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- (71) Applicant (for all designated States except US): **INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)** [FR/FR]; 101, Rue de Tolbiac, F-75013 Paris (FR).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **DAUSSE, Eric** [FR/FR]; U869 Inserm IECB, Université de Bordeaux 2 rue Robert Escarpit, F-33607 Pessac Cedex (FR). **DI PRIMO, Carmelo** [FR/FR]; U869 Inserm IECB, Université de Bordeaux 2 rue Robert Escarpit, F-33607 Pessac Cedex (FR). **CHEVET, Eric** [FR/FR]; Inserm U1053 Université Bordeaux 2 146 rue Léo Saignat, F-33000 Bordeaux (FR). **TAOUJI, Said** [FR/FR]; Inserm U1053 Université Bordeaux 2 146 rue Léo Saignat, F-33000 Bordeaux (FR). **TOULME, Jean-Jacques** [FR/FR]; U869 Inserm IECB, Université de Bordeaux 2 rue Robert Escarpit, F-33607 Pessac Cedex (FR).
- (74) Agents: **DOMENEGO, Bertrand** et al.; 2, place d'Estienne d'Orves, F-75009 Paris (FR).
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(54) Title: METHODS FOR IDENTIFYING APTAMERS

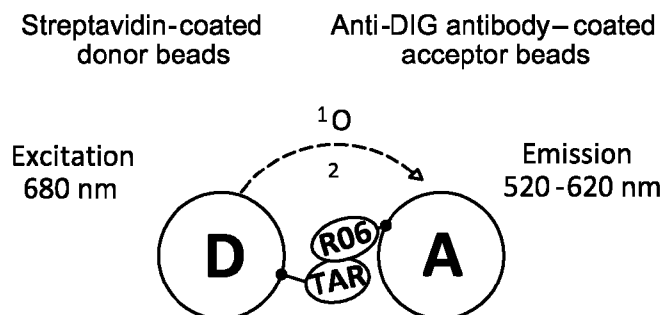


FIG.1

(57) Abstract: The present invention relates to a method for identifying aptamers directed against a target molecule.

Methods for identifying aptamers

The present invention relates to a method for identifying aptamers directed
5 against a target molecule.

Aptamers are DNA or RNA oligomers selected from random pools on the basis
of their ability to bind other molecules (Ellington *et al* (1990) *Nature* **346** (6287): 818,
Robertson and Joyce (1990) *Nature* **344** (6265): 467, Tuerk and Gold (1990) *Science* **249**
10 (4968): 505). To date, aptamers have been selected against many different types of
targets: small organic compounds, proteins, nucleic acids and complex scaffolds such as
live cells (Dausse *et al.* (2009) *Curr. Opin. Pharmacol* **9**(5): 602, Hall *et al.* (2009) *Curr.*
Protoc. Mol. Biol. Chapter **24**, Unit 24 (3)). These molecules compete with antibodies in
terms of binding properties, specificity of recognition and potential uses in medicine and
technology.

15 Aptamers are generally obtained by systematic evolution of ligands by
exponential enrichment (SELEX) (Gold *et al.* (1997) *Proc. Natl. Acad. Sci. USA* **94** (1): 89)
even though selection without any amplification step (non-SELEX) has also been
described (Javaherian *et al.* (2009) *Nucleic Acids Res* **37** (8): e62).

The current approaches require sequencing of the selected sequences at the
20 end of directed evolution, followed by a limiting step based on sequence comparison and
individual evaluation of a few candidates for the identification of the suitable aptamers.
This makes SELEX a slow process and prevents whole procedure automation.
Furthermore, this may lead to the loss of orphan candidates that could represent strong
binders but could be masked by efficiently amplified poor binders.

25 As a consequence, a method leading to an increased rate in aptamer discovery
and amenable to automation would constitute a significant advance in the field.

In this study, a functional screen downstream of the SELEX pipeline was
developed, based on a luminescent oxygen channelling immunoassay (LOCI), like the
AlphaScreen® technology. LOCI is based on Donor (D, photosensitizer) and Acceptor (A,
30 chemiluminescer) microbeads, coated with two molecules of interest, susceptible to bind
to each other (**Figure 1**). Laser excitation of the D beads causes ambient oxygen to be
converted to the singlet state by the photosensitizer. Singlet oxygen species activate in
turn chemiluminescent agents on the A bead. Upon activation, the chemiluminescent
agent emits light which is detected by the photo-detector in a microplate reader. A signal
35 is produced when the A and D beads are brought into proximity (<200 nm), thus reporting
for the interaction between the partners captured on the two beads (Taouji *et al.* (2009)
Curr Genomics **10**(2):93).

Here, the inventors showed for the first time that it was possible to develop a quick method for the identification of aptamers starting from a mixture of candidate nucleic acids and using a LOCI-type technique. Besides, this method allows for screening a larger number of candidates than the current procedures do and is amenable to automation.

5 More specifically, the inventors developed a method that allows the primary screening of aptamer candidates based on the blind determination of their binding properties rather than on sequence homology.

Detailed description of the invention

10 The present invention relates to a method for identifying an aptamer directed against a target molecule comprising the following steps:

a. obtaining a candidate enriched mixture of nucleic acids directed against the target molecule by:

15 i) contacting a mixture of candidate nucleic acids with the target molecule wherein nucleic acids having a strongest affinity to the target molecule relative to the candidate mixture may be partitioned from the remainder of the candidate nucleic acid mixture,

ii) partitioning the nucleic acids with strong affinity for the target molecule from the remainder of the candidate nucleic acid mixture; and

20 iii) amplifying the nucleic acid with strong affinity to yield a candidate enriched mixture of nucleic acids,

b. isolating each of the candidate nucleic acids from the candidate enriched mixture,

c. binding each candidate nucleic acid to one member of a first binding pair, which forms a bound candidate nucleic acid,

25 d. providing the target molecule bound to one member of a second binding pair, forming a bound target,

e. contacting the bound candidate nucleic acid with a first particle bound to the second member of the first binding pair, possibly via an intermediary compound,

30 f. contacting the bound target with a second particle bound to the second member of the second binding pair, possibly via an intermediary compound,

wherein said first and second particles are respectively a donor and an acceptor particle, or said first and second particles are respectively an acceptor and a donor particle,

g. identifying the aptamer nucleic acid candidates by:

35 i) detecting if an energy and/or chemical transfer occurs between the donor and acceptor particles, and

ii) identifying the candidate nucleic acid as an aptamer directed against the target molecule if energy transfer is detected.

Aptamers and target molecules

5 "Aptamers" are nucleic acids that exhibit specificity and affinity for a target molecule.

In accordance with the invention, the "aptamers directed against a target molecule" have specificity and affinity for this target molecule.

As used herein, "specificity" refers to the ability of the nucleic acid to distinguish in
10 a reasonably unique way between the target molecule and any other molecules.

The "affinity" of the nucleic acid for its target molecule corresponds to stability of the complex between the two and can be expressed as the equilibrium dissociation constant (K_D). The techniques used to measure affinity are well-known by the skilled person. They can be, for example by Surface Plasmon Resonance analyses as described
15 in example 3.

The affinity depends on the nature of the nucleic acid and of the target molecule. The one skilled in the art is able to determine the desired conditions depending on the tested nucleic acids and target molecules. More precisely, the one skilled in the art is able to define the sufficient level of affinity for obtaining the desired aptamers using the process
20 used to conduct step a. For instance, as disclosed herein, the one skilled in the art may perform the known SELEX method for step a. in the usual conditions and with a suitable affinity, *i.e.* to obtain a candidate enriched mixture containing the nucleic acids having a strong affinity (those having the strongest affinity in the starting mixture). Preferentially, the specificity and affinity of an aptamer according to the invention for its target are
25 comparable or higher to those of an antibody directed against this target.

In accordance with the invention, the nucleic acid can be of any type, it can notably be natural or synthetic, DNA or RNA, single or double-stranded, preferably single-stranded. In particular, where the nucleic acid is synthetic, it can comprise non-natural modifications of the bases or bonds, in particular for increasing the resistance to
30 degradation of the nucleic acid. Preferably, the nucleic acid according to the invention is a RNA.

"Candidate nucleic acids" is used herein to define nucleic acids which are potentially able to act as aptamers of a target molecule.

A "mixture of candidate nucleic acid" or "candidate nucleic acid mixture" is a
35 mixture of nucleic acids differing in sequences, from which one wants to select a desired ligand.

The source of a candidate mixture can be from naturally-occurring nucleic acids or fragments thereof, chemically synthesized nucleic acids, enzymatically synthesized nucleic acids or nucleic acids made by a combination of the foregoing techniques. The candidate mixture generally includes regions of fixed sequences (*i.e.*, each of the members of the candidate mixture contains the same sequences in the same location). The fixed sequence regions can be chosen to assist in the amplification steps described below. Preferentially, the mixtures of candidate nucleic acids originate from libraries of nucleic acids obtained as described in Dausse *et al* (2005) *Meth Mol Biol* **288**: 391-410.

As intended herein, a "candidate enriched mixture of nucleic acids" is a mixture of candidate nucleic acids which is enhanced in those candidate nucleic acids with the strongest affinities to the target molecule.

In accordance with the invention, a "target molecule" can be any molecule against which aptamers may be directed. Preferentially, a target molecule is a protein, a nucleic acid (DNA or RNA) or a small organic molecule.

In another embodiment the target molecule is a mixture of target molecules. The mixture may contain 2, 3, 4, 5 or more target molecules.

These target molecules may all be of the same kind; for example, they can all be nucleic acids or proteins or small organic molecules. Alternatively, the target molecules comprised within the mixture of target molecules can be of different kinds, for example a mixture of proteins and nucleic acids and might eventually interact with each other; in this case the target molecule will be a complex.

Obtention of a candidate enriched mixture of nucleic acids directed against the target molecule

The candidate enriched mixtures of nucleic acids can be produced by any methods known by the skilled person. In particular, they can be obtained by a SELEX-type method.

The SELEX method involves the combination of a selection of nucleic acid candidates which bind to a target molecule with an amplification of those selected nucleic acids. Iterative cycling of the selection/amplification steps allows selection of nucleic acids which bind most strongly to the target from a pool which contains a very large number of nucleic acids.

For example, the SELEX method (hereinafter termed SELEX), was first described in U.S. application Ser. No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands By Exponential Enrichment," now abandoned. U.S. Pat. No.

5,475,096, entitled "Nucleic Acid Ligands," and U.S. Pat. No. 5,270,163, entitled "Methods for Identifying Nucleic Acid Ligands," also disclose the basic SELEX process.

The SELEX-type process as used in a method according to the invention may, for example, be defined by the following series of steps:

5 i) Contacting a mixture of candidate nucleic acids with the target molecule; nucleic acids having a strongest affinity to the target molecule relative to the candidate mixture may be partitioned from the remainder of the candidate nucleic acid mixture. Preferably, the mixture is contacted with the selected target molecule under conditions suitable for binding to occur between them. Under these circumstances, complexes between the
10 target molecule and the nucleic acids having the strongest affinity for the target molecule can be formed.

ii) Partitioning the nucleic acids with the strongest affinity for the target molecule from the remainder of the candidate mixture. At this step, the nucleic acids with the strongest affinity for the target molecule are partitioned from those nucleic acids with
15 lesser affinity to the target molecule.

iii) Amplifying the nucleic acids with the strongest affinity to the target molecule to yield a candidate enriched mixture of nucleic acids. In this step, those nucleic acids selected during partitioning as having a relatively higher affinity to the target molecule are amplified to create a new candidate mixture that is enriched in nucleic acids having a
20 relatively higher affinity for the target.

In an embodiment according to the invention, the partitioning and amplifying steps above can be repeated (cycling) so that the newly formed candidate mixture contains fewer unique sequences and the average degree of affinity of the nucleic acid mixture to the target is increased.

25 "Partitioning" means any process whereby nucleic acid candidates bound to target molecules, identified herein as candidate-target complexes, can be separated from nucleic acids not bound to target molecules. Partitioning can be accomplished by various methods known in the art. For example, candidate-target complexes can be bound to nitrocellulose filters while unbound candidates are not. Columns which specifically retain
30 candidate-target complexes can be used for partitioning. Liquid-liquid partition can also be used as well as filtration gel retardation, affinity chromatography and density gradient centrifugation.

Preferentially, the partitioning can be performed by attaching the target molecules on magnetic beads followed by binding of the nucleic acids to the target molecules and
35 subsequent separation of the magnetic beads/target molecules/nucleic acids particles. Several different methods of automated separation of magnetic beads are known from the

art. The first method is to insert a magnetic or magnetizable device into the medium containing the magnetic beads, binding the magnetic beads to the magnetic or magnetizable device, and remove the magnetic or magnetizable device. In a second method the separation of medium and the magnetic particles, both aspirated into a pipette tip, is facilitated by a magnetic or magnetizable device which is brought into spatial proximity to the pipette tip. The choice of the partitioning method will depend on the properties of the target and of the candidate-target complexes and can be made according to principles known to those of ordinary skill in the art.

Preferably, the partitioning can be performed by magnetic beads separation. The 3' end biotinylated targets were immobilized on magnetic streptavidin beads and incubated with the RNA pool. The incubation may be performed at room temperature, i.e. between 20°C to 30°C, preferably between 22°C to 25°C.

After the candidate nucleic acids bound to the target molecules have been separated from those which have remained unbound, the next step in partitioning is to separate them from the target molecules. Thus, the candidate nucleic acids can be separated by heating in water at a temperature sufficient to allow separation of the species. Alternatively separation can be achieved by addition of a denaturing agent. Bound candidates can also be collected by competition with the free target. For example, the candidate nucleic acids can be separated by heating in water for one minute at 75°C as described in particular in the examples below. A mixture of nucleic acids with increased affinity to the target molecule is thus obtained.

After partitioning, the candidate nucleic acids with high affinity may be amplified. As intended herein "amplifying" means any process or combination of process steps that increases the amount or number of copies of a molecule or class of molecules.

The amplification step can be performed by various methods which are well known to the person skilled in the art.

A method for amplifying DNA molecules can be, for example, the polymerase chain reaction (PCR). In its basic form, PCR amplification involves repeated cycles of replication of a desired single-stranded DNA (or cDNA copy of an RNA) using specific oligonucleotides complementary to the 3' and 5' ends of the single stranded DNA as primers, achieving primer extension with a DNA polymerase followed by DNA denaturation. The products generated by extension from one primer serve as templates for extension from the other primer. Descriptions of PCR methods are found in Saiki *et al.* (1985) *Science* **230**:1350-1354 or Saiki *et al.* (1986) *Nature* **324**:163-166.

Methods for amplifying RNA molecules are well known from the person skilled in the art. For example, amplification can be carried out by a sequence of three reactions:

making cDNA copies of selected RNAs (using reverse transcriptase), using the polymerase chain reaction to increase the copy number of each cDNA, and transcribing the cDNA copies to obtain RNA molecules having the same sequences as the selected RNAs.

5 In accordance with the invention, the candidate nucleic acids are preferably amplified with the help of oligonucleotides capable of hybridizing to fixed sequences common to these nucleic acids.

10 In accordance with the invention, an amplification step is preferentially carried out on the mixture of nucleic acids with increased affinity obtained during the partitioning step to yield a candidate enriched mixture of nucleic acids.

The relative concentrations of target molecules to nucleic acid employed to achieve the desired partitioning will depend for example on the nature of the target molecule, on the strength of the binding interaction and on the buffer used. The relative concentrations needed to achieve the desired partitioning result can be readily determined empirically without undue experimentation.

15 Cycling (repetition) of the partitioning /amplification procedure can be continued until a selected goal is achieved. For example, cycling can be continued until a desired level of binding of the nucleic acids in the test mixture is achieved or until a minimum number of nucleic acid components of the mixture is obtained. It could be desired to continue cycling until no further improvement of binding is achieved.

20 The number of cycles to be carried out is preferably below 100, more preferably below 10. According to one way of performing the invention, the number of cycles is 7. According to another way of performing the invention, the number of cycles is less than 7, preferentially equal to 6, 5, 4, 3, 2 or 1 cycle(s).

25

Isolation of the nucleic acid candidates from the candidate enriched mixture of nucleic acids

Each of the candidate nucleic acids can be isolated from the candidate enriched mixture of nucleic acids or from mixture of nucleic acids with increased affinity. Preferably, it is isolated by cloning it into a vector suitable to be transferred in a host cell.

30

Methods for cloning nucleic acids and transferring vectors in host cells are well known from the person skilled in the art (Molecular Cloning : a laboratory Manual, Joseph Sambrook, David William Russell- Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 3rd edition, January 15, 2001).

35

As a vector, use may be done of the pCR^R2.1-TOPO vector (Invitrogen, Carlsbad, USA).

Preferably, selected sequences were amplified by PCR with a final 7 to 10 minutes extension step and cloned in using the TOPO TA cloning kit[®] (Invitrogen).

5 The term "transferred" according to the invention means the introduction of a "foreign" DNA or RNA sequence into a cell. The cell according to the invention may, for example, be a bacterial cell, a yeast cell or an animal cell like a mammal cell or an avian cell. Preferably, the cell according to the invention is a bacterial cell, more preferably an *Escherichia coli* cell.

10 The transferred methods are well-known by the skilled person. Preferentially, transferred is carried out by transformation, electroporation or conjugation.

The candidate nucleic acids sequence is preferably introduced into the cell by a vector.

15 An amplification step is preferably be performed after cloning of the candidate nucleic acids.

The amplification step can be performed by various methods which are well known to the person skilled in the art.

20 As described above, a method for amplifying DNA molecules can be, for example, the polymerase chain reaction (PCR). In its basic form, PCR amplification involves repeated cycles of replication of a desired single-stranded DNA (or cDNA copy of an RNA) using specific oligonucleotides complementary to the 3' and 5' ends of the single stranded DNA as primers, achieving primer extension with a DNA polymerase followed by DNA denaturation. The products generated by extension from one primer serve as templates for extension from the other primer. Descriptions of PCR methods are found in
25 Saiki *et al.* (1985) *Science* **230**:1350-1354 or Saiki *et al.* (1986) *Nature* **324**:163-166.

30 Methods for amplifying RNA molecules are well known from the person skilled in the art. For example, amplification can be carried out by a sequence of three reactions: making cDNA copies of selected RNAs (using reverse transcriptase), using the polymerase chain reaction to increase the copy number of each cDNA, and transcribing the cDNA copies to obtain RNA molecules having the same sequences as the selected RNAs.

In accordance with the invention, the candidate nucleic acids are preferably amplified with the help of oligonucleotides capable of hybridizing to fixed sequences common to these nucleic acids.

35 The amplification step performed after cloning of the candidate nucleic acids may, preferably, be directly performed on the transformed colonies (colony PCR). For example,

colonies are sampled with a sterile toothpick and a small quantity of cells is transferred into a PCR mix. Alternatively, amplification can be carried out on the recombinant vector containing the candidate nucleic acid after the vectors are isolated from the host cells. Techniques for isolating recombinant vectors from host cells are well known by the skilled person, for example it can be achieved with the help of a miniprep kit. Preferentially, the PCR is performed directly on the colonies.

In a preferred way to perform the invention, the oligonucleotide used for amplifying the candidate nucleic acids carries a sequence which is not complementary to that of the candidate nucleic acids and which will lead to the copies of the candidate nucleic acids being labelled. For example, the oligonucleotide carries a repeated sequence such as poly A, poly T, poly G or poly C which leads to the candidate nucleic acids carrying a poly T, poly A, poly C or poly G sequence. Preferably, the oligonucleotide carries a poly A sequence. The copies of the candidate nucleic acids obtained after amplification by such an oligonucleotide are said to be bound to a poly T tail.

Particles

As intended herein, "particles" refers to a particulate water-insoluble polymeric material which remains in suspension. Preferentially, particles of the invention are beads. The beads of the invention can be 20 nm to 20 mm, more preferably 100 to 1000 nm, in diameter. Still more preferably, the beads are of 200 nm diameter.

The beads are preferably made of latex that is of a substituted polyethylene such as the following: polystyrene-butadiene, polyacrylamide polystyrene, polystyrene with amino groups, poly-acrylic acid, polymethacrylic acid, acrylonitrile-butadiene, styrene copolymers, polyvinyl acetate-acrylate, polyvinyl pyridine, vinyl-chloride acrylate copolymers, and the like.

The particles according to the invention are said to be donor particles if they contain or are bound to a compound which emits an energy and/or a chemical event when submitted to an excitation such as an irradiation, a chemi-excitation, an electrochemical activation or the like. They are said to be acceptor particles if they contain or are bound to a compound which is susceptible to an energy and/or chemical event linked to the energy and/or a chemical event issued from the donor particles, and emits a detectable signal.

Such particles can be commercially available particles.

In a first embodiment, the donor particle contains or is bound to a photosensitizer and the acceptor particle contains or is bound to a chemiluminescent.

For the purpose of this invention, "photosensitizer" refers to a molecule which can be excited to a metastable state, usually a triplet state. When it is in the proximity of

molecular oxygen, it can directly or indirectly transfer its energy to the oxygen with simultaneous excitation of the oxygen to a highly reactive excited state, often referred to as singlet state oxygen.

The photosensitizer will usually be excited by the absorption of light but may also be excited by chemi-excitation, electrochemical activation or by other means. Preferably, excitation of the photosensitizer is caused by irradiation with light from an external source. The photosensitizer according to the invention will preferably have an absorption maximum in the wavelength range of 250-1100 nm, preferably 300-1000 nm, and more preferably 450-950 nm, with an extinction coefficient at its absorbance maximum greater than $500 \text{ M}^{-1}\text{cm}^{-1}$, preferably at least $5000 \text{ M}^{-1}\text{cm}^{-1}$, more preferably at least $50,000 \text{ M}^{-1}\text{cm}^{-1}$. The lifetime of the excited state, usually a triplet state produced following absorption of light by the photosensitizer, will usually be at least 100 nsec, preferably at least 1 microsecond in the absence of oxygen. In general, the lifetime must be sufficiently long to permit the energy transfer to oxygen, which will normally be present at concentrations in the range of 10^{-5} to 10^{-2}M (depending on the medium).

The photosensitizer of the instant invention is preferably relatively photostable and will not react efficiently with the singlet state molecular oxygen so generated.

Typical photosensitizers include ketones such as benzophenone and 9-thioxanthone; xanthenes such as eosin and rose bengal; polyaromatic compounds such as buckminsterfullerene and 9,10-dibromoanthracene; porphyrins including metallo-porphyrins such as hematoporphyrin and chlorophyllis; oxazines; cyanines; squarate dyes; phthalocyanine; naphthalocyanines; merocyanines; thiazines such as methylene blue. Preferably, the photosensitizer according to the invention is phthalocyanine.

As intended herein, "chemiluminescent" refers to a photoactivable substance that undergoes a chemical reaction with singlet state oxygen to form a metastable reaction product that is capable of decomposition with the simultaneous or subsequent emission of light, usually in the wavelength range of 250 to 1200 nm. The chemiluminescent of interest will preferably emit at a wavelength above 300 nm, preferably above 500 nm, and more preferably above 550 nm, even more preferably above 600 nm. Chemiluminescents that are preferred in accordance with the present invention comprise rubrene, enol ethers, enamines, 9-alkylidene-N-alkylacridans, arylvinylethers, dioxenes, arylimidazoles, 9-alkylidenexanthenes and lucigenin, luminol and other phthalhydrazides, firefly luciferin, aquaphorin, luminal. Beads called ALPHALISA beads can also be used and contains europium cryptate which is disrupted upon excitation and emits with a narrow bandwidth. Preferably, the chemiluminescent according to the invention is rubrene.

In a preferred embodiment, the photosensitizer is phthalocyanin and the chemiluminescent is rubrene.

In another embodiment, the donor particle contains or is bound to a donor fluorophore and the acceptor particle contains or is bound to an acceptor fluorophore.

5 The pairs of fluorophores are preferentially chosen so that their excitation spectra do not or very little overlap and so that the emission spectrum of the donor fluorophore overlaps at least in part the excitation spectrum of the acceptor fluorophore. The fluorophore donor/fluorophore acceptor pairs can be, for example, Blue Fluorescent protein (BFP) / Green Fluorescent protein (GFP), Cyan Fluorescent protein (CFP) / Yellow
10 Fluorescent protein (YFP), GFP/cyanine 3. Alternatively, fluorescein can be the donor when the fluorophore acceptor is chosen amongst eosin, chlorofluorescein or Texas red.

The association of the photosensitizer, chemiluminescent or fluorophore with the beads used in the present invention may involve incorporation during formation of the particles by polymerization but will usually involve incorporation into preformed particles,
15 usually by non-covalent association to the particles. The methods for producing such particles are well-known by the skilled person.

In an embodiment, the first particle is a donor particle and the second particle is an acceptor particle. In another embodiment, the first particle is an acceptor particle and the second particle is a donor particle.

Binding pairs

According to the invention, each candidate nucleic acid is bound to one member of a first binding pair, and each target molecule is bound to one member of a second binding pair.

25 A first particle is bound to the second member of the first binding pair, optionally via an intermediary compound.

A second particle is bound to the second member of the second binding pair, optionally via an intermediary compound.

The binding pairs according to the invention comprise two molecules capable of
30 interacting with each other. Thus, a binding pair can be an antigen-antibody pair, for example a digoxigenin/anti-digoxigenin antibody pair. Further binding pairs can be biotin/streptavidin, hormone/hormone receptor, IgG/protein A.

Alternatively, the binding pair can be a polynucleotide pair such as DNA-DNA, DNA-RNA, for example the binding pair could be two sequences complementary one to
35 another such as a polyA and a polyT sequence, or can be a Locked Nucleic Acid (LNA) anchor.

The binding pairs according to the invention are chosen amongst the above-mentioned pairs so that they are all different from each other. Thus, the members of the binding pairs according to the invention cannot interact.

A first or second member of a binding pair may be any one of the two different members of the pair, in any order. Thus, for example, if the binding pair is biotin/streptavidin, when the first member of the pair is biotin, the second member of the pair is streptavidin. Conversely, in the case where the first member of the pair is streptavidin, the second member of the binding pair is biotin.

The member of the binding pair which is attached to the particle may be bound by physical adsorption on the surface of the particle or may be covalently bonded to the particle. The methods to obtain such particles are well known by the skilled person. They may be commercially available.

The target molecule bound to one member of a second binding pair may be obtained by any methods known by the person skilled in the art, for example, they can be commercially available. Alternatively, the bound target molecule is provided by binding the target molecule to one member of a second binding pair.

The members of the binding pairs can be bound to the candidate nucleic acids and to the target molecule in a covalent or non-covalent manner. The methods to be used for binding the members of the binding pairs to the candidate nucleic acids and to the target particle depend on the nature of the target molecule and of the members of the binding pairs. They are well known from the skilled person.

The term « intermediary compound » is used to qualify any compound allowing an indirect interaction between two species, e.g. between the first particle and the second member of the first binding pair and between the second particle and the second member of the second binding pair. This linker can be of nucleotide origin in the case of RNA aptamers. Preferentially, the intermediary compound is an oligonucleotide or a protein, more preferentially, it is an oligonucleotide.

In a preferred embodiment, the first particle is bound to the second member of the first binding pair via the intermediary compound to which it is linked by a member of a third binding pair.

In a further preferred embodiment, the second particle is bound to the second member of the second binding pair via the intermediary compound to which it is linked by a member of a third binding pair.

According to these two preferred embodiments:

- the first particle or the second particle is bound to a first member of a third binding pair, and

- the intermediary compound is simultaneously bound to the second member of the first binding pair or of the second binding pair, and to the second member of the third binding pair. The interaction of the two members of the third binding pair thus allows the binding of the first particle or of the second particle to the intermediary compound and, thus, indirectly, to the second member of the first or second binding pair.

Preferentially, the first binding pair is the poly A/poly T sequence. The candidate nucleic acid can be bound to a member of this first binding pair by PCR amplification with the help of an oligonucleotide comprising either a poly T sequence or a poly A sequence.

Alternatively, in a preferred embodiment, the first binding pair is the poly A/poly T sequence and the intermediary compound is an oligonucleotide bound to a poly T or a poly A sequence. Preferentially, the oligonucleotide is synthesized in such a manner as to comprise a poly T or a poly A sequence.

Preferentially, the first binding pair is a poly A/poly T sequence and the nucleic acid is bound to a poly A sequence and the intermediary compound is an oligonucleotide comprising a poly T sequence.

In a preferred way to perform the invention,

- the first binding pair is a poly A/poly T sequence,
- the second binding pair is biotin/streptavidin,
- the third binding pair is digoxigenin/anti-digoxigenin antibody, and
- the intermediary compound is an oligonucleotide.

Thus, the intermediary compound is simultaneously linked to a member of the poly A/poly T sequence pair and to a member of the digoxigenin/antidigoxigenin pair.

In another preferred embodiment, the first particle is bound to the second member of the first binding pair via a first intermediary compound to which it is linked by a member of a third binding pair, and the second particle is bound to the second member of the second binding pair via a second intermediary compound to which it is linked by a member of a fourth binding pair.

According to this preferred embodiment:

- the first particle is bound to a first member of a third binding pair, and
- the second particle is bound to a first member of a fourth binding pair, and
- the first intermediary compound is simultaneously bound to the second member of the first binding pair and to the second member of the third binding pair. The interaction of the two members of the third binding pair thus allows the binding of the first particle to the intermediary compound and, thus, indirectly, to the second member of the first binding pair, and

- the second intermediary compound is simultaneously bound to the second member of the second binding pair and to the second member of the fourth binding pair. The interaction of the two members of the fourth binding pair thus allows the binding of the second particle to the intermediary compound and, thus, indirectly, to the second member of the second binding pair.

Preferentially, the first binding pair is the poly A/poly T sequence. The candidate nucleic acid can be bound to a member of this first binding pair by PCR amplification with the help of an oligonucleotide comprising either a poly T sequence or a poly A sequence.

Preferentially, the second binding pair is the poly A/poly T sequence. The target molecule can be bound to a member of this second binding pair by PCR amplification with the help of an oligonucleotide comprising either a poly T sequence or a poly A sequence.

Alternatively, in a preferred embodiment, the first binding pair is the poly A/poly T sequence, the second binding pair is the poly A/poly T sequence and the first and second intermediary compound are oligonucleotides bound to a poly T or a poly A sequence. Preferentially, the oligonucleotide is synthesized in such a manner as to comprise a poly T or a poly A sequence.

Preferentially, the first binding pair is a poly A/poly T sequence and the nucleic acid is bound to a poly A sequence, and the second binding pair is a poly A/poly T sequence and the target molecule is bound to a poly A sequence, and the first and second intermediary compound are oligonucleotides comprising a poly T sequence.

In a preferred way to perform the invention,

- the first binding pair is a poly A/poly T sequence,
- the second binding pair is a poly A/poly T sequence,
- the third binding pair is biotin/streptavidin,
- the fourth binding pair is digoxigenin/anti-digoxigenin antibody,
- the first and second intermediary compounds are oligonucleotides.

Thus, the first intermediary compound is simultaneously linked to a member of a poly A/poly T sequence pair and to a member of the biotin/streptavidin pair and the second intermediary compound is simultaneously linked to a member of a poly A/poly T sequence pair and to a member of the digoxigenin/anti-digoxigenin pair.

Contacting the nucleic acid candidate, the molecular target, the first particle and the second particle

According to the invention the bound candidate nucleic acid is contacted with a first particle bound to the second member of the first binding pair, optionally via an

intermediary compound and the bound target is contacted with a second particle bound to the second member of the second binding pair, optionally via an intermediary compound.

The candidate nucleic acids, the target molecules and the first and second particles which are thus bound are put into contact and this can be done in any order. For example, it is possible to mix two of them and add the other two separately or together. One may equally mix three of the partners and add the fourth one subsequently. It is also possible to mix the four partners simultaneously.

Preferably, the four partners are mixed simultaneously. Alternatively, the candidate nucleic acids and the target molecules are mixed before adding the first and second beads.

Preferably, the partners are put into contact in a buffer which helps the formation of interactions between them. The composition of such a buffer can be determined by the person skilled in the art. An example of such a buffer is notably described in example 2 or 3.

Preferably, the partners are put into contact on a microwell titer plate, each well containing a candidate nucleic acid, a target molecule, a first and a second particle. The binding time can easily be experimentally determined by the skilled person, taking into account the nature of the binding pairs and of the target molecule, the kind of buffer used and the number of partners which are contacted at the same time. For example, the binding time can be of less than 15 minutes, less than 30 minutes, less than an hour or less than two hours. Accordingly, the target molecule and the candidate nucleic acids can be contacted a first time for 45 minutes and, then again, for 45 minutes in the presence of the first and second beads as notably described in example 2 or 3.

Identifying the aptamer nucleic acid candidates

The aptamer nucleic acid candidates are identified by methods such as the Luminescent Oxygen Channeling Immunoassay (LOCI) or the Fluorescent Resonance Energy Transfer (FRET), preferably they are identified by a LOCI-type method, in particular by an Alphascreen®-type method.

Alphascreen® is based on Donor (D, photosensitizer) and Acceptor (A, chemiluminescer) microbeads, coated with two molecules of interest, susceptible of binding to each other. Laser excitation of the D beads causes ambient oxygen to be converted to the singlet state by the photosensitizer. Singlet oxygen species in turn activate chemiluminescent agents on the A beads. Upon activation, the chemiluminescent agent emits light which is detected by the photo-detector in a microplate reader. A signal is produced when the A and D beads are brought into proximity (<200 nm) thus reporting

for the interaction between the partners captured on the two beads (Taouji *et al.* (2009) *Curr Genomics* **10**(2):93).

The binding of a candidate nucleic acid already linked to a donor bead or to an acceptor bead, to a target molecule already linked to the other one of the donor or acceptor beads will result in the beads being brought into proximity and, therefore in an energy transfer.

Preferably the binding of the candidate nucleic acid to the target molecule will result in bringing the donor and acceptor particle at a distance less than 200 nm from each other.

The energy transfer between the donor and acceptor molecules is due to the production of singlet state oxygen after activation of the donor particle.

This energy transfer is preferentially detected by measuring the luminescence produced by the acceptor particle after activation by the singlet oxygen. The luminescence can for example be measured with a photodetector in a microwell plate reader, such as the EnVision® multilabel plate reader from Perkin-Elmer as described in the examples.

In a still preferred embodiment, the donor beads which contain phthalocyanine are submitted to a laser excitation at 680 nm, inducing the conversion of ambient oxygen to a singlet state oxygen. The singlet state oxygen reacts with the chemiluminescent agent on an acceptor bead containing rubren. Upon energy transfer, activated rubrene emits light at 520-620 which is detected by the photodetector in a microplate reader.

If the donor and acceptor beads are bound to fluorophores, the energy transfer amounts to fluorescence being emitted upon excitation by a first donor fluorophore, towards a second acceptor fluorophore. The acceptor fluorophore is, thus, activated and emits a second fluorescence. The fluorescence of the second acceptor fluorophore can be measured for example by a spectrofluorometer.

If energy transfer is detected, it is considered that the candidate nucleic acid has bound the target molecule; the candidate nucleic acid is, thus, an aptamer of this target molecule.

Mixtures of target molecules

The method according to the invention can be performed on a mixture of target molecules.

The nucleic acids with high affinity to target molecules in the mixture are then partitioned in step a. iii) and they are identified as aptamers against a target molecule in step f.

In a preferred embodiment, the nucleic acid aptamers directed against the mixture of target molecules are submitted again to steps c. to f. in the presence of each one of the target molecules of the mixture individually, in order to identify those which are directed against either one of the target molecules present in said mixture.

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Brief description of the drawings

Figure 1 depicts the scheme of the assay setup using a digoxigenin-tagged aptamer (R06) and a biotinylated target RNA hairpin (TAR). The association of the two components is detected by using both Donor streptavidin (D) and Acceptor anti-digoxigenin (A) coated AlphaScreen® beads. The production of singlet oxygen upon laser excitation by D-phthalocyanin is monitored by the fluorescence emission of A-rubrene beads.

Figure 2 depicts the AlphaScreen® signal obtained (in cpsx10⁻⁵, vertically) in function of the concentration of dig-R06 added to A and D beads (in nM horizontally) for different biot-TAR concentrations (from 0 to 40 nM as indicated to the right).

Figure 3 depicts secondary structures and/or sequences of the top part of the trans-activating responsive (TAR) RNA element of HIV-1 TAR (biot-TAR), 5' end-extended TAR (rA-TAR), RNA aptamer R06 (dig-R06), domain II of the HCV Internal Ribosome Entry Site (biot-DII : SEQ ID NO: 10), primer anchor (dig-primer). The latter was synthesized with 2'-O-methyl residues except at two positions (underlined) where Locked Nucleic Acid (LNA) residues were introduced to promote hybridization and to increase complex stability. Oligod(T₃CT₂₁) anchor was used for capturing rA-TAR or the candidates from the SELEX M1 and M2. The former were captured with biot-dT and the latter with dig-dT oligonucleotides.

Figure 4 depicts the competition assay setup. The assay was carried out as described in Figure 1 in the presence of untagged R06 (fR06).

Figure 5 depicts the normalized AlphaScreen® signal (in %, vertically) obtained in function of increasing concentrations of fR06 (in nM, horizontally) added to the reaction containing 10 nM of dig-R06 and 40 nM of biot-TAR. Data are presented as percent of maximal signal (mean ± SD) and are representative of at least 3 independent experiments carried out in triplicate.

Figure 6 depicts the Rop binding assay to the TAR-R06 kissing-complex. AlphaScreen® signal was obtained with a constant amount of biotinylated TAR and digoxigenated R06 (40 nM and 10 nM, respectively) in the presence of increasing concentrations of the *E. coli*

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Rop protein. Data are representative of 3 independent experiments carried out in triplicate.

Figure 7 depicts the AlphaScreen® signal obtained (in cpsx10⁻⁵, vertically) in function of the concentration of Rop (in nM, horizontally) in the absence (circle) or in the presence of 3 mM MgCl₂ (triangles). Two recordings were performed after 1h (open signs) or 17h incubation (closed signs). The experiment was repeated three times in duplicate, the mean ±SD is shown.

Figure 8 depicts the AlphaScreen® signal obtained (in cpsx10⁻⁵, vertically) in function of the concentration of BioT-dT (in nM, horizontally). TAR-R06 aptamer complexes were monitored as in Figure 1 except that a 3' extended TAR (rA-TAR) was immobilized on the beads through a biotinylated anchor oligonucleotide (biot-dT). Increasing concentrations of biot-dT are incubated in the presence of 40nM rA-TAR and 10 nM dig-R06. The results are representative of three independent experiments carried out in triplicate (mean ±SD). Maximal AlphaScreen® signals are obtained for stoichiometric concentrations of biot-dT and rA-TAR.

Figure 9 depicts the AlphaScreen® signal obtained (in cpsx10⁻⁵, vertically) in function of the number of selex round (horizontally) for monitoring of the evolution of SELEX pools to the HCV IRES domain II (DII IRES). AlphaScreen® signals are reported for each population as the mean of three independent experiments ±SD carried out in duplicate.

Figure 10 depicts the AlphaScreen®-based analysis of individual candidates issued from SELEX M1 against a mixed target population a, b and c. Error bars (horizontal bars) represent the mean ± SD values of three distinct AlphaScreen® signal measurement for each SELEX population.

Figure 11 depicts the AlphaScreen®-based analysis of individual candidates issued from SELEX M2 against the single target x. Error bars (horizontal bars) represent the mean ± SD values of three distinct AlphaScreen® signal measurement for each SELEX population. The 75 percentile above and below the average is shown.

Figure 12 depicts the HAPIscreen flowchart. The different steps of the HAPIscreen methodology are indicated (right) in comparison to that of the standard SELEX method (left).

EXAMPLES

METHODS

Oligonucleotide synthesis and purification - DNA primers and the biotinylated DNA anchor (biot-dT: SEQ ID NO: 1) purchased from Sigma or MWG Biotech were purified by HPLC. All RNA targets and the digoxigenin 2'-O-methyl-LNA anchor (dig-

primer: SEQ ID NO: 2) were chemically synthesized on an Expedite 8908 synthesizer (Applied Biosystems, USA) and purified by electrophoresis on denaturing 20% polyacrylamide, 7M urea gels. RNA candidates were synthesized by *in vitro* transcription, using T7 RNA polymerase.

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AlphaScreen® assays - The AlphaScreen® technology was used to assess the interaction between candidate oligonucleotides derived from SELEX experiments, and biotinylated target. Binding assays were performed by using white 384-well Optiplates (Perkin- Elmer) in a total volume of 25 µl. The AlphaScreen® reagents (anti-dig-coated acceptor beads and streptavidin-coated donor beads) were obtained from Perkin-Elmer. biot-TAR (SEQ ID NO: 3) and dig-R06 (SEQ ID NO: 4) (oligonucleotide sequences are shown in **Figure 3**) were prepared in a 10 mM sodium phosphate buffer, pH 7.2 at 20 °C, containing 140 mM potassium chloride, 20 mM sodium chloride and 3 mM magnesium chloride. Prior to the experiments, the RNA samples were heated in this buffer at 95 °C for 1 min and 30 s and cooled down on ice for 10 min. The protein ROP, purified as previously described (Di Primo and Lebars *Anal Biochem* **368** (2), 148 (2007), was prepared in this buffer with or without magnesium chloride. For the analysis of SELEX populations, denaturation of the candidate aptamers and targets was performed in water prior to the reaction with the anchor (biot-dT : SEQ ID NO: 1). Denaturation and re-folding of candidate aptamers and targets were achieved by incubation in water at 65°C for 3 min or 80°C for 1 min, respectively. After denaturation, candidate aptamers and targets were quickly cooled down to 4°C for 3 min and then equilibrated at room temperature (RT) for 5 min before adding the selection buffer (20 mM Na acetate, 140 mM K acetate, 3 mM Mg acetate, 20 mM HEPES ; pH 7.4). An equal volume of each partner candidate and target (5µl) was incubated at final concentration of 0.2 µM and 0.625 µM respectively for 45 min at RT. In parallel, acceptor beads (20 µg/ml) were incubated with dig-primer (0.625 µM) for 1h incubation at room temperature in the selection buffer. Then, 10 µl of each of the interacting partners were added to the plate, after 45 min incubation at RT, 5 µl of donor beads were added to the mixture at a 20 µg/ml concentration. All manipulations involving AlphaScreen® beads were performed under subdued lighting. The plates were allowed to incubate either 1 h or overnight in the dark at room temperature. Light signal was detected by using an EnVision® multilabel plate reader from Perkin-Elmer.

In vitro selection - The RNA library used for the selection was obtained by transcription of the DNA library (5'GTGTGACCGACCGTGGTGC-N30-GCAGTGAAGGCTGGTAACC (SEQ ID NO:5) as previously described (Dausse *et al*

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(2005) *Meth Mol Biol* **288**: 391). Two different primers P20 5'GTGTGACCGACCGTGGTGC (SEQ ID NO:6) and 3'SL containing the T7 transcription promoter (underlined) 5'-TAATACGACTCACTATAGGTTACCAGCCTTCACTGC (SEQ ID: N°7) were used for PCR amplification. Selection steps were performed in the SELEX buffer (20 mM HEPES, pH 7.4 at 23°C, 20 mM sodium acetate, 140 mM potassium acetate and 3 mM magnesium acetate) at 23°C on an automated workstation (Tecan freedom evo 150). All steps (magnetic bead separation, vacuum purification, PCR amplification and transcription) were carried out in microplates. Two parallel SELEX, each against 3 target premiRs, constituting mixtures M1 and M2, were performed on the automated workstation. For each SELEX, 3 µM of the RNA library was heated at 80°C for 1 min, cooled at 4°C for 3 min, placed at room temperature for 5 min and mixed for the counter-selection with streptavidin-coated beads (50 µg of Streptavidin MagneSphere® Paramagnetic Particles from Promega or 500 µg of Dynabeads M-280). RNA candidates not retained by the beads were then mixed and incubated for 10 min with 10 pmol of 3 different 3'-end biotinylated pre-miRs that were previously immobilized on streptavidin beads. Unbound RNA was removed and the beads were washed twice with 100 µl of the SELEX buffer. The bound RNA candidates were eluted from the pre-miRs by heating for 1 min at 75°C in 50 µl of water. RNA candidates were reverse-transcribed with 200 units of M-MLV reverse transcriptase RNase H⁻ Point mutant (Promega) for 50 min at 50°C. The cDNA was amplified by PCR at 63°C with 20 units of the DNA polymerase AmpliTaq Gold™ (Perkin Elmer) and the two primers P20 and 3'SL at 2 µM, during 25 cycles. RNA candidates were obtained by *in vitro* transcription of the PCR products with the Ampliscribe T7 transcription kit from Epicentre Biotechnologies. After 2 first manual and 5 automated rounds of selection against pre-miRs, carried out on an Evo150 (Tecan) in house-assembled robot, selected candidates were cloned using the TOPO TA cloning kit (Invitrogen).

The principle of this cloning relies on topoisomerase activated vectors that can incorporate any type of compatible cDNA (see Invitrogen TOPO cloning strategies <http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/PCR-cloning/PCRC-Misc/The-Technology-Behind-TOPO-Cloning.html>).

Synthesis and capture of the candidate nucleic acids - In order to generate candidates for high throughput screening, a second automated workstation was set up (Tecan freedom Evo 200), equipped with a thermal cycler, an orbital shaker, a magnetic particle separation module and a vacuum separation module. Three hundred and eighty four clones (192 clones from either M1 or M2 populations) from round 7 were produced

blindly on this second workstation. Candidates were directly amplified from colonies with a 5' end oligod(T₍₂₁₎CT₍₃₎) (underlined) lengthened

P20 5'-TTTTTTTTTTTTTTTTTTTTCTTTGTGTGACCGACCGTGGTGC (SEQ ID: NO:8) and the 3'SL 5'TAATACGACTCACTATAGGGTTACCAGCCTTCACTGC (SEQ ID: NO:7) primers allowing the addition of an oligodA/T extension to the PCR products. RNAs produced by transcription of these PCR amplifications contained a 3' end oligorA tail that was used to capture them for the AlphaScreen® tests with a digoxigenin-conjugated oligonucleotide (dig-dT: SEQ ID NO: 1) (**Figure 3**).

Sequencing of the candidate nucleic acids - Candidates were sequenced by using the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions.

Surface Plasmon Resonance analyses – SPR experiments were performed with a BIAcore 3000 apparatus. The biotinylated pre-miRs were immobilised at 50 nM (300 to 400 RU) on a SA (BIAcore) or SAD200m sensor chip (XanTech Bioanalytics; Germany) coated with streptavidin. Aptamers were injected at 500 nM in the SELEX buffer at a 20 µl/min flow-rate and at 23 °C. After each injection of the candidates, the target-surface was regenerated with a 1 min pulse of a mixture containing 40% formamide, 30 mM EDTA and 3.6 M urea prepared in milliQ water. The sensorgrams were analysed with the BIAeval software 4.1 (Di Primo C, Lebars I, *Anal Biochem* 2007, 368:148-155).

RESULTS

EXAMPLE 1: AlphaScreen® is suitable to monitor the interaction between an aptamer and its target.

In the proof of concept phase, it was focused on a loop-loop RNA-RNA complex that was previously characterized in great details (Ducongé *et al* (2000) *J Biol Chem* **275**(28): 21287, Van Melckebeke *et al.* (2008) *Proc Natl Acad Sci USA* **105**(27): 9210, Lebars *et al.* (2008) *Nucleic Acids Res* **36**(22):7146). AlphaScreen® was used to quantify the interaction between the *trans*-activating responsive (TAR) RNA element of HIV-1 and a RNA aptamer, R06 identified from a random library of oligonucleotides (**Figures 1 to 3**). These two oligoribonucleotides adopt a stem-loop structure, display complementary sequences in the apical loop (**Figure 3**) and were demonstrated to give rise to loop-loop (also called kissing) interactions. Increasing concentrations (0-40 nM) of digoxigenin-coupled R06 (dig-R06 : SEQ ID NO: 4) were incubated in the presence of increasing

concentrations of biotin-coupled TAR (biot-TAR : SEQ ID NO: 3), and constant amounts of streptavidin-D and anti-Dig-A beads. Such titration experiments were carried out at various biot-TAR concentrations ranging from 0 to 40 nM (**Figure 2**). This revealed typical increasing AlphaScreen® signals reaching a maximum for 40 nM biot-TAR and 10 nM dig-R06. Indeed as the anti-Dig-A-bead amount remains constant, addition of dig-R06 reaches a point at which it is no longer captured; consequently the excess of free dig-R06 competes with immobilized R06 for interacting with TAR captured on D-beads. This results in a decrease in fluorescence signal.

To demonstrate the specificity of the interaction, a competition assay was carried out in which increasing concentrations of unconjugated (free) R06 (fR06) were incubated in the presence of 10 nM dig-R06 and 40 nM biot-TAR (**Figure 4**). As expected AlphaScreen® signal decrease correlated with increasing concentration of fR06, leading to an apparent EC50 value of 16.7 nM $\pm$ 1.7 (**Figure 5**), a value reflecting an affinity in the same order of magnitude as that calculated using Surface Plasmon Resonance (SPR) (Ducongé *et al* (2000) *J Biol Chem* **275**(28): 21287; Lebars *et al* (2008) *Nucleic Acid Res* **36**: 7146).

Using thermal denaturation monitored by UV spectroscopy, SPR and gel-shift assays, it was previously shown that magnesium ions stabilize the TAR-R06 complex (Ducongé *et al* (2000) *J Biol Chem* **275**(28): 21287). In addition, we also used the E.coli protein ROP, which is involved in the control of the ColE1 plasmid copy number and specifically recognizes this loop-loop complex (Darfeuille *et al.* (2001) *Nucleosides Nucleotides Nucleic Acids* **20**(4-7): 441, Di primo and Lebars (2007) *Anal Biochem* **368**(2): 148). We previously demonstrated that ROP was able to bind to the TAR-R06 complex (Di Primo *et al* (2007) *Anal Biochem* **368**: 148; Watrin *et al* (2009) *Biochemistry* **48**: 6278). In the above described AlphaScreen® assay, increasing concentrations of ROP were added to biot-TAR-dig-R06 complexes in the absence or in the presence of 3 mM MgCl₂. The addition of 1mM ROP resulted in increased AlphaScreen® signal indicating the formation of a highly stable ROP-R06-TAR kissing complex (**Figure 6**). A ~10 fold increased signal was observed in the presence of 3 mM MgCl₂ (**Figure 7**). Noteworthy, an increased AlphaScreen® signal was also observed following 17h incubation for ROP concentrations higher than 200nM either in the absence or in the presence of magnesium (**Figure 7**). These experiments demonstrate that this methodology provides signals correlated to the affinity of the complex. Indeed, no signal was detected for R06 variants that do not complex with TAR, whereas conditions known to increase the interaction (addition of magnesium or ROP protein) resulted in increased fluorescence signals. These results demonstrate that AlphaScreen® is suitable to monitor the interaction between an aptamer and its target and translates directly the affinity of the complex into a fluorescence signal.

EXAMPLE 2: AlphaScreen®-based approaches could be undertaken for screening large collections of selected candidates using a unique anchor oligonucleotide.

The objective was then to adapt this approach to the screening of large pools of SELEX-derived sequences. To this end, it was necessary to capture every selected candidate on the A beads. This was achieved through the use of a biotinylated anchor complementary to a pre-determined sequence on the candidates. Indeed every candidate contains fixed sequences flanking the random region, used in the SELEX process for the amplification of the selected candidates. An oligomer complementary to one of the flank efficiently allows for the capture of every candidate, at least those for which this region is not involved in a strong intramolecular interaction. In order to validate this approach a biotinylated oligod(T₃CT₂₁) (biot-dT : SEQ ID NO: 1) (**Figure 3**) was assayed in the presence of 10 nM dig-R06 and 40 nM TAR bearing an oligor(A₂₁GA₃) tail (rA-TAR : SEQ ID NO: 9) (**Figure 3**). Increasing concentrations of biot-dT led to a maximal AlphaScreen® signal for rA-TAR concentrations ranging from 40 to 80 nM, in accordance with a stoichiometric association between TAR and the biot-dT anchor (**Figure 8**) and the respective Donor and Acceptor bead capture capacities under the current assay conditions. Therefore the anchor-based methodology allows for monitoring aptamer-target interactions. Such a strategy was then used for monitoring the evolution of populations derived from 7 rounds of RNA SELEX against the domain II of the HCV mRNA internal ribosome entry site (Da Rocha *et al.* (2004) *Biochem Biophys Res Commun* **322**: 820-826). This domain that folds as a hairpin with a 7 nt loop, was used as the target. To monitor the evolution of the RNA pool binding properties, candidates from the library (T0) and from rounds one to seven (T1-T7) were trapped on the acceptor beads using a digoxigenin-conjugated oligonucleotide, dig-primer (SEQ ID NO: 2 and **Figure 3**) complementary to their common 5' end. AlphaScreen® signals obtained with D-beads carrying a 19 nucleotides long hairpin corresponding to the top part of DII were detected from round 4 and further increased at subsequent rounds, thus indicating the progressive enrichment of the population in strong binders (**Figure 9**) in agreement with band shift assays (Da Rocha Gomes *et al* (2004) *Biochem Biophys Res Commun* **322**: 820) and SPR experiments carried out with bimolecular complexes formed between the immobilized HCV target and the SELEX pools. These results demonstrate that AlphaScreen®-based approaches (HAPIscreen) could be undertaken for screening large collections of selected candidates using a unique anchor oligonucleotide.

EXAMPLE 3: HAPIScreen allows for primary screening of aptamer candidates

HAPIScreen was then used for evaluating the outcomes of a SELEX experiment carried out using a RNA library against pre-microRNAs (pre-miRs). Pre-miRs display more or less perfect hairpin structures that are matured into functional miRs. Aptamers raised against pre-miRs are consequently anticipated to modulate miR interaction with proteins involved in the maturation process and impact on their regulatory function. SELEX was performed against two mixtures M1 and M2 of three human pre-miRs each (a, b and c and x, y and z, respectively). At the end of seven SELEX rounds, the selected candidates were cloned and produced. Using the same HAPIScreen assay design as above, candidates were trapped on the acceptor beads using dig oligod(T₃CT₂₁) (dig-dT : SEQ ID NO: 1) (**Figure 3**) complementary to their identical 3' end and individually assayed against each individual biotin-tagged target. 192 clones derived from the 7th rounds-enriched populations against either M1 and M2 were screened in duplicate blindly against either the premiR x (from M2) or the mixture of the three baits a, b, c (M1) or against the premiR x alone, respectively. It should be pointed out that the second experiment aims at identifying the partner actually targeted by a given aptamer selected against the mixture; repeating this experiment with immobilized y or z would allow the complete assignment of aptamers and targets. These experiments were carried out in a 384-well plate format and led to the identification of hits in both RNA populations (**Figures 10 and 11**). Candidates from each selection were picked according to their high AlphaScreen® signal (**Figures 10 and 11**) and tested using SPR. In the latter experiments, biotinylated pre-miRs a, b, c and x were individually immobilized on their respective sensor chip flow cells on which candidates were injected. Nine out of 12 (SELEX to x from M2) and 7 out of 13 (SELEX to a, b or c from M1) displayed K_D values lower than or equal to 30 nM for unique-based or mixture-based target assays, respectively. There was a fair agreement between both SPR and the AlphaScreen® analyses even though the order of the signals was not rigorously the same with the two techniques. We also tested the behaviour of 5 candidates that generated a low AlphaScreen® signal using SPR. This revealed that 4 out of the 5 candidates gave a weak or no resonance signal (not shown).

These high affinity aptamers were cloned and sequenced. As usual for *in vitro* selection we picked sequences displaying a high degree of similarity and identical motifs. Sequence differences likely account for the slight variation observed in AlphaScreen® or SPR signals. Finally, using MFold (Mfold web server for nucleic acid folding and hybridization prediction (2003) *Nucleic Acid Res* **31**(13): 3406-15), aptamers from SELEX

against M2 were predicted to adopt a consensus hairpin structure with a loop containing several nucleotides complementary to pre-miR loops. This suggested the formation of loop-loop aptamer/premiR complexes as previously described for TAR aptamers (Ducongé *et al* (2000) *J Biol Chem* **275**(28): 21287). Using a similar approach, aptamers derived from SELEX against M1 also showed sequences complementary to premiR target loop, allowing for the formation of 8 potential adjacent base pairs but were not predicted to fold as hairpins.

In conclusion, we have developed a method that overcomes the bias traditionally encountered in SELEX experiments. Usually candidates selected at the end of the process are cloned and sequenced. Sequences and/or predicted secondary structures are then compared in order to generate families and to choose few representatives that are then individually produced and characterized to identify aptamers (**Figure 12**). HAPIScreen bypasses the sequence comparison steps and directly allows for blind screening of aptamer candidates based on their exclusive binding properties rather than on sequence homologies. We demonstrate that HAPIScreen is a high throughput technology that can be used to analyze large collections of candidates. We used a 384-well plate format but HAPIScreen could as well be adapted to a 1536-well plate format. In addition, as the SELEX can be run simultaneously against a mixture of targets and the AlphaScreen® analysis can be carried out against individual targets this predicts an increased discovery rate of aptamers. Moreover HAPIScreen is fully amenable to automation (currently the SELEX and the AlphaScreen® steps are independently automated) and can be adapted for a wide range of targets due to the availability of different tags/beads. HAPIScreen also proved to be faster than traditional SELEX approaches as the process time was shortened by at least 50% from the isolated SELEX population to the identification of high affinity binders. The preparation of hundreds of candidates being achieved on an automated platform, this step is not time consuming. Finally, HAPIScreen potentially increases the chance of selecting orphan candidates (i.e. poorly amplified) by allowing the evaluation of larger aptamer collections. HAPIScreen therefore represents a major step forward in aptamer discovery and identification.

CLAIMS

1. Method for identifying an aptamer directed against a target molecule comprising the following steps:

5 a. obtaining a candidate enriched mixture of nucleic acids directed against the target molecule by:

i) contacting a mixture of candidate nucleic acids with the target molecule wherein nucleic acids having a strongest affinity to the target molecule relative to the candidate mixture may be partitioned from the remainder of the candidate nucleic acid mixture,

10 ii) partitioning the nucleic acids with strong affinity for the target molecule from the remainder of the candidate nucleic acid mixture; and

iii) amplifying the nucleic acid with strong affinity to yield a candidate enriched mixture of nucleic acids,

b. isolating each of the candidate nucleic acids from the candidate enriched mixture,

15 c. binding each candidate nucleic acid to one member of a first binding pair, which forms a bound candidate nucleic acid

d. providing the target molecule bound to one member of a second binding pair, forming a bound target

20 e. contacting the bound candidate nucleic acid with a first particle bound to the second member of the first binding pair, optionnally via an intermediary compound,

f. contacting the bound target with a second particle bound to the second member of the second binding pair, optionnally via an intermediary compound,

wherein said first and second particles are respectively a donor and an acceptor particle, or said first and second particles are respectively an acceptor and a donor particle,

25 g. identifying the aptamer nucleic acid candidates by:

i) detecting if an energy transfer occurs between the donor and acceptor particles, and

30 ii) identifying the nucleic acid candidate as an aptamer directed against the target molecule if energy transfer is detected.

2. A method according to claim 1, wherein the first particle is bound to the second member of the first binding pair via an intermediary compound to which it is linked by a third binding pair.

3. A method according to claim 1 or 2, wherein the second particle is bound to the second member of the second binding pair via an intermediary compound to which it is linked by a third binding pair.

5 4. A method according to any one of claims 1 to 3, wherein the first particle is bound to the second member of the first binding pair via a first intermediary compound to which it is linked by a third binding pair and the second particle is bound to the second member of the second binding pair via a second intermediary compound to which it is linked by a fourth binding pair.

10 5. Method according to any one of claims 1 to 4, wherein the donor particle contains or is bound to a photosensitizer, and the acceptor particle contains or is bound to a chemiluminescent.

15 6. Method according to any one of claims 1 to 4, wherein the donor particle contains or is bound to a donor fluorophore, and the acceptor particle contains or is bound to an acceptor fluorophore.

20 7. Method according to any one of claims 1 to 6, wherein the binding pair is streptavidin/biotin, digoxigenin/anti-digoxigenin antibody or polyA/poly T sequence.

8. Method according to anyone of claims 1 to 6, wherein the first binding pair consists in a polyA/polyT sequence.

25 9. Method according to claim 8, wherein the candidate nucleic acid is bound to a polyA sequence by PCR amplification with an oligonucleotide comprising a polyT sequence.

10. Method according to claims 8 or 9, wherein the intermediary compound is an oligonucleotide which comprises a poly T sequence.

30 .
11. Method according to any one of claims 1 to 3 and 5 to 10, wherein,
- the first binding pair is poly A/poly T sequence,
- the second binding pair is biotin/streptavidin,
- the third binding pair is digoxigenin/anti-digoxigenin antibody, and
35 - the intermediary compound comprises an oligonucleotide.

12. Method according to any one of claims 1, 2 and 4 to 10, wherein,

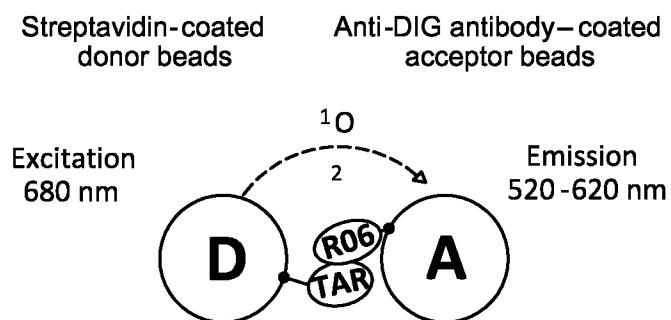
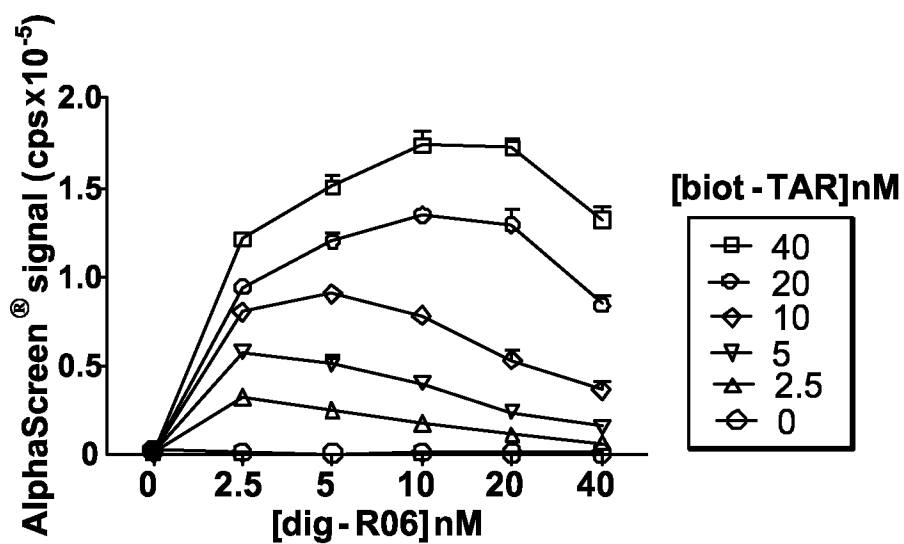
- the first binding pair is poly A/poly T sequence,
- the second binding pair is poly A/poly T sequence,
- the third binding pair is biotin/streptavidin,
- 5 - the fourth binding pair is digoxigenin/anti-digoxigenin antibody, and
- the first and second intermediary compounds comprise an oligonucleotide.

13. Method according to any one of claims 1 to 12, wherein step a. is carried out on a mixture of target molecules.

14. Method according to claim 13, wherein the nucleic acid aptamers directed against the mixture of target molecules are submitted again to steps c. to f. in the presence of each one of the target molecules of the mixture individually, in order to identify those which are directed against either one of the target molecules present in said mixture.

15. Method according to anyone of claims 1 to 14, wherein the candidate nucleic acid is not sequenced between steps a and f.

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FIG.1FIG.2

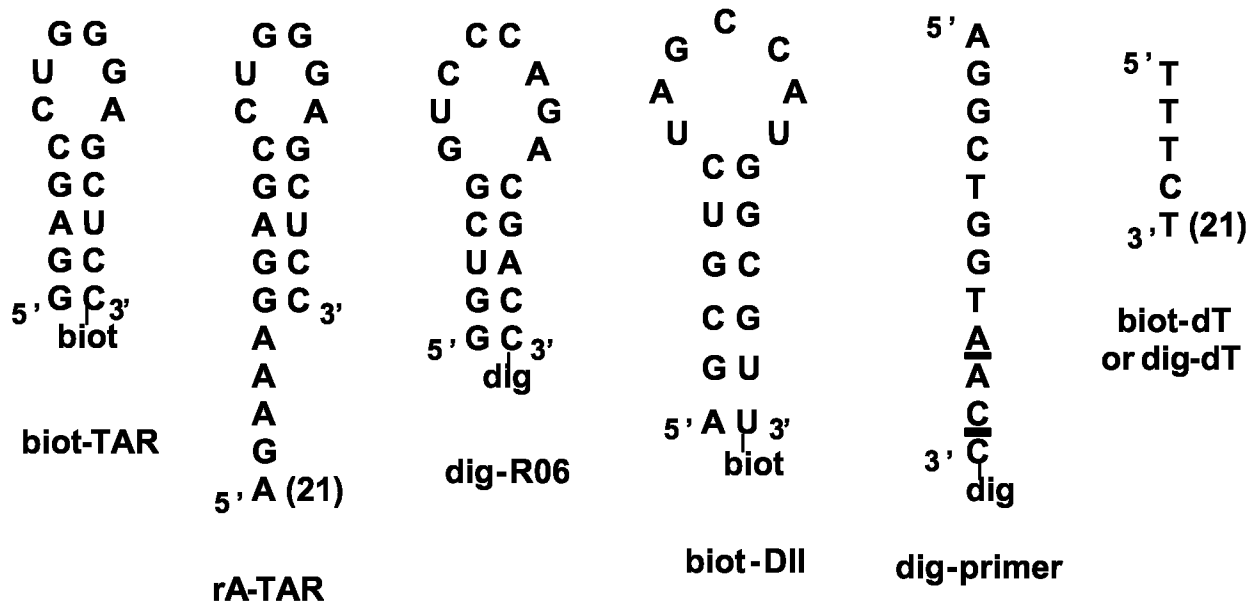
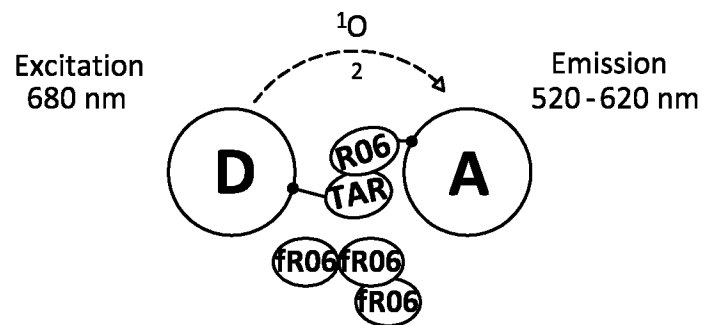
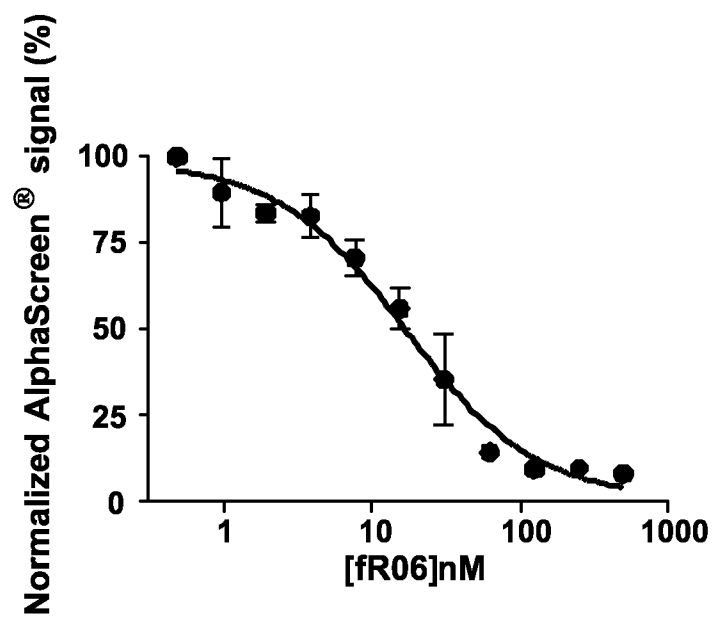
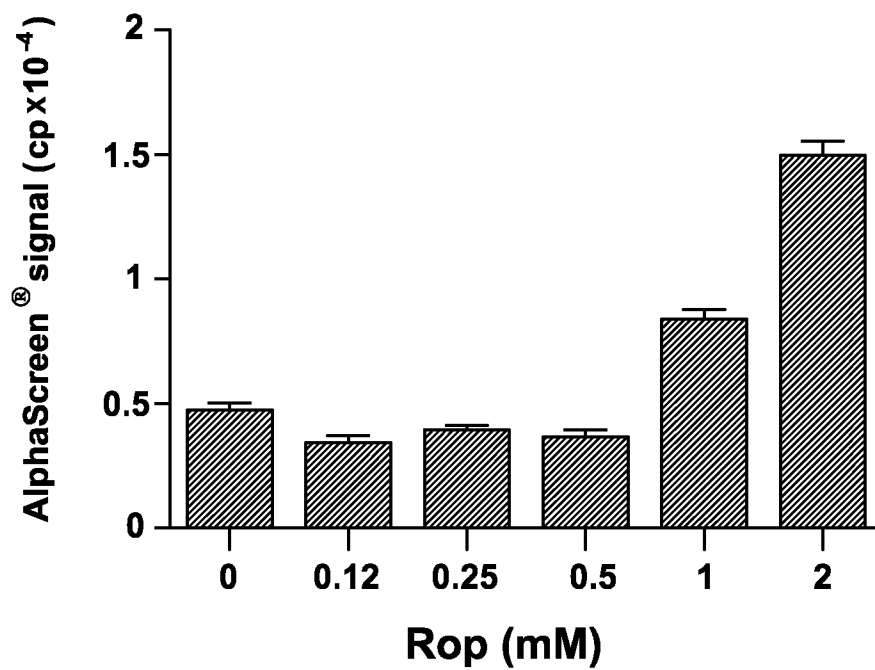
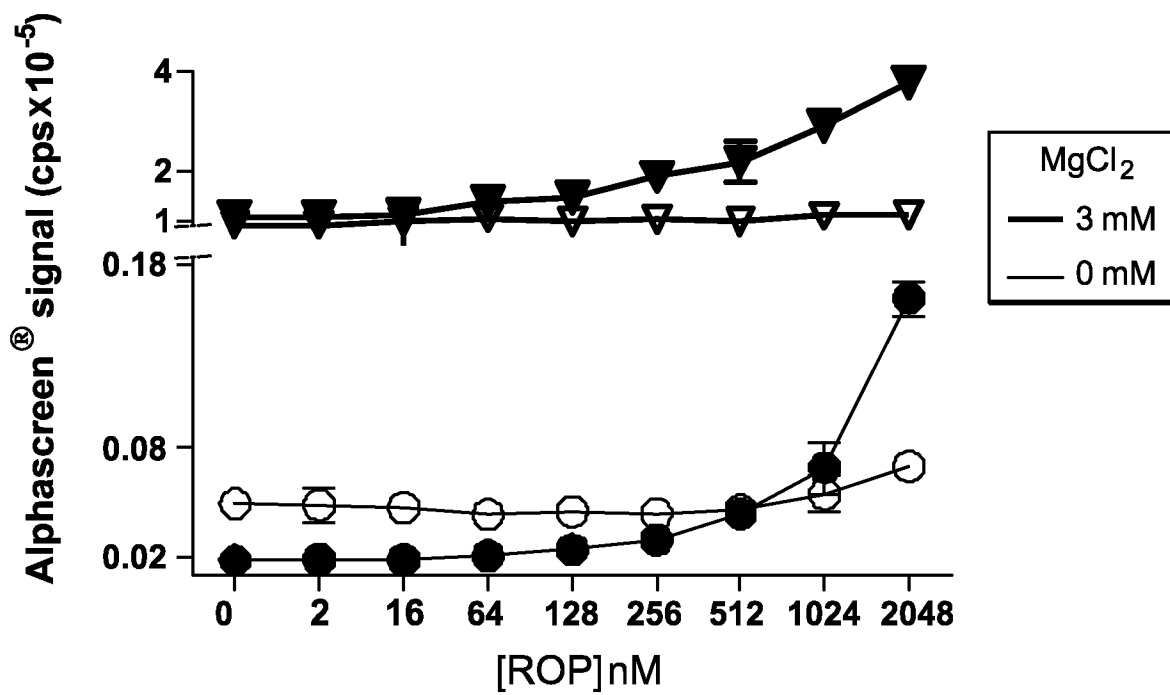


FIG.3

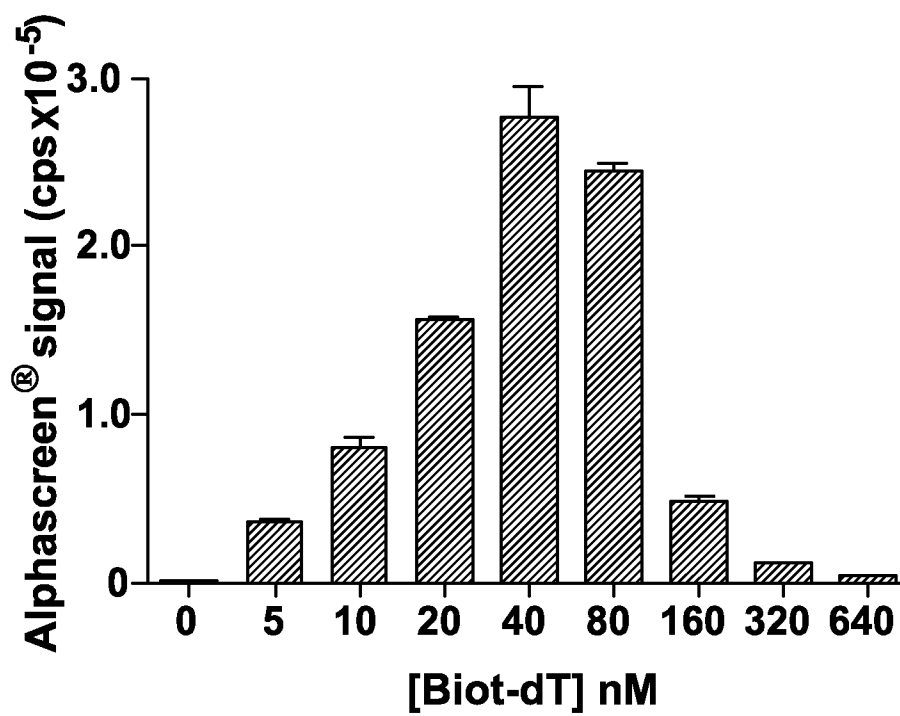
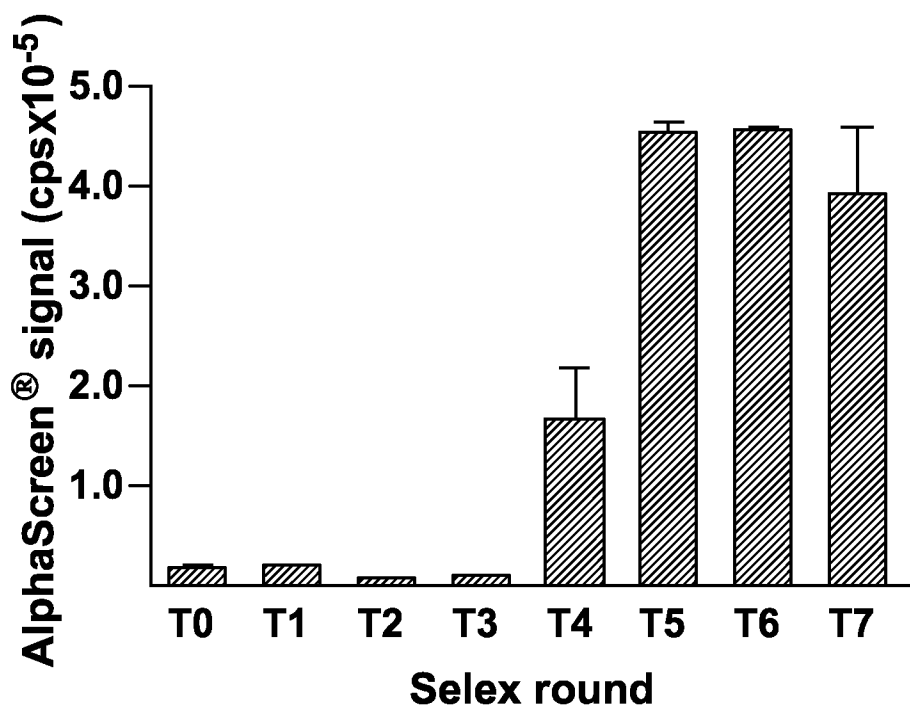
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FIG.4FIG.5

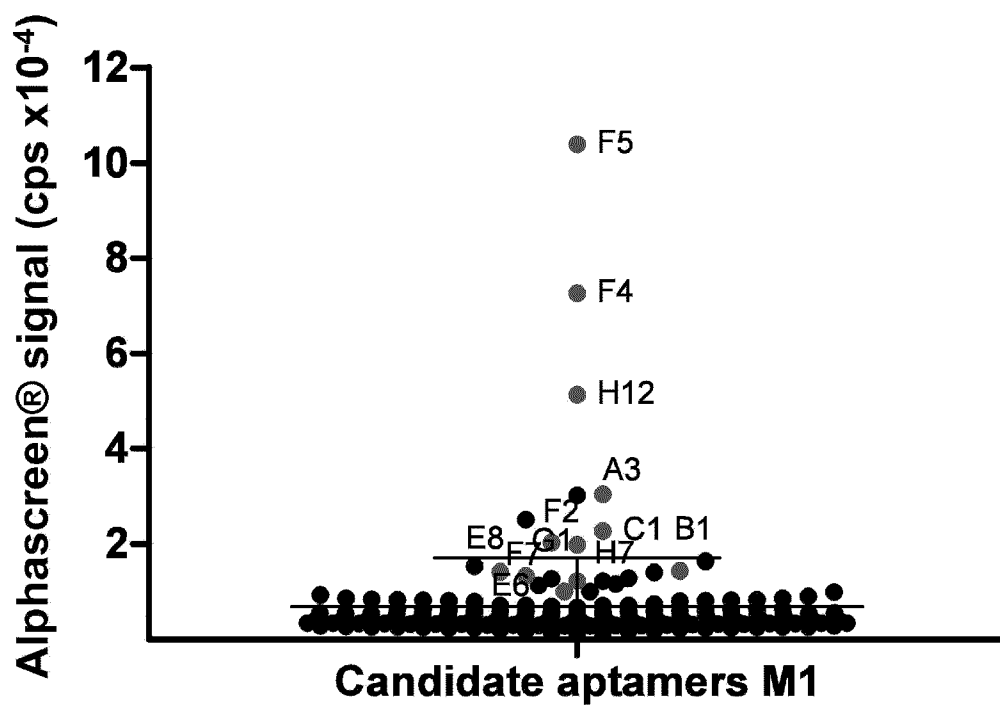
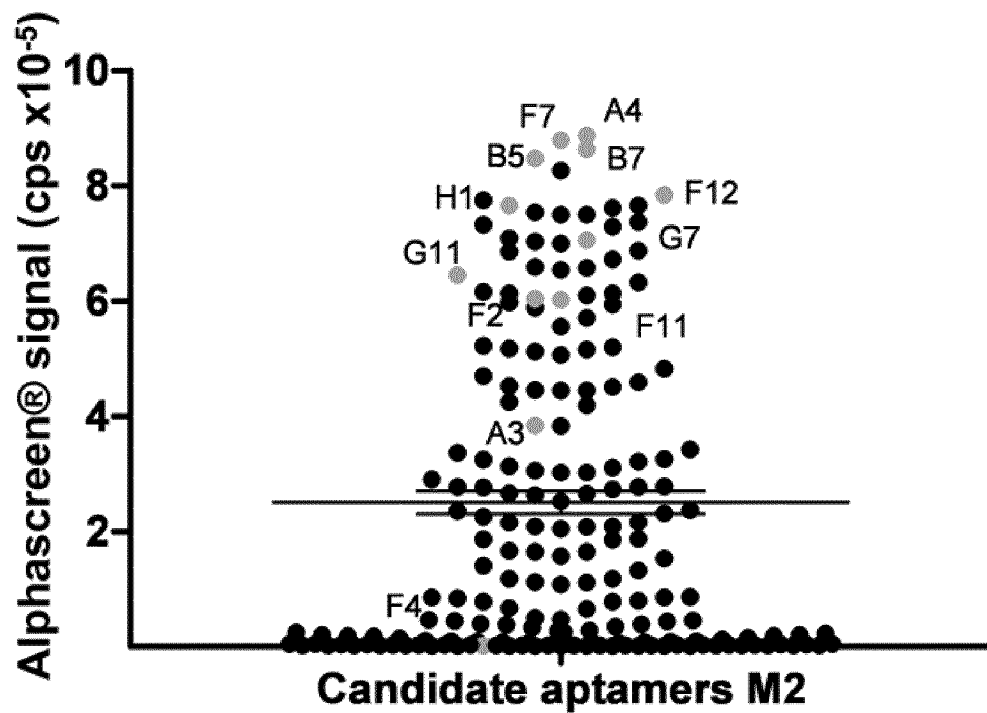
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FIG.6FIG.7

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FIG.8FIG.9

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FIG.10FIG.11

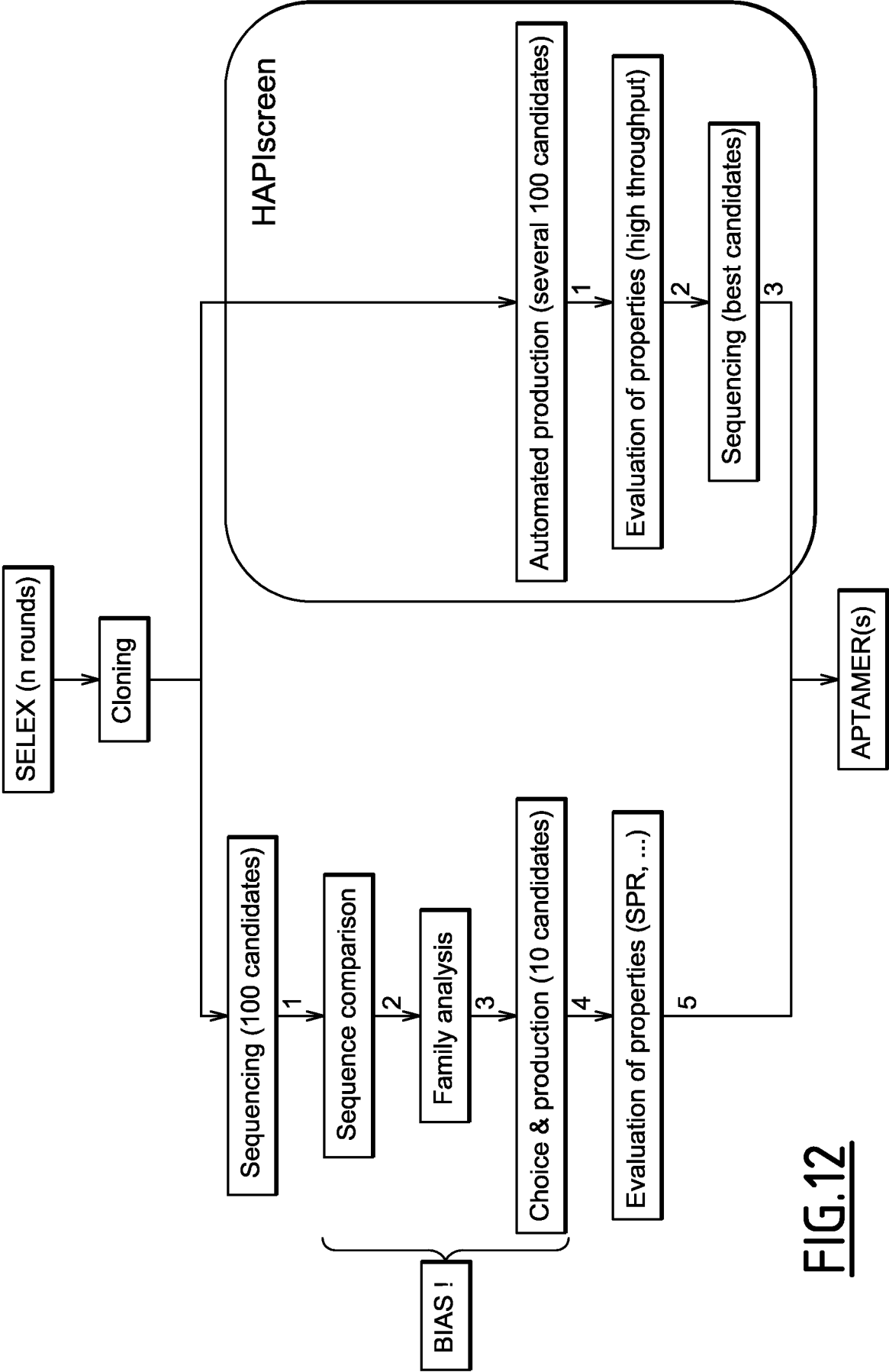


FIG.12

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2012/058283

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)

☐	on paper
☒	in electronic form
 - b. (time)

☒	in the international application as filed
☐	together with the international application in electronic form
☐	subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/058283

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/10 C12N15/115
ADD.

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)
C12Q C12N

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2008/048230 A2 (UNIV EMORY [US]; NIE SHUMING [US]; AGRAWAL AMIT [US]; CHUN YANG ZHANG) 24 April 2008 (2008-04-24) abstract; claims 1,3,13-16 -----	1-15
A	WO 2010/047797 A2 (UNIV CITY [US]; JOHNSON LAWRENCE W [US]; ZHANG CHUN-YANG [US]) 29 April 2010 (2010-04-29) abstract page 5; claims 15,16 ----- -/--	1-15



Further documents are listed in the continuation of Box C.



See patent family annex.

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"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

1 August 2012

Date of mailing of the international search report

09/08/2012

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European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Costa Roldán, Nuria

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/058283

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZHANG HAIYAN ET AL: "Development of smart nanoparticle-aptamer sensing technology.", FARADAY DISCUSSIONS 2011 LNKD-PUBMED:21413189, vol. 149, 2011, pages 319-332 ; DIS, XP009152582, ISSN: 1359-6640 abstract page 325	1-15
A	----- ALGAR W R ET AL: "Beyond labels: A review of the application of quantum dots as integrated components of assays, bioprobes, and biosensors utilizing optical transduction", ANALYTICA CHIMICA ACTA 2010 ELSEVIER NLD LNKD- DOI:10.1016/J.ACA.2010.05.026, vol. 673, no. 1, July 2010 (2010-07), pages 1-25, XP027112281, ISSN: 0003-2670 abstract page 7 - page 9	1-15
A	----- TAOUJI SAID ET AL: "Current Screens Based on the AlphaScreen (TM) Technology for Deciphering Cell Signalling Pathways", CURRENT GENOMICS, vol. 10, no. 2, April 2009 (2009-04), pages 93-101, XP002660263, ISSN: 1389-2029 the whole document	1-15
T	----- DAUSSE ERIC ET AL: "HAPIScreen, a method for high-throughput aptamer identification", JOURNAL OF NANOBIO TECHNOLOGY, vol. 9, June 2011 (2011-06), XP002660264, the whole document	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/058283

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008048230 A2	24-04-2008	US 2010304358 A1 WO 2008048230 A2	02-12-2010 24-04-2008
WO 2010047797 A2	29-04-2010	US 2011287557 A1 WO 2010047797 A2	24-11-2011 29-04-2010