Supporting Information

A *Thermus thermophilus* Argonaute (TtAgo) cleavage-aided isothermal amplification strategy (TAC-IAS) for precise detection of locus-specific DNA methylation

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1. Experimental section

Materials and reagents. *Thermus thermophilus* Argonaute (TtAgo), Vent (exo-) DNA polymerase, Nt.BstNBI nicking enzyme, and extreme thermostable single-stranded DNA-binding protein (ET SSB), along with the reaction buffers, were obtained from New England Biolabs (USA). Betaine was supplied by Sigma-Aldrich (Shanghai, China). SYBR Green I was obtained from Zeesan Biotechnology (Xiamen, China). SYBR™ Gold Nucleic Acid Gel Stain and DEPC water were purchased from Invitrogen. Target M and target N sequences, designed based on the BRCA1 promoter, were obtained from Integrated DNA Technologies (USA). Other oligonucleotide sequences were purchased from Takara Biotechnology (Dalian, China). Detailed oligonucleotide sequences are shown in Table S1.

Table S1. Sequences of target DNA, DNA probes and templates used in this work.

Name	Sequence
target M	5′-ATTCTGAGAGGCTGCTGCTTAG^{5m}CGGTAGCCCCTTGGTTTC^{5m}CG TGGCAA^{5m}CGGAAAAG^{5m}CG^{5m}CGGGAATTACAGATAAATTAAAACTG ^{5m}CGACTG^{5m}CG^{5m}CGG^{5m}CGTGAGCT^{5m}CGCTGAGACTTCCTGGA^{5m}CG GGG-3′
target N	5′-ATTCTGAGAGGCTGCTGCTTAGCGGTAGCCCCTTGGTTTCCG TGGCAACGGAAAAGCGCGGGAATTACAGATAAATTAAAACTG CGACTGCGCGGCGTGAGCTCGCTGAGACTTCCTGGACGGGG-3′
guide DNA (gDNA) [a]	5′-PO_4-TTA ATT CCC GCG CTT T-PO_4-3′
EXPAR -template (EXPAR-Tem)	5′-CGC TTT TCC GTT ACC ACG AAA ACT TCA GAC TCC GCT TT **X'** **Y** T CCG TTA CCA CGA AAA CTT TTT ddC-3′ **X'**
DNA-5mC	5′-CCAGGTCCCACAGATCTATCACC^{5m}CGGGGCTCTTCAAACTCTG CAGG-3′
DNA-C	5′-CCAGGTCCCACAGATCTATCACCCGGGGCTCTTCAAACTCTG CAGG-3′
guide DNA-1 (gDNA-1) [a]	5′-PO_4-TAA AAA CCC CGA ATA A-PO_4-3′
EXPAR -template-1	5′-GAA TAA TAA ATC TAT AAA ACC TAA T TCA GAC TCG AAT **Z'** **Y** AAT AAA TCT ATA AAA CCT AAT T TTT ddC-3′ **Z'**

Note: [a] The gDNA probe was phosphorylated at both the 5′ and 3′ termini. The 3′ end of the EXPAR-template was blocked with 3′-dideoxy-C (-ddC). In the EXPAR-Tem, the sequences of X' is complementary to the newly exposed 3′-

end sequence of the disconnected target M (denoted as X), and Y represents the Nt.BstNBI recognition sequence. Similarly, in EXPAR-template-1, Z' represents the complementary sequence of the 3′ end sequence of the DNA-5mC cleavage product, while Y is the recognition site of the Nt.BstNBI.

Human genomic DNA extraction and processing. Genomic DNA was extracted from whole blood samples collected from healthy volunteers in our laboratory utilizing the TIANamp Genomic DNA Kit, and the amount of genomic DNA was quantified using Nanodrop One. The genomic DNA was equally apportioned into two fractions, one of which was subjected to M.SssI methyltransferase treatment while the other remained untreated, resulting in both methylated and unmethylated genomic DNA samples (detailed in supporting information). Both samples were treated with the EZ DNA Methylation-Gold Kit for bisulfite conversion, and then were fragmented using a Covaris ML230 Focused-ultrasonicator to avoid DNA supercoiling during heating. Before with these generated DNA as targets for TtAgo cleavage reaction, they were heat-denatured and immediately placed on ice.

Standard procedures of TAC-IAS assay. A 4 μL reaction mixture, which was composed of 500 nM gDNA probe, 1×ThermoPol reaction buffer, 50 nM TtAgo, 0.8 M Betaine and 6 mM $MgSO_4$ was first pre-incubated at 75 °C for 20 min. It was then mixed with different concentrations of DNA target (target M or target N) or genome DNA samples. TtAgo cleavage was performed at 80 °C for 1 hour. TtAgo activity was subsequently terminated by adding Proteinase K, followed by incubating at 56 °C for 15 min and 95 °C for 10 min.

Next, two separate mixtures (A and B) were prepared for the EXPAR reaction. Mixture A contained 0.5× NEBuffer r3.1, 10 nM EXPAR-Tem and 100 μM dNTPs. Mixture B was made up of 1× ThermoPol buffer, 0.12 U Vent (exo-) DNA polymerase, 10 ng ET SSB, 5 U Nt.BstNBI nicking enzyme and SYBR Green I (0.4 ng/μL). The cleavage product (1 μL) was mixed with mixture A and heated at 94 °C for 2 min, followed by incubating at 55 °C for 5 min. The mixture was then combined with mixture B resulting in a final 10 μL solution. The amplification reaction was carried out at 60 °C and real-time fluorescence was monitored using the Step-One Real-Time PCR system.

2. Effect of the amount of TtAgo on the detection of DNA methylation

TtAgo has a significant influence on the gDNA-directed cleavage reaction, as the cleavage product serves as the start-up key for EXPAR. The effect of TtAgo dosage ranging from 10 nM to

250 nM was investigated on the detection of methylated DNA with the proposed TAC-IAS method. The point of inflection (POI) values, which was the reaction time with the maximum slope of the fluorescent curves, were recorded for quantitative analysis of DNA target. As exhibited in Fig. S1a, in the presence of 10 nM TtAgo, the fluorescence amplification curve generated by 2 fM target M was virtually indistinguishable from that of the blank control. It should be attributed that the amount of TtAgo was insufficient to effectively perform the DNA cleavage reaction to trigger EXPAR. With the increase of TtAgo, as low as 2 fM of target M could be clearly detected. When 50 nM TtAgo was employed (Fig. S1b), the difference in POI values between 2 pM target M and target N reached its maximum. It was evident that an excessively high concentration of TtAgo (250 nM) could result in non-specific and undesirable digestion in Fig. S1c. Hence, taking into the comprehensive consideration of sensitivity and specificity, 50 nM of TtAgo was selected for the TtAgo-EXPAR assay.

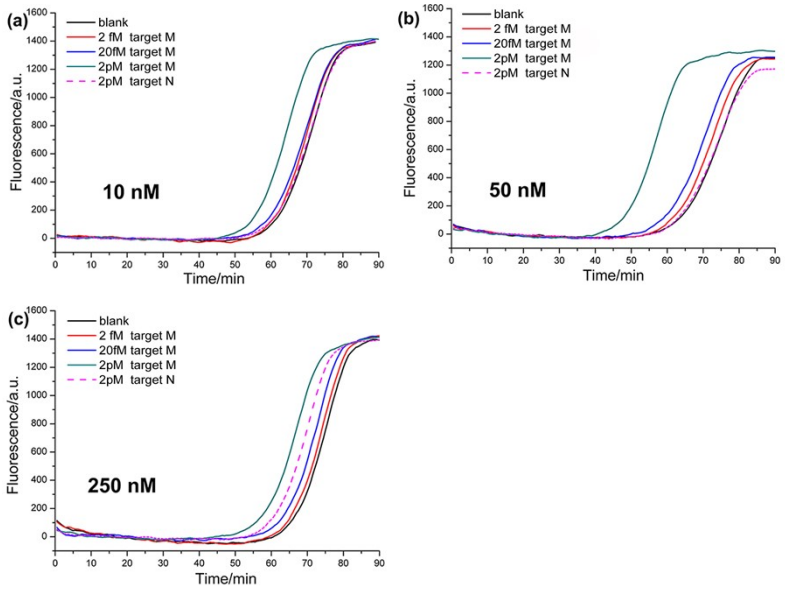

Fig. S1 Effect of the amount of TtAgo on the detection of DNA methylation. The real-time fluorescence curves were produced by blank control, 2 fM target M, 20 fM target M, 2 pM target M and 2 pM target N respectively in each image. The amount of TtAgo was (a) 10 nM, (b) 50 nM, and (c) 250 nM.

3. The influence of the concentration of gDNA on DNA methylation assay

The concentration of gDNA was also a critical factor in achieving the highly sensitive and specific detection of DNA methylation with the proposed TAC-IAS method. To investigate the effect of gDNA concentration on TtAgo cleavage, we performed the TAC-IAS assay employing gDNA

concentrations of 50 nM, 500 nM and 1 μM in the cleavage reaction. As shown in Fig. S2, when the concentration of gDNA ranged from 50 nM to 1 μM, the fluorescence responses of 2 fM target M could be clearly detected. Nevertheless, a discernible non-specific amplification signal was observed at 2 pM of target N in the presence of 50 nM gDNA. It was suggested that this low concentration of gDNA might be insufficient to effectively suppress interference from target N. When the concentration of the gDNA was 500 nM in Fig. S2b, the ΔPOI values of 2 pM target M and N reached maximum. If the dosage of gDNA was further increased to 1 μM, the 2 pM target N would once again generate the abundant nonspecific interference signal, indicating that 1 μM gDNA was excessive to lead to nonspecific digestion of target N. Therefore, 500 nM gDNA was employed as the optimal gDNA concentration for TtAgo-assisted DNA cleavage strategy.

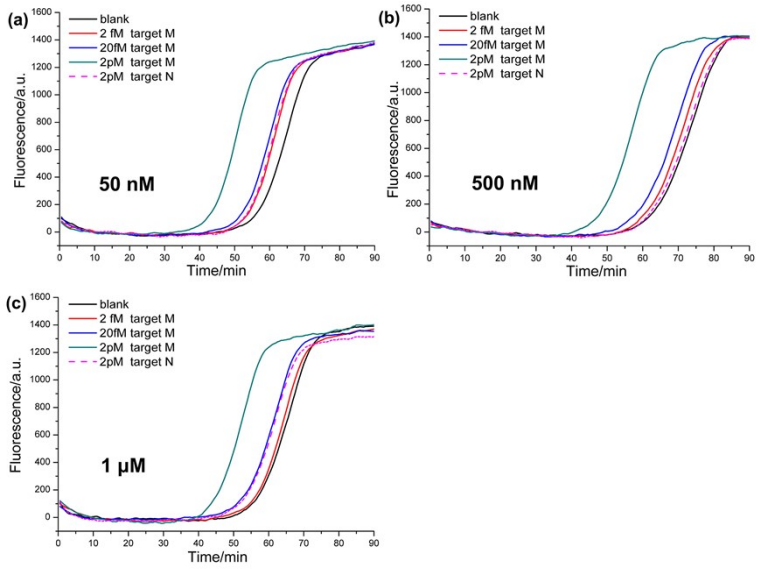

Fig. S2 The influence of the concentration of gDNA on DNA methylation assay. The real-time fluorescence curves were induced by blank control, 2 fM target M, 20 fM target M, 2 pM target M and 2 pM target N respectively in each image. The concentration of gDNA was (a) 50 nM, (b) 500 nM, and (c) 1 μM.

4. Reaction buffer optimization in the TtAgo-assisted DNA cleavage

We tested restriction endonuclease activity of TtAgo in various buffers of DNA cleavage reaction. According to the design principle, gDNA was incubated with TtAgo protein to activate its endonuclease activity, and site-specific DNA cleavage was initiated via base-pairing between target M with small gDNA. Due to the mismatches between gDNA and bisulfite-treated target N, TtAgo-based DNA cleavage was unable to proceed, resulting in maintaining the integrity of target N.

Therefore, the specific cleavage ability of TtAgo was particularly important for DNA methylation detection, which might vary in different reaction buffers. The buffer compositions were displayed as follows: Buffer 1 was the 1x Thermol Buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 @25 °C) supplied with TtAgo. A higher concentration of Mg^{2+} (4 mM and 8 mM) was contained in Buffer 2 and Buffer 3, and betaine (0.8 M) was introduced into Buffer 4 and Buffer 5 on the basis of the above buffer. As illustrated in Fig. S3, TtAgo exhibited efficient cleavage in buffer 1, but a small portion of target M remained uncleaved. It was known that enzyme activity increased with higher concentrations of Mg^{2+}. The addition of Mg^{2+} and betaine improved the efficiency of DNA cleavage to some extent in Fig. S3. TtAgo demonstrated optimal performance in Buffer 5 (1x Thermol Buffer with 6 mM Mg^{2+} and 0.8 M betaine), achieving nearly 100% cleaving efficiency for the gDNA complementary target M while the target N remained intact at 80 °C, which was consistent with earlier study [18].

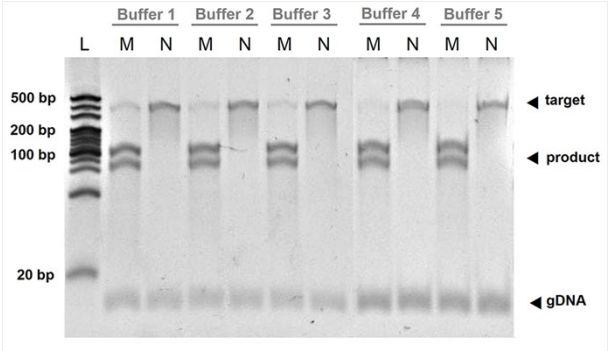

Fig. S3 Electropherograms of TtAgo cleavage products of target M and target N after incubation in different buffer components for 60 min. TtAgo:gDNA:target =1:10:1 (0.2 μM : 2 μM : 0.2 μM).

5. Optimization of the TtAgo-assisted DNA cleavage time

As depicted in Fig.S4, the efficiency of TtAgo-based DNA cleavage remained consistently high with extended incubation times, where TtAgo exhibits excellent performance in precise cleavage. In order to ensure sufficient cleavage for the accurate detection of DNA methylation, an incubation period of 60 min at 80 °C was selected for this assay.

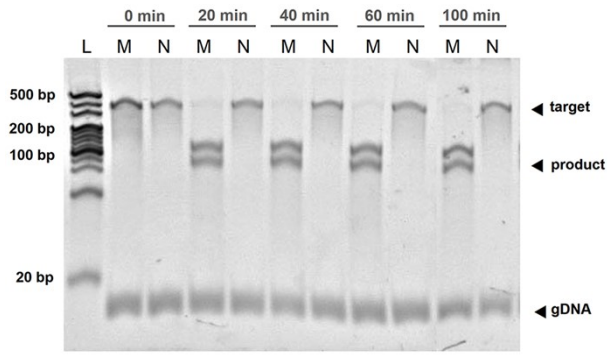

Fig. S4 Electropherograms of TtAgo cleavage products of target M and target N with different cleavage time. TtAgo:gDNA:target =1:10:1 (0.2 μM : 2 μM : 0.2 μM).

6. Evaluation of the practicability of TAC-IAS

To validate the practicability of the TAC-IAS assay, genomic DNA was extracted from whole blood samples collected from healthy volunteers in our laboratory. The genomic DNA was then equally divided into two parts, one of which was treated with M.SssI methyltransferase (+) while the other part remained untreated. The detailed protocol was following that the extracted genomic DNA was mixed with 16 U M.SssI, 0.64 mM S-adenosylmethionine (SAM) and 1x reaction buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9 @ 25 °C) with a final volume of 20 μL. The mixture was first incubated at 37 °C for 5 h, then 16 U of M.SssI and 0.64 mM of SAM were re-added in the reaction system and incubated at 37 °C for another 12 h to ensure that all the CpG dinucleotide sites of target DNA were comprehensively methylated. Subsequently, the reaction was terminated by heating at 65 °C for 20 min to inactivate M.SssI.

The treated genomic DNA samples underwent bisulfite conversion and then sequenced to confirm the methylation status of the detected site in *BRCA1* gene. The PCR reaction was performed in 40 μL mixture containing 2 U Taq Hot Start DNA polymerase, 400 nM Forward Primer (GGT AGA TTG GGT GGT TAA TTT AGA GTT T) and Reverse Primer (TAT CTA AAA AAC CCC ACA ACC TAT C), 0.2 mM dNTPs and PCR reaction buffer (10 mM Tri-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3 @25 °C). The PCR procedure was as follows: 5 min at 94 °C, followed by 30 cycles of 94 °C for 20 s, 57 °C for 20 s and 72 °C for 30 s, finally incubated at 72 °C for 10 min. Then PCR products were finally sent to Sangon Biotech. (Shanghai, China) for sequencing. The results demonstrated that all CpG sites in (+) M.SssI genomic DNA were fully methylated, whereas (-) M.SssI genomic DNA were unmethylated (Fig. S5). This confirmed the successful preparation of

both methylated and unmethylated genomic DNA samples.

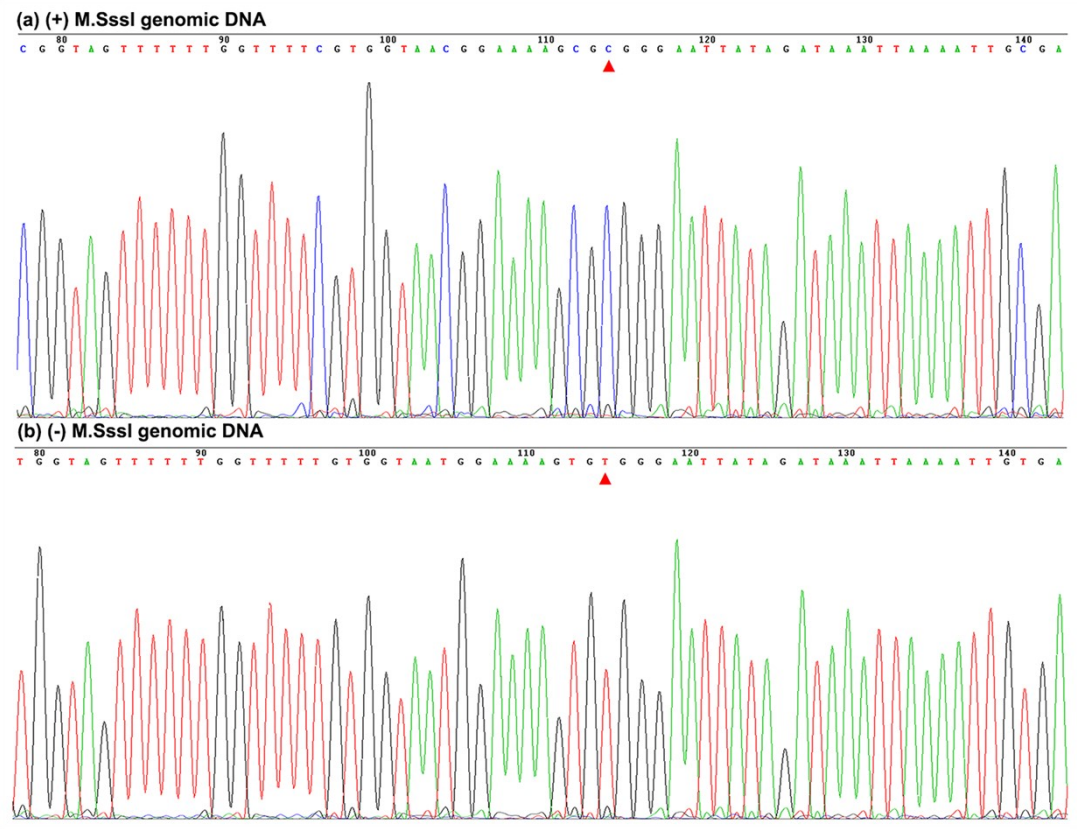

Fig. S5 The sequencing results of the *BRCA1* gene in genomic DNA. (a) The sequencing result of genomic DNA treated with M.SssI methyltransferase; (b) the sequencing result of genomic DNA without M.SssI methyltransferase.

Both the methylated and unmethylated genomic DNA samples were subjected to bisulfite conversion and fragmentation, and then the samples with 100-fold dilution were incubated with gDNA to proceed TAC-IAS. As depicted in Fig. S6, a clear positive fluorescence signal was observed in the methylated genomic DNA sample, while the fluorescence signal from the unmethylated genomic DNA overlapped with that of the blank control. The amount of target M in the methylated genomic DNA sample was determined to be 20.7 fM according to the corresponding calibration curve in Fig.3b. To further confirm the result, 100 fM of standard target M was pre-spiked with the methylated genomic DNA for further testing. The concentration of target M in this spiked sample was determined to be 125 fM, resulting in a recovery rate of 104.3%. Therefore, these results suggest that the TAC-IAS strategy is appropriate and feasible for the quantitative detection of locus-specific DNA methylation in real biological samples.

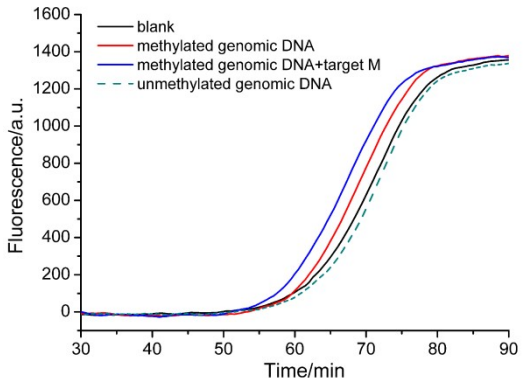

Fig. S6 The real-time fluorescence curves produced by methylated genomic DNA, methylated genomic DNA with 100 fM target M, unmethylated genomic DNA and blank control.

7. The comparison of the proposed TAC-IAS approach with other widely used DNA methylation assays

Table S2. The comparison of the proposed TAC-IAS approach with other widely used DNA methylation assays.

Detection strategy	Recognition sites	Operation and procedures	Single CpG	Detection range and detection limit	Specificity	Reference
Methylation specific PCR	No	Methylation of primer binding sites was amplified by PCR after bisulfite conversion	No	1 ng	0.1%	S1
Sensitive Melting Analysis after Real Time Methylation Specific PCR	No	MSP assay and the amplicon by high-resolution melting (HRM)	No	25 ng	0.1%	S2
Thermodynamics-guided strand-displacement-based DNA probe	No	Bisulfite conversion; PCR amplification and fluorescent probe detection	No	/	10%	S3
MutS-based methylation-specific PCR (MB-MSP)	No	MutS protein combined with MSP and inhibited the non-specific amplification	No	/	0.5%	S4
Surface-enhanced Raman scattering via ligase chain reaction	No	Bisulfite PCR; LCR reaction and SERS detection	Yes	0.5 pM -500 pM 0.5 pM	10%	S5

CRISPR/Cas12a-Triggered CHA with methylation-sensitive restriction enzyme	5′-CCGC-3′	AciI cleave nonmethylated targets; CRISPR/Cas12a recognize the site-specific methylated DNA and trigger CHA reaction	Yes	10 fM-1×10^7 fM 2.02 fM	/	S6
HpaII-assisted loop-mediated isothermal amplification	5′-CCGG-3′	HpaII digested nonmethylated fragments; LAMP reaction with methylated DNA	Yes	10 aM-10 pM	0.1%	S7
HpaII-based microfluidic chip-based digital PCR	5′-CCGG-3′	HpaII digested nonmethylated fragments; microfluidic digital PCR chip (Taqman probe)	Yes	10 copies/μL	0.52%	S8
HhaI-assisted RPA coupling with CRISPR/Cas12a	5′-GCGC-3′	HhaI cleaved; RPA amplification; Lambda digested and CRISPR/Cas12a system	Yes	2 fM to 20 pM 0.98 fM	0.05%	S9
GlaI-rolling circle amplification (RCA)	5′-GmCGmC-3′	GlaI methylation specific digestion; ligation reaction and followed RCA	Yes	50 copies/μL	1%	S10
GlaI–EXPAR–Cas 12a platform	5′-GmCGmC-3′	GlaI cleaved methylated target; the released fragement triggered EXPAR reaction and CRISPR/Cas12a detection	Yes	3 fM-6 pM 1.25 fM	0.1%	S11
Oxidation damage base-based fluorescent probe	No	Bisulfite treatment; recycle cleavage of oxidation damage base modified fluorescent probe with hOGG1	Yes	100 fM-100 nM 34.58 fM	0.01%	S12
Ligation-based PCR	No	Ligation with methylation-specific oligonucleotide probes and PCR reaction	Yes	50 ng	1%	S13
Dendritic DNA nanostructure combined with ligase reaction	No	Dendritic DNA nanostructure and signal-enriching polystyrene microbeads with ligation reaction	Yes	1 fM-100 nM 0.4 fM	/	S14
TtAgo cleavage - aided isothermal amplification (TAC-IAS)	No	TtAgo digested methylated DNA and the released product triggered EXPAR reaction	Yes	2 fM-200 pM	0.1%	This work

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