IN THE UNITED STATES DISTRICT COURT

FOR THE DISTRICT OF DELAWARE

CAREDX, INC. and THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY, Plaintiffs v. NATERA, INC., Defendant.	C.A. No. _____ **JURY TRIAL DEMANDED**

COMPLAINT

Plaintiffs CareDx, Inc. ("CareDx") and The Board Of Trustees Of The Leland Stanford Junior University ("Stanford," and collectively with CareDx "Plaintiffs"), for their complaint against Defendant Natera, Inc. ("Natera"), hereby allege as follows:

NATURE OF THE ACTION

1. Years ago, researchers at Stanford University invented a method for determining organ transplant rejection. It allowed doctors to assess rejection through blood tests and without invasive biopsies. This method is intended to saves lives, minimize patient pain and stress, and cuts the healthcare costs of treating transplant patients.

2. Stanford University secured the patents to its researchers' invention and licensed the patents exclusively to CareDx. CareDx then brought this invention out of the lab and into the clinical setting, helping leading transplant centers around the country treat patients. CareDx has worked hard on this effort, investing substantially to make this technology widely available.

3. Now, years after Stanford researchers and CareDx put in the research and development work to invent this new method and bring it to the clinical setting, Natera uses CareDx's licensed technology without permission in violation of the patent laws. Natera must be held accountable.

4. Accordingly, this is an action for patent infringement arising under the patent laws of the United States, Title 35, United States Code, against Defendant Natera.

5. CareDx brings this action to halt Natera's infringement of CareDx's rights under the Patent Laws of the United States 35 U.S.C. § 1, et seq., which arise under U.S. Patent Nos. 9,845,497 ("the '497 patent") (attached as Exhibit 1) and 8,703,652 ("the '652 patent") (attached as Exhibit 2).

PARTIES

6. CareDx is a corporation organized and existing under the laws of the state of Delaware, having its principal place of business at 3260 Bayshore Blvd., Brisbane, CA 94005.

7. CareDx was formed in 1998 by pioneers in molecular diagnostics. Since its inception, CareDx has focused its expertise on the discovery, development and commercialization of clinically differentiated, high-value solutions for organ transplant recipients. It was the first company to develop and commercialize non-invasive transplant surveillance testing to monitor transplant recipients' immune status with the aim to improve long-term patient outcomes.

8. Today, CareDx markets and sells AlloSure® ("AlloSure"). AlloSure uses advanced DNA sequencing methods to quantify donor-derived cell-free DNA (dd-cfDNA) in transplant recipients without having to conduct separate genotyping. Measuring dd-cfDNA in a transplant recipient's blood enables early detection of kidney transplant rejection and facilitates personalized immunosuppressive treatment. AlloSure has helped numerous nephrologists manage their patients' post-transplant care, while avoiding the high costs and added risks of renal biopsies.

9. Stanford is a trust possessing corporate powers that is organized under the laws of California, with a principal place of business at the Office of the President, Building 10 Main Quad, Stanford, California 94305. Stanford is the patent owner and licensor for the '497 and '652 patents and is joined in the infringement action for these patents because it is a necessary party.

10. On information and belief, Natera is a corporation organized and existing under the laws of the state of Delaware, having its principal place of business at 201 Industrial Road, Suite 410, San Carlos, CA 94070. Natera markets and sells a Kidney Transplant Rejection Test, also described as an "organ transplant rejection assay" and "allograft rejection" test, which it performs at its CLIA-certified laboratory in San Carlos, CA. Exhibit 3.

JURISDICTION AND VENUE

11. This action arises under the patent laws of the United States, 35 U.S.C. §§ 100, *et seq.*, and this Court has jurisdiction over the subject matter of this action under 28 U.S.C. §§ 1331, 1338(a), 2201 and 2202.

12. Venue is proper in this Court under 28 U.S.C. §§ 1391 and 1400(b).

13. This Court has jurisdiction over Natera because Natera is a Delaware corporation.

14. This Court also has jurisdiction over Natera because, upon information and belief, Natera, directly or indirectly, uses, offers for sale, and/or sells the Kidney Transplant Rejection Test throughout the United States and in this judicial district.

BACKGROUND

15. Plaintiffs repeat and re-allege the foregoing paragraphs as if set forth specifically herein.

16. On information and belief, in the mid-2018 time frame Natera began preparing to develop and commercialize a Kidney Transplant Rejection Test. According to a Natera press

release, "Natera's organ transplant rejection assay is designed to detect active allograft rejection in patients who have undergone renal (kidney) transplantation. The assay works by measuring the fraction of donor-derived cell-free DNA (dd-cfDNA) in the recipient's blood, which can spike relative to background cfDNA when the transplanted organ is injured due to immune rejection. The assay leverages Natera's core single nucleotide polymorphism (SNP)-based massively multiplexed PCR (mmPCR) technology, to more accurately measure dd-cfDNA levels without the need for donor genotyping, and it has been clinically validated for test performance independent of donor type, rejection type, and clinical presentation." Exhibit 3.

17. On December 5, 2018, scientists affiliated with Natera listed a clinical trial titled, "Utility of a Novel Dd-cfDNA Test to Detect Injury in Renal Post-Transplant Patients" on the National Institute of Health (NIH) website: clinicaltrials.gov. Exhibit 4. The clinical trial listing states that the intervention/treatment is a diagnostic test called "Natera KidneyScan" and describes the test as "Natera's novel SNP-based mmPCR-NGS test that measures dd-cfDNA" in a transplant recipients' blood to diagnose kidney transplant rejection.

18. On December 23, 2018, scientists affiliated with Natera published an article entitled, "Optimizing Detection of Kidney Transplant Injury by Assessment of Donor-Derived Cell-Free DNA Via Massively Multiplex PCR" (attached hereto as Exhibit 5) in the Journal of Clinical Medicine. On information and belief, this article describes the methodology of Natera's Kidney Transplant Rejection Test. Natera's January 7, 2019 press release confirms that the purpose of the study was to clinically validate the Kidney Transplant Rejection Test methodology: a "donor-derived cell-free DNA (dd-cfDNA) test for active allograft rejection in kidney transplant recipients" which uses "Natera's core single nucleotide polymorphism (SNP)-based massively multiplexed PCR (mmPCR) technology." Exhibit 6.

19. On February 1, 2019, Natera publicly announced a partnership to begin distributing "Natera's kidney transplant rejection test in the United States in collaboration with the company's direct sales team." Exhibit 3. As Natera's CEO explained, the partnership would permit it to "accelerate" Natera's entry into the market. *Id.*

20. Natera infringes, literally or under the doctrine of equivalents, the '497 patent through its activities connected to its performance of the Kidney Transplant Rejection Test and all variants of the Kidney Transplant Rejection Test. For instance, representative Claim 1 of the '497 patent is listed below:

> 1. A method of detecting donor-specific circulating cell-free nucleic acids in a solid organ transplant recipient, the method comprising:
>
> (a) genotyping a solid organ transplant donor to obtain a single nucleotide polymorphism (SNP) profile of the solid organ transplant donor;
>
> (b) genotyping a solid organ transplant recipient to obtain a SNP profile of the solid organ transplant recipient, wherein the solid organ transplant recipient is selected from the group consisting of: a kidney transplant, a heart transplant, a liver transplant, a pancreas transplant, a lung transplant, a skin transplant, and any combination thereof;
>
> (c) obtaining a biological sample from the solid organ transplant recipient after the solid organ transplant recipient has received the solid organ transplant from the solid organ transplant donor, wherein the biological sample is selected from the group consisting of blood, serum and plasma, and wherein the biological sample comprises circulating cell-free nucleic acids from the solid organ transplant; and
>
> (d) determining an amount of donor-specific circulating cell-free nucleic acids from the solid organ transplant in the biological sample by detecting a homozygous or a heterozygous SNP within the donor-specific circulating cell-free nucleic acids from the solid organ transplant in at least one assay, wherein the at least one assay comprises high-throughput sequencing or digital polymerase chain reaction (dPCR), and wherein the at least one assay detects the donor-specific circulating cell-free nucleic acids from the solid organ transplant when the donor-specific circulating cell-free nucleic acids make up at least 0.03% of the total circulating cell-free nucleic acids in the biological sample.

21. Performance of Natera's Kidney Transplant Rejection Test and all variants thereof leads to infringement of this claim in the following way. First, a plasma sample containing cell-free DNA (cfDNA) from a kidney transplant recipient (post-transplant) is genotyped to obtain a SNP profile. This involves three steps: (i) cfDNA is extracted from the recipient's plasma sample, (ii) cfDNA is amplified via massively multiplex PCR (mmPCR), and (iii) the amplified SNPs are then subject to next generation sequencing (NGS). Finally, the amount of donor-derived cfDNA (dd-cfDNA) in the post-transplant recipient's plasma is determined by detecting a homozygous or a heterozygous SNP within the donor-specific circulating cell-free nucleic acids. *See* Exhibit 5; Exhibit 6.

22. As an example, attached hereto as Exhibit 7 is a preliminary and exemplary claim chart detailing Natera's infringement of multiple claims of the '497 patent. This chart is not intended to limit Plaintiffs' right to modify this chart or any other claim chart or allege that other activities of Natera infringe the identified claims or any other claims of the '497 patent or any other patents.

23. Natera infringes, literally or under the doctrine of equivalents, the '652 patent through its activities connected to its performance of the Kidney Transplant Rejection Test and all variants thereof. For instance, representative Claim 1 of the '652 patent is listed below:

> 1. A method for detecting transplant rejection, graft dysfunction, or organ failure, the method comprising:
>
> (a) providing a sample comprising cell-free nucleic acids from a subject who has received a transplant from a donor;
>
> (b) obtaining a genotype of donor-specific polymorphisms or a genotype of subject-specific polymorphisms, or obtaining both a genotype of donor-specific polymorphisms and subject-specific polymorphisms, to establish a polymorphism profile for detecting donor cell-free nucleic acids, wherein at least one single nucleotide polymorphism (SNP) is homozygous for the subject if the genotype comprises subject-specific polymorphisms comprising SNPs;

(c) multiplex sequencing of the cell-free nucleic acids in the sample followed by analysis of the sequencing results using the polymorphism profile to detect donor cell-free nucleic acids and subject cell-free nucleic acids; and

(d) diagnosing, predicting, or monitoring a transplant status or outcome of the subject who has received the transplant by determining a quantity of the donor cell-free nucleic acids based on the detection of the donor cell-free nucleic acids and subject cell-free nucleic acids by the multiplexed sequencing, wherein an increase in the quantity of the donor cell-free nucleic acids over time is indicative of transplant rejection, graft dysfunction or organ failure, and wherein sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV).

24. Performance of Natera's Kidney Transplant Rejection Test and all variants thereof leads to infringement of this claim in the following way. First, a plasma sample containing cell-free DNA (cfDNA) from a kidney transplant recipient (post-transplant) is genotyped to obtain a SNP profile. This involves three steps: (i) cfDNA is extracted from the recipient's plasma sample, (ii) cfDNA is amplified via massively multiplex PCR (mmPCR), and (iii) the amplified SNPs are then subject to next generation sequencing (NGS). Finally, a quantity of donor-derived cfDNA (dd-cfDNA) in the post-transplant recipient's plasma is determined. Active rejection (AR) can be detected with at least 89% sensitivity. *See* Exhibit 5; Exhibit 6.

25. As an example, attached hereto as Exhibit 8 is a preliminary and exemplary claim chart detailing Natera's infringement of multiple claims of the '652 patent. This chart is not intended to limit Plaintiffs' right to modify this chart or any other claim chart or allege that other activities of Natera infringe the identified claims or any other claims of the '652 patent or any other patents.

COUNT I

(Infringement of U.S. Patent No. 9,845,497)

26. Plaintiffs repeat and re-allege the foregoing paragraphs as if set forth specifically herein.

27. On December 19, 2017, the United States Patent and Trademark Office duly and legally issued U.S. Patent No. 9,845,497 (the "'497 patent"), entitled "Non-Invasive Diagnosis of Graft Rejection in Organ Transplant Patients."

28. Stephen R. Quake, Ph.D., Thomas M. Snyder, Ph.D., and Hannah Valantine, M.D. are the sole and true inventors of the '497 patent. By operation of law and as a result of written assignment agreements, Stanford obtained the entire right, title, and interest to and in the '497 patent.

29. Pursuant to license agreements with Stanford, CareDx obtained an exclusive license to the '497 patent in the field of non-invasive monitoring of organ transplant rejection through cell-free DNA analysis.

30. On information and belief, Natera has infringed and continues to infringe the '497 patent pursuant to 35 U.S.C. § 271(a), literally or under the doctrine of equivalents, by performing within the United States without authority the Kidney Transplant Rejection Test. As an example, attached as Exhibit 7 is a preliminary and exemplary claim chart detailing Natera's infringement of the '497 patent. This chart is not intended to limit Plaintiffs' right to modify the chart or allege that other activities of Natera infringe the identified claims or any other claims of the '497 patent or any other patents.

31. Exhibit 7 is hereby incorporated by reference in its entirety. Each claim element in Exhibit 7 that is mapped to the Kidney Transplant Rejection Test shall be considered an allegation within the meaning of the Federal Rules of Civil Procedure and therefore a response to each allegation is required.

COUNT II

(Infringement of U.S. Patent No. 8,703,652)

32. Plaintiffs repeat and re-allege the foregoing paragraphs as if set forth specifically herein.

33. On April 22, 2014, the United States Patent and Trademark Office duly and legally issued U.S. Patent No. 8,703,652, entitled "Non-Invasive Diagnosis of Graft Rejection in Organ Transplant Patients."

34. Stephen R. Quake, Ph.D., Thomas M. Snyder, Ph.D., and Hannah Valantine, M.D. are the sole and true inventors of the '652 patent. By operation of law and as a result of written assignment agreements, Stanford obtained the entire right, title, and interest to and in the '652 patent.

35. Pursuant to license agreements with Stanford, CareDx obtained an exclusive license to the '652 patent in the field of non-invasive monitoring of organ transplant rejection through cell-free DNA analysis.

36. On information and belief, Natera has infringed and continues to infringe the '652 patent pursuant to 35 U.S.C. § 271(a), literally or under the doctrine of equivalents, by performing within the United States without authority the Kidney Transplant Rejection Test. As an example, attached as Exhibit 8 is a preliminary and exemplary claim chart detailing Natera's infringement of these claims of the '652 patent. This chart is not intended to limit Plaintiffs' right to modify the chart or allege that other activities of Natera infringe the identified claims or any other claims of the '652 patent or any other patents.

37. Exhibit 8 is hereby incorporated by reference in its entirety. Each claim element in Exhibit 8 that is mapped to Natera's Kidney Transplant Rejection Test shall be considered an

allegation within the meaning of the Federal Rules of Civil Procedure and therefore a response to each allegation is required.

JURY DEMAND

38. CareDx and Stanford demand a jury trial on all issues so triable.

PRAYER FOR RELIEF

WHEREFORE, CareDx and Stanford pray that this Court grant the following relief:

A. A judgment that Natera has infringed the ’497 patent and/or the ’652 patent and that the ’497 patent and/or the ’652 patent are valid.

B. Damages or other monetary relief, including, but not limited to, costs and pre- and post-judgment interest, to Plaintiffs;

C. An order enjoining Natera and its officers, directors, agents, servants, affiliates, employees, divisions, branches, subsidiaries, parents, and all others acting in active concert therewith from further infringement of the ’497 patent and/or the ’652 patent;

D. Such further and other relief as this Court deems proper and just, including, but not limited to, a determination that this is an exceptional case under 35 U.S.C. § 285 and an award of attorneys’ fees and costs to Plaintiffs in this action.

Dated: March 26, 2019

Respectfully submitted,

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EXHIBIT 1

US009845497B2

(12) **United States Patent**
Quake et al.

(10) **Patent No.: US 9,845,497 B2**
(45) **Date of Patent: *Dec. 19, 2017**

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

(71) Applicant: **The Board of Trustees of the Leland Stanford Junior University**, Palo Alto, CA (US)

(72) Inventors: **Stephen R. Quake**, Stanford, CA (US); **Thomas M. Snyder**, Palo Alto, CA (US); **Hannah Valantine**, Stanford, CA (US)

(73) Assignee: **The Board of Trustees of the Leland Stanford Junior University**, Stanford, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 452 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **14/188,455**

(22) Filed: **Feb. 24, 2014**

(65) **Prior Publication Data**

US 2015/0337361 A1 Nov. 26, 2015

Related U.S. Application Data

(63) Continuation of application No. 13/508,318, filed as application No. PCT/US2010/055604 on Nov. 5, 2010, now Pat. No. 8,703,652.

(60) Provisional application No. 61/280,674, filed on Nov. 6, 2009.

(51) **Int. Cl.**

C12Q 1/68	(2006.01)
C40B 30/00	(2006.01)
G06F 19/18	(2011.01)
G01N 33/48	(2006.01)

(52) **U.S. Cl.**

CPC ***C12Q 1/686*** (2013.01); ***C12Q 1/6837*** (2013.01); ***C12Q 1/6869*** (2013.01); ***G06F 19/18*** (2013.01); *C12Q 1/6883* (2013.01); *C12Q 2600/106* (2013.01)

(58) **Field of Classification Search**

CPC C12Q 1/68

See application file for complete search history.

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(57) **ABSTRACT**

The invention provides methods, devices, compositions and kits for diagnosing or predicting transplant status or outcome in a subject who has received a transplant.

33 Claims, 6 Drawing Sheets

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Figure 1

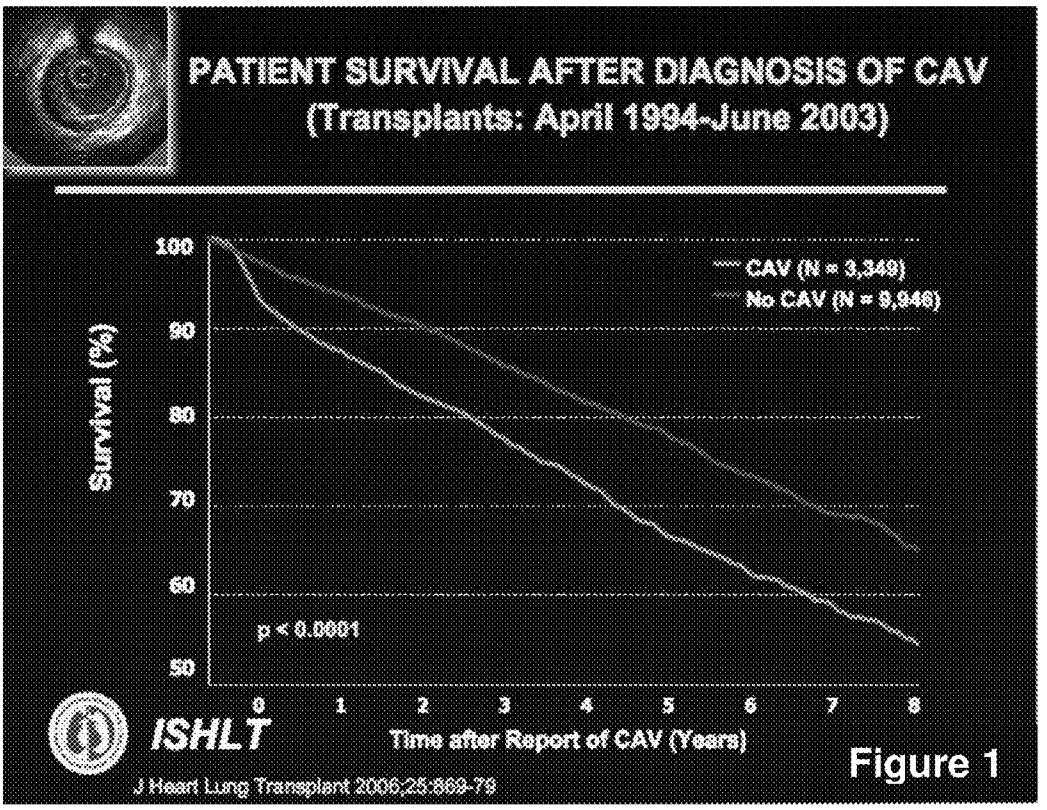

PATIENT SURVIVAL AFTER DIAGNOSIS OF CAV
(Transplants: April 1994-June 2003)
CAV (N = 3,349)
No CAV (N = 9,946)
Survival (%)
100
90
80
70
60
50
p < 0.0001
0
1
2
3
4
5
6
7
8
Time after Report of CAV (Years)
ISHLT
J Heart Lung Transplant 2006;25:869-79
Figure 1

Figure 2

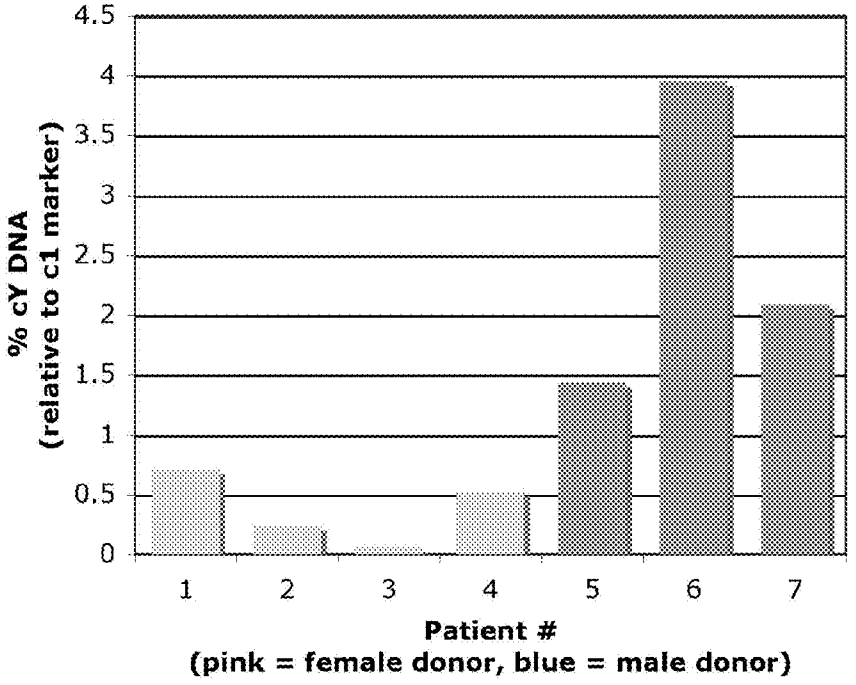

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
% cY DNA
(relative to c1 marker)
1
2
3
4
5
6
7
Patient #
(pink = female donor, blue = male donor)

Figure 3

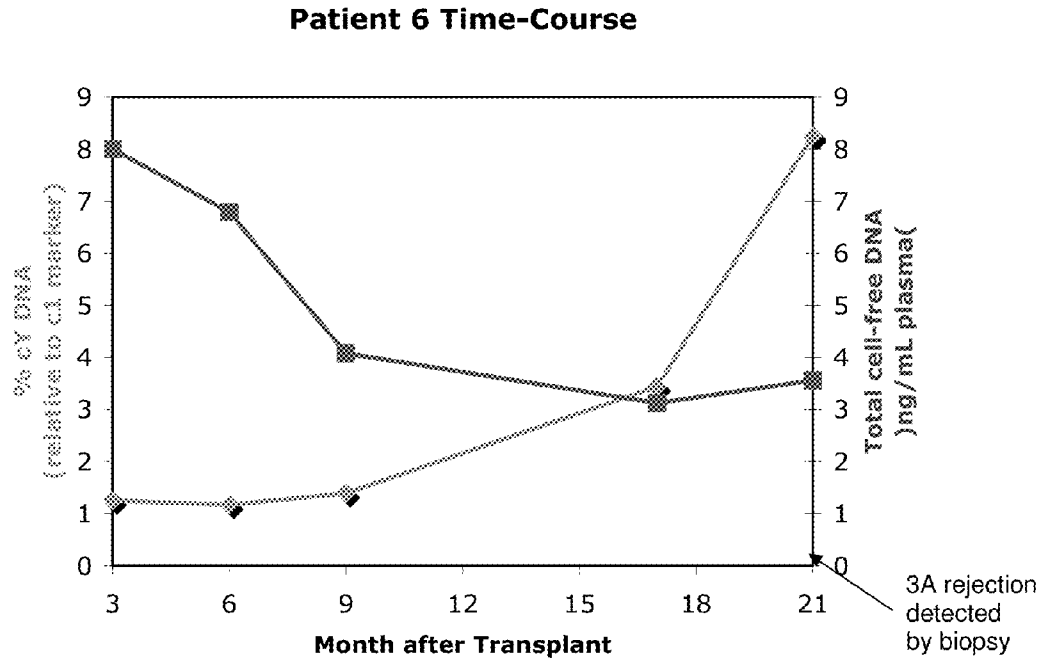

Patient 6 Time-Course
% cY DNA (relative to c1 marker)
Total cell-free DNA)ng/mL plasma(
Month after Transplant
3A rejection detected by biopsy
0
1
2
3
4
5
6
7
8
9
3
6
9
12
15
18
21

Figure 4

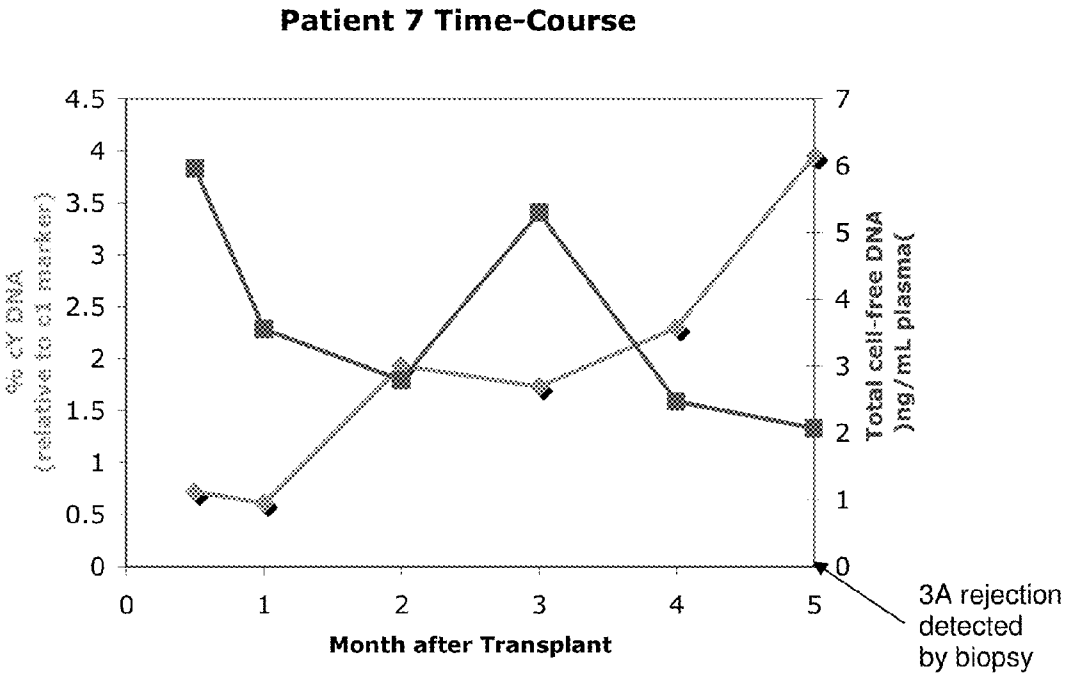

Patient 7 Time-Course
% cY DNA (relative to c1 marker)
Total cell-free DNA)ng/mL plasma(
Month after Transplant
3A rejection detected by biopsy
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
0
1
2
3
4
5
6
7
0
1
2
3
4
5

Figure 5

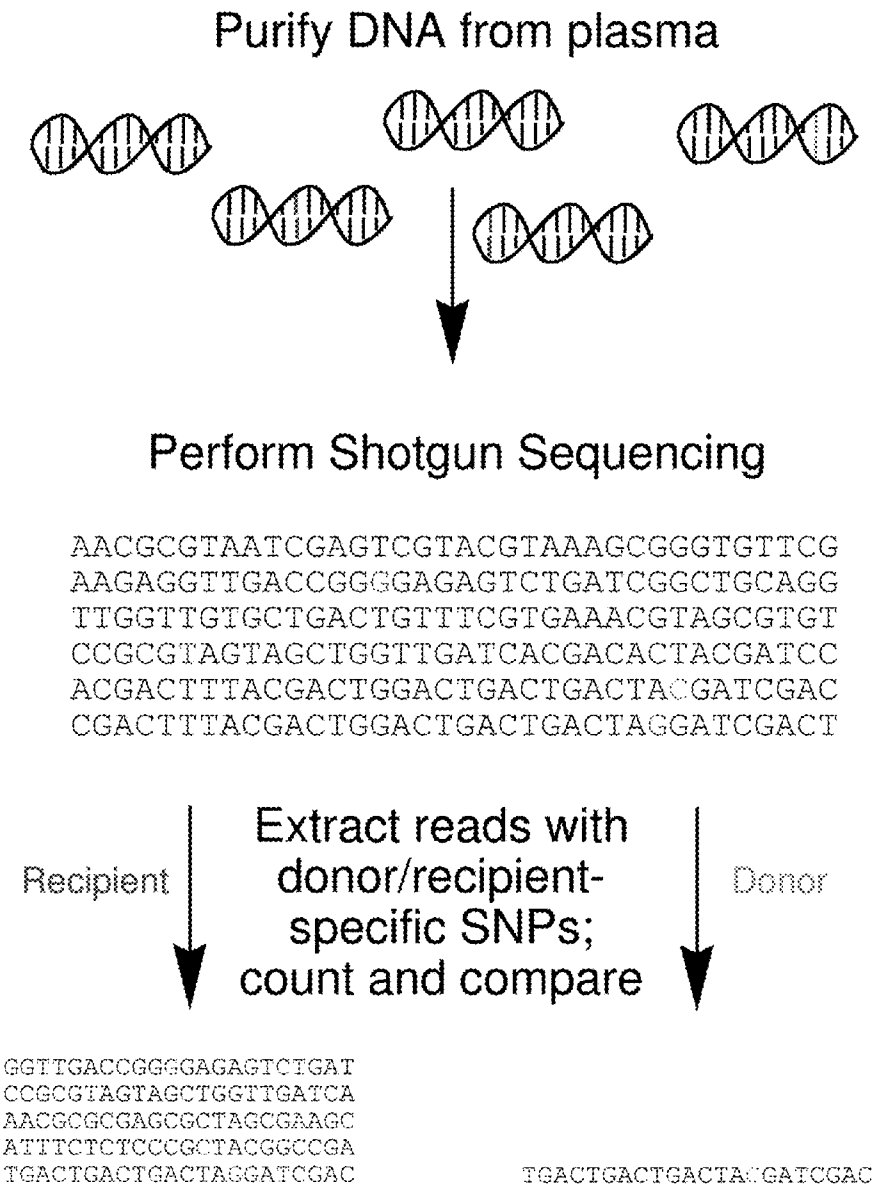
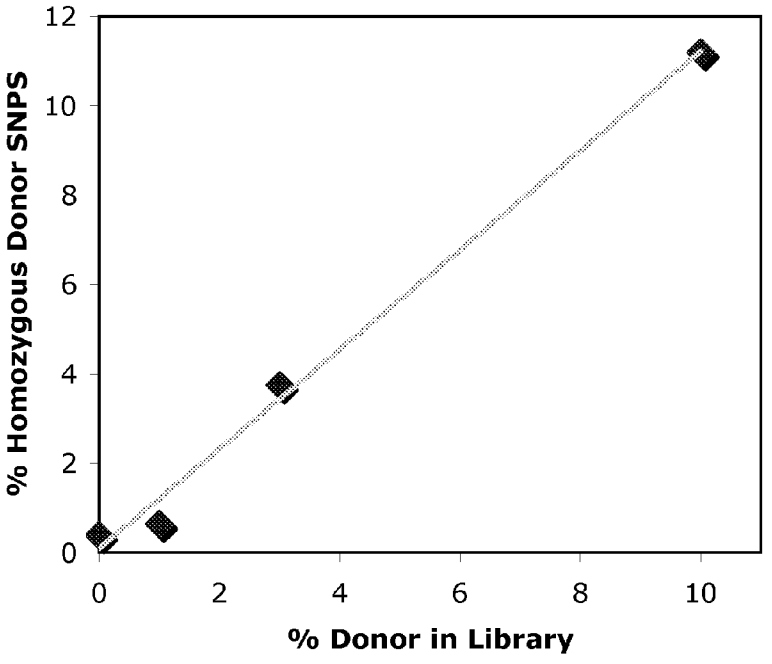

Purify DNA from plasma
Perform Shotgun Sequencing
AACGCGTAATCGAGTCGTACGTAAAGCGGGTGTTCG
AAGAGGTTGACCGGGGAGAGTCTGATCGGCTGCAGG
TTGGTTGTGCTGACTGTTTCGTGAAACGTAGCGTGT
CCGCGTAGTAGCTGGTTGATCACGACACTACGATCC
ACGACTTTACGACTGGACTGACTGACTACGATCGAC
CGACTTTACGACTGGACTGACTGACTAGGATCGACT
Recipient
Extract reads with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

Figure 6A

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

Figure 6B



NON-INVASIVE DIAGNOSIS OF GRAFT REJECTION IN ORGAN TRANSPLANT PATIENTS

GOVERNMENT RIGHTS

This invention was made with Government support under contracts HL099995 and OD000251 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

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.

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

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.

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.

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

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.

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.

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.

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.

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.

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

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.

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

INCORPORATION BY REFERENCE

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

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:

FIG. **1** shows patient survival after diagnosis of CAV.

FIG. **2** shows detection of donor DNA in patients receiving gender mismatched transplants.

FIG. **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.

FIG. **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.

FIG. **5** depicts in one embodiments of the invention a general strategy to monitor all transplant patients

FIGS. **6**A-**6**B show sequencing results comparing four levels of substitutions of donor DNA into recipient DNA.

DETAILED DESCRIPTION OF THE INVENTION

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.

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.

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.

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.

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.

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.

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

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.

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.

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.

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.

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

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.

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

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

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). (FIG. **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 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.

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.

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

a. Circulating Nucleic Acids

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 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))

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.

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

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 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))

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 (FIG. **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 FIGS. **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.

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))

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.

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 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.

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).

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.

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

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, 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 Ser. No. 09/790,673. Other devices can separate cells from the bloodstream, see Demirci U, Toner M., Direct etch method for microfluidic channel and nano-height 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.

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.

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.

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.

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 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

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.

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

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.

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.

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.

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 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.

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.

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.

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.

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.

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; 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.

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.

As used herein the term "diagnose" or "diagnosis" of a transplant status or outcome includes predicting or diagnosing the transplant status or outcome, determining predispo-

sition 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

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.

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.

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).

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.

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.

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 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.

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. Preamplification 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, 5 ng, 1 ng, 500 pg, 200 pg, 100 pg, 50 pg, 40 pg, 30 pg, 20 p, 10 pg, 5 pg, or 1 pg or between 1 5 μg, 5 10 μg, or 10 50 μg.

a. PCR

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.

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 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

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.

In some embodiments, high-throughput sequencing involves the use of technology available by Helicos BioSciences Corporation (Cambridge, Mass.) 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.

In some embodiments, high-throughput sequencing involves the use of technology available by 454 Lifesciences, Inc. (Branford, Conn.) such as the Pico Titer Plate device which includes a fiber optic plate that transmits chemiluminescent 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.

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

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 U.S. Pat. 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.

In some embodiments of this aspect, high-throughput sequencing of RNA or DNA can take place using Any-Dot.chips (Genovoxx, Germany), which allows for the monitoring of biological processes (e.g., miRNA expression or allele variability (SNP detection). In particular, the Any-Dot-chips allow for 10×-50× 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.

Other high-throughput sequencing systems include those disclosed in Venter, J., et al. Science 16 Feb. 2001; Adams, M. et al, Science 24 Mar. 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.

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

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.

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 $\epsilon/3$. 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 $\epsilon^2/9$ or less than 0.003%.

FIG. **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 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

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 U.S. Pat. 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 U.S. Pat. Nos. 7,035,740; 7,033,754; 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; 6,355,431 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 Nov. 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 J E, et al., Methods Enzymol 303: 3-18, 1999; Pabon C, et al., Biotechniques 3 1(4): 8769, 2001; Van Gelder R N, et a]., Proc Natl Acad Sci USA 87: 1663-7 (1990); and Murray, S S. BMC Genetics B(SupplI):SX5 (2005).

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, Bioinformatics 21: 2123-5, 2005; and Leykin et a]., BMC Genet. 6:7, 2005; and in U.S. Pat. No. 5,733,729.

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. August 1; 10(16): 1657-64, 2001: Maneesh Jain, Ph.D., et al. Genetic Engineering News V24: No. 18, 2004; and Fakhrai-Rad H, el al. Genome Res. July; 14(7):1404-12, 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.

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 U.S. Pat. Nos. 7,040,959; 7,035,740; 7,033, 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

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).

Another method contemplated by the present invention to detect and/or quantify target nucleic acids involves the use of nanoreporters as described in U.S. Pat. No. 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", U.S. application Ser. No. 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".

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

e. Labels

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™), carboxy tetramethylrhodamine (TAMRA™), carboxy-X-rhodamine (ROX™), LIZ™, VIC™, NED™, PET™, 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.

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

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.

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, 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.

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.

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.

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.

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.

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 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).

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.

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.

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

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.

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 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.

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.

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.

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.

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.

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.

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%.

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.

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

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.

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.

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.

The kits may comprise reagents such as buffers, and H_2O. 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.

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.

Computer Program

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).

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.

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.

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.

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

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.

While blood transfusions/male child birth are known mechanisms to have detectable cY signature in a female patient, FIG. **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.

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 (FIG. **3**). The results in FIG. **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

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 (FIG. **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

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.

Example 2: Genotyping of Transplant Donor and Transplant Recipient

FIG. **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

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 ½ of total donor fraction), (ii) approximately 160,000 homozygous donor SNPs.

Sequencing Results:

4 lanes of Illumina sequencing are used to compare 4 different levels of substitution of Donor DNA into Recipient DNA (See FIG. **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.

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.

What is claimed is:

1. A method of detecting donor-specific circulating cell-free nucleic acids in a solid organ transplant recipient, the method comprising:

(a) genotyping a solid organ transplant donor to obtain a single nucleotide polymorphism (SNP) profile of the solid organ transplant donor;

(b) genotyping a solid organ transplant recipient to obtain a SNP profile of the solid organ transplant recipient, wherein the solid organ transplant recipient is selected from the group consisting of: a kidney transplant, a heart transplant, a liver transplant, a pancreas transplant, a lung transplant, a skin transplant, and any combination thereof;

(c) obtaining a biological sample from the solid organ transplant recipient after the solid organ transplant recipient has received the solid organ transplant from the solid organ transplant donor, wherein the biological sample is selected from the group consisting of blood, serum and plasma, and wherein the biological sample comprises circulating cell-free nucleic acids from the solid organ transplant; and

(d) determining an amount of donor-specific circulating cell-free nucleic acids from the solid organ transplant in the biological sample by detecting a homozygous or a heterozygous SNP within the donor-specific circulating cell-free nucleic acids from the solid organ transplant in at least one assay, wherein the at least one assay comprises high-throughput sequencing or digital polymerase chain reaction (dPCR), and

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tggttgatca cgacactacg atccacgact ttacgactgg actgactgac tacgatcgac 180

cgactttacg actggactga ctgactagga tcgact 216

<210> SEQ ID NO 2
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<400> SEQUENCE: 2

ggttgaccgg gagagtctga tccgcgtagt agctggttga tcaaacgcgc gagcgctagc 60

gaagcatttc tctcccgcta cggccgatga ctgactgact aggatcgac 109

<210> SEQ ID NO 3
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<213> ORGANISM: H. sapiens

<400> SEQUENCE: 3

tgactgactg acacgatcga c 21

wherein the at least one assay detects the donor-specific circulating cell-free nucleic acids from the solid organ transplant when the donor-specific circulating cell-free nucleic acids make up at least 0.03% of the total circulating cell-free nucleic acids in the biological sample.

2. The method of claim **1**, wherein the biological sample is blood.

3. The method of claim **1**, wherein the at least one assay is a high-throughput sequencing assay that generates at least 1,000 sequence reads per hour.

4. The method of claim **3**, wherein the high-throughput sequencing assay comprises a next-generation sequencing assay.

5. The method of claim **4**, wherein the high-throughput sequencing assay generates sequencing reads of at least 36 bases.

6. The method of claim **1**, wherein at least ten different homozygous or heterozygous SNPs are detected.

7. The method of claim **1**, further comprising a step of administering a therapeutic regimen to treat the solid organ transplant recipient.

8. The method of claim **7**, wherein the therapeutic regimen comprises administering an immunosuppressant treatment.

9. The method of claim **1**, wherein the solid organ transplant recipient is a human subject.

10. The method of claim **1**, wherein an amplification reaction is performed on the donor-specific circulating cell-free nucleic acids in the biological sample prior to determining the amount of donor-specific circulating cell-free nucleic acids from the solid organ transplant in the biological sample.

11. The method of claim **3**, wherein the high-throughput sequencing assay is a shotgun sequencing assay.

12. The method of claim **1**, wherein the donor-specific circulating cell-free nucleic acids from the solid organ transplant in the biological sample are DNA, RNA, mRNA, miRNA, double-stranded DNA, single-stranded DNA, single-stranded DNA hairpins, DNA/RNA hybrids, RNA hairpins, or a combination thereof.

13. The method of claim **1**, wherein the donor-specific circulating cell-free nucleic acids from the solid organ transplant in the biological sample are RNA.

14. The method of claim **13**, wherein the donor-specific circulating cell-free nucleic acids from the solid organ transplant are organ-specific RNA transcripts.

15. The method of claim **1**, further comprising genotyping the solid organ transplant donor, the solid organ transplant recipient, or both the solid organ transplant donor and the solid organ transplant recipient prior to the determining in step (d).

16. The method of claim **1**, further comprising genotyping the solid organ transplant donor, the solid organ transplant recipient, or both the solid organ transplant donor and the solid organ transplant recipient simultaneously with the determining in step (d).

17. The method of claim **1**, wherein the SNP profile comprises informative homozygous and heterozygous SNPs.

18. The method of claim **17**, wherein the method further comprises multiplying the number of informative heterozygous SNP molecules from the solid organ transplant by a factor of two.

19. The method of claim **1**, wherein sensitivity of the method is greater than 56%.

20. The method of claim **1**, wherein the determining comprises detecting donor-specific circulating cell-free nucleic acids from the solid organ transplant wherein the donor-specific circulating cell-free nucleic acids from the solid organ transplant make up between 0.03% and 8.0% of the total circulating cell-free nucleic acids in the biological sample.

21. The method of claim **1**, wherein the high-throughput sequencing assay has a sequencing error rate of less than 1.5% for detecting donor-specific circulating cell-free nucleic acids from the solid organ transplant.

22. The method of claim **1**, wherein the high-throughput sequencing assay has resequencing error rate of less than 0.003% for detecting donor-specific circulating cell-free nucleic acids from the solid organ transplant.

23. The method of claim **1**, wherein the detecting of the homozygous or heterozygous SNP comprises using a quality score.

24. The method of claim **1**, wherein the homozygous or heterozygous SNP comprises one or more base changes selected from the list consisting of an insertion, a repeat, and a deletion.

25. The method of claim **1**, wherein the homozygous or heterozygous SNP comprises a marker having at least two alleles, each occurring at a frequency greater than 1% of the population.

26. The method of claim **1**, wherein the high-throughput sequencing assay comprises mapping one or more of the donor-specific circulating cell-free nucleic acids from the solid organ transplant in the biological sample with a genome sequence of the solid organ transplant donor.

27. The method of claim **1**, wherein the biological sample is plasma.

28. The method of claim **1**, wherein the biological sample is serum.

29. The method of claim **8**, wherein the immunosuppressant regimen is selected from the group consisting of rapamycin, cyclosporin A, and anti-CD40L monoclonal antibody.

30. The method of claim **1**, wherein the method comprises using a computer to access data reflecting the amount of donor-specific circulating cell-free nucleic acids from the solid organ transplant in the biological sample.

31. The method of claim **1**, wherein the at least one assay detects the donor-specific circulating cell-free nucleic acids from the solid organ transplant when the donor-specific circulating cell-free nucleic acids make up less than 8% of the total circulating cell-free nucleic acids in the biological sample.

32. The method of claim **1**, wherein the at least one assay detects the donor-specific circulating cell-free nucleic acids from the solid organ transplant when the donor-specific circulating cell-free nucleic acids make up 2% of the total circulating cell-free nucleic acids in the biological sample.

33. The method of claim **1**, wherein the at least one assay detects the donor-specific circulating cell-free nucleic acids from the solid organ transplant when the donor-specific circulating cell-free nucleic acids make up 0.03% of the total circulating cell-free nucleic acids in the biological sample.

* * * * *

EXHIBIT 2

US008703652B2

(12) **United States Patent**
Quake et al.

(10) **Patent No.: US 8,703,652 B2**
(45) **Date of Patent: Apr. 22, 2014**

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

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(73) Assignee: **The Board of Trustees of the Leland Stanford Junior University**, Palo Alto, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

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(86) PCT No.: **PCT/US2010/055604**

§ 371 (c)(1),
(2), (4) Date: **Jul. 19, 2012**

(87) PCT Pub. No.: **WO2011/057061**

PCT Pub. Date: **May 12, 2011**

(65) **Prior Publication Data**

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Related U.S. Application Data

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(51) **Int. Cl.**
C12Q 1/68 (2006.01)
C40B 30/04 (2006.01)

(52) **U.S. Cl.**
USPC **506/9**; 435/6.11; 435/6.12

(58) **Field of Classification Search**
CPC C12Q 1/68; C40B 30/04
USPC 506/9; 435/6.12, 6.11
See application file for complete search history.

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(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.

16 Claims, 6 Drawing Sheets

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Figure 1

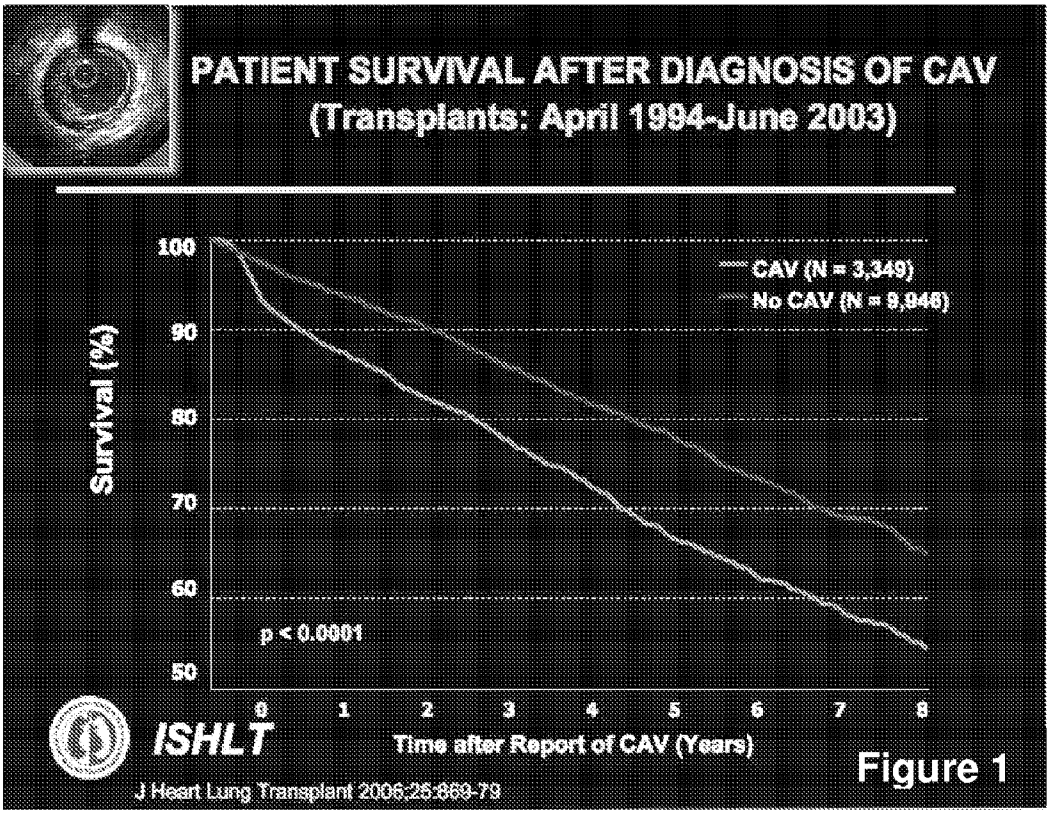

PATIENT SURVIVAL AFTER DIAGNOSIS OF CAV
(Transplants: April 1994-June 2003)
Survival (%)
100
90
80
70
60
50
CAV (N = 3,349)
No CAV (N = 9,946)
p < 0.0001
0
1
2
3
4
5
6
7
8
Time after Report of CAV (Years)
ISHLT
J Heart Lung Transplant 2006;25:869-79
Figure 1

Figure 2

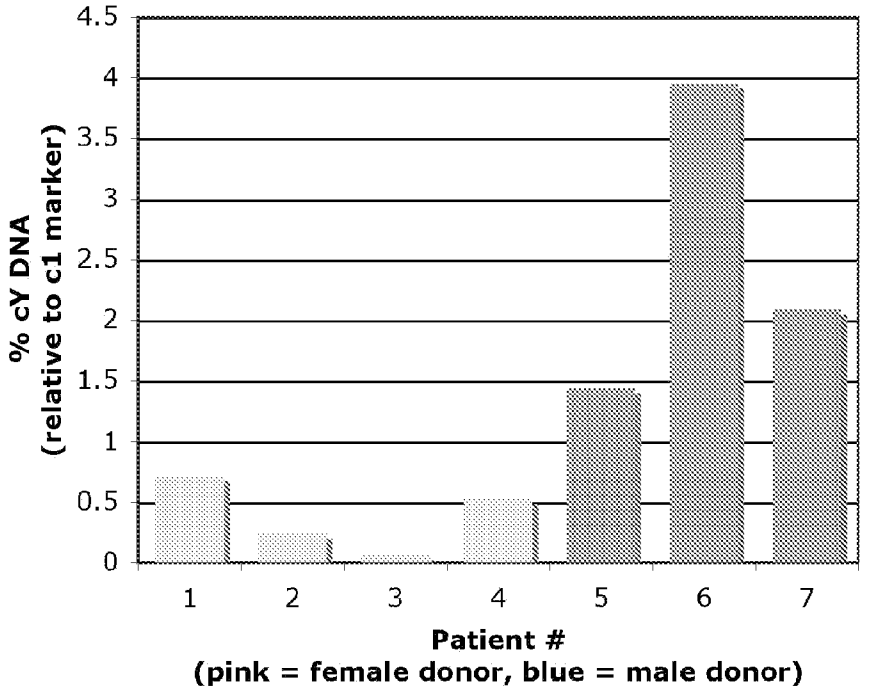

4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
% cY DNA
(relative to c1 marker)
1
2
3
4
5
6
7
Patient #
(pink = female donor, blue = male donor)

Figure 3

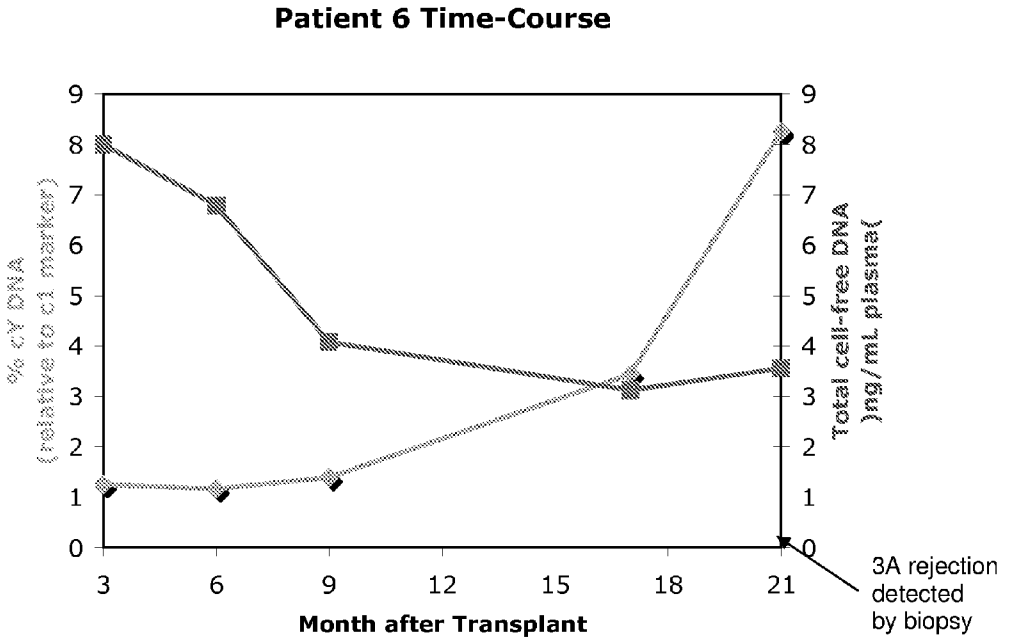

Patient 6 Time-Course
% cY DNA (relative to c1 marker)
Total cell-free DNA)ng/mL plasma(
0
1
2
3
4
5
6
7
8
9
3
6
9
12
15
18
21
Month after Transplant
3A rejection detected by biopsy

Figure 4

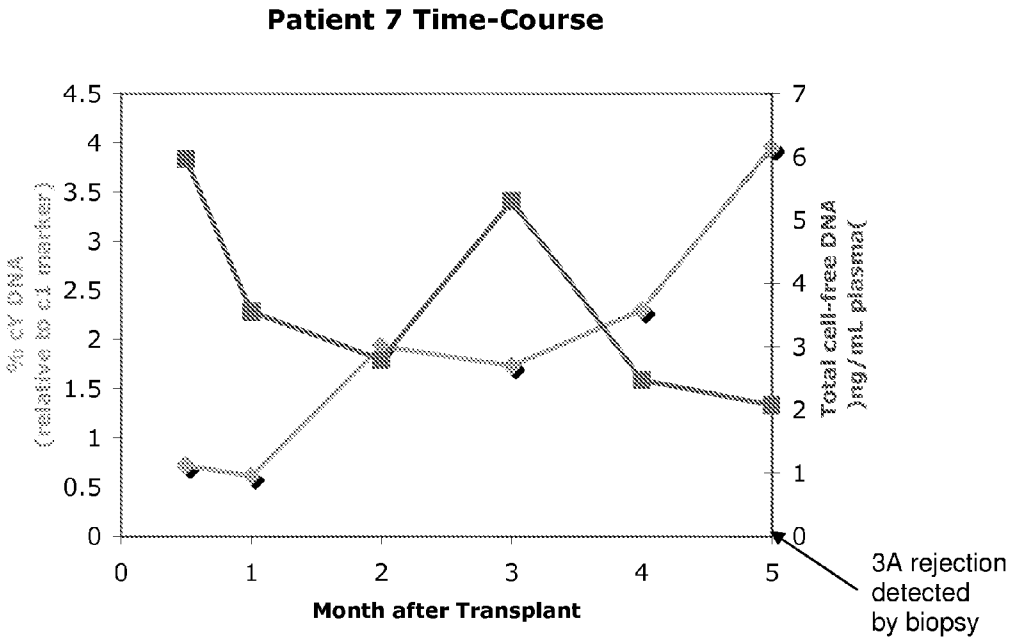

Patient 7 Time-Course
% cY DNA (relative to c1 marker)
Total cell-free DNA)ng/mL plasma(
Month after Transplant
3A rejection detected by biopsy
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
0
1
2
3
4
5
6
7

Figure 5

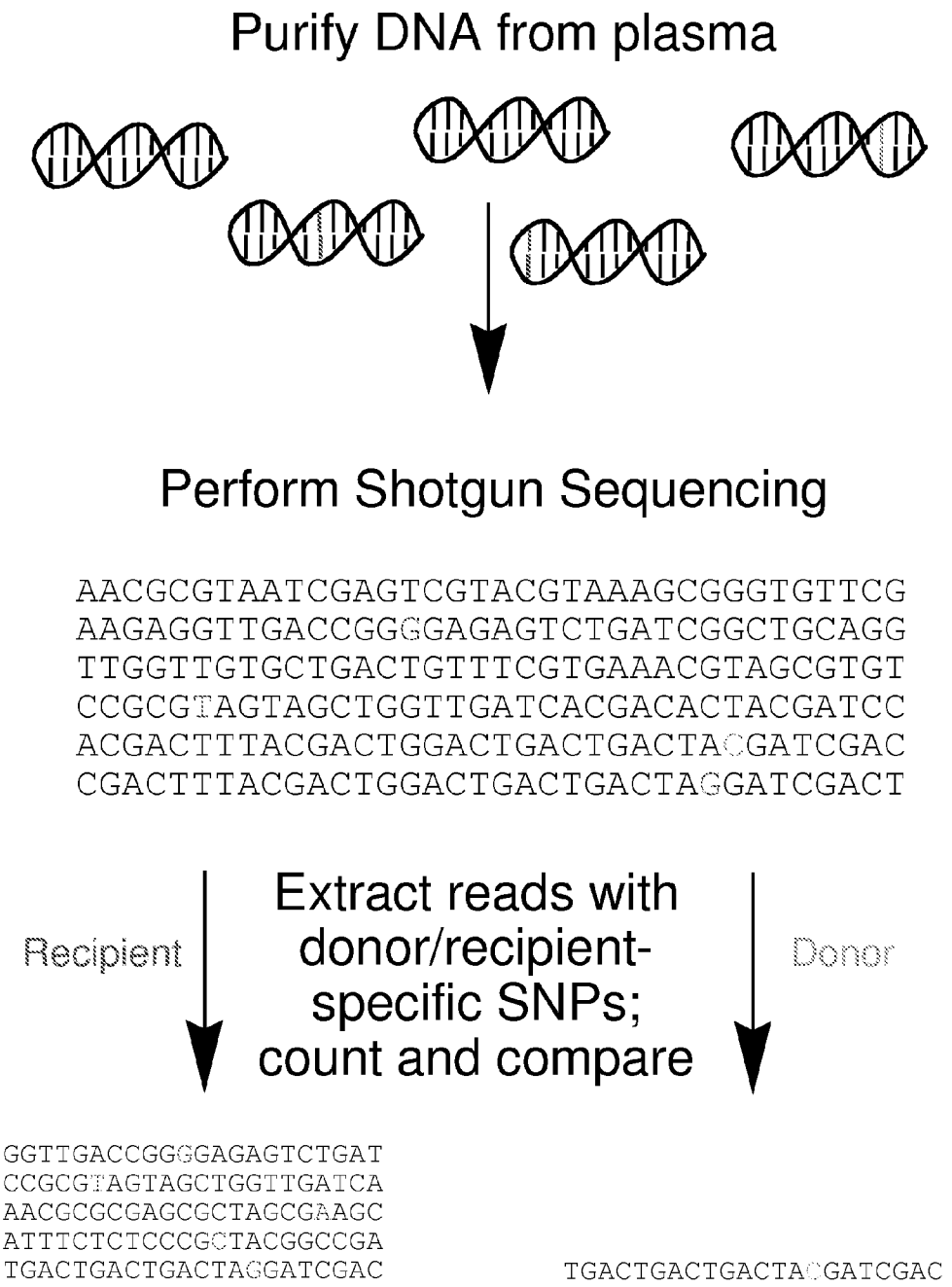
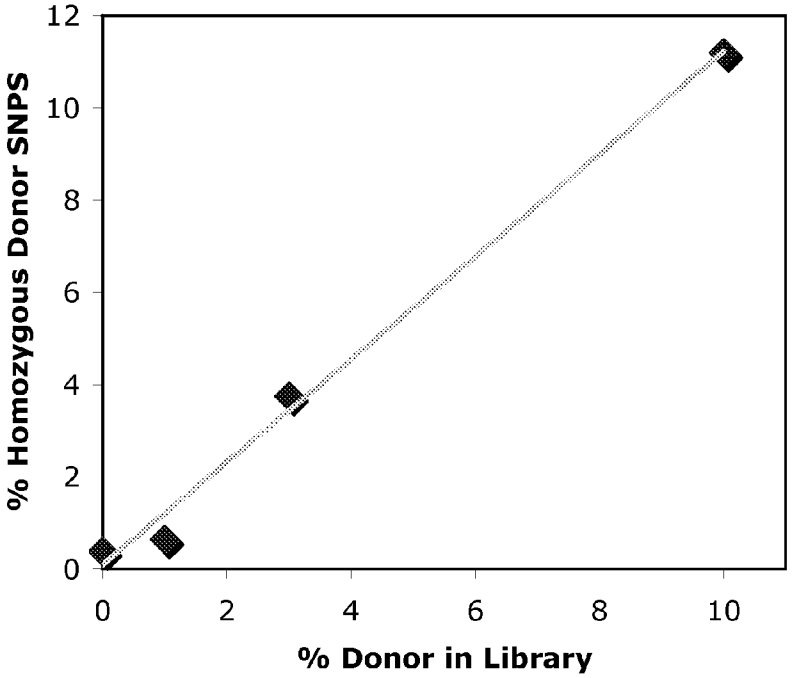

Purify DNA from plasma
Perform Shotgun Sequencing
AACGCGTAATCGAGTCGTACGTAAAGCGGGTGTTCG
AAGAGGTTGACCGGGGAGAGTCTGATCGGCTGCAGG
TTGGTTGTGCTGACTGTTTCGTGAAACGTAGCGTGT
CCGCGTAGTAGCTGGTTGATCACGACACTACGATCC
ACGACTTTACGACTGGACTGACTGACTACGATCGAC
CGACTTTACGACTGGACTGACTGACTAGGATCGACT
Recipient
Extract reads with 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.



NON-INVASIVE DIAGNOSIS OF GRAFT REJECTION IN ORGAN TRANSPLANT PATIENTS

GOVERNMENT RIGHTS

This invention was made with Government support under contracts HL099995 and OD000251 awarded by the National Institutes of Health. The Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

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.

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

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.

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.

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

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.

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.

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.

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.

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.

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

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.

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

INCORPORATION BY REFERENCE

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

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:

FIG. **1** shows patient survival after diagnosis of CAV.

FIG. **2** shows detection of donor DNA in patients receiving gender mismatched transplants.

FIG. **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.

FIG. **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.

FIG. **5** depicts in one embodiments of the invention a general strategy to monitor all transplant patients

FIG. **6** shows sequencing results comparing four levels of substitutions of donor DNA into recipient DNA.

DETAILED DESCRIPTION OF THE INVENTION

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.

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.

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.

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.

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.

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.

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

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.

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.

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, perioperative ischemia, reperfusion injury, hypertension, physiological stress, injuries due to reactive oxygen species and injuries caused by pharmaceutical agents.

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.

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

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.

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 (RI. Freshney, ed. (1987)).

Introduction

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

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). (FIG. 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 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.

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.

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

a. Circulating Nucleic Acids

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 can-

cer, 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 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))

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.

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

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

Example 1 examined gender-mismatched heart transplant recipients and applied digital PCR (Warren, L., Bryder, D., Weissman, LL., 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 (FIG. **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 FIGS. **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.

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))

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.

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 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, tet-

ranucleotide repeats, simple sequence repeats, and insertion elements such as Alu. In some embodiments, the set of markers comprises SNPs.

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).

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.

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

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, 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 Ser. No. 09/790,673. Other devices can separate cells from the bloodstream, see Demirci U, Toner M., Direct etch method for microfluidic channel and nano-height 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.

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.

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.

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.

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 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

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.

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

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.

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.

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.

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 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.

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.

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.

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.

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.

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; 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.

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.

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

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.

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.

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).

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.

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.

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 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.

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. Preamplification 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, 5 ng, 1 ng, 500 pg, 200 pg, 100 pg, 50 pg, 40 pg, 30 pg, 20 p, 10 pg, 5 pg, or 1 pg or between 1 5 μg, 5 10 μg, or 10 50 μg.

a. PCR

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.

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 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, L 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

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.

In some embodiments, high-throughput sequencing involves the use of technology available by Helicos BioSciences Corporation (Cambridge, Mass.) 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.

In some embodiments, high-throughput sequencing involves the use of technology available by 454 Lifesciences, Inc. (Branford, Conn.) 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.

Methods for using bead amplification followed by fiber optics detection are described in Marguiles, M., et al. "Genome sequencing in microfabricated high-density pricoliter reactors", Nature, doi: 10.1038/nature03959; and well as in US Publication Application Nos. 200200 12930; 20030058629; 20030 1001 02; 20030 148344; 20040248 161; 200500795 10,20050 124022; and 20060078909.

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 U.S. Pat. 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.

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 10×-50× 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.

Other high-throughput sequencing systems include those disclosed in Venter, J., et al. Science 16 Feb. 2001; Adams, M. et al, Science 24 Mar. 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.

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

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.

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 $\epsilon/3$. 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 $\epsilon^2/9$ or less than 0.003%.

FIG. **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 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

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 U.S. Pat. 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 U.S. Pat. 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; 6,355,431 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 Nov. 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 J E, et al., Methods Enzymol 303: 3-18, 1999; Pabon C, et al., Biotechniques 3 1(4): 8769, 2001; Van Gelder R N, et al., Proc Natl Acad Sci USA 87: 1663-7 (1990); and Murray, S S. BMC Genetics B(SupplI):SX5 (2005).

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, Bioinformatics 21:2123-5, 2005; and Leykin et al., BMC Genet. 6:7, 2005; and in U.S. Pat. No. 5,733,729.

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 21 (6), 673-8, 2003; Faham M, et al. Hum Mol Genet. August 1; 10(16): 1657-64, 2001: Maneesh Jain, Ph.D., et al. Genetic Engineering News V24: No. 18, 2004; and Fakhrai-Rad H, el al. Genome Res. July; 14(7):1404-12, 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.

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 U.S. Pat. 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

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).

Another method contemplated by the present invention to detect and/or quantify target nucleic acids involves the use of nanoreporters as described in U.S. Pat. No. 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", U.S. application Ser. No. 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",

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

e. Labels

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™), carboxy tetramethylrhodamine (TAMRA™), carboxy-X-rhodamine (ROX™), LIZ™, VIC™, NED™, PET™, 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.

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

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.

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, 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.

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.

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.

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.

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.

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 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).

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.

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.

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

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.

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 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.

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.

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.

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.

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.

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.

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%.

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.

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

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.

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.

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.

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.

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.

Computer Program

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).

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.

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.

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.

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

Using digital PCR as described before (Warren, L., Bryder, D., Weissman, L 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.

While blood transfusions/male child birth are known mechanisms to have detectable cY signature in a female patient, FIG. **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.

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 (FIG. **3**). The results in FIG. **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

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 (FIG. **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

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.

Example 2

Genotyping of Transplant Donor and Transplant Recipient

FIG. **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

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 ½ of total donor fraction), (ii) approximately 160,000 homozygous donor SNPs.

Sequencing Results:

4 lanes of Illumina sequencing are used to compare 4 different levels of substitution of Donor DNA into Recipient DNA (See FIG. **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.

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.

SEQUENCE LISTING

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<400> SEQUENCE: 1

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atcggctgca ggttggttgt gctgactgtt tcgtgaaacg tacgctgtcc gcgtagtagc 120

tggttgatca cgacactacg atccacgact ttacgactgg actgactgac tacgatcgac 180

cgactttacg actggactga ctgactagga tcgact 216

<210> SEQ ID NO 2
<211> LENGTH: 109
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<400> SEQUENCE: 2

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gaagcatttc tctcccgcta cggccgatga ctgactgact aggatcgac 109

<210> SEQ ID NO 3
<211> LENGTH: 21
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<213> ORGANISM: H. sapiens

<400> SEQUENCE: 3

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What is claimed is:

1. A method for detecting transplant rejection, graft dysfunction, or organ failure, the method comprising:

(a) providing a sample comprising cell-free nucleic acids from a subject who has received a transplant from a donor;

(b) obtaining a genotype of donor-specific polymorphisms or a genotype of subject-specific polymorphisms, or obtaining both a genotype of donor-specific polymorphisms and subject-specific polymorphisms, to establish a polymorphism profile for detecting donor cell-free nucleic acids, wherein at least one single nucleotide polymorphism (SNP) is homozygous for the subject if the genotype comprises subject-specific polymorphisms comprising SNPs;

(c) multiplex sequencing of the cell-free nucleic acids in the sample followed by analysis of the sequencing results using the polymorphism profile to detect donor cell-free nucleic acids and subject cell-free nucleic acids; and

(d) diagnosing, predicting, or monitoring a transplant status or outcome of the subject who has received the transplant by determining a quantity of the donor cell-free nucleic acids based on the detection of the donor cell-free nucleic acids and subject cell-free nucleic acids by the multiplexed sequencing, wherein an increase in the quantity of the donor cell-free nucleic acids over time is indicative of transplant rejection, graft dysfunction or organ failure, and wherein sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV).

2. The method of claim **1**, wherein the polymorphism profile comprises one or more genetic variations selected from single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, insertion elements, insertions, repeats, or deletions.

3. The method of claim **1**, wherein the cell-free nucleic acids are deoxyribonucleic acid (DNA).

4. The method of claim **1**, wherein the multiplexed sequencing comprises shotgun sequencing.

5. The method of claim **1**, wherein the multiplexed sequencing occurs in a single container.

6. The method of claim **1**, wherein the multiplexed sequencing comprises sequencing at least ten different nucleic acids.

7. The method of claim **1** further comprising administering an immunosuppressive drug.

8. The method of claim **1**, wherein the diagnosing, predicting, or monitoring transplant status or outcome comprises treating a transplant rejection in a subject in need thereof.

9. The method of claim **1**, wherein the diagnosing, predicting, or monitoring transplant status or outcome comprises determining, modifying, or maintaining an immunosuppressive regimen.

10. The method of claim **1** further comprising conducting an assay selected from: digital polymerase chain reaction (PCR), real-time polymerase chain reaction (RT-PCR), array, or any combination thereof.

11. The method of claim **1**, wherein the polymorphism profile comprises at least one single nucleotide polymorphism.

12. The method of claim **1**, wherein the sample is blood or serum.

13. The method of claim **1**, wherein the sample is urine or stool.

14. The method of **1**, wherein the transplant is selected from the group consisting of: kidney transplant, pancreas transplant, liver transplant, heart transplant, lung transplant, intestine transplant, pancreas after kidney transplant, and simultaneous pancreas-kidney transplant.

15. The method of claim **1**, wherein the transplant is a heart transplant or kidney transplant.

16. The method of claim **1**, wherein the cell-free nucleic acids are ribonucleic acid (RNA).

* * * * *

EXHIBIT 3

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News Release

Natera Announces Agreement with One Lambda to Co-Distribute Its Kidney Transplant Rejection Test

Agreement Enhances Commercial Presence in Organ Transplant Centers

SAN CARLOS, Calif., Feb. 1, 2019 /PRNewswire/ -- Natera, Inc. (https://c212.net/c/link/?t=0&l=en&o=2363243-1&h=441237491&u=http%3A%2F%2Fwww.natera.com%2F&a=Natera%2C+Inc.) (NASDAQ: NTRA), a leader in non-invasive genetic testing and the analysis of cell-free DNA, today announced a partnership with Thermo Fisher Scientific's One Lambda brand, a global leader in human leukocyte antigen (HLA) typing and antibody monitoring assays for transplantation, to co-distribute Natera's kidney transplant rejection test in the United States in collaboration with the company's direct sales team.

"We are excited to partner with One Lambda, a pioneer in transplant diagnostics," said Steve Chapman, CEO, Natera. "This partnership will help accelerate our entry into this new market by leveraging the commercial infrastructure of a highly respected and well-established leader in the field, along with our direct sales team."

"Natera's donor-derived cell-free DNA test complements our existing transplant offerings and enables us to provide a more advanced portfolio for monitoring kidney rejection," said Parisa Khosropour, President, Transplant Diagnostics, Thermo Fisher Scientific. "We look forward to offering our customers a test that improves upon currently available options and provides unique performance advantages in detecting T-cell mediated rejection and subclinical acute rejection, both of which may have a more positive impact on patient outcomes."

About Natera's dd-cfDNA Organ Transplant Assay

Natera's organ transplant rejection assay is designed to detect active allograft rejection in patients who have undergone kidney transplantation. The assay works by measuring the fraction of donor-derived cell-free DNA (dd-cfDNA) in the recipient's blood, which can spike relative to normal cfDNA when the transplanted organ is injured due to immune rejection. The assay leverages Natera's core single nucleotide polymorphism (SNP)-based massively multiplexed PCR (mmPCR) technology to accurately measure dd-cfDNA levels without the need for donor genotyping, and it has been clinically validated with test performance independent of donor type, rejection type, and clinical presentation.

In a recent study published in the *Journal of Clinical Medicine*, Natera's assay detected acute rejection (AR) with 89% sensitivity and 0.87 area under the curve (AUC).[1] This test performance compares favorably to the current standard of care, which is based on serial measurements of serum creatinine; and it compares favorably against competition, which in a 2017 study reported 59% sensitivity and 0.74 AUC.[2] The recent study also had two novel, clinically significant findings relative to previously published studies of dd-cfDNA. The Natera dd-cfDNA assay was able to accurately detect TCMR (T-cell mediated rejection), a common and treatable form of active rejection, and subclinical acute rejection.[1] No other dd-cfDNA assay has been shown to detect TCMR or validated to detect subclinical AR, which occurs in 20-25% of patients in the first two years post-transplant,[3] and which is considered a major driver of graft failure.

About One Lambda

One Lambda, a Thermo Fisher Scientific brand, is the global leader in HLA and antibody monitoring assays for transplantation. Known for its commitment to quality, service, and innovation, the company develops and distributes several lines of HLA typing and antibody monitoring tests utilizing serological, molecular, flow, solid phase & NGS technologies. In addition, One Lambda also provides laboratory instrumentation and computer software that are used to simplify and automate testing procedures and final test evaluations. For more information, please visit www.onelambda.com (https://c212.net/c/link/?t=0&l=en&o=2363243-1&h=1743076005&u=http%3A%2F%2Fwww.onelambda.com%2F&a=www.onelambda.com).

About Natera

Natera (https://c212.net/c/link/?t=0&l=en&o=2363243-1&h=1048191798&u=http%3A%2F%2Fwww.natera.com%2F&a=Natera) is a global leader in cell-free DNA testing. The mission of the company is to transform the management of diseases worldwide. Natera operates an ISO 13485-certified and CAP-accredited laboratory certified under the Clinical Laboratory Improvement Amendments (CLIA) in San Carlos, Calif. It offers a host of proprietary genetic testing services to inform physicians who care for pregnant women, researchers in cancer including biopharmaceutical companies, and genetic laboratories through its cloud-based software platform. Follow Natera on LinkedIn (https://c212.net/c/link/?t=0&l=en&o=2363243-1&h=4034764291&u=https%3A%2F%2Fwww.linkedin.com%2Fcompany%2Fnatera%2F&a=LinkedIn) and Twitter (https://c212.net/c/link/?t=0&l=en&o=2363243-1&h=1295433236&u=https%3A%2F%2Ftwitter.com%2FNateraGenetics&a=Twitter).

Natera Forward-Looking Statements

All statements other than statements of historical facts contained in this press release are forward-looking statements and are not a representation that Natera's plans, estimates, or expectations will be achieved. These forward-looking statements represent Natera's expectations as of the date of this press release, and Natera disclaims any obligation to update the forward-looking statements. These forward-looking statements are subject to known and unknown risks and uncertainties that may cause actual results to differ materially, including with respect to our efforts to develop and commercialize new product offerings, our ability to successfully increase demand for and grow revenues for our product offerings, whether the results of clinical studies will support the use of our product offerings, our expectations of the reliability, accuracy and performance of our screening tests, or of the benefits of our screening tests and product offerings to patients, providers and payers. Additional risks and uncertainties are discussed in greater detail in "Risk Factors" in Natera's recent filings on Forms 10-K and 10-Q and in other filings Natera makes with the SEC from time to time. These documents are available at www.natera.com/investors (https://c212.net/c/link/?t=0&l=en&o=2363243-1&h=2006750296&u=http%3A%2F%2Fwww.natera.com%2Finvestors&a=www.natera.com%2Finvestors) and www.sec.gov (https://c212.net/c/link/?t=0&l=en&o=2363243-1&h=3902011912&u=http%3A%2F%2Fwww.sec.gov%2F&a=www.sec.gov).

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References

1. Sigdel TK, Archila FA, Constantin T, et al. Optimizing detection of kidney transplant injury by assessment of donor-derived cell-free DNA via massively multiplex PCR. *J Clin Med*. 2019;8(1):19.
2. Bloom RD, Bromberg JS, Poggio ED, et al. Cell-free DNA and active rejection in kidney allografts. *J Am Soc Nephrol*. 2017;28(7):2221-2232. doi: 10.1681/ASN.2016091034.
3. Choi BS, Shin MJ, Shin SJ, et al. Clinical significance of an early protocol biopsy in living-donor renal transplantation: ten-year experience at a single center. *Am J Transplant*. 2005;6:1354-1360.

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SOURCE Natera, Inc.

EXHIBIT 4

Utility of a Novel Dd-cfDNA Test to Detect Injury in Renal Post-Transplant Patients (QIDNEY)

ClinicalTrials.gov Identifier: NCT03765203

Recruitment Status : Recruiting
First Posted : December 5, 2018
Last Update Posted : December 5, 2018

See **Contacts and Locations**

Sponsor:
Qure Healthcare, LLC

Collaborator:
Natera, Inc.

Information provided by (Responsible Party):
Mary Tran, Qure Healthcare, LLC

Study Details | Tabular View | No Results Posted | Disclaimer | How to Read a Study Record

Study Description

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Brief Summary:

Detecting allograft injury and rejection is critical to preventing graft loss. The current standard of care (SoC) relies on serum creatinine (SC) and biopsy to monitor for and identify kidney injury earlier. SC has poor specificity and sensitivity and response to rejection is often delayed. Protocol biopsy is more

accurate but involves the risk of complications. A more definitive, less invasive method for monitoring injury and early rejection is needed.

We report on the clinical utility of donor-derived cell-free DNA (dd-cfDNA) in transplant recipients' blood, measured using a novel SNP-based mmPCR NGS methodology, to diagnose allograft injury/rejection. In this study, investigators will measure how use of dd-cfDNA changes clinical practice.

Condition or disease	Intervention/treatment	Phase
Transplant;Failure,Kidney	Diagnostic Test: Natera KidneyScan Other: Clinical Performance and Value Vignettes	Not Applicable

Detailed Description:

Five-year kidney allograft survival rates are estimated to be as low as 71.6%. A leading cause for the high prevalence of graft loss is the delay in detecting allograft injury from active rejection, when early diagnosis and intervention presents the greatest chance of preserving kidney function. Despite the frequent testing called for by care protocols, low levels of injury can go undetected due to the low specificity and sensitivity of current, standard testing methods: checking creatinine and immunosuppressive drug levels. More definitive graft biopsies are an option, but they are invasive, expensive and can even put the patient at risk for graft loss and other complications, making it undesirable as a frequent monitoring test.

Donor-derived cell-free DNA (dd-cfDNA) detected in the blood of transplant recipients has been shown to be a non-invasive diagnostic marker for allograft injury/rejection. Natera, Inc. has recently developed a novel single nucleotide polymorphism (SNP)-based mmPCR NGS methodology to measure dd-cfDNA in kidney transplant recipients for the detection of allograft injury and rejection. As a growing leader in the diagnostic space, Natera has commissioned a randomized controlled trial to determine the clinical utility of its dd-cfDNA detection methodology for practicing nephrologists treating kidney allograft patients. This study is expected to fill a gap in the evidence base on the clinical utility of dd-cfDNA testing for allograft rejection.

The study is a pre-post, two round controlled trial of care practices in a nationally representative sample of practicing nephrologists randomly assigned to a control or an intervention arm. All participants will be asked to propose care for a total of 6 CPV simulated patients who are adults aged 30-75; three or more months post-transplant; and presenting with signs, symptoms and laboratory findings suggestive of allograft rejection. Each assessment round will consist of 3 simulated patients. In between assessment rounds, participants randomized into the intervention arm will receive educational materials on the new allograft rejection test.

Investigators will assess whether practicing nephrologists more effectively identify and manage patients with possible kidney allograft rejection when given access to Natera's novel SNP-based mmPCR-NGS

test that measures dd-cfDNA, and, whether those behavioral changes improves patient management and optimizes resource utilization.

Study Design

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Study Type :	Interventional (Clinical Trial)
Estimated Enrollment :	170 participants
Allocation:	Randomized
Intervention Model:	Parallel Assignment
Intervention Model Description:	The study is a pre-post, two round controlled trial of care practices in a nationally representative sample of practicing nephrologists randomly assigned to a control or an intervention arm. All providers will be asked to propose care for a total of 6 CPV simulated patients who are adults aged 30-75; three or more months post-transplant; and presenting with signs, symptoms and laboratory findings suggestive of allograft rejection. Each assessment round will consist of 3 simulated patients. In between assessment rounds, physicians randomized into the intervention arm will receive educational materials on the new allograft rejection test.
Masking:	None (Open Label)
Primary Purpose:	Other
Official Title:	Prospective, Randomized Controlled Trial Using CPV Vignettes to Assess the Clinical Utility of Natera Dd-cfDNA Test to Detect Allograft in Post-Transplant Patients
Actual Study Start Date :	November 5, 2018
Estimated Primary Completion Date :	February 1, 2019
Estimated Study Completion Date :	March 30, 2019

Arms and Interventions

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Arm	Intervention/treatment
Active Comparator: Control Control participants will care for the same set of CPV patients as the intervention arm, but will not have knowledge of or access to Natera's dd-cfDNA test results. Investigators will compare control participants' clinical recommendations to those in the intervention arm.	Other: Clinical Performance and Value Vignettes Online renal allograft simulated patients Other Name: CPVs
Experimental: Intervention	Diagnostic Test: Natera KidneyScan

Intervention participants will care for the same set of CPV patients as the control arm, but will be educated on and given access to Natera's dd-cfDNA test results. Investigators will compare intervention participants' clinical recommendations to those in the control arm.	Online educational materials on Natera Kidneyscan (dd-cfDNA) and sample test results for simulated patients Other: Clinical Performance and Value Vignettes Online renal allograft simulated patients Other Name: CPVs

Outcome Measures

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Primary Outcome Measures :

1. Diagnosis-Treatment [Time Frame: 3 months]

   Difference-in-differences regression analysis between the control and the intervention group's identification and treatment of hyperglycemia, as measured by the participants diagnostic and treatment CPV case domain scores. In each domain of a CPV (history, physical exam, workup, diagnosis and treatment), participants' care recommendations are evaluated against evidence-based care scoring criteria which can sum to a high potential score of up to 100% in each domain.

Secondary Outcome Measures :

1. Quality of Care: CPV scores [Time Frame: 3 months]

   Difference-in-differences regression analysis between the control and the intervention group's overall quality of care scores. In each CPV case, participants' care recommendations are evaluated against evidence-based care scoring criteria which can sum to a high potential score of up to 100% in each case.

2. Workup Costs [Time Frame: 3 months]

   Difference-in-differences regression analysis between between the control and the intervention group in the average cost of diagnostic tests ordered.

Eligibility Criteria

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Information from the National Library of Medicine NIH NLM

Choosing to participate in a study is an important personal decision. Talk with your doctor and family members or friends about deciding to join a study. To learn more about this study, you or your doctor may contact the study research staff using the contacts provided below. For general information, Learn About Clinical Studies.

Ages Eligible for Study:	Child, Adult, Older Adult
Sexes Eligible for Study:	All
Accepts Healthy Volunteers:	Yes

Criteria

Inclusion Criteria:

- A minimum of 2 years post-residency but no more than 40 years in practice
- Board-certified in internal medicine
- Completion of a nephrology fellowship
- In a private solo or multi-group practice
- Minimum threshold of 5 post-kidney transplant (KT) patients currently seen monthly
- Informed, signed and voluntarily consented to be in the study

Exclusion Criteria:

- Not board certified in internal medicine
- Have practiced as a board-certified physician for less than 2 or greater than 40 years
- See <5 post-transplant patients monthly
- Non-English speaking
- Unable to access the internet

Contacts and Locations

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Information from the National Library of Medicine NIH NLM

To learn more about this study, you or your doctor may contact the study research staff using the contact information provided by the sponsor.

Please refer to this study by its ClinicalTrials.gov identifier (NCT number): ***NCT03765203***

Locations

United States, California

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More Information

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Responsible Party: Mary Tran, John W Peabody, MD PhD, Qure Healthcare, LLC
ClinicalTrials.gov Identifier: NCT03765203 History of Changes
Other Study ID Numbers: Pro00030299
First Posted: December 5, 2018 Key Record Dates
Last Update Posted: December 5, 2018
Last Verified: December 2018

Individual Participant Data (IPD) Sharing Statement:
Plan to Share IPD: No

Studies a U.S. FDA-regulated Drug Product: No
Studies a U.S. FDA-regulated Device Product: No

Additional relevant MeSH terms:
Renal Insufficiency
Kidney Diseases
Urologic Diseases

EXHIBIT 5

Journal of
Clinical Medicine

MDPI

Article

Optimizing Detection of Kidney Transplant Injury by Assessment of Donor-Derived Cell-Free DNA via Massively Multiplex PCR

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‡ At time of study.

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Abstract: Standard noninvasive methods for detecting renal allograft rejection and injury have poor sensitivity and specificity. Plasma donor-derived cell-free DNA (dd-cfDNA) has been reported to accurately detect allograft rejection and injury in transplant recipients and shown to discriminate rejection from stable organ function in kidney transplant recipients. This study used a novel single nucleotide polymorphism (SNP)-based massively multiplexed PCR (mmPCR) methodology to measure dd-cfDNA in various types of renal transplant recipients for the detection of allograft rejection/injury without prior knowledge of donor genotypes. A total of 300 plasma samples (217 biopsy-matched: 38 with active rejection (AR), 72 borderline rejection (BL), 82 with stable allografts (STA), and 25 with other injury (OI)) were collected from 193 unique renal transplant patients; dd- cfDNA was processed by mmPCR targeting 13,392 SNPs. Median dd-cfDNA was significantly higher in samples with biopsy-proven AR (2.3%) versus BL (0.6%), OI (0.7%), and STA (0.4%) ($p < 0.0001$ all comparisons). The SNP-based dd-cfDNA assay discriminated active from non-rejection status with an area under the curve (AUC) of 0.87, 88.7% sensitivity (95% CI, 77.7–99.8%) and 72.6% specificity (95% CI, 65.4–79.8%) at a prespecified cutoff (>1% dd-cfDNA). Of 13 patients with AR findings at a routine protocol biopsy six-months post transplantation, 12 (92%) were detected positive by dd-cfDNA. This SNP-based dd-cfDNA assay detected allograft rejection with superior performance compared with the current standard of care. These data support the feasibility of using this assay to detect disease prior to renal failure and optimize patient management in the case of allograft injury.

Keywords: cfDNA; kidney transplantation; rejection

1. Introduction

Precision medicine and personalized tailoring of immunosuppressive drug regimens can improve the current state of organ transplant management [1]. Transplantation injuries may be delayed in detection, and therefore treated ineffectively, because diagnosis can be difficult and biopsy, an invasive and potentially morbid procedure, may be inconclusive. Though advances in immunosuppressive drugs, organ procurement methods, and human leukocyte antigen-typing have lowered the number of clinical- and biopsy-confirmed rejection episodes, sub-clinical rejection of kidney grafts remains a significant risk [2,3]. Kidney transplant management is particularly challenging owing to the lack of sensitivity and specificity of the serum creatinine assay, which, in addition to the late detection of transplant injuries, makes immunosuppression dosage and adjustment far from personalized [4,5]. Therefore, rapid and non-invasive detection and prediction of allograft injury/rejection holds promise for improving the post-transplantation management of patients who have received kidney allografts.

Diagnosis of renal transplant rejection is generally dependent on an increase in serum creatinine levels or its algorithmic derivative, estimated glomerular filtration rate (eGFR), which indicates altered renal filtration functioning. Methods of estimating kidney rejection in allograft recipients based on serum creatinine or eGFR, however, lack sufficient accuracy. Since there are many causes of the baseline drift in altered renal filtering in these patients, biopsy is required for definitive diagnosis. However, biopsies are invasive and costly procedures, which limit their use in clinical practice. Furthermore, biopsy results are often plagued by expert reader variance and can lead to delayed diagnosis of active rejection, after which irreversible organ damage may have occurred [6,7]. There is a current unmet need for a rapid, accurate, and noninvasive approach to detecting allograft rejection and/or injury—one which may require integration of the current "gold" standard morphological assessments with modern molecular diagnostic tools [8].

Donor-derived cell-free DNA (dd-cfDNA) detected in the blood of transplant recipients has been reported as a noninvasive marker to diagnose allograft injury/rejection [9–12], and holds promise for producing faster and more quantitative results compared with current diagnostic options. Recently, it was demonstrated that plasma dd-cfDNA fraction, typically between 0.3% and 1.2% in stable patients [13], can discriminate active rejection status from stable organ function in kidney transplant recipients [14]. Previously we validated the clinical application of a targeted, single nucleotide polymorphism (SNP)-based cell-free assay targeting greater than 10,000 loci as a successful screening tool for the detection of fetal chromosomal abnormalities [15–17] and show here that a similar approach targeting 13,392 SNPs can be used to evaluate differences in donor cfDNA burden in different transplant rejection injuries over time. This study uses a novel SNP-based mmPCR-next generation sequencing (NGS) methodology to measure dd-cfDNA in renal transplant recipients for the detection of allograft rejection/injury without prior knowledge of donor genotypes.

2. Materials and Methods

2.1. Study Design

This was a retrospective analysis of blood samples from kidney transplant recipients who had transplant surgeries at the University of California at San Francisco (USCF) Medical Center. The study was approved by the institutional review board at the UCSF Medical Center. All patients provided written informed consent to participate in the research, in full adherence to the Declaration of Helsinki. The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the Declaration of Istanbul on Organ Trafficking and Transplant Tourism.

2.2. Study Population and Samples

Male and female adult or young-adult patients received a kidney from related or unrelated living donors, or unrelated deceased donors. Plasma samples were obtained from an existing biorepository; time points of patient blood draw following transplantation surgery were either at the time of an

allograft biopsy or at various pre-specified time intervals based on lab protocols. Typically, samples were biopsy-matched and had blood drawn at the time of clinical dysfunction and biopsy or at the time of protocol biopsy (at which time most patients did not have clinical dysfunction). In addition, some patients had serial post transplantation blood drawn as part of routine Internal Review Board approved bio-sampling studies. The selection of study samples was based on (a) adequate plasma being available, and (b) if the sample was associated with biopsy information. Among the full 300 sample cohort, 72.3% were drawn on the day of biopsy. Patients without biopsy-matched samples were excluded from the primary analyses.

2.3. Biopsy Samples

All kidney biopsies were analyzed in a blinded manner by a UCSF pathologist and were graded by the 2017 Banff classification [18] for active rejection (AR); intragraft C4d stains were performed [19] to assess for acute humoral rejection [20]. Biopsies were not done in cases of active urinary tract infection (UTI) or other infections. Transplant "injury" was defined as a >20% increase in serum creatinine from its previous steady-state baseline value and an associated biopsy that was classified as either active rejection (AR), borderline rejection (BL), or other injury (OI) (e.g., drug toxicity, viral infection). Active rejection was defined, at minimum, by the following criteria: (1) T-cell-mediated rejection (TCMR) consisting of either a tubulitis (t) score >2 accompanied by an interstitial inflammation (i) score >2 or vascular changes (v) score >0; (2) C4d positive antibody-mediated rejection (ABMR) consisting of positive donor specific antibodies (DSA) with a glomerulitis (g) score >0/or peritubular capillaritis score (ptc) >0 or v > 0 with unexplained acute tubular necrosis/thrombotic micro angiopathy (ATN/TMA) with C4d = 2; or (3) C4d negative ABMR consisting of positive DSA with unexplained ATN/TMA with g + ptc $\geq$2 and C4d is either 0 or 1. Borderline change (BL) was defined by t1 + i0, or t1 + i1, or t2 + i0 without explained cause (e.g., polyomavirus-associated nephropathy (PVAN)/infectious cause/ATN). Other criteria used for BL changes were g > 0 and/or ptc > 0, or v > 0 without DSA, or C4d or positive DSA, or positive C4d without nonzero g or ptc scores. Normal (STA) allografts were defined by an absence of significant injury pathology as defined by Banff schema.

2.4. dd-cfDNA Measurement in Blood Samples

Cell-free DNA was extracted from plasma samples using the QIAamp Circulating Nucleic Acid Kit (Qiagen) and quantified on the LabChip NGS 5k kit (Perkin Elmer, Waltham, MA, USA) following manufacturer's instructions. Cell-free DNA was input into library preparation using the Natera Library Prep kit as previously described [21], with a modification of 18 cycles of library amplification to plateau the libraries. Purified libraries were quantified using LabChip NGS 5k as previously described [21]. Target enrichment was accomplished using massively multiplexed-PCR (mmPCR) using a modified version of a previously described method [22], with 13,392 single nucleotide polymorphisms (SNPs) targeted. Amplicons were then sequenced on an Illumina HiSeq 2500 Rapid Run, 50 cycles single end, with 10–11 million reads per sample.

2.5. Statistical Analyses of dd-cfDNA and eGFR

In each sample, dd-cfDNA was measured and correlated with rejection status, and results were compared with eGFR. Where applicable, all statistical tests were two sided. Significance was set at $p < 0.05$. Because the distribution of dd-cfDNA in patients was severely skewed among the groups, data were analyzed using a Kruskal–Wallis rank sum test followed by Dunn multiple comparison tests with Holm correction [23,24]. eGFR (serum creatinine in mg/dL) was calculated as described previously for adult [25] and pediatric patients [26]. Briefly, eGFR = 186 $\times$ Serum Creatinine$^{-1.154}$ $\times$ Age$^{-0.203}$ $\times$ (1.210 if Black) $\times$ (0.742 if Female).

To evaluate the performance of dd-cfDNA and eGFR (mL/min/1.73m^2) as rejection markers, samples were separated into an AR group and a non-rejection group (BL + STA + OI). Using this

categorization, the following predetermined cut-offs were used to classify a sample as AR: >1% for dd-cfDNA [14] and <60.0 for eGFR [27].

To calculate the performance parameters of each marker (sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and area under the curve (AUC)), a bootstrap method was used to account for repeated measurements within a patient [28]. Briefly, at each bootstrap step, a single sample was selected from each patient; by assuming independence among patients, the performance parameters and their standard errors were calculated. This was repeated 10,000 times; final confidence intervals were calculated using the bootstrap mean for the parameter with the average of the bootstrap standard errors with standard normal quantiles. Standard errors for sensitivity and specificity were calculated assuming a binomial distribution; for PPV and NPV a normal approximation was used; and for AUC the DeLong method was used. Performance was calculated for all samples with a matched biopsy, including the sub-cohort consisting of samples drawn at the same time as a protocol biopsy.

Differences in dd-cfDNA levels by donor type (living related, living non-related, and deceased non-related) were also evaluated. Significance was determined using the Kruskal–Wallis rank sum test as described above. Inter- and intra-variability in dd-cfDNA over time was evaluated using a mixed effects model with a logarithmic transformation on dd-cfDNA [29]; 95% confidence intervals (CI) for the intra- and inter-patient standard deviations were calculated using a likelihood profile method.

Post hoc analyses evaluated (a) different dd-cfDNA thresholds to maximize NPV (Table S1) and (b) combined dd-cfDNA and eGFR to define an empirical rejection zone that may improve the PPV for AR diagnosis (Figure S1).

All analyses were done using R 3.3.2 using the FSA (for Dunn tests), lme4 (for mixed effect modeling) and pROC (for AUC calculations) packages.

3. Results

3.1. Patients and Blood Samples

A total of 300 plasma samples were collected from 193 unique renal transplant recipients. Of these, 23 samples from 15 patients did not meet inclusion criteria and were excluded from analyses; this included samples collected within three days from transplant (15), and samples unable to be sequenced (8). Of the remaining 277 samples, 217 were biopsy-matched, including 38 collected from patients with biopsy-proven active rejection (AR), 72 with biopsy-proven borderline rejection (BL), 82 normal, stable allografts (STA), and 25 with a biopsy that indicated other injury (OI) (Figure 1). Of the 178 unique patients included in the study, 20% (35) were under 18 years of age; 30% (54) were between 18 and 40 years, and 50% (89) were older than 40 years of age at the time of first blood sample.

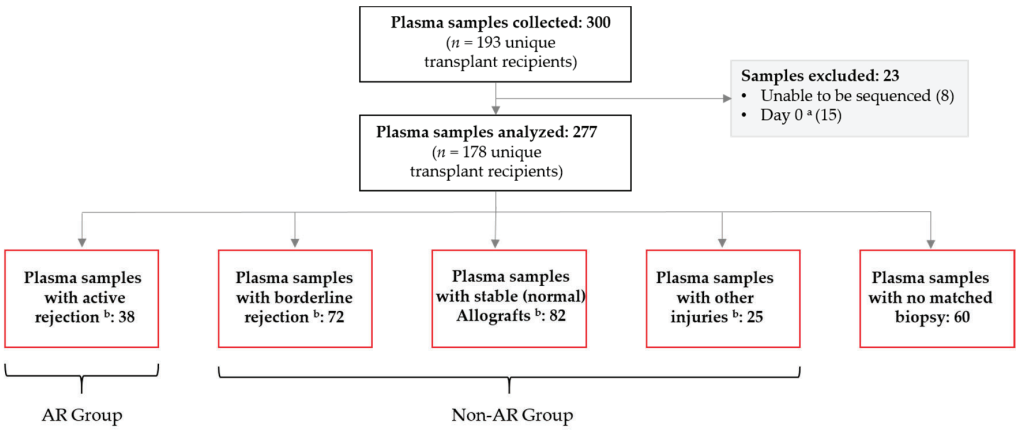


Figure 1. Plasma sample breakdown. AR, active rejection. [a] Collected within three days post transplantation; [b] samples drawn on the day of biopsy (i.e., were biopsy-matched).

Published data have shown that dd-cfDNA fractions in patients with AR are significantly higher than patients with non-rejection; however, these data have shown an inability of dd-cfDNA to detect all types of AR, specifically failing to detect TCMR [14]. In this data set, the performance of the assay to detect rejection was evaluated for all types of rejection combined (ABMR, TCMR), based on the assumption that elevated dd-cfDNA levels are indicative of ongoing damage to the transplanted organ, irrespective of the underlying biology of rejection. Therefore, the ability of the assay to detect AR versus non-rejection was calculated, where non-rejection was defined as all specimens that were classified as STA, BL, or OI. Additionally, the performance of the assay to discriminate AR from complete absence of injury (STA) was also evaluated. A summary of demographic information and sample characteristics are provided in Table 1. All pathology samples were read at UCSF by a single renal pathologist and rated according to the recently updated Banff criteria [18].

Table 1. Demographics and characteristics [a].

Phenotype Characteristic	Active Rejection (38 Samples)	Non-Rejection			
		Stable (82 Samples)	Borderline AR (72 Samples)	Other Injury (25 Samples) [b]	Combined (179 Samples)
Recipient age, year * (p-value < 0.0001)					
(0, 18)	0 (0)	44 (53.7)	1 (1.4)	4 (16.0)	49 (27.4)
(18, 40)	10 (26.3)	32 (39.0)	18 (18.0)	8 (32.0)	58 (32.4)
(40, 80)	28 (73.7)	6 (7.3)	53 (73.6)	13 (52.0)	72 (40.2)
Mean ± SD	47.91 ± 14.31	20.04 ± 11.97	47.88 ± 13.24	44.75 ± 23.73	34.65 ± 19.87
Median	49.13	19.96	47.46	40.97	31.33
Range	23–76	3–70	5–74	3–80	3–80
Male/female, no. (%) (p-value = 0.5988)					
Male	17 (44.7)	48 (58.5)	40 (55.6)	15 (60)	103 (57.5)
Female	21 (55.3)	34 (41.5)	32 (44.4)	10 (40)	76 (42.5)
Ethnicity, no. (%) (p-value = 1)					
Hispanic or Latino	13 (34.2)	28 (34.1)	24 (33.3)	10 (40)	62 (34.6)
Not Hispanic or Latino	25 (65.8)	54 (65.9)	48 (66.7)	15 (60)	117 (65.4)
Race groups, no. (%) (p-value = 0.4695)					
White or Caucasian	10 (26.6)	42 (51.2)	16 (22.2)	6 (24)	64 (35.8)
Black or African American	6 (15.8)	7 (8.5)	14 (19.4)	4 (16)	25 (14.0)
Asian or Pacific Islander	8 (21.1)	4 (4.9)	15 (20.8)	4 (16)	23 (12.8)
Other/Not reported	14 (36.8)	29 (35.4)	27 (37.8)	11 (44.0)	67 (37.4)
Recipient weight, kg (p-value = 0.6039)					
Mean ± SD	76.22 ± 19.7	70.9 ± 8.8	79.18 ± 18.7	78.33 ± 17.1	78.1 ± 17.6
Median	72.5	73.0	78.0	76.0	76.0
Range	45–119	52–81	46–134	47–109	46–134
Unknown	6	72	7	7	86
DSA positive, no. (%) (p-value = 0.1928)					
Yes	15 (39.5)	0 (0)	18 (25)	2 (8)	20 (11.2)
No	21 (55.3)	0 (0)	48 (66.7)	3 (12)	51 (28.5)
Not recorded	2 (5.3)	82 (100)	6 (8.3)	20 (80)	108 (60.3)
Indication for renal transplantation, no. (%) (p-value = 0.4869)					
Glomerulonephritis	5 (13.2)	6 (7.3)	4 (5.6)	1 (4)	11 (6.1)
Focal segmental glomerulosclerosis	5 (13.2)	5 (6.1)	6 (8.3)	2 (8)	13 (7.3)
Diabetes mellitus	5 (13.2)	3 (3.7)	15 (20.8)	5 (20)	23 (12.8)
Thin basement membrane nephropathy	0 (0)	0 (0)	2 (2.8)	0 (0)	2 (1.1)
Polycystic kidney disease	3 (7.9)	2 (2.4)	7 (9.7)	1 (4)	10 (5.6)
Solitary kidney	0 (0)	0 (0)	3 (4.2)	0 (0)	3 (1.7)
Hypertension	4 (10.5)	2 (2.4)	13 (18.1)	3 (12)	18 (10.1)
IgA nephropathy	3 (7.9)	0 (0)	7 (9.7)	1 (4)	8 (4.5)
Lupus nephritis	2 (5.3)	0 (0)	0 (0)	0 (0)	0 (0.0)
ANCA—vasculitis	1 (2.6)	0 (0)	2 (2.8)	0 (0)	2 (1.1)
Other/Unknown	10 (26.3)	64 (78.1)	13 (18.1)	12 (48)	89 (49.7)

Table 1. *Cont.*

Phenotype Characteristic	Active Rejection (38 Samples)	Non-Rejection			
		Stable (82 Samples)	Borderline AR (72 Samples)	Other Injury (25 Samples) [b]	Combined (179 Samples)
Donor source *, no. (%) (p-value < 0.0001)					
Living related	1 (2.8)	2 (2.4)	9 (12.5)	3 (12)	14 (7.8)
Living unrelated	2 (5.3)	50 (61)	18 (25)	7 (28)	75 (41.9)
Deceased unrelated	35 (92.1)	30 (36.6)	45 (62.5)	15 (60)	90 (50.3)

* Indicates the association with AR status (AR/non rejection) was statistically significant ($p < 0.001$). Categorical variables were tested using Fisher's exact test for count data, and numerical variables were tested using a likelihood ratio test based on a logistic regression. [a] Characteristics and demographic information are based on all samples drawn on the day of biopsy; data reflects multiple samples for some patients. [b] Other injuries included: chronic allograft nephropathy (10 samples), drug toxicity (11 samples), BK nephritis (1 sample), acute tubular necrosis (1 sample), transplant glomerulopathy (1 sample), and post borderline-TCMR (1 sample). DSA, donor specific antibodies; AR, active rejection.

3.2. dd-cfDNA and eGFR in Kidney Transplant Recipients

The amount of dd-cfDNA was significantly higher in the circulating plasma of the AR group (median = 2.32%) compared with the non-rejection group (median = 0.47%, $p < 0.0001$) (Table 2, Figure S2). Additionally, the median level of dd-cfDNA was significantly higher in the AR group compared with all three individual non-rejection subgroups: BL group (0.58%), STA group (0.40%), and OI (0.67%, all comparisons, adj. $p < 0.0001$) (Figure 2A, Table S2). That the dd-cfDNA burden was higher in the AR group as compared to the BL group indicates that dd-cfDNA fraction may be used to track the evolution of early injury to more established rejection, as well as any subsequent recovery. The differences between the levels of dd-cfDNA between any of the non-rejection subgroups (STA, BL, and OI) were not significant (Figure 2A; Table S2).

In contrast to dd-cfDNA, eGFR scores did not have as much discriminatory ability for differentiating AR and individual non-rejection groups (Table 2, Figure S2). Overall, the median eGFR score in the AR group (45.67) was significantly lower than that observed in the non-rejection group (76.6, $p < 0.0001$) (Table 2 and Table S2, Figure S2) and even lower compared to the STA group alone (104.5, adj. $p < 0.0001$) (Table 2 and Table S2, Figure 2B). However, unlike the dd-cfDNA results, there was no difference in median eGFR scores between the AR and BL groups (45.67 vs. 55.99, adj. $p = 0.461$) (Table 2 and Table S2; Figure 2B). Additionally, compared with the STA group, eGFR levels were significantly higher in the BL (55.99, adj. $p < 0.0001$) and OI (57.4, adj. $p < 0.0001$) groups (Table 2 and Table S2, Figure 2B).

Table 2. Summary statistics for donor-derived cell-free DNA (dd-cfDNA) and estimated glomerular filtration rate (eGFR) variables across AR and non-rejection groups.

Parameter	Active Rejection	Non-Rejection			
		Stable	Borderline AR	Other Injury	Combined
dd-cfDNA					
Number of samples (%)	38 (17.5)	82 (37.8)	72 (33.2)	25 (11.5)	179 (82.5)
Mean (SD)	4.64 (5.45)	0.90 (1.36)	0.95 (1.31)	0.89 (0.91)	0.92 (1.28)
Median (range)	2.32 (0.1–23.9)	0.4 (0.03–6.8)	0.58 (0.02–6.7)	0.67 (0.08–3.69)	0.47 (0.04–6.78)
eGFR					
Number of samples (%)	38 (17.5)	82 (37.8)	72 (33.2)	25 (11.5)	179 (82.5)
Score mean (SD)	49.0 (22.4)	99.5 (16.1)	55.9 (21.4)	63.8 (29.0)	77.0 (8.45)
Score median (range)	45.67 (8.0–100.4)	104.5 (47.4–131.1)	55.99 (6.4–109.4)	57.4 (25.0–116.9)	76.06 (6.4–131.1)

AR, active rejection.

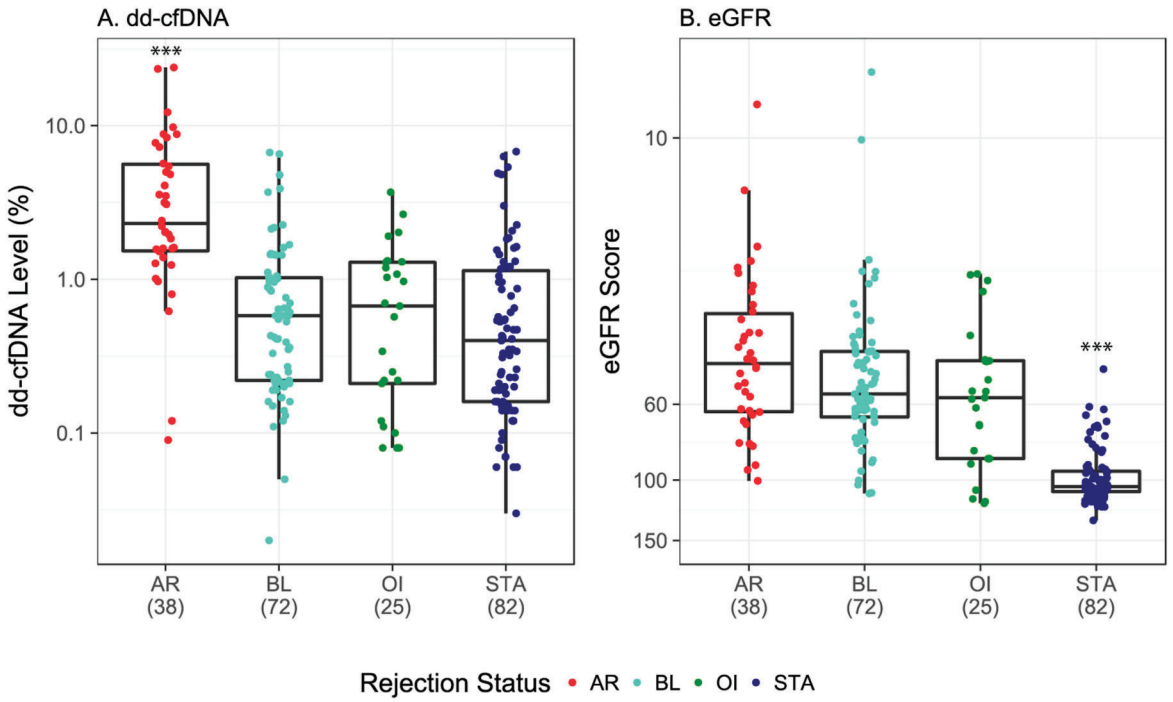


Figure 2. Discrimination of active rejection by dd-cfDNA versus eGFR. (**A**) and (**B**): Boxes indicate interquartile range (25th to 75th percentile); horizontal lines in boxes represent medians; each dot depicts one sample. *p*-values for dd-cfDNA and eGFR adjusted using Kruskal–Wallis rank sum test followed by Dunn multiple comparison tests with Holm correction. *** indicates adj. $p < 0.0001$ from all other group comparisons (see Table S2). AR, active rejection; BL, borderline; OI, other injury; STA, stable; dd-cfDNA, donor-derived cell-free DNA; eGFR, estimate glomerular filtration rate.

3.3. Performance Estimates for Discriminatory Ability of Tests

With a dd-cfDNA cutoff of >1%, the mmPCR-NGS method had an 88.7% sensitivity (95% CI, 77.7–99.8%) and 72.6% specificity (95% CI, 65.4–79.8%) for detection of AR. Sensitivity and specificity values are shown over the range of dd-cfDNA cutoffs in Figure 3A. The AUC was 0.87 (95% CI, 0.80–0.95). Based on a 25% prevalence of rejection in an at-risk population, the positive predictive value (PPV) was projected to be 52.0% (95% CI, 44.7–59.2%) and the negative predictive value (NPV) was projected to be 95.1% (95% CI, 90.5–99.7%).

Sensitivity and specificity were lower using eGFR (Figure 3B). Using an eGFR cutoff score <60 for AR, sensitivity and specificity values were 67.8% (95% CI, 51.3–84.2%) and 65.3% (57.6–73.0%), respectively, with an AUC of 0.74 (0.66–0.83). The projected PPV and NPV values of eGFR were 39.4% (31.6–47.3%) and 85.9% (75.9–92.2%), respectively.

As a post hoc analysis, we also evaluated a combination of eGFR with dd-cfDNA. Although we do not have a large number of samples to train a combined model, we can still see potential empirical rejection zones. Samples with a very high eGFR score, for example, tend to correspond to non-rejection samples (Figure S1). Defining the active rejection zone to be dd-cfDNA level >1% and eGFR <100, and non-rejection to be dd-cfDNA level <1% or eGFR >100, the combined dd-cfDNA and eGFR markers correctly classified 32/38 (84.2%) AR samples, and 145/179 (81.0%) non-rejection samples. Meanwhile at an equivalent specificity of 81.0%, (using a cut off of 1.3% dd-cfDNA) the sensitivity of the dd-cfDNA marker alone was 82.3%. Therefore the combined biomarker approach appeared to add little or no value over cfDNA alone.

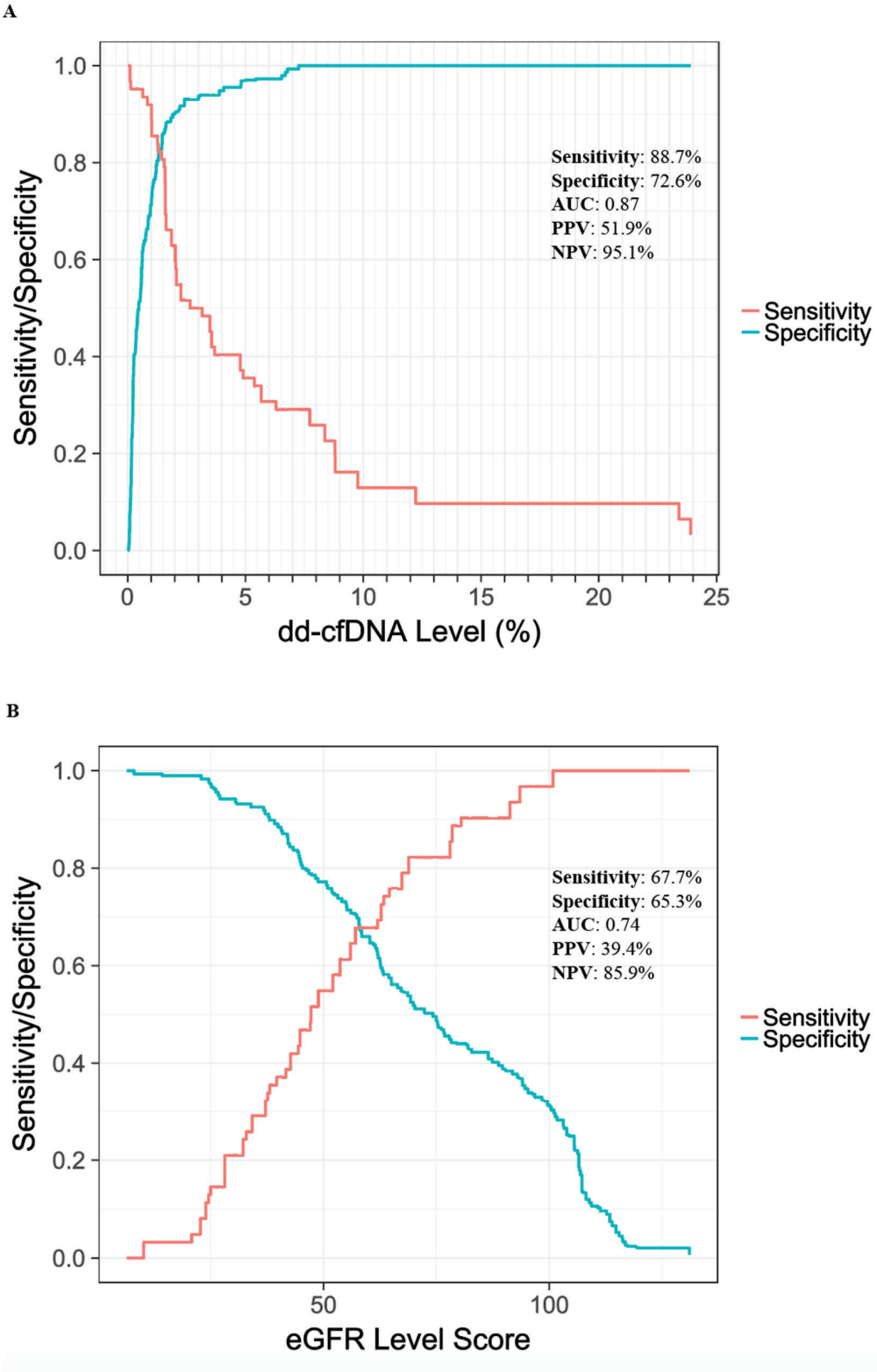


Figure 3. Predictive statistics for active rejection versus non-rejection. Sensitivity (red line) and specificity (blue line) are depicted over the observed range of dd-cfDNA levels (**A**) and eGFR scores (**B**). Reported sensitivity and specificity correspond to cutoffs of 1% for dd-cfDNA and a score of 60 for eGFR. PPV and NPV are based on a 25% AR prevalence. AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value.

3.4. dd-cfDNA Performance in Unique Biopsy-Confirmed Subgroups

Among the biopsy-matched samples, 103 (47.5%) were biopsied for clinical reasons, whereas 114 (52.5%) were biopsied according to protocol (Table 3 and Table S3). Figure 4 depicts sample dd-cfDNA levels among all subgroups; 85 (39.2%) had dd-cfDNA levels >1%. Of those, 22 (25.9%) were STA; the remainder were AR (33 (38.8%)), OI (10 (11.8%)), or BL (20 (23.5%)). Of the individual groups, 33 (86.8%) of the total AR samples and 22 (26.8%) of the total STA samples had dd-cfDNA levels above 1%. In comparison, 20 (27.8%) of the total BL samples and 10 (40.0%) of the total OI samples had dd-cfDNA levels above 1%.

Table 3. Cohort breakdown into for-cause and protocol biopsy.

Rejection Status	Biopsy Reason	Total	Median	Low	High	Mean	SD
AR	For-cause	25	2.04	0.09	23.9	3.85	4.81
	Protocol	13	3.56	0.12	23.4	6.16	6.44
BL	For-cause	39	0.64	0.02	6.54	1.07	1.32
	Protocol	33	0.33	0.05	6.69	0.82	1.30
OI	For-cause	12	0.865	0.08	3.69	1.03	1.02
	Protocol	13	0.25	0.08	2.65	0.76	0.82
STA	For-cause	27	0.54	0.12	5.38	1.12	1.36
	Protocol	55	0.26	0.03	6.78	0.80	1.37

AR, active rejection; BL, borderline; OI, other injury; STA, stable.

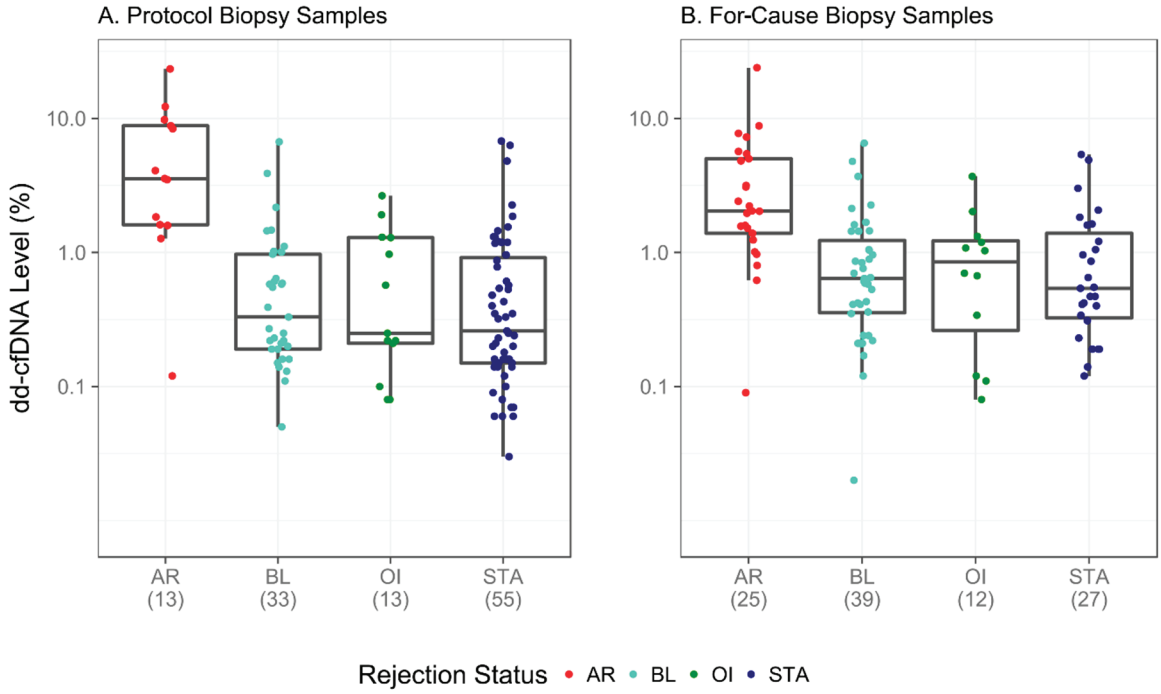


Figure 4. Discrimination of active rejection by dd-cfDNA in biopsy-matched samples stratified by biopsy type. The number of samples per group and the distribution of their dd-cfDNA levels are depicted for protocol biopsy (**A**) and for-cause biopsy (**B**) samples. Boxes indicate inter-quartile range, horizontal lines represent medians. AR, active rejection; BL, borderline; OI, other injury; STA, stable.

Figure 4 shows assay performance for the subset of samples drawn at the time of a for-cause biopsy (4A) and protocol biopsy (4B); performance shown in protocol biopsies is expected to reflect performance when the assay is used in routine surveillance, that is, when there are no signs of renal injury. This cohort of 114 samples showed a 92.3% sensitivity (95% CI, 64.0–99.8%) and 75.2% specificity (95% CI, 65.7–83.3%) for detection of AR. The AUC was 0.89 (95% CI, 0.76–0.99). Based on a 25% prevalence of rejection in an at-risk population, the positive predictive value (PPV) was projected to be

55.4% (95% CI, 46.2–64.7%) and the negative predictive value (NPV) was projected to be 96.7% (95% CI, 90.6–99.9%).

Sensitivity, specificity, PPV and NPV were also calculated at different dd-cfDNA level rejection cutoffs. Table S1 shows the metrics at 0.6%, 0.8%, 1.0%, 1.2%, 1.4%, and 1.6%. Raising the cutoff has the effect of improving the specificity and the PPV; lowering the cutoff improves sensitivity and NPV.

3.5. Relationship Between dd-cfDNA and Rejection Type

Of the 38 samples with biopsy-proven AR, 16 were classified as either ABMR or ABMR and borderline T-cell-mediated rejection (bTCMR); 12 had a combination of both ABMR and TCMR; 10 were classified as either TCMR or TCMR and bABMR. In addition, 13 and 59 BL samples were classified as bAMBR and bTCMR, respectively. Figure 5 shows the relationship between dd-cfDNA level and type of rejection (for groups with known ABMR or known TCMR). Median dd-cfDNA did not differ significantly between AMBR (2.2%), ABMR/TCMR (2.6%), or TCMR (2.7%) groups ($p = 0.855$) (Table S4). The study contained a range of pathologies, and the data indicate that this assay, unlike other published studies measuring cfDNA by other assays [14], is robust to different rejection types (Table S5). The dd-cfDNA breakdown of bABMR and bTCMR samples are depicted in Figure S3.

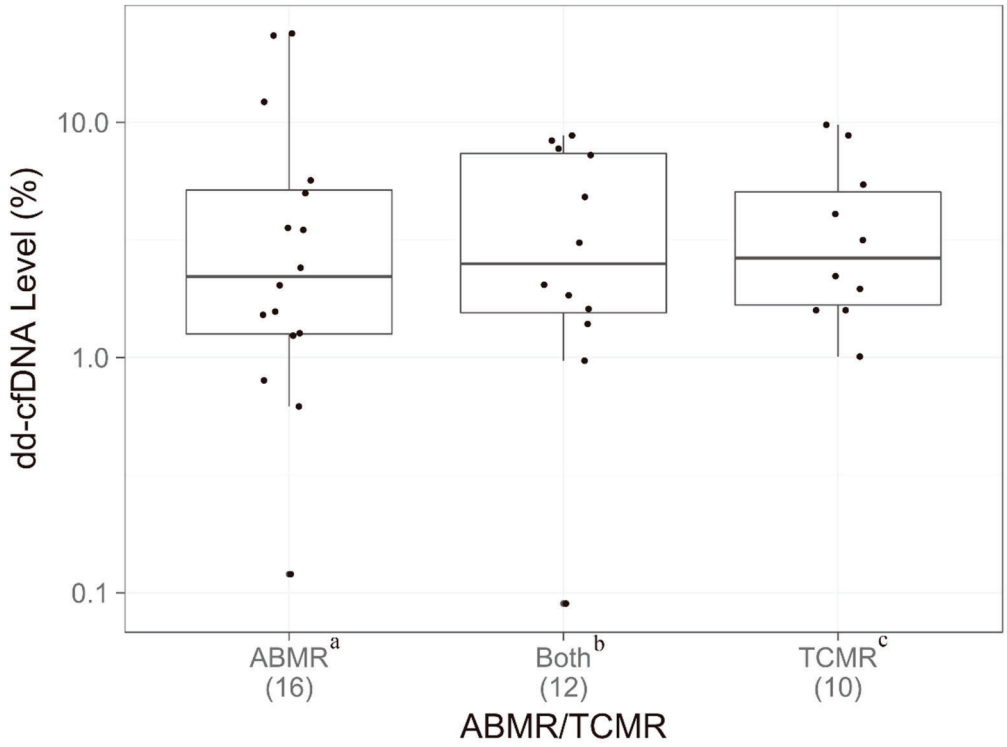
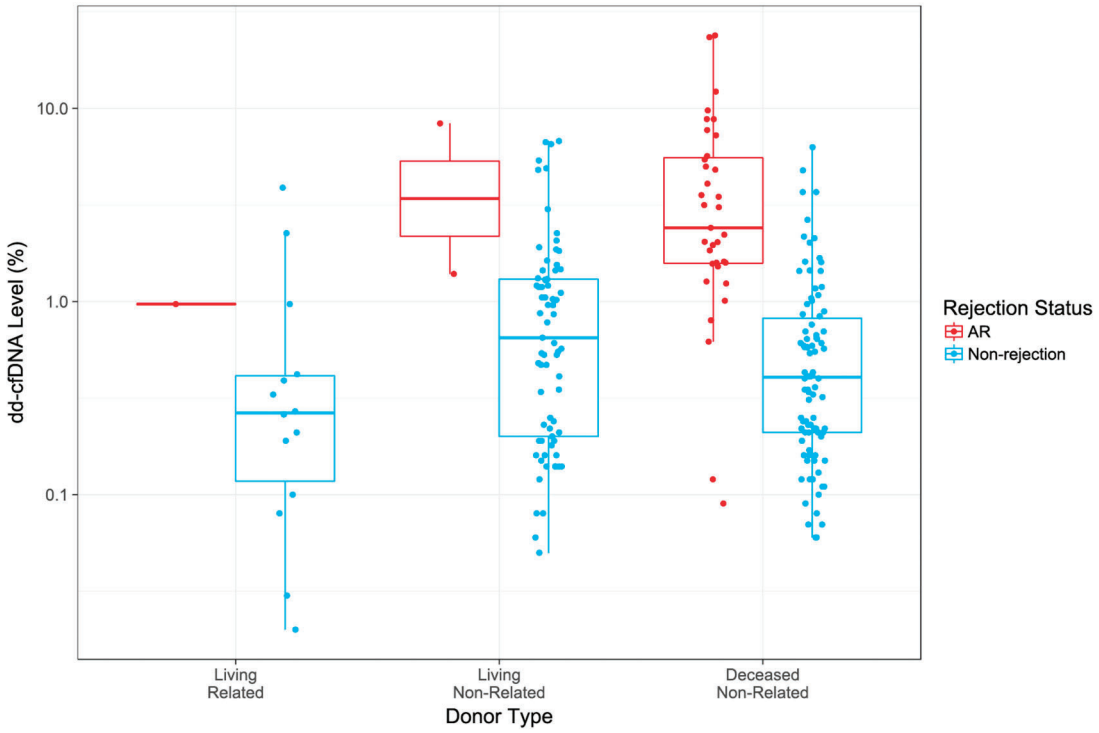


Figure 5. dd-cfDNA as a function of antibody-mediated—versus T-cell—mediated rejection. Boxes indicate interquartile range (25th to 75th percentile); horizontal lines in boxes represent medians; dots indicate all individual data points. *p*-values for dd-cfDNA adjusted using Kruskal–Wallis rank sum test. [a] Samples assigned ABMR or ABMR and bTCMR. [b] Samples assigned ABMR and TCMR. [c] Samples assigned TCMR or TCMR and bABMR. ABMR, antibody-mediated rejection; TCMR, T-cell-mediated rejection.

3.6. dd-cfDNA Levels by Donor Type

To assess the relationship between dd-cfDNA and donor type (living related, living non-related, and deceased non-related) a linear mixed-effects model was constructed using a log transformed dd-cfDNA as the response and donor type as the predictor for the non-rejection group. The log-transformation was applied to satisfy the model's assumptions. The test was limited to the non-rejection group due to the limited number of AR samples in two groups (living related and living non-related). An ANOVA

Wald-test with Kenward–Roger approximation for the degrees of freedom showed significance ($p = 0.045$). Tukey's post-hoc test was used to determine the difference among the three groups: none of the post-hoc tests demonstrated any association (Figure 6, Table S6). It is possible that the overall effect is driven by a sub-category of the non-rejection group (STA, BL, or OI) or the effect between the groups is smaller than detectable with the current sample size [30].

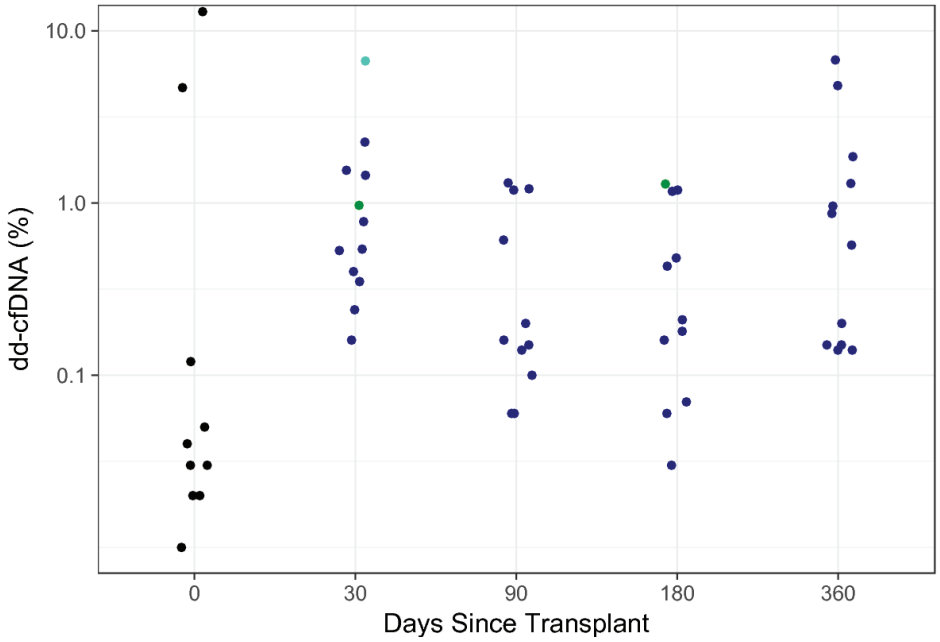


Comparison	P-value
Living related versus living non-related	0.0623
Living related versus deceased non-related	0.3728
Living non-related versus deceased non-related	0.1803

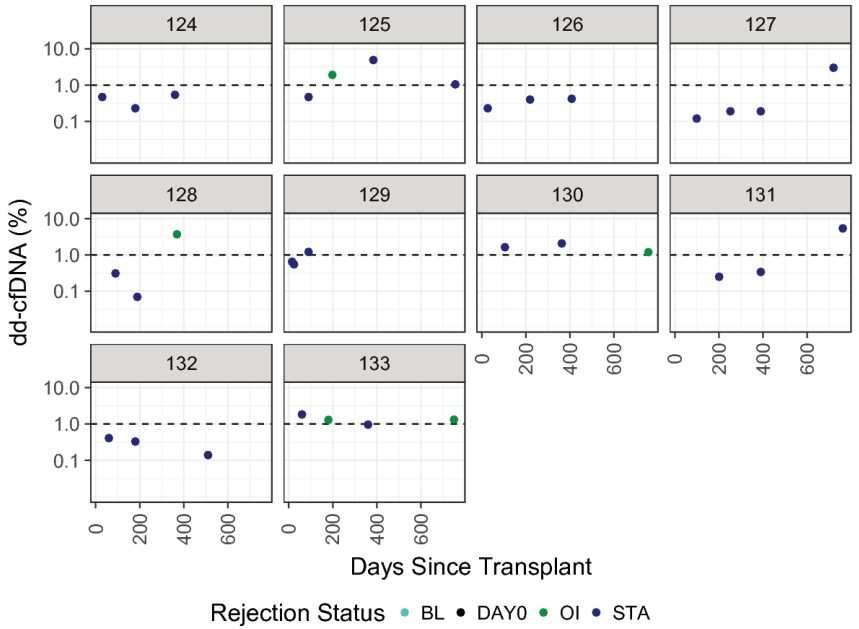
Figure 6. Relationship between dd-cfDNA and donor type. Boxes indicate inter-quartile range, horizontal lines represent medians. *p*-values for dd-cfDNA an ANOVA Wald-test with Kenward–Roger approximation for the degrees of freedom was followed by Tukey's post-hoc test. AR, active rejection.

3.7. dd-cfDNA Variability over Time

Two analyses were designed to evaluate the natural variability in dd-cfDNA over time in biopsy-matched, non-rejection patients. The first sub-analysis was a cross-sectional analysis of 60 plasma samples from 60 different patients, collected immediately following surgery (within three days ("Day 0")) or at 1, 3, 6, or 12 months post-surgery. Among these STA patients, dd-cfDNA levels were lower at month 0 than subsequent time points; however, for most of these STA samples dd-cfDNA levels were <1% across all time points (Figure 7A). No association was observed between Day 0 samples and the other time points, although the overall distribution of dd-cfDNA levels in the Day 0 group appears lower in comparison (Figure 7A).

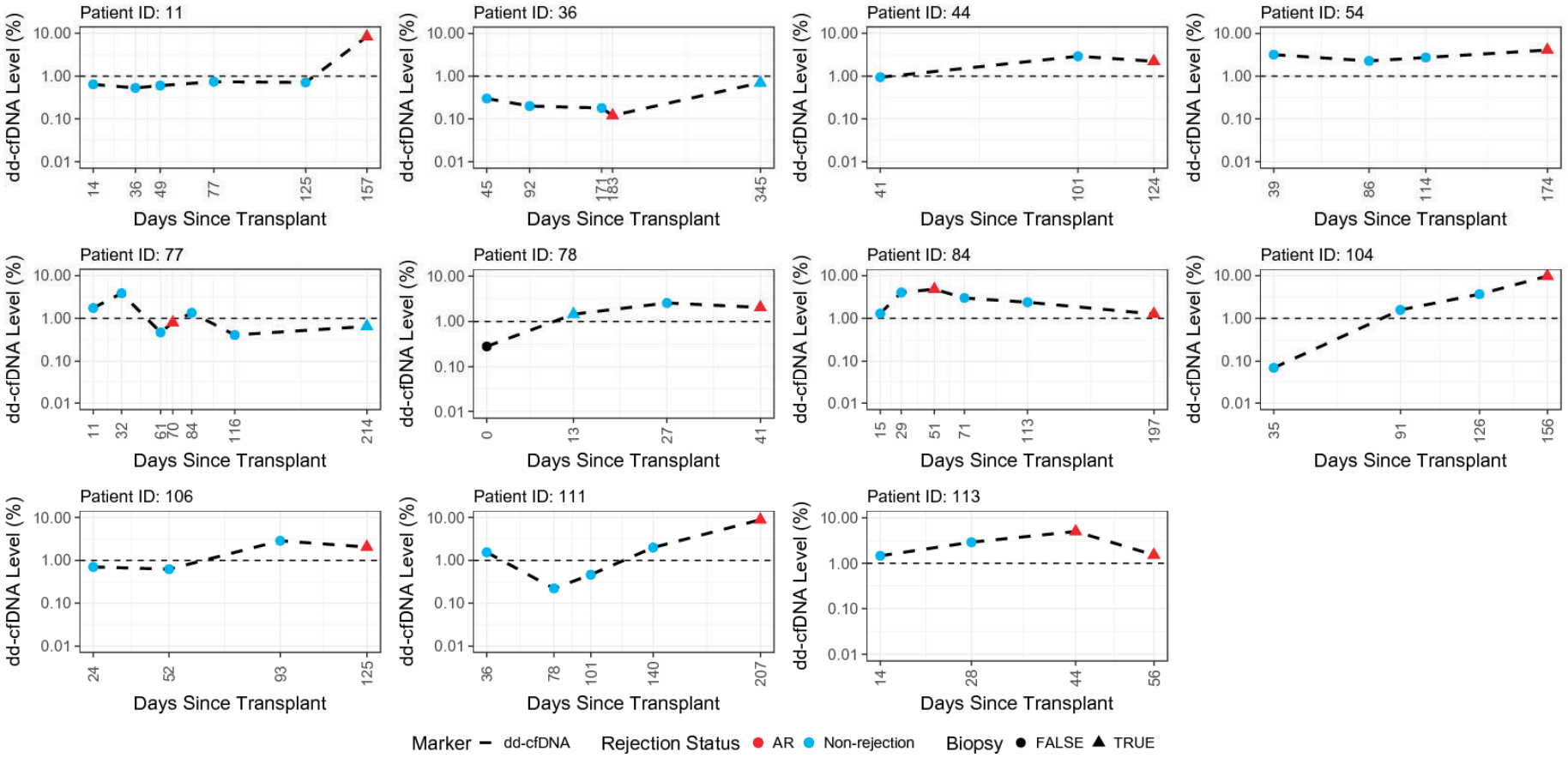


(**A**)



(**B**)

Figure 7. *Cont.*



(C)

Figure 7. Variability in dd-cfDNA in non-rejection patients over time. (**A**) Inter-patient variability (60 samples from 60 patients over time); (**B**) intra-patient variability (samples from the same 10 patients over time); (**C**) change in dd-cfDNA levels over time in patients with active rejection. AR, active rejection; BL, borderline; OI, other injury; STA, stable.

To evaluate the normal intra-patient variation in donor fraction, the second sub-analysis longitudinally assessed 10 individual patients across four time points (varying between about 1 month and 1 year post transplantation (minimum interval: 11 days, maximum interval: 345 days)). Overall, organ injury occurred at dd-cfDNA levels above 1% (Figure 7B). The inter-patient standard deviation within this cohort was 0.16 (95% CI, 0.0–0.37) and the intra-patient standard deviation was 0.42 (95% CI, 0.32–0.56). The intraclass-correlation coefficient was low (0.1193), which suggests that the variability in these data are mostly due to intra-patient variation. Figure 7C depicts all available longitudinal data among patients that experienced a rejection. In 9/11 patients, dd-cfDNA levels were above 1% prior to rejection.

4. Discussion

In this study, median dd-cfDNA was significantly higher in the AR group (2.32%) versus the non-rejection group (0.47%; $p < 0.0001$). Analysis of performance estimates demonstrated that the mmPCR-NGS method was able to discriminate active from non-rejection status with an AUC of 0.87 and high sensitivity (88.7%) and specificity (72.6%) at an AR cutoff of >1% dd-cfDNA. Based on a 25% prevalence of rejection, projected PPV and NPV were 52.0% and 95.1%, respectively. In contrast, eGFR scores were generally less discriminatory, with a 67.7% sensitivity and 65.3% specificity, and projected PPV and NPV of 39.4% and 85.9%, respectively. Therefore, if eGFR measurements were used as the sole clinical decision point, about 1 in 7 patients found to be at low risk of rejection would actually be experiencing rejection, and would not be referred for an indication biopsy—this is in comparison to the projected NPV for dd-cfDNA that suggests that only 1 in 20 patients would miss an indication biopsy where it might be clinically necessary. Taken together, the superior performance of this SNP-based dd-cfDNA assay over that of the current standard of care for the evaluation of allograft rejection holds promise for enabling patients a greater opportunity for timely therapy in the case of an allograft injury.

Levels of dd-cfDNA also provided discrimination of AR from the three non-rejection subgroups (STA, BL, and OI); median dd-cfDNA levels were significantly higher for samples with biopsy-proven AR (2.3%) versus BL (0.6%), OI (0.7%), and STA (0.4%). In a post hoc analysis, we examined the ability of dd-cfDNA combined with eGFR to predict rejection status (AR/non-rejection) in biopsy matched samples (Figure S1). This combined approach correctly classified 32/38 (84.2%) AR and 145/179 (81.0%) non-rejection samples, though in a head-to-head comparison it showed little to no improvement over dd-cfDNA alone. Combining dd-cfDNA with other markers may provide improved predictive value, but this was outside the scope of this study. Also of note, while both dd-cfDNA and eGFR can be used to differentiate AR and STA cases, the BL and OI samples stratify differently: they tend to aggregate with STA when using dd-cfDNA and with AR when using eGFR. This suggests that dd-cfDNA could be used together with eGFR to differentiate patients into three groups—STA patients, AR patients, and patients experiencing BL or OI.

In a recent study that amplified hundreds of target SNPs in dd-cfDNA to detect active rejection in kidney allografts, that method was able to discriminate AR from non-rejection with an AUC of 0.74, 59% sensitivity, and 85% specificity [14]. In comparison with that study, the novel dd-cfDNA test described in the current study showed a higher AUC value (0.87) as well as greater sensitivity (89%). On the other hand, specificity (73%) was slightly lower in the current study, partly driven by the fact that a majority of the "false positives" were cases with BL and OI indicating some form of organ injury. The predefined analysis in this study used 1% dd-cfDNA cutoff, based on prior experience [14]; however, as a different sensitivity/specificity tradeoff may be optimal in different use cases, performance was calculated, in a post hoc fashion, for additional cfDNA cutoffs: 0.6%, 0.8%, 1.2%, 1.4%, and 1.6 % (Table S1).

Another important finding of this study was that the fraction of dd-cfDNA did not differ between ABMR and TCMR groups, with dd-cfDNA levels of 2.2% and 2.7%, respectively. These results are novel considering that a previously conducted study by Bloom et al. (2017), which used a different assay, found significantly higher dd-cfDNA levels for ABMR (2.9%) than for TCMR ($\leq$1.2%) [14],

showing a lower ability to detect T-cell mediated rejections. Though the assay used in that study also measured dd-cfDNA, the methods used by the two assays differ greatly. It is unclear whether that test could not differentiate AR from non-rejection in cases of TCMR or if the result was due to the smaller sample size of that group in that study (n = 11). Regardless, it appears that dd-cfDNA measurements based on the mmPCR assay in this study can accurately discriminate AR from non-rejection across a range of pathologies, including both acute and chronic findings, in both the ABMR and TCMR groups. An additional finding in this study is that borderline, or early rejection injury, has a lower burden of dd-cfDNA than more established injury, making it possible to use this sensitive assay to track evolution of, or recovery from, AR.

One barrier to widespread clinical use of dd-cfDNA as a diagnostic tool for monitoring organ transplant has been the limitations in measuring dd-cfDNA in certain cases, such as when the donor genotype is unknown or when the donor is a close relative. Given the design of the assay used here, it is possible to quantify dd-cfDNA without prior recipient or donor genotyping. Further, there is no need for a computational adjustment based on whether the donor is related to the recipient. In this study, evaluation of dd-cfDNA levels by donor type revealed that regardless of donor type (living related, living non-related, deceased non-related), dd-cfDNA levels were similar across all donor types within in the AR and non-rejection categories.

A limitation of this study is that it was a retrospective analysis of archived samples from a single center. However, the central geographical area enabled all biopsies to be performed by a single pathologist, which may have helped minimize variability in biopsy classification; further, all experimenters were kept blinded during the process of data generation. The retrospective study design may have led to differences in patient characteristics across the rejection groups; for example, the STA group was enriched with younger patients who may be better suited immunologically to tolerate transplanted organs compared to older-aged patients. However, these age differences likely did not affect the validity of the study findings.

A strength of this study is the large number of samples drawn at the time of a protocol biopsy. Performance of the assay among samples drawn at the time of a protocol biopsy are more reflective of expected performance during routine use of the assay, where there are no overt signs of injury; this is in contrast to for-cause biopsies, which are performed in a high-risk cohort where there are peripheral signs of organ injury. In this study, more than half (53%, 114/217) of the biopsy-matched samples were performed on protocol. The assay showed better performance in this cohort, with a sensitivity of 92.3%, specificity of 75.2%, and AUC of 0.89%. This data suggests that application of the dd-cfDNA assay in a clinical setting could potentially reduce the need for protocol biopsies.

Another strength is the variety of patient samples in the non-rejection group, which comprised not only STA, but also BL and OI samples. This allowed for additional analyses in this study, which found that dd-cfDNA was significantly different in the AR group versus BL and OI groups. Additional sub-analyses by type of AR (ABMR and TCMR), as well as by donor type, demonstrated that dd-cfDNA levels were able to discriminate AR versus non-rejection in a variety of rejection and patient types. Further, the SNP-based mmPCR methodology underlying this assay has been extensively validated in the context of prenatal testing, and has been used to determine the DNA fraction of the minor constituent in a clinical setting in over a million maternal/fetal DNA samples. Finally, the inclusion of longitudinal data enabled a unique evaluation of the natural variability of dd-cfDNA in transplant patients over time. Inter-patient variability data demonstrated that between 0 and 12 months post-surgery, most patients with STA biopsies had dd-cfDNA levels below 1%, and most patients with a positive biopsy had a positive dd-cfDNA test at a time point prior to the positive biopsy. Taken together, this suggests that this mmPCR assay may be used for routine monitoring, to determine whether a renal transplant patient is experiencing organ injury that may require a change in management.

5. Conclusions

In conclusion, this study validates the use of dd-cfDNA in the blood as an accurate marker of kidney injury/rejection across a range of pathologies with acute and chronic findings. This rapid, accurate, and noninvasive technology allows for detection of significant renal injury in patients better than the current standard of care, with the potential for better patient management, more targeted biopsies, and improved renal allograft function and survival.

Supplementary Materials: The Tables S1–S6 and Figures S1–S3 are available online at http://www.mdpi.com/2077-0383/8/1/19/s1.

Author Contributions: Conceptualization, S.M. and M.M.S.; Data curation, F.A.A., M.M.S., T.K.S., R.D.S., C.C.-O., S.-C.H., T.C., J.L., I.D., P.T., S.A.P., and S.N.; Formal analysis, F.A.A. and M.M.S.; Funding acquisition, S.M. and M.M.S.; Investigation, T.K.S., S.M., and M.M.S.; Methodology (cfDNA), F.A.A., T.C., A.R., and B.Z.; Methodology (study design), M.M.S.; Project administration, S.A.P.; Resources, T.K.S., M.M.S., and P.R.B.; Software, F.A.A. and E.K.; Supervision, Z.P.D., A.R., S.S., B.Z., M.M.S., P.R.B., and S.M.; Validation, M.M.S., T.C., and E.K.; Visualization, A.R., M.M.S., and S.M.; Writing—original draft, S.A.P.; Writing—review & editing, F.A.A., T.K.S., T.C., S.A.P., J.L., P.T., S.N., Z.P.D., A.R., S.S., R.D.S., C.C.-O., S.-C.H., B.Z., P.R.B., and M.M.S.

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EXHIBIT 6

News Release

Natera Announces Publication of Kidney Transplant Validation Study, Demonstrating Superior Data in Detection of Clinical and Subclinical Rejection

Represents Successful Achievement of All 2018 Commercialization Milestones, on Path to 2019 Launch

SAN CARLOS, Calif., Jan. 7, 2019 /PRNewswire/ -- Natera, Inc. (https://c212.net/c/link/?t=0&l=en&o=2339767-1&h=3123807725&u=http%3A%2F%2Fwww.natera.com%2F&a=Natera%2C+Inc.) (NASDAQ: NTRA), a leader in cell-free DNA, today announced clinical validation study results published in the *Journal of Clinical Medicine*,[1] demonstrating the highly accurate performance of its donor-derived cell-free DNA (dd-cfDNA) test for active allograft rejection in kidney transplant recipients, including higher sensitivity and nearly 18% higher area under the curve (AUC) than the competitive dd-cfDNA assay.[1,2] The study also reports the first accurate detection of T-cell mediated rejection (TCMR) and subclinical rejection. This marks the successful completion of all 2018 commercialization milestones, and is in line with the company's plan to secure Medicare coverage and commercially launch its test in 2019.

The blinded study, conducted in collaboration with the University of California, San Francisco (UCSF), leveraged Natera's massively-multiplexed PCR (mmPCR) technology to measure dd-cfDNA levels in plasma collected on the same day as a kidney biopsy from 193 unique kidney transplant recipients. The primary analysis focused on 217 plasma samples from 178 unique kidney transplant patients, including 38 cases of histologically-confirmed active rejection (AR), making it the

largest published study of its kind, with approximately two times more patients and 40 percent more affected cases of AR than the next largest study.[2] Another strength of the study is its broad ethnic diversity, which is important because kidney transplant assessment and biomarker performance are known to vary by ethnicity.

In the study, Natera's assay detected AR with 89% sensitivity and 0.87 AUC. This test performance compared favorably to the current standard of care, eGFR (estimated glomerular filtration rate), which is a clinically accepted but inaccurate biomarker for AR. The study results also showed higher sensitivity (89% vs. 59%) and higher AUC (0.87 vs. 0.74) than the competitive dd-cfDNA assay.[2] This superior data may be due to differences in the analytical performance and underlying technology behind the assays.

The new study also had two novel, clinically significant findings:

- **TCMR detection:** Test performance was independent of rejection type, including antibody-mediated rejection (ABMR, 16 cases), T-cell mediated rejection (TCMR, 10 cases), and combinations of the two (ABMR/TCMR, 12 cases). By contrast, previous dd-cfDNA studies reported a poor ability to detect TCMR, which represents approximately one third of all AR diagnoses, and more than half of the AR cases in certain patient subgroups.[3]
- **Subclinical AR detection:** Test performance was also independent of clinical presentation, demonstrating high accuracy in detecting both clinical and subclinical AR. The study was unique in that 13 of the 38 AR cases were diagnosed using protocol (or surveillance) biopsies, in contrast to the other 25 cases diagnosed using for-cause (or clinically-indicated) biopsies. The 13 cases are considered "subclinical AR," because the patients otherwise had stable renal function based on serum creatinine, showing no clinical signs of rejection. In the study, Natera's assay detected the subclinical AR cases with 92% sensitivity and 75% specificity. No other dd-cfDNA assay has been validated to detect subclinical AR, which occurs in 20-25% of patients in the first two years post-transplant,[4] and which is considered a major driver of graft failure.

According to Paul Billings, M.D., Ph.D., Natera's Chief Medical Officer and Senior Vice President of Medical Affairs, Natera's dd-cfDNA-based assay is designed to help physicians detect active rejection events earlier, avoid unnecessary biopsies, and better optimize immunosuppression levels. "This published study adds to the mounting body of evidence showing the validity of dd-cfDNA in detecting active allograft rejection," Billings said.

"With this publication, we have achieved all of the company's 2018 milestones related to our transplant business, including the attainment of a Z-code, completion of analytical and clinical validation studies, and completion of the CLIA validation," said Steve Chapman, Natera's CEO. "This is in line with Natera's history of successful execution with regard to commercializing novel clinical assays."

There are more than 190,000 people living with a kidney transplant in the U.S.[5] and roughly 20,000 new kidney transplant surgeries performed each year.[6] It is estimated that 20-30 percent of organ transplants fail within five years and approximately 50 percent fail within 10 years.[7,8] The current tools for diagnosing organ transplant rejection are either invasive (biopsies) or inaccurate (serum creatinine), creating a strong unmet need for better diagnostic tools to improve patient management and outcomes.

About Natera's dd-cfDNA Organ Transplant Assay

Natera's organ transplant rejection assay is designed to detect active allograft rejection in patients who have undergone renal (kidney) transplantation. The assay works by measuring the fraction of donor-derived cell-free DNA (dd-cfDNA) in the recipient's blood, which can spike relative to background cfDNA when the transplanted organ is injured due to immune rejection. The assay leverages Natera's core single nucleotide polymorphism (SNP)-based massively multiplexed PCR (mmPCR) technology, to more accurately measure dd-cfDNA levels without the need for donor genotyping, and it has been clinically validated for test performance independent of donor type, rejection type, and clinical presentation.

About Natera

Natera (https://c212.net/c/link/?t=0&l=en&o=2339767-1&h=2651265320&u=http%3A%2F%2Fwww.natera.com%2F&a=Natera) is a global leader in cell-free DNA testing. The

mission of the company is to transform the management of diseases worldwide. Natera operates an ISO 13485-certified and CAP-accredited laboratory certified under the Clinical Laboratory Improvement Amendments (CLIA) in San Carlos, Calif. It offers a host of proprietary genetic testing services to inform physicians who care for pregnant women, researchers in cancer including bio pharmaceutical companies, and genetic laboratories through its cloud-based software platform. For more information, visit natera.com (https://c212.net/c/link/?t=0&l=en&o=2339767-1&h=3905460127&u=http%3A%2F%2Fwww.natera.com%2F&a=natera.com). Follow Natera on LinkedIn (https://c212.net/c/link/?t=0&l=en&o=2339767-1&h=1342219293&u=https%3A%2F%2Fwww.linkedin.com%2Fcompany%2Fnatera%2F&a=LinkedIn) and (https://c212.net/c/link/?t=0&l=en&o=2339767-1&h=1788793007&u=https%3A%2F%2Ftwitter.com%2FNateraGenetics&a=%C2%A0)Twitter (https://c212.net/c/link/?t=0&l=en&o=2339767-1&h=3981149194&u=https%3A%2F%2Ftwitter.com%2FNateraGenetics&a=Twitter).

Forward-Looking Statements

All statements other than statements of historical facts contained in this press release are forward-looking statements and are not a representation that Natera's plans, estimates, or expectations will be achieved. These forward-looking statements represent Natera's expectations as of the date of this press release, and Natera disclaims any obligation to update the forward-looking statements. These forward-looking statements are subject to known and unknown risks and uncertainties that may cause actual results to differ materially, including with respect to our efforts to develop and commercialize new product offerings, our ability to successfully increase demand for and grow revenues for our product offerings, whether the results of clinical studies will support the use of our product offerings, our expectations of the reliability, accuracy and performance of our tests, or of the benefits of our tests and product offerings to patients, providers and payers. Additional risks and uncertainties are discussed in greater detail in "Risk Factors" in Natera's recent filings on Forms 10-K and 10-Q and in other filings Natera makes with the SEC from time to time. These documents are available at www.natera.com/investors (https://c212.net/c/link/?t=0&l=en&o=2339767-1&h=3621891654&u=http%3A%2F%2Fwww.natera.com%2Finvestors&a=www.natera.com%2Finvestors) and (https://c212.net/c/link/?t=0&l=en&o=2339767-1&h=2760063341&u=https%3A%2F%2Fwww.sec.gov%2F&a=%C2%A0)www.sec.gov (https://c212.net/c/link/?t=0&l=en&o=2339767-1&h=1223606294&u=http%3A%2F%2Fwww.sec.gov%2F&a=www.sec.gov). (https://c212.net/c/link/?t=0&l=en&o=2339767-1&h=1826827854&u=https%3A%2F%2Fwww.sec.gov%2F&a=.)

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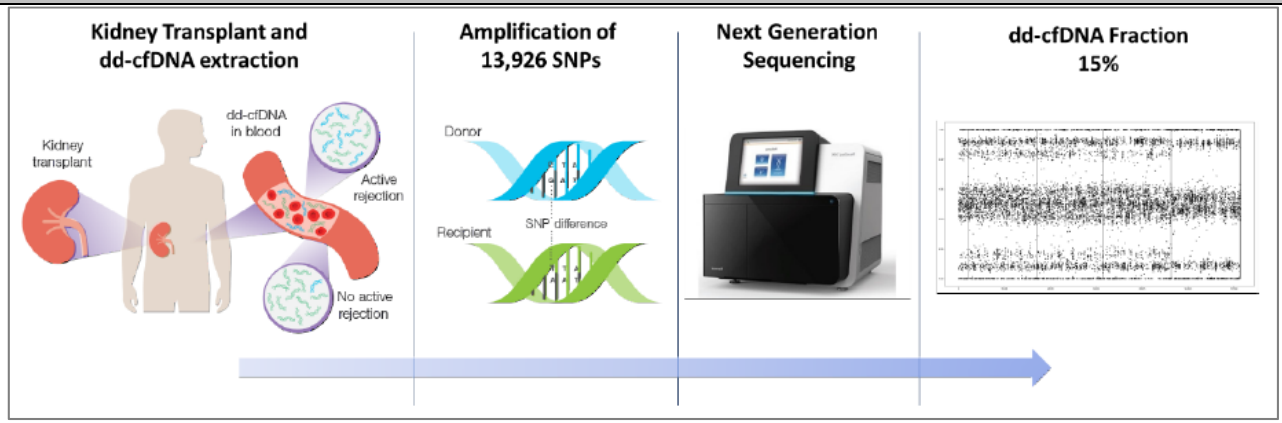
SOURCE Natera

EXHIBIT 7

US 9,845,497
Infringement Analysis re: Natera

'497 Claim Language	**Infringement Support**
A method of detecting donor-specific circulating cell-free nucleic acids in a solid organ transplant recipient, the method comprising:	To the extent that the preamble is considered a claim limitation, the ClinicalTrials.gov description of Natera's KidneyScan test states that it is intended to "measure dd [donor-derived] –cf [cell free] DNA in kidney transplant recipients for the detection of allograft injury and rejection." Donor-derived cell-free DNA (dd-cfDNA) detected in the blood of transplant recipients has been shown to be a non-invasive diagnostic marker for allograft injury/rejection. Natera, Inc. has recently developed a novel single nucleotide polymorphism (SNP)-based mmPCR NGS methodology to measure dd-cfDNA in kidney transplant recipients for the detection of allograft injury and rejection. As a growing leader in the diagnostic space, Natera has commissioned a randomized controlled trial to determine the clinical utility of its dd-cfDNA detection methodology for practicing nephrologists treating kidney allograft patients. This study is expected to fill a gap in the evidence base on the clinical utility of dd-cfDNA testing for allograft rejection. Ex. 4: *Utility of a Novel Dd-cfDNA Test to Detect Injury in Renal Post-Transplant Patients*, Natera KidneyScan, https://clinicaltrials.gov/ct2/show/NCT03765203?term=Natera&rank=2. Figure 1 of Natera's 2019 publication in the journal *Transplantation* illustrates Natera's Kidney Transplant Rejection Test methodology:

'497 Claim Language	Infringement Support
	 "Figure 1: Workflow of a clinical grade next generation sequencing assay. Donor-derived-cfDNA is released from renal allograft into circulation; blood is drawn and centrifuged, and plasma is isolated. cfDNA is extracted from plasma samples and used for library preparation followed by targeted PCR amplification of 13,926 SNPs, performed using mmPCR. Amplicons are sequenced on a next-generation sequencer, and sequencing data is analyzed using a maximum likelihood estimate method to give a dd-cfDNA fraction, which is reported to the physician." Ex. 10: Altug et al., *Analytical Validation of a Single-Nucleotide Polymorphism-Based Donor-Derived Cell-Free DNA Assay for Detecting Rejection in Kidney Transplant Patients*, TRANSPLANTATION (2019) (forthcoming).
(a) genotyping a solid organ transplant donor to obtain a single nucleotide polymorphism (SNP) profile of the solid organ transplant donor;	Natera's abstract from The Transplantation Society 27th International Congress states that evaluation of dd-cfDNA "requires differentiation of donor/recipient DNA by sequencing" and explains that Natera's method "measure[s] thousands of informative SNPs to assess dd-cfDNA with high accuracy in a selected cohort of kidney tx patients."

'497 Claim Language	Infringement Support
	Introduction: Plasma donor-derived cell-free DNA (dd-cfDNA) has been implicated as a noninvasive marker for transplant (tx) rejection. dd-cfDNA evaluation requires differentiation of donor/recipient DNA by sequencing; recent studies have amplified hundreds of target SNPs to detect active rejection in kidney allografts with 59.3% sensitivity and 84.7% specificity. We measure thousands of informative SNPs to assess dd-cfDNA with high accuracy in a selected cohort of kidney tx patients having contemporaneous tx biopsies scored for presence and type of Banff-graded T cell-/antibody-mediated–rejection (TCMR/ABMR) and borderline rejection (BL). Ex. 9: Sigdel et al., *Plasma Donor-Derived Cell-Free DNA Quantification by Massively Multiplex PCR Distinguishes Kidney Transplant Acute Rejection*, 102 TRANSPLANTATION (2018).
(b) genotyping a solid organ transplant recipient to obtain a SNP profile of the solid organ transplant recipient, wherein the solid organ transplant recipient is selected from the group consisting of: a kidney transplant, a heart transplant, a liver transplant, a pancreas transplant, a lung transplant, a skin transplant, and any combination thereof;	As described for subpart 1(a), the Natera abstract states that evaluation of dd-cfDNA "requires differentiation of donor/recipient DNA by sequencing" and explains that Natera's method "measure[s] thousands of informative SNPs to assess dd-cfDNA with high accuracy in a selected cohort of kidney tx patients." Introduction: Plasma donor-derived cell-free DNA (dd-cfDNA) has been implicated as a noninvasive marker for transplant (tx) rejection. dd-cfDNA evaluation requires differentiation of donor/recipient DNA by sequencing; recent studies have amplified hundreds of target SNPs to detect active rejection in kidney allografts with 59.3% sensitivity and 84.7% specificity. We measure thousands of informative SNPs to assess dd-cfDNA with high accuracy in a selected cohort of kidney tx patients having contemporaneous tx biopsies scored for presence and type of Banff-graded T cell-/antibody-mediated–rejection (TCMR/ABMR) and borderline rejection (BL). Ex. 9: Sigdel et al., *Plasma Donor-Derived Cell-Free DNA Quantification by Massively Multiplex PCR Distinguishes Kidney Transplant Acute Rejection*, 102 TRANSPLANTATION (2018). In Natera's method, for "each sample, DF was estimated based on the minor allele frequencies measured for all SNPs where the recipient was estimated to be homozygous." Ex. 10 Altug et al., *Analytical Validation of a Single-Nucleotide Polymorphism-Based Donor-Derived Cell-Free DNA*

'497 Claim Language	**Infringement Support**
	Assay for Detecting Rejection in Kidney Transplant Patients, TRANSPLANTATION (2019) (forthcoming).
(c) obtaining a biological sample from the solid organ transplant recipient after the solid organ transplant recipient has received the solid organ transplant from the solid organ transplant donor, wherein the biological sample is selected from the group consisting of blood, serum and plasma, and wherein the biological sample comprises circulating cell-free nucleic acids from the solid organ transplant; and	According to the Natera abstract, the Natera test detects "for the presence and type of Banff-graded T cell-/antibody-mediated-rejection (TCMR/ABMR) and borderline rejection (BL)" in a "selected cohort of kidney tx patients." Ex. 9: Sigdel et al., *Plasma Donor-Derived Cell-Free DNA Quantification by Massively Multiplex PCR Distinguishes Kidney Transplant Acute Rejection*, 102 TRANSPLANTATION (2018). This method is performed using "[p]lasma donor-derived cell-free DNA (dd-cfDNA)" as a "noninvasive marker for transplant (tx) rejection." In the Materials and Methods section, these samples are described as "plasma samples" from "187 unique patients." The test assesses dd-cfDNA. Introduction: Plasma donor-derived cell-free DNA (dd-cfDNA) has been implicated as a noninvasive marker for transplant (tx) rejection. dd-cfDNA evaluation requires differentiation of donor/recipient DNA by sequencing; recent studies have amplified hundreds of target SNPs to detect active rejection in kidney allografts with 59.3% sensitivity and 84.7% specificity. We measure thousands of informative SNPs to assess dd-cfDNA with high accuracy in a selected cohort of kidney tx patients having contemporaneous tx biopsies scored for presence and type of Banff-graded T cell-/antibody-mediated–rejection (TCMR/ABMR) and borderline rejection (BL). Materials and Methods: 292 unique plasma samples from 187 unique patients were categorized as stable (STA; n=73), acute rejection (AR; n=52), other injury (OI; n=85), or BL (n=82), and processed by massively Ex. 9: Sigdel et al., *Plasma Donor-Derived Cell-Free DNA Quantification by Massively Multiplex PCR Distinguishes Kidney Transplant Acute Rejection*, 102 TRANSPLANTATION (2018). In another study, Natera extracted plasma from whole blood samples taken from kidney transplant recipients (post-transplant).

'497 Claim Language	**Infringement Support**
	"Whole blood samples (20 mL) were collected from healthy volunteers (n=31) and transplant patients (n=6) in Cell-Free DNA BCT tubes . . . Plasma (5-10 mL) was isolated from blood after centrifugation at 3220 x g for 30 minutes at 22°C and stored at -80°C . . . Cell-free DNA (cfDNA) was extracted using either Natera's in-house spin column-based chemistry for extraction (San Carlos, CA) or QIAamp circulating nucleic acid kit (Qiagen, Germatown, MD) and were used as either blanks (n=15) for LoB or plasma mixture samples (n=16)." Ex. 10 Altug et al., *Analytical Validation of a Single-Nucleotide Polymorphism-Based Donor-Derived Cell-Free DNA Assay for Detecting Rejection in Kidney Transplant Patients*, TRANSPLANTATION (2019) (forthcoming).
(d) determining an amount of donor-specific circulating cell-free nucleic acids from the solid organ transplant in the biological sample by detecting a homozygous or a heterozygous SNP within the donor-specific circulating cell-free nucleic acids from the solid organ transplant in at least one assay, wherein the at least one assay comprises high throughput sequencing or digital polymerase chain reaction (dPCR), and	Using Natera's method, the samples are "processed by massively multiplex PCR," where the PCR was "targeting SNPs," such that analysis of the sequencing results is done using the polymorphism profile. Materials and Methods: 292 unique plasma samples from 187 unique patients were categorized as stable (STA; n=73), acute rejection (AR; n=52), other injury (OI; n=85), or BL (n=82), and processed by massively multiplex PCR targeting 13,392 SNPs. Cross-sectional samples were obtained from AR and OI patients (other causes of graft dysfunction were drug toxicity [n=18], acute tubular necrosis [n=2], BK nephritis [n=4], chronic allograft nephropathy [CAN; n=57], and tx glomerulpathy [n=3]). AR was scored by Banff for TCMR (n=95), ABMR (n=37), and BL. 41 patients contributed 3–4 samples each (146 samples total) over 12–24 months for longitudinal assessment. dd-cfDNA performance was evaluated by ROC with inclusion of eGFR in the prediction model. Calculations were determined using 95% confidence. Ex. 9: Sigdel et al., *Plasma Donor-Derived Cell-Free DNA Quantification by Massively Multiplex PCR Distinguishes Kidney Transplant Acute Rejection*, 102 TRANSPLANTATION (2018). The ClinicalTrials.gov site specifies that the tests are performed using "(SNP)-based mm [massively multiplexed] PCR NGS [Next Generation Sequencing] methodology," a high-throughput sequencing method.

'497 Claim Language	Infringement Support
	Donor-derived cell-free DNA (dd-cfDNA) detected in the blood of transplant recipients has been shown to be a non-invasive diagnostic marker for allograft injury/rejection. Natera, Inc. has recently developed a novel single nucleotide polymorphism (SNP)-based mmPCR NGS methodology to measure dd-cfDNA in kidney transplant recipients for the detection of allograft injury and rejection. As a growing leader in the diagnostic space, Natera has commissioned a randomized controlled trial to determine the clinical utility of its dd-cfDNA detection methodology for practicing nephrologists treating kidney allograft patients. This study is expected to fill a gap in the evidence base on the clinical utility of dd-cfDNA testing for allograft rejection. Ex. 4: *Utility of a Novel Dd-cfDNA Test to Detect Injury in Renal Post-Transplant Patients*, Natera KidneyScan, https://clinicaltrials.gov/ct2/show/NCT03765203?term=Natera&rank=2. Another study describes Natera's methodology as follows: "a novel single nucleotide polymorphism (SNP)-based massively multiplexed PCR (mmPCR) methodology to measure dd-cfDNA in various types of renal transplant recipients for the detection of allograft rejection/injury without prior knowledge of donor genotypes." Ex. 5: Sigdel et al., *Optimizing Detection of Kidney Transplant Injury by Assessment of Donor-Derived Cell-Free DNA via Massively Multiplex PCR*, 8 J. CLIN. MED. 1 (2019). In Natera's method, "donor genotypes were represented by a probability model that incorporated both population-based prior probabilities31 and the observed allele ratios. No heuristic adjustment was needed for related donors because the algorithm does not incorporate prior assumptions regarding the level of genotype concordance between the recipient and the donor." Ex. 10: Altug et al., *Analytical Validation of a Single-Nucleotide Polymorphism-Based Donor-Derived Cell-Free DNA Assay for Detecting Rejection in Kidney Transplant Patients*, TRANSPLANTATION (2019) (forthcoming).
wherein the at least one assay detects the donor-specific circulating cell-free nucleic acids from the solid	Figure 1 of a Natera abstract shows that % dd cf-DNA measurements are made at very low donor DNA fractions.

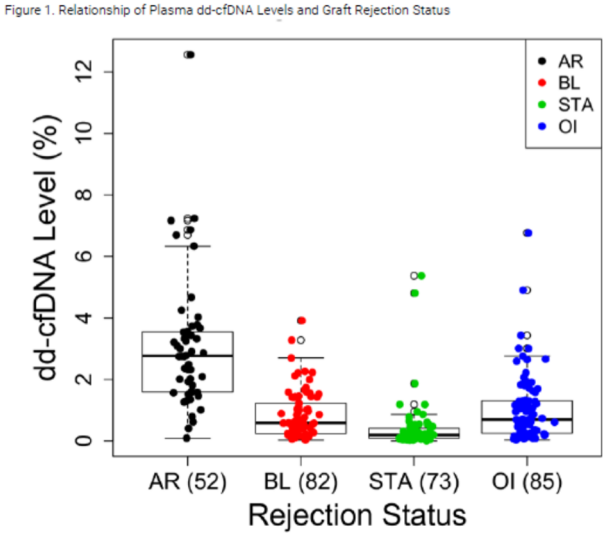
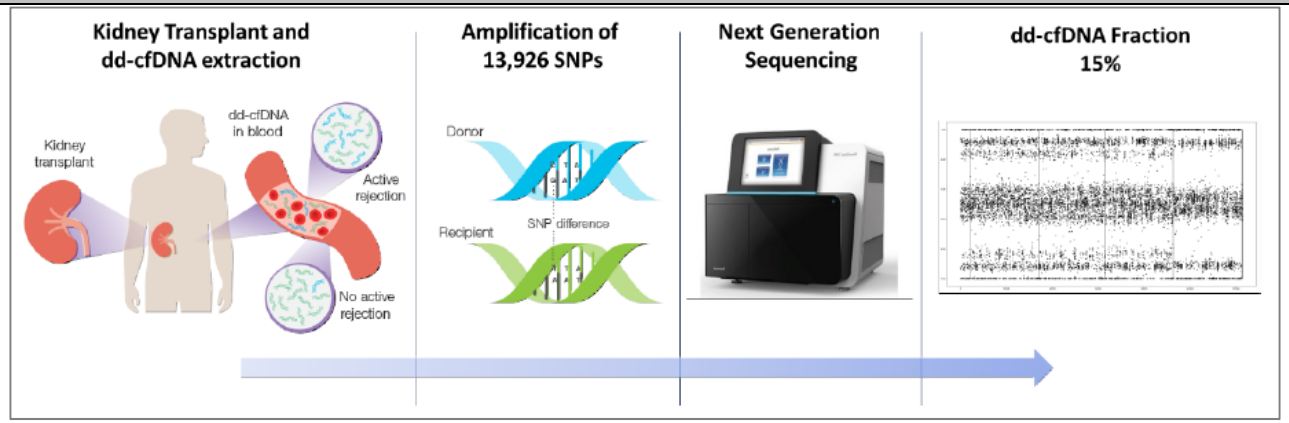
'497 Claim Language	**Infringement Support**
organ transplant when the donor-specific circulating cell-free nucleic acids make up at least 0.03% of the total circulating cell-free nucleic acids in the biological sample.	 Ex. 9: Sigdel et al., *Plasma Donor-Derived Cell-Free DNA Quantification by Massively Multiplex PCR Distinguishes Kidney Transplant Acute Rejection*, 102 TRANSPLANTATION (2018).

EXHIBIT 8

US 8,703,652
Infringement Analysis re: Natera

'652 Claim Language	**Infringement Support**
1. A method for detecting transplant rejection, graft dysfunction, or organ failure, the method comprising:	To the extent that the preamble is considered a claim limitation, the ClinicalTrials.gov description of Natera's KidneyScan test states that it is intended to "measure dd-cfDNA in kidney transplant recipients for the detection of allograft injury and rejection." Donor-derived cell-free DNA (dd-cfDNA) detected in the blood of transplant recipients has been shown to be a non-invasive diagnostic marker for allograft injury/rejection. Natera, Inc. has recently developed a novel single nucleotide polymorphism (SNP)-based mmPCR NGS methodology to measure dd-cfDNA in kidney transplant recipients for the detection of allograft injury and rejection. As a growing leader in the diagnostic space, Natera has commissioned a randomized controlled trial to determine the clinical utility of its dd-cfDNA detection methodology for practicing nephrologists treating kidney allograft patients. This study is expected to fill a gap in the evidence base on the clinical utility of dd-cfDNA testing for allograft rejection. Ex. 4: *Utility of a Novel Dd-cfDNA Test to Detect Injury in Renal Post-Transplant Patients*, Natera KidneyScan, https://clinicaltrials.gov/ct2/show/NCT03765203?term=Natera&rank=2. Figure 1 of Natera's 2019 publication in the journal *Transplantation* illustrates Natera's Kidney Transplant Rejection Test methodology:

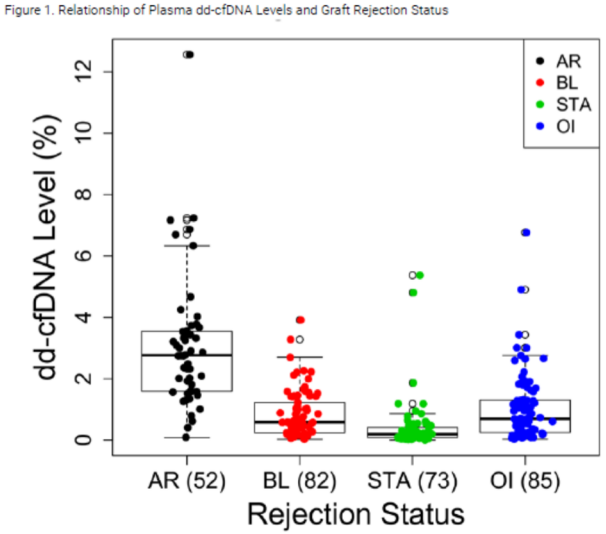
'652 Claim Language	Infringement Support
	 "Figure 1: Workflow of a clinical grade next generation sequencing assay. Donor-derived-cfDNA is released from renal allograft into circulation; blood is drawn and centrifuged, and plasma is isolated. cfDNA is extracted from plasma samples and used for library preparation followed by targeted PCR amplification of 13,926 SNPs, performed using mmPCR. Amplicons are sequenced on a next-generation sequencer, and sequencing data is analyzed using a maximum likelihood estimate method to give a dd-cfDNA fraction, which is reported to the physician." Ex. 10 Altug et al., *Analytical Validation of a Single-Nucleotide Polymorphism-Based Donor-Derived Cell-Free DNA Assay for Detecting Rejection in Kidney Transplant Patients*, TRANSPLANTATION (2019) (forthcoming).
(a) providing a sample comprising cell-free nucleic acids from a subject who has received a transplant from a donor;	According to Natera's abstract from The Transplantation Society 27th International Congress, the Natera test detects "for the presence and type of Banff-graded T cell-/antibody-mediated-rejection (TCMR/ABMR) and borderline rejection (BL)" in a "selected cohort of kidney tx patients." This method is performed using "[p]lasma donor-derived cell-free DNA (dd-cfDNA)" as a "noninvasive marker for transplant (tx) rejection." In the Materials and Methods section, these samples are described as "plasma samples" from "187 unique patients."

'652 Claim Language	Infringement Support
	Introduction: Plasma donor-derived cell-free DNA (dd-cfDNA) has been implicated as a noninvasive marker for transplant (tx) rejection. dd-cfDNA evaluation requires differentiation of donor/recipient DNA by sequencing; recent studies have amplified hundreds of target SNPs to detect active rejection in kidney allografts with 59.3% sensitivity and 84.7% specificity. We measure thousands of informative SNPs to assess dd-cfDNA with high accuracy in a selected cohort of kidney tx patients having contemporaneous tx biopsies scored for presence and type of Banff-graded T cell-/antibody-mediated–rejection (TCMR/ABMR) and borderline rejection (BL). Ex. 9: Sigdel et al., *Plasma Donor-Derived Cell-Free DNA Quantification by Massively Multiplex PCR Distinguishes Kidney Transplant Acute Rejection*, 102 TRANSPLANTATION (2018). In another study, Natera extracted plasma from whole blood samples taken from kidney transplant recipients (post-transplant). "Whole blood samples (20 mL) were collected from healthy volunteers (n=31) and transplant patients (n=6) in Cell-Free DNA BCT tubes . . . Plasma (5-10 mL) was isolated from blood after centrifugation at 3220 x g for 30 minutes at 22°C and stored at -80°C . . . Cell-free DNA (cfDNA) was extracted using either Natera's in-house spin column-based chemistry for extraction (San Carlos, CA) or QIAamp circulating nucleic acid kit (Qiagen, Germatown, MD) and were used as either blanks (n=15) for LoB or plasma mixture samples (n=16)." Ex. 10 Altug et al., *Analytical Validation of a Single-Nucleotide Polymorphism-Based Donor-Derived Cell-Free DNA Assay for Detecting Rejection in Kidney Transplant Patients*, TRANSPLANTATION (2019) (forthcoming).
(b) obtaining a genotype of donor-specific polymorphisms or a genotype of subject-specific polymorphisms, or	The Natera abstract states that evaluation of dd-cfDNA "requires differentiation of donor/recipient DNA by sequencing" and explains that Natera's method "measure[s] thousands of informative SNPs to assess dd-cfDNA with high accuracy in a selected cohort of kidney tx patients."

'652 Claim Language	Infringement Support
obtaining both a genotype of donor-specific polymorphisms and Subject-specific polymorphisms, to establish a polymorphism profile for detecting donor cell-free nucleic acids, wherein at least one single nucleotide polymorphism (SNP) is homozygous for the subject if the genotype comprises Subject-specific polymorphisms comprising SNPs;	> **Introduction**: Plasma donor-derived cell-free DNA (dd-cfDNA) has been implicated as a noninvasive marker for transplant (tx) rejection. dd-cfDNA evaluation requires differentiation of donor/recipient DNA by sequencing; recent studies have amplified hundreds of target SNPs to detect active rejection in kidney allografts with 59.3% sensitivity and 84.7% specificity. We measure thousands of informative SNPs to assess dd-cfDNA with high accuracy in a selected cohort of kidney tx patients having contemporaneous tx biopsies scored for presence and type of Banff-graded T cell-/antibody-mediated–rejection (TCMR/ABMR) and borderline rejection (BL). Ex. 9: Sigdel et al., *Plasma Donor-Derived Cell-Free DNA Quantification by Massively Multiplex PCR Distinguishes Kidney Transplant Acute Rejection*, 102 TRANSPLANTATION (2018). The ClinicalTrials.gov description confirms that Natera's KidneyScan test is "SNP-based." > Investigators will assess whether practicing nephrologists more effectively identify and manage patients with possible kidney allograft rejection when given access to Natera's novel SNP-based mmPCR-NGS test that measures dd-cfDNA, and, whether those behavioral changes improves patient management and optimizes resource utilization. Ex. 4: *Utility of a Novel Dd-cfDNA Test to Detect Injury in Renal Post-Transplant Patients*, Natera KidneyScan, https://clinicaltrials.gov/ct2/show/NCT03765203?term=Natera&rank=2. In Natera's method, for "each sample, DF was estimated based on the minor allele frequencies measured for all SNPs where the recipient was estimated to be homozygous." Additionally, "donor genotypes were represented by a probability model that incorporated both population-based prior probabilities31 and the observed allele ratios. No heuristic adjustment was needed for related donors because the algorithm does not incorporate prior assumptions regarding the level of genotype concordance between the recipient and the donor." Ex. 10: Altug et al., *Analytical Validation of a Single-Nucleotide Polymorphism-Based Donor-Derived Cell-Free DNA Assay for Detecting Rejection in Kidney Transplant Patients*, TRANSPLANTATION (2019) (forthcoming).

'652 Claim Language	**Infringement Support**
(c) multiplex sequencing of the cell-free nucleic acids in the sample followed by analysis of the sequencing results using the polymorphism profile to detect donor cell-free nucleic acids and subject cell-free nucleic acids; and	Using Natera's method, the samples are "processed by massively multiplex PCR," where the PCR was "targeting SNPs," such that analysis of the sequencing results is done using the polymorphism profile. **Materials and Methods**: 292 unique plasma samples from 187 unique patients were categorized as stable (STA; n=73), acute rejection (AR; n=52), other injury (OI; n=85), or BL (n=82), and processed by massively multiplex PCR targeting 13,392 SNPs. Cross-sectional samples were obtained from AR and OI patients (other causes of graft dysfunction were drug toxicity [n=18], acute tubular necrosis [n=2], BK nephritis [n=4], chronic allograft nephropathy [CAN; n=57], and tx glomerulpathy [n=3]). AR was scored by Banff for TCMR (n=95), ABMR (n=37), and BL. 41 patients contributed 3–4 samples each (146 samples total) over 12–24 months for longitudinal assessment. dd-cfDNA performance was evaluated by ROC with inclusion of eGFR in the prediction model. Calculations were determined using 95% confidence. Ex. 9: Sigdel et al., *Plasma Donor-Derived Cell-Free DNA Quantification by Massively Multiplex PCR Distinguishes Kidney Transplant Acute Rejection*, 102 TRANSPLANTATION (2018). Another study describes Natera's methodology as follows: "a novel single nucleotide polymorphism (SNP)-based massively multiplexed PCR (mmPCR) methodology to measure dd-cfDNA in various types of renal transplant recipients for the detection of allograft rejection/injury without prior knowledge of donor genotypes." Ex. 5: Sigdel et al., *Optimizing Detection of Kidney Transplant Injury by Assessment of Donor-Derived Cell-Free DNA via Massively Multiplex PCR*, 8 J. CLIN. MED. 1 (2019). In Natera's method, for "each sample, DF was estimated based on the minor allele frequencies measured for all SNPs where the recipient was estimated to be homozygous." Additionally, "donor genotypes were represented by a probability model that incorporated both population-based prior probabilities31 and the observed allele ratios. No heuristic adjustment was needed for related donors because the algorithm does not incorporate prior assumptions regarding the level of genotype concordance between the recipient and the donor." Ex. 10: Altug et al., *Analytical Validation of a Single-Nucleotide Polymorphism-Based Donor-Derived Cell-Free DNA Assay for Detecting Rejection in Kidney Transplant Patients*, TRANSPLANTATION (2019) (forthcoming).

'652 Claim Language	**Infringement Support**
(d) diagnosing, predicting, or monitoring a transplant status or outcome of the subject who has received the transplant by determining a quantity of the donor cell-free nucleic acids based on the detection of the donor cell free nucleic acids and subject cell-free nucleic acids by the multiplexed sequencing, wherein an increase in the quantity of the donor cell-free nucleic acids over time is indicative of transplant rejection, graft dysfunction or organ failure, and wherein sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV).	The Natera abstract describes that "41 patients contributed 3-4 samples each (146 samples total) over 12-24 months for longitudinal assessment," allowing for the measurement of the change in the quantity of donor cell-free nucleic acids over time. (n=95), ABMR (n=37), and BL. 41 patients contributed 3–4 samples each (146 samples total) over 12–24 months for longitudinal assessment. dd-cfDNA performance was evaluated by ROC with inclusion of eGFR in the prediction model. Calculations were determined using 95% confidence. Ex. 9: Sigdel et al., *Plasma Donor-Derived Cell-Free DNA Quantification by Massively Multiplex PCR Distinguishes Kidney Transplant Acute Rejection*, 102 TRANSPLANTATION (2018). Another study confirmed that Natera's "mmPCR assay may be used for routine monitoring, to determine whether a renal transplant patient is experiencing organ injury that may require a change in management." Ex. 5: Sigdel et al., *Optimizing Detection of Kidney Transplant Injury by Assessment of Donor-Derived Cell-Free DNA via Massively Multiplex PCR*, 8 J. CLIN. MED. 1 (2019). Figure 1 of a Natera abstract shows a graph of the quantified levels of donor-derived cfDNA according to rejection status of the patient.

'652 Claim Language	Infringement Support
	 Ex. 9: Sigdel et al., *Plasma Donor-Derived Cell-Free DNA Quantification by Massively Multiplex PCR Distinguishes Kidney Transplant Acute Rejection*, 102 TRANSPLANTATION (2018). Natera shows that their results are able to detect acute rejection (AR) with 91.8% sensitivity. The confidence interval for measures of sensitivity for the assays was between 92.7 and 100.

'652 Claim Language	Infringement Support
	Results: dd-cfDNA circulatory burden was significantly higher in AR (3.075±2.136%) compared to STA (0.428±0.851%; p<0.0001) and OI (1.051±1.112%; p<0.0001) (Figure 1). dd-cfDNA was also higher in Banff-confirmed AR over BL rejection (0.834 ±0.765%; p<0.0001), with no difference in burden observed for TCMR and ABMR (3.003 ±2.292% and 3.185 ±1.931%, respectively; p=0.5203). To compare dd-cfDNA to eGFR score, samples with available eGFR score were used (STA, n=7; AR, n=52). Using a cutoff of >1% dd-cfDNA, AR was detected with 91.8% sensitivity (CI 80.4–97.7) and 100% specificity (CI 59–100). Area under the curve (AUC) of 0.985 showed strong AR detection power of dd-cfDNA. Using a logistic regression integrating both dd-cfDNA and eGFR with a >50% probability cutoff, classification of samples was 100% accurate. Estimated CI for sensitivity and specificity were (92.7–100) and (59–100), respectively, with AUC of 1 (compared with AUC of 0.79 using eGFR alone). Ex. 9: Sigdel et al., *Plasma Donor-Derived Cell-Free DNA Quantification by Massively Multiplex PCR Distinguishes Kidney Transplant Acute Rejection*, 102 TRANSPLANTATION (2018). Another Natera study showed their methodology could detect acute rejection (AR) with 89% sensitivity. "The SNP-based dd-cfDNA assay discriminated active from non-rejection status with an area under the curve (AUC) of 0.87, 88.7% sensitivity (95% CI, 77.7–99.8%) and 72.6% specificity (95% CI, 65.4–79.8%) at a prespecified cutoff (>1% dd-cfDNA)." Ex. 6: Sigdel et al., *Optimizing Detection of Kidney Transplant Injury by Assessment of Donor-Derived Cell-Free DNA via Massively Multiplex PCR*, 8 J. CLIN. MED. 1 (2019).

EXHIBIT 9

Immune Monitoring (Videos Available)

Tuesday July 03, 2018 from 09:45 to 11:15

Room: N-106

420.1 Plasma donor-derived cell-free DNA quantification by massively multiplex PCR distinguishes kidney transplant acute rejection

Minnie M. Sarwal, United States
Professor of Surgery
Division of Transplant Surgery
University of California San Francisco (UCSF)

Abstract

Plasma Donor-Derived Cell-Free DNA Quantification by massively multiplex PCR Distinguishes Kidney Transplant Acute Rejection

Tara Sigdel[1], Felipe Archila[2], Samantha Navarro[2], Bernhard Zimmermann[2], Solomon Moshkevich[2], Minnie Sarwal[1].

[1]Dept of Surgery, University of California San Francisco, San Francisco, CA, United States; [2]Natera, Inc., San Carlos, CA, United States

Introduction: Plasma donor-derived cell-free DNA (dd-cfDNA) has been implicated as a noninvasive marker for transplant (tx) rejection. dd-cfDNA evaluation requires differentiation of donor/recipient DNA by sequencing; recent studies have amplified hundreds of target SNPs to detect active rejection in kidney allografts with 59.3% sensitivity and 84.7% specificity. We measure thousands of informative SNPs to assess dd-cfDNA with high accuracy in a selected cohort of kidney tx patients having contemporaneous tx biopsies scored for presence and type of Banff-graded T cell-/antibody-mediated–rejection (TCMR/ABMR) and borderline rejection (BL).

Materials and Methods: 292 unique plasma samples from 187 unique patients were categorized as stable (STA; n=73), acute rejection (AR; n=52), other injury (OI; n=85), or BL (n=82), and processed by massively

multiplex qPCR targeting 15,372 SNPs. Cross-sectional samples were obtained from AR and CI patients (other causes of graft dysfunction were drug toxicity [n=18], acute tubular necrosis [n=2], BK nephritis [n=4], chronic allograft nephropathy [CAN; n=57], and tx glomerulpathy [n=3]). AR was scored by Banff for TCMR (n=95), ABMR (n=37), and BL. 41 patients contributed 3–4 samples each (146 samples total) over 12–24 months for longitudinal assessment. dd-cfDNA performance was evaluated by ROC with inclusion of eGFR in the prediction model. Calculations were determined using 95% confidence.

Results: dd-cfDNA circulatory burden was significantly higher in AR (3.075±2.136%) compared to STA (0.428±0.851%; p<0.0001) and OI (1.051±1.112%; p<0.0001) (Figure 1). dd-cfDNA was also higher in Banff-confirmed AR over BL rejection (0.834 ±0.765%; p<0.0001), with no difference in burden observed for TCMR and ABMR (3.003 ±2.292% and 3.185 ±1.931%, respectively; p=0.5203).

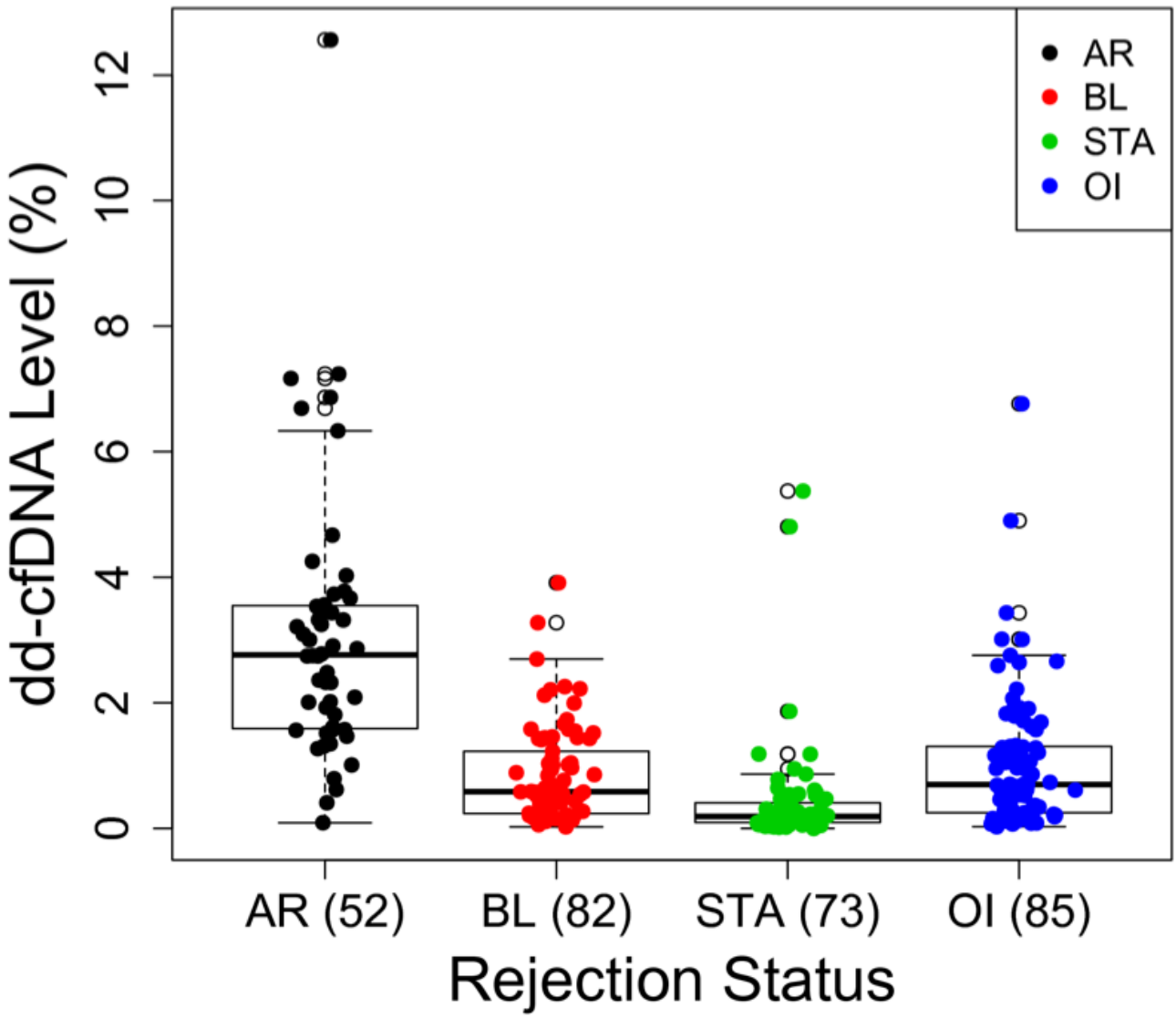
To compare dd-cfDNA to eGFR score, samples with available eGFR score were used (STA, n=7; AR, n=52). Using a cutoff of >1% dd-cfDNA, AR was detected with 91.8% sensitivity (CI 80.4–97.7) and 100% specificity (CI 59–100). Area under the curve (AUC) of 0.985 showed strong AR detection power of dd-cfDNA. Using a logistic regression integrating both dd-cfDNA and eGFR with a >50% probability cutoff, classification of samples was 100% accurate. Estimated CI for sensitivity and specificity were (92.7–100) and (59–100), respectively, with AUC of 1 (compared with AUC of 0.79 using eGFR alone).

Discussion: The novel SNP-based mmPCR assay enabled rapid detection of dd-cfDNA without need for sequencing or laborious analytics. Irrespective of rejection type, the assay observed a threshold for STA patients and an exponential increase in kidney injury burden in CAN and BL rejection with much greater burden in AR; taken together, these data suggest that combined dd-cfDNA and eGFR markers can accurately assess AR risk in kidney tx recipients.

Conclusion: This technology may provide a less invasive and more sensitive approach to monitoring the

health of kidney allografts.

Figure 1. Relationship of Plasma dd-cfDNA Levels and Graft Rejection Status



Natera, Inc..

Presentations by Minnie M. Sarwal

Mon-02 from 13:30 - 14:15 in room Retiro Room (located next to the exhibit room)

Lunch and Learn 1

Precision Transplant medicine: what's next?

Sat-30 from 16:45 - 18:45 in room N-104

Clinical Science: Precision Thinking (Videos Available)

Biomarkers of graft function & failure

Tue-03 from 09:45 - 11:15 in room N-106

Immune Monitoring (Videos Available)

Plasma donor-derived cell-free DNA quantification by massively multiplexed PCR distinguishes kidney transplant acute rejection

Tue-03 from 08:30 - 09:30 in room N-102

Immunosuppression 1 (Videos Available)

The results of the PRISM (prediction of rejection in sensitized patient blood samples) trial with a novel bioassay

27th International Congress of The Transplantation Society

Email: info@tts.guide

+1.514.874-1717

EXHIBIT 10

ACCEPTED – 2/1/2019 in Transplantation

Analytical Validation of a Single-Nucleotide Polymorphism-Based Donor-Derived Cell-Free DNA Assay for Detecting Rejection in Kidney Transplant Patients

Yücel Altuğ, PhD,* Nathan Liang, BA,* Rosalyn Ram, PhD,* Harini Ravi, PhD, Ebad Ahmed, PhD, Maxim Brevnov, PhD, Ryan Swenerton, PhD, Bernhard Zimmermann, PhD, Meenakshi Malhotra, PhD, Zachary P. Demko, PhD, Paul R. Billings, MD, PhD, Allison Ryan, PhD‡

**denotes equal contribution*

Natera, Inc., San Carlos, CA

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Authorship:

- **Conceptualization and study design:** YA, RR, RKS, NL, BZ, AR, EA, HR, MB, PB
- **Investigation and research performance:** YA, RR, RKS, NL, EA, HR
- **Data acquisition and data analysis/interpretation:** YA, RR, NL, AR, EA, HR
- **Statistical analysis:** YA, EA, AR
- **Resources and analytical tools:** YA, EA, PB
- **Manuscript drafting:** YA, RR, NL, AR, MM, ZD, HR
- **Manuscript revision and intellectual contribution:** YA, RR, RKS, NL, BZ, AR, MM, ZD, EA, HR, MB, PB
- **Supervision:** RKS, BZ, AR, ZD, HR, MB, PB

Disclosure: All authors are employees of Natera, Inc. with stock/options to own stock in the company

Funding: This study was funded by Natera, Inc.

Abbreviations

AR, active rejection

AUC, area under the curve

cfDNA, cell-free DNA

CLSI, clinical and laboratory standards institute

CI, confidence interval

CV, coefficient of variation

DNA, deoxyribonucleic acid

dd-cfDNA, Donor-derived cell-free DNA

ddPCR, digital droplet PCR

DF, dd-cfDNA fraction

eGFR, estimated glomerular filtration rate

gDNA, genomic DNA

IRB, Institutional review board

KT, Kidney transplant

LoB, limit of blank

LoD, limit of detection

LoQ, limit of quantitation

mmPCR, massively multiplexed-PCR

NGS, next generation sequencing

QC, quality check

SCr, serum creatinine

SNP, single-nucleotide polymorphism

Abstract

Background: Early detection of rejection in kidney transplant recipients holds the promise to improve clinical outcomes. Development and implementation of more accurate, non-invasive methods to detect allograft rejection remains an ongoing challenge. The limitations of existing allograft surveillance methods present an opportunity for donor-derived cell-free DNA (dd-cfDNA), which can accurately and rapidly differentiate patients with allograft rejection from patients with stable organ function.

Methods: This study evaluated the analytical performance of a massively multiplexed PCR (mmPCR) assay that targets 13,926 single nucleotide polymorphisms (SNPs), characterized and validated using 66 unique samples with 1064 replicates, including cell line-derived reference samples, plasma-derived mixtures, and transplant patient samples. The dd-cfDNA fraction was quantified in both related and unrelated donor-recipient pairs.

Results: The dd-cfDNA assay showed a limit of blank (LoB) of 0.11% and limit of detection/quantitation (LoD/LoQ) of 0.15% for unrelated donors, and LoB of 0.23%, and LoD/LoQ of 0.29% for related donors. All other metrics (linearity, accuracy and precision) were observed to be equivalent between unrelated and related donors. The measurement precision of coefficient of variation was 1.8% (repeatability, 0.6% dd-cfDNA) and was less than 5% for all of the different reproducibility measures.

Conclusions: This study validates the performance of a SNP-based mmPCR assay to detect the dd-cfDNA fraction with improved precision over currently available tests, regardless of donor-recipient relationships.

1.0 Introduction

Kidney transplantation is the best option for patients with end-stage renal disease.[1] According to United Network for Organ Sharing, more than 19,000 kidneys were transplanted in the United States in 2016 and approximately 200,000 patients are living with a functional kidney transplant (KT).[1] Despite life-long immunosuppressive maintenance regimens designed to optimize the therapeutic outcome,[2] approximately 20-30% of patients experience overall renal graft failure within the first 5 years,[3] and only 55% of transplanted kidneys survive to 10 years.[4,5] Thus, a compelling need exists for new strategies to avoid or minimize acute/subclinical rejection episodes, nephrotoxicity, other co-morbidities, and otherwise improve clinical outcomes.[6] Optimal implementation of new methods would require a simple, accurate way to monitor allograft health, allowing early detection of treatable pathology and with the goal of preventing graft loss by optimizing immunosuppressive regimens. Current clinical options to monitor allograft rejection in transplant recipients, most notably biopsies and assessing dynamic changes in serum creatinine (SCr), have significant drawbacks.

Biopsy with detailed pathology is the “gold-standard” for the diagnosis of active rejection (AR). Although some centers recommend asymptomatic surveillance “protocol” biopsies, their clinical utility is significantly limited due to invasiveness, cost, inadequate sampling, and poor reproducibility.[7-11] “For-cause” biopsies, typically ordered in response to changes in clinical symptoms and declining renal function, e.g. rising SCr and proteinuria, share similar limitations, and are often performed only after substantial allograft injury.[12] Subclinical rejection without significant changes in renal function or

proteinuria is predicted by previous active events and rising donor specific antibody titers but requires biopsy for confirmation.[13]

SCr levels are commonly used to screen patients for AR and indicate when biopsy and histological evaluation of renal tissue is warranted.[8,14] Although easy to measure, SCr is a poor marker due to its low sensitivity and specificity. Furthermore, it is a lagging indicator of renal injury;[15] by the time SCr levels increase, the allograft may have undergone severe and irreversible damage.[6,16] Thus, there is a need for a simple, non-invasive, highly accurate assay that can detect ongoing AR.

Donor-derived cell-free DNA (dd-cfDNA) found in the plasma of transplant patients is a proven non-invasive biomarker for KT rejection.[2,9,14,17-19] dd-cfDNA has also been utilized in assessing graft function in other organ transplants (liver, heart, lung and bone marrow).[2,20-26] We have previously demonstrated accurate quantification of cfDNA mixture proportions using a single-nucleotide polymorphism (SNP)-based massively multiplexed-PCR (mmPCR) methodology in the prenatal and oncology contexts.[27-30] Leveraging this technology, we have developed a non-invasive assay that estimates dd-cfDNA fraction (DF) in KT recipients by measuring the allele frequency at 13,926 SNPs chosen to maximize informative genotypes across ethnicities. A recent clinical validation study demonstrated the ability of this method to discriminate AR from non-rejection with a sensitivity of 88.7%, specificity of 73.2%, and area under the curve (AUC) of 0.87 using a DF cut-off of 1%.[14] In the current study, we analytically validate our clinical-grade next generation sequencing (NGS) assay by determining the limit of blank (LoB), limit of detection (LoD), limit of quantitation (LoQ), linearity, precision (reproducibility

and repeatability) and accuracy in measuring the DF in KT recipients in both related and unrelated donors.

2.0 Materials and Methods

2.1 Samples

2.1.1 Plasma mixture samples

Whole blood samples (20 mL) were collected from healthy volunteers (n=31) and transplant patients (n=6) in Cell-Free DNA BCT tubes (Streck, Omaha, NE) in accordance with the institutional review board (IRB)–approved protocol (Ethical and Independent IRB, Corte Madera, CA; approval number: IRB00007807, protocol number: 18-141), and the declaration of Helsinki. All participants provided signed informed consents. Plasma (5-10 mL) was isolated from blood after centrifugation at 3220 x *g* for 30 minutes at 22°C and stored at -80°C. Cell-free DNA (cfDNA) was extracted using either Natera's in-house spin column-based chemistry for extraction (San Carlos, CA) or QIAamp circulating nucleic acid kit (Qiagen, Germatown, MD) and were used as either blanks (n=15) for LoB or plasma mixture samples (n=16). Plasma mixture samples were developed from 3 unrelated (1 male designated donor and 3 female designated recipient, Stem Express, Folsom, CA) and 6 related (3 mother-son pairs, 2 brother-sister pairs, and 1 uncle-niece pair) binary mixture samples. cfDNA concentration of plasma mixture samples was quantified using Quant-iT or Qubit dsDNA kits (ThermoFisher, Carlsbad, CA).

2.1.2 Reference samples (cell-line derived)

Reference samples were procured from SeraCare Life Sciences (Milford, MA) and were developed by mixing genomic DNA (gDNA) from 5 different cell lines to generate 3 binary female (recipient)/male (donor) reference mixtures; 1 related and 2 unrelated, at specific targeted DFs (0%, 0.1%, 0.3%, 0.6%, 1.2%, 2.4%, 5%, 10%, and 15%). The DF in each reference mixture was verified by digital droplet PCR (ddPCR) by SeraCare. The gDNA mixtures were sheared by sonication and size selected to mimic expected cfDNA fragments of 160 base pairs by SeraCare. cfDNA concentration of the reference samples was quantified using Quant-iT or Qubit dsDNA kits (ThermoFisher, Carlsbad, CA).

2.2 Targeted amplification, SNP Selection, Sequencing Data Analysis and Quality Control

All samples were used as input for library preparation followed by targeted PCR amplification.[27] Targeted amplification was achieved by performing mmPCR as previously described, with a modification to the primer pool, which targeted 13,926 SNP positions (**Figure 1**).[29] Bi-allelic SNPs were selected on chromosomes 2, 13, 18, 21, 22 and X although only chromosomes 2, 13, 18 and 21 were included in the DF analysis. To ensure accurate DF estimate regardless of patient ethnicity, SNPs were required to have high minor allele frequency across the major ethnic groups as defined in the 1000 Genomes project.[31]

The PCR amplicons generated after targeted amplification were barcoded and combined to generate 32-plex pools, which were sequenced using NGS technology (Illumina NextSeq 500 instrument, 50 cycles, single end reads). Approximately 940 DNA copies were sequenced per locus. Sequenced reads were demultiplexed and mapped to the hg19 reference genome using Novoalign version 2.3.4 (*http://www.novocraft.com/products/novoalign/*). Bases with Phred quality score <30 and reads with mapping quality score <30 were filtered. Multiple quality checks (QCs) (cluster density, mapping rate, etc.) were applied to the sequencing run and each sample was confirmed to have the desired number of reads (8 million) after filtering. Any pool failing sequencing run QCs was re-sequenced. Any sample that failed to produce the necessary number of reads was removed from the analysis.

2.3 dd-cfDNA Fraction Calculation

For each sample, DF was estimated based on the minor allele frequencies measured for all SNPs where the recipient was estimated to be homozygous. The DF calculation is a maximum likelihood estimate over a search range from 0.01% to 25% at increments of 0.01%. While the technology places no upper limit on the dynamic range of the assay, 25% was chosen for this study based on the DF ranges observed in KT patients. Our approach did not include a separate donor sample, and donor genotypes were represented by a probability model that incorporated both population-based prior probabilities[31] and the observed allele ratios. No heuristic adjustment was needed for related donors because the algorithm does not incorporate prior assumptions regarding the level of genotype concordance between the recipient and the donor. Instead, the

corresponding genotype inheritance constraints were incorporated into the donor genotype probability model when the donor and recipient were related. This estimate mode was referred as "related estimate" and the unconstrained estimate was referred as "unrelated estimate".

2.4 Experimental Plan and Statistical Analysis

To evaluate analytical performance of the test, LoB, LoD, LoQ, linearity, precision, and accuracy were measured based on Clinical and Laboratory Standards Institute (CLSI) guidelines (EP-17A2, EP05-A3).[32,33] Experimental design with sample type, input mass, DFs, and number of measurements for each study is listed in **Table 1**. All samples were tested with a minimum input amount of 15 ng, and run in triplicates, except for clinical samples, which were tested in duplicates (***Supplementary Section I: Table S1***). Statistical analysis was performed using Python programming language (Python Software Foundation, version 3.6, *https://www.python.org/*).

2.4.1 Limit of Blank

LoB was established using reference samples (blanks or single genome), obtained from SeraCare (n=5), and plasma samples (n=15) collected from healthy blood donors with no history of organ transplant or recent blood transfusion **(Table 1)**. Reference samples were prepared at different targeted library input amounts to mimic the expected range of cfDNA yields achieved from 20 mL blood collections. Plasma samples were used at their unadjusted concentrations to reflect the variable cfDNA yields typical from real samples. In compliance with CLSI guidelines (EP-17A2)[32], samples were tested in

triplicate on 3 different days with 2 different sequencing reagent lots that consisted of at least 60 measurements per lot.

LoB is defined as the empirical 95th percentile value measured from a set of blank (no-analyte) samples. The calculation is performed for the reference samples and plasma samples from each reagent lot (lots 1 and 2), and for each DF estimation method, i.e., unrelated and related. For each estimation method, the final LoB was the maximum of the two per-lot results.

2.4.2 Limit of Detection and Limit of Quantitation

LoD and LoQ were measured using both reference samples and plasma mixture samples from healthy volunteers at different cfDNA input amounts. LoD was measured at the three lowest DFs by 2 operators on different days using different reagents lots and sequencing instruments. LoQ analysis was performed on the same samples as LoD with additional replicates at higher DFs (**Table 1**).

LoD was calculated following the parametric estimate method specified in EP-17A2,[32] which computes LoD by adding a standard deviation term to the LoB. LoDs for reference samples and plasma mixture samples for each reagent lot were calculated for each DF estimation method by combining the corresponding LoBs and standard deviation measurements. Similar to LoB, for each estimation method, the final LoD value was calculated using the maximum value of lot 1 and 2, calculated with the corresponding method. Furthermore, LoDs were also calculated separately for each DF estimation method for plasma mixture samples and reference samples, at each input amount.

An appropriate LoQ assessment was selected based on the quantification requirements of the test process. LoQ is defined as the lowest DF at which a sufficient relative measurement precision is achieved, lower bounded by the LoD. We defined sufficient relative measurement precision as 20% coefficient of variation (CV). The relationship between DF and its CV was modeled as CV = a + b*exp(-c*DF), where the model parameters a, b and c are estimated from the data using a non-linear least squares procedure. The CV model (described by parameters a, b, c) was estimated for each DF estimate method, and used to evaluate the LoQ criterion above. This model-based approach requires inclusion of higher DF measurements for the LoQ assessment in order to ensure convergence to an appropriate constant value at high DF. In line with the aforementioned LoD calculation, LoQs for reference samples and plasma mixture samples were calculated for each reagent lot and DF estimation method. The final LoQ value was calculated from the maximum of the values of lot 1 and 2, calculated with the corresponding method. LoQs were also calculated for each DF estimation method separately for plasma mixture samples and for reference samples at each input amount.

2.4.3 Linearity and Accuracy

Linearity and accuracy were measured using the same sample set as described for LoQ, with the accuracy measurement restricted to reference samples only. Linearity was evaluated based on the R^2 value produced by a standard linear regression analysis of the relationship between measured DF and targeted mixture fractions for each DF estimation method. Linearity was evaluated for both reference and plasma mixture samples separately for each DF estimation method. Accuracy was evaluated based on

the linear regression analysis of the relationship between measured DF and the orthogonal ddPCR measurement for each DF estimation method.

2.4.4 Precision

Precision was measured by testing reproducibility (inter-run) and repeatability (intra-run) across reference and transplant samples. Matched blood draws (4 tubes per patient) from transplant recipients were run in duplicates and were evaluated for reproducibility in clinical samples. Reproducibility samples were processed by 2 different operators on 8 different days (24 runs across 23 days) with 3 reagent lots, and 17 sequencing instruments. Repeatability was determined by measuring variability between technical replicates of samples measured under similar conditions. One related (mother-son) reference mixture at 2 DFs was assayed by a single operator, reagent lot, and instrument.

Repeatability, defined as the CV measured across the set of replicates at a single targeted DF, under matched conditions was calculated once at 0.6% and once at 2.4% DF. Reproducibility was also measured using CV, calculated separately for each combination of DNA input amount and mixture fraction.

3.0 Results

3.1 Limit of Blank

LoB was calculated using 64 measurements from lot 1 and 2 each. The LoB was found to be 0.11% for the unrelated donor estimate and 0.23% for the related donor estimate. Evaluation of plasma sample measurements only (60 measurements total, combined across both lots) resulted in LoB of 0.04% (unrelated) and 0.08% (related), suggesting a significantly lower LoB in plasma samples when compared with reference samples. **Figure 2** shows histograms of the relevant DF measurements broken down by method and lot (***Supplementary Section II: Figure S1, Table S2 and Table S3).***

3.2 Limit of Detection and Limit of Quantitation

LoD was calculated from 168 and 220 measurements from unrelated and related samples, respectively, resulting in LoD of 0.15% (unrelated) and 0.29% (related). One sample was excluded from the analysis due to failed QC. The difference in LoD for related versus unrelated donors was approximately equal to the difference in corresponding LoB, meaning that the measurement variance near the LoD was approximately the same in the two methods. The LoD was not significantly different at the different DNA input amounts (***Supplementary Section III: Figure S2, Figure S3 and Table S4***). Restricting the measurements to plasma-mixture samples yielded lower estimated LoD: 0.05% (unrelated) and 0.11% (related), although the number of measurements performed was less (54, related and 60, unrelated) than suggested by the guidelines (***Supplementary Section III: Table S5***).

LoQ was calculated from 381 and 412 measurements from unrelated and related samples, respectively, after exclusion of 5 samples that failed QC. Upper LoQ is the

largest DF tested, which is 15%. The empirical CVs were all found to be less than 20% at each targeted DF, including reference and plasma mixture samples. Empirical CVs and the resulting parametric models are shown in **Figure 3**. The modeled CVs were also found to be less than 20% for all DFs greater than or equal to the LoD. Thus, the LoQ is equal to the LoD for all relevant scenarios. This was observed to be true when the analysis was restricted to plasma mixture samples only, as well as to reference samples only, at each input amount (***Supplementary Section IV: Figure S4, Figure S5 and Table S6***). For ease of reference, **Table 2** summarizes the results of LoB, LoD and LoQ.

3.3 Linearity, Accuracy and Precision

Linearity was measured from 381 unrelated and 412 related samples, after removal of 5 samples that failed QC. Accuracy was measured from the subset of these (reference samples) for which ddPCR-measured DF was available as a reference: 285 unrelated and 349 related samples, excluding 4 that failed QC. Linearity was also evaluated for 6 clinical transplant patient samples using 12 measurements, all of which passed QC. The individual measurements and linear regression lines are shown in **Figure 4** (linearity) and **Figure 5** (accuracy). **Figure 6** shows the measured DFs from Lot 2 plotted against those from Lot 1, and the linear regression line for clinical transplant patient samples.

Linearity was measured by linear regression against the targeted DF and accuracy was measured by linear regression against the ddPCR-measured DF. Linearity for clinical transplant patient samples was measured by a linear regression of the measured DF

from Lot 2 plotted against those from Lot 1. The linear regression results are shown in **Table 3**. The DF measurement was observed to be highly linear (R^2 greater than 0.99 in all models) and accurate (slope approximately 1, intercept approximately zero) with no significant difference between related and unrelated donors. Linearity and accuracy analyses restricted to plasma mixture and reference samples only are provided in the supplementary material (***Supplementary Section V: Figure S6, Figure S7 and Table S7; Section VI: Figure S8 and Table S8***).

Precision was estimated by evaluating CV in two scenarios: repeatability within a single set of conditions, and reproducibility across a varied set of conditions. CV calculations combined samples with related and unrelated donors. Repeatability was measured at two targeted DFs (0.6% and 2.4%), each using 64 reference sample measurements with all samples passing QC.

Per-input reproducibility was calculated by using 498 measurements, excluding 6 samples that failed QC. Per-lot reproducibility was calculated from a subset of the aforementioned samples, whose cardinality was 374, excluding 4 samples that failed QC. Reproducibility of DF in clinical transplant patient samples was calculated using the aforementioned 12 measurements. The estimated CVs, along with 95% confidence interval (CI) are provided in **Table 4**. Finally, for clinical transplant patient samples, 100% concordance of clinical calls was observed (95% CI: 54.07% - 100%) between replicates. *(**Supplementary Section VII: Figure S9**)*.

4.0 Discussion

Early detection of rejection in KT recipients holds promise for improved outcomes, but this goal remains unmet due to the unavailability of accurate, non-invasive methods to detect allograft rejection before substantial injury has occurred. Given the limitations associated with current allograft monitoring practices, most notably SCr and biopsy, there exists an opportunity to develop better tools for early detection of allograft rejection.

Several studies have shown the clinical relevance of non-invasive dd-cfDNA assays, which give a quantitative assessment of the likelihood of AR in recipients of KT.[14,18] In 2017, Bloom et al. using a different targeted NGS approach, correlated plasma DF and rejection status in 107 biopsy-matched specimens, demonstrating a significant difference in median DF between cases with AR (1.6%) and non-rejection (0.3%), (P<0.001) using a predefined cut-off of >1%, with sensitivity and specificity of 59% and 85%, respectively.[18] A more recent study, Sigdel et al., evaluated DF in 217 biopsy-matched plasma samples and showed the superior performance of dd-cfDNA in differentiating AR (including subclinical rejection) from non-rejection (including stable, borderline and other injury cases) compared with estimated glomerular filtration rate (eGFR), with a sensitivity of 88.7% vs. 67.7%, specificity of 72.6% vs. 65.3% and AUCs of 0.87 vs. 0.74, respectively.[14] The study showed similar performance of the dd-cfDNA assay in protocol and for-cause biopsies, as well as the ability to detect both antibody-mediated and T cell-mediated rejection.[14] The assay did not need prior genotype information and was robust to different donor-recipient relationships.

The current study addresses the analytical validity of the DF quantification method used in Sigdel et al. Patients with DF of 1% or more are classified as “at increased risk of organ rejection”;[14] analytical performance should be interpreted in the context of accurately classifying a sample with respect to that threshold. In this study, LoD and LoQ were shown to be 0.15% for unrelated donors and 0.29% for related donors, indicating an ability to accurately quantify DF at a level significantly lower than the classified threshold. When analysis was restricted to plasma mixture samples, which are more reflective of clinical samples, the LoD and LoQ were observed to be significantly lower (0.05% LoD and LoQ for unrelated donors). This difference can be partly attributed to the significantly higher per-base insert error rate in reference samples compared with plasma samples in the LoB calculation (0.001355 versus 0.001170, $P<0.0001$, independent t-test). Contrived reference samples are commonly substituted for plasma samples in analytical testing and this difference in error rate is negligible in evaluations other than LoB. Analytical performance of a different SNP-based assay[2] for measuring DF in KT recipients reported similar LoD and LoQ (0.15%, and 0.2%), respectively but did not report any distinction between reference and plasma samples although they were both evaluated.

The current method was also confirmed to have high accuracy based on linear regression analysis, comparing measurements on more than 600 samples to an orthogonal DF measurement, ddPCR. Performance was evaluated with respect to a range of DNA input masses, selected to represent the expected distribution of cfDNA yields achieved from the clinical protocol-specified 20 mL blood collections. No detectable performance difference was observed at different DNA input levels. Precision

studies showed that the DF measurement was stable across intra-run and inter-run replicates, across multiple lots of critical reagents, and between repeat (concurrent) blood draws from the same patient. This indicates that the test is appropriate for large-scale implementation in a clinical lab setting.

This assay achieved superior measurement precision close to the classification range, as compared to a previously published assay,[2] with a CV of 1.85% vs. 9.2% (within run, at 0.6% DF and a CV of 1.99% (across runs, at 45 ng input) vs. 4.5% (across runs, at 60 ng input). The approximately 5-fold difference in CV measured within-run at a DF relatively close to the classification threshold indicates this assay has improved precision; taken in combination with the higher AUC demonstrated previously,[14] this suggests that the higher precision may have a positive impact on clinical accuracy. Several factors may account for this improvement in performance. The library preparation step reduces variability caused by the long DNA fragments. Also, the very large number of targeted SNPs (13,926) and the probability model for donor genotypes enable an accurate DF estimation independent of the degree of relationship between the donor and recipient. This is important due to the concern that the higher rate of genotype concordance (implying a lower rate of informative genotypes) in a related donor scenario might limit the accuracy of DF estimates. This study tested a large number of mixture sample replicates from mother-child and other related donor pairings and showed a LoB that was higher in related donor pairs, which led to correspondingly higher LoD, though these limits were still substantially below the assay's clinical threshold. All of the other metrics including linearity, accuracy and the various precision metrics were equivalent between related and unrelated donor pairs, showing that the

quantitative performance of the test is not meaningfully impacted by the reduced number of informative genotypes. The previously published method addressed this concern through in-silico estimates but did not confirm test performance on reference samples or plasma mixture samples from related individuals.[2]

Several other methods have been used to measure dd-cfDNA levels in transplant recipients. Beck et al. described a fast, inexpensive ddPCR method and demonstrated its use in heart, liver and kidney transplant patients; the CV was shown to range from 4% to 14%, and precision was not measured below 2%, which is above the cut off used in the current study.[20] DeVlaminck et al. described a shotgun NGS method, which was shown to detect mild and moderate-to-severe rejection events with an AUC of 0.75; the method required prior genotyping of the donor and recipient prior to transplant.[21,24] Sharon et al. recently described another shotgun NGS method that overcomes the need for prior genotyping of the donor, though not the recipient, that estimated dd-cfDNA in both related and unrelated donor-recipients.[25] None of these methods have been validated for clinical use. The SNP-based mmPCR assay described in the current study does not require prior genotyping of either donor or recipient and detects dd-cfDNA fraction with high precision (a CV of 2%), irrespective of the donor-recipient relationship. Though this study has only validated the assay for use in kidney transplant, we expect that it will show clinical value in other organ transplants such as heart, liver and bone marrow.

5.0 Conclusions

With an unacceptably high rate of allograft rejection in KT recipients, a paradigm-shift in the management of renal allograft health is overdue. Routine measurement of dd-cfDNA, with its ability to detect rejection early and non-invasively, represents a key strategy to improve clinical outcomes. Ongoing registry studies seek to demonstrate the efficacy of dd-cfDNA to detect allograft rejection, and its corresponding utility for optimizing biopsy use and immunosuppressive regimens and improving graft survival rates. This study demonstrates the analytical validity of an accurate, non-invasive SNP-based dd-cfDNA assay. Taken alongside the clinical validity of this assay, demonstrated by Sigdel et al.,[14] this heralds a new diagnostic tool for nephrologists with the promise of better patient management and outcomes.

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Tables

Table 1: Experimental design

Performance Metric	Sample Type	Input mass (ng)	Sample mixtures	dd-cfDNA fractions (%)	Number of Measurements	Total Measurements
LoB	Reference (n=5 blanks)	15, 30, 45	N/A	N/A	68	128
	Plasma (n=15)	Variable	N/A	N/A	60	
LoD	Reference	15, 30, 45	1: Related	0.1, 0.3, 0.6	166	389
			2: Unrelated		108	
	Plasma mixtures (n=16)	15	3: Unrelated	0.1, 0.3, 0.6	60	
		Variable	6: Related		55	
LoQ, Linearity	Reference	15, 30, 45	1: Related	0.1, 0.3, 0.6, 1.2, 2.4, 5, 10, 15	350	798
			2: Unrelated		288	
	Plasma mixtures (n=16)	Variable	6: Related	0.1, 0.3, 0.6, 1.2	64	
		15	3: Unrelated	0.1, 0.3, 0.6, 1.2, 2.4, 5, 10	96	
Accuracy	Reference	15, 30, 45	1: Related	0.1, 0.3, 0.6, 1.2,	350	638

			2: Unrelated	2.4, 5, 10, 15	288	
Reproducibility	Reference	15, 30, 45	1: Related	0.1, 0.3, 0.6, 1.2, 2.4, 5, 10	336	516
			2: Unrelated		168	
	Transplant Patient (n=6)	Variable	4: Related	Variable	12	
			2: Unrelated			
Repeatability	Reference	30	1: Related	0.6, 2.4	128	128

LoB, limit of blank; LoD, limit of detection; LoQ, limit of quantitation; N/A, Not applicable

Table 2. LoB, LoD, and LoQ values for each estimation method and various sample types

Performance Metric, Estimation Method	Combined Samples[a] (%)	Plasma and/or Plasma Mixture Samples Only (%)
LoB, Related	0.23	0.08
LoB, Unrelated	0.11	0.04
LoD, Related	0.29	0.11
LoD, Unrelated	0.15	0.05
LoQ, Related	0.29	0.11

LoQ, Unrelated	0.15	0.05

LoB, limit of blank; LoD, limit of detection; LoQ, limit of quantitation

[a]Combined samples include reference and plasma samples for LoB calculation; reference and plasma mixture samples for LoD and LoQ calculation

Table 3: Linear regression results for linearity and accuracy, including 95% confidence intervals

Performance Metric, Data set	Linearity and Accuracy Parameters		
	Slope	**Intercept**	**R^2**
Accuracy, Combined	1.0591 (0.9763, 1.1418)	0.0001 (-0.0045, 0.0047)	0.9988 (0.9987, 0.9990)
Accuracy, Related	1.0333 (0.9241, 1.1425)	-0.0001 (-0.0047, 0.0046)	0.9989 (0.9986, 0.9990)
Accuracy, Unrelated	1.0664 (0.9416, 1.1912)	0.0008 (-0.0076, 0.0092)	0.9997 (0.9997, 0.9998)
Linearity, Combined	1.0516 (0.9781, 1.1251)	0.0004 (-0.0033, 0.0042)	0.9968 (0.9964, 0.9972)
Linearity, Related	0.9852 (0.8895, 1.0809)	0.0008 (-0.0031, 0.0047)	0.9991 (0.9989, 0.9992)
Linearity, Unrelated	1.0813 (0.9721, 1.1906)	0.0006 (-0.0060, 0.0071)	0.9995 (0.9994, 0.9996)

Performance Metric, Data set	Linearity and Accuracy Parameters		
	Slope	**Intercept**	**R^2**
Linearity, Transplant patient samples	1.0125 (-0.3932, 2.4183)	-0.0002 (-0.0121, 0.0117)	0.9998 (0.9984, 1.0000)

Table 4: Estimated CV's, including 95% confidence intervals, for repeatability and reproducibility for different scenarios.

Performance Metric	CV (%)
Repeatability - 0.6% DF	1.85 (1.34, 2.73)
Repeatability - 2.4% DF	1.22 (0.88, 1.80)
Per Input Reproducibility - 15ng	3.10 (1.58, 4.37)
Per Input Reproducibility - 30ng	3.07 (1.42, 4.50)
Per Input Reproducibility - 45ng	1.99 (1.10, 2.75)

Performance Metric	CV (%)
Per Lot Reproducibility - Lot 1	3.99 (2.42, 5.41)
Per Lot Reproducibility - Lot 2	4.44 (2.69, 6.02)
Reproducibility, Transplant Patient Samples	4.29 (0.65, 6.86)

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Figures

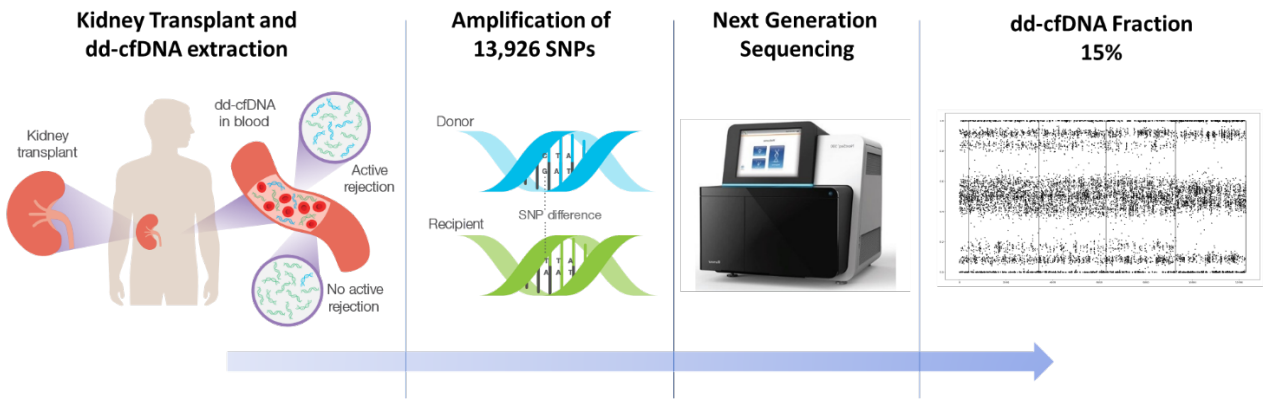


Figure 1: Workflow of a clinical grade next generation sequencing assay. Donor-derived-cfDNA is released from renal allograft into circulation; blood is drawn and centrifuged, and plasma is isolated. cfDNA is extracted from plasma samples and used for library preparation followed by targeted PCR amplification of 13,926 SNPs, performed using mmPCR. Amplicons are sequenced on a next-generation sequencer, and sequencing data is analyzed using a maximum likelihood estimate method to give a dd-cfDNA fraction, which is reported to the physician.

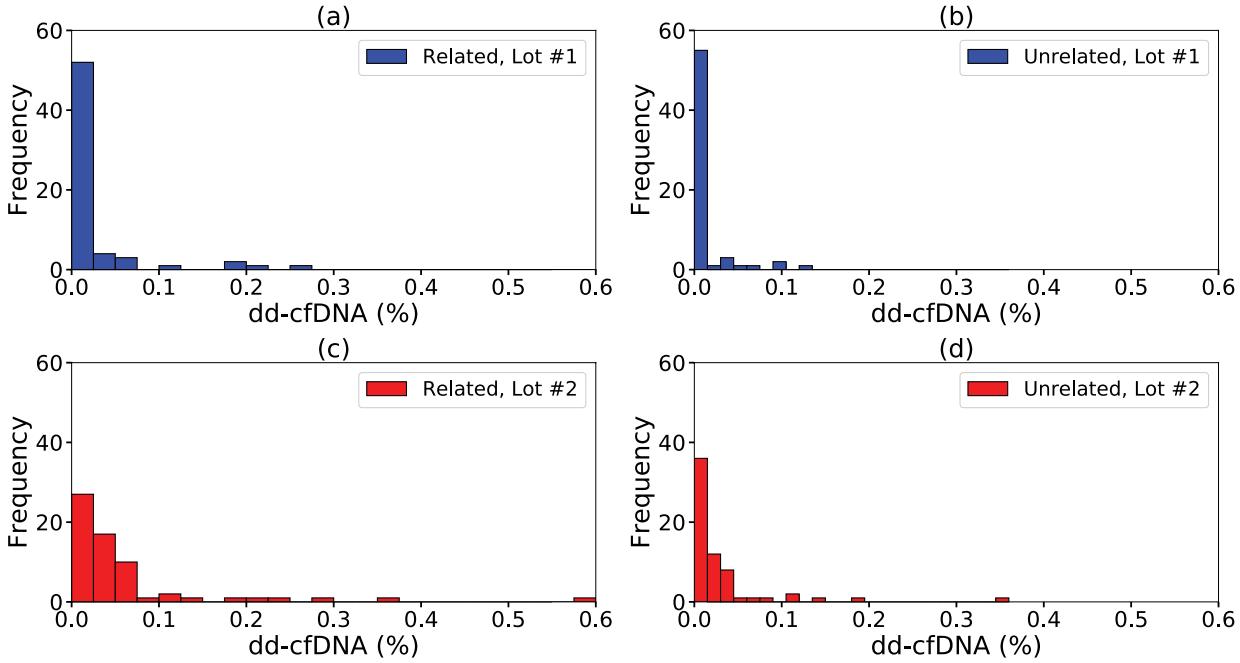


Figure 2: Histograms of measured dd-cfDNA for LoB analysis: (a) Related method, Lot 1 (b) Unrelated method, Lot 1 (c) Related method, Lot 2 (d) Unrelated method, Lot 2

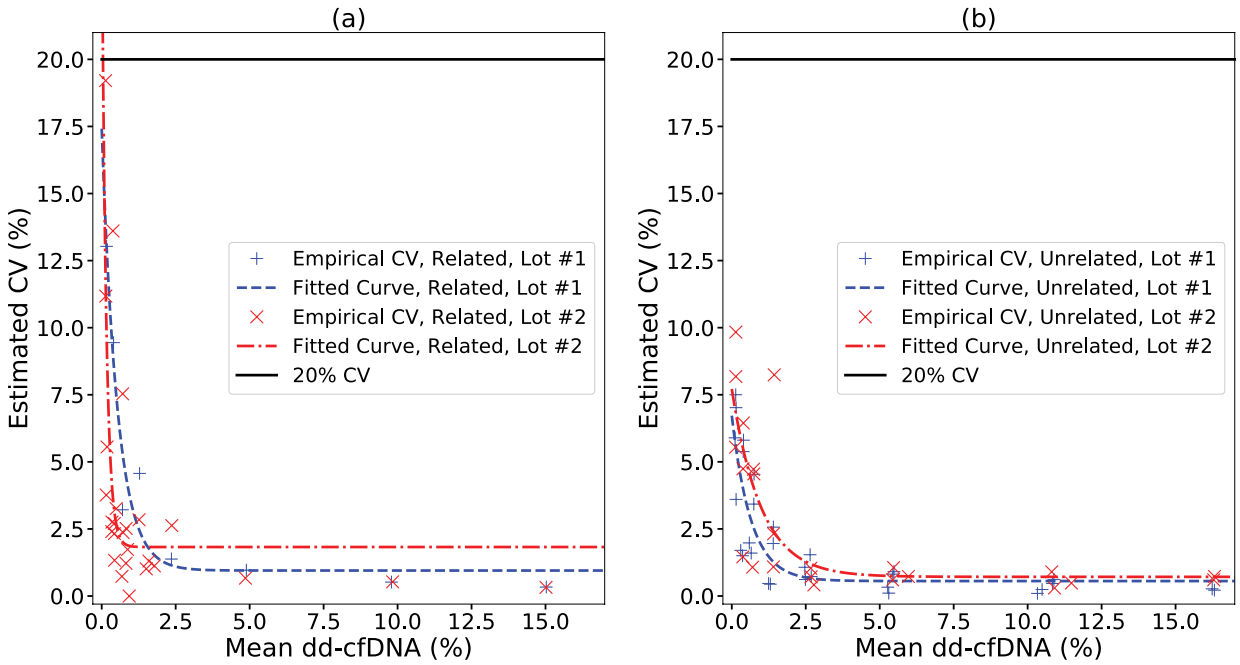


Figure 3: Measured percent CV values as a function of the corresponding percent empirical means for LoQ analysis: (a) related samples (b) unrelated samples

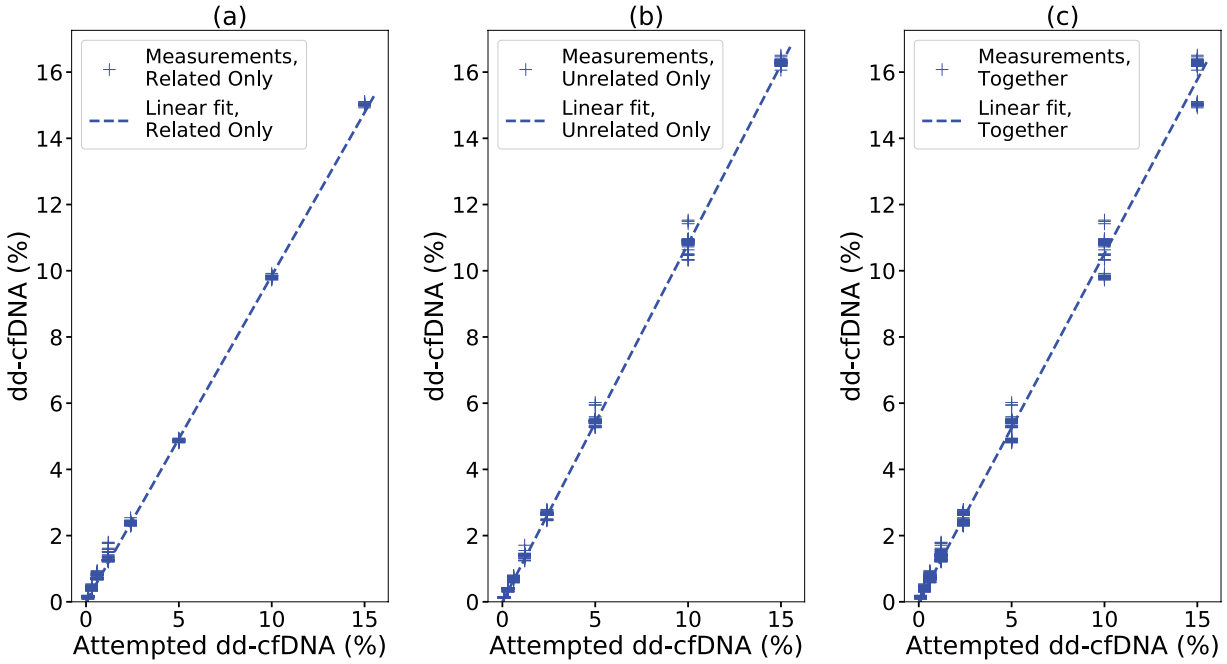


Figure 4: Measured dd-cfDNA as a function of the corresponding attempted dd-cfDNA, along with the calculated linear fit for linearity analysis: (a) Related only (b) Unrelated only (c) Related and unrelated cases together

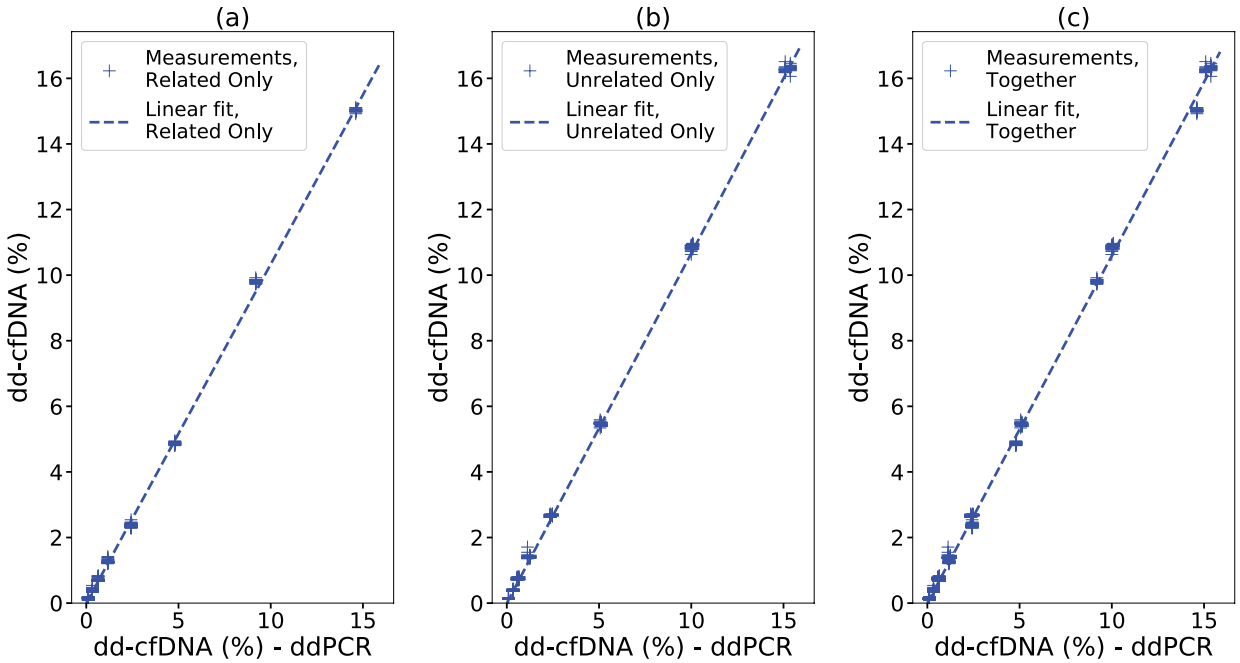


Figure 5: Measured dd-cfDNA as a function of the corresponding ddPCR values, along with the calculated linear fit for accuracy analysis: (a) Related only (b) Unrelated only (c) Related and unrelated cases together

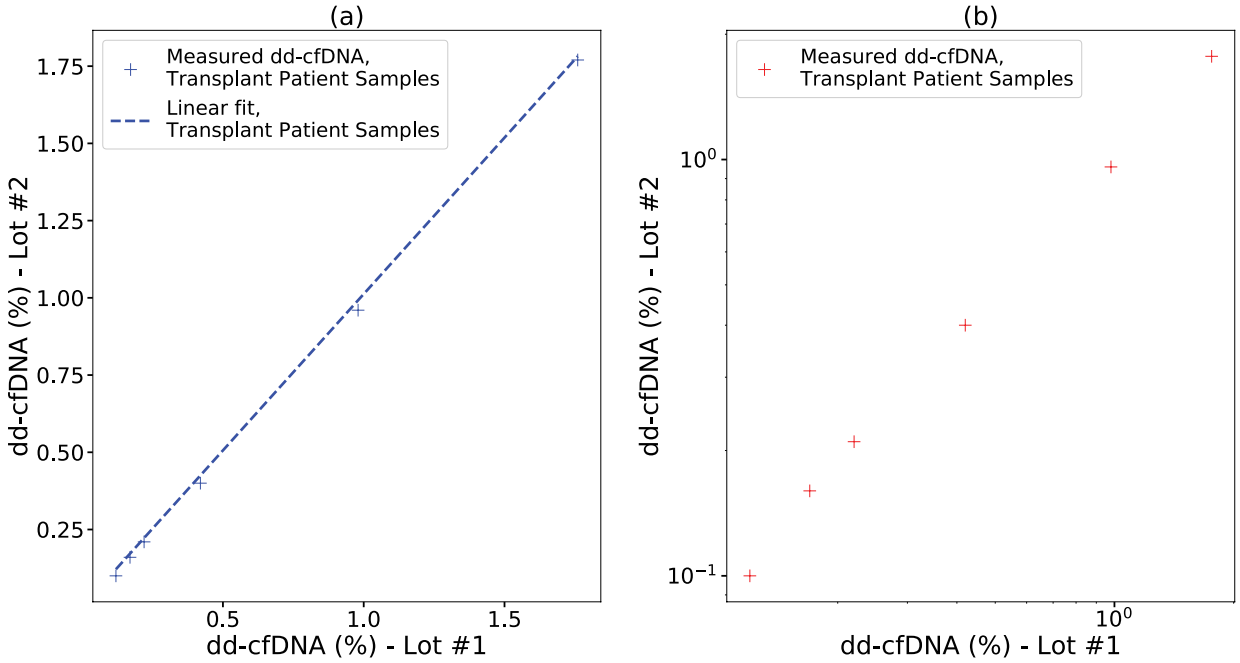


Figure 6: Measured dd-cfDNA from the replicates of Lot 2 as a function of the replicate values from Lot 1: (a) On linear scale, along with the calculated linear fit (b) On log-log scale

Supplementary Information

Section I.

Table S1: Detailed Experimental Design

Performance Metric	Sample	Sample Mixtures	Related/ Unrelated	Input Mass (ng)	dd-cfDNA fraction (%)	Number of Replicates	Number of Measurements	Total Measurements
LoB	Reference (n=4, blank)	N/A	N/A	15	N/A	3	12	128
	Reference	N/A	N/A	15	N/A	11	11	

	(n=1, blank)							
	Reference (n=5 blanks)	N/A	N/A	30	N/A	3	15	
	Reference (n=5 blanks)	N/A	N/A	45	N/A	6	30	
	Plasma (n=10)	N/A	N/A	Variable	N/A	3	30	
	Plasma (n=5)	N/A	N/A	Variable	N/A	6	30	
LoD	Reference	1	Related	15,45	0.1, 0.3, 0.6	6	36	389
		1	Related	30	0.1, 0.3, 0.6	22	66	
		1	Related	30	0.6	64	64	
		2	Unrelated	15,30,45	0.1, 0.3, 0.6	6	108	
	Plasma mixtures	3	Unrelated	15	0.1	8	24	
		3	Unrelated	15	0.3,0.6	6	36	
		3	Related	Variable	0.1,0.3,0.6	3	27	
		2	Related	Variable	0.3,0.6	4	16	
		1	Related	Variable	0.3,0.6	6	12	
LoQ, Linearity	Reference	1	Related	15,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0, 15.0	6	96	798
		1	Related	30	0.1, 0.3, 0.6, 1.2	22	88	
		1	Related	30	2.4	20	20	
		1	Related	30	5.0, 10.0, 15.0	6	18	
		2	Unrelated	15,30,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0, 15.0	6	288	
		1	Related	30	0.6,2.4	64	128	
	Plasma mixtures	3	Unrelated	15	0.1	8	24	
		3	Unrelated	15	0.3,0.6	6	36	

		3	Unrelated	15	1.2,2.4,5.0,10.0	3	36	
		3	Related	Variable	0.1,1.2	3	18	
		2	Related	Variable	0.3,0.6	4	16	
		1	Related	Variable	0.3,0.6	6	12	
		3	Related	Variable	0.3,0.6	3	18	
Accuracy	Reference	1	Related	15,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0, 15.0	6	96	638
		1	Related	30	0.1, 0.3, 0.6, 1.2	22	88	
		1	Related	30	2.4	20	20	
		1	Related	30	5.0, 10.0, 15.0	6	18	
		2	Unrelated	15,30,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0, 15.0	6	288	
		1	Related	30	0.6,2.4	64	128	
Reproducibility, Per Input	Reference	1	Related	15	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0	12	84	504
		1	Related	30,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0	6	84	
		2	Unrelated	15	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0	12	168	
		2	Unrelated	30,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0	6	168	
Reproducibility, Per Lot	Reference	1	Related	15,30,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0	6	126	378

		2	Unrelated	15,30,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0	6	252	
Linearity, Reproducibility	Transplant Patient Samples (n=6)	4	Related	Variable	Var	2	8	12
		2	Unrelated	Variable	Var	2	4	
Repeatability	Reference	1	Related	30	0.6,2.4	64	128	128

N/A, Not applicable

Section II.

LoB Analysis: Tables S2 summarizes the mean, median, and standard deviation values of the measured dd-cfDNA fractions for each lot and mode of test:

Table S2: Mean, median and standard deviation values of measured dd-cfDNA for related and unrelated cases for Lots 1 and 2.

LoB – Statistics, estimation methods	Lot 1	Lot 2
Mean, Related (%)	0.03	0.06
Mean Unrelated (%)	0.02	0.03
Median, Related (%)	0.01	0.03
Median, Unrelated (%)	0.01	0.01
Standard Deviation, Related (%)	0.05	0.1
Standard Deviation, Unrelated (%)	0.02	0.05

In order to demonstrate the performance of the test for reference and plasma samples separately, LoB was computed for each case by using 60 and 68 measurements obtained from plasma and reference samples, respectively. In order to increase the sample size, lots were not distinguished. **Figure S1** and **Table S3** depicts the histograms and LoB values for each sample type and estimation method, respectively.

Figure S1: Histograms of measured dd-cfDNA for LoB analysis: (a) related method, reference samples (b) unrelated method, reference samples, (c) related method, plasma samples and (d) unrelated method, plasma samples.

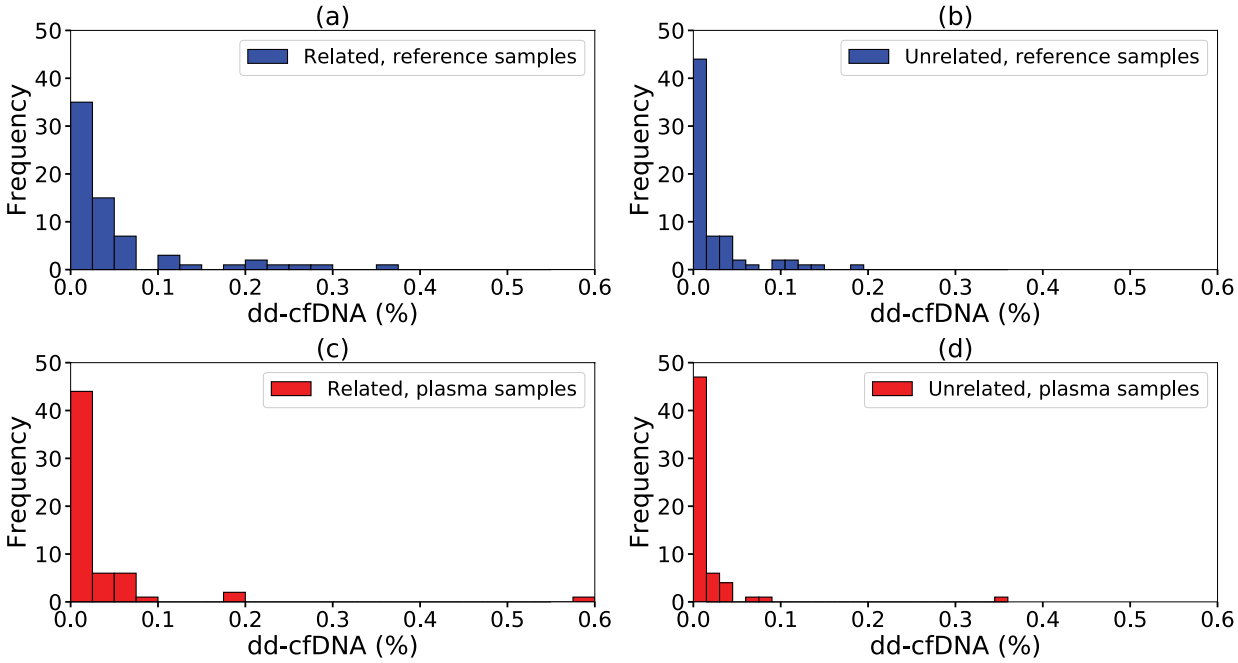


Table S3: LoB values for related and unrelated estimation methods for reference and plasma samples.

LoB, Estimation Methods	Reference samples	Plasma samples
Related (%)	0.23	0.08
Unrelated (%)	0.11	0.04

Section III.

LoD Analysis: The parametric LoD computation method necessitates that: (i) The measurements from low-level samples, approximately follows a Gaussian distribution, (ii) The empirical standard deviations of the described samples, approximately remain constant as a function of empirical mean. **Figure S2** depicts the histograms of centered, measured DFs for each lot and each test mode. **Figure S3** shows empirical standard deviation as a function of empirical mean for both lots and test modes. **Figures S2** and **S3** demonstrate that these two conditions are satisfied for both related and unrelated low-level samples.

Figure S2: Histograms of centered, measured dd-cfDNA for LoD analysis: (a) related samples from Lot 1, (b) related samples from Lot 2, (c) unrelated samples from Lot 1, and (d) unrelated samples from Lot 2.

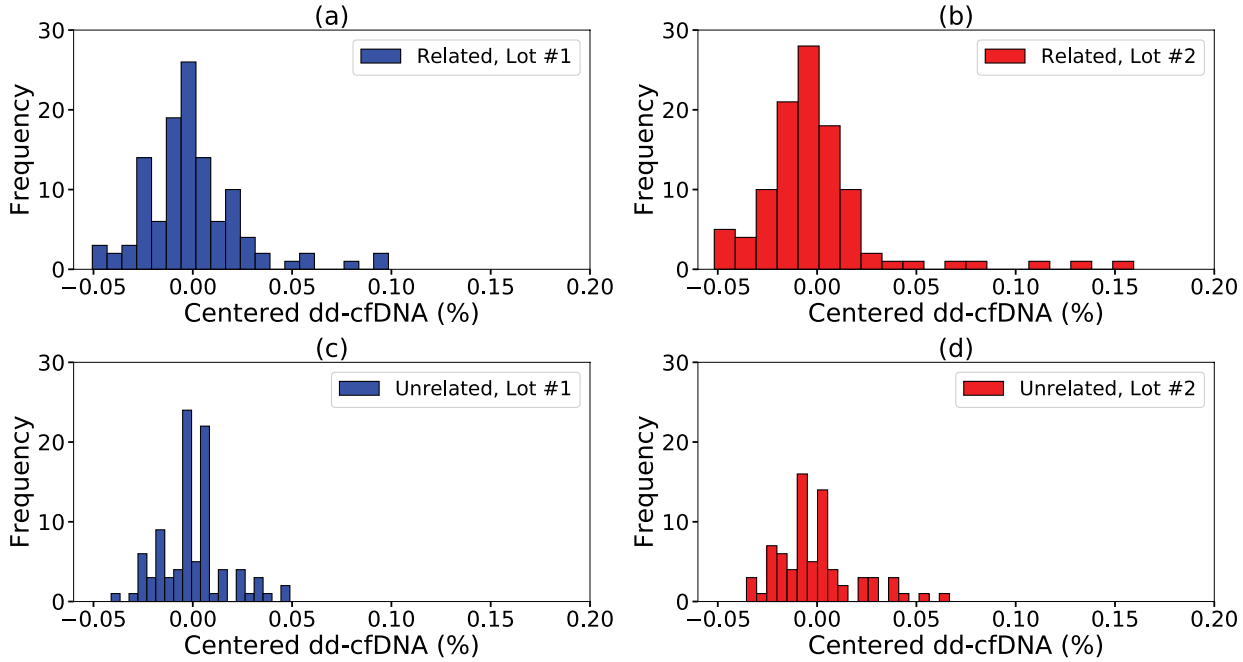
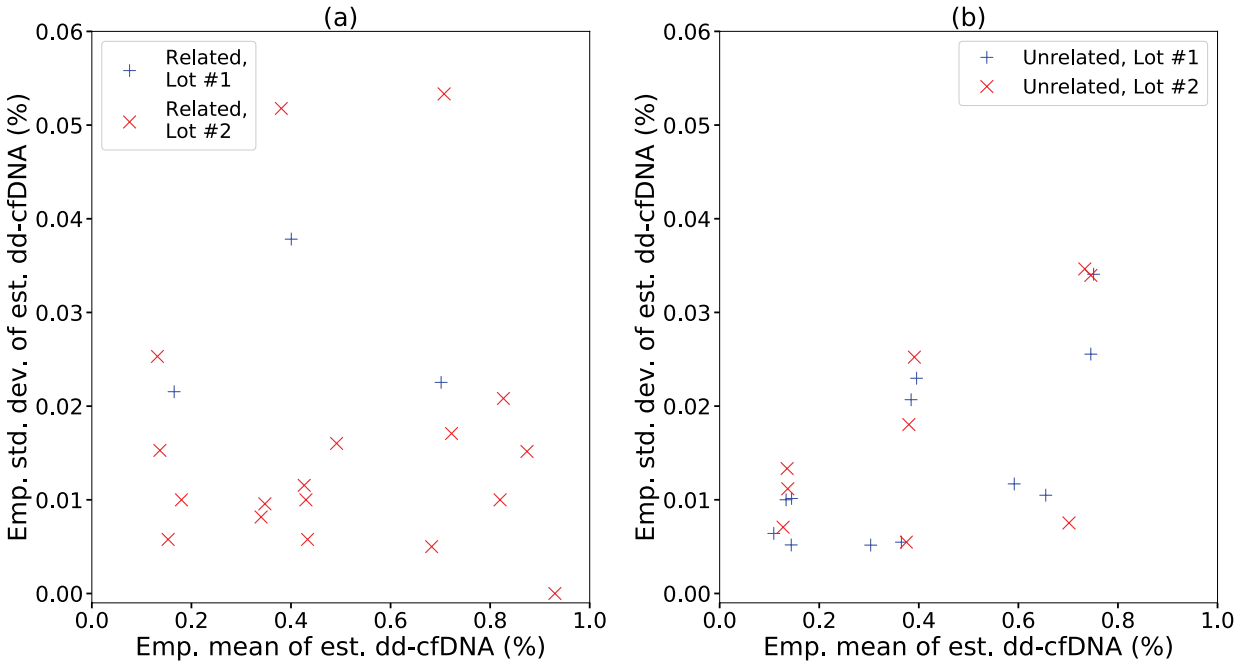


Figure S3: Empirical standard deviations as a function of the corresponding empirical means for LoD analysis: (a) related samples from Lot 1 and Lot 2, (b) unrelated samples from Lot 1 and Lot 2.



In order to demonstrate LoD for reference and plasma mixture samples separately, and to observe the effect of input amount on reference samples, LoD analysis for these sets of samples was carried out separately, by using their corresponding LoB values. Further, for reference samples, 18 related and 36 unrelated measurements were used at 15 ng and 45 ng inputs; 130 related and 36 unrelated measurements were used at 30 ng input. **Table S4** and **S5** provides a breakdown of the computed LoD values with respect to their estimation method and input amount for reference samples and for plasma mixture samples, respectively.

Table S4: LoD values for related and unrelated estimation methods, for reference samples at 15, 30, and 45ng inputs.

Reference samples, Estimation Methods	LoD		
	15ng	30ng	45ng
Related (%)	0.28	0.26	0.25
Unrelated (%)	0.13	0.13	0.12

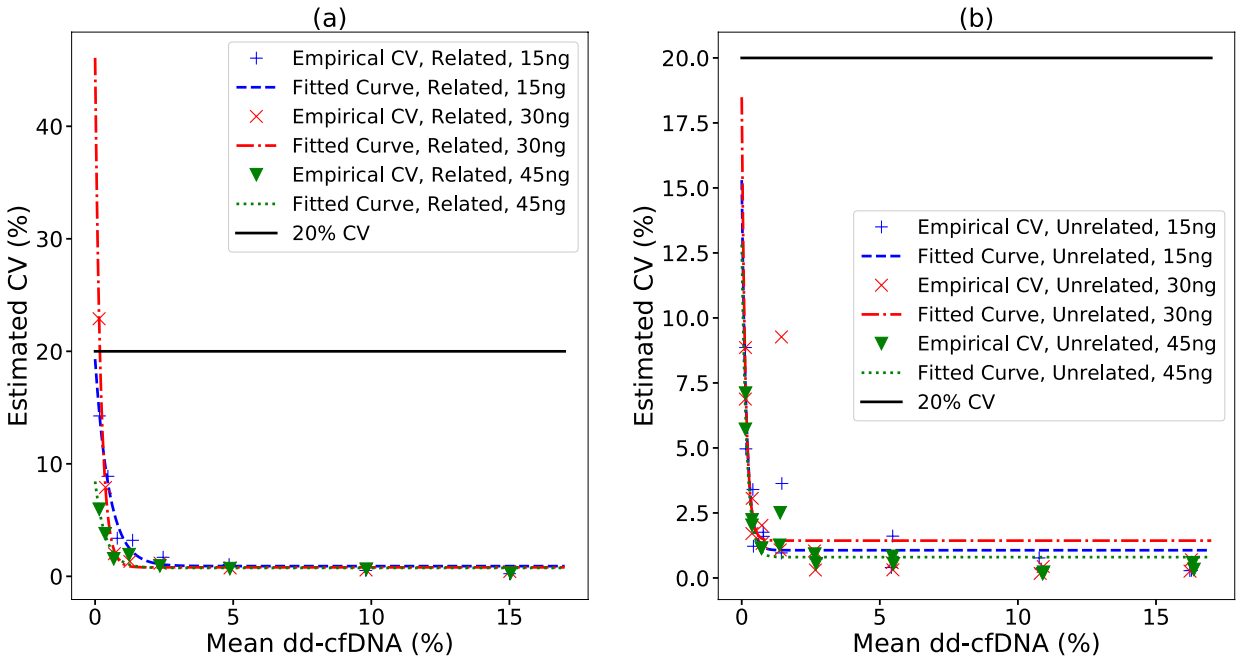
Table S5: LoD values for related and unrelated estimation methods, for plasma mixture samples

Plasma mixture samples, Estimation Methods	LoD
Related (%)	0.11
Unrelated (%)	0.05

Section IV.

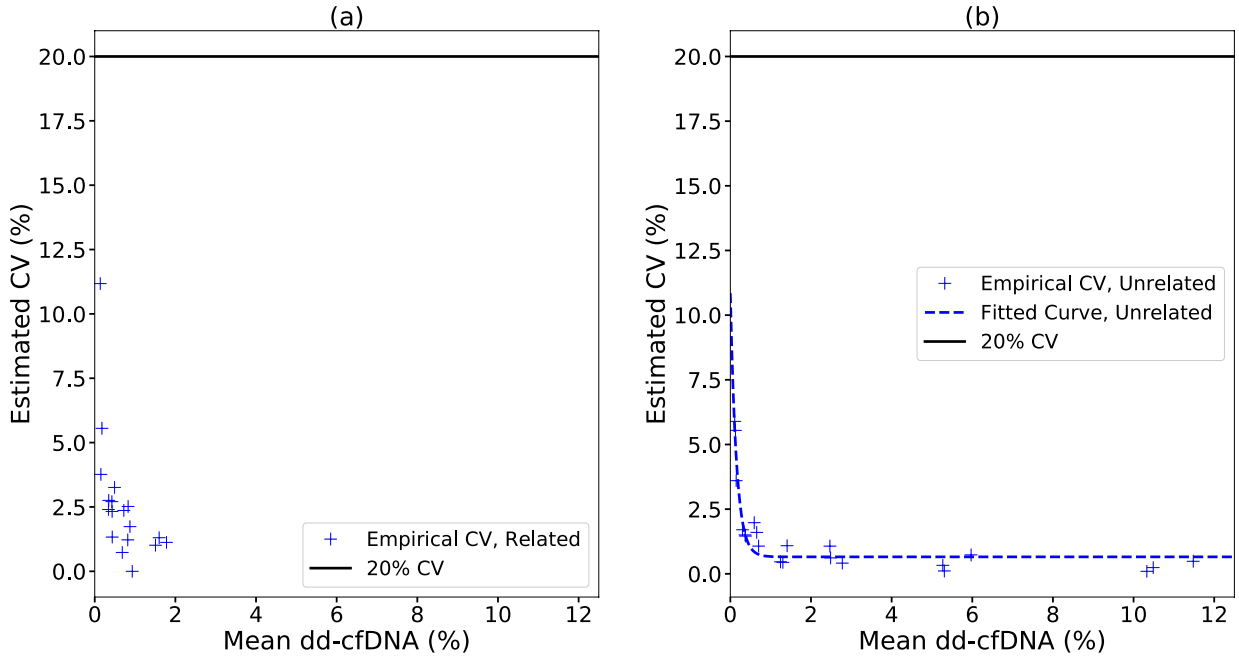
LoQ Analysis: Similar to LoD analysis, LoQ was evaluated for reference samples, which were further partitioned with respect to their input amounts. As depicted in **Figure S4**, all the measured CV values for all the spike levels tested were below 20% cut-off for related samples at all input levels, as well as related samples at 15 and 45 ng input levels. Thus, for all the cases, lower LoQ was equal to LoD, by definition. For related samples with 30 ng input level, fitted curve intersects 20% CV level at approximately 0.174%, which was lower than the corresponding LoD, i.e., 0.26%, for this case. Thus, the lower LoQ is again equal to LoD, by definition.

Figure S4: Measured percent CV values as a function of the corresponding percent empirical means, particularized with respect to input amount, for LoQ analysis of reference samples: (a) related samples (b) unrelated samples



Further, LoQ values for plasma mixture samples were also computed (**Figure S5)**. For both cases, a lower LoQ was observed equal to the corresponding LoD. It should be noted that the exponential-fit for **Figure S5 (a)** was not accurate, which limited readability of the graph, and hence omitted. The main reason for the inaccuracy of the fit was the lack of higher DF level samples, compared to all other scenarios. This exclusion, however, did not affect our inference about the lower LoQ, since all the estimated CV values were well below 20% cut-off.

Figure S5: Measured percent CV values as a function of the corresponding percent empirical means for LoQ analysis of plasma mixture samples: (a) related (b) unrelated.



Finally, **Table S6** summarizes the estimated parameters of the non-linear fit for CV in all different scenarios:

Table S6: Estimated parameters of the exponentially decaying model of the CV for every scenario - LoQ.

LoQ, Data Set, Estimation Method	Estimated Parameters		
	a	b	c
Combined, Lot 1, Related,	0.950216	16.4685	1.88562
Combined, Lot 2, Related,	1.82651	24.2948	6.82745
Combined, Lot 1, Unrelated,	0.557873	6.16417	1.53284
Combined, Lot 2, Unrelated,	0.715364	7.00144	1.00344
Reference samples, 15ng, Related,	0.907757	18.3994	1.97114
Reference samples, 30ng, Related,	0.798892	45.2805	4.943
Reference samples, 45ng, Related,	0.746606	7.69009	2.62489
Reference samples, 15ng, Unrelated,	1.06598	14.2357	6.04647
Reference samples, 30ng, Unrelated,	1.4362	17.0526	7.3715
Reference samples, 45ng, Unrelated,	0.801393	12.0185	5.69333
Plasma mixture samples, Related	1.88546	13275.4	53.5112
Plasma mixture samples, Unrelated	0.654995	10.1971	6.67823

Section V.

Linearity Analysis: Similar to previous performance metrics, linearity analyses for reference and plasma mixture samples are reported separately. Specifically, analysis for reference samples used 349 related and 285 unrelated measurements, whereas analysis for plasma mixture samples used 63 related and 96 unrelated measurements. **Figure S6** depicts the individual measurements and linear regression lines for reference samples. Similarly, **Figure S7** depicts the individual measurements and linear regression lines for plasma mixture samples. **Table S7** lists corresponding linear regression results for reference and plasma mixture samples, respectively.

Figure S6: Measured dd-cfDNA as a function of the corresponding attempted (targeted) spike levels, along with the calculated linear fit, for linearity analysis of reference samples: (a) related only (b) unrelated only (c) related and unrelated cases together.

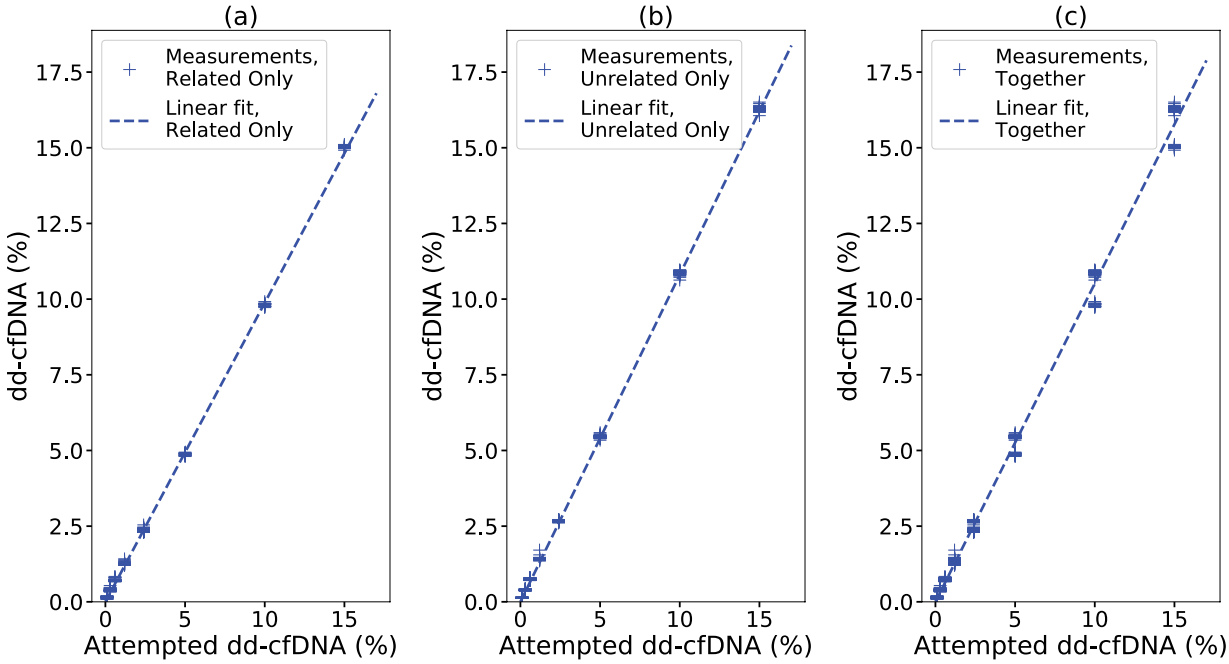


Figure S7: Measured dd-cfDNA as a function of the corresponding attempted (targeted) spike levels, along with the calculated linear fit, for linearity analysis of plasma mixture samples: (a) related only (b) unrelated only (c) related and unrelated cases together.

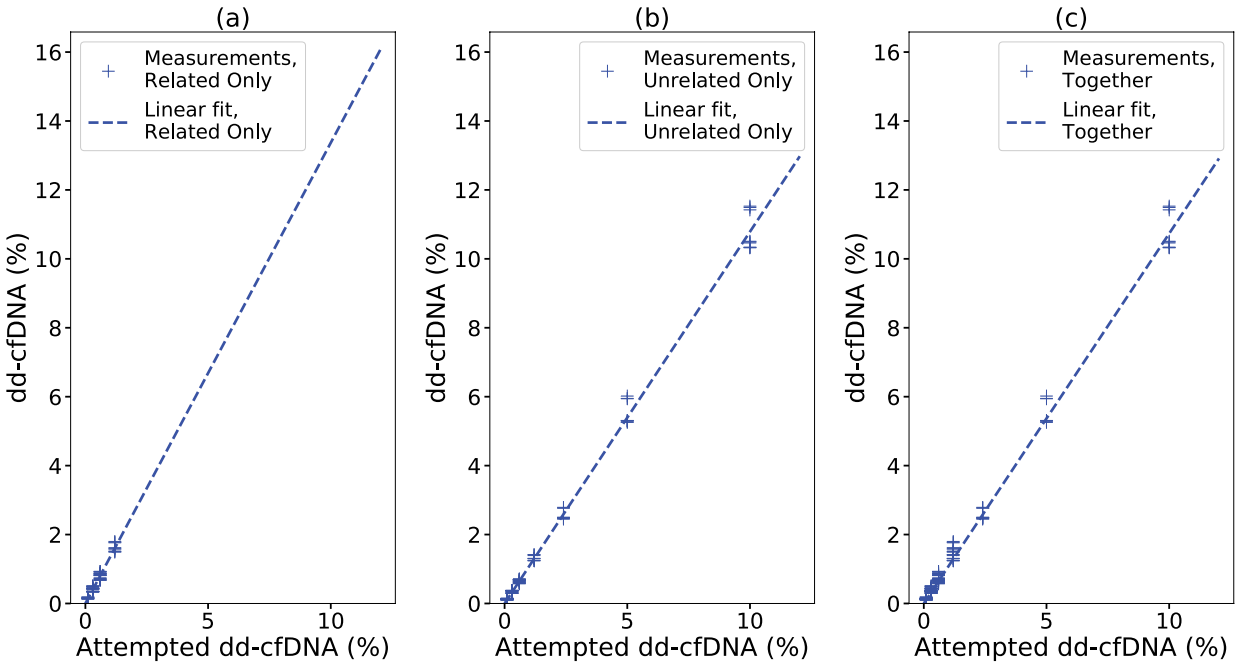


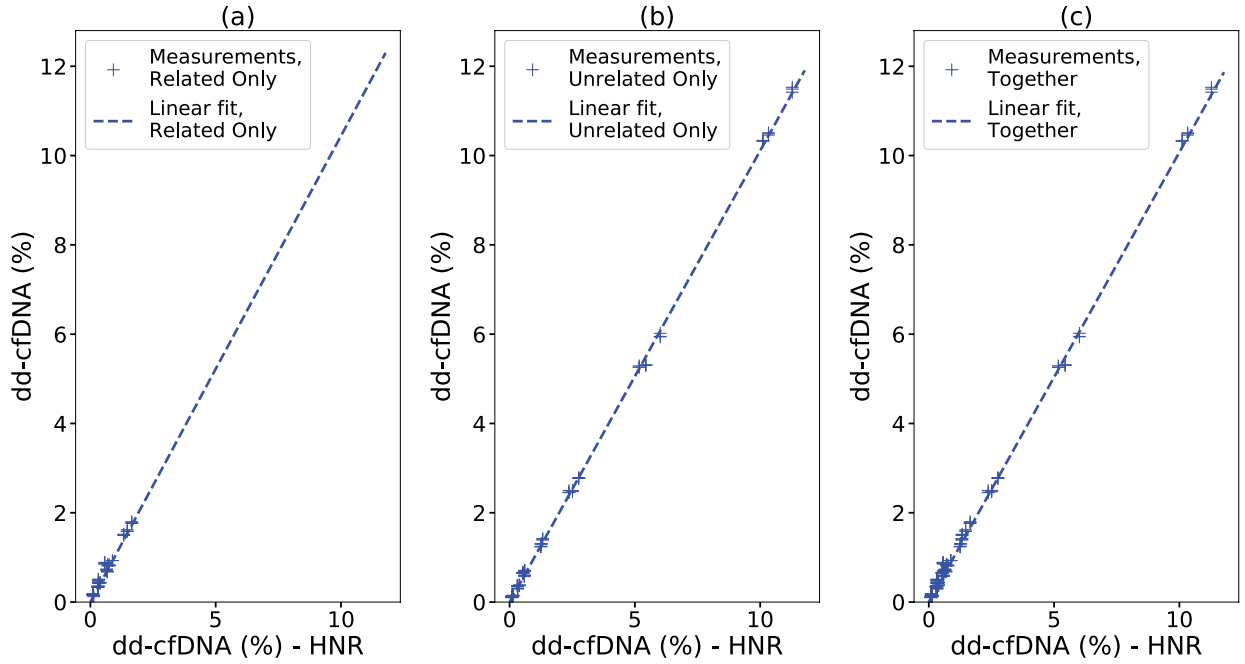
Table S7: Linear regression results for linearity of reference samples and plasma mixture samples, including 95% confidence intervals.

Sample type	Linearity Parameters		
Reference samples	**Slope**	**Intercept**	**R^2**
Unrelated	1.0804 (0.9540, 1.2069)	0.0007 (-0.0077, 0.0091)	0.99989 (0.99986,0.99992)
Related	0.9876 (0.8833, 1.0920)	0.0005 (-0.0041, 0.0052)	0.9994 (0.9974, 0.9995)
Related + Unrelated	1.0515 (0.9693, 1.1338)	0.0003 (-0.0043, 0.0049)	0.9969 (0.9964, 0.9974)
Plasma mixture samples	**Slope**	**Intercept**	**R^2**
Unrelated	1.0787 (0.8574, 1.300)	0.0002 (-0.0076, 0.0080)	0.9962 (0.9943, 0.9975)
Related	1.3368 (0.9895, 1.6841)	0.0001 (-0.0020, 0.0022)	0.9713 (0.9528, 0.9965)
Related + Unrelated	1.0734 (0.9038, 1.2430)	0.0008 (-0.0039, 0.0055)	0.9953 (0.9935, 0.9965)

Section VI.

Accuracy Analysis: In order to demonstrate the accuracy for plasma mixture samples, a DF estimated by using SNP's from homologous non-recombining region in lieu of ddPCR for reference samples. The rationale of using this method as a more precise alternative to the conventional DF estimate is as follows: the non-recombining nature ensures that the targets in this region have the property of X chromosome always provides the reference allele and the Y chromosome always provides the mutant allele. Thus, the allele ratio reflects the ratio of X and Y chromosomes in the sample. This observation, coupled with the fact that plasma mixture samples are designed to have a female background with a male spike-in, which implies that the allele ratio is directly proportional to the half of DF. Hence, DF can be estimated without the background interference. The analysis was carried out using 63 related and 96 unrelated plasma mixture sample measurements, which excludes one sample that failed QC. The individual measurements and linear regression lines are shown in Figure S8, and the corresponding linear regression results are shown in Table S8. It is anticipated that the relative wider confidence intervals for plasma mixture sample estimates compared to their reference sample counterparts might be due to the relatively smaller sample size of the former compared to the latter.

Figure S8: Measured dd-cfDNA (y-axis) as a function of the corresponding dd-cfDNA values measured using homologous non-recombining region (x-axis), along with the calculated linear fit, for accuracy analysis of plasma mixture samples: (a) related only (b) unrelated only (c) related and unrelated cases together



HNR, Homologous non-recombining region

Table S8: Linear regression results for accuracy of plasma mixture samples, including 95% confidence intervals.

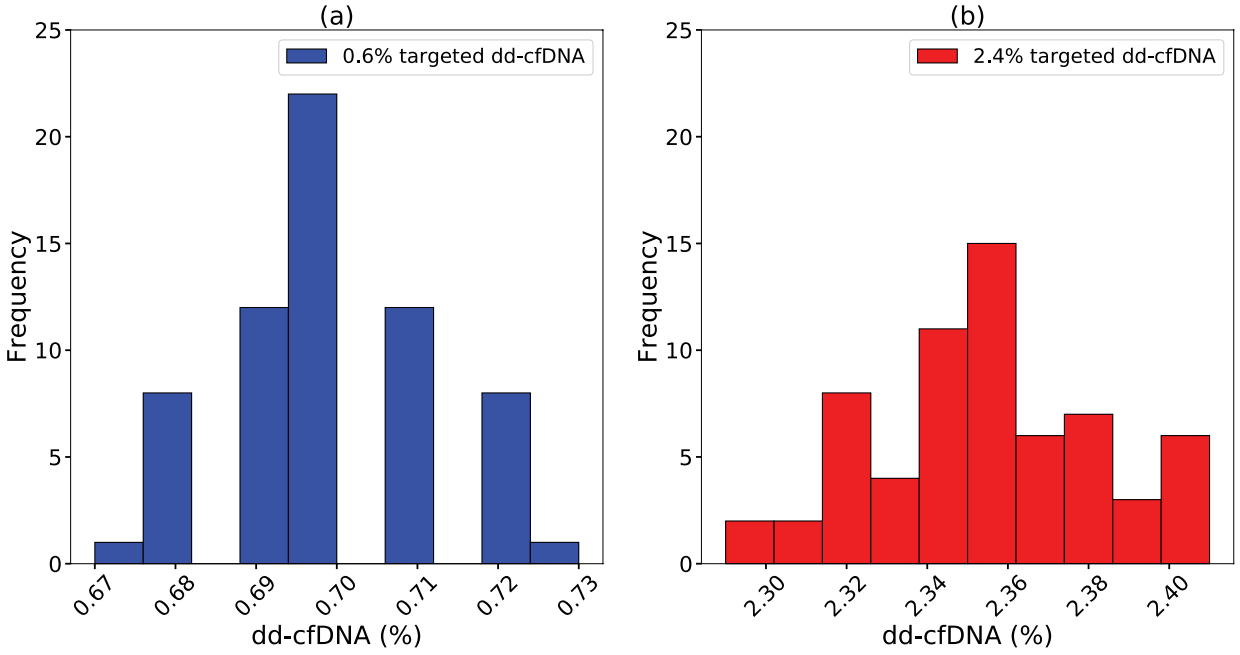
Plasma mixture samples	Accuracy		
	Slope	Intercept	R^2
Unrelated	1.0108 (0.8038, 1.2179)	0.0002 (-0.0076, 0.0080)	0.9996 (0.9994, 0.9997)
Related	1.0440 (0.7727, 1.3153)	0.0007 (-0.0012, 0.0027)	0.9706 (0.9517, 0.9993)

Plasma mixture samples	Accuracy		
	Slope	Intercept	R^2
Combined	1.0073 (0.8484, 1.1662)	0.0005 (-0.0042, 0.0053)	0.9991 (0.9987, 0.9993)

Section VII.

Precision Analysis: In order to compute the confidence intervals on the estimated CV's for repeatability analysis, the classical bounds of McKay was used based on a chi-squared approximation.[1] The derivation of these bounds assumes that the underlying measurements from which CV is estimated are realizations from a Gaussian distribution. **Figure S9** illustrates that the said assumption was justified in our case.

Figure S9: Histograms of measured dd-cfDNA for repeatability analysis: (a) 0.6% targeted dd-cfDNA (b) 2.4% targeted dd-cfDNA



It should be noted that chi-squared approximation-based bounds used in repeatability analysis is not suitable to compute the confidence intervals of the estimated CV's for reproducibility analysis. The reason is that the underlying measurements from which CV value is estimated do not follow a Gaussian distribution, due to the broad range of underlying DFs. Thus, confidence intervals by a standard bootstrapping technique was computed. Because of the inherent stochasticity of the approach, the values may slightly vary for each trial of the method.

Confidence intervals of the estimated concordance between clinical samples was computed via Clopper-Pearson method for binomial proportions. Specifically, the closed-form expression of the said method for 100% observed success rate was used.

Reference

1. McKay AT. Distribution of the Coefficient of Variation and the Extended "t" Distribution. *Journal of the Royal Statistical Society.* 1932;95(4):695-698.