Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Site-specific polymerase incorporation of consecutive ligand-containing nucleotides for multiple metal-mediated base pairing

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1. Experimental methods

Materials and equipment.

All natural DNA strands, including 6-carboxyfluorescein (FAM)-labelled strands, a substrate strands containing a riboadenosine (rA), were purchased from Japan Bio Service Co., Ltd. (Saitama, Japan) at HPLC purification grade. Hydroxypyridone nucleoside triphosphate (d**H**TP) was synthesised according to the reported procedure.[1] Natural nucleoside triphosphates (dNTPs) were purchased from Toyobo Co., Ltd. Sulfolobus DNA polymerase IV (Dpo4), T4 DNA ligase and a ligation buffer (NEBNext® Quick Ligation Reaction Buffer) were purchased from New England Biolabs, Inc. $CuSO_4 \cdot 5H_2O$ (99.5% purity), $MnCl_2 \cdot 4H_2O$ (99%) and polyethylene glycol 6,000 (PEG6000, for molecular biology) were purchased from FUJIFILM Wako Pure Chemical Industries and used without further purification. The 5′-phosphrlylated strands used for the ligation reaction were purchased from Japan Bio Service Co., Ltd. (Saitama, Japan) or prepared by using T4 Polynucleotide Kinase (T4 PNK, New England Biolabs). The FAM-labelled single-stranded DNA ladder marker was prepared by the treatment of a 5′-FAM-labelled T_{38} oligomer with DNase I (Promega).

Denaturing polyacrylamide gel electrophoresis (PAGE) was carried out with 20% polyacrylamide gel containing 7 M urea. The gels were analysed using Gel Doc EZ Imager and Image Lab software (Bio-Rad).

Consecutive incorporation of dHTP by Dpo4 polymerase

A FAM-labelled primer and a template strands were mixed in a 1:1.5 molar ratio in a buffer (20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.1% Triton® X-100, 6% PEG6000) and annealed prior to the reaction (85 °C → 4 °C, 1.0 °C min^{-1}). After the addition of d**H**TP, Dpo4 polymerase and $MnCl_2$, the reaction mixture was incubated at 37 °C or 55 °C. The optimised final concentration of each component was as follows: [primer] = 1.0 μM, [template] = 1.5 μM, [d**H**TP] = 15 μM (15 equiv.), [$MnCl_2$] = 0.20 mM and [Dpo4 polymerase] = 0.04 U μL^{-1}. The reaction was quenched by adding a 3:1 mixture of 7 M urea and a loading solution (30% glycerol, 0.25% bromophenol blue), and the mixture was immediately heated at 95 °C for 10 min. The products were analysed by denaturing PAGE.

Polymerase synthesis of artificial DNA strands containing consecutive H nucleotides

A primer and a template strands were mixed in a buffer (20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.1% Triton® X-100, 6% PEG6000) and annealed prior to the reaction (85 °C → 4 °C, 1.0 °C min^{-1}). After d**H**TP, Dpo4 polymerase and $MnCl_2$ were added, the mixture was incubated at 55 °C for 4 h. The concentration of each component was as follows: [primer] = 3.0 μM, [template] = 4.5 μM, [d**H**TP] = 45 μM (15 equiv.), [$MnCl_2$] = 0.20 mM and [Dpo4 polymerase] = 0.12 U μL^{-1}. After the incubation, dNTPs and $MnCl_2$ were added to the reaction mixture, which was further incubated at 37 °C for 20 h. The final concentration of each component was as follows: [primer] = 2.0 μM, [template] = 3.0 μM, [d**H**TP] = 30 μM (15 equiv.), [dNTPs] = 200 μM (100 equiv.), [$MnCl_2$] = 0.20 mM and [Dpo4 polymerase] = 0.08 U μL^{-1}. The reaction was quenched by adding the loading buffer containing 7 M urea, and the mixture was immediately heated at 95 °C for 10 min. The reaction progress was analysed by denaturing PAGE.

Ligation of a strand bearing consecutive H nucleotides by T4 DNA ligase.

A primer and a template strands were mixed in a buffer (20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.1% Triton® X-100, 6% PEG6000) and annealed prior to the reaction (85 °C → 4 °C, 1.0 °C min^{-1}). After the addition of d**H**TP, Dpo4 polymerase and $MnCl_2$, the mixture was incubated at 55 °C for 8 h. The product strand containing **H** nucleotides (acceptor strand) and the template were isolated by isopropanol precipitation. After the addition of a 5′-phosphorylated donor strand, the mixture was annealed (85 °C → 4 °C, 1.0 °C min^{-1}) with in a ligation buffer (50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1.0 mM rATP, 10 mM dithiothreitol (DTT), 10% PEG6000). T4 DNA ligase was added, and then the mixture was incubated at 37 °C for 48 h. The final concentration of each component was as follows: [acceptor] = [template] = 10 μM, [donor] = 12 μM, [T4 DNA ligase] = 50 U μL^{-1}. The reaction was stopped by adding the loading buffer containing 7 M urea, and the mixture was immediately heated at 95 °C for 10 min. The products were analysed by denaturing PAGE.

MALDI-MS analysis.

The measurement was carried out in the same manner as described previously.[2] After quenching the enzymatic reaction by adding EDTA, the sample was subjected to isopropanol precipitation and desalted by gel filtration. After the treatment with a cation-exchange resin

(Dowex 50W×8, NH_4^+-form), mass spectra were measured using a mixture of 3-hydroxypicolinic acid (3-HPA) and ammonium citrate as a matrix. The DNA strands isolated by denaturing PAGE were also analysed according to the same procedure.

Enzymatic synthesis of H3-DNAzyme.

An acceptor strand, two phosphorylated donor strands and a template strand were combined in a reaction buffer (20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.1% Triton® X-100, 6% PEG6000). The mixture was annealed prior to the reaction (85 °C → 4 °C, 1.0 °C min^{-1}). After adding d**H**TP, Dpo4 polymerase and $MnCl_2$, the reaction mixture was incubated at 55 °C for 43 h. The final concentration of each component was as follows: [acceptor] = [donor] = [template] = 1.0 μM, [d**H**TP] = 30 μM, [$MnCl_2$] = 0.20 mM and [Dpo4 polymerase] = 0.04 U μL^{-1}. The enzymatic reaction was quenched by adding an equal volume of 50 mM EDTA and heating at 95 °C for 10 min.

The resulting DNA strands were isolated by isopropanol precipitation, and then annealed in a ligation buffer (50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM rATP, 10 mM dithiothreitol (DTT), 10% PEG6000) (85 °C → 4 °C, 1.0 °C min^{-1}). After adding T4 DNA ligase, the mixture was incubated at 37 °C for 24 h. The final concentration of each component was as follows: [acceptor] = [donor] = [template] = 10 μM, [T4 DNA ligase] = 50 U μL^{-1}. The reaction was quenched by adding EDTA and heating at 95 °C for 10 min. The product was purified by denaturing PAGE, gel filtration (Sephadex G-25 Fine, GE Healthcare) and isopropanol precipitation.

RNA-cleaving reaction by H3-DNAzyme.

A **H3**-DNAzyme strand (1.0 μM) was annealed (85 °C → 25 °C, 1.0 °C min^{-1}) in a reaction buffer (10 mM HEPES (pH 7.0), 1 M NaCl, 10 mM $MgCl_2$) in the presence or absence of $CuSO_4$ (1.0 equiv. to a **H**–**H** pair). The RNA-cleaving reaction was initiated by adding a 5′-FAM-labelled substrate strand. The final concentration of each component was as follows: [DNAzyme] = 1.0 μM, [substrate] = 10 μM (10 equiv.), [$CuSO_4$] = 0 or 3.0 μM. The reaction mixture was incubated at 25 °C. Aliquots of the mixture were taken at the predetermined time point, and the reaction was stopped by adding a 3:1 mixture of 7 M urea and the loading buffer. The progress of the DNAzyme reaction was analysed by denaturing PAGE. The percentage of the cleaved product (*F*) was calculated as follows:

F (%) = I_c / (I_c + I_u) × 100,

where I_c and I_u are the band intensities of the cleaved product and the uncleaved substrate, respectively. The apparent first-order rate constants (k_{obs}) were calculated from the initial velocity determined from the time points when F was less than 20%.

2. Supporting figures

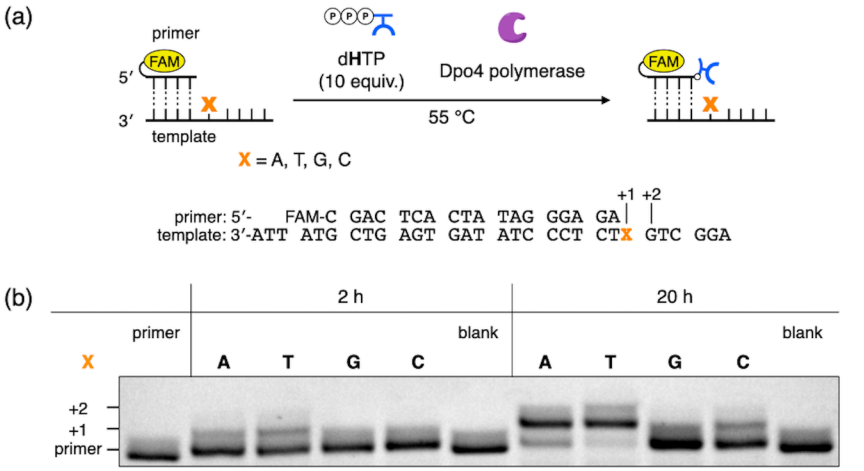

Figure S1. Enzymatic incorporation of a **H** nucleotide by Dpo4 polymerase with varying the opposite natural nucleotide (**X**). (a) Reaction scheme. [primer] = 1.0 μM, [template] = 1.5 μM, [d**H**TP] = 10 μM, [Dpo4 polymerase] = 0.04 U μL^{-1} in 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.1% Triton® X-100, 6% PEG6000, 55 °C, 2 or 20 h. (b) Denaturing PAGE analysis. FAM detection. d**H**TP was incorporated by Dpo4 polymerase when the opposite template base was A or T, as is the case in the incorporation by Klenow fragment exo– (KF exo–).[2]

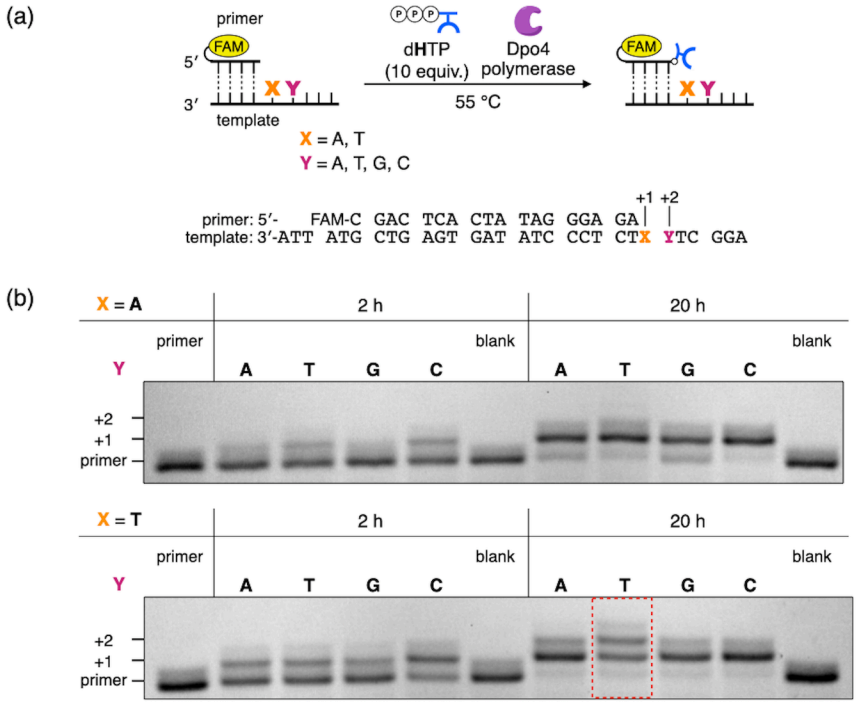

Figure S2. Enzymatic incorporation of multiple **H** nucleotides by Dpo4 polymerase with varying the nucleotide at the +2 position (**Y**). (a) Reaction scheme. [primer] = 1.0 μM, [template] = 1.5 μM, [d**H**TP] = 10 μM, [Dpo4 polymerase] = 0.04 U μL^{-1} in 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.1% Triton® X-100, 6% PEG6000, 55 °C, 2 or 20 h. (b) Denaturing PAGE analysis. FAM detection. Two **H** nucleotides were incorporated to the sites opposite the tandem T bases (**X**, **Y** = T).

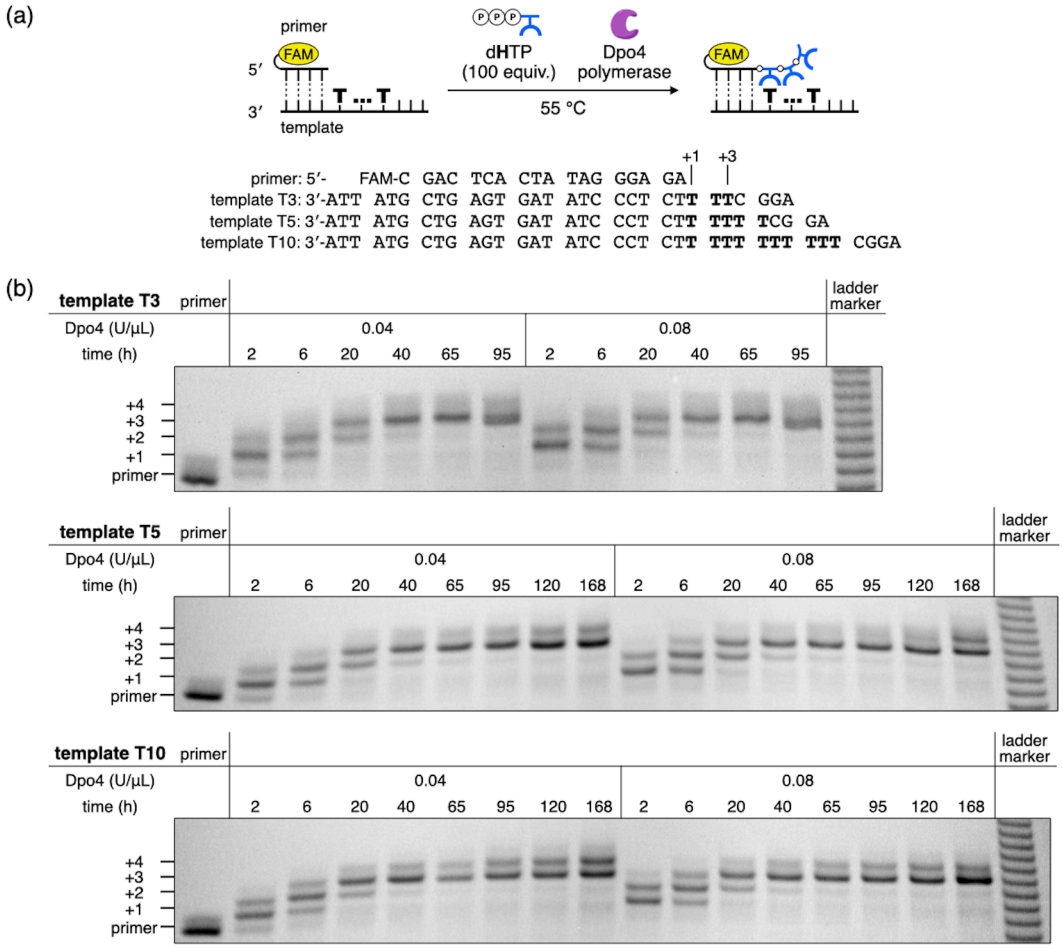

Figure S3. Enzymatic incorporation of multiple **H** nucleotides by Dpo4 polymerase using template strands having consecutive thymidine nucleotides (**T**). (a) Reaction scheme. [primer] = 1.0 μM, [template] = 1.5 μM, [d**H**TP] = 100 μM, [Dpo4 polymerase] = 0.04, 0.08 U μL^{-1} in 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.1% Triton® X-100, 6% PEG6000, 55 °C. (b) Denaturing PAGE analysis. FAM detection. When template T3 was used, three **H** nucleotides were appended to the primer in an almost quantitative manner. With templates T5 and T10, the incorporation of the fourth **H** was observed, but it was too slow to complete even after 168 h. The further extension was not observed even when the amount of Dpo4 polymerase was increased.

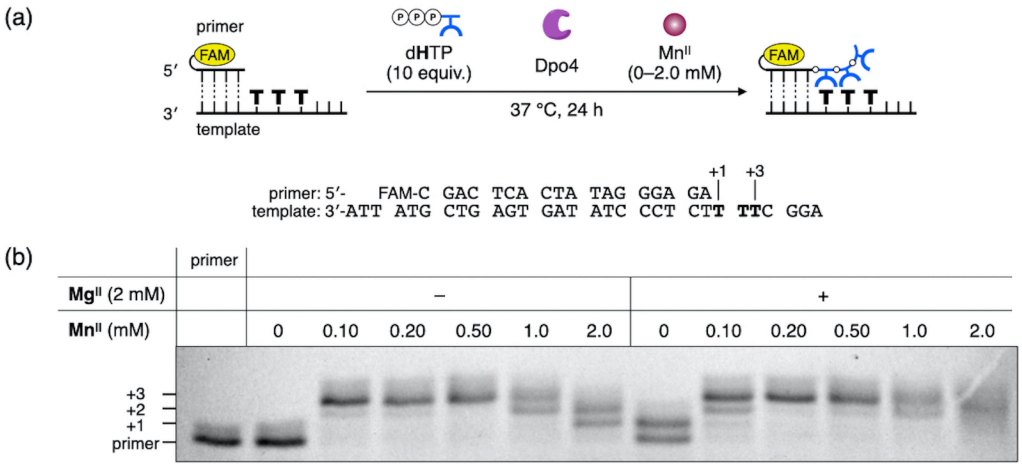

Figure S4. Enzymatic incorporation of consecutive **H** nucleotides by Dpo4 polymerase in the presence of different concentrations of Mn^{II} ions. (a) Reaction scheme. [primer] = 1.0 μM, [template] = 1.5 μM, [d**H**TP] = 10 μM (10 equiv.), [Dpo4 polymerase] = 0.04 U μL^{-1}, [$MnCl_2$] = 0–2.0 mM in 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0 or 2.0 mM $MgSO_4$, 0.1% Triton® X-100, 6% PEG6000, 37 °C, 24 h. (b) Denaturing PAGE analysis. FAM detection. The reaction was examined both in the absence and presence of Mg^{II} ions. The addition of Mn^{II} ions greatly accelerated the consecutive incorporation of d**H**TP. The optimal concentration of Mn^{II} was found to be 0.20 to 0.50 mM.

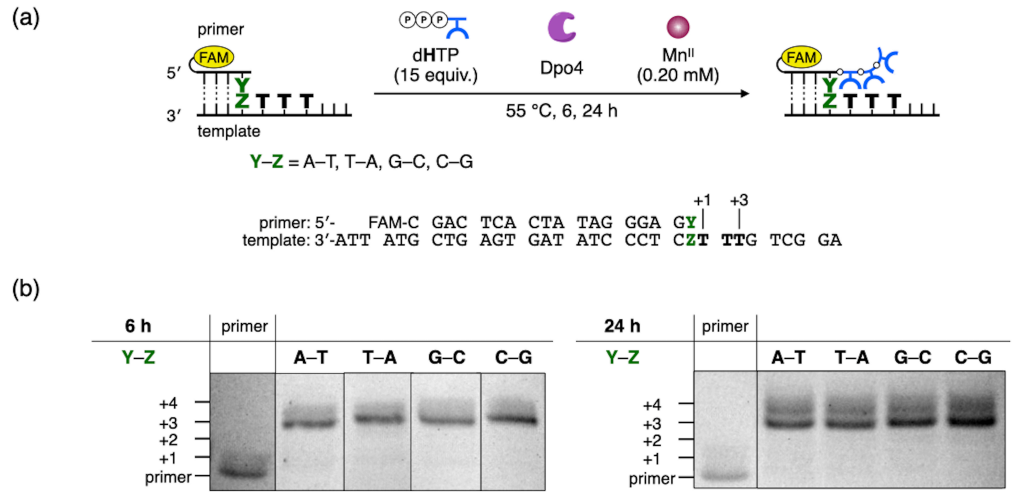

Figure S5. Polymerase incorporation of consecutive **H** nucleotides with varying the primer terminus base pair (**Y**–**Z**). (a) Reaction scheme. [primer] = 1.0 μM, [template] = 1.5 μM, [d**H**TP] = 15 μM, [Dpo4 polymerase] = 0.04 U μL^{-1} in 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.20 mM $MnCl_2$, 0.1% Triton® X-100, 6% PEG6000, 55 °C, 6 or 24 h. (b) Denaturing PAGE analysis. FAM detection. Three **H** nucleotides were consecutively incorporated regardless of the primer terminus base pair.

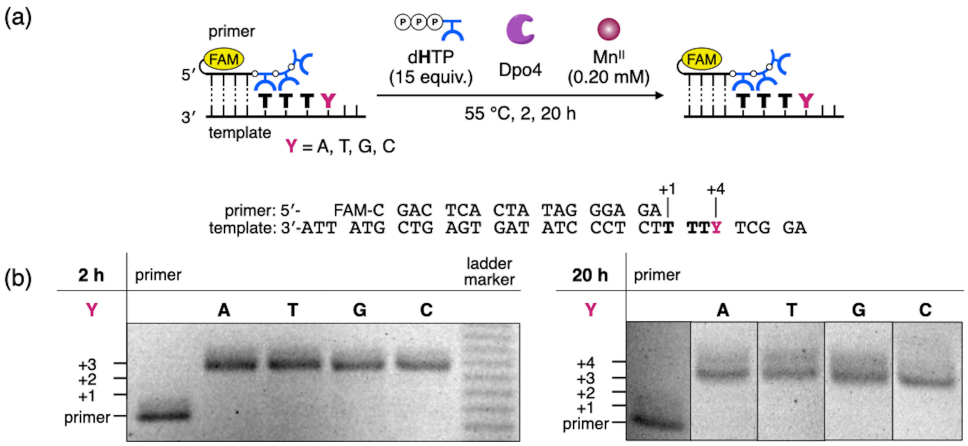

Figure S6. Polymerase incorporation of consecutive **H** nucleotides with varying the downstream template nucleotides at the +4 position (**Y**). (a) Reaction scheme. [primer] = 1.0 μM, [template] = 1.5 μM, [d**H**TP] = 15 μM, [Dpo4 polymerase] = 0.04 U μL^{-1} in 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.20 mM $MnCl_2$, 0.1% Triton® X-100, 6% PEG6000, 55 °C, 2 or 20 h. (b) Denaturing PAGE analysis. FAM detection. The downstream nucleotides had no effects on the incorporation of consecutive **H** nucleotides.

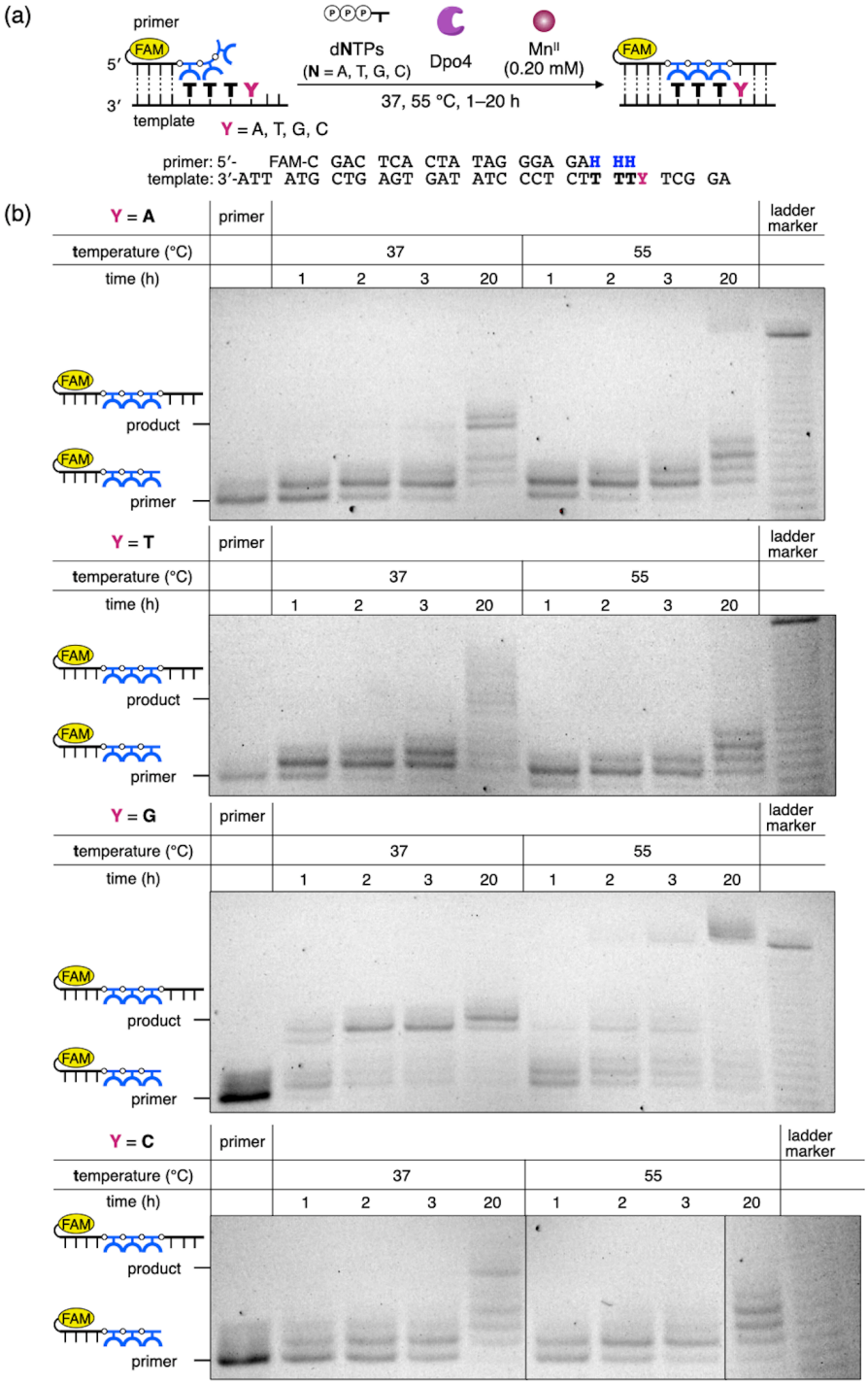

Figure S7. Enzymatic elongation of DNA strands after consecutive **H** nucleotides with varying the downstream template nucleotides (**Y**). (a) Reaction scheme. [primer] = 2.0 μM, [template] = 3.0 μM, [dNTPs] = 200 μM each, [Dpo4 polymerase] = 0.08 U μL^{-1} in 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.20 mM $MnCl_2$, 0.1% Triton® X-100, 6% PEG6000, 37 or 55 °C. (b) Denaturing PAGE analysis. FAM detection. When the downstream template nucleotide was G (**Y** = G), natural nucleotides (dNTPs) were efficiently incorporated even after **H** nucleotides, and the full-length product was obtained in a high yield. The reaction temperature of 37 °C was more suitable for the extension after **H** nucleotides than an optimal temperature of Dpo4 (55 °C), at which the termination or over-elongation of the **H**-bearing primer was observed.

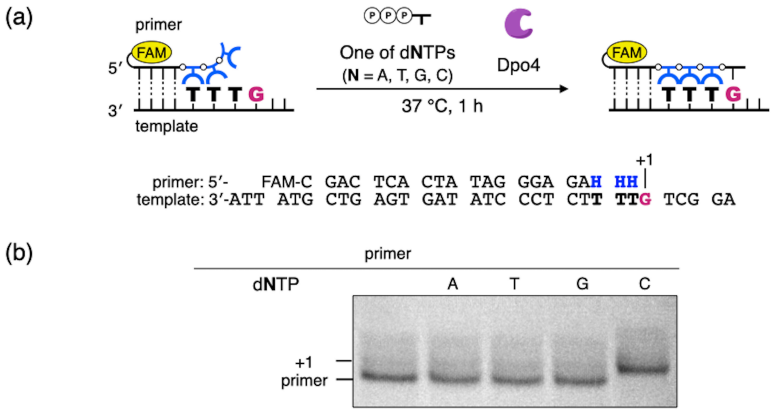

Figure S8. Polymerase incorporation of a natural nucleotide after consecutive **H** nucleotides. (a) Reaction scheme. [primer] = 2.0 μM, [template] = 3.0 μM, [dNTP] = 200 μM, [Dpo4 polymerase] = 0.08 U μL^{-1} in 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.20 mM $MnCl_2$, 0.1% Triton® X-100, 6% PEG6000, 37 °C, 1 h. (b) Denaturing PAGE analysis. FAM detection. Only dCTP was appended to the primer, showing that Dpo4 polymerase incorporates a nucleotide that forms a correctly matched base pair (i.e. G–C base pair) after the consecutive **H** nucleotides.

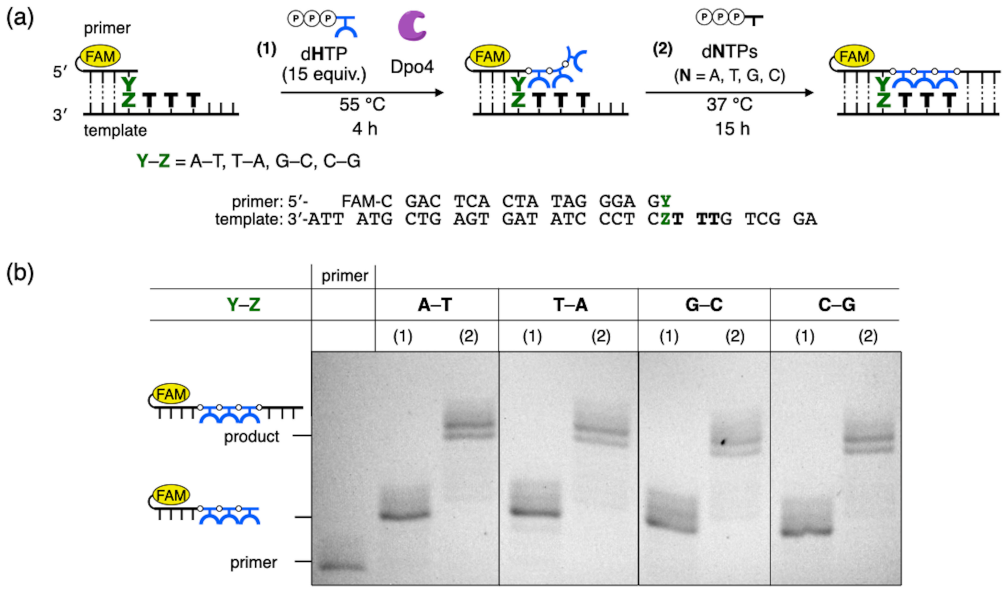

Figure S9. Enzymatic synthesis of DNA strands possessing consecutive **H** nucleotides with varying the primer terminus base pair (**Y**–**Z**). (a) Reaction scheme. (step 1) [primer] = 3.0 μM, [template] = 4.5 μM, [d**H**TP] = 45 μM, [Dpo4 polymerase] = 0.16 U μL^{-1}, 55 °C, 4 h; (step 2) [primer] = 2.0 μM, [template] = 3.0 μM, [d**H**TP] = 30 μM, [dNTPs] = 400 μM each, [Dpo4 polymerase] = 0.08 U μL^{-1}, 37 °C, 15 h. In 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.20 mM $MnCl_2$, 0.1% Triton® X-100, 6% PEG6000. (b) Denaturing PAGE analysis. FAM detection. The two-step primer extension afforded the full-length strand having three **H** nucleotides at an internal position regardless of the primer terminus base pair.

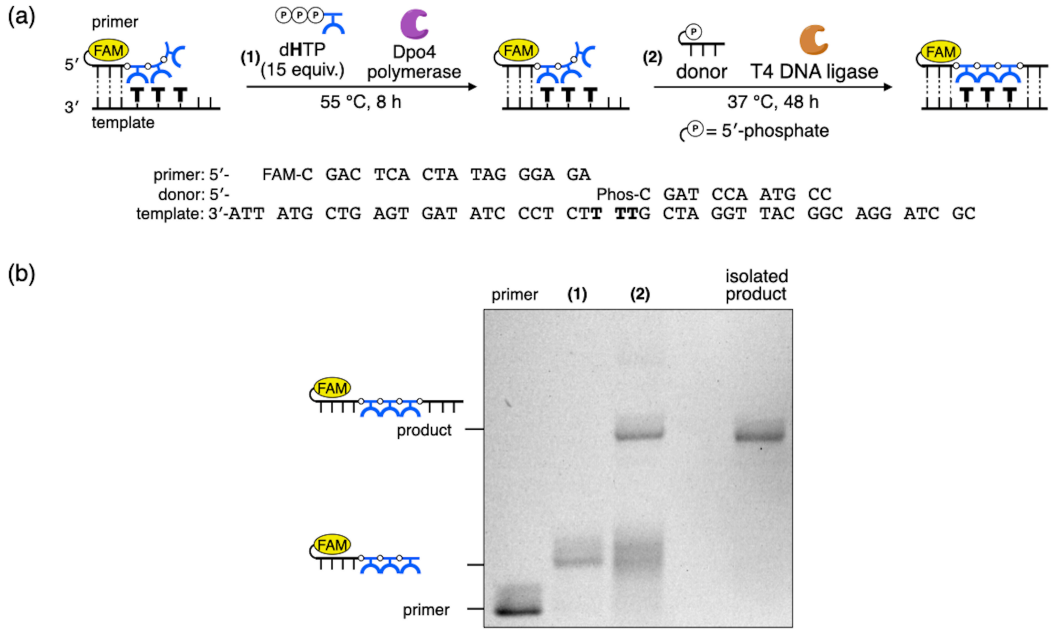

Figure S10. Enzymatic synthesis of DNA strands possessing consecutive **H** nucleotides by ligation with T4 DNA ligase. (a) Reaction scheme. (step 1) [primer] = [template] = 1.0 μM, [d**H**TP] = 15 μM, [Dpo4 polymerase] = 0.04 U μL^{-1} in 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.20 mM $MnCl_2$, 0.1% Triton® X-100, 6% PEG6000. 55 °C, 8 h. (step 2) [primer] = [template] = 10 μM, [donor] = 12 μM, [T4 DNA ligase] = 50 U μL^{-1} in 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1.0 mM rATP, 10 mM DTT, 10% PEG6000. 37 °C, 48 h. (b) Denaturing PAGE analysis. FAM detection.

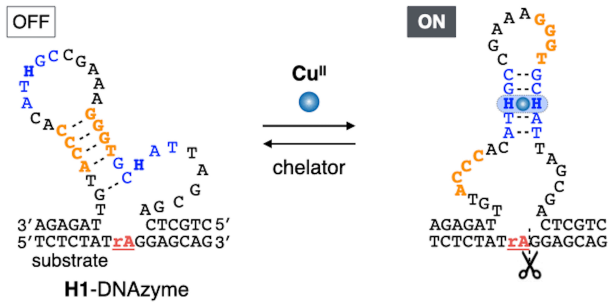

Figure S11. Design of the previously developed **H1**-DNAzyme, which has one **H**–Cu^{II}–**H** base pair.[3]

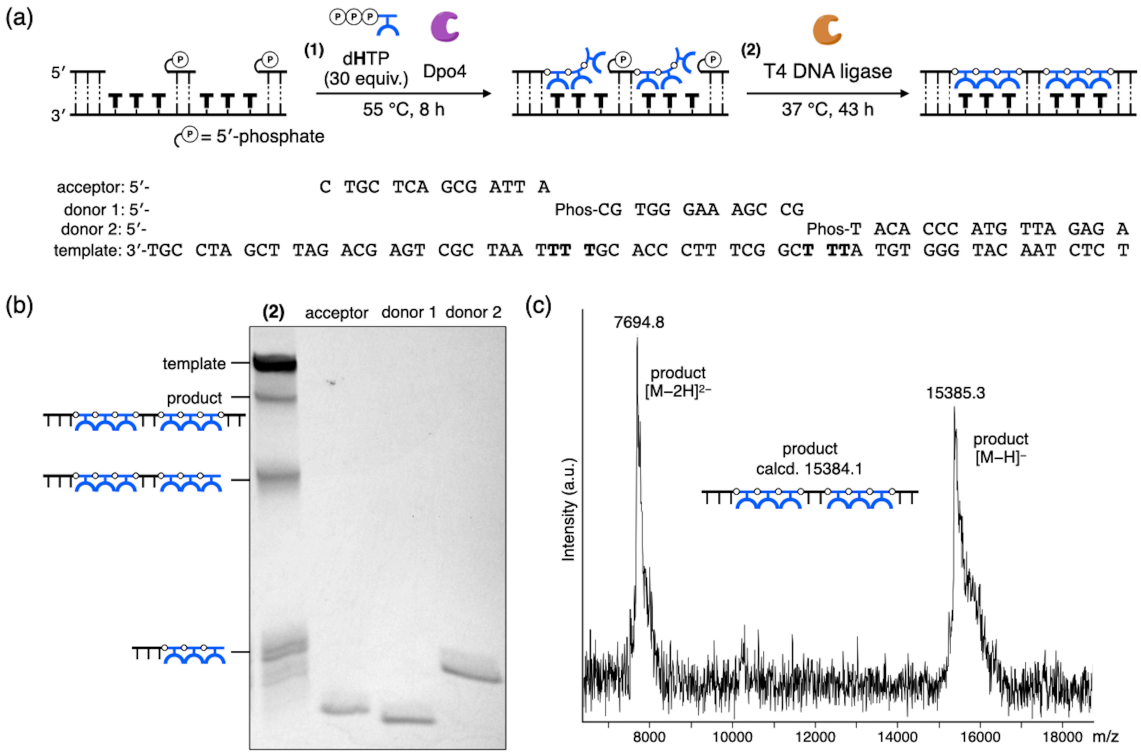

Figure S12. Enzymatic synthesis of **H3**-DNAzyme. (a) Reaction scheme. (step 1) [DNA strands] = 1.0 μM each, [d**H**TP] = 30 μM, [Dpo4 polymerase] = 0.04 U μL^{-1} in 20 mM Tris–HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2.0 mM $MgSO_4$, 0.20 mM $MnCl_2$, 0.1% Triton® X-100, 6% PEG6000. 55 °C, 8 h. (step 2) [DNA strands] = 10 μM each, [T4 DNA ligase] = 50 U μL^{-1} in 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1.0 mM rATP, 10 mM DTT, 10% PEG6000. 37 °C, 43 h. (b) Denaturing PAGE analysis. SYBR Gold staining. It should be noted that the ligation reaction proceeded regardless of the 5′-terminus of the donor strands (i.e., C and T). (c) MALDI-TOF mass analysis of the isolated product. Negative mode. Sodium adducts were also observed.

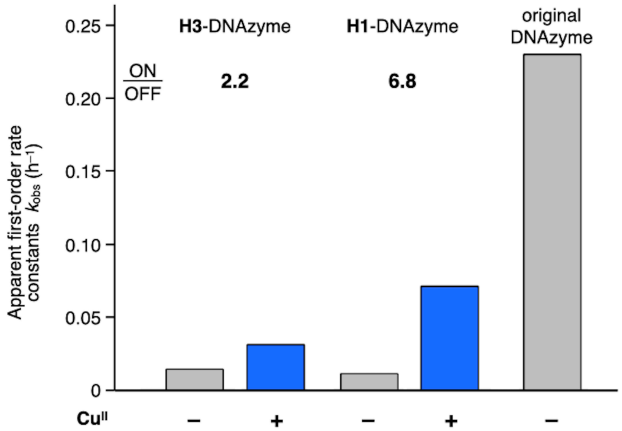

Figure S13. Apparent first-order rate constants (k_{obs}) of the RNA-cleaving reaction catalysed by the Cu^{II}-responsive DNAzymes in the presence and in the absence of Cu^{II} ions (1 equiv. for a **H**–**H** pair).

3. References

1. T. Kobayashi, Y. Takezawa, A. Sakamoto and M. Shionoya, *Chem. Commun.*, 2016, **52**, 3762–3765.
2. Y. Takezawa, T. Nakama and M. Shionoya, *J. Am. Chem. Soc.*, 2019, **141**, 19342–19350.
3. T. Nakama, Y. Takezawa, D. Sasaki and M. Shionoya, *J. Am. Chem. Soc.*, 2020, **142**, 10153–10162.