Electronic Supplementary Material (ESI) for Analytical Methods.

Performance of a template enhanced hybridization processes in biological media for detection of a breast cancer biomarker

Reyhaneh Tavakoli-Koopaei[1], Fatemeh Javadi-Zarnaghi[1,*], Maedeh Aghahosseini[1], Manoochehr Tavassoli[1], Mohammad Javad Rasaee[2].

1. Department of Cell and Molecular Biology & Microbiology, Faculty of Biological Science and Technology, University of Isfahan, Isfahan, Iran.
2. Department of Medical Biotechnology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

*Corresponding author: fa.javadi@sci.ui.ac.ir

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Supplementary table SI. The raw data of the target titration experiments for validation of LOB and LOD.

	hemin	IS3+IS9+hemin	IS3+IS9+hemin+ target			
Target concentration	0	0	50	100	250	500
A460 nm repeat 1	0.35	1.22	2.13	2.978	3.01	3.19
A460 nm repeat 2	0.38	0.91	1.67	1.6	1.67	2.27
A460 nm repeat 3	0.36	1.82	1.87	2.113	2.78	3.75
average	0.363333	1.316667	1.89	2.230333	2.483333	3.07
SD	0.015275	0.462637	0.230651	0.696453	0.719321	0.747262

$$LOB = mean_{no\ target} + 1.645 \times SD_{no\ target}$$

$$LOB = 1.31 + 1.645 \times 0.46 = 2.07$$

$$LOD = LOB + 1.645 \times SD_{low\ concentration\ targets}$$

$$LOD = 2.07 + 1.645 \times 0.23 = 2.45$$

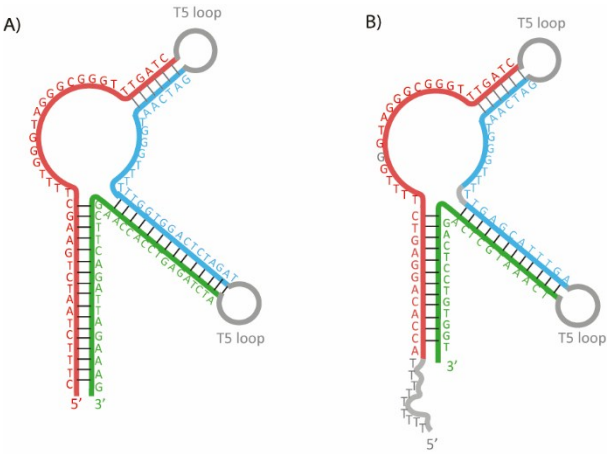
The analysis showed that the signal intensity of the limit of detection must be above 2.45 a.u. which was on average resulted from 250 nM target. Thus 250 nM target was taken as the LOD of the system.

Supplementary Table SII. The sequence strands and the programs at DINAmelt webserver that have been used for calculation of binding energies and Tms of each stem and the trimolecular complex. TSM: Two-state melting (hybridization) for 2.5 µM DNA at 37 °C with 100 mM NaCl, TSF: Two-state folding for linear DNA at 37 °C with 100 mM NaCl.

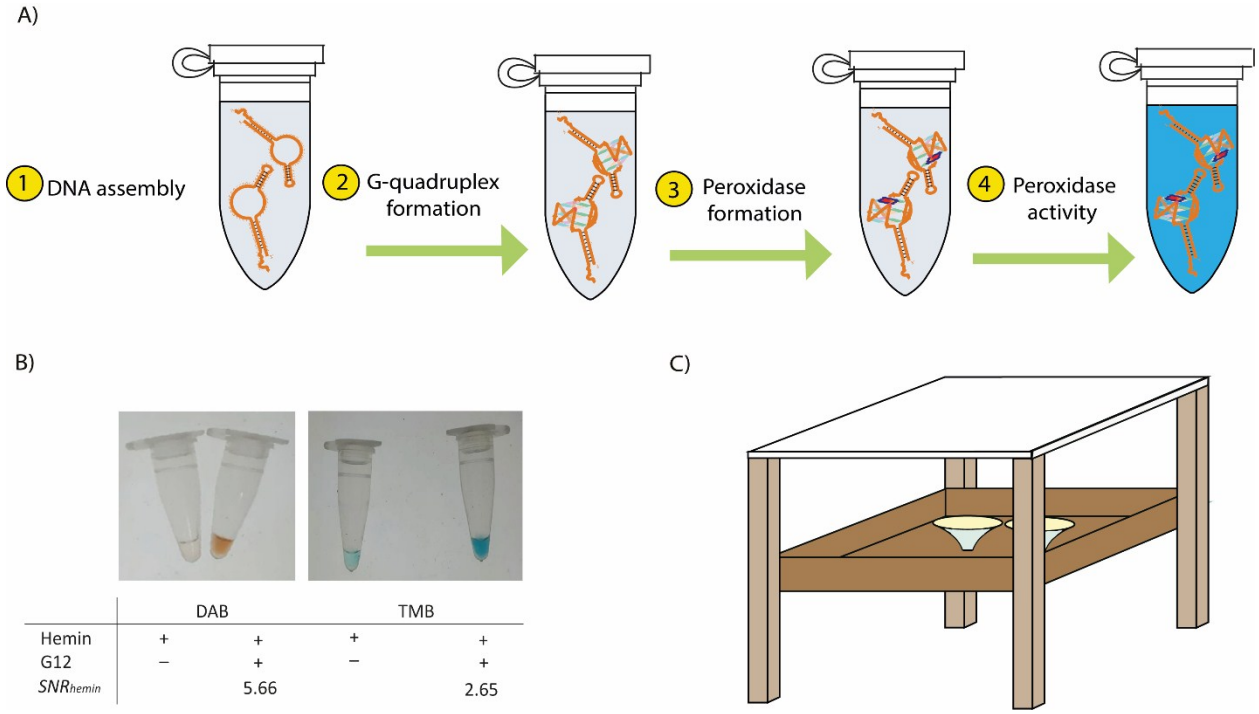
	Stem	Input sequence	Program from DINAmelt webserver	ΔG, Kcal/mol	Tm, °C
Nakayama et. al.	I	Strand 1: TTGATC Strand 2: GATCAA	TSM	-2.6	-6.9
	II	Strand 1: CTTTCTAATCTGAAGC Strand2: GCTTCAGATTAGAAAG	TSM	-12.3	46.3
	III	Strand 1: TTGGTGGACTCTAGAT Strand 2: ATCTAGAGTCCACCAA	TSM	-13.7	50.6
	Trimolecular complex	CTTTCTAATCTGAAGCTTTTGGGTAGGGCGGGTTTGATCTTTTTGA TCAATGGGTTTTTTGGTGGACTCTAGATTTTTTATCTAGAGTCCAC CAAGCTTCAGATTAGAAAG	TSF	-19.5	59.8
This report	I	Strand 1: TTGATC Strand 2: GATCAA	TSM	-2.6	-6.9
	II	Strand 1: ACCACAGGAGTC Strand2: GACTCCTGTGGT	TSM	-11.2	45.6
	III	Strand 1: TGAGCATTTGA Strand 2: TCAAATGCTCA	TSM	-8.9	37.5
	Trimolecular complex	ACCACAGGAGTCTTTTGGGTAGGGCGGGTTTGATCTTTTTGATCA ATGGGTTTTTGAGCATTTGATTTTTTCAAATGCTCAGACTCCTGTG GT	TSF	-13.4	57.7

Supplementary Note SI. In the report by Nakayama et al., the TeHyP assay was designed to be performed in trimolecular format. Intermolecular split-G quadruplex was engineered to be formed only in the presence of target nucleic acids. Two DNA strands, one containing three times three guanines in a row (long strand; probe B5) and one containing one time three consecutive guanines (short strand; probe A4) were designed to be minimally paired in the absence of the target. In the presence of the target nucleic acid, the two probes were recruited on the target via extended binding arms and thus were brought to efficient vicinity for formation of an active G-quadruplex.

In the proposed TeHyP assay of Nakayama et. al. each probe contained a short binding arm with 6 nucleotides (nt) length that were complementary to the binding arm of the other probe (forming stem I in Fig 2A). The ΔG of the hybridization of the two strands via stem I was only -2.6 Kcal/mol that led to the calculated Tm value below 0 °C. Thus the length and sequence of the stem I did not support adequate biding energy to efficiently combine the two strands. Each probe had an extended arm to support partial binding to the target via stem II and stem III in Fig 2B and 2C. Stem II provided binding to the 16 nt of the 3' end of the target while stem III was complementary to the rest i.e. 16 nt of the 5' end. Combination of the two probes provided full hybridization chance for the target. The binding energy for the hybridization of stem II and III were -12.3 Kcal/mol and -13.7 Kcal/mol that provided a calculated Tm of 46.3 °C and 50.6 °C for each stem respectively, attained by two-state hybridization protocol at DINAmelt . In fact, the binding of the target to both strands would release a higher free energy cumulatively and thus the overall melting point for the target bound to both half sites would be above the Tm of each binding arm, separately. In another words, the real Tm of the stem II and stem III would be above the calculated values since stem II and III are somewhat resembling a single 32 nt long double stranded DNA with a loop placed just in the middle. To the best knowledge of the authors of this report, there is no algorithm available for direct analysis of such circumstances (analysis of the binding energies and Tm values of trimolecular complex species). Additionally, available methods most likely calculate based on duplex formation and thermodynamics of formation of G-quartets and its competition with the double strand formation are not considered in any algorithm. Thus, with the available methods the most realistic model of the binding energy of the trimolecular complex (containing the two probes and the target DNA) was analyzed here by the two-state folding protocol at DINAmelt webserver, assuming placement of T5 loops between the separate strands, i.e. assuming the trimolecular complex to be unimolecular species (see supplementary Table SII and supplementary Fig SI for detailed DNA sequences and programs that have been used in this study for analysis of thermodynamic values). The Tm of the trimolecular complex was thus hypothesized as ca. 59.8 °C with the binding energy of ca. -19.5 Kcal/mol(Fig 2D).

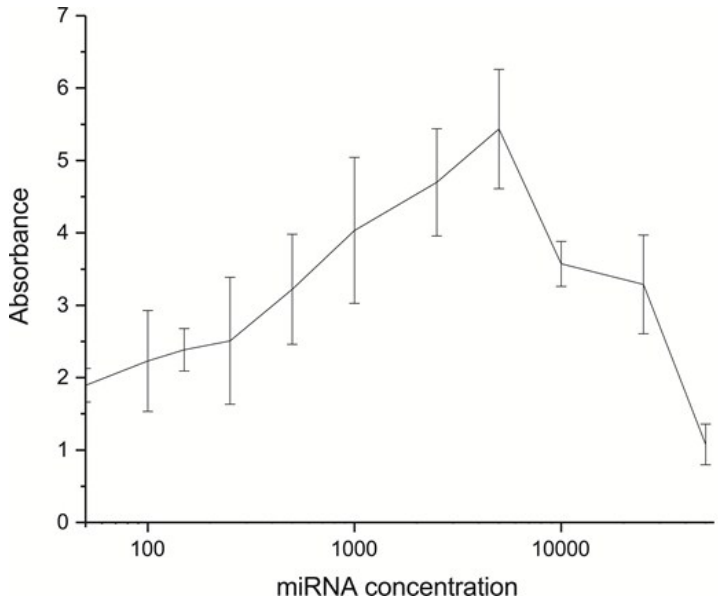


Supplementary Fig SI. A schematic representation of the DNA sequences that have been analyzed as a unimolecular complex in two state folding (TSF) program of DINAmelt as a replacement for the trimolecular complex. A) for Nakayama et. al. B) for the mir-105 TeHyP.



	DAB		TMB	
Hemin	+	+	+	+
G12	–	+	–	+
SNR_{hemin}		5.66		2.65

Supplementary Figure SII. The workflow for the unimolecular reactions. B) A comparison of the SNR_{hemin} for unimolecular format assays by TMB and DAB applying the G12 after 5 min C) Schematic drawing for the photography chamber used in this study.

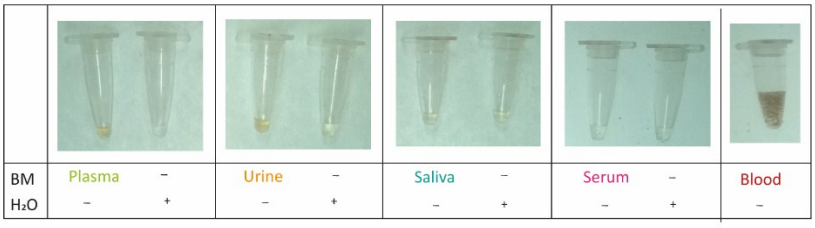


Supplementary Fig SIII. Continues x-axis representation of the same data of Fig 3B.

A)

Tris-HCl pH 7.9 (1M)	0.5 µl
NH_4Cl (2.5M)	0.6 µl
H_2O/Biological Media (BM)	7.5 µl
Incubation at 25°C for 15 minutes	
Incubation on ice for 15 minutes	
Hemin (10µM)	0.5 µl
Incubation at 25°C for 30 minutes	
DAB (20mM)	0.5 µl
H_2O_2 (35%)	0.5 µl
Final volume 10 µl	

B)



Supplementary Fig SIV. A) The protocol of control reactions in presence of biological samples. The final concentrations were 50 mM Tris-HCl pH 7.9, 150 mM NH_4Cl 0.5 µM hemin, 1 mM DAB and 1.75% H_2O_2. B) The photos of the tubes containing reactions of A.

A)

Tris-HCl pH 7.9 (1M)	0.5 µl
NH_4Cl (2.5M)	0.6 µl
IS3	
IS9	
miRNA	

Annealing 5 minutes at 95°C

	I	II	III	IV	V
BM %	0%	10%	20%	30%	45%
Plasma	0 µl	1 µl	2 µl	3 µl	4.5 µl
Urine	0 µl	1 µl	2 µl	3 µl	4.5 µl
Saliva	0 µl	1 µl	2 µl	3 µl	4.5 µl
Serum	0 µl	1 µl	2 µl	3 µl	4.5 µl
Blood	0 µl	1 µl	2 µl	3 µl	4.5 µl

Incubation at 25°C for 15 minutes

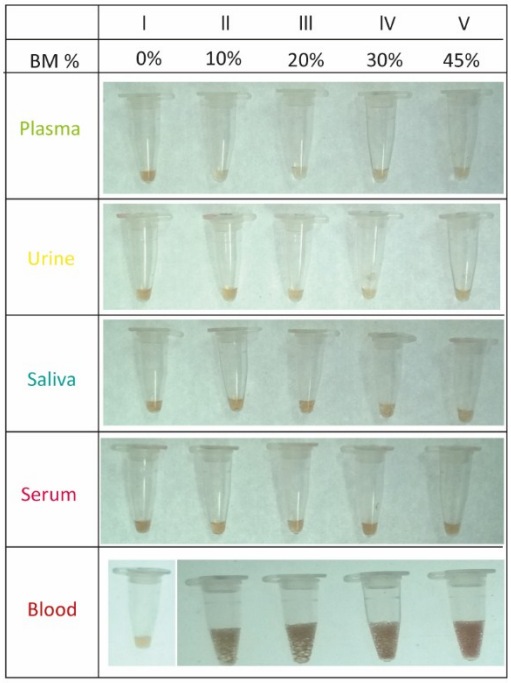
Incubation on ice for 15 minutes

Hemin (10µM)	0.5 µl

Incubation at 25°C for 30 minutes

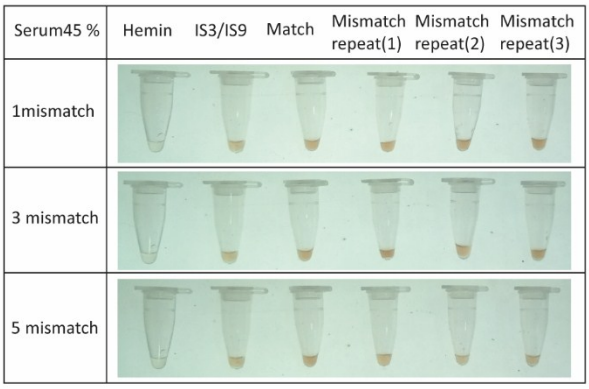
DAB (20mM)	0.5 µl
H_2O_2 (35%)	0.5 µl
Final valume	10 µl

B)



Supplementary Fig SV. A) The protocol of TeHyP performance in presence of biological samples. The final concentrations were 50 mM Tris-HCl pH 7.9 150 mM NH_4Cl, 5 µM IS3, 2.5µM IS9, 0.25µM target, 0.5 µM hemin, 1 mM DAB and 1.75% H_2O_2 and 0, 10, 20, 30 and 45% of biological samples. The biological samples and water had been added according to the red table for the reactions of I-V. B) the photos of the tubes containing reactions of A.

A)



B)

	Average of PA	SD
Hemin control	0.48	0.13
IS3/IS9	0.9	0.3
Match	3.02	0.38
1 Mismatch	3.09	0.31
3 Mismatch	3.92	0.55
5 Mismatch	1.99	0.05

Supplementary Fig SVI. A) Representative photos of the performance of the TeHyP in presence of 45% serum for the target and its mutants. B) The average peroxidase mimic activity and standard deviation of the peroxidase mimic activities of the TeHyP assay for the target and its mutants.