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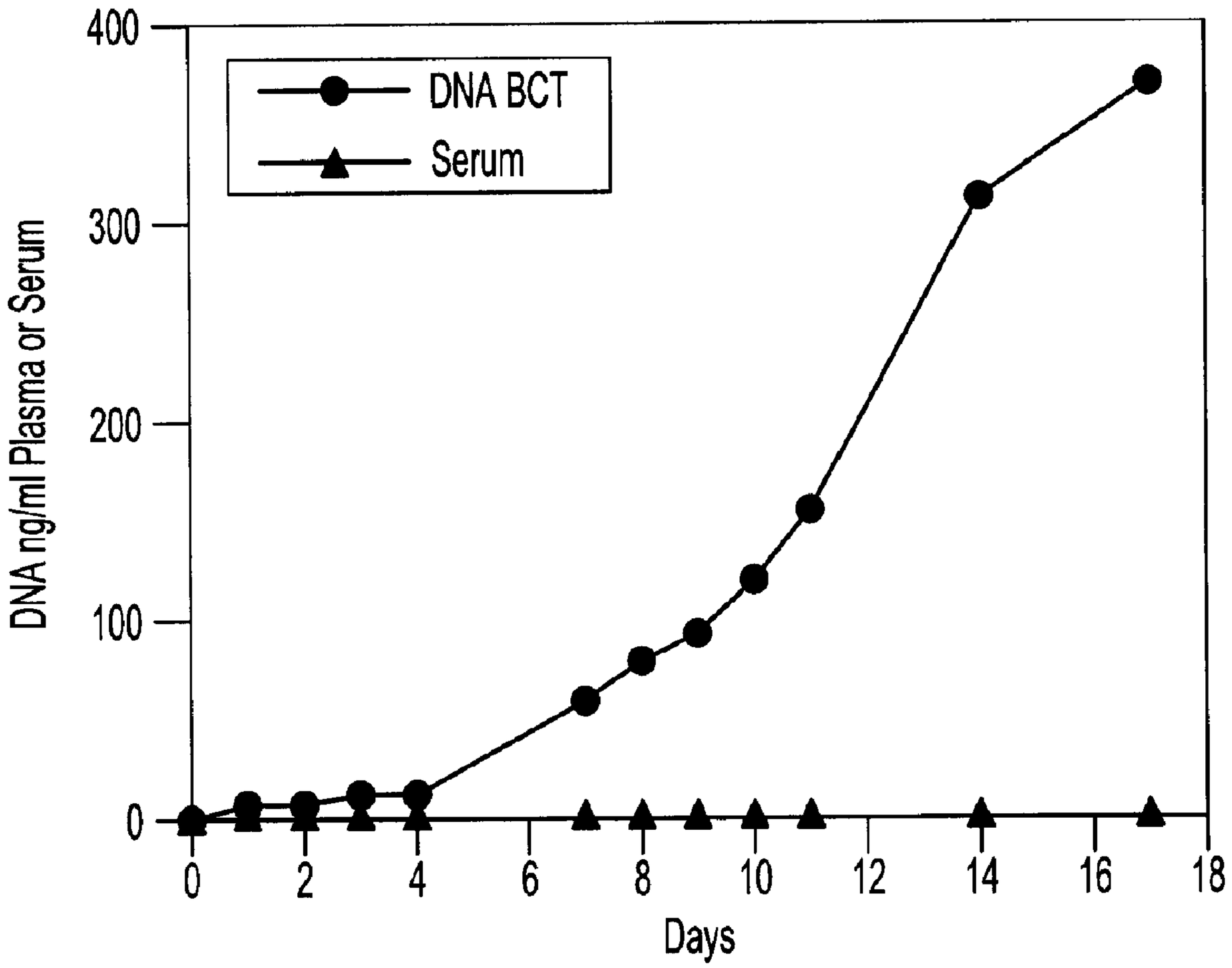
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(72) **Inventeurs/Inventors:**
FERNANDO, M. ROHAN, US;
CHAO-WEI CHEN, KATE, US

(73) **Propriétaire/Owner:**
STRECK, INC., US

(74) **Agent:** MCCARTHY TETRAULT LLP

(54) **Titre : PRESERVATION DES ACIDES NUCLEIQUES FOETAUX DANS LE PLASMA MATERNEL**
(54) **Title: PRESERVATION OF FETAL NUCLEIC ACIDS IN MATERNAL PLASMA**



(57) **Abrégé/Abstract:**
A method for preserving and processing fetal nucleic acids located within maternal plasma is disclosed, wherein a sample of maternal blood containing fetal nucleic acids is treated to reduce both cell lysis of the maternal blood cells and deoxyribonuclease (DNase) and ribonuclease (RNase) activity within the fetal nucleic acids. The treatment of the sample aids in increasing the amount of fetal nucleic acids that can be identified and tested while maintaining the structure and integrity of the fetal nucleic acids.

PRESERVATION OF FETAL NUCLEIC ACIDS IN MATERNAL PLASMA

ABSTRACT

A method for preserving and processing fetal nucleic acids located within maternal plasma is disclosed, wherein a sample of maternal blood containing fetal nucleic acids is treated to reduce both cell lysis of the maternal blood cells and deoxyribonuclease (DNase) and ribonuclease (RNase) activity within the fetal nucleic acids. The treatment of the sample aids in increasing the amount of fetal nucleic acids that can be identified and tested while maintaining the structure and integrity of the fetal nucleic acids.

PRESERVATION OF FETAL NUCLEIC ACIDS IN MATERNAL PLASMA

FIELD OF THE INVENTION

[0001] This invention relates to prenatal diagnosis of fetal abnormalities and more particularly to the preservation of fetal nucleic acids within a maternal blood sample.

BACKGROUND OF THE INVENTION

[0002] The demonstration by Leon et al. *Cancer Res* 37 (1977) 646-650 that cell-free plasma DNA is elevated in cancer patients paved the way for the present day interest in cell-free plasma nucleic acid in disease diagnosis. Relatively recently, Lo et al. *Lancet* 350 (1997) 485-487 have identified the existence of circulating cell-free fetal nucleic acids in maternal plasma. Since this work, a number of studies have demonstrated that cell-free fetal nucleic acids present in maternal plasma can be used in non-invasive prenatal diagnosis.

[0003] The analysis of nucleic acids can serve as a predictor of patient vulnerabilities by identifying chromosomes and corresponding genes that represent possible disease-related issues for a patient or a patient's offspring. Research has provided the chromosomal locations of many hereditary diseases and also the genotype or chromosomal mutation that corresponds with the disease. As the genetic markers for these hereditary diseases are ascertained, there is a parallel interest identifying patients that carry these genetic traits, especially when such diseases may only manifest in a patient's offspring. Further, hereditary diseases may only affect a child if both parents carry a necessary allele. In the interest of identifying the offspring that may be stricken with a fatal or debilitating hereditary condition, prenatal testing has become a much more routine practice. However, the difficulty in obtaining the genetic material of a fetus has presented a number of barriers to testing for the many known genetic markers for hereditary disease.

[0004] The most thorough and accurate prenatal screening procedures for fetal abnormalities generally involve invasive techniques such as amniocentesis and chorionic villus sampling. While providing reliable results, these procedures

are often regarded as carrying a substantial potential risk of pregnancy complications due to their invasive nature. In recent years, the identification of fetal nucleic acids within maternal blood has led to extensive research with a focus on isolating such fetal DNA and RNA to test for any number of fetal abnormalities. Such testing desirably is performed using only a maternal blood sample thereby eliminating the need for the invasive testing procedures. Unfortunately, it has proved challenging to isolate fetal nucleic acids from maternal nucleic acids.

[0005] More specifically, in order to obtain consistent and reliable results from the testing of fetal nucleic acids within maternal blood, it is important to both distinguish the fetal nucleic acids from the maternal nucleic acids and to preserve the structural integrity of the fetal nucleic acids. Traditionally, the first step of isolating cell-free nucleic acid from blood is obtaining either serum or plasma and then isolating the cell-free nucleic acids within the serum or plasma. However, serum is generally not suitable for cell-free nucleic acid isolation since blood clotting processes release cellular nucleic acids which contaminate cell-free plasma DNA (see Figure 1) as well as other deleterious substances that may destabilize the nucleated blood cells. Therefore efforts have been directed also at plasma as preferred starting material for cell-free nucleic acid isolation. Under such an approach, efforts at plasma separation from blood have been carried out to obtain a cell-free plasma sample. Unfortunately, this is frequently a tedious and time consuming multi-step process as it is important to use carefully controlled conditions to prevent cell breakage during centrifugation which will contaminate the cell-free nucleic acids with cellular nucleic acids released during breakage. Another important consideration is that cellular nucleic acid releases into plasma due to cell breakage during ex vivo incubation, typically within a relatively short period of time from a blood draw event. Once maternal cell lysis begins, the lysed maternal cells release additional nucleic acids which become mixed with the cell-free fetal nucleic acids and it becomes increasingly difficult to recover the fetal nucleic acids for testing. Further, the amount and recoverability of available cell-free DNA will decrease substantially over a relatively short period

of time due to degradation (e.g., from deoxyribonuclease (DNase) or ribonuclease (RNase) activity) of fetal cell-free DNA (which reduces the already finite supply of fetal DNA that can be recovered for analysis). For example, after a period of about 36 hours, an untreated sample is expected to be sufficiently corrupt that it would not lead to reliable or conclusive analysis. Thus, cell-free nucleic acids desirably are isolated as soon as plasma is separated or the plasma may be frozen at -80°C until the nucleic acids can be isolated. This too imposes practical constraints upon processing. It would therefore be of great benefit to develop sample processing techniques that would increase the amount of fetal nucleic acids (DNA and/or RNA) recoverable from maternal plasma, making the isolation and testing of the fetal nucleic acids more reliable and consequently improving the diagnostic capabilities of the fetal nucleic acids.

[0006] The problems generally associated with the isolation of cell-free nucleic acids include the time consuming and tedious nature of traditional isolation protocols and the requirement that blood samples be processed immediately in an effort to avoid maternal cell lysis. Often, maternal blood samples are immediately treated to remove all maternal cells and the resulting plasma is frozen. However, this process is lengthy and often cell lysis begins before cells are removed. Further, any protocols for removing the maternal cells, including centrifuging the maternal cells out of the sample and plasma freezing may have deleterious effects on the fetal nucleic acids.

[0007] In an effort to counter these problems and avoid cell degradation, blood samples have been subjected to a protocol which includes contacting the samples with formaldehyde. Formaldehyde is often used to stabilize cell membranes and its use could therefore reduce maternal cell lysis. Formaldehyde has also been thought to inhibit DNase and RNase thereby increasing the preservation and stability of the cell-free fetal nucleic acids. Studies by Dhallan et al. *JAMA* 291 (2004) 1114-1119 have demonstrated a decrease in cell lysis and a substantial increase in the amount of recoverable cell-free fetal nucleic acids. However, other studies have countered this data indicating that the formaldehyde does not have the desired effect. Most recently, Zhang et al., *Clinical Chimica*

Acta 397 (2008) 60-64, determined that the effect of formaldehyde on the percentage of fetal DNA in maternal plasma depends on processing time, wherein formaldehyde has little to no effect on samples processed at 6 hours, but has substantial preservation effect on samples processed at 36 hours. More particularly, samples contacted with formaldehyde and processed at 36 hours were found have reduced cell lysis and increased inhibition of plasma DNase activity. The use of formaldehyde for such purposes is discussed in U.S. Patent Nos. 7,332,277 and 7,442,506.

[0008] The potential for unreliability and toxicity considerations attendant with formaldehyde processing make its use for maternal plasma preservation undesirable. Given the immense discrepancies regarding the use of formaldehyde for fetal DNA sample preservation, there remains a need for a processing protocol that will consistently reduce one or any combination of maternal cell lysis and DNase and/or RNase activity within maternal plasma samples. It is further desired that such protocol allow for increased sample storage time, so that samples can be taken from a pregnant patient and subsequently stored or sent to a remote location for testing without fear of reduced integrity of the fetal nucleic acids.

[0009] A number of patent documents address such processes for the stabilizing, identification and testing of fetal cells and/or nucleic acids located within blood. See, generally, U.S. Patent Nos. 5,447,842; 5,457,024; 5,861,253; 6,258,540; 6,617,170; 6,821,789; 7,332,277; 7,442,506 and U.S. Patent Publication Nos. 2007/0111233; 2007/0134658; 2007/0202525; 2008/0020390; and 2008/0108071. Further, a substantial amount of academic research has been published in regard to fetal cell-free DNA and associated topics.

[0010] Notwithstanding the above, there remains a need for fetal nucleic acid isolation and preservation methods that are simplified and less time consuming. It is further desirable that these methods increase the amount of recovered fetal DNA and RNA from maternal plasma (e.g., as compared with methods that do not employ the teachings herein) while maintaining the integrity of the DNA and RNA and producing reliable diagnostic results. Efforts to increase

the reliability and consistency of fetal nucleic acid analysis include treating a maternal blood sample so that the amount of viable fetal DNA and/or RNA recovered is increased. The concentration of cell-free fetal DNA found within samples of maternal plasma at the time of blood draw generally ranges from 3.4% to 6.2% of the total amount of the cell-free DNA that is present in the plasma, depending on duration of gestation.

[0011] The present invention addresses the need for an efficient and consistent method of preserving and testing fetal nucleic acids from within maternal plasma. By providing an improved method for the reduction of maternal cell lysis and nuclease activity, the present invention includes a protocol that increases the amount of recoverable fetal nucleic acids thereby improving the diagnostic reliability of the fetal nucleic acids. The present invention helps prevent contamination of plasma cell-free nucleic acids with cellular nucleic acids that are released from damaged cells. The present invention further helps to inhibit nuclease activity to protect the integrity of the cell-free plasma nucleic acid. The stabilizing of the nucleated blood cells within a blood sample makes it no longer necessary to separate plasma immediately after blood draw. The present invention may further allow for blood samples to be stored at room temperature for up to about 14 days without compromising the integrity of the cell-free nucleic acids present in the plasma and without contaminating the sample with cellular nucleic acids originating from lysed cells. The present invention may also make it possible to avoid any freezing of the plasma and/or contact with any formaldehyde.

[0012] One advantage of the present invention is the possibility for essentially simultaneous stabilizing of both the nucleated blood cells and cell-free nucleic acids. This helps to prevent cellular genomic nucleic acids (e.g., maternal cellular genomic nucleic acids) from being released into plasma, and further diluting the fetal nucleic acids (and associated biomarkers) of interest, while also maintaining the structural integrity of the fetal nucleic acids. An additional possible advantage of the present invention lies in its ability to maintain relative amounts of fetal nucleic acids. In vivo there is constant replenishment of the fetal

nucleic acids to maintain a consistent amount of fetal nucleic acids but upon blood draw the fetal nucleic acid amounts will deteriorate without replenishment. The teachings of the present invention also contemplate the possibility to arrest the degradation of the fetal nucleic acids post-blood draw.

SUMMARY OF THE INVENTION

[0013] In a first aspect, the present invention contemplates a non-invasive prenatal screening method for the identification of fetal characteristics. The method includes the steps of: contacting a drawn maternal blood sample that includes a plurality of blood cells with a nucleic acid protective agent in an amount and time sufficient so that the blood cells are substantially prevented from (i) releasing genomic nucleic acids into the blood sample and from (ii) experiencing nuclease activity that degrades fetal nucleic acid; isolating fetal nucleic acids from the maternal blood sample; and analyzing the isolated fetal nucleic acids to identify a fetal characteristic.

[0014] The nucleic acid protective agent may include a formaldehyde releaser preservative agent such as one selected from the group consisting of: diazolidinyl urea, imidazolidinyl urea, dimethoylol-5,5dimethylhydantoin, dimethylol urea, 2-bromo-2.-nitropropane-1,3-diol, oxazolidines, sodium hydroxymethyl glycinate, 5-hydroxymethoxymethyl-1-1aza-3,7-dioxabicyclo [3.3.0]octane, 5-hydroxymethyl-1-1aza-3,7dioxabicyclo[3.3.0]octane, 5-hydroxypoly[methyleneoxy]methyl-1-1aza-3, 7dioxabicyclo[3.3.0]octane, quaternary adamantine and any combination thereof. The concentration of the preservative agent prior to the contacting step may be between about 0.1 g/ml and about 3 g/ml. The concentration of the preservative agent prior to the contacting step may be between about 0.4 g/ml and about 0.8 g/ml. The concentration of the preservative agent prior to the contacting step may be a concentration at which cross-linking of nucleic acids and proteins is observed, as indicated by agarose gel electrophoresis. The amount of the preservative agent in a treated sample may be less than about 20 mg/ ml of the blood sample.

[0015] The isolating step may include isolating nucleic acid from maternal

plasma and isolating the fetal nucleic acid in the absence of any cell. Either or both of the isolating or analyzing steps may occur at least 2 hours, 7 days, or even 14 days after the blood sample is drawn. Either or both of the isolating or analyzing steps may occur without and/or prior to any freezing the blood sample or any of its constituents (e.g. to a temperature colder than about -30°C (more preferably colder than about -70°C)). In one embodiment the isolating step occurs without prior freezing of the blood sample. In another embodiment, the analyzing steps occurs without prior freezing of the fetal nucleic acids isolated therefrom, In another embodiment both steps can occur without prior freezing of the respective material.

[0016] The fetal nucleic acid may be DNA, RNA or both. The analyzing step, the isolating step or both may include a step of contacting the fetal nucleic acid with an enzyme, an amplifier or both. The contacting step may take place in a blood collection tube into which the blood sample is drawn (e.g., while the blood sample is entering a blood collection tube). The contacting step may take place as the blood sample is drawn. The contacting step may be sufficient so that after a period of at least 7 days (or even 14 days) from the time the blood sample is drawn, the amount of fetal nucleic acid is at least about 90% of the amount of fetal nucleic acid at the time the blood sample is drawn. The contacting step may be sufficient so that after a period of at least 7 days from the time the blood sample is drawn, the amount of fetal nucleic acid present in the sample is about 100% of the amount of fetal nucleic acid present in the sample at the time the blood sample is drawn. The contacting step may be sufficient so that after a period of at least about 14 days from the time the blood sample is drawn, the concentration of fetal nucleic acid relative to the total nucleic acid in the blood sample that is present is at least about 10 to at least about 50 times the amount of fetal nucleic acid that would be present in the absence of the contacting step.

[0017] The protective agent may include a nuclease inhibitor selected from the group consisting of: diethyl pyrocarbonate, ethanol, aurintricarboxylic acid (ATA), formamide, vanadyl-ribonucleoside complexes, macaloid, ethylenediamine tetraacetic acid (EDTA), proteinase K, heparin, hydroxylamine-

oxygen-cupric ion, bentonite, ammonium sulfate, dithiothreitol (DTT), beta-mercaptoethanol, cysteine, dithioerythritol, tris (2-carboxyethyl) phosphene hydrochloride, a divalent cation such as Mg^{+2}, Mn^{+2}, Zn^{+2}, Fe^{+2}, Ca^{+2}, Cu^{+2} and any combination thereof. The protective agent may include an anticoagulant selected from the group consisting of heparin, ethylenediamine tetraacetic acid, citrate, oxalate, and any combination thereof. The protective agent may include a preservative agent and an anticoagulant. The protective agent may include imidazolidinyl urea and ethylenediamine tetraacetic acid.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] FIG. 1 is an illustrative graphic representation showing the relative amounts of cellular DNA present as a result of cell leakage within two blood samples stored at room temperature over time; there is seen a plot of "DNA BCT" data that extends substantially entirely along the x-axis at a y-axis (0 DNA) value of zero (0).

[0019] FIG. 2 is an illustrative graphic representation showing the relative amounts of Y-chromosomal DNA present as a result of male white blood cell leakage within two female blood samples over time; again there is seen a plot of "cell-free DNA BCT" that extends substantially entirely along the x-axis at a y-axis value of zero (0) DNA.

[0020] FIG. 3 is an illustrative graphic representation showing the relative amounts of cell-free DNA present within blood samples over time using lambda DNA as a marker.

[0021] FIG. 4 is an illustrative graphic representation showing the relative amounts of cell-free fetal DNA present within two blood samples over time using the RASSF1A promoter region as a marker.

[0022] FIG. 5 is an illustrative graphic representation showing the relative amounts of plasma DNA over time in a blood sample drawn into standard K_3EDTA tubes. In each box-plot, the total amount of cell-free plasma DNA is represented as genome equivalents per milliliter of plasma (GE/ ml). The line inside of the box indicates the median value. The limits of the box represent the

75th and 25th percentiles. The upper and lower error bars indicate the 10th and 90th percentiles, respectively. The uppermost and lowermost dots indicate the maximum and minimum values. The y-axis is in logarithmic scale.

[0023] FIG. 6 is an illustrative graphic representation showing the relative amounts of plasma DNA over time in a blood sample drawn into a device of the present teachings. In each box-plot, the total amount of cell-free plasma DNA is represented as genome equivalents per milliliter of plasma (GE/ ml). The line inside of the box indicates the median value. The limits of the box represent the 75th and 25th percentiles. The upper and lower error bars indicate the 10th and 90th percentiles, respectively. The uppermost and lowermost dots indicate the maximum and minimum values. The y-axis is in logarithmic scale.

[0024] FIG. 7 is an illustrative graphic representation showing the relative amounts of fetal cell-free DNA over time in a blood sample drawn into standard K_3EDTA tubes. In each box plot, the percentage of cell-free plasma DNA is represented as genome equivalents per milliliter of plasma (GE/ ml). The line inside of the box indicates the median value. The limits of the box represent the 75th and 25th percentiles. The upper and lower error bars indicate the 10th and 90th percentiles, respectively. The upper most and lower most dots indicate the maximum and minimum values. The y-axis is in logarithmic scale. Over time, a statistically significant decrease in the percentage of fetal cell-free DNA is seen only in K_3EDTA tubes (*P< 0.05, **$P \leq 0.01$ by paired Student's t test).

[0025] FIG. 8 is an illustrative graphic representation showing the relative amounts of fetal cell-free DNA over time in a blood sample drawn into a device of the present teachings. In each box plot, the percentage of cell-free plasma DNA is represented as genome equivalents per milliliter of plasma (GE/ ml). The line inside of the box indicates the median value. The limits of the box represent the 75th and 25th percentiles. The upper and lower error bars indicate the 10th and 90th percentiles, respectively. The upper most and lower most dots indicate the maximum and minimum values. The y-axis is in logarithmic scale. Over time, a statistically significant decrease in the percentage of fetal cell-free DNA is seen only in K_3EDTA tubes (*P< 0.05, **$P \leq 0.01$ by paired Student's t test).

[0026] FIG. 9 is an illustrative graphic representation showing amplification of fetal cell-free DNA from maternal plasma by whole genome amplification (WGA). One aliquot (without amplification) is used directly to quantify (by real-time PCR) the Y chromosomal SRY sequence from maternal plasma (an indicator of fetal DNA in maternal plasma). The other aliquot (with amplification) is subjected to WGA and then SRY sequence quantification by real-time PCR is performed. Enrichment in fetal cell-free DNA from maternal plasma by eighty fold is observed with WGA. A plasmid DNA construct containing a single copy of the Y chromosomal SRY sequence is used to plot the standard curve for the quantification.

DETAILED DESCRIPTION

[0027] In general, the invention herein contemplates a method of prenatal screening which includes the isolation and preservation of fetal nucleic acids located within maternal blood. A unique preservation step acts to increase the amount of recoverable fetal nucleic acids thereby improving the diagnostic capabilities of the fetal DNA and RNA.

[0028] More particularly, the present invention provides a method for the isolation of fetal nucleic acids including a preservation step that includes contacting a maternal blood sample with a protective agent. The nucleic acid may be DNA or RNA or any combination thereof. The fetal nucleic acid may be cell-free DNA or RNA. The samples from which the nucleic acids may be isolated include any maternal blood sample. The fetal nucleic acids may be located in maternal plasma. The method disclosed herein allows for the efficient isolation and preservation of fetal nucleic acids while avoiding confusion with maternal nucleic acids that enter a blood sample due to maternal cell lysis after blood draw.

[0029] The process for improved fetal nucleic acid isolation from a maternal blood sample begins by contacting a blood sample with a protective agent containing an active ingredient to maintain the integrity of the components within the sample. Ingredients that may be used include, but are not limited to,

diazolidinyl urea, imidazolidinyl urea, dimethoylol-5,5dimethylhydantoin, dimethylol urea, 2-bromo-2.-nitropropane-1,3-diol, oxazolidines, sodium hydroxymethyl glycinate, 5-hydroxymethoxymethyl-1-1aza-3,7-dioxabicyclo[3.3.0]octane, 5-hydroxymethyl-1-1aza-3,7dioxabicyclo[3.3.0]octane, 5-hydroxypoly[methyleneoxy]methyl-1-1aza-3, 7dioxabicyclo [3.3.0]octane, quaternary adamantine, 2-aminoacetic acid or any combination thereof. Preferred ingredients are selected from the group consisting of diazolidinyl urea (DU), imidazolidinyl urea (IDU), and any combination thereof.

[0030] The protective agent may consist essentially of the active ingredient. It may be at least about 10%, 50%, or even 80% by volume of the protective agent. For instance, the amount of active ingredient within the protective agent used may be generally about 100 to about 800 grams per liter. The amount of active ingredient within the protective agent may be at least about 25 grams per liter or even 50 grams per liter. The amount of active ingredient within the protective agent may be less than about 1500 grams per liter or even 1200 grams per liter. For example, the protective agent may comprise about 0.05 to about 0.4 grams of a formaldehyde releaser preservation agent (e.g., IDU) per 0.2 ml of the total protective agent.

[0031] As used throughout the present teachings, the protective agent composition preferably is substantially non-toxic. For example, the methods herein (and compositions used herein) may be free of separately adding and/or handling of any materially significant concentration (e.g., less than about 1% by weight, more preferably less than about 2000 parts per million, more preferably less than about 1000 parts per million, and still more preferably less than about 500 parts per million) of formaldehyde and/or paraformaldehyde prior to any contact with a blood product sample.

[0032] The protective agent may include a nuclease inhibitor in a suitable amount to prevent DNase and RNase activity from further decreasing (e.g. by at least about 10% by weight, and more preferably at least about 50% by weight) the quality and amount of fetal nucleic acids recoverable from the blood sample as compared with a sample that does not include a nuclease inhibitor. Nuclease

inhibitors that may be used include, but are not limited to diethyl pyrocarbonate, ethanol, aurintricarboxylic acid (ATA), formamide, vanadyl-ribonucleoside complexes, macaloid, ethylenediamine tetraacetic acid (EDTA), proteinase K, heparin, hydroxylamine-oxygen-cupric ion, bentonite, ammonium sulfate, dithiothreitol (DTT), beta-mercaptoethanol, cysteine, dithioerythritol, tris (2-carboxyethyl) phosphene hydrochloride, or a divalent cation such as Mg^{+2}, Mn^{+2}, Zn^{+2}, Fe^{+2}, Ca^{+2}, Cu^{+2} or any combination thereof. Further, the protective agent may be substantially free of guanidinium salts, sodium dodecyl sulfate (SDS), or any combination thereof.

[0033] The initial contacting of the blood sample may be for a time sufficient to inhibit one or both of maternal cell lysis, nuclease activity, or any combination thereof. Contacting may occur for at least about 10 seconds, more preferably at least about 1 minute, still more preferably at least about 2 minutes. Contacting can occur for longer periods of time. For example, contacting may be commenced substantially contemporaneously from the time of blood draw (e.g., within less than about 10 minutes of the blood draw) and it may last until nucleic acids are isolated, screened, and/or tested. The contacting step may also be employed to provide a sample with a longer shelf life. Thus, it is possible that a lapse of time of at least about 2 hours, more preferably at least about 6 hours, at least about 24 hours, at least about 7 days or even at least about 14 days can elapse between the time of blood draw (which may be substantially contemporaneous with the contacting step), and the time of any testing or screening of the sample, and/or isolation of the nucleic acids.

[0034] The protective agent may comprise an active agent in solution. Suitable solvents include water, saline, dimethylsulfoxide, alcohol and mixtures thereof. The protective agent may comprise diazolidinyl urea (DU) and/or imidazolidinyl urea (IDU) in a buffered salt solution. The protective agent may further comprise EDTA and 2-aminoacetic acid. Alternatively, the protective agent may contain only a fixative (e.g., an active ingredient) and may be free of any additional additives.

[0035] The amount of any active ingredient within the protective agent may

generally be about 10% to about 90% by weight. The active ingredient or fixative may comprise about 70% to about 90% by weight of the protective agent. The protective agent may further contain an anticoagulant such as about 5% to about 20% by weight EDTA. The protective agent may contain about 10% by weight EDTA. The protective agent may include from about 1% to about 40% by weight of nuclease inhibitor.

[0036] The amount of active ingredient or fixative (e.g. the formaldehyde releaser) relative to the amount of EDTA may be about 1 to about 10 parts (more preferably about 2 to about 8 parts) by weight of fixative to about 1 part by weight EDTA. The amount of protective agent within a tube prior to blood draw may be about 0.05 to about 1.0 ml and more preferably about 0.1 to about 0.3 ml.

[0037] The combination of an active ingredient or fixative (e.g. the formaldehyde releaser) and anticoagulant within the protective agent results in improved ability to maintain the amount and quality of fetal DNA within a maternal blood sample. These results are believed unexpected and superior to results obtained by the use of only the fixative or only the anticoagulant. Therefore it is believed that a synergistic effect may occur when both a fixative and anticoagulant are combined. The compositions disclosed herein specifically envision the possibility to include the combination of a formaldehyde releaser and an anticoagulant.

[0038] The protective agent may be located within a specialized device, wherein the protective agent is already present in the device prior to addition of the blood sample, such as that disclosed in U.S. Patent Publication No. 2004/0137417. The device may be an evacuated collection container, usually a tube. The tube may be made of a transparent material that will also resist adherence of the cells within a given sample. The interior wall of the tube may be coated or otherwise treated to modify its surface characteristics, such as to render it more hydrophobic and/or more hydrophilic, over all or a portion of its surface. The tube may have an interior wall flame sprayed, subjected to corona discharge, plasma treated, coated or otherwise treated. The tube may be treated by contacting an interior wall with a substance so that the nucleic acids of interest

will resist adhering to the tube walls. The surface of the tube may be modified to provide a dual functionality that simultaneously provides an appropriate balance of desired hydrophilicity and hydrophobicity, to allow collection of blood, dispersion of the protective agent disclosed herein, while resisting adhesion of nucleic acids to the inner wall of the blood collection tube.

[0039] It is possible that any coating may be a functionalized polymeric coating that includes a first polymer and one or more second monomeric and/or polymeric functionalities that are different from (e.g., chemically different from) the first polymer. The coating may include one or more co-polymers (e.g., block copolymer, graft copolymer, or otherwise). For example, it may include a copolymer that includes a first hydrophobic polymeric portion, and a second hydrophilic polymeric portion. The coating may be a water based coating. The coating may optionally include an adhesion promoter. The coating may be applied in any suitable manner, it may be sprayed, dipped, swabbed, or otherwise applied onto some or all of the interior of the blood collection tube. The coating may also be applied in the presence of heat. Preferably any coating applied to the inner wall of a blood collection tube will form a sufficiently tenacious bond with the glass (e.g., borosilicate glass) or other material (e.g., polymeric material) of the tube so that it will not erode or otherwise get removed from the inner wall. Examples of suitable polymeric coatings may include silicon containing polymers (e.g., silanes, siloxanes, or otherwise); polyolefins such as polyethylene or polypropylene; polyethylene terephthalate; fluorinated polymers (e.g., polytetrafluoroethylene); polyvinyl chloride, polystyrene or any combination thereof. Examples of teachings that may be employed to coat an interior of a blood collection tube may be found in U.S. Patent Nos. 6,551,267; 6,077,235; 5,257,633; and 5,213,765.

[0040] The tube as described above may preferably include an anticoagulant agent and an active ingredient such as a fixative agent including but not limited to those active ingredients disclosed herein. The tube may also may further include a nuclease inhibitor. Preferably, the compounds included in the tube are in an amount sufficient to preserve maternal cell morphology and

prevent cell degradation while also preventing deleterious DNase and RNase activity within the fetal cell-free nucleic acids. However, the amount of protective agent may also be sufficiently small so that any consequential dilution of the sample is substantially avoided, and cell-free nucleic acids in the sample are not materially diluted. A blood sample may be fixed simultaneously as it is drawn into the specialized tube. The tube may also be coated over an exterior wall with a protective coating (e.g., a containment barrier that helps control glass shard fragmentation) such as that disclosed in U.S Patent No. 7,419,832.

[0041] Additionally, the protective agent may be in a highly viscous or substantially solid state, such that (for example) it can be used effectively as a substantially solid state coating. Examples of such substantially solid state preservatives can be found in commonly owned co-pending U.S. Application Serial No. 12/646,204, filed December 23, 2009. Liquid removal techniques can be performed on the protective agent in order to obtain a substantially solid state protective agent. Liquid removal conditions may be such that they result in removal of at least about 50% by weight, at least about 75% by weight, or at least about 85% by weight of the original amount of the dispensed liquid protective agent. Liquid removal conditions may be such that they result in removal of sufficient liquid so that the resulting composition is in the form of a film, gel or other substantially solid or highly viscous layer. For example it may result in a substantially immobile coating (preferably a coating that can be re-dissolved or otherwise dispersed upon contact with a blood product sample). It is possible that lyophilization or other techniques may be employed for realizing a substantially solid form of the protective agent (e.g., in the form of one or more pellet). Thus, liquid removal conditions may be such that they result in a material that upon contact with the sample under consideration (e.g., a maternal blood sample) the protective agent will disperse in the sample, and substantially preserve components (e.g., cell-free nucleic acids) in the sample. Liquid removal conditions may be such that they result in a remaining composition that is substantially free of crystallinity; has a viscosity that is sufficiently high that the remaining composition is substantially immobile at ambient temperature (e.g., it

does not exhibit any visibly detectable (as seen by the naked eye) flow when held in a storage device at room temperature on an incline of at least about 45° for at least one hour); or both. A colorant may also be employed.

[0042] As discussed herein, contacting a maternal blood or plasma sample with the protective agent allows the sample to be stored for a period of time prior to isolating and testing the fetal nucleic acids. More preferably, a maternal blood or plasma sample may be drawn at one location (e.g., a health care facility), contacted with the protective agent, and later transported to a different remote location (e.g., a laboratory, such as one that is separately housed at a distance of at least about 1 km, 2 km, 3 km, or further away from the draw site) for the nucleic acid isolation and testing process. Fetal nucleic acids may be isolated from the maternal blood or plasma sample and tested for various fetal characteristics (including but not limited to chromosomal abnormalities) at the remote location and the resulting diagnostic information may then be reported to the site of the original blood draw. The fetal nucleic acid isolation process may be performed at one remote location and the resulting information can be analyzed to identify fetal characteristics including chromosomal abnormalities at a third location. Moreover, the results of the fetal nucleic acid isolation process may be sent back to the site of the initial blood draw and analyzed there. The resulting diagnostic information may then be sent to a third location or back to the remote location or the site of the initial blood draw.

[0043] At any time after the initial contact of the maternal blood or plasma sample with the protective agent, the sample can be treated to isolate the cell-free fetal nucleic acids located within the maternal blood. The nucleic acids may be isolated using any isolation method including those methods disclosed in commonly owned United States Application Publication No. 2009/0081687 Preferably, the maternal blood cells will stay generally intact, so that maternal nucleic acids are not released into the sample from broken blood cells, making isolation of the fetal nucleic acids more difficult. The fixative acts to prevent cell lysis so that the maternal cells remain intact and substantially all maternal nucleic acids remain intra-cellular to avoid unwanted contamination of the cell-free fetal

nucleic acids.

[0044] After the fetal nucleic acids have been isolated, they can be tested to identify various fetal characteristics including but not limited to sex of the fetus, preeclampsia in the mother, rhesus status of the fetus and the presence of any chromosomal abnormalities including but not limited to any chromosomal inversions, translocations, aneuploidies, other mutations, or any combination thereof. The methods herein thus further contemplate a step of nucleic acid testing. Testing of the fetal nucleic acids can be performed using any nucleic acid testing method including, but not limited to polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR), quantitative real time polymerase chain reaction (Q-PCR), gel electrophoresis, capillary electrophoresis, mass spectrometry, fluorescence detection, ultraviolet spectrometry, DNA hybridization, allele specific polymerase chain reaction, polymerase cycling assembly (PCA), asymmetric polymerase chain reaction, linear after the exponential polymerase chain reaction (LATE-PCR), helicase-dependent amplification (HDA), hot-start polymerase chain reaction, intersequence-specific polymerase chain reaction (ISSR), inverse polymerase chain reaction, ligation mediated polymerase chain reaction, methylation specific polymerase chain reaction (MSP), multiplex polymerase chain reaction, nested polymerase chain reaction, solid phase polymerase chain reaction, or any combination thereof.

[0045] One aspect of the teachings herein contemplates a method for isolating and testing cell-free fetal DNA from maternal plasma. The method may be performed on a single sample or on a multitude of samples (e.g., in a multi-well plate). The method may include contacting the maternal plasma sample with a protective agent. The protective agent may include a fixative as previously discussed so that the maternal cells remain intact throughout the blood draw and DNA isolation process. The protective agent may further include a DNase inhibitor to maintain the structural integrity of the fetal DNA. After contacting the maternal plasma sample with the protective agent, the sample may be centrifuged to separate the plasma and the supernatant is discarded. By

contacting a maternal blood sample with the protective agent, the blood sample does not necessarily require immediate processing and may be stored for a prolonged period, such as up to about 14 days or longer at room temperature. Thus the inventions herein contemplate one or more steps of storing and/or otherwise waiting a relatively lengthy period from the time of blood draw and/or contacting until the time of screening, testing or other analysis.

[0046] Once, the sample is processed, an appropriate concentration of an agent for inducing precipitation (e.g., a composition of salt and/or alcohol) may be added to precipitate the fetal DNA containing material. An organic or other compound such as a phenol derivative or the like may be added to remove any remaining protein contaminants. Any protein contaminants that still remain may be removed by adding additional amounts of an organic or other compound such as a phenol derivative or the like. After centrifugation, ethanol may be added and the sample centrifuged again. Any remaining liquid may be removed from the sample so only the fetal DNA will remain. The finished product of isolated fetal DNA may then be contacted with a buffer.

[0047] One or more steps of incubation may be performed. Incubation may occur on ice or at any temperature between -30°C and 70°C. For example, a sample may be incubated at about -20°C. A sample may also be stored at room temperature and thus substantially free of freezing upon blood draw.

[0048] Centrifugation may be performed at a suitable rate. For example, centrifugation may be done at about 500 to about 20,000 rpm. Centrifugation may occur at about 1,000 to 16,000 rpm. Centrifugation may be performed at about room temperature or cooler. For example, it may be performed at about 1-20°C, or still more specifically at about 4-9°C.

[0049] The following illustrates how a blood collection device in accordance with the present teachings can preserve fetal cell-free DNA and help minimize the cell-free DNA background in maternal plasma at ambient temperature. As will be seen, blood samples are drawn from healthy pregnant donors into (i) standard K_3EDTA (sold under the name BD Vacutainer® by Becton Dickinson of Franklin Lakes, New Jersey) blood collection tubes and (ii) blood collection tubes

containing the protective agent taught herein ("the protective agent of the present teachings"), and kept at ambient temperature. For example, the protective agent of the present teachings may include about 500 g/L IDU, about 81 g/L Tripotassium EDTA, and about 47 g/l glycine. The protective agent of the present teachings may be placed within a tube so that the tube contains about 0.20 ml of the protective agent. The tube containing the protective agent may receive about 10 ml of patient blood. The patient blood may be drawn directly into the tube containing the protective agent. It is believed that results shown will vary by about ± 25% of that described across a range of about 300 to about 700 g/L IDU (with similar results expected for other formaldehyde releasers described herein) and from about 60 to about 100 g/L Tripotassium EDTA, and about 20 to about 60 g/L glycine. The protective agent may include roughly about 6 parts by weight IDU per about 1 part by weight EDTA, and roughly about 10 parts by weight IDU per about 1 part glycine. The protective agent may include about 80% by volume of IDU, 12.8% by volume Tripotassium EDTA, and 7.25 by volume glycine. An example of a commercially available tube in accordance with the present teachings is sold under the name Cell-Free DNA BCT by Streck, Inc., Omaha, Nebraska.

[0050] For comparison purposes, a blood sample that is not treated with the compositions disclosed herein is centrifuged to cause plasma separation and cell-free DNA is extracted. Cell-free DNA from plasma is quantified by quantitative real-time PCR. These maternal blood samples (drawn into standard K_3EDTA tubes) show a steady reduction in the amount of fetal cell-free DNA during an extended time period (e.g., 36 hours, 7 days, 2 weeks etc.) at ambient temperature. Conversely, blood drawn into a device containing the protective agent of the present teachings shows no change in the amount of fetal cell-free DNA over the same time period.

[0051] Using maternal plasma stored in a device containing the protective agent of the present teachings for an extended period, fetal cell-free DNA may be amplified at least 10-fold (e.g., 80-fold) using whole genome amplification at the end of the extended period, and there is sufficient quantity of DNA available for

meaningful analysis. Thus, use of the protective agent of the present teachings makes it possible to preserve fetal cell-free DNA for extended times as well as minimize any post-sampling maternal cell-free DNA background. Preserved in this way, fetal cell-free DNA can be amplified by whole genome amplification technology for producing sufficient amounts of fetal nucleic acids as a starting material for nucleic acid-based prenatal diagnostic tests.

[0052] For the discussion that follows, there is envisioned a protocol that employs some or all of the following steps, following direct draw of a blood sample into an evacuated blood collection tube. In accordance with the present invention, the blood sample may be contacted by a protective agent such as those protective agents described herein. The processing of nucleic acids for analysis may include a step of purifying the nucleic acids and amplifying the nucleic acids.

[0053] Samples of the treated blood (e.g., one and one half ml aliquots of blood) may be removed from each tube periodically; cell-free plasma DNA may be purified; primers and probes for the real-time PCR quantification of one or more antibody or protein sequences (e.g., β-actin, SRY, RASSF1A and/or other markers for fetal DNA) may be prepared; real-time PCR quantification of one or more antibody or protein sequences (e.g., β-actin, SRY, RASSF1A and/or other markers for fetal DNA) may be carried out; re-suspended plasma DNA may be treated with a restriction enzyme; a promoter region sequence (such as those associated with the fetal DNA markers discussed herein) may be used as a universal marker for fetal DNA; a suitable amplifier may be used to amplify fetal cell-free plasma DNA obtained from maternal blood stored in the device of the present teachings; or statistical analysis may be carried out.

[0054] Primers and probes for the real-time PCR quantification of certain antibody or protein sequences discussed herein (e.g., β-actin, RASSF1A and/or other markers for fetal DNA) may be prepared in accordance with art-disclosed teachings, such as described by Chan et al. *Clinical Chemistry* 52:2211-2218 (2006). Primers for the Y-chromosomal sex determining region (SRY) may be prepared in accordance with art-disclosed teachings, such as Lee et al., *Blood*

93:3127-3139. An example probe that may be used for the quantification of SRY sequence is 6FAM-ATG GCT CTA GAG AAT CCC AGA ATG CGA AAC TCA GAG A-TAMRA. Commercially available primers, probes and PCR master mix, (e.g., TaqMan® Universal PCR master mix) may be purchased from Applied Biosystems, Foster City, CA. Plasmid DNA constructs may be prepared so that each contains a single copy of the antibody or protein sequences discussed herein (β-actin, RASSF1A, SRY, and/or other fetal DNA markers). These plasmid constructs may be used to plot the standard curves.

[0055] After re-suspension of the plasma DNA, the plasma may be treated with a restriction enzyme (e.g., 25 U of BstUI restriction enzyme, available from New England Biolabs, Ipswich, MA) in accordance with art-disclosed teachings, such as described by Chan et al. *Clinical Chemistry* 52:2211-2218 (2006).

[0056] Following re-suspension, a suitable amplifier (e.g., a QIAGEN REPLI-g® UltraFast Mini whole genome amplification kit available from QIAGEN, Inc., Valencia, California) may be used for the step of amplifying the fetal cell-free plasma DNA obtained from maternal blood stored in the device of the present teachings. Purified cell-free DNA is prepared from a volume of plasma (e.g., at least about 100 µl, or less than about 800 µl) as described above, but is re-suspended in a small volume (e.g., at least about 0.05 µl, or less than about 10 µl) and amplified using the kit according to the manufacturer's instructions. After amplification, the sample may be diluted (e.g., by about 25-fold) prior to PCR analysis.

[0057] In verifying the protective capabilities of the compositions disclosed herein, standard K_3EDTA blood collection tubes are thus compared against tubes containing the protective agent of the present teachings, which thus contains a composition that stabilizes nucleated blood cells and inhibits plasma nucleases. In the examples and results discussed below, statistical analysis is carried out using software available at the *Tools for Science* website of the Physics Department, College of Saint Benedict Saint John's University, St. Joseph, Minnesota (http://www.physics.csbsju.edu/). Paired Student's t test is used and $P < 0.05$ is considered statistically significant.

Example 1

[0058] Blood samples are taken from a female donor and a male donor. The female blood sample is transferred into two tubes, tube A containing about 500 g/L IDU, about 80 g/L Tripotassium EDTA, and about 50 g/L glycine and tube B containing only the Tripotassium EDTA. Both tubes are stored at room temperature. White blood cells from the male blood sample are isolated and spiked into both tube A and tube B. 3ml of blood are taken from each tube on day 0, day 1, day 2, day 3, day 4, day 7 and day 11. Each sample is centrifuged at room temperature at 800 g for 10 minutes and the upper plasma layer is transferred to a new tube and further centrifuged at 1500 g at room temperature for 10 minutes. The free circulating DNA in each tube is then purified using the NucleoSpin® Plasma XS kit available from Macherey-Nagel Inc., Bethlehem, Pennsylvania. The samples are then amplified by Real Time PCR amplification of a fragment of the Y-chromosome (using iQ SYBR Green Supermix reagents available from BIO-RAD Laboratories (Hercules, California)). Any rupture of the male white blood cells during sample processing will cause Y-chromosomal DNA to be detectable within the female blood sample. Tube A shows no Y-chromosomal DNA presence within the plasma sample, while the amount of Y-chromosomal DNA identified in tube B increases at each measurement, indicating male white blood cell rupture in tube B. The expected results of this example are shown in graphic format at Figure 2, and supports that use of the compositions disclosed herein are capable of substantially preventing lysis of the blood cells spiked into the samples.

Example 2

[0059] Blood samples from the same donor are drawn into two different types of blood collection tubes. One tube contains 500 g/L IDU, 81 g/L Tripotassium EDTA and 47 g/L glycine. The other tube contains only Heparin. All samples are centrifuged at 2100 g for 30 minutes at room temperature to separate the plasma. The plasma is then transferred to new tubes and non-human (lambda) DNA is then spiked into the plasma tubes. The spiked samples are then stored at room temperature for 0, 1, 2, 3, 4, 7, 11, and 14 days. Free

circulating DNA is purified using the QIAamp® DNA Blood Mini Kit available from QIAGEN Inc. (Valencia, CA). DNA is extracted from each plasma sample. The samples are then amplified by Quantitative PCR (using iQ SYBR Green Supermix reagents available from BIO-RAD Laboratories (Hercules, California)) to identify the amount of lambda DNA present. Results show a consistent relative percentage of lambda DNA presence at each measurement, indicating little if any decline in the percentage of cell-free DNA in the plasma samples contacted by both IDU and Tripotassium EDTA. The amount of lambda DNA decreases at every consecutive measurement in those samples contacted with only Heparin, indicating a gradual decline in the relative percentage of cell-free DNA. The expected results of this example are shown in graphic format at Figure 3. This example confirms that the compositions of the present invention are able to maintain the integrity and amount of DNA present in a blood sample.

Example 3

[0060] Two maternal blood samples from the same donor are drawn into two separate blood collection tubes. One tube contains about 500 g/L IDU, about 80 g/L Tripotassium EDTA, and about 50 g/L glycine. The other tube contains only the Tripotassium EDTA. Both tubes are stored at room temperature and 1ml aliquots of blood are removed from each tube on day 0, day 7, and day 14 and plasma is separated. All samples are centrifuged at 800 g for 10 minutes at room temperature to separate the plasma. The plasma is then transferred into new tubes and centrifuged at 1500 g for 10 minutes at room temperature. Free circulating DNA is purified using the NucleoSpin® Plasma XS kit available from Macherey-Nagel Inc., Bethlehem, Pennsylvania. DNA is extracted from each plasma sample and eluted in 60µl of elution buffer. An amount of 32µl of eluted DNA is digested with 40 U of BstU I enzyme at 60° for 6 hours. The samples are then amplified by Real Time PCR (using TaqMan® RT PCR reagents available from Applied Biosystems, Foster City, California) using primers for RASSF1A promoter region. Results show a consistent relative percentage of RASSF1A presence at each measurement, indicating little if any decline in the percentage of fetal cell-free DNA in the maternal plasma samples contacted by both IDU and

Tripotassium EDTA. The amount of RASSF1A decreases at every consecutive measurement in those samples contacted with only the Tripotassium EDTA, indicating a gradual decline in the relative percentage of fetal cell-free DNA. The results of this example are shown in graphic format at Figure 4.

[0061] Figure 5 shows the expected result of ex-vivo incubation of maternal blood when drawn into standard K_3EDTA tubes on the cell-free DNA concentration in plasma. Initially (3 hrs), the median cell-free DNA concentration is found to be 762 genome equivalents per ml of plasma (GE/ ml) which increased markedly over time. Compared to the initial 3 hrs value, statistically significant increases are observed in the cell-free DNA concentration at 24 hrs ($P < 0.05$), 48 hrs ($P < 0.05$), 72 hrs ($P < 0.05$), 7 days ($P < 0.05$) and 14 days ($P \leq 0.001$). This steady increase may reflect the lysis of nucleated blood cells and the subsequent release of cellular genomic DNA into the plasma that continued for 14 days.

[0062] Figure 6 illustrates the expected effect of ex-vivo incubation of maternal blood drawn into tubes containing the protective agent of the present teachings on the plasma cell-free DNA concentration. Here, an initial median cell-free DNA concentration of 672 GE/ ml does not increase significantly throughout the entire 14 day experimental period, indicating that an enhanced integrity of nucleated blood cells is observed. After 3 hrs of incubation, a comparison of the plasma cell-free DNA concentration in K_3EDTA tubes and tubes containing the protective agent of the present teachings showed a statistically significant difference ($P < 0.05$). The mean cell-free DNA concentration in K_3EDTA tubes is 6341 GE/ ml whereas it is 680 GE/ ml in those tubes containing the protective agent of the present teachings. The higher cell-free plasma DNA concentration in the K_3EDTA tube compared to those tubes containing the protective agent of the present teachings indicates that cellular DNA is released into plasma by nucleated blood cell lysis.

[0063] Figure 7 shows the expected effect of ex vivo incubation on the fetal cell-free DNA in maternal plasma in K_3EDTA blood collection tubes. A statistically significant decrease in the percentage of fetal cell-free DNA is observed. This

downward trend in the median values of the percentage of fetal cell-free DNA; 4.05, 1.33, 0.45, 0.11, 0.10 and 0.023 (at 3 hrs, 24 hrs, 48 hrs, 72 hrs, 7 days and 14 days, respectively), demonstrates that K_3EDTA tubes are not capable of maintaining the fetal cell-free DNA percentage in maternal plasma at a constant level.

[0064] Figure 8 shows the expected effect of ex-vivo incubation on the fetal cell-free DNA in maternal plasma when contacted by the protective agent of the present teachings. Here, the percentage of fetal cell-free DNA does not change significantly in the trend of median values; 6.8, 5.5, 6.2, 6.1, 6.8 and 6.5 at 3 hrs, 24 hrs, 48 hrs, 72 hrs, 7 days and 14 days, respectively.

[0065] In further testing, donor plasma that had tested positive for the Y chromosome (data not shown) was used for whole genome amplification (WGA). By real-time PCR, 398 SRY DNA copies are detected without WGA, whereas 32,300 SRY DNA copies are detected following WGA. This represents enrichment in fetal cell-free DNA by at least about 10 fold, 20 fold, 40 fold or even 80 fold from maternal plasma that had been stored in a device containing the protective agent of the present teachings at ambient temperature for the same period (e.g., 2 weeks) (Figure 9).

[0066] As discussed in reference to Figure 5, maternal blood collected into standard K_3EDTA tubes shows a 7-fold and 98-fold increase in total cell-free DNA in maternal plasma at 24 hrs and 14 days as compared to 3 hrs, respectively. However, total cell-free DNA concentration is constant at ambient temperature for up to 14 days in maternal blood contacted by the protective agent of the present teachings (Figure 6). Without intending to be bound by theory, this indicates that the chemicals present in the protective agent of the present teachings are able to fix the nucleated blood cells thereby preventing apoptosis, cell death and cell lysis associated cellular genomic DNA release into plasma. A comparison of total cell-free DNA concentrations in K_3EDTA and the device of the present teachings at initial time point (3 hrs) show a statistically significant difference. The mean total cell-free DNA concentration in K_3EDTA tube at 3 hrs is 6341 GE/ ml, whereas in a tube containing the protective agent of

the present teachings, it is only 680 GE/ ml. This multi-fold (e.g., 9-fold) increase in total cell-free DNA concentration in the samples contacted by only K_3EDTA as compared to samples contacted by the protective agent of the present teachings may result from increased cellular genomic DNA release from nucleated blood cell apoptosis, death and lysis during post blood draw ex vivo incubation and sample processing, and may be substantially avoided by use of the teachings herein.

[0067] Figure 7 shows the expected effect of ex vivo incubation (of blood drawn into K_3EDTA tube) on fetal cell-free DNA percentage in maternal blood plasma. There is a statistically significant decrease in fetal cell-free DNA percentage over time. The major contributor to this steady decrease in fetal cell-free DNA percentage may be the increased background maternal cell-free DNA, as fetal cell-free DNA degradation may occur due to nuclease action. In contrast, as shown in Figure 8, fetal cell-free DNA percentage of maternal blood contacted by the protective agent of the present teachings may be substantially constant over time at ambient temperature. It is believed that this protective effect may be the result of the chemicals present in the protective agent of the present teachings that stabilize blood cells preventing cellular DNA release as well as nuclease inhibitory activity that protect fetal cell-free DNA from degradation. Thus, the teachings herein contemplate treating a sample in a matter such that limits the deleterious effects of DNase and RNase on the fetal nucleic acids present in the plasma.

[0068] As evidenced by the examples and testing results disclosed herein, fetal cell-free DNA found in maternal blood plasma is a valuable source for noninvasive prenatal diagnosis. However, a major factor that limits the effective use of fetal cell-free DNA in nucleic acid-based prenatal testing is that the total DNA concentration present in maternal plasma, comes largely from the mother herself. Thus, samples may be free of cell-free DNA attributable to apoptosis, cell death and lysis of nucleated maternal blood cells. This may be the case after about 4 hours from blood draw, 6 hours from blood draw, or even 24 hours from blood draw.

[0069] When the integrity of a DNA target is compromised, the targeted DNA sequence fails to be amplified. Since most of the DNA-based prenatal diagnostic tests depend on subsequent DNA amplification, it is important to protect the integrity of rare DNA targets such as fetal cell-free DNA during all pre-analytical procedures. Here, fetal cell-free DNA percentage is determined by real-time quantitative PCR. Figure 8 shows that fetal cell-free DNA percentage stays substantially constant for up to 14 days and provides evidence that the protective agent of the present teachings can protect the integrity of fetal cell-free DNA at ambient temperature for up to 14 days.

[0070] One of the factors that limit the use of fetal cell-free DNA in maternal plasma in noninvasive prenatal diagnosis is its low relative level in maternal plasma. Therefore, we amplify fetal cell-free DNA in maternal plasma by whole genome amplification (WGA). A first trimester pregnant donor with a male fetus is identified by amplifying Y chromosomal SRY region sequence. Amplification of cell-free plasma DNA obtained from this donor by WGA and subsequent detection of Y chromosomal SRY region sequence by real-time PCR shows a multi-fold (e.g., at least 10, 20, 40 or ~80-fold) increase in fetal cell-free DNA concentration (Figure 9). When cell-free DNA for WGA isolated from blood contacted by the protective agent of the present teachings and stored at ambient temperature for 14 days, results are expected to provide strong evidence that the protective agent of the present teachings is able to preserve the integrity of long fetal cell-free DNA molecules that are required for WGA.

[0071] This new methodology can be used to circumvent many existing pre-analytical issues that can affect the detection of fetal cell-free DNA in maternal blood. Since the protective agent of the present teachings stabilizes nucleated blood cells and inhibits plasma nucleases, it is possible to store maternal blood samples in a device containing the protective agent of the present teachings at ambient temperature for up to 14 days without any increase in background maternal cell-free DNA concentration and without any alteration in cell-free DNA integrity. Methods herein contemplate such storing. The methods herein also contemplate using the device of the present teachings to draw maternal blood for

noninvasive prenatal diagnosis when blood drawing and nucleic acid testing are not done at the same location. The methods herein thus may be free of any step of immediate separation of plasma after blood draw, freezing of the plasma (e.g., at -80^{0}C) or both, for shipping. The methods herein may also be free of any use of magnetic beads or particles of any kind. The methods herein may be free of any addition of formaldehyde to the blood sample immediately following the blood draw.

[0072] The examples and testing results discussed above demonstrate an unexpected synergistic effect occurring only in blood samples contacted by both a fixative and an anticoagulant, or more specifically, by IDU, EDTA and glycine. Maternal blood samples contacted by only a fixative or only an anticoagulant do not demonstrate the ability to maintain the integrity of the maternal blood cells or the integrity of the fetal nucleic acids. The combined effect of the IDU, EDTA and glycine far exceeds any expectations based on the effect, or lack thereof, of the IDU or EDTA or glycine used alone.

[0073] It will be appreciated that concentrates or dilutions of the amounts recited herein may be employed. In general, the relative proportions of the ingredients recited will remain the same. Thus, by way of example, if the teachings call for 30 parts by weight of a Component A, and 10 parts by weight of a Component B, the skilled artisan will recognize that such teachings also constitute a teaching of the use of Component A and Component B in a relative ratio of 3:1. Teachings of concentrations in the examples may be varied within about 25% (or higher) of the stated values and similar results are expected. Moreover, such compositions of the examples may be employed successfully in the present methods to isolate fetal nucleic acids (e.g., cell-free fetal DNA).

[0074] It will be appreciated that the above is by way of illustration only. Other ingredients may be employed in any of the compositions disclosed herein, as desired, to achieve the desired resulting characteristics. Examples of other ingredients that may be employed include antibiotics, anesthetics, antihistamines, preservatives, surfactants, antioxidants, unconjugated bile acids, mold inhibitors, nucleic acids, pH adjusters, osmolarity adjusters, or any

combination thereof.

[0075] The explanations and illustrations presented herein are intended to acquaint others skilled in the art with the invention, its principles, and its practical application. Those skilled in the art may adapt and apply the invention in its numerous forms, as may be best suited to the requirements of a particular use. Accordingly, the specific embodiments of the present invention as set forth are not intended as being exhaustive or limiting of the invention. The scope of the invention should, therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. Other combinations are also possible as will be gleaned from the following claims.

Claims

We claim:

1. A non-invasive prenatal screening method for the identification of fetal characteristics, comprising the steps of:

a. contacting a drawn maternal blood sample from a pregnant individual that includes a plurality of blood cells with a nucleic acid protective agent in an amount and time sufficient so that the blood cells are substantially prevented from (i) releasing genomic nucleic acids into the maternal blood sample and from (ii) experiencing nuclease activity that degrades fetal nucleic acid, wherein the nucleic acid protective agent consists essentially of imidazolidinyl urea, glycine and an anticoagulant;

b. isolating fetal nucleic acids from the maternal blood sample; and

c. analyzing the isolated fetal nucleic acids to identify a fetal characteristic.

2. The method of claim 1, wherein the analyzing step, the isolating step or both includes a step of contacting the fetal nucleic acid with an enzyme, an amplifier or both.

3. The method of claim 1 or claim 2, wherein the contacting step takes place in a blood collection tube into which the maternal blood sample is drawn.

4. The method of any one of claims 1 to 3, including:

isolating and quantifying fetal nucleic acids from the maternal blood sample upon blood draw;

storing the maternal blood sample in contact with the nucleic acid protective agent for at least seven days;

isolating and quantifying fetal nucleic acids from the maternal blood sample;

wherein after the at least 7 days from the time the maternal blood sample is drawn, the amount of fetal nucleic acid is at least 90% of the amount of fetal nucleic acid at the time the maternal blood sample is drawn.

5. The method of any one of claims 1 to 3, including:

isolating and quantifying fetal nucleic acids from the maternal blood sample upon blood draw;

storing the maternal blood sample in contact with the nucleic acid protective agent for at least seven days;

isolating and quantifying fetal nucleic acids from the maternal blood sample;

wherein after the at least 7 days from the time the maternal blood sample is drawn, the amount of fetal nucleic acid present in the sample is about 100% of the amount of fetal nucleic acid present in the sample at the time the maternal blood sample is drawn.

6. The method of any one of claims 1 to 3, including:

isolating and quantifying fetal nucleic acids from the maternal blood sample upon blood draw;

storing the maternal blood sample in contact with the nucleic acid protective agent for at least 14 days;

isolating and quantifying fetal nucleic acids from the maternal blood sample;

wherein after the at least 14 days from the time the maternal blood sample is drawn, the concentration of fetal nucleic acid relative to the total nucleic acid in the maternal blood sample that is present is at least 10 times the amount of fetal nucleic acid that would be present in the absence of the contacting step.

7. The method of any one of claims 1 to 3, including:

isolating and quantifying fetal nucleic acids from the maternal blood sample upon blood draw;

storing the maternal blood sample in contact with the nucleic acid protective agent for at least 14 days;

isolating and quantifying fetal nucleic acids from the maternal blood sample;

wherein after the at least 14 days from the time the maternal blood sample is drawn, the concentration of fetal nucleic acid relative to the total nucleic acid in the maternal blood sample that is present is at least 20 to 50 times the amount of fetal nucleic acid that would be present in the absence of the contacting step.

8. The method of any one of claims 1 to 7, wherein the anticoagulant is selected from the group consisting of heparin, ethylenediamine tetraacetic acid, citrate, and oxalate, and any combination thereof.

9. The method of any one of claims 1 to 8, wherein the anticoagulant is ethylenediamine tetraacetic acid.

10. The method of any one of claims 1 to 9, wherein a tube is preloaded with a protective agent consisting essentially of imidazolidinyl urea, ethylenediamine tetraacetic acid, and glycine and the maternal blood sample from a pregnant patient is drawn into the tube and fetal DNA located within the maternal blood sample is analyzed at least three hours post blood draw, wherein the amount of fetal DNA located within the maternal blood sample is at least 90% of the amount of fetal nucleic acid at the time the maternal blood sample is drawn.

11. The method of any one of claims 1 to 10, wherein the concentration of the imidazolidinyl urea prior to the contacting step is between about 0.1 g/ml and about 3 g/ml.

12. The method of any one of claims 1 to 11, wherein the concentration of the imidazolidinyl urea prior to the contacting step is between about 0.4 g/ml and about 0.8 g/ml.

13. The method of any one of claims 1 to 12, wherein the concentration of the protective agent prior to the contacting step is a concentration at which cross-linking of nucleic acids and proteins is observed, as indicated by agarose gel electrophoresis.

14. The method of any one of claims 1 to 13, wherein the amount of imidazolidinyl urea is less than about 20 mg/ml of the maternal blood sample.

15. The method of any one of claims 1 to 14, wherein either or both of the isolating or analyzing steps occurs without freezing the maternal blood sample.

16. The method of any one of claims 1 to 9 or any one of claims 11 to 15, wherein the nucleic acid protective agent is located into a tube that includes a polymeric coating.

17. The method of any one of claims 1 to 9 or any one of claims 11 to 15, wherein the nucleic acid protective agent is located into a tube that includes a silicon-containing polymeric coating.

18. The method of any one of claims 1 to 9 or any one of claims 11 to 15, wherein the nucleic acid protective agent is located into a polymeric tube.

19. The method of any one of claims 1 to 18, wherein the amount of imidazolidinyl urea relative to the amount of anticoagulant is 1 to 10 parts by weight of imidazolidinyl urea to 1 part by weight anticoagulant.

20. The method of any one of claims 1 to 19, wherein the amount of imidazolidinyl urea relative to the amount of anticoagulant is 2 to 8 parts by weight of imidazolidinyl urea to 1 part by weight anticoagulant.

21. The method of any one of claims 1 to 20, wherein the amount of imidazolidinyl urea is 300 to 700 g/L IDU and from 60 to 100 g/L anticoagulant, and 20 to 60 g/L glycine.

22. The method of any one of claims 1 to 21, wherein the protective agent includes 6 parts by weight imidazolidinyl urea per 1 part by weight anticoagulant, and 10 parts by weight imidazolidinyl urea per 1 part glycine.

23. The method of any one of claims 1 to 22, wherein the protective agent includes 80% by volume of imidazolidinyl urea, 12.8% by volume anticoagulant, and 7.25 by volume glycine.

24. The method of any one of claims 1 to 23, wherein the protective agent is substantially free of any formaldehyde or paraformaldehyde prior to contact with the maternal blood sample.

25. The method of any one of claims 1 to 24, wherein one or more nucleic acids in the maternal blood sample is amplified by a polymerase chain reaction protocol post blood draw.

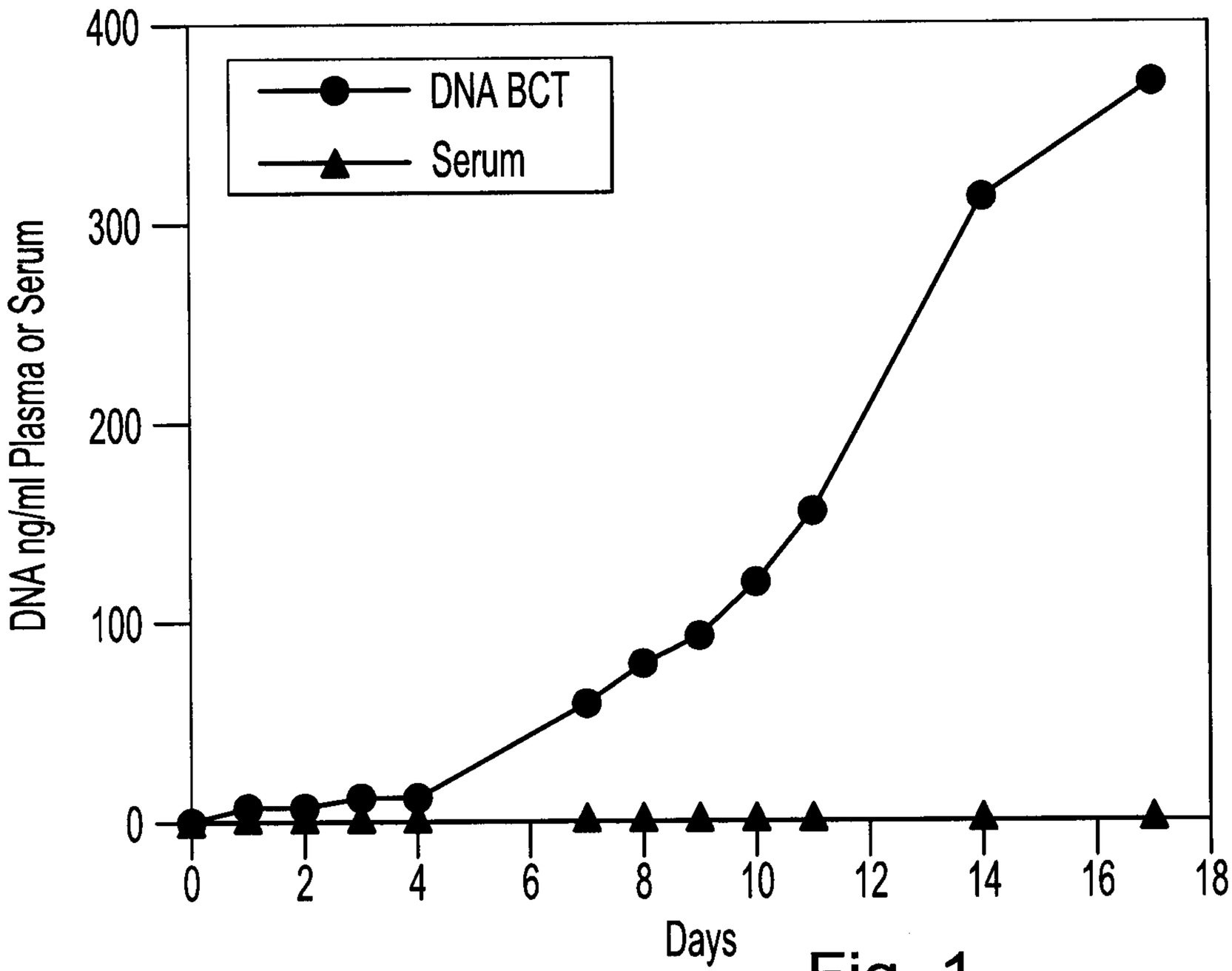

400
300
200
100
0
DNA ng/ml Plasma or Serum
DNA BCT
Serum
0
2
4
6
8
10
12
14
16
18
Days

Fig - 1

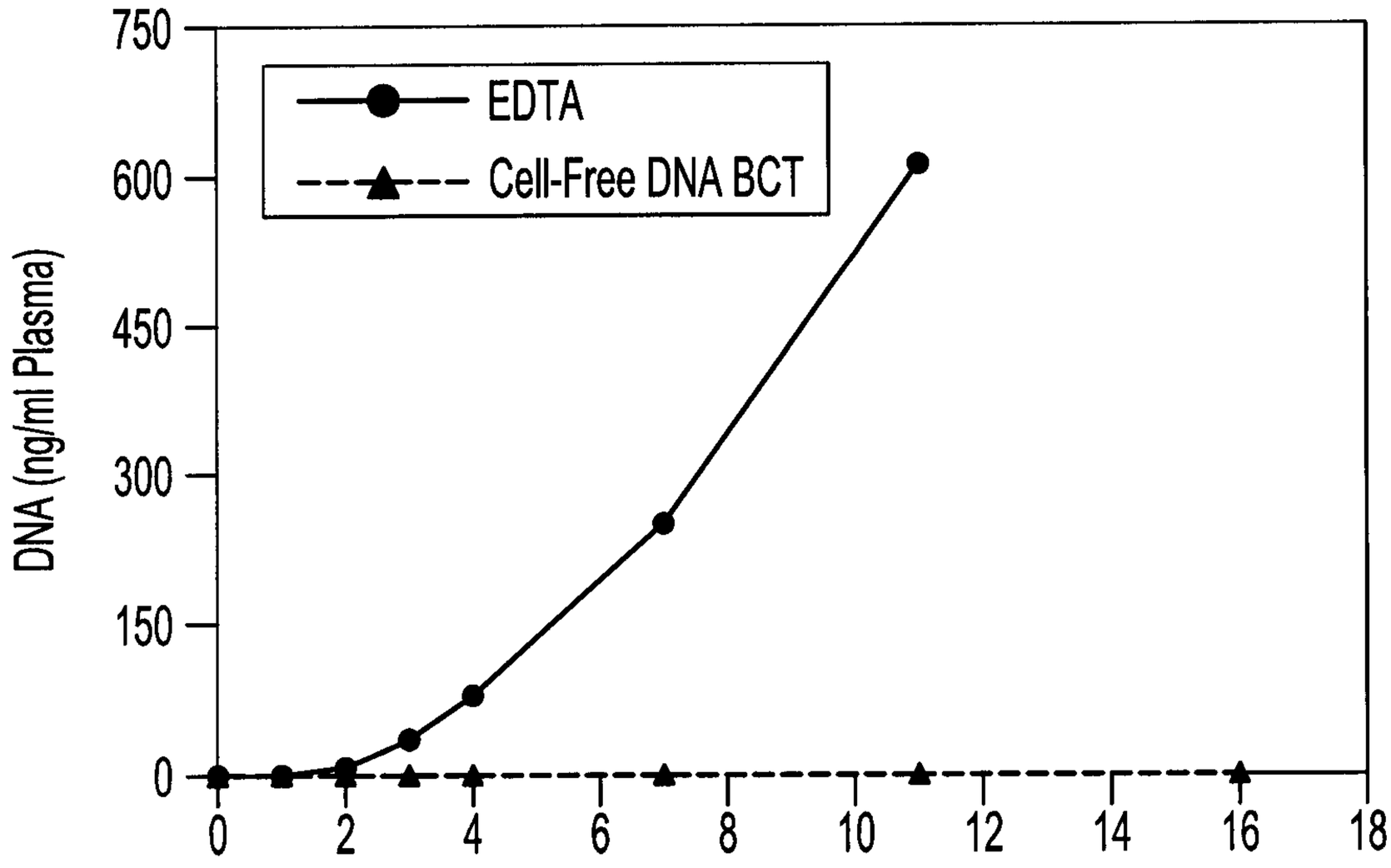

750
600
450
300
150
0
DNA (ng/ml Plasma)
EDTA
Cell-Free DNA BCT
0
2
4
6
8
10
12
14
16
18
Days

Fig - 2

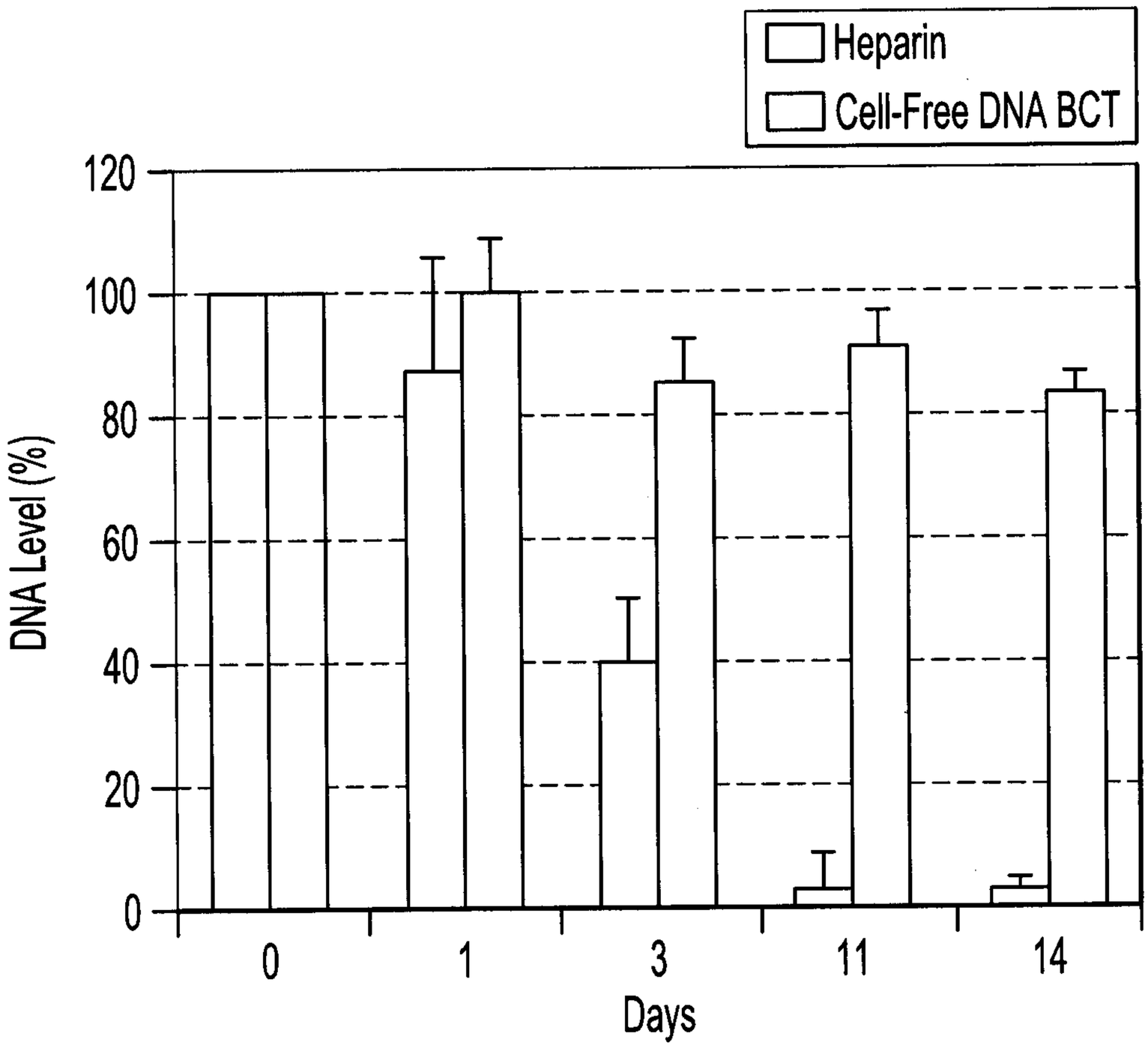

Heparin
Cell-Free DNA BCT
120
100
80
60
40
20
0
DNA Level (%)
0
1
3
11
14
Days

Fig - 3

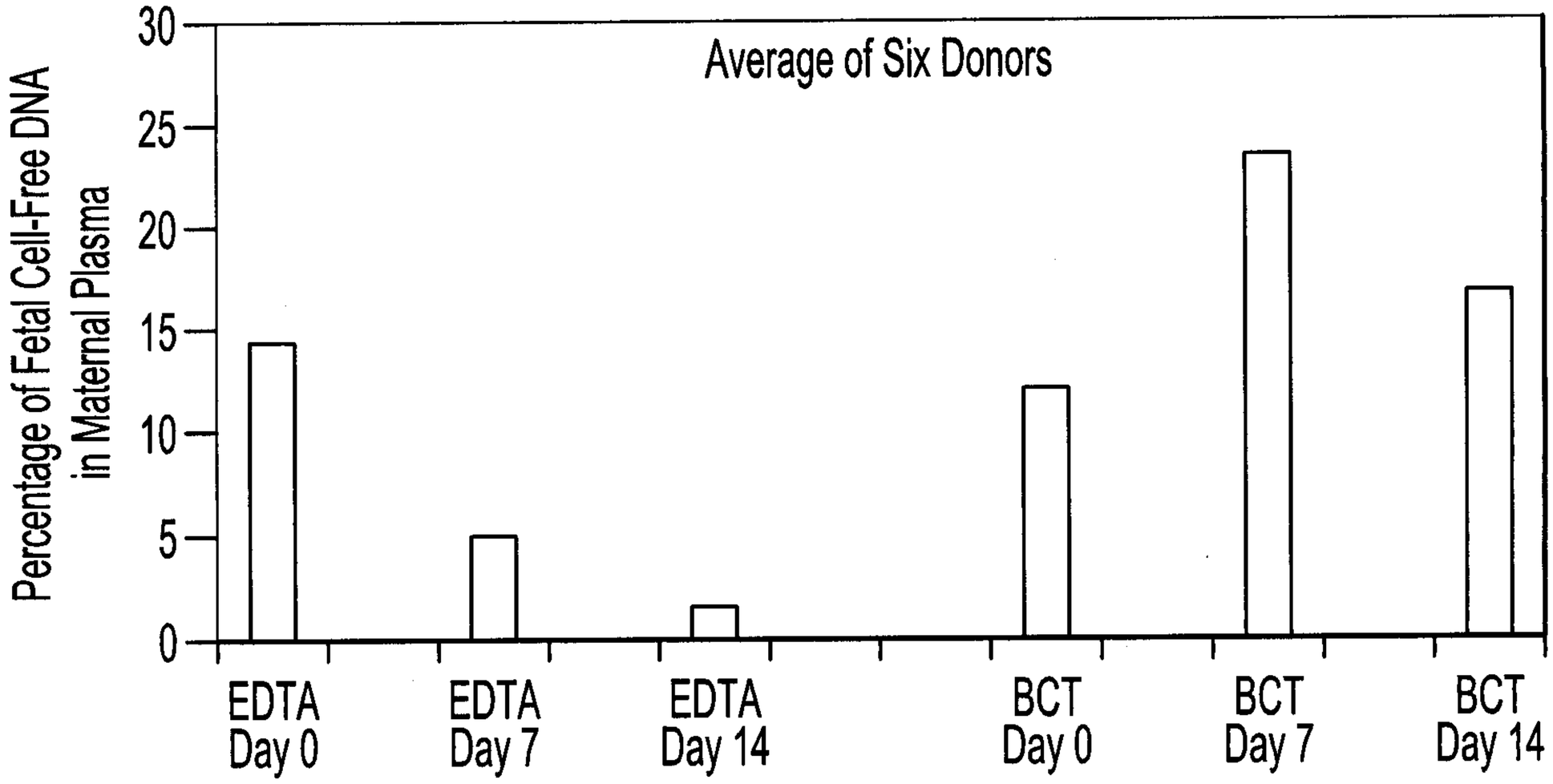

Average of Six Donors
30
25
20
15
10
5
0
Percentage of Fetal Cell-Free DNA in Maternal Plasma
EDTA Day 0
EDTA Day 7
EDTA Day 14
BCT Day 0
BCT Day 7
BCT Day 14

Fig - 4

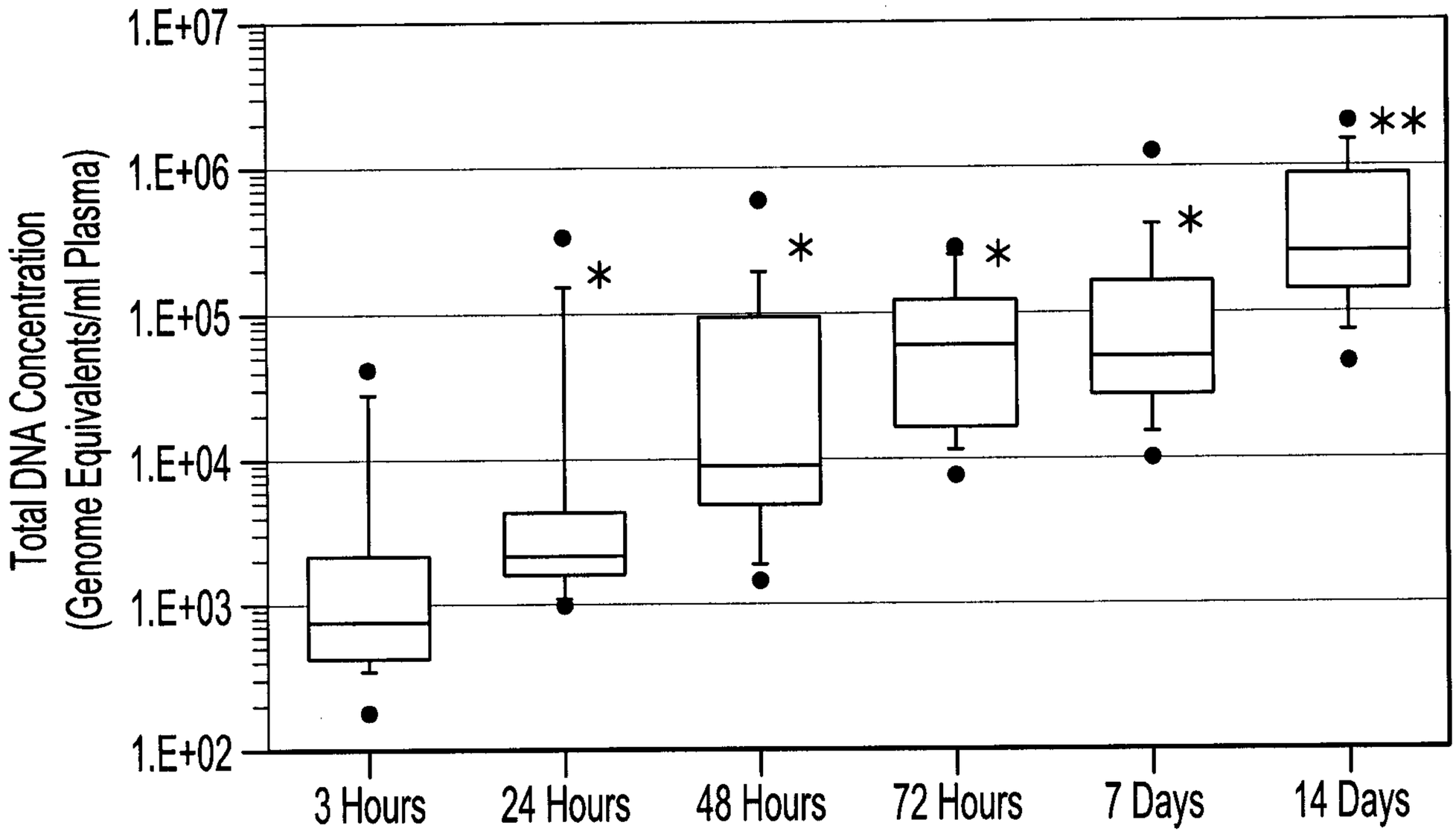

1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
Total DNA Concentration
(Genome Equivalents/ml Plasma)
3 Hours
24 Hours
48 Hours
72 Hours
7 Days
14 Days
Time from Collection

Fig-5

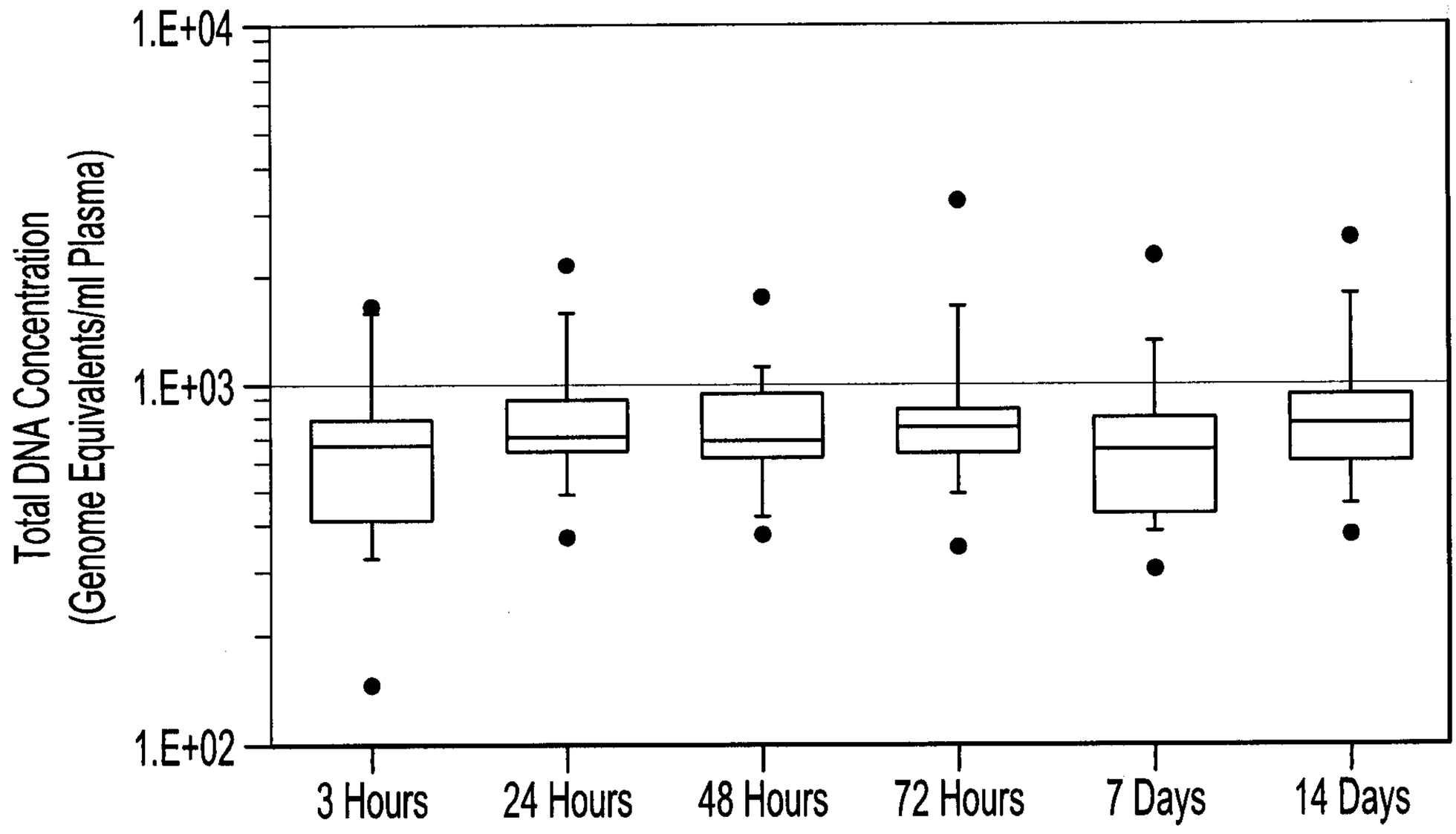

1.E+04
1.E+03
1.E+02
Total DNA Concentration
(Genome Equivalents/ml Plasma)
3 Hours
24 Hours
48 Hours
72 Hours
7 Days
14 Days
Time from Collection

Fig-6

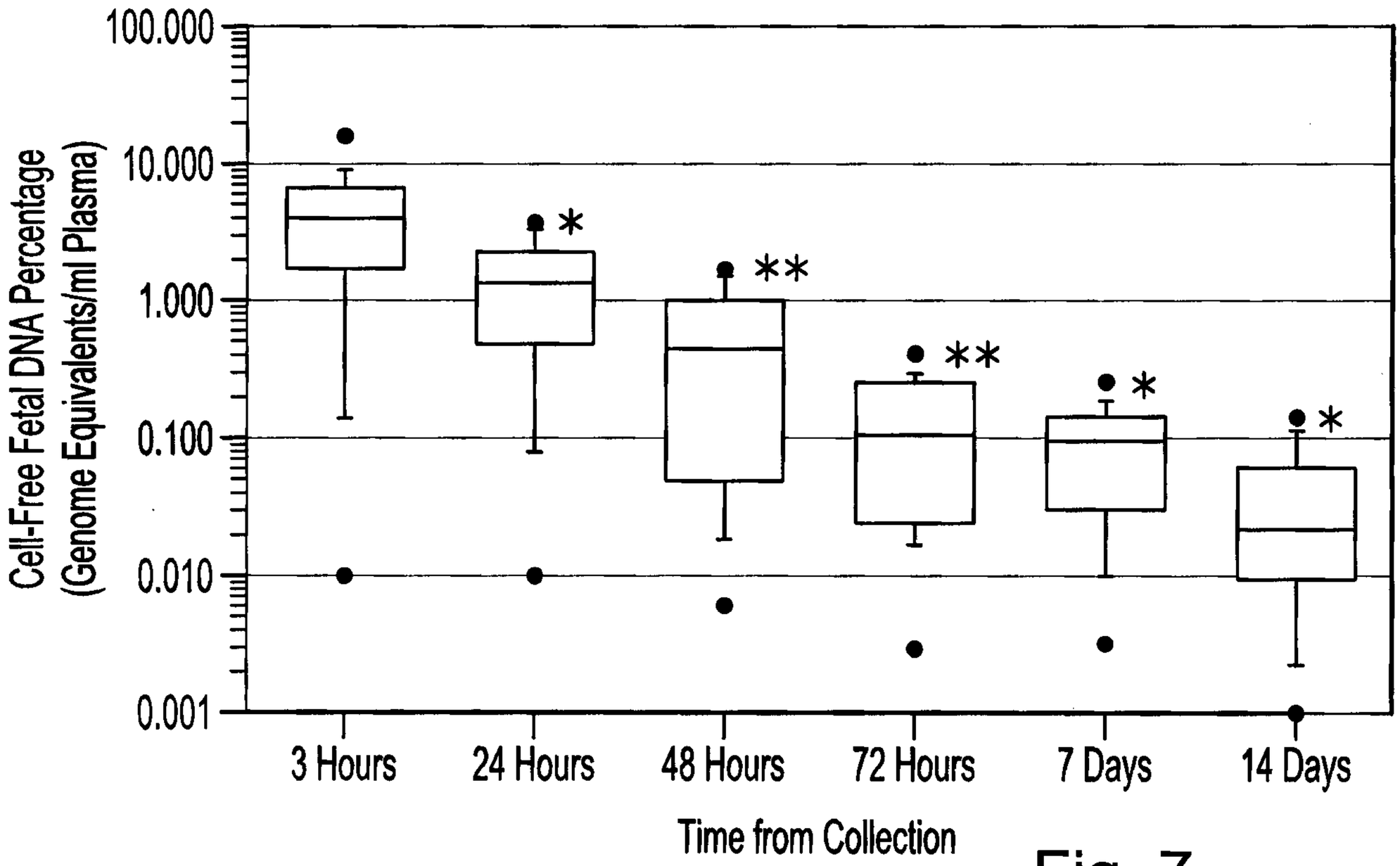

100.000
10.000
1.000
0.100
0.010
0.001
Cell-Free Fetal DNA Percentage
(Genome Equivalents/ml Plasma)
3 Hours
24 Hours
48 Hours
72 Hours
7 Days
14 Days
Time from Collection

Fig-7

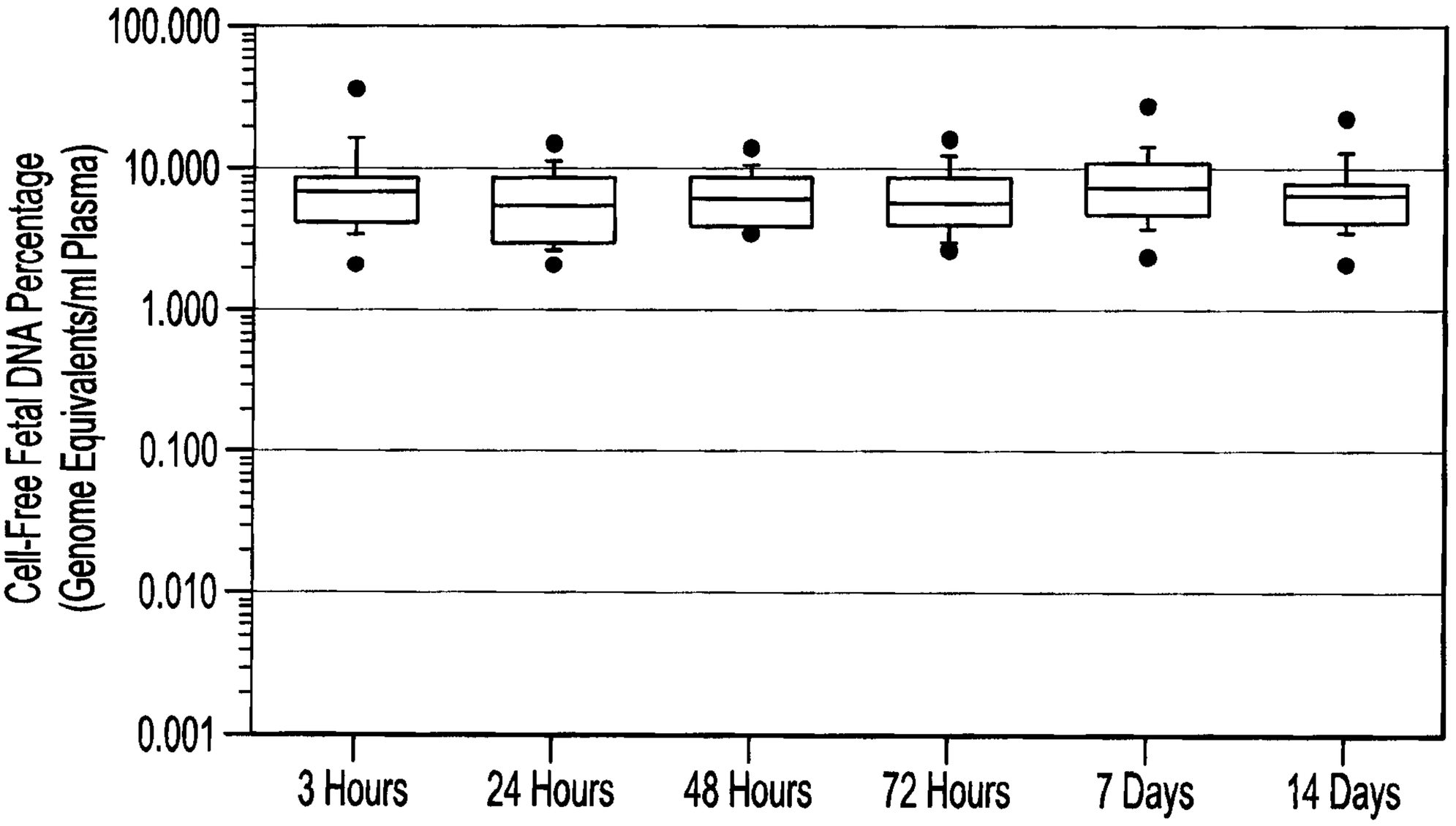

100.000
10.000
1.000
0.100
0.010
0.001
Cell-Free Fetal DNA Percentage
(Genome Equivalents/ml Plasma)
3 Hours
24 Hours
48 Hours
72 Hours
7 Days
14 Days
Time from Collection

Fig-8

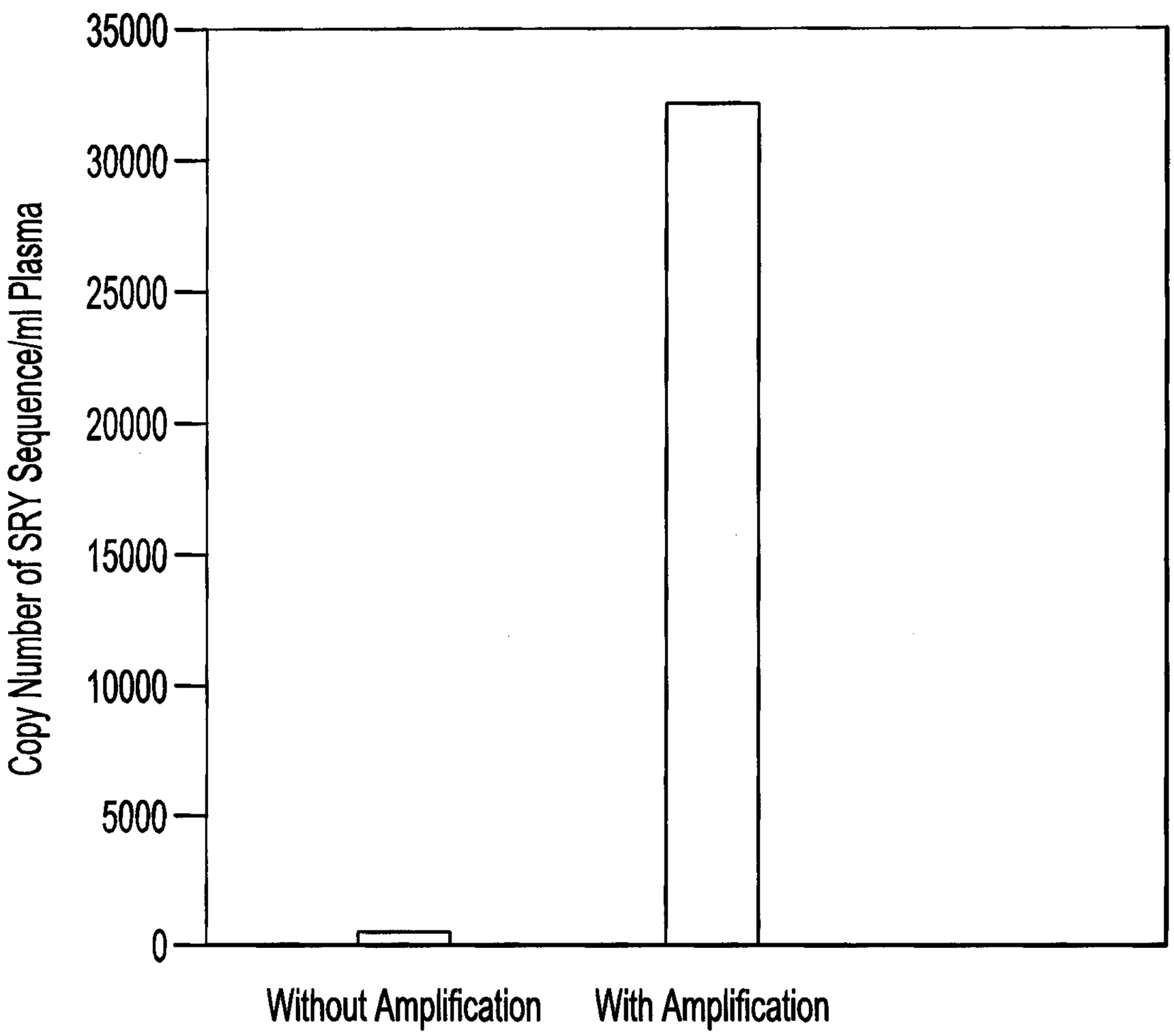
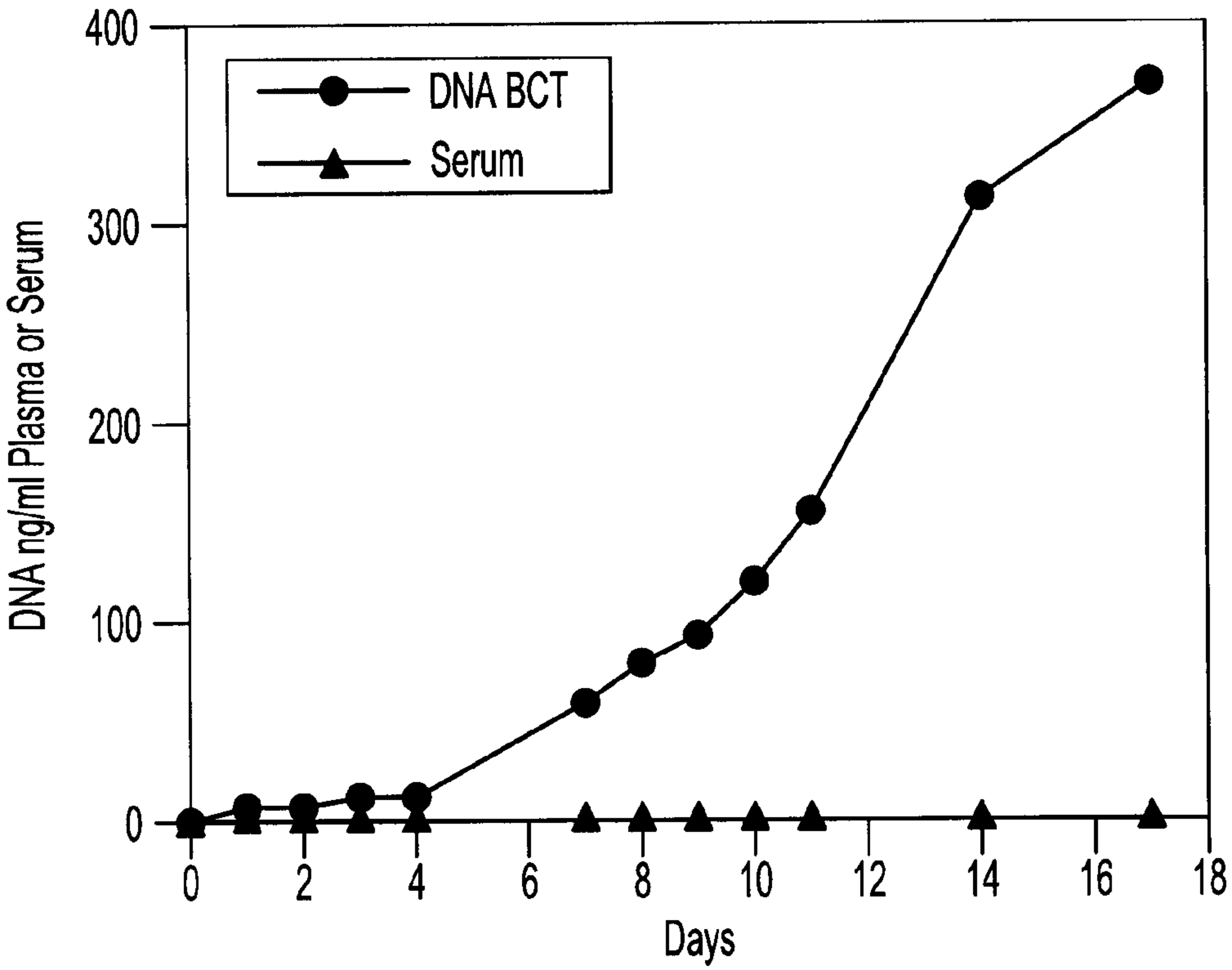

35000
30000
25000
20000
15000
10000
5000
0
Copy Number of SRY Sequence/ml Plasma
Without Amplification
With Amplification

Fig-9


DNA BCT
Serum
DNA ng/ml Plasma or Serum
400
300
200
100
0
0
2
4
6
8
10
12
14
16
18
Days