Laboratory validation of CE-marked AlloSeq cfDNA to measure donor-derived cell-free DNA in transplant patients.

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Introduction

In solid organ transplant recipients, circulating donor-derived cell-free DNA (dd-cfDNA) is a noninvasive biomarker of transplant allograft injury[1,2] that may enable more frequent, quantitative, and safer assessment of allograft rejection and injury status. We have developed a targeted next-generation sequencing (NGS) assay that utilizes 202 single-nucleotide polymorphisms located throughout the genome to measure the dd-cfDNA in plasma of transplant recipients. The assay combines a clinical laboratory compatible protocol with streamlined workflow and a fully automated analysis software to accurately quantify dd-cfDNA without separate genotyping of either donor or recipient. Performance validation was conducted with more than 40 samples across a total of 30 runs and established a Limit of Detection (LoD) of 0.23%. Limit of Blank (LoB) and limit of Quantification (LoQ) were determined at 0.18% and 0.23%, respectively. %CV for within run and between run of < 15% when tested with 1% dd-cfDNA. In tests conducted in parallel across three independent laboratories, AlloSeq cfDNA accurately and reproducibly quantified the fraction of dd-cfDNA in 12 samples of various types, including raw plasma samples that were extracted on site.

Methods and Materials

dd-cfDNA Quantification Assay

The concept of the CareDx AlloSeq cfDNA assay is centered around a single multiplex PCR followed by sequencing to determine the fraction of donor-specific nucleotides at 202 selected SNP loci, allowing the quantification of dd-cfDNA with or without prior genotyping of the donor or recipient (figure 1).

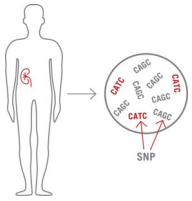

Figure 1. Relative quantification of dd-cfDNA (in red), based on SNP sequence.

The kit includes a locus-specific oligonucleotide primer pool to amplify the targeted regions of interest. In combination with index adapters, a unique cycling protocol amplifies the locus-specific regions and indexes the libraries concurrently. Indexed samples are subsequently pooled and purified together to prepare for sequencing.

After sequencing is complete, an analysis report provides precise percentage of dd-cfDNA present in each sample, in less than 24 hours after sample receipt and cfDNA extraction. Library preparation is completed within four hours of which only one hour is hands on (figure 2).

Figure 2. Concurrent locus-specific and indexing PCR requires minimal hands on time. (TAT: Turn Around Time; HOT: Hands-On Time).

Analysis Software

Results are generated automatically by AlloSeq cfDNA software from the sequencer-generated FastQ file. Multiple recipients can be analyzed in parallel, but the AlloSeq cfDNA Windows software design also allows monitoring %dd-cfDNA levels in a same recipient over time. During monitoring, one or more samples are taken post-transplant. The software calculates %dd-cfDNA from these post-transplant samples and displays the results in a report to compare levels from different samples (figure 4) to support building longitudinal studies. A recipient-only sample can be taken from the recipient prior to transplant (or from a different tissue) and can be included in this calculation. Details of the analysis output is also available for each sample (figure 5) to access sample quality metrics, facilitating potential troubleshooting.

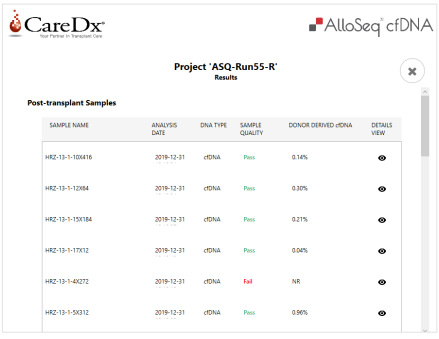

Figure 4. Software interface allows comparison between different timepoints for longitudinal studies. The results page for a project enumerates essential data and analytical results about post-transplant and recipient-only samples in a single, interactive display. The essential data for post-transplant samples are the sample name, collection date, %dd-cfDNA, and whether the quality metrics with a surpass a set of quality thresholds.

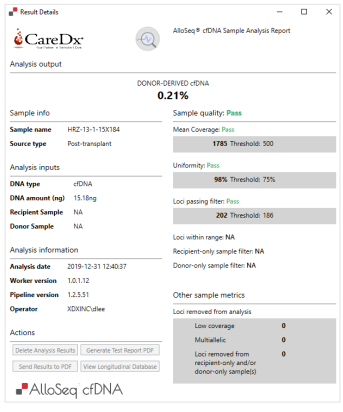

Figure 5. Details of a timepoint with quality control metrics.
The user can view additional data about each sample by clicking a button that displays a sample details dialog box. This dialog shows additional data about the quality metrics and which failed the quality thresholds.

Results

During this study across three independent laboratories, a total of 238 libraries were generated using a DNA input of 10ng with a first pass rate of 96.6% (232/238). Analytical performance was confirmed and measurement reproducibility between sites was evaluated across the entire workflow, from blood same to %dd-cfDNA results, in two experiments.
In the first experiment, two independent cfDNA extractions (Extraction A and Extraction B) were carried out on a same plasma sample from each of four kidney transplant patients at CareDx. cfDNA samples from extraction A were tested at CareDx and cfDNA samples from extraction B were sent to three different laboratories for AlloSeq cfDNA testing (figure 6a). No significant difference in dd-cfDNA % were observed between the two independent extractions.

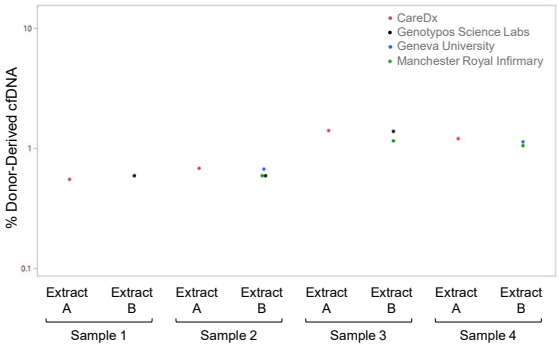

Figure 6a. Relative quantification across sites on cfDNA from two different extraction of the same blood sample. Runs which did not pass Quality metrics are not shown

Next, two plasma samples (tubes A & B) were obtained from each of six kidney transplant patients and were processed for cfDNA extraction at four different laboratories (figure 6b). Again, no significant differences were observed between tubes A (purified and tested at CareDx) and tubes B (purified and tested at the external laboratories), demonstrating the high reproducibility of the results generated by the AlloSeq cfDNA solution.

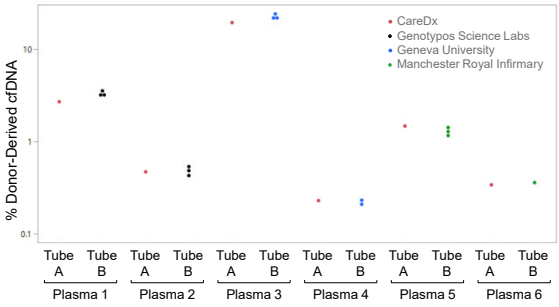

Figure 6b. Relative quantification across sites on cfDNA from two different blood samples from the same patient. Experiments were run in triplicates. Runs which did not pass Quality metrics are not shown

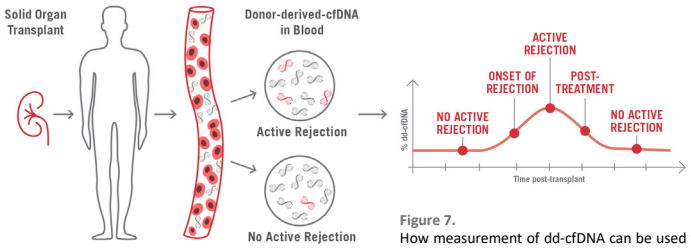

Figure 7.
How measurement of dd-cfDNA can be used for graft monitoring.

Conclusions

This noninvasive streamlined sequencing assay requires minimal hands-on time and can be completed within 24 hours post obtaining recipients cfDNA, on site, providing a critical practical turnaround time preferred in post transplantation surveillance (figure 7). AlloSeq cfDNA is a CE mark approved solution, making it available to transplant patients and clinicians around the world.

References

1. Am J Transplant 2019, 5. doi: 10.1111/ajt.15339.
2. J Am Soc Nephrol 2017, 28: 2221-2232.

AlloSeq® cfDNA

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