WHITE PAPER

# NGS whole-genome sequencing provides informative NIPT results

## A comparative analysis of commercially available methods for NIPT

# Introduction

Noninvasive prenatal testing (NIPT) represents a major advance in prenatal screening, providing accurate information about fetal aneuploidy status as early as 9-10 weeks gestation using a single maternal blood draw.[1] With NIPT, pregnant women can now screen for fetal chromosomal abnormalities, such as Down syndrome (trisomy 21), Edwards syndrome (trisomy 18), Patau syndrome (trisomy 13), and sex chromosome–related syndromes such as Klinefelter and Turner syndromes. Next-generation sequencing (NGS) is a trusted and proven method for performing NIPT. In fact, 95% of NIPT samples in published studies are run on Illumina systems with Illumina NGS technology.[2] This white paper provides a comparison of NGS vs. other NIPT methods currently available.

# Background of cfDNA screening

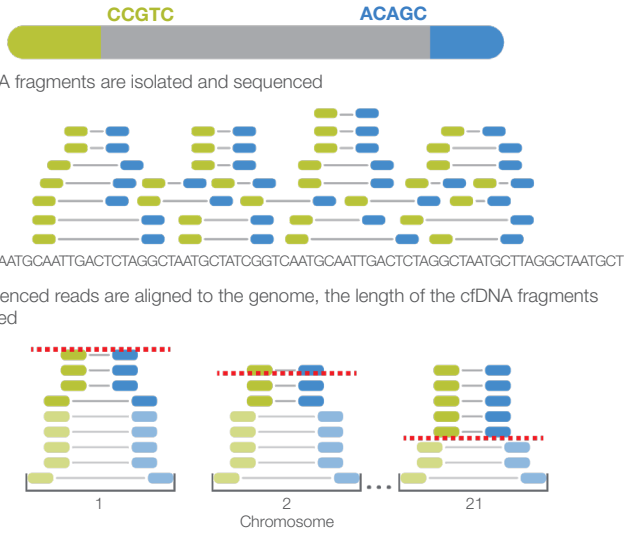
Circulating cell-free DNA (cfDNA), from both the fetus* and the mother, is found in maternal blood. Approximately 3-13% of the cfDNA circulating in maternal blood is from the fetus. NGS, which uses millions of sequence reads per sample, can detect and measure aneuploidy within this mixed sample.[3,4] Quantitative differences in cfDNA in maternal blood can be used to distinguish fetuses affected with trisomy 21 (and other fetal aneuploidies) from those that are unaffected. In clinical trials in the general obstetrical population, prenatal screening by cfDNA has been demonstrated to have significantly lower false positive rates and higher positive predictive values for trisomies 21 and 18 than standard screening.[1]

# NIPT technologies

NIPT using NGS technology provides informative results with a comprehensive view across the entire genome vs. targeted sequencing or array-based platforms. Targeted technologies include single nucleotide polymorphism (SNP) analysis, microarray analysis, and rolling circle amplification. With targeted approaches, only limited regions of select chromosomes are analyzed. These approaches have additional steps and employ more rounds of amplification than whole-genome sequencing methods, introducing a more complicated workflow.

*Fetal or fetus in this document refers to the fetal component of cfDNA derived from placental trophoblasts that are released into maternal circulation.

**NIPT and Whole-Genome Sequencing**

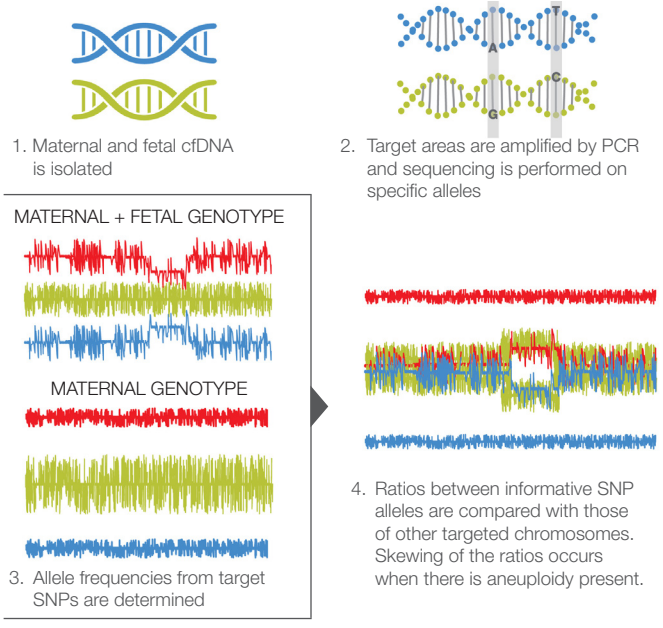



1. cfDNA fragments are isolated and sequenced
2. Sequenced reads are aligned to the genome, the length of the cfDNA fragments inferred
3. Reads are sorted by chromosomes, with shorter cfDNA fragments known to be enriched with fetal cfDNA. Deviations from expected fragment counts indicates aneuploidy.*

*This image represents rendition and does not imply that each chromosome is evaluated by one read.

SNP and microarray analysis both employ polymerase chain reaction (PCR). PCR can be used with NGS, however this will extend turn-around time (TAT) and add more complexity. PCR bias can lead to uneven coverage. A PCR-free workflow eliminates the need for pre- and post-PCR lab space, allowing steps to be performed in a single area. NGS without PCR improves workflow and allows for better TAT. NGS technology can also be scaled easily to accommodate the needs of a growing lab, allowing the best test option to be selected that meets local guidelines and coverage policies.

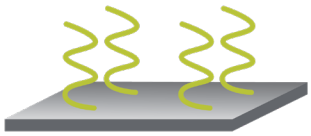
**SNP Analysis**



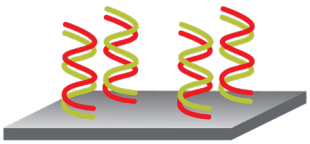

1. Maternal and fetal cfDNA is isolated
2. Target areas are amplified by PCR and sequencing is performed on specific alleles
3. Allele frequencies from target SNPs are determined
4. Ratios between informative SNP alleles are compared with those of other targeted chromosomes. Skewing of the ratios occurs when there is aneuploidy present.

### Microarray Analysis

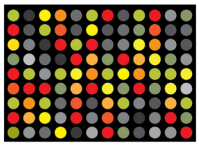
1. Target cfDNA fragments are labeled with a fluorescent tag



2. Complementary probes are bound on the microarry



3. Target DNA is hybridized to complementary probes on a microarray

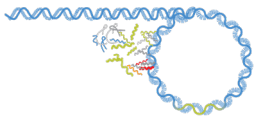


4. Fluorescence is compared to a reference target chromosomes. Deviations in fluorescence counts indicate aneuploidy.

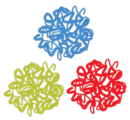
### Rolling Circle Amplification

1. Target cfDNA fragments are isolated

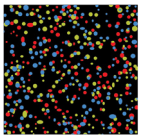
2. Fragments hybridize to specific probes and create circular DNA templates



3. Circular DNA templates undergo rolling circle amplification



4. The rolling circle products collapse into submicron nanoballs, each having a unique fluorescent tag



5. Fluorescence is compared to a reference target chromosome. Deviations in fluorescence counts indicate aneuploidy.

## NIPT market landscape for in-lab solutions

| | METHODOLOGY | | | | |
|---|---|---|---|---|---|
| | NGS [5] | Targeted/array[6] | Targeted/SNP[7] | Targeted/RCA[8] | NGS w/PCR [9] |
| Fetal fraction detection | Dynamic | Fixed | Fixed | None | Dynamic |
| End-to-end workflow | ✓ | ✗ | ✗ | ✓ | ✓ |
| PCR-free DNA preparation | ✓ | ✗ | ✗ | ✓ | ✗ |
| Detection of sex chromosome aneuploidies | ✓ | ✓ | ✓ | ✗ | ✓ |
| Detection of all autosomes | ✓ | ✗ | ✗ | ✗ | ✓ |
| Detection of partial dups/dels ≥7Mb for all autosomes | ✓ | ✗ | ✗ | ✗ | ✓ |
| Detection of select microdeletions | ✓ | ✓ | ✓ | ✗ | ✓ |

**NGS:** next-generation sequencing | **SNP:** single nucleotide polymorphism | **RCA:** rolling circle amplification | **PCR:** polymerase chain reaction

The methodologies shown above do not represent a comprehensive list. There may be other NIPT service providers depending on geography. This chart is informational only. Failure rates are representative of each manufacturer's clinical validation study design and are not meant to provide comparative insight.

# NIPT performance | Clinical experience studies

NGS using whole-genome sequencing is a trusted methodology with a broad publication history. The performance characteristics are well-established. Over 2.1 million NIPT samples in published studies have been run on Illumina NGS technology.[2] Below is a sample of recent published clinical studies.

| PUBLICATION [10-14] | KEY OBSERVATIONS |
|---|---|
| Borth H, Teubert A, Glaubitz R, et al. Analysis of cell-free DNA in a consecutive series of 13,607 routine cases for the detection of fetal chromosomal aneuploidies in a single center in Germany. *Arch Gynecol* Obstet.2021;303(6):1407-1414. doi:10.1007/s00404-020-05856-0111 | For trisomies 21, 18, 13, and monosomy X, sensitivities were ≥98.89% and specificities were ≥99.73%. An overall positive predictive value of 84.8% was observed. |
| Eiben, B., Borth, H., Kutur, N. et al. Clinical experience with noninvasive prenatal testing in Germany: analysis of over 500 high-risk cases for trisomy 21, 18,13, and monosomy X. *Current Obstetrics and Gynecology Reports*. 2021. 5, 1-7. | NGS-based NIPT method can reliably identify the most frequent aneuploidies in an experience with over 40,000 clinical NIPT cases.<br>Dynamic FF threshold allows trisomy cases to be detected, which would otherwise be missed using a strict FF cut-off. |
| van Prooyen Schuurman L, Sistermans EA, Van Opstal D, et al. Clinical impact of additional findings detected by genome-wide non-invasive prenatal testing: Follow-up results of the TRIDENT-2 study. Am *J Hum Genet*. 2022;109(6):1140-1152. doi:10.1016/j.ajhg.2022.04.018 | Most fetal chromosomal aberrations detected by NIPT are pathogenic and associated with severe clinical phenotypes.<br>Confined placental mosaicism (CPM) is significantly associated with adverse perinatal outcomes such as preeclampsia. |
| Soster E, Boomer T, Hicks S, et al. Three years of clinical experience with a genome-wide cfDNA screening test for aneuploidies and copy-number variants. *Genet Med*. 2021;23(7):1349-1355. doi:10.1038/s41436-021-01135-8111 | A positive predictive value (PPV) of 72.6% for genome-wide partial deletions and duplications and 22.4% for rare autosomal trisomies was observed.<br>Twenty-five percent of the positive results would have been missed with traditional cfDNA screening. |
| Rafalko J, Soster E, Caldwell S, et al. Genome-wide cell-free DNA screening: a focus on copy-number variants. *Genet Med*. 2021;23(10):1847-1853. doi:10.1038/s41436-021-01227-5 | Overall PPV for partial deletion and duplication cases with diagnostic outcomes was >70%. |

## Dynamic fetal fraction measurement

Different NIPT approaches vary in the way they evaluate fetal fraction (FF) and use FF information in sample reporting. Some NIPT technologies, such as microarray and SNP-based sequencing approaches, use a fixed FF cut-off for sample reporting that can lead to higher test failure rates. In contrast, some whole-genome sequencing-based technologies use a dynamic threshold, providing an alternative to strict FF cut-offs.

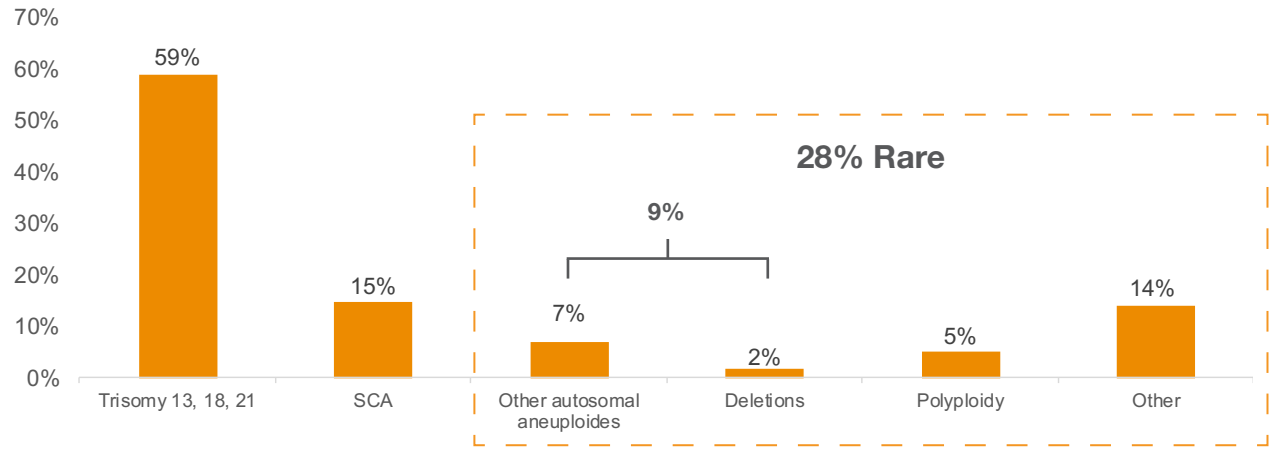
Strict fetal fraction cut offs of 4% may exclude patients with the highest risk of aneuploidy. Trisomy 21[15] is associated with increased fetal fraction, whereas trisomies 13 and 18 are associated with a decrease in fetal fraction. Norton et al.[16] reported aneuploidy in 4.7% of pregnancies that did not yield a cfDNA result due to FF < 4%, compared with 0.4% of those that did, indicating a tenfold increase in aneuploidy incidence. Similar data have also been reported by Pergament et al.[17]. Samples with a FF of less than 3.4% are nearly 9 times more likely to be aneuploid vs. those above this FF threshold. These studies underscore the importance of determining the actual limit of detection for each specific NIPT methodology, rather than using a theoretically determined fixed FF cut-off for all cfDNA testing methodologies.

## Genome-wide screening

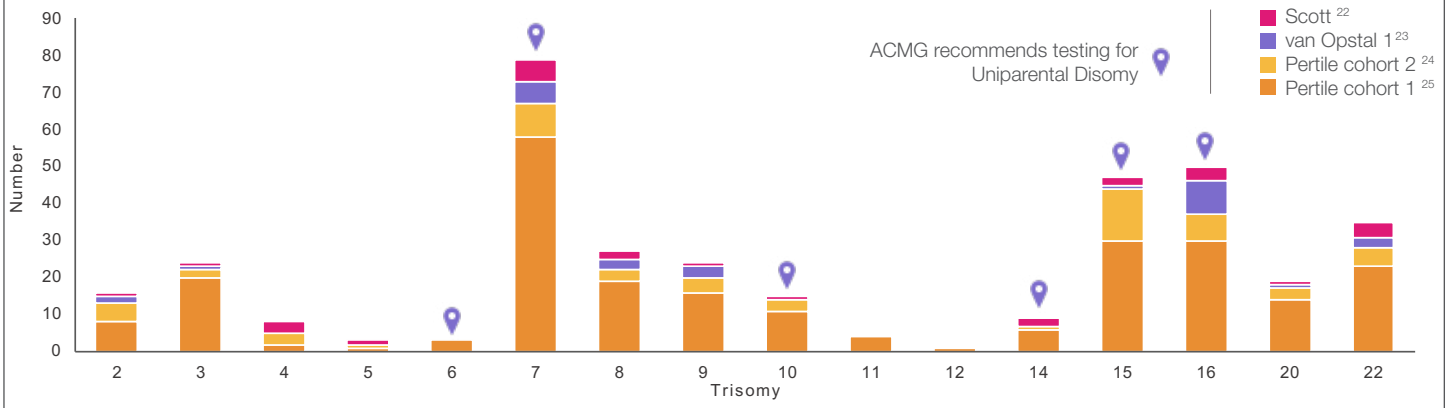
NIPT using NGS allows testing for all autosomes and sex chromosomes. Screening is not limited to the common trisomies. Genome-wide screening has the ability to provide information about common trisomies, rare autosomal aneuploidies (RAAs), sex chromosome aneuploidies (SCA), and partial deletions and duplications of all autosomes. RAAs are not as rare as the name implies. In a retrospective study of 2.4M live born infants, fetal deaths beyond 20 weeks, and pregnancy terminations for congenital anomalies, chromosomal anomalies were reported in 0.44% of the cases.[18] Of the 0.44%,17% were chromosomal abnormalities other than the three classic trisomies or SCAs and detectable by karyotyping.[18] This number is almost as frequent as the incidence of T18 and T13 combined.[18]

In a retrospective study of 90K prenatal early pregnancy amniotic fluid and chorionic villi (CV) samples, chromosomal anomalies were present in 8.6% of the CV samples, with 28% of these being rare chromosome anomalies and 9% of the rare anomalies being RAAs or deletions.[19]

RAAs are associated with several adverse pregnancy outcomes, including miscarriage, fetal growth restriction, stillbirth, clinically relevant uniparental disomy (UPD), spontaneous preterm birth and abnormal fetal karyotype or phenotype.[20] While ultrasound is an important screening test used in conjunction with NIPT, not all RAAs and partial deletions and duplications of all autosomes are associated with fetal structural anomalies visible on ultrasound. Many ultrasound findings are only visible later in pregnancy.[21]




**Relative frequency of chromosomal anomalies in CV samples—**Data from a retrospective study of 90K samples with ~84K samples analyzed by standard karyotype: (73,268 amniotic fluid, 10,511 CV) taken in early pregnancy from patients with an elevated risk for a chromosomal anomalies.[19]




**Rare autosomal aneuploidies may be present with almost all chromosomes**

## Key takeaways
## Technology

- NGS (without PCR) allows for optimal workflow and rapid TAT.
- NGS technology can be scaled easily to accommodate the needs of a growing lab, allowing the best test option to be selected that meets local guidelines and coverage policies.
- Whole-genome sequencing-based technologies use a dynamic threshold, provide an alternative to strict FF cut-offs

## Key takeaways
## Clinical considerations

- NGS using whole-genome sequencing for NIPT is a trusted methodology with a broad publication history.
- Genome-wide screening can provide information about common trisomies, rare autosomal aneuploidies (RAAs), sex chromosome aneuploidies (SCA), and copy number variations.
- RAAs are associated with several adverse pregnancy outcomes, including miscarriage, fetal growth restriction, stillbirth, clinically relevant uniparental disomy (UPD), spontaneous preterm birth and abnormal fetal karyotype or phenotype.

**LIMITATIONS OF THE TEST—**Noninvasive prenatal testing (NIPT) based on cell-free DNA analysis from maternal blood is a screening test; it is not diagnostic. False positive and false negative results do occur. Test results must not be used as the sole basis for diagnosis. Further confirmatory testing is necessary prior to making any irreversible pregnancy decision. A negative result does not eliminate the possibility that the pregnancy has a chromosomal or sub chromosomal abnormality. This test does not screen for polyploidy (eg, triploidy), birth defects such as open neural tube defects, single gene disorders, or other conditions,such as autism. There is a small possibility that the test results might not reflect the chromosomal status of the fetus, but may instead reflect chromosomal changes in the placenta (confined placental mosaicism, CPM) or the mother that may or may not have clinical significance

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