Proficiency Testing for and Surveillance of Heart Transplant Health Using Donor-Derived Cell-Free DNA

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Introduction

Cell-free DNA (cfDNA) has been widely adopted as a diagnostic biomarker in prenatal testing and is being investigated for application in screening and recurrence testing in oncology and organ transplantation. cfDNA comprises nucleosomal protected genomic segments, most being short (~160bp) DNA fragments released as a consequence of cell death. cfDNA is readily obtained from plasma and can be quantified despite the low overall abundance and even lower abundance of the subset of molecules targeted for distinct quantification.

Following solid organ transplantation, ongoing monitoring of allograft health is required for individualization and optimization of immunosuppressive therapy. Under-immunosuppression can lead to rejection of the transplanted organ and over-immunosuppression can lead to complications such as infection and cancer. For heart transplant recipients, non-invasive monitoring for acute cellular rejection (ACR) using the FDA-cleared gene expression test, AlloMap, has been widely adopted. However, there are no similar tests available for other solid organ transplants and AlloMap does not address all of the needs for heart transplant monitoring. Donor-derived cell-free DNA (dd-cfDNA) is a biomarker of organ transplant rejection and the resulting allograft damage with potential utility in all solid organ transplant recipients (DeVlaminck et al, Science Transl Med 2014; Gielis et al, Am J Transp 2015; DeVlaminck et al, PNAS 2015). cfDNA has been measured in transplantation using methods ranging from shotgun sequencing to digital PCR, each of which requires prior knowledge of the donor and recipient genotypes.

We developed an assay using Next Generation Sequencing (NGS) to measure differential allele contributions in a panel of amplified SNPs to quantify dd-cfDNA in recipients without prior knowledge of donor or recipient genotype and with potential applicability across organ transplantation. The complexity of NGS assays for cell-free DNA presents multiple challenges encompassing rapidly evolving sequencing technology, quantification of trace amounts of cfDNA and customized computational analysis. For advanced sequencing assays, CAP has identified laboratory, computation, and interpretation as three processes that merit discrete validation. We have validated each of these three process areas for the dd-cfDNA assay. The main objectives of the studies reported here were to 1) characterize analytical performance of the test, 2) determine the utility of dd-cfDNA in heart transplant recipients with stable allograft function, and 3) determine the utility in recipients who experienced rejection.

Materials and Methods

dd-cfDNA Assay Design

266 SNPs with high minor allele frequency, low amplification error, low linkage, minimal polymorphism, and unbiased regarding ancestral heritage were selected for amplification and sequencing. cfDNA extracted from 1.25 ml plasma or reference materials (described below) were amplified using limited complexity multiplexes (1-11 targets per amplification) on the Fluidigm Access Array system. Index sequences were added to each sample and the sample qualified by capillary electrophoresis. Quantified sample amplicons were pooled in equimolar amounts and sequenced on an Illumina MiSeq. An analysis pipeline incorporating open source and custom NGS bioinformatics tools was used to align reads to the SNP regions and determine the number of reads representing each SNP allele. For each sample, SNPs with a minimum of 1000 reads were used in the custom algorithm developed to determine the contribution of donor-derived sequences and calculate the percent of dd-cfDNA.

Assay Validation & Performance Characterization

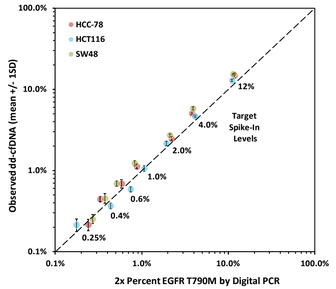
Assay performance was characterized using reference materials consisting of cell line genomic DNA fragmented by sonication to the approximate size of cfDNA extracted from plasma. Multiple reference material panels were assembled with a 'donor' cell line (RKO with EGFR T790M) and different 'recipient' cell lines (SW48, HCT116, and HCC-78). Trace amounts of 'donor' DNA were mixed into 'recipient' DNA at targeted spike-in levels ranging from 0.2% to 12%, and then further defined by digital PCR of the single-copy EGFR T790M mutation in the 'donor' DNA. The reference materials were developed due to the low abundance of cfDNA in human fluids and the difficulty of obtaining a sustainable source of rigorously characterized patient-relevant material. Reference material panels were run at 3ng, 8ng, and 60ng to represent the minimum, 10th percentile, and 80th percentile mass of cfDNA recovered from 1.25ml plasma of transplant recipients.

Accuracy, linearity, precision (across runs, days, and operators), were characterized using the reference panels. The analytical performance was assessed according to standard CLSI guidance (EP17). Linearity was further compared across the quantifiable range using a small number of reference samples assembled from cfDNA extracted from plasma from different normal healthy volunteers. (data not shown)

Clinical-Grade Next Generation Sequencing

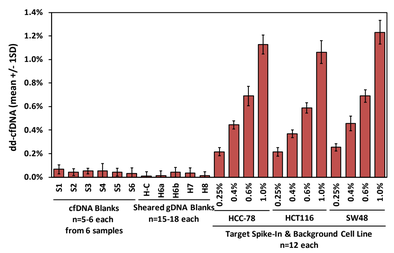
The low level of dd-cfDNA found in plasma of transplant recipients requires high precision amplification and sequencing. The validity of a subset of 266 SNPs of the NGS assay was established by comparison to results from an Illumina CytoSNP-850k SNP array performed on reference material. Additionally, the Genome-in-a-Bottle and NIST sample (RM 8398) was tested and the results compared to the established sequences. (data not shown)

Results: 1. The dd-cfDNA Assay Identifies Low Amounts of 'donor' cfDNA with High Linearity and Accuracy



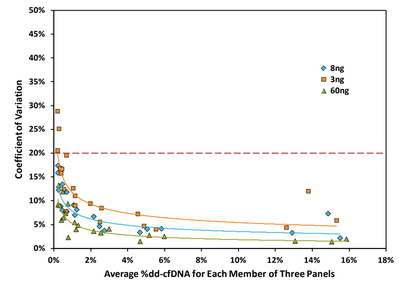
Targeted amplification followed by NGS was used to determine the percent of 'donor' genome present (y-axis) for all three reference material panels (each panel shown in a different color). Results from digital PCR targeting the single-copy EGFR T790M in the 'donor' genome were multiplied by two to represent the genome percentage (x-axis). Target spike-in levels are labeled. Results from 8ng input DNA are shown. This mass represents an amount lower than in 90% of clinical samples, and at the 0.4% spike-in level results in approximately 11 copies of 'donor' genome. A line fit to these points has a slope of 1.27, a y-intercept of 0.0006, and an R^2 value of 0.992. These data indicate a linear and accurate assay with minimal proportional or systematic bias.

2. Very Low Levels of dd-cfDNA are Distinguished From Blank Samples



The dd-cfDNA levels in the lowest spike-in reference materials are reproducibly quantified and differentiated from blank samples. Data from the four lowest levels of all three reference panels are shown. 15-18 replicates of sheared genomic DNA from each cell line (without spike-in) and 5 or 6 replicates of cfDNA from 6 normal healthy volunteers (blanks) are all significantly lower. The Lower Limit of Detection (LLOD) was defined by the level at which 95% of Reference Standard replicates are detected as different from blank. The LLOD is 0.4% dd-cfDNA.

3. Precision is High Across the Quantifiable Range



Replicate runs of reference material were performed on different days by different operators across multiple instruments. 12 replicates of each panel member at 8ng input mass, 12 replicates at 3ng, and 6 replicates at 60ng input mass were used to determine the coefficient of variation (CV = SD/mean×100) for each of seven members from three panels. The Lower Limit of Quantification (LLOQ) was defined as the lowest level of %dd-cfDNA measured at or above the LLOD at which the CV was less than 20%. All replicate sets with target concentration at least 0.4% had a CV less than 20%. Therefore the LLOQ 0.4%

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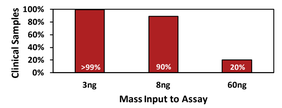
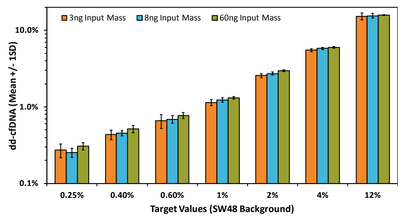
4. Clinical Samples Have Sufficient cfDNA to Be Measured by the Assay

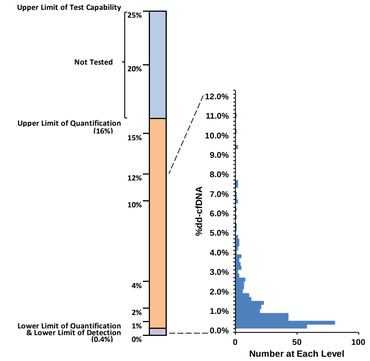


Of 210 heart transplant samples, 99.5% have cfDNA concentrations greater than 3ng, 90% greater than 8ng, and 20% greater than 60ng per 1.25ml plasma, the input amount for the dd-cfDNA test.



All three reference materials were tested, only SW48 is shown. The assay produces equivalently accurate and sufficiently reproducible results at 3ng, 8ng, and 60ng.

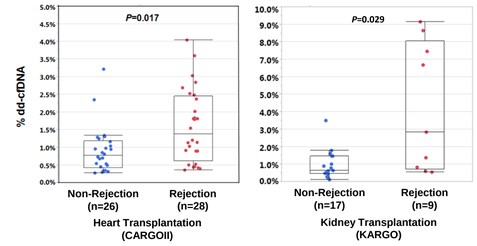
5. dd-cfDNA Assay Performance Characteristics Match the Distribution of dd-cfDNA in Plasma from Heart and Kidney Transplant Recipients



The LLOD (0.4%) and the LLOQ (0.4%) were described in Figures 2 and 3. The Upper Limit of Quantification was defined by the highest reference material with a CV of replicates <20%; the highest tested was 16%. This assay has an upper limit of 25% dd-cfDNA based on the methods used.

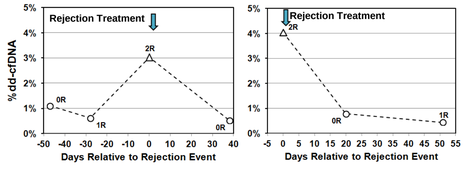
The performance of the assay matches the distribution of dd-cfDNA for transplant recipient samples. The dd-cfDNA distribution of 390 samples from 142 patients is shown in the histogram. The linear range of the %dd-cfDNA assay matches the physiological range for plasma from heart and kidney transplant patients.

6. Increased dd-cfDNA Correlates with Biopsy-proven Transplant Rejection



dd-cfDNA was measured in patient samples from two clinical studies. Samples from patients experiencing moderate-severe rejection as determined by biopsy pathology were compared to samples from patients with no evidence of rejection. Mean %dd-cfDNA in rejection-associated samples is 1.9-fold (heart) or 4.4-fold (kidney) higher than non-rejection.

7. dd-cfDNA Decreases Following Successful Rejection Treatment



dd-cfDNA was measured in longitudinal samples from two heart transplant patients (CARGOII). The dd-cfDNA is reduced following treatment (3 doses of 250mg prednisone at the time shown by the blue arrow). Individual visits are shown as circles (no rejection or mild rejection) or triangles (moderate or severe rejection) and are annotated with the biopsy pathology grade (ISHLT 2004 system). Successful treatment was defined by pathology grade improvement to 0R (no histopathologic damage observed).

Conclusions

The precision and accuracy of the clinical-grade NGS dd-cfDNA assay for use in organ transplantation was demonstrated using custom designed reference material panels independently verified by digital PCR. Results from a large set of clinical samples are well within the performance characteristics of the assay, with critical differences between rejection and non-rejection within the linear range. Longitudinal evaluation of dd-cfDNA suggests utility of this biomarker for monitoring treatment effectiveness in addition to identifying acute cellular rejection. Ongoing studies are evaluating the application of dd-cfDNA to additional organ health monitoring in transplantation.

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