Supplementary Information

Development of a novel MSRE-dPCR assay for non-invasive prenatal testing of Trisomy 21

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Figure S1

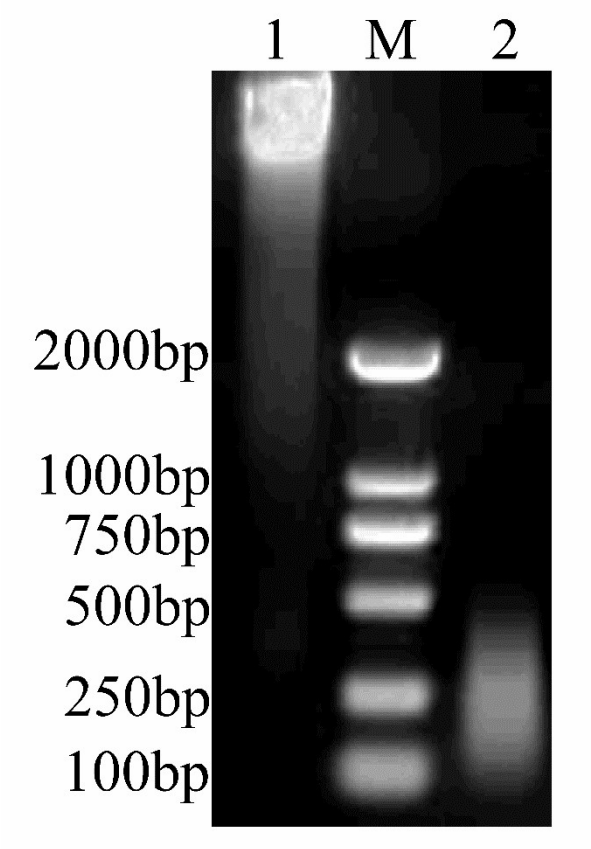

Figure S1 DNA fragments obtained by ultrasonic fragmentation methods. Lane 1: Original genomic DNA whose length was mostly above 2000 bp; Lane 2: DNA fragments whose length were generally around 250 bp; Lane M: DNA Marker.

Figure S2

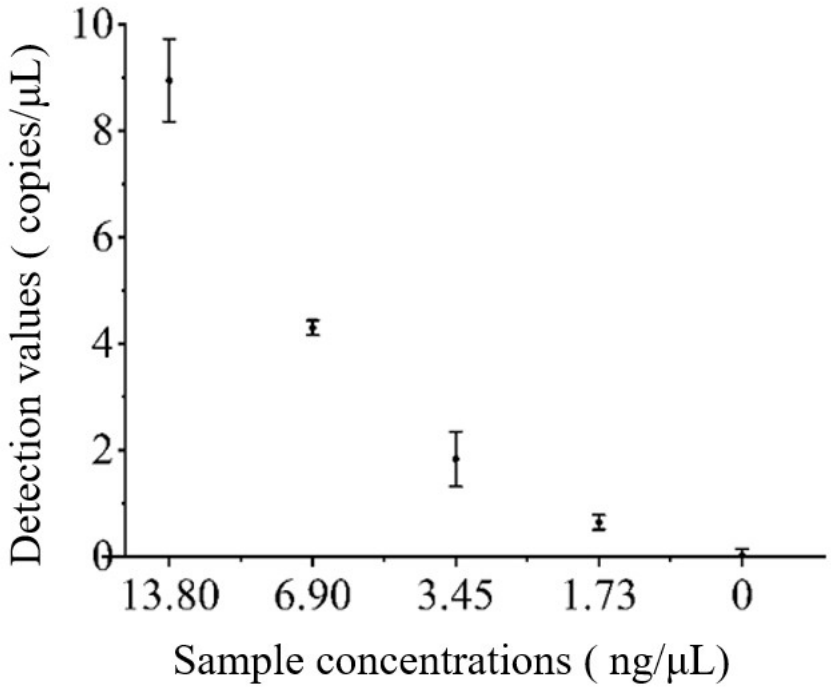

Figure S2 dPCR analysis of simulated samples with gradient concentrations, using AATBC as an example.

Figure S3

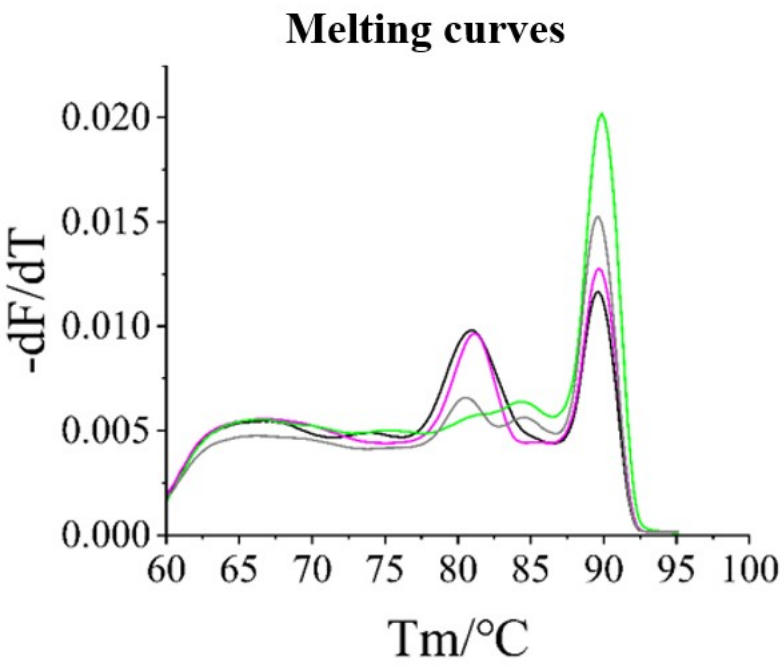

Figure S3 Melting curves for the amplification of RIPK4. Multiple peaks on a melting curve indicated the occurrence of non-specific amplification.

Table S1 Clinical samples used for the establishment of methods

No.	Age	Normal or T21	Pregnancy weeks	Sample type
HF1	34	Normal	20.2	Amniotic fluid
HF2	20	Normal	19.5	
HF3	25	Normal	20.0	
HF4	25	Normal	18.6	
HF5	30	Normal	19.2	
TF1	36	T21	19.5	
TF2	34	T21	20.4	
TF3	27	T21	20.0	
TF4	28	T21	19.1	
TF5	43	T21	18.4	
TF6	30	T21	21.1	
M1	36	Normal	14.6	Peripheral blood
M2	34	Normal	18.4	
M3	23	T21	16.6	
M4	28	T21	17.0	
M5	29	Normal	15.0	
M6	27	Normal	17.0	
M7	27	Normal	17.5	
M8	33	Normal	17.1	
M9	30	Normal	17.6	
M10	31	Normal	18.1	

Table S2 Samples preparation for each stage

Stages	Sample Types	Composition / Description
Initial screening of DMRs [a]	Normal	Mixture of genomic DNA from M1, M2, M5 (1:1:1)
	T21	Mixture of genomic DNA from TF1, TF2, TF3 (1:1:1)
Further Screening of DMRs [b]	Normal	Fragmented genomic DNA from M4, M5, and M6, respectively
	T21	Fragmented genomic DNA from TF4, TF5, and TF6, respectively
Establishment of MSRE-dPCR techniques [c]	Normal	Mixing maternal DNA (from samples M5 to M10) and fetal DNA (from samples HF2 to HF5) at a 9.09% mass ratio (fetal/total, m/m) to prepare 12 samples: M5+HF2 M6+HF2 M8+HF2 M9+HF2 M10+HF2 M5+HF4 M5+HF3 M6+HF3 M8+HF3 M9+HF3 M10+HF3 M5+HF5
	T21	Mixing maternal DNA (from samples M5 to M10) and fetal DNA (from samples TF2 to TF5) at a 9.09% mass ratio (fetal/total, m/m) to prepare 12 samples: M5+TF5 M6+TF5 M8+TF5 M9+TF5 M10+TF5 M5+TF4 M5+TF3 M6+TF3 M8+TF3 M9+TF3 M10+TF3 M5+TF2
Detection limit analysis [d]	Normal (pooled)	Mixture of Fragmented genomic DNA from HF1 and M5 at ratios of 2.44, 4.76, 9.09, and 16.7% [HF1/(M5+HF1), m/m]
	T21 (pooled)	Mixture of Fragmented genomic DNA from M5 and TF1 at ratios of 2.44, 4.76, 9.09, and 16.7% [TF1/(M5+TF1), m/m]

[a]Initial screening was performed using intact, high-molecular-weight genomic DNA

[b]Further screening used sonicated DNA (~250 bp) to better approximate fragment size considerations.

[c]Simulated samples were prepared by mixing sheared maternal (M) and fetal (HF/TF) DNA at the indicated mass ratios to mimic a specific fetal DNA fraction (FF).

[d] Detection limit analysis used simulated samples with varying FF to determine the minimum detectable fraction

All the DNA was extracted from blood cells or amniotic fluid cells *via* a Dzup Genomic DNA Isolation Reagent Kit

Table S3 Primers used for the initial screening of potential DMRs.

Site ID	Forward primer(5'->3'）	Reverse primer (5'->3'）
S1	AGCTGGCACCCGCTGG	GTGTGGGGTTGCACGCG
S2	**GCGCCTAGCACAGACTCTTC**	**TCAAATACCCGCACAGTTCA**
S3	**ACATTTTCCTCTCGCTCTGC**	**CACTCAGGGTCCTCAGTTCC**
S4	**AGACCTCCGGTTTCTGGTTT**	**TTCCAGAACTTCCCAGCAAC**
S5	**ACACTTCCTCTGCACCTGCT**	**GGATCCGGGCTATACTCACA**
S6	ACTCAGTGGTGTCGCCTCG	CGTCCCCAACCATCCAAG
S7	GAGCCAGGTCTGTTCTCCACG	AGCCGATGCCCTTGTTGC
S8	GGGCAGCCAACAGCCAGTA	ATCCCTACCCCTCCCCACC
S9	GGGGAGGGGTAGGGATGGT	AGCCGATGCCCTTGTTGC
S10	CGCCGTCAGCTCAGTGGG	CCTGGATGCGGAGGGTGAG
S11	CCTCCAGCCAGCCCAGCAGA	ACCCGCGACAAGGCACCCA
S12	GAGGTTGCCATGAGCCGAGA	GTGAGGGTGGCCGCACCT
S13	GCCCCGCCTCTTCCTATA	TTTGTGCCACGGTTCCTAA
S14	AGAACTCCGCCGTTCCTGG	AAAGAACCGCAATAAGCTCCC
S15	ATCAGTTCCTAAGATCGGTTCAAA	AATAGATAGCCCATAGGCACAAA
S16	TGCTGCCAAATTCAAAGCTAAA	CACACCCTCAAATCACTGGACTA
S17	TACAGAAACAAATTCAGGGAAGG	ACAACCTGGTGAATCAGCAAACA
S18	TTTTGGGCCACATCATTCT	GCTGGGATTACAGGCGTGAG
S19	AAAACTGGTGTCCCAAATGC	AGTGGGTAGCAAGGTACTCGTC
S20	CGCTGCGCTCCTGCACA	CGCTCGGCTTCACTCGC
S21	TGTTTCTTATTTCCTGAAGGGTTTG	AAATGGGCAACGTGGAGGC
S22	CCTAAGACCTTTGCCTAGCTTCT	TCCTTTATGGGATTTCTGTTGTG
S23	AGCAGACACGGGCTCGGT	CGGTGCTCAGCAACCACC
S24	ATTACAGGCATAAGCCACCG	GGCTCAGCATCCCACTTCC
S25	CAGGAGGCTGAGGCAGGG	GGTACAGGTTGGGAGTTTGGG
S26	TACGCGGGTAGGAGGGAG	GTCAGGCCGAGGGGAATC
S27	GCATGCAGACAGGAGGGC	CGGTGATTTTTGGAGGGA
S28	GGGGACAGGAAAGAGGAAGTGAC	ACAGAGCAGGATAAAAAGTGGGG
S29	GAGACTGCAGGGGTGAAATAAA	AGCATCGACTGGCTTGAGAA
S30	**TAGTAAGGGAGGGCCGAGAA**	**CAGAGCCAGAACGCACAGAG**
S31	**TTACTGCTCCCTTTCATCATTTC**	**TGTGAAGAAAACATCCTGATCCT**
S32	**TCTGACCCAGTCCGACTCCTC**	**CTTAGGGAACTGACAAAGGCAAC**
S33	GGTTCGTGCACTCGCTGAG	GCCTTCTCCTTCTGGAACATCT
S34	TTCGTTATTGTAAATAAAACGGTTCC	CCTGGCTAGAGCGGTGTCC
S35	TGCAGCATGTGCCAGGTG	CAGCGCTCGATCAGGTCC
S36	**AGATAGAGCTGGAGTTCCCCG**	**CCCTTATGACAATCATGTGCGT**
S37	CGCACGGCCTACTCAAGTCTC	TGGCGTCGGAGGTGAGGC
S38	**TAAGGTGGATCCGTTTGAGG**	**TTCGGGCTTTCAGTTAGGTG**
S39	**CACACGCACACAGGACGCCAC**	**GGAAACCAGCCCGCACAGGAA**
S40	ACTGTCAAGGTTAGTCCAGCAAT	GCTCAATTCTAGCACTTTGGGTG

S41	**AAGTTCTCCTTTCGTGTGTGGG**	**GGCTGTGGTCTGTAAATTGGTA**
S42	ACCGGGCCTTCTGTCTGTC	TCTGCACGCTTCAATCCTTC
S43	GTTGGGATTACAGGCGTGAG	CTCTGCTGCCAGGGAAAA
S44	CAGCCCTAAGTTCTATTCACCCT	AGCTGCTGAAGTCGCAGTTTATT
S45	ACCTGGGGACTTTGGCTTATG	CAATCTTTGATTTTCCTTCCTTACAC
S46	GAGCCCGGGAGCCTCT	CCCAGAACAGGGACAGGAC
S47	**CCTTTGTTCTCATAAAGCAGCAG**	**CTCAGGACCCTTCCCCAGTG**
S48	GTGGTTGTTTCCCATACCTACTATTC	ATCTCCATTCTGCTGGCATTTCT
S49	ACATGCATTATCTTTCAAACATGT	GGCTCCGCAGAATCCAGGTC
S50	GTTCAGGCAAAGGCAGCAGT	AGGTGGGTGATGACAAGCAAA
S51	CCTAGCATTGCCATAACAAACTG	AGCGAGGAAGGATTCTCCATTA
S52	TCGGATCTGAGGTGCTGCA	CCATATCTGCGAAGACCCTTATT
S53	GCATCACCGCCTCCCACAGA	TACCCAGGGCTCGGCACCAA
S54	GGCTTCGGCCCTTGTCA	AAGGAAGGCTGGGTCCATG
S55	CAATGGGGTGATAAGAGACAAAA	GGGAAAGATCCAGAAAGGGTAGT
S56	TGCTGGGATGATGGTGCTT	GCCTTCTGGTGGCTAGGGA
S57	CGGGTTGAGTGCCTCCTGT	TTGTGGTCGGCCTTTGCTG
S58	CCCCATCACCAACTTCACTC	GAAACTGAGTCTCTCGCAAGG
S59	GCTGGACCAGAAAGTGTTGAG	GTGTGCTGCTTTGCAATGTG
S60	AATCAGCACCTTTTCAAAATCAAC	ATAAAGCATGGGTTCTATGGGATA
S61	**GGTCGAGTTTTTGGTGGTGT**	**CCACCGTCACTGTTCCTAGA**
S62	GAGCGTCTCTTGTTGTTCACCC	TCTTCTCACCATCCACTTCTGC
S63	**GGGCTTGGGGGGTTTCTG**	**TTCTCTCCTCCTCGGGGG**
S64	**TGAGCTCACAGGTCTGGAAA**	**CCCCACAGGGTTCTGGTAAT**

The primers selected for T21 detection were shown in bold. All these primers were designed for preliminary screening to assess the general usability of candidate DMR regions. Their initial design did not impose strict constraints on amplicon size.

Table S4 Primers used in further screening of DMRs.

DMRs	Related gene	Forward primer(5'->3'）	Reverse primer (5'->3'）
S2	*SIM2*	GAAAAAACCTCGCCCGCA	TCCTCCCAAGCACCCCTG
	SIM2	TGAGTGAAAAAACCTCGCCCG	ATGTTCCTCCCAAGCACCCCT
	SIM2	CATCTAGCACCCGACCACCTC	TCTGTAATTCCCCAAAGCCTC
S3	*ERG*	ACCTTTGCATGTGAGAGGCA	AGCTGGGACCGGAATGTACG
	ERG	TTGCAAACTAGATGGGAGGA	GTCGAGCCAGCGTCTTTTAT
	ERG	**ACCTTTGCATGTGAGAGGCA**	**GTCGAGCCAGCGTCTTTTAT**
S4	***CD48***	AGCACATGCTTCCTTCCAGA	AAGTTCCGTTTTTAGCCCCG
S5	*FAIM3*	TGGAGGATCCGGGCTATACTC	TGGCTGACACAACCACTGTGG
S30	*SIM2*	GCTGTTCGCTGTTTGAGGGA	CCGGTTTAAAGGTGGCTGAG
	SIM2	**GCTGTTCGCTGTTTGAGGGA**	**CAGAACGCACAGAGCCGGTT**
	SIM2	AAGATGCGAAGTGGTGGGTGT	AATGCCTGGAGACTTGCTTTC
	SIM2	ACCGCTCAGCCACCTTTAA	ACTCGGGGCCCAGCAATTC
S31	*NRIP1*	TTCTTTTAAGATGCTCTCCATCTC	AATTACTGGTGTAGTTCTCTGGGA
S32	*RIPK4*	CCCCAAGAGGGACGAGA	CCCGACCACAGGCTCCG
	RIPK4	**TGACCCAGTCCGACTCCTCA**	**TCCAAAGAGACCGACCCGC**
	RIPK4	GCGGGTCGGTCTCTTTGGA	TCATGAGCAAGGCGCTTGG
S36	***ETS2***	**CACAGACAATTAGCTGGGGC**	**CAGTTGCGCTGCTTATCACC**
S38	*OLIG2*	GCCCTCACTTCCCACTCGTT	TGGCATTCTAGAGGCTTCGG
S39	***C2CD2***	**CTCCCTTGAAACTTTTGTGCTT**	**TTGGTCGTGGATATACTCAGCA**
S41	*TSPEAR2*	TTAGGAAATCTGGTTTACAAACAAA	TCTAATGCGGATTCACTCACTCAAG
	TSPEAR2	AAACAAAATCTCTGTTCGAAGGG	TAATGCGGATTCACTCACTCAAG
S47	*SIK1*	AGGATGGGGTAAGGATGGAT	GTCTGCCCCCTCCTCAGGAC

	SIK1	AACCTGGCGGAGGATGGG	GGCCGGGCTCAGTCACTG
	SIK1	CGGGCGGTGCTGAGACT	AGGTGGCCCCGTGGTCT
	SIK1	CTGGCGGAGGATGGGGTA	GGCCGGGCTCAGTCACTG
	SIK1	CGGAGGATGGGGTAAGGA	GGCCGGGCTCAGTCACTG
S61	***DSCAM***	**GAGTTTTTGGTGGTGTTGAGCG**	**GGAGCAGCCTGAGGATCTTAGA**
S63	***UMODL1***	**GCAGTTCAGCCCAACAGTTTCT**	**CCACCCACATTCTGCTTGACTC**
S64	***AATBC***	**TGGAAATGGTCTGAATAGAAAGGA**	**AATATCAATAAAAACCCCACAGGG**

The primers selected for T21 detection were shown in bold. Primers in Table S3 but unsuitable for short cfDNA fragments were re-engineered to enhance detection specificity and efficiency.

Table S5 Clinical samples used for the validation of MSRE-dPCR

No.	Age	Normal or T21	Pregnancy weeks	Sample type
V1	35	Normal	15.4	Peripheral plasma
V2	36	Normal	14.9	
V3	32	Normal	15.1	
V4	33	Normal	16.1	
V5	33	Normal	14.7	
V6	23	T21	16.6	
V7	28	T21	17.0	
V8	36	T21	19.5	
V9	34	T21	20.4	
V10	27	T21	20.0	
V11	28	T21	19.1	
V12	27	Normal	14.7	
V13	40	Normal	15.6	
V14	43	T21	18.4	
V15	33	T21	18.4	
V16	34	Normal	14.9	
V17	38	Normal	17	
V18	37	Normal	16.3	

Due to the difficulty in obtaining T21 samples, 5 mL plasma was obtained from 5 previous T21 pregnancies and a new case. These samples were disarranged by the doctors and given to the authors without labeling.

Table S6 Distinguishing performance of MSRE-qPCR/dPCR for 24 simulated samples

Types	No.	MSRE-qPCR		MSRE-dPCR	
		Predicted values	Accuracy	Predicted values	Accuracy
Normal	1	4.32	√	2.13	√
	2	3.61	√	2.76	√
	3	4.15	√	3.54	√
	4	3.17	√	2.48	√
	5	3.23	√	2.71	√
	6	4.09	√	3.22	√
	7	5.71	×	2.69	√
	8	4.58	√	3.82	√
	9	2.09	√	3.91	√
	10	3.88	√	3.24	√
	11	2.60	√	3.35	√
	12	3.37	√	2.96	√
T21	1	7.21	√	4.32	√
	2	6.12	√	5.13	√
	3	3.46	×	4.81	√
	4	6.22	√	4.72	√
	5	6.76	√	4.23	√
	6	6.40	√	4.68	√
	7	4.90	×	7.35	√
	8	6.24	√	4.44	√
	9	7.71	√	6.39	√
	10	6.11	√	5.03	√
	11	9.46	√	4.61	√
	12	9.35	√	4.61	√