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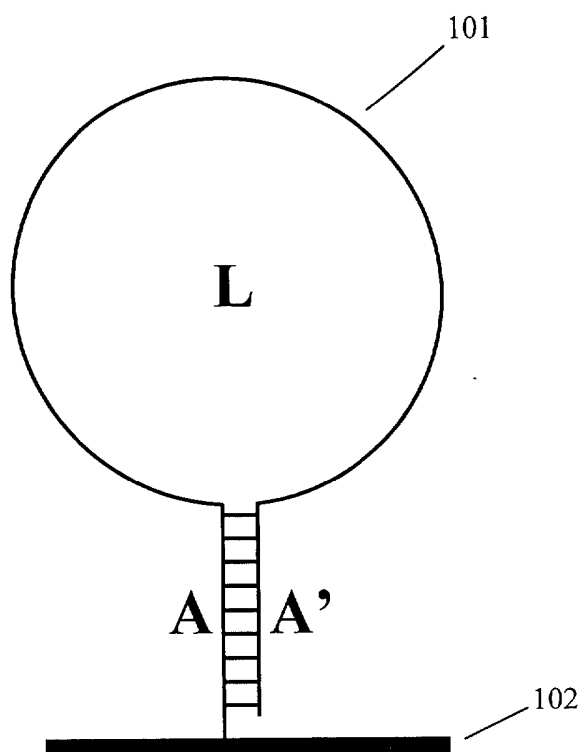
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(54) Title: NUCLEIC ACID LIGANDS WITH INTRAMOLECULAR DUPLEXES



(57) Abstract: The present invention provides methods and reagents for obtaining nucleic acid ligands by the SELEX process. In particular, the present invention provides methods and reagents for obtaining nucleic acid ligands of general composition 5' A-L-AI 31, wherein L forms the target-binding region of the nucleic acid ligand, and wherein sequence A is complementary in sequence to sequence A', such that A and A' are capable of forming an intramolecular duplex. The nucleic acid ligands of the invention are obtained by performing the SELEX process or the photoSELEX process using a candidate mixture of nucleic acids, each nucleic acid in the candidate mixture comprising the sequence 51 A-R-A' 3', wherein R is a region of at least partially-randomized sequence.

WO 03/014369 A1



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NUCLEIC ACID LIGANDS WITH INTRAMOLECULAR DUPLEXES

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FIELD OF THE INVENTION

This invention is directed to a method for the generation of nucleic acid ligands having specific functions against target molecules using the SELEX process.

10 BACKGROUND OF THE INVENTION

The dogma for many years was that nucleic acids had primarily an informational role. Through a method known as Systematic Evolution of Ligands by EXponential enrichment, termed the SELEX process, it has become clear that nucleic acids have three dimensional structural diversity not unlike proteins. The SELEX process is a method for the in vitro evolution of nucleic acid molecules with highly specific binding to target molecules and is described in United States Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by EXponential Enrichment," now abandoned, United States Patent No. 5,475,096 entitled "Nucleic Acid Ligands", United States Patent No. 5,270,163 (see also WO 91/19813) entitled "Nucleic Acid Ligands" each of which is specifically incorporated by reference herein. Each of these patents and applications, collectively referred to herein as the SELEX Patent Applications, describes a fundamentally novel method for making a nucleic acid ligand to any desired target molecule. The SELEX process provides a class of products which are referred to as nucleic acid ligands or aptamers, each having a unique sequence, and which has the property of binding specifically to a desired target compound or molecule. Each SELEX-identified nucleic acid ligand is a specific ligand of a given target compound or molecule. The SELEX process is based on the unique insight that nucleic acids have sufficient capacity for forming a variety of two- and three-dimensional structures and sufficient chemical versatility available within their monomers to act as ligands (form specific binding pairs) with virtually any chemical compound, whether monomeric or polymeric. Molecules of any size or composition can serve as targets. The SELEX method applied to the application of high affinity binding involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of nucleic acids, preferably comprising a segment of randomized sequence, the SELEX method includes steps of contacting the mixture

with the target under conditions favorable for binding, partitioning unbound nucleic acids from those nucleic acids which have bound specifically to target molecules, dissociating the nucleic acid-target complexes, amplifying the nucleic acids dissociated from the nucleic acid-target complexes to yield a ligand-enriched mixture of nucleic acids, then reiterating the steps of
5 binding, partitioning, dissociating and amplifying through as many cycles as desired to yield highly specific high affinity nucleic acid ligands to the target molecule.

It has been recognized by the present inventors that the SELEX method demonstrates that nucleic acids as chemical compounds can form a wide array of shapes, sizes and configurations, and are capable of a far broader repertoire of binding and other functions than
10 those displayed by nucleic acids in biological systems.

The present inventors have recognized that SELEX or SELEX-like processes could be used to identify nucleic acids which can facilitate any chosen reaction in a manner similar to that in which nucleic acid ligands can be identified for any given target. In theory, within a candidate mixture of approximately 10^{13} to 10^{18} nucleic acids, the present inventors postulate
15 that at least one nucleic acid exists with the appropriate shape to facilitate each of a broad variety of physical and chemical interactions.

United States Patent Application Serial No. 08/123,935, filed September 17, 1993, and United States Patent Application Serial No. 08/443,959 filed May 18, 1995, both entitled "Photoselection of Nucleic Acid Ligands," and both now abandoned, and United States Patent
20 No. 5,763,177, United States Patent No. 6,001,577, WO 95/08003, United States Patent Application Serial No. 09/459,553, filed December 13, 1999, United States Patent Application Serial No. 09/619,213, filed July 17, 2000, and United States Patent Application Serial No. 09/723,718, filed November 28, 2000, each of which is entitled "Systematic Evolution of Nucleic Acid Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and
25 Solution SELEX," and United States Provisional Patent Application Serial No. 60/278,354, filed March 22, 2001, entitled "The photoSELEX process: photocrosslinking to target in solution," all describe a SELEX process-based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or photocrosslinking to and/or photoinactivating a target molecule. The resulting nucleic acid ligands are often referred to as
30 "photoaptamers." These patents and patent applications are referred to in this application collectively as "the photoSELEX process applications." In the photoSELEX process variation of the SELEX process, a modified nucleotide activated by absorption of light is incorporated in place of a native base in either RNA- or in ssDNA-randomized oligonucleotide libraries.

United States Patent No. 5,580,737 entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, termed Counter-SELEX. United States Patent No. 5,567,588 entitled "Systematic Evolution of
5 Ligands by EXponential Enrichment: Solution SELEX," describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. United States Patent No. 5,496,938 entitled "Nucleic Acid Ligands to HIV-RT and HIV-1 Rev," describes methods for obtaining improved nucleic acid ligands after SELEX has been performed. United States Patent No. 5,705,337 entitled
10 "Systematic Evolution of Ligands by Exponential Enrichment: Chemi-SELEX," describes methods for covalently linking a ligand to its target.

The SELEX method encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved in vivo stability or improved delivery characteristics. Examples of such
15 modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX process-identified nucleic acid ligands containing modified nucleotides are described in United States Patent No. 5,660,985 entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides," that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. United States Patent
20 No. 5,580,737, supra, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH₂), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of 2' Modified Pyrimidine Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

25 The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent No. 5,637,459 entitled "Systematic Evolution of Ligands by EXponential Enrichment: Chimeric SELEX," and United States Patent No. 5,683,867 entitled "Systematic Evolution of Ligands by EXponential Enrichment: Blended SELEX," respectively. These
30 applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

The SELEX method further encompasses combining selected nucleic acid ligands with lipophilic compounds or non-immunogenic, high molecular weight compounds in a diagnostic or therapeutic complex as described in United States Patent No. 6,011,020 entitled "Nucleic Acid Complexes".

5 The SELEX process has been adapted in order to allow the high-throughput, automated generation of high affinity nucleic acid ligands to targets of interest. Methods and apparatus for automated generation of nucleic acid ligands are described in United States Patent Application Serial No. 09/232,946, filed January 19, 1999, United States Patent Application Serial No. 09/356,233 filed July 16, 1999, United States Patent Application Serial No.
10 09/616,284, filed July 14, 2000, and United States Patent Application Serial No. 09/815,171, filed March 22, 2001, each of which is entitled "Methods and Apparatus for the Automated Generation of Nucleic Acid Ligands." These patent applications are collectively referred to as "the automated SELEX process applications."

One potential problem encountered in the diagnostic use of nucleic acids is that
15 oligonucleotides in their phosphodiester form may be quickly degraded in body fluids by intracellular and extracellular enzymes such as endonucleases and exonucleases before the desired effect is manifest. Certain chemical modifications of the nucleic acid ligand can be made to increase the in vivo stability of the nucleic acid ligand or to enhance or to mediate the delivery of the nucleic acid ligand. See, e.g., U.S. Patent Application Serial No. 08/117,991,
20 filed September 9, 1993, now abandoned, and United States Patent No. 5,660,985, both entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides", and U.S. Patent Application Serial No. 09/362,578 filed July 28, 1999, entitled "Transcription-free SELEX", each of which is specifically incorporated herein by reference. Modifications of the nucleic acid ligands contemplated in these applications include, but are not limited to, those which
25 provide other chemical groups that incorporate additional charge, polarizability, hydrophobicity, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine,
30 substitution of 5-bromo or 5-iodo-uracil; backbone modifications, phosphorothioate or alkyl phosphate modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping. In preferred embodiments of the instant invention, the nucleic

acid ligands are DNA molecules that are modified with a photoreactive group on the 5-position of pyrimidine residues. The modifications can be pre- or post-SELEX process modifications.

Each of the above described patent applications, many of which describe modifications of the basic SELEX procedure, are specifically incorporated by reference herein in their entirety.

Nucleic acid ligands may be attached to planar solid supports to form microarrays. Such microarrays (also commonly referred to as "biochips"), and methods for their manufacture and use, are described in United States Patent Application Serial No. 08/990,436, filed December 15, 1997, United States Patent Application Serial No. 08/211,680, filed December 14, 1998, now abandoned, Patent Cooperation Treaty Application Serial No. PCT/US98/26515, filed December 14, 1998, and United States Patent Application Serial No. 09/581,465, filed June 12, 2000, each of which is entitled "Nucleic Acid Ligand Diagnostic Biochip." These patent applications are collectively referred to as "the biochip applications." In preferred embodiments, nucleic acid ligands of targets implicated in disease are attached to a planar solid support in an array format, and the solid support is then contacted with a bodily fluid suspected of containing those targets. Quantitation of the level of target binding to each nucleic acid ligand is then used to provide diagnostic or prognostic medical information. In particularly preferred embodiments, each nucleic acid ligand in the array is generated using the photoSELEX process as detailed in the photoSELEX process applications. After such nucleic acid ligands have been contacted with the fluid to be assayed, they are photoactivated and the solid support is washed under very stringent conditions (preferably under conditions that denature nucleic acids) in order to remove non-specifically bound molecules; bound target is not removed because it is covalently crosslinked to nucleic acid ligand via the photoreactive group. For protein targets, target quantitation can then be achieved by using a reagent that labels all proteins with a detectable group, such as a fluorescent group.

Nucleic acid ligands can be attached to solid supports by a number of methods well known in the art, and detailed in the biochip applications. Preferably, the 5' or 3' terminus of the nucleic acid ligand is covalently-coupled to a derivatized solid support.

One potential problem of using nucleic acid ligands in such diagnostic applications is that occasionally a nucleic acid ligand might non-specifically bind molecules other than its cognate target contained in bodily fluids. It is believed that single-stranded nucleic acid has the potential to be bound by certain proteins, such as heparin-binding proteins (including Platelet Derived Growth Factor (PDGF)), that have a general affinity for polyelectrolytes; hence,

single-stranded regions of nucleic acid ligands might be expected to bind the same proteins non-specifically. Some single-stranded regions in nucleic acid ligands will be required for target binding, but other regions will be dispensable. In particular, the 5' or 3' terminal portion that is used to attach the nucleic acid ligand to a solid support is typically single-stranded, but dispensable for target binding. In very rare cases involving photocrosslinkable nucleic acid ligands, a protein that non-specifically binds single-stranded nucleic acid may be sufficiently close to a photoreactive group to become photocrosslinked to the nucleic acid ligand. If this occurs, then even stringent washing of the solid support will not remove the non-specifically bound protein, and quantitation of target binding to the nucleic acid ligand will be inaccurate. Although these problems are expected to occur very infrequently, it would be desirable nonetheless to have a method for obtaining nucleic acid ligands that do not have single stranded regions at their 5' or 3' ends.

SUMMARY OF THE INVENTION

The present invention provides nucleic acid ligands of general composition 5' A-L-A' 3', wherein L comprises the target-binding or target-reacting portion of the nucleic acid ligand, and wherein A and A' are flanking mutually-complementary sequences that can base-pair with one another to form an intramolecular duplex. In turn, the 5' and/or the 3' terminus of the nucleic acid ligand may be bound to a solid support to form a spatially-localized nucleic acid ligand. A plurality of such bound nucleic acids on a solid support constitutes a nucleic acid ligand array or biochip. The resulting nucleic acid ligands, attached to the solid support via the intramolecular duplex, minimize the presence of single-stranded nucleic acid that is not essential for target binding. Furthermore, the length of the intramolecular duplex can be chosen such that the distance between the target-binding portion of the nucleic acid ligand and the solid support is optimal for target binding.

The invention also includes methods and reagents for generating nucleic acid ligands of composition 5' A-L-A' 3' by the SELEX process. Specifically, the invention includes a method for the identification of a nucleic acid ligand from a candidate mixture of nucleic acids, each said nucleic acid in said candidate mixture comprising the sequence 5' A-R-A' 3', wherein R is at least partially-randomized sequence, and wherein A and A' are fixed sequence regions complementary in sequence to one another that are capable of forming an intramolecular duplex, the method comprising the steps:

a) contacting the candidate mixture with the target wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;

5 b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and

c) amplifying the increased affinity nucleic acids;
wherein a nucleic acid ligand is identified in which A and A' are capable of forming an intramolecular duplex.

The invention also provides photocrosslinking nucleic acid ligands of the general
10 composition 5' A-L-A' 3', wherein the target-binding region L comprises one or more photoreactive groups, and wherein A and A' are flanking mutually-complementary sequences that can base-pair with one another to form an intramolecular duplex. In turn, the 5' and/or the 3' terminus of the nucleic acid ligand may be bound to a solid support to form a spatially-localized photocrosslinking nucleic acid ligand. A plurality of such bound photocrosslinking
15 nucleic acids on a solid support constitutes a nucleic acid ligand array or biochip. The photocrosslinking nucleic acid ligands of the instant invention are generated by the process of identifying a nucleic acid ligand of a target from a candidate mixture of nucleic acids comprising the sequence 5' A-R-A' 3', wherein R is at least partially-randomized sequence comprising one or more photoreactive groups, said process comprising:

20 a) contacting said candidate mixture with said target molecule, wherein nucleic acid sequences having increased affinity to the target molecule relative to the candidate mixture form nucleic acid-target molecule complexes;

b) irradiating said candidate mixture, wherein said nucleic acid-target molecule complexes photocrosslink;

25 c) partitioning the crosslinked nucleic acid-target molecule complexes from free nucleic acids in the candidate mixture; and

d) identifying the nucleic acid sequences that photocrosslinked to the target molecule.

30 BRIEF DESCRIPTION OF THE DRAWING

Figure 1 illustrates schematically the attachment to a solid surface 102 of a nucleic acid 101 of composition 5' A-L-A' 3', wherein L comprises the target-binding portion of the nucleic acid ligand, wherein A and A' are flanking mutually-complementary sequences that can base-

pair with one another to form an intramolecular duplex, and wherein A is attached to a solid support.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 The central method utilized herein for identifying nucleic acid ligands with an intramolecular duplex formed between sequences at the 5' and 3' ends is called the SELEX process, an acronym for Systematic Evolution of Ligands by Exponential enrichment.

Definitions

10 Various terms are used herein to refer to aspects of the present invention. To aid in the clarification of the description of the components of this invention, the following definitions are provided:

As used herein, "nucleic acid ligand" is a non-naturally occurring nucleic acid having a desirable action on a target. Nucleic acid ligands are often referred to as "aptamers". The term
15 aptamer is used interchangeably with nucleic acid ligand throughout this application. A desirable action includes, but is not limited to, binding of the target, catalytically changing the target, reacting with the target in a way which modifies/alters the target or the functional activity of the target, covalently attaching to the target as in a suicide inhibitor, facilitating the reaction between the target and another molecule. In certain preferred embodiments, the action
20 is specific binding affinity for a target molecule, such target molecule being a three dimensional chemical structure other than a polynucleotide that binds to the nucleic acid ligand through a mechanism which predominantly depends on Watson/Crick base pairing or triple helix binding, wherein the nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule. Nucleic acid ligands include
25 nucleic acids that are identified from a candidate mixture of nucleic acids, said nucleic acid ligand being a ligand of a given target, by the method comprising: a) contacting the candidate mixture with the target, wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture; b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture;
30 and c) amplifying the increased affinity nucleic acids to yield a ligand-enriched mixture of nucleic acids.

As used herein, "candidate mixture" is a mixture of nucleic acids comprised of differing regions of sequence from which 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. In preferred embodiments of this invention, each nucleic acid in a candidate mixture comprises an at least-partially randomized sequence flanked on either side by mutually complementary fixed sequences that can form an intramolecular duplex. In a preferred embodiment, each nucleic acid has additional fixed sequences on either side of an at least-partially randomized sequence region to facilitate the amplification process. In especially preferred embodiments, fixed terminal "tail" sequences are added to the fixed sequence regions and primers used for amplification in order to prevent the formation of high molecular weight "parasites" of the amplification process, which are believed to result from rare mispriming events. Such "tails" obviate the need to size fractionate amplification products at the conclusion of each round of the SELEX process or the photoSELEX process, and are therefore especially useful in the automated SELEX process and the automated photoSELEX process. Methods for preventing "parasite" formation are described in United States Patent Application Serial No. 09/616,284, filed July 14, 2000 and United States Patent Application Serial No. 09/815,171, filed March 22, 2001, both entitled "Method and Apparatus for the Automated Generation of Nucleic Acid Ligands," and both incorporated herein by reference in their entirety.

As used herein, "nucleic acid" means either DNA, RNA, single-stranded or double-stranded, and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or to the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil; backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. An example of a backbone modified nucleic acid included in the instant invention is peptide nucleic acid (PNA). Modifications can also include 3' and 5' modifications such as capping.

"SELEX" methodology involves the combination of selection of nucleic acid ligands which interact with a target in a desirable manner, for example binding to a protein, with amplification of those selected nucleic acids. Optional iterative cycling of the selection/amplification steps allows selection of one or a small number of nucleic acids which

interact most favorably with the target from a pool which contains a very large number of nucleic acids. Cycling of the selection/amplification procedure is continued until a selected goal is achieved. The SELEX methodology is described in the SELEX Patent Applications.

"SELEX target" or "target molecule" or "target" refers herein to any compound upon which a nucleic acid can act in a predetermined desirable manner. A SELEX target molecule can be a protein, peptide, nucleic acid, carbohydrate, lipid, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, pathogen, toxic substance, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, cell, tissue, etc., without limitation. Virtually any chemical or biological effector would be a suitable SELEX target. Molecules of any size can serve as SELEX targets. A target can also be modified in certain ways to enhance the likelihood of an interaction between the target and the nucleic acid.

"Tissue target" or "tissue" refers herein to a certain subset of the SELEX targets described above. According to this definition, tissues are macromolecules in a heterogeneous environment. As used herein, tissue refers to a single cell type, a collection of cell types, an aggregate of cells, or an aggregate of macromolecules. This differs from simpler SELEX targets which are typically isolated soluble molecules, such as proteins. In the preferred embodiment, tissues are insoluble macromolecules which are orders of magnitude larger than simpler SELEX targets. Tissues are complex targets made up of numerous macromolecules, each macromolecule having numerous potential epitopes. The different macromolecules which comprise the numerous epitopes can be proteins, lipids, carbohydrates, etc., or combinations thereof. Tissues are generally a physical array of macromolecules that can be either fluid or rigid, both in terms of structure and composition. Extracellular matrix is an example of a more rigid tissue, both structurally and compositionally, while a membrane bilayer is more fluid in structure and composition. Tissues are generally not soluble and remain in solid phase, and thus partitioning can be accomplished relatively easily. Tissue includes, but is not limited to, an aggregate of cells usually of a particular kind together with their intercellular substance that form one of the structural materials commonly used to denote the general cellular fabric of a given organ, e.g., kidney tissue, brain tissue. The four general classes of tissues are epithelial tissue, connective tissue, nerve tissue and muscle tissue.

Examples of tissues which fall within this definition include, but are not limited to, heterogeneous aggregates of macromolecule such as fibrin clots which are acellular; homogeneous or heterogeneous aggregates of cells; higher ordered structures containing cells which have a specific function, such as organs, tumors, lymph nodes, arteries, etc.; and

individual cells. Tissues or cells can be in their natural environment, isolated, or in tissue culture. The tissue can be intact or modified. The modification can include numerous changes such as transformation, transfection, activation, and substructure isolation, e.g., cell membranes, cell nuclei, cell organelles, etc.

5 Sources of the tissue, cell or subcellular structures can be obtained from prokaryotes as well as eukaryotes. This includes human, animal, plant, bacterial, fungal and viral structures.

As used herein, "solid support" is defined as any surface to which molecules may be attached through either covalent or non-covalent bonds. This includes, but is not limited to, membranes, microtiter plates, magnetic beads, charged paper, nylon, Langmuir-Bodgett films,
10 functionalized glass, germanium, silicon, PTFE, polystyrene, gallium arsenide, gold, and silver. Any other material known in the art that is capable of having functional groups such as amino, carboxyl, thiol or hydroxyl incorporated on its surface, is also contemplated. This includes surfaces with any topology, including, but not limited to, spherical surfaces and grooved surfaces.

15 As used herein, "biological fluid" refers to any biological substance, including but not limited to, blood (including whole blood, leukocytes prepared by lysis of red blood cells, peripheral blood mononuclear cells, plasma, and serum), sputum, urine, semen, cerebrospinal fluid, bronchial aspirate, sweat, feces, synovial fluid, macerated tissue, and tissue extracts. Biological fluid typically contains cells and their associated molecules, soluble factors, small
20 molecules and other substances.

As used herein, "photoaptamers" refers to nucleic acid ligands that contain a photoreactive group that can crosslink with the target. Suitable photoreactive groups include, but are not limited to, 5-bromouracil, 5-iodouracil, 5-bromovinyluracil, 5-iodovinyluracil, 5-azidouracil, 4-thiouracil, 5-bromocytosine, 5-iodocytosine, 5-bromovinylcytosine, 5-
25 iodovinylcytosine, 5-azidocytosine, 8-azidoadenine, 8-bromoadenine, 8-iodoadenine, 8-azidoguanine, 8-bromoguanine, 8-iodoguanine, 8-azidohypoxanthine, 8-bromohypoxanthine, 8-iodohypoxanthine, 8-azidoxanthine, 8-bromoxanthine, 8-iodoxanthine, 5-bromodeoxyuracil, 8-bromo-2'-deoxyadenine, 5-iodo-2'-deoxyuracil, 5-iodo-2'-deoxycytosine, 5-[(4-azidophenacyl)thio]cytosine, 5-[(4-azidophenacyl)thio]uracil, 7-deaza-7-iodoadenine, 7-deaza-
30 7-iodoguanine, 7-deaza-7-bromoadenine, and 7-deaza-7-bromoguanine. An ideal photoaptamer will react rapidly and in high yield with its target and will display little cross-reaction with non-targets.

Note that throughout this application, various references are cited. Every reference cited herein is specifically incorporated in its entirety.

A. The SELEX Process Methodology

5 In the preferred embodiment, the nucleic acid ligands of the present invention are derived from the SELEX process methodology. The SELEX process is described in United States Patent Application Serial No. 07/536,428, entitled "Systematic Evolution of Ligands by Exponential Enrichment," now abandoned, United States Patent No. 5,475,096 entitled "Nucleic Acid Ligands," and in United States Patent No. 5,270,163 (see also, WO 91/19813)
10 entitled "Nucleic Acid Ligands." These applications, each specifically incorporated herein by reference, are collectively called the SELEX Patent Applications.

 The SELEX process provides a class of products which are nucleic acid molecules, each having a unique sequence, and each of which has the property of binding specifically to a desired target compound or molecule. Target molecules are preferably proteins, but can also
15 include among others carbohydrates, peptidoglycans and a variety of small molecules. SELEX methodology can also be used to target biological structures, such as cell surfaces or viruses, through specific interaction with a molecule that is an integral part of that biological structure.

 In its most basic form, the SELEX process may be defined by the following series of steps:

20 1) A candidate mixture of nucleic acids of differing sequence is prepared. 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) and regions of randomized sequences. The fixed sequence regions are chosen either: (a) to assist in the amplification steps described below, (b) to mimic a sequence known to bind to the target, (c) to
25 enhance the concentration of a given structural arrangement of the nucleic acids in the candidate mixture, and/or (d) to function as "tails" that prevent high molecular weight parasites of the amplification process from forming in the absence of size fractionation of amplifications products, as described above. The randomized sequences can be totally randomized (i.e., the probability of finding a base at any position being one in four) or only partially randomized
30 (e.g., the probability of finding a base at any location can be selected at any level between 0 and 100 percent).

 2) The candidate mixture is contacted with the selected target under conditions favorable for binding between the target and members of the candidate mixture. Under these

circumstances, the interaction between the target and the nucleic acids of the candidate mixture can be considered as forming nucleic acid-target pairs between the target and those nucleic acids having the strongest affinity for the target.

3) The nucleic acids with the highest affinity for the target are partitioned from those nucleic acids with lesser affinity to the target. Because only an extremely small number of sequences (and possibly only one molecule of nucleic acid) corresponding to the highest affinity nucleic acids exist in the candidate mixture, it is generally desirable to set the partitioning criteria so that a significant amount of the nucleic acids in the candidate mixture (approximately 5-50%) are retained during partitioning.

4) Those nucleic acids selected during partitioning as having the relatively higher affinity for the target are then amplified to create a new candidate mixture that is enriched in nucleic acids having a relatively higher affinity for the target.

5) By repeating the partitioning and amplifying steps above, the newly formed candidate mixture contains fewer and fewer unique sequences, and the average degree of affinity of the nucleic acids to the target will generally increase. Taken to its extreme, the SELEX process will yield a candidate mixture containing one or a small number of unique nucleic acids representing those nucleic acids from the original candidate mixture having the highest affinity to the target molecule.

Many modifications of the basic SELEX process are enabled by those patent applications and patents referred to within the "Background of the Invention." Other modifications are known to one of ordinary skill in the art. Such modifications may be made post-SELEX process (modification of previously identified unmodified ligands) or by incorporation into the SELEX process.

B. Generation of Nucleic Acid Ligands with Intramolecular Duplexes Formed Between 5' and 3' Fixed Sequence Regions

In one embodiment of the invention, the candidate nucleic acid mixture comprises fixed sequence regions that are complementary to one another. By way of example only, a candidate nucleic acid mixture can be prepared in which an at least-partially random sequence region is flanked 5' by the fixed sequence:

5'-GGCTGA-3'

and 3' by the fixed sequence:

5'-TCAGCC-3'

Below the melting temperature (T_m) of these sequences, the 5' fixed sequence will base-pair with the 3' fixed sequence to form an intramolecular duplex. Each nucleic acid in the candidate mixture will therefore comprise an intramolecular duplex "stem" formed by the complementary fixed regions, and a region comprising the at least partially-randomized random sequence that connects the strands of the "stem". The exact structure of the at least partially-randomized region of each nucleic acid in the candidate mixture, and hence its propensity for target binding, will depend on the sequence of the random region. We refer to this candidate mixture as "intramolecular duplex candidate mixture." The intramolecular duplex candidate mixture has the general composition 5' A-R-A' 3', wherein R is the at least-partially randomized sequence region, and A and A' are the complementary fixed sequence regions.

The intramolecular duplex candidate mixture can be used to perform the SELEX process as described in the SELEX patent applications. This leads to the generation of nucleic acid ligands of general composition 5' A-L-A' 3' wherein L comprises the target-binding portion of the nucleic acid ligand.

In some embodiments of the invention, the intramolecular candidate mixture is amplified during the SELEX process by the Polymerase Chain Reaction (PCR) with primers complementary in sequence to A and A'. If A-A' intramolecular duplexes and intermolecular duplexes (between the A sequence of one nucleic acid and the A' sequence of a second nucleic acid) form during the PCR procedure, then primer annealing will be inefficient, resulting in a low yield of amplified product. In order to prevent the formation of intramolecular and intermolecular duplexes during primer annealing, the PCR procedure is preferably carried out with a concentration of primer sufficiently high to insure that fixed regions of each nucleic acid in the intramolecular duplex candidate mixture are more likely to anneal to primer than to one another.

In preferred embodiments of the invention, the candidate mixture comprises additional fixed sequence regions 5' of A and 3' of A'. These additional fixed sequence regions serve as primer annealing sites for the amplification of the intramolecular duplex candidate mixture. Such candidate mixture can be represented as 5' P₁-A-R-A'-P₂ 3', wherein P₁ and P₂ are the fixed sequence regions used for PCR amplification, R is the at least-partially randomized region, and A and A' form the intramolecular duplex. Preferably, the fixed sequence regions P₁ and P₂ used for amplification have higher T_m 's than the fixed sequence regions A and A' that form the intramolecular duplex. This enables the intramolecular duplex candidate mixture to be amplified by PCR at an annealing/extension temperature above the T_m of the intramolecular

duplex, thereby preventing the formation of intramolecular and intermolecular duplexes by A and A' during PCR.

When the SELEX process is performed according to the abovementioned embodiments with an intramolecular duplex candidate mixture, nucleic acids ligands will be obtained in which the target binding by the L region can take place in the context of an intramolecular duplex formed between the complementary fixed sequence regions A and A'. In other words, the L region can adopt its target-binding conformation when (and in some cases, only if) the flanking complementary fixed regions A and A' of the nucleic acid ligand form an intramolecular duplex. The nucleic acid ligand thus identified can be attached, either covalently or non-covalently, to a solid support via the A-A' intramolecular duplex. The 5' end of A and/or the 3' end of A' can be attached to the solid support. Any suitable technique known in the art for the attachment of nucleic acids to solid supports is contemplated by the instant invention.

FIGURE 1 illustrates the attachment of a nucleic acid ligand of the instant invention via its 5' terminus to a solid support. Note that in this figure, target binding region L is shown schematically as a loop. The exact conformation of region L in any given nucleic acid ligand will depend upon its sequence, and may comprise one or more intramolecular duplexes.

In embodiments in which additional fixed sequences (such as P₁ and P₂ above) are present 5' and 3' of the complementary fixed sequences A and A', the nucleic acid ligands, once identified, can be synthesized without the additional fixed sequences by standard nucleic acid synthesis techniques. For example, in embodiments in which the intramolecular candidate mixture is 5' P₁-A-R-A'-P₂ 3', the nucleic acid ligands identified can be represented as 5' P₁-A-L-A'-P₂ 3'. The identified nucleic acid ligands can then be synthesized lacking the sequences P₁ and P₂. This allows the nucleic acid ligand to be attached to a solid support via the intramolecular duplex between A and A'.

Relative to single-stranded nucleic acid, duplex nucleic acid has a defined, rigid structure. The intramolecular duplex formed by A and A', when anchored to a solid support, can thus be thought of as a "scaffold" upon which the target-binding L region the nucleic acid ligand is displayed. Given the comparative rigidity and uniformity of the intramolecular duplex, target binding will be more efficient and reproducible than if attachment occurs through single-stranded nucleic acid. Moreover, by minimizing the amount of dispensable single-stranded nucleic acid, the nucleic acid ligands of the instant invention are less likely to

bind proteins non-specifically than are nucleic acid ligands that have single-stranded 5' and 3' regions.

In preferred embodiments, the length of the A-A' intramolecular duplex, and hence the distance between the target-binding L region of the nucleic acid ligand and the solid support, is selected to allow for optimal target binding. Once a nucleic acid ligand has been identified by the methods provided herein, it can then be synthesized by standard nucleic acid synthesis techniques known in the art. The A-A' duplex can be either truncated or extended relative to the original candidate mixture by deleting or adding complementary nucleotides respectively to A and A' during synthesis. Because the A-A' duplex merely provides a scaffold upon which the target-binding L region of the nucleic acid ligand is displayed, the exact sequence of the duplex is unimportant.

In another aspect of the invention, the methods and reagents of the instant invention are used to perform the photoSELEX process. The photoSELEX process is described in great detail in the photoSELEX process applications mentioned above. Once identified by the photoSELEX process, nucleic acid ligands can then be synthesized such that photoreactive nucleotides are incorporated only into the L region. If the A-A' intramolecular duplex of the resulting nucleic acid ligand non-specifically binds some protein (which in itself will be a much rarer event than for single-stranded nucleic acid), then that protein will not become photocrosslinked to the duplex, and so can be removed by post-photoactivation washing.

As described above, nucleic acid ligands sometimes are degraded in bodily fluids by exonucleases and endonucleases. Nucleic acid ligands identified according to the methods of the instant invention can be synthesized with peptide linkages (instead of phosphodiester linkages) between the nucleotides or ribonucleotides that form each strand of the intramolecular duplex. Nucleic acid with such peptide linkages is referred to as peptide nucleic acid (PNA) and is well known in the art. Preferably, the three nucleotides or ribonucleotides at the 3' terminus and the three nucleotides or ribonucleotides at the 5' terminus are synthesized with peptide linkages. The presence of PNA at the terminus of the intramolecular duplex prevents the digestion of the nucleic acid ligand at this location by exonucleases and endonucleases as these enzymes are unable to cleave peptide linkages.

C. Use of Intramolecular Duplex Candidate Mixtures in the Automated SELEX Process

As described in the abovementioned automated SELEX applications, the SELEX process or the photoSELEX process can be automated to generate nucleic acid ligands with

little or no operator intervention. In its most basic embodiment, the automated SELEX process method involves:

- a) contacting a candidate mixture of nucleic acid ligands in a containment vessel with a target molecule that is associated with a solid support;
- 5 b) incubating the candidate mixture and the solid support in the containment vessel at a predetermined temperature to allow candidate nucleic acid ligands to interact with the target;
- c) partitioning the solid support with bound target and associated nucleic acid ligands away from the candidate mixture;
- 10 d) optionally washing the solid support under predetermined conditions to remove nucleic acid that are associated non-specifically with the solid support or the containment vessel;
- e) releasing from the solid support the nucleic acid ligands that interact specifically with the target;
- 15 f) amplifying, purifying and quantifying the released nucleic acid ligands;
- g) repeating steps (a)-(f) a predetermined number of times; and
- h) isolating the resulting nucleic acid ligands.

wherein steps (a)-(g) are performed automatically by the computer-controlled robotic manipulator.

20 In its most basic embodiment, the automated photoSELEX process involves the steps:

- a) contacting a candidate mixture of nucleic acids with a target, each nucleic acid comprising one or more photoreactive groups, wherein nucleic acids having an increased affinity to the target relative to the candidate mixture form nucleic acid-target complexes with the target;
- 25 b) irradiating said complexes, wherein said nucleic acid-target complexes photocrosslink;
- c) partitioning the photocrosslinked nucleic acid-target complexes from the remainder of said candidate mixture; and
- d) identifying a nucleic acid ligand that photocrosslinked to the target; wherein
- 30 steps a)-c) are performed automatically by the computer-controlled robotic manipulator.

It is specifically contemplated that the intramolecular duplex candidate mixtures of the instant invention be used to perform the automated SELEX process and the automated photoSELEX process. When combined with the automated SELEX process and the automated

photoSELEX process, the methods of the instant invention will greatly facilitate the efficient generation of large number of nucleic acid ligands that can be attached to solid supports in a reproducible and uniform manner.

After multiple rounds of the automated SELEX process, the dominant nucleic acid product occasionally comprises high molecular weight nucleic acids without ligand activity. While not wishing to be bound by any particular theory, it is believed that these nucleic acid species--which we term "parasites"--result from rare mispriming events that occur during PCR. These mispriming events are believed to occur when rare candidate nucleic acid ligands contain a sequence in their random regions that is complementary in sequence to the 3' fixed sequence used for PCR amplification. If the 3' fixed sequence folds back over this complementary sequence in the random region, a self-priming intramolecular duplex may form. This structure can extended by Taq polymerase to form a longer product during PCR amplification. Alternatively, the 3' fixed sequence of another candidate nucleic acid ligand can form an intermolecular duplex with the complementary sequence in the random region, and the 3' end of the former candidate nucleic acid can be extended by Taq polymerase to form a longer product. A series of either of the events will produce parasites with a variable number of repeats. Once these parasites have formed, they will anneal promiscuously with other nucleic acids, including the correct products, leading to the formation of ever-larger parasites through 3' end extension. As parasites grow, they contain more and more primer binding sites, allowing them to be efficiently amplified during the PCR process at the expense of bona fide nucleic acid ligands. In the most extreme cases, nucleic acid ligand products are sometimes eliminated from the candidate mixture of nucleic acid ligands that contains a parasite.

Parasites most commonly form and grow during the later cycles of PCR where the concentration of free primer no longer exceeds the concentration of the product. Once a parasite has formed in an automated SELEX experiment, it contaminates the entire laboratory environment. Whenever automated SELEX experiments are performed using the same primer set, the parasite is efficiently amplified, grows by promiscuous annealing as described above, and quickly overwhelms the SELEX process.

It is possible to delay or prevent parasites from dominating the automated SELEX process by size-fractionating PCR products using acrylamide gel electrophoresis before beginning the next round of the SELEX process. However, it is extremely cumbersome to automate this gel electrophoresis step because of the difficulties well known in the art in

automating gel loading and band excision. Moreover, because gel electrophoresis is time-consuming, it would be the rate limiting step in any SELEX process that employs it.

In United States Patent Application Serial No. 09/616,284, filed July 14, 2000, and United States Patent Application Serial No. 09/815,171, filed March 22, 2001, each entitled
5 "Method and Apparatus for the Automated Generation of Nucleic Acid Ligands," methods and reagents are described that prevent parasites from arising during the automated SELEX process without requiring the use of size-fractionation procedures. Specifically, the likelihood that parasites will form is reduced by adding sequences to the 5' termini of the PCR primers, wherein the added sequence have melting temperature (T_m) values lower than the PCR
10 annealing temperature. At the annealing temperature, these added sequences are unstable, whereas the primers anneal to the fixed sequence regions of the candidate nucleic acids. We refer to these unstable sequences that are added to the 5' end of primers as "tails." For example, PCR can be performed with one primer linked to a tail sequence ATATATAT, and the other linked to the tail sequence TTTTTTTT. The correct PCR product will have
15 ATATATAT on the 3' terminus of one strand and AAAAAAAA on the 3' terminus of the other strand. At a typical PCR annealing temperature of 60°C, the tail sequences AAAAAAAA and ATATATAT will not anneal intra- or intermolecularly to the random regions of candidate nucleic acid ligands that fortuitously contain just those sequences. It will be recognized by those skilled in the art that other sequences with low T_m may also be used.

20 Similarly, if the random region contains a sequence complementary both to the primer and the tail, then there is a chance that an intramolecular or intermolecular duplex may form during the PCR annealing and extension step. The primer and its complement will form a duplex, but the 3' end of the duplex (at which extension must occur) will be unstable at the PCR annealing temperature because of the presence of the tail sequence. Because polymerase
25 absolutely requires a base-paired 3' terminal nucleotide in order to begin extension, polymerase extension of this structure will be an extremely rare event.

In preferred embodiments of the instant invention, the 5' ends of the primers used for PCR amplification of the intramolecular duplex candidate mixture are attached to tail
30 sequences selected according to the abovementioned criteria. In addition, the intramolecular duplex candidate mixture used to initiate the automated SELEX process also preferably has tail sequences attached to both the 5' and 3' termini. Each tail sequence is chosen to have a lower T_m than the any of the fixed sequence regions in the candidate mixture (e.g., lower than the T_m of A/A', and, if present, lower than the T_m s of P_1 and P_2). For example, a suitable

intramolecular duplex candidate mixture can be represented by the formula 5' T₁-P₁-A-R-A'-P₂-T₂ 3', wherein T₁ and T₂ are tail sequences, P₁ and P₂ are fixed sequence regions used for PCR amplification, A and A' are complementary fixed sequences that are capable of forming the intramolecular duplex, and R is the at least partially-randomized sequence region.

5 Following identification of a nucleic acid through the automated SELEX process or the automated photoSELEX process, the nucleic acid ligand can be synthesized without the tail sequences or the primer annealing sequences used for amplification. In embodiments of the invention in which the automated photoSELEX process is performed with tailed primers and a tailed candidate mixture, the tail sequences will preferably not contain any photoreactive
10 groups.

What is claimed is:

1. A method for the identification of a nucleic acid ligand of a target from a candidate mixture of nucleic acids, each said nucleic acid in said candidate mixture comprising the sequence 5' A-R-A' 3', wherein R is at least partially-randomized sequence, and wherein A and A' are fixed sequence regions complementary in sequence to one another that are capable of forming an intramolecular duplex, the method comprising the steps:

a) contacting the candidate mixture with the target wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;

b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and

c) amplifying the increased affinity nucleic acids;

wherein a nucleic acid ligand of said target is identified in which A and A' are capable of forming an intramolecular duplex.

2. The method of claim 1 further comprising step:

d) repeating steps a) through c) using the ligand enriched mixture of each successive repeat as many times as required to yield a desired level of increased ligand enrichment.

3. The method of claim 2, wherein steps a) through c) are performed by a computer-controlled robotic manipulator.

4. A method for identifying a nucleic acid ligand that photocrosslinks to a target molecule from a candidate mixture of nucleic acids, each nucleic acid in said candidate mixture comprising the sequence 5' A-R-A' 3', wherein R is at least partially-randomized sequence comprising photoreactive groups, and wherein A and A' are fixed sequence regions complementary in sequence to one another that are capable of forming an intramolecular duplex, said method comprising:

a) contacting said candidate mixture with said target molecule, wherein nucleic acid sequences having increased affinity to the target molecule relative to the candidate mixture form nucleic acid-target molecule complexes;

b) irradiating said candidate mixture, wherein said nucleic acid-target molecule complexes photocrosslink;

c) partitioning the crosslinked nucleic acid-target molecule complexes from free nucleic acids in the candidate mixture; and

5 d) identifying the nucleic acid sequences that photocrosslinked to the target molecule;

wherein a photocrosslinking nucleic acid ligand of said target is identified in which A and A' are capable of forming an intramolecular duplex.

10 5. The method of claim 4 further comprising the step:

e) repeating steps a) through c); and

f) amplifying the nucleic acids that photocrosslinked to the target molecule to yield a mixture of nucleic acids enriched in sequences that are capable of photocrosslinking the target molecule.

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6. The method of claim 4 wherein steps a) through c) and f) are performed by a computer-controlled robotic manipulator.

7. A nucleic acid ligand comprised of a non-naturally occurring nucleic acid having a specific binding affinity for a target molecule, such target molecule being a three dimensional chemical structure other than a polynucleotide that binds to said nucleic acid ligand through a mechanism which predominantly depends on Watson/Crick base pairing or triple helix binding, wherein said nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule, said nucleic acid ligand comprising the sequence 5' A-L-A', wherein L is the target binding region, and wherein A and A' are fixed sequence regions complementary in sequence to one another that are capable of forming an intramolecular duplex, said nucleic acid ligand obtained by the process of identifying a nucleic acid ligand of a target from a candidate mixture of nucleic acids comprised of nucleic acids each comprising the sequence 5' A-R-A' 3', wherein R is at least partially-randomized sequence, said process comprising:

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a) contacting the candidate mixture with the target molecule, wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture;

b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and

c) amplifying the increased affinity nucleic acids to yield a ligand-enriched mixture of nucleic acids;

5 whereby a nucleic acid ligand of the target compound may be identified in which A and A' are capable of forming an intramolecular duplex.

8. A nucleic acid ligand comprised of a non-naturally occurring nucleic acid having a specific binding affinity for a target molecule, such target molecule being a three dimensional
10 chemical structure other than a polynucleotide that binds to said nucleic acid ligand through a mechanism which predominantly depends on Watson/Crick base pairing or triple helix binding, wherein said nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule, said nucleic acid ligand comprising the sequence 5' A-L-A', wherein L is the target binding region, and wherein A and A' are fixed sequence regions
15 complementary in sequence to one another that are capable of forming an intramolecular duplex.

9. A method for the identification of a nucleic acid ligand of a target from a candidate mixture of nucleic acids, each said nucleic acid in said candidate mixture comprising the
20 sequence 5' P₁-A-R-A'-P₂ 3', wherein P₁ and P₂ are primer annealing sites, R is at least partially-randomized sequence, and wherein A and A' are fixed sequences complementary to one another and capable of forming an intramolecular duplex;

a) contacting the candidate mixture with the target wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the
25 remainder of the candidate mixture;

b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture;

c) amplifying the increased affinity nucleic acids using the polymerase chain reaction (PCR) with primers complementary to P₁ and P₂ to yield a ligand enriched mixture of
30 nucleic acids, wherein the 5' ends of said primers are attached to tail sequences having a lower melting temperature (T_m) than said primers, wherein the polymerase chain reaction comprises a denaturation step, a primer annealing step, and a primer extension step, and wherein said primer annealing step and said primer extension step are performed at a temperature higher

than the melting temperature of said tail sequences and higher than the melting temperature of A and A';

d) repeating steps a) through c) using the ligand enriched mixture of each successive repeat as many times as required to yield a desired level of increased ligand enrichment; and

e) optionally removing the sequences P₁ and P₂ from nucleic acids in the ligand enriched mixture with the desired level of increased ligand enrichment;

wherein a nucleic acid ligand is identified in which A and A' are capable of forming an intramolecular duplex.

10. The method of claim 9 wherein steps a) through c) are performed by a computer-controlled robotic manipulator.

11. A method for identifying a photocrosslinking nucleic acid ligand of a target molecule from a candidate mixture of nucleic acids, each nucleic acid in said candidate mixture comprising the sequence 5' P₁-A-R-A'-P₂ 3', wherein P₁ and P₂ are primer annealing sites, R is at least partially-randomized sequence comprising one or more photoreactive groups, and A and A' are fixed sequences complementary to one another and capable of forming an intramolecular duplex; the method comprising the steps:

a) contacting said candidate mixture with said target molecule, wherein nucleic acid sequences having increased affinity to the target molecule relative to the candidate mixture form nucleic acid-target molecule complexes;

b) irradiating said candidate mixture, wherein said nucleic acid-target molecule complexes photocrosslink;

c) partitioning the crosslinked nucleic acid-target molecule complexes from free nucleic acids in the candidate mixture; and

d) amplifying the increased affinity nucleic acids using the polymerase chain reaction (PCR) with primers complementary to P₁ and P₂ to yield a ligand enriched mixture of nucleic acids, wherein the 5' ends of said primers are attached to tail sequences having a lower melting temperature (T_m) than said primers, wherein the polymerase chain reaction comprises a denaturation step, a primer annealing step, and a primer extension step, and wherein said primer annealing step and said primer extension step are performed at a temperature higher

than the melting temperature of said tail sequences and higher than the melting temperature of A and A'; and

e) repeating steps a) through d) using the ligand enriched mixture of each successive repeat as many times as required to yield a desired level of increased ligand enrichment;

f) optionally removing the sequences P₁ and P₂ from nucleic acids in the ligand enriched mixture with the desired level of increased ligand enrichment;

wherein a photocrosslinking nucleic acid ligand of said target is identified in which A and A' are capable of forming an intramolecular duplex.

12. The method of claim 11 wherein steps a) through d) are performed by a computer-controlled robotic manipulator.

13. A nucleic acid ligand that photocrosslinks to a target molecule, said nucleic acid ligand comprised of a non-naturally occurring nucleic acid having a specific binding affinity for said target molecule, wherein said target molecule is not a nucleic acid binding molecule, and wherein said nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule, said nucleic acid ligand comprising the sequence 5' A-L-A' 3', wherein L is the target-binding region comprising one or more photoreactive groups, and wherein A and A' are fixed sequence regions complementary in sequence to one another that are capable of forming an intramolecular duplex.

14. A nucleic acid ligand that photocrosslinks to a target molecule, said nucleic acid ligand comprised of a non-naturally occurring nucleic acid having a specific binding affinity for said target molecule, wherein said target molecule is not a nucleic acid binding molecule, and wherein said nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule, said nucleic acid ligand comprising the sequence 5' A-L-A' 3', wherein L is the target-binding region comprising one or more photoreactive groups, and wherein A and A' are fixed sequence regions complementary in sequence to one another that are capable of forming an intramolecular duplex, said nucleic acid ligand obtained by the process of identifying a nucleic acid ligand of a target from a candidate mixture of nucleic acids comprising the sequence 5' A-R-A' 3', wherein R is at least partially-randomized sequence comprising one or more photoreactive groups, said process comprising:

- a) contacting said candidate mixture with said target molecule, wherein nucleic acid sequences having increased affinity to the target molecule relative to the candidate mixture form nucleic acid-target molecule complexes;
- b) irradiating said candidate mixture, wherein said nucleic acid-target molecule
5 complexes photocrosslink;
- c) partitioning the crosslinked nucleic acid-target molecule complexes from free nucleic acids in the candidate mixture; and
- d) identifying the nucleic acid sequences that photocrosslinked to the target molecule.

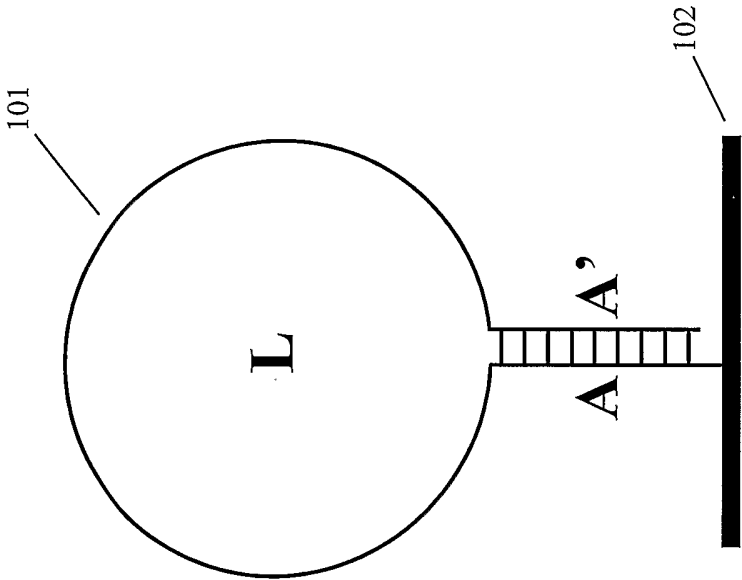


FIGURE 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/27085

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12P 1/68; C12Q 19/34; C07H 21/04, 21/02

US CL : 435/6, 92.1; 536/22.1

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)

U.S. : 435/6, 92.1; 536/22.1

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)

WEST, DIALOG: nucleic, ligand, aptamer, intramolecular, stem-loop, photoreactive, photocrosslink, computer, robot

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP 2001103975 A (PENMECCHA et al.) 17 April, 2001, abstract and structures pages 2 and 5	1-14
Y	US 6,001,577 A (GOLD et al.) 14 December 1999, col. 59-64, claims 1-16	11-14



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
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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	

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