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(54) **METHODS FOR TESTING MILK**

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(57) **ABSTRACT**

The disclosure is related generally to methods for testing mammary fluid (including milk) to establish or confirm the identity of the donor of the mammary fluid. Such methods are useful in the milk-bank business to improve safety.

**51 Claims, No Drawings**

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# METHODS FOR TESTING MILK

**Matter enclosed in heavy brackets [ ] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue; a claim printed with strikethrough indicates that the claim was canceled, disclaimed, or held invalid by a prior post-patent action or proceeding.**

## CROSS REFERENCE TO RELATED APPLICATIONS

This application is a *reissue of U.S. application Ser. No. 13/529,678, filed Aug. 23, 2012, now U.S. Pat. No. 8,628,921, which is a* continuation of U.S. application Ser. No. 13/079,932, filed Apr. 5, 2011, now U.S. Pat. No. 8,278,046, which is a continuation of U.S. application Ser. No. 12/052,253, filed Mar. 20, 2008, now U.S. Pat. No. 7,943,315, which is a continuation-in-part and claims priority to the international application PCT/US2006/036827 with an international filing date of Sep. 20, 2006, which, in turn, claims priority under 35 U.S.C. [§119] *§ 119* from Provisional Application Ser. Nos. 60/719,317, filed Sep. 20, 2005, and 60/731,428, filed Oct. 28, 2005*; U.S. application Ser. No. 13/592,678 is also a continuation-in-part of U.S. application Ser. No. 11/947,580, filed Nov. 29, 2007*. The disclosures of all of each of which are incorporated herein by reference in their entireties.

## TECHNICAL FIELD

The methods featured herein are related generally to methods of testing mammary fluid (including milk) to establish or confirm the identity of the donor of the mammary fluid.

## BACKGROUND

Unlike blood donors, who give their donation under the direct supervision of the blood bank personnel, human breast milk donors tend to pump their milk for donation at home or other locations convenient to them and then often store the breast milk in their freezers until they have accumulated enough to bring to the donation center. Thus, in the absence of direct supervision of the donations, questions may arise as to the provenance of the donated breast milk.

In order to establish that the breast milk provided by a donor is, in fact, exclusively from that human female donor, some form of testing to establish donor identity should occur.

Many different methods of DNA typing are known for identifying or typing specimens from humans. Such methods include short tandem repeats ("STR"), microsatellite repeats or simple sequence repeats ("SSR") analysis of human DNA; analysis of multiple human genes and analysis of human lymphocyte antigen (HLA) genes and loci by polymerase chain reaction (PCR) analysis, restriction length polymorphism analysis and other methods.

It is known that humans possess antigens which are specific to that individual. For example, the human leukocyte antigens (HLA) have been used in the past for typing tissue for transplantation.

Such typing methods, among others, can be used to test for donor identity in the methods featured herein.

## SUMMARY

The methods and systems featured herein relate to diagnosing or screening mammary fluid from any number of mammalian organisms. In one aspect, the invention provides methods and systems for diagnosing or screening human milk samples to confirm that the milk is from a defined source.

The methods include obtaining a donated reference sample from a potential mammary fluid donor, e.g., a human breast milk donor. The sample can be analyzed at or around the time of obtaining the sample for one or more markers that can identify the potential donor. Alternatively, or in addition, the sample can be stored and analyzed for identifying markers at a later time. When the potential mammary fluid donor expresses the mammary fluid and donates the fluid (e.g., by bringing or sending the fluid to the donation center), the mammary fluid can be analyzed for the same marker or markers as the donor's reference sample. The match between the markers (and lack of any additional unmatched markers) would indicate that the donated milk comes from the same individual as the one who donated the reference sample. Lack of a match (or presence of additional unmatched markers) would indicate that the donated mammary fluid either comes from a non-tested donor or has been contaminated with fluid from a non-tested donor.

The testing of the reference sample and of the donated mammary fluid can be carried out at the donation facility and/or milk processing facility. The results of the reference sample tests can be stored and compared against any future donations by the same donor.

Testing donors to confirm their identity improves safety of donated milk. It ensures the provenance of the donated milk, which as discussed above, is most often donated without supervision by the donor center. Testing donor identity by the methods featured herein allows for multiple donations by the same donor, whose identity can be confirmed at the time of each donation. The donor can live at any distance from the donation and/or processing facilities, as she can send her milk at long distances, and her identity can be confirmed based on reference samples or reference tests stored at the donation and/or processing facility.

The mammary fluid tested by the methods featured herein can be processed for further use. The donation facility and milk processing facility can be the same or different facility. The donated milk can be processed, e.g., to obtain human milk fortifiers, standardized human milk formulations; human lipid products, and/or compositions for total parenteral nutrition.

In one aspect, a method of determining whether a donated mammary fluid was obtained from a specific subject is featured. The method includes: (a) testing a donated biological sample from the specific subject to obtain at least one reference identity marker profile for at least one marker; (b) testing a sample of the donated mammary fluid to obtain at least one identity marker profile for the at least one marker in step (a); and (c) comparing the identity marker profiles, wherein a match between the identity marker profiles indicates that the mammary fluid was obtained from the specific subject.

Embodiments can include one or more of the following features.

The method can further include: (d) processing the donated mammary fluid whose identity marker profile has been matched with a reference identity marker profile. The mammary fluid is human breast milk. Processing can include generating a pasteurized milk composition for administration to a human infant. The processing can include: filtering the milk; heat-treating the milk; separating the milk into cream and skim; adding a portion of the cream to the skim; and pasteurizing.

The processed and pasteurized milk composition can include: a human protein constituent of about 35-85 mg/mL; a human fat constituent of about 60-110 mg/mL; and a human carbohydrate constituent of about 60-140 mg/mL, and optionally, one or more constituents selected from the group consisting of: calcium, chloride, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc.

The processed and pasteurized milk composition can include: a human protein constituent of about 11-20 mg/mL; a human fat constituent of about 35-55 mg/mL; and a human carbohydrate constituent of about 70-120 mg/mL, and, optionally, one or more components selected from the group consisting of: calcium, chloride, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc.

The processing can include separating the milk into a cream portion and a skim portion, processing the cream portion, and pasteurizing the cream portion.

The testing of the mammary fluid sample and the testing of the biological sample can include a nucleic acid typing, e.g., STR analysis, HLA analysis, multiple gene analysis, and a combination thereof.

The donated mammary fluid can be frozen, and the method can include obtaining the mammary fluid sample by drilling a core through the frozen fluid. Alternatively, or in addition, the method can include obtaining the mammary fluid sample by scraping the surface of the frozen mammary fluid.

The method can further include isolating the mammary fluid prior to step (b).

The mammary fluid sample can include a mixture of one or more mammary fluid samples. The testing of the mammary fluid sample and the testing of the biological sample can include antibody testing to obtain a self-antigen profile. The sample of the donated mammary fluid can include a selected solid fraction of the fluid. The identity profiles can include peptide markers. The donated biological sample can be, e.g., milk, saliva, buccal cell, hair root, and blood.

A lack of a match between the identity marker profiles indicates contamination of the mammary fluid by another mammal.

Steps (a) through (c) can be carried out at a human breast milk donation center or at a milk processing facility.

In another aspect the disclosure is related to a method for determining whether breast milk was obtained from a specific human comprising testing a sample of the breast milk to obtain an identity marker profile and testing a biological sample from the human to obtain a reference identity marker profile and comparing the identity marker profiles.

The disclosure provides a method for determining whether breast milk was obtained from a desired source or specific human comprising nucleic acid typing of a sample of the breast milk to obtain a DNA type profile and nucleic acid typing of a biological sample from the human to obtain a reference DNA type profile and comparing the DNA type profiles.

In one embodiment, the method of nucleic acid typing of the biological sample from the human is selected from STR analysis, HLA analysis or multiple gene analysis. It is further contemplated that nucleic acid typing method used for the breast milk sample is the same as that used for the biological sample. It is contemplated that the loci/alleles used for the DNA type profile will be the same for both the reference DNA type profile and the breast milk sample DNA type profile.

In one embodiment the breast milk will be frozen. It is further contemplated that the method for obtaining the breast milk sample from the frozen breast milk will be by drilling a core through the frozen breast milk. Alternatively, it is contemplated that the breast milk sample may be obtained by scraping the surface of the frozen breast milk.



In another embodiment the breast milk will be liquid. It is contemplated that the method for obtaining the breast milk sample will be by isolating the breast milk sample by pipette or other means.

In another embodiment the breast milk samples may be combined or mixed prior to nucleic acid typing.

The disclosure also provides a method for determining whether breast milk was obtained from a defined source (e.g., a specific human) comprising testing of a sample of the breast milk to obtain a self-antigen profile and testing a biological sample from the human to obtain a reference self-antigen profile and comparing the self-antigen profiles.

The disclosure also provides an article of manufacture or kit comprising a container, a label on the container and a reagent for detecting or measuring identity markers, wherein the label on the container indicates that the reagent can be used to determine the identity marker profile of breast milk.In one embodiment, the reagent comprises PCR materials (a set of primers, DNA polymerase and 4 nucleoside triphosphates) that hybridize with the gene or loci thereof. The kit may further comprise additional components, such as reagents, for detecting or measuring the detectable entity or providing a control. Other reagents used for hybridization, prehybridization, DNA extraction, visualization and the like may also be included, if desired. In another embodiment, the regent is an antibody for detecting self-antigens.

Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of skill in the art to which this invention belongs.

One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of this invention. Indeed, the invention is no way limited to the methods and materials described herein. For purposes of the methods featured herein, the following terms are defined.

"Mammary fluid" includes breast milk and/or colostrum expressed from lactating female subjects. Whole mammary fluid, selected liquid or solid fractions of the mammary fluid, whole cells or cellular constituents, proteins, glycoproteins, peptides, nucleotides (including DNA and RNA polynucleotides) and other like biochemical and molecular constituents of the mammary fluid can be used in the present methods. The mammary fluid may be obtained from any number of species of female subjects including, but not limited to, humans, bovines, goats and the like.

"Identity marker" includes a marker that can be used to identify an individual subject from other subjects in a population. Such markers are present in the cells found in mammary fluid. Such markers could include, but are not limited to, genes, alleles, loci, antigens polypeptides or peptides.

An "identity marker profile" comprises a profile of a number of identity markers. The profile identifies the individual human or subject from other humans with a sufficient degree of certainty. It is contemplated that the identity marker profile identifies at least one human from 100,000 humans, or 1 human from 1 million humans or 1 human from 5 million humans.

"Nucleic acid typing" refers to a method of determining the DNA type profile of a biological or milk sample. Such methods include, but are not limited to: STR analysis, HLA

analysis or multiple gene analysis of genes/alleles/loci present in a polynucleotide sample of the biological or milk sample.

"DNA type profile" refers to a profile of a human's or subject's genomic DNA, which is sufficient to distinguish the individual human or subject from other humans with a sufficient degree of certainty. It is contemplated that the DNA profile identifies at least one human from 100,000 humans, or 1 human from 1 million humans or 1 human from 5 million humans. Generally the methods featured herein involve identifying alleles of at least 5 loci/genes or at least 10 loci/genes or at least 13 loci/gene.

An "allele" comprises one of the different nucleic acid sequences of a gene at a particular locus on a chromosome. One or more genetic differences can constitute an allele. Examples of HLA allele sequences are set out in Mason and Parham (1998) Tissue Antigens 51:417-66, which list HLA-A, HLA-B, and HLA-C alleles and Marsh et al (1992); and Hum. Immunol. 35:1, which list MLA Class II alleles for DRA, DRB, DQA1, DQB1, DPA1, and DPB1.

A "locus" comprises a discrete location on a chromosome. The loci may be part of a gene or part of repeat sequence. Exemplary human leukocyte antigens (HLAs) loci are the class I MHC genes designated HLA-A, HLA-B and HLA-C; nonclassical class I genes including HLA-E, HLA-F, HLA-G, HLA-H, HLA-J and HLA-X, MIC; and class II genes such as HLA-DP, HLA-DQ and HLA-DR. Exemplary STR loci are: CSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, DI8S51, D21S11, DYS19, F13A1, FES/FPS, FGA, HPRTB, THO1, TPDX, DYS388, DYS391, DYS392, DYS393, D2S1391, D18S535, D2S.1338, D19S433, D6S477, D1S518, D14S306, D22S684, F13B, CD4, D12S391, D10S220 and D7S523 (see, e.g., U.S. Pat. No. 6,090,558).

A method of HLA analysis or human leukocyte antigen analysis is a method that permits the determination or assignment of one or more genetically distinct human leukocyte antigen (HLA) genetic polymorphisms by any number of methods known in the art. Some methods contemplated are described below.

A method of STR analysis is a method that permits the determination or assignment of one or more genetically distinct STR genetic polymorphisms by any number of methods known in the art. Some methods contemplated are described herein.

A method of multiple gene analysis is a method that permits the determination or assignment of one or more genetically distinct genetic polymorphisms of human genes by any number of methods known in the art. Such genes may or may not include the HLA genes. Some methods contemplated are described herein.

A number of amplification techniques are known in the art. Amplifying refers to a reaction wherein a template nucleic acid, or portions thereof, is duplicated at least once. Such amplification techniques include arithmetic, logarithmic, or exponential amplification. The amplification of a nucleic acid can take place using any nucleic acid amplification system, both isothermal and thermal gradient based including, but not limited to, polymerase chain reaction (PCR), reverse-transcription-polymerase chain reaction (RT-PCR), ligase chain reaction (LCR), self-sustained sequence reaction (3SR), and transcription mediated amplifications (TMA). Typical nucleic acid amplification mixtures (e.g., PCR reaction mixture) include a nucleic acid template that is to be amplified, a nucleic acid polymerase, nucleic acid primer sequence(s), nucleotide triphosphates, and a buffer containing all of the ion species required for the amplification reaction.

Amplification products obtained from an amplification reaction typically comprise a single stranded or double stranded DNA or RNA or any other nucleic acid products of isothermal and thermal gradient amplification reactions that include PCR, LCR, and the like.

A "template nucleic acid" refers to a nucleic acid polymer that is sought to be copied or amplified. The template nucleic acid(s) can be isolated or purified from a cell, tissue, and the like. The template nucleic acid can comprise genomic DNA, eDNA, RNA, or the like.

Primers are used in some amplification techniques. A primer comprises an oligonucleotide used in an amplification reaction (e.g., PCR) to amplify a target nucleic acid. The primer is typically single stranded. The primer may be from about 5 to 30 nucleic acids in length, more commonly from about 10 to 25 nucleic acids in length.

An STR locus-specific primer is an oligonucleotide that hybridizes to a nucleic acid target variant that defines or partially defines that particular STR locus.

An HLA allele-specific primer is an oligonucleotide that hybridizes to a nucleic acid target variant that defines or partially defines that particular HLA allele.

HLA locus-specific primer is an oligonucleotide that permits the amplification of an HLA locus or that can hybridize specifically to an HLA locus.

An allele-specific primer is an oligonucleotide that hybridizes to a target nucleic acid variant that defines or partially defines that particular gene allele.

A locus-specific primer is an oligonucleotide that permits the amplification of a gene locus or that can hybridize specifically to a gene locus.

A forward primer and a reverse primer constitute a pair of primers that can bind to a template nucleic acid and under proper amplification conditions produce an amplification product. If the forward primer is binding to the sense strand then the reverse primer is binding to antisense strand. Alternatively, if the forward primer is binding to the antisense strand then the reverse primer is binding to sense strand. In essence, the forward or reverse primer can bind to either strand so long as the other reverse or forward primer binds to the opposite strand.

Any number of detectable labels can be used to detect a target nucleic acid by use of amplification or other techniques. A detectable label refers to a moiety that is attached through covalent or non-covalent techniques to an oligonucleotide or other detection agent. Examples of detectable labels include a radioactive moiety, a fluorescent moiety, a chemiluminescent moiety, a chromogenic moiety and the like. Fluorescent moieties comprise chemical entities that accepts radiant energy of one wavelength and emits radiant energy at a second wavelength.

Various hybridization techniques can be used in the methods described herein. Hybridizing or hybridization refers to the binding or duplexing of a molecule to a substantially complementary polynucleotide or fragment through bonding via base pairing. Hybridization typically involves the formation of hydrogen bonds between nucleotides in one nucleic acid and a complementary second nucleic acid. Methods of hybridization can include highly stringent, moderately stringent, or low stringency conditions.

The term "stringent conditions" refers to conditions under which a capture oligonucleotide, oligonucleotide or amplification product will hybridize to its target nucleic acid.

"Stringent hybridization conditions" or "highly stringent conditions" are sequence dependent and will be different with different environmental parameters (e.g., salt concentrations, and presence of organics). Generally, stringent conditions are selected to be about 5° C. to 20° C. lower than the thermal melting point ($T_m$) for a specific nucleic acid at a defined ionic strength and pH. Stringent conditions are about 5° C. to 10° C. lower than the thermal melting point for a specific nucleic acid bound to a complementary nucleic acid. The $T_m$ is the temperature (under defined ionic strength and pH) at which 50% of a nucleic acid hybridizes to a matched probe. Longer oligonucleotides hybridize at higher temperatures. Typically, stringent conditions will be those in which the salt concentration comprises about 0.01 to 1.0 M Na (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 300 C for short probes (e.g., 10 to 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. An extensive guide to the hybridization and washing of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes parts I and II, Elsevier, N.Y.; Choo (ed) (1994) Methods Molecular Biology Volume 33, In Situ Hybridization Protocols, Humana Press Inc., New Jersey; Sambrook et al., Molecular Cloning, A Laboratory Manual (2nd ed. 1989); Current Protocols in Molecular Biology, Ausubel et al., eds., (1994).

Hybridization conditions for a particular probe, primer and target are readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of single stranded nucleic acids to anneal with a complementary strands present in an environment below their inciting temperature. The higher the degree of desired homology between a probe and hybridizable target, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so.

Hybridization wash conditions are ordinarily determined empirically for hybridization of each probe or set of primers to a corresponding target nucleic acid. The target nucleic acid and probes/primers are first hybridized (typically under stringent hybridization conditions) and then washed with buffers containing successively lower concentrations of salts, or higher concentrations of detergents, or at increasing temperatures until the signal to noise ratio for specific to non-specific hybridization is high enough to facilitate detection of specific hybridization. Stringent temperature conditions will usually include temperatures in excess of about 30° C. more usually in excess of about 37° C., and occasionally in excess of about 45° C. Stringent salt conditions will ordinarily be less than about 1000 mM, usually less than about 500 mM, more usually less than about 400 mM, typically less than about 300 mM, typically less than about 200 mM, and more typically less than about 150 mM. However, the combination of parameters is more important than the measure of any single parameter. See, e.g., Wetmur et al., J. Mol Biol 31:349-70 (1966), and Wetmur, Critical Reviews Biochemistry and Molecular Biology 26 (34):227-59 (1991).

In one embodiment, highly stringent conditions comprise hybridization in 50% formamide, 6×SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5× Denhardt's solution, sonicated salmon sperm DNA (100/μg/ml), 0.5% SDS, and 10% dextran sulfate at 420 C, with washes at 42° C. in 2×SSC (sodium chloride/sodium citrate) and 0.1% SDS, followed by a high-stringency wash comprising of 0.2×SSC containing 0.1% SDS at 42° C.

The terms "complement," "complementarity" or "complementary," as used herein, are used to describe single-stranded polynucleotides related by the rules of antiparallel base-pairing. For example, the sequence 5'-CTAGT-3' is completely complementary to the sequence 5'-ACTAG-3'. Complementarity may be "partial," where the base pairing is less than 100%, or complementarity may be "complete" or "total," implying perfect 100% antiparallel complementation between two polynucleotides. By convention in the art, single-stranded nucleic acid molecules are written with their 5' ends to the left, and their 3' ends to the right.

The term "complementary base pair" refers to a pair of bases (nucleotides) each in a separate nucleic acid in which each base of the pair is hydrogen bonded to the other. A "classical" (Watson-Crick) base pair contains one purine and one pyrimidine; adenine pairs specifically with thymine (A-T), guanine with cytosine (G-C), uracil with adenine (U-A). The two bases in a classical base pair are said to be complementary to each other.

"Substantially complementary" between a probe or primer nucleic acid and a target nucleic acid embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired degree of hybridization and identification of hybridized target polynucleotides.

A "capture oligonucleotide" useful for identification of a target nucleic acid refers to a nucleic acid or fragment that can hybridize to a polynucleotide, oligonucleotide, amplification product, or the like, and has the ability to be immobilized to a solid phase. A capture oligonucleotide typically hybridizes to at least a portion of an amplification product containing complementary sequences under stringent conditions.

An "HLA locus-specific capture oligonucleotide" is a capture oligonucleotide that is complementary to and hybridizes to a conserved region of an HLA locus. For example, the capture oligonucleotide can be specific for the HLA-A locus and will hybridize to one or more conserved regions or subsequences of the HLA-A locus.

Similarly, an "STR locus-specific capture oligonucleotide" is a capture oligonucleotide that is complementary to and hybridizes to a conserved region of an STR locus. A locus-specific capture oligonucleotide is a capture oligonucleotide that is complementary to and hybridizes to a conserved region of a genetic locus.

A capture oligonucleotide is typically immobilized on a solid phase directly or indirectly. Such immobilization may be through covalent and/or non-covalent bonds.

The term "amplicon", is used herein to mean a population of DNA molecules that has been produced by amplification, e.g., by PCR.

The term "subject" refers to a lactating mammalian subject. The subject may be a human, bovine, goat and the like. For example, the screening as to the origination of milk products from non-human mammals may be important for the tracing of products to a particular bovine, for example, for FDA or other purposes.

"Mutation" as used herein sometimes refers to a functional polymorphism that occurs in the population, and is strongly correlated to a gene. "Mutation" is also used herein

to refer to a specific site and type of functional polymorphism, without reference to the degree of risk that particular mutation poses to an individual for a particular disease.

A "self-antigen" is an antigen which identifies the individual subject from other subjects in the population. Exemplary self-antigens include the major histocompatibility antigens (MHC) antigens or the blood type antigens (ABO).

A "self antigen profile" means a profile of a human's or subject's self-antigens which is sufficient to distinguish the individual subject from other subjects with a sufficient degree of certainty.

The term "antibody" is used in its broadest sense and covers polyclonal antibodies, monoclonal antibodies, single chain antibodies and antibody fragments.

*This disclosure features human milk compositions, e.g., pasteurized human milk compositions, and methods of making and using such compositions. The compositions include human milk fortifiers (e.g., PROLACTPLUS™ Human Milk Fortifiers, e.g., PROLACT+4™, PROLACT+6™, PROLACT+8™, and/or PROLACT+10™), which are produced from human milk and contain various concentrations of nutritional components, e.g., protein, fat, carbohydrates, vitamins, and/or minerals. These fortifiers can be added to the milk of a nursing mother to provide an optimal nutritional content of the milk for, e.g., a preterm infant. Depending on the content of mother's own milk, various concentrations of the fortifiers can be added to mother's milk. For example, the protein concentration of the mother's milk can be increased with the use of the fortifier. As mentioned above, the fortifiers of the present disclosure are generated from human milk and, therefore, provide infants with human-derived nutrients.*

*The disclosure also features standardized human milk formulations (exemplified by PROLACT20™, NEO20™, and/or PROLACT24), which are produced from human milk. Methods of making and using such compositions are also described herein. These standardized human milk formulations can be used to feed, e.g., preterm infants, without mixing them with other fortifiers or milk. They provide a nutritional human-derived formulation and can substitute for mother's milk. Human milk formulations can contain various caloric contents, for example, PROLACT24™ (a full-feed whole milk product) can contain about 24 Cal/oz or about 81 Cal/100 mL.*

*The methods featured herein are used to process large volumes of donor milk, e.g., about 75-2,000 liters/lot of starting material.*

*In one aspect, the disclosure features a pasteurized human milk composition that includes: a human protein constituent of about 35-85 mg/mL; a human fat constituent of about 60-110 mg/mL; and a human carbohydrate constituent of about 60-140 mg/mL. The carbohydrate constituent can include lactose. The composition can further include IgA and/or one or more constituents selected from the group consisting of: calcium, chloride, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc. In one embodiment, the composition can be mixed with raw human milk to provide a nutritional composition, wherein the raw human milk comprises about 80%, about 70%, about 60%, or about 50% of the nutritional composition.*

*Embodiments can include one or more of the following features.*

*In one embodiment, the composition can include the protein constituent of about 55-65 mg/mL; the fat constituent of about 85-95 mg/mL; and the carbohydrate constituent of about 70-120 mg/mL. The carbohydrate constituent can include lactose. The composition can further include IgA and/or one or more constituents selected from the group consisting of: calcium (e.g., at a concentration of about 4.0-5.5 mg/mL or at 2.00-2.90 mg/mL); chloride (e.g., at a concentration of about 0.35-0.95 mg/mL or at about 0.175-0.475 mg/mL); copper (e.g., at a concentration of about 0.0005-0.0021 mg/mL or at about 0.00025-0.001 mg/mL); iron (e.g., at a concentration of about 0.001-0.007 mg/mL or at about 0.0005-0.0025 mg/mL); magnesium (e.g., at a concentration of about 0.180-0.292 mg/mL or at about 0.090-0.170 mg/mL); manganese (e.g., at a concentration of about 0.010-0.092 mcg/mL or at about 0.005-0.046 mcg/mL;); phosphorus (e.g., at a concentration of about 2.00-3.05 mg/mL or at about 1.00-2.90 mg/mL, e.g., at about 1.00-1.50 mg/mL); potassium (e.g., at a concentration of about 1.90-2.18 mg/mL or at about 0.95-1.41 mg/mL); sodium (e.g., at a concentration of about 0.75-0.96 mg/mL or at about 0.375-0.608 mg/mL); and zinc (e.g., at a concentration of about 0.0200-0.0369 mg/mL or at about 0.010-0.0198 mg/mL). In one embodiment, the composition can be mixed with raw human milk to provide a nutritional composition, wherein the raw human milk comprises about 80%, about 70%, about 60%, or about 50% of the nutritional composition.*

*In another aspect, the disclosure features a pasteurized human milk composition that includes: a human protein constituent of about 11-20 mg/mL, e.g., about 11-13 mg/mL; a human fat constituent of about 35-55 mg/mL; and a human carbohydrate constituent of about 70-120 mg/mL, e.g., about 80-105 mg/mL. The carbohydrate constituent can include lactose. The caloric content of the composition can be about 0.64 to about 1.10 Cal/mL.*

*Embodiments can include one or more of the following features.*

*In one embodiment, the pasteurized human milk composition can further include one or more of the following components: calcium (e.g., at a concentration of about 0.40-1.50 mg/mL); chloride (e.g., at a concentration of about 0.30-0.80 mg/mL); copper (e.g., at a concentration of about 0.0005-0.0021 mg/mL); iron (e.g., at a concentration of about 0.001-0.005 mg/mL); magnesium (e.g., at a concentration of about 0.03-0.13 mg/mL); manganese (e.g., at a concentration of about 0.01-0.092 mcg/mL); phosphorus (e.g., at a concentration of about 0.15-0.631 mg/mL); potassium (e.g., at a concentration of about 0.60-1.20 mg/mL); sodium (e.g., at a concentration of about 0.20-0.60 mg/mL); and/or zinc (e.g., at a concentration of about 0.0025-0.0120 mg/mL).*

*The disclosure also features method of making various human milk compositions.*

*In one aspect, the disclosure features a method for obtaining a pasteurized human milk composition. The method includes: (a) genetically screening human milk for one or more viruses; (b) filtering the milk; (c) heat-treating the milk, e.g., at about 63° C. or greater for about 30 minutes; (d) separating the milk into cream and skim; (e) adding a portion of the cream to the skim; and (f) pasteurizing.*

*Embodiments include one or more of the following features.*

*In one embodiment, the method can further include filtering the skim through filters after step (d), e.g., to filter the water out of the skim. After filtering the skim after step (d), the filters used in the filtering can be washed to obtain a post wash solution. The post wash solution can be added to the skim.*

*The genetic screening in step (a) can be polymerase chain reaction and/or can include screening for one or more viruses, e.g., HIV-1, HBV, and/or HCV. The milk can be*

*filtered through an about 200 micron screen in step (b). The method can further include running cream, e.g., about 30-50% of cream, through a separator following step (d). The composition of post wash and skim can include about 7.0-7.2% of protein.*

*The method can further include carrying out mineral analysis of the portion of the composition obtained after step (e). The method can also include adding to the composition obtained after step (e) one or more minerals selected from the group consisting of: calcium, chloride, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc. Adding of the one or more minerals can include heating the composition.*

*The method can also include cooling the composition after step (f), carrying out biological testing of a portion of the composition after step (f), and/or carrying out nutritional testing of a portion of the composition after step (f).*

*The human milk of step (a) can be pooled human milk. The methods featured herein can be carried out with large volumes of the starting material, e.g., human milk, e.g., pooled human milk. The volumes can be in the range of about 75-2,000 liters/lot of starting material.*

*The composition obtained after step (f) can include about 8.5-9.5% fat, about 6.3-7.0% protein, and about 8.0-10.5% lactose.*

*In another aspect, the disclosure features a method for obtaining a pasteurized human milk composition. The method includes: (a) genetically screening human milk for one or more viruses; (b) filtering the milk; (c) adding cream; and (d) pasteurizing.*

*Embodiments can include one or more of the following features.*

*In one embodiment, the genetic screening in step (a) can be polymerase chain reaction. The genetic screening can include screening for one or more viruses, e.g., HIV-1, HBV, and/or HCV.*

*The milk can be filtered through an about 200 micron screen in step (b). The method can further include ultra filtering the whole milk after step (b) through filters. The filters used during ultra filtering can be post washed.*

*The composition can be cooled after step (d). Biological and/or nutritional testing of the composition can be carried out after step (d).*

*Human milk of step (a) can be pooled human milk. The methods featured herein can be carried out with large volumes of the starting material, e.g., human milk, e.g., pooled human milk. The volumes can be in the range of about 75-2,000 liters/lot of starting material.*

*The method can also include adding to the composition obtained after step (c) one or more minerals selected from the group consisting of: calcium, chloride, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc. In one embodiment, the composition obtained after step (d) can include about 11-20 mg/mL protein, about 35-55 mg/mL fat, and about 70-120 mg/mL carbohydrates.*

*In another aspect, the disclosure features a kit that includes the pasteurized human milk compositions featured herein (e.g., a fortifier) and a graduated container (e.g., a bottle, a syringe, and a can) for mixing the featured compositions with raw human milk.*

*In yet another aspect, the disclosure features a method of obtaining a nutritional milk composition. The method includes adding the pasteurized human milk compositions featured herein (e.g., fortifiers) to raw human milk, thereby increasing the nutritional concentration of the raw human milk. The caloric composition of the raw human milk can be increased by about 2-10 Cal/oz.*

*In another aspect, the disclosure features a method of providing supplemental nutrients to a premature human infant, the method comprising adding the compositions (fortifiers) featured herein to raw human milk to obtain a mixture and administering the mixture to the premature infant.*

*The terms "premature", "preterm" and "low-birth-weight (LBW)" infants are used interchangeably and refer to infants born less than 37 weeks gestational age and/or with birth weights less than 2500 gm.*

*By "whole milk" is meant milk from which no fat has been removed.*

*By "bioburden" is meant microbiological contaminants and pathogens (generally living) that can be present in milk, e.g., viruses, bacteria, mold, fungus and the like.*

*The compositions of the present disclosure are generated from human donor milk, e.g., pooled milk, which undergoes rigorous genetic screening, processing (e.g., to concentrate nutrients in the fortifier compositions, and/or to reduce bioburden), and pasteurization. The milk can be supplemented with various minerals and/or vitamins. Thus, the disclosure also features methods of obtaining and processing milk from human donors.*

The details of one or more embodiments of the methods featured herein are set forth in the description below. Other features, objects, and advantages of the methods will be apparent from the description and the claims.

All patents, patent applications, and references cited herein are incorporated in their entireties by reference.

## DETAILED DESCRIPTION

In one aspect, the methods featured herein are used to determine milk origination. For example, in order to ensure that human breast milk received from a specific human actually comes from that human, methods of identity testing are needed on samples of milk received from each human. Testing donors to confirm their identity improves safety of donated milk. It ensures the provenance of the donated milk, which as discussed above, is most often donated without supervision of personnel of the organization that will be receiving the milk, e.g., a milk bank center. Testing donor identity by the methods featured herein allows for multiple donations by the same donor, whose identity can be confirmed at the time of each donation. The donor can live at any distance from the donation and/or processing facilities, as she can send her milk at long distances, and her identity can be confirmed based on reference samples or reference test results stored at the donation and/or processing facility.

As part of the qualification process for donating milk, each potential milk donor will be identified by biological methods (e.g., biological fingerprinting, as described herein). The identifying characteristics of the individual (i.e., at least one identity marker) will also be present in the donor's milk. Such characteristics will be used to match the donated milk with a specific donor.

Obtaining a Reference Biological Sample

The methods featured herein include, inter alia, obtaining at least one donated reference sample from a potential mammary fluid donor, e.g., a human breast milk donor. Such sample may be obtained by methods known in the art such as, but not limited to, a cheek swab sample of cells, or a drawn blood sample, milk, saliva, hair roots, or other convenient tissue. Samples of reference donor nucleic acids (e.g., genomic DNA) can be isolated from any convenient biological sample including, but not limited to, milk, saliva, buccal cells, hair roots, blood, and any other suitable cell or

tissue sample with intact interphase nuclei or metaphase cells. The sample is labeled with a unique reference number. The sample can be analyzed at or around the time of obtaining the sample for one or more markers that can identify the potential donor. Results of the analysis can be stored, e.g., on a computer-readable medium. Alternatively, or in addition, the sample can be stored and analyzed for identifying markers at a later time.

It is contemplated that the biological reference sample may be DNA typed by methods known in the art such as STR analysis of STR loci, HLA analysis of HLA loci or multiple gene analysis of individual genes/alleles (further discussed below). The DNA-type profile of the reference sample is recorded and stored, e.g., on a computer-readable medium.

It is further contemplated that the biological reference sample may be tested for self-antigens using antibodies known in the art or other methods to determine a self-antigen profile. The antigen (or another peptide) profile can be recorded and stored, e.g., on a computer-readable medium.

Testing a Sample of Donated Mammary Fluid

A subject desiring to donate mammary fluid will express the mammary fluid (breast milk) using standard procedures. The mammary fluid is typically collected in containers useful for shipping and storage. The mammary fluid can be frozen prior to donation. The mammary fluid may be frozen at the donation facility or processing facility for later analysis and use or analyzed without freezing. One or more of the containers with donated fluid can be used for obtaining a test sample. The test sample is taken for identification of one or more identity markers.

Methods of obtaining a sample of expressed frozen fluid include a stainless steel boring tool used to drill a core the entire length of the container. Alternatively, a sample may be scraped from the surface of the frozen mammary fluid. The container may contain a separate portion for collection of a sample of the expressed mammary fluid, and this portion may be removed as the sample for testing. Where the mammary fluid is in liquid form it is contemplated that the method for obtaining the test sample will be by pipette or other means.

A sample of the donated the mammary fluid is analyzed for the same marker or markers as the donor's reference sample. The marker profiles of the reference biological sample and of the donated mammary fluid are compared. The match between the markers (and lack of any additional unmatched markers) would indicate that the donated milk comes from the same individual as the one who donated the reference sample. Lack of a match (or presence of additional unmatched markers) would indicate that the donated mammary fluid either comes from a non-tested donor or has been contaminated with fluid from a non-tested donor.

The donated mammary fluid sample and the donated reference biological sample can be tested for more than one marker. For example, each sample can be tested for multiple DNA markers and/or peptide markers. Both samples, however, need to be tested for at least some of the same markers in order to compare the markers from each sample.

Thus, the reference sample and the donated mammary fluid sample may be tested for the presence of differing identity marker profiles. If there are no identity marker profiles other than the identity marker profile from the expected subject, it generally indicates that there was no fluid (e.g., milk) from other humans or animals contaminating the donated mammary fluid. If there are signals other than the expected signal for that subject, the results are indicative of contamination. Such contamination will result in the mammary fluid (e.g., milk) failing the testing.

The testing of the reference sample and of the donated mammary fluid can be carried out at the donation facility and/or milk processing facility. The results of the reference sample tests can be stored and compared against any future donations by the same donor.

It is contemplated that samples from a number of milk containers from the same subject may be pooled for identity marker testing. It is contemplated that at least 2 samples, at least 5 samples or at least 8 samples may be pooled for testing.

It is contemplated that the test sample of the donated mammary fluid may be tested by nucleic acid typing using methods known in the art, such as, STR analysis of STR loci, HLA analysis of HLA loci or multiple gene analysis of individual genes/alleles to obtain the DNA-type of the milk sample. The donated mammary fluid can also be tested for peptide profiles, e.g., antigen profile.

The DNA-type or another biological profile (i.e., identity profile (s)) of the donated mammary fluid test sample (s) will be compared to the reference DNA-type or another biological profile for the putative donor. A match or identity of the DNA-type or biological profile will indicate that the mammary fluid was obtained from a same (i.e., a specified subject).

Use of the Donated and Tested Mammary Fluid

The mammary fluid tested by the methods featured herein can be processed for further use. The donation facility and milk processing facility can be the same or different facility. The donated milk whose provenance has been confirmed can be processed, e.g., to obtain human milk fortifiers, standardized human milk formulations, and/or human lipid compositions. As discussed above, testing the mammary fluid to confirm the identity of the donor ensures safety of the mammary fluid and any products derived from such fluid.

Processing of human milk to obtain human milk fortifiers (e.g., PROLACTPLUS™ Human Milk Fortifiers, e.g., PROLACT+4™, PROLACT+6™, PROLACT+8™, and/or PROLACT+10™, which are produced from human milk and contain various concentrations of nutritional components) and the compositions of the fortifiers are described in U.S. patent application Ser. No. 11/947,580, filed on Nov. 29, 2007, the contents of which are incorporated herein in their entirety. These fortifiers can be added to the milk of a nursing mother to provide an optimal nutritional content of the milk for, e.g., a preterm infant. Depending on the content of mother's own milk, various concentrations of the fortifiers can be added to mother's milk.

Methods of obtaining standardized human milk formulations (exemplified by PROLACT20™, NEO20™, and/or PROLACT24) and formulations themselves are also discussed in U.S. patent application Ser. No. 11/947,580, filed on Nov. 29, 2007, the contents of which are incorporated herein in their entirety. These standardized human milk formulations can be used to feed, e.g., preterm infants, without mixing them with other fortifiers or mills. They provide a nutritional human-derived formulation and can substitute for mother's milk.

Compositions that include lipids from human milk, methods of obtaining such compositions, and methods of using such compositions to provide nutrition to patients are described in PCT Application PCT/US07/86973 filed on Dec. 10, 2007, the contents of which are incorporated herein in their entirety.

Methods of obtaining other nutritional compositions from human milk that can be used with the methods featured

herein are discussed in U.S. patent application Ser. No. 11/012,611, filed on Dec. 14, 2004, and published as U.S. 2005/0100634 on May 12, 2005, the contents of which are incorporated herein in their entirety.

Processing of milk that has been tested with the methods featured herein can be carried out with large volumes of donor milk, e.g., about 75 liters/lot to about 2,000 liters/lot of starting material.

The methods featured herein can also be integrated with methods of facilitating collection and distribution of human milk over a computer network, e.g., as described in U.S. patent application Ser. No. 11/526,127, filed on Sep. 22, 2006, and published as U.S. 2007/0098863 on May 3, 2007; and in U.S. patent application Ser. No. 11/679,546, filed on Feb. 27, 2007, and published as U.S. 2007/0203802 on Aug. 30, 2007. The contents of both applications are incorporated herein in their entireties.

*As discussed above, donor milk is screened to ensure the identity of the donors and reduce the possibility of contamination. Donor milk is pooled and further screened (step 1), e.g., genetically screened (e.g., by PCR). The screening can identify, e.g., viruses, e.g., HIV-1, HBV, and/or HCV. Milk that tests positive is discarded. After the screening, the composition undergoes filtering (step 2). The milk is filtered through about a 200 micron screen and then ultrafiltered. During ultrafiltration, water is filtered out of the milk (and is referred to as permeate) and the filters are postwashed using the permeate. Post wash solution is added to the milk to recover any lost protein and increase the concentration of the protein to, e.g., about 1.2% to about 1.5%. Cream from another lot (e.g., excess cream from a previous fortifier lot) is added in step 3 to increase the caloric content. At this stage of the process, the composition generally contains: about 3.5% to 5.5% of fat; about 1.1% to 1.3% of protein; and about 8% to 10.5% of carbohydrates, e.g., lactose.*

*At this stage, the composition can be frozen and thawed out for further processing later.*

*Optionally, if the human milk formulation is to be fortified with minerals, a mineral analysis of the composition is carried out after step 3. Once the mineral content is known, a desired amount of minerals can be added to achieve target values.*

*In step 4, the composition is pasteurized. Pasteurization methods are known in the art. For example, the product can be pasteurized in a tank that is jacketed. Hot glycol can be use to heat up the tank. The product temperature can be about 63° C. or greater and the air temperature above the product about 66° C. or greater. The product is pasteurized for a minimum of about 30 minutes. Other pasteurizing techniques are known in the art.*

*After cooling to about 2 to 8° C., the product is filled into containers of desired volumes and various samples of the human milk formulation are taken for nutritional and bioburden analysis. The nutritional analysis ensures proper content of the composition. A label generated for each container reflects the nutritional analysis. The bioburden analysis tests for presence of contaminants, e.g., total aerobic count, B. cereus, E. coli, Coliform, Pseudomonas, Salmonella, Staphylococcus, yeast, and/or mold. The product is packaged and shipped once the analysis is complete and desired results are achieved.*

*The standardized human milk formulations featured herein can be used in lieu of mother's own milk to feed the infants, e.g., premature infants. They include various nutritional components for infant growth and development.*

*In one embodiment, the standardized human milk formulation can include: a human protein constituent of about 11-20 mg/mL; a human fat constituent of about 35-55 mg/mL; and a human carbohydrate constituent of about 70-120 mg/mL. In a particular embodiment, the formulation can contain: a human protein constituent of about 11-13 mg/mL; a human fat constituent of about 35-55 mg/mL; and a human carbohydrate constituent of about 80-105 mg/mL. The total caloric content of the formulations can be, e.g., from about 0.68 Cal/mL to about 0.96 Cal/mL.*

*The milk formulation can be supplemented with vitamins and/or minerals. In one embodiment, the composition can include: calcium concentration of about 0.40-1.50 mg/mL; chloride concentration of about 0.30-0.80 mg/mL; copper concentration of about 0.0005-0.0021 mg/mL; iron concentration of about 0.001-0.005 mg/mL; magnesium concentration of about 0.03-0.13 mg/mL; manganese concentration of about 0.01-0.092 mg/mL; phosphorus concentration of about 0.15-0.631 mg/mL (e.g., about 0.15-0.60 mg/mL); potassium concentration of about 0.60-1.20 mg/mL; sodium concentration of about 0.20-0.60 mg/mL; and zinc concentration of about 0.0025-0.0120 mg/mL.*

*The human milk formulations can contain various caloric content, e.g., 67 Kcal/dL (20 Calorie per ounce), and 81 Kcal/dL (24 Calorie per ounce). An exemplary human milk formulation (e.g., PROLACT24™) can include the following constituents: human milk, calcium glycerophosphate, potassium citrate, calcium gluconate, calcium carbonate, magnesium phosphate, sodium chloride, sodium citrate, zinc sulfate, cupric sulfate, and manganese sulfate. This exemplary composition can have the following characteristics per 100 ml: about 81 Cal; about 4.4 g of total fat; about 20.3 mg of sodium; about 60.3 mg of potassium; about 8 g total carbohydrates; about 5-9 g of sugars; about 2.3 g of protein; about 180-250 IU of Vitamin A; less than about 1.0 mg of Vitamin C; about 40.0-150.0 mg of calcium; about 100-150 mcg of iron; about 15-50 mg of phosphorus; about 3-10 mg of magnesium; about 25-75.0 mg of chloride; about 1.2 mcg of zinc; about 140-190 mcg of copper; less than about 60.2 mcg of manganese; and Osmolarity of about 322 mOsm/Kg $H_2O$. Milk formulations with other constituents and constituents of different concentrations are encompassed by this disclosure.*

*The osmolality of human milk fortifiers and standardized milk formulations featured herein can affect adsorption, absorption, and digestion of the compositions. High osmolality, e.g., above about 400 mOsm/Kg $H_2O$, has been associated with increased rates of necrotizing enterocolitis (NEC), a gastrointestinal disease that affects neonates (see, e.g., Srinivasan et al., Arch. Dis. Child Fetal Neonatal Ed. 89:514-17, 2004). The osmolality of the human milk composition and fortifier (once mixed with raw milk) of the disclosure is typically less than about 400 mOsm/Kg $H_2O$. Typically the osmolality is from about 310 mOsm/Kg of water to about 380 mOsm/Kg of water. The osmolality can be adjusted by methods known in the art.*

Nucleic Acid Identity Marker Profiles

As discussed above, samples of reference donor nucleic acids (e.g., genomic DNA) are isolated from any convenient biological sample including, but not limited to, milk, saliva, buccal cells, hair roots, blood, and any other suitable cell or tissue sample with intact interphase nuclei or metaphase cells.

Methods for isolation of nucleic acids (e.g., genomic DNA) from these various sources are described in, for example, Kirby, DNA Fingerprinting, An Introduction, W. H. Freeman & Co. New York (1992). Nucleic acids (e.g., genomic DNA) can also be isolated from cultured primary

or secondary cell cultures or from transformed cell lines derived from any of the aforementioned tissue samples.

Samples of RNA can also be used. RNA can be isolated as described in Sambrook et al., supra, RNA can be total cellular RNA, mRNA, poly A+ RNA, or any combination thereof. For best results, the RNA is purified, but can also be unpurified cytoplasmic RNA. RNA can be reverse transcribed to form DNA which is then used as the amplification template, such that PCR indirectly amplifies a specific population of RNA transcripts. See, e.g., Sambrook et al., supra, and Berg et al., Hum. Genet. 85:655-658 (1990).

Short tandem repeat (STR) DNA markers, also referred to as microsatellites or simple sequence repeats (SSRs) or DNA tandem nucleotide repeat ("DTNR"), comprise tandem repeated DNA sequences with a core repeat of 2-6 base pairs (bp). STR markers are readily amplified during PCR by using primers that bind in conserved regions of the genome flanking the repeat region.

Commonly sized repeats include dinucleotides, trinucleotides, tetranucleotides and larger. The number of repeats occurring at a particular genetic locus varies from a few to hundreds depending on the locus and the individual. The sequence and base composition of repeats can vary significantly, including a lack of consistency within a particular nucleotide repeat locus. Thousands of STR loci have been identified in the human genome and have been predicted to occur as frequently as once every 15 kb. Population studies have been undertaken on dozens of these STR markers as well as extensive validation studies in forensic laboratories. Specific primer sequences located in the regions flanking the DNA tandem repeat region have been used to amplify alleles from STR loci via the polymerase chain reaction ("PCR"). The PCR products include the polymorphic repeat regions, which vary in length depending on the number of repeats or partial repeats, and the flanking regions, which are typically of constant length and sequence between samples.

The number of repeats present for a particular individual at a particular locus is described as the allele value for the locus. Because most chromosomes are present in pairs, PCR amplifications of a single locus commonly yields two different sized PCR products representing two different repeat numbers or allele values. The range of possible repeat numbers for a given locus, determined through experimental sampling of the population, is defined as the allele range, and may vary for each locus, e.g., 7 to 15 alleles. The allele PCR product size range (allele size range) for a given locus is defined by the placement of the two PCR primers relative to the repeat region and the allele range. The sequences in regions flanking each locus must be fairly conserved in order for the primers to anneal effectively and initiate PCR amplification. For purposes of genetic analysis di-, tri-, and tetranucleotide repeats in the range of 5 to 50 are typically utilized in screens. Forensic laboratories use tetranucleotide loci (i.e., 4 bp in the repeat) due to the lower amount of "stutter" produced during PCR (Stutter products are additional peaks that can complicate the interpretation of DNA mixtures by appearing in front of regular allele peaks). The number of repeats can vary from 3 or 4 repeats to more than 50 repeats with extremely polymorphic markers. The number of repeats and hence the size of the PCR product, may vary among samples in a population making STR markers useful in identity testing of genetic mapping studies.

There are 13 core STR loci identified in the United States CODIS database. These STR loci are THO1, TPDX, CSF1PO, VWA, FGA, D3S1358, D5S818, D7S820, D13S317, D16S539, D8S1179, D18S51 and D21S11. The sex-typing marker amelogenin, is also included in the STR multiplexes that cover the 13 core STR loci. The 13 CODIS STR loci are covered by the Profiler Plus™ and COfiler™ kits from Applied Biosystems (ABI) (Foster City, Calif.). It is contemplated that the following STR loci may be used in this invention: CSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, DYS19, F13A1, FESfFPS, FGA, HPRTB, THO1, TPDX, DYS388, DYS391, DYS392, DYS393, D2S1391, D18S535, D2S1338, D19S433, D6S477, D1S518, D14S306, D22S684, F13B, CD4, D12S391, D10S220 and D7S523 (the sequence of each loci is incorporated herein by reference). With the exception of D3 S1358, sequences for the STR loci of this invention are accessible to the general public through GenBank (see U.S. Pat. No. 6,090,558, incorporated herein by reference). Other STR loci have been developed by commercial manufacturers and studied extensively by forensic scientists. These include all of the GenePrint™ tetranucleotide STR systems from Promega Corporation (Madison Wis.).

Many different primers have been designed for various STR loci and reported in the literature. These primers anneal to DNA segments outside the DNA tandem repeat region to produce PCR products containing the tandem repeat region. These primers were designed with polyacrylamide gel electrophoretic separation in mind as a method of detection/measurement, because DNA separations have traditionally been performed by slab gel or capillary electrophoresis. STR multiplex analysis is usually performed with PCR amplification and detection of multiple markers. STR multiplexing is most commonly performed using spectrally distinguishable fluorescent tags and/or non-overlapping PCR product sizes. Multiplex STR amplification in one or two PCR reactions with fluorescently labeled primers and measurement with gel or capillary electrophoresis separation and laser induced fluorescence detection is a standard method. The STR alleles from these multiplexed PCR products typically range in size from 100-800 bp with commercially available lots.

Gel-based systems are capable of multiplexing the analysis of 2 or more STR loci using two approaches. The first approach is to size partition the different PCR product loci. Size partitioning involves designing the PCR primers used to amplify different loci so that the allele PCR product size range for each locus covers a different and separable part of the gel size spectrum. As an example, the PCR primers for Locus A might be designed so that the allele size range is from 250 to 300 nucleotides, while the primers for Locus B are designed to produce an allele size range from 340 to 410 nucleotides.

The second approach to multiplexing 2 or more STR loci on gel-based systems is the use of spectroscopic partitioning. Current state of the art for gel-based systems involves the use of fluorescent dyes as specific spectroscopic markers for different PCR amplified loci. Different chromophores that emit light at different color wavelengths provide a method for differential detection of two different PCR products even if they are exactly the same size, thus 2 or more loci can produce PCR products with allele size ranges that overlap. For example, Locus A with a green fluorescent tag produces an allele size range from 250 to 300 nucleotides, while Locus B with a red fluorescent tag produces an allele size range of 270 to 330 nucleotides. A scanning, laser-excited fluorescence detection device monitors the wavelength of emissions and assigns different PCR product sizes, and their corresponding allele values, to their specific loci based on their fluorescent color.

It is contemplated that a mass spectrometry approach to STR typing and analysis, examining smaller nucleic acid oligomers may be used because the sensitivity of detection and mass resolution are superior with smaller oligomers. Application of STR analysis to time of flight-mass spectrometry (TOF-MS) requires the development of primer sets that produce small PCR products 50 to 160 nucleotides in length, typically about 50 to 100 nucleotides in length. Amplified nucleic acids may also be used to generate single stranded products that are in the desired size range for TOF-MS analysis by extending a primer in the presence of a chain termination reagent. A typical class of chain termination reagent commonly used by those of skill in the art is the dideoxynucleotide triphosphates. Again, application of STR analysis to TOF-MS requires that the primer be extended to generate products of 50 to 160 nucleotides in size, and typically about 50 to 100 nucleotides in length (see U.S. Pat. No. 6,090,558 incorporated by reference).

A biotinylated cleavable oligonucleotide is used as a primer in each assay and is incorporated through standard nucleic acid amplification (i.e., PCR) methodologies into the final product which is measured in the mass spectrometer. This process is described in, for example, U.S. Pat. No. 5,700,642 and U.S. Pat. No. 6,090,558 (see also Butler et al., International Journal of Legal Medicine 112(1) 45-59 (1998)). The STR assay involves a PCR amplification step where one of the primers is replaced by a cleavable biotinylated primer. The biotinylated PCR product is then captured on streptavidin-coated magnetic beads for post-PCR sample cleanup and salt removal, followed by mass spectrometry analysis.

Single nucleotide polymorphisms (SNPs) represent another form of DNA variation that is useful for human identity testing. SNPs are the most frequent form of DNA sequence variation in the genomes of organisms and are becoming increasingly popular genetic markers for genome mapping studies and medical diagnostics. SNPs are typically bi-allelic with two possible nucleotides (nt) or alleles at a particular site in the genome. Because SNPs are less polymorphic (i.e., have fewer alleles) than the currently used STR markers, more SNP markers are required to obtain the same level of discrimination between samples. Approximately 30-50 unlinked SNPs may be required to obtain the matching probabilities of 1 in 100 billion as seen with the 13 CODIS STRs.

A SNP assay typically involves a three-step process: (1) PCR amplification (2) phosphatase removal of nucleotides, and (3) primer extension using a biotinylated cleavable primer with dideoxynucleotides for single-base addition of the nucleotide (s) complementary to the one(s) at the SNP site (Li et al., Electrophoresis 20(6): 1258-1265 (1999)).

Simultaneous analysis of multiple SNP markers (i.e. multiplexing) is possible by simply putting the cleavage sites at different positions in the various primers so that they do not overlap on a mass scale.

The most common means for amplification is polymerase chain reaction (PCR), as described in U.S. Pat. Nos. 4,683,195, 4,683,202, 4,965,188 each of which is hereby incorporated by reference in its entirety. If PCR is used to amplify the target regions in blood cells, heparinized whole blood should be drawn in a sealed vacuum tube kept separated from other samples and handled with clean gloves. For best results, blood should be processed immediately after collection; if this is impossible, it should be kept in a sealed container at about 4° C. until use. Cells in other physiological fluids may also be assayed. When using any of these fluids, the cells in the fluid should be separated from the fluid component by centrifugation.

To amplify a target nucleic acid sequence in a sample by PCR, the sequence must be accessible to the components of the amplification system. One method of isolating target DNA is crude extraction which is useful for relatively large samples. Briefly, mononuclear cells from samples of blood, buccal cells, or the like are isolated. The pellets are stored frozen at −200C until used (U.S. Patent Publication No. 20040253594 which is incorporated by reference).

The pellets may be resuspended in lysis solution from the PUREGENE® DNA isolation kit (Cat#D-5000, GENTRA, Minneapolis, Minn.) containing 100/ug/ml of proteinase K. After incubating at 55° C. overnight, DNA extraction is performed according to manufacturers recommendations. The DNA samples are resuspended in aqueous solution and stored at −200C.

When the sample contains a large number of cells, extraction may be accomplished by methods as described in Higuchi, "Simple and Rapid Preparation of Samples for PCR", in PCR Technology, p. 31-43 Ehrlich, H. A. (ed.), Stockton Press, New York.

A relatively easy procedure for extracting DNA for PCR is a salting out procedure adapted from the method described by Miller et al., Nucleic Acids Res. 16:1215 (1988), which is incorporated herein by reference. Nucleated cells are resuspended in 3 ml of lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM Na2 EDTA, pH 8.2). Fifty μl of a 20 mg/ml solution of proteinase K and 200 ul of a 20% SDS solution are added to the cells and then incubated at 37° C. overnight. Following adequate digestion, one ml of a 6M NaCl solution is added to the sample and vigorously mixed. The resulting solution is centrifuged for 15 minutes at 3000 rpm. The pellet contains the precipitated cellular proteins, while the supernatant contains the DNA. The supernatant is removed to a 15 ml tube that contains 4 ml of isopropanol. The contents of the tube are mixed gently until the water and the alcohol phases have mixed and a white DNA precipitate has formed. The DNA precipitate is placed in distilled water and dissolved. (U.S. Patent Publication No. 20040253594 which is incorporated by reference).

Kits for the extraction of high-molecular weight DNA for PCR include PUREGENE® DNA Isolation kit (D-5000) GENTRA, a Genomic Isolation Kit A. S.A. P.® (Boehringer Mannheim, Indianapolis, Ind.), Genomic DNA Isolation System (GIBCO BRL, Gaithdrsburg, Md.), ELU-QUIK® DNA Purification Kit (Schleicher & Schnell, Keene, N.H.), DNA Extraction Kit (Stratagene, LaJolla, Calif.), TURBOGEN® Isolation Kit (Invitrogen, San Diego, Calif.), and the like. Use of these kits according to the manufacturer's instructions is generally acceptable for purification of DNA prior to practicing the methods of the invention (U.S. Patent Publication No. 20040253594 which is incorporated by reference).

The concentration and purity of the extracted DNA can be determined by spectrophotometric analysis of the absorbance of a diluted aliquot at 260 nm and 280 nm.

After extraction of the DNA, PCR amplification may proceed. The first step of each cycle of the PCR involves the separation of the nucleic acid duplex formed by the primer extension. Once the strands are separated, the next step in PCR involves hybridizing the separated strands with primers that flank the target sequence. The primers are then extended to form complementary copies of the target strands. For successful PCR amplification, the primers are designed so that the position at which each primer hybridizes along a

duplex sequence is such that an extension product synthesized from one primer, when separated from the template (complement), serves as a template for the extension of the other primer. The cycle of denaturation, hybridization, and extension is repeated as many times as necessary to obtain the desired amount of amplified nucleic acid (U.S. Patent Publication No. 20040253594 which is incorporated by reference).

In one embodiment of PCR amplification, strand separation is achieved by heating the reaction to a sufficiently high temperature for a sufficient time to cause the denaturation of the duplex but not to cause an irreversible denaturation of the polymerase (see U.S. Pat. No. 4,965,188, incorporated herein by reference). Typical heat denaturation involves temperatures ranging from about 80° C. to about 105° C. for times ranging from seconds to minutes. Strand separation, however, can be accomplished by any suitable denaturing method including physical, chemical, or enzymatic means. Strand separation may be induced by a helicase, for example, or an enzyme capable of exhibiting helicase activity. For example, the enzyme RecA has helicase activity in the presence of ATP. The reaction conditions suitable for strand separation by helicases are known in the" art (see Kuhn et al., 1979, CSH-Quantitative Biology, 43:63-67; and Radding, 1982, Ann. Rev, Genetics 16:405-437, incorporated by reference).

Template-dependent extension of primers in PCR is catalyzed by a polymerizing agent in the presence of adequate amounts of four deoxyribonucleotide triphosphates (typically dATP, dGTP, dCTP, and dTTP) in a reaction medium comprised of the appropriate salts, metal cations, and pH buffering systems. Suitable polymerizing agents are enzymes known to catalyze template-dependent DNA synthesis. In some cases, the target regions may encode at least a portion of a protein expressed by the cell. In this instance, mRNA may be used for amplification of the target region. Alternatively, PCR can be used to generate a cDNA library from RNA for further amplification, the initial template for primer extension is RNA. Polymerizing agents suitable for synthesizing a complementary copy-DNA (cDNA) sequence from the RNA template are reverse transcriptase (RT), such as avian myeloblastosis virus RT, Moloney murine leukemia virus RT, or Thermus thermophilus (Tth) DNA polymerase, a thermostable DNA polymerase with reverse transcriptase activity marketed by Perkin Elmer Cetus, Inc. Typically, the genomic RNA template is heat degraded during the first denaturation step after the initial reverse transcription step leaving only DNA template. Suitable polymerases for use with a DNA template include, for example, E. coli DNA polymerase I or its Klenow fragment, T4 DNA polymerase, Tth polymerase, and Taq polymerase, a heat-stable DNA polymerase isolated from Thermus aquaticus and commercially available from Perkin Elmer Cetus, Inc. The latter enzyme is widely used in the amplification and sequencing of nucleic acids. The reaction conditions for using Taq polymerase are known in the art (U.S. Patent Publication No. 20040253594 which is incorporated by reference).

Allele-specific PCR differentiates between target regions differing in the presence or absence of a variation or polymorphism. PCR amplification primers are chosen which bind only to certain alleles of the target sequence. This method is described by Gibbs, Nucleic Acid Res. 17:2437-2448 (1989) (U.S. Patent Publication No. 20040253594 which is incorporated by reference).

Further diagnostic screening methods employ the allele-specific oligonucleotide (ASO) screening methods, as described by Saiki et al., Nature 324:163-166 (1986). Oligonucleotides with one or more base pair mismatches are generated for any particular allele. ASO screening methods detect mismatches between variant target genomic or PCR amplified DNA and non-mutant oligonucleotides, showing decreased binding of the oligonucleotide relative to a mutant oligonucleotide. Oligonucleotide probes can be designed that under low stringency will bind to both polymorphic forms of the allele, but which at high stringency, bind to the allele to which they correspond. Alternatively, stringency conditions can be devised in which an essentially binary response is obtained, i.e., an ASO corresponding to a variant form of the target gene will hybridize to that allele, and not to the wild-type allele (U.S. Patent Publication No. 20040253594 which is incorporated by reference).

Target regions of a subject's DNA can be compared with the mammary fluid sample by ligase-mediated allele detection. Ligase may also be used to detect point mutations in the ligation amplification reaction described in Wu and Wallace., Genomics 4:560-569 (1989). The ligation amplification reaction (LAR) utilizes amplification of specific DNA sequence using sequential rounds of template dependent ligation as described in Barany, Proc. Nat. Acad. Sci. 88:189-193 (1990) and U.S. Patent Publication No. 20040253594 which are incorporated by reference.

Amplification products generated using the polymerase chain reaction can be analyzed by the use of denaturing gradient gel electrophoresis. Different alleles can be identified based on the different sequence-dependent melting properties and electrophoretic migration of DNA in solution. DNA molecules melt in segments, termed melting domains, under conditions of increased temperature or denaturation. Each melting domain melts cooperatively at a distinct, base-specific melting temperature (TM). Melting domains are at least 20 base pairs in length, and may be up to several hundred base pairs in length.

Differentiation between alleles based on sequence specific melting domain differences can be assessed using polyacrylamide gel electrophoresis, as described in Myers et al., Chapter 7 of Erlich, ed., PCR Technology, W.H. Freeman and Co., New York (1989) incorporated by reference.

Generally, a target region to be analyzed by denaturing gradient gel electrophoresis is amplified using PCR primers flanking the target region. The amplified PCR product is applied to a polyacrylamide gel with a linear denaturing gradient as described in Myers et al., Meth. Enzymol. 155:501-527 (1986), and Myers et al., in Genomic Analysis, A Practical Approach, K. Davies Ed. IRL Press Limited, Oxford, pp. 95-139 (1988). The electrophoresis system is maintained at a temperature slightly below the Tm of the melting domains of the target sequences.

In an alternative method of denaturing gradient gel electrophoresis, the target sequences may be initially attached to a stretch of GC nucleotides, termed a GC clamp, as described by Myers in Chapter 7 of Erlich, PCT Technology Stockton Press, It is contemplated that at least 80% of the nucleotides in the GC clamp are either guanine or cytosine. The GC clamp may be at least 30 bases long. This method is particularly suited to target sequences with high Tm's.

Generally, the target region is amplified by the polymerase chain reaction as described above. One of the oligonucleotide PCR primers carries at its 5' end, the GC clamp region, at least 30 bases of the GC rich sequence, which is incorporated into the 5' end of the target region during amplification. The resulting amplified target region is run on an electrophoresis gel under denaturing gradient conditions as described above. Nucleic acid fragments dif-

fering by a single base change will migrate through the gel to different positions, which may be visualized by ethidium bromide staining.

Temperature gradient gel electrophoresis (TGGE) is based on the same underlying principles as denaturing gradient gel electrophoresis, except the denaturing gradient is produced by differences in temperature instead of differences in the concentration of a chemical denaturant. Standard TGGE utilizes an electrophoresis apparatus with a temperature gradient running along the electrophoresis path. As samples migrate through a gel with a uniform concentration of a chemical denaturant, they encounter increasing temperatures. An alternative method of TGGE, temporal temperature gradient gel electrophoresis (TTGE or tTGGE) uses a steadily increasing temperature of the entire electrophoresis gel to achieve the same result. As the samples migrate through the gel the temperature of the entire gel increases, leading the samples to encounter increasing temperature as they migrate through the gel. Preparation of samples, including PCR amplification with incorporation of a GC clamp, and visualization of products are the same as for denaturing gradient gel electrophoresis (see, e.g., U.S. Patent Application No. 20040253594).

The human leukocyte antigen complex (also known as the major histocompatibility complex) spans approximately 3.5 million base pairs on the short arm of chromosome 6. It is divisible into 3 separate regions which contain the class I, the class II and the class III genes. In humans, the class I HLA complex is about 2000 kb long and contains about 20 genes. Within the class I region exist genes encoding the well characterized class I MHC molecules designated HLA-A, HLA-B and HLA-C. In addition, there are nonclassical class I genes that include HLA-E, HLA-F, HLA-G, HLA-H, HLA-J and JLA-X as well as a new family known as MIC. The class II region contains three genes known as the HLA-DP, HLA-DQ and HLA-DR loci. These genes encode the chains of the classical class II MHC molecules designated HLA-DR, DP and DQ. In humans, nonclassical genes designated DM, DN and DO have also been identified within class II. The class III region contains a heterogeneous collection of more than 36 genes. Several complete components are encoded by three genes including the TNFs (see, e.g., U.S. Pat. No. 6,670,124 incorporated by reference).

Any given copy of human chromosome 6 can contain many different alternative versions of each of the preceding genes and thus can yield proteins with distinctly different sequences. The loci constituting the MHC are highly polymorphic, that is, many forms of the gene or alleles exist at each locus. Several hundred different allelic variants of class I and class II MHC molecules have been identified in humans. However, any one individual only expresses up to 6 different class I molecules and up to 12 different class II molecules.

The foregoing regions play a major role in determining whether transplanted tissue will be accepted as self (histocompatible) or rejected as foreign (histoincompatible). For instance, within the class H region, three loci, i.e., HLA-DR, DQ and DP are known to express functional products. Pairs of A and B genes within these three loci encode heterodimeric protein products which are multi-allelic and allorcactive. In addition, combinations of epitopes on DR and/or DQ molecules are recognized by alloreactive T cells. This reactivity has been used to define "Dw" types by cellular assays based upon the mixed lymphocyte reaction (MLR). It is contemplated that matching of the HLA type of the reference biological sample with the mammary fluid sample may be used to determine whether the mammary fluid sample originated from the donor.

One nucleic acid typing method for the identification of these alleles has been restriction fragment length polymorphism (RFLP) analysis discussed herein (see, also, U.S. Pat. No. 6,670,124).

In addition to restriction fragment length polymorphism (RFLP), another approach is the hybridization of PCR amplified products with sequence-specific oligonucleotide probes (PCR-SSO) to distinguish between HLA alleles (see, Tiercy et al., (1990) Blood Review 4:9-15). This method requires a PCR product of the HLA locus of interest be produced and then dotted onto nitrocellulose membranes or strips. Then each membrane is hybridized with a sequence specific probe, washed, and then analyzed by exposure to x-ray film or by colorimetric assay depending on the method of detection. Similarly to the PCR-SSP methodology, probes are made to the allelic polymorphic area responsible for the different HLA alleles. Each sample must be hybridized and probed at least 100-200 different times for a complete Class I and II typing. Hybridization and detection methods for PCR-SSO typing include the use of nonradioactive labeled probes, microplate formats, and the like (see, e.g., Saiki et al. (1989) Proc. Natl. Acad. Sci., U.S.A. 86: 6230-6234; Erlich et al. (1991) Eur. J. Immunogenet. 18(1-2): 33-55; Kawasaki et al. (1993) Methods Enzymol. 218:369-381), and automated large scale HLA class II typing (see, e.g., U.S. Pat. No. 6,670,124).

Another typing method comprises sequence specific primer amplification (PCR-SSP) which may be used in the methods of the invention (see, Olemp and Zetterquist (1992) Tissue Antigens 39: 225-235). In PCR-SSP, allelic sequence specific primers amplify only the complementary template allele, allowing genetic variability to be detected with a high degree of resolution. This method allow determination of HLA type simply by whether or not amplification products (collectively called an "amplicon") are present or absent following PCR. In PCR-SSP, detection of the amplification products is usually done by agarose gel electrophoresis followed by ethidium bromide (EtBr) staining of the gel (see, e.g., U.S. Pat. No. 6,670,124).

Another HLA typing method is SSCP—Single-Stranded Conformational Polymorphism. Briefly, single stranded PCR products of the different HLA loci are run on non-denaturing Polyacrylamide Gel Electrophoresis (PAGE). The single strands will migrate to a unique location based on their base pair composition. By comparison with known standards, a typing can be deduced. It is the only method that can determine true homozygosity, (see, e.g., U.S. Pat. No. 6,670,124) (Orita et al., Proc. Nat. Acad. Sci 86:2766-2770 (1989)).

The identification of a DNA sequence can be made without an amplification step, based on polymorphisms including restriction fragment length polymorphisms ("RFLP") in a subject. Hybridization probes are generally oligonucleotides which bind through complementary base pairing to all or part of a target nucleic acid. Probes typically bind target sequences lacking complete complementarity with the probe sequence depending on the stringency of the hybridization conditions. The probes are typically labeled directly or indirectly, such that by assaying for the presence or absence of the probe, one can detect the presence or absence of the target sequence. Direct labeling methods include radioisotope labeling, such as with 32P or 35S. Indirect labeling methods include fluorescent tags, biotin complexes which may be bound to avidin or streptavidin, or

peptide or protein tags. Visual detection methods include photoluminescents, Texas red, rhodamine and its derivatives, red leuco dye and 3,3',5,5'-tetra-methylbenzidine (TMB), fluorescein, and its derivatives, dansyl, umbelliferone and the like or with horse radish peroxidase, alkaline phosphatase and the like (see, e.g., U.S. Patent Publication No. 20040253594, U.S. Patent Publication No. 20050123947, which are incorporated by reference).

One or more additional restriction enzymes and/or probes and/or primers can be used. Additional enzymes, constructed probes, and primers can be determined by routine experimentation by those of ordinary skill in the art and are intended to be within the scope of the invention.

Although the methods described herein may be in terms of the use of a single restriction enzyme and a single set of primers, the methods are not so limited. One or more additional restriction enzymes and/or probes and/or primers can be used, if desired. Additional enzymes, constructed probes and primers can be determined through routine experimentation, combined with the teachings provided and incorporated herein.

The reagents suitable for applying the methods of the invention may be packaged into convenient kits. The kits provide the necessary materials, packaged into suitable containers. Typically, the reagent is a PCR set (a set of primers, DNA polymerase and 4 nucleoside triphosphates) that hybridize with the gene or loci thereof. Typically, the PCR set is included in the kit. Typically, the kit further comprises additional means, such as reagents, for detecting or measuring the detectable entity or providing a control. Other reagents used for hybridization, prehybridization, DNA extraction, visualization etc. may also be included, if desired.

Other Identity Markers Profiles

It is further contemplated that the mammary fluid sample may be tested for self-antigens (or other peptides and polypeptides) present in the mammary fluid to establish a self-antigen profile (identity marker profile). The self-antigen profile of the mammary fluid sample will be compared to the reference self-antigen profile for the individual human. A match or identity of the self-antigen profile will indicate that the mammary fluid was obtained from the specific subject.

The various antigens that determine self are encoded by more than 40 different loci, such as the major histocompatibility complex (MHC), also called the human leukocyte antigen (HLA) locus, and the blood group antigens, such as ABO.

Methods are known in the art for screening humans for ABO blood group type. The blood-group antigens are expressed on red blood cells, epithelial cells and endothelial cells.

Testing for HLA type can be conducted by methods known in the art such as serological and cellular typing.

It is contemplated that the antigens could be identified by a microcytotoxicity test. In this test, white blood cells are distributed in a microtiter plate and monoclonal antibodies specific for class I and class II MHC antigens are added to different wells. Thereafter, complement is added to the wells and cytotoxicity is assessed by uptake or exclusion to various dyes (e.g., trypan blue or eosin Y) by the cells. If the white blood cells express the MHC antigen for a particular monoclonal antibody, then the cells will be lysed on addition of complement and these dead cells will take up the dye (see, Terasaki and McClelland, (1964) Nature, 204:998 and U.S. Pat. No. 6,670,124). HLA typing based on antibody-mediated microcytotoxicity can thus indicate the presence or absence of various MHC alleles (See Kuby Immunology 4th Ed., Freeman and Company, pp 520-522).

The detection of antigens may be selected from, but is not limited to, enzyme-linked immunosorbent assay, solid phase radiobinding immunoassays where the antibodies may be directed against soluble antigens or cell surface antigens, autoradiography, competitive binding radioimmunoassay, immunoradiometric assay (IRMA) electron microscopy, peroxidase antiperoxidase (PAP) labeling, fluorescent microscopy, alkaline phosphatase labeling and peroxidase labeling.

In the case where the detection method (s) use optical microscopy, the cells from the biological sample or the mammary fluid sample are mounted and fixed on a microscope slide. In this case, the step of detecting the labelled antibody is detecting a resulting colouration of the self-antigen with an optical microscope (see, e.g., U.S. Pat. No. 6,376,201).

The following example provides an embodiment of the methods described herein and should not be understood as restrictive.

## Example 1

### Testing of a Human Breast Milk Donor

A woman who wishes to donate her breast milk will provide a biological reference sample prior to (or at the time of) her first donation. The biological sample will include a convenient tissue type, e.g., blood, cheek cell, hair etc. The sample will be donated under supervision of another individual(s), e.g., bank milk personnel. The sample will be labeled for later reference. The reference sample will be tested for a specific marker profile, e.g., nucleic acid and/or peptide profile. The sample will be tested for one or more markers. Results of the tests will be stored, e.g., on a computer-readable medium for future reference. Any remaining sample will be stored. The woman can also be screened (using the reference sample or another sample) for, e.g., drug use, viruses, bacteria, parasites, and fungi etc., to determine her health. The woman will be given a label corresponding to the reference sample to keep with her and use with her donated milk.

Alternatively, the sample will be stored without testing, and will be tested at a later date, for example, together with the donated breast milk.

The woman will express her milk for donation and either forward the milk to the milk bank or processing facility or store the milk in her refrigerator, e.g., the freezer, for donation with other samples at a later date. The donated milk will be labeled with the label given to the woman and matching the reference sample.

A sample of the donated milk that arrives at the milk bank or processing facility will be tested for at least one of the same markers as the reference sample. The marker profile of the reference sample will be compared to the marker profile of the donated milk sample. If the profiles will match, the identity of the donor will be confirmed. If the profiles will not match, the results will be an indication that the donated milk is contaminated with another woman's milk or that it does not come from the woman whose reference sample was taken.

The milk whose provenance (i.e., origin) will be confirmed by the matched profiles will be further processed, e.g., pasteurized, e.g., into human milk fortifiers, standardized human milk compositions, and/or human lipid compositions. Such compositions will be administered to human

infants, e.g., premature infants, whose mothers may not be able to provide them with adequate nutrition.

The reference sample and/or results of the reference sample tests will be stored for any future donation by the corresponding mother.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

What is claimed is:

**1**. A method for determining whether a donated **[**mammary fluid**]** *human breast milk* was obtained from a specific subject, *wherein the specific subject is a qualified donor*, the method comprising:

(a) testing a donated biological sample from the specific subject to obtain at least one reference identity marker profile for at least one *identity* marker, *wherein the specific subject has been identified as a qualified donor by testing the specific subject for absence of chronic illness*;

(b) testing a sample of the donated **[**mammary fluid**]** *human breast milk* to obtain at least one identity marker profile for the at least one marker in step (a);

(c) comparing the identity marker profiles *obtained in step* (*a*) *and* (*b*),

wherein, *if one or more identity markers in the biological sample match one or more identity markers in the donated human breast milk, such* a match **[**between the identity marker profiles**]** indicates that the **[**mammary fluid**]** *donated human breast milk* was obtained from the **[**specific subject**]** *qualified door*; and

(d) processing the donated **[**mammary fluid**]** *human breast milk from the qualified door* whose identity marker profile has been matched with a reference identity marker profile, wherein the processed donated **[**mammary fluid**]** *human breast milk* comprises *a caloric content of 20 Cal/ounce;* a human protein constituent of 11-20 mg/mL; a human fat constituent of 35-55 mg/mL; and a human carbohydrate constituent of 70-120 mg/mL.

**[2**. The method of claim **1**, wherein the mammary fluid is human breast milk.**]**

**3**. The method of claim **1** wherein the processing comprises:

(a) filtering the milk;

(b) heat-treating the milk;

(c) separating the milk into cream and skim;

(d) adding a portion of the cream to the skim; and

(e) pasteurizing.

**[4**. The method of claim **1**, wherein the composition further comprises one or more constituents selected from the group consisting of: calcium, chloride, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc.**]**

**5**. The method of claim **1**, wherein processing comprises separating the milk into a cream portion and a skim portion, processing the cream portion, and pasteurizing the cream portion.

**6**. The method of claim **1**, further comprising nucleic acid typing, wherein the nucleic acid typing comprises a method selected from the group consisting of: STR analysis, HLA analysis, multiple gene analysis, and a combination thereof.

**7**. The method of claim **1**, wherein the donated **[**mammary fluid**]** *breast milk* is frozen.

**8**. The method of claim **1**, wherein the **[**mammary fluid**]** *breast milk* sample comprises a mixture of one or more **[**mammary fluid**]** *breast milk* samples.

**9**. The method of claim **1**, wherein the donated biological sample is selected from a group consisting of: milk, saliva, buccal cell, hair root, and blood.

**10**. The method of claim **1**, wherein steps (a) through (c) are carried out at a human breast milk donation center or at a milk processing facility.

**11**. The method of claim **1**, wherein steps (a) and (b) are carried out at different facilities.

**12**. The method of claim **11**, wherein step (a) is carried out at a human breast milk donation facility and step (b) is carried out at a milk processing facility.

**13**. A method for processing a donated human breast milk obtained from a specific, *qualified donor* subject comprising:

(a) *screening a donated biological sample from the subject for the absence of chronic illness;*

(*b*) testing a donated biological sample from the specific subject to obtain at least one reference identity marker profile for at least one marker;

(**[**b**]** *c*) testing a sample of the donated human breast milk to obtain at least one identity marker profile for the at least one marker in step (**[**a**]** *b*);

(**[**c**]** *d*) comparing the identity marker profiles of steps (**[**a**]** *b*) and (**[**b**]** *c*), wherein, *if one or more identity markers in the biological sample of step* (*b*) *match one or more identity markers in the donated human breast milk of step* (*c*), *such* a match **[**between the identity marker profiles**]** indicates that the donated human breast milk was obtained from the specific subject;

(**[**d**]** *e*) *selecting for* processing the donated human breast milk *from the subject screened for the absence of chronic illness and* whose identity marker profile has been matched with a reference identity marker profile, *wherein the selected subject is a qualified donor; and*

(*f*) *processing the donated human breast milk*, wherein the processing comprises:

(i) filtering the donated human breast milk; (ii) heat treating the donated human breast milk;

(iii) separating the donated human breast milk into cream and skim; (iv) adding a portion of the cream to the skim to form a human milk composition; and

(v) pasteurizing the human milk composition to produce a processed human breast milk composition;

and wherein the processed donated human breast milk comprises *a caloric content of 20 Cal/ounce;* a human protein constituent of 11-20 mg/mL; a human fat constituent of 35-55 mg/mL; and a human carbohydrate constituent of 70-120 mg/mL.

**14**. A processed human milk composition suitable for administration to an infant made by the process of claim **13**.

**[15**. The method of claim **13**, wherein the method further comprises adding to the processed human breast milk one or more constituents selected from the group consisting of: calcium, chloride, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc.**]**

**16**. The method of claim **13**, wherein the testing of the donated human breast milk of step (**[**b**]** *c*) and the testing of the donated biological sample of step (**[**a**]** *b*) comprises nucleic acid typing selected from the group consisting of: STR analysis, HLA analysis, multiple gene analysis, and a combination thereof.

**17**. The method of claim **13**, wherein the donated biological sample *of step* (*a*) *and/or the donated biological*

*sample of step* (*b*) is selected from a group consisting of: milk, saliva, buccal cell, hair root, and blood.

**18**. The method of claim **13**, wherein steps (**[**a**]** *b*) through (**[**c**]** *e*) are carried out at a human breast milk donation center or at a milk processing facility.

**19**. The method of claim **13**, wherein steps (**[**a**]** *b*) and (**[**b**]** *c*) are carried out at different facilities.

**20**. The method of claim **19**, wherein step (**[**a**]** *b*) is carried out at a human breast milk donation facility and step (**[**b**]** *c*) is carried out at a milk processing facility.

*21. The method of claim 1, wherein the human protein constituent comprises about 11-13 mg/mL.*

*22. The method of claim 1, wherein the human carbohydrate constituent comprises about 80-105 mg/mL.*

*23. The method of claim 1, wherein the processed donated breast milk of step* (*d*) *does not comprise added non-human derived nutritional components.*

*24. The method of claim 1, wherein the method comprises pooling the donated breast milk of step* (*c*) *whose identity marker profile has been matched with the reference identity marker profile, with other donated breast milk samples that have been matched with the reference identity marker profile to obtain a pool of identity matched breast milk.*

*25. The method of claim 24, wherein the pool of identity matched breast milk is in a volume of at least about 75 liters/lot to about 2,000 liters/lot.*

*26. The method of claim 1, wherein the processed breast milk of step* (*d*) *has an osmolality of less than about 400 mOsm/kg* $H_2O$.

*27. The method of claim 1, wherein the processing of step* (*d*) *comprises filtering the donated breast milk by ultrafiltration.*

*28. The method of claim 27, wherein the ultrafiltration filters out water, and wherein the processing step further comprises washing the filters used during the ultrafiltration with the water obtained by the ultrafiltration.*

*29. The method of claim 26, wherein the processing of step* (*d*) *further comprises reducing the bioburden of the human donor milk.*

*30. The method of claim 1, wherein the processing of step* (*d*) *comprises concentrating the nutrients in the donated breast milk.*

*31. The method of claim 30, wherein the processing of step* (*d*) *further comprises reducing the bioburden of the human donor milk.*

*32. The method of claim 13, wherein the processed donated human breast milk comprises no added non-human derived nutritional components.*

*33. The method of claim 13, wherein the pool of identity matched human donor milk is in a volume of at least about 75 liters/lot to about 2,000 liters/lot.*

*34. The method of claim 13, wherein the processed milk has on osmolality of less than about 400 mOsm/kg* $H_2O$.

*35. The method of claim 13, wherein the processing step comprises filtering the human donor milk.*

*36. The method of claim 13, wherein the processing step comprises filtering the human donor milk by ultrafiltration.*

*37. The method of claim 36, wherein the ultrafiltration filters out water, and wherein the processing step further comprises washing the filters used during the ultrafiltration with the water obtained by the ultrafiltration.*

*38. The method of claim 13, wherein the processing step comprises concentrating the nutrients in the human donor milk.*

*39. The method of claim 38, wherein the processing step further comprises reducing the bioburden of the human donor milk.*

*40. The method of claim 35, wherein the processing step further comprises reducing the bioburden of the human donor milk.*

*41. The method of claim 13, wherein the human protein constituent comprises about 11-13 mg/mL.*

*42. The method of claim 13, wherein the human carbohydrate constituent comprises about 80-105 mg/mL.*

*43. A method for making concentrated, bioburden reduced, identity-matched and health screened human donor milk from a qualified human milk donor, wherein the human donor milk is safe and provides standardized nutrition, the method comprising:*

(*a*) *screening a subject for drug use and/or chronic illness by testing a biological sample from the subject;*

(*b*) *selecting a qualified donor, wherein the subject is a qualified donor if the biological sample of step* (*a*) *did not test positive for drug use or chronic illness;*

(*c*) *testing a donated biological sample from the qualified donor to obtain at least one DNA marker profile for at least one DNA marker;*

(*d*) *testing a sample of donated human breast milk from the qualified donor to obtain at least one DNA marker profile for the at least one DNA marker in step* (*c*);

(*e*) *comparing the DNA marker profiles obtained in* (*c*) *and* (*d*), *wherein, if DNA markers in the biological sample of step* (*c*) *match DNA markers in the donated human breast milk of step* (*d*), *such match indicates that the donated human breast milk was obtained from the specific, health screened, qualified donor, thereby obtaining identity matched, health screened donor milk from a qualified human milk donor;*

(*d*) *pooling the identity matched, health screened donor milk obtained in step* (*d*) *with other identity matched, health screened donor milk from other qualified donors to obtain a pool of identity matched, health screened human donor milk from qualified donors, comprising at least about 75 liters, wherein the pool of identity matched, health screened donor milk is not nutritionally sufficient for preterm infants;*

(*e*) *concentrating the pool of non-nutritionally sufficient, identity matched, health screened human donor milk from qualified donors to comprise a human protein constituent of 11-13 mg/mL; a human fat constituent of 35-55 mg/mL; a human carbohydrate constituent of 70-120 mg/mL; a caloric content of 20 Cal/ounce; and no added non-human derived vitamins or minerals to obtain a pool of concentrated identity matched donor milk from qualified donors with an osmolality of less than about 400 mOsm/Kg* $H_2O$; *and*

(*f*) *treating the pool of concentrated, identity-matched, health screened donor milk obtained in* (*e*) *to reduce the bioburden; thereby obtaining concentrated, bioburden reduced, identity-matched, health screened donor milk from qualified donors.*

*44. The method of claim 43, wherein the nucleic acid typing comprises STR analysis, HLA analysis, multiple gene analysis, or a combination thereof.*

*45. The method of claim 43, wherein the donated biological sample of step* (*a*) *and/or* (*c*) *is milk, saliva, buccal cell, hair root, or blood.*

*46. A processed human milk composition suitable for administration to an infant made by the process of claim 43.*

*47. The processed human milk composition of claim 46, wherein the infant is a preterm infant.*

*48. The method of claim 43, wherein the concentrating step comprises filtering the human donor milk.*

*49. The method of claim 48, wherein the filtering step comprises ultrafiltering the human donor milk.*

*50. The method of claim 49, wherein the ultrafiltration filters out water, and wherein the processing step further comprises washing the filters used during the ultrafiltration with the water obtained by the ultrafiltration.*

*51. The method of claim 1, wherein the one or more identity markers comprise DNA.*

*52. The method of claim 51, wherein the one or more identity markers comprise multiple DNA markers.*

*53. The method of claim 1, wherein the specific subject has been identified as a qualified donor by further screening for drug use.*

*54. The method of claim 1, wherein the specific subject has been identified as a qualified donor by interview and/or by biological sample testing for viral contamination.*

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