Journal of Medical Diagnostic Methods
ISSN: 2168-9784
OPEN ACCESS Freely available online
Research Article

Analytical Performance Validation of a Multi-Biomarker Test for Assessment of Allograft Rejection in Renal Transplant Recipients

Zawada RJX[1], Valdivieso K[1], Katzenbach P[1], Chalasani K[1], Mark C[1], Hongo D[1], Lugtu G[1], Chen E[1], Huang SJ[1], Wexler DS[1], Yang JYC[1,2], Sarwal R[1,2], Sarwal MM[1,2], Nolan N[1*]

[1]*NephroSant Inc. 150 North Hill Drive, Suite 10, Brisbane, CA 94005, USA*

[2]*Department of Surgery, University of California San Francisco, San Francisco, CA 94143, USA*

ABSTRACT

A novel algorithm has been developed for a multi-biomarker urine test, as a clinical tool to provide a quantitative measurement for the noninvasive detection of renal transplant rejection. The analytical performance of the six urinary biomarkers (Cell-Free DNA (cfDNA), Methylated cfDNA (m-cfDNA), Clusterin, CXCL10, Creatinine, and Total Protein) was assessed for sensitivity, specificity, and reproducibility as per Clinical Laboratory Improvement Amendments (CLIA) recommended guidelines. The characterization analysis indicated robust analytical performance across all six biomarkers contributing to the composite renal transplant rejection Q-score. This test was designed be used as a surveillance tool to accurately and reliably assess the rejection status of kidney transplant patients. The potential clinical utility of the test includes early detection, proactive management of graft rejection, and ultimately controlling subclinical intragraft inflammation thereby prolonging graft survival.

Keywords: Acute kidney rejection; T cell-mediated rejection; Antibody-mediated rejection; Cell-free DNA; Kidney transplant; QSant

INTRODUCTION

Following kidney transplants, it is essential to monitor for evidence of rejection to reduce the risk of graft loss. The current gold standard for detecting kidney transplant rejection is measuring serum creatinine levels followed by confirmation through a kidney tissue biopsy. However, the elevation of serum creatinine levels is not necessarily specific to kidney rejection and is a late indicator where there can be greater than 60% kidney injury before creatinine levels start to rise [1]. Kidney biopsies are more sensitive than serum creatinine, but they can still miss over 30% of transplant rejections since they are site specific and many kidney diseases have specific foci of diseased tissue and do not affect all regions of the kidney uniformly [2]. Moreover, biopsies are invasive, can lead to other complications, and are expensive, with a cost of nearly $4000 per biopsy [3]. More sensitive and less invasive tools for the monitoring of kidney rejection in a transplant early when tissue damage is still reversible is a critical unmet need.

Here we report the analytical performance of a highly sensitive, specific, and non-invasive urine test for early detection of kidney rejection before current diagnosis by serum creatinine elevation. The test is comprised of a panel of six biomarkers to compute a predictive injury Q-score that quantifies the risk level of kidney allograft rejection. This means that physicians can reduce the number of unnecessary biopsies but still detect clinical and subclinical transplant rejections early, to provide patient-specific treatments to prevent the progression of kidney injury and allograft loss.

The six biomarkers utilized by the algorithm, cfDNA, m-cfDNA, Clusterin, CXCL10, Creatinine, and Total Protein, were initially selected based on previous urine genomic, metabolomic, and proteomic studies that showed that these biomarkers could track kidney injury from different renal subcompartments [4,5]. Cell-free DNA (cfDNA) are small DNA fragments found circulating in blood and other bodily fluids and can also be excreted in urine. In healthy individuals, the levels of cfDNA are generally low; however, during pregnancy, illness, and exacerbation of tissue (intensive exercise or injury) the levels of cfDNA generally increase. After organ transplantation, Donor-Derived Cell-free DNA (dd-cfDNA) can be detected in the recipient's blood and urine. The dd-cfDNA kinetics seems to follow an L-shaped curve with high percentages in the immediate post-engraftment phase followed by a swift decrease to a stable baseline level [6]. Similar dd-cfDNA

Correspondence to: Niamh Nolan, NephroSant Inc. 150 North Hill Drive, Suite 10, Brisbane, CA 94005, USA, Telephone: +14084396648; E-mail: nnolan@nephrosant.com

Received: October 07, 2020; **Accepted:** October 15, 2020; **Published:** October 22, 2020

Citation: Zawada RJX, Valdivieso K, Katzenbach P, Chalasani K, Mark C, Hongo D, et al. (2020) Analytical Performance Validation of a Multi-Biomarker Test for Assessment of Allograft Rejection in Renal Transplant Recipients. J Med Diagn Meth 9:301. doi: 10.35248/2168-9784.2020.9.301

kinetic curves have been observed in plasma and urine from kidney transplant recipients. Existing literature shows that dd-cfDNA has high negative predictive value for ruling out transplant rejection and can better support the decision to use biopsies to confirm rejection in solid organ transplants [6]. In a recent study that looked into monitoring methods for active rejection in kidney allografts, researchers found that the dd-cfDNA level discriminated between Antibody-Mediated Rejection (ABMR) and controls (no rejection histologically), $p<0.001$ (receiver operating characteristic Area Under the Curve (AUC), 0.74; 95% Confidence Interval (95% CI), 0.61 to 0.86) [7,8]. Further, when cfDNA level is used in conjunction with Donor Specific Antibodies (DSA) monitoring, the detection of ABMR is improved with an AUC, 0.86 [9]. However, since only a small fraction of the total cfDNA in circulating blood is graft-derived, quantification of dd-cfDNA from blood is challenging; whereas, cfDNA in urine is primarily from the transplanted kidney [10]. Therefore, monitoring cfDNA in urine promises to be a more robust and sensitive means to reflect kidney injury following transplantation. The methylated fraction of cfDNA (m-cfDNA) is also included in the panel as it can further augment detection of renal parenchymal injury [11].

C-X-C motif chemokine 10 (CXCL10), also known as Interferon Gamma-Induced Protein 10 (IP-10) or small-inducible cytokine B10, is a protein belonging to the CXC chemokine family that in humans is encoded by the CXCL10 gene. Existing literature demonstrates that CXCL10 is a reliable marker of both renal and transplant inflammation and, thus, is a good marker for monitoring renal injury in patients post-kidney transplant [12–14].

Clusterin is a disulfide-linked heterodimeric protein associated with the clearance of cellular debris and apoptosis and was first identified in ram rete testis fluid where it showed signs of clustering with rat sertoli cells and erythrocytes, hence its name. Based on existing literature, clusterin is a good biomarker for monitoring kidney tubular injury in transplant patients [15].

Creatinine is a breakdown product of creatine phosphate in muscle and is produced at a relatively constant rate, depending on muscle mass. Creatinine is removed from the blood chiefly by the kidneys, primarily by glomerular filtration, but also by proximal tubular secretion. Existing literature demonstrates that while serum creatinine is a good indicator of clinically symptomatic rejection, creatinine measurements are often subject to variability and their results should be interpreted with caution. Studies have indicated that normalized levels of a biomarker reflecting tubular injury can be influenced by dynamic changes in the urine creatinine excretion rate when the GFR changes [16]. Thus, despite creatinine having poor precision in the monitoring of kidney function, it is still useful as a 'normalizing biomarker' in urine [17]. Measuring spot urine creatinine normalizes protein excretion to the glomerular filtration rate, such that the urine protein–creatinine ratio is not subject to variation due to hydration status. Therefore, urine creatinine was included for data normalization and control for hydration status and diurnal variation [16,18].

Existing literature demonstrates urinary total protein to be a reliable marker for the monitoring of kidney injury in patients post-transplant. As mentioned earlier, measurements of urinary total protein are always normalized to creatinine (known as the protein/creatinine ratio or PCR or Proteinuria) as measuring spot urine creatinine normalizes protein excretion to the glomerular filtration rate; and studies have shown proteinuria to have a strong independent association with AKI [19]. Total protein was selected as a late marker of glomerular injury [20,21].

Combining biomarkers that provide independent value for predictive and diagnostic purposes is well-established to increase the predictive capabilities for a given indication, especially for complex diseases that have multiple root causes and phenotypic differences. Transplant rejection is one such indication, given the different presentations (ABMR, TCMR, mixed) resulting from distinct types of immunological responses and the molecular heterogeneity of rejection types [22]. The six biomarkers described above were selected based on genomic, epigenomic, proteomic, and metabolomic studies specifically looking at kidney transplant rejection and capture the wide variety of immunologic presentations and kidney sub-compartment injuries that would be expected in rejection episodes of the kidney. Indeed, these biomarkers have been shown to provide independent value to the prediction and diagnosis of kidney transplant rejection. To increase the predictive power of monitoring these biomarkers, a composite Q-score ranging from 0 to 100 on these biomarkers using a Random Forest bootstrap model was developed. From a training set of 111 urine samples that were tested with an allograft biopsy that included both stable (STA) and acute rejection (AR) patients, an optimal threshold Q-score was found that maximized sensitivity and specificity (94.9% and 100%, respectively) for detection of rejection. This threshold was then applied to two validation sets totaling 162 samples and the observed sensitivity and specificity of the aggregate was found to be 95.2% and 95.9%, respectively [23]. Thus, tracking the levels of these six biomarkers that make up the QSant panel in urine can clearly be a powerful tool for monitoring allograft rejection in kidney transplant patients.

This report evaluates the analytical performance of the assays for the biomarkers that make up the QSant panel. Since the Creatinine and Total Protein assays used for the QSant panel are already Food and Drug Administration (FDA) approved and have had their analytical performance already analyzed and validated, this paper will focus on the analytical performance of the cfDNA, m-cfDNA, CXCL10 and Clusterin assays.

MATERIALS AND METHODS

Biomarker panel quantification protocols

The QSant algorithm utilized measurements of six urinary biomarkers: cfDNA, Methylated cfDNA (m-cfDNA), CXCL10, Clusterin, Creatinine, and Total Protein. Briefly, cfDNA was extracted from urine using QIAamp Circulating Nucleic Acid Kit (Qiagen, Germantown, MD, USA). The extracted DNA was coated on a high binding 96-well plate for 2 hours at room temperature. The plate was then washed with Phosphate-Buffered Saline (PBS) and blocked with 5% Bovine Serum Albumin (BSA) in PBS for one hour and washed again. Next the plate was incubated with a proprietary biotinylated probe complementary to the ALU human element for 1 hour and then washed. Streptavidin-HRP (R and D Systems, Minneapolis, MN, USA) and SuperSignal™ ELISA Femto Substrate (Thermo Fisher Scientific, Waltham, MA, USA) were used for luminescent detection and quantitation. The reported cfDNA values were dilution-adjusted and reported as Genomic

Equivalents (GE) per mL, where one GE is equivalent to 6.6 pg of human DNA. A standard curve is generated from fragmented Human Mixed Genomic DNA standard (Promega, Madison, WI, USA).

Similarly, m-cfDNA was extracted from urine using QIAamp Circulating Nucleic Acid Kit (Qiagen). The extracted DNA was coated on a high binding 96-well plate for 2 hours at room temperature. The plate was then washed with PBS and blocked with 5% BSA in PBS for one hour and washed again. Next, the plate was incubated for 1 hour at room temperature with an anti-5-methylcytosine antibody (Thermo Fisher Scientific) and then washed. Subsequently, the plate was incubated for 1 hour at room temperature with Goat anti-Rabbit Poly-HRP Secondary Antibody (Thermo Fisher Scientific) and then washed. Finally, SuperSignalTM ELISA Femto Substrate (Thermo Fisher Scientific) was used for luminescent detection and quantitation. The reported m-cfDNA values were dilution-adjusted and reported as ng/mL. A standard curve was generated from fragmented methylated DNA standard (Thermo Fisher Scientific).

CXCL10 and Clusterin were measured with customized versions of ELISA Kits (R and D Systems, Minneapolis, MN, USA). Creatinine and Total Protein were measured on a Beckman Coulter AU400 analyzer using the Creatinine (OSR6178) and Total Protein (OSR6270) reagents.

Process control urines

In addition to the Standard Curve Calibrators, a set of two or three well-characterized urine samples were also included in every test run.

Samples were collected midstream in sterile containers and centrifuged at 2000 xg at 4 degrees Celsius for 30 minutes within three hours of collection. The supernatant was aliquoted, and stored at –80 degrees Celsius until further use. For the cfDNA and m-cfDNA assays, urine pools were prepared from clinically asymptomatic or "Normal" (NL) individuals, as well as individuals previously diagnosed with Chronic Kidney Disease (CKD). For the Clusterin assay, a "high" control from a pool of CKD donors and "low" control from a pool of normal donors were sourced from BioIVT. A "medium" control for the Clusterin assay was also prepared by mixing equal parts of the "high" and "low" controls. For CXCL10, a "high" control from a pool of CKD donors was also used, as well as high and low controls sourced from R&D Systems. These samples served as longitudinal measures of test performance for each analyte.

Curve-fitting and concentration calculation

Each of the four assays characterized in this manuscript utilized a 4-Parameter Logistic (4-PL) model (see below) to generate a calibration curve relating the raw signal observed for each step in the standard curve dilution series to the known analyte concentration. The signal(s) obtained for individual analytes from test specimens were then plotted back against the 4-PL derived calibration curve for that analyte to compute their concentration.

$$Concentration = D + \frac{A - D}{1 + \left(\frac{X}{C}\right)^{B}}$$

Where:

A: Signal associated with lowest analyte concentration

B: Hill slope

C: Analyte concentration inflexion point (C50)

D: Signal associated with high analyte concentration

X: Signal associated with individual analyte measurement

Sensitivity: Limit of Blank (LOB), Limit-of-Detection (LOD) and Upper Limit of Quantitation (ULOQ)

To assess the analytical sensitivity (LOB and LOD) of the processes underlying the measurement of each of the analytes included in the QiSant test, a series of studies utilizing either elution buffer for the cfDNA and m-cfDNA assays or urine-based diluents for the CXCL10 and Clusterin assays (aka "Blanks") and known concentrations of purified forms of each analyte were used. Measurements were carried out on at least ten plates and by at least two operators for each analyte.

These measurements were, in turn, used to establish a LOB for each analyte such that there is less than a 5% chance that specimen containing measurable analyte would return a valuable indicative of an absence in analyte. The LOB is expressed in concentration:

$$LOB = Blank_{Mean} + 2 \times Blank_{StDev}$$

where $Blank_{Mean}$ is the average signal detected for the "Blanks" and $Blank_{StDev}$ is the standard deviation of the signals detected for the "Blanks".

These measurements were also used to establish a LOD for each analyte such that the chance that specimen lacking measurable analyte would return a value indicative of the presence of analyte. The LOD is expressed in concentration:

$$LOD = Blank_{Mean} + 3 \times Blank_{StDev}$$

The ULOQ was set for each assay as the concentration of the highest process control or standard that had an acceptable Co-Efficient of Variation (CV) and exhibited an interpolated concentration with a 70-130% recovery of the expected concentration.

Analytical linearity

Analytical linearity of the analytes included in the QSant test was determined by serially diluting known concentrations of analytes across the anticipated Analytical Measurement Range (AMR) in either an elution buffer (cfDNA and m-cfDNA) or a urine-based (CXCL10 and Clusterin) matrix. For all assays, serial 2-fold points were prepared for each respective assay. Individual analyte values collected from at least ten replicate runs performed by multiple operators were used to calculate percent recovery. Percent recovery was calculated for each dilution step according to the formula (%Recovery=Observed Concentration/Known Spike Concentration × 100%). Those analyte concentrations exhibiting 70%-130% recovery with an acceptable Co-Efficient of Variation (CV) established the Analytical Measuring Range (AMR) for each analyte.

Analytical precision (reproducibility)

To assess the analytical precision (%CV) of the assays underlying the measurement of each of the four analytes included in the QiSant test, different specimen types were utilized across at least

ten plates by multiple operators. For cfDNA and m-cfDNA, Process Control batches from a known CKD donor and known "Normal" donor were used. For CXCL10, Process Controls prepared from purified recombinant proteins were used, as well as CKD patient urine. For Clusterin, Process Controls secured from urine of CKD patients and Normal patients were used separately and mixed half and half for an intermediate control.

The raw signal and calculated concentrations associated with three intraplate replicates of two or three Process Controls were collected from at least ten independent runs. These studies yielded a total of at least fifteen inter-plate replicates for each Process Control run by at least two operators.

Interfering substances

Interfering substances for protein and creatinine can be found in literature, including instrument-reagent manufacturer studies; however, established interfering substances were not found for cfDNA, m-cfDNA, Clusterin, and CXCL10, which is a limitation for the test at this time. Semi-quantitative information of common analytes (leukocytes, nitrites, urobilinogen, pH, blood, ketones, bilirubin, and glucose) is screened at sample receipt and reported for the sample, to aid in the determination of kidney rejection in conjunction with the Q-score and other laboratory and clinical findings for the patient. A study was performed for the potential interference of blood, which is determined to be the highest potential source of contamination. Whole blood samples from 3 separate donors were obtained. Synthetic urine (for Clusterin and CXCL10) and urine from a CKD donor (for cfDNA and m-cfDNA) were used for the study.

In addition to a baseline/non-spiked urine, three levels of whole blood were spiked into the synthetic urine and CKD donor urine: Low (0.001%, ~50 ery/µL); Medium (0.002%, ~100 ery/µL) and high (0.005%, ~250 ery/µL). For Clusterin and CXCL10, high, medium, and low levels of purified recombinant standard were spiked into each of the three donor blood sample sets and also into a set of non-spiked synthetic urine (four samples per blood spike and three blood levels for twelve total samples per blood donor). For the cfDNA and m-cfDNA assays, the three levels of blood from one blood donor were spiked into a CKD donor's urine prior to processing the urine samples. A level of interfering substance exhibiting greater than a 30% change in analyte concentration was deemed as interfering with the analytical measurement of that analyte and, hence, was set as a pre-analytical exclusion criterion for specimens submitted for testing. This degree of change was selected as it exceeds the variation deemed acceptable during routine repeated testing of any given sample. The %Change in the measured concentration in the presence of a potential interfering substance was computed as follows:

$$\%Change = \frac{|Concentration_{Plus\ Interferent} - Concentration_{No\ Interferent}|}{Concentration_{No\ Interferent}} \times 100\%$$

RESULTS

Interfering substances

cfDNA: Standard curves ranging from 666,667 GE/mL down to 10,417 GE/mL in two-fold dilution steps were run on fourteen plates by two separate operators. A similar curve shape was observed on all fourteen plates. The average RLUs for all the standard replicates were plotted and is depicted in Figure 1. The percent recoveries of the interpolated values for the six highest standards was between 70%-130% on each plate. The lowest standard (10,417 GE/mL) was not consistently interpolated and must be considered outside the AMR. Moreover, although we consistently interpolated the 2nd lowest standard (20,833 GE/mL), the LOD computed from the standard deviation of the blanks was slightly higher at 22,950 GE/mL, so this was designated as the lower limit of the AMR. Since the urine is concentrated 20-fold during processing and then diluted two-fold for the cfDNA assay for a final concentrating factor of 10, this range would correspond to 2,295 to 66,667 GE/mL in raw urine.

m-cfDNA: Standard curves ranging from 625 ng/mL down to 10 ng/mL in 2-fold dilution steps were run on thirteen plates by two separate operators. The average RLUs for all the standard replicates from the thirteen plates was plotted and is depicted in Figure 2. The percent recoveries of the interpolated values for all the standards was between 80%-120% on each plate with acceptable precision. Thus, we can confidently set the AMR to be between 10 and 625 ng/mL. Since the urine is concentrated 10-fold during processing and assay, this range would correspond to 1 to 62.5 ng/mL in raw urine.

CXCL10: Standard curves ranging from 500 pg/mL down to

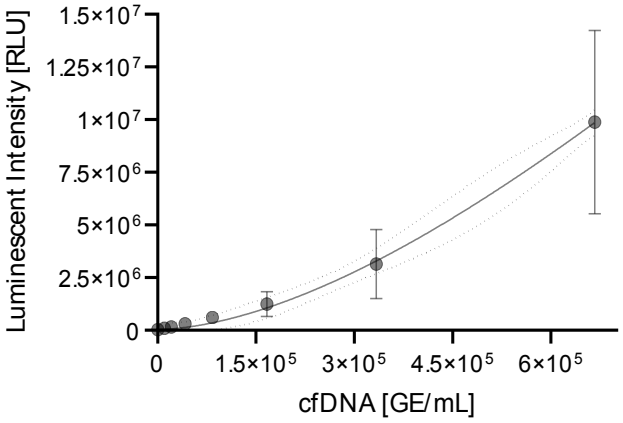

Figure 1: The average RLUs for each cfDNA standard from fourteen different plates (forty-two replicates per standard) run by two separate operators plotted versus the cfDNA concentrations. Goodness of fit was found to have a R2=0.7870.

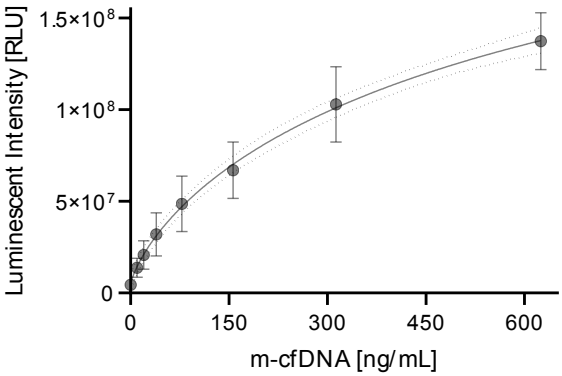

Figure 2: The average RLUs for each m-cfDNA standard from thirteen different plates (thirty-nine replicates per standard) run by two separate operators plotted versus the m-cfDNA concentrations. Goodness of fit was found to have a R2=0.9224.

7.8 pg/mL in 2-fold dilution steps were run on ten plates by two separate operators. A plot depicting all ten of the standard curves along with a plot representing the average of the standard curves are presented in Figure 3. The percent recoveries of the interpolated values for the six highest standards was between 90%-110% on each plate. The %Recovery of the interpolated value for the lowest standard (7.8 pg/mL) was between 88%-112% for every plate, but one for which it had a %Recovery of 74%. Acceptable precision was observed at all standard curve concentration levels. Thus, we can confidently set the AMR to be between 7.8 pg/mL and 500 pg/mL.

Clusterin: Standard curves ranging from 200 ng/mL down to 3.125 ng/mL in 2-fold dilution steps were run on eighteen plates by two separate operators. A plot depicting all eighteen of the standard curves along with a plot representing the average of the standard curves are presented in Figure 4. The percent recoveries of the interpolated values for the six highest standards was between 90%-110% on each plate. The %Recovery of the interpolated value for the lowest standard (3.125 ng/mL) was between 80%-120% for every plate and all standard curve concentration levels showed acceptable precision. Thus, we can confidently set the AMR to be between 3.125 ng/mL and 200 ng/mL. Since the urine samples are diluted 4-fold prior to assaying, this range corresponds to 12.5 ng/mL to 800 ng/mL in urine.

Analytical precision (reproducibility)

cfDNA: Two levels of process controls (High and Low) were added to ten separate plates and run by two different operators. Table 1 summarizes the average intra-plate and inter-plate results.

m-cfDNA: Two levels of process controls (High and Low) were added to ten separate plates and run by two different operators. Table 2 summarizes the average intra-plate and inter-plate results. The inter-plate %CV for the high process control was only 11.3% but was over 30% for the low process control. However, this high %CV was heavily influenced by a single plate on which the measured concentration for low process control was less than half of the average measured concentration from the other nine plates. Indeed, if the low process control from this single plate was excluded, the %CV would drop to 30%. Moreover, this high %CV did not adversely affect the calculated Q-score (see Q-score reproducibility section).

CXCL10: Three levels of quality controls (High, Medium, and Low) and one process control were added to ten separate plates and run by at least two different operators. Table 3 summarizes the average intra-plate and inter-plate results. The inter-plate %CV's were all well below the prescribed 30% limit.

Clusterin: Three levels of process controls (High, Medium, and Low) were added to sixteen separate plates and run by four

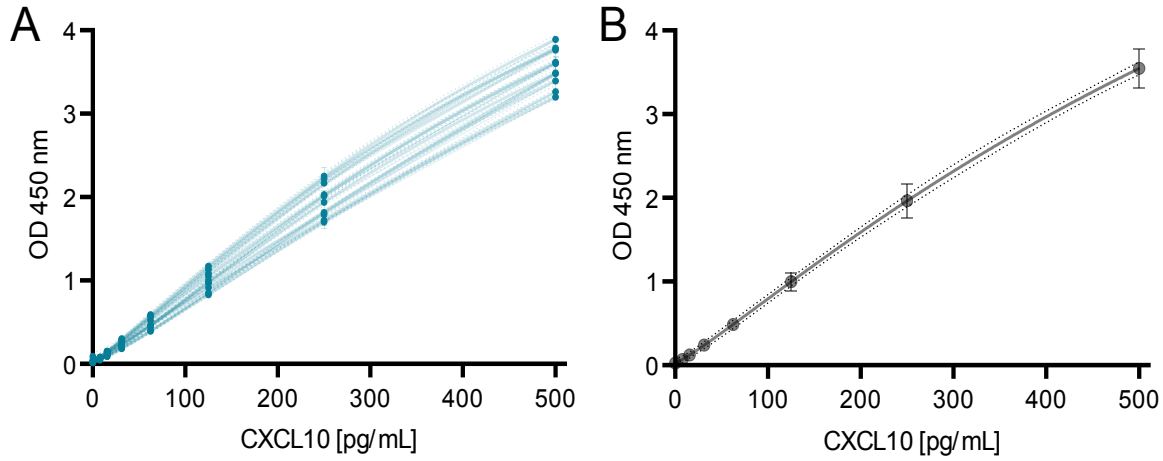

Figure 3: (a) CXCL10 standard curves from ten separate plates and two separate operators plotted individually and (b) from the compiled averages of all plates. Each standard was run in duplicate on each plate for a total of 20 replicates plotted. Goodness of fit was found to have a R2=0.9906.

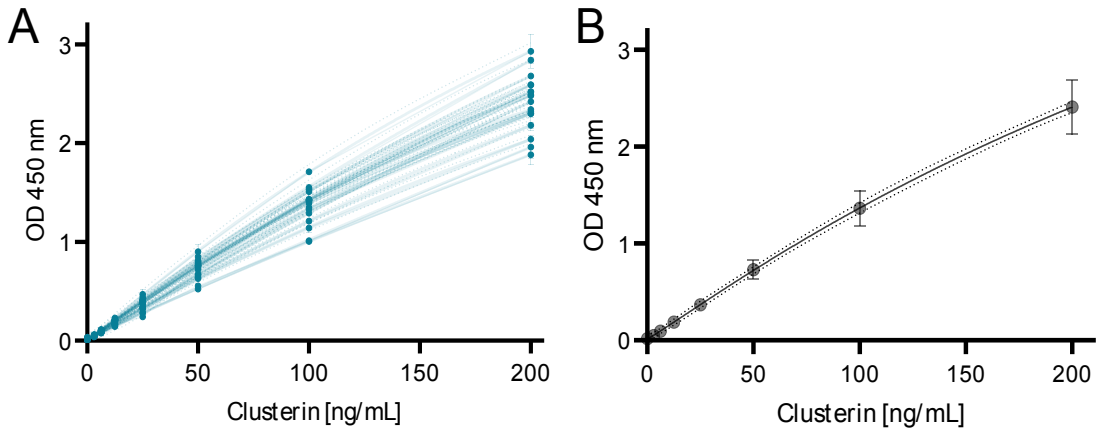

Figure 4: (a) Clusterin standard curves from eighteen separate plates and four separate operators plotted individually and (b) from the compiled averages of all plates. Each standard was run in duplicate on each plate for a total of 36 replicates plotted. Goodness of fit was found to have a R2=0.9765.

Table 1: Results for the cfDNA concentrations in urine of the cfDNA controls. This table summarizes the average intra-plate and inter-plate results over ten separate plates and two separate operators.

Process control	Avg. conc. (GE/mL)	StDev (GE/mL)	Intra %CV	Inter %CV
High (H4)	22739	5262	6.2	23.1
Low (H5)	7156	2204	8.6	30.8

Table 2: Results for m-cfDNA concentrations in urine of the m-cfDNA Controls. This table summarizes the average intra-plate and inter-plate results over twelve separate plates and two separate operators.

Process control	Avg. conc. (ng/mL)	StDev (ng/mL)	Intra %CV	Inter %CV
High (H6)	56.5	6.4	6.2	11.3
Low (H5)	16.4	5.9	5.7	36.2

Table 3: Results for CXCL10 controls. This table summarizes the average intra-plate and inter-plate results over ten separate plates and two separate operators.

Controls	Avg. conc. (pg/mL)	StDev (pg/mL)	Intra %CV	Inter %CV
High	215.9	25.5	3.9	11.8
Medium	109.2	16	8.5	14.7
Low	39	5.7	5.1	14.6
Process control	20.8	5.6	11.8	26.7

Table 4: Results for Clusterin controls. This table summarizes the average intra-plate and inter-plate results over sixteen separate plates and four separate operators.

Process control	Avg. conc. (ng/mL)	StDev (ng/mL)	Intra %CV	Inter %CV
High	102	21.3	3.6	20.9
Medium	55	11.3	6.5	20.6
Low	13.5	2.1	6.1	15.8

different operators. Table 4 summarizes the average intra-plate and inter-plate results. The inter-plate %CV's were all well below the prescribed 30% limit.

Sensitivity, LOB, LOD, and ULOQ

cfDNA: From fourteen plates of standards run for cfDNA by two separate operators, the LOB was found to be 17,990 GE/mL and the LOD was found to be 22,950 GE/mL for the DNA extractions. For the source urine which is concentrated twenty-fold during the extraction process and then diluted two-fold for the assay (equivalent of a ten-fold concentration in the assay), the LOB would be 1,799 GE/mL and the LOD would be 2,295 GE/mL. The ULOQ was 666,667 GE/mL for a DNA extraction which would correspond to 66,667 GE/mL in raw urine.

m-cfDNA: From thirteen plates of standards run for m-cfDNA by two separate operators, the LOB was found to be 3.1 ng/mL and the LOD was found to be 5.3 ng/mL for the DNA extractions. For the source urine which is concentrated twenty-fold during the extraction process and then diluted two-fold for the assay (equivalent of a ten-fold concentration in the assay), the LOB would be 0.31 ng/mL and the LOD would be 0.53 ng/mL. The ULOQ was 625 ng/mL for a DNA extraction which would correspond to 62.5 ng/mL in raw urine.

CXCL10: Ten separate plates with CXCL10 standard curves were run. The data from all plates were compiled to determine the LOB (4.4 pg/mL) and LOD (6.1 pg/mL). The ULOQ was set at the highest concentration of the standard curve, 500 pg/mL.

Clusterin: Eighteen separate plates with Clusterin standard curves were run. The data from all plates were compiled to determine the LOB (1.9 ng/mL) and LOD (2.6 ng/mL). For the source urine which is diluted 4-fold for this assay, the LOB would be 7.6 ng/mL and the LOD would be 10.4 ng/mL. The ULOQ was set at the highest concentration of the standard curve, 200 ng/mL which would correspond to 800 ng/mL in raw urine.

Interfering substances

cfDNA: Three levels of blood were spiked into urine from a CKD donor and from a normal donor prior to processing. The spiked urines and unspiked control urine were extracted and assayed for cfDNA three separate times. The average % change of the cfDNA concentration computed from the three separate assay runs for the three levels of blood ranged from 1.8 to 18.6% in the CKD donor's urine and from 27.3 to 42.6% in the normal donor's urine. However, it must be noted that the cfDNA level in the normal donor's urine was below the LOD of the assay and that the % change in the RLU signals ranged from 9.0 to 14.5%, thus, the high % change in the concentration measurement was due largely to the low levels of cfDNA. Acceptable % change (30%) was selected to not exceed the variation deemed acceptable during routine repeated testing of samples. Therefore, we can rule out blood as an interfering substance for the cfDNA assay.

m-cfDNA: Three levels of blood were spiked into urine from a CKD donor and from a normal donor prior to processing. The spiked urines and unspiked control urine were extracted and

assayed for m-cfDNA three separate times. The average %Change of the m-cfDNA concentration computed from the three separate assay runs for the three levels of blood ranged from 13.4 to 26.2% in the CKD donor's urine and from 2.1% to 26.2% in the normal donor's urine. Thus, we can rule out blood as an interfering substance for the m-cfDNA assay.

CXCL10: Blood from three different "normal" donors was spiked into synthetic urine at three different levels. Each blood spike level was then spiked with three different concentrations of CXCL10: 500, 125, and 15.625 pg/mL. These same concentrations were also spiked into synthetic urine without any blood. The % recovery for each CXCL10 spike was computed by dividing the concentration observed for the blood spiked sample by the concentration measured in the non-blood sample. A percent recovery between 95%-115% was observed for each spike and each blood sample. Thus, we can rule out blood as an interfering substance for the CXCL10 assay.

Clusterin: Blood from three different "normal" donors was spiked into synthetic urine at three different levels. Each blood spike level was then spiked with three different concentrations of Clusterin: 200 ng/mL, 50 ng/mL, and 6.25 ng/mL. These same concentrations were also spiked into synthetic urine without any blood. The % recovery for each Clusterin spike was computed by dividing the concentration observed for the blood spiked sample by the concentration measured in the non-blood sample. Except for the 6.25 ng/mL spike, the lowest spiked concentration, for one of the high-level blood spikes, a percent recovery between 80%-125% was observed for 35 of the 36 spikes. The one sample outside of this range averaged 137% recovery across three plates. However, the spiked concentration of Clusterin in this sample was only 2× higher than the LOD of the assay, so the inherent higher degree of percent variability for even small changes in such low concentrations is likely a major cause for the observed high percent recovery. Thus, we can with a high degree of confidence rule out blood as an interfering substance for the Clusterin assay.

DISCUSSION

The QSant algorithm was developed for a test panel of six biomarkers considered to be key indicators of kidney injury. Maintaining consistent, predictable assay performance for individual analytes is critical for reporting consistent and accurate results for an individual patient. The studies described herein have established a baseline for monitoring the assay panel quality in a clinical reference laboratory. Two of the assays in the test panel were IVD kits previously approved by the FDA and had their analytical performance characterized and validated by the manufacturer. The four remaining tests comprising the test panel examined here met the predefined analytical performance criteria required for consistent Q-Score calculations.

The validated clinical performance of the QSant test has been described elsewhere in two separate clinical studies. The first study by Yang et al. demonstrated a sensitivity of 95.2% and specificity of 95.9% for distinguishing between stable and acute rejection patients [23]. The Nolan et al. study reproduced this clinical performance [24]. Because the QSant test provides a quantitative rejection risk estimate, the larger the Q-Score, the higher the patient's rejection risk. Therefore, acceptable analytical reproducibility of the assays are critical for accurately assigning rejection risk level. Having demonstrated acceptable analytical performance for the assays comprising the QSant test, the current work enhances the confidence clinicians can place in the Q-Score to aid their clinical decisions.

CONCLUSION

We have demonstrated acceptable analytical performance of the QSant panel of assays using CLSI guidelines, thus validating its analytical capability for assessing the health of a transplanted kidney to provide early detection, proactive management of graft rejection, control of subclinical intra-graft inflammation, and reduce the use of invasive biopsies. This test has the potential to provide a powerful noninvasive monitoring tool to assist doctors in providing the proper treatments to prolong graft survival.

AUTHOR CONTRIBUTIONS

Zawada RJX, Sarwal MM and Nolan N: Study concept and design; Zawada RJX, Valdivieso K, Katzenbach P, Chalasani K, Mark C, Yang JYC and Nolan N: Methodology development; Hongo D, Huang SJ, Lugtu G, and Chen E: Clinical laboratory assay support; Wexler DS and Yang JYC: Statistical analysis; Yang JYC: Visualization; Zawada RJX: Writing-original draft preparation; Valdivieso K, Wexler DS, Sarwal MM, Yang JYC, Sarwal R, Hongo D and Nolan N: Writing-review and editing; Zawada RJX, Nolan N and Valdivieso K: Project administration and support. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was funded by NephroSant Inc. Brisbane, CA, USA.

ACKNOWLEDGEMENTS

We are grateful for the help from physicians, clinical coordinators, research personnel, patients, and patient families. We also are grateful to Lucy H. Lu for her suggestions and review of the manuscript.

CONFLICT OF INTEREST

Zawada RJX, Valdivieso K, Katzenbach P, Chalasani K, Mark C, Lugtu G, Chen E, and Nolan N are employees of NephroSant Inc. Hongo D and Yang JYC are consultants of NephroSant Inc. Sarwal MM is the Founder of NephroSant, and is on the FDA Science Board and consults or has recently consulted or received sponsored research funds from Bristol-Myers Squibb, Natera, Astellas Pharma, Genentech, and Jazz Pharmaceuticals.

REFERENCES

1. Baum N, Dichoso CC, Carlton CE. Blood urea nitrogen and serum creatinine physiology and interpretations. Urology. 1975;5:583588.
2. Nicholson ML, Wheatley TJ, Doughman TM, White SA, Morgan JDT, Veitch PS, et al. A prospective randomized trial of three different sizes of core-cutting needle for renal transplant biopsy. Kidney Int. 2000;58:390-395.
3. Schmid A. Percutaneous renal transplant biopsy: Is the safety profile adequate for short-term postprocedure monitoring? Transpl Int. 2016;29:165-166.
4. Watson D, Yang JYC, Sarwal RD, Sigdel TK, Liberto JM, Damm I, et al. A novel multi-biomarker assay for non-invasive detection and quantitative monitoring of kidney injury. J Clin Med. 2019;8:1-17.

5. Yang JYC, Sarwal RD, Sigdel TK, Damm I, Rosenbaum B, Liberto JM, et al. A urine score for noninvasive accurate diagnosis and prediction of kidney transplant rejection. Sci Transl Med. 2020;12:1-11.

6. De Vlaminck I, Valantine HA, Snyder TM, Strehl C, Cohen G, Luikart H, et al. Circulating cell-free DNA enables noninvasive diagnosis of heart transplant rejection. Sci Transl Med. 2014;6:241ra77-241ra77.

7. De Vlaminck I, Martin L, Kertesz M, Patel K, Kowarsky M, Strehl C, et al. Noninvasive monitoring of infection and rejection after lung transplantation. Proc Natl Acad Sci. 2015;112:13336-13341.

8. Bloom RD, Bromberg JS, Poggio ED, Bunnapradist S, Langone AJ, Sood P, et al. Cell-free DNA and active rejection in kidney allografts. J Am Soc Nephrol. 2017;28:2221-2232.

9. Zaky ZS, Grskovic M, Langone A, Matas AJ, Sharfuddin A, Fischbach B, et al. Biological variation of donor-derived cell-free DNA in renal transplant recipients: clinical implications. J Appl Lab Med An AACC Publ. 2018;2:309-321.

10. Jordan SC, Bunnapradist S, Bromberg JS, Langone AJ, Hiller D, Yee JP, et al. Donor-derived cell-free DNA identifies antibody-mediated rejection in donor specific antibody positive kidney transplant recipients. Transplant Direct. 2018;4:e379.

11. Yang JYC, Sarwal MM. Transplant genetics and genomics. Nat Rev Genet. 2017;18:309-326.

12. Shield PG, McGuinness D, Eriksson M, Kooman JP, Stenvinkel P. The role of epigenetics in renal ageing. Nat Rev Nephrol. 2017;13:471.

13. Yang JYC, Verleden SE, Zarinsefat A, Vanaudenaerde BM, Vos R, Verleden GM, et al. Cell-free DNA and CXCL10 derived from bronchoalveolar lavage predict lung transplant survival. J Clin Med. 2019;8:9.

14. Rabant M, Amrouche L, Lebreton X, Aulagnon F, Benon A, Sauvaget V, et al. Urinary C-X-C motif chemokine 10 independently improves the noninvasive diagnosis of antibody-mediated kidney allograft rejection. J Am Soc Nephrol. 2015;26:2840-2851.

15. Schaub S, Nickerson P, Rush D, Mayr M, Hess C, Golian M, et al. Urinary CXCL9 and CXCL10 levels correlate with the extent of subclinical tubulitis. Am J Transplant. 2009;9:1347-1353.

16. Zhou X, Ma B, Lin Z, Qu Z, Huo Y, Wang J, et al. Evaluation of the usefulness of novel biomarkers for drug-induced acute kidney injury in beagle dogs. Toxicol Appl Pharmacol. 2014;280:30-35.

17. Waikar SS, Sabbisetti VS, Bonventre JV. Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. Kidney Int. 2010;78:486-494.

18. Tonomura Y, Uehara T, Yamamoto E, Torii M, Matsubara M. Decrease in urinary creatinine in acute kidney injury influences diagnostic value of urinary biomarker-to-creatinine ratio in rats. Toxicol. 2011;290:241-248.

19. Heymsfield SB, Arteaga C, McManus C, Smith J, Moffitt S. Measurement of muscle mass in humans: validity of the 24-hour urinary creatinine method. Am J Clin Nutr. 1983;37:478-494.

20. Grams ME, Astor BC, Bash LD, Matsushita K, Wang Y, Coresh J. Albuminuria and estimated glomerular filtration rate independently associate with acute kidney injury. J Am Soc Nephrol. 2010;21:1757-1764.

21. Loupy A, Vernerey D, Tinel C, Aubert O, Van Huyen JPD, Rabant M, et al. Subclinical rejection phenotypes at 1 year post-transplant and outcome of kidney allografts. J Am Soc Nephrol. 2015;26:1721-1731.

22. Rush DN, Nickerson P, Gough J, McKenna R, Grimm P, Cheang M, et al. Beneficial effects of treatment of early subclinical rejection: A randomized study. J Am Soc Nephrol. 1998;9:2129-2134.

23. Sarwal M, Chua M, Kambham N, Hsieh S, Satterwhite T, Masek M, et al. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. N Engl J Med. 2003;349:125-138.

24. Nolan N, Lu LH, Valdivieso K, Mani R, Hytopoulos E, Katzenbach P, et al. Clinical and analytical validation of a novel urine-based test for the detection of allograft rejection in renal transplant patients. J Clin Med. 2020;9:2325.