Analytical Performance of the Kidney Solid Organ Response Test (kSORT™)

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Introduction

The field of renal transplantation has progressed considerably in the past half-century largely due to an improved understanding of the role of the immune system in allograft rejection, the interpretation of the molecular mechanisms underlying graft failure, and better management of immunosuppression[1,2]. However, due to the dynamic immune response of post renal transplant patients, events such as acute rejection (AR) remain an obstacle occurring in approximately 15%–20% of patients using the current standard of care for immunosuppression[3].

Currently, the gold standard for detection of graft injury is an invasive biopsy following an increase in serum creatinine. However, biopsy histology is subject to sampling error and increased creatinine is insensitive, occurring only after graft damage has occurred[3–5]. Rejection events lead to chronic graft injury and require cost-intensive care, reduce the quality of life of patients, and may ultimately result in total graft loss. The development of a sensitive, specific, and noninvasive test for assessing the immune risk of post-transplant is, therefore, a critical and currently unmet need in transplantation.

Despite the application of high-throughput discovery methods for detection of disease-specific biomarkers, there is ambiguity about the path to developing a sensitive and specific assay that can be used in practice for the prediction of rejection events and patient care management[6,7]. Diagnostic precision is likely to be derived through an integrated approach comprised of molecular, morphological, serological, and clinical variables. kSORT™, the kidney Solid Organ Response Test, is a non-invasive, whole blood derived, molecular expression assay that can establish an immune risk index for enhanced post-transplant surveillance of graft health and immune quiescence in immunosuppressed, renal transplant patients[3,6]. In conjunction with standard clinical care guidelines, kSORT™ assists in assessing the overall dynamic immune risk profile of post-renal transplant patients thereby improving risk stratification and patient management.

To date, kSORT™ has been offered only by the research laboratory that developed the test (The Sarwal Lab, 513 Parnassus Ave. Med Sciences Bldg., Room S1268, San Francisco, CA 94143) for select research and further development purposes. While the clinical utility of kSORT™ is still being established in ongoing clinical trials, a sensitive and non-invasive method to identify post-transplant patients at high risk of graft injury or over immunosuppression continues to be an unmet need in renal transplantation. Immucor DX, an independent clinical laboratory (CLIA# 23D1054909; Grand Rapids, MI) will make kSORT™ widely available as a Laboratory Developed Test (LDT) for use as a diagnostic immune surveillance tool in conjunction with the current standards of care. As a CLIA certified, CAP accredited laboratory the analytical performance of kSORT™ was evaluated and determined to be acceptable for patient testing by Immucor DX.

Analytical Performance of the Kidney Solid Organ Response Test (kSORT™)

Methods

kSORT™ is a quantitative, real-time PCR (qPCR) method that analyzes the relative expression of a specific, proprietary gene set in peripheral blood which have been shown to play a role in the immune response[3,7].

Peripheral blood specimens for kSORT™ are collected in PAXgene Blood RNA Tubes (PreAnalytiX; Hombrechtikon, Switzerland). The PAXgene tube contains a proprietary reagent that protects RNA from degradation thereby allowing for prolonged storage and transportation of the specimen[8]. A PAXgene specimen is stable for 3 days at 15-25°C, 5 days at 2-8°C, and up to 8 years at <-20°C[9,10]. RNA is extracted from the PAXgene specimen manually using the PAXgene Blood RNA Kit (PreAnalytiX; Hombrechtikon, Switzerland). The RNA yield (ng/mL) and purity (AD260/280) are measured for each specimen.

The next step is reverse transcription using 50ng of RNA and SuperScript VILO MasterMix (ThermoFisher Scientific; Waltham, MA). In this step, the RNA is converted to cDNA using random primers contained in the VILO MasterMix. In addition to the specimens, Human XpressRef Universal Total RNA is reverse transcribed and serves as a positive control for the reverse transcription and qPCR.

Gene expression of each of the target genes is measured using TaqMan primer/probe sets (ThermoFisher Scientific; Waltham, MA). The gene expression of the target genes is normalized to expression of 18S ribosomal RNA, which serves as the internal control, yielding a delta threshold cycle (dCT). Relative gene expression is then determined by comparison to the Human XpressRef Universal Total RNA to yield a delta delta threshold cycle (ddCT).

The raw ddCT data generated by kSORT™ is analyzed using a proprietary centroid-based algorithm (kSAS), which provides a qualitative result that can be reported to clinicians. kSAS utilizes 13 models, each with different combinations of 12 genes from the kSORT™ gene panel. A reference centroid for each gene was predetermined using biopsy-confirmed specimens for patients with rejection (HIGH RISK) and stable patients (LOW RISK), resulting in a reference vector for HIGH and LOW RISK. A reference centroid is a predetermined ddCT value for each gene and a reference vector is a combination of these reference centroids of the 12 genes in each of the 13 models, for HIGH and LOW RISK.

kSAS calculates Pearson correlation coefficients from the comparison of the HIGH and LOW reference centroids for each model with ddCT values for each corresponding model of the sample being tested. Two correlation coefficients are obtained for each model of each sample, one for comparison of each model of the sample vector with each model of the HIGH RISK reference vector and one for comparison of each model of the sample vector with each model of the LOW RISK reference vector. A model score for each model is then calculated by subtracting the Pearson correlation of the sample comparison with LOW RISK vector from the Pearson correlation of the sample comparison with the HIGH RISK vector and multiplying the difference by 10. This model score is then compared to predetermined model score confidence limits. If the model score lies within these confidence limits, then the sample is assigned model call = 0. If the model score lies outside of these confidence limits and is positive, then the sample is assigned model call = +1. If the model score lies outside of these confidence limits and is negative, then the sample is assigned model call = -1. Model calls for the 13 models of a sample are then added to determine the kSAS score, which can range from -13 to +13. Samples with a kSAS score of < -9 are predicted to have a LOW IMMUNE RISK INDEX while samples with a kSAS score > 9 are predicted to have a HIGH IMMUNE RISK INDEX. For kSAS scores between -8 and 8, inclusive, the result is classified as indeterminate (IND).

Analytical Performance of the Kidney Solid Organ Response Test (kSORT™)

Results

The analytical performance of kSORT™ was evaluated in the Immucor DX Laboratory The parameters assessed were accuracy, precision, sensitivity, and specificity.

Analytical accuracy is defined as the closeness of agreement between a test result and an accepted reference value. The analytical accuracy of kSORT™ was determined by concordance with the research laboratory that originally developed thekSORT™ test (The Sarwal Lab) and biopsy results. The accuracy study included 100 previously extracted and characterized RNA specimens sent to ImmucorDX by The Sarwal Lab in a blinded fashion, 84 of which had biopsies for correlation. There was substantial correlation between Immucor DX and The Sarwal Lab as indicated by a Cohen's Kappa of 0.7911(Table 1a). There was 87% agreement between the Immucor DX results and the biopsy correlated results (Table 1b). There was a kSORT™ indeterminate rate of 11%. Excluding indeterminate results, the diagnostic sensitivity was 100% and the diagnostic specificity was 96% in the 84 biopsy correlated specimens (Table 2).

Table 1a.

Immucor DX	The Sarwal Lab				
		HIGH	IND	LOW	*Total*
	HIGH	33	4	0	*37*
	IND	0	4	6	*10*
	LOW	0	2	51	*53*
	Total	*33*	*10*	*57*	*100*

Table 1b.

Immucor DX	The Sarwal Lab			
		AR	NO AR	*Total*
	HIGH	26	2	*28*
	IND	2	7	*9*
	LOW	0	47	*47*
	Total	*28*	*56*	*84*

Table 2.

Agreegment Rate	87%
IND Rate	11%
Sensitivity	100%
Specificity	96%

Precision refers to the agreement between independent test results obtained from the same set of samples under prescribed conditions. The variability of test results is described by coefficient of variation (% CV) for quantitative results or agreement for qualitative results.

The precision, or variability, of kSORT™ was evaluated using whole blood from six (6) non-transplanted subjects collected in PAXgene tubes. The six samples were selected to cover all possible outcomes of the test with two being indeterminate (IND), one LOW RISK, and three HIGH RISK specimens. The six samples were extracted and cDNA generated on day 1. The cDNA was stored at 2-8°C and kSORT™ was run each day for five (5) days with all samples on the same plate. The kSAS score and qualitative results were compared across the (5) days.

Analytical Performance of the Kidney Solid Organ Response Test (kSORT™)

The precision study demonstrates that across the 6 samples tested over 5 days the average % CV for the kSAS score was 15% and the average agreement of the qualitative results was 94% (Table 3).

Table 3.

Sample ID	kSAS Score %CV	Qualitative Result	Qualitative % Agreement
1	25%	IND	100%
2	17%	IND	100%
3	8%	LOW	100%
4	10%	HIGH	83%
5	22%	HIGH	83%
6	9%	HIGH	100%
MEAN	15%		94%

The analytical sensitivity of kSORT™ encompasses the RNA yield of the extraction and the limit of detection of the PCR amplification. To determine the efficiency of the extraction process, blank samples were spiked with 1ug, 5ug, 10ug, and 20ug of Control RNA (Qiagen). After extraction, the samples were quantified by Nanodrop. The efficiency of extraction was linear across the concentrations tested (Fig.1). The average recovery from spiked blank samples was 51% with an average CV of 7%.

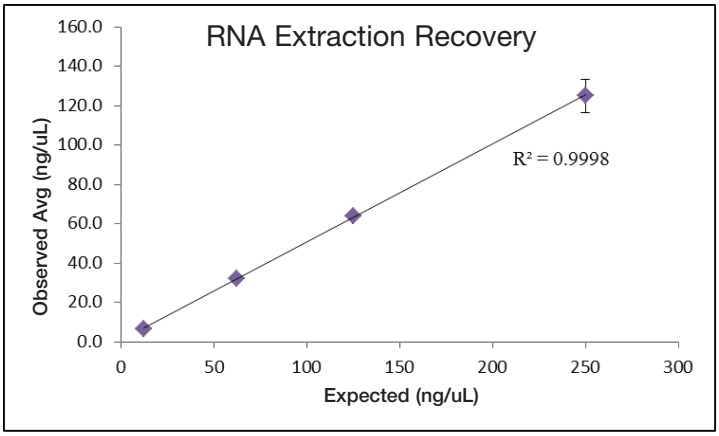

Figure 1. Observed recovered RNA concentration (Y-axis) compared to expected recovered RNA concentration (X-axis). Data are expressed as mean ± standard deviation.

Analytical Performance of the Kidney Solid Organ Response Test (kSORT™)

Additionally, PAXgene samples from three (3) healthy subjects were spiked with 10ug of Control RNA (Qiagen). After extraction, the samples were quantified by Nanodrop. The RNA recovery was consistent between spiked blanks and spiked patient samples. On average, the RNA recovery from spiked patient samples was 52% with a minimum of 44% and a maximum recovery of 69%.

To determine the yield and purity of RNA from the extraction process, PAXgene tubes were collected from 20 healthy subjects and were extracted and quantitated. The yield ranged from 40-182ng/mL with a purity of 2.12±0.02 (A260/280). The same 20 samples were run through kSORT™ both with and without a reverse transcription (RT) step to determine the level of genomic DNA (gDNA) contamination. Samples run without an RT step will not generate cDNA and, thus, no amplification during qPCR. In the no RT test runs all genes were undetectable with the exception of RHEB, which was detected at >35Ct and was considered acceptable. 18S was detected in all samples at an average Ct of 32.4 (SD=1.5, %CV=4.7%) in the no RT test runs; however, this is 22Ct greater than was observed in patient samples, which is indicative of a ~1,000,000X lower starting template concentration and is considered acceptable.

To determine the minimum input cDNA concentration for maximum PCR efficiency, four (4) patient samples were run with different inputs of RNA: 10ng, 20ng, 30ng, 40ng, 50ng, 100ng, 200ng, and 400ng. The Ct values for two targets, 18S and MAPK9, were plotted to determine LOD as well as assay efficiency (Fig. 2). The assay is able to detect targets down to 10ng of input; however the highest efficiency of the assay occurs with a 50ng input.

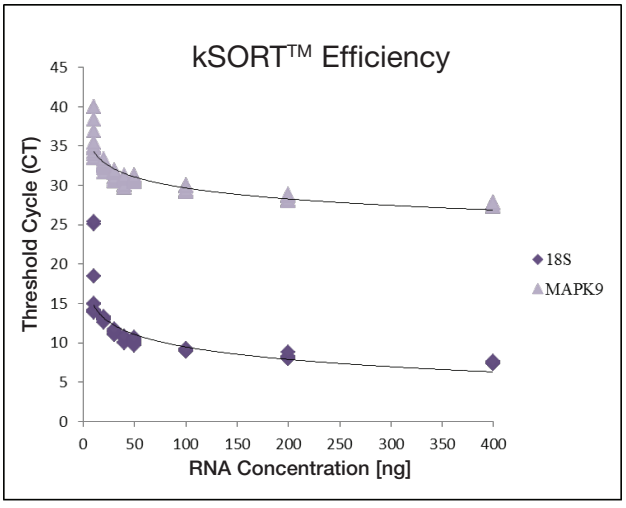

Figure 2. The mean Ct (y-axis) for 18S and MAPK9 at eight different RNA inputs (x-axis). The Logarithmic trendline reveals the efficiency of the assay.

Interference sources for kSORT™ include contamination, sample handling, exogenous substances, and other admixtures. The interference study consisted of 16 patient samples in duplicate. The duplicate PaxGene tubes were spiked with known levels of potential interferences. Each potential interfering substance was tested in duplicate. The interference study yielded 100% concordance for creatinine, bilirubin, triglycerides, tacrolimus, prednisone, and IVIg. Results were also acceptable for sirolimus and rituximab in that one sample for each was concordant and the second sample yielded indeterminate results.

Analytical Performance of the Kidney Solid Organ Response Test (kSORT™)

Discussion

The analytical performance of kSORT™ was evaluated for the first time by Immucor DX, a CLIA certified, CAP accredited independent clinical laboratory. All parameters, including accuracy, precision, sensitivity, and specificity, were well within acceptable parameters and kSORT™ has been validated to be acceptable for patient testing.

To date, the "gold standard" for detection of graft injury and rejection has been biopsy. However, biopsy is an invasive technique that is known to have a high failure rate[4]. Biopsy is subject to sampling error as well as subjective divergence based on the reader[4,12]. Further, biopsy can detect subclinical graft injury due to immune response, infection, or nephrotoxicity as a result of immunosuppression[13,14]. All of these factors contribute to a failure rate (combination of false positives and false negatives) of approximately 30%[4,12–14]. Serum creatinine, which is the other marker that has traditionally been used to detect renal graft injury, also lacks sensitivity and specificity. Additionally, significant damage to the graft must occur before there is a shift in serum creatinine levels. Cumulatively, all of these factors indicate that these markers are insufficient for early detection of graft injury that would allow for intervention to prevent or reverse the damage.

The lack of a sensitive and specific test for graft injury post-transplant has driven great interest in molecular biomarkers for post-transplant surveillance. kSORT™ was developed for this specific purpose. Previous studies have shown proof of concept that the kSORT™ gene set is associated with rejection and graft injury and for the use of a centroid based algorithm in producing qualitative results[3,7]. Furthermore, data from these studies suggest that kSORT™ can detect an immune response that may lead to either cellular and humoral rejection weeks to months before rejection is confirmed by biopsy[15]. The performance of kSORT™ in prior studies was not affected by age or time post-transplantation[3,7]. Validation of the analytical performance of kSORT™ was performed by Immucor DX.

Validation of kSORT™ performance included an evaluation of accuracy, precision, sensitivity and specificity. Analytical accuracy analysis yielded an acceptable Cohen's Kappa of 0.79 for correlation between ImmucorDx and The Sarwal Lab and 87% agreement between ImmucorDx results and biopsy results. The assay gives an indeterminate rate of 11%; sensitivity and specificity are 100% and 96% respectively after excluding indeterminate results. In two cases where kSORT™ classified HIGH RISK specimens among negative biopsies. It has been suggested by previous studies that kSORT™ can detect graft inflammation before subclinical rejection is detected on a biopsy[3]. Therefore, it is possible that those two specimens may indeed have immune activation and graft inflammation, which may be indicative of early injury. The two specimens are part of the ongoing, prospective SAILOR study (Gothenburg, Sweden). Precision analysis demonstrates average %CV of 15% for the kSAS score and an average agreement of 93% for qualitative results across six samples tested over 5 days. Interference analysis demonstrated that creatinine, bilirubin, triglycerides, tacrolimus, prednisone, and IVIg do not interfere with the analytical performance of kSORT™. The parameters evaluated for the analytical performance of kSORT™ by Immucor DX were all well within acceptable limits.

kSORT™ is a multi-gene assay utilizing qPCR to measure relative expression levels of circulating RNA transcripts in peripheral blood. The relative expression of the gene panel is then subjected to a proprietary algorithm, which assigns the patient an IMMUNE RISK INDEX that may be indicative of graft inflammation and/or early graft injury. kSORT™ provides risk stratification assessment of renal transplant patients and can be used as an informative decision-making tool by clinicians for immunosuppressive therapy and management of patient care. kSORT™ represents the introduction and promise of personalized, precision medicine in transplantation.

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