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(54) Title: MULTIPLE GENETIC MARKER SELECTION AND AMPLIFICATION

(57) Abstract: Methods of selection and amplification of genetic markers for genetic testing are provided, and in particular, to facilitate multiplex PCR amplification from limiting amounts of target nucleic acid (i.e. 1 ng, or less, nucleic acid or the amount of nucleic acid contained in 200 or fewer cells). The method of selecting a plurality of genetic markers as targets for nucleic acid amplification includes the step of selecting each of said plurality of genetic markers according to a heterozygosity index, wherein the heterozygosity index is 0.5 or greater. The method of nucleic acid sequence amplification includes a step of using a nucleic acid sequence amplification technique and at least nine primer pairs in combination to amplify a plurality of respective genetic markers from a limiting amount of nucleic acid samples. The methods of the invention are generally applicable to improved genetic diagnostic and screening methods such as prenatal diagnostic testing, fetal sex determination and genetic identification, such as by DNA fingerprinting, in organisms such as bacteria, humans and other animals where available target nucleic acid is limiting. The nucleic acid amplification method of the invention is also applicable to forensic analysis of degraded, old, ancient, difficult and or other low-abundance samples that have hitherto been difficult to amplify and identify.

TITLE

MULTIPLE GENETIC MARKER SELECTION AND AMPLIFICATION

FIELD OF THE INVENTION

THIS INVENTION relates to selection and amplification of genetic markers for genetic analysis. More particularly, this invention relates to selection of genetic markers and primers to facilitate multiplex PCR amplification from limiting amounts of nucleic acid. The methods of the invention are generally applicable to improved genetic testing methods including but limited to diagnostics and screening, for example prenatal diagnostic testing, fetal sex determination and genetic identification, such as by DNA fingerprinting, in organisms including but not limited to bacteria, plants, humans and other animals where available nucleic acid is limiting. The nucleic acid amplification method of the invention is also applicable to by genetic identification of degraded, old, ancient, difficult and or low-abundance samples that have hitherto been difficult to amplify, detect and/or identify.

BACKGROUND OF THE INVENTION

Conventional genetic analysis generally consists of a single test on large amounts of sample, for example diagnosis of ΔF508 (major cystic fibrosis mutation) from DNA extracted from a relatively large volume (5 mL) of blood.

Multiple diagnoses by amplification techniques such as multiplex polymerase chain reaction (PCR) have been obtained from large samples such as for forensic DNA fingerprinting, usually requiring a minimum of 200 cells. Or alternatively, single or few diagnoses have been obtained from small samples such as single cells, for example, in pre-implantation genetic diagnosis.

One system that has been used for multiple diagnoses, particularly in prenatal diagnosis, is quantitative PCR using polymorphic short tandem repeats (STRs; Mansfield, 1993, Hum Mol Genet. **2** 43-50).

Quantitative PCR accurately determines the amount of PCR product from each allele permitting the ratio of product quantity between alleles to be calculated thus determining aneuploidy status. Even though quantitative PCR was first described in the early 1990s, there are few reports in clinical prenatal diagnosis (Adinolfi *et al.*, 1995. Bioessays **17** 661-664.).

A modification of this quantitative PCR technique known as multiplex fluorescent PCR (MF-PCR) has demonstrated the feasibility of using quantitative PCR in clinical prenatal diagnosis even though several thousand cells are still required (Findlay *et al.*, 1994, Human Reproduction **9** 23; Findlay *et al.*, 1998a, Prenatal Diagnosis **18** 1413-1421; Findlay, 1998, Single cell PCR: Theory. Practice and Applications. Clinical Applications In PCR. Edited by Y-M.D. Lo, published by Humana Press; Findlay *et al.*, 1998b, Molecular Pathology **51** 164-168; Findlay *et al.*, 1998c, Journal Assisted Reproduction and Genetics **15** 265-274; Findlay *et al.*, 1998d, Clinical Genetics **53** 92-95; Toth *et al.*, 1998, American Journal of Obstetrics **178** 1101-1102; Tóth *et al.*, 1997, American Journal of Human Genetics **61** 807; Verma *et al.*, 1998, Lancet 4;352 (9121) 9-12; Pertl *et al.*, 1999a, J. Med. Genet **36** 300-3; Pertl *et al.*, 1999b, Molecular Human Reproduction **5** 1176-9; Schmidt *et al.*, 2000, Mol. Hum. Reprod **6** 855-860).

Four main types of STR signal are obtained depending on trisomy status, three of which are shown in Figure 1. Trisomic samples produce either a triallelic signal (three peaks of similar size) seen in Panel 1, or a diallelic or double-dose signal (two peaks, one of which is approximately twice the size of the other) seen in Panel 2. Disomic samples produce a heterozygous signal (double peaks of similar size) seen in Panel 3. Homozygous signal showing a single peak (not shown) are regarded as uninformative, as they can be obtained from both disomic and trisomic samples.

Diallelic signals are partially informative with triallelic signals being the most informative.

Multiplex fluorescent PCR provides two main advantages. Firstly, the multiplex system provides multiple diagnoses, using either linked markers to confirm results or to expand the scope of the test to multiple chromosomes or perform other diagnoses. Secondly, the use of fluorescent markers significantly increases the threshold of detection almost 1000 fold (Findlay *et al.*, 1995, Human Reproduction **10** 1005). Generally, however, it has not been possible to use these techniques to diagnose trisomies at or near the single cell level (Findlay, 1998, *supra*; Findlay *et al.*, 1998a & 1998c, *supra*; Findlay *et al.*, 1999, Journal of Assisted Reproduction and Genetics **16** 199-206).

Fluorescent, multiplex PCR has been shown a reliable and accurate method for determining sex (Salido *et al.*, 1992, Am. J Human genetics **50** 303; Findlay *et al.*, 1994a, Human Reproduction, **9** 23; Findlay et al., 1994b, Advances in Gene Technology: Molecular Biology and Human Genetic Disease. Vol 5, page 62. Findlay *et al.*, 1995, Human Reproduction **10** 1005-1013; Findlay *et al.*, 1998c, *supra* diagnosing genetic diseases such as cystic fibrosis (Findlay *et al.*, 1995, *supra*), detecting chromosomal aneuploidies and in genetic analyses for genetic identification, such as typically referred to as DNA fingerprinting (Findlay *et al.*, 1997, Nature **389** 355-356).

The reliability and accuracy rates of fluorescent PCR compare very well with other diagnostic techniques including fluorescent in *situ* hybridisation (FISH) and Primed In Situ Synthesis (PRINS; Findlay *et al.*, 1998, J. Assisted Reproduction & Genetics **15** 257).

OBJECT OF THE INVENTION

The present inventors have sought to improve the quality of information obtainable from amplification of multiple genetic markers in applications relating to genetic testing and identification, such as in prenatal diagnosis, genetic disease screening and testing and DNA fingerprinting, such as in forensics. A problem associated with improving the performance of diagnostic nucleic acid sequence amplification, particularly from limiting amounts of nucleic acid template, is that the more genetic markers are amplified in a multiplex reaction, the more compromised is the quality of the information obtained from the reaction. To solve this problem, the present inventors have improved the selection of genetic markers that can be amplified in combination, the number of genetic markers that can be amplified in combination and the efficiency of amplification from limiting amounts of nucleic acid template

It is therefore an object of the invention to provide improved genetic marker selection.

It also an object of the invention to provide improved multiplex nucleic acid sequence amplification, particularly from limiting amounts of nucleic acid template.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a method of selecting a plurality of genetic markers as targets for nucleic acid sequence amplification, said method including the step of selecting each of said plurality of genetic markers according to a heterozygosity index, wherein said heterozygosity index is 0.5 or greater.

Preferably, said heterozygosity index is 0.7 or greater.

More preferably, said heterozygosity index is 0.9 or greater.

In another aspect, the invention provides a method of producing one or more primers for amplification of each of a plurality of genetic markers selected according to the first aspect of the invention, said method including the step of selecting a nucleotide sequence for each of said one or more primers so that upon amplification of said genetic marker using said one or more primers, a resultant amplification product has a molecular size in the range 50-3000 bp.

Preferably, the molecular size is in the range 50-1000 bp.

More preferably the molecular size is in the range 80-500 bp.

Even more preferably the molecular size is in the range 100-400 bp.

Primers constructed according to this aspect may be degenerate or non-degenerate as is well understood in the art.

In yet another aspect, the invention provides a method of nucleic acid sequence amplification including the step of using a nucleic acid sequence amplification technique and at least nine primer pairs in combination to amplify a plurality of respective genetic markers from a limiting amount of nucleic acid sample.

In particular embodiments, at least ten, eleven, twelve, thirteen, fourteen, fifteen and sixteen primer pairs are used to amplify said respective genetic markers.

Preferably, each said primer pair amplifies a respective genetic marker.

Preferably, nucleic acid sequence amplification is performed using PCR.

Preferably, PCR is fluorescent multiplex PCR.

Throughout this specification, unless the context requires otherwise, the words "comprise", "comprises" and "comprising" will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

BRIEF DESCRIPTION OF THE FIGURES AND TABLES

Reference is now made to non-limiting embodiments of the present invention described by way of example with reference to the accompanying figures and tables.

TABLE 1: Examples of genetic markers and primers and, in selected examples, heterozygosity indices (labelled as "heter" for convenience). Expected amplification product sizes may be obtained by consulting databases such as Genbank using the accession number or marker designation listed. The molecular fragment sizes and corresponding primer sequences listed in Table 1 may be readily altered or manipulated by persons skilled in the art. Accordingly, Table 1 provides non-limiting examples of primers and resultant amplification fragment sizes applicable to each genetic marker. A reference to the DYS14 marker is Lo *et al.*, 1993, Hum. Genet. **90** 483. Primers are attributed SEQ ID NOS:1-92 in order of appearance in Table 1.

TABLE 2: Non-limiting examples of fluorescently-labeled primers and corresponding genetic markers applicable to multiple genetic diagnoses.

TABLE 3: Non-limiting examples of fluorescently-labeled primers and corresponding genetic markers applicable to DNA fingerprinting.

TABLE 4: Comparison of FISH, PRINS and fluorescent multiplex PCR techniques for preimplantation genetic diagnosis (PGD).

TABLE 5: Single cell DNA fingerprinting improvements. The new method is that described herein; the published method is that described in Findlay *et al.*, 1997, Nature **389** 355-356. [1]Full profiles provide highest possible specificity. However as more STR markers are added it becomes more likely that one or more will fail or be compromised. Although higher full-profile values are generally better, the inclusion of additional markers with higher specificity more than compensates; for example add four markers but lose one = net gain of three. The "acceptable profile" category is much more important and significant. [2]Surplus alleles. Additional alleles in conjunction with true alleles. Causes a marker to be defined as uninformative. Lower percentages are therefore better. [3]False alleles. Additional alleles in place of true allele. Extra-allelic peaks caused by contamination, somatic mutation or PCR generated non-allelic peak artefacts. False alleles may result in misdiagnosis and must therefore be minimized as much as possible. In this improved system, such false alleles occur rarely (<1%). More

than 2 additional peaks in a profile or from 18 negatives were not observed. When false alleles are observed the marker, but not the profile, is defined as uninformative. Lower values are therefore better. [4]Allele dropout results in reduction of specificity. Lower values are again better. However as more STR markers are added it becomes more likely that one or more individual alleles will fail. Again the inclusion of additional markers more than compensates. For example add four markers but lose one = net gain of three.

TABLE 6a and 6b: Example demonstrating twin heterozygosity. Table 6a shows allele sizes obtained for each marker. It can be seen that the genetic identification allele sizes for twin 1 are identical to that of twin 2 thus indicating that the twins are identical twins. Table 6b demonstrates maternal or paternal derivation of each allele thus indicating maternity and paternity.

TABLE 7: Example demonstrating twin heterozygosity. Table 7 shows allele sizes obtained for each marker. It can be seen that the genetic identification allele sizes for twin 1 are different to that of twin 2 thus indicating that the twins are not identical twins.

FIG. 1: Quantitative fluorescent PCR in prenatal diagnosis. Panel two shows 2:1 ratio or diallelic signal.

FIG. 2: Multiplex fluorescent PCR from a single cell sample with nine out of nine genetic markers present.

FIG. 3: Limited nucleic acid template sample subjected to eleven (11) primer set multiplex PCR. In this example 10 of 11 markers were amplified.

FIG. 4: Eleven (11) primer set multiplex PCR on single diploid cell. 11 of 11 markers were amplified

FIG. 5: Eleven (11) primer set multiplex PCR on single sperm (haploid cell). Note all markers are homozygous, which is indicative of haploid cells. 11 of 11 markers were amplified

FIG. 6: Sixteen (16) primer multiplex PCR on single diploid cell. 16 of 16 markers amplified

FIG. 7: Genetic identification of single fetal cell isolated from PAP smears using nine (9) primer pairs. 9 of 9 markers amplified. Maternal genetic identification is also

shown to demonstrate that both fetal signal and maternal signals share common alleles (indicating maternity), but the fetal cell has inherited other alleles from a paternal source, consistent with Mendelian inheritance.

FIG. 8: Genetic identification of single fetal cell isolated from PAP smear using eleven (11) primer pairs.

FIG. 9: Nine (9) primer pair multiplex PCR demonstrating twin heterozygosity using limited amount of amniotic fluid. Results indicate both twins identical.

FIG. 10: Nine (9) primer pair multiplex PCR demonstrating twin heterozygosity using limited amount of amniotic fluid. Results indicate both twins non-identical.

FIG. 11: Multiplex fluorescent PCR from a single cell sample with nine (9) out of nine (9) genetic markers present.

FIG. 12: DNA fingerprint obtained from hairshaft using eleven (11) primer fluorescent multiplex PCR.

FIG. 13: Ten (10) primer pair fluorescent multiplex PCR demonstrating genetic diagnosis of trisomy status from limited amount of amniotic fluid.

FIG. 14: Ten (10) primer pair multiplex demonstrating simultaneous diagnosis of single-gene defect (cystic fibrosis), sex, trisomy status and genetic identification.

DETAILED DESCRIPTION OF THE INVENTION

The invention described herein relates to nucleic acid sequence amplification of multiple genetic markers, and methods of selecting genetic markers to improve the efficiency of marker amplification.

With regard to genetic marker selection, the present invention is based on the realization that there are two main considerations when selecting a genetic marker for nucleic acid sequence amplification.

- *Heterozygosity*. Heterozygosity is defined as the presence of different alleles of a gene at one or more loci. Heterozygosity occurs when a diploid organism or cell has inherited different alleles at a particular locus from each parent. Heterozygosity index is a measure of the likelihood of marker alleles being different within individuals i.e. having two alleles rather than one. For example alleles from markers with low heterozygosity are more likely to be identical or be homozygous within

an individual or population. Markers with higher heterozygosities are more likely to provide triallelic (most informative) results (see Figure 1), if the sample is trisomic. It is therefore necessary to choose markers with as high heterozygosity (dp) values as possible.

- *Fragment size*. The optimal fragment size window is between 100-400bp although 80bp to 500bp, 50bp to 1000bp or even 50 bp to 3000 bp can be used. Fluorescent systems for fragment detection have increasingly limited detection when fragment size is less than 80bp due to interference from primer dimer. Fragment sizes that are large, (e.g. greater than 500bp), even though they may not accurately be sized can still be used to identify multiple peaks and triallelic results. In general the larger the fragment size the more time it takes for results to be obtained. As most diagnostic laboratories require results as quickly as possible, smaller fragments would therefore be most preferred.

Additional considerations include:-

- *Appropriate chromosome*. It is necessary to choose a marker that will accurately reflect the test being performed. For example, if one is attempting to determine the number of copies of chromosome 21, a marker on chromosome 21 is most likely to be the most appropriate.
- *Fluorescent labeling*. Using fluorescent labeled primers to combine markers in a multiplex with markers of similar or overlapping fragment ranges. The choice of fluorescent label is very important since marker allele sets can overlap with each other. Overlap with another marker would make the marker of limited value since each marker may then be indistinguishable from the other. For example if one marker was heterozygous and the other homozygous this would show as a triallelic response incorrectly indicating a trisomy. When marker size sets do overlap, the marker could be labelled with a differently coloured fluorochrome thus allowing identification of each marker.

The present invention therefore provides a substantial improvement in the efficiency of genetic marker selection such as for the purposes of selecting STRs that

allow PCR amplification of multiple genetic markers for applications including genetic identification (for example, human embryo identification and forensics), genetic diagnosis and screening (for example, pre-implantation genetic diagnosis after IVF and from fetal cells obtained from cervical smears, CVS or amniocentesis), although without limitation thereto.

Furthermore, the present invention provides multiplex PCR amplification using at least nine primer pairs to amplify discrete genetic markers from limiting amounts of nucleic acid sample in a highly efficient manner. For example, nine (9) informative genetic markers were successfully amplified in 69 of 69 multiplex amplifications using nine (9) primer pairs and nucleic acid samples from single buccal cells. The present invention also contemplates amplification of up to and in excess of sixteen (16) genetic markers as will be described in more detail hereinafter.

It will be apparent to the skilled person that the present invention is broadly applicable to "*genetic analysis*". As used herein, genetic analysis and genetic diagnosis are used interchangeably and broadly cover detection, analysis, identification and/or characterization of genetic material and includes and encompasses terms such as, but not limited to, genetic identification, genetic diagnosis, genetic screening, genotyping and DNA fingerprinting which are variously used throughout this specification.

For the purposes of this invention, by "*isolated*" is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state. Isolated material may be in native or recombinant form.

The term "*nucleic acid*" as used herein designates single-or double-stranded mRNA, RNA, cRNA, RNAi and DNA, said DNA inclusive of cDNA and genomic DNA.

A "*polynucleotide*" is a nucleic acid having eighty (80) or more contiguous nucleotides, while an "*oligonucleotide*" has less than eighty (80) contiguous nucleotides.

By "*genetic marker*" or "*marker*" is meant any locus or region of a genome. The genetic marker may be a coding or non-coding region of a genome. For example, genetic markers may be coding regions of genes, non-coding regions of genes such as introns or promoters, or intervening sequences between genes such as those that include tandem repeat sequences, for example satellites, microsatellites, short tandem repeats (STRs) and minisatellites, although without limitation thereto.

A "*probe*" may be a single or double-stranded oligonucleotide or polynucleotide, suitably labeled for the purpose of detecting complementary sequences in Northern or Southern blotting, for example.

Nucleic acid amplification techniques are well known to the skilled addressee, and include polymerase chain reaction (PCR) and ligase chain reaction (LCR) as for example described in Chapter 15 of Ausubel *et al.* CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (John Wiley & Sons NY, 1995-1999); strand displacement amplification (SDA) as for example described in U.S. Patent No 5,422,252; rolling circle replication (RCR) as for example described in Liu *et al.*, 1996, J. Am. Chem. Soc. **118** 1587 and International application WO 92/01813 and by Lizardi *et al.*, in International Application WO 97/19193; nucleic acid sequence-based amplification (NASBA) as for example described by Sooknanan *et al.*,1994, Biotechniques **17** 1077; and Q-β replicase amplification as for example described by Tyagi *et al.*, 1996, Proc. Natl. Acad. Sci. USA **93** 5395.

The abovementioned are examples of nucleic acid sequence amplification techniques but are not presented as an exhaustive list of techniques. Persons skilled in the art will be well aware of a variety of other applicable techniques as well as variations and modifications to the techniques described herein.

As used herein, "*multiplex amplification*" or "*multiplex PCR*" refers to amplification of a plurality of genetic markers in a single amplification reaction.

In particular embodiments, the invention provides "*fluorescent PCR*". This system uses fluorescent primers and an automated analyser such as a DNA sequencer to detect PCR product (Tracy & Mulcahy, 1991, Biotechniques **11** 68-75). Fluorescent PCR has improved both the accuracy and sensitivity of PCR for genotyping (Ziegle *et*

al., 1992, Genomics, **14** 1026-1031; Kimpton *et al.,* 1993, PCR Methods and Applications **3** 13-22).

In particular embodiments fluorescent amplification products are electrophoresed using gel or capillary systems and pass a scanning laser beam, which induces the tagged amplification product to fluoresce. The DNA sequencer combined with appropriate software is generally known as a "Genescanner". Stored data can then be analysed to provide product sizes and the relative amount of amplification product in each sample.

A preferred nucleic acid sequence amplification technique is PCR.

As used herein, an "*amplification product*" refers to a nucleic acid product generated by a nucleic acid amplification technique.

A "*primer*" is usually a single-stranded oligonucleotide, preferably having 12-50 contiguous nucleotides which, for example, is capable of annealing to a complementary nucleic acid "template" and being extended in a template-dependent fashion by the action of a DNA polymerase such as *Taq* polymerase, RNA-dependent DNA polymerase or Sequenase™.

Non-limiting examples of primers that may be used in combination are primers capable of amplifying genetic markers (STR loci) index listed in Table 1 .

For fluorescent PCR, each said primer may be fluorescently-labeled to produce a fluorescently-labeled primer pair. Fluorescent labels are well known in the art and include but are not limited to TET, FAM, HEX as for example described in Table 2. Other fluorescent labels potentially useful according to the invention include but are not limited to CyDyes™ such as Cy2, Cy3, C3.5, and Cy5.

Non-limiting examples of fluorescent-labeled primers are set forth in Table 2.

Primer synthesis and incorporation of fluorescent labels are well known in the art and labeled, synthetic primers are readily available from commercial sources. However, an example of primer synthesis methodology is provided in Chapter 2..11 of Current Protocols in Molecular Biology Ausubel *et al.* Eds (John Wiley & Sons, NY, 1996-2001).

It will be appreciated by the skilled that the present invention is particularly suited to selection of genetic markers and corresponding primers for the purposes of nucleic acid sequence amplification from limiting amounts of nucleic acid.

As used herein, a "*limiting amount of nucleic acid*" is an amount of nucleic acid used in a nucleic acid sequence amplification reaction less than 1 ng, preferably less than 500 pg, more preferably less than 200 pg, even more preferably less than 50 pg and in particular embodiments, about 3-6 pg.

It should also be appreciated that a limiting amount of nucleic acid may also relate to the number of cells containing the nucleic acid sample used for amplification.

Preferably, the limiting amount of target nucleic acid is obtained from less than 200 cells, more preferably from less than 100 cells, more preferably from less than 50 cells and even more preferably from less than 20 cells.

In particularly preferred embodiments, the limiting amount of nucleic acid is isolated from no more than ten cells, or from a single cell.

For example, the present inventors have performed multiplex PCR amplification from a single, haploid sperm cell which comprises about 3-6 pg of DNA.

Although the invention also contemplates use of nucleic acid other than DNA, preferably the nucleic acid is DNA.

More preferably, the nucleic acid is genomic DNA.

Suitable sources of cells from which DNA may be obtained include, but are not limited to, buccal cells, sperm cells, hair follicle cells, skin cells, epithelial cells, nucleated cells circulating in blood, embryonic cells, fetal cells such as obtained from fetal blood, CVS or amniocentesis samples or cervical (PAP) smears, corneal cells, cell or tissue biopsies, or any other cells or tissues from which genetic material can be obtained.

In one embodiment, the invention is applicable to genetic analysis from small numbers of cells such as for the purposes of prenatal diagnostic testing or screening, fetal sex determination and genetic identification by DNA fingerprinting.

Cellular sources such as described above may be of any organism inclusive of plants, bacteria and animals.

Preferred sources of nucleic acids are mammals, preferably humans.

The invention also contemplates genetic analysis of non-human samples such as from cows, sheep, horses, pigs and the like, although without limitation thereto.

Also contemplated are other vertebrate sources of nucleic acid such as birds. Non-limiting examples are domestic birds such as chickens, ducks and geese.

The skilled person will also appreciate that nucleic acids may be obtained from non-cellular sources such as viruses.

It will also be understood that nucleic acids are not necessarily directly obtained from their cellular or non-cellular source organism. As will be apparent from this specification, nucleic acids may be obtained from objects such as paper, documents, clothing, bedding, motor vehicles, physical fingerprints, weapons, furniture and building fixtures and a variety of substrates such as biological fluids and ink although without limitation thereto, such as for the purposes of genetic identification.

For the purposes of obtaining nucleic acid samples for amplification, cells or nucleic acid material may or may not require isolation by techniques as are well known in the art. These include but are not limited to densitometric separation such as by gradient centrifugation through media such as Metrizamide™, Ficoll™ and Percoll™, biochips, micro-manipulation, pulse field separation, differential lysis and antibody-mediated isolation such as by panning, magnetic bead separation or flow-cytometric (FACS) sorting.

An example of densitometric cell separation is provided in Albright *et al.*, 1986, Cytometry 7 536, where the use of centrifugal separation of cells in sputum specimens is described.

Isolation of antibody-labeled cells by magnetic bead separation is well known in the art for which kits are commercially available (for example the Dynal MPC cell separation kit).

With regard to FACS sorting, generally applicable flow cytometry methods are described in Practical Flow Cytometry (Second Edition) by Howard M. Shapiro and in Chapter 5 of Current Protocols in Immunology, Coligan *et al.* Eds (John Wiley & Sons NY, 1995-2001

Prenatal diagnosis using nucleic acids isolated from fetal cells

In a particular embodiment, the invention provides a method of prenatal genetic diagnosis of a fetus wherein a nucleic acid sample for amplification is obtained from one or more fetal cells isolated from a pregnant individual (*i.e* the mother). As used herein, the word "fetal" includes embryonic cells at any developmental stage from any species.

Fetal cells may be isolated by any cell isolation method.

Said one or more fetal cells may be isolated from any pregnant mammal.

Preferably, said one or more fetal cells are isolated from a pregnant human.

When a fetus is at increased risk for genetic defects such as chromosomal anomalies, typically prenatal diagnosis is by invasive procedures such as either chorionic villus sampling (CVS) in the late 1st trimester or amniocentesis in the 2nd trimester of pregnancy. By the third trimester, a combination of CVS and amniocentesis, or even fetal blood sampling, may be necessary.

A rapid, less-invasive and low cost method of prenatal diagnosis involves genetic diagnosis from fetal cells shed into the cervical sump at 6-20 weeks of gestation. These samples are obtained from the cervix by cytobrush in a manner similar to a PAP smear which is similar to but less invasive than invasive transcervical sampling. Although promising, there are four major difficulties. Firstly the need to obtain the large numbers of fetal cells normally required for genetic analysis. Secondly the isolation of fetal cells from the cervical sample is extremely difficult as recent results suggest that fetal cells could be isolated and diagnosed in only ~22% of cases due to the presence of "contaminating" maternal cells. Previous approaches have generally concentrated on isolating fetal cells by morphology or cell sorting. Unfortunately, morphology grading is extremely time-consuming, expensive and generally unreliable and inaccurate. Alternative cell sorting techniques involve antibody-labelled slides to capture fetal cells, which is generally unspecific and can result in major maternal contamination and misdiagnosis or insufficient fetal cells. Thirdly the difficulty of obtaining genetic diagnosis from small cell numbers. Although fetal cells have been identified in cervical samples (mainly by identifying male cells within the sample) aneuploidy screening (the primary reason for prenatal diagnosis)

cannot usually be performed nor diagnosis made if the fetus is female. Finally, although sampling is theoretically much safer than CVS and amniocentesis as PAP smears have been taken during pregnancy for many years, relative safety remains to be fully evaluated.

According to this embodiment of the present invention, it is preferred that said fetal cells are present in a maternal cavity, such as the uterus, or endocervical canal sample, particularly a transcervical sample. Methods of isolating fetal cells include but are not limited to cervical cotton swab, cytobrush, aspiration of cervical mucus, lavage of the endocervical canal and uterine lavage. Samples can be obtained from transcervical aspiration of mucus from just above the internal os or the lower uterine cavity. Another isolation method which may be used is lavage which is generally conducted with a saline wash, but other isotonic solutions are suitable. Typically, endocervical lavage with 5-10ml or intrauterine lavage with 10-20 ml saline provides sufficient fetal cells upon separation from maternal cells. The sample may be collected using a combination of methods.

Preferably, cell samples are isolated from a female human in the first trimester of pregnancy or when the fetus is between 6 to 20 weeks gestation.

Preferably, to aid fetal cell separation, clumps of cells are preferably treated to obtain a suspension of single cells. The clumps may be separated by techniques known to a skilled person, such as enzymatic, chemical or mechanical separation. For example, enzymatic separation may utilise protease or trypsin. Chemical separation may utilise acetyl cysteine and mechanical separation may involve gentle teasing, aspiration or micromanipulation.

The number of fetal cells in the sample varies depending on factors including the age of the fetus, method of sampling, number and frequency of samplings, the volume of washing in each lavage where lavage is used and the volume aspirated.

Maternal uterine cavity or endocervical canal samples typically contain at least two main types of nucleated fetal cells: cytotrophoblasts and syncytiotrophoblasts cells.

Fetal cells can be isolated either by selecting fetal cells from maternal cells (positive selection) or isolating the maternal cells from the fetal cells (negative

selection) or most preferably a combination of both. Preferably, the nucleated fetal cells are retained in the purified sample.

When 100% purity is desired, one method to isolate fetal cells is micromanipulation. In another method, for example, cell suspensions containing an individual cell per a preselected volume of suspension medium can be prepared by limiting dilution. Drops containing individual cells can placed in suitable container (e.g. 96 well plates) and examined visually with a fluorescent microscope to identify single-labelled (or unlabelled) cells.

For PCR analysis, analysis can be performed using a single, identified fetal cell. Alternatively, ways can be envisaged of identifying monozygosity (indicative of the presence of a monogenic disease) in a mixed cell population containing minimal fetal material including as few as one fetal cell in 100 cells. Following sorting, the separated cells can be washed twice in a physiologic buffer and resuspended in an appropriate medium for any subsequent analysis to be performed on the cells.

Following recovery or isolation methods, the fetal cells can be used in the same manner as fetal cells obtained by other methods such as amniocentesis and chorionic villus biopsy. The cells can be used as a source of DNA for analysis of the fetal alleles, as by polymerase chain amplification for example. PCR analysis methods may be used to detect, for example, fetal sex, beta thalassemia, phenylketonuria (PKU), and Duchennes muscular dystrophy without limitation thereto.

Alternatively, the cells can be cultured in a similar manner as material biopsied for karyotyping analyses. However, the incubation period may be significantly shortened if a DNA content of greater than or equal to 2C is used as a selection criterion.

In another embodiment, the present invention provides a method of prenatal analysis using nucleic acids isolated from fetal cells isolated by but not limited to invasive procedures such as from fetal blood, amniocentesis or CVS.

Although widespread, conventional cytogenetic diagnoses from amniotic fluid have significant disadvantages including high cost and significant delay (~2 weeks) which results in a lengthy wait for reassurance for a healthy pregnancy or the prospect of a late termination for the parents.

Alternative sampling methods such as CVS or diagnostic techniques such as FISH (Fluorescent in situ hybridisation) can provide more rapid results. However CVS has higher miscarriage risk; requires higher technical skill and requires physical referral to specialised tertiary centres with additional inconvenience, stress, and costs to families and Health Care. FISH has interpretation difficulties and higher failure rate when samples are small; high cost; limited throughput and is labour intensive (~10 samples per day) and has limited analysis potential (~5 of the common chromosomes defects).

An alternative diagnostic method is quantitative PCR using polymorphic short tandem repeats (STRs) to accurately determine PCR product ratio from each allele and thus aneuploidy status. However the high numbers of cells required and profile interpretation difficulties result in few clinical applications.

Multiplex fluorescent PCR is a viable alternative for clinical prenatal diagnosis. MF-PCR techniques are becoming adopted overseas in both public and private laboratories.

However these techniques have had several limitations:

1. Limited marker sets with limited diagnostic capability which, for example, generally only determine aneuploidy and/or sex.
2. Large amount of samples required as DNA extraction is required.
3. Samples are not genetically identified as being fetal thus having. potential for misdiagnosis from contamination.
4. Additional cost and time for DNA extraction.
5. Low range of diagnoses available.

The present invention in one embodiment provides improved multiplex nucleic acid sequence amplification on limited samples to overcome these difficulties. This invention in one embodiment will significantly improve diagnostic confidence, capability as well as reduce cost and time.

Preferably, cell samples are isolated from a female human in the first trimester of pregnancy or when the fetus is between 6 to 20 weeks gestation and may consist of amniotic fluid or samples from the chorionic villi.

The number of fetal cells in the sample varies depending on factors including the age of the fetus, method of sampling, skill of operator, number and frequency of samplings, the amount of sample obtained in each procedure and the volume aspirated.

Amplification methods such as PCR analysis may be used to detect, for example, fetal sex, beta thalassemia, phenylketonuria (PKU), and Duchennes muscular dystrophy without limitation thereto.

Forensic samples

In another embodiment, the present invention relates to genetic analysis or genetic identification by "DNA profiling" or commonly known as DNA fingerprinting of samples. In this regard, cellular and/or non-cellular nucleic acid samples can be obtained from a variety of sources including but not limited to forensic samples (such as clothing, bedding, motor vehicles, physical fingerprints, weapons, furniture and building fixtures and a variety of substrates such as biological fluids and ink), documents or other substrates such as ink or paper and ink derived therefrom, archaeological or other old or ancient samples, samples obtained for the purposes of personal identification, biological samples or clinical samples such as used for genetic identification, testing, screening and/or diagnosis of genetic diseases, sexing and detection of chromosomal abnormalities.

DNA profiling is an extremely powerful method for forensic identification with current prior art achieving power of discrimination in excess of 1 in 10 billion.

However, many forensic PCR methods require many hundreds of cells to maintain the necessary high rates of reliability and accuracy. Forensics identification has been attempted on smaller samples with limited success. For example, STR profiling systems have been applied to low cell samples such as cigarette butts (Torre and Gino, 1996, J Forensic Sci. **41** 658-9) and from cells left on pens, car keys, etc (van Oorschot and Jones, 1997, Nature **387** 767). However, these systems either still require similarly large amounts of DNA for high reliability, or as cell numbers decrease have fewer STR markers therefore markedly decreased discriminating power and reliability.

A particular problem applies in rape cases, particularly multiple rape where semen from multiple sources are present. Conventional forensic analysis requires a clean uncontaminated sample to obtain a DNA fingerprint but, as the semen may be a

mixed sample (for example, from each assailant, or assailant and male partner), definitive DNA fingerprints are usually not possible. This leads to a failed forensic test, with the result that there may be insufficient evidence for the prosecution or defence.

The present invention provides an improved method whereby genetic identification by DNA fingerprinting can now be obtained from small samples and or single cells such as single sperm to determine their origin and thus identify each assailant.

In another embodiment, single cells may be obtained from samples which have too few cells for conventional profiling; from samples contaminated by blood or other cell types; from archived cases; old previously solved or unsolved cases; and from physical fingerprints. The single cell DNA fingerprint test described here could be applied for genetic identification to a wide variety of samples and sample types including but not limited to smudged physical fingerprints, single flakes of dandruff, as well as small samples left on weapons, vehicles and other objects.

Preimplantation Genetic Diagnosis

IVF success rates have remained relatively constant at only ~10-20% per embryo transferred. This may be because a sizeable number of human embryos are chromosomally or otherwise abnormal and therefore unable to implant, or form or maintain a pregnancy.

It has been possible since 1990 to diagnose genetic defects from single embryonic cells removed from embryos (preimplantation genetic diagnosis (PGD) also alternatively known as preimplantation diagnosis (PID)). There are three main applications for PGD: sex, single gene defects and aneuploidy e.g. trisomy diagnosis. In general FISH (fluorescent in *situ* hybridisation) is used for sex or aneuploidies and PCR for single gene defects.

Single cell fluorescent PCR has previously shown to be highly reliable (97%), highly accurate (97%), rapid (6hrs) and wide ranging (simultaneous diagnoses of sex, single gene defects and trisomies) (Findlay *et al.*, 1995, Human Reproduction **10** 1609-1618). However such testing has been limited to a limited number (upto and including 8) of markers, which limits use.

Single cell fluorescent PCR can also determine a DNA fingerprint from a single cell therefore minimising the risk of misdiagnosis due to contamination (Findlay *et al.*, 1995, Human Reproduction **10** 1005-1013; Findlay, 1996, Human Reproduction Update **2** 137-152; Findlay *et al.*, 1997, Nature **389** 355-356; Henderson *et al.*, 2001, Cornea **20** 400-403). Again such testing has been limited to a limited number (up to and including 8) of markers, which severely limits use in genetic identification particularly since a major source of contamination is parental DNA which share common alleles with the embryonic cell thus significantly decreasing specificity of discrimination of the DNA fingerprint. Fluorescent PCR can be favourably compared to other techniques as shown in Table 4.

DNA fingerprinting of embryonic cells allows individual embryos to can be genetically "tracked" from the 6-8-cell stage to birth and beyond. This makes it possible to determine which pregnancy resulted from which embryo. The present invention allows DNA fingerprinting to be performed on single cells with a specificity greater than 10 billion to 1, far in excess of any other single cell genotyping system and far in excess of prior art (Findlay *et al.*, 1997, Nature **389** 355-356) single cell DNA fingerprinting at ~100 million to 1. In the case of IVF, this embodiment of the present invention provides but is not limited to: a clinical tool that provides a quality control mechanism; patient reassurance that correct embryos are identified for transfer; determination of separate pregnancy rates in multiple embryo transfer e.g. when different treatments such as ICSI and non-ICSI embryos are transferred together; minimization of contamination rate in pre-implantation genetic diagnosis using PCR; as a research tool for example in comparing embryonic phenotype with genotype; and can be used to determine heterozygosity of twins in multiple births.

Furthermore, the method of the invention may be used to provide accurate and absolute correlation of embryo quality with pregnancy and might be used to accurately compare differing culture conditions. For example embryos cultured in two different media can be transferred to the same woman and an accurate pregnancy rate per media derived.

Patient reassurance is also improved by the PCR method of the invention by confirming that embryos transferred are genetically derived from parents.

It should also be appreciated by the skilled that the invention may be used for genetic analysis such as PGD or prenatal diagnosis or screening from non-human sources. Such non-limiting examples include PGD or genetic screening of an increased number of a wide variety of genetic traits to improve qualities from domestic animals such as cattle.

It should also be appreciated by the skilled that the invention may be used for a wide variety or purposes and applications where samples are limited in amount or availability and/or where maximal information from genetic testing is required or useful.

So that the invention may be more readily understood and put into practical effect, the skilled person is referred to the following non-limiting examples.

EXAMPLES

Heterozygosity Index

The eukaryotic genome is densely populated with islands of short sequences that are repeated over and over in small to large arrays called minisatellites and microsatellites. Another term commonly used to describe these sequences is variable number tandem repeats or VNTRs.

For a given repetitive locus, the number of repeats is highly variable among individuals and heterozygosity is high (i.e. the number of repeats at the locus is usually different on the two pairs of chromosomes of one individual). Analysing the number of repeats at one or more such loci provides a highly sensitive measure of individual identity and is the preferred technique for forensic DNA typing as means of genetic identification.

Tandem repetitive sequences are classified into three major groups:

1. Satellites are very highly repetitive with repeat lengths of one to several thousand base pairs. These sequences typically are organized as large (up to 100 million bp) clusters in the heterochromatic regions of chromosomes, near centrosomes and telomeres; these are also found abundantly on the Y chromosome.
2. Minisatellites are moderately repetitive, tandemly repeated arrays of moderately-sized (9 to 100 bp, but usually about 15 bp) repeats, generally

involving mean array lengths of 0.5 to 30 kb. They are found in euchromatic regions of the genome of vertebrates, fungi and plants and are highly variable in array size.

3. Microsatellites are moderately repetitive, and composed of arrays of short (2-6 bp) repeats found in vertebrate, insect and plant genomes. The human genome contains at least 30,000 microsatellite loci located in euchromatin. Copy numbers are characteristically variable within a population, typically with mean array sizes on the order of 10 to 100.

In general, satellite DNAs show exceptional variability among individuals, particularly with regard to the number of repeats at a given locus. Microsatellite loci are highly polymorphic sequences elements in the human genome, and delineating the repeat lengths of these loci is the basis of most DNA typing systems used in forensic medicine.

Heterozygosity is defined as the presence of different alleles of a gene at one or more loci. Heterozygosity occurs when a diploid organism or cell has inherited different alleles at a particular locus from each parent. Both cases result in mixtures of DNA sequences that have important applications in fields such as forensics, pathology, genetic diagnosis, and evolutionary genetics.

Genetic marker and primer selection

Polymorphism in a population is due to the existence of different genetic variants. The basis of variation is thus the number of polymorphic loci together with the number of alleles and their frequency distributions in a population. Based on this concept, markers in Table 1 were checked for the number of different alleles and genetic diversity, both by determining allele frequencies and from data provided publicly through public databases such as GenBank. Markers with higher heterozygosity rates (highly variable) are selected in preference.

PCR protocol for sexing, chromosome 21, 18 and 13 detection

A limited number of cells were isolated from amniotic fluid. A mastermix containing the reagents required for the PCR is made up under aseptic conditions. The mastermix contains enough reagents for a number of 25µl reactions. The primers

together with an indication of fluorescent labels for each primer are shown in Table 2, and the composition of the mastermix, per reaction, is as follows:-

Reagents	Amount/µl
• 10x PCR buffer	2.5
• $MgCl_2$	1.5 (1.5mM concentration)
• dNTP's	4.0 (1.25mM concentration)
• Taq	0.24 (5U per µl)
• Distilled water to make up to 24µl	

The mastermix is mixed thoroughly and added to template, or if using a plate, the mastermix is aliquoted and template added to it. The tubes/plate was placed on a thermal cycler and subjected to the following PCR program:-

1. 95°C for 15 minutes
2. 94°C for 30 seconds
3. 59°C for 45 seconds
4. 72°C for 60 seconds
5. Goto step 2, 39 times
6. 72°C for 10 minutes
7. 4°C hold
8. End

The PCR uses no oil overlay, as the heated lid of the PCR is sufficient. The PCR is taken off the block and stored at 4°C until required for electrophoresis.

DNA fingerprinting of single/small numbers of cells

Cell lysis protocol

1. After cell isolation, single or small numbers of cells are stored at –80°C until needed.
2. Lysis is carried out by adding 1µl of Lysis Buffer (200mM KOH, 50mM DTT) to the cell or cells.
3. The mixture containing the cell or cells is spun down with the buffer and heated to 65°C for 10 minutes.

4. 1µl of Neutralising Buffer (300mM KCl, 900mM Tris-HCl pH 8.3, 200mM HCl) is added to the cell or cells.
5. The cell mixture is spun down and is ready for PCR or stored at -80°C until needed.

PCR protocol

A mastermix containing the reagents required for the PCR is made up under aseptic conditions. The mastermix contains enough reagents for a number of 25µl reactions. The primers together with an indication of fluorescent labels for each primer are shown in Table 3 and the composition of the mastermix, per reaction, is as follows:-

Reagents	Amount/µl
• 10x PCR buffer	2.5
• $MgCl_2$	1.5 (1.5mM concentration)
• dNTP's	4.0 (1.25mM concentration)
• Taq	0.24 (5U per µl)
• Distilled water to make up to 24µl	

The mastermix is mixed thoroughly and added to 1µl of template, or if using a plate, the mastermix is aliquoted and the template is added to it.

The tubes/plate is placed on a thermal cycler and subjected to the following program:-

1. 95°C for 14 minutes
2. 94°C for 60 seconds
3. 57°C for 60 seconds
4. 72°C for 60 seconds
5. Goto step 2, 44 times
6. 72°C for 10 minutes
7. 4°C for ever
8. End

The PCR uses no oil overlay, as the heated lid of the PCR is sufficient. The PCR is taken off the block and stored at 4°C until required for electrophoresis.

Multiplex PCR from a single cell sample with nine out of nine genetic markers present

Single cells were isolated by micro-manipulation from buccal cell samples Genetic markers are AMEL (1), D13S631 (2), D13S258 (3), D18S851 (4), D18S391 (5), DYS14 (6), D21S11 (7), D21S1411 (8) & D21S1412 (9) as shown in Figure 2.

Primer concentrations were: -

AMEL	3.5 pmole
D13S631	9 pmole
D13S258	6 pmole
D18S851	5 pmole
D18S391	10 pmole
DYS14	4 pmole
D21S11	7 pmole
D21S1411	10 pmole
D21S1412	13 pmole

PCR cycling programme for the PCR in Figure 2 was:

a. 95°C for 15 minutes
b. 94°C for 30 seconds
c. 59°C for 45 seconds
d. 72°C for 60 seconds
e. Go to 2, 39 times
f. 72°C for 10 minutes
g. Hold at 4°C.

The following PCR conditions are used for all single-cell and low copy analysis unless stated differently.

Single cell PCR was conducted in 0.2 ml tubes on a PTC 200 DNA engine (MJ research, Geneworks). Master-mix consisted of 2 units taq polymerase (Amplitaq Gold (Applied Biosystems)); 1x PCR buffer and 1.5mM Magnesium Chloride (provided with the Amplitaq Gold); dNTP's (0.2mM concentration) and fluorescent and non-fluorescent primers at varying concentrations. The master-mix was made up to 24µl using Milli-Q sterilised water. Single cells were isolated using a drawn glass pipette whilst spread in a 30mm plastic Petri dish in Phosphate buffered saline (PBS) (without Magnesium) (Gibco Brl), ~ 1µl of PBS drawn with the single cell. DNA analysis was

performed using DNA sequencers such as ABI 377 or Megabace 1000 using standard protocols.

Low copy number sample subjected to 10 primer set multiplex PCR

Figure 3 shows a low copy number sample subjected to 10 primer set multiplex. Single cells were isolated by micro-manipulation from buccal cell samples. The PCR amplified genetic markers are AMEL (1), D13S631 (2), D18S851 (3), DYS14 (4), D18S391 (5), D13S317 (6), D21S11 (7), D13S258 (8), D18S51 (9), D21S1412 (10). In this example 10 of 10 markers amplified successfully.

PCR cycling programme for PCR for Figure 3:-

a. 95°C for 15 minutes
b. 94°C for 30 seconds
c. 59°C for 45 seconds
d. 72°C for 60 seconds
e. Go to 2, 39 times
f. 72°C for 10 minutes
g. Hold at 4°C.

Primer concentrations were: -

AMEL	1.4 pmole
D13S631	6 pmole
D18S851	5 pmole
D13S258	6 pmole
D18S391	11 pmole
DYS14	4 pmole
D21S11	8 pmole
D18S51	3 pmole
D21S1412	12 pmole
D13S317	8 pmole

11 primer set multiplex PCR on single diploid cell

Figure 4 shows an electrophorogram of eleven genetic markers AMEL, THO, D21S11, D18S51, VWA, FGA, D3S1358, D5S818, D7S820, CSF and TPOX amplified

from DNA template obtained from a single cell isolated from a buccal cell sample. In this example 11 of 11 markers amplified successfully.

PCR cycling parameters were:-

1) 95°C for 10 minutes
2) 94°C for 60 seconds
3) 57°C for 60 seconds
4) 72°C for 60 seconds
5) Go to 2, 44 times
6) 72°C for 10 minutes
7) Hold at 4°C

Single cell samples are added to 1ul of lysis buffer (200mM KOH/50mM DTT), heated to 65°C for 10 minutes. 1ul of neutralising buffer (300mM KCl/900mM Tris-HCl, ph8.3/200mM HCl) was then added.

PCR was conducted in 0.2 ml tubes on a PTC 200 DNA engine (MJ research, Geneworks). Master-mix for the PCR was made using 1.2 units Hot Start taq (Qiagen) in all single cell and amniotic samples unless otherwise stated. 1x PCR buffer and 1.5mM Magnesium Chloride was added. dNTP's were added to reach 0.2mM concentration. Primers were added as described for each individual case. The master-mix was made up to 24µl using Milli-Q sterilised water. Single cells were picked using a drawn glass pipette whilst spread in a 30mm plastic Petri dish in Phosphate buffered saline (PBS) (without Magnesium) (Gibco Brl), ~ 1µl of PBS drawn with the single cell.

PCR primer concentrations:-

Marker	pmol/rxn
AMEL	3.5
THO	6.0
D21S11	16.0
D18S51	16.0
VWA	30.0
FGA	6.0
D3S1358	10.0

D5S818	8.0
D7S820	10.0
CSF	8.0
TPOX	3.0

12 primer set multiplex PCR on single sperm (haploid cell)

The electropherogram shown in Figure 5 shows the results of multiplex PCR amplification from DNA template obtained from a single sperm cell. In this example 12 of 12 markers amplified successfully.

Primer concentrations were:-

Marker	pmol/rxn
AMEL(1)	3.5
THO(2)	6.0
D21S11 (3)	16.0
D18S51(4)	6.5
VWA(5)	3.6
FGA(6)	8.0
D3S1358 (7)	6.0
D5S818 (8)	10.0
D7S820 (9)	10.0
CSF (10)	6.0
TPOX (11)	6.0
D13S317 (12)	6.0

The single cells were subjected to lysis prior to PCR. Each single cell had 5µl of 0.624 mg/ml Proteinase K. The single cells were then subjected to the following heating program

1) 50°C for 30 minutes
2) 95°C for 15 minutes

The cells were then ready for master-mix to be added and subsequent following PCR program.

1) 95°C for 1 minutes
2) 94°C for 40 seconds

3) 57°C for 60 seconds
4) 72°C for 40 seconds
5) Go to 2, 44 times
6) Hold at 4°C

Single cell PCR was conducted in 0.2 ml tubes on a PTC 200 DNA engine (MJ research, Geneworks). Master-mix for the PCR was made using 1.8U of Hot Star Taq (Quigen) per reaction in all single cell. 1x PCR buffer (which contains 1.5mM Magnesium Chloride) were added (provided with the Hot start taq). dNTP's were added to reach 0.2mM concentration. Primers were added as described below. The master-mix was made up to 17.5µl using Milli-Q sterilised water. Single cells were picked using a drawn glass pipette whilst spread in a 30mm plastic Petri dish in Phosphate buffered saline (PBS) (without Magnesium) (Gibco Brl), ~ 1µl of PBS drawn with the single cell.

16 primer multiplex PCR on single diploid cell (16 simultaneous marker sets)

Figure 6 shows an electropherogram that demonstrates successful amplification of sixteen (16) genetic markers. In this example 16 of 16 markers amplified successfully. Single cells obtained from buccal cell samples were subjected to lysis prior to PCR. Each single cell had 5µl of 0.624 mg/ml Proteinase K. The single cells were then subjected to the following heating program:

a) 50°C for 30 minutes
b) 95°C for 15 minutes

The cells were then ready for master-mix to be added and subsequent following PCR program.

a) 95°C for 1 minutes
b) 94°C for 40 seconds
c) 57°C for 60 seconds
d) 72°C for 40 seconds
e) Go to (b), 44 times
f) Hold at 4°C

Single cell PCR was conducted in 0.2 ml tubes on a PTC 200 DNA engine (MJ research, Geneworks). Master-mix for the PCR was made using 1.8U of Hot Star Taq (Quigen) per reaction in all single cell. 1x PCR buffer (which contains 1.5mM Magnesium Chloride) were added (provided with the Hot star taq). dNTP's were added to reach 0.2mM concentration. Primers were added as described below. The master-mix was made up to 17.5µl using Milli-Q sterilised water. Single cells were picked using a drawn glass pipette whilst spread in a 30mm plastic Petri dish in Phosphate buffered saline (PBS) (without Magnesium) (Gibco Brl), ~ 1µl of PBS drawn with the single cell.

Primer concentrations in pmoles are as follows: -

AMEL	4
THO1	12
D21S11	8
D18S51	4
FGA	4
D3S1358	4
D5S818	8
D7S820	8
CSF1PO	8
D13S631	6
D13S317	6
D18S851	5
DYS14	4
D13S258	6
D21S1412	10
TPOX	2

Genetic identification of an isolated fetal cell from PAP smear using nine primer pair multiplex PCR

The electropherogram in Figure 7 shows the result of amplification of nine (9) genetic markers by multiplex PCR amplification from a single fetal cell isolated from a PAP smear.In this example 9 of 9 markers amplified successfully.

PCR conditions were:-

a) 94°C for 2 minutes

b) 94°C for 30 seconds

c) 57°C for 60 seconds

d) 68°C for 30 seconds

e) Go to (b), 45 times

f) 72°C for 10 minutes

g) Hold at 4°C

Isolated single cell samples were fixed then lysed by alkaline lysis using standard techniques before PCR processing.

PCR was conducted in 0.2 ml tubes on a PTC 200 DNA engine (MJ research, Geneworks). Master-mix for the PCR was made using AccuPrime Taq (Invitrogen); 1x PCR buffer and 1.5mM Magnesium Chloride; dNTP's were added to reach 0.2mM concentration. Primers were added as described. The master-mix was made up to 24µl using Milli-Q sterilised water.

The concentration of each set of primer was:-

Marker	pmol/rxn
AMEL	4
THO	12.0
D21S11	8
D18S51	4
FGA	4.0
D3S1358	4
D5S818	8
CSF	8
D7	8

Genetic identification of an isolated fetal cell from PAP smear using eleven primer pair multiplex PCR

The results of multiplex amplification of eleven, respective genetic markers from a single fetal cell using eleven primer pairs are shown in Figure 8. In this example 11 of 11 markers amplified successfully.

The primer concentrations were:-

Marker	pmoles
AMEL	3.5
THO	6.0
D21S11	16.0
D18S51	16.0
VWA	30.0
FGA	6.0
D3S1358	10.0
D5S818	8.0
D7S820	10.0
CSF1PO	8.0
TPOX	3.0

Each single cell was treated with 5 μL of 0.624 mg/ml Proteinase K.

The single cells were then subjected to the following heating program:

a) 50°C for 30 minutes

b) 95°C for 15 minutes

The cells were then ready for master-mix to be added and subsequent thermal cycling conditions as follows:-.

a) 95°C for 1 minutes

b) 94°C for 40 seconds

c) 57°C for 60 seconds

d) 72°C for 40 seconds

e) Go to (b), 44 times

f) Hold at 4°C.

Single cell PCR was conducted in 0.2 ml tubes on a PTC 200 DNA engine (MJresearch, Geneworks). Master-mix for the PCR was made using 1.2 U of Hot Star Taq (Quiagen) per reaction in all single cell. 1x PCR buffer (which contains 1.5mM Magnesium Chloride) were added (provided with the Hot star taq). dNTP's were added to reach 0.2mM concentration. Primers were added as described above. The master-mix was made up to 17.5ul using Milli-Q sterilised water. Single cells were picked using a

drawn glass pipette whilst spread in a 30mm plastic Petri dish in Phosphate buffered saline (PBS) (without Magnesium) (Gibco Brl) Approximately 1 µL of PBS was drawn with the single cellll.

Heterozygosity of twins (DNA fingerprinting of twins in utero at 12 weeks gestation) using 9 primer multiplex PCR

The results of multiplex PCR analysis of identical (monozygotic) twins are summarized in Table 6 and Figure 9; the results of multiplex PCR analysis of non-identical (dizygotic) twins are summarized in Table 7 and Figure 10.In this example 9 of 9 markers amplified successfully.

PCR cycling parameters were:-

a) 95°C for 10 minutes

b) 94°C for 60 seconds

c) 57°C for 60 seconds

d) 72°C for 60 seconds

e) Go to (b), 44 times

f) 72°C for 10 minutes

g) Hold at 4°C

Samples from a limited amount of amniotic fluid were added to 1ul of lysis buffer, heated to 65°C for 10 minutes. 1ul of neutralising buffer was then added.

PCR was conducted in 0.2 ml tubes on a PTC 200 DNA engine (MJ research, Geneworks). Master-mix for the PCR was made using 1.2 units Hot Start taq (Qiagen). 1x PCR buffer and 1.5mM Magnesium Chloride were added. dNTP's were added to reach 0.2mM concentration. Primers were added as described below. The master-mix was made up to 24µl using Milli-Q sterilised water.

The primer concentrations were:-

Marker	pmol/rxn
AMEL	3.5
THO	6.0
D21S11	16.0
D18S51	13.0
FGA	6.0

D3S1358	1.6
D5S818	1.5
CSF	12.0
TPOX	0.4

Genetic identification of single amniotic cell samples

The electropherogram in Figure 11 shows a 9 primer set multiplex from a single amniotic cell that has a 1 in 9 billion chance of two individuals having the same genetic fingerprint.

PCR cycling programme

a) 95°C for 10 minutes
b) 94°C for 60 seconds
c) 57°C for 60 seconds
d) 72°C for 60 seconds
e) Go to (b), 44 times
f) 72°C for 10 minutes
g) Hold at 4°C

Samples were added to 1ul of lysis buffer, heated to 65°C for 10 minutes. 1ul of neutralising buffer was then added.

PCR was conducted in 0.2 ml tubes on a PTC 200 DNA engine (MJ research, Geneworks). Master-mix for the PCR was made using 1.2 units Hot Start taq (Qiagen) in all single cell and amniotic samples unless otherwise stated. 1x PCR buffer and 1.5mM Magnesium Chloride were added. dNTP's were added to reach 0.2mM concentration. Primers were added as described below. The master-mix was made up to 24µl using Milli-Q sterilised water. Single cells were picked using a drawn glass pipette whilst spread in a 30mm plastic Petri dish in Phosphate buffered saline (PBS) (without Magnesium) (Gibco Brl), ~ 1µl of PBS drawn with the single cell.

PCR primer concentrations were:-

Marker	pmol/rxn
AMEL	3.5
THO	6.0
D21S11	16.0

D18S51	13.0
FGA	6.0
D3S1358	1.6
D5S818	1.5
CSF	12.0
TPOX	0.4

11 primer multiplex PCR amplification from hair shaft

As shown in Figure 12, DNA profiles were successfully obtained from hair roots using 11 primer sets to amplify the genetic markers listed hereinafter.

The primer concentrations were:-

Marker	pmol/rxn
AMEL	3.5
THO	6.0
D21S11	16.0
D18S51	16.0
VWA	30.0
FGA	6.0
D3S1358	10.0
D5S818	10.0
D7S820	10.0
CSF	13.0
TPOX	3.0

Simultaneous diagnosis and confirmation of chromosome status

Figure 13 shows a amniotic low copy number sample subjected to 10 primer set multiplex. The PCR amplified genetic markers were AMEL, D13S631, D18S851, DYS14, D18S391, D13S317, D21S11, D13S258, D18S51, D21S1412

PCR cycling parameters were:-

a. 95°C for 15 minutes
b. 94°C for 30 seconds
c. 59°C for 45 seconds
d. 72°C for 60 seconds

e. Go to b, 39 times

f. 72°C for 10 minutes

g. Hold at 4°C.

An amniotic cell suspension was added at 1.5µl (Stored in PBS) and run in a 96 well 200µl plate on a PTC 200 DNA engine (MJ research, Geneworks). Master-mix for the PCR was made using 1.2 units of Amplitaq Gold (Applied Biosystems) in all single cell and amniotic samples unless otherwise stated. 1x PCR buffer and 1.5mM Magnesium Chloride were added. dNTP's were added to reach 0.2mM concentration. Primers were added as described below. The master-mix was made up to 24µl using Milli-Q sterilised water.

Primer concentrations: -

AMEL	1.4 pmole
D13S631	6 pmole
D18S851	5 pmole
D13S258	6 pmole
D18S391	11 pmole
DYS14	4 pmole
D21S11	8 pmole
D18S51	3 pmole
D21S1412	12 pmole
D13S317	8 pmole

Increasing the number of simultaneous genetic diagnoses

Application of multiple genetic tests according to the invention may be applied, for example, to genetic analysis such as prenatal diagnosis using nucleic acids from isolated fetal cells such as from PAP smears, amniotic fluid or other nucleic acids such as free fetal DNA in maternal blood supply.

Figure 14 shows an electropherogram of a 10 primer set multiplex PCR consisting of simultaneous detection of:

1. Single gene defect status (cystic fibrosis Delta 508 marker)
2. Sexing status (male)
3. Trisomy status (trisomy detection)

4. DNA fingerprint

The DNA was obtained using PCR on a single buccal cell This PCR contains CF1, AMEL, D13S631, D18S851, DYS14, D13S391, D13S317, D21S11, D13S258 & D18S51

Primer concentrations were (per reaction): -

AMEL	1.4 pmole
D13S631	6 pmole
D18S851	5 pmole
D13S258	6 pmole
D18S51	2.6 pmole
DYS14	4 pmole
D21S11	9 pmole
D18S391	8 pmole
D13S317	5 pmole
CF	5 pmol

PCR cycling parameters were:-

a. 95°C for 15 minutes
b. 94°C for 30 seconds
c. 59°C for 45 seconds
d. 72°C for 60 seconds
e. Go to 2, 39 times
f. 72°C for 10 minutes
g. Hold at 4°C.

The single buccal cell was added at 1.5µl (Stored in PBS) and run on a PTC 200 DNA engine (MJ research, Geneworks). Master-mix for the PCR was made using 1.2 units of Amplitaq Gold (Applied Biosystems).. 1x PCR buffer and 1.5mM Magnesium Chloride were added . dNTP's were added to reach 0.2mM concentration. Primers were added as described for each individual case. The master-mix was made up to 24µl using Milli-Q sterilised water

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or

specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scopes of the present invention.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference.

TABLE 1

STR database

marker	ALIAS	GenBank No.	Position	heter	PRIMER SEQUENCE
D13S241	UT556	L17673	13pter	0.83	CCA GGC ACT TTG GGA GGC TG ACC CAC TGT ATC CTG GGC A
D13S242	UT557	L18329	13q21.2	0.83	ATT GCA CCC CAT CCT GGG TCC TTT TCC TAC CAT TTG CAT
D13S243	UT558	L18330	13cen-13q12.1	0.75	ACT GTA CTT CTG CCT GGG C TTT TGT AAT GCC TCA ACC ATG
D13S248	UT1213	L15541	13q32-13q34	0.83	ACT TAA ATG TCC ATC AAT AAA T TGA TTG GCT TTT TTT ACT TAC
D13S251	UT1329	L16338	13q31-13q32	0.75	CAC ATA GCT TAT TGT TGT TGC GTT ATC TGT GAG CAA ATA CAG
D13S253	UT1378	L16396	13q22-13q32	0.75	CTC AAG GGA TGT TAA CAC AC AGG AGG AAA AAG TGG AGA AG
D13S254	UT1585	L18690	13q31-13q32	0.86	TGA ACT CCG GCC TGG GTG A TTT TGG AGC TGG GGA TGT C
D13S256	UT2120	L17977	13q14.1-13q22	0.91	CCT GGG CAA CAA GAG CAA A AGC AGA GAG ACA TAA TTG TG
D13S257	UT2119	L18729	13q14.1-13q21.1	0.86	CAA CAA GAG CAA AAC TCC AT AAG CAC ATA AGT TGG TAT GAA
D13S258	UT2413	L18095	13q21.2-13q31	0.88	ACC TGC CAA ATT TTA CCA GG GAC AGA GAG AGG GAA TAA ACC
D13S303	UT936	L31309	13q22-13q31	0.75	ACA TCG CTC CTT ACC CCA TC TGT ACC CAT TAA CCA TCC CCA
D13S631	UT7403	L18392	13q31-13q32	0.94	GGC AAC AAG AGC AAA ACT CT TAG CCC TCA CCA TGA TTG G
D18S51	UT574	L18333	18q21.33-18q21.33	0.86	GAG CCA TGT TCA TGC CAC TG CAA ACC CGA CTA CCA GCA AC
D18S378	UT485	L16262	18P11.22-18P11.22	0.75	AGC CTG GGT GAC AGA GCA A ACA GGG AAA GCT GGG GGA T
D18S382	UT600	L16292	18pter-18pter	0.8	CAT CCA TCC ATC CTT CCA C TGT GCT GGT ATT ACA GGC G
D18S386	UT754	L18400	18q22.1-18q22.2	0.88	TCA GGA GAA TCA CTT GGA AC TCC ATG AAG TAG CTA AGC AG
D18S390	UT1227	L15542	18q22.3-18q23	0.75	TAA CCA AAG CAA ATC CCT GG CAC TTA CAC TGT TAT CCT GG
D18S391	UT1302	L16384	18pter-18p11.22	0.75	CTG GTT TTC GTC TTG AGA AG CAC TAT TCC CAT CTG AGT CA
D18S814	UT1248	L17776	18pter-18pter	0.73	CTT CCC TGG GTA TCA AGA CT TCC CAC TAT ATG TAT GTT CAC C
D18S815	UT1438	L17819	18pter-18qter	0.75	GGC TGA GAC AGG AGA ATC AC CTC ACC AGG ATT TCC TTG C
D18S819	UT7251	L30411	18pter-18qter	0.75	ACC ACA GTT ACT AAG ATG TAA

					GCC TCC AGA AAA AAT TTC CA
D18S851	SHGC 4561	G08002	18pter-18qter		CTG TCC TCT AGG CTC ATT TAG C
					TTA TGA AGC AGT GAT GCC AA
D21S11	VS17T3	M84567	21q21-21q21	0.9	GTG AGT CAA TTC CCC AAG
					GTT GTA TTA GTC AAT GTT CTC C
D21S1240	UT656	L18360	21pter-21qter	0.5	GAG ACG GTA GGA AAA GGA G
					AGC CAA GTT CGA GCC ACT G
D21S1244	UT761	L16331	21q21-21q22.1	0.8	GTC CCC ATA TTG ATA AAC TAT T
					ATG AAT AGG GGA TAT GCT GG
D21S1413	UT7582	L30513	21pter-21pter	0.875	TTG CAG GGA AAC CAC AGT T
					TCC TTG GAA TAA ATT CCC GG
D21S1412	UT6930	L29680	21pter-21pter	0.8	CGG AGG TTG CAG TGA GTT G
					GGG AAG GCT ATG GAG GAG A
D21S1411	UT1355	L17803	21pter-21pter	0.933	ATG ATG AAT GCA TAG ATG GAT G
					AAT GTG TGT CCT TCC AGG C
PENTA E	PAUL1	AC027004	21q	0.88	TCC AGC CTA GGT GAC AGA GC
					TGC CTA AAC CTA TGG TCA TAA CG
AMEL		M55418	Xp22.31-p22.1	n/a	CCC TGG GCT CTG TAA AGA ATA GTG
					ATC AGA GCT TAA ACT GGG AAG CTG
HUMTHO		D00269	11p15-15.5	0.76	GCT TCC GAG TGC AGG TCA CA
					CAG CTG CCC TAG TCA GCA C
TPOX		M68651	2p23-2pter	0.65	CAC TAG CAC CCA GAA CCG TC
					CCT TGT CAG CGT TTA TTT GCC
VWA		M25858	12p12-pter	0.83	CCC TAG TGG ATG ATA AGA ATA ATC AGT ATG
					GGA CAG ATG ATA AAT ACA TAG GAT GGA TGG
D3S1358		11449919	3p	0.78	ACT GCA GTC CAA TCT GGG T
					ATG AAA TCA ACA GAG GCT TG
D5S818		G08446	5q21-q31	0.71	GGG TGA TTT TCC TCT TTG GT
					TGA TTC CAA TCA TAG CCA CA
D7S820		G08616	7q	0.79	TGT CAT AGT TTA GAA CGA ACT AAC G
					CTG AGG TAT CAA AAA CTC AGA GG
CSF1PO	U63963	X14720	5q33.3-34	0.78	AAC CTG AGT CTG CCA AGG ACT AGC
					TTC CAC ACA CCA CTG GCC ATC TTC
FGA		M64982	4q28	0.86	GCC CCA TAG GTT TTG AAC TCA
					TGA TTT GTC TGT AAT TGC CAG C
D13S317		G09017	13q22-q31	0.71	ACA GAA GTC TGG GAT GTG GA
					GCC CAA AAA GAC AGA CAG AA
DYS14				n/a	CTT TCC ACA GCC ACA TTT GTC
					CAT CCA GAG CGT CCC TGG CTT
CF					GTT TTC CTG GAT TAT GCC TGG GCA
					GTT GGC ATG CTT TGA TGA CGC TTC
D16S539					GAT CCC AAG CTC TTC CTC TT
					ACG TTT GTG TGT GCA TCT GT
D16S690					GCA CAG CTT CCT GAT CTG A
					TCA CAC AAC CCA CAG AGA A

D22S417	CCT GGG AAG TTA AGA CTG C TCT ACC GCT TAT TTC TTC CCT
D22S526	AGA GCA AGA CTC TGT CTC AAC A TTC TCC TTC ACT TTC TGC CAT G

TABLE 2

Primer set	Fluorescent Dye	pmoles
Amelogenin	FAM	Variable from 1-40
DYS14	FAM	Variable from 1-40
D21S11	FAM or TET	Variable from 1-40
D13S631	HEX	Variable from 1-40
D13S258	HEX	Variable from 1-40
D18S51	FAM	Variable from 1-40
D18S851	FAM	Variable from 1-40
D18S391	HEX	Variable from 1-40
D13S317	TET	Variable from 1-40
D21S1413	HEX	Variable from 1-40
D21S1412	TET	Variable from 1-40
D21S1411	FAM	Variable from 1-40
Penta E	TET	Variable from 1-40
D16S539	TET	Variable from 1-40
D16S690	TET	Variable from 1-40
D22S417	FAM	Variable from 1-40
D22S526	FAM	Variable from 1-40
D18S378	HEX	Variable from 1-40
CF	HEX	Variable from 1-40

TABLE 3

Primer set	Fluorescent Dye	pmoles
Amelogenin	FAM	Variable from 1-40
HUMTHO	FAM	Variable from 1-40
D21S11	FAM	Variable from 1-40
D18S51	FAM	Variable from 1-40
VWA	HEX	Variable from 1-40
FGA	HEX	Variable from 1-40
D3S1358	FAM	Variable from 1-40
D5S818	TET	Variable from 1-40
D7S820	TET	Variable from 1-40
CSF1PO	HEX	Variable from 1-40
TPOX	TET	Variable from 1-40

TABLE 4

	Fluorescent PCR	**FISH**	**Conventional PCR**	**PRINS**
High reliability	97%	86%	84% for CF	91%
High accuracy	97-99% 97% for (Carrier) in CF	>95%	79% (Unaffect) & 66% (Carrier) for CF	25%
Rapid diagnosis	6 hrs	~4 hrs	8-10 hrs	6 hrs
Diagnosis of sex	Yes	Yes	Possible but poor reliability & accuracy	Yes
Diagnosis of single-gene defects	Yes	No	Yes	No
Diagnosis of trisomies	Yes	Yes	Very poor	Yes
Simultaneous confirmation of diagnosis	Yes	No	No	No
DNA fingerprinting	Yes, high specificity >1 in 10 billion.	No	Limited	No
Detection of contamination	Yes	No	Very limited	No
Simultaneous multiple diagnoses	Sex, CF, trisomies & DNA fingerprint	Trisomies	No	No
No of chromosomes simultaneously analysed	16	3-5	1	3

TABLE 5

	Published protocol	New protocol	Comment
Number of cells	226	114	
Reliability	91% (206/226)	95% (108/114)	Higher is better
Amp failure	9% (10/226)	5% (6/114)	Lower is better
Contamination (>1 surplus allele)	0	1% (1/108)	Lower is better
Full STR profile	55% (114/206)	29% (31/108)	Higher is better[1]
Acceptable profile (≥4 STRs)	70% (144/206)	100% (108/108)	Higher is better
Partial profile (1-3 STRs)	30% (62/206)	0	Lower is better
Surplus alleles[2]	14% (28/206)	6% (6/108)	Lower is better
False alleles[3]	5% (11/206)	1% (1/108)	Lower is better
Allele dropout	43% (88/206)	67% (72/108)	Lower is better[4]
Sex diagnosis	84% (172/206)	95% (102/108)	Higher is better
No of STRs in PCR	6	10	Higher is better
Mean STRs/cell	4.5 (estimated)	8.9	Higher is better
Estimated informativity (full)	>100 million	> 5 billion	Higher is better

TABLE 6

	Mother		Father		Twin 1		Twin 2	
AMEL	X	X	X	Y	X	X	X	X
TPOX	113	113	113	113	113	113	113	113
D3S1358	125	130	130	134	125	130	125	130
D5S818	150	150	141	158	141	150	141	150
HUMTHO	170	170	173	173	170	173	170	173
FGA	198	202	189	193	189	202	189	202
D21S11	225	229	239	239	225	239	225	239
D18S51	290	298	294	298	290	298	290	298
CSF1PO	315	315	311	319	315	319	315	319

Table 6b

	Mother		Father		Twin 1		Twin 2	
AMEL			X	Y		X		X
TPOX			113	113		113		113
D3S1358			130	134		130		130
D5S818			141	158	141		141	
HUMTHO			173	173		173		173
FGA			189	193	189		189	
D21S11			239	239		239		239
D18S51			294	298		298		298
CSF1PO			311	319		319		319

Maternal

Paternally derived

TABLE 7

	Maternal	Twin 1		Twin 2	
AMEL			X		X
TPOX		113		113	
D3S1358			135		135
VWA			153		153
HUMTHO			158		174
FGA			210		210
D7S820		203			207
D21S11		239			239
D18S51		290			287
CSF1PO		312	316		312
D13S631		120		120	
D18S851			154		158
D13S317			179	179	
D18S391			183		183
D13S258		239			235
D21S1413		157		157	
D21S1411		288	312	300	

Maternal	
Either	
Presumptive Paternal	

CLAIMS

1. A method of selecting a plurality of genetic markers as targets for nucleic acid sequence amplification, said method including the step of selecting each of said plurality of genetic markers according to a heterozygosity index, wherein said heterozygosity index is 0.5 or greater.

2. The method of Claim 1, wherein said heterozygosity index is 0.7 or greater.

3. The method of Claim 1, wherein said heterozygosity index is 0.9 or greater.

4. The method of Claim 1 further including the step of selecting one or more primers for each respective said genetic marker so that upon amplification of said genetic marker using said one or more primers, a resultant amplification product has a molecular size in the range 50-3000 bp.

5. The method of Claim 4, the molecular size is in the range 50-1000 bp.

6. The method of Claim 4, wherein the molecular size is in the range 80-500 bp.

7. The method of Claim 4, wherein the molecular size is in the range 100-400 bp.

8. The method of Claim 1, wherein each of said genetic markers is a short tandem repeat (STR).

9. A method of nucleic acid sequence amplification, said method including the step of using a nucleic acid sequence amplification technique and at least nine primer pairs in combination to amplify a plurality of respective genetic markers from a limiting amount of nucleic acid sample.

10. The method of Claim 9, wherein at least ten primer pairs are used to amplify said respective genetic markers.

11. The method of Claim 9, wherein at least eleven primer pairs to amplify said respective genetic markers.

12. The method of Claim 9, wherein at least twelve primer pairs are used to amplify said respective genetic markers.

13. The method of Claim 9, wherein at least thirteen primer pairs are used to amplify said respective genetic markers.

14. The method of Claim 9, wherein at least fourteen primer pairs are used to amplify said respective genetic markers.

15. The method of Claim 9, wherein at least fifteen primer pairs are used to amplify said respective genetic markers.

16. The method of Claim 9, wherein sixteen primer pairs are used in combination to amplify said respective genetic markers.

17. The method of Claim 9 wherein the nucleic acid sample has less than 1 ng of DNA.

18. The method of Claim 9 wherein the nucleic acid sample has less than 500 pg of DNA.

19. The method of Claim 9, wherein the nucleic acid sample has less than 200 pg of DNA.

20. The method of Claim 9, wherein the nucleic acid sample has 3-6 pg of target DNA.

21. The method of Claim 9, wherein said nucleic acid sample comprises DNA isolated from less than 200 cells, or an equivalent amount of DNA.

22. The method of Claim 9, wherein said nucleic acid sample comprises DNA isolated from less than 100 cells or an equivalent amount of DNA

23. The method of Claim 9, wherein said nucleic acid sample comprises DNA isolated from less than 50 cells or an equivalent amount of DNA

24. The method of Claim 9, wherein said nucleic acid sample comprises DNA isolated from less than 10 cells or an equivalent amount of DNA

25. The method of Claim 9 wherein the nucleic acid sample comprises DNA isolated from a single cell, or an equivalent amount of DNA.

26. The method of Claim 25 wherein the single cell is a fetal cell.

27. The method of Claim 26, wherein the fetal cell is isolated from a PAP smear.

28. The method of Claim 26, wherein the fetal cell is obtained by CVS, amniocentesis or is obtained from fetal blood.

29. The method of Claim 25 wherein the single cell is a human embryonic cell.

30. The method of Claim 25 wherein the single cell is a haploid cell.

31. The method of Claim 30, wherein the haploid cell is a spermatozoa.

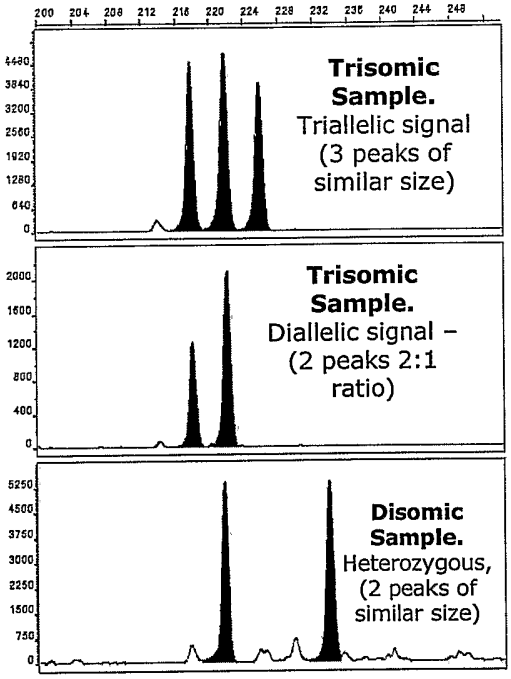
32. The method of Claim 25 wherein the single cell is a human epithelial cell.

33. The method of Claim 25, wherein the single cell is obtained from a non-human mammal.

34. The method of Claim 9 wherein nucleic acid sequence amplification is PCR.

35. The method of Claim 34 wherein PCR is fluorescent multiplex PCR.

36. The method of Claim 9, wherein the primers are selected from the group consisting of SEQ ID NOS: 1-92.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3840
3200
2560
1920
1280
640
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Trisomic Sample. Triallelic signal (3 peaks of similar size)
2000
1600
1200
800
400
0
Trisomic Sample. Diallelic signal – (2 peaks 2:1 ratio)
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4500
3750
3000
2250
1500
750
0
Disomic Sample. Heterozygous, (2 peaks of similar size)

FIG. 1

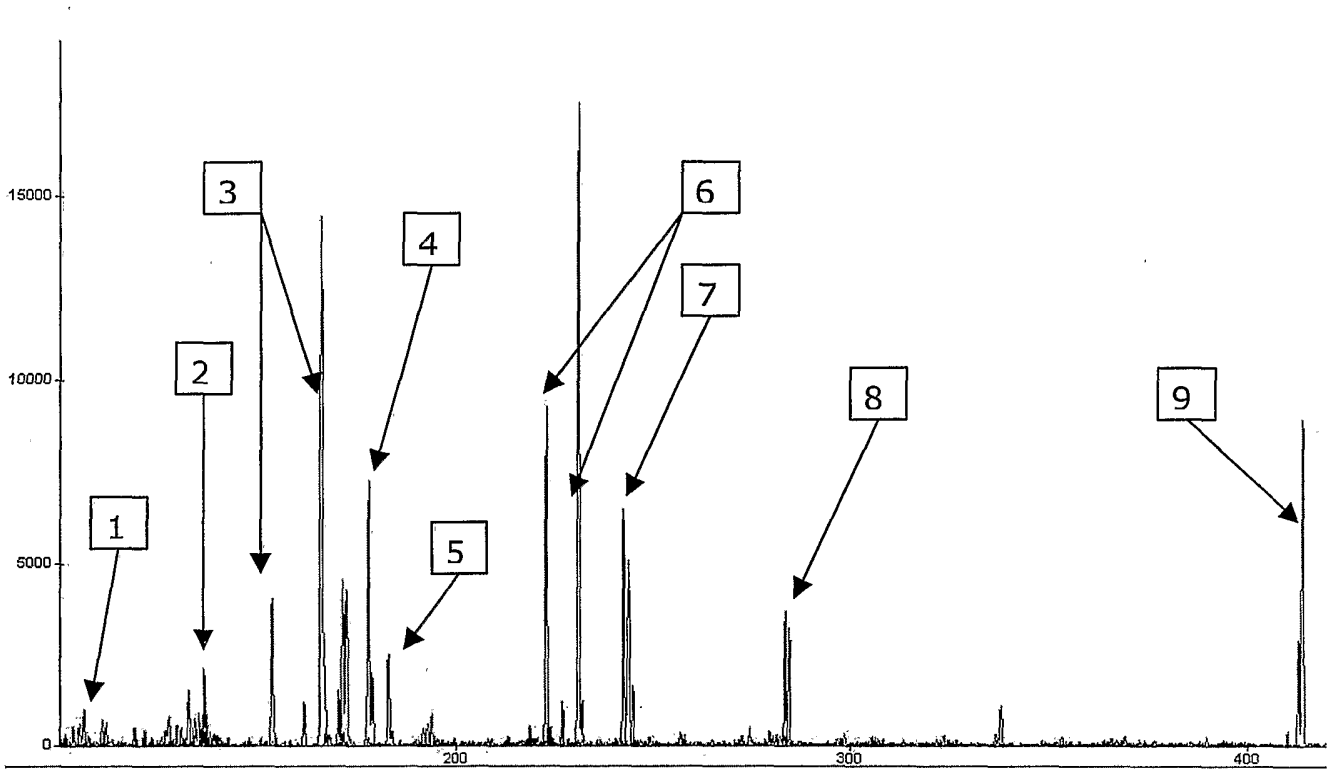


FIG. 2

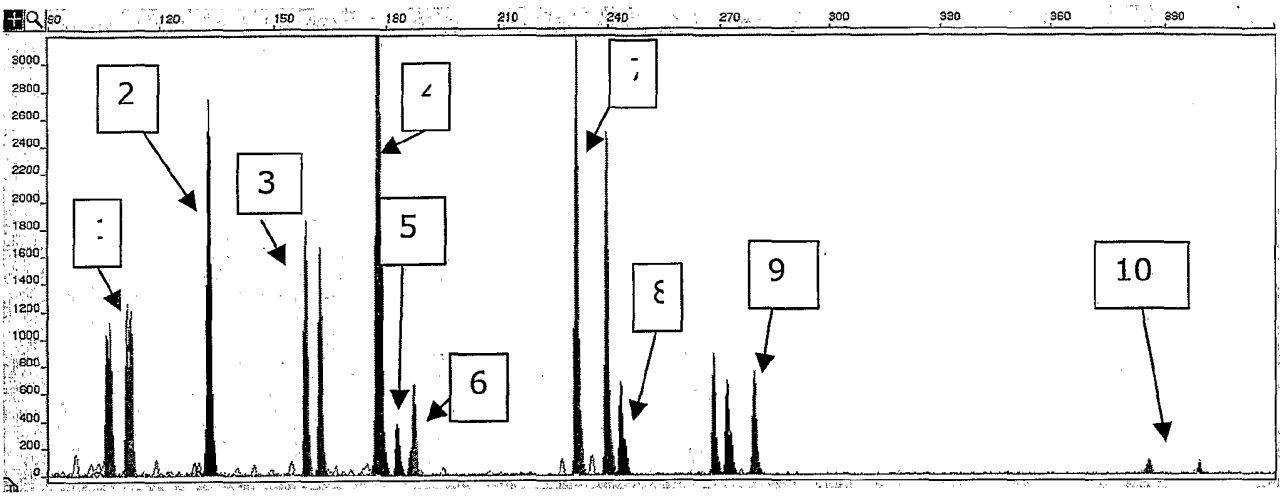


FIG. 3

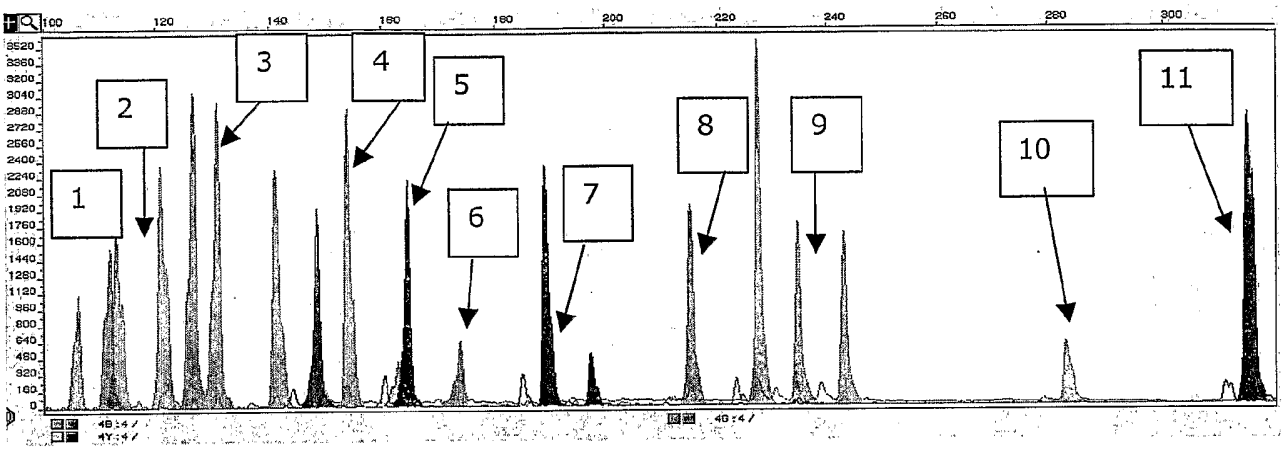
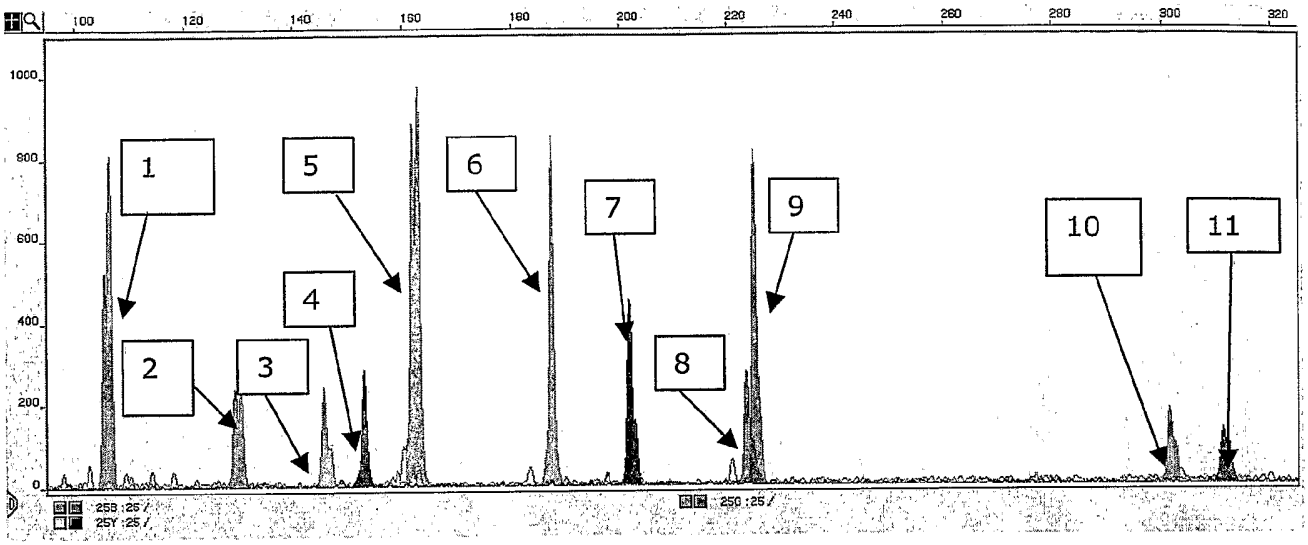


FIG. 4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180
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220
240
260
280
300
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400
200
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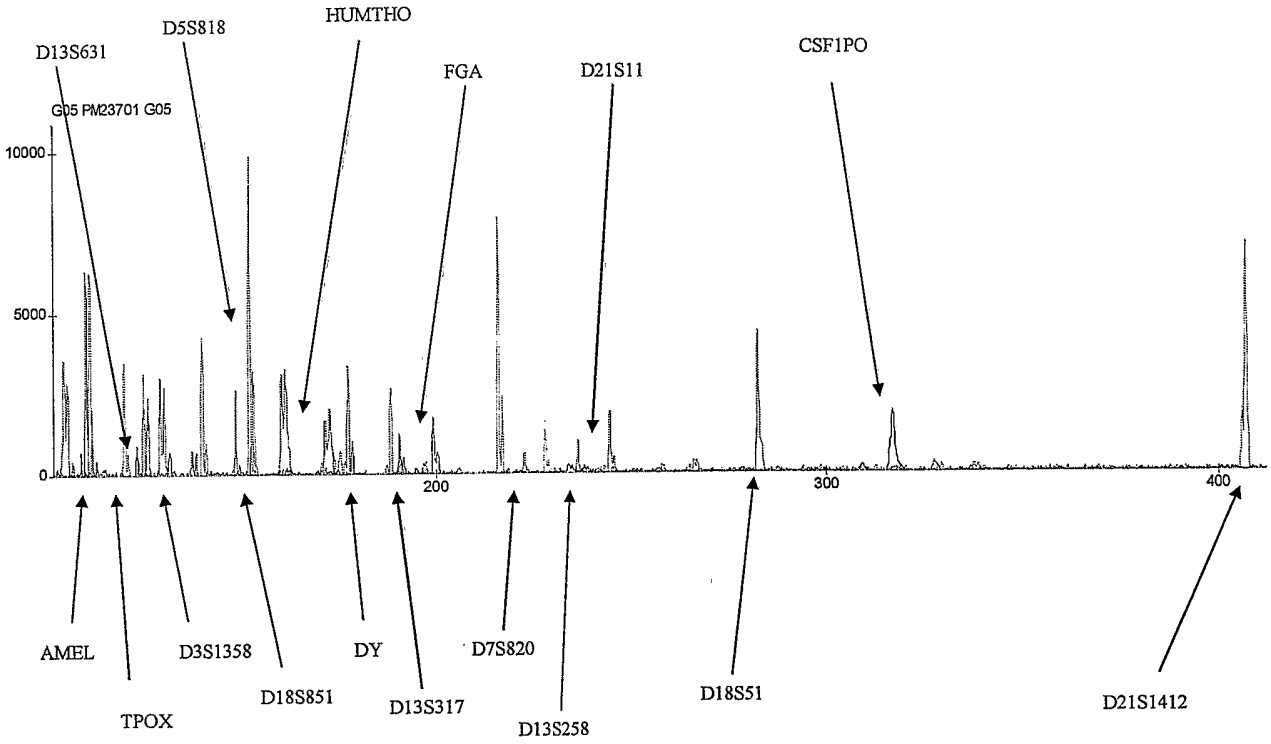
1
2
3
4
5
6
7
8
9
10
11

FIG. 5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FGA
D21S11
CSF1PO
G05 PM23701 G05
10000
5000
0
200
300
400
AMEL
TPOX
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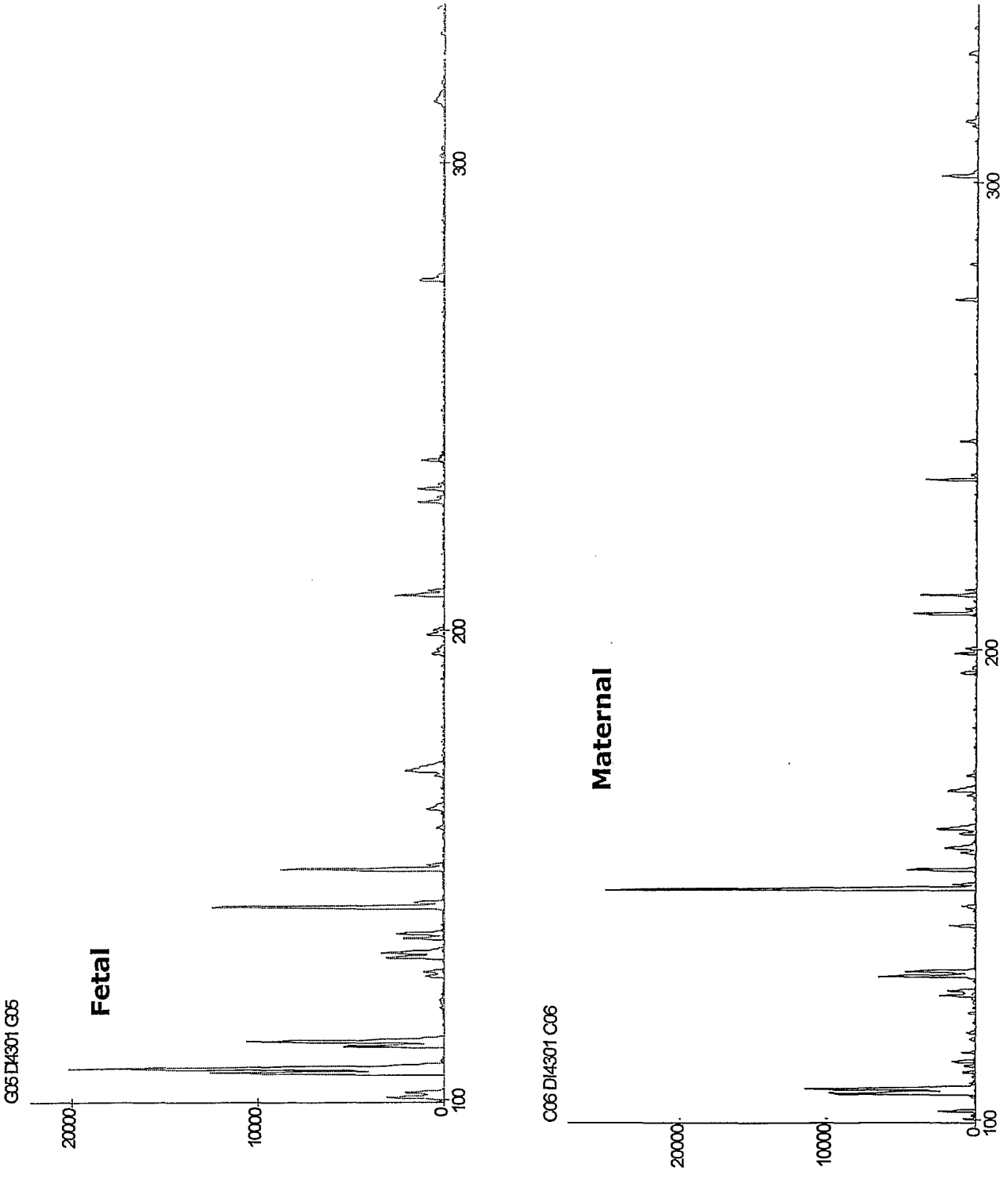
D18S851
DY
D13S317
D7S820
D13S258
D18S51
D21S1412

FIG. 6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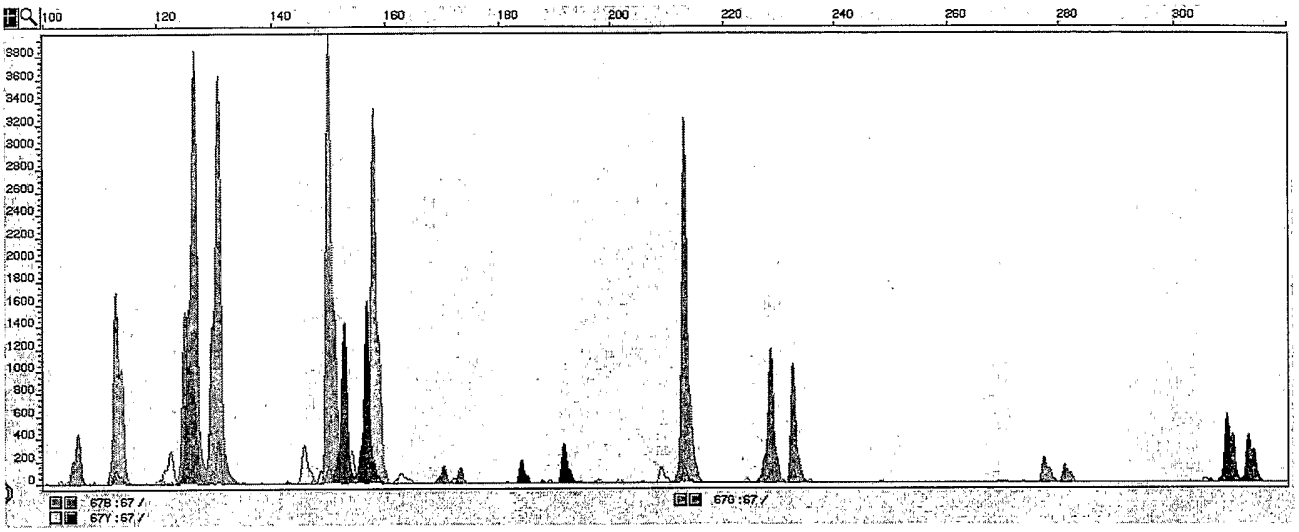
Fetal
20000
10000
0
100
200
300
C06 DI4301 C06
Maternal
20000
10000
0
100
200
300

FIG. 7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300
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3600
3400
3200
3000
2800
2600
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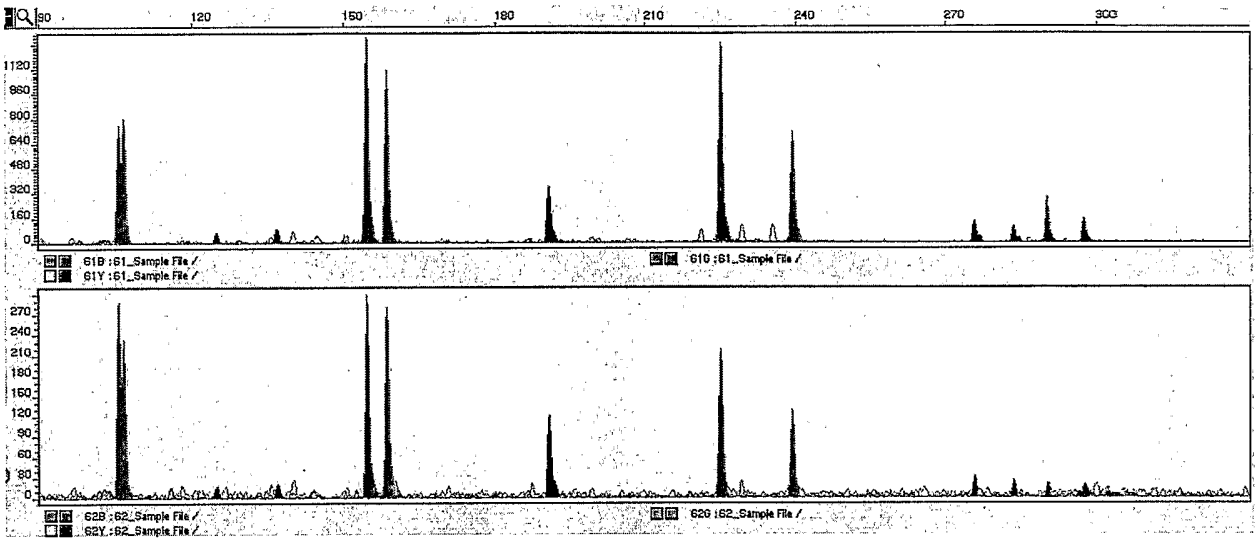
400
200
0
678 :67
67Y :67
670 :67

FIG. 8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150
180
210
240
270
300
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61Y :61_Sample File
61G :61_Sample File
62B :62_Sample File
62Y :62_Sample File
62G :62_Sample File

FIG. 9

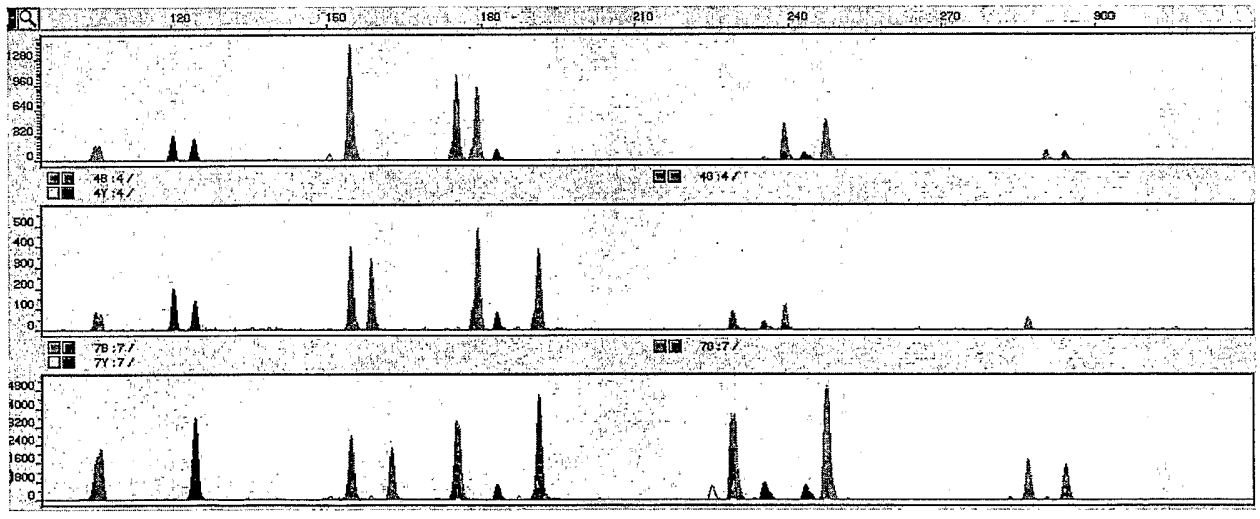
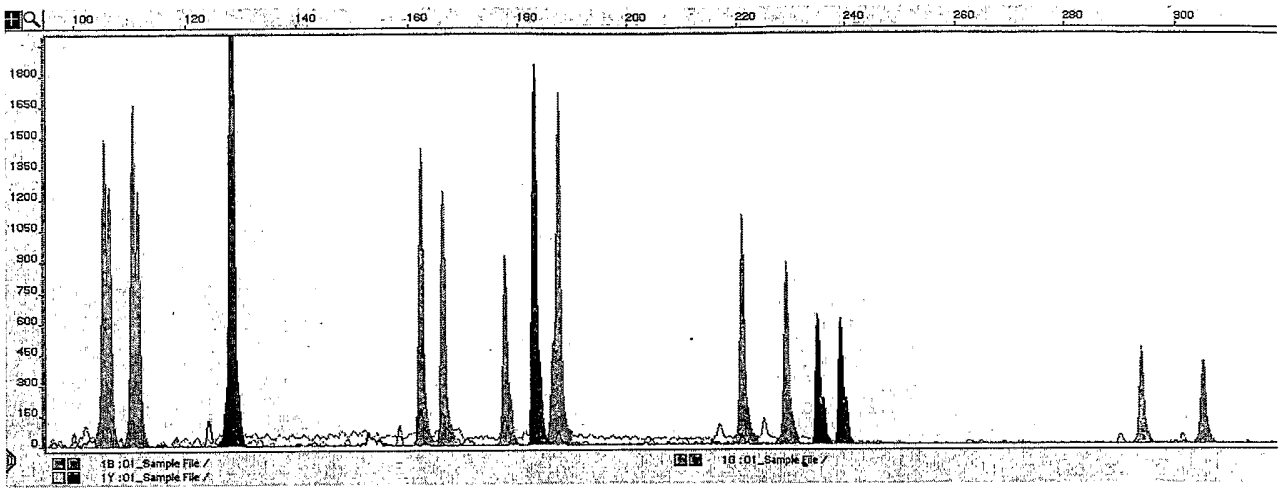

120
150
180
210
240
270
300
4B :4
4Y :4
4G :4
7B :7
7Y :7
7G :7

FIG. 10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120
140
160
180
200
220
240
260
280
300
1800
1650
1500
1350
1200
1050
900
750
600
450
300
150
0
1B :01_Sample File /:
1Y :01_Sample File /:
1G :01_Sample File /

FIG. 11

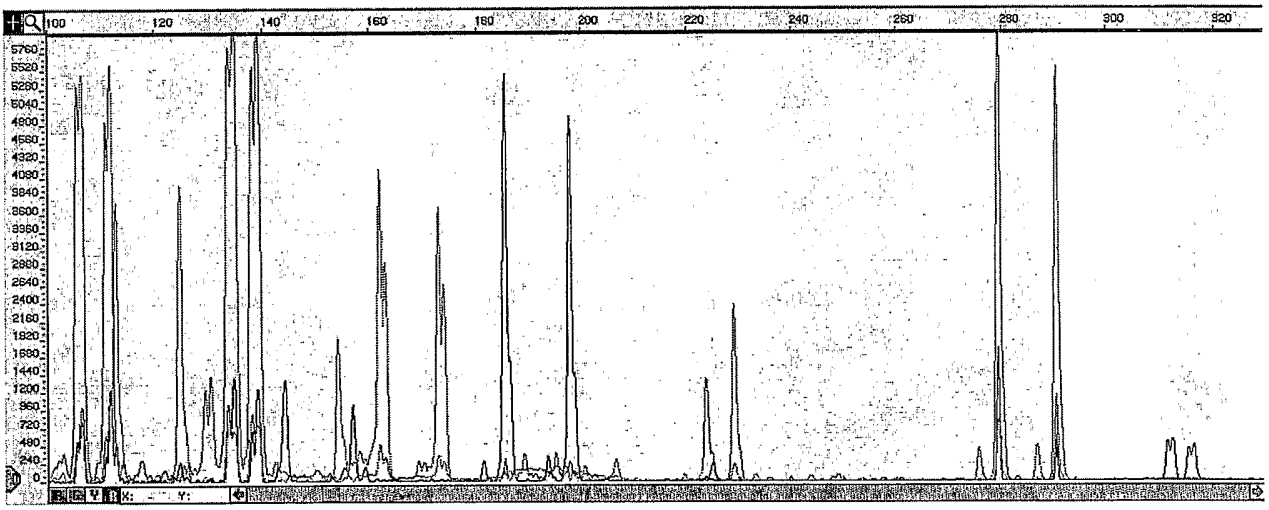
100
120
140
160
180
200
220
240
260
280
300
320
5760
5520
5280
5040
4800
4560
4320
4080
3840
3600
3360
3120
2880
2640
2400
2160
1920
1680
1440
1200
960
720
480
240
0
X:
Y:

FIG. 12

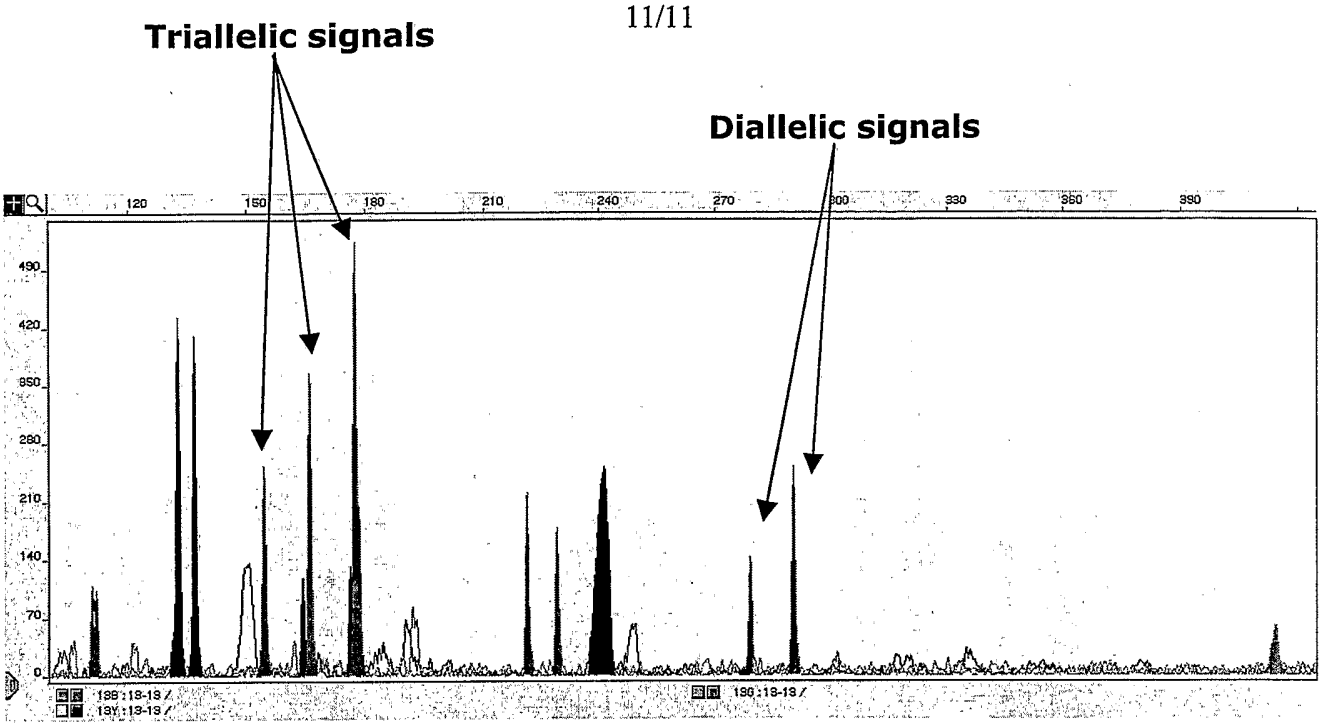

FIG. 13

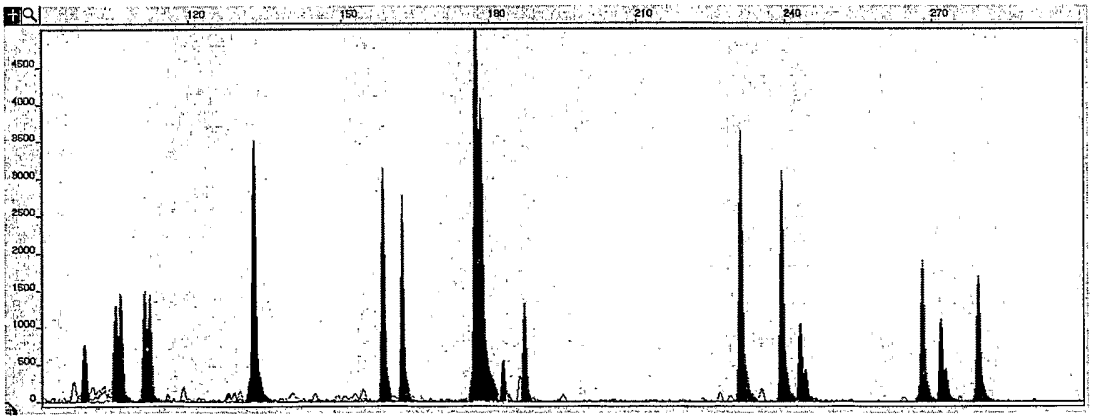

FIG. 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01388

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12Q 1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Derwent (JAPIO and WPAT), PUBMED, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	*Am. J. Hum. Genet.* (1995), **57**, 619-628, "A Collection of Ordered Tetranucleotide-Repeat Markers from the Human Genome", The Utah Marker Development Group (see entire document, in particular the abstract, page 621 column 1, page 625 column 2, page 626 column 1)	1-8
X	*Croatian Medical Journal* (June 2001), **42** (3), 260-266, "DNA typing from Skeletal Remains: Evaluation of Multiplex and Megaplex STR Systems on DNA Isolated from Bone and Teeth Samples", Antonio Alonso *et al.* (see entire document, in particular page 261 column 1, page 262 column 2)	9-25, 34-35
X	*Journal of Forensic Sciences* (1999), 44 (1), 167-170, "Allele Distribution at Nine STR Loci-D3S1358, vWA, FGA, TH01, TPOX, CSF1PO, D5S818, D13S317 and D7S820-in the Japanese Population by Multiplex PCR and Capillary Electrophoresis", Toshimichi Yamamoto *et al.* (see entire document, in particular page 167)	1-2, 4-8

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
20 December 2002

Date of mailing of the international search report
- 7 JAN 2003

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01388

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	*Electrophoresis* (1999), **20**, 1717-1721, "Ancient DNA profiling by megaplex amplifications", Susanne Hummel *et al.* (see entire document, in particular page 1717 column 2,page 1721 column 1	9, 34-36
X	*Int. J. Leg. Med* (1995), **107**, 246-249, "Swiss population data and forensic efficiency values on 3 tetrameric short tandem repeat loci-HUMTH01, TPOX, and CSF1P0-derived using a STR multiplex system", M.N. Hochmeister *et al.* (see entire document, in particular the abstract and page 248)	1, 4-8
X	*Human Biology*, (April 2001), **73** (2), 175-190, "Genomic Diversity at Thirteen Short Tandem Repeat Loci in a Substructured Caste Population, Golla, of Southern Andhra Pradesh, India", B. Mohan Reddy *et al.* (see entire document, in particular the abstract, Results(Allelic Distributions) Table 2 and Discussion)	1, 4-8
X, O	"Development of New STRs for Forensic Casework: Criteria for Selectio, Sequencing & Population Data and Forensic Validation" Angel Carracedo and M.V. Lareu in Genetic Identity Conference Proceedings Ninth International Symposium on Human Identification - 1998 Oral Presentations, (see entire presentation, in particular "Selection of STRS", Figure 1) (http://www.promega.com/geneticidproc/ussymp9proc/default.htm)	1-8
X	*Journal of Forensic Sciences*, (1999), **44** (2), 392-395, "Maine Caucasian Population DNA Database Using Twelve Short Tandem Repeat Loci", Timothy D. Kupferschmid *et al.* (see entire document, in particular page 392 column 1)	9, 17, 21, 34-35
X	*Genome Research* (1995), **5**, 488-493, "A Simplified Procedure for Developing Multipllex PCRs", Anthony P. Shuber *et al.* (see entire document, in particular Table 1 (CFTR) 15-plex primer sequences, page 492 column 2)	9-15, 34
X	*Croatian Medical Journal* (June 2001), **42** (3), 239-243, "Comparison of PowerPlex™ 16, PowerPlex™1.1/2.1, and ABI Amp*fl*STR™ Profiler Plus™/COfiler™ for Forensic Use", Christine S. Tomsey *et al.* (see entire document)	9-19, 21-24, 34-36
X, Y	International Congress Series (1998), 1167 (*Progress in Forensic Genetics 7*), (Proceedings of the 17th International ISFH Congress, Oslo, 2-6 September 1997), Society for Forensic Haemogenetics. Congress B. Olaisen, B. Brinkmann and P.J. Lincoln (Editors), Elsevier Science B.V., pages 198-200, "Further Exploration of New STRs of Interest for Forensic Genetic Analalysis", M.V. Lareu *et al.* (see entire document)	1-8
Y	WO 99/04034 A1 (Procrea Biosciences Inc.) 28 January 1999 (see entire document, in particular page 10)	1-8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01388

Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The following two groups of claims are considered to relate to separate inventions;
Claims 1-8 are directed to a method of selecting a plurality of genetic markers as targets for nucleic acid sequence amplification. The markers being selected such that their heterozygosity index is 0.5 or greater.
Claims 9-36 are directed to a method of nucleic acid sequence amplification. The method being characterized as including at least nine primer pairs in combination to amplify a plurality of respective genetic markers from a limiting amount of nuclei acid sample.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU02/01388

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	99/04034	AU	83297/98	EP	1002130	US	6060243

END OF ANNEX