# Chapter 4

# Successful Noninvasive Trisomy 18 Detection Using Single Molecule Sequencing

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## *Abstract*

**Background:** Noninvasive trisomy 21 detection using massively parallel sequencing is achievable with high diagnostic sensitivity and low false positive rates. Detection of fetal trisomy 18 and 13 has been reported as well, but seems to be less accurate using this approach. Reduced accuracy can be explained by PCR introduced guanine-cytosine (GC) bias influencing sequencing data. Previously, we demonstrated that sequence data generated by single molecule sequencing show virtually no GC bias and result in a more pronounced noninvasive detection of fetal trisomy 21. In this study, single molecule sequencing was used for noninvasive detection of trisomy 18 and 13.

**Methods:** Single molecule sequencing was performed on the Helicos platform using free DNA isolated from maternal plasma from 11 weeks of gestation onwards (n=17). Relative sequence tag density ratios were calculated against male control plasma samples and results were compared to those of previous karyotyping.

**Results:** All trisomy 18 fetuses were identified correctly with a diagnostic sensitivity and specificity of 100%. However, low diagnostic sensitivity and specificity was observed for fetal trisomy 13 detection.

**Conclusions:** We successfully applied single molecule sequencing in combination with relative sequence tag density calculations for noninvasive trisomy 18 detection using free DNA from maternal plasma. However, noninvasive trisomy 13 detection was not accurate and seemed to be influenced by more than just GC content.

Recent large studies have confirmed that noninvasive prenatal diagnosis (NIPD) for fetal aneuploidies is achievable (Fan *et al.*, 2008; Chiu *et al.*, 2008; Chiu *et al.*, 2011a; Ehrich *et al.*, 2011; Palomaki *et al.*, 2011; Lau *et al.*, 2012a; Bianchi *et al.*, 2012). Using massively parallel sequencing (MPS) and subsequent quantification of chromosome specific sequences, overrepresentation of a specific chromosome can be determined with high diagnostic accuracy. Successful detection of fetal trisomy 21 (T21) in maternal plasma was shown in several clinical validation studies (Chiu *et al.*, 2011a; Palomaki *et al.*, 2011; Ehrich *et al.*, 2011; Lau *et al.*, 2012a; Bianchi *et al.*, 2012). For noninvasive detection of trisomy 18 (Edwards Syndrome, T18) and trisomy 13 (Patau Syndrome, T13), however, it seems to be more difficult to achieve similar results (Chen *et al.*, 2011; Lau *et al.*, 2012a; Palomaki *et al.*, 2012; Bianchi *et al.*, 2012). Although theoretically molecules from different regions of a genome should be sequenced uniformly by MPS, preferential amplification of sequences, depending on different guanine-cytosine (GC) content, has been observed (Dohm *et al.*, 2008; Fan *et al.*, 2008; van den Oever *et al.*, 2012). In contrast to an average GC content of chromosome 21, chromosomes 13 and 18 have a relatively low GC content (Fan *et al.*, 2008; van den Oever *et al.*, 2012). Therefore, non-uniform amplification of these chromosomes could occur on PCR based MPS platforms. As a result, several studies have used specific algorithms or internal references to correct for GC content to optimize noninvasive detection rates for T18 and T13 (Chen *et al.*, 2011; Palomaki *et al.*, 2012; Sparks *et al.*, 2012a; Lau *et al.*, 2012b).



We previously demonstrated that sequence data generated by single molecule sequencing show virtually no GC bias (van den Oever *et al.*, 2012). This specific method of sequencing requires no PCR amplification step during sample preparation or during flow cell processing and results in a more pronounced noninvasive detection of T21. Therefore, this approach could also be applicable for the detection of other common fetal aneuploidies such as T18 and T13.

To test this hypothesis, a retrospective study was performed on first and second trimester pregnant women with an increased risk for fetal aneuploidy based on previous serum screening and/or ultrasound results. Maternal peripheral blood samples were collected in EDTA coated tubes and processed within 24 hrs after collection. All blood samples were drawn at a median gestational age of 12 weeks + 6 days (range 11w +4d to 22w +1d, see Table 1) prior to an invasive procedure, except for one sample, which was obtained 6 days after amniocentesis. Plasma was obtained by double centrifugation of the blood samples and stored at -80$^{o}$C until further processing. Material from all invasive procedures was sent to our cytogenetics laboratory for karyotyping as the gold standard.

A total of 21 plasma samples were used in this study. Four plasma control samples from anonymous male blood donors and 17 samples of singleton pregnancies (Table 1), consisting of 9 cases of T18 (2 female and 7 male fetuses), 4 cases of T13 (2 female and 2 male fetuses), and 4 euploid pregnancies (all male fetuses) were included. All maternal blood samples were processed within 24 hrs after collection. Cell-free DNA was isolated from plasma using the EZ1 Virus Mini Kit v2.0. For quality control purposes, fetal sex and the total amount of free DNA in maternal plasma were determined by Real-Time Taqman PCR assays as described previously (Boon *et al.*, 2007; van den Oever *et al.*, 2012). In addition, using this data, the percentage of cell-free fetal DNA (cffDNA) for male pregnancies was estimated (van den Oever *et al.*, 2012). All samples were de-identified to the investigators before sample preparation and data analysis. Libraries were prepared according to manufacturer's ChipSeq protocol and a standard 120-cycle sequencing run was performed on the Helicos platform (Helicos BioSciences, www.helicosbio.com).

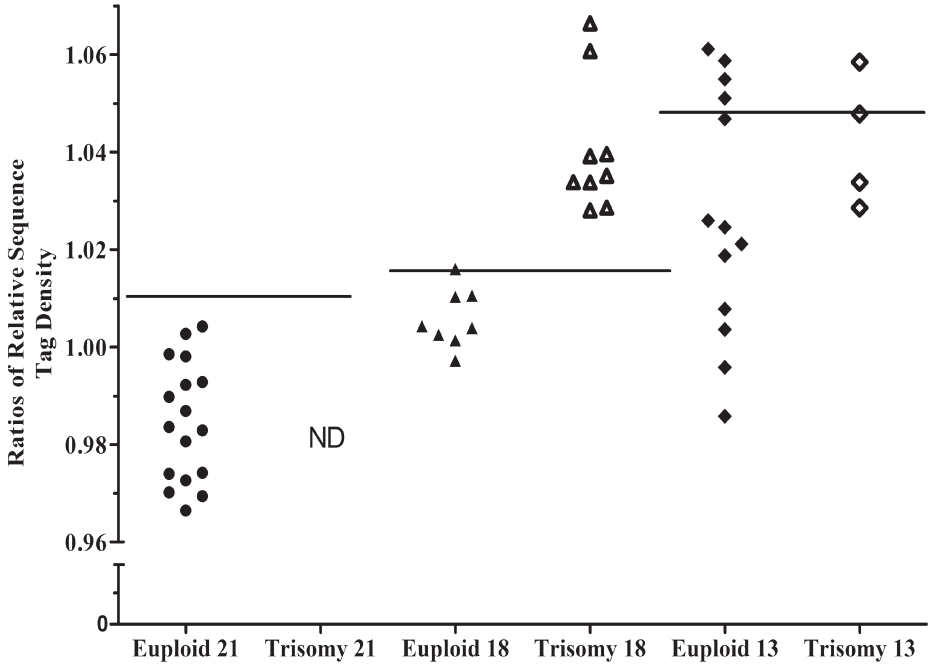


**Figure 1. Ratios of normalized Relative Sequence Tag Density for noninvasive fetal aneuploidy detection.**
Ratios were calculated against anonymous male plasma controls (n=4). Samples are divided in either disomic (closed symbols) or trisomic (open symbols) for that specific chromosome. Chromosome 21 is displayed as circles, chromosome 18 as triangles and chromosome 13 as diamonds. 99% Confidence intervals for disomic samples were calculated for each chromosome and upper boundaries are depicted in the graph as a line. Euploid fetuses (n=4), T18 fetuses (n=9), T13 fetuses (n=4). ND: Not determined.

Raw data analysis was performed with the HeliSphere software package. Ratio calculations and statistics were executed as described previously (van den Oever *et al.*, 2012). In short, for fetal trisomy detection, ratios of relative sequence tag density (RSTD) were calculated by dividing the normalized total summed number of reads for each sample by the normalized mean of male plasma controls for each chromosome of interest. After alignment against hg19 reference genome and filtering of gaps and repeats a mean of $1.21x10^6 + 0.69x10^6$ (SD) reads, with a median of $1.12x10^6$, were obtained per sample. In 11 maternal plasma samples from women carrying a male fetus, the percentage of cffDNA was estimated, resulting in a mean percentage of 11 % (Table 1).

For noninvasive T18 detection we showed that using RSTD calculations for chromosome 18, all T18 samples (n=9) were correctly identified as being aneuploid and all euploid controls and T13 samples as being disomic for chromosome 18. When constructing a 99% confidence interval from all samples disomic for chromosome 18 (n=8), all T18 samples were outside the upper boundary of the 99% CI [0.991, 1.016], while all euploid controls and T13 samples were on or below this upper boundary (Fig. 1). For noninvasive T13 detection, only 1 out of 4 T13 samples was correctly identified. False positive results (4/13) were observed in both euploid (n=2) and T18 (n=2) samples when using RSTD ratio and 99% CI calculations for chromosome

13, resulting in a diagnostic sensitivity and specificity of 25% and 69% respectively (Fig. 1). As a control we calculated RSTD ratios for chromosome 21 for all samples tested in this study (n=17) using the 99% CI previously published (VAN DEN OEVER *et al.*, 2012). All samples tested in this study were indeed identified as disomic for chromosome 21 (Fig 1). When calculating a 99% CI using RSTD results from this study a similar upper boundary was obtained, thus confirming this result.

As a follow up on noninvasive T21 detection using single molecule sequencing, in the present study we demonstrated successful noninvasive detection of T18 (100% diagnostic sensitivity and specificity) using free DNA from maternal plasma from 11w + 4d of gestation onwards. The mean percentage of cffDNA in maternal plasma in the first trimester was 4.03% and we observed an increase in fetal fraction during the second trimester, with a mean percentage of 21.1%. This observation is concordant with previous reports (LUN *et al.*, 2008a; LO *et al.*, 1998). Even though the percentage increased, we still observed quite a large range between individuals with an approximate 4-fold change for the second trimester pregnancies, up to a 13-fold difference between first trimester samples.



Compared to noninvasive detection of T18, our data showed low diagnostic sensitivity and specificity for detection of T13 using single molecule sequencing. Previous publications from other groups also reported reduced diagnostic sensitivity and/or specificity for noninvasive T13 detection (CHEN *et al.*, 2011; LAU *et al.*, 2012b; PALOMAKI *et al.*, 2012; BIANCHI *et al.*, 2012). However, the values were not as low as observed in this study. Furthermore, in these cases it was thought to be related to the GC content of chromosome 13 given that PCR based Next Generation Sequencing (NGS) platforms were used. As shown in our previous study, data for chromosome 13 are biased on such platforms (VAN DEN OEVER *et al.*, 2012). Chromosome 13, compared to 18 and 21, has the lowest GC content of all three (38.5%) (DUNHAM *et al.*, 2004). This low GC content could be reason for a misrepresentation of the amount of sequencing reads coming from these PCR based NGS platforms. However, in the current study, single molecule sequencing results were not influenced by a chromosome's GC content, implying that other factors might be involved in lowering the diagnostic sensitivity and specificity for noninvasive trisomy 13 detection.

The fetal contribution of free DNA in maternal plasma is derived from syncytiotrophoblasts undergoing apoptosis (ALBERRY *et al.*, 2007). Placental apoptosis is a naturally occurring process during gestation in both normal and abnormal pregnancies, resulting in fragmented fetal DNA circulating in the maternal circulation (HEAZELL *et al.*, 2008; ALBERRY *et al.*, 2007). Some studies have demonstrated the difference in size between fetal and maternal free DNA fragments and have even shown that the entire fetal genome is present (LO *et al.*, 2010). However, virtually no studies have considered that fetal DNA from chromosomes of different sizes and/or those of differing GC contents may fragment at different rates. Considering that chromosome 13 is the largest acrocentric chromosome with the lowest gene density among all human chromosomes (DUNHAM *et al.*, 2004), its stability may differ from chromosome 18 and 21. A less stable chromosome is hypothesized to degrade faster, which could lead to a skewed number of DNA fragments from this particular chromosome in maternal plasma. Also, several segmental duplications with at least 90% homology and regions with a high SNP density due to the presence of paralogous sequence variants have been shown for chromosome 13 (DUNHAM *et al.*, 2004). This may influence data analysis, resulting in improper assignment of reads to a certain chromosome during alignment. Which factors exactly play a role is not clear at this point and needs to be studied in more detail; however, our study suggests that sequencing

**Table 1. Overview of included maternal plasma samples.**

| *Sample number* | *Karyotype* | *Maternal age, years* | *Indication* | *GA Blood* | *CVS/ Amnio* | *SRY (pg/ 10 µL)* |
|---|---|---|---|---|---|---|
| 1 | 46,XY | 28 | Ultrasound abnormality | 12 w + 1d | CVS | 2 |
| 2 | 46,XY | 33 | Ultrasound abnormality | 12 w + 1d | CVS | 4 |
| 3 | 46,XY | 30 | Increased NT/ serum screening | 12 w + 6d | CVS | 11 |
| 4 | 46,XY | 30 | Ultrasound abnormality | 13 w + 5d | CVS | 10 |
| 5 | 47,XY,+18 | 44 | Ultrasound abnormality | 11 w + 4d | CVS | 5 |
| 6 | 47,XY,+18 | 44 | Ultrasound abnormality | 11 w + 6d | CVS | 5 |
| 7* | 47,XY,+18 | 33 | Ultrasound abnormality | 22 w + 1d | Amnio | 5 |
| 8 | 47,XY,+18 | 39 | Ultrasound abnormality | 13 w + 1d | CVS | 9 |
| 9 | 47,XY,+18 | 42 | Ultrasound abnormality | 11 w + 6d | CVS | ND |
| 10 | 47,XY,+18 | 39 | Ultrasound abnormality | 21 w + 3d | Amnio | 6 |
| 11 | 47,XY,+18 | 38 | Increased NT/ serum screening | 12 w + 6d | CVS | 14 |
| 12 | 47,XX,+18 | 36 | Ultrasound abnormality | 13 w + 3d | CVS | ND |
| 13 | 47,XX,+18 | 41 | Ultrasound abnormality | 13 w + 4d | CVS | ND |
| 14 | 47,XX,+13 | 37 | Advanced maternal age | 11 w + 4d | CVS | ND |
| 15 | 47,XX,+13 | 35 | Ultrasound abnormality | 15 w +2d | Amnio | ND |
| 16 | 47,XY,+13 | 37 | Ultrasound abnormality | 12 w + 3d | CVS | 4 |
| 17 | 47,XY,+13 | 29 | Ultrasound abnormality | 12 w + 3d | CVS | 3 |

**Table 1:** GA blood, gestational age at the time of blood collection depicted as weeks + days; amnio, amniocentesis; CVS, chorionic villus sampling; NT, nuchal translucency measurement; SRY, fetal DNA concentrations of the SRY gene were determined by quantitative real-time Taqman PCR. *This blood sample was obtained 6 days after amniocentesis. Other blood samples were obtained prior to the invasive procedure.

data are influenced by more than just GC content alone. Data analysis for noninvasive fetal trisomy 13 detection may therefore require a different approach.

In summary, we demonstrate successful noninvasive T18 detection using a combination of single molecule sequencing and relative sequence tag density ratio calculations, while non-invasive T13 detection is not accurate using this approach.

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