Chapter 18

In Vitro Selection of Metal Ion-Selective DNAzymes

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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.

Key words: DNAzyme, In vitro selection, Functional DNA, Deoxyribozyme, Catalytic DNA, Metal ions, Bioinorganic chemistry

1. Introduction

Discovered by Breaker and Joyce (1), DNAzymes demonstrate that DNA is capable of much more than passive genetic 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 ribozymes. 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

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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+} (10, 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, DNAzyme-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. In vitro selection is a powerful method to achieve the goal. During in vitro selection, a DNA pool of ~10^{14} sequences is incubated with a solution of the selection target under application-relevant conditions (1, 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 sequences in those families, are 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 DNAzyme consists of an enzyme strand that hybridizes to a substrate strand containing a ribonucleotide in its center. The phosphodiester bond in RNA is ~10^5 times more readily hydrolyzed than that in DNA, making it the most susceptible region of the substrate (16). The DNAzyme 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 DNAzyme 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

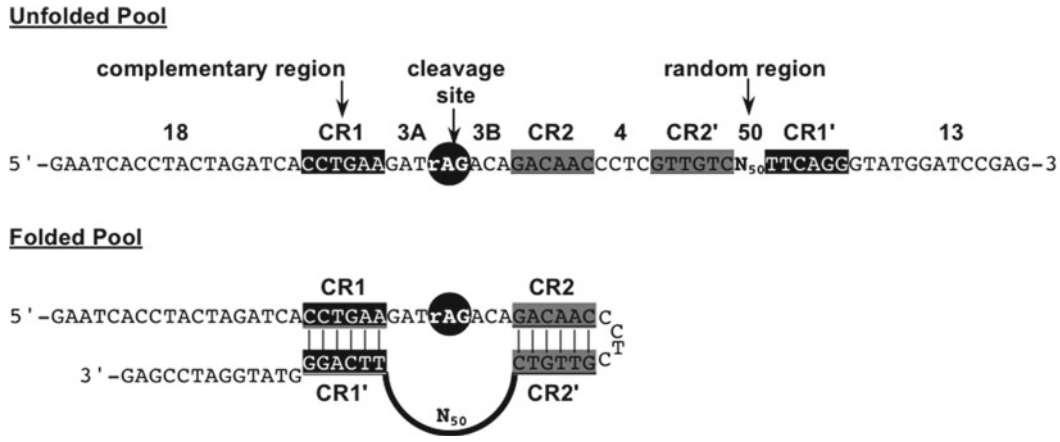

Fig. 1. During the selection process, the pool is a single-stranded DNA that folds back upon itself, as shown here. The pool has two sets of complementary regions (CR1, CR2, and their complements) that makes this folding possible. A cleavage site with an RNA nucleotide adjacent to a DNA nucleotide is marked here, as well as the 50-base random region. The pool should be designed so that its most energetically favorable state is the folded structure shown here, where the random region has full access to the cleavage site.

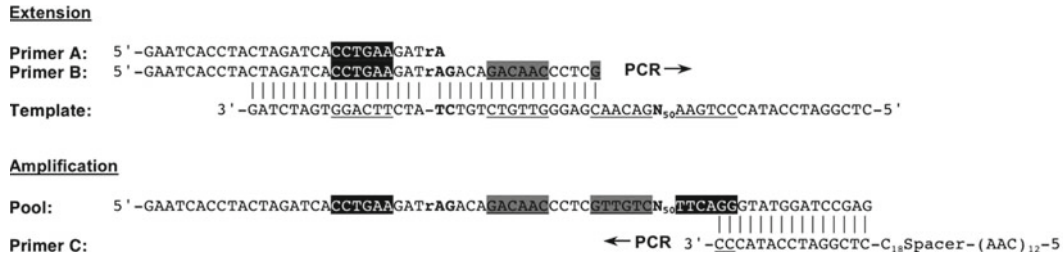

Fig. 2. Four oligonucleotides are used to construct or regenerate the pool. Primer A is a truncated version of Primer B, and both primers are complementary to the template. Primer C, on the other hand, is complementary to the pool, and contains a Stop *Taq* region (C_{18} spacer + AAC repeats) so its complementary product can be distinguished from the pool when PCR products are purified by PAGE.

Primers A–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 25 nucleotide random region was used, the entire sequence space could theoretically be covered if a selection began with a 2 nmol 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) (21). These two types of selection rounds can be used in conjunction with selection pressures to select robust DNAzymes. A round can be made more stringent by decreasing the incubation time and/or the analyte concentration. When varying these characteristics, the results from previous selections should be taken into account. It is recommended the pool's activity be assessed multiple times throughout 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-, 200-, and 1,000-μL barrier pipette tips.
2. 200-μL Seal-Rite thin-wall PCR tubes.
3. 1.7-mL microtubes.
4. 10× PCR buffer without $MgCl_2$ (see Note 2).
5. 2 mM dNTP solution (see Note 3).
6. 50 mM $MgCl_2$.
7. Primer B, Primer C, and Template (see Note 4).
8. [α ^{32}P]-deoxyadeonisine-5′-triphosphate.
9. Millipore water (see Note 5).
10. Platinum *Taq* DNA Polymerase.
11. C1000 PCR thermocycler (Bio-Rad, Hercules, CA).
12. 3 M sodium acetate (pH 5.2; made from sodium acetate dihydrate).
13. Ethanol.
14. Vacufage plus vacuum concentrator (Eppendorf, Hauppauge, NY).

2.3. 5′-γ ^{32}P Labeling of DNA Pool

1. DNA.
2. [γ^{32}P]-deoxyadeonisine-5′-triphosphate.
3. 10× forward reaction buffer (New England Biolabs, Ipswich, MA).
4. T4 Kinase.
5. Sep-Pak Plus® C18 cartridges (Waters Corporation, Milford, MA).
6. Acetonitrile.
7. Methanol.
8. 2 M ammonium acetate.
9. Vacufage plus vacuum concentrator (Eppendorf, Hauppauge, NY).

2.4. Polyacrylamide Gel Electrophoresis

1. Tris[hydroxymethyl]aminomethane.
2. Boric acid, crystalline powder, electrophoresis grade.
3. Ethylenediaminetetraacetic acid, disodium salt, dihydrate, crystalline powder, electrophoresis grade.
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).
6. Tetramethylethylenediamine (TEMED).
7. 40% acrylamide/bisacrylamide (3.3% C).
8. Urea, high purity grade.
9. Gel electrophoresis apparatus.
10. Electrophoresis power supply.
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).
14. 3 M sodium acetate (pH 5.2).
15. Ethanol.
16. Vacufage plus vacuum concentrator (Eppendorf, Hauppauge, NY).

2.5. Positive In Vitro Selection

1. 2× selection solution (unique to each selection; see the details in Subheading 3).
2. Loading buffer (8 M urea, 50 mM EDTA, 1× TBE).
3. Bromophenol blue, electrophoresis purity.
4. Xylene cyanol FF, electrophoresis purity.
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 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.
2. 10× PCR buffer.
3. 2 mM dNTP solution.
4. 50 mM $MgCl_2$.
5. Primer A, Primer C, and template.
6. [α ^{32}P]-deoxyadeonisine-5′-triphosphate.
7. Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA).

2.7. Activity Assays

1. 96-Well plate.
2. 96-Well adhesive plate cover.
3. 2× Selection solution (unique to each selection; see the details in Subheading 3).
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).

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).

Table 1
Oligonucleotides used to construct or regenerate the selection pool

DNA sequence	Purpose	Length (nt)	Complementary to	Note
Primer A	5′-GAATCACCTACTAGATCACCTGAAGAT**rAG**ACAGACAACCCTCG-3′			
	Forward primer; introduce the ribonucleotide	28	Template	Ends with the ribonucleotide
Primer B	5′-GAATCACCTACTAGATCACCTGAAGAT**rA**-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′-CTCGGATCCATACCCTGAA**N50**GACAACGAGGGTTGTCTGTCTATCTTCAGGTGATCTAG-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

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 20 PCR tubes, each containing 99 μL of solution.
3. Add 1 μL of *Taq* to each tube.
4. Place the tubes in a PCR thermocycler and proceed with the protocol shown in Table 4 (see Note 12). At the eighth PCR step shown in Table 4, add 13.4 μL 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 μL of solution.
6. Ethanol precipitate the random pool by adding 28.4 μL of 3 M sodium acetate, pH 5.2 (10% of the DNA solution's volume) and 710 μL of 200 proof ethanol (250% of the DNA solution's volume). Pipette to mix, and place in a −80°C freezer for 1 h. Centrifuge at 17 krpm (≈32,000 × *g*) for 30 min 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 Vacufage until dry (see Note 13).

3.3. 5′-γ^{32}P Labeling of DNA Pool

1. Make the solution described in Table 5.
2. Heat the solution at 37°C for 1.5 h 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 μL of water to the DNA labeling solution, then load this solution on the Sep Park 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).

Table 2
Extension solution (make two)

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
Amplification solution (make one)

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
PCR protocol for pool generation

Step	Temperature (°C)	Time (min)	Note
1	95	3	
2	52	1.5	
3	72	1	
4	93	1	
5	52	1	
6	72	1	
7			Run PCR steps 4 through 6 (93˚, 52˚, 72˚) six times
8	85	3	Add amplification solution
9	93	1	
10	52	1	
11	72	1	
12			Run PCR steps 9 through 11 (93˚, 52˚, 72˚) six times
13	72	3	

Table 5
5′-γ ^{32}P DNA labeling solution

10 μM of the DNA to be labeled	2 μL
10× Forward reaction buffer	2 μL
γ ^{32}P dATP	3 μL
Water	10.5 μL
T4 kinase	2.5 μL

9. Dissolve the two samples in water and reconstitute by vortexing and spinning down.

3.4. Polyacrylamide Gel Electrophoresis

1. Prepare a 10% gel stock solution. Combine 250 mL of 40% acrylamide/bisacrylamide, 480 g urea, and 100 mL of 10× TBE. Add enough water to make 1 L and stir until dissolved. Filter through a 0.25-μm filter and store in an airtight container (see Note 16).
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 min, 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 min 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 h 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.
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 h.
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 h.
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 Subheading 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.
2. 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.
3. Choose an incubation time for the first selection. 1–5 h is a usual incubation time for the first selection round.
4. Dissolve the random pool in 2× selection buffer. Denature at 95°C for 3 min and anneal by allowing the DNA to cool to room temperature over a 30-min period.
5. Spin down the sample, then incubate it with an equal volume of 2× metal solution.

6. After the time for the selection has elapsed, add an equal volume of loading buffer to the reaction.
7. 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).
8. Extract the cleaved pool from the gel by soaking it, then ethanol precipitate and lyophilize it as described in Subheading 3.4.
9. Dissolve the lyophilized, cleaved pool in water (~60 μL). 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.

Table 6
PCR1 solution: PCR extension of cleaved product

DNA product	30 μL
10× PCR buffer	10 μL
2 mM dNTP solution	10 μL
50 mM $MgCl_2$	3 μL
10 μM Primer A	4 μL
10 μM Primer C	4 μL
H_2O	38 μL
Taq polymerase	1 μL

Table 7
PCR2 solution: PCR amplification of cleaved product

PCR1 product	10 μL
10× PCR buffer	10 μL
2 mM dNTP solution	10 μL
50 mM $MgCl_2$	3 μL
10 μM Primer B	5 μL
10 μM Primer C	2.5 μL
H_2O	57.5 μL
Taq polymerase	1 μL

Table 8
PCR conditions to regenerate pool

Step	Temperature (°C)	Time (min)	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 19 steps for PCR2
11	72	3	

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 Subheading 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 Subheading 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 Subheading 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 20 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.

Table 9
PCR2*: labeling of a DNA population for an activity assay

PCR1 product	10 μL
10× PCR buffer	10 μL
2 mM dNTP solution	3 μL
50 mM $MgCl_2$	3 μL
10 μM Primer B	1 μL
10 μM Primer C	0.5 μL
10 μM P3*	5 μL
Water	66.5 μL
Taq polymerase	1 μL

7. Dissolve the DNA population in 2× 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.
8. Denature at 95°C for 3 min and anneal by allowing the solution to cool to room temperature over a 30-min 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 h).
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 $\%Pt = \%P_0 + \%P\infty\ (1 - e^{-kt})$, where t is the reaction time in minutes, $\%Pt$ 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.

4. Notes

1. Any 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 min, and then in 3 M NaOH for 2 min, 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 ~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.
2. This solution is included with every *Taq* DNA polymerase purchase.
3. Dilute from the 10 mM solution provided by New England Biolabs.
4. DNA 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.

5. The water used for all experiments was purified using a Milli-Q system (Millipore, Billerica, MA).
6. The 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.
7. These 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.
8. It has been found that the 16 choices (rAA, rAC, rAG, rAT, etc.) vary in their cleavage rate, Pb^{2+} sensitivity, and susceptibility to producing the common 8–17 DNAzyme motif.
9. Use "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.
10. You can alter the %GC content for your own advantage during this process as well.
11. When 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.
12. These 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.
13. To 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.
14. Squeeze 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.
15. This typically is an overnight process, because of the large amount of liquid.
16. Do 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.

17. Plates should be soaked in detergent (such as RBS 35® detergent (Pierce, Rockford, IL)) between uses, if contaminated with radioactive material.
18. Air 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.
19. Binder 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.
20. Because 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 min, 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.
21. Adjust 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.
22. The upper band is the sequence complementary to the pool, which contains the C_{18} Stop *Taq* sequence.
23. This 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.
24. Commonly, the cleaved product will not be visible for the first few rounds. Thus, the marker allows one to cut out the product.
25. When 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.

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

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