INFORMATION SHEET TO THE NON-INVASIVE PRENATAL SCREENING TEST FOR ANEUPLOIDIES, STRUCTURAL CHROMOSOMAL ALTERATIONS AND FETAL GENETIC DISEASES BY ANALYSIS OF FETAL DNA FROM MATERNAL BLOOD

Purpose of the PrenatalSafe® test

PrenatalSafe® is a **non-invasive** prenatal test which, by analysing circulating cell-free fetal DNA isolated from a sample of maternal blood, evaluates the presence of common fetal aneuploidies in pregnancy, such as those related to chromosome **21 (Down syndrome)**, chromosome **18 (Edwards syndrome)**, chromosome **13 (Patau's syndrome)** and the sex chromosomes **(X** and **Y**), such as for example **Turner syndrome** (X chromosome monosomy). The test also provides for a level of investigation that allows evaluating aneuploidies and structural chromosomal alterations on each chromosome, with results very similar to the analysis of the fetal karyotype by invasive prenatal diagnosis techniques.

The **PrenatalSafe® 3** test evaluates the aneuploidies of chromosomes **21, 18, 13** and includes the determination of fetal sex (optional).

The **PrenatalSafe® 5** test evaluates the aneuploidies of chromosomes **21, 18, 13**, of the sex chromosomes (**X** and **Y**) and includes the determination of fetal sex (optional). **Remember that this analysis cannot be prescribed for bichorial twin pregnancies.**

The **PrenatalSafe® 5 DiGeorge** test evaluates the aneuploidies of chromosomes **21, 18, 13**, of the sex chromosomes (**X and Y**) and includes the determination of fetal sex (optional) in conjunction with the search for 22q11.2 microdeletion **Remember that this analysis cannot be prescribed for bichorial twin pregnancies.**

The **PrenatalSafe® Plus** test, in addition to the aneuploidies of chromosomes **21, 18, 13** and of the sex chromosomes (**X** and **Y**), also evaluates the **trisomy of chromosomes 9 and 16** (optional) and allows detecting the presence in the fetus of submicroscopic **structural** chromosomal alterations, such as some common **microdeletion** syndromes. The test also includes the determination of fetal sex (optional). **Remember that this analysis cannot be prescribed for bichorial twin pregnancies.**

The **PrenatalSafe® Karyo** test enables detecting aneuploidies and fetal structural alterations on each chromosome, with results very similar to the **fetal karyotype determination** performed using invasive prenatal diagnosis techniques. The test includes the determination of fetal sex (optional).

The **PrenatalSafe® Karyo Plus** test is an evolution of the **PrenatalSafe® Karyo** test and to the potential of the latter adds the possibility of detecting the presence in the fetus of submicroscopic **structural** chromosome alterations, such as some common microdeletion syndromes. The test includes the determination of fetal sex (optional). **Remember that this analysis cannot be prescribed for bichorial twin pregnancies.**

The **PrenatalSafe® Complete** test adds to the potential of **PrenatalSafe® Karyo** and **PrenatalSafe® Karyo Plus** the possibility of detecting **genetic diseases with hereditary transmission** in the fetus (e.g. **Cystic Fibrosis, Beta Thalassemia**, etc.) or with **de-novo onset** (e.g. **Achondroplasia, Hypochondroplasia, Noonan Syndrome, Craniosynostosis, etc.**).
The **PrenatalSafe® Complete** and **PrenatalSafe® Complete Plus** test, consisting of combining the **PrenatalSafe® Karyo and PrenatalSafe® Karyo Plus** test with the **GeneSafe™ Complete** test, provides the **most in-depth level of information** that can be obtained during pregnancy through a non-invasive prenatal screening test.

The **PrenatalSafe®** tests can be integrated with the **RhSafe®** test, a non-invasive prenatal test that, by analysing fetal DNA isolated from a blood sample of the expectant mother, determines the fetal Rh(D) factor. The **RhSafe®** test is optional and is performed (on request) in Rh(D) negative pregnant women with an Rh(D) positive male partner.

Chromosomal aneuploidies evaluated by the PrenatalSafe® test

TRISOMY 21: It is caused by the presence of an extra copy of chromosome 21 and is also known as **Down syndrome**. It is the most common genetic cause of mental retardation and is estimated to be present in **1/700 births**, although the risk that a woman may become pregnant with trisomy 21 is closely related to her age at the time of conception and may therefore vary over the life of a woman.

TRISOMY 18: It is caused by the presence of an extra copy of chromosome 18. Also known as **Edwards Syndrome**, it is linked to a high risk of abortion and causes severe mental retardation. Newborns with trisomy 18 often have congenital heart defects, as well as other pathological conditions that reduce their life expectancy. It is estimated that trisomy 18 is present in **1/5,000 births**, although the risk that a woman may become pregnant with trisomy 18 is closely related to her age at the time of conception and may therefore vary over the life of a woman.

TRISOMY 13: It is caused by the presence of an extra copy of chromosome 13. Also known as Patau syndrome, it is linked to a high risk of abortion. Newborns with trisomy 13 may have heart defects and other pathological conditions, which means that survival beyond the age of one year is rare. It is estimated that trisomy 13 is present in **1/16,000 births**,

although the risk that a woman may become pregnant with trisomy 13 is closely related to her age at the time of conception and may therefore vary over the life of a woman.

Sex chromosome aneuploidies

PrenatalSafe® analyses the sex chromosomes (**X** and **Y**) providing information about the fetal sex and aneuploidies of these chromosomes. The test is able to identify several aneuploidies of the sex chromosomes, the most frequent one of which is **Monosomy X** which is clinically linked to a condition known as Turner syndrome. **Turner syndrome**, by definition, affects only females who have short stature and reproductive difficulties in association with other, not constant, clinical manifestations such as congenital heart disease, neurosensory hypoacusis, renal abnormalities such as horseshoe kidney and predisposition to some autoimmune diseases (thyroiditis, diabetes, coeliac disease, etc.). Other sex chromosome aneuploidies that can be found with the test are **Trisomy X** (XXX), **Klinefelter syndrome** (XXY) and **Jacobs syndrome** (XYY).

PrenatalSafe® 5 DiGeorge is able to detect the trisomies of chromosomes 13, 18, 21 and the aneuploidies of the sex chromosomes, together with the search for deletion of chromosome 22 in the q11.2 region, which causes DiGeorge syndrome. DiGeorge syndrome is a complex condition characterized by the combination of various manifestations: congenital heart disease, palate defects (cleft) and developmental delay/ intellectual disability being the most frequent in conjunction with hypoplasia of the thymus and immunodeficiency, and characteristic facial features. The worldwide incidence is 1/2,000-1/4,000 live births.

PrenatalSafe® Plus can also highlight the **trisomy** of **chromosomes 9 and 16** (optional). Complete trisomies of these two chromosomes are generally not compatible with life and often result in spontaneous abortions, while mosaic forms may be viable.

The **PrenatalSafe® Karyo** test, in addition to the most common fetal aneuploidies identifiable in pregnancy, allows analysing all the chromosomes and therefore detecting even the rarest aneuploidies.

Examples of less frequent chromosomal aneuploidies detectable with PrenatalSafe® Karyo:

- **Trisomy 1**
- **Trisomy 4**
- **Trisomy 5**
- **Trisomy 7**
- **Trisomy 9***
- **Trisomy 12**
- **Trisomy 16***
- **Trisomy 22***

* With greater incidence among the less frequent fetal aneuploidies.

Structural chromosome alterations (deletions and segmental duplications) evaluated by the PrenatalSafe® Karyo test:

With **PrenatalSafe® Karyo** it is also possible to detect **structural alterations** of all the chromosomes of the fetus. Structural abnormalities originate from the breakage of one or more chromosomes and, since these breakages can theoretically occur anywhere in the genome, the number of potential rearrangements is practically infinite. Such alterations cause the loss/acquisition of genetic material; therefore, they are generally identified in subjects with varying clinical manifestations. The main types of structural abnormalities are:

1) **Deletions:** consist of the loss of a segment of a chromosome, which may be terminal or interstitial. Deletion syndromes usually involve relatively large segments of a chromosome (> 10 Mb = Megabases).
2) **Duplications:** consist of the presence of 3 copies of a segment of a chromosome and therefore constitute partial trisomies.
3) **Complex rearrangements:** consist of the presence of deletions/duplications of two or more chromosomal segments. They may originate from conception or be the consequence of the imbalance of a balanced translocation present in a parent (**unbalanced translocation**).

Microdeletion syndromes evaluated by the PrenatalSafe® Plus test

PrenatalSafe® Plus provides for the possibility of performing a second level investigation, which allows detecting **6** of the most common **microdeletion syndromes** in the fetus.
Microdeletion syndromes are chromosomal abnormalities characterized by the loss of a small chromosomal tract and, consequently, of the genes located on that chromosomal fragment. These alterations cause syndromes of varying clinical importance depending on the chromosome involved, the chromosomal region involved and its size.

The main microdeletion syndromes investigated include:

- **DiGeorge syndrome** (22q11.2 deletion): DiGeorge syndrome is a complex condition characterized by the combination of various manifestations: congenital heart disease, palate defects (cleft) and developmental delay/ intellectual disability being the most frequent in conjunction with hypoplasia of the thymus and immunodeficiency, and characteristic facial features. The worldwide incidence is **1/2,000-1/4,000 live births**.

- **Cri-du-chat syndrome** (5p deletion): is a chromosomal disease caused by the deletion of a variable portion of the short arm of chromosome 5 (5p-). The main clinical signs include a high-pitched monotonous cry (hence the name of "cat cry" syndrome), microcephaly, characteristic facial features and severe psychomotor delay and intellectual disability. The worldwide incidence varies between **1/15,000 and 1/50,000 live births**.

- **Prader-Willi/ Angelman syndrome** (15q11.2 deletion): these two syndromes involve the same chromosomal tract (critical region 15q11.2-q13) but have different manifestations depending on whether the chromosome concerned is of maternal or paternal origin.
  Prader-Willi syndrome is a condition mainly characterized by low stature, obesity, muscle hypotonia, endocrinological alterations, facial dysmorphisms and psychomotor developmental delay. This syndrome affects **1/25,000 births**.
  Angelman syndrome is a neurological disease characterized by severe psychomotor delay and intellectual disability, and characteristic facial dysmorphisms. Its prevalence is estimated to be between **1/10,000 and 1/20,000**.

- **1p36 deletion syndrome:** is a chromosomal abnormality caused by a partial heterozygous deletion of the distal part of the short arm of chromosome 1, with breaking points between 1p36.13 and 1p36.33. It is characterized by typical facial dysmorphisms, hypotonia, developmental delay, cognitive impairment, convulsions, heart disease, deafness and prenatal-onset growth retardation. It is considered one of the most common chromosomal deletion syndromes, with an incidence of **1/5,000-10,000 live births**.

- **Wolf-Hirschhorn syndrome** (4p deletion): is a developmental disease, determined by a deletion of the short arm of chromosome 4 (region 4p16.3) and characterized by characteristic craniofacial signs, prenatal and postnatal growth retardation, cognitive impairment, severe psychomotor developmental delay, convulsions and hypotonia. The prevalence is **1/50,000 births** and affects females more often than males (2:1).

It is advisable to use **PrenatalSafe® Plus in certain clinical settings** (e.g. ultrasound doubts suggestive of chromosomal microdeletion syndrome) for which a second level diagnostic investigation is indicated.

Microdeletion syndromes evaluated by the PrenatalSafe® Karyo Plus test

PrenatalSafe® Karyo Plus allows detecting the presence in the fetus of **9 microdeletion syndromes**:

Microdeletion syndrome	Chromosomal region	Prevalence (at birth)
DiGeorge syndrome	deletion 22q11.2	1/2,000 – 1/4,000
Cri-du-chat syndrome	deletion 5p15.3	1/15,000 – 1/50,000
Prader-Willi syndrome	deletion 15q11.2	1/25,000
Angelman syndrome	deletion 15q11.2	1/10,000 – 1/20,000
1p36 deletion syndrome	deletion 1p36	1/5,000 – 1/10,000
Wolf-Hirschhorn syndrome	deletion 4p16.3	1/20,000 – 1/50,000
Jacobsen's syndrome	deletion 11q23-q24.3	1/100,000
Langer-Giedion syndrome	deletion 8q24.11-q24.13	1/200,000
Smith-Magenis syndrome	deletion 17p11.2	1/15,000 – 1/25,000

● **Jacobsen syndrome** is caused by a deletion of the long arm of chromosome 11 (11q23). It is characterized by developmental delay, characteristic facies, haemorrhagic diseases and some behavioural disorders. The prevalence is **1/100,000 births**.

● **Langer-Giedion syndrome** or trichorhinophalangeal syndrome type 2 is caused by a microdeletion of the long arm of chromosome 8 (region 8q24.11-q24.13), which leads to the loss of at least two genes: TRPS1 and EXT1. It is characterized by cognitive impairment associated with various abnormalities, including redundant skin, multiple cartilage exostosis, characteristic facies and cone-shaped phalangeal epiphysis. Growth retardation, microcephaly, hypotonia and hearing problems have also been described.

● **Smith-Magenis syndrome** is a complex genetic disease with Mild to moderate cognitive impairment, significant delay in speech, reduced sensitivity to pain, peripheral neuropathy, (characteristic) sleep disorders and disruptive behaviour (whims/temper tantrums, constant search for attention, aggressiveness, disobedience, distraction and self-harm behaviours). The worldwide prevalence is **1/15,000-25,000 births**. SMS is a sporadic disease caused by 17p11.2 deletion of the RAI1 gene (retinoic acid-induced 1; 90%) or by mutation of the gene itself (10%).

It is advisable to use **PrenatalSafe® Karyo Plus only in certain clinical settings** (e.g. ultrasound doubts suggestive of chromosomal microdeletion syndrome) for which a second level diagnostic investigation is justified. Microdeletion syndromes are generally sporadic, i.e. they occur on a single conception as a random event. More rarely and only for some of the conditions identifiable with **PrenatalSafe® Karyo Plus** microdeletions are transmitted by one of the two parents.

Genetic diseases investigated by the GeneSafe™ Complete test

The **GeneSafe™ Complete** test screens the fetus for both **genetic diseases with hereditary transmission** (**GeneSafe™ *Inherited***) and with de-novo onset (***GeneSafe™ de novo***).
In particular, the **GeneSafe™ *Inherited*** test allows detecting variants in **4 genes** responsible for **4 genetic diseases** with autosomal-recessive transmission frequently found in the Italian population, such as **Cystic Fibrosis**, **Sickle Cell Anaemia**, **Beta Thalassemia** and the most common form of **Hereditary Deafness**. The genes investigated by the **GeneSafe™ *Inherited*** test and their genetic diseases are shown in the table below.

Table 1: List of genes investigated by the GeneSafe™ Inherited test and associated diseases

Hereditary genetic diseases identified by GeneSafe™ *Inherited*	Gene
Cystic Fibrosis	**CFTR**
Beta Thalassemia	**HBB**
Sickle Cell Anaemia	**HBB**
Autosomal recessive hereditary deafness type 1A	**CX26 (GJB2)**
Autosomal recessive hereditary deafness type 1B	**CX30 (GJB6)**

The **GeneSafe™ *de novo*** test detects variants in **25 genes** associated with **44 monogenic diseases** not inherited from parents, but which may occur randomly for the first time in the fetus and in these cases called *de novo*. These variants are not usually included in pre-conception screening tests performed on parents as they are not hereditary. The above *de novo* variants can result in **skeletal dysplasias, heart defects, multiple congenital abnormalities and/or intellectual impairment** in the child. The genes investigated by the **GeneSafe™ *de novo*** test and their genetic diseases are shown in the table below.
The diseases investigated by the **GeneSafe™ *de novo*** test are often not detectable in the first-trimester fetal ultrasound scan (some are detectable by ultrasound only in the second or third trimester) and are independent of maternal age. The **GeneSafe™ *de novo*** test can identify certain genetic diseases that may be associated with **advanced paternal age** (e.g., Achondroplasia, Pfeiffer syndrome, Apert syndrome, Crouzon syndrome, Osteogenesis Imperfecta, etc.), as they are caused by genetic errors that arise during the spermatogenesis process.

Table 2: List of genetic diseases with de novo onset investigated by the GeneSafe™ de novo test

Syndromic diseases	
Alagille syndrome	**JAG1**
CHARGE syndrome	**CHD7**
Cornelia de Lange type 5 syndrome	**HDAC8**
Cornelia de Lange type 1 syndrome	**NIPBL**
Rett syndrome	**MECP2**
Sotos type 1 syndrome	**NSD1**
Bohring-Opitz syndrome	**ASXL1**
Schinzel-Giedion syndrome	**SETBP1**
Holoprosencephaly	**SIX3**
Craniosynostosis	
Antley-Bixler syndrome without genital abnormalities or steroidogenesis disorders	**FGFR2**
Apert syndrome	
Crouzon syndrome	
Jackson-Weiss syndrome	
Pfeiffer syndrome, type 1	
Pfeiffer syndrome, type 2	
Pfeiffer syndrome, type 3	
Noonan syndrome	
Cardio facio cutaneous syndrome (CFS) type 1	**BRAF**
Noonan-like syndrome with or without juvenile myelomonocytic leukaemia	**CBL**
Noonan syndrome	**KRAS**
Cardio facio cutaneous syndrome (CFS) type e 3	**MAP2K1**
Cardio facio cutaneous syndrome (CFS) type 4	**MAP2K2**
Noonan syndrome 6	**NRAS**
Noonan syndrome 1/ LEOPARD syndrome 1	**PTPN11**
Noonan syndrome 5/LEOPARD syndrome 2	**RAF1**
Noonan syndrome 8	**RIT1**
Noonan-like syndrome with loose anagen hair	**SHOC2**
Noonan syndrome 4	**SOS1**
Skeletal diseases	
Achondrogenesis type 2	**COL2A1**
Achondroplasia	**FGFR3**
CATSHL syndrome	
Crouzon syndrome with acanthosis nigricans	
Hypochondroplasia	
Muenke syndrome	
Thanatophoric dysplasia, type I	
Thanatophoric dysplasia, type II	
Ehlers-Danlos syndrome, classical	**COL1A1**
Ehlers-Danlos syndrome, type VIIA	
Osteogenesis imperfecta, type I	
Osteogenesis imperfecta, type II	
Osteogenesis imperfecta, type III	
Osteogenesis imperfecta, type IV	
Ehlers-Danlos syndrome, cardiac-valvular form	**COL1A2**
Ehlers-Danlos syndrome, type VIIB	
Osteogenesis imperfecta, type II	
Osteogenesis imperfecta, type III	
Osteogenesis imperfecta, type IV	

Who can take the PrenatalSafe® test

All pregnant women with a gestational age of at least **10 weeks**. The test can be carried out in the case of both single and twin pregnancies, obtained either by natural conception or by both homologous or heterologous assisted reproductive techniques (ART).

How the PrenatalSafe® test is performed

During pregnancy, some fragments of fetal DNA circulate in the maternal blood. Fetal DNA is detectable from week 5 of gestation and its concentration increases in the following weeks, disappearing immediately after delivery. The amount of fetal DNA circulating from week 9-10 of gestation is sufficient to ensure the test high specificity and sensitivity. The test is carried out by drawing a blood sample of the expectant mother with a gestational age of at least **10 weeks**. The test is performed by **analyzing circulating free DNA (cfDNA)** in maternal blood using the innovative **massively parallel sequencing (MPS)** technology of the **entire fetal genome**, using ILLUMINA Next Generation Sequencing (NGS) sequencers. The chromosomal sequences are then quantified through **advanced bioinformatics analysis**, to determine the presence of possible fetal chromosomal aneuploidies, identified by supernumerary sequences that can be aligned to a specific chromosome. Similarly, the analysis is carried out to detect pathological variants that cause inherited or de novo genetic diseases.

Results that can be obtained with the PrenatalSafe® test

"POSITIVE" - Aneuploidy or structural chromosomal alteration detected: indicates that the test has produced a result consistent with an aneuploidy or fetal structural chromosomal abnormality of one (or more) of the chromosomes investigated. This result, however, is not diagnostic and therefore does not guarantee that the fetus has such a condition. The recommended follow-up is an invasive prenatal diagnosis test, such as chorionic villus sampling (CVS) or amniocentesis. The geneticist of Eurofins Genoma (or in general genetic counselor), in the genetic consultation, will explain the test result in detail and recommend confirming the result with invasive prenatal diagnosis. Under no circumstances can you avail yourself of Italian Law 194/78 on the voluntary interruption of pregnancy without having first confirmed the test result by amniocentesis or villocentesis (in case of test perform in Italy).
"NEGATIVE" - Aneuploidy or structural chromosomal alteration not detected: indicates that the test did not detect any aneuploidies or structural chromosomal alterations at the level of the examined chromosomes. This result, however, does not guarantee that the fetus does not carry these abnormalities. In fact, due to placental physiology, the result obtained might not reflect the true chromosomal state of the fetus.
In some cases (approximately 1%) the test could produce a **non-optimal** or **inconclusive result**. In such cases, the expectant mother will be asked for a new blood sample in order to repeat the test. Even after its repetition, the test might not produce a conclusive result. In these cases, it is recommended to use alternative prenatal diagnosis methods, such as amniocentesis or CVS, since in scientific literature an increase in the incidence of fetal aneuploidies has been reported in samples with inconclusive results, for example due to a low fetal fraction.
In other cases, the test could give a result indicating **suspected** fetal chromosomal aneuploidy (**borderline result**). In this case, it will be recommended to confirm the result by invasive prenatal diagnosis, as for the positive result.

The fetal sex will be stated in the medical report if it is requested.
In twin pregnancies, one result will be reported for both fetuses. In these cases, the fetal sex is stated as male or female, based on the presence or absence of the Y chromosome.

Results that can be obtained with the GeneSafe™ Complete test

"POSITIVE": indicates that the test **has detected** one or more variants at the level of one or more of the genes investigated. In the case of diseases investigated with **GeneSafe™ *de novo***, the finding of a pathogenetic variant indicates that the fetus presents a high risk for the specific associated disease, but does not guarantee that the fetus has such a condition. In the case of diseases investigated with **GeneSafe™ *Inherited***, the finding of two pathogenetic variants or result consistent with homozygosity for one variant indicates that the fetus presents a high risk for the specific associated disease, but does not guarantee that the fetus has such a condition. Again, in the case of the diseases investigated with **GeneSafe™ *Inherited*** it is possible to find only one pathogenetic variant, consistent with a carrier state, in relation to the limits of the method (see section entitled Limits of the GeneSafe test). In the event of a high risk, the recommended follow-up is an invasive prenatal diagnosis test, such as villocentesis or amniocentesis. The geneticist of Eurofins Genoma (or in general a genetic counsellor), in the genetic consultation, will explain the test result in detail and recommend confirming the result with invasive prenatal diagnosis. Under no circumstances can you avail yourself of Italian Law 194/78 on the voluntary interruption of pregnancy without having first confirmed the test result by amniocentesis or villocentesis (in case of test perform in Italy).
The **GeneSafe™** test only identifies variants **with a known pathogenic effect**. The test does not search for benign variants, that is those without a benign significance, and variants of uncertain clinical significance (VoUS**)**, that is those not yet known or characterized by the medical-scientific community.
"NEGATIVE": indicates that the test **did not detect** any variant, de novo or inherited from parents, in the fetus with a known pathological significance in the genes examined. This result is consistent with a **low risk,** i.e., it considerably reduces the chances that the fetus has the genetic diseases examined, but cannot guarantee that the fetus does not have the above-mentioned diseases. In some cases (approximately 1%) the test could produce a **non-optimal or inconclusive result**. In such cases, the expectant mother will be asked for a new blood sample in order to repeat

the test. In other cases, in order to obtain an optimal interpretation of the results, it is necessary to examine a paternal sample as well. There is no specific reporting for this latter test.

Accuracy of the PrenatalSafe® test

In validation studies, the test demonstrated a reliability greater than **99%** in detecting common chromosomal aneuploidies in pregnancy (**trisomy 21, trisomy 18, trisomy 13, sex chromosome aneuploidies**), with false positive and false negative percentages <1%. Although the test error is limited, it does however exist. The performance of the **PrenatalSafe®** test is given below:

Table 3. Performance relating to NIPT of common aneuploidies, SCAs and other abnormalities in 71883 pregnancies

Positive cases *n*= 1011 Total follow-up *n*=868	Trisomy 21	Trisomy 18	Trisomy 13	SCA	Other abnormalities*	Overall performance
True positives	437	93	37	156	58	781
False positives	3	1	8	17	54	83
True negatives	71392	71775	71828	65598	46577	70872
False negatives	2	0	0	1	1	4
Sensitivity (95% CI)	**99.54%** (98.36% - 99.94%)	**100%** (96.11% - 100.00%)	**100%** (90.51% - 100.00%)	**99.36%** (96.50% - 99.98%)	**98.31%** (90.91% - 99.96%)	**99.49%** (98.70% - 99.86%)
Specificity (95% CI)	**100%** (99.99% - 100.00%)	**100%** (99.99% - 100.00%)	**99.99%** (99.98% - 100.00%)	**99.97%** (99.96% - 99.99%)	**99.88%** (99.98% - 99.99%)	**99.88%** (99.86% - 99.91%)
PPV (95% CI)	**99.32%** (97.92% - 99.78%)	**98.94%** (92.91% - 99.85%)	**82.22%** (69.82% - 90.24%)	**90.17%** (85.08% - 93.66%)	**51.79%** (45.08% - 58.42%)	**90.39%** (88.36% - 92.11%)
NPV (95% CI)	**100%** (99.99% - 100.00%)	**100%** (99.99% - 100.00%)	**100%** (99.99% - 100.00%)	**100%** (99.99% - 100.00%)	**100%** (99.99% - 100.00%)	**99.99%** (99.99% -100.00%)

CI: Confidence Intervals; SCA: Sex chromosome aneuploidies.
Positive cases without follow-up excluded from the count of positives in Table 3 (No.): T21 (49); T18 (14); T13 (10); SCA (36); other abnormalities (34).
***Rare autosomal aneuploidies, segmental abnormalities and microdeletions are included.**

Table 4. Performance relating to NIPT of sex chromosome aneuploidies

Sex chromosome aneuploidies	X0	XXX	XXY	XYY
True positives	52	27	51	26
False positives	13	0	3	1
True negatives	65724	65775	65747	65776
False negatives	1	0	0	0
Sensitivity (95% CI)	**98.11%** (89.93% - 99.95%)	**100%** (87.23% - 100.00%)	**100%** (93.02% - 100.00%)	**100%** (86.77% - 100.00%)
Specificity (95% CI)	**99.98%** (99.97% - 99.99%)	**100%** (99.99%- 100.00%)	**99.99%** (99.99% - 100.00%)	**99.99%** (99.99% - 100.00%)
PPV (95% CI)	**80%** (69.88% - 87.34%)	**100%** (99.99% - 100.00%)	**94.44%** (84.57% - 98.14%)	**96.3%** (78.55% - 99.46%)
NPV (95% CI)	**100%** (99.99% - 100.00%)	**100%** (99.99% - 100.00%)	**100%** (99.99% - 100.00%)	**100%** (99.99% - 100.00%)

CI: Confidence intervals; SCA: Sex chromosome aneuploidies. Positive cases without follow-up excluded from the count of positives in Table 4 (No.): X0 (18); XXX (6); XXY (7); XYY (5).

Table 5. Performance relating to NIPT of rare autosomal aneuploidies, segmental abnormalities and microdeletions

Other abnormalities	RAA	Segmental abnormalities (>7 Mb)	Microdeletions* (Segmental abnormalities <7 Mb)
True positives	33	20	5
False positives	36	16	2
True negatives	46630	46681	28743
False negatives	0	0	1
Sensitivity (95%CI)	**100%** (89.42% - 100.00%)	**100%** (83.16% - 100.00%)	**83.33%** (35.88% - 99.58%)
Specificity (95%CI)	**99.92%** (99.89% - 99.95%)	**99.97%** (99.96%- 99.99%)	**99.99%** (99.99% - 100.00%)
PPV (95%CI)	**47.83%** (39.81% - 55.96%)	**55.56%** (43.37%- 67.11%)	**71.43%** (37.40% - 91.27%)
NPV (95%CI)	**100%** (99.99% - 100.00%)	**100%** (99.99% - 100.00%)	**100%** (99.99% - 100.00%)

CI: Confidence intervals; RAA: Rare chromosomal abnormalities.
Positive cases without follow-up excluded from the count of positives in Table 5 (No.): RAA (25); Segmental abnormalities >7Mb (7); Microdeletions (2).
***Investigated microdeletion syndromes: DiGeorge syndrome, Cri-du-chat syndrome, Prader-Willi syndrome, Angelman Syndrome, 1p36 deletion syndrome, Wolf-Hirschhorn syndrome, Jacobsen syndrome, Langer-Jeidion syndrome and Smith-Magenis syndrome.**

Limits of the PrenatalSafe® test

Non-invasive prenatal testing of circulating cell-free fetal DNA isolated from a sample of maternal blood is a **screening test** and although this test is very accurate, **the results are not diagnostic** and are to be evaluated in the context of the expectant mother's clinical picture and family medical history. In addition, the test is not a substitute for invasive prenatal diagnosis (villocentesis or amniocentesis).

The test has been validated on single and twin pregnancies, monozygotic or dizygotic, starting from week 10 of gestation.

The test cannot rule out the presence of all fetal chromosomal abnormalities. **PrenatalSafe® 3** evaluates only the aneuploidies borne by chromosomes 13, 18, 21, **PrenatalSafe® 5** also evaluates the aneuploidies of the sex chromosomes (X and Y); **PrenatalSafe® Plus** also evaluates the aneuploidies of chromosomes 9 and 16; the aneuploidies of other chromosomes are only identifiable with the **PrenatalSafe® Karyo** test.

The **PrenatalSafe® Karyo** test detects **92.6%** of the fetal chromosomal abnormalities detectable prior to birth and **96.2%** of those found at birth.

The **PrenatalSafe® Karyo Plus** test detects **95.5%** of fetal chromosomal abnormalities detectable prior to birth and 99.1% of those found at birth.

The **PrenatalSafe® test** is not able to detect balanced chromosomal rearrangements, fetal and/or placental chromosomal mosaics (i.e. the presence of two cell lines with a different chromosomal arrangement), single nucleotide variants, methylation defects and polyploidies. The test does not detect any malformations or defects not specifically searched for. In particular, the test does not detect the presence of hereditary genetic diseases with Mendelian transmission.

Partial alterations in the chromosomes analysed and structural chromosome alterations can only be detected with the **PrenatalSafe® Karyo** tests. The estimated resolution limit of the test is **10 Mb**, comparable to that of the cytogenetic (traditional) karyotype at 400 bands (approximately 7-10 Mb). The **PrenatalSafe® Karyo Plus** test has an estimated resolution limit of **7 Mb** and detects structural chromosome alterations at a resolution of approximately **3 Mb** at the level of the chromosomal regions associated with the microdeletion syndromes investigated.

In **bichorial twin pregnancies** it is not possible to distinguish the condition of the individual fetus, to evaluate the aneuploidies of the sex chromosomes, or to detect the presence of microdeletions. However, the presence/absence of the Y chromosome can be detected. If the presence of the Y chromosome is detected, it is not possible to discern whether only one or both fetuses are male. In pregnancies that have started as twins or multiple, followed by the abortion of one or more fetuses with reabsorption of the gestational chamber (**vanishing twin**), cell-free fetal DNA of the aborted fetus may also be present in the maternal blood. This could interfere with the quality of the results, with consequent false positive results if the cause of the abortion was due to the presence in the above-mentioned fetus of chromosomal aneuploidies affecting one of the chromosomes investigated. Similarly, there may be a mismatch in sex results (e.g., male sex diagnosis, where the presence of the Y chromosome originates from the DNA of the aborted fetus).

The existence of a tumour condition in the expectant mother could produce false positive test results.
The test is based on the quantification of the cell-free fetal DNA fragments circulating in the maternal blood, which are placental in origin. Therefore, due to conditions of chromosomal mosaicism (frequency: 1-2%) there may be discrepancies in the results (false positives or false negatives) that justify the sensitivity and specificity of the test <100%. In particular, the test could produce a positive result (aneuploidy detected), but this chromosomal abnormality could be confined to the placenta due to the **chromosomal mosaicism** and therefore the fetus could have a normal karyotype at the time of invasive prenatal diagnosis (false positive). On the other hand, the test may produce a negative result (aneuploidy not detected), but due to the chromosomal mosaicism the fetal DNA without aneuploidy could be confined to the placenta and therefore the fetus could have an aneuploid karyotype at the time of invasive prenatal diagnosis (false negative) or after birth.
Fetal sex is referred to as male or female, based on the presence or absence of the Y chromosome, but it gives no information on the presence or absence of the SRY gene.

Pregnancies with ultrasound findings suggestive of fetal disease should be studied with other types of prenatal investigations, such as molecular fetal karyotype on chorionic villi or amniotic fluid.
The test may detect chromosomal abnormalities of the expectant mother in a homogeneous or mosaic form with a consequent reduction in test accuracy. If the pregnant woman has a chromosomal abnormality, the test cannot give any information about its presence in the fetus and it may be advisable to evaluate the opportunity to proceed with invasive diagnostics as well as further investigations on the expectant mother.

A **"NEGATIVE - Aneuploidy or structural chromosomal alteration not detected"** result significantly reduces the chances that the fetus has an aneuploidy or structural chromosome alteration at the level of the examined chromosomes, but it cannot guarantee that the chromosomes are actually normal or that the fetus is healthy.
Due to the above limits, in the event of a positive result, it is recommended to have a consultation with a geneticist and confirm the result by analysing the karyotype on amniotic fluid.

GeneSafe™ test limits

This test evaluates only the genetic diseases and genes listed in Table 1 (**GeneSafe™ inherited**) and in Table 2 (**GeneSafe™ de novo**). The test does not detect any genetic disease or genes not specifically investigated. The test is also unable to detect:

- variants located in the intronic regions over ± 5 nucleotides from the exon/intron boundaries;
- deletions, inversions or duplications greater than 20 bp;
- mosaicisms.

The test might not detect low percentage insertions/deletions at polynucleotide regions consisting of a repeated series of the same nucleotide in sequence. In the case of biological parents both carrying the same genetic variant in the genes included in the GeneSafe™ Inherited test, a reduction in reliability is possible, in relation to the percentage of fetal fraction found and further investigation by invasive means may be suggested.
GeneSafe™ is a **screening test** and is not a diagnostic test. Although this test is very accurate, **the results are not diagnostic** and are to be evaluated in the context of the expectant mother's clinical picture and family medical history. In addition, the test is not a substitute for invasive prenatal diagnosis (villocentesis or amniocentesis).
The test has been validated on single or twin pregnancies, monozygotic or dizygotic, with at least 10 weeks of gestation.

In **twin pregnancies** it is not possible to distinguish the condition of the individual fetus. In pregnancies that have started as twins or multiple, followed by the spontaneous abortion of one or more fetuses with reabsorption of the gestational chamber (**vanishing twin**), cell-free fetal DNA of the aborted fetus may also be present in the maternal blood. This could interfere with the quality of the results, producing false positives/false negatives. The existence of a tumour condition in the pregnant woman could produce false positive test results due to circulating tumour DNA (ctDNA) variants at the level of the genes involved in the carcinogenesis process (e.g. BRAF, KRAS, NRAS).
A **"NEGATIVE" - Low risk"** result considerably reduces the chances that the fetus has the genetic diseases examined but cannot guarantee that the fetus is healthy.
It is not possible to perform this test in women who carry a mutation in the genes investigated in Table 2 (**GeneSafe™ *de novo***).

The **GeneSafe™** test only identifies variants **with a known pathogenic effect**. The test does not search for benign variants, that is those without a benign significance, and variants of uncertain clinical significance (VoUS**)**, that is those not yet known or characterized by the medical-scientific community. The interpretation of the genetic variants is based on the latest knowledge available at the time of analysis. This interpretation could change in the future

according to new scientific and medical information on the structure of the genome and influence the evaluation of the variants.
Due to the above limits, it is recommended to have a consultation with a geneticist in the event of a positive result.

PrenatalSafe®: Reporting Times

The estimated reporting times vary depending on the type of test requested:

Type of test	FAST procedure	STANDARD procedure
PrenatalSAFE® 3	**3 days**	**3 days**
PrenatalSAFE® 5	**3 days**	**3 days**
PrenatalSAFE® 5 DiGeorge	**5 days**	**7 days**
PrenatalSAFE® Plus	**5 days**	**7 days**
PrenatalSAFE® Karyo	**4 days**	**5 days**
PrenatalSAFE® Karyo Plus	**5 days**	**7 days**
PrenatalSAFE® COMPLETE	**4 (10-15) days*** **	**5 (10-15) days *** **
PrenatalSAFE® COMPLETE Plus	**5 (10-15) days *** **	**7 (10-15) days *** **
GeneSAFE™		**10-15 days**

*** The **PrenatalSafe® COMPLETE** test report consists of two different reports: the first one refers to the non-invasive analysis of the fetal karyotype that will be provided in 4 business days and the second one is related to inherited and/or de novo genetic diseases that will be available between 10 and 15 business days.
The reporting times indicated above, however, are not mandatory and could be extended in the event of repeating the test, biological mother carrying recessive disease (investigated in the **GeneSafe™ *Inherited*** test), non-optimal results, in-depth test analysis or interpretative doubts.

Alternatives to the PrenatalSafe® test: invasive prenatal diagnosis

Non-invasive prenatal testing of fetal DNA in maternal blood is just one of the options for pregnant women to determine the presence of fetal chromosomal abnormalities during pregnancy.
There are several other tests that can be carried out during pregnancy, in particular the study of the karyotype by cytogenetic analysis (traditional fetal karyotype) or by array (molecular fetal karyotype) can be carried out by sampling the amniotic fluid or chorionic villus. Chorionic villus sampling (placental tissue which, although separated from the fetus, contains the same DNA) or villocentesis is carried out between week 11 and 12 of gestation and consists in transabdominal ultrasound-guided sampling of chorionic villi. This sampling involves a risk of abortion. Amniotic fluid sampling or amniocentesis is performed by ultrasound-guided transabdominal puncture between week 16 and week 18 of pregnancy and carries a risk of abortion.
On the sampling of the chorionic villi and amniotic fluid, it is possible to investigate not only the fetal karyotype but also other genetic conditions. The indications for these investigations may be different and should be discussed with the attending physician and/or in the course of a dedicated genetic consultation.

Comparison of the *detection rate* between PrenatalSafe® and fetal karyotype

The **PrenatalSafe® 3 and 5** tests enable detecting respectively **71% and 83.1%** of the chromosomal abnormalities found in pregnancy. With the **PrenatalSafe® Plus** test the detection rate becomes **86%. PrenatalSafe® Karyo**, on the other hand, enables detecting **92.6%** of the chromosomal abnormalities that can be detected during pregnancy and **96.2%** of those found at birth.

The **PrenatalSafe® Karyo Plus** test detects **95.5%** of fetal chromosomal abnormalities detectable prior to birth and 99.1% of those detected at birth, reaching a detection rate level very similar to that of the **traditional fetal karyotype (96.9%)**, obtained by invasive prenatal diagnosis techniques. The **molecular fetal karyotype** – array-CGH performed on fetal cells taken with amniocentesis or villocentesis, enables detecting **99.8%** of the chromosomal abnormalities found in pregnancy. (Wellesley, D, et al., 2012; Wapner et al., 2012; Fiorentino et al., 2011; 2013).

The GeneSafe™ test as a supplement to the PrenatalSafe® test

The **GeneSafe™** test provides information on the presence/absence of variants in specific monogenic diseases found in the fetus but does not provide any information with respect to fetal aneuploidies or structural chromosomal abnormalities. In order to obtain as much information as possible, using non-invasive prenatal screening techniques, it is helpful to combine the **GeneSafe™** test with the **PrenatalSafe® Karyo** test. The latter test is able to detect aneuploidies and chromosomal structural abnormalities on the whole fetal karyotype, providing very similar information to that obtainable by invasive prenatal analysis.

Additional analysis to the PrenatalSafe® test

With a specific request it is possible to associate the **PrenatalSafe®** test with the **RhSafe®** test as well.
The RhSafe® test is a non-invasive prenatal test that, by analysing fetal DNA isolated from a blood sample of the expectant mother, determines the fetal Rh(D) factor. The test applies only to pregnancies with expectant mothers Rh (D) negative and partners Rh (D) positive.

The Rhesus Factor (Rh)

Human red blood cells have on their surface the antigens responsible for the AB0 blood groups and the antigens that make up the **Rhesus Factor (Rh)**. Among Rh factor antigens, D is the clinically most important one. Subjects presenting the D antigen on the surface of the red blood cells are defined as **Rh positive** and subjects not presenting it are **Rh negative** (approximately 15% of the Caucasian population).
RhD plays a key role in what is called **maternal-fetal incompatibility**. If the fetus is **Rh(D)** positive **(Rh+)** and the expectant mother **Rh(D) negative (Rh-)** it is likely that the red blood cells of the fetus with the Rh antigen will enter the maternal blood stream. Without the appropriate precautions during pregnancy, there is a risk that the pregnant woman will develop an immune reaction producing antibodies against the red blood cells of the fetus (**sensitization or alloimmunization**), which will remain in her blood. In the event of a second pregnancy with a positive Rh(D) fetus, maternal antibodies against the fetal erythrocyte antigens may pass through the placenta and attack the fetal red blood cells, which will then be destroyed as they are recognized as “foreign”. This then determines the so-called **haemolytic disease of the fetus and newborn (HDFN)**.
Maternal-fetal incompatibility does not occur when both parents are Rh negative, or if the mother is Rh positive and the father is Rh negative.

How to avoid the risk of sensitization

To prevent sensitization, the pregnant woman may undergo immunoprophylaxis with **anti-D immunoglobulins**, during pregnancy and after delivery.

The benefits of the RhSafe® test?

The **non-invasive** test for early determination of the fetal **Rh (D) factor**, using cell-free fetal DNA analysis in maternal blood, is a reliable and useful test that has become routine in the management of pregnancies characterized by maternal-fetal incompatibility.
The test helps **identify pregnancies at risk** for haemolytic disease of the fetus and newborn and thus reduce the use of anti-D prophylaxis in cases where the fetus is Rh(D) negative like the mother.

Who can take the RhSafe® test?

All pregnant women Rh (D) negative with a gestational age of at least **10 weeks**. The **RHSafe®** test is performed (on request) only in pregnancies with expectant mother Rh(D) negative and male partner Rh(D) positive. Medical reports certifying the couple’s Rh factor must be presented in order to perform the test. Should these medical reports not be presented within 30 days of the date of acceptance of the sample, the test will not be performed.

How is the RhSafe® test performed?

Cell-free **fetal DNA** is initially isolated from the plasma component of the maternal blood. Subsequently, through a highly sensitive advanced technological process, the DNA is **amplified by a Real time PCR technique**. Automated genetic analysis identifies the presence/absence of DNA sequences belonging to the RHD gene and therefore enables defining whether the fetus is Rh(D) positive (presence of the RHD gene) or negative (absence of the RHD gene) in Rh(D) negative women.

Waiting times for the results

The estimated reporting time is approximately 10 business days. These terms, however, are not mandatory and may be extended in the event of repeating the test, suboptimal results, in-depth analysis or interpretative doubts.

Limits of the RhSafe® test

The RhSafe® test is a screening test and is not a diagnostic test. The test is not a substitute for invasive prenatal diagnosis (villocentesis or amniocentesis). A “NEGATIVE - Absence of the RHD gene” result greatly reduces the chances that the fetus is RhD positive but cannot guarantee that this result is consistent with the actual fetal genotype. The absence of the signal related to the amplification of the RHD gene may be due to the absence or low amount of detectable fetal DNA in the maternal blood or its degradation. The reliability of the result is therefore correlated to the amount of fetal DNA available in the test sample, which depends on the patient's gestation week and the quality of the fetal DNA in the biological sample. Some fragments of the RHD gene may be found in pregnant women with the Rh- phenotype, this may occur due to a pseudogene (RHDΨ) or a d(C)ce haplotype variant, both present mainly in the African population. These genotypescan give false positives with genetic analysis of the RHD. Due to the presence of localized variants at the primer annealing sites, false negatives are also possible, especially in less studied populations. For this reason, it is always recommended to perform the RhD genotype at the time of delivery. This test cannot be performed on RhD Positive pregnant women. In the case of a twin pregnancy, a positive result does not define whether one or both fetuses have the RHD gene. However, there is a risk of fetal maternal immunization even if only one of the fetuses is RhD positive. The sensitivity and specificity of the test are greater than 99% [Runkel et al., BMC Pregnancy and Childbirth (2020) 20:83]. The estimated risk of diagnostic error is approximately 1%.

Pre-Test Information Sheet

Our centre offers the pre-test information service free of charge, both by telephone and at the offices of the laboratories of the Eurofins Genoma Group, to explain the aims of the test, its benefits, its limits, and the results that can be obtained to the expectant mother.

Genetic Consultation

Our centre offers free genetic consulting services, both pre-test and post-test, at the offices of the laboratories of the Eurofins Genoma Group, in order to explain to the expectant mother, the aims of the test, the results that can be obtained and the results that emerged upon completion of the test, in particular in cases of pathological findings, with a high risk of chromosomal disease.

Privacy

All your data will be treated with extreme confidentiality and according to the applicable laws on Privacy (EU Reg. 679/ 2016). The results of the tests will be communicated only to the health-care operators involved in carrying out the test or to the geneticist (if necessary). In addition, the test results can be issued to those who, by law, can have access to them.

Storage of samples

Biological samples are identified with a barcode and alpha-numeric ID, so no identification data is linked to the specimen tube. It is therefore impossible for anyone to be able to trace personal data.