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Supporting Information

Enzymatic Synthesis of Nucleic Acids with Defined Regioisomeric 2′-5′ Linkages

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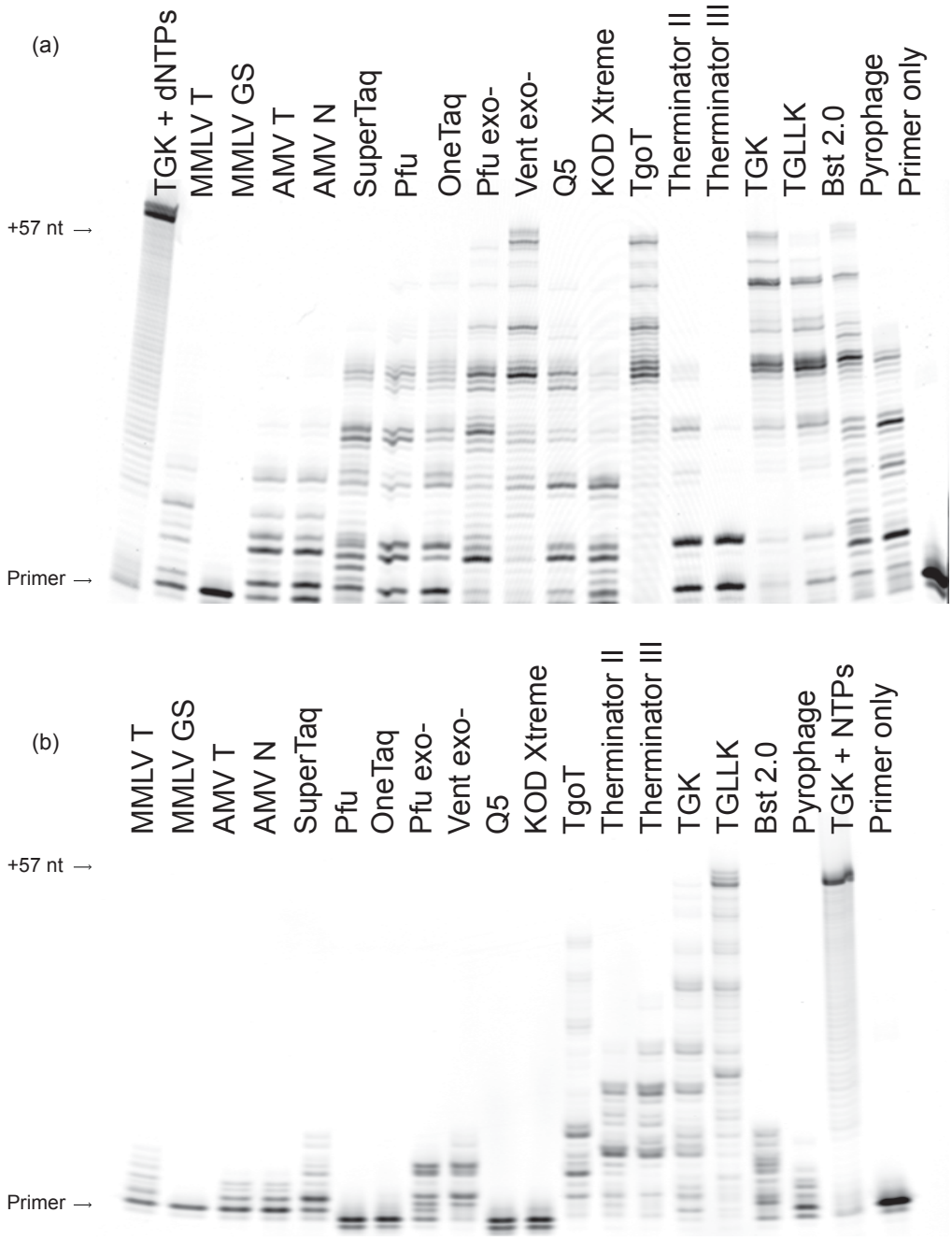

Figure S1: Polymerase screening for (a) 3'dG / (b) 3'OMe-G incorporation.

A range of polymerases were tested for their ability to synthesize mixed backbone polymers from either dHTP / 3'dGTP or HTP / 3'O-Me-GTP. Reaction conditions, polymerase vendors and primer/template sequences are listed in the experimental section. MMLV T = Transcriptor RT. MMLV GS = GoScript RT. AMV T = Thermoscript RT. AMV N = AMV (NEB).

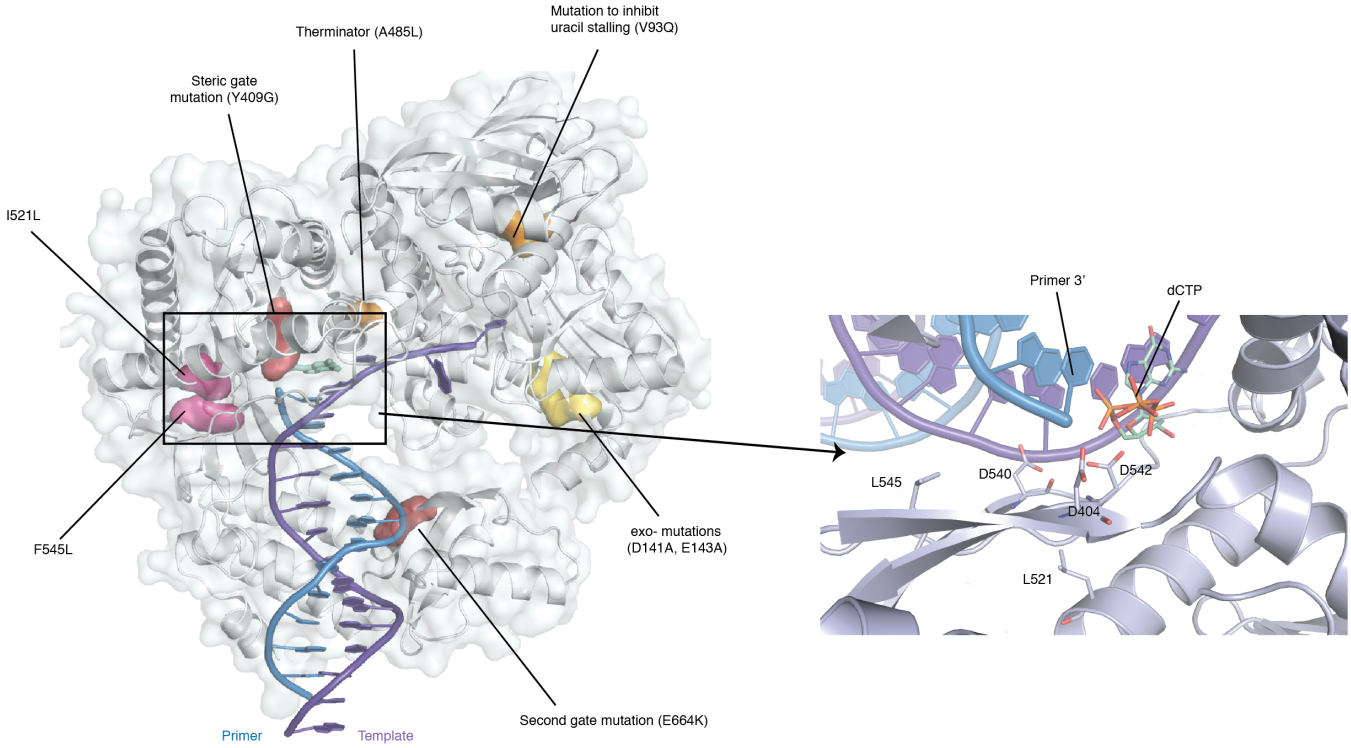

Figure S2: A polymerase (TGLLK) capable of synthesizing mixed 2'-5' / 3'-5' backbone nucleic acids.

Model of TGLLK based on apo Tgo structure (PDB: 1TGO[1]) with DNA modeled by alignment with *Eschericia coli* DNA pol II (PDB: 3MAQ[2]); all numbering is from Tgo. Figures were generated using MacPyMOL 1.7.6.0 and labeled in Adobe Illustrator CS6. (a) TGLLK consists Tgo V93Q, D141A, E143A, Y409G, A485L, I521L, F545L, E664K. V93Q[3] inhibits template uracil stalling, D141A/E143A inactivate 3'-5' exonuclease activity, Y409G reduces steric clashes with incoming nucleotide triphosphates with bulky 2' substitutions, A485L[4] (Therminator) enhances substrate promiscuity, I521L[5] enables reverse transcription by Tgo and enhances incorporation of unnatural nucleotides, F545L[6] also enhances incorporation of unnatural nucleotides and E664K[7] enables processive synthesis with A-form polymers. (b) A close-up of the active site with L521 and F545 shown in stick representation. Some residues have been hidden for clarity. Both 521 and 545 are in close proximity of the catalytic triad (D404, D540, D542) and are thought to enhance substrate promiscuity through effects on active site dynamics and/or geometry.

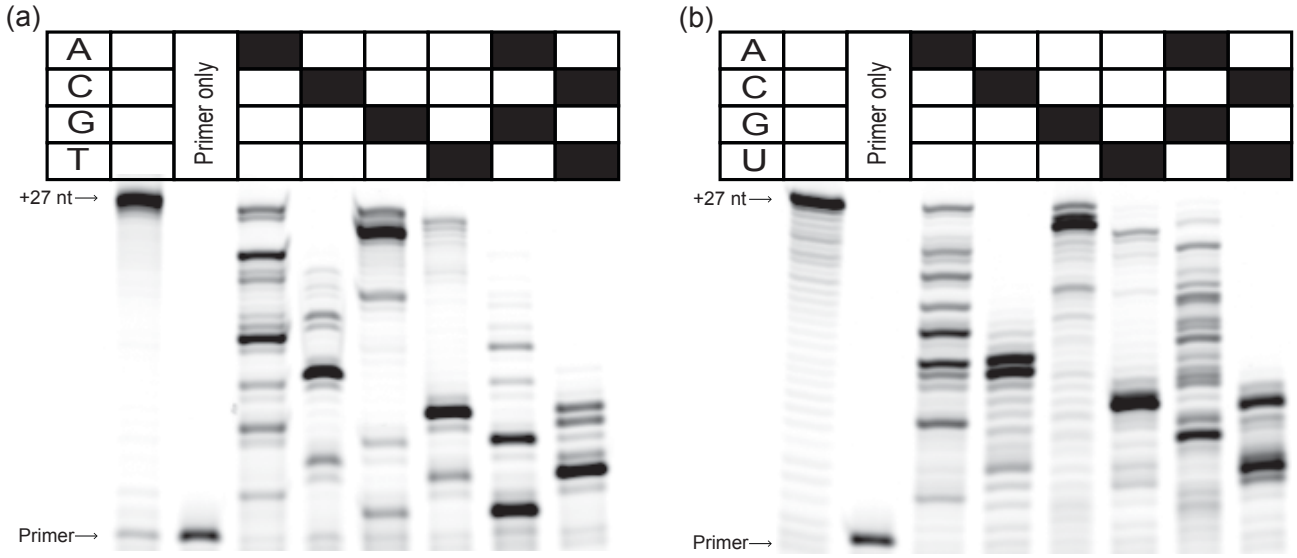

Figure S3: Polymerase-directed synthesis of mixed backbone polymers.

Black boxes mark 3'dNTPs (a) or 3'OMe-NTPs (b). All other nucleotide triphosphates are natural dNTPs (a) or NTPs (b). Reactions consisted 1 x Thermopol buffer, 400 nM primer Cy5-fd, 800 nM template Temp-Nshort2, 300 nM polymerase TGLLK and 0.25 mM each d/NTP. 5 µL reactions were incubated for a single cycle of 3 min 95°C, 5 min 50°C, 2 h 65°C and quenched with 2 vol 98% formamide/10 mM EDTA.

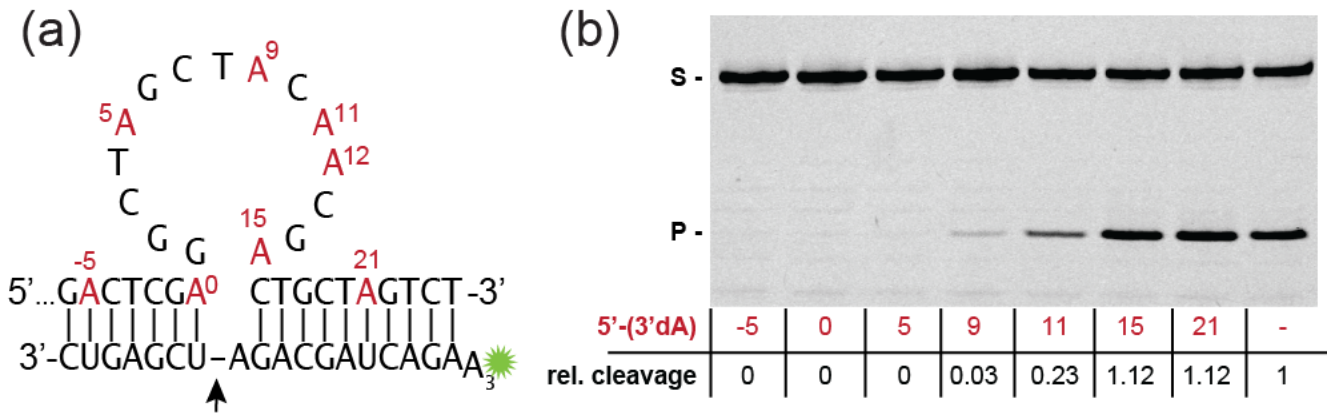

Figure S4: 3'dA primer scanning of 10-23 DNAzyme

(a) 10-23 DNAzyme / substrate pair used in this study. (b) Cleavage (relative to wild type) of the fluorescent RNA substrate (200 nM) after 1 h at 37°C by different DNAzyme variants (40 nM) containing 2'-5' linkages following all A nucleotides after A-5, A0, A5, A9, A11, A15 or A21.

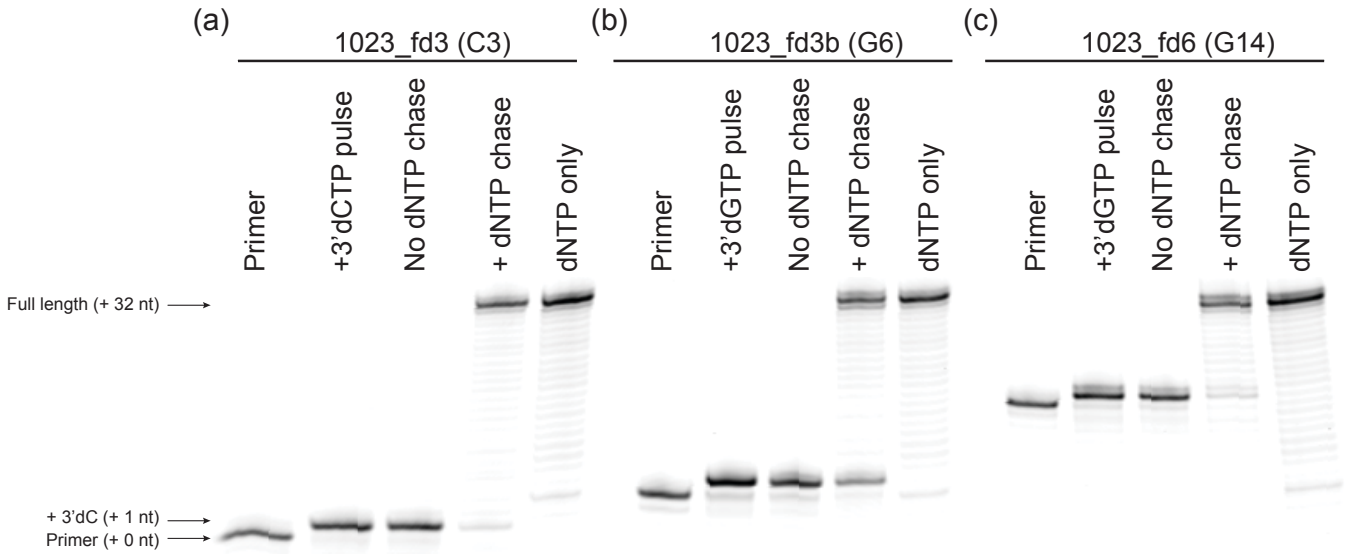

Figure S5: "Pulse-chase" synthesis of position-selective 2'-5' DNA linkages.

(a) Lane 1 (Primer) shows primer 1023_fd3, which encodes C3 as the first incorporation. Lane 2 (+3'dCTP pulse) shows the primer after incubation with TGLLK and 3'dCTP only, resulting in complete extension of the primer to +1 nt. Lane 3 (No dNTP chase) shows the reaction from Lane 2 after shrimp alkaline phosphatase treatment and extended incubation at 65°C, which results in no further primer extension. Lane 4 (+dNTP chase) is as lane 3 except supplemented with dNTPs after shrimp alkaline phosphatase treatment, resulting in full length 10-23 with a site-specific 2'-5' linkage (in this case, between C3 and T4). Lane 5 (dNTP only) shows extension of the primer with dNTPs only (positive control). Arrows mark (from bottom to top) unextended primer, +1 nt after incubation with 3'dCTP and full length extension (+32 nt). (b) as (a) except primer 1023_fd3B and 3'dGTP were used to insert a 2'-5' linkage between G6 and C7. (c) as (a) except primer 1023_fd6 and 3'dGTP were used to insert a specific 2'-5' linkage between G14 and A15.

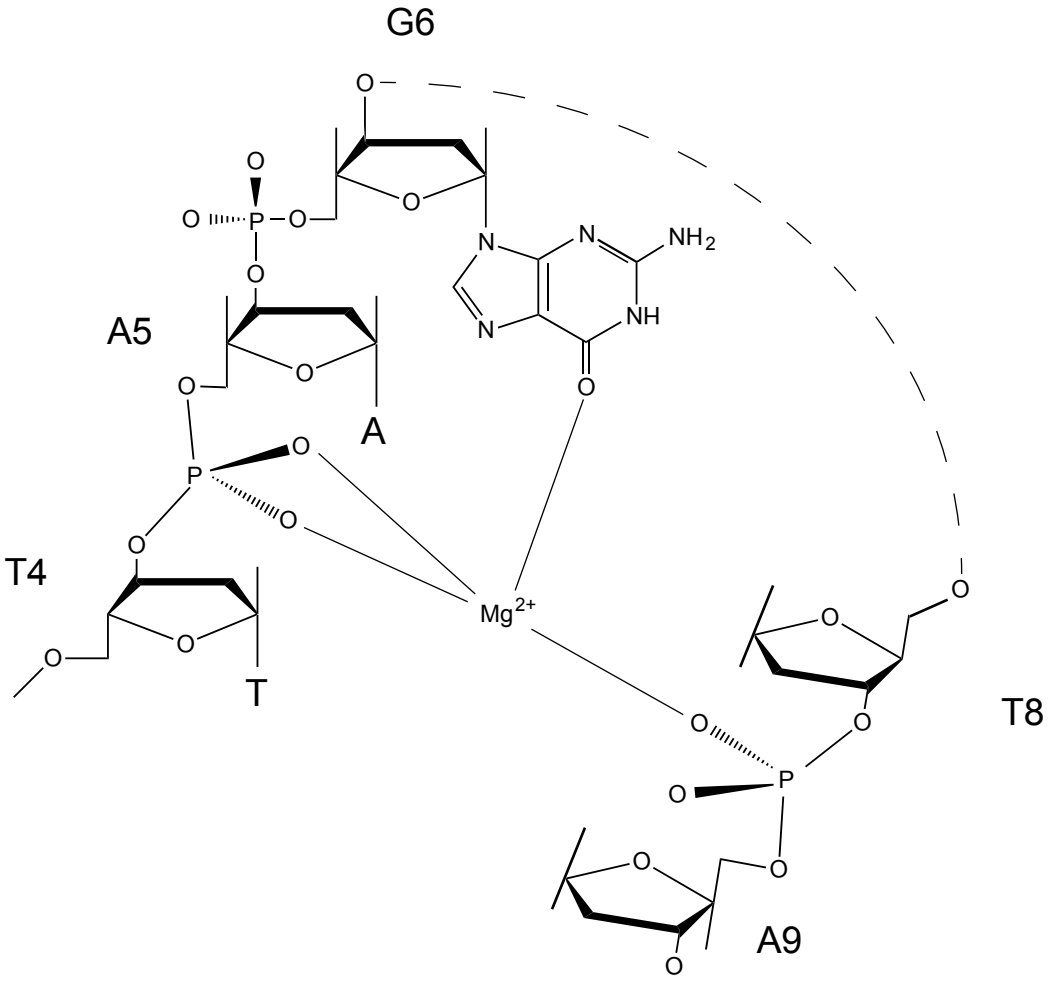

Figure S6: Proposed 10-23 Mg^{2+} binding site.

Mg^{2+} binding site as proposed by Nawrot et al[8]. We find position-selective insertion of a 2'-5' linkage between T4-A5 substantially reduces DNAzyme activity (to <10% wild type activity), suggesting this linkage may be critical to catalytic activity.

Table S1. Observed misincorporation rates

Sample	Nucleotides sequenced	Global misincorporation rate	3’d/3’OMe misincorporation rate
3’dA	3.58×10^{6}	7.98×10^{-3}	2.63×10^{-2}
3’dG	3.24×10^{6}	5.08×10^{-4}	2.93×10^{-3}
3’O-Me-A	78.42×10^{6}	7.18×10^{-4}	1.17×10^{-3}
3’O-Me-G	45.22×10^{6}	1.74×10^{-4}	3.82×10^{-3}

Observed misincorporation rates for reactions containing 250 μM 3’dATP, 25 μM dCTP, 25 μM dGTP, 25 μM dTTP (3'dA), 25 μM dATP, 25 μM dCTP, 250 μM 3’dGTP, 25 μM dTTP (3'dG), 250 μM each 3’O-methyl-ATP, CTP, GTP, UTP (3’OMe-A) or 250 μM each ATP, CTP, 3’O-methyl-GTP, UTP (3'OMe-G). All figures are given to 2 decimal places.

Table S2. Oligonucleotide sequences (5' -> 3') used for 10-23 primer scanning.
Grey indicates the FD1 primer binding site not relevant for 10-23 activity, green the substrate binding arms, and blue the catalytic core. The cleavage site in the RNA substrate is shown in red.

1023_TempFD1_A15	AGACTAGCAGTCGTTGTAGCTAGCCTCGAGTCTGGCAAACGCTAATAAGGGGAAAAAAAAA AAAAAA
1023_FD1	/5TYE665/CCCCTTATTAGCGTTTGCCA
1023_FD2	/5TYE665/CCCCTTATTAGCGTTTGCCAGACTCG
1023_FD_G1	/5TYE665/CCCCTTATTAGCGTTTGCCAGACTCGA
1023_FD_G2	/5TYE665/CCCCTTATTAGCGTTTGCCAGACTCGAG
1023_FD3	/5TYE665/CCCCTTATTAGCGTTTGCCAGACTCGAGG
1023_FD_T4	/5TYE665/CCCCTTATTAGCGTTTGCCAGACTCGAGGC
1023_FD3b	/5TYE665/CCCCTTATTAGCGTTTGCCAGACTCGAGGCTA
1023_FD4	/5TYE665/CCCCTTATTAGCGTTTGCCAGACTCGAGGCTAG
1023_FD_T8	/5TYE665/CCCCTTATTAGCGTTTGCCAGACTCGAGGCTAGC
1023_FD5	/5TYE665/CCCCTTATTAGCGTTTGCCAGACTCGAGGCTAGCTAC
1023_FD6	/5TYE665/CCCCTTATTAGCGTTTGCCAGACTCGAGGCTAGCTACAAC
1023_FD7	/5TYE665/CCCCTTATTAGCGTTTGCCAGACTCGAGGCTAGCTACAACGA
1023_FL	/5TYE665/CCCCTTATTAGCGTTTGCCAGACTCGAGGCTAGCTACAACGACTGCTAGTCT
1023_sub	/5-6FAM/rArArArArGrArCrUrArGrCrArGrArUrCrGrArGrUrC

Experimental Section

Materials

All oligonucleotides were purchased from IDT (Coralville, IA, USA), apart from oligonucleotides containing 2'-5' linkages which were from ChemGenes Corp (Wilmington, MA, USA). 3'dNTPs (cat # N-3001, N-3002, N-3003, N-3004) and 3'O-methyl-NTPs (cat # N-1056, N-1057, N-1058, N-1059) were purchased from TriLink Biotechnologies (San Diego, CA, USA). dNTPs were from Bioline and NTPs were from Roche.

Polymerase mutation, expression and purification

Mutations were introduced by iPCR using primers RT521Lba2 (GAGTCAGGTCTCAGGCAGTACATCGAG ACTACGATAAGGG) and RT520fo (GAGTCAGGTCTCCTGCCTGCCCCAAGCGGTAACGCTC) for I521L and F545LbaBsa (GAGTCAGGTCTCGGATTTCTGGCAACAATACCTGGAGCGGACG) and F544foBsa (GAGTCAGGTCTCAAATCCATCTGTGTCCGCGTAGAGGAC) for F545L. iPCR was carried out using Q5 Hot Start High Fidelity DNA Polymerase (NEB), the reactions purified using NucleoSpin columns (Machery-Nagel), dual digested using DpnI (NEB) and BsaI (NEB) to remove template DNA and generate complementary sticky ends, purified using NucleoSpin columns (Machery-Nagel), ligated using T4 DNA ligase (NEB), phenol:chloroform extracted, isopropanol precipitated and transformed into T7 Express LysY competent cells (NEB). Mutations were confirmed by sequencing of clones.

All Tgo-based polymerases (TgoT (Tgo: V93Q, D141A, E143A, A485L), TGK (TgoT: Y409G, E664K), TGLLK (TGK: I521L, F545L)) were expressed and purified as previously described[9].

Deep sequencing and analysis

All deep sequencing was carried out on an Illumina MiSeq. Libraries were prepared by PCR using KOD Hot Start DNA Polymerase (Merck Millipore) to append barcodes and sequencing primers. Libraries were gel purified on 3.5% agarose gels (TopVision Low Melting Point Agarose, Life Technologies) stained with SYBR safe (Life Technologies) and cleaned up using NucleoSpin columns (Machery-Nagel). Libraries were then quantified using NEBNext Library Quant Kit for Illumina (NEB) on a RotorGene 6000 (QIAGEN). Typically we set up MiSeq runs using a 150 cycle v3 reagent kit, with 8 pM pooled libraries and 20% phiX and the 'Generate FASTQ' workflow.

FASTQ files were quality-filtered, trimmed and barcode split using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html). These processed reads were then aligned to a reference sequence by the Burrows-Wheeler Aligner (BWA, http://bio-bwa.sourceforge.net/bwa.shtml [10]) using the BWA backtrack algorithm. The SAMtools suite (http://samtools.sourceforge.net/ [11]) was used to assemble the aligned reads into sorted BAM files, which were then used to generate pileup files via the mpileup command (option -d was increased to 10,000,000). Pileup files were interrogated using a custom Perl script, miCount.pl (available as an additional supplementary file), to give both positional and aggregate frequencies of bases and indels. These data were used to quantify misincorporation and indel

rates in Microsoft Excel. Expected errors from AMV RT and Taq PCR were subtracted as previously[9], using published values for Taq[12] and AMV[13].

Polymerase screening

The full panel of polymerases screened for ability to synthesize 2'-5' linkages was: Transcriptor Reverse Transcriptase (Roche), GoScript Reverse Transcriptase (Promega), Thermoscript Reverse Transcriptase (Life Technologies), AMV Reverse Transcriptase (NEB), SuperTaq DNA Polymerase (Life Technologies), Pfu Turbo (Agilent), Exo- Pfu DNA Polymerase (Agilent), OneTaq DNA Polymerase (NEB), Q5 Hot Start High Fidelity DNA polymerase (NEB), KOD Xtreme DNA Polymerase (Merck Millipore), TgoT[9] (a Therminator DNA Polymerase homologue), Therminator II DNA Polymerase (NEB), Therminator III DNA Polymerase (NEB), TGK[9], TGLLK (this publication), Bst2.0 (NEB) and PyroPhage 3173 DNA Polymerase-WT (Lucigen). All reactions were set up using 1 x of the supplied buffer (Thermopol (NEB) was used for TgoT, TGK and TGLLK) and the recommended xNTP nucleotide and polymerase concentrations. Polymerases expressed and purified in-house were (TgoT, TGK, TGLLK) were used at 300 nM. An equimolar mix of dATP, dCTP, 3'dGTP and dTTP was used for the 3'dGTP screen or of ATP, CTP, 3'O-Me-GTP and UTP for the 3'O-Me-GTP screen. All reactions were 5 µL and contained 0.2 µM primer Cy5-fd (5'-Cy5-CCCCTTATTAGCGTTTGCCA-3') and 0.4 µM template TempN (5'-GATGCTGGACCAGATAAGCACTTAGC
CACGTAGTGCTGTTCGGTAATCGATCTGGCAAACGCTAATAAGGGG-3', primer binding site underlined) or TempNshort2 which is truncated 30 nt from the 5'. All reactions were heated to 95°C for 5 min with no enzyme and cooled to room temperature. Hot start polymerases (OneTaq DNA Polymerase (NEB), Q5 Hot Start High Fidelity DNA polymerase (NEB), KOD Xtreme DNA Polymerase (Merck Millipore)) were also heated to 95°C for 5 min and cooled to room temperature. Following polymerase addition, the two MMLV enzymes (Transcriptor Reverse Transcriptase (Roche) and GoScript Reverse Transcriptase (Promega)) and the two AMV enzymes (Thermoscript Reverse Transcriptase (Life Technologies), AMV Reverse Transcriptase (NEB)) were incubated for 1 h 42°C, 1 h 55°C (2 h total extension). All other reactions were incubated for 2 h 65°C. All reactions were quenched in 2 volumes 98% loading buffer (98 % formamide, 10 mM EDTA, bromophenol blue) and run on 15 % polyacrylamide / 8 M urea gels in 1 x TBE.

Enzymatic synthesis of oligonucleotides with heterogeneous 2'-5' / 3'-5' backbone regiochemistry

For simplicity TGLLK was used for all experiments. Typical primer extension conditions for experimental gels were 1 x Thermopol reaction buffer (NEB), 0.25 mM each xNTP, 100 nM primer, 200 nM template, 300 nM TGLLK. For synthesis of larger amounts of oligonucleotide 1 µM primer, 2 µM template and 1.3 µM TGLLK was used. Typically a complete reaction was made up on ice and incubated 5 m 95°C, 5 m 50°C, 2 h 65°C (this step was extended to 6 or 12 h for preparative reactions).

Template strands were removed either by using 5' phosphorylated templates and digestion with λ-exonuclease (Lucigen) followed by urea/polyacrylamide gel purification or through use of templates with 20 nt poly(dA) overhangs and urea/polyacrylamide gel purification.

Reverse transcription of oligonucleotides with heterogeneous 2'-5' / 3'-5' backbone regio-chemistry

Enzymatically synthesised and gel purified oligonucleotides were reverse transcribed using GoTaq (Promega) or AMV Reverse Transcriptase (NEB).

The 2'-5' / 3'-5' DNA 10-23 DNAzymes used for cleavage reactions were poly(dA) tailed using Terminal Transferase (NEB) according to the manufacturer's instructions for 30 min 37°C and cleaned up using a NucleoSpin column (Machery Nagel). Reverse transcription was carried out using 1 x GoTaq Hot Start Green Master Mix with 1 µM primer CDSIII-dT_{30} (5'-ATTCTAGAGGCCGAGGCGGCCGACATGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'), 0.2 µM template for 95°C 5 min, 50°C 30 min. After this the reverse transcriptions were supplemented to 1 x Exonuclease I reaction buffer and 1 µL Exonuclease I (Thermo Scientific) added and incubated for 45 min 37°C, 20 min 80°C. PCR was carried out using primers CDSIII (5'-ATTCTAGAGGCCGAGGCGGCCGACATG-3') and fd (5'-CCCCTTATTAGCGTTTGCCA -3')

3'-O-methyl-RNA / RNA was reverse transcribed using AMV (NEB). In this case the primer was DNase resistant (OMe_tag4fd5F 5'-GTCGGATCCGTTTAAGCTAGGCCCCTTATTAGCGTT*TGCCA*-3', where all nucleotides are 2'O-methyl-RNA except the last 5 (italicized) which are 2'fluoro-DNA) and the template (HH38v2_test1b
5'-AGAACAGAGTAGAGAGCGAGAAGGATCAGATTTCGTCTCGAAAGACTCATCAGGAGTCAGTGGC
AAACGCTAATAAGGGGAAAAAAAAAAAAAA-3') encoded a direct RT priming site. After synthesis the reaction was supplemented to 1 x TURBO DNase buffer, 5 µL TURBO DNase (Life Technologies) was added and incubated for 2 h 37°C to degrade the template prior to gel purification. The purified product was suspended in 10 µL 10 mM Tris•HCl pH 8.8. An 8.5 µL reverse transcription reaction consisting 1 µL of the above purified reaction, 2 mM dNTPs and 1 µM primer LMB3+_test1b (5'-CAGGAAACAGCTATGACAAAAGAACAGAGTAGAGAGCGAGA-3') was heated to 95°C for 5 min and snap cooled on ice prior to the addition of 1 µL 10x AMV buffer and 0.5 µL (5 U) AMV RT (NEB). The reaction was incubated for 15 min 37°C, 15 min 55°C, 5 min 95°C and Exonuclease I treated as for 2'-5'-DNA containing reactions. PCR was carried out using outnesting tags tag4 (5'-GTCGGATCCGTTTAAGCTAGG-3') and LMB3+ (5'-CAGGAAACAGCTATGACAAA-3') to ensure specificity.

Primer scanning of 10-23 DNAzymes.

Variants of a 10-23 DNAzyme (1023_FL, Table S1) containing either 3'dA or 3'dG nucleotides were synthesized from template 1023_TempFD1_A15 (Table S1) using either TGK (3'dG) or TGLLK (3'dA) and 5'-TYE-665 labeled primers 1023_FD1 to 1023_FD7 (3'dA) or 1023_FD3, 1023_FD5, and 1023_FD7 (3'dG). Synthesis was performed in 1x Thermopol buffer (NEB) supplemented with 2 mM $MgSO_4$ and either 200 µM 2'dBTPs (3'dA) or 200 µM 2'dHTPs (3'dG), 400 µM of the respective 3'dRTP, 3 µM template, and 0.5 µM polymerase. All reactions were incubated 5 min 95°C, 5 min 50°C, 12 h 65°C. Full-length DNAzymes were purified using conventional gel purification after 8 M urea/PAGE. For each preparation, a 3'-5' "wild type" 10-23 control was synthesized and purified using the same protocol.

RNA-cleavage activity of the different 10-23 variants was probed under multi-turnover conditions using 40 nM of each 10-23 DNAzyme variant and 200 nM of the 5'-FAM labeled 1023_sub RNA substrate (Table S1). 10-23 variants and 1023_sub were pre-incubated separately at 37°C in 50 mM Tris□HCl pH 8.3, 100 mM $MgCl_2$, and 25 mM NaCl for 2 min before mixing. For each time point a 2 µL sample of the reaction was removed and mixed with 8 µL quenching mix (86% formamide, 60 mM EDTA pH 8, 0.05% bromphenol blue). Cleavage was monitored by urea–PAGE of 0.2 pmol fluorescently labeled 1023_sub followed by analysis using a Typhoon Trio scanner (GE Healthcare). The fractional intensities of the starting material and cleavage product were determined using ImageQuant TL (GE Healthcare) and plotted using GraphPad Prism 6 (GraphPad Software).

Position-selective 2'-5' linkage synthesis (Figure S5)

DNAzymes with single internal 2'-5' linkages were generated by incubating a primer that encoded the desired 3' modification as the first incorporation (e.g. 1023_FD3, which terminates at G2 meaning the first incorporation is C3) with template 1023_TempFD1_A15 with 600 nM TGLLK and 200 µM 3'dCTP (in this case) for 2 min 95°C, 15 min 50°C. The reaction was then cooled, supplemented with 0.1 U/µL Shrimp Alkaline Phosphatase (NEB) and incubated for 30 min 37°C, 5 min 65°C prior to addition of dNTPs to 200 µM each and incubation for 5 min 50°C, 1 h 65°C and gel purification as described above. Incorporation of only a single G nucleotide at position G1/G2 was possible as incorporation of two consecutive Gs does occur after a "running start" when the polymerase has already incorporated a stretch of natural dNTPs or NTPs before the duplet.

RNA-cleavage activity of the different 10-23 variants was performed as described in the previous section. For each variant the amount of cleavage after 1 h of incubation was quantified in three independent experiments and normalized against the amount of cleavage by the wild type DNAzyme (N = 6). The standard deviations Δr of this relative activity r were determined by

$$\Delta r = |r| \sqrt{\left(\frac{\Delta c_{wt}}{c_{wt}}\right)^2 + \left(\frac{\Delta c_{var}}{c_{var}}\right)^2}$$

where c_{wt} is the average amount of cleavage by the wild type DNAzyme (with the standard deviation Δc_{wt}) and c_{var} the average amount of cleavage by the variant tested (with the standard deviation Δc_{var}).

HPLC-Analysis

HPLC standards of the 5' 6-carboxyfluorescein labeled oligonucleotide 5'-(6-FAM)-ACCGCCGCGTCA*TTC-3' (where * marks the site with either 3'-5' or 2'-5' linkage) were purchased from ChemGenes. The same sequence was synthesized enzymatically using TGLLK from the template 5'-GAATGACGCGGCGGTAAAAAAAAAAAAAAA-3' (primer binding site underlined) using the 5' 6-carboxyfluorescein labeled primer 6-FAM-ACCGCCGCG. A typical 500 µL reaction contained 1 x Thermopol reaction buffer (NEB), 1.5 µM primer, 1.5 µM template, 1.5 µM TGLLK, 25 µM 2'dYTPs, and 250 µM 3'dATP or 2'dATP. Reactions were incubated for 1 min 95°C, 40 s 40°C, and 1 h 65°C. To remove any non-templated 3' overhangs created by TGLLK, synthesis reactions containing 2'dATP were further

incubated with T4-DNA Polymerase (15 U, 10 min at 12°C) followed by quenching of all reactions by adding EDTA to a final concentration of 10 mM. Note that T4 DNA Polymerase treatment was not used in 3'dATP containing reactions as this would lead to a general decrease in the amount of full-length product. For gel purification of full-length product, the chemically synthesized 3'dA standard was used as size-marker during the preparative 8 M urea/PAGE step.

Chemically and enzymatically synthesized standards were analyzed by strong anion exchange chromatography (SAX-HPLC) using a Varian Prostar system (Agilent, USA) with a DNAPac PA200 column (Dionex/Thermo, USA) under denaturing conditions sufficient to resolve linkage regioisomers[14]. For each run 20 µL of a sample containing a total concentration of 15 nM RNA in H_2O was injected onto a column equilibrated in 10 mM sodium phosphate buffer (pH 11.5). Regioisomers were eluted in a linear gradient (0.4 M to 0.9 M NaCl over 14 min, flow rate 1.5 ml min^{-1}). Fluorescence was detected using a 122 fluorometer (Gilson, USA) set to excitation 488 nm, emission 520 nm for carboxyfluorescein (6FAM)-labeled RNA.

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