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(57) Abrégé/Abstract:
The present invention relates to a method for detecting fetal Down syndrome (Trisomy 21), trisomy 13, trisomy 18 and other chromosomal anomalies during prenatal screening by analyzing a dried blood sample from a pregnant woman. More particularly the present invention relates to a method for improving detection efficiency in screening for the anomalies by measuring the amount of the free beta human chorionic gonadotropin (HCG) and nicked or fragmented or aberrant forms of free beta (HCG), all of which are referenced throughout this application as free beta (HCG) in dried blood samples from pregnant women.

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**(54) Title:** DOWN SYNDROME SCREENING METHOD UTILIZING DRIED BLOOD SAMPLES

**(57) Abstract**

The present invention relates to a method for detecting fetal Down syndrome (Trisomy 21), trisomy 13, trisomy 18 and other chromosomal anomalies during prenatal screening by analyzing a dried blood sample from a pregnant woman. More particularly the present invention relates to a method for improving detection efficiency in screening for the anomalies by measuring the amount of the free beta human chorionic gonadotropin (HCG) and nicked or fragmented or aberrant forms of free beta (HCG), all of which are referenced throughout this application as free beta (HCG) in dried blood samples from pregnant women.

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# DOWN SYNDROME SCREENING METHOD

# UTILIZING DRIED BLOOD SAMPLES

## FIELD OF THE INVENTION

The present invention relates to a method for detecting fetal Down syndrome (Trisomy 21), Trisomy 13, Trisomy 18, Turners syndrome and other chromosomal anomalies during prenatal screening by analyzing a dried blood sample from a pregnant woman. More particularly the present invention relates to a method for improving detection efficiency in prenatal screening for Down syndrome by measuring the amount of free beta (human chorionic gonadotropin "HCG") and nicked or fragmented or aberrant forms of free beta

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(HCG) all of which are referenced throughout this application as free beta (HCG) in dried blood samples from pregnant women.

## BACKGROUND OF THE INVENTION

Down syndrome, also referred to as Trisomy 21, is the most common congenital cause of severe mental retardation. Generally, fetal Down syndrome can be determined by diagnostic procedures including amniocentesis or chorionic villus sampling and karyotyping. However, these diagnostic procedures are invasive and involve risk to the woman and the fetus. For this and other reasons, amniocentesis or chorionic villus sampling and karyotyping are not routinely performed during all pregnancies. Instead, one or more screening methods may be utilized to determine when the risk to the pregnancy warrants the risk of undergoing an invasive diagnostic procedure.

The incidence of Down syndrome increases significantly with increasing maternal age. Historically, the prenatal detection of Down syndrome has focused on pregnant women at and over the age of 35, at which ages the risks of Down syndrome approach or exceed the risks of diagnostic procedures utilized to detect fetal Down syndrome. Therefore the standard method of prenatal screening has involved selecting women for diagnostic amniocentesis on the basis of maternal age. Age, however, is an inadequate screening criterion in that only about 20% of all Down syndrome pregnancies can be detected by carrying out amniocentesis and karyotyping on the 5% of pregnant women most at risk, that is, those aged 35 years or greater. And, because in actual clinical practice only about half of the women aged 35 years or greater undergo amniocentesis and karyotyping, fewer than 10% of Down syndrome pregnancies are prenatally detected.

In 1984 an association between lowered maternal blood alpha-fetoprotein (AFP) levels and fetal Down syndrome was discovered. It was noted that other chromosomal trisomies, in particular Trisomy 13 and Trisomy 18, were also associated with lowered maternal blood AFP levels. The incidence of these additional chromosomal trisomies (1 in 5000 pregnancies and 1 in 6600 pregnancies, respectively) is significantly lower than the general a priori risk associated with Trisomy 21 (Down syndrome, 1 in 800 pregnancies). However, because of the association of these other chromosomal trisomies with lowered MSAFP levels, and elevated or depressed free beta (HCG) levels, such abnormalities will also be detected within a screening protocol utilizing maternal blood AFP and free beta (HCG) and possibly additional markers described herein. It is obvious to those skilled in the art that in using the protocol described herein for Trisomy 21, the detection of Trisomy 13, Trisomy 18, Turners syndrome and other chromosomal anomalies may also be accomplished.

The association between lowered maternal blood AFP levels and fetal Down syndrome presented the opportunity to use a non-invasive blood screening test in the detection of Down syndrome cases in young, apparently unaffected families where approximately 80% of Down syndrome cases occur. It is estimated that the use of a screening test based on low maternal blood AFP (as a screening marker) would lead to the prenatal detection of approximately 20% of all cases of fetal Down syndrome. Another method for screening involves measuring the level of unconjugated estriol (UE) in maternal blood.

U.S. Patent No. 4,874,693 to Bogart discloses an association between elevated maternal blood HCG levels and elevated maternal blood levels of the alpha subunit of HCG, during the 18th - 25th weeks of pregnancy, and fetal Down syndrome. In the Bogart patent it is

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estimated that the use of elevated maternal blood HCG levels and elevated maternal blood levels of the alpha subunit of HCG in a screening protocol, would detect a greater percentage of chromosomally abnormal fetuses than the use of AFP or UE alone. In a paper entitled "Human Chorionic Gonadotropin Levels in Pregnancies with Aneuploid Fetuses" (Bogart et al., Prenatal Diagnosis, Vol. 9, 379-384 (1989)) Bogart discloses that a screening method utilizing HCG and the alpha subunit of HCG is not useful at 9-11 weeks gestation (the first trimester of pregnancy) for selecting pregnancies at risk for fetal aneuploidy (including Down syndrome).

In my co-pending U.S. patent applications, serial number 07/868,160, filed April 14, 1992, serial number 07/709,019 filed May 31, 1991 and serial number 07/420,775, filed October 12, 1989, I describe Down syndrome screening methods utilizing free beta (HCG) (and nicked or fragmented or aberrant forms of free beta (HCG)) that detect a greater percentage of chromosomally abnormal fetuses than heretofore utilized screening methods. These applications also describe an apparatus that may be advantageously utilized by laboratories to analyze samples in a screening protocol. The disclosure of each application is hereby incorporated herein by reference.

As described in these applications, a particularly effective Down syndrome screening method includes measuring a pregnant woman's maternal blood level of free beta (HCG), and comparing the measured level of free beta (HCG) to reference data including the levels of free beta (HCG) in (1) pregnant woman carrying normal fetuses and (2) pregnant woman carrying Down syndrome fetuses. I also describe how Down syndrome screening may be further enhanced by utilizing a screening method that includes comparing the pregnant woman's maternal blood level of AFP and the pregnant

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U.S. Patent Nos. 5,316,953; 5,258,907 and 5,324,668 describe Down syndrome screening methods utilizing free beta (HCG) (and nicked or fragmented or aberrant forms of free beta (HCG)) that detect a greater percentage of chromosomally abnormal fetuses than heretofore utilized screening methods. These patents also describe an apparatus that may be advantageously utilized by laboratories to analyze samples in a screening protocol.

As described in these U.S. patents, a particularly effective Down syndrome screening method includes measuring a pregnant woman's maternal blood level of free beta (HCG), and comparing the measured level of free beta (HCG) to reference data including the levels of free beta (HCG) in (1) pregnant woman carrying normal fetuses and (2) pregnant woman carrying Down syndrome fetuses. I also describe how Down syndrome screening may be further enhanced by utilizing a screening method that includes comparing the pregnant woman's maternal blood level of AFP and the pregnant

woman's gestational age to reference data. This comparison is advantageously accomplished using a multivariate discriminant analysis technique. Discriminant analysis is a generally known approach to multivariate analysis involving the separation of a population into two or more groups on the basis of probability. A general discussion of discriminant analysis can be found in Marketing Research; Churchill, G.A.; Dryden, 1976; Chapter 15, pages 530 - 543. The use of multivariate discriminant analysis to compare the pregnant woman's levels of free beta (HCG) an AFP, and her gestational age to reference data detects a greater percentage, with a lower false positive rate, of fetal Down syndrome cases than any other known screening method for the prenatal detection of Down syndrome.

As described in the preceding paragraphs, prenatal screening methods generally include an analysis of the pregnant woman's blood. Generally, this blood sample is taken from the pregnant woman at her physician's office, or in a similar clinical setting then transported to a clinical laboratory for analysis. The blood sample may also be stored for analysis or transport at a later time.

In many instances the clinical setting is in a different physical location than the clinical laboratory where the blood is to be analyzed. For example, the clinical setting where the blood sample is drawn from the pregnant woman may be located in a city or population center and the laboratory located in a suburban or rural setting and in some instances in another state.

In generally utilized screening methods, the blood sample taken from the pregnant woman is transported, and/or stored for later analysis, in liquid form in a vial or test tube. Additionally, the test tubes must be sealed to prevent contamination of the sample and

also to prevent evaporation of the sample prior to analysis. Transporting and storing blood samples in liquid form has many disadvantages. Special handling techniques must generally be utilized to avoid breaking the test tube or vial including packaging the tubes or vials in shock absorbent packages. It is customary to place the tubes in leak proof bags. Generally, to avoid spills the packages must also be designed so that the test tubes remain upright. The net result can be bulky packages that require large storage or shipment areas.

It is also known that blood borne pathogens may be transmitted by liquid blood samples due to splashes, needle sticks, broken test tubes etc that may occur when the blood sample is taken, stored and/or transported with containers used for handling liquid blood. Additionally, it is generally known that certain constituents of the blood will degrade in vitro if not maintained at cool temperatures.

## SUMMARY OF THE INVENTION

According to the method of the present invention, blood is collected from a pregnant woman to be screened during a time period selected from the group consisting of the first, second and third trimesters of pregnancy. Than the blood is transferred to filter paper where the blood dries. The result is a spot, or spots, of dried blood on the filter paper.

The filter paper containing the blood spot is then analyzed to determine the pregnant woman's level of the analyte, or analytes, utilized in the screening protocol. The filter paper containing the blood spot may be stored and/or transported prior to analysis. In a preferred embodiment of the present invention the blood spot is analyzed to determine the pregnant woman's level of free beta (HCG) and/or alpha fetoprotein (AFP).

Although the method of the present invention is described herein with reference to blood, the method may also be advantageously utilized with other bodily fluids, including urine. For example, drops of urine, or other bodily fluid, from a patient could be placed on a specimen card and dried. The dried spots could then be analyzed by conventional immunological techniques, known to those of ordinary skill in the art, in a manner similar to the manner described herein with reference to the analysis of dried blood spots.

In the preferred method of the present invention the dried blood spot is analyzed to determine the pregnant woman's level of free beta (HCG) by eluting the dried blood spot and determining the free beta (HCG) level by conventional immunological methods. The level of free beta (HCG) is then compared to a set of reference data to determine the patient's risk of carrying a fetus with Down syndrome. To improve detection efficiency, the level of free beta (HCG) and the gestational age can be compared to a set of reference data. To further improve detection efficiency, the dried blood spot may be analyzed to determine the pregnant woman's maternal blood levels of free beta (HCG) and AFP (referred to as "analytes" or "markers"). The levels of each marker are then compared to a set of reference data to determine the patient's risk of carrying a fetus with Down syndrome or other chromosomal anomaly.

A multivariate discriminant analysis technique is advantageously used to compare the levels of the markers to a set of reference data. More particularly, a patient specific risk is then calculated using Bayes rule, the patient's a priori risk, and the relative frequencies for unaffected and affected pregnancies which are determined by incorporating the log of the patient's quantitative levels of each marker into the probability density functions for the reference data

developed using multivariate discriminant analysis. If the patient's risk of carrying a fetus with Down syndrome is greater than a given risk cut-off level, the patient should be counseled about further diagnostic tests to confirm the presence of Down syndrome and other chromosomal anomalies.

Similarly, if the method of the present invention is utilized in a screening protocol for trisomy 13, trisomy 18, Turner's syndrome or other chromosomal anomalies, the patient's risk of carrying a fetus with the anomaly may be determined using a multivariate discriminant analysis technique whereby the level of free beta (HCG) and AFP are compared to a set of reference data.

Incorporating gestational age as a marker along with the level of free beta (HCG) and the level of AFP will further improve detection efficiency. Since the maternal blood level of free beta (HCG) and the maternal blood level of AFP for a number of samples tend to be distributed according to a log-gaussian distribution curve, the greatest detection efficiency can be achieved by incorporating the log of the patient's quantitative levels of each marker and gestational age into the probability density functions for the reference data developed using multivariate discriminant analysis.

Further details and advantages of a screening protocol utilizing free beta HCG, and other screening protocols advantageous for use with dried blood or urine spots are set forth in U.S. Patent No. 5,316,953 and No. 5,324,667 entitled "Method and Apparatus for Detecting Down Syndrome by Non-Invasive Maternal Blood Screening".

The technique of "spotting" blood on filter paper is generally known. For example, many hospitals utilize dried blood specimens in neonatal screening programs. The heel of a new born infant is pricked after birth and a drop of blood is obtained on filter paper. After the drop of blood dries, the filter paper may be delivered by conventional surface mails to clinical laboratories for analysis. However, to the best of my knowledge dried blood samples have not been previously utilized in a prenatal Down syndrome screening program utilizing free beta (HCG). A particular advantage of the method of the present invention is that the level of free beta (HCG) resists the effects of dimer HCG degradation irrespective of the temperature at which the dried blood spot is stored. This may lead to better detection efficiency.

The method of the present invention also provides numerous advantages in the transport and storage of blood samples. The dried blood spot samples on filter paper take up much less space than liquid blood samples in test tubes. Thus less space is needed to store the samples, and the samples may be shipped by conventional mails or delivery services in small packages. Other advantages in shipping dried blood spots on filter paper in comparison with liquid blood samples in tubes or vials are readily apparent to those of skill in the art

A further advantage of using dried blood spots is that in the method of the present invention a smaller volume of blood is collected from the pregnant woman than in the case where a liquid blood sample is to be analyzed. Since less blood is needed, it is possible to make the collection technique less invasive to, and potentially less painful for, the pregnant woman.

An advantage of the preferred method, utilizing free beta (HCG), is that it correctly predicts a higher percentage of fetal Down syndrome cases, with a lesser

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false positive rate than other known methods and processes.

Other advantages of the present invention will become clear from the following more detailed description and the following examples.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a Table, referred to in Example 2, showing the level of significance of individual markers for Trisomy 21.

FIG. 2 is a table, referred to in Example 2, showing Down syndrome screening efficiency of individual markers.

FIG. 3 is a table, referred to in Example 2, showing Down syndrome screening efficiency of composite markers.

FIG. 4 is a table, referred to in Example 2, showing proportion of Down syndrome cases above given percentiles of the distribution of free beta (HCG) in unaffected pregnancies

FIG. 5 is a table, referred to in Example 2, showing Down syndrome efficiency of individual markers.

FIG. 6 is a table, referred to in Example 2, showing Down syndrome screening efficiency of log AFP and log free beta (HCG) as a composite marker at different gestational age ranges.

FIG. 7 is a table, referred to in Example 2, showing projected Down syndrome screening efficiency of AFP, free beta (HCG) and maternal age across the U.S.A.

FIG. 8, referred to in Example 2, shows the levels of free beta (HCG) in cases of trisomy 21 in relation to various percentiles of the unaffected pregnancies.

FIG. 9, referred to in Example 2, shows the distributions of free beta (HCG) levels.

FIG. 10 is a Table, referred to in Example 3, showing Down syndrome screening efficiency for a variety of combinations of markers.

FIG. 11, referred to in Example 4, shows the distribution of free beta (HCG) levels in patient samples.

FIG. 12, referred to in Example 5, shows the distribution of free beta (HCG) levels in patient samples.

FIG. 13, referred to in Example 6, shows the distribution of free beta (HCG) levels in patient samples.

FIG. 14 depicts a sample specimen card in front view.

FIG. 15 depicts a sample specimen card in back view.

FIG. 16 depicts a site for obtaining a blood sample.

FIG. 17, referred to in Example 7, is a chart depicting the change in measured levels of free beta (HCG) in dried blood samples, over time, at various storage conditions.

FIG. 18, referred to in Example 7, is a chart depicting the change in measured levels of free beta (HCG) in liquid blood samples, over time, at various storage conditions.

## DETAILED DESCRIPTION OF THE INVENTION

According to the method of the present invention a maternal blood sample is taken from a pregnant woman and dried on filter paper. The filter paper, which is also referred to as a specimen collection card, is commercially available from a variety of sources, including Whatman, Inc., located in Hillsborough, Oregon and Schleicher & Schuell, located in Keene, New Hampshire. Generally a 3 inch by 4 inch, or a 5 inch by 7 inch card is utilized to collect the samples, however the filter paper may be any size without affecting the method of the present invention. Preferably, the size of the filter paper is convenient

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for transporting, storing and/or indexing the dried blood samples. The filter paper may advantageously be of sufficient size to enable a technician or nurse to write the pregnant woman's name or other identifier as well as other information such as the date the sample was collected on the paper. In a preferred embodiment of the present invention the filter paper (specimen collection card) is a Schleicher & Schuell #903™ 3 inch by 4 inch card pre-printed with circles to provide locations for the blood spots (application sites) and spaces for the technician or nurse collecting the blood sample to enter the patient's identification number, birth date, the date of collection of the sample and the physician's name.

This type of specimen card is shown in Figures 14 and 15. As shown in Figure 14, the front 500, of specimen card 501 has the outlines of five circles 502. These circles provide reference points for the technician or nurse collecting the blood sample. As set forth below, it is preferred that the technician or nurse attempt to locate the dried blood specimens within the circles 502. The front, 500 of specimen card 501 may also contain preprinted locations for the technician or nurse to enter information about the patient from whom the blood is collected. As shown in Figure 14, this information may include the patient's name, 504 and identification, 506.

Figure 15 shows the back side 510, of specimen card 501. As shown in Figure 15 the back of the specimen card may also contain outlines of the circles, 502 to assist the technician or nurse in locating the blood samples on the card.

The amount of blood taken from the pregnant woman should be sufficient to produce at least one spot on the filter paper approximately 10 millimeters in diameter. It is generally advantageous however to produce more than one dried blood spot from the

pregnant woman. In a preferred embodiment of the present invention the amount of blood taken from the pregnant woman is sufficient to produce six spots approximately 10 mm in diameter on the filter paper. It will be understood by those of ordinary skill in the art that the number of blood spots produced on a single piece of filter paper depends on the dimensions of the filter paper and the requirements of the physician and the clinical laboratory that will be analyzing the blood.

A variety of techniques for "spotting" blood on filter paper are known to the art. The choice of the particular technique utilized to produce the blood spots is a matter of choice to the person collecting the sample. Generally, a convenient site on the pregnant woman, preferably a finger tip, toe or ear lobe, is sterilized and then pricked with a sterile lancet. Lancets are commercially available from a variety of sources. An especially useful lancet is the Tenderlett™ lancet manufactured and sold by Technidyne, Corporation in New Jersey.

The drops of blood that form at the pricked site may be allowed to drip onto the filter paper to form the blood spots. Alternatively the pricked site may be placed in contact with the filter paper to produce the blood spots. The blood should dry on the filter paper prior to transport and/or storage.

In a preferred embodiment of the present invention the blood spots are collected in the following manner on a Schleicher & Schuell #903™ 3 inch by 4 inch card preprinted with six circles approximately 10 mm in diameter such as depicted in Figures 14 and 15. First the preprinted sections, 504 and 506 in Figure 14, of the card seeking information concerning the pregnant woman and other details are filled out. Preferably a finger tip is chosen as the site that will be pricked to draw the pregnant woman's blood. The fingertip is

warmed by the technician or nurse collecting the sample by rubbing the area. Next the skin around the site to be pricked is cleaned using an alcohol swab and the skin is allowed to air dry. A Tenderlett™ lancet or similar device is removed from its package and firmly positioned at the incision site. The fingertip is pricked (incised) using the lancet to a depth that will ensure sufficient blood flow. The preferred point of incision is shown in Figure 16.

The first drop of blood is wiped away with sterile gauze. Holding the finger flat against a table with the incision site up the filter paper is brought up to the incision site. Before allowing excess pooled blood to run down the finger in one step the paper is brought in contact with the site and blood is allowed to soak through the paper. The paper is positioned so that the incision site contacts a portion of the paper approximately corresponding to the center of one of the six preprinted circles, 502 in Figures 14 and 15. The paper is then removed from contact with the incision site, repositioned and then retouched to the incision site 3 to 5 times to produce a total of six blood spots roughly positioned over the preprinted application areas on the card. Gentle massage around the incision site may be utilized to increase the blood flow if necessary to produce a total of four to six blood spots. After four to six spots have been made the incision is closed by applying a sterile gauze and pressure. The incision site may then be bandaged if necessary.

The blood spots are allowed to air dry at ambient temperature (65°-80° F) in a flat horizontal position generally for a minimum of 2 hours. The filter paper should not be stacked until the blood spots are dry to avoid cross contamination or leaching of the samples. After drying, the filter paper may be placed in an envelope and transported to the laboratory where the

blood spots will be analyzed, or stored for future transport.

While the foregoing paragraphs contain a detailed description of a preferred method for collecting samples, the method of the present invention should not be construed as limited to this method. Other methods for collecting dried blood samples are known to physicians, technicians, nurses and other medical personnel and are suitable for use in the method of the present invention.

The dried blood spots contained on the filter paper may be analyzed to screen the pregnant woman for Down syndrome, and/or other chromosomal anomalies i.e. to determine whether the pregnant woman's risk of carrying a fetus with Down syndrome or another chromosomal anomaly warrants further invasive testing. The blood spots may be analyzed by common laboratory techniques that generally include eluting the blood sample to determine the level of the analyte or analytes of interest. The term analyte refers to a molecule or substance whose concentration or level in the pregnant woman's blood is being determined. In a preferred method of the present invention the dried blood spot is analyzed by eluting the spot as part of the immunological assay technique utilized to determine the concentration of the analyte in the blood.

In more detail, this preferred method utilizes the following assay procedure.

1. A wash solution is prepared by dissolving 5 PBS (phosphate buffered saline) tablets in 1000 milliliters of distilled or de-ionized water. 0.5 ml of Tween*20, a detergent manufactured and sold by Sigma Chemical Company, located in St. Louis, Missouri, are added to complete the wash solution.

2. Utilizing a single well punch/direct transfer to elution plate device, two 4 mm discs/elution plate well are punched from two of the dried blood spots.

*Trade-mark

3. 15 ml of assay buffer are added to a designated reagent trough and then 135 µl of assay buffer are added to the punched discs in each elution plate well.

4. The elution plate is covered and placed on a plate shaker, such as the Titertek* plate shaker manufactured by Flow Laboratories, of McLean, Virginia. The elution plate is shaken at the high speed setting for 30 ± 2 minutes.

5. The eluted material in the elution plate wells is then transferred to micro test tubes using a pipettor. The remaining procedures are set forth with reference to a standard 8 well by 12 well assay plate.

6. An additional 15 ml of assay buffer are added to the reagent trough and 110 µl of the buffer are transferred to each well of a monoclonal "capture" antibody-coated assay plate.

7. 20 µl of the eluted solution from each of the micro test tubes is transferred to a designated well in the antibody-coated assay plate.

8. The assay plate is covered and then shaken on a plate shaker at medium speed for 30 ± 5 seconds.

9. The assay plate is then placed on a platform rotator at 200 RPM for approximately 60 ± 5 minutes.

10. Each well of the assay plate is then washed 5 times with a wash solution to remove the solution remaining in each well.

11. A first working reagent ("working reagent 1" containing labeling antibodies for the monoclonal antibodies on the coated plate is prepared by mixing 20 µl of each antibody in 12 ml of assay buffer.

12. 100 µl of the working reagent 1 is transferred to all of the assay plate wells. The plates are covered and allowed to incubate for 30 ± 2 minutes on a platform rotator at 200 RPM.

13. Each well of the assay plate is then washed 5 times with a wash solution to remove the solution remaining in each well.

*Trade-mark

14. A second working reagent ("working reagent 2") containing "signal" compounds for the labeling antibodies is prepared by mixing 12 ml of assay buffer and 10 $\mu$l of the signal compound for each of the labeling antibodies.

15. 100 $\mu$l of working reagent 2 are added to each of well of the assay plate. The plate is then incubated for approximately 10 $\pm$ 0.5 minutes on a platform rotator at 200 RPM.

16. Each well of the assay plate is then washed 5 times with a wash solution to remove the solution remaining in each well.

17. A phosphatase solution, or a similar solution, that will react with working reagent 2 to produce a color change is prepared. In the case of a phosphatase solution, 15 ml of the phosphatase substrate solution are mixed with 3 phosphatase substrate tablets. In the case of a peroxidase solution, 6 ml of peroxidase substrate are added to 6 ml of peroxidase solution.

18. 100 $\mu$l of the phosphatase or peroxidase solution is added to each well. The plate is then incubated for 15 $\pm$ 1 minutes on a platform rotator at 200 RPM.

19. 100 $\mu$l of phosphatase or peroxidase stop solution is then added to each well to stop further color change.

20. The absorbance of each well is read utilizing a spectrophotometer to determine the amount of analyte in each blood sample. Generally the absorbance is read at 450 nm or 450/690 nm.

21. Each well of the assay plate is then washed 5 times with a wash solution to remove the solution remaining in each well.

22. If a dual analyte assay technique has been utilized, the amount of the second analyte in each blood sample is determined by repeating steps 17-20. Generally a peroxidase or similar solution different

from the first solution prepared in step 17 is utilized.

Although the foregoing steps set forth a preferred procedure for analyzing the blood spots to determine the level of selected analytes in the pregnant woman's blood, other techniques may be utilized. It is believed that analysis of the blood spots is within the ability of those of ordinary skill in the art including, but not limited to, laboratory scientists and their assistants.

The conventional immunological techniques suitable for use in the present invention also include the use of biosensors. A biosensor generally comprises a biologically-derived sensing element, such as an antibody, linked to a transducer that can produce an electrical signal or other observable readout. When a certain amount of the substance to be measured, for example free beta (HCG) or a form thereof, contacts the sensing element a reaction takes place and the transducer translates the reaction into a change of color, fluorecence, temperature, electrical current or other electro-chemical signal. A biosensor that analyzes the dried blood or urine spot is within the scope of the present invention.

In one embodiment of the present invention, the maternal blood level of free beta (HCG) is then measured by analyzing the blood spots. The maternal blood level of free beta (HCG) is then compared to a set of reference data to determine whether the patient is at an increased risk of carrying a fetus with Down syndrome. To increase detection efficiency, both gestational age and the maternal blood level of free beta (HCG) may be compared to a set of reference data to determine whether the patient is at increased risk of carrying a fetus with Down syndrome.

Although any of the known analytical methods for measuring the maternal blood level of free beta (HCG)

will function in the present invention, as obvious to one skilled in the art, the analytical method used for free beta (HCG) must be the same method used to generate the reference data for free beta HCG. If a new analytical method is used for free beta (HCG), a new set of reference data, based on data developed with the method, must be generated. Thus, the technique utilized to analyze the blood spots should be the same for the reference data and the samples to be screened.

It is also generally understood that in generating antibodies specific for free beta (HCG), some antibodies will be specific for the protein and some will be specific for carbohydrate associated antigenic sites. The measurement of the level of free beta (HCG) referred to throughout the description of the invention includes using antibodies specific for either the protein or the carbohydrate associated antigenic sites or any other site on free beta (HCG).

It is further understood by those of ordinary skill in the art, that while the alpha subunit of (HCG) is encoded by a single gene, free beta (HCG) is encoded by a complex family of at least seven very similar genes or pseudogenes. For example, see "Human chorionic gonadotropin beta-subunit is encoded by at least eight genes arranged in tandem and inverted pairs," Boorstein, Vamvakopoules, & Fiddes; Nature Vol 300, 2 December 1982. It is known that only three of the seven free beta (HCG) genes are expressed in the normal placental production of free beta (HCG). For example, see "Fragmentation of the Beta Subunit of Human Chorionic Gonadotropin Produced by Choriocarcinoma; Nishimura, Ide, Utsunomiya, Kitajima, Yuki, and Mochizuki; Endocrinology, Vol. 123, No. 1, 1988. Whether these same three genes are expressed in disease states such as during the presence

of fetal Down syndrome, has not been determined. It is, therefore, possible that multiple forms of free beta (HCG) with small differences in amino acid sequences, or other small differences, may be synthesized. It is further possible that in Down syndrome, one or more of the free beta (HCG) genes are expressed, thereby producing a unique variant or variants (previously referenced as nicked or fragmented or aberrant forms) of free beta (HCG). According to the present invention these variants could be measured by conventional immunological techniques for measuring free beta (HCG). An assay produced to measure the specific free beta (HCG) variant, or variants, associated with Down syndrome may result in even further enhancement of detection efficiency.

We have effectively used assay techniques to measure free beta (HCG) to distinguish between Trisomy 21 affected and unaffected pregnancies. Detection efficiency for Trisomy 21 as high as 83% has been achieved. As is well known to those skilled in the art, the use of antibodies to quantitate specific analytes may result in degrees of cross-reactivity with a distinct yet similar substance. Hence, the distinction between affected and unaffected cases may be influenced by the presence of an aberrant form of free beta (HCG) which, because of some degree of cross-reactivity with the antibodies being used, is being detected. An aberrant form of free beta (HCG) may be designated as a new biochemical substance. Indeed, information from the scientific literature indicates that aberrant forms of free beta (HCG) have been recognized (for example, see Nishimura et al. infra.)

Trisomy 21 affected cases may also be characterized by an aberrant form of free beta (HCG) in which case those skilled in the art will be capable of developing specific antibodies to such aberrant forms

which may result in a further enhancement of detection efficiency for this syndrome.

Alternatively, Down syndrome affected cases may also be characterized by a fragmented form (or fragment) of free beta (HCG) comprising an incomplete portion of the amino acids that comprise free beta (HCG). As will be understood by those of ordinary skill in the art, assays utilized to measure free beta (HCG) will also detect fragments of free beta (HCG) if the epitope, or epitopes, utilized in the assays are present in the fragment of free beta (HCG).

One such aberrant or fragmented form of free beta (HCG) may be referred to as "nicked" free beta (HCG). In nicked free beta (HCG) peptide linkages are missing between amino acids in free beta (HCG). In known forms of nicked free beta (HCG) peptide linkages can be missing between residues 44 and 45 or between residues 47 and 48. Techniques have been developed to measure forms of nicked free beta (HCG). As explained above, these techniques may be utilized in the method of the present invention to screen for fetal Down syndrome by measuring the maternal blood level of nicked free beta (HCG) or the combined blood level of nicked free beta (HCG) and free beta (HCG).

As illustrated in Example 9, certain immunoassays utilized in the measurement of free beta (HCG) measure "nicked" free beta (HCG). The method of the present invention includes the use of such immunoassays. As illustrated in the Examples, a screening protocol for Down syndrome utilizing immunoassays that measure the level of free beta (HCG) and nicked free beta (HCG) in a pregnant woman's blood, may have a detection efficiency of approximately 80%.

Additionally, it is generally known that certain fragments of free beta (HCG) may be excreted in a pregnant woman's urine. One such fragment is commonly referred to as the "beta core fragment" and comprises

the 6th - 40th amino acid residues disulfide linked to the 55th - 92nd amino acid residues that make up the complete free beta (HCG) molecule. It is possible that an assay designed to measure the beta core fragment of free beta (HCG) in a pregnant woman's urine could be utilized the method of the present invention wherein dried urine spots are analyzed.

The reference data reflects the maternal blood level of free beta (HCG) for pregnant women carrying fetuses with Down syndrome (also referred to as affected) and/or the maternal blood level of free beta (HCG) for pregnant women carrying normal fetuses (also referred to as unaffected). As will be generally understood by those of skill in the art, methods for screening for fetal Down syndrome are processes of decision making by comparison. For any decision making process, reference values based on patients having the disease or condition of interest and/or patients not having the disease or condition of interest are needed. In the present invention the reference values are the maternal blood level of the measured marker or markers, for example, free beta (HCG), in both pregnant women carrying Down syndrome fetuses and pregnant women carrying normal fetuses. A set of reference data is established by collecting the reference values for a number of samples. As will be obvious to those of skill in the art, the set of reference data will improve by including increasing numbers of reference values.

To determine whether the patient is at increased risk of carrying a fetus with Down syndrome, a cut-off must be established. It is obvious to those skilled in the art that a cut-off established to determine whether a patient is at increased risk of carrying a fetus with Trisomy 13 or Trisomy 18 may also be effective in identifying cases of trisomy 21. This cut-off may be established by the laboratory, the physician or on a

case by case basis by each patient. The cut-off level can be based on several criteria including the number of women who would go on for further invasive diagnostic testing, the average risk of carrying a Down syndrome fetus to all the women who go on for further invasive diagnostic testing, a decision that any woman whose patient specific risk is greater than a certain risk level such as 1 in 400 should go on for further invasive diagnostic testing or other criteria known to those skilled in the art. The cut-off level could be established using a number of methods, including: percentiles, mean plus or minus standard deviation(s); multiples of median value; patient specific risk or other methods known to those who are skilled in the art.

In another embodiment of the present invention, which results in a detection of a greater number of the cases of fetal Down syndrome, the blood spots are analyzed for both free beta (HCG) and AFP utilizing a dual analyte assay. Although any of the known analytical methods for measuring the maternal blood levels of these analytes will function in the present invention, as obvious to one skilled in the art, the analytical method used for each marker must be the same method used to generate the reference data for the particular marker. If a new analytical method is used for a particular marker, a new set of reference data, based on data developed with the method, must be generated.

The level of free beta (HCG) and AFP in the blood spot is then compared to reference data to determine the pregnant woman's risk of carrying a fetus with Down syndrome. To further improve detection efficiency, gestational age is included in this comparison.

Preferably, a patient specific risk of carrying a fetus with Down syndrome is calculated using Bayes rule, the patients a priori risk, and the relative

frequencies for unaffected and affected pregnancies which are determined by incorporating the patient's quantitative levels on each analyte (free beta (HCG) and AFP) along with the patient's gestational age, into the probability density functions developed for the reference data using multivariate discriminant analysis. The multivariate discriminant analysis can be performed on the commercially available computer program statistical package Statistical Analysis System (manufactured and sold by SAS Institute Inc.) or by other methods of multivariate statistical analysis or other statistical software packages known to those skilled in the art.

The probability density function provides a method for comparing the patient's level of each analyte to a set of reference data. One type of probability density function is set forth below, although as will be obvious to one skilled in the art, other probability density functions will perform similarly, and therefore perform adequately in the present invention.

Formula for Risk of Down syndrome

$$\frac{(1 / \sqrt{|COV|}) * EXP(-.5(Xa-Ma)^{T} cov^{-1}(Xa-Ma)) * Prior\ Risk}{[(1/\sqrt{|COV|}) * EXP(-.5(Xa-Ma)^{T} cov^{-1}(Xa-Ma)) * Prior\ Risk + (1/\sqrt{|COV|}) * EXP(-.5(Xu-Mu)^{T} cov^{-1}(Xu-Mu)) * (1 - Prior\ Risk)}$$

The subscript "a" refers to the affected cases

The subscript "u" refers to the unaffected cases

(X - M) is a vector where each element is the level of each variable minus the mean of the variable.

$cov^{-1}$ is the inverse of the pooled covariance matrix of the affected and unaffected of all of the variables in the model

$(X - M)^{T}$ is the transpose of the (X-M) vector.

EXP refers to the exponential function.

|COV| refers to the determinant of the covariance matrix of all the variables in the model for the reference data.

As obvious to those skilled in the art, individual covariance matrices for unaffected and affected pregnancies can be substituted for the pooled covariance matrix. The formula for the Risk of Down syndrome would then become:

$$\frac{(1 / \sqrt{|COVa|}) * EXP(-.5(Xa-Ma)^{T} COVa^{-1}(Xa-Ma)) * Prior\ Risk}{[(1/\sqrt{|COVa|}) * EXP(-.5(Xa-Ma)^{T} COVa^{-1}(Xa-Ma)) * (Prior\ Risk) + (1/\sqrt{|COVu|}) * EXP(-.5(Xu-Mu)^{T} COVu^{-1} Xu-Mu)) * (1 - Prior\ Risk)]}$$

For the purposes of the discriminant analysis an assumption is made as to the prior probability of Down syndrome in the general unselected population. Generally, the prior probability is approximately 1 in 800. For the multivariate discriminant analysis a decision is made as to what risk cut- off level constitutes a positive test result. For example, if it is desirable to perform further diagnostic tests on a pregnant woman who has a 1 in 400 or greater possibility of carrying a Down syndrome fetus, then when the results of the discriminant analysis indicate that a pregnant woman has a 1 in 400 or greater possibility of carrying a Down syndrome fetus, the pregnant woman is considered to have a positive test result. If a positive test result is indicated, the patient should be counseled about further diagnostic tests to confirm the presence of Down syndrome.

As will be apparent to a person of skill in the art, other statistical and mathematical techniques for calculating the reference parameters, other than a linear discriminant analysis procedure, can also be used.

As set forth above, in a preferred embodiment of the present invention the dried maternal blood samples are analyzed to determine the maternal blood levels of free beta (HCG), and AFP. A patient specific risk of carrying a fetus with Down syndrome is then calculated

using Bayes rule, the patient's a priori risk, and the relative frequencies for unaffected and affected pregnancies which are determined by incorporating the patient's quantitative levels of each marker along with the patient's gestational age, into the probability density functions developed for the reference data using multivariate discriminant analysis. To further increase detection efficiency, the log of the patient's quantitive levels of free beta (HCG) and AFP, along with the patients gestational age, are incorporated into the probability density functions developed for the reference data using multivariate discriminant analysis. The multivariate discriminant analysis can be performed on the commercially available computer program statistical package Statistical Analysis System (manufactured and sold by SAS Institute Inc.) or by other methods of multivariate statistical analysis or other statistical software packages known to those skilled in the art.

For the purposes of the discriminant analysis an assumption is made as to the prior probability of Down syndrome in the general unselected population. Generally, the prior probability is approximately 1 in 800. For the multivariate discriminant analysis a decision is made as to what risk cutoff level constitutes a positive test result. For example, if it is desirable to perform further diagnostic tests on a pregnant woman who has a 1 in 400 or greater possibility of carrying a Down syndrome fetus, then when the results of the discriminant analysis indicate that a pregnant woman has a 1 in 400 or greater possibility of carrying a Down syndrome fetus, the pregnant woman is considered to have a positive test result. If a positive test result is indicated, the patient should be counseled about further diagnostic tests to confirm the presence of Down syndrome.

As obvious to one skilled in the art, in any of the embodiments discussed above, changing the risk cut-off level of a positive or using different a priori risks which may apply to different subgroups in the population, could change the results of the discriminant analysis for each patient.

The present invention is not limited to the embodiments discussed above but rather includes all of the possible embodiments and combination of markers disclosed in the following examples.

EXAMPLE 1

Over 400 patient samples were utilized to study the relationship of fetal Down syndrome to the maternal blood levels of free beta (HCG) in conjunction with maternal serum AFP (MSAFP), UE, and intact HCG. These samples included 25 maternal blood samples from pregnant women known to be carrying fetuses with Down syndrome and control samples matched to the affected cases. In this example liquid blood samples were analyzed.

For each blood sample quantitative levels of AFP, the intact HCG molecule, free beta (HCG), and UE (hereinafter each is referred to as a Marker) were determined by the following assay techniques:

| Marker | Assay Technique |
|---|---|
| MSAFP | Enzyme linked immunosorbent assay (ELISA) |
| UE | Radioimmunoassay |
| Intact HCG | Bead type ELISA |
| free beta (HCG) | ELISA |

The level of each Marker became a variable in the stepwise discriminant procedure and the linear discriminant procedure on the commercially available computer software statistical package Statistical

Analysis System (SAS Institute Inc.) to generate a set of reference data. The ratio of free beta (HCG) to the intact HCG molecule and the patients gestational age were also incorporated as variables. The stepwise discriminant procedure determined that all of the variables could be incorporated into the linear discriminant procedure. The linear discriminant procedure was then performed on each variable separately and on different combinations of variables. The results of these discriminant analyses are summarized in the chart below. Sensitivity is the percentage of fetal Down syndrome cases which show a positive test result. False positives is the percentage of normal fetuses which show a positive test result.

| VARIABLE | SENSITIVITY | FALSE POSITIVES |
|---|---|---|
| MSAFP | 15.4 % | 4.2 % |
| UE | 15.4% | 2.8% |
| Intact HCG | 37% | 8.6% |
| MSAFP, UE Intact HCG | 50.0% | 7.2% |
| free beta (HCG) +Intact HCG | 60.0% | 8.5% |
| Composite w/o ratio | 76% | 5.3% |
| Composite w/o UE | 76% | 5.3% |
| Composite | 80% | 4.3% |
| Composite w/o free beta HCG | 60.0% | 5.3% |
| * log intact HCG + (log free beta (HCG) + intact HCG) | 68% | 7.6% |
| *log intact HCG, log MSAFP (log free beta (HCG) + intact HCG) | 88% | 7.4% |

Composite = MSAFP + free beta (HCG) + Intact HCG + UE + Ratio Gestational age is incorporated along with each variable Risk cut-off level = 1 in 400 except (*) which is 1 in 365.

As obvious to one skilled in the art, changing the risk cut-off level of a positive, or use of different a priori risks which may apply to different subgroups in the population will change the results of the discriminant procedure for the patient.

EXAMPLE 2

Over 550 patient samples were utilized to study the relationship of fetal Down syndrome to the maternal blood levels of free beta HCG. Initially 29 samples from pregnant women known to be carrying fetuses with Down syndrome and 520 unaffected samples matched for gestational age (same week), maternal age (within 3 years) and freezer storage time (within one month) were analyzed. All samples were from singleton, non-diabetic, white gravid women. In this Example each of the samples analyzed was a liquid blood sample.

In order to avoid training set bias in estimates of screening efficiency, the concept of a validation set was used. A validation set is a set of data independent of the reference data set. The results from patients in the validation set are not used in establishing reference data. Rather, results from patients in the validation set are compared to the reference data set to determine screening efficiency. This second validation set consisted of 26 additional, confirmed cases of trisomy 21 (55 cases total) and a randomly selected group of 159 control samples. The control samples were similarly drawn from singleton, non-diabetic, white gravid women.

The total study consisted of 4388 determinations on 7 different assays of the maternal blood levels of the markers a set forth below:

| Marker | Assay |
|---|---|
| MSAFP | ELISA (enzyme linked immunosorbent assay) |
| Intact HCG | ELISA |
| Intact hcg + free beta HCG | ELISA |
| free beta HCG | RIA (radio immunoassay) |
| free alpha-HCG | RIA |
| UE | 2 methods, Enzyme Immunoassay & RIA |

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The level of each Marker became a variable in the stepwise discriminant procedure and the linear discriminant procedure on the commercially available computer software statistical package Statistical Analysis System (SAS Institute Inc.) to generate a set of reference data. Gestational age wac also incorporated as a variable. The linear discriminant procedure was then performed on each variable separately and or different combinations of variables. Patients were classified as being affected or unaffected based on a Down syndrome risk cut-off of 1 in 365. Unaffected cases that were classified as affected were considered false positive. Each patient's risk of Down syndrome was calculated using Bayes' rule, the multivariate normal probability density functions for affected and unaffected cases, and a general a priori risk of 1 in 800. A pooled covariance matrix was used for each probability density function.

The results found in Tables 1 through 3, shown in FIGS. 1 - 3, relate to the initial study set. Results in tables 5 - 7, FIGS. 5 - 7, are based on the classification of patients in the validation set. Table 4, shown in FIG. 4, and FIGS. 8 and 9 are based on the initial study set and all affected cases.

The results from the assay procedures were analyzed to determine whether there existed significant differences in the levels of each marker between affected and unaffected cases. Table 1 (FIG. 1) indicates that affecteds were significantly different from unaffected in all but U.E. Additionally, the false positive rate and detection efficiency; of each marker was determined as shown in Table 2 (FIG. 2). The highest detection efficiency was achieved with an HCG assay measuring both the intact molecule and free beta (HCG). A further enhancement in detection efficiency was observed by combining individual markers

into composites. The composite containing the HCG assay, which measured both the intact HCG molecule and free beta (HCG), produced the highest detection efficiency among the composites as shown in Table 3 (FIG. 3).

The evaluation of the beta and alpha subunit of HCG individually showed that no significant differences existed between affected and unaffected cases for the alpha subunit (p .23) while a significant increase in free beta (HCG) was observed in affecteds (p= .001). As generally known in the art, p measures the strength of evidence in scientific studies by indicating the probability that a result at least as extreme as that observed would occur by chance. The lower the p value the stronger the evidence that the observation is not a chance occurrence.

FIG. 8 shows the 10th, 50th and 90th percentiles of free beta (HCG) by gestational age. A continuing downward trend by gestational age in unaffected pregnancies is observed. Analysis of free beta (HCG) levels in cases of fetal Down syndrome, as shown in Table 4 (FIG. 4), reveals that 86% fall above the median of unaffected.

The levels of free beta (HCG) in both the affected and unaffected cases fit a log Gaussian distribution (p= .78 and .86). FIG. 9 illustrates these distributions. Table 5 (FIG. 5) provides detection efficiency data on free beta (HCG) and free alpha (HCG) individually. The high detection efficiency of free beta (HCG) is shown in Table 5.

An even higher detection efficiency was achieved with a composite of AFP and free beta (HCG). By incorporating the log of the level of free beta (HCG) and the log of the level of MSAF into the linear discriminant procedure on the commercially available computer software statistical package Statistical Analysis System (SAS Institute Inc.), as described

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above, a superior detection efficiency was achieved as shown in Table 6 (FIG. 6). A high detection efficiency would also be achieved using the level of free beta (HCG) and the level of AFP, as opposed to the log of each.

Further analysis of the data noted that both AFP and free beta (HCG) are independent of maternal age (p = .8394 and .5214 using Kruskal-Wallis tests across four different maternal age groups for AFP and free beta (HCG) respectively, (ages = 30, 31 - 35, 36 - 40, and 40). Additionally the correlation (r) of the levels of free beta (HCG) and AFP was not significantly different from zero (r = .04, p = .39 and r = -.06, p = .81 for unaffected and affected cases respectively).

The fundamental observation found in our data confirms the fact that free beta (HCG) contributes the highest detection efficiency for Down syndrome. In fact, the use of an assay measuring solely free beta (HCG) produced a detection efficiency and false positive rate of 65.4% and 5.2% respectively. These rates are comparable to those reported by others utilizing a combination of three assays. Thus, as previously set forth, reducing the number of assays is an advantage of the present invention.

Our findings on the contribution of free beta (HCG) are based on the following: (a) the single best contributor to detection efficiency for Down syndrome was an assay for free beta (HCG), (b) an assay for the intact HCG molecule yields substantially lower detection rates, (c) an assay which measures a combination of the intact HCG molecule and free beta (HCG) yields a higher detection efficiency than an assay measuring the intact HCG molecule alone.

It is established that the risk of fetal Down syndrome increases with maternal age. Therefore, as described above, to produce patient-specific risks for clinical use of the present invention a maternal age

specific a priori risk is incorporated into the multivariate discriminant analysis procedure. Since both AFP and free beta (HCG) levels are independent of maternal age, we have analyzed our data to see how many unaffected and affected women would have positive results given an a priori risk for each individual age. The foregoing information was used to project, based upon the maternal age distribution of live births in the United States, the false positive and sensitivity rate for comprehensive nationwide screening within the United States. As shown in Table 7 (FIG. 7) the projections indicate that it is possible to achieve a detection rate of 80%, with 5% false positives.

The samples described in Example 2 were further analyzed to discover the detection efficiency of other combinations of the markers. More particularly, the linear discriminant procedure of Example 2, with the same risk cut-off and a priori risk level, was performed on different combinations of the markers, multiples of the median (MOM) for the marker and logs of the markers, with and without the incorporation of gestational age. The linear discriminant procedure was performed using both the reference data and the validation data. The results are summarized in Table 8 in FIG. 10.

EXAMPLE 3

The following example illustrates the preparation of a one step free beta (HCG) assay and a two step free beta (HCG) assay.

**Preparation of a one step free beta (HCG) assay**

1. A ninety-six well microtiter plate is coated with a catching antibody that is specific to the free beta human chorionic gonadotropin (HCG) molecule. The antibody may be either monoclonal or polyclonal. The concentration of antibody used to coat the plate is 0.8

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micrograms per well but could be different, if desired. The plate is incubated at 4°C for at least 16 hours.

2. The plate is washed with a phosphate buffered saline solution of Ph 7.2 that contains 0.05% Tween 20. Other suitable wash buffers may be employed. The plate is then blocked with a solution containing 3% hydrolyzed animal protein and 0.05% Tween 20 in a phosphate buffered saline solution of Ph 7.2. Other solutions, familiar to those skill in the art, such as a 1% bovine serum albumin solution, may b used. Three hundred microliters of the blocking solution is added to each well and the plate is allowed to incubate for an hour at ambient temperature. Other blocking procedures are also viable, e.g. glazing.

3. The plate is then washed, as described earlier, and 100 microliters of assay buffer containing a biotinylated antibody specific to the free beta (HCG) is added to each well. The assay buffer used is 3% hydrolyzed animal protein and 0.05% Tween*20 in a phosphate buffered saline solution of Ph 7.2, but may be any of a number of suitable solutions known to those skilled in the art. The antibody may be monoclonal or polyclonal and, depending on the preference of the operator, may be conjugated to a substance other than biotin, such as horseradish peroxidase or alkaline phosphatase. The concentration of the antibody in the assay buffer may be adjusted to obtain optimal absorbance values.

4. Twenty microliters of sample is then added to each well. The sample may be: assay buffer, run as blank to verify the performance of the assay; a solution of free beta (HCG) used to standardize values of unknown samples; or a serum sample from second trimester gravid-woman. The plate is vortexed for 30 seconds and

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then placed on a rotator at 200 rpm, where it incubates for 30 minutes at ambient temperature.

5. The plate is then washed as described previously. One hundred microliters of assay buffer containing streptavidin conjugated to horseradish peroxidase is then added to each well. This step is not required if the second antibody used i conjugated to a substance other than biotin. The concentration of streptavidin-peroxidase in assay buffer is 2.0 micrograms per milliliter. The plate is placed on a rotator at 200 rpm for 5 minutes at ambient temperature.

6. The plate is then washed as described previously. One hundred microliters of an o-phenylendiamine substrate solution is added to each well. This substrate solution may alternatively be any one of a number appropriate dyes known to those skilled in the art and depends upon what substance is conjugated to the second antibody. The plate is placed on a rotator at 200 rpm and incubated at ambient temperature in the dark for 8 minutes.

7. One hundred microliters of dilute (1.0 N) sulfuric acid is then added to each well to stop the reaction of the substrate.

8. The absorbance of each well is determined spectrophotometrically at 492 nm.

**Preparation of Two-step beta (HCG) Assay**

1. A ninety-six well microtiter plate is coated with a catching antibody that is specific to the free beta (HCG) molecule. The antibody may be either monoclonal or polyclonal. The concentration of antibody used to coat the plate is 0.8 micrograms per well but may be

different, if desired. The plate is incubated at 4°C for at least 16 hours.

2. The plate is washed with a phosphate buffered saline solution of Ph 7.2 that contains 0.05% Tween*20. Other suitable wash buffers may be employed. The plate is then blocked with a solution containing 3% hydrolyzed animal protein and 0.05% Tween*20 in a phosphate buffered saline solution of Ph 7.2. Other solutions, familiar to those skilled in the art, such as a 1% bovine serum albumin solution, may be used. Three hundred microliters of the blocking solution is added to each well and the plate is allowed to incubate for on hour at ambient temperature. Other blocking procedures, such as glazing, may be employed.

3. The plate is then washed, as described earlier, and 100 microliters of an assay buffer is added to each well. The assay buffer used is 3% hydrolyzed animal protein and 0.05% Tween 20 in a phosphate buffered saline solution of Ph 7.2, but may be any of a number of suitable solutions known to those skilled in the art.

4. Twenty microliters of sample is then added to each well. The sample may be: assay buffer, run as the blank to verify the performance of the assay; a solution of free beta (HCG) used to standardize values of unknown samples; or a serum sample from second trimester gravid woman. The plate is vortexed for 30 seconds and then placed on a rotator at 200 rpm, where it incubates for 30 minutes at ambient temperature.

5. The plate is then washed, as described previously, and lo microliters of assay buffer containing a biotinylated antibody specific to the free beta (HCG) is added to each well The antibody may be monoclonal or

*Trade-mark

polyclonal and, depending or the preference of the operator, may be conjugated to a substance other than biotin, such as horseradish peroxidase or alkaline-phosphatase. The concentration of the antibody may adjusted to obtain optimal absorbance values. The plate is vortexed for 30 seconds and then placed on a rotator at 200 rpm where it incubates for 30 minutes at ambient temperature.

6. The plate is then washed as described previously. One hundred microliters, of assay buffer containing streptavidin conjugated to horseradish peroxidase is then added to each well. This step is not required if the second antibody used is conjugated to a substance other than biotin. The concentration of streptavidin-peroxidase in assay buffer is 2.0 micrograms per milliliter. The plate is placed on a rotator @ 200 rpm for 5 minutes at ambient temperature.

7. The plate is then washed as described previously. One hundred microliters of an o-phenylenediamine solution is added to each well. This substrate solution may alternatively be an one of a number appropriate dyes known to those skilled in the art and depends upon what substance is conjugated to the second antibody. The plate is placed on a rotator at 200 rpm and incubated at ambient temperature in the dark for 8 minutes.

8. One hundred microliters of dilute (1.0 N) sulfuric acid then added to each well to stop the reaction of the substrate.

9. The absorbance of each well is determined spectrophotometrically at 492 nm.

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These two assays were used in carrying out the method of the present invention. One hundred seventy eight (178) patient liquid blood samples were utilized to study the relationship of fetal Down syndrome to the maternal blood levels of free beta HCG. Twenty six (26) samples from pregnant women known to be carrying fetuses with Down syndrome and 152 unknown unaffected samples were analyzed. All samples were from singleton, nondiabetic, white gravid women.

The patient samples were then analyzed for quantitative levels of MSAFP, by an ELISA assay, and levels of free beta (HCG) by the one step assay and the two step assay independently. The level of MSAFP and the level of free beta (HCG) by each assay then became a variable in the linear discriminant procedure on the commercially available computer software statistical package Statistical Analysis System to generate a set of reference data. The patient's gestational age was also incorporated as a variable in the discriminant procedure. The results of these discriminant analyses, for all gestational ages, and for gestational ages between 14 and 16 weeks are summarized below.

| | ALL WEEKS | | | |
|---|---|---|---|---|
| | False Positive | Detection Efficiency | Controls | Affected |
| Log (beta-1) | 6.6% | 69.2% | 152 | 26 |
| Log (beta-1) + Log (AFP) | 5.9% | 72.0% | 152 | 25 |
| Log (beta-2) | 8.2% | 33.3% | 138 | 18 |
| Log (beta-2) + Log (AFP) | 10.1% | 64.7% | 138 | 17 |
| Log (beta-2)* | 9.6% | 33.3% | 136 | 18 |
| Log (beta-2) + Log (AFP)* | 10.3% | 52.9% | 136 | 17 |

| | Weeks 14-16 | | | |
|---|---|---|---|---|
| | False Positive | Detection Efficiency | Controls | Affected |
| Log (beta-1) | 5.8 | 68.4% | 104 | 19 |
| Log (beta-1) + Log (AFP) | 4.8% | 73.7% | 104 | 19 |
| Log(beta-2) | 7.1% | 45.4% | 98 | 11 |
| Log(beta-2) + Log (AFP) | 9.2% | 63.6% | 98 | 11 |
| Log (beta-2)* | 10.4% | 54.6% | 96 | 11 |
| Log (beta-2) + Log (AFP)* | 8.3% | 63.6% | 96 | 11 |

Note: All analyses included gestational age
* In the analyses with a * next to them 2 outliers with values of 260 and 316 were removed.

The suffix -1 means one-step procedure, -2 means two-step procedure.

Detection Efficiency refers to the percentage of fetal Down Syndrome cases which show a positive test result.

False Positive refers to the percentage of normal fetuses which show a positive test result.

Controls refers to the number of unaffected samples analyzed.

Affected refers to the number of affected samples analyzed.

Using a combination of the one-step assay for free beta (HCG) and AFP yields the highest detection efficiency with the lowest false positive rate for all weeks of gestation and for 14-16 weeks gestation.

Our findings support the performance of Down syndrome screening in a feasible and effective fashion within a prenatal serum screening protocol which includes: (a) non-invasive techniques (b) high detection efficiency with low false positive rates, (c) use of markers which are largely independent of each other (d) absence of unwieldy restrictions in blood

sampling (time of day, diet, personal habits, etc.) and (e) compatibility with other prenatal screening services.

Example 4

This example illustrates the use of the method of the present invention in screening for Down syndrome during the first trimester of pregnancy. Maternal serum samples from 150 patients were utilized. Each patient was carrying a fetus that, at the time the sample was taken, was between 9 and 13 weeks of gestational age. 139 of the patients were carrying a fetus without Down syndrome, referred to herein as "controls" and 11 patients were carrying a fetus with Down syndrome. Each of the patients was a non-diabetic, white, woman with a singleton pregnancy.

The level of free beta in each of the 150 maternal serum samples was analyzed utilizing the two step free beta (hCG) assay described in Example 3. The results of this analysis are shown graphically in Figure 11. As shown in Figure 15, the median level of free beta in the maternal serum of patients carrying a fetus with Down syndrome was 2.45 MOM (multiples of the median) greater than the median level of free beta in the maternal serum of the controls. This level of free beta (HCG) in patients carrying a fetus with Down syndrome is significantly greater than the level of free beta (HCG) in the controls. Thus, the free beta protein is significantly elevated in the first trimester when Down syndrome is present.

Example 5

This example also illustrates the use of the method of the present invention in screening for Down syndrome during the first trimester of pregnancy. Maternal serum samples from 277 patients were utilized. Each patient was carrying a fetus that, at the time the

sample was taken, was between 7 and 13 weeks of gestational age. 264 of the patients were carrying a fetus without Down syndrome, referred to herein as "controls" and 13 patients were carrying a fetus with Down syndrome. Each of the patients was a non-diabetic, white, woman with a singleton pregnancy.

The level of free beta (HCG) in each of the 277 maternal serum samples was analyzed utilizing the two step free beta (HCG) hCG assay described in Example 3. The results of this analysis are shown graphically in Figure 12. As shown in Figure 16, the median level of free beta (HCG) in the maternal serum of patients carrying a fetus with Down syndrome was 1.82 MOM (multiples of the median) greater than the median level of free beta in the maternal serum of the controls. This level of free beta (HCG) in patients carrying a fetus with Down syndrome is significantly greater than the level of free beta (HCG) in the controls. Thus, the free beta protein is significantly elevated in the first trimester when Down syndrome is present.

Example 6

This example further illustrates the use of the method of the present invention in screening for Down syndrome during the first trimester of pregnancy. Maternal serum samples were drawn from 392 patients who had chosen to undergo a chorionic villus sample (CVS) procedure. The CVS procedure is a clinical technique wherein a sample of chorionic villi is obtained for chromosomal analysis karotyping. The serum samples utilized in this example were drawn from the patients prior to the patients undergoing the CVS procedure. Usually, this technique is utilized with patients 35 years old, or older, a group that is known to have a higher incidence of carrying a fetus with Down syndrome, or utilized with patients having a family history of chromosomal abnormalities.

Each patient was carrying a fetus that, at the time the sample was taken, was between 9 and 13 weeks of gestational age. 386 of the patients were carrying a fetus without Down syndrome, referred to herein as "controls" and 6 patients were carrying a fetus with Down syndrome. Each of the patients was a non-diabetic, white, woman with a singleton pregnancy.

The level of free beta (HCG) in each of the 392 maternal serum samples was analyzed utilizing the two step free beta (HCG) assay described in Example 3. The results of this analysis are shown graphically in Figure 13. As shown in Figure 17, the median level of free beta (HCG) in the maternal serum of patients carrying a fetus with Down syndrome was 2.94 MOM (multiples of the median) greater than the median level of free beta (HCG) in the maternal serum of the controls. This level of free beta (HCG) in patients carrying a fetus with Down syndrome is significantly greater than the level of free beta (HCG) in the controls. Thus, the free beta protein is significantly elevated in the first trimester when Down syndrome is present.

The Examples further illustrate that the method of the present invention, as described herein, may be utilized to non-invasively screen for Down syndrome during the first or second trimester of pregnancy. As used herein, the first trimester of pregnancy refers to the time period prior to 14 weeks of gestational age. Similarly, the second trimester of pregnancy refers to the time period between 14 and 26 weeks of gestational age.

For example, a laboratory could generate a set of gestational week specific reference data containing the levels of free beta (HCG) in maternal serum samples obtained during the first trimester of pregnancy. This

reference data can be generated in a manner comparable to the manner set forth in the Examples.

To screen for fetal Down syndrome, the laboratory would measure and compare the level of free beta (HCG) in a blood sample obtained from a patient during the first trimester of pregnancy to the reference data to determine whether the patient is at sufficient risk of carrying a fetus with Down syndrome to warrant further testing. As set forth herein, this determination by the laboratory requires a comparison of the patient's a posteriori (after screening) risk of carrying a fetus with Down syndrome with an established cut-off level which reflects the risk to the patient and fetus of an invasive procedure to obtain fetal cells for chromosomal analysis.

A laboratory could decide to use a multiples of the median approach to determine a positive screening result. For example, using the data set forth in Example 4 as a set of reference data, the reference data would indicate that the median level of free beta (HCG) is 2.45 times greater in women carrying Down syndrome fetuses than in women carrying unaffected fetuses. Using a multiples of the median approach to screening, if the level of free beta (HCG) in the patient's sample is greater than a chosen cut-off level such as 2.5 multiples of the median greater than the level of free beta (HCG) in the controls, the laboratory would recommend that the pregnant woman undergo further invasive testing to diagnose whether her fetus has Down syndrome.

It should be noted that this screening method, relying on a Multiples of the Median approach for a single analyte, may not be as accurate as a screening procedure utilizing multiple analytes and a multivariate discriminant analysis approach, such as that described in Example 1.

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Example 7

This example compares the measured level of free beta (HCG) in liquid and dried blood samples stored for 5 days at 4° C and at room temperature (approximately 20° C).

Liquid blood samples were obtained from pregnant women and the level of free beta (HCG) was measured by the assay techniques described herein. Six aliquots from each liquid sample were spotted on each of two specimen cards. The remaining liquid blood sample was separated into two parts.

One specimen card from each liquid blood sample and one of the two parts of the remaining liquid blood sample was stored in a refrigerator at 4° C. The other specimen card, and the other part of the remaining liquid blood sample was stored at room temperature.

At approximately the same time each day, for the next 5 days, a blood sample from each specimen card and both parts of the remaining liquid sample was analyzed for free beta (HCG) content. In order to reduce any errors attributable to the assay procedure, the liquid blood sample was spotted onto a specimen card prior to analysis. The analysis of the dried blood spots was performed in the manner described herein.

The results are depicted graphically in Figures 17 and 18. As shown in these figures, the measured level of free beta (HCG) in dried blood samples, stored at room temperature, remained more constant than the measured level of free beta (HCG) in liquid blood samples stored at room temperature.

Example 8

This example compares a screening method utilizing liquid blood versus a screening method utilizing dried blood spots. The screening methods utilized in each case were the methods described herein, namely

comparing the maternal blood level of free beta (HCG) and AFP and gestational age to reference data.

In this example, the offices of selected physicians, cooperating in a dried blood spot experimental protocol forwarded maternal blood samples to my laboratory as dried blood spots. These dried blood samples were evaluated as described herein to determine the pregnant women at increased risk of carrying a fetus with Down syndrome.

The results using the dried blood spot samples were compared to a similar population of pregnant women in which blood samples were collected as a liquid and evaluated as described in Example 3 herein.

The results were as follows:

PAPER SPOT VS. LIQUID BLOOD
DOWNS SCREENING

| | PAPER SPOT | | LIQUID BLOOD | |
|---|---|---|---|---|
| | Number | At Risk | Number | At Risk |
| **Sample** | **69** | **2.9%** | **250** | **7.2%** |

This indicates that for a similar population of pregnant women, the number of pregnant women designated as being at increased risk of carrying of fetus with Down syndrome based on analysis of a dried blood spot was significantly reduced in comparison with the analysis of a liquid blood sample.

Example 9

This example illustrates that certain immunoassays utilized in the measurement of free beta (HCG) will also measure "nicked" free beta (HCG).

Nicked free beta (HCG) was prepared as follows. 100 µl of 212 µg/ml hCG ß-subunit (Scripps Catalog Number C0904) manufactured and sold by Scripps Laboratories, LaHoya California diluted in deionized water was added to 100 µl of hLE (0.3 unit) (hLE =

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human leucocyte elastase) prepared in 0.1 M Tris-HCl, ph 8.5 containing 0.5 M NaCl and 1 g Brij per liter, manufactured and sold by Sigma Chemical Company, St Louis, Mo.. The mixture was incubated at 37º C. Samples were drawn at 0, 60, 120 and 180 minutes from the mixture. 10 µl of 10 mg/ml soybean trypsin inhibitor was added to each sample to stop the reaction between the ß HCG and the hLE, thereby stopping the formation of further nicked free beta (HCG). The four samples, one of free beta (HCG) (0 minute sample) and three containing nicked free beta (HCG) (60, 120 and 180 minute samples), were analyzed as follows.

Seven additional concentrations of each sample were made by diluting the initial sample by half, in a stepwise fashion, using PBS (phosphate buffer saline solution) pH 7.2 containing stabilizer and preservative. Thus eight concentrations of each sample were prepared, 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.5625%, 0.78125% (% = %initial sample). The eight concentrations of each sample (0, 60, 120 and 180 minutes) were analyzed twice utilizing the two step free beta (HCG) assay described herein in Example 3, with two different pairs of catching/detecting antibodies. The results were as follows.

In each assay, a slight elevation occurred in measured level of free beta (HCG) in the samples incubated for 60, 120 and 180 minutes with hLE in comparison with the measured level of free beta (HCG) in the sample incubated for 0 minutes. The average % change for each assay was 102.1% (range 96.2% - 107.6%) and 112.4% (range 106.3% to 121.7%) respectively for 180 minute samples versus the 0 minute samples for the eight concentrations tested.

In the first antibody pair, recognition of an increasing nicked free beta (HCG) percentage in the samples did not alter the degree of immunoreactivity to free beta (HCG) significantly. The second antibody

pair had more affinity for nicked free beta (HCG) than the first antibody pair. In the case of the second antibody pair, the degree of change was increased over the span from time 0 minutes to 60 minutes (110.5% change); and 0 to 120 minutes (115.1% change). The % change at 180 minutes did not increase versus the % change at 120 minutes. The average percent change for the second antibody pair was 112.7%. Thus, the first antibody pair is equally reactive to free beta (HCG) and nicked free beta (HCG) and the second antibody pair is more reactive (+12%) to nicked free beta (HCG) than the first antibody pair.

These results provide support for the use of an assay that measures both nicked free beta (HCG) and free beta (HCG) in a screening protocol for Down Syndrome. The use of such an assay, in the method of the present invention, should be expected to provide detection efficiencies of approximately 80%.

Our results indicate that it is possible to achieve higher detection rates in Down syndrome screening while conducting fewer biochemical analyses than proposed by others. The use of highly effective markers in Down syndrome screening can serve to provide non-invasive screening information during the early antenatal period to the highest proportion of families affected by this most common cause of severe mental retardation.

As obvious to one skilled in the art changing the risk cut-off level of a positive, or using different a priori risks which may apply to different subgroups in the population, will change the results of the discriminant procedure for the patient.

Accordingly, it should be clearly understood that the present invention includes all modifications falling within the scope of the following claims.

61293-339



CLAIMS:

1. A screening method for determining a pregnant woman's risk of carrying a fetus with Down syndrome comprising:

obtaining a blood sample from the pregnant woman during a time period selected from the group consisting of the first trimester of pregnancy, the second trimester of pregnancy and the third trimester of pregnancy, on filter paper and allowing the blood sample to dry to form a dried blood spot;

determining the pregnant woman's maternal blood level of free beta (human chorionic gonadotropin (HCG)) from the dried blood spot; and

comparing the level of free beta (HCG) to reference values of the level for free beta (HCG) during the time period in: (1) pregnant women carrying a fetus with Down syndrome and (2) pregnant women carrying normal fetuses, the comparison being indicative of the pregnant woman's risk of carrying a fetus with Down syndrome, wherein a higher level of free beta (HCG) is indicative of a higher probability of carrying a fetus with Down syndrome.

2. The method of claim 1, which further comprises:

determining the pregnant woman's maternal blood level of alpha-fetoprotein (AFP) from the dried blood spot and incorporating the woman's blood level of AFP and reference values of the levels AFP during the time period in: (1) pregnant women carrying a fetus with Down syndrome and (2) pregnant women carrying normal fetuses, into the comparison wherein a lower level of AFP is indicative of a higher probability of risk.

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3. A method for determining the risk that a pregnant woman is carrying a fetus with Down syndrome comprising:

obtaining a blood sample from the pregnant woman during a time period selected from the group consisting of the first trimester of pregnancy, the second trimester of pregnancy and the third trimester of pregnancy, on filter paper and allowing the blood sample to dry to form a dried blood spot;

determining the pregnant woman's maternal blood level of an analyte selected from the group consisting of the protein portion of free beta (human chorionic gonadotropin (HCG)); carbohydrate portion of free beta (HCG); and the portion of free beta (HCG) located at about the junction of the carbohydrate and the protein portions, from the dried blood spot; and

comparing the level of the analyte to reference data containing reference values of the level for the analyte during the time period in: (1) pregnant women carrying a fetus with Down syndrome and (2) pregnant women carrying normal fetuses, the comparison being indicative of the pregnant woman's risk wherein a higher level of the analyte is indicative of a higher probability of carrying a fetus with Down syndrome.

4. The method of claim 3, which further comprises:

determining the pregnant woman's maternal blood level of alpha-fetoprotein (AFP) from the dried blood spot and incorporating the woman's blood level of AFP and reference values of the levels AFP during the time period in: (1) pregnant women carrying a fetus with Down syndrome and (2) pregnant women carrying normal fetuses, into the

comparison wherein a lower level of AFP is indicative of a higher probability of risk.

5. The method of claim 1 wherein the free beta (HCG) is an aberrant form of free beta (HCG).

6. The method of claim 2 wherein the free beta (HCG) is an aberrant form of free beta (HCG).

7. A method for determining the risk that a pregnant woman is carrying a fetus with Down syndrome comprising:

obtaining a blood sample from the pregnant woman during a time period selected from the group consisting of the first trimester of pregnancy, the second trimester of pregnancy and the third trimester of pregnancy, on filter paper and allowing the blood sample to dry to form a dried blood spot;

determining the pregnant woman's maternal blood level of a fragment of free beta (human chorionic gonadotropin (HCG)) from the dried blood spot; and

comparing the measurement of the level of the fragment of free beta (HCG) to reference data containing reference values of the level of the fragment of free beta (HCG) during the time period in: (1) pregnant women carrying a fetus with Down syndrome and (2) pregnant women carrying normal fetuses, the comparison being indicative of the pregnant woman's risk wherein a higher level of the fragment of free beta (HCG) is indicative of a higher probability of carrying a fetus with Down syndrome.

8. The method of any one of claims 1 to 7, wherein the filter paper is a specimen collection card having, on a front side, a plurality of pre-printed circles for the blood

spots and pre-printed locations for entering information about the pregnant woman.

9. Th method of claim 8, wherein the specimen card also has outlines of the circles on a back side.

10. A screening method for determining a pregnant woman's risk of carrying a fetus with chromosomal trisomy selected from Trisomy 18 and Trisomy 13, comprising:

obtaining a blood sample from the pregnant woman during a time period selected from the group consisting of the first trimester of pregnancy, the second trimester of pregnancy and the third trimester of pregnancy, on filter paper and allowing the blood sample to dry to form a dried blood spot;

determining the pregnant woman's maternal blood level of free beta (human chorionic gonadotropin (HCG)) from the dried blood spot; and

comparing the level of free beta (HCG) to reference values of the level for free beta (HCG) during the time period in: (1) pregnant women carrying a fetus with the chromosomal trisomy and (2) pregnant women carrying normal fetuses, the comparison being indicative of the pregnant woman's risk of carrying a fetus with the chromosomal trisomy, wherein a higher level of free beta (HCG) is indicative of a higher probability of carrying a fetus with the chromosomal trisomy.

11. The method of claim 10, wherein the chromosomal trisomy is Trisomy 18.

12. The method of claim 10 or 11, wherein the free beta (HCG) is an aberrant form of free beta (HCG).

13. The method of claim 12, wherein the aberrant form of free beta (HCG) is nicked free beta (HCG).

14. A screening method for determining a pregnant woman's risk of carrying a fetus with Down syndrome comprising:

obtaining a urine sample from the pregnant woman during a time period selected from the group consisting of the first trimester of pregnancy, the second trimester of pregnancy and the third trimester of pregnancy, on filter paper and allowing the urine sample to dry to form a dried urine spot;

determining the pregnant woman's urine level of the beta core fragment of free beta (human chorionic gonadotropin (HCG)) from the dried urine spot; and

comparing the level of beta core fragment of free beta (HCG) to reference values of the level for the beta core fragment of free beta (HCG) during the time period in: (1) pregnant women carrying a fetus with Down syndrome and (2) pregnant women carrying normal fetuses, the comparison being indicative of the pregnant woman's risk of carrying a fetus with Down syndrome, wherein a higher level of the beta core fragment of free beta (HCG) is indicative of a higher probability of carrying a fetus with Down syndrome.

15. A screening method for determining a pregnant woman's risk of carrying a fetus with chromosomal trisomy selected from Trisomy 18 and Trisomy 13, comprising:

obtaining a urine sample from the pregnant woman during a time period selected from the group consisting of the first trimester of pregnancy, the second trimester of pregnancy and the third trimester of pregnancy, on filter

paper and allowing the urine sample to dry to form a dried urine spot;

determining the pregnant woman's urine level of beta core fragment of free beta (human chorionic gonadotropin (HCG)) from the dried urine spot; and

comparing the level of beta core fragment of free beta (HCG) to reference values of the level for beta core fragment of free beta (HCG) during the time period in: (1) pregnant women carrying a fetus with the chromosomal trisomy and (2) pregnant women carrying normal fetuses, the comparison being indicative of the pregnant woman's risk of carrying a fetus with the chromosomal trisomy, wherein a higher level of beta core fragment of free beta (HCG) is indicative of a higher probability of carrying a fetus with the chromosomal trisomy.

16. The method of claim 15, wherein the chromosomal trisomy is Trisomy 18.

SMART & BIGGAR

OTTAWA, CANADA

PATENT AGENTS

| MARKER | P VALVE |
|---|---|
| U E (EIA) | .14 |
| U E (RIA) | .66 |
| INTACT hCG | .0002 |
| AFP | .0365 |
| INTACT+ β hCG | <.0001 |

FIG. 1

| ANALYTES | FALSE POSITIVE | DETECTION EFFICIENCY | CONTROLS/ AFFECTED |
|---|---|---|---|
| U E (RIA) | 2.4 | 15.4 | 495/26 |
| U E (EIA) | 4.4 | 10.3 | 520/29 |
| LOG INTACT hCG | 7.4 | 33.3 | 489/27 |
| LOG AFP | 5.0 | 26.9 | 424/26 |
| LOG INTACT+ β hCG | 9.6 | 56.0 | 437/25 |

FIG. 2

| ANALYTES | FALSE POSITIVE | DETECTION EFFICIENCY | CONTROLS/ AFFECTED |
|---|---|---|---|
| 1 LOG AFP<br>LOG INTACT+ β hCG<br>U E | 8.5 | 64.0 | 422/25 |
| 2 LOG AFP<br>LOG INTACT hCG<br>U E | 8.4 | 57.7 | 419/26 |

FIG. 3

| PERCENTILE OF UNAFFECTED PREGNANCIES | DOWN SYNDROME CASES |
|---|---|
| > 5 | 98 |
| >10 | 98 |
| >25 | 90 |
| >50 | 86 |
| >75 | 79 |
| >90 | 62 |
| >95 | 36 |
| >99 | 31 |

FIG. 4

| ANALYTE | FALSE POSITIVE | DETECTION EFFICIENCY | CONTROLS/ AFFECTED |
|---|---|---|---|
| LOG β SUBUNIT | 5.2 | 65.4 | 154/26 |
| LOG α SUBUNIT | 0.0 | 0.0 | 157/17 |

FIG. 5

| GESTAGIONAL AGE RANGE | FALSE POSITIVE | DETECTION EFFICIENCY | CONTROLS/ AFFECTED |
|---|---|---|---|
| ALL WEEKS | 3.2 | 73.1 | 154/26 |
| < 17 WEEKS | 3.8 | 83.3 | 79/18 |
| >= 17 WEEKS | 1.3 | 50.0 | 75/8 |

FIG. 6

| RISK CUT-OFF | FALSE POSITIVE | DETECTION EFFICIENCY |
|---|---|---|
| 1 IN 400 | 5.0 | 80 |
| 1 IN 350 | 4.2 | 76 |
| 1 IN 300 | 3.5 | 73 |
| 1 IN 250 | 2.6 | 67 |
| 1 IN 200 | 1.6 | 60 |
| 1 IN 150 | 0.9 | 55 |
| 1 IN 100 | 0.5 | 45 |

FIG. 7

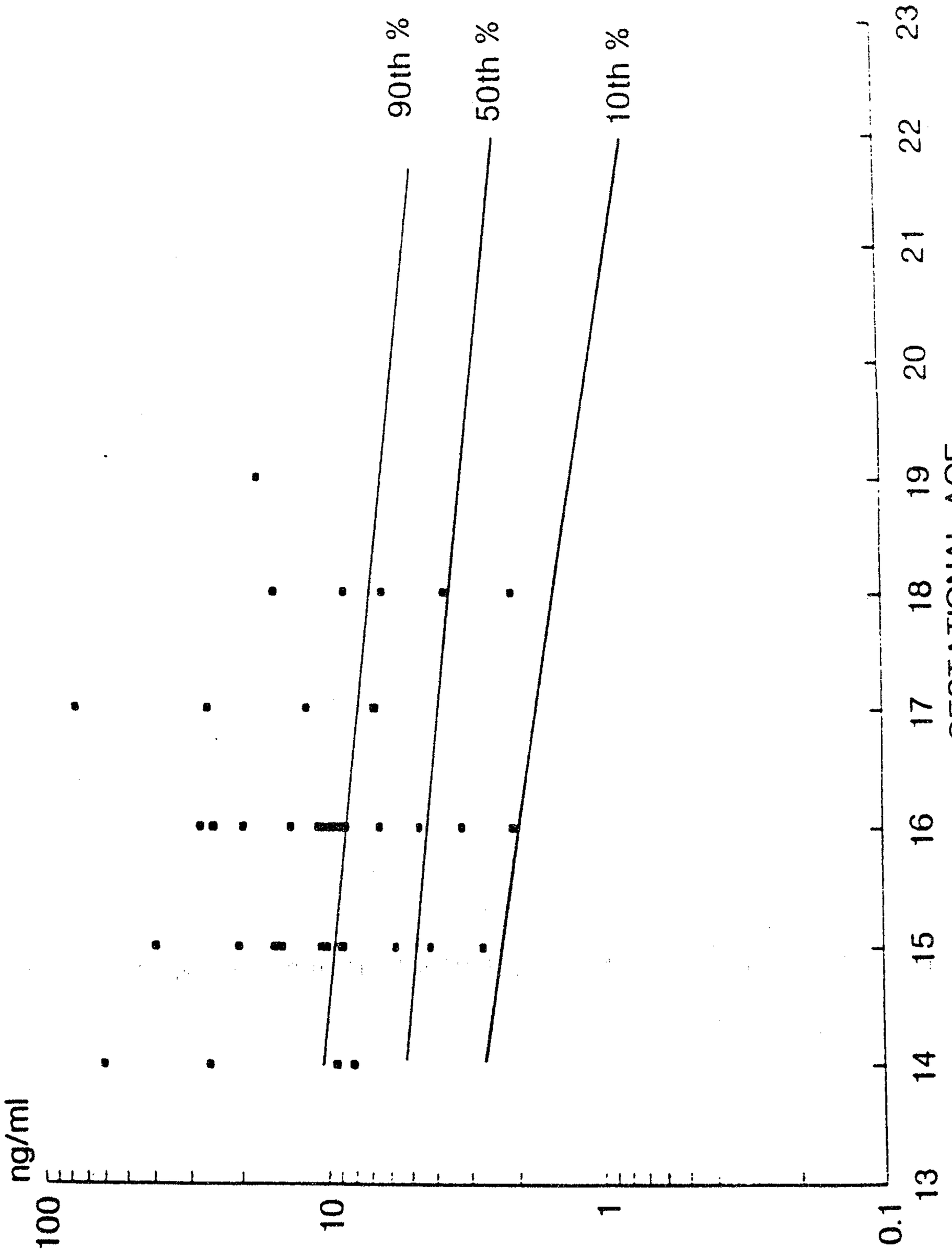

90th %
50th %
10th %
ng/ml
100
10
1
0.1
15
16
17
18
19
20
21
22
23
GESTATIONAL AGE
13
14

FIG. 8

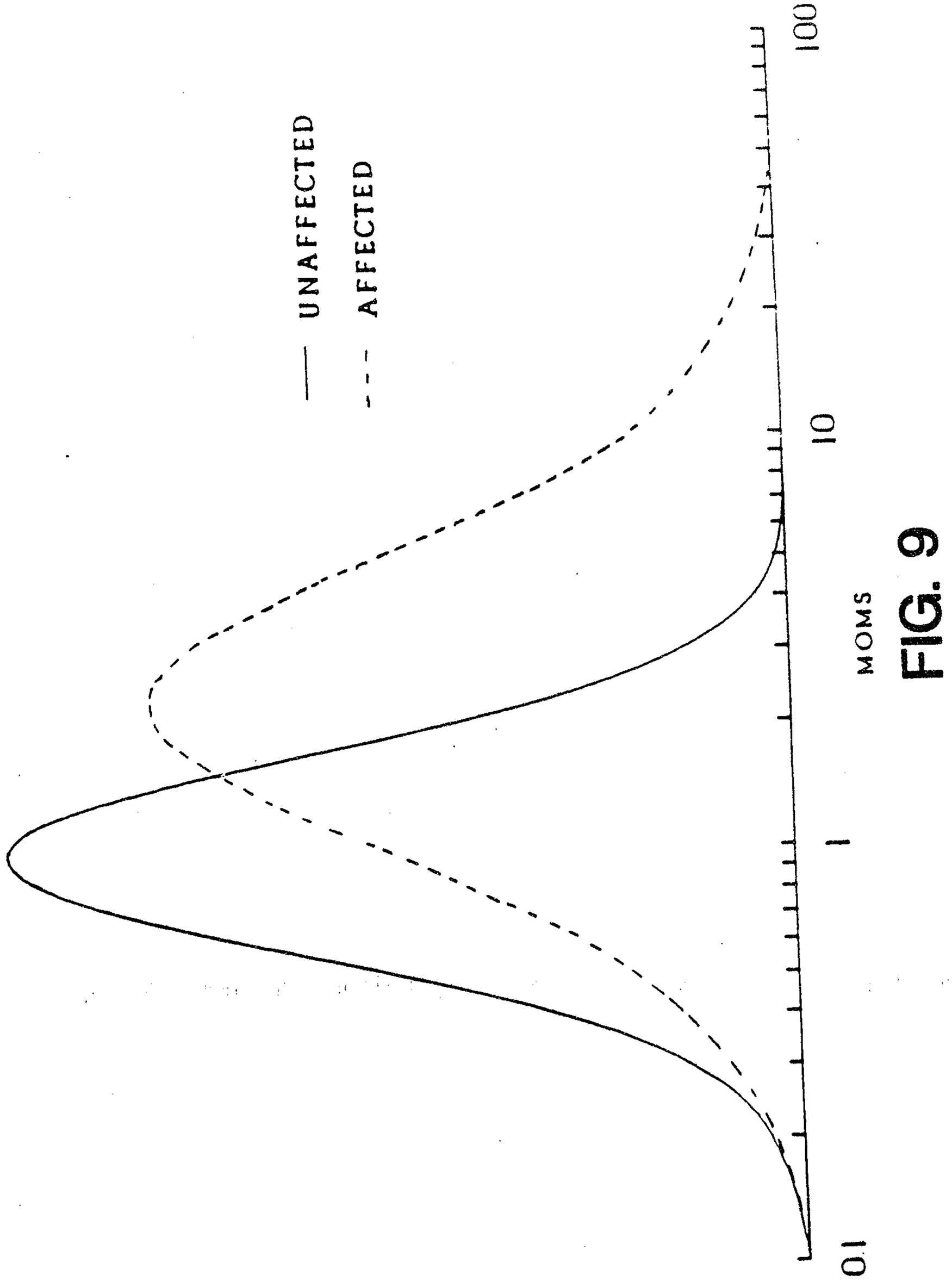



FIG. 9

TABLE 8

| MARKER(S) | FALSE POS. RATE(%) | SENSITIVITY (%) |
|---|---|---|
| FREE β (NO LOG. NO GA) | 5.7 | 69.2 |
| LOG FREE β (NO GA) | 5.9 | 69.2 |
| FREE β , GA | 5.7 | 69.2 |
| LOG FREE β, AFP IN LOG MOMs | 3.9 | 73.1 |
| FREE β, AFP IN LOG MOMs | 1.3 | 73.1 |
| FREE β , GA, LOG AFP | 5.2 | 73.1 |
| FREE β , LOG AFP | 1.3 | 73.1 |
| FREE β IN LOG MOMS, AFP IN LOG MOMS | 3.9 | 69.2 |
| LOG FREE β , LOG AFP | 3.3 | 69.2 |
| LOG AFP, LOG INTACT+ β , LOG α , GA | 9.6 | 52.9 |
| LOG INTACT hCG, GA, LOG INTACT+ β , LOG α | 26.1 | 52.9 |
| LOG AFP, LOG INTACT hCG, GA, LOG INTACT+ β , LOG α | 20.4 | 52.9 |

FIG. 10

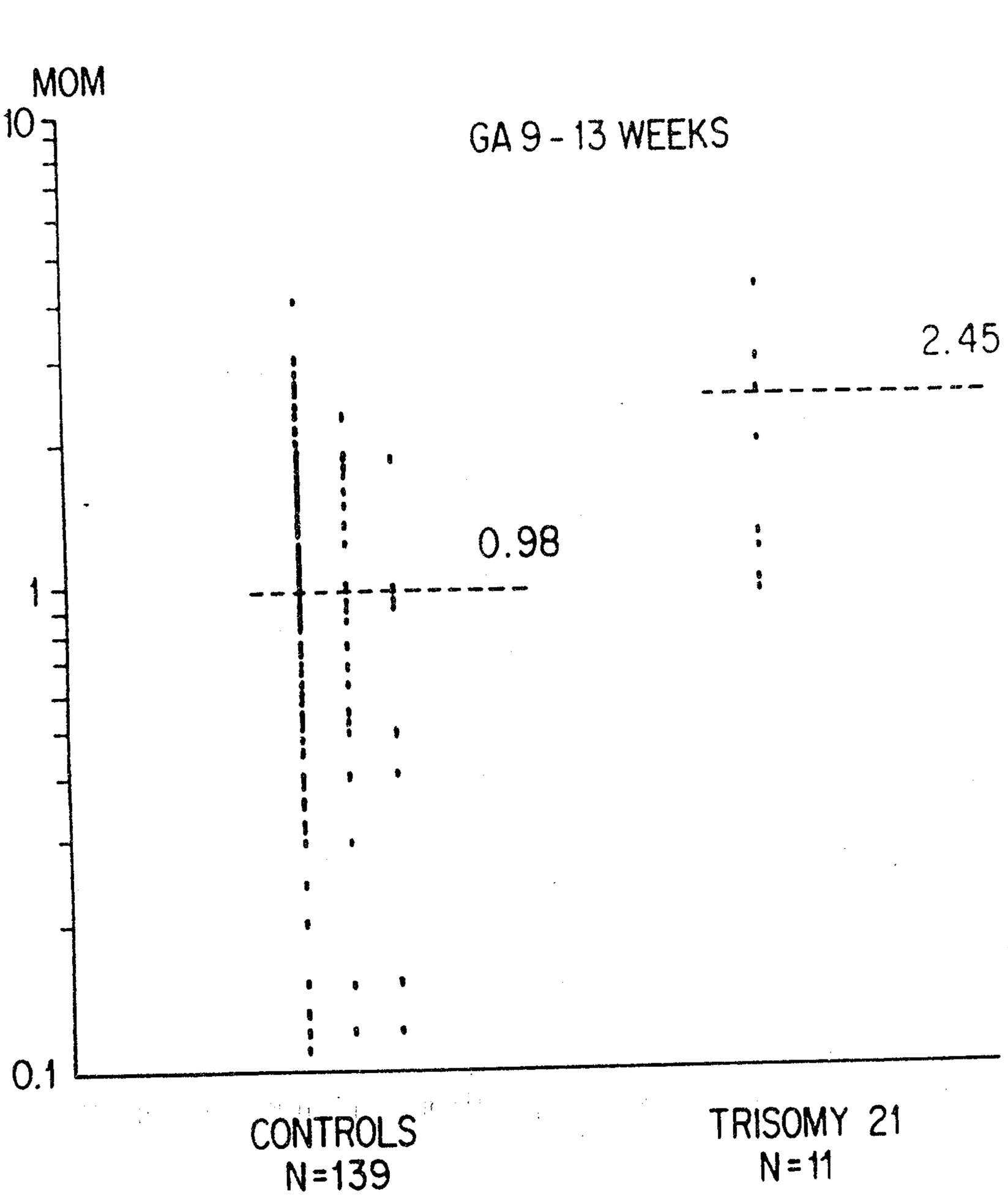



FIG. 11

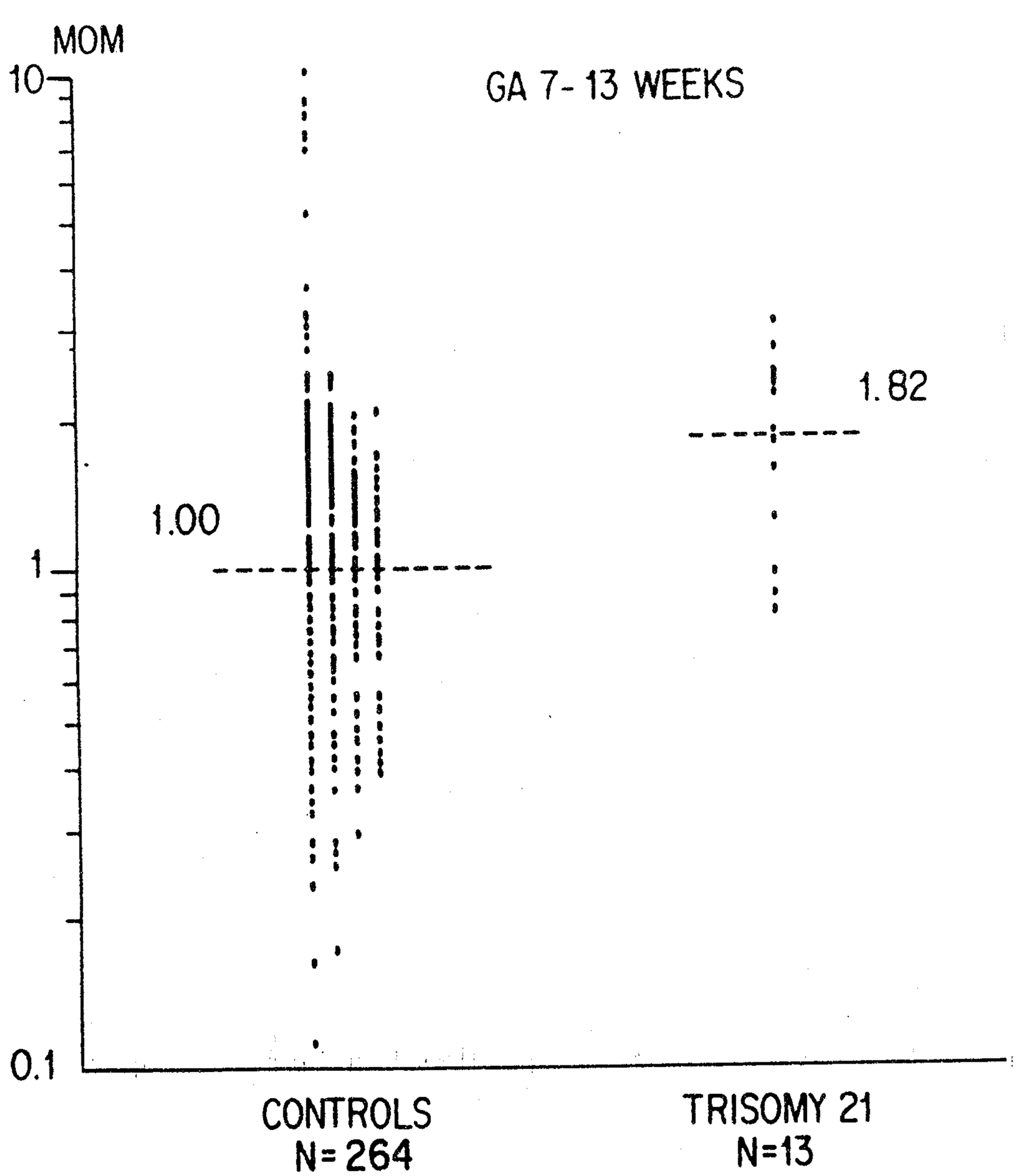



FIG. 12

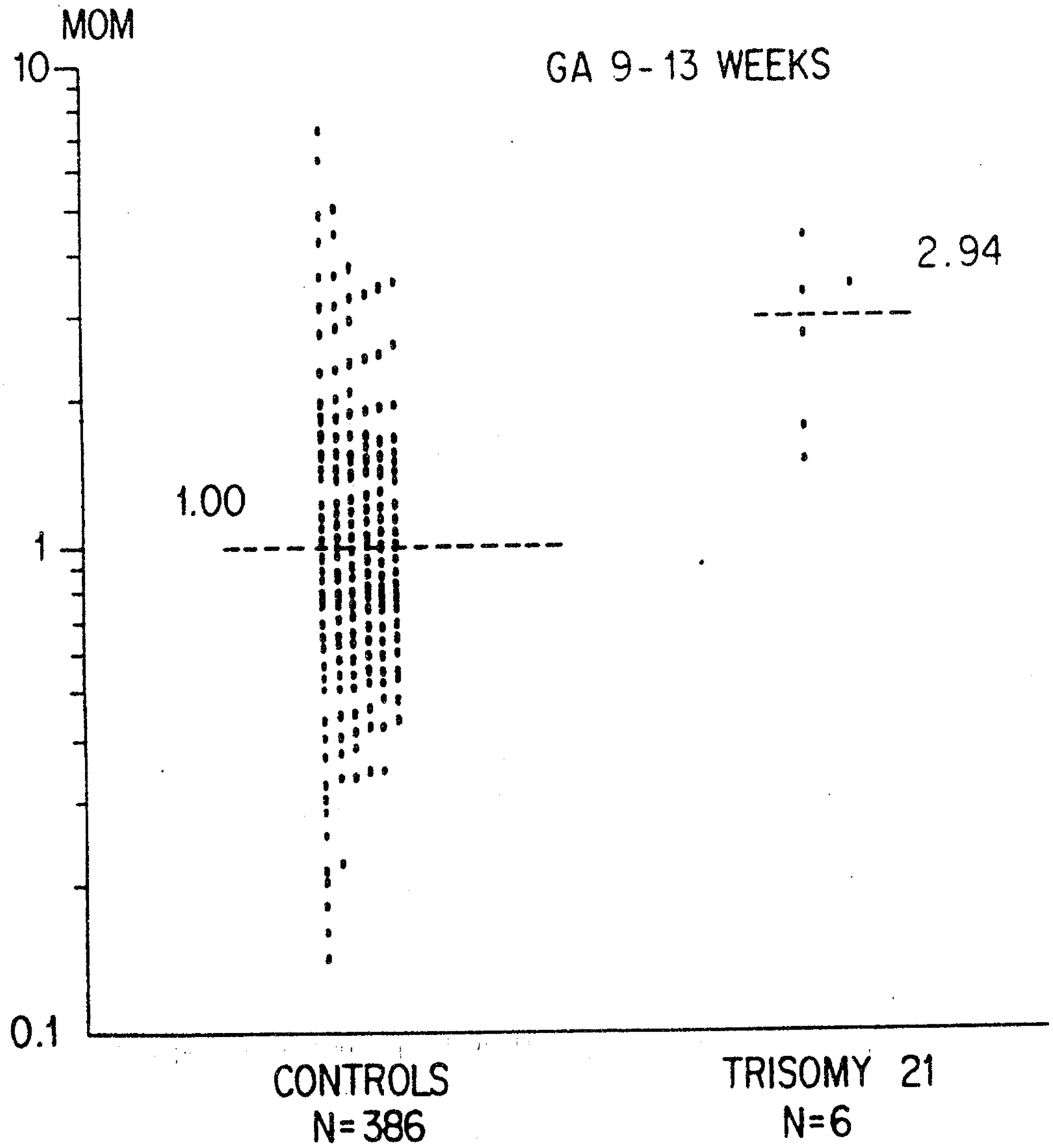



FIG. 13

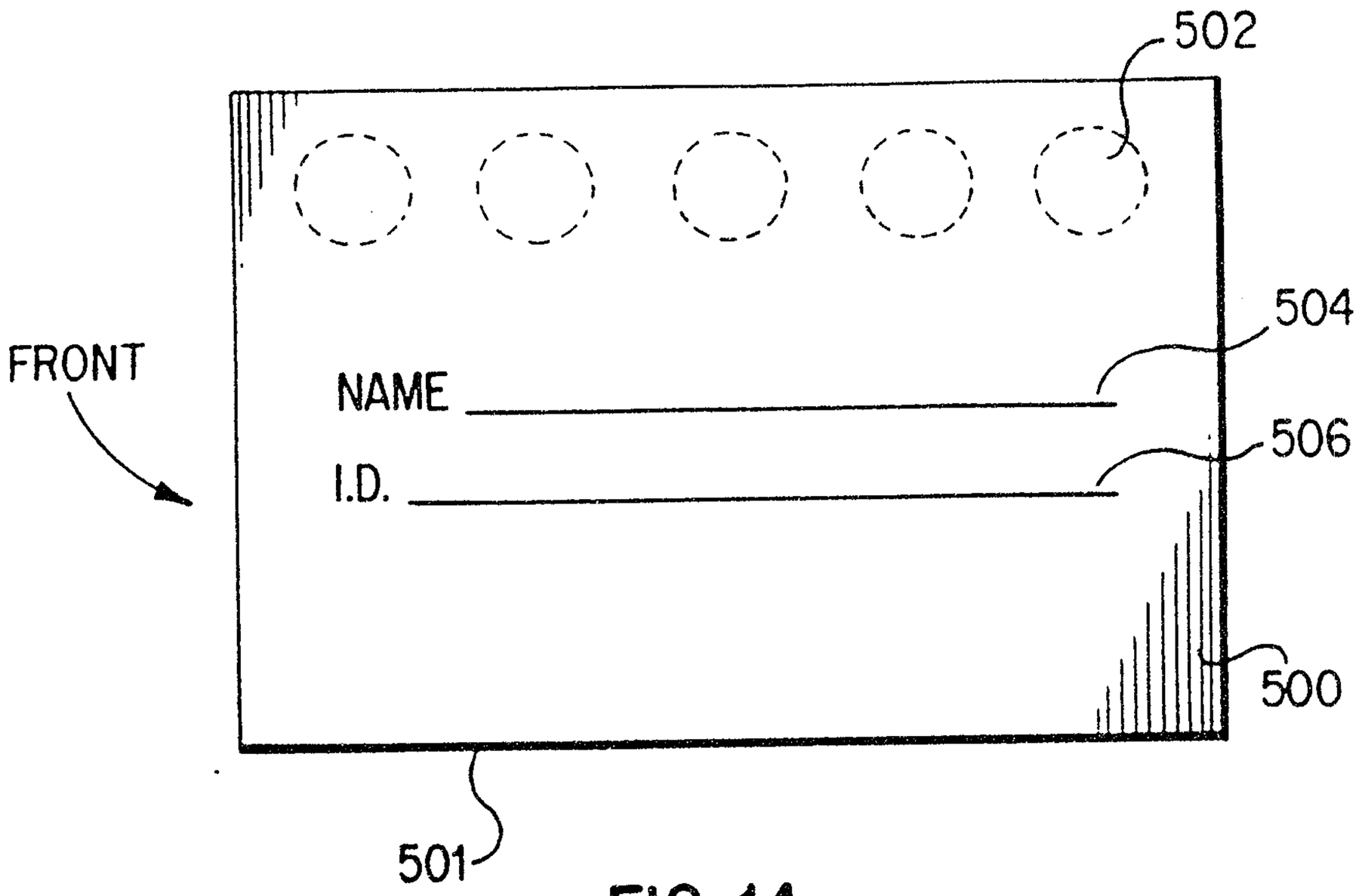



FIG. 14

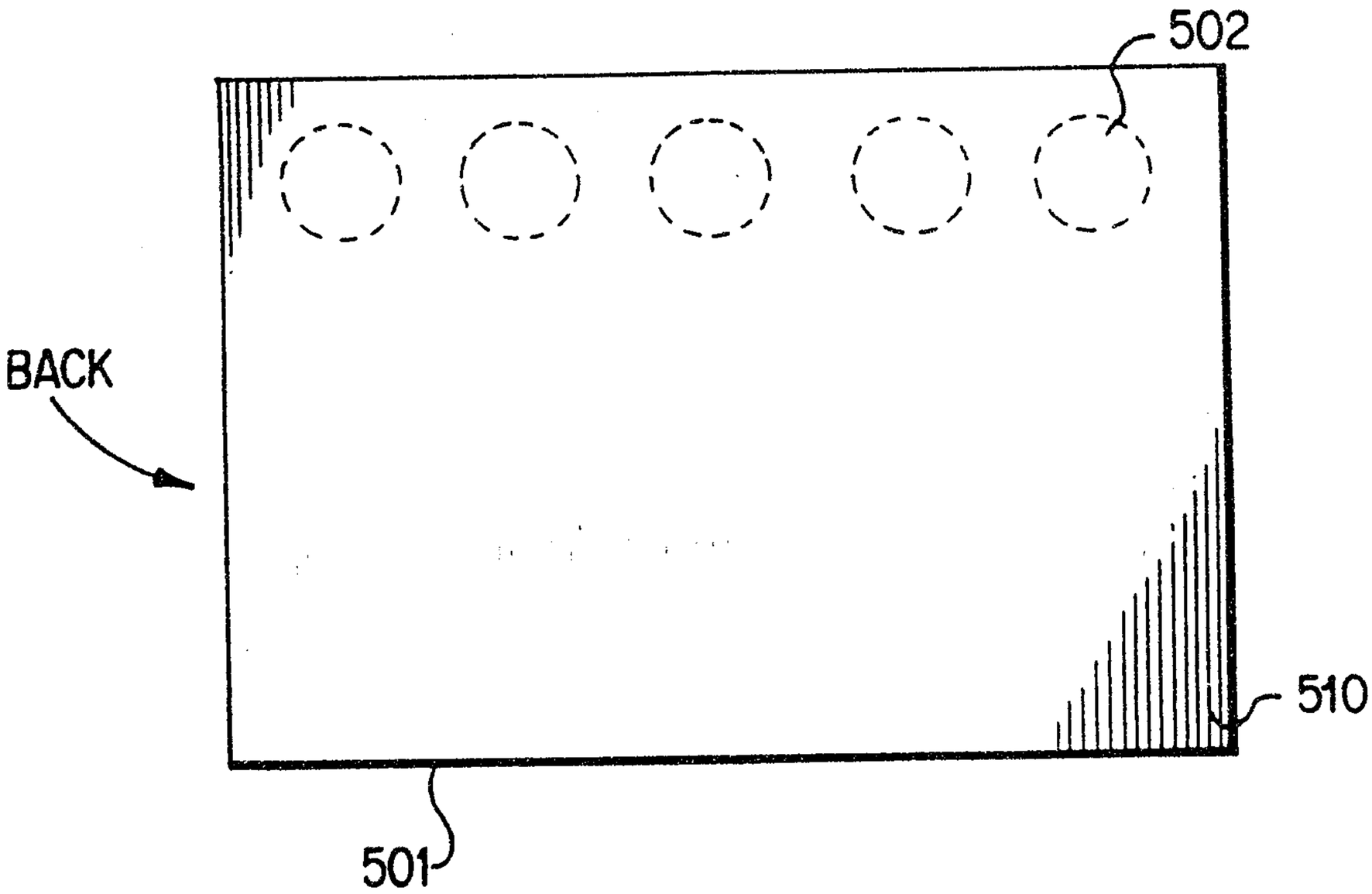



FIG. 15

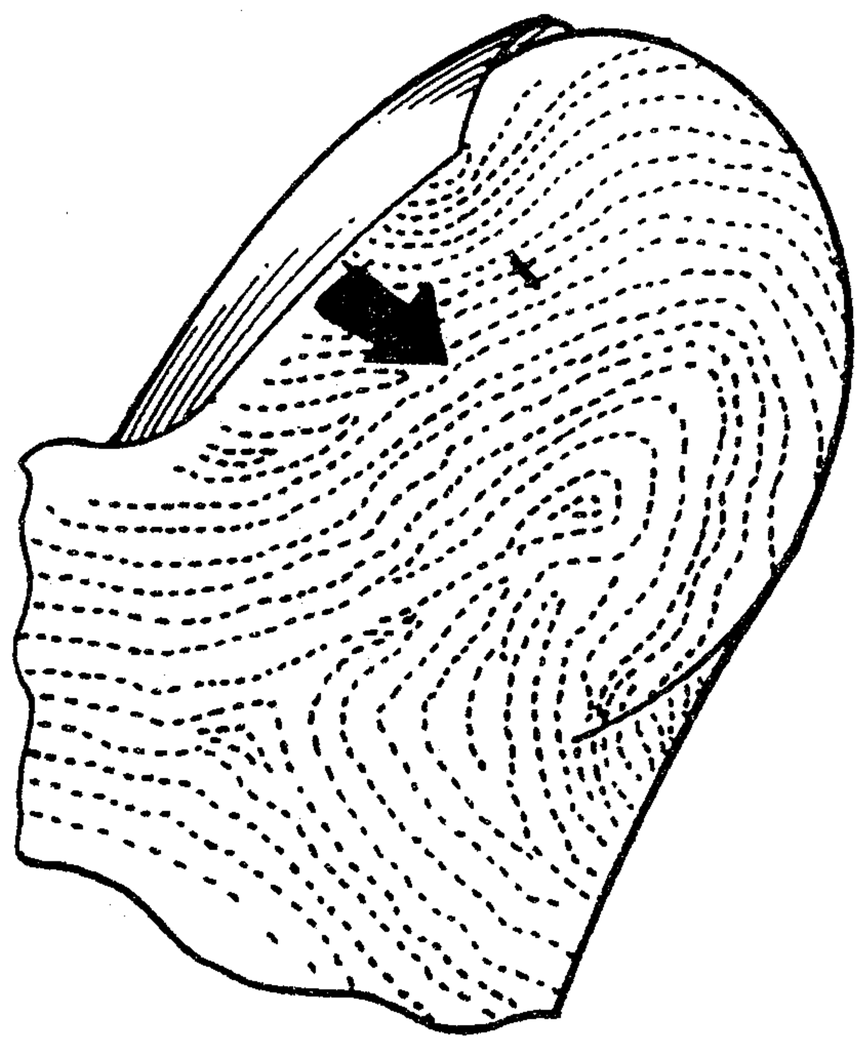


FIG. 16

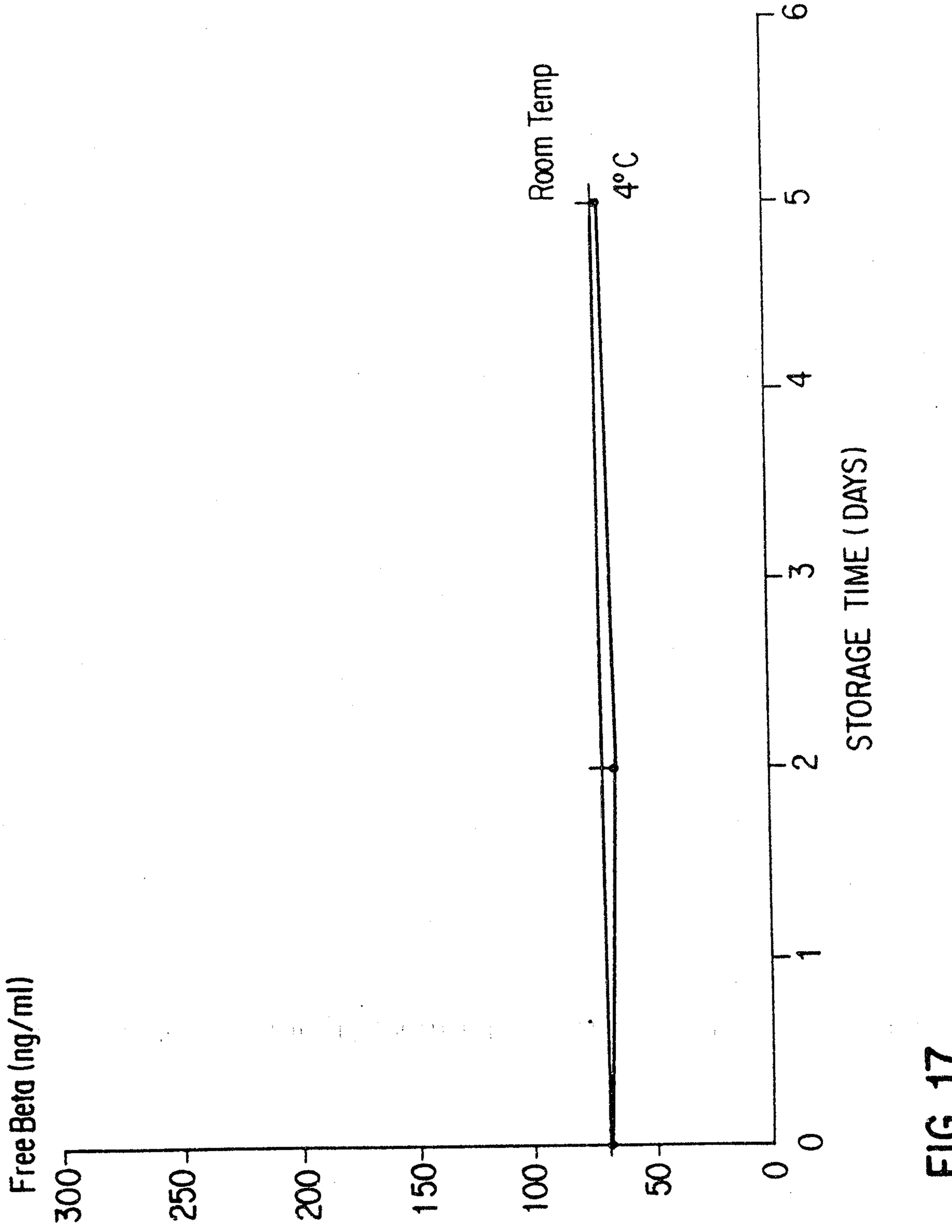

Free Beta (ng/ml)
300
250
200
150
100
50
0
0
1
2
3
STORAGE TIME (DAYS)
4
5
6
4°C
Room Temp

FIG. 17

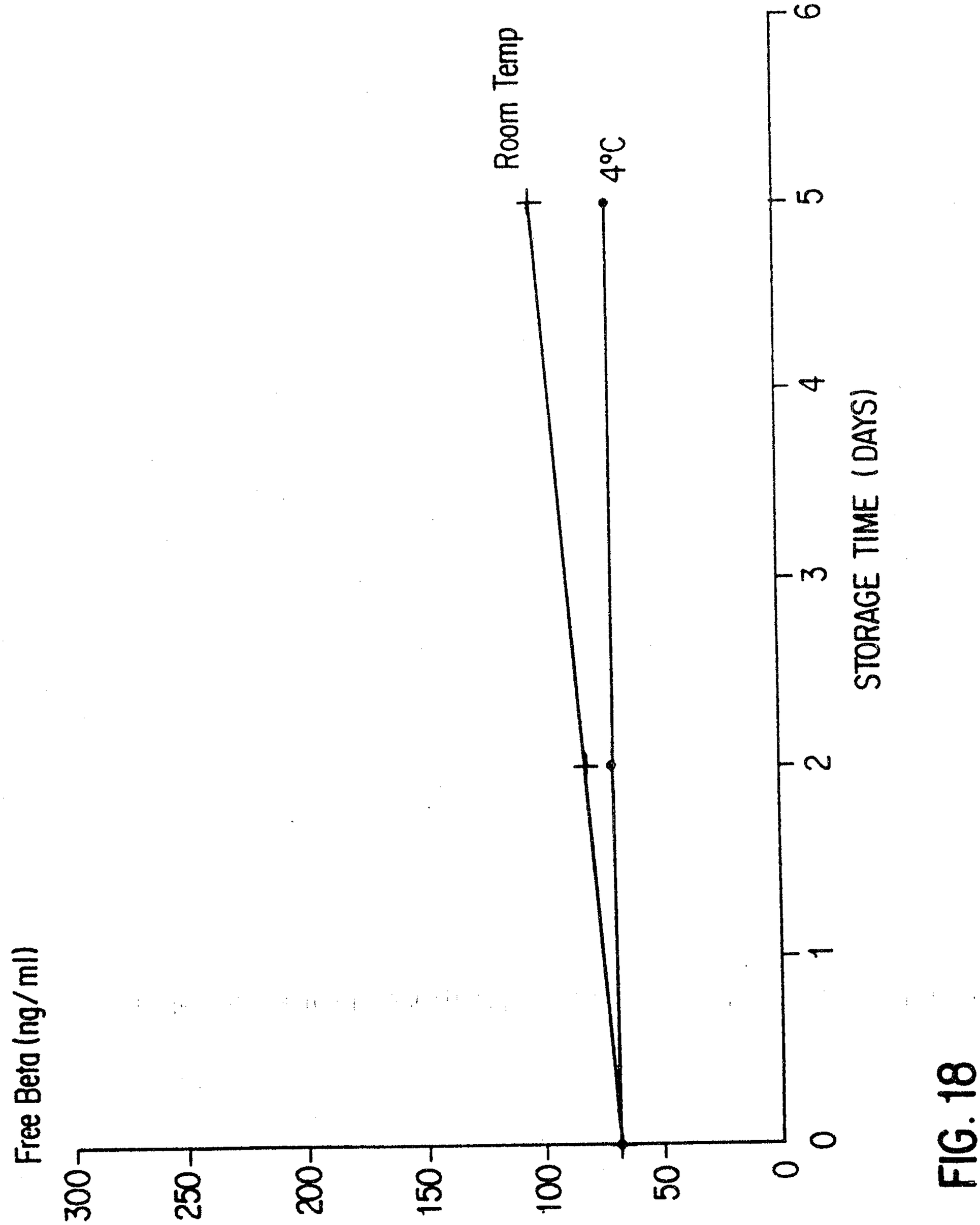

Free Beta (ng/ml)
300
250
200
150
100
50
0
0
1
2
3
4
5
6
STORAGE TIME (DAYS)
Room Temp
4°C

FIG. 18