Electronic Supplementary Information

Ultra-specific genotyping of single nucleotide variants by ligase-based loop-mediated isothermal amplification coupled with modified ligation probe

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1. The sequences used in this study

Table S1. The DNA sequences used in this study

Name	sequence (5'-3')
mutDNA	GCCTGTCCTGGGAGAGAC**T**GGCGCACAGAGGAAGAG
wtDNA	GCCTGTCCTGGGAGAGAC**C**GGCGCACAGAGGAAGAG
Probe A	PO_4-GTCTCTCCCAGGACAGGCTTTTATCGTCGTGACTG TTTGTAATAGGACAGAGCCCCGCACTTTCAGTCACGACGAT
Probe mB	CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGATTTT CCTCTGCTGTCGTTTTCTCTTCCTCTGTGCGCCA
Probe mB-2	CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGATTTT CCTCTGCTGTCGTTTTCTCTTCCTCTGTGCGC***G***A
Probe mB-3	CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGATTTT CCTCTGCTGTCGTTTTCTCTTCCTCTGTGCG***G***CA
Probe mB-4	CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGATTTT CCTCTGCTGTCGTTTTCTCTTCCTCTGTGC***C***CCA
Probe wB-3	CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGATTTT CCTCTGCTGTCGTTTTCTCTTCCTCTGTGCG***G***CG
FP	ATCGTCGTGACTGAAAGTGCGGGGCTCTGTCCTATTAC
BP	CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGA

Note: The underlined bold letters in red color were highlighted the base at the mutation site in mutDNA and wtDNA, respectively. A series of artificial mismatched bases introduced in Probe mB (corresponding to Probe mB-2, Probe mB-3 and Probe mB-4) and Probe wB-3 were also highlighted with underlined bold italics.

2. Effect of the amount of FP and BP on AML-LAMP assay

The amount of primers (forward primer (FP) and backward primer (BP)) could directly affect the amplification efficiency. Therefore, the effect of the amount of FP and BP on the AMP-LAMP-based genotyping assay was investigated. The different concentrations of mutDNA and wtDNA were

simultaneously detected with different amounts of primers ranging from 0.4 μM to 1.2 μM. As depicted in Fig. S1a, when less than 0.4 μM FP and BP was employed, almost no fluorescence signal produced by 10 aM mutDNA was detected within the reaction time of 70 min, indicating that the amount of primers was insufficient for the LAMP reaction. When the amount of FP and BP was increased to 0.8 μM and 1.2 μM (Fig. S1b and c), the AMP-LAMP reaction was gradually accelerated with the increase of primers dosage. Nonetheless, the difference of POI values between 1 fM mutDNA and wtDNA was significantly decreased. One could see from Fig. S1b that the difference of POI values produced by 1 fM mutDNA and wtDNA reached the maximum with 0.8 μM FP and BP. In this regard, the amount of 0.8 μM FP and BP was chosen as the optimized amount for the genotyping assay.

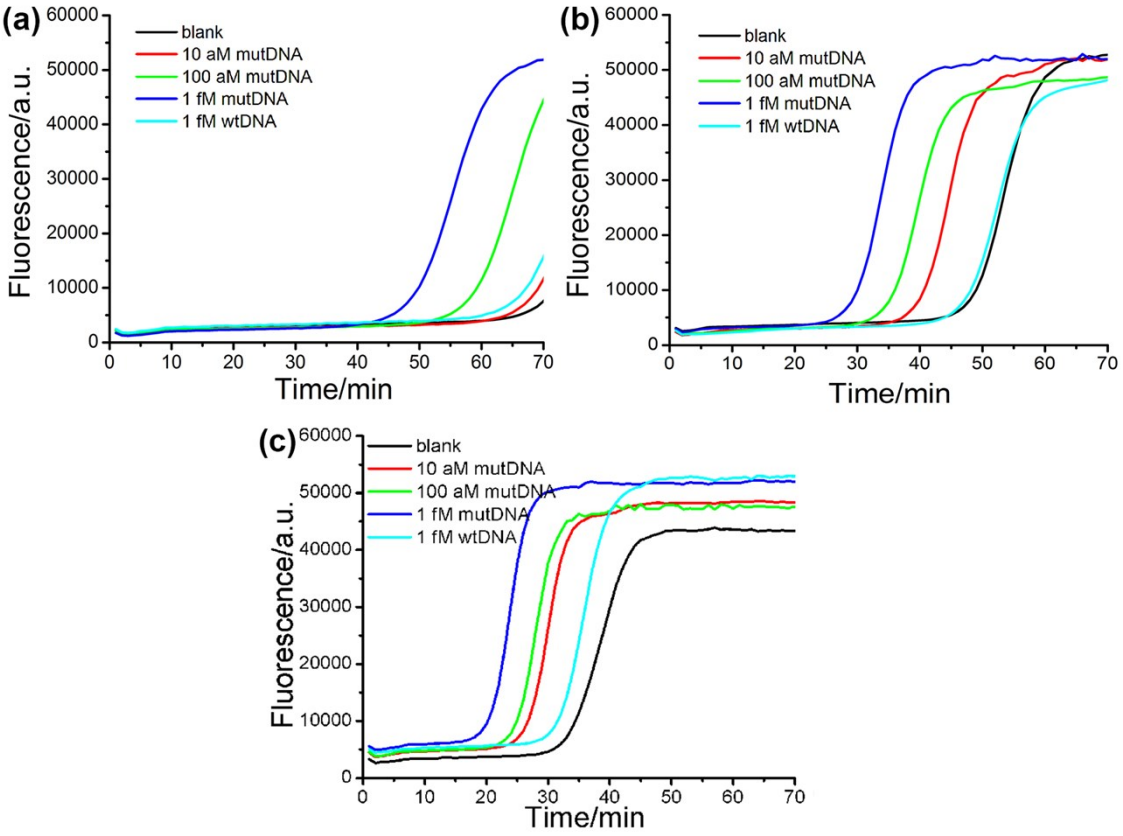

Fig. S1 The effect of the amount of FP and BP on the AML-LAMP assay. The real-time fluorescence curves produced by mutDNA of 0 (blank), 10 aM, 100 aM, 1 fM and 1 fM wtDNA with different FP and BP concentrations of (a) 0.4 μM, (b) 0.8 μM and (c) 1.2 μM, respectively.

3. Optimization of the concentration of Probe A and Probe mB-3

The concentration of Probe A and Probe mB-3 was another critical factor for the highly selective discrimination of the single base variation. The ligation product of the double stem-loop DNA with

Probe A and Probe mB-3 was the essential element for the subsequent LAMP amplification. In order to achieve sensitive and specific detection of SNVs, the concentration of Probe A and Probe mB-3 in the range of 0.1 nM to 5 nM was investigated and optimized. As depicted in Fig. S2a, 10 aM mutDNA could not be detected when 0.1 nM ligation probe was employed, which should be attributed that the low concentration of probes resulted in less ligation products, thus could not produce the detectable signal in LAMP amplification, particularly for low concentration of mutDNA. When the concentration of probes was more than 1 nM, as shown in the Fig. S2b-d that as low as 10 aM mutDNA could be clearly determined. Notably, with the concentration of 2 nM, the difference of POI value between 1 fM mutDNA and wtDNA reached the maximum. However, when the concentration was further elevated to 5 nM (Fig. S2d), the undesirably nonspecific amplification aroused by 1 fM wtDNA increased remarkably, indicating that excessive probes would cause much template-independent ligation and then produced more nonspecific amplification signal. Therefore, 2 nM of Probe A and Probe mB-3 was employed for the AMP-LAMP genotyping assay in this work.

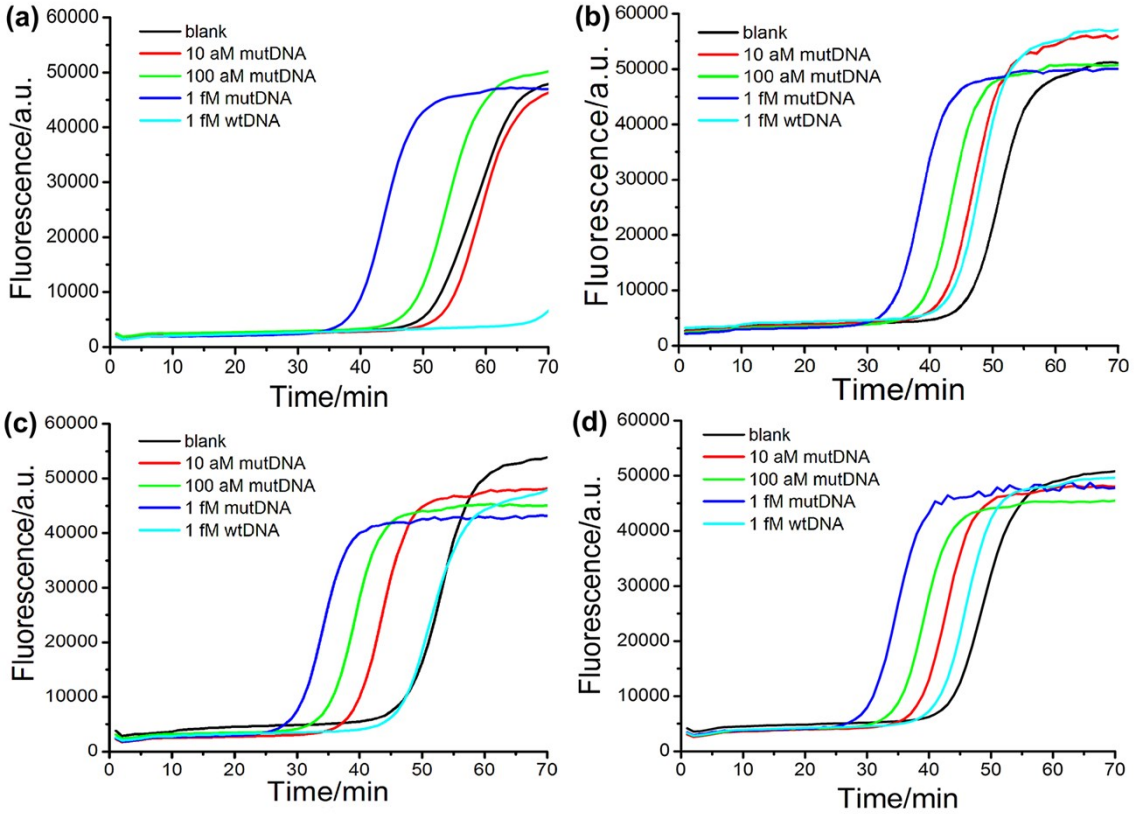

Fig. S2 The influence of the concentration of the Probe A and Probe mB-3 on the AMP-LAMP-based genotyping assay. Real-time fluorescence curves produced by the mutDNA and wtDNA were recorded with different concentrations of Probe A and Probe mB-3 of (a) 0.1 nM, (b) 1 nM, (c) 2 nM, and (d) 5 nM, respectively.

4. Determination of genomic DNA extracted from the human whole blood

To validate the robustness and practicability of the proposed AML-LAMP SNV assay in real biological samples, we have collected blood samples from 13 ovarian cancer, 4 benign ovarian tumor patients and a healthy volunteer. Then it was applied to directly detect the extracted genomic DNA from whole blood with mutant probes (Probe A and Probe mB-3) or wild-type probes (Probe A and Probe wB-3). Before with the genomic DNA as the template of ligation reaction, it should be heat-denatured at 95 °C for 3 min and then placed on ice immediately, then the single-stranded genomic DNA directly acted as the template for the ligation reaction. As depicted in Fig. S3a, when Probe A and Probe mB-3 were used to detect genomic DNA, no effective real-time fluorescence signal could be observed by all the patients and healthy people, indicating that target mutDNA could not be detected in the genome extracts. On the contrary, genomic DNA of all the samples could produce the well-defined real-time fluorescence signals compared with the blank control by using Probe A and Probe wB-3 (w represented wild-type-the 3′ terminal base in Probe wB-3 was complementary to wtDNA, the sequence was listed in Table S1) to perform the assay (Fig. S3b). The above results suggested that all the extracted genomic DNA were wild-type. Moreover, as little as 100 pg genomic DNA could be obviously detected, and a good linear relationship between the POI values and the -lg of genomic DNA amounts (ranging from 100 pg to 100 ng) was obtained in Fig. S3c-d.

To further verify its potential clinical applicability, the proposed assay was also applied to detect spiked mutDNA in 10 ng genomic DNA. As illustrated in Fig. S4, the fluorescence curve produced by 10 ng genomic DNA was the same as that of blank control because the detection site in p53 gene was wild-type in genomic DNA. In order to verify whether the various co-existing sequences in the complex genomic DNA would interfere with the detection of mutDNA, different amounts of synthetic mutDNA were spiked into 10 ng genomic DNA. Then, such spiked samples were respectively examined by the AML-LAMP assay. At the same time, the series dilutions of synthetic mutDNA without genomic DNA were also tested for comparison. It can be seen from Fig. S4 that the fluorescence curves gradually appear earlier with the increasing dosage of spiked mutDNA in the genomic DNA. More importantly, the fluorescence curves aroused by the different amounts of spiked mutDNA in the genomic DNA were basically the same as those of without genomic DNA. Therefore, the proposed method is of great potential for detecting mutDNA with different abundances in complex samples, since the complicated wtDNA matrixes would not interfere with the mutDNA assay.

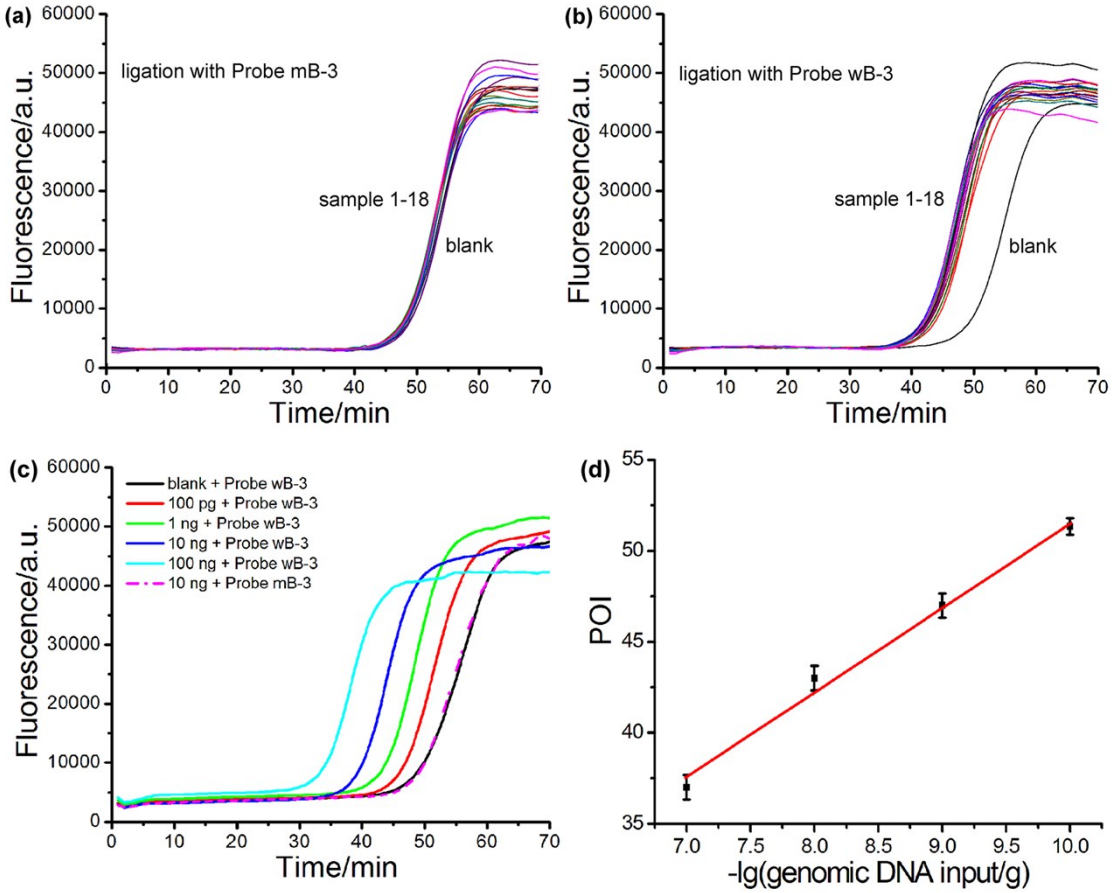

Fig. S3 The real-time fluorescence curves produced by all the extracted genomic DNA and blank control (without genomic DNA) with mutant probes of Probe A and Probe mB-3 (a) and wild-type probes of Probe A and Probe wB-3 (b); (c) The fluorescence responses of different amounts of genomic DNA (100 pg to 100 ng) with specific wild-type probes and 10 ng genomic DNA with mutant probes; (d) The relationship between POI values and -lg of the amount of genomic DNA. Error bars were calculated from three replicate measurements.

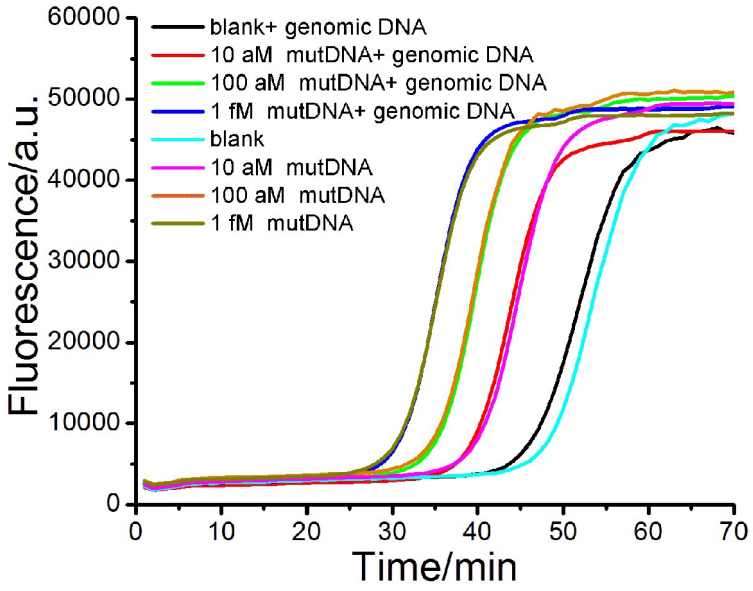

Fig. S4 The real-time fluorescence curves of synthetic mutDNA and the mixture of genomic DNA and synthetic mutDNA. The concentration of synthetic mutDNA from right to left was 0, 10 aM, 100 aM and 1 fM, respectively. The experiment was performed according to the experimental section.

5. The sequencing result of the extracted genomic DNA

The target gene in extracted genomic DNA was pre-amplified before sequencing. The PCR amplification was performed in 20 μL mixture containing 1 U Taq Hot Strat DNA polymerase, 200 nM primer PU8 (AGG ACC TGA TTT CCT TAC TGC CTC T) and primer PD8 (GTC CTG CTT GCT TAC CTC GCT TAG T), 0.2 mM dNTPs and PCR reaction buffer (10 mM Tri-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3 @25 ºC). The PCR system was first heated for 5 min at 94 ºC, followed by 30 cycles of 94 ºC for 20 s, 58 ºC for 20 s and 72 ºC for 30 s. Then the PCR products were finally sent to Sangon Biotech. (Shanghai, China) for sequencing.

The sequencing results shown that the SNV detection site was C, indicating the target gene of all the extracted genomic DNA was wild-type DNA, as shown in Fig. S5, which were consistent with the result by the AML-LAMP strategy in Fig. S3. It further proved that the AML-LAMP-based genotyping assay was credible and effective.

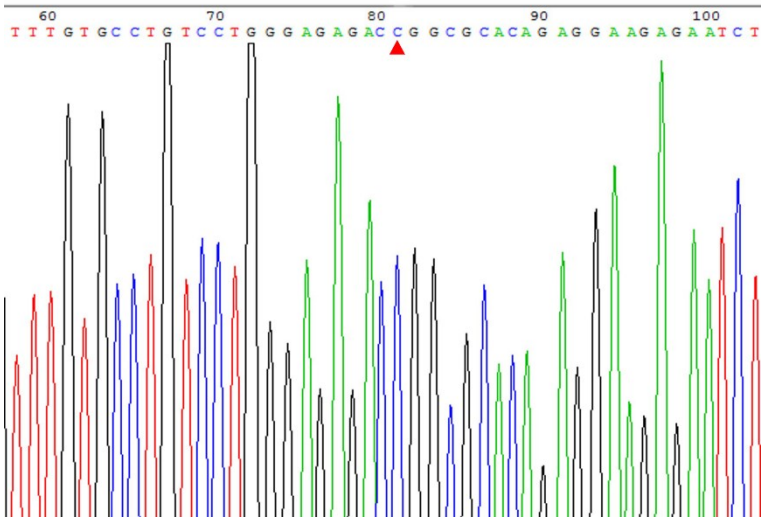

Fig. S5 The sequencing result of the genomic DNA region of exon 8 of p53 gene, where the sequence of the SNV detection site was marked with the triangle.

6. Comparison of the widely used methods for SNPs assay

Table S2. The comparison of the widely used methods for SNPs assay.

Detection strategy	Temperature control	Sensitivity	Specificity	Reference
Allele specific PCR by using cationic conjugated polymers	Thermal cycle	None	2%	8
Bioluminometic assay with modified primer extension reaction	Thermal cycle	14 amol	2%	9
Branch migration based selective PCR	Thermal cycle	None	0.1%	S1
Ligation-based PCR with Taqman probes	Thermal cycle	None	0.1%~0.01%	15
Ligase chain reaction with cationic conjugated polymers (CCPs)	Thermal cycle	1 fM (600 zmol)	1%	20
LCR-based electrochemical biosensor	Thermal cycle	10 aM	0.1%	S2
Ligation-mediated SDA with chemiluminescence detection	isothermal	0.1 fM	0.6%	22
LCR-coupled RCA assay with RNA FRET probes	isothermal	10 aM	1%	23
ligase-based isothermally exponential amplification	isothermal	10 aM	0.1%	24
RCA combined with gold nanoparticle aggregates	isothermal	70 fM	0.01%	S3
LAMP with one-step strand displacement reporters	isothermal	~2 aM	5%	35
T7 exonuclease mediated isothermal amplification	isothermal	50 nM	0.2%	S4
Ligase-based LAMP coupled with modified ligation probe	isothermal	10 aM (0.1 zmol)	0.01%	This work

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