Supporting Information

G/T Mismatch Discrimination by positioning the mismatch site close to the catalytic core of a 10-23 DNAzyme sensor

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Methods and Materials

Assembling of DNM. To perform annealing, machine parts T1m and T2m (DNMm) or T1c and T2c (DNMc) were added in equal amounts (Table S5) to a reaction buffer consisting of 50 mM HEPES (pH 7.4) (Helicon, Russia), 150 mM KCl (LenReactiv, Russia), 15 mM NaCl (LenReactiv, Russia) and 50 mM $MgCl_2$ (LenReactiv, Russia) for DNMm and DNMc. The resulting solution was then placed into a beaker containing 500 mL preboiled water. The temperature was then gradually decreased overnight until it reached room temperature. Annealing efficiency was tested using native gel electrophoresis.

Native polyacrylamide gel electrophoresis. Native 10% polyacrylamide gel was prepared according to the following tables, for the evaluation of DNMm and DNMc assembly. Electrophoresis proceeded at 80 V for a duration of 120 min. Following electrophoresis, the gel underwent incubation in GelRed (Biotium, USA) solution. Visualization and analysis were performed utilizing the GelDoc Go and Image Lab (Bio-Rad, USA) gel visualization system.

Fluorescence assay. In this experiment, synthetic DNA analyte at concentration of 1 nM was incubated in the 50mM $MgCl_2$ containing reaction buffer (Table S2) in the presence of 200 nM F-sub, 20 nM of the free arm and 5 nM of the annealed DNMm or DNMc, respectively (Table S5). The experiment was incubated in a waterbath at 45^{o}C for 3 h. Once the incubation was over sample fluorescence was measured using excitation/emission wavelengths of 480/525 nm on Spark fluorescent plate reader (Tecan, Switzerland). Controls in the absence of a cleaving agent and/or analyte molecule were added in all experiments.

Kinetics of Fsub cleavage. In this experiment, synthetic DNA analyte at concentration of 1 nM was incubated in the 50mM $MgCl_2$ containing reaction buffer (Table S2) in the presence of 200 nM F-sub, 20 nM of the free arm and 5 nM of the annealed DNMm or DNMc, respectively (Table S5). The experiment was incubated in a waterbath at 45^{o}C for 3 h. At the following time points – 0, 5, 10, 20, 40, 60, 90, 120, 150, and 180 min – each sample was vortexed and 50mkl of the reaction solution were taken for fluorescence measurement using excitation/emission wavelengths of 480/525 nm on Spark fluorescent plate reader (Tecan, Switzerland). Controls in the absence of a cleaving agent and/or analyte molecule were added in all experiments.

Limit of Detection. To evaluate the limit of detection (LOD) for both DNMm and DNMc, a series of KRAS-A analyte concentrations ranging from 0.5 pM to 1000 pM was tested. All samples were incubated in the 50mM $MgCl_2$ containing reaction buffer (Table S2) in the presence of 200 nM F-sub, 20 nM of the free arm and 5 nM of the annealed DNMs. A control sample without analyte was also included to establish the background signal. All samples were then incubated in a water bath at 45 °C for 3 hours. Once the incubation was over sample fluorescence was measured using excitation/emission wavelengths of 480/525 nm on Spark fluorescent plate reader (Tecan, Switzerland).

Table S1. Constituents of the native 10% polyacrylamide gel for a single gel (10 mL).

Reagents	Volume
TBE 10x	1 mL
40% AA:BA	2.5 mL
10% APS (Helicon, Russia)	80 µL
TEMED (Helicon, Russia)	8 µL
H_2O	Till 10 mL

Table S2. Reaction buffer components and their concentrations.

Reagent	Concentration
$MgCl_2$ (LenReactiv, Russia)	50 mM
KCl (LenReactiv, Russia)	150 mM
NaCl (LenReactiv, Russia)	15 mM
HEPES pH (7.4) (Helicon, Russia)	50 mM

Oligonucleotides used in this study

Table S3. List of oligonucleotides used in this study and their sequences. Oligonucleotides containing FAM, BHQ1 or HEG linker were purchased from DNAsynthesis (Moscow, Russia); the rest were purchased from Evrogen (Moscow, Russia).

Name	Sequences 5’ -> 3’
F-sub	CGGT ACAT TGTA GAAG TTAA GGTT^{FAM} TCCTC gu CCCT GGGCA -BHQ1
KRAS-G	GAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTC

KRAS-A	GAATATAAACTTGTGGTAGTTGGAGCTGGTGACGTAGGCAAGAGTGCCTTGACGATACAGCTAATTC
DNMm	
T1m	TAAGTTAACAGAGGCATGTTACGCATAGTCTCATAGTGTA TTT TTT AGGCACTCTTGC**ACAACGA**GAGGAAACCTT
T2m	GAATTAGCTGTATCGTCA TTT TTT TACACTATGAGACTATGCGTAACATGCCTCTGTTAACTTA TTT TTT GCTCCAACTACCACAAGTT
Arm3m v1	TGCCCAGGG**AGGCTAGCT**CTACGTCACCA
Arm3m v2	TGCCCAGGG**AGGCTAGCT**CTACGTCACCG
Arm3m v3	TGCCCAGGG**AGGCTAGCT**CTACGTCACTA
Arm3m v4	TGCCCAGGG**AGGCTAGCT**CTACGTCATCA
Arm3m v5	TGCCCAGGG**AGGCTAGCT**CTACGTCAGCA
Arm3m v6	TGCCCAGGG**AGGCTAGCT**CTACGTCAACA
DNMc	
T1c	TAAGTTAACAGAGGCATGTTACGCATAGTCTCATAGTGTA /HEG/ CACTCTTGCCTACG**ACAACGAG**AGGAAACCTT
T2c	TAGCTGTATCGTCAAGG /HEG/ TACACTATGAGACTATGCGTAACATGCCTCTGTTAACTTA /HEG/ TACCACAAGTTTATATTC
Arm3c v1	TGCCCAGGG**AGGCTAGCT**TCACCAGCTCCAAC
Arm3c v2	TGCCCAGGG**AGGCTAGCT**TCACCTGCTCCAAC
Arm3c v3	TGCCCAGGG**AGGCTAGCT**TCATCAGCTCCAAC
Arm3c v4	TGCCCAGGG**AGGCTAGCT**TTACCAGCTCCAAC
Arm3c v5	TGCCCAGGG**AGGCTAGCT**TCCCCAGCTCCAAC
Arm3c v6	TGCCCAGGG**AGGCTAGCT**TCGCCAGCTCCAAC

Bold nucleotides are the Dz 10-23 catalytic core; Underlined nucleotides are F-sub binding arms; Nucleotides in blue represent analyte-binding arms; Nucleotides in red represent the SNV site; Nucleotides in green represent the secondary mismatch site; FAM - fluorescein amidites; BHQ1 – black hole quencher 1; ribonucleotides are in lower case.

ΔΔG between fully mitched miR17 and mismatched miR20 and the biDz sensor.

Table S4. Difference of the predicted free-energy between fully mitched miR17 and mismatched miR20 and the biDz sensor. IDT's OligoAnalyzer tool was used to predict ΔGs. Parameters used: SpecSheet, DNA, [oligo] = 0.01 µM, $[Na^+]$ = 165 mM, $[Mg^{2+}]$ = 50 mM, [dNTPs] = 0 µM, Hetero-dimer.

Arm	*ΔG [kcal/mol]*		*ΔΔG [kcal/mol]*
	miR17	*miR20*	
Arm1	-20.51	-18.56	1.95
Arm2	-18.38	-16.43	1.95

DNM assembly assessment

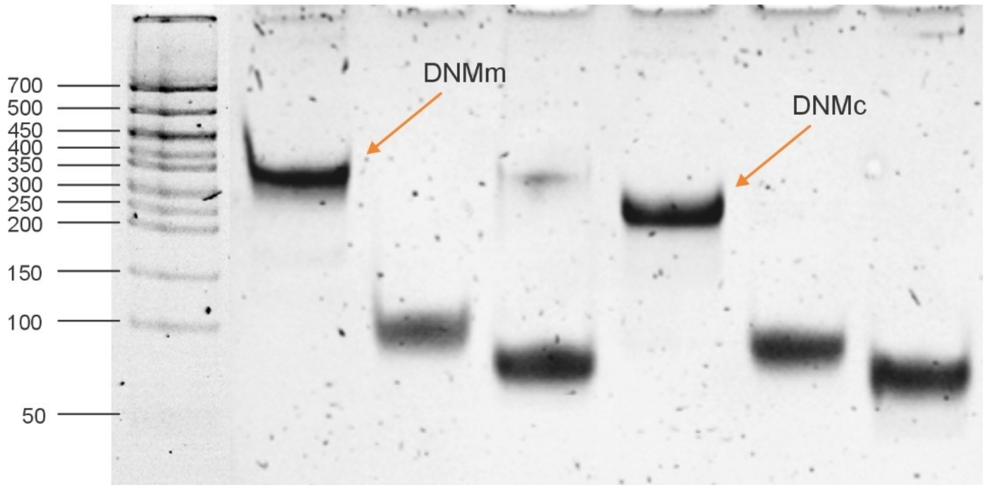


Figure S1. Assembly of DNMm and DNMc tested by native PAGE containing 2 mM Mg^{2+}. Each lane respectively contains: L – 50bp+ molecular ladder (Evrogen, Russia), 2 – DNMm assembled, 3 – T2m, 4 – T1m, 5 – DNMc assembled, 6 – T2c, 7 – T1c.

Multiple turnover experiment over time

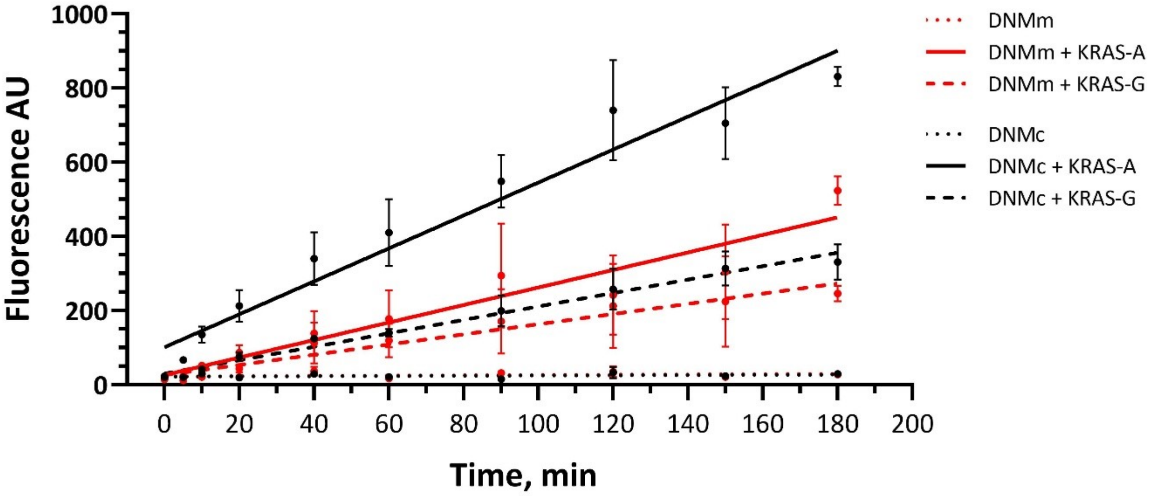
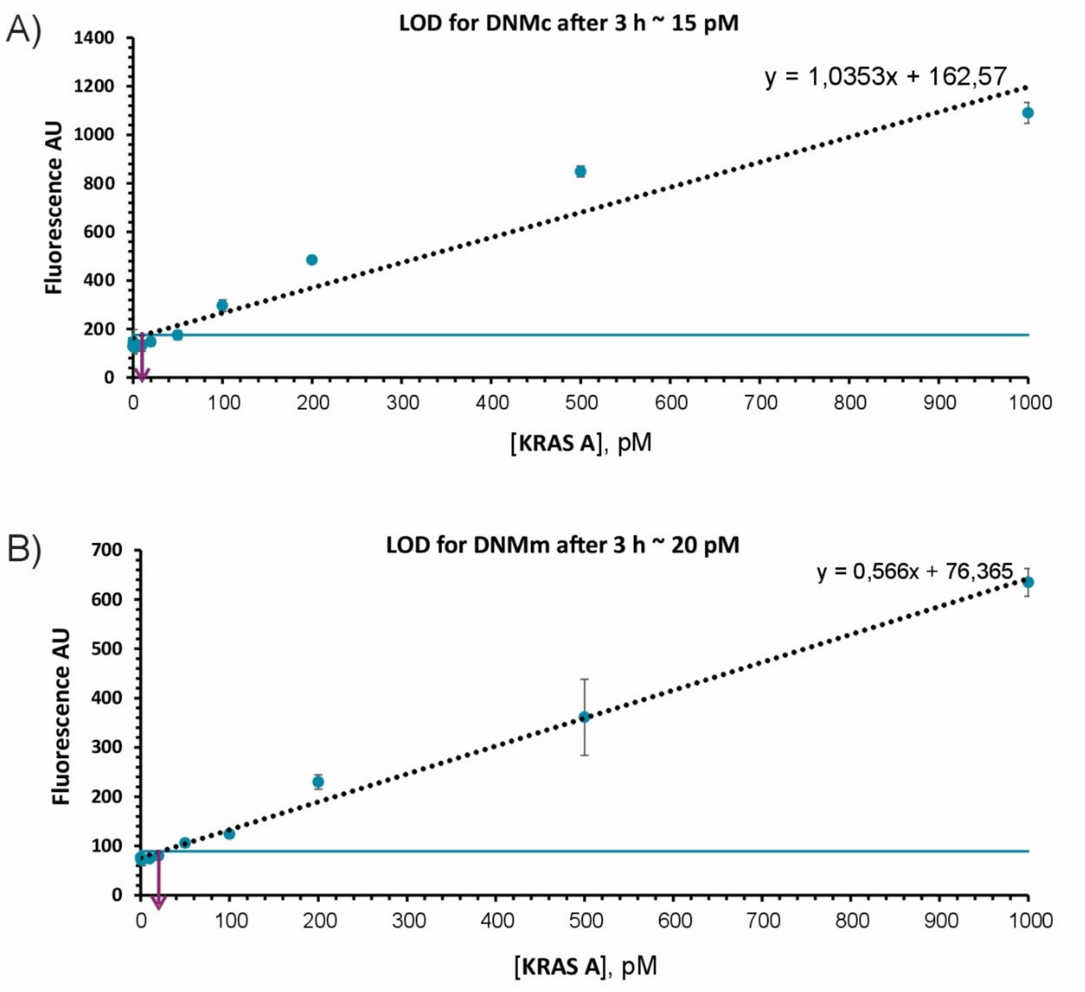


Figure S2. Steady-state kinetics of F-sub cleavage by both DNMm and DNMc, in complex with Arm3m v1 and Arm3c v1 respectively, and in the presence of KRAS-A and KRAS-G. The experiment was conducted under multiple-turnover conditions over 10 time points (0, 5, 10, 20, 40, 60, 90, 120, 150, and 180 min). The experiment was done in triplicates in the presence of 50mM $MgCl_2$, incubated at 45ºC. The graph represents the linear fit for each complex. The relative rate of each reaction is shown in the table below.

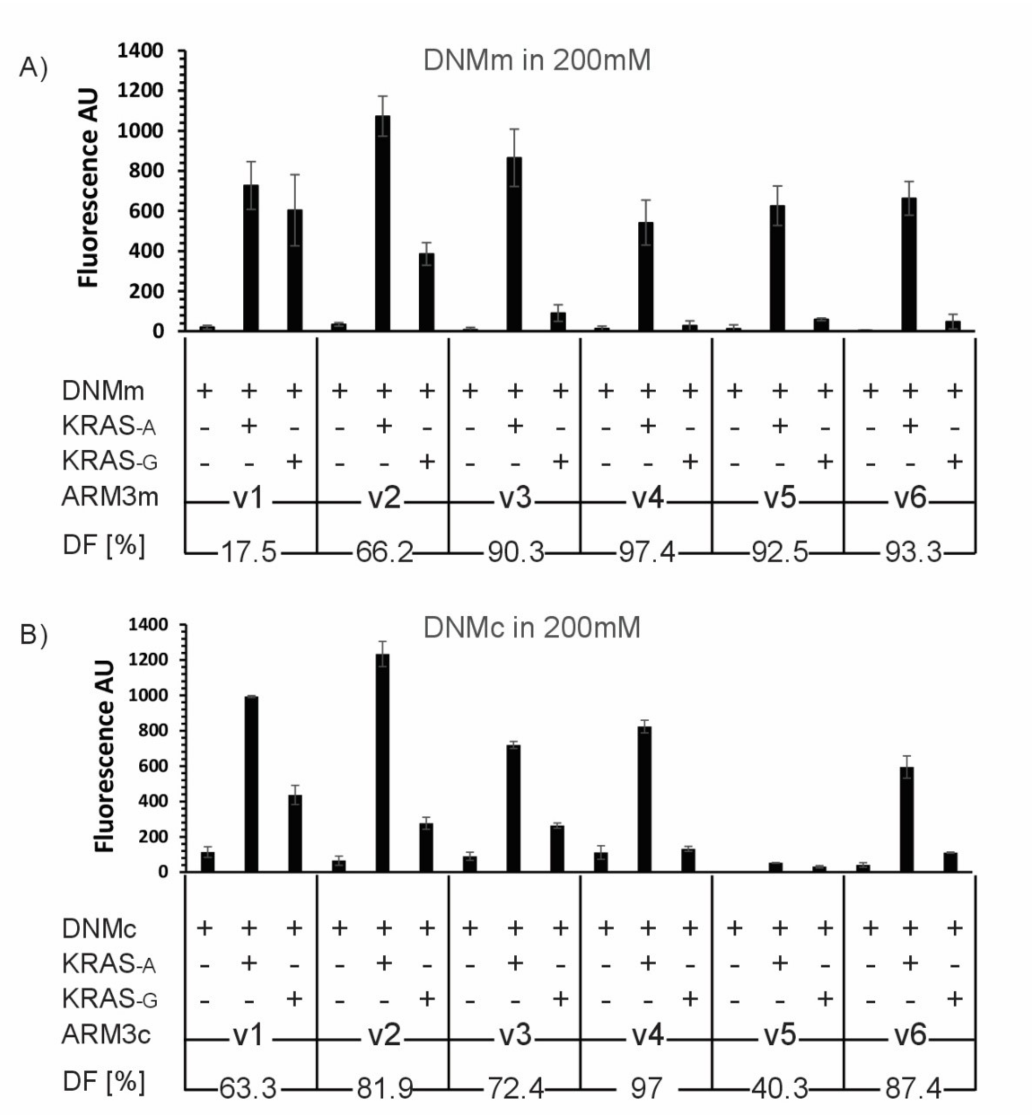
Table S5. Relative rate of F-sub cleavage in the presence of each complex indicated below.

		DNMm	*DNMm + KRAS-A*	*DNMm + KRAS-G*	*DNMc*	*DNMc + KRAS-A*	*DNMc + KRAS-G*
Relative rate AU*min^{-1}		0,039	2,361	1,371	0,030	4,442	1,811



Limit of Detection

Figure S3. A) Limit of detection (LOD) of KRAS-A by DNMm. A series of KRAS-A analyte concentrations ranging from 0.5 pM to 1000 pM was tested and the LOD was determined to be approximately 20 pM. B) Limit of detection (LOD) of KRAS-A by DNMc. A series of KRAS-A analyte concentrations ranging from 0.5 pM to 1000 pM was tested and the LOD was determined to be approximately 15 pM. For LOD determination, the procedure followed previously described guidelines [1]. First, the standard deviation (σ) of the background sample was calculated. A threshold was then defined on the LOD plot as the background fluorescence of the DNA machine plus three times the standard deviation (y = background + 3σ, blue line). The point where this threshold intersected the calibration curve was projected onto the x-axis (purple arrow), and this value was taken as the LOD.



Impact of a higher concentration of Magnesium on specificity

Figure S4. Comparison between DNMm and DNMc G→A differentiation power in the presence of 200 mM $MgCl_2$. Higher concentration of $MgCl_2$ increases the activity of the complexes in the presence of both fully matched and mismatched analytes, reducing specificity in comparison with 50 mM $MgCl_2$ concentration. Overall, the specificity trend among all arm seemed similar and comparable to experiment conducted in 50 mM $MgCl_2$. A) Fluorescence assay; comparison between different Arm3m variants for the DNMm. DNMm v1 shows a DF of 17.5%. Arm3m v4 and Arm3m v6 still display the highest and second highest specificities among all variants. All samples were incubated for 3 h at 45ºC. B) Fluorescence assay; comparison between different free Arm variants for the DNMc. DNMc v1 is more than three times more specific than DNMm v1, with a DF of 63.3%. Arm3c v6 in the presence of

200 mM $MgCl_2$ displays a similar behavior as Arm3m v6 in the presence of 50 mM $MgCl_2$, by showing increased activity and specificity in comparison with Arm 3c v5, which incorporated the secondary mismatch in the same position. Interestingly, in this case too, the G/T mismatch of Arm3c v6 is known to stabilize the complex in the CGC/GTG triplet context.[2] The C/T mismatch of Arm3c v5 on the hand is significantly destabilizing, which can explain the near activity of the complex even at 200 mM $MgCl_2$. All samples were incubated for 3 h at 45ºC.

References

1 Daniel. MacDougall, W. B. Crummett and . et al., *Anal. Chem.*, 1980, **52**, 2242–2249.
2 J. S. Jr and D. Hicks, *Annual Review of Biophysics*, 2004, **33**, 415–440.