Electronic Supplementary Information

Precise quantitation and sensitive detection of copy number within genetic variations using ligation-mediated droplet digital PCR in plasma

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1. The sequences of the nucleic acids used in this work.

Table S1. Sequences information for the DNA oligonucleotides and TaqMan probes of the ligation-ddPCR assay.

Name	Sequence（5'-3'）
Target DNA sequence: (MRGPRX1: Sequence ID: NC 000010.11)	CCTGCAAAGTAGGAAAACATCATCACAGGATAGAGGATTTTAGAGATGGTATGGG GGATACTGATGAAGCTTAA
Probe A	PO_4-CCTCTATCCTGTGATGATGTTTTCCCACACTCTATGCTTGCTACCGTCG
Probe B	GGGATACTGGAACCTGATGATGACGTATCCCCCATACCATCTCTAAAAT
TaqMan probe-FAM	6FAM-TCTCTAAAATCCTCTATCCT–MGBNFQ
Reference DNA sequence: (RPP30: Sequence ID: NC 000011.10)	CGGTGTTTGCAGATTTGGACCTGCGAGCGGGTTCTGACCTGAAGGCTCTGCGCGG ACTTGTGGAGACAGCCGC
Probe A-re	PO_4-GAACCCGCTCGCAGGTCCAACACACTCTATGCTTGCTACCGTCG
Probe B-re	GGGATACTGGAACCTGATGATGACCGCGCAGAGCCTTCAGGTCA
TaqMan probe-VIC	VIC-TCAGGTCAGAACCCGC–MGBNFQ
Universal forward primer	CGACGGTAGCAAGCATAGAGTGTG
Universal reverse primer	GGGATACTGGAACCTGATGATGAC
The letter "re" indicated probes are related with "reference gene"	

Table S2. Sequences information for MSPA assay.

Gene	Method	Name	Sequence（5'-3'）
MRGPRX1	MSPA assay	Forward primer-MR	TTAAGCTTCATCAGTATCCCCCA
		Reverse primer-MR	CAAAGTAGGAAAACATCATCACAGGA
		TaqMan probe-MR	6FAM-ACCATCTCTAAAATCCT-MGBNFQ
RPP30	MSPA assay	Forward primer-RP	GATTTGGACCTGCGAGCG
		Reverse primer-RP	GCGGCTGTCTCCACAAGT
		TaqMan probe-RP	VIC-CTGACCTGAAGGCTCT-MGBNFQ
The letter “MSPA assay” indicated “multiplex specific primers amplification” method.			

2. Detailed information for the ddPCR-based assay according to dMIQE guidelines.

Table S3. Detailed information for the ddPCR-based assay according to guidelines of the dMIQE checklist.

ITEM TO CHECK	IMPORTANCE	CHECK-LIST	COMMENT
EXPERIMENTAL DESIGN			
Definition of experimental and control groups	E	+	With/without synthetic target DNA; with/without plasma dilution
Number within each group	E	+	3 replicates per group
Assay carried out by core lab or investigator's lab?	D	+	Investigator’s lab
SAMPLE			
Sample information	E	+	Target DNA: MRGPRX1 (Sequence ID: NC 000010.11) Reference DNA: RPP30 (Sequence ID: NC 000011.10)

Volume/mass of sample processed	E	+	Different concentrations of DNA, or 2 µL of circulating-free plasma (see Experimental Section and Supporting Information)
If frozen - how and how quickly?	E	+	DNA stored at -20 °C Plasma stored at -80 °C
Sample storage conditions and duration	E	+	Synthetic DNA: -20 °C, RNase free water, 1 year; Extracted genomic DNA: -20 °C, RNase free water, 1 year; Plasma: -80 °C, 1 year.
NUCLEIC ACID EXTRACTION			
Template structural information	E	+	See table S1
Quantification-instrument/method	E	+	NanoDrop, spectrometrically
Manufacturer of reagents used and catalogue number	E	+	CEM, K562, BALL-1 and Leukocyte: Genomic DNA extracted by TIANamp Genomic DNA kit (TIANGEN BIOTECH, cat: DP304)
ddPCR TARGET INFORMATION			
Sequence accession number	E	+	The sequence of the ligated probes is artificially and specifically designed according to the locus-specific of target DNA
Amplicon length	E	+	Target DNA-templated: 98 bp
Other homologs?	D	+	No homologs
Sequence alignment	D	+	PrimerBLAST
Secondary structure analysis of amplicon and GC content	E	+	Only probes, primers and TaqMan probes
ddPCR OLIGONUCLEOTIDES			
Primer sequences	E	+	see Table S1 and Table S2
Probe sequences	D	+	see Table S1 and Table S2
Location and identity of any modifications	E	+	TaqMan probe-FAM: 5'-6FAM fluorophore; 3'-MGBNFQ, TaqMan probe-VIC: 5'-VIC fluorophore; 3'-MGBNFQ, Probe A/A-re/A-CC/A-BR: 5'-phosphate group
Manufacturer of oligonucleotides	D	+	TaqMan probe: Applied Biosystems; DNA and phosphorylation-modified probes and other oligonucleotides: Takara Biotechnology Co., Ltd.
Purification method	D	+	PAGE
ddPCR PROTOCOL			
Reaction volume and amount of DNA	E	+	20 µL, containing 2 µL ligation products and 18 µL PCR reaction mixture
Primer, TaqMan probe, Mg^{2+} and dNTP concentrations	E	+	Primer: 500 nM; TaqMan Probes: 250 nM; Mg^{2+} and dNTP: proprietary (Bio-Rad)
Polymerase identity and concentration	E	-	proprietary (Bio-Rad)
Buffer/kit catalogue number and manufacturer	E	+	2x ddPCR Supermix for Probes (no dUTP), catalog: 186-3010 (Bio-Rad)
Exact chemical constitution of the buffer	D	-	proprietary (Bio-Rad)
Plates/tubes manufacturer and catalog number	D	+	PCR: twin. tec 96 well plates, catalog: 0030 128. 605 (Eppendorf)
Complete thermocycling parameters	E	+	see Experimental Section in the manuscript
Reaction setup, gravimetric or volumetric dilutions (manual/robotic)	D	+	Manual volumetric dilutions
Total PCR reaction volume prepared	D	+	20 µL
Partition number, individual partition volume	E	+	~20,000 droplets, ~0.95 nL

Total volume of the partitions measured (effective reaction size)	E	+	~14,500 droplets × 0.95 nL = 13.8 μL
Comprehensive details and appropriate use of controls	E	+	Controls: without adding synthetic DNA, extracted genomic DNA or plasma
Manufacturer of dPCR instrument	E	+	Bio-Rad (QX200)
ddPCR VALIDATION			
Optimization data for the assay	E	+	Ligation time, ligation temperature, annealing-extended temperature and thermal cycle number
Comparison validation	E	+	Data validation of sensitivity and small concentration variation by ddPCR and qPCR, respectively. Meanwhile，comparison between the proposed assay and MSPA assay
DATA ANALYSIS			
dPCR analysis program (source, version)	E	+	Quantasoft Version: 1.7.4.0917 (Bio-Rad).
Results of no-template controls	E	+	Blank assay of every data
Where appropriate, justification of number and choice of reference genes	E	+	Reference genes previously optimized with target DNA
Repeatability (intraassay variation)	E	+	3 independent repetitions for each sample
Experimental variance or CI	E	+	error bar provided for each data point
Statistical methods used for analysis	E	+	Mean and relative standard deviation, arithmetic statistical method of the proprietary Quantasoft analysis software

E, Essential information; D, Desirable information; +/-, applied/not applied.

3. Optimization of ligation reaction temperature.

Ligation reaction is important step for the proposed assay, two pairs of probes (A/B and A-re/B-re are related with MRGPRX1 and RPP30, respectively) are specifically hybridized with locus-specific of the both genes, and then probes are effectively ligated with each other. The difference of specific hybridization region between ligation probes and corresponding genes would affect hybridization stability at the same temperature, because the difference of oligonucleotides sequence caused the variance of melting temperature at complementary sites. The difference of efficiency for specific hybridization further influenced ligation reaction efficiency, as well as sensitivity and precision for copy number detection.

In order to evaluate the effect of the ligation temperature in the ligation reaction, MRGPRX1 and RPP30 as a proof-of-concept genes are introduced (the detailed information of is listed on Table S1). They are diluted into the same concentration gradient, and then added into same ligation reaction mixture to assess consistency of ligation efficiency in double assays. The ligation reaction and subsequent ddPCR process are performed following the foregoing protocol in the Experiment Section only by varying the ligation reaction temperatures. As shown in Fig. S1a, S1b and S1e, when 63 ^{o}C and 60 ^{o}C are respectively selected as ligation reaction temperatures, the positive droplet number which target DNA as template-ligated is less than that of the reference DNA, because higher ligation temperatures went against hybridization between target DNA and target-specific sequences of probes. Nevertheless, as depicted in Fig. S1d and S1e, target and reference DNA as template-ligated produced fewer positive droplets number when 55 ^{o}C is selected, since lower temperature is bad for catalyzing of Amp-Ligase. Notably, as shown in Fig. S1c and S1e, the number of

both positive droplet is closed, the used of 58 °C ligation temperature can observably balance hybridization stability between multiplex specific probes and corresponding target/reference DNA, as well as 58 °C is appropriate catalytic temperature for Amp-Ligase. Hence, 58 °C is used to be the optimum for the assay.

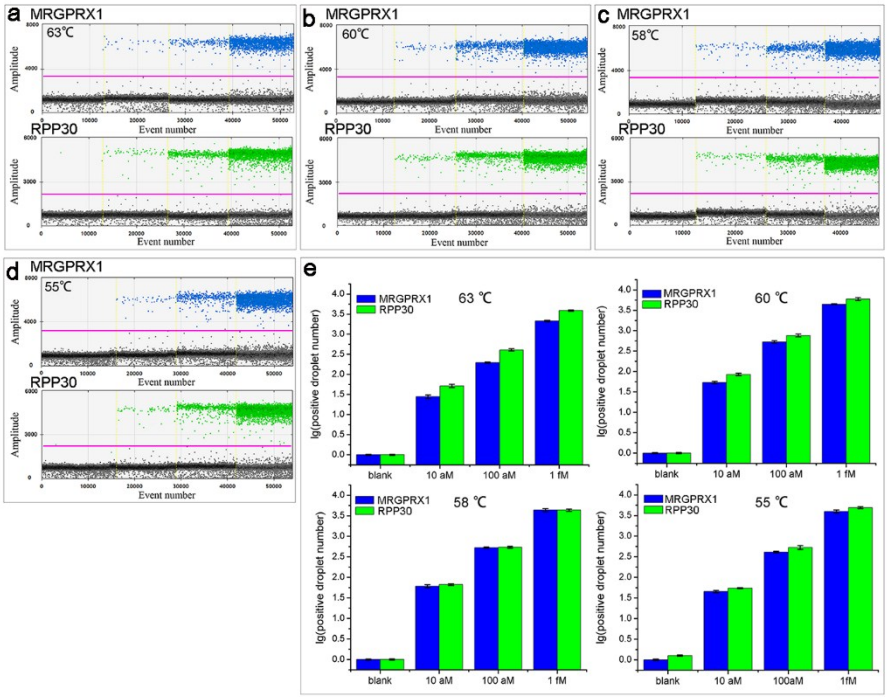

Fig. S1 The positive droplets produced by MRGPRX1 and RPP30 are displayed at different ligation reaction temperatures of a) 63 °C, b) 60 °C, c) 58 °C and d) 55 °C. e) The number of positive droplets with different ligation reaction temperature is recorded, and the histograms represents the relationship between lg of positive droplet number and different sample concentrations. Each ddPCR assay with same concentration gradient of target and reference DNA. From left to right in the each image, the concentration of target and reference DNA is 0, 10 aM, 100 aM, 1 fM, respectively. Blank represents without DNA.

4. Optimization of the ligation reaction time.

To find the optimal ligation reaction efficiency, our proposed assay has optimize the ligation reaction time by explored the relationship of ligation reaction time and positive droplet number. 100 aM target DNA as ligation template is used to complete the ligation reaction under different ligation time (from 0 to 35 min). The detailed information of ligation reaction and ddPCR is described in the Experimental Section except the different ligation reaction time. As shown in Fig. S2a and S2c, the number of positive droplet by 100 aM target DNA increased rapidly with the increase of ligation time from 0 to 25 min. After 25 min, the positive droplet number has not obviously changed. Meanwhile, the Fig. S2b shown that the positive droplet number of blank control (without target DNA) is nonexistent with increase of ligation reaction time, the result demonstrated nonspecific ligation and amplification are kept a low level in this work.

According to the above consequence, 30 min is enough to complete the ligation reaction. Hence, 30 min is applied to ligation reaction as the optimal ligation reaction time.

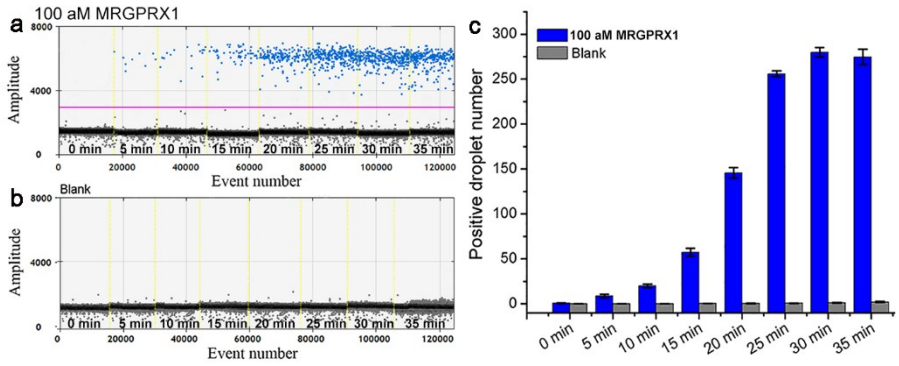

Fig. S2 The effect of ligation reaction time for proposed assay. a) The positive droplet produced by 100 aM target DNA with different time in ligation reaction. b) Different blank control assays with different ligation reaction time. c) The histogram of relationship between different ligation reaction time and each corresponding positive droplet number. The ligation reaction time from left to right is 0, 5 min, 10 min, 15 min, 20 min, 25 min, 30 min and 35 min, respectively.

5. Optimization of the thermal cycle number in the ddPCR process.

The thermal cycle number is a key parameter for ddPCR assay, because the ddPCR-based assay is depended on end-point detection. The unsuitable thermal cycle number can affect the sensitive for target DNA. So, we have optimized the number of thermal cycle to choose optimal conditions. As shown in Fig. S3a, when 30 thermal cycles are applied for ddPCR assay, the distinction of fluorescence amplitude of positive droplets is closed to that of negative droplets, which should be possibly attributed to inadequate PCR amplification at low thermal cycles. When the number of thermal cycle increased from 35 to 40, the distinction of fluorescence amplitude of positive and negative droplets become more and more obvious due to high PCR efficiency (Fig. S3b and S3c). However, as shown in Fig. S3d, the thermal cycle number is raised to 45, the result has not changed dramatically comparing with previous phenomenon. So, 40 thermal cycles are chosen in this work.

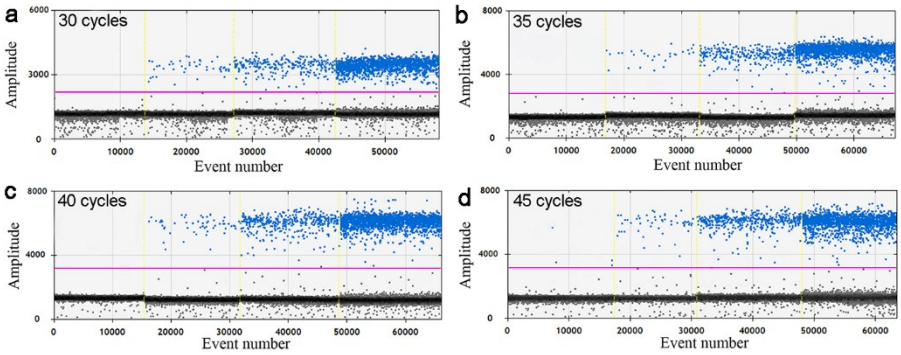

Fig. S3 The positive droplets produced by MRGPRX1 with different concentration are shown in each image with thermal cycles of a) 30, b) 35, c) 40 and d) 45, respectively. The concentration from left to right is 0 aM, 10 aM, 100 aM and 1 fM.

6. Optimization of the annealing-extension temperature.

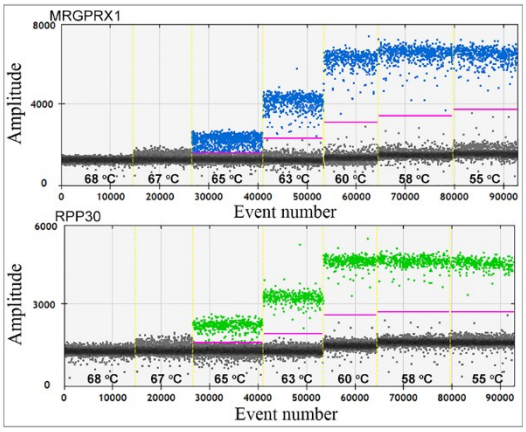

Fig. S4 The positive droplets produces by 100 aM MRGPRX1 and RPP30 gene is displayed at different annealing-extension temperature containing 68 °C, 67 °C, 65 °C, 63 °C, 60 °C, 58 °C and 55 °C, respectively.

The annealing-extension temperature can affect the catalytic activity of DNA polymerase, as well as hybridization stability between TaqMan probes (FAM-modified and VIC-modified) and corresponding products-amplified because of different melting temperature. Catalytic activity and hybridization stability are important factors for the amplification efficiency of ddPCR in the dispersive droplets, and further influence the distinction of fluorescence amplitude between positive and negative droplets. Considering with the difference of hybridization stability between TaqMan probes and corresponding target/reference DNA at same annealing-extension temperature maybe cause different result, 100 aM of the MRGPRX1 gene and 100 aM RPP30 gene are detected simultaneously for investigation of optimal annealing-extension temperature, furthermore, ligation reaction and ddPCR are performed following the Experiment Section except for the variations of annealing-extension temperature ranging from 68-55 °C. As shown in Fig. S4, there is not apparent separation between the positive droplets and negative droplets from 68-65 °C due to low catalytic activity of DNA polymerase and hybridization stability, it cannot achieves sensitive and precise detection for MRGPRX1 and RPP30 gene. The catalytic activity and hybridization stability increase with the decrease of annealing-extension temperature. When the annealing-extension temperature decrease from 63-55 °C, the amplitude of fluorescence signals which spring from the both genes are raised at the same time. Specifically, 60 °C is selected as annealing-extension temperature, the result shows the optimal distinction of fluorescence amplitude between positive and negative droplets, moreover, the amplitude of negative droplet kept a low level compared with others which came from lower temperature (58 °C and 55 °C). According to the consequence displayed in Fig. S4, 60 °C is chosen as optimal experiment condition, which may lead to higher sensitivity and precision for ligation-based ddPCR CNV assay.

7. The comparison of different analytical methods.

Table S4. The comparison of the proposed assay with representative methods for CNV analysis.

Detection strategy	Detection method	Detection limit（copy number）	Reference
DNA microarrays for comparative genomic hybridization （CGH） based on degenerate oligonucleotide-primers PCR	Array-based CGH	2.6×10^3	Ref. S2

Multiplex ligation-based probe amplification (MLPA) based on fluorescence quantitative PCR	qPCR	590	Ref. S3
Methylation-specific MLPA	qPCR	590	Ref. S4
Multiplex analysis of copy number variation with next-generation sequence (NGS)	NGS	148	Ref. S5
Amplicon-based next-generation sequence	NGS	590	Ref. S6
Analysis of copy number using high-density SNP array	SNP-array	7.2×10^3	Ref. S7
This work	ddPCR	~7	

8. Comparison of the ligation-based ddPCR assay with the real-time quantitative PCR.

For the detection sensitivity, MRGPRX1 gene as the template-ligated is adopted in this study. When the ligation reaction is completed according to standard procedure in the Experimental Section, the ligation products carry out the following real-time quantitative PCR with a final volume of 20 μL mainly included 500 nM primers, 250 nM TaqMan probe-FAM, 2 μL ligation products, 1 U Taq Hot Start DNA Polymerase, 10 μM dNTPs and reaction buffer (20 mM Tris-HCl, pH 8.4, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$ and 20 mM KCl). The PCR is performed with a TL988 real-time fluorescence quantitative PCR system by incubating at 94 °C for 5 min followed by 50 cycles of 94 °C for 30 s, 60 °C for 40 s.

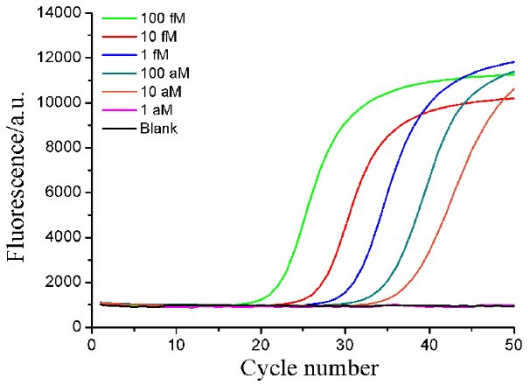

Fig. S5 The fluorescence quantitative curves produced by the real-time fluorescence quantitative PCR with the different target DNA concentrations.

As shown in Fig. 2, the positive droplets of 1 aM target DNA can be obviously distinguished from the large number of negative droplets. However, while one can see from Fig. S5, the fluorescence signal produced by 1 aM target DNA is almost the same as the blank control and without exponential amplification, just 10 aM target DNA can be detected. So, the result obviously demonstrates that the Ligation-ddPCR assay for target DNA is 10-flod higher detection sensitivity than that of the conventional real-time quantitative PCR assay.

Furthermore, a reliable and ideal copy number detection assay needs a high precision, which should enable the accurate and precise detection of target gene even with slight copy number changes. Both Ligation-ddPCR assay and real-time fluorescence quantitative PCR assay for MRGPRX1 detection with slight concentration change are compared in this study. As demonstrated in Fig. S6a and b, the twice number of positive droplet is gradually increased with the

increase of two-fold target DNA concentration (from 10 aM to 640 aM). Meanwhile, an excellent linear relationship is established between the lg of target DNA concentrations (lg C_{DNA}/aM, aM = M$\times 10^{-18}$) and the lg of corresponding positive droplet number, and the correlation coefficient is 0.9999. In Fig. S6b, the relative standard deviation (RSD) values of each data point from three parallel testing represents by error bars, and all of error bars are less than 1.32 %.

By contrast, the Fig. S6c and d display the detection results of the same target DNA sample by the real-time fluorescence quantitative PCR method. The result demonstrates that the fluorescence curves produced by target DNA sample with two-fold concentration variation is close with each other, so it is difficult to distinguish the threshold cycle of each fluorescence curve. Therefore, within the not so well linear relationship (r = 0.9698) between the lg of the target DNA concentration and the threshold cycle (Ct), the much larger error bars in Figure S6d demonstrates that the ability of precise quantitation with slight concentration variation by RT-qPCR is weaker than that of ddPCR assay.

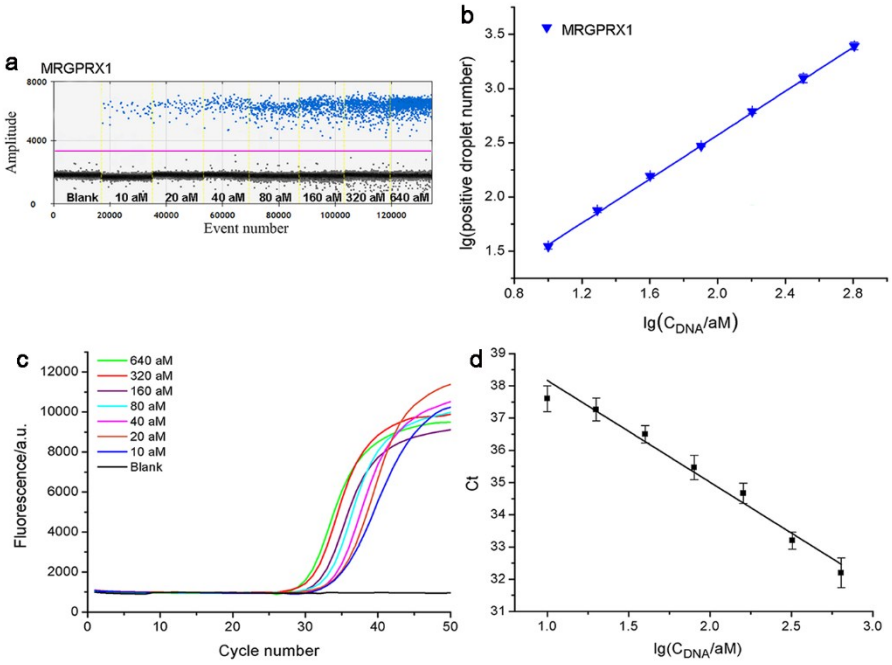

Fig. S6 Detection of the copy number of MRGPRX1 with small concentration variations by dd-PCR and real-time fluorescence quantitative PCR assay, respectively. a) The concentration left to right is 0, 10 aM, 20 aM, 40 aM, 80 aM, 160 aM, 320 aM and 640 aM, respectively. b) The plots represent the linear relationship between lg of target DNA concentration and lg of the positive droplet number. c) The fluorescence quantitative curves produced by quantitative PCR with same concentration gradient. d) The linear relationship between Ct values and lg of target DNA concentration. Error bars of every point represent the relative standard deviation from three parallel testing.

All the above results reliably illustrate that the proposed ligation-based ddPCR assay is more sensitive and precision than conventional fluorescence quantitative PCR. It is more applicable for the precise detection of copy number with slight copy number change in biological samples.

9. Validation of the reliability of the Ligation-ddPCR assay.

For validation of reliability, human brain genomic DNA (New England Biolabs, USA) as standard biological sample is tested respectively by proposed assay and standard multiplex specific primers amplification (MSRA) method.

In the MSPA assay, genomic DNA is digested with 2 units of Rsal restriction enzyme and 1 μL of 10X CutSmart buffer in 10 μL at 37 °C for 60 min. And then 14.3 ng and 143 ng of products-digested are respectively assayed in 20 μL ddPCR mixture containing corresponding specific primers and TaqMan probes of MRGPRX1 and RPP30 (Table S2). The thermal cycling process is same as the proposed assay in the ddPCR reaction.

On the other hand, the ligation-based ddPCR assay is performed according to Experimental Section. Human brain genomic DNA with different concentrations are directly added into each ligation reaction mixture. Finally, 14.3 ng and 143 ng of human brain genomic DNA can be detected by ddPCR assay.

By comparing of both detection methods, as can be seen from Fig. S7a that the positive droplets from Ligation-ddPCR and MSPA assay can be clearly distinguished from blank control. As can be seen from Fig. S7b, the relationship between lg of positive droplet number (left y-axis) and different sample concentration of the both methods shown that the number of positive droplets from MRGPRX1 or RPP30 exhibits consistent results under the same sample concentration (14.3 ng or 143 ng). Meanwhile, the uniform copy number of MRGPRX1 (3 copies per genomic DNA) is obtained regardless of the sample concentration (right y-axis). The results validate that the copy number can be reliably detected with the proposed assay.

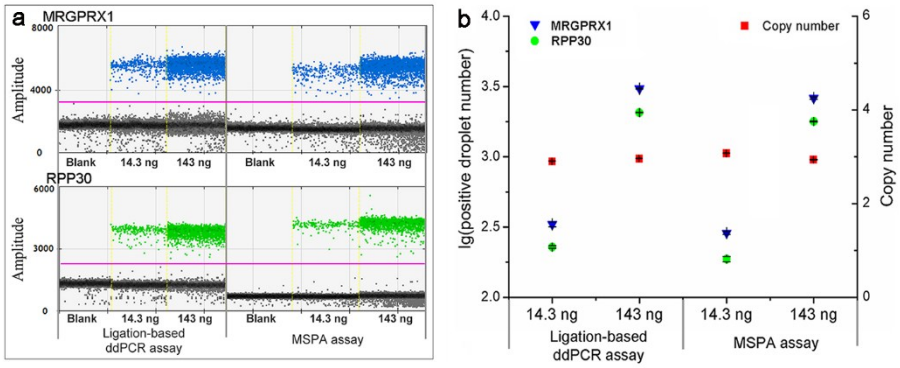

Fig. S7 Validation reliability of the proposed assay. a) The positive droplets produced by MRGPRX1 and RPP30 from human brain genomic DNA with different concentrations. b) The graph shows the relationships between different concentrations (x-axis) and lg of positive droplet number (left y-axis), as well as the copy number of MRGPRX1 (right y-axis). Both of the assays are respectively carried out with the same concentration of human brain genomic DNA, but the blank control had not any DNA.

10. The copy number detection in various cells.

Since the Ligation-ddPCR assay shows high sensitivity, high precision and reliability, we further use the method to detect copy number of MRGPRX1 within the genomic DNA extracted respectively from CEM, K562, BALL-1 and leukocyte of leukemia patient. The details of cells culture and genomic DNA extraction according to Experiment Section in the Manuscript. 2 μL of genomic DNA is added into ligation reaction as template of ligation reaction. As shown in Fig. S8, all of the cells extract is detected by three replicate tests, both of the positive droplets can effectively distinguish with corresponding negative droplets, in addition, the blank control keep extremely low level.

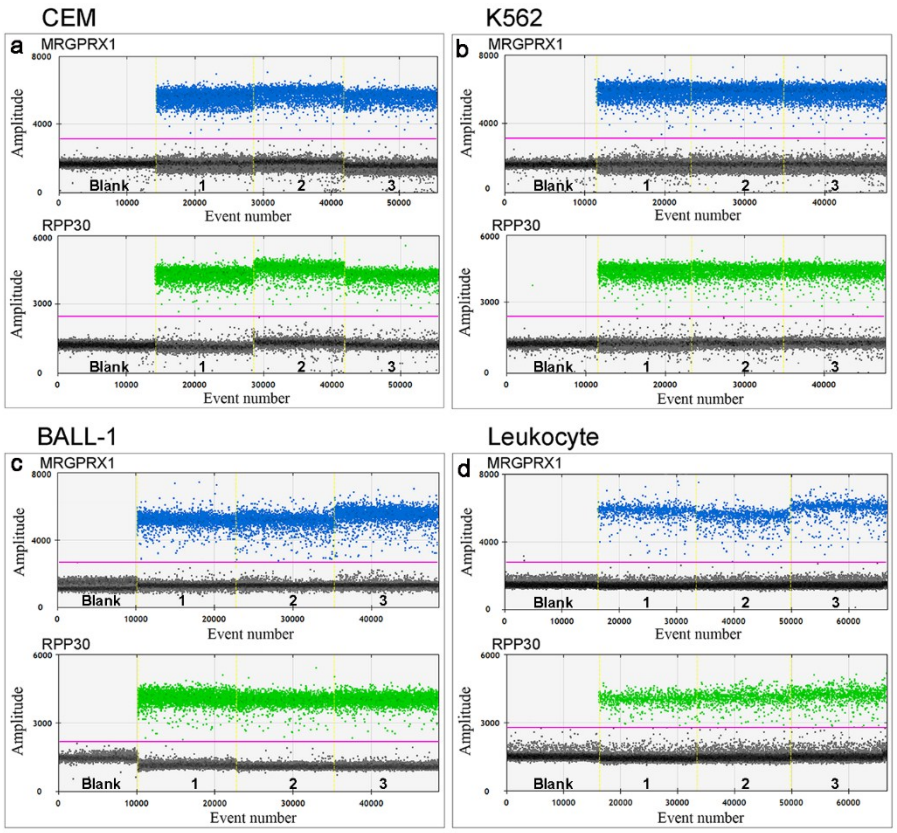

Fig. S8 The copy number detection of MRGPRX1 in cells. From a) to d), both of the positive droplets produced by MRGPRX1 and RPP30. The target genes with final concentration of 164 ng, 172 ng, 131 ng and 83 ng are detected from the genomic DNA of CEM, K562, BALL-1 and leukocyte cells, respectively. Blank without any genomic DNA, 1-3 are three replicate trials.

11. The detection of copy number of MRGPRX1 in plasmas.

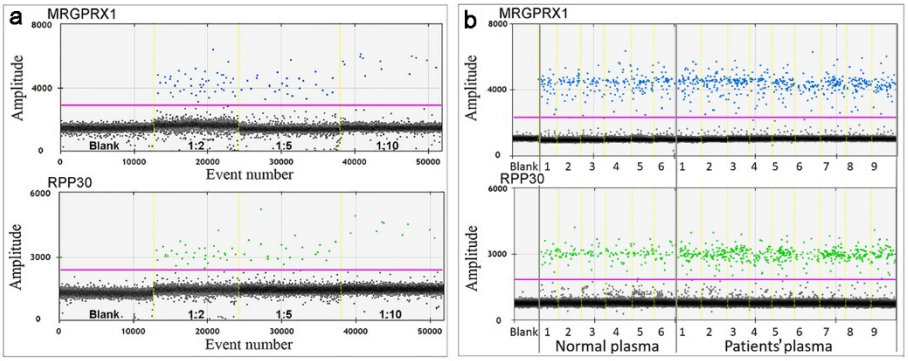

Fig. S9 The detection of copy number of MRGPRX1 in plasmas. a) The detection of copy number of MRGPRX1 in plasmas from normal human. The dilution ratio of the plasma is 1:2, 1:5 and 1:10, respectively. b) The comparison of the copy number of MRGPRX1 in the plasmas of six healthy volunteers and nine leukemia patients, 1:5 of the dilution is used in this test. Each sample is tested 3 times, one of which is shown in Fig. S9b.

12. The comparison of copy number of MRGPRX1 for the detection of cells and plasmas.

Table S5. The copy number change of MRGPRX1 from different cells and plasmas.

Detection gene	Sample	Name	Detection method	Copy number
Target DNA (MRGPRX1: Sequence ID: NC 000010.11) Reference DNA (RPP30: Sequence ID: NC 000011.10)	Cell	CEM	Genomic DNA is extracted and detected by ddPCR	2
		K562		4
		BALL-1		2
		Leukocyte		2
	Standard genomic DNA sample	human brain genomic DNA	Genomic DNA is detected by ddPCR	3
	Plasma	Normal human plasma	Plasma is simply treated and detected by ddPCR	3
		Plasma from patient with acute B-lymphatic leukemia		2

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