapid Detection and Traceability of Cyclospora cayetanensis in Fresh Berries and Berry Farm Soils. *Foods* **9**, 261 (2020).

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5 da Silva, A. J. *et al.* Detection of Cyclospora Cayetanensis in Food and Clinical Samples Using a Gelified Real-Time Pcr as Say. *Am J Trop Med Hyg* **95**, 175-175 (2017).

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8 Tucker, M. S., Khan, A., Jenkins, M. C., Dubey, J. P. & Rosenthal, B. M. Hastening progress in Cyclospora requires studying Eimeria surrogates. *Microorganisms* **10**, 1977 (2022).

Annexure 1 – Tables and Figures

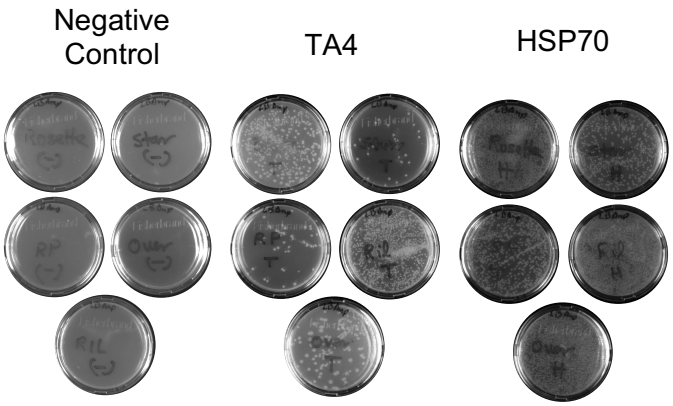

Fig. 1. *Cyclospora cayetanensis* genes cloning and transformation into *E. coli* BL21 strains.

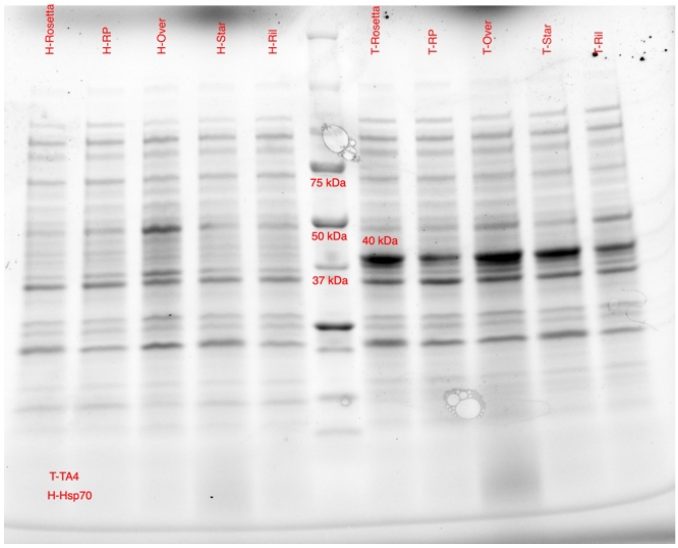

Fig. 2. Screening of different *E. coli* BL21 strains for maximum overexpression. SDS-PAGE profile of *C. cayetanensis* specific proteins (TA4 and HSP70) overexpression in five different strains of *E. coli* BL21.

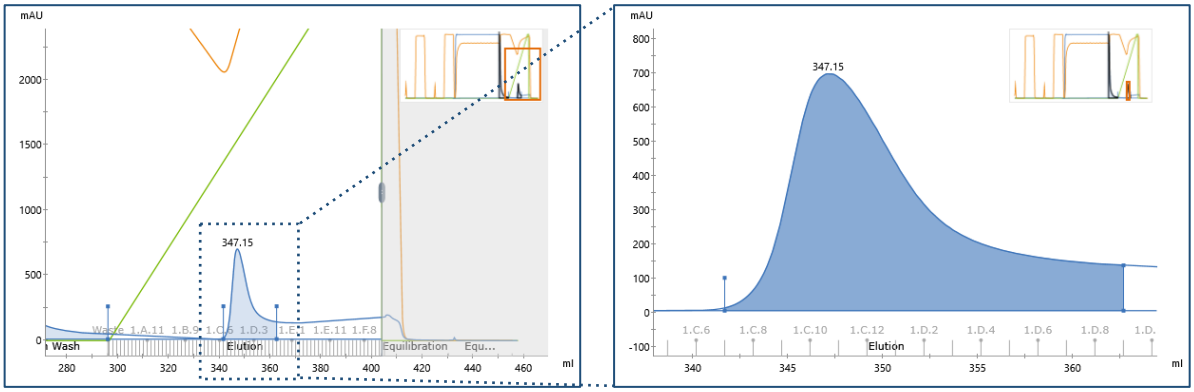

Fig. 3. Purification of TA4 protein using His-Trap column. Selected *E. coli* BL21 were induced to overexpressTA4.

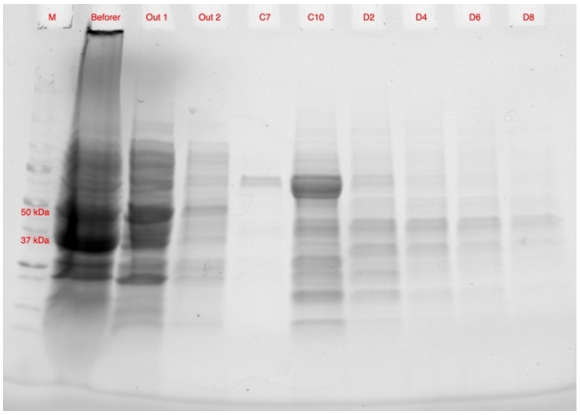

Fig. 4. TA4 purification. SDS-PAGE profile of various protein fractions before and after purification using HIS-Trap chromatography.

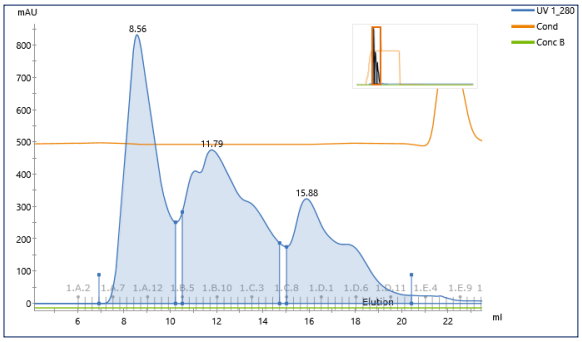

Fig. 5. TA4 purification. Chromatography profile of various eluted protein fractions using gel filtration chromatography with Superdex® 200.

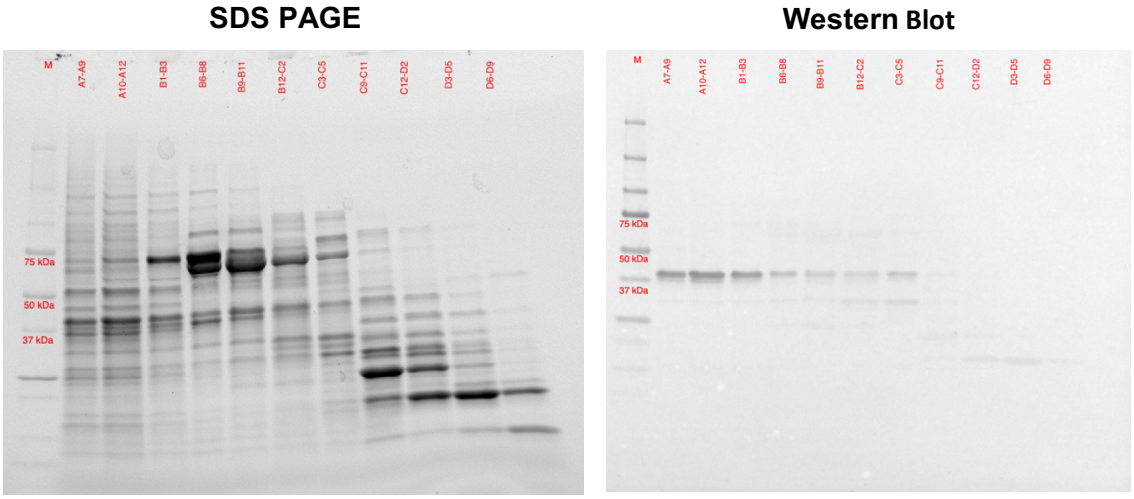

Fig. 6. TA4 purification. Western Blot analysis of different eluted fractions to identify *E. coli* expressed *C. cayetanensis* TA4 protein, using TB induction medium.

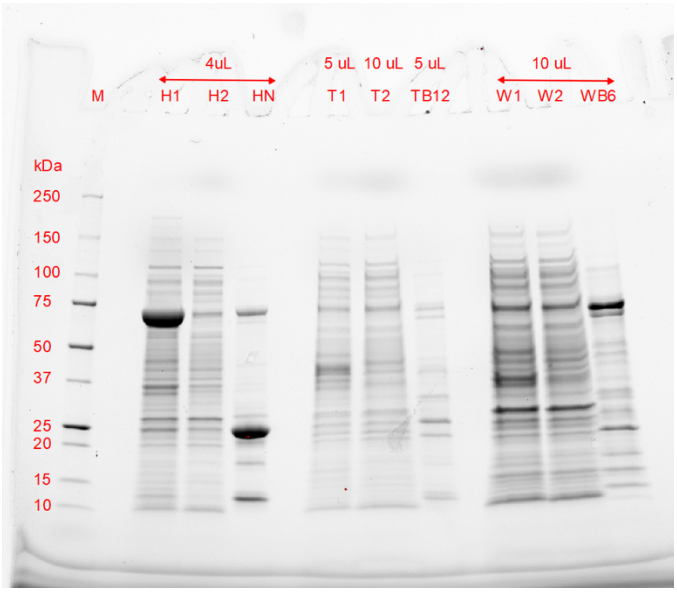

Fig. 7. HSP70, TA4 and Wall Protein 2 purification. SDS PAGE analysis of HSP70, TA4 and Wall Protein 2 fractions overexpressed in *E. coli* followed by HIS-Trap purification.

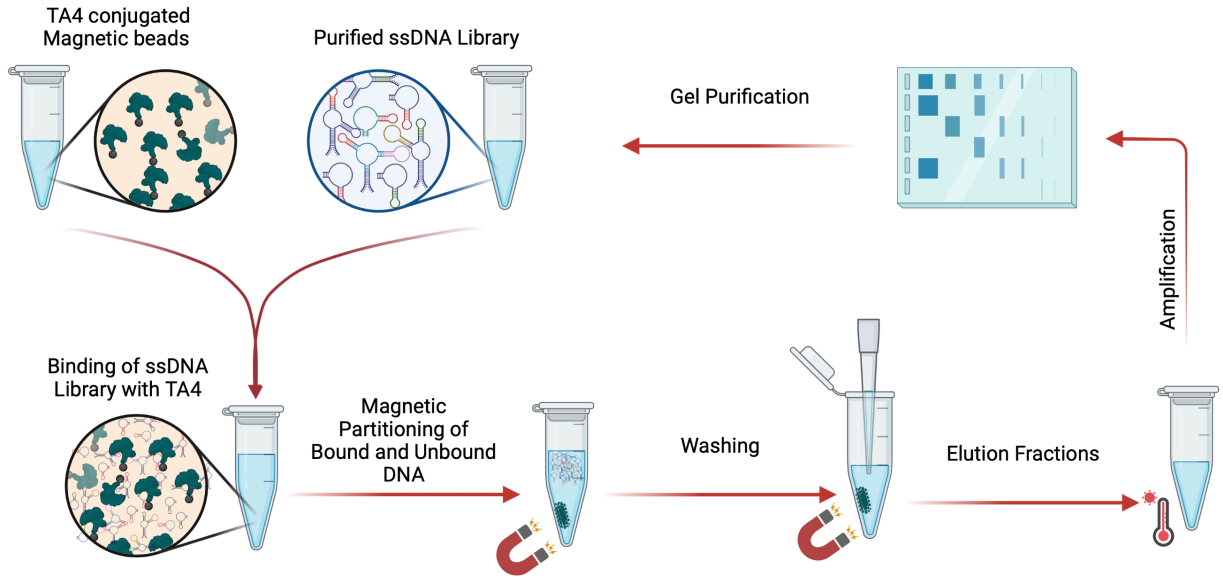

Fig 8. The positive SELEX strategy employed to select an aptamer that specifically binds to the TA4 antigen of *Cyclospora cayetanensis*. The figure illustrates the process of incubating the ssDNA library or the last eluent from the previous SELEX round with TA4 conjugated magnetic beads. The unbound oligonucleotides were removed, and the bound oligonucleotides were eluted from the beads. The eluted oligonucleotides were amplified through preparative PCR to generate the next round's ssDNA library. This process was repeated for several rounds, enriching for aptamers that specifically bound to the TA4 antigen of *Cyclospora cayetanensis*.

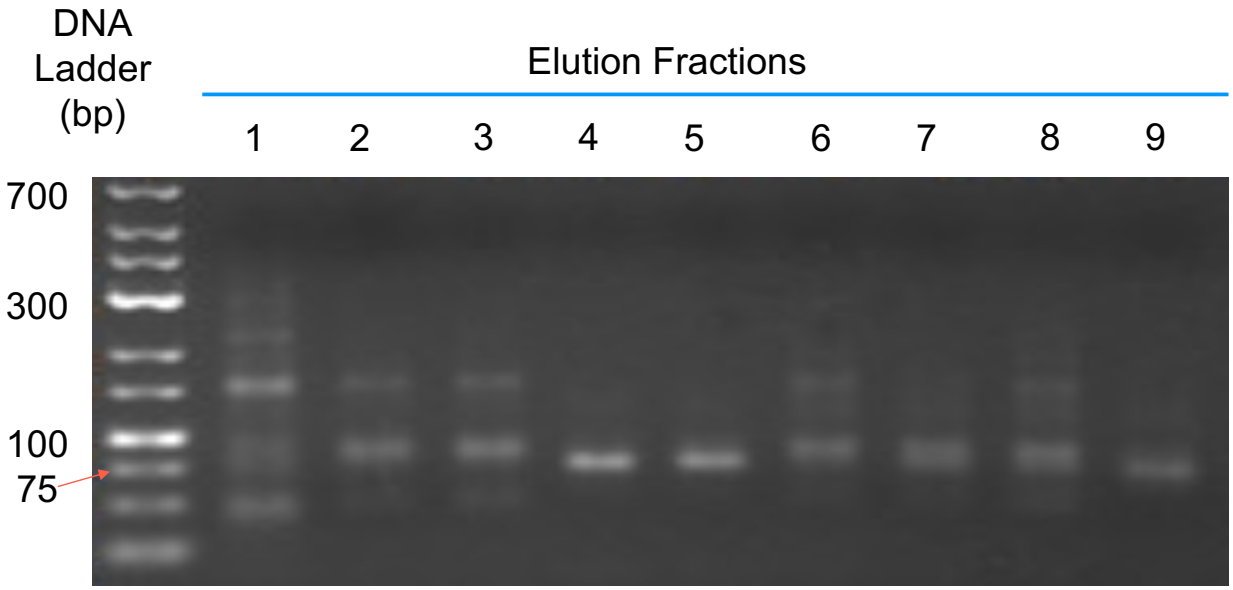

Fig 9. The gel electrophoresis image of the PCR amplified eluates obtained during the first round of the SELEX process.

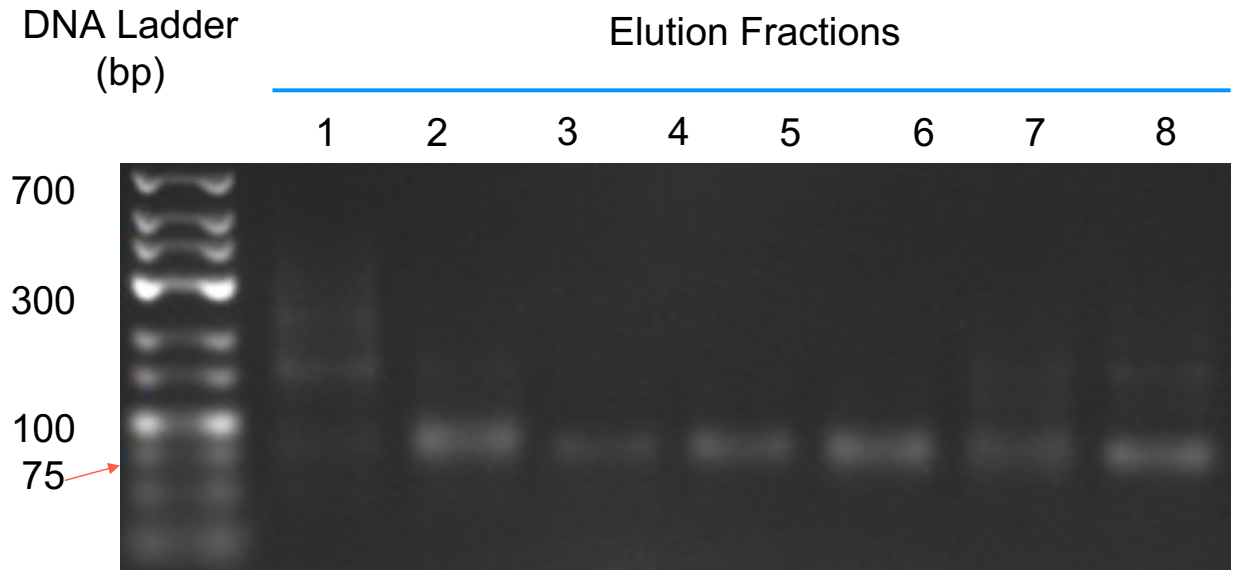

Fig 10. The gel electrophoresis image of the PCR amplified eluates obtained during the second round of the SELEX process.

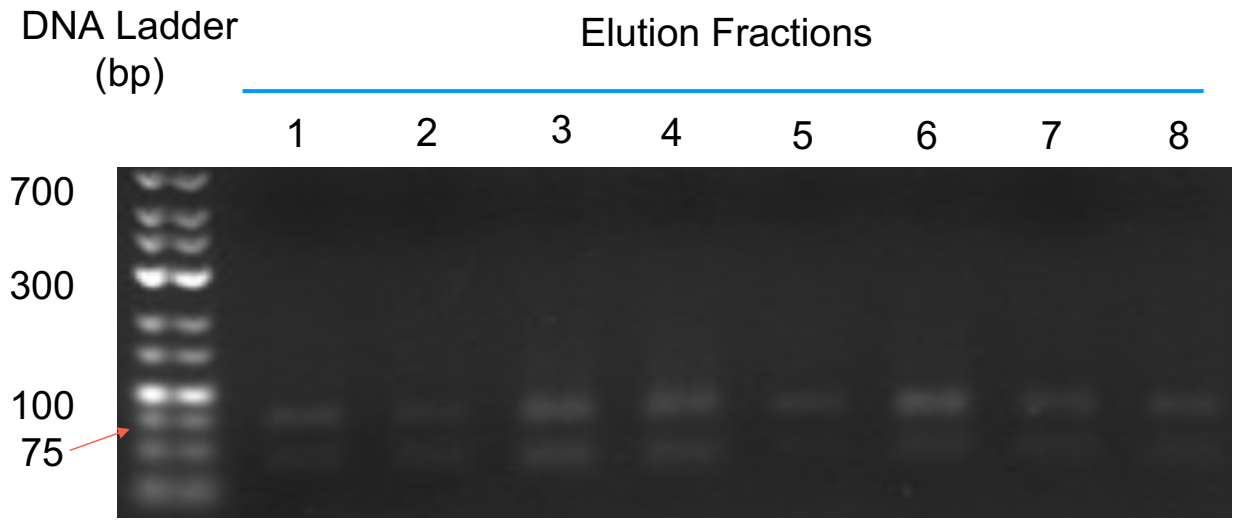

Fig 11. The gel electrophoresis image of the PCR amplified eluates obtained during the third round of the SELEX process.

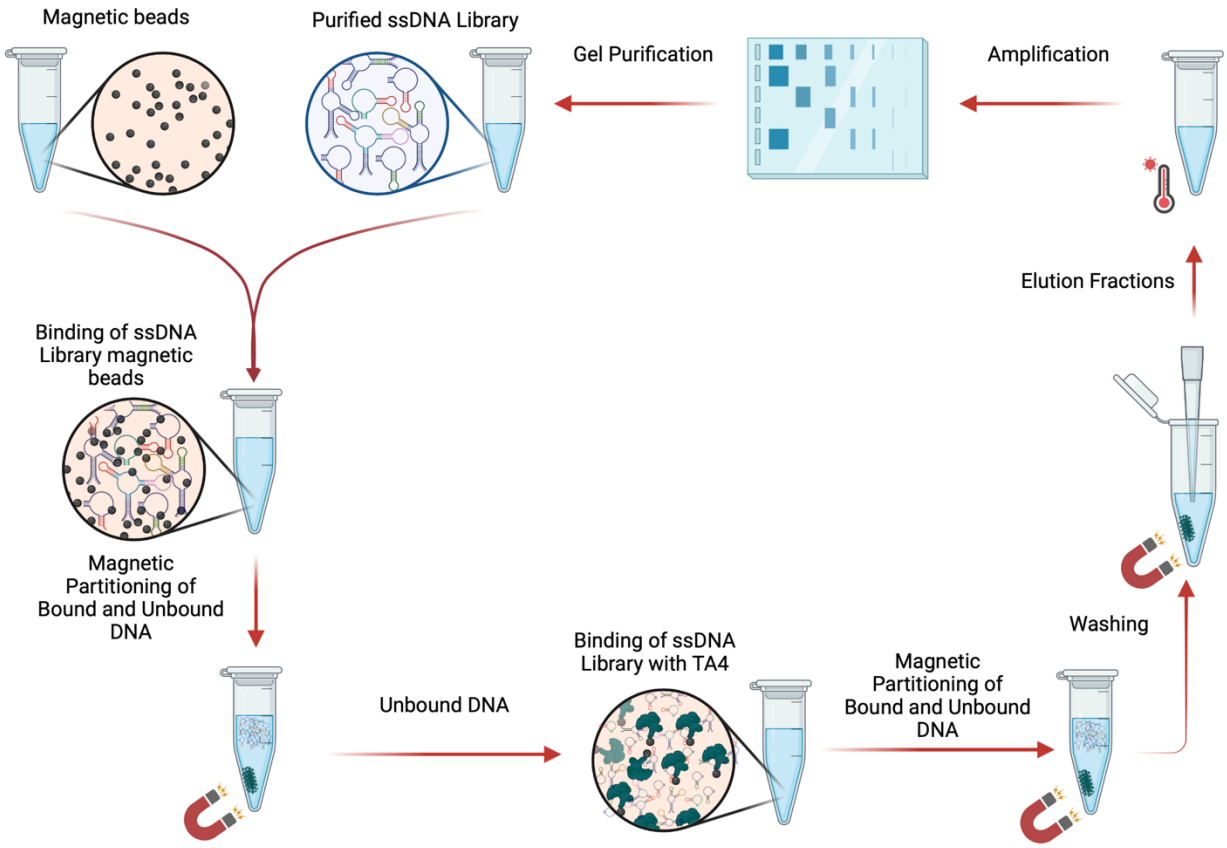

Fig 12. The negative SELEX strategy employed to select an aptamer that specifically binds to the TA4 antigen of *Cyclospora cayetanensis*. The figure depicts the process of preincubating the ssDNA library or the last eluent from the previous SELEX round with pristine magnetic beads to remove any nonspecific binders. This step ensured that only ssDNA molecules that did not bind to the pristine magnetic beads were used for the subsequent positive selection rounds.

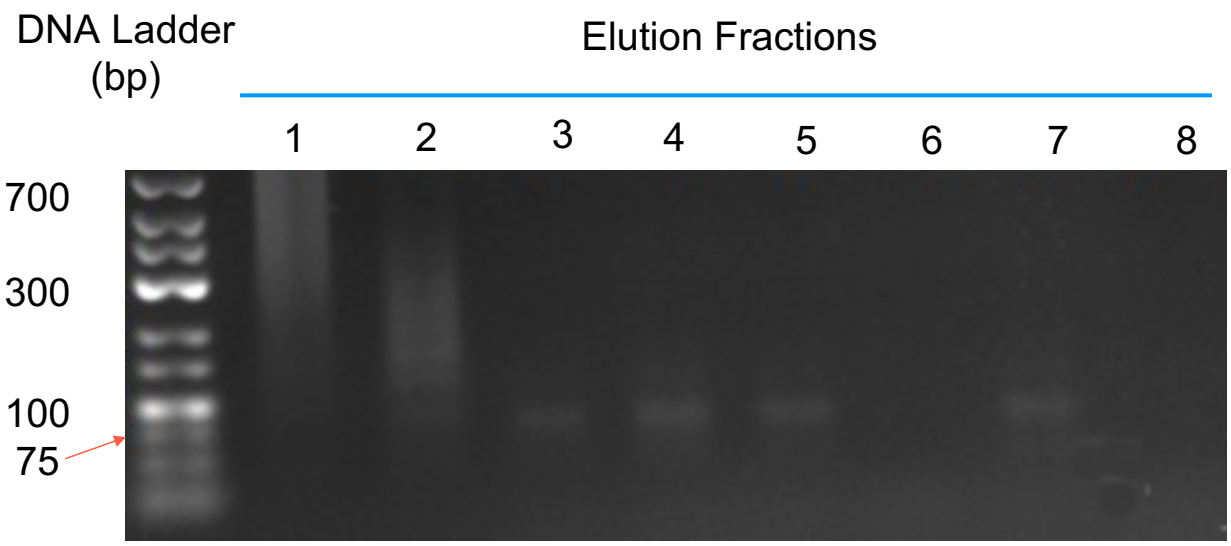

Fig 13. The gel electrophoresis image of the PCR amplified eluates obtained during the fourth round of the SELEX process.

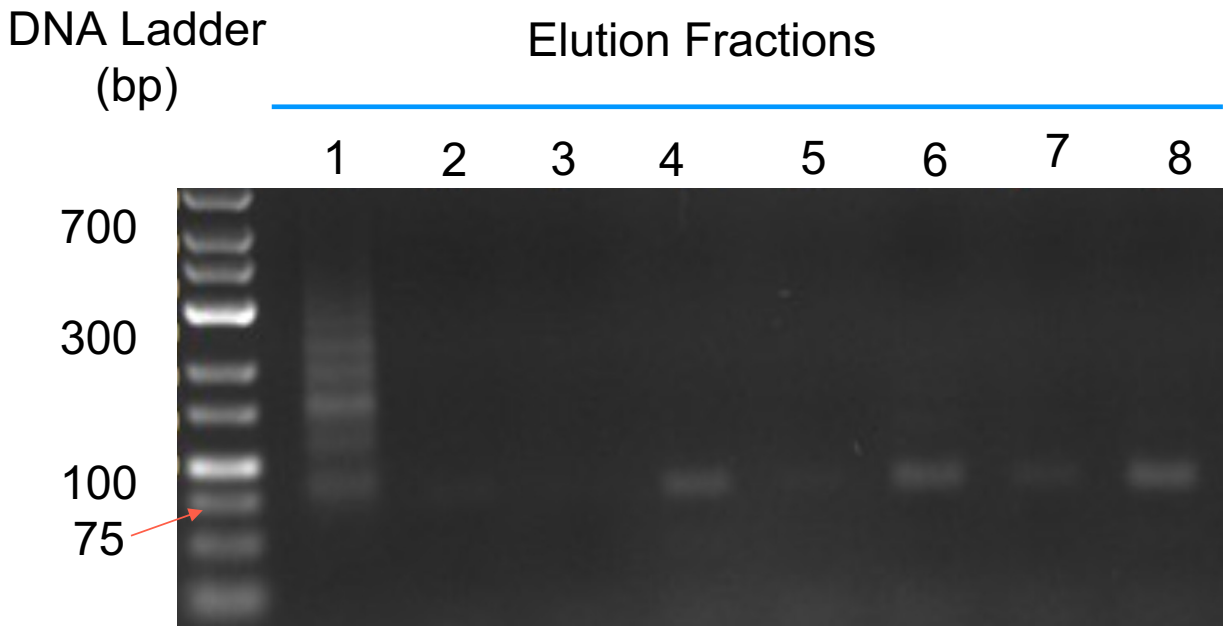

Fig 14. The gel electrophoresis image of the PCR amplified eluates obtained during the fifth round of the SELEX process.

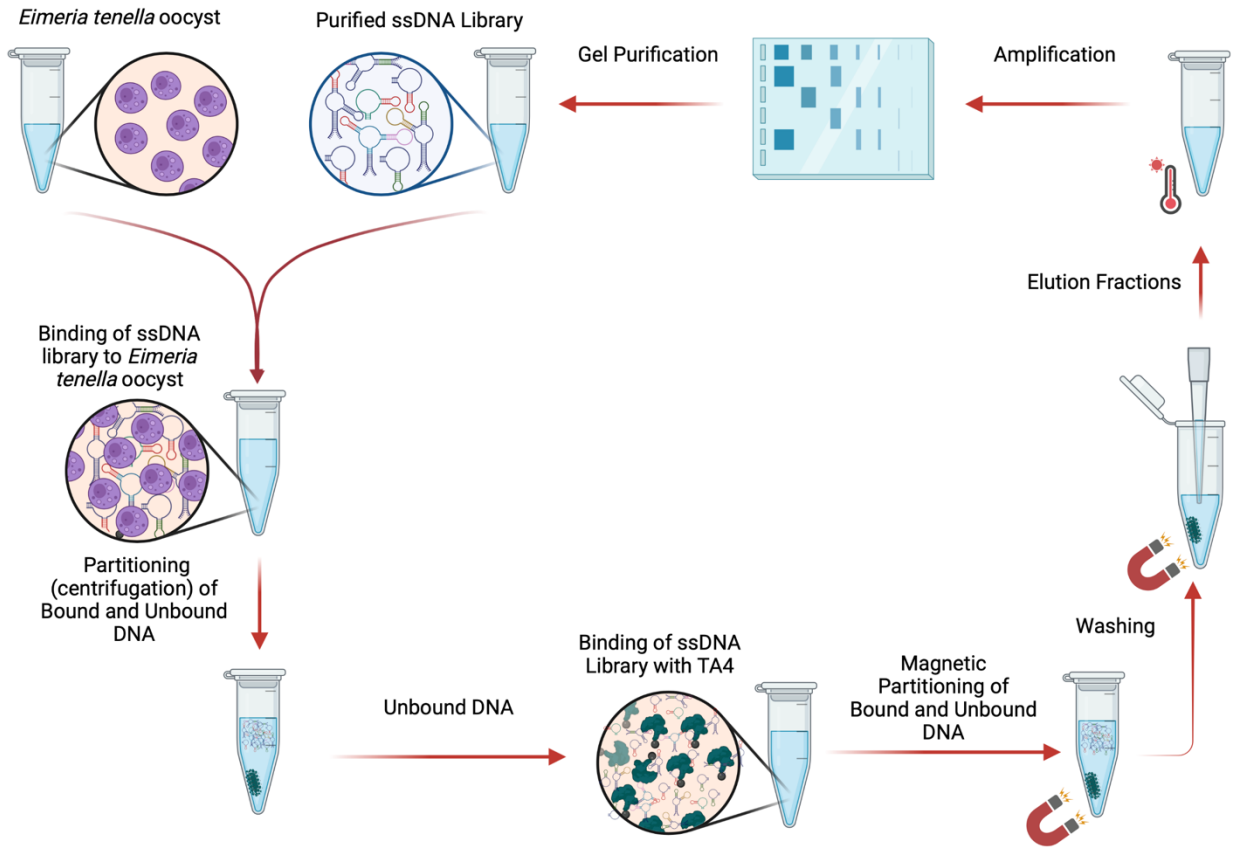

Fig 15. The counter SELEX strategy employed to select an aptamer that specifically binds to the TA4 antigen of *Cyclospora cayetanensis*. The figure illustrates the process of preincubating the ssDNA library or the last eluent from the previous SELEX round with Eimeria tenella oocysts to remove any aptamers that cross-reacted with the *Eimeria tenella* oocysts. The unbound ssDNA molecules were then used for subsequent positive selection rounds with TA4 conjugated magnetic beads. Through this approach, aptamers that specifically bound to the TA4 antigen of *Cyclospora cayetanensis* and did not cross-react with the *Eimeria tenella* oocysts were enriched.

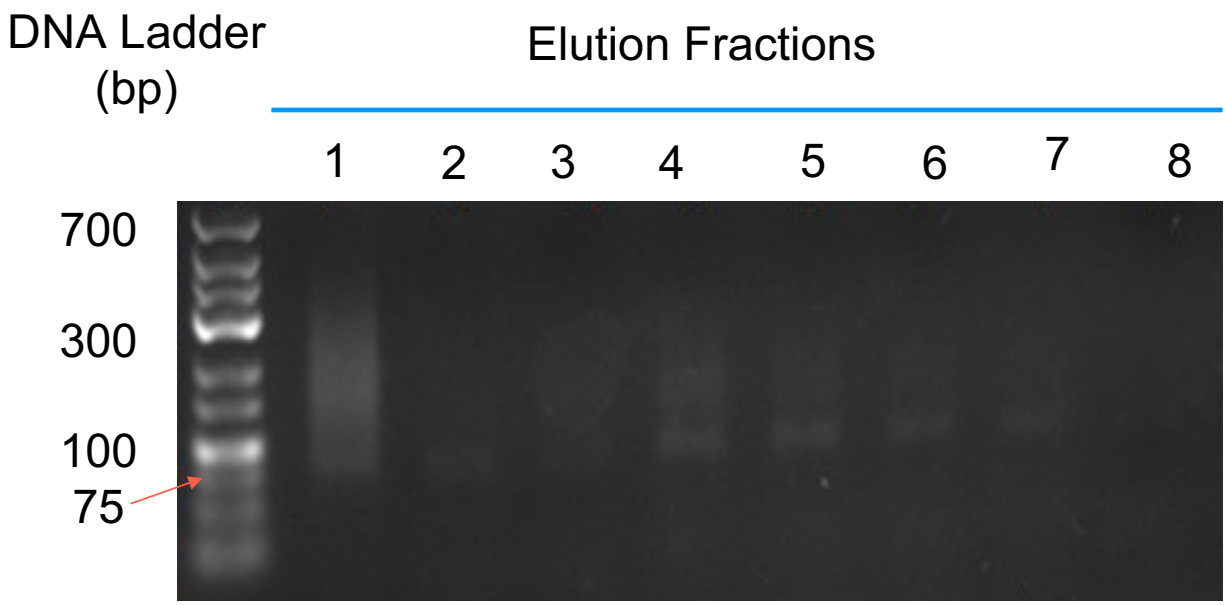

Fig 16. The gel electrophoresis image of the PCR amplified eluates obtained during the sixth round of the SELEX process.

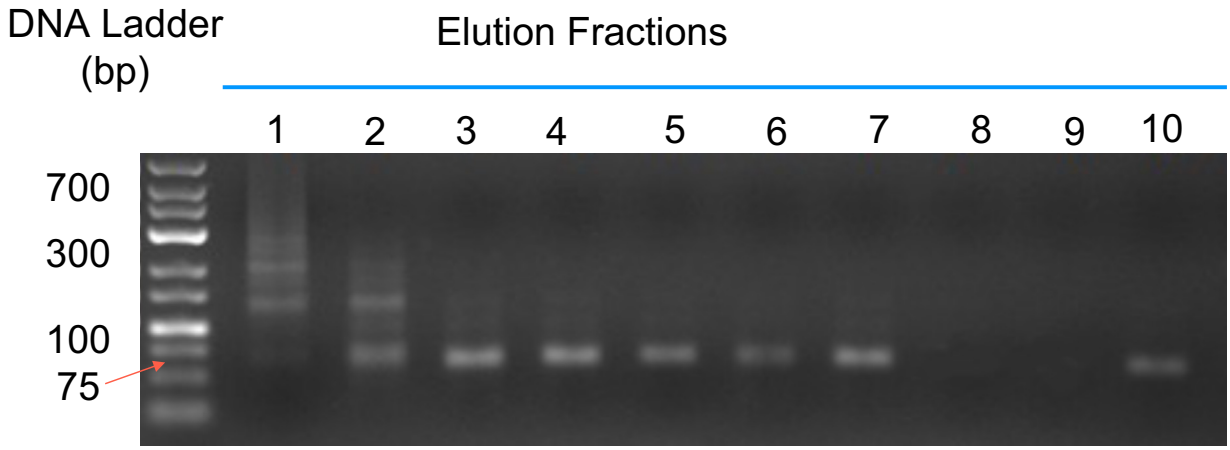

Fig 17. The gel electrophoresis image of the PCR amplified eluates obtained during the seventh round of the SELEX process.

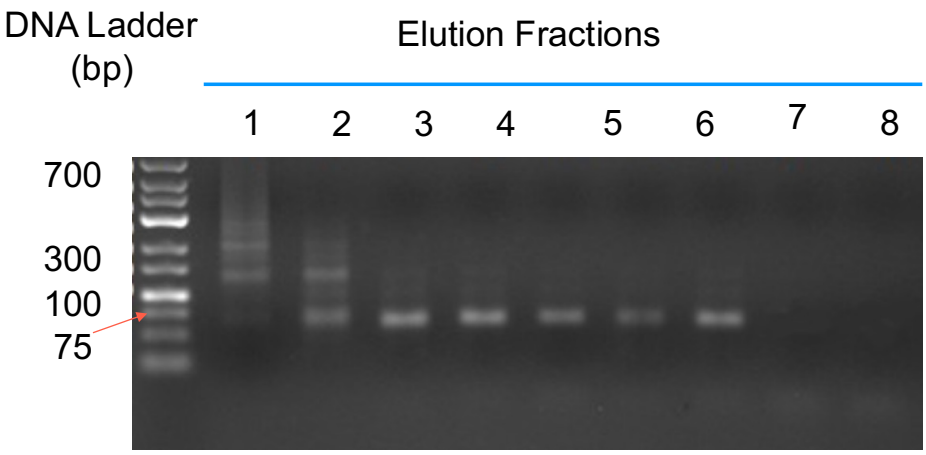

Fig 18. The gel electrophoresis image of the PCR amplified eluates was obtained during the eighth round of the SELEX process.

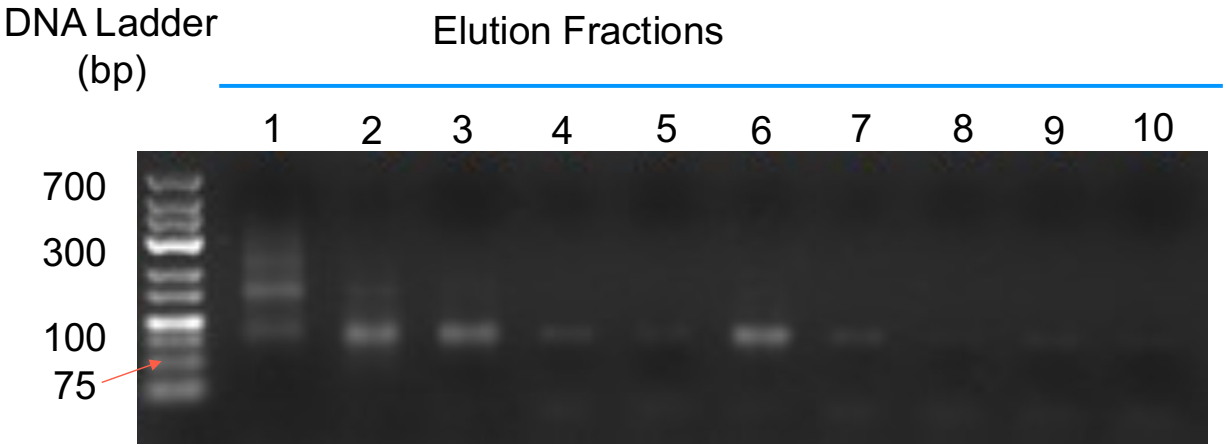

Fig 19. The gel electrophoresis image of the PCR amplified eluates was obtained during the 9th round of the SELEX.

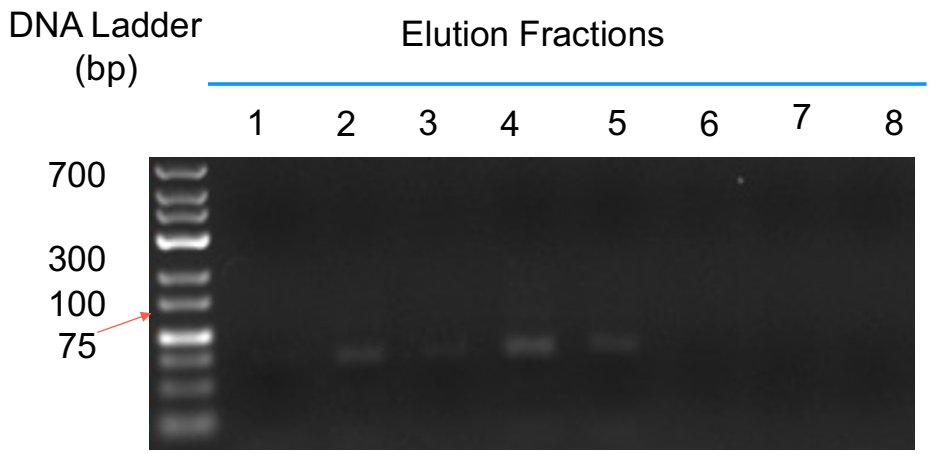

Fig 20. The gel electrophoresis image of the PCR amplified eluates was obtained during the 10th round of the SELEX.

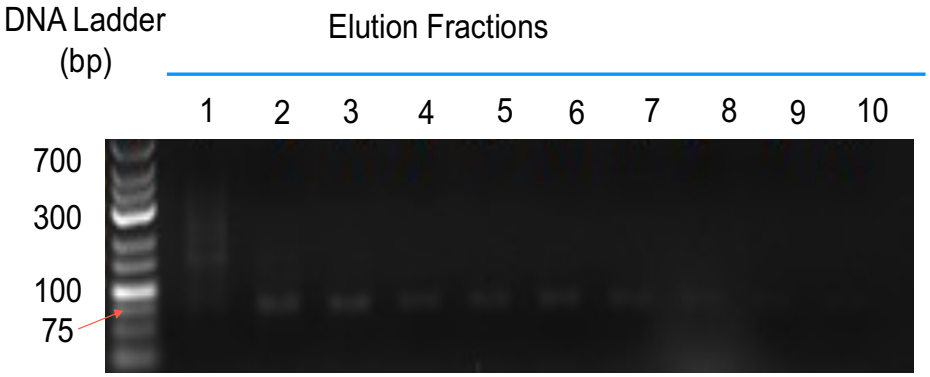

Fig 21. The gel electrophoresis image of the PCR amplified eluates was obtained during the 11th round of the SELEX.

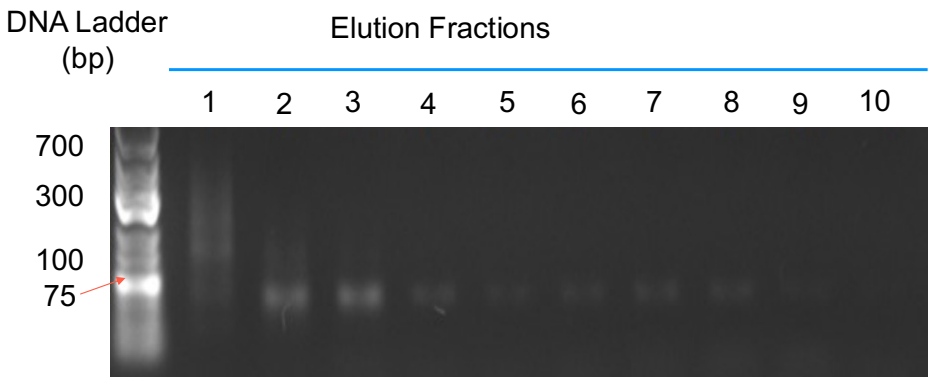

Fig 22. The gel electrophoresis image of the PCR amplified eluates was obtained during the 12th round of the SELEX.

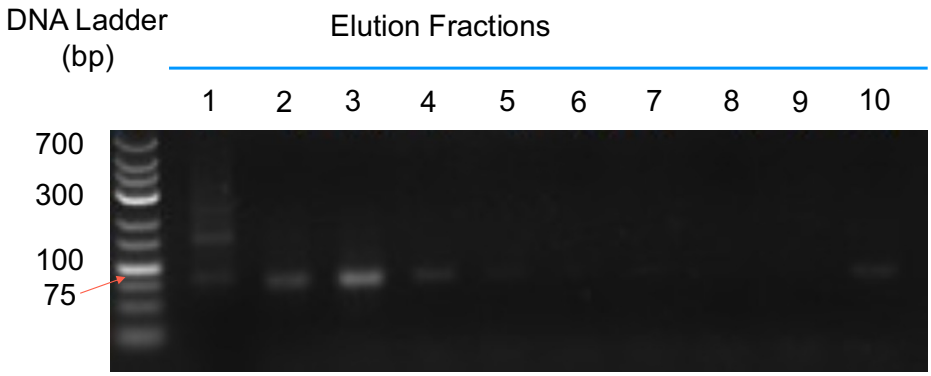

Fig 23. The gel electrophoresis image of the PCR amplified eluates was obtained during the 13th round of the SELEX.

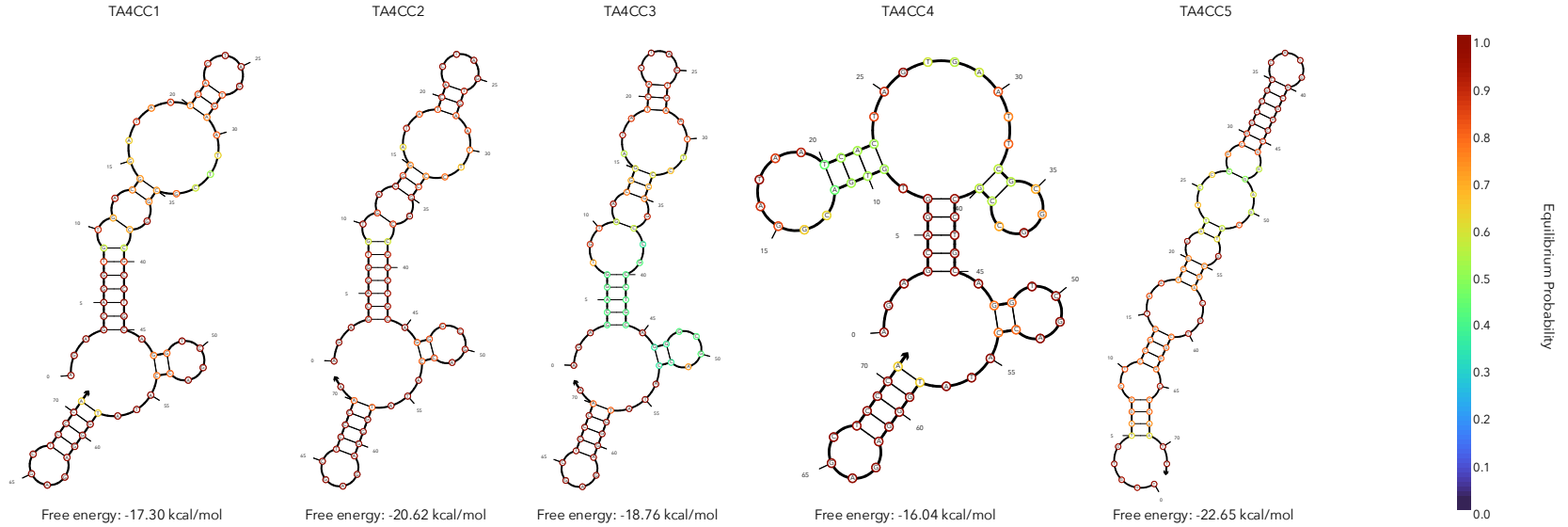

Fig 24. The secondary structure of the five aptamers, specific to TA4 was selected after the last SELEX round.

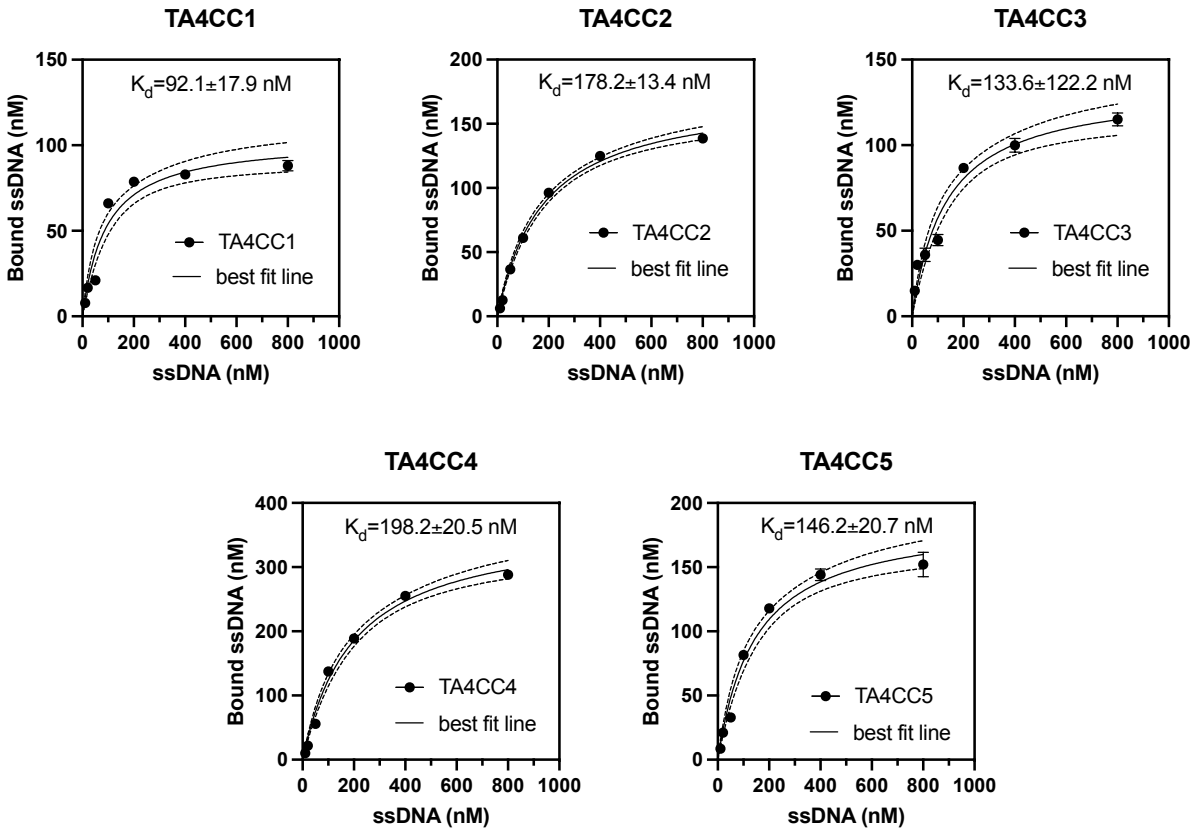

Fig 25. The binding affinity constant (K_d) of the respective TA4 aptamers. To determine K_D, $1x10^7$ TA4-coated magnetic beads were incubated with 100 µL of 10-800 nM aptamer (labeled at the 5' end with 6-carboxyfluorescein) for 1 h at room temperature. Unbound aptamers were collected by five quick washings with binding buffer; Bound aptamers were collected after incubating the beads with eluting buffer at 90°C for 10 min with mild shaking. The fluorescence of unbound and bound aptamers was measured at the excitation wavelength (494 nm) and the emission wavelength (520 nm) of 6-carboxyfluorescein.

Table 1: Sequences of the selected aptamers and respective binding affinity constant (Kd)

Aptamers	Sequence (5'→3')	Length (bp)	K_d (nM)
TA4CC1	AGAGCAGGTGTGACGGA TAATCACTAGTGAATTCG CGGCCGCCTGCAGGTCG ACCATATGGGAGAGCTC CCA	72	K_d=92.1±17.9 nM
TA4CC2	TGGTGTTGGCTCCCGTA AATCGAATTCCCGCGGC CGCCATGGCGGCCGGGA GCATGCGACGTCGGGCC CAAT	72	K_d=178.2±13.4 nM
TA4CC3	AGAGCAGGTGTGACGGA TATCACTAGTGAATTCCC GGCCGCCTGCAGGGCGA CCATATGGGAGAGCTCC CA	71	K_d=133.6±122.2 nM
TA4CC4	AGAGCAGGTGTGACGGA TAATCACTAGTGAATTCG CGGCCGCCTGCAGGTCG ACCATATGGGAGAGCTC CCA	72	K_d=198.2±20.5 nM
TA4CC5	TGGTGTTGGCTCCCGTA ATCGAATTCCCGCGGCC GCCATGGCGGCCGGGA GCATGCGACGTCGGGCC CAAT	71	K_d=146.2±20.7 nM

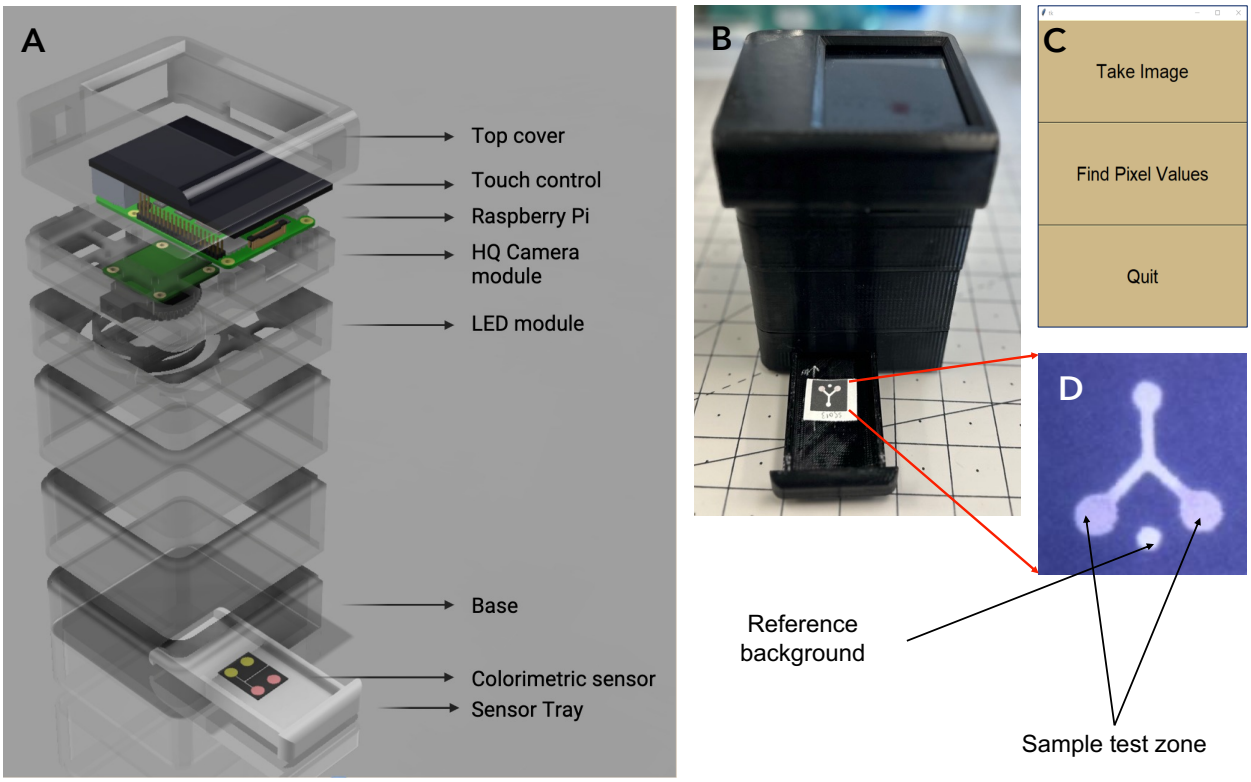

Fig 26. Schematic (A) of the 3D printed PURE-SCAN device (B) developed for detecting *Cyclospora cayetanensis*. The device consists of a Raspberry Pi 4B and a high-quality camera system with LED lights. The paper-based colorimetric sensors used in the device change their native red/reddish-pink color (analyte absent) to blue/blueish-gray in the presence of *Cyclospora cayetanensis* in the test sample. The device takes an optimally illuminated image of the paper-based μPAD (D) colorimetric sensors and processes it to create a calibration curve for quantitatively determining the concentration of contaminating pathogen(s) in the test samples. The program responsible for image analysis is written in Python and contains two main functions, accessible by graphical user interface (C) buttons.

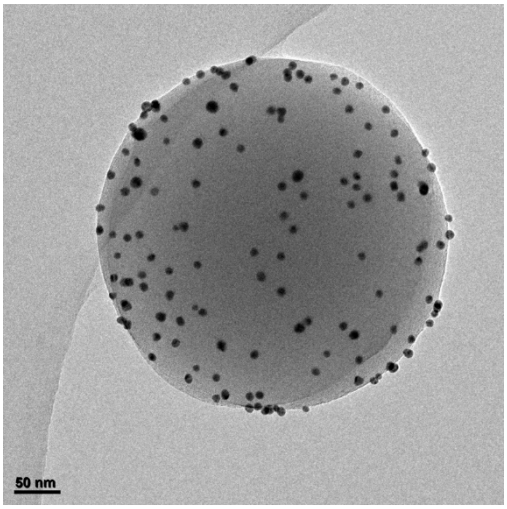

Fig 27. TEM images of the prepared polystyrene-gold nanoparticles.

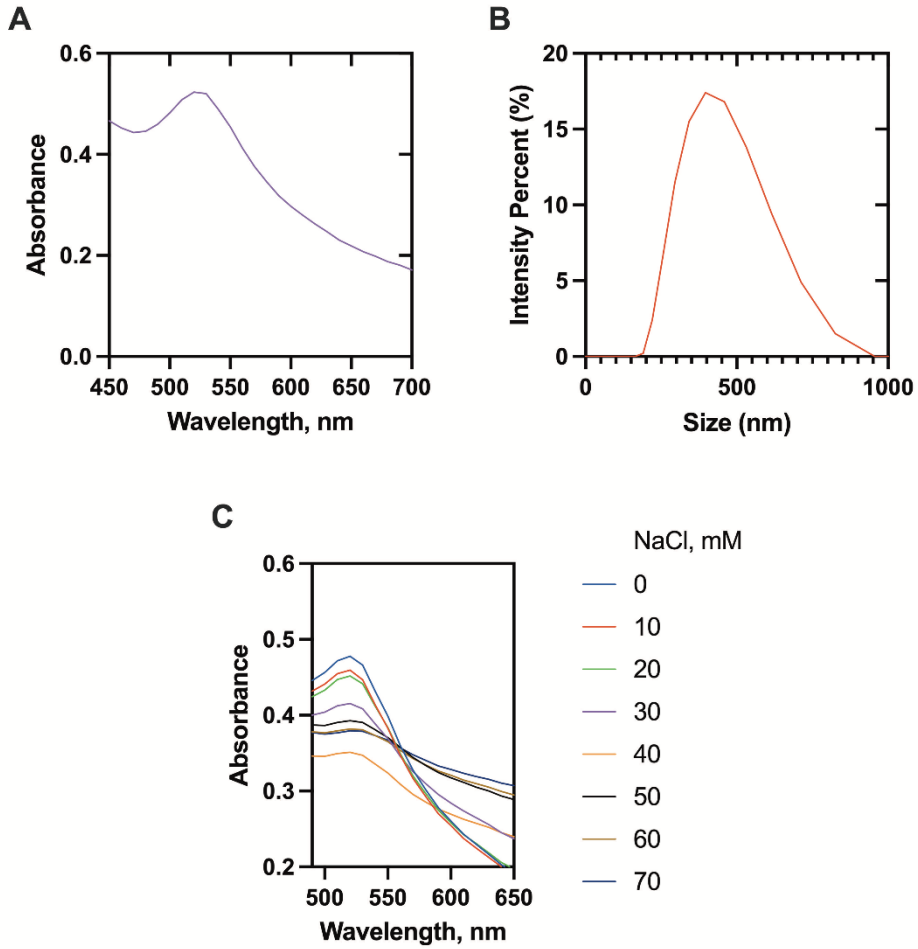

Fig 28. Characterization of the prepared PS-AuNP. A. UV-Visible absorption spectra; B. Particle size distribution; C. Salt aggregation test using increasing concentration of sodium chloride.

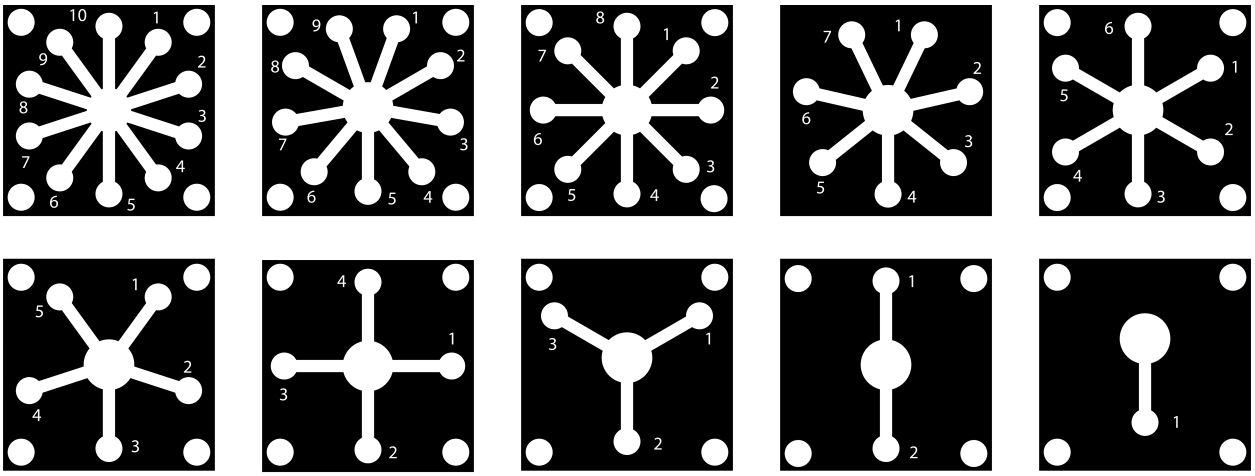

Fig 29. Design of various paper-based microfluidic analytical devices being tested in this project.