Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Rapid and highly specific detection of site-specific 5-hydroxymethylcytosine based on peroxotungstate oxidation and mismatch ligation-based LAMP

Zhenhao Zhang[ab], Tong He[ab], Yan Qi[a], Yuxuan Dai[a], Kejing Lao[*a] and Xingchun Gou[*a]

Table of contents:

1. Materials and Reagents
2. General procedures
Table S1. Sequences of the synthetic oligonucleotides used in the experiments
Figure S1. The schematic representation of LAMP assay.
Figure S2. Dynamic range and sensitivity of ligation-based LAMP assay
Figure S3. Effect of the concentration of DNA Probes on the mismatch ligation-based LAMP
Figure S4. Influence of the amount of Ampligase on mismatch ligation reaction
Figure S5. Optimization of the ligation temperature of mismatch ligation reaction
Figure S6. Analysis of oxidation and ligation efficiency of mismatch ligation assay

1. Materials and Reagents

Bst DNA Polymerase (Large Fragment) and ThermoPol® Reaction Buffer were purchased from New England Biolabs (Beverly, MA, USA). Ampligase (Thermostable DNA ligase) was purchased from Epicentre Biotechnologies (Wisconsin, USA). Potassium tungstate (K_2O_4W) were ordered from Aladdin Industrial Corporation (shanghai, China). Dr.GenTLE® Precipitation Carrier, nuclease-free water and dNTP mixture (2.5 mM each) were purchased from TaKaRa Biotechnology Co., Ltd (Dalian, China). SYBR Green I (20× stock solution in DMSO,) was obtained from Xiamen Bio-Vision Biotechnology (Xiamen, China). All oligonucleotides (Table S1) containing modified bases were synthesized and purified by TaKaRa Biotechnology Co., Ltd (Dalian, China). All other reagents were analytical reagent grade and used as purchased without further purification.

2. General procedures

2.1 Preparation of Dinuclear peroxotungstate ($K_2[\{W(=O)(O_2)_2(H_2O)\}2(\mu\text{-}O)]\cdot 2H_2O$)

Dinuclear peroxotungstate was synthesized according to the procedure in ref. 25 (G. Hayashi, K. Koyama, H. Shiota, A. Kamio, T. Umeda, G. Nagae, H. Aburatani, A. Okamoto, J. Am. Chem. Soc. 2016, 138, 14178-14181), and characterized by elemental analysis, IR, UV/V, and X-ray crystallographic structural analysis according to the reported procedures (K. Kamata, K. Yamaguchi, N. Mizuno, Chem. Eur. J., 2004, 10, 4728).

2.2 Oxidation of targets by peroxotungstate

Firstly, the synthetic dsDNAs targets (1uM) were diluted to 100 nM，10 nM，1 nM，100 pM，10 pM，1 pM, 100 fM. Secondly, 1μL of the diluted sample was added into 9ul of the mixture, respectively. Mixture contains 5 mM peroxotungstate, 1.4 M urea, 100 mM sodium chloride and 50 mM sodium phosphate (pH 7.0). Incubation conditions were as follows: 10 cycles of 90 °C for 1 min and 60 °C for 10 min. The product was purified by ethanol precipitation with the help of Dr.GenTLE® Precipitation carrier and dissolved in 10 μL nuclease-free water.

2.3.1 Ligation reaction and mismatch ligation reaction

The mixture (total volume 9 μL) of ligation reaction consisted of 2 nM probe A, 2 nM probe B, 0.02 U Ampligase, Ampligase buffer (20 mM Tris-HCl, 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM NAD and 0.1% Triton X-100, pH 8.3). The mixture of mismatch ligation reaction was the same as the ligation reaction, except that the probe B1 was replaced with the probe B. The appropriate amounts of treated synthetic dsDNAs or genomic DNA samples were added in the mixture to give a final volume of 10 μL. The reaction was carried out through the following program: 94 ℃ for 3 min and 20 cycles of 94 ℃ for 30 s and 58 ℃ for 1 min. After the ligation reaction, the products were immediately put on ice.

2.3.2 Gap ligation reaction

The mixture of gap ligation reaction (total volume 9 μL) contains 2 nM probe A, 2 nM probe B3, 1 uM dATP, 0.02 U Ampligase, Ampligase buffer (20 mM Tris-HCl, 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM NAD, 0.1% Triton X-100, pH 8.3), 0.06 U jumpStart™ Taq DNA polymerase and PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin). The procedure of gap ligation-based LAMP was same to 2.3.1

2.4 LAMP reaction

LAMP reaction mixture contains 4 U *Bst* DNA Polymerase, Large Fragment, ThermoPol reaction buffer (20 mM Tris-HCl, 10 mM KCl, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, pH 8.8), 0.4 μM FIP and 0.4 μM BIP,

200 μM dNTPs, 0.4×SYBR Green I. 2 μL of the ligated products were added to LAMP reaction mixture bring the final volume to 10 μL. Each reaction mixture was then mixed thoroughly and immediately transferred into the 96 well plates. LAMP reaction was carried out in the StepOne Real-Time PCR System (Applied Biosystems, USA). The reaction temperature was 65℃ and the real-time fluorescence intensity was monitored at intervals of 1 min.

2.5 Mouse brain genomic DNA analysis

The mouse brain tissue (≤25 mg) was minced into small pieces and placed in a 1.5ml centrifuge tube. Genomic DNA was extracted from mouse brain tissue using the DNeasy Blood & Tissue Kit (Qiagen, Germany). Genomic DNA quality was assessed by PCR and agarose gel electrophoresis with the following PCR primers:

Forward primer sequence: TCCTTACCCTGAATGACTCC

Reverse primer sequence: CAACCCACACTATTCCCTTG

The analysis method of mouse brain genomic DNA includes three protocols, they are peroxytungstate oxidation, mismatch ligation and LAMP reaction. These protocols are the same as the model experiments and can refer to protocols 2.2, 2.3.1 and 2.4.

Table S1. The sequences of target DNA, DNA probes used for ligation reaction and the primers for LAMP amplification.

Name	Sequence (5′-3′)
Target C	5′-CCAGGTCCCACAGATCTATCACCCGGGGCTCTTCAAACTCTGCAGG -3′
Target 5mC	5′-CCAGGTCCCACAGATCTATCACC5mCGGGGCTCTTCAAACTCTGCAGG -3′
Target 5hmC	5′-CCAGGTCCCACAGATCTATCACC5hmCGGGGCTCTTCAAACTCTGCAGG -3′
Target T	5′-CCAGGTCCCACAGATCTATCACCTGGGGCTCTTCAAACTCTGCAGG -3′
Complementary sequences	5′-CCTGCAGAGTTTGAAGAGCCCCGGGTGATAGATCTGTGGGACCTGG-3′
Probe A	5′-Phosphate-GGTGATAGATCTGTGGGACCTTTTATCGTCGTGACTGTTTGTAATAGGACAGAGCCCCGCACTTTCAGTCACGACGAT-3′
Probe B1	5′-CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGATTTTCCTCTGCTGTCGTTTTAGAGTTTGAAGAGCCCCA-3′
Probe B	5′-CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGATTTTCCTCTGCTGTCGTTTTAGAGTTTGAAGAGCCGCA-3′
Probe B2	5′-CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGATTTTCCTCTGCTGTCGTTTTAGAGTTTGAAGAGCCCC-3′
BIP	5′-CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGA-3′
FIP	5′-ATCGTCGTGACTGAAAGTGCGGGGCTCTGTCCTATTAC-3′

Note. The information of target DNA and genomic DNA is from low density lipoprotein receptor-related protein gene (Lrp1, intron) in chromosome 5 of Mus musculus strain C57BL/6J. The detection site is located in locus 35096044 of the Lrp1 gene. The underlined blue bases were the detected sites in the sequences. The probe A

was modified with a phosphate group at its 5′-end. Furthermore, yellow base G is mismatch base with the target sequence which is the third position from the 3′-end of the probe B and the red base A is the base of the complementary of target 5hmC after oxidation

The mechanism of LAMP

The structure 1 is added to the LAMP amplification mixtures for amplification and real-time fluorescence detection in step-one fluorescence system. The LAMP amplification mixtures contain universal primers (forward inner primer (FIP), backward inner primer (BIP)), dNTPs, Bst DNA polymerase, glycine betaine, SYBR Green I and NEB buffer. SYBR Green I is selected as the fluorescent dye.

The mechanism of LAMP is schematically illustrated in Figure 1b. In the LAMP, probe A and probe BX can bind to target sequence and form a double stem-loop structure 1 which is the initiate substance of LAMP. Probe BX represents probe B, probe B1 or probe B2, in different ligation strategies, respectively. The detailed sequences of probe A and probe BX are shown in Table S1. In the presence of Bst DNA polymerase and dNTPs, the 3′-end of structure 1 can extend and form B1C and B2C that was the complementary sequence of B1 and B2, respectively. Meanwhile, FIP hybridizes to F1 and F2 in the structure 1 and extends. With the extension of FIP, the extended sequence of structure 1 is replaced and released to form a stem-loop of B1C and B2C at the 3′ end. With the 3′-end of B2C extending, the product of FIP extension is replaced to form a double stem-loop structure 2. The 3′-end of structure 2 can extend and form F1 and F2 that was the complementary sequence of F1C and F2C, respectively. Simultaneously, BIP hybridizes to B1C and B2C to perform the primer extension. With the extension of BIP at the 3′end, the extended sequence of structure 2 is replaced and released to form a stem-loop of F1 and F2. With the 3′-end of F1 extending, the product of BIP extension is replaced and released a double stem-loop structure 1. Thus, FIP and BIP, respectively, can be constantly hybridized to the stem-loop structures and to form more and more stem-loop structures. The detailed sequences of FIP and BIP are displayed in Table S1. The process can be repeated continuously and leads to exponential amplification of DNA.

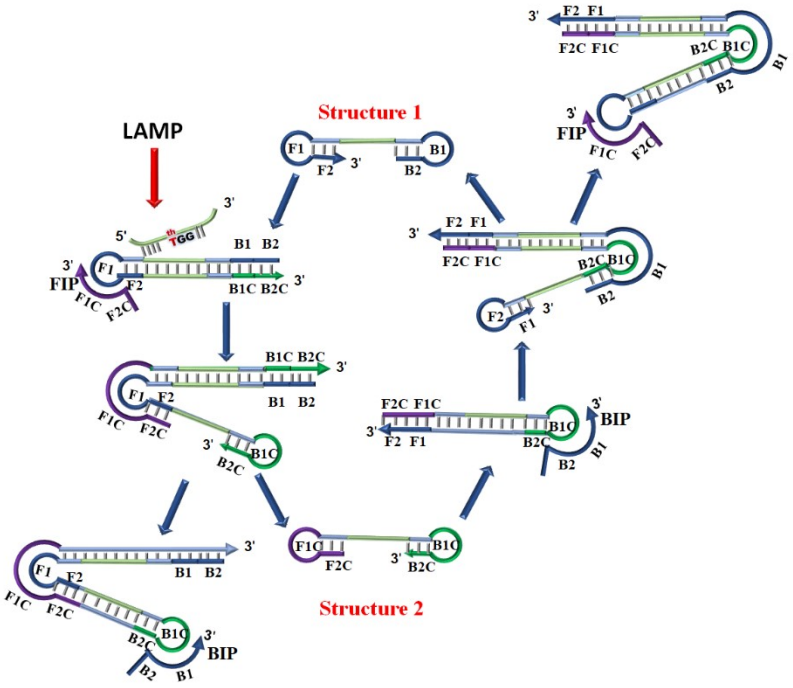

Figure S1. The schematic representation of LAMP assay.

The ligation-based LAMP strategy

As shown in Figure S2, we first investigate the sensitivity and specificity of the ligation strategy in which the probe B1 was designed an adenine (A) at 3′end. Probe A and probe B1 completely bound to half of the target 5hmC, respectively and formed structure 1 by catalysis of Ampligase. But the probes cannot be ligated relying on target C and 5mC due to the mismatch at the 3′-end of probe B1. The application of this probe B1 could achieve high ligation efficiency, which could lead to high sensitivity and stability, and as low as treated 200 aM of target 5hmC can be detected. The POI values were linearly dependent on the lg of treated target 5hmC concentration in the range from 200 aM to 200 pM. The POI values, namely, the times corresponding to the maximum slope in each real time fluorescence curve, are recorded for the quantitative determination of target. The correlation equation was POI = -41.7-6.35 lgC_{5hmC} and the corresponding correlation coefficient R was 0.997(Figure 2a-2b). To evaluate the specificity of the ligation-based LAMP assay, 20 fM of targets C, 5mC and 5hmC are tested in the same conditions. According to correlation equation shown in Figure S2b, the interference for the detection of the 5hmC by the signals produced by target C and target 5mC is estimated to be 4.5 and 9.8 %, respectively. This method has high sensitivity but the specificity of this strategy is not ideal.

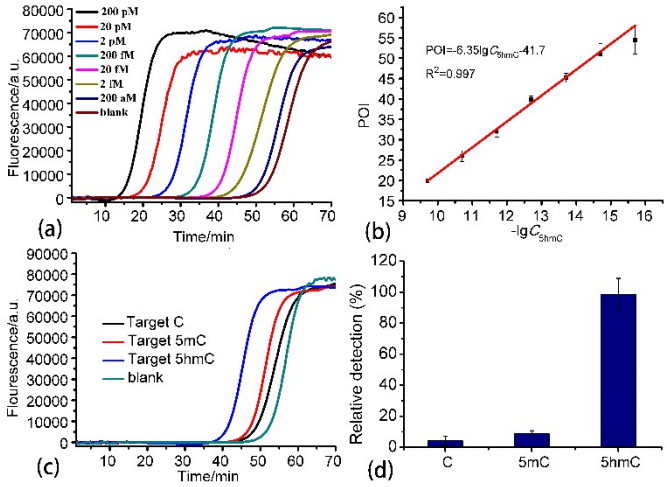

Figure S2 Dynamic range and sensitivity of ligation-based LAMP assay. (a) Real time amplification curve produced by synthetic target 5hmC from right to left, the concentration of target 5hmC successively was blank, 200 aM, 2 fM, 20 fM, 200 fM, 2 pM, 20 pM, 200 pM. (b) The relationship between POI and lg of target 5hmC concentration (M). (c) Real time amplification curve produced by 20 fM synthetic target C, target 5mC and target 5hmC.

Effect of the concentration of DNA Probes on the mismatch ligation-based LAMP

The concentration of the probe A and probe B were critical factors to affect the formation of the double stem-loop structure that was the essential element for the subsequent LAMP amplification. So the amount of probes (keep the same concentration in this study) was optimized with the different concentrations in the range of 400 pM to 10 nM. As depicted in Figure S3a and S3b, when the concentration of probe A / probe B was increase from 400 pM to 2 nM, the fluorescence signal of target 5hmC had been significantly advance but target C and 5mC have slight change. The reason might be that low concentration of probes would lead to low efficiency of ligation, particularly, at the low levels of the target 5hmC. However, with increasing the concentration of probes to 10 nM (Figure S1c), the ligation reaction was obviously accelerated and difference between target 5hmC and C, 5hmC and 5mC were significantly decreased, respectively. This indicated that too excessive probes probably caused much target-independent nonspecific ligation reaction. Therefore, 2 nM of probe A / probe B was employed for the mismatch ligation-based LAMP in this work.

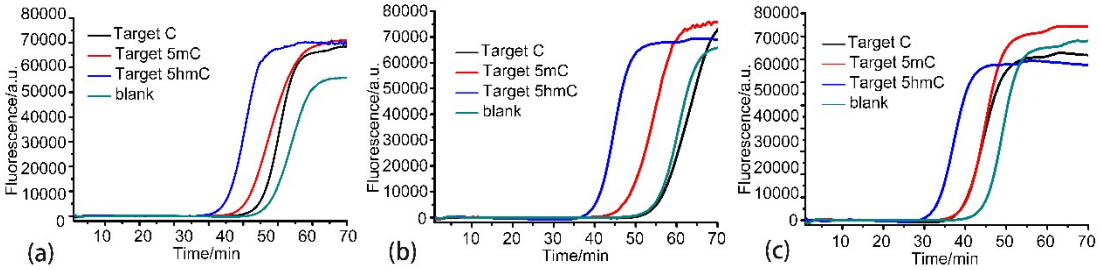

Figure S3. Real-time fluorescence curves produced by 200 fM of synthetic target C, target 5mC and target 5hmC, respectively. The concentration of probe A and probe B were used in the ligation reaction as (a) 400 pM, (b) 2 nM, (c) 10 nM, respectively.

Influence of the amount of Ampligase on mismatch ligation reaction

The ligation reaction relied on the catalysis of the Ampligase with DNA ligation activity. In the mismatch ligation-based LAMP, the ligation efficiency was imperatively affected by the amount of Ampligase. The effect of different amounts of Ampligase ranging from 0.002 U to 0.2 U was investigated. As shown in Figure S4, when 0.002 U of Ampligase was employed, there was almost no amplification fluorescence signal of blank was detected within the reaction time of 70 min, but at the expense of loss sensitivity. This indicated that the amount of Ampligase was insufficient to produce efficient DNA ligation. As the amount of enzyme increased from 0.02 U to 0.2 U, the reaction was gradually accelerated. Nonetheless, we found that difference among target 5hmC and C and 5mC firstly increased and then decreased and reached the maximum with 0.02 U Ampligase. In this regard, the amount of 0.02 U Ampligase was chosen as the optimal amount for the mismatch ligation-based LAMP.

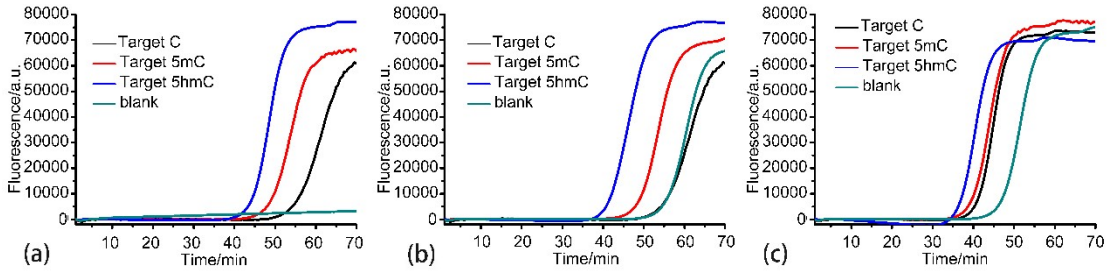

Figure S4. Real-time fluorescence curves produced by 200 fM of synthetic target C, target 5mC and target 5hmC, respectively. The amounts of Ampligase were applied in the reaction as (a) 0.002 U, (b) 0.02 U, (c) 0.2 U, respectively.

Optimization of the ligation temperature of mismatch ligation reaction

To investigate the influence of the ligation temperature on the mismatch ligation-based LAMP, the reaction was performed at the temperature of 54 °C, 58 °C, and 62 °C, respectively. As shown in Figure S5a and S5b, relative to 58°C, the ligation reaction was accelerated at 54°C, and the ΔPOI values between 5hmC and C, 5hmC and 5mC decreased, respectively. It was probably because low temperature causes non-specific ligation. With the increasing temperature to 62 °C (Figure S5c), no detectable blank signals are observed and the reaction time was delayed, which may be attributed to the low ligation efficiency at 62 ℃. The ligation efficiency of target 5hmC at 62 ℃ was lower than 58 ℃, and lead to low sensitivity for detection of 5hmC. When the ligation reaction was conducted at 58 °C (Figure S5b), it could achieve the highest specificity under high sensitivity conditions. So, the 58 ℃ was chosen as the optimal temperature for ligation reaction.

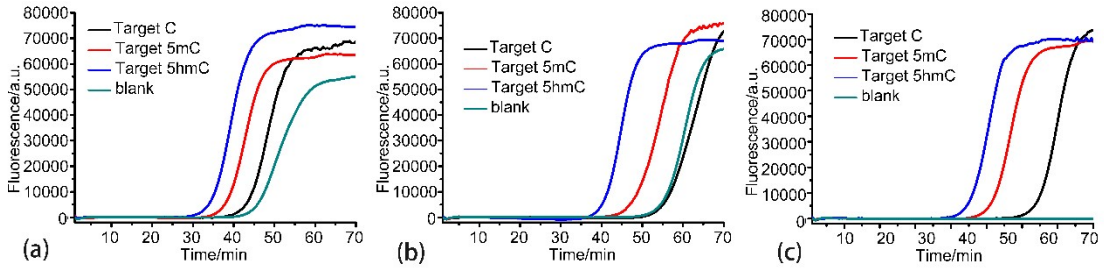

Figure S5. Real-time fluorescence curves produced by 200 fM of synthetic target C, target 5mC and target 5hmC, respectively. The ligation reactions were performed at (a) 54 ℃, (b) 58 ℃ and (c) 62 ℃, respectively.

Analysis of oxidation and ligation efficiency of mismatch ligation assay

In order to analyze the oxidative and ligation efficiency, we synthesized a target sequence with a detection site of T and the mismatched ligation reaction was performed after processing. The relationship between the POI value and the amount of synthetic target T was shown in Figure S6, the POI values were linearly dependent on the lg of concentrations of target T in the range from 2 fM to 200 pM. The correlation equation was POI =- 5.607 lgC_T -31.59 and the corresponding correlation coefficient R was 0.9996.

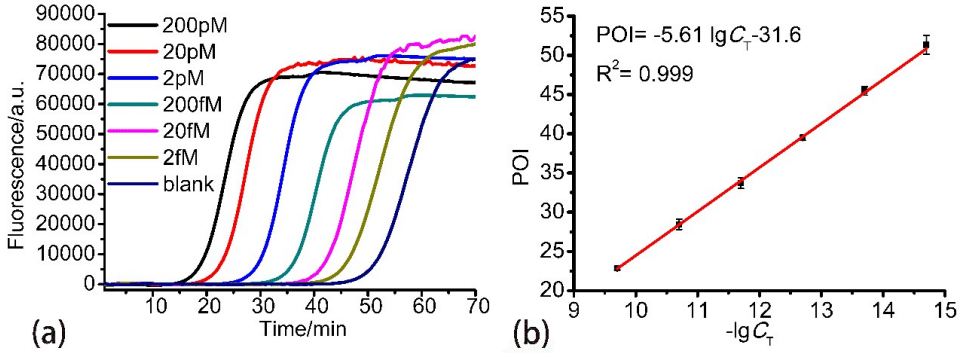

Figure S6. Dynamic range and sensitivity of LAMP based on mismatch ligation assay. (a) Amplification plot of synthetic target T over 6 orders of magnitude. Each reaction was run in triplicate. Blank was a control reaction which was detected with the same procedures but without target T. Except blank, from right to left, the concentration of target T successively was 2 fM, 20 fM, 200 fM, 2 pM, 20 pM, 200 pM. (b) The relationship between POI and lg of target T concentration (M).