



Validation of a Clinical-Grade Assay to Measure Donor-Derived Cell-Free DNA in Solid Organ Transplant Recipients



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The use of circulating cell-free DNA (cfDNA) as a biomarker in transplant recipients offers advantages over invasive tissue biopsy as a quantitative measure for detection of transplant rejection and immunosuppression optimization. However, the fraction of donor-derived cfDNA (dd-cfDNA) in transplant recipient plasma is low and challenging to quantify. Previously reported methods to measure dd-cfDNA require donor and recipient genotyping, which is impractical in clinical settings and adds cost. We developed a targeted next-generation sequencing assay that uses 266 single-nucleotide polymorphisms to accurately quantify dd-cfDNA in transplant recipients without separate genotyping. Analytical performance of the assay was characterized and validated using 1117 samples comprising the National Institute for Standards and Technology Genome in a Bottle human reference genome, independently validated reference materials, and clinical samples. The assay quantifies the fraction of dd-cfDNA in both unrelated and related donor-recipient pairs. The dd-cfDNA assay can reliably measure dd-cfDNA (limit of blank, 0.10%; limit of detection, 0.16%; limit of quantification, 0.20%) across the linear quantifiable range (0.2% to 16%) with across-run CVs of 6.8%. Precision was also evaluated for independently processed clinical sample replicates and is similar to across-run precision. Application of the assay to clinical samples from heart transplant recipients demonstrated increased levels of dd-cfDNA in patients with biopsy-confirmed rejection and decreased levels of dd-cfDNA after successful rejection treatment. This noninvasive clinical-grade sequencing assay can be completed within 3 days, providing the practical turnaround time preferred for transplanted organ surveillance. (*J Mol Diagn* 2016, 18: 890–902; <http://dx.doi.org/10.1016/j.jmoldx.2016.07.003>)

Transplantation is often the most effective treatment option for patients with end-stage organ disease. Organ transplant patients require lifelong immunosuppression that necessitates a finely tuned therapeutic strategy. Suboptimal dosing and the threat of allograft rejection must be balanced with increased risks of nephrotoxicity, diabetes, cardiovascular complications, infections, and cancer that may result from life-long immunosuppression. In this regard, a significant unmet medical need exists for diagnostic tools, preferably noninvasive, to assist clinicians who monitor the health of the transplanted organ and manage immunosuppressive therapy to improve short- and long-term outcomes. One of

the common procedures used to monitor organ health is biopsy, which is invasive, subject to interpretation bias, costly, and, at times associated with significant adverse events.

Plasma cell-free DNA (cfDNA) has been described as a biomarker for prenatal testing, cancer, and organ transplantation,^{1–4} each indication presenting different clinical

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and technological challenges. The presence of allograft (ie, donor-derived) cfDNA (dd-cfDNA) circulating in the blood of transplant recipients was first detected with PCR amplification of Y-chromosome genes in sex-mismatched transplant recipients.⁵ Since then, multiple studies, using various procedures, have shown that allograft DNA is detectable and can be quantified as a fraction of total cfDNA in recipient's plasma,^{6–11} serum,^{12,13} and urine.^{14,15} Most of these studies exploit the genetic polymorphisms that differ between the donor and recipient, such as human leukocyte antigen mismatch,¹² differences in copy number deletions,¹⁶ and single-nucleotide polymorphisms (SNPs).^{7–11} Whether based on SNPs, human leukocyte antigen, or other genetic differences, using digital PCR,^{9,17} next-generation sequencing (NGS),^{7,8,11} or other methods, all previously described measurements of dd-cfDNA rely on knowledge of donor and recipient genotypes, which requires significant time and cost and depends on the availability of donor genetic material.

To investigate the role of cfDNA as a biomarker of organ injury associated with acute rejection, Snyder et al⁸ analyzed dd-cfDNA levels in heart transplant patients and found significantly increased levels of dd-cfDNA in patients with biopsy-proven acute cellular rejection ($2.75\% \pm 1.81\%$, SD) compared to patients without evidence of rejection ($0.92\% \pm 1.16\%$, SD). At the threshold of 1.70%, receiver operating characteristic analysis demonstrated sensitivity of 83% and specificity of 84%. Similar results were obtained in a subsequent single-center prospective study, in which heart transplant patients with either acute cellular or antibody-mediated rejection exhibited significantly higher dd-cfDNA levels compared to stable transplant recipients.⁷ The levels of dd-cfDNA increased with severity of rejection, with area under curve of 0.60 for distinguishing mild, 0.83 for moderate-to-severe, and 0.95 for severe rejection events, each compared to the absence of rejection. Furthermore, dd-cfDNA was found to be elevated up to 5 months before the biopsy-proven rejection event, suggesting a potential value of dd-cfDNA as an early diagnostic marker of transplant rejection.⁷ Increased levels of dd-cfDNA were also found to be associated with biopsy-proven rejection in separate studies of pediatric and adult heart transplant recipients.^{10,18}

In addition to heart transplant recipients, elevated dd-cfDNA was found to be associated with rejection in other transplanted organ settings. In stable liver transplant recipients, dd-cfDNA levels were between 5% and 10%, whereas in two cases of rejection dd-cfDNA levels increased to 55% and 60%.⁹ In kidney, urinary dd-cfDNA was found to be elevated with both acute rejection and BK virus nephropathy, suggesting the role for urinary dd-cfDNA as a biomarker of acute injury of the donor organ.¹⁴ In a study of lung transplant recipients, increased levels of dd-cfDNA in plasma correlated with severity of transbronchial biopsy grades, with comparison of moderate-to-severe acute rejection versus absence of rejection yielding an area under curve of 0.9.¹¹

Beyond its value as a biomarker of rejection, dd-cfDNA may be used as a guiding sensor for personalized immunosuppressive treatment. For example, Oellerich et al¹⁷ compared blood trough tacrolimus concentrations to dd-cfDNA levels in 10 liver transplant patients and found lower amounts of dd-cfDNA were associated with higher tacrolimus concentrations. The possible role of dd-cfDNA in establishing minimal effective trough tacrolimus concentrations suggests the wider potential of cfDNA in monitoring of allograft health and detecting allograft injury during optimization of immunosuppression.

Despite much progress in the methods to detect and quantify dd-cfDNA, technical limitations have prevented clinical implementation of dd-cfDNA as a diagnostic test. Some methods used to measure dd-cfDNA levels are only suitable for female recipients with transplanted organs from males.^{5,6,13–15} Other methods require prior recipient and donor genotyping, are time consuming, or are costly.^{7–9,11} In addition, each of these studies used research-grade tools to measure dd-cfDNA. Robust clinical validity requires the use of an assay with rigorously established analytical performance. Once clinically validated, studies that establish the clinical utility can be performed by actively managing patients according to the outcome of the biomarker.

Herein, we describe a targeted amplification, clinical-grade NGS assay to quantify dd-cfDNA in transplant recipients without the need for separate recipient or donor genotyping. A panel of 266 SNPs was selected based on allele frequency across ancestral heritage groups, sequencing accuracy, and lack of linkage. In addition, the absence of genetic association of these SNPs with common complex diseases avoids the challenges of reporting incidental findings. Genomic regions encompassing each SNP position are amplified, and each of the alleles is quantified by NGS. The recipient genotype is determined at each SNP position, and the relative fraction of dd-cfDNA is computed on a validated analysis pipeline incorporating open source and custom bioinformatic tools. The validity of the test was demonstrated by establishing the sensitivity and reproducibility of the test on independent reference materials, and clinical performance was demonstrated using heart transplant recipient plasma.

Materials and Methods

Plasma Samples

Blood samples from healthy, nontransplant volunteers and transplant patients were collected in 10-mL Cell-Free DNA BCT tubes (Streck, Omaha, NE) and shipped to CareDx, Inc. (Brisbane, CA), at ambient temperature in insulated packaging to minimize temperature fluctuation. On arrival, and within 7 days post-draw, plasma was separated by centrifugation at $1600 \times g$ for 20 minutes, followed by a second centrifugation at $16,000 \times g$ for 10 minutes, and either plasma was stored at -80°C or cfDNA was extracted

immediately using the Circulating Nucleic Acid kit (Qiagen, Redwood City, CA).

For the Cardiac Allograft Rejection Gene Expression Observational (CARGO) II study, plasma from heart transplant patients was drawn into a BD Vacutainer PPT Plasma Preparation Tube (BD Biosciences, San Jose, CA), separated by centrifugation, and stored at -80°C . cfDNA was extracted from thawed plasma using the Circulating Nucleic Acid kit (Qiagen) and concentrated by centrifugal vacuum concentration.

All transplant patient samples were collected in accordance with institutional review board–approved study designs with appropriate informed consent (full list of participating institutions is available in [Supplemental Table S1](#)). The CARGO II study was designed to provide independent evidence of the clinical performance of the noninvasive gene-expression profiling (AlloMap) test (<https://clinicaltrials.gov>; trial identifier NCT00761787). Utility of Donor-Derived Cell-Free DNA in Association with Gene-Expression Profiling (AlloMap) in Heart Transplant Recipients (D-OAR; <https://clinicaltrials.gov>; trial identifier NCT02178943) is an observational study to assess the use of dd-cfDNA separately and in association with gene-expression profiling (AlloMap) in heart transplant recipients. Circulating Donor-Derived Cell-Free DNA in Blood for Diagnosing Acute Rejection in Kidney Transplant Recipients (DART; <https://clinicaltrials.gov>; trial identifier NCT02424227) is a prospective, multicenter, observational study to assess circulating dd-cfDNA in blood for diagnosing acute rejection in kidney transplant recipients.

Reference Materials

Reference materials were developed by mixing cell line genomic DNA (gDNA) fragmented by sonication (Covaris SE220, Woburn, MA) to approximately 160-bp fragments to simulate the size profile of cell-free DNA (Horizon Discovery, Cambridge, UK). Three separate reference material panels were constructed using a donor cell line (RKO containing a single copy of *EGFR T790M* gene) and three recipient cell lines (SW48, HCT116, and HCC-78). Trace amounts of the donor DNA were mixed with the recipient DNA at target levels ranging from 0.25% to 12% to simulate different amounts of cfDNA originating from donor. The amount of donor DNA was verified by Horizon Discovery using digital PCR and a SNP genotyping assay for *EGFR T790M* (Life Technologies, Carlsbad, CA) on a Biorad QX100 platform (BioRad, Hercules, CA).

The four cell lines were genotyped with the Infinium CytoSNP-850K panel (Illumina, San Diego, CA). The chip-based SNP genotyping provided an independent source of comparison genotypes for the SNPs evaluated in the assay on these same cell lines.

National Institute for Standards and Technology Genome in a Bottle reference NA12878 (RM 8398) was obtained from the National Institute of Standards and Technology

and used to verify the accuracy of the sequence of each SNP region and requisite SNP genotypes.

Three cfDNA reference sample panels were each constructed by mixing cfDNAs from plasma samples collected in Streck BCT from two healthy volunteers (one donor and one recipient) and serial dilutions of that sample with recipient cfDNA. cfDNA panel 1 was diluted 3-, 6-, 12-, 20-, and 30-fold. cfDNA panels 2 and 3 were diluted 6-, 20-, and 30-fold.

SNP Selection and Primer Design

For the dd-cfDNA assay to be applicable to different transplant recipients without requiring separate genotyping of either donor or recipient, SNPs were selected to ensure that the same SNP panel could be used for individuals with different ancestral heritages. We included 85 of 92 SNPs previously identified as suitable for differentiating between any two unrelated individuals.¹⁹ This combination of SNPs has an extremely low probability for two unrelated individuals from across the globe having identical genotypes. All SNPs have an average heterozygosity >0.4 , and the F_{ST} values are all <0.06 on 44 tested populations, making these a universally applicable panel irrespective of ethnicity or ancestry. We selected an additional 181 SNPs based on the following criteria: minor allele frequency >0.4 , alleles with known low polymerase error, high coverage in the dbSNP database (>1000 counts; <http://www.ncbi.nlm.nih.gov/SNP>), low linkage (>500 -kb apart), no more than one additional SNP with minor allele frequency >0.1 in the amplicon, and no known association with disease. Primers encompassing these 266 SNPs were designed with GC content of $<66\%$ and the median amplicon length of 109 nucleotides (minimum, 100 nucleotides; and maximum, 130 nucleotides). Primer sequences are included in [Supplemental Table S2](#).

Targeted Amplification and Sequencing

The dd-cfDNA assay is based on targeted amplification of DNA regions harboring 266 SNPs and the measurement by NGS of each allele contribution at each SNP position. cfDNA extracted from 1.25 mL plasma or reference materials (described above, used at 3, 8, or 60 ng) was preamplified in a single multiplex reaction with 266 primer pairs for 15 cycles. Preamplified material was further amplified using 48 limited complexity multiplexes (1 to 11 targets per reaction) on the Access Array microfluidic system (Fluidigm, South San Francisco, CA). Index sequences and Illumina sequencing adapters were added to each sample DNA by PCR, and the sample was qualified and quantified by capillary electrophoresis. Up to 16 amplified samples were pooled in equimolar amounts, purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA), and sequenced on an Illumina MiSeq instrument.

Sequencing Data Analysis and SNP Allele Counting

The bioinformatic pipeline is hosted on a cloud-based genome informatics and data management platform (DNAnexus, Mountain View, CA) to provide a secure environment to host the clinical sequencing data, an extensible pipeline that can handle increased capacity without modification, and a sealed package to run the validated process that is fixed. Quality control (QC) processes and metrics were built around the pipeline to ensure proper upload, processing, and results generation. Each component was verified and documented in standard operating procedures for critical parameters, and a set of critical files for verification and testing was established.

The SNP allele counting pipeline included open source and custom software. Bcl files were transferred to the cloud environment and processed to FASTQ files. FASTQ files were trimmed by TrimGalore! version 0.3.7 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore) to remove barcode sequences (trimming quality minimum = 30). Trimmed FASTQ files were aligned to a custom reference specifically designed for the assay. The custom reference was generated by concatenating sections of genome comprising the targeted amplicons and 500-base flanking regions on both sides of each amplicon using the hg19 assembly (Supplemental Appendix S1). Alignment was accomplished with BWA MEM version 0.7.9 (<http://bio-bwa.sourceforge.net>) using Illumina 1.3 to 1.6 format. SAM files were generated as intermediate output files of this step. Samtools version 0.1.19 (<http://samtools.sourceforge.net>) was used to convert SAM files into BAM files, followed by BAM file sorting and indexing. Pileup was accomplished by using mpileup of Samtools. Minimum base quality of 30 and maximum number of reads per SNP of 25,000 were used. Raw pileup files were produced as input to a subsequent step of allele counting and tabulation. Each pileup file contained base calls and base qualities for each position sequenced in the target amplicons for each sample. Each raw pileup file was parsed and reformatted into a VCF file and pileup summary file as the final output of the SNP allele counting pipeline.

Percent dd-cfDNA Calculation

The counts generated by NGS for each allele at each SNP locus were examined, and the allele with a higher number of counts was assigned primary and the other alternate designation. SNPs with total coverage $<1000\times$ or with more than two alleles were excluded from further analysis. The alternate allele frequency was calculated as the count of alternate alleles/the total count of alternate and primary alleles. Background levels of alternate allele, resulting from amplification or sequencing error, were subtracted from the alternate allele frequency at each SNP site. The amount of background subtracted from the allele frequency was 4.14 multiplied by the background signal observed across non-SNP positions.

The value of 4.14 was empirically determined following the observation that error rate at SNP positions is typically higher than at non-SNP positions. The amount of background subtracted was generally 0.03% to 0.05%.

The recipient genotype at each SNP was inferred from the background-corrected alternate allele frequency. A statistical procedure was used to infer a recipient heterozygous cutoff in the range between 10% and 25%. SNPs that had alternate allele frequency greater than or equal to a given cutoff were called as recipient heterozygous, and SNPs that had an alternate allele frequency less than the cutoff were called as recipient homozygous. The SNPs called as recipient heterozygous were not used further to estimate % dd-cfDNA. A variable recipient heterozygous cutoff was necessary to produce a high accuracy estimate for samples with low and high donor concentrations.

The percent dd-cfDNA was estimated from the background-corrected alternate allele frequencies of the recipient homozygous SNPs. To further reduce the influence of outliers, the highest 5% of SNPs called as recipient homozygous were discarded and the mean alternate allele frequency of the remaining SNPs was calculated. Finally, the percent dd-cfDNA was estimated by multiplying the mean donor allele frequency with a multiplier based on the level of relatedness between a donor and recipient. The multiplier was determined based on the 5% fraction of discarded SNPs and the expected distribution of donor genotypes among the recipient homozygous SNPs (Table 1).

Sample Quality Control

To ensure accurate and robust dd-cfDNA reported results, several quality control metrics were developed, and for each metric a cutoff value was chosen, below or above which no result was returned (Table 1): i) coverage variability, defined as the CV of the total read counts across all SNPs; ii) the fraction of recipient homozygous SNPs; iii) the number of recipient homozygous SNPs; iv) background; and v) fraction of SNPs with only one allele present.

Analytical Validation Plan and Analysis

A set of studies were performed to establish the analytical performance characteristics of the dd-cfDNA assay, including determining limit of blank (LOB), lower limit of detection (LOD), lower limit of quantification (LOQ), linear range, accuracy, precision, and process reproducibility. The upper limit of detection was not addressed in this study because the percent dd-cfDNA calculation has a set upper limit of detection at 25% dd-cfDNA. LOD and LOQ refer to lower limit of detection and lower limit of quantification, respectively. Samples (1115; 168 unique) were tested by four different operators using two microfluidic systems and four sequencing instruments, with each replicate run on a different day as follows: i) For LOB: six replicates of each of the four fragmented gDNAs (with no donor genome

Table 1 Expected SNP Homozygosity and Quality Control Metric Cutoffs for Different Degrees of Relatedness between a Donor and a Recipient

Donor/recipient relationship	% Recipient homozygous SNPs with allele identical to donor	% dd-cfDNA calculation multiplier	Maximum coverage variability cutoff	Acceptable fraction of recipient homozygous SNPs	Minimum No. of recipient homozygous SNPs	Maximum background	Minimum fraction of SNPs with only one allele
Unrelated	25	2.11	0.8	>0.3 and <0.7	60	0.1	0.1
Cousins and great aunts/great uncles	31.25	2.45	0.8	>0.3 and <0.7	65	0.1	0.125
Grandparents/aunts/uncles/half siblings	37.5	2.92	0.8	>0.3 and <0.7	72	0.1	0.15
Parent/child	50	4.22	0.8	>0.3 and <0.7	90	0.1	0.2
Siblings	56.25	4.75	0.8	>0.3 and <0.7	103	0.1	0.225

dd-cfDNA, donor-derived cell-free DNA; SNP, single-nucleotide polymorphism.

added) at three different input masses, 3, 8, and 60 ng (72 samples total); 26 replicates of HCT116 fragmented gDNA at 8 ng and six replicates of each of the 15 cfDNA samples obtained from healthy volunteers (90 samples total). All of these samples have only one genotype and therefore are blank for cfDNA from a second or donor genome. Input mass for the healthy volunteer cfDNA samples was not controlled so that samples tested reflect the range of yields obtained. The input mass for these samples varied among donors, but the within-donor replicates had the same input mass. ii) For LOD, LOQ, and Linearity: twelve replicates of each sample from the three reference panels at input mass of 3 and 8 ng for each spiked-in level, and six replicates of each sample from the three reference panels at input mass of 60 ng for each spiked-in level. In addition, six replicates of each of the three cfDNA reference panels at 8 ng input mass for each spiked-in level. iii) Within-Run Precision: duplicates at two targeted spiked-in levels, 0.60% and 2.00%, within each of 24 runs. iv) Across-Run Precision: twelve replicates of each sample from the three reference panels at input mass of 3 and 8 ng for each spiked-in level, and six replicates of each sample from the three reference panels at input mass of 60 ng for each spiked-in level. For clinical samples, blood was collected in two Cell-Free DNA BCT tubes (Streck, Omaha, NE) and processed independently by different operators on different days.

Statistical analysis was performed using R version 3.2.0, 64 bit (The R Foundation for Statistical Computing, Vienna, Austria). Analytical parameters were calculated using guidance provided by the Clinical and Laboratory Standards Institute guidance document EP17-A2.²⁰

LOB estimates were calculated using the nonparametric method recommended in EP17-A2 via the R quantile function. All replicates of blank samples passing sample QC metrics were included for analysis. Limit of detection estimates were calculated as follows: $LOD = LOB + c_p SD_L$, where SD_L is the standard deviation of all replicates pooled across unique low-level

samples, and c_p is a multiplier reflecting the degrees of freedom of SD_L . No normality transformation was required before applying this method. Accuracy and linearity analyses using spiked-in samples required the reference, or truth, values to be greater than or equal to the limit of quantification, and to have measured dd-cfDNA values greater than or equal to the limit of detection. Accuracy and linearity analyses using Streck samples required only the latter restriction. To properly reflect run-to-run variability, all accuracy and linearity parameters are calculated by first fitting simple linear regression models within a run, and then averaging across runs.

LOD Assessment for Related Donor and Recipient Pairs

Because of the limited availability of reference material from transplant recipients with related donors, for related donor and recipient pairs, the LOB and LOD were estimated using the alternate allele frequency information from individual SNPs adjusted for genotype frequencies based on degree of relatedness. This estimation was performed using fragmented gDNA panels at input mass of 3 and 8 ng.

First, a set of SNPs that had no alternate alleles for individual samples was identified. These were either homozygous SNPs from blank samples or homozygous SNPs with known identical genotype between the donor and the recipient. Second, a zero cutoff was calculated as the 95th percentile of a normal distribution fit to the low alternate allele frequencies of these homozygous SNPs without a donor alternate allele and with alternate allele frequency <0.25%. The zero cutoff was 0.145%. Third, the fraction of recipient homozygous SNPs with alternate allele frequency higher than the zero cutoff was calculated for each of 180 blank samples (no spike-in) from the fragmented gDNA panels and healthy volunteer cfDNA. The 95th percentile of the fraction higher than the zero cutoff from these samples was 0.163. This is the fraction non-zero LOB that was subsequently used to define the LOD for

samples with related donor and recipient. Fourth, the fraction of non-zero recipient homozygous SNPs (alternate allele frequency higher than the zero cutoff) was calculated for each sample from reference panels. Because the percentage of recipient homozygous SNPs that is expected to carry the same allele as a donor SNP in unrelated donor/recipient pairs is 25% (Table 1), the maximum average fraction of non-zero SNPs in unrelated pairs is 0.75. The fraction of non-zero SNPs for different related donor/recipient pairs was modeled based on the expected genetic similarity and the maximum average non-zero SNPs for each sample from reference panels. Finally, an LOD for related donor/recipient pairs was defined based on these data. LOD is defined (Clinical and Laboratory Standards Institute EP17-A2) as the minimum sample amount at which 95% of the samples are detectable. Therefore, LOD for related donor/recipient pairs was defined as the sample with lowest percent dd-cfDNA for which 95% of the repeats are higher than the LOB.

Process Validation

The full assay process was validated end-to-end by running 45 mock samples, both spiked-in samples and

blanks, from sample accessioning through the laboratory workflow, data analysis, and reporting. Subsets of these samples were assigned to commonly encountered workflow scenarios and tested to ensure that samples could be reliably tracked and tested and results reported. Scenarios included discrepant sample information handling, retesting, and reanalysis.

Results

Clinical-Grade NGS Assay

We developed a clinical-grade NGS assay to measure the fraction of dd-cfDNA in transplant patients (Figure 1). cfDNA is purified from plasma, and selected SNPs are amplified and sequenced to establish the allele counts for each of the two possible alleles for each SNP. The relative proportions of donor and recipient alleles are assigned and calculated from the sequence data and used to estimate the overall proportion of cfDNA from the donor. The assay does not rely on prior genotyping of donor nor recipient. Percentage of donor-derived cfDNA is computed on a validated cloud-based analysis pipeline incorporating open source and custom bioinformatic tools. Results are verified

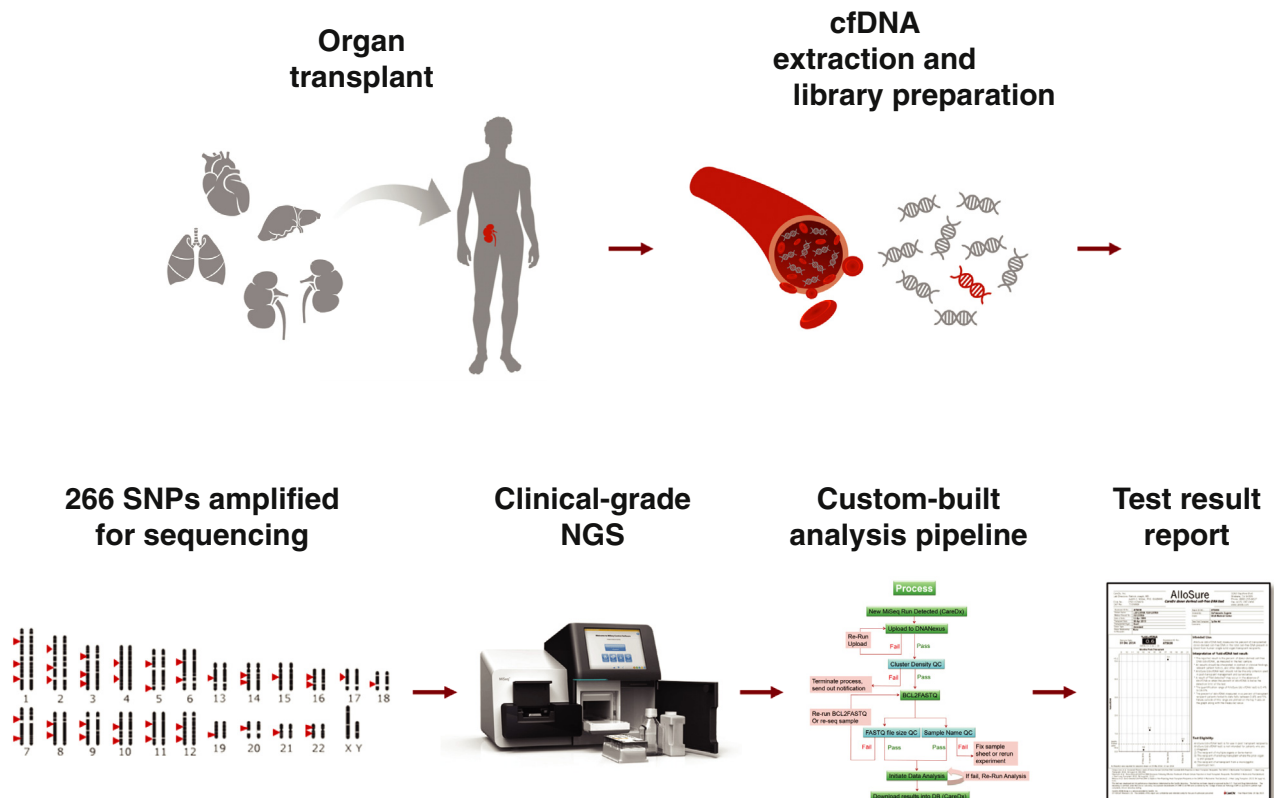


Figure 1 Clinical-grade dd-cfDNA assay workflow. Blood is drawn from a transplant recipient and delivered to the Clinical Laboratory Improvement Amendment laboratory in cfDNA preservation tubes. cfDNA is extracted and next-generation sequencing (NGS) library is generated by PCR amplification of a select panel of 266 single-nucleotide polymorphisms (SNPs). Libraries are sequenced and data are analyzed by custom-built bioinformatic pipeline hosted on a cloud-based genome data management platform. Relative fraction of dd-cfDNA is computed, results are verified by quality control software, and a summary report is automatically generated. All steps are performed using an established documented laboratory workflow and locked algorithms for sequence analysis, % dd-cfDNA computation, and quality control.

by custom quality control software, and a summary is automatically generated. All steps are performed using an established laboratory workflow and locked algorithms for sequence analysis and % dd-cfDNA computation. The low level of dd-cfDNA found in plasma of transplant recipients requires high precision amplification and sequencing. The validity of the NGS assay was established by comparison to results from an 850,000 SNP array performed on reference standards. In addition, methods-based proficiency testing was performed using the Genome-in-a-Bottle and National Institute for Standards and Technology sample (RM 8398), and the results corresponded to the established sequences (data not shown). After the test development, the standard operating procedures, analysis pipeline, QC parameters, and data analysis plan were determined and locked. Analytical validation testing was performed in Clinical Laboratory Improvement Amendment–certified and College of American Pathologists–accredited laboratory in a version-controlled and procedure-locked laboratory workflow, including results generation by the NGS analysis pipeline, % dd-cfDNA calculation, and application of QC metrics.

Reference Materials

Analytical validation of dd-cfDNA using transplant recipient plasma is hampered by the low abundance of cfDNA in blood and the difficulty in obtaining a sustainable source of patient-derived material spanning various predetermined fractions of dd-cfDNA in total cfDNA. Reference materials were therefore designed and developed to represent predefined percentages of dd-cfDNA across a dynamic range previously observed in

transplant recipients, 0.25% to 12%.^{7–9} Availability of large quantities of these reference standards allowed for multiple replicate testing at each point in the dynamic range at each of three different total input masses to assess assay precision and variability.

The amount of total cfDNA in plasma varies among transplant recipients. The distribution of total cfDNA amounts in transplant populations was characterized using purified cfDNA from 238 heart and 185 kidney transplant recipients from D-OAR and DART studies, respectively (Figure 2). Reference panels were diluted to three different input masses, 3, 8, and 60 ng, representing the 1st, 17th, and 86th percentile, respectively, of the mass input of cfDNA from clinical samples. Data for 8 ng are presented below, and data for 3 and 60 ng are in Supplemental Figure S1.

Performance Characterization

To characterize the performance of the dd-cfDNA assay, including determining LOB, LOD, LOQ, linearity, accuracy, and precision, 1026 samples (101 unique) were tested. On average, 238 of 266 SNPs assayed per sample had coverage $>1000\times$ and no more than two alleles and were, therefore, included in the analysis. Of samples, 42 (4%) failed during processing or sample QC and were therefore not included in the analysis.

In accordance with the Clinical and Laboratory Standards Institute EP17-A2, LOB was empirically determined as the 95th percentile of 180 single-genome–containing (blank for a donor genome) samples to equal 0.10% dd-cfDNA (Figure 3A). The median % dd-cfDNA value for blank samples was 0.02%.

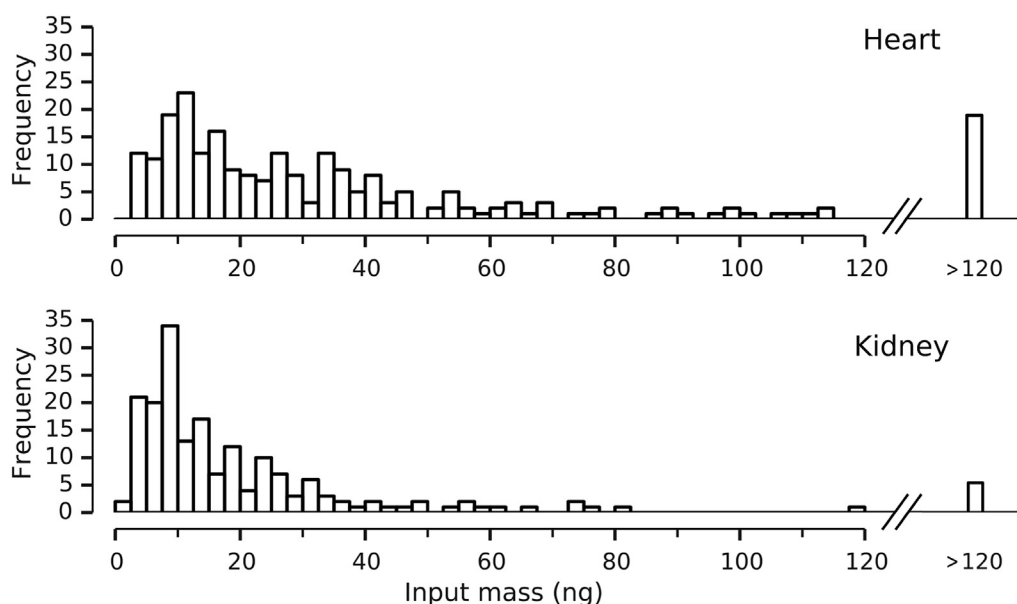


Figure 2 Total cfDNA assay input amounts in transplant recipients. Distribution of cfDNA assay input mass (ng) for 238 heart (top panel) and 185 kidney (bottom panel) transplant patients. cfDNA mass was determined by real-time quantitative PCR (qPCR) using assays designed for *PDCD1* and *ERCC5* genes. These qPCR assays are 106 and 103 nucleotides, respectively, matching the length of the amplicons in the dd-cfDNA assay.

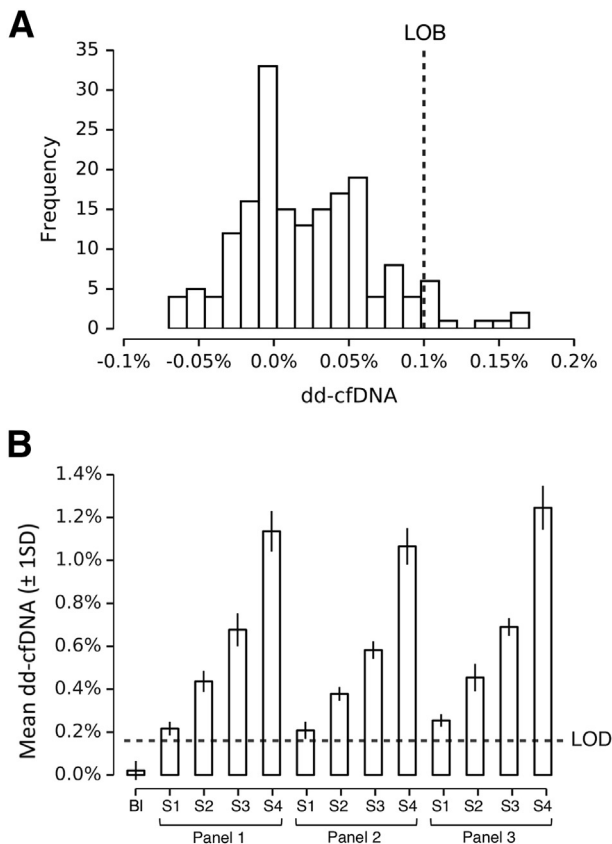


Figure 3 dd-cfDNA assay limit of blank (LOB) and limit of detection (LOD). **A:** LOB (dotted line) was determined as the 95th percentile of % dd-cfDNA for 180 blank samples (no donor genome present). **B:** Mean % dd-cfDNA for the four lowest levels (S1, S2, S3, and S4, 9 to 12 replicates each) of all three reference panels at 8-ng input mass are shown. Dotted line represents LOD, 0.16%. Error bars represent SD (**B**). $n = 180$ blank samples (**A** and **B**); $n = 319$ total samples (**B**). Bl, blank.

LOD is dependent on both the number of genome copies in the assay and the number of SNPs that differ between donor and recipient. For 8 ng input mass of sample with unrelated donor-recipient pair, LOD was 0.16%; for 3 and 60 ng input mass, LOD was 0.19% and 0.15%, respectively (Figure 3B and Supplemental Figure S1, A and B). Three nanograms of sample with 0.20% dd-cfDNA contains approximately 2 genome equivalents of dd-cfDNA; thus, the assay can distinguish between 0 and 2 genome equivalents of dd-cfDNA (Supplemental Table S3). For samples where donor and recipient DNA differ in the least number of SNPs (ie, where donor and recipient are siblings), LOD is 0.22% and 0.28% for 8 and 3 ng of input, respectively (Figure 4 and Supplemental Figure S1C).

The precision of the assay was determined across 12 (for 3 and 8 ng input mass) or six (for 60 ng input mass) replicates of the reference samples, and the limits of that precision were identified to determine the LOQ. Figure 5A shows the precision profile for all samples. As expected, higher fractions of dd-cfDNA yield lower CVs, with a mean CV of 7.2% across all samples tested. The LOQ is defined as the lowest level of % dd-cfDNA measured at or above the

LOD at which the CV is <20%. As all of the samples tested at 8 ng input mass had a CV <20%, for unrelated donor/recipient pairs the LOQ equals the % dd-cfDNA for the lowest sample tested, 0.20%. The LOQ for highly related donor/recipient pair is equal to LOD, 0.22% (Table 2). The highest % dd-cfDNA sample tested measured 16% dd-cfDNA and had a CV <20%. Therefore, the upper limit of quantification is 16%. The upper limit of detection is dictated by the assay design as 25% dd-cfDNA.

The accuracy of the assay was established by comparing measured % dd-cfDNA results to the digital PCR results obtained by the manufacturer of the reference materials (Figure 5B). To assess the linearity of the dd-cfDNA assay within the quantitative range, a simple linear regression was fitted for each panel, input amount, and testing batch, and inferred across batches (Figure 5B). The mean of within-batch slopes is 1.275, mean of y-intercepts is -0.00083, and mean

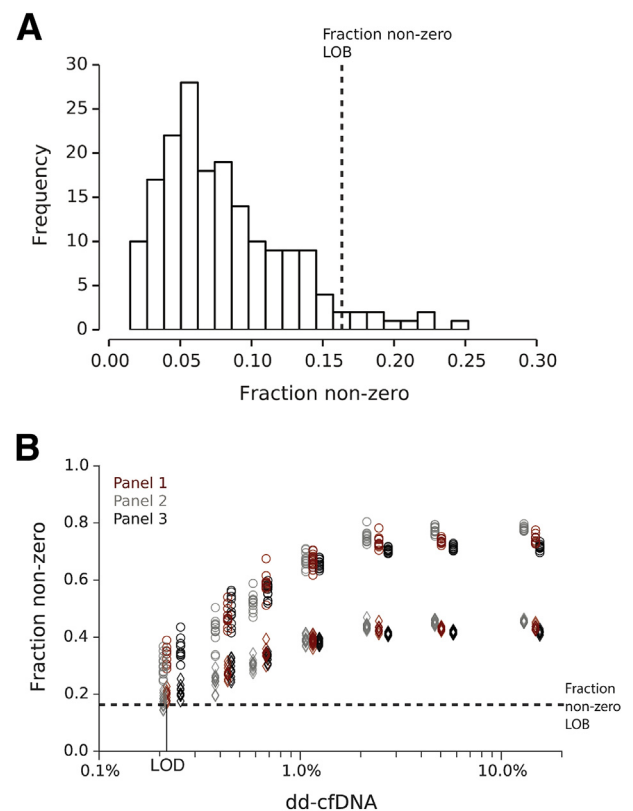


Figure 4 Limit of detection (LOD) for dd-cfDNA from transplant recipients with closely related donors. **A:** Fraction non-zero limit of blank (LOB) is determined as the 95th percentile of the fraction of recipient homozygous single-nucleotide polymorphisms (SNPs) with alternate allele frequency higher than the zero cutoff for 180 blank samples (0.163, dotted line). **B:** The fraction of non-zero recipient homozygous SNPs (alternate allele frequency higher than the zero cutoff) is shown for each sample from reference panels at 8-ng input mass (circle; red, gray, and black for panels 1, 2, and 3, respectively). The fraction of non-zero SNPs for closely related donor-recipient pairs (siblings) was modeled based on Hardy-Weinberg principle (diamonds). The sample with the lowest % dd-cfDNA for which 95% of the repeats are higher than the LOB represents the LOD for the samples from closely related donor and recipient, 0.22% (vertical line).

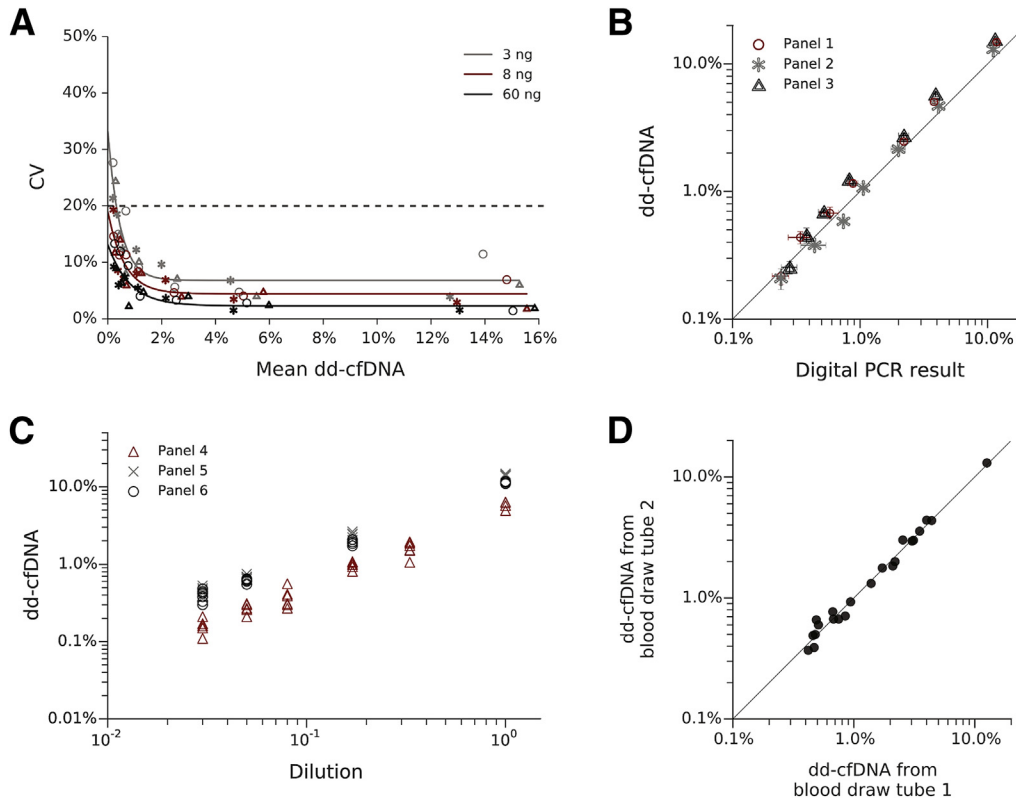


Figure 5 Analytical performance of dd-cfDNA assay. **A:** Assay precision and limit of quantification (LOQ). Twelve replicates of each panel member at 3 (gray) and 8 ng (red) input mass, and 6 replicates at 60 ng (black) input mass were tested on different days by different operators across multiple instruments. The CV ($CV = SD/mean \times 100$) for each of seven members from each of three panels is shown [panel 1 (circles), 2 (stars), and 3 (triangles)]. An exponential decay function was chosen as a model for the relationship between CV and mean dd-cfDNA spiked-in percentage, such that $CV \approx \alpha + \beta \cdot \exp(-\gamma \cdot \text{dd-cfDNA})$. This model was fit using nonlinear least squares. The LOQ was defined as the lowest level of % dd-cfDNA measured at or above the limit of detection at which the CV was $<20\%$. For 3-, 8-, and 60-ng mass input, LOQ is 0.37%, 0.22%, and 0.2%, respectively. **Dotted line** represents 20% CV. **B:** Assay linearity. Results from 8-ng input mass are shown relative to orthogonal validation of reference material by digital PCR of the *EGFR T790M* gene [panel 1 (circles), 2 (stars), and 3 (triangles)]. Digital PCR result was multiplied by 2 to correct for single-gene versus two-copy genome measurement. Vertical error bars represent SD of the % dd-cfDNA, horizontal error bars represent SD of digital PCR assay. Limited replicates of digital PCR at low dilutions resulted in large measurement error. **C:** Assay linearity in cfDNA sample mixtures from healthy volunteers diluted with recipient cfDNA. x Axis denotes fold dilution of the initial cfDNA mixture, which is set to 1 (10^0). **D:** High assay reproducibility in clinical samples. Blood was collected in two separate blood collection tubes from the same venipuncture from each of 22 transplant recipients. cfDNA was extracted from plasma and % dd-cfDNA assayed across multiple days, reagents, and operators.

R^2 value is 0.99819. These data indicate a linear and accurate assay with minimal proportional or systematic bias.

To further validate the performance of the assay, reference samples were constructed from cfDNA extracted from

plasma drawn from normal healthy volunteers using Streck BCT tubes (Figure 5C). Three sets of cfDNA samples were prepared by mixing cfDNAs from two healthy volunteers and performing serial dilutions of the sample with recipient cfDNA. Linearity of the assay was confirmed by linear

Table 2 dd-cfDNA Assay Performance Characteristics (% dd-cfDNA)

Performance characteristic	dd-cfDNA input		
	3 ng	8 ng	60 ng
LOB	0.10	0.10	0.10
LOD			
Unrelated/distantly related	0.19	0.16	0.15
Closely related	0.28	0.22	0.20
LOQ			
Unrelated/distantly related	0.37	0.20	0.20
Closely related	0.37	0.22	0.20

dd-cfDNA, donor-derived cell-free DNA; LOB, limit of blank; LOD, lower limit of detection; LOQ, lower limit of quantification.

Table 3 dd-cfDNA Assay Precision

Assay precision	Sample(s)	Mean CV (%)
Within run	0.6% dd-cfDNA	9.2
	2% dd-cfDNA	4.6
Across runs	3-ng input	9.9
	(0.37%–15.5% dd-cfDNA)	
	8-ng input	6.8
	(0.2%–15.5% dd-cfDNA)	
	60-ng input	4.5
	(0.2%–15.5% dd-cfDNA)	
Between clinical samples	$<2\%$ dd-cfDNA	7.7
	$\geq 2\%$ dd-cfDNA	4.5

dd-cfDNA, donor-derived cell-free DNA.

regression fit to six replicates of these reference sample sets, calculated as above for the reference materials, with mean R^2 value of 0.9914934 (Figure 5C).

Finally, assay precision was assessed across the quantifiable range within run, across runs, and in clinical samples derived from heart transplant recipients (Table 3). Within-run mean CV was determined based on 22 runs with paired 0.6% and 2% dd-cfDNA samples at 8-ng input mass. Across-run CVs were computed for the quantifiable range for 3-, 8-, and 60-ng input mass. Blood was drawn and cfDNA derived from two independent cfDNA preservation tubes (Streck) from 22 transplant recipients. The % dd-cfDNA was determined in independent runs, and results compared (Figure 5D). The average CV for samples <2% dd-cfDNA was 7.7%; and for samples >2% dd-cfDNA, 4.5%. These data indicate a highly reproducible and robust assay.

Process Validation

Rigorous process validations demonstrated a robust assay system that accurately tracked and analyzed samples from accessioning through the laboratory workflow, analysis pipeline, and reporting. Summarized results of the accessioning, testing, and reporting scenarios tested are shown in Supplemental Table S4.

Distribution of dd-cfDNA in Plasma from Transplant Recipients

Previous studies have described levels of % dd-cfDNA in most transplant patients to vary between 0% and 2%.^{7,8} A total of 185 plasma samples collected from 101 heart transplant patients in the multicenter observational CARGO II study were analyzed for dd-cfDNA. The performance and the linear range of the % dd-cfDNA assay matched the range for dd-cfDNA present in plasma from this representative set of heart transplant patient samples (Figure 6A).

Increased dd-cfDNA Correlates with Biopsy-Proven Transplant Rejection

To assess the validity of dd-cfDNA as a biomarker of acute cellular rejection in organ transplant recipients, plasma levels of dd-cfDNA were determined in heart transplant recipients with a biopsy-confirmed rejection and compared to levels of dd-cfDNA in nonrejecting heart transplant recipients. Fifty-three samples were selected from the CARGO II study based on clinical data that defined the samples and consensus pathologist reading of endomyocardial biopsy specimens. Rejection was defined as agreement among two or more pathologists that the biopsy grade was 2R or 3R, according to the International Society for Heart and Lung Transplantation guidelines.²¹ Samples collected from patients without rejection (quiescent) were selected to match the

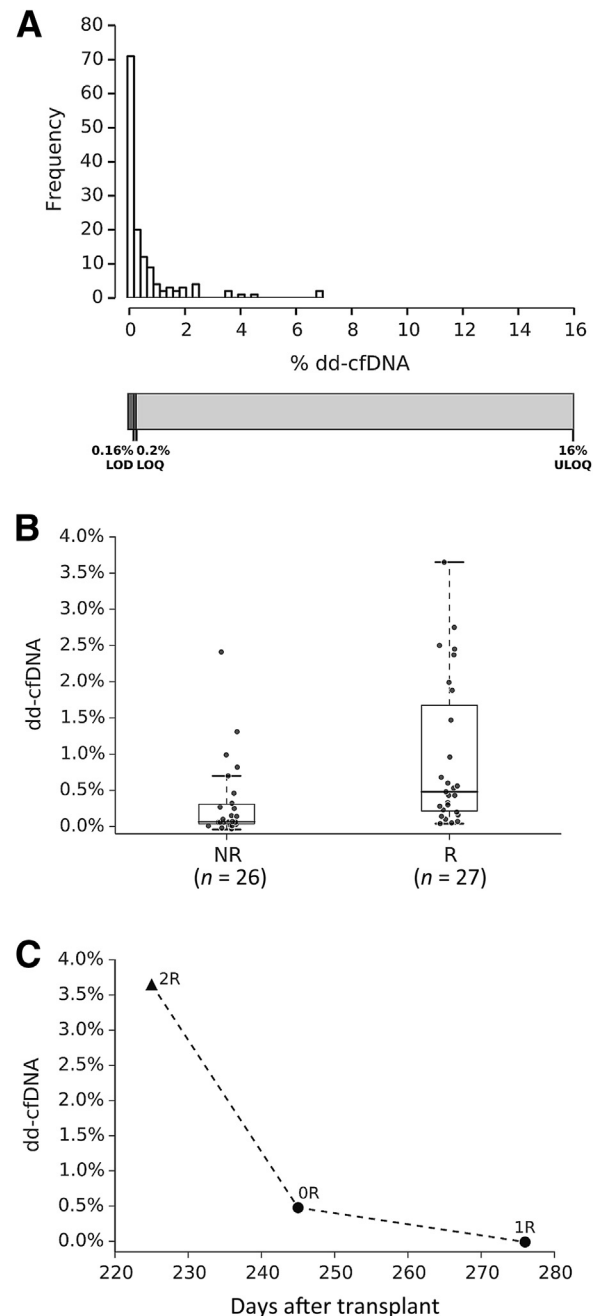


Figure 6 Assay performance and validation in clinical samples. **A:** The linear range of the assay matches the physiological range of % dd-cfDNA for 136 transplant recipient samples from the Cardiac Allograft Rejection Gene Expression Observational (CARGO) II study. **B:** dd-cfDNA levels in patients experiencing moderate-severe rejection as determined by biopsy pathology (R) compared to samples from patients with no evidence of rejection (NR). Mean and median % dd-cfDNA in rejection-associated samples are 2.97- and 7.38-fold higher, respectively, than in nonrejection. **C:** dd-cfDNA was measured in three longitudinal samples from a heart transplant patient (CARGO II). Individual visits are shown as circles (no rejection or mild rejection) or triangle (moderate or severe rejection) and are annotated with the biopsy pathology grade (International Society for Heart and Lung Transplantation 2004 system). The dd-cfDNA is reduced after treatment (three doses of 250-mg prednisone at the time of rejection). $P = 0.008$ by t -test (B). LOD, lower limit of detection; LOQ, lower limit of quantification; ULOQ, upper limit of quantification.

demographic metrics and days after transplant of the rejection set. Nonrejection was defined as consensus among three or more pathologists that the endomyocardial biopsy was grade 0R. A *t*-test demonstrated a statistical difference between rejection and quiescent samples ($P = 0.008$) (Figure 6B). In longitudinal samples from a heart transplant patient in which dd-cfDNA was elevated at time of biopsy-proven rejection, dd-cfDNA levels decreased after rejection treatment with immunosuppressive drug (Figure 6C). These data suggest utility of this biomarker for monitoring treatment effectiveness in addition to identifying acute cellular rejection.

Discussion

Sensitive noninvasive diagnostic tests for early detection of transplanted organ injury and guided adjustment of immunosuppression remain unmet clinical needs in solid organ transplantation. The most common diagnostic method for acute cellular rejection and antibody-mediated rejection, a biopsy of the organ for histopathological interpretation, is not optimal: it is invasive, dependent on heterogeneous sampling of tissue, and susceptible to substantial interobserver variability.^{22,23} In a large representative multicenter heart transplant study, CARGO II, the overall concordance of biopsy interpretation among cardiac pathologists was found to be 71%, and the positive agreement for biopsy specimens assigned a moderate or severe rejection grade ($\geq 2R$) was $< 30\%$.²² Substantial improvements in short-term transplant survival have been realized; however, long-term survival has remained essentially unaltered. Long-term survival has been compromised because of nephrotoxicity, cardiovascular complications, diabetes, infections, and malignancies resulting from chronic and potent immunosuppression. dd-cfDNA in the recipient's blood and urine is a promising, potentially pan-organ biomarker for monitoring the health of solid organ transplants.^{4–15} Although cfDNA assays for research purposes have been described, a clinical-grade assay has not been reported. In this study, we validated an NGS-based targeted amplification assay that measures the fraction of dd-cfDNA. Validation was informed by NGS guidelines and recommendations from professional organizations (College of American Pathologists, American College of Medical Genetics, Association for Molecular Pathology, American Society for Human Genetics); and performed in a Clinical Laboratory Improvement Amendment–regulated laboratory in accordance with the NGS checklist established by the College of American Pathologists.^{24–26}

The overall performance of the assay was high: sensitive to low levels of dd-cfDNA with precise quantification at those levels. For input mass representing most transplant samples, the LOD was 0.16%, which is lower than previously described cfDNA assays.^{27,28} To obtain sufficient material for validation replicate testing, we designed

reference standards derived from mixtures of fragmented cell line gDNA. Linearity studies using both the reference materials and serially diluted spiked-in samples constructed from blood drawn in Streck tubes showed the same assay performance, confirming the appropriateness of the cell line mixtures as reference materials. The amount of donor DNA in reference standards was estimated by digital PCR using an assay for a point mutation in the *EGFR* gene (T790M). % dd-cfDNA measurements are highly reproducible, both within and between runs. As expected, we observed tighter CVs for samples with higher fraction of dd-cfDNA and those with larger input mass, likely because of larger absolute number of dd-cfDNA molecules in both cases. The variability observed likely stems from sampling bias for low input and low % dd-cfDNA samples, polymerase errors during SNP amplification and sequencing library generation, and errors in NGS process itself.²⁹ The use of high-fidelity polymerase and relatively short read paired-end sequencing (2×82) in this assay minimizes the library preparation and sequencing errors.

By design, the assay detects trace amounts of a second genome in the low levels of cfDNA present in plasma. During the validation and post-validation testing, we did not observe cross-contaminated samples, with blank samples tested measuring lower than the LOD. Cross-contamination of human genomic samples has been reported as an issue in clinical NGS testing settings.³⁰ To detect possible contamination, each test run includes a blank control sample and two control reference samples with known amounts of dd-cfDNA; detection of any dd-cfDNA in control blank sample or increased % dd-cfDNA in reference samples invalidates the entire test run and no results are reported. In addition, each sample is expected to contain a certain number of SNPs that share the same allele between the recipient and donor (based on the level of genetic relatedness) (Table 1); if fewer than the expected number of such SNPs is detected, sample will fail the QC criteria and no result will be reported. National Institute for Standards and Technology Genome in a Bottle reference NA12878 (RM 8398) was used to verify the accuracy of the amplification and sequencing by examining each SNP region and requisite SNP genotypes and comparing them to established values.³¹

Although not limited by the need for donor or recipient genotypes, the design of this dd-cfDNA does lead to several limitations that affect the population that can be served. The upper limit of detection is 25% dd-cfDNA by design. Although there are no reports of dd-cfDNA levels this high in heart or kidney transplant recipients, baseline levels appear to be higher in lung and liver transplant recipients.^{9,11} We continue to evaluate samples from different organ transplants to better define this limit. As with all tests dependent on genomic differences, identification of dd-cfDNA in identical twin donor/recipient pairs is not possible; and the results may be impaired in the donor/recipient siblings from consanguineous marriages. For dual organ transplants from a single donor, the assay identifies

graft injury, but does not distinguish the affected organ. Last, multiple organ transplants (from different donors) require the differentiation of the genotype among several genomes, one recipient and multiple donors. Our current algorithm does not address this constraint and therefore the assay is currently limited to single-organ, single-donor transplants. In multiple organ transplants where donor DNA for each additional transplanted organ is available, it may be possible to modify the assay such that the separately determined donor genotype(s) is (are) used to determine the level of dd-cfDNA in organ-specific manner.

Preliminary clinical validity of this assay is demonstrated in heart transplant recipients with samples from patients with biopsy-proven rejection having significantly increased % dd-cfDNA compared to biopsy-proven quiescent patients. In addition, the levels and distribution of dd-cfDNA in heart transplant recipients reported herein is in accordance with levels and distribution reported previously.^{7,8,10} Although absolute % dd-cfDNA levels may differ among types of assays with varying levels of analytical validation, the ratio of mean rejection over mean nonrejection samples is remarkably similar ($2.9\times$ for the assay reported herein and by Snyder et al⁸). Additional clinical validity and clinical utility studies are needed for dd-cfDNA to be widely implemented in clinical practice. Two studies, D-OAR and DART, have been initiated in conjunction with large numbers of clinical centers. Early collection of samples in these studies was used herein to assess the distribution of cfDNA mass collected from transplant recipients. Ongoing collections will be used in case-control analyses to establish clinical validity for dd-cfDNA and define the distribution of % dd-cfDNA in the reference population. The validation approach for multiple organ transplants will be dependent on the information necessary to differentiate signals. Multiple organ transplant from the same donor (eg, heart/lung) may require validation of different thresholds because of the baseline level of contribution from two organs. The situation of organs from multiple donors will require validation of the analytical methods to disambiguate two donors, but may be bridged to single-organ, single-donor clinical validations.

On collection of evidence in support of correlation of dd-cfDNA with organ injury in clinical validity studies in additional organs, there are several potential indications of use that merit clinical utility studies. First, informing biopsy decisions through enhanced interpretation of histological findings of biopsy, to provide alternative to biopsy if the benefit and risk of biopsy are not clearly supported, and to reduce morbidity and increase cost-effectiveness of transplant recipient surveillance. Second, detection of subclinical organ injury to improve long-term outcomes and survival. Third, immunosuppressive therapy optimization by noninvasive monitoring and thereby reduction of infections and malignancies, and improved long-term survival. The different types of organ transplants and relatively small number of transplants and long-term nature of outcomes

challenge conventional trial designs. For example, we anticipate that the thresholds and baselines may vary among the different types of organ transplants, or for multiorgan transplants. In this regard, an adaptive strategy, as suggested for staged adoption of drugs, merits consideration.³² The proposed adaptive strategy recognizes that evidence is a continuum and may therefore facilitate timely pairing of available evidence and clinical unmet need of specific transplant patients.

In summary, we have developed and analytically validated a clinical-grade NGS test to measure the fraction of dd-cfDNA in plasma of solid organ transplant patients. This test is applicable to single-organ donor-recipient pairs and does not depend on separate determination of either donor or recipient genotypes. We further report preliminary clinical validity results of the assay for heart transplant. Additional prospective clinical validity and utility studies will inform how this clinical tool can be used to manage transplant patients.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.jmoldx.2016.07.003>.

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