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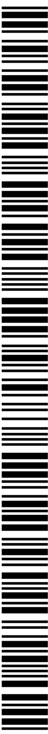

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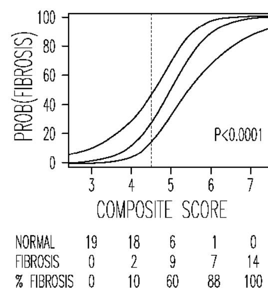

Fig. 4A

(57) Abstract: Measurement of mRNAs in urinary cells offers a noninvasive means of diagnosing fibrosis in kidneys. One aspect of the invention is a method that includes: (a) measuring quantities of vimentin mRNA, NKCC2 mRNA, and E-cadherin mRNA in a test sample of cells obtained from urine; and (b) determining whether the vimentin mRNA quantity is higher, the NKCC2 mRNA quantity is lower, or the E-cadherin mRNA is higher than in healthy urinary cells; and thereby detecting that the sample is a fibrotic kidney sample. Step (a) can also include measuring the quantity of RNA expressed by a housekeeping gene (e.g., 18S rRNA). The quantities of vimentin mRNA, NKCC2 mRNA, and E-cadherin mRNA can be normalized against the quantity of housekeeping gene RNA.

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NON-INVASIVE METHOD FOR DIAGNOSING RENAL FIBROSIS

This application claims benefit of the filing date of U.S. Provisional Patent Application No. 61/647,347, filed May 15, 2012, the contents of which are specifically incorporated herein in their entirety.

This invention was made with Government support under Grant Number
5 2R37-A1051652 awarded by the National Institute of Allergy and Infectious Disease. The United States Government has certain rights in the invention.

BACKGROUND

Renal allograft fibrosis is currently identified using the invasive allograft
10 biopsy procedure in patients with worsening renal function. However, many challenges exist including early diagnosis of fibrosis (see, e.g., Arias et al., *Transplantation* 91:4 (2011)) and neither serum creatinine nor estimated glomerular filtration rate appears to be an accurate indicator of fibrosis (Yilmaz et al., *Transpl Int* 20: 608 (2007)). Moreover, the biopsy procedure is costly,
15 complications still occur, sampling errors may bias the diagnosis, and inter-observer variability in grading of biopsies remains a challenge (Huraib et al., *Am J Kidney Dis* 14:13 (1989); Beckingham et al., *Br J Urol* 73: 13 (1994); Benfield et al., *Transplantation* 67: 544 (1999); Sorof et al., *Transplantation* 60: 1215 (1995); Colvin et al., *J Am Soc Nephrol* 8: 1930 (1997); Nicholson et al.,
20 *Kidney Int* 58: 390 (2000); Joh et al. *Clin Transplant* 20 Suppl 15: 53 (2006)).

SUMMARY

A noninvasive test for the diagnosis of renal (kidney) fibrosis is provided herein. Instead of invasive biopsy extraction, urinary samples can be used to
25 assess the propensity for developing renal fibrosis, to assess the severity of renal fibrosis, and/or to monitor the progression of kidney fibrosis in a subject. For example, about 50% of kidney transplants are currently lost due to patient death with a functioning graft. The potent immunosuppressive regimens used to date increase cardiovascular risk factors such as hypertension and
30 hypercholesterolemia and increase malignancy development (9), which may contribute to transplant patient death rates. Over-immunosuppression may also increase the risk for developing opportunistic infections, which may further complicate transplant management. The invention provides a non-invasive

method of detecting a transplant related disease that can be performed repeatedly and analyzed quickly without the increased risk of an invasive procedure. Hence, one of the advantages of the methods and devices described herein their non-invasive, which permit repeated risk-free testing.

5 One aspect of the invention is a method that includes: (a) measuring quantities of vimentin mRNA, NKCC2 mRNA, and E-cadherin mRNA in a test sample of cells obtained from urine; and (b) determining whether the vimentin mRNA quantity is higher, the NKCC2 mRNA quantity is lower, or the E-cadherin mRNA is higher than in healthy urinary cells; and thereby detecting 10 that the sample is a fibrotic kidney sample. Step (a) can also include measuring the quantity of RNA expressed by a housekeeping gene (e.g., 18S rRNA). The quantities of vimentin mRNA, NKCC2 mRNA, and E-cadherin mRNA can be normalized against the quantity of housekeeping gene RNA. Methods for assigning a composite score regarding the expression values are also 15 described herein, which can facilitate identification of fibrotic test samples and subjects that can benefit from treatment.

DESCRIPTION OF THE FIGURES

FIG. 1A and 1B1-1B22 show steps involved in generating Discovery and 20 Validation sets based upon differential expression of urinary mRNAs and the differential expression of mRNAs in fibrosis and normal renal biopsies. FIG. 1A is a flow chart illustrating the discovery and validation of urinary mRNA profiles. One hundred fourteen renal allograft recipients (48 with biopsies showing fibrosis and 66 with normal biopsy results) were rank ordered within 25 group (Fibrosis group or Normal Biopsy group) by the copy number of 18S rRNA and partitioned into triplets. Within each triplet, the first and third patients were assigned to the Discovery set and the second patient was assigned to the Validation set, resulting in the two sets being exactly matched on fibrosis status and very closely matched on 18S rRNA copy number. Twice as many patients 30 were assigned to the Discovery set in order to enhance statistical power for the exploratory analyses which included a procedure to protect against the risk of a Type I error. FIG. 1B1-1B22 illustrate that urinary cell mRNAs are differentially expressed in fibrosis tissues versus normal tissues by graphically illustrating the $\log_{10}$ normalized mRNA quantities from fibrotic and normal

tissues. FIG. 1B1shows vimentin expression in normal and fibrosis biopsies. FIG. 1B2 shows S100A4 expression in normal and fibrosis biopsies. FIG. 1B3 shows α-SMA expression in normal and fibrosis biopsies. FIG. 1B4 shows fibronectin1 expression in normal and fibrosis biopsies. FIG. 1B5 shows TIMP1
5 expression in normal and fibrosis biopsies. FIG. 1B6 shows PAI1 expression in normal and fibrosis biopsies. FIG. 1B7 shows collagen 1A1 expression in normal and fibrosis biopsies. FIG. 1B8 shows NKCC2 expression in normal and fibrosis biopsies. FIG. 1B9 shows E-cadherin expression in normal and fibrosis biopsies. FIG. 1B10 shows USAG1 expression in normal and fibrosis biopsies.
10 FIG. 1B11 shows FGF2 expression in normal and fibrosis biopsies. FIG. 1B12 shows TGFβ1 expression in normal and fibrosis biopsies. FIG. 1B13 shows ITGB6 expression in normal and fibrosis biopsies. FIG. 1B14 shows CTGF expression in normal and fibrosis biopsies. FIG. 1B15 shows BMP7 expression in normal and fibrosis biopsies. FIG. 1B16 shows HGF expression in normal and
15 fibrosis biopsies. FIG. 1B17 shows FOX P3 expression in normal and fibrosis biopsies. FIG. 1B18 shows CTLA4 expression in normal and fibrosis biopsies. FIG. 1B19 shows CD103 expression in normal and fibrosis biopsies. FIG. 1B20 shows perforin expression in normal and fibrosis biopsies. FIG. 1B21 shows CD25 expression in normal and fibrosis biopsies. FIG. 1B22 shows granzyme B
20 expression in normal and fibrosis biopsies.

FIG. 2A1-2A12 and 2B1-2B10 illustrate that the levels of twelve of twenty-two mRNAs analyzed in urinary cell samples appear to be significantly associated with the diagnosis of fibrosis when using the Holm modified Bonferoni procedure (Holm, *Journal of Statistics* 6: 65 (1979)) to control the
25 risk of a Type I error. FIG. 2A1-2A12 graphically illustrates that the $\log_{10}$ expression values of 12 genes in urinary cells are predictive of fibrosis (A1=vimentin; A2=HGF; A3=αSMA; A4=fibronectin; A5=perforin; A6=PAI1; A7=TGFβ1; A8=TIMP1; A9=granzyme B; A10=FSP1; A11=CD103; A12=collagen 1A1). The predicted probability of fibrosis as a function of
30 urinary cell mRNA copy number in the Discovery set, for the locally weighted scatterplot smoothing (LOESS) model and the piece-wise linear logistic regression model, after controlling for 18S rRNA copy number. Urine samples were collected from 32 renal transplant recipients with graft dysfunction and biopsy-confirmed fibrosis and 44 recipients with stable allograft function and

normal allograft biopsy, and levels of mRNA in urinary cells were measured with the use of pre-amplification enhanced kinetic quantitative PCR assays. FIG. 2B1-2B10 illustrates the predicted probability of fibrosis (Y-axis), controlling for 18S rRNA, of ten genes as a function of individual $\log_{10}$-transformed mRNA copy numbers (X-axis) (B1=BMP7; B2=CTGF; B3=CTLA4; B4=FGF2; B5=CD25; B6=FOXP3; B7=USAG1; B8=E-cadherin; B9=ITGB6; and B10=NKCC2). Each plot shows the LOESS model's predicted probabilities (dotted line), their 95% confidence interval (shaded area) and the logistic regression model's predicted probabilities (solid line). As indicated by the data in FIG. 2B1-2B10, the ten mRNAs tested and evaluated as described are apparently are not significantly correlated with a fibrosis diagnosis. Thus, according to the logistic models, the levels of twelve of the twenty-two mRNAs (vimentin, HGF, α-SMA, fibronectin 1, perforin, PAI1, TGFβ1, TIMP1, granzyme B, FSP1,CD103, and collagen 1A1) were significantly (P-values <0.05 with modified Bonferroni correction) associated with the diagnosis of fibrosis. Adjusted P-value for each parametric model is shown. The number of stable patients, number of fibrosis patients, and percentage of fibrosis patients within categories of the mRNA measure appear in each plot.

FIG. 3A-3D shows the final model derived from the Discovery Set for the diagnosis of fibrosis. FIG. 3A shows the probability of fibrosis in view of log vimentin expression in urinary cells. FIG. 3B shows the probability of fibrosis in view of log NKCC2 expression in urinary cells. FIG. 3C shows the probability of fibrosis in view of log E-cadherin expression in urinary cells. Fig. 3D shows the parameter estimates for the model, including terms accounting for the relationships, including non-linear relationships, between the RNA expression levels and diagnosis.

FIG. 4A-4D illustrates the relationship of the composite score to fibrosis in the Discovery set (FIG. 4A), receiver operating characteristics (ROC) curve analysis of the composite score in the Discovery set (FIG. 4B) and the Validation set (FIG. 4C) and the predicted and observed number of transplant recipients with fibrosis for each sextile of the composite score within the Discovery and Validation sets (FIG. 4D).

FIG. 5 graphically illustrates the mean level (and 95%CI) of the 4-gene composite score by fibrosis grade. Kidney allograft biopsies were classified

as normal, mild fibrosis (grade I, <25% of cortical area), moderate (grade II, 26-50% of cortical area), or severe (grade III, >50% of cortical area). The mean (and 95%CI) composite scores derived from urinary cell vimentin, NKCC2 and E-cadherin mRNA levels and 18S rRNA level were significantly different across the four groups (P<0.0001, one-way ANOVA). Pair-wise comparisons revealed that the mean composite score of normal biopsies was significantly different from those of mild fibrosis (P=0.0002, Tukey's honestly significant differences criterion), moderate fibrosis (P<0.0001) and severe fibrosis (P<0.0001). Within the fibrosis group however the mean composite scores were not significantly different (mild vs. moderate [P=0.64], mild vs. severe [P=0.65] and moderate vs. severe [P=0.99]). Values under each biopsy diagnosis show the number of kidney graft recipients from whom urine samples were collected for the measurement of urinary cell mRNA.

DETAILED DESCRIPTION

Kidney fibrosis can accurately and less invasively be detected, monitored and evaluated by use of the methods and devices described herein. As demonstrated herein vimentin, NKCC2 and E-cadherin mRNA levels as well as the 18S rRNA level were significantly different in urinary sample cells of subjects with kidney fibrosis than in healthy subjects. Moreover, the severity of kidney fibrosis directly correlates with the degree to which the quantities of these four RNAs in the test sample differ from control RNA quantities. The control RNA quantities are the quantities of the same RNAs from healthy subject(s) who do not have renal fibrosis.

Thus, a four-gene method involving measurement of levels of mRNA for vimentin, NKCC2, and E-cadherin, as well as 18S rRNA, is an accurate, parsimonious, diagnostic model of kidney fibrosis, having 93.8% sensitivity and 84.1% specificity (P<0.0001) in a Discovery set. In an independent validation set, this same model predicted the presence of allograft fibrosis with 77.3% sensitivity and 87.5% specificity (P<0.0001).

Vimentin

Vimentin is a type III intermediate filament protein that is expressed in mesenchymal cells, where it serves as a major cytoskeletal component. Vimentin plays a significant role in supporting and anchoring the position of the organelles in the cytosol.

In the 4-gene diagnostic signature defined herein, vimentin had the strongest association with the allograft fibrosis diagnosis. Ivaska et al. (*Exp Cell Res* 313:2050 (2007)) have reviewed the dynamic nature of vimentin expression and the role of this evolutionarily conserved protein in cell adhesion, migration and signaling. Whereas healthy renal tubular cells do no express vimentin protein, injured ones are decorated by vimentin. Vimentin-expressing regenerating renal tubular cells have been reported by Nakatsuji et al. (*Virchows Arch* 433: 359 (1998); see also, Bielesz et al., *J Clin Invest* 120: 4040 (2010); Hertig et al., *J Am Soc Nephrol* 19: 1584 (2008)).

Nucleic acid and protein sequences for vimentin are available, for example, in the sequence database maintained by the National Center for Biotechnology Information (see website at www.ncbi.nlm.nih.gov/). One example of a human vimentin nucleic acid sequence is available as accession number NM_003380.3 (GI:240849334), provided below as SEQ ID NO:1.

```
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 41 ACCCACCGCG CCCTCGTTCG CCTCTTCTCC GGGAGCCAGT
 81 CCGCGCCACC GCCGCCGCCC AGGCCATCGC CACCCTCCGC
121 AGCCATGTCC ACCAGGTCCG TGTCCTCGTC CTCCTACCGC
161 AGGATGTTCG GCGGCCCGGG CACCGCGAGC CGGCCGAGCT
201 CCAGCCGGAG CTACGTGACT ACGTCCACCC GCACCTACAG
241 CCTGGGCAGC GCGCTGCGCC CCAGCACCAG CCGCAGCCTC
281 TACGCCTCGT CCCCGGGCGG CGTGTATGCC ACGCGCTCCT
321 CTGCCGTGCG CCTGCGGAGC AGCGTGCCCG GGGTGCGGCT
361 CCTGCAGGAC TCGGTGGACT TCTCGCTGGC CGACGCCATC
401 AACACCGAGT TCAAGAACAC CCGCACCAAC GAGAAGGTGG
441 AGCTGCAGGA GCTGAATGAC CGCTTCGCCA ACTACATCGA
481 CAAGGTGCGC TTCCTGGAGC AGCAGAATAA GATCCTGCTG
521 GCCGAGCTCG AGCAGCTCAA GGGCCAAGGC AAGTCGCGCC
561 TGGGGGACCT CTACGAGGAG GAGATGCGGG AGCTGCGCCG
601 GCAGGTGGAC CAGCTAACCA ACGACAAAGC CCGCGTCGAG
641 GTGGAGCGCG ACAACCTGGC CGAGGACATC ATGCGCCTCC
681 GGGAGAAATT GCAGGAGGAG ATGCTTCAGA GAGAGGAAGC
721 CGAAAACACC CTGCAATCTT TCAGACAGGA TGTTGACAAT
761 GCGTCTCTGG CACGTCTTGA CCTTGAACGC AAAGTGGAAT
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 801 CTTTGCAAGA AGAGATTGCC TTTTTGAAGA AACTCCACGA
 841 AGAGGAAATC CAGGAGCTGC AGGCTCAGAT TCAGGAACAG
 881 CATGTCCAAA TCGATGTGGA TGTTTCCAAG CCTGACCTCA
 921 CGGCTGCCCT GCGTGACGTA CGTCAGCAAT ATGAAAGTGT
 961 GGCTGCCAAG AACCTGCAGG AGGCAGAAGA ATGGTACAAA
1001 TCCAAGTTTG CTGACCTCTC TGAGGCTGCC AACCGGAACA
1041 ATGACGCCCT GCGCCAGGCA AAGCAGGAGT CCACTGAGTA
1081 CCGGAGACAG GTGCAGTCCC TCACCTGTGA AGTGGATGCC
1121 CTTAAAGGAA CCAATGAGTC CCTGGAACGC CAGATGCGTG
1161 AAATGGAAGA GAACTTTGCC GTTGAAGCTG CTAACTACCA
1201 AGACACTATT GGCCGCCTGC AGGATGAGAT TCAGAATATG
1241 AAGGAGGAAA TGGCTCGTCA CCTTCGTGAA TACCAAGACC
1281 TGCTCAATGT TAAGATGGCC CTTGACATTG AGATTGCCAC
1321 CTACAGGAAG CTGCTGGAAG GCGAGGAGAG CAGGATTTCT
1361 CTGCCTCTTC CAAACTTTTC CTCCCTGAAC CTGAGGGAAA
1401 CTAATCTGGA TTCACTCCCT CTGGTTGATA CCCACTCAAA
1441 AAGGACACTT CTGATTAAGA CGGTTGAAAC TAGAGATGGA
1481 CAGGTTATCA ACGAAACTTC TCAGCATCAC GATGACCTTG
1521 AATAAAAATT GCACACACTC AGTGCAGCAA TATATTACCA
1561 GCAAGAATAA AAAAGAAATC CATATCTTAA AGAAACAGCT
1601 TTCAAGTGCC TTTCTGCAGT TTTTCAGGAG CGCAAGATAG
1641 ATTTGGAATA GGAATAAGCT CTAGTTCTTA ACAACCGACA
1681 CTCCTACAAG ATTTAGAAAA AAGTTTACAA CATAATCTAG
1721 TTTACAGAAA AATCTTGTGC TAGAATACTT TTTAAAAGGT
1781 ATTTTGAATA CCATTAAAAC TGCTTTTTTT TTTCCAGCAA
1801 GTATCCAACC AACTTGGTTC TGCTTCAATA AATCTTTGGA
1841 AAAACTC
```

The human protein encoded by the vimentin nucleic acid shown above as SEQ ID NO:1 has an amino acid sequence with SEQ ID NO:2, shown below.

```
  1 MSTRSVSSSS YRRMFGGPGT ASRPSSSRSY VTTSTRTYSL
 41 GSALRPSTSR SLYASSPGGV YATRSSAVRL RSSVPGVRLL
 81 QDSVDFSLAD AINTEFKNTR TNEKVELQEL NDRFANYIDK
121 VRFLEQQNKI LLAELEQLKG QGKSRLGDLY EEEMRELRRQ
161 VDQLTNDKAR VEVERDNLAE DIMRLREKLQ EEMLQREEAE
201 NTLQSFRQDV DNASLARLDL ERKVESLQEE IAFLKKLHEE
241 EIQELQAQIQ EQHVQIDVDV SKPDLTAALR DVRQQYESVA
281 AKNLQEAEEW YKSKFADLSE AANRNNDALR QAKQESTEYR
321 RQVQSLTCEV DALKGTNESL ERQMREMEEN FAVEAANYQD
361 TIGRLQDEIQ NMKEEMARHL REYQDLLNVK MALDIEIATY
401 RKLLEGEESR ISLPLPNFSS LNLRETNLDS LPLVDTHSKR
411 TLLIKTVETR DGQVINETSQ HHDDLE
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Another example of a human vimentin nucleic acid sequence is available as accession number NM_003380.3 (GI:240849334), provided below as SEQ ID NO:3.

```
   1 GCCTCTCCAA AGGCTGCAGA AGTTTCTTGC TAACAAAAAG
  41 TCCGCACATT CGAGCAAAGA CAGGCTTTAG CGAGTTATTA
  81 AAAACTTAGG GGCGCTCTTG TCCCCCACAG GGCCCGACCG
 121 CACACAGCAA GGCGATGGCC CAGCTGTAAG TTGGTAGCAC
 161 TGAGAACTAG CAGCGCGCGC GGAGCCCGCT GAGACTTGAA
 201 TCAATCTGGT CTAACGGTTT CCCCTAAACC GCTAGGAGCC
 241 CTCAATCGGC GGGACAGCAG GGCGCGTCCT CTGCCACTCT
 281 CGCTCCGAGG TCCCCGCGCC AGAGACGCAG CCGCGCTCCC
 321 ACCACCCACA CCCACCGCGC CCTCGTTCGC CTCTTCTCCG
 361 GGAGCCAGTC CGCGCCACCG CCGCCGCCCA GGCCATCGCC
 401 ACCCTCCGCA GCCATGTCCA CCAGGTCCGT GTCCTCGTCC
 441 TCCTACCGCA GGATGTTCGG CGGCCCGGGC ACCGCGAGCC
 481 GGCCGAGCTC CAGCCGGAGC TACGTGACTA CGTCCACCCG
 521 CACCTACAGC CTGGGCAGCG CGCTGCGCCC CAGCACCAGC
 561 CGCAGCCTCT ACGCCTCGTC CCCGGGCGGC GTGTATGCCA
 601 CGCGCTCCTC TGCCGTGCGC CTGCGGAGCA GCGTGCCCGG
 641 GGTGCGGCTC CTGCAGGACT CGGTGGACTT CTCGCTGGCC
 681 GACGCCATCA ACACCGAGTT CAAGAACACC CGCACCAACG
 721 AGAAGGTGGA GCTGCAGGAG CTGAATGACC GCTTCGCCAA
 761 CTACATCGAC AAGGTGCGCT TCCTGGAGCA GCAGAATAAG
 801 ATCCTGCTGG CCGAGCTCGA GCAGCTCAAG GGCCAAGGCA
 841 AGTCGCGCCT GGGGGACCTC TACGAGGAGG AGATGCGGGA
 881 GCTGCGCCGG CAGGTGGACC AGCTAACCAA CGACAAAGCC
 921 CGCGTCGAGG TGGAGCGCGA CAACCTGGCC GAGGACATCA
 961 TGCGCCTCCG GGAGAAATTG CAGGAGGAGA TGCTTCAGAG
1001 AGAGGAAGCC GAAAACACCC TGCAATCTTT CAGACAGGAT
1041 GTTGACAATG CGTCTCTGGC ACGTCTTGAC CTTGAACGCA
1081 AAGTGGAATC TTTGCAAGAA GAGATTGCCT TTTTGAAGAA
1121 ACTCCACGAA GAGGAAATCC AGGAGCTGCA GGCTCAGATT
1181 CAGGAACAGC ATGTCCAAAT CGATGTGGAT GTTTCCAAGC
1201 CTGACCTCAC GGCTGCCCTG CGTGACGTAC GTCAGCAATA
1241 TGAAAGTGTG GCTGCCAAGA ACCTGCAGGA GGCAGAAGAA
1281 TGGTACAAAT CCAAGTTTGC TGACCTCTCT GAGGCTGCCA
1321 ACCGGAACAA TGACGCCCTG CGCCAGGCAA AGCAGGAGTC
1361 CACTGAGTAC CGGAGACAGG TGCAGTCCCT CACCTGTGAA
1401 GTGGATGCCC TTAAAGGAAC CAATGAGTCC CTGGAACGCC
1441 AGATGCGTGA AATGGAAGAG AACTTTGCCG TTGAAGCTGC
1481 TAACTACCAA GACACTATTG GCCGCCTGCA GGATGAGATT
1521 CAGAATATGA AGGAGGAAAT GGCTCGTCAC CTTCGTGAAT
1561 ACCAAGACCT GCTCAATGTT AAGATGGCCC TTGACATTGA
1601 GATTGCCACC TACAGGAAGC TGCTGGAAGG CGAGGAGAGC
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1641 AGGATTTCTC TGCCTCTTCC AAACTTTTCC TCCCTGAACC
1681 TGAGGGAAAC TAATCTGGAT TCACTCCCTC TGGTTGATAC
1721 CCACTCAAAA AGGACACTTC TGATTAAGAC GGTTGAAACT
1761 AGAGATGGAC AGGTTATCAA CGAAACTTCT CAGCATCACG
1801 ATGACCTTGA ATAAAAATTG CACACACTCA GTGCAGCAAT
1841 ATATTACCAG CAAGAATAAA AAAGAAATCC ATATCTTAAA
1881 GAAACAGCTT TCAAGTGCCT TTCTGCAGTT TTTCAGGAGC
1921 GCAAGATAGA TTTGGAATAG GAATAAGCTC TAGTTCTTAA
1961 CAACCGACAC TCCTACAAGA TTTAGAAAAA AGTTTACAAC
2001 ATAATCTAGT TTACAGAAAA ATCTTGTGCT AGAATACTTT
2041 TTAAAAGGTA TTTTGAATAC CATTAAAACT GCTTTTTTTT
2081 TTCCAGCAAG TATCCAACCA ACTTGGTTCT GCTTCAATAA
2121 ATCTTTGGAA AAACTCAAAA AAAAAAAAAA A
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The human protein encoded by the vimentin nucleic acid shown above as SEQ ID NO:3 has an amino acid sequence with NCBI accession number NP_003371.2 (GI:62414289), shown below as SEQ ID NO:4.

```
  1 MSTRSVSSSS YRRMFGGPGT ASRPSSSRSY VTTSTRTYSL
 41 GSALRPSTSR SLYASSPGGV YATRSSAVRL RSSVPGVRLL
 81 QDSVDFSLAD AINTEFKNTR TNEKVELQEL NDRFANYIDK
121 VRFLEQQNKI LLAELEQLKG QGKSRLGDLY EEEMRELRRQ
161 VDQLTNDKAR VEVERDNLAE DIMRLREKLQ EEMLQREEAE
201 NTLQSFRQDV DNASLARLDL ERKVESLQEE IAFLKKLHEE
241 EIQELQAQIQ EQHVQIDVDV SKPDLTAALR DVRQQYESVA
281 AKNLQEAEEW YKSKFADLSE AANRNNDALR QAKQESTEYR
321 RQVQSLTCEV DALKGTNESL ERQMREMEEN FAVEAANYQD
361 TIGRLQDEIQ NMKEEMARHL REYQDLLNVK MALDIEIATY
401 RKLLEGEESR ISLPLPNFSS LNLRETNLDS LPLVDTHSKR
421LIKTVETR DGQVINETSQ HHDDLE
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Urinary cell levels of vimentin mRNA were significantly associated with the presence of kidney fibrosis ($P<0.0001$, logistic regression model). The predicted probability of fibrosis (Y-axis) as a function of vimentin log10-transformed mRNA copy numbers (X-axis) is shown in FIG. 2A. The vimentin plot shows the LOESS model's predicted probability (dotted line), its 95% confidence interval (shaded area) and the logistic regression model's predicted probabilities (solid line). The parameter estimates for the 4-gene model including terms accounting for the relationships, including non-linear relationships, between the mRNAs and diagnosis are provided in FIG. 3D.

Any probe or primer that is specific for vimentin can be used in the methods and devices described herein. Examples are provided herein.

NKCC2

The Na-K-Cl cotransporter (NKCC, SLC12A2) is a protein that aids in the active transport of sodium, potassium, and chloride into and out of cells. There are two varieties, or isoforms, of this membrane transport protein, called NKCC1 and NKCC2.

Nucleic acid and protein sequences for NKCC2 are available, for example, in the sequence database maintained by the National Center for Biotechnology Information (see website at www.ncbi.nlm.nih.gov/). One example of a human NKCC2 nucleic acid sequence is available as accession number BC040138.2 (GI:34193025), provided below as SEQ ID NO:5.

```
   1 CTTTGAAGAA CATCCTGAAG ATTATATCGG AGACAATATA
  41 TCAAGAATCT ATTTATTGAA TCATCTAGAA CAAAAGCCAG
  61 GAGCTCCCTA ATGGAAGCAC ATTAGTGTTT ATTTTGATGA
 121 AGAAATATAT AGATTTTTTA AAACAACCAC AAAGTAGATA
 161 GCTCAGTAAA AAATCAATTT TGGAAGATGT CACTGAACAA
 201 CTCTTCCAAT GTATTTCTGG ATTCAGTGCC CAGTAATACC
 241 AATCGCTTTC AAGTTAGTGT CATAAATGAG AACCATGAGA
 281 GCAGTGCAGC TGCAGATGAC AATACTGACC CACCACATTA
 321 TGAAGAAACC TCTTTTGGGG ATGAAGCTCA GAAAAGACTC
 361 AGAATCAGCT TTAGGCCTGG GAATCAGGAG TGCTATGACA
 401 ATTTCCTCCA AAGTGGAGAA ACTGCTAAAA CAGATGCCAG
 441 TTTTCACGCT TATGATTCTC ACACAAACAC ATACTATCTA
 481 CAAACTTTTG GCCACAACAC CATGGATGCC GTTCCCAAGA
 521 TAGAGTACTA TCGTAACACC GGCAGCATCA GTGGGCCCAA
 561 GGTCAACCGA CCCAGCCTGC TTGAGATTCA CGAGCAACTC
 601 GCAAAGAATG TGGCAGTCAC CCCAAGTTCA GCTGACAGAG
 641 TTGCTAACGG TGATGGGATA CCTGGAGATG AACAAGCTGA
 661 AAATAAGGAA GATGATCAAG CTGGTGTTGT GAAGTTTGGA
 721 TGGGTGAAAG GTGTGCTGGT AAGATGCATG CTGAACATCT
 761 GGGGAGTCAT GCTCTTCATT CGCCTCTCCT GGATTGTTGG
 801 AGAAGCTGGA ATTGGTCTTG GAGTTCTCAT AATTCTTCTT
 841 TCCACCATGG TAACTTCTAT TACTGGGTTG TCAACTTCTG
 881 CGATAGCAAC TAACGGGTTT GTTCGTGGAG GTGGGGCCTA
 921 CTATCTTATT TCCAGAAGTT TAGGGCCCGA GTTCGGTGGG
 961 TCAATAGGCC TGATCTTTGC TTTTGCTAAT GCAGTGGCTG
1001 TTGCTATGTA TGTGGTGGGA TTTGCTGAGA CTGTAGTAGA
1041 TCTTCTTAAG GAGAGTGATT CGATGATGGT GGATCCAACC
1081 AATGACATCC GGATTATAGG CTCCATCACA GTGGTGATTC
1121 TTCTAGGAAT TTCAGTAGCT GGAATGGAAT GGGAGGCAAA
1161 GGCCCAAGTC ATTCTTCTGG TCATTCTTCT AATTGCTATT
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1201 GCAAACTTCT TCATTGGAAC TGTCATTCCA TCCAACAATG
1241 AGAAAAAGTC CAGAGGTTTC TTTAATTACC AAGCATCAAT
1281 ATTTGCAGAA AACTTTGGGC CACGCTTCAC AAAGGGTGAA
1321 GGCTTCTTCT CTGTCTTTGC CATTTTTTTC CCAGCAGCTA
1361 CTGGGATTCT TGCTGGTGCC AATATCTCAG GAGATTTGGA
1401 GGCACTGAGG AAACAAGGAG CTTCACCTCT CCCTCAAGGA
1441 GCTCAGAGTC GAAGGAGGAG ACAGACTTCC CTTATATGAA
1481 TTAGAACAAG CAAGAGTAGA ATCAAGTGCA AAGGAAAGAG
1521 GAAGCAGAAA TTGCCTGTCC CCTCAAAAAG TAAAGGAAGA
1561 CTTTCAGAAG AGGGGACACT CAATCCAGGT TTTGAGGGAT
1601 GAACAGGAGT TTGCCGACAG GACAAAGAAG AGACGGACAT
1641 TTGAAACAGA AGGAATGGGA TGTAAGAAGG CACCAAGAAA
1681 GATGCTGCTA ATGAGAATTA TTTTATGTGC AGAGTAGTGT
1721 ATGTAATCCT TCATTAATAT ATTAATAAAC ATATTTATAA
1761 ATAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAA
```

The protein encoded by the NKCC2 nucleic acid with SEQ ID NO:5 has NCBI accession number AAH40138.1 (GI:25304083) and the following human amino acid sequence (SEQ ID NO: 6).

```
  1 MSLNNSSNVF LDSVPSNTNR FQVSVINENH ESSAAADDNT
 41 DPPHYEETSF GDEAQKRLRI SFRPGNQECY DNFLQSGETA
 81 KTDASFHAYD SHTNTYYLQT FGHNTMDAVP KIEYYRNTGS
121 ISGPKVNRPS LLEIHEQLAK NVAVTPSSAD RVANGDGIPG
161 DEQAENKEDD QAGVVKFGWV KGVLVRCMLN IWGVMLFIRL
201 SWIVGEAGIG LGVLIILLST MVTSITGLST SAIATNGFVR
241 GGGAYYLISR SLGPEFGGSI GLIFAFANAV AVAMYVVGFA
281 ETVVDLLKES DSMMVDPTND IRIIGSITVV ILLGISVAGM
321 EWEAKAQVIL LVILLIAIAN FFIGTVIPSN NEKKSRGFFN
361 YQASIFAENF GPRFTKGEGF FSVFAIFFPA ATGILAGANI
401 SGDLEALRKQ GASPLPQGAQ SRRRRQTSLI
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Even though initial analysis indicated that NKCC2 was not amongst the twelve genes that initially appeared to be more correlated with fibrosis (FIG. 2A), quantification of NKCC2 mRNA levels along with vimentin, E-cadherin and 18S rRNA provided the most accurate, parsimonious, diagnostic model of allograft fibrosis with 93.8% sensitivity and 84.1% specificity ($P<0.0001$).

Additional mRNAs such as HGF ($P<0.0001$), α-SMA ($P<0.0001$), fibronectin 1 ($P<0.0001$), perforin ($P=0.0002$), PAI1 ($P=0.0002$), TGFß1 ($P=0.0004$), TIMP1 ($P=0.0009$), granzyme B ($P=0.0009$), FSP1 ($P=0.006$), CD103 ($P=0.02$), and collagen 1A1 ($P=0.04$) were also associated with fibrosis.

Surprisingly, once vimentin mRNA levels were entered into the four-gene model that included analysis of levels of mRNA for vimentin, NKCC2, and E-cadherin, with 18S rRNA, none of the mRNA levels increased the accuracy of diagnosis of fibrosis. The four gene signature was robustly validated using an independent set of urine samples (the validation set) that were not used in the discovery of the four gene diagnosis model.

The parameter estimates for the four-gene model, including terms accounting for non-linear relationships between the mRNA levels and diagnosis are provided in FIG. 3. As shown, the propensity for development of kidney fibrosis is inversely proportional to NKCC2 expression. In other words, the propensity for development of kidney fibrosis is higher when NKCC2 expression is lower.

Any probe or primer that is specific for NKCC2 can be used in the methods and devices described herein. Examples are provided herein.

E-cadherin

Cadherins (named for "calcium-dependent adhesion") are a class of type-1 transmembrane proteins. They play important roles in cell adhesion, ensuring that cells within tissues are bound together. They are dependent on calcium (Ca2+) ions to function, hence their name. E-cadherin is found in epithelial tissue.

Nucleic acid and protein sequences for E-cadherin are available, for example, in the sequence database maintained by the National Center for Biotechnology Information (see website at www.ncbi.nlm.nih.gov/). One example of a human E-cadherin nucleic acid sequence is available as accession number XM_007840.5 (GI:15316186), provided below as SEQ ID NO:7.

```
  1 AGTGAATTTT GAAGATTGCA CCGGTCGACA AAGGACAGCC
 41 TATTTTTCCC TCGACACCCG ATTCAAAGTG GGCACAGATG
 81 GTGTGATTAC AGTCAAAAGG CCTCTACGGT TTCATAACCC
121 ACAGATCCAT TTCTTGGTCT ACGCCTGGGA CTCCACCTAC
161 AGAAAGTTTT CCACCAAAGT CACGCTGAAT ACAGTGGGGC
201 ACCACCACCG CCCCCCGCCC CATCAGGCCT CCGTTTCTGG
241 AATCCAAGCA GAATTGCTCA CATTTCCCAA CTCCTCTCCT
281 GGCCTCAGAA GACAGAAGAG AGACTGGGTT ATTCCTCCCA
321 TCAGCTGCCC AGAAAATGAA AAAGGCCCAT TTCCTAAAAA
361 CCTGGTTCAG ATCAAATCCA ACAAAGACAA AGAAGGCAAG
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 401 GTTTTCTACA GCATCACTGG CCAAGGAGCT GACACACCCC
 441 CTGTTGGTGT CTTTATTATT GAAAGAGAAA CAGGATGGCT
 481 GAAGGTGACA GAGCCTCTGG ATAGAGAACG CATTGCCACA
 521 TACACTCTCT TCTCTCACGC TGTGTCATCC AACGGGAATG
 561 CAGTTGAGGA TCCAATGGAG ATTTTGATCA CGGTAACCGA
 601 TCAGAATGAC AACAAGCCCG AATTCACCCA GGAGGTCTTT
 641 AAGGGGTCTG TCATGGAAGG TGCTCTTCCA GGAACCTCTG
 681 TGATGGAGGT CACAGCCACA GACGCGGACG ATGATGTGAA
 721 CACCTACAAT GCCGCCATCG CTTACACCAT CCTCAGCCAA
 761 GATCCTGAGC TCCCTGACAA AAATATGTTC ACCATTAACA
 801 GGAACACAGG AGTCATCAGT GTGGTCACCA CTGGGCTGGA
 841 CCGAGAGAGT TTCCCTACGT ATACCCTGGT GGTTCAAGCT
 881 GCTGACCTTC AAGGTGAGGG GTTAAGCACA ACAGCAACAG
 921 CTGTGATCAC AGTCACTGAC ACCAACGATA ATCCTCCGAT
 961 CTTCAATCCC ACCACGTACA AGGGTCAGGT GCCTGAGAAC
1001 GAGGCTAACG TCGTAATCAC CACACTGAAA GTGACTGATG
1041 CTGATGCCCC CAATACCCCA GCGTGGGAGG CTGTATACAC
1081 CATATTGAAT GATGATGGTG GACAATTTGT CGTCACCACA
1121 AATCCAGTGA ACAACGATGG CATTTTGAAA ACAGCAAAGG
1161 GCTTGGATTT TGAGGCCAAG CAGCAGTACA TTCTACACGT
1201 AGCAGTGACG AATGTGGTAC CTTTTGAGGT CTCTCTCACC
1241 ACCTCCACAG CCACCGTCAC CGTGGATGTG CTGGATGTGA
1281 ATGAAGCCCC CATCTTTGTG CCTCCTGAAA AGAGAGTGGA
1321 AGTGTCCGAG GACTTTGGCG TGGGCCAGGA AATCACATCC
1361 TACACTGCCC AGGAGCCAGA CACATTTATG GAACAGAAAA
1401 TAACATATCG GATTTGGAGA GACACTGCCA ACTGGCTGGA
1441 GATTAATCCG GACACTGGTG CCATTTCCAC TCGGGCTGAG
1481 CTGGACAGGG AGGATTTTGA GCACGTGAAG AACAGCACGT
1521 ACACAGCCCT AATCATAGCT ACAGACAATG GTTCTCCAGT
1561 TGCTACTGGA ACAGGGACAC TTCTGCTGAT CCTGTCTGAT
1601 GTGAATGACA ACGCCCCCAT ACCAGAACCT CGAACTATAT
1641 TCTTCTGTGA GAGGAATCCA AAGCCTCAGG TCATAAACAT
1681 CATTGATGCA GACCTTCCTC CCAATACATC TCCCTTCACA
1721 GCAGAACTAA CACACGGGGC GAGTGCCAAC TGGACCATTC
1761 AGTACAACGA CCCAACCCAA GAATCTATCA TTTTGAAGCC
1801 AAAGATGGCC TTAGAGGTGG GTGACTACAA AATCAATCTC
1841 AAGCTCATGG ATAACCAGAA TAAAGACCAA GTGACCACCT
1881 TAGAGGTCAG CGTGTGTGAC TGTGAAGGGG CCGCTGGCGT
1921 CTGTAGGAAG GCACAGCCTG TCGAAGCAGG ATTGCAAATT
1961 CCTGCCATTC TGGGGATTCT TGGAGGAATT CTTGCTTTGC
2001 TAATTCTGAT TCTGCTGCTC TTGCTGTTTC TTCGGAGGAG
2041 AGCGGTGGTC AAAGAGCCCT TACTGCCCCC AGAGGATGAC
2081 ACCCGGGACA ACGTTTATTA CTATGATGAA GAAGGAGGCG
2121 GAGAAGAGGA CCAGGACTTT GACTTGAGCC AGCTGCACAG
2161 GGGCCTGGAC GCTCGGCCTG AAGTGACTCG TAACGACGTT
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2201 GCACCAACCC TCATGAGTGT CCCCCGGTAT CTTCCCCGCC
2241 CTGCCAATCC CGATGAAATT GGAAATTTTA TTGATGAAAA
2281 TCTGAAAGCG GCTGATACTG ACCCCACAGC CCCGCCTTAT
2321 GATTCTCTGC TCGTGTTTGA CTATGAAGGA AGCGGTTCCG
2361 AAGCTGCTAG TCTGAGCTCC CTGAACTCCT CAGAGTCAGA
2401 CAAAGACCAG GACTATGACT ACTTGAACGA ATGGGGCAAT
2441 CGCTTCAAGA AGCTGGCTGA CATGTACGGA GGCGGCGAGG
2481 ACGACTAGGG GACTCGAGAG AGGCGGGCCC CAGACCCATG
2521 TGCTGGGAAA TGCAGAAATC ACGTTGCTGG TGGTTTT
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The protein encoded by the E-cadherin nucleic acid with SEQ ID NO:7 has the following human amino acid sequence (SEQ ID NO: 8).

```
  1 MEILITVTDQ NDNKPEFTQE VFKGSVMEGA LPGTSVMEVT
 41 ATDADDDVNT YNAAIAYTIL SQDPELPDKN MFTINRNTGV
 81 ISVVTTGLDR ESFPTYTLVV QAADLQGEGL STTATAVITV
121 TDTNDNPPIF NPTTYKGQVP ENEANVVITT LKVTDADAPN
161 TPAWEAVYTI LNDDGGQFVV TTNPVNNDGI LKTAKGLDFE
201 AKQQYILHVA VTNVVPFEVS LTTSTATVTV DVLDVNEAPI
241 FVPPEKRVEV SEDFGVGQEI TSYTAQEPDT FMEQKITYRI
281 WRDTANWLEI NPDTGAISTR AELDREDFEH VKNSTYTALI
321 IATDNGSPVA TGTGTLLLIL SDVNDNAPIP EPRTIFFCER
361 NPKPQVINII DADLPPNTSP FTAELTHGAS ANWTIQYNDP
401 TQESIILKPK MALEVGDYKI NLKLMDNQNK DQVTTLEVSV
441 CDCEGAAGVC RKAQPVEAGL QIPAILGILG GILALLILIL
481 LLLLFLRRRA VVKEPLLPPE DDTRDNVYYY DEEGGGEEDQ
521 DFDLSQLHRG LDARPEVTRN DVAPTLMSVP RYLPRPANPD
561 EIGNFIDENL KAADTDPTAP PYDSLLVFDY EGSGSEAASL
601 SSLNSSESDK DQDYDYLNEW GNRFKKLADM YGGGEDD
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Another example of a human E-cadherin nucleic acid sequence is available as accession number NM_004360.3 (GI:169790842), provided below as SEQ ID NO:9.

```
  1 AGTGGCGTCG GAACTGCAAA GCACCTGTGA GCTTGCGGAA
 41 GTCAGTTCAG ACTCCAGCCC GCTCCAGCCC GGCCCGACCC
 81 GACCGCACCC GGCGCCTGCC CTCGCTCGGC GTCCCCGGCC
121 AGCCATGGGC CCTTGGAGCC GCAGCCTCTC GGCGCTGCTG
161 CTGCTGCTGC AGGTCTCCTC TTGGCTCTGC CAGGAGCCGG
201 AGCCCTGCCA CCCTGGCTTT GACGCCGAGA GCTACACGTT
241 CACGGTGCCC CGGCGCCACC TGGAGAGAGG CCGCGTCCTG
281 GGCAGAGTGA ATTTTGAAGA TTGCACCGGT CGACAAAGGA
321 CAGCCTATTT TTCCCTCGAC ACCCGATTCA AAGTGGGCAC
361 AGATGGTGTG ATTACAGTCA AAAGGCCTCT ACGGTTTCAT
401 AACCCACAGA TCCATTTCTT GGTCTACGCC TGGGACTCCA
441 CCTACAGAAA GTTTTCCACC AAAGTCACGC TGAATACAGT
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 481 GGGGCACCAC CACCGCCCCC CGCCCCATCA GGCCTCCGTT
 521 TCTGGAATCC AAGCAGAATT GCTCACATTT CCCAACTCCT
 561 CTCCTGGCCT CAGAAGACAG AAGAGAGACT GGGTTATTCC
 601 TCCCATCAGC TGCCCAGAAA ATGAAAAAGG CCCATTTCCT
 641 AAAAACCTGG TTCAGATCAA ATCCAACAAA GACAAAGAAG
 681 GCAAGGTTTT CTACAGCATC ACTGGCCAAG GAGCTGACAC
 721 ACCCCCTGTT GGTGTCTTTA TTATTGAAAG AGAAACAGGA
 761 TGGCTGAAGG TGACAGAGCC TCTGGATAGA GAACGCATTG
 801 CCACATACAC TCTCTTCTCT CACGCTGTGT CATCCAACGG
 841 GAATGCAGTT GAGGATCCAA TGGAGATTTT GATCACGGTA
 881 ACCGATCAGA ATGACAACAA GCCCGAATTC ACCCAGGAGG
 921 TCTTTAAGGG GTCTGTCATG GAAGGTGCTC TTCCAGGAAC
 961 CTCTGTGATG GAGGTCACAG CCACAGACGC GGACGATGAT
1001 GTGAACACCT ACAATGCCGC CATCGCTTAC ACCATCCTCA
1041 GCCAAGATCC TGAGCTCCCT GACAAAAATA TGTTCACCAT
1081 TAACAGGAAC ACAGGAGTCA TCAGTGTGGT CACCACTGGG
1121 CTGGACCGAG AGAGTTTCCC TACGTATACC CTGGTGGTTC
1161 AAGCTGCTGA CCTTCAAGGT GAGGGGTTAA GCACAACAGC
1201 AACAGCTGTG ATCACAGTCA CTGACACCAA CGATAATCCT
1241 CCGATCTTCA ATCCCACCAC GTACAAGGGT CAGGTGCCTG
1281 AGAACGAGGC TAACGTCGTA ATCACCACAC TGAAAGTGAC
1321 TGATGCTGAT GCCCCCAATA CCCCAGCGTG GGAGGCTGTA
1361 TACACCATAT TGAATGATGA TGGTGGACAA TTTGTCGTCA
1401 CCACAAATCC AGTGAACAAC GATGGCATTT TGAAAACAGC
1441 AAAGGGCTTG GATTTTGAGG CCAAGCAGCA GTACATTCTA
1481 CACGTAGCAG TGACGAATGT GGTACCTTTT GAGGTCTCTC
1521 TCACCACCTC CACAGCCACC GTCACCGTGG ATGTGCTGGA
1561 TGTGAATGAA GCCCCCATCT TTGTGCCTCC TGAAAAGAGA
1601 GTGGAAGTGT CCGAGGACTT TGGCGTGGGC CAGGAAATCA
1641 CATCCTACAC TGCCCAGGAG CCAGACACAT TTATGGAACA
1681 GAAAATAACA TATCGGATTT GGAGAGACAC TGCCAACTGG
1721 CTGGAGATTA ATCCGGACAC TGGTGCCATT TCCACTCGGG
1761 CTGAGCTGGA CAGGGAGGAT TTTGAGCACG TGAAGAACAG
1801 CACGTACACA GCCCTAATCA TAGCTACAGA CAATGGTTCT
1841 CCAGTTGCTA CTGGAACAGG GACACTTCTG CTGATCCTGT
1881 CTGATGTGAA TGACAACGCC CCCATACCAG AACCTCGAAC
1921 TATATTCTTC TGTGAGAGGA ATCCAAAGCC TCAGGTCATA
1961 AACATCATTG ATGCAGACCT TCCTCCCAAT ACATCTCCCT
2001 TCACAGCAGA ACTAACACAC GGGGCGAGTG CCAACTGGAC
2041 CATTCAGTAC AACGACCCAA CCCAAGAATC TATCATTTTG
2081 AAGCCAAAGA TGGCCTTAGA GGTGGGTGAC TACAAAATCA
2121 ATCTCAAGCT CATGGATAAC CAGAATAAAG ACCAAGTGAC
2161 CACCTTAGAG GTCAGCGTGT GTGACTGTGA AGGGGCCGCT
2201 GGCGTCTGTA GGAAGGCACA GCCTGTCGAA GCAGGATTGC
2241 AAATTCCTGC CATTCTGGGG ATTCTTGGAG GAATTCTTGC
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     2281 TTTGCTAATT CTGATTCTGC TGCTCTTGCT GTTTCTTCGG
     2321 AGGAGAGCGG TGGTCAAAGA GCCCTTACTG CCCCCAGAGG
     2361 ATGACACCCG GGACAACGTT TATTACTATG ATGAAGAAGG
     2401 AGGCGGAGAA GAGGACCAGG ACTTTGACTT GAGCCAGCTG
5    2441 CACAGGGGCC TGGACGCTCG GCCTGAAGTG ACTCGTAACG
     2481 ACGTTGCACC AACCCTCATG AGTGTCCCCC GGTATCTTCC
     2521 CCGCCCTGCC AATCCCGATG AAATTGGAAA TTTTATTGAT
     2561 GAAAATCTGA AAGCGGCTGA TACTGACCCC ACAGCCCCGC
     2601 CTTATGATTC TCTGCTCGTG TTTGACTATG AAGGAAGCGG
10   2641 TTCCGAAGCT GCTAGTCTGA GCTCCCTGAA CTCCTCAGAG
     2681 TCAGACAAAG ACCAGGACTA TGACTACTTG AACGAATGGG
     2721 GCAATCGCTT CAAGAAGCTG GCTGACATGT ACGGAGGCGG
     2761 CGAGGACGAC TAGGGGACTC GAGAGAGGCG GGCCCCAGAC
     2801 CCATGTGCTG GGAAATGCAG AAATCACGTT GCTGGTGGTT
15   2841 TTTCAGCTCC CTTCCCTTGA GATGAGTTTC TGGGGAAAAA
     2881 AAAGAGACTG GTTAGTGATG CAGTTAGTAT AGCTTTATAC
     2921 TCTCTCCACT TTATAGCTCT AATAAGTTTG TGTTAGAAAA
     2961 GTTTCGACTT ATTTCTTAAA GCTTTTTTTT TTTTCCCATC
     3001 ACTCTTTACA TGGTGGTGAT GTCCAAAAGA TACCCAAATT
20   3041 TTAATATTCC AGAAGAACAA CTTTAGCATC AGAAGGTTCA
     3081 CCCAGCACCT TGCAGATTTT CTTAAGGAAT TTTGTCTCAC
     3121 TTTTAAAAAG AAGGGGAGAA GTCAGCTACT CTAGTTCTGT
     3161 TGTTTTGTGT ATATAATTTT TTAAAAAAAA TTTGTGTGCT
     3201 TCTGCTCATT ACTACACTGG TGTGTCCCTC TGCCTTTTTT
25   3241 TTTTTTTTAA GACAGGGTCT CATTCTATCG GCCAGGCTGG
     3281 AGTGCAGTGG TGCAATCACA GCTCACTGCA GCCTTGTCCT
     3321 CCCAGGCTCA AGCTATCCTT GCACCTCAGC CTCCCAAGTA
     3361 GCTGGGACCA CAGGCATGCA CCACTACGCA TGACTAATTT
     3401 TTTAAATATT TGAGACGGGG TCTCCCTGTG TTACCCAGGC
30   3441 TGGTCTCAAA CTCCTGGGCT CAAGTGATCC TCCCATCTTG
     3481 GCCTCCCAGA GTATTGGGAT TACAGACATG AGCCACTGCA
     3521 CCTGCCCAGC TCCCCAACTC CCTGCCATTT TTTAAGAGAC
     3561 AGTTTCGCTC CATCGCCCAG GCCTGGGATG CAGTGATGTG
     3601 ATCATAGCTC ACTGTAACCT CAAACTCTGG GGCTCAAGCA
35   3641 GTTCTCCCAC CAGCCTCCTT TTTATTTTTT TGTACAGATG
     3681 GGGTCTTGCT ATGTTGCCCA AGCTGGTCTT AAACTCCTGG
     3721 CCTCAAGCAA TCCTTCTGCC TTGGCCCCCC AAAGTGCTGG
     3861 GATTGTGGGC ATGAGCTGCT GTGCCCAGCC TCCATGTTTT
     3801 AATATCAACT CTCACTCCTG AATTCAGTTG CTTTGCCCAA
40   3841 GATAGGAGTT CTCTGATGCA GAAATTATTG GGCTCTTTTA
     3881 GGGTAAGAAG TTTGTGTCTT TGTCTGGCCA CATCTTGACT
     3921 AGGTATTGTC TACTCTGAAG ACCTTTAATG GCTTCCCTCT
     3961 TTCATCTCCT GAGTATGTAA CTTGCAATGG GCAGCTATCC
     4001 AGTGACTTGT TCTGAGTAAG TGTGTTCATT AATGTTTATT
45   4041 TAGCTCTGAA GCAAGAGTGA TATACTCCAG GACTTAGAAT
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4081 AGTGCCTAAA GTGCTGCAGC CAAAGACAGA GCGGAACTAT
4121 GAAAAGTGGG CTTGGAGATG GCAGGAGAGC TTGTCATTGA
4161 GCCTGGCAAT TTAGCAAACT GATGCTGAGG ATGATTGAGG
4201 TGGGTCTACC TCATCTCTGA AAATTCTGGA AGGAATGGAG
4241 GAGTCTCAAC ATGTGTTTCT GACACAAGAT CCGTGGTTTG
4281 TACTCAAAGC CCAGAATCCC CAAGTGCCTG CTTTTGATGA
4321 TGTCTACAGA AAATGCTGGC TGAGCTGAAC ACATTTGCCC
4361 AATTCCAGGT GTGCACAGAA AACCGAGAAT ATTCAAAATT
4401 CCAAATTTTT TTCTTAGGAG CAAGAAGAAA ATGTGGCCCT
4441 AAAGGGGGTT AGTTGAGGGG TAGGGGGTAG TGAGGATCTT
4481 GATTTGGATC TCTTTTTATT TAAATGTGAA TTTCAACTTT
4521 TGACAATCAA AGAAAAGACT TTTGTTGAAA TAGCTTTACT
4561 GTTTCTCAAG TGTTTTGGAG AAAAAAATCA ACCCTGCAAT
4601 CACTTTTTGG AATTGTCTTG ATTTTTCGGC AGTTCAAGCT
4641 ATATCGAATA TAGTTCTGTG TAGAGAATGT CACTGTAGTT
4681 TTGAGTGTAT ACATGTGTGG GTGCTGATAA TTGTGTATTT
4721 TCTTTGGGGG TGGAAAAGGA AAACAATTCA AGCTGAGAAA
4761 AGTATTCTCA AAGATGCATT TTTATAAATT TTATTAAACA
4801 ATTTTGTTAA ACCAT
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The protein encoded by the E-cadherin nucleic acid with SEQ ID NO:9 has a human amino acid sequence with NCBI accession number NP_004351.1 (GI:4757960), which is provided below as SEQ ID NO: 10.

```
  1 MGPWSRSLSA LLLLLQVSSW LCQEPEPCHP GFDAESYTFT
 41 VPRRHLERGR VLGRVNFEDC TGRQRTAYFS LDTRFKVGTD
 61 GVITVKRPLR FHNPQIHFLV YAWDSTYRKF STKVTLNTVG
121 HHHRPPPHQA SVSGIQAELL TFPNSSPGLR RQKRDWVIPP
161 ISCPENEKGP FPKNLVQIKS NKDKEGKVFY SITGQGADTP
201 PVGVFIIERE TGWLKVTEPL DRERIATYTL FSHAVSSNGN
241 AVEDPMEILI TVTDQNDNKP EFTQEVFKGS VMEGALPGTS
281 VMEVTATDAD DDVNTYNAAI AYTILSQDPE LPDKNMFTIN
321 RNTGVISVVT TGLDRESFPT YTLVVQAADL QGEGLSTTAT
361 AVITVTDTND NPPIFNPTTY KGQVPENEAN VVITTLKVTD
401 ADAPNTPAWE AVYTILNDDG GQFVVTTNPV NNDGILKTAK
441 GLDFEAKQQY ILHVAVTNVV PFEVSLTTST ATVTVDVLDV
481 NEAPIFVPPE KRVEVSEDFG VGQEITSYTA QEPDTFMEQK
521 ITYRIWRDTA NWLEINPDTG AISTRAELDR EDFEHVKNST
561 YTALIIATDN GSPVATGTGT LLLILSDVND NAPIPEPRTI
601 FFCERNPKPQ VINIIDADLP PNTSPFTAEL THGASANWTI
641 QYNDPTQESI ILKPKMALEV GDYKINLKLM DNQNKDQVTT
681 LEVSVCDCEG AAGVCRKAQP VEAGLQIPAI LGILGGILAL
721 LILILLLLLF LRRRAVVKEP LLPPEDDTRD NVYYYDEEGG
761 GEEDQDFDLS QLHRGLDARP EVTRNDVAPT LMSVPRYLPR
801 PANPDEIGNF IDENLKAADT DPTAPPYDSL LVFDYEGSGS
841 EAASLSSLNS SESDKDQDYD YLNEWGNRFK KLADMYGGGE
881 DD
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Even though initial analysis indicated that E-cadherin was not amongst the twelve genes that initially appeared to be more correlated with fibrosis (FIG. 2A), quantification of E-cadherin mRNA levels along with vimentin, NKCC2 and 18S rRNA provided the most accurate, parsimonious, diagnostic model of allograft fibrosis with 93.8% sensitivity and 84.1% specificity ($P<0.0001$).

It is surprising that despite initial correlation of fibrosis with HGF ($P<0.0001$), α-SMA ($P<0.0001$), fibronectin 1 ($P<0.0001$), perforin ($P=0.0002$), PAI1 ($P=0.0002$), TGFß1 ($P=0.0004$), TIMP1 ($P=0.0009$), granzyme B ($P=0.0009$), FSP1 ($P=0.006$), CD103 ($P=0.02$), and collagen 1A1 ($P=0.04$), a four-gene model that included analysis of levels of mRNA for vimentin, NKCC2, E-cadherin and 18S rRNA was more accurate and diagnostic of kidney fibrosis. In the independent validation set, this four-gene model predicted the presence of allograft fibrosis with 77.3% sensitivity and 87.5% specificity ($P<0.0001$).

The parameter estimates for the four-gene model including terms accounting for the relationships, including non-linear relationships, between the mRNAs and diagnosis are provided in FIG. 3. As shown, the propensity for development of kidney fibrosis is proportional to E-cadherin expression. In other words, the propensity for development of kidney fibrosis is higher when E-cadherin expression increases.

Any probe or primer that is specific for E-cadherin can be used in the methods and devices described herein. Examples are provided herein.

18S rRNA

Expression levels of a housekeeping gene can be measured and used to normalize the quantities of the other mRNAs measured. The 18S ribosomal RNA (abbreviated 18S rRNA) is one convenient gene whose expression can be employed for such normalization. The 18S rRNA is a part of the ribosomal RNA. The S in 18S represents Svedberg units. 18S rRNA is a component of the small eukaryotic ribosomal subunit (40S). 18S rRNA is the structural RNA for the small component of eukaryotic cytoplasmic ribosomes, and thus one of the basic components of all eukaryotic cells.

Nucleic acid sequences for rRNA are available, for example, in the sequence database maintained by the National Center for Biotechnology Information (see website at www.ncbi.nlm.nih.gov/). One example of a human rRNA nucleic acid sequence is available as accession number K03432.1 (GI:337377), provided below as SEQ ID NO:11.

```
   1 CGCTGCTCCT CCCGTCGCCG TCCGGGCCCG TCCGTCCGTC
  41 CGTCCGTCGT CCTCCTCGCT NNNNCGGGGC GCCGGGCCCG
  61 TCCTCACNGG CCCCCGNNNN NGTCCNGGCC CGTCGGGGCC
 121 TCGCCGCGCT CTACCTTACC TACCTGGTTG ATCCTGCCAG
 161 TAGCATATGC TTGTCTCAAA GATTAAGCCA TGCATGTCTA
 201 AGTACGCACG GCCGGTACAG TGAAACTGCG AATGGCTCAT
 241 TAAATCAGTT ATGGTTCCTT TGGTCGCTCG CTCCTCTCCT
 281 ACTTGGATAA CTGTGGTAAT TCTAGAGCTA ATACATGCCG
 301 ACGGGCGCTG ACCCCCTTCG CGGGGGGGAT GCGTGCATTT
 361 ATCAGATCAA AACCAACCCG GTCAGCCCCT CTCCGGCCCC
 401 GGCCGGGGGG CGGGCGCCGG CGGCTTTGGT GACTCTAGAT
 441 AACCTCGGGC CGATCGCACG CCCCCCGTGG CGGCGACGAC
 481 CCATTCGAAC GTCTGCCCTA TCAACTTTCG ATGGTAGTCG
 521 CCGTGCCTAC CATGGTGACC ACGGGTGACG GGGAATCAGG
 561 GTTCGATTCC GGAGAGGGAG CCTGAGAAAC GGCTACCACA
 601 TCCAAGGAAG GCAGCAGGCG CGCAAATTAC CCACTCCCGA
 641 CCCGGGGAGG TAGTGACGAA AAATAACAAT ACAGGACTCT
 681 TTCGAGGCCC TGTAATTGGA ATGAGTCCAC TTTAAATCCT
 721 TTAACGAGGA TCCATTGGAG GGCAAGTCTG GTGCCAGCAG
 761 CCGCGGTAAT TCCAGCTCCA ATAGCGTATA TTAAAGTTGC
 801 TGCAGTTAAA AAGCTCGTAG TTGGATCTTG GGAGCGGGCG
 841 GGCGGTCCGC CGCGAGGCGA GCCACCGCCC GTCCCCGCCC
 881 CTTGCCTCTC GGCGCCCCCT CGATGCTCTT AGCTGAGTGT
 921 CCCGCGGGGC CCGAAGCGTT TACTTTGAAA AAATTAGAGT
 961 GTTCAAAGCA GGCCCGAGCC GCCTGGATAC CGCAGCTAGG
1001 AATAATGGAA TAGGACCGCG GTTCTATTTT GTTGGTTTTC
1041 GGAACTGAGG CCATGATTAA GAGGGACGGC CGGGGGCATT
1081 CGTATTGCGC CGCTAGAGGT GAAATTCCTT GGACCGGCGC
1121 AAGACGGACC AGAGCGAAAG CATTTGCCAA GAATGTTTTC
1161 ATTAATCAAG AACGAAAGTC GGAGGTTCGA AGACGATCAG
1201 ATACCGTCGT AGTTCCGACC ATAAACGATG CCGACCGGCG
1241 ATGCGGCGGC GTTATTCCCA TGACCCGCCG GGCAGCTTCC
1281 GGGAAACCAA AGTCTTTGGG TTCCGGGGGG AGTATGGTTG
1321 CAAAGCTGAA ACTTAAAGGA ATTGACGGAA GGGCACCACC
1361 AGGAGTGGAG CCTGCGGCTT AATTTGACTC AACACGGGAA
1401 ACCTCACCCG GCCCGGACAC GGACAGGATT GACAGATTGA
1441 TAGCTCTTTC TCGATTCCGT GGGTGGTGGT GCATGGCCGT
1481 TCTTAGTTGG TGGAGCGATT TGTCTGGTTA ATTCCGATAA
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1521 CGAACGAGAC TCTGGCATGC TAACTAGTTA CGCGACCCCC
1561 GAGCGGTCGG CGTCCCCCAA CTTCTTAGAG GGACAAGTGG
1601 CGTTCAGCCA CCCGAGATTG AGCAATAACA GGTCTGTGAT
1641 GCCCTTAGAT GTCCGGGGCT GCACGCGCGC TACACTGACT
1681 GGCTCAGCGT GTGCCTACCC TACGCCGGCA GGCGCGGGTA
1721 ACCCGTTGAA CCCCATTCGT GATGGGGATC GGGGATTGCA
1761 ATTATTCCCC ATGAACGAGG AATTCCCAGT AAGTGCGGGT
1801 CATAAGCTTG CGTTGATTAA GTCCCTGCCC TTTGTACACA
1841 CCGCCCGTCG CTACTACCGA TTGGATGGTT TAGTGAGGCC
1881 CTCGGATCGG CCCCGCCGGG GTCGGCCCAC GGCCCTGGCG
1921 GAGCGCTGAG AAGACGGTCG AACTTGACTA TCTAGAGGAA
1961 GTAAAAGTCG TAACAAGGTT TCCGTAGGTG AACCTGCGGA
2001 AGGATCATTA ACGGAGCCCG GACGGCGGCC CGCGGCGGCG
2041 CCGCGCCGCG CTTCCCTCCG CACACCCACC CCCCCACCGC
2081 GACGGCGCGT GCGGGCGGGG CCGTGCCCGT TCGTTCGCTC
2121 GCTCGTTCGT TCGCCGCCCG GCCCGGCCGC GAGAGCCGAG
2161 AACTCGGGAG GGAGACGGGG GAGAGAGAGA GAGAGAGAGA
2201 GAGAGAGAGA GAGAGAGAGA GAAAGAAGGG CGTGT
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The 18S rRNA expression can be used as a normalizing factor for amount and quality of total RNA isolated from the urinary cells. For example, the quantities of vimentin, NKCC2, and E-cadherin mRNAs can be divided by the quantity of 18S rRNA to remove sample-to-sample variability caused by factors other than those relating to expression levels (e.g., variation in cell numbers in the test sample). Surprisingly, the levels of 18S rRNA also contribute to the accuracy of diagnosis.

Assays for Detecting and Quantifying RNA

Any technique known to one of skill in the art for detecting and measuring RNA expression levels can be used in accordance with the methods described herein. Non-limiting examples of such techniques include reverse transcription, polymerase chain reaction pre-amplification, real-time quantitative polymerase chain reaction, microarray analysis, Northern blotting, nuclease protection assays, RNA fingerprinting, polymerase chain reaction, ligase chain reaction, Qbeta replicase, isothermal amplification method, strand displacement amplification, transcription based amplification systems, quantitative nucleic acid amplification assays (e.g., polymerase chain reaction assays), combined reverse transcription/nucleic acid amplification, nuclease protection (SI nuclease

or RNAse protection assays), Serial Analysis Gene Expression (SAGE), next generation sequencing, gene expression microarray, as well as other methods.

Nucleic acids the can hybridize RNAs of vimentin, NKCC2, E-cadherin and one or more housekeeping genes (e.g., 18S rRNA) can be used as probes or primers for quantifying these RNAs. For example, the probes and/or primers can selectively hybridize to a nucleic acid encoding any of the polypeptides with SEQ ID NO:2, 4, 6, 8 and/or 10 sequence. When 18S rRNA levels are quantified the probes and/or primers can selectively hybridize to a nucleic acid that has at least 90% or at least 95% sequence identity or sequence complementarity to any of SEQ ID NO:11. Similarly, probes and/or primers for vimentin, NKCC2, and E-cadherin can have at least 90% or at least 95% sequence identity or sequence complementarity to any of SEQ ID NO:1, 3, 5, 7, and/or 9. Examples of primers and/or probes are provided in Table 2. For example, primers or probes for vimentin can include any of SEQ ID NO:12-14, or a combination thereof. Examples of NKCC2 probes or primers can include any of SEQ ID NO: 69-71, or a combination thereof. Examples of E-cadherin probes or primers can include any of SEQ ID NO: 75-77, or a combination thereof. Examples of 18S rRNA probes or primers can include any of SEQ ID NO: 78-81, or a combination thereof.

A "probe or primer" as used herein refers to one or more nucleic acids that may be used to detect one or more RNA type (e.g. vimentin, NKCC2, E-cadherin and a housekeeping RNA such as 18S rRNA). Detection may be, for example, through amplification as in PCR, RT-PCR, quantitative PCR or through hybridization, or through selective destruction and protection, as in assays based on the selective enzymatic degradation of single or double stranded nucleic acids, or by detecting mRNA. Probes and/or primers can be labeled with one or more fluorescent, radioactive, quenchers, or other detectable moieties (including enzymes). Probes may be any size so long as the probe is sufficiently large to selectively detect the desired gene or be amplified.

Primers can be polynucleotides or oligonucleotides capable of being extended in a primer extension reaction at their 3' end. In order for an oligonucleotide to serve as a primer, it typically is sufficiently complementary in sequence to be capable of forming a double-stranded structure with the template, or target, under the conditions employed. Establishing such conditions typically involves selection of solvent and salt concentration, incubation temperatures,

incubation times, assay reagents and stabilization factors known to those in the art. The term primer or primer oligonucleotide refers to an oligonucleotide as defined herein, which is capable of acting as a point of initiation of synthesis when employed under conditions in which synthesis of a primer extension product that is complementary to a nucleic acid strand is induced, as, for example, in a cDNA or DNA replication reaction such as a PCR reaction. Like non-primer oligonucleotides, primer oligonucleotides can be labeled according to any technique known in the art, such as with radioactive atoms, fluorescent labels, enzymatic labels, proteins, haptens, antibodies, sequence tags, mass label or the like. Such labels may be employed by associating them, for example, with the 5' terminus of a primer by a plurality of techniques known in the art. Such labels may also act as capture moieties. A probe or primer may be in solution, as would be typical for multiplex PCR, or a probe or primer may be adhered to a solid surface, as in an array or microarray. Compounds such as peptide nucleic acids (PNAs) can be used instead of nucleic acids to hybridize to the RNAs. In addition, probes may contain rare or unnatural nucleic acids such as inosine.

Such a RNA or DNA (or fragments therefore) may serve as a probe, for example, when it is at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or at least 16 consecutive nucleotides in length. In some embodiments, the probe is about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21 or about 22 consecutive nucleotides in length. In further embodiments, the probe may be at least 20, at least 30, at least 50, or at least 70 consecutive nucleotides in length. The primers and/or probes can be less than about 80, less than about 70, less than about 60, less than about 50, less than about 45, less than about 40, less than about 39, less than about 38, less than about 37, less than about 36, less than about 35, less than about 34, less than about 33, less than about 32, less than about 31, or less than about 30 consecutive nucleotides in length.

During quantification probes and primers can be hybridized to vimentin, NKCC2, E-cadherin and housekeeping (e.g. 18S rRNA) RNAs. Hybridization reactions can be performed under conditions of different "stringency". The stringency of a hybridization reaction includes the difficulty with which any two nucleic acid molecules will hybridize to one another. Under stringent conditions, nucleic acid molecules at least 60%, 65%, 70%, 75% identical (or

complementary) to each other remain hybridized to each other, whereas molecules with low percent identity do not remain hybridized. As the hybridization conditions become more stringent, the percent sequence identity or percent sequence complementarity between nucleic acid hybrids increases.
5 Under highly stringent conditions, nucleic acid molecules at least 90%, 92%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical (or complementary) to each other remain hybridized to each other, whereas molecules with low percent identity cannot remain hybridized.

A preferred, non-limiting example of stringent hybridization conditions
10 are hybridization in 6 X sodium chloride/sodium citrate (SSC) at about 45ºC., followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50ºC. A non-limiting example of highly stringent hybridization conditions is hybridization in 6 X sodium chloride/sodium citrate (SSC) at about 45ºC., followed by one or more washes in 0.2 X SSC, 0.1% SDS at 60ºC., or preferably at 65 ºC.

15 When hybridization occurs in an antiparallel configuration between two single-stranded polynucleotides, the reaction is called "annealing" and those polynucleotides are described as "complementary". A double-stranded polynucleotide can be "complementary" or "homologous" to another polynucleotide, if hybridization can occur between one of the strands of the first
20 polynucleotide and the second. "Complementarity" or "homology" (the degree that one polynucleotide is complementary with another) is quantifiable in terms of the proportion of bases in opposing strands that are expected to hydrogen bond with each other, according to generally accepted base-pairing rules.

The probe can be labeled by any of the many different methods known to
25 those skilled in this art. The labels most commonly employed for these studies are radioactive elements, enzymes, chemicals that fluoresce when exposed to ultraviolet light, and others. A number of fluorescent materials are known and can be utilized as labels. These include, but are not limited to, fluorescein, rhodamine, auramine, Texas Red, AMCA blue and Lucifer Yellow. The
30 radioactive label can be detected by any of the currently available counting procedures. Non-limiting examples of isotopes include ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Ci, ^{51}Cr, ^{57}Co, ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re.

Enzyme labels are likewise useful, and can be detected by any of the presently utilized colorimetric, spectrophotometric, fluorospectrophotometric,

amperometric or gasometric techniques. The enzyme is conjugated to the selected particle by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Any enzymes known to one of skill in the art can be utilized. Examples of such enzymes include, but are not limited to, 5 peroxidase, beta-D-galactosidase, urease, glucose oxidase plus peroxidase and alkaline phosphatase. U.S. Pat. Nos. 3,654,090, 3,850,752, and 4,016,043 are referred to by way of example for their disclosure of alternate labeling material and methods.

Quantification of RNA levels is typically performed in solution. As 10 described herein such quantification of a plurality of RNAs is informative for identifying whether a sample is diagnostic of fibrosis, determining whether a sample exhibits progression of a fibrotic disease or condition, and, whether a sample is diagnostic of the severity of a fibrotic condition (i.e., are prognosis-informative for a particular patient subset).

15 Quantitative reverse transcriptase PCR (qRT-PCR) can also be used to determine the expression profiles of RNA genes (see, e.g., U.S. Patent Application Publication No. 2005/0048542A1). The first step in gene expression profiling by RT-PCR is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in a PCR reaction. The two 20 most commonly used reverse transcriptases are avilo myeloblastosis virus reverse transcriptase (AMV-RT) and Moloney murine leukemia virus reverse transcriptase (MLV-RT). The reverse transcription step is typically primed using specific primers, random hexamers, or oligo-dT primers, depending on the circumstances and the goal of expression profiling. For example, extracted RNA 25 can be reverse-transcribed using a GeneAmp RNA PCR kit (Perkin Elmer, Calif., USA), following the manufacturer's instructions. The derived cDNA can then be used as a template in the subsequent PCR reaction.

Although the PCR step can use a variety of thermostable DNA-dependent DNA polymerases, commonly employed polymerases include the Taq 30 DNA polymerase, which has a 5′-3′ nuclease activity but lacks a 3′-5′ proofreading endonuclease activity. TaqMan® PCR typically utilizes the 5′-nuclease activity of Taq or Tth polymerase to hydrolyze a hybridization probe bound to its target amplicon, but any enzyme with similar or equivalent 5′ nuclease activity can be used. Two oligonucleotide primers are used to generate

an amplicon typical of a PCR reaction. A third oligonucleotide, or probe, is designed to detect nucleotide sequence located between the two PCR primers. The probe is non-extendible by Taq DNA polymerase enzyme, and is labeled with a reporter fluorescent dye and a quencher fluorescent dye. Any laser-induced emission from the reporter dye is quenched by the quenching dye when the two dyes are located close together as they are on the probe. During the amplification reaction, the Taq DNA polymerase enzyme cleaves the probe in a template-dependent manner. The resultant probe fragments disassociate in solution, and signal from the released reporter dye is free from the quenching effect of the second fluorophore. One molecule of reporter dye is liberated for each new molecule synthesized, and detection of the unquenched reporter dye provides the basis for quantitative interpretation of the data.

TaqMan® RT-PCR can be performed using commercially available equipment, such as, for example, ABI PRISM 7700™. Sequence Detection System™ (Perkin-Elmer-Applied Biosystems, Foster City, Calif., USA), or Lightcycler (Roche Molecular Biochemicals, Mannheim, Germany). In one embodiment, the 5′ nuclease procedure is run on a real-time quantitative PCR device such as the ABI PRISM 7700™ Sequence Detection System™. The system consists of a thermocycler, laser, charge-coupled device (CCD), camera and computer. The system includes software for running the instrument and for analyzing the data.

In some embodiments, the quantitative RT-PCR assay data are presented as Ct values, also referred to as ΔCt thresholds. The ΔCt (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross a detectable threshold. The ΔCt is a measure of when the amount of RNA expressed exceeds background levels. Ct threshold levels are inversely proportional to the amount of target nucleic acid in the sample (i.e., the lower the Ct threshold the greater the amount of target nucleic acid in the sample). Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The point when the fluorescent signal is first recorded as statistically significant is the threshold cycle (ΔCt).

To minimize errors and the effect of sample-to-sample variation, RT-PCR is often performed using an internal standard. The ideal internal standard is

expressed at a constant level among different tissues, and is unaffected by the experimental treatment. RNAs most frequently used to normalize patterns of gene expression are mRNAs for the housekeeping genes glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and β-actin.

5 A more recent variation of the RT-PCR technique is the real time quantitative PCR, which measures PCR product accumulation through a dual-labeled fluorogenic probe (i.e., TaqMan® probe). Real time PCR is compatible both with quantitative competitive PCR, where internal competitor for each target sequence is used for normalization, and with quantitative comparative 10 PCR using a normalization gene contained within the sample, or a housekeeping gene for RT-PCR. For further details see, e.g. Held et al., Genome Research 6:986-994 (1996).

Polynucleotide microarrays generally have probes bound to a solid surface. Microarrays can be used to simultaneously measure whether or not any 15 of several RNAs are expressed. A standard Northern blot assay can be used to ascertain an RNA size, and the relative amounts of RNA in a sample, in accordance with conventional Northern hybridization techniques known to those persons of ordinary skill in the art. In Northern blots, RNA samples are first separated by size via electrophoresis in an agarose gel under denaturing 20 conditions. The RNA is then transferred to a membrane, crosslinked and hybridized with a labeled probe. Nonisotopic or high specific activity radiolabeled probes can be used including random-primed, nick-translated, or PCR-generated DNA probes, in vitro transcribed RNA probes, and oligonucleotides. Additionally, sequences with only partial homology (e.g., a 25 RNA from a different species or genomic DNA fragments that might contain an exon) may be used as probes. The labeled probe can be a labeled cDNA; a full-length, single stranded labeled RNA or DNA, or a labeled fragment of that RNA or DNA sequence.

Nuclease protection assays such as ribonuclease protection assays and S1 30 nuclease assays can be used to detect and quantify specific RNAs. In nuclease protection assays, an antisense probe (labeled with, e.g., radiolabeled or nonisotopic) hybridizes in solution to an RNA sample. Following hybridization, single-stranded, unhybridized probe and RNA are degraded by nucleases. An acrylamide gel is used to separate the remaining protected fragments. Typically,

solution hybridization is more efficient than membrane-based hybridization, and it can accommodate up to 100 μg of sample RNA, compared with the 20-30 μg maximum of blot hybridizations.

A ribonuclease protection assay employs RNA probes. Oligonucleotides and other single-stranded DNA probes can only be used in assays containing S1 nuclease. The single-stranded, antisense probe must typically be completely homologous to target RNA to prevent cleavage of the probe:target hybrid by nuclease.

Serial Analysis Gene Expression (SAGE), which is described in e.g., Velculescu et al., 1995, *Science* 270:484-7; Carulli, et al., 1998, *Journal of Cellular Biochemistry Supplements* 30/31:286-96, can also be used to determine RNA abundances in a cell sample.

Transcript levels can be calculated by a standard curve method, and mRNA copy numbers can be normalized against 18S rRNA copy numbers, by dividing the number of mRNA copies by the number of 18S rRNA copies. For example, the number of mRNA copies in 1 μg of RNA can be divided by the number of 18S rRNA copies in 1 femtogram (fg) of RNA.

As described herein, the distribution of each mRNA, as well as the 18S rRNA, exhibited considerable positive skewness, which can be substantially reduced by use of a log transformation. For example, the number of mRNA copies normalized against rRNA can be converted to the $\log_{10}$ values. These $\log_{10}$ values can be used in the 4-gene model to predict the propensity of a subject to develop kidney fibrosis, to predict the severity of a kidney fibrosis disease, and/or to evaluate the progression of a kidney fibrosis disease.

The process for converting into vimentin, NKCC2 and E-cadherin mRNA quantities and the 18S rRNA quantity into a composite score for the diagnosis of fibrosis involves, dividing the 18S rRNA quantity by 10^5 (i.e., 100,000). The composite score can be calculated as follows.

$$\begin{aligned}\text{Composite Score} = \; & 36.10283 + [-15.84215*\log_{10}(18s)] \\ & + 1.56907*\log_{10}(18s)*\log_{10}(18s) \\ & + 5.11698*\max[0, \log_{10}(\text{Vimentin}) - 5.6] \\ & + [-1.44145* \log_{10}(\text{NKCC2})] \\ & + 3.31357*\min[3.1, \log_{10}(\text{E-cadherin})]\end{aligned}$$

where,

$$\begin{aligned}\max[0, \log_{10}(\text{Vimentin}) - 5.6] &= 0 \text{ whenever } \log_{10}(\text{Vimentin}) \text{ is} \leq 5.6 \text{ and} \\ &= \log_{10}(\text{Vimentin}) - 5.6 \text{ whenever } \log_{10}(\text{Vimentin}) \text{ is} > 5.6;\end{aligned}$$

$\min[3.1, \log_{10}(\text{E-cadherin})] = \log_{10}(\text{E-cadherin})$ whenever $\log_{10}(\text{E-cadherin}) < 3.1$
and $= 3.1$ whenever $\log_{10}(\text{E-cadherin}) \geq 3.1$;

and * signifies multiplication.

5

The $\log_{10}$ variables are defined as follows:

$\log_{10}$(18s RNA) is $\log_{10}$ of 18S RNA/100,000 quantity in the test sample;

$\log_{10}$(Vimentin) is $\log_{10}$ of normalized vimentin mRNA quantity in the test sample;

10 $\log_{10}$(NKCC2)] is $\log_{10}$ of normalized NKCC2 mRNA quantity in the test sample; and

$\log_{10}$(E-cadherin) is $\log_{10}$ of normalized E-cadherin mRNA quantity in the test sample.

In calculating the composite score, the vimentin mRNA quantity, the 15 NKCC2 mRNA quantity, and the E-cadherin mRNA quantity can be divided by the 18S RNA quantity multiplied by 100,000 before generating the $\log_{10}$(Vimentin), the $\log_{10}$(NKCC2), and the $\log_{10}$(E-cadherin) values, respectively. This generates normalized values of these mRNA quantities.

Normalized Vimentin mRNA quantity: $\dfrac{\text{measured vimentin mRNA quantity}}{\text{Measured 18S rRNA quantity / 100,000}}$

20

Normalized NKCC2 mRNA quantity: $\dfrac{\text{measured NKCC2 mRNA quantity}}{\text{Measured 18S rRNA quantity / 100,000}}$

25 Normalized E-cadherin mRNA quantity: $\dfrac{\text{measured E-cadherin mRNA quantity}}{\text{Measured 18S rRNA quantity / 100,000}}$

The composite score varies from about 1 to 8, where a normal (healthy) composite score is about 3.5. A test sample with a composite score of 4.7 or 30 more indicates that a subject has fibrosis. For example, a test sample with a composite score from about 4.7 to about 6.5 indicates a subject has mild to moderate fibrosis. A test sample with a composite score from about 6.5 or more indicates a subject has moderate to severe fibrosis.

Therefore, kidney fibrosis can be diagnosed using the methods described 35 herein.

Kidney Fibrosis

Human kidney disease can evolve from various origins including kidney transplantation, glomerular nephritis, nephritis associated with systemic lupus, cancer, physical obstructions, toxins, metabolic disease and immunological diseases, all of which may culminate in kidney fibrosis. Different types of insults can therefore converge on a single genetic program resulting in two hallmarks of fibrosis: the proliferation of fibroblasts and overproduction by them of various protein components of connective tissue. In addition, thickening of the basal membrane in the glomeruli accompanies interstitial fibrosis and culminates in glomerulosclerosis.

The severity of kidney fibrosis disease can be described by the grade of disease. Fibrosis grade I is assigned when less than about 25% of the kidney cortical area is fibrotic (mild fibrosis). Fibrosis grade II is assigned when about 26-50% of the kidney cortical area is fibrotic (moderate fibrosis). Fibrosis grade III is assigned when greater than about 50% of the kidney cortical area is fibrotic (severe fibrosis). Those with substantially no evidence of fibrosis have a normal biopsy and exhibit substantially no fibrosis.

Fibrotic diseases are generally characterized by the excess deposition of a fibrous material within the extracellular matrix, which contributes to abnormal changes in tissue architecture and interferes with normal organ function. Tissues damaged by trauma respond by the initiation of a wound-healing program. Fibrosis, a type of disorder characterized by excessive scarring, occurs when the normal self-limiting process of wound healing response is disturbed, and causes excessive production and deposition of collagen. As a result, normal organ tissue is replaced with scar tissue, which eventually leads to the functional failure of the organ.

Treatment

When kidney fibrosis is detected in a test sample, the subject from which the sample was obtained can be treated. Such treatment can include administration of any therapeutic agent useful for treatment of kidney fibrosis. Such therapeutic agents can include agents that treat the underlying cause(s) of kidney fibrosis, that delay the progression of kidney fibrosis, or ameliorate the symptoms of kidney fibrosis. For example, therapeutic agents that can be employed include anti-inflammatory agents, anti-coagulants, antioxidants, blood pressure medications, angiotensin-converting enzyme inhibitors (ACEIs), angiotensin AT1 receptor blockers, connective tissue growth factor (CTGF) inhibitors, antifibrotic agents (e.g., pirfenidone or tranilast), and the like.

Treatment can also include kidney transplantation.

Kits

The methods described herein may be performed by utilizing pre-packaged diagnostic kits that include devices and reagents for performing any of the methods described herein.

For example, a kit can be made and/or used for detecting kidney fibrotic diseases or disorders in a subject, where the kit includes (i) reagents for conducting a method of the invention and (ii) instructions for its use. The kits may include a device for calculating a composite score. Such a device can be a calculator, computer or minicomputer with software for performing the calculation of composite score.

The kits may be conveniently used, e.g., in clinical settings, to monitor kidney function, to detect kidney dysfunction, and to screen, monitor and diagnose transplant recipients for transplant health or the development of transplant related disease.

A variety of reagents can be included in the kits. For example, the nucleic acids (e.g., primers and/or probes) for quantification RNA levels of vimentin, NKCC2, E-cadherin, and a housekeeping gene (e.g., 18S rRNA) can be provided in separate vials, compartments, or areas of a microarray. The kits can therefore include nucleic acid primers for amplifying and quantifying the RNA levels, as well as enzymes for performing the amplification. Enzymes can also be provided in separate vials, or compartments of a container. Such enzymes can

include reverse transcriptases, thermally stable DNA polymerases and the like. The kits can also include nucleotides, stabilizing agents, RNase inhibitors, protease inhibitors, and buffers useful in the method of the invention as well as electrophoretic markers such as a 200 bp ladder. The kit will also include
5 detailed instructions for carrying out the methods of the invention.

Definitions

As used herein, the term “fibrosis” refers to the formation or development of excess fibrous connective tissue in an organ or tissue as a
10 reparative or reactive process, as opposed to a formation of fibrous tissue as a normal constituent of an organ or tissue.

A diagnostic biomarker is described by its sensitivity, specificity and its receiver operating characteristics (ROC) curve. ROC-analysis allows finding the best cut-off value to assign the test result to be ‘positive’ or ‘negative’. For
15 clinical decision-making, it is more important to know the positive (PPV; ‘true positives’) and negative predictive value (NPV; ‘true negatives’) than its sensitivity and specificity. This calculation then allows determination of how many ‘false positive’ and ‘false negative’ results the test produces. These numbers should be as low as possible, because they represent the patients that
20 are wrongly assigned to have either a ‘positive’ or a ‘negative’ test. Besides the given and constant factors that affect sensitivity and the specificity of a diagnostic test, the prevalence of the target disease in the screened population largely influences the PPV, the NPV, the number of ‘false positives’ and the number of ‘false negatives’. Therefore, these values should always be calculated
25 based on the ‘true prevalence’ of the disease in the screened population rather than from a selected population, which may over- or underestimate the ‘true prevalence’ and consequently lead to wrongly calculated PPV and NPV (64).

A prognostic biomarker should preferably ‘predict’ the outcome of a particular condition. Prediction requires the further criterion of showing that
30 changes in the value have consequential changes in the outcome. Many prognostic biomarkers used to date only ‘correlate’ with an outcome (e.g. C-reactive protein and risk of acute myocardial infarction), fewer ‘predict’ (e.g. smoking and risk of lung cancer or acute myocardial infarction).

As used herein, “obtaining a test sample” involves removing a sample of tissue or fluid from a patient, receiving a sample of tissue or fluid from a patient, receiving a patient’s tissue or fluid sample from a physician, receiving a patient’s tissue or fluid sample via mail delivery and/or removing a patient’s tissue or fluid sample from a storage apparatus (e.g., a refrigerator or freezer) or a facility. Thus, obtaining a test sample can involve removal or receipt of the test sample directly from the patient, but obtaining a test sample can also include receipt of a test sample indirectly from a medical worker, from a storage apparatus/facility, from a mail delivery service after transportation from a medical facility, and any combination thereof. The test sample can therefore originate in one location, and be transported to another location where it is received and tested. Any of these activities or combinations of activities involves “obtaining a test sample.” The test sample can be body fluid or a tissue sample. For example, the test sample can be a urine sample or a kidney biopsy.

As used herein the phrase “determining whether a test dataset of expression levels within a test sample from the patient is significantly within a fibrosis dataset or within a non-fibrosis dataset” can involve actual measurement of test dataset expression levels, i.e., quantifying the expression levels of vimentin, NKCC2, E-cadherin, and/or 18S rRNA in a test sample from the patient and then assessing whether the those test dataset expression levels are significantly (e.g., statistically significantly) within a fibrosis dataset or within a non-fibrosis dataset. In some cases, the phrase “determining whether a test dataset of expression levels within a test sample from the patient is significantly within a fibrosis dataset or within a non-fibrosis dataset” involves obtaining measurements of test dataset expression levels by directing another person or entity to make those measurements, and then assessing whether the those test dataset expression levels are significantly (e.g., statistically significantly) within a fibrosis dataset or within a non-fibrosis dataset. In further embodiments, the phrase “determining whether a test dataset of expression levels within a test sample from the patient is significantly within a fibrosis dataset or within a non-fibrosis dataset” involves obtaining measurements of test dataset expression levels by directing another person or entity to make those measurements, and having that other person or entity assess whether the those test dataset expression levels are significantly (e.g., statistically significantly) within a fibrosis dataset

or within a non-fibrosis dataset. The other (second) person or entity can then report to the person or entity that requested the determination and/or assessment. Thus, the determining step can be performed directly by one person or entity; or alternatively, the determining step can be performed indirectly by a second person or entity who is acting at the request of a first person or entity. The first person or entity can assess whether the test dataset expression levels are significantly (e.g., statistically significantly) within a fibrosis dataset or within a non-fibrosis dataset. Alternatively, the first person or entity can direct the second person or entity to assess whether the test dataset expression levels are significantly (e.g., statistically significantly) within a fibrosis dataset or within a non-fibrosis dataset.

As used herein, the term "acute rejection" (e.g., of a transplant) refers to a rejection of a transplanted organ developing after the first 5-60 post-transplant days. It is generally a manifestation of cell-mediated immune injury. It is believed that both delayed hypersensitivity and cytotoxicity mechanisms are involved. The immune injury is directed against HLA, and possibly other cell-specific antigens expressed by the tubular epithelium and vascular endothelium.

As used herein, the term "chronic rejection" (e.g., of a transplant) represents a consequence of combined immunological injury and non-immunological damage (e.g. from hypertensive nephrosclerosis, or nephrotoxicity of immuno-suppressants like cyclosporine A), occurring months or years after transplantation and ultimately leading to fibrosis and sclerosis of the allograft, associated with progressive loss of organ function.

Treatment" refers to both therapeutic treatment, and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those prone to have the disorder, or those in whom the disorder is to be prevented.

"Subject" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the subject is human.

The present description is further illustrated by the following examples, which should not be construed as limiting in any way. The contents of all cited references (including literature references, issued patents, published patent

applications as cited throughout this application) are hereby expressly incorporated by reference.

Example 1. Renal Allograft histology and urine collection

Study cohort and renal allograft histology. The for-cause biopsy group consisted of 48 subjects with graft dysfunction and biopsy-confirmed tubulointerstitial fibrosis (Fibrosis biopsy group, N=48) and the protocol biopsy group included 66 subjects with stable allograft function and normal allograft biopsy (Normal biopsy group, N=66).

The biopsy specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin, periodic acid–Schiff, and Masson's trichrome stains. Cryostat or paraffin sections of the for-cause biopsies were examined for C4d deposition with the use of anti-human C4d antibody. In addition to screening for the presence or absence of fibrosis and the grading of fibrosis, the allograft biopsies were also classified using the Banff 07 updated version of Banff 97 diagnostic categories and using the Banff schema 66 allograft biopsies were classified as Normal, and 48 biopsies with fibrosis were classified as IF/TA, no evidence of any specific etiology (N= 30), chronic antibody-mediated rejection (N=6), chronic active T-cell mediated rejection (N=6), and the remaining 6 with fibrosis were also classified as having diabetic nephropathy (N=4) or recurrent glomerular disease (N=2).

The allograft fibrosis biopsies were also scored for concurrent inflammation as indicated by cellular infiltration within non-fibrotic areas of cortical interstitium. Among the 48 patients with allograft fibrosis, 32 biopsies from 32 patients showed no inflammation (inflammation score=0) and 16 biopsies from 16 patients displayed both fibrosis and inflammation. Inflammation was graded as 1 when 10-25% of cortical interstitium was involved (N=8 biopsies), 2 when 26-50% of cortical interstitium was involved N= 2 biopsies) or 3 when greater than 50% cortical interstitium was involved , N= 6 biopsies). All biopsies were classified by a pathologist blinded to the molecular study results.

Urine collection. One hundred and four of the 114 urine specimens for the mRNA profiling study were collected within 24 hours of the biopsy procedure, 8 within 7 days and the remaining 2 specimens within 15 days.

These time lines refer to the time intervals between the biopsy procedure and urine specimen collection and not to the time interval between the time the urine was collected and when it was centrifuged to obtain the urine pellet prepared for RNA isolation.

5 Urine was centrifuged at 2,000g for 30 minutes and the cell pellet was prepared within 4 hours of urine collection. RNAlater (50μl) was then added to the urine pellet and stored at -80^{0}C prior to isolation of RNA. RNA was extracted from the pellet using the RNeasy mini kit (Qiagen) and reverse-transcribed to cDNA using TaqMan® Reverse Transcription Reagents (Applied 10 Biosystems).

Example 2. Study Cohorts for the Discovery Set and Validation Set

Urine samples were examined from 114 kidney transplant recipients who had undergone either a diagnostic (for-cause) renal allograft biopsy or a 15 scheduled (protocol) biopsy. The biopsies were examined for the presence or absence of tubulointerstitial fibrosis or inflammation, and classified according to the Banff schema (Solez et al., Am J Transplant 8: 753 (2008)) by a pathologist blinded to the mRNA results.

Prior to data analysis, the 114 urine samples were assigned, at a 2:1 ratio, 20 to a Discovery set of 76 samples (32 samples from 32 recipients with renal allograft biopsies showing fibrosis and 44 samples from 44 recipients with normal biopsy results) and an independent Validation set of 38 samples (16 samples from 16 recipients with biopsies showing fibrosis and 22 samples from 22 recipients with normal biopsy results). FIG. 1A outlines the process employed. 25 Neither the recipients' characteristics nor the transplant or renal allograft related variables differed between those assigned to the Discovery set or the Validation set (Table 1). The risk factors for fibrosis such as acute rejection and deceased donor grafts however were more frequent in the fibrosis biopsy group compared to the normal biopsy group.

Table 1: Characteristics of the Renal Allograft Recipients

	Discovery Set (N=76)				Validation Set (N=38)				P[a]
Parameter	All Patients (N=76)	Fibrosis Biopsy Group (N=32)	Normal Biopsy Group (N=44)	P[a] Fibrosis vs. Normal	All Patients (N=38)	Fibrosis Biopsy Group (N=16)	Normal Biopsy Group (N=22)	P[a] Fibrosis vs. Normal	Discovery Set vs. Validation Set
Recipient Characteristics									
Age (mean±SD, years)	46±13	46±14	46±12	0.88	44±10	44±9	44±12	0.80	0.40
Gender (Male/female)	37/39	17/15	20/24	0.50	21/17	8/8	13/9	0.57	0.50
Ethnicity (White/Black/Other)	26/19/31	12/7/13	14/12/18	0.82	13/11/14	4/9/3	9/2/11	0.006	0.88
Cause of End Stage Renal Disease, N (%)				0.18				0.29	0.20
Glomerulonephritis	18 (24)	10 (31)	8 (18)		7 (18)	4 (25)	3 (14)		
Diabetes	21 (28)	9 (28)	12 (27)		6 (16)	1 (6)	5 (23)		
Cystic/hereditary/ Congenital	11 (15)	4 (13)	7 (16)		10 (26)	4 (25)	6 (27)		
Secondary Glomerulonephritis	4 (5)	1 (3)	3 (7)		4 (11)	3 (19)	1 (5)		
Hypertension	6 (8)	1 (3)	5 (11)		6 (16)	3 (19)	3 (14)		
Interstitial nephritis	4 (5)	3 (9)	1 (2)		0 (0)	0 (0)	0 (0)		
Miscellaneous Conditions	2 (3)	2 (6)	0 (0)		1 (3)	1 (6)	0 (0)		
Neoplasm	0 (0)	0 (0)	0 (0)		1 (3)	0 (0)	1 (5)		
Etiology uncertain	10 (13)	2 (6)	8 (18)		3 (8)	0 (0)	3 (14)		
Peak pre-transplant HLA class I or II PRA (mean±SD, %)[b]	17.6 ±23.7	18.8 ±25.3	17.0 ±23.0	0.77	26.4 ±28.3	34.8 ±33.1	20.3 ±23.1	0.12	0.09

Peak pre-transplant HLA class I or II PRA ≥50%, n (%)	6 (9)	2 (9)	4 (9	0.95	6 (16)	4 (25)	2 (9)	0.18	0.29
Transplant variables									
Deceased donor, N (%)	26 (34)	19 (59)	7 (16)	<0.0001	18 (47)	12 (75)	6 (27)	0.004	0.17
Donor age (mean±SD, years)	44±11	48±11	42±10	0.03	45±14	49±13	42±14	0.12	0.65
HLA mismatches (mean±SD)	3.5 ±2.0	3.8 ±2.2	3.4 ±1.8	0.41	3.3 ±1.8	4.6 ±1.4	2.4 ±1.5	<0.0001	0.57
Cold ischemia time (deceased donor grafts only, mean±SD, hours)	20.8 ±7.6	20.5 ±8.1	21.3 ±7.1	0.83	22.2 ±5.0	22.9 ±5.5	20.7 ±3.6	0.39	0.51
Delayed graft function[c], N (%)									
Deceased donor grafts	8/26 (39)	6/19 (32)	2/7 (29)	0.88	6/18 (33)	5/12 (42)	1/6 (17)	0.28	0.85
History of acute rejection before biopsy, N (%)	9 (12)	7 (22)	2 (5)	0.02	9 (24)	8 (50)	1 (5)	0.001	0.10
History of BK virus nephropathy before biopsy, N (%)	2 (3)	2 (6)	0 (0)	0.09	2 (5)	2 (13)	0 (0)	0.09	0.47
Graft failure within 12 months after biopsy, N (%)	13 (17)	13 (41)	0 (0)	<0.0001	8 (21)	7 (44)	1 (5)	0.003	0.61
Renal allograft variables									
Time of biopsy (mean±SD, months since transplant)	25.4 ±53.1	54.4 ±72.7	4.3 ±4.4	<0.0001	14.1 ±23.3	29.2 ±29.9	3.0 ±3.8	0.0002	0.21
Serum creatinine at biopsy (mean±SD, mg/dL)	2.1 ±1.5	3.2 ±1.9	1.3 ±0.4	<0.0001	2.3 ±1.5	3.3 ±1.8	1.5 ±0.4	<0.0001	0.63
eGFR at biopsy (mean±SD, mL/min/1.73m^2)	45.1 ±20.6	26.2 ±13.2	58.5 ±13.1	<0.0001	42.8 ±19.5	29.1 ±19.0	52.7 ±12.9	<0.0001	0.56
Urinary protein:creatinine ratio[d] at biopsy	1.4 ±2.9	3.6 ±4.3	0.3 ±0.3	<0.0001	0.5 ±0.9	1.2± 1.4	0.2 ±0.1	0.004	0.11

Allograft fibrosis grade	Discovery Set (N=76)			Validation Set (N=38)		
	All patients (n=76)	Fibrosis biopsy group (n=32)	Normal biopsy group (n=44)	All patients (n=38)	Fibrosis biopsy group (n=16)	Normal biopsy group (n=22)
No fibrosis, N (%)	44 (58)	0 (0)	44 (100) 0	22 (58)	0 (0)	22 (100)
Grade I (<25% of cortical area), N (%)	7 (9)	7 (22)	(0) 0	2 (5)	2 (13)	0 (0)
Grade II (26-50% of cortical area), N (%)	9 (12)	9 (28)	(0) 0 (0)	7 (18)	7 (44)	0 (0)
Grade III (>50% of cortical area), N (%)	16 (21)	16 (50)		7 (18)	7 (44)	0 (0)
Mean±SD fibrosis grade	1.0±1.2	2.3±0.8	0.0±0.0	1.0±1.2	2.3±0.7	0.0±0.0
Banff Classification categories[e]						
Normal	39 (51)	0 (0)	39 (89)	19 (50)	0 (0)	19 (86)
Chronic active antibody-mediated rejection	4 (5)	4 (13)	0 (0)	2 (5)	2 (13)	0 (0)
Chronic active T-cell mediated rejection	3 (4)	3 (9)	0 (0)	3 (8)	3 (19)	0 (0)
IF/TA, no evidence of any specific etiology	22 (29)	22 (69)	0 (0)	8 (21)	8 (50)	0 (0)
Other	8 (11)	3 (9)[f]	5 (11)[g]	6 (16)	3 (19)[f]	3(19)[g]

a: P-values determined by Chi-square or Fisher's exact tests for categorical variables or independent samples T-test for continuous variables.
b: Panel reactive antibodies (PRA) directed to the HLA class I or II antibodies were identified using the complement dependent cytotoxicity assay, and PRA value was available in 67 of 76 patients in the Discovery set (23 of 32 patients in the fibrosis biopsy group and 44 of 44 patients in the normal biopsy group) and 38 of 38 patients in the Validation set.
c: Defined by the need for hemodialysis in the first week post-transplantation
d: Urinary protein:creatinine ratio is the urinary protein concentration (mg/dL) divided by the urinary creatinine concentration (mg/dL) in a random urine specimen.
e: In addition to screening the allograft biopsies for the presence or absence of tubulointerstial fibrosis and grading the extent of fibrosis, presence or absence of inflammation, the allograft biopsies were also classified using the Banff 07 updated version of the Banff 97 diagnostic categories (21). All 6 biopsies classified as chronic antibody mediated rejection were positive for C4d deposition; cryostat or paraffin sections of the for-cause biopsies were examined for C4d deposition with the use of anti-human C4d antibody. All other biopsies in the fibrosis group were negative for C4d deposition.
f: 6 biopsies in the Other diagnosis category; 3 in the Discovery set and 3 in the Validation set include diabetic nephropathy (N=4), and glomerulonephritis recurrence (N=2).
g: 8 biopsies in the Other diagnosis category; 5 in the Discovery set and 3 in the Validation set includes vascular changes but no interstitial fibrosis.

Example 3. Diagnostic Value of Individual mRNA Levels in the Discovery Set

This study employed the pre-amplification enhanced kinetic quantitative PCR assay for the absolute quantification of mRNAs in the urine of renal allograft recipients reported in (Muthukumar et al., N Engl J Med 353: 2342 (2005)). This method is in frequent use in the inventor's laboratory. This assay enables measurement of a large number of mRNAs using a very small quantity of cDNA.

Urine was centrifuged at 2000g for 30 min within 4 hr of collection. RNA was extracted from the pellet using the RNeasy mini kit (Qiagen, Valencia, CA) and reverse transcribed to complementary DNA using TaqMan Reverse Transcription Reagents (Applied Biosystems). PCR analysis involved a preamplification step, followed by quantification of mRNA with an ABI Prism 7500 Fast detection system (Applied Biosystems). Transcript levels were calculated by a standard curve method (Anglicheau et al., Prov. Natl. Acad. Sci. USA 106: 5330 (2009)). The sequence and location of the gene specific oligonucleotide primers and TaqMan probes designed for quantifying the mRNAs in the PCR assays are listed in Table 2.

Table 2. Oligonucleotide primers and probes used in kinetic quantitative polymerase chain reaction assays for the quantification of mRNAs.

Gene	Accession number	Sequence	Location	SEQ ID NO:
Vimentin	NM_003380.2	Sense: 5' TCAGAGAGAGGAAGCCGAAAAC 3'	706-727	12
		Antisense: 5' CCAGAGACGCATTGTCAACATC 3'	770-749	13
		Probe: 5' FAM CCCTGCAATCTTTCAGAC MGB 3'	729-746	14
HGF	BC063485	Sense: 5' CAAATGTCAGCCCTGGAGTTC 3'	526-546	15
		Antisense: 5' CTGTAGGTCTTTACCCCGATAGCT 3'	604-581	16
		Probe: 5' FAM ATGATACCACACGAACACAGCTTTTTGCC TAMARA 3'	548-576	17
α-SMA	NM_001613	Sense: 5' TGGGACGACATGGAAAAGATC 3'	288-308	18
		Antisense: 5' CAGGGTGGGATGCTCTTCAG 3'	365-346	19
		Probe: 5' FAM CCACTCTTTCTACAATGAGCTTCGTGTTGCC TAMRA 3'	314-344	20
Fibronectin 1	XM_055254	Sense: 5' GAAAGTACACCTGTTGTCATTCAACA 3'	2582-2607	21
		Antisense: 5' ACCTTCACGTCTGTCACTTCCA 3'	2688-2666	22
		Probe: 5' FAM CCACTGGCACCCCACGCTCA TAMRA 3'	2613-2632	23
PAI1	NM_000602.1	Sense: 5' AATCAGACGGCAGCACTGTCT 3'	716-736	24
		Antisense: 5' GGGCGTGGTGAACTCAGTATAGT 3'	792-770	25
		Probe: 5' FAM TGTGCCCATGATGGC MGB 3'	738-752	26
Perforin	M28393	Sense: 5' GGACCAGTACAGCTTCAGCACTG 3'	492-514	27

Gene	Accession number	Sequence	Location	SEQ ID NO:
		Antisense: 5' GCCCTCTTGAAGTCAGGGTG 3'	587-568	28
		Probe: 5' FAM TGCCGCTTCTACAGTTTCCATGTGGTACAC TAMRA 3'	526-555	29
TGFI31	NM_000660	Sense: 5' GCGTGCTAATGGTGGAAACC 3'	1170-1189	30
		Antisense: 5' CGGAGCTCTGATGTGTTGAAGA 3'	1263-1242	31
		Probe: 5’ FAM ACAACGAAATCTATGACAAGTTCAAGCAG AGTACACA TAMRA 3’	1191-1227	32
TIMP1	NM003254	Sense: 5’ GACGGCCTTCTGCAATTCC 3’	288-306	33
		Antisense: 5’ GTATAAGGTGGTCTGGTTGACTTCTG 3’	366-341	34
		Probe: 5’ FAM AGGGCCAAGTTCGTGG MGB 3’	319-334	35
Granzyme B	J04071	Sense: 5' GCGAATCTGACTTACGCCATTATT 3'	534-557	36
		Antisense: 5' CAAGAGGGCCTCCAGAGTCC 3'	638-619	37
		Probe: 5’ FAM CCCACGCACAACTCAATGGTACTGTCG TAMRA 3’	559-585	38
FSP1	CR450345.1	Sense: 5’ AGGAGCTGCTGACCCGG 3’	104-120	39
		Antisense: 5’ GCTTCATCTGTCCTTTTCCCC 3’	158-138	40
		Probe: 5’ FAM CTGCCCAGCTTCT MGB 3’	124-136	41
CD103	XM_008508	Sense: 5' CGTGCTCAGCTCCCTTCTG 3'	211-229	42
		Antisense: 5' CCTGGTGTCCTCTTGGTTCTG 3'	297-277	43
		Probe: 5’ FAM ACCAAGACCCCAGCACCAACCATACCT TAMRA 3’	231-257	44
Collagen 1A1	NM_000088.3	Sense: 5' CCAGAAGAACTGGTACATCAGCAA 3'	4050-4073	45
		Antisense: 5' CGCCATACTCGAACTGGAATC 3'	4144-4124	46

Gene	Accession number	Sequence	Location	SEQ ID NO:
		Probe: 5’ FAM ACAAGAGGCATGTCTGG MGB 3’	4085-4101	47
BMP7	NM_001719.1	Sense: 5' GCTTCGTCAACCTCGTGGAA 3’	526-545	48
		Antisense: 5’ CAAACCGGAACTCTCGATGGT 3’	597-577	49
		Probe: 5’ FAM ATGACAAGGAATTCTTCCACCCACGCTAC TAMRA 3’	547-575	50
CTLA4	BC074893	Sense: 5’ CGCCATACTACCTGGGCATAG 3’	441-461	51
		Antisense: 5 GATCCAGAGGAGGAAGTCAGAATC 3’	529-506	52
		Probe: 5’ FAM CAGATTTATGTAATTGATCCAGAAC CGTGCCC TAMRA 3’	473-504	53
CTGF	NM_001901	Sense: 5’ TGTGTGACGAGCCCAAGGA 3’	639-657	54
		Antisense: 5’ TAGTTGGGTCTGGGCCAAAC 3’	725-706	55
		Probe: 5’ FAM CCTGCCCTCGCGGCTTACCG TAMRA 3’	674-693	56
FGF2	NM_002006.3	Sense: 5’ CCGACGGCCGAGTTGAC 3’	601-617	57
		Antisense: 5’ TAACGGTTAGCACACACTCCTTTG 3’	712-689	58
		Probe: 5’ FAM ACCCTCACATCAAGCTACAACTTCAA GCAGAA TAMRA 3’	637-668	59
CD25	NM_000417	Sense: 5’ GACTGCTCACGTTCATCATGGT 3’	185-206	60
		Antisense: 5’ AATGTGGCGTGTGGGATCTC 3’	266-247	61
		Probe: 5' FAM AGAGCTCTGTGACGATGACCCGCC TAMRA 3'	222-245	62
FoxP3	NM_014009	Sense: 5’ GAGAAGCTGAGTGCCATGCA 3’	939-958	63
		Antisense: 5’ GGAGCCCTTGTCGGATGAT 3’	1025-1007	64
		Probe: 5’ FAM TGCCATTTTCCCAGCCAGGTGG TAMRA 3’	962-983	65
USAG1	NM_015464	Sense: 5' TGGAGGCAGGCATTTCAGTAA 3'	364-366	66
		Antisense: 5' TTCCCGGCAACCCACTT 3'	412-396	67

Gene	Accession number	Sequence	Location	SEQ ID NO:
		Probe: 5' FAM CCCGAGTGTTCCGATCCAGTCCAGT TAMRA 3'	392-368	68
NKCC2	BC040138.2	Sense: 5' TCACGAGCAACTCGCAAAGA 3'	588-607	69
		Antisense: 5' TCCCATCACCGTTAGCAACTC 3'	658-638	70
		Probe: 5' FAM TGTGGCAGTCACCCCAAGTTCAGC TAMRA 3'	609-632	71
ITGB6	NM_000888.3	Sense: 5' GGATTGAACTGCTTTGCCTGTT 3'	21-42	72
		Antisense: 5' GGCACAGCCACCTTGTACGT 3'	69-88	73
		Probe: 5' FAM TTTCTATTTCTAGGAAGGAATG MGB 3'	44-65	74
E-cadherin	XM_007840	Sense: 5' TGAGTGTCCCCCGGTATCTTC 3'	2469-2489	75
		Antisense: 5' CAGCCGCTTTCAGATTTTCAT 3'	2549-2529	76
		Probe: 5' FAM CCTGCCAATCCCGATGAAATTGGAAAT TAMRA 3'	2495-2521	77
18S rRNA	K03432	Sense: 5' GCCCGAAGCGTTTACTTTGA 3'	929-948	78
		Antisense: 5' TCCATTATTCCTAGCTGCGGTATC 3'	1009-986	79
		Probe: 5' FAM AAAGCAGGCCCGAGCCGCC TAMRA 3'	965-983	80

Pre-amplification enhanced real-time quantitative PCR Assay. Oligonucleotide primers and fluorogenic probes were designed for the measurement of levels of mRNAs (Table 2). encoding proteins implicated in fibrosis, extracellular matrix accumulation, and/or EMT (TGFß1, integrin ß6 [ITGB6], fibroblast growth factor-2 [FGF2], connective tissue growth factor [CTGF], PAI1, tissue inhibitor of metalloproteinases-1 [TIMP1], fibronectin 1, collagen 1A1, E-cadherin, BMP7 and HGF). Also measured were mRNAs for proteins expressed in renal tubular epithelial cells (NKCC2 found on the apical membrane of the thick ascending limb of loop of Henley, and uterine sensitization associated gene 1 [USAG1] expressed in distal collecting tubules), mesenchymal cells (vimentin, FSP1, α-smooth muscle actin [α-SMA]), and effector and/or regulatory T lymphocytes (perforin, granzyme B, CD25, CD103, FoxP3, CTLA4).

PCR analysis was performed by a two-step process, a preamplification step followed by measurement of mRNA with an ABI Prism 7500 Fast detection system. A pre-amplification protocol that allows quantification of these 22 mRNAs from small amounts of cDNA was developed. The pre-amplification reaction for each sample was set up in a 0.2 ml PCR tube with a final reaction volume of 10 µl containing 3.0 µl cDNA (from reverse transcription of 1 µg total RNA in 100 µl buffer), 1.0 µl 10x buffer, 1.0 µl MgCl2 (25 mM), 0.25 µl 4x dNTP (10 mM each), 0.25 µl Ampli-Taq gold (5 U/µl), 0.15 µl primer mix per gene (50 µM sense and 50 µM antisense primer) and water to final volume of 10 µl. Following vortexing, the PCR was set up using a Veriti thermal cycler (Applied Biosystems) and the 10-cycle PCR reaction profile consisted of an initial hold at 95°C for 10 min, denaturing at 95°C for 15 seconds and primer annealing and extension at 60°C for 1 min. At the end of 10 cycles, 140 µl of TE buffer was added to the PCR reaction and 2.5 µl of diluted PCR amplicons were then used for quantification of mRNA using the real-time quantitative PCR assay.

Transcript levels (copy number/µg total RNA) were calculated by a standard curve method and all analyses of mRNA copy numbers statistically controlled for the copy number of the reference gene 18S ribosomal RNA (rRNA).

The LOESS (locally weighted scatterplot smoothing) method was employed in the discovery phase of the analysis to initially examine the bivariate relationship of each mRNA measure to diagnosis in the Discovery set comprised of 32 renal transplant recipients with biopsy-confirmed fibrosis and 44 recipients with normal allograft biopsy results, controlling for the quadratic relationship of 18S rRNA. Logistic regression analysis was then used to parsimoniously model each relationship as a piece-wise linear model.

Advantage of the LOESS Model. LOESS (locally weighted scatterplot smoothing) is a powerful tool to elucidate the potentially non-linear relationship between two variables since it has the advantage of fitting segments of data without pre-specifying a specific, usually linear, global function. Importantly, a threshold effect at which the risk for an outcome increases can be ascertained.

Definition of Parsimonious Model. A parsimonious model is a model that contains the fewest number of predictor variables for a given outcome, without compromising the model's prediction accuracy. In essence, it balances the trade-off between simplicity (simpler is better) and the incremental increase in prediction accuracy that is obtained by including more predictors in a model. The analyses of levels of 22 mRNAs measured in this study showed that the diagnostic accuracy of the 4-gene model (vimentin, 18S, NKCC2 and E-cadherin) is not significantly improved by inclusion of the levels of any or all of the remaining 18 mRNAs that were measured. Thus, the 4-gene model is the parsimonious model in this study.

FIG. 1B shows that up to 22 genes are differentially express in renal fibrosis tissues obtained by biopsy compared to normal renal biopsy tissues.

FIG. 2A illustrates that the levels of twelve of the twenty-two mRNAs measured from urinary cells are significantly associated with the diagnosis of fibrosis after using the Holm modified (Holm, *Scandinavian Journal of Statistics* 6: 65 (1979)) Bonferoni procedure to control the risk of a Type I error. The lack of association between the remaining 10 mRNAs and allograft diagnosis is shown in FIG. 2B.

Receiver-Operating-Characteristic (ROC) Curve Analysis. Analysis involving ROC curve demonstrated that allograft fibrosis can be predicted accurately using urinary cell levels of mRNA for vimentin (area under the curve [AUC] and 95% confidence intervals = 0.90, 0.82-0.97), HGF (0.91, 0.84-0.98),

α-SMA (0.88, 0.80-0.95), fibronectin 1 (0.83, 0.73-0.93), perforin (0.83, 0.74-0.93), TGFß1 (0.82, 0.72- 0.92), TIMP1 (0.81, 0.71-0.90), granzyme B (0.82, 0.71-0.92), FSP1 (0.81, 0.71-0.91), PAI1 (0.79, 0.68-0.90), collagen 1A1 (0.77, 0.66-0.88) or CD103 (0.76, 0.65-0.87).

5

Example 4. Multigene Prediction Model of Fibrosis Diagnosis in the Discovery Set

It was determined useful to build a multigene prediction model of fibrosis around vimentin in view of biologic properties of vimentin and data from pre-
10 clinical models that vimentin is over-expressed preceding and/or during fibrosis and the clinical observation that vimentin expression in the 3-month protocol biopsies of renal allografts is associated with fibrosis score at 12 months. Accordingly, a LOESS model was once again estimated and corresponding piece-wise linear model for the relationship of each mRNA measure to fibrosis,
15 this time controlling for vimentin mRNA level and the quadratic relationship of 18S rRNA level. These analyses showed that after controlling for vimentin mRNA levels, the levels of other mRNAs (HGF, TGFß1, fibronectin 1, PAI1, FSP1, collagen 1A1, α-SMA, CD103, granzyme B or perforin) that were initially significantly associated with fibrosis were no longer significant
20 (P>0.05), whereas the mRNAs for NKCC2 and E-cadherin became significantly associated with the diagnosis (Figure 3). Based on these findings, a 4-gene diagnostic model that included vimentin, NKCC2, E-cadherin and 18S rRNA was developed. The parameter estimates for the model, provided in Figure 3, include terms accounting for the relationships, including non-linear relationships, between
25 the mRNAs and diagnosis.

The composite score based on this model was highly associated with the diagnosis of fibrosis (Figure 4A). The ROC curve (Figure 4B) shows, for various levels of this composite score, the fraction of true positive results (sensitivity) and false positive results (1-specificity) for distinguishing recipients
30 with allograft fibrosis from recipients with normal biopsy results. The AUC was 0.95 (95%CI: 0.90 to 0.99, P<0.0001), and a significant improvement (P<0.05) over the AUC for vimentin and 18S only. Using the optimal cut-point of 4.5 (the cut-point yielding the highest combined sensitivity and specificity), the

composite score predicted fibrosis with a specificity of 84.1% (95%CI: 73.3 to 94.9%) and a sensitivity of 93.8% (95%CI: 85.4.0 to 99.9%) (Figure 4B).

Example 5. Independent Validation of the Diagnostic Signature

5 The final diagnostic equation predicting fibrosis in the Discovery set was then validated in an independent Validation set of 38 renal transplant recipients consisting of 16 patients with biopsy-proven fibrosis and 22 recipients with normal allograft biopsy results (Table 1). Figure 4C shows the ROC curve of this equation based on urinary cell levels of vimentin, NKCC2 and E-cadherin 10 mRNAs and 18S rRNA level for the diagnosis of fibrosis. This 4-gene classifier could diagnose fibrosis in the Validation set with high accuracy and the AUC for the diagnosis of fibrosis in the independent Validation set was 0.89 (95%CI: 0.78 to 0.99, P<0.0001) (Figure 4C). At the composite score cut-point of 4.5 (the same cut-point used in the Discovery set), fibrosis was diagnosed in the Validation set 15 with a specificity of 77.3% (95%CI: 59.8 to 94.8%) and a sensitivity of 87.5% (95%CI: 71.3 to 99.9%)

In this study the fit of the predictor model was also examined by dividing the Discovery and Validation sets into sextiles of the composite score and examining the predicted and observed number of transplant recipients with 20 fibrosis, separately for each sets, for each sextile (Figure 4D). Based on the Hosmer-Lemeshow test, the fit between the observed and the predicted number of subjects with fibrosis in each of the sextiles was excellent (P=0.69) in the Discovery set (left half of Figure 4D). For the Validation set (right half of Figure 4D), the P-value was 0.04, suggesting a good fit, given that this set was not 25 involved in the estimation of the model.

Serum creatinine levels were higher in the fibrosis group compared to the normal biopsy group (P<0.0001, Table 1). The study assessed whether the composite score independently differentiates the fibrosis and stable patient groups after controlling for serum creatinine. This analysis showed that the 30 composite score is statistically significant and a slightly stronger predictor of group status (Fibrosis vs. Normal) than serum creatinine (each P<0.0001, controlling for the other).

This study also explored whether graft dysfunction, independent of fibrosis, was associated with the composite score. The log mean composite score

of the 4-gene signature was 4.58 (95%CI: 3.52 to 5.64) in the acute tubular necrosis (ATN) group with graft dysfunction (N=9 patients) and 6.49 (95%CI: 5.96 to 7.02) in the fibrosis group with graft dysfunction (N=48 patients) (P=0.01). In addition, the composite score for the ATN group was not significantly different from that of normal biopsy group (N=66) with normal graft function (P=0.12). Whether the time to biopsy was associated with the diagnostic signature (composite score) was also investigated. This analyses showed that there was no significant association between the diagnostic signature and time to biopsy; Pearson correlation coefficient r=0.17, P=0.24 in the fibrosis biopsy group (N=48) and r=0.23, P=0.07 in the normal biopsy group (N=66).

Example 6. Fibrosis Grades and the 4-Gene Composite Score

Whether this 4-gene composite score could strongly discriminate patients with differing degrees of fibrosis from patients with no evidence of fibrosis was also investigated. This analysis revealed that the log mean composite score derived from urinary cell vimentin, NKCC2 and E-cadherin mRNA levels and 18S rRNA level was significantly different among the four groups (fibrosis grades I [<25% of cortical area], II [26-50%], and III [>50%] and those with no evidence of fibrosis, P<0.0001, one-way ANOVA) (FIG. 5). Pair-wise comparisons revealed that the mean composite score of normal biopsies were significantly different from that of grade I fibrosis (P=0.0002), grade II fibrosis (P<0.0001) and grade III fibrosis (P<0.0001). The mean composite score however did not differ significantly among the three grades of fibrosis (P=0.58).

Example 7. Allograft Fibrosis with Concurrent Inflammation and the 4-Gene Composite Score

Among the 48 patients with allograft fibrosis, 32 biopsies from 32 patients showed no inflammation and 16 biopsies from 16 patients displayed both fibrosis and inflammation. The log mean composite score was 7.5 ±2.3 in the 16 urine samples from patients with both fibrosis and inflammation and 5.9 ±1.3 score in the 32 urine samples from patients with fibrosis only and without concurrent inflammation (P=0.003).

Example 8. Statistical analysis

The 114 patients (48 recipients with allograft fibrosis and 66 recipients with normal biopsies) were rank ordered within group by the copy number of 18S rRNA and partitioned into consecutive triplets. Within each triplet, the first and third patients were assigned to the Discovery set and the second patient was assigned to the Validation set, resulting in the two sets being exactly matched on fibrosis status and very closely matched on 18S. Twice as many patients were assigned to the Discovery set in order to enhance statistical power for the exploratory analyses which included a procedure to protect against the risk of a Type I error.

The distribution of each mRNA, as well as 18S rRNA, exhibited considerable positive skewness, which was substantially reduced by use of a log transformation. LOESS methods were used to examine the relationship of the mRNA measures to diagnosis (Fibrosis vs. Normal). An initial LOESS model revealed a U-shaped relationship of 18S to diagnosis that was well represented by a quadratic function. Then a GAM (generalized additive model) (Hastie & Tibshirani, *Statistical Science* 1: 297 (1986); Hastie & Tibshirani, eds. GENERALIZED ADDITIVE MODELS, New York: Chapman & Hall (1990)) procedure was used to fit an additive LOESS model of the relationship of each individual mRNA measure with diagnosis while statistically controlling for the quadratic effect of 18S. The smoothing parameter for the LOESS model was determined using the generalized cross validation criterion, but restricted to DF<5). After reviewing the smoothed relationship, a piece-wise linear logistic regression spline model was fit that closely approximated the LOESS-smoothed relationship. Plots are presented where the parametric model of the relationship of mRNA level to the probability of being in the Fibrosis group is superimposed on the LOESS model. Also presented are the AUC and its 95% confidence interval for each logistic model. Significance levels of the 22 parametric models were adjusted for the experiment-wise risk of a Type I error using Holm's modified (27) Bonferroni method. Based on the results, one mRNA was chosen to be definitely included in the final model, and then repeated the above process for the remaining 21 mRNA measures to determine which if any could further improve the prediction of fibrosis diagnosis. This stepwise process was repeated until, after 3 steps, no further mRNA measures significantly

improved the prediction model. The ROC curve for the final model and its AUC are presented.

In the Validation phase, the final prediction equation from the Discovery phase was used to calculate composite scores for those in the Validation set. A logistic regression analysis predicting fibrosis diagnosis from this single composite score was estimated to test the significance of the prediction equation. The ROC curve for the prediction equation and its AUC for the Validation set are presented. Finally, the Discovery and Validation sets were each divided into sextiles and an exact test version of the Hosmer-Lemeshow test (Hosmer & Lemeshow, APPLIED LOGISTIC REGRESSION, New York: John Wiley & Sons (1989) was used to assess the fit of the equation in both the Discovery and Validation sets.

All analyses were performed using SAS, version 9.2 (Cary, NC).

The process for converting into vimentin, NKCC2 and E-cadherin mRNA quantities and the 18S rRNA quantity into a composite score for the diagnosis of fibrosis involves, dividing the 18S rRNA quantity by 10^5 (i.e., 100,000). The composite score can be calculated as follows.

$$\text{Composite Score} = 36.10283 + [-15.84215*\log_{10}(18s)] + 1.56907*\log_{10}(18s)*\log_{10}(18s) + 5.11698*\max[0, \log_{10}(\text{Vimentin}) - 5.6] + [-1.44145*\log_{10}(\text{NKCC2})] + 3.31357*\min[3.1, \log_{10}(\text{E-cadherin})]$$

where,

$\max[0, \log_{10}(\text{Vimentin}) - 5.6] = 0$ whenever $\log_{10}(\text{Vimentin})$ is ≤ 5.6 and $= \log_{10}(\text{Vimentin}) - 5.6$ whenever $\log_{10}(\text{Vimentin})$ is >5.6;

$\min[3.1, \log_{10}(\text{Ecadherin})] = \log_{10}(\text{E-cadherin})$ whenever $\log_{10}(\text{E-cadherin})<3.1$ and $= 3.1$ whenever $\log_{10}(\text{E-cadherin}) \geq 3.1$;

and * signifies multiplication.

The $\log_{10}$ variables are defined as follows:

$\log_{10}$(18s RNA) is $\log_{10}$ of 18S RNA/100,000 quantity in the test sample;

$\log_{10}$(Vimentin) is $\log_{10}$ of normalized vimentin mRNA quantity in the test sample;

$\log_{10}$(NKCC2)] is $\log_{10}$ of normalized NKCC2 mRNA quantity in the test sample; and

$\log_{10}$(E-cadherin) is $\log_{10}$ of E-cadherin mRNA quantity in the test sample.

In calculating the composite score, the vimentin mRNA quantity, the NKCC2 mRNA quantity, and the E-cadherin mRNA quantity can be divided by the 18S RNA quantity multiplied by 100,000 before generating the $\log_{10}$(Vimentin), the $\log_{10}$(NKCC2), and the $\log_{10}$(E-cadherin) values, respectively. This generates normalized values of these mRNA quantities.

$$\text{Vimentin mRNA quantity can equal: } \frac{\text{measured vimentin mRNA quantity}}{\text{Measured 18S rRNA quantity/ 100,000}}$$

$$\text{NKCC2 mRNA quantity can equal: } \frac{\text{measured NKCC2 mRNA quantity}}{\text{Measured 18S rRNA quantity / 100,000}}$$

$$\text{E-cadherin mRNA quantity can equal: } \frac{\text{measured E-cadherin mRNA quantity}}{\text{Measured 18S rRNA quantity / 100,000}}$$

The composite score varies from about 1 to 8, where a normal (healthy) composite score is about 3.5. A test sample with a composite score of above 4.7 indicates that the subject has fibrosis. For example, a test score from about 4.7 to about 6.5 indicates a subject has mild to moderate fibrosis. A test sample with a composite score from about 6.5 or more indicates a subject has moderate to severe fibrosis.

Therefore, kidney fibrosis can be diagnosed using the methods described herein.

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All patents and publications referenced or mentioned herein are indicative of the levels of skill of those skilled in the art to which the invention pertains, and each such referenced patent or publication is hereby specifically incorporated by reference to the same extent as if it had been incorporated by
25 reference in its entirety individually or set forth herein in its entirety. Applicants reserve the right to physically incorporate into this specification any and all materials and information from any such cited patents or publications.

The specific methods, devices and compositions described herein are representative of preferred embodiments and are exemplary and not intended as
30 limitations on the scope of the invention. Other objects, aspects, and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed
35 herein without departing from the scope and spirit of the invention.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitation or limitations, which is not specifically disclosed herein as essential. The methods and processes illustratively described herein suitably may be practiced in differing orders of steps, and the methods and processes are not necessarily restricted to the orders of steps indicated herein or in the claims.

As used herein and in the appended claims, the singular forms “a,” “an,” and “the” include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to “a reactor” or “a mixer” or “a feedstream” includes a plurality of such reactors, mixers or feedstreams (for example, a series of reactors, mixers or feedstreams), and so forth. In this document, the term “or” is used to refer to a nonexclusive or, such that “A or B” includes “A but not B,” “B but not A,” and “A and B,” unless otherwise indicated.

Under no circumstances may the patent be interpreted to be limited to the specific examples or embodiments or methods specifically disclosed herein. Under no circumstances may the patent be interpreted to be limited by any statement made by any Examiner or any other official or employee of the Patent and Trademark Office unless such statement is specifically and without qualification or reservation expressly adopted in a responsive writing by Applicants.

The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intent in the use of such terms and expressions to exclude any equivalent of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention as claimed. Thus, it will be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims and statements of the invention.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

The following statements describe some of the elements or features of the invention.

Statements:

1. A method comprising:
   (a) measuring quantities of vimentin mRNA, NKCC2 mRNA, and E-cadherin mRNA in a test sample of cells obtained from urine;
   (b) determining whether the vimentin mRNA quantity is higher, the NKCC2 mRNA quantity is lower or the E-cadherin mRNA is higher than in healthy urinary cells; and

   thereby detecting that the sample is a fibrotic kidney sample.
2. The method of statement 1, further comprising measuring a housekeeping gene RNA quantity.
3. The method of statement 1 or 2, further comprising measuring a housekeeping gene mRNA quantity, and normalizing the vimentin mRNA quantity, the NKCC2 mRNA quantity, or the E-cadherin mRNA against the housekeeping gene mRNA quantity.
4. The method of statement 1 or 2, further comprising measuring a housekeeping gene mRNA quantity, and dividing the vimentin mRNA quantity, the NKCC2 mRNA quantity, or the E-cadherin mRNA by the housekeeping gene mRNA quantity.
5. The method of any of statements of 2-4, wherein the housekeeping gene is 18S rRNA, actin mRNA, histone mRNA, ribosomal protein mRNA, myosin mRNA, cytochrome c mRNA, β2-microglobulin mRNA, or major histocompatibility complex mRNA.

6. The method of any of statements of 2-5, wherein the housekeeping gene is 18S rRNA.
7. The method of any of statements 1-6, wherein the vimentin mRNA quantity is divided by the quantity of 18S rRNA/100,000 in the sample to generate a normalized vimentin mRNA value.
8. The method of any of statements 1-7, wherein the vimentin mRNA quantity is divided by the quantity of 18S rRNA/100,000 in the sample to generate a normalized vimentin mRNA value, which is converted into a $\log_{10}$ normalized vimentin mRNA value.
9. The method of any of statements 1-8, wherein detecting that the sample is a fibrotic kidney sample comprises determining that the $\log_{10}$ normalized vimentin mRNA value is at least 5.2, or at least 5.3, or at least 5.4, or at least 5.5, or at least 5.6 (as illustrated in FIG. 3A).
10. The method of any of statements 1-9, wherein the NKCC2 mRNA quantity is divided by the quantity of 18S rRNA/100,000 in the sample to generate a normalized NKCC2 mRNA value.
11. The method of any of statements 1-10, wherein the NKCC2 mRNA quantity is divided by the quantity of 18S rRNA/100,000 in the sample to generate a normalized NKCC2 mRNA value, which is converted into a $\log_{10}$ normalized NKCC2 mRNA value.
12. The method of any of statements 1-11, wherein detecting that the sample is a fibrotic kidney sample comprises determining that the $\log_{10}$ normalized NKCC2 mRNA value is 2.5 or less, or 2.4 or less, or 2.3 or less, or 2.2 or less, or 2.1 or less, or 2.0 or less (as illustrated in FIG. 3B).
13. The method of any of statements 1-12, wherein the E-cadherin mRNA quantity is divided by the quantity of 18S rRNA/100,000 in the sample to generate a normalized E-cadherin mRNA value.
14. The method of any of statements 1-13, wherein the E-cadherin mRNA quantity is divided by the quantity of 18S rRNA/100,000 in the sample to generate a normalized E-cadherin mRNA value, which is converted into a $\log_{10}$ normalized E-cadherin mRNA value.
15. The method of any of statements 1-14, wherein detecting that the sample is a fibrotic kidney sample comprises determining that the $\log_{10}$ normalized E-

cadherin mRNA value is at least 2.7, or at least 2.8, or at least 2.9, or at least 3.0, or at least 3.1 (as illustrated in FIG. 3C).

16. The method of any of statements 1-15, wherein measuring quantities of vimentin mRNA, NKCC2 mRNA, and E-cadherin mRNA comprises reverse transcription, polymerase chain reaction preamplification, real-time quantitative polymerase chain reaction, microarray analysis, Northern blotting, nuclease protection assays, RNA fingerprinting, ligase chain reaction, Qbeta replicase, isothermal amplification method, strand displacement amplification, transcription based amplification systems, quantitative nucleic acid amplification, combined reverse transcription/nucleic acid amplification, nuclease protection, Serial Analysis Gene Expression (SAGE), next generation sequencing, gene expression microarray, and combinations thereof.
17. The method of any of statements 1-16, wherein measuring quantities of vimentin mRNA, NKCC2 mRNA, and E-cadherin mRNA comprises reverse transcription, polymerase chain reaction preamplification, real-time quantitative polymerase chain reaction, or a combination thereof.
18. The method of any of statements 1-17, wherein a nucleic acid measuring quantities of vimentin mRNA, NKCC2 mRNA, and E-cadherin mRNA comprises use of a probe or primer that can stringently hybridizes to a nucleic acid encoding an amino acid sequence selected from the group consisting of SEQ ID NO:2, 4, 6, 8, 10 or a combination thereof.
19. The method of any of statements 1-18, wherein a nucleic acid measuring quantities of vimentin mRNA, NKCC2 mRNA, and E-cadherin mRNA comprises use of a probe or primer that can stringently hybridizes to a nucleic acid comprising any of SEQ ID NO:1, 3, 5, 7, 9, 12, 13, 14, 69, 70, 71, 75, 76, 77, or a combination thereof.
20. The method of any of statements 1-19, wherein a nucleic acid measuring quantities of 18S rRNA comprises use of a probe or primer that can stringently hybridize to a nucleic acid comprising any of SEQ ID NO: 11, 78, 79, 81, or a combination thereof.
21. The method of any of statements 1-20, wherein a nucleic acid measuring quantities of vimentin mRNA, NKCC2 mRNA, and E-cadherin mRNA comprises use of a probe or primer that has at least 90% or at least 95%

sequence identity or sequence complementarity to any of SEQ ID NO:1, 3, 5, 7, 9, 12, 13, 14, 69, 70, 71, 75, 76, 77, or a combination thereof.

22. The method of any of statements 1-21, wherein a nucleic acid measuring quantities of 18S rRNA comprises use of a probe or primer that has at least 90% or at least 95% sequence identity or sequence complementarity to any of SEQ ID NO: 11, 78, 79, 81, or a combination thereof.

23. The method of any of statements 1-22, further comprising assigning a composite score calculated as follows:

$$\begin{aligned}\text{Composite Score} = \; & 36.10283 + [-15.84215*\log_{10}(18s)] \\ & + 1.56907*\log_{10}(18s)*\log_{10}(18s) \\ & + 5.11698*\max[0, \log_{10}(\text{Vimentin}) - 5.6] \\ & + [-1.44145*\log_{10}(\text{NKCC2})] \\ & + 3.31357*\min[3.1, \log_{10}(\text{E-cadherin})]\end{aligned}$$

where,

$$\max[0, \log_{10}(\text{Vimentin}) - 5.6] = 0 \text{ whenever } \log_{10}(\text{Vimentin}) \text{ is} \leq 5.6 \text{ and } = \log_{10}(\text{Vimentin}) - 5.6 \text{ whenever } \log_{10}(\text{Vimentin}) \text{ is} > 5.6;$$

$$\min[3.1, \log_{10}(\text{E-cadherin})] = \log_{10}(\text{E-cadherin}) \text{ whenever } \log_{10}(\text{E-cadherin}) < 3.1 \text{ and } = 3.1 \text{ whenever } \log_{10}(\text{E-cadherin}) \geq 3.1;$$

and * signifies multiplication.

24. The method of statement 23, wherein:

$\log_{10}$(18s RNA) is $\log_{10}$ of 18S RNA quantity/100,000 in the test sample;

$\log_{10}$(Vimentin) is $\log_{10}$ of the normalized vimentin mRNA quantity in the test sample;

$\log_{10}$(NKCC2)] is $\log_{10}$ of the normalized NKCC2 mRNA quantity in the test sample; and

$\log_{10}$(E-cadherin) is $\log_{10}$ of the normalized E-cadherin mRNA quantity in the test sample.

25. The method of statement 23 or 24, wherein the vimentin mRNA quantity, the NKCC2 mRNA quantity, and the E-cadherin mRNA quantity is divided by the 18S rRNA quantity divided by 100,000 (i.e., [18S rRNA]/100,000) before generating the $\log_{10}$(Vimentin), the $\log_{10}$(NKCC2) , and the $\log_{10}$(E-cadherin) values, respectively.

26. The method of any of statements 23-25, wherein the composite score varies from about 1 to 8, and a normal (healthy) composite score is about 3.5.
27. The method of any of statements 23-26, wherein a test sample with a composite score of about 4.7 indicates a subject has fibrosis.
28. The method of any of statements 23-27, wherein a test sample with a composite score of about 4.7 to about 6.5 indicates a subject has mild to moderate fibrosis; and/or a test sample with a composite score of about 6.5 or more indicates a subject has moderate to severe fibrosis.
29. The method of any of statements 1-28, further comprising treating a subject from which the fibrotic kidney sample was obtained.
30. The method of any of statements 1-29, further comprising treating a subject from which the fibrotic kidney sample was obtained by administering a therapeutic agent to the subject.
31. The method of any of statements 1-30, further comprising treating a subject from which the fibrotic kidney sample was obtained by administering to the subject a therapeutic agent selected from an agent that treats the underlying cause(s) of kidney fibrosis, an agent that delays the progression of kidney fibrosis, an agent that ameliorates the symptoms of kidney fibrosis, or a combination thereof.
32. The method of any of statements 1-31, further comprising replacing a kidney in a subject from which the fibrotic kidney sample was obtained.
33. A method comprising:
    (a) measuring quantities of vimentin RNA, NKCC2 RNA, E-cadherin RNA and 18S rRNA in a test sample of cells obtained from a subject's urine to generate a vimentin RNA quantity value [vimentin], a NKCC2 RNA quantity value [NKCC2], an E-cadherin RNA quantity value [E-cadherin], and an 18S rRNA quantity value [18S rRNA];
    (b) identifying the subject as a patient who would benefit from treatment of kidney fibrosis when:
        (i) the $\log_{10}$([vimentin] / [18S rRNA/ 10^5]) value is greater than about 5.0;
        (ii) the $\log_{10}$([NKCC2] / [18S rRNA] / 10^5) value is less than about 3.0; or

(iii) the $\log_{10}$([E-cadherin] / [18S rRNA/ 10^5]) value is greater than about 3.0.

34. The method of statement 33, further comprising treating the patient who would benefit from treatment of kidney fibrosis.
35. The method of statement 33 or 34, further comprising treating the patient who would benefit from treatment of kidney fibrosis by administering a therapeutic agent to the patient.
36. The method of any of statements 33-35, further comprising treating the patient who would benefit from treatment of kidney fibrosis by administering to the patient a therapeutic agent selected from an agent that treats the underlying cause(s) of kidney fibrosis, an agent that delays the progression of kidney fibrosis, an agent that ameliorates the symptoms of kidney fibrosis, or a combination thereof.
37. The method of any of statements 33-36, further comprising replacing a kidney in the patient who would benefit from treatment of kidney fibrosis.
38. The method of any of statements 33-37, further comprising any of the methods of statements 1-32.
39. A method comprising: treating kidney fibrosis in a subject when a sample of urinary cells from the subject has a vimentin mRNA quantity that is higher, a NKCC2 mRNA quantity that is lower, or an E-cadherin mRNA that is higher than in healthy urinary cells.
40. A method comprising: treating kidney fibrosis in a subject when a sample of urinary cells from the subject has:
    (i) a $\log_{10}$([vimentin] / [18S rRNA]) value is greater than about 5.0;
    (ii) a $\log_{10}$([NKCC2] / [18S rRNA]) value is less than about 3.0;
    (iii) a $\log_{10}$([E-cadherin] / [18S rRNA]) value is greater than about 3.0; or
    (iv) a combination thereof.
41. A method comprising: treating kidney fibrosis in a subject when a sample of urinary cells from the subject has:
    (i) a $\log_{10}$([vimentin] / [18S rRNA / 10^5]) value greater than about 5.0;

(ii) a $\log_{10}$([NKCC2] / [18S rRNA / 10^5]) value less than about 3.0;

(iii) a $\log_{10}$([E-cadherin] / [18S rRNA / 10^5]) value greater than about 3.0; or

5 (iv) a combination thereof.

42. The method of any of statements 39-41, further comprising any of the methods of statements 1-32.

10 The claims summarize features of the invention.

WHAT IS CLAIMED:

1. A method comprising:
   a) measuring quantities of vimentin mRNA, NKCC2 mRNA, and E-cadherin mRNA in a test sample of cells obtained from urine;
5 b) determining whether the vimentin mRNA quantity is higher, the NKCC2 mRNA quantity is lower, or the E-cadherin mRNA is higher than in healthy urinary cells; and

   thereby detecting whether or not the sample is a fibrotic kidney sample.
2. The method of claim 1, further comprising measuring a housekeeping gene
10 RNA quantity.
3. The method of claim 1 or 2, further comprising measuring a housekeeping gene mRNA quantity, and normalizing the vimentin mRNA quantity, the NKCC2 mRNA quantity, or the E-cadherin mRNA against the housekeeping gene mRNA quantity.
15 4. The method of claim 2 or 3, wherein the housekeeping gene is 18S rRNA, actin mRNA, histone mRNA, ribosomal protein mRNA, myosin mRNA, cytochrome c mRNA, β2-microglobulin mRNA, or major histocompatibility complex mRNA.
5. The method of any of claims of 2-4, wherein the housekeeping gene is 18S
20 rRNA.
6. The method of any of claims 1-5, wherein the vimentin mRNA quantity is divided by the quantity of 18S rRNA/100,000 in the sample to generate a normalized vimentin mRNA value.
7. The method of any of claims 1-6, wherein the vimentin mRNA quantity is
25 divided by the quantity of 18S rRNA/100,000 in the sample to generate a normalized vimentin mRNA value, which is converted into a log10 normalized vimentin mRNA value.
8. The method of any of claims 1-7, wherein detecting that the sample is a fibrotic kidney sample comprises determining that the $\log_{10}$ normalized
30 vimentin mRNA value is at least 5.2.
9. The method of any of claims 1-8, wherein the NKCC2 mRNA quantity is divided by the quantity of 18S rRNA/100,000 in the sample to generate a normalized NKCC2 mRNA value.

10. The method of any of claims 1-9, wherein the NKCC2 mRNA quantity is divided by the quantity of 18S rRNA/100,000 in the sample to generate a normalized NKCC2 mRNA value, which is converted into a $\log_{10}$ normalized NKCC2 mRNA value.
5 11. The method of any of claims 1-10, wherein detecting that the sample is a fibrotic kidney sample comprises determining that the $\log_{10}$ normalized NKCC2 mRNA value is 2.5 or less.
12. The method of any of claims 1-11, wherein the E-cadherin mRNA quantity is divided by the quantity of 18S rRNA/100,000 in the sample to generate a
10 normalized E-cadherin mRNA value.
13. The method of any of claims 1-12, wherein the E-cadherin mRNA quantity is divided by the quantity of 18S rRNA/100,000 in the sample to generate a normalized E-cadherin mRNA value, which is converted into a $\log_{10}$ normalized E-cadherin mRNA value.
15 14. The method of any of claims 1-13, wherein detecting that the sample is a fibrotic kidney sample comprises determining that the $\log_{10}$ normalized E-cadherin mRNA value is at least 2.7.
15. The method of any of claims 1-14, wherein measuring quantities of vimentin mRNA, NKCC2 mRNA, and E-cadherin mRNA comprises
20 reverse transcription, polymerase chain reaction preamplification, real-time quantitative polymerase chain reaction, microarray analysis, Northern blotting, nuclease protection assays, RNA fingerprinting, ligase chain reaction, Qbeta replicase, isothermal amplification method, strand displacement amplification, transcription based amplification systems,
25 quantitative nucleic acid amplification, combined reverse transcription/nucleic acid amplification, nuclease protection, Serial Analysis Gene Expression (SAGE), next generation sequencing, gene expression microarray, and combinations thereof.
16. The method of any of claims 1-15, wherein a nucleic acid measuring
30 quantities of vimentin mRNA, NKCC2 mRNA, and E-cadherin mRNA comprises use of a probe or primer that can stringently hybridizes to a nucleic acid encoding an amino acid sequence selected from the group consisting of SEQ ID NO:2, 4, 6, 8, 10 or a combination thereof.

17. The method of any of claims 1-16, wherein a nucleic acid measuring quantities of 18S rRNA comprises use of a probe or primer that can stringently hybridize to a nucleic acid comprising any of SEQ ID NO: 11, 78, 79, 81, or a combination thereof.

5 18. The method of any of claims 1-17, further comprising:

(a) measuring quantities of vimentin RNA, NKCC2 RNA, E-cadherin RNA and 18S rRNA in a test sample of cells obtained from a subject's urine to generate a vimentin RNA quantity value [vimentin], a NKCC2 RNA quantity value [NKCC2], an E-cadherin RNA quantity value [E-cadherin],

10 and an 18S rRNA quantity value [18S rRNA];

(b) identifying the subject as a patient who would benefit from treatment of kidney fibrosis when:

(ii) the $\log_{10}$([vimentin] / [18S rRNA / 10^5]) value is greater than about 5.0;

15 (iii) the $\log_{10}$([NKCC2] / [18S rRNA / 10^5]) value is less than about 3.0; or

(iv) the $\log_{10}$([E-cadherin] / [18S rRNA / 10^5]) value is greater than about 3.0.

20 19. The method of any of claims 1-18, further comprising assigning a composite score to the test sample, which is calculated as follows:

Composite Score = 36.10283 + [-15.84215*$\log_{10}$(18s)]
+ 1.56907*$\log_{10}$(18s)*$\log_{10}$(18s)
+ 5.11698*max[0, $\log_{10}$(Vimentin) - 5.6]
25 + [-1.44145* $\log_{10}$(NKCC2)]
+ 3.31357*min[3.1, $\log_{10}$(E-cadherin)]

where,
$\log_{10}$(18s RNA) is $\log_{10}$ of 18S RNA quantity/10^5 in the test sample;
30 $\log_{10}$(Vimentin) is $\log_{10}$ of normalized vimentin mRNA quantity in the test sample;
$\log_{10}$(NKCC2)] is $\log_{10}$ of normalized NKCC2 mRNA quantity in the test sample;
$\log_{10}$(E-cadherin) is $\log_{10}$ of normalized E-cadherin mRNA quantity in the test
35 sample.

max[0, $\log_{10}$(Vimentin) - 5.6] = 0 whenever $\log_{10}$(Vimentin) is $\leq$ 5.6 and
= $\log_{10}$(Vimentin) - 5.6 whenever $\log_{10}$(Vimentin) is >5.6;

min[3.1, $\log_{10}$(E-cadherin)] = $\log_{10}$(E-cadherin) whenever $\log_{10}$(E-cadherin)<3.1 and = 3.1 whenever $\log_{10}$(E-cadherin) $\geq$ 3.1; and

* signifies multiplication.

5

20. The method of claim 19, wherein the composite score varies from about 1 to 8, and a normal composite score is about 3.5.
21. The method of claim 19 or 20, wherein a test sample with a composite score of about 4.7 indicates a subject has fibrosis.
22. The method of any of claims 1-21, further comprising treating a subject from which the fibrotic kidney sample was obtained. 10
23. A method comprising: treating kidney fibrosis in a subject when a test sample of urinary cells from the subject has a vimentin mRNA quantity that is higher, a NKCC2 mRNA quantity that is lower, or an E-cadherin mRNA that is higher than in healthy urinary cells. 15
24. A method comprising: treating kidney fibrosis in a subject when a test sample of urinary cells from the subject has:
    (i) the $\log_{10}$([vimentin] / [18S rRNA] / 10^5) value is greater than about 5.0;
    (ii) the $\log_{10}$([NKCC2] / [18S rRNA] / 10^5) value is less than about 3.0; or 20
    (iii) the $\log_{10}$([E-cadherin] / [18S rRNA] / 10^5) value is greater than about 3.0.
25. A method comprising: treating kidney fibrosis in a subject when a test sample of urinary cells from the subject has a composite score that is calculated as follows: 25

Composite Score = 36.10283 + [-15.84215*$\log_{10}$(18s RNA)]
+ 1.56907*$\log_{10}$(18s RNA)*$\log_{10}$(18s RNA)
+ 5.11698*max[0, $\log_{10}$(Vimentin) - 5.6] 30
+ [-1.44145* $\log_{10}$(NKCC2)]
+ 3.31357*min[3.1, $\log_{10}$(E-cadherin)]

where,
$\log_{10}$(18s RNA) is $\log_{10}$ of 18S RNA quantity/10^5 in the test sample; 35
$\log_{10}$(Vimentin) is $\log_{10}$ of a normalized vimentin mRNA quantity in the test sample;
$\log_{10}$(NKCC2)] is $\log_{10}$ of a normalized NKCC2 mRNA quantity in the test sample;

$\log_{10}$(E-cadherin) is $\log_{10}$ of a normalized E-cadherin mRNA quantity in the test sample;

max[0, $\log_{10}$(Vimentin) - 5.6] = 0 whenever $\log_{10}$(Vimentin) is $\leq$ 5.6 and
= $\log_{10}$(Vimentin) - 5.6 whenever $\log_{10}$(Vimentin) is >5.6;

5

min[3.1, $\log_{10}$(E-cadherin)] = $\log_{10}$(E-cadherin) whenever $\log_{10}$(E-cadherin)<3.1 and = 3.1 whenever $\log_{10}$(E-cadherin) ≥ 3.1; and

* signifies multiplication.

10

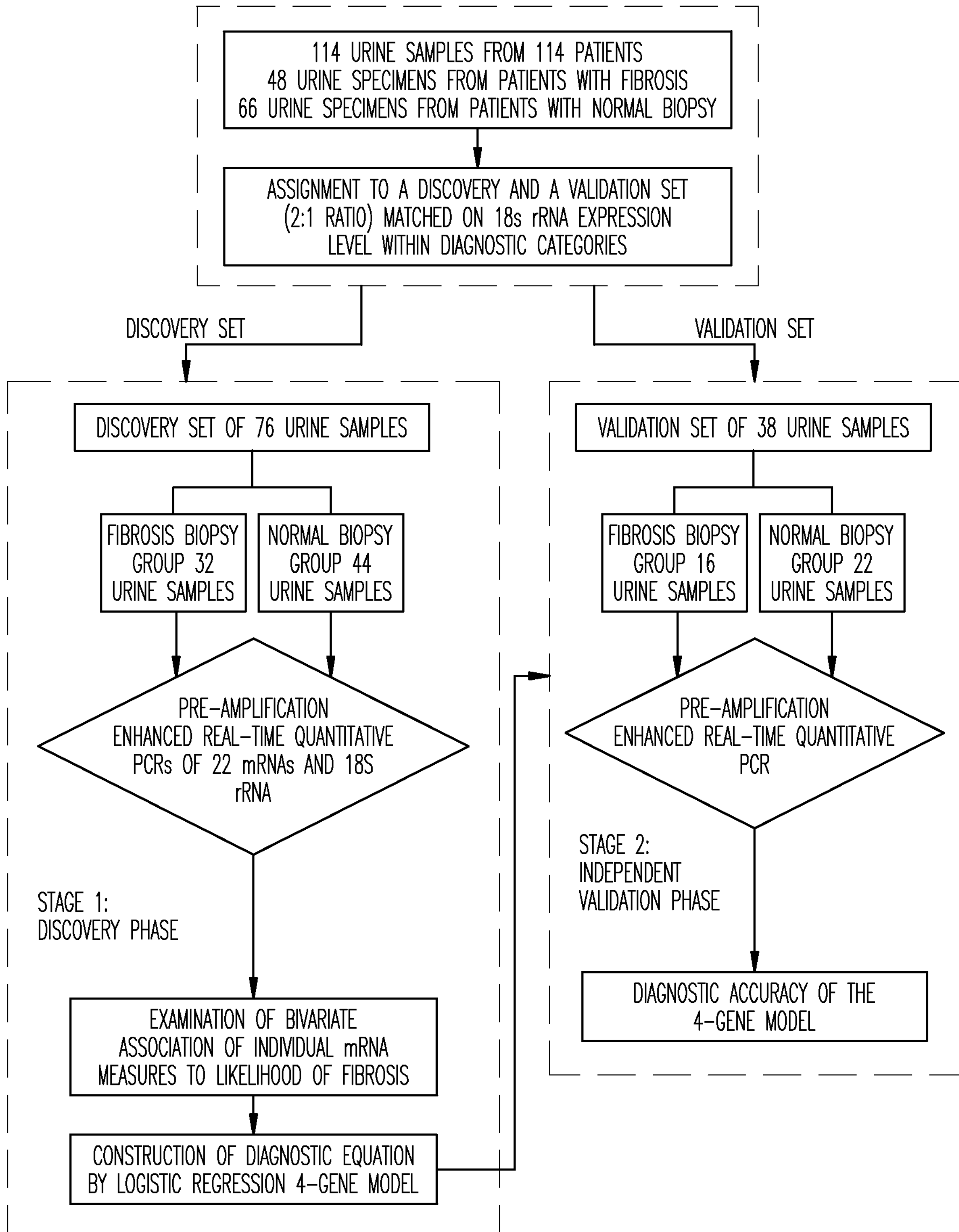


Fig. 1A

2/22

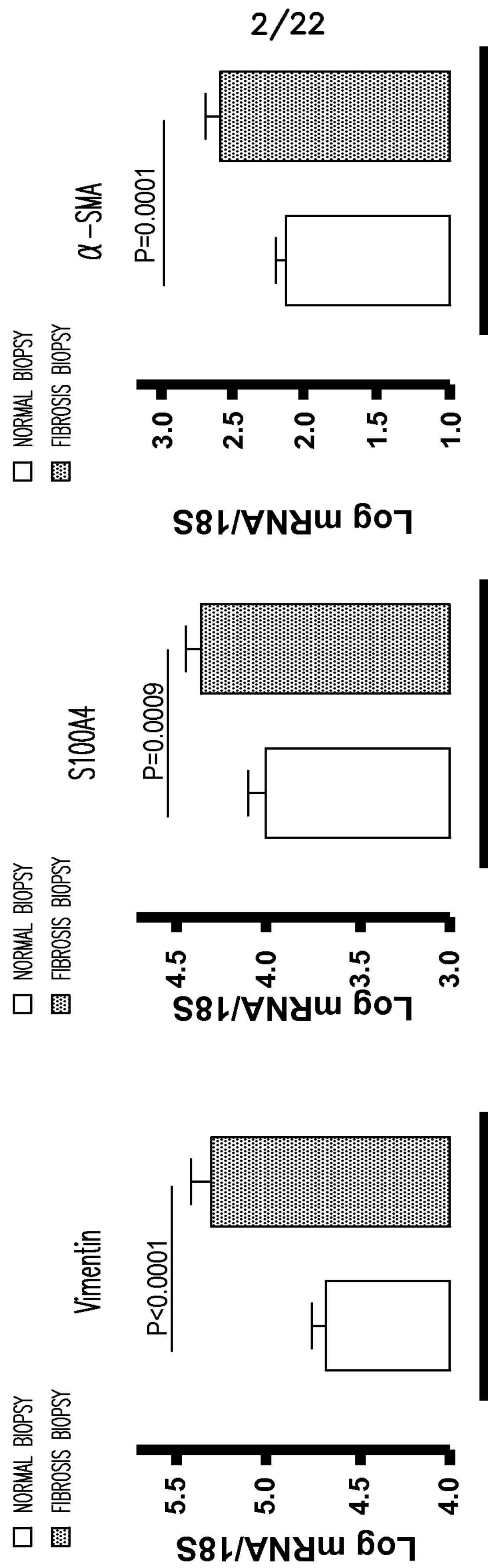


Fig. 1B1

Fig. 1B2

Fig. 1B3

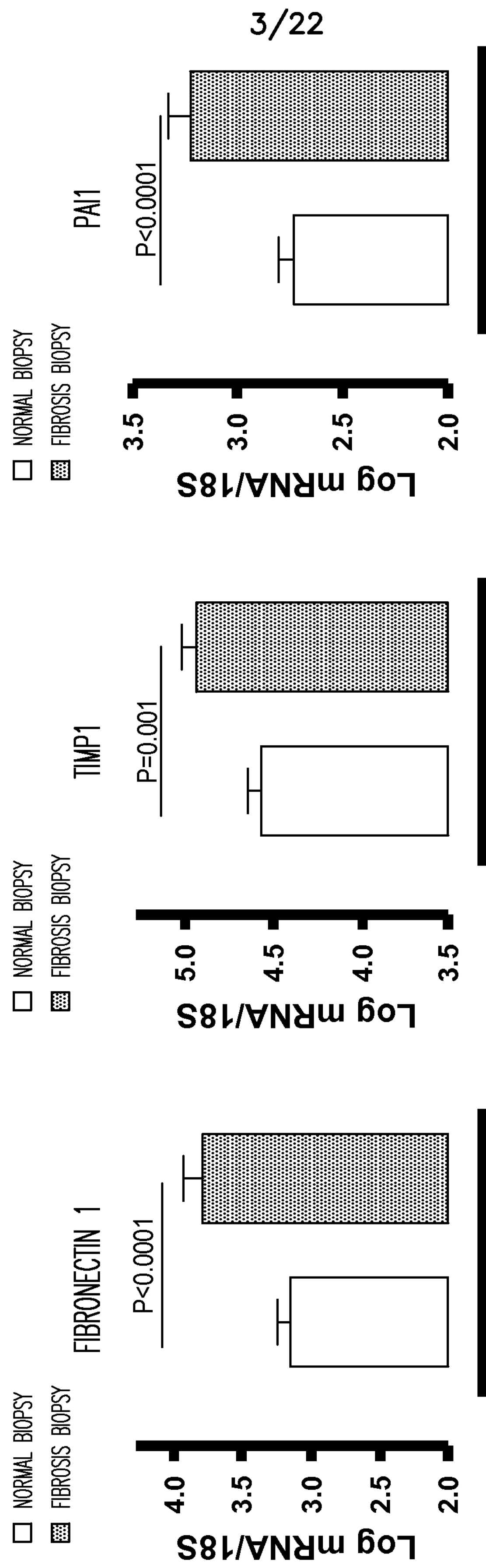


Fig. 1B4

Fig. 1B5

Fig. 1B6

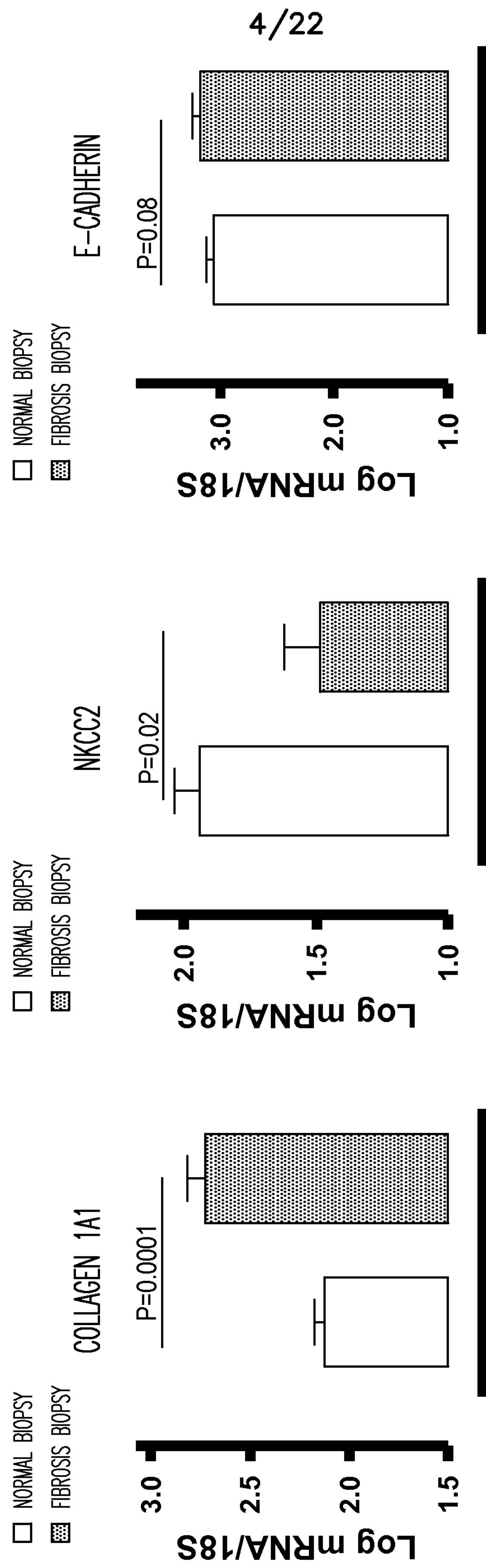


Fig. 1B7

Fig. 1B8

Fig. 1B9

5/22

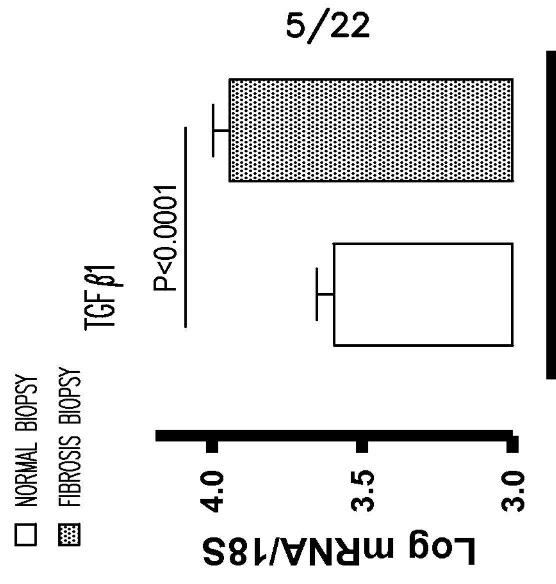


Fig. 1B12

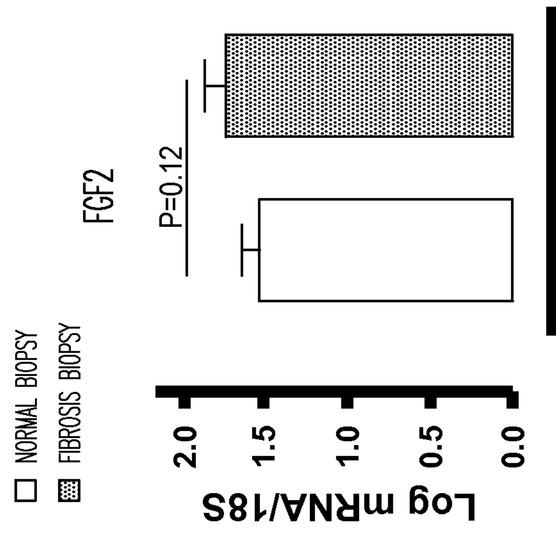


Fig. 1B11

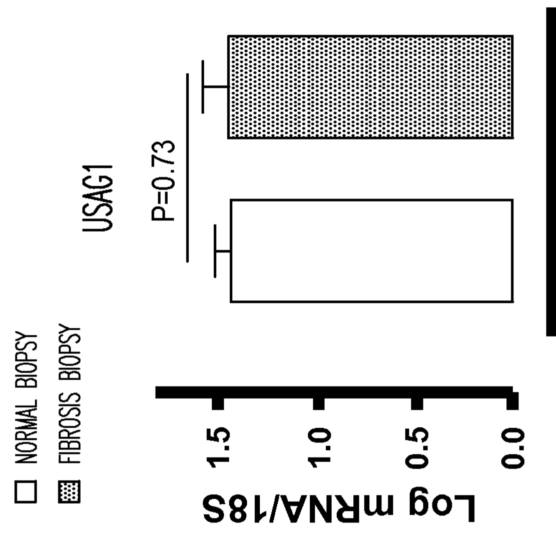


Fig. 1B10

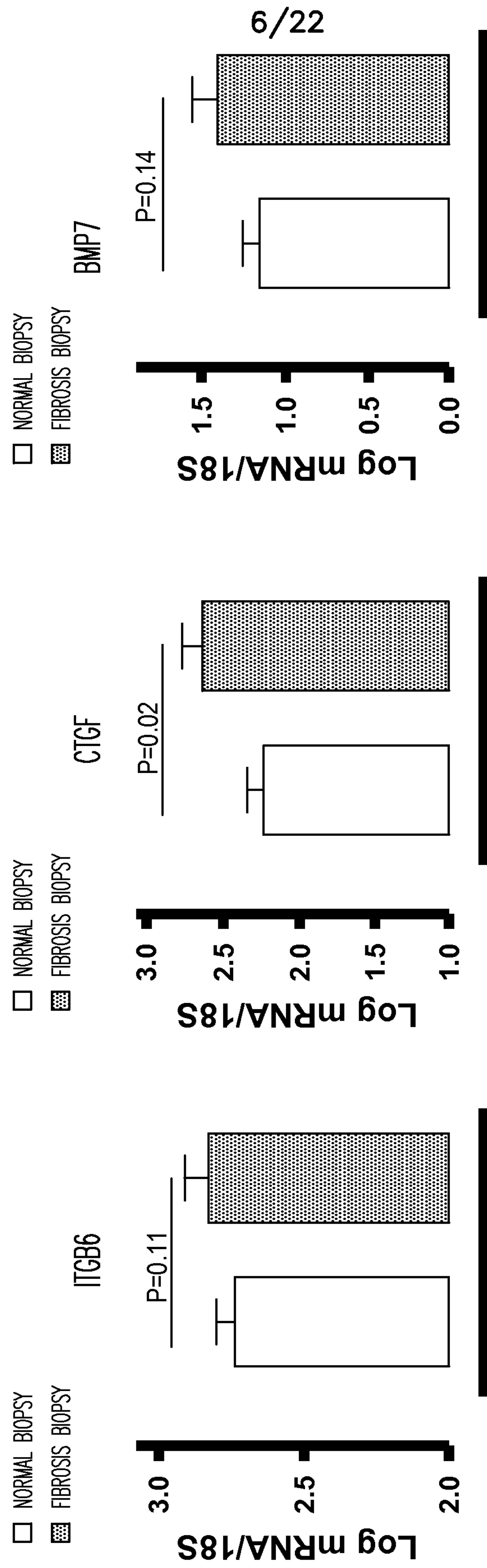


Fig. 1B13

Fig. 1B14

Fig. 1B15

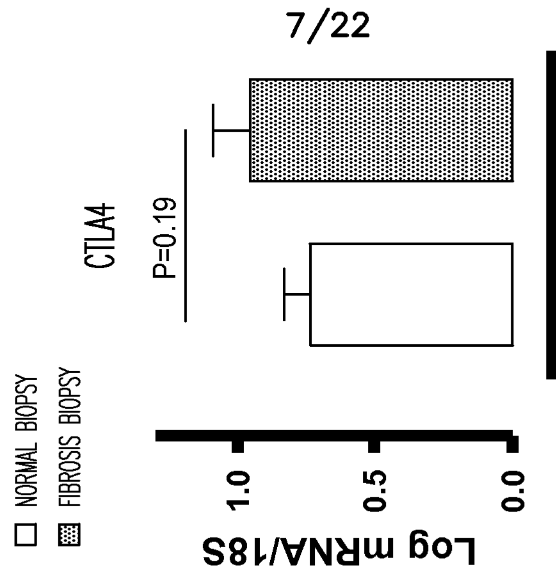


Fig. 1B18

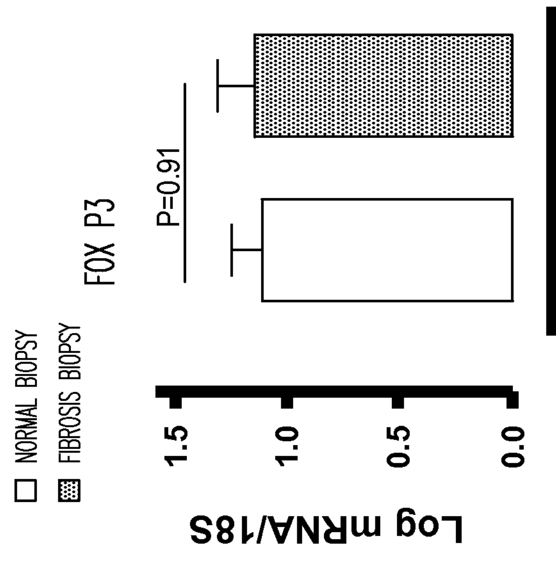


Fig. 1B17

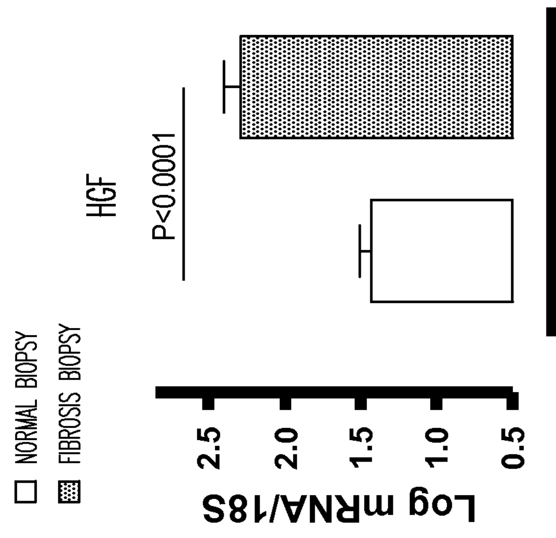


Fig. 1B16

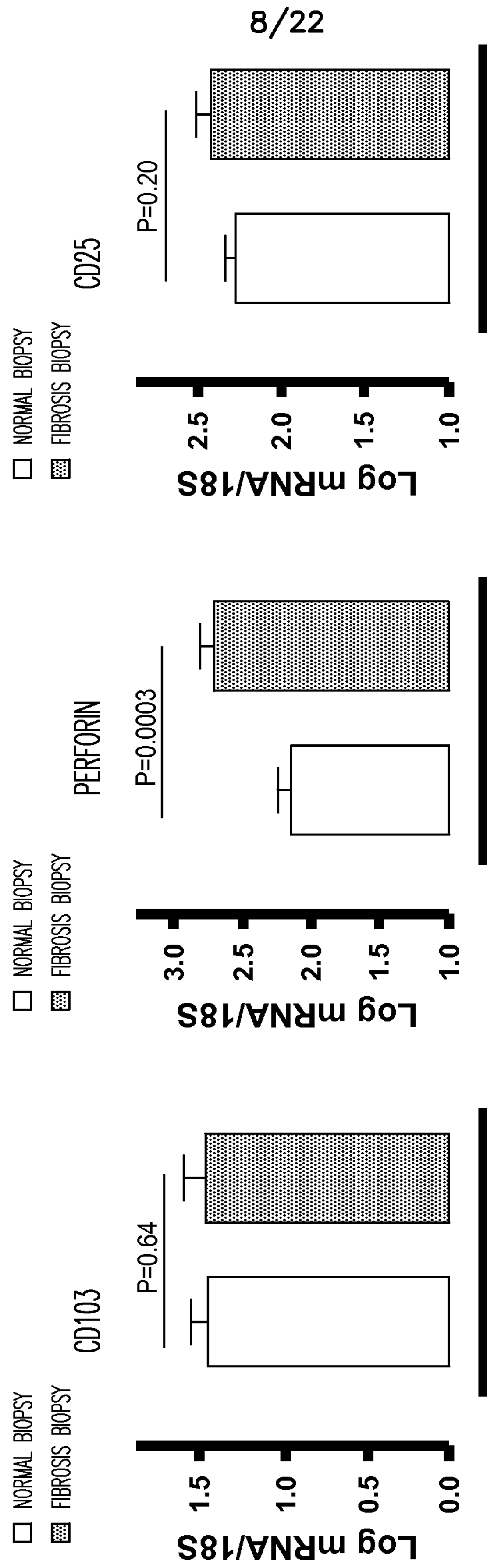


Fig. 1B19

Fig. 1B20

Fig. 1B21

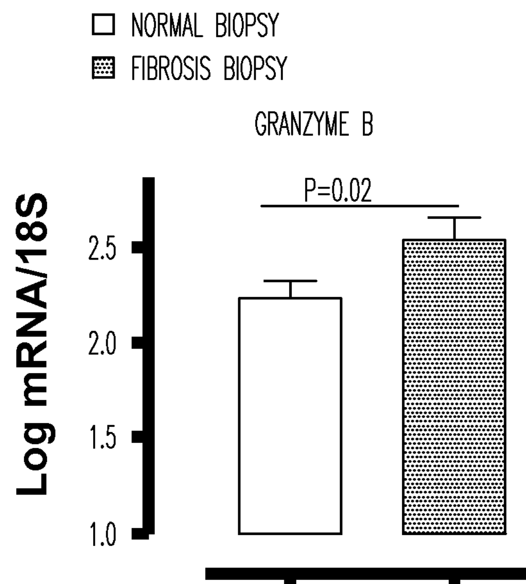


Fig.1B22

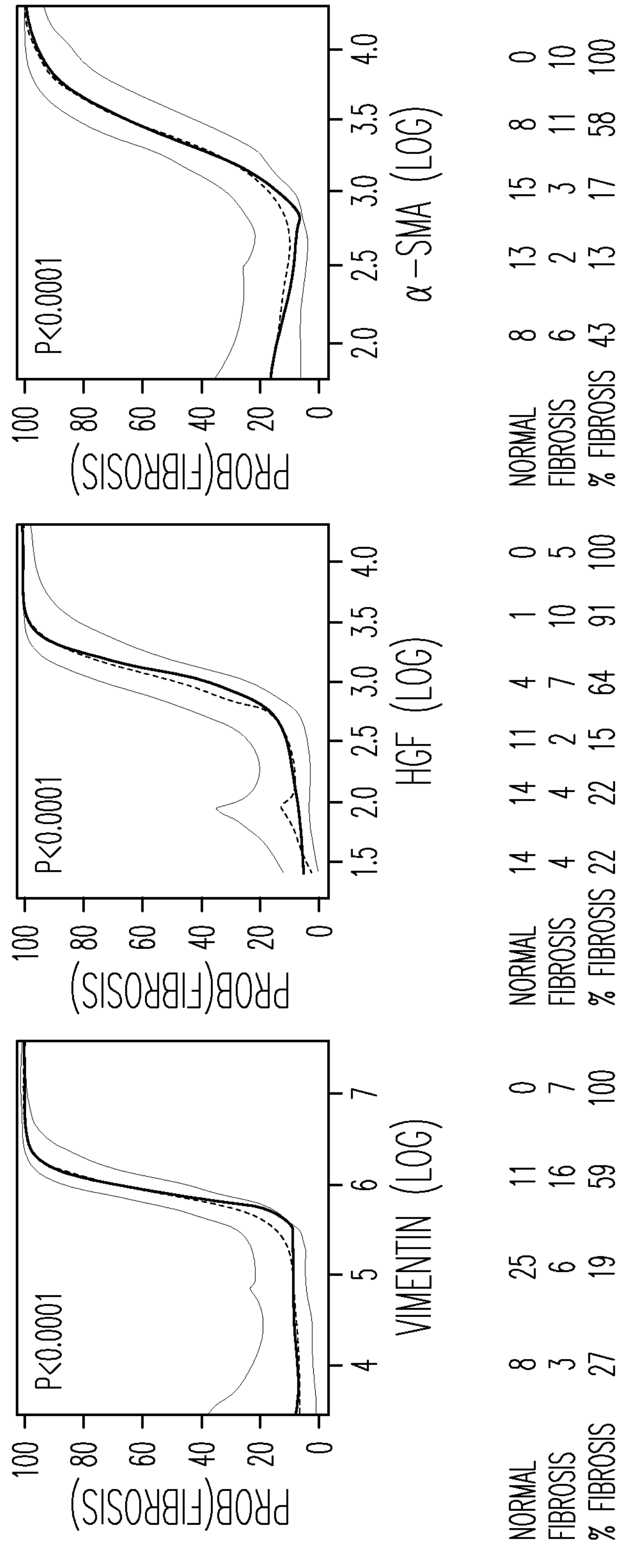


Fig.2A1

Fig.2A2

Fig.2A3

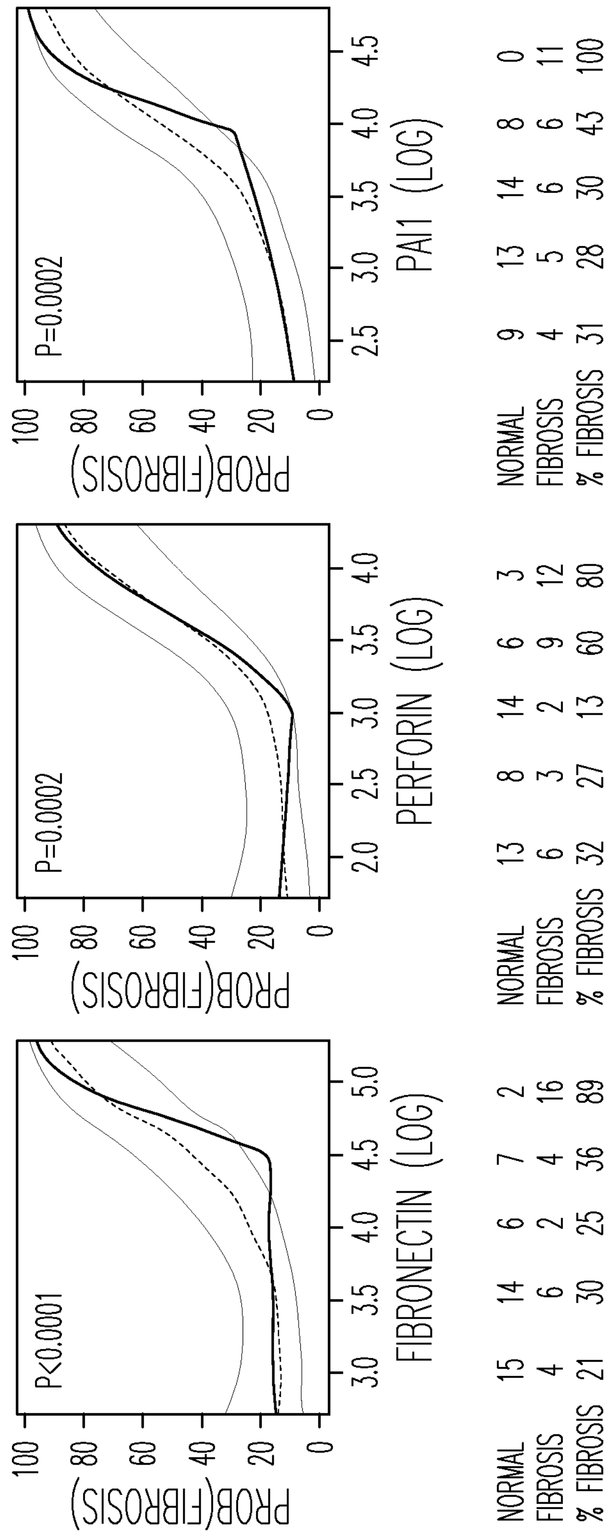


Fig.2A4

Fig.2A5

Fig.2A6

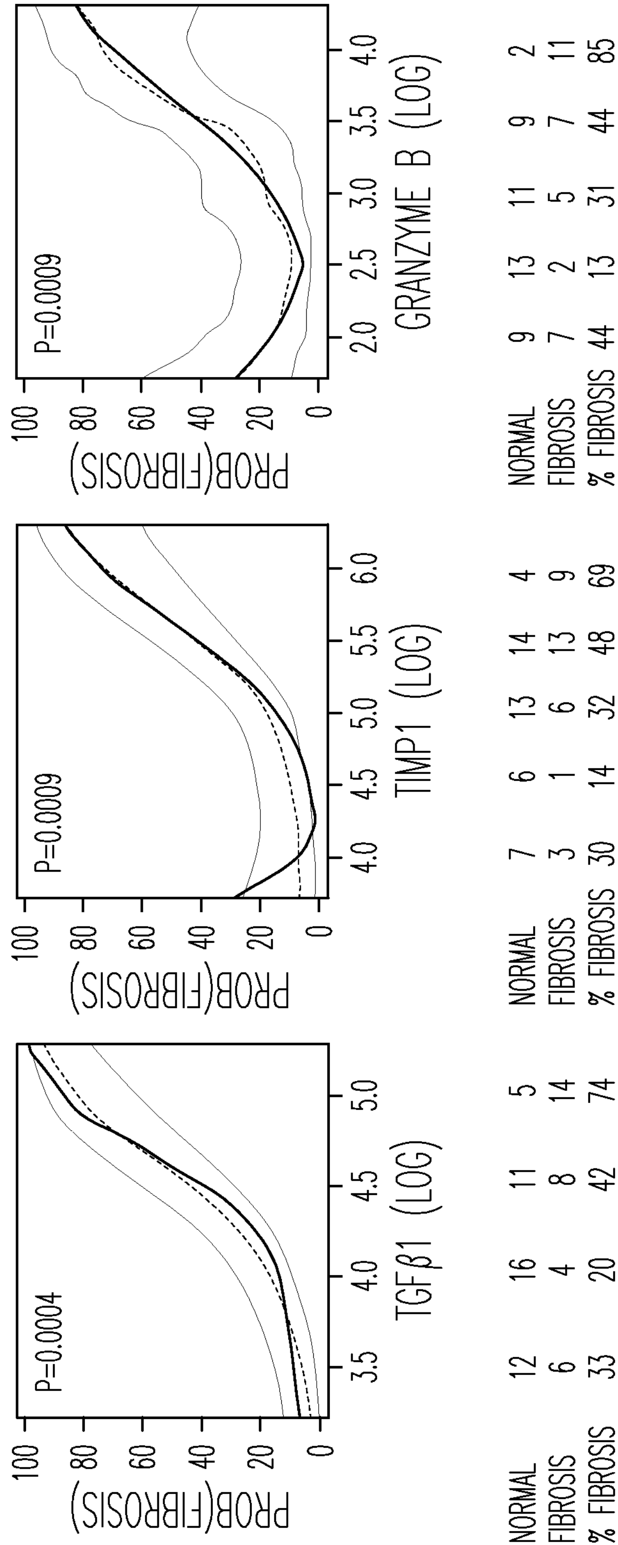


Fig.2A7

Fig.2A8

Fig.2A9

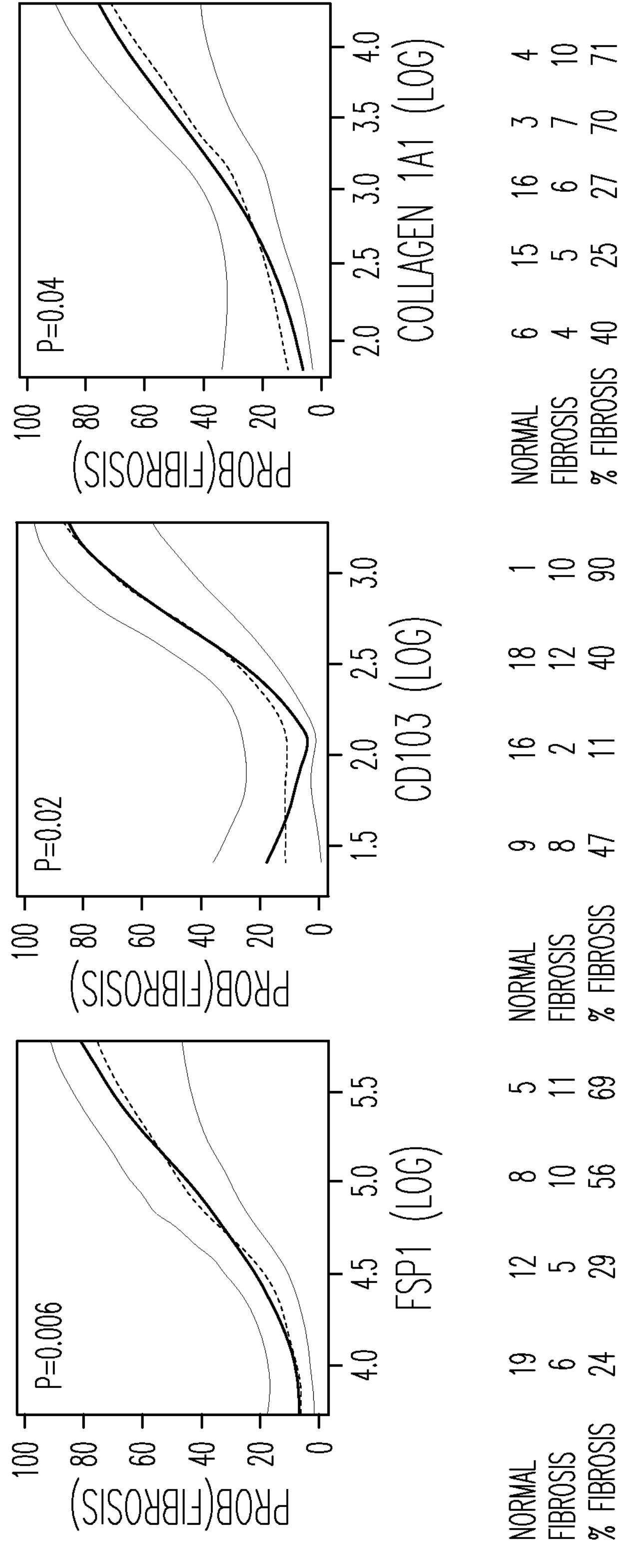


Fig.2A10

Fig.2A11

Fig.2A12

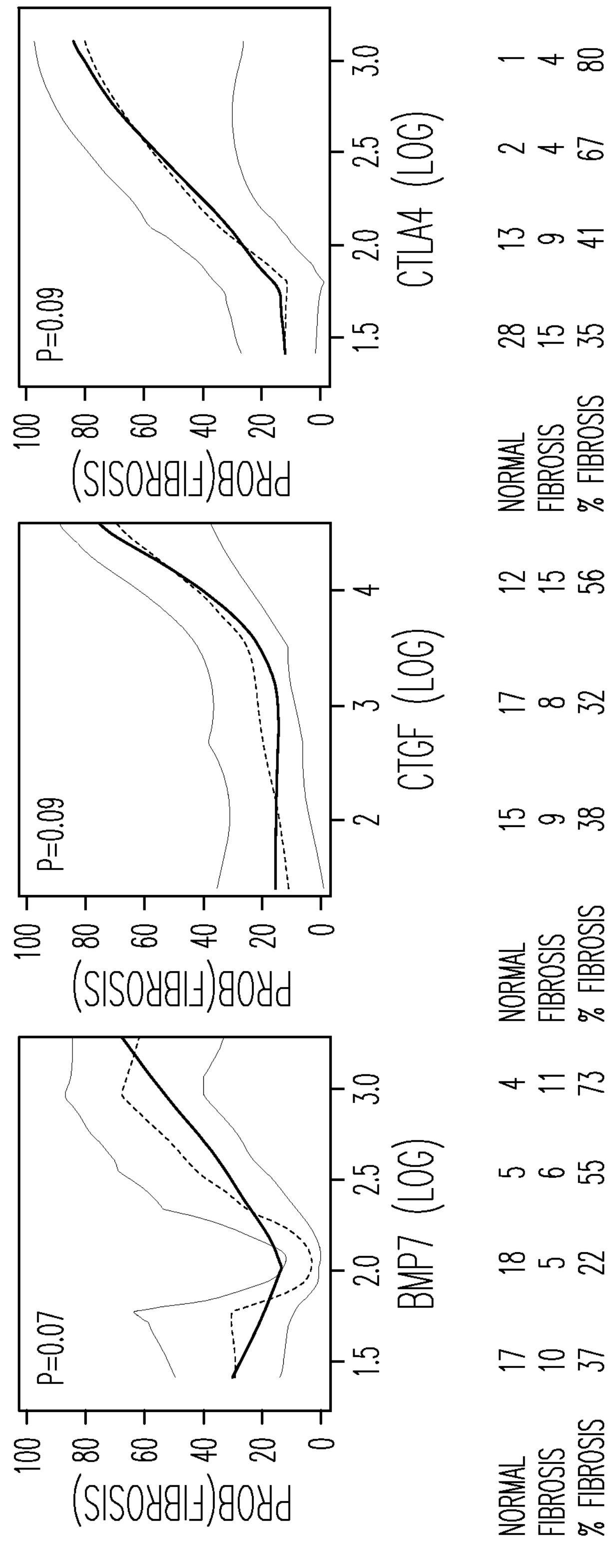


Fig.2B1

Fig.2B2

Fig.2B3

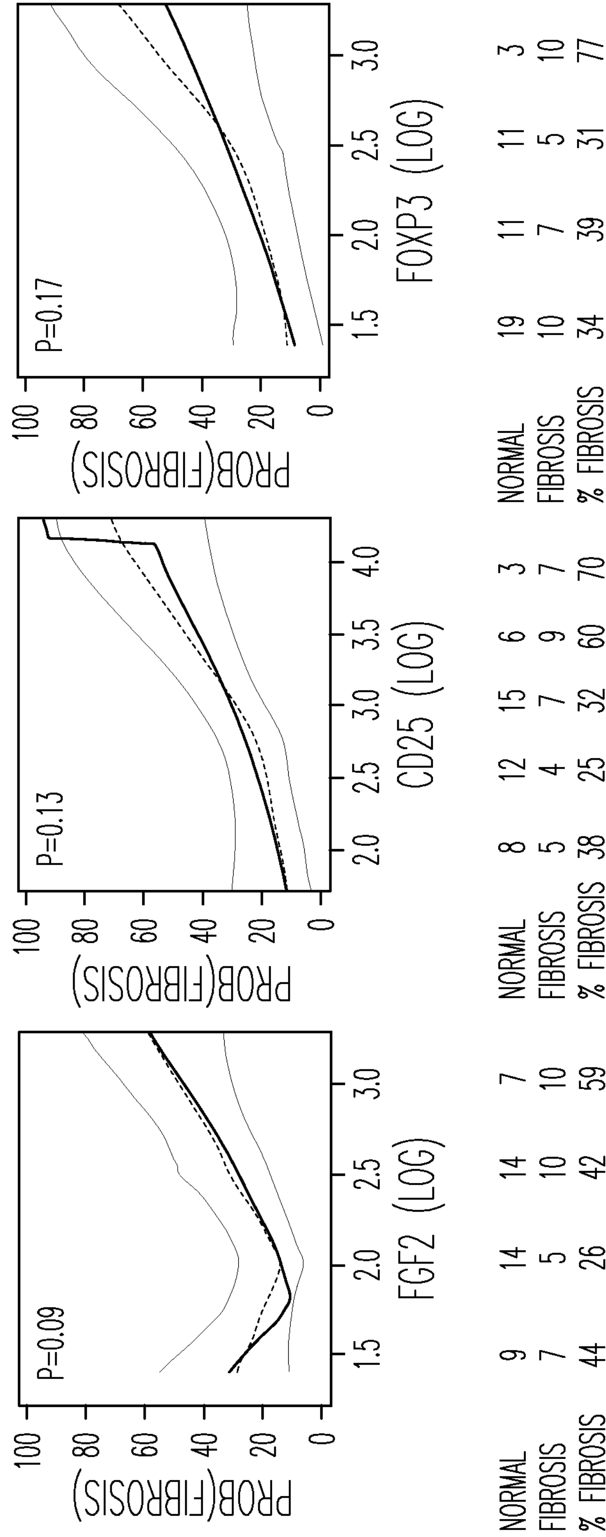


Fig.2B4

Fig.2B5

Fig.2B6

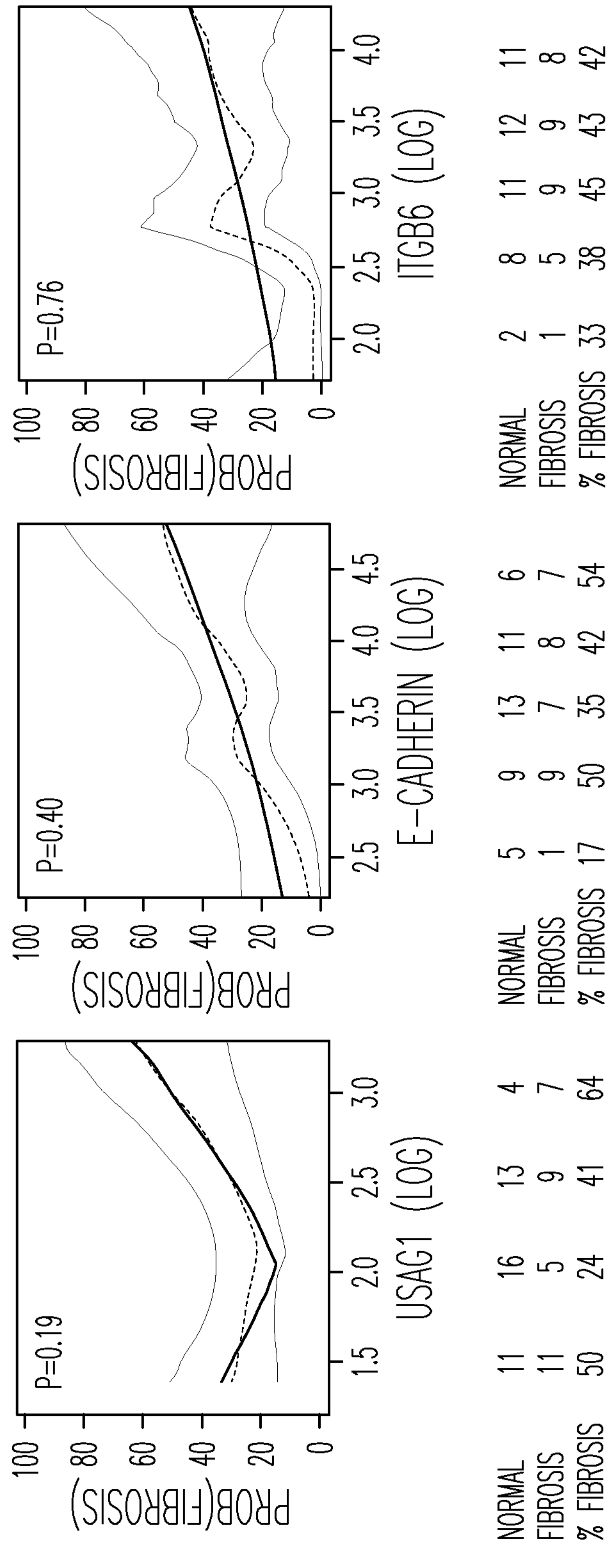
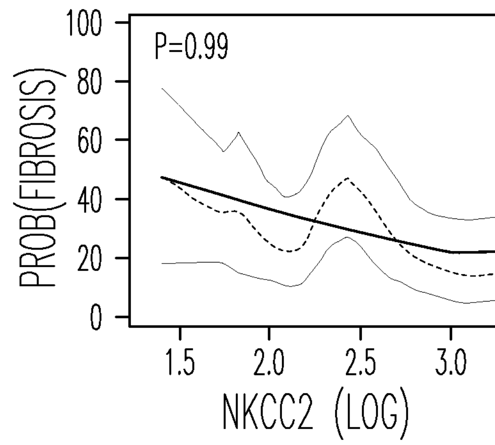


Fig.2B7

Fig.2B8

Fig.2B9



NORMAL	4	14	11	15
FIBROSIS	6	9	11	6
% FIBROSIS	60	39	50	29

Fig.2B10

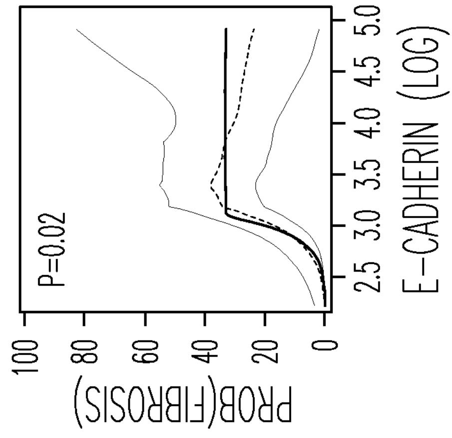


Fig.3C

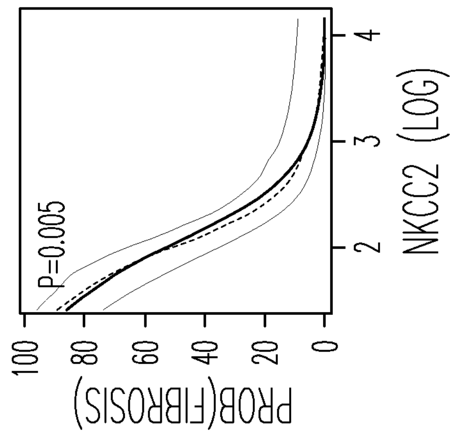


Fig.3B

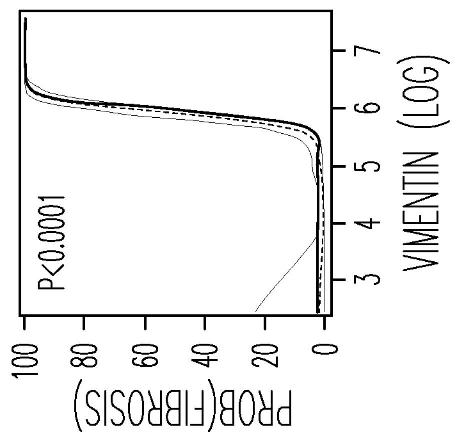


Fig.3A

VARIABLE	B	SE	95%CI	P
LOG_{10}(18S rRNA)	−15.84	7.41	−30.37 TO −1.31	0.03
LOG_{10}(18S rRNA)^2	1.57	0.81	−0.02 TO 3.16	0.05
MAX (0, LOG_{10}(VIMENTIN)−5.6)	5.12	1.31	2.55 TO 7.68	0.0001
LOG_{10}(NKCC2)	−1.44	0.51	−2.44 TO −0.44	0.005
MIN (3.1 LOG_{10}(E−CADHERIN))	3.31	1.44	0.49 TO 6.14	0.02
CONSTANT	36.10			

Fig.3D

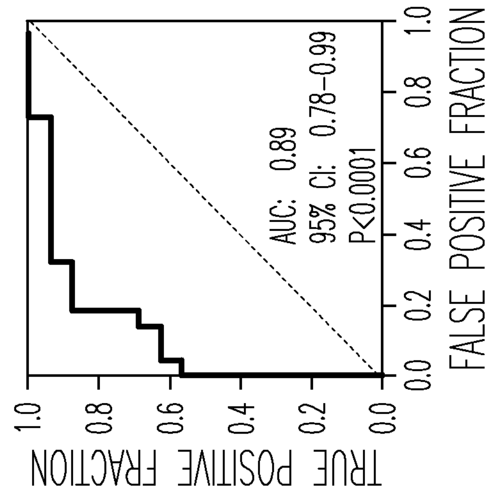


Fig. 4C

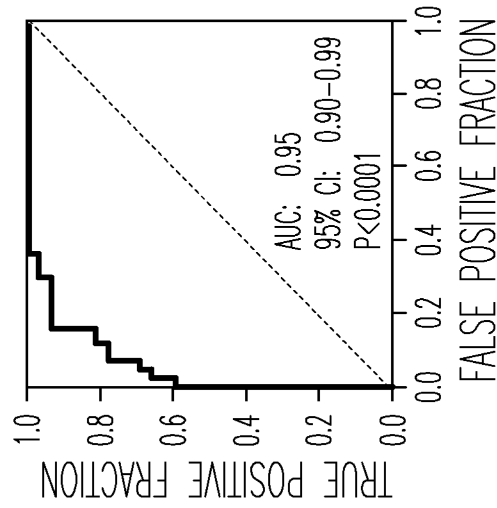


Fig. 4B

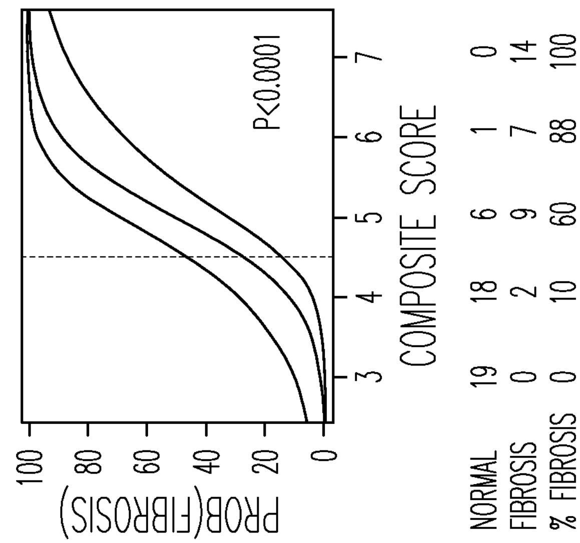


Fig. 4A

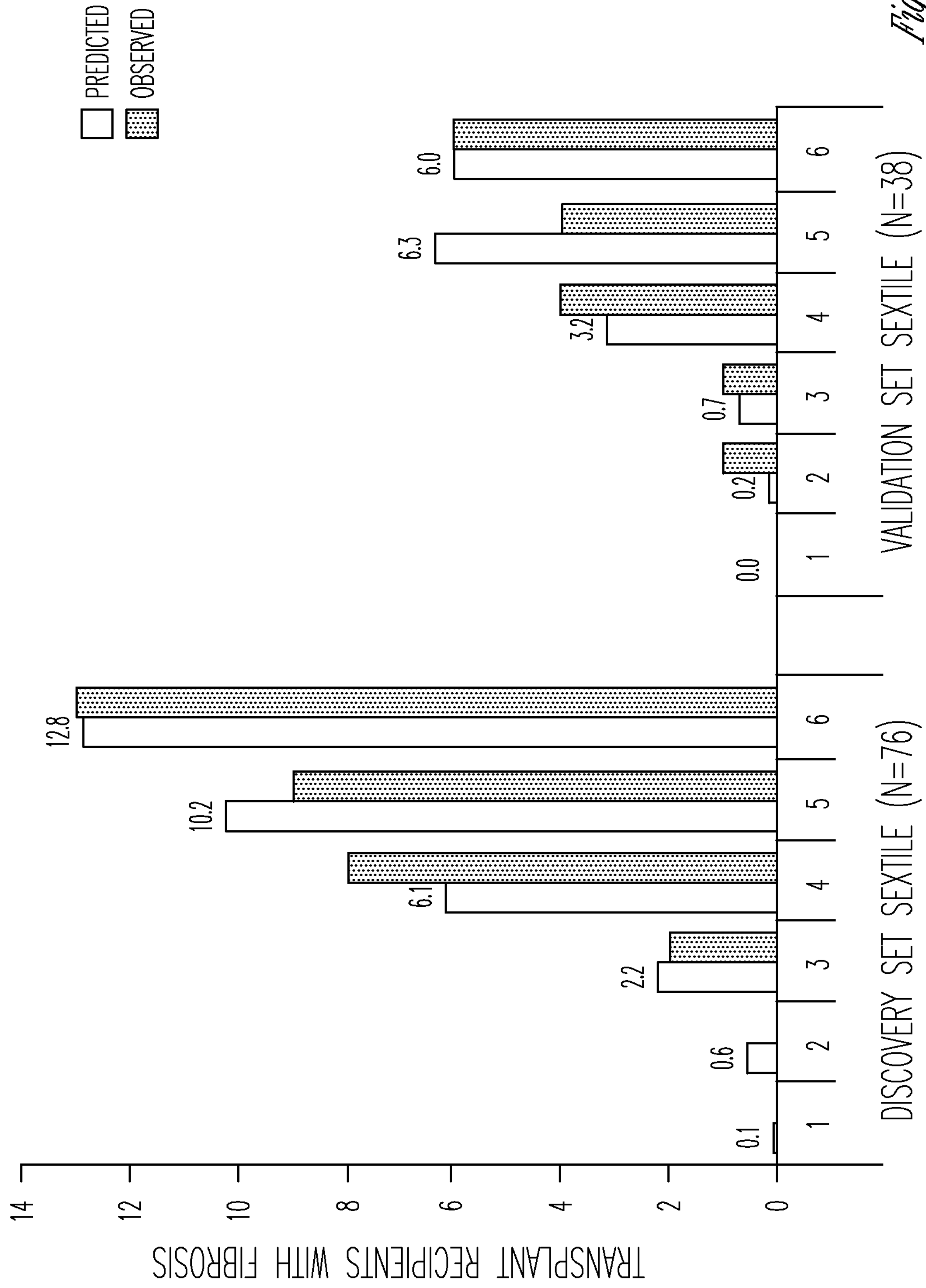


Fig. 4D

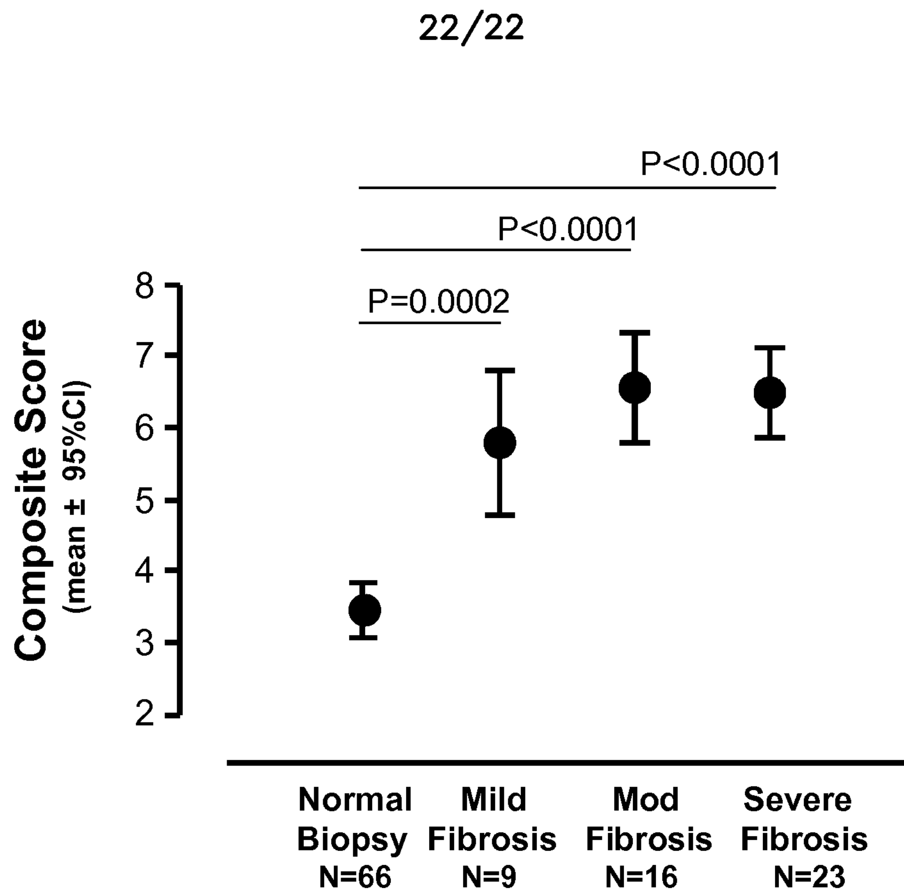


Fig.5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/041206

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/68 (2013.01)
USPC - 435/6.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 35/12; C12N 5/0686, 15/09; C12Q 1/68; G01N 33/68, 33/5023 (2013.01)
USPC - 435/6.1, 6.13, 6.17, 325, 375; 514/15.4, 44R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C12N 2310/141; C12Q 1/6883, 2600/158; G01N 2333/78, 2800/347 (2013.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatBase, Google Patents, Google, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	ANGLICHEAU et al. 'Epithelial-Mesenchymal Transition (EMT) and Fibrosis Are Rapidly Induced during Acute Rejection (AR) of Human Renal Allografts.' Poster presentation (F-PO2089). J AM Soc Nephrol 20: 2009. entire document	1-3 -------- 24, 25
X	WO 2011/054893 A2 (HOGABOAM et al) 12 May 2011 (12.05.2011) entire document	23
Y	US 2011/0288134 A1 (MAKSUMOVA et al) 24 November 2011 (24.11.2011) entire document	24, 25
A	CHENG et al. 'Connective Tissue Growth Factor is a Biomarker and Mediator of Kidney Allograft Fibrosis.' AM J Transplant. 6(10): Pages 2292-22306. 04 August 2006. entire document	1-3, 23-25
A	KANEYAMA et al. 'Tranilast modulates fibrosis, epithelial-mesenchymal transition and peritubular capillary injury in unilateral ureteral obstruction rats.' Pathology 42(6): Pages 564-573. October 2010. entire document	1-3, 23-25
P, X	ANGLICHEAU et al. 'Discovery and Validation of a Molecular Signature for the Noninvasive Diagnosis of Human Renal Allograft Fibrosis.' Transplantation. 93(11): Pages 1136-1146. 15 June 2012. entire document	1-3, 23-25

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 September 2013	11 OCT 2013

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-22
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.