

Original Article

Count-based size-correction analysis of maternal plasma DNA for improved noninvasive prenatal detection of fetal trisomies 13, 18, and 21

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Abstract: Purpose: Our goal was to derive more sensitive and accurate Z-scores based on combined DNA count- and size-based algorithms to advance molecular diagnostics for noninvasive prenatal testing of fetal trisomies. Methods: We included 180 cases at high risk for fetal aneuploidy who underwent amniotic fluid cytogenetic analysis. We calculated their traditional count-based Z-scores, as well as their 100-, 130- and 150-, and 166-bp size-corrected Z-scores, and determined each Z-score's reliability based on its comparison to the cases' cytogenetic results. Results: We detected for trisomies 13, 18, or 21 among the 180 cases in our study by amniotic testing and DNA sequence analysis. None trisomies 13 was detected, while 1 case of trisomies 18 and 3 cases of trisomies 21 were found. The sensitivity, specificity, and accuracy of traditional count-based Z-scores were 75%, 98.86%, and 98.33%, respectively, while these rates increased to 80%, 99.43%, and 99.44% with a count-based 100- and 166-bp size correction. Moreover, the sensitivity, specificity, and accuracy of count-based Z-scores with 130- and 150-bp size corrections were 100%, and neither of these algorithms yielded false positive trisomies, unlike other evaluated size correction Z-scores. Conclusions: Count-based Z-scores with 130- and 150-bp size corrections more robustly predict fetal trisomies than count-based methods alone or those combined with other size-correction cutoffs, such as 166 bp. These testing parameters may enhance the utility of DNA sequence-based methods for noninvasive prenatal detection of fetal trisomies.

Keywords: Fetal aneuploidy, trisomy, noninvasive prenatal testing, Z-score, crossover study

Introduction

Using maternal plasma fetal DNA for noninvasive prenatal testing (NIPT) is a dynamic area in research [1, 2] and is possible due to the presence of cell-free fetal DNA in the plasma of pregnant women [3]. The traditional way to distinguish fetal aneuploidy from euploidy is based on tag counting analysis that yields a DNA count-based Z-score that relies on genomic representations of the test and reference chromosomes [4].

Cell-free DNA molecules are typically ≤ 200 bp [5], and circulating fetal DNA molecules are generally smaller than maternal DNA molecules. The distinctive difference between maternal and fetal DNA in plasma relates to fetal DNA's reduced proportion of molecules ≥ 166 bp and corresponding increased propor-

tion of molecules ≤ 150 bp [6]. A DNA size-based Z-score, an alternative to count-based methods for detecting fetal aneuploidy that relies on this difference, has been reported [7]. This more recent size-based approach relies on plasma DNA fragment size and compares proportions of fragment sizes from reference chromosomal DNA with those from the patient.

The size-based algorithm to detect fetal aneuploidy has demonstrated clinical effectiveness, although combining DNA count- and size-based analyses may yield more robust prenatal test results. However, few reports have relied on crossover studies to compare derived Z-scores with amniotic fluid cytogenetic results. Thus, we devised a novel method for deriving Z-scores from combined count- and size-based algorithms and used crossover study data to evaluate its reliability and potential for advancing

Novel Z-score detection of fetal trisomies

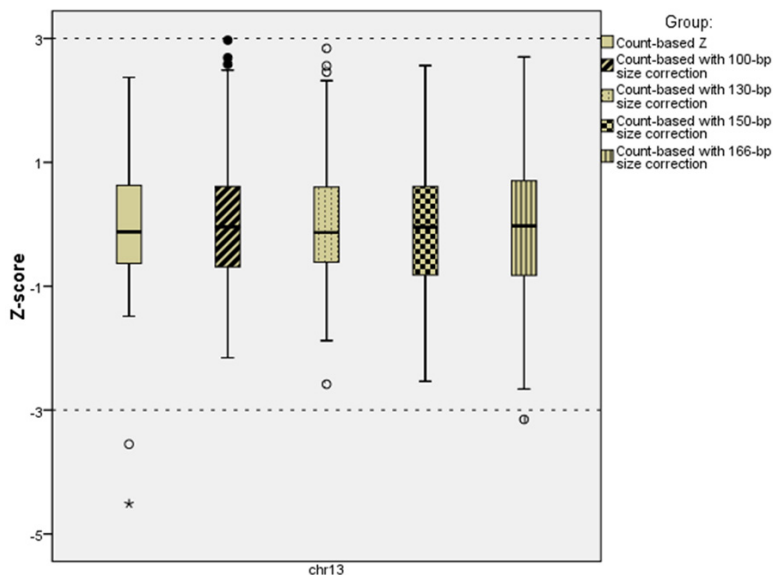


Figure 1. Detection of fetal trisomy 13 using the traditional count-based Z-scores and count-based Z-scores with 100-, 130-, 150-, and 166-bp size correction. Dashed lines indicate Z-score cutoffs.

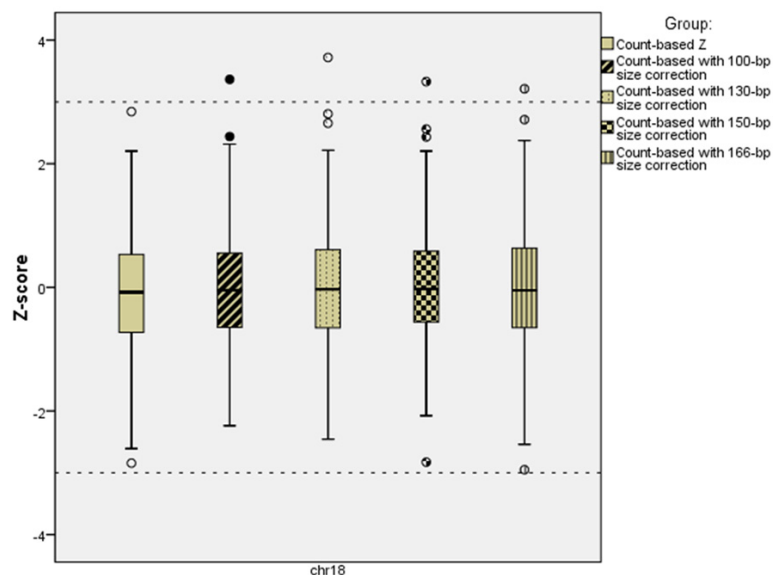


Figure 2. Detection of fetal trisomy 18 using the traditional count-based Z-scores and count-based Z-scores with 100-, 130-, 150-, and 166-bp size correction. Dashed lines indicate Z-score cutoffs.

noninvasive prenatal detection of fetal trisomies.

Materials and methods

Participants

We included 180 cases at high risk of fetal aneuploidy due to one or more risk factors,

including advanced maternal age (≥ 35 years), positive serum analyte screen, or abnormal fetal ultrasound [8]. The study proposal was approved by the hospital ethical committee. Informed consent was obtained from all individual participants in the study. Results of the study were fully disclosed and explained to all participants.

Experimental design

Participants in Group 1 gave maternal plasma for noninvasive prenatal crossover testing in triplicate, which involved amniotic fluid studies and DNA sequence-based Z-score calculations. Participants in Group 2, who were blinded to Group 1, underwent only amniotic fluid studies and served as reference controls.

Massively parallel sequencing

We selected a standard procedure for DNA sequencing preparation [9] and performed massively parallel DNA sequencing with an Ion Proton™ Sequencer (ThermoFisher Scientific, Waltham, MA). All sequenced reads were aligned to the non-repeat-masked human reference genome (hg19) with the Short Oligonucleotide Alignment Program 2.

Tag counting analysis

We calculated means and standard deviations of genomic representation (GR) in the tested chromosome (GR_chrN) of the reference controls and computed a count-based Z-score for each chromosome in each test sample using the following equation [4]:

$$\text{Count-based Z-score}_{\text{chrN}} = (\text{GR}_{\text{chrN}}_{\text{sample}} - \text{mean GR}_{\text{chrN}}_{\text{ref}}) / \text{SD GR}_{\text{chrN}}_{\text{ref}}$$

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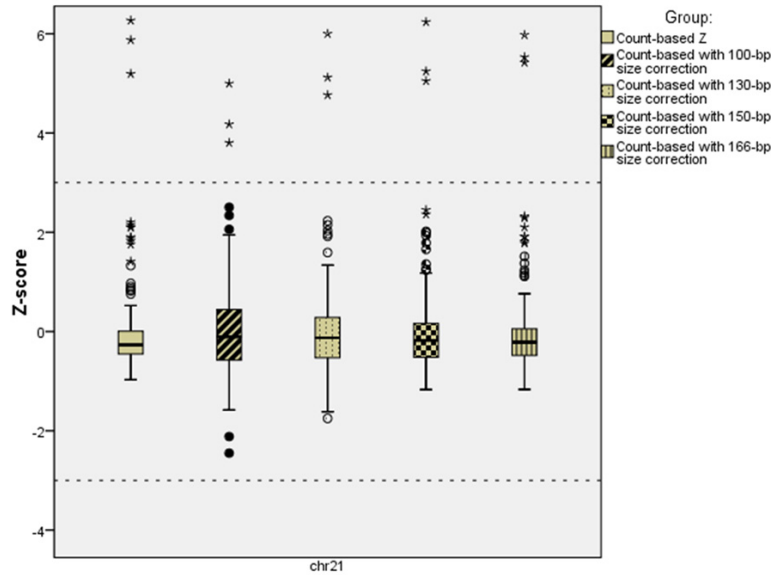


Figure 3. Detection of fetal trisomy 13 using the traditional count-based Z-scores and count-based Z-scores with 100-, 130-, 150-, and 166-bp size correction. Dashed lines indicate Z-score cutoffs.

proportion (Pf) was calculated with the following equation:

$$Pf = \frac{P_{less_150 \text{ bp_chrN}}}{P_{less_150 \text{ bp_chrRef}}}$$

Count-based 150-bp size-corrected GR values were computed similarly as in the traditional tag counting analysis and were defined as:

$$GRp = GR * Pf$$

Therefore, the count-based 150-bp size-corrected Z-score was calculated as:

$$Z\text{-score}_{chrN} = \frac{(GRp_{chrN_sample} - \text{mean } GRp_{chrN_ref}) / SD \text{ } GRp_{chrN_ref}}$$

Table 1. Amniotic fluid cytogenetic-based detection of aneuploidies in our study participants

	Trisomy 13	Trisomy 18	Trisomy 21	Others	Total
Karyotype	0	1	3	176	180
FISH	0	1	3	176	180

FISH, fluorescence *in situ* hybridization.

Size-based analysis

We calculated differences (ΔF_{chrN}) in the proportions of short DNA ($P_{less_150 \text{ pb}}$) between the target and reference chromosomes using the following equation [7]:

$$\Delta F_{chrN} = P_{less_150 \text{ bp_chrN}} - P_{less_150 \text{ bp_chrRef}}$$

Next, we calculated means and standard deviations of the ΔF_{chrN_sample} and obtained the size-based Z-score for each chromosome as follows:

$$\text{Size-based } Z\text{-score}_{chrN} = \frac{(\Delta F_{chrN_sample} - \text{mean } \Delta F_{chrN_ref}) / SD \text{ } \Delta F_{chrN_ref}}$$

Count-based analysis with 150-bp size correction

The proportions of short DNA ($P_{less_150 \text{ pb}}$) in the target and references chromosomes were denoted as “ $P_{less_150 \text{ bp_chrN}}$ ” and “ $P_{less_150 \text{ bp_chrRef}}$,” and their correction

Count-based GR values with 100-, 130-, and 166-bp size-correction analysis were obtained in a similar manner.

Amniotic fluid cytogenetics

Amniocytes of all 180 cases were cultured, harvested, and karyotyped. In parallel, fluorescence *in situ* hybridization (FISH) using chromosome-specific fluorescently-tagged probes provided a rapid method for confirming fetal trisomies 13, 18, and 21.

Outcome analysis

Statistical analysis was performed with SPSS version 21 (IBM Analytics, Armonk, NY) using a Z-score cutoff of >3.

Results

Fetal aneuploidy was detected in all 180 cases aneuploidy by DNA sequencing. Resulting sequences data were analyzed in triplicate for fetal aneuploidy using the traditional count-based and count-based 100-, 130-, 150-, and 166-bp size-correction algorithms in turn. Calculated Z-scores for fetal trisomies 13, 18, and 21 based on DNA sequencing are shown in **Figures 1-3**, respectively.

Fetal aneuploidy was detected in all 180 cases aneuploidy by DNA sequencing. Resulting

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Table 2. Comparison of traditional and novel Z-score algorithms for detecting fetal trisomies 13, 18, and 21 based on amniotic fluid cytogenetic analysis as the “gold standard” reference

	Sensitivity	Specificity	Accuracy
Count-based Z-score	75%	98.86%	98.33%
Count-based with 100-bp size correction	80%	99.43%	99.44%
Count-based with 130-bp size correction	100%	100%	100%
Count-based with 150-bp size correction	100%	100%	100%
Count-based with 166-bp size correction	80%	99.43%	99.44%

sequences data were analyzed in triplicate for fetal aneuploidy using the traditional count-based and count-based 100-, 130-, 150-, and 166-bp size-correction algorithms in turn. Calculated Z-scores for Data from the amniotic fluid cytogenetic analysis as the gold standard were unblinded. All karyotype results were confirmed by FISH (**Table 1**). None trisomies 13 was detected, while 1 case of trisomies 18 and 3 cases of trisomies 21 were found. Karyotypes for each of the representative trisomies 18 and 21 are shown in [Figure S1](#).

Comparisons of the sensitivity, specificity, and accuracy of each Z-score type regarding detection of trisomies 13, 18, and 21 are shown in **Table 2**.

Count-based 130- and 150-bp size-correction Z-scores yielded more accurate detection than 100- and 166-bp and size-correction Z-scores, as no false positives were observed from these two models. In contrast, false positives were detected in the traditional count-based and count-based 100- and 166-bp size-correction analyses. All count-based size-correction Z-scores exhibited higher sensitivity, specificity, and accuracy than traditional count-based Z-scores. These results indicate the nearly 100% predictive value of certain count-based size-correction Z-scores when detecting fetal trisomies in clinical practice.

Discussion

The proportion of fetal DNA in maternal plasma is an important factor affecting the accuracy of noninvasive prenatal testing [10]. Because of this lower proportion and NIPT's associated lower sensitivity, many current NIPT protocols choose a lower fetal DNA fraction detection cutoff of 3-4% [11]. Although the utility of DNA size-based Z-score analysis in detecting fetal

aneuploidies has already been established [7], we wanted to maximize this data set's importance in clinical practice by applying both tag counting and size profiling for more robust detection of these abnormalities.

We had considered performing DNA sequence analyses with a simple “OR” operator

but found this results in decreased specificity, while the “AND” operator decreases sensitivity. Therefore, this study aimed to derive a novel approach for calculating Z-scores combining multiple algorithms simultaneously. Based on the principle of NIPT, we assumed that the presence of trisomy(ies) N , values of the chromosome N 's GR and the Pf of short DNA between chromosome N and other autosomes will increase. Therefore, GR multiplied by Pf will significantly increase if both GR and Pf increase, which could improve sensitivity of detection. We designed the current study to verify this relationship and found that count-based size-correction Z-scores demonstrated greater detection sensitivity than traditional count-based Z-scores.

As described previously, fetal DNA has an increased proportion of molecules shorter than 150 bp and a lower proportion of molecules larger than 166 bp compared to the mother [6]. To this end, others have reported various cutoffs less than 160 bp for DNA size-based Z-scores [5], including 140, 145, 150, and 155 bp. In our study, 150-bp size correction had the greatest predictive value of all size cutoffs tested. We used a crossover test approach to confirm that both 130- and 150-bp size-correction Z-scores more accurately detected trisomies 13, 18, and 21 than traditional count-based Z-scores with other size corrections. Though a larger clinical sample study is needed, we predict that count-based size-correction Z-scores with 130- and 150-bp cutoffs enhance the sensitivity and specificity of DNA sequence-based trisomy detection and can expand the clinical spectrum of NIPT. In the future, we plan to increase the sample size of our study and evaluate the utility of other cutoff values to advance molecular diagnostic techniques of noninvasive prenatal genetic testing.

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Disclosure of conflict of interest

None.

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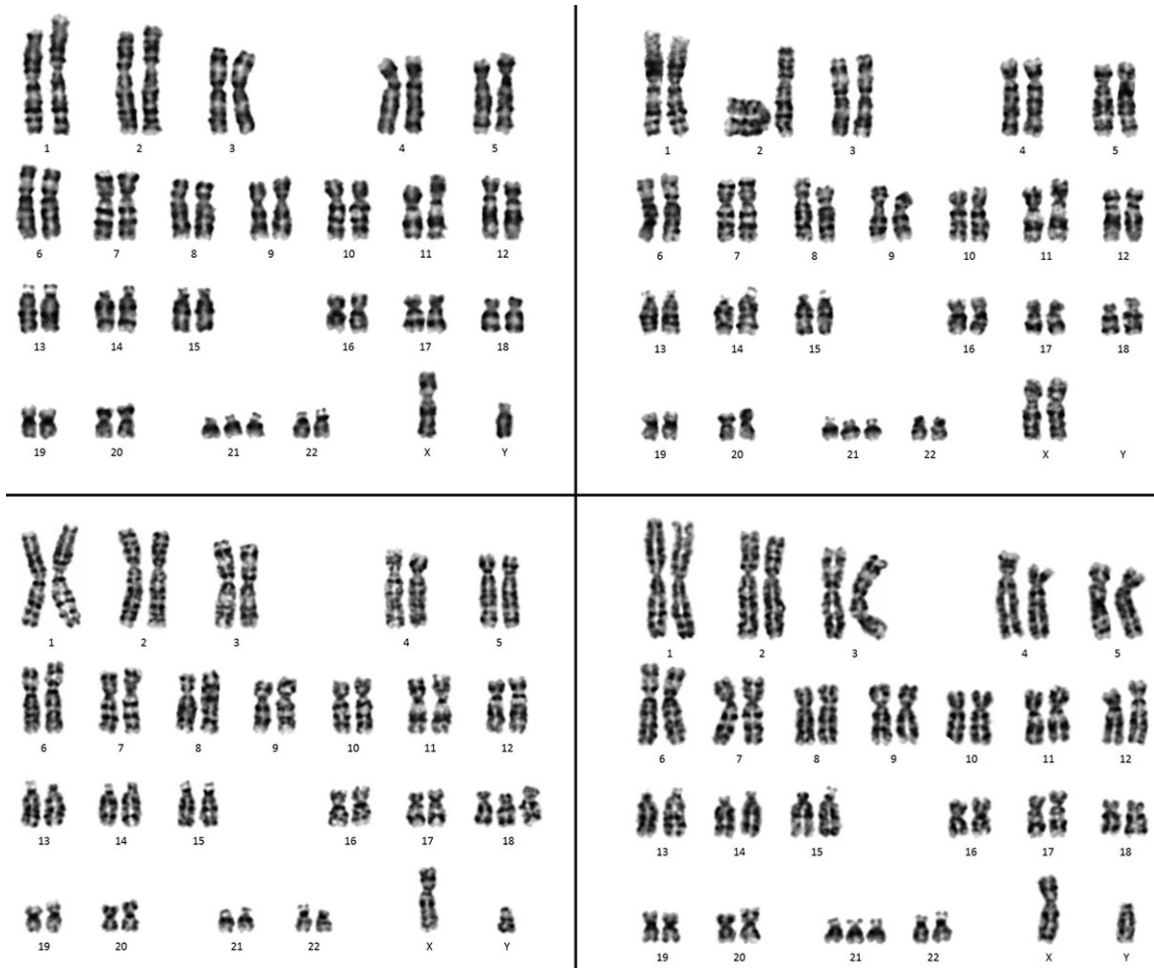


Figure S1. Karyotypes for each of the representative trisomies 18 and 21.