Chapter 10

In Vitro Selection of Deoxyribozymes for the Detection of RNA Modifications

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Abstract

Deoxyribozymes are artificially evolved DNA molecules with catalytic abilities. RNA-cleaving deoxyribozymes have been recognized as an efficient tool for detection of modifications in target RNAs and provide an alternative to traditional and modern methods for detection of ribose or nucleobase methylation. However, there are only few examples of DNA enzymes that specifically reveal the presence of a certain type of modification, including N^6-methyladenosine, and the knowledge about how DNA enzymes recognize modified RNAs is still extremely limited. Therefore, DNA enzymes cannot be easily engineered for the analysis of desired RNA modifications, but are instead identified by in vitro selection from random DNA libraries using synthetic modified RNA substrates. This protocol describes a general in vitro selection stagtegy to evolve new RNA-cleaving DNA enzymes that can efficiently differentiate modified RNA substrates from their unmodified counterpart.

Key words RNA, Deoxyribozymes, Modified RNA nucleotides, Catalytic DNA, Epitranscriptomics, In vitro selection, RNA cleavage

1 Introduction

Cellular RNAs can be modified posttranscriptionally through chemical modifications on nucleobases or the ribose-phosphate backbone. The flourishing field of "epitranscriptomics" explores the modifications that are functionally relevant to RNA structure, stability, base pairing, and binding potential to proteins and other ligands [1, 2]. Besides the reversible chemical modifications of DNA and proteins, posttranscriptional RNA modifications provide another layer of regulation for gene expression [3]. Although modified nucleotides are found in many different types of coding and noncoding transcripts and the precise roles are far from being completely understood, several tRNA and rRNA modifications are expected to play specific structural and functional roles [4]. For example, since ribosomal rRNA modifications are installed early in rRNA processing, it is possible that they assist RNA folding

or are involved in the recruitment of chaperone proteins [5]. Interestingly, many ribosomal RNA modifications are evolutionary conserved but individual modifications are often not essential, while a global lack of rRNA modifications diminishes viability. Recently it was found that some rRNA modifications are installed substoichiometrically, resulting in different subpopulations of ribosomes with altered fitness or fine-tuned activity for translation of specific mRNA [6, 7].

Although more than 120 different modifications in various types of RNAs are known for decades, the field is still lagging on technologies for transcriptome-wide mapping for most of these modifications. Several techniques such as two-dimensional thin layer chromatography (2D-TLC), high-resolution liquid chromatography coupled to mass spectrometry (HPLC-MS), methylated RNA immunoprecipitation, and reverse transcriptase–based signatures followed by deep sequencing have been used to map modifications in RNA [8–12]. These techniques, however, suffer from various limitations such as the loss of sequence information upon digestion into mononucleotides as required for TLC and MS approaches, and from low specificity and selectivity of antibodies used for immunoprecipitation. Therefore a combination of different methods is necessary to obtain reliable insights into presence, distribution and abundance of certain modified nucleotides, and simple analytical tools are needed for validation [13].

Deoxyribozymes (alternatively called DNA enzymes) are attractive tools to expand the repertoire of methods for detecting RNA modifications in a sequence-specific manner. Deoxyribozymes are in vitro selected DNA molecules that have the potential to catalyze various chemical reactions, including protein modifications, DNA/RNA ligation, and DNA/RNA cleavage [14–16]. Among these, DNA enzymes that catalyze a site-specific RNA cleavage reaction are the most prominent group [17, 18].

Deoxyribozymes have a catalytic core that originates from a random region of 20–40 nucleotides and that is flanked by two binding arms complementary to the RNA substrate. DNA enzymes require metal ions (Mg^{2+}, Mn^{2+}, Zn^{2+}) for their catalytic activity. RNA-cleaving deoxyribozymes catalyze the cleavage reaction by mediating the attack of the 2′-hydroxyl group onto the adjacent phosphodiester linkage, which results in the formation of 2′,3′-cyclic phosphate and 5′-hydroxyl termini [18, 19]. This reaction gives the possibility to detect modifications that directly block the functional group in the cleavage reaction, such as 2′-O-methylation. In fact, this resulted in the first application of DNA enzymes to analyze ribosomal RNA modifications [20]. Alternatively, deoxyribozymes have been used to detect RNA modifications on the 5′-terminus of the cleavage site. DNA enzymes 8–17 and 10–23 [21] oriented to cleave various dinucleotide junctions served as tool for cleavage of phosphodiester linkage followed by radioactive

labeling and 2D TLC for site-specific analysis of pseudouridine [22], one of the most important ribonucleotide modifications in rRNA. Recently, deoxyribozymes have been explored to sense other modifications such as N^6-methyladenosine (m^{6}A) and N^6-isopentenyladenosine (i^{6}A) [23, 24]. The m^{6}A-sensitive DNA enzymes have been shown to be generally applicable to analyze the presence of m^{6}A in DGACH sequence motifs, as demonstrated for lncRNAs and a set of C/D box snoRNAs that function as guides for 2′-O ribose methylation of ribosomal RNA. These recent examples demonstrated that catalytic DNA can likely be developed for various modifications as a tool to differentiate modified from unmodified RNA in a sequence-specific manner.

Deoxyribozymes are identified through in vitro selection based on the systematic evolution of ligands by exponential enrichment (SELEX) technique from a random pool of DNA through repetitive cycles of selection and amplification as shown in Fig. 1.

This chapter gives a detailed protocol for the gel-based SELEX technique to evolve catalytically active DNA species from a random pool that is capable of specifically detecting RNA modification, thus leading to an enhanced/reduced cleavage of the target RNA depending on the modification state. The in vitro selection cycle begins with the ligation of the DNA pool to the RNA substrate containing the desired RNA modification by T4 DNA ligase using a
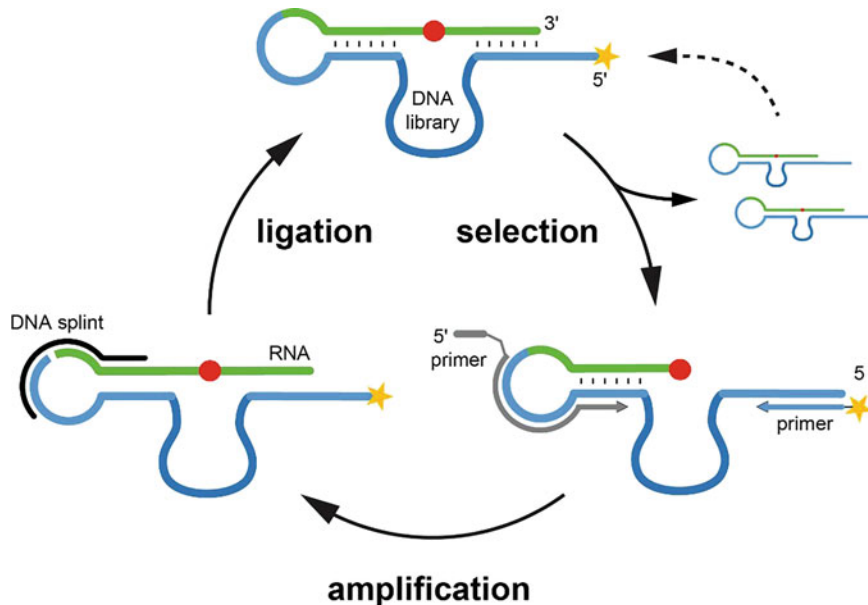

Fig. 1 Overview of the in vitro selection scheme for the generation of modification-sensitive RNA cleaving deoxyribozymes. The red dot represents the modified nucleotide in the RNA substrate (green), in between Watson–Crick-base-paired regions. The constant regions of the DNA are shown in light blue, and the random region of the DNA library in dark blue. The yellow star represents a 5′-label on the DNA facilitating detection on PAGE

DNA splint. The ligated product is then incubated with Mg^{2+} at 37 °C to initiate the cleavage reaction. The active fraction is isolated by PAGE due to the change in size. Active DNA enzymes are then amplified through PCR using a 5'-labeled forward primer (fluorescein or ^{32}P-labeled for detection of bands on PAGE) and a tailed reverse primer containing a nonextendable ethylene glycol spacer to allow separation of sense and antisense strands. After isolation of the single-stranded PCR product through denaturing PAGE, the enriched active species are ligated to the RNA substrate to initiate the next round of selection. To increase the specificity of the resulting deoxyribozymes, negative selection rounds are introduced, in which the DNA library is challenged with the unmodified RNA. Other factors that can be adapted to enhance specificity are metal ion concentration, selection time and temperature. After several rounds of in vitro selection, the enriched pool is tested for its ability to discriminate modified from unmodified RNA. Finally, individual deoxyribozymes are identified by traditional cloning and Sanger sequencing and/or from Illumina NGS datasets that allow a deeper analysis of the enrichment of certain sequence motifs. New candidate DNA enzymes are characterized by analyzing cleavage kinetics for modified and unmodified RNA substrates and mutational analyses are performed to identify key sequence motifs responsible for recognition of the RNA modification. In exceptional cases, the modified nucleotide in the RNA may lead to switch in the cleavage site of the endonuclease deoxyribozyme, providing an additional opportunity for quantitative readout of the modification level [24].

2   Materials

2.1   Oligonucleotides

1. Use ultrapure water for all oligonucleotides, buffers, and reactions.

2. Purify oligonucleotides by denaturing PAGE before use and store all oligonucleotide solutions and buffers at −20 °C.

3. RNA substrates with and without modification (for counter selection), prepared by solid-phase synthesis on 0.5–1 μmol scale, using commercially available or in-house synthesized phosphoramidites of modified nucleotides. A fraction of RNA substrate is labeled at 3'-end or 5'-end for kinetic assays.

4. Deoxyribozyme library: 0.5 μmol synthesis scale, 100 μM stock solution.

5. Primers: 5'-fluorescein labeled forward primer and PEG$_3$-linked tailed reverse primer. 0.5 μmol synthesis scale, 100 μM solution.

6. Splint DNA for ligation of DNA library to RNA substrate. 0.5 μmol synthesis scale, 100 μM solution.

2.2 Denaturing Polyacrylamide Gel Electrophoresis

1. $10\times$ TBE buffer: 89 mM Tris–HCl, pH 8.0 at 25 °C, 89 mM boric acid, 50 mM EDTA.

2. Acrylamide gel stock solution: acrylamide solution (10% and 20%), 7 M Urea, $1 \times$ TBE (*see* **Note 1**).

3. 25% ammonium persulfate (APS). Store at 4 °C.

4. N,N,N',N'-tetramethyl ethylenediamine (TEMED). Store at 4 °C.

5. PAGE loading buffer: 70% formamide, $1\times$ TBE buffer, 50 mM EDTA, 0.4 mM bromophenol blue, 0.4 mM xylene cyanol, pH 8.0.

6. Elution buffer: 10 mM Tris, 1 mM EDTA, 300 mM NaCl, pH 8.0.

7. Absolute ethanol and 70% ethanol. Store at −20 °C.

8. Glass plates (20 cm × 20 cm and 20 cm × 30 cm), combs, and spacers.

2.3 In Vitro Selection

2.3.1 Phosphorylation of RNA Selection Substrates

1. T4 PNK enzyme (10 U/μL).

2. T4 PNK buffer A: 500 mM Tris–HCl, 10 mM $MgCl_2$, 500 mM DTT, 1 mM spermidine.

3. 10 mM ATP.

4. Roti® phenol–chloroform–isoamyl alcohol for extraction of RNA.

5. Roti® chloroform.

2.3.2 Splint Ligation

1. T4 DNA ligase enzyme (5 U/μL). Store at −20 °C.

2. T4 DNA ligase buffer: ($10\times$) 400 mM Tris–HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8. Store at −20 °C.

3. $10\times$ Annealing buffer: 40 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0.

2.3.3 Selection Step

1. $10\times$ Selection buffer: 500 mM Tris–HCl, 1500 mM NaCl, pH 7.5.

2. 400 mM $MgCl_2$ (stock solution).

2.3.4 PCR Amplification

1. DreamTaq DNA polymerase. Store at −20 °C.

2. DreamTaq buffer®: ($10\times$) 20 mM $MgCl_2$, KCl, $(NH_4)_2SO_4$. Store at −20 °C.

3. dNTPs mixture (20 mM each dNTP).

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2.3.5   Kinetic
Characterization of
Deoxyribozymes

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1. 10× Kinetic assay buffer: 500 mM Tris–HCl, 1500 mM NaCl, pH 7.5.

2. 400 mM MgCl$_2$ (stock solution).

3. Quench buffer: 70% formamide, 1× TBE buffer, 50 mM EDTA, pH 8.0.

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3   Methods

3.1   DNA Library Design

1. Select RNA sequence with target modification, inspired by natural sequence context of the modification, for example, in tRNAs or rRNA (or conserved motifs such as DRACH for m^{6}A).

2. For DNA library, select a random region of about twenty nucleotides (*see* **Note 2**). Add flanking sequence complementary to RNA substrate upstream and downstream of the catalytic core, maintain two nucleotides in the vicinity of modified nucleotide unpaired, and add 3′-overhang for splinted ligation to RNA substrate (*see* Fig. 1).

3. Splint DNA should be complementary to 3′-end of deoxyribozyme library and 5′-end of RNA substrate (*see* **Note 3**).

4. Synthesize or custom order appropriately designed deoxyribozyme library (0.5 μmol scale), RNA substrate with and without desired modification, splint DNA and primers.

3.2   Denaturing Polyacrylamide Gel Electrophoresis

Purification of ligated DNA–RNA product, separation of active and inactive fraction of the DNA library, and separation of PCR products into single strands is performed by denaturing polyacrylamide gel electrophoresis.

1. Calculate the volume (×) of gel solution needed to pour gel of desired size, that is, 40 mL for 20 × 20 cm gel size.

2. Assemble the glass plates with spacer of 0.4 mm thickness.

3. Mix × mL of gel solution of desired percentage with ×/400 mL of 25% APS and ×/2000 mL of TEMED.

4. Pour the gel and insert the comb with well size of 3–4 cm.

5. Allow the gel to polymerize for 30 min.

6. Remove the comb and rinse wells thoroughly with ultrapure water.

7. Assemble the gel apparatus and prerun the gel for 30 min to equilibrate the temperature. Set the power to a constant value of 25–35 W depending on the size of the glass plates (25 W for 20 cm × 20 cm and 35 W for 20 cm × 30 cm).

8. Heat the sample for 2 min at 95 °C and then allow it to cool down to 25 °C for 5 min.

9. Switch off the power supply and wash the wells thoroughly with 1× TBE buffer and load the samples to the gel.

10. Run the gel at the same setting as used for prerun for 1–2 h until bromophenol blue reaches the bottom of the gel.

11. Disassemble the gel and transfer the gel between two layers of Saran Wrap.

12. Visualize the bands using fluorescein gel documentation (Gel Doc) imager or UV-transilluminator.

13. Cut the bands with a scalpel and transfer the gel piece to a 1.5 mL test tube for recovery of DNA by crush and soak.

14. Crush the gel pieces by centrifugation at 21,000 rcf for 5 min.

15. Add elution buffer to completely immerse the gel and incubate it at 37 °C for 4 h at 700 rpm.

16. After incubation, transfer the elution buffer to another test tube.

17. Precipitate the oligonucleotide by adding 3 volumes of ethanol.

18. Mix properly by vortex and freeze the sample using liquid nitrogen.

19. Centrifuge for 30 min at 4 °C.

20. Carefully separate the supernatant and wash the pellet with 75 μL of 70% ethanol.

21. Centrifuge for 10 min at 4 °C and dry the pellet in vacuum.

22. Dissolve the pellet in an appropriate volume of ultrapure water.

3.3 In Vitro Selection

3.3.1 Phosphorylation of RNA Selection Substrates

In order to ligate RNA selection substrates with DNA library, RNA has to be phosphorylated at 5′-end.

1. Calculate the concentration of RNA and take 5 nmol of the RNA in 0.7-mL test tube.

2. Add 5 μL of 10× PNK buffer A, 5 μL of ATP (10 mM), 4 μL of T4 PNK enzyme (10u/μL) and adjust the volume to 50 μL.

3. Incubate the reaction mixture at 37 °C for 5 h.

4. Afterward, dilute the reaction mixture to 300 μL with 1× elution buffer.

5. Add equal volume of Roti® phenol–chloroform–isoamyl alcohol to the reaction mixture and vortex it for 1 min followed by centrifugation for 1 min.

6. Carefully separate the supernatant and transfer it to a new test tube.

7. Add equal volume of chloroform and vortex the sample for 1 min followed by centrifugation for 1 min.

8. Carefully separate the supernatant and add 3 volumes of chilled absolute ethanol.

9. Vortex to mix properly and freeze the sample using liquid nitrogen.

10. Centrifuge the sample for 30 min at 4 °C.

11. Carefully separate the supernatant and wash pellet with 75 μL of 70% ethanol.

12. Centrifuge the sample for 10 min at 4 °C and dry the pellet in vacuum.

13. Dissolve the pellet in an appropriate volume (50 μL) of ultra-pure water.

3.3.2 Splint Ligation of RNA Substrate to Deoxyribozyme Selection Pool

Ligation of ssRNA substrate with ssDNA library is facilitated by complementary DNA splint in the presence of T4 DNA ligase.

1. Take 5′-phosphorylated RNA (2 nmol), DNA selection pool (1.6 nmol) and complementary DNA splint (1.8 nmol) in a total volume of 10 μL.

2. Add 1.5 μL of 10× annealing buffer and incubate the reaction mixture at 95 °C for 4 min. After denaturation, allow the solution to cool down to 25 °C for 15 min.

3. Add 1.5 μL of ligase buffer, 2 μL of T4 DNA ligase (5 U/μL) and incubate the sample at 37 °C for 16 h.

4. Add 15 μL of PAGE loading buffer to the reaction mixture.

5. Purify the ligated product (DNA–RNA hybrid) using denaturing PAGE as mentioned under Subheading 3.2.

3.3.3 DNA-Catalyzed Cleavage of DNA–RNA Hybrids (Key Selection Step)

Selection of active DNA enzymes is based on a change in size upon cleavage of the RNA substrate in the vicinity of the modified nucleotide.

1. Take 250 pmol (in a total volume of 7.5 μL) of DNA–RNA hybrid in the first round of selection, and approximately 10–30 pmol in further rounds.

2. Add 1 μL of 10× selection buffer and incubate at 95 °C for 4 min and at 25 °C for 15 min.

3. Add $MgCl_2$ to a final concentration of 5 mM in a final volume of 10 μL and incubate at 37 °C for 16 h.

4. Add 10 μL of PAGE loading dye to the reaction mixture.

5. Separate the active fraction of DNA enzymes using denaturing PAGE and determine the area corresponding to cleaved product by size marker (*see* **Note 4**).

6. Cut the bands and extract the DNA enzymes as mentioned under Subheading 3.2.

7. Dissolve the pellet in 30 μL of ultrapure water.

3.3.4 PCR Amplification of Active DNA Enzymes

The cleaved fraction of the DNA library containing active DNA enzymes is amplified through two subsequent asymmetric PCRs. To visualize the PCR product, the forward primer is labeled with fluorescein at 5′ end and reverse primer has a tail connected through PEG_3 linker to separate sense from antisense strand by denaturing PAGE. Forward primer and reverse primer are used in a ratio of 4:1 to preferentially amplify forward strand with fluorescein label.

PCR-I

1. Use 30 μL of the solution from the previous step (containing active DNA enzymes) as template.
2. Assemble the reaction: 30 μL of DNA template, forward primer (100 pmol), reverse primer (25 pmol), 0.625 μL of 20 mM dNTP mixture, 5 μL of 10× Dream taq buffer, 0.25 μL of Dream taq DNA polymerase (5 U/μL) and water up to 50 μL.
3. PCR conditions are: 95 °C for 4 min [10× (95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min), 72 °C for 5 min].
4. Use an aliquot of the sample for PCR-II and store the rest at −20 °C.

PCR-II

1. Take an aliquot of PCR-I product (2–5 μL) as the template.
2. Assemble the reaction: 5 μL of DNA template, forward primer (200 pmol), reverse primer (50 pmol), 1.25 μL of 20 mM dNTP mixture, 10 μL of 10× Dream taq buffer, 0.5 μL of Dream taq DNA polymerase (5 U/μL), and water up to 100 μL.
3. PCR conditions are: 95 °C for 4 min [30× (95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min), 72 °C for 5 min].
4. Purify the PCR product using denaturing PAGE.
5. Isolate the fluorescein labeled forward strand by extraction and precipitation as described under Subheading 3.2.

3.3.5 Continuation of Selection and Identification of Deoxyribozyme Sequences

Take the single-stranded PCR product from the previous step and ligate it to the RNA substrate using the DNA splint (*see* **Note 3**) and T4 DNA ligase, essentially as described under Subheading 3.3.2, but on a smaller scale (i.e., use only 100 pmol of RNA and 75 pmol of DNA splint in a final volume of 10 μL for the ligation reaction). The isolated ligation product is then subjected to the next round of incubation as described under Subheading 3.3.3.

After 6–8 rounds of in vitro selection, include negative selection rounds to enrich DNA enzymes having high selectivity toward modified RNA and eliminate DNA enzymes that can cleave both modified and unmodified RNA. For this purpose, ligate the DNA pool with the unmodified analog of the RNA substrate. During the selection step, perform all steps in identical manner and analyze the
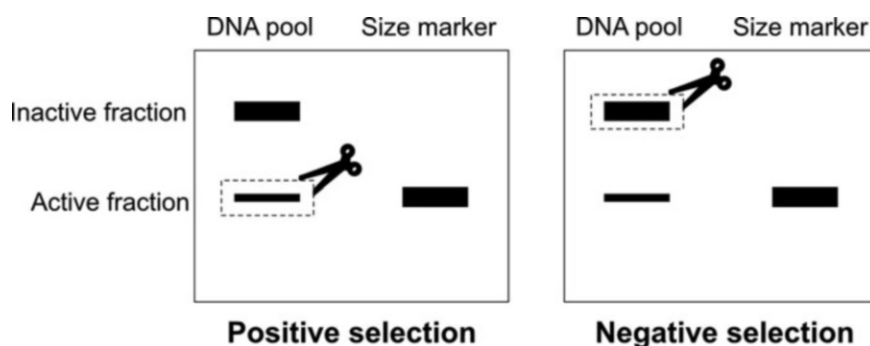
Fig. 2 Schematic presentation of polyacrylamide gel electrophoresis (PAGE) for positive and negative selection rounds. The diagram presents the area of the gel from which the corresponding cleaved (positive selection round) or uncleaved fraction (negative selection round) is isolated

catalytic activity of the DNA pool by denaturing PAGE. Note that the uncleaved fraction on gel corresponds to the specific DNA enzymes that remain inactive toward unmodified substrate (*see* **Note 5**). The band signal observed at the height of the size marker corresponds to non-specific DNA enzymes that can cleave both modified and unmodified substrates. Therefore, in the negative selection round, the uncleaved fraction is isolated from the gel (Fig. 2) and amplified by PCR as described above. Each negative round is followed by a positive round (during these positive rounds, increase the stringency of the selection by decreasing the incubation time, i.e., 6 h, 3 h, and 1 h) and continue the cycle at least for four rounds of alternating negative and positive selections.

Afterward, amplify the active DNA pool through PCR. Due to non–template-dependent terminal transferase activity of *Taq* polymerase, the product will contain a 3′-A overhang that allows TOPO-TA cloning (commercially available kit). Select the individual clones and subject them to Sanger sequencing. Each sequence obtained from Sanger Sequencing data corresponds to individual deoxyribozyme candidates. These sequences are then synthesized by solid-phase syntheses and their catalytic potential and discrimination power for RNA modification are assessed by kinetic assays.

3.4 Kinetic Characterization of Deoxyribozymes

Kinetic characterization analyzes the *trans*-activity of individual deoxyribozyme to cleave modified and unmodified RNA (that are not ligated to the DNA library). Therefore, for each deoxyribozyme, the assay is performed in parallel with both, modified and unmodified RNA substrates of otherwise the same sequence. This allows to compare the cleavage rates and to determine calibration curves that are needed for quantitative estimation of modification levels.

1. Take 100 pmol of deoxyribozyme, 10 pmol of fluorescent labeled RNA substrate and adjust to a final volume of 7.5 μL.

2. Incubate at 95 °C for 4 min and 25 °C for 15 min.

3. Add 1 μL of 10× selection buffer and 0.5 μL of 400 mM MgCl$_2$ (final concentration 20 M) to start the reaction.

4. Incubate at 37 °C and take aliquots (1 μL) at different time points (e.g., 0, 10 min, 30 min, 60 min, 120 min, 180 min, 360 min).

5. Quench the reaction with 4 μL of quench buffer and analyze by denaturing PAGE. Take an image of the gel on a gel documentation system, determine the band intensities of cleaved and uncleaved RNA, and calculate cleavage yield and the observed rate constant (k_{obs}) (*see* **Note 6**).

4 Notes

1. For all in-vitro selection steps in this protocol use 10% acrylamide gel solution and for the analysis of *trans*-activity of individual DNA enzymes use 20% acrylamide stock solution.

2. For new selections, keep the catalytic core sequence completely random. The choice of 20 randomized positions allows a large fraction of the sequence space to be covered in one experiment. Larger random regions of 40 or more nucleotides have also be used successfully in earlier deoxyribozyme selections. To improve the selectivity of the obtained catalysts, reselection can be initiated from partially randomized DNA libraries. Also, the number and identity of the unpaired nucleotides around the desired cleavage site next to the modified nucleotide can be varied.

3. After PCR amplification, DNA library has 3′-A overhang due to the non-template dependent terminal transferase activity of *Taq* polymerase which reduces ligation efficiency. To overcome this problem, insert an extra T to the splint to base pair with 3′-A overhang and use it for all further rounds of selection starting from round 2, following a strategy introduced by [25].

4. Size marker is the oligonucleotide corresponding to the length of product size expected to be obtained after the selection round. It is prepared by ligating only the 5′-part of the RNA sequence without modification (corresponding to the expected cleavage product) to the DNA library. However, note that the selection strategy does not enforce a particular cleavage site, but due to the small number of 2–3 unpaired RNA nucleotides around the modification, cleavage usually happens near the modified base in RNA. The exact cleavage site then needs to be identified for each obtained deoxyribozyme by analyzing the size of the cleavage product next to an alkaline hydrolysis ladder.

5. As described in this protocol, the obtained DNA enzymes are trained to recognize the modified nucleotides in the positive selection round, that is, preferentially cleave the RNA substrate only when it contains the modification, but leaves unmodified RNA intact. However, the strategy can also be reversed, such that a modified nucleotide completely inhibits the DNA-catalyzed cleavage of the RNA target. For such a scenario, the positive selection is performed with unmodified RNA, and the modified RNA is used in the negative selection round. In this way, DNA enzymes were identified that are strongly inhibited by m^6A [23].

6. Analyze the cleavage yield by determining fluorescence intensities of the corresponding bands. Plot cleavage yield versus time (min). Determine k_{obs} (observed cleavage rate) and, Υ_{max} (maximum yield) by using the first order kinetic equation: $\Upsilon = \Upsilon_{max} \times (1 - e^{-k_{obs} \times t})$.

Acknowledgments

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