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Research Article

The Evolution and Innovation of Donor-Derived Cell-Free DNA Testing in Transplantation

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ABSTRACT

Background: Improvements to the donor-derived cell-free DNA measurement were implemented in the AlloSure assay to achieve faster turnaround time, streamlined laboratory workflow, and a lower limit of detection. Analytical validation established the performance characteristics. Clinical samples were used to demonstrate equivalence to the original assay.

Materials and Methods: AlloSure 3.0 was implemented with a single multiplex library preparation and single read sequencing to streamline the workflow and decrease processing time. The analytical performance of the assay was characterized according to CLSI methods.

Results: AlloSure 3.0 performance is improved relative to the performance of the original AlloSure assay. The reportable range is from 0.12% to 16% donor-derived cell-free DNA (dd-cfDNA). The precision has also improved, with run-to-run coefficient of variation of 2.7%. Following these improvements to the assay, we established 99% concordance to the original version of AlloSure for clinical samples.

Conclusion: Ongoing improvements to the donor-derived cell-free DNA methods resulted in a larger reportable range, decreased turnaround time, and improved precision, while maintaining the same accuracy as demonstrated with the initial release of AlloSure. The improved performance enables greater range for evaluation of dynamic changes that have been demonstrated to inform on low-grade T cell mediated rejectionl.

Keywords: Cell free DNA; Transplantation; Organ rejection; Analytical validation

INTRODUCTION

AlloSure was introduced to the transplant community with rigorous analytical and clinical validation in kidney transplantation [1,2]. Measurement of donor-derived cell-free DNA (dd-cfDNA) by AlloSure enables non-invasive surveillance for graft rejection in kidney transplant recipients based on the initial clinical validation while incorporating the knowledge of the biological variability within kidney transplant recipients and the baseline AlloSure results. Biological variability and baseline (0.21%) were determined from 380 samples from 93 patients that had no evidence of rejection or other graft injury [3]. No-Rejection (NR) status as demonstrated by biopsy had an AlloSure result indistinguishable from a population without any evidence of or concern for rejection. The consistency between AlloSure from NR [2] and from stable-healthy recipients [3] suggests that clinicians and patients can have confidence that a change greater than the 61% change attributed to biological variability will indicate change from healthy to a position of concern for graft health [3]. Additional data supporting clinical utility in borderline and TCMR1A kidney allograft rejection suggested clinically significant changes at 0.5%. These findings support the transition from a discrete threshold to a continuum of change, making the Relative Change Value (RCV) integral for AlloSure interpretation in addition to the absolute threshold [4,5].

AlloSure has also been demonstrated to correlate with rejection in heart transplantation, but with an even lower baseline (0.07%) and a 0.15% threshold for rejection, which is much lower than in kidney [6,7]. These results emphasized the need for a precise, accurate, and reliable result in heart transplantation. Data for lung transplant recipients have also demonstrated the importance of using AlloSure for non-invasive surveillance for rejection [8]. Some lung transplant centers transitioned from bronchoscopy-based biopsy to non-invasive surveillance using AlloSure during

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the Coronavirus Disease-2019 (COVID-19) pandemic due the demands on the bronchoscopy suite and the desire to minimize the interaction of immunosuppressed patients with the healthcare system [9].

Analytical performance must be established to enable individual test results to be confidently reported and interpreted. Standard methods for analytical validation of diagnostic tests have been established and are published by the clinical laboratory standards institute [10]. As illustrated in (Figure 1), there is a level below which the result is indistinguishable from the noise of the measurement system; this level is called the Limit of Detection (LOD). For dd-cfDNA tests with a sufficiently low LOD, a value below the LOD is a good result, as it is quite low and indicates a lack of graft injury. To compute the LOD, the Limit of Blank (LOB) is determined from a set of blank samples (only one genome, no donor), which produce only values that are generated by the noise and measurement error in the system. The LOB is most commonly the 95th percentile of the blanks. To the LOB is added a multiple of measurement variance for the lowest sample for which the values were above the LOB. This defines the LOD as a value that can be confidently stated is measurable and reportable. The LOD is commonly used as the bottom of the reportable range. Patient results below the LOD are reported and used for patient management as "< 0.12%" or similar. However, when a study is performed on a large number of samples, original results from the samples in the group that are below the LOD can be used in group analyses. Each measurement, no matter how large or small, carries with it an amount of uncertainty. This uncertainty becomes part of the overall group median or mean and measures of variability, but with sufficient numbers, an accurate estimate of a median for such a group is possible. The uncertainty of measurements below the LOD requires reporting "< 0.12%" for an individual sample, however such values can be incorporated into calculations for study groups with multiple samples.

To provide a best-in-class analytically validated assay, we have continued to improve the analytical performance of AlloSure, improving the workflow, and enabling efficient turnaround of results. These improvements have been made while maintaining a robust high-quality output that continues to inform clinicians on how best to manage patients with the same results. The established accuracy and reliability was unchanged to enable continued use of the product that has become the standard for non-invasive surveillance for rejection in kidney transplantation.

MATERIALS AND METHODS

Assay

This AlloSure 3.0 assay evaluates a total of 405 Single Nucleotide Polymorphisms (SNPs) by next-generation sequencing, using PCR conditions optimized for multiplexing. The assay is run on the Illumina NextSeq 550 using either mid- or high-output flow cells. The refined methods do not require pre-amplification, which contributes to improving the turnaround time of the test. Aside from the analysis of a larger set of SNPs, the bioinformatics pipeline and computation of the AlloSure results remain unchanged from the original AlloSure assay. As with the original AlloSure assay, AlloSure 3.0 is run in a CLIA/CAP-accredited laboratory (CareDx, Brisbane CA).

The components of the multiplex master mix were based on the preamplification conditions used for the original AlloSure assay [1]. The components were optimized for the AlloSure application, including magnesium chloride concentration, enzyme concentration, dNTP concentration, PCR enhancers, DMSO, and primer concentration. The final conditions and chemistry for the library preparation amplification increased hybridization specificity and melting temperature, resulting in improved assay performance. Final reaction conditions use the Roche FastSart High Fidelity Reaction Buffer, final 4.48 mM $MgCl_2$, 4% DMSO, 200 µM each dNTPs, 35 mM TMAC, 0.2 U/µl FastStart High Fidelity Enzyme, 50% volume cfDNA, and PCR primers at concentrations ranging from 50-180 nM. The PCR amplification library preparation conditions were updated to 95°C for 2 min, 30 cycles of 95°C, 60°C, and 72°C for 30 seconds each, followed by 72°C for 4 minutes.

The furthest distance of the SNP from the sequencing primer is 55 nucleotides, therefore a sequencing read length was optimized to enable enables fewer sequencing cycles and minimize the total sequencing time. No differences were observed in the metrics such as coverage variability or uniformity under the final conditions (single read, 66 cycles) when compared to paired-end reads with up to 82 cycles as used for the original AlloSure assay.

Uniformity is a measure of the read depth variation among the SNPs in the assay. The goal during development was to maximize the fraction of SNPs with normalized reads between 0.5 X to 2 X mean read. Normalized reads for each SNP were calculated using the ratio of SNP read to mean read of the sample for the purpose of the uniformity metric. A second form of uniformity measured during development was coverage variability. Coverage variability is the coefficient of variation of the read depth, calculated by dividing standard deviation of the read depth among the SNPs by the mean read depth.

Study samples

Reference materials were generated and orthogonally characterized by Horizon Discovery as described in Grskovic et al. [1]. Three panels (P9, P11, and P12) were used with target references ranging from 0.10% to 20%. Blank samples are defined as samples with only a single genome represented; they do not contain a second or "donor" genome. These were either reference materials with only the base cell line genome, genomic DNA from cell lines, or cfDNA isolated from individuals without a transplant or other source of a second genome, such as pregnancy. Kidney transplant recipient samples (195) were used to generate data for the clinical accuracy of the test. The kidney transplant samples were selected from commercial testing based on the original AlloSure assay result, then completely de-identified before testing with AlloSure 3.0 for the concordance.

Validation study design and analysis

To establish the analytical performance of AlloSure 3.0, nine independent runs were performed from existing template material. 530 analytical samples were run using two independent sequencers and multiple operators. For the determination of LOB 206 samples were run across the eight runs, 107 cfDNA (no transplant blanks) 93 gDNA; and 6 reference samples with only one genome. The LOB was computed by the non-parametric method recommended

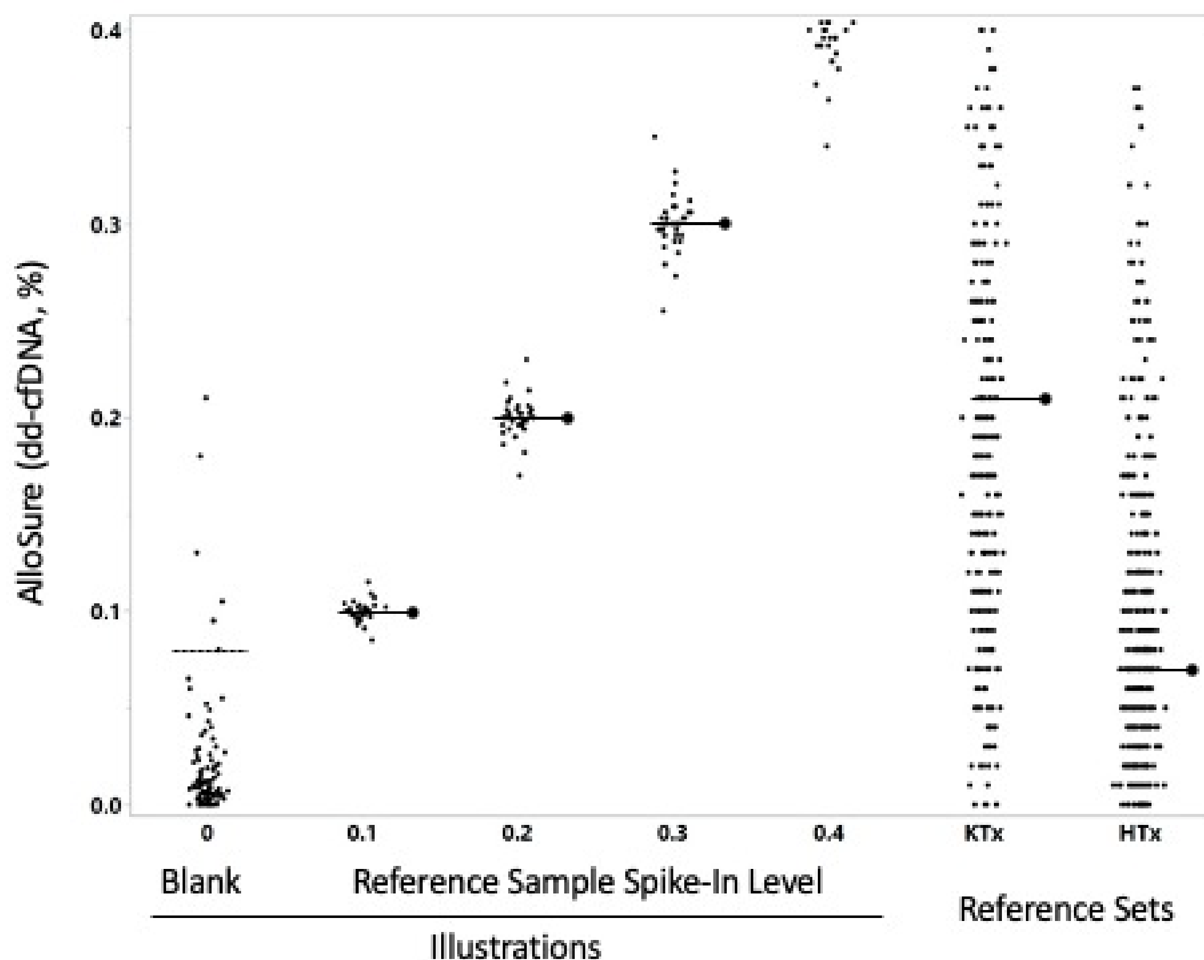

Figure 1: Examples of analytical performance metrics. Five leftmost representative datasets created for illustration, and are not measured values. Blanks show the 95th percentile of 100 samples at 0.8% (dashed line) to demonstrate the calculation of LOB. For 0.1% through 0.4% spike-in reference samples, 33 replicates are shown, each with a mean at the target spike-in value and a CV of 5.2% around each mean. The variance of the lowest measured reference sample (0.1%, since values are all above the LOB) is used to calculate the LOD per CLSI standard methods. Actual reference set data for kidney and heart transplantation [2, 6] are shown on the right side of the plot. Only two digits past the decimal are output for AlloSure, so results are in bands for hundredths of a percent dd-cfDNA. Medians are indicated by a black line with a bubble on the end.

in CLSI document EP-17-A2, the 95th percentile of the set of all available blanks [10].

For the determination of LOB precision, and linearity, 274 samples were evaluated across 8 runs. They were across three different panels of reference samples independently created and characterized as described in Grskovic et al. [1]. For LOD, the lowest reference sample with a value from the original AlloSure assay measured above the LOB was used, with 13 replicates of each reference sample. The LOD was computed in accordance with CLSI EP-17-A2 [10].

Inter-run precision was determined based on a separate set of runs of the reference panels across the reportable range. The CVs for the individual reference samples were determined from 13 replicate runs on 13 days with four operators and on two sequencing instruments. The mean CV was computed for all reference samples within the critical decision range (0.2% to 16%). Intra-run precision was determined in a separate study using 64 replicates of each reference sample at 0.6% and 2.4%.

Clinical validation was determined as concordance with the results generated in the original AlloSure assay by re-testing 195 unique samples on both the original AlloSure assay and on AlloSure 3.0.

RESULTS

A full analytical validation was performed for AlloSure 3.0 to characterize the performance. The study design was similar to that used for the original AlloSure assay analytical validation [1]. Several parameters did not need to be updated since the process impacting those metrics remained the same. The studies demonstrating the lack of impact from interfering substance were not repeated since the enzymatic reactions taking place with the initial extracted cfDNA was the same as the original AlloSure [1].

The updated chemistry and associated PCR primer sets were tested to ensure uniformity of the results prior to analytical validation. High uniformity ensures that the read depth per SNP is sufficiently uniform across the full set of SNPs and within an acceptable distribution. A highly uniform assay has low percentage of poor amplifying SNPs and a low percentage of outlier high amplifying SNPs. AlloSure 3.0 has a mean uniformity of 83%.

Although various data on assay composition or NGS metrics can be reported, especially for development, a properly performed analytical validation is the best indicator of the analytical performance of the assay. The results of the analytical performance

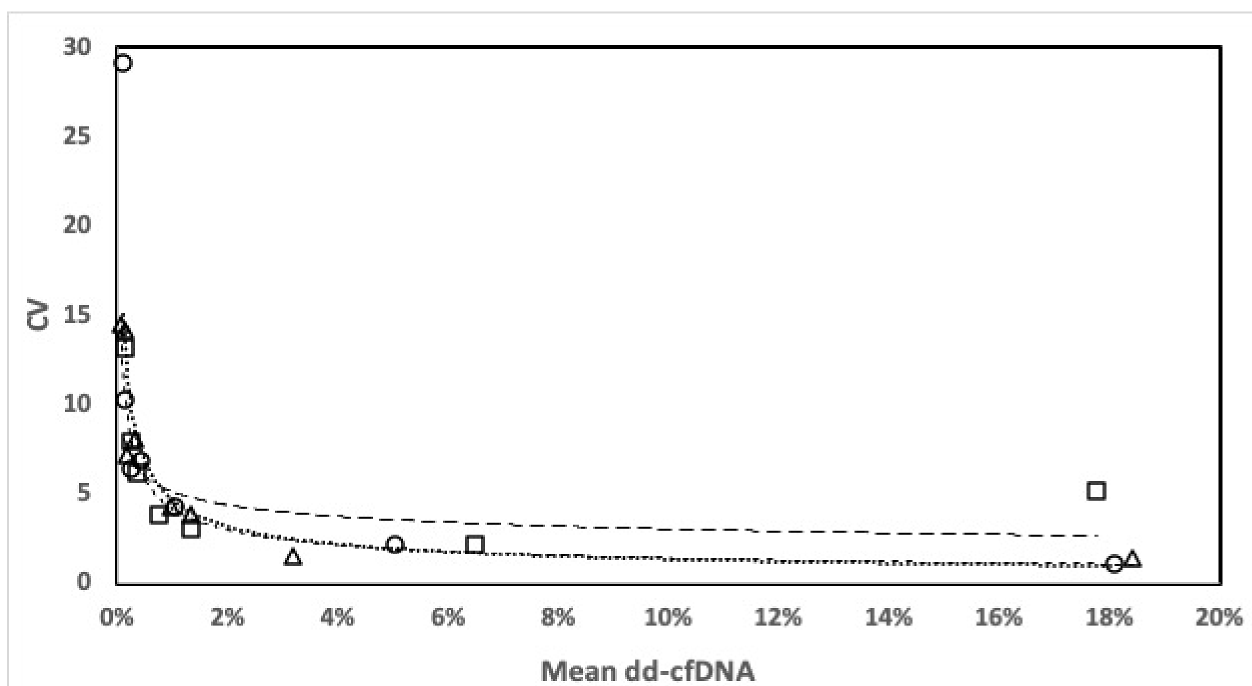

Figure 2: Precision across the range of reference materials tested. For each reference in each panel, a point is plotted at the mean and CV of 5-19 replicate runs. A fit line is drawn for each panel. Three panels of Horizon materials were tested, shown by the square and dashed line, triangle and solid line, and circle with dotted line. The CV is well below 10% throughout the range for decision making.

Table 1: Performance characterization of AlloSure 3.0.

Metric	Value
LOB	0.08% dd-cfDNA
LOD	0.12% dd-cfDNA
LOD (closely related donor)	0.18% dd-cfDNA
CV (inter-run for 0.2%-16%)	2.7%
CV (intra-run at 0.6%)	1.60%
CV (intra-run at 2.4%)	2.40%
Concordance	99%

characterization of AlloSure 3.0 are shown (Table 1). The LOB was 0.08%, based on a set of 206 samples (107 cfDNA, 93 g DNA, 6 reference samples). The LOD was determined to be 0.12% (95% CI 0-0.15%) using the 0.1% reference material sample. No changes were made to the AlloSure assay that would impact the upper end of the reportable range, therefore the reportable range for AlloSure 3.0 is 0.12% to 16% per CLSI EP-17-A2 recommendations [10]. AlloSure results that pass all quality control metrics and are outside the reportable range are reported as "< 0.12%" or ">16%". For recipients with a close relationship with the donor, (sibling, parent/child) the LOD is 0.18%, calculated according to the methods previously described [1].

The precision of the assay was determined using 216 reference samples from the full range of target concentrations of dd-cfDNA. The inter-run precision is plotted (Figure 2), showing that the Coefficient of Variation (CV) is very low throughout the range of the assay, rising only due to very low numbers of molecules associated with percentages below one half of one percent dd-cfDNA. The inter-run CV is 2.7% across the 13 runs on two instruments with multiple operators and 13 different days. The intra-run precision was determined with 64 replicates each of a reference sample at 0.6% and another at 2.4%. The intra-run CV was only 1.6% in the 0.6% reference sample and 2.4% in the 2.4% reference sample.

The accuracy of the assay for determination of clinical results was assessed by a concordance study comparing the results from the original AlloSure runs on commercial kidney transplant samples with those measured using AlloSure 3.0. 195 clinical samples were run to determine the concordance of AlloSure 3.0 results to the results from the original AlloSure version to achieve the same result, either below or above 1%. Overall the concordance was 99%.

DISCUSSION

The analytical performance of AlloSure 3.0 is improved over the original AlloSure assay. The original AlloSure assay analytical performance study set standards for analytical validation of a donor-derived cell-free DNA assay [1]. The validation of AlloSure 3.0 was performed to the same rigor to ensure consistent clinical assay performance.

Incremental advances to laboratory tests are routinely implemented to enable higher throughput, faster turnaround time, higher quality, or cost efficiency. AlloSure 3.0 was developed to streamline the laboratory workflow and enable higher throughput while improving turnaround time and maintaining performance. The improved chemistry and sequencing improved performance, lowering the LOD for unrelated donors from 0.19% to 0.12%. Although the qualitative "< 0.12%" result is valuable in transplantation as a result confirming very low levels of dd-cfDNA, newer clinical studies are indicating more attention should be paid to low levels and changes within lower ranges of dd-cfDNA. Stites et al., showed that an AlloSure threshold of 0.5% in kidney transplantation could differentiate between TCMR with poorer outcomes and those with better outcomes [5]. Khush et al., performed a clinical validation of AlloSure use in heart transplantation and identified that the

threshold for identification of rejection should be in the range of 0.15% to 0.25% [6].

CONCLUSION

The improved AlloSure assay was put into use in 2019 and provides equivalent results to prior versions, but with a streamlined workflow in the laboratory and the added benefit of an expanded reportable range at the low end where it matters most for heart and kidney transplantation. Continuous improvement will enable better patient management using the latest in non-invasive diagnostic tools.

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CONFLICT OF INTEREST

All authors were employees of CareDx at the time of the study.

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