

## Value of the TruGraf Blood Test as a Biomarker for Monitoring Renal Transplant Recipients

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### Abstract

Kidney transplantation is the optimal treatment for many patients with end-stage kidney disease. The short-term outcomes of kidney transplantation have improved in the past few decades as a result of development of newer immunosuppressive drugs, more effective anti-microbial prophylaxis, and improved surgical techniques; however, long-term outcomes remain suboptimal. Routine post-transplant monitoring consists of the measurement of serum creatinine (SCr) and immunosuppressive drug levels. However, both are insensitive and non-specific markers of graft damage.

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### Introduction

Routine surveillance (protocol) biopsies have been used in some centers to monitor patients with stable renal function following kidney transplantation, but these are expensive, invasive, pose significant logistical issues and are subject to variability of interpretation, thus limiting wider application. The current standard of care in monitoring patients following kidney transplantation ranges from not using surveillance biopsies at all, using them only in patients at high immunologic risk, to routine use (protocol biopsies) in all patients. For these reasons, repetitive surveillance biopsies are not a practical approach to monitoring after renal transplantation [1-6].

Subclinical acute rejection (subAR) is the presence of histological features of acute rejection on renal biopsy in the absence of a decline in renal function. SubAR is present in approximately 25% of surveillance biopsies in renal transplant recipients with stable renal function [7]. Therefore, roughly 75% of surveillance biopsies could be avoided if there was a validated and reliable biomarker test that would distinguish patients with stable renal function who had a quiescent immune profile from those with immune activation. A validated test to measure and monitor the adequacy of immunosuppression is needed in order to prevent over-immunosuppression, which may result in opportunistic infections, malignancy and drug toxicities (such as nephrotoxicity and new-onset of diabetes after transplantation/NODAT, and under-immunosuppression with resulting acute rejection [8]. The need for robust, multicenter validation biomarker studies

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for monitoring the complexities of immunosuppressive therapy, and, thereby, improving long-term results in transplant recipients is well-documented [9,10]. Genomic biomarkers in the blood or urine hold the promise for non-invasive immune monitoring that better inform patient management and monitoring following renal transplantation.

### Discussion

Molecular biomarkers have been studied in the graft, urine and blood of kidney transplant recipients [11-15]. DNA microarrays have been used to analyze tissue biopsies of kidney transplant

recipients to detect gene expression profiles associated with rejection [12]. The urinary cell mRNA profile has been used for the assessment of acute cellular rejection in kidney allograft recipients [13]. The kSORT assay measures gene expression of a 17-gene set via quantitative real-time PCR (qPCR) to detect renal transplant patients at high risk for acute rejection [14]. However, none of these tests are routinely used in a clinical setting because the tissue test requires a biopsy, and the others have yet to be validated in biopsy-proven samples. What's more, none of these serum tests have been shown to detect subAR in patients with stable SCr levels. The recently introduced AlloSure test measures donor-derived cell-free DNA (dd-cf DNA) in kidney transplant recipients [15]. While the test has a high predictive value for detection of antibody-mediated rejection (AMR), it was shown to be associated with the presence of donor-specific antibody (DSA), which is a diagnostic criterion for the diagnosis of AMR in the Banff classification [16]. In a separate publication a dd-cf DNA level above 1.2% was reported as being out of range and potentially abnormal, with approximately 96% of the samples exhibiting dd-cfDNA values below 1.0% indicating they are normal [17]. This data demonstrates that AlloSure is unsuitable for identifying the 25% of subjects with stable renal function who are experiencing subAR.

The TruGraf test (Transplant Genomics Inc., Mansfield, MA) described in this paper was developed in our Clinical Laboratory Improvement Amendments (CLIA) approved laboratory in Pleasanton, CA. TruGraf is a blood-based assay that provides non-invasive, accurate detection of adequacy of immunosuppression in kidney transplant recipients. Microarray analysis was used to study gene expression. The test is based on analysis of gene-expression "signatures" in the peripheral blood that can differentiate a state of immune quiescence, indicating an adequate state of immunosuppression, referred to as Transplant eXcellence (TX) from not-TX, an indication of suboptimal immunosuppression or immune activation. The aim of the TruGraf test is assist the physician in the assessment of whether the current level of immunosuppression is adequate and to help guide personalized treatment plans, thereby protecting the function and prolonging graft survival in each individual patient. This paper describes clinical validation studies involving samples from four different transplant centers that demonstrate the performance of the TruGraf blood test.

The probesets selected for the TruGraf Classifier were selected not only for the altered gene expression associated with graft rejection, they were also selected on the basis of robust performance, i.e., probesets that were determined to create a lot of signal "noise" were eliminated from contention as those probesets would not provide a stable signature for routine use in a CLIA setting. The TruGraf Classifier is designed up front to minimize the occurrence of probeset signals being out-of-range.

Details of the analytical and clinical validation have been published previously [18]. The performance statistics were calculated on the basis of comparisons of TruGraf results with concurrent biopsy phenotype results taken from specimens used to run validation studies. Each run has several in-process controls run concurrently

with the samples on the run. The Affymetrix Genechip workflow has external RNA controls that monitor labeling and hybridization reactions. Final QC metrics (post-hybridization) include correct classification of run controls, GAPDH Ratio results and review of the Affymetrix external RNA control results prior to the release of results [18]. Labeling and hybridization control intensities were used to generate the descriptive statistics for this study cohort. In the external controls, the coefficient of variation for all RNA controls was <10%, indicating a high degree of reproducibility [18]. A degree of interpatient variability is inherent in the microarray analysis process (mainly due to differences in *in vitro* transcript (IVT) labeling reactions and hybridization); therefore, a whole assay control (WAC) was included on each run [18].

As a part of our initial validation, a cohort of samples was tested with varying lots of reagent in order to further understand the contribution of processing variability over time. Specimen requirements for the TruGraf assay are peripheral blood collected in the BD PAXgene Blood RNA system. Biological replicate samples were processed that had individual PAXgene tubes subjected to various preanalytical conditions including elevated temperatures (> 400C) or extended periods at ambient temperatures (> 3 days) prior to RNA extraction. Specimens arriving at the CLIA Lab that do not meet preanalytic processing criteria were rejected for analysis as the quality of RNA obtained was found to be insufficient [18].

The classifier comprised of 210 probesets that mapped to 161 fully annotated genes. Utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) [19], the pathways that they mapped to were: central carbon metabolism in cancer, AMPK signaling, Fc gamma R-mediated phagocytosis, aldosterone-regulated sodium reabsorption, type II diabetes mellitus and regulation of lipolysis in adipocytes.

The original version of the TruGraf test utilized a classifier developed using the Support Vector Machines (SVM) algorithm to identify genes specific to each phenotype [20]. Further analysis of gene specific data indicated that variable genes, based on the high range of their signals, were confounding the performance of the assay. Further modification used the Random Forest algorithm to select component genes enabled more detailed assessment of each gene's contribution to the test result thereby optimizing assay performance. In this assay TX was considered to be the positive result. A threshold of 0.5 enhanced performance with regards accuracy, positive predictive value (PPV) and sensitivity of the TX phenotype [21]. We subsequently modified our approach to analyzing data generated by the TruGraf test by changing the data interpretation so that we consider not-TX to be the positive/disease class. TruGraf test provides 73% concordance of results with diagnoses based on other clinical data in 105 subjects from the four transplant centers. All patients were monitored with serial serum creatinine levels; in addition, transplant biopsy results were available in 44 subjects. Stability of renal function and histologic findings were used in correlating TruGraf test results. Sensitivity of the test was measured at 81%, specificity 70%, PPV 47%, and NPV 92%, with a false negative rate of 19%. These results are particularly impressive given that

TruGraf is a minimally invasive blood test, and the gold standard histology against which TruGraf is compared is known to be imperfect.

## Conclusion

TruGraf is a qualitative assay designed to rule in or rule out immune quiescence. A TruGraf blood test reported as “TX” would indicate, with a high degree of probability that the individual kidney transplant recipient does indeed have a phenotype of immune quiescence. Such a result would allow the treating physicians to consider following such a patient without doing an invasive surveillance biopsy and to consider reducing the doses of immunosuppressive drugs in conjunction with serial TruGraf monitoring. A follow-up signature of “TX” would reassure the clinician that a lower level of immunosuppression is, indeed, adequate. Alternatively, should the signature change to “not-TX”, whether in the process of monitoring a patient with stable

renal function, or following reduction in immunosuppression, this would serve as a warning sign to monitor the patient more closely with more frequent TruGraf testing, or perhaps to reverse the reduction in immunosuppression, and if indicated, to perform a transplant biopsy.

In the cohort described above, the TruGraf test results could have supported decisions to avoid invasive, costly, logistically challenging and risky surveillance biopsies, or could be used to increase confidence in the results of histology by providing an independent measure in the clinical assessment of the patient. At present, there are still no other blood or urine tests that have been demonstrated to provide any indication in apparently stable patients as to whether or not they are truly immune quiescent; TruGraf is first non-invasive test that offers an alternative or complement to surveillance biopsies to help support physician decisions regarding optimization of therapy in kidney transplant recipients.

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