Supplementary Information (SI) for Analyst.
This journal is © The Royal Society of Chemistry 2026

Supporting Information

DNA nanotechnology for nucleic acid analysis: Detection of RNA and dsDNA amplicon using multifunctional DNAzyme nanomachine (DNM)

Muhannad Ateiah[1,2], Sofia S. Zablotskaya[1], Maria S. Rubel[1,2], and Dmitry M. Kolpashchikov[3,4,*]

Affiliations

[1]– Center for Molecular and Biological Technologies, ITMO University, Lomonosova 9, St Petersburg 191002, Russia

[2]– Laboratory of Amyloid biology, St Petersburg State University, Universitetskaya enb., 7-9, St Petersburg 199034, Russia

[3]– Chemistry Department, University of Central Florida, Orlando, Florida 32816-2366, United States

[4]– Burnett School of Biomedical Sciences, University of Central Florida, Orlando, Florida 32816, United States

[*]Address correspondence to: Dmitry M. Kolpashchikov, E.mail: dmitry.kolpashchikov@ucf.edu

Table of content

1. **Materials and instrumentation.**
2. **Detailed Experimental Procedures**
3. **Table S1. Sequences of oligonucleotides**
4. **Table S2. 6-DNM oligonucleotides' binding melting temperature**
5. **Detailed structure of the DNA-RNA 120 analyte**
6. **Gel electrophoresis and dsDNA quantification**
7. **Detailed structure of the DNM machines design and assembly, and LOD.**
8. **LOD of 6-DNM with RNA and dsDNA amplicons**
9. **Table S3. ΔG calculation of DNM–Nucleic Acid complex formation**
10. **Reference**

1. Materials and instrumentation. DNAse/RNAse-free water was purchased from Qiagen (Hilden, Germany) and used for all the stock solutions of oligonucleotides. MQ water was purchased from Millipore RiOs-DI 3 Smart and used for buffers and solutions. Fluorogenic substrates (F-sub) was synthesized and HPLC purified by Genterra (Moscow, Russia). All oligonucleotides with HEG modification were synthesized and purified by DNK-syntez (Moscow, Russia). All other oligonucleotides (see Table S1 for sequences) were obtained from Evrogen (Moscow, Russia). The oligonucleotides were dissolved in DNAse/RNAse-free water and stored at –20 °C. The fluorescence intensities were measured at 520 nm (excitation wavelength at 480 nm). Excitation and emission slits were both 10 nm (Spark Multimode Microplate Reader Tecan, Spark- 10M). The data was processed using Microsoft Excel.

2. Detailed Experimental Procedures

2.1. Assembling of DNMs. Stock solutions of DNMs were prepared by annealing 1 μM of the specified tile strands (see Table S1) in reaction buffer composed of 50 mM HEPES (pH 7.4), 200 mM $MgCl_2$, 150 mM KCl, and 15 mM NaCl. Annealing was performed by mixing equimolar concentrations of the component strands, heating the mixture at 95 °C for 5 min, and allowing it to cool slowly to room temperature overnight. For 6-DNM, strands T1_6, T2_6, T3_6, and T4_6 were used; for 5-DNM: T1_5, T2_5, and T3_5; for 4-DNM: T1_4 and T2_4; and for 3-DNM: T1_3 and T2_3. Final working concentrations were: 1 nM Dza with 20 nM Dzb, 5 nM 6-DNM with 20 nM Arm3_6, 1 nM 5-DNM with 20 nM Arm2_5, 3 nM 4-DNM with 20 nM Arm3_4, and 1 nM 3-DNM with 20 nM Arm2_3. These concentrations of DNM and Arm 2 or Arm3 were found to be optimal in terms of ssDNA analyte dependent response over the background (data not shown).

2.2. General fluorescent assay for measuring limit of detection (LOD). Each sample prepared in 150 µL of reaction buffer (50 mM HEPES, pH 7.4, 200 mM $MgCl_2$, 150 mM KCl, 15 mM NaCl) contained 200 nM F_sub, the specific concentration of assembled DNM and free arm mentioned in 2.1, and target analyte in concentrations ranged from 0 to 800 pM of ssDNA_120. All samples were incubated at 55 °C for 30, 60 or 180 min followed by fluorescent measurement at 520 nm (λex = 480 nm). The reaction buffer composition was optimized in earlier studies [1-3] to ensure robust catalytic activity and reliable signal generation and was used consistently across all experiments to enable direct comparison between different DNM architectures.

2.3. PCR. ssDNA_142_2 was added to the samples and PCR amplified (CFX96 Touch RealTime PCR Bio-Rad) using the following temperature profiles: initial denaturation 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 17 s, extension at 72 °C for 30 s, and final extension at 72 °C for 5 min (1 cycle). The experimental tube contained 1pg of ssDNA. Negative control PCR «no DNA» contained all the components except the template. The reaction mixture consists of buffer $(NH_4)_2SO_4$, $MgCl_2$, primers (Primer_F; Primer_R_T7), dNTPs, Taq – polymerase, nuclease-free water (Invitrogen). The concentration of DNA amplicons was estimated as described in Figure S7. For Limit-of-Detection experiments 1 ng– 104 ng was used. Mismatched dsDNA amplicons were prepared using the same PCR protocol, substituting the template with ssDNA_142_MM.

2.4 *In* vitro transcription. Purified dsDNA template (Section 2.3) was obtained using the Evrogen CleanUp Standard Kit, following the manufacturer's protocol. *In* vitro transcription was performed using the Biolabmix T7 RNA polymerase kit in a total volume of 50 µL. Each reaction contained 1× transcription buffer, 1× DTT, 1 mM of each rNTP, 300 ng of dsDNA template, 3 U of T7 RNA polymerase, 50 U of RNase inhibitor (Biolabmix), and nuclease-free water (Invitrogen). Reactions were incubated overnight at 37 °C in a water bath. Following transcription, DNA templates were removed by treatment with DNase I (Thermo Scientific RNase-free DNase I kit) according to the manufacturer's instructions. RNA concentration was quantified using Equalbit RNA BR Assay Kit (Vazyme, China) following the manufacturer's instructions. For the Limit of detection experiments 1 pM – 1000 pM of RNA was used.

2.5 Limit of detection determination. LOD was determined according to the earlier established guidelines using the following method. First, the standard deviation (σ) of the background tube was calculated, and a threshold line was created at the y-axis on the resulting graph of LOD at y = (DNM background fluorescence + 3.σ background). The LOD was then determined by projecting the intersection point of the threshold line with the resulting trendline onto the x-axis.

3. Table S1. Sequences of oligonucleotides

Name	Sequence	Purification
ssDNA_120	AAAACCGTCTGTTCTAATAGCGATGACGGATAAATCAGATAGCAGATGACCACCAT AGGACCACCATTAGGAGAAAATAGACCATGGCCCAATAGCATAGCATAGACGATA AAAAAAAAAAA	**PAGE**
ssDNA_T7_template	TTTTTTTTTTTTATCGTCTATGCTATGCTATTGGGCCATGGTCTATTTTCTCCTAATGG TGGTCCTATGGTGGTCATCTGCTATCTGATTTATCCGTCATCGCTATTAGAACAGAC GGTTTTCCCTATAGTGAGTCGTATTA	**PAGE**
ssDNA_T7_template _MM	TTTTTTTTTTTTATCGTCTATGCTATGCTATTGGGCCATGGTCTATTTTCTCTTAATGG TGGTCCTATGGTGGTCATCTGCTATCTGATTTATCCGTCATCGCTATTAGAACAGAC GGTTTTCCCTATAGTGAGTCGTATTA	**PAGE**
Dza_BiDz	TGCCCAGGG ***AGGCTAGCT***GGTCCTATGGTGGTCATCTGCTATCTGATT	**PAGE**
Dzb_BiDz	TGCTATTGGGCCATGGTCTATTTTCTCCTAATGGT ***ACAACGA***GAGGAAACCTT	**PAGE**
T1_3-DNM	TCTAGCGTAGTCACTATATTAGAGTCGAATATGTGAAGACCT	**PAGE**
T2_3-DNM	TGCCCAGGG***AGGCTAGCT***TCCTATGGTGGTCATCTGC/HEG/AGGTCTTCACATATT CGACTCTAATATAGTGACTACGCTAGA/HEG/ATGCTATGCTATTGGGCCAT	**PAGE**
Arm 2_3-DNM	GGTCTATTTTCTCCTAATGGTGG ***ACAACGA***GAGGAAACCTT	**PAGE**
T1_4-DNM	TTTTTTTTTTTTATCGTCTATGCTA/HEG/TTCAGAGTCAAGACCTACGCTCTGCCACT CGTAACTGTGACTTGACCATCGGTCTCGCAGTCCTAA/HEG/TATCCGTCATCGCTAT TAGAACAGACGGT	**PAGE**
T2_4-DNM	TTAGGACTGCGAGACCGATGGTCAAGTCACAGTTACGAGTGGCAGAGCGTAGGTC TTGACTCTGAA/HEG/TGCTATTGGGCCATGGTCTATTTTCTCCTAATGGT ***ACAACGA***GAGGAAACCTT	**PAGE**
Arm 3_4-DNM	TGCCCAGGG ***AGGCTAGCT***GGTCCTATGGTGGTCATCTGCTATCTGATT	**PAGE**
T1_5-DNM	GTATCGTCCACTTCAGCAGTTTAGGCCGTACTGTTATCATTAGACTCGAATATGTGA AGACCT	**PAGE**
T2_5-DNM	TGCCCAGGG***AGGCTAGCT***TCCTATGGTGGTCATCTG/HEG/AACTGCTGAAGTGGA CGATAC/HEG/GCTATTAGAACAGACGGTTTT	**PAGE**
T3_5-DNM	TATGCTATTGGGCCATGG/HEG/AGGTCTTCACATATTCGAGTCTAATGATAACAGT ACGGCCTA/HEG/CTATCTGATTTATCCGTCATC	**PAGE**
Arm 2_5-DNM	TCTATTTTCTCCTAATGGTGG ***ACAACGA***GAGGAAACCTT	**PAGE**
T1_6-DNM	CTACTACGTTTTGATGAGGTAACATGGTTTGTATGTTCGAGTTCATCTGGTATGTCG AGCATTGACTGATGACATGCT	**PAGE**
T2_6-DNM	TGCCCAGGG***AGGCTAGCT***TCCTATGGTGGTCATCTG/HEG/TACCTCATCAAAACGT AGTAG/HEG/GCTATTAGAACAGACGGTTTT	**PAGE**
T3_6-DNM	TATGCTATTGGGCCATGG/HEG/GCTCGACATACCAGATGAACTCGAACATACAAA CCATGT/HEG/CTATCTGATTTATCCGTCATC	**PAGE**
T4_6-DNM	TTTTTTTTTTTTATCGTCTATGCT/HEG/AGCATGTCATCAGTCAAT	**PAGE**
Arm 3_6-DNM	TCTATTTTCTCCTAATGGTGG ***ACAACGA***GAGGAAACCTT	**PAGE**
Arm3_2_6-DNM	ATTTTCTCCTAATGGTGG ***ACAACGA***GAGGAAACCTT	**PAGE**
F-sub	AAGGTT/dT-FAM/TCCTC gu CCCTGGGCA/BHQ1	**HPLC**
Primer_F	TTTTTTTTTTTTATCGTCTATG	**PAGE**
Primer_R_T7	TAATACGACTCACTATAGGGAAAACCGTCTGTTC	**PAGE**

RNA nucleotides are in low case; Bold and italic nucleotides are the Dz 10-23 catalytic core; underlined nucleotides are F-sub-l binding arms; FAM - fluorescein amidites; BHQ1 – black hole quencher 1; HEG (17-O-(4,4'-Dimethoxytrityl)-hexaethyleneglycol, 1-[(2-cyanoethyl)- (N,N-diisopropyl)]-phosphoramidite); PAGE: polyacrylamide gel electrophoresis. MM – mismatched analyte.

4. Table S2. 6-DNM oligonucleotides' binding melting temperature

Oligonucleotides	Melting temperature
T4_6 (by Arm 1)	59.6 °C
T3_6 (by Arm2)	60.2 °C
Arm3_6	61.3 °C
Arm3_6_2	58 °C
T3_6 (by Arm4)	59.4 °C
T2_6 (by Arm5)	57.7 °C
T2_6 (by Arm6)	60.6 °C
T1with T2	62.4 °C
T1with T3	80.3 °C
T1 with T4	59.6 °C

The sequences of the arms are shown in Figures S6.

5. Detailed structure of DNA-RNA 120 analyte

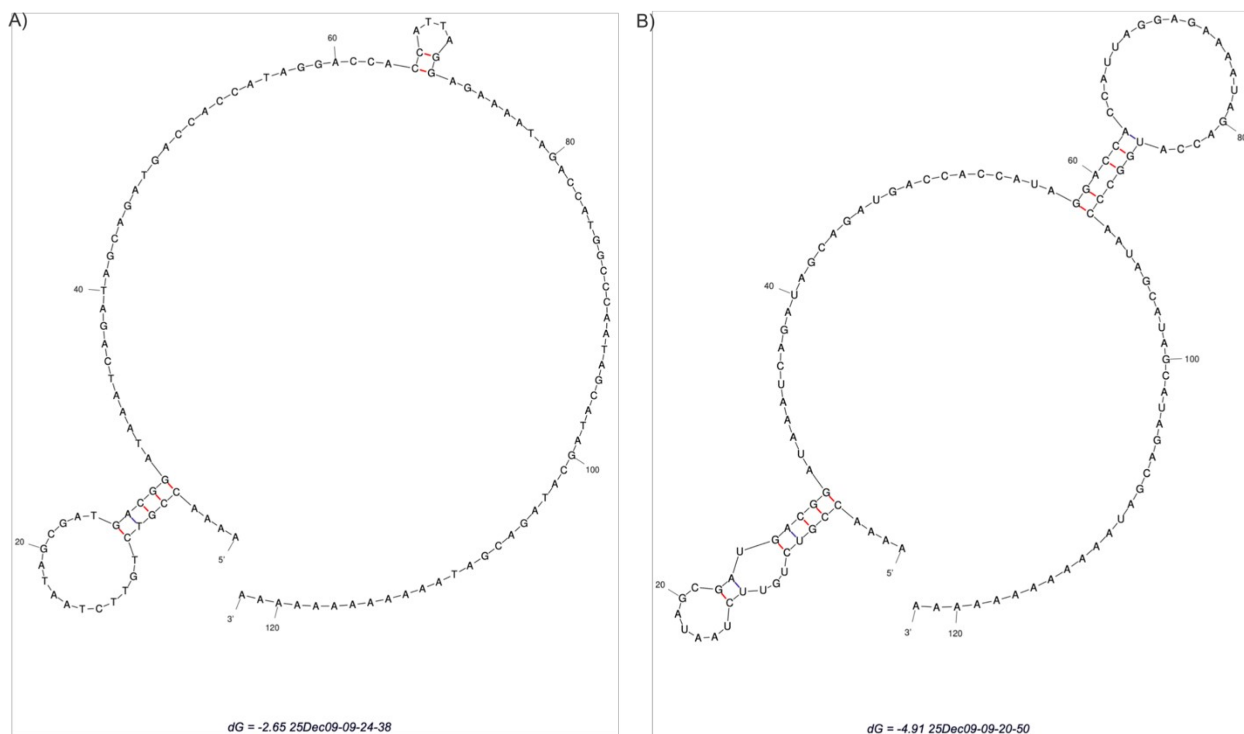

Figure S1. Structures of DNA-RNA 120 analyte used in this study. A) Predicted secondary structure of synthetic DNA-120 analyte (Table S1) by Mfold at 55 °C in the 215 mM Na^{+}, 200 mM Mg^{2+}. **B)** Predicted secondary structure of synthetic RNA-120 analyte by Mfold at 55 °C.

6. Gel electrophoresis

6.1 Analysis of PCR amplicons and dsDNA quantification. PCR samples were analyzed in a 2,0 % agarose gel using a Thermo Scientific EasyCast B1a mini gel electrophoresis system. DNA ladder Evrogen 50+ bp and 4×dye DNA Loading Dye were purchased from Evrogen (Moscow, Russia). The samples were run at 100 V for 20 min followed by staining in GelRed for 15 min. The volume of samples was 0.5; 1; 2; 5; 7; 10 µL (Fig. S7A), respectively. For analysis of concentrations, gel densitometry was used (Fig 7A and B).

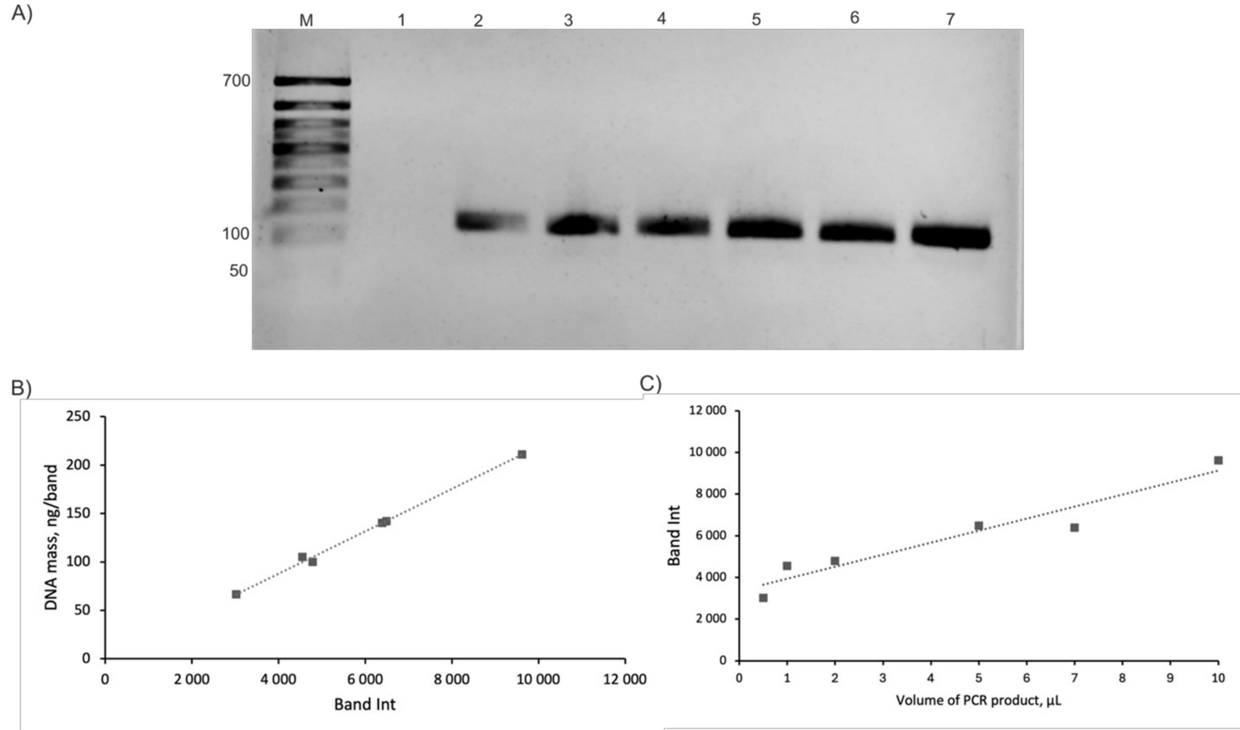

Figure S2. **Agarose gel electrophoresis analysis of dsDNA amplicons.** Marker (M) - ladder (Evrogen 50+ bp DNA Marker). Lane 1: PCR "no DNA" negative control. Lanes 2-7: amplification of product with various volumes: 0,5; 1; 2; 5; 7; 10 µL. According to our estimation, the concentration of dsDNA amplicons was 322.4 nM.

6.2 Analysis of RNA analyte by PAGE gel electrophoresis. RNA analyte was analyzed in a 12% PAGE gel using a

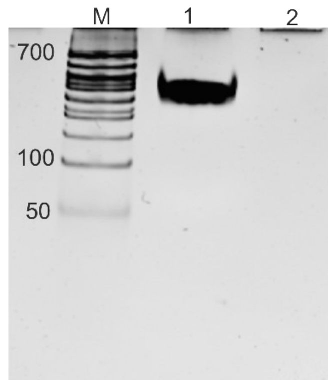

Figure S3. PAGE electrophoresis analysis of RNA analyte. Marker (M) - ladder (Evrogen 50+ bp DNA Marker). Lane 1: RNA analyte. Lane 2: negative control. The samples were run at 75 V for 80 min followed by staining in GelRed for 15 min.

7. Detailed structure of the DNM machines design and assembly, and LOD.

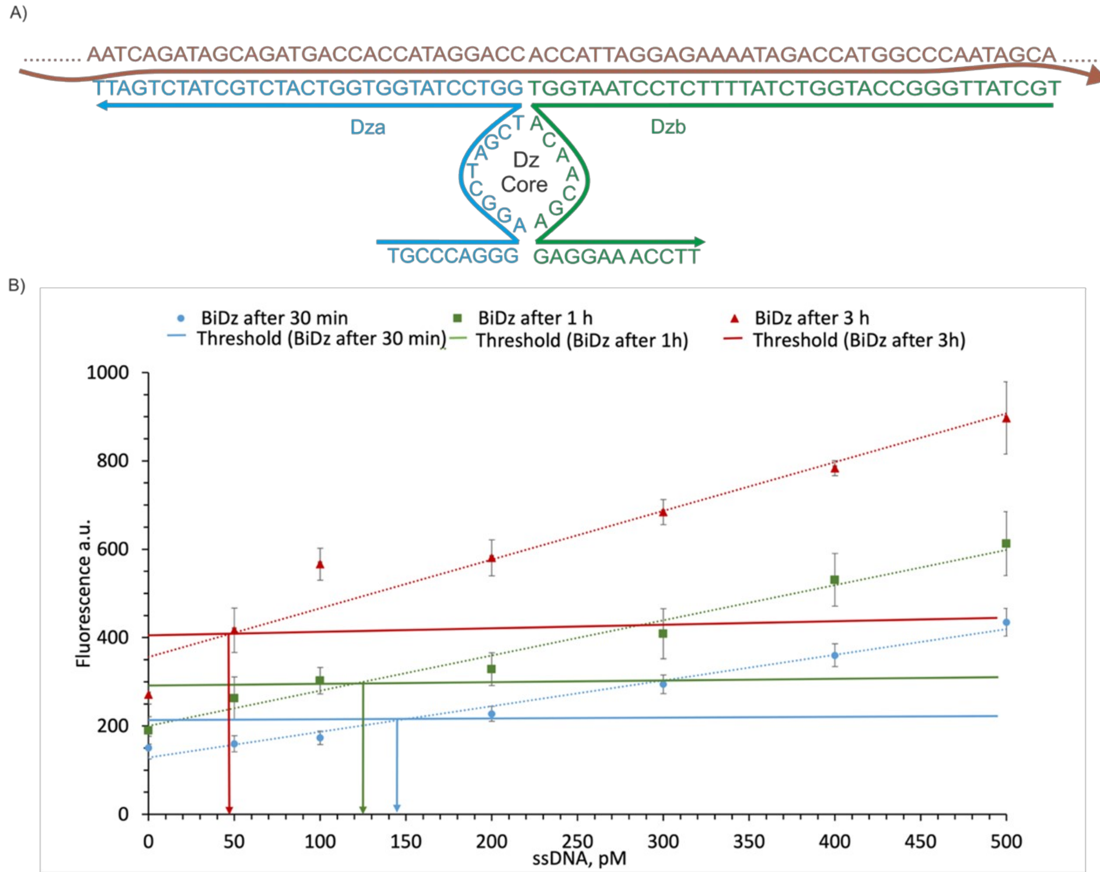

Figure S4. Structures of BiDz used in this study. A) The BiDz in complex with ssDNA_120 synthetic analyte. BiDz consists of 2 parts: Dza and Dz. **B)** LOD of BiDz after 30, 60, and 180 min of incubation at 55 °C for synthetic ssDNA. The data for LOD determination for BiDz is average values of three independent measurements. Trendlines are shown in blue (30 min), green (60 min), and red (180 min). Threshold lines correspond to the background average plus three standard deviations. The error bars are one standard deviation of the average.

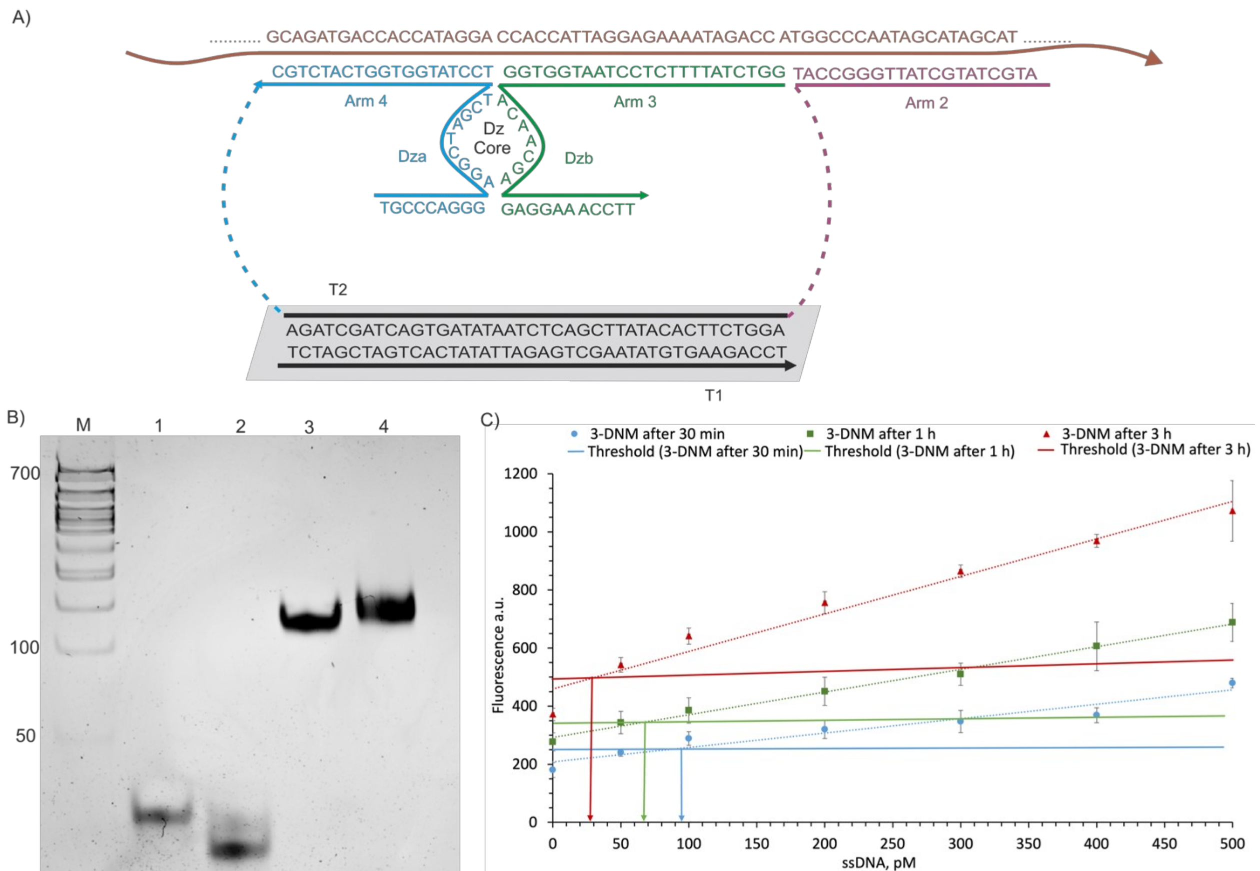

Figure S5. Structures of 3-DNA nanomachine used in this study. A) The 3-DNM machine is in complex with ssDNA_120 synthetic analyte. 3-DNM consists of 2 major parts, T1, T2 and the free separated Arm 2. T1 has no analyte-binding arm and is part of the DNA scaffold. T2 has two analyte-binding arms A1 and A3connected with HEG linkers to the DNA scaffold. **B)** Analysis of 3-DNM association in 12% Native PAGE. M (Marker) – Ladder (Evrogen 50+ bp DNA Marker), 1– Free Arm 2; 2 – Tile stand T1; 3 – T2; 4 – 3-DNM (T1, T2 strands annealed). The assembling of 3-DNM is described in Detailed Experimental Procedures (2.1). The samples were separated in 12% Native PAGE at 85 V during 75 min followed by staining in GelRed for 15 min. **C)** LOD of 3-DNM after 30, 60, and 180 min of incubation at 55 °C for synthetic ssDNA. The data for LOD determination for 3-DNM is average values of three independent measurements. Trendlines are shown in blue (30 min), green (60 min), and red (180 min). Threshold lines correspond to the background average plus three standard deviations. The error bars are one standard deviation of the average.

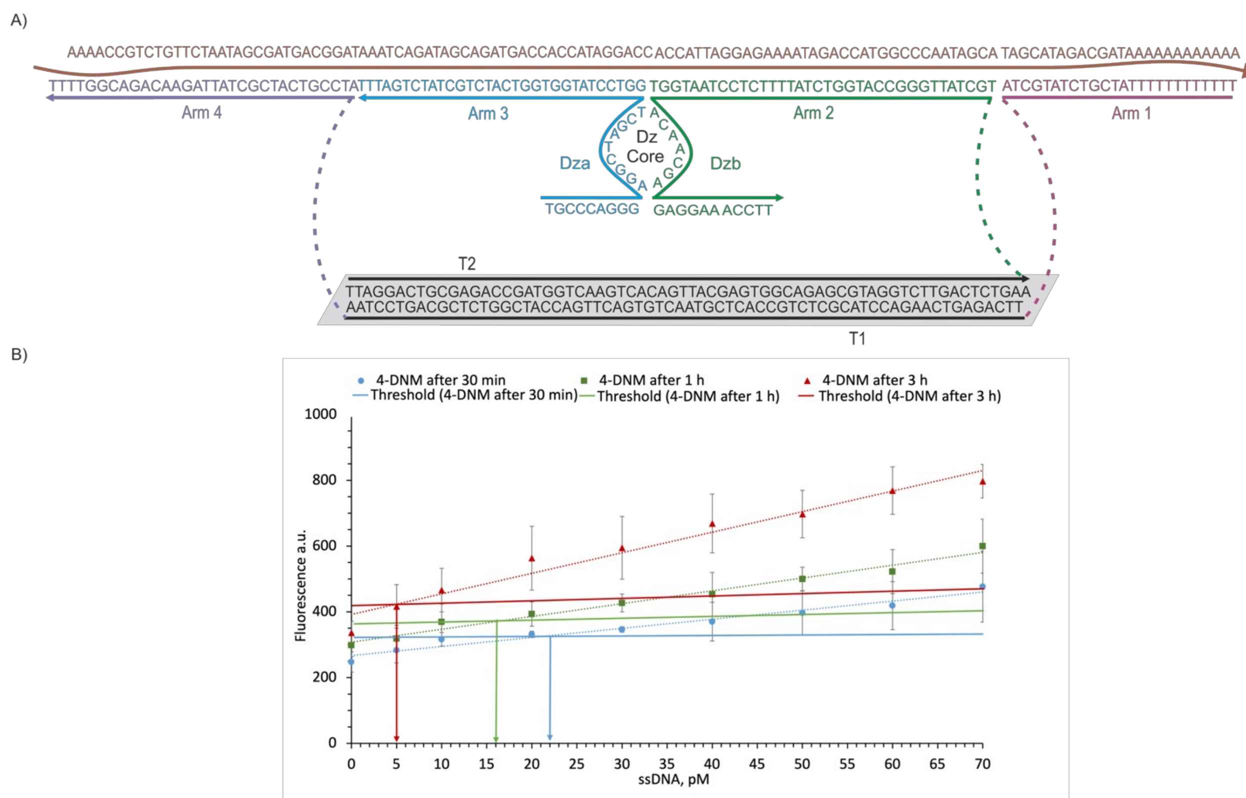

Figure S6. Structures of 4-DNA nanomachine used in this study. A) The 4-DNM machine in complex with ssDNA_120 synthetic analyte. 4-DNM consists of 2 major parts, T1, T2 and the free separated Arm 3. T1 contains Arm 1 and Arm 4 linked by a HEG linker to the DNA scaffold. T2 has one analyte-binding arms A3 connected with HEG linkers to the DNA scaffold. **B)** LOD of 4-DNM after 30, 60, and 180 min of incubation at 55 °C for synthetic ssDNA. The data for LOD determination for 4-DNM is average values of three independent measurements. Trendlines are shown in blue (30 min), green (60 min), and red (180 min). Threshold lines correspond to the background average plus three standard deviations. The error bars are one standard deviation of the average.

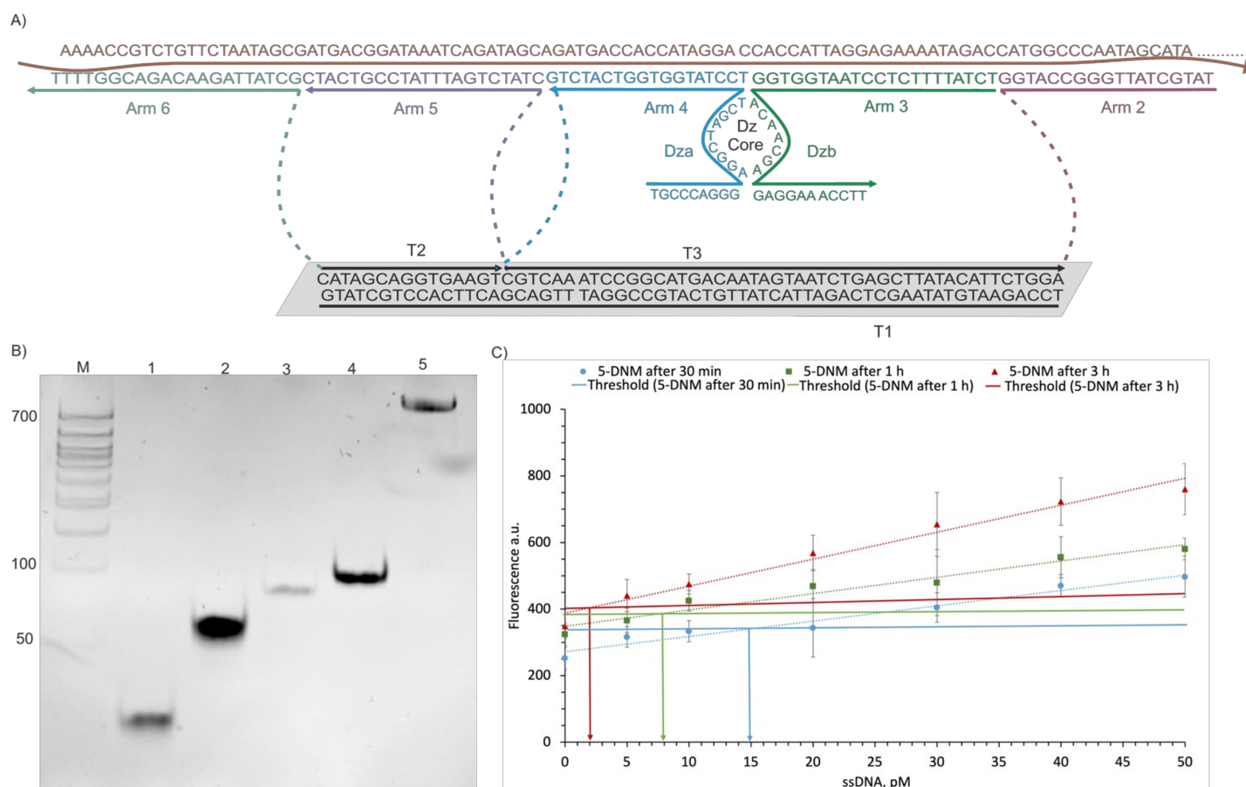

Figure S7. Structures of 5-DNA nanomachine used in this study. A) The 5-DNM machine in complex with ssDNA_120 synthetic analyte. 5-DNM consists of 3 major parts, T1, T2, T3 and the free separated Arm 2. T1 has no analyte-binding arm and is part of the DNA scaffold. T2 contains Arm 1 and Arm 4 linked by a HEG linker to the DNA scaffold. T3 has two analyte-binding arms, A3 and A5, connected with HEG linkers to the DNA scaffold. **B)** Analysis of 5-DNM association in 12% Native PAGE. M (Marker) – Ladder (Evrogen 50+ bp DNA Marker), 1 – Free Arm 2;2 – Tile stand T1; 3 – T2; 4 – T3; 5 – 5-DNM (T1, T2, T3 strands annealed). The assembling of 5-DNM was described in Detailed Experimental Procedures (2.1). The samples were separated in 12% Native PAGE at 85 V during 75 min followed by staining in GelRed for 15 min. **C)** LOD of 5-DNM after 30, 60, and 180 min of incubation at 55 °C for synthetic ssDNA. The data for LOD determination for 5-DNM is average values of three independent measurements. Trendlines are shown in blue (30 min), green (60 min), and red (180 min). Threshold lines correspond to the background average plus three standard deviations. The error bars are one standard deviation of the average.

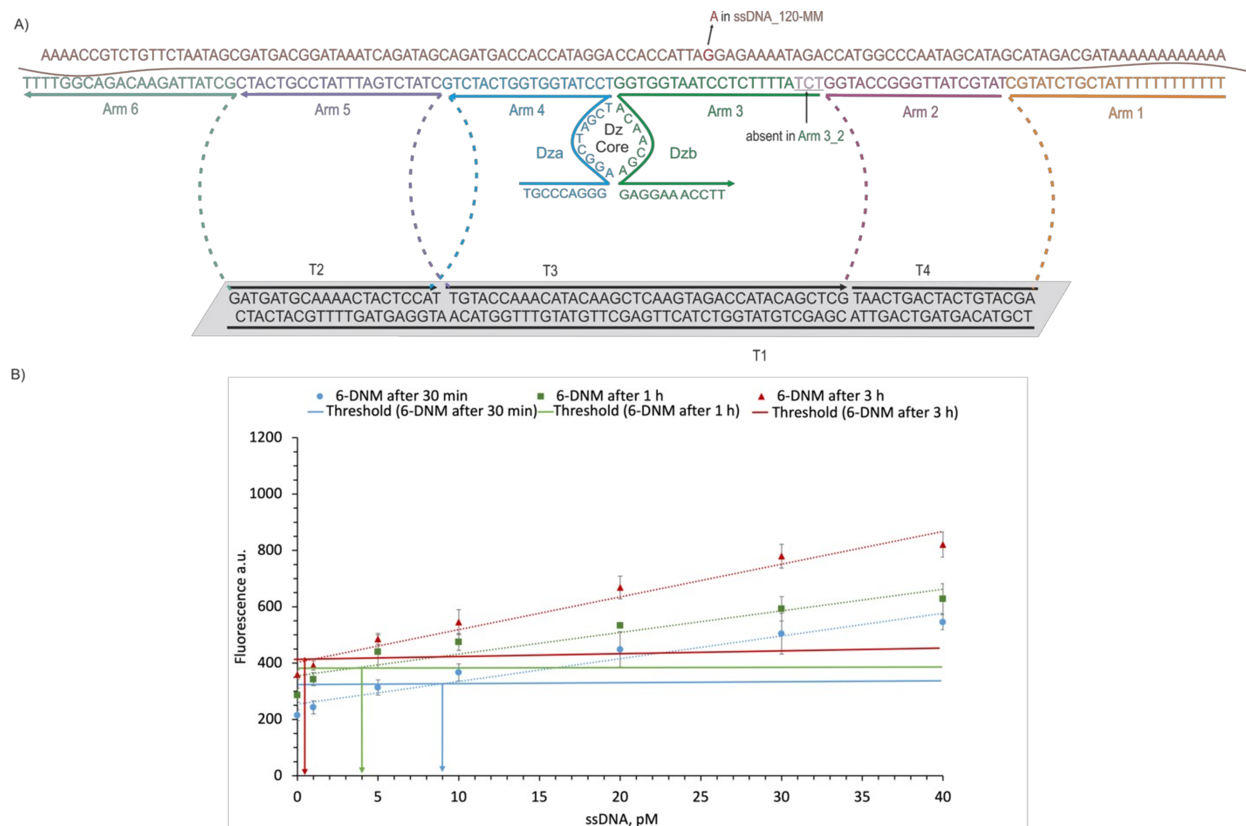

Figure S8. Structures of 6- DNA nanomachine used in this study. **A)** The 6-DNM machine in complex with ssDNA_120 synthetic analyte. 6-DNM consists of 4 major parts, T1, T2, T3, T4 and the free separated Arm 3. T1 has no analyte-binding arm and is part of the DNA scaffold. T2 contains Arm 4 and Arm 6 linked by a HEG linker to the DNA scaffold. T3 has two analyte-binding arms, A3 and A5, connected with HEG linkers to the DNA scaffold. T4 has one analyte binding arm A1. **B)** LOD of 6-DNM after 30, 60, and 180 min of incubation at 55 °C for synthetic ssDNA. The data for LOD determination for 6-DNM is average values of three independent measurements. Trendlines are shown in blue (30 min), green (60 min), and red (180 min). Threshold lines correspond to the background average plus three standard deviations. The error bars are one standard deviation of the average.

8. LOD of 6-DNM with RNA and dsDNA amplicons.

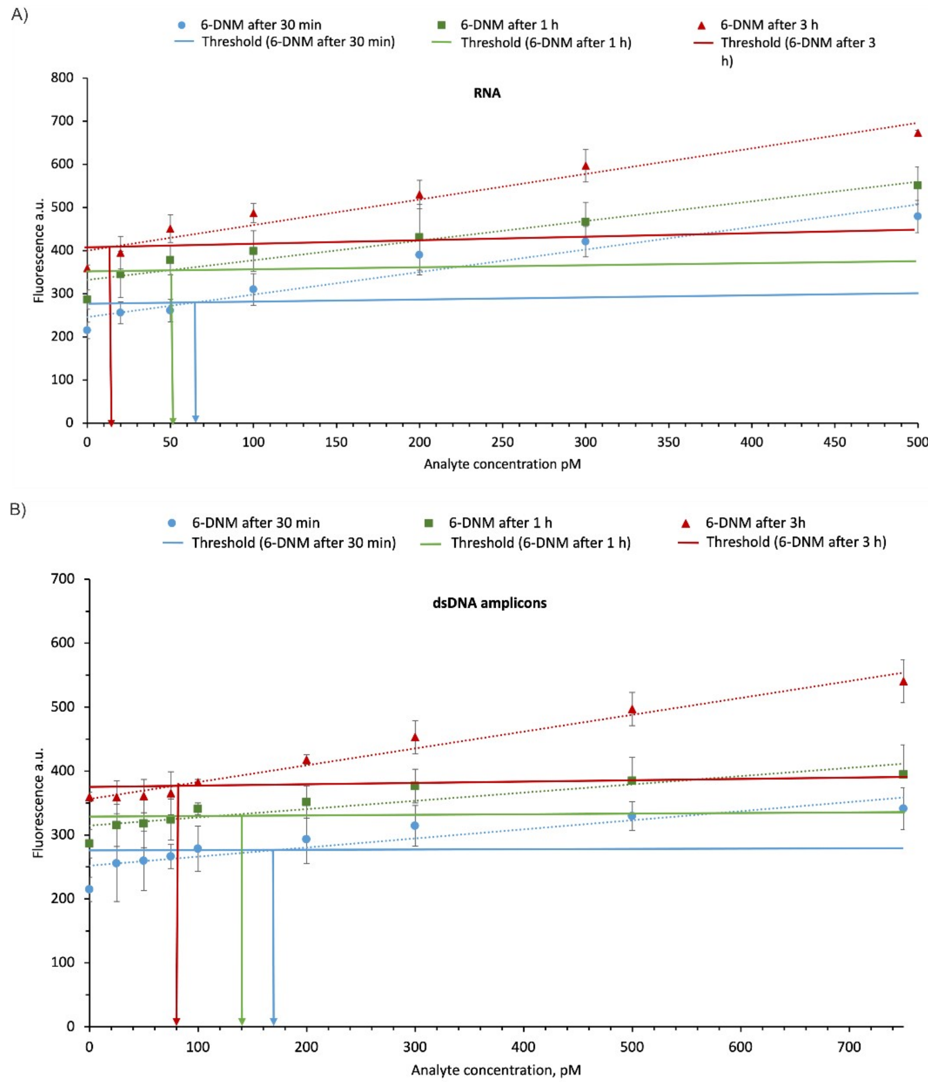

Figure S9. A) LOD of 6-DNM after 30, 60, and 180 min of incubation at 55 °C for synthetic RNA. The data for LOD determination for 6-DNM is average values of three independent measurements. Trendlines are shown in blue (30 min), green (60 min), and red (180 min). Threshold lines correspond to the background average plus three standard deviations. The error bars are one standard deviation of the average. **B)** LOD of 6-DNM after 30, 60, and 180 min of incubation at 55 °C for dsDNA amplicons. The data for LOD determination for 6-DNM is average values of three independent measurements. Trendlines are shown in blue (30 min), green (60 min), and red (180 min). Threshold lines correspond to the background average plus three standard deviations. The error bars are one standard deviation of the average.

9. Table S3. ΔG calculation of DNM–Nucleic Acid complex formation

	Name	Sequence 5′ ⟶ 3′	Complementary	ΔG (kcal.mol^{-1})
self-folding	ssDNA _120 (Top strand)	see Table S1	–	-2,62
	ssDNA_120 (Bottom strand)	see Table S1	–	-2,48
	RNA-120	see Table S1	–	-13,69
	dsDNA 120		–	-115,9
6-DNM	Arm1	TTTTTTTTTTTTTATCGTCTATGCT	AGCATAGACGATAAAAAAAAAAAAA	-19,37
	Arm2	TATGCTATTGGGCCATGG	CCATGGCCCAATAGCATA	-19,63
	Arm 3	TCTATTTTCTCCTAATGGTGG	CCACCATTAGGAGAAAATAGA	-19,72
	Arm 4	TCCTATGGTGGTCATCTG	CAGATGACCACCATAGGA	-18,15
	Arm 5	CTATCTGATTTATCCGTCATC	GATGACGGATAAATCAGATAG	-18,08
	Arm 6	GCTATTAGAACAGACGGTTTT	AAAACCGTCTGTTCTAATAGC	-19,45
	ΔG of arms binding = -114,4			
6-DNM_ self-folding	T1_6-DNM	see Table S1	–	-1,99
	T2_6-DNM	see Table S1	–	-2,41
	T3_6-DNM	see Table S1	–	-0,52
	T4_6-DNM	see Table S1	–	-1,79
	Arm 3_6-DNM	see Table S1	–	-0,01
	ΔG of 6-DNM_self-folding = -6,72			
ΔG of sensor–complex formation = ΔG arms binding – ΔG sensor self-folding - ΔG ssDNA self-folding (or RNA or dsDNA) **ΔG of 6-DNM–ssDNA complex= -105,06** **ΔG of 6-DNM–RNA complex = -93,99** **ΔG of 6-DNM–dsDNA complex= +12,51**				
4-DNM	Arm 1	TTTTTTTTTTTTTATCGTCTATGCTA	TAGCATAGACGATAAAAAAAAAAAAA	-20,38
	Arm 2	TGCTATTGGGCCATGGTCTATTTTCT CCTAATGGT	ACCATTAGGAGAAAATAGACCATGG CCCAATAGCA	-34,59
	Arm 3	GGTCCTATGGTGGTCATCTGCTATCT GATT	AATCAGATAGCAGATGACCACCATA GGACC	-29,68
	Arm 4	TATCCGTCATCGCTATTAGAACAGAC GGT	ACCGTCTGTTCTAATAGCGATGACGG ATA	-29,83
	ΔG of arms binding = -114,48			
4-DNM_ self-folding	T1_4-DNM	see Table S1	–	-5,45
	T2_4-DNM	see Table S1	–	-5,88
	Arm 3_4-DNM	see Table S1	–	-0,56
	ΔG of 4-DNM_self-folding = -11,89			
ΔG of 4-DNM–ssDNA complex= -99,97 **ΔG of 4-DNM–RNA complex = -88,9** **ΔG of 4-DNM–dsDNA complex= +17,6**				

BiDz	Dza	GGTCCTATGGTGGTCATCTGCTATCT GATT	AATCAGATAGCAGATGACCACCATA GGACC	-29,68
	Dzb	TGCTATTGGGCCATGGTCTATTTTCT CCTAATGGT	ACCATTAGGAGAAAATAGACCATGG CCCAATAGCA	-34,59
	ΔG of arms binding = -64,27			
BiDz_ self-folding	Dza	see Table S1	–	-0,56
	Dzb	see Table S1	–	-0,93
	ΔG of BiDz_self-folding = -1,49			
ΔG of BiDz–ssDNA complex= -60,16 **ΔG of BiDz –RNA complex = -49,09** **ΔG of BiDz –dsDNA complex= +57,41**				

ΔG values were calculated using the NUPACK and UNAFold web servers under the following conditions: oligonucleotide concentration, 20 nM; Na^+ concentration, 215 mM; Mg^{2+} concentration, 200 mM; temperature, 55 °C. Mg^{2+} and Na^+ concentration reflects the experimental assay conditions required for 10-23 DNAzyme catalysis.

10. References

[1] Hussein Z., Golovina L. A., Alaji M., Nour M.A.Y., Kolpashchikov D. M., Komissarov A. B., El-Deeb A. A. *Chembiochem.* 2024, 25, e202400572.

[2] Hussein Z., Nour M. A. Y., Kozlova A. V., Kolpashchikov D. M., Komissarov A. B., El-Deeb A. A. *Anal Chem.* 2023, 95, 18667-18672.

[3] El-Deeb A. A., Zablotskaya S. S., Rubel M. S., Nour M. A. Y., Kozlovskaya L. I., Shtro A. A., Komissarov A. B., Kolpashchikov D. M. *ChemMedChem.* 2022, 17, e202200382.