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Hui Lisa (Orcid ID: 0000-0002-9720-3562)

Title: Fetal fraction and noninvasive prenatal testing: What clinicians need to know

Authors

Lisa Hui MBBS PhD[1,2,3,4]

Diana W Bianchi MD[5,6]

Affiliations

1. Reproductive Epidemiology group, Murdoch Children's Research Institute, Parkville VIC Australia
2. Department of Obstetrics and Gynaecology, University of Melbourne, Parkville VIC Australia
3. Department of Perinatal Medicine, Mercy Hospital for Women, Heidelberg VIC, Australia
4. Department of Obstetrics and Gynaecology, Northern Health, Epping VIC, Australia
5. Prenatal Genomics and Therapy Section, Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA
6. *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA

ABSTRACT

The fetal fraction (FF) is a function of both biological factors and bioinformatics algorithms used to interpret DNA sequencing results. It is an essential quality control component of noninvasive prenatal testing (NIPT) results. Clinicians need to understand the biological influences on FF to be able to provide optimal post-test counseling and clinical management. There are many different technologies available for the measurement of FF. Clinicians do not need to know the details behind the bioinformatics algorithms of FF measurements, but they do need to

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appreciate the significant variations between the different sequencing technologies used by different laboratories. There is no universal FF threshold that is applicable across all platforms and there have not been any differences demonstrated in NIPT performance by sequencing platform or method of FF calculation. Importantly, while FF should be routinely measured, there is not yet a consensus as to whether it should be routinely reported. The clinician should know what to expect from a standard test report and whether reasons for failed NIPT results are revealed. Emerging solutions to the challenges of samples with low FF should reduce rates of failed NIPT in the future. In the meantime, having a "plan B" prepared for those patients for whom NIPT is unsuccessful is essential in today's clinical practice.

Bullet points

What do we already know?

- Fetal fraction (FF) is a crucial quality control parameter for interpretation of noninvasive prenatal testing (NIPT) results;
- There are highly variable laboratory practices for FF measurement and reporting;
- In some studies, failed NIPT results due to low FF are associated with an increased risk of aneuploidy.

What does this paper add?

- This paper summarizes the relevant biological and technical aspects of FF measurement for the clinician;
- The clinical management of women with failed NIPT results due to low FF should include detailed fetal ultrasound examination and an individualized discussion of the available options including diagnostic testing, NIPT redraw, and alternative screening tests.

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INTRODUCTION

Over the past decade the advent of non-invasive prenatal testing (NIPT) has necessitated the rapid education of all clinicians involved in prenatal care. Not only did NIPT abruptly displace longstanding practices in aneuploidy screening – it was also the first clinical test based on the fundamentally novel principle of analyzing circulating cell free (cf) DNA. Clinicians have therefore had to promptly implement a new screening test, as well as grasping the new biology and technology that underlies its performance.

CfDNA refers to the DNA that exists as short fragments in plasma or other body fluids, which is distinct from the DNA contained within the nucleus of an intact cell. These cfDNA fragments are released from all organs during a range of cellular processes, including apoptosis, necrosis and microparticle secretion.[1] Circulating plasma cfDNA has been intensively studied for a wide range of noninvasive "liquid biopsy" applications in oncology and general medicine but has had the most impactful translational success in prenatal screening for fetal aneuploidy.[2]

Maternal plasma cfDNA contains both maternal and fetal sources of cfDNA (Figure 1). The source of fetal DNA is the trophoblast,[3-6] while the predominant source of maternal DNA is the hematopoietic system.[7,8] All maternal organs contribute some cfDNA into maternal plasma, including solid tumors.[9]

NIPT for fetal aneuploidy employs high throughput sequencing methods ("next generation sequencing") to count the proportional representation of each chromosome in the plasma cfDNA.[10] In the nonpregnant state, the proportional representation of each chromosome in the plasma cfDNA reflects the size of the chromosome and the karyotype of the individual. In a euploid pregnant woman, a deviation from the expected chromosome profile in plasma cfDNA due to excess or deficient cfDNA fragments from a particular chromosome suggests the presence of fetal trisomy or monosomy, respectively. A statistically-significant variation in cfDNA

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fragment counts for a particular chromosome - commonly defined as a z-score > 3 – constitutes a high-risk result.

Whole chromosome imbalances, as well as sub-chromosomal copy number variants (CNVs), can also be detected using cfDNA. These include genome-wide CNVs as small as 3 Mb in size[11-13] and targeted microdeletion syndromes such as 5p– (cri du chat), 22q11.2– (Di George syndrome), 15q– (Prader-Willi/Angelman syndrome), 4p– (Wolf-Hirschhorn syndrome), 11q– (Jacobsen syndrome), 8q– (Langer-Giedion syndrome), and 1p36–.[14-16]

What is FF?

Fetal fraction (FF) is the percentage of total maternal plasma cfDNA that is of fetoplacental origin [FF= fetal cfDNA/(fetal cfDNA + maternal cfDNA). It is therefore a function of both maternal and fetal cfDNA levels in the maternal plasma. The "fetal" DNA is actually placental.[17] Between 10 and 20 weeks of gestation, the average FF is 10-15%.[18,19]

Why is FF important with regard to NIPT performance?

FF measurement is a crucial component of sample quality control and statistical confidence.[20] Measuring the FF ensures that placental cfDNA is detectable in the maternal plasma in sufficient quantity to generate a meaningful result.[21] The minimum FF threshold for adequate NIPT performance varies by assay (discussed below), but is typically between 2-4%. With a higher FF, there is greater statistical separation of aneuploid and euploid pregnancies, providing greater confidence in the final result (Figure 2). Detection rates decline with low FFs, such that a "no call" may be issued if the FF is below the lower limit of detection for that laboratory. Higher sequencing depths can be used to compensate for very low FFs, but this has significant cost implications.

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What is the significance of high fetal fraction?

There has been considerable interest in determining whether high levels of total fetal cfDNA may be an early predictor for placental pathologies such as spontaneous preterm birth[22] and preeclampsia.[23] Results, however, have been inconsistent, with other studies showing no relationship between elevated placental cfDNA levels and pregnancy complications.[24,25] There is currently no validated clinical test using absolute placental cfDNA levels or FF to predict pregnancy outcomes.

What is the significance of low fetal fraction?

Low FF can result in a test failure or a "no call" result. The rate of "no call" results range from 1-8% depending on the assay technology[26], with low FF being the most common cause. Approximately 50-60% of women with a failed NIPT result will have success with a second blood draw.[27,28] However, the major concern with a failed result due to low FF is that this has been associated with a higher risk of aneuploidy across several sequencing platforms, ranging from 2.7% to 23.3%.[29-31]

The association between low FF and an increased risk of aneuploidy generates clinical concern after a failed NIPT result. For clinicians to understand the significance of low FF, they need to understand the biological factors that can affect FF and the bioinformatics behind the interpretation algorithms.

1. THE BIOLOGY OF FETAL FRACTION

There is large interindividual variation in FF, with many biological factors influencing the maternal and placental cfDNA contributions. Any conditions that increase the maternal contribution and/or reduce the placental contribution may lower the FF. Table 1 summarizes some of the established feto-placental and maternal influences on FF.

Maternal component

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The most well-recognized and clinically significant influence on FF is maternal weight. The proportion of women with FF < 4% increases with maternal weight, estimated at 7%, 11% and 50% of women weighing 100kg, 110kg and 160kg respectively.[18] This FF decline with maternal weight is thought to be due to an increase in maternal cfDNA contribution, possibly due to adipocyte inflammation and necrosis,[32] with or without a simultaneous reduction in placental contribution.[20]

Maternal cfDNA levels are also increased by inflammatory conditions such as systemic lupus erythematosus[33] and by B12 deficiency.[34] Women with active autoimmune disease such as autoimmune neutropenia have been reported to experience repeated NIPT failures that appear to be resolved by suppression of disease activity.[35,36] There are also emerging reports linking low molecular weight heparin (LMWH) with NIPT failures due to low FF.[37,38] Failures due to low FF may occur in up to 18% of women on therapy, with a significant difference from untreated women even after controlling for maternal weight and hypertension.[37] Adjusting the timing of blood sample collection to immediately prior to the next dose may improve the success of a redraw in these women.[39] However, a conflicting report claims that the relationship between low FF and LMWH is due to the underlying disease rather than the treatment itself.[40] The exact mechanism of the interaction between LWMH and NIPT failures remain to be elucidated.

Fetoplacental component

FF is affected by different fetal aneuploidies.[18,27,30,41] The higher FF associated with trisomy 21 is advantageous for the performance of NIPT for this condition. In contrast, trisomies 13 and 18 are associated with lower median FFs (approximately 0.7 MoM on SNP-based assay[30]) and lower overall detection rates compared with trisomy 21.[42] Digynic triploidy is associated with extremely low FF (measurements < 0.5th percentile after correction for maternal weight and gestation).[43] Aneuploidies reported after a failed NIPT result include: triploidy, trisomy 13, trisomy 18, trisomy 21, trisomy 16 mosaic, and monosomy X.[29,30]

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It is now possible to measure the relative contribution of a trisomic chromosome to cfDNA and compare it to the overall fetal fraction. This is called the **"trisomic fraction"**.[44] In a fetus with a high risk NIPT result for trisomy, the specific cfDNA fraction contributed by the trisomic chromosome may guide interpretation of the abnormal result. When the trisomic fraction is much less than the FF, it suggests the presence of placental mosaicism (i.e., the number of trisomy 21 cells is less than the total placental cells contributing to FF). Conversely, if the trisomic fraction is much higher than the FF, then maternal mosaicism could be suspected as it suggests a maternal source of cfDNA (Figure 3).[44,45] When the trisomic fraction approximates the fetal fraction, it suggests true aneuploidy. Furthermore, the risk of adverse pregnancy outcomes, including miscarriage, fetal death, fetal growth restriction, true fetal mosaicism, and uniparental disomy appears to be increased for this group.[45]

Multiple gestation

The available data from multiple gestations suggest that NIPT performs as well in twins as in singletons, though the point estimates are less precise due to smaller numbers.[42] For comparable accuracy, the 'per fetus' fetal fraction should be the same as that required for singletons. One might expect that women carrying twins would have twice as much circulating placental DNA, but that is not the case. Both dichorionic and monochorionic pregnancies, have lower 'per fetus' FFs than singletons[46] though arguably, only total fetal cfDNA is relevant in monochorionic twins because they are almost always monozygotic with concordant karyotypes. SNP-based cfDNA analysis has shown that the combined FFs for dizygous and monozygous fetuses were 35% and 26% greater than singletons, respectively. However, the 'per fetus' FF in dizygous twins is 32% lower than singletons.[47]
Test failures due to low FF are accordingly generally higher in twins, and vary by chorionicity and cfDNA platform. Reported test failure rates due to low FF in targeted cfDNA assays are: 1.8% (433/23495) for singletons, 4.1% (5/122) for monochorionic twins, and 8.7% (70/806) for dichorionic diamniotic twins.[48] For a SNP-based cfDNA

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assay with a 2.8% FF limit of detection, test failure rates are 1.7% (2102/121,446) for singletons, 0.8% (11/1,454) for monozygous twins, and 5.6% (178/3,161) for dizygous twins.

Determining whether a twin pregnancy with a high risk NIPT result is discordant or concordant for aneuploidy can be estimated using the 'trisomic fraction' approach described above. Where the trisomic fraction is approximately half of the total fetal fraction, this would suggest one euploid and one trisomic fetus, whereas twins with a trisomic fraction equal to the total FF would be suspected to be concordant for trisomy.[44]

The interpretation of NIPT results is complicated in the event of a single fetal demise in a twin gestation. The placental territory of a demised fetus may continue to release cfDNA into maternal blood for more than three months, which may cause a false positive result if the demised twin was aneuploid.[46,49] It has also been reported that the changes in FF following single fetal demise are unpredictable, and not amenable to assigning a "safe" waiting period before performing NIPT.[48] Therefore, cfDNA is not recommended for aneuploidy screening at any interval after single twin demise.

2. CLINICAL LABORATORY ASPECTS OF FETAL FRACTION

Differences in single nucleotide polymorphisms (SNP) between the mother and the fetus can be used to distinguish fetal cfDNA from maternal cfDNA, thereby facilitating measurement of FF.[50,51] However, accumulating knowledge about the unique biological characteristics of fetal cfDNA enable it to be distinguished from maternal DNA without knowledge of specific genotype or SNP differences. Placental cfDNA fragments are now known to be shorter, differentially-methylated and have a different nucleosome 'footprint' compared with cfNA of maternal origin.[52,53]

What methods are used to measure FF?

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With the maturing knowledge base in the field and the recognition of the importance of FF for quality control, many NIPT providers routinely measure FF. However, the methods used to measure FF vary considerably and are not directly comparable, leading to calls for industry standardization in methods and reporting.[54,55] While Y chromosome-based methods appear to be the closest to an accepted "gold standard" in this field[56-58], these are obviously limited by their applicability to only those pregnancies with a male fetus. Some advocate using a combination of methods to check for the presence of fetal cfDNA and FF calculation for female and male bearing pregnancies.[57] Examples of current FF measurement approaches are provided in Table 2.

Bioinformatics tools profoundly influence FF measurement. They have been previously thoroughly reviewed by Peng and colleagues.[59] Several attempts to compare different FF algorithms have been published that highlight the significant variation between methods.[56,60] It is now apparent that individual clinical laboratories should optimize their platform-specific FF methods according to their lower limit of detection (LOD), and not rely on arbitrary FF cutoff values for quality control.

How does setting a cut-off value affect interpretation of NIPT results?

Optimizing the limit of detection involves a trade-off between higher statistical confidence and minimizing "no call" results. If an overly stringent FF cutoff is used, this could lead to more frequent "no call" results, with the associated health care costs of medical consultations, additional risk evaluation and possibly, invasive testing. In large population-based observational study involving three different NIPT platforms, when test failures were included with high risk calls as "screen positives", the screen positive rate of NIPT was only 0.5% lower than combined first trimester screening (2.4% vs 2.9%).[61]

What can be done to reduce "no call" results on samples with low FF?

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Addressing the problem of low FF is a high priority. Many approaches have been proposed to enrich fetal fraction by optimizing sequencing conditions, capitalizing on the biological differences between fetal and maternal DNA, and/or developing new statistical algorithms, including:

1. Deeper sequencing[62] or improving sequencing efficiency of existing methods[50]
2. Enrichment of fetal cfDNA based on their smaller fragment size using magnetic beads[62] or e-gel electrophoresis[64]
3. cfDNA recovery and repair with a commercial DNA kit used in forensics and archaeology[65]
4. Statistical algorithms to identify pregnancies at increased risk of trisomy 18, 13 or triploidy after a failed result due to low FF.[66]

Should clinical laboratories routinely report the FF to clinicians?

Laboratory reporting practices vary substantially with regard to FF. A workshop hosted by the Laboratory Methods Special Interest Group at the 2016 meeting of the International Society of Prenatal Diagnosis (ISPD) could not reach agreement on whether FF should be routinely reported. Only 11 out of the 43 participating laboratories included FF on their submitted examples of NIPT reports; a further laboratory reported only that the FF was 'good'. As no consensus was reached, no clear guidance on FF reporting arose from the workshop.[55]

A subsequent international pilot study of external quality assessment for NIPT also found a large variation in the content of reports, with key information frequently omitted or difficult to identify. Of 40 participants in the first pilot group, FF was reported by 51%, with 40% failing to report the fraction or whether it was measured at all. The remaining 9% of laboratories only reported FF for the "no abnormality detected" cases, or if it was insufficient to meet internal quality control standards.[67]

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The American College of Medical Genetics and Genomics (ACMGG) recommends that all laboratories establish and monitor analytical and clinical validity for fetal fraction, *and* include a clearly visible fetal fraction on NIPT reports.[68] The ACMGG also recommends that all laboratories should specify the reason for a no-call when reporting noninvasive prenatal screening results. In a recent evaluation of commercial laboratory practices in the United States, nine of 10 participating companies provided FF in their reports but only five specified a reason for a no-call result.[69]

It remains debatable whether it is useful to provide a FF percentage to clinicians, given the significant variation in measurement between technologies, lack of universal FF cutoff, and laboratory-specific limits of detection. Some methodologies may be reliable with FFs as low as 2% while others use a fixed cutoff of 4%.[70] Furthermore, some laboratories use multiple methods to determine FF, depending on fetal sex, so a single number may not be meaningful to a clinician. Ultimately, the intent of FF reporting is to provide assurance that fetal DNA has been detected and that the level was sufficient to pass laboratory internal quality assurance standards. If the FF is reported, its significance in relation to the test result should be made clear.[55]

3. 'PLAN B': CLINICAL MANAGEMENT AFTER A FAILED RESULT

It is recommended that women whose cfDNA screening results are not reported, indeterminate, or uninterpretable, receive further genetic counseling and be offered comprehensive ultrasound evaluation and diagnostic testing because of an increased risk of aneuploidy.[71]

Should women have a redraw, another aneuploidy screening method, or a diagnostic testing after a failed result due to low FF?

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All of these options should be discussed with the woman, incorporating consideration of factors such as gestational age, background risk of aneuploidy, results of detailed fetal ultrasound examination, and patient preferences. Women should be made aware of the increased risk of aneuploidy after a failed result (in the absence of other obvious causes, such as incorrect dates), and the 40-50% failure rate of a repeat blood sample. A detailed ultrasound examination should be performed, and diagnostic testing recommended if a fetal abnormality is present. If the ultrasound is normal and the woman wishes to avoid diagnostic testing, she could be offered an alternative screening test (ideally the first trimester combined test), or a repeat blood draw for NIPT. If the woman elects to have repeat NIPT between 10-13 weeks, storing a serum sample at the time of the second NIPT blood draw will ensure that an opportunity for first trimester combined screening will not be missed should the second NIPT attempt fail.

For women with an increased risk of test failure (e.g., maternal weight $\geq$ 100kg) delaying NIPT collection until the time of a 12 week scan would be prudent to reduce NIPT failures due to borderline FF at 10-11 weeks. This would also provide an opportunity for a good early fetal morphological assessment with transvaginal ultrasound examination. If there are no fetal abnormalities seen at 12 weeks that would prompt an offer of prenatal diagnostic testing, then NIPT can be performed, with a back-up serum sample for the first trimester combined test collected in case of NIPT failure. While PaPP-A and free bHCG are positively correlated with FF (Table 1), there are no data on whether prior failed NIPT due to low FF affects the performance of the first trimester combined test or second trimester serum screening.

Summary: The 3 “B”s – biology, bioinformatics, and a plan “B”

FF is an essential quality control component of NIPT that is intimately related to both biology and technology. Clinicians need to understand the **biological influences** on

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FF to avoid test failures wherever possible, and to manage test failures due to low FF rationally (Box 1). FF can now also provide useful clinical information in the case of a high-risk result, with regard to the likelihood of placental mosaicism, maternal CNV or discordant aneuploidy in twins.

There are many different technologies available for NIPT and FF measurement. Clinicians do not need to know the details behind **bioinformatics** algorithms used to measure FF, but they do need to appreciate the significant variation between sequencing laboratories and that methods used are not directly comparable. There is no universal fixed FF threshold that is applicable across all platforms and there have not been any demonstrated differences in NIPT performance by platform or FF method.[72]

Importantly, routine *measurement* of FF should be a factor in choosing an NIPT provider, although routine *reporting* of FF is still under debate. The clinician should know what to expect from a standard test report and whether reasons for failed NIPT are revealed (Box 2). Emerging solutions to the challenges of samples with low FF should reduce rates of failed NIPT results in the future. In the meantime, having a "**plan B**" prepared for those patients for whom NIPT is unsuccessful is essential in today's clinical practice.

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Table 1. Biological influences on fetal fraction

	Effect on fetal fraction	References
Feto-placental factors		
Gestational age (GA)	Increases with GA	27
Crown rump length (CRL)	Increases with CRL	18
Mosaicism	Decreases	44
Fetal aneuploidy	Variable	41, 27, 31, 18
Triploidy	Decreases	43
Multiple pregnancy	Total FF increases, but FF decreases per fetus	47,48
Maternal factors		
Maternal weight	Decreases with increasing maternal weight	18, 27, 19
Maternal autoimmune disease	Decreases with active maternal disease	33, 35, 36
Low molecular weight heparin	Possible decrease	37, 38, 39,40
Serum PaPP-A	Increases	18, 73
Serum free beta-HCG	Increases	18, 74
Ethnicity	Variable	18, 48, 75
Assisted reproductive technology conception	Decreases	74
Parity	Decreases	48, 75
Maternal Age	Decreases	48, 75

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Table 2. Technical approaches used to measure FF

Method	Algorithm name	Selected references
Sex chromosome based		
Y-chromosome based		52, 76, 77, 78
	DFRAG	56, 57
		78
	cSMART	78
X-chromosome based	PREFACE	80
Genotype-independent methods		
Sequence read count	SeqFF	56, 81
Nucleosome profile	SANEFALCON	82
Fragment sized-based		83, 84
MPS deduction - sequence counts	Fetal Quant	85
Methylation differences		53, 86
SNP-based		
Polymorphic loci quantified with microarray or sequencing	DANSR	48, 51, 58
SNP loci		47, 50
Insertion/deletion polymorphisms		87

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A fetal fraction toolbox for clinicians

Box 1. Questions to ask yourself

What is my patient's weight?

Are there any possible relevant maternal medical conditions?

Is my patient on low molecular weight heparin?

Was this pregnancy conceived by assisted reproductive technology?

Have gestation and viability been determined with ultrasound examination?

Is this a multiple pregnancy? Is it dichorionic or monochorionic?

Are there sonographic features to suggest triploidy or aneuploidy?

Box 2. Questions to ask the clinical laboratory providing your NIPT results

Do you routinely measure FF as part of quality control procedures?

Has your laboratory established its own limit of detection?

Do you report FF on the clinical reports? If so, do you explain the significance of the FF to the clinician?

What is the rate of NIPT failures based on low FF? For singletons and twins?

Do you calculate per fetus FF for twins? Do you use chorionicity information?

Do you provide clinicians with the reason for a failed NIPT result?

What is the rate of successful repeat blood draws for women with a failed result?

What other support to you provide to clinicians and patients after a failed result?

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Figure Legends

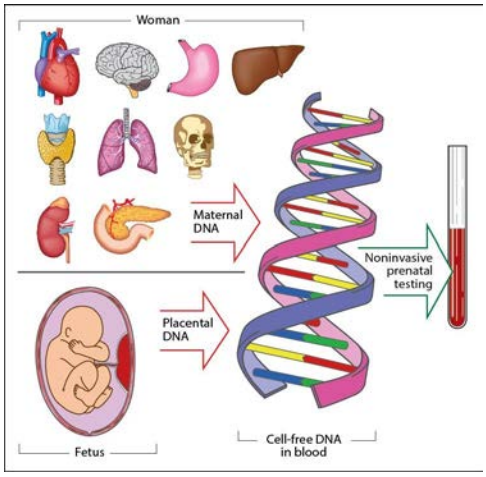
Figure 1. Multiple organ sources of cell-free DNA in maternal plasma

Figure 2. Relationship between fetal fraction and chromosome 21 z-score in euploid and pregnancies with Down syndrome.
The large open circles indicate chromosome z-scores in first and second trimester pregnancies with Down syndrome. As fetal fraction increases, the average z-score also increases. The line indicates the change in the average z-score by fetal fraction. All but four of the 212 cases are above a z-score of 3, and these four occur at fetal fractions of 7% or less. Small open circles indicate chromosome 21 z-scores in 1484 euploid pregnancies. The z-scores are centered on zero and generally fall between −3 and +3. Only three euploid pregnancies fall above a z-score of 3 (arrows), and these are not associated with lower fetal fractions. Reproduced from Canick et al. 2013 with permissions from Wiley-Blackwell Publishing.[20]

Figure 3. Normalized chromosomal read count in relation to the fetal fraction
Normalized chromosomal read count in relation to the fetal fraction (SeqFF) for chromosomes 13 (A), 18 (B), 21 (C) and 7 (D). The X axis depicts the fetal fraction and the Y axis the normalized chromosomal read count. Horizontal and diagonal lines, respectively, mark the predicted normal and trisomic normalized read counts (full lines) and ±3 SD as measured in the normal cases (X-axis and dashed lines). Values within 3 SD from expected trisomy counts are plotted as blue triangles. Values outside the area delineated by the X-axis and dashed lines represent cases with |Z| > 3 and |TriZ| > 3. These are plotted as red circles and indicate pregnancies at risk for fetoplacental mosaicism. The arrows indicate a set of discordant twins. Reproduced from Brison et al. 2019 with permission from Wiley-Blackwell Publishing.[44]

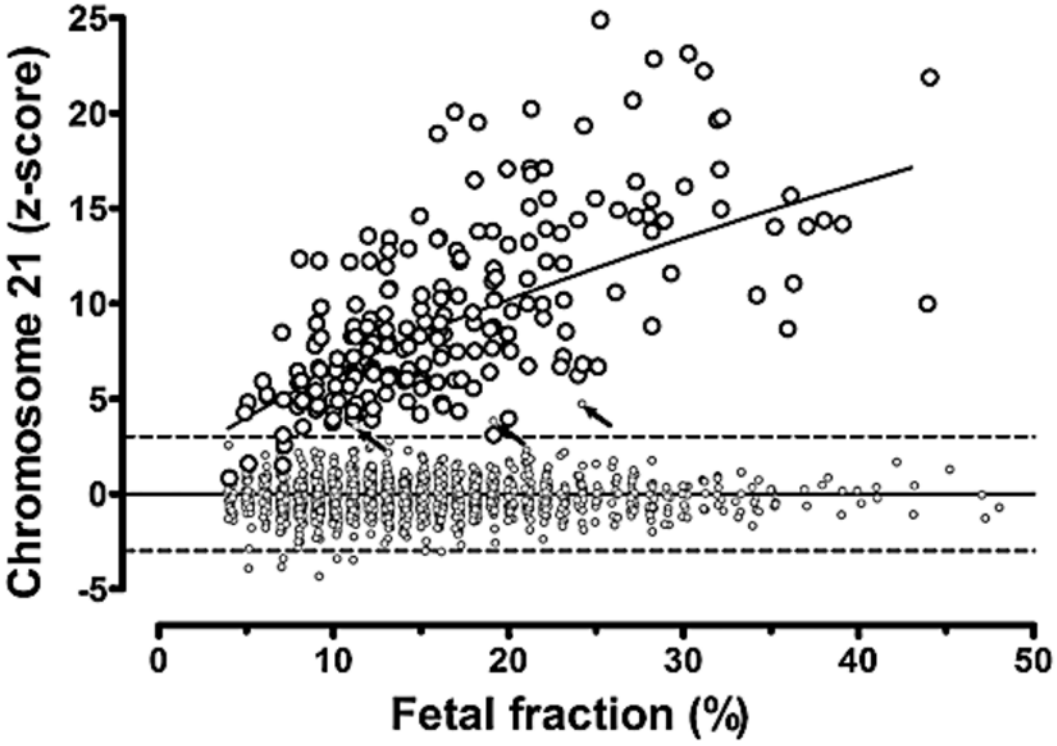
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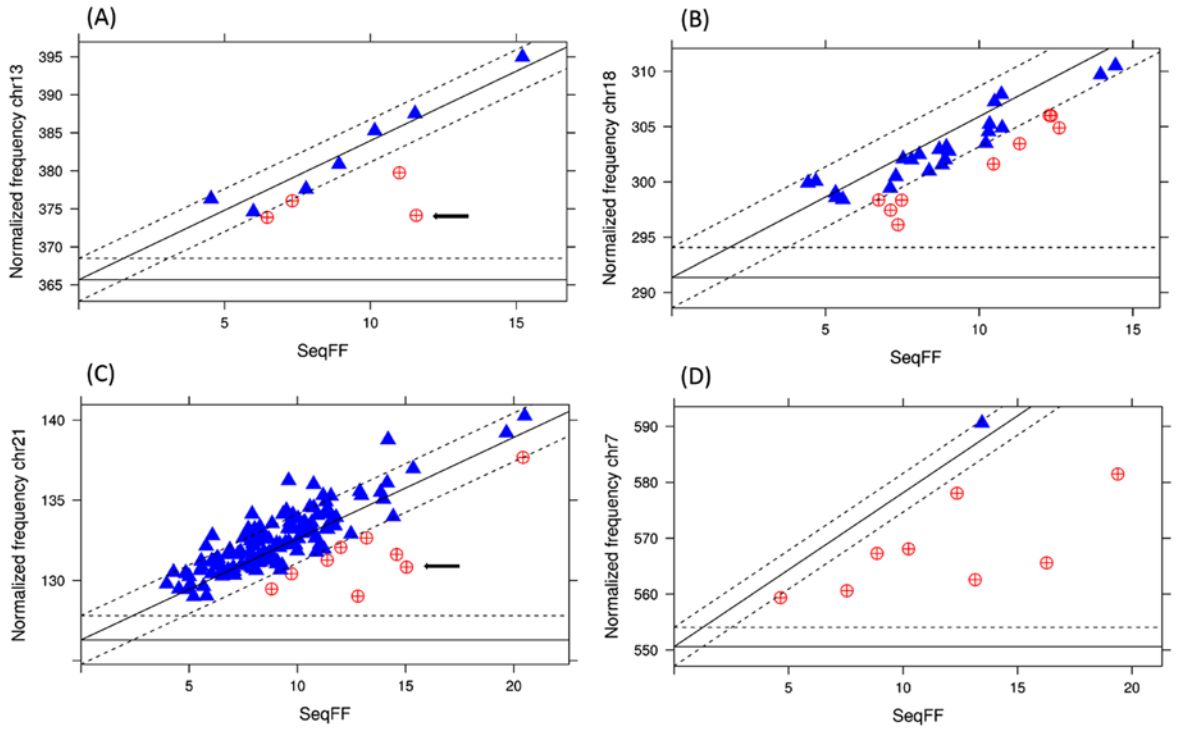
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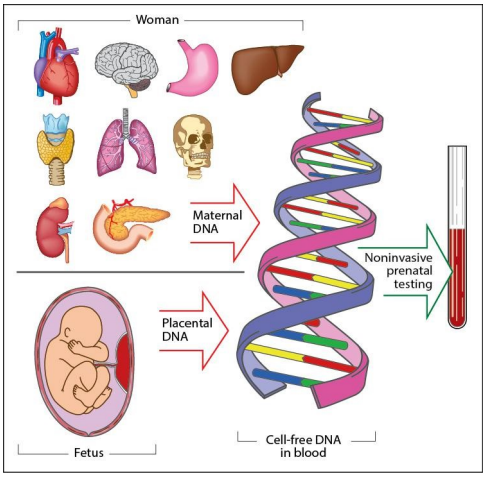
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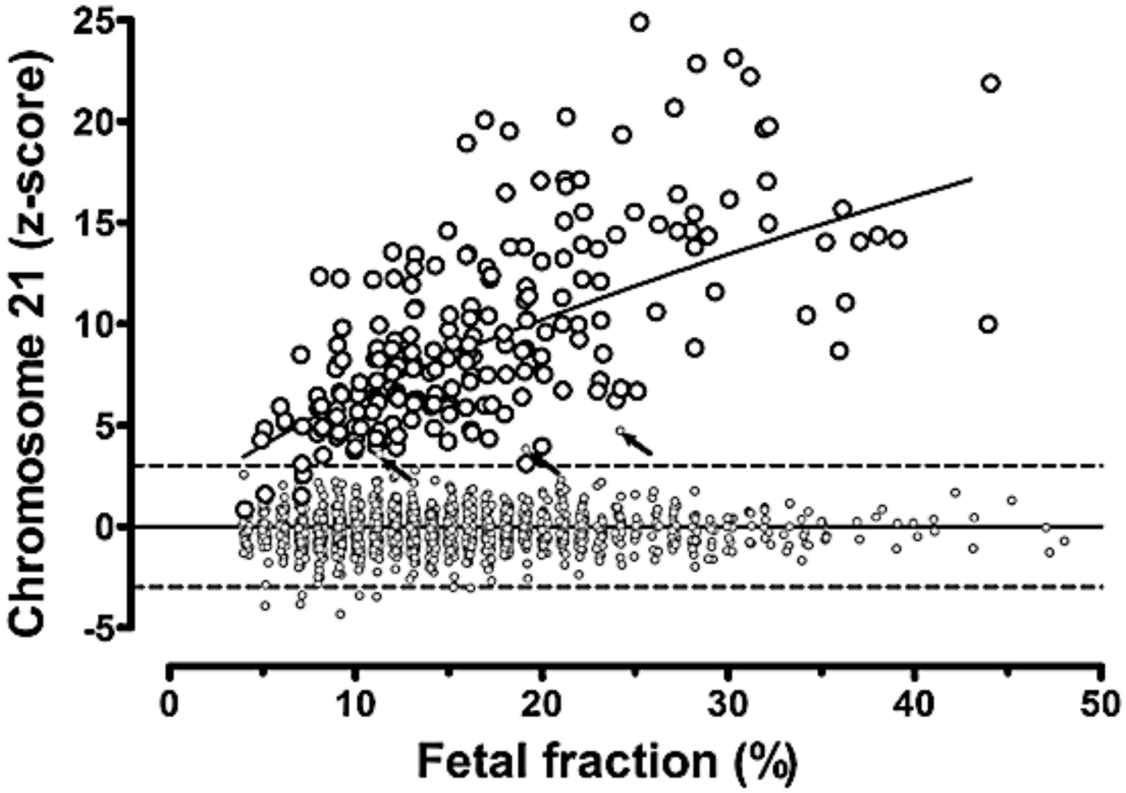
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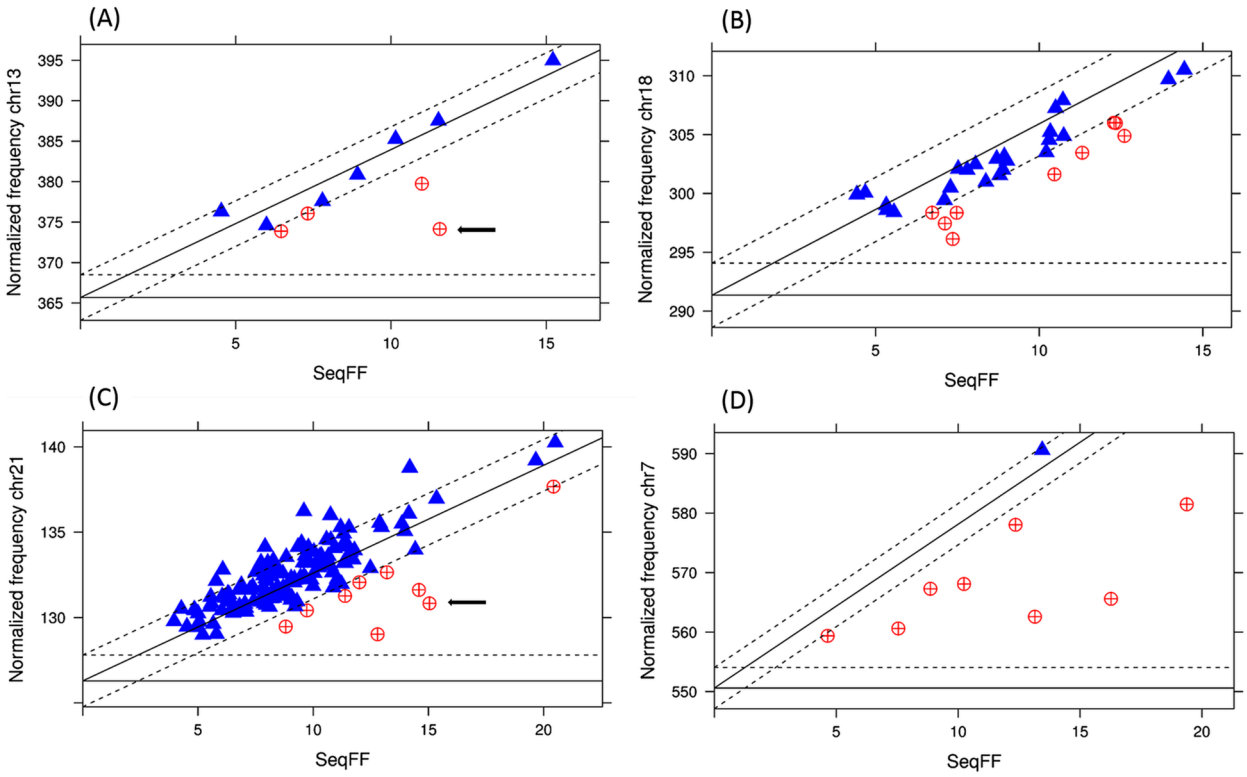
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