Received: 4 March 2024 | Revised: 19 April 2024 | Accepted: 29 April 2024

DOI: 10.1111/tan.15518

ORIGINAL ARTICLE

HLA Immune Response Genetics WILEY

Multi-centre analytical performance verification of an IVD assay to quantify donor-derived cell-free DNA in solid organ transplant recipients

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Donor-derived cell-free DNA (dd-cfDNA) has been widely studied as biomarker for non-invasive allograft rejection monitoring. Earlier rejection detection enables more prompt diagnosis and intervention, ultimately improving patient treatment and outcomes. This multi-centre study aims to verify analytical performance of a next-generation sequencing-based dd-cfDNA assay at end-user environments. Three independent laboratories received the same experimental design and 16 blinded samples to perform cfDNA extraction and the dd-cfDNA assay workflow. dd-cfDNA results were compared between sites and against manufacturer validation to evaluate concordance, reproducibility, repeatability and verify analytical performance. A total of 247 sample libraries were generated across 18 runs, with completion time of <24 h. A 96.0% first pass rate highlighted minimal failures. Overall observed versus expected dd-cfDNA results demonstrated good concordance and a strong positive correlation with linear least squares regression $r^2 = 0.9989$, and high repeatability and reproducibility within and between sites, respectively ($p > 0.05$).

Abbreviations: ANOVA, analysis of variance; ASTS, American Society of Transplant Surgeons; CI, confidence interval; CSLI, Clinical and Laboratory Standards Institute; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibodies; ESOT, European Society for Organ Transplantation; IRB, institutional review board; ISHLT, International Society for Heart and Lung Transplantation; IVD, in-vitro diagnostic; LOB, limit of blank; LOD, limit of detection; LOQ, limit of quantification; NGS, next-generation sequencing; PCR, polymerase chain reaction; QC, quality control; QF-PCR, quantitative fluorescence polymerase chain reaction; RMS, root mean square; SC, serum creatinine; SD, standard deviation; SNPs, single-nucleotide polymorphisms; SOT, solid organ transplant; STR, short tandem repeat.

Sílvia Casas and Narin S. Tangprasertchai are co-first authors.

Manufacturer validation established limit of blank 0.18%, limit of detection 0.23% and limit of quantification 0.23%, and results from independent sites verified those limits. Parallel analyses illustrated no significant difference ($p = 0.951$) between dd-cfDNA results with or without recipient genotype. The dd-cfDNA assay evaluated here has been verified as a reliable method for efficient, reproducible dd-cfDNA quantification in plasma from solid organ transplant recipients without requiring genotyping. Implementation of onsite dd-cfDNA testing at clinical laboratories could facilitate earlier detection of allograft injury, bearing great potential for patient care.

KEYWORDS

allograft, AlloSeq, cfDNA, dd-cfDNA, liquid biopsy, NGS, SNPs, transplant biomarker, transplant surveillance, transplantation

1 | INTRODUCTION

Monitoring solid organ transplant (SOT) recipients presents an extreme challenge. All transplant recipients harbour high risk of developing malignancies, allograft malfunction or rejection and infections.[1] Immunosuppression must be closely monitored to balance allograft health with immunodeficiency effects like infections or cancer, as well as adverse effects of immunosuppressive therapy like renal failure or diabetes.[2] Histological analysis of transplant tissue recovered by biopsy is the gold standard to diagnose potential transplant rejection. However, biopsy is highly invasive, expensive, inappropriate for frequent assessment, bears procedure-related risks and is highly subjective.[3,4] Consequently, non-invasive approaches offer welcome alternatives for allograft surveillance via the measurement of routine clinical biochemistry biomarkers such as serum creatinine (SC) for kidney or donor-specific antibodies (DSA) for SOT.[5,6] These biomarkers allow routine monitoring, but with limitations; SC levels have low sensitivity and specificity and increase after serious kidney tissue damage,[7] while DSA measurement is challenging and has a poor predictive value.[8]

Donor-derived cell-free DNA (dd-cfDNA) in transplant recipients' blood has been proposed as a non-invasive marker for allograft injury to aid in surveillance and rejection risk assessment.[9] It is well established that cfDNA circulates in the blood stream from normal cell death mechanisms[10,11] and is increased in certain disease states.[12] Solid organ allografts typically release cfDNA into the recipient's body and levels increase under abnormal circumstances, including graft malfunction and rejection. dd-cfDNA was first assessed in female transplant recipients with male donors,[9] allowing quantification of dd-cfDNA by polymerase chain reaction (PCR) amplification of Y-chromosome genes.[9] Many studies have since shown that dd-cfDNA levels above certain thresholds are associated with rejection and other sources of allograft injury.[13–22] Non-invasive post-transplant surveillance, such as quantification of dd-cfDNA in blood, has now been recommended by the International Society for Heart and Lung Transplantation, the American Society of Transplant Surgeons and the European Society for Organ Transplantation guidelines and consensus documents to monitor for allograft rejection, ultimately reducing biopsies and helping to prioritise hospital visits.[23–25]

Donor cfDNA that is genetically distinct from the recipient's (i.e., excluding cases where donor and recipient are monozygotic twins) can be detected and quantified, making it a promising biomarker for routine, real-time allograft monitoring. Several recent validation trials have demonstrated a strong correlation between dd-cfDNA levels and early allograft rejection, by using single-nucleotide polymorphisms (SNPs) to characterise dd-cfDNA with next-generation sequencing (NGS) methods.[13,14,18] For best dd-cfDNA assay performance, SNP selection should include genome-wide coverage, multi-ethnic representation, high uniformity and statistical sufficiency to distinguish even genetically related donors and recipients.[18] Of focus in this study is a commercially available NGS assay that targets 202 bi-allelic SNPs throughout the genome to measure dd-cfDNA in transplant recipients. Several studies have examined the clinical utility of this assay in transplant surveillance, initially presenting single-centre experiences and demonstrating the robustness and value of implementing dd-cfDNA clinical laboratory NGS testing.[26–36] However, evaluation of analytical performance metrics among different users has not yet been reported.

Herein are the results of a multi-centre verification study assessing the analytical performance of a

commercially available, in vitro diagnostic (IVD), NGS-based assay for dd-cfDNA quantification. The study, conducted within an end-user environment, assessed performance verification data regarding assay concordance, reproducibility and repeatability among laboratory users. These data may contribute to cover unmet need for building confidence in standard use of local laboratory dd-cfDNA run and promote broader adoption of such testing in clinical laboratories.

2 | METHODS

2.1 | Samples

This multi-centre study was conducted at three independent molecular diagnostic laboratories in Greece, the United Kingdom and Switzerland. All sites received the same study protocol and a set of 16 blinded samples. Samples included cfDNA reference samples, transplant patient cfDNA samples and transplant patient plasma samples (Table 1). Cell line genomic DNA was fragmented by sonication to approximately 160 bp fragments to mimic cfDNA size, and single- and two-genome reference samples were characterised using digital PCR and a SNP genotyping assay as previously described (Horizon Discovery, Waterbeach, UK)[18] (Table 1). Expected dd-cfDNA results for post-transplant samples covered a dynamic range of 0.23%–19.29%, representing what has been reported from solid organ recipient populations.[9,16] Due to the use of unidentified leftover human specimens for analytical verification of this dd-cfDNA assay, this study qualified as exempt from the need for institutional review board (IRB) review by an external IRB committee, in accordance with 45 CFR 46.104(d)(4(ii)) regulation. All researchers complied with relevant state and federal regulations.

2.2 | Plasma isolation and cfDNA extraction

Blood was drawn into cell-free DNA BCT (Streck, La Vista, NE, USA) and plasma isolated using the Double Spin 2 protocol. cfDNA was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Germantown, MD, USA).

Duplicate cfDNA extractions (cfDNA A and B) were performed on plasma samples from four kidney transplant patients by the assay manufacturer. cfNDA A was processed by the manufacturer and cfDNA B was sent to participating laboratories. Two plasma samples (Tubes A and B) were isolated from each of six kidney transplant patients and cfDNA was extracted at the manufacturer (Tube A) or participating laboratories (Tube B). Patient samples were de-identified prior to use and considered remnants of specimens collected for routine clinical care of analysis that would have been otherwise discarded, according to the federal Common Rule, 45 CFR 46 subpart A.

TABLE 1 Reference material and post-transplant sample characteristics.

Sample name	Sample category	Sample type	Expected % dd-cfDNA
CTRL-A	Reference	cfDNA	0.00
CTRL-C	Reference	cfDNA	0.00
CTRL-E	Reference	cfDNA	0.00
CTRL-G	Reference	cfDNA	0.00
CTRL-I	Reference	cfDNA	0.32
CTRL-K	Reference	cfDNA	0.31
B-03	Post-transplant	Extracted cfDNA	0.55
B-04	Post-transplant	Extracted cfDNA	0.68
B-05	Post-transplant	Extracted cfDNA	1.40
B-06	Post-transplant	Extracted cfDNA	1.20
B-S1-01	Post-transplant	Plasma	0.34
B-S1-02	Post-transplant	Plasma	1.47
B-S3-01	Post-transplant	Plasma	0.23
B-S3-02	Post-transplant	Plasma	19.29
B-S4-01	Post-transplant	Plasma	0.47
B-S4-02	Post-transplant	Plasma	2.69

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

2.3 | dd-cfDNA assay

The percentage of dd-cfDNA (% dd-cfDNA) was measured for all samples using AlloSeq cfDNA (CareDx, Brisbane, CA, USA) following manufacturer instructions. The assay uses custom SNP primers and index adapters with a proprietary thermocycling protocol to simultaneously amplify SNP regions and index libraries. Indexed samples are subsequently pooled and purified in preparation for sequencing. A 10 ng cfDNA samples were amplified and indexed through multiplex PCR using a primer pool for 202 SNPs and designated index pairs. Resulting PCR products were sequenced on MiSeq sequencing systems with MiSeq Reagent Kit v3 (Illumina), and sequencing data were analysed with AlloSeq cfDNA Software v1.0.0. Genetic relationship between donor and recipient was entered into AlloSeq cfDNA software, and the automatic % dd-cfDNA calculation was adjusted accordingly. In addition to % dd-cfDNA, AlloSeq cfDNA quality control (QC) metrics for all loci, mean coverage, uniformity and locus count were monitored. First pass rate, the percentage of samples yielding dd-cfDNA results on the first attempt, was also calculated.

2.4 | Analytical performance analysis

Replicates of each unique sample were designed to generate sufficient data for evaluating variability and verifying analytical performance metrics, with even distribution across sites. Results from the same samples tested at three different sites were analysed to characterise intersite variability. Expected % dd-cfDNA was defined as % dd-cfDNA determined during manufacturer validation. Percent difference was defined as the difference between observed and expected results divided by the expected result and evaluated using a one-sample *t*-test. Reproducibility was analysed by comparing mean and standard deviation (SD) with one-way analysis of variance nonparametrical Kruskal–Wallis test. The Bartlett test was used to evaluate equality of variance both between and within sites per sample.

Analytical performance characteristics limit of blank (LOB), lower limit of detection (LOD) and lower limit of quantification (LOQ) were established previously by the manufacturer following Clinical and Laboratory Standards Institute (CSLI) guidelines.[37] LOB verification included 20 replicates and required that no more than 3 measurements out of 20 could exceed the claimed LOB. LOD verification included 25 replicates and required that no more than 3 measurements out of 25 fall below the claimed LOB. LOQ verification included 25 replicates and required that no more than 3 measurements out of 25 fall outside the acceptable range, defined as mean $\pm$ 2 $\times$ SD for <1% dd-cfDNA.

2.5 | Recipient genotype analysis

AlloSeq cfDNA has been validated for measuring intervals 0% to 100% with recipient or donor genotype and 0% to <50% without.[38] Analyses with and without recipient genotype were performed to evaluate accuracy for samples with expected results in the validated measuring interval.

CTRL-G was used as the recipient genotype sample for CTRL-K, prepared as a mixture of majority CTRL-G and another, genetically unrelated sample. Each site performed five runs with five replicates of CTRL-K, analysed both with and without CTRL-G recipient genotype using AlloSeq cfDNA software, and mean and SD were calculated.

3 | RESULTS

3.1 | First pass rate

A total of 247 sample libraries were generated using 10 ng DNA input, following the manufacturer's validated protocol, across 18 runs. First pass rate was 96.0% (237 out of 247), and no common mode was observed among the 10 QC failures.

3.2 | Variability

Reproducibility of AlloSeq cfDNA across participating laboratories was evaluated based on analysis of positive control samples CTRL-I and CTRL-K (Table 1) provided to each site. Each laboratory generated 25 results for each sample, with 5 replicates in each of 5 runs. Characterisation of these data revealed no significant differences in mean, intersite SD, or intrarun SD (*p*-values 0.794 and 0.499 for mean, 0.316 and 0.072 for intersite SD and 0.248 and 0.287 for intrarun RMS, for CTRL-I and CTRL-K, respectively; Table 2). Owing to rather low SD in two cases, CTRL-I for site B and CTRL-K for site C, significant differences were observed in the interrun SD comparison (*p*-values <0.001 and 0.022 for CTRL-I and CTRL-K, respectively; Table 2). Overall coefficient of variation were 12.6%–22.5% for intersite variability, 9.4%–16.3% for intrarun variability and 0.0%–20.3% for interrun variability (Table 2).

Variability was evaluated for both cfDNA and plasma samples. The manufacturer performed AlloSeq cfDNA and sent duplicate cfDNA samples to participant laboratories (B03, B04, B05 and B06; Table 1). Results from

TABLE 2 Analysis of dd-cfDNA assay results intersite, intrarun and interrun variability, for 25 replicates generated by each site.

				Intersite			Intrarun			Interrun		
CTRL	Site	Mean	Mean, *p*-value	SD	SD, *p*-value	CV	RMS error	RMS error, *p*-value	CV	SD	SD, *p*-value	CV
CTRL-I	A	0.32	0.794	0.07	0.316	20.2%	0.04	0.248	12.6%	0.05	<0.001	15.8%
	B	0.32		0.05		16.0%	0.05		16.3%	0.00		0.0%
	C	0.33		0.07		22.5%	0.05		16.2%	0.05		16.4%
CTRL-K	A	0.42	0.499	0.08	0.072	18.3%	0.05	0.287	12.0%	0.06	0.022	15.1%
	B	0.45		0.09		20.8%	0.04		9.4%	0.09		20.3%
	C	0.43		0.05		12.6%	0.05		12.3%	0.01		2.6%

Abbreviations: CV, coefficient of variation; dd-cfDNA, donor-derived cell-free DNA; RMS, root mean square; SD, standard deviation.

TABLE 3 dd-cfDNA results for two different cfDNA samples from the same patient, A by the manufacturer and B by test sites.

A	B-03 ($n = 1$)	B-04 ($n = 3$)	B-05 ($n = 2$)	B-06 ($n = 2$)	Mean ± SD	Difference (percent difference)	CV (%)
0.65	0.59				-	−0.06 (−9.23%)	-
0.75		0.59 0.59 0.67			0.62 ± 0.05	−0.13 (−17.78%)	8%
1.52			1.15 1.38		1.26 ± 0.17	−0.26 (−16.78%)	13%
1.24				1.05 1.13	1.09 ± 0.05	−0.15 (−12.10%)	5%

Abbreviation: CV, coefficient of variation; dd-cfDNA, donor-derived cell-free DNA; SD, standard deviation.

TABLE 4 dd-cfDNA results for two different plasma samples from the same patient, A by the manufacturer and B by test sites.

A	B-S1-01 ($n = 1$)	B-S1-02 ($n = 3$)	B-S3-01 ($n = 2$)	B-S3-02 ($n = 3$)	B-S4-01 ($n = 3$)	B-S4-02 ($n = 3$)	Mean ± SD	Difference (percent difference)	CV (%)
0.24	0.36						-	0.12 (50.00%)	-
1.31		1.16 1.26 1.34					1.25 ± 0.09	−0.06 (−4.33%)	7%
0.23			0.21 0.22				0.22 ± 0.01	−0.02 (−6.52%)	4%
22.69				21.50 21.80 22.27			21.86 ± 0.39	−0.83 (−3.67%)	2%
0.53					0.43 0.48 0.49		0.47 ± 0.03	−0.06 (−11.95%)	7%
2.97						3.12 3.26 3.31	3.23 ± 0.09	0.26 (8.75%)	3%

Abbreviation: CV, coefficient of variation; dd-cfDNA, donor-derived cell-free DNA; SD, standard deviation.

these cfDNA samples across the three independent laboratories showed no practically significant difference compared with those generated by the manufacturer (Table 3). The manufacturer performed cfDNA extraction and AlloSeq cfDNA and sent duplicate plasma samples to participant laboratories (B-S1-01, B-S1-02, B-S3-01, B-S3-02, B-S4-01 and B-S4-02; Table 1). No practically significant differences were observed in % dd-cfDNA results across sites (Table 4), demonstrating highly reproducible AlloSeq cfDNA results.

3.3 | Concordance

A total of 176 measurements were collected for samples with expected % dd-cfDNA ≥ LOD of 0.23% (reference and post-transplant samples, Table 1), and 75 results from CTRL-K were ultimately excluded from concordance analysis, as discussed below. Expected versus observed dd-cfDNA, percent difference versus expected dd-cfDNA and observed difference versus expected dd-cfDNA results for the remaining dataset, 101 data points including replicates from all sites are shown in Figure 1. Linear least squares regression showed strong positive correlation with r^2 of 0.9989, slope of 1.1357 and intercept of 0.0599 (Figure 1A). As expected, greater percent differences were observed for lower expected % dd-cfDNA (Figure 1B) and greater observed differences for higher expected % dd-cfDNA (Figure 1C). Given limited availability of post-transplant samples in the range of 3%–19% dd-cfDNA, a separate regression was performed for samples between 0%–3% to investigate potential bias from samples >19% (Figure S1). Weighted least squares regression for the full expected versus observed dataset and standard regression for the full expected versus mean observed dataset were also evaluated. These additional analyses yielded results similar to the regression shown in Figure 1A, supporting validity of the findings presented here.

Forty-three measurements exceeded 35% difference, 7 from CTRL-I and 37 from CTRL-K. Investigation into the difference distribution for these samples is shown in Figure 2. Analysis of CTRL-I results revealed that all test sites yielded mean 0.32% dd-cfDNA with SD 0.06%, demonstrating good concordance with the expected value of 0.32%. Individual site means were 0.32%, 0.32% and 0.33%, confirming high precision and reproducibility between independent laboratories. The mean % difference was −5.17% with 95% confidence interval (CI) of −9.39% to −0.94%, showing acceptable variation within 10% difference (Figure 2A).

Analysis of CTRL-K results revealed that all test sites yielded mean 0.43% dd-cfDNA with SD 0.08%, demonstrating poor concordance with the expected value of 0.31%. Individual site means were 0.42%, 0.45% and 0.43%, demonstrating no significant differences between the three sites (p-value 0.49) and confirming high precision and reproducibility between independent laboratories. However, the mean % difference was 40.04% with 95% CI of 34.37%–45.72%, highlighting a major discrepancy (Figure 2B). In light of this observation, the manufacturer collected additional data on CTRL-K and obtained mean 0.42% dd-cfDNA with SD 0.05% across six replicates and two runs (Figure 2C). This updated result was consistent with those reported from test sites, and the significant change upon repeating at a later time suggests a problem with the sample itself. Considering this inconsistency and implications of questionable sample integrity, CTRL-K was excluded from concordance analysis.

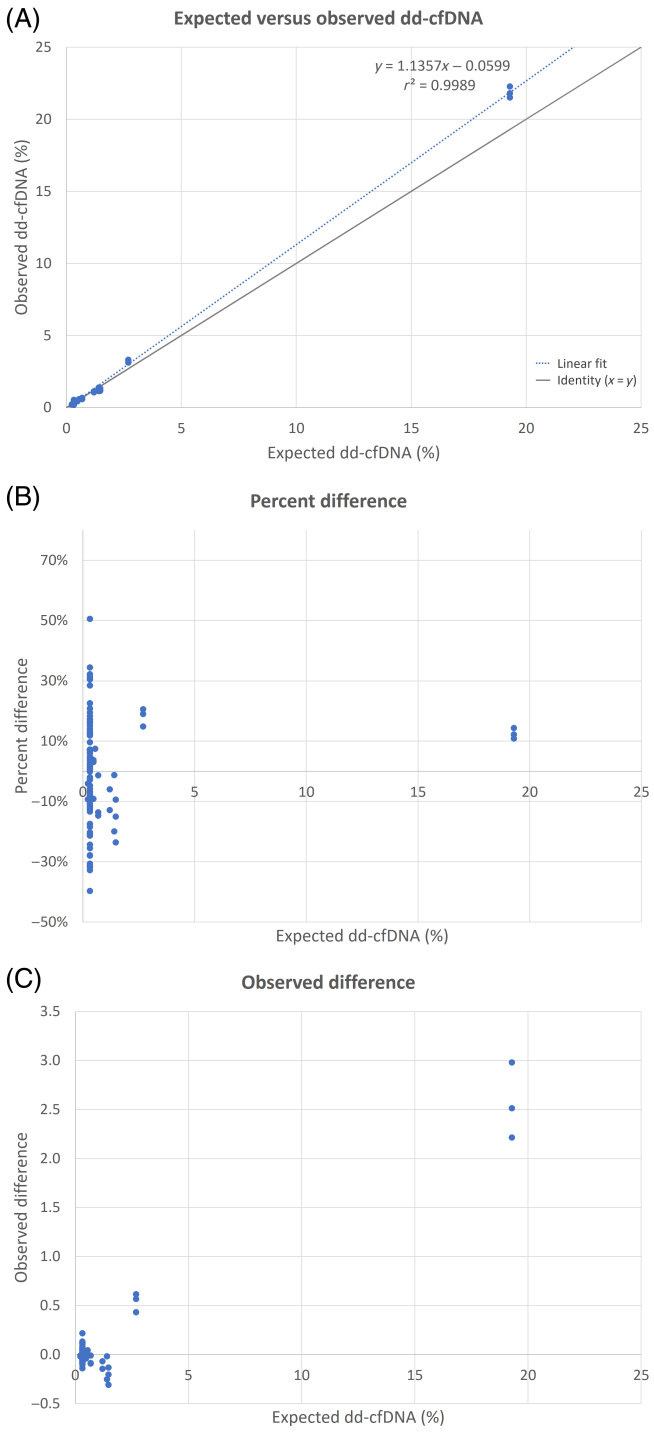

FIGURE 1 (A) Expected versus observed donor-derived cell-free DNA (dd-cfDNA) results with linear least squares regression (dashed blue) and identity (solid grey) lines. (B) Percent difference versus expected dd-cfDNA. (C) Observed difference versus expected dd-cfDNA.

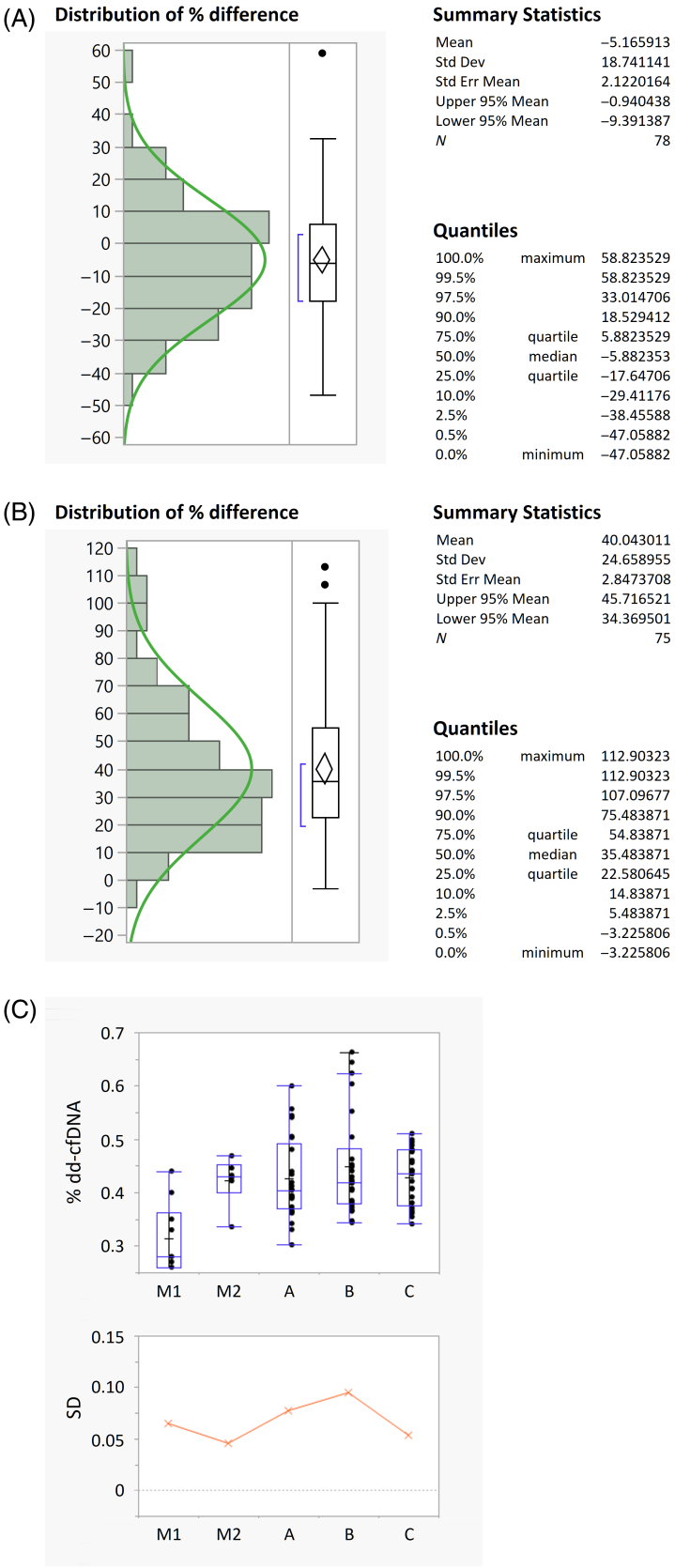

FIGURE 2 Distribution of the difference between observed and expected % donor-derived cell-free DNA (dd-cfDNA) results for (A) CTRL-I and (B) CTRL-K. Percent differences (green bars), distributions (green lines) and mean and interquartile ranges (box plots) are shown. (C) Results for CTRL-K from all sites: manufacturer before external testing (M1), manufacturer after external testing (M2) and test sites (A, B and C). Mean and interquartile ranges (box plots) are shown.

3.4 | Analytical performance

AlloSeq cfDNA LOB, LOD and LOQ were established as 0.18%, 0.23% and 0.23%, respectively, by the manufacturer's internal validation. These limits were verified by end users at three external test sites, following CLSI guidelines.[37]

Blank control samples (CTRL-A, CTRL-C, CTRL-E and CTRL-G) were used to verify the claimed LOB 0.18%

at 10 ng input. Twenty replicates were run at each site; per CLSI guidelines,[37] no more than 3 measurements out of 20 may exceed the claimed LOB. Site A had one measurement exceeding the claimed LOB (CTRL-G 0.21% dd-cfDNA), site B had one (CTRL-G 0.34% dd-cfDNA) and site C had two (CTRL-A 0.19% dd-cfDNA and CTRL-G 0.20% dd-cfDNA). Three total sample measurements did not pass QC metrics. Even considering these QC failures, results from all sites successfully verified the claimed LOB.

A control sample with an expected % dd-cfDNA close to the claimed LOD 0.23% was used for verification. For this study, no reference materials were available with expected % dd-cfDNA equal to the claimed LOD, so CTRL-I with expected % dd-cfDNA 0.32% was chosen. Twenty-five replicates were run at each site; per CLSI guidelines,[37] no more than 3 measurements out of 25 may fall below the claimed LOB. Across all sites, no measurements fell below the claimed LOB, passing LOD verification.

A control sample with an expected % dd-cfDNA close to the claimed LOQ 0.23% was used for verification. For this study, no reference materials were available with expected % dd-cfDNA equal to the claimed LOQ, so CTRL-I with expected % dd-cfDNA 0.32% was chosen. Twenty-five replicates were run at each site; per CLSI guidelines,[37] the acceptable range was defined by mean $\pm 2 \times$ SD, and no more than 3 measurements out of 25 may fall outside the acceptable range. The mean % dd-cfDNA for CTRL-I was 0.32% across all sites, with SD 0.06%, making the acceptable range 0.20%–0.45%. Site A had one measurement (0.45%) outside the acceptable range, and site B had none. Site C had 3 QC failures that yielded no usable measurement, so additional replicates were added; 2 measurements (0.18%, 0.54%) out of 28 fell outside the acceptable range. All sites successfully verified the claimed LOQ.

3.5 | Recipient genotype effect

AlloSeq cfDNA analyses across all runs yielded resulting mean 0.42% dd-cfDNA with 95% CI 0.40%–0.43% with recipient genotype and 0.41%–0.44% without. No significant differences were observed overall (p-value 0.14). Results were also reproducible across the three laboratory sites, with no differences between mean dd-cfDNA with or without recipient genotype. Site A yielded mean 0.43% dd-cfDNA (p-value 0.22), site B 0.40% (p-value 0.17) and site C 0.43% (p-value 0.17). Results are shown in Table S1.

4 | DISCUSSION

During this study, three independent laboratories in different countries received the same experimental design, blinded reference materials and blinded post-transplant samples to perform NGS-based dd-cfDNA testing at local laboratory with a commercially available IVD assay. Analytical performance metrics claimed by the manufacturer (LOB 0.18%, LOD 0.23% and LOQ 0.23%) were successfully verified by results generated at these sites. dd-cfDNA results from test sites demonstrated a strong positive correlation and good concordance with expected results from the manufacturer, including cfDNA extracted by the manufacturer and by participating laboratories. The NGS-based assay for dd-cfDNA quantification evaluated here demonstrated high reproducibility between sites across the entire workflow.

Given the sensitivity of NGS-based assays and detection capabilities below the current established limits, the assessed dd-cfDNA assay has the potential to achieve even lower limits. Analytical performance characteristics may be enhanced with greater cfDNA input or protocols optimised for higher efficiency or purity, which may be explored with future validation. Factors like quality and quantity of input material, amplification efficiency, library purity and sequencing coverage also contribute to overall assay performance and generation of high-quality results. Every laboratory should perform verification upon implementing any new manufacturer-validated assay, including characterisation of relevant analytical performance metrics. Any modifications to the validated workflow require subsequent validation by the laboratory, following accreditation body recommendations. The availability of external QC schemes for dd-cfDNA may be useful for guiding and improving testing proficiency at accreditated laboratories, programs that are not yet offered by service providers.

The high reproducibility of this NGS dd-cfDNA assay demonstrated both within and between different sites and concordance with manufacturer results not only ensure the reliability of results but also enable standardisation across multiple locations. With mean >1000× coverage (>500× required), 98% mean uniformity (>75% expected) and 202 informative SNPs (>186 required), independent sites succeeded with a strong positive correlation between observed and expected % dd-cfDNA for the same set of blinded samples, yielding linear least squares regression with r^2 of 0.9989, slope of 1.1357 and intercept of 0.0599. This is the first reported multi-centre analytical performance verification study for this dd-cfDNA assay and offers a comprehensive overview of reproducibility and concordance. By utilising kit-based NGS dd-cfDNA assays, laboratories can streamline their testing processes and achieve consistency in results interpretation, irrespective of their location.

Today, most dd-cfDNA testing platforms are centralised send-out systems, posing a significant disadvantage for clinical sites abroad. A broadly used centralised NGS

testing service sharing the same fundamental biochemistry employs 405 SNPs and requires independent library amplification and indexing reactions,[18] while the NGS assay kit studied here uses 202 SNPs and a single PCR step. Concordance between the centralised test and the kit-based assay, with $r^2 = 0.9136$ for clinical samples, $r^2 = 0.9458$ for analytical samples near LOD and $r^2 = 0.9991$ in linearity analysis up to 70% (unpublished observations from manufacturer), demonstrates high consistency of results. High coefficient of correlation with respect to the predicate centralised dd-cfDNA assay has also been observed at end-user level based on the results from a large kidney transplant cohort multi-centre study (data not shown, submitted for publication). This kit assay has also been compared with alternative methods, such as short tandem repeat genotyping by quantitative fluorescence PCR (QF-PCR),[17] droplet digital PCR,[39] and high-throughput sequencing[40] using clinical samples. dd-cfDNA results from these different methods showed mutual agreement with high coefficients of correlation, except agreement between QF-PCR and NGS <5%, owing to limited sensitivity of QF-PCR and greater sensitivity of NGS in this range.[17]

Transplant biopsies have been the gold standard for detecting allograft injury, but the invasive procedure has well-known limitations, including notorious subjectivity.[3,4] Non-invasive diagnostic methods to monitor for damage after organ transplantation have recently emerged into clinical practice and show great potential. In SOT recipients, dd-cfDNA is an effective, minimally-invasive biomarker of transplant allograft injury that enables frequent, safe and quantitative surveillance. Timely, non-invasive detection of increased dd-cfDNA levels in SOT recipients could reduce unnecessary biopsies[41–44] and allow clinicians to promptly modify immunosuppression.[45–47]

The dd-cfDNA assay evaluated here is a targeted NGS assay utilising 202 SNPs throughout the genome to quantify dd-cfDNA from transplant recipient blood. This verification study was conducted by laboratory end users, including performing the streamlined protocol compatible with clinical laboratory practices and running the automatic analysis software to quantify dd-cfDNA without genotyping,[48] with turnaround time of under 24 h from cfDNA sample to result,[38] enabling efficient post-transplant surveillance. dd-cfDNA cut-offs for risk of allograft injury differ between organs, established by various dd-cfDNA clinical validity studies as 0.20% for heart,[20] originally 1%[13] and later redefined as 0.5%[14,22] for kidney, 0.85% for lung,[15,19] and 10% for liver.[17,21] Independent studies using the dd-cfDNA assay kit assessed in this study have shown thresholds for kidney,[26,27,29,31,33,34] heart,[36] lung,[35] and liver[17] consistent with those established values, highlighting assay reliability.

Novel molecular diagnostic tools are more complex than many other non-invasive tests, like SC or cardiac and liver enzymes, and a thorough understanding is crucial for clinicians to assess appropriateness, clinical utility and cost effectiveness of NGS assays for transplant patients. The dd-cfDNA assay of focus here leverages a panel of curated SNPs with the power of NGS to enable rapid dd-cfDNA quantification in transplant recipients with genetically distinct donors, without requiring genotyping. This study demonstrates that this NGS-based assay accurately quantifies dd-cfDNA, a leading indicator of graft injury, with high repeatability and reproducibility, providing critical information for optimum transplant patient management.

AUTHOR CONTRIBUTIONS

S.C. initiated writing of the original draft. S.C. and T.V. handled project administration. N.S.T. and J.L. collaborated on formal analysis. J.L. validated the data analysis. N.S.T reviewed the original draft and composed the remainder of the manuscript. K.O., S.M., Z.C.S., S.S., N.D.B., P.C. and J.V. performed the investigation. All authors contributed to manuscript review.

CONFLICT OF INTEREST STATEMENT

S.C., N.S.T., J.L. and T.V. are CareDx full time employees with stock plan benefits. N.D.B. has received advisory board honorarium from CareDx. All the other authors declared no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from CareDx Inc. Restrictions apply to the availability of these data, which were used under license for this study. Data are available from the author(s) with the permission of CareDx Inc.

FUNDING INFORMATION

CareDx funded with the laboratory reagents necessary for the testing included in the study.

ORCID

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SUPPORTING INFORMATION

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How to cite this article: Casas S, Tangprasertchai NS, Oikonomaki K, et al. Multi-centre analytical performance verification of an IVD assay to quantify donor-derived cell-free DNA in solid organ transplant recipients. *HLA*. 2024;103(5):e15518. doi:10.1111/tan.15518