Supplementary Information

One-step digestion-ligation-activation universal strategy for ultrasensitive detection of DNA methylation

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2 Experimental Section

2.1 Materials and apparatus

All the oligonucleotides listed in Table S1 were synthesized by Sangon Biotech (Shanghai, China). Betaine, dNTPs, TE buffer and DEPC-treated water were supplied by Sangon Biotech (Shanghai, China). Ezup Column Animal Genomic DNA Purification Kit and PAGE related reagents (Agarose, Acryl/Bis 30% Solution (29:1), nucleic acid stain, TBE buffers, loading buffers and DNA marker) were supplied by Sangon Biotech (Shanghai, China). RNase-Free ddH_2O was purchased from Tiangen. T4 DNA ligase, HhaI and Bst 2.0 DNA polymerase were obtained from New England Biolabs (USA). BstUI was available from Thermo scientific. Cas12a (Cpf1) was obtained from Guangzhou Meige Biological Technology.

Table S1 The DNA sequences used in this study

Name	Sequence (5'-3' direction)
Methylated target	GCAGTTGGGCTCmCGmCGCmCGTGGAGCAGCAGCAGCTCCGC-NH_2
Unmethylated target	GCAGTTGGGCTCCGCGCCGTGGAGCAGCAGCAGCTCCGC-NH_2
padlock probe	P-CTGCTCCACGGCGCGGAGCCTTTTCGACACGACACGATTTTGGAACTCTGCTCGACGGATTAAAAAATAAGAGATAATAAGAGATTTTTGGAGCTGCTG
SLP	ATCGTCGTGACTGTTTTCCCTAACCCTAACCCTAACCCTTTTCAGTCACGACGATTTTTAATAAGAGATAATAAGAGAT
FIP	CGACACGACACGAAAAAAATCCGTCGAGCAGAGTTCC
BIP	ATCGTCGTGACTGTTTTCCCTAACCCTAACCCTAACCC
crRNA	UAAUUUCUACUAAGUGUAGAUAAUAAGAGAUAAUAAGAGAU
Random 1	AAAAAAAAAAAAAAAAAAAAAAAACCTCAGCAACCCCTCAGCGCGGAGGGCAGCGGCGAGAAAAA
Random 2	TGGCTGGTGGGCAGCGGGTCGmCGmCGGAGGGCAGCGGCGAG
Random 3	GGGCTGCTTCmCGGCTGGTGCCCCC
Random 4	CATTGAGCTGmCGGGAGCTGGCACCmCGCTGGGmCGmCGCTGGGAAGGGCmCGCACCmCGGCTGGAGmCGTGCCAAmCGmCGCTGmCGCATmCGmCGmCGGGGCACmCGmCGTGCAACCCCACAmCGGCAGCTGGTCCCTG

"p" and "NH_2" mean the phosphate and amidogen modification.

DNA and RNA sequences used in this study. CpG methylation sites sensitive to restriction digestion are highlighted in red. The complementary regions between the

padlock probe and target DNA are shown in blue. The green sequence represents the protospacer generated after RLAMP amplification, which is recognized by the crRNA spacer sequence (red) for Cas12a activation.

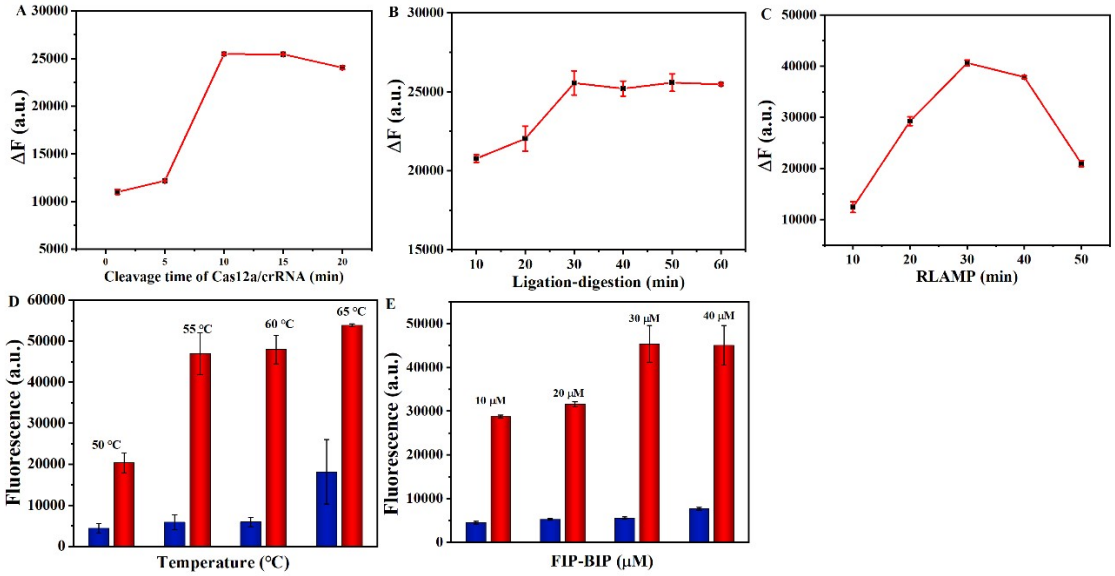

Figure S1. Optimizations of DNA methylation detection by the proposed method. (A) Optimization of CRISPR/Cas12a cleavage time; (B) Optimization of simultaneously ligation–digestion reaction time; (C) Optimization of RLAMP amplification time; (D) Optimization of RLAMP reaction temperature; (E) Optimization of inner primer (FIP/BIP) concentration.

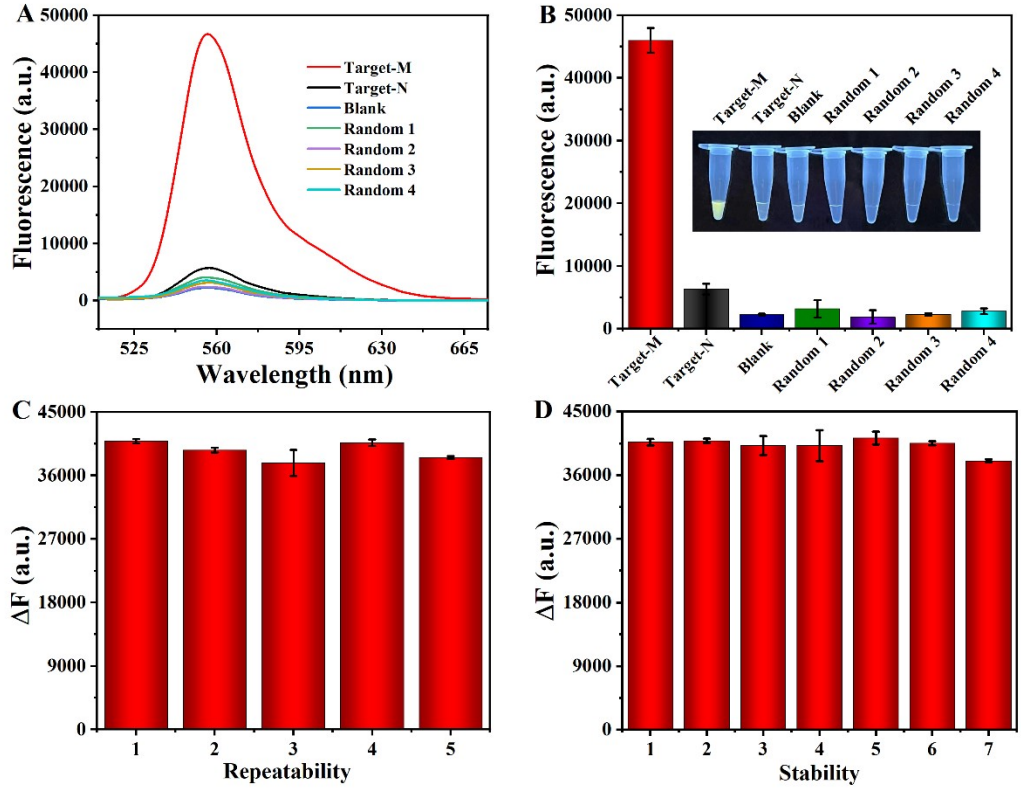

Figure S2. Evaluation of analytical performance of the DL-RLAMP/Cas12a assay. (A) Selectivity evaluation toward methylated target DNA; (B) Anti-interference selectivity analysis using non-methylated DNA controls; (C) Repeatability assessment of the DL-RLAMP/Cas12a assay;(D) Stability evaluation.

Table S2 Comparison with other methods for methylation detection.

method	signal amplification	detection range	LOD	Ref.
Electrochemistry	Bi_2S_3 nanorods as photoelectric conversion materials	10^{-13} to 10^{-9} M	10^{-13} to 10^{-9} M	[32]
Electrochemistry	Aminated rGO	50 fM to 100 pM	12 fM	[33]
Colorimetry	AuNP-based ligase chain reaction	0.01 fM to 1 fM	0.01 fM	[34]
Colorimetry	the peroxidase-mimetic activity of mesoporous iron oxide	-	10%	[35]
SERS	wrapped graphene-Ag array	5×10^{-3} – 5 ng μL^{-1}	0.5 ± 0.1 pg μL^{-1} (4.5 pmol L^{-1}) (0.1%)	[36]
SERS	ligase chain reaction	0-500 pM	0.5 pM	[37]
Fluorescence	MutS-Methylation specific PCR	-	~(0.5%)	[38]
Fluorescence	dendritic DNA nanostructure in combination with ligase reaction	10^{-15} to10^{-7} M	0.4 fM	[39]
Fluorescence	DL-RLAMP/Cas12a	2 fM ~ 200 pM	0.18 fM (0.1%)	this work
Electrochemistry	Graphene Oxide Combining with Restriction Endonuclease	0.1 to 450 U mL^{-1}	(0.05 ± 0.02) U mL^{-1}（6 h）	[40]
Electrochemistry	quantum dot signaling tracer	1 to 100 U·mL^{-1}	1 U·mL^{-1}（3 h）	[41]
Colorimetry	nicking enzyme amplification and the use of gold nanoparticles conjugated to graphene oxide	0.2 to 60 U·mL^{-1}	67 U·mL-1 （7 h）	[42]
Colorimetry	PER/functionalized hemin/G-quadruplex DNAzyme	1 to 7.5 U·mL^{-1}	0.3 U/mL （3 h）	[43]
SERS	RCA/silver core-gold satellite nanocomposites	0.05 – 50 U·mL^{-1}	2.8×10-3 (16.4 h)	[44]
Fluorescence	Cascade Signal Amplification	0.1 – 10 U·mL^{-1}	0.002 (4h)	[45]
Fluorescence	hyperbranched rolling circle amplification	2.5–70 U·mL^{-1}	1.8 （11 h）	[46]
Fluorescence	Methylation blocks cascade displacement amplification	2–40 U·mL^{-1}	0.67 U mL^{-1} （90 min）	[47]
Fluorescence	DL-RLAMP/Cas12a	0 ~ 80 U·mL^{-1}	6.92×10^{-4} U mL^{-1} 100 min	this work

Assay time represents total experimental duration excluding sample preparation.

[32] Yin Huanshun, Sun Bing, Zhou Yunlei, Wang Mo, Xu Zhenning, Fu Zhengliang, Ai Shiyun. A new strategy for methylated DNA detection based on photoelectrochemical immunosensor using Bi_2S_3 nanorods, methyl bonding domain protein and anti-his tag antibody [J]. Biosensors & Bioelectronics, 2014, 51: 103-108.

[33] Safarzadeh Mina, Suhail Ahmed, Sethi Jagriti, Sattar Anas, Jenkins David, Pan Genhua. A Label-Free DNA-Immunosensor Based on Aminated rGO Electrode for the Quantification of DNA Methylation [J]. Nanomaterials, 2021, 11(4): 985.

[34] Su Fengxia, Wang Limei, Sun Yueying, Liu Chenghui, Duan Xinrui, Li Zhengping. Highly sensitive detection of CpG methylation in genomic DNA by AuNP-based colorimetric assay with ligase chain reaction [J]. Chemical Communications, 2015, 51(16): 3371-3374.

[35] Bhattacharjee Ripon, Tanaka Shunsuke, Moriam Sofia, Masud Mostafa Kamal, Lin Jianjian, Alshehri Saad M., Ahamad Tansir, Salunkhe Rahul R., Nam-Trung Nguyen, Yamauchi Yusuke, Hossain Md Shahriar A., Shiddiky Muhammad J. A. Porous nanozymes: the peroxidase-mimetic activity of mesoporous iron oxide for the colorimetric and electrochemical detection of global DNA methylation [J]. Journal of Materials Chemistry B, 2018, 6(29): 4783-4791.

[36] Ouyang Lei, Hu Yaowu, Zhu Lihua, Cheng Gary J., Irudayaraj Joseph. A reusable laser wrapped graphene-Ag array based SERS sensor for trace detection of genomic DNA methylation [J]. Biosensors & Bioelectronics, 2017, 92: 755-562.

[37] Wang Yuling, Wee Eugene J. H., Trau Matt. Highly sensitive DNA methylation analysis at CpG resolution by surface-enhanced Raman scattering via ligase chain reaction [J]. Chemical Communications, 2015, 51(54): 10953-10956.

[38] Zhang Lei, Zhang Wei, Mu Yaoqin, Hu Hao, Dong Kejun, Wen Xu, Ye Zhengxin, Sun Qiangqiang, Yan Bei, Mao Zenghui, Xiao Xianjin. Ultrasensitive and Quantitative DNA Methylation Detection Method Based on the MutS Protein [J]. Analytical Chemistry, 2023, 95(51): 18828-18835.

[39] Zhang Shu, Huang Jian, Lu Jingrun, Liu Min, Li Yan, Fang Lichao, Huang Hui, Huang Jianjun, Mo Fei, Zheng Junsong. A novel fluorescent biosensor based on dendritic DNA nanostructure in combination with ligase reaction for ultrasensitive detection of DNA methylation [J]. Journal of Nanobiotechnology, 2019, 17(1): 121.

[40] Li Wen, Wu Ping, Zhang Hui, Cai Chenxin. Signal Amplification of Graphene Oxide Combining with Restriction Endonuclease for Site-Specific Determination of DNA Methylation and Assay of Methyltransferase Activity [J]. Analytical Chemistry, 2012, 84(17): 7583-7590.

[41] Baek Songyi, Won Byoung Yeon, Park Ki Soo, Park Hyun Gyu. An electrochemical one-step system for assaying methyltransferase activity based on transport of a quantum dot signaling tracer [J]. Biosensors & Bioelectronics, 2013, 49: 542-546.

[42] Li Zhi-Mei, Zhang Xiao, Pi Ting, Bu Jin, Deng Rui-Hong, Chi Bao-Zhu, Zheng Xiang-Juan. Colorimetric determination of the activity of methyltransferase based on nicking enzyme amplification and the use of gold nanoparticles conjugated to graphene oxide [J]. Microchimica

Acta, 2019, 186(8): 594.

[43] Yuan Wenxu, Xiao Kaiting, Liu Xingxing, Lai Yanming, Luo Fazeng, Xiao Wei, Wu Jinjun, Pan Pan, Li Yongkui, Xiao Heng. A programmable DNA nanodevice for colorimetric detection of DNA methyltransferase activity using functionalized hemin/G-quadruplex DNAzyme [J]. Analytica Chimica Acta, 2023, 1273: 341559.

[44] Chen Runzhi, Shi Huayi, Meng Xinyu, Su Yuanyuan, Wang Houyu, He Yao. Dual-Amplification Strategy-Based SERS Chip for Sensitive and Reproducible Detection of DNA Methyltransferase Activity in Human Serum [J]. Analytical Chemistry, 2019, 91(5): 3597-3603.

[45] Liu Yunlong, Tu Yuanbiao, Wu Haiping, Zhang Hang, Chen Honghong, Zhou Guohua, Wang Peng, Gu Yueqing. A renewable DNA biosensor for sensitive detection of DNA methyltransferase activity based on cascade signal amplification [J]. Sensors and Actuators B-Chemical, 2020, 313: 128029.

[46] Chen Liping, Zhang Ying, Xia Qian, Luo Fang, Guo Longhua, Qiu Bin, Lin Zhenyu. Fluorescence biosensor for DNA methyltransferase activity and related inhibitor detection based on methylation-sensitive cleavage primer triggered hyperbranched rolling circle ampli fication [J]. Analytica Chimica Acta, 2020, 1122: 1-8.

[47] Wen Qilin, Li Dandan, Xi Huai, Huang Guidan, Zhu Wenyuan. Methylation-blocked cascade strand displacement amplification for rapid and sensitive fluorescence detection of DNA methyltransferase activity [J]. Journal of Pharmaceutical and Biomedical Analysis, 2022, 219: 114935.