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Generation of a potential biocatalytic DNA sequence for acceleration of Diels-Alder cycloadditions

This work was performed in collaboration with Colette Dalton at the University of Strathclyde.

5.1 Introduction

Investigation into the literature describing Diels-Alder cycloadditions of oligonucleotides unveiled a report detailing the isolation of an RNA sequence which had been shown to accelerate Diels-Alder reactions of anthracene modified oligoribonucleotides by approximately 20, 000-fold. [(1)] With this in mind, it was decided to attempt the generation of a DNA sequence with similar biocatalytic properties in terms of the Diels-Alder reaction.

The ability for RNA sequences to behave as biological catalysts is well established. The first reports of natural RNA sequences with enzymatic functionality, [(2) (3)] also known as ribozymes, were laterally followed by reports of artificial ribozymes, generated through *in vitro* evolution, which could catalyse a broader range of reactions. [(4) (5)] These reactions were, however, related only to the biological nature of nucleic acids. Later studies revealed the catalytic activity of RNA sequences, isolated from combinatorial libraries, in organic chemistry reactions such as

aminoacylation, [6] *N*- alkylation, [7] *N*- glycosidic bond formation, [8] amide bond formation [9] and, significant to this research, Diels-Alder cycloaddition [1] [10].

To date, no DNA enzyme of natural origin has ever been reported. [11] The first report of a DNA enzyme, generated using *in vitro* techniques, was by Breaker and Joyce [12] whereby a 38-nucleotide DNA sequence was used to catalyse the Pb^{2+}-dependent cleavage of an RNA phosphoester. Since then, a DNA catalyst for acceleration of Diels-Alder cycloaddition has been reported. [13] However, this DNA is not a biocatalytic aptamer in the true sense, being double stranded and dependent upon a copper complex of 4,4′-dimethyl-2,2′-bipyridine as a cofactor. As such, the focus of this work was generation of a single stranded biocatalytic DNA sequence for acceleration of Diels-Alder cycloadditions, independent of a cofactor.

5.1.1 In vitro sequence selection

A common method for selection of nucleic acid sequences which bind specifically to a target molecule involves affinity chromatography, using a solid matrix onto which the target molecule of interest is immobilised. [14] (**Fig. 5.1**) A DNA aptamer library is applied to the matrix, such that sequences with an affinity for the target molecule bind to the matrix and can then be isolated from unbound sequences *via* a washing cycle. The bound sequences are then competitively eluted from the matrix using a buffer containing a higher concentration of free target molecule. The collected sequences are then amplified enzymatically (PCR), before undergoing the entire cycle again. Following a number of cycles it is possible to obtain a library enriched with nucleic acid sequences with an affinity for the desired target. Analysis of this library in order to elucidate specific sequence structures then allows for identification of common structural motifs. This system is often termed SELEX (Systematic Evolution of Ligands by Exponential Enrichment). [15]

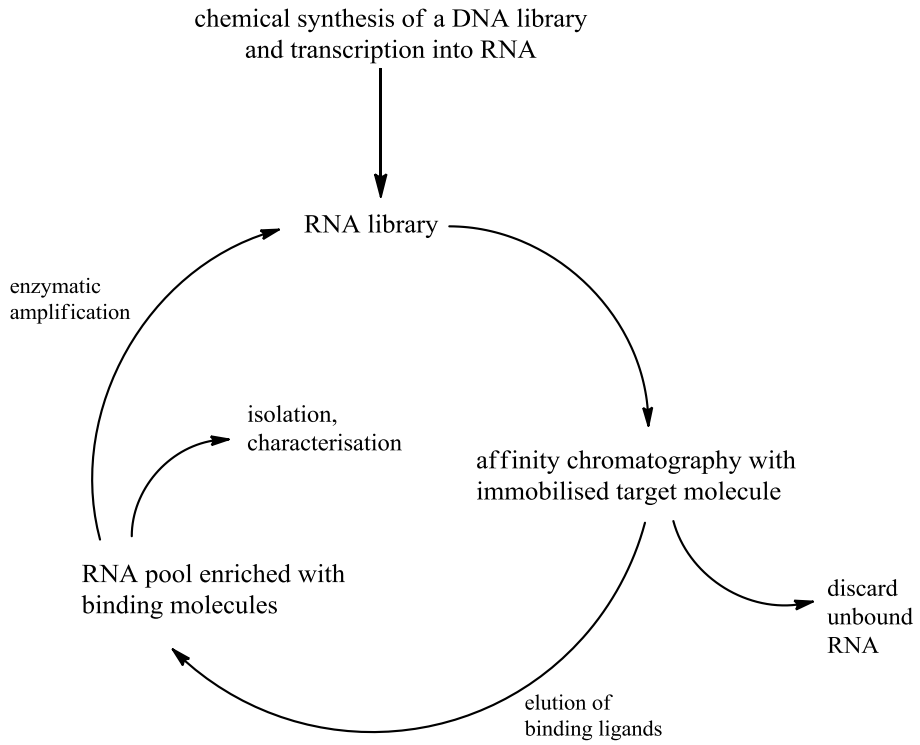


Figure 5.1 – *In vitro selection of RNA ligands with specific binding properties* [16]

Jaschke *et al.* [16] describe a similar methodology for isolation of an RNA sequence motif shown to accelerate Diels-Alder cycloadditions 20,000-fold. A library of RNA sequences, each modified with anthracene *via* a PEG linker were reacted with biotin maleimide. Biotinylated reaction products were isolated using a streptavidin matrix. The bound sequences were amplified then the cycle was repeated through 10 rounds. Analysis of the resultant isolated sequences allowed for identification of a common biocatalytic RNA motif.

In the search for a DNA aptamer for biocatalysis of Diels-Alder cycloaddition, it was decided to adopt a similar approach. It was proposed that a cyclohexadienyl modified DNA aptamer library should be reacted with biotin maleimide, prebound to magnetic streptavidin beads. Washing to remove all unreacted sequences would be followed by enzymatic amplification of the bound sequences. Repeating the cycle, each time with a shortened reaction time for the Diels-Alder cycloaddition, should result in generation of a small library, enriched with sequences with biocatalytic properties for the Diels-Alder reaction. Elucidation of sequence structure should then allow for identification of a common potential biocatalytic motif.

5.2 Results and discussion

5.2.1 Generation of a cyclohexadienyl modified aptamer library

The first step in generation of a DNA sequence for catalysis of Diels-Alder cycloadditions was production of a dienyl functionalised aptamer library for participation in the Diels-Alder, SELEX-type process. Since previous work had shown cyclohexadiene to be optimal for Diels-Alder cycloadditions of 5'- dienyl modified oligonucleotides, this was the diene of choice for synthesis of a dienyl modified aptamer library. This was achieved by 5'- modification of a reverse primer sequence, oligo sequence (**1**), using the cyclohexadienyl modified phosphoramidite, as described previously (see 3.2.2 – *Synthesis of 5'- dienyl modified oligonucleotides*), oligo sequence (**1**) being complementary to the primer binding region of the aptamer library to be generated:

Oligonucleotide sequence 1: 5'- X TCC ACG TTT TCC
CAG TCA GAC GTA A -3'

Where X is the cyclohexadienyl modification. Successful synthesis of the 5'- cyclohexadienyl modified reverse primer sequence was confirmed by MALDI-TOF mass analysis (**Table 5.1**).

Calc'd M	**MALDI-TOF MS Found $[M + H]^+$**
7868.0	7871.0

Table 5.1 – *Mass spectroscopic characterisation of 5'- cyclohexadienyl modified reverse primer oligo sequence* ***(1)***

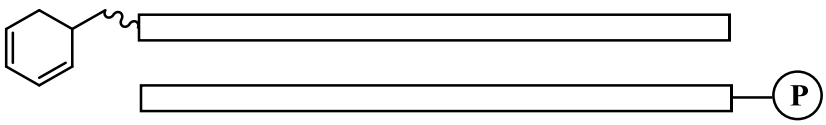


Figure 5.2 – *PCR product; double stranded DNA modified with a phosphate group at the 5'- terminus of one strand, and with the cyclohexadienyl modification at the 5'- terminus of the complimentary strand*

Using the dienyl modified reverse primer sequence, a polymerase chain reaction (PCR) was performed in order to produce double stranded DNA modified with a phosphate group at the 5'- terminus of one strand, and with the cyclohexadienyl modification at the 5'- terminus of the complementary strand (**Figure 5.2**).

Generation of a single stranded DNA aptamer library, suitable for introduction into a SELEX process was performed using standard procedures developed in-house. [17] First, gel electrophoresis was used to assess the effectiveness of the PCR generation of cyclohexadienyl modified double stranded DNA. A potential difference is applied across an agarose gel stained with ethidium bromide gel. Over a period of time, DNA applied to the gel migrates towards the positive electrode by virtue of its electrostatic interaction with the anionic charges on the DNA phosphate backbone. The porous nature of the gel is such that smaller DNA fragments migrate across a greater distance than larger fragments, resulting in sequence separation according to size. After developing the gel, comparison of the bands observed with a standard allows identification and relative quantification of the desired sequence. Following this procedure, the band identified to contain the sequence of desired length was removed from the gel; the sequence was then purified from the gel component using silica membrane columns.

To render the double stranded DNA single stranded, it was treated with a λ exonuclease enzyme, which digests the phosphorylated strand whilst leaving the cyclohexadienyl modified strand untouched (**Fig. 5.3**).

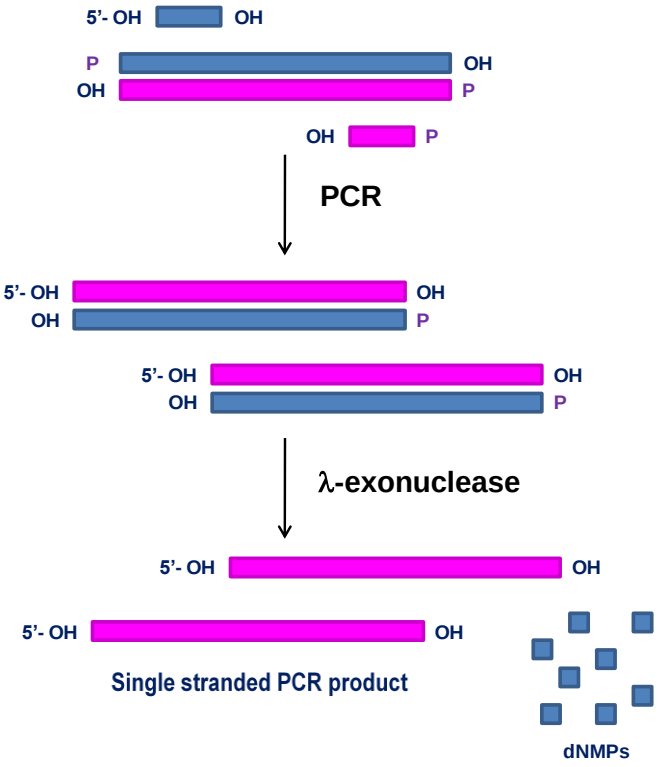


Figure 5.3 – *Schematic of λ exonuclease process*

The final step in generation of a cyclohexadienyl modified DNA aptamer library was purification of the single stranded DNA. This was achieved by ethanol precipitation, whereby large DNA fragments are isolated from smaller fragments and salt ions by precipitation out of solution using ethanol. Now available was a library of pure, single stranded, cyclohexadienyl modified DNA sequences for use in a SELEX-type process for selection of a DNA sequence with biocatalytic properties for the Diels-Alder reaction.

5.2.2 Selection of a biocatalytic DNA aptamer sequence for acceleration of Diels-Alder cycloadditions

5.2.2.1 Diels-Alder SELEX process

The SELEX process used for selection of a DNA sequence for biocatalysis of Diels-Alder cycloadditions differs from a typical SELEX process in that the binding process involves formation of a covalent bond, rather than the usual weaker binding

interactions formed through electrostatic, hydrophobic and Van der Waals interactions.

In the first step of the SELEX process a counter SELEX was carried out to rule out the possibility of sequence binding to the solid matrix (magnetic streptavidin beads). The cyclohexadienyl modified DNA aptamer library was incubated with beads alone to ensure that any sequences with affinity would not be taken into the first round of SELEX.

The dienophile target molecule to be used in the SELEX process was maleimide. Maleimide was attached to the solid matrix (magnetic streptavidin beads) using biotin maleimide, making use of the known strong interaction between biotin and streptavidin. The malemido functionalised beads were exposed to the cyclohexadienyl modified DNA aptamer library for a fixed period of time at room temperature then washed to remove any unbound sequences. The covalent nature of sequence binding to the solid matrix *via* the Diels-Alder reaction was such that PCR amplification had to be performed on the streptavidin beads themselves. This meant that for some rounds of the SELEX process, two PCR cycles had to be performed before evidence of sequence binding could be observed by gel electrophoresis.

A SELEX programme was designed such that the exposure time of the cyclohexadienyl modified DNA aptamer library to the maleimido modified matrix was decreased from 2 hours to 15 minutes, the idea being that only sequences with biocatalytic properties would react with the matrix within the shortened time periods. For each time interval, the SELEX cycle was performed twice. A summary of the results is shown in **Table 5.2**.

The gel electrophoresis data obtained as a result of the SELEX programme shows that, in the presence of sequences in the cyclohexadienyl modified DNA library, it is possible for the Diels-Alder reaction to take place at room temperature in only 15 minutes. This is a significant improvement on the previous results of Diels-Alder cycloaddition of oligonucleotides to maleimido modified TAT peptide, whereby conditions of 40 ^{o}C, overnight were required for the reaction to take place.

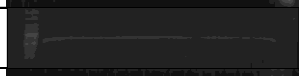
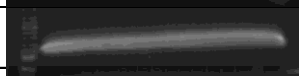
SELEX	Incubation time (mins)	No.of PCR s	Gel Image
Counter	60	-	
1	120	1	
2	120	1	
3	60	1	
4	60	2	
5	30	2	
6	30	2	
Counter	60	-	
7	15	2	
8	15	2	

Table 5.2 – *Summary of results of Diels-Alder SELEX programme*

It should be noted though that there may have been traces of Mg^{2+} present in each SELEX cycle, contained in the binding buffer used to wash the functionalised beads, and that being a Lewis acid, its presence may be necessary for the Diels-Alder reaction to proceed. This is an issue that will require further investigation.

5.2.2.2 Cloning and sequence analysis

The next step in this work was to isolate a potentially biocatalytic sequence from the now enriched cyclohexadienyl modified aptamer library to use in test Diels-Alder cycloadditions. This was achieved by cloning. In this step, the pool of aptamer library molecules are separated into individual clones, grown and processed to allow for sequence analysis.

Cloning was performed by Colette Dalton at the University of Strathclyde, using a commercially available kit.

Sequencing was perfomed by The College of Life Sciences Cloning Service, University of Dundee. A total of 24 plasmid samples were sent for sequencing. Five samples failed to be sequenced successfully; this could have been as a result of a number of different factors including low DNA concentration, complex DNA

secondary structure or interference from salt ions. Analysis of the sequencing results was performed by Colette Dalton using sequence alignment software (Multalin). [18]

From the data obtained, six sets of sequences (each containing 2 or 3 samples) were identified as showing common structural motifs. From these, DNA sequence (**1**) was selected at random to be used in test Diels-Alder cycloadditions to assess its biocatalytic potential.

DNA Sequence 1: AGG CAA GGT ACA GCG GGG GTT GCG GGT CAG GTC GTG TGT GTG TGG GGT GTC CCG TGC GGT

As the results of this work stand, it is not yet possible to conclude that this sequence is a biocatalyst for the Diels-Alder cycloaddition – it is only potentially biocatalytic, with a turnover of only one cycloadduct product per DNA strand. To determine whether or not the sequence is truly biocatalytic, experiments involving addition of only a catalytic amount to a series of sample Diels-Alder cycloadditions are required. This work will be performed by Colette Dalton and is ongoing.

5.3 Conclusions

A cyclohexadienyl modified DNA aptamer library was generated by way of synthesis of a 5'-cyclohexadienyl modified complementary primer sequence, using chemistry established in previous work, for use in PCR to generate a library of double stranded DNA. Standard purification and enzyme digestion procedures were used to generate a library of single stranded, cyclohexadienyl modified DNA aptamers.

This aptamer library was used in a Diels-Alder SELEX-type process, being exposed to a maleimido modified streptavidin matrix over incrementally decreasing periods of time. Amplification of the library at each round of the SELEX cycle was achieved by performing PCR on the streptavidin bead matrix. This was due to the covalent bond formed between aptamer sequences and the maleimido modified matrix, brought about as a result of the Diels-Alder reaction.

Using this SELEX process it was possible to isolate a small library of sequences with the capability to catalyse Diels-Alder cycloaddition such that the reaction took place in only 15 minutes at room temperature. This was a marked improvement on any results achieved previously in work using Diels-Alder cycloadditions. However, the possible contribution to cycloaddition catalysis of traces of Mg^{2+} from the binding buffer used in the SELEX process needs to be investigated.

Using cloning and sequencing procedures it was then possible to isolate a small number of sequences with a common structural motif from this library. One of these sequences was selected to be used in test Diels-Alder reactions to assess its true biocatalytic potential.

5.4 Experimental

5.4.1 Generation of a cyclohexadienyl modified aptamer library

5.4.1.1 Synthesis of a 5'- cyclohexadienyl modified complementary primer sequence

Synthesis of a cyclohexadienyl modified phosphoramidite and the proceeding synthesis of 5'-cyclohexadienyl modified oligonucleotide sequence (**1**), complementary to the primer binding region of the aptamer library, was achieved as described previously *(Chapter 2 – Conjugation of 5'-dienyl modified oligonucleotides to Tat peptide via Diels-Alder cycloaddition).*

5.4.2 PCR for generation of double stranded DNA library

This work was performed in collaboration with Colette Dalton at the University of Strathclyde.

Five PCR mixtures and one negative control were prepared through the use of a master mix (**Table 5.2** and **Table 5.3**). The master mix contains all necessary

reagents to carry out the PCR except the template. This is used to help prevent contamination and for ease of preparation.

	Volume (μl)		
	PCR	Master	Negative
1. DEPC water	20.5	123.0	22.5
2. buffer B	25.0	150.0	25
3. KOD	0.5	3.0	0.5
4. oligonucleotide sequence (**1**)	1.0	6.0	1.0
5. U fwd primer	1.0	6.0	1.0
6. template	2.0	-	-

Table 5.2 – *Volumes of PCR solutions used to make master, PCR and negative control mixtures*

Component	Notes
1. DEPC water	PCR water, nuclease free (Bioline)
2. buffer B	2x PCR PreMix (Epicentre)*
3. KOD polymerase	1 u / μL hotstart proof reading enzyme (Novagen) #
4. oligonucleotide sequence (**1**)	100 pmol / μL 5' cyclohexadiene modified complementary primer sequence
5. U fwd primer	100 pmol / μL 5' phosphate (MWG)
6. template	10 pmol / μL 60 base random region (MWG)

Table 5.3 – *Details of PCR solutions used*

** 100 mM Tris-HCl, pH 8.3, 100 mM KCl, 400 μM each dNTP, 3 –7 mM $MgCl_2$.*

 # The KOD stock is expressed in units (1u.μl^{-1}). "One unit is defined as the amount of enzyme that will catalyse the incorporation of 10 nmol of dNTP into acid insoluble form in 30 minutes at 75℃ in a reaction containing 20 mM Tris-HCl (pH 7.5 at 25℃), 8 mM MgCl, 0.5 mM DTT, 50 μg / ml BSA, 150 μM each dATP, dCTP, dGTP,

dTTP (a mix of unlabelled and [^{3}H]-dTTP) and activated calf thymus DNA." Definition provided by Novagen®.

48 µl master mix was aliquotted into PCR tubes. To the negative control was added DEPC water (2.0 µl). To tubes 1 – 5 was added the template (2.0 µl). The samples were then run on the thermocycler (Minicycler ^{TM} MT Research) under the following conditions:

10 mins	@ 94 ^oC	
20 secs	@ 94 ^oC	x 15
20 secs	@ 53 ^oC	
20 secs	@ 72 ^oC	
30 secs	@ 94 ^oC	
1 min	@ 53 ^oC	
5 mins	@ 72 ^oC	
2 hrs	@ 4 ^oC	

5.4.2.1 Gel electrophoresis and purification

All gels used throughout this work (unless otherwise stated) were 2 % w/v of agarose in 1 x Tris Borate EDTA (TBE) buffer (Sigma). Once set, the gel was pre-soaked in ethidium bromide (0.5 µg ml^{-1} in TBE buffer) for 30 mins and placed in an electrophoresis tank containing 1 x TBE buffer. The PCR products were combined and 10 µl mixture was retained as stock. The remainder was loaded in a well with 1/10 volume of tricolour loading buffer (Bioline). 7.5 µl Gel Pilot 50 ladder (Qiagen) was also run on the gel as this provided a size reference. This ladder contains a bright band at 100 base pairs (bp), which was appropriate for the size of the aptamer library being generated as part of this work. The electrophoresis was run at 120 Volts for 50 mins.

The gel was photographed using a gel imager (BioDoc-It™ Imaging System), a 2 UV transilluminator (UVP, Cambridge), on the higher wavelength (365 nm) to preserve DNA integrity. To extract the DNA, the gel was visualised using a transilluminator (UVP) on a low power setting, for the same reason. The band at ~ 100 bp was excised using a scalpel and placed in a sterile pre-weighed 20 ml Sterilin tube. The gel was purified using 6 columns (depending on weight) from a QIAquick gel extraction kit (Qiagen) according to the manufacturer's guidelines. Samples were then pooled.

5.4.3 Rendering DNA single stranded

The following mixture was made up in a small PCR tube:

ds DNA = volume from purification columns e.g. 200 µl

enzyme = 1.0 µl

buffer = 1/10 of total volume e.g. 22 µl

The sample was placed in the thermocycler and subjected to the following conditions:

30 mins @ 37 °C

10 mins @ 72 °C

5.4.3.1 Ethanol precipitation

The following mixture was made up in an eppendorf:

ss DNA = from strandase (1 vol) e.g. 200 µl

NaAc buffer (3 M) = 1 / 10 vol e.g. 20 µl

Gen Elute LPA = 1 µl

Ethanol (100 %) = 2.5 vol e.g. 500 µl

The mixture was vortexed thoroughly and then centrifuged for 10 mins @ 13 000 rpm. The supernatant was removed, care being taken not to disturb the pellet. To the mixture was added 70 % (v/v) ethanol (1 ml) then the mixture was centrifuged for 5mins @ 13 000 rpm. The supernatant was removed then 100 % ethanol (500 μl) was added. The mixture was centrifuged for 5 mins @ 13 000 rpm. The supernatant was removed, the mixture was centrifuged for a further 1 min @ 13 000 rpm, then the supernatant was removed once again. The mixture in the eppendrof was left to air dry for 5 mins. The pellet was resuspended in in DEPC water (100 – 200 μl).

5.4.4 Diels-Alder SELEX process

5.4.4.1 Counter SELEX

375 μl magnetic streptavidin beads (New England Biolabs, USA) were washed (x 3) with sterile binding buffer (500 μl , 20 mM Tris HCl, 0.3 M NaCl, 5 mM $MgCl_2$, pH 7.6, sterilised using 0.2 μm syringe filter (Elkay)). The cyclohexadienyl modified DNA aptamer library solution was made up to 300 μl with binding buffer. The washed beads were resuspended in the DNA solution and incubated for 1 hour. Following separation by application of a specialised magnet, the supernatant was removed and added directly to biotin maleimide functionalised beads. *This procedure was carried out before both SELEX 1 and SELEX 7.*

5.4.4.2 Diels-Alder SELEX

375 μl streptavidin beads were washed (x 3) with binding buffer (500 μl). The beads were resuspend beads in biotin malemide solution (100 μl, 15 μM in binding buffer) and incubated for 30 mins. Following separation by application of a magnet, the beads were washed (x 3) with binding buffer (300 μl) to remove any unbound biotin malemide. The counter SELEX supernatant (or DNA aptamer library from previous SELEX) was added to the beads and incubated for X mins (according to **Table 5.5**). Following separation by application of a magnet, the supernatant was removed and the beads were washed (x 3) with binding buffer (300 μl). The beads were resuspended in DEPC water (100 μl) and used as template for PCR.

After the completion of each round of SELEX, PCR, gel electophoresis, conversion to single stranded DNA and ethanol precipitation had to carried out. This was performed as described previously, with the only difference being the volumes used in the PCR mixture (**Table 5.6**). 80 μl master mix was aliquotted into six PCR tubes. DEPC water (20 μl) was added to the negative control while the template (20 μl beads), from the SELEX cycle, were added to tubes 1 – 5.

Type	Incubation time (mins)
Counter SELEX	60
SELEX 1	120
SELEX 2	120
SELEX 3	60
SELEX 4	60
SELEX 5	30
SELEX 6	30
Counter SELEX	60
SELEX 7	15
SELEX 8	15
SELEX 9	5
SELEX 10	5

Table 5.5 – *Outline of SELEX*

	Volume (μl)		
	PCR	Master	Negative
1. DEPC water	25	150	45
2. Buffer B	50	300	50
3. KOD	1	6	1
4. Victoria Rev	2	12	2
5. U Fwd P	2	12	2
6. Template	20	-	-

Table 5.6 – *Volumes of PCR solutions used to make master, PCR and negative control mixtures as part of SELEX process*

5.4.5 Cloning and sequence analysis

This work was performed by Colette Dalton at the University of Strathclyde.

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