

(19) World Intellectual Property Organization  
International Bureau



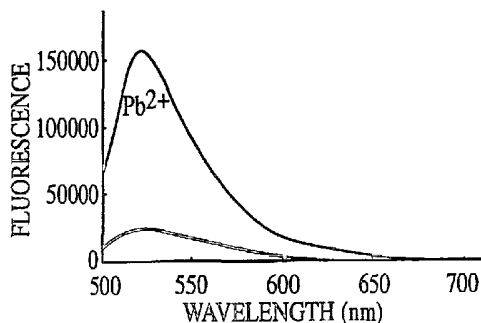
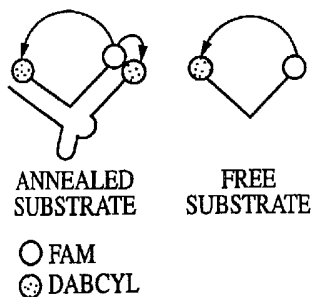
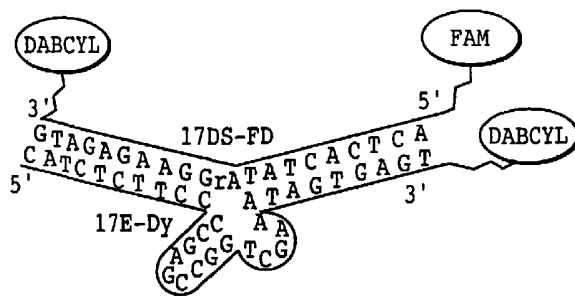
(43) International Publication Date  
20 November 2003 (20.11.2003)

PCT

(10) International Publication Number  
WO 03/095648 A1

- (51) International Patent Classification<sup>7</sup>: C12N 15/11, C12Q 1/68
- (21) International Application Number: PCT/US03/08483
- (22) International Filing Date: 18 March 2003 (18.03.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
10/144,094 10 May 2002 (10.05.2002) US
- (71) Applicant: BOARD OF TRUSTEES OF THE UNIVERSITY OF ILLINOIS [US/US]; 352 Henry Administration Building, 506 W. Wright, Urbana, IL 61801 (US).
- (72) Inventors: LU, Yi; 2172 Prairie Meadow Drive, Champaign, IL 61822 (US). LIU, Juewen; 1009 W. Stoughton Street, Urbana, IL 61801 (US).
- (74) Agent: RAUCH, Paul, E.; Sonnenschein Nath & Rosenthal, P.O. Box 061080, Wacker Drive Station, Sears Tower, Chicago, IL 60606-1080 (US).
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**  
— with international search report  
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: NEW FLUORESCENCE BASED BIOSENSOR



WO 03/095648 A1

(57) Abstract: A novel biosensor comprises at least one fluorophore and at least two quenchers, and is capable of selectively and specifically detecting the presence of an ion in the presence of other ions.

## NEW FLUORESCENCE BASED BIOSENSOR

## FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The U.S. Government may have rights in the present invention pursuant to the terms of grant number DEFG02-01ER63179 awarded by the Department of Energy.

## 5 BACKGROUND

Many metals pose a risk as environmental contaminants. A well-known example is lead. Low level lead exposure can lead to a number of adverse health effects, with as many as 9-25% of pre-school children presently at risk.

10 Approximately twenty-two million old houses in the United States alone have lead paint (Schwartz & Levin, 1991; Rabinowitz *et al.*, 1985). Although leaded paints and gasoline have been banned, lead can accumulate in soils or sediments for long periods of time (Marcus & Elias, 1995; Bogden & Louria, 1975). The level of lead in the blood considered toxic is  $\geq 10\mu\text{g/dL}$  (480nM). Current methods for lead analysis, such as atomic absorption spectrometry, inductively coupled plasma mass  
15 spectrometry, and anodic stripping voltammetry, are complex, expensive and often require sophisticated equipment, sample pre-treatment and skilled operators.

Simple, rapid, inexpensive, selective and sensitive methods that permit real time detection of  $\text{Pb}^{2+}$  and other metal ions are very important in the fields of environmental monitoring, clinical toxicology, wastewater treatment, and industrial  
20 process monitoring and can lead to preventative measures or at least lower risks associated with metal contaminants. Furthermore, methods are needed for monitoring free or bioavailable, instead of total, metal ions in industrial and biological systems.

Fluorescence spectroscopy is a technique well suited for detection of very small concentrations of analytes. Fluorescence provides significant signal  
25 amplification, since a single fluorophore can absorb and emit many photons, leading to strong signals even at very low concentrations. In addition, the fluorescence time-scale is fast enough to allow real-time monitoring of concentration fluctuations. Fluorescent properties only respond to changes related to the fluorophore, and therefore can be highly selective. Also, fluorometers, for measuring fluorescence  
30 signals, are commercially available. Fluorescent detection is also compatible with

fiber-optic technology and well suited for *in vivo* imaging applications. Several fluorescence-related parameters can be assessed for purposes of sensing, detecting, identifying or quantifying a target analyte, including fluorescence intensity, emission or excitation wavelength, fluorescence lifetime and anisotropy.

5 For example, bioaffinity sensors, labeled with fluorophores, have been used to detect DNA hybridization and single-nucleotide polymorphisms (Didenko, 2001). Specifically, molecular beacon, a DNA hairpin structure, is labeled with both a fluorophore and quencher (Tyagi & Kramer, 1996). In the absence of target DNA, the hairpin structure is closed and due to the close proximity of the fluorophore and  
10 quencher, fluorescence is quenched. However, in the presence of a complementary DNA strand, the hairpin secondary structure is destroyed and the fluorescence is released without quenching. Multiple DNA strands may be detected at the same time by placing a quencher on one end of the molecular beacon DNA strand and two fluorophores (a donor fluorophore and an acceptor fluorophore) on the other end  
15 (Tyagi & Kramer, 1998; 2000). This design, based on fluorescence resonance energy transfer (FRET), quenches fluorescence of the fluorophores in the absence of complementary DNA due to the hairpin structure being closed. However, upon hybridization of the molecular beacon and the complementary DNA, the secondary structure is destroyed and the donor fluorophore transfers energy to the acceptor  
20 fluorophore, resulting in fluorescence. Molecular beacon can be designed to target different DNA sequences by constructing complementary DNA strand hairpins, each with a different acceptor fluorophore, while keeping the donor fluorophore the same.

Biosensors, devices capable of detecting target ions using biological reactions, in contrast to bioaffinity sensors, can be modified to utilize fluorescence for detecting,  
25 identifying or quantifying target ions, which can act as catalysts of the biosensor. These modified biosensors, called fluorosensors, are highly sensitive. For example, many fluorescent chemosensors, including fluorophore-labeled organic chelators (Rurack, *et al.*, 2000; Hennrich *et al.*, 1999; Winkler *et al.*, 1998; Oehme & Wolfbeis, 1997) and peptides (Walkup & Imperiali, 1996; Deo & Godwin, 2000; Pearce *et al.*,  
30 1998), have been developed for metal ion detection. These ion sensors are usually composed of an ion-binding motif and a fluorophore. Metal detection using these fluorescent chemosensors relies on the modulation of the fluorescent properties of the

fluorophore by the metal-binding event. Detection limits on the level of micromolar and even nanomolar concentrations have been achieved for heavy metal ions including  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Hg^{2+}$ ,  $Cd^{2+}$  and  $Ag^+$ .

5 Recently, the molecular recognition and catalytic function of nucleic acids have been extensively explored. This exploration has led to the development of aptamers and nucleic acid enzymes, which can be used as biosensors. Aptamers are single-stranded oligonucleotides derived from an *in vitro* evolution protocol called systematic evolution of ligands by exponential enrichment (SELEX). Nucleic acid aptamers can selectively bind to non-nucleic acid targets, such as small organic  
10 molecules or proteins, with affinities as high as  $10^{-14}$  M (Uphoff *et al.*, 1996; Famulok, 1999). Most aptamers undergo a conformational change when binding their cognate ligands. With this property, several DNA and RNA aptamers have been engineered to sense L-adenosine or thrombin through an internally labeled fluorescent reporter group (Jhaveri *et al.*, 2000). Thus, the conformational change in the aptamer  
15 upon binding leads to a change in fluorescence. Nucleic acid enzymes, molecules capable of catalyzing a chemical reaction, may be specifically designed through *in vitro* selection. (Breaker & Joyce, 1994; Breaker, 1997). Allosteric ribozymes (or aptazymes), which combine the features of both aptamer and catalytic RNA, also hold promise for sensing small molecules (Potyrailo *et al.*, 1998; Koizumi *et al.*, 1999;  
20 Robertson & Ellington, 1999, 2000). Their reactivity is modulated through the conformational changes caused by the binding of small organic molecules to an allosteric aptamer domain. Therefore, the signal of ligand binding can be transformed into a signal related to chemical reaction.

## SUMMARY

25 In a first aspect, the present invention is a method of detecting an ion in the presence of other ions, in a sample. The method comprises: forming a mixture of a nucleic acid enzyme including at least one quencher, a substrate and the sample, to produce a product; and detecting the presence of the product. The substrate is a nucleic acid sequence including a ribonucleotide, at least one quencher and at least  
30 one fluorophore.

In a second aspect, the present invention is a method of determining the concentration of an ion in the presence of other ions, in a sample, comprising: forming a mixture of a nucleic acid enzyme comprising at least one quencher, a substrate comprising a ribonucleotide, at least one quencher and at least one fluorophore, and the sample, to produce a product; and measuring the amount of product produced.

In a third aspect, the present invention is a biosensor, capable of detecting the presence of an ion in the presence of other ions, comprising: a nucleic acid enzyme which includes at least one quencher, and a substrate which includes a ribonucleotide, at least one quencher and at least one fluorophore.

A "nucleic acid enzyme" is a nucleic acid molecule that catalyzes a chemical reaction. The nucleic acid enzyme may be covalently linked with one or more other molecules yet remain a nucleic acid enzyme. Examples of other molecules include dyes, quenchers, proteins, and solid supports. The nucleic acid enzyme may be entirely made up of ribonucleotides, deoxyribonucleotides, or a combination of ribo- and deoxyribonucleotides.

A "sample" may be any solution that may contain an ion (before or after pre-treatment). The sample may contain an unknown concentration of an ion. For example, the sample may be paint that is tested for lead content. The sample may be diluted yet still remain a sample. The sample may be obtained from the natural environment, such as a lake, pond, or ocean, an industrial environment, such as a pool or waste stream, a research lab, a common household, or a biological environment, such as blood. Of course, sample is not limited to the taking of an aliquot of solution but also includes the solution itself. For example, a biosensor may be placed into a body of water to measure for contaminants. In such instance, the sample may comprise the body of water or a particular area of the body of water. Alternatively, a solution may be flowed over the biosensor without an aliquot being taken. Furthermore, the sample may contain a solid or be produced by dissolving a solid to produce a solution. For example, the solution may contain soil from weapon sites or chemical plants.

"Measuring an amount of the product produced" includes measuring the result of the production of a product by an enzyme. For example, in an embodiment where the substrate comprises a quencher and fluorophore and the enzyme comprises a

second quencher, and cleavage of the substrate by the enzyme leads to dissociation of the product from the enzyme, "measuring an amount of the product produced" includes detecting the increase of fluorescence. Thus, one is measuring the product by detecting its inability to quench fluorescence.

5 "Forming a mixture" includes placing the sample, a substrate and an enzyme in proximity such that an ion in the sample could be used as a cofactor. "Forming a mixture" includes such acts as pipetting a sample onto a solid support or into a tube or well containing the nucleic acid enzyme. Alternatively, the enzyme may be brought to the sample. For example, the enzyme may be placed into a stream to monitor for  
10 the presence of a contaminant.

#### BRIEF DESCRIPTION THE DRAWINGS

**FIG. 1.** Selection scheme for RNA-cleaving deoxyribozymes. **FIG. 1A.** (SEQ ID NO: 12) Starting pool of random-sequenced DNAs, engineered to contain two substrate-binding domains. Each member of the pool contains a 5'-terminal biotin (encircled B), a single embedded ribonucleotide (rA) and a 40-nucleotide random sequence domain (N40). **FIG. 1B.** Selective amplification scheme for isolation of DNA that catalyzes the metal cofactor ( $\text{Co}^{2+}$  or  $\text{Zn}^{2+}$ ) dependent cleavage of an RNA phosphodiester.

**FIG. 2.** (SEQ ID NOS 13-23, respectively, in order of appearance) Sequence classes of the cloned Zn-DNA with clone numbers shown on the left, highly conserved sequences in bold, covariant nucleotides underlined, and 5'- and the 3'- primer binding sequences shown in italics.

**FIG 3.** (SEQ ID NOS 24-42, respectively, in order of appearance) Sequence classes of the cloned Co-DNA with clone-numbers listed on the left and 5' and the 3' primer binding sequences in italics.

**FIG. 4.** (SEQ ID NOS 43-70, respectively, in order of appearance) Sequence alignment of the N40 region of the reselected Zn-DNAs with wild-type sequence listed on top, followed by reselected Zn-DNA sequences showing only point mutations. Shown on the left are clone-numbers and rate constants ( $k_{\text{obs}}$ ) of several reselected Zn-DNA in  $100 \mu\text{M Zn}^{2+}$  are shown on the right.

**FIG. 5.** (SEQ ID NOS 1 & 2) Proposed secondary structure of the Zn(II)-dependent *trans*-cleaving deoxyribozyme.

**FIG. 6.** Sequences and proposed secondary-structures of several RNA-cleaving deoxyribozymes. **FIG. 6A** (SEQ ID NOS 71 & 72) and **FIG. 6B** (SEQ ID NOS 73 & 74). The deoxyribozyme selected using  $Mg^{2+}$  or  $Pb^{2+}$  as cofactor (Breaker & Joyce, 1994, 1995). **FIG. 6C** (SEQ ID NOS 75 & 76) and **FIG. 6D** (SEQ ID NOS 77 & 78). The 10-23 and the 8-17 deoxyribozymes selected in  $Mg^{2+}$  to cleave all-RNA substrate (Santoro & Joyce, 1997). **FIG. 6E** (SEQ ID NOS 79 & 80). A deoxyribozyme selected using L-histidine as cofactor. **FIG. 6F** (SEQ ID NOS 81 & 82). The 17E deoxyribozyme selected in  $Zn^{2+}$ . In each structure, the upper strand is the substrate and the lower strand is the enzyme. Arrows identify the site of RNA transesterification.

**FIG. 7.** Comparison of G3 deoxyribozyme with class II Co-DNA. **FIG. 7A.** (SEQ ID NO: 83) The predicted secondary structure of the G3 deoxyribozyme (Geyer & Sen, 1997) with X representing variable sequences. The boxed region was also found in class II Co-DNA. **FIG. 7B.** (SEQ ID NO: 84) The minimal structure motif of the class II Co-DNA predicted by *mfold* program with arrows indicating cleavage sites.

**FIG. 8.** Steady-state fluorescence spectra of the substrate (Rh-17DS) alone (I), after annealing to the deoxyribozyme (17E-Dy) (II), and 15 min after adding 500 nM  $Pb(OAc)_2$  (III).

**FIG. 9.**  $Pb^{2+}$  sensitive biosensor. **FIG. 9A.** Selectivity and sensitivity of biosensor for  $Pb^{2+}$  at room temperature. **FIG. 9B.** Quantification of **FIG. 9A.** **FIG. 9C.** Time dependent curve illustrating fluorescence intensity increase for 500 nM divalent ions.  $Pb^{2+}$  curve is represented by the upper curve of dots. Other six metal ions,  $Co^{2+}$ ,  $Mg^{2+}$ ,  $Zn^{2+}$ ,  $Cd^{2+}$ ,  $Mn^{2+}$ ,  $Ni^{2+}$  are in the baseline level.

**FIG 10.** Dependence of  $v_{fluo}$  on the concentration of  $Pb^{2+}$  or  $Co^{2+}$ . **FIG 10A.** The initial rate ( $v_{fluo}$ ) increased with the concentration of  $Pb^{2+}$  (◆) and  $Co^{2+}$  (■) over a range of three orders of magnitude. **FIG 10B.** At low concentrations,  $v_{fluo}$  increased linearly with  $Pb^{2+}$  (◆) or  $Co^{2+}$  (■) concentration.

**FIG. 11.** DNA chips for ion sensing. **FIG. 11A.** The array of deoxyribozymes with different metal specificity and affinity on the DNA chip for metal ion sensing. **FIG. 11B.** Quantitative and qualitative detection of metal ions using the metal ion-sensing deoxyribozyme chip with the z-axis representing fluorescence intensity change upon the exposure of the chip to the sample under examination.

**FIG. 12.** Design of a biosensor of U.S. Application Serial No. 09/605,558. **FIG. 12A** The 3' end of the substrate is labeled with the fluorophore TAMRA and the 3' end of the enzyme is labeled with the quencher DABCYL.  $Pb^{2+}$  acts as a cofactor of this enzyme-substrate duplex, cleaving the substrate at the position of rA. **FIG. 12B** Representation of the biosensor system at room temperature, where the substrate and enzyme are poorly annealed and free substrate increases background fluorescence signal, making detection signal relatively weaker. **FIG. 12C** Room temperature fluorescence spectra for 1:1 substrate enzyme ratio in the absence of  $Pb^{2+}$  (lower curve) and in the presence of  $Pb^{2+}$  (upper curve). The fluorescence increase is only 60%.

**FIG. 13.** Design of a biosensor with at least 2 quencher molecules and at least one fluorophore molecule. **FIG. 13A** The biosensor has a quencher molecule (DABCYL) located on the 3' end of both the substrate and enzyme and a fluorophore (FAM) on the 5' end of the enzyme. **FIG. 13B** Representation of the biosensor at room temperature, where regardless of hybridization between the enzyme and substrate, fluorescence is quenched in the absence of cleavage of the substrate. **FIG. 13C** Room temperature fluorescence spectra for 1:1 substrate enzyme ratio in the absence of  $Pb^{2+}$  (lower curve) and in the presence of  $Pb^{2+}$  (upper curve). The fluorescence is increased 660% over the background fluorescence signal, which is a more than 10 fold improvement over the biosensor design of Figure 12.

**FIG. 14.** Image comparison for the performance of the biosensor disclosed herein and the biosensor disclosed in U.S. Application Serial No. 09/605,558. (Images from a fluorescence image reader (Fuji)).



## DETAILED DESCRIPTION

The present invention makes use of the discovery that including a second quencher can dramatically reduce background fluorescence signal in a biosensor system at room temperature, and thereby enhance sensitivity for ion detection.

5 U.S. Application Serial No. 09/605,558, describes a combination of a nucleic acid enzyme, including a quencher, and a nucleic acid substrate, including a fluorophore. This previous biosensor comprises a fluorophore and quencher arranged in proximity such that prior to cleavage the fluorophore and quencher are proximal to one another and fluorescence intensity is decreased by the quencher. Upon binding  
10 of a specifically recognized ion, for example  $Pb^{2+}$ , cleavage occurs and the fluorophore and quencher are separated, leading to an increase in fluorescence intensity, which may then be detected. However, at room temperature (around 23°C), due to the relatively low hybridization temperature of the enzyme-substrate duplex (around 35°C), a fraction of the duplex melts, resulting in free substrate labeled with a  
15 fluorophore, which leads to a high level of background fluorescence signal. To overcome this problem, Application Serial No. 09/605,558 describes using the enzyme-substrate duplex at low temperatures, around 4°C, to promote annealing of the enzyme and substrate. At 4°C, this enzyme-substrate duplex yields a 400% increase in fluorescence signal intensity, compared to only 60% increase in signal  
20 intensity at room temperature (Fig. 12C, 13C and 14). This significant decrease in signal intensity greatly detracts from the sensitivity and interpretability of the results of such a test.

One method for overcoming increased background signal due to increased free substrate at higher temperatures would be to increase the hybridization strength of the  
25 recognition arms, thereby making the substrate-enzyme duplex more stable. While this method would decrease background fluorescence signal at higher temperatures, it would also greatly increase the reaction time due to slow release of the cleaved substrate recognition arms. The present invention avoids both problems by adding a quencher to the substrate on the end opposite the fluorophore. This design  
30 successfully prevents significant levels of background fluorescence because when the substrate is poorly annealed to the enzyme it forms a random coil so that the end-to-end distance is much shorter than in the fully stretched, annealed state, resulting in

significant energy transfer from fluorophore to quencher, thereby significantly decreasing any detectable background fluorescence signals.

The present invention has much less background fluorescence. For example, in one embodiment, selectivity for  $Pb^{2+}$  was increased 10 fold at room temperature over the  $Pb^{2+}$  sensitive biosensor of U.S. Application Serial No. 09/605,558, which itself has selectivity for  $Pb^{2+}$  more than 80 fold over other divalent metal ions with high sensitivity (660% signal increase over background fluorescence signal of the new biosensor compared to 60% signal increase over background of the biosensor of U.S. Application Serial No. 09/605,558, at room temperature) (Figure 14). Such selectivity and sensitivity provide for qualitative and quantitative detection of ions over a concentration range of several orders of magnitude. The new biosensor also provides easily interpretable results, by lowering background fluorescence signals to almost zero. The fluorescence domain of this biosensor may be decoupled from the ion-recognition/catalysis domain, and therefore the sensitivity and signal over background ratio of this system may be manipulated by a careful choice of fluorophores and by performing *in vitro* selection of ion-binding domains to not only keep sequences reactive with the ion of choice, but also remove sequences that also respond to other ions.

The present invention provides a simple, rapid, inexpensive, selective and sensitive method for detecting the presence of an ion, with background fluorescence signal near zero and effective at any temperature, and is an important and useful tool in preventing or at least lowering health and environmental risks associated with environmental contaminants.

DNA is stable, inexpensive and easily adaptable to optical fiber and chip technology for device manufacture. The attachment of DNA enzymes to optical fibers or chips allows regeneration of the sensors by washing away the cleavage products and adding new substrates. Finally, sequences specific for other ions and with various detection ranges may be isolated by varying the selection conditions, providing for a highly sensitive and selective fluorosensor system.

#### Nucleic Acid Enzymes

A growing number of nucleic acid enzymes have been discovered or developed showing a great diversity in catalytic activity (Table 1 and Table 2). Many,

if not all, of the enzymes are dependent on one or more ion cofactors. *In vitro* selection may be used to “enhance” selectivity and sensitivity for a particular ion. Such enzymes find particular utility in the compositions and methods of the present invention. For example, nucleic acid enzymes that catalyze molecular association (ligation, phosphorylation, and amide bond formation) or dissociation (cleavage or transfer) are particularly useful.

In preferred embodiments, a nucleic acid enzyme that catalyzes the cleavage of a nucleic acid in the presence of an ion is used. The nucleic acid enzyme may be RNA (ribozyme), DNA (deoxyribozyme), a DNA/RNA hybrid enzyme, or a peptide nucleic acid (PNA) enzyme. PNAs comprise a polyamide backbone and the bases found in naturally occurring nucleosides and are commercially available, e.g., from Biosearch, Inc. (Bedford, Mass.).

Ribozymes that may be used in the present invention include, but are not limited to, group I and group II introns, the RNA component of the bacterial ribonuclease P, hammerhead, hairpin, hepatitis delta virus and Neurospora VS ribozymes. Also included are *in vitro* selected ribozymes, such as those isolated by Tang and Breaker (2000).

One limitation of using a ribozyme is that they tend to be less stable than deoxyribozymes. Thus, in preferred embodiments, the nucleic acid enzyme is a deoxyribozyme. Preferred deoxyribozymes include those shown in FIG. 6A-6F and deoxyribozymes with extended chemical functionality (Santoro *et al.*, 2000).

Table 1.

Reactions catalyzed by ribozymes that were isolated from *in vitro* selection experiments.

Reaction	$k_{cat}$ ( $\text{min}^{-1}$ )	$K_m$ ( $\mu\text{M}$ )	$k_{cat}/k_{uncat}^a$	Reference
<i>Phosphoester centers</i>				
Cleavage	0.1	0.03	$10^5$	Vaish, 1998
Transfer	0.3	0.02	$10^{13}$	Tsang, 1996
Ligation	100	9	$10^9$	Ekland, 1995

Phosphorylation	0.3	40	$>10^5$	Lorsch, 1994
Mononucleotide polymerization	0.3	5000	$>10^7$	Ekland, 1996
<i>Carbon centers</i>				
Aminoacylation	1	9000	$10^6$	Illangasekare, 1997
Aminoacyl ester hydrolysis	0.02	0.5	10	Piccirilli, 1992
Aminoacyl transfer	0.2	0.05	$10^3$	Lohse, 1996
<i>N</i> -alkylation	0.6	1000	$10^7$	Wilson, 1995
<i>S</i> -alkylation	$4 \times 10^{-3}$	370	$10^3$	Wecker, 1996
Amide bond cleavage	$1 \times 10^{-5}$		$10^2$	Dai, 1995
Amide bond formation	0.04	2	$10^5$	Wiegand, 1997
Peptide bond formation	0.05	200	$10^6$	Zhang, 1997
Diels-Alder cycloaddition	$>0.1$	$>500$	$10^3$	Tarasow, 1997
<i>Others</i>				
Biphenyl isomerization	$3 \times 10^{-5}$	500	$10^2$	Prudent, 1994
Porphyrin metallation	0.9	10	$10^3$	Conn, 1996

<sup>a</sup> Reactions catalyzed by ribozymes that were isolated from *in vitro* selection experiments.  $k_{cat}/k_{uncat}$  is the rate enhancement over uncatalyzed reaction.

5

**Table 2.**  
**Deoxyribozymes isolated through *in vitro* selection.**

Reaction	Cofactor	$k_{max}(\text{min}^{-1})^a$	$k_{cat}/k_{uncat}$	Reference
RNA transesterification	$\text{Pb}^{2+}$	1	$10^5$	Breaker, 1994
	$\text{Mg}^{2+}$	0.01	$10^5$	Breaker, 1995
	$\text{Ca}^{2+}$	0.08	$10^5$	Faulhammer, 1997
	$\text{Mg}^{2+}$	10	$>10^5$	Santoro, 1997

	None	0.01	$10^8$	Geyer, 1997
	L-histidine	0.2	$10^6$	Roth, 1998
	Zn <sup>2+</sup>	~40	$>10^5$	Li, J., 2000
DNA cleavage	Cu <sup>2+</sup>	0.2	$>10^6$	Carmi, 1996
DNA ligation	Cu <sup>2+</sup> or Zn <sup>2+</sup>	0.07	$10^5$	Cuenod, 1995
DNA phosphorylation	Ca <sup>2+</sup>	0.01	$10^9$	Li, Y., 1999
5',5'-pyrophosphate formation	Cu <sup>2+</sup>	$5 \times 10^{-1}$	$>10^{10}$	Li, Y., 2000
Porphyrin methalation	None	1.3	$10^3$	Li, Y., 1996

<sup>a</sup>  $k_{\max}$  is the maximal rate constant obtained under optimized conditions.

An advantage of ribozymes and deoxyribozymes is that they may be produced and reproduced using biological enzymes and appropriate templates. However, the present invention is not limited to ribozymes and deoxyribozymes. Nucleic acid enzymes that are produced by chemical oligosynthesis methods are also included. Thus, nucleic acids including nucleotides containing modified bases, phosphate, or sugars may be used in the compositions and methods of the present invention. Modified bases are well known in the art and include inosine, nebularine, 2-aminopurine riboside, N<sup>7</sup>-denzaadenosine, and O<sup>6</sup>-methylguanosine (Earnshaw & Gait, 1998). Modified sugars and phosphates are also well known and include 2'-deoxynucleoside, abasic, propyl, phosphorothioate, and 2'-O-allyl nucleoside (Earnshaw & Gait, 1998). DNA/RNA hybrids and PNAs may be used in the compositions and methods of the present invention. The stability of PNAs and relative resistance to cellular nucleases make PNA enzymes amenable to *in vivo* applications.

In certain embodiments, the substrate for the nucleic acid enzyme and the enzyme itself are contained in the same nucleic acid strand. Such enzymes are *cis*-acting enzymes. Examples include the Zn<sup>2+</sup>-dependent deoxyribozymes (Zn-DNA) created in Example 1 (FIG. 1A and FIG. 2).

In preferred embodiments, the nucleic acid enzyme cleaves a nucleic acid strand that is separate from the strand comprising the enzyme (*trans*-acting). One advantage of utilizing *trans*-activity is that, after cleavage, the product is removed and

additional substrate may be cleaved by the enzymatic strand. A preferred nucleic acid enzyme is 5'-CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT-3' (17E; FIG. 5; SEQ ID NO:1). The corresponding preferred substrate to 17E is 5'-

ACTCACTATrAGGAAGAGATG-3' (17DS; FIG. 5; SEQ ID NO:2), where rA denotes a single ribonucleotide.

It may be beneficial to use directed mutation to change one or more properties of a nucleic acid enzyme or its substrate. Using 17E and 17DS-FD as an example, one may wish to alter the avidity of the two arms of the hybridized enzyme and substrate. The "arms" are those areas displaying Watson-Crick basepairing in FIG. 5. To alter avidity, one may increase or decrease the length of the arms. Increasing the length of the arms increases the number of Watson-Crick bonds, thus increasing the avidity. The opposite is true for decreasing the length of the arms. Decreasing the avidity of the arms facilitates the removal of substrate from the enzyme, thus allowing faster enzymatic turnover.

Another method of decreasing avidity includes creating mismatches between the enzyme and the substrate. Alternatively, the G-C content of the arms may be altered. Of course, the effect of any directed change should be monitored to ensure that the enzyme retains its desired activity, including ion sensitivity and selectivity. In light of the present disclosure, one of skill in the art would understand how to monitor for a desired enzymatic activity. For example, to ensure that the mutated enzyme maintained sensitivity and selectivity for  $Pb^{2+}$ , one would test to determine if the mutated enzyme remained reactive in the presence of lead (sensitivity) and maintained its lower level of activity in the presence of other ions (selectivity).

The nucleic acid enzyme is sensitive and selective for a single ion. The ion may be any anion, for example, arsenate ( $AsO_4^{3-}$ ), or cation. The ion may be monovalent, divalent, trivalent, or polyvalent. Examples of monovalent cations include  $K^+$ ,  $Na^+$ ,  $Li^+$ ,  $Tl^+$ ,  $NH_4^+$  and  $Ag^+$ . Examples of divalent cations include  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Mn^{2+}$ ,  $Co^{2+}$ ,  $Ni^{2+}$ ,  $Zn^{2+}$ ,  $Cd^{2+}$ ,  $Cu^{2+}$ ,  $Pb^{2+}$ ,  $Hg^{2+}$ ,  $Pt^{2+}$ ,  $Ra^{2+}$ ,  $Ba^{2+}$ ,  $UO_2^{2+}$  and  $Sr^{2+}$ . Examples of trivalent cations include  $Co^{3+}$ ,  $Cr^{3+}$ , and lanthanide ions ( $Ln^{3+}$ ). Polyvalent cations include  $Ce^{4+}$ ,  $Cr^{6+}$ , spermine, and spermidine. The ion detected by the biosensor also includes ions having a metal in a variety of oxidation states. Examples include K(I), Na(I), Li(I), Tl(I), Ag(I), Hg(I), Mg(II), Ca(II), Mn(II),

Co(II), Ni(II), Zn(II), Cd(II), Pb(II), Hg(II), Pt(II), Ra(II), Ba(II), Sr(II), Co(III), Cr(III), Ln(III), Ce(IV), Cr(VI) and U(VI).

The biosensors of the present invention may be used to monitor contaminants in the environment; in such a case preferred ions are those that are toxic to living organisms, *e.g.*, Ag<sup>+</sup>, Pb<sup>2+</sup> and Hg<sup>2+</sup>.

Often the nucleic acid enzymes that have activity with one ion also have at least some activity with one or more other ions. Such multi-sensitive enzymes may still be used in the compositions and methods of the present invention. However, it should be understood that use of a multi-sensitive enzyme may lead to uncertainty as to which of the ions is present. In such cases, measuring the rate of enzymatic activity, using serial dilutions, or using an array of nucleic acid enzymes may be helpful in deciphering which ion is present.

#### *In vitro* Selection of Nucleic Acid Enzymes

Many nucleic acid enzymes that are dependent on ions, particularly metal ions, for activity are known in the art (Breaker & Joyce, 1994; Pan & Uhlenbeck, 1992; Cuenoud & Szostak, 1995; Carmi *et al.*, 1996; Li *et al.*, 2000; Santoro *et al.*, 2000). In light of the present disclosure, one of skill in the art would understand how to utilize a known nucleic acid enzyme in the methods and biosensors of the present invention. Furthermore, the present invention may include a nucleic acid enzyme created by *in vitro* selection. Methods of *in vitro* selection of nucleic acid enzymes are known in the art and described herein.

*In vitro* selection is a technique in which RNA or DNA molecules with certain functions are isolated from a large number of sequence variants through multiple cycles of selection and amplification (Joyce, 1994; Chapman *et al.*, 1994). The concept of *in vitro* selection of catalytic RNA molecules was first introduced in the late 1980's. Since then, it has been widely applied to obtain ribozymes with maximized activities or novel catalytic abilities, and to identify oligonucleotides (called aptamers) that bind to certain proteins or small molecules with high affinity. The process for aptamers selection is sometimes referred as systematic evolution of ligands by exponential enrichment (SELEX)(Tuerk & Gold, 1990).

The first catalytic DNA (deoxyribozyme) was isolated by Breaker and Joyce in 1994 through *in vitro* selection. This deoxyribozyme is able to catalyze phosphodiester cleavage reaction in the presence of  $Pb^{2+}$ . Unlike RNA-based catalysts, DNA molecules with catalytic functions have not been encountered in nature, where DNA exists primarily as base-paired duplex and serves mainly as the carrier of genetic information. The identification of DNA molecules with catalytic functions further demonstrated the power of *in vitro* selection.

*In vitro* selection is typically initiated with a large collection of randomized sequences. A typical DNA or RNA library for selection contains  $10^{13}$ - $10^{16}$  sequence variants. The construction of a completely randomized pool is accomplished by chemical synthesis of a set of degenerated oligonucleotides using standard phosphoramidite chemistry. The 3'-phosphoramidite compounds of four nucleosides (A, C, G, and T) are premixed before being supplied to an automated DNA synthesizer to produce oligonucleotides. By controlling the ratio of four phosphoroamidites, the identity at each nucleotide position can be either completely random, i.e. with equal chance for each base, or biased toward a single base. Other strategies for creating a randomized DNA library include applying mutagenic polymerase chain reaction (PCR) and template-directed mutagenesis (Tsang and Joyce, 1996; Cadwell and Joyce, 1992, 1994). For the purpose of *in vitro* selection of functional RNA molecules, the randomized DNA library is converted to an RNA library through *in vitro* transcription.

*In vitro* selection takes advantage of a unique property of RNA and DNA, i.e., the same molecule can possess both genotype (coding information) and phenotype (encoded function). The DNA or RNA molecules in the randomized library are screened simultaneously. Those sequences that exhibit a desired function (phenotype) are separated from the inactive molecules. Usually the separation is performed through affinity column chromatography, being linked to or released from a solid support, gel electrophoresis separation, or selective amplification of a tagged reaction intermediate. The genotype of the active molecules are then copied and amplified, normally through polymerase chain reaction (PCR) for DNA or isothermal amplification reaction for RNA (Guatelli *et al.*, 1990). Mutations can be performed with mutagenic PCR to reintroduce diversity to the evolving system. These three



steps- selection, amplification and mutation, are repeated, often with increasing selection stringency, until sequences with the desired activity dominate the pool.

Novel nucleic acid enzymes isolated from random sequences *in vitro* have extended the catalytic repertoire of RNA and DNA far beyond what has been found in nature. The selected ribozymes are capable of catalyzing a wide range of reactions at both phosphate and non-phosphate centers (Table 1). The reactions that are catalyzed by deoxyribozymes are less diverse, compared with the ribozymes (Table 2).

However, the catalytic rate ( $k_{cat}$ ) of most deoxyribozymes is comparable to that of the ribozymes catalyzing the same reaction. In certain cases, the catalytic efficiency ( $k_{cat}/K_m$ ) of nucleic acid enzymes even exceeds that of the protein enzymes.

*In vitro* selection can be used to change the ion specificity or binding affinity of existing ribozymes, or to obtain nucleic acid enzymes specific for desired ions. For example, *in vitro*-selected variants of the group I intron (Lehman & Joyce, 1993) and the RNase P ribozyme (Frank & Pace, 1997) have greatly improved activity in  $Ca^{2+}$ , which is not an active metal ion cofactor for native ribozymes. The  $Mg^{2+}$  concentration required for optimal hammerhead ribozyme activity has been lowered using *in vitro* selection to improve the enzyme performance under physiological conditions (Conaty *et al.*, 1999; Zillman *et al.*, 1997). Breaker and Joyce have isolated several RNA-cleaving deoxyribozymes using  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ , or  $Pb^{2+}$  as the cofactor (Breaker & Joyce, 1994, 1995). Only the sequence and structure of the  $Pb^{2+}$ -dependent and the  $Mg^{2+}$ -dependent deoxyribozymes were reported (FIG. 6A and 6B). Other examples of metal-specific RNA/DNA enzymes obtained through *in vitro* selection include a  $Pb^{2+}$ -specific RNA-cleaving ribozyme (called leadzyme) (Pan & Uhlenbeck, 1992), a  $Cu^{2+}$ -specific DNA-cleaving deoxyribozyme (Carmi *et al.*, 1996), and a DNA ligase active in  $Zn^{2+}$  and  $Cu^{2+}$  (Cuonod & Szostak, 1995).

Often nucleic acid enzymes developed for a specific metal ion by *in vitro* selection will have activity in the presence of other metal ions. For example, 17E deoxyribozyme was developed by *in vitro* selection for activity in the presence of  $Zn^{2+}$ . Surprisingly, the enzyme showed greater activity in the presence of  $Pb^{2+}$  than  $Zn^{2+}$ . Thus, although produced in a process looking for  $Zn^{2+}$ -related activity, 17E may be used as a sensitive and selective sensor of  $Pb^{2+}$ .

To produce nucleic acid enzymes with greater selectivity, a negative selection step may be included in the process. For Example,  $Pb^{2+}$ -specific deoxyribozymes may be isolated using a similar selection scheme as for the selection of  $Co^{2+}$ - and  $Zn^{2+}$ -dependent DNA enzymes described in Example 1. In order to obtain  
5 deoxyribozymes with high specificity for  $Pb^{2+}$ , negative-selections may be carried out in addition to the positive selections in the presence of  $Pb^{2+}$ .

For negative selection, the DNA pool is selected against a "metal soup", which contains various divalent metal ions (e.g.  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ ,  $Cd^{2+}$ ,  $Co^{2+}$ ,  $Cu^{2+}$ , etc.). Those sequences that undergo self-cleavage in the presence of divalent metal  
10 ions other than  $Pb^{2+}$  are then washed off the column. The remaining sequences are further selected with  $Pb^{2+}$  as the cofactor.  $Pb^{2+}$ -dependent deoxyribozymes with different affinities for  $Pb^{2+}$  can be obtained by controlling the reaction stringency ( $Pb^{2+}$  concentration).

#### 15 Fluorophores and Quenchers

Any chemical reaction that leads to a fluorescent or chemiluminescent signal may be used in the compositions and methods of the present invention. In preferred  
embodiments, fluorophores are used to measure enzymatic activity and, thus, detect the presence of a particular ion. Essentially any fluorophore may be used, including  
20 BODIPY, fluorescein, fluorescein substitutes (Alexa Fluor dye, Oregon green dye), long wavelength dyes, and UV-excited fluorophores. These and additional fluorophores are listed in Fluorescent and Luminescent Probes for Biological Activity. A Practical Guide to Technology for Quantitative Real-Time Analysis,  
Second Ed. W.T. Mason, ed. Academic Press (1999) (incorporated herein by  
25 reference). In preferred embodiments, the fluorophore is 6-carboxyfluorescein (FAM). FAM has an excitation range of 460-500 nm.

A quencher is a molecule that absorbs the energy of the excited fluorophore. Close proximity of a fluorophore and a quencher allow for the energy to be  
transferred from the fluorophore to the quencher. By absorbing this energy, the  
30 quencher prevents the fluorophore from releasing the energy in the form of a photon, thereby preventing fluorescence.

Quenchers may be categorized as non-fluorescent and fluorescent quenchers. Non-fluorescent quenchers are capable of quenching the fluorescence of a wide variety of fluorophores. Generally, non-fluorescent quenchers absorb energy from the fluorophore and release the energy as heat. Examples of non-fluorescent quenchers include 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL), QSY-7, and QSY-33.

Fluorescent quenchers tend to be specific to fluorophores that emit at a specific wavelength range. Fluorescent quenchers often involve fluorescence resonance energy transfer (FRET). In many instances the fluorescent quencher molecule is also a fluorophore. In such cases, close proximity of the fluorophore and fluorescent quencher is indicated by a decrease in fluorescence of the "fluorophore" and an increase in fluorescence of the fluorescent quencher. Commonly used fluorescent fluorophore pairs (fluorophore/fluorescent quencher) include fluorescein/tetramethylrhodamine, IAEDANS/fluorescein, fluorescein/fluorescein, and BODIPY FL/ BODIPY FL.

When choosing a fluorophore, a quencher, or where to position these molecules, it is important to consider, and preferably to test, the effect of the fluorophore or quencher on the enzymatic activity of the nucleic acid enzyme. Also, it is preferable that the fluorophore display a high quantum yield and energy transfer efficiency. Long-wavelength (excitation and emission) fluorophores are preferred because of less interference from other absorbing species. The fluorophore should also be less sensitive to pH change or to non-specific quenching by metal ions or other species.

Methods and devices for detecting fluorescence are well developed. Essentially any instrument or method for detecting fluorescent emissions may be used. For example, WO 99/27351 (incorporated herein in its entirety) describes a monolithic bioelectrical device comprising a bioreporter and an optical application specific integrated circuit (OASIC). The device allows remote sampling for the presence of substances in solution.

Furthermore, the fluorescence may be measured by a number of different modes. Examples include fluorescence intensity, lifetime, and anisotropy in either steady state or kinetic rate change modes (Lakowicz, 1999).

Sometimes other factors in a solution such as pH, salt concentration or ionic strength, or viscosity will have an effect on fluorescence, and may even affect the hybridization of the substrate and enzyme. Therefore, in preferred methods, controls are run to determine if the solution itself, regardless of enzymatic activity, is altering the fluorescence. Such controls include the use of non-cleavable substrates and or substrate without the presence of enzyme.

### Biosensors

A biosensor is a device which is capable of detecting target analytes by utilizing biological reactions. The biosensor of the present invention is quite different from a bioaffinity sensor, which relies on specific binding and recognition events of target DNA sequences, because a biosensor takes advantage of its own catalytic activities, caused by a target analyte or ion.

For example, described herein are biosensors which are nucleic acid enzymes that are dependent on the presence of a specific ion for activity. Using fluorophores or fluorophore/quencher labeling, it is possible to measure enzymatic activity, even in real time. These qualities make the compositions of the present invention excellent for use in biosensors, which are useful for detecting the presence of a target ion in the presence of other ions.

A key to biosensor detection methods is to minimize background fluorescence signals by maintaining the fluorophore and quenchers in close proximity in the absence of cleavage. Therefore the fluorophore could be linked essentially anywhere on the substrate and quenchers could be linked essentially anywhere on the substrate and enzyme, as long as the fluorophore is in close proximity to at least one of the quenchers prior to cleavage. By close proximity, it is meant that they are situated such that the quencher is able to function (*i.e.*, where efficiency of energy transfer between the quencher and the fluorophore is 50% or more), and preferably are less than a distance of 20 nucleic acid bases or 70 angstroms. For example, a fluorophore may be linked to one end of the substrate in the substrate-enzyme duplex, while a quencher is linked to the opposite end of the substrate and a second quencher is linked to the end of the enzyme which hybridizes with the fluorophore labeled end of the substrate. (Fig. 13A) This configuration provides the advantage of continually

Sometimes other factors in a solution such as pH, salt concentration or ionic strength, or viscosity will have an effect on fluorescence, and may even affect the hybridization of the substrate and enzyme. Therefore, in preferred methods, controls are run to determine if the solution itself, regardless of enzymatic activity, is altering the fluorescence. Such controls include the use of non-cleavable substrates and or substrate without the presence of enzyme.

### Biosensors

A biosensor is a device which is capable of detecting target analytes by utilizing biological reactions. The biosensor of the present invention is quite different from a bioaffinity sensor, which relies on specific binding and recognition events of target DNA sequences, because a biosensor takes advantage of its own catalytic activities, caused by a target analyte or ion.

For example, described herein are biosensors which are nucleic acid enzymes that are dependent on the presence of a specific ion for activity. Using fluorophores or fluorophore/quencher labeling, it is possible to measure enzymatic activity, even in real time. These qualities make the compositions of the present invention excellent for use in biosensors, which are useful for detecting the presence of a target ion in the presence of other ions.

A key to biosensor detection methods is to minimize background fluorescence signals by maintaining the fluorophore and quenchers in close proximity in the absence of cleavage. Therefore the fluorophore could be linked essentially anywhere on the substrate and quenchers could be linked essentially anywhere on the substrate and enzyme, as long as the fluorophore is in close proximity to at least one of the quenchers prior to cleavage. By close proximity, it is meant that they are situated such that the quencher is able to function (*i.e.*, where efficiency of energy transfer between the quencher and the fluorophore is 50% or more), and preferably are less than a distance of 20 nucleic acid bases or 70 angstroms. For example, a fluorophore may be linked to one end of the substrate in the substrate-enzyme duplex, while a quencher is linked to the opposite end of the substrate and a second quencher is linked to the end of the enzyme which hybridizes with the fluorophore labeled end of the substrate. (Fig. 13A) This configuration provides the advantage of continually

keeping the fluorophore, in the absence of cleavage, proximal to a quencher, regardless of hybridization of the substrate-enzyme duplex, thereby eliminating nearly all background fluorescence signals. In the presence of the target ion the substrate is cleaved and the product disassociates from the enzyme. Dissociation of the product  
5 removes the fluorophore from the vicinity of the quenchers, leading to an increase in fluorescence (FIG. 8).

It should be appreciated that the design of the present invention relies on the polymer end-to-end distance distribution. Therefore it may not be general for long strand polymers. However, in such long strand polymers, the quencher may be placed  
10 in the middle of the polymer or any other appropriate position, thereby eliminating the problem of being too distant.

It should also be appreciated that FRET can be used for sensing, detecting, identifying or quantifying a target ion in the present invention by using a fluorescent quencher instead of a non-fluorescent quencher.

In light of the present disclosure, one of ordinary skill in the art would know how to modify the nucleic acid biosensors to include nucleic acid enzymes. For example, a biosensor of the present invention may comprise a nucleic acid enzyme labeled with a fluorescent quencher, a substrate labeled with a fluorophore and a second fluorescent quencher, and a device to detect fluorescence such as a  
15 fluorescence microscope or a fluorometer. In a method using this embodiment, the enzyme and substrate are contacted with a sample suspected of containing an ion to which the enzyme is sensitive. Fluorescence is measured and compared to a control wherein the ion is absent. Change in fluorescence is indicative of the presence of the ion.  
20

Of course, many variants of even this simple embodiment are included within the scope of the invention. Such variants include placing the enzyme, substrate, and sample in the well of a microtiter plate and measuring fluorescence with a microtiter plate reader. In another variation, the enzyme is attached to a solid support. When the enzyme is attached to a solid support, it is preferable that a linker is used. An  
25 exemplary linking system is biotin/streptavidin. For example, the biotin molecule may be linked to the enzyme and a plate may be coated with streptavidin. When  
30

linking an enzyme to a solid support, it is important to determine the effect of linkage on the enzymatic activity of the enzyme.

In an alternative embodiment, the solid support may be a bead and fluorescence measured using a flow cytometer. In embodiments having the enzyme attached to a solid support, the biosensor may be reusable. Old substrate and sample  
5 is removed, leaving the enzyme in place. New substrate and sample may then be added.

In another embodiment, the nucleic acid enzyme may be used in conjunction with fiber-optics (Lee & Walt, 2000). The nucleic acid enzyme may be immobilized  
10 on the surface of silica microspheres and distributed in microwells on the distal tip of an imaging fiber. The imaging fiber may then be coupled to an epifluorescence microscope system.

In certain embodiments, the biosensor will comprise an array of nucleic acid enzymes. The arrays of the present invention provide for the simultaneous screening  
15 of a variety of ions by nucleic acid enzymes. The array may contain as little as 2 or as many as 10,000 different nucleic acid enzymes. Of course, any integer in between may be used. Preferably, each individual nucleic acid enzyme has a measurable difference in specificity or affinity for at least one ion compared to at least one other nucleic acid enzyme within the array.

In preferred embodiments, the array is a high-density array like those used in  
20 DNA-chip technologies. Methods of forming high density arrays of nucleic acids with a minimal number of synthetic steps are known (U.S. Pat. No. 6,040,138). The nucleic acid array can be synthesized on a solid support by a variety of methods, including light-directed chemical coupling, and mechanically directed coupling (U.S.  
25 Pat. No. 5,143,854; WO 90/15070; WO 92/10092; WO 93/09668). Using this approach, one heterogenous array of polymers is converted, through simultaneous coupling at a number of reaction sites, into a different heterogenous array.

The light-directed combinatorial synthesis of nucleic acid arrays on a glass  
30 surface uses automated phosphoramidite chemistry and chip masking techniques. In one specific implementation, a glass surface is derivatized with a silane reagent containing a functional group, e.g., a hydroxyl or amine group blocked by a photolabile protecting group. Photolysis through a photolithographic mask is used

selectively to expose functional groups which are then ready to react with incoming 5'-photoprotected nucleoside phosphoramidites. The phosphoramidites react only with those sites which are illuminated (and thus exposed by removal of the photolabile blocking group). Thus, the phosphoramidites only add to those areas  
5 selectively exposed from the preceding step. These steps are repeated until the desired array of sequences have been synthesized on the solid surface. Combinatorial synthesis of different nucleic acid analogues at different locations on the array is determined by the pattern of illumination during synthesis and the order of addition of coupling reagents.

10 In the event that a PNA is used in the procedure, it is generally inappropriate to use phosphoramidite chemistry to perform the synthetic steps, since the monomers do not attach to one another via a phosphate linkage. Instead, peptide synthetic methods are substituted (U.S. Pat. No. 5,143,854).

15 In addition to the foregoing, additional methods which can be used to generate an array of nucleic acids on a single solid support are known (For example, WO 93/09668). In these methods, reagents are delivered to the solid support by either (1) flowing within a channel defined on predefined regions or (2) "spotting" on predefined regions. However, other approaches, as well as combinations of spotting and flowing, may be employed. In each instance, certain activated regions of the  
20 solid support are mechanically separated from other regions when the monomer solutions are delivered to the various reaction sites.

25 A typical "flow channel" method applied to the nucleic acid enzyme arrays of the present invention can generally be described as follows. Diverse nucleic acid sequences are synthesized at selected regions of a solid support by forming flow channels on a surface of the solid support through which appropriate reagents flow or in which appropriate reagents are placed. For example, assume a monomer "A" is to be bound to the solid support in a first group of selected regions. If necessary, all or part of the surface of the solid support in all or a part of the selected regions is activated for binding by, for example, flowing appropriate reagents through all or  
30 some of the channels, or by washing the entire solid support with appropriate reagents. After placement of a channel block on the surface of the solid support, a reagent having the monomer A flows through or is placed in all or some of the



channel(s). The channels provide fluid contact to the first selected regions, thereby binding the monomer A on the solid support directly or indirectly (via a spacer) in the first selected regions.

5           Thereafter, a monomer B is coupled to second selected regions, some of which may be included among the first selected regions. The second selected regions will be in fluid contact with a second flow channel(s) through translation, rotation, or replacement of the channel block on the surface of the solid support; through opening or closing a selected valve; or through deposition of a layer of chemical or photoresist. If necessary, a step is performed for activating at least the second  
10           regions. Thereafter, the monomer B is flowed through or placed in the second flow channel(s), binding monomer B at the second selected locations. In this particular example, the resulting sequences bound to the solid support at this stage of processing will be, for example, A, B, and AB. The process is repeated to form a vast array of nucleic acid enzymes of desired length and sequence at known locations on the solid  
15           support.

          After the solid support is activated, monomer A can be flowed through some of the channels, monomer B can be flowed through other channels, a monomer C can be flowed through still other channels, etc. In this manner, many or all of the reaction regions are reacted with a monomer before the channel block must be moved or the  
20           solid support must be washed and/or reactivated. By making use of many or all of the available reaction regions simultaneously, the number of washing and activation steps can be minimized.

          There are alternative methods of forming channels or otherwise protecting a portion of the surface of the solid support. For example, according to some  
25           embodiments, a protective coating such as a hydrophilic or hydrophobic coating (depending upon the nature of the solvent) is utilized over portions of the solid support to be protected, sometimes in combination with materials that facilitate wetting by the reactant solution in other regions. In this manner, the flowing solutions are further prevented from passing outside of their designated flow paths.

30           The "spotting" methods of preparing nucleic acid arrays can be implemented in much the same manner as the flow channel methods. For example, a monomer A can be delivered to and coupled with a first group of reaction regions which have been

appropriately activated. Thereafter, a monomer B can be delivered to and reacted with a second group of activated reaction regions. Unlike the flow channel embodiments described above, reactants are delivered by directly depositing (rather than flowing) relatively small quantities of them in selected regions. In some steps, of course, the entire solid support surface can be sprayed or otherwise coated with a solution. In preferred embodiments, a dispenser moves from region to region, depositing only as much monomer as necessary at each stop. Typical dispensers include a micropipette to deliver the monomer solution to the solid support and a robotic system to control the position of the micropipette with respect to the solid support. In other embodiments, the dispenser includes a series of tubes, a manifold, an array of pipettes, or the like so that various reagents can be delivered to the reaction regions simultaneously.

The biosensors of the array may be selective for a single type of ion or each biosensor may be selective for a different type of ion. The substrates for the nucleic acid enzymes of the array may be labeled with a single fluorophore or with different fluorophores. For example, a biosensor, selective for the presence of  $Pb^{2+}$ , may be designed to emit a certain fluorescence, such as FAM, in the presence of  $Pb^{2+}$ . An array may be covered with this biosensor. Another example would include an array comprising several biosensors, where one is selective for the presence  $Zn^{2+}$ , another is selective for the presence of  $Pb^{2+}$ , and a third biosensor is selective for the presence of  $Co^{2+}$ . Each of these three biosensors of the array may be designed to emit a single type of fluorescence, such as FAM, in the presence of each respective specific ion or each of these three biosensors may be designed to emit a different type of fluorescence in the presence of each respective specific ion. Thus depending on design of the biosensor, the array may: (1) generally report a single product, indicating the presence or concentration of a single specific ion type; (2) generally report a single product, indicating the presence of numerous different specific ion types; or (3) specifically report different products, indicating the presence of numerous different specific ion types.

Methods of detecting fluorescent signals on a DNA chip are well known to those of skill in the art. In a preferred embodiment, the nucleic acid enzyme array is excited with a light source at the excitation wavelength of the particular fluorescent

label and the resulting fluorescence at the emission wavelength is detected. In a particularly preferred embodiment, the excitation light source is a laser appropriate for the excitation of the fluorescent label.

5 A confocal microscope may be automated with a computer-controlled stage to automatically scan the entire high density array. Similarly, the microscope may be equipped with a phototransducer (e.g., a photomultiplier, a solid state array, a CCD camera, etc.) attached to an automated data acquisition system to automatically record the fluorescence signal produced by each nucleic acid enzyme on the array. Such automated systems are described at length in U.S. Pat. No: 5,143,854 and PCT  
10 application 20 92/10092.

## EXAMPLES

The following examples are included to demonstrate embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors  
15 to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain like or similar results without departing from the spirit and scope of the invention.

### Example 1 – *In vitro* selection of a ion-dependent deoxyribozyme

This example demonstrates a method of creating nucleic acid enzymes that are dependent on the presence of an ion for activity. More specifically, use of a partially  
25 random DNA library to obtain deoxyribozymes that cleave RNA in the presence of  $Zn^{2+}$  or  $Co^{2+}$  is shown.

#### Materials and Methods used in this Example

##### Oligonucleotides

DNA oligonucleotides were purchased from Integrated DNA Technologies Inc. Sequences of the random DNA template and the primers (P1, P2 and P3) used in  
30 PCR amplifications are listed below:

*P1*: 5'-GTGCCAAGCTTACCG-3' (SEQ ID NO:3)

**P2:** 5'-CTGCAGAATTCTAATACGACTCACTATAGGAAGAGATGGCGAC-3'

(SEQ ID NO:4)

**P3:** 5'-GGGACGAATTCTAATACGACTCACTATrA-3' (SEQ ID NO:5)

**Template for random DNA pool:**

5 5'-GTGCCAAGCTTACCGTCAC-N40-GAGATCTCGCCATCTCTTCCT  
ATAGTGAGTCGTATTAG-3' (SEQ ID NO:6)

Primer P1b and P3b are the 5'-biotinylated version of primers P1 and P3.

Primer P1a and P3a were prepared by 5'-labeling P1 and P3 with [ $\alpha$ - $^{32}$ P] ATP

(Amersham) and T4 polynucleotide kinase (Gibco). The DNA/RNA chimeric

10 substrate (17DS) for *trans*-cleavage assays has the sequence 5'-

ACTCACTATrAGGAAGAGATG-3' (SEQ ID NO:2), where rA denotes a single

ribonucleotide. The all-RNA substrate (17RS) with the same sequence was purchased

from Dharmacon Research Inc. The *trans*-cleaving deoxyribozyme 17E has the

sequence 5'-CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT-3' (SEQ ID

15 NO:1). The deoxyribozyme named 17E1 is a variant of 17E with the sequence 5'-

CATCTCTTTTGTGTCAGCGACTCGAAATAGTGA GT-3' (SEQ ID NO:7). All

oligonucleotides were purified using denaturing polyacrylamide gel electrophoresis

and desalted with the SepPak nucleic acid purification cartridges (Waters) before use.

#### Preparation of Random DNA Pool

20 The initial pool for DNA selection was prepared by template-directed extension

followed by PCR amplification. The extension was carried out with 200 pmol of

DNA template containing a 40-nucleotide random sequence region, and 400 pmol of

primer P3b in 20 $\times$  100  $\mu$ l reaction mixtures for four thermal-cycles (1 min at 92°C, 1

min at 52°C, and 1 min at 72°C). Reaction buffer also included 0.05 U/ $\mu$ l *Taq*

25 polymerase (Gibco), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl (pH 8.3 at 25°C),

0.01% gelatin and 0.2 mM of each dNTP. Subsequently, 1 nmol each of P1 and P3b

were added to the extension product to allow four more cycles of PCR amplification.

The products were precipitated with ethanol and dissolved in 0.5 ml of buffer A,

which contains 50 mM HEPES (pH 7.0), 500 mM (for Zn-DNA selection) or 1 M (for

30 Co-DNA selection) NaCl. About 20  $\mu$ M EDTA was also added to the buffer to

chelate trace amount of divalent metal ion contaminants.

### In Vitro Selection

The random DNA pool was immobilized on a NeutrAvidin column (Pierce) by incubating with the column materials for 30 minutes. The mixture was gently vortex-mixed a few times during the incubation. The unbound DNA strands were eluted with at least  $5 \times 100 \mu\text{l}$  of buffer A. The non-biotinylated strands of immobilized DNA were washed off the column with  $5 \times 100 \mu\text{l}$  of freshly prepared 0.2 M NaOH and  $20 \mu\text{M}$  EDTA. The column was then neutralized with  $5 \times 100 \mu\text{l}$  of buffer A. The cleavage reaction was carried out by incubating the immobilized single-stranded DNA containing the single ribonucleotide (rA) with  $3 \times 20 \mu\text{l}$  of reaction buffer (buffer A plus 1 mM  $\text{ZnCl}_2$  or  $\text{CoCl}_2$ ) over 1h. The eluted DNA molecules were pooled and precipitated with ethanol. A fraction of the selected DNA was amplified in 100  $\mu\text{l}$  PCR reaction with 40 pmol each of primers P1 and P2 over 10-20 thermal cycles. One tenth of the PCR product was further amplified for six cycles with 50 pmol of primers P1 and P3b. The final PCR product was ethanol precipitated and used to initiate the next round of selection. During the selection of Zn(II)-dependent deoxyribozymes (called Zn-DNA hereafter), the concentration of  $\text{ZnCl}_2$  was kept constant at  $100 \mu\text{M}$  in the reaction buffer for the following rounds of selection. Reaction time was gradually decreased from 1 h to 30 s within 12 rounds of selection. For the selection of Co(II)-dependent deoxyribozymes (called Co-DNA hereafter), the concentration of  $\text{CoCl}_2$  was gradually decreased from 1 mM to  $100 \mu\text{M}$  and the reaction time from 1 h to 1 min within 10 rounds of selection. The twelfth generation of selectively amplified Zn-DNA and the tenth generation of Co-DNA were cloned using TA-TOPO Cloning Kit (Invitrogen) and sequenced with T7 Sequenase 2.0 Quick-denatured Plasmid Sequencing Kit (Amersham).

### Reselection

Based on the sequence of class I Zn-DNA or Co-DNA, partially degenerate DNA template libraries for reselection were synthesized (Integrated DNA Technology Inc.) with 20% degeneracy at the N40 region. In other words, during the oligonucleotide synthesis of the N40 region, the wild type sequence was introduced at a probability of 80% at each position, while the other three nucleotides each occurred at a probability of 6.67%. The reselection pool was prepared with 10 pmol of template and 100 pmol of primers P1 and P3b using the same protocol previously

described. With 10 pmol (number of molecules  $S = 6 \times 10^{12}$ ) of partially randomized template, the statistic parameters of the DNA library used for reselection were calculated based on the following equations.

$$P(k,n,d) = [n!/(n-k)!k!]d^k(1-d)^{n-k} \quad (1)$$

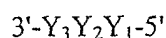
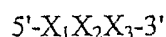
$$N(k) = [n!/(n-k)!k!]3^k \quad (2)$$

$$C(n,k) = SP(k,n,d)/N(k) \quad (3)$$

$P(k,n,d)$  is the probability of having  $k$  mutations within  $n$  (number of randomized positions,  $n = 40$ ) nucleotide positions that have been randomized at a degeneracy of  $d$ .  $N(k)$  is the number of distinct sequences that have  $k$  mutations with respect to the prototype sequence.  $C(n,k)$  is the number of copies for each sequence that has  $k$  mutations. The reselection pool was expected to contain the wild type sequence, all possible sequences with 1-8 point mutations, and a sampling of the sequences with  $>8$  point mutations. More than half of the population contains  $\geq 8$  point-mutations. The protocol for reselection was the same as the primary selection, except that the reaction time was decreased from 20 min to 1 min and the concentration of  $ZnCl_2$  or  $CoCl_2$  was decreased from 20  $\mu M$  to 5  $\mu M$  over six generations. The sixth generation of reselected Zn- or Co-DNA were cloned and sequenced as previously described.

#### Kinetic Assays of the Reselected *Cis*-cleaving DNA

The 5'  $^{32}P$ -labeled precursor DNA for *cis*-cleavage assay was prepared by PCR-amplification of the selected DNA population or the cloned DNA plasmid with primer 1b and 3a. The double-stranded product was immobilized on a NeutrAvidin column through the biotin moiety on primer P1b. The catalytic strand of DNA was eluted off the column with  $3 \times 20 \mu l$  freshly prepared 0.2 N NaOH and neutralized with 8  $\mu l$  of 3 M sodium acetate (pH 5.3) in the presence of 50  $\mu g/ml$  bovine serum albumin (Sigma). Following ethanol precipitation, the single-stranded DNA was purified on an 8% denaturing polyacrylamide gel and desalted with SepPak nucleic acid purification cartridge. Bovine serum albumin (50  $\mu g/ml$ ) was added to the gel-soaking buffer (0.2 M NaCl, 20  $\mu M$  EDTA, 10 mM Tris-HCl, pH 7.5) to prevent the DNA from adhering to the tube. The concentration of the DNA was determined by scintillation counting the radioactivity.



The secondary structures of the sequenced Zn-DNA were predicted using Zuker's *DNA mfold* program (see <http://mfold.wustl.edu/~folder/dna/form1.cgi>) through minimization of folding energy. The most stable structures predicted for those containing Region-20nt all contained a similar structure motif. This common motif consists of a pistol-shaped three-way helical junction formed by a 3 bp hairpin, an 8 bp hairpin and a double helix linking to the rest of the molecule. The 3 bp hairpin and its adjacent single-stranded regions are part of the Region-20nt. The ribonucleic adenosine is unpaired and positioned opposite of the 3 bp hairpin.

After reselection, twenty-eight Zn-DNA clones were sequenced (FIG. 4). When compared with the parental wild type sequence (class I Zn-DNA), the reselected Zn-DNA contained point mutations mostly outside of Region-20nt. About one third of these sequences have a T → A mutation at position 73, converting the T-T mismatch in the wild type sequence to a Watson-Crick base pair. In one fourth of the reselected DNAs, the 5 nucleotide single-stranded bulge of the three-way junction has the sequence 5'-ACGAA-3', corresponding to 5'-TCGAA-3' in the wild type. Clone #17 (named ZnR17) of the reselected Zn-DNA is most active under selection conditions (FIG. 4). Structural analysis of ZnR17 revealed two completed base-paired helices in the three-way junction. Therefore, it was engineered into a *trans*-cleaving deoxyribozyme by deleting the sequences outside of the three-way junction and the loop of the 8 bp hairpin. Such truncation resulted in two individual stands, which hybridize with each other through two 9-10 bp helices. The strand containing the single ribonucleotide residue (rA) is considered as the substrate (named 17DS), while the other strand as the enzyme (named 17E). The catalytic core, which was highly conserved during selection, consists of a 3 bp hairpin and a 5 nt single-stranded bulge (FIG. 5).

Although ZnR17 was selected in Zn<sup>2+</sup>, it does not contain structure motifs that were discovered in several Zn(II)-binding RNA molecules (Ciesiolka *et al.*, 1995; Ciesiolka & Yarus, 1996). However, the conserved region of ZnR17 is very similar to that of the 8-17 deoxyribozymes selected by Santoro and Joyce using Mg<sup>2+</sup> as cofactor (Santoro & Joyce, 1997). The unpaired bulge region in the 8-17 DNA

Both substrates are covalently linked at the 5' end with 6-carboxyfluorescein (FAM) through NHS-ester conjugation and at the 3' end with DABCYL via CPG phosphoramidite. The deoxyribozyme (17E-Dy) is labeled at the 3'-end with DABCYL via CPG phosphoramidite and has the sequence 5'-  
5 CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGT-3' (SEQ ID NO:1). All the oligonucleotides were purified by denaturing 20% polyacrylamide gel electrophoresis to ensure 100% labeling with the fluorescent dyes.

#### Fluorescence Spectroscopy

10 The enzyme-substrate complex was prepared with 50 nM each of 17E-Dy and Rh-17DS-FD in 50 mM NaCl and 50 mM Tris acetate (TA) buffer (pH 7.2) with a volume of 600  $\mu$ l. The sample was heated at 80°C for 5 min and cooled to 4°C slowly to anneal the enzyme and substrate strands together. Fluorescence signal was collected by a FLA-3000G Multi-purpose 3-laser scanner for Fluorescence,  
15 Radioactivity and Macro Arrays (Fuji). The excitation laser wavelength was set at 473 nm and the filter was set at 520 nm to monitor the fluorescence of fluorescein. Steady state and slow-kinetic fluorescence were collected using a SLM 8000S photon counting fluorometer at ambient temperature. Excitation wavelength was fixed at 473 nm and emission was scanned from 500 to 650 nm. Polarization artifacts were  
20 avoided by using "magic angle" conditions. The steady-state emission spectra were collected from 460 to 500 nm ( $\lambda_{\text{ex}} = 473$  nm). The time-dependent DNA enzyme catalyzed substrate cleavage was monitored at 473 nm at 2 s intervals. To initiate the reaction, 1 - 2  $\mu$ l of concentrated divalent metal ion solution was injected into the cuvette using a 10  $\mu$ l syringe while the DNA sample in the cuvette was constantly  
25 stirred.

#### DNA-based sensor of metal ions

An *in vitro* selected DNA enzyme from Example 1 (termed 17E) that is capable of cleaving a lone RNA linkage within a DNA substrate (termed 17DS-FD) (FIG. 13A) was chosen for use as a DNA-based, fluorescent biosensor of metal ions.  
30 Assays of this enzyme indicate a highly  $\text{Pb}^{2+}$  dependent activity with  $k_{\text{obs}} = 6.5 \text{ min}^{-1}$  at pH 6.0 and  $K_{\text{apparent}} = 13.5 \text{ }\mu\text{M}$ . The biosensor was constructed by dual labeling the 5'-end of the substrate with the fluorophore 6-carboxyfluorescein (FAM) and the 3'-



end of the enzyme strand with DABCYL. This dual labeled substrate is named 17DS-FD. The 3'-end of the enzyme (17E) is also labeled with DABCYL. DABCYL serves as a universal fluorescence quencher. Steady-state fluorescence spectra were obtained by exciting the sample at 473 nm and scanning its emission from 500 to 650 nm.

When the substrate (17DS-FD) was hybridized to the enzyme strand (17E-Dy), the fluorescence of FAM was further quenched by the nearby additional DABCYL (FIG. 13B). Upon addition of  $Pb^{2+}$ , this quenching was eliminated and the fluorescence of FAM increased by ~660% over background fluorescence signals. Little change in the fluorescence signal occurred with addition of  $Pb^{2+}$  to the substrate alone or to the complex of the enzyme and a non-cleavable DNA substrate with identical sequence. These findings show that the change in fluorescent signal with 17DS-FD/17E-Dy results from a DNA enzyme-catalyzed substrate cleavage, followed by product release.

The substrate cleavage reaction was monitored in real time with fluorescence spectroscopy. Like the ratiometric, anisotropy, or lifetime-based method, kinetic fluorescence measurement is independent of sampling conditions such as illumination intensity and sample thickness (Oehme & Wolfbeis, 1997). In order to determine the selectivity of the catalytic DNA sensor, a fluorescence image reader (Fuji) was used for real time monitoring of the cleavage reaction and product release using 7 different divalent metal ions. The activity of  $Co^{2+}$ ,  $Mg^{2+}$ ,  $Zn^{2+}$ ,  $Mn^{2+}$ ,  $Cd^{2+}$  and  $Ni^{2+}$  in cleaving the substrate strand was compared with  $Pb^{2+}$  cleavage activity. These metal ions were chosen for comparison because in previous assays they demonstrated relatively high cleavage rates of 17E, while other metal ions were almost unreactive. The excitation laser wavelength of the fluorescence image reader was set to 473 nm and an emission filter was used to cut the wavelength to shorter than 520 nm. A 96-well plate was used as a reaction container. The first well of each row was set as an internal standard to quantify intensity and compare different scans. Therefore 5  $\mu$ L of water were pipetted into the first well and 5  $\mu$ L of the appropriate divalent metal ion were pipetted into the remaining wells.

Many different methods, such as comparing the cleavage rate constant or comparing initial reaction rate, may be used to assay the cleavage activity of the metal

ions. One easy, practical way to monitor the reaction is by looking at the fluorescence intensity at a specific time interval, which conveniently eliminates the need for complicated data processing. Using this method, it was found that the fluorescence intensity after a 2 minute interval, showed high selectivity of the biosensor for  $\text{Pb}^{2+}$ .  
5 This selectivity is shown in Figure 9A. Four different concentrations of metal ions were monitored using this method and in each case,  $\text{Pb}^{2+}$  gave the highest fluorescence increase, indicating the fastest cleavage. To present data in a quantitative way, the darkness of each well was quantified and plotted in Figure 9B. Besides  $\text{Pb}^{2+}$ , only  $\text{Zn}^{2+}$  and  $\text{Co}^{2+}$  showed any fluorescence increase at the 5  $\mu\text{M}$  level.

10 Cleavage kinetics may be fitted into an exponential increase to a maximum wherein the initial stage of cleavage is considered linear. When comparing the relative fluorescence increases, the time interval does not have to be 2 minutes; any quantity of time in the linear range is suitable, and so long as it is kept the same for all the metal ions, the results should be consistent. Using this method, the present  
15 biosensor shows very high selectivity. For example, at low metal ion concentrations (500 nM),  $\text{Pb}^{2+}$  is the only metal ion which causes the biosensor to produce a fluorescent signal. Figure 9C shows the cleavage kinetics for all seven metal ions in a time course of 90 minutes.  $\text{Pb}^{2+}$  was the only ion to produce a fluorescence signal; all other metal ions produced signals similar to the background, demonstrating that the  
20 signal response to  $\text{Pb}^{2+}$  was not affected by the presence of equal amounts of other ions, indicating that this biosensor is well suited for selective monitoring of  $\text{Pb}^{2+}$  in the presence of other metal ions.

The  $\text{Pb}^{2+}$  detection range is from 100 nM to 5  $\mu\text{M}$ , if the fluorescence increase after 2 minutes of reaction is counted. However, when lead concentration is higher  
25 than 5  $\mu\text{M}$ , the signal is saturated. To avoid saturation of the signal, dilution must be done on more concentrated  $\text{Pb}^{2+}$  samples before an accurate concentration can be derived. Respectively,  $\text{Zn}^{2+}$  and  $\text{Co}^{2+}$  showed the second and third highest signal response, and are considered interference ions. However, when the concentration is below 5  $\mu\text{M}$ , the biosensor has almost no response to them.

30  
**Fluorescence of the new biosensor versus fluorescence of the biosensor disclosed in U.S. Application Serial No. 09/605,558**

When the temperature is increased from 4°C to room temperature (23°C), the biosensor of U.S. Application Serial No. 09/605,558 shows a significant decrease in fluorescence signal (from 400% signal to background ratio to 60% signal to background ratio), due to the partial “melting” of the substrate-enzyme duplex.

5 Therefore when utilizing these biosensors, it is important to know the melting profile of the enzyme-substrate duplex. Two methods may be used to determine the melting temperature of the duplex. One method is based on the hyperchromatic property of DNA. By monitoring the absorption at 260 nm with increases of temperature, the melting temperature can be obtained. Using this method, the melting temperature of  
10 17DS-FD / 17E-DY duplex was determined to be 35°C.

A second method for determining melting temperatures takes advantage of the fluorescent properties of the 17DS-FD / 17E-DY duplex. The substrate strand used is non-cleavable FI-17DDS (17DDS with a FAM attached to the 5' end), and the enzyme strand is 17E-DY (17E with DABCYL attached to the 3' end). When the two  
15 strands are annealed, the fluorescence from FAM is quenched by DABCYL. The fluorescence is recovered when the duplex melts. By monitoring the FAM fluorescence at 520 nm, the melting curve of the DNA can be acquired. The melting temperature determined by this method is 34°C. The results from the two different methods are similar, indicating that the coupling of the fluorophore to the DNA does  
20 not change the stability of the duplex.

### **Example 3 – DNA chip comprising an array of nucleic acid enzymes**

This prophetic example describes the production of and use of a DNA chip for sensing ions, in particular heavy metal ions.

25 The first step towards the application of deoxyribozymes in heavy metal sensing is to obtain various deoxyribozymes with different metal specificity and affinity. *In vitro* selection will be carried out to isolate a variety of deoxyribozymes. A detailed description of the selection protocol can be found in Example 1. Each family of deoxyribozyme will be specific for different divalent metal ions (e.g. Pb<sup>2+</sup>,  
30 Hg<sup>2+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, Cd<sup>2+</sup>, Ni<sup>2+</sup>, Mn<sup>2+</sup>, etc). Within each family, different sequences will have different affinities of the specified metal ion.

5           These deoxyribozymes and their substrates will then be arrayed onto a DNA chip with one dimension for metal ion specificity and the other for affinity of the corresponding metal (FIG. 11). The enzyme strands immobilized on the chip at 3'-ends can be synthesized on the chip using photolithographic methods (Fodor *et al.*, 1991; Pease *et al.*, 1994) or can be synthesized off-chip and then attached to the chip using various methods (Joos *et al.*, 1997; O'Donnell-Maloney *et al.*, 1997; Guschin *et al.*, 1997). The 3'-ends of the enzyme and substrate strands will be labeled with a fluorescence quencher, which can be a fluorescent or non-fluorescent moiety. The 5' end of the substrate will be labeled with a fluorophore. Guanidine base may be used, for example, as an efficient quencher of fluorescein.

10           Hybridization of the enzyme and substrate will result in the quenching of the donor fluorescence. Upon exposure to the sample containing the active metal ion, the substrate will be cleaved and products will dissociate, resulting in strong fluorescence of the dye attached to the enzyme strand. The metal ion species can be qualitatively identified based on the metal specificity of different families of deoxyribozymes. A hypothetical sample result is shown in FIG. 11B. The pattern of fluorescence intensity shows that there are three kinds of metal (M1, M4, and M6) in the sample.

15           The concentration of the metal ion under inspection can be quantified with deoxyribozymes with different metal affinity. Given a certain concentration of the metal ion, different sequences within the same family will have different cleavage efficiencies due to their different thresholds in response to the metal concentration. The metal concentration applied may exceed the saturation concentration of those having higher affinity; therefore full cleavage will occur within a certain time and present strong fluorescence. On the other hand, the substrates of those with lower affinity will only be partly cleaved and emit weaker fluorescence. The sample hypothetical result shown in FIG. 11B shows high (c1), medium (c4), and low (c6) concentrations of M1, M4, and M6, respectively.

20           The fluorescence patterns with respect to different deoxyribozyme sequences will be compared with standard calibration maps. After de-convolution of the fluorescence pattern, direct information can be obtained about the identity and concentration of metal ions in the samples.

25

30

## REFERENCES

- Bogden, J.D.; Louria, D.B. *Bull. Environ. Contam. Toxicol.* **1975**, *14*:289-94.
- Breaker, R. R.; Joyce, G. F. *Chem. Biol.* **1995**, *2*, 655-660.
- Breaker, R.R. & Joyce, G.F. A DNA enzyme that cleaves RNA. *Chem. Biol.* **1**, 223-  
5 229 (1994).
- Breaker, R.R. DNA enzymes. *Nat. Biotechnol.* **15**, 427-431 (1997).
- Cadwell, R. C.; Joyce, G. F. *PCR Methods Appl.* **1992**, *2*, 28-33.
- Cadwell, R. C.; Joyce, G. F. *PCR Methods Appl.* **1994**, *3*, S136-S140.
- Carmi, N., Shultz, L.A. & Breaker, R.R. In vitro selection of self-cleaving DNAs.  
10 *Chem. Biol.* **3**, 1039-1046 (1996).
- Chapman, K. B.; Szostak, J. W. *Curr. Opin. Struct. Biol.* **1994**, *4*, 618-622.
- Ciesiolka, J.; Gorski, J.; Yarus, M. *RNA* **1995**, *1*, 538-550.
- Ciesiolka, J.; Yarus, M. *RNA* **1996**, *2*, 785-793.
- Conaty, J.; Hendry, P.; Lockett, T. *Nucleic Acids Res.* **1999**, *27*, 2400-2407.
- 15 Conn, M. M.; Prudent, J. R.; Schultz, P. G. *J. Am. Chem. Soc.* **1996**, *118*, 7012-7013.
- Cuenoud, B. & Szostak, J.W. A DNA metalloenzyme with DNA ligase activity.  
*Nature* **375**, 611-614 (1995).
- Czarnik, A.W. Desperately seeking sensors. *Chem. Biol.* **2**, 423-428 (1995).
- Dai, X.; De Mesmaeker, A.; Joyce, G. F. *Science* **1995**, *267*, 237-240.
- 20 Deo, S. & Godwin, H.A. A Selective, Ratiometric Fluorescent Sensor for Pb<sup>2+</sup>. **2000**,  
*J. Am. Chem. Soc.* **122**, 174-175.
- Didenko, V.V. *BioTechniques* **2001**, *31*:1106-21.
- Earnshaw & Gait, "Modified Oligoribonucleotides as site-specific probes of RNA  
structure and function," *Biopolymers* (John Wiley & Sons, Inc.) **48**:39-55,  
25 1998.
- Ekland, E. H.; Szostak, J. W.; Bartel, D. P. *Science* **1995**, *269*, 364-370.
- Ekland, E. H.; Bartel, D. P. *Nature* **1996**, *382*, 373-376.
- Famulok, M. *Curr. Opin. Struct. Biol.* **1999**, *9*, 324-329.
- Faulhammer, D.; Famulok, M. *Angew. Chem., Int. Ed. Engl.* **1997**, *35*, 2837-2841.
- 30 Fodor, S.P.A., Read, J.L., Pirrung, M.C., Stryer, L., Lu, A.T. & Solas, D. (1991).  
Light-directed, spatially addressable parallel chemical synthesis. *Science* **251**:  
767-773.

- Frank, D. N.; Pace, N. R. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 14355-14360.
- Geyer, C. R.; Sen, D. *Chem. Biol.* **1997**, *4*, 579-593.
- Godwin, H.A. & Berg, J.M. A Fluorescent Zinc Probe Based on Metal-Induced Peptide Folding. *J. Am. Chem. Soc.* **118**, 6514-6515 (1996).
- Guschin, D., Yershov, G., Zaslavsky, A., Gemmell, A., Shick, V., Proudnikov, D., Arenkov, P. & Mirzabekov, A. (1997). Manual manufacturing of oligonucleotide, DNA, and protein microchips. *Anal. Biochem.* **250**: 203-211.
- Hennrich, G.; Sonnenschein, H.; Resch-Genger, U. *J. Am. Chem. Soc.* **1999**, *121*, 5073-5074.
- Illangasekare, M.; Yarus, M. *J. Mol. Biol.* **1997**, *268*, 631-639.
- Imperiali, B., Pearce, D.A., Sohna, J.-E., Walkup, G. & Torrado, A. Peptide platforms for metal ion sensing. *Proc. SPIE-Int. Soc. Opt. Eng.* **3858**, 135-143 (1999).
- Jhaveri, *et al.*, Designed Signaling Aptamers that Transduce Molecular Recognition to Changes in Fluorescence Intensity, *Journal of the American Chemical Society*; **2000**; *122*(11); 2469-2473.
- Joos, B., Kuster, H. & Cone, R. (1997). Covalent attachment of hybridizable oligonucleotides to glass supports. *Anal. Biochem.* **247**: 96-101.
- Joyce, G. F. *Curr. Opin. Struct. Biol.* **1994**, *4*, 331-336.
- Koizumi, M.; Soukup, G. A.; Kerr, J. N. Q.; Breaker, R. R. *Nat. Struct. Biol.* **1999**, *6*, 1062-1071.
- Lakowicz, J. R. In *Principles of Fluorescence Spectroscopy*; 2nd ed.; Kluwer Academic/Plenum: New York, 1999.
- Lee, M., & Walt, D. R. A fiber-optic microarray biosensor using aptamers as receptors. *Anal Biochem* **282**(1):142-146, 2000.
- Lehman, N.; Joyce, G. F. *Nature* **1993**, *361*, 182-185.
- Li, J., Zheng, W., Kwon, A.H. & Lu, Y. In vitro selection and characterization of a highly efficient Zn(II)-dependent RNA-cleaving deoxyribozyme. *Nucleic Acids Res.* **28**, 481-488 (2000).
- Li, Y.; Sen, D. *Nat. Struct. Biol.* **1996**, *3*, 743-747.
- Li, Y.; Breaker, R. R. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96*, 2746-2751.
- Li, Y.; Liu, Y.; Breaker, R. R. *Biochemistry* **2000**, *39*, 3106-3114.
- Lohse, P. A.; Szostak, J. W. *Nature* **1996**, *381*, 442-444.

- Lorsch, J. R.; Szostak, J. W. *Nature* **1994**, *371*, 31-36.
- Marcus, A.H.; Elias, R.W. *ASTM Spec. Tech. Publ.* **1995**, *STP 1226*: 12-23.
- Miyawaki, A., *et al.* Fluorescent indicators for Ca<sup>2+</sup> based on green fluorescent proteins and calmodulin. *Nature* **388**, 882-887 (1997).
- 5 O'Donnell-Maloney, M.J., Tang, K., Koester, H., Smith, C.L. & Cantor, C.R. (1997). High-Density, Covalent Attachment of DNA to Silicon Wafers for Analysis by MALDI-TOF Mass Spectrometry. *Anal. Chem.* *69*: 2438-2443.
- Oehme, I. & Wolfbeis, O.S. Optical sensors for determination of heavy metal ions. *Mikrochim. Acta* **126**, 177-192 (1997).
- 10 Pan, T. & Uhlenbeck, O.C. A small metalloribozyme with a two-step mechanism. *Nature* **358**, 560-563 (1992).
- Pan, T.; Dichtl, B.; Uhlenbeck, O. C. *Biochemistry* **1994**, *33*, 9561-9565.
- Pearce, D. A.; Walkup, G. K.; Imperiali, B. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1963-1968.
- 15 Pease, A.C., Solas, D., Sullivan, E.J., Cronin, M.T., Holmes, C.P. & Fodor, S.P.A. (1994). Light-generated oligonucleotide arrays for rapid DNA sequence analysis. *Proc. Natl. Acad. Sci. U. S. A.* *91*: 5022-5026.
- Piccirilli, J. A.; McConnell, T. S.; Zaug, A. J.; Noller, H. F.; Cech, T. R. *Science* **1992**, *256*, 1420-1424.
- 20 Pley, H. W.; Flaherty, K. M.; McKay, D. B. *Nature* **1994**, *372*, 68-74.
- Potyrailo, R. A.; Conrad, R. C.; Ellington, A. D.; Hieftje, G. M. *Anal. Chem.* **1998**, *70*, 3419-3425.
- Potyrailo, R.A., Conrad, R.C., Ellington, A.D. & Hieftje, G.M. (1999). Adapting Selected Nucleic Acid Ligands (Aptamers) to Biosensors. *Anal. Chem.* *70*: 3419-3425.
- 25 Prudent, J. R.; Uno, T.; Schultz, P. G. *Science* **1994**, *264*, 1924-1927.
- Rabinowitz, M.; Leviton, A.; Bellinger, D. *Am. Jour. Public Health Field.* **1985**, *Apr* *75*: 403-4.
- Robertson, M. P.; Ellington, A. D. *Nat. Biotechnol.* **1999**, *17*, 62-66.
- 30 Robertson, M. P.; Ellington, A. D. *Nucleic Acids Res.* **2000**, *28*, 1751-1759.
- Roth, A.; Breaker, R. R. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 6027-6031.

- Rurack, K., Kollmannsberger, M., Resch-Genger, U. & Daub, J. A Selective and Sensitive Fluoroionophore for HgII, AgI, and CuII with Virtually Decoupled Fluorophore and Receptor Units. *J. Am. Chem. Soc.* **122**, 968-969 (2000).
- Santoro, S. W.; Joyce, G. F. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94*, 4262-4266.
- 5 Santoro, S.W., Joyce, G.F., Sakthivel, K., Gramatikova, S. & Barbas, C.F., III RNA Cleavage by a DNA Enzyme with Extended Chemical Functionality. *J. Am. Chem. Soc.* **122**, 2433-2439 (2000).
- Schwartz, J.; Levin, R. *Env. Research Field.* **1991**, *Feb 54*: 1-7.
- Scott, W. G.; Finch, J. T.; Klug, A. *Cell* **1995**, *81*, 991-1002.
- 10 Tang and Breaker, *Proc. Natl. Acad. Sci. USA*, **97**, 5784-5789 (2000).
- Tarasow, T. M.; Tarasow, S. L.; Eaton, B. E. *Nature* **1997**, *389*, 54-57.
- Thompson, R.B., Maliwal, B.P., Fellicia, V.L., Fierke, C.A. & McCall, K. Determination of Picomolar Concentrations of Metal Ions Using Fluorescence Anisotropy: Biosensing with a "Reagentless" Enzyme Transducer. *Anal. Chem.* **70**, 4717-4723 (1998).
- 15 Tsang, J.; Joyce, G. F. *Methods Enzymol.* **1996**, *267*, 410-426.
- Tsien, R.Y. Fluorescent and photochemical probes of dynamic biochemical signals inside living cells. in *Fluorescent Chemosensors for Ion and Molecule Recognition* (ed. Czarnik, A.W.) 130-46 (American Chemical Society, Washington, DC, 1993).
- 20 Tuerk, C.; Gold, L. *Science* **1990**, *249*, 505-510.
- Tyagi S.; Kramer, F.R. *Nat. Biotechnol.* **1996** *14*, 303.
- Tyagi, S.; Bratu, D.P.; Kramer, F.R. *Nat. Biotechnol.* **1998**, *16*:49-58.
- Tyagi, S.; Marras, S.A.E.; Kramer, F.R. *Nat. Biotechnol.* **2000**, *18*:1191-6.
- 25 Uphoff, K. W.; Bell, S. D.; Ellington, A. D. *Curr. Opin. Struct. Biol.* **1996**, *6*, 281-288.
- Vaish, N. K.; Heaton, P. A.; Fedorova, O.; Eckstein, F. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 2158-2162.
- Walkup, G.K. & Imperiali, B. Design and Evaluation of a Peptidyl Fluorescent Chemosensor for Divalent Zinc. *J. Am. Chem. Soc.* **118**, 3053-3054 (1996).
- 30 Wecker, M.; Smith, D.; Gold, L. *RNA* **1996**, *2*, 982-994.
- Wiegand, T. W.; Janssen, R. C.; Eaton, B. E. *Chem. Biol.* **1997**, *4*, 675-683.



Wilson, C.; Szostak, J. W. *Nature* **1995**, *374*, 777-782.

Winkler, J.D., Bowen, C.M. & Michelet, V. Photodynamic Fluorescent Metal Ion Sensors with Parts per Billion Sensitivity. *J. Am. Chem. Soc.* **120**, 3237-3242 (1998).

5 Wittmann, C., Riedel, K. & Schmid, R.D. Microbial and Enzyme sensors for environmental monitoring. *Handb. Biosens. Electron. Noses*, 299-332 (1997).

Zhang, B.; Cech, T. R. *Nature* **1997**, *390*, 96-100.

Zillmann, M.; Limauro, S. E.; Goodchild, J. *RNA* **1997**, *3*, 734-747.

## CLAIMS

1. A method of detecting the presence of an ion in the presence of other ions, in a sample, comprising:
- 5 forming a mixture comprising:
- (1) a nucleic acid enzyme,
  - (2) the sample, and
  - (3) a substrate,
- to produce a product from the mixture; and
- 10 determining the presence of the product;
- wherein the enzyme comprises at least one quencher and is dependent on the ion to produce the product from the substrate, and
- the substrate comprises a ribonucleotide, at least one fluorophore and at least one quencher.
- 15
2. The method of claim 1, wherein the nucleic acid enzyme and the substrate comprise separate nucleic acid strands.
3. The method of claim 1, wherein a 5' end of the substrate comprises a first fluorophore and a 3' end of the substrate comprises a first quencher for the fluorophore and wherein a 3' end of the enzyme comprises a second quencher for the fluorophore.
- 20
4. The method of claim 3, wherein the fluorophore is 6-carboxyfluorescein (FAM) and wherein the first and second quenchers are 4-(4'-(dimethylaminophenylazo)benzoic acid) (DABCYL).
- 25
5. The method of claim 1, wherein the enzyme is linked to a support.
- 30
6. The method of claim 1, wherein the substrate comprises a nucleic acid of SEQ ID NO: 2.

7. The method of claim 1, wherein the enzyme comprises a nucleic acid of SEQ ID NO:1.
- 5 8. The method of claim 1, wherein the ion is  $\text{AsO}_4^{3-}$ .
9. The method of claim 1, wherein the ion is selected from the group consisting of  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Li}^+$ ,  $\text{Tl}^+$ ,  $\text{NH}_4^+$  and  $\text{Ag}^+$ .
- 10 10. The method of claim 1, wherein the ion comprises a member selected from the group consisting of K(I), Na(I), Li(I), Tl(I), Ag(I), Hg(I), Mg(II), Ca(II), Mn(II), Co(II), Ni(II), Zn(II), Cd(II), Pb(II), Hg(II), Pt(II), Ra(II), Ba(II), Sr(II), Co(III), Cr(III), Ln(III), Ce(IV), Cr(VI) and U(VI).
- 15 11. The method of claim 1, wherein the ion is selected from the group consisting of  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Hg}_2^{2+}$ ,  $\text{Pt}^{2+}$ ,  $\text{Ra}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{UO}_2^{2+}$  and  $\text{Sr}^{2+}$ .
12. The method of claim 11, wherein the ion is  $\text{Pb}^{2+}$ .
- 20 13. The method of claim 1, wherein the ion is selected from the group consisting of  $\text{Co}^{3+}$ ,  $\text{Cr}^{3+}$  and  $\text{Ln}^{3+}$ .
14. The method of claim 1, wherein the ion is selected from the group consisting of  $\text{Ce}^{4+}$ ,  $\text{Cr}^{6+}$ , spermine and spermidine.
- 25 15. The method of claim 1, wherein the ion is a metal ion.
16. The method of claim 15, wherein the metal ion is  $\text{Ag}^+$ .
- 30 17. The method of claim 15, wherein the metal ion is  $\text{Hg}^{2+}$ .

18. The method of claim 1, wherein the sample suspected of containing the ion comprises a bodily fluid.
19. The method of claim 18, wherein the bodily fluid is blood.
- 5 20. The method of claim 1, wherein an array of nucleic acid enzymes comprises the nucleic acid enzyme.
- 10 21. A method of determining the concentration of an ion in the presence of other ions, in a sample, comprising:  
detecting the ion in the sample by the method of claim 1; and  
measuring an amount of the product produced.
- 15 22. The method of claim 21, wherein the nucleic acid enzyme and the substrate comprise separate nucleic acid strands.
- 20 23. The method of claim 21, wherein a 5' end of the substrate comprises a first fluorophore and a 3' end of the substrate comprises a first quencher for the fluorophore and wherein a 3' end of the enzyme comprises a second quencher for the fluorophore.
- 25 24. The method of claim 23, wherein the fluorophore is 6-carboxyfluorescein (FAM) and wherein the first and second quenchers are 4-(4'-(dimethylaminophenylazo)benzoic acid) (DABCYL).
- 30 25. The method of claim 21, wherein the enzyme is linked to a support.
26. The method of claim 21, wherein the substrate comprises a nucleic acid of SEQ ID NO: 2.
27. The method of claim 21, wherein the enzyme comprises a nucleic acid of SEQ ID NO:1.

28. The method of claim 21, wherein the ion is  $\text{AsO}_4^{3-}$ .
29. The method of claim 21, wherein the ion is selected from the group consisting of  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Li}^+$ ,  $\text{Tl}^+$ ,  $\text{NH}_4^+$  and  $\text{Ag}^+$ .
- 5 30. The method of claim 21, wherein the ion comprises a member selected from the group consisting of K(I), Na(I), Li(I), Tl(I), Ag(I), Hg(I), Mg(II), Ca(II), Mn(II), Co(II), Ni(II), Zn(II), Cd(II), Pb(II), Hg(II), Pt(II), Ra(II), Ba(II), Sr(II), Co(III), Cr(III), Ln(III), Ce(IV), Cr(VI) and U(VI).
- 10 31. The method of claim 21, wherein the ion is selected from the group consisting of  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Hg}_2^{2+}$ ,  $\text{Pt}^{2+}$ ,  $\text{Ra}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{UO}_2^{2+}$  and  $\text{Sr}^{2+}$ .
- 15 32. The method of claim 31, wherein the ion is  $\text{Pb}^{2+}$ .
33. The method of claim 21, wherein the ion is selected from the group consisting of  $\text{Co}^{3+}$ ,  $\text{Cr}^{3+}$  and  $\text{Ln}^{3+}$ .
- 20 34. The method of claim 21, wherein the ion is selected from the group consisting of  $\text{Ce}^{4+}$ ,  $\text{Cr}^{6+}$ , spermine and spermidine.
35. The method of claim 21, wherein the ion is a metal ion.
- 25 36. The method of claim 35, wherein the metal ion is  $\text{Ag}^+$ .
37. The method of claim 35, wherein the metal ion is  $\text{Hg}^{2+}$ .
38. The method of claim 21, wherein the sample suspected of containing the ion comprises a bodily fluid.
- 30 39. The method of claim 38, wherein the bodily fluid is blood.

40. The method of claim 21, wherein an array of nucleic acid enzymes comprises the nucleic acid enzyme.
- 5 41. A biosensor, capable of detecting the presence of an ion in the presence of other ions, in a sample, comprising:
- (a) a nucleic acid enzyme, and
  - (b) a substrate,
- 10 wherein the enzyme comprises at least one quencher and is dependent on the ion to produce a product from the substrate; and the substrate comprises a ribonucleotide, at least one quencher and at least one fluorophore.
- 15 42. The biosensor of claim 41, wherein the nucleic acid enzyme and the substrate comprise separate nucleic acid strands.
- 20 43. The biosensor of claim 41, wherein a 5' end of the substrate comprises a first fluorophore and a 3' end of the substrate comprises a first quencher for the fluorophore and wherein a 3' end of the enzyme comprise a second quencher for the fluorophore.
- 25 44. The biosensor of claim 43, wherein the fluorophore is 6-carboxyfluorescein (FAM) and wherein the first and second quenchers are 4-(4'-(dimethylaminophenylazo)benzoic acid) (DABCYL).
- 30 45. The biosensor of claim 41, wherein the enzyme is linked to a support.
46. The biosensor of claim 41, wherein the substrate comprises a nucleic acid of SEQ ID NO:2.
47. The biosensor of claim 41, wherein the enzyme comprises a nucleic acid of SEQ ID NO:1.

48. The biosensor of claim 41, wherein the ion is  $\text{AsO}_4^{3-}$ .
49. The biosensor of claim 41, wherein the ion is selected from the group consisting of  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Li}^+$ ,  $\text{Tl}^+$ ,  $\text{NH}_4^+$  and  $\text{Ag}^+$ .
- 5  
50. The biosensor of claim 41, wherein the ion comprises a member selected from the group consisting of  $\text{K(I)}$ ,  $\text{Na(I)}$ ,  $\text{Li(I)}$ ,  $\text{Tl(I)}$ ,  $\text{Ag(I)}$ ,  $\text{Hg(I)}$ ,  $\text{Mg(II)}$ ,  $\text{Ca(II)}$ ,  $\text{Mn(II)}$ ,  $\text{Co(II)}$ ,  $\text{Ni(II)}$ ,  $\text{Zn(II)}$ ,  $\text{Cd(II)}$ ,  $\text{Pb(II)}$ ,  $\text{Hg(II)}$ ,  $\text{Pt(II)}$ ,  $\text{Ra(II)}$ ,  $\text{Ba(II)}$ ,  $\text{Sr(II)}$ ,  $\text{Co(III)}$ ,  $\text{Cr(III)}$ ,  $\text{Ln(III)}$ ,  $\text{Ce(IV)}$ ,  $\text{Cr(VI)}$  and  $\text{U(VI)}$ .
- 10  
51. The biosensor of claim 41, wherein the ion is selected from the group consisting of  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Hg}_2^{2+}$ ,  $\text{Pt}^{2+}$ ,  $\text{Ra}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{UO}_2^{2+}$  and  $\text{Sr}^{2+}$ .
- 15  
52. The biosensor of claim 51, wherein the ion is  $\text{Pb}^{2+}$ .
53. The biosensor of claim 41, wherein the ion is selected from the group consisting of  $\text{Co}^{3+}$ ,  $\text{Cr}^{3+}$  and  $\text{Ln}^{3+}$ .
- 20  
54. The biosensor of claim 41, wherein the ion is selected from the group consisting of  $\text{Ce}^{4+}$ ,  $\text{Cr}^{6+}$ , spermine and spermidine.
55. The biosensor of claim 41, wherein the ion is a metal ion.
- 25  
56. The biosensor of claim 55, wherein the metal ion is  $\text{Ag}^+$ .
57. The biosensor of claim 55, wherein the metal ion is  $\text{Hg}^{2+}$ .
58. The biosensor of claim 41, wherein the sample suspected of containing the ion comprises a bodily fluid.
- 30  
59. The biosensor of claim 58, wherein the bodily fluid is blood.

60. The biosensor of claim 41, wherein an array of nucleic acid enzymes comprises the nucleic acid enzyme.
- 5 61. The biosensor of claim 60, wherein the array comprises a plurality of different nucleic acid enzymes, and wherein each different nucleic acid enzyme is dependent on a different ion to produce the same or different products.



1/14

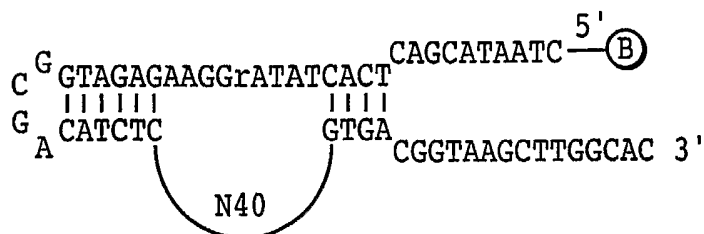


FIG. 1A

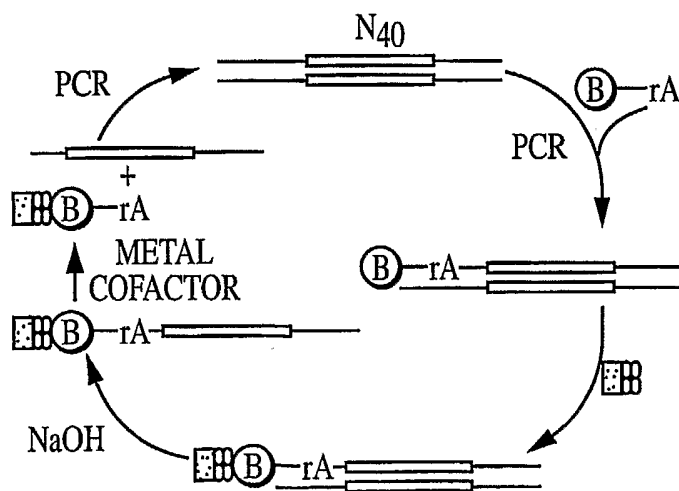


FIG. 1B

2/14

Zn-DNA

5' - CTGCAGAATTCTAATACGACTCATATAGGAAGAGATGGCGAC

CLASS I (USED FOR RESELECTION)

#5,6,7,9,21,25,29,43,47

ATCTC TTTTGTCAGCGACTCGAAATAGTGTGTTGAAGCAGCTCTA GTGAC

CLASS II

#2,10,17,20,24,31,37,39

AGCCA -TAGTTCTACCAGCGGTTCGAAATAGTGAAGTGTTCGTGA CTATC#3 GGCCA -TAGTTCTACCAGCGGTTCGAAATAGTGAAGTGTTCGTGA CTATC#4 GCCAGATTAGTTCTACCAGCGGTTCGAAATAGTGAAGTGTTCGTGA CTATC

CLASS III

#15,18,19,34,35,38,50

ATCTC CAAAGATGCCAGCATGCTATTCTCCGAGCCGGTCGAAATA GTGAC#14 ATCTC CAAAGATGCCTGCATGCTATTCTCCGAGCCGGTCGAAATA GTGAC

UNCLASSIFIED

#36 ATCTC GTCTCCGAGCCGGTCGAAATAGTCAGGTGTTTCTATTCCG GTGAC#40 ATCAC CTTCTCCGAGCCGGTCGAAATAGTAGTTTTTAGTATATCT GTGAC

#42 ATCTC AGGTGTTGGCTGCTCTCGCGGTGGCGAGAGGTAGGGTGAT GTGAC

GGTAAGCTTGGCAC-3'

FIG. 2

3/14

Co-DNA

5' - CTGCAGAATTCTAATACGACGCACTATAGGAAGAGATGGCGAC

CLASS I (USED FOR RESELECTION)

#18,15,34

ATCTC TTGTATTAGCTACACTGTTAGTGGATCGGGTCTAATCTCG GTGAC  
 #1 GTCTC TTGTATTAGCTACACTGTTAGTGGATCGGGTCTAATCTCG GTGAC  
 #25 ATCTC CTGTATTAGCTACACTGTTAGTGGATCGGGTCTAATCTCG GTGAC  
 #16 ATCTC TTGTATTAGCTACACTGTTAGTGGGAACGTTATCAT-TCG GTGAC

CLASS II

#2,4,7,23,26

ATCTC TTGACCCAAGAAGGGGTGTCAATCTAATCCGT CAACCATG  
 #8 ATCTC TTGACCCAAGAAGGGGTGTCAATCAAATCCGT CAACCATG  
 #17 ATCTC TTGACCCAAGAAGGGGTGTCAATCTAATCCGTACAACCATG ACGGTAA  
 #27 ATCTC TTGACCCAAGAAGGGGTGTCAATCTAATCCGT CAAGGATG CCGTAA

CLASS III

#5 ATCTC AGGTGTTGGCTGCTCCCGCGGTGGCGGGAGGTAGGGTGAT GTGAC  
 #11 ATCTC AGGTGTTGGCATCTCCCGCGGTGGCGAGAGGTAGGGTGAT GTGAC  
 #6 ATCTC AGGTGTTGGCTGCTCTCGCGGTGGCGAGAGGTAGGGTCAT GTGAC

UNCLASSIFIED

#21 ATCTC GCAGTCGAAGCTTCACTGTTAGTGC GGACGGGTAGACTTC GTGAC  
 #29 ATTTT TTCTGAATCCTCAATGTTAGTGGACCTAGTCGTAGTCGAT GTGAC  
 #12 ATCTC GGAGCCAGTTAGCATAATCTTCTGAATCCTCAATGTTAGT GTGAC  
 #10 ATCTC GGTGTTGGCTGGATAGAGCCGGTAGGCCCTATCGTAGGGT GTGAC  
 #1 GTCTC TTTTGTCCGCGACTCGAAATAGTGTGTTGAAGCAGCTCTA GTGAC  
 #28 AGCCA TAGTTCTACCAGCGGTTTCGAAATAGTGAAGTGTTCGTGACTATCG GTA

GGTAAGCTTGGCAC-3'

FIG. 3

4/14

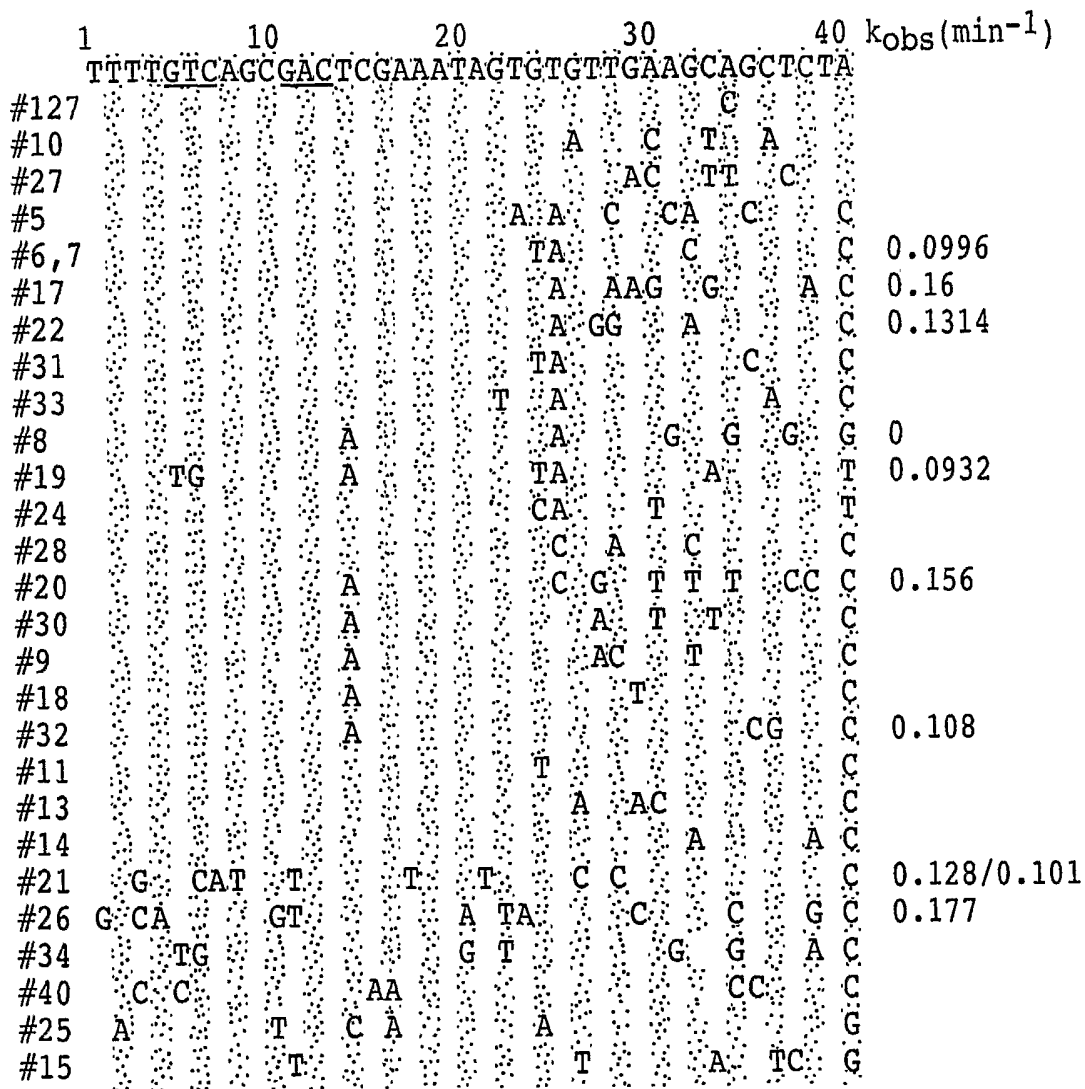


FIG. 4

5/14

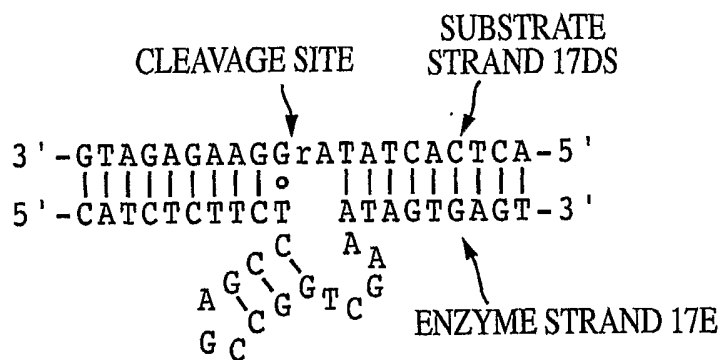


FIG. 5

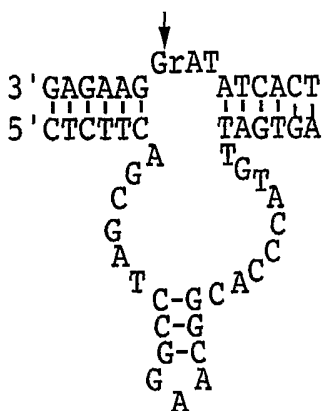


FIG. 6A

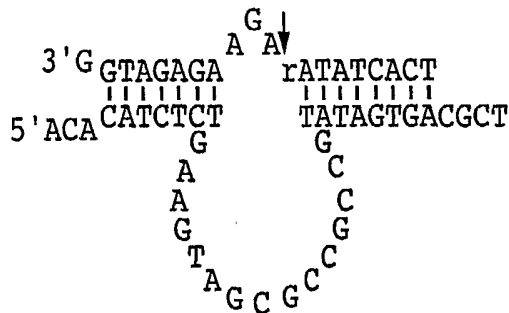


FIG. 6B

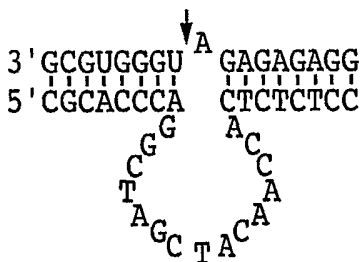


FIG. 6C

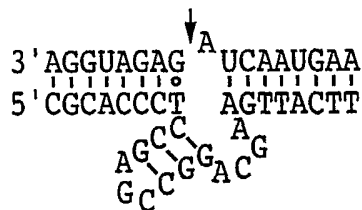


FIG. 6D

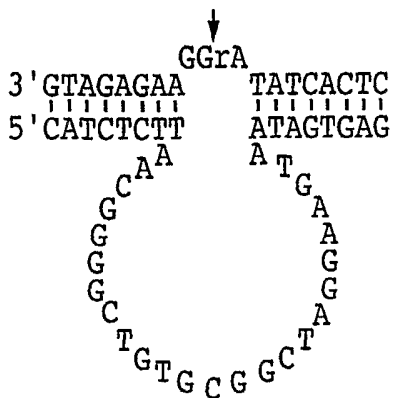


FIG. 6E

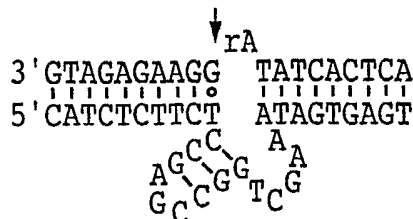


FIG. 6F



8/14

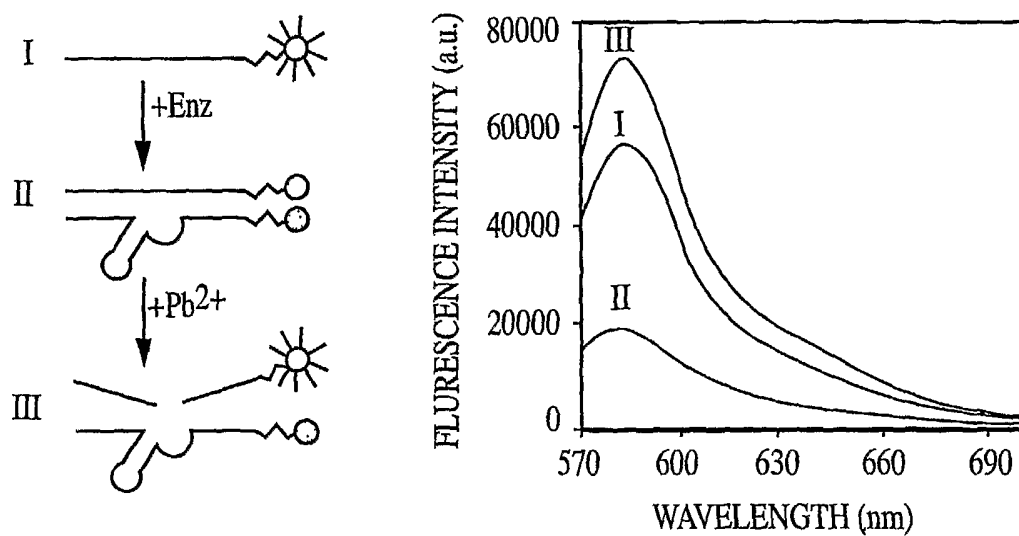


FIG. 8



9/14

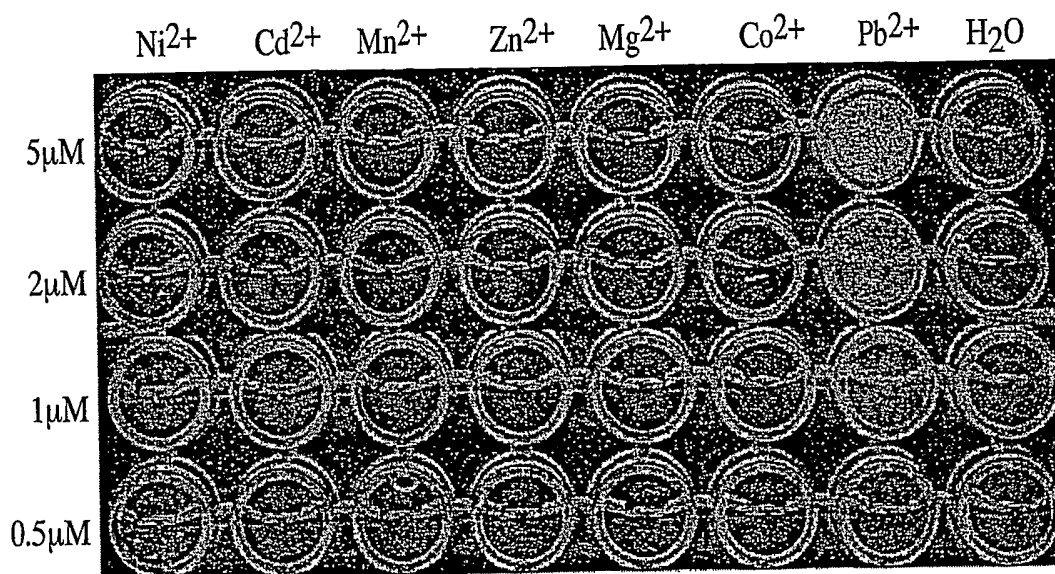


FIG. 9A

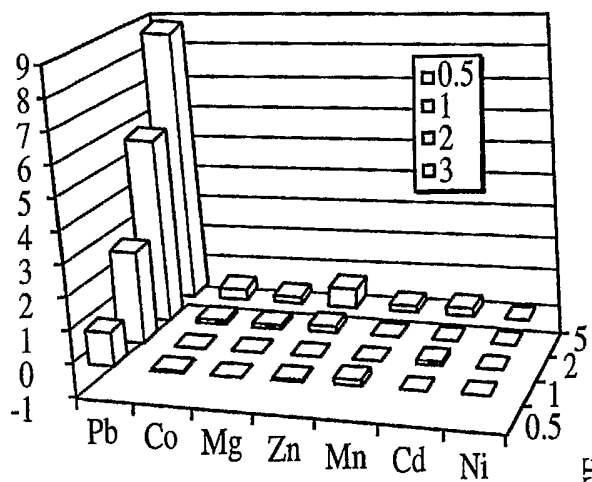
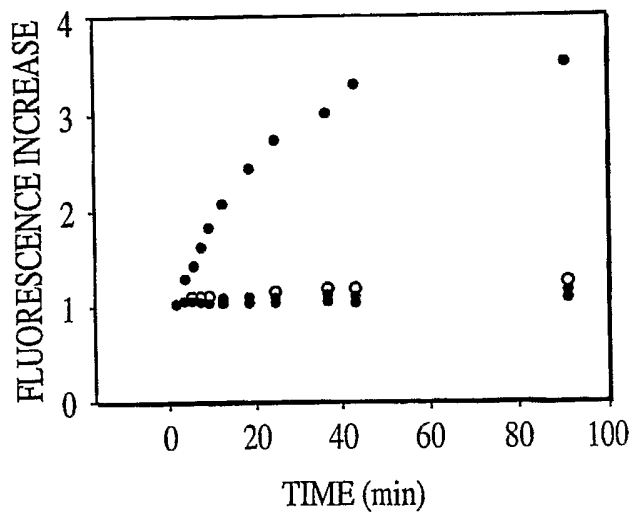


FIG. 9B



10/14

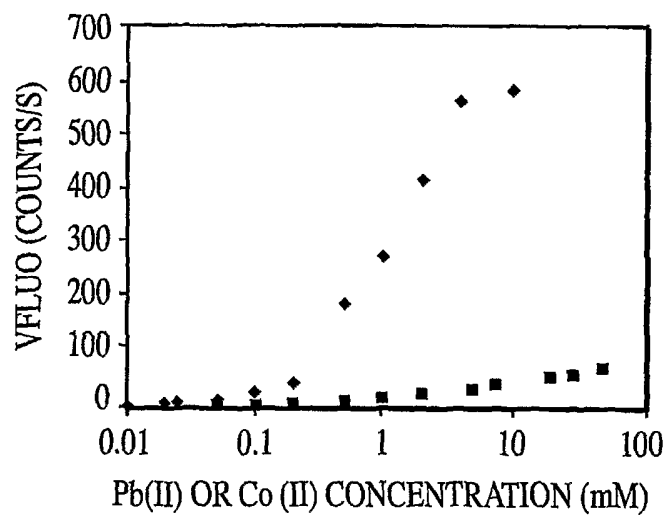


FIG. 10A

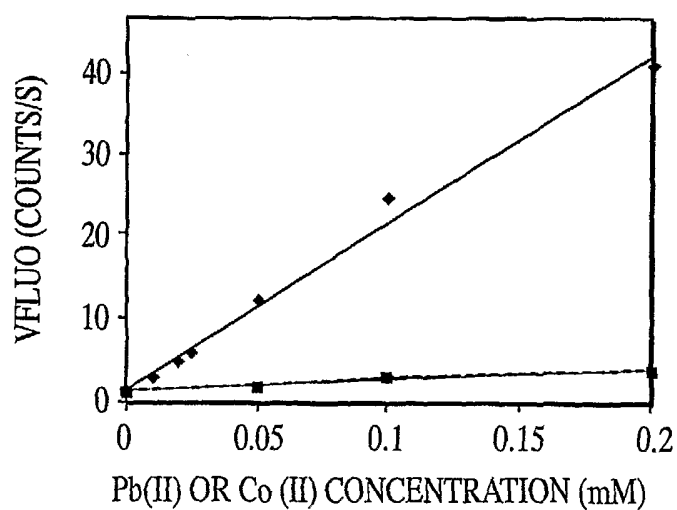


FIG. 10B

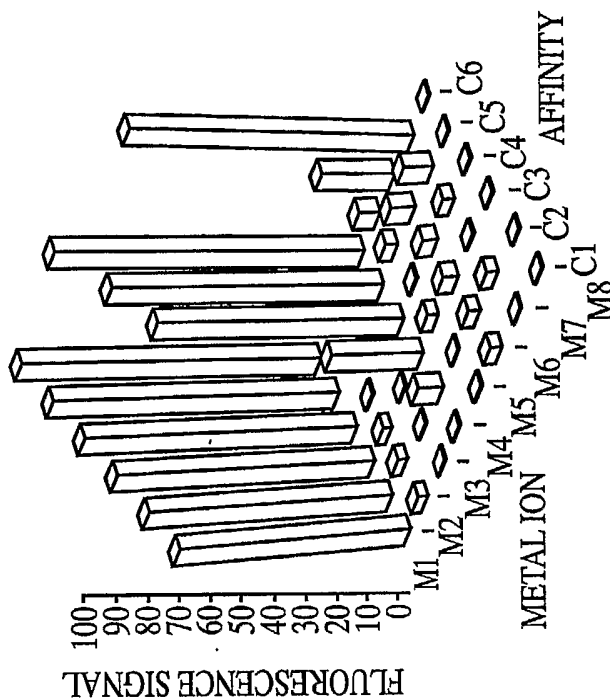


FIG. 11B

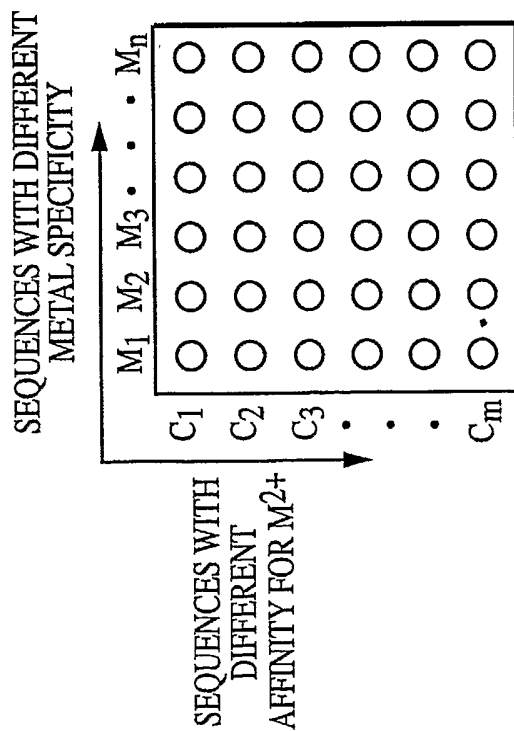


FIG. 11A

12/14

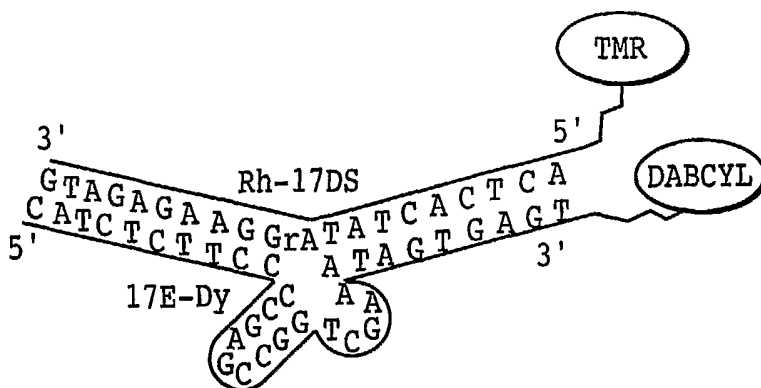


FIG. 12A

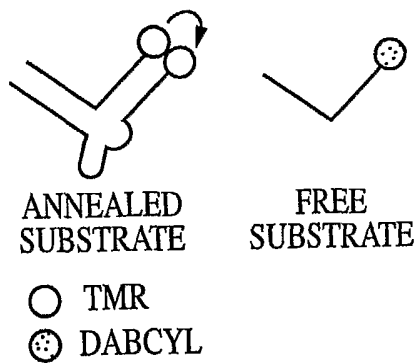


FIG. 12B

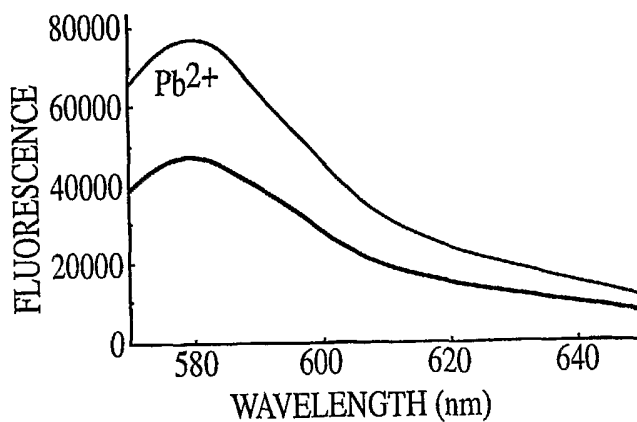


FIG. 12C

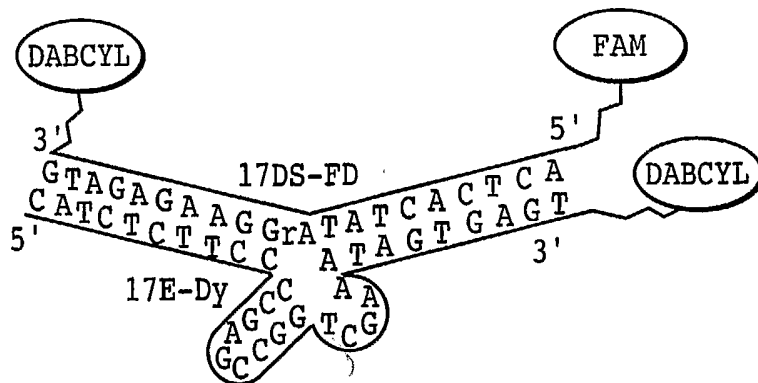


FIG. 13A

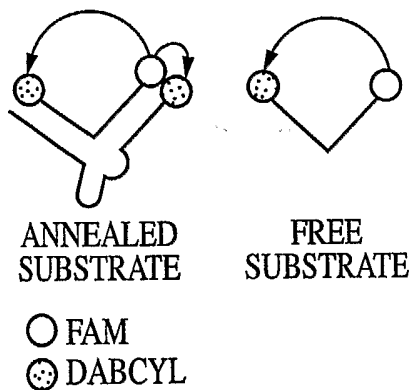


FIG. 13B

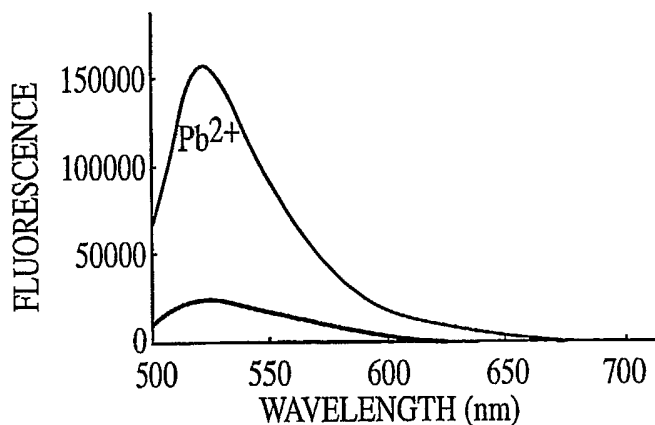


FIG. 13C

14/14



DUAL DABCYL  
FAM 6uM  
Pb 2 MIN

BLANK

TMR 6uM  
Pb 2 MIN

BLANK

FIG. 14

## SEQUENCE LISTING

5 <110> LU, YI  
LIU, Juewen

<120> NEW FLUORESCENCE BASED BIOSENSOR

<130> 10322/44

10 <140> not assigned  
<141> 2002-05-10

<160> 84

15 <170> PatentIn Ver. 2.1

<210> 1  
<211> 33  
<212> DNA  
20 <213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Trans-cleaving  
25 deoxyribozyme 17E

<400> 1  
catctcttct ccgagccggt cgaatagtg agt 33

30 <210> 2  
<211> 20  
<212> DNA  
<213> Artificial Sequence

35 <220>  
<223> Description of Artificial Sequence: Synthetic chimeric  
substrate

40 <220>  
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
substrate

45 <400> 2  
actcactata ggaagagatg 20

<210> 3  
<211> 15  
50 <212> DNA  
<213> Artificial Sequence

<220>  
55 <223> Description of Artificial Sequence: Primer

<400> 3

gtgccaagct taccg 15

5 <210> 4  
 <211> 43  
 <212> DNA  
 <213> Artificial Sequence

10 <220>  
 <223> Description of Artificial Sequence: Primer

<400> 4 43  
 ctgcagaatt ctaatacgac tcactatagg aagagatggc gac

15 <210> 5  
 <211> 28  
 <212> DNA  
 <213> Artificial Sequence

20 <220>  
 <223> Description of Artificial Sequence: Primer

25 <220>  
 <223> Description of Combined DNA/RNA Molecule: Primer

<400> 5 28  
 gggacgaatt ctaatacgac tcactata

30 <210> 6  
 <211> 97  
 <212> DNA  
 <213> Artificial Sequence

35 <220>  
 <223> Description of Artificial Sequence: DNA Template

40 <220>  
 <221> modified\_base  
 <222> (20)..(59)  
 <223> variable nucleotides

45 <400> 6 60  
 gtgccaagct taccgtcacn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnng 60  
 agatctcgcc atctcttct atagtgagtc gtattag 97

50 <210> 7  
 <211> 33  
 <212> DNA  
 <213> Artificial Sequence

55 <220>  
 <223> Description of Artificial Sequence: Variant of  
 deoxyribozyme named 17E1

<400> 7



catctctttt gtcagcgact cgaaatagtg agt

33

5 <210> 8  
 <211> 20  
 <212> DNA  
 <213> Artificial Sequence

10 <220>  
 <223> Description of Artificial Sequence: Zn-DNA

15 <220>  
 <221> modified\_base  
 <222> (2)..(4)  
 <223> variable base complementary to positions 8-10

20 <220>  
 <221> modified\_base  
 <222> (8)..(10)  
 <223> variable base complementary to positions 2-4

<400> 8  
 tnnnagcnnn tcgaaatagt 20

25 <210> 9  
 <211> 15  
 <212> DNA  
 <213> Artificial Sequence

30 <220>  
 <223> Description of Artificial Sequence: Zn-DNA

35 <220>  
 <221> modified\_base  
 <222> (2)..(4)  
 <223> variable base complementary to positions 8-10

40 <220>  
 <221> modified\_base  
 <222> (8)..(10)  
 <223> variable base complementary to positions 2-4

45 <400> 9  
 tnnnagcnnn acgaa 15

50 <210> 10  
 <211> 15  
 <212> DNA  
 <213> Artificial Sequence

55 <220>  
 <223> Description of Artificial Sequence: Class II  
 Co-DNA

<400> 10  
 acccaagaag ggggtg 15

5 <210> 11  
 <211> 20  
 <212> DNA  
 <213> Artificial Sequence

10 <220>  
 <223> Description of Artificial Sequence: Rh-17DDS

<400> 11  
 actcactata ggaagagatg 20

15 <210> 12  
 <211> 97  
 <212> DNA  
 <213> Artificial Sequence

20 <220>  
 <223> Description of Artificial Sequence: Synthetic chimeric  
 substrate

25 <220>  
 <223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
 substrate

30 <220>  
 <221> modified\_base  
 <222> (39)..(78)  
 <223> variable nucleotides

35 <400> 12  
 ctaatacgac tcactatagg aagagatggc gacatctcnn nnnnnnnnnn nnnnnnnnnn 60  
 nnnnnnnnnn nnnnnnnngt gacggtaagc ttggcac 97

40 <210> 13  
 <211> 43  
 <212> DNA  
 <213> Artificial Sequence

45 <220>  
 <223> Description of Artificial Sequence: Zn-DNA

<400> 13  
 ctgcagaatt ctaatacgac tcactatagg aagagatggc gac 43

50 <210> 14  
 <211> 50  
 <212> DNA  
 <213> Artificial Sequence

55 <220>  
 <223> Description of Artificial Sequence: Zn-DNA

<400> 14

atctcttttg tcagcgactc gaaatagtgt gttgaagcag ctctagtgac 50

5 <210> 15  
<211> 49  
<212> DNA  
<213> Artificial Sequence

10 <220>  
<223> Description of Artificial Sequence: Zn-DNA

<400> 15  
agccatagtt ctaccagcgg ttcgaaatag tgaagtgttc gtgactatc 49

15 <210> 16  
<211> 49  
<212> DNA  
<213> Artificial Sequence

20 <220>  
<223> Description of Artificial Sequence: Zn-DNA

25 <400> 16  
ggccatagtt ctaccagcgg ttcgaaatag tgaagtgttc gtgactatc 49

30 <210> 17  
<211> 51  
<212> DNA  
<213> Artificial Sequence

35 <220>  
<223> Description of Artificial Sequence: Zn-DNA

<400> 17  
gccagattag ttctaccagc gtttcgaaat agtgaaatgt tcgtgactat c 51

40 <210> 18  
<211> 50  
<212> DNA  
<213> Artificial Sequence

45 <220>  
<223> Description of Artificial Sequence: Zn-DNA

50 <400> 18  
atctccaaag atgccagcat gctattctcc gagccggtcg aaatagtgac 50

55 <210> 19  
<211> 50  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Zn-DNA

<400> 19  
5 atctccaaag atgcoctgcat gctattctcc gagccggtcg aaatagtgac 50

<210> 20  
<211> 50  
10 <212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Zn-DNA

15 <400> 20  
atctcgtctc cgagccggtc gaaatagtca ggtgtttcta ttcgggtgac 50

<210> 21  
20 <211> 50  
<212> DNA  
<213> Artificial Sequence

<220>  
25 <223> Description of Artificial Sequence: Zn-DNA

<400> 21  
30 atcaccttct ccgagccggt cgaaatagta gtttttagta tatctgtgac 50

<210> 22  
<211> 50  
35 <212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Zn-DNA

40 <400> 22  
atctcaggtg ttggctgctc tcgcggtggc gagaggtagg gtgatgtgac 50

<210> 23  
45 <211> 14  
<212> DNA  
<213> Artificial Sequence

<220>  
50 <223> Description of Artificial Sequence: Zn-DNA

<400> 23  
ggtaagcttg gcac 14

<210> 24  
55 <211> 43  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Co-DNA

5 <400> 24  
ctgcagaatt ctaatacgac gcactatagg aagagatggc gac 43

10 <210> 25  
<211> 50  
<212> DNA  
<213> Artificial Sequence

15 <220>  
<223> Description of Artificial Sequence: Co-DNA

<400> 25  
atctcttgta ttagctacac tgttagtgga tcgggtctaa tctcggtgac 50

20 <210> 26  
<211> 50  
<212> DNA  
<213> Artificial Sequence

25 <220>  
<223> Description of Artificial Sequence: Co-DNA

30 <400> 26  
gtctcttgta ttagctacac tgttagtgga tcgggtctaa tctcggtgac 50

35 <210> 27  
<211> 50  
<212> DNA  
<213> Artificial Sequence

40 <220>  
<223> Description of Artificial Sequence: Co-DNA

<400> 27  
atctcttgta ttagctacac tgttagtgga tcgggtctaa tctcggtgac 50

45 <210> 28  
<211> 49  
<212> DNA  
<213> Artificial Sequence

50 <220>  
<223> Description of Artificial Sequence: Co-DNA

<400> 28  
atctcttgta ttagctacac tgttagtggg aacgttatca ttcggtgac 49

55 <210> 29  
<211> 45

<212> DNA  
<213> Artificial Sequence

5 <220>  
<223> Description of Artificial Sequence: Co-DNA

<400> 29  
atctcttgac ccaagaagg ggtgcaatct aatccgtcaa ccatg 45

10 <210> 30  
<211> 45  
<212> DNA  
<213> Artificial Sequence

15 <220>  
<223> Description of Artificial Sequence: Co-DNA

20 <400> 30  
atctcttgac ccaagaagg ggtgcaatca aatccgtcaa ccatg 45

<210> 31  
<211> 54  
25 <212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Co-DNA

30 <400> 31  
atctcttgac ccaagaagg ggtgcaatct aatccgtaca accatgacgg taag 54

35 <210> 32  
<211> 52  
<212> DNA  
<213> Artificial Sequence

40 <220>  
<223> Description of Artificial Sequence: Co-DNA

<400> 32  
45 atctcttgac ccaagaagg ggtgcaatct aatccgtcaa ggatgacggta ag 52

<210> 33  
<211> 50  
50 <212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Co-DNA

55 <400> 33  
atctcagggtg ttggctgctc ccgcggtggc gggaggtagg gtgatgtgac 50

5 <210> 34  
<211> 50  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Co-DNA

10 <400> 34  
atctcaggtagg ttggcatctc ccgcggtggc gagaggtagg gtgatgtgac 50

15 <210> 35  
<211> 50  
<212> DNA  
<213> Artificial Sequence

20 <220>  
<223> Description of Artificial Sequence: Co-DNA

<400> 35  
atctcaggtagg ttggctgctc tcgcggtggc gagaggtagg gtcatgtgac 50

25 <210> 36  
<211> 50  
<212> DNA  
<213> Artificial Sequence

30 <220>  
<223> Description of Artificial Sequence: Co-DNA

35 <400> 36  
atctcgcagtagt cgaagcttca ctgtagtgac ggacgggtag acttcgtgac 50

40 <210> 37  
<211> 50  
<212> DNA  
<213> Artificial Sequence

45 <220>  
<223> Description of Artificial Sequence: Co-DNA

<400> 37  
atctcttctg aatcctcaat gttagtggac ctagtctgtag tcgatgtgac 50

50 <210> 38  
<211> 50  
<212> DNA  
<213> Artificial Sequence

55 <220>  
<223> Description of Artificial Sequence: Co-DNA

<400> 38

atctcggagc cagtttagcat aatcttctga atcctcaatg ttagtgtgac 50

5 <210> 39  
<211> 50  
<212> DNA  
<213> Artificial Sequence

10 <220>  
<223> Description of Artificial Sequence: Co-DNA

<400> 39  
atctcgggtgt tggctggata gagccggtag gccctatcgt aggggtgac 50

15 <210> 40  
<211> 50  
<212> DNA  
<213> Artificial Sequence

20 <220>  
<223> Description of Artificial Sequence: Co-DNA

25 <400> 40  
gtctcttttg tccgcgactc gaaatagtgt gttgaagcag ctctagtgac 50

30 <210> 41  
<211> 54  
<212> DNA  
<213> Artificial Sequence

35 <220>  
<223> Description of Artificial Sequence: Co-DNA

<400> 41  
agccatagtt ctaccagcgg ttcgaaatag tgaagtgttc gtgactatcg gtaa 54

40 <210> 42  
<211> 14  
<212> DNA  
<213> Artificial Sequence

45 <220>  
<223> Description of Artificial Sequence: Co-DNA

50 <400> 42  
ggtaagcttg gcac 14

55 <210> 43  
<211> 40  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Zn-DNA



5 <400> 43  
ttttgtcagc gactcgaaat agtgtgttga agcagctcta 40

<210> 44  
<211> 40  
<212> DNA  
<213> Artificial Sequence

10 <220>  
<223> Description of Artificial Sequence: Zn-DNA

15 <400> 44  
ttttgtcagc gactcgaaat agtgtgttga agccgctcta 40

<210> 45  
<211> 40  
<212> DNA  
<213> Artificial Sequence

20 <220>  
<223> Description of Artificial Sequence: Zn-DNA

25 <400> 45  
ttttgtcagc gactcgaaat agtgtattgc agtagatcta 40

30 <210> 46  
<211> 40  
<212> DNA  
<213> Artificial Sequence

35 <220>  
<223> Description of Artificial Sequence: Zn-DNA

40 <400> 46  
ttttgtcagc gactcgaaat agtgtgttac agttgcccta 40

<210> 47  
<211> 40  
<212> DNA  
<213> Artificial Sequence

45 <220>  
<223> Description of Artificial Sequence: Zn-DNA

50 <400> 47  
ttttgtcagc gactcgaaat agagagtcga cacacctctc 40

55 <210> 48  
<211> 40  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Zn-DNA

5 <400> 48  
ttttgtcagc gactcgaaat agttagttga accagctctc 40

10 <210> 49  
<211> 40  
<212> DNA  
<213> Artificial Sequence

15 <220>  
<223> Description of Artificial Sequence: Zn-DNA

<400> 49  
ttttgtcagc gactcgaaat agtgagtaag aggagctatc 40

20 <210> 50  
<211> 40  
<212> DNA  
<213> Artificial Sequence

25 <220>  
<223> Description of Artificial Sequence: Zn-DNA

30 <400> 50  
ttttgtcagc gactcgaaat agtgagggga aacagctctc 40

35 <210> 51  
<211> 39  
<212> DNA  
<213> Artificial Sequence

40 <220>  
<223> Description of Artificial Sequence: Zn-DNA

<400> 51  
ttttgtcagc gactcgaaat agttagttga acacctctc 39

45 <210> 52  
<211> 40  
<212> DNA  
<213> Artificial Sequence

50 <220>  
<223> Description of Artificial Sequence: Zn-DNA

55 <400> 52  
ttttgtcagc gactcgaaat attgagttga agcagatctc 40

<210> 53  
<211> 40

<212> DNA  
<213> Artificial Sequence

5 <220>  
<223> Description of Artificial Sequence: Zn-DNA

<400> 53  
ttttgtcagc gacacgaaat agtgagttga ggcggcgctg 40

10 <210> 54  
<211> 40  
<212> DNA  
<213> Artificial Sequence

15 <220>  
<223> Description of Artificial Sequence: Zn-DNA

20 <400> 54  
ttttgtcagc gacacgaaat agttagttga agaagctctt 40

25 <210> 55  
<211> 40  
<212> DNA  
<213> Artificial Sequence

30 <220>  
<223> Description of Artificial Sequence: Zn-DNA

<400> 55  
ttttgtcagc gactcgaaat agtcagttgt agcagctctt 40

35 <210> 56  
<211> 40  
<212> DNA  
<213> Artificial Sequence

40 <220>  
<223> Description of Artificial Sequence: Zn-DNA

45 <400> 56  
ttttgtcagc gactcgaaat agtgcgtaga accagctctc 40

50 <210> 57  
<211> 40  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Zn-DNA

55 <400> 57  
ttttgtcagc gacacgaaat agtgcggtgt atctgcctc 40

5  
<210> 58  
<211> 40  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Zn-DNA  
  
10 <400> 58  
ttttgtcagc gacacgaaat agtgtgatgt agtagctctc 40  
  
15 <210> 59  
<211> 38  
<212> DNA  
<213> Artificial Sequence  
  
20 <220>  
<223> Description of Artificial Sequence: Zn-DNA  
  
<400> 59  
ttttgtcagc gacacgaaat agtgtgacga atcatctc 38  
  
25 <210> 60  
<211> 39  
<212> DNA  
<213> Artificial Sequence  
  
30 <220>  
<223> Description of Artificial Sequence: Zn-DNA  
  
35 <400> 60  
ttttgtcagc gacacgaaat agtgtgttta agcgcctctc 39  
  
40 <210> 61  
<211> 40  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Zn-DNA  
  
45 <400> 61  
ttttgtcagc gacacgaaat agtgtgttga agcagctctc 40  
  
50 <210> 62  
<211> 40  
<212> DNA  
<213> Artificial Sequence  
  
55 <220>  
<223> Description of Artificial Sequence: Zn-DNA  
  
<400> 62  
ttttgtcagc gactcgaaat agtttgttga agcagctctc 40

5 <210> 63  
<211> 40  
<212> DNA  
<213> Artificial Sequence

<220>  
10 <223> Description of Artificial Sequence: Zn-DNA

<400> 63  
ttttgtcagc gactcgaaat agtgtattac agcagctctc 40

15 <210> 64  
<211> 40  
<212> DNA  
<213> Artificial Sequence

20 <220>  
<223> Description of Artificial Sequence: Zn-DNA

<400> 64  
25 ttttgtcagc gactcgaaat agtgtgttga aacagctatc 40

<210> 65  
<211> 40  
<212> DNA  
30 <213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Zn-DNA

35 <400> 65  
tttgtcatgc tactcgtaat tgtgtctcga agcagctctc 40

40 <210> 66  
<211> 40  
<212> DNA  
<213> Artificial Sequence

45 <220>  
<223> Description of Artificial Sequence: Zn-DNA

<400> 66  
gtcagtcagg tactcgaaaa atagtgttca agccgctgtc 40

50

55 <210> 67  
<211> 40  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Zn-DNA

5 <400> 67  
tttttgcagc gactcgaaag attgtggtga ggcggctatc 40

10 <210> 68  
<211> 40  
<212> DNA  
<213> Artificial Sequence

15 <220>  
<223> Description of Artificial Sequence: Zn-DNA

20 <400> 68  
ttctctcagc gactaaaaat agtgtggtga agcccctctc 40

25 <210> 69  
<211> 40  
<212> DNA  
<213> Artificial Sequence

30 <220>  
<223> Description of Artificial Sequence: Zn-DNA

35 <400> 69  
tattgtcagt gaccctaaat agtatggtga agcagctctg 40

40 <210> 70  
<211> 39  
<212> DNA  
<213> Artificial Sequence

45 <220>  
<223> Description of Artificial Sequence: Zn-DNA

50 <400> 70  
ttttgtcagc tactgaaata gtgttttgaa gaagtcctg 39

55 <210> 71  
<211> 15  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic chimeric  
substrate

<220>  
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
substrate

55 <400> 71  
tcactatagg aagag 15

5 <210> 72  
<211> 36  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> Description of Artificial Sequence: Synthetic chimeric  
substrate

10 <220>  
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
substrate

15 <400> 72  
ctcttcagcg atccggaacg gcacccatgt tagtga 36

20 <210> 73  
<211> 19  
<212> DNA  
<213> Artificial Sequence

25 <220>  
<223> Description of Artificial Sequence: Synthetic chimeric  
substrate

30 <220>  
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
substrate

<400> 73  
tcactataag aagagatgg 19

35 <210> 74  
<211> 37  
<212> DNA  
<213> Artificial Sequence

40 <220>  
<223> Description of Artificial Sequence: Synthetic chimeric  
substrate

45 <220>  
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
substrate

50 <400> 74  
acacatctct gaagtagcgc cgccgtatag tgacgct 37

55 <210> 75  
<211> 17  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Synthetic chimeric

substrate

5 <220>  
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
substrate

<400> 75 17  
ggagagagau gggugcg

10 <210> 76  
<211> 31  
<212> DNA  
<213> Artificial Sequence

15 <220>  
<223> Description of Artificial Sequence: Synthetic chimeric  
substrate

20 <220>  
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
substrate

25 <400> 76 31  
cgcaccagg ctagctacaa cgactctctc c

30 <210> 77  
<211> 17  
<212> DNA  
<213> Artificial Sequence

35 <220>  
<223> Description of Artificial Sequence: Synthetic chimeric  
substrate

40 <220>  
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
substrate

<400> 77 17  
aaguaacuag agaugga

45 <210> 78  
<211> 29  
<212> DNA  
<213> Artificial Sequence

50 <220>  
<223> Description of Artificial Sequence: Synthetic chimeric  
substrate

55 <220>  
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
substrate

<400> 78



cgcacctcc gagccggacg aagttactt 29

5 <210> 79  
<211> 19  
<212> DNA  
<213> Artificial Sequence

10 <220>  
<223> Description of Artificial Sequence: Synthetic chimeric  
substrate

15 <220>  
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
substrate

<400> 79  
ctcactatag gaagagatg 19

20 <210> 80  
<211> 41  
<212> DNA  
<213> Artificial Sequence

25 <220>  
<223> Description of Artificial Sequence: Synthetic chimeric  
substrate

30 <220>  
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
substrate

35 <400> 80  
catctcttaa cggggctgtg cggctaggaa gtaatagtga g 41

40 <210> 81  
<211> 20  
<212> DNA  
<213> Artificial Sequence

45 <220>  
<223> Description of Artificial Sequence: Synthetic chimeric  
substrate

50 <220>  
<223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
substrate

<400> 81  
actcactata ggaagagatg 20

55 <210> 82  
<211> 33  
<212> DNA  
<213> Artificial Sequence

5 <220>  
 <223> Description of Artificial Sequence: Synthetic chimeric  
 substrate

10 <220>  
 <223> Description of Combined DNA/RNA Molecule: Synthetic chimeric  
 substrate

15 <400> 82  
 catctcttct ccgagccggt cgaaatagtg agt 33

20 <210> 83  
 <211> 107  
 <212> DNA  
 <213> Artificial Sequence

25 <220>  
 <223> Description of Artificial Sequence: Predicted  
 secondary structure of the G3 deoxyribozyme

30 <220>  
 <221> modified\_base  
 <222> (67)  
 <223> variable nucleotide

35 <220>  
 <221> modified\_base  
 <222> (69)..(74)  
 <223> variable nucleotide

40 <220>  
 <221> modified\_base  
 <222> (80)..(82)  
 <223> variable nucleotide

45 <220>  
 <221> modified\_base  
 <222> (85)..(89)  
 <223> variable nucleotide

50 <400> 83  
 gggacgaatt ctaatacgac tcactatagg aagagatggc gacaactctt tacccaagaa 60  
 ggggtgngnn nnnngctacn nnatnnnnt gacggtagct tggcacc 107

55 <210> 84  
 <211> 45  
 <212> DNA  
 <213> Artificial Sequence

60 <220>  
 <223> Description of Artificial Sequence: Co-DNA

65 <400> 84  
 cactatagga agagatggcg acatctcttg acccaagaag ggggtg 45

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 03/08483

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12N15/11 C12Q1/68

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)

IPC 7 C12N C12Q

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 practical, search terms used)

BIOSIS, EPO-Internal, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 02 00006 A (TRUSTEES OF THE UNIVERSITY OF) 3 January 2002 (2002-01-03) the whole document	1-61
Y	JENNE A: "REAL-TIME CHARACTERIZATION OF RIBOZYMES BY FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)" ANGEWANDTE CHEMIE, INTERNATIONAL EDITION, VERLAG CHEMIE, WEINHEIM, DE, vol. 38, no. 9, 3 May 1999 (1999-05-03), pages 1300-1303, XP002111691 ISSN: 0570-0833 the whole document	1-61
A	DE 199 15 141 A (ARTUS GES FUER MOLEKULARBIOLOG) 28 September 2000 (2000-09-28) page 2, line 14 -page 5, line 6; figure 16	1-61
	-/--	

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

3 November 2003

Date of mailing of the international search report

10.11.03

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Rutz, B

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 03/08483

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 47704 A (JENNE ANDREAS ;FAMULOK MICHAEL (DE)) 23 September 1999 (1999-09-23) page 14, line 3 -page 15, line 5; figures 1,3 ---	1-61
A	JENNE A ET AL: "Rapid identification and characterization of hammerhead-ribozyme inhibitors using fluorescence-based technology" NATURE BIOTECHNOLOGY, NATURE PUBLISHING, US, vol. 19, no. 1, January 2001 (2001-01), pages 56-61, XP002225624 ISSN: 1087-0156 ---	
A	SINGH K K ET AL: "FLUORESCENCE POLARIZATION FOR MONITORING RIBOZYME REACTIONS IN REAL TIME" BIOTECHNIQUES, EATON PUBLISHING, NATICK, US, vol. 29, no. 2, August 2000 (2000-08), pages 344-351, XP001093639 ISSN: 0736-6205 -----	

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 03/08483

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
WO 0200006	A	03-01-2002	AU	7684301 A	08-01-2002
			WO	0200006 A2	03-01-2002
DE 19915141	A	28-09-2000	DE	19915141 A1	28-09-2000
			AU	5981799 A	16-10-2000
			WO	0058505 A1	05-10-2000
			EP	1165841 A1	02-01-2002
WO 9947704	A	23-09-1999	DE	19811618 C1	31-08-2000
			WO	9947704 A1	23-09-1999
			EP	1064405 A1	03-01-2001
			US	2003008315 A1	09-01-2003
			US	6451535 B1	17-09-2002