Analytical and Clinical Validation of a Molecular Diagnostic Signature in Kidney Transplant Recipients

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Abstract

Context: The TruGraf test is a blood-based assay that provides non-invasive, accurate assessment of adequacy of immunosuppression in kidney transplant recipients. TruGraf relies on gene-expression “signatures” that differentiate a state of Transplant eXcellence (TX, indicating adequately immunosuppressed) from not-TX.

Objective: To evaluate the performance of the TruGraf test.

Design: Analytical performance studies to characterize stability of RNA in blood during collection and shipment, analytical sensitivity (input RNA concentration), analytical specificity (interfering substances) and assay performance (clinical validity, and intra-assay, inter-assay, inter-laboratory reproducibility).

Results: Total RNA extracted from whole blood specimens collected in PAXgene Blood RNA tubes was stable up to 3 days at room temperature (stable RNA yield). Under routine ambient shipping conditions, storage and shipping temperatures did not affect results. However, specimen shipments exposed to temperatures >40°C or to ambient temperatures for >3 days were unacceptable for processing. Analytical sensitivity studies demonstrated tolerance to variation in RNA input (50 to 400 ng per 3’ IVT (in vitro transcript labeling reaction). Specificity studies using genomic DNA spiked into 3’ IVT reactions at 10-20% demonstrated negligible assay interference. The test was reproducible across operators, runs, reagent lots, and laboratories. External validation demonstrated that the TruGraf blood test accurately classified patients in 72% of 295 samples.

Conclusions: Analytical sensitivity, analytical specificity, robustness, quality control, and clinical validity of the TruGraf blood test were successfully verified, indicating its suitability for clinical use.

Keywords: Kidney transplant recipients; Gene expression signatures; Immunosuppression; TruGraf blood test

Introduction

The survival benefits of solid organ transplants in the United States are well documented [1]. Improvements in immunosuppression, better anti-infective agents, and improved ancillary care have resulted in significant improvements in short-term outcomes; however, there has been little improvement in long-term graft loss [2-4]. Data from the Organ Procurement Transplant Network/Scientific Registry of Transplant Recipients (OPTN/SRTR) 2015 Annual Data Report indicate that the kidney graft failure rate for deceased donor transplants is 4.8% at 6 months, 6.4% at 1 year, 14.6% at 3 years, 26.3% at 5 years and 52.84% at 10 years [5]. Over the past two decades, attrition rates in the first year post-transplant have shown significant improvement across all subgroups; however, there have been only minor improvements in attrition rates beyond the first year [3].

Post-transplant monitoring relies on serial serum creatinine (Scr) measurements and “protocol” or surveillance biopsies. However the Scr level is a highly insensitive indicator of the degree of damage in the kidney, has a lag time of weeks to months while on-going damage is occurring and changes are non-specific with regards to the cause of the injury. Surveillance biopsies are performed infrequently and with variable frequency after kidney transplantation. Biopsies, other than for-cause, are almost non-existent after two years, and when performed are invasive, expensive, and subject to inter-grader variability of approximately 30% [6]; therefore, performing invasive biopsies is not suitable for frequent monitoring. Currently, there is no validated test to measure or monitor the adequacy of immunosuppression, the failure of which may result in over-immunosuppression and opportunistic infections, or under-immunosuppression and acute rejection [7]. Recent reviews have highlighted the need for robust multicenter validation studies while underscoring the potential for biomarker monitoring of immunosuppressive therapy and transplant outcomes [8,9]. Molecular biomarkers have been studied in the graft, urine and blood of kidney transplant recipients [10-13].

Microarray analysis, a widely used technology for studying gene expression, is not routinely used as a diagnostic tool. Numerous studies have shown that microarray analysis results in improved diagnosis and risk stratification for conditions such as breast cancer [14].

In studies of diagnostic accuracy, the outcomes from a new test under evaluation ("new-test") is compared with outcomes from the reference standard, both measured in subjects who are suspected of
having the condition of interest [15]. New-test may refer to the methods used to obtain additional information on a patient's health status, including information from history and physical examination, laboratory tests, imaging tests, function tests and histopathology. The results of new-test may prompt clinical actions, such as further diagnostic testing, or the initiation or modification of treatment [15]. The term accuracy refers to the amount of agreement between the information from the new-test under evaluation and the reference standard. Diagnostic accuracy can be expressed in many ways, including sensitivity and specificity, likelihood ratios, diagnostic odds ratio, and the area under a receiver operator characteristic (ROC) curve [15]. Positive predictive value (PPV) and negative predictive value (NPV) should also be considered in evaluating the new-test [16].

The goal of method validation in the molecular diagnostics laboratory is to ensure that a given test is ready for implementation in the clinical laboratory, and that the analytical performance of this Laboratory Developed Test (LDT) is comparable between testing laboratories.

To reach that goal, each step of the testing process must be carefully evaluated and documented. TruGraf (Transplant Genomics Inc., Mansfield, MA) is a blood-based assay that provides non-invasive, accurate detection of adequacy of immunosuppression in kidney transplant recipients. TruGraf relies on analysis of gene-expression "signatures" (reference profiles of the expression levels of many genes associated with a given phenotype) that can differentiate a state of Transplant eXcellence (TX, indicating adequately immunosuppressed) from not-TX. With the current standards for monitoring after kidney transplantation, significant tissue injury can progress for months to years without being detected or treated accordingly and result ultimately in graft failure and return to dialysis or death. Through differential diagnosis of TX versus not-TX, the TruGraf blood test provides a noninvasive tool to support physicians in maintaining levels of effective immunosuppression and to help guide personalized treatment plans. Significant healthcare savings could be realized by optimizing each individual patient's therapy by ensuring adequacy of immunosuppression to protect the function and prolong the survival of their graft. This manuscript provides the first description of the analytical validation studies supporting the use of the TruGraf blood test as performed in the Transplant Genomics Inc Clinical Laboratory Improvement Amendments (CLIA) Laboratory.

Materials and Methods

The various steps involved in performing the TruGraf blood test are described below. At a high level, blood samples are obtained from patients, and RNA is extracted, amplified and hybridized to DNA microarrays. Arrays are washed, stained and scanned to detect levels of hybridization of sample RNA to specific oligonucleotide probes. A proprietary algorithm is used to analyze the pattern of hybridization, compare the results with a reference dataset, and generate a qualitative result of "TX" or "not-TX". The results of the TruGraf blood test may be used by a physician, in the context of other clinical information available, to assess whether or not a kidney transplant recipient is adequately immunosuppressed.

RNA extraction, amplification and hybridization

Total RNA was extracted from PAXgene Blood RNA (IVD) tubes (Qiagen, Valencia, CA). PAXgene tubes were processed using PAXgene Blood micro RNA (miRNA) reagents on the QIAcube instrument (Qiagen, Valencia, CA). Total RNA yield and concentration were determined using the Nanodrop 8000 and quality was determined using the Bioanalyzer RNA Nano system (Agilent Technologies, Santa Clara, CA) to generate an RNA integrity number (RIN) [17]. RNA yield and quality thresholds were established and used as acceptance criteria for downstream sample processing. The Affymetrix 3' IVT (in vitro transcript) PLUS labeling system was used to perform in vitro transcription and labeling reactions (3' IVT) on globin-reduced RNA with a reaction input of 200ng (Affymetrix, Santa Clara, CA). Samples were fragmented and a final pre-hybridization RNA quality check was performed on labeled cRNA as well as the fragmented final cRNA product. Hybridization cocktails were prepared with an input of 7.5 µg of biotin-labeled cRNA. Array hybridization and subsequent washing, staining and array scanning steps were completed on Affymetrix HG-U133+ arrays using the standard GeneTitan Gene Expression array workflow (Affymetrix, Santa Clara, CA). A whole assay control (WAC) consisting of RNA from a subject with a known TruGraf response processed from RNA extraction through GeneTitan processing (as utilized in addition to no sample (Nuclease free water) and Affymetrix External RNA controls (Poly A RNA, B2 Oligo and 20x Hybridization Controls) as in-process controls for the RNA labeling and hybridization reactions. Raw expression data files (CEL), an ASCII text file used by Affymetrix software, and generated by the GeneTitan were processed for Quality Control (QC) metrics using the Affymetrix Expression Console software (build 1.4.1.46, Affymetrix). Predefined specifications for yield, array data quality and control sample classifier results were used as acceptance criteria prior to sample data being analyzed on the TruGraf Classifier.

The analytical validation studies performed, including the sample source, the study design and the data evaluated, are summarized in Table 1.

| Study             | Sample source    | Design summary                                                                 | Data evaluated                                      |
|-------------------|------------------|-------------------------------------------------------------------------------|----------------------------------------------------|
| Analytical Sensitivity - LOD | HeLa Control RNA | LOD testing was performed on a dilution series (4 dilutions of 3' IVT Labeling reaction input concentrations and 4 Hybridization reaction input concentrations) of HeLa Control RNA samples. Sample data analysis was performed on the Affymetrix Expression Console software. | In-roces QC Points: NanoDrop and RIN values. Hyb QC Results – includes RLE values and signal boxplots, background levels. Labeling and Hyb Control acceptability, GAPDH signal intensity, GAPD3 3-5 Ratio, Pearson correlations. LOD – 4 sample input concentrations for both the IVT Labeling and hybridization reactions. |
### Table 1: Summary of analytical validation studies.

| Analytical Specificity | Preanalytical Factors | Reproducibility – Intra-assay | Reproducibility – Intermediate Precision | TruGraf™ Classifier Bioinformatics |
|------------------------|-----------------------|-------------------------------|----------------------------------------|-----------------------------------|
| HeLa Control RNA       | PAXGene Blood RNA derived from 3 normal, non-transplant subjects (Sufficient blood was collected to allow for replicates of samples to be run). | PAXGene Blood RNA derived from normal, non-transplant subjects (Sufficient blood was collected to allow for replicates of samples to be run). | PAXGene Blood RNA derived from normal, non-transplant subjects (Sufficient blood was collected to allow for replicates of samples to be run). | Raw data files (.CEL) |
| RNA from the HeLa Control supplied with the 3’ IVT was spiked with genomic DNA and processed thru array Hyb on the GeneTitan. Sample data analysis was performed on the Affymetrix Expression Console software. | Normal subject (NS) blood specimens in PAXgene tubes were obtained by the TGI CLIA Lab. Specimens were subject to varying preanalytic conditions and extracted. Downstream GLOBINclear, 3’ IVT and array hybridization processing were performed on a single run. | Normal subject (NS) blood specimens in PAXgene tubes were obtained by the TGI CLIA Lab. Multiple replicates of 4 patient samples were processed on a single run. | Normal subject (NS) blood specimens in PAXgene tubes were obtained by the TGI CLIA Lab. Replicates of 4 patient samples were run in duplicate on each of 8 separate runs. | Internal Validation – performed on ~120 data files from the original discovery data set. External / Independent Validation – 130 data files processed independent of the discovery set. |
| In-process QC Points: NanoDrop and RIN values. Hyb QC Results – includes RLE values and signal boxplots, background levels, Labeling and Hyb Control acceptability, GAPDH signal intensity, GAPDH 3-5 Ratio, Pearson correlations. Array CEL file data was analyzed on the TruGraf™ Classifier algorithm to generate IQ/IA scores. | In-process QC Points: NanoDrop and RIN values. Hyb QC Results – RLE values and signal boxplots, background levels, Labeling and Hyb Control acceptability, GAPDH signal intensity, GAPDH 3-5 Ratio, Pearson correlations. Array CEL file data was analyzed on the Affymetrix Expression Console software to generate Hyb QC data and on the TruGraf™ Classifier algorithm to generate IQ/IA scores. | In-process QC Points: NanoDrop and RIN values. Hyb QC Results – RLE values and signal boxplots, background levels, Labeling and Hyb Control acceptability, GAPDH signal intensity, GAPDH 3-5 Ratio, Pearson correlations. Array CEL file data was analyzed on the Affymetrix Expression Console software to generate hybridization metrics. Descriptive statistics were evaluated for reproducibility and precision. | In-process QC Points: NanoDrop and RIN values. Hyb QC Results – RLE values and signal boxplots, background levels, Labeling and Hyb Control acceptability, GAPDH signal intensity, GAPDH 3-5 Ratio, Pearson correlations. Array CEL file data was analyzed using RNA data from the Affymetrix Expression Console to generate hybridization metrics. Descriptive statistics were evaluated for reproducibility and precision. | Array CEL files were analyzed using the TruGraf(v0.6) Classifier. |

**Abbreviations**

- LOD: Limit of Detection
- IVT: In vitro Transcription reaction
- RIN: RNA Integrity Number
- Hyb QC: Hybridization Quality Control
- GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
- RLE: Relative logarithmic expression
- CEL file: CEL is an ASCII text file used by Affymetrix software
- gDNA: genomic DNA
The TruGraf classifier

The TruGraf classifier is based on a locked Support Vector Machine (SVM) model using the e1071 package, subsequently adapted for use in the R statistical computing environment. The Cohort 1/Validation Set was used to perform an internal validation of the classifier using the “leave-one-out” cross-validation and bootstrap resampling methods. External validation testing was performed on a second cohort of independent samples that were not used in the training dataset. Blood samples obtained from patients with biopsy-confirmed TX or not-TX phenotypes were used to perform this analysis (Table 2).

| Samples | Discovery | Cohort 1 | Cohort 2 |
|---------|-----------|----------|----------|
| TX      | 238       | 61       | 81       |
| Not-TX  | 260       | 65       | 49       |
| Total   | 498       | 126      | 130      |

Table 2: Discovery and validation data sets.

Results

Preanalytic conditions

One of the advantages of using the PAXgene Blood RNA system is that samples can be shipped overnight at ambient temperature without negative consequences for RNA quality. We examined the effects of potential shipping scenarios during the course of our validation in order to establish specimen acceptability criteria. The main indicator used to assess the quality of labeled RNA transcripts is the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This gene is expressed in most cell types at relatively consistent levels. The Affymetrix HG-U133+ chips include GAPDH probesets for use as assay control metrics. By comparing the signal intensities from the 3’ probes to the 5’ probes, it is possible to obtain a post-hybridization (“post-chip”) measure of the integrity of labeled cRNA. If the resulting ratios are high, this indicates the presence of truncated transcripts. Using the GAPDH ratio as a quality indicator, we determined that samples that were subjected to elevated temperatures (>40°C) or extended periods at ambient temperature (>3 days) prior to RNA extraction yielded degraded RNA unsuitable for downstream processing. Results from this cohort were used to establish specimen rejection criteria (samples held at ambient temperature for >3 days, or samples subjected to elevated temperatures, such as samples that might get lost or delayed during shipping). Figure 1 shows the GAPDH ratio metrics for samples subjected to elevated preanalytic temperatures (sample aliquots E and F), as well as samples held at ambient temperature for longer than 3 days prior to extraction (sample aliquots C and D).

In addition to GAPDH ratio data; the relative logarithmic expression (RLE) is a relative value measure of the deviation of a single array signal compared to a group average which is commonly used to assess variation in microarray studies. Low RLE values are characteristic of high reproducibility and unusually high values indicate outliers. 22 RLE signal data for various samples on this cohort confirm the limits of preanalytic conditions (Figure 2). In this figure, the RLE Boxplots for samples subjected to elevated preanalytic temperatures (sample aliquots E and F), as well as samples held at ambient temperature for longer than 3 days prior to extraction (sample aliquots C and D) showed greater signal variation when compared to samples exposed to routine preanalytical conditions.

Figure 1: Pre-analytical effects of temperature and incubation length on RNA integrity showing glyceraldehyde 3-phosphate dehydrogenase (GAPDH) ratio metrics for samples subjected to elevated temperatures (E and F), and samples held at ambient temperature for >3 days prior to extraction (C and D).

Figure 2: Relative log expression (RLE) Boxplots of pre-analytical samples illustrating that samples subjected to elevated temperatures (T4E and T4F), and held at ambient temperature for >3 days (T4D) had greater signal variation compared to routine samples. Abbreviations: Pt 1, Pt 2, Pt 3 = patients 1, 2, 3; A-L = replicate specimens.

Pearson's correlation data obtained by comparing signal data for all HG U133 Plus probesets again demonstrates the effect of subjecting samples to elevated temperatures or samples held for >3 days prior to extraction; r<0.900 compared to other samples in the cohort (Figure 3).
The RNA extraction process removes heme and DNA as a potential interfering substance that might be present as a result of deviations from the standard RNA extraction process. We designed the samples processed to test the effects of 10-30% (by reaction input) genomic DNA spiked into the labeling reaction. The effects of the varied reaction input conditions were assessed through QC metrics. Probeset signal intensity variance, as expressed by CV% was reviewed for TruGraf classifier – informative probesets. These data indicate that 10-20% genomic DNA contamination of sample RNA does not interfere with individual probeset intensity. When the percentage of contaminating genomic DNA reached 30% individual probeset signal intensities showed increased variance as seen by higher CVs (Figure 4).

### Analytical sensitivity: RNA input

Two limit of detection (LOD) reactions were tested. Group T1A consisted of a set of 4 HeLa Control samples, run in duplicate designed to span the 3' IVT reaction input range from 50 ng to 400 ng of globin-reduced RNA. Affymetrix recommends a 3' IVT process input RNA range of 50 ng to 500 ng per 3' IVT reaction. Samples in this group had their hybridization reaction inputs normalized to 7.5 µg per reaction, creating a group of samples whose results were informative of the effects of differing RNA labeling reaction concentrations.

Group T1B consisted of a set of 4 HeLa Control samples, run in duplicate designed to span a hybridization reaction input range from 3 ug to 10 ug of fragmented, labeled cRNA. Affymetrix recommends a hybridization reaction input of 7.5 ug per hybridization reaction (the range of inputs for this group would therefore represent 40 to 133% of the recommended range). Samples in this group had their 3' IVT reaction inputs set at 200 ng/reaction, creating a group of samples whose results were informative of the effects of differing hybridization reaction concentrations. Review of the GAPDH 3' to 5' Ratio housekeeping gene metric for samples in this cohort demonstrated values ranging from 1.08 to 1.29 for these samples, indicating high quality labeled cRNA. QC analysis of sample data (using Affymetrix Expression Console software build 1.4.1.46) allowed for visualization of clear outliers, especially for the hybridization reaction extremes. Both RLE mean data and RLE signal boxplots indicate that hybridization reaction inputs of 3 µg and 10 µg per reaction result in outliers (Figures 2 and 3). Review of the signal intensity data for 3'IVT labeling and hybridization controls was similarly informative as decreased performance was seen for the higher concentration reactions for both labeling and hybridization reactions.

### Analytical specificity: genomic DNA

The Affymetrix Gene Expression analysis workflow has several different purification steps designed to eliminate interfering substances. The RNA extraction process removes heme and DNA as a part of RNA purification process; the globin reduction process removes globin RNA and the purification step at the conclusion of the 3'IVT Labeling process removes unincorporated label and "left over" reagents, yielding pure labeled cRNA. Genomic DNA was tested as a potential interfering substance that might be present as a result of deviations from the standard RNA extraction process. We designed the samples processed to test the effects of 10-30% (by reaction input) genomic DNA spiked into the labeling reaction. The effects of the varied reaction input conditions were assessed through QC metrics. Probeset signal intensity variance, as expressed by CV% was reviewed for TruGraf classifier – informative probesets. These data indicate that 10-20% genomic DNA contamination of sample RNA does not interfere with individual probeset intensity. When the percentage of contaminating genomic DNA reached 30% individual probeset signal intensities showed increased variance as seen by higher CVs (Figure 4).

### Accuracy and reportable range

Independent clinical validation of the performance of the TruGraf assay was completed on a total of 295 patient samples collected in 3 sample cohorts of 126, 130 and 39 samples. Comparison of the TruGraf molecular phenotype measured from blood was made to the histological phenotype reported for a tissue biopsy collected at the same time as the corresponding blood sample was drawn. Results are shown in Table 3. Accuracy of the TruGraf blood test was 72% (95% confidence interval=+0.01%). The sensitivity of 78% and positive predictive value (PPV) of 89% indicate that a "true TX" will be identified as TX positive in a high proportion of the intended clinical patient population using the TruGraf test.

### Table 3

| True Phenotype | n       | TruGraf TX | TruGraf not TX |
|----------------|---------|------------|----------------|
| TX             | 163     | 127        | 36             |
| Not TX         | 132     | 47         | 85             |
| TOTAL          | 295     | 174        | 121            |

**Statistics** (n = 295)

- **Accuracy**: 72% (212/295)
- **Sensitivity**: 78%
Specificity 65%  
PPV 89%  
NPV 45%  

**Table 3:** Accuracy statistics *population prevalence of TX assumed to be 7.*

**Assay reproducibility**

CEL file data for samples tested on the precision cohorts was analyzed using the Affymetrix Gene Console software. PLIER (Probe Logarithmic Intensity Error) analysis was performed to generate QC metric data. Data for the internal and external RNA Controls was used to assess technical performance of sample processing [18]. Descriptive statistics for external RNA Controls were used to assess precision for hybridization (Hyb) (20X Hyb Controls) and labeling (PolyA IVT Controls). Distribution statistics for average GAPDH signal intensity as well as the GAPDH 3’ to 5’ ratio were also used as internal sample metrics.

The samples processed on the Intra-run precision run were processed with the same lot numbers of reagents throughout the processing workflow. The samples were hybridized on the same Affymetrix HG-U133 Plus array plate. This created a group of 16 samples on this run demonstrated control intensities within ± 2.5 S.D. of the mean intensities. Table 4 demonstrates the CV’s for all external RNA controls were less than 10% indicating a high degree of reproducibility.

| Control | PolyA-LYS | PolyA-PHE | PolyA-THR | PolyA-DAB | Hyb bioB | Hyb bioC | Hyb bioD | Hyb Cre |
|---------|-----------|-----------|-----------|-----------|----------|----------|----------|---------|
| With-in Run | 9.5% | 6.6% | 6.7% | 3.4% | 4.1% | 3.0% | 1.7% | 0.6% |
| Between Run | 14.4% | 10.8% | 12.1% | 7.3% | 4.5% | 3.5% | 2.5% | 1.4% |

**Table 4:** Average coefficient of variation (CV) for external RNA controls (within-run and between run).

Samples processed as part of the intermediate precision cohort were processed on 8 separate runs utilizing several reagent lots (including HG-U133 Plus lots) systematically changed to capture variation throughout the processing workflow. Review of mean intensity values for the Poly A and hybridization controls displayed the appropriate pattern of increasing signal values; reflecting the increase in relative concentration of the controls. All samples on these runs demonstrated control intensities within +2.5 S.D. of the mean intensities (Table 5). The CVs for the PolyA Controls (external RNA Labeling Controls) were <15%, with the LYS Control (present in copy number ratio of 1:100,000) showing the highest CV and the DAP Control (present in copy number ratio of 1:6,667) showing a CV below 10%. External hybridization RNA control CVs were less than 5% indicating a high degree of reproducibility; the highest CV was seen for the bioB Control, which is present at a concentration of 1.5 pM.

Figure 5 displays a graphical view of variation of representative housekeeping and control probeset intensities. Note that probeset intensities are fairly tightly clustered regardless of lot-to-lot reagent changes.

| Validation Study | Qiagen RNA Kit | Ambion GLOBINclear | Affy 3’ Kit | AFFY HWS Kit | HG-U133 Array | Affy Wash A | Affy Wash B |
|------------------|----------------|--------------------|-------------|--------------|---------------|-------------|-------------|
| T5               | Baseline/Lot1  | Baseline/Lot1      | Baseline/Lot1 | Baseline/Lot1 | Baseline/Lot1 | Baseline/Lot1 | Baseline/Lot1 |
| T6A              | Baseline/Lot1  | Baseline/Lot1      | Baseline/Lot1 | Baseline/Lot1 | Baseline/Lot1 | Baseline/Lot1 | Baseline/Lot1 |
| T6B              | Lot 2 Qiagen RNA | Baseline/Lot1     | Baseline/Lot1 | Baseline/Lot1 | Baseline/Lot1 | Baseline/Lot1 | Baseline/Lot1 |
| T6C              | 148052863       | Lot 2 Ambion GLOBINclear | Baseline/Lot1 | Baseline/Lot1 | Baseline/Lot1 | Baseline/Lot1 | Baseline/Lot1 |
| T6D              | Baseline/Lot1  | Baseline/Lot1      | Lot 2 Affy 3’ IVT Reagents | Baseline/Lot1 | Baseline/Lot1 | Lot 2 Wash Buffers | Lot 2 Wash Buffers |
| T6E              | Baseline/Lot1  | Baseline/Lot1      | Lot 2 Affy 3’ IVT Reagents | Baseline/Lot1 | Lot 2 Arrays | Lot 2 Wash Buffers | Lot 2 Wash Buffers |
| T6F              | Baseline/Lot1  | Baseline/Lot1      | Baseline/Lot1 | Baseline/Lot1 | Baseline/Lot1 | Lot 2 Wash Buffers | Lot 2 Wash Buffers |
| T6H              | Baseline/Lot1  | Baseline/Lot1      | Baseline/Lot1 | Baseline/Lot1 | Lot 2 Arrays | Lot 2 Wash Buffers | Lot 2 Wash Buffers |
Bioinformatic Validation – Internal and External Validation

The Internal validation of the classifier was performed with two methods: (i) leave-one-out cross-validation and (ii) bootstrap resampling. The leave-one-out cross-validation method iteratively removes each sample from the population, trains the model on the remaining samples, and predicts the class of the excluded sample. The accuracy is then calculated as a percentage of correctly classified samples in the discovery dataset. Each bootstrap resampling is a random subset of samples from the discovery dataset where some samples were represented more than once. Each bootstrapped dataset is then used to train a model that is tested on the samples not found in the bootstrap resampling population. On average, ~63% of the samples in the discovery dataset will be found in a given bootstrap iteration and the testing results are used to calculate an estimated error based on the number resampling iterations (i.e., 500. The “632+” adjustment is then applied to the estimated error) [19].

The External validation was performed on two independent cohorts of TX and not-TX samples that were not used in the training dataset (Table 2). Each validation run of the bioinformatic pipeline used a set of sequentially named (i.e., S001 to S126) .CEL files as input and delivered the output as a tab-delimited text file with three fields for each of the samples. These fields are the sample name (i.e., S001 to S126), the classification of the sample, and an internal (non-reportable) score associated with the classification.

Result comparisons were based on use of the histologically determined phenotype from the sample’s matched biopsy. Additionally, the algorithm installation in the TGI CLIA Lab was tested by running CEL files from the validation cohort #1 and the independent cohort #2 (Table 2) and comparing the results output to the previous algorithm output generated during the internal validation. Interpretation calls and score outputs of the external validation testing were compared to results generated during internal testing. Additional statistics (accuracy, sensitivity, and specificity) were calculated for the entire validation cohort and compared to the previous results (Table 6). Each of these metrics were calculated for the validation cohorts using TX or not-TX as the reference range.

Error rates were determined by comparing molecular phenotype results to the histological phenotypes of a matched biopsy obtained at the time of PAXgene blood sampling. Error rates for each of the validation cohorts were calculated using the following equation: 100% - % calculated accuracy.

| Metric       | Discovery | Cohort 1       | Cohort 2       |
|--------------|-----------|----------------|----------------|
| Accuracy     | 77.4%     | 77.0% (97/126) | 71.5% (93/130) |
| Sensitivity  | 69.2%     | 70.9%          | 82.4%          |
In this report, we have demonstrated both the analytical validity and clinical validity of the TruGraf blood test and the robust nature of the assay utilizing a number of different metrics. The goal of method validation in the molecular diagnostics laboratory is to ensure that a given test is ready for implementation in the clinical laboratory. To reach that goal, multiple steps in the testing process have been carefully evaluated and documented. Clinical validity has been demonstrated based on a high correlation between TruGraf results and the current gold standard of care for assessing adequacy of immunosuppression, namely histology on tissue samples collected by biopsy of the transplanted kidney.

Predictive performance of the assay has been demonstrated in multiple cohorts, and at a level relevant to how the test will be used in the clinical situation. TruGraf is a blood-based assay that provides a non-invasive and an accurate assessment of adequacy of immunosuppression in kidney transplant recipients. TruGraf relies on analysis of gene-expression signatures that profile the expression levels of many genes associated with a given phenotype, thereby differentiating a state of Transplant eXcellence (TX), indicating adequately immunosuppressed) from not-TX. With the current standards for monitoring after kidney transplantation, significant tissue injury can progress for a prolonged period of time without being detected or treated accordingly, and result ultimately in graft failure and return to dialysis or death. TruGraf blood testing allows noninvasive serial monitoring of kidney transplant patients to detect indicators of adequacy of immunosuppression, which previously was only possible with insensitive trailing biomarkers of damage already done, or invasive procedures not suitable for serial monitoring. Many transplant centers perform invasive protocol biopsies with the inherent assumption that a patient will benefit, when, in fact, approximately 80% of these patients, all of whom have no signs of renal dysfunction, are determined to be immune quiescent. The primary intended use for the TruGraf test will thus be on patients with stable renal function after transplantation to confirm (rule in) a blood gene expression profile consistent with a state of sufficient or over-immunosuppression (TX). Considered in the context of the rest of the clinical information available to the physician, a TX result may support a decision to avoid costly and invasive protocol biopsies on the vast majority of patients who would not benefit from them. We have previously described the potential economic benefits of using the test in place of protocol biopsies [20]. Physicians will be able to use TruGraf results in combination with other laboratory test results and other clinical findings to help develop an individualized treatment plan based on each patient’s unique biology and immune activity levels.

Through differential diagnosis of Transplant eXcellence, TruGraf provides a noninvasive tool to support physicians in maintaining effective levels of immunosuppression and help guide personalized treatment plans. In the process, patients will be spared unnecessary protocol biopsies, the healthcare system will realize significant economic benefits, and the ability to intervene early with therapies to fend off clinical acute rejection may provide the added benefit of improving long-term outcomes.

**Conclusion**

In conclusion, the analytical sensitivity, analytical specificity, robustness, quality control and clinical validity of the TruGraf assay were successfully verified in these studies.

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The authors of this manuscript have the following conflicts of interest to disclose: SMK, JIF and MMA are founding scientists and have stock ownership in Transplant Genomics Inc. MRF, DP, MM, SR, AV, NB, PL, and DL are full-time employees at Transplant Genomics Inc. The other authors have no conflicts of interest to disclose.

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