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(54) Title: NON-INVASIVE DIAGNOSIS OF GRAFT REJECTION IN ORGAN TRANSPLANT PATIENTS

(57) Abstract: The disclosure provides methods, devices, compositions and kits for diagnosing or predicting transplant status or outcome in a subject who has received a transplant. The methods comprise determining the presence or absence of one or more nucleic acids from a donor transplant, wherein said one or more nucleic acids from said donor are identified based on a predetermined marker profile, and diagnosing or predicting transplant status or outcome based on the presence or absence of said one or more nucleic acids.

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NON-INVASIVE DIAGNOSIS OF GRAFT REJECTION IN ORGAN TRANSPLANT PATIENTS

BACKGROUND OF THE INVENTION

[0001] Organ transplantation is an important medical procedure which saves lives in cases where a patient has organ failure or disablement, and it is now possible to transplant many organs including heart, lungs, kidney, and liver. In some cases, the transplanted organ is rejected by the recipient patient, which creates a life-threatening situation. Monitoring the patient for rejection is difficult and expensive, often requiring invasive procedures. Furthermore, current surveillance methods lack adequate sensitivity.

[0002] The present invention resolves these problems by providing non-invasive methods of monitoring organ transplant patients for rejection that are sensitive, rapid and inexpensive.

SUMMARY OF THE INVENTION

[0003] The invention provides methods, devices, compositions and kits for diagnosing and/or predicting transplant status or outcome in a subject who has received a transplant. In some embodiments, the invention provides methods of diagnosing or predicting transplant status or outcome comprising the steps of: (i) providing a sample from a subject who has received a transplant from a donor; (ii) determining the presence or absence of one or more nucleic acids from the donor transplant, where the one or more nucleic acids from the donor are identified based on a predetermined marker profile; and (iii) diagnosing or predicting transplant status or outcome based on the presence or absence of the one or more nucleic acids.

[0004] In some embodiments, the transplant status or outcome comprises rejection, tolerance, non-rejection based allograft injury, transplant function, transplant survival, chronic transplant s injury, or titer pharmacological immunosuppression. In some embodiments, the non-rejection based allograft injury is selected from the group of ischemic injury, virus infection, peri-operative ischemia, reperfusion injury, hypertension, physiological stress, injuries due to reactive oxygen species and injuries caused by pharmaceutical agents.

[0005] In some embodiments, the sample is selected from the group consisting of blood, serum, urine, and stool. In some embodiments, the marker profile is a polymorphic marker profile. In some embodiments, the polymorphic marker profile comprises one or more single nucleotide polymorphisms (SNP's), one or more restriction fragment length polymorphisms (RFLP's), one or more short tandem repeats (STRs), one or more variable number of tandem repeats (VNTR's), one or more hypervariable regions, one or more minisatellites, one or more dinucleotide repeats, one or more trinucleotide repeats, one or more tetranucleotide repeats, one or more simple sequence repeats,

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or one or more insertion elements. In some embodiments, the polymorphic marker profile comprises one or more SNPs

[0006] In some embodiments, the marker profile is determined by genotyping the transplant donor. In some embodiments, the methods further comprise genotyping the subject receiving the transplant. In some embodiments, the methods further comprise establishing a profile of markers, where the markers are distinguishable between the transplant donor and the subject receiving the transplant. In some embodiments, the genotyping is performed by a method selected from the group consisting of sequencing, nucleic acid array and PCR.

[0007] In any of the embodiments described herein, the transplant graft maybe any solid organ and skin transplant. In some embodiments, the transplant is selected from the group consisting of kidney transplant, heart transplant, liver transplant, pancreas transplant, lung transplant, intestine transplant and skin transplant.

[0008] In some embodiments, the nucleic acid is selected from the group consisting of double-stranded DNA, single-stranded DNA, single-stranded DNA hairpins, DNA/RNA hybrids, RNA and RNA hairpins. In some embodiments, the nucleic acid is selected from the group consisting of double-stranded DNA, single-stranded DNA and cDNA. In some embodiments, the nucleic acid is mRNA. In some embodiments, the nucleic acid is obtained from circulating donor cells. In some embodiments, the nucleic acid is circulating cell-free DNA.

[0009] In some embodiments, the presence or absence of the one or more nucleic acids is determined by a method selected from the group consisting of sequencing, nucleic acid array and PCR. In some embodiments, the sequencing is shotgun sequencing. In some embodiments, the array is a DNA array. In some embodiments, the DNA array is a polymorphism array. In some embodiments, the polymorphism array is a SNP array.

[0010] In some embodiments, the methods further comprise quantitating the one or more nucleic acids. In some embodiments, the amount of the one or more nucleic acids is indicative of transplant status or outcome. In some embodiments, the amount of the one or more nucleic acids above a predetermined threshold value is indicative of a transplant status or outcome. In some embodiments, the threshold is a normative value for clinically stable post-transplantation patients with no evidence of transplant rejection or other pathologies. In some embodiments, there are different predetermined threshold values for different transplant outcomes or status. In some embodiments, temporal differences in the amount of the one or more nucleic acids are indicative of a transplant status or outcome.

[0011] In some embodiments, the methods described herein have at least 56 % sensitivity. In some embodiments, the methods described herein have at least 78 % sensitivity. In some embodiments, the methods described herein have a specificity of about 70% to about 100%. In some embodiments, the methods described herein have a specificity of about 80% to about 100%. In some

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embodiments, the methods described herein a specificity of about 90% to about 100%. In some embodiments, the methods described herein have a specificity of about 100%.

[0012] In some embodiments, the invention provides computer readable mediums comprising: a set of instructions recorded thereon to cause a computer to perform the steps of: (i) receiving data from one or more nucleic acids detected in a sample from a subject who has received transplant from a donor, where the one or more nucleic acids are nucleic acids from the donor transplant, and where the one or more nucleic acids from the donor are identified based on a predetermined marker profile; and (ii) diagnosing or predicting transplant status or outcome based on the presence or absence of the one or more nucleic acids.

[0013] In some embodiments, the invention provides reagents and kits thereof for practicing one or more of the methods described herein.

INCORPORATION BY REFERENCE

[0014] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0016] **Figure 1** shows patient survival after diagnosis of CAV.

[0017] **Figure 2** shows detection of donor DNA in patients receiving gender mismatched transplants.

[0018] **Figure 3** shows a time course study for detection of donor DNA in a transplant patient that received a gender mismatched transplant and suffered a 3A rejection episode.

[0019] **Figure 4** shows a time course study for detection of donor DNA in a transplant patient that received a gender mismatched transplant and suffered a 3A rejection episode.

[0020] **Figure 5** depicts in one embodiments of the invention a general strategy to monitor all transplant patients

[0021] **Figure 6** shows sequencing results comparing four levels of substitutions of donor DNA into recipient DNA.

DETAILED DESCRIPTION OF THE INVENTION

[0022] Reference will now be made in detail to particularly preferred embodiments of the invention. Examples of the preferred embodiments are illustrated in the following Examples section.

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[0023] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents and publications referred to herein are incorporated by reference in their entirety.

[0024] Methods, devices, compositions and kits are provided for diagnosing or predicting transplant status or outcome in a subject who has received a transplant. The transplant status or outcome may comprise rejection, tolerance, non-rejection based transplant injury, transplant function, transplant survival, chronic transplant injury, or titer pharmacological immunosuppression.

[0025] This invention describes sensitive and non-invasive methods, devices, compositions and kits for monitoring organ transplant patients, and/or for diagnosing or predicting transplant status or outcome (e.g. transplant rejection). In some embodiments, the methods, devices, compositions and kits are used to establish a genotype for both the donor and the recipient before transplantation to enable the detection of donor-specific nucleic acids such as DNA or RNA in bodily fluids such as blood or urine from the organ recipient after transplantation.

[0026] In some embodiments, the invention provides methods of determining whether a patient or subject is displaying transplant tolerance. The term “transplant tolerance” includes when the subject does not reject a graft organ, tissue or cell(s) that has been introduced into/onto the subject. In other words, the subject tolerates or maintains the organ, tissue or cell(s) that has been transplanted to it. The term “patient” or “subject” as used herein includes humans as well as other mammals.

[0027] In some embodiments the invention provides methods for diagnosis or prediction of transplant rejection. The term “transplant rejection” encompasses both acute and chronic transplant rejection. "Acute rejection or AR" is the rejection by the immune system of a tissue transplant recipient when the transplanted tissue is immunologically foreign. Acute rejection is characterized by infiltration of the transplanted tissue by immune cells of the recipient, which carry out their effector function and destroy the transplanted tissue. The onset of acute rejection is rapid and generally occurs in humans within a few weeks after transplant surgery. Generally, acute rejection can be inhibited or suppressed with immunosuppressive drugs such as rapamycin, cyclosporin A, anti-CD40L monoclonal antibody and the like.

[0028] "Chronic transplant rejection or CR" generally occurs in humans within several months to years after engraftment, even in the presence of successful immunosuppression of acute rejection. Fibrosis is a common factor in chronic rejection of all types of organ transplants. Chronic rejection can typically be described by a range of specific disorders that are characteristic of the particular organ. For example, in lung transplants, such disorders include fibroproliferative destruction of the airway (bronchiolitis obliterans); in heart transplants or transplants of cardiac tissue, such as valve replacements, such disorders include fibrotic atherosclerosis; in kidney transplants, such disorders include, obstructive nephropathy, nephrosclerorsis, tubulointerstitial nephropathy; and in liver transplants, such disorders include disappearing bile duct syndrome. Chronic rejection can also be

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characterized by ischemic insult, denervation of the transplanted tissue, hyperlipidemia and hypertension associated with immunosuppressive drugs.

[0029] In some embodiments, the invention further includes methods for determining an immunosuppressive regimen for a subject who has received a transplant, e.g., an allograft.

[0030] Certain embodiments of the invention provide methods of predicting transplant survival in a subject that has received a transplant. The invention provides methods of diagnosing or predicting whether a transplant in a transplant patient or subject will survive or be lost. In certain embodiments, the invention provides methods of diagnosing or predicting the presence of long-term graft survival. By "long-term" graft survival is meant graft survival for at least about 5 years beyond current sampling, despite the occurrence of one or more prior episodes of acute rejection. In certain embodiments, transplant survival is determined for patients in which at least one episode of acute rejection has occurred. As such, these embodiments provide methods of determining or predicting transplant survival following acute rejection. Transplant survival is determined or predicted in certain embodiments in the context of transplant therapy, e.g., immunosuppressive therapy, where immunosuppressive therapies are known in the art. In yet other embodiments, methods of determining the class and/or severity of acute rejection (and not just the presence thereof) are provided.

[0031] In some embodiments, the invention provides methods for diagnosis or prediction of non-rejection based transplant injury. Examples of non-rejection based graft injury include, but are not limited to, ischemic injury, virus infection, peri-operative ischemia, reperfusion injury, hypertension, physiological stress, injuries due to reactive oxygen species and injuries caused by pharmaceutical agents.

[0032] As in known in the transplantation field, the transplant organ, tissue or cell(s) may be allogeneic or xenogeneic, such that the grafts may be allografts or xenografts. A feature of the graft tolerant phenotype detected or identified by the subject methods is that it is a phenotype which occurs without immunosuppressive therapy, i.e., it is present in a host that is not undergoing immunosuppressive therapy such that immunosuppressive agents are not being administered to the host. The transplant graft maybe any solid organ and skin transplant. Examples of organ transplants that can be analyzed by the methods described herein include but are not limited to kidney transplant, pancreas transplant, liver transplant, heart transplant, lung transplant, intestine transplant, pancreas after kidney transplant, and simultaneous pancreas-kidney transplant.

[0033] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or

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both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0034] Certain ranges are presented herein with numerical values being preceded by the term "about." The term "about" is used herein to provide literal support for the exact number that it precedes, as well as a number that is near to or approximately the number that the term precedes. In determining whether a number is near to or approximately a specifically recited number, the near or approximating unrecited number may be a number which, in the context in which it is presented, provides the substantial equivalent of the specifically recited number.

[0035] The practice of the present invention employs, unless otherwise indicated, conventional techniques of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which are within the skill of the art. See Sambrook, Fritsch and Maniatis, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd edition (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel, et al. eds., (1987)); the series METHODS IN ENZYMOLOGY (Academic Press, Inc.): PCR 2: A PRACTICAL APPROACH (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) ANTIBODIES, A LABORATORY MANUAL, and ANIMAL CELL CULTURE (R.I. Freshney, ed. (1987)).

Introduction

[0036] Methods, devices, compositions and kits are provided for diagnosing or predicting transplant status or outcome in a subject who has received a transplant.

[0037] As mention above, monitoring transplant patients for transplant status or outcome is difficult and expensive, often requiring non-sensitive and invasive procedures. For instance, in heart transplant patients acute rejection surveillance requires serial endomyocardial biopsies that are routinely performed at weekly and monthly intervals during the initial year after transplant, with a total of 6-8 biopsies in most patients. Advances in immunosuppression, rejection surveillance, and early recognition and treatment of life-threatening infections have led to continuous improvements in early outcomes after cardiac transplantation. (Taylor, D.O., et al., *J Heart Lung Transplant*, **27**, 943-956 (2008)) However, there has not been a similar improvement in late mortality, which is largely attributable to cardiac allograft vasculopathy (CAV). (Figure 1) Today, CAV remains the major cause of late graft failure and death amongst the nearly 22,000 living heart transplant recipients in the United States. Early detection of CAV, prior to the development of angiographically apparent disease, graft dysfunction, or symptom onset is important because patient mortality after detection by coronary angiography (the standard of care) is unacceptably high, with 2-year mortality rates of 50% having been reported. Current surveillance methods for CAV lack adequate sensitivity or require invasive procedures and the most commonly applied method, coronary angiography, lacks sensitivity (Kobashigawa, J.A., et al., J Am Coll Cardiol, 45, 1532-1537 (2005)). Delayed diagnosis due to underestimation of disease severity is a feature of coronary angiography that is largely overcome by

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intravascular ultrasound (IVUS). (Fitzgerald, P.J., et al., *Circulation*, **86**, 154-158 (1992)) However, both of these invasive left-heart, arterial catheter methods are costly, resource intensive, and associated with significant risk of morbidity and patient discomfort. Early detection of CAV, prior to the development of angiographically apparent disease, graft dysfunction, or symptom onset is crucial to guide the appropriate use of emerging therapies that retard and occasionally reverse progression of CAV. The development of markers for early, non-invasive, safe, and cost-effective detection of acute rejection and CAV, and their rapid translation to a practical and reliable test that can be used in the clinic represents a major unmet medical need for the nearly 22,000 living heart transplant recipients in the United States, and a similar number worldwide.

[0038] The pressing need for early diagnosis and risk stratification is further underscored by recent studies demonstrating delayed progression and/or reversal of CAV following intervention with newer immunosuppressive regimens. Since the use of these newer therapies are encumbered by adverse effects, drug interactions, and cost, it is important to identity the patients in whom the benefits outweigh the risks. Aside from its impact on mortality and morbidity, CAV surveillance is costly in terms of resource utilization and potential for patient complications. Given the current standard of care to perform annual coronary angiography for the initial five years after heart transplantation, each patient surviving to year 5 will have received 4 angiograms for an average fully loaded cost of $25,000 per angiogram. Since the 5-year survival rate after heart transplantation is 72%, approximately 1,440 patients out of the 2,000 patients receiving heart transplants each year will undergo 4 procedures for a total of at least 5,760 procedures. At an average cost of $25,000 per coronary angiogram, this will amount to $144,000,000 per year in healthcare dollars for monitoring patients after heart transplantation. A non-invasive test that identifies the patients at low risk of CAV would mean that coronary angiography could be safely avoided in this group, thereby considerably reducing the cost of their long-term management.

[0039] The same difficulties and expenses are experienced by patients receiving other type of transplants.

a. Circulating Nucleic Acids

[0040] Circulating, or cell-free, DNA was first detected in human blood plasma in 1948. (Mandel, P. Metais, P., C R Acad. Sci. Paris, 142, 241-243 (1948)) Since then, its connection to disease has been established in several areas. (Tong, Y.K. Lo, Y.M., Clin Chim Acta, 363, 187-196 (2006)) Studies reveal that much of the circulating nucleic acids in blood arise from necrotic or apoptotic cells (Giacona, M.B., et al., Pancreas, 17, 89-97 (1998)) and greatly elevated levels of nucleic acids from apoptosis is observed in diseases such as cancer. (Giacona, M.B., et al., Pancreas, 17, 89-97 (1998); Fournie, G.J., et al., Cancer Lett, 91, 221-227 (1995)) Particularly for cancer, where the circulating DNA bears hallmark signs of the disease including mutations in oncogenes, microsatellite alterations, and, for certain cancers, viral genomic sequences, DNA or RNA in plasma has become increasingly studied as a potential biomarker for disease. For example, Diehl et al recently demonstrated that a

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quantitative assay for low levels of circulating tumor DNA in total circulating DNA could serve as a better marker for detecting the relapse of colorectal cancer compared with carcinoembryonic antigen, the standard biomarker used clinically. (Diehl, F., et al., Proc Natl Acad Sci, 102, 16368-16373 (2005); Diehl, F., et al., Nat Med, 14, 985-990 (2008)) Maheswaran et al reported the use of genotyping of circulating cells in plasma to detect activating mutations in epidermal growth factor receptors in lung cancer patients that would affect drug treatment. (Maheswaran, S., et al., N Engl J Med, 359, 366-377 (2008)) These results collectively establish both circulating DNA, either free in plasma or from circulating cells, as a useful species in cancer detection and treatment. Circulating DNA has also been useful in healthy patients for fetal diagnostics, with fetal DNA circulating in maternal blood serving as a marker for gender, rhesus D status, fetal aneuploidy, and sex-linked disorders. Fan et al recently demonstrated a strategy for detecting fetal aneuploidy by shotgun sequencing of cell-free DNA taken from a maternal blood sample, a methodology that can replace more invasive and risky techniques such as amniocentesis or chorionic villus sampling. (Fan, H.C., Blumenfeld, Y.J., Chitkara, U., Hudgins, L., Quake, S.R., Proc Natl Acad Sci, 105, 16266-16271 (2008))

[0041] In all these applications of circulating nucleic acids, the presence of sequences differing from a patient's normal genotype has been used to detect disease. In cancer, mutations of genes are a tell-tale sign of the advance of the disease; in fetal diagnostics, the detection of sequences specific to the fetus compared to maternal DNA allows for analysis of the health of the fetus.

[0042] In some embodiments, the invention provides non-invasive diagnostics exists for organ transplant patients where sequences from the organ donor, otherwise "foreign" to the patient, can be quantitated specifically. Without intending to be limited to any theory, as cell-free DNA or RNA often arises from apoptotic cells, the relative amount of donor-specific sequences in circulating nucleic acids should provide a predictive measure of on-coming organ failure in transplant patients for many types of solid organ transplantation including, but not limited to, heart, lung, liver, and kidney.

b. Circulating Nucleic Acids and Transplant Rejection

[0043] In some embodiments, the invention provides methods, devices, compositions and kits for detection and/or quantitating circulating nucleic acids, either free in plasma or from circulating cells, for the diagnosis, prognosis, detection and/or treatment of a transplant status or outcome. There have been claims of detection of donor-DNA in sex-mismatched liver and kidney transplant patients; conventional PCR was used to search for Y chromosome sequences from male donors in the blood of female patients. (Lo, Y.M., et al., Lancet, 351, 1329-1330 (1998) However, in a follow-on study Y-chromosome specific sequences were not detected above background in 16 out of 18 patients using a more accurate quantitative polymerase chain reaction (qPCR) assay. (Lui, Y.Y., et al., Clin Chem, 49, 495-496 (2003)) In renal transplantation, urine samples of similarly sex-mismatched transplant patients were analyzed and Y chromosomal DNA was detected in patients immediately after

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transplantation as well as during graft rejection episodes. (Zhang, J., et al., Clin Chem, 45, 1741-1746 (1999) ; Zhong, X.Y., et al., Ann N Y Acad Sci, 945, 250-257 (2001))

[0044] Example 1 examined gender-mismatched heart transplant recipients and applied digital PCR (Warren, L., Bryder, D., Weissman, I.L., Quake, S.R., Proc Natl Acad Sci, 103, 17807-17812 (2006); Fan, H.C. Quake, S.R., Anal Chem, 79, 7576-7579 (2007)) to detect the level of donor-derived chromosome Y signal in plasma samples taken at the same time that an endomyocardial biopsy determined a grade 3A or 3B rejection episode. While there was not any significant chromosome Y signal detected from four control female-to-female transplant patients, 1.5-8% total genomic fraction for chromosome Y signals at the rejection time points was observed for three male-to-female transplant patients across four rejection episodes (Figure 2). A time-course study for one of these patients revealed that the level of chromosome Y detected in plasma was neglible in plasma at three months prior to rejection, but increased >10-fold to 2% of total genomic fraction at the time a biopsy determined rejection (See Figures 3 and 4). Collectively, these results establish that for heart transplant patients, donor-derived DNA present in plasma can serve as a potential marker for the onset of organ failure.

[0045] While each of these studies demonstrates donor-DNA in bodily fluids for different solid organ transplants, they are all limited to the special case of females receiving organs from males and will not work for females receiving from females, males receiving from males, or males receiving from females. Further problems with this strategy arise from the prevalence of microchimerism in female patients where past male pregnancies or blood transfusions may lead to Y-chromosome specific signals from sources other than the transplanted organ. (Hubacek, J.A., Vymetalova, Y., Bohuslavova, R., Kocik, M., Malek, I., Transplant Proc, 39, 1593-1595 (2007); Vymetalova, Y., et al., Transplant Proc, 40, 3685-3687 (2008)) The detection of donor-specific human leukocyte antigen (HLA) alleles in circulating DNA has been considered as a signal for organ rejection, specifically for kidney and pancreas transplant patients. (Gadi, V.K., Nelson, J.L., Boespflug, N.D., Guthrie, K.A., Kuhr, C.S., Clin Chem, 52, 379-382 (2006)) However, this strategy will also be limited by the inability to distinguish HLA alleles between all donors and recipients, particularly for common HLA types, and the potential complication of microchimerism such as from blood transfusions. (Baxter-Lowe, L.A. Busch, M.P., Clin Chem, 52, 559-561 (2006))

[0046] In some embodiments, the invention provides a universal approach to noninvasive detection of graft rejection in transplant patients which circumvents the potential problems of microchimerism from DNA from other foreign sources and is general for all organ recipients without consideration of gender. In some embodiments, a genetic fingerprint is generated for the donor organ. This approach allows for a reliable identification of sequences arising solely from the organ transplantation that can be made in a manner that is independent of the genders of donor and recipient.

[0047] In some embodiments, both the donor and recipient will be genotyped prior to transplantation. Examples of methods that can be used to genotyped the transplant donor and the

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transplant recipient include, but are not limited to, whole genome sequencing, exome sequencing, or polymorphisms arrays (e.g., SNP arrays). A set of relevant and distinguishable markers between the two sources is established. In some embodiments, the set of markers comprises a set of polymorphic markers. Polymorphic markers include single nucleotide polymorphisms (SNP's), restriction fragment length polymorphisms (RFLP's), short tandem repeats (STRs), variable number of tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, and insertion elements such as Alu. In some embodiments, the set of markers comprises SNPs.

[0048] Following transplantation, bodily fluid such as blood can be drawn from the patient and analyzed for markers. Examples of bodily fluids include, but are not limited to, smears, sputum, biopsies, secretions, cerebrospinal fluid, bile, blood, lymph fluid, saliva, and urine. Detection, identification and/or quantitation of the donor-specific markers (e.g. polymorphic markers such as SNPs) can be performed using real-time PCR, chips (e.g., SNP chips), high-throughput shotgun sequencing of circulating nucleic acids (e.g. cell-free DNA), as well as other methods known in the art including the methods described herein. The proportion of donor nucleic acids can be monitored over time and an increase in this proportion can be used to determine transplant status or outcome (e.g. transplant rejection).

[0049] In some embodiments, where the transplant is a xenotransplant, detection, identification and/or quantitation of the donor-specific markers can be performed by mapping one or more nucleic acids (e.g., DNA) to the genome of the specie use to determine whether the one or more nucleic acids come from the transplant donor. Polymorphic markers as described above can also be used where the transplant is a xenotransplant.

[0050] In any of the embodiments described herein, the transplant graft can be any solid organ or skin transplant. Examples of organ transplants that can be analyzed by the methods described herein include but are not limited to kidney transplant, pancreas transplant, liver transplant, heart transplant, lung transplant, intestine transplant, pancreas after kidney transplant, and simultaneous pancreas-kidney transplant.

Samples

[0051] In some embodiments, the methods described herein involve performing one or more genetic analyses or detection steps on nucleic acids. In some embodiments target nucleic acids are from a sample obtained from a subject that has received a transplant. Such subject can be a human or a domesticated animal such as a cow, chicken, pig, horse, rabbit, dog, cat, or goat. In some embodiments, the cells used in the present invention are taken from a patient. Samples derived from an animal, e.g., human, can include, for example whole blood, sweat, tears, saliva, ear flow, sputum, lymph, bone marrow suspension, lymph, urine, saliva, semen, vaginal flow, cerebrospinal fluid, brain fluid, ascites, milk, secretions of the respiratory, intestinal or genitourinary tracts fluid, a lavage of a tissue or organ (e.g. lung) or tissue which has been removed from organs, such as breast, lung,

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intestine, skin, cervix, prostate, pancreas, heart, liver and stomach. For example, a tissue sample can comprise a region of functionally related cells or adjacent cells. Such samples can comprise complex populations of cells, which can be assayed as a population, or separated into sub-populations. Such cellular and acellular samples can be separated by centrifugation, elutriation, density gradient separation, apheresis, affinity selection, panning, FACS, centrifugation with Hypaque, etc. By using antibodies specific for markers identified with particular cell types, a relatively homogeneous population of cells may be obtained. Alternatively, a heterogeneous cell population can be used. Cells can also be separated by using filters. For example, whole blood can also be applied to filters that are engineered to contain pore sizes that select for the desired cell type or class. Cells can be filtered out of diluted, whole blood following the lysis of red blood cells by using filters with pore sizes between 5 to 10 μm, as disclosed in U.S. Patent Application No. 09/790,673. Other devices can separate cells from the bloodstream, see Demirci U, Toner M., Direct etch method for microfluidic channel and nanoheight post-fabrication by picoliter droplets, Applied Physics Letters 2006; 88 (5), 053117; and Irimia D, Geba D, Toner M., Universal microfluidic gradient generator, Analytical Chemistry 2006; 78: 3472-3477. Once a sample is obtained, it can be used directly, frozen, or maintained in appropriate culture medium for short periods of time. Methods to isolate one or more cells for use according to the methods of this invention are performed according to standard techniques and protocols well-established in the art.

[0052] To obtain a blood sample, any technique known in the art may be used, e.g. a syringe or other vacuum suction device. A blood sample can be optionally pre-treated or processed prior to enrichment. Examples of pre-treatment steps include the addition of a reagent such as a stabilizer, a preservative, a fixant, a lysing reagent, a diluent, an anti-apoptotic reagent, an anti-coagulation reagent, an anti-thrombotic reagent, magnetic property regulating reagent, a buffering reagent, an osmolality regulating reagent, a pH regulating reagent, and/or a cross-linking reagent.

[0053] When a blood sample is obtained, a preservative such an anti-coagulation agent and/or a stabilizer can be added to the sample prior to enrichment. This allows for extended time for analysis/detection. Thus, a sample, such as a blood sample, can be analyzed under any of the methods and systems herein within 1 week, 6 days, 5 days, 4 days, 3 days, 2 days, 1 day, 12 hrs, 6 hrs, 3 hrs, 2 hrs, or 1 hr from the time the sample is obtained.

[0054] In some embodiments, a blood sample can be combined with an agent that selectively lyses one or more cells or components in a blood sample. For example platelets and/or enucleated red blood cells are selectively lysed to generate a sample enriched in nucleated cells. The cells of interest can subsequently be separated from the sample using methods known in the art.

[0055] When obtaining a sample from a subject (e.g., blood sample), the amount can vary depending upon subject size and the condition being screened. In some embodiments, up to 50, 40, 30, 20, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 mL of a sample is obtained. In some embodiments, 1-50, 2-40, 3-30, or 4-20

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mL of sample is obtained. In some embodiments, more than 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 mL of a sample is obtained.

Nucleic Acids

[0056] Nucleic acids from samples that can be analyzed by the methods herein include: double-stranded DNA, single-stranded DNA, single-stranded DNA hairpins, DNA/RNA hybrids, RNA (e.g. mRNA or miRNA) and RNA hairpins. Examples of genetic analyses that can be performed on nucleic acids include e.g., sequencing, SNP detection, STR detection, RNA expression analysis, and gene expression.

[0057] In some embodiments, less than 1 pg, 5pg, 10 pg, 20 pg, 30 pg, 40 pg, 50 pg, 100 pg, 200 pg, 500 pg, 1 ng , 5ng, 10 ng, 20 ng, 30 ng, 40 ng, 50 ng, 100 ng, 200 ng, 500 ng, 1ug, 5ug, 10 ug, 20 ug, 30 ug, 40 ug, 50 ug, 100 ug, 200 ug, 500 ug or 1 mg of nucleic acids are obtained from the sample for further genetic analysis. In some cases, about 1-5 pg, 5-10 pg, 10- 100 pg, 100 pg- 1 ng, 1 -5 ng, 5 - 10 ng, 10 - 100 ng, 100 ng- 1ug of nucleic acids are obtained from the sample for further genetic analysis.

[0058] In some embodiments, the methods described herein are used to detect and/or quantified a target nucleic acid molecule. In some embodiments, the methods described herein are used to detect and/or quantified multiple target nucleic acid molecules. The methods described herein can analyzed at least 1; 2; 3; 4; 5; 10, 20; 50; 100; 200; 500; 1,000; 2,000; 5,000; 10,000, 20,000; 50,000; 100,000; 200,000; 300,000; 400,000; 500,000; 600,000; 700,000; 800,000; 900,000; 1,000,000; 2,000,000 or 3,000,000 different target nucleic acids.

[0059] In some embodiments, the methods described herein are used to distinguish between target nucleic acids that differ from another nucleic acid by 1 nt. In some embodiments, the methods described herein are used to distinguish between target nucleic acids that differ from another nucleic acid by 1 nt or more than 1, 2, 3, 5, 10, 15, 20, 21, 22, 24, 25, 30 nt.

[0060] In some embodiments, the methods described herein are used to detect and/or quantify genomic DNA regions. In some embodiments, the methods described herein can discriminate and quantitate genomic DNA regions. The methods described herein can discriminate and quantitate at least 1; 2; 3; 4; 5; 10, 20; 50; 100; 200; 500; 1,000; 2,000; 5,000; 10,000, 20,000; 50,000; 100,000; 200,000; 300,000; 400,000; 500,000; 600,000; 700,000; 800,000; 900,000; 1,000,000; 2,000,000 or 3,000,000 different genomic DNA regions. The methods described herein can discriminate and quantitate genomic DNA regions varying by 1 nt or more than 1, 2, 3, 5, 10, 15, 20, 21, 22, 24, 25, 30 nt.

[0061] In some embodiments, the methods described herein are used to detect and/or quantify genomic DNA regions such as a region containing a DNA polymorphism. A polymorphism refers to the occurrence of two or more genetically determined alternative sequences or alleles in a population. A polymorphic marker or site is the locus at which divergence occurs. Preferred markers have at least two alleles, each occurring at a frequency of preferably greater than 1%, and more preferably greater

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than 10% or 20% of a selected population. A polymorphism may comprise one or more base changes, an insertion, a repeat, or a deletion. A polymorphic locus may be as small as one base pair. Polymorphic markers include single nucleotide polymorphisms (SNP's), restriction fragment length polymorphisms (RFLP's), short tandem repeats (STRs), variable number of tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, and insertion elements such as Alu. A polymorphism between two nucleic acids can occur naturally, or be caused by exposure to or contact with chemicals, enzymes, or other agents, or exposure to agents that cause damage to nucleic acids, for example, ultraviolet radiation, mutagens or carcinogens. In some embodiments, the methods described herein can discriminate and quantitate a DNA region containing a DNA polymorphism. The methods described herein can discriminate and quantitate of at least 1; 2; 3; 4; 5; 10, 20; 50; 100; 200; 500; 1,000; 2,000; 5,000; 10,000, 20,000; 50,000; 100,000; 200,000; 300,000; 400,000; 500,000; 600,000; 700,000; 800,000; 900,000; 1,000,000; 2,000,000 or 3,000,000 DNA polymorphism.

[0062] In some embodiments, the methods described herein can discriminate and quantitate at least 1; 2; 3; 4; 5; 10; 20; 50; 100; 200; 500; 1,000; 2,000; 5,000; 10,000; 20,000; 50,000; 100,000; 200,000; 300,000; 400,000; 500,000; 600,000; 700,000; 800,000; 900,000; 1,000,000; 2,000,000 or 3,000,000 different polymorphic markers.

[0063] In some embodiments, the methods described herein can discriminate and quantitate at least 1; 2; 3; 4; 5; 10; 20; 50; 100; 200; 500; 1,000; 2,000; 5,000; 10,000; 20,000; 50,000; 100,000; 200,000; 300,000; 400,000; 500,000; 600,000; 700,000; 800,000; 900,000; 1,000,000; 2,000,000 or 3,000,000 different SNPs.

[0064] In some embodiments, the methods described herein are used to detect and/or quantify gene expression. In some embodiments, the methods described herein provide high discriminative and quantitative analysis of multiples genes. The methods described herein can discriminate and quantitate the expression of at least 1, 2, 3, 4, 5, 10, 20, 50, 100, 200, 500, 1,000, 2,000, 5,000, 10,000, 20,000, 50,000, 100,000, different target nucleic acids.

[0065] In some embodiments, the methods described herein are used to detect and/or quantify gene expression of genes with similar sequences. The methods described herein can discriminate and quantitate the expression of genes varying by 1 nt or more than 1, 2, 3, 4, 5, 10, 12, 15, 20, 21, 22, 24, 25, 30 nt.

[0066] In some embodiments, the methods described herein are used to detect and/or quantify genomic DNA regions by mapping the region to the genome of a species in the case where the transplant donor and the transplant recipient are not from the same species (e.g., xenotransplants). In some embodiments, the methods described herein can discriminate and quantitate a DNA region from a species. The methods described herein can discriminate and quantitate of at least 1; 2; 3; 4; 5; 10, 20; 50; 100; 200; 500; 1,000; 2,000; 5,000; 10,000, 20,000; 50,000; 100,000; 200,000; 300,000;

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400,000; 500,000; 600,000; 700,000; 800,000; 900,000; 1,000,000; 2,000,000 or 3,000,000 DNA regions from a species.

[0067] In some embodiments, the methods described herein are used for diagnosing or predicting transplant status or outcome (e.g. transplant rejection). In some embodiments, the methods described herein are used to detect and/or quantify target nucleic acids to determine whether a patient or subject is displaying transplant tolerance. In some embodiments, the methods described herein are used to detect and/or quantify target nucleic acids for diagnosis or prediction of transplant rejection. In some embodiments, the methods described herein are used to detect and/or quantify target nucleic acids for determining an immunosuppressive regimen for a subject who has received a transplant, e.g., an allograft. In some embodiments, the methods described herein are used to detect and/or quantify target nucleic acids to predict transplant survival in a subject that have received a transplant. The invention provides methods of diagnosing or predicting whether a transplant in a transplant patient or subject will survive or be lost. In certain embodiments, the methods described herein are used to detect and/or quantify target nucleic acids to diagnose or predict the presence of long-term graft survival. In some embodiments, the methods described herein are used to detect and/or quantify target nucleic acids for diagnosis or prediction of non-rejection based transplant injury. Examples of non-rejection based graft injury include, but are not limited to, ischemic injury, virus infection, peri-operative ischemia, reperfusion injury, hypertension, physiological stress, injuries due to reactive oxygen species and injuries caused by pharmaceutical agents.

[0068] As used herein the term "diagnose" or "diagnosis" of a transplant status or outcome includes predicting or diagnosing the transplant status or outcome, determining predisposition to a transplant status or outcome, monitoring treatment of transplant patient, diagnosing a therapeutic response of transplant patient, and prognosis of transplant status or outcome, transplant progression, and response to particular treatment.

Donor Organ Nucleic Acid Detection and Analysis

[0069] In some embodiments, the methods, devices, compositions and kits are used to establish a genotype for both the donor and the recipient before transplantation to enable the detection of donor-specific nucleic acids such as DNA or RNA in bodily fluids such as blood or urine from the organ recipient after transplantation. This approach allows for a reliable identification of sequences arising solely from the organ transplantation that can be made in a manner that is independent of the genders of donor and recipient.

[0070] In some embodiments, a genetic fingerprint is generated for the donor organ. Both the donor and recipient will be genotyped prior to transplantation. Genotyping of transplant donors and transplant recipients establishes a profile, using distinguishable markers, for detecting donor nucleic acids (e.g. circulating cell-free nucleic acid or nucleic acids from circulating donor cells). In some embodiments, for xenotransplants, nucleic acids from the donors can be mapped to the genome of the donor species.

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[0071] Following transplantation, samples as described above can be drawn from the patient and analyzed for markers. The proportion of donor nucleic acids can be monitored over time and an increase in this proportion can be used to determine transplant status or outcome (e.g. transplant rejection).

[0072] In some embodiments, genotyping comprises detection and quantitation of nucleic acids from circulating transplant donor cells or circulating cell-free nucleic acids. Examples of nucleic acids include, but are not limited to double-stranded DNA, single-stranded DNA, single-stranded DNA hairpins, DNA/RNA hybrids, RNA (e.g. mRNA or miRNA) and RNA hairpins. In some embodiments, the nucleic acid is DNA. In some embodiments, the nucleic acid is RNA. For instance, cell-free RNA is also present in human plasma (Tong, Y.K. Lo, Y.M., Clin Chim Acta, 363, 187-196 (2006)) and cDNA sequencing of organ-specific transcripts provides another option to detect donor-specific nucleic acids arising from cells in the transplanted organ. In some embodiments, nucleic acids collected from circulating cells in the blood are used.

[0073] In some embodiments, genotyping comprises detection and quantitation of polymorphic markers. Examples of polymorphic markers include single nucleotide polymorphisms (SNP's), restriction fragment length polymorphisms (RFLP's), variable number of tandem repeats (VNTR's), short tandem repeats (STRs), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, and insertion elements such as Alu. In some embodiments, genotyping comprises detection and quantitation of STRs. In some embodiments, genotyping comprises detection and quantitation of VNTRs.

[0074] In some embodiments, genotyping comprises detection and quantitation of SNPs. Without intending to be limited to any theory, any donor and recipient will vary at roughly three million SNP positions if fully genotyped. Usable SNPs must be homozygous for the recipient and ideally homozygous for the donor as well. While the majority of these positions will contain SNPs that are heterozygous for either the donor or the recipient, over 10% (or hundreds of thousands) will be homozygous for both donor and recipient meaning a direct read of that SNP position can distinguish donor DNA from recipient DNA. For example, after genotyping a transplant donor and transplant recipient, using existing genotyping platforms know in the art including the one described herein, one could identify approximately 1.2 million total variations between a transplant donor and transplant recipient. Usable SNPs may comprise approximately 500,000 heterozygous donor SNPs and approximately 160,000 homozygous donor SNPs. Companies (such as Applied Biosystems, Inc.) currently offer both standard and custom-designed TaqMan probe sets for SNP genotyping that can in principle target any desired SNP position for a PCR-based assay (Livak, K. L., Marmaro, J., Todd, J. A., Nature Genetics, 9, 341-342 (1995); De La Vefa, F. M., Lazaruk, K. D., Rhodes, M. D., Wenz, M. H., Mutation Research, 573, 111-135 (2005)). With such a large pool of potential SNPs to choose from, a usable subset of existing or custom probes can be selected to serve as the probe set for any donor/recipient pair. In some embodiments, digital PCR or real-time PCR performed on the nucleic

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acids recovered from plasma or other biological samples will directly quantitate the percentage of donor-specific species seen in the sample. In some embodiments, sequencing performed on the nucleic acid recovered from plasma or other biological samples will directly quantitate the percentage of donor-specific species seen in the sample. In some embodiments, arrays can be used on the nucleic acids recovered from plasma or other biological samples to directly quantitate the percentage of donor-specific species seen in the sample.

[0075] Due to the low number of expected reads for any individual nucleic acid (e.g. SNP) in patient samples, some preamplification of the sample material may be required before analysis to increase signal levels, but using either preamplification, sampling more target nucleic acid positions (e.g. SNP positions), or both, will provide a reliable read-out of the transplant donor nucleic acid fraction. Pre-amplification can be preformed using any suitable method known in the art such as multiple displacement amplification (MDA) (Gonzalez et al. Envircon Microbiol; 7(7); 1024-8 (2005)) or amplification with outer primers in a nested PCR approach. This permits detection and analysis of donor nucleic acids even if the total amount of donor nucleic acid in the sample (e.g. blood from transplant patient) is only up to 1 μg, 500 ng, 200 ng, 100 ng, 50 ng, 40 ng, 30 ng, 20 ng, 10 ng, 5ng, 1 ng , 500 pg, 200 pg, 100 pg, 50 pg, 40 pg, 30 pg, 20 p, 10 pg, 5pg, or 1 pg or between 1 5 μg, 5 10 μg, or 10 50 μg.

a. PCR

[0076] Genotyping donor and recipient nucleic acids, and/or detection, identification and/or quantitation of the donor-specific nucleic acids after transplantation (e.g. polymorphic markers such as SNPs) can be performed by PCR. Examples of PCR techniques that can be used to detect, identify and/or quantitate the donor-specific nucleic acids include, but are not limited, to quantitative PCR, quantitative fluorescent PCR (QF-PCR), multiplex fluorescent PCR (MF-PCR), real time PCR (RT-PCR), single cell PCR, restriction fragment length polymorphism PCR (PCR-RFLP), PCR-RFLP/RT-PCR-RFLP, hot start PCR, nested PCR, in situ polonony PCR, in situ rolling circle amplification (RCA), bridge PCR, picotiter PCR and emulsion PCR. Other suitable amplification methods include the ligase chain reaction (LCR), transcription amplification, self-sustained sequence replication, selective amplification of target polynucleotide sequences, consensus sequence primed polymerase chain reaction (CP-PCR), arbitrarily primed polymerase chain reaction (AP-PCR), degenerate oligonucleotide-primed PCR (DOP-PCR) and nucleic acid based sequence amplification (NABSA). Other amplification methods that may be used to amplify specific polymorphic loci include those described in, U.S. Pat. Nos. 5,242,794, 5,494,810, 4,988,617 and 6,582,938. In some embodiments, Detection, identification and/or quantitation of the donor-specific nucleic acids (e.g. polymorphic markers such as SNPs) is performed by real-time PCR.

[0077] In some embodiments, digital PCR or real time PCR to quantitate the presence of specific polymorphisms that have already been identified in the initial genotyping step pre-transplantation. Compared with the quantitative PCR techniques used in some of the earlier cited work, digital PCR

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is a much more accurate and reliable method to quantitate nucleic acid species including rare nucleic acid species, and does not require a specific gender relationship between donor and recipient. (Warren, L., Bryder, D., Weissman, I.L., Quake, S.R., Proc Natl Acad Sci, 103, 17807-17812 (2006)). In some embodiments, digital PCR or real-time PCR assays can be used to quantitate the fraction of donor DNA in a transplant patient using probes targeted to several SNPs.

b. Sequencing

[0078] Genotyping donor and recipient nucleic acids, and/or detection, identification and/or quantitation of the donor-specific nucleic acids after transplantation (e.g. polymorphic markers such as SNPs) can be performed by sequencing such as whole genome sequencing or exome sequencing. Sequencing can be accomplished through classic Sanger sequencing methods which are well known in the art. Sequence can also be accomplished using high-throughput systems some of which allow detection of a sequenced nucleotide immediately after or upon its incorporation into a growing strand, i.e., detection of sequence in red time or substantially real time. In some cases, high throughput sequencing generates at least 1,000, at least 5,000, at least 10,000, at least 20,000, at least 30,000, at least 40,000, at least 50,000, at least 100,000 or at least 500,000 sequence reads per hour; with each read being at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 120 or at least 150 bases per read. Sequencing can be preformed using nucleic acids described herein such as genomic DNA, cDNA derived from RNA transcripts or RNA as a template.

[0079] In some embodiments, high-throughput sequencing involves the use of technology available by Helicos BioSciences Corporation (Cambridge, Massachusetts) such as the Single Molecule Sequencing by Synthesis (SMSS) method. SMSS is unique because it allows for sequencing the entire human genome with no pre amplification step needed. Thus, distortion and nonlinearity in the measurement of nucleic acids are reduced. This sequencing method also allows for detection of a SNP nucleotide in a sequence in substantially real time or real time. Finally, as mentioned above, SMSS is powerful because, like the MIP technology, it does not require a pre amplification step prior to hybridization. In fact, SMSS does not require any amplification. SMSS is described in part in US Publication Application Nos. 2006002471 I; 20060024678; 20060012793; 20060012784; and 20050100932.

[0080] In some embodiments, high-throughput sequencing involves the use of technology available by 454 Lifesciences, Inc. (Branford, Connecticut) such as the Pico Titer Plate device which includes a fiber optic plate that transmits chemiluninescent signal generated by the sequencing reaction to be recorded by a CCD camera in the instrument. This use of fiber optics allows for the detection of a minimum of 20 million base pairs in 4.5 hours.

[0081] Methods for using bead amplification followed by fiber optics detection are described in Marguiles, M., et al. "Genome sequencing in microfabricated high-density pricolitre reactors", Nature, doi: 10.1038/nature03959; and well as in US Publication Application Nos. 200200 12930;

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20030058629; 20030 1001 02; 20030 148344 ; 20040248 161 ; 200500795 10,20050 124022; and 20060078909.

[0082] In some embodiments, high-throughput sequencing is performed using Clonal Single Molecule Array (Solexa, Inc.) or sequencing-by-synthesis (SBS) utilizing reversible terminator chemistry. These technologies are described in part in US Patent Nos. 6,969,488; 6,897,023; 6,833,246; 6,787,308; and US Publication Application Nos. 200401061 30; 20030064398; 20030022207; and Constans, A,, The Scientist 2003, 17(13):36.

[0083] In some embodiments of this aspect, high-throughput sequencing of RNA or DNA can take place using AnyDot.chips (Genovoxx, Germany), which allows for the monitoring of biological processes (e.g., miRNA expression or allele variability (SNP detection). In particular, the AnyDot-chips allow for 10x - 50x enhancement of nucleotide fluorescence signal detection. AnyDot.chips and methods for using them are described in part in International Publication Application Nos. WO 02088382, WO 03020968, WO 0303 1947, WO 2005044836, PCTEP 05105657, PCMEP 05105655; and German Patent Application Nos. DE 101 49 786, DE 102 14 395, DE 103 56 837, DE 10 2004 009 704, DE 10 2004 025 696, DE 10 2004 025 746, DE 10 2004 025 694, DE 10 2004 025 695, DE 10 2004 025 744, DE 10 2004 025 745, and DE 10 2005 012 301.

[0084] Other high-throughput sequencing systems include those disclosed in Venter, J., et al. Science 16 February 2001; Adams, M. et al, Science 24 March 2000; and M. J, Levene, et al. Science 299:682-686, January 2003; as well as US Publication Application No. 20030044781 and 2006/0078937. Overall such system involve sequencing a target nucleic acid molecule having a plurality of bases by the temporal addition of bases via a polymerization reaction that is measured on a molecule of nucleic acid, i e., the activity of a nucleic acid polymerizing enzyme on the template nucleic acid molecule to be sequenced is followed in real time. Sequence can then be deduced by identifying which base is being incorporated into the growing complementary strand of the target nucleic acid by the catalytic activity of the nucleic acid polymerizing enzyme at each step in the sequence of base additions. A polymerase on the target nucleic acid molecule complex is provided in a position suitable lo move along the target nucleic acid molecule and extend the oligonucleotide primer at an active site. A plurality of labeled types of nucleotide analogs are provided proximate to the active site, with each distinguishably type of nucleotide analog being complementary to a different nucleotide in the target nucleic acid sequence. The growing nucleic acid strand is extended by using the polymerase to add a nucleotide analog to the nucleic acid strand at the active site, where the nucleotide analog being added is complementary to the nucleotide of the target nucleic acid at the active site. The nucleotide analog added to the oligonucleotide primer as a result of the polymerizing step is identified. The steps of providing labeled nucleotide analogs, polymerizing the growing nucleic acid strand, and identifying the added nucleotide analog are repeated so that the nucleic acid strand is further extended and the sequence of the target nucleic acid is determined.

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[0085] In some embodiments, shotgun sequencing is performed. In shotgun sequencing, DNA is broken up randomly into numerous small segments, which are sequenced using the chain termination method to obtain reads. Multiple overlapping reads for the target DNA are obtained by performing several rounds of this fragmentation and sequencing. Computer programs then use the overlapping ends of different reads to assemble them into a continuous sequence

[0086] In some embodiments, the invention provides methods for detection and quantitation of SNPs using sequencing. In this case, one can estimate the sensitivity of detection. There are two components to sensitivity: (i) the number of molecules analyzed (depth of sequencing) and (ii) the error rate of the sequencing process. Regarding the depth of sequencing, a frequent estimate for the variation between individuals is that about one base per thousand differs. Currently, sequencers such as the Illumina Genome Analyzer have read lengths exceeding 36 base pairs. Without intending to be limited to any theory or specific embodiment, this means that roughly one in 30 molecules analyzed will have a potential SNP. While the fraction of donor DNA in the recipient blood is currently not well determined and will depend on organ type, one can take 1% as a baseline estimate based on the literature and applicants own studies with heart transplant patients. At this fraction of donor DNA, approximately one in 3,000 molecules analyzed will be from the donor and informative about donor genotype. On the Genome Analyzer one can obtain about 10 million molecules per analysis channel and there are 8 analysis channels per instrument run. Therefore, if one sample is loaded per channel, one should be able to detect about 3,000 molecules that can be identified as from the donor in origin, more than enough to make a precise determination of the fraction of donor DNA using the above parameters. If one wants to establish a lower limit of sensitivity for this method by requiring at least 100 donor molecules to be detected, then it should have a sensitivity capable of detecting donor molecules when the donor fraction is as low as 0.03%. Higher sensitivity can be achieved simply by sequencing more molecules, i.e. using more channels.

[0087] The sequencing error rate also affects the sensitivity of this technique. For an average error rate of ε, the chance of a single SNP being accidentally identified as of donor origin as a result of a mis-read is roughly $\varepsilon/3$. For each individual read, this establishes a lower limit of sensitivity of one's ability to determine whether the read is due to donor or recipient. Typical sequencing error rates for base substitutions vary between platforms, but are between 0.5-1.5%. This places a potential limit on sensitivity of 0.16 to 0.50%. However, it is possible to systematically lower the sequencing error rate by resequencing the sample template multiple times, as has been demonstrated by Helicos BioSciences (Harris, T.D., et al., Science, 320, 106-109 (2008)). A single application of resequencing would reduce the expected error rate of donor SNP detection to $\varepsilon^2/9$ or less than .003%.

[0088] Figure 5 shows in one embodiments of the inventions a general strategy for monitor all patients, (i.e., not just female patients receiving male organs), to determine a transplants status or outcome. Genotyping of donor and recipient can establish a single nucleotide polymorphism (SNP) profile for detecting donor DNA. Shotgun sequencing of cell-free DNA in plasma, with analysis of

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observed unique SNPs, allows quantitation of % donor DNA. While any single SNP may be difficult to detect with so little DNA in plasma, with hundred of thousands or more signals to consider, high sensitivity should be possible.

c. Arrays

[0089] Genotyping donor and recipient nucleic acids, and/or detection, identification and/or quantitation of the donor-specific nucleic acids after transplantation (e.g. polymorphic markers such as SNPs) can be performed using arrays (e.g. SNPs arrays). Results can be visualized using a scanner that enables the viewing of intensity of data collected and software to detect and quantify nucleic acid. Such methods are disclosed in part US Patent No. 6,505,125. Another method contemplated by the present invention to detect and quantify nucleic acids involves the use of bead as is commercially available by Illumina, Inc. (San Diego) and as described in US Patent Nos. 7,035,740; 7033,754; 7,025,935, 6,998,274; 6, 942,968; 6,913,884; 6,890,764; 6,890,741; 6,858,394; 6,812,005; 6,770,441; 6,620,584; G,544,732; 6,429,027; 6,396,995; 6,355,43 1 and US Publication Application Nos. 20060019258; 0050266432; 20050244870; 20050216207; 20050181394; 20050164246; 20040224353; 20040185482; 20030198573; 20030175773; 20030003490; 20020187515; and 20020177141; and in B. E. Stranger, et al., Public Library of Science-Genetics, I (6), December 2005; Jingli Cai, el al., Stem Cells, published online November 17, 2005; C.M. Schwartz, et al., Stem Cells and Development, f 4, 517-534, 2005; Barnes, M., J. el al., Nucleic Acids Research, 33 (1 81, 5914-5923, October 2005; and Bibikova M, et al. Clinical Chemistry, Volume 50,No.12, 2384-2386, December 2004. Additional description for preparing RNA for bead arrays is described in Kacharmina JE, et al., Methods Enzymol303: 3-18, 1999; Pabon C, et al., Biotechniques 3 l(4): 8769,2001; Van Gelder RN, et a]., Proc Natl Acad Sci USA 87: 1663-7 (1990); and Murray, SS. BMC Genetics B(SupplI):SX5 (2005).

[0090] When analyzing SNP according to the methods described herein, the transplant donor and/or recipient nucleic acids can be labeled and hybridized with a DNA microarray (e.g., 100K Set Array or other array). Results can be visualized using a scanner that enables the viewing of intensity of data collected and software "calls" the SNP present at each of the positions analyzed. Computer implemented methods for determining genotype using data h m mapping arrays are disclosed, for example, in Liu, et al., Bioinformatics 19:2397-2403,2003; and Di et al., Bioinformatics 21: 1958-63,2005. Computer implemented methods for linkage analysis using mapping array data are disclosed, for example, in Ruschendorf and Nusnberg, Bioinfonnatics 21 :2I23-5,2005; and Leykin et a]., BMC Genet. 6:7, 2005; and in US Patent No. 5,733,729.

[0091] In some embodiments of this aspect, genotyping microarrays that are used to detect SNPs can be used in combination with molecular inversion probes (MIPS) as described in Hardenbol et al., Genome Res. 15(2) :269-275,2005, Hardenbol, P. et al. Nature Biotechnology 2 1 (6), 673-8,2003; Faham M, et al. Hum Mol Genet. Aug 1; 10(16): 1657-64,200 1: Maneesh Jain, Ph.D., et al. Genetic Engineering News V24: No. 18, 2004; and Fakhrai-Rad H, el al. Genome Res. Jul; 14(7):1404-12,

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2004; and in U.S. Pat. No. 5,858,412. Universal tag arrays and reagent kits for performing such locus specific genotyping using panels of custom MIPs are available from Affymetrix and ParAllele. MIP technology involves the use enzymological reactions that can score up to 10,000: 20,000, 50,000; 100,000; 200,000; 500,000; 1,000,000; 2,000,000 or 5,000,000 SNPs (target nucleic acids) in a single assay. The enzymological reactions are insensitive to crossreactivity among multiple probe molecules and there is no need for pre-amplification prior to hybridization of the probe with the genomic DNA. In any of the embodiments, the target nucleic acid(s) or SNPs can be obtained from a single cell.

[0092] Another method contemplated by the present invention to detect target nucleic acids involves the use of bead arrays (e.g., such as one commercially available by Illumina, Inc.) as described in US Patent Nos. 7,040,959; 7,035,740; 7033,754; 7,025,935, 6,998,274; 6,942,968; 6,913,884; 6,890,764; 6,890,741; 6,858,394; 6,846,460; 6,812,005; 6,770,441; 6,663,832; 5,520,584; 6,544,732; 6,429,027; 6,396,995; 6,355,431 m d US Publication Application Nos. 20060019258; 20050266432; 20050244870; 20050216207; 20050181394; 20050164246; 20040224353; 20040185482; 20030198573; 200301 75773; 20030003490; 200201 8751 5; and 20020177141; as well as Shen, R., et al. Mutation Research 573 70-82 (2005).

d. Other techniques

[0093] In some of the embodiment herein, nucleic acids are quantified. Methods for quantifying nucleic acids are known in the art and include, but are not limited to, gas chromatography, supercritical fluid chromatography, liquid chromatography (including partition chromatography, adsorption chromatography, ion exchange chromatography, size exclusion chromatography, thin-layer chromatography, and affinity chromatography), electrophoresis (including capillary electrophoresis, capillary zone electrophoresis, capillary isoelectric focusing, capillary electrochromatography, micellar electrokinetic capillary chromatography, isotachophoresis, transient isotachophoresis and capillary gel electrophoresis), comparative genomic hybridization (CGH), microarrays, bead arrays, and high-throughput genotyping such as with the use of molecular inversion probe (MIP).

[0094] Another method contemplated by the present invention to detect and/or quantify target nucleic acids involves the use of nanoreporters as described in US patent 7,473,767 entitled "Methods for detection and quantification of analytes in complex mixtures", US patent publication no. 2007/0166708 entitled "Methods for detection and quantification of analytes in complex mixtures", US application number 11/645,270 entitled "Compositions comprising oriented, immobilized macromolecules and methods for their preparation", PCT application no US06/049274 entitled "Nanoreporters and methods of manufacturing and use thereof",

[0095] Quantification of target nucleic acid can be used to determine the percentage of donor nucleic acids such as DNA.

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e. Labels

[0096] Detection and/or quantification of target nucleic acids can be done using fluorescent dyes known in the art. Fluorescent dyes may typically be divided into families, such as fluorescein and its derivatives; rhodamine and its derivatives; cyanine and its derivatives; coumarin and its derivatives; Cascade Blue™ and its derivatives; Lucifer Yellow and its derivatives; BODIPY and its derivatives; and the like. Exemplary fluorophores include indocarbocyanine (C3), indodicarbocyanine (C5), Cy3, Cy3.5, Cy5, Cy5.5, Cy7, Texas Red, Pacific Blue, Oregon Green 488, Alexa fluor®-355, Alexa Fluor 488, Alexa Fluor 532, Alexa Fluor 546, Alexa Fluor-555, Alexa Fluor 568, Alexa Fluor 594, Alexa Fluor 647, Alexa Fluor 660, Alexa Fluor 680, JOE, Lissamine, Rhodamine Green, BODIPY, fluorescein isothiocyanate (FITC), carboxy-fluorescein (FAM), phycoerythrin, rhodamine, dichlororhodamine (dRhodamine.TM.), carboxy tetramethylrhodamine (TAMRA.TM.), carboxy-X-rhodamine (ROX.TM.), LIZ.TM., VIC.TM., NED.TM., PET.TM., SYBR, PicoGreen, RiboGreen, and the like. Descriptions of fluorophores and their use, can be found in, among other places, R. Haugland, Handbook of Fluorescent Probes and Research Products, 9.sup.th ed. (2002), Molecular Probes, Eugene, Oreg.; M. Schena, Microarray Analysis (2003), John Wiley & Sons, Hoboken, N.J.; Synthetic Medicinal Chemistry 2003/2004 Catalog, Berry and Associates, Ann Arbor, Mich.; G. Hermanson, Bioconjugate Techniques, Academic Press (1996); and Glen Research 2002 Catalog, Sterling, Va. Near-infrared dyes are expressly within the intended meaning of the terms fluorophore and fluorescent reporter group.

[0097] In another aspect of the invention, a branched-DNA (bDNA) approach is used to increase the detection sensitivity. In some embodiments, bDNA approach is applied to an array detection assay. The array detection assay can be any array assay known in the art, including the array assays described herein. bDNA approach amplifies the signals through a branched DNA that are attached by tens or hundreds of alkaline phosphatase molecules. Thus, the signals are significantly amplified while the fidelity of the original nucleic acid target abundance is maintained.

Methods

[0098] In one aspect the invention provides methods for the diagnosis or prediction of transplant status or outcome in a subject who has received a transplant. The transplant status or outcome may comprise rejection, tolerance, non-rejection based transplant injury, transplant function, transplant survival, chronic transplant injury, or titer pharmacological immunosuppression. Examples of non-rejection based allograft injury include, but are not limited to, ischemic injury, virus infection, peri-operative ischemia, reperfusion injury, hypertension, physiological stress, injuries due to reactive oxygen species and injuries caused by pharmaceutical agents. The transplant status or outcome may comprise vascular complications or neoplastic involvement of the transplanted organ.

[0099] In some embodiments, the invention provides methods of diagnosing or predicting transplant status or outcome comprising the steps of: (i) providing a sample from a subject who has received a transplant from a donor; (ii) determining the presence or absence of one or more nucleic acids from

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the donor transplant, wherein the one or more nucleic acids from the donor are identified based on a predetermined marker profile; and (iii) diagnosing or predicting transplant status or outcome based on the presence or absence of the one or more nucleic acids from said donor.

[00100] In some embodiments, the methods of the invention are used to establish a genotype for both the donor and the recipient before transplantation. In some embodiments, the genotyping of both the donor and the recipient before transplantation enables the detection of donor-specific nucleic acids such as DNA or RNA in bodily fluids as described herein (e.g., blood or urine) from the organ recipient after transplantation. In some embodiments a marker profile for the donor is determined based on the genotyping of the transplant donor. In some embodiments, a marker profile is determined for the transplant recipient based on the genotyping of the transplant recipient. In some embodiments, a marker profile is established by selecting markers that are distinguishable between the transplant donor and the subject receiving the transplant. This approach allows for a reliable identification of nucleic acids arising solely from the organ transplantation that can be made in a manner that is independent of the genders of donor and recipient.

[00101] Genotyping of the transplant donor and/or the transplant recipient may be performed by any suitable method known in the art including those described herein such as sequencing, nucleic acid array or PCR. In some embodiments, genotyping of the transplant donor and/or the transplant recipient is performed by shotgun sequencing. In some embodiments, genotyping of the transplant donor and/or the transplant recipient is performed using a DNA array. In some embodiments, genotyping of the transplant donor and/or the transplant recipient is performed using a polymorphism array such as a SNP array.

[00102] In some embodiments, the marker profile is a polymorphic marker profile. Polymorphic marker profile may comprise one or more single nucleotide polymorphisms (SNP's), one or more restriction fragment length polymorphisms (RFLP's), one or more short tandem repeats (STRs), one or more variable number of tandem repeats (VNTR's), one or more hypervariable regions, one or more minisatellites, one or more dinucleotide repeats, one or more trinucleotide repeats, one or more tetranucleotide repeats, one or more simple sequence repeats, or one or more insertion elements. In some embodiments, the marker profile comprises at least 1; 2; 3; 4; 5; 10; 20; 50; 100; 200; 500; 1,000; 2,000; 5,000; 10,000; 20,000; 50,000; 100,000; 200,000; 300,000; 400,000; 500,000; 600,000; 700,000; 800,000; 900,000; 1,000,000; 2,000,000 or 3,000,000 different polymorphic markers.

[00103] In some embodiments, the polymorphic marker profile comprises one or more SNPs. In some embodiments, the marker profile comprises at least 1; 2; 3; 4; 5; 10; 20; 50; 100; 200; 500; 1,000; 2,000; 5,000; 10,000; 20,000; 50,000; 100,000; 200,000; 300,000; 400,000; 500,000; 600,000; 700,000; 800,000; 900,000; 1,000,000; 2,000,000 or 3,000,000 different SNPs.

[00104] Following transplantation, samples as described above can be drawn from the patient and analyzed for the presence or absence of one or more nucleic acids from the transplant donor. In some embodiments, the sample is blood, plasma, serum or urine. The proportion and/or amount of donor

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nucleic acids can be monitored over time and an increase in this proportion can be used to determine transplant status or outcome (e.g. transplant rejection).

[00105] The presence or absence of one or more nucleic acids from the transplant donor in the transplant recipient may be determined by any suitable method known in the art including those described herein such as sequencing, nucleic acid arrays or PCR. In some embodiments, the presence or absence of one or more nucleic acids from the transplant donor in the transplant recipient is determined by shotgun sequencing. In some embodiments, the presence or absence of one or more nucleic acids from the transplant donor in the transplant recipient is determined using a DNA array. In some embodiments, the presence or absence of one or more nucleic acids from the transplant donor in the transplant recipient is determined using a polymorphism array such as a SNP array.

[00106] In some embodiments, where the transplant is a xenotransplant, detection, identification and/or quantitation of the donor-specific markers can be performed by mapping one or more nucleic acids (e.g., DNA) to the genome of the specie use to determine whether the one or more nucleic acids come from the transplant donor. Polymorphic markers as described above can also be used where the transplant is a xenotransplant.

[00107] In some embodiments, the presence or absence of circulating DNA or RNA from a transplant donor in a transplant recipient is used to determine the transplant status or outcome. The DNA can be double-stranded DNA, single-stranded DNA, single-stranded DNA hairpins, or cDNA. The RNA can be single stranded RNA or RNA hairpins. In some embodiments, the presence or absence of circulating DNA/RNA hybrids from a transplant donor in a transplant recipient is used to determine the transplant status or outcome. In some embodiments, the presence or absence of circulating mRNA from a transplant donor in a transplant recipient is used to determine the transplant status or outcome. In some embodiments, the presence or absence of circulating DNA from a transplant donor in a transplant recipient is used to determine the transplant status or outcome. In some embodiments, cDNA is used to determine the transplant status or outcome. The DNA or RNA can be obtained from circulating donor cells. Alternative, the DNA or RNA can be circulating cell-free DNA or circulating cell-free RNA

[00108] In any of the embodiments described herein, the transplant graft maybe any solid organ and skin transplant. Examples of transplants, whose transplant status or outcome could be determined by the methods described herein, include but are not limited to, kidney transplant, heart transplant, liver transplant, pancreas transplant, lung transplant, intestine transplant and skin transplant.

[00109] In some embodiments, the invention provides methods of determining whether a patient or subject is displaying transplant tolerance. In some embodiments the invention provides methods for diagnosis or prediction of transplant rejection. The term "transplant rejection" encompasses both acute and chronic transplant rejection. In some embodiments, the invention further includes methods for determining an immunosuppressive regimen for a subject who has received a transplant, e.g., an allograft. In some embodiments, the invention further includes methods for determining the

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effectiveness of an immunosuppressive regimen for a subject who has received a transplant. Certain embodiments of the invention provide methods of predicting transplant survival in a subject that has received a transplant. The invention provides methods of diagnosing or predicting whether a transplant in a transplant patient or subject will survive or be lost. In certain embodiments, the invention provides methods of diagnosing or predicting the presence of long-term graft survival. In some embodiments, the invention provides methods for diagnosis or prediction of non-rejection based transplant injury. Examples of non-rejection based graft injury include, but are not limited to, ischemic injury, virus infection, peri-operative ischemia, reperfusion injury, hypertension, physiological stress, injuries due to reactive oxygen species and injuries caused by pharmaceutical agents. In some embodiments, the invention provides methods for diagnosis or prediction of vascular complications or neoplastic involvement of the transplanted organ.

[00110] In some embodiments, the amount of one or more nucleic acids from the transplant donor in a sample from the transplant recipient is used to determine the transplant status or outcome. Thus, in some embodiments, the methods of the invention further comprise quantitating the one or more nucleic acids from the transplant donor. In some embodiments, the amount of one or more nucleic acids from the donor sample is determined as a percentage of total the nucleic acids in the sample. In some embodiments, the amount of one or more nucleic acids from the donor sample is determined as a ratio of the total nucleic acids in the sample. In some embodiments, the amount of one or more nucleic acids from the donor sample is determined as a ratio or percentage compared to one or more reference nucleic acids in the sample. For instance, the amount of one or more nucleic acids from the transplant donor can be determined to be 10% of the total nucleic acids in the sample. Alternatively, the amount of one or more nucleic acids from the transplant donor can be at a ratio of 1:10 compared to total nucleic acids in the sample. Further, the amount of one or more nucleic acids from the transplant donor can be determined to be 10% or at a ratio of 1:10 of a reference gene such a β-globin. In some embodiments, the amount of one or more nucleic acids from the transplant donor can be determined as a concentration. For example, the amount of one or more nucleic acids from the donor sample can be determined to be 1 ug/mL.

[00111] In some embodiments, the amount of one or more nucleic acids from the transplant donor above a predetermined threshold value is indicative of a transplant status or outcome. For example, the normative values for clinically stable post-transplantation patients with no evidence of graft rejection or other pathologies can be determined. An increase in the amount of one or more nucleic acids from the transplant donor above the normative values for clinically stable post-transplantation patients could indicate a change in transplant status or outcome such as transplant rejection or transplant injury. On the other hand, an amount of one or more nucleic acids from the transplant donor below or at the normative values for clinically stable post-transplantation patients could indicate graft tolerance or graft survival.

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[00112] In some embodiments, different predetermined threshold values are indicative of different transplant outcomes or status. For example, as discussed above, an increase in the amount of one or more nucleic acids from the transplant donor above the normative values for clinically stable post-transplantation patients could indicate a change in transplant status or outcome such as transplant rejection or transplant injury. However, an increase in the amount of one or more nucleic acids from the transplant donor above the normative values for clinically stable post-transplantation patients but below a predetermined threshold level could indicate a less serious condition such as a viral infection rather than transplant rejection. An increase in the amount of one or more nucleic acids from the transplant donor above a higher threshold could indicate transplant rejection.

[00113] In some embodiments, temporal differences in the amount of said one or more nucleic acids from the transplant donor are indicative of a transplant status or outcome. For instance, a transplant patient can be monitored over time to determine the amount of one or more nucleic acids from the transplant donor. A temporary increase in the amount of one or more nucleic acids from the transplant donor, which subsequently return to normal values, might indicate a less serious condition rather than transplant rejection. On the other hand, a sustained increase in the amount one or more nucleic acids from the transplant donor might indicate a serious condition such as transplant rejection.

[00114] In some embodiments, temporal differences in the amount of said one or more nucleic acids from the transplant donor can be used to monitor effectiveness of an immunosuppressant treatment or to select an immunosuppressant treatment. For instance, the amount of one or more nucleic acids from the transplant donor can be determined before and after an immunosuppressant treatment. A decrease in the one or more nucleic acids from the transplant donor after treatment may indicate that the treatment was successful in preventing transplant rejection. Additionally, the amount of one or more nucleic acids from the transplant donor can be used to choose between immunosuppressant treatments, for examples, immunosuppressant treatments of different strengths. For example, a higher amount in one or more nucleic acids from the transplant donor may indicate that there is a need of a very potent immunosuppressant, whereas a lower amount in one or more nucleic acids from the transplant donor may indicate that a less potent immunosuppressant may be used.

[00115] The invention provides methods that sensitive and specific. In some embodiments, the methods described herein for diagnosing or predicting transplant status or outcome have at least 56 %, 60%, 70%, 80%, 90%, 95% or 100% sensitivity. In some embodiments, the methods described herein have at least 56 % sensitivity. In some embodiments, the methods described herein have at least 78 % sensitivity. In some embodiments, the methods described herein have a specificity of about 70% to about 100%. In some embodiments, the methods described herein have a specificity of about 80% to about 100%. In some embodiments, the methods described herein have a specificity of about 90% to about 100%. In some embodiments, the methods described herein have a specificity of about 100%.

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[00116] Also provided herein are methods for screening and identifying markers recognizing a donor nucleic acid that can be useful in the methods described herein, e.g. diagnosing or predicting transplant status or outcome. In some embodiments, the donor nucleic acid is cell-free DNA or DNA isolated from circulating donor cells.

[00117] Donor nucleic acid can be identified by the methods described herein including the methods described in the Examples. After identifying these, then one could look at the donor nucleic acids and examine them for their correlation with transplant status and outcomes such as chronic graft injury, rejection, and tolerance. In some embodiments, the longitudinal change of donor nucleic acids is studied. If clinically significant, these levels could be followed to titer pharmacological immunosuppression, or could be studied as a target for depletion.

Kits

[00118] Also provided are reagents and kits thereof for practicing one or more of the above-described methods. The subject reagents and kits thereof may vary greatly. Reagents of interest include reagents specifically designed for use in production of the above-described: (i) genotyping of a transplant donor and a transplant recipient; (ii) identification of marker profiles; and (ii) detection and/or quantitation of one or more nucleic acids from a transplant donor in a sample obtained from a transplant recipient.

[00119] One type of such reagents are one or more probes or an array of probes to genotype and/or to detect and/or to quantitate one or more nucleic acids. A variety of different array formats are known in the art, with a wide variety of different probe structures, substrate compositions and attachment technologies.

[00120] The kits of the subject invention may include the above-described arrays. Such kits may additionally comprise one or more therapeutic agents. The kit may further comprise a software package for data analysis, which may include reference profiles for comparison with the test profile.

[00121] The kits may comprise reagents such as buffers, and H_20. The kits may comprise reagents necessary to perform nucleic acid extraction and/or nucleic acid detection using the methods described herein such as PCR and sequencing.

[00122] Such kits may also include information, such as scientific literature references, package insert materials, clinical trial results, and/or summaries of these and the like, which indicate or establish the activities and/or advantages of the composition, and/or which describe dosing, administration, side effects, drug interactions, or other information useful to the health care provider. Such kits may also include instructions to access a database. Such information may be based on the results of various studies, for example, studies using experimental animals involving in vivo models and studies based on human clinical trials. Kits described herein can be provided, marketed and/or promoted to health providers, including physicians, nurses, pharmacists, formulary officials, and the like. Kits may also, in some embodiments, be marketed directly to the consumer.

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Computer Program

[00123] Any of the methods above can be performed by a computer program product that comprises a computer executable logic that is recorded on a computer readable medium. For example, the computer program can execute some or all of the following functions: (i) controlling isolation of nucleic acids from a sample, (ii) pre-amplifying nucleic acids from the sample, (iii) amplifying, sequencing or arraying specific polymorphic regions in the sample, (iv) identifying and quantifying a marker profile in the sample, (v) comparing data on marker profile detected from the sample with a predetermined threshold, (vi) determining a transplant status or outcome , (vi) declaring normal or abnormal transplant status or outcome. In particular, the computer executable logic can analyze data on the detection and quantity of polymorphism(s) (e.g. SNPs).

[00124] The computer executable logic can work in any computer that may be any of a variety of types of general-purpose computers such as a personal computer, network server, workstation, or other computer platform now or later developed. In some embodiments, a computer program product is described comprising a computer usable medium having the computer executable logic (computer software program, including program code) stored therein. The computer executable logic can be executed by a processor, causing the processor to perform functions described herein. In other embodiments, some functions are implemented primarily in hardware using, for example, a hardware state machine. Implementation of the hardware state machine so as to perform the functions described herein will be apparent to those skilled in the relevant arts.

[00125] The program can provide a method of evaluating a transplant status or outcome in a transplant recipient by accessing data that reflects the genotyping of the transplant donor and the transplant patient, and/or the presence or absence of one or more nucleic acids from the transplant donor in the circulation of the transplant patient post-transplantation.

[00126] In one embodiment, the computer executing the computer logic of the invention may also include a digital input device such as a scanner. The digital input device can provide information on a nucleic acid, e.g., polymorphism levels/quantity. For example, a scanner of this invention can provide an image of the polymorphism (e.g., SNPs) according to method herein. For instance, a scanner can provide an image by detecting fluorescent, radioactive, or other emission; by detecting transmitted, reflected, or scattered radiation; by detecting electromagnetic properties or other characteristics; or by other techniques. The data detected is typically stored in a memory device in the form of a data file. In one embodiment, a scanner may identify one or more labeled targets. For instance, a first DNA polymorphism may be labeled with a first dye that fluoresces at a particular characteristic frequency, or narrow band of frequencies, in response to an excitation source of a particular frequency. A second DNA polymorphism may be labeled with a second dye that fluoresces at a different characteristic frequency. The excitation sources for the second dye may, but need not, have a different excitation frequency than the source that excites the first dye, e.g., the excitation sources could be the same, or different, lasers.

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[00127] In some embodiments, the invention provides a computer readable medium comprising a set of instructions recorded thereon to cause a computer to perform the steps of (i) receiving data from one or more nucleic acids detected in a sample from a subject who has received transplant from a donor, wherein said one or more nucleic acids are nucleic acids from said donor transplant, and wherein said one or more nucleic acids from said donor are identified based on a predetermined marker profile; and (ii) diagnosing or predicting transplant status or outcome based on the presence or absence of the one or more nucleic acids.

EXAMPLES

Example 1: Detection of Donor DNA in Organ Transplant Recipients

[00128] Using digital PCR as described before (Warren, L., Bryder, D., Weissman, I.L., Quake, S.R., Proc Natl Acad Sci, 103, 17807-17812 (2006); Fan, H.C. Quake, S.R., Anal Chem, 79, 7576-7579 (2007)), the amount of chromosome Y and chromosome 1 markers were quantitated for female patients receiving either male or female hearts in plasma samples taken at the same time that an endomyocardial biopsy determined a grade 3A or 3B rejection episode.

[00129] While blood transfusions/male child birth are known mechanisms to have detectable cY signature in a female patient, Figure 2 shows that the overall levels of cY are uniformly higher for patients receiving hearts from male donors. No significant chromosome Y signal from four control female-to-female transplant patients was detected. On the other hand, 1.5-8% total genomic fraction for chromosome Y signals was observed at the rejection time points for three male-to-female transplant patients across four rejection episodes.

[00130] Levels of chromosome Y in plasma were monitored at several time points following transplantation for some of these patients, and compared with biopsy time points for organ rejection. For patient 6, a 3A grade rejection was detected after biopsy 21 months after transplant. The level of chromosome Y detected in plasma was neglible in plasma at three months prior to rejection, but increased >10-fold to 2% of total genomic fraction at the time a biopsy determined rejection. The highest levels of cY in the plasma DNA are seen at this time (Figure 3). The results in Figure 3 suggest that the overall levels of cell-free DNA in the plasma are not diagnostic of organ failure and do not track the "donor-specific" DNA signal

[00131] Similar trends were observed for another patient that had cY levels increasing at 5 months after transplant when a biopsy detected a grade 3A rejection (Figure 4). The percentage of cY (or % "Donor") DNA is increasing before and highest at rejection time. Like above, the amount of total cell-free DNA does not seem diagnostic for heart rejection

[00132] Collectively, these results establish that for heart transplant patients, donor-derived DNA present in plasma can serve as a potential marker for the onset of organ failure.

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Example 2: Genotyping of Transplant Donor and Transplant Recipient

[00133] Figure 5 shows a general strategy to monitor all transplant patients. Genotyping of donor and recipient can establish a single nucleotide polymorphism (SNP) profile for detecting donor DNA. Shotgun sequencing of cell-free DNA in plasma, with analysis of observed unique SNPs, allows quantitation of % Donor DNA in the sample. While any single SNP may be difficult to detect with so little DNA in plasma, with hundred of thousands or more signals to consider, high sensitivity should be possible

[00134] Libraries of mixed genotypes can be created using two CEU (Mormon, Utah) HapMap lines. Approximately 1.2 million total variations between these two individuals were already established using existing genotyping platforms (e.g., Illumina Golden Gate). Usable SNPs must be homozygous for the recipient and ideally homozygous for the donor as well. Usable SNPs comprise: (i) approximately 500,000 heterozygous donor SNPs (count will be 1/2 of total donor fraction), (ii) approximately 160,000 homozygous donor SNPs.

[00135] *Sequencing Results*: 4 lanes of Illumina sequencing are used to compare 4 different levels of substitution of Donor DNA into Recipient DNA (See Figure 6). Error rate of sequencing is currently ~0.3-0.5% for base substitution. The use of quality scores for improved filtering of SNP calls, or the use of resequencing, should reduce error rate and increase sensitivity. The use of more SNP locations (from full genotyping) should also improve yield of signal with no change in protocol.

[00136] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

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WHAT IS CLAIMED IS:

1. A method of diagnosing or predicting transplant status or outcome comprising:

providing a sample from a subject who has received a transplant from a donor;

determining the presence or absence of one or more nucleic acids from said donor transplant, wherein said one or more nucleic acids from said donor are identified based on a predetermined marker profile; and

diagnosing or predicting transplant status or outcome based on the presence or absence of said one or more nucleic acids.

2. The method of claim 1 wherein said transplant status or outcome comprises rejection, tolerance, non-rejection based allograft injury, transplant function, transplant survival, chronic transplant s injury, or titer pharmacological immunosuppression.

3. The method of claim 2 wherein said non-rejection based allograft injury is selected from the group of ischemic injury, virus infection, peri-operative ischemia, reperfusion injury, hypertension, physiological stress, injuries due to reactive oxygen species and injuries caused by pharmaceutical agents.

4. The method of claim 1 wherein said sample is selected from the group consisting of blood, serum, urine, and stool.

5. The method of claim 1 wherein said marker profile is a polymorphic marker profile.

6. The method of claim 1 wherein said polymorphic marker profile comprises one or more single nucleotide polymorphisms (SNP's), one or more restriction fragment length polymorphisms (RFLP's), one or more short tandem repeats (STRs), one or more variable number of tandem repeats (VNTR's), one or more hypervariable regions, one or more minisatellites, one or more dinucleotide repeats, one or more trinucleotide repeats, one or more tetranucleotide repeats, one or more simple sequence repeats, or one or more insertion elements.

7. The method of claim 1 wherein said polymorphic marker profile comprises one or more SNPs

8. The method of claim 1 wherein said transplant is selected from the group consisting of kidney transplant, heart transplant, liver transplant, pancreas transplant, lung transplant, intestine transplant and skin transplant.

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9. The method of claim 1 wherein said nucleic acid is selected from the group consisting of double-stranded DNA, single-stranded DNA, single-stranded DNA hairpins, DNA/RNA hybrids, RNA and RNA hairpins.

10. The method of claim 1 wherein said nucleic acid is selected from the group consisting of double-stranded DNA, single-stranded DNA and cDNA.

11. The method of claim 1 wherein said nucleic acid is mRNA.

12. The method of any of the claims 9 to 11 wherein said nucleic acid is obtained from circulating donor cells.

13. The method of claim 1, wherein said nucleic acid is circulating cell-free DNA.

14. The method of claim 1, wherein the presence or absence of said one or more nucleic acids is determined by a method selected from the group consisting of sequencing, nucleic acid array and PCR.

15. The method of claim 14 wherein said sequencing is shotgun sequencing.

16. The method of claim 14 wherein said array is a DNA array.

17. The method of claim 16 wherein said DNA array is a polymorphism array.

18. The method of claim 17 wherein said polymorphism array is a SNP array.

19. The method of claim 1, further comprising quantitating said one or more nucleic acids.

20. The method of claim 19, wherein the amount of said one or more nucleic acids is indicative of transplant status or outcome.

21. The method of claim 20, wherein the amount of said one or more nucleic acids above a predetermined threshold value is indicative of a transplant status or outcome.

22. The method of claim 21, wherein said threshold is a normative value for clinically stable post-transplantation patients with no evidence of transplant rejection or other pathologies.

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23. The method of claim 21, wherein there are different predetermined threshold values for different transplant outcomes or status.

24. The method of claim 20 wherein temporal differences in the amount of said one or more nucleic acids are indicative of a transplant status or outcome.

25. The method of claim 1, wherein said marker profile is determined by genotyping said transplant donor.

26. The method of claim 25, further comprising genotyping said subject receiving said transplant.

27. The method of claim 26, further comprising establishing a profile of markers, wherein said markers are distinguishable between said transplant donor and said subject receiving said transplant.

28. The method of any of the claims 25 to 27 wherein said genotyping is performed by a method selected from the group consisting of sequencing, nucleic acid array and PCR.

29. The method of claim 1 wherein said method has at least 56 % sensitivity.

30. The method of claim 1 wherein said method has at least 78 % sensitivity.

31. The method of claim 1 wherein said method has a specificity of about 70% to about 100%.

32. The method of claim 1 wherein said method has a specificity of about 80% to about 100%.

33. The method of claim 1 wherein said method has a specificity of about 90% to about 100%.

34. The method of claim 1 wherein said method has a specificity of about 100%.

35. A computer readable medium comprising:

a set of instructions recorded thereon to cause a computer to perform the steps of:

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(i) receiving data from one or more nucleic acids detected in a sample from a subject who has received transplant from a donor, wherein said one or more nucleic acids are nucleic acids from said donor transplant, and wherein said one or more nucleic acids from said donor are identified based on a predetermined marker profile; and

(ii) diagnosing or predicting transplant status or outcome based on the presence or absence of said one or more nucleic acids.

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Foresight v Personalis

Figure 1

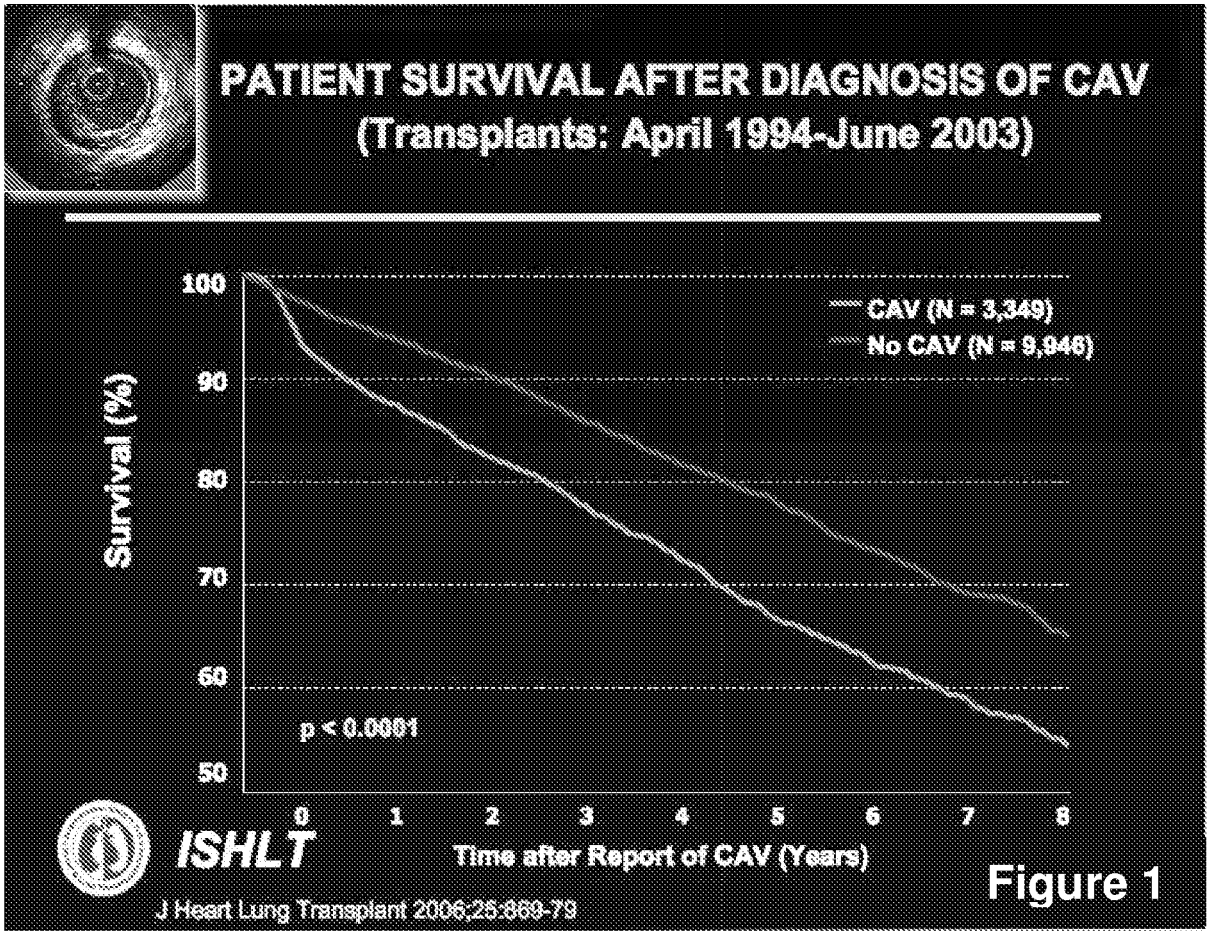

PATIENT SURVIVAL AFTER DIAGNOSIS OF CAV
(Transplants: April 1994-June 2003)
CAV (N = 3,349)
No CAV (N = 9,946)
Survival (%)
100
90
80
70
60
50
p < 0.0001
0
1
2
3
4
5
6
7
8
Time after Report of CAV (Years)
ISHLT
J Heart Lung Transplant 2006;25:869-79
Figure 1

Figure 2

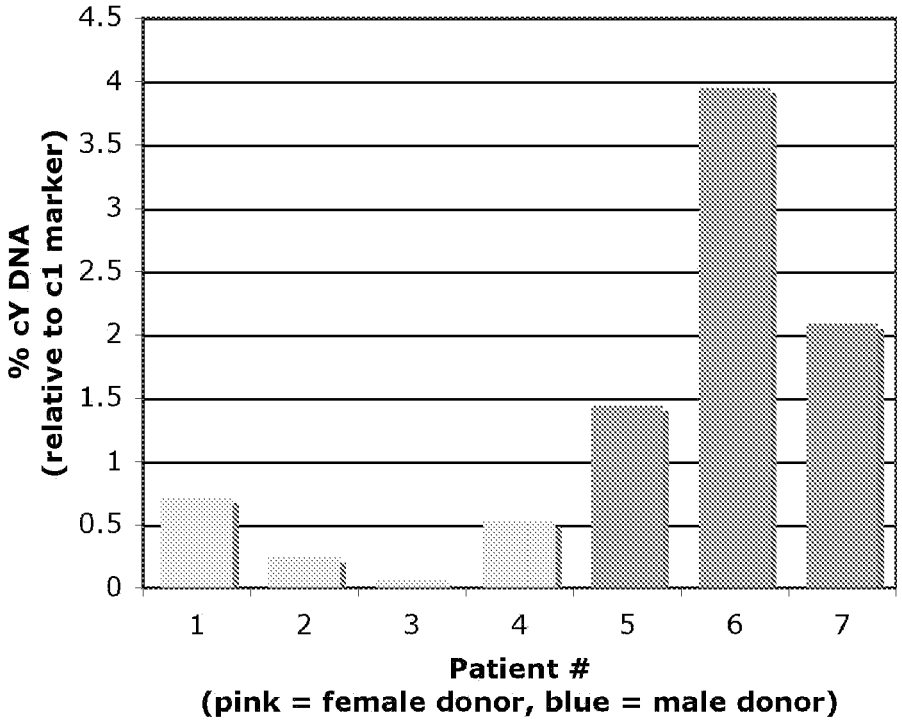

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
% cY DNA
(relative to c1 marker)
1
2
3
4
5
6
7
Patient #
(pink = female donor, blue = male donor)

Figure 3

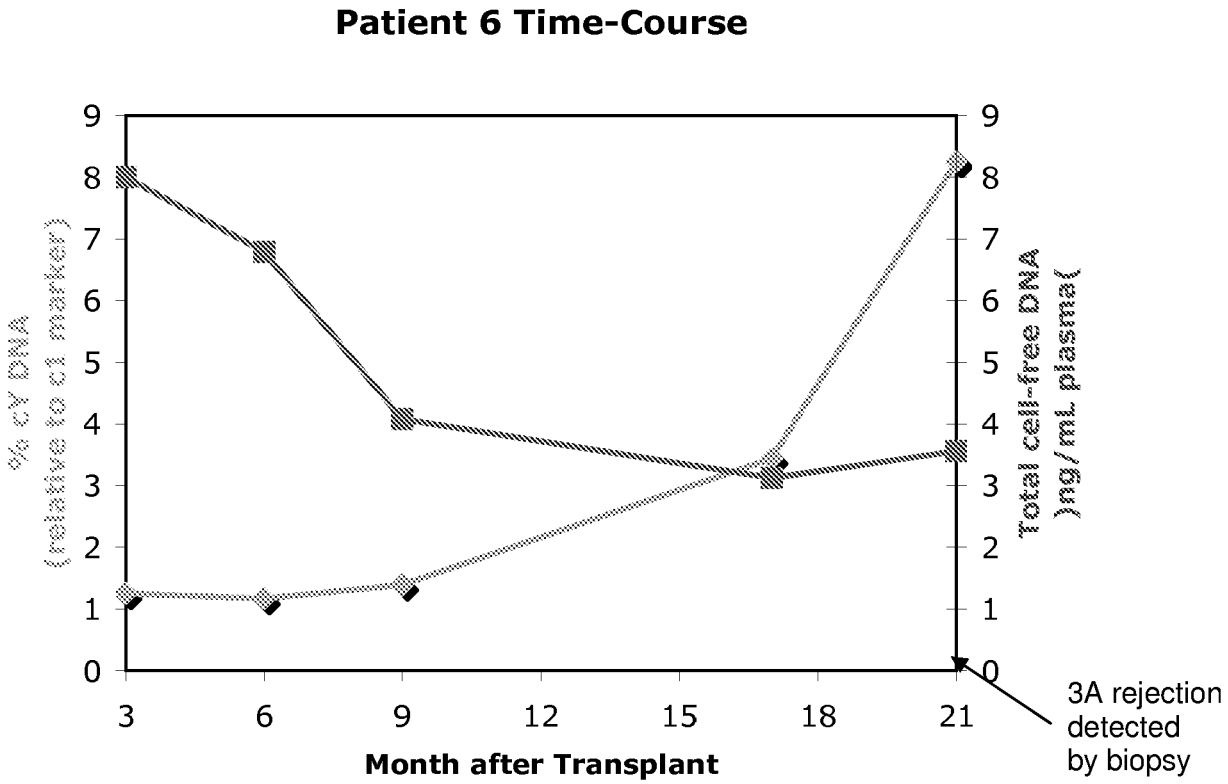

Patient 6 Time-Course
% cY DNA
(relative to c1 marker)
Total cell-free DNA
)ng/mL plasma(
Month after Transplant
0
1
2
3
4
5
6
7
8
9
3
6
9
12
15
18
21
3A rejection
detected
by biopsy

Figure 4

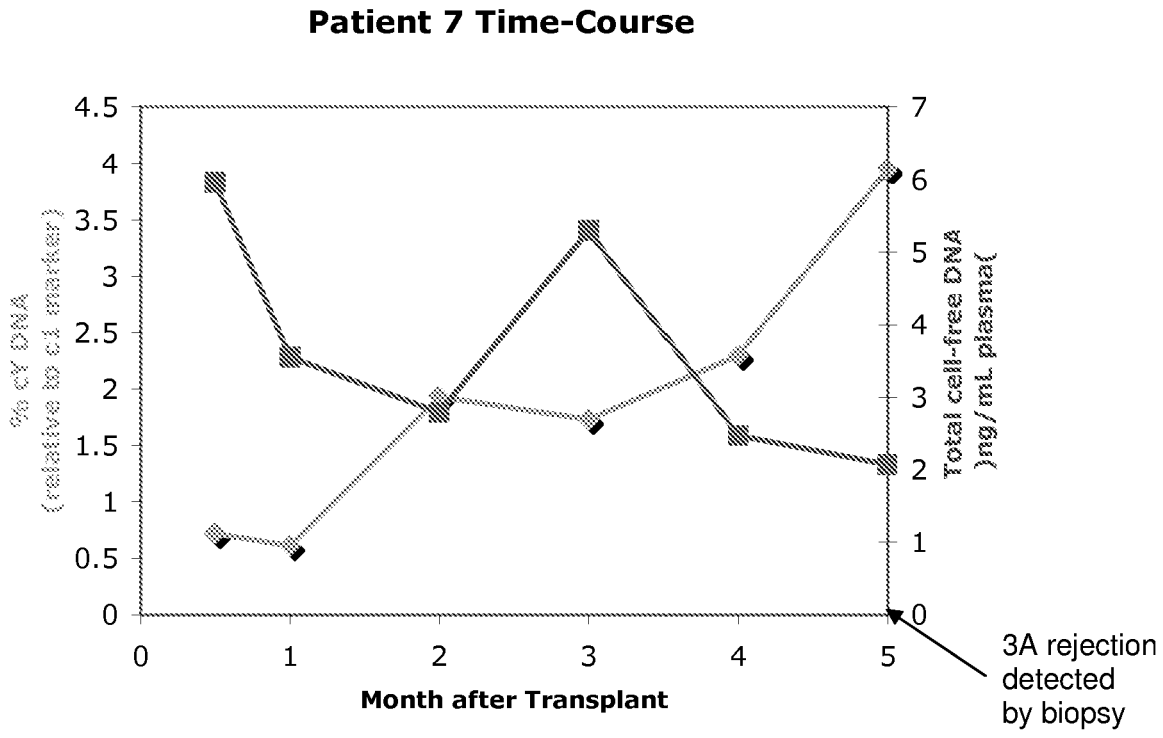

Patient 7 Time-Course
% cY DNA
(relative to c1 marker)
Total cell-free DNA
)ng/mL plasma(
Month after Transplant
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
7
6
5
4
3
2
1
0
0
1
2
3
4
5
3A rejection
detected
by biopsy

Figure 5

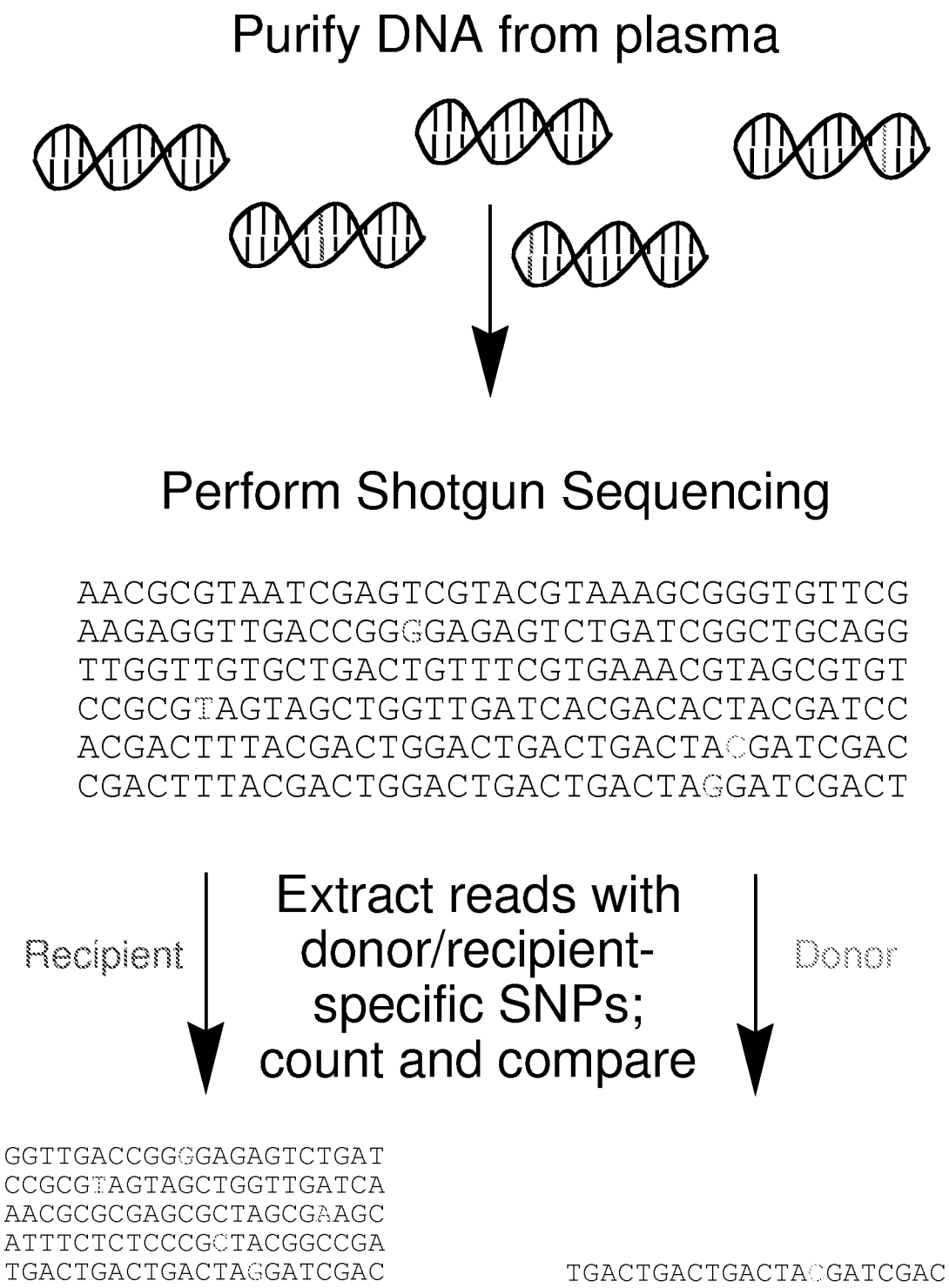
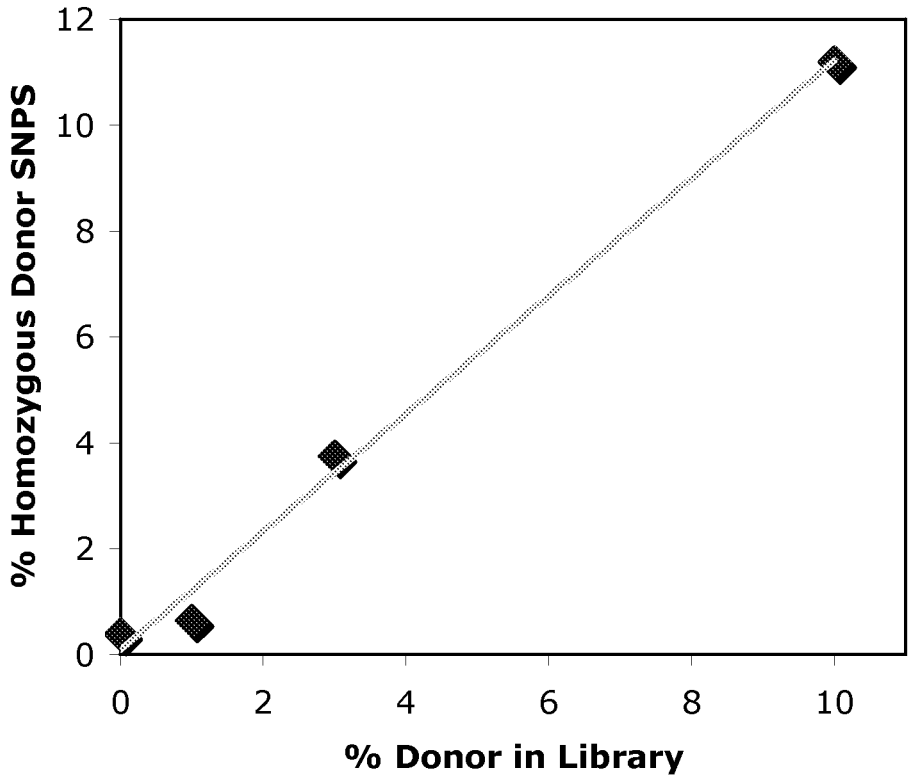

Purify DNA from plasma
Perform Shotgun Sequencing
AACGCGTAATCGAGTCGTACGTAAAGCGGGTGTTCG
AAGAGGTTGACCGGGGAGAGTCTGATCGGCTGCAGG
TTGGTTGTGCTGACTGTTTCGTGAAACGTAGCGTGT
CCGCGTAGTAGCTGGTTGATCACGACACTACGATCC
ACGACTTTACGACTGGACTGACTGACTACGATCGAC
CGACTTTACGACTGGACTGACTGACTAGGATCGACT
Recipient
Extract reads with donor/recipient-specific SNPs; count and compare
Donor
GGTTGACCGGGGAGAGTCTGAT
CCGCGTAGTAGCTGGTTGATCA
AACGCGCGAGCGCTAGCGAAGC
ATTTCTCTCCCGCTACGGCCGA
TGACTGACTGACTAGGATCGAC
TGACTGACTGACTACGATCGAC

Figure 6

A.

Library	0% Donor	1% Donor	3% Donor	10% Donor
Total Unique Aligned Reads	8747074	9340382	5444089	8485355
Total w/ SNPs	80301	84525	40161	71286
# Homo for Recip.	19882	20571	9269	15367
# Homo for Donor	77	133	360	1936

B.



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/55604

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2010.01)
USPC - 435/6 .
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12Q 1/68 (2010.01)
USPC - 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
435/7.23,7.24

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST - DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=YES; OP=ADJ; Google Scholar
search terms: transplan$, allotransplan$, xenotransplan$, allograf$, graft$, status, outcome, prognos$, risk, rejec$, toleran$, profil$, marker, biomarker, snp, polymorphis$, repeats, insertion elements, hypervariable, genotyp$, sequencing, sequenced, pcr, polymerase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ------ Y	US 2009/0263800 A1 (WOHLGEMUTH et al.) 22 October 2009 (22.10.2009) para [0010]-[0012]; [0019]; [0021]-[0023]; [0026]; [0040]; [0044]; [0047]; [0051]; [0116]-[0118]; [0122]; [0159]; [0168]; [0178]; [0200]; [0224]; [0233]; [0285]-[0290]; [0293]; [0299]; [0300]; [0306]; [0307]; [0336]; [0340]; [0342]; [0367]; [0401]-[0405]; [0409]; [0440]; [0761]; [0819]; [0820].	1-12, 14, 16-35 ------ 13, 15
Y	US RE39920 E (UMANSKY et al.) 13 November 2007 (13.11.2007) abstracy; col. 27, ln 65-col. 28, ln 7.	13
Y	US 2009/0221620 A1 (LUKE et al.) 03 September 2009 (03.09.2009) para [0130]; [0131].	15

☐ Further documents are listed in the continuation of Box C. ☐

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

Date of the actual completion of the international search	Date of mailing of the international search report
12 January 2011 (12.01.2011)	26 JAN 2011
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)