Ultrasound Obstet Gynecol 2014; **44**: 25–30
Published online 12 June 2014 in Wiley Online Library (wileyonlinelibrary.com). **DOI:** 10.1002/uog.13377

Genome-wide normalized score: a novel algorithm to detect fetal trisomy 21 during non-invasive prenatal testing

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KEYWORDS: genome-wide; MPSS; NIPT; plasma DNA; statistical algorithm

ABSTRACT

Objectives *Non-invasive prenatal testing for fetal trisomy 21 (T21) by massively parallel shotgun sequencing (MPSS) is available for clinical use but its efficacy is limited by several factors, e.g. the proportion of cell-free fetal DNA in maternal plasma and sequencing depth. Existing algorithms discard DNA reads from the chromosomes for which testing is not being performed (i.e. those other than chromosome 21) and are thus more susceptible to diluted fetal DNA and limited sequencing depth. We aimed to describe and evaluate a novel algorithm for aneuploidy detection (genome-wide normalized score (GWNS)), which normalizes read counts by the proportions of DNA fragments from chromosome 21 in normal controls.*

Methods *We assessed the GWNS approach by comparison with two existing algorithms, i.e. Z-score and normalized chromosome value (NCV), using theoretical approximations and computer simulations in a set of 86 cases (64 euploid and 22 T21 cases). We then validated GWNS by studying an expanded set of clinical samples (*n = *208). Finally, dilution experiments were undertaken to compare performance of the three algorithms (Z-score, NCV, GWNS) when fetal DNA concentration was low.*

Results *At fixed levels of significance and power, GWNS required a smaller fetal DNA proportion and fewer total MPSS reads compared to Z-score or NCV. In dilution experiments, GWNS also outperformed the other two methods by reaching the correct diagnosis with the lowest range of fetal DNA concentrations (GWNS, 3.83–4.75%; Z-score, 4.75–5.22%; NCV, 6.47–8.58%).*

Conclusion *Our results demonstrate that GWNS is comparable to Z-score and NCV methods regarding the performance of detecting fetal T21. Dilution experiments suggest that GWNS may perform better than the other methods when fetal fraction is low.* Copyright © 2014 ISUOG. Published by John Wiley & Sons Ltd.

INTRODUCTION

Recent development of massively parallel shotgun sequencing (MPSS) technologies enables researchers to detect fetal aneuploidies from maternal plasma; thus, the use of invasive procedures, which carry a small but significant risk of miscarriage, can be reduced[1–8]. The number of MPSS reads from distinct chromosomes constitutes relatively fixed ratios[9]. These ratios depend on a variety of factors, e.g. total length, proportion of repeat sequences and guanine-cytosine (GC) content of each chromosome.

A popular method of non-invasive prenatal testing (NIPT) for trisomy 21 (T21) using circulating fetal DNA in maternal plasma was described in 2008[6]. Each sample gave rise to a ratio of the number of reads belonging to chromosome 21 to the total read number (denoted as y_{21}), and the mean and standard deviation of y_{21} among control samples with normal karyotype were evaluated. The y_{21} value of a sample with unknown fetal karyotype was then normalized by the control mean and standard deviation to form z_{21} (equation 1). *Z*-scores above a threshold value were labeled as T21, as disproportionally more reads were from chromosome 21. *Z*-scores depend solely on chromosome 21 read numbers and thus are sensitive to variations in chromosome 21 reads.

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Accepted: 26 March 2014

Sehnert *et al.*[8] proposed an alternative algorithm by calculating the ratio of the chromosome 21 read number to the read number of a reference chromosome 9. The algorithm was termed the normalized chromosome value (NCV). NCV improves utilization of information but still does not take into account information from all remaining chromosomes.

We propose here a novel algorithm to fully utilize read counts from all 22 autosomes. The DNA read proportion of each chromosome constitutes a robust ratio among normal controls. We normalized the proportion of DNA reads originating from a specific chromosome (e.g. chromosome 21) using its corresponding ratio from normal controls. We call this method the genome-wide normalized score (GWNS). If the test sample has a normal karyotype, its GWNS for each chromosome is close to 1. Conversely, deviations in GWNS from 1 likely arise from aneuploidy. We compared the T21 detection performances of these three methods (*Z*-score, NCV and GWNS) in this study.

MATERIALS AND METHODS

Three methods of trisomy detection

Z-score

The ratio of chromosome k read number to the total read number was denoted as y_k. For a sample with unknown fetal karyotype, y_k was normalized by the mean and standard deviation acquired from normal controls:

$$z_k = \frac{y_k - \mathrm{E}\left[y_k|\text{normal control}\right]}{\sqrt{\mathrm{var}\ \left[y_k|\text{normal control}\right]}} \quad (1)$$

Chiu *et al.*[6] assumed that $z_k \sim N(0,1)$ and employed z_{21} to quantify deviation of the chromosome 21 read counts from normal controls.

Normalized chromosome value (NCV)

Because z_k depends only on the read number of the target chromosome, it is sensitive to its fluctuation. Sehnert *et al.*[8] replaced the denominator of y_k with the read number from a reference chromosome R and defined the ratio as $s_k = \frac{y_k}{y_R}$. The reference chromosome is supposed to remain euploid across all samples. They chose chromosome 9 as the reference for chromosome 21. Analogous to *Z*-scores, the ratio was normalized by the mean and standard deviation acquired from normal controls:

$$\xi_k \equiv \frac{s_k - \mathrm{E}\left[s_k|\text{normal control}\right]}{\sqrt{\mathrm{var}\ \left[s_k|\text{normal control}\right]}} \quad (2)$$

They called ξ_k NCV, assumed $\xi_k \sim N(0,1)$ and employed ξ_k to detect T21 as well as other aneuploidies.

Genome-wide normalized score (GWNS)

If counts from all chromosomes are to be used they must be transformed into comparable values. Among euploid samples, the read number from each chromosome should constitute a relatively robust ratio. Consequently, the read number ratio of chromosome k (y_k) normalized by the robust ratio should be close to 1 for all chromosomes among all euploid samples. Deviation of the normalized scores from 1 indicates an aneuploidy such as T21. Since normalized scores become comparable for all chromosomes, the significance of T21 deviation can be quantified by the normalized scores from all chromosomes instead of chromosome 21 and/or chromosome 9 alone. Specifically, these ratios were denoted as m_k ($k = 1, \ldots, 22$) where $\sum_{k=1}^{2z} m_k = 1$. m_k is proportional to the length of chromosome k if all reads are uniquely mappable and uniformly sampled from all chromosomes. In reality, m_k also depends on other factors such as GC content and repeat sequence distributions in the genome. In each control sample the ratio of each chromosome read number is normalized by m_k:

$$r_k^i = \frac{y_k^i}{m_k} \quad (3)$$

where y_k^i denotes the ratio of chromosome k reads in sample i. Normalized ratios of all chromosomes are comparable since their means are all equal to 1. Consequently, we can exploit data from all chromosomes to detect aneuploidies such as T21. For each case with an unknown fetal karyotype, we evaluated the normalized ratio of chromosome 21 reads and denoted it as r_{21}^{new}. We counted the fraction of r_k^i's in the control samples that exceed r_{21}^{new} and used it as the *P*-value of the GWNS in T21 detection.

Statistical comparison of trisomy detection methods

For each detection method, accuracy – significance (1 – type I error rate or 1 – false-positive rate) and power (1 – type II error rate or 1 – false-negative rate) are positively correlated with both fetal DNA proportions and total MPSS read numbers. Different combinations of fetal DNA proportions and total read numbers may achieve the same level of accuracy. We term the union of all these combinations an 'isoquality curve' in the two-dimensional parameter space. With a fixed level of significance and power, we compared positions of the isoquality curves among three different methods: *Z*-score, NCV and GWNS. To justify the use of GWNS before being applied to real samples, we did theoretical approximations (Appendix S1), followed by simulations on a set of simulated data as well as real MPSS data (Appendix S2).

In addition to isoquality curves, we compared detection accuracy of three methods using receiver–operating characteristics (ROC) curves. We generated ROC curves by varying detection thresholds for diluted and undiluted samples, and reported the areas under the ROC curves (AUC).

Clinical validation of GWNS

Further details are provided in Appendix S3.

Samples

Peripheral blood samples were collected from a cohort of 208 women with singleton pregnancies and plasma DNAs were extracted and used for MPSS analysis (Table S1). To evaluate GWNS, reference data from euploid pregnancies must be established in advance. In our samples, 55 normal pregnancies were randomly selected as reference controls and the remaining 128 disomy 21 (D21, including 124 euploid and four T18 cases) and 25 T21 pregnancies were used as the test sample set. Additionally, to evaluate the minimum fraction of fetal DNA that would be detected in GWNS, 14 serially diluted plasma DNA libraries derived from four T21 pregnancies (Figure S1) were also included for analysis.

MPSS

Approximately 1 ng of plasma DNA was used for DNA library construction with the beta chromatin immune-precipitation sequencing (ChIP-Seq) sample preparation kit (Illumina, Inc., CA, USA) with minor modifications. Adapter-ligated DNA fragments were first amplified for 4-cycle polymerase chain reaction (PCR) (consecutively, 10 s at 98 °C, 30 s at 63 °C and 1 min at 72 °C) and recovered after agarose (3%) gel electrophoresis. Selected DNA libraries were additionally amplified for 12-cycle PCR. Libraries were sequenced with single-end 50 cycles on GAIIx (Illumina) following an 8-plex/lane protocol.

Bioinformatic analysis

Qualified 50-bp single-end reads were aligned to the human reference genome (hg19) using the Burrows–Wheeler aligner[10]. Duplicate and imperfectly mapped reads were removed. Only reads that were unambiguously mapped to the human genome, without any mismatch, were retained for further analysis. The effect of GC bias was corrected by LOESS regression[9]. Normalized read counts were used to detect fetal T21 by applying GWNS and two previously reported methods, *Z*-score[6] and NCV[8]. In GWNS analysis, the fixed constant m_k (Table S2) was derived from read fractions of all autosomes of the 55 reference controls.

RESULTS

Comparison of ROC curves among three detection methods

The three methods yielded perfect ROC curves (AUC 1.0, or 100% sensitivity and specificity were simultaneously achieved) on undiluted samples (Figure S2). By pooling samples with dilution treatments, ROC curves (Figure 1) were degraded, yet still nearly perfect. The AUCs obtained with the three methods were 0.9794 (*Z*-score), 0.9762 (NCV) and 0.9794 (GWNS). Prediction accuracy was sensitive to total read numbers and fetal DNA fractions. We varied combinations of six possible total read numbers and 22 possible fetal DNA fractions and compared AUC orders of the three methods in all parameter combinations among simulated data and reads resampled from experimental data.

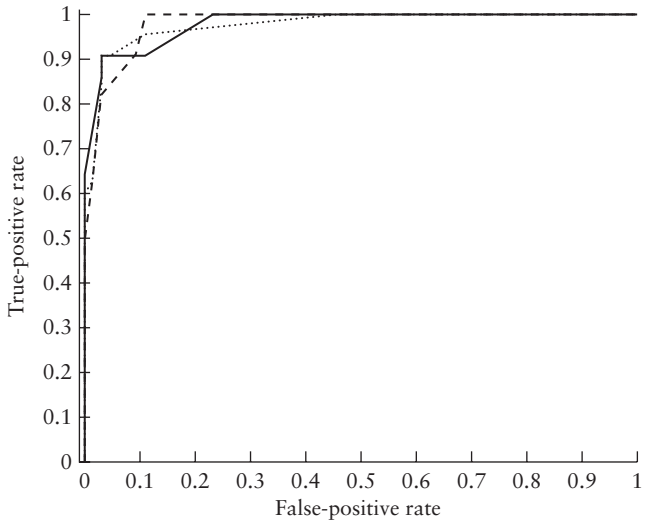


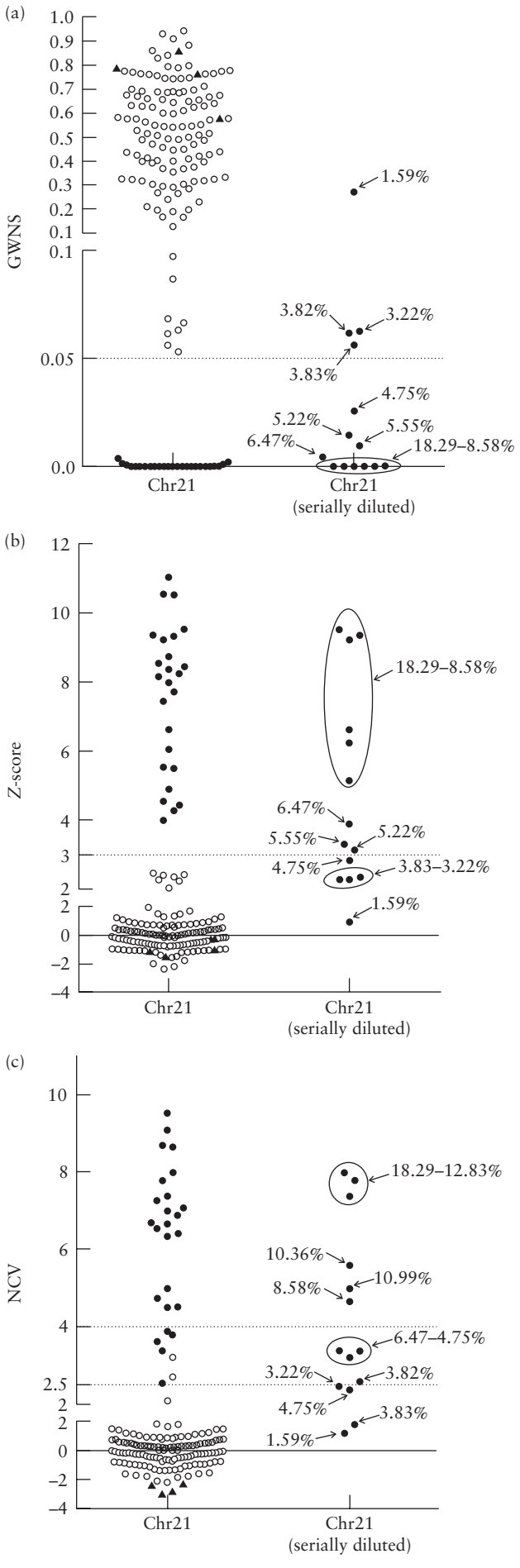
Figure 1 Receiver–operating characteristics (ROC) curves of three detection methods for trisomy 21 applied to a diluted dataset of 86 clinical test samples (64 euploid and 22 trisomy 21 pregnancies) (Table S2.1 in Appendix S2). The areas under the ROC curve for the *Z*-score (——), normalized chromosome value (·····) and genome-wide normalized score (- - -) methods were 0.9794, 0.9762 and 0.9794, respectively.

Table 1 reports the numbers (and percentage) of parameter combinations from simulation and resampled experimental data for which each of the three methods prevailed in terms of AUCs. On both simulated and resampled data, GWNS prevailed in the highest numbers

Table 1 Prediction accuracy* for each method (*Z*-score, normalized chromosome value (NCV) and genome-wide normalized score (GWNS)) based on simulated and resampled experimental data

Dataset	*Combinations* (n *(%))*
Simulated data	
Z-scores	32 (24)
NCV	28 (21)
GWNS	46 (35)
Experimental data	
Z-scores	87 (33)
NCV	42 (16)
GWNS	100 (38)

*Prediction accuracy was measured by the area under the receiver–operating characteristics curve (AUC). Each entry specifies the number (percentage) of combinations (six total read numbers, 22 fetal DNA fractions) for which each method achieved the best performance in terms of AUC. There were 132 parameter combinations in simulation studies. In real experimental data analysis, an additional parameter of the number of resampled data points (1 or 10) was introduced. Hence, there was a total of 264 parameter combinations.



of parameter combinations compared with others. For instance, on resampled data GWNS had higher AUCs than did other methods in 100 (38%) parameter combinations, whereas *Z*-scores and NCV were dominant in 87 (33%) and 42 (16%) parameter combinations, respectively.

Clinical validation of GWNS

By applying the 8-plex/lane protocol on GAIIx, we obtained 3–4 million 50-bp sequencing reads per sample. After trimming 2–5% low-quality sequences and 1–2% duplicate reads, approximately 85–89% of total reads mapped uniquely to the human genome without any mismatch. With GC correction using Loess (local regression), *c.*1.4% of reads mapped to chromosome 21 in the 55 reference controls. To determine chromosome status in a test plasma DNA, the *P*-value (i.e. GWNS) of the genome-wide normalized ratio of chromosome 21 was calculated. We set a cut-off value of $P = 0.05$ to signify a difference of a chromosome of a test sample from 95% CIs of the same chromosome in the disomy cases. Figure 2 shows the results for aneuploidy analysis of chromosome 21 in two sample sets: 153 test samples and 14 serially diluted T21 samples (see Materials and Methods). In the first sample set, all 25 T21 cases had a *P*-value < 0.05 (range, 0–0.0037) and D21 cases ($n = 128$) had a *P*-value > 0.05 (range, 0.069–0.916). Therefore, under the defined criterion of aneuploidy, $P < 0.05$, sensitivity and specificity of GWNS to detect T21 were all 100%. The results were comparable to those of *Z*-score, and superior to those of NCV for which four samples with values of $2.5 < \text{NCV} < 4.0$ were classified as 'no call' (Figure 2). In the second sample set, it was not surprising to find that not all samples were correctly classified as T21 since samples were serially diluted by maternal blood cell DNA, in which fetal DNA fractions varied from 1.59% to 18.29% (Figure 2). Using GWNS, samples with 4.75% fetal DNA fractions were correctly identified with $P < 0.05$, whereas, using *Z*-score and NCV, the minimum fractions of fetal DNA that could be detected were *c.* 4.75–5.22% and 6.47–8.58%, respectively, if the reported criteria of $|Z\text{-score}| > 3$ and $\text{NCV} > 4$ for classification of affected cases were adopted (Figure 2).

Figure 2 Trisomy 21 (T21) screening by three detection methods for samples of a clinical plasma DNA set (128 disomy 21 (124 euploid plus four T18) and 25 T21 pregnancies) and a set of serially diluted T21 plasma DNA samples (14 samples including fetal DNA fractions ranging from 18.29 to 1.59%, with the fetal fraction for each diluted DNA mixture indicated in the figure; see Appendix S3 and Figure S1 for details). Dotted lines indicate thresholds for diagnosis of affected fetuses using: (a) genome-wide normalized score (GWNS) ($P < 0.05$), (b) *Z*-score ($|Z\text{-score}| > 3$) and (c) normalized chromosome value (NCV) ($\text{NCV} > 4$) methods. Note that $2.5 < \text{NCV} < 4$ was classified as 'no call'[8]. ○, normal euploidy; ▲, trisomy 18; ●, trisomy 21.

DISCUSSION

Non-invasive prenatal screening of fetal aneuploidy by maternal plasma DNA sequencing has become clinically available for pregnant women but test performance is profoundly limited by counting statistics[11]. This study demonstrates that use of complete sequencing information from all chromosome reads increased detection sensitivity for fetal aneuploidy. Overall, *Z*-score and GWNS perform comparably with MPSS experimental data, and both methods are superior to NCV. However, GWNS requires smaller total read numbers and fetal DNA proportions in order to reach the same levels of type I (false-positive) and type II (false-negative) errors. This advantage can be crucial when samples are collected in early gestation or at limited sequencing depth. GWNS requires fewer fetal DNA fractions or total read numbers to achieve the same accuracy level (Appendices S1 and S2). On analysis of diluted samples and simulation experiments, GWNS also outperformed the other two methods in terms of AUCs in the highest number of total read number/fetal DNA fraction combinations (Figure 1 and Table 1). Furthermore, using GWNS, we have correctly diagnosed dozens of T21 and hundreds of D21 pregnancies with as few as 3–4 million sequencing reads per sample (Figure 2).

As shown in Appendices S1 and S2, for each detection method, accuracy is positively correlated with both fetal DNA fractions and total read numbers. Higher quantities of fetal DNA or total read numbers better reflect chromosome status and thus would improve accuracy rates. However, plasma samples with higher fetal DNA amounts are not always available from early and even mid-gestation pregnancies because fetal DNA represents only a fraction (average, 3–11%) of maternal plasma DNA[12,13], and there is almost no difference in the fraction of fetal DNA during first- and second-trimester pregnancies[13]. A low fetal DNA fraction (< 4%, or even sometimes in the range of 4–7%) apparently affects detection accuracy and is one of the most common causes of assay failure[14,15]. A marked increase in fetal DNA fractions was found only in the third trimester[13]. Yet, tests performed within this gestational-age window provided no benefit for early detection of fetal aneuploidies.

Alternatively, accurate detection of fetal aneuploidies can be achieved by increasing total read numbers. Using sequences that allow one or two mismatches for genomic alignment, or MPSS with deeper coverage, are practical approaches to meeting this goal. However, our MPSS experimental data showed that 89–91% of the trimmed reads are uniquely mapped to single positions of the human genome without any mismatch. Thus, incorporation of 'imperfectly matched reads' contributes only slightly to the number of aligned reads. Deeper sequencing can definitely provide more usable reads for analysis, but an increase in read numbers also elevates test cost and sequencing time. Therefore, introduction of a new statistical algorithm, as described in this study, is more economical and thus will be practical for sensitive detection of fetal aneuploidy.

We observed that GWNS correctly distinguished between all our samples of T21 and D21, even in cases with very low fetal DNA fractions. By using serially diluted T21 plasma DNA, we estimated that 4.75% of fetal DNA fraction is sufficient for T21 detection using GWNS (Figure 2a). The results support the performance of MPSS across a broad gestational age range. After 10 weeks of gestation, when the majority of pregnant women tend to accept testing, fetal DNA fractions are usually large enough for testing[13]. Conversely, for correct detection of T21 by other methods, higher fetal DNA fractions are frequent requisites[13,14]. In the same dataset that we tested using GWNS, the minimum fractions of fetal DNA that could be correctly detected by *Z*-score and NCV were estimated to be *c.* 4.75–5.22% and 6.47–8.58%, respectively (Figures 2b and c).

The demand for a higher fetal DNA fraction for T21 detection may be attributed to the statistics used in the previously reported algorithms. Both *Z*-score and NCV are based on *Z* statistics which quantify deviation in read ratio of chromosome(s) of interest (e.g. chromosome 21) from the normal control. In normal pregnancies, because the read ratio of chromosome 21 seems consistent, *Z* statistics present no response to the fetal DNA fractions[16]. In other words, not only euploid samples but also aneuploid samples with modest fetal DNA fractions, may lead to low *Z*-scores and NCV values that theoretically indicate euploidy (Figures 2b and c). On the contrary, sensitive detection of T21 with lower fetal DNA fractions indicated that GWNS was responsive to fetal DNA fractions in both euploid and aneuploid pregnancies (Figure 2a). We consider this characteristic to be important with regards to clinical testing.

An additional advantage of GWNS is that it normalizes the DNA read proportion of each chromosome by its fixed ratio acquired from a collection of normal samples, whereas most existing computational methods focus on chromosomes with observed aneuploidy (e.g. chromosome 21) and discard information from DNA reads of other chromosomes. In this study, the normalized ratios of distinct chromosomes are directly comparable because ratios are all centered around 1 amongst normal fetuses. Consequently, we can exploit data from all chromosomes to detect fetal aneuploidies of each chromosomal pair.

ACKNOWLEDGMENTS

We would like to thank Wen-Hsiang Lin and Mei-Hui Lee for help in sample collection and genetic counseling. This study was sponsored by research grants from Changhua Christian Hospital (CCH), Taiwan [101-CCH-IRP-40 to G.C.M. and 102-CCH-IRP-034 to M.C.] and the Chang Gung Memorial Hospital (CGMH), Taiwan [101-1237A3 to P.J.C.].

Disclosures

Y. S. Lin is an employee and holds equity in Welgene biotech company. M. Chen and G. C. Ma are collaborating with the Welgene biotech company. Part of the content

of this study has been included in filing for patents to the Taiwanese government in September, 2013 (Taiwan is an official member of World Trade Organization).

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Appendix S1 Theoretical approximations of three algorithms: Z-score, normalized chromosome value (NCV) and genome-wide normalized score (GWNS).

Appendix S2 Simulation analysis of the isoquality curves.

Appendix S3 Clinical validation of GWNS.

Figure S1 Simple linear regression of fetal DNA fractions and serially diluted plasma DNA libraries in four trisomy 21 pregnancies.

Figure S2 ROC curves of three detection methods on undiluted samples.

Table S1 Profiles of 208 pregnancies with maternal plasma DNA sequencing.

Table S2 Fixed constant m_k values used for GNWS analysis.