

Supplementary Material

One-step colorimetric genotyping of single nucleotide polymorphism using probe-enhanced loop-mediated isothermal amplification (PE-LAMP)

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Table S1. The sequences used in our study

SNP and gene	Primers and probes	Sequence(5'-3')
rs3741219 (H19)	F1C-F2	GTCACCCGGCCCAGATGGAGCAGTACGAGTGTGC
		GTGAG
	B1C-B2	CTGTGTGCCCCGAGGCCTCAGCTCCGTGATGTCGG
		TCG
	F3	GGAGACGGCCTTGAGTCT
	B3	GGGCGTAATGGAATGCTTGA
	LP(G)	CCTGCGCAGGC
	LP(A)	CCTGCAACAGGC
	PCR forward	CTCCTCCAGCGGGATGA
	PCR reverse	CACATCATGCCCACAGCC
rs1045642 (MDR1)	F1C-F2	GCAGTCAAACAGGATGGGCTCCGAATGTTCAGTG
		GCTCCGA
	B1C-B2	TTGCCTATGGAGACAACAGCCGGTATGTTGGCCTC
		CTTTGCT
	F3	CAGCTGCTTGATGGCAAAG
	B3	CAGTGA CTGATGAAGGCA
	LPC	AGAGATCGTGAGG
	LPT	AGAGATTGTGAGG
	PCR forward	TGTGCTGGTCCTGAAGTTG
	PCR reverse	AGTGTGGCCAGATGCTTG

rs4244285	F1C-F2	AGCTCTGGTTGTAATTTAAACTACAATAAAAATTTC
(CYP2C19)		CCCATC
	B1C-B2	GATATGCAATAATTTCCCACTATCTTCATAAAAGC
		AAGGTT
	F3	TCAGAGAATTACTACACATG
	B3	ACTTTCTCCAAAATATCACT
	LPG	TTTCCC G GGAAC
	LPA	TTTCCC A GGAAC
	PCR forward	CAACCAGAGCTTGGCATATTGT
	PCR reverse	GCATTACTCCTTGACCTGT

F1C-F2 and B1C-B2 are inner primers. F3 and B3 are outer primers.

The SNP position in probes is highlighted in bold, and the mutant allele is highlighted in red and bold.

The PCR reverse primers were used to direct sequencing.

Table S2. The length and T_m value of the probes used in our experiments

Probes	Sequence (5'-3')	Length (nt)	T _m value (°C)
loop primer	CGGCCGGGCCCTGC G	15	67.5
13nt LP(G)	CCCTGC G CAGGCA	13	45.3
13nt LP(A)	CCCTGC A CAGGCA	13	42.1
12nt LP(G)	CCCTGC G CAGGC	12	44.1
12nt LP(A)	CCCTGC A CAGGC	12	40.7
11nt LP(G)	CCTGC G CAGGC	11	38.9
11nt LP(A)	CCTGC A CAGGC	11	38.2
5' (G) LP	G CAGGCACTTG	11	36.0
5' (A) LP	A CAGGCACTTG	11	34.0
3' (G) LP	CGGGCCCTGC G	11	42.6
3' (A) LP	CGGGCCCTGC A	11	38.9
LPC	AGAGAT C GTGAGG	13	40.0
LPT	AGAGAT T GTGAGG	13	38.0
LPG	TTTCCC G GGAAC	12	38.0
LPA	TTTCCC A GGAAC	12	36.2

The SNP position in probes was highlighted in red and bold.

It was worth noting that LPs used to investigate the positional effect both had the T_m value around 38°C.

The temperature of the probe-enhanced LAMP reactions for these three SNPs was 62°C.

Table S3. positional effect of targeted nucleotide in LP

LP	position	T _m (°C)	mutant template (C)		Wild-type template (T)	
			T _q (min)	ΔT _q (min)	T _q (min)	ΔT _q (min)
5' (G) LP	5'-end	36.0	41.0	>44	>85	>39.3
5' (A) LP	5'-end	34.0	>85		45.7	
3' (G) LP	3'-end	42.6	35.1	43.9	68.2	31
3' (A) LP	3'-end	38.9	79.0		37.2	
11nt LP(G)	central	38.9	32.4	49.9	80.9	47.9
11nt LP(A)	central	38.2	82.3		33.0	
Without LP	-	-	>85	-	>85	-

The whole reaction time was fixed at 85 min with 10⁴ copies of templates.

The ΔT_q in the table is the time window between specific amplification and non-specific amplification when amplifying the same template.

The time window obtained by the LP with the SNP at the central was wider than that obtained by the others LP.

Table S4. Comparison of analytical performances in typing SNP between PCR-based methods and LAMP-based methods

Methods	Sensitivity (copies)	accuracy	Instrument requirement	Operation	Measurement Time (min)
qPCR (HRM)	1000	high	+++	one-step	90
<i>(J Mol Diagn 2013, 15: 600-606)</i>					
Pyrosequencing	1000	high	+++	Two-step	90
LAMP-Invader-AuNP	1000	high	+	Two-step	90
<i>(Biosens Bioelectron. 2017 Apr 15;90:388-393)</i>					
AS-LAMP	100	moderate	++	One-step	40
<i>(Biosens Bioelectron. 2018 Sep 15;115:70-76)</i>					
PE-LAMP	1000	high	+	One-step	60

Measurement time includes amplification and signal readout.

The HRM is short for high resolution melting analysis.

Two-step operation means the amplification and signal readout are separate.

+: few; ++: medium; +++: high

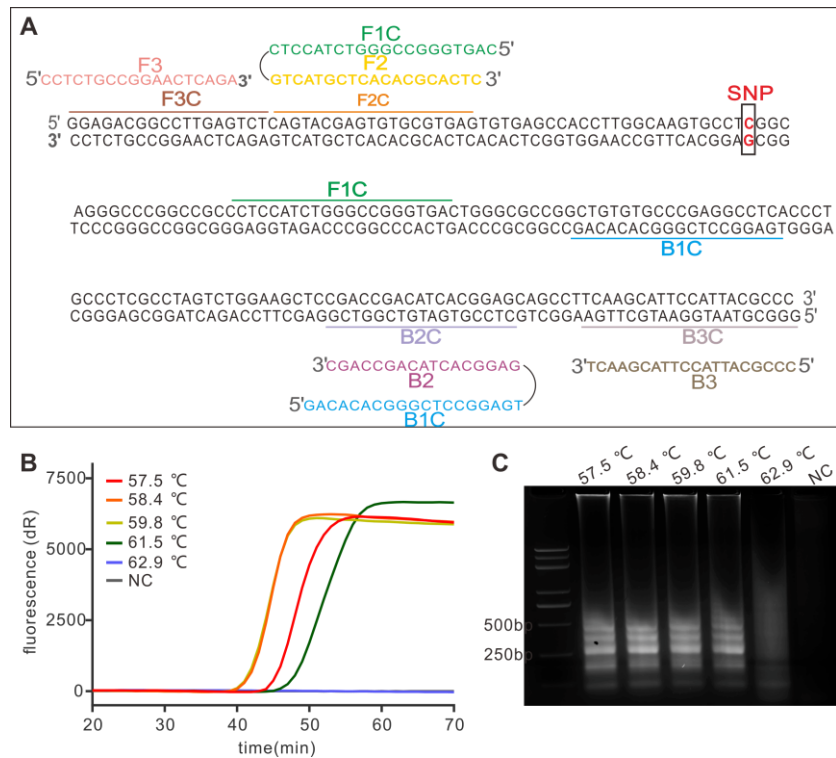


Figure S1 Primer design and optimization of reaction temperature. (A) The primer design for SNP rs3741219. (B) the real-time fluorescent LAMP without loop primer under a temperature gradient ranging from 57.5 °C to 62.9 °C. (C) Gel electrophoresis analysis of LAMP products. The concentration of the plasmid template was 10^7 copies. Even though the reaction showed a better amplification result under the temperature around 59 °C, the higher temperature (62 °C) was chosen for the following assay due to the SNP genotyping was based on the temperature hybridization of a specific probe.

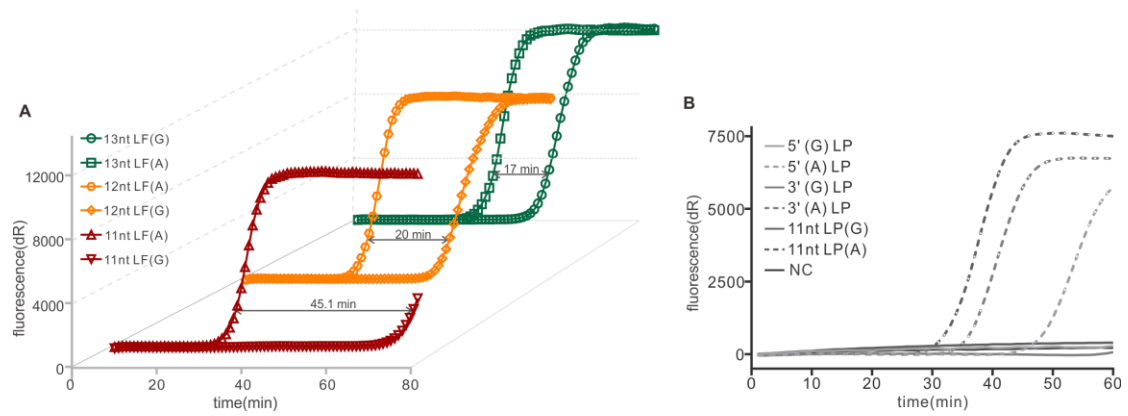


Figure S2 Selection of LP using wild-type plasmid. (A) Selection of the LP with proper length using wild-type plasmid (rs3741219 T). (B) Investigating the positional effect of the mutant in LP. When using the wild-type template as the target to be detected, the 11nt LP with the mutant in the center was able to obtain the optimal discrimination compared to other LPs. The result was consistent with the reaction using mutant template (rs3741219 C).

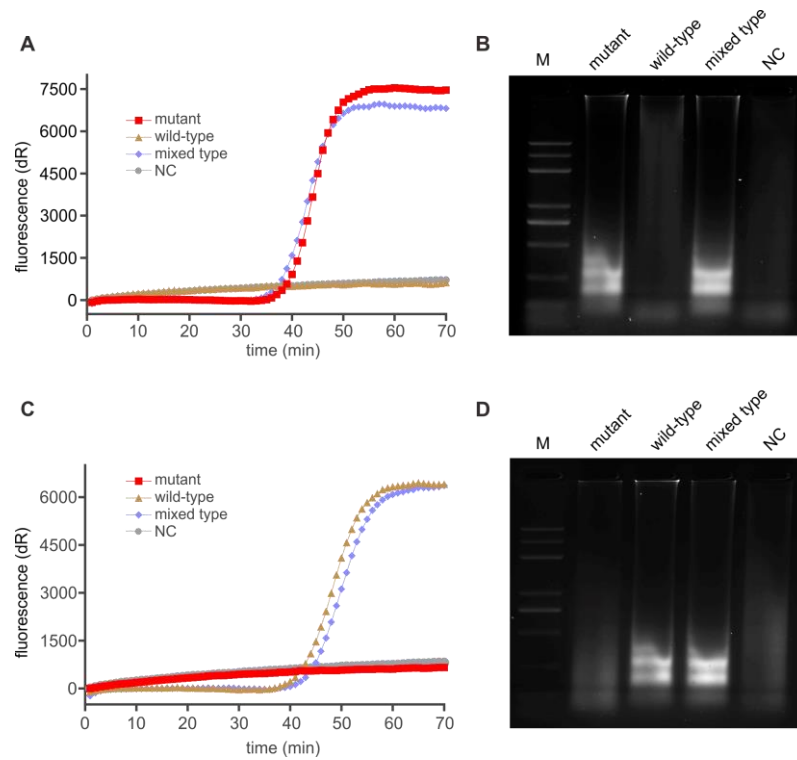


Figure S3 Detection of different targets using specific LP. (A) Using 11nt LP(G) to detect mutant, wild-type and mixed type templates. (B) Gel electrophoresis analysis of detection using 11nt LP(G). (C) Using 11nt LP(A) to detect mutant, wild-type and mixed type templates. (D) Gel electrophoresis analysis of detection using 11nt LP(A). M: DNA marker.

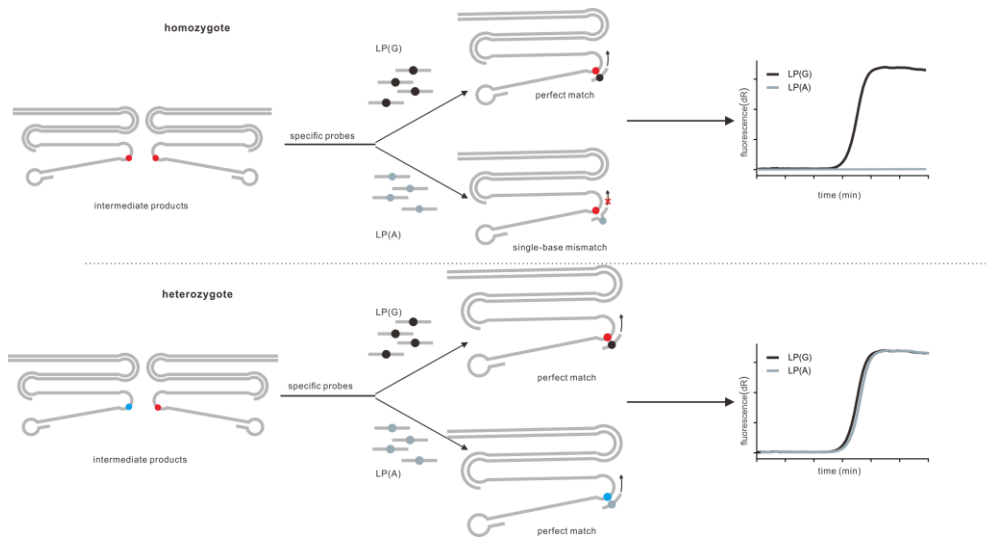


Figure S4. Illustration of genotyping using PE-LAMP. When using the PE-LAMP to genotype an undefined target, we design two LPs, differing in only one base, to detect the same template at two parallel reaction tubes. When the target is a homozygote, there will be a great difference in amplification efficiency between the parallel reactions and the fluorescence result resemble the upper in Fig. S4. When it comes to the heterozygote, there will only a slight or even no discrepancy between two reactions, like the bottom in Fig. S4. Based on the results of amplification, we could easily discriminate different type of an individual's SNP genotype. Additionally, the genotyping results could be reflected in a visualized manner with the help of colorimetric indicator.

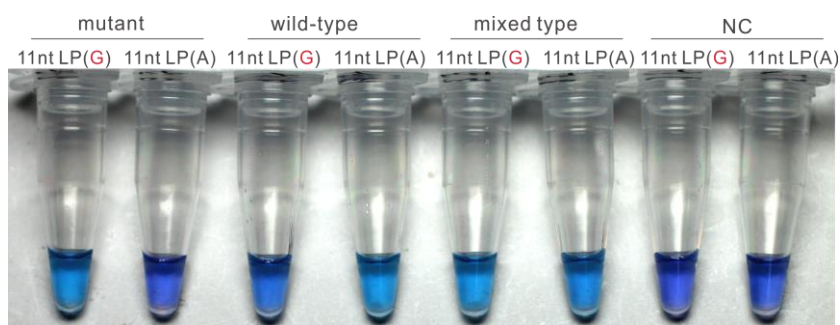


Figure S5 Using pre-added **HNB to colorimetrically type plasmid templates. 120 μ M**

Hydroxy naphthol blue (HNB) was added to the LAMP solution before the amplification.

HNB is the most reported indicator used in LAMP reaction, which is a colorimetric indicator

for Mg²⁺ ion with the color change from violet to sky blue as the Mg²⁺ ion concentration

decreases. When the LAMP proceeds, the by-product pyrophosphate could form an

insoluble complex with Mg²⁺, thus the concentration of Mg²⁺ decreases along with reaction

process.

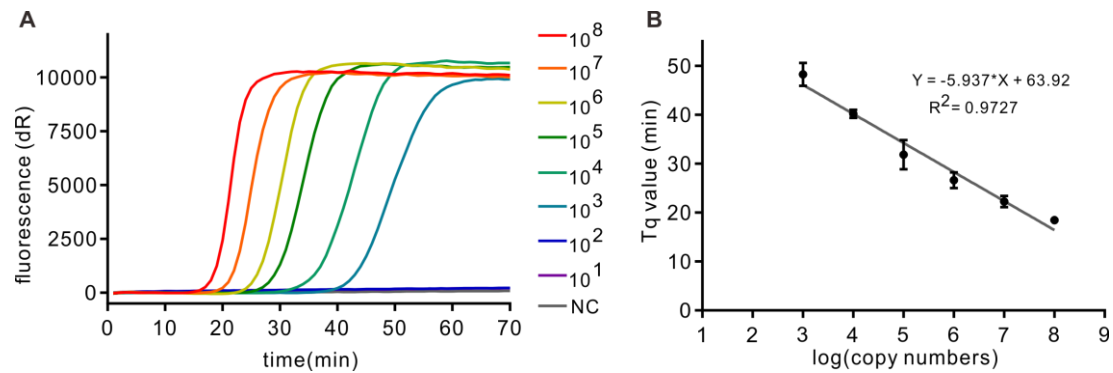


Figure S6 The sensitivity of PE-LAMP using wild-type plasmid. (A) Sensitivity of the real-time fluorescent LAMP using serially diluted wild-type plasmid as template. (B) The linear relationship between the Tq value and the logarithm of the copy number in the range of 10^8 copies to 10^3 copies. The error bars are the standard deviation of three repetitive measurements. The sensitivity of the method was about 10^3 copies. This was not like the previously reported LAMP reactions, which could even detect less than 10 copies of targets. However, the human genome concentration of routine extraction of from saliva, blood and tissue was generally 10^4 - 10^5 copies per microliter. In clinical use, 10^4 - 10^5 copies of genome are sufficient for the routine diagnostic. Thus, the sensitivity of our strategy was enough to detect clinical sample.

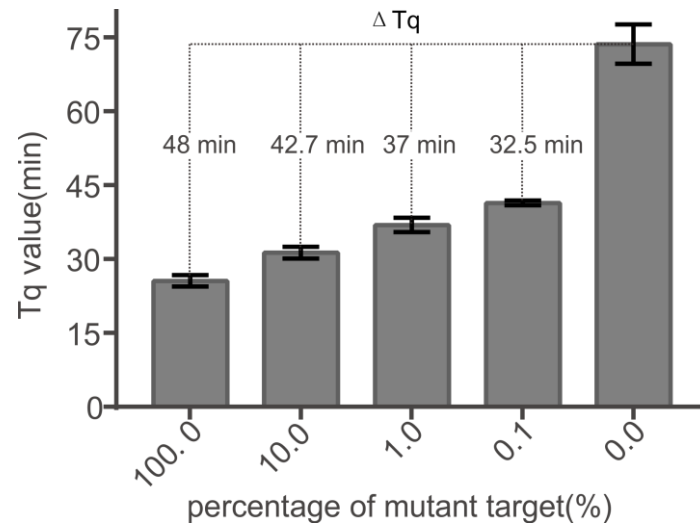


Figure S7 The Tq values of PE-LAMP using the mutant-harboring templates. The error bars are the standard deviations of three repetitive experiments. The mutant target was mixed with wild-type plasmid in different ratios (100%, 10%, 1%, 0.1 and 0%). The total number of the template was 10^6 copies. The ΔTq was defined as the time gap between the mutant-harboring reaction and the background (0% mutant). As we can see from the column picture, there was a huge time gap (more than 30min) between the positive reaction and the background, which demonstrate the good specificity of the method in detecting the low-abundance target.

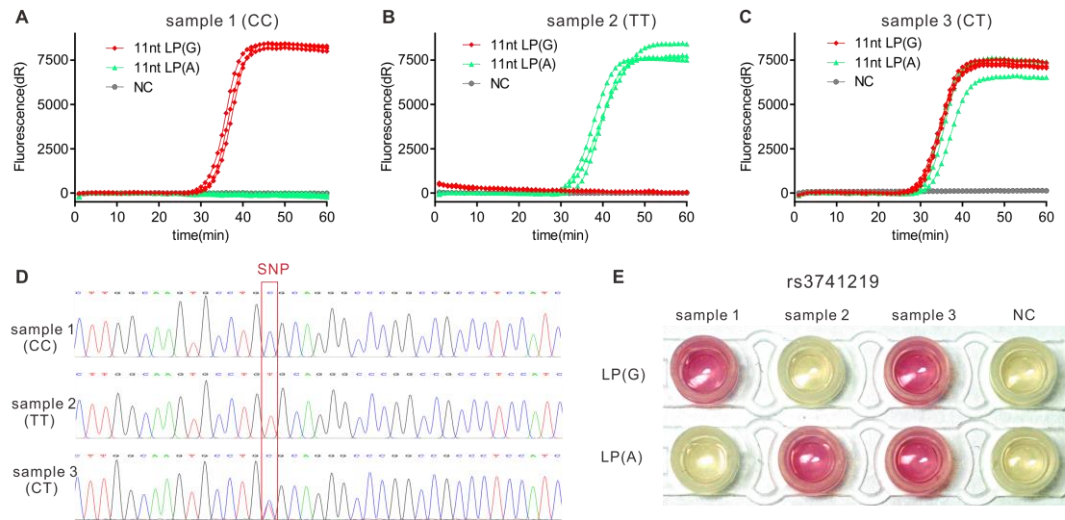


Figure S8 Validation of the PE-LAMP in genotyping real sample. (A) Genotyping the sample 1 (homozygote of rs3741219 CC). (B) Genotyping the sample 2 (homozygote of rs3741219 TT). (C) Genotyping the sample 3 (heterozygote of rs3741219 CT). (D) the sequence results of sample 1,2 and 3. (E) Colorimetric genotyping. Real-time LAMP curves as well as the colorimetric assay results, corresponding to the sequencing results, showed that the probe-enhanced LAMP assay was available to genotype homozygotes and heterozygotes of rs3741219 in an hour when using saliva lysates as the template. The colorimetric typing reactions were carried out with one microliter of simply treated saliva lysate in a conventional thermal cycler and the results were photographed at the end of the reaction (60min). Compared to the traditional genotyping methods, the probe-enhanced LAMP assay has a shorter readout time and the operation is less complicated. The isothermal condition and colorimetric reporting system in our strategy are much suitable for on-site genotyping. The one-step colorimetric genotyping is favorable in on-site diagnosis and the only required equipment is a portable thermal controller that is cheaper and more convenient than the

currently used thermal cycler. The whole assay could be finished in 75 min including sample processing(15min) and the colorimetric readout (60min).

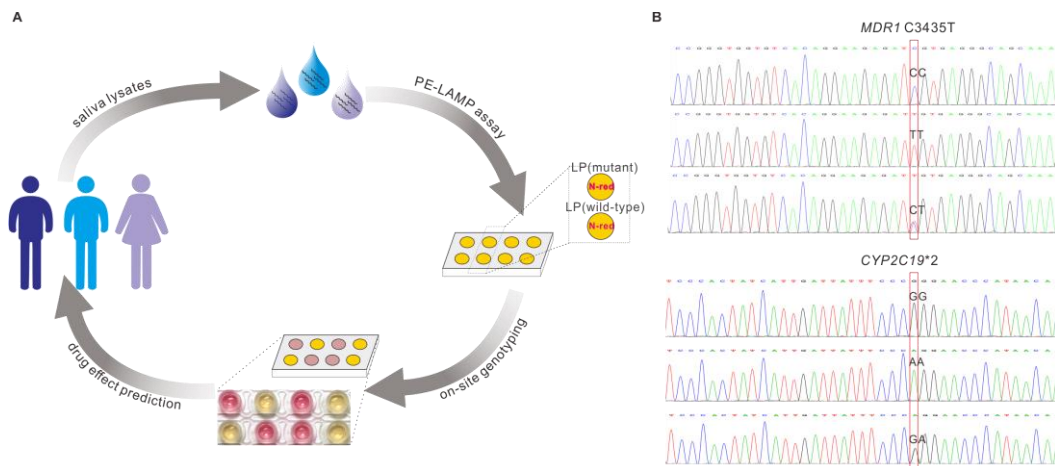


Figure S9 Application of PE-LAMP to pharmacogenomic test. (A) The workflow of pharmacogenomic use of PE-LAMP assay. (B) The sequencing results of homozygotes and heterozygotes of MDR1 C3435T (upper) and CYP2C19*2 (bottom). We enrolled 15 individuals and sequenced their genotype at these two SNP sites, then the PE-LAMP was used to genotype these individuals. The pharmacogenomic test could be carried out in a single-step manner, which makes the PE-LAMP an effective tool in on-site detection. The saliva sample was simply treated by the fast extraction kit that is commercially available and then the saliva lysate was subjected to genotyping by PE-LAMP.

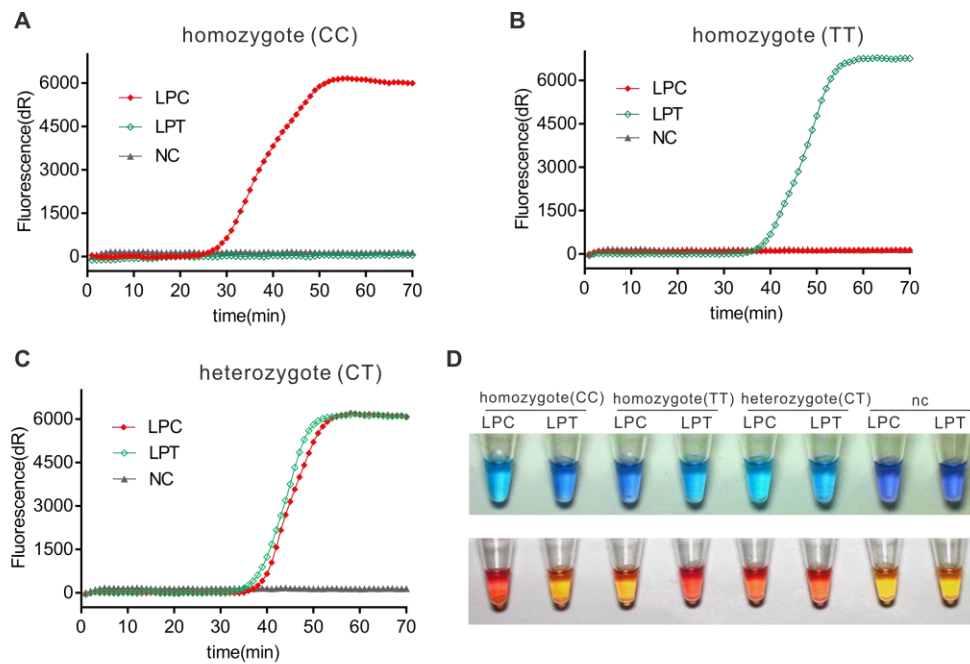


Figure S10 The PE-LAMP for genotyping *MDR1* C3435T. (A) Genotyping the homozygote *MDR1* 3435CC. (B) Genotyping the homozygote *MDR1* 3435TT. (C) Genotyping the heterozygote *MDR1* 3435 CT. (D) Colorimetric genotyping of *MDR1* C3435T. The *MDR1* C3435T is the typical germline mutation used in the pharmacogenomic study. Apart from the antiplatelet drug Clopidogrel, *MDR1* C3435T is also involved in transportation of many clinical used drugs including Simvastatin, Methotrexate, and Aliskiren.

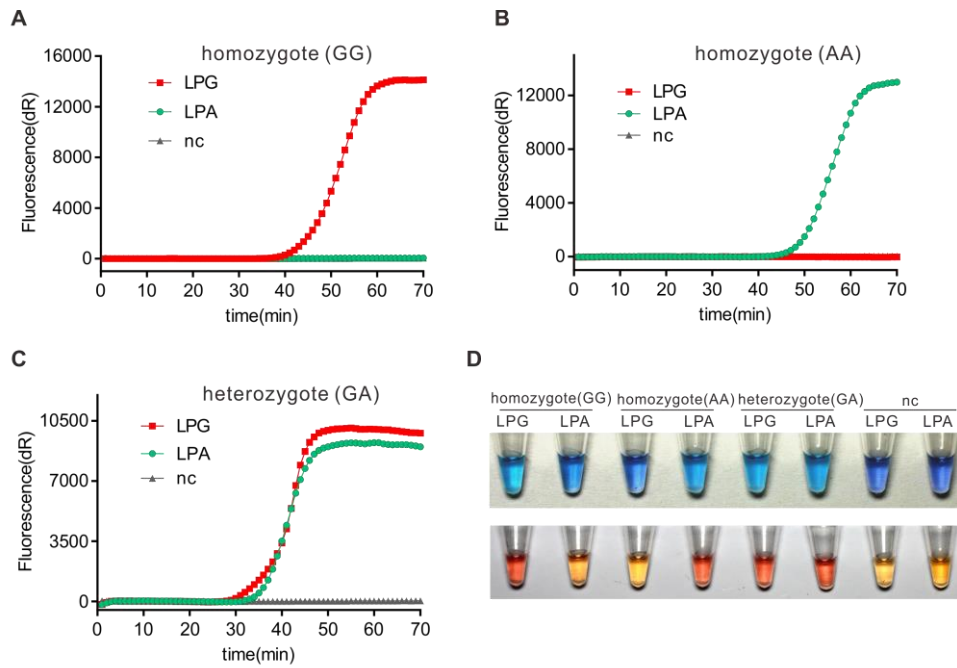


Figure S11 The PE-LAMP for genotyping *CYP2C192.** (A) Genotyping the homozygote *CYP2C19**2 GG. (B) Genotyping the homozygote *CYP2C19**2 AA. (C) Genotyping the heterozygote *CYP2C19**2 GA. (D) Colorimetric genotyping of *CYP2C19**2. Apart from the antiplatelet drug clopidogrel, *CYP2C19**2 is also associated with the drug effect of Voriconazole, Sertraline and Amitriptyline and so on.

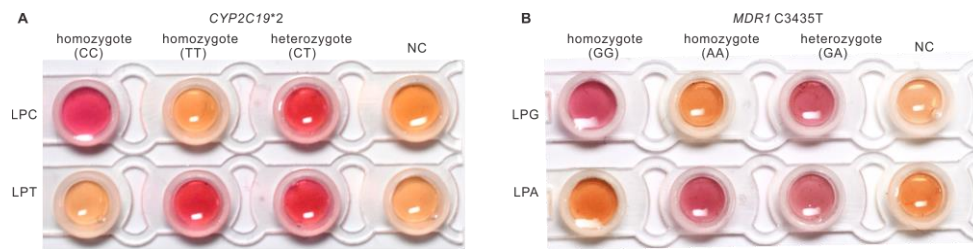


Figure S12 The PE-LAMP based genotyping using simply treated whole blood samples. (A) Genotyping of *CYP2C19**2. (B) Genotyping of *MDR1* C3435T. The whole blood samples were collected in EDTA-coated tubes from those consent-informed candidates. Then 50 μ L of the whole blood was mixed with the extraction buffer in the ratio of 1:1, followed by adding 100 μ L neutralization buffer (Sigma-Aldrich Corporation, USA). Finally, 1 μ L of simply treated blood sample was added to the reaction tube for colorimetric genotyping. It is worth noting that the genotyping of simply treated blood sample took more time than saliva sample due to the inhibitory substance contained in the blood lysate.

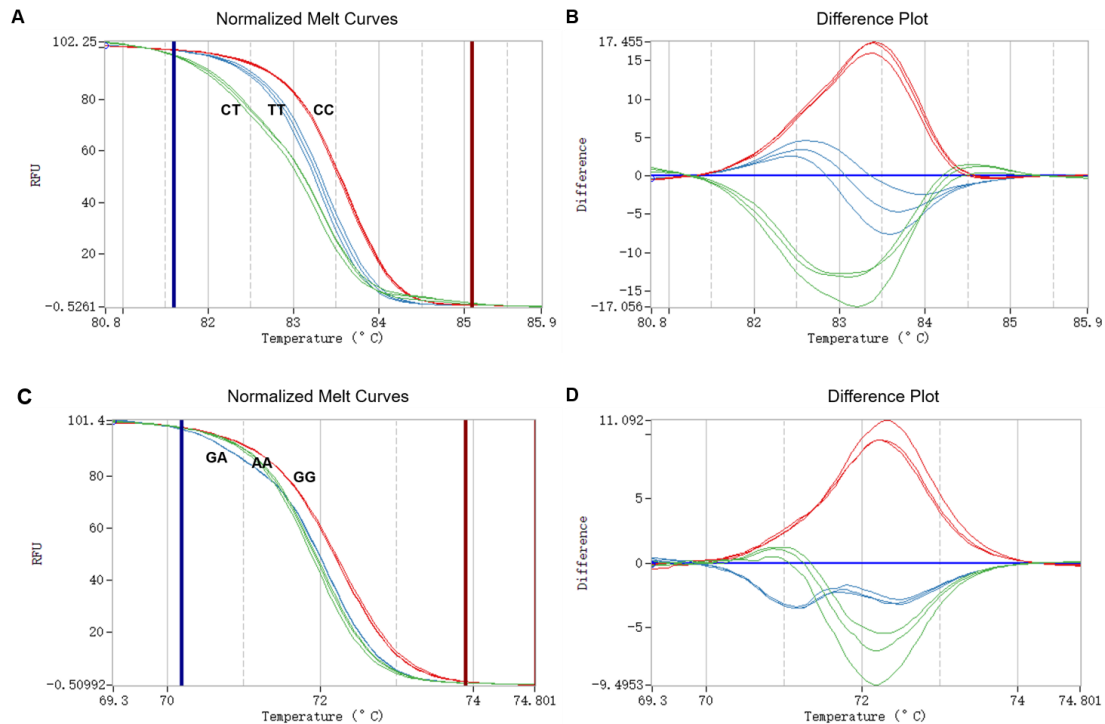


Figure S13 The HRM based genotyping using real-life samples. (A) Melt-curve profiles of *MDR1* C3435T. (B) Melt-curve profiles of *CYP2C19*2*. (C) visual identification of clusters for *MDR1* C3435T, the HRM Analysis software generates a difference curve for each sample. (D) visual identification of clusters for *CYP2C19*2*, the HRM Analysis software generates a difference curve for each sample. The HRM analysis was carried out on a Thermo Scientific PikoReal real-time PCR system (Thermo Fisher Scientific Corporation, USA) by an initial denaturation at 95 °C for 2 min followed by 40 cycles, each comprising 10 s at 95 °C and 30 s at 60 °C. Then the PCR products were slowly heated over the range from 65 °C to 95 °C, rising 0.1 °C per sec. According to the HRM analysis, different genotypes of *MDR1* C3435T and *CYP2C19*2* were accurately determined. However, it took more than 1 hour and a half to finish the whole assay; and the high reliance on the sophisticated instrument limited the HRM analysis to the laboratory with specialized facilities. In contrast, the measurement time of PE-LAMP was about 1 hour and less requirement of instrument made it more user-friendly.