Electronic Supplementary Information

Ultrasensitive Genotyping with Target-Specifically Generated Circular Templates and RNA FRET Probes

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1. Experimental Section

Reagents and instruments

Ampligase (thermostable DNA ligase) and phi29 DNA Polymerase were purchased from Epicentre Technologies (Madison, WI, USA). RNase H, Exonuclease I and Exonuclease III were purchased from New England Biolabs. Ribonuclease (RNase) inhibitor, dNTPs, RNase-free water was obtained from Takara Biotechnology Co., Ltd. (Dalian, China). The ligation reaction was carried out with a 2720 Thermocycler (Applied Biosystems, USA). Fluorescence detection was conducted with either a LS-55 model spectrofluorimeter (PerkinElmer Co., USA) or a StepOne Real-Time PCR System (Applied Biosystems, USA). All of the DNA and RNA oligonucleotides were synthesized and purified by Integrated DNA Technologies (USA), and all the sequences are listed in Table S1.

Ligation of padlock probes

For ligation reactions, 1.0 U of Ampligase was added to a 10 μL reaction mixture containing ligation buffer (20 mM Tris–HCl, pH 8.3, 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM nicotinamide adenine dinucleotide (NAD), 0.01% Triton X-100), 20 nM padlock probe, and an appropriate amount of target DNA in one tube. After incubated at 95 ºC for 3 min to denature the target DNA and padlock probe, ligation was performed at 70 ºC for 30 min. The thermal cycle ligation of padlock probes was conducted with 30 cycles of 95 ºC for 30 s and 70 ºC for 1 min.

LCR amplification and ligation of padlock probes

LCR amplification was performed in 10 μL of ligation buffer (20 mM Tris–HCl, pH 8.3, 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM nicotinamide adenine dinucleotide (NAD), 0.01% Triton X-100) containing 20 nM probe X', 20 nM probe Y', 5 nM probe X, 5 nM probe Y, 1.0 U of Ampligase, and appropriate amount of DNA targets or genomic DNA. After an initial incubation at 95 ºC for 3 min, LCR amplification was performed with 15 thermal cycles of 95 ºC for 30 s and 60 ºC for 1 min. Then 20 nM padlock probe was added and the ligation reaction was carried out with the same 15 thermal cycles. Next, 10 U of Exo I and 100 U Exo III were added into the reaction mixture to degrade the unreacted probes by incubation at 37 ºC for 90 min and terminated by incubation at 85 ºC for 20 min.

RCA amplification and fluorescence measurement

Aliquots of 2 μL product of above ligation reaction was transferred to the 10 μL RCA reaction mixture containing the reaction buffer (80 mM Tris-HCl, pH 7.5, 100 mM KCl, 20 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 8 mM DTT), 2.5 mM of each dNTP, 1.0 μM primer, 0.8 μM RNA FRET probe, 10 U of phi29 DNA polymerase, and 2.5 U of RNase H. The RCA reactions were performed at 37 ºC. The real-time fluorescence intensity was monitored and recorded at intervals of 2 min with a StepOne Real-Time PCR System. For end-point fluorescence measurement, the RCA reactions were performed at 37 ºC for 6 h and then terminated by incubation at 85 ºC for 20 min. Aliquots of 10 μL RCA product was diluted to 110 μL with TAE buffer (pH 8.5). The fluorescence spectra were measured on a LS-55 spectrofluorimeter (PerkinElmer) equipped with a xenon lamp. The excitation wavelength was 490 nm, and the spectra were recorded between 510 and 650 nm.

Human Genomic DNA Sample Analysis

Human genomic DNA was extracted from whole blood of a healthy volunteer in our laboratory using a TIANamp Genomic DNA Kit (TIANGEN Biotechnologies) according to the manufacture's protocol. The extracted genomic DNA was quantified from the absorption at 260 nm with a UV-Vis spectrophotometer (Beijing Purkinje General Instrument Co. Ltd). Prior to use, the genomic DNA was denatured at 95 ºC for 5 min then cooled on ice. The next ligation and amplification operation procedure was the same as the method of LCR coupled RCA described above. The genomic DNA-induced fluorescence signal of RCA reaction was recorded by a StepOne Real-Time PCR System as described above.

Table S1. Oligonucleotide sequences

Name	Sequences (5'-3' direction)
[a] mutDNA	TTTGTGCCTGTCCTGGGAGAGAC**T**GGCGCACAGAGGAAGAGAATCT
[b] wtDNA	TTTGTGCCTGTCCTGGGAGAGAC**C**GGCGCACAGAGGAAGAGAATCT
mutDNA-specific padlock probe	phosphate-GTCTCTCCCAGGACAGGCTTTTGATCACAGTTTACGGTTTAGCATAACTCTACT

	ATCATTTACTTTACGATTTCGGCTCTTCCTCTGTGCGCC**A**
wtDNA-specific padlock probe	phosphate-GTCTCTCCCAGGACAGGCTTTTGATCACAGTTTACGGTTTAGCATAACTCTACT ATCATTTACTTTACGATTTCGGCTCTTCCTCTGTGCGCC**G**
Primer	TAAACCGTAAACTGTGATCA
[c] RNA FRET probe	FAM-rArTrArArCrTrCrTrArCrTrArTrC-BHQ1
probe X	phosphate-GTCTCTCCCAGGACAGGC
probe Y	CTCTTCCTCTGTGCGCC**A**
probe Y-w	CTCTTCCTCTGTGCGCC**G**
probe X'	TTTGTGCCTGTCCTGGGAGAGAC
probe Y'	phosphate-**T**GGCGCACAGAGGAAGAGAATCT
probe Y'-w	phosphate-**C**GGCGCACAGAGGAAGAGAATCT
	[d]ATCTACTGGGACGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGA GAC***C***GGCGCACAGAGGAAGAGAATCTCCGCAAGA
	[e]TCTTGCGGAGATTCTCTTCCTCTGTGCGCC***G***GTCTCTCCCAGGACAGGCACAAA CACGCACCTCAAAGCTGTTCCGTCCCAGTAGAT

[Note] [a,b] The underlined bold letters indicate the base at mutant site in mutDNA and wtDNA, respectively. [c] The letter "r" indicates "ribonucleotide". The PCR-based DNA Sequencing results for exon 8 in p53 gene of human genomic DNA extracted from whole blood are listed as d and e. [d] The sequence of upper DNA strand. The underlined sequence is the same as that of synthetic wtDNA. [e] The sequence of lower DNA strand. The primers for PCR amplification of exon 8 in p53 gene are PU8: 5'-AGG ACC TGA TTT CCT TAC TGC CTC T-3', and PD8: 5'-GTC CTG CTT GCT TAC CTC GCT TAG T-3'. The DNA Sequencing was accomplished by Shanghai Sangon Biotech (Shanghai, China).

2. Influence of the concentration of RNA FRET probe on the RCA-based assay

In this work, we have invented a new RNA FRET probe for detection of RCA products, which can form the mechanism of target-dependent signal amplification with the digestion of RNase H. Thus, the detection sensitivity of the RCA-based assay can be greatly improved. The concentration of the RNA FRET probe used is a key factor to affect the RCA-based

assay. Therefore, the influence of the concentration of the RNA FRET probe ranging from 0.2 μM to 1.8 μM on the RCA-based assay is investigated through detection of blank, mutDNA and wtDNA with the RCA-based assay. As depicted in Fig. S1, all of the fluorescence signals produced by blank, mutDNA and wtDNA are gradually increased with increase of the concentration of RNA FRET probe. When the RNA FRET probe concentration is 0.8 μM, by using mutDNA-specific padlock probe, the fluorescence intensities produced by the blank and wtDNA keep at a low level. Meanwhile, 1 pM mutDNA and 10 pM mutDNA can produce sufficient fluorescence intensities and adequate signal difference. Therefore, 0.8 μM RNA FRET probe is selected for the RCA-based assay.

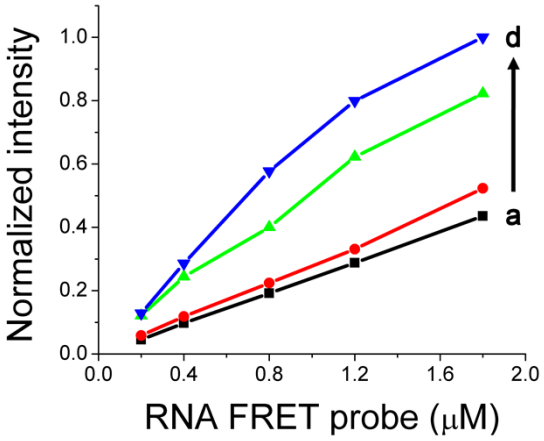

Fig. S1. The influence of the concentration of RNA FRET probe in the range of 0.2~1.8 μM on the RCA-based assay. The fluorescence signals were produced respectively by (a) blank, (b) 10 pM wtDNA (c) 1 pM mutDNA, and (d) 10 pM mutDNA with the RCA-based assay by using mutDNA-specific padlock probe. Except the concentration of RNA FRET probe, other experiment conditions were the same as described in Experimental Section. The maximum fluorescence intensity was normalized to 1.0 in order to compare easily. The fluorescence intensities were monitored with end-point fluorescence measurement manner.

3. Effect of the amount of RNase H on the RCA-based assay

The signal amplification is based on the digestion of RNA FRET probe catalyzed by RNase H. The amount of RNase H is another critical parameter for the RCA-based assay. The effect of the amount of RNase H is studied in the range of 1.0~10.0 U by detection of blank, wtDNA and mutDNA with the RCA-based assay by using mutDNA-specific padlock

probe, and the experimental results are presented in Fig. S2. There is almost no change of the blank signal as increasing the amount of RNase H. The fluorescence intensities produced by 10 pM wtDNA are gradually increased with increase of the amount of RNase H, but keep a low intensity when the amount is less than 5 U. On the other hand, the fluorescence intensities produced by mutDNA rapidly increase when the amount of RNase H is increased from 1.0 U to 2.5 U, and the signal increase become rather slow when the RNase H is increased from 2.5 U to 5 U. Finally, no increase can be observed when the RNase H is greater than 5 U. In order to achieve high sensitivity and selectivity and to save the cost, 2.5 U of RNase H is used for the RCA-based assay.

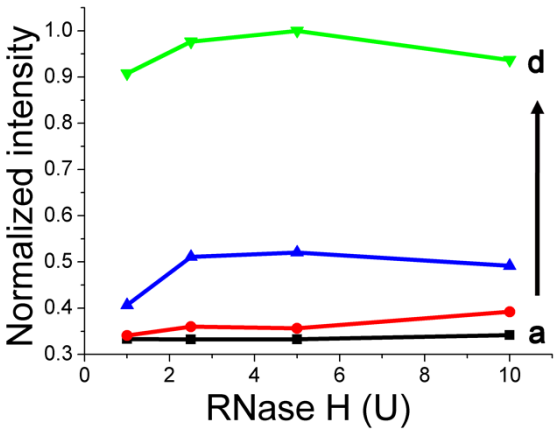

Fig. S2. The effect of the amount of RNase H in the range of 1.0 U~10 U on the RCA-based assay. The fluorescence signals were produced respectively by (a) blank, (b) 10 pM wtDNA (c) 1 pM mutDNA, and (d) 10 pM mutDNA with the RCA-based assay by using mutDNA-specific padlock probe. Except the amount of RNase H, other experiment conditions were the same as described in Experimental Section. The maximum fluorescence intensity was normalized to 1.0 in order to compare easily. The fluorescence intensities were monitored with end-point fluorescence measurement manner.

4. Optimization of the thermal cycle number of LCR for the LCR-coupled RCA assay

The thermal cycle number of the LCR is closely related to the sensitivity and selectivity of the LCR-coupled RCA assay. The optimization of the thermal cycle number of the LCR is investigated through detection of blank and mutDNA with the LCR-coupled RCA assay by using mutDNA-specific LCR probes and padlock probe. As shown in Fig. S3, when the

thermal cycle number of LCR is increased from 5 to 15, the fluorescence intensities produced by mutDNA and the difference among blank, 10 aM, 1 fM and 10 fM mutDNA are increased with the thermal cycle number, and reach their maximum when 15 cycles is used. Meanwhile, the blank does not increase obviously with increasing the thermal cycle number. These results indicate that the detection sensitivity of the LCR-coupled RCA assay can be improved but the nonspecific amplification of LCR can be kept at a low level with increasing the thermal cycle number. However, when the thermal cycle number is increased to 20 (Fig. S3(4)), the blank rapidly increases and the difference between the blank and the fluorescence produced by mutDNA is reduced compare to the results shown in Fig. S3(3) by using 15 cycles, indicating that the nonspecific amplification of LCR significantly increases and the detection sensitivity decreases. Therefore, 15 thermal cycles for LCR is considered to be the optimal for the LCR-coupled RCA assay.

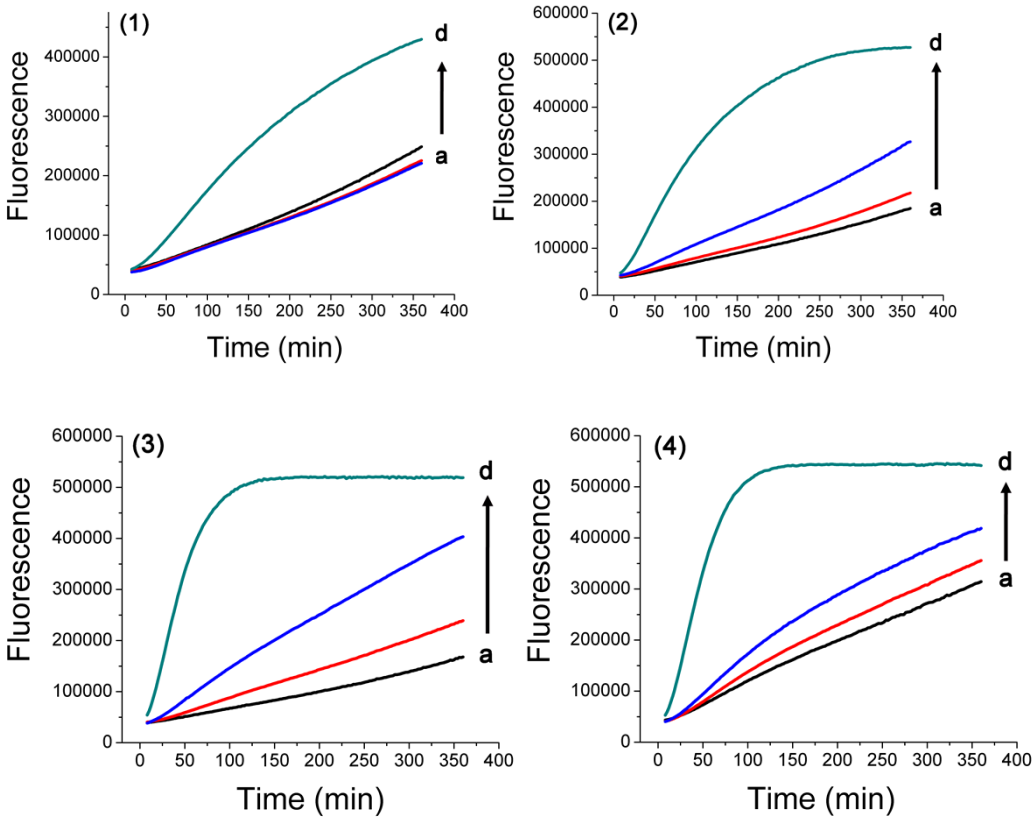

Fig. S3. The real-time fluorescence curves with the LCR-coupled RCA assay by using mutDNA-specific LCR probes and padlock probe. The thermal cycle number of LCR was (1) 5, (2) 10, (3) 15, and (4) 20, respectively. The real-time fluorescence curves were

respectively produced by (a) blank, (b) 10 aM mutDNA, (c) 1 fM mutDNA, and (d) 10 fM mutDNA. Other experiment conditions were the same as described in Experimental Section.

5. Optimization of the thermal cycle number of padlock probe ligation for the LCR-coupled RCA assay

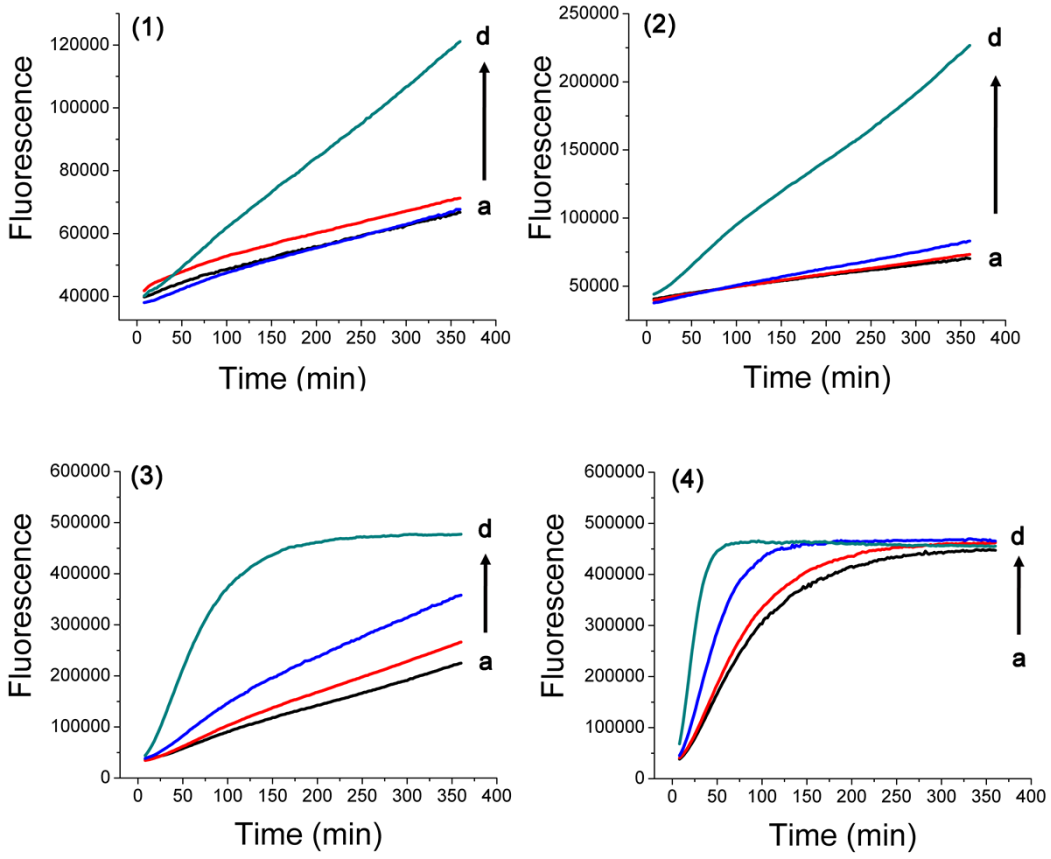

Fig. S4. The real-time fluorescence curves with the LCR-coupled RCA assay by using mutDNA-specific LCR probes and padlock probe. The thermal cycle number of padlock probe ligation is (1) 5, (2) 10, (3) 15, and (4) 20, respectively. The real-time fluorescence curves were respectively produced by (a) blank, (b) 10 aM mutDNA, (c) 1 fM mutDNA, and (d) 10 fM mutDNA. Other experiment conditions were the same as described in Experiment Section.

Similar to the thermal cycle number of LCR, the thermal cycle number of padlock probe ligation is another critical parameter for the LCR-coupled RCA assay. We also carry out the optimization studies of the thermal cycle number of padlock probe ligation through detection

of blank and mutDNA by using mutDNA-specific LCR probes and padlock probe with the LCR-coupled RCA assay. As depicted in Fig. S4, when 5 thermal cycles are used, the fluorescence signals produced by 10 aM mutDNA and 1 fM mutDNA cannot be separated from the blank. When the thermal cycle number is increased to 10, the mutDNA can be detected only when its concentration is greater than 1 fM. When the padlock probe ligation is performed with 15 thermal cycles, as low as 10 aM mutDNA can be well detected and the difference between blank and the fluorescence signals produced by mutDNA reaches the maximum. However, when 20 thermal cycles are used for the padlock probe ligation (Fig. S4(4)), the blank can be rapidly increased and the difference between blank and the fluorescence signals produced by mutDNA is significantly reduced, indicating great increase of the nonspecific amplification. Therefore, 15 is selected as the optimized thermal cycle number for padlock probe ligation.

6. The real-time fluorescence curves of LCR-coupled RCA assay for the detection of genomic DNA

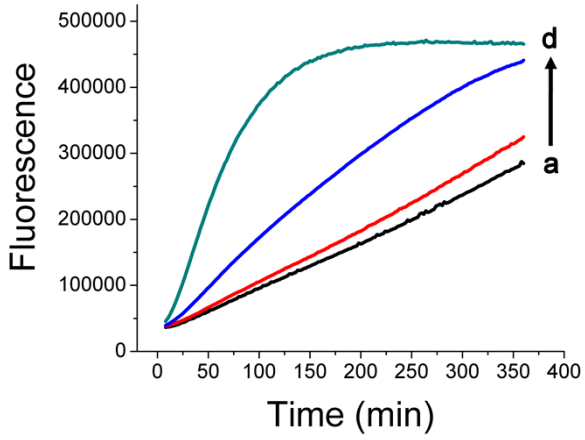

Fig. S5. Real-time fluorescence curves with the LCR-coupled RCA assay by using wtDNA-specific LCR probes and padlock probe produced by (a) blank, (b) 100 pg genomic DNA, (c) 1 ng genomic DNA, and (d) 10 ng genomic DNA.