PrenaTest

The non-invasive molecular genetic prenatal test for fetal trisomy 13, 18 and 21 using next generation sequencing and z-score calculation following DNA isolation from maternal plasma

Information for health care professionals
References

1. Lo et al., Lancet 1997; 350:485-487
3. Lo et al., Am J Hum Genet 1999; 64:218-224
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5. Eiben et al., medgen 2011; 23:453-456
7. Deutsches Ärzteblatt 2003; 9:AS83
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Using next generation sequencing technologies, the non-invasive molecular-genetic PrenaTest® is capable of detecting the most common autosomal trisomies (trisomy 21, 18 and 13) in maternal blood with a high degree of accuracy. The PrenaTest® is, therefore, a non-invasive molecular-genetic prenatal diagnostic procedure which supplements conventional prenatal diagnostic methods. Unlike invasive methods like amniocentesis, it does not carry the risk of procedure-related fetal losses. The PrenaTest® is solely intended for use with women who are in the 12th week of pregnancy or later, and who carry an elevated risk of fetal trisomy 13, 18 or 21.

Scientific Basis

The PrenaTest® method centres on the analysis of cell-free DNA (cfDNA) in the pregnant woman’s blood. These small fragments of genetic material are not contained within cells, but circulate freely in the mother’s bloodstream; they include maternal DNA as well as between 2-40% (on average around 10%) fetal DNA (cell-free fetal DNA, or cffDNA). The genetic material stems from dead placental cells, and is continually released into the pregnant woman’s bloodstream (Fig. 1).

![Figure 1 Origins of cffDNA](image-url)
Regulatory requirements for NIPT-based aneuploidy testing in Europe

NIPT-based aneuploidy tests for trisomy 21 fall under the European Union Directive on In-vitro Diagnostics 98/79/EG* (List B, Appendix II, IVD products), and under the corresponding national laws.

This means:

- The data analysis pipeline must be CE-marked under the supervision of a Notified Body.
- The company offering the test must have a comprehensive quality assurance system in place (EN ISO 13485).

The PrenaTest® is currently the only test that meets these regulatory requirements, making it the only non-invasive molecular genetic blood test which is currently marketable as per the European Union Directive on In-vitro Diagnostics.

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A sample calculation illustrating the proportional relationships involved

If, for example, a plasma sample taken from a pregnant woman contained 100 chromosome 21-specific fragments, then with a euploid fetus, around 90 fragments would be of maternal and around 10 of fetal origin. If a comparable plasma sample were taken from a woman pregnant with a fetus with trisomy 21, then 90 of 105 chromosome 21-specific fragments would be of maternal and 15 of fetal origin. The PrenaTest® can reliably detect differences of 1:1.05.
For what types of pregnant women is the PrenaTest® suitable?

Positioning statements by national and international scientific associations

Current positioning statements by national and international gynaecological and prenatal-diagnostics associations (see box) declare non-invasive prenatal testing (NIPT) to be an advanced non-invasive testing method with high accuracy. Even so, it is currently classified as ‘not fully diagnostic’. Furthermore, it should not be used in place of first-trimester ultrasounds with nuchal translucency (NT) measurement, as a thicker NT can also indicate the presence of other chromosomal aberrations or fetal malformations. NIPT should always be performed in conjunction with comprehensive counselling and diagnostics.

In Germany, counselling is mandatory according to the Genetic Diagnostics Act (GenDG), which requires that the patient be thoroughly informed and given non-directive counselling on human genetics before a predictive prenatal genetic examination can be performed. The woman must also receive genetic counselling after test results are in. Corresponding laws in Austria and Switzerland are, respectively, the Gene Technology Act (GTG) and the Federal Law on Genetic Testing in Humans (GUMG).

The consensus among scientific associations is that NIPT technology should be viewed positively, as an alternative to direct invasive testing of pregnant women at risk of fetal trisomy. For example, Fetal Medicine Foundation (FMF) UK guidelines currently recommend offering invasive diagnostics to pregnant women with risk levels of 1:300 or higher after first-trimester screening (FTS).4 FMF Germany sets this risk group as 1:150 or higher, and also defines an intermediate risk range of 1:500 to 1:151, for whom additional second-trimester ultrasounds are recommended to detect any possible anomalies.5

In this respect, the use of NIPT for pregnant women with elevated risk has the potential to prevent procedure related fetal losses, because it is a further aid to decide for or against invasive procedures.

Incorporating NIPT into prenatal risk assessment

In a 2012 publication6, Benn et al. provided model calculations showing how such decision-making aids could be used in practice. Based on the sensitivity and specificity of the three large studies on NIPT and trisomy 21 which had been published at that time, they calculated that positive NIPT results were associated with a 290-fold increase in incidence of fetal trisomy 21, while negative NIPT results were associated with a 110-fold decrease in incidence. For example, a pregnant woman whose first-trimester screening indicated a 1:100 risk of fetal trisomy 21 would then have a risk level of 3:1 following positive NIPT results - giving a clear indication for invasive diagnostics to clarify the results. With negative

Positioning statements/guidelines on NIPT

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<td>Association of Privately Practising Prenatal Medical Professionals BVNP <a href="http://www.bvnp.de">www.bvnp.de</a></td>
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<td>The American College of Obstetricians and Gynecologists Committee on Genetics and The Society for Maternal-Fetal Medicine Publications Committee ACOG <a href="http://www.acog.org">www.acog.org</a></td>
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NIPT results, the woman’s risk level would be adjusted to 1:11000; this dramatic reduction in risk level would make it possible to skip invasive diagnostics. Based on these results, the authors conclude that the NIPT is a useful secondary test for fetal trisomy 21 in women with pregnancies at risk according to conventional first-trimester screenings.

NIPT applications as recommended by the ACOG

The ACOG’s positioning statement defines a somewhat broader range of indications for NIPT:
- as a primary screening test for pregnant women with elevated risk of fetal aneuploidy, and
- as a follow-up test after a positive first-trimester screening.

In practice, this means that pregnant women who are older or have a significant hereditary risk of a particular trisomy can undergo NIPT even without prior first-trimester screening. The test also represents an additional option if first-trimester ultrasounds reveal potential trisomy 13, 18, or 21-related abnormalities, or if serum tests indicate anything unusual.

Clinical trials have validated the PrenaTest® for use with certain types of pregnancies with elevated risk, especially those involving one or more of the following risk factors: advanced maternal age, abnormal serum markers, suspicious sonographic findings, or structural or numerical chromosomal aberrations in one parent. Prior first-trimester screening was not required. The women included in the studies were in Gestational Weeks 11(+0) to 32(+1). The study results are discussed in more detail on page 11.

NIPT applications as recommended by the GfH

Although scientific associations advise against using NIPT with pregnant women without elevated risk of the trisomies in question until the appropriate “low-risk collective” clinical trials have been performed, the GfH brings an additional aspect into this discussion:
- “With non-invasive prenatal diagnostic methods, there is no longer a need to weigh intervention-related risks against the probability of the child having an illness or health condition; as such, NIPT examinations should be made available to all pregnant women, or no pregnant woman should be denied one.”

German regulations and guidelines

Guidelines on prenatal diagnosis of illnesses and predispositions to health conditions clearly indicate that the treating physician is obligated to inform the pregnant woman
- “…of the possibility of diagnosing defects of the fetus.”
- “The potential danger invasive prenatal diagnostic procedures pose to the child necessitates that all low-risk diagnostic options be fully exhausted”.

The treating physician is thus obliged to inform every pregnant woman not only about invasive diagnostic options, but also about NIPT.

Section 10.12 of the Guidelines on Human Genetic Diagnostics and Genetic Counselling, issued by the German Human Genetics Society and the German Professional Association of Human Geneticists⁸, make the following express requirements of the physician:
- “Patients must be given access to all available information and data collected for medical purposes, and to all findings which enable the patient to make an independent decision.”
- Section 2.1 expressly notes that the patient’s subjective concerns may also constitute a medical indication.

To undergo the PrenaTest®, a pregnant woman must:
- receive comprehensive information in Germany from a qualified physician as per the Genetic Diagnosis Act (GenDG) as well as the Genetic Diagnostics Commission guidelines, and undergo non-directive counselling on human genetics (should blood samples be taken in other countries, the corresponding national laws apply).
- be in the 12th week of pregnancy (Gestational Week 11+0) or later.
- exhibit one of the following indications for performing the PrenaTest®:
  - Advanced age (35 years or older)
  - Fetal ultrasonographic findings indicating an increased risk of trisomy 13, 18 or 21
  - Suspicious serum markers for trisomy 13, 18, or 21
  - Trisomy 13, 18 or 21 in family history or in a previous pregnancy
  - Other medical reasons (joint decision between doctor and patient)
What requirements must doctors meet if they wish to offer the PrenaTest®?

In accordance with §10 GenDG, patients in Germany must be given non-directive counseling before and after the PrenaTest® is performed. As such, the PrenaTest® can generally only be offered by doctors who fulfil the requirements listed under § 7, paragraphs 1 and 3 — in other words, human genetics specialists, or other doctors who (having acquired specialist qualification or additional licensing) are qualified to perform genetic testing in their speciality areas. An exception here would be a situation where the pregnant woman has been referred to another qualified physician for genetic counselling. In any case, when PrenaTest® is ordered, written documentation must be on hand that genetic counselling has been performed in accordance with the applicable national laws. Please contact us if you are interested in offering the PrenaTest®.

How does the PrenaTest® work?

1. After the patient has received genetic counselling and granted consent, the doctor draws 2x10 ml venous blood using special blood collection tubes (Cell-Free DNA™ BCT by Streck Innovations, Inc.) which are provided by LifeCodexx.

2. The blood samples are sent by courier to the LifeCodexx diagnostic laboratory in Konstanz, Germany.

3. Laboratory work begins by isolating the blood plasma.

4. Next, the cell-free genetic material (cfDNA) is extracted from the plasma. The cfDNA is not further separated into maternal and fetal DNA—further analysis is always performed using the mixture of maternal and fetal cell-free DNA. For the analysis to be successful, the mixture must contain ≥4% fetal cell-free DNA mixed in with the maternal DNA. Should fetal DNA comprise less than 4% of the cell-free DNA mixture, it is recommended that new blood samples be taken later on in the pregnancy.

5. The recovered DNA is present in only minute quantities; after determining its percentage of fetal DNA, a so-called genomic library is generated and reproduced so that it can be analysed.

6. This is followed by sequencing using state-of-the-art analysis equipment (DNA sequencing using the massive parallel sequencing (MPS) method, with the help of Illumina HiSeq2000 next generation sequencing technology).

7. The sequences are evaluated using PrenaTest® DAP.plus Software (see page 8). A number of other quality standards must be achieved, such as minimum and maximum numbers (10-30 million) of generated sequences, or reads. These are prerequisites for performing the final analysis and interpreting the values—in other words, for reporting test results.
Evaluation using PrenaTest® DAP.plus software

PrenaTest® DAP.plus is a specially developed and CE-marked data analysis pipeline which first sorts the generated sequences (read) according to defined quality criteria (see Fig. 2) and chromosome numbers (see Fig. 3). After that, it measures the amount of genetic material present for chromosomes 13, 18 and 21 (see Fig. 4).

**Per sample (single-read sequencing):**

- The programme generates around 14.8 million 36 bp reads (thereby covering around 8% of the human genome);
- Of which around 6.8 million reads only match a single point on the human reference genome (unique reads);
- Of which around 5.7 million reads fit the human genome exactly, with no deviation (unique without mismatch);
- Euploid: of which around 70,000 belong to chromosome 21 (1.2%);
- T21: of which around 73,000 belong to chromosome 21 (1.32%)

*Figure 2  Sorting the resulting sequences (reads)*
While performing quantification, the software also takes into account the fact that different chromosomes have different proportions of G-C base pairs (in contrast to A-T base pairs). When these molecular biological techniques are applied, GC-rich sequences behave differently from AT-rich ones in that sequenced DNA no longer contains these sections in the same proportions as the original genetic material did – sequencing “biases” the DNA towards higher proportions of GC-rich sections. The programme’s “GC normalisation” process corrects this bias bioinformatically.

Chromosome 21 contains GC- and AT-rich sections in approximately equal measure, so this normalisation process is not critical for chromosome 21 analysis; chromosome 13, on the other hand, has significantly higher GC proportions, so GC normalisation is vitally important to accurate trisomy 13 analysis. Similarly, trisomy 18 analysis is only possible thanks to GC normalisation.

The amount of genetic material corresponding to the chromosomes in question, in relation to the total number of genetic sequences meeting the given quality criteria, is expressed as percentages of “chromosomal representation” – for example, 1.25% Chr21 in Figure 2. As the studies used to validate the PrenaTest® were designed to detect trisomy 21, and trisomy 21 also represented the highest number of cases (41), the following explanations will use trisomy 21 as an example. The validated algorithm is also used to determine whether the amount of chromosome 21 DNA present is higher than what would be found in a euploid chromosome set. Normal levels can be represented as a Gaussian bell curve (see Fig. 5).
A statistical value known as a \textit{z-score} is calculated for the sample in question. The \textit{z-score} represents how much the percentage of chromosome-21 sequences in the tested sample differs from the median levels in a reference sample, expressed in terms of median absolute deviation (MAD) (see Fig. 5).

This \textit{z-score} is calculated using the following formula:

\[
\text{z}(\text{Chr n})_{\text{sample}} = \frac{\%\text{(Chr n)}_{\text{sample}} \text{ – Median (Chr n)}_{\text{reference}}}{\text{MAD (Chr n)}_{\text{reference}}}
\]

\textit{z-scores} lower than 3 are typically seen in pregnancies without fetal trisomy 21 – in these pregnancies, \textit{z-score} distribution corresponds to normal Gaussian distribution (Fig. 5). A \textit{z-score} $\geq 3$ means that the value measured for the sample exceeds the median absolute deviation (MAD) of the reference set by more than three standard deviations, meaning chromosome 21 is represented in statistically abnormal quantities — which typically indicates the presence of trisomy 21.

Besides the 41 cases of trisomy 21 mentioned, the validation study also used the PrenaTest\textsuperscript{®} DAP.plus data analysis pipeline to analyse 5 cases of trisomy 13 and 8 cases of trisomy 18.

### Reporting the results

LifeCodexx AG sends the responsible physician the patient’s PrenaTest\textsuperscript{®} results in the form of separate \textit{z-scores} for chromosomes 13, 18 and 21. An interpretive statement is provided for each \textit{z-score} value, such as: “For the sample examined, the resultant \textit{z-scores} are within/outside the normal range. The test result thus does not indicate/indicate the presence of fetal trisomy 21, 18 or 13.”

Diagnosis and the associated genetic counselling in accordance with §10 GenDG remains the sole responsibility of the physician — that is, the responsible physician can use the corresponding \textit{z-scores} to either confirm or rule out the presence of trisomy 13, 18 or 21 with a high degree of accuracy.
Significance of the testing method

The PrenaTest®'s significance has been evaluated through clinical studies in which LifeCodexx was substantially involved (publication pending).

A total of 468 blood samples were analysed and evaluated; these included 41 cases of trisomy 21, eight cases of trisomy 18 and five cases of trisomy 13. A total of 466 blood samples (>99%) were correctly classified using the PrenaTest®; one case of trisomy 21 was not detected (false negative), and one case of trisomy 18 was detected but not actually present (false positive). This translates to an 98.1% overall detection rate among all samples, with a false-positive rate of 0.2%.

LifeCodexx will relay additional details regarding the study as soon as the scientific data has been published.

The PrenaTest® does not make it possible to draw any conclusions as regards structural chromosomal aberrations — it only allows the quantitative measurement of fetal trisomies 21, 18 and 13. Whereas a large number of cases have statistically demonstrated the test’s sensitivity and specificity in detecting trisomy 21, the numbers of trisomy 13 and 18 cases tested are insufficient to draw conclusions on its sensitivity and specificity in these areas.

In rare cases, fetal trisomies can be caused by smaller structural aberrations affecting chromosome 21, 18 or 13; such cases cannot be detected using this molecular genetic test. Furthermore, the cell-free fetal DNA examined in the PrenaTest® originates from the trophoblast cells and as such it is only possible to achieve a level of diagnostic certainty close to that attained via direct chorionic villus sampling. Consequently, mosaics or fetoplacental discrepancies in trisomies 21, 18 or 13 are not recognisable. In the event of a fetoplacental discrepancy, this can also mean that the PrenaTest® result is not representative for the unborn child. The PrenaTest® is also currently not suitable for testing multiple pregnancies. All other chromosomal abnormalities and genetic disorders fall outside the scope of this testing method, so no conclusions may be drawn in this regard.

Figure 7
Fetal trisomy 21, 18 and 13 detection rates — comparison of invasive and non-invasive methods

First-trimester screening, nuchal translucency measurement, and determination of maternal serum markers; 5% false positive rate vs. PrenaTest®; 0.2% false positive rate vs. Karyotyping
## Practical procedure for performing the PrenaTest®

<table>
<thead>
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<th>Description</th>
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<tr>
<td>1</td>
<td>PrenaTest® sets ordered through <a href="mailto:info@lifecodexx.com">info@lifecodexx.com</a></td>
</tr>
<tr>
<td>2</td>
<td>Patient provided with information and genetic counselling</td>
</tr>
<tr>
<td>3</td>
<td>Appropriate forms filled out; copies made for doctor and patient</td>
</tr>
</tbody>
</table>
| 4    | Blood drawn using special blood collection system (included)  
Return box packed and stored in refrigerator (4-10 °C) |
| 5    | Samples sent to LifeCodexx: direct delivery within one working day |
| 6    | Sample usually analysed within two weeks (10 working days) |
| 7    | Results report sent to medical partners by fax and by mail |
| 8    | Results communicated to patient by physician responsible (GenDG: restricted to physicians) |