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Research Paper

Segmental duplication as potential biomarkers for non-invasive prenatal testing of aneuploidies

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ABSTRACT

Background: Segmental duplication (SD) regions are distinct targets for aneuploidy detection owing to the virtual elimination of amplification bias. The difficulty of searching SD sequences for assay design has hampered their applications.
Methods: We developed a computational program, ChAPDes, which integrates SD searching, refinement, and design of specific PCR primer/probe sets in a pipeline to remove most of the manual work. The generated primer/probe sets were first tested in a multiplex multicolour melting curve analysis for the detection of five common aneuploidies. The primer/probe sets were then tested in a digital PCR assay for the detection of trisomy 21. Finally, a digital PCR protocol was established to quantify maternal plasma DNA sequences for the non-invasive prenatal detection of fetal trisomy 21.
Findings: ChAPDes could output 21,772 candidate primer/probe sets for trisomy 13, 18, 21 and sex chromosome aneuploidies within 2 working days. Clinical evaluation of the multiplex multicolour melting curve analysis involving 463 fetal genomic DNA samples revealed a sensitivity of 100% and specificity of 99.64% in comparison with the reference methods. Using the established digital PCR protocol, we correctly identified two trisomy 21 fetuses and thirteen euploid foetuses from the maternal plasma samples.
Interpretation: The combination of ChAPDes with digital PCR detection could facilitate the use of SD as potential biomarkers for the non-invasive prenatal testing of fetal chromosomal aneuploidies.

1. Introduction

Segmental duplications (SDs) are duplicated blocks of genomic DNA typically ranging in size from 1 to 200 kb (IHGSC 2001), which have a sequence identity of at least 90%. Both experimental and in silico analyses initially suggested that 5–6% of human euchromatin is composed of SDs. They contribute to gene dosage imbalance associated with disease [1,2] and are 10 times more likely to contribute to normal copy number variation (CNV) [3]. They are also a reservoir for gene innovations associated with species adaptations [4,5]. The size, copy number, and sequence identity of SDs suggest that they are usually the last regions of the genome to be sequenced and assembled, often using large-insert bacterial artificial chromosomes [6,7]. Despite the increasing interest in their structure, functions, and homology [8], the clinical use of SDs remains largely unexplored.

One attractive use of SDs is as a biomarker for aneuploidy detection [9–12]. For this purpose, a pair of SDs sequences located in the query chromosome and reference chromosome is amplified. Because of their high similarity, the two sequences can be virtually equally amplified by PCR using one pair of primers and their original copy number ratio remains unchanged after amplification. The relative copy number of the amplified products can be estimated by a variety of detection methods. For example, using high resolution melting (HRM) analysis, aneuploidy associated dosage abnormalities produced different ratios of similar amplicons, yielding melting curves that were detectably different from those of samples from unaffected individuals [9]. In another example, by using a set of SD sequences differed by amplicon length, simultaneous detection of aneuploidy of five chromosomes could be achieved by the capillary electrophoresis fluorescence signals following a multiplex PCR [11,12]. The so-called quantitative fluorescent PCR (QF-PCR) method even allowed mosaic

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Research in context

Evidence before this study

Determining fetal chromosomal aneuploidy is crucial for both pregnancy management and genetic counseling. PCR-based approaches have been widely used for aneuploidy determination in prenatal diagnosis, however, few are directly used for non-invasive prenatal testing. Segmental duplication (SD) regions are unique biomarkers for aneuploidy detection owing to the virtual elimination of PCR-derived amplification bias by using a common pair of primers to amplify two similar sequences differing by one or a few nucleotides. It is nevertheless difficult to search and validate SD sequences for such purposes. Also, robust and accurate detection tools remain to be integrated with SD biomarkers for improved aneuploidy detection beyond invasive prenatal diagnosis.

Added value of this study

We developed a computational program whereby available SD regions can be processed and analyzed efficiently for their potential use as biomarkers of the aneuploidy of interest. For the five common aneuploidies, i.e., trisomy 13, 18, 21, and two sex chromosome aneuploidies, a total of 21,772 candidate SD biomarker sequences together with their corresponding primer/probe sets were generated. The primer/probe sets were tested using a real-time PCR-based multicolour melting curve analysis for simultaneous detection of the five common aneuploidies, and yielded 100% clinical sensitivity and 99.64% specificity when subjected to a clinical evaluation. Following the observations that the SD biomarkers for aneuploidy could be better detected by digital PCR with improved accuracy, we established a noninvasive prenatal testing protocol for trisomy 21 and attained 100% concordance with next generation sequencing.

Implications of all the available evidence

Our study confirmed that SD regions are preferred biomarkers for aneuploidy detection and in particular SD-based digital PCR could find potential use for NIPT of trisomy. A similar strategy can be applied to other chromosomal abnormality and genetic disorders.

samples to be correctly identified. Alternatively, by choosing multiple pairs of SD sequences with single-nucleotide differences, multicolour melting curve analysis (MMCA) using differently fluorophore-labelled probes allowed multiple aneuploidies to be determined in single reaction [10]. Collectively, the SD-based aneuploidy assays showed rapidness, accuracy, and flexibility, and they could potentially provide a better alternative to conventional methods.

Despite the advantages mentioned above, one fundamental barrier for developing an SD-based aneuploidy assay is the difficulty of finding the candidate SD sequences. Indeed, it is an uneasy task to manually search for the candidates among more than 50,000 human SD sequences, many of which are unsuitable for this aim, such as those occurring in multiple locations (>2), in tandem, or intrachromosomally. Moreover, depending on the detection principle, the two candidate SD sequences representing their respective chromosomes must contain a variant site to be easily differentiated, e.g., length difference or single nucleotide variant. Importantly, the two sequences should be amplified by a common pair of primers at identical efficiency. Current SD-based aneuploidy assays are mostly accomplished by empirical experiences that rely on a small number of reported SD regions, which prevents the development of those assays that might need a large number of SD sequences. Furthermore, the lack of a thorough analysis cause incidences of unreported single nucleotide polymorphisms (SNPs) or CNVs within the target regions, leading to misclassification of the results and finally ambiguous or false findings.

To overcome the aforementioned difficulties and further expand applications of SDs in aneuploidy detection, we developed a computational program to assist efficient search, refinement, and design of specific PCR primer/probe sets for the detection of five common aneuploidies. The candidate primer/probe sets were first verified using MMCA assays in the detection of the five common aneuploidies. Furthermore, for the first time, digital PCR was introduced for the detection to obtain absolute quantitative results. We showed that droplet digital PCR (ddPCR) rendered a successful SD-based non-invasive prenatal testing (NIPT) for trisomy 21. All the assays were validated with clinical samples for prenatal diagnosis or NIPT.

2. Methods

2.1. The methodology overview

As illustrated in the flowchart (Fig. 1), our study consisted of three major parts. First, a novel computational program, namely ChAPDes, was compiled for automatic primer/probe design on the basis of human genome SDs and eventually provided a list of candidate primer/probe sets for five common aneuploidies, i.e., trisomy 13, 18, 21, X, and Y. Second, an MMCA assay was established to verify the performance of the candidate primer/probe sets in the detection of the aneuploidies from genomic DNA samples (gDNA), i.e., an invasive assay. Subsequently, a ddPCR assay was developed to detect trisomy 21 from plasma cell-free DNA samples (cfDNA), i.e., a non-invasive assay. Third, clinical studies for both assays were performed in their respective cohorts including the amniotic fluid (the AF-cohort), chorionic villus sampling (the CVS-cohort), and the NIPT-cohort.

2.2. Automatic primer/probe design

The candidate primer/probe sets for aneuploidy detection should meet the following criteria: (i) the primer pairs should be highly specific for both the target chromosome (the abnormal chromosome to be tested, e.g., chromosome 21 for trisomy 21) and the corresponding reference chromosome (the normal autosomes other than the target chromosome); (ii) to ensure consistent binding affinity, the primer-binding regions of the target chromosome and the reference chromosome should contain no SNP sites; (iii) the probe-binding regions of the target chromosome and the reference chromosome should have one or two nucleotides difference to ensure differential hybridization efficiency. These criteria are not easily satisfied by the simple application of current primer design methods. Therefore, we developed a computational program, ChAPDes, which integrated several state-of-art bioinformatics tools. For the convenience of users, we have packed the program and deposited it on GitHub for free access (https://github.com/crystal525/The-Chromosome-Aneuploidy-Primer-Designer.git).

2.2.1. Data collection and k-mers generation

The SD sequences and the corresponding human genome sequences (hg19, Genome Reference Consortium Human Build 37) were downloaded from the various data sources (Supplementary Table S1). Of 51,600 distinct SD sequences derived from the Segmental Duplication Database [13], the overlapping ones were first discarded. Then, a list of forward and backward pairs of 20–25 base pairs (bp) k-mer (the size of normal primers) were extracted from the SDs of target chromosomes by sliding word windows from both sequence flanks. The k-mer pairs containing either highly variable region

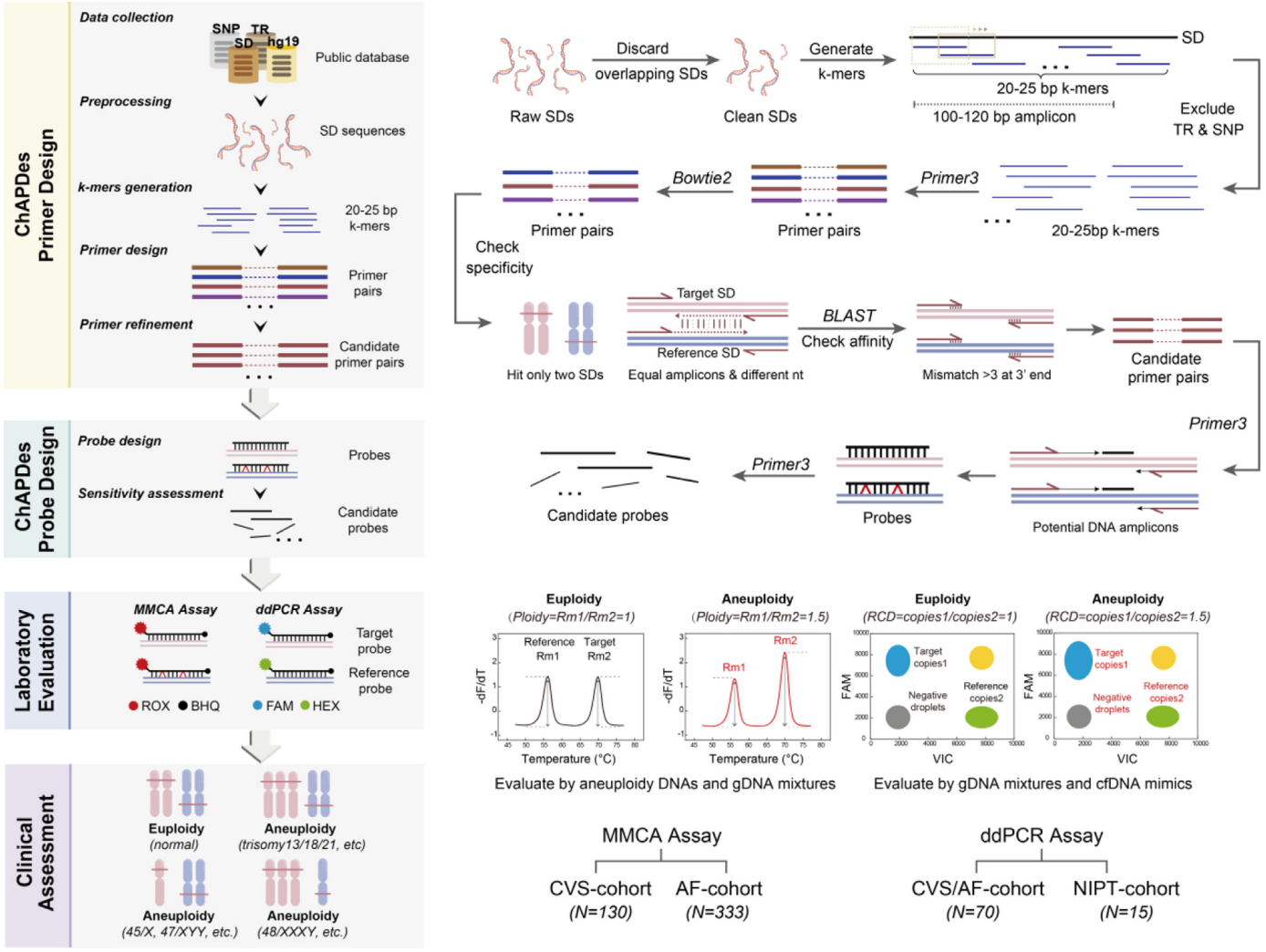

Fig. 1. Schematic overview of the study workflow. The ChAPDes program starts with input of raw SD sequences and ends with output of a list of candidate primer pairs and probes. The primer/probe sets were tested with MMCA and ddPCR assays for the detection aneuploidies. Both assays were evaluated with clinical samples obtained from the respective cohorts.

(average SNP frequency of East Asia > 1%, according to the 1000Genomes VCF hg19) or tandem repeats (TRs according to the hg19 GRCh37) were discarded. The remaining k-mer pairs were retained for further primer design.

2.2.2. Primer design based on k-mers

Next, we selected the potential primer pairs from the k-mer pairs using the "check_primers" function of Primer3_core program [14]. The Primer3_core main program adopted the boulder-IO format for k-mer input and primer output. The minimum, optimum, and maximum melting temperatures for a primer were set at 57 °C, 60 °C, and 63 °C, respectively. The maximum melting temperature difference between the pair of primers was set at 3 °C. The minimum, optimum, and maximum percentage of Gs/Cs were set at 20%, 50%, and 80%, respectively. A list of distinct primer pairs was thus obtained for autosomal aneuploidies 13, 18, and 21 and sex chromosome aneuploidies.

2.2.3. Evaluation of primer specificity

Then, we used both Bowtie2 [15] and BLAST [16] to check the specificity of the primers obtained above to the target and its paired SD sequences. Bowtie2 is an ultrafast and memory-efficient tool for aligning sequencing reads to long reference sequences. Before sequence alignment, the primer pair sequences were transformed from the boulder-IO format to the FASTA format, preserving the forward and reverse information. For each primer pair, the sequences were mapped against the human genome (hg19) using the Bowtie2 (version 2.3.3.1; parameters: –very-sensitive and maximum mismatch penalty = 1). Bowtie2 output the alignment results in SAM file, which were interpreted by the SAMtools software (version 2.3.0+) [17]. After checking the output SAM files, we only chose the primer pairs that satisfied the following criteria: (a) the pair of primers hit one target SD and one reference SD only, and the two SDs were located on different chromosomes. (b) the expected PCR amplicons of target and reference SDs were nearly equal in size of 100–120 bp. (c) the amplicons derived from target and reference SDs had different nucleotide compositions. The composition difference endowed the designed probes with different hybridization efficiency to the amplicons. As a result, a list of potential primer pairs specific for different aneuploidies was obtained.

2.2.4. Evaluation of primer binding affinity

Next, we refined the primer pairs by excluding those that had comparatively weak DNA template binding affinity. Based on the experimental experience, the mismatches at the 3′ terminal of the primer may lead to instable template DNA binding. Hence, the BLAST tool was adopted to map the primer sequences against the human genome (hg19) again. The BLAST parameters were preset as follows: the maximum mismatch ratio = 35%, the Maximum Expect

Value = 30,000, the Word Size = 7, and the ratio of Match Reward/Mismatch Reward penalty = 1:1. We discarded the primers if there were > 2 mismatches in the last five nucleotides to the 3′ terminal. The BLAST results were output in a tab-delimited text format. After the refinement, we obtained a list of candidate primer pairs with high sequence specificity and binding affinity.

2.2.5. Probe design and sensitivity assessment

Introduction of a probe can substantially improve the specificity of PCR assay. In this study, we used the "pick_hyb_probe_only" function of Primer3 to design TaqMan probes based on the amplicons encompassed by the selected primer pairs. The minimum, optimum, and maximum melting temperatures were set at 67 °C, 70 °C, and 73 °C respectively. The minimum, optimum, and maximum percentages of Gs/Cs were set at 35, 50 and 65%. The minimum, optimum, and maximum lengths of hybridization probe were set at 30, 33, and 35 bp. The Primer3 output a list of potential probes for every primer pairs if available. Subsequently, we checked the probe specificity to the target SD and the reference SD by the "check_primers" module of Primer3_core program, taking the primers, the probes, and the potential amplicons as the input. The parameters were set as follows: the minimum, optimum, and maximum melting temperatures for a primer were set at 57 °C, 60 °C, and 63 °C, respectively. The maximum melting temperature difference between the primer pairs was set at 3 °C. The minimum, optimum, and maximum percentages of Gs/Cs were set at 20%, 50%, and 80%, respectively. The refinement eventually yielded a list of candidate probes, along with the responding primer pairs, for the aneuploidy assays.

2.3. Aneuploidy detection by MMCA

The MMCA is a strategy for variant detection based on melting temperature of thermal denaturation of probe-target hybrid that has been previously described [18]. We developed an MMCA assay for aneuploidy detection. Both the target and reference SDs were amplified and detected using program-designed primer/probe sets, and the chromosomal ploidy was assessed by the MMCA method.

2.3.1. Determination of chromosomal ploidy from gDNA by MMCA

The MMCA reaction was performed on a SLAN-96S real-time PCR detection system (Zeesan Biotech, Xiamen, China) in a 25-μL mix that contained 1 × PCR master mix buffer (Takara Biomedical Technology, Dalian, China), 4.0 mmol/L $MgCl_2$, 0.04 mmol/L dNTPs, 2.0 U of hot-start Taq polymerase (Takara), 0.05 to 0.08 mmol/L limiting primers, 0.5 to 0.8 mmol/L excess primers, 0.4 mmol/L probes, and 5 μL of extracted gDNA. After a denaturation step at 95 °C for 5 min, a touchdown program was performed with 10 cycles at 95 °C for 15 s, 65 °C for 15 s (1 °C per cycle), and 76 °C for 20 s, followed by 40 cycles at 95 °C for 15 s, 55 °C for 15 s, and 76 °C for 20 s. The melting curve analysis started with a denaturation step of 95 °C for 1 min, a hybridization step of 37 °C for 3 min, and a continuous temperature increase from 40 °C to 85 °C at a ramp rate of 0.04 °C/s. The fluorescence data from the FAM, HEX, and ROX channels were recorded at the annealing step of every 40 cycles and at each step of the continuous temperature increase during the melting curve analysis procedure. The melting curves were obtained by plotting the negative variation of fluorescence signal with respect to temperature (dF/dT) versus temperature. The corresponding temperature (Tm) and relative fluorescence of melting (Rm) values were determined automatically by the embedded SLAN8.2 software.

The chromosome ploidy was calculated by the ratio of *Rm1* to *Rm2*, corresponding to the target chromosome and reference chromosome, respectively. For normal euploidy, the ploidy was 2:2. For aneuploidies, a deviation in ploidy was expected. For example, the ploidy for trisomy 21 would be 3:2 theoretically, yielding a 1.5-fold difference from the normal euploid. To eliminate the system error, the ploidy was further normalised to that of the control sample. In clinical assessment, Z-score represented the number of standard deviations away from the mean of euploid sample datasets. Three standard deviations were used as the cut-off Z-score to classify samples as euploid or aneuploid.

$$Ploidy = \frac{Rm1(target\ chromosome)}{Rm2(competitor\ chromosome)} \quad (1)$$

$$Normalized\ Ploidy = \frac{Ploidy\ of\ the\ unkonw\ sample}{Ploidy\ of\ the\ control\ sample} \quad (2)$$

$$Z - score = \frac{X - X}{Standard\ Deviation} \quad (3)$$

2.3.2. Analytical evaluation of MMCA aneuploidy detection

The evaluation was undertaken using purified gDNA of different aneuploidies (Coriell Institute for Medical Research, Camden, NJ). The aneuploidy types included trisomy 21 (T21, NA01921), trisomy 18 (T18, NA02732), trisomy 13 (T13, NA02948), 45/X (NA01176), 48/XXXX (NA01416), 47/XXY (NA03102), 47/XYY (NA09326), and 49/XXXXY (NA00326). As the normal control, whole blood samples were collected from the laboratorians themselves. The human gDNA was extracted using the Lab-Aid 824 automatic DNA extraction system (Zeesan Biotech, Xiamen, China). The concentration of gDNA samples was determined by ND-2000 UV−VIS spectrophotometer (NanoDrop Technologies Inc, Wilmington, USA). To study the resolution of the assay for chimeric samples, a series of mimic gDNA mixtures was prepared containing different percentages of aneuploidy DNA (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%). In the cases of 45/X, 48/XXXX, 47/XXY, 47/XYY, and 49/XXXXY, only 50% mixtures were prepared. Each chimeric sample was detected in four replicates. The detection limit of the assay was defined as the lowest concentration of euploidy with the Z-score smaller than three times of the average standard deviation (n = 4).

2.3.3. Clinical evaluation of MMCA aneuploidy detection

Two clinical cohorts were recruited for prenatal diagnosis from the Suzhou Municipal Hospital: a cohort of 333 pregnant women who underwent amniotic fluid collection (the AF-cohort) and a cohort of 130 pregnant women who underwent chorionic villus sampling (the CVS-cohort). For the AF-cohort, the uncultured amniotic fluid was collected after 15 weeks of gestation. For the CVS-cohort, the chorionic villus cells were collected between 10 and 14 weeks of gestation. Both operations followed the standard clinical practices. The human gDNA was extracted using the automatic magnetic beads approach. The ploidies of both cohorts were also determined by G-banded karyotyping or chromosome microarray analysis (CMA), and the results were compared with MMCA assay in a double-blind manner.

2.4. Aneuploidy detection by ddPCR

We also developed a ddPCR assay for non-invasive aneuploidy detection based on plasma cfDNA. Both the target and reference SDs were amplified by the selected program-designed primer/probe sets, and the chromosomal ploidy was assessed by the ddPCR method. The ddPCR is a strategy for absolute quantification of nucleic acids, which has been previously used for relative chromosome dosage analysis [19].

2.4.1. Determination of chromosomal ploidy from cfDNA by ddPCR

The preamplification reaction was performed to reduce standard deviation of ddPCR detection by increasing the number of the positive droplets. Preamplification was carried out using 1 × PCR buffer, 5.0 mmol/L $MgCl_2$, 0.03 mmol/L dNTPs, 2.0 U of hot-start Taq

polymerase (Takara), 0.05 mmol/L of the pooled primer pairs, and 1−10 ng input cfDNA in a 50 μL reaction. The PCR procedure began with the enzyme activation and template denaturation at 95 °C for 5 min, followed by 8 cycles of denaturation at 94 °C for 20 s and annealing at 58 °C for 2 min, and it was finally held at 72 °C for 2 min. The ddPCR was performed in eight separate reactions in parallel using the ddPCR System (TargetingOne, Beijing, China). A 30-μL ddPCR mix was prepared with 15 μL of Probe ddPCR SuperMix (TargetingOne), 0.8 μmol/L upstream and downstream primers, 0.25 μmol/L FAM- and HEX- labelled 5′-hydrolysis probes each, and 5 μL of template. The formed microemulsion mixture was subjected to amplification on a PCR thermal cycler (LongGene, Hangzhou, China). The reaction program started with the enzyme activation and template denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 94 °C for 30 s and annealing at 58 °C for 60 s, and ended with heat preservation at 12 °C. The reaction was then transferred to the Chip Reader (TargetingOne) for droplet readout. The raw data were analyzed using the TargetingOne ddPCR Analyzer. The relative chromosome dosage (RCD) was calculated by:

$$RCD = \frac{Copy\ number\ of\ target\ chromosome}{Copy\ number\ of\ competitor\ chromosome} \quad (4)$$

Theoretically, the RCD value is 1.0 when the sample is euploid, otherwise it can be regarded as aneuploidy. For example, the RCD became 1.05 when the maternal plasma contained 10% of fetal cfDNA from a trisomy 21 fetus. The RCD value is dependent on the fraction of fetal cfDNA in maternal plasma. The Z-score of three standard deviations was calculated to determine the disease status of a sample based on the RCD value.

2.4.2. Preparing gDNA mixtures and cfDNA mimics

A series of mimic gDNA mixtures was prepared containing different percentages of aneuploidy DNA (0, 5, 10 and 100%). To simulate cfDNA in plasma, the gDNA mixtures were fragmented into 100−200 bp by sonication using M220 Focused-ultrasonicator (Covaris, Woburn, MA). The DNA integrity was subsequently verified by capillary gel electrophoresis system Qsep100™ (BiOptic Inc, Taiwan, China).

2.4.3. Non-invasive prenatal testing of trisomy 21 by the ddPCR-based RCD method

A small cohort of 15 pregnant women who were carrying a fetus with a gestational age of 14−20 weeks were recruited for non-invasive detection of trisomy 21 (the NIPT-cohort) from the Suzhou Municipal Hospital. Plasma was separated by centrifugation at 1600 g for 10 min, transferred to new microcentrifuge tubes, and centrifuged at 16,000 g for 10 min to remove cell debris at room temperature. The plasma samples were stored at −80 °C before extraction cfDNA was extracted. The cfDNA was isolated from 1 mL of each plasma sample by the Apostle MiniMax™ High Efficiency cfDNA Isolation Kit (Apostle Inc, San Jose, CA) in line with the manufacturer's instructions. The cfDNA was quantified using Qubit 3.0 fluorometer (Thermo Fisher Scientific, Shanghai, China). The cfDNA was suspended in a final volume of 30 μL and stored at −20 °C before use. The aneuploidy status of cohort members was compared with the results obtained from the next generation sequencing (NGS) in a blinded manner.

2.5. Statistical analysis

Statistical analysis was determined by GraphPad Prism version 8.0 (GraphPad Software, Inc., CA, USA) or Origin version 8.0 (OriginLab, MA, USA) using tests as stated in the figure legends. Significant differences between two groups were determined by the unpaired Student's *t*-test. All reported p-values were two-sided, and a p-value less than 0.05 was considered statistically significant.

2.6. Ethics statement

The collection of specimens and associated clinical data used in this study were approved by the Research Ethics Committee of Suzhou Municipal Hospital (J1315009). All of the experiments were performed in accordance with the Declaration of Helsinki and informed consent was obtained from the individual patients or their guardians.

2.7. Role of funders

Funders provide financial support for this study, and did not participate in study design, data collection, data analyses, interpretation, or writing of report. The corresponding author had full access to all of the data and the final responsibility, with the agreement of all authors, for the decision to submit for publication.

3. Results

3.1. ChAPDes-derived primer/probe sets

For every aneuploidy examined in this study, a list of candidate primer/probe sets was generated by ChAPDes. The intermediate results are summarized in Table 1, which contains the starting dataset size of SDs, the pre-processed dataset size, the number of program-designed primer pairs, the number of refined primer pairs, and the computational time. The final candidate primer/probe sets for every aneuploidy are deposited in the supplementary tab-delimitated files (Additional file 1). In these files, comprehensive information, including sequence ID, chromosome, coordinates in the genome, forward primer sequence (its *Tm* value, GC%, and self-complementarity score), reverse primer sequence (its *Tm* value, GC%, and self-complementarity score), *Tm* difference between the forward primer and the reverse primer (Diff *Tm*), and probe sequence (its *Tm* value, GC%, and self-complementarity score) are given for each of the primer/probe sets. In total, 21,772 candidate primer/probe sets were output for trisomy 13 (5024), trisomy 18 (4023), trisomy 21 (5722), 45/X and 47/XXX (294), 47/XXY (2390), and 47/XYY (4319).

Table 1
Summary of diagnostics primers selecting results.

Chromosome Aneuploidies	Target Chr	Reference Chr	k-mers of SDs	Preprocessed k-mers of SDs	Number of Primer Pairs	Number of Primer Pairs (Refinement)	Running Time(h:m:s)
Trisomy 13	13	Autosome	195,407,982	77,418,420	6373,173	5024	48:07:45
Trisomy 18	18	Autosome	75,389,832	34,023,103	1726,336	4023	21:42:45
Trisomy 21	21	Autosome	44,139,060	18,219,351	1244,469	5722	30:31:29
45/X,47/XXX	X	Autosome	130,265,502	45,596,452	2577,004	294	20:53:52
47/XXY	Y	Autosome	134,403,234	45,170,031	2243,010	2390	17:25:42
47/XYY	Y	X	134,403,234	45,170,031	2243,010	4319	51:28:26

Chr: Chromosome, SDs: Segmental duplications.

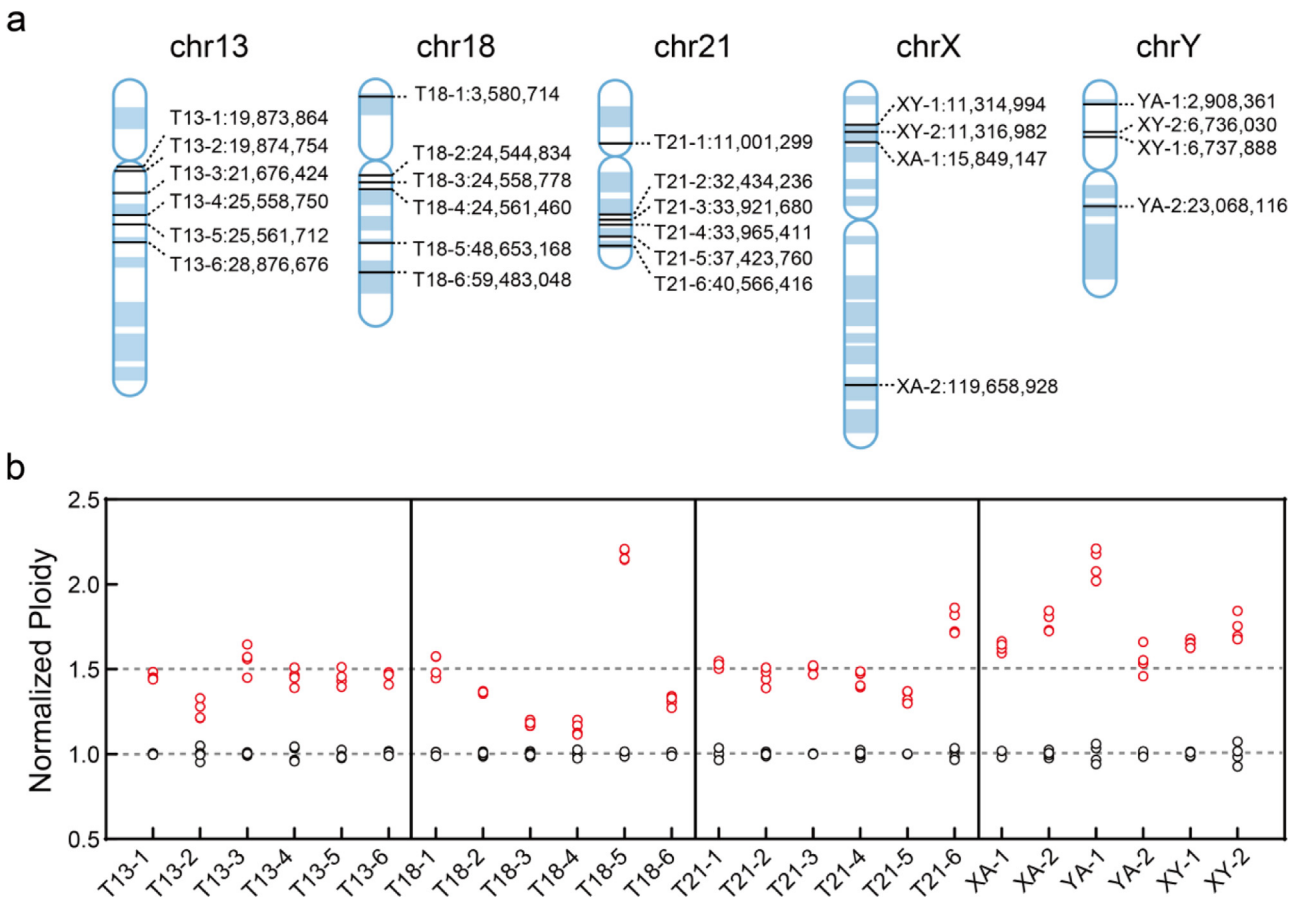

Fig. 2. Evaluation of the primer/probe sets for the detection of five aneuploidies by MMCA. (a) The positions of the forward primers on the target chromosomes chosen for aneuploidy detection. Each number given represents the position of the first 5′-end nucleotide of the forward primer. (b) The performance of primer/probe sets tested by MMCA as indicated by the normalised ploidy values. The black circles stand for normal DNA samples, and the red ones stand for the aneuploidy samples (Student's *t*-test, $p < 0.01$).

3.2. Aneuploidy detection by MMCA

To evaluate the performance of ChAPDes derived primer/probe, six sets were randomly chosen for each SD and used to establish an MMCA assay. The MMCA assay was used to test the performance of the primer/probe sets by analysing both normal and aneuploidy reference DNA samples. From the relative peak height of the melting curves of both the target and the reference chromosome (Supplementary Fig. S1 and Table S2), the normalised ploidy value of each sample was calculated. The results showed that all of the primer/probe sets could discriminate normal from aneuploidy DNA, but with different discriminating power (Fig. 2). For example, the six candidate primer/probe sets for trisomy 13 showed remarkable discrimination between normal and trisomy samples (Student's *t*-test, $p < 0.01$). However, the ploidy values varied from 1.21 to 1.65 for the trisomy DNA. Comparatively, the ploidy values for normal DNA kept consistent at nearly 1.0. The T13−1 set displayed consistent performance in multiple assays with a ploidy value close to 1.5 for trisomy and a ploidy value close to 1.0 for the normal. Thus, it was chosen as the optimal set for trisomy 13 assay. In the same way, we determined the optimum primer/probe sets for other aneuploidies. They were T13−1, T18−2, T21−3, XA-2, YA-2, and XY-1 for trisomy 13, trisomy 18, trisomy 21, 45/X, 47/XXX, 47/XYY, and 47/XXY, respectively.

By using these optimum primer/probe sets, a two-reaction MMCA assay was established to detect the aneuploidies included in this study (Fig. 3a). We then examined the discrimination power of the MMCA assay by analysing the chimeric samples containing different percentages of aneuploidy DNA. The results showed that 10% mixtures could be reliably detected from the normal sample (Fig. 3b), indicating the potential of MMCA assay for detecting samples containing maternal DNA contamination. The detection limit was determined to be 25 ng of aneuploidy gDNA per reaction (Fig. 3c), suggesting that this assay could well satisfy most of the normal amounts of aneuploidy gDNA extracted from amniotic fluid or chorionic villus cells. To evaluate the clinical performance of the assay, 333 amniotic fluid samples and 130 chorionic villus cells samples were analyzed. In total, 53 trisomies (including all three investigated types of trisomy) and 37 sex aneuploidies (including four types of aneuploidy of sex chromosomes) were detected. When using G-banded karyotyping and CMA as the gold standard, the MMCA assay had three false positive samples detected, achieving an overall specificity of 99.64% and sensitivity of 100% (Table 2).

3.3. Aneuploidy detection by ddPCR using gDNA

Unlike MMCA, ddPCR system could only detect a pair of target and reference chromosomes in one reaction. Using trisomy 21 as a model target, the performance of the previously chosen primer/probe sets was individually evaluated by analysing different percentages of gDNA mixtures. Of the six SDs, T21−1, T21−2, T21−4 and T21−5 were able to differentiate 5% mimic gDNA mixtures from the normal (0%) (Supplementary Fig. S2 and Table S3). The reproducibility of ddPCR was evaluated by detecting six normal samples using the four primer/probe sets. With the exception of T21−4, all the other sets could give reproducible results (Fig. 4a). The T21−1 set was then chosen to detect 70 clinical samples (including 52 amniotic fluid samples and 18 chorionic villus cells samples). The results showed that all the samples were correctly detected when compared with the reference

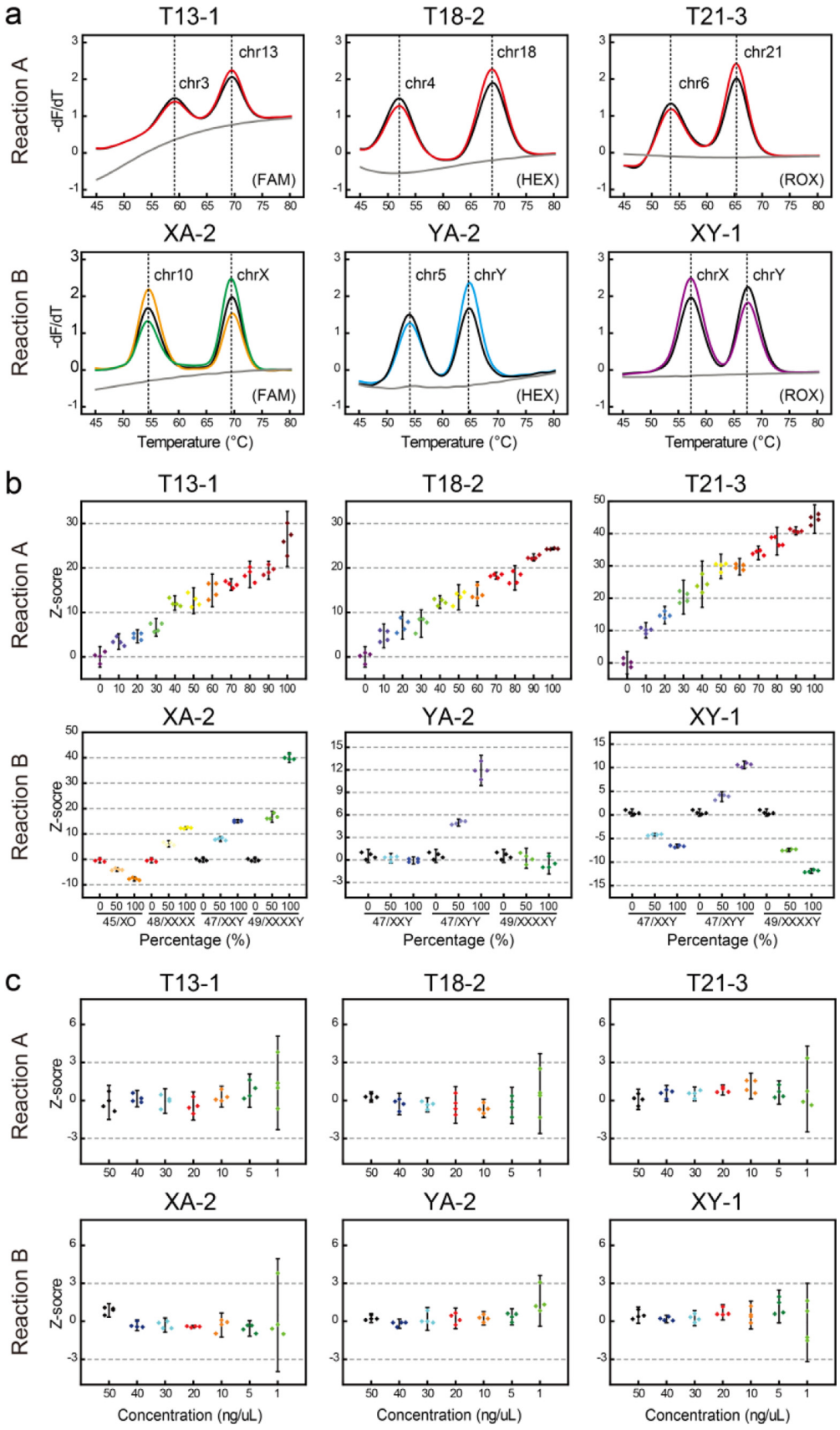

Fig. 3. The performance of MMCA-based aneuploidy assay. (a) The melting curves of the MMCA assay obtained from gDNA sample of varied aneuploidy status, including three common types of trisomy (red), and four sex chromosome aneuploidies: Turner syndrome (orange), Triple X syndrome (green), Superman syndrome (blue), and Klinefelter syndrome (purple). The normal gDNA control was shown as black lines and the NTC was shown as gray lines. (b) Discrimination power of the MMCA assay in the detection of aneuploidies. In the cases of trisomy, the gDNA mixtures contain 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% of trisomy gDNA, respectively. For sex chromosome aneuploidies, the gDNA mixtures contain 0%, 50%, and 100% of aneuploidy gDNAs, respectively. (c) The stability of the MMCA assay at different input gDNA. Normal gDNA samples containing 50, 40, 30, 20, 10, 5, 1.0 ng/μL, respectively, were tested each in four replicates (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Table 2
Aneuploidy detection of AF-cohort and CVS-cohort by MMCA.

Chromosome Aneuploidies	TP	TN	FP	FN	PPV	NPV	Specificity	Sensitivity
T21	34	429	1	0	97.14%	100.00%	99.77%	100.00%
T18	14	449	0	0	100.00%	100.00%	100.00%	100.00%
T13	5	458	2	0	71.43%	100.00%	99.57%	100.00%
ACAs	53	410	3	0	94.64%	100.00%	99.27%	100.00%
45/X	26	437	0	0	100.00%	100.00%	100.00%	100.00%
47/XXX	2	461	0	0	100.00%	100.00%	100.00%	100.00%
47/XXY	8	455	0	0	100.00%	100.00%	100.00%	100.00%
47/XYY	1	462	0	0	100.00%	100.00%	100.00%	100.00%
SCAs	37	426	0	0	100.00%	100.00%	100.00%	100.00%
Grand total	90	836	3	0	96.77%	100.00%	99.64%	100.00%

ACAs: Autosome Chromosome aneuploidies, SCAs: Sex Chromosome aneuploidies, T13: Trisomy 13, T18: Trisomy 18, T21: Trisomy 21. TP: true positive, TN: true negative, FP: false positive, FN: false negative, NPV: negative predictive value, PPV: positive predictive value. Sensitivity=TP/(TP+FN), Specificity=TN/(TN+FP).

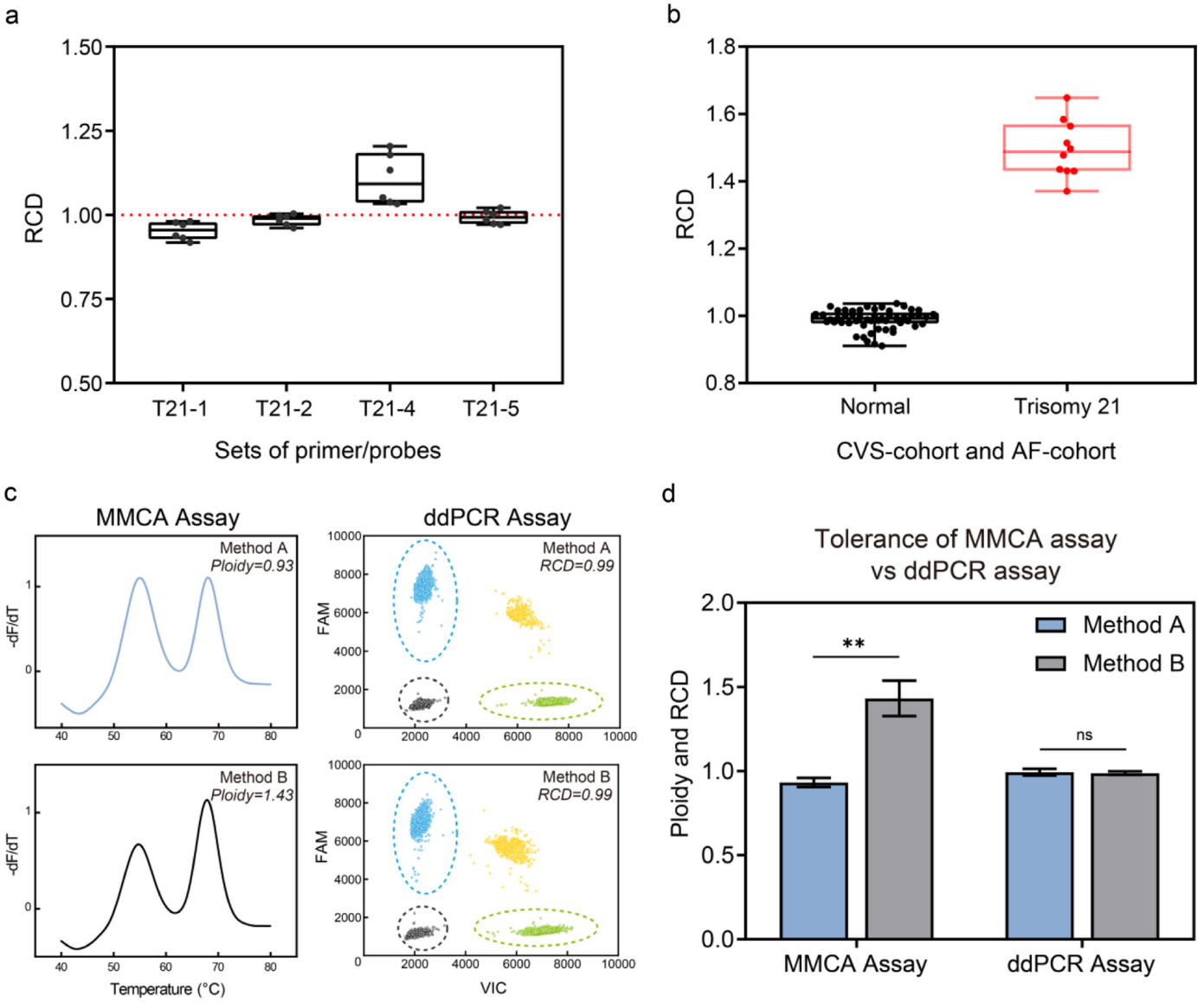

Fig. 4. The robustness of ddPCR in the detection of trisomy 21. (a) The detection results of four primer/probe sets from the normal gDNA tested in six healthy individual samples. (b) The detection results of the ddPCR assay from 60 normal and 10 trisomy 21 gDNA samples. (c) The raw data of the MMCA and ddPCR obtained from healthy individual gDNA samples by paramagnetic particle method (Method A) and phenol-chloroform method (Method B). (d) The average *Rm* ratio of ploidy and average RCD of the two assays obtained from four healthy individual gDNA samples by the two extraction methods, where "**" represents $p < 0.01$ and "ns" represents no significant difference. Data shown are mean ± standard deviation.

methods, and the obtained RCD values were close to the theoretical ones (Fig. 4b). Notably, when the three false-positive samples previously detected by MMCA were subjected to the ddPCR assay, all of them were correctly detected irrespective of the primer/probe set of SDs (Supplementary Fig. S3). Further studies showed that, ddPCR could always give correct results regardless of the gDNA extraction methods whereas the MMCA results could alter with the extraction method (Fig. 4c and d). Therefore, ddPCR detection is more tolerant to DNA impurities than the MMCA method.

3.4. Aneuploidy detection by ddPCR using cfDNA

The above results encouraged us to extend ddPCR assay to plasma cfDNA-based NIPT, which is now routinely accomplished by NGS but with lengthy, complex, and expensive procedures. A major barrier for the use of ddPCR is the low content of fetal cfDNA in plasma, which may cause bias in RCD quantification. According to the mathematical model of the relationships between precision, dynamic range, number of partitions, and sensitivity in digital PCR based on Poisson distribution [20], our calculation showed that detection of trisomy 21 in maternal plasma containing a fraction of 4% fetal cfDNA at a statistically significant RCD with 95% confidence interval needed at least 400,000 total droplets and 89,257.4 positive copies (Fig. 5a). To satisfy these demands, eight reactions were required for a ddPCR assay to detect one sample in order to reach the total droplets number using the ddPCR system in this study (50,000 droplets per reaction). Additionally, assuming that 1 mL of plasma was used for testing, the amount of extracted cfDNA was about 1−10 ng. Considering the low limit of input cfDNA (1 ng, ~300 copies of human genome equivalent), the preamplification step was necessary to increase the copy number of input cfDNA to at least 10,000 (89,257.4/8) copies per reaction.

Following the above demands, cfDNA obtained from four healthy individuals was preamplified by eight PCR cycles, followed by ddPCR in duplicate using four sets of primer/probe (T21−1, T21−2, T21−4, and T21−5). The resulting RCD of T21−1 or T21−5 was found to have smaller standard deviation and thus more stable than that of T21−2 or T21−4 (Supplementary Fig. S4a). The RCD of the combined two sets (T21−1 and T21−5) was more stable than that of the combined four sets (Supplementary Fig. S4b). Consequently, the combined T21−1 and T21−5 sets were used to detect trisomy 21 in plasma cfDNA by eight ddPCR reactions. The discrimination ability of the assay was examined by detecting varied fraction of fetal cfDNA, which was simulated by mixing fragmented gDNA containing 0, 5, 10, and 100% trisomy 21. The assay was able to discriminate 5% trisomy 21 from 0% ($p < 0.05$) and 10% trisomy 21 from 0% ($p < 0.001$) (Fig. 5b). These results demonstrated that the two-step, eight-reaction ddPCR assay could potentially detect the status of the fetus ploidy using cfDNA in maternal plasma. The entire assay included cfDNA extraction (1 h), an eight-cycle preamplification step (0.5 h), and eight-reaction ddPCR detection (2.5 h) using the combined T21−1 and T21−5 primer/probe sets, and each in four replicates.

Finally, we applied the ddPCR assay to a cohort of 15 pregnant women who were carrying a fetus with a gestational age of 14−20 weeks. Their fetus trisomy status had been tested using a routine NGS protocol. The assay results were compared to those of NGS-based NIPT assays in a double-bind manner. Of the 15 samples, two trisomy 21 samples were unequivocally different from the other 13 normal samples (Fig. 5c and Table 3). These results were 100% concordant with NGS-based NIPT.

4. Discussion

The uniqueness of SD sequences-based PCR assays lies in the use of a common pair of primers to amplify a pair of SD sequences located in the respective target and the reference chromosomes. Thus, virtually identical amplification efficiency for both sequences can be achieved even when multiple primers are present or even when the reaction conditions fluctuate [21]. This is critical in the particular case of aneuploidy detection, where a small amplification bias might mitigate the small copy number differences between euploidy and aneuploidy. Such an advantage of SD sequences has been increasingly recognised by a variety of PCR assays for aneuploidy detection [22,23].

Traditionally, development of an SD-based aneuploidy assay begins with manual searching for two SD sequences of high similarity by aligning against the human genome to ensure that one copy is located on the target chromosome, while the other is located on a reference chromosome other than the target chromosome. The similarity and difference between the two SD sequences should be carefully balanced because identical regions are required for primer design, whereas the length or composition differences are needed for differentiation analysis. Furthermore, unwanted polymorphisms, such as SNPs and CNVs, in the amplicon regions should be excluded bioinformatically. Finally, the primers designed for the selected SD sequences should be subjected to extensive evaluation in silico or experimentally. Overall, it can take months to output one candidate primer sets for aneuploidy detection manually. Consequently, existing assays often use a few common SD sequences for aneuploidy detection even if they do not fully satisfy the stringent criteria stated above [9−12].

ChAPDes integrates all the steps described above in a pipeline to remove most of the manual work. Significantly shortened time in obtaining primer/probe sets for SDs has been achieved. For example, for trisomy 21 detection, up to 5722 primer/probe sets could be generated within two working days (< 31 h). Although ChAPDes pipeline was developed to output primer/probe sets for the five common aneuploidies, it can be custom designed for other chromosomes or even certain genes. This can be simply done by changing the target chromosome SD sequences or the designated gene sequences.

The usefulness of the ChAPDes-derived primer/probe sets was demonstrated in the detection of five common aneuploidies using the MMCA approach, which has been used previously for SD-based aneuploidy detection [10]. We showed that all the candidate primer/probe sets randomly chosen were successfully applied for the detection of the five aneuploidies. When evaluated with samples from a cohort of 463 pregnant women, the established MMCA assay displayed specificity of 99.64% and sensitivity of 100% with reference to both karyotyping and CMA. It is worth noting that MMCA assay and other existing assays using SDs are all based on analog rather than digital signals. The results of this type of assay essentially indicate the relative signal difference rather than the relative copy number difference. Therefore, any signal fluctuation that occurs may compromise the final results. We noticed that three samples were detected as false positive by MMCA due to the change in the relative height of the peaks caused by different DNA extraction methods. In fact, the peak height ratio varied with different primer/probe sets in the detection of all the aneuploidies (Supplementary Fig. S1). One additional confounding variable in the MMCA assay is the stronger inhibition of the probes on the matched amplicon compared with the mismatched one, causing imbalanced amplification of the two sequences. Therefore, a normalization step is required before the ploidy is given. Similarly, HRM results are sensitive to DNA impurity, quantity, and reaction conditions [9]. In the QF-PCR, the capillary electropherogram of each peak, i.e., peak shape, height, and time, can be influenced by external voltage and electrophoresis condition as well as the service time of the capillary [11,12]. Therefore, we speculate that all the existing methods relying on analog signals could have a risk of failure despite their ease of use and wide access.

A digital detection system like ddPCR could make better use of the ChAPDes-derived SD primer/probe sets for aneuploidy detection. In the ddPCR system, the proportion of fluorescent partitions, through Poisson statistics, is used to calculate the target concentration within a well-defined confidence interval. Due to the concentration and

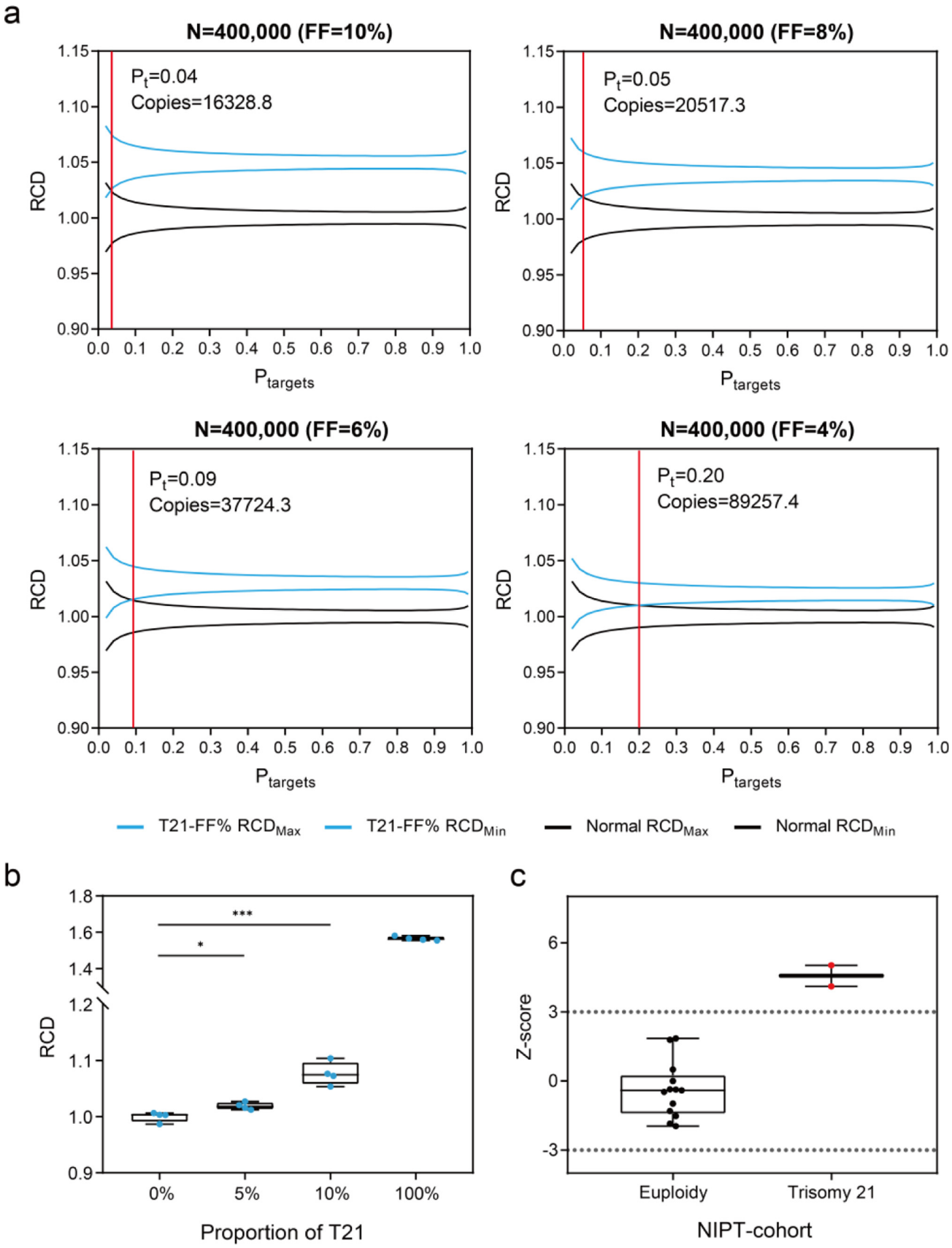

Fig. 5. Theoretical analysis and evaluation of ddPCR for NIPT of trisomy 21. (a) The precision of dynamic range related to the number of total droplets (N) and the fraction of fetal cf-DNA (FF). Based on Poisson distribution, theoretical 95% confidence bounds for a range of RCD when the number of droplets (N) were 400,000. Theoretical 95% confidence bounds for a euploid sample (RCD = 1), analyzed with T21 sample containing 4% of FF (RCD = 1.02), T21 sample containing 6% of FF (RCD = 1.03), T21 sample containing 10% of FF (RCD = 1.05), respectively. For each FF, the point of intersection between the upper bound for the euploid and the lower bound for the T21 sample is indicated by a red line. (b) The RCD obtained by ddPCR from cfDNA samples containing varied percentages of trisomy 21 in four replicates. The RCD difference between two mixtures was determined by the Student's *t*-test, where "*" represents $p < 0.05$ and "***" represents $p < 0.001$. (c) The ddPCR NIPT results from a cohort of 15 pregnant women. Z-score plus three times standard deviations is used to classify between euploid and aneuploid.

Table 3
Noninvasive aneuploidy detection of NIPT-cohort by ddPCR.

Sample	Gestationalage	Fetal fraction*(%)	Input DNA(ng)	RCD	Z-score	dPCR	NGS
122,045	15+2	6.14	3.36	1.3191	8.1673	T21	T21
10,002	14+2	9.15	4.00	1.3492	6.7864	T21	T21
13,035	14+3	6.99	2.80	1.1415	−1.3553	E	E
13,036	16+1	5.76	3.35	1.1234	−2.1850	E	E
13,037	19+5	11.93	2.22	1.2006	1.3529	E	E
13,038	17+5	3.87	1.37	1.1720	0.0438	E	E
13,039	14+3	7.24	2.31	1.1711	0.0007	E	E
13,040	18+4	6.16	2.08	1.1342	−1.6912	E	E
13,043	16+5	12.24	3.52	1.1202	−2.3329	E	E
13,044	16+1	11.24	1.90	1.1521	−0.8688	E	E
13,015	17+1	8.23	2.50	1.1687	−0.1103	E	E
13,016	16+4	5.34	1.32	1.2449	1.8467	E	E
13,020	14+1	8.92	1.73	1.1843	0.6071	E	E
13,022	16+3	9.57	1.82	1.1724	0.0587	E	E
13,023	17+3	13.03	1.92	1.2429	1.7854	E	E

* The fetal fraction was obtained from NGS. E Euploidy, T21 Trisomy 21.

purification effects, ddPCR substantially improves precision in counting single molecules and resolves a small number of copies in the presence of inhibitors or other populations [24,25]. Introduction of ddPCR to SD-based PCR would improve the performance of aneuploidy detection due to the synergetic effect: On the one hand, ddPCR could provide absolute quantitative results, and on the other hand, the two SD sequences could retain their original copy number ratio after PCR due to the identical amplification efficiency.

The improved performance of ddPCR is reflected in the better accuracy and precision when compared with MMCA [26]. We showed that the three aneuploidy false positive samples (two with trisomy 13, and one with trisomy 21) from the MMCA assay were all correctly detected by ddPCR regardless of the primer/probe sets (Supplementary Figure S3), and ddPCR provided consistent results irrespective of the DNA extraction methods (Fig. 4c and d). Using the described protocol, ddPCR could reliably differentiate 1.05-fold dosage variation in the detection of trisomy 21 (Fig. 5a). When applied to NIPT of a cohort of 15 pregnant women who were carrying a fetus with a gestational age of 14−20 weeks, two trisomy 21 samples were unequivocally identified from the other 13 normal samples, demonstrating a 100% concordance with the NGS results (Fig. 5c and Table 3). These results revealed that integration of ddPCR with SD sequences enabled NIPT of the fetus aneuploidy.

Despite the excellent performance of SD sequences for aneuploidy detection, their real implementation as biomarkers for trisomy detection ether invasively or non-invasively in clinical condition warrants further validation. Because such aneuploidy detections rely solely on the certain differences in the paired SD sequences, any unknown polymorphism that occur in these sequences of individuals might distort the analysis. Therefore, an aneuploidy detection based on a single pair of SD sequences can be potentially problematic. In this context, the primer/probe sets chosen in this study cannot guarantee their practical use before a large-scale validation study is conducted. This issue, nevertheless, can be overcome by choosing multiple SD sequences as targets as already found in other biomarkers. Thus, ChAPDes, a program that can automatically generate a large number of SD primer/probe sets, could expedite the final use of SDs for aneuploidy detection in clinical settings.

Nearly a decade ago, the use of digital PCR for NIPT of aneuploidy was first proposed [19,27]. However, until recently, few studies have been evaluated with real clinical samples, and the accuracy of these studies is limited [28−30]. These studies used regular gene sequences rather than SD sequences as targets, resulting in biased amplification of target and reference chromosomes, untrue ratio of the target to reference ratio, and uncertain aneuploidy classification. Thus, by changing to SD sequences, it can be expected that the robustness of ddPCR-based NIPT for triploids could be significantly improved [31]. Moreover, when multiple SD sequences obtained with ChAPDes are used as targets for multi-color ddPCR and even for NGS, which represents a particularly powerful form of dPCR in that hundreds of millions of template molecules can be analyzed one by one, it can be anticipated that even lower fractions of fetal DNA could be analyzed and thus earlier pregnancy NIPT might be realized. Beyond these common aneuploidies, other chromosome aneuploidies, microdeletions, and microduplication syndromes could also be detected in the NIPT once their SD sequences are available.

Declaration of Competing Interest

The authors declare no competing interests.

Contributions

Xinwen Chen, Yifan Li, Qingge Li and Zhi-Liang Ji developed and designed the methodology. Ting Wang and Yinghua Liu provided study materials and clinical samples. Xinwen Chen, Qiuying Huang, Yafang Wang and Ying Liu has verified the underlying data. Yifan Li, Xingming Lin and Qiushun He designed the computer program and implemented the computer code. All authors contributed to the acquisition, analysis, or interpretation of data. All authors contributed to revision of the manuscript. All authors read and approved the final manuscript.

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Data sharing statement

The Segmental Duplication Database from She X et al. was downloaded at https://humanparalogy.gs.washington.edu/. The ChAPDes program has been packed and the software deposited on GitHub (https://github.com/crystal525/The-Chromosome-Aneuploidy-

Primer-Designer.git) for free access. ChAPDes generated data presented in this paper can be found on Additional file 1.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ebiom.2021.103535.

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