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A method for improving the accuracy of non-invasive prenatal screening by cell-free foetal DNA size selection

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ABSTRACT

Background: Non-invasive prenatal screening (NIPS) using cell-free foetal DNA (cfDNA) has been widely used for identifying common foetal aneuploidies (e.g. trisomy 21 (T21), trisomy (T18) and trisomy 13 (T13)) in clinical practice. The sensitivity and specificity of NIPS exceeds 99%, but the positive prediction value (PPV) is approximately 70% (combined T21, T18 and T13). Thus, some 30% of pregnant women who have positive NIPS results are eventually identified as normal by amniocentesis. These women therefore must undertake needless invasive tests and risk miscarrying healthy babies because of false positive NIPS results.

Methods: In order to achieve higher accuracy, we amended the standard NIPS (s-NIPS) protocol with an additional cfDNA size selecting step in agarose-electrophoresis. The advantage of the new method (named e-NIPS) was validated by comparing the results of e-NIPS and s-NIPS using 114 retrospective cases selected from 15,930 cases.

Results: Our results showed that the foetal cfDNA fraction can be enriched significantly by a size selection step. With this modification, all 98 negative cases and 9 of 11 false positive cases of s-NIPS were correctly identified by e-NIPS, resulting in an increased PPV from 71% to 77%. Additionally, a simulation test showed that e-NIPS is more reliable than s-NIPS, especially when the foetal cfDNA concentration and sequencing coverage are low.

Conclusion: cfDNA size selection is an important step in improving the accuracy of non-invasive prenatal screening for chromosomal abnormalities.

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KEYWORDS
Non-invasive prenatal screening; cfDNA size selection; agarose gel electrophoresis; low-coverage sequencing

Introduction

Non-invasive prenatal screening (NIPS) has been widely used in clinical practice to detect foetal aneuploidies (such as T21, T18 and T13) using whole-genomic sequencing cell-free DNA (cfDNA) from maternal blood. With a sensitivity and specificity <99%, it is a viable alternate to traditional screening methods, and a routine prenatal screening option recommend by both the American Congress of Obstetricians and Gynecologists (ACOG) and the American College of Medical Genetics and Genomics (ACMG) [1,2]. However, there is considerable room for improvement of the method. For example, an ACOG report suggests that the positive predictive value (PPV) of NIPS for pregnant women aged 40 years old is 70% (T21:87%, T18:68% and T13:57%) [1]. For younger pregnant women (age of 25), the PPV falls to 18% (T21:33%, T18:13% and T13:9%). In our clinical practice of 15,930 cases, the PPV of the standard NIPS protocol is also nearly 70% (Table 1), consistent with previous studies [3–6]. Thus, nearly 30% of positive NIPS results are in fact incorrect, and these patients must adopt needless invasive testing and take the unnecessary risk of miscarriage.

The most likely major reason for the low PPV arises from the mixed nature of cfDNAs (consisting of both maternal and foetal DNA) that is sequenced by next-generation sequencing technologies. In maternal plasma, only 10–20% of cfDNAs are from the foetus at 10–20 weeks of gestation [7]. Furthermore, foetal cfDNAs may have different karyotypes in conditions such as confined placental mosaicism (CPM) and true foetal mosaicisms (TFM) [8–10]. Thus, the percentage of foetal cfDNA may be much lower than 10–20%, and the screening result of NIPS can be more easily falsified by contaminating cfDNA from the placenta and elsewhere. Therefore, increasing the percentage of genuinely foetal cfDNA within a total cfDNA preparation is the key to improving the accuracy of NIPS.

Previous studies indicate that <80% of cfDNA derived from pregnant women were 20 bp longer than foetal cfDNA [11,12]. Therefore, it is possible to enrich cfDNA by size-selection using simple DNA electrophoresis before making a DNA sequencing library. We reasoned that this should reduce the interference from maternal cfDNA or CMP and so improve the signal-to-noise ratio.

Accordingly, using material from 114 retrospective cases, we set out to improve the standard NIPS protocol (s-NIPs) with an additional DNA sizing selection step to enrich short cfDNA fragments, a new protocol we

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describe as enriched-NIPS (e-NIPS). In addition, to validate our new protocol, we performed simulation experiments using artificial samples to test for any advantage(s) of e-NIPS regarding foetal aneuploidy identification. This in part addresses the problems encountered when the foetal cfDNA concentration and sequencing coverage are low.

Methods

The workflow of s-NIPs and e-NIPS are illustrated in Figure 1. The protocol was approved by the Medical Ethics Committee of The Affiliated Suzhou Hospital of Nanjing Medical University (NJMU-2015-014). All patients provided written informed consent prior to participation, which included a summary of the testing process, potential benefits and limitations of testing and possible testing outcomes. Venous blood (10 ml) was taken into a K_2EDTA vacutainer and centrifuged twice at 1600*g* for 10 min at 4 °C. Plasma was collected and stored at −20 °C. cfDNA was extracted from 600 µl plasma (Micro DNA Purification kit, Tiangen Biotech, Beijing, China) according to the manufacturer's instructions, and quantified by Qubit 3.0 (Lift Technologies, Carlsbad, U.S.A.). The extracted cfDNA was used to generate a library by PCR using a specific kit for detecting foetal aneuploidies (T21, T18 and T13) (DAAN Gene, Zhongshan, China).

cfDNA size selection and sequencing library generation was as follows. The cfDNA was enriched first by agarose gel electrophoresis in 48 V DC and 15 min. Then, the target foetal cfDNA ranging from 120 to 150 bp in length was recycled from pre-made holes in gel [13] (Figure 1). The gel image was captured in real time by E-Gel® Safe ImagerTM Real-time Transilluminator. Recycled foetal cfDNAs were used to generate a sequencing library by microemulsions PCR (Ion OneTouch 2, Lift technologies, Carlsbad, U.S.A.). The methods of each step in NIPS were performed according to the manufacturer's protocol for the detection of foetal aneuploidies (DAAN Gene, China).

Sequencing and reads alignment were as follows. The prepared sequencing library was loaded into an Ion PI HiQ chip and sequenced with 'single-end' model in Ion Torrent (Lift technologies, Carlsbad, U.S.A.). The raw data were analysed automatically via platform-specific pipeline software of Ion Torrent (Torrent Suite, version 2.0.1) after filtering out adapters and low-quality reads. The software of TMAP (https://github.com/iontorrent/TMAP) was used to align reads to human reference genome (hg19) with the default parameters.

Data processing of sequencing was as follows. To calculate the unique mapped reads count for each chromosome, the genome was partitioned into 50 kb non-overlapping bins and the reads count of each bin counted and corrected by LOESS regression according to the guanine/cytosine content of each bin [14]. The corrected reads count of each bin was used to calculate the percentage of each chromosome in total reads. Finally, The *Z* score of each chromosome obtained according to the reported method of Wang et al. [15]. According to the percentage of *Y* chromosome in total unique reads, the foetal cfDNA concentration was estimated for a male foetus [15]. All data are presented as mean with standard deviation (SD) and student's *t*-test was used for statistical comparisons.

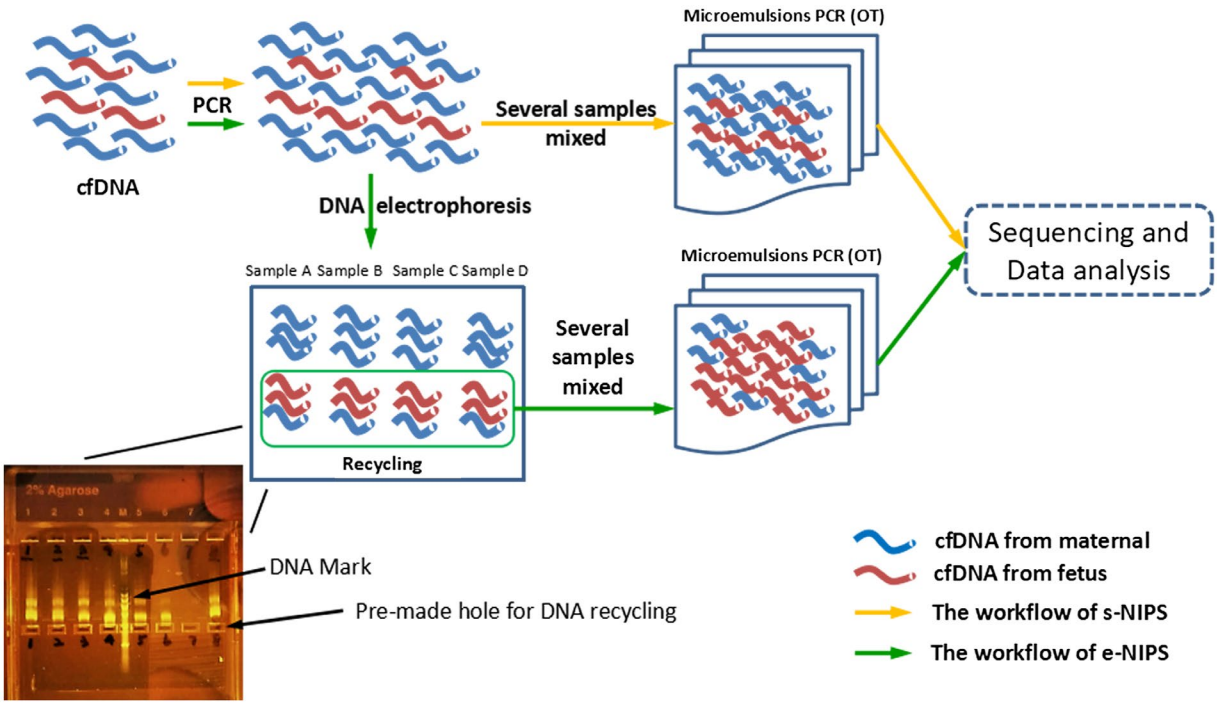


Figure 1. The workflow of s-NIPS and e-NIPS.

Results

A total of 15,930 cases, comprising 15,779 true negative cases, 28 false positive cases, 94 true positive cases and a false negative case, were tested with the s-NIPS method following amniocentesis for validation. The specificity and sensitivity of s-NIPS in all 15,930 cases were over 98% (Table 1). The combined positive predictive value (PPV) for foetal aneuploidies T21, T18 and T13 was 71%, calculated by dividing the sum of the number of true positive cases by the sum of the number of detected positive cases (Table 1).

In order to determine whether e-NIPS has a higher PPV and specificity than s-NIPs, we selected 114 samples including all positive, false positive and false negative cases and randomly selected 98 negative cases to re-analyse by e-NIPS method. The mean (standard deviation) gestation of these pregnancies was 129 (7) days, 54 were male and 62 were female. The mean (standard deviation) proportion of foetal cfDNA determined by s-NIPS was 12.6% (5.3%), whereas in e-NIPS it was 30.6% (11.2%) ($P < 0.001$). However, the difference of initial concentrations of cfDNA between s-NIPS (0.069 (0.017) μg/μl) and e-NIPS (0.067 (0.018) μg/μl) is not significant ($P = 0.41$).

The foetal cfDNA concentration of male foetuses determined by e-NIPS is increased approximately 2.5 times than in s-NIPS. The median (interquartile range) for the foetal cell-free DNA rate in s-NIPS was 12 (8-15)%, and was 29 (22-27)% in e-NIPS analyses (p<0.0001) (Figure 2(a)). The negative cases (98) and true positive cases (6) were correctly identified by new method and the most of false positive cases (9 out of 11) were categorized correctly as negative (Figure 2(B)). In all false positive cases, three out of four cases of T21 were correctly identified (accuracy: 75%), five false positive cases of T18 were discovered (accuracy: 100%) and half of false positive cases of T13 also detected (accuracy: 50%). The TPPV of 15,930 cases improved from 71% to 77% and the PPV of T21, T18 and T13 also improved to 3, 13 and 1%, respectively (Table 1). The results suggest that e-NIPS achieve a higher PPV than s-NIPs.

The false negative case had a classic characteristic of Down's syndrome (T21) in the second trimester and was reported with prenatal ultrasonography at 24 weeks of gestation: the foetal nasal bone is absent (Figure 3(C)). Using e-NIPS, we identified the case as T21 correctly and the *Z* score of chromosome 21 was significantly increased from 2.6 (s-NIPs) to 5.2 (e-NIPS) which is over the cut-off value (*Z* score = 3). It means that the false negative case has been correctly detected by e-NIPS and the result also confirmed by karyotype analysis (47XY + 21) using foetal peripheral blood at seven days after birth (Figure 3(A) and (B)). To investigate whether the false negative case can be corrected by deeper sequencing in s-NIPs, we repeated the s-NIPs experiment for the sample and increased sequencing depth by 10 times (from 4×10^6 to 4×10^7 reads). However, the *Z* scores on chromosome 21 increased only from 2.7 to 2.8 without significant improvements (Figure 3(A)). These results suggest that the gel electrophoresis-based DNA size selection is more efficient than increased sequencing depth to improve the accuracy of s-NIPS. The low foetal cfDNA concentration is a major reason for false s-NIPS results and the method of e-NIPS is more robust than s-NIPs in countering interference from maternal cfDNA.

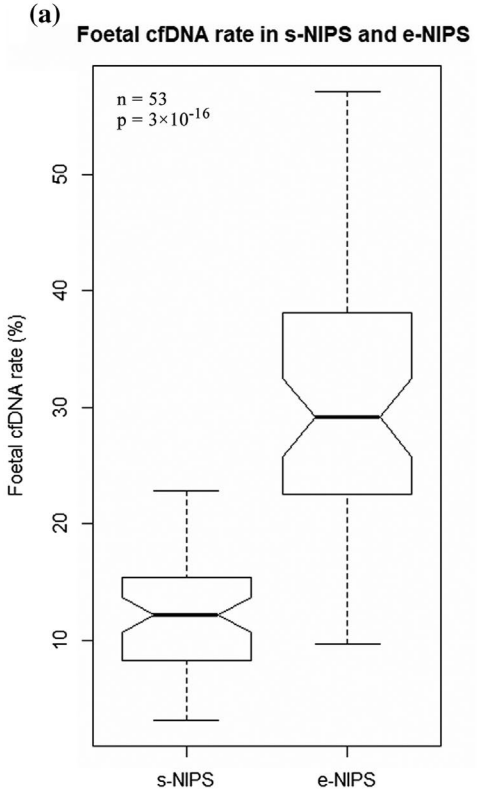
In general, the lower limit of foetal cfDNA concentration in s-NIPs is 3% [16–18]. To compare the performance of e-NIPS and s-NIPs where there is a low concentration of foetal cfDNA, we made two artificial samples by mixing the plasma of positive case (patient) into a negative case according to the mixture ratio of 2% (sample A) and 3% (sample B), respectively. All samples were tested by both s-NIPs and e-NIPS. As Figure 3(D) shows, in e-NIPS, the distribution of cfDNA length between 120 and 140 bp. In s-NIPs, they were enriched at 155–165 bp. The result showed that e-NIPS can correctly identify both sample A and sample B as positive cases, whilst s-NIPs failed in sample A detection. Furthermore, e-NIPS *Z* scores on chromosome 21 were significant higher than s-NIPS (Figure 3(E)).

Table 1. The information of cases in 15,930 s-NIPS cases and 114 selected e-NIPS cases.

	T21	T18	T13
15,930 s-NIPS cases			
True positive	74	16	4
False positive	11	11	16
False negative	1	0	0
PPV	87% (90%)	59% (72%)	20% (21%)
Sensitivity	98.6% (100%)	100%	100%
Specificity	99.93% (99.95%)	99.93% (99.96%)	99.9% (99.91%)
114 e-NIPS cases			
True positive	4 (4/100%)	1 (1/100%)	1 (1/100%)
False positive	4 (3/75%)	5 (5/100%)	2 (1/50%)
False negative	1 (1/100%)	0	0

PPV – positive predictive value. There were 15,797 negative s-NIPS cases and 98 negative e-NIPS cases. Upper panel: result of 15,930 cases using s-NIPS (figures in parenthesis are the adjusted e-NIPS adjusted. result. For example, '87% (90%)' means that the PPV of T21 has been adjusted from 87% to 90% by e-NIPS. Lower panel: the number of cases, the number/true positive ratio of identified cases of e-NIPS were listed and marked by parenthesis. For example, '4 (3/75%)' means that four false positive cases of T21 were selected in which three of them were identified by e-NIPS correctly therefore the true positive ratio of it is 75%.

(a)



(b)

ID	e-NIPT	Amniocentesis	s-NIPT	
			+/-	Type
C150601011	-	-	+	T21
C150810001	-	-	+	
C150917035	-	-	+	
C151113015	+	-	+	
C150401013	+	+	+	
C150814044	+	+	+	
C150909036	+	+	+	
C150819020	+	+	+	
C151014053	-	-	+	T18
C150815004	-	-	+	
C150909018	-	-	+	
C151012006	-	-	+	
C151015020	-	-	+	
C150424004	+	+	+	
C150807042	-	-	+	T13
C150820004	+	-	+	
C150718003	+	+	+	
C170221060	+	+	-	-

Figure 2. Summary of cfDNA concentration and results of e-NIPS, s-NIPS and amniocentesis experiments. The final concentration of cfDNA in sequencing library in s-NIPS and e-NIPS (left panel). Results of e-NIPS, s-NIPS and amniocentesis for 18 positive cases. Positive results are marked by plus (+); the negative results by minus (–) (right panel).

Discussion

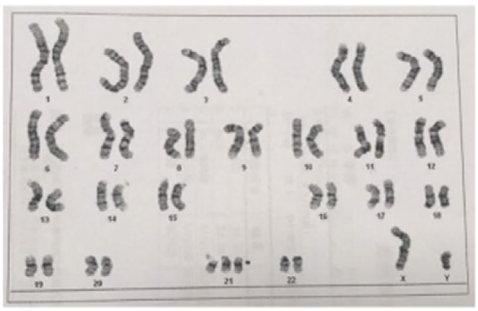
We report an improvement to the standard NIPS method (s-NIPs) by adding an additional step prior to the preparation of a DNA sequencing library, intending to enrich foetal cfDNA from total cfDNA. We name this enriching amendment e-NIPS. With the DNA size selection step, the foetal cfDNA in the sequencing library was enriched 2.5 times compared to regular s-NIPS protocol, which results in the higher signal to noise ratio in NIPS data analysis, especially when the foetal cfDNA concentration or the sequencing depth is low. Our statistical analyses results highlight the clear advantage of e-NIPS in foetal aneuploidies detection with higher accuracy and sensitivity of NIPS. Additionally, e-NIPS can be implemented easily in a clinical laboratory because the platform of DNA electrophoresis is an essential method in most PCR grade laboratories worldwide. Compared with similar findings [19,20], we provide a workable proposal to improve the accurate of NIPS by cfDNA size selection and its performance and conditions of application were evaluated and demonstrated. Finally, we confirm that the size of foetal cfDNA is smaller than maternal cfDNA and suggest that incompletely digested cfDNA may be a reason why the size of maternal cfDNA is larger than foetal cfDNA. Because the short foetal cfDNAs, which are more easily split from a nucleosome than longer ones *in vivo* [21], are more likely to cross the placenta into maternal peripheral blood by material transport in intervillous spaces. Therefore, the digesting of foetal cfDNA is more complete than that of maternal cfDNA, but the mass of the foetal cfDNA is markedly less than the quantity of maternal cfDNA [22,23]. The larger and more rapid release of maternal cfDNA from the placenta and other tissues may easily lead to incomplete digestion, and so result in the larger size of the cfDNA in maternal peripheral blood.

Although our study suggests that e-NIPS is a very promising amendment to s-NIPS, its performance need be further validated in a larger sample set. Another potential issue regarding e-NIPS is that the DNA electrophoresis and size selection step increases the possibility of cross contamination and should be performed carefully, and increased attention should be paid to this step to increase recycling rate. It is important to pay close attention to the cfDNA band's position in the gel to determine the most appropriate and opportune time points of the recycling, as this will have a bearing on the possibility of failure in recycling. Using affinity columns to purify small foetal cfDNA may be a better way to overcome these shortcomings. Additionally, the PPV for

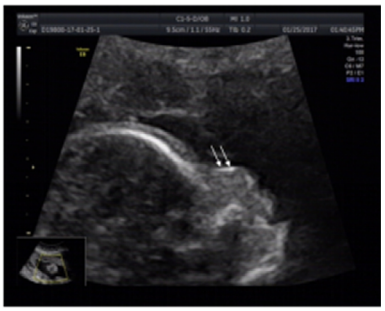
(A)

	T21	T18	T13	Unique reads	Fetal%
s-NIPS	2.71	0.46	0.28	40M	17%
s-NIPS	2.81	-0.15	-0.09	4.2M	17%
e-NIPS	5.21	0.18	0.32	4.1M	44%

(B)



(C)



(D)

(E)

	T21	T18	T13	Unique reads	SMR	Fetal%
s-NIPS	2.62	1.99	2.95	3.2M	2%	2.9%
e-NIPS	10.43	-2.63	1.14	3.4M	2%	7.9%
s-NIPS	4.61	1.82	-0.10	3.8M	3%	4.7%
e-NIPS	16.04	-1.34	-0.16	3.2M	3%	12%

SMR: Simulated mosaicisms reate

Figure 3. Detailed information for the false negative case and two artificial cases. (A) The detection result of the false negative case by e-NIPS and s-NIPS. (B) The karyotype analysis of foetal peripheral blood. (C) The image of B-mode ultrasound at 24 weeks of gestation. The white arrow marks the location of nasal bone. (D) Reads length distribution in the method of e-NIPS and s-NIPS. (E) Detection result of artificial cases by the method of e-NIPS and s-NIPS.

the determination of other chromosomal abnormalities, especially the abnormalities of sex chromosome, should be estimated by e-NIPS in a larger sample set, and a computational method should be carefully devised to estimate the percentage of X chromosomes and Y chromosomes in a preparation.

In summary, this work represents an advance in the practice of biomedical science as it demonstrates the advantage of e-NIPS over s-NIPS in the detection of common aneuploidies (T21, T18 and T13), and proves that cfDNA size selecting is helpful in achieving robust and higher accuracy NIPS screening results.

Summary table

What is known about this subject

- Non-invasive prenatal screening (NIPS) based on cell-free foetal DNA (cfDNA) is the best method for identifying common foetal aneuploidies such as T21, T18 and T13, and is widely used in clinical practice.
- The sensitivity and specificity of NIPS is <99%, but the total positive prediction value (PPV) of foetal aneuploidies (combined T21, T18 and T13) is only 70%.
- At least 25% positive or likely positive patients of NIPS have to be checked via invasive testing, and so take the additional risk that invasive tests may cause procedure-related pregnancy loss.

What this study adds

- cfDNA size selection significant enriches foetal cfDNA in sequencing libraries, therefore achieving a higher signal to noise ratio.
- e-NIPS has better PPV and performance than s-NIPS in detecting common foetal aneuploidies (T21, T18 and T13).

Disclosure statement

No potential conflict of interest was reported by the authors.

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