In vitro Evolution of an RNA-Cleaving DNA Enzyme into an RNA Ligase Switches the Selectivity from 3'–5' to 2'–5'

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Figures in this Supporting information are prefixed by the letter X (e.g., Figure X1) to distinguish them from the manuscript Figures. All references cited by number are from the manuscript. See the manuscript's Experimental Section and (more comprehensively) ref. 8 for full experimental details.

Calculations of extent of pool randomization

The parent 8–17 DNA enzyme sequence is 13 nucleotides long, and the parent 10–23 sequence is 15 nucleotides long. To plan preparation of the partially randomized deoxyribozyme strands for the selection strategy (see ref. 8), we calculated the distribution of nucleotide changes per molecule, relative to the parent sequence, as a function of the fraction parent nucleotide at each position. Let x = fraction "correct" nucleotide at each individual position (x has the same value for each nucleotide position in a particular selection pool). We define P(n) = the probability of having a total of n changes relative to the parent sequence. It is readily shown that $P(n) = x^{m-n} \bullet (1-x)^n \bullet {}_mC_n$, where m = the length of the sequence and ${}_mC_n$ denotes the combinatorial function of m objects taken n at a time. For the parent 8–17 enzyme, m = 13, and for 10–23, m = 15, and thus P(n) is completely determined by x for each deoxyribozyme. Calculated values of P(n) plotted versus n for various values of x are shown in Figure X1.

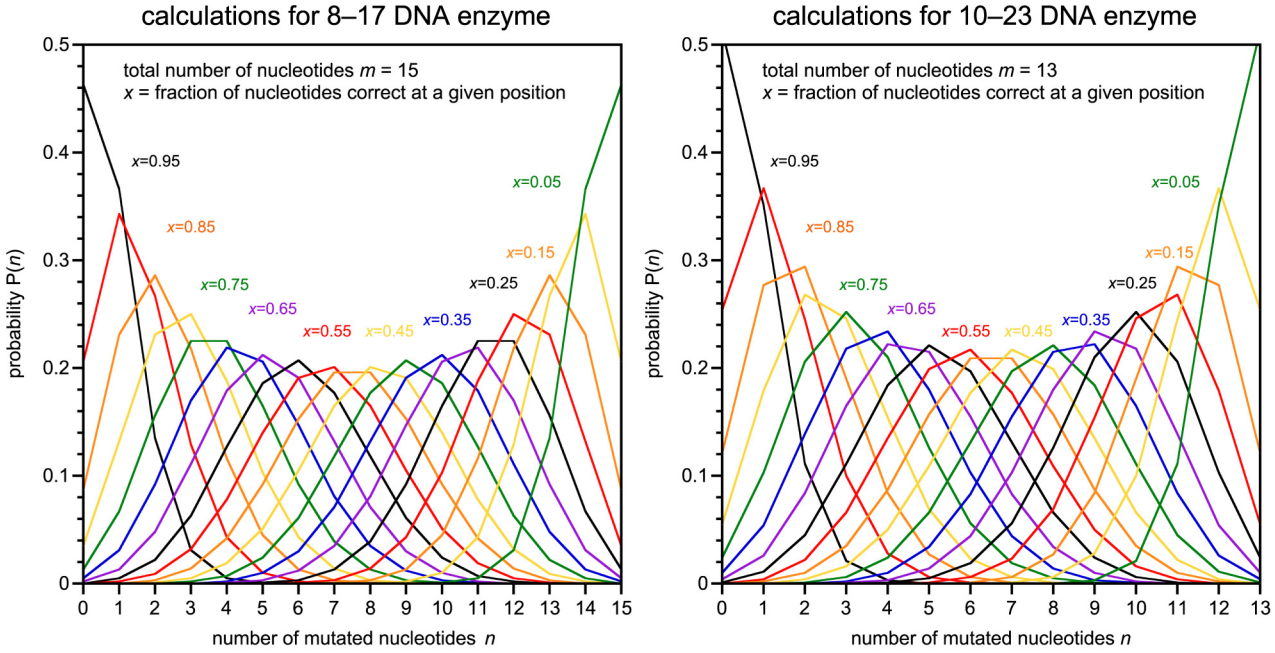

Figure X1. Calculation of probability distribution of number of nucleotide changes n as a function of fraction correct nucleotide x for the random pool DNAs. See text for explanation.

Based on these plots, we chose $x = 0.80$ for the "low randomization" pools and $x = 0.50$ for the "high randomization" pools. For the 8–17 enzyme, these values of x correspond to 2-3 and 6-7 nucleotide changes per molecule as the most likely (mode) values for the "low" and "high" pools, respectively. For the 10–23 enzyme, the most likely values are 2-3 and 7-8 changes. However, it is important to note that there are a significant number of molecules in each pool with many more mutated nucleotides than the most likely value (i.e., the curves have long tails out to higher values of n).

Because each nucleoside phosphoramidite (T, G, C, A) does not couple with equal efficiency during solid-phase DNA synthesis, the four phosphoramidites were mixed in unequal ratios to obtain the desired values of x, such that there was equal likelihood of incorporating any of the three "incorrect" nucleotides (specifically, probability x of inserting the correct nucleotide, and probability $x/3$ of incorporating each of the three incorrect nucleotides). Using the manufacturer's values for relative coupling efficiencies (T 0.30, G 0.26, C 0.24, and A 0.20), the phosphoramidite mixes were prepared as shown in Table X1 (the same mixes were used for the 8–17 and 10–23 pools).

low randomization:	**T**	**G**	**C**	**A**
T*	755	73	79	94
G*	57	787	71	85
C*	54	62	804	80
A*	47	54	58	841
high randomization:	**T**	**G**	**C**	**A**
T*	435	167	181	217
G*	139	480	173	208
C*	135	156	507	203
A*	127	146	158	569

Table X1. Phosphoramidite mixes used to obtain the partially randomized DNA pools with desired ratios of incorporated nucleotides. An asterisk indicates the parent nucleotide. For example, A* denotes that A is found in the parent sequence; where A is found in the parent enzyme, the A* mix was used at that particular position. Tabulated under the boldface letters are relative volumes of 0.1 M stock solutions of each phosphoramidite, where the volumes sum to 1000 for each row (stock solutions were prepared in 1-ml amounts using the indicated values in μL).

Although only the 13- or 15-base "enzyme region" of manuscript Figure 1A was subject to partial randomization in the deoxyribozyme strand, the cloned deoxyribozymes had a varying number of nucleotide changes in the substrate binding arms near the ligation site (compare Figure 1A and Figure 3A). We attribute these changes to errors introduced by *Taq* polymerase during the selection rounds. While not initially sought, these changes may be beneficial. For example, the 7Q10 enzyme has three changes out of seven for the binding arm DNA nucleotides shown in Figure 1A. Also, 7Q5 has a deletion, again attributed to *Taq* polymerase.

RNA ligation experiments at 37 °C

RNA ligation experiments with individual deoxyribozymes were performed in the intermolecular format of Figure X2A, where L denotes the left-hand 17-mer RNA substrate, R is the 17-mer right-hand RNA substrate, and E is the DNA enzyme. Ligation assays were performed with L:E:R ratios of (<1):3:6, typically 5:15:30 pmol in 10-μL reactions. For details of the experiments, see ref. 8. Representative ligation experiments for 7P4, 7Q2, 7Q5, and 7Q10 are shown in Figure X3. Kinetics data with fits from these and analogous experiments are shown as Figure 3C of the manuscript.

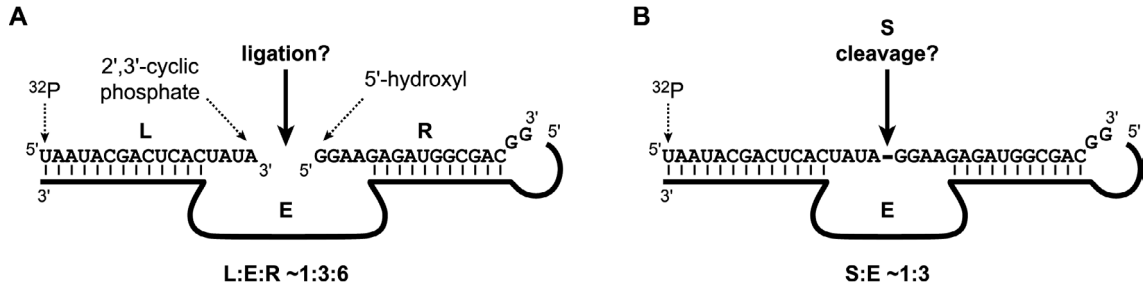

Figure X2. Format of the ligation (A) and cleavage (B) experiments for individual deoxyribozyme clones. The vertical lines indicate canonical Watson-Crick base pairs (the DNA complement sequence is omitted for clarity). L and R denote the left-hand and right-hand RNA substrates for ligation; S is the substrate for cleavage (S can be either 3'–5' or 2'–5' linked at the indicated A↓G site); and E indicates the DNA enzyme.

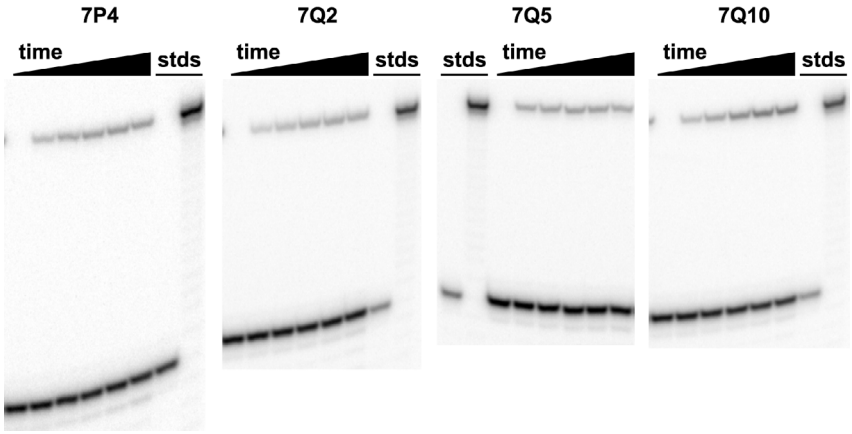

Figure X3. Representative RNA ligation experiments for the 7P4, 7Q2, 7Q5, and 7Q10 deoxyribozymes. Conditions: 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl, 40 mM $MgCl_2$, 37 °C, 10 μL reaction volume; aliquots of 1 μL withdrawn at appropriate timepoints and quenched onto a solution of 80% formamide, 1× TB (89 mM each Tris and boric acid, pH 8.3), 50 mM EDTA containing 0.25% each bromophenol blue and xylene cyanol. Timepoints at 0, 1.5, 3, 6, 12, and 24 h after annealing (95 °C for 3 min in 5 mM HEPES, pH 7.5, 15 mM NaCl, 0.1 mM EDTA, then cooling on ice 5 min), adjustment of salts as above (without Mg^{2+}), equilibration at 37 °C for 2 min, and initiation by addition of $MgCl_2$ to 40 mM.

Because the ligation rate of 7Q5 in particular is higher than for the other three deoxyribozymes, we re-determined its ligation rate taking faster timepoints. The experiment is shown in Figure X4; the k_{obs} is the same as found from the data in Figure X3.

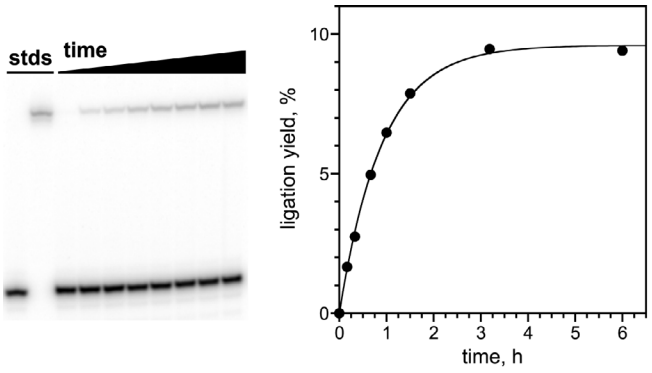

Figure X4. Re-determination of the ligation rate for deoxyribozyme 7Q5 with faster timepoints. Assay as in Figure X3, except timepoints at 0, 10, 20, 40 min; 1, 1.5, 3, and 6 h. Kinetic data: k_{obs}, 1.1 h^{-1}.

RNA cleavage experiments at 37 °C

The cleavage experiments were performed in the format of Figure X2B, where S denotes the 5'-^{32}P-labeled 34-mer RNA substrate strand containing either a 3'–5' or a 2'–5' linkage at the indicated junction. The assays were performed with S:E ratio of 1:3, specifically 5:15 pmol in 10-μL reactions. For details of the experiments, see ref. 8. The cleavage experiments for 7P4, 7Q2, 7Q5, and 7Q10 are shown in Figure X5A for a 2'–5' RNA substrate and Figure X5B for a 3'–5' RNA substrate. Kinetic data from the experiments of Figure X5A are shown as Figure 6B of the manuscript (cleavage of 2'–5' linkages). In contrast, the results reveal that 3'–5' linkages are not detectably cleaved (<5%) by any of the new DNA enzymes.

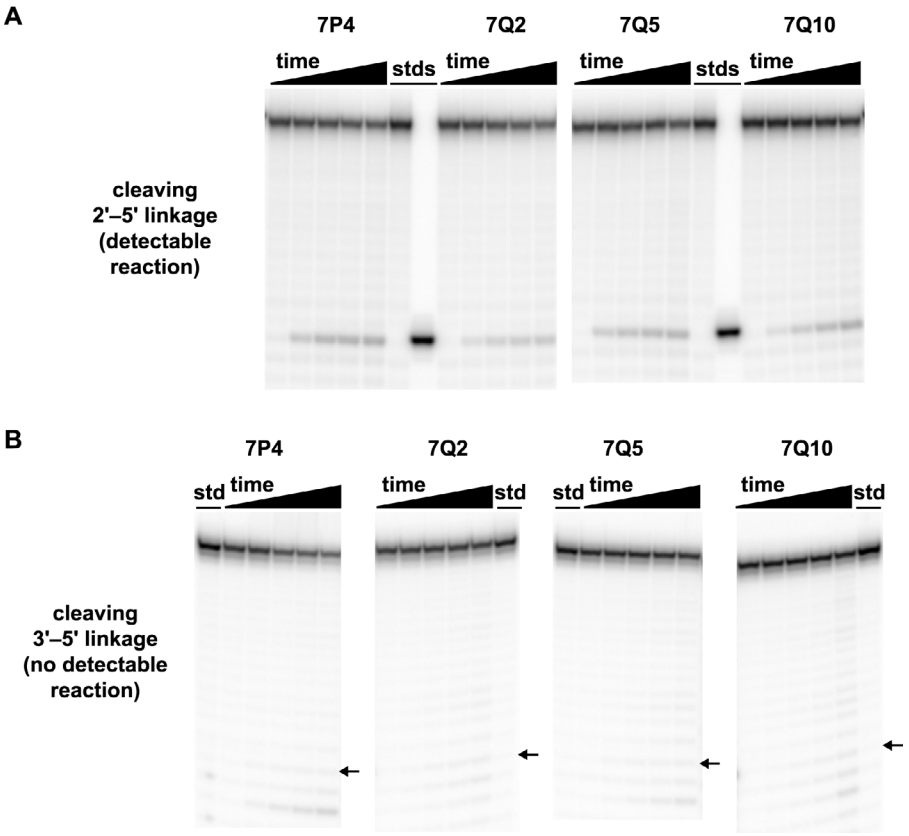

Figure X5. RNA cleavage experiments for the 7P4, 7Q2, 7Q5, and 7Q10 deoxyribozymes. (A) Cleaving a 2'–5' RNA linkage. See manuscript Figure 6B for plots of the data. (B) Cleaving a 3'–5' RNA linkage. Conditions: 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl, 40 mM $MgCl_2$, 37 °C, 10 μL reaction volume; aliquots of 1 μL withdrawn at appropriate timepoints and quenched onto a solution of 80% formamide, 1× TB (89 mM each Tris and boric acid, pH 8.3), 50 mM EDTA containing 0.25% each bromophenol blue and xylene cyanol. Timepoints at 0, 2, 6, 12, and 24 h; see Figure X3 caption for annealing and initiation conditions. For the gel images in panel B, the ligation standard is shown; the cleavage standard was on the other side of the gel, at the position indicated by the arrowhead.

RNA ligation experiments at 10 °C

Ligation experiments at 10 °C were performed to check if the rates and/or yields improved at lower temperature. The results are shown in Figure X6 and indicate that the lower temperature does *not* enhance RNA ligation by the new DNA enzymes.

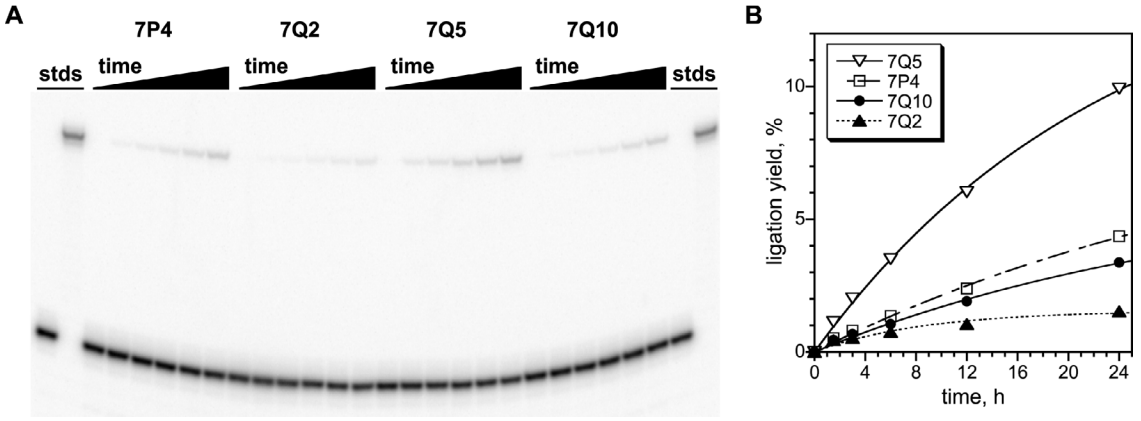

Figure X6. Ligation experiments with the new deoxyribozymes at 10 °C. Experiments as in Figure X3 except for temperature. (A) Gel image; timepoints at 0, 1.5, 3, 6, 12, and 24 h. (B) Kinetic data. k_{obs}, h^{-1}: 7Q5, 0.043; 7P4, 0.026; 7Q2, 0.12 (note very low yield); 7Q10, 0.031.

Determination of $K_{d,app}(Mg^{2+})$ for the 7Q10 DNA enzyme

The ligation assays were repeated at various $[Mg^{2+}]$ for the 7Q10 DNA enzyme (Figure X7).

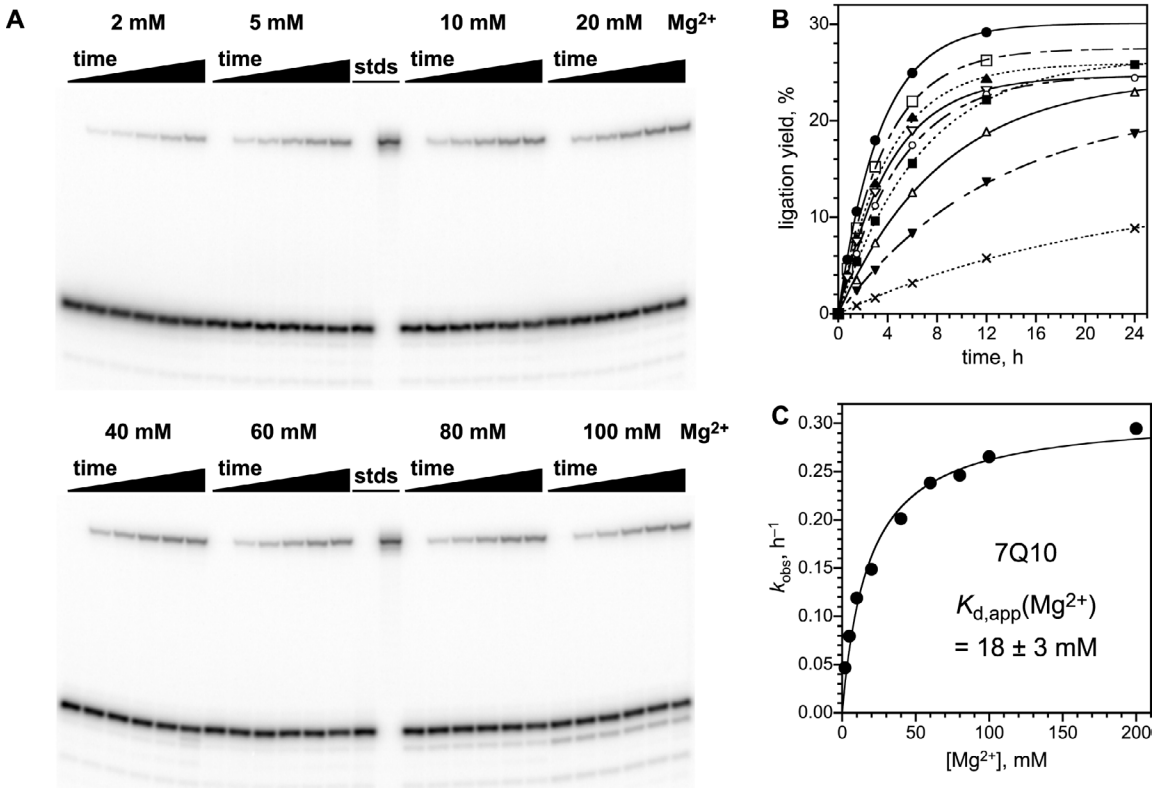

Figure X7. Determination of $K_{d,app}(Mg^{2+})$ for the 7Q10 deoxyribozyme. (A) Gel images for $[Mg^{2+}]$ = 2 mM to 100 mM. (B) Fits to data. Top-most data was at 200 mM Mg^{2+}; others were at 100 to 2 mM Mg^{2+} top to bottom as in panel A. C, Fit to determine the value of $K_{d,app}(Mg^{2+})$.

Ligation assays with 7Q10 and 7P4 using various metal ions

The ligation assays of Figure X3 were repeated using 7Q10 and 7P4 with various metal ions, specifically 10 mM EDTA (i.e., no divalent metal ion); 40 mM $MgCl_2$ (positive control), 40 mM $CaCl_2$, 40 mM $SrCl_2$, 10 mM $MnCl_2$, 1 mM $ZnCl_2$, 5 μM $CuCl_2$, or 4 mM $Co(NH_3)_6^{3+}$•$3Cl^-$ (the latter with 10 mM EDTA). The data are shown in Figure X8 and show that both deoxyribozymes require divalent metal ions for activity. They each perform well with Mg^{2+}, Ca^{2+} and Mn^{2+} but not with the other tested metals. 7Q10 and 7P4 each show trace activity with Sr^{2+}, and 7Q10 shows trace activity with Zn^{2+}, but these activities are barely detectable above background.

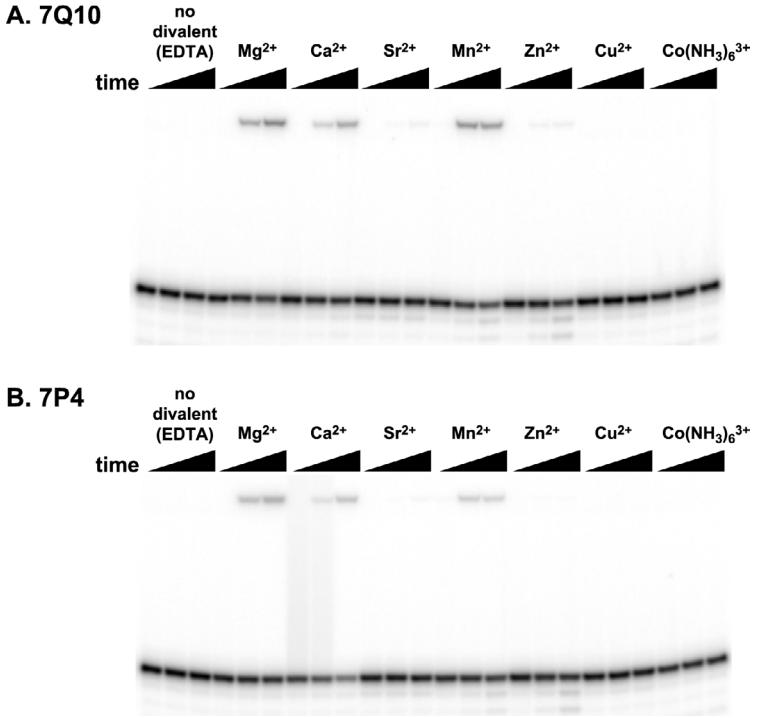

Figure X8. Assaying RNA ligation activity of deoxyribozymes with various metal ions. (A) 7Q10. (B) 7P4. Concentrations of metal ions are given in text. Timepoints for 7Q10: 0, 3, 12 h; for 7P4: 0, 2, 8 h. Percent ligation at 12 h for 7Q10: EDTA (background), 0.07, Mg^{2+}, 22.4; Ca^{2+}, 11.4; Sr^{2+}, 0.5; Mn^{2+}, 20.7, Zn^{2+}, 0.7. Percent ligation at 8 h for 7P4: EDTA (background), 0.06, Mg^{2+}, 12.4; Ca^{2+}, 12.7; Sr^{2+}, 0.23; Mn^{2+}, 4.2, Zn^{2+}, 0.14.

Assaying the ligation junctions: 3'–5' or 2'–5' ?

Control experiments using the 8–17 deoxyribozyme (which cleaves 3'–5' but not 2'–5' linked RNA) and using alkaline hydrolysis at 100 mM Mg^{2+} in the presence of the full DNA complement (which cleaves 2'–5' but not 3'–5' linked RNA) are shown in Figure X9. These control experiments validate that this two-part assay unambiguously distinguishes between 3'–5' and 2'–5' linked RNA products. For details of the assays, see ref. 8.

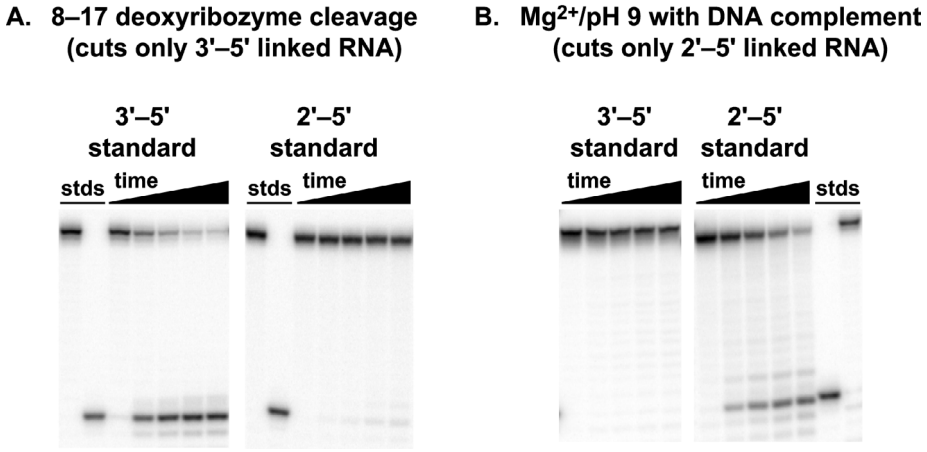

Figure X9. Control experiments to validate the two-part assay for distinguishing 3'–5' and 2'–5' linked RNA products. The substrates were 34-mer standard RNAs linked by either a 3'–5' or a 2'–5' bond (see Figure X2 for sequence). (A) Cleavage with the 8–17 deoxyribozyme; this assay is selective for 3'–5' linkages. Timepoints: 0, 20 min; 1, 4, 7 h. (B) Cleavage at 100 mM Mg^{2+} and pH 9 in the presence of fully complementary DNA; this assay is selective for 2'–5' linkages. Timepoints: 0, 2, 6, 13, 24 h. The calculated $t_{1/2}$ of ~11 h matches that described in ref. 14b. See also ref. 8 for details of these assays.

The RNA products from each of the new deoxyribozymes 7P4, 7Q2, 7Q5, and 7Q10 were assayed by the same two methods, with results shown in Figure X10. These data clearly indicate that the products have <5% 3'–5' linkage at the newly formed phosphodiester bond. Part of these data (for 7Q10) are shown as manuscript Figure 5.

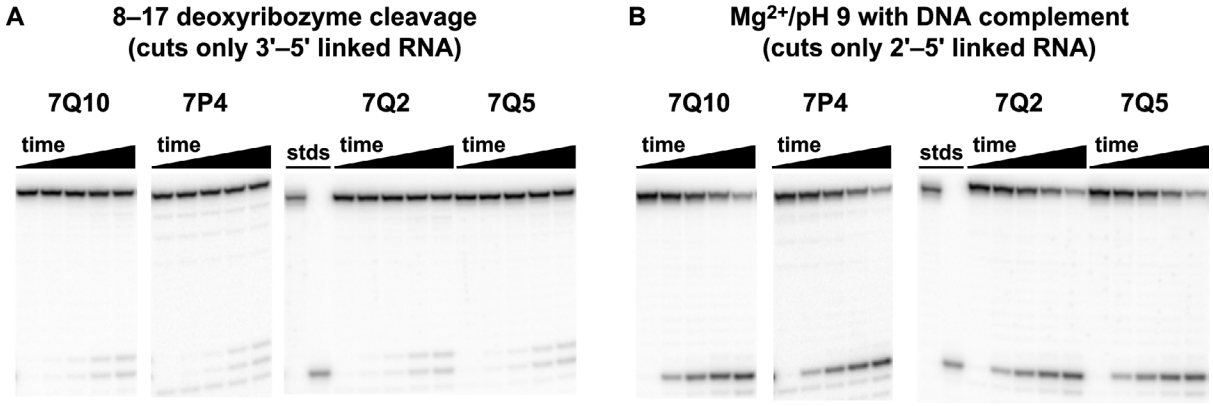

Figure X10. Assaying the ligation junctions created by the new deoxyribozymes 7P4, 7Q2, 7Q5, and 7Q10, using the two assays as described in text. (A) The 8–17 deoxyribozyme assay. (B) The Mg^{2+}/pH 9/complementary DNA assay. Timepoints for A: 0, 20 min; 1, 4, 7 h. Timepoints for B: 0, 2, 6, 13, 24 h.

Similar assays were performed using the products from the 8–17 selection round 7 pools and 10–23 selection round 9 pools, after first 5'-^{32}P-radiolabeling then removing the DNA from the ligated RNAs with DNase. Because DNase does not cleave the last few deoxynucleotides close to the junction with RNA, the assayed material is slightly longer (and with a ragged 3'-end) relative to the 34-mer RNA standard. The assays are shown in Figure X11 and reveal that each pool's products have <5% 3'–5' linkage at the newly formed bond. Note that for the 10–23 selection pools (Figure X11B), the assay for cleaving 3'–5' linkages used an appropriate 10–23 enzyme rather than an 8–17 enzyme, because the 10–23 enzyme is required to cleave any newly formed 3'–5' A↓U bonds (refs. 8 and 9). The assay with the 10–23 enzyme itself gives a small amount of cleavage at long timepoints of Figure X11B, which we attribute to slow cleavage of a 2'–5' A↓U phosphodiester bond by the 10–23 enzyme. The rate of this cleavage is ~0.05 h^{-1}, which is orders of magnitude slower than that of a 3'–5' linkage by 10–23 (refs. 8 –10).

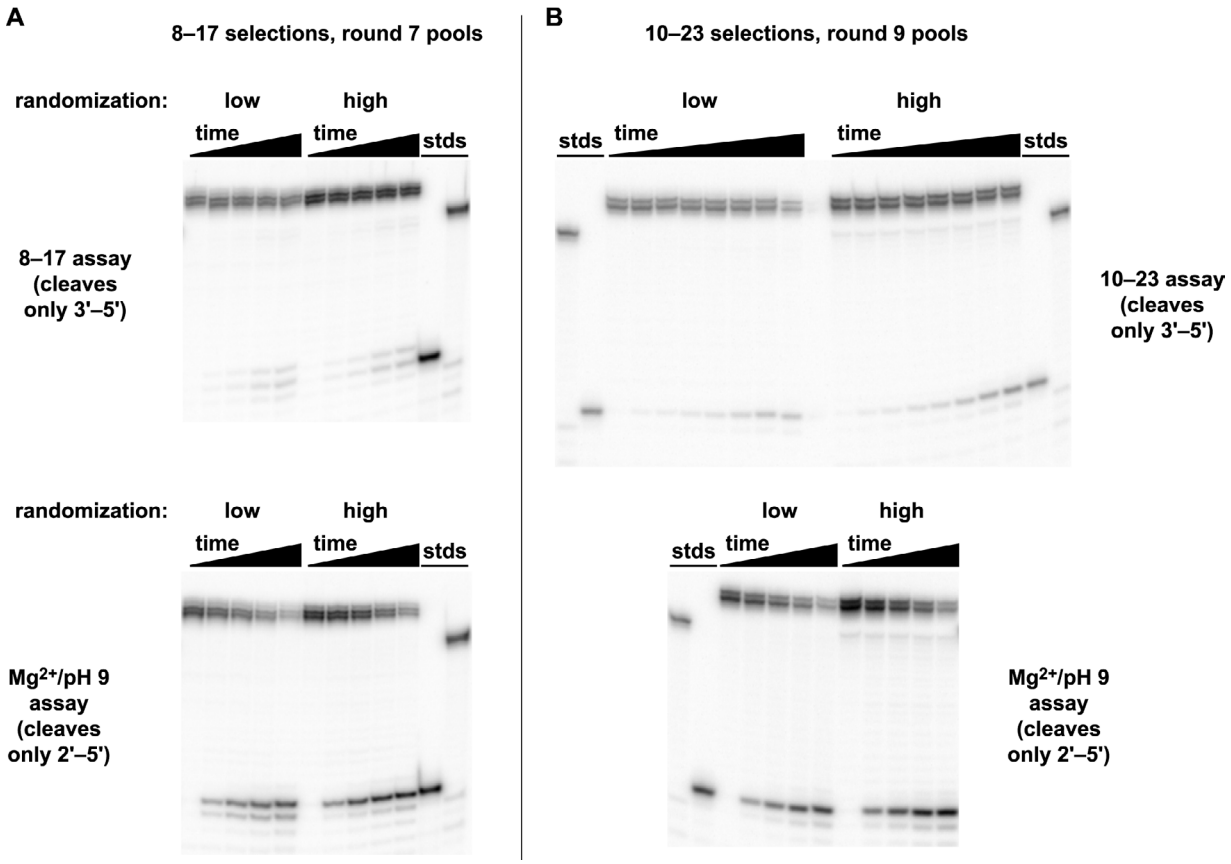

Figure X11. Assaying the ligation junctions created by the new deoxyribozymes, using the uncloned pools from round 7 of the 8–17 selections or round 9 of the 10–23 selections. (A) The 8–17 selections round 7 pools. Upper assay timepoints are 0, 20 min, 1, 4 and 7 h; lower assay timepoints are 0, 2, 6, 13, and 24 h. (B) The 10–23 selections round 9 pools. Upper assay timepoints are 0, 10, 20, 40 min, 1, 2, 4 and 7 h; lower assay timepoints are 0, 2, 6, 13, and 24 h.