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HAL Authorization

1 **Selection of spore-specific aptamers for *Geobacillus stearothermophilus*, a food spoilage**
2 **bacterium**

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12 **Abstract:** Due to their ability to form extremely heat resistant spores, anaerobic bacteria are responsible for
13 frequent food spoilage. The development of rapid and specific methods for the detection and quantification of
14 spore contamination is therefore of major interest. In this paper, we describe for the first time the selection of
15 aptamers specific to spores of *Geobacillus stearothermophilus* (*Gbs*), which induce flat sour spoilage in vegetable
16 cans. Eighteen Spore-SELEX cycles were performed including 4 counter-selections with 12 bacteria commonly
17 found in cannery. To optimise candidate amplification, PCR in emulsion was performed, and high-throughput
18 sequencing analysis was applied to follow candidate evolution. Sequencing of aptamers from cycle 18 revealed 43
19 overrepresented sequences whose copy number exceeds 0.15% of the total obtained sequences. Within this group,
20 the A01 aptamer presented a much higher enrichment with a relative abundance of 17.71%. Affinity and specificity
21 for *Gbs* spores of the 10 most abundant candidates at cycle 18 were confirmed by PCR assay based on aptamer-
22 spore complex formation and filtration step. Obtaining these aptamers is the starting point for the future
23 development of biosensors dedicated to the detection of *Gbs* spores.

24 **Highlights:**

- 25 - *G. stearothermophilus* spore-specific aptamers were selected by Spore-SELEX
26 - Next Generation Sequencing data analysis identified 43 candidate sequences
27 - Spore binding affinity was confirmed for top 10 candidates

28 **Keywords:** Aptamers; Spore-SELEX; *Geobacillus stearothermophilus*; Food spoilage

29 **Declarations of interest:** none

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40 1. INTRODUCTION

41 The development of spore-forming thermophilic bacteria in canned food may result in food spoilage and food
42 poisoning [1]. The thermophilic bacteria species usually found in those products are either facultative anaerobes
43 such as *Geobacillus stearothermophilus* (*Gbs*) or obligate anaerobes such as *Thermoanaerobacterium*
44 *thermosaccharolyticum* and *Moorella thermoacetica* [1,2]. These species are non-pathogenic Gram-positive
45 bacteria which are able to grow in high temperature environments. Their spores are extremely heat resistant, with
46 a decimal reduction time of a few minutes at 121 °C [1,3]. In food production, growth of *Gbs* spores results in flat
47 sour spoilage due to acid production associated to little or no gas production [4,5] while thermophilic obligate
48 anaerobes usually produce abundant gas, responsible for can swelling [1]. These spoilages are mainly observed in
49 low acidic food such as milk, vegetables, meat and marine products under improper storage conditions. The others
50 most frequent spore forming bacteria identified in canned food belong to the *Bacillus*, *Thermoanaerobacter*,
51 *Caldanaerobius*, *Anoxybacillus*, *Paenibacillus* and *Clostridium* genera [6,7].

52 Standardised heat treatments are generally applied to prevent canned food spoilage. Their effectiveness can be
53 verified by carrying out stability controls on the finished products. The Codex Alimentarius, a collection of
54 internationally adopted food standards and related texts designed to protect the health of consumers, defines an
55 essential incubation period for international shipments of canned foods. The containers must be incubated for 10-
56 14 days at 37°C or 10 days at 35°C. These controls are also subject to regional or national standards. For example,
57 stability tests for canned products are defined by the French standards NF V08-401 [8] as reference method and
58 NF V08-408 [9] as routine method, consisting in food product incubation for 21 days or 7 days respectively at
59 32°C, 37°C or 55°C. Following this incubation step, various observations are performed such as aspect, odour,
60 texture, pH measurement and microscopic examination. Specific detection of spore-forming bacteria species
61 involves classical plate count method. These traditional culture-based assays require multiple steps and are guided
62 by different standards such as the following French standards NF V08-405 [10], NF V08-407 [11], NF V08-250
63 [12], NF V08-602 [13]. These methods allow the detection of 4 groups of spore-forming bacteria including aerobic
64 mesophilic and thermophilic, and anaerobic mesophilic and thermophilic, using two culture media (either
65 BromoCresol Purple agar (BCP) or Meat Liver Agar (MLA)) and two incubation temperatures (37 °C or 55 °C).
66 The incubation times are comprised between 2 and 5 days depending on the analysed group. These methods require
67 specific equipment, are labour intensive, time consuming and are not always successful if spores fail to germinate
68 [14].

69 Faster and more sensitive detection methods have been recently developed based on molecular techniques. Among
70 these methods, Polymerase Chain Reaction (PCR) has been the subject of considerable attention. Real-time
71 quantitative PCR (qPCR) is now regarded as a robust method for the detection and quantification of
72 microorganisms. Its main advantages over conventional culture methods are related to the rapidity, sensitivity, and
73 specificity of the method, and the possibility of simultaneous detection of different species [15]. However, PCR
74 methods are not really specific for bacterial spores detection as they are based on the recognition of genetic
75 material, which does not differ between spores and vegetative forms [14]. Only the detection of non-spore forming

76 foodborne pathogens by PCR is governed by ISO standards (ISO 22174:2005 [16], ISO 20836:2021 [17], ISO
77 20837:2006 [18], ISO 20838:2006 [19]).

78 Consequently, the development of reliable alternative methods for the detection and quantification of
79 microbiological risk linked to spore contamination has become of major interest. For such purposes, biosensors
80 appear as promising tools as they allow rapid and efficient diagnosis, they are easy to operate, and they can be
81 miniaturised [20,21]. These analytical devices are made of the tight association of a biorecognition molecule, often
82 called bioreceptor, and a physical transducer allowing the conversion of the recognition event into a measurable
83 signal. The bioreceptor is the key element since its properties condition the sensitivity and the selectivity of the
84 biosensor [22,23]. Biosensors dedicated to bacteria detection very often involve antibodies able to specifically
85 recognise determinants present on the bacterial cell surface. However, it is often difficult to design antibodies that
86 are able to discriminate between vegetative and spore forms of the same bacteria. In some cases, the knowledge
87 of the composition of cell surfaces can facilitate the development of specific antibodies [24,25]. For instance,
88 antibodies have been produced for the spores of *B. anthracis* and *B. cereus*, for which a specific oligosaccharide
89 named anthrose, present in the exosporium, has been identified [26]. However, external structures are not so well
90 deciphered for all the spore forming bacteria, thus hampering the development of appropriate antibodies.

91 One way to tackle selectivity problems could consist in using aptamers as bioreceptors. Aptamers are short
92 synthetic single-stranded oligonucleotides (20-100 nucleotides) that are able to bind to a target with high affinity
93 and specificity thanks to their three-dimensional structures. Aptamer selection occurs *in vitro* through an iterative
94 process termed Systematic Evolution of Ligands by EXponential enrichment (SELEX), which can be adapted to
95 the needs of the intended application. This protocol has been used for the selection of aptamers against a wide
96 range of targets, from small molecules [27–29] to whole cells [30], protozoan parasites [31] or pathogen bacteria
97 [32–34]. To date, specific aptamers against bacterial spores have been selected for *B. anthracis* [35,36], *B.*
98 *thuringiensis* [37], *Alicyclobacillus acidoterrestris* [38], and *B. cereus* [39].

99 However, only a few aptamers against bacterial spores have been used for analytical purposes. In 2007, an assay
100 was developed for the detection of *B. thuringiensis* spores based on the fluorescence observed upon binding of an
101 aptamer-quantum dot conjugate to spores [40]. In 2017, Fischer et al. integrated an aptamer recognising *A.*
102 *acidoterrestris* spores into a Lateral Flow Assay (LFA) [41]. More recently, a colorimetric paper chip was
103 developed in 2020 for the detection of *B. thuringiensis* spores [42]. These analytical methods were all tested only
104 under laboratory conditions and in buffered solutions.

105 The aim of this work was to select aptamers able to bind with spores of *Gbs*, which is with *M. thermoacetica* one
106 of the two strains most frequently found in spoiled canned food [7]. For this purpose, a SELEX protocol was
107 carried out using spores produced from a *Gbs* strain isolated from green beans sample, and including
108 numerous counter-selection cycles either with vegetative *Gbs* cells, or spore suspensions from 12 others spore
109 forming bacteria. PCR was performed in emulsion (emPCR) for a better amplification of DNA from random
110 libraries. Next Generation Sequencing (NGS) was then applied to allow a deeper candidate analysis and to monitor
111 DNA aptamer evolution over cycles. The interaction of spores with the most frequently selected aptamers was
112 then confirmed by aptamer-target complex formation confirmation by PCR.

113 2. MATERIALS AND METHODS

114 **2.1. Reagents, buffers and culture media**

115 All reagents and chemicals used were of either analytical grade or HPLC grade. All oligonucleotides, including
116 single stranded DNAs (ssDNAs) library, aptamers and primers, were purchased from Microsynth AG (Balgach,
117 Switzerland). The ssDNA library contained a central randomised sequence of 40 nucleotides flanked by two 18
118 nucleotides primer-binding regions for SELEX PCR amplification: 5'-CATCCGTCACACCTGCTC-40N-
119 GGTGTTGGCTCCCGTATC-3'. Two different primers were used for SELEX PCR amplification: *Gbs*-F 5'-
120 CATCCGTCACACCTGCTC-3', and *Gbs*-R 5'-biotin-GATACGGGAGCCAACACC-3'. Two other primers
121 were used for NGS analysis: NGS-F 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCATCCGTC
122 ACACCTGCTC-3' and NGS-R 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGATACGGG
123 AGCCAACACC-3'. The aptamer used as negative control for spore-aptamer binding assays was designed as
124 follow: 5'-CATCCGTCACACCTGCTCGTCAGCGATCAGTTAGCTAGAAGCGGTGTTGGCTCCCGTATC-
125 3'. The working solution of ssDNA library was prepared at 1 nM in the selection buffer (100 mM NaCl, 20 mM
126 Tris HCl pH 7.6, 5 mM KCl, 1 mM CaCl₂, 5 mM MgCl₂). All PCR amplifications were carried out with the Go
127 Taq® G2 Flexi PCR kits from Promega (Charbonnières les Bains, France).

128 **2.2. Bacterial strain and spore production**

129 All strains used in this study were isolated from canned food and identified using 16S sequencing by the French
130 Technical Centre for the Preservation of Agricultural Products (CTCPA). A primary revivification culture was
131 incubated during 48 h at 55 °C in Brain-Heart Infusion (BHI) broth. This primary culture was used as an inoculum
132 for the scale-up multiplication culture that was incubated for 48 h at 55 °C in BHI broth. This suspension was
133 designed as vegetative cell suspension. To produce spores, 1 mL of vegetative cell suspension was inoculated on
134 *Gbs* sporulation medium agar plates (GSM), composed of meat extract (10 g/L), yeast extract (2 g/L), type E agar
135 (15 g/L), and MnSO₄ monohydrate (0.04 g/L). The plates were incubated at 55 °C for at least 3 days. Sporulation,
136 following by phase-contrast microscopy counting (Olympus BX50), was stopped when free spores represented
137 more than 90% of cells. Spores were then harvested by pouring distilled sterile water into the plates and gently
138 scraping their surface, and the obtained suspensions were centrifuged at 6 000 g for 20 min at 4 °C. The resulting
139 pellet was resuspended in 5 mL of sterile water, heated 10 min at 100 °C to kill vegetative cells, cooled and stored
140 at 4 °C until use.

141 Spore production from other bacterial strains used for counter-selections were performed similarly but using their
142 corresponding medium (Table 1). All strains were isolated from spoiled canned food and identified by the CTCPA.

143 **2.3. Enumeration method**

144 To estimate bacterial concentration, ten-fold dilution series were prepared from vegetative cell suspensions in
145 tryptone salt broth, and 1 mL of each dilution was spread onto agar plates containing various media following
146 incubation at the appropriate temperature (Table 1). Colonies Forming Units (CFU) were then enumerated.
147 Counting of spores was carried out using the same protocol with a preheating step of 10 min at 100 °C, performed
148 prior to enumeration, to eliminate vegetative cells and induce germination.

149 **2.4. Purification of *Gbs* spore suspension**

150 To optimise the purity of *Gbs* spore suspension, an additional step was added. The remaining vegetative cells and
151 cellular debris were eliminated by three washing steps followed by centrifugation at 200 g for 5 min at 4 °C. The
152 obtained pellet was resuspended in cold sterile water. Spore purity was estimated after each washing step by phase-
153 contrast microscopy. After the three washing steps, spore concentration was enumerated as described before.

154 **2.5. *In vitro* selection of *Gbs* spore aptamer**

155 The SELEX protocol used in this work was adapted from the one described by Hänniger et al. [38] with some
156 modifications (Fig. 1).

157 The first four cycles started by a counter-selection step against *Gbs* vegetative cells suspension. Before use, the
158 ssDNA library was heated at 95 °C for 5 min, cooled on ice for 5 min and kept at room temperature for 20 min to
159 allow the formation of folded DNA structures. Denaturation step was performed at the beginning of each SELEX
160 cycle. For the first cycle, 100 µL of 1 nM denatured ssDNA library were incubated for 60 min with *Gbs* vegetative
161 cells suspension at a final concentration of 10⁵ CFU/mL. After the binding reaction, the mixture was centrifuged
162 during 12 min at 10 500 g. The pellet containing vegetative cells-bound oligonucleotides was discarded, and the
163 supernatant was used for the next SELEX step. After a new denaturation step, the collected oligonucleotides were
164 incubated for 60 min with purified *Gbs* spores at a final concentration of 10⁵ spores/mL. A centrifugation was then
165 performed during 12 min at 10 500 g, the supernatant was discarded, and the pellet was washed several times with
166 200 µL of binding-and-washing buffer (BW: 1 mM EDTA, 2 M NaCl, 10 mM Tris-HCl, pH 7.5), and resuspended
167 in 35 µL of sterile nuclease-free water. A heating step of 5 min at 95 °C was performed to separate the spores from
168 the oligonucleotides, followed by a quick centrifugation (10 s) at 10 500 g. The aptamer candidates were finally
169 collected in the supernatant.

170 Amplification of ssDNAs was achieved by emPCR, carried out using the Micellula DNA Emulsion and
171 Purification Kit (EUR_x, Gdańsk, Poland). The PCR mixture for amplifying ssDNA candidates contained 0.025 U
172 of DNA Polymerase, 1X of buffer, 2 mM of MgCl₂, 0.8 mM of dNTPs and 4 µM of each primer *Gbs*-F and *Gbs*-
173 R. Positive and negative emPCR controls were performed using ssDNAs from the starting library and ultrapure
174 water, respectively.

175 Oil-phase was prepared separately according to Micellula protocol and kept on ice. PCR mixture was added in the
176 cold oil-phase and mixed vigorously for 5 min at room temperature to create emulsion. The mixture was divided
177 into several 50 µL aliquots and emPCR was carried out under the following conditions: preheat at 95 °C for 5 min,
178 30 cycles of 95 °C for 30 s, 61.5 °C for 30 s, 72 °C for 30 s and final elongation at 72 °C for 5 min. Finally, the
179 emulsion was broken using methanol, the oil-phase was discarded and emPCR product was purified using
180 Micellula kit.

181 A strand separation step was mandatory to retrieve only ssDNA aptamers. For this purpose, 100 µL of streptavidin
182 magnetic beads (Dynabeads M-270 Streptavidin, Fisher Scientifics S.A.S.) were incubated under agitation with
183 biotinylated emPCR products in BW buffer. After 15 min, the supernatant was discarded, the beads were
184 resuspended in 80 µL of sterile distilled water and heated during 10 min at 95 °C for emPCR products denaturation.
185 The tube was immediately centrifuged for 10 s at 10 500 g and placed on a magnetic rack. The hot supernatant
186 containing the desired aptamers was collected and kept at 4 °C until the following cycle.

187 The monitoring of the SELEX process was assessed by 7% denaturing PolyAcrylamide Gel Electrophoresis
188 (PAGE). Thus, the emPCR products after purification and after strand separation were deposited as well as the
189 emPCR positive and negative controls. The bands were revealed by post staining using 3X GelRed® Nucleic Acid
190 Stain.

191 Subsequent selection cycles were performed with decreasing the amount of input target (from 20 µL to 15 µL
192 spore solution) and incubation times (from 60 to 10 min) and increasing the number of washing steps (from 1 to
193 3). All conditions are summarised in Fig. 1.

194 Elimination of non-specific binding sequences was performed by carrying out from cycle 15 several counter-
195 selection cycles with mixtures of non-target spores, as performed with vegetative *Gbs* cells. Each bacterial strain
196 was used at 10⁶ spores/mL and exposed to the aptamer candidate pool for 60 min. Mixture specie compositions
197 used in counter-selection cycles are described in Table 1. 4 cycles with counter-selection were carried out
198 following the previous 14 classical cycles, bringing the total number of SELEX cycles to 18.

199 **2.6. Aptamer identification**

200 Aptamer candidate diversity was monitored using a high throughput sequencing of emPCR products after strand
201 separation obtained upon cycles 8, 10, 12, 14, 16 and 18. A two-step procedure was used to amplify the emPCR
202 products after the strand separation step of the six selected SELEX cycles. In the first step, a PCR was done using
203 the NGS-F and NGS-R primers under the following conditions: 1 U of DNA Polymerase, 1X of buffer, 2 mM of
204 MgCl₂, 0.2 mM of dNTPs and 0.2 µM of each primer NGS-F and NGS-R in a total volume of 50 µL. After a
205 preheat step at 95 °C for 5 min; 10 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s were carried out
206 followed by a final elongation step at 72 °C for 5 min. The second barcoding step, size amplicons verification and
207 normalization were performed by the Bio-Environment platform (University of Perpignan). Diversity of the
208 aptamer candidates from the different cycles was assessed by Illumina 2x250 base-pair MiSeq sequencing run.

209 **2.7. Sequencing data analysis**

210 The sequencing data were first analysed with FastQC software and confirmed using QIIME 2. The frequency of
211 occurrence of sequences, as well as the sequence categories were expressed as a percentage of the total number of
212 reads in each SELEX run (% of total reads). Over-represented sequences, considered to represent more than 0.1%
213 of all aptamers sequenced in each run, were listed for each sequenced run. Multiple sequence alignment was carried
214 out using ClustalW. Alignment and phylogenetic reconstructions were performed using the "build" function of
215 ETE3 3.1.2 [43] as implemented on the GenomeNet (<https://www.genome.jp/tools/ete/>). The tree was constructed
216 using FastTree v2.1.8 with default parameters [44]. Secondary structure and free energy estimation of the aptamers
217 were predicted using the NUPACK web application [45].

218 **2.8. Spore-aptamer binding assays**

219 The recognition capacity for the target was evaluated using the 10 most abundant aptamers of cycle 18. Briefly,
220 0.1 µM of denatured A01 to A10 aptamers was incubated for 60 min with 10⁷ *Gbs* spores in 1 mL of selection
221 buffer under agitation. Then, the volume was adjusted to 10 mL with selection buffer and the suspension was
222 immediately filtered through a Nuclepore Track-Etch Membrane (PC MB 47 mm 0.2 µm, Whatman) to eliminate

223 unbound aptamers. The filter was then washed with 10 mL of selection buffer, transferred in a new tube with 1
224 mL physiological water and strongly agitated for 2 h to transfer the spore-aptamer complex into the liquid phase.

225 The resulting retentate was amplified by PCR to reveal the presence of bound aptamers. The PCR
226 mixture contained 0.025 U of DNA Polymerase, 1X of buffer, 0.1 mM of MgCl₂, 0.2 mM of dNTPs and 0.1 μM
227 of each primer in a total volume of 50 μL. 20 μL of retentate was added to this mix and PCR was carried out under
228 the following conditions: preheat at 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 15 s and
229 final elongation at 72 °C for 5 min. The PCR products were then analysed by 7% PAGE.

230 Several negative controls were performed in technical triplicates to ensure the validity of the results. Firstly, to
231 verify that aptamers are not retained by the filters, 0.1 μM of each aptamer were incubated without spore
232 suspension, then filtered under the same conditions as described before, and the retentate was submitted to PCR
233 amplification.

234 Then, the specificity of the 10 most abundant aptamers was controlled by carrying out the same experiments using
235 *Paenibacillus sp.* spores instead of *Gbs* spores.

236 Finally, to verify that the aptamers were not retained during the filtration process due to spore obstruction on the
237 filter membrane surface, an aptamer having the two nucleotides primer-binding sequences and a random internal
238 sequence of 24 nucleotides was designed. 0.1 μM of this aptamer was incubated with *Gbs* spores suspension as
239 described above, and the retentate was submitted to PCR amplification.

240 **3. RESULTS**

241 **3.1. Preparation of bacterial spore suspensions**

242 In a first time, spore suspensions of *Gbs* and of the different strains used in the counter-selection cycles were
243 produced on agar plates under appropriate conditions and temperature. Spores concentrations were then
244 determined using a culturable enumeration method (Table 1). The concentrations obtained from the spore
245 suspensions intended for the counter-selection cycles allowed their use at a final concentration of 10⁶ spores/mL.

246 A specific protocol of purification was applied to *Gbs* spore suspension to increase the specificity of candidate
247 aptamers for spore binding rather than to vegetative forms. The resulting spore concentration after purification was
248 5.4 10⁶ spores/mL, which allowed this suspension to be used during the SELEX at a final concentration of 10⁵
249 spores/mL.

250 **3.2. Spore-SELEX**

251 The SELEX protocol for the selection of aptamers specific to *Gbs* spores was conducted over a total of 18 cycles.
252 The first cycle consisted in a counter-selection with *Gbs* vegetative, allowing elimination of DNA sequences
253 specific to these forms. Similarly, to increase aptamer specificity, the last 4 cycles (cycles 15 to 18) were preceded
254 by counter-selection phases performed with a mixture of various bacterial spores frequently found in the food
255 environment (Table 1). In addition, working conditions were modified throughout the SELEX process to increase
256 the stringency and improve selection pressure and screening efficiency, and finally retain the aptamers presenting
257 the highest affinity (Fig. 1). The monitoring of the SELEX process was done by electrophoresis at the end of each

258 cycle to confirm the size and abundance of the selected DNAs. emPCR products were deposited on PAGE gels
259 before and after the strand separation step as well as the emPCR positive and negative controls. As a reminder, 76
260 nucleotides-long DNA were used in the starting library. Migration patterns obtained before and after strand
261 separation were identical, and were also similar to this obtained for the positive control, as shown in Fig. S1 for
262 cycles 8 and 9. Nevertheless, some differences in length of emPCR products compared to the positive control
263 appeared since cycle 11, with additional shorter fragments that became more and more visible until they became
264 the majority at cycle 14 (data not shown). In any case, no band has ever been observed for the negative controls.
265 Those observations were constant all over the SELEX cycles, validating each cycle before proceeding to the next.
266 To allow a complete analysis of the aptamers and to follow their evolution during the SELEX, DNA candidates
267 obtained at the end of cycles 8, 10, 12, 14, 16 and 18 were sequenced by NGS. This method generates between
268 912,496 and 1,585,742 sequences per cycle for a total of 8,001,215 sequences.

269 Nucleotide sequence analysis aimed to characterise the most widely represented sequences that should correspond
270 to the best candidates in terms of target recognition capacity. Indeed, a large number of copies of a single sequence
271 reflects its better conservation during the selection process and thus its possible good affinity for the target. It is
272 therefore important to classify the data obtained during sequencing in order to highlight the most frequently
273 encountered nucleotide sequences. Thus, the sequences were grouped into five frequency categories according to
274 their number of reads: from 1 to 9, from 10 to 99, from 100 to 999, from 1,000 to 9,999 and more than 10,000
275 times. This data processing was adapted from Kolm et al. [46].

276 Analysis of the NGS data showed that sequence enrichment was already observable in cycle 8, where sequences
277 comprised between 1,000 to 9,999 reads were already present (1.65%) (Fig. 2A). For the cycles 8 and 10, the
278 sequences counted 9 times or less are in a majority with 60.7% and 53.4%, respectively. The proportion of this
279 category of sequences decreased over the SELEX cycles, reaching 17.5% in cycle 18. Similarly, the sequences
280 with a number of reads comprised between 10 and 99 were shown to decrease from 27.7% in cycle 8 to 9.4% in
281 cycle 18. Sequences with a number of reads between 100 and 999 were observed at a maximum frequency of 14%,
282 after a minimum of 10.0% observed in cycle 8. Conversely, the frequencies of the sequences with a number of
283 reads between 1,000 and 9,999 increased during the SELEX, from 1.7% in cycle 8 to 16.4% in cycle 18. Finally,
284 the most significant increase was observed for the category of sequences having more than 10,000 reads. Although
285 it was not present in cycle 8, this category appeared in cycle 10 with a frequency of 1.9% and increased rapidly to
286 become a large majority in cycle 18 (43.6%).

287 The analysis of the sequencing data also permits monitoring the evolution of the selected DNAs length along the
288 SELEX process. These data confirmed the results of emPCR product size observed with PAGE analysis. Fig. 2B
289 shows the distribution of sequenced DNAs grouped into length categories at the end of cycles 8, 10, 12, 14, 16 and
290 18. Similarly to the previous analysis, obtained values were normalised to the total number of reads in each SELEX
291 run. Up to cycle 10, fragments between 75 and 79 nucleotides were by far the most representative. In subsequent
292 cycles, shorter fragments emerged at the expense of longer ones, and the 75-79 category almost disappeared in
293 cycle 18, with an abundance of only 1.98%. Interesting to note that the category that emerged most rapidly from
294 cycle 12 consisted of fragments of 55 to 59 nucleotides, followed by those with sizes between 60 and 64
295 nucleotides. In cycle 18, those two categories represented respectively 53.43% and 39.10% of the total sequences.

296 Surprisingly, the intermediate categories with a length between 65 and 74 nucleotides were globally not very
297 abundant, with a maximum of 17.17% in cycle 16.

298 A sequence table was then generated including sequences with a copy number exceeding 0.15% of the total
299 sequences obtained in each cycle. Due to the enrichment of DNAs during SELEX, the cycle with the most of these
300 sequences is cycle 18 with 43 candidates. Those sequences were ranked according to their frequency, and their
301 evolution was tracked from the first sequenced cycle to the last one. The abundances throughout the SELEX cycles
302 of the 10 most represented aptamers in cycle 18 are presented in Fig. 3.

303 These data show that the percentage of abundance of those 10 aptamers globally increased during SELEX. The
304 A01 aptamer was by far the most abundant from cycles 14 to 18, accounting for 17.71% of all the sequenced
305 ssDNAs in cycle 18 and thus being more than 5 times more abundant than A02 aptamer in cycle 18. Furthermore,
306 it is important to note that the abundance of A01 aptamer did not decrease during the counter-selection cycles
307 (cycles 15 to 18), suggesting that this sequence is specific to *Gbs* spores and is not likely to bind to other spores
308 used in counter-selections.

309 In contrast, a drop in abundance was observed for the A10 aptamer between cycles 16 and 18, suggesting that this
310 sequence could be specific to at least one of the spores used in counter-selections. This aptamer was also included
311 in the list of candidate sequences for further testing as it showed high abundance in all other cycles, being even
312 the most abundant up to cycle 12.

313 **3.3. Alignment of sequences obtained in cycle 18**

314 In order to study the similarities between the over-represented sequences highlighted by the NGS data, an
315 alignment of the 43 selected aptamers was performed and represented as a phylogenetic tree (Fig. 4).

316 As can be seen in Fig. 4, the 43 candidates are divided into 5 heterogeneous main branches. The 10 most
317 represented aptamers in cycle 18 are fairly evenly distributed among these branches. Thus, the aptamers A01 and
318 A02 belong to the same family, as for A05 and A08, A03 and A09 and A04, A06 and A07. Only the sequence
319 A10 is separated in a branch of only two candidates.

320 In the first family, 13 sequences are included in a phylogenetic group with closely related individuals. This set of
321 sequences, including the A01 aptamer, is referred to as the A01 cluster, and those 13 candidates are present among
322 the 43 most represented sequences in cycle 18. Alignment of these sequences (Fig. 5) shows that they differ from
323 each other by only one nucleotide, placed at different positions. Together, these aptamers represent 21.8% of the
324 sequences obtained in cycle 18.

325 The A01 sequence, used here as reference, displays a high cytosine ratio of 42%. No deletions or additions are
326 observed among the A01 cluster but only replacements. 6 of the 12 replacements involve cytosines that are
327 systematically substituted by thymines. Although they have very similar sequences, the aptamers of the A01 cluster
328 show very different percentages of abundance at cycle 18. For instance, the second highest ranked aptamer in this
329 cluster after A01 is A11, showing an abundance of only 0.59%, being 30 times lower than A01. A prediction of
330 their most likely secondary structures by NUPACK allowed visualising the opening of a loop structure due to the
331 replacement of a guanine by a cytosine at position 36 (Fig. 6).

332 The 2D structure analysis of the cluster A01 aptamers also showed that certain patterns are highly conserved. This
333 is the case for the stem-loop structure composed of bases 4 to 29, which is identical in 12 of the 13 sequences, as
334 A17 aptamer displays a very similar structure whose stem is shortened by 1 base.

335 Alignment and comparison of the secondary structures of the first 10 aptamers obtained in cycle 18 were also
336 performed (Fig. S2 and S3). The analysis of conserved patterns among these sequences was made difficult by the
337 variable size of the aptamers.

338 **3.4. Affinity tests**

339 To access the recognition capacity of the 10 most abundant aptamers of cycle 18, each of them was incubated with
340 the same amount of *Gbs* spores for 1 hour, following by a 0.2 μm filtration step of the resulting retentate in order
341 to separate the spores-aptamer complex from the unbound aptamers. Bound aptamers were then revealed by PCR
342 amplification.

343 The designed protocol was first validated by several assays. It was first checked that the used membrane did not
344 retain the aptamers in the absence of spores. As shown in Fig. 7a, lane C1, no PCR product was observed in
345 samples when filtration assays were performed only with aptamers. Migration pattern revealed only the presence
346 of the primers at the bottom of the gel. This condition was tested three times in independent trials.

347 The risk of membrane saturation by spores, resulting in an unspecific retention of aptamers, was also assessed in
348 three independent trials using a 60 nucleotides ssDNA whose sequence was not found among the 8,001,215
349 sequences obtained by NGS. Absence of PCR amplification from the resulting retentate obtained after incubation
350 of this aptamer with *Gbs* spores validated our protocol, as shown in Fig. 7a, lane C2.

351 The developed protocol was then applied to the 10 most abundant aptamers. The results of the assays on aptamers
352 A01 to A10 are shown in Fig. 7b. The presence of PCR products in all cases demonstrated that these aptamers are
353 able to bind to *Gbs* spores during incubation time. Differences in migration height were assigned to differences in
354 aptamer sizes.

355 In order to test the specificity of the selected aptamers, similar assays were carried out with *Paenibacillus sp.*
356 spores. As shown in Fig. 7c, no amplification of the aptamers was observed, confirming that the tested aptamers
357 were not able to bind to *Paenibacillus sp.* as they do to *Gbs* spores.

358 **4. DISCUSSION**

359 In order to be able to develop specific detection tools for *Gbs* spores, recognition elements must be identified. To
360 our knowledge, no antibody or other specific element has been designed to date against these spores. This study
361 therefore focused on the development of aptamers specific to *Gbs* spores. Different variations of SELEX protocol
362 have been described in literature, some of them using whole cells as target, as proposed for the spore-SELEX [38].
363 One of the strengths of this protocol is the use of emulsion PCR for the amplification step of the candidates selected
364 during the cycles. Indeed, the formation of by-products during the PCR amplification step inhibits product
365 generation and therefore limits the application of conventional PCR in aptamer selection [47,48]. Emulsion PCR,
366 as a single-molecule PCR, allows the amplification of complex DNA libraries whilst avoiding the appearance of
367 chimeric products [49,50].

368 Due to the considerable progress in NGS technologies over the last decade, Sanger sequencing of aptamers
369 obtained after cloning is being gradually replaced by high-throughput sequencing [51–53]. Indeed, this technology
370 allows for a very large amount of data to be obtained from different cycles. This allows monitoring of the SELEX
371 protocol as it provides maximum information on the evolution of the pool and is very useful for understanding the
372 process and progression of SELEX [54]. The data collected during the various sequencing cycles show that the
373 level of overexpression of the candidate sequences evolves more significantly after a dozen SELEX cycles. This
374 confirms that the choice of the number of cycles carried out in this study is relevant.

375 In our study, the secondary structure predictions did not identify any conserved structures or motifs among the 10
376 most abundant sequences in cycle 18. Similar results have been recently observed for the aptamers most frequently
377 identified, following NGS analysis against *Enterococcus faecalis* [46] and against *Escherichia coli* and *Listeria*
378 *monocytogenes* [55]. Nevertheless, our sequences show great structural complexity with hairpin loops, internal
379 loops and bulges. It is recognised that the key molecular interactions that confer the specificity of aptamers to their
380 targets include precise stacking of flat moieties, electrostatic and van der Waals interactions, and hydrogen bonding
381 [56,57]. The high structural diversity observed among the selected aptamers increases the probability of the
382 presence of such molecular interactions. In particular, it is well known that stem regions play an important role in
383 binding the target [58]. Moreover, this high diversity of structural features may suggest that the obtained aptamers
384 presumably bind to various spore surface areas and not a specific spore determinant.

385 The results obtained also showed the presence of a cluster of candidate sequences differing from each other by
386 only one base. This suggests that all these sequences could be able to recognise the same target. However, it is
387 probable that the mutations observed among these sequences impact their recognition efficiency, which could
388 explain the overexpression level variability between A01 cluster candidates. In contrast, the locations without
389 mutations, such as the first 25 bases, could reveal the regions essential for target recognition. Analysis of the 2D
390 structures of the A01 cluster shows that the stem-loop structure generated by the first 25 bases is conserved in 12
391 sequences out of 13 (Fig. 6).

392 NGS data showed that the level of overexpression of certain sequences, such as A10, decreases during counter-
393 selection cycles. It can be expected that the A10 aptamer would be able to bind to an element shared by *Gbs* spores
394 and spores of one or more of the species exposed in cycles 17 and/or 18, *i.e.*, *M. thermoacetica*, *C. sporogenes*,
395 *Anoxybacillus sp.*, *Paenibacillus sp.*, *Paenibacillus polymyxa*. However, affinity tests showed that aptamer A10
396 did not seem to be able to bind to *Paenibacillus sp.* spores. It would therefore be interesting to carry out the same
397 affinity tests with the other spores mentioned. Indeed, an aptamer that is not only specific to *Gbs* but able to target
398 a wider range of spores could be suitable for the development of an analytical tool that recognises a variety of
399 bacterial spores. There are already aptamers capable of recognising groups of bacteria, presumably based on the
400 presence of common structural compounds on their surfaces. This is the case of aptamers published by Shin et al
401 in 2019 being able to target several Gram-negative bacteria [59].

402 Affinity tests were carried out for the 10 most abundant aptamers in cycle 18 with a method adapted from Tuerk
403 and Gold [60]. Due to the nature of these tests, they provided qualitative but not quantitative results. These tests
404 were performed to evaluate the ability of the aptamers to recognise or not *Gbs* spores under the conditions tested.
405 However, it is not possible to quantitatively compare the aptamers with each other or to determine an
406 association/dissociation constant by this method. In our study, the results showed that all 10 aptamers tested are

407 able to bind to *Gbs* spores. Furthermore, cross-reactivity tests showed no interaction with *Peanibacillus sp.* spores
408 under the same conditions.

409 More advanced affinity tests could allow the quantitative characterisation of the recognition capacity of the
410 aptamers selected in this study before applying them to further research. Several biophysical instruments or
411 techniques could be investigated to measure aptamer-target binding affinity [61,62]. For instance, Microscale
412 Thermophoresis (MST) [63,64] and Quartz Crystal Microbalance (QCM) [65,66] are two methods that could allow
413 us to measure dissociation constants (Kd) for our aptamers towards *Gbs* spores.

414 5. CONCLUSION

415 This work reported for the first time the possibility of isolating aptamers specific to *Gbs* spores using spore-SELEX
416 protocol. An aptamer cluster of 13 over-represented sequences was identified containing the aptamer A01, alone
417 representing 18% of the aptamers present in cycle 18. Qualitative binding experiments confirmed that the isolated
418 aptamers were able to specifically bind to *Gbs* spores. These aptamers are thus promising candidates for their
419 integration as biorecognition molecules in analytical devices. Several types of biosensors can be considered, based
420 on different transduction methods. Paper chip systems will be first explored due to their simple setup and
421 compatibility to field use. A previous work has demonstrated the feasibility of capturing bacterial spores of
422 *Bacillus thuringiensis*, on the surface of polyvinylidene fluoride (PVDF) paper strips [42]. The possibility of
423 electrochemical transduction methods will be also explored based on the works of Mazzaracchio et al. [67], who
424 described the impedimetric detection of *Bacillus cereus* spores with a detection limit of only 3.10^3 CFU/mL. Thus,
425 the implementation of the selected *Gbs* aptamers in a biosensor system will offer a new analytical tool, available
426 for food quality analysis.

427 Compliance with Ethical Standards

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442 NP, LB and TN conceived and directed research. GD wrote the manuscript. All authors read, amended and
443 approved the manuscript.

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649

650 Figure captions

651 **Fig. 1** Schematic illustration of aptamer selection for *Gbs* spores by spore-SELEX protocol

652 **Fig. 2** NGS analysis of the aptamer population. **a** Frequencies of sequences that were counted 1 to 9, 10 to 99, 100
 653 to 999, 1,000 to 9,999 and more than 10,000 times in the SELEX cycles 8, 10, 12, 14, 16 and 18. **b** Frequencies of
 654 the sequences with lengths ranging from 75 to 79, 70 to 74, 65 to 69, 60 to 64, 55 to 59, and lower than 54
 655 nucleotides in SELEX cycles 8, 10, 12, 14, 16 and 18

656 **Fig. 3** Evolution of the abundance over the SELEX course of the main aptamers found in cycle 18. Dot lines
 657 between points are shown for a better visibility of aptamer abundance evolutions over sequenced cycles

658 **Fig. 4** Phylogenetic tree resulting from the alignment of the 43 overrepresented sequences from the NGS analysis

659 **Fig. 5** Aptamer sequences of the A01 cluster. Nucleotides differing from the sequence of A01 aptamer are shown
 660 in red. Conserved regions complementary to the primers used for emPCR are represented in green

661 **Fig. 6** 2D structures of A01 cluster aptamers modelled by NUPACK web application (parameters: 25 °C, 100 mM
 662 Na⁺, 5 mM Mg²⁺). The nucleotides that differ between sequences are shown in red

663 **Fig. 7** Polyacrylamide gel electrophoresis analysis of PCR products from retentate obtained after filtration. **a** C+:
 664 PCR positive control; C1: assay performed with aptamers only; C2: assay performed with *Gbs* spores and non-
 665 specific aptamer. **b** Retentates after incubation of aptamers A01 to A10 with *Gbs* spores. **c** Retentates after
 666 incubation of aptamers A01 to A10 with *Paenibacillus sp.* spores

667 Table

668 **Table 1** Bacteria species used during selection and counter-selection cycles. Medium, incubation temperatures
 669 and concentrations of initial spore suspensions are indicated

Bacteria species	SELEX cycle	Medium	Incubation temperature	Spore concentration (spores/mL)
<i>Geobacillus stearothermophilus</i>	1-18	GSM ^a	55 °C	9,90E+08
<i>Thermoanaerobacterium thermosaccharolyticum/bryantii</i>	15	MLA ^b	55 °C	3,00E+08
<i>Thermoanaerobacterium sp.</i>	15	MLA ^b	55 °C	5,50E+07
<i>Bacillus coagulans</i>	16	BCP agar ^c	37 °C	2,40E+08
<i>Bacillus cereus</i>	16	BCP agar ^c	37 °C	1,60E+09

<i>Bacillus subtilis</i>	16	BCP agar ^c	37 °C	7,40E+09
<i>Bacillus licheniformis</i>	16	BCP agar ^c	37 °C	7,00E+10
<i>Bacillus smithii</i>	16	BCP agar ^c	55 °C	3,60E+09
<i>Moorella thermoacetica</i>	17	MLA ^b	55 °C	1,90E+08
<i>Clostridium sporogenes</i>	17	MLA ^b	37 °C	2,70E+08
<i>Anoxybacillus sp.</i>	18	BCP agar ^c	55 °C	2,90E+09
<i>Paenibacillus sp.</i>	18	BCP agar ^c	37 °C	8,40E+09
<i>Paenibacillus polymyxa</i>	18	BCP agar ^c	37 °C	5,60E+08

^a GSM: Gbs Sporulation Medium, see detailed composition in section "Bacterial strain and spore production"

^b MLA: Meat Liver Agar

^c BCP agar: Bromocresol purple lactose agar

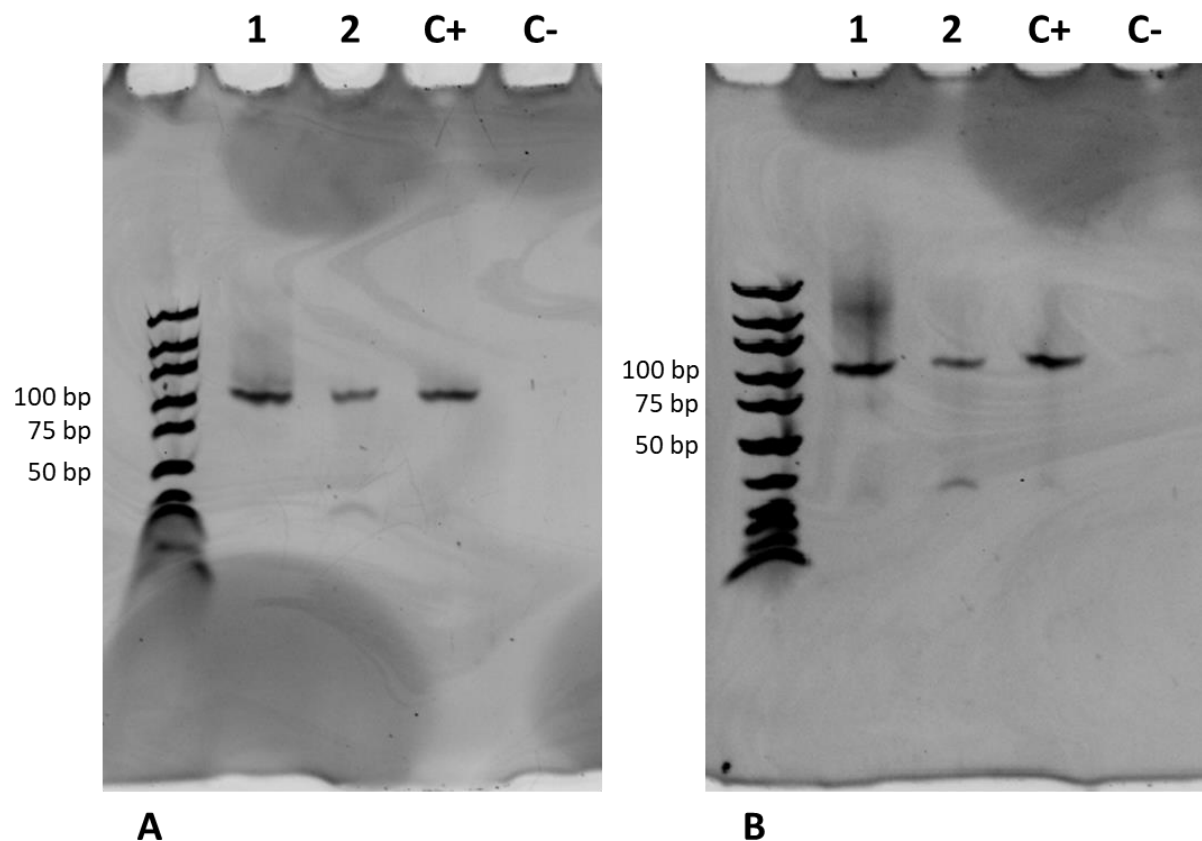
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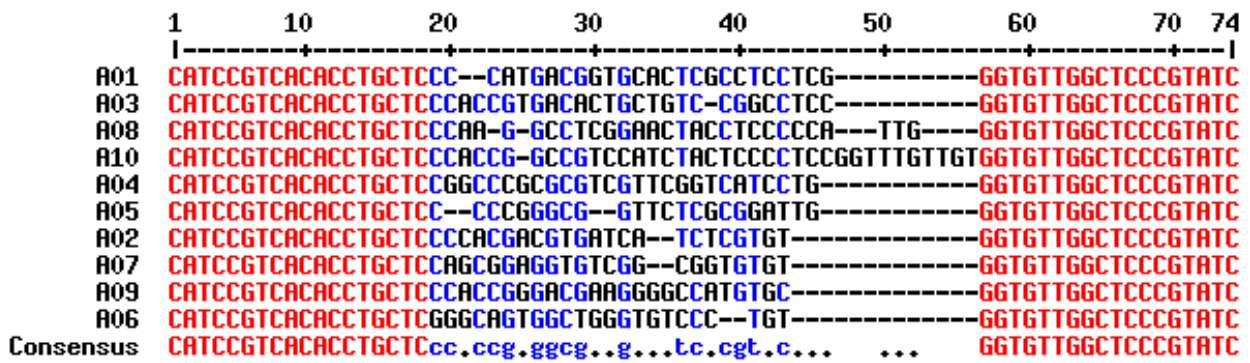
673 SUPPLEMENTARY MATERIAL

674 Figure S1



675 **Fig. S1** Denaturant polyacrylamide gel electrophoresis of emPCR products obtained after the SELEX cycles N°8
 676 (A) and N°9 (B). Lane 1: amplification product of resulting DNAs at the end of SELEX cycle. Line 2: amplification
 677 product of resulting DNAs at the end of SELEX cycle after strand separation. Lines C+ and C-: positive and
 678 negative emPCR control, respectively

679 Figure S2



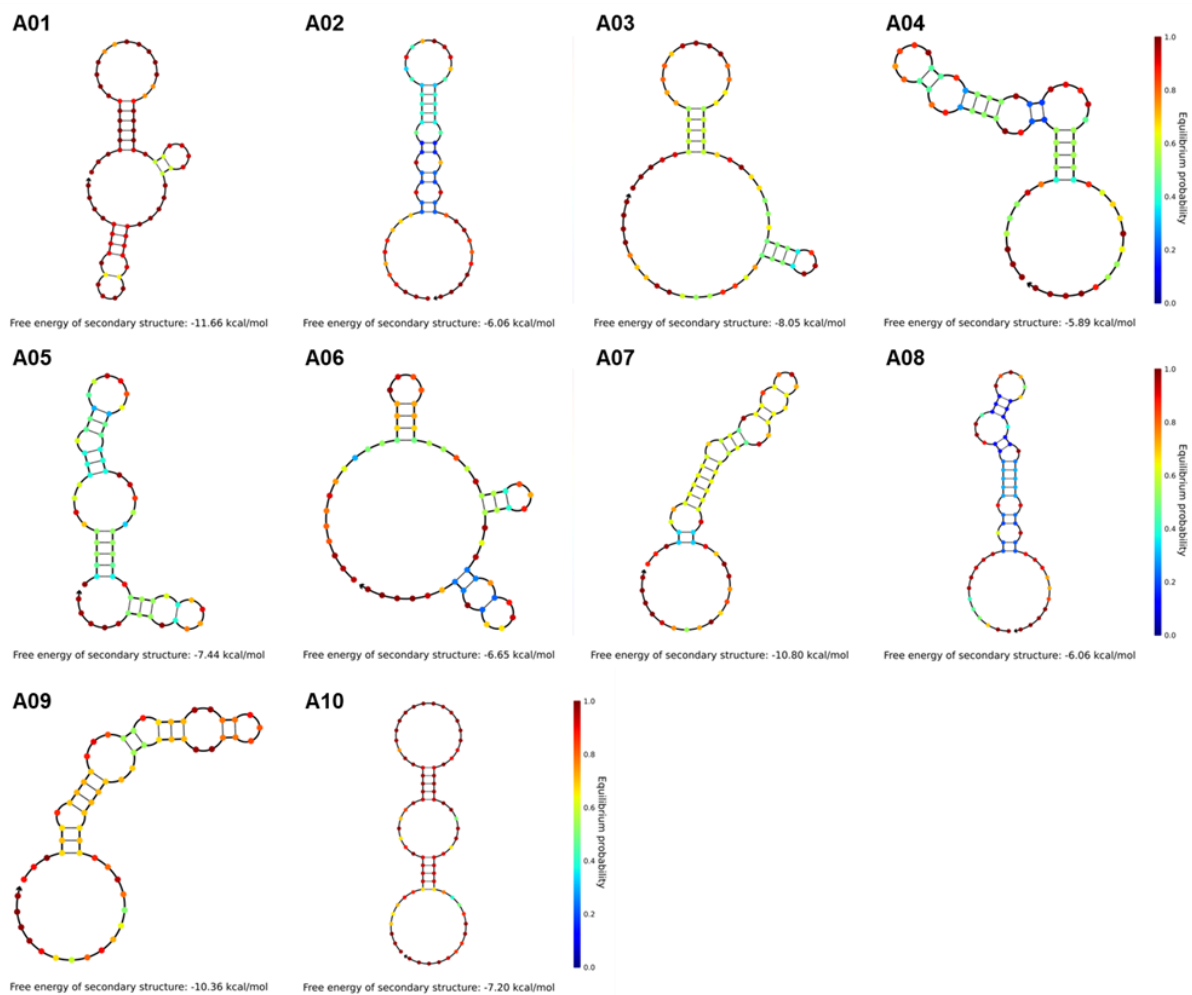
680 **Fig. S2** Sequence alignments of aptamers A01 to A10. Alignment was done with the MultAlin software (Multiple
 681 sequence alignment with hierarchical clustering, F. CORPET, 1988, Nucl. Acids Res., 16 (22), 10881-10890)

682

683 Figure S3

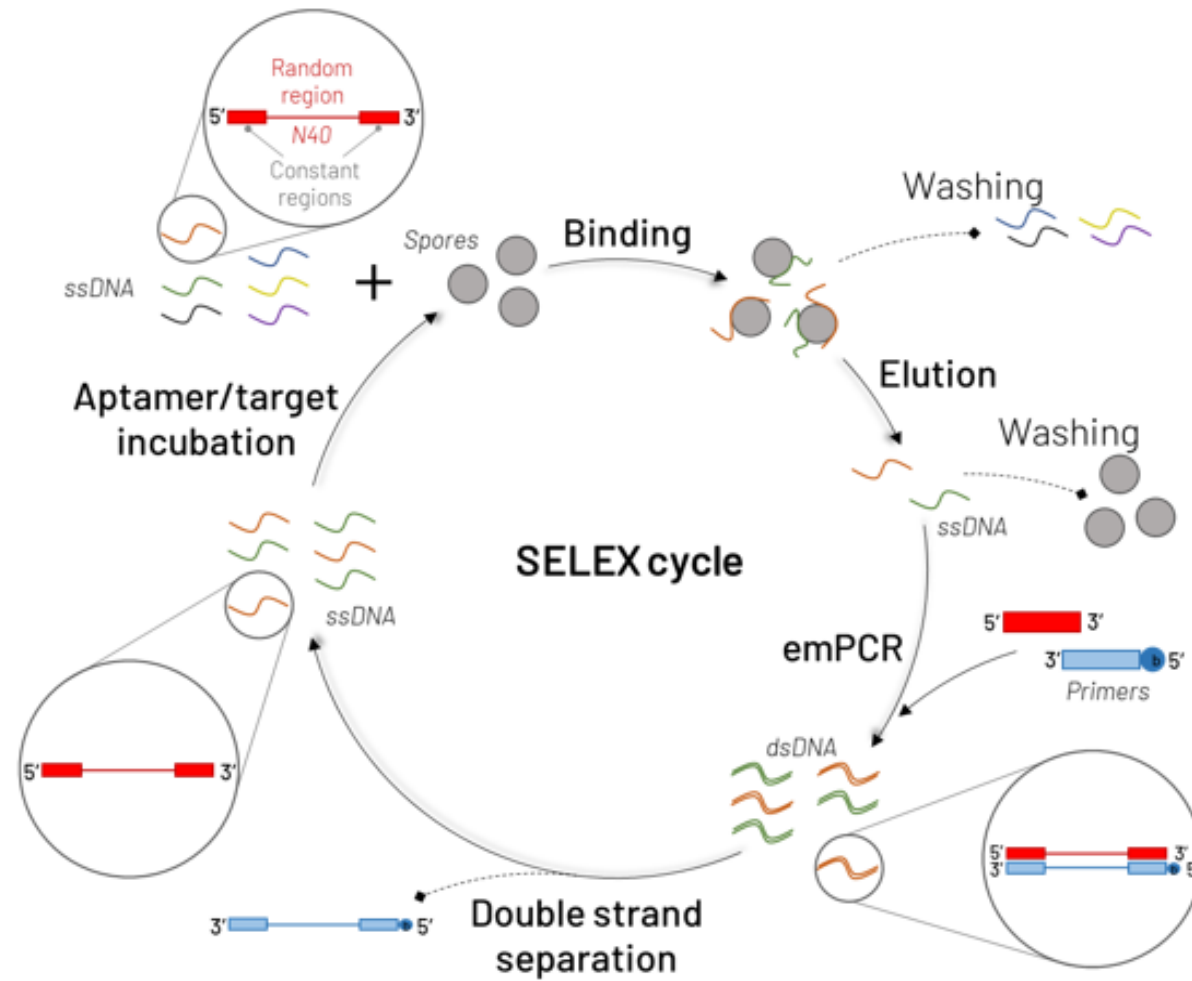
684 **Fig. S3** 2D structures of aptamers A01 to A10 modelled by NUPACK web application (parameters: 25 °C, 100
 685 mM Na⁺, 5 mM Mg²⁺)

686



687

Color should be used



- Selection pressure increased over the Spores-SELEX cycles
- Counter-selection with *Gbs* vegetative cells in the four first cycles
- Counter-selection with non-target spores in the four last cycles
- Selection success monitored by PAGE electrophoresis

SELEX cycle	Spore volume (μL)	Incubation time (min)	Washing step
1-4	20	60	1
5, 6	20	40	1
7	20	35	1
8	20	30	2
9	20	25	2
10	20	20	2
11	15	15	2
12-14	15	15	3
15-18	10	10	3

Fig. 2

Color should be used

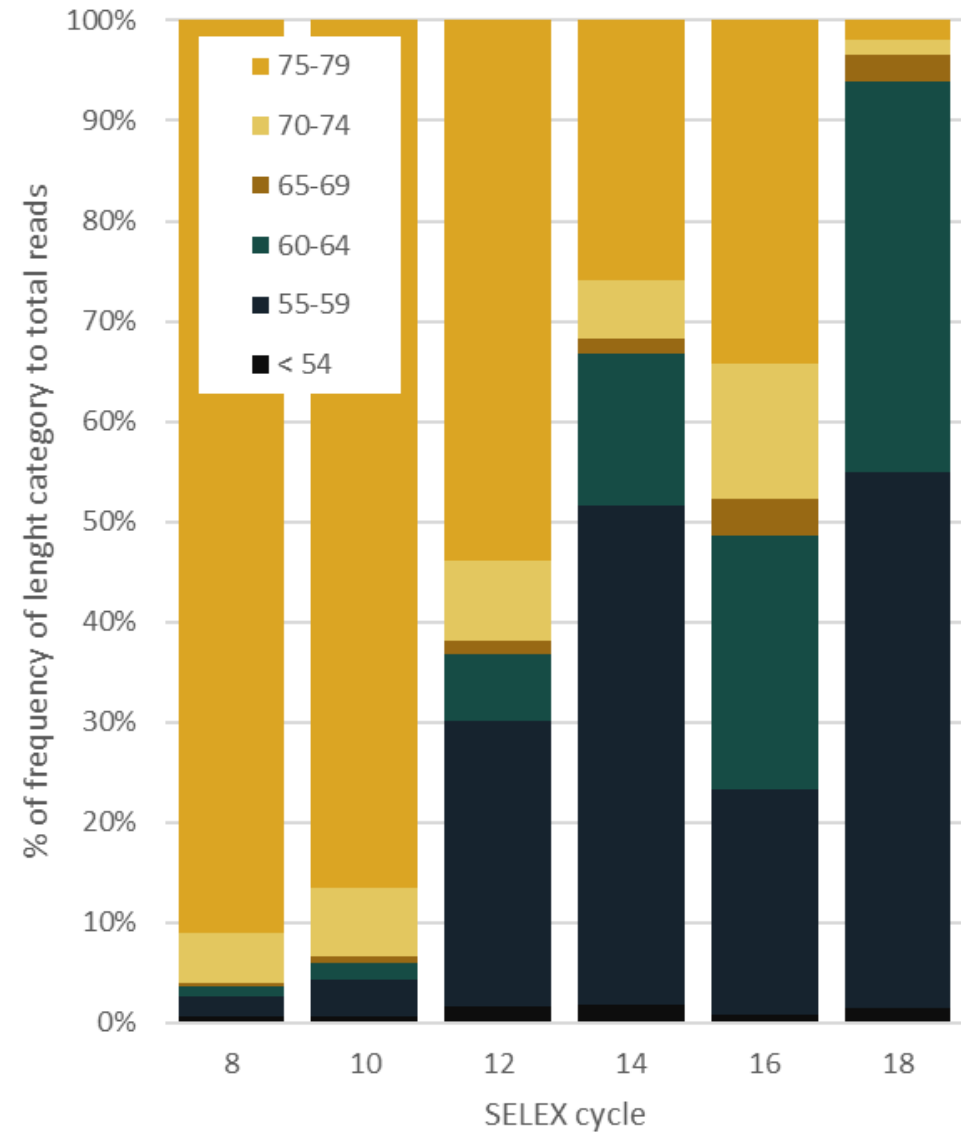
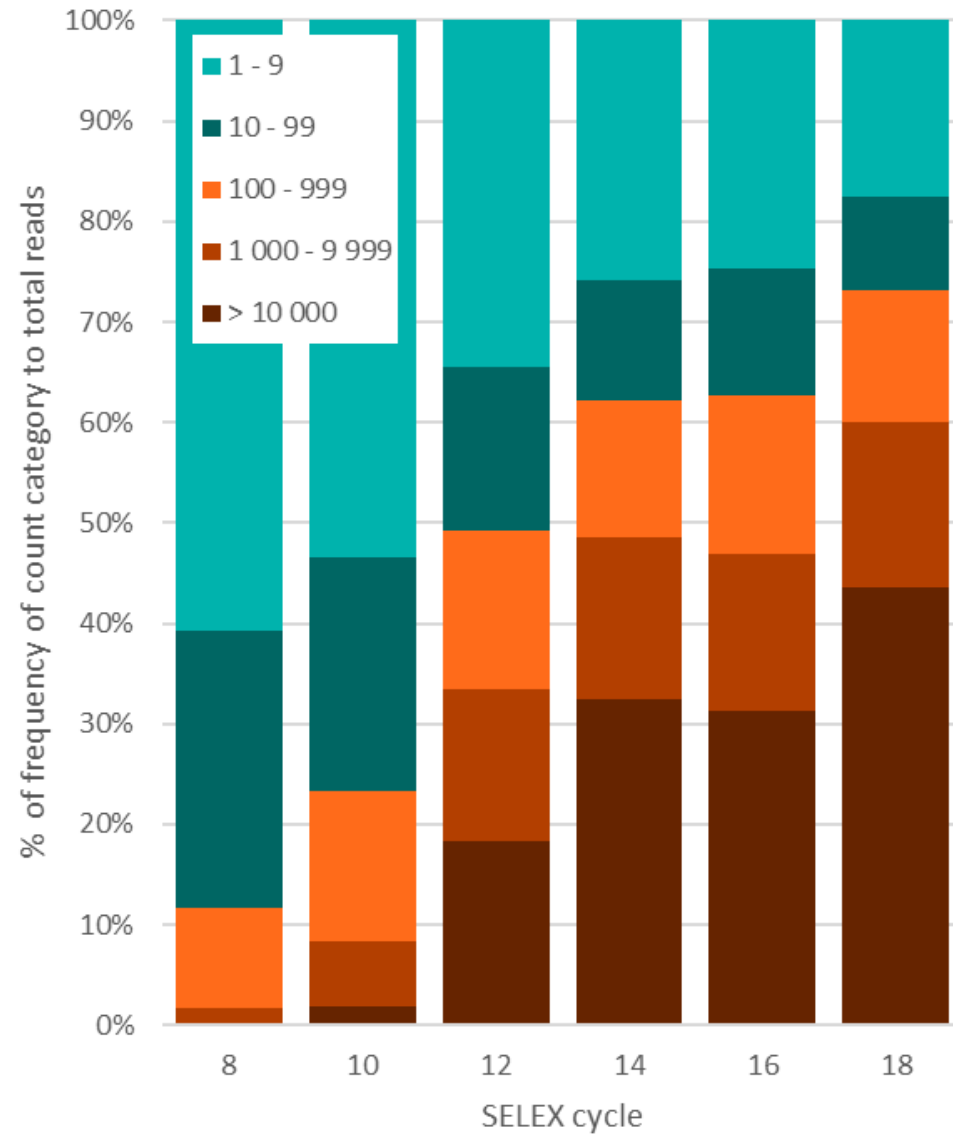


Fig. 3

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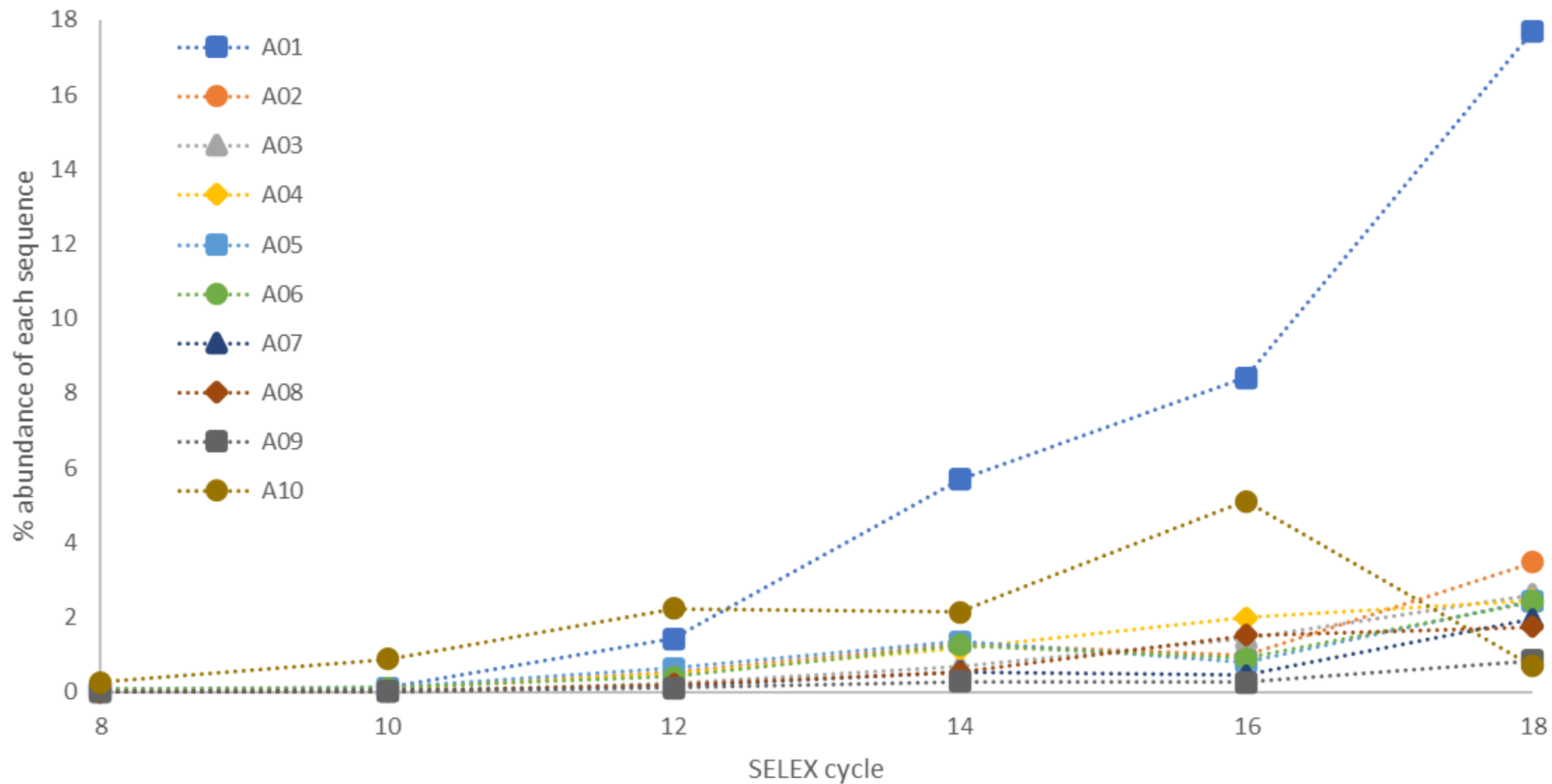


Fig. 4

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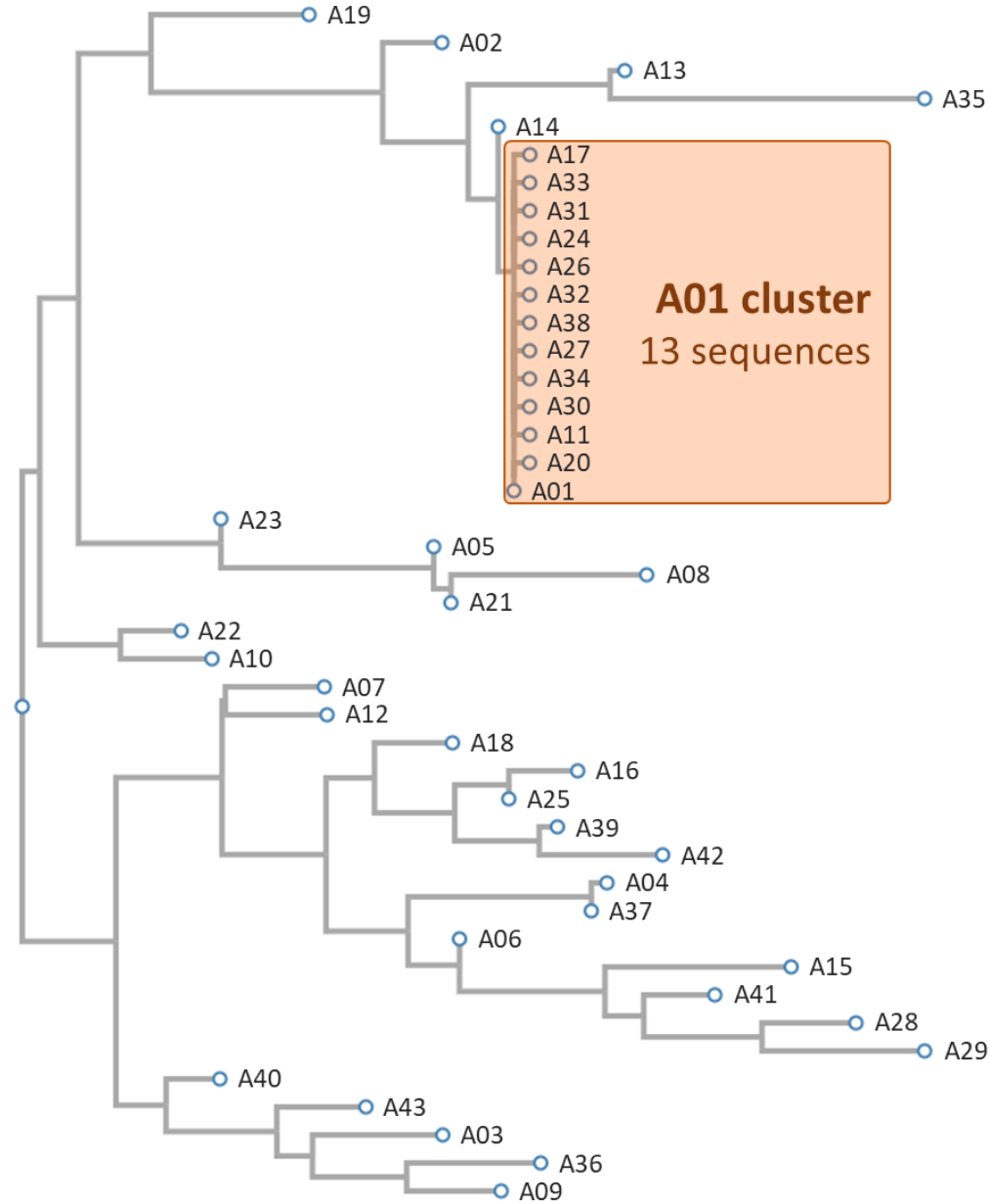


Fig. 5

Color should be used

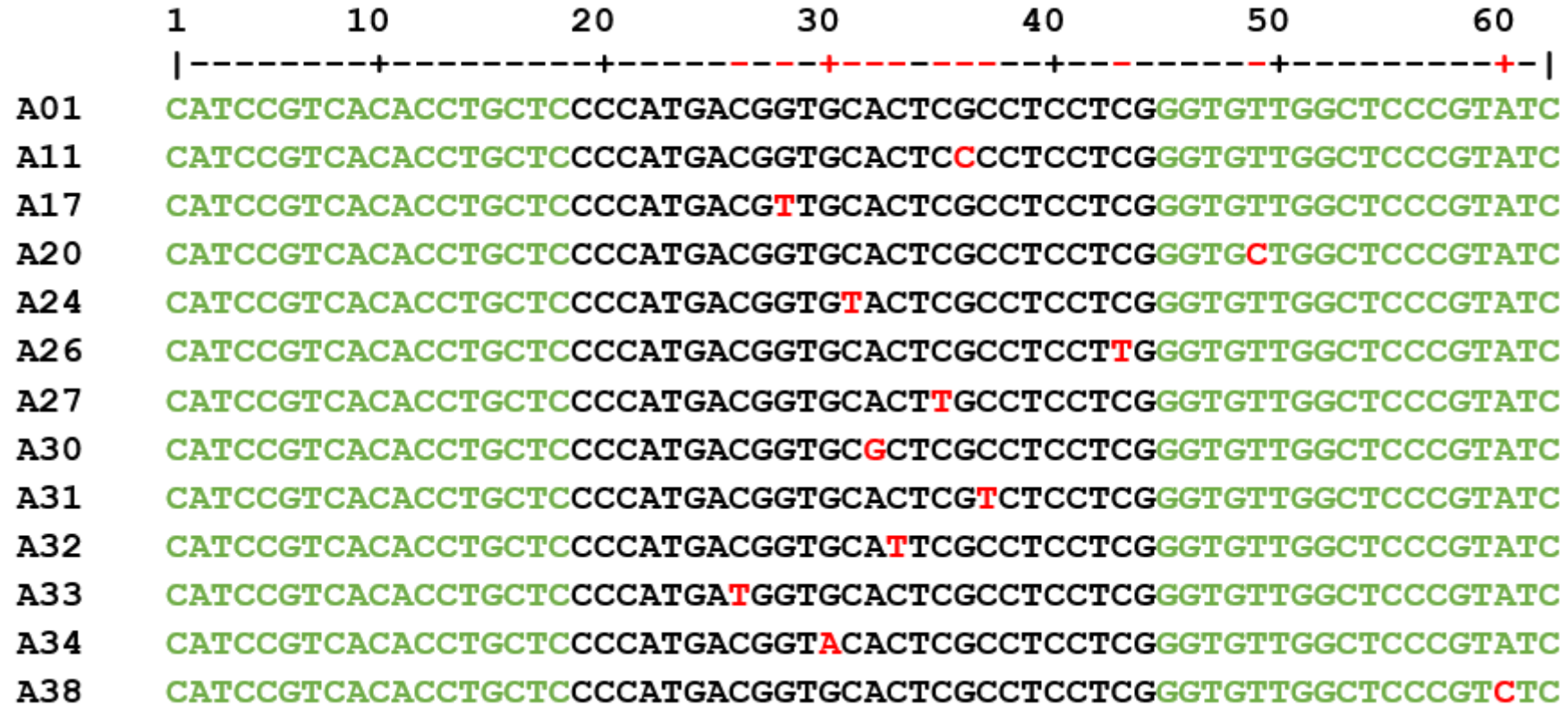
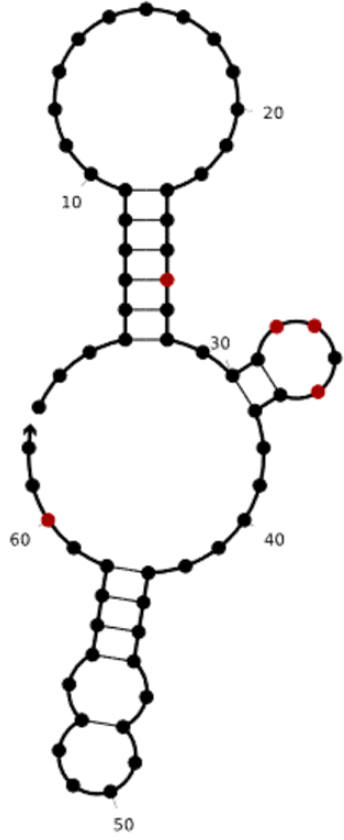


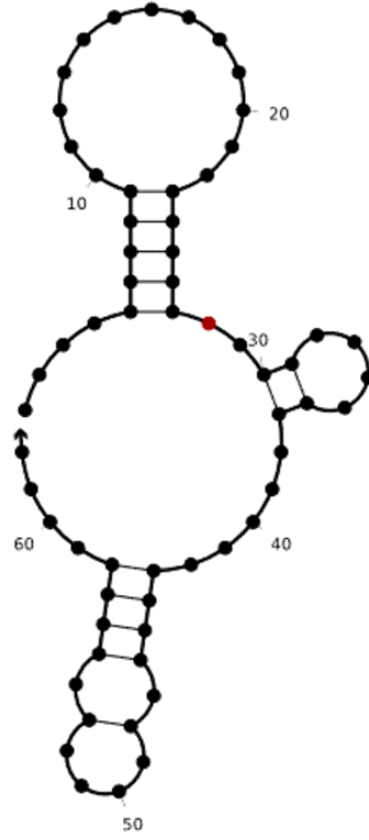
Fig. 6

Color should be used

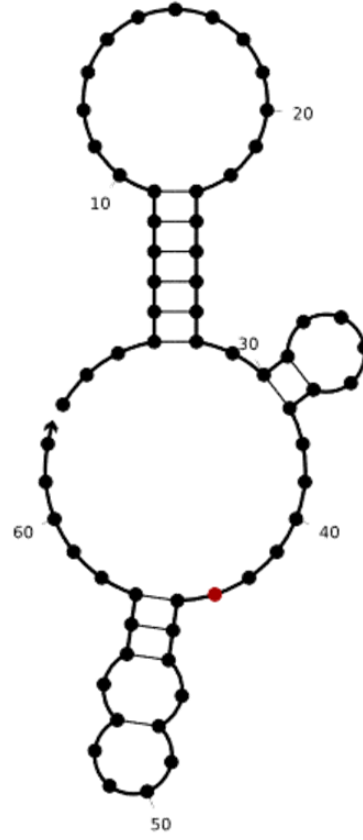
A01; A27; A30;
A32; A33; A38



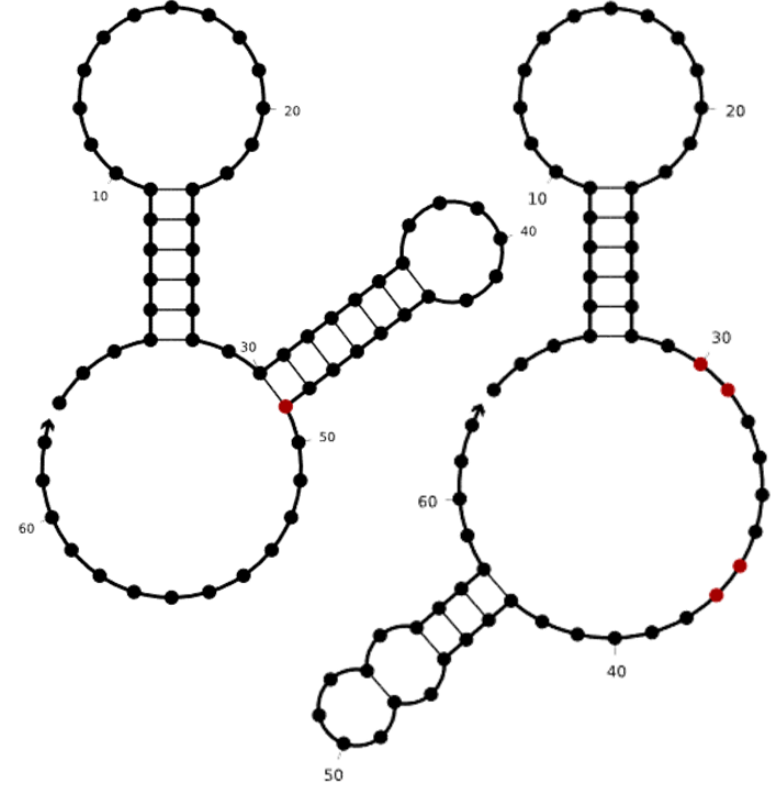
A17



A26



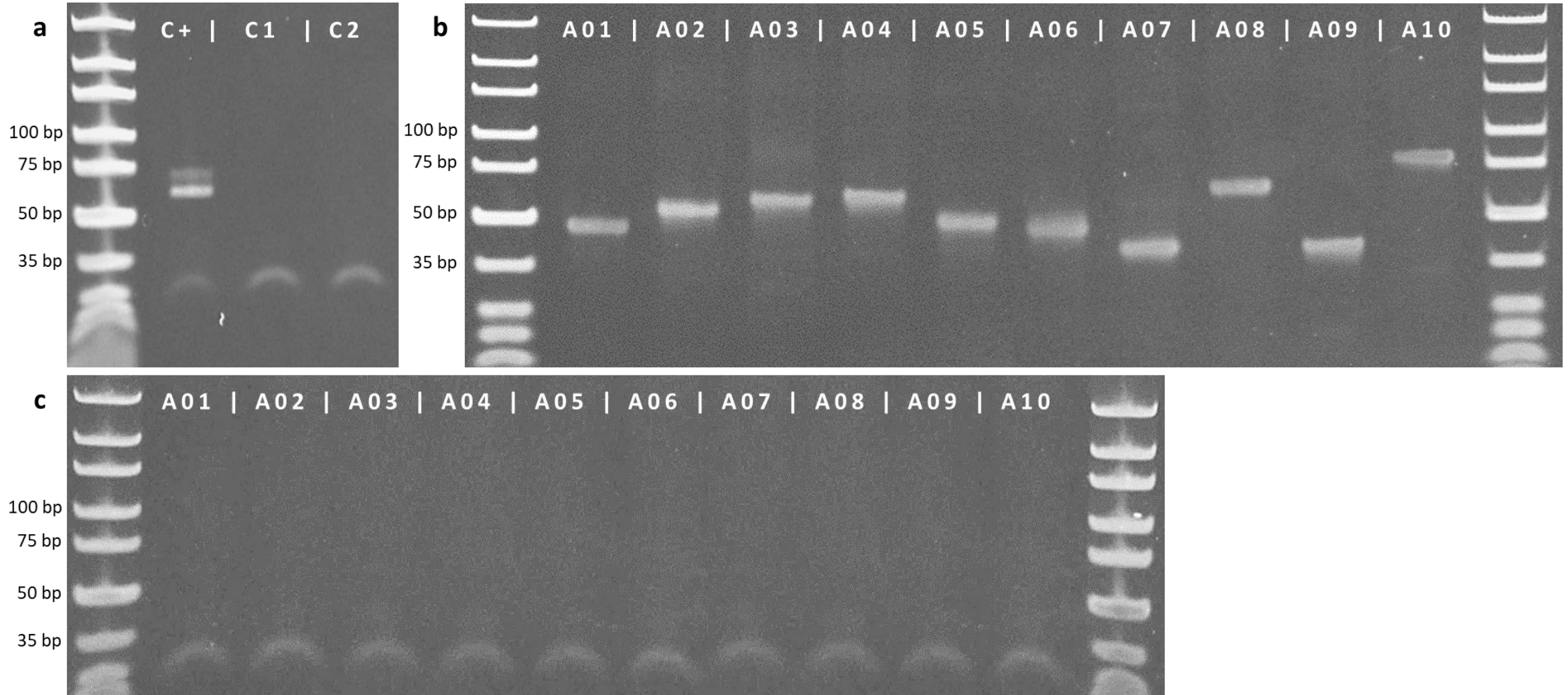
A20



A11; A24;
A31; A34

Fig. 7

Color should be used



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Author Contribution Statement: GD carried out the research and analyzed the data. NP, LB and TN conceived and directed research. GD, LB and TN wrote the manuscript. All authors read, amended and approved the manuscript.

Declaration of Interest

We, as authors, declared that there is no financial and personal interest that could affect our objectivity. We also declared that there is no potential competing interests.