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A highly selective DNAzyme sensor for mercury cations

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Materials: dA^{im}TP was synthesized according to a literature procedure[1] and dU^{aa}TP was purchased from Sigma-Aldrich. All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA) and were purified by 10-15% 8 M urea denaturing PAGE. Ultrapure dNTPs were obtained from Fermentas, while Sequenase Version 2.0 and Single Stranded DNA binding protein were purchased from GE Healthcare. Lambda exonuclease, *Taq* DNA polymerase and Vent exo(-) DNA polymerase were obtained from New England Biolabs. Streptavidin magnetic particles were purchased from Roche. The nucleoside triphosphate dGTP α-[^{32}P] was purchased from Perkin Elmer. pGEM-T-Easy Vector Systems kit was obtained from Promega. $LiClO_4$ was purchased from J.T. Baker. $Hg(OAc)_2$ and all buffer and metal salts were obtained from Aldrich, save for $FeSO_4 \cdot 7H_2O$, $Fe(NO_3)_3 \cdot 9H_2O$, $Pb(NO_3)_2$, $UO_2(OAc)_2 \cdot 2H_2O$, $CoCl_2 \cdot 7H_2O$ and $CuSO_4$ which were purchased from Fisher.

Oligonucleotides (shown 5' to 3'): biotin-T_{20}GCGTGCC**rC**GTCTGTTGGGCCC **1**, GAGCTCGCGGGGCGTGCN$_{40}$CTGTTGGTAGGGCCCAACAGACG **2**, phosphate-CGTCTGTTGGGCCCTACCA **3**, GAGCTCGCGGGGCGTGC **4**, phosphate-ACGACACAGAGCGTGCCCGTCTGTTGGGCCCTACCA **5**, TTTTTTTTTTTTTTTTTTTTGAGCTCGCGGGGCGTGC **6**, phosphate-TAATACGACTCACTATAGGGAGCTCGCGGGGCGTGC **7**, GGGCGTGCTACGGCAGGGCGGGCGCGCGAGGCCGTCACACACGTGTGCTATT GGTAGGGCCCAACAGACGGGCACGCTCGTGTCGT **8**.

Buffers : **1** Mg^{2+}-buffer: 25 mM sodium cacodylate (pH 7.5), 5 mM $MgCl_2$, 200 mM NaCl, **2** Hg^{2+}-cleavage-buffer: a 0.1 M solution of $Hg(OAc)_2$ (freshly prepared, 0.319 g in 10 ml H_2O) is first 100-fold diluted in buffer **1** (1 mM solution) and then 200-fold diluted in buffer **1** to give the final cleavage buffer: 5 μM Hg^{2+}, 5 mM $MgCl_2$, 200 mM NaCl, 25 mM cacodylate (pH 7.5), **3** No metal-buffer: 25 mM sodium cacodylate (pH 7.5), 1 mM EDTA, 200 mM NaCl, **4** Zn^{2+}/Cu^{2+}-buffer: 5 μM Zn^{2+}, 5 μM Cu^{2+}, 5 mM $MgCl_2$, 200 mM NaCl, 25 mM cacodylate (pH 7.5), **5** elution buffer: 1% $LiClO_4$/Tris-HCl 10 mM (pH 8) in water.

***In vitro* selection** : 30 pmol of oligonucleotide **1** (5'-biotin-T_{20}GCGTGCC**rC**GTCTGTTGGGCCC-3') were annealed to 30 pmol of template DNA

(T_{20}GAGCTCGCGGGGCGTGCN$_{40}$CTGTTGGTAGGGCCCAACAGACG prepared by nested PCR using primers **5** and **6**) then enzymatically polymerized at 37°C for 3h using 9.1 units of Sequenase in a mixture containing Single Stranded Binding protein (SSB, 5 units), 5mM DTT, 250 μM dA^{im}TP, 50 μM of each dU^{aa}TP, dCTP, dGTP and trace amounts of dGTP α-[^{32}P]. The reaction was quenched by adding EDTA (25 mM final). The extension product was immobilized on 50 μl of pre-washed magnetic streptavidin particles by incubating at room temperature for 30 min. After 2 short washes with 100 μl TEN buffer, the template strand was removed by five washes of 100 μl NaOH 0.1M, EDTA 1 mM, followed by a neutralization wash of 200 μl cacodylate 25 mM (pH 6) and one 100 μl water wash. For the first four rounds of selection, the particles were suspended in 100 μl buffer **1** at room temperature for 30 min. For rounds 5 to 7, the negative selection consisted in incubating the particles in 100?μl buffer **1** at room temperature for 4 hours. For the three final rounds, the particles were first suspended in 100 μl buffer **3** at room temperature for 2 hours. After decanting, the particles were incubated in 100 μl buffer **1** at room temperature for 2 hours and then in 100 μl buffer **4** for 2 hours. For the positive selection, the particles were suspended in 100 μl buffer **2** at room temperature. The reaction time was decreased from 60 min down to 1 min and ten selection rounds were carried out. Following magnetization, the supernatant was precipitated (1% $LiClO_4$ in acetone), washed (EtOH), resuspended and resolved by 7% 8 M urea denaturing PAGE.

The species corresponding to the cleaved product was eluted using buffer **5**, precipitated and desalted. The PCR amplification of the resulting modified DNA followed the nested double PCR amplification method outlined in the selection of 9_{25}-11. In the first amplification step, the modified DNA was PCR amplified using primers **3** and **4** and an internal label (10 μCi dGTP α-[^{32}P]) for 30 cycles (15 seconds at 54°C, 40 seconds at 75°C and 15 seconds at 95°C). The reaction buffer included 0.07 units/μl Vent(exo-) DNA polymerase, 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 3 mM $MgSO_4$, 0.1% gelatin, 7 μM oligonucleotides and 0.3 mM of each natural dNTP. Prior to purification by 10% 8 M urea denaturing PAGE, the amplicon was treated with lambda exonuclease (. An aliquot was then further amplified using primers **5** and **6** with 0.1 unit/μl Vent(exo-) DNA polymerase over 30 PCR cycles. The resulting product was precipitated (phenol-chloroform followed by an EtOH wash) and the phosphorylated strand was digested using lambda

exonuclease. The single stranded DNA product was then purified by 10% 8 M urea denaturing mini-PAGE and identified by UV-shadowing.

Cloning of cDNAs: The 10^{th} generation of the selection was amplified using *Taq* DNA polymerase with primers **3** and **7** to produce PCR products with 3'-A overhangs. These amplicons were then TA cloned using the pGEM-T-Easy Vector Systems kit and were used to transform *E.coli* DH10B via electroporation. The transformation was plated on LB Agar containing 100 mg/l ampicillin. White colonies were picked and used to inoculate 1 ml of TB containing Plasmid Miniprep Kit and were subjected to restriction digest using EcoR I. Plasmids containing a single insert of the correct size (as controlled by 2% agarose gels), were submitted for sequencing. 60 random clones were then PCR amplified using 0.1 unit/µl *Taq* DNA polymerase with primers **5** and **6**. The resulting products were hen purified by 10% 8 M urea denaturing mini-PAGE and their concentration determined to be ~30 pmol by measuring their A_{260}. 5 pmol of each individual clones were used as templates to synthesize modified DNA, which was then immobilized on streptavidin magnetic particles. Following 2 short washes with 100?µl TEN buffer, the template strands were removed by three washes of 100?µl NaOH 0.1M, EDTA 1 mM, followed by a neutralization wash of 200 µl cacodylate 25 mM (pH 6) and one 100 µl water wash. Half of the single stranded modified DNA bound on the magnetic particles, was incubated in 30 µl buffer **2**. The other half was treated with 30 µl buffer **2** containing 200 nM Hg^{2+}. Three time points were taken (5, 30 and 60 min) and resolved by 7% 8 M urea denaturing PAGE. The Nucleic Acid Protein Service Unit of UBC carried out the sequencing of the most active clones using an SP6 sequencing primer. Synthetic oligonucleotides corresponding to the various clones were used as templates to synthesize modified DNA. After immobilizing the thus produced DNA on streptavidin magnetic particles and removing the template strand as described previously, the catalysts were incubated in buffers **1** to **4**. Clone #13 showed the best selectivity and activity and was thus, fully investigated for activity as reported herein.

Kinetic analysis of intramolecular cleavage: Following 5 washes with 100 µl NaOH 0.1M, EDTA 1 mM, one neutralization with 200 µl cacodylate 25 mM (pH 6) and one short water wash (100 µl), the avidin-bound modified DNA was incubated in 90 µl of buffer **2** containing varying Hg^{2+}-concentrations. 5 µl of the slurries were

quenched in 15 µl formamide (containing biotin (1 mM), EDTA (25 mM), 0.01% bromophenol blue and 0.01% xylene cyanole). Samples were then heated (95°C, 5 min), cooled (0°C), magnetized and resolved by 7% 8 M urea denaturing PAGE. Visualization was carried out by means of a phosphorimager (Amersham Typhoon 9200) and polygons were drawn around the bands corresponding to the cleaved and uncleaved species. The data of the cleavage reactions were then fitted to first-order reactions with Sigmaplot 2001 (version 7.101) using equation (1):

$$P_t = P_8 \cdot (1-e^{-kt}) \qquad (1)$$

Where P_t and P_8 are the fractions cleaved at time t and the end point of the reaction respectively and k is the first-order rate constant. At least three independent sets of data were collected.

Metal selectivity : 36 pmol of primer **1** containing the embedded ribose, rC, (5'-biotin-T_{20}GCGTGCC**rC**GTCTGTTGGGCCC-3') was annealed to 30 pmol of synthetic template 10-13, then enzymatically polymerized at 37°C for 3h using Sequenase 2.0 in a mixture containing Single Stranded Binding protein (SSB, 5 units), 250 µM $dA^{im}TP$, 50?µM of each $dU^{aa}TP$, dCTP, dGTP and trace amounts of dGTP α-[^{32}P]. The reaction was quenched by adding EDTA (25 mM final). The extension product was immobilized on 50 ?l of pre-washed magnetic streptavidin particles (Roche) by incubating at room temperature for 30 min. The template strand was then removed by five washes of 100?µl NaOH 0.1M, EDTA 1 mM, followed by a neutralization wash of 200 µl cacodylate 25 mM (pH 6) and one 100 µl water wash. The slurry of streptavidin particles in water was then divided in an appropriate amount of tubes and decanted. The reactions were then initiated by adding 20 µl of 1x metal cation solutions. At two time points (5 and 60 min), 5 µl of the slurries were quenched in 15 µl formamide (containing biotin (1 mM), EDTA (25 mM), 0.01% bromophenol blue and 0.01% xylene cyanole). Samples were then heated (95°C, 5 min), cooled (0°C), magnetized and resolved by 7% 8 M urea denaturing PAGE.

Salts used in this work: $FeSO_4 \cdot 7H_2O$, $Fe(NO_3)_3 \cdot 9H_2O$, $BaCl_3$, $UO_2(OAc)_2 \cdot 2H_2O$, $Pb(NO_3)_2$, $MnCl_2$, $CeCl_3 \cdot 7H_2O$, $CoCl_2 \cdot 7H_2O$, $SmCl_3 \cdot 6H_2O$, $EuCl_3 \cdot xH_2O$, $YbCl_3 \cdot 6H_2O$, $NiCl_2$, $Cd(NO_3)_2$, $CaCl_2$, $CuSO_4$ and $ZnSO_4$.

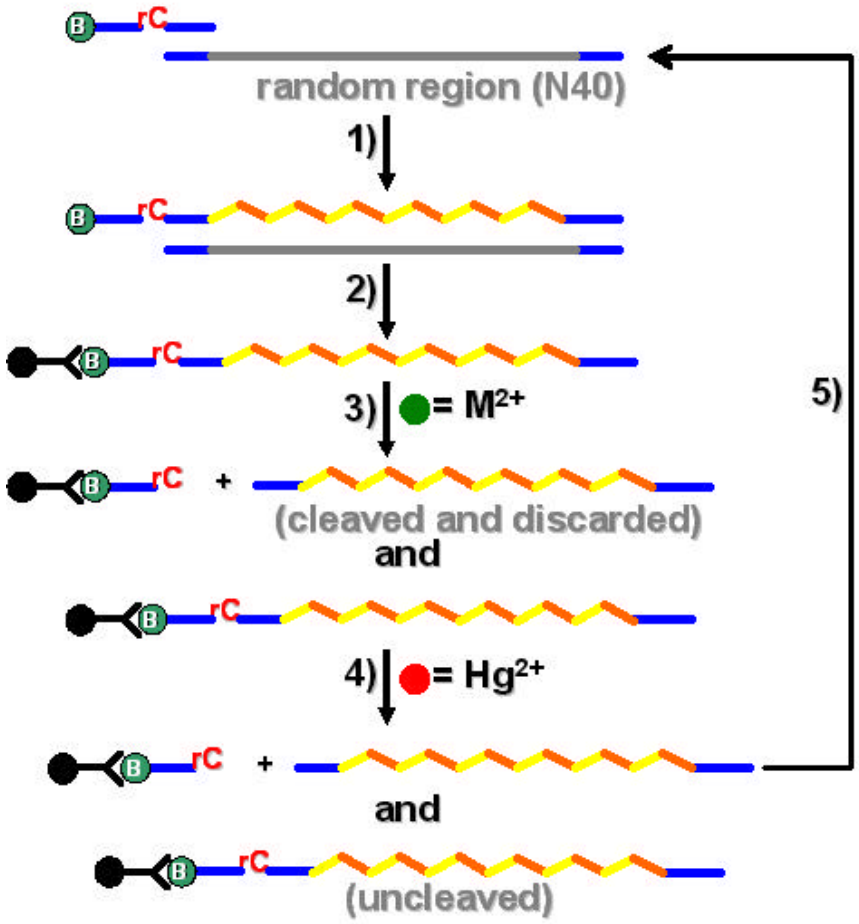

Figure S1. Selection strategy for the modified DNAzyme acting as a mercury sensor: 1) polymerization of the two modified nucleoside triphosphates and the remaining two natural triphosphates along a template containing 40 degenerate positions; 2) Following immobilization on streptavidin magnetic beads, the non-biotinylated strand was removed; 3) the pool was subjected to a counter-selection using various divalent metal cations (Zn^{2+}, Cu^{2+} and Mg^{2+}); the active species were discarded; 4) positive selection reaction: 5 µM Hg^{2+}, 5 mM Mg^{2+}, 200 mM NaCl, 25 mM Na-cacodylate, pH 7.5, 60 min to 1 min, rt; 5) the cleaved DNAs that are enriched for the recognition of mercury cations were amplified using a nested double amplification procedure[2]

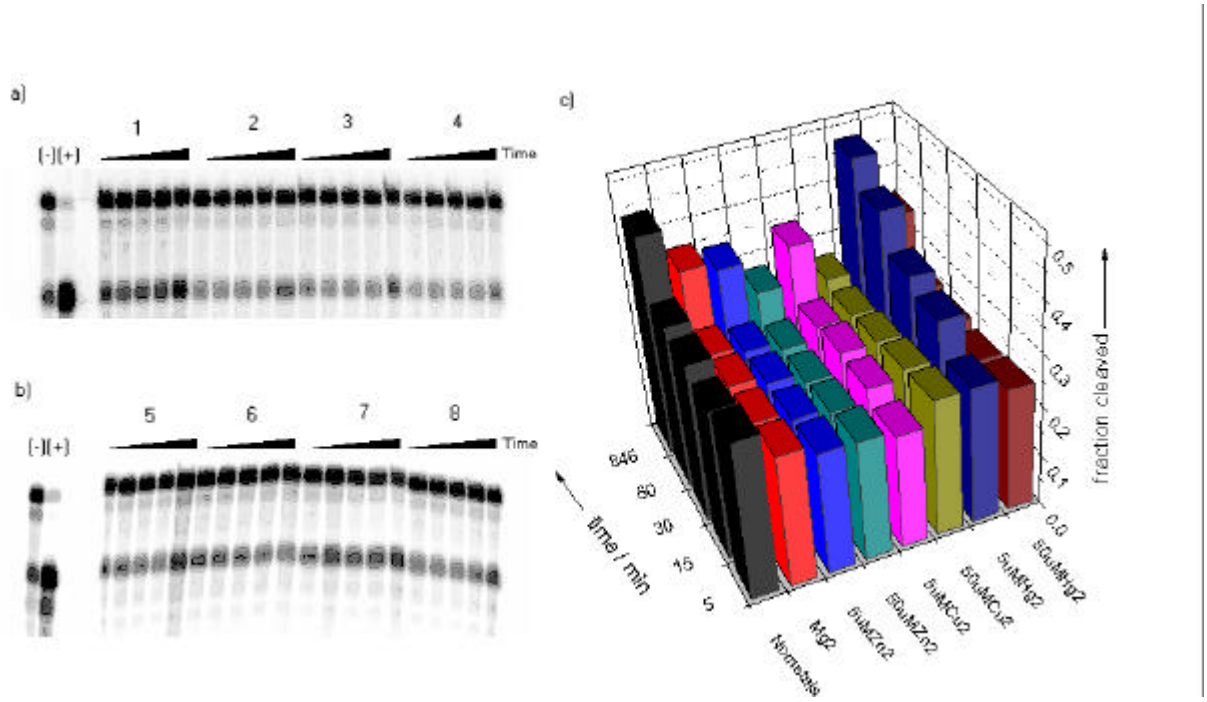

Figure S2. a) Behavior of the DNA population of round 7 in the absence of any metal cation (1); with Mg^{2+} alone (2); in the presence of 5 and 50 μM Zn^{2+} (3 and 4 respectively). b) Effect of 5 and 50 μM Cu^{2+} (5 and 6 respectively) and 5 and 50 μM Hg^{2+} (7 and 8 respectively) on generation 7. All gels are PAGE 7%, (+) and (-) represent the standards with and without RNase A treatment; c) Fraction cleaved as a function of time and condition applied.

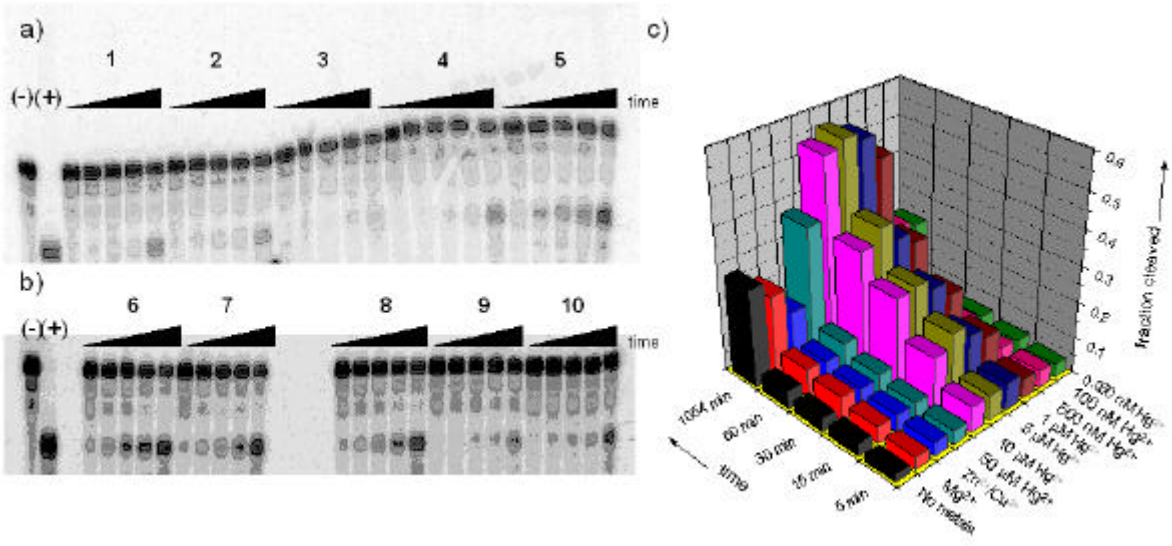

Figure S3. a) Behavior of the DNA population of round 10 in the absence of any metal cation (1); with Mg^{2+} alone (2); in the presence of 5 μM Zn^{2+} and Cu^{2+} (3); with 50 and 10 μM Hg^{2+} (4 and 5 respectively). b) Effect of 5 and 1 μM Hg^{2+} (6 and 7 respectively); 500 and 100 nM Hg^{2+} (8 and 9 respectively) and 100 nM Hg^{2+} (10) on generation 10. All gels are PAGE 7%, (+) and (-) represent the standards with and without RNase A treatment; c) Fraction cleaved as a function of time and condition applied.

#3 CAC AGU GUG UGA GGC ACU GUA CGG UGA GUG GUG CUU

#5 UUC UCA UCC GUA GUG AGG GAC GCG GCG CUC CCC CGU U

#8 CAU AGU GCG UGA GGC CCG CCC ACU CCC CGU CGU GGU A

#9 UUC UCA UCC GUA GUG AGG GAC GCG GCG CUC CCC CGU U

#10 GCA CAG UGU GUG AGG CAU GUG CGA GUG UGC UGU CUC G

#13 CAC ACG UGU GUG ACG GCC UCG CGC GCC CGC CCU GCC GUA

#17 CAU AGU GCG UGA GGC GCG UCU GCA GCG UGG UGG GUU U

#21 CAC AGU UGU GUG AUG GCU GGG CAG CCA GCG GUG GUC A

#24 CAU AGU GCG UGA GGC UUG CGC UGU CAG CGG UCG

#31 CAC AGU UGU GUG AGG CGC GUG UAC AGU GCG GCG GGU CU

#34 CAC AGU UGU GUG AUG GCU GGC UCU GCA GCC CGC UGG GU

#40 CAC ACG UGU GUG AGG CAU GCG CUC ACU GCC GUG GGU CU

#41 CAC AGU GUG UGA GGU UCG CCU CUG CCU CCC UGC UAC A

#42 CAU AGU GCG UGA GGC ACG GUU ACC GCC GUG GUG UGU

#49 CAC AGU GUG UGA GGC GUG CGC GAG UGG UUA GUG UCC UG

Figure S4. Sequence alignment obtained for the 15 most active clones of the Hg^{2+}-selection. Only the N_{40} random region is shown (written in the 5' to 3' orientation).

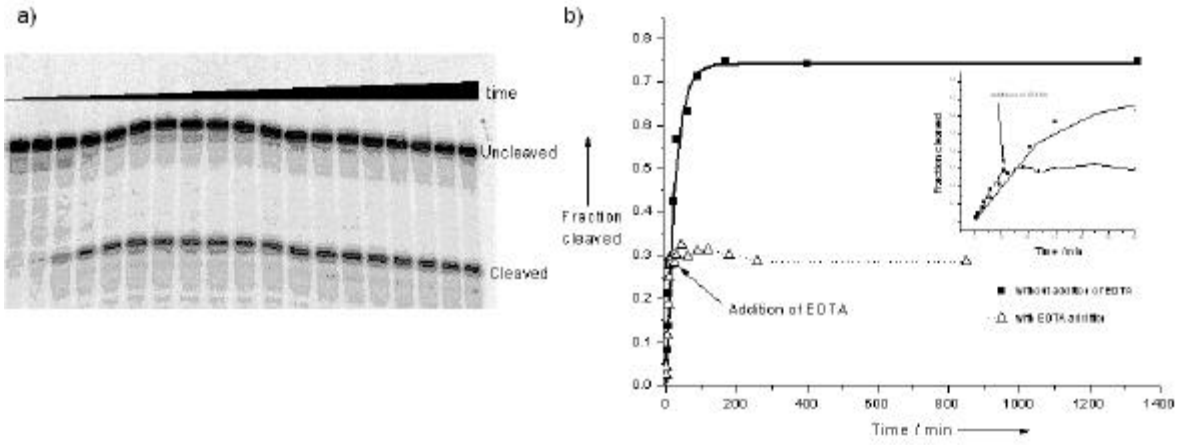

Figure S5. Delayed addition of EDTA. a) Gel image of the effect of addition of EDTA 0.5 M (14 mM final concentration) to a reaction initiated by the addition of 5 μM Hg^{2+} after 12 min. b) Graphical analysis of the assay without (!) EDTA and with (8) EDTA. Inset: close-up view on the evolution of the cleaved fraction for time points below 60 min.

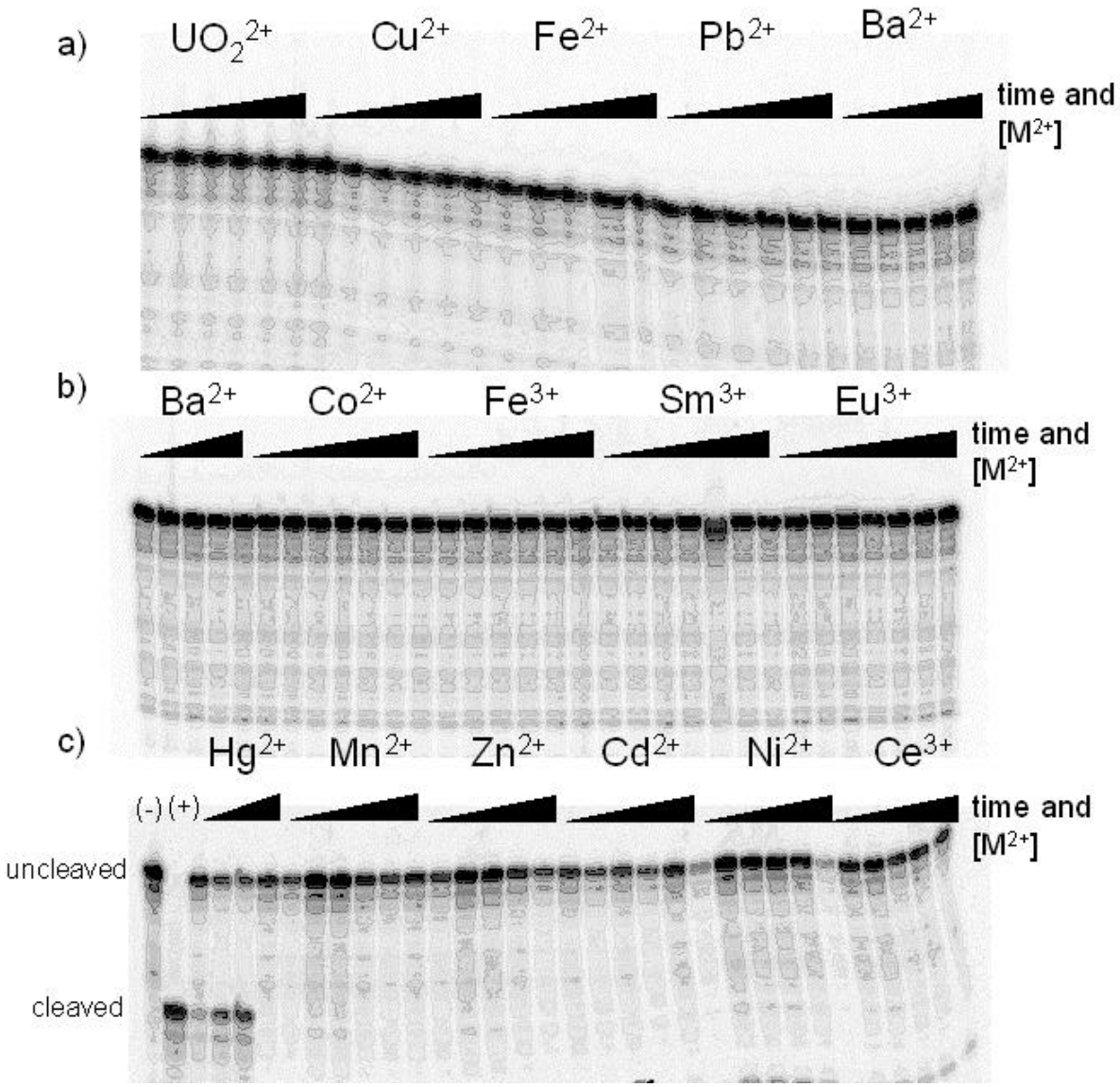

Figure S6. Effect of 14 (out of 16) competing metals. a) Effect of UO_2^{2+}, Cu^{2+}, Fe^{2+}, Pb^{2+} and Ba^{2+}. b) Effect of Ba^{2+}, Co^{2+}, Fe^{3+}, Sm^{3+} and Eu^{3+}. c) Controls without (-) and with (+) RNase A, effect of 5 μM Hg^{2+} (control reaction) and effect of Mn^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+} and Ce^{3+}. All gel images are PAGE 7%. For each metal, three different concentrations were investigated (1, 20 and 100 μM) at two different time points (5 and 60 min), which corresponds to the ordering on the gels.

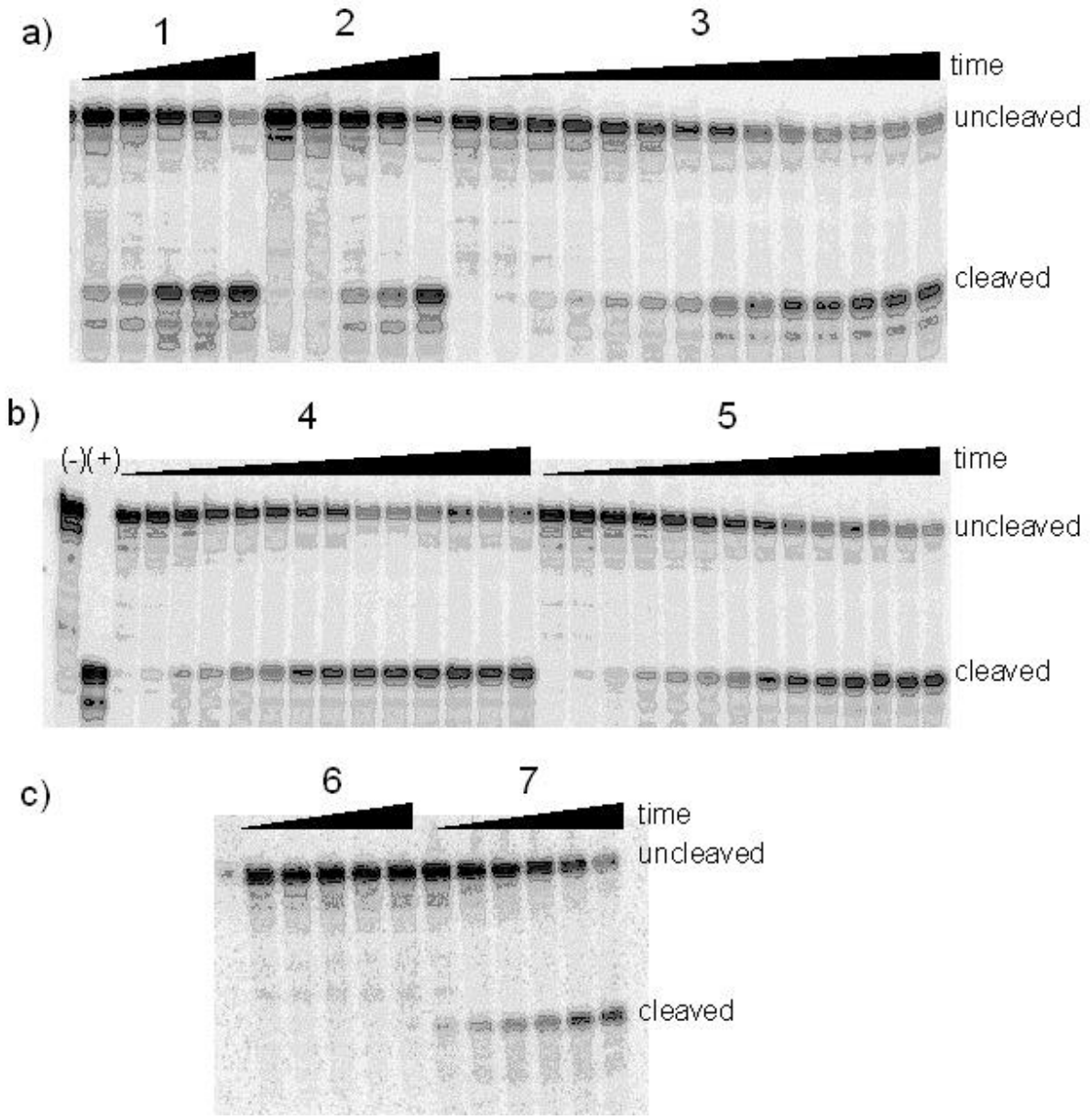

Figure S7. a) Reaction in the presence of 5 μM Hg^{2+} and 25 mM Mg^{2+} (1); reaction in the presence of 5 μM Hg^{2+} and 5 mM Mn^{2+} (2); reaction with 15 μM Hg^{2+} (3). b) Reactions initiated by the addition of 5 μM Hg^{2+} with 5 mM Mg^{2+} (4) and in the absence of Mg^{2+} (5). c) replacement of Mg2+ for Zn2+ and $[Co(NH_3)_6]^{3+}$ (6 and 7 respectively) in the presence of 5 μM Hg^{2+}. All gel images are PAGE 7%.

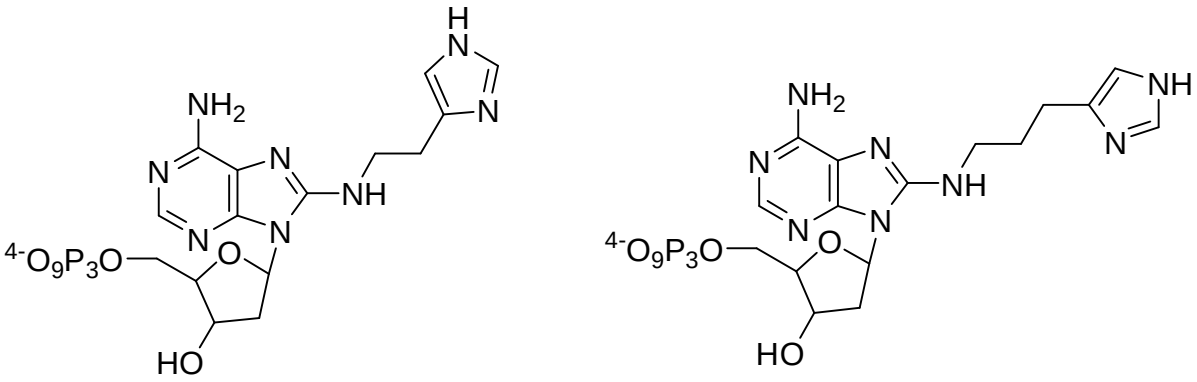

Figure S8. Structures of 8-(2-(4-Imidazolyl)-ethylamino)-2'-deoxyriboadenosine-5'-triphosphate (dA^{im}TP) and 8-(2-(4-Imidazolyl)-propylamino)-2'-deoxyriboadenosine-5'-triphosphate (dA^{hom}TP).[1, 3]

References:

[1] D. M. Perrin, T. Garestier, C. Hélène, *Nucleosides Nucleotides* **1999**, *18*, 377-391.
[2] D. M. Perrin, T. Garestier, C. Hélène, *J. Am. Chem. Soc.* **2001**, *123*, 1556-1563.
[3] C. Lam, D. M. Perrin, Manuscript in preparation.