Donor-derived cell-free DNA: clinical applications for the diagnosis of rejection

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Key Points

- **Donor-derived cell-free DNA (dd-cfDNA) is a noninvasive biomarker of organ rejection in solid organ transplant recipients**
- **Validation of four independent dd-cfDNA assays yielded similar clinical performance parameters suggesting generalized clinical applicability, although importantly direct assay comparisons are required for conclusive demonstration.**
- **Any cause of injury to the donated organ (rejection, infection or toxicity) may result in elevated levels of dd-cfDNA, thus emphasizing the need for comprehensive diagnostic approaches for optimal outcomes.**
- **Future innovations may allow combining dd-cfDNA assays and broad-spectrum pathogen detection into a single assay.**

Abstract

Immunological rejection presents one of the main obstacles to the long-term success of organ transplantation. Accurate, noninvasive laboratory assays are emerging as an alternative to biopsies for diagnosis of rejection. One of the most promising assays makes use of the proportion of donor-derived cell-free DNA (dd-cfDNA) as an indicator of organ damage, primarily due to rejection but also potentially due to infection or drug toxicity. Reviewing data published to date demonstrates that measurement of dd-cfDNA has broad clinical utility as a rejection biomarker.

Introduction

While the clinical outcomes of solid organ transplant (SOT) have improved steadily for decades, median survival rates range from 5 to 15 years, depending primarily on the organ transplanted. In 2017, 10-year all-cause graft failure was 49.7% for deceased donor kidney recipients and 34.1% for living donor kidney transplants (Hart et al., 2019). Rejection of the transplanted organ remains one of the key challenges to long-term survival. The current standard method of allograft rejection diagnosis is by organ biopsy, an invasive technique, suffers from high cost, subjective interpretation and multiple potentially serious complications. Over a period of many years, various biomarkers (e.g. cytokines, metabolites, gene expression profiles) have been proposed to noninvasively monitor for rejection, but none have demonstrated high levels of diagnostic accuracy or been applicable to multiple transplanted organs. Notably, AlloMap® (CareDx®) has been used for rejection diagnosis in heart transplant recipients for nearly a decade. AlloMap® is an FDA-cleared assay (although not sold as a kit) which measures increases in a panel of genes by quantitative RT-PCR (Deng et al., 2006). Another gene expression panel, TruGraf® (TGI), was recently validated to monitor subclinical acute rejection in kidney transplant recipients (Marsh et al., 2019).

Quantification of donor-derived cell-free DNA (dd-cfDNA) in a transplant recipient's plasma by molecular methods has emerged as a tool to monitor transplant patients for rejection (Synder et al., 2011; De Vlaminck et al., 2015; Gielis et al., 2015). These methods use single nucleotide polymorphisms (SNPs) present in genes of the organ donor compared to those of the recipient to estimate the fraction of circulating cfDNA originating from the donor. Cell-free DNA is present at low levels in the plasma of all individuals, but damage to the donated organ due to any cause (rejection, infection or toxicity) increases the number of molecules of dd-cfDNA relative to recipient cfDNA in transplant patients, as shown in **Figure 1**. Next generation sequencing (NGS) allows precise, reproducible quantification of these two fractions primarily due to assessment of a large number of SNPs and sequencing a large number of individual molecules. Specialized and complex bioinformatics analysis is required to derive the final result, which is expressed as a percentage of donor-derived molecules relative to the background of recipient cfDNA.

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A) Sequencing results from a transplant recipient with no rejection

TAATCGACTTCATGGATCTA
ATGCTACAGATTATAAATCT
CCATACTGGATACCTAGATC
GATCATACCTACTATATTAC
AAATACTAACTACTGTACTA
TTACGACTACAGATAAGCAG
TACTCTAGGATACGCCATAC
CATGGACTACATCTATTACG
TGAACTACTTAGTATCATAC
ATATGGCTACAGATAGCAGA

B) Sequencing results from a transplant recipient with significant rejection (donor SNPS shown in red):

TAATCGACTTCATAGATCTA
ATGCTACAGATTATAAATCT
CCATACTGGATACCTAGATC
GATCATACCTACTATATTAC
AAATACTAACTACTGTACTA
TTACGACTACAGATAAGCAG
TACTCTAGGATACGCCATAC
CATGGACTACATCTATTACG
TGAACTACTTAGTATCATAC
ATATGGCTACAGAAAAGCAGA

Figure 1. Illustration of the method for diagnosing rejection from cell-free DNA. **Panel A** represents a patient with no detectible rejection. Potential informative SNPs are bolded and underlined, but in this sequencing result all originate from the recipient (black letters). **Panel B** represents a patient with significant rejection. Donor SNPs in this sequencing result are shown as red; of ten potential informative SNPs, two originate from the donor for a dd-cfDNA value of 20%.

The use of dd-cfDNA has significant potential advantages in both sensitivity and diagnostic accuracy, as well as being noninvasive and broadly applicable to different organ transplants (e.g. lung, liver, kidney, heart, and pancreas). Although organ-specific cutoffs are likely required, a single assay would have the ability to detect rejection for any transplanted organ. Other causes of donated organ dysfunction (e.g. infection, toxicity) will also result in elevated levels of dd-cfDNA, thus emphasizing the need for comprehensive diagnostic approaches for optimal outcomes.

Performance of Viracor TRAC™ assay

Viracor TRAC™ (Transplant Rejection Allograft Check) dd-cfDNA assay is based on low-coverage whole genome sequencing and queries >100,000 SNPs throughout the genome to accurately quantifying the dd-cfDNA percentage (Sharon et al., 2017). Analytical validation demonstrated that Viracor TRAC™ assay accurately detect dd-cfDNA with a notably large linear range (0.50% to 60% dd-cfDNA). Key TRAC analytical validation results are shown in **Table 1**.

Table 1. Analytical validation summary of Viracor TRAC™ dd-cfDNA assay

Performance parameter	Value
Limit of blank	0.21% dd-cfDNA
Lower limit of detection	0.32% dd-cfDNA
Lower limit of quantification	0.50% dd-cfDNA
Upper limit of quantification	60.00% dd-cfDNA
Linearity (0.50% to 60% dd-cfDNA)	Slope = 0.99, y-intercept = 0.06, r2≥ 0.99
Precision	Intra-assay precision CV=0.55%, 2.28%, and 5.82% for high, medium, and low samples, respectively. Inter-assay precision CV=3.68%, 5.85%, and 7.12% for high, medium, and low samples, respectively

Following analytical validation, 77 plasma biorepository samples from 25 kidney transplant recipients were assayed. Samples were collected from one day prior to transplant to 1,146 days post-transplant, with a mean of approximately 3 samples analyzed per kidney transplant recipient. When rejection was suspected, the status at the time of sample collection was established by renal biopsy (“for cause” biopsies) with histopathology assigned according to the Banff classification of renal allograft pathology. Of the 77 samples tested, 55 were collected during a period free of acute rejection (AR). A total of 3 samples were collected during borderline rejection and grouped with those characterized as free of AR. A total of 15 samples were collected during a period of biopsy-proven AR (humoral and/or cellular); 4 samples were collected during a period of BKV associated nephropathy and were grouped with those characterized as AR. Results were analyzed as a single time point. A summary of results is shown in **Table 2**. Receiver operator characteristic (ROC) curve analysis was performed (**Figure 2**), demonstrating an area under the curve (AUC) of 0.85 (95% CI 0.77 – 0.93, P < 0.001). Using the ROC results, a cutoff of >0.69% for positivity was established. At this cutoff, the assay sensitivity, specificity, positive predictive value and negative predictive value were 57.9%, 84.5%, 55% and 86%, respectively, for results from a single time point.

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Table 2. Summary of % dd-cfDNA results by rejection status at a cutoff of 0.69%

	Rejection[1]	Control[2]
Test positive	**12**	**8**
Test negative	**7**	**50**

[1]Includes samples from subjects with BK virus associated nephropathy
[2]Includes samples from subjects with borderline rejection biopsy results

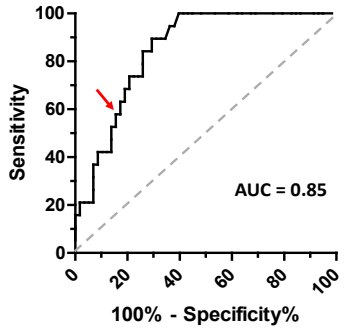

Figure 2. Receiver operator characteristic (ROC) curve for TRAC using clinical (biorepository) samples. The red arrow represents the optimized cutoff and the dashed grey line represents an area under the curve (AUC) of 0.50.

Review of dd-cfDNA assays

Validation results for additional dd-cfDNA assays have recently been published. AlloSure® (CareDx®) is an amplicon based dd-cfDNA assay that queries 266 SNPs (Grskovic et al., 2016). A multisite clinical validation was performed on 102 subjects providing 107 samples; 27 of the samples were collected during a period of rejection (Bloom et al., 2017). From this study, a cutoff value of 1.0% was established. Diagnostic performance was strongest for antibody mediated rejection (ABMR), although T cell mediated rejection (TCMR) graded >1B was also effectively differentiated from controls in this study as well. While an independent external validation confirmed the ability of AlloSure® to diagnose ABMR (ROC AUC = 0.82), TCMR could not be differentiated from controls (ROC AUC = 0.42) in this study (Huang et al., 2019).

Validation of second amplicon-based dd-cfDNA assay (Prospera™, Natera®) was published in 2018 (Sigdel et al., 2018). Prospera™ is based on high-level multiplex PCR amplification with assessment of up to 13,392 SNPs by NGS. The study for Prospera™ was performed retrospectively on 193 subjects providing 300 samples; 38 of the samples were collected during a period of rejection. From this study, a cutoff value of 1.0%, which is identical to AlloSure®, was established. Interestingly, this study demonstrated very similar dd-cfDNA diagnostic performance for samples collected at the same time as either surveillance or for-cause biopsies.

Finally, a method using droplet digital quantitative PCR (dd-qPCR) method (TheraSure™) was recently validated for detection of rejection (Oellerich et al., 2019). As with NGS methods, dd-qPCR depends on identification of SNPs to differentiate donor and recipient cfDNA molecules but rather than sequencing to identify the donor/recipient origin of cfDNA molecules, specific qPCR primers selectively amplify in a proportionate manner. This study demonstrated performance that was similar overall to NGS methods, but also found a moderate improvement in diagnostic performance for analysis based on absolute quantification of dd-cfDNA (expressed in copies/mL) relative to analysis of the percentage of dd-cfDNA.

Overall, validation results of each assay reported to date show comparable results despite differences in platforms and methods. Importantly, the data available strongly demonstrate that measurement of dd-cfDNA at a single time point is highly quantitative within the range of the assay. A summary of clinical validation values for each assay reported to date in kidney transplant recipients is shown in **Table 3**. Given the variation in rejection prevalence in these studies, the most applicable comparison is shown in the final columns of **Table 3** in which the PPV and NPV values are adjusted to a single rejection prevalence value (15%). This uniformity supports the concept that accurate measurement of the target analyte (dd-cfDNA) results in very similar diagnostic outcomes.

Table 3. Summary of clinical performance for dd-cfDNA assays in kidney transplant recipients

				Reported		Adjusted to 15% prevalence	
Test	**Sensitivity**	**Specificity**	**AUC[1]**	**PPV[2]**	**NPV[3]**	**PPV**	**NPV**
Allosure®	59.0%	85.0%	0.74	61.0%	84.0%	40.9%	92.1%
Prospera™	88.7%	72.6%	0.87	52.0%	95.1%	36.3%	97.3%
TheraSure™	73.0%	73.0%	0.83	13.0%	98.0%	32.3%	93.9%
TRAC™	57.9%	84.5%	0.85	55.0%	86.0%	39.7%	91.9%

[1]AUC, area under the curve from Receiver Operator Curve analysis
[2]PPV, positive predictive value
[3]NPV, negative predictive value

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Conclusion

The clinical performance of Viracor's dd-cfDNA assay was demonstrated to be comparable in results to other recently published assays. Data from four independent dd-cfDNA assays demonstrates remarkably consistent clinical performance parameters, suggesting generalized clinical applicability. However, direct comparison of assay performance for a single sample set has not yet been reported and this type of evaluation would be required to reliably assess the similarity of results. Evaluation of dd-cfDNA provides physicians treating kidney transplant recipients with valuable information regarding organ damage. Additional noninvasive infectious disease testing (e.g. BKV qPCR) and therapeutic drug monitoring will then provide a more comprehensive picture upon which additional diagnostic testing, such as invasive biopsies, and treatment can be based.

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