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Clinical Validation of an Immune Quiescence Gene Expression Signature in Kidney Transplantation

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Key Points:

*AlloMap Kidney is a gene expression profile developed using candidate genes from the AlloMap assay broadly used in heart transplantation

*AlloMap Kidney was validated to differentiate quiescence from rejection in two independent sample sets using a quantitative scale

*Blood cell gene expression and dd-cfDNA contribute independent signals and inform on different aspects of allograft rejection

Abstract:

Background: Despite advances in immune suppression, kidney allograft rejection and other injuries remain a significant clinical concern, particularly with regards to long-term allograft survival. Evaluation of immune activity can provide information about rejection status and help guide interventions to extend allograft life. Here we describe the validation of a blood gene expression classifier developed to differentiate immune quiescence from both T cell mediated rejection (TCMR) and antibody-mediated rejection (ABMR). Methods: A five-gene classifier (DCAF12, MARCH8, FLT3, IL1R2, and PDCD1) was developed on 56 peripheral blood samples and validated on two sample sets independent of the training cohort. The primary validation set comprised 98 quiescence samples and 18 rejection samples: 7 TCMR, 10 ABMR, and 1 mixed rejection. The second validation set included 8 quiescence and 11 rejections: 7 TCMR, 2 ABMR, and 2 mixed. AlloSure donor derived cell-free DNA was also evaluated. Results: AlloMap Kidney classifier scores in the primary validation set differed significantly between quiescence (median 9.49, IQR 7.68-11.53) and rejection (median 13.09, IQR 11.25-15.28), $p < 0.001$. In the second validation set, the cohorts were statistically different ($p = 0.028$) and the medians were similar to the primary validation set. The AUC for discriminating rejection from quiescence was 0.786 for the primary validation and 0.800 for the second validation. AlloMap Kidney results were not significantly correlated with AlloSure, although both were elevated in rejection. The ability to discriminate rejection from quiescence was improved when AlloSure and AlloMap Kidney were used together (AUC 0.894). Conclusion: Validation of AlloMap Kidney demonstrated the ability to differentiate between rejection and immune quiescence using a range of scores. The diagnostic performance suggests that assessment of the mechanisms of immunological activity is complementary to allograft injury information derived from AlloSure dd-cfDNA. Together, these biomarkers offer a more comprehensive assessment of allograft health and immune quiescence.

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Clinical Validation of an Immune Quiescence Gene Expression Signature in Kidney Transplantation

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KEY POINTS

- AlloMap Kidney is a gene expression profile developed using candidate genes from the AlloMap assay broadly used in heart transplantation
- AlloMap Kidney was validated to differentiate quiescence from rejection in two independent sample sets using a quantitative scale
- Blood cell gene expression and dd-cfDNA contribute independent signals and inform on different aspects of allograft rejection

ABSTRACT

Background: Despite advances in immune suppression, kidney allograft rejection and other injuries remain a significant clinical concern, particularly with regards to long-term allograft survival. Evaluation of immune activity can provide information about rejection status and help guide interventions to extend allograft life. Here we describe the validation of a blood gene expression classifier developed to differentiate immune quiescence from both T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR).

Methods: A five-gene classifier (DCAF12, MARCH8, FLT3, IL1R2, and PDCD1) was developed on 56 peripheral blood samples and validated on two sample sets independent of the training cohort. The primary validation set comprised 98 quiescence samples and 18 rejection samples: 7 TCMR, 10 ABMR, and 1 mixed rejection. The second validation set included 8 quiescence and 11 rejections: 7 TCMR, 2 ABMR, and 2 mixed. AlloSure donor-derived cell-free DNA was also evaluated.

Results: AlloMap Kidney classifier scores in the primary validation set differed significantly between quiescence (median 9.49, IQR 7.68–11.53) and rejection (median 13.09, IQR 11.25–15.28), $p < 0.001$. In the second validation set, the cohorts were statistically different ($p = 0.028$) and the medians were similar to the primary validation set. The AUC for discriminating rejection from quiescence was 0.786 for the primary validation and 0.800 for the second validation. AlloMap Kidney results were not significantly correlated with AlloSure, although both were elevated in rejection. The ability to discriminate rejection from quiescence was improved when AlloSure and AlloMap Kidney were used together (AUC 0.894).

Conclusion: Validation of AlloMap Kidney demonstrated the ability to differentiate between rejection and immune quiescence using a range of scores. The diagnostic performance suggests that assessment of the mechanisms of immunological activity is complementary to allograft injury information derived from AlloSure dd-cfDNA. Together, these biomarkers offer a more comprehensive assessment of allograft health and immune quiescence.

INTRODUCTION

Despite modern immune suppression regimens, kidney allograft rejection continues to be both a common occurrence and the primary driver of unacceptably high graft failure rates (1-3). Ten percent of kidney transplant recipients experience allograft rejection in the first year after transplant (4). Although allograft biopsy is the current standard for diagnosis of rejection, optimizing the appropriateness of biopsies by non-invasive techniques is imperative due to the invasive nature of the procedure and the associated risk, as well as the sampling error and subjective nature of histopathologic interpretation. Analysis of large series of renal transplant protocol biopsies demonstrated a 1.9% major complication rate and a 4.7% risk of gross hematuria (5, 6). Additionally, up to 15% of biopsies yield an inadequate specimen, exposing patients to procedural risk without diagnostic benefit (7). The ability to enhance the timing and diagnostic yield of biopsies could meaningfully improve post-transplant outcomes. In addition, methods for assessing response to rejection treatment and return to baseline allograft function frequently rely on additional follow-up biopsies, all associated with risks, expense, inconvenience, and diagnostic failure.

Serum creatinine is commonly used to assess kidney function as a screen for rejection. However, allograft damage sufficient to impair renal function is often irreversible (8) and serum creatinine has repeatedly been shown to be a poorly sensitive or specific indicator of rejection (9). Robust diagnostic and prognostic biomarkers that provide evidence of graft rejection ahead of pathological findings are needed to help guide clinical management of transplant patients. Among the best studied advanced biomarkers in transplantation is the plasma level of donor-derived cell-free DNA (dd-cfDNA) used to assess allograft injury (9-12). dd-cfDNA has gained significant adoption since it became available as a

clinically validated test (12), with numerous studies demonstrating clinical utility in a broad array of contexts (10, 11, 13-15).

Additional assays are those that use gene expression of circulating immune cells to evaluate immune activity. One example of a broadly integrated gene expression profile assay is AlloMap, available as a surveillance tool for heart transplant recipients since 2005 (16). The assay methodology has not changed since validation and subsequent publication of clinical utility (17); the high negative predictive value (NPV) has enabled avoidance of biopsies for over 15 years by the heart transplant community (18). Gene expression profiling of the immune system in kidney transplantation, however, has been elusive as a consistently reliable and reproducible measure of rejection. Several plasma gene expression panels have been published (19-22), one of which is commercially available and designed for use specifically in place of protocol biopsy (19). Another has faced concerns after independent studies were unable to replicate the validation (20, 23). Other signatures with a strong association with fibrosis (21) or specific to antibody-mediated rejection (ABMR) (22) have not yet achieved routine clinical use.

A focused determination of the immune state as active compared to quiescent can help to assess the likelihood of allograft rejection. We describe validation of a blood gene expression profile (GEP) that can stratify samples according to likelihood of immune quiescence versus T cell-mediated rejection (TCMR) or ABMR. Building on the extensively demonstrated utility of the AlloMap gene set (17), we developed a novel classifier for kidney allograft rejection, condensing the 11 informative genes from AlloMap to a 5-gene subset. The AlloMap Kidney classifier was validated using two sample sets independent of the training set and the performance was evaluated in the primary validation both independently and in conjunction with AlloSure dd-cfDNA.

MATERIALS AND METHODS

Study Design

Three sets of data were used in this study. The training set and the primary validation set were randomly-assigned (per cohort) distinct sets of patients from the DART study (ClincalTrials.gov NCT02424227), a multicenter, prospective, observational study to collect plasma in Streck Cell-Free DNA BCT for the

purpose of dd-cfDNA measurement and whole blood RNA in PAXgene tubes for gene expression profiling (9). The institutional review board (IRB) at each site approved the study and all the patients provided written informed consent. The study sponsor provided the statistical analysis, data management, and clinical operations coordination. A second validation set was used to further validate the performance of AlloMap Kidney. These samples, from Montefiore Medical Center, Bronx, NY, were from patients not included in the DART study. Samples were derived from IRB-approved study of "Immune monitoring of Kidney Transplant recipients" (IRB No. 09-06-174). The clinical and research activities reported are consistent with the Principles of the Declaration of Istanbul as outlined in the "Declaration of Istanbul on Organ Trafficking and Transplant Tourism".

Patients and Samples

Patient samples were assigned to two general cohorts: Rejection or Quiescence. Each cohort was defined by established Banff criteria (24) and each contained subgroups as follows. The rejection samples comprised TCMR, ABMR, or mixed rejection (meeting criteria for both ABMR and TCMR). The quiescence samples were one of three types: healthy stable (HS), which had no clinical or laboratory indicators of concern for the graft (and therefore no clinically-indicated biopsy) and a low level of dd-cfDNA as measured by AlloSure (< 0.5%); non-rejection (NR), which were determined to not have signs of rejection upon pathologist review following a clinically-indicated biopsy; and protocol non-rejection (pNR), which were determined to not have signs of rejection upon pathologist review following a protocol surveillance biopsy. Because it is standard to include the best-defined members of the two main cohorts when training a classifier, the Rejection sets for training and the primary validation set included TCMRIA, IB, IIA, and IIB along with the ABMR. Borderline TCMR samples were a part of the Rejection group in the second validation set. For ABMR, acute/active and chronic, active ABMR were included.

RNA Purification

The DART PAXgene blood tubes were collected alongside Streck BCT plasma, shipped at ambient temperature, received within 3 days, and stored at -80°C until RNA extraction. After thawing, RNA was purified using the QIASymphony PAXgene Blood RNA kit (QIAGEN, Cat. No. 762635) on

the QIASymphony SP system following the manufacturer's instructions. The second validation set samples were extracted manually using PAXgene blood RNA kits (QIAGEN, Cat. No. 762164) according to the manufacturer's instructions. The concentration and purity of the extracted RNA samples were determined by spectrophotometry. Samples were also analyzed for integrity by capillary electrophoresis (TapeStation, Agilent).

qRT-PCR Methodology

The purified RNA samples were subjected to qRT-PCR in the CareDx CLIA laboratory as described (16). The qPCR for each gene was run in triplicates and the raw C_T values (threshold cycle; at which probe fluorescence reaches the measurement threshold) were used to calculate a smoothed mean C_T. The mean C_T was then used for the development of the AlloMap Kidney signature as described below without using additional data processing methods or procedures from AlloMap Heart.

Classifier Training

The mean C_T for the candidate test genes was normalized against six reference genes (**Supplemental Table S2**), which were selected based on their stability in this sample set using a scheme similar to what was described previously (16). The normalized results were assessed for statistical significance in a univariate model. Six genes identified as statistically significant were then cross-validated via bootstrapping and leave-one-out validations. The five genes that passed these internal validations were grouped into three clusters based on their normalized C_T level across the full set of training samples. Each cluster has a pairwise correlation coefficient above 0.6. A multivariate model that integrates the normalized expression of the five genes was built to optimize performance to differentiate rejection from quiescence in the training sample set.

RNA-seq Methodology

RNA-seq was chosen as a validation and testing platform to enable improved detection of low-expression genes, higher reproducibility, and accurate measurement of gene expression changes that can be readily expanded to additional gene sets and classifiers. A targeted RNA sequencing (RNA-seq) panel (QIAseq, QIAGEN), which includes the five informative genes and 15 reference genes (**Supplemental Tables S1 and S2**) as well as genomic DNA contamination controls and spike-in

controls, was developed and optimized for PAXgene blood RNA samples on an RNA-seq platform using molecular tags (25). Single-read sequencing was performed on Illumina NextSeq 550. Primary analysis of the sequencing data was performed using the QIAGEN GeneGlobe QIAseq bioinformatics pipeline for adaptor trimming, read mapping, quality checks, and computing the molecular tag counts (MTs) for the targeted transcripts. As the MTs are directly correlated with the initial copy number of the input RNA, a conversion could be defined and tested (**Supplementary Material**) to convert MTs to a C_T value that would match C_T generated on the same sample by qPCR. The corresponding C_T number was derived using the equation $X_0 = E_{amp}^{(b-C_T)}$, where E_{amp} is the exponential amplification value, b is the y-intercept of log(copies) vs C_T, using the average amplification efficiency of 98%, and b = 39 for the AlloMap qRT-PCR tests. The C_T values for the informative genes were normalized using the average C_T of the reference genes. The normalized C_T numbers were then used to compute the AlloMap Kidney score using the locked classifier algorithm trained on the qRT-PCR data. AlloSure measurement of dd-cfDNA was performed as previously described (26).

Statistical Analysis

The analysis of differences between groups was performed using an unpaired t-test; performance metrics were calculated using standard methods in JMP version 13. ROC curves were generated using the pROC package in R (version 4.0.5). To generate ROC plots for combined AlloMap Kidney and AlloSure data, the AlloSure score was log transformed then converted to the standard normal distribution. The AlloMap Kidney score was converted to the standard normal distribution similarly. The two converted scores were added arithmetically to create a single result for each sample to plot the ROC.

RESULTS

The AlloMap Heart gene set was developed using peripheral blood mononuclear cells (PBMC) and comprises genes implicated in diverse immune pathways; therefore, this was chosen as a source of candidate genes for development of a classifier in kidney transplantation (**Supplemental Table S1**) (16). Due to the complexity of purifying PBMCs at the time of collection, whole blood samples were collected in PAXgene tubes from kidney transplant recipients. Gene expression data were generated for the

11 AlloMap Heart genes from a subset of the DART study patients designated as the training sample set, with 38 samples from 22 patients classified as quiescence (HS with AlloSure < 1%) and 18 samples from 16 patients classified as rejection (7 TCMR, 8 ABMR, 3 mixed). The only demographic differences between the cohorts in the training sample set were that the Quiescence cohort were earlier post-transplant and that the Rejection groups had higher serum creatinine and lower eGFR, as expected. No differences were observed in race, gender, type of transplant, recipient or donor cytomegalovirus (CMV) serology, HLA mismatches, panel reactive antibodies, induction therapy, or maintenance immunosuppression (**Table 1**).

Given the differences in sample type and the transplanted organ, a new gene expression classifier was developed starting from the AlloMap Heart gene set. Of the 11 informative genes included in AlloMap Heart (16), six were significantly different between the Quiescence and Rejection cohorts in the training set in a univariate model ($P < 0.02$, **Supplementary Table S1**). Bootstrap and leave-one-out testing within the training set indicated that five of the six genes were used in more than 75% of instances; subsequent stepwise selection yielded three important clusters with five genes. These genes represent biological functions related to immune response pathways: DCAF12 and MARCH8 are involved in modulating immune reactivity, FLT3 and IL1R2 are steroid-responsive genes, and PDCD1 is expressed on activated T lymphocytes (**Supplemental Table S1)** (27). In the training set the AlloMap Kidney classifier readily distinguished rejection from quiescence, $P < 0.001$, and with an area under the receiver operating characteristics curve (AUC) of 0.939 (95% CI 0.889–0.991).

A second set of DART samples from patients not contributing to the training sample set was used as the primary validation set. These 99 unique patients contributed 98 quiescence samples (22 HS, 29 pNR, and 47 NR) and 18 rejection samples (7 TCMR, 10 ABMR, and one mixed rejection) (**Table 1**). The only demographic differences between the cohorts in the primary validation set were that the Rejection cohort were younger and had fewer HLA class 1 mismatches. There is not a statistical difference in time post-transplant between the Rejection and Quiescence cohorts. The Rejection cohort had higher serum creatinine levels and lower estimated glomerular filtration rate (eGFR) than the Quiescence cohort, as expected. No differences were observed in race, gender, type of transplant, time

post-transplant, HLA class II mismatches, panel reactive antibodies, induction therapy, or maintenance immunosuppression. This set of independent samples validated that the AlloMap Kidney classifier distinguished quiescence (median 9.49; IQR 7.68–11.53) from rejection ($p < 0.001$; median = 13.09; IQR = 11.25–15.28), (**Figure 1a**). The medians for each of the sample groups were: HS = 10.04 (8.38–11.85), pNR = 8.73 (7.45–11.13), NR = 10.07 (8.05–12.14), TCMR = 15.09 (11.99–17.42), ABMR = 11.48 (10.95–13.68), and mixed = 14.33. Each of the three defined Quiescence groups was significantly different from the Rejection cohort (P-values for rejection vs HS = 0.003, rejection vs pNR < 0.001, rejection vs NR < 0.001) (**Figure 1b**). Each of the defined types of rejection was different from the Quiescence cohort (**Figure 1b**), with p-values of 0.028 and 0.001 for ABMR and TCMR vs Quiescence, respectively. Although insufficient numbers were available for a robust analysis of the association of AlloMap Kidney scores with TCMR grade, the data suggest that higher grades of TCMR may have higher scores (**Figure 1c**). The AUC for quiescence versus rejection in the primary validation set was 0.786 (95% CI 0.661–0.911), demonstrating the excellent performance of the GEP across the score range (**Figure 1d**).

To further validate the performance of AlloMap Kidney, a set of samples from a center not included in the DART study was also evaluated. The Quiescence cohort in this set included eight NR, while the Rejection cohort contained 11 samples (2 ABMR, 7 TCMR, 2 mixed). In this set, AlloMap Kidney scores were significantly different between quiescence (NR) and Rejection groups ($p = 0.028$, **Figure 2a**). Both the TCMR and the ABMR samples had elevated scores relative to the NR group (**Figure 2b**). The TCMR group consisted of five IIA rejections and two borderline rejections, with median AlloMap Kidney score of 11.85 (11.26–12.67). The AUC for discriminating samples with rejection in this sample set was 0.796 (95% CI 0.571–1) (**Figure 2c**).

We also assessed the performance of AlloMap Kidney in the combined validation sets to provide an analysis with a larger number of rejection samples. In the combined analysis, the scores for the NR group (median 10.19, IQR 7.64–12.09) were significantly lower than the scores for the Rejection cohort (median 12.43, IQR 11.12–14.29), $P<0.001$. All three Rejection groups showed elevated scores: TCMR (median 12.55, IQR 11.52–16.25, n = 14), ABMR (median 11.48, IQR 10.95–14.06, n = 12),

and mixed rejection (median 12.72, IQR 11.12–14.33, n = 3) **(Figure 3b**). Analysis of the combined independent validation sets resulted in an AUC of 0.779 (95% CI 0.686–0.871) for Quiescence vs Rejection cohorts (**Figure 3c**).

NPV and positive predictive value (PPV) were determined at prevalence levels of 10% and 25%, representing the estimated prevalence of rejection on first-year surveillance and clinically indicated biopsies, respectively (4, 9). The single-center sample set contained only NR in the Quiescent cohort; therefore, the performance of the classifier to differentiate the full quiescent group from biopsy-defined rejection was assessed on the DART validation set. **Figure 4** shows plots of sensitivity, specificity, PPV, and NPV of the classifier. For all performance metrics, the data are shown with either the full Quiescence cohort (HS, NR, and pNR) or with only the NR group. Sensitivity did not change as the Rejection cohort remained the same, but specificity, NPV, and PPV are dependent on the choice of quiescence cohort samples. The threshold used for this binary performance characterization was 11.5, at which the AlloMap Kidney score achieved the maximum accuracy for sensitivity and specificity (**Figure 4**). At the 11.5 score, AlloMap Kidney had a PPV of 23.2% and an NPV of 95.3% at 10% prevalence and a PPV of 47.6% and an NPV of 87.2% at 25% prevalence to discriminate rejection from quiescence.

For samples in the DART study, plasma was also collected to measure dd-cfDNA using AlloSure. AlloSure is highly associated with graft injury (9); the signal is hypothesized to be different from that of the AlloMap Kidney signature, although both are correlated with rejection. **Figure 5a** shows the data for all quiescence and rejection cohorts (mixed rejection was included in the ABMR group). There was a weak correlation between AlloSure and AlloMap Kidney (R = 0.15, P=0.233). However, samples with AlloSure results of 1% or above had higher AlloMap Kidney scores. Several TCMR samples with AlloSure between 0.5% and 1% had very high AlloMap Kidney scores, suggesting a role for AlloMap Kidney to inform on which of these intermediate scores likely correlate with rejection (11). To examine the potential of coupled testing in post-transplant care, a combined score was derived with equal weighting of AlloSure and AlloMap Kidney. The range of this combined score can be envisioned along the diagonal from lower left in the plot in **Figure 5a** to the upper right. These data were used to generate an ROC plot for the combined score, which was compared to the ROC plots for AlloSure or AlloMap

Kidney alone in the same sample set (**Figure 5b**). These data showed a superior performance for the combined use of AlloMap Kidney and AlloSure vs AlloMap Kidney alone ($p = 0.005$).

DISCUSSION

Factors predisposing the development of active rejection have been extensively studied, with the recipient immune system proving to be a key intermediary in many relevant processes, including ischemia-reperfusion injury, infection, and response to immunosuppression. Uncontrolled inflammation in kidney allografts leads to the chronic damage and progressive fibrosis that accounts for the majority of long-term allograft loss (28, 29). Genetic predictors of active rejection have also been described in recent years, some of which implicate immune activity. Taken in concert, these data suggest that monitoring gene expression in peripheral blood immune cells may lead to earlier or more sensitive detection of AR (19, 20, 30).

The objective of the current study was to validate a classifier that discriminates immune quiescence from kidney allograft rejection. Rather than novel discovery from the whole transcriptome, this classifier was developed using a candidate gene approach using the AlloMap Heart genes, which have been implicated in immune responses or regulation and therefore presented a rational starting point for developing a quiescence signature for kidney transplantation. Furthermore, we elected to measure whole blood gene transcripts rather than looking specifically at the PBMC subset, the method employed by the AlloMap Heart assay. Collection of whole blood is significantly less complicated than PBMC collection, enabling a more streamlined workflow at the collection site. After accurately measuring the expression of these candidate genes, we identified five that could discriminate kidney rejection using a training sample set from the DART study that was collected in PAXgene tubes. This classifier was tested using two validation sets: the primary validation set comprised independent patients from DART and the second validation set was from a single center. Both sample sets demonstrate the validity of the classifier to discriminate biopsy-defined rejection from quiescence. Classifier scores were statistically significantly different between the rejection cohort and the quiescence cohort as well as between the quiescence cohort and either TCMR or ABMR. Results in the three subgroups of quiescence (HS, pNR, NR) were each statistically different from those seen in rejection. In both validation sets, the AUC demonstrated

excellent diagnostic performance. The classifier had an NPV of over 95% in a surveillance population (10% prevalence) based on a score of 11.5, chosen for maximal sensitivity and specificity.

The limitations of the current study include the number of rejection samples and limited scope of the patient population. To generate training and validation sets from completely independent patients, we were limited to only 18 rejections samples in each set, randomly selected to generate the two independent sets. Despite these small numbers, independent validation sets of TCMR and ABMR, as well as mixed rejection, demonstrated excellent diagnostic performance in all comparisons. Further, a second independent validation set recapitulated the performance of the classifier in the primary validation set. Although three different types of quiescence samples (NR, pNR, and HS) were included in this study, there may be other subsets of the target patient population that should be independently characterized on this classifier in future studies, such as infection, interstitial fibrosis and tubular atrophy (IF/TA), drug toxicity, BKV nephropathy, and recurrent or de-novo glomerular disease. Each of these could have been undiagnosed in the HS set, which serves as an indicator of a surveillance population at large. However, clinicians may still wish to biopsy to identify some of these pathologies in patients with high suspicion. The results presented here represent assessment of the status of the allograft at the time of the AlloMap Kidney testing rather than long-term graft survival. A correlation with long-term outcomes necessarily requires large, prospective studies and does not detract from the demonstrated capability of AlloMap Kidney to provide non-invasive assessment of current rejection status. Lastly, our approach necessarily limited the scope of possible genes and therefore may not have included the best genes for discrimination of rejection from immune quiescence. With further sample availability from ongoing studies, we anticipate a robust sample set to potentially expand the clinical utility of the assay with expanded gene sets.

One strength of the current study is that we started with a well-validated gene set and test condition. The gene set from the FDA-cleared AlloMap Heart test has proven robust and clinically relevant for over 15 years in heart transplantation (17). In contrast, within kidney transplantation, previously developed gene expression profiling assays have limited overall use in more narrow clinical indications such as replacing surveillance biopsies or assessment of fibrosis (19, 21).

In contrast to the AlloMap Kidney measure of immune activity, the use of AlloSure to quantify dd-cfDNA provides insight into molecular injury in the allograft. Prior experience with combined testing in heart transplantation suggested that gene expression measurement in immune cells provides a complementary signal that can provide added insight into allograft rejection (31). Gene expression profiling may discriminate between types of allograft injury, including drug toxicity that can lead to injury in the absence of rejection. Therefore, there is potential added value when the two tests are combined. Indeed, when the DART validation set was analyzed using both AlloMap Kidney and AlloSure, a higher diagnostic performance was observed. Only a single rejection sample was below the nominal threshold for both tests. The details of the pathology report for this sample were independently reviewed and despite being called TCMR1A by the pathologist at the treating center, the biopsy only show focal mild tubulitis. Based on Banff 2019, this biopsy would not even meet the criteria for borderline TCMR; it would be called acute tubular injury based on the pathology report. A post-hoc re-analysis of the AlloMap Kidney AUC with this sample in the NR set produced a value of 0.83. Future studies will better define how these complementary signals can enhance diagnostic assessment and management of kidney transplant recipients. In the primary validation cohort, AlloMap Kidney scores appear higher in TCMR than in ABMR, whereas AlloSure scores have been reported higher with ABMR (9). The coupled use of these assays may allow non-invasive discrimination of the type of rejection. The high negative predictive value of AlloMap Kidney makes it an ideal assay for integration into routine post-transplant care, allowing minimization of biopsies in the same manner as AlloMap Heart has in heart transplantation for over 15 years. This may be complemented by the AlloSure signal indicating injury, thus improving the PPV. Future analysis of larger datasets of paired data will provide detailed performance of the use of both tests together.

Immune activity biomarkers can strengthen the high negative predictive value of existing markers, allowing the confidence to rule out pathology by identifying those who are immunoquiescent. These types of markers also open the prospect of managing immunomodulation. Reducing medication dose for patients who are adequately immunosuppressed and increasing in those patients who are not may lead to improved outcomes for both patient populations. The combination of allograft injury (AlloSure

dd-cfDNA) and immune activation markers (AlloMap Kidney GEP) also take us in the direction of non-invasive characterization of underlying pathology and one step closer to offering a true liquid biopsy for monitoring allograft health. With uncontrolled inflammation playing such a fundamental role across the spectrum of allograft loss, non-invasive characterization of both injury and gene expression testing presents an attractive paradigm shift towards transplant precision medicine.

DISCLOSURES

H. Xu, X. Jin, DH, S. Dholakia, and R. Woodward are all employees of CareDx.

DA^2, DA^3, DA^4, DCB, and JSB were DART investigators.

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AUTHOR CONTRIBUTIONS

RNW, HX, and SD drafted the initial manuscript, and all authors were critical to revision and provided final approval for publication. EA, MRW, DCB, HX, SD, RNW, and JSB substantially contributed to conceiving the design of these studies, HX, RNW, and XJ were significant contributors to acquisition and analysis of the data, while EA, MRW, SB, DCB, RDS, AL, AD, JX, SD, RNW, and JSB were critical to interpretation of the data.

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TABLE

Table 1. Clinical characteristics of the DART analysis groups.

Clinical Characteristic		Training			Validation				
		HS AS < 1%	R	P-Value	NR	HS	pNR	R	P-Value (NR vs R)
Number of Patients		22	16		44	20	19	16	
Number of Samples		38	18		47	22	29	18	
Race, n (%)				0.353					0.629
	Black	13 (34.2)	8 (44.4)		14 (29.8)	0	8 (27.6)	4 (22.2)	
	White	21 (55.3)	9 (50.0)		25 (53.2)	15 (68.2)	19 (65.5)	13 (72.2)	
	Native	0	1 (5.6)		0	0	1 (3.5)	0	
	Hispanic/Latino	3 (7.9)	0		5 (10.6)	4 (18.2)	1 (3.5)	1 (5.6)	
	Asian	0	0		1 (2.1)	1 (4.6)	0	0	
	Other	1 (2.6)	0		2 (4.3)	2 (9.1)	0	0	
Sex, n (%)				0.883					0.309
	Men	24 (63.2)	11 (61.1)		30 (63.8)	14 (63.6)	21 (72.4)	9 (50.0)	
	Women	14 (36.8)	7 (38.9)		17 (36.2)	8 (36.4)	8 (27.6)	9 (50.0)	
Age at Enrollment (Years)		48.8 ±13.7	47.9 ±17.8	0.850	50.9 ±14.5	54.9 ±11.8	53.8 ±12.7	39.4 ±10.9	0.001
Post-Transplant (Days)		94.5 ±55.3	1091 ±1071	<0.001	965 ±1360	244 ±200	242 ±192	1322 ±1213	0.323
CMV Serologic Status, n (%)				0.050					0.074
	D-/R+	10 (26.3)	3 (16.7)		13 (27.7)	7 (31.8)	12 (41.4)	3 (16.7)	
	D+/R+	14 (36.8)	3 (16.7)		14 (29.8)	7 (31.8)	7 (24.1)	3 (16.7)	
	D-/R-	7 (18.4)	2 (11.1)		8 (17.0)	3 (13.6)	7 (24.1)	4 (22.2)	
	D+/R-	4 (10.5)	3 (16.7)		5 (10.6)	2 (9.1)	3 (10.3)	0	
	Unknown	3 (7.9)	7 (38.9)		7 (14.9)	3 (13.6)	0	8 (44.4)	
Donor Type, n (%)				0.474					0.115
	Deceased	21 (55.3)	13 (72.2)		28 (59.6)	13 (59.1)	15 (51.7)	11 (61.1)	
	Living - Unrelated	4 (10.5)	1 (5.6)		14 (29.8)	3 (13.6)	8 (27.6)	2 (11.1)	
	Living - Related	13 (34.2)	4 (22.2)		5 (10.6)	6 (27.3)	6 (20.1)	5 (27.8)	
Creatinine		1.46 ±0.53	2.47 ±1.15	<0.001	2.27 ±1.49	1.36 ±0.53	1.61 ±0.57	2.83 ±0.91	0.084
eGFR		55.0 ±14.9	33.6 ±13.1	<0.001	38.9 ±19.8	56.3 ±19.6	48.2 ±13.2	25.9 ±11.3	0.002
HLA Class 1 No. of Mismatches (A, B)		2.7 ±1.1	3.1 ±1.3	0.300	2.5 ±1.3	2.1 ±1.1	2.9 ±1.2	1.7 ±1.1	0.020
HLA Class 2 No. of Mismatches (DR)		1.2 ±0.6	1.2 ±0.5	0.947	1.1 ±0.8	1.1 ±0.8	1.2 ±0.7	0.9 ±0.8	0.317
PRA Class I	Mean PRA	1.72	1.73	0.995	24.1	10.8	9.03	13.4	0.172
	Samples	36	15		39	16	29	15	
PRA Class II	Mean PRA	4.67	18.1	0.120	14.8	10.9	2.9	20.9	0.524
	Samples	36	14		39	16	29	15	
Induction				0.052					0.464
patients (%)	ATG	2 (9.1)	8 (50)		9 (20.5)	2 (10)	0	5 (31.3)	
	Campath	3 (13.6)	3 (18.8)		9 (20.5)	1 (5)	0	2 (12.5)	
	Simulect	2 (9.1)	0		0	1 (5)	0	1 (6.3)	
	Other	3 (13.6)	3 (18.8)		8 (18.2)	2 (10)	0	3 (18.8)	
	None	14 (64.6)	5 (31.3)		23 (52.3)	15 (75)	19 (100)	8 (50)	
Immunosuppression				0.262					0.519
samples (%)	Cyclosporin	2 (5.3)	2 (11.1)		5 (10.6)	1 (4.5)	0	2 (11.1)	
	Tacrolimus	35 (92.1)	16 (88.9)		38 (80.9)	20 (20)	27 (93.1)	15 (83.3)	
	Mycophenolate	34 (89.5)	14 (77.78)		40 (85.1)	20 (90.9)	28 (96.7)	15 (83.3)	
	Prednisone	17 (44.7)	14 (77.78)		28 (59.6)	16 (72.7)	10 (34.5)	13 (72.2)	
	Rapamycin	2 (5.3)	1 (5.6)		3 (6.4)	0	0	2 (11.1)	
	Azathioprine	2 (5.3)	1 (5.6)		3 (6.4)	0	1 (3.4)	1 (5.6)	
	Belatacept	1 (2.6)	0		2 (4.3)	0	2 (6.9)	0	
	Other	0	3 (16.7)		0	0	0	2 (11.1)	

FIGURE LEGENDS

Figure 1: AlloMap Kidney classifier differentiates quiescence from rejection in the primary independent validation set. (a) Box and whisker plots show that biopsy-defined rejections (n=18) were significantly different from quiescence (n=98). (b) No difference among quiescence subgroups, including NR, protocol NR, and healthy stable; all three were significantly lower than TCMR and ABMR (n=22 HS, 29 pNR, 47 NR, 7 TCMR, 10 ABMR, 1 mixed). (c) TCMR results stratified by grade suggest a trend for AlloMap Kidney and TCMR grade. (d) ROC plot for the primary validation set with rejection compared to quiescence (red line) or NR (green line). Unpaired Student's t-test.

Figure 2. AlloMap Kidney differentiates quiescence from rejection in a single-center validation set. (a) Biopsy-defined rejection (n=11) is significantly different from biopsy-defined no-rejection (n=8). (b) All three rejection groups are elevated relative to NR (n = 8 NR, 7 TCMR, 2 ABMR, 2 mixed). (c) ROC plot for the second validation set NR vs rejection (TCMR, ABMR, and mixed). Unpaired Student's t-test.

Figure 3. The combined validation sets discriminate quiescence from rejection. (a) Quiescence (n=55) vs rejection (n=29) for the full combined datasets. (b) NR vs each type of rejection (n = 55 NR, 14 TCMR, 12 ABMR, 3 mixed). (c) ROC of the combined data. Red = Quiescence (n=106) vs rejection, AUC = 0.779. Green = NR vs rejection, AUC = 0.776. Unpaired Student's t test.

Figure 4. Performance characteristics for AlloMap Kidney across the range of scores. (a) Sensitivity (circles) and specificity (triangles) for NR vs rejection. (b) Sensitivity (circles) and specificity (triangles) for quiescence vs rejection. (c) NPV for NR vs rejection. (d) NPV for quiescence vs rejection. (e) PPV for NR vs rejection. (f) PPV for quiescence vs rejection. For NPV and PPV, 25% prevalence shown in filled symbols, 10% prevalence open symbols.

Figure 5. The combination of AlloMap Kidney with AlloSure has greater discriminating ability than either alone. (a) Plot of all quiescence and rejection points from the validation set for both AlloMap Kidney (y-axis) and AlloSure (x-axis). Circles are all types of quiescence (NR, HS, pNR). Orange triangles = TCMR. Blue squares = ABMR. b) ROC plots of a linear combination of AlloMap Kidney and AlloSure scores as shown in (a). Blue is the combined score (AUC 0.894), AlloMap Kidney alone shown in green (AUC 0.768), AlloSure alone shown in red (AUC 0.85).

FIGURE 1

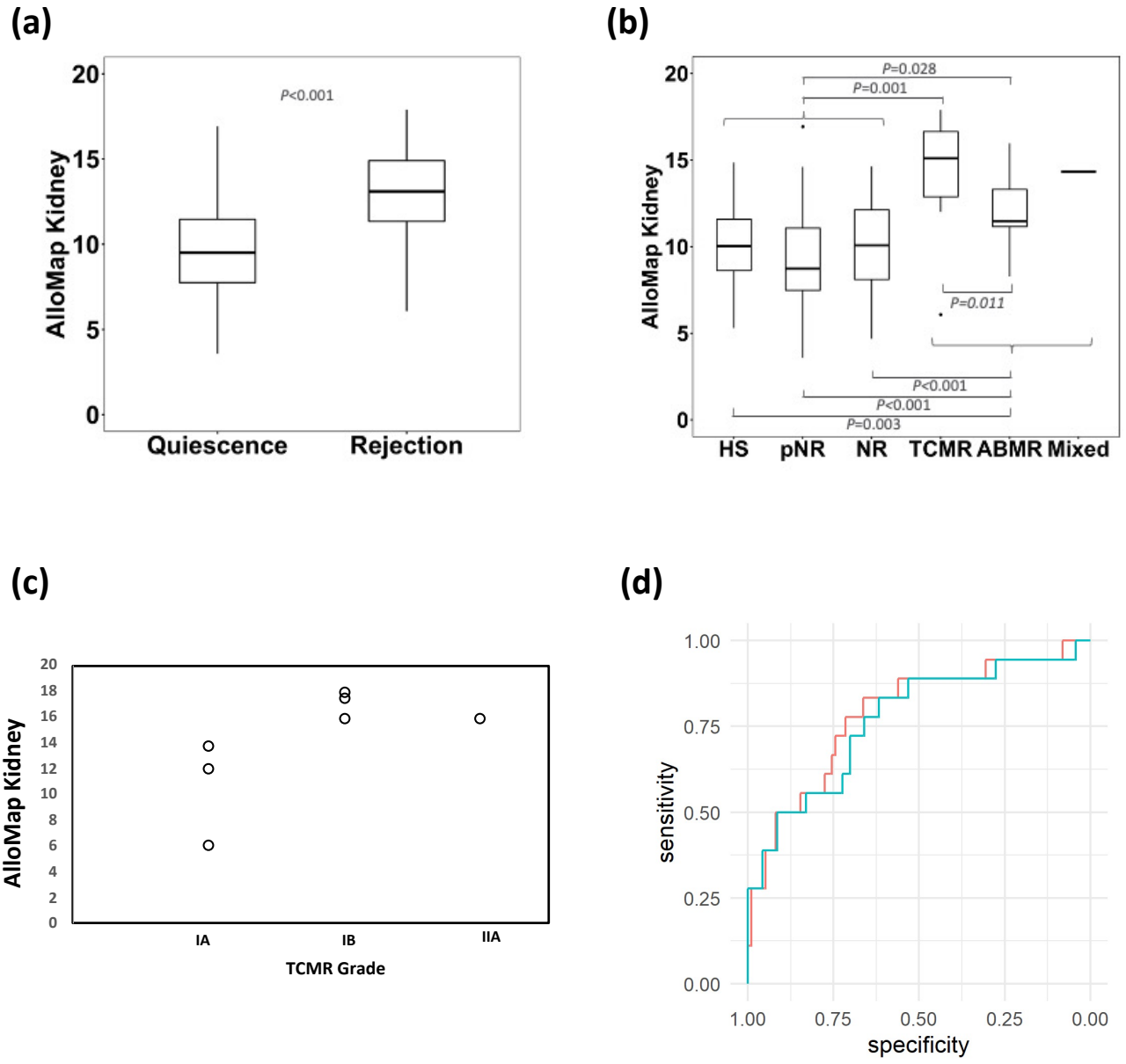

(a)
AlloMap Kidney
P<0.001
Quiescence
Rejection
(b)
AlloMap Kidney
P=0.028
P=0.001
P=0.011
P<0.001
P<0.001
P=0.003
HS
pNR
NR
TCMR
ABMR
Mixed
(c)
AlloMap Kidney
IA
IB
IIA
TCMR Grade
(d)
sensitivity
specificity
1.00
0.75
0.50
0.25
0.00

FIGURE 2

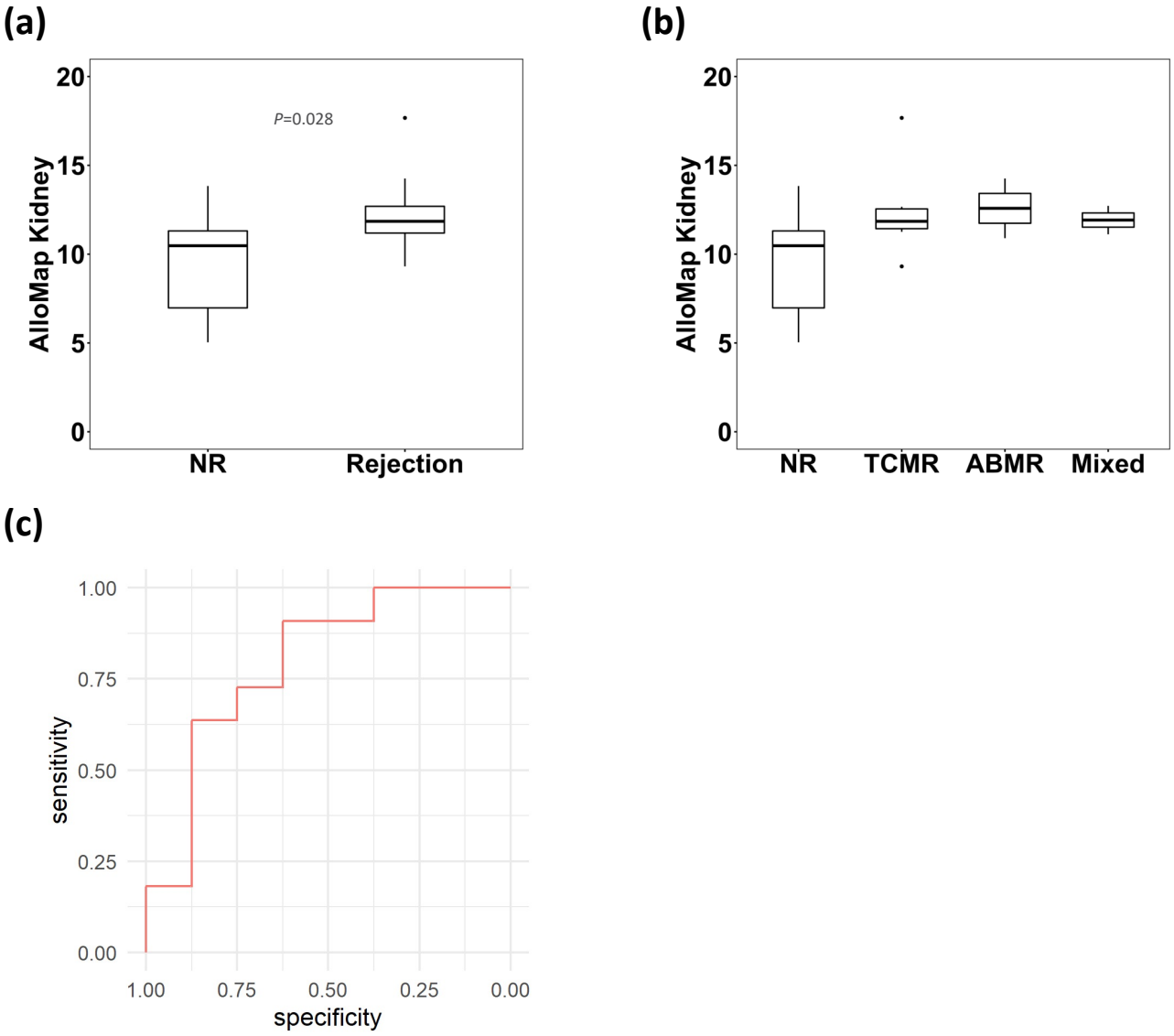

(a)
AlloMap Kidney
20
15
10
5
0
P=0.028
NR
Rejection
(b)
AlloMap Kidney
20
15
10
5
0
NR
TCMR
ABMR
Mixed
(c)
sensitivity
1.00
0.75
0.50
0.25
0.00
1.00
0.75
0.50
0.25
0.00
specificity

FIGURE 3

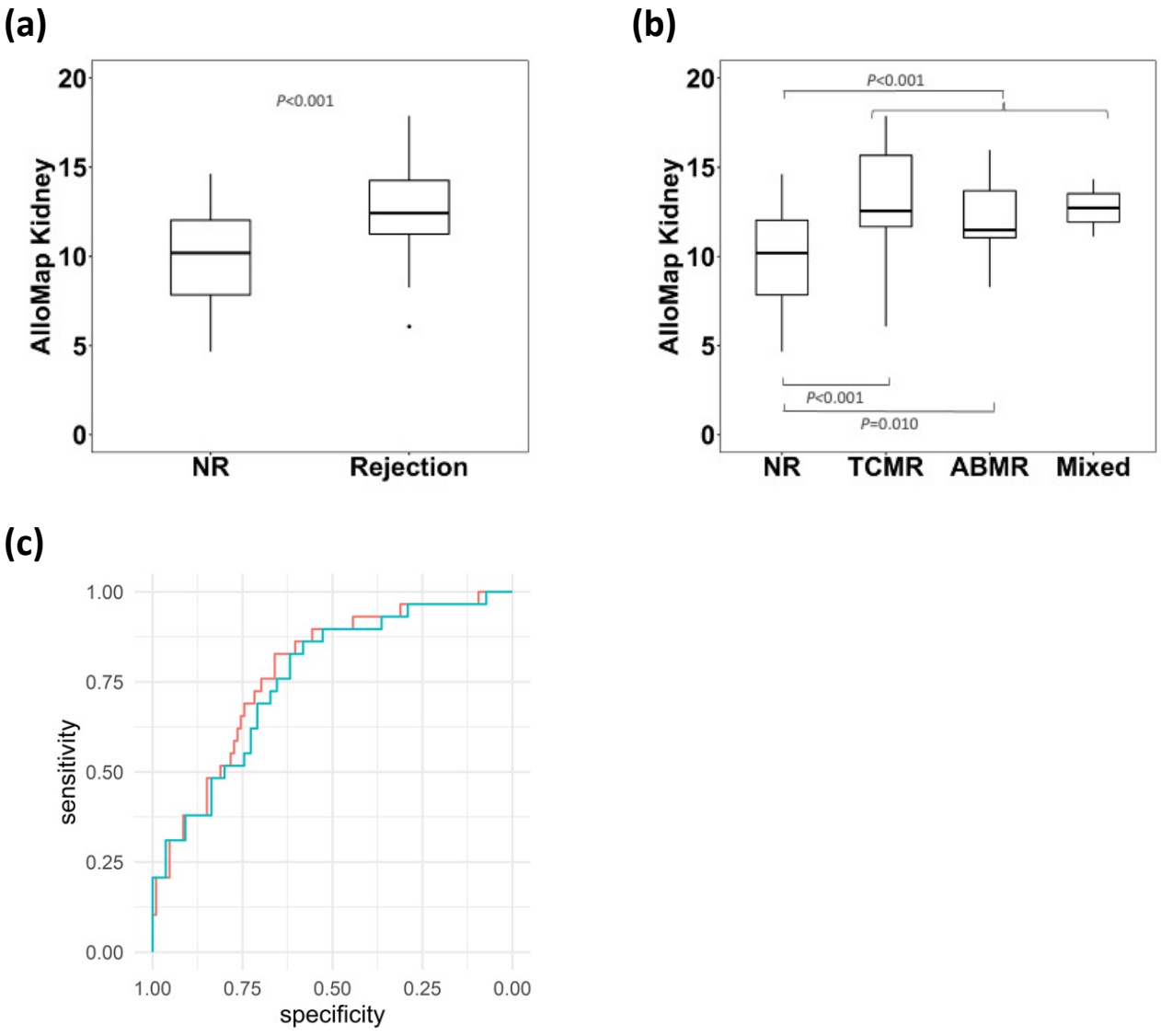

(a)
AlloMap Kidney
20
15
10
5
0
P<0.001
NR
Rejection
(b)
AlloMap Kidney
20
15
10
5
0
P<0.001
P<0.001
P=0.010
NR
TCMR
ABMR
Mixed
(c)
sensitivity
1.00
0.75
0.50
0.25
0.00
1.00
0.75
0.50
0.25
0.00
specificity

FIGURE 4

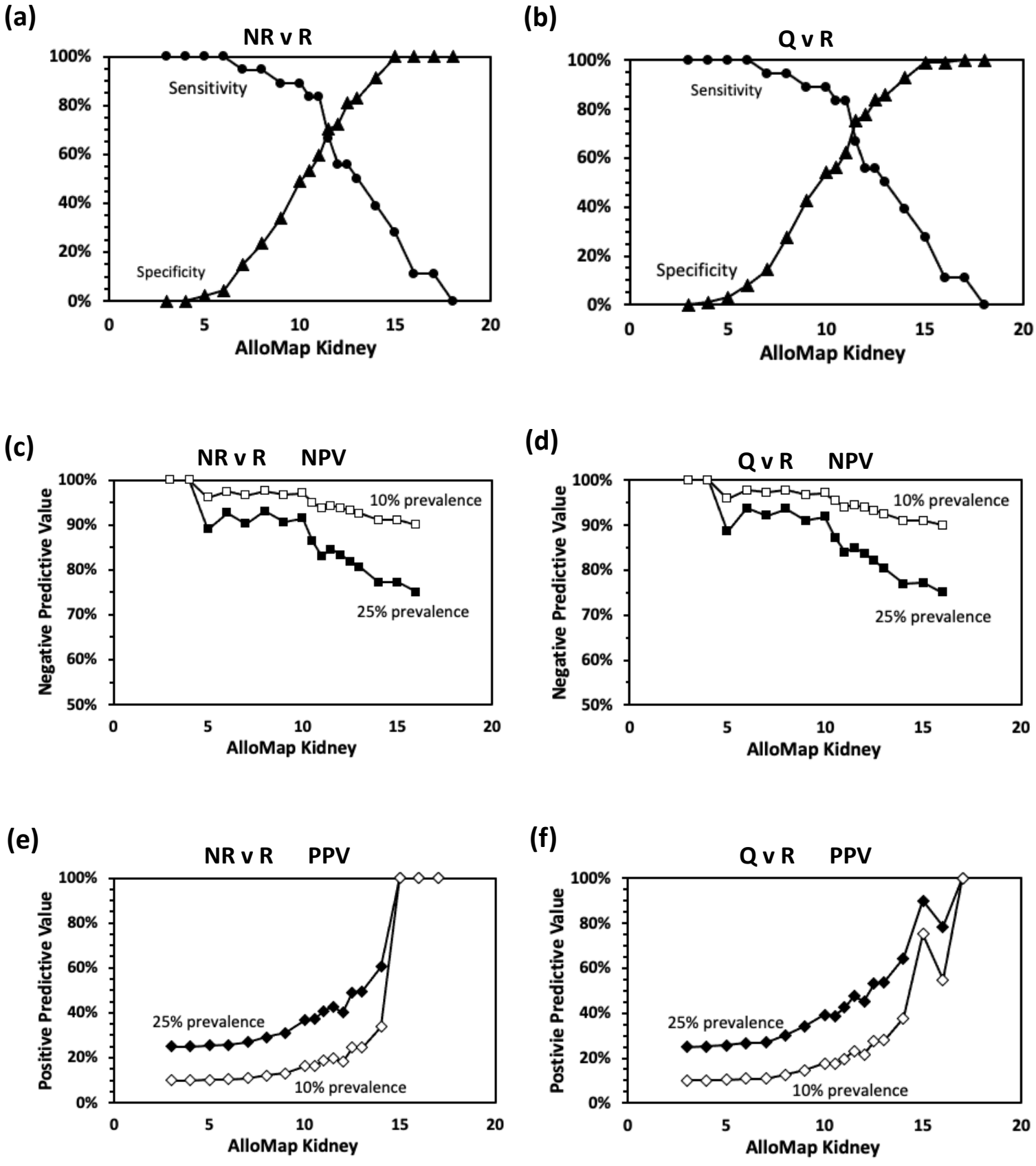
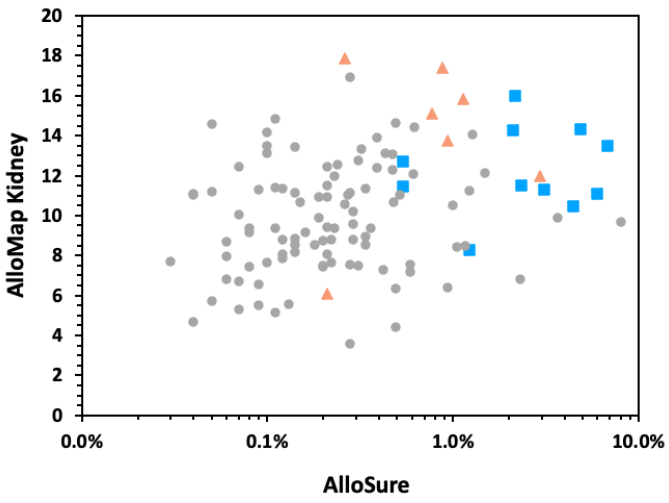
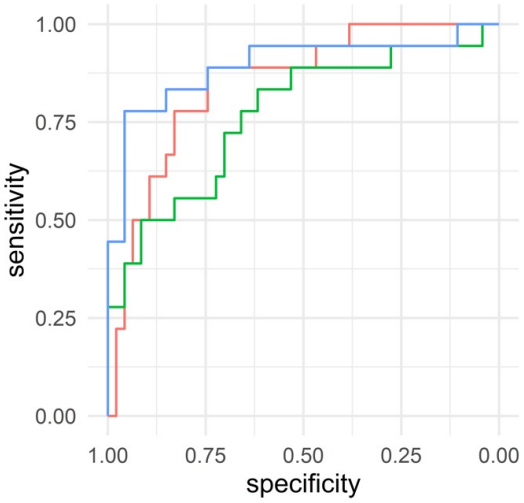

(a)
NR v R
100%
80%
60%
40%
20%
0%
Sensitivity
Specificity
0
5
10
15
20
AlloMap Kidney
(b)
Q v R
100%
80%
60%
40%
20%
0%
Sensitivity
Specificity
0
5
10
15
20
AlloMap Kidney
(c)
NR v R NPV
Negative Predictive Value
100%
90%
80%
70%
60%
50%
10% prevalence
25% prevalence
0
5
10
15
20
AlloMap Kidney
(d)
Q v R NPV
Negative Predictive Value
100%
90%
80%
70%
60%
50%
10% prevalence
25% prevalence
0
5
10
15
20
AlloMap Kidney
(e)
NR v R PPV
Positive Predictive Value
100%
80%
60%
40%
20%
0%
25% prevalence
10% prevalence
0
5
10
15
20
AlloMap Kidney
(f)
Q v R PPV
Postivie Predictive Value
100%
80%
60%
40%
20%
0%
25% prevalence
10% prevalence
0
5
10
15
20
AlloMap Kidney

FIGURE 5

(a)


AlloMap Kidney
AlloSure
0
2
4
6
8
10
12
14
16
18
20
0.0%
0.1%
1.0%
10.0%

(b)


sensitivity
specificity
0.00
0.25
0.50
0.75
1.00
1.00
0.75
0.50
0.25
0.00

SUPPLEMENTAL MATERIAL

Supplemental Material Table of Contents

I. Supplemental Methods

For AlloMap Kidney, an RNA-seq platform incorporating unique molecular tags was used to enable improved detection of low-expression genes, higher reproducibility, and accurate measurement of gene expression changes that can be readily expanded to additional gene sets and classifiers. The training data were generated on the AlloMap Heart qPCR platform. As both platforms measure the level of RNA in the same starting material, a conversion equation was defined based on the principles of the two methods. The conversion assumes that for the qPCR platform there is 98% PCR amplification efficiency and that a C_T of 39 is equivalent to one starting molecule (internal data at CareDx). For RNA-seq, the molecule counts were converted to C_T, then used in the AlloMap Kidney classifier as trained on qRT-PCR results. This conversion of the raw RNA-seq data before application to the classifier enabled the use of the locked classifier algorithm without any modification. To ensure reliable results, the conversion equations were tested using DART samples analyzed on both platforms that were not members of the sets used for training or testing the classifier but covered the critical range of the test results. The results generated from the original method (qRT-PCR) and the final test method (RNA-seq) on the same samples showed 92% correlation, demonstrating the validity of the conversion (**Supplemental Figure S1**).

The single-center sample set from Albert Einstein Medical Center was processed by extracting the RNA from the PAXgene tubes using manual spin columns. Due to the authors' experience with varying RNA transcript levels across extraction methods, the manual methods and the automated methods

were first compared on an independent sample set. Forty samples with paired PAXgene tubes (same venipuncture) from DART and two other studies in the biobanks at CareDx were extracted by the two methods and a conversion equation defined for the small overall difference **(Supplemental Figure S2**). After this conversion was determined and locked, the single-center validation set samples were subsequently run and the conversion was then applied as part of the data analysis to generate AlloMap Kidney results.

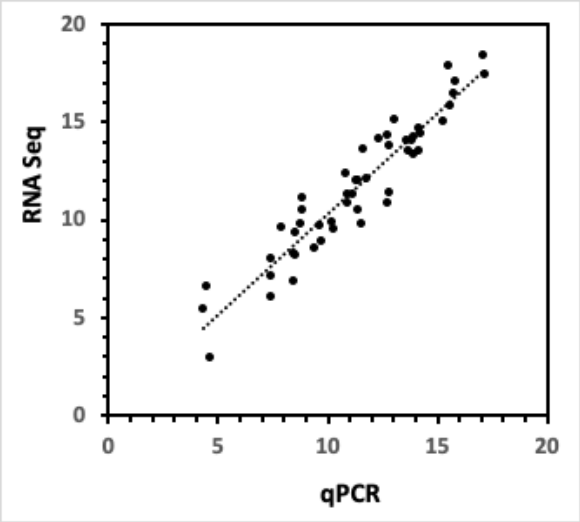


Figure S1: Conversion of AlloMap Kidney classifier trained on qPCR data to produce the same results from RNA-seq data.

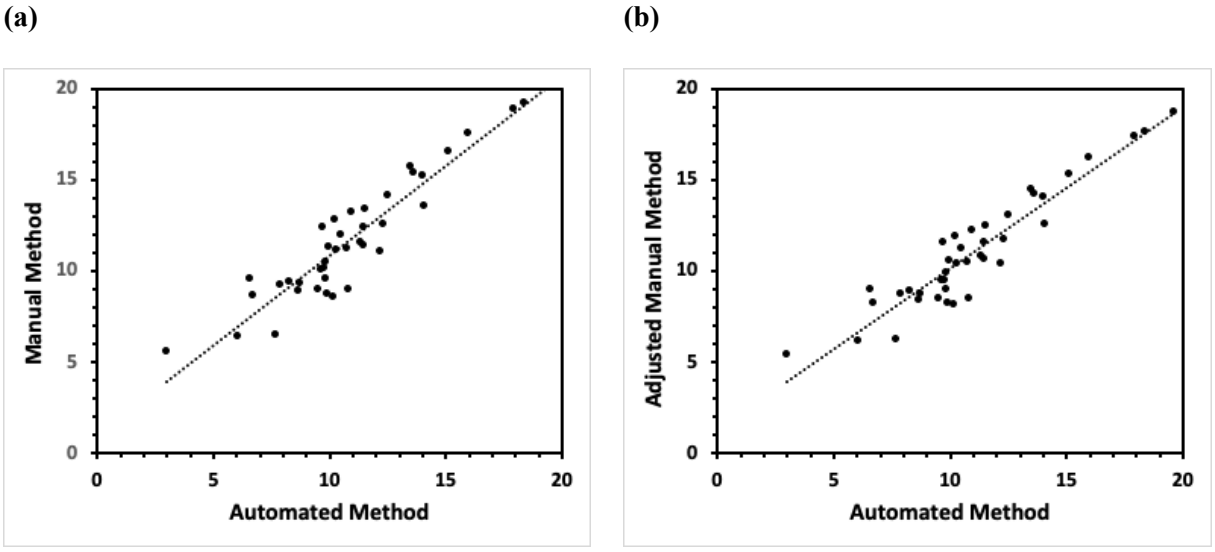


Figure S2: Comparison of AlloMap Kidney data from manual and automated extraction methods on the same 40 samples. **(a)** before adjustment, mean difference 0.7951, intercept = 0.422, slope – 0.897. **(b)** after adjustment, mean difference -0.0015, intercept -0.001, slope = 1.000.

II. Supplemental Table S1

Table S1. Overview of the AlloMap genes. Utility is from Dedrick et al., 2007. Gene names and function from GeneCards (https://www.genecards.org/). Significance in training is the p-value of t-test for individual genes in the training sample set.

Gene		Associated Pathway	Gene Name	Alternative Symbol(s)	Function	Significance in Training
AlloMap Kidney	AlloMap Heart					
PDCD1	PDCD1	T-cell priming	Programmed cell death 1	PD-1, SLEB2	Immune inhibitory receptor. Regulates T cell functions.	0.002
MARCH8	MARCH8	Proliferation and mobilization of blood cells	Membrane Associated Ring-CH-Type Finger 8	MARCHF8, MIR	Ubiquitin ligase, induces internalization of membrane glycoprotens	0.005
DCAF12	WDR40A	Proliferation and mobilization of blood cells	DDB1 and CUL4 Associated Factor 12	WDR40A, KIAA1892	Regulates activity of culling RING E3 ligases	<0.001
IL1R2	IL1R2	Steroid Responsive	Interleukin 1 receptor type 2	IL1RB, CD121b	IL-1a, IL1B and IL1R1 decoy receptor inihibits signaling	0.019
FLT3	FLT3	Steroid Responsive	FMS Related Receptor Tyrosine Kinase 3	FLT3	Regulates hematopoiesis by activating pathways involved in apoptosis, proliferation, and differentiation	0.017
	ITGA4	T-cell priming	Integrin alpha-4	ITGA4	T cell motility and adhesion	0.176
	ITGAM	T-cell priming	Integrin alpha-M	MAC-1, CD11b, CR3A	Alpha subunit of MAC-1 involved in cell trafficking	0.662
	PF4	Platelet Activation	Platelet factor 4	CXCL4	Platelet aggregation, chemotactic for numerous cell types	0.017
	C6orf25	Platelet Activation	G6b inhibitory receptor	G6b, MPIG6B	MHC class III Inhibitory receptor of the Ig superfamily	0.420
	SEM7A	Lymphocyte activation	Semaphorin 7A	SEMAL	Marker of activated lymphocytes.	0.169
	RHOU	Leukocytes migrating into tissues	Ras homolog gene family member U	ARHU	Rho GPT-ase amiliy involved in cytoskeleton ogranization	0.435

III. Supplemental Table S2

Table S2. List of the AlloMap reference genes. In both cases, reference genes were chosen as invariant in a set of NR or Q samples. Gene names from GeneCards (https://www.genecards.org/).

Gene		Gene Name
AlloMap Kidney Training	AlloMap Kidney Validation	
ERCC5		ERCC Excision Repair 5, Endonuclease cell death 1
GABPB2	GABPB2	GA Binding Protein Transcription Factor Subunit Beta 2
CCDC159		Coiled-Coil Domain Containing Protein 159
GPI		Glucose-6-Phosphate Isomerase
RPLP1		Ribosomal Protein Lateral Stalk Subunit P1
GUSB	GUSB	Glucuronidase Beta
	DECR1	2,4-Dienoyl-CoA Reductase 1
	EWSR1	EWS RNA Binding Protein 1
	GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
	HSP90AB1	Heat Shock Protein 90 Alpha Family Class B Member 1
	MAP3K3	Mitogen-Activated Protein Kinase Kinase Kinase 3
	MAPK9	Mitogen-Activated Protein Kinase 9
	NONO	Non-POU Domain Containing Octamer Binding
	RXRB	Retinoid X Receptor Beta
	SDHA	Succinate Dehydrogenase Complex Flavoprotein Subunit A
	SRRM1	Serine And Arginine Repetitive Matrix 1
	TBC1D10B	TBC1 Domain Family Member 10B
	TBP	TATA-Box Binding Protein
	TOP2B	DNA Topoisomerase II Beta