Published in final edited form as:

Methods Mol Biol. 2012 ; 848: 297–316. doi:10.1007/978-1-61779-545-9_18.

***In Vitro* Selection of Metal-Ion-Selective DNazymes**

Hannah E. Ihms and Yi Lu

Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA,

Phone: +217-333-2619

Yi Lu: yi-lu@illinois.edu

Abstract

The discovery of DNazymes that can catalyze a wide range of reactions in the presence of metal ions is important on both fundamental and practical levels; it advances our understanding of metal-nucleic acid interactions and allows for the design of highly sensitive and selective metal-ion sensors. A crucial factor in this success is a technique known as *in vitro* selection, which can rapidly select metal-specific RNA-cleaving DNazymes. *In vitro* selection is an iterative process where a DNA pool containing a random region is incubated with the target metal ion. Those DNA sequences that catalyze the preferred reaction (the “winners”) are amplified and carried on to the next step, where the selection is carried out under more stringent conditions. In this way, the selection pool becomes enriched with DNazymes that exhibit desirable activity and selectivity. The method described can be applied to isolate DNazymes selective to many different types of metal ions or different oxidation states of the same metal ion.

Keywords

DNzyme; *in vitro* selection; functional DNA; deoxyribozyme; catalytic DNA; metal ions; bioinorganic chemistry

1. Introduction

Discovered by Breaker and Joyce in 1994 (1), DNazymes demonstrate that DNA is capable of much more than passive information-storage. DNazymes, also known as deoxyribozymes or catalytic DNA, are strands of DNA that catalyze reactions such as RNA cleavage, porphyrin metallation, and DNA adenylation (2-8). Though DNazymes can exhibit a wide range of structural diversity (9), DNA has fewer functional groups than protein or RNA enzymes. By making use of metal cofactors, however, DNazymes can expand their activity. That anionic DNA could bind cationic metal ions was predictable; that it could do so selectively, and that these metal ions would enhance the DNA's catalytic activity was not predicted. *In vitro* selection has now been used to obtain DNazymes with high selectivity toward Pb^{2+} , Mg^{2+} , Co^{2+} and UO_2^{2+} . Since the mechanisms of these DNazymes remains largely unknown, biochemical and biophysical studies are underway to increase the fundamental understanding of metal-to-nucleic acids interactions. Even without a detailed knowledge of their principle of action, DNazymes with high selectivities have

been converted into fluorescent (10), colorimetric (11), and electrochemical (12) sensors for metal ions such as Pb^{2+} (13) and UO_2^{2+} (14). Since *in vitro* selection can isolate DNazymes that bind a variety of metal ions, or even different oxidation states of the same metal ion, it is one of the most general approaches for developing metal sensors. As a further benefit, DNazymes can withstand harsh conditions including multiple denaturing and annealing steps. Therefore, DNzyme-based sensors can be tailored for a wide range of applications such as environmental monitoring and biomedical diagnostics. Because DNazymes are important on both fundamental and practical levels, it is desirable to obtain new DNazymes for novel targets. During *in vitro* selection, a DNA pool of $\approx 10^{14}$ sequences is incubated with a solution of the selection target under application-relevant conditions (15). In iterative rounds, the “winner” strands which catalyze the desired reaction in the presence of the target metal ion are amplified, enriching them in the pool. When the ensemble activity of the pool reaches a desirable level, the pool is sequenced, and the sequences are sorted into families based on their sequence similarities. The most active families, then the most active individual sequence in those families is isolated. After characterizing this active sequence to determine its sensitivity and selectivity, it can be developed into a sensor for the target metal ion by functionalizing it with a fluorophore and quencher, nanoparticles, or electrochemical tags.

RNA-cleaving DNazymes are one class of DNazymes that are commonly selected for using *in vitro* selection. This type of DNzyme consists of an enzyme strand that hybridizes to a substrate strand containing a ribonucleotide in its center. The phosphodiester bond in RNA is $\approx 10^5$ times more readily hydrolyzed than a nucleotide-nucleotide bond, making it the most susceptible region of the substrate (16). The DNzyme hybridizes to its substrate in such a way that the catalytic core of the enzyme strand can access the junction of the ribonucleotide and its 3'-adjacent base, and catalyze the cleavage of the phosphodiester bond. During the selection process, a single strand of DNA containing both the fixed substrate strand and a random region to give rise to the DNzyme is generated through the polymerase chain reaction (PCR). The pool contains primer-binding regions for PCR amplification. During each selection round, the pool is incubated with the target analyte, then the cleaved product “winners” are isolated, amplified, and regenerated.

In the method described below, four different oligonucleotides are used to construct or regenerate the pool: template, and Primers A, B, and C (see Figs. 1 and 2). The template is a shortened and complementary version of the final random pool, and Primers A and B are forward primers of two different lengths that introduce the ribonucleotide. Primer C is a reverse primer containing a Stop *Taq* sequence that makes its PCR product longer than the desired pool; these two strands can thus be separated from one other during polyacrylamide gel electrophoresis.

There are many choices involved in designing an *in vitro* selection method. The oligonucleotides required to construct or regenerate the pool should be carefully designed to avoid homo- or heterodimers and undesirable secondary structure; the primers should have compatible melting temperatures. If the primer-binding regions contain extensive amounts of secondary structure, or if they can dimerize, it will make PCR less efficient. The number of nucleotides to include in the random region must be decided. The smaller the number of

random nucleotides, the higher the probability that the entire sequence space will be probed. For example, if a twenty-five nucleotide random region was used, the entire sequence space could theoretically be covered if a selection began with a 2 nmole pool. On the other hand, it might not be necessary to probe all of sequence space to find a desired DNAzyme. Catalytic cores range in size, and with a longer random region, a desired sequence could appear multiple times in the random region (17), and a selection could easily become biased toward shorter motifs, because of their prevalence. This could explain the high incidence of a DNAzyme motif known as the “8-17.” Many selections carried out by different labs have produced this motif. It can be a source of difficulty because it is often Pb^{2+} -sensitive, and it is often highly desirable to select a DNAzyme that is not sensitive to Pb^{2+} . Thus, various research labs have devised strategies to limit the incidence of the 8-17 motif. A final consideration in sequence design is that while all four nucleotides can be equally distributed in the random region, it can also be enriched with nucleotides of interest (18).

The selection buffer must contain the target metal ion in a stabilized form and available for the DNAzyme to bind in an application-relevant manner. Another crucial choice is the dinucleotide junction defining the cleavage site. RNA-cleaving DNAzymes can be selected to cleave any dinucleotide junction, but the global folding (19) and rate of cleavage is highly dependent on the identity of the two bases (20). In addition, the concentration of the selection buffer as well as the incubation time should be decreased as the selection continues; as the selection conditions become more stringent, the “winners” will be narrowed down to only include sequences with high catalytic rates.

In vitro selection provides not only a method of searching for a DNAzyme with preferred activity in the presence of a target analyte (positive selections); it also allows a pool to be biased against undesirable target analytes (negative selections). These two types of selection rounds can be used in conjunction with selection pressures to select robust DNAzymes. Deciding how to vary the selection pressures of incubation time and analyte concentration should take into account what is known about the dynamics of a pool during selection and the results from previous selections (21). It is recommended to increase the stringency of the selection rounds during the course of the selection, that the pool's activity be assessed at multiple points during the selection, and that the activities of major and minor sequences obtained from the selection be evaluated (22).

Single nucleotide mutations can substantially alter the selectivity and sensitivity of DNAzymes (23), and once an analyte-specific sequence has been discovered through *in vitro* selection, its sequence can be subsequently fine-tuned.

2. Materials

2.1. Design of DNAzyme Selection Pool

1. Random DNA Generator, <<http://www.faculty.ucr.edu/~mmaduro/random.htm>> (The Morris Maduro Lab, University of California at Riverside, Riverside, CA)
2. OligoAnalyzer, <<http://www.idtdna.com/analyzer/Applications/OligoAnalyzer>> (Integrated DNA Technologies, Coralville, IA)

3. Mfold <<http://mfold.ma.albany.edu/?q=mfold>> (The RNA Institute, University at Albany State University of New York)

2.2. Pool Generation (see Note 1)

1. 20 μ L, 200 μ L, and 1000 μ L barrier pipet tips (Denville Scientific, South Plainfield NJ)
2. 200 μ L Seal-Rite thin-wall PCR tubes (USA Scientific Inc., Orlando, FL)
3. 1.7 mL microtubes (Axygen, Union City, CA)
4. 10 $\times$ PCR buffer without $MgCl_2$ (Invitrogen, Carlsbad, CA) (*see* Note 2).
5. 2 mM dNTP solution (New England Biolabs, Ipswich, MA) (*see* Note 3).
6. 50 mM $MgCl_2$ (New England Biolabs, Ipswich, MA)
7. Primer B, Primer C, and Template (custom orders from Integrated DNA Technologies, Coralville, IA) (*see* Note 4).
8. [α ^{32}P]-deoxyadenosine-5'-triphosphate (PerkinElmer, Waltham, MA)
9. Millipore water (*see* Note 5).
10. Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA)
11. C1000 PCR thermocycler (Bio-Rad, Hercules, CA)
12. 3 M sodium acetate (pH 5.2; made from sodium acetate dihydrate, Sigma-Aldrich, St. Louis, MO)
13. 200 proof ethanol (Decon Laboratories, Inc., King of Prussia, PA)
14. Vacufuge plus vacuum concentrator (Eppendorf, Hauppauge, NY)

2.3. 5'- γ ^{32}P Labeling of DNA Pool

1. DNA (Integrated DNA Technologies, Coralville, IA)
2. [γ ^{32}P]-deoxyadenosine-5'-triphosphate (PerkinElmer, Waltham, MA)
3. 10 $\times$ forward reaction buffer (New England Biolabs, Ipswich, MA)

¹Any tube to be used for the long-term storage of any solution should be soaked in 10% nitric acid overnight and triple-rinsed with Millipore water to prevent metal ions from leaching out of the plastic into the solution. Use ultrapure materials for all solutions, and adjust their pH using a sterilized pH meter and ultrapure acid or base, such as that available from Alfa Aesar (Ward Hill, MA). After calibrating the pH meter, it can be sterilized by soaking it in 1 M HCl for 2 minutes, and then in 3 M NaOH for 2 minutes, rinsing it in Millipore water after each soak. Any solution that does not contain EDTA should be treated with Chelex 100 sodium form beads (Sigma-Aldrich, St. Louis, MO) overnight to remove any trace divalent cations. To treat a solution, add $\approx$ 1 g of sodium Chelex beads per 100 mL of solution, and stir overnight. The solution should be stirred fast enough for the beads to circulate, but not so rapidly that the beads beat against the side of the container and are damaged. After treating the solution, remove the Chelex beads by filtering the solution. Since Chelex treatment can alter the pH of the solution, check the pH of the solution and readjust it, if necessary.

²This solution is included with every *Taq* DNA polymerase purchase.

³Dilute from the 10 mM solution provided by New England Biolabs.

⁴DNA can be ordered at different purity levels, with the two most common levels being standard desalted (low purity) and HPLC-purified (high purity). All DNA should be purified before use, if not by HPLC, then by PAGE-purifying desalted DNA. The choice between these levels is individual, with convenience lying on the side of HPLC-purified DNA, and economy on the side of desalted DNA.

⁵The water used for all experiments was purified using a Milli-Q system (Millipore, Billerica, MA).

4. T4 Kinase (New England Biolabs, Ipswich, MA)
5. Sep-Pak Plus® C18 cartridges (Waters Corporation, Milford, MA)
6. Acetonitrile (Fisher Scientific, Pittsburgh, PA)
7. Methanol (Fisher Scientific, Pittsburgh, PA)
8. 2 M ammonium acetate (solid from Sigma-Aldrich, St. Louis, MO)
9. Vacufuge plus vacuum concentrator (Eppendorf, Hauppauge, NY)

2.4. Polyacrylamide Gel Electrophoresis (PAGE)

1. Tris[hydroxymethyl]aminomethane (USB Corporation, Cleveland, OH)
2. Boric acid, crystalline powder, electrophoresis grade (Fisher Scientific, Pittsburgh, PA)
3. Ethylenediaminetetraacetic acid, disodium salt, dihydrate, crystalline powder, electrophoresis grade (EDTA; Fisher Scientific, Pittsburgh, PA)
4. 10× TBE buffer (1.78 M Tris, 1.78 M boric acid, 0.05 M EDTA) (*see* Note 6).
5. 25% (w/v) ammonium persulfate (APS) (solid APS from Fisher Scientific, Pittsburgh, PA)
6. Tetramethylethylenediamine (TEMED; Fisher Scientific, Pittsburgh, PA)
7. 40% acrylamide/bisacrylamide (3.3% C) (Bio-Rad, Hercules, CA)
8. Urea, high purity grade (USB Corporation, Cleveland, OH)
9. Gel electrophoresis apparatus (Thermo Scientific Owl, Rochester, NY)
10. Electrophoresis power supply (Amersham Biosciences, Piscataway, NJ)
11. Maximum Resolution (MR) film (Eastman KODAK Co., Rochester, NY)
12. FUTURA 200K™ Automatic X-ray film processor (Fischer Industries, Inc., Geneva, IL)
13. Soak solution (10 mM Tris, 1 mM EDTA, 300 mM NaCl, pH 7.5; NaCl from Sigma-Aldrich, St. Louis, MO)
14. 3 M sodium acetate (pH 5.2; solid sodium acetate from Fisher, Pittsburgh, PA)
15. 200 proof ethanol (Decon Laboratories, Inc., King of Prussia, PA)
16. Vacufuge plus vacuum concentrator (Eppendorf, Hauppauge, NY)

2.5. Positive *In Vitro* Selection

1. 2× selection solution (unique to each selection; see the details in the Methods section)

⁶The urea in this solution is prone to precipitate over time, so its shelf-life is only about a month. It is easiest to remove solid urea from the sample bottle by adding nitric acid, then rinsing.

2. Loading buffer (8 M urea, 50 mM EDTA, 1 × TBE)
3. Bromophenol blue, electrophoresis purity (Bio-Rad Laboratories, Hercules, CA)
4. Xylene cyanol FF, electrophoresis purity (Bio-Rad Laboratories, Hercules, CA)
5. Loading buffer with dye (same as the loading buffer listed above, but with 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol)
6. 117 nt and 89 nt DNA Markers (Integrated DNA Technologies, Coralville, IA) (*see* Note 7).

2.6. Pool Regeneration

1. 200 µL Seal-Rite thin-wall PCR tubes (USA Scientific Inc., Orlando, FL)
2. 10× PCR buffer (Invitrogen, Carlsbad, CA)
3. 2 mM dNTP solution (New England Biolabs, Ipswich, MA)
4. 50 mM MgCl₂ (New England Biolabs, Ipswich, MA)
5. Primer A, Primer C, and Template (custom orders from Integrated DNA Technologies, Coralville, IA)
6. [α ³²P]-deoxyadenosine-5'-triphosphate (PerkinElmer, Waltham, MA)
7. Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA)

2.7. Activity Assays

1. 96-well plate (Thermo Scientific, Rochester, NY)
2. 96-well adhesive plate cover (PGC Scientifics, Frederick MD)
3. 2× selection solution (unique to each selection; see the details in the Methods section)
4. Loading buffer with dye (8 M urea, 50 mM EDTA, 1 × TBE, 0.3% (w/v) bromophenol blue and 0.3% (w/v) xylene cyanol)
5. Plastic transparency sheet
6. Phosphorimager screen and cassette (Amersham Biosciences, Piscataway, NJ)
7. Storm 430 Phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA)
8. ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA)
9. OriginPro software (OriginLab, Northampton, MA)

⁷These markers are the length of the intact pool (117 nt), and the length of the cleaved pool (89 nt). Choose random sequences of DNA that will not form homo- or heterodimers with the random pool.

3. Methods

3.1. Design of DNAzyme Selection Pool

1. Choose a length of random region, and decide whether or not to include secondary structure in it.
2. Choose a ribonucleotide-nucleotide cleavage junction (*see* Note 8).
3. Design the sequence of the random pool. As shown in Fig. 1, the random pool is 117 nucleotides long, and is designed so that as the six-base complementary regions anneal (CR1 binding to CR1' and CR2 binding to CR2'), the pool folds back on itself, holding the cleavage site in place across from the random region that will give rise to the DNAzyme. Using the Random DNA Generator, choose 6-base complementary regions for the pool. Using Fig. 1 as a guide, design the rest of the pool. (Note: the individual bases shown in Fig. 1 show one possible pool design, and this protocol describes how to choose a new pool design).
4. Use OligoAnalyzer and/or mfold to analyze the sequences generated (*see* Note 9). For parameters, use room temperature and the monovalent and divalent concentrations of metal ions present during PCR. See if the sequence's most stable predicted secondary structure shows the pool folding as shown in Fig. 1, and if the sequence forms homodimers. If it is prone to form unwanted secondary structures with high melting temperature or homodimers, identify the bases giving rise to the problem, use OligoAnalyzer to generate a new sequence, and try again (*see* Note 10).
5. Using the results from the previous step, and referencing Fig. 2 and Table 1, design the four oligonucleotides that will be used to construct or regenerate the random pool.
6. Use OligoAnalyzer to analyze these four oligonucleotides. Ensure that the primers have similar melting temperatures, and that they do not form homodimers, heterodimers, or unwanted secondary structures.

3.2. Pool Generation (*see* Note 11)

1. Make two batches of extension solution, and one batch of amplification solution, following Tables 2 and 3.
2. Aliquot the two batches of extension solution into twenty PCR tubes, each containing 99 μ L of solution.
3. Add 1 μ L of *Taq* to each tube.

⁸It has been found that the sixteen choices (rAA, rAC, rAG, rAT, etc.) vary in their cleavage rate, Pb²⁺ sensitivity, and susceptibility to producing the common 8-17 DNAzyme motif.

⁹Use "N" for the random region, but include only five nucleotides in the random region, to separate the parts of the pool surrounding the random region, but not to generate a large number of side products from the random region binding to itself. The random region will interact with itself, but the point of this step is to ensure that there is minimal unwanted interactions between the primer-binding regions.

¹⁰You can alter the % GC content for your own advantage during this process as well.

¹¹When working with radiolabeled DNA, standard radiation safety procedures should be followed, and all waste should be labeled and disposed of properly. Always use filtered pipettes for radioactive solutions.

4. Place the tubes in a PCR thermocycler and proceed with the protocol shown in Table 4 (*see* Note 12). At step 8, add 13.4 μ L of amplification solution to each tube.
5. Divide the contents of all 20 PCR tubes into eight 1.5 mL centrifuge tubes, with each containing 284 μ L of solution.
6. Ethanol precipitate the random pool by adding 28.4 μ L of 3 M sodium acetate, pH 5.2 (10% of the DNA solution's volume) and 710 μ L of 200 proof ethanol (250% of the DNA solution's volume). Pipette to mix, and place in a -80°C freezer for one hour. Centrifuge at 17 krpm for 30 minutes at -4°C.
7. Pipette off and discard the supernatant, being careful not to disturb the pellet DNA on the side of the tube.
8. Lyophilize in the Vacufuge until dry (*see* Note 13).

3.3. 5'- γ ³²P Labeling of DNA Pool

1. Make the solution described in Table 5.
2. Heat the solution at 37°C for 1.5 hours in a PCR thermocycler.
3. Desalt the solution using a Sep-Pak Plus[®] C18 cartridge. Prepare the cartridge by washing it with 10 mL 95% (w/w) acetonitrile, 10 mL (1:1:1 acetonitrile:methanol:water), 20 mL water, and 10 mL 2 M ammonium acetate (*see* Note 14).
4. Add 200 μ L of water to the DNA labeling solution, then load this solution on the Sep Pak cartridge.
5. Wash the sample-loaded cartridge with 20 mL of water.
6. Elute the radiolabeled DNA in 2 mL of 1:1:1 acetonitrile:methanol:water.
7. Uncap the solution and immerse it in liquid nitrogen to flash freeze it.
8. Lyophilize it to dryness (*see* Note 15).
9. Dissolve the two samples in water and reconstitute by vortexing and spinning down.

3.4. Polyacrylamide Gel Electrophoresis (PAGE)

1. Prepare a 10% gel stock solution. Combine 250 mL of 40% acrylamide/bisacrylamide, 480 g urea, and 100 mL of 10 $\times$ TBE. Add enough water to make 1 L and stir until dissolved. Filter through a 0.25 μ m filter and store in an airtight container (*see* Note 16).

¹²These PCR conditions should be optimized for the DNA sequences used in each selection. An efficient way to do this is realtime PCR (RT-PCR). Two of the most important parameters to optimize are the annealing temperature and number of PCR cycles.

¹³To protect a sample from dust, punch a hole in a 1.5 mL tube cap with the needle. Cut the cap off, and snap it onto the sample tube.

¹⁴Squeeze the solution through the cartridge at a rate that is dropwise; do not push air through the cartridge or pull air back into the cartridge.

¹⁵This typically is an overnight process, because of the large amount of liquid.

¹⁶Do not refrigerate, because this would cause the urea to precipitate. Note: unpolymerized acrylamide/bisacrylamide is a carcinogen. Handle with care, and change your gloves after working with it.

2. Dilute 10× TBE to make 1× TBE.
3. Place spacers between two glass plates, and tape the two sides and bottom with gel tape (*see* Note 17). Cast a 10% gel by adding 45 µL of TEMED and 45 µL of 25% APS to 35 mL of 10% PAGE gel stock in an Erlenmeyer flask. Swirl to dissolve, and pour between the two glass plates (*see* Note 18). Insert a well comb, and clamp the sides with binder clips (*see* Note 19). Allow the gel to polymerize for ≈ 45 minutes, or until the gel is firm to the touch.
4. Cut the bottom tape from the gel, and carefully rinse the gel to remove any unpolymerized gel.
5. Place the gel on the gel rack, add an aluminum plate to evenly distribute the heat generated during electrophoresis, and pour 1× TBE into the top and bottom buffer reservoirs.
6. Pre-run the gel for 30 minutes at 25 W.
7. Meanwhile, dissolve the lyophilized DNA in water and combine the DNA from all the tubes together (*see* Note 20).
8. Add an equal volume of loading buffer to the reconstituted sample, and, after its pre-run is over, load it on the 10% gel.
9. Run radiolabeled markers alongside the pool (*see* Note 21).
10. Run the gel for 1.5 hours at 25 W.
11. Remove the gel from the rack, remove the top plate, and wrap the gel and bottom plate in plastic wrap. Place a clean plate on top of this, and place in a metal cassette.
12. In a dark developing room, remove the top plate, and place an X-ray film on top of the plastic wrap to expose it to the radiolabeled DNA. With a razor blade, cut an “x” in two opposing corners so that the film and gel can be reoriented later. Replace the top plate, place the assembly back into the metal cassette, and wrap the cassette in a black sleeve.
13. After exposing the film, develop it.

¹⁷Plates should be soaked in detergent (such as RBS 35® detergent (Pierce, Rockford, IL)) between uses, if contaminated with radioactive material.

¹⁸Air bubbles should be carefully removed during this process. First, after pouring the gel solution between the plates, hold the gel vertically and tap it on the bench to remove air bubbles. Then, when inserting the well comb, use it to “scoop out” air bubbles as they rise to the top.

¹⁹Binder clips, such as are available at office stores, are used. It should be noted that four clips are often used, with two on either side, directly across from one another. If the gel is unevenly clamped, it can cause the gel to polymerize crookedly, wreaking havoc on the gel as it runs.

²⁰Because the DNA can be spread around all sides of the tube, it is important to use a large enough volume of water when reconstituting the sample, and to vortex it thoroughly. On the other hand, it is also best to keep the sample as concentrated as possible at all steps. To accomplish this, add ≈200 µL of water to the first tube, vortex it for 2 minutes, spin it down, and transfer the volume to the next tube. Continue this with all of the sample tubes, then lyophilize the sample to concentrate the sample to ≈50 µL.

²¹Adjust the amount of radiolabeled marker so it is of approximately the same activity as the sample. The two marker strands should be labeled separately, and Sep-paked together. The two fractions can be combined, and reconstituted in 100 µL of water. Typically, when fresh, 1 µL of this solution can be added to 10 µL of loading solution with dye, and run in a well alongside the sample. As the marker decays, increase the amount used, being sure that the solution loaded is at least 50% loading solution.

14. Place the film on top of a light box, and place the gel/glass plate assembly on top of the film. Realign the film to the gel using the “x”s cut earlier.
15. With a fresh razor blade, cut out the lower band that runs alongside the upper, 117mer marker (*see* Note 22).
16. Place this gel slice in a 1.5 mL tube, add 1 mL of soak solution, and allow it to soak for 2 hours.
17. After this time, pipette off the first soak batch and transfer it to another tube. Add another 1 mL of soak solution to the gel slice, and allow it to soak for another 2 hours.
18. Pipette off this second solution, combine it with the first batch of soak solution, then ethanol precipitate and lyophilize it per the directions in Section 3.2.

3.5. Positive *In Vitro* Selection (*see* Note 23)

1. Choose the incubation solution pH based on the solubility, stability, and speciation of the metal, and the application the DNAzyme is intended for. Take into account the air sensitivity of the metal in solution, and other factors influencing its stability. If the metal is not stable in solution, make it fresh before use each time. If the metal solution is stable, make a concentrated solution, aliquot it, and freeze it. If the metal ion target is not very soluble, consider including a chelator such as citrate. Include monovalent cations to stabilize the random pool, and buffer the solution to the appropriate pH. Common solution concentrations are 100-500 mM NaCl, and 25-50 mM buffer.

1. When carrying out a selection round, choose a metal concentration higher than that intended for the final application, to increase the probability of the metal ion binding to the target analyte. Common starting concentrations for 2× metal solutions are 1-20 mM.
2. Choose an incubation time for the first selection. 1-5 hours is a usual incubation time for the first selection round.
3. Dissolve the random pool in 2× selection buffer. Denature at 95°C for 3 minutes and anneal by allowing the DNA to cool to room temperature over a 30-minute period.
4. Spin down the sample, then incubate it with an equal volume of 2× metal solution.
5. After the time for the selection has elapsed, add an equal volume of loading buffer to the reaction.
6. Gel purify the selection reaction on a 10% denaturing PAGE gel. Run this against radiolabeled markers, and cut out the lower band corresponding to the cleaved, 89-nt product (*see* Note 24).

²²The upper band is the sequence complementary to the pool, which contains the C₁₈ Stop *Taq* sequence.

²³This section describes a positive selection round. If a negative selection round is to be undertaken, change the metal solution to be incubated with to a metal that you do not desire your pool to be sensitive to. Then, instead of excising and purifying the cleaved pool, excise and purify the uncleaved pool.

²⁴Commonly, the cleaved product will not be visible for the first few rounds. Thus, the marker allows one to cut out the product.

7. Extract the cleaved pool from the gel by soaking it, then ethanol precipitate and lyophilize it as described in Section 3.4.
8. Dissolve the lyophilized, cleaved pool in water ($\approx 60 \mu\text{L}$). Freeze half of it for future use and regenerate the rest to take on to the next round of selection.

3.6. Pool Regeneration

1. Prepare the solutions described in Tables 6 and 7.
2. Perform PCR1 with the solution described above, following the protocol outlined in Table 8.
3. Add 10 μL of the PCR1 product to the PCR2 solution. Following the protocol in Table 8, perform PCR2 with this solution. Freeze the remaining PCR1 volume at -80°C .
4. Purify the PCR2 product on a 10% PAGE gel as described in Section 3.4, but this time at step 14 cut out the regenerated pool that runs alongside the 89 nt marker. Extract, ethanol precipitate, and lyophilize the pool.
5. Use this DNA population for the next round of selection. Repeat the steps in Section 3.5, changing the selection pressures (reaction time and target analyte concentration) as desired.

3.7. Activity Assays (see Note 25)

1. Following the protocol in Section 3.3, 5'- γ ^{32}P label 2 μL of a 10 μM solution (20 pmol) of Primer B. (This radiolabeled primer is now referred to as Primer B*) (*see* Note 26).
2. Dissolve the labeled primer in 20 μL water.
3. Label the DNA population from the round of interest by preparing the solution described in Table 9.
4. Run twenty cycles of PCR (PCR2 as described before).
5. Purify the labeled pool by 10% PAGE. Only one band -- the strand incorporating P3* -- will be visible; the complementary strand will not be visible. Cut out this band and soak as before to extract the DNA.
6. Sep-Pak purify the DNA and lyophilize.
7. Dissolve the DNA population in 2 $\times$ selection buffer. Allow for at least 30 μL of solution for each reaction, and ensure that you have at least two reactions: one with the metal solution used during selection rounds, and a control with water.

²⁵When working with radiolabeled DNA, standard radiation safety procedures should be followed, and all waste should be labeled and disposed of properly. Always use filtered pipettes for radioactive solutions.

²⁶To assess the pool's activity at any given selection round, the DNA pool can be γ -labeled with ^{32}P . This is done instead of incorporating the α -labeled dATP into the strand PCR because a 5'-labeled oligonucleotide is uniformly labeled and can be used in quantitative evaluations while oligonucleotides labeled by the random incorporation of a radioactive nucleotide vary in their radioactivity and cannot be used quantitatively.

8. Denature at 95°C for 3 minutes and anneal by allowing the solution to cool to room temperature over a 30-minute time period.
9. Prepare a 96-well plate with a separate well for each time point to be taken. Pipette 10 µL of loading buffer with dyes into each time-point well, and pipette 60 µL of 2× metal solution into another well, as well as 60 µL of water into a final well. Finally, pipette 30 µL of the prepared pool solution for each reaction.
10. Initiate the reaction by adding 30 µL of the 2× metal solution to the 30 µL of labeled pool solution. Then initiate the control reaction by adding 30 µL of water to the second 30 µL aliquot of labeled pool solution.
11. At predetermined time points, withdraw 5 µL of the metal reaction mixture and add it to 10 µL of loading buffer with dyes. Do the same with the control reaction.
12. Separate the uncleaved pool and product on a 20% PAGE gel (32 W, 2.5 hours).
13. Remove the gel from the rack, transfer it from the glass plate to a plastic transparency sheet, and wrap the gel and plastic sheet in plastic wrap.
14. Expose the gel by placing it in a phosphorimager cassette (*see* Note 27).
15. Image using Molecular Dynamics Storm 430 Phosphorimager.
16. Analyze the fraction of pool cleavage using ImageQuant software. Place boxes of the same area around the uncleaved and cleaved pool bands, and a background position. In a spreadsheet, background subtract the intensity of each band, and compute the percentage of the pool cleaved at each time point in minutes.
17. Plot kinetic curves using OriginPro and fit to the equation $\%P_t = \%P_0 + \%P_\infty (1 - e^{-kt})$, where t is the reaction time in minutes, $\%P_t$ is the percent product at time t , $\%P_0$ is the initial percent product ($t = 0$), $\%P_\infty$ is percent product at the end point of the reaction ($t = \infty$), and k is the observed rate of cleavage.

Acknowledgments

The authors would like to thank Andrea K. Brown, Debapriya Mazumdar, Nandini Nagraj, Tian Lan, and Seyed-Fakhreddin Torabi for developing and fine-tuning this method. This work was supported by the U.S. National Institutes of Health (ES016865), Department of Energy (DE-FG02-08ER64568), and the U.S. National Science Foundation (CTS-0120978 and DMI-0328162).

References

1. Breaker R, Joyce G. A DNA Enzyme that Cleaves RNA. *Chem Biol.* 1994; 1:223–229. [PubMed: 9383394]
2. Li Y, Sen D. A Catalytic DNA for Porphyrin Metallation. *Nat Struct Biol.* 1996; 3:743–747. [PubMed: 8784345]
3. Geyer C, Sen D. Evidence for the Metal-cofactor Independence of an RNA Phosphodiester-Cleaving DNA Enzyme. *Chem Biol.* 1997; 4:579–593. [PubMed: 9281526]
4. Li Y, Liu Y, Breaker R. Capping DNA with DNA. *Biochem.* 2000; 39:3106–3114. [PubMed: 10715132]

²⁷The exposure time depends on the radioactivity of the sample.

5. Schlosser K, Li Y. Biologically Inspired Synthetic Enzymes Made from DNA. *Chem Biol.* 2009; 16:311–322. [PubMed: 19318212]
6. Silverman S. Catalytic DNA (Deoxyribozymes) for Synthetic Applications—Current Abilities and Future Prospects. *Chem Commun.* 2008:3467–3485.
7. Höbartner C, Silverman S. Recent Advances in DNA Catalysis. *Biopolymers.* 2007; 87:279–292. [PubMed: 17647280]
8. Franzen S. Expanding the Catalytic Repertoire of Ribozymes and Deoxyribozymes Beyond RNA Substrates. *Curr Opin Mol Ther.* 2010; 12:223–232. [PubMed: 20373266]
9. McManus S, Li Y. The Structural Diversity of Deoxyribozymes. *Molecules.* 2010; 15:6269–6284. [PubMed: 20877222]
10. Lan T, Furuya K, Lu Y. A Highly Selective Lead Sensor Based on a Classic Lead DNAzyme. *Chem Commun.* 2010; 46:3896–3898.
11. Mazumdar D, Liu J, Lu G, Zhou J, Lu Y. Easy-to-use Dipstick Tests for Detection of Lead in Paints using Non-cross-linked Gold Nanoparticle-DNAzyme Conjugates. *Chem Commun.* 2010; 46:1416–1418.
12. Xiao Y, Rowe A, Plaxco K. Electrochemical Detection of Parts-per-billion Lead via an Electrode-Bound DNAzyme Assembly. *J Am Chem Soc.* 2007; 129:262–263. [PubMed: 17212391]
13. Li J, Lu Y. A Highly Sensitive and Selective Catalytic DNA Biosensor for Lead Ions. *J Am Chem Soc.* 2000; 122:10466–10467.
14. Liu J, Brown A, Meng X, Cropek D, Istok J, Watson D, Lu Y. A Catalytic Beacon Sensor for Uranium with Parts-per-trillion Sensitivity and Millionfold Selectivity. *P Natl Acad Sci USA.* 2007; 104:2056–2061.
15. Tuerk C, Gold L. Systematic Evolution of Ligands by Exponential Enrichment: RNA Ligands to Bacteriophage T4 DNA Polymerase. *Science.* 1990; 249:505–510. [PubMed: 2200121]
16. Li Y, Breaker R. Kinetics of RNA Degradation by Specific Base Catalysis of Transesterification Involving the 2'-Hydroxyl Group. *J Am Chem Soc.* 1999; 121:5364–5372.
17. Vant-Hull, B.; Gold, L.; Zichi, D. Theoretical Principles of In Vitro Selection using Combinatorial Nucleic Acid Libraries. In: Egli, M.; Herdewijn, P.; Matusda, A.; Sangyi, Y., editors. *Current Protocols in Nucleic Acid Chemistry.* Wiley; New York: 2000.
18. Schlosser K, Li Y. DNAzyme-mediated Catalysis with Only Guanosine and Cytidine Nucleotides. *Nucleic Acids Res.* 2009; 37:413–420. [PubMed: 19050014]
19. Lam J, Li Y. Influence of Cleavage Site on Global Folding of an RNA-Cleaving DNAzyme. *Chem Bio Chem.* 2010; 11:1710–1719.
20. Schlosser K, Li Y. A Versatile Endoribonuclease Mimic Made of DNA: Characteristics and Applications of the 8–17 RNA-Cleaving DNAzyme. *Chem Bio Chem.* 2010; 11:866–879.
21. Silverman S. Deoxyribozymes: Selection Design and Serendipity in the Development of DNA Catalysts. *Acc Chem Res.* 2009; 42:1521–1523. [PubMed: 19572701]
22. Schlosser K, Lam J, Li Y. A Genotype-to-Phenotype Map of *In Vitro* Selected RNA-cleaving DNAzymes: Implications for Accessing the Target. *Nucleic Acids Res.* 2009; 37:3545–3557. [PubMed: 19357090]
23. Lam J, Withers J, Li Y. A Complex RNA-Cleaving DNAzyme That Can Efficiently Cleave a Pyrimidine–Pyrimidine Junction. *J Mol Bio.* 2010; 400:689–701. [PubMed: 20630470]

Fig. 1. During the selection process, the pool is a single-stranded DNA that folds back upon itself, as shown here. The pool has two sets of complementary regions (CR1, CR2, and their complements) that makes this folding possible. A cleavage site with an RNA nucleotide adjacent to a DNA nucleotide is marked here, as well as the fifty-base random region. The pool should be designed so that its most energetically favorable state is the folded structure shown here, giving the random region where a DNAzyme is to be selected full access to the cleavage site.

Extension

Primer A: 5'-GAATCACCTACTAGATCACTGAAGATrA
Primer B: 5'-GAATCACCTACTAGATCACTGAAGATrAGACAGACAAACCTCg PCR →
Template: 3'-GATCTAGTGGACTTCTA-TCTGTCTGTTGGGAGCAACAGN₅₀AAGTCCCATACCTAGGCTC-5'

Amplification

Pool: 5'-GAATCACCTACTAGATCACTGAAGATrAGACAGACAAACCTCgTTGTCTTCAGGSTATGGATCCGAG
Primer C: ← PCR 3'-CCCATACCTAGGCTC-C₁₈Spacer-(AAC)₁₂-5'

Fig. 2.

Four oligonucleotides are used to construct or regenerate the pool. Primer A is a truncated version of Primer B, and both primers are complementary to the template. Primer C, on the other hand, is complementary to the pool, and contains a Stop *Taq* region (C₁₈ spacer + AAC repeats) so its complementary product can be distinguished from the pool when PCR products are purified by PAGE.

Table 1

The random pool used for selection is produced from four oligonucleotides. An example is shown here; a unique pool can be designed for each selection performed in parallel, to prevent cross-contamination.

DNA Sequence	Purpose	Length (nt)	Complementary to	Note
Primer A	5'-GAATCACCTACTAGATCACCTGAAGAGATrAGACAGACAACCCCTCG-3'			
	Forward primer; introduce the ribonucleotide	28	Template	Ends with the ribonucleotide
Primer B	5'-GAATCACCTACTAGATCACCTGAAGAGATrA-3'			
	Forward primer; introduce the ribonucleotide; elongate a truncated pool	43	Template	Is identical to Primer A, except it continues past the ribonucleotide
Primer C	5'-(AAC) ₁₂ -C ₁₈ Spacer-CTCGGATCCATACCC-3'			
	Reverse primer	51+C ₁₈ sequence	Random Pool	Includes a C ₁₈ Stop Taq sequence to distinguish the pool from its reverse complement during PAGE
Template	5'-CTCGGATCCATACCCTGAAN50GACAACGAGGGTTGTCTATCTTCAGGGTGATCTAG-3'			
	Provide a basis for the random pool	107	Random Pool	The template is shorter than the random pool because the primers add length to it

Table 2

This solution is used to construct a pool from the template and primer strands.

10× PCR buffer	105 µL
2 mM dNTP solution	105 µL
50 mM MgCl ₂	31.5 µL
100 µM N ₅₀ Template	1.05 µL
10 µM Primer B	21 µL
Water	776 µL

Table 3

Once the pool has been generated, this solution is used to amplify it.

10× PCR buffer	26.4 μL
2 mM dNTP solution	26.4 μL
50 mM MgCl ₂	7.92 μL
10 μM Primer B	57 μL
10 μM Primer C	154 μL
[α- ³² P]-dATP	2 μL

Table 4

This PCR protocol generates a pool to be used in subsequent selection rounds. It should be optimized for each random pool.

Step	Temperature (°C)	Time (minutes)	Note
1	95°	3	
2	52°	1.5	
3	72°	1	
4	93°	1	
5	52°	1	
6	72°	1	
7			Go to step 4 six times
8	85°	3	Add amplification solution
9	93°	1	
10	52°	1	
11	72°	1	
12			Go to step 9 six times
13	72°	3	

Table 5

γ -labeled DNA used during activity assays can be prepared using this solution.

10 μ M of the DNA to be labeled	2 μ L
10 $\times$ forward reaction buffer	2 μ L
γ 32 P dATP	3 μ L
water	10.5 μ L
T4 kinase	2.5 μ L

Table 6

This solution is used to regenerate a cleaved pool which has been incubated with its target metal ion, separated from other products by PAGE, and extracted into buffer.

DNA product	30 μ L
10 $\times$ PCR buffer	10 μ L
2 mM dNTP solution	10 μ L
50 mM MgCl ₂	3 μ L
10 μ M Primer A	4 μ L
10 μ M Primer C	4 μ L
H ₂ O	38 μ L
<i>Taq</i> polymerase	1 μ L

Table 7

One-tenth of the PCR1 reaction is carried on to the second round of PCR, and the remainder is frozen down for future use. This step significantly increases the copy numbers of selection “winners.”

PCR1 product	10 μ L
10 $\times$ PCR buffer	10 μ L
2 mM dNTP solution	10 μ L
50 mM MgCl ₂	3 μ L
10 μ M Primer B	5 μ L
10 μ M Primer C	2.5 μ L
H ₂ O	57.5 μ L
<i>Taq</i> polymerase	1 μ L

Table 8

The same PCR conditions are used for PCR1 and PCR2, and the two protocols differ only by the number of cycles performed. As with the pool generation protocol, this PCR step should be optimized for each random pool.

Step	Temperature (°C)	Time (minutes)	Note
1	95°	3	
2	52°	0.5	
3	72°	1	
4	93°	0.5	
5	52°	0.5	
6	72°	1	
7			Go to step 4 nine times for PCR1 or nineteen steps for PCR2
11	72°	3	

Table 9

To assess a selection's progress, activity assays are performed. This solution is used to label the pool for these activity assays.

PCR1 product	10 μ L
10 $\times$ PCR buffer	10 μ L
2 mM dNTP solution	3 μ L
50 mM MgCl ₂	3 μ L
10 μ M Primer B	1 μ L
10 μ M Primer C	0.5 μ L
10 μ M P3*	5 μ L
water	66.5 μ L
<i>Taq</i> polymerase	1 μ L