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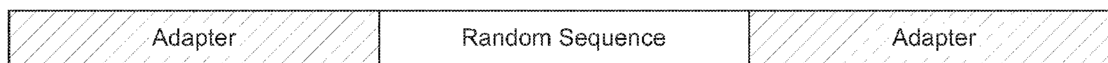


Fig. 1

**(57) Abstract:** The present disclosure relates to aptamers for detection of an analyte in a sample; a substrate having attached thereto the aptamers according to the invention; a kit of parts for the detection of an analyte in a sample; and a method for the detection of an analyte in a sample comprising the use of said aptamers.

WO 2019/067383 A1

# Aptamer Screening

## Technical field

The present disclosure relates to aptamers for the detection of an analyte in a sample; a substrate having attached thereto the aptamers according to the invention; a kit of parts for the detection of an analyte in a sample comprising said aptamers; and a method for the detection of an analyte in a sample comprising the use of said aptamers.

## Background

Aptamers are synthetic receptors that can adopt local or global conformations that enable them to bind molecular analytes with high affinity. Aptamers are generally considered as biopolymers and can be made from natural or unnatural oligonucleotides, amino acids, or hybrid structures Currently, a large number of generated aptamers can bind various targets, ranging from simple inorganic molecules to large protein complexes, and entire cells. In fact, aptamers are generally nucleotide analogues of antibodies, but aptamer generation is significantly easier and cheaper than the production of antibodies.

A common feature or strategy in aptamer generation, and to identify high affinity aptamer candidates, is the ability to identify high-affinity molecules within a large library of structures by “directed evolution,” in which candidate aptamers are subjected to multiple cycles of chemical selection steps, followed by self-amplification and further screening. The process, systematic evolution of ligands by exponential enrichment (SELEX), involves use of a library of randomly generated oligonucleotides which undergoes a process of sequential selection (typically 15-20 iterations) to generate aptamers having high affinity for target molecules[1, 2].

Specifically, to identify aptamers for soluble targets of interest, an aptamer library of randomly generated sequences of fixed length flanked by constant 5' and 3' primer ends (known as adapter sequences, figure 1) are typically bound to a solid substrate via base pairing of the adapter sequence with surface-tethered oligonucleotide primers. The annealed complex is then washed with the target molecules of interest, with any oligomers that do not bind to the aptamer released into the wash. The bound sequences

are then eluted and amplified by PCR to prepare for subsequent rounds of selection. The process is then repeated 15-20 times with a gradual reduction of target concentration thereby increasing the stringency of the elution conditions to identify the tightest-binding sequences. Any aptamers of high affinity can be identified based on consensus sequences found in members of the final aptamer library via DNA sequencing.

However, this process has several associated problems including aptamer cross-reactivity wherein aptamers that recognize particular targets can also bind to molecules with a similar structure. Further, it is often the case that the selected aptamers are not those with highest affinity and specificity due to a suboptimal SELEX stringency protocol.

Having performed meta-analysis of aptamers obtained using SELEX, we have found that the adapter sequence used to anchor the aptamer library to the solid substrate can also contribute to the recognition of target molecules. It therefore follows that the current methodology does not make use of the full binding capacity of the aptamer library and so the interrogatory potential of said library is not fully utilised, with a concomitant reduction in potential.

We therefore herein disclose an improved method that increases the structural diversity of aptamers that bind a given target molecule or molecules. Moreover, increasing the interrogatory diversity of the aptamer binding sequences results in the identification of aptamers that have higher affinity and/or specificity for their target molecule(s).

## Statements of invention

According to a first aspect of the invention there is provided a method for identifying an aptamer molecule capable of binding a target analyte in a sample comprising:

providing a plurality of test aptamer molecules each comprising a sequence of base units having at a first end a first adapter sequence common to all or a majority of the aptamers and at a second end a second adapter sequence common to all or a majority of the aptamers,

providing a substrate comprising surface tethered sequences complementary or substantially complementary to said first and/or said second adapter sequence(s); and wherein said method comprises the steps of:

i) hybridising the test aptamer molecules to said surface tethered sequences complementary to said first adapter sequence;

ii) exposing said hybridised aptamers to a sample under conditions whereby target analytes in said sample that have target analyte binding sites for an aptamer, bind any one or more of said test hybridised aptamer molecules;

iii) eluting or extracting at least the aptamer molecules hybridised to said surface tethered sequences and bound by said target analytes;

iv) hybridising the eluted aptamer molecules of step iii) to said surface tethered sequences complementary to said second adapter sequence;

v) exposing said hybridised aptamers of step iv) to the said sample or a further sample comprising target molecules under conditions whereby target analytes in said sample that have target analyte binding sites for an aptamer bind any one or more of said test hybridised aptamer molecules;

vi) eluting or extracting at least the aptamer molecules hybridised to said surface tethered sequences and bound by said target analytes; and

vii) optionally, repeating steps i)-iii) and/or iv)-vi) ; and

viii) concluding said aptamers of steps vi) or vii) are capable of identifying the target analyte(s) in the sample.

Reference herein to an analyte is any selected target the aptamer can recognise but most typically is selected from the group comprising: protein, enzyme, antigen, receptor, hormone, metabolite, an organic molecule and carbohydrate.

As will be appreciated by those skilled in the art, the purpose of the invention is to identify aptamer molecules from a number of test aptamer molecules to determine those with the

best binding affinity for a given one or more target analyte(s) in a sample. Therefore, preferably, said test aptamer molecules form part of a library of aptamers suitable for screening according to the method of the invention. Accordingly, ideally, steps i) – iii) and/or iv) – vi); or i) – vi) are repeated a number of times, such as 5-20 times, including all intervals therein and ideally, 10-15 times.

In a preferred method of the invention said adapter sequences at either end of said aptamer are different from each other.

Reference herein to substrate refers to any material or coating to which sequences can be tethered such as, but not limited to, silver, gold, aluminium, copper, platinum, iron and silica metals/metalloids or an alloy thereof or a polymer-coated version thereof, or indeed a polymer material.

The method disclosed herein relies upon the sequential binding of a pair of adapter sequences of the test aptamers to surface tethered sequences, and therefore requires the sequential hybridisation of said adapter sequences to the surface tethered sequences. As will be appreciated by those skilled in the art, this can be achieved by numerous means such as the provision of different adapter sequences that vary in their sequences such that their hybridisation properties differ whereby sequential hybridisation of said adapter sequences to the surface tethered sequences is controlled by sequential changes in reaction conditions. Alternatively, or additionally, in yet a preferred method of the invention said first and second surface tethered sequences are provided on separate substrates thus the method steps i) and iv) involve the use of a first substrate comprising a first set of surface tethered sequences complementary or substantially complementary to the first adapter sequence and a second substrate comprising a second set of surface tethered sequences complementary or substantially complementary to the second adapter sequence, respectively.

Ideally, said first and said second substrates are made from the same material. Alternatively, said first and second substrates are made from different materials.

In yet a further preferred method of the invention, a dissociation step is provided following the elution or extraction step of iii) and/or vi) above whereby the eluted or extracted aptamer is separated from the bound target analyte(s). Additionally, or alternatively, an amplification step is provided following the elution or extraction step of iii) and/or vi) whereby, in the former case, the eluted or extracted aptamer(s) are amplified before the subsequent hybridisation.

In a preferred embodiment of the invention said base units are nucleotides, ideally, selected from the group comprising: ssDNA and ssRNA, more ideally still comprising natural and/or unnatural nucleotides.

Moreover, said sequence of base units preferably comprises biopolymers known to bind DNA or RNA such as, but not limited to, oligonucleotides, peptide nucleic acid, locked nucleic acid and oligodeoxynucleotides such as deoxyribonucleic acid or ribonucleic acid.

It has been found that the hybridisation of the aptamers to the target is influenced by both the sequence of base units and the adapter sequences. By utilising both adapter sequences for target analyte recognition one can achieve increased target diversity and also permit the possibility of binding multiple sites on the same target, such that they interact to form secondary structures. As will be appreciated by those skilled in the art this can, in part, be influenced by the size of the sequence of base units forming the aptamer; where a short sequence of base units is used, both adapter ends may hybridise and bind the same target molecule leading to multiple binding of the same target analyte at the same site or in close proximity to one another. Therefore, according to this preferred embodiment said base units are between 5-40 nucleotides in length and most ideally 15-35 nucleotides in length and even more ideally 20-30 nucleotides in length.

Alternatively, in the case wherein a long sequence of base units is used, each adapter sequence may lead to the binding of two or more different sites on the same target. As will be appreciated by those skilled in the art, such arrangement permits the identification of aptamer molecules exhibiting increased affinity for their respective target molecules by

reducing the dissociation rate of the aptamer-target complex. Therefore, according to this preferred embodiment said base units are between 25-100 nucleotides in length and most ideally 30-75 nucleotides in length and even more ideally 35-50 nucleotides in length.

As will be apparent, the methodology disclosed herein uses a double hybridisation selection step to identify the best aptamer molecules wherein each adapter sequence is, in turn, hybridised to surface tethered sequences (and/or substrate) and the aptamer is tested for binding to the target analyte. As will be appreciated by those skilled in the art, this raises the further possibility of using a different target analyte site in the first and second hybridisation steps such that the first adapter sequence is tested for its ability to bind a first target analyte site and the second adapter sequence is tested for its ability to bind a second target analyte site, or even a different target analyte. This will thus lead to the identification of aptamer molecules capable of simultaneously binding two target sites of interest (within the same target or two different targets) giving rise to bifunctional aptamer molecules.

Accordingly, in yet a further preferred embodiment of the invention said method comprises identifying an aptamer molecule capable of binding at least two target analytes in a sample and/or at least two target analyte sites in a single target in a sample wherein the target analyte or the target analyte site of step ii) differs from the target analyte or the target analyte site of step v).

In yet a further preferred method of the invention said target analyte(s) or said target analyte site(s) is/are any binding partner the aptamer can recognise but most typically is selected from the group comprising: protein, enzyme, antigen, receptor, hormone, metabolite, an organic molecule and carbohydrate.

Accordingly, as described above, the invention concerns the identification of aptamers with the best binding affinity/specificity for at least one target analyte or analyte site, the invention employs the sequential binding of different parts of the aptamer, i.e. using at

least a part of the adapter sequences at both ends of the aptamer, thus leading to the identification of aptamers with superior affinity for target analytes or analyte sites.

Further, utilising at least a part of the adapter sequences at both end of the aptamer has been found to increase the diversity of base sequences involved in analyte binding leading to isolation of aptamers with unique recognition sequences not otherwise obtainable by conventional SELEX. Without wishing to be bound by theory, it is thought that this process maximises utilisation of the oligonucleotide adapter space to create unique recognition sequences, some of which may be superior in binding affinity versus those generated by standard protocols. Further, it is thought that by adopting this process aptamers can be found that are capable of binding multiple target sites thereby promoting formation of secondary structure that leads to multiple binding motifs that are multivariant in nature, thus offering aptamers with superior binding capabilities.

According to a yet further aspect of the invention there is provided an aptamer comprising:

a) a sequence of base units;
b) a first adapter sequence at a first end of said base units; and
c) a second adapter sequence at a second end of said base units.

In a preferred embodiment said base units are between 5-40 nucleotides in length and most ideally 15-35 nucleotides in length and even more ideally 20-30 nucleotides in length.

Alternatively, said base units are between 25-100 nucleotides in length and most ideally 30-75 nucleotides in length and even more ideally 35-50 nucleotides in length.

Yet more preferable, said first adapter sequence and said second different adapter are different from each other, ideally but not exclusively, in terms of sequence.

According to a yet further aspect of the invention there is provided a library of aptamers comprising:

a plurality of test aptamers at least one of which, and preferably a number of which such as a majority or even all, has/have a different sequence of base units with respect to other aptamers in said library; but common to all or a majority of the aptamers is a first adapter sequence at a first end of said aptamer and a second adapter sequence at a second end of said aptamer.

In a preferred library of the invention said first and second adapter sequences are placed at the same end of each of the same or similar aptamers or at different/opposite ends of the same or similar aptamers.

Yet more preferable, said first adapter sequence and said second different adapter are different from each other, ideally, but not exclusively, in terms of sequence.

According to yet a further aspect of the invention there is provided a kit of parts for identifying an aptamer molecule capable of binding a target analyte in a sample comprising:

> a plurality of test aptamer molecules each comprising a sequence of base units having at a first end a first adapter sequence common to all or a majority of the aptamers and at a second end a second adapter sequence common to all or a majority of the aptamers; and/or
>
> a plurality of test aptamers at least one of which, and preferably a number of which, has/have a different sequence of base units with respect to other aptamers in said library; but common to all or a majority of the aptamers is a first adapter sequence at a first end of said aptamer and a second adapter sequence at a second end of said aptamer; and
>
> at least one substrate comprising at least one surface tethered sequence complementary or substantially complementary to said first and/or said second adapter sequence.

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the

word “comprises”, or variations such as “comprises” or “comprising” is used in an inclusive sense i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

All references, including any patent or patent application, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. Further, no admission is made that any of the prior art constitutes part of the common general knowledge in the art.

Preferred features of each aspect of the invention may be as described in connection with any of the other aspects.

Other features of the present invention will become apparent from the following examples. Generally speaking, the invention extends to any novel one, or any novel combination, of the features disclosed in this specification (including the accompanying claims and drawings). Thus, features, integers, characteristics, compounds or chemical moieties described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein, unless incompatible therewith.

Moreover, unless stated otherwise, any feature disclosed herein may be replaced by an alternative feature serving the same or a similar purpose.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

An embodiment of the present invention will now be described by way of example only with reference to the following wherein:

**Figure 1**. Schematic illustration of an aptamer according to the invention comprising a first and a second outer adapter sequence and positioned therebetween a random sequence of nucleotides whose binding affinity for a target analyte is to be tested.

**Figure 2.** Schematic illustration of the selection method according to the invention wherein aptamers according to the invention and so comprising a first and a second outer adapter sequence are exposed to test or target material after which they are eluted or extracted from their substrate before being bound to a second substrate and then exposed to the same or a different test or target material, after which the process is repeated using test or target material that is less and less concentrated thus effectively increasing the stringency of the assay until only those aptamers with the strongest binding affinity will recognise test or target material. Beads 1 and 2 provide two alternative anchoring surfaces for aptamer libraries. These two types of beads are used in alternating orders during SELEX to encompass both ends of aptamers (5' and 3') in target recognition. Although not shown, PCR amplification of aptamers that bound test or target material was undertaken prior to sequential binding to beads 1 or 2.

**Figure 3 (a).** Shows a consensus sequence of random regions (52 bp) of aptamers.

**Figure 3 (b).** Shows alignment results of 10 randomly selected colonies.

The consensus sequence binding affinities to cortisol can be determined using BLI (Bio-layer Interferometry).

**Methods and Materials**

**SELEX**

SELEX (systematic evolution of ligands by exponential enrichment), was used to generate DNA aptamers that can bind specifically to a target analyte. Generally, a single-stranded DNA (ssDNA) library pool was incubated with the target analytes. Binding sequences are selected and amplified by PCR, e.g. using biotin-labelled primers. This is followed by removal of antisense strands to generate an ssDNA pool for subsequent rounds of selection. The enrichment of the selected pools can be monitored by flow cytometry binding assays, with selected pools having increased fluorescence compared with the unselected DNA library.

The random aptamer library ($N_{30}$) and primers were purchased from Trilinkbiotech. The structure of the aptamer library attached to the beads is:

5' TAG GGA AGA GAA GGA CAT ATG AT- $N_{30}$-TTG ACT AGT ACA TGA CCA CTT GA 3'

With the possible primers that could be used reading:

FORWARD - 5' TAG GGA AGA GAA GGA CAT ATG AT 3'
REVERSE - 5' TCA AGT GGT CAT GTA CTA GTC AA 3'
FORWARD Biotin - 5' (Biotin BB™) TAG GGA AGA GAA GGA CAT ATG AT 3'
REVERSE Biotin - 5' (Biotin BB™) TCA AGT GGT CAT GTA CTA GTC AA 3'

The buffers used were:

Wash/Binding (W&B) buffer (10mM Tris-HCl, pH 7.5, 1mM EDTA and 2M NaCl);
SELEX Buffer (150 mmol $L^{-1}$ NaCl, 3 mmol $L^{-1}$ KCl, 25 mmol $L^{-1}$ Tris, 0.05% Tween 20); and
TE buffer (10mM Tris-HCl, 1mM EDTA).

Streptavidin labelled magnetic Dynabeads M-270 (Thermo Fisher Scientific) were used to immobilize the aptamer library via streptavidin-biotin interactions for each round of the SELEX; with the use of a strong magnet to separate the beads by pull downs. Cortisol (Sigma-Aldrich, >98%) and progesterone (Sigma-Aldrich, >99%) were used as the positive and negative analytes to be used in the rounds of the process.

## Detailed description

## Immobilization of Primers and Library:

### 1. Dynabead Wash

A 1.5 mL eppedndorff tube containing a 500µL aliquot of M-270 Dynabeads was placed over a magnet until said Dynabeads have been pulled down to the bottom of the tube. Then the 500µL of storage buffer was pipetted off the Dynabeads, retaining the Dyanbeads within the tube. The Dynabeads were then washed with W&B buffer. Specifically, 500µL of W&B buffer was applied and the tube vortexed until the solution is a homogenous orange/brown colour and there was no sediment remaining in the tube. The beads were then pulled down again using a magnet. This wash process was carried out in total 3 times.

### 2. Dynabead Coating with Biotinylated Reverse Primers

Biotinylated reverse primer (1 nmole) was added to the 500 µl washed Dynabead solution and the mixture was incubated for 10 minutes with gentle agitation at room temperature. The beads were then washed a total of three times using with W&B buffer using the same method as described above. Following the final application of fresh W&B buffer to this point the beads and primers can be produced in bulk and stored at 4°C.

**Hybridization of Aptamer Library.**

To hybridize the library, an aliquot of 100µL of primer-attached Dynabeads was placed in a separate eppendorff tube. Again, a magnet is used to pull down the beads to the tip of the tube (darkest in color point). The 100µL of W&B buffer is then removed by pipette. 100µL of SELEX buffer is then added followed by gentle vortexing of the tube until the solution became homogenous. The Dynabeads were washed three times and stored in SELEX buffer. 5µL of 0.5 µM of the $N_{30}$ library was then added and the solution was gently mixed by vortexing. The mixture was then heated to 95°C for 5 minutes to allow for the aptamer library to anneal to the primers. The solution was then flash cooled on ice for 15 minutes until the tube reached approx. 4°C. The solution was then gently rotated on a

rocking platform for 30 minutes at room temperature. The resultant hybridized library can be stored at 4 °C briefly prior to use.

**SELEX Selection Rounds**

The selection rounds used were both positive (cortisol) and negative (progesterone). The positive was the main analyte used within this process, as it represents the target substrate. For these selection rounds the concentration of cortisol was initially high and steadily decreased before a negative selection round (progesterone) was completed followed by further positive selection rounds in which the concentration of the target substrate (cortisol) was steadily increased. The use of such a gradient allows for the total specific binding affinity of the aptamer library to increase with every round. The table below displays the concentration of cortisol (positive) or progesterone (negative) used as screening substrate for each of SELEX selection rounds 1 to 11.

| SELEX Round | Cortisol µM | Progesterone µM |
|---|---|---|
| 1 | 100 | 0 |
| 2 | 75 | 0 |
| 3 | 50 | 0 |
| 4 | 25 | 0 |
| 5 | 0 | 5 |
| 6 | 25 | 0 |
| 7 | 50 | 0 |
| 8 | 75 | 0 |
| 9 | 100 | 0 |
| 10 | 0 | 10 |
| 11 | 100 | 0 |

Each SELEX selection round was completed using the following general procedure:

1. The hybridized library was washed with SELEX buffer twice to remove any free DNA fragments. The sample is subjected to gentle agitation for 5 minutes per wash to ensure that the DNA is not perturbed (e.g., sheared) by, e.g. vortexing;
2. After, washing twice, fresh SELEX buffer is applied for a third time along with the screening substrate (i.e. cortisol (positive) or progesterone (negative)). The mixture is then incubated at room temperature with gentle agitation for 30 minutes;
3. The incubated mixture is then subjected to a magnetic pull down and the supernatant is removed and collected to use for the PCR (PCR template). The beads are then washed 3 times with SELEX buffer.

**Polymerase Chain Reaction (PCR)**

The PCR step used for this process was primarily high fidelity PCR using Phusion polymerase (NEB). Later a mutagenic step was added to increase the diversity of aptamer library. After each SELEX round, a PCR reaction (50 µl) was setup using the following recipe:

Each reaction included 5X PCR reaction Buffer, 10mM dNTPs, 10 nmoles primers (reverse biotinylated for ease of separation), 4 % PCR reaction volume for the DNA template volume and 0.5µL Phusion polymerase. The PCR was run over 36 cycles with initial hold at 98°C (30s), denaturing at 98°C (10s), annealing at 60°C (30s), extension at 72°C (30s), final extension at 72°C (60s) and then finally hold at 4°C.

The PCR product was then analysed using gel electrophoresis (15% DNA-PAGE gel). Two control samples will be loaded simultaneously: 1. a DNA template free PCR sample and 2. DNA template.

**Elution and Precipitation of Library**

A fresh aliquot of Dynabeads (100 µl) is washed 3 times using W&B buffer. The Dynabeads are then incubated with 40µL of the PCR product that includes the reverse biotinylated primer. The sample is then gently vortexed until the solution is homogenous.

The sample is then incubated with gentle agitation for 15 minutes at room temperature. The sample is then washed with W&B buffer three times.

114µL of 0.125mM NaOH is then added to the sample followed by gentle agitation (no longer than two minutes) to induce DNA strand dissociation. After that, a magnetic pull down of the beads is carried out and the supernatant (~ 100 µl) is transferred to another tube containing a neutralizing solution (3mM Sodium Acetate and TE buffer, 250µL). The collected beads are washed again with NaOH buffer and the supernatant collected and neutralized as before. The supernatant solutions from both washes are then pooled together.

The volume of the pooled supernatant is noted and an equal volume of isopropanol is added to the tube followed by centrifugation for 15 mins at 15,00rpm, 4°C. Upon completion of centrifugation, most of the solution is removed and one equal volume of ice-cooled 70% ethanol is added to the tube before a further centrifugation step (5 mins at 15,000 rpm, 4°C). The ethanol is then pipetted from the tube and the tube is placed in a heated plate (45°C) for 15 mins to remove any remaining ethanol. Following this the next generation DNA library can be resuspeneded in 50 µl of TE buffer. The DNA library concentration is then measured using UV-Vis and adjusted to 5µM. The library is then ready for a further round of SELEX.

The SELEX selection round may be repeated several times (e.g. 15-20 times) before the library is annealed to T vectors (T-easy, Promega), transformed into *E coli* (XL Blue Ultra II, Stratagene) and the selected aptamer sequences subjected to Sanger sequencing to determine the sequence of the aptamers.

**Results**

A15-step double-ended SELEX using cortisol as the target molecule was performed, and the sequence of a new cortisol aptamer was identified by performing the conventional blue-white colony selection approach[3] followed by DNA sequencing. Ten white colonies were selected and DNA Sanger sequencing performed. Among them, the consensus sequence shown below was found.

(GGGCCGCGACAACGATGTGTTGTCTCGGAGGGTAATTCGGCACATGCGGTCC) as shown in Figure 3a. The full sequence including the adaptor was

ATACCAGCTTATTCAATTGGGCCGCGACAACGATGTGTTGTCTCGGAGGGTAATTC GGCACATGCGGTCCAGATAGTAAGTGCAATCT, where Adaptor sequence is underlined).

Three out of 10 selected colonies exhibit identical sequences to the consensus sequence. Excellent sequence convergence was observed on most of the random sequence sites, except a few sites as shown in Fig. 3b. This shows the novel method of the invention can be reliably used to identify new aptamers that bear surprisingly similar sequence structure.

**Conclusion**

Low target specificity is one of the major limitations of aptamer use in various biosensing applications. Our new screening tool is expected to address that limitation by increasing the specificity of selected aptamers on the basis of using aptamers that have two target recognition motifs, whether that is in respect of two target recognition motifs for a single analyte or two target recognition motifs in respect of two different analytes.

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## Claims

1. A method for identifying an aptamer molecule capable of binding a target analyte in a sample comprising:

providing a plurality of test aptamer molecules each comprising a sequence of base units having at a first end a first adapter sequence common to all or a majority of the aptamers and at a second end a second adapter sequence common to all or a majority of the aptamers,

providing a substrate comprising surface tethered sequences complementary or substantially complementary to said first and/or said second adapter sequence(s); and wherein said method comprises the steps of:

i) hybridising the test aptamer molecules to said surface tethered sequences complementary to said first adapter sequence;

ii) exposing said hybridised aptamers to a sample under conditions whereby target analytes in said sample that have target analyte binding sites for an aptamer, bind any one or more of said test hybridised aptamer molecules;

iii) eluting or extracting at least the aptamer molecules hybridised to said surface tethered sequences and bound by said target analytes;

iv) hybridising the eluted aptamer molecules of step iii) to said surface tethered sequences complementary to said second adapter sequence;

v) exposing said hybridised aptamers of step iv) to the said sample or a further sample comprising target molecules under conditions whereby target analytes in said sample that have target analyte binding sites for an aptamer bind any one or more of said test hybridised aptamer molecules;

vi) eluting or extracting at least the aptamer molecules hybridised to said surface tethered sequences and bound by said target analytes; and

vii) optionally, repeating steps i)-iii) and/or iv)-vi); and

viii) concluding said aptamers of steps vi) or vii) are capable of identifying the target analyte(s) in the sample.

2. The method according to claim 1 wherein said test aptamer molecules form part of a library of aptamers.
3. The method according to claim 1 or claims 2 wherein steps i) – iii) and/or iv) – vi) are repeated a number of times.
4. The method according to any preceding claim wherein said adapter sequences at either end of said aptamer are different from each other.
5. The method according to any preceding claim wherein said substrate is a material or coating selected form the list comprising: silver, gold, aluminium, copper, platinum, iron and silica metals/metalloids or an alloy thereof or a polymer-coated version thereof.
6. The method according to any preceding claim wherein said substrate comprises surface tethered sequences complementary or substantially complementary to said first and said second adapter sequences.
7. The method according to any one of claims 1-6 wherein said first and second surface tethered sequences are provided on separate substrates.
8. The method according to claim 7 wherein step i) involves the use of a first substrate comprising a first set of surface tethered sequences complementary or substantially complementary to the first adapter sequence and step iv) involves the use of a second substrate comprising a second set of surface tethered sequences complementary or substantially complementary to the second adapter sequence.
9. The method according to claim 7 or claim 8 wherein said first and said second substrates are made from the same material or said first and second substrates are made from different materials.
10. The method according to any preceding claim wherein a dissociation step is provided following the elution or extraction step of iii) and/or vi) above whereby the eluted or extracted aptamer is separated from the bound target analyte(s).
11. The method according to any preceding claim wherein an amplification step is provided following the elution or extraction step of iii) and/or vi).

12. The method according to any preceding claim wherein said base units are nucleotides, ideally, selected from the group comprising: ssDNA and ssRNA comprising natural and/or unnatural nucleotides.
13. The method according to any preceding claim wherein said base units preferably comprises biopolymers known to bind DNA or RNA such as, but not limited to, oligonucleotides, peptide nucleic acid, locked nucleic acid, oligodeoxynucleotides such as deoxyribonucleic acid or ribonucleic acid.
14. The method according to any preceding claim wherein said base units are between 5-40 nucleotides in length such as 15-35 nucleotides in length or 20-30 nucleotides in length.
15. The method according to any one of claims 1-13 wherein said base units are between 25-100 nucleotides in length such as 30-75 nucleotides in length or 35-50 nucleotides in length.
16. The method according to any preceding claim wherein said method comprises identifying an aptamer molecule capable of binding at least two target analytes in a sample and/or at least two target analyte sites in a single target in a sample wherein the target analyte or the target analyte site of step ii) differs from the target analyte or the target analyte site of step v).
17. An aptamer comprising:

a) a sequence of base units;

b) a first adapter sequence at a first end of said base units; and

c) a second adapter sequence at a second end of said base units.

18. The aptamer according to claim 17 wherein said base units are selected from the group comprising: 5-40 nucleotides in length, 15-35 nucleotides in length, 20-30 nucleotides in length, 25-100 nucleotides in length, 30-75 nucleotides in length and 35-50 nucleotides in length.

19. The aptamer according to claim 17 or claim 18 wherein said first adapter sequence and said second different adapter are different from each other.

20. A library of aptamers comprising:

a plurality of test aptamers at least one of which, and preferably a number of which, has/have a different sequence of base units with respect to other aptamers in said library; but common to all or a majority of the aptamers is a first adapter sequence at a first end of said aptamer and a second adapter sequence at a second end of said aptamer.

21. The library according to claim 20 wherein said first and second adapter sequences are placed at the same end of each of the same or similar aptamers or at different/opposite ends of the same or similar aptamers.

22. The library according to claim 20 or 21 wherein said first adapter sequence and said second different adapter are different from each other.

23. A kit of parts for identifying an aptamer molecule capable of binding a target analyte in a sample comprising:

a plurality of test aptamer molecules each comprising a sequence of base units having at a first end a first adapter sequence common to all or a majority of the aptamers and at a second end a second adapter sequence common to all or a majority of the aptamers; and/or

a plurality of test aptamers at least one of which, and preferably a number of which, has/have a different sequence of base units with respect to other aptamers in said library; but common to all or a majority of the aptamers is a first adapter sequence at a first end of said aptamer and a second adapter sequence at a second end of said aptamer; and

at least one substrate comprising at least one surface tethered sequence complementary or substantially complementary to said first and/or said second adapter sequence.

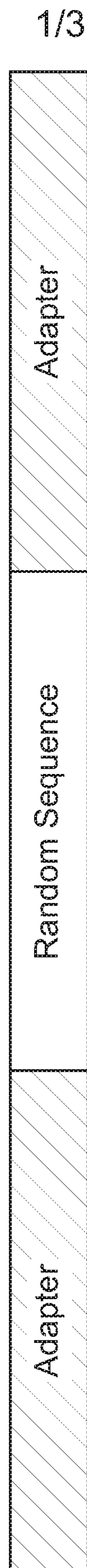


Fig. 1

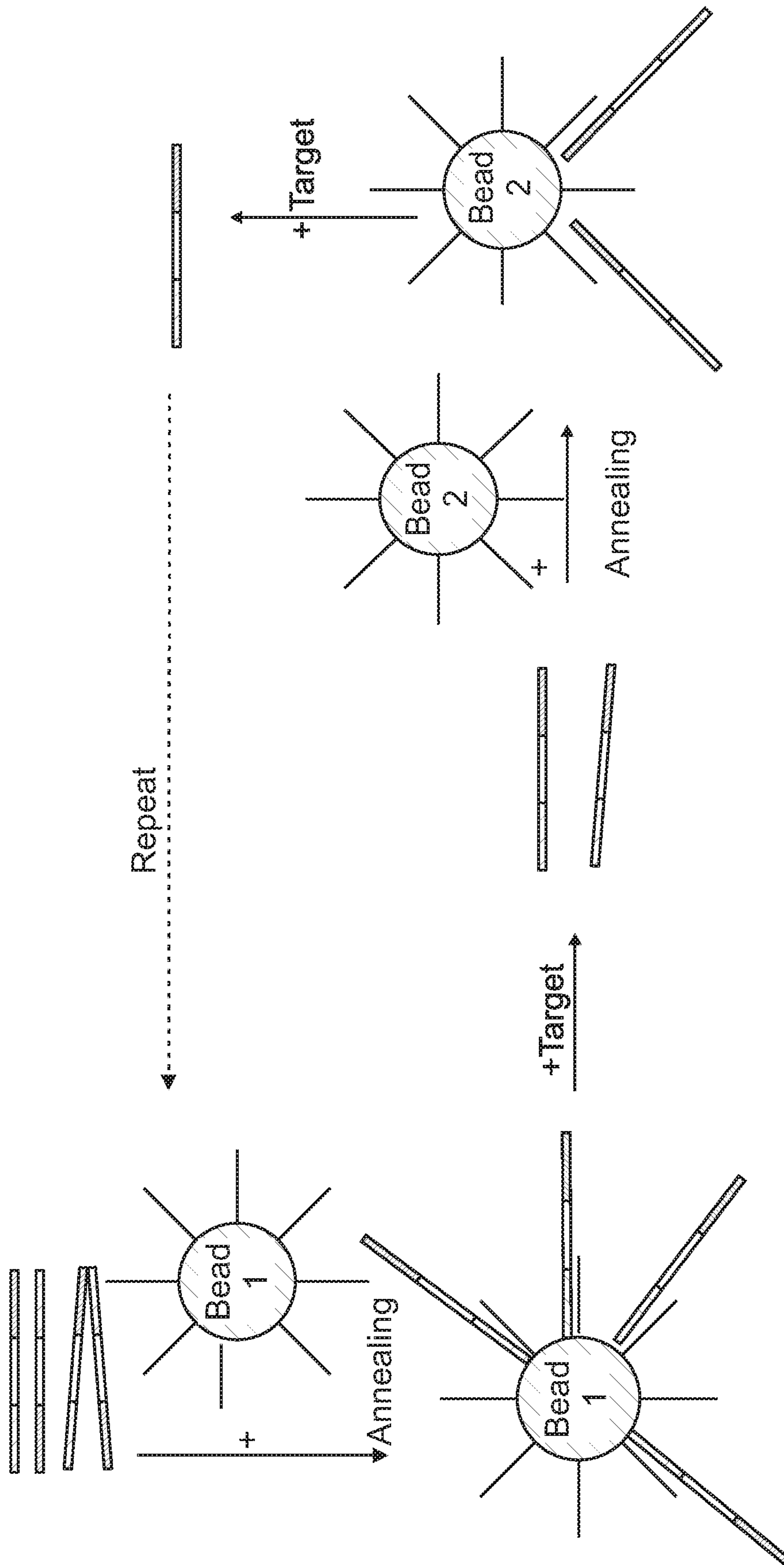


Fig. 2

3/3

a

bits

5' 3'

b

Adaptor 1

Adaptor 2

Fig. 3

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/52525

A. CLASSIFICATION OF SUBJECT MATTER
IPC - C12Q 1/68; C12N 15/115; C40B 40/06 (2018.01)
CPC - C12Q 1/6811, 6813, 6837, 6855; C12N 15/115; C40B 40/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | (RAY, P et al.) Cell-SELEX Identifies a (Sticky) RNA Aptamer Sequence. Journal of Nucleic Acids. 17 January, 2017; Vol. 2017, No. 4943072; pages 1-10; page 2, column 2, paragraph 3; page 8, column 1, paragraph 3; DOI: 10.1155/2017/4943072 | 17-18, 19/17-18, 20-21, 22/20-21 |
| A | US 2015/064696 A1 (NEC CORPORATION) 05 March, 2015; claim 1 | 1-2, 3/1-2, 23 |
| A | WO 2014/100434 A1 (CARIS SCIENCE, INC.) 26 June, 2014; paragraph [00229] | 1-2, 3/1-2, 23 |
| A | WO 2011/068909 A2 (BRIGHAM AND WOMEN'S HOSPITAL, INC.) 09 June, 2011; figures 5A-B; page 8, paragraph 8 | 1-2, 3/1-2, 23 |
| A | (LIU, Q et al.) Aptamer-Conjugated Nanomaterials for Specific Cancer Cell Recognition and Targeted Cancer Therapy. NPG Asia Materials. 11 April, 2014; Vol. 6, No. 2; pages 1-10; page 3, column 1, paragraph 2; DOI: 10.1038/am.2014.12 | 1-2, 3/1-2, 23 |
| A | (ABDELSAYED, MM et al.) Multiplex Aptamer Discovery through Apta-Seq and Its Application to ATP Aptamers Derived from Human-Genomic SELEX. ACS Chemical Biology. 11 July, 2017; Vol. 12, No. 8; pages 1-19; page 9, paragraph 1; DOI: 10.1021/acschembio.7b00001 | 1-2, 3/1-2, 23 |

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 21 December 2018 (21.12.2018) | 15 JAN 2019 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| Mail Stop PCT, Attn: ISA/US, Commissioner for Patents<br>P.O. Box 1450, Alexandria, Virginia 22313-1450<br>Facsimile No. 571-273-8300 | Shane Thomas<br>PCT Helpdesk: 571-272-4300<br>PCT OSP: 571-272-7774 |

## INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/52525

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.