Electronic Supplementary Information

A Multiplex Droplet Digital PCR Assay for Non-invasive Prenatal Testing of Fetal Aneuploidy

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Supplementary Methods

S1. Quantitative real-time PCR

Quantitative real-time PCR was performed using an ABI 7500 real-time PCR System (Applied Biosystems, CA). All reactions were prepared in 10 µl with final concentrations of 1× TaqMan Genotyping Master Mix (Thermo Fisher Scientific, MA), 200 nM forward and reverse primers, and 100 nM universal probes, using the following cycling conditions: 25°C for 10 min, 95°C for 10 min, 40 cycles of 94°C for 20 sec and 60°C for 60 sec, 72°C for 5 min, 98°C for 10 min, and finally hold at 12°C.

S2. The calculation of the amount of input DNA corresponding to the required number of target DNA molecules

The amount of input DNA was calculated using

$$\text{Input DNA}_{\text{reference}} = \frac{\text{required number of target DNA molecules}_{\text{reference}}}{2} \times 6.4\ (\text{pg}), \quad (1)$$

$$\text{Input DNA}_{\text{aneuploidy}} = \frac{\text{required number of target DNA molecules}_{\text{aneuploidy}}}{2(1 - f) + 3f} \times [(1 - f) \times 6.4 + f \times 6.5]\ (\text{pg}), \quad (2)$$

and

$$\text{Input DNA} = \max\left(\text{Input DNA}_{\text{reference}}, \text{Input DNA}_{\text{aneuploidy}}\right), \quad (3)$$

where f is the fraction of cffDNA in maternal plasma. According to the previous studies[1, 2], human diploid cell, trisomy 21 cell, and trisomy 18 cell contain approximately 6.4 pg, 6.5 pg, and 6.5 pg genomic DNA, respectively.

S3. The calculation of the required number of primer pairs

The number of target DNA molecules for 14 ng of input DNA is

$$\frac{14000}{6.4} \times 2 = 4375,$$

human diploid cell contains approximately 6.4 pg genomic DNA [1, 2].

According to the statistical analysis, 78605 of target DNA molecules for reference chromosome is needed to reliably test the samples containing 4% of cffDNA. So, the required number of primer pairs is

$$\frac{78605}{4375} \approx 18.$$

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Supplementary Results

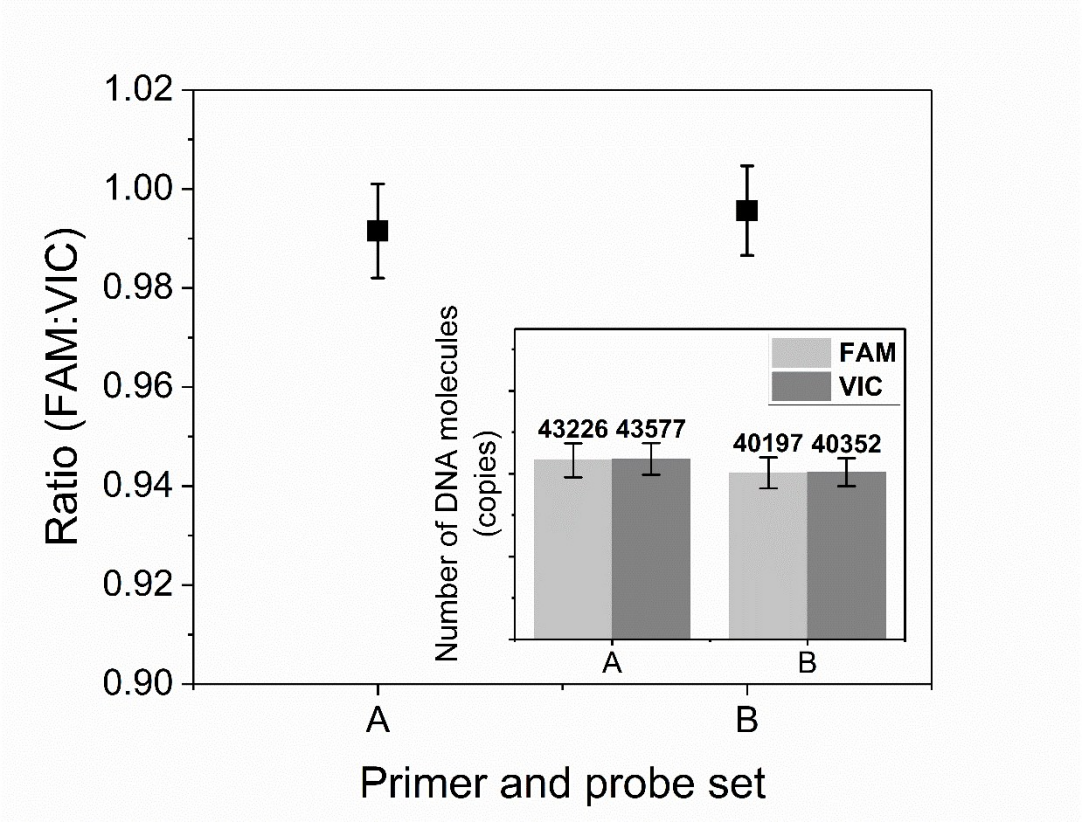

Figure S1. The validation of the reference primer and probe set (chr1 to chr5, chr10, and chr11). We used 10ng of fragmented normal genomic DNA to validate the primer and probe sets; four replicates were performed for each group. Set A: chr21 (FAM) and chr18 (VIC), set B: chr21 (FAM) and chr1 to chr5, chr10 and chr11 (VIC).

Table S1. The requirements for accurately classifying a sample as euploid or aneuploid with a 0.95 probability (N = 5 million).

Fraction of cffDNA	The required number of target DNA molecules (copies)		The amount of input
(%)	Reference chromosome	Aneuploid chromosome	DNA (ng)
4	78605	80177	252
5	50351	51610	161
8	19825	20618	64
10	12772	13411	41

Table S2. Information of gene locations and sequences of primers and probes (chr21 and chr18).

Gene location [a]	Primer sequence (5'-3')	Amplicon length (bp)
Chromosome 21		
14099141-14099218	F: ctaggagactgtccctgagctt R: agggggaacatagaggcttg	78
15634475-15634559	F: gggatagttgaatggggatgt R: agggattgtggagtttggtg	85
16912895-16912966	F: gcttctgggatgacacacg R: cacatgatattgtgaaaagcaagtt	72
18597570-18597636	F: tgaattagttgcaaggtggttct R: ccgagaagaagaaggattgaca	67
23717711-23717786	F: tcttccctccccttggaa R: gcctggtttctccatagaatca	76
24207756-24207825	F: cataattcccactgtggtggtat R: gcagagcccccatgaatta	70
25698963-25699026	F: agagctgataccattgcttgc R: aagtgctgccatctttcctg	64
25988208-25988278	F: ggccagagctaaagaagcaa R: ctgtacatgatgtcccccatt	71
26492402-26492463	F: tgagggaaatagaggtgctca R: tgtgtcattgtttgccaattc	62
27800999-27801086	F: acgccaccatgcctagtta R: tgaggtcgggaattcaagg	88
29174833-29174909	F: tgtgtggaacaactggagaaa R: cactgaaaaatttaaagttaggacga	77
31033224-31033289	F: tcccttagcctaggttatctagca R: ccatcaagcattagagacaaagg	66
31509622-31509681	F: gcatcaaggacccacagag R: cagggcattggaactgtctt	60
36205063-36205124	F: acagggtgctgttgtcactg R: agcaccgctttggtacaatc	62
37959129-37959206	F: agacttcttcgagaattgtgtaattg R: aattgatgtctgtttgagttgatttta	78
41284470-41284545	F: ccactgaaattattctaactagccaaa R: tgtggtttctgtaggaaagaagg	76
42234238-42234310	F: cttaacacctggagtcccaact R: tctgagcgattgcaaagaga	73
42923863-42923928	F: tgggtgaggtactgaaactctct R: caaaagactcggtttatttccag	66
44441621-44441686	F: gcagtccgtagggctcag	66

Gene location [a]	Primer sequence (5'-3')	Amplicon length (bp)
	R: agaacaagggcacagctgac	
45098547-45098621	F: ccctggaacaaaactgttgag R: gaaactgaggcccagcact	75
Chromosome 18		
1248496-1248575	F: ccatctccataacccaaatacc R: ccttgcaaacctcatgttga	80
5147229-5147324	F: aatgaccaagaagccaccat R: gcagttgccttaaacaaggtatatta	96
8473597-8473674	F: gagaaacctgcccacaagat R: tctcctgttcggttataatgtcc	78
8870993-8871084	F: cctgtttgttctttaatagcctcat R: tggcaaatttcaatgatgtcac	92
13150636-13150721	F: gcatcatcggatggttttg R: ccttgctacttctaggctaacca	86
21382385-21382445	F: tcagaagggtagggtcttgg R: tggctaatgatactcaaagggtct	61
23485174-23485251	F: ggagcaaagggaaaacagg R: tgccaattaaacatgctagacaa	78
24943949-24944019	F: tgaccagaggagcctggtag R: ttcatcttcaaagagctccaga	71
37487343-37487441	F: cctgggagacagaaactctcag R: ggagagagatttctccgaagc	99
37782135-37782224	F: gggtctccactctgatgatttg R: tgggaacttaatcccaaaattaac	90
48190141-48190240	F: tgggagtgcagtgtctgtct R: acacttagtcttcttcttgctcctg	100
48852421-48852513	F: catgactcaggtaggcttgct R: caagaggccgggataaagat	93
50208031-50208106	F: aacttgcaaggttttcactgg R: tgggccatgctgtattcat	76
51129532-51129592	F: tgagctcagggtggaaagag R: caagggattacgcatgcac	61
51295343-51295441	F: catggttctgcaggctatacaa R: ttgtcccctccaaatgtcag	99
51951491-51951550	F: aagacaggagagcgaggtga R: ttctgttaataaggcccatgc	60
52280540-52280623	F: aaacattggaatcagactgaggtag R: agaagttacaacttacccacgctta	84
56126533-56126611	F: ccctcacatcccttccaac R: ccctgcattaacccctcag	79

Gene location [a]	Primer sequence (5'-3')	Amplicon length (bp)
56554079-56554149	F: tttctgactctgcaatctgctta R: cacagaggtgaaggcacaaa	71
61875811-61875887	F: caagggctcaggtcttcatt R: caggaagggacgaaggtg	77
Chromosome	**Probe sequence (5'-3') [b]**	**Probe length (nt)**
21	5'-FAM/C+C+CT+G+CCT+CT/3'-IABkFQ	10
18	5'-YAkYel/C+C+CA+C+CTC+CA/3'-IABkFQ	10

[a] Gene location refers to the human genome assembly version hg19/Genome Reference Consortium Human Build 37 (GRCh37)

[b] plus sign "+" represents locked nucleic acid (LNA)

Table S3. Information of gene locations and sequences of primers and probes (chr1 to chr5, chr10, and chr11).

Chromosome	Gene location [a]	Primer sequence (5'-3')	Amplicon length (bp)
chr1	32118909-32118986	F: tgaggtgctgagtagaacttgaac R: gctagcatgatgcagtaggaga	78
chr1	43801802-43801867	F: cctccccacaactcacctc R: gccaattctaggaggcttgac	66
chr1	163805637-163805704	F: ggatggctcagcacacact R: tgggactcattattccatacaaac	68
chr1	181586708-181586783	F: ccaatccccctatcaagaact R: ggcacccctcactgctataa	76
chr2	158985736-158985805	F: gctatctggattcccctggt R: ggtagacgatggagccttgt	70
chr3	70123869-70123936	F: tggtttgtacatagtgtgcatgtc R: gatgttcaatgatgctaaagccta	68
chr3	82481661-82481721	F: ggtataatcacctcccaaacca R: gaagtcctaaccccagtgtga	61
chr3	99791421-99791484	F: aactgtcaaccgaccatcg R: ttgttgcaaagaagactgtacca	64
chr4	17441176-17441250	F: tgtgacccagacaccttgac R: tgaccctccaaccctcataa	75
chr4	40928392-40928460	F: gcactgaccttccaattcct R: ggcaatttgggattggagta	69
chr4	55780242-55780309	F: gacctctgggtccctcactt R: ttgtcatttcaaacacaaggcta	68
chr5	5865548-5865615	F: aaaatacttgtctgcttctgcgta R: agactggcttctatgaagatatgga	68
chr5	11631974-11632051	F: caagagtgccaatccaaaaga R: cccaggcaacacatttatacc	78
chr5	59134034-59134101	F: tccttcatcttcacccaagg R: tctgcatgactgtagggttctg	68
chr5	71674073-71674143	F: gtgcccacaccactttctg R: ccagatgaagaaaatggagacc	71
chr5	83165836-83165911	F: tcaaagactttaagtaaaatgggctac R: tcttcatgtactcgcaacttcaa	76
chr10	10806742-10806817	F: gtctcccccactttctaggc R: tgctgcagaagatgtacaagc	76
chr10	59552066-59552135	F: ggccacctgaaagatatgga R: aggcaacctaaagccagttg	70

Chromosome	Gene location [a]	Primer sequence (5'-3')	Amplicon length (bp)
chr10	120784836-120784929	F: aggagagggctcctcgtg R: gtgctttggctccgtcag	70
chr11	126417993-126418054	F: gcccctcagtcagaacaaat R: ggtacagatgacaggctcgtg	62
Probe sequence (5'-3') [b]		**Probe length (nt)**	
5'-YAkYel/C+C+CA+C+CTC+CA/3'-IABkFQ		10	

[a] Gene location refers to the human genome assembly version hg19/Genome Reference Consortium Human Build 37 (GRCh37)

[b] plus sign "+" represents locked nucleic acid (LNA)

Table S4. Information of clinical samples for training test.

Sample	Gestational age	Gender [a]	Input DNA (ng)	Total copy number		$R_{21:18}$	ddPCR result [b]	NGS result
				chr21	chr18			
T1	14w	M	1.793	9902	10297	0.9616	E	E
T2	20w	M	2.268	10252	10737	0.9548	E	E
T3	16w	F	2.387	11453	11678	0.9807	E	E
T4	14w+3	M	2.257	12043	12483	0.9648	E	E
T5	16w+3	M	2.992	12686	12689	0.9998	E	E
T6	16w+2	F	3.218	22496	22774	0.9878	E	E
T7	16w	F	4.288	23162	24092	0.9614	E	E
T8	26w	M	4.460	24341	24231	1.0045	E	E
T9	18w	M	4.493	24701	26405	0.9355	E	E
T10	18w	M	5.519	24852	25176	0.9871	E	E
T11	16w	M	4.720	25520	26079	0.9786	E	E
T12	18w	M	4.784	26016	26287	0.9897	E	E
T13	16w	M	5.281	27193	27797	0.9783	E	E
T14	17w	M	4.979	27546	27985	0.9843	E	E
T15	19w	F	6.696	35915	35569	1.0097	E	E
T16	18w	M	7.420	37640	37847	0.9945	E	E
T17	17w	F	6.340	37955	38162	0.9946	E	E
T18	18w+4	F	8.867	43602	44737	0.9746	E	E
T19	20w	M	8.510	44149	44344	0.9956	E	E
T20	13w	M	8.100	44192	45228	0.9771	E	E
T21	22w	F	8.618	46088	46621	0.9886	E	E
T22	19w	F	8.532	46504	47660	0.9757	E	E
T23	17w	M	8.413	46779	46840	0.9987	E	E
T24	14w+5	M	8.446	46879	47255	0.9920	E	E
T25	17w	M	9.601	47305	47335	0.9994	E	E
T26	19w	M	7.690	47353	47771	0.9912	E	E
T27	18w+2	F	9.688	47541	47856	0.9934	E	E
T28	19w+4	F	8.618	47567	48290	0.9850	E	E
T29	18w+5	M	8.597	47719	48078	0.9925	E	E
T30	13w+5	F	9.601	52640	52791	0.9971	E	E

[a] F, female; M, male.

[b] E, euploidy.