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(54) **Diagnosing and monitoring cardiac allograft rejection based on marker expression level**

Diagnose und Überwachung von Herz-Allotransplantatabstossung mittels Markerexpressionsniveau

Diagnostic et surveillance du rejet d'une allogreffe cardiaque basés sur le niveau d'expression d'un marqueur

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(73) Proprietor: **XDx, Inc.**
San Francisco CA 94080 (US)

(72) Inventors:
- **Wohlgemuth, Jay**
  Menlo Park, CA 94303 (US)
- **Fry, Kirk**
  Palo Alto, CA 94303 (US)
- **Woodward, Robert**
  Pleasanton, CA 94558 (US)
- **Ly, Ngoc**
  San Bruno, CA 94066 (US)
- **Prentice, James**
  San Francisco, 94027 (US)
- **Morris, MacDonald**
  Atherton, CA 94027 (US)
- **Rosenberg, Steven**
  Oakland, CA 94682 (US)

(74) Representative: **Holmberg, Martin Tor**
Bergenstråhle & Lindvall AB
P.O. Box 17704
118 93 Stockholm (SE)

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Description

Related Applications

[0001] This application claims priority to U.S. Application No. 10/131,831, filed April 24, 2002, and U.S. Application No. 10/325,899, filed December 20, 2002.

Field of the Invention

[0002] This invention is in the field of expression profiling following organ transplantation.

Background of the Invention

[0003] Many of the current shortcomings in diagnosis, prognosis, risk stratification and treatment of disease can be approached through the identification of the molecular mechanisms underlying a disease and through the discovery of nucleotide sequences (or sets of nucleotide sequences) whose expression patterns predict the occurrence or progression of disease states, or predict a patient's response to a particular therapeutic intervention. In particular, identification of nucleotide sequences and sets of nucleotide sequences with such predictive value from cells and tissues that are readily accessible would be extremely valuable. For example, peripheral blood is attainable from all patients and can easily be obtained at multiple time points at low cost. This is a desirable contrast to most other cell and tissue types, which are less readily accessible, or accessible only through invasive and aversive procedures. In addition, the various cell types present in circulating blood are ideal for expression profiling experiments as the many cell types in the blood specimen can be easily separated if desired prior to analysis of gene expression. While blood provides a very attractive substrate for the study of diseases using expression profiling techniques, and for the development of diagnostic technologies and the identification of therapeutic targets, the value of expression profiling in blood samples rests on the degree to which changes in gene expression in these cell types are associated with a predisposition to, and pathogenesis and progression of a disease.

[0004] Hematopoiesis is the development and maturation of all cell types of the blood. These include erythrocytes, platelets and leukocytes. Leukocytes are further subdivided into granulocytes (neutrophils, eosinophils, basophils) and mononuclear cells (monocytes, lymphocytes). These cells develop and mature from precursor cells to replenish the circulating pool and to respond to insults and challenges to the system. This occurs in the bone marrow, spleen, thymus, liver, lymph nodes, mucosal associated lymphoid tissue (MALT) and peripheral blood.

[0005] Precursor cells differentiate into immature forms of each lineage and these immature cells develop further into mature cells. This process occurs under the influence and direction of hematopoietic growth factors. When hematopoiesis is stimulated, there is an increase in the number of immature cells in the peripheral blood and in some cases, precursor cells are found at increased frequency. For example, CD34+ cells (hematopoietic stem cells) may increase in frequency in the peripheral blood with an insult to the immune system. For neutrophils, "band" forms are increased, for erythrocytes, reticulocytes or nucleated red cells are seen. Lymphocytes are preceeded by lymphoblasts (immature lymphocytes).

[0006] It may be an important clinical goal to measure the rate of production of blood cells of a variety of lineages. Hematological disorders involving over or under production of various blood cells may be treated pharmacologically. For example, anemia (low red blood cells) may be treated with erythropoietin (a hematopoietic growth factor) and response to this therapy can be assessed by measuring RBC production rates. Low neutrophils counts can be treated by administration of G-CSF and this therapy may be monitored by measuring neutrophil production rates. Alternatively, the diagnosis of blood cell disorders is greatly facilitated by determination of lineage specific production rates. For example, anemia (low RBCs) may be caused by decreased cellular production or increased destruction of cells. In the latter case, the rate of cellular production will be increased rather than decreased and the therapeutic implications are very different. Further discussion of the clinical uses of measures of blood cell production rates is given in below.

[0007] Assessment of blood cell production rates may be useful for diagnosis and management of non-hematological disorders. In particular, acute allograft rejection diagnosis and monitoring may benefit from such an approach. Current diagnosis and monitoring of acute allograft rejection is achieved through invasive allograft biopsy and assessment of the biopsy histology. This approach is sub-optimal because of expense of the procedure, cost, pain and discomfort of the patient, the need for trained physician operators, the risk of complications of the procedure, the lack of insight into the functioning of the immune system and variability of pathological assessment. In addition, biopsy can diagnose acute allograft rejection only after significant cellular infiltration into the allograft has occurred. At this point, the process has already caused damage to the allograft. For all these reasons, a simple blood test that can diagnose and monitor acute rejection at an earlier stage in the process is needed. Allograft rejection depends on the presence of functioning cells of the immune system. In addition, the process of rejection may cause activation of hematopoiesis. Finally, effective immunosuppressive therapy to treat or prevent acute rejection may suppress hematopoiesis. For these reasons, as-

sessment of hematopoietic cellular production rates may be useful in the diagnosis and monitoring of acute rejection.

[0008] Current techniques for measuring cellular development and production rates are inadequate. The most common approach is to measure the number of mature cells of a lineage of interest over time. For example, if a patient is being treated for anemia (low red blood cell counts), then the physician will order a blood cell count to assess the number of red blood cells (RBCs) in circulation. For this to be effective, the physician must measure the cell count over time and may have to wait 2-4 weeks before being able to assess response to therapy. The same limitation is true for assessment of any cell lineage in the blood.

[0009] An alternative approach is to count the number of immature cells in the peripheral blood by counting them under the microscope. This may allow a more rapid assessment of cellular production rates, but is limited by the need for assessment by a skilled hematologist, observer variability and the inability to distinguish all precursor cells on the basis of morphology alone.

[0010] Bone marrow biopsy is the gold standard for assessment of cellular production rates. In addition to the limitations of the need for skilled physicians, reader variability and the lack of sensitivity of morphology alone, the technique is also limited by the expense, discomfort to the patient and need for a prolonged visit to a medical center. Thus there is a need for a reliable, rapid means for measuring the rate of hematopoeisis in a patient.

[0011] In addition to the relationship between hematopoiesis and variety of disease processes, there is an extensive literature supporting the role of leukocytes, e.g., T-and B-lymphocytes, monocytes and granulocytes, including neutrophils, in a wide range of disease processes, including such broad classes as cardiovascular diseases; inflammatory, autoimmune and rheumatic diseases, infectious diseases, transplant rejection, cancer and malignancy, and endocrine diseases. For example, among cardiovascular diseases, such commonly occurring diseases as atherosclerosis, restenosis, transplant vasculopathy and acute coronary syndromes all demonstrate significant T cell involvement (Smith-Norowitz et al. (1999) Clin Immunol 93:168-175; Jude et al. (1994) Circulation 90:1662-8; Belch et al. (1997) Circulation 95:2027-31). These diseases are now recognized as manifestations of chronic inflammatory disorders resulting from an ongoing response to an injury process in the arterial tree (Ross et al. (1999) Ann Thorac Surg 67:1428-33). Differential expression of lymphocyte, monocyte and neutrophil genes and their products has been demonstrated clearly in the literature. Particularly interesting are examples of differential expression in circulating cells of the immune system that demonstrate specificity for a particular disease, such as arteriosclerosis, as opposed to a generalized association with other inflammatory diseases, or for example, with unstable angina rather than quiescent coronary disease.

[0012] A number of individual genes, e.g., CD11b/CD18 (Kassirer et al. (1999) Am Heart J 138:555-9); leukocyte elastase (Amaro et al. (1995) Eur Heart J 16:615-22; and CD40L (Aukrust et al. (1999) Circulation 100:614-20) demonstrate some degree of sensitivity and specificity as markers of various vascular diseases. In addition, the identification of differentially expressed target and fingerprint genes isolated from purified populations of monocytes manipulated in various in vitro paradigms has been proposed for the diagnosis and monitoring of a range of cardiovascular diseases, see, e.g., US Patents Numbers 6,048,709; 6,087,477; 6,099,823; and 6,124,433 "COMPOSITIONS AND METHODS FOR THE TREATMENT AND DIAGNOSIS OF CARDIOVASCULAR DISEASE" to Falb (*see also*, WO 97/30065). Lockhart, in US Patent Number 6,033,860 "EXPRESSION PROFILES IN ADULT AND FETAL ORGANS" proposes the use of expression profiles for a subset of identified genes in the identification of tissue samples, and the monitoring of drug effects.

[0013] MORGUN A. et al (TRANSPLANTATION PROCEEDINGS, vol. 1/02, no. 33, 1 February 2001) describes the identification of gene expression markers in blood and correlation with rejection of cardiac allografts. The markers identified are interferon gamma, TIRC7, perforin and Granzyme B.

[0014] The accuracy of technologies based on expression profiling for the diagnosis, prognosis, and monitoring of disease would be dramatically increased if numerous differentially expressed nucleotide sequences, each with a measure of specificity for a disease in question, could be identified and assayed in a concerted manner. PCT application WO 02/057414 "LEUKOCYTE EXPRESSION PROFILING" to Wohlgemuth identifies one such set of differentially expressed nucleotides.

[0015] In order to achieve this improved accuracy, the sets of nucleotide sequences once identified need to be validated to identify those differentially expressed nucleotides within a given set that are most useful for diagnosis, prognosis, and monitoring of disease. The present invention addresses these and other needs, and applies to transplant rejection and detection of the rate of hematopoeisis for which differential regulation of genes, or other nucleotide sequences, of peripheral blood can be demonstrated.

Summary of the Invention

[0016] In order to meet these needs, the present disclosure is thus directed to a system for detecting differential gene expression.

[0017] In a further variation, the invention is directed to a method of diagnosing or monitoring cardiac transplant rejection in a patient by detecting the expression level of one or more genes in the patient to diagnose or monitor cardiac

transplant rejection in the patient wherein the one or more genes include a nucleotide sequence selected according to SEQ ID NO:67,

[0018] In another aspect, the methods of diagnosing or monitoring transplant rejection include detecting the expression level of at least two of the genes. In another variation, methods of diagnosing or monitoring transplant rejection include detecting the expression level of at least ten of the genes. In a further variation, the methods of diagnosing or monitoring transplant rejection include detecting the expression level of at least one hundred of the genes. In still a further variation, the methods of diagnosing or monitoring transplant rejection include detecting the expression level of all the listed genes.

[0019] In another variation, transplant rejection may be heart transplant rejection

[0020] In another aspect, the methods of detecting transplant rejection include detecting the expression level by measuring the RNA level expressed by one or more genes. The method may further including isolating RNA from the patient prior to detecting the RNA level expressed by the one or more genes.

[0021] In one variation, the RNA level is detected by PCR. In a still further variation, the PCR uses primers consisting of nucleotide sequences selected from the group consisting of SEQ ID NO:731, SEQ ID NO:1062, SEQ ID NO:1704, SEQ ID NO:1952, SEQ ID NO:1393, SEQ ID NO:2200.

[0022] The RNA level may be detected by hybridization to the probes. In a further variation, the RNA level is detected by hybridization to an oligonucleotide. Examples of oligonucleotide include oligonucleotides having a nucleotide sequence according to SEQ ID NO:67,

[0023] The oligonucleotide has a nucleotide sequence consisting of SEQ ID NO: 107. The oligonucleotide may be DNA, RNA, cDNA, PNA, genomic DNA, or synthetic oligonucleotides.

[0024] In another aspect, the methods of detecting transplant rejection include detecting the expression level by measuring one or more proteins expressed by the one or more genes. In one variation, the one or more proteins include an amino acid sequence according to SEQ ID NO:2463,

[0025] The method of diagnosing or monitoring cardiac transplant rejection in a patient further provides for detecting the expression level of one or more genes in the patient to diagnose or monitor cardiac transplant rejection in the patient by measuring one or more proteins expressed by the one or more genes. The one or more proteins may include an amino acid sequence according to SEQ ID NO.2463,

[0026] Alternatively, the expression level of the one or more genes may be detected by measuring one or more proteins expressed by one or more genes, and one or more proteins expressed by one or more additional genes. In one variation, the one or more proteins expressed by the one or more genes include an amino acid sequence according to SEQ ID NO:2463,

[0027] Protein detection may be accomplished by measuring serum. In another variation, the protein is a cell surface protein. In a further variation, the measuring includes using a fluorescent activated cell sorter.

[0028] In another aspect, the disclosure is directed to a substantially purified oligonucleotide having the nucleotide sequence according to SEQ ID NO:67, and substantially purified oligonulcleotides having at least 90% sequence identity to an oligonucleotide having the nucleotide sequence according to SEQ ID NO:67,

[0029] In a further aspect, the disclosure is directed to a substantially purified oligonucleotide that hybridizes at high stringency to an oligonucleotide having the nucleotide sequence according to SEQ ID. NO:67,

[0030] The sequences may be used as diagnostic oligonucleotides for transplant rejection and/or cardiac transplant rejection. The oligonucleotide may have nucleotide sequence including DNA, cDNA, PNA, genomic DNA, or synthetic oligonucleotides.

[0031] In another aspect, the disclosure is directed to a method of diagnosing or monitoring transplant rejection in a patient wherein the expression level of one or more genes in a patient's bodily fluid is detected. In a further variation, the bodily fluid is peripheral blood.

[0032] In another aspect, the disclosure is directed to a method of diagnosing or monitoring transplant rejection in a patient, comprising detecting the expression level of four or more genes in the patient to diagnose or monitor transplant rejection in the patient wherein the four or more genes include a nucleotide sequence according to SEQ ID NO:67,

[0033] In a further aspect, the disclosure is also directed to a system for detecting gene expression in body fluid including at least two isolated polynucleotides wherein the isolated polynucleotides detect expression of a gene wherein the gene includes a nucleotide sequence according to SEQ ID NO:67, and the gene is differentially expressed in body fluid in an individual rejecting a transplanted organ compared to the expression of the gene in leukocytes in an individual not rejecting a transplanted organ.

[0034] In another aspect, the disclosure is directed to a system for detecting gene expression in body fluid including at least two isolated polynucleotides wherein the isolated polynucleotides detect expression of a gene wherein the gene includes a nucleotide sequence according to SEQ ID NO:67, and the gene expression is related to the rate of hematopoiesis or the distribution of hematopoeitic cells along their maturation pathway.

Brief Description of the Sequence Listing

[0035]

SEQ ID's 1-332 are 50mer oligonucleotides corresponding to gene expression markers for diagnosis and monitoring of allograft rejection and other disorders.
SEQ ID's 333-664 are Reference mRNA sequences for genes identified by probes 1-332.
SEQ ID's 665-995 are a first set of Left PCR primers for genes 1-332.
SEQ ID's 996-1326 are a first set of Right PCR primers for genes 1-332.
SEQ ID's 1327-1657 are Taqman probes for the first set PCR primers for genes 1-332.
SEQ ID's 1658-1903 are a second alternative set of left PCR primers for selected genes 1-332
SEQ ID's 1904-2151 are a second alternative set of right PCR primers for selected genes 1-332
SEQ ID's 2152-2399 are Taqman probes for the second alternative set of PCR primers for selected genes 1-332.
SEQ ID's 2400-2626 are Proteins encoded by mRNA's from genes identified in 1-332.
SEQ ID's 2627-2795 are 50mer oligonucleotide array probes used to identify genes in Figure 7 and Tables 6 and 8.
SEQ ID's 2796-2924 are reference mRNA sequences for genes in Table 8 which show altered expression in renal transplantation and rejection.
SEQ ID's 2925-3015 are proteins coded by genes which show altered expression in Table 8.
SEQ ID's 3016-3081 are 50mer oligonucleotide array probes and used to identify genes in the Examples.
SEQ ID's 3082-3107 are genes and primers discussed in the Examples.
SEQ ID's 3108-3117 are mRNAs from human genes in which regulation is altered upon CMV infection.

Brief Description of the Figures

[0036]

Figure 1: Figure 1 is a schematic flow chart illustrating a schematic instruction set for characterization of the nucleotide sequence and/or the predicted protein sequence of novel nucleotide sequences.
Figure 2: Figure 2 depicts the components of an automated RNA preparation machine.
Figure 3 shows the results of six hybridizations on a mini array graphed (n=6 for each column). The error bars are the SEM. This experiment shows that the average signal from AP prepared RNA is 47% of the average signal from GS prepared RNA for both Cy3 and Cy5.
Figure 4 shows the average background subtracted signal for each of nine leukocyte-specific genes on a mini array. This average is for 3-6 of the above-described hybridizations for each gene. The error bars are the SEM.
Figure 5 shows the ratio of Cy3 to Cy5 signal for a number of genes. After normalization, this ratio corrects for variability among hybridizations and allows comparison between experiments done at different times. The ratio is calculated as the Cy3 background subtracted signal divided by the Cy5 background subtracted signal. Each bar is the average for 3-6 hybridizations. The error bars are SEM.
Figure 6 shows data median Cy3 background subtracted signals for control RNAs using mini arrays.
Figure 7: Cardiac Allograft rejection diagnostic genes.

A. Example of rejection and no-rejection samples expression data for 5 marker genes. For each sample, the associated rejection grades are shown as are the expression ratios for 5 differentially expressed genes. The genes are identified by the SEQ ID number for the oligonucleotide. The average fold difference between grade 0 and grade 3A samples is calculated at the bottom.
B. CART classification model. Decision tree for a 3 gene classification model for diagnosis of cardiac rejection. In the first step, expression of gene 223 is used to divide the patients to 2 branches. The remaining samples in each branch are then further divided by one remaining gene. The samples are classified as either rejection or no rejection. 1 no rejection sample is misclassified as a rejection sample.
C. Surrogates for the CART classification model. For each of the 3 splitter genes in the CART rejection model described in the example, 5 top surrogate genes are listed that were identified by the CART algorithm

Figure 8: Validation of differential expression of a gene discovered using microarrays using real-time PCR
Figure 8A. The Ct for each patient sample on multiple assays is shown along with the Ct in the R50 control RNA. Triangles represent -RT (reverse transcriptase) controls.
Figure 8B. The fold difference between the expression of Granzyme B and an Actin reference is shown for 3 samples from patients with and without CMV disease.
Figure 9: Endpoint testing of PCR primers

Electrophoresis and microfluidics are used to assess the product of gene specific PCR primers. β-GUS gel image. Lane 3 is the image for primers F178 and R242. Lanes 2 and 1 correspond to the no-template control and -RT control, respectively.

The electropherogram of β-GUS primers F178 and R242, a graphical representation of Lane 3 from the gel image.

β-Actin gel image. Lane 3 is the image for primers F75 and R178. Lanes 2 and 1 correspond to the no-template control and -RT control, respectively.

The electropherogram of β-Actin primers F75 and R178, a graphical representation of Lave 3 from the gel image.

Figure 10: PCR Primer efficiency testing. A standard curve of Ct versus log of the starting RNA amount is shown for 2 genes.

Figure 11: Real-time PCR control gene analysis

11 candidate control genes were tested using real-time PCR on 6 whole blood samples (PAX) paired with 6 mononuclear samples (CPT) from the same patient. Each sample was tested twice. For each gene, the variability of the gene across the samples is shown on the vertical axis (top graph). The average Ct value for each gene is also shown (bottom graph). 2ug RNA was used for PAX samples and 0.5 ug total RNA was used for the mononuclear samples (CPT).

Figure 12: Rejection marker discovery by co-expression with established marker Microarrays were used to measure expression of genes SEQ ID 85 and 302 in samples derived from 240 transplant recipients. For each sample, the expression measurement for 85 is plotted against 302.

Figure 13: ROC (receiver operator characteristics) curve for a 3-gene PCR assay for diagnosis of rejection (see example 17). The Sensitivity and False Positive Rate for each test cutoff is shown.

Brief Description of the Tables

[0037]

Table 1: Table 1 lists diseases or conditions amenable to study by leukocyte profiling.

Table 2: Transplant Markers

A. Transplant Genes: Genes useful for monitoring of allograft rejection are listed in this here. The gene symbol and name are given. SEQ ID 50mer is the sequence ID of a 50mer oligonucleotide that is specific for the gene. The NCBI Unigene number (HS) from (Build 160, 16 Feb 2003) is given as is an accession number (ACC) from (Genbank Release 135, 15 April 2003) for an RNA or cDNA is Genbank that corresponds to the gene. The sequence identified by the ACC number is in the sequence listing (SEQ ID RNA/cDNA).

B. Microarray Data: SEQ ID 50mer, Gene, Gene Name, ACC and SEQ ID RNA/cDNA are given for each gene as in A (above). Each identified gene has a Non-Parametric Score and Median Rank in NR given from the non-parametric analysis of the data. The genes are ranked from highest to lowest scoring. Down Regulated genes are noted with a 1 in this column.

C. PCR Primers: Primers and probes for real-time PCR assays for each gene are given along with their SEQ ID #s. Each gene has 1 or 2 sets of a forward and reverse PCR primer and a hybridization probe for detection in TaqMan or similar assays.

D. PCR Data: Real-time PCR data was generated on a set of transplant samples using sybr green technology as described in the text. For each gene the number of samples (n) used in the analysis is given. An odds ratio and the p-values for a Fisher test and t-test are given for the comparison of acute rejection samples is given (see text).

E. Transplant proteins: For each gene, the corresponding protein in the RefSeq data base (Genbank Release 135, 18 April 2003) is given (RefSeq Peptide Accession #) along the the SEQ ID for that protein for the sequence listing.

Table 3: Viral gene for arrays. Viral genomes were used to design oligonucleotides for the microarrays. The accession numbers for the viral genomes used are given, along with the gene name and location of the region used for oligonucleotide design.

Table 4. Dependent variables for discovery of gene expression markers of cardiac allograft rejection. A stable Grade 0 is a Grade 0 biopsy in a patient who does not experience rejection with the subsequent biopsy. HG or highest grade means that the higher of the biopsy grades from the centralized and local pathologists was used for a definition of the dependent variable.

Table 5: Real-time PCR assay reporter and quencher dyes. Various combinations of reporter and quencher dyes are useful for real-time PCR assays. Reporter and quencher dyes work optimally in specific combinations defined by their spectra. For each reporter, appropriate choices for quencher dyes are given.

Table 6: Rejection marker PCR assay results
Results of real-time PCR assays are listed for the comparison of rejection samples to no rejection samples. The fold change is given for expression of each gene in rejection/no rejection samples. The p-value for the t-test comparing the rejection and no rejection classes is given.

Table 7: Summary results of array rejection significance analysis. Summary results are given for correlation analysis of leukocyte gene expression to acute rejection using significance analysis for microarrays (SAM). Five analyses are described. The ISHLT grades used to define the rejection and no rejection classes are given. In each case the highest grade from three pathology reading was taken for analysis. All samples are used for two analyses. The other analyses reduce redundancy of patients used in the analysis by using only one sample per patient ("Non-redundant") or using only one sample per patient within a given class ("Non-redundant within class"). The number of samples used in the analysis is given and the lowest false detection rate (FDR) achieved is noted.

Table 8: Renal tissue rejection array significance analysis. Genes are listed that were identified as upregulated using microarrays on renal tissue with acute rejection versus controls. Significance analysis for microarrays (SAM) was used to determine the false detection rate for each gene (FDR). Genes with known expression in leukocytes are noted in the table.

Table 9: Rejection marker sequence analysis. For 63 of the allograft rejection markers listed in Table 2, an analysis of the gene sequence was done. The genes and proteins are identified by accession numbers. The cellular localization of each gene is described as either secreted, nuclear, mitochondrial, cytoplasmic or cellular membrane. The function of the gene is also described.

Table 10: Gene expression markers for immature cells of a variety of lineages are given in Table 10 by way of example

Table 11: Changes in the rate of hematopoiesis have been correlated to a number of disease states and other pathologies. Examples of such conditions are listed in Table 11.

Table 12: This table lists the oligonucleotides and associated genes identified as having value for the diagnosis and monitoring of CMV infection. The first column gives the SEQ ID that corresponds to the oligonuclotide in the sequence listing. The unigene number, genebank accession and GI number are also given for each sequence when known. The name of the gene associated with the accession number is noted. The strand is noted as -1 or 1, meaning that the probe was designed from the complement of the sequence (-1) or directly from the sequence (1). Next, the nucleotide sequence of each probe is also given. For each gene, the false detection rate (FDR) from the significance analsysis described in example 7 is given if applicable. WBC is the white blood cell count. WPT is the number of weeks past transplant.

Detailed Description

Definitions

[0038] Unless defined otherwise, all scientific and technical terms are understood to have the same meaning as commonly used in the art to which they pertain. For the purpose of the present invention, the following terms are defined below.

[0039] In the context of the invention, the term "gene expression system" refers to any system, device or means to detect gene expression and includes diagnostic agents, candidate libraries, oligonucleotide sets or probe sets.

[0040] The term "monitoring" is used herein to describe the use of gene sets to provide useful information about an individual or an individual's health or disease status. "Monitoring" can include, determination of prognosis, risk-stratification, selection of drug therapy, assessment of ongoing drug therapy, prediction of outcomes, determining response to therapy, diagnosis of a disease or disease complication, following progression of a disease or providing any information relating to a patients health status over time, selecting patients most likely to benefit from experimental therapies with known molecular mechanisms of action, selecting patients most likely to benefit from approved drugs with known molecular mechanisms where that mechanism may be important in a small subset of a disease for which the medication may not have a label, screening a patient population to help decide on a more invasive/expensive test, for example a cascade of tests from a non-invasive blood test to a more invasive option such as biopsy, or testing to assess side effects of drugs used to treat another indication.

[0041] The term "diagnostic oligonucleotide set" generally refers to a set of two or more oligonucleotides that, when evaluated for differential expression of their products, collectively yields predictive data. Such predictive data typically relates to diagnosis, prognosis, monitoring of therapeutic outcomes, and the like. In general, the components of a diagnostic oligonucleotide set are distinguished from nucleotide sequences that are evaluated by analysis of the DNA to directly determine the genotype of an individual as it correlates with a specified trait or phenotype, such as a disease, in that it is the pattern of expression of the components of the diagnostic nucleotide set, rather than mutation or polymorphism of the DNA sequence that provides predictive value. It will be understood that a particular component (or member) of a diagnostic nucleotide set can, in some cases, also present one or more mutations, or polymorphisms that

are amenable to direct genotyping by any of a variety of well known analysis methods, e.g., Southern blotting, RFLP, AFLP, SSCP, SNP, and the like.

[0042] A "disease specific target oligonucleotide sequence" is a gene or other oligonucleotide that encodes a polypeptide, most typically a protein, or a subunit of a multi-subunit protein, that is a therapeutic target for a disease, or group of diseases.

[0043] A "candidate library" or a "candidate oligonucleotide library" refers to a collection of oligonucleotide sequences (or gene sequences) that by one or more criteria have an increased probability ofbeing associated with a particular disease or group of diseases. The criteria can be, for example, a differential expression pattern in a disease state or in activated or resting leukocytes in vitro as reported in the scientific or technical literature, tissue specific expression as reported in a sequence database, differential expression in a tissue or cell type of interest, or the like. Typically, a candidate library has at least 2 members or components; more typically, the library has in excess of about 10, or about 100, or about 1000, or even more, members or components.

[0044] The term "disease criterion" is used herein to designate an indicator of a disease, such as a diagnostic factor, a prognostic factor, a factor indicated by a medical or family history, a genetic factor, or a symptom, as well as an overt or confirmed diagnosis of a disease associated with several indicators such as those selected from the above list. A disease criterian includes data describing a patient's health status, including retrospective or prospective health data, e.g. in the form of the patient's medical history, laboratory test results, diagnostic test result, clinical events, medications, lists, response(s) to treatment and risk factors, etc.

[0045] The terms "molecular signature" or "expression profile" refers to the collection of expression values for a plurality (e.g., at least 2, but frequently about 10, about 100, about 1000, or more) of members of a candidate library. In many cases, the molecular signature represents the expression pattern for all of the nucleotide sequences in a library or array of candidate or diagnostic nucleotide sequences or genes. Alternatively, the molecular signature represents the expression pattern for one or more subsets of the candidate library. The term "oligonucleotide" refers to two or more nucleotides. Nucleotides may be DNA or RNA, naturally occurring or synthetic.

[0046] The term "healthy individual," as used herein, is relative to a specified disease or disease criterion. That is, the individual does not exhibit the specified disease criterion or is not diagnosed with the specified disease. It will be understood, that the individual in question, can, of course, exhibit symptoms, or possess various indicator factors for another disease.

[0047] Similarly, an "individual diagnosed with a disease" refers to an individual diagnosed with a specified disease (or disease criterion). Such an individual may, or may not, also exhibit a disease criterion associated with, or be diagnosed with another (related or unrelated) disease.

[0048] An "array" is a spatially or logically organized collection, e.g., of oligonucleotide sequences or nucleotide sequence products such as RNA or proteins encoded by an oligonucleotide sequence. In some embodiments, an array includes antibodies or other binding reagents specific for products of a candidate library.

[0049] When referring to a pattern of expression, a "qualitative" difference in gene expression refers to a difference that is not assigned a relative value. That is, such a difference is designated by an "all or nothing" valuation. Such an all or nothing variation can be, for example, expression above or below a threshold of detection (an on/off pattern of expression). Alternatively, a qualitative difference can refer to expression of different types of expression products, e.g., different alleles (e.g., a mutant or polymorphic allele), variants (including sequence variants as well as post-translationally modified variants), etc.

[0050] In contrast, a "quantitative" difference, when referring to a pattern of gene expression, refers to a difference in expression that can be assigned a value on a graduated scale, (e.g., a 0-5 or 1-10 scale, a + - +++ scale, a grade 1-grade 5 scale, or the like; it will be understood that the numbers selected for illustration are entirely arbitrary and in no-way are meant to be interpreted to limit the invention).

Gene Expression Systems

[0051] The disclosure is directed to a gene expression system having one or more DNA molecules wherein the one or more DNA molecules has a nucleotide sequence which detects expression of a gene corresponding to the oligonucleotides depicted in the Sequence Listing. In one format, the oligonucleotide detects expression of a gene that is differentially expressed in leukocytes. The gene expression system may be a candidate library, a diagnostic agent, a diagnostic oligonucleotide set or a diagnostic probe set. The DNA molecules may be genomic DNA, protein nucleic acid (PNA), cDNA or synthetic oligonucleotides. Following the procedures taught herein, one can identify sequences of interest for analyzing gene expression in leukocytes. Such sequences may be predictive of a disease state.

Diagnostic oligonucleotides of the invention

[0052] The disclosure is directed to diagnostic nucleotide set(s) comprising members of the leukocyte candidate library

listed in Table 2, Table 8, and in the Sequence Listing, for which a correlation exists between the health status of an individual, the individual's expression of RNA or protein products corresponding to the nucleotide sequence, and the diagnosis and prognosis of transplant rejection. In some instances, only one oligonucleotide is necessary for such detection. Members of a diagnostic oligonucleotide set may be identified by any means capable of detecting expression of RNA or protein products, including but not limited to differential expression screening, PCR, RT-PCR, SAGE analysis, high-throughput sequencing, microarrays, liquid or other arrays, protein-based methods (e.g., western blotting, proteomics, and other methods described herein), and data mining methods, as further described herein.

[0053] Also disclosed is a diagnostic oligonucleotide set that comprises at least two oligonucleotide sequences listed in Table 2, Table 8, or the Sequence Listing which are differentially expressed in leukocytes in an individual with at least one disease criterion for at least one leukocyte-implicated disease relative to the expression in individual without the at least one disease criterion, wherein expression of the two or more nucleotide sequences is correlated with at least one disease criterion, as described below.

[0054] Further disclosed is a diagnostic nucleotide set that comprises at least one oligonucleotide having an oligonucleotide sequence listed in Table 2, Table 8, or the Sequence Listing which is differentially expressed, and further wherein the differential expression/correlation has not previously been described. In some embodiments, the diagnostic nucleotide set is immobilized on an array.

[0055] In one embodiment, diagnostic nucleotides (or nucleotide sets) are related to the members of the leukocyte candidate library listed in Table 2, Table 8, or in the Sequence Listing, for which a correlation exists between the health status, diagnosis and prognosis of transplant rejection (or disease criterion) of an individual. The diagnostic nucleotides are partially or totally contained in (or derived from) full-length gene sequences (or predicted full-length gene sequences) for the members of the candidate library listed in Table 2, Table 8, and the sequence listing. In some cases, oligonucleotide sequences are designed from EST or Chromosomal sequences from a public database. In these cases the full-length gene sequences may not be known. Full-length sequences in these cases can be predicted using gene prediction algorithms. Alternatively the full-length can be determined by cloning and sequencing the full-length gene or genes that contain the sequence of interest using standard molecular biology approaches described here. The same is true for olignonucleotides designed from our sequencing of cDNA libraries where the cDNA does not match any sequence in the public databases.

[0056] The diagnostic nucleotides may also be derived from other genes that are coexpressed with the correlated sequence or full-length gene. Genes may share expression patterns because they are regulated in the same molecular pathway. Because of the similarity of expression behavior genes are identified as surrogates in that they can substitute for a diagnostic gene in a diagnostic gene set. Example 4 demonstrates the discovery of surrogates from the data and the sequence listing identifies and gives the sequence for surrogates for cardiac diagnostic genes.

[0057] As used herein the term "gene cluster" or "cluster" refers to a group of genes related by expression pattern. In other words, a cluster of genes is a group of genes with similar regulation across different conditions, such as graft non-rejection verus graft rejection. The expression profile for each gene in a cluster should be correlated with the expression profile of at least one other gene in that cluster. Correlation may be evaluated using a variety of statistical methods. As used herein the term "surrogate" refers to a gene with an expression profile such that it can substitute for a diagnostic gene in a diagnostic assay. Such genes are often members of the same gene cluster as the diagnostic gene. For each member of a diagnostic gene set, a set of potential surrogates can be identified through identification of genes with similar expression patterns as described below.

[0058] Many statistical analyses produce a correlation coefficient to describe the relatedness between two gene expression patterns. Patterns may be considered correlated if the correlation coefficient is greater than or equal to 0.8. In preferred embodiments, the correlation coefficient should be greater than 0.85, 0.9 or 0.95. Other statistical methods produce a measure of mutual information to describe the relatedness between two gene expression patterns. Patterns may be considered correlated if the normalized mutual information value is greater than or equal to 0.7. In preferred embodiments, the normalized mutual information value should be greater than 0.8, 0.9 or 0.95. Patterns may also be considered similar if they cluster closely upon hierarchical clustering of gene expression data (Eisen et al. 1998). Similar patterns may be those genes that are among the 1, 2, 5, 10, 20, 50 or 100 nearest neighbors in a hierarchical clustering or have a similarity score (Eisen et al. 1998) of > 0.5, 0.7, 0.8, 0.9, 0.95 or 0.99. Similar patterns may also be identified as those genes found to be surrogates in a classification tree by CART (Breiman et al. 1994). Often, but not always, members of a gene cluster have similar biological functions in addition to similar gene expression patterns.

[0059] Correlated genes, clusters and surrogates are identified for the diagnostic genes of the disclosure. These surrogates may be used as diagnostic, genes in an assay instead of, or in addition to, the diagnostic genes for which they are surrogates.

[0060] The disclosure is also directed to diagnostic probe sets. It is understood that a probe includes any reagent capable of specifically identifying a nucleotide sequence of the diagnostic nucleotide set, including but not limited to amplified DNA, amplified RNA, cDNA, synthetic oligonucleotide, partial or full-length nucleic acid sequences. In addition, the probe may identify the protein product of a diagnostic nucleotide sequence, including, for example, antibodies and

other affinity reagents.

[0061] It is also understood that each probe can correspond to one gene, or multiple probes can correspond to one gene, or both, or one probe can correspond to more than one gene.

[0062] Homologs and variants of the disclosed nucleic acid molecules may be used in the present disclosure. Homologs and variants of these nucleic acid molecules will possess a relatively high degree of sequence identity when aligned using standard methods. The sequences encompassed by the disclosure have at least 40-50, 50-60, 70-80, 80-85, 85-90, 90-95 or 95-100% sequence identity to the sequences disclosed herein.

[0063] It is understood that for expression profiling, variations in the disclosed sequences will still permit detection of gene expression. The degree of sequence identity required to detect gene expression varies depending on the length of the oligomer. For a 60 mer, 6-8 random mutations or 6-8 random deletions in a 60 mer do not affect gene expression detection. Hughes, TR, et al. "Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. Nature Biotechnology, 19:343-347(2001). As the length of the DNA sequence is increased, the number of mutations or deletions permitted while still allowing gene expression detection is increased.

[0064] As will be appreciated by those skilled in the art, the sequences of the present invention may contain sequencing errors. That is, there may be incorrect nucleotides, frameshifts, unknown nucleotides, or other types of sequencing errors in any of the sequences; however, the correct sequences will fall within the homology and stringency definitions herein.

[0065] The minimum length of an oligonucleotide probe necessary for specific hybridization in the human genome can be estimated using two approaches. The first method uses a statistical argument that the probe will be unique in the human genome by chance. Briefly, the number of independent perfect matches (Po) expected for an oligonucleotide of length L in a genome of complexity C can be calculated from the equation (Laird CD, Chromosoma 32:378 (1971):

$$Po=(1/4)^{L} * 2C$$

[0066] In the case of mammalian genomes, 2C = ~3.6 X 10^9, and an oligonucleotide of 14-15 nucleotides is expected to be represented only once in the genome. However, the distribution of nucleotides in the coding sequence of mammalian genomes is nonrandom (Lathe, R. J. Mol. Biol. 183:1 (1985) and longer oligonucleotides may be preferred in order to in increase the specificity of hybridization. In practical terms, this works out to probes that are 19-40 nucleotides long (Sambrook J et al., infra). The second method for estimating the length of a specific probe is to use a probe long enough to hybridize under the chosen conditions and use a computer to search for that sequence or close matches to the sequence in the human genome and choose a unique match. Probe sequences are chosen based on the desired hybridization properties as described in Chapter 11 of Sambrook et al, infra. The PRIMER3 program is useful for designing these probes (S. Rozen and H. Skaletsky 1996,1997; Primer3 code available at the web site located at genome.wi.mit.edu/genome_software/other/primer3.html). The sequences of these probes are then compared pair wise against a database of the human genome sequences using a program such as BLAST or MEGABLAST (Madden, T.L et al.(1996) Meth. Enzymol. 266:131-141). Since most of the human genome is now contained in the database, the number of matches will be determined. Probe sequences are chosen that are unique to the desired target sequence.

[0067] In some embodiments, a diagnostic probe set is immobilized on an array. The array is optionally comprises one or more of: a chip array, a plate array, a bead array, a pin array, a membrane array, a solid surface array, a liquid array, an oligonucleotide array, a polynucleotide array or a cDNA array, a microtiter plate, a pin array, a bead array, a membrane or a chip.

[0068] In some embodiments, the leukocyte-implicated disease is selected from the diseases listed in Table 1. In other embodiments, In some embodiments, the disease is atherosclerosis or cardiac allograft rejection. In other embodiments, the disease is congestive heart failure, angina, and myocardial infarction.

[0069] In some embodiments, diagnostic nucleotides of the invention are used as a diagnostic gene set in combination with genes that are know to be associated with a disease state ("known markers"). The use of the diagnostic nucleotides in combination with the known markers can provide information that is not obtainable through the known markers alone. The known markers include those identified by the prior art listing provided.

Hematopoeisis

[0070] The present disclosure is also directed to methods of measurement of the rate of hematopoiesis using the diagnostic oligonucleotides of the invention and measurement of the rates of hematopoesis by any technique as a method for the monitoring and diagnosis of transplant rejection. Precursor and immature cells often have cell specific phenotypic markers. These are genes and/or proteins that expressed in a restricted manner in immature or precursor cells. This expression decreases with maturation. Gene expression markers for immature cells of a variety of lineages are given in Table 10 below by way of example.

Table 10:

Gene	Cell type
CD10	B-lymphoblasts
RAG1	B-lymphoblasts
RAG2	B-lymphoblasts
NF-E2	Platelets/Megakaryocyte/Erythroid
GATA-1	Platelets/Megakaryocyte
GP IIb	Platelets
pf4	Platelets
EPO-R	Erythroblast
Band 4.1	Erythrocyte
ALAS2	Erythroid specific heme biosynthesis
hemoglobin chains	Erythocyte
2,3-BPG mutase	Erythrocyte
CD16b	Neutrophil
LAP	Neutrophil
CD16	NK cells
CD159a	NK cells

[0071] By measuring the levels of these and other genes in peripheral blood samples, an assessment of the number and proportion of immature or precursor cells can be made. Of particular use is RNA quantification in erythrocytes and platelets. These cells are anucleated in their mature forms. During development, platelets pinch off of a megakaryocyte and take a compliment of RNA without a nucleus. This RNA is quickly consumed by the platelet. Erythrocytes start as nucleated cells, but the nucleus extrudes toward the end of the maturation process. These cells have RNA which is rapidly consumed within the first 2 days of the cells 120 day life span.

[0072] For these anucleated cell types, gene expression markers must be specific only to the cell line (and not the immature form) to be useful as measures of cellular production rates. Genes specific to the lineage vs. other blood cell types will serve as markers of cellular production rates when measured on the RNA level. This is because RNA is specific to immature forms in these cases. For example, hemoglobin is specific to erythrocytes, but hemoglobin RNA is specific to newly produced erythrocytes. Therefore, if the rate of production of erythrocytes increases, so will the level of a lineage specific RNA (e.g., hemoglobin).

[0073] Hematopoietic growth factors and cytokines have incomplete lineage specificity. G-CSF is administered to patient with low granulocyte counts and the effect is a stimulation of all lineages (granulocytes, erythrocytes, platelets, etc...). Hemolytic anemia leads to increased production of multiple cell lineages although the only lineage in increased demand is the erythrocyte. Because of this lack of specificity of hematopoietic responses, erythrocyte and platelet production rates may serve as surrogates of increased production of lymphocyte lineages. Using RBCs and platelets production rates as surrogates for lymphocyte lineages may be useful because of the lack of a nucleus in these cells and the ease of measuring cellular production rates by simply measuring lineage specific RNA levels.

[0074] Hematopoieis rates can be measured using gene expression profiling of peripheral blood. RBC and platelet specific genes provide unique opportunity for this because of their lack of a nucleus and kinetics. New cells = new / much more RNA from these cell types in peripheral blood. Immature lymphocytes may be even more specific for immune activation and rejection. Cell specific markers of lymphocyte precursors were identified (aka lymphoblasts) see below. Granulocyte precursors and markers of megakaryocytes or premature forms of any blood cells may be useful in this regard.

Applications for measuring the rate of hematopoiesis

[0075] Changes in the rate of hematopoiesis have been correlated to a number of disease states and other pathologies. Examples of such conditions are listed in Table 11. One of skill in the art would be aware of other such conditions. In addition, one aspect of the present invention is the identification of the linkage between changes in the rate of hemat-

opoiesis. The methods of the present invention directed to measuring the rates of hematopoiesis can therefore be applied to the diagnosis and monitoring of a number of disease states and other pathologies. In addition, these methods can be beneficial in determining appropriate therapies for patients.

Table: 11

Disorder / condition	Cell type	Cell production	Therapy
Anemia-Iron Erythrocyte Deficiency		Decreased	Iron
Anemia - B 12, Folate deficiency	Erythrocyte	Decreased	B12, Folate
Anemia- Aplastic	Erythrocyte	Decreased	Epogen, transfusion
Anemia - hemolytic	Erythrocyte	Increased	Immunosuppression, Splenectomy
Anemia - Renal failure	Erythrocyte	Decreased	Erythropoietin
Anemia - Chronic disease	Erythrocyte	Decreased	Treat underlying cause
Polycythemia rubra vera	Erythrocyte	Increased	
Idiophic Thrrombocytopenic purpura	Platelet	Increased	Immunosuppression, Splenectomy
Thrombotic Thrombocytopenic purpura	Platelet	Increased or decreased	Immunosuppression, plasmapheresis
Essential thrombocytosis	Platelet	Increased	
Leukemia	All lineages, variable	Increase, decreased or abnomal	Chemotherapy, BMT
Cytopenias due to immunosupression	All lineages, variable	Decreased	Epo, neupogen
Cytopenias due to Chemotherapy	All lineages, variable	Decreased	Epo, GCSF, GMCSF
GVHD	All lineages, variable	Decreased	Immunosuppression
Myelodysplasia	All lineages, variable	Decreased, increased or abnormal	Chemo?
Allograft rejection	Lymphocytes, All lineages	Increased	Immunosuppression
Autoimmune diseases (many)	Lymphocytes, All lineages	Increased	Immunosuppression

[0076] The methods disclosed are also useful for monitoring treatment regimens of diseases or other pathologies which are correlated with changes in the rate of hematopoiesis. Furthermore, the methods may be used to monitor treatment with agents that affect the rate of hematopoiesis. One of skill in the art is aware of many such agents. The following agents are examples of such.

[0077] Erythropoietin is a growth factor that is used to treat a variety of anemias that are due to decreased red cell production. Monitoring of red cell production by gene expression or other means may improve dosing and provide a means for earlier assessment of response to therapy for this expensive drug.

[0078] Neupogen (G-CSF) is used for the treatment of low neutrophil counts (neutropenia) usually related to immunosuppression or chemotherapy. Monitoring neutrophil production by gene expression testing or another means may improve dosing, patient selection, and shorten duration of therapy.

[0079] Prednisone / Immunosuppression - One of most common side effects of immunosuppression is suppression of hematopoiesis. This may occur in any cell lineage. Gene expression monitoring or other measures of hematopoietic rates could be used to monitor regularly for cytopenias in a particular cell line and the information could be used to modify

dosing, modify therapy or add a specific hematologic growth factor. Following cell counts themselves is less sensitive and results in the need for prolonged trials of therapies at a given dose before efficacy and toxicity can be assessed.

[0080] Monitoring of chemotherapeutic agents -Most chemotherapy agents suppress the bone marrow for some or all lineages. Gene expression testing or other means of assessing hematopoietic rates could be used to monitor regularly for cytopenias in a particular cell line and use information to modify dosing, modify therapy or add a specific hematologic growth factor.

General Molecular Biology References

[0081] In the context of the invention, nucleic acids and/or proteins are manipulated according to well known molecular biology techniques. Detailed protocols for numerous such procedures are described in, e.g., in Ausubel et al. Current Protocols in Molecular Biology (supplemented through 2000) John Wiley & Sons, New York ("Ausubel"); Sambrook et al. Molecular Cloning - A Laboratory Manual (2nd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989 ("Sambrook"), and Berger and Kimmel Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, CA ("Berger").

[0082] In addition to the above references, protocols for in vitro amplification techniques, such as the polymerase chain reaction (PCR), the ligase chain reaction (LCR), Q-replicase amplification, and other RNA polymerase mediated techniques (e.g., NASBA), useful e.g., for amplifying cDNA probes of the invention, are found in Mullis et al. (1987) U.S. Patent No. 4,683,202; PCR Protocols A Guide to Methods and Applications (Innis et al. eds) Academic Press Inc. San Diego, CA (1990) ("Innis"); Arnheim and Levinson (1990) C&EN 36; The Journal Of NIH Research (1991) 3:81; Kwoh et al. (1989) Proc Natl Acad Sci USA 86, 1173; Guatelli et al. (1990) Proc Natl Acad Sci USA 87:1874; Lomell et al. (1989) J Clin Chem 35:1826; Landegren et al. (1988) Science 241:1077; Van Brunt (1990) Biotechnology 8:291; Wu and Wallace (1989) Gene 4: 560; Barringer et al. (1990) Gene 89:117, and Sooknanan and Malek (1995) Biotechnology 13:563. Additional methods, useful for cloning nucleic acids in the context of the present invention, include Wallace et al. U.S. Pat. No. 5,426,039. Improved methods of amplifying large nucleic acids by PCR are summarized in Cheng et al. (1994) Nature 369:684 and the references therein.

[0083] Certain polynucleotides of the invention, e.g., oligonucleotides can be synthesized utilizing various solid-phase strategies involving mononucleotide- and/or trinucleotide-based phosphoramidite coupling chemistry. For example, nucleic acid sequences can be synthesized by the sequential addition of activated monomers and/or trimers to an elongating polynucleotide chain. See e.g., Caruthers, M.H. et al. (1992) Meth Enzymol 211:3.

[0084] In lieu of synthesizing the desired sequences, essentially any nucleic acid can be custom ordered from any of a variety of commercial sources, such as The Midland Certified Reagent Company, The Great American Gene Company ExpressGen, Inc., Operon Technologies, Inc. and many others.

[0085] Similarly, commercial sources for nucleic acid and protein microarrays are available, and include, e.g., Agilent Technologies, Palo Alto, CA Affymetrix, Santa Clara,CA ; and others.

[0086] One area of relevance to the present invention is hybridization of oligonucleotides. Those of skill in the art differentiate hybridization conditions based upon the stringency of hybridization. For example, highly stringent conditions could include hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65° C, and washing in 0.1XSSC/0.1% SDS at 68° C. (Ausubel F. M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3). Moderate stringency conditions could include, e.g., washing in 0.2XSSC/0.1% SDS at 42°C. (Ausubel et al., 1989, supra).

The disclosure also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the DNA sequences of the present disclosure. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances wherein the nucleic acid molecules are deoxyoligonucleotides ("oligos"), highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37°C. (for 14-base oligos), 48°C. (for 17-base oligos), 55°C. (for 20-base oligos), and 60°C. (for 23-base oligos). These nucleic acid molecules may act as target nucleotide sequence antisense molecules, useful, for example, in target nucleotide sequence regulation and/or as antisense primers in amplification reactions of target nucleotide sequence nucleic acid sequences. Further, such sequences may be used as part of ribozyme and/or triple helix sequences, also useful for target nucleotide sequence regulation. Still further, such molecules may be used as components of diagnostic methods whereby the presence of a disease-causing allele, may be detected.

Identification of diagnostic nucleotide sets

Candidate library

[0087] Libraries of candidates that are differentially expressed in leukocytes are substrates for the identification and evaluation of diagnostic oligonucleotide sets and disease specific target nucleotide sequences.

[0088] The term leukocyte is used generically to refer to any nucleated blood cell that is not a nucleated erythrocyte. More specifically, leukocytes can be subdivided into two broad classes. The first class includes granulocytes, including, most prevalently, neutrophils, as well as eosinophils and basophils at low frequency. The second class, the non-granular or mononuclear leukocytes, includes monocytes and lymphocytes (e.g., T cells and B cells). There is an extensive literature in the art implicating leukocytes, e.g., neutrophils, monocytes and lymphocytes in a wide variety of disease processes, including inflammatory and rheumatic diseases, neurodegenerative diseases (such as Alzheimer's dementia), cardiovascular disease, endocrine diseases, transplant rejection, malignancy and infectious diseases, and other diseases listed in Table 1. Mononuclear cells are involved in the chronic immune response, while granulocytes, which make up approximately 60% of the leukocytes, have a non-specific and stereotyped response to acute inflammatory stimuli and often have a life span of only 24 hours.

[0089] In addition to their widespread involvement and/or implication in numerous disease related processes, leukocytes are particularly attractive substrates for clinical and experimental evaluation for a variety of reasons. Most importantly, they are readily accessible at low cost from essentially every potential subject. Collection is minimally invasive and associated with little pain, disability or recovery time. Collection can be performed by minimally trained personnel (e.g., phlebotomists, medical technicians, etc.) in a variety of clinical and non-clinical settings without significant technological expenditure. Additionally, leukocytes are renewable, and thus available at multiple time points for a single subject.

Assembly of an initial candidate library

[0090] The initial candidate library was assembled from a combination of "mining" publication and sequence databases and construction of a differential expression library. Candidate oligonucleotide sequences in the library may be represented by a full-length or partial nucleic acid sequence, deoxyribonucleic acid (DNA) sequence, cDNA sequence, RNA sequence, synthetic oligonucleotides, etc. The nucleic acid sequence can be at least 19 nucleotides in length, at least 25 nucleotides, at least 40 nucleotides, at least 100 nucleotides, or larger. Alternatively, the protein product of a candidate nucleotide sequence may be represented in a candidate library using standard methods, as further described below. In selecting and validatating diagnostic oligonucleotides, an initial library of 8,031 candidate oligonucleotide sequences using nucleic acid sequences of 50 nucleotides in length was constructed as described below.

Candidate nucleotide library

[0091] We identified members of an initial candidate nucleotide library that are differentially expressed in activated leukocytes and resting leukocytes. From that initial candidate nucleotide library, a pool of candidates was selected as listed in Table 2, Table 8, and the seuqnce listing. Accordingly, the disclosure provides the candidate leukocyte nucleotide library comprising the nucleotide sequences listed in Table 2, Table 8, and in the sequence listing. In another embodiment, the disclosure provides an candidate library comprising at least one nucleotide sequence listed in Tables 2 and 8 and the sequence listing. In another embodiment, the disclosure provides an candidate library comprising at least two nucleotide sequences listed in Tables 2 and 8 and the sequence listing. In another embodiment, the at least two nucleotide sequence are at least 19 nucleotides in length, at least 35 nucleotides, at least 40 nucleotides or at least 100 nucleotides. In some embodiments, the nucleotide sequences comprises deoxyribonucleic acid (DNA) sequence, ribonucleic acid (RNA) sequence, synthetic oligonucleotide sequence, or genomic DNA sequence. It is understood that the nucleotide sequences may each correspond to one gene, or that several nucleotide sequences may correspond to one gene, or both.

[0092] The disclosure also provides probes to the candidate nucleotide library. The probes can comprise at least two nucleotide sequences listed in Table 2, Table 8, or the sequence listing which are differentially expressed in leukocytes in an individual with a least one disease criterion for at least one leukocyte-related disease and in leukocytes in an individual without the at least one disease criterion, wherein expression of the two or more nucleotide sequences is correlated with at least one disease criterion. It is understood that a probe may detect either the RNA expression or protein product expression of the candidate nucleotide library. Alternatively, or in addition, a probe can detect a genotype associated with a candidate nucleotide sequence, as further described below. The probes for the candidate nucleotide library can also be immobilized on an array.

[0093] The candidate nucleotide library disclosed is useful in identifying diagnostic nucleotide sets of the disclosure. The candidate nucleotide sequences may be further characterized, and may be identified as a disease target nucleotide sequence and/or a novel nucleotide sequence, as described below. The candidate nucleotide sequences may also be suitable for use as imaging reagents, as described below.

Detection of non-leukocyte expressed genes

[0094] When measuring gene expression levels in a blood sample, RNAs may be measured that are not derived from

leukocytes. Examples are viral genes, free RNAs that have been released from damaged non-leukocyte cell types or RNA from circulating non-leukocyte cell types. For example, in the process of acute allograft rejection, tissue damage may result in release of allograft cells or RNAs derived from allograft cells into the circulation. In the case of cardiac allografts, such transcripts may be specific to muscle (myoglobin) or to cardiac muscle (Troponin I, Toponin T, CK-MB). Presence of cardiac specific mRNAs in peripheral blood may indicate ongoing or recent cardiac cellular damage (resulting from acute rejection). Therefore, such genes may be excellent diagnostic markers for allograft rejection.

Generation of Expression Patterns

RNA, DNA or protein sample procurement

[0095] Following identification or assembly of a library of differentially expressed candidate nucleotide sequences, leukocyte expression profiles corresponding to multiple members of the candidate library are obtained. Leukocyte samples from one or more subjects are obtained by standard methods. Most typically, these methods involve trans-cutaneous venous sampling of peripheral blood. While sampling of circulating leukocytes from whole blood from the peripheral vasculature is generally the simplest, least invasive, and lowest cost alternative, it will be appreciated that numerous alternative sampling procedures exist, and are favorably employed in some circumstances. No pertinent distinction exists, in fact, between leukocytes sampled from the peripheral vasculature, and those obtained, e.g., from a central line, from a central artery, or indeed from a cardiac catheter, or during a surgical procedure which accesses the central vasculature. In addition, other body fluids and tissues that are, at least in part, composed of leukocytes are also desirable leukocyte samples. For example, fluid samples obtained from the lung during bronchoscopy may be rich in leukocytes, and amenable to expression profiling in the context of the invention, e.g., for the diagnosis, prognosis, or monitoring of lung transplant rejection, inflammatory lung diseases or infectious lung disease. Fluid samples from other tissues, e.g., obtained by endoscopy of the colon, sinuses, esophagus, stomach, small bowel, pancreatic duct, biliary tree, bladder, ureter, vagina, cervix or uterus, etc., are also suitable. Samples may also be obtained other sources containing leukocytes, e.g., from urine, bile, cerebrospinal fluid, feces, gastric or intestinal secretions, semen, or solid organ or joint biopsies.

[0096] Most frequently, mixed populations of leukocytes, such as are found in whole blood are utilized in the methods of the present invention. A crude separation, e.g., of mixed leukocytes from red blood cells, and/or concentration, e.g., over a sucrose, percoll or ficoll gradient, or by other methods known in the art, can be employed to facilitate the recovery of RNA or protein expression products at sufficient concentrations, and to reduce non-specific background. In some instances, it can be desirable to purify sub-populations of leukocytes, and methods for doing so, such as density or affinity gradients, flow cytometry, fluorescence Activated Cell Sorting (FACS), immuno-magnetic separation, "panning," and the like, are described in the available literature and below.

Obtaining DNA, RNA and protein samples for expression profiling

[0097] Expression patterns can be evaluated at the level of DNA, or RNA or protein products. For example, a variety of techniques are available for the isolation of RNA from whole blood. Any technique that allows isolation of mRNA from cells (in the presence or absence of rRNA and tRNA) can be utilized. In brief, one method that allows reliable isolation of total RNA suitable for subsequent gene expression analysis, is described as follows. Peripheral blood (either venous or arterial) is drawn from a subject, into one or more sterile, endotoxin free, tubes containing an anticoagulant (e.g., EDTA, citrate, heparin, etc.). Typically, the sample is divided into at least two portions. One portion, e.g., of 5-8 ml of whole blood is frozen and stored for future analysis, e.g., of DNA or protein. A second portion, e.g., of approximately 8 ml whole blood is processed for isolation of total RNA by any of a variety of techniques as described in, e.g, Sambook, Ausubel, below, as well as U.S. Patent Numbers: 5,728,822 and 4,843,155.

[0098] Typically, a subject sample of mononuclear leukocytes obtained from about 8 ml of whole blood, a quantity readily available from an adult human subject under most circumstances, yields 5-20 μg of total RNA. This amount is ample, e.g., for labeling and hybridization to at least two probe arrays. Labeled probes for analysis of expression patterns of nucleotides of the candidate libraries are prepared from the subject's sample of RNA using standard methods. In many cases, cDNA is synthesized from total RNA using a polyT primer and labeled, e.g., radioactive or fluorescent, nucleotides. The resulting labeled cDNA is then hybridized to probes corresponding to members of the candidate nucleotide library, and expression data is obtained for each nucleotide sequence in the library. RNA isolated from subject samples (e.g., peripheral blood leukocytes, or leukocytes obtained from other biological fluids and samples) is next used for analysis of expression patterns of nucleotides of the candidate libraries.

[0099] In some cases, however, the amount of RNA that is extracted from the leukocyte sample is limiting, and amplification of the RNA is desirable. Amplification may be accomplished by increasing the efficiency of probe labeling, or by amplifying the RNA sample prior to labeling. It is appreciated that care must be taken to select an amplification procedure that does not introduce any bias (with respect to gene expression levels) during the amplification process.

[0100] Several methods are available that increase the signal from limiting amounts of RNA, e.g. use of the Clontech (Glass Fluorescent Labeling Kit) or Stratagene (Fairplay Microarray Labeling Kit), or the Micromax kit (New England Nuclear, Inc.). Alternatively, cDNA is synthesized from RNA using a T7- polyT primer, in the absence of label, and DNA dendrimers from Genisphere (3DNA Submicro) are hybridized to the poly T sequence on the primer, or to a different "capture sequence" which is complementary to a fluorescently labeled sequence. Each 3DNA molecule has 250 fluorescent molecules and therefore can strongly label each cDNA.

[0101] Alternatively, the RNA sample is amplified prior to labeling. For example, linear amplification may be performed, as described in U.S. Patent No. 6,132,997. A T7-polyT primer is used to generate the cDNA copy of the RNA. A second DNA strand is then made to complete the substrate for amplification. The T7 promoter incorporated into the primer is used by a T7 polymerase to produce numerous antisense copies of the original RNA. Fluorescent dye labeled nucleotides are directly incorporated into the RNA. Alternatively, amino allyl labeled nucleotides are incorporated into the RNA, and then fluorescent dyes are chemically coupled to the amino allyl groups, as described in Hughes. Other exemplary methods for amplification are described below.

[0102] It is appreciated that the RNA isolated must contain RNA derived from leukocytes, but may also contain RNA from other cell types to a variable degree. Additionally, the isolated RNA may come from subsets of leukocytes, e.g. monocytes and/or T-lymphocytes, as described above. Such consideration of cell type used for the derivation of RNA depend on the method of expression profiling used. Subsets of leukocytes can be obtained by fluorescence activated cell sorting (FACS), microfluidics cell seperation systems or a variety of other methods. Cell sorting may be necessary for the discovery of diagnostic gene sets, for the implementation of gene sets as products or both. Cell sorting can be achieved with a variety of technologies (See Galbraith et al. 1999, Cantor et al. 1975, see also the technology of Guava Technologies, Hayward, CA).

[0103] DNA samples may be obtained for analysis of the presence of DNA mutations, single nucleotide polymorphisms (SNPs), or other polymorphisms. DNA is isolated using standard techniques, e.g. *Maniatus, supra.*

[0104] Expression of products of candidate nucleotides may also be assessed using proteomics. Protein(s) are detected in samples of patient serum or from leukocyte cellular protein. Serum is prepared by centrifugation of whole blood, using standard methods. Proteins present in the serum may have been produced from any of a variety of leukocytes and non-leukocyte cells, and include secreted proteins from leukocytes. Alternatively, leukocytes or a desired sub-population of leukocytes are prepared as described above. Cellular protein is prepared from leukocyte samples using methods well known in the art, e.g., Trizol (Invitrogen Life Technologies, cat # 15596108; Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156; Simms, D., Cizdziel, P.E., and Chomczynski, P. (1993) Focus® 15, 99; Chomczynski, P., Bowers-Finn, R., and Sabatini, L. (1987) J. ofNIH Res. 6, 83; Chomczynski, P. (1993) Bio/Techniques 15, 532; Bracete, A.M., Fox, D.K., and Simms, D. (1998) Focus 20, 82; Sewall, A. and McRae, S. (1998) Focus 20, 36; Anal Biochem 1984 Apr:138(1):141-3, A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids; Wessel D, Flugge UI. (1984) Anal Biochem 1984 Apr;138(1):141-143.

[0105] The assay itself may be a cell sorting assay in which cells are sorted and/or counted based on cell surface expression of a protein marker. (See Cantor et al. 1975, Galbraith et al. 1999)

Obtaining expression patterns

[0106] Expression patterns, or profiles, of a plurality of nucleotides corresponding to members of the candidate library are then evaluated in one or more samples of leukocytes. Typically, the leukocytes are derived from patient peripheral blood samples, although, as indicated above, many other sample sources are also suitable. These expression patterns constitute set of relative or absolute expression values for a some number of RNAs or protein products corresponding to the plurality of nucleotide sequences evaluated, which is referred to herein as the subject's "expression profile" for those nucleotide sequences. While expression patterns for as few as one independent member of the candidate library can be obtained, it is generally preferable to obtain expression patterns corresponding to a larger number of nucleotide sequences, e.g., about 2, about 5, about 10, about 20, about 50, about 100, about 200, about 500, or about 1000, or more. The expression pattern for each differentially expressed component member of the library provides a finite specificity and sensitivity with respect to predictive value, e.g., for diagnosis, prognosis, monitoring, and the like.

Clinical Studies, Data and Patient Groups

[0107] For the purpose of discussion, the term subject, or subject sample of leukocytes, refers to an individual regardless of health and/or disease status. A subject can be a patient, a study participant, a control subject, a screening subject, or any other class of individual from whom a leukocyte sample is obtained and assessed in the context of the invention. Accordingly, a subject can be diagnosed with a disease, can present with one or more symptom of a disease, or a predisposing factor, such as a family (genetic) or medical history (medical) factor, for a disease, or the like. Alternatively, a subject can be healthy with respect to any of the aforementioned factors or criteria. It will be appreciated that the term

"healthy" as used herein, is relative to a specified disease, or disease factor, or disease criterion, as the term "healthy" cannot be defmed to correspond to any absolute evaluation or status. Thus, an individual defined as healthy with reference to any specified disease or disease criterion, can in fact be diagnosed with any other one or more disease, or exhibit any other one or more disease criterion.

Methods for obtaining expression data

[0108] Numerous methods for obtaining expression data are known, and any one or more of these techniques, singly or in combination, are suitable for determining expression profiles in the context of the present invention. For example, expression patterns can be evaluated by northern analysis, PCR, RT-PCR, Taq Man analysis, FRET detection, monitoring one or more molecular beacon, hybridization to an oligonucleotide array, hybridization to a cDNA array, hybridization to a polynucleotide array, hybridization to a liquid microarray, hybridization to a microelectric array, molecular beacons, cDNA sequencing, clone hybridization, cDNA fragment fingerprinting, serial analysis of gene expression (SAGE), subtractive hybridization, differential display and/or differential screening (*see,* e.g., Lockhart and Winzeler (2000) Nature 405:827-836, and references cited therein).

[0109] For example, specific PCR primers are designed to a member(s) of an candidate nucleotide library. cDNA is prepared from subject sample RNA by reverse transcription from a poly-dT oligonucleotide primer, and subjected to PCR. Double stranded cDNA may be prepared using primers suitable for reverse transcription of the PCR product, followed by amplification of the cDNA using in vitro transcription. The product of in vitro transcription is a sense-RNA corresponding to the original member(s) of the candidate library. PCR product may be also be evaluated in a number of ways known in the art, including real-time assessment using detection of labeled primers, e.g. TaqMan or molecular beacon probes. Technology platforms suitable for analysis of PCR products include the ABI 7700, 5700, or 7000 Sequence Detection Systems (Applied Biosystems, Foster City, CA), the MJ Research Opticon (MJ Research, Waltham, MA), the Roche Light Cycler (Roche Diagnositics, Indianapolis, IN), the Stratagene MX4000 (Stratagene, La Jolla, CA), and the Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA). Alternatively, molecular beacons are used to detect presence of a nucleic acid sequence in an unamplified RNA or cDNA sample, or following amplification of the sequence using any method, e.g. IVT (In Vitro transcription) or NASBA (nucleic acid sequence based amplification). Molecular beacons are designed with sequences complementary to member(s) of an candidate nucleotide library, and are linked to fluorescent labels. Each probe has a different fluorescent label with non-overlapping emission wavelengths. For example, expression of ten genes may be assessed using ten different sequence-specific molecular beacons.

[0110] Alternatively, or in addition, molecular beacons are used to assess expression of multiple nucleotide sequences at once. Molecular beacons with sequence complimentary to the members of a diagnostic nucleotide set are designed and linked to fluorescent labels. Each fluorescent label used must have a non-overlapping emission wavelength. For example, 10 nucleotide sequences can be assessed by hybridizing 10 sequence specific molecular beacons (each labeled with a different fluorescent molecule) to an amplified or un-amplified RNA or cDNA sample. Such an assay bypasses the need for sample labeling procedures.

[0111] Alternatively, or in addition bead arrays can be used to assess expression of multiple sequences at once. See, e.g, LabMAP 100, Luminex Corp, Austin, Texas). Alternatively, or in addition electric arrays are used to assess expression of multiple sequences, as exemplified by the e-Sensor technology of Motorola (Chicago, III.) or Nanochip technology ofNanogen (San Diego, CA.)

[0112] Of course, the particular method elected will be dependent on such factors as quantity of RNA recovered, practitioner preference, available reagents and equipment, detectors, and the like. Typically, however, the elected method(s) will be appropriate for processing the number of samples and probes of interest. Methods for high-throughput expression analysis are discussed below.

[0113] Alternatively, expression at the level of protein products of gene expression is performed. For example, protein expression, in a sample of leukocytes, can be evaluated by one or more method selected from among: western analysis, two-dimensional gel analysis, chromatographic separation, mass spectrometric detection, protein-fusion reporter constructs, colorimetric assays, binding to a protein array and characterization of polysomal mRNA. One particularly favorable approach involves binding of labeled protein expression products to an array of antibodies specific for members of the candidate library. Methods for producing and evaluating antibodies are widespread in the art, *see,* e.g., Coligan, *supra*; and Harlow and Lane (1989) Antibodies: A Laboratory Manual, Cold Spring Harbor Press, NY ("Harlow and Lane"). Additional details regarding a variety of immunological and immunoassay procedures adaptable to the present invention by selection of antibody reagents specific for the products of candidate nucleotide sequences can be found in, e.g., Stites and Terr (eds.)(1991) Basic and Clinical Immunology, 7th ed., and Paul, *supra.* Another approach uses systems for performing desorption spectrometry. Commercially available systems, e.g., from Ciphergen Biosystems, Inc. (Fremont, CA) are particularly well suited to quantitative analysis of protein expression. Indeed, Protein Chip® arrays (*see,* e.g., the web site cipliergen.com) used in desorption spectrometry approaches provide arrays for detection of protein expression. Alternatively, affinity reagents, e.g., antibodies, small molecules, etc.) are developed that recognize epitopes

of the protein product. Affinity assays are used in protein array assays, e.g. to detect the presence or absence of particular proteins. Alternatively, affinity reagents are used to detect expression using the methods described above. In the case of a protein that is expressed on the cell surface of leukocytes, labeled affinity reagents are bound to populations of leukocytes, and leukocytes expressing the protein are identified and counted using fluorescent activated cell sorting (FACS).

[0114] It is appreciated that the methods of expression evaluation discussed herein, although discussed in the context of discovery of diagnostic nucleotide sets, are equally applicable for expression evaluation when using diagnostic nucleotide sets for, e.g. diagnosis of diseases, as further discussed below.

High Throughput Expression Assays

[0115] A number of suitable high throughput formats exist for evaluating gene expression. Typically, the term high throughput refers to a format that performs at least about 100 assays, or at least about 500 assays, or at least about 1000 assays, or at least about 5000 assays, or at least about 10,000 assays, or more per day. When enumerating assays, either the number of samples or the number of candidate nucleotide sequences evaluated can be considered. For example, a northern analysis of, e.g., about 100 samples performed in a gridded array, e.g., a dot blot, using a single probe corresponding to an candidate nucleotide sequence can be considered a high throughput assay. More typically, however, such an assay is performed as a series of duplicate blots, each evaluated with a distinct probe corresponding to a different member of the candidate library. Alternatively, methods that simultaneously evaluate expression of about 100 or more candidate nucleotide sequences in one or more samples, or in multiple samples, are considered high throughput.

[0116] Numerous technological platforms for performing high throughput expression analysis are known. Generally, such methods involve a logical or physical array of either the subject samples, or the candidate library, or both. Common array formats include both liquid and solid phase arrays. For example, assays employing liquid phase arrays, e.g., for hybridization of nucleic acids, binding of antibodies or other receptors to ligand, etc., can be performed in multiwell, or microtiter, plates. Microtiter plates with 96, 384 or 1536 wells are widely available, and even higher numbers of wells, e.g, 3456 and 9600 can be used. In general, the choice of microtiter plates is determined by the methods and equipment, e.g., robotic handling and loading systems, used for sample preparation and analysis. Exemplary systems include, e.g., the ORCA™ system from Beckman-Coulter, Inc. (Fullerton, CA) and the Zymate systems from Zymark Corporation (Hopkinton, MA).

[0117] Alternatively, a variety of solid phase arrays can favorably be employed in to determine expression patterns in the context of the invention. Exemplary formats include membrane or filter arrays (e.g, nitrocellulose, nylon), pin arrays, and bead arrays (e.g., in a liquid "slurry"). Typically, probes corresponding to nucleic acid or protein reagents that specifically interact with (e.g., hybridize to or bind to) an expression product corresponding to a member of the candidate library are immobilized, for example by direct or indirect cross-linking, to the solid support. Essentially any solid support capable of withstanding the reagents and conditions necessary for performing the particular expression assay can be utilized. For example, functionalized glass, silicon, silicon dioxide, modified silicon, any of a variety of polymers, such as (poly)tetrafluoroethylene, (poly)vinylidenedifluoride, polystyrene, polycarbonate, or combinations thereof can all serve as the substrate for a solid phase array.

[0118] In a preferred embodiment, the array is a "chip" composed, e.g., of one of the above specified materials. Polynucleotide.probes, e.g., RNA or DNA, such as cDNA, synthetic oligonucleotides, and the like, or binding proteins such as antibodies, that specifically interact with expression products of individual components of the candidate library are affixed to the chip in a logically ordered manner, i.e., in an array. In addition, any molecule with a specific affinity for either the sense or anti-sense sequence of the marker nucleotide sequence (depending on the design of the sample labeling), can be fixed to the array surface without loss of specific affinity for the marker and can be obtained and produced for array production, for example, proteins that specifically recognize the specific nucleic acid sequence of the marker, ribozymes, peptide nucleic acids (PNA), or other chemicals or molecules with specific affinity.

[0119] Detailed discussion of methods for linking nucleic acids and proteins to a chip substrate, are found in, e.g., US Patent No. 5,143,854 "LARGE SCALE PHOTOLITHOGRAPHIC SOLID PHASE SYNTHESIS OF POLYPEPTIDES AND RECEPTOR BINDING SCREENING THEREOF" to Pimmg et al., issued, September 1, 1992; US Patent No. 5,837,832 "ARRAYS OF NUCLEIC ACID PROBES ON BIOLOGICAL CHIPS" to Chee et al., issued November 17, 1998; US Patent No. 6,087,112 "ARRAYS WITH MODIFIED OLIGONUCLEOTIDE AND POLYNUCLEOTIDE COMPOSITIONS" to Dale, issued July 11, 2000; US Patent No. 5,215,882 "METHOD OF IMMOBILIZING NUCLEIC ACID ON A SOLID SUBSTRATE FOR USE IN NUCLEIC ACID HYBRIDIZATION ASSAYS" to Bahl et al., issued June 1, 1993; US Patent No. 5,707,807 "MOLECULAR INDEXING FOR EXPRESSED GENE ANALYSIS" to Kato, issued January 13, 1998; US Patent No. 5,807,522 "METHODS FOR FABRICATING MICROARRAYS OF BIOLOGICAL SAMPLES" to Brown et al., issued September 15, 1998; US Patent No. 5,958,342 "JET DROPLET DEVICE" to Gamble et al., issued Sept. 28, 1999; US Patent 5,994,076 "METHODS OF ASSAYING DIFFERENTIAL EXPRESSION" to Chenchik et al., issued Nov. 30, 1999;

US Patent No. 6,004,755 "QUANTITATIVE MICROARRAY HYBRIDIZATION ASSAYS" to Wang, issued Dec. 21,1999; US Patent No. 6,048,695 "CHEMICALLY MODIFIED NUCLEIC ACIDS AND METHOD FOR COUPLING NUCLEIC ACIDS TO SOLID SUPPORT" to Bradley et al., issued April 11, 2000; US Patent No. 6,060,240 "METHODS FOR MEASURING RELATIVE AMOUNTS OF NUCLEIC ACIDS IN A COMPLEX MIXTURE AND RETRIEVAL OF SPECIFIC SEQUENCES THEREFROM" to Kamb et al., issued May 9, 2000; US Patent No. 6,090,556 "METHOD FOR QUANTITATIVELY DETERMINING THE EXPRESSION OF A GENE" to Kato, issued July 18, 2000; and US Patent 6,040,138 "EXPRESSION MONITORING BY HYBRIDIZATION TO HIGH DENSITY OLIGONUCLEOTIDE ARRAYS" to Lockhart et al., issued March 21, 2000.

[0120] For example, cDNA inserts corresponding to candidate nucleotide sequences, in a standard TA cloning vector are amplified by a polymerase chain reaction for approximately 30-40 cycles. The amplified PCR products are then arrayed onto a glass support by any of a variety of well known techniques, e.g., the VSLIPS™ technology described in US Patent No. 5,143,854. RNA, or cDNA corresponding to RNA, isolated from a subject sample of leukocytes is labeled, e.g., with a fluorescent tag, and a solution containing the RNA (or cDNA) is incubated under conditions favorable for hybridization, with the "probe" chip. Following incubation, and washing to eliminate non-specific hybridization, the labeled nucleic acid bound to the chip is detected qualitatively or quantitatively, and the resulting expression profile for the corresponding candidate nucleotide sequences is recorded. It is appreciated that the probe used for diagnostic purposes may be identical to the probe used during diagnostic nucleotide sequence discovery and validation. Alternatively, the probe sequence may be different than the sequence used in diagnostic nucleotide sequence discovery and validation. Multiple cDNAs from a nucleotide sequence that are non-overlapping or partially overlapping may also be used.

[0121] In another approach, oligonucleotides corresponding to members of an candidate nucleotide library are synthesized and spotted onto an array. Alternatively, oligonucleotides are synthesized onto the array using methods known in the art, e.g. Hughes, et al. *supra.* The oligonucleotide is designed to be complementary to any portion of the candidate nucleotide sequence. In addition, in the context of expression analysis for, e.g. diagnostic use of diagnostic nucleotide sets, an oligonucleotide can be designed to exhibit particular hybridization characteristics, or to exhibit a particular specificity and/or sensitivity, as further described below.

[0122] Hybridization signal may be amplified using methods known in the art, and as described herein, for example use of the Clontech kit (Glass Fluorescent Labeling Kit), Stratagene kit (Fairplay Microarray Labeling Kit), the Micromax kit (New England Nuclear, Inc.), the Genisphere kit (3DNA Submicro), linear amplification, e.g. as described in U.S. Patent No. 6,132,997 or described in Hughes, TR, et al., Nature Biotechnology, 19:343-347 (2001) and/or Westin et al. Nat Biotech. 18:199-204.

[0123] Alternatively, fluorescently labeled cDNA are hybridized directly to the microarray using methods known in the art. For example, labeled cDNA are generated by reverse transcription using Cy3- and Cy5-conjugated deoxynucleotides, and the reaction products purified using standard methods. It is appreciated that the methods for signal amplification of expression data useful for identifying diagnostic nucleotide sets are also useful for amplification of expression data for diagnostic purposes.

[0124] Microarray expression may be detected by scanning the microarray with a variety of laser or CCD-based scanners, and extracting features with numerous software packages, for example, Imagene (Biodiscovery), Feature Extraction (Agilent), Scanalyze (Eisen, M. 1999. SCANALYZE User Manual; Stanford Univ., Stanford, CA. Ver 2.32.), GenePix (Axon Instruments).

[0125] In another approach, hybridization to microelectric arrays is performed, e.g. as described in Umek et al (2001) J Mol Diagn. 3:74-84. An affinity probe, e.g. DNA, is deposited on a metal surface. The metal surface underlying each probe is connected to a metal wire and electrical signal detection system Unlabelled RNA or cDNA is hybridized to the array, or alternatively, RNA or cDNA sample is amplified before hybridization, e.g. by PCR. Specific hybridization of sample RNA or cDNA results in generation of an electrical signal, which is transmitted to a detector. See Westin (2000) Nat Biotech. 18:199-204 (describing anchored multiplex amplification of a microelectronic chip array); Edman (1997) NAR 25:4907-14; Vignali (2000) J Immunol Methods 243:243-55.

[0126] In another approach, a microfluidics chip is used for RNA sample preparation and analysis. This approach increases efficiency because sample preparation and analysis are streamlined. Briefly, microfluidics may be used to sort specific leukocyte sub-populations prior to RNA preparation and analysis. Microfluidics chips are also useful for, e.g., RNA preparation, and reactions involving RNA (reverse transcription, RT-PCR). Briefly, a small volume of whole, anti-coagulated blood is loaded onto a microfluidics chip, for example chips available from Caliper (Mountain View, CA) or Nanogen (San Diego, CA.) A microfluidics chip may contain channels and reservoirs in which cells are moved and reactions are performed. Mechanical, electrical, magnetic, gravitational, centrifugal or other forces are used to move the cells and to expose them to reagents. For example, cells of whole blood are moved into a chamber containing hypotonic saline, which results in selective lysis of red blood cells after a 20-minute incubation. Next, the remaining cells (leukocytes) are moved into a wash chamber and finally, moved into a chamber containing a lysis buffer such as guanidine isothyocyanate. The leukocyte cell lysate is further processed for RNA isolation in the chip, or is then removed for further processing, for example, RNA extraction by standard methods. Alternatively, the microfluidics chip is a circular disk

containing ficoll or another density reagent. The blood sample is injected into the center of the disc, the disc is rotated at a speed that generates a centrifugal force appropriate for density gradient separation of mononuclear cells, and the separated mononuclear cells are then harvested for further analysis or processing.

[0127] It is understood that the methods of expression evaluation, above, although discussed in the context of discovery of diagnostic nucleotide sets, are also applicable for expression evaluation when using diagnostic nucleotide sets for, e.g. diagnosis of diseases, as further discussed below.

Evaluation of expression patterns

[0128] Expression patterns can be evaluated by qualitative and/or quantitative measures. Certain of the above described techniques for evaluating gene expression (as RNA or protein products) yield data that are predominantly qualitative in nature. That is, the methods detect differences in expression that classify expression into distinct modes without providing significant information regarding quantitative aspects of expression. For example, a technique can be described as a qualitative technique if it detects the presence or absence of expression of an candidate nucleotide sequence, i.e., an on/off pattern of expression. Alternatively, a qualitative technique measures the presence (and/or absence) of different alleles, or variants, of a gene product.

[0129] In contrast, some methods provide data that characterizes expression in a quantitative manner. That is, the methods relate expression on a numerical scale, e.g., a scale of 0-5, a scale of 1-10, a scale of + - +++, from grade 1 to grade 5, a grade from a to z, or the like. It will be understood that the numerical, and symbolic examples provided are arbitrary, and that any graduated scale (or any symbolic representation of a graduated scale) can be employed in the context of the present invention to describe quantitative differences in nucleotide sequence expression. Typically, such methods yield information corresponding to a relative increase or decrease in expression.

[0130] Any method that yields either quantitative or qualitative expression data is suitable for evaluating expression of candidate nucleotide sequence in a subject sample of leukocytes. In some cases, e.g., when multiple methods are employed to determine expression patterns for a plurality of candidate nucleotide sequences, the recovered data, e.g., the expression profile, for the nucleotide sequences is a combination of quantitative and qualitative data.

[0131] In some applications, expression of the plurality of candidate nucleotide sequences is evaluated sequentially. This is typically the case for methods that can be characterized as low- to moderate-throughput. In contrast, as the throughput of the elected assay increases, expression for the plurality of candidate nucleotide sequences in a sample or multiple samples of leukocytes, is assayed simultaneously. Again, the methods (and throughput) are largely determined by the individual practitioner, although, typically, it is preferable to employ methods that permit rapid, e.g. automated or partially automated, preparation and detection, on a scale that is time-efficient and cost-effective.

[0132] It is understood that the preceding discussion, while directed at the assessment of expression of the members of candidate libraries, is also applies to the assessment of the expression of members of diagnostic nucleotide sets, as further discussed below.

Genotyping

[0133] In addition to, or in conjunction with the correlation of expression profiles and clinical data, it is often desirable to correlate expression patterns with the subject's genotype at one or more genetic loci. The selected loci can be, for example, chromosomal loci corresponding to one or more member of the candidate library, polymorphic alleles for marker loci, or alternative disease related loci (not contributing to the candidate library) known to be, or putatively associated with, a disease (or disease criterion). Indeed, it will be appreciated, that where a (polymorphic) allele at a locus is linked to a disease (or to a predisposition to a disease), the presence of the allele can itself be a disease criterion.

[0134] Numerous well known methods exist for evaluating the genotype of an individual, including southern analysis, restriction fragment length polymorphism (RFLP) analysis, polymerase chain reaction (PCR), amplification length polymorphism (AFLP) analysis, single stranded conformation polymorphism (SSCP) analysis, single nucleotide polymorphism (SNP) analysis (e.g., via PCR, Taqman or molecular beacons), among many other useful methods. Many such procedures are readily adaptable to high throughput and/or automated (or semi-automated) sample preparation and analysis methods. Most, can be performed on nucleic acid samples recovered via simple procedures from the same sample of leukocytes as yielded the material for expression profiling. Exemplary techniques are described in, e.g., Sambrook, and Ausubel, *supra.*

Identification of the diagnostic nucleotide sets

[0135] Identification of diagnostic nucleotide sets and disease specific target nucleotide sequence proceeds by correlating the leukocyte expression profiles with data regarding the subject's health status to produce a data set designated a "molecular signature." Examples of data regarding a patient's health status, also termed "disease criteria(ion)", is

described below and in the Section titled "selected diseases," below. Methods useful for correlation analysis are further described elsewhere in the specification.

[0136] Generally, relevant data regarding the subject's health status includes retrospective or prospective health data, e.g., in the form of the subject's medical history, as provided by the subject, physician or third party, such as, medical diagnoses, laboratory test results, diagnostic test results, clinical events, or medication lists, as further described below. Such data may include information regarding a patient's response to treatment and/or a particular medication and data regarding the presence of previously characterized "risk factors." For example, cigarette smoking and obesity are previously identified risk factors for heart disease. Further examples of health status information, including diseases and disease criteria, is described in the section titled Selected diseases, below.

[0137] Typically, the data describes prior events and evaluations (i.e., retrospective data). However, it is envisioned that data collected subsequent to the sampling (i.e., prospective data) can also be correlated with the expression profile. The tissue sampled, e.g., peripheral blood, bronchial lavage, etc., can be obtained at one or more multiple time points and subject data is considered retrospective or prospective with respect to the time of sample procurement.

[0138] Data collected at multiple time points, called "longitudinal data", is often useful, and thus, the invention encompasses the analysis of patient data collected from the same patient at different time points. Analysis of paired samples, such as samples from a patient at different time, allows identification of differences that are specifically related to the disease state since the genetic variability specific to the patient is controlled for by the comparison. Additionally, other variables that exist between patients may be controlled for in this way, for example, the presence or absence of inflammatory diseases (e.g., rheumatoid arthritis) the use of medications that may effect leukocyte gene expression, the presence or absence of co-morbid conditions, etc. Methods for analysis of paired samples are further described below. Moreover, the analysis of a pattern of expression profiles (generated by collecting multiple expression profiles) provides information relating to changes in expression level over time, and may permit the determination of a rate of change, a trajectory, or an expression curve. Two longitudinal samples may provide information on the change in expression of a gene over time, while three longitudinal samples may be necessary to determine the "trajectory" of expression of a gene. Such information may be relevant to the diagnosis of a disease. For example, the expression of a gene may vary from individual to individual, but a clinical event, for example , a heart attack, may cause the level of expression to double in each patient. In this example, clinically interesting information is gleaned from the change in expression level, as opposed to the absolute level of expression in each individual.

[0139] When a single patient sample is obtained, it may still be desirable to compare the expression profile of that sample to some reference expression profile. In this case, one can determine the change of expression between the patient's sample and a reference expression profile that is appropriate for that patient and the medical condition in question. For example, a reference expression profile can be determined for all patients without the disease criterion in question who have similar characteristics, such as age, sex, race, diagnoses etc.

[0140] Generally, small sample sizes of 20-100 samples are used to identify a diagnostic nucleotide set. Larger sample sizes are generally necessary to validate the diagnostic nucleotide set for use in large and varied patient populations, as further described below. For example, extension of gene expression correlations to varied ethnic groups, demographic groups, nations, peoples or races may require expression correlation experiments on the population of interest.

Expression Reference Standards

[0141] Expression profiles derived from a patient (i.e., subjects diagnosed with, or exhibiting symptoms of, or exhibiting a disease criterion, or under a doctor's care for a disease) sample are compared to a control or standard expression RNA to facilitate comparison of expression profiles (e.g. of a set of candidate nucleotide sequences) from a group of patients relative to each other (i.e., from one patient in the group to other patients in the group, or to patients in another group).

[0142] The reference RNA used should have desirable features of low cost and simplicity of production on a large scale. Additionally, the reference RNA should contain measurable amounts of as many of the genes of the candidate library as possible.

[0143] For example, in one approach to identifying diagnostic nucleotide sets, expression profiles derived from patient samples are compared to a expression reference "standard." Standard expression reference can be, for example, RNA derived from resting cultured leukocytes or commercially available reference RNA, such as Universal reference RNA from Stratagene. See Nature, V406, 8-17-00, p. 747-752. Use of an expression reference standard is particularly useful when the expression of large numbers of nucleotide sequences is assayed, e.g. in an array, and in certain other applications, e.g. qualitative PCR, RT-PCR, etc., where it is desirable to compare a sample profile to a standard profile, and/or when large numbers of expression profiles, e.g. a patient population, are to be compared. Generally, an expression reference standard should be available in large quantities, should be a good substrate for amplification and labeling reactions, and should be capable of detecting a large percentage of candidate nucleic acids using suitable expression profiling technology.

[0144] Alternatively, or in addition, the expression profile derived from a patient sample is compared with the expression of an internal reference control gene, for example, β-actin or CD4. The relative expression of the profiled genes and the internal reference control gene (from the same individual) is obtained. An internal reference control may also be used with a reference RNA. For example, an expression profile for "gene 1" and the gene encoding CD4 can be determined in a patient sample and in a reference RNA. The expression of each gene can be expressed as the "relative" ratio of expression the gene in the patient sample compared with expression of the gene in the reference RNA. The expression ratio (sample/reference) for gene 1 may be divided by the expression ration for CD4 (sample/reference) and thus the relative expression of gene 1 to CD4 is obtained.

[0145] The disclosure also provides a buffy coat control RNA useful for expression profiling, and a method of using control RNA produced from a population of buffy coat cells, the white blood cell layer derived from the centrifugation of whole blood. Buffy coat contains all white blood cells, including granulocytes, mononuclear cells and platelets. The disclosure also provides a method of preparing control RNA from buffy coat cells for use in expression profile analysis of leukocytes. Buffy coat fractions are obtained, e.g. from a blood bank or directly from individuals, preferably from a large number of individuals such that bias from individual samples is avoided and so that the RNA sample represents an average expression of a healthy population. Buffy coat fractions from about 50 or about 100, or more individuals are preferred. 10 ml buffy coat from each individual is used. Buffy coat samples are treated with an erthythrocyte lysis buffer, so that erthythrocytes are selectively removed. The leukocytes of the buffy coat layer are collected by centrifugation. Alternatively, the buffy cell sample can be further enriched for a particular leukocyte sub-populations, e.g. mononuclear cells, T-lymphocytes, etc. To enrich for mononuclear cells, the buffy cell pellet, above, is diluted in PBS (phosphate buffered saline) and loaded onto a non-polystyrene tube containing a polysucrose and sodium diatrizoate solution adjusted to a density of 1.077+/-0.001 g/ml. To enrich for T-lymphocvtes. 45 ml of whole blood is treated with RosetteSep (Stem Cell Technologies), and incubated at room temperature for 20 minutes. The mixture is diluted with an equal volume of PBS plus 2% FBS and mixed by inversion. 30 ml of diluted mixture is layered on top of 15 ml DML medium (Stem Cell Technologies). The tube is centrifuged at 1200 x g, and the enriched cell layer at the plasma : medium interface is removed, washed with PBS + 2% FBS, and cells collected by centrifugation at 1200 x g. The cell pellet is treated with 5 ml of erythrocyte lysis buffer (EL buffer, Qiagen) for 10 minutes on ice, and enriched T-lymphoctes are collected by centrifugation.

[0146] In addition or alternatively, the buffy cells (whole buffy coat or sub-population, e.g. mononuclear fraction) can be cultured *in vitro* and subjected to stimulation with cytokines or activating chemicals such as phorbol esters or ionomycin. Such stimuli may increase expression of nucleotide sequences that are expressed in activated immune cells and might be of interest for leukocyte expression profiling experiments.

[0147] Following sub-population selection and/or further treatment, e.g. stimulation as described above, RNA is prepared using standard methods. For example, cells are pelleted and lysed with a phenol/guanidinium thiocyanate and RNA is prepared. RNA can also be isolated using a silica gel-based purification column or the column method can be used on RNA isolated by the phenol/guanidinium thiocyanate method. RNA from individual buffy coat samples can be pooled during this process, so that the resulting reference RNA represents the RNA of many individuals and individual bias is minimized or eliminated. In addition, a new batch of buffy coat reference RNA can be directly compared to the last batch to ensure similar expression pattern from one batch to another, using methods of collecting and comparing expression profiles described above/below. One or more expression reference controls are used in an experiment. For example, RNA derived from one or more of the following sources can be used as controls for an experiment: stimulated or unstimulated whole buffy coat, stimulated or unstimulated peripheral mononuclear cells, or stimulated or unstimulated T-lymphocytes.

[0148] Alternatively, the expression reference standard can be derived from any subject or class of subjects including healthy subjects or subjects diagnosed with the same or a different disease or disease criterion. Expression profiles from subjects in two distinct classes are compared to determine which subset of nucleotide sequences in the candidate library best distinguish between the two subject classes, as further discussed below. It will be appreciated that in the present context, the term "distinct classes" is relevant to at least one distinguishable criterion relevant to a disease of interest, a "disease criterion." The classes can, of course, demonstrate significant overlap (or identity) with respect to other disease criteria, or with respect to disease diagnoses, prognoses, or the like. The mode of discovery involves, e.g., comparing the molecular signature of different subject classes to each other (such as patient to control, patients with a first diagnosis to patients with a second diagnosis, etc.) or by comparing the molecular signatures of a single individual taken at different time points. The invention can be applied to a broad range of diseases, disease criteria, conditions and other clinical and/or epidemiological questions, as further discussed above/below.

[0149] It is appreciated that while the present discussion pertains to the use of expression reference controls while identifying diagnostic nucleotide sets, expression reference controls are also useful during use of diagnostic nucleotide sets, e.g. use of a diagnostic nucleotide set for diagnosis of a disease, as further described below.

Analysis of expression profiles

[0150] In order to facilitate ready access, e.g., for comparison, review, recovery, and/or modification, the molecular signatures/expression profiles are typically recorded in a database. Most typically, the database is a relational database accessible by a computational device, although other formats, e.g., manually accessible indexed files of expression profiles as photographs, analogue or digital imaging readouts, spreadsheets, etc. can be used. Further details regarding preferred embodiments are provided below. Regardless of whether the expression patterns initially recorded are analog or digital in nature and/or whether they represent quantitative or qualitative differences in expression, the expression patterns, expression profiles (collective expression patterns), and molecular signatures (correlated expression patterns) are stored digitally and accessed via a database. Typically, the database is compiled and maintained at a central facility, with access being available locally and/or remotely.

[0151] As additional samples are obtained, and their expression profiles determined and correlated with relevant subject data, the ensuing molecular signatures are likewise recorded in the database. However, rather than each subsequent addition being added in an essentially passive manner in which the data from one sample has little relation to data from a second (prior or subsequent) sample, the algorithms optionally additionally query additional samples against the existing database to further refine the association between a molecular signature and disease criterion. Furthermore, the data set comprising the one (or more) molecular signatures is optionally queried against an expanding set of additional or other disease criteria. The use of the database in integrated systems and web embodiments is further described below.

Analysis of expression profile data from arrays

[0152] Expression data is analyzed using methods well known in the art, including the software packages Imagene (Biodiscovery, Marina del Rey, CA), Feature Extraction Software (Agilent, Palo Alto, CA), and Scanalyze (Stanford University). In the discussion that follows, a "feature" refers to an individual spot of DNA on an array. Each gene may be represented by more than one feature. For example, hybridized microarrays are scanned and analyzed on an Axon Instruments scanner using GenePix 3.0 software (Axon Instruments, Union City, CA). The data extracted by GenePix is used for all downstream quality control and expression evaluation. The data is derived as follows. The data for all features flagged as "not found" by the software is removed from the dataset for individual hybridizations. The "not found" flag by GenePix indicates that the software was unable to discriminate the feature from the background. Each feature is examined to determine the value of its signal. The median pixel intensity of the background (B_n) is subtracted from the median pixel intensity of the feature (F_n) to produce the background-subtracted signal (hereinafter, "BGSS"). The BGSS is divided by the standard deviation of the background pixels to provide the signal-to-noise ratio (hereinafter, "S/N"). Features with a S/N of three or greater in both the Cy3 channel (corresponding to the sample RNA) and Cy5 channel (corresponding to the reference RNA) are used for further analysis (hereinafter denoted "useable features"). Alternatively, different S/Ns are used for selecting expression data for an analysis. For example, only expression data with signal to noise ratios > 3 might be used in an analysis. Alternatively, features with S/N values < 3 may be flagged as such and included in the analysis. Such flagged data sets include more values and may allow one to discover expression markers that would be missed otherwise. However, such data sets may have a higher variablilty than filtered data, which may decrease significance of findings or performance of correlation statistics.

[0153] For each usable feature (i), the expression level (e) is expressed as the logarithm of the ratio (R) of the Background Subtracted Signal (hereinafter "BGSS") for the Cy3 (sample RNA) channel divided by the BGSS for the Cy5 channel (reference RNA). This "log ratio" value is used for comparison to other experiments.

$$R_i = \frac{BGSS_{sample}}{BGSS_{reference}} \qquad (0.1)$$

$$e_i = \log r_i \qquad (0.2)$$

[0154] Variation in signal across hybridizations may be caused by a number of factors affecting hybridization, DNA spotting, wash conditions, and labeling efficiency.

[0155] A single reference RNA may be used with all of the experimental RNAs, permitting multiple comparisons in addition to individual comparisons. By comparing sample RNAs to the same reference, the gene expression levels from each sample are compared across arrays, permitting the use of a consistent denominator for our experimental ratios.

[0156] Alternative methods of analyzing the data may involve 1) using the sample channel without normalization by

the reference channel, 2) using an intensity-dependent normalization based on the reference which provides a greater correction when the signal in the reference channel is large, 3) using the data without background subtraction or subtracting an empirically derived function of the background intensity rather than the background itself.

Scaling

[0157] The data may be scaled (normalized) to control for labeling and hybridization variability within the experiment, using methods known in the art. Scaling is desirable because it facilitates the comparison of data between different experiments, patients, etc. Generally the BGSS are scaled to a factor such as the median, the mean, the trimmed mean, and percentile. Additional methods of scaling include: to scale between 0 and 1, to subtract the mean, or to subtract the median.

[0158] Scaling is also performed by comparison to expression patterns obtained using a common reference RNA, as described in greater detail above. As with other scaling methods, the reference RNA facilitates multiple comparisons of the expression data, e.g., between patients, between samples, etc. Use of a reference RNA provides consistent denominator for experimental ratios.

[0159] In addition to the use of a reference RNA, individual expression levels may be adjusted to correct for differences in labeling efficiency between different hybridization experiments, allowing direct comparison between experiments with different overall signal intensities, for example. A scaling factor (*a*) may be used to adjust individual expression levels as follows. The median of the scaling factor (*a*), for example, BGSS, is determined for the set of all features with a S/N greater than three. Next, the $BGSS_i$ (the BGSS for each feature "i") is divided by the median for all features (*a*), generating a scaled ratio. The scaled ration is used to determine the expression value for the feature (e_i), or the log ratio.

$$S_i = \frac{BGSS_i}{a} \qquad (0.3)$$

$$e_i = \log\left(\frac{Cy3S_i}{Cy5S_i}\right) \qquad (0.4)$$

[0160] In addition, or alternatively, control features are used to normalize the data for labeling and hybridization variability within the experiment. Control feature may be cDNA for genes from the plant, *Arabidopsis thaliana,* that are included when spotting the mini-array. Equal amounts of RNA complementary to control cDNAs are added to each of the samples before they were labeled. Using the signal from these control genes, a normalization constant (*L*) is determined according to the following formula:

$$L_j = \frac{\frac{\sum_{i=1}^{N} BGSS_{j,i}}{N}}{\frac{\sum_{j=1}^{K}\frac{\sum_{i=1}^{N} BGSS_{j,i}}{N}}{K}}$$

where $BGSS_i$ is the signal for a specific feature, *N* is the number *of A. thaliana* control features, *K* is the number of hybridizations, and L_j is the normalization constant for each individual hybridization.

[0161] Using the formula above, the mean for all control features of a particular hybridization and dye (e.g., Cy3) is calculated. The control feature means for all Cy3 hybridizations are averaged, and the control feature mean in one hybridization divided by the average of all hybridizations to generate a normalization constant for that particular Cy3 hybridization (L_j), which is used as *a* in equation (0.3). The same normalization steps may be performed for Cy3 and Cy5 values.

[0162] An alternative scaling method can also be used. The log of the ratio of Green/Red is determined for all features. The median log ratio value for all features is determined. The feature values are then scaled using the following formula:

Log_Scaled_Feature_Ratio Log_feature_Ratio - Median_Log_Ratio.

[0163] Many additional methods for normalization exist and can be applied to the data. In one method, the average ratio of Cy3 BGSS / Cy5 BGSS is determined for all features on an array. This ratio is then scaled to some arbitrary number, such as 1 or some other number. The ratio for each probe is then multiplied by the scaling factor required to bring the average ratio to the chosen level. This is performed for each array in an analysis. Alternatively, the ratios are normalized to the average ratio across all arrays in an analysis. Other methods of normalization include forcing the distribution of signal strengths of the various arrays into greater agreement by transforming them to match certain points (quartiles, or deciles, etc.) in a standard distribution, or in the most extreme case using the rank of the signal of each oligonucleotide relative to the other oligonucleotides on the array.

[0164] If multiple features are used per gene sequence or oligonucleotide, these repeats can be used to derive an average expression value for each gene. If some of the replicate features are of poor qualitay and don't meet requirements for analysis, the remaining features can be used to represent the gene or gene sequence.

Correlation analysis

[0165] Correlation analysis is performed to determine which array probes have expression behavior that best distinguishes or serves as markers for relevant groups of samples representing a particular clinical condition. Correlation analysis, or comparison among samples representing different disease criteria (e.g., clinical conditions), is performed using standard statistical methods. Numerous algorithms are useful for correlation analysis of expression data, and the selection of algorithms depends in part on the data analysis to be performed. For example, algorithms can be used to identify the single most informative gene with expression behavior that reliably classifies samples, or to identify all the genes useful to classify samples. Alternatively, algorithms can be applied that determine which set of 2 or more genes have collective expression behavior that accurately classifies samples. The use of multiple expression markers for diagnostics may overcome the variability in expression of a gene between individuals, or overcome the variability intrinsic to the assay. Multiple expression markers may include redundant markers (surrogates), in that two or more genes or probes may provide the same information with respect to diagnosis. This may occur, for example, when two or more genes or gene probes are coordinately expressed. For diagnostic application, it may be appropriate to utilize a gene and one or more of its surrogates in the assay. This redundancy may overcome failures (technical or biological) of a single marker to distinguish samples. Alternatively, one or more surrogates may have properties that make them more suitable for assay development, such as a higher baseline level of expression, better cell specificity, a higher fold change between sample groups or more specific sequence for the design of PCR primers or complimentary probes. It will be appreciated that while the discussion above pertains to the analysis of RNA expression profiles the discussion is equally applicable to the analysis of profiles of proteins or other molecular markers.

[0166] Prior to analysis, expression profile data may be formatted or prepared for analysis using methods known in the art. For example, often the log ratio of scaled expression data for every array probe is calculated using the following formula:

log(Cy3 BGSS/Cy5 BGSS)

,where Cy 3 signal corresponds to the expression of the gene in the clinical sample, and Cy5 signal corresponds to expression of the gene in the reference RNA.

[0167] Data may be further filtered depending on the specific analysis to be done as noted below. For example, filtering may be aimed at selecting only samples with expression above a certain level, or probes with variability above a certain level between sample sets.

[0168] The following non-limiting discussion consider several statistical methods known in the art. Briefly, the t-test and ANOVA are used to identify single genes with expression differences between or among populations, respectively. Multivariate methods are used to identify a set of two or more genes for which expression discriminates between two disease states more specifically than expression of any single gene.

t-test

[0169] The simplest measure of a difference between two groups is the Student's t test. See, e.g., Welsh et al. (2001) Proc Natl Acad Sci USA 98:1176-81 (demonstrating the use of an unpaired Student's t-test for the discovery of differential gene expression in ovarian cancer samples and control tissue samples). The t- test assumes equal variance and normally distributed data. This test identifies the probability that there is a difference in expression of a single gene between two groups of samples. The number of samples within each group that is required to achieve statistical significance is dependent upon the variation among the samples within each group. The standard formula for a t-test is:

$$t(e_i) = \frac{\bar{e}_{i,c} - \bar{e}_{i,t}}{\sqrt{(s^2_{i,c}/n_c) + (s^2_{i,t}/n_t)}}, \qquad (0.5)$$

where $\bar{e}_i$ is the difference between the mean expression level of gene i in groups c and t, $s_{i,c}$ is the variance of gene x in group c and $s_{i,t}$ is the variance of gene x in group t. n_c and n_t are the numbers of samples in groups c and t.

[0170] The combination of the t statistic and the degrees of freedom [min(n_t, n_c)-1] provides a p value, the probability of rejecting the null hypothesis. A p-value of ≤0.01, signifying a 99 percent probability the mean expression levels are different between the two groups (a 1% chance that the mean expression levels are in fact not different and that the observed difference occurred by statistical chance), is often considered acceptable.

[0171] When performing tests on a large scale, for example, on a large dataset of about 8000 genes, a correction factor must be included to adjust for the number of individual tests being performed. The most common and simplest correction is the Bonferroni correction for multiple tests, which divides the p-value by the number of tests run. Using this test on an 8000 member dataset indicates that a p value of ≤0.00000125 is required to identify genes that are likely to be truly different between the two test conditions.

Significance analysis for microarrays (SAM)

[0172] significance analysis for microarrays (SAM) (Tusher 2001) is a method through which genes with a correlation between their expression values and the response vector are statistically discovered and assigned a statistical significance. The ratio of false significant to significant genes is the False Discovery Rate (FDR). This means that for each threshold there are a set of genes which are called significant, and the FDR gives a confidence level for this claim. If a gene is called differentially expressed between 2 classes by SAM, with a FDR of 5%, there is a 95% chance that the gene is actually differentially expressed between the classes. SAM takes intoaccount the variability and large number of variables of microarrays. SAM will identiy genes that are most globally differentially expressed between the classes. Thus, important genes for identifying and classifying outlier samples or patients may not be identified by SAM.

Non-Parametric Tests

[0173] Wilcoxon's signed ranks method is one example of a non-parametric test and is utilized for paired comparisons. See e.g., Sokal and Rohlf (1987) Introduction to Biostatistics 2nd edition, WH Freeman, New York. At least 6 pairs are necessary to apply this statistic. This test is useful for analysis of paired expression data (for example, a set of patients who have cardiac transplant biopsy on 2 occasions and have a grade 0 on one occasion and a grade 3A on another). The Fisher Exact Test with a threshold and the Mann-Whitney Test are other non-parametric tests that may be used.

ANOVA

[0174] Differences in gene expression across multiple related groups may be assessed using an Analysis of Variance (ANOVA), a method well known in the art (Michelson and Schofield, 1996).

Multivariate analysis

[0175] Many algorithms suitable for multivariate analysis are known in the art. Generally, a set of two or more genes for which expression discriminates between two disease states more specifically than expression of any single gene is identified by searching through the possible combinations of genes using a criterion for discrimination, for example the expression of gene X must increase from normal 300 percent, while the expression of genes Y and Z must decrease from normal by 75 percent. Ordinarily, the search starts with a single gene, then adds the next best fit at each step of the search. Alternatively, the search starts with all of the genes and genes that do not aid in the discrimination are eliminated step-wise.

Paired samples

[0176] Paired samples, or samples collected at different time-points from the same patient, are often useful, as described above. For example, use of paired samples permits the reduction of variation due to genetic variation among individuals. In addition, the use of paired samples has a statistical significance, in that data derived from paired samples can be calculated in a different manner that recognizes the reduced variability. For example, the formula for a t-test for

paired samples is:

$$t(e_x) = \frac{\overline{D}_{e_x}}{\sqrt{\frac{\sum D^2 - (\sum D)^2 / b}{b-1}}}, \qquad (0.5)$$

where D is the difference between each set of paired samples and b is the number of sample pairs. $\overline{D}$ is the mean of the differences between the members of the pairs. In this test, only the differences between the paired samples are considered, then grouped together (as opposed to taking all possible differences between groups, as would be the case with an ordinary t-test). Additional statistical tests useful with paired data, e.g., ANOVA and Wilcoxon's signed rank test, are discussed above.

Diagnostic classification

[0177] Once a discriminating set of genes is identified, the diagnostic classifier (a mathematical function that assigns samples to diagnostic categories based on expression data) is applied to unknown sample expression levels.

[0178] Methods that can be used for this analysis include the following non-limiting list:

[0179] CLEAVER is an algorithm used for classification of useful expression profile data. See Raychaudhuri et al. (2001) Trends Biotechnol 19:189-193. CLEAVER uses positive training samples (e.g., expression profiles from samples known to be derived from a particular patient or sample diagnostic category, disease or disease criteria), negative training samples (e.g., expression profiles from samples known not to be derived from a particular patient or sample diagnostic category, disease or disease criteria) and test samples (e.g., expression profiles obtained from a patient), and determines whether the test sample correlates with the particular disease or disease criteria, or does not correlate with a particular disease or disease criteria. CLEAVER also generates a list of the 20 most predictive genes for classification.

[0180] Artificial neural networks (hereinafter, "ANN") can be used to recognize patterns in complex data sets and can discover expression criteria that classify samples into more than 2 groups. The use of artificial neural networks for discovery of gene expression diagnostics for cancers using expression data generated by oligonucleotide expression microarrays is demonstrated by Khan et al. (2001) Nature Med. 7:673-9. Khan found that 96 genes provided 0% error rate in classification of the tumors. The most important of these genes for classification was then determined by measuring the sensitivity of the classification to a change in expression of each gene. Hierarchical clustering using the 96 genes results in correct grouping of the cancers into diagnostic categories.

[0181] Golub uses cDNA microarrays and a distinction calculation to identify genes with expression behavior that distinguishes myeloid and lymphoid leukemias. See Golub et al. (1999) Science 286:531-7. Self organizing maps were used for new class discovery. Cross validation was done with a "leave one out" analysis. 50 genes were identified as useful markers. This was reduced to as few as 10 genes with equivalent diagnostic accuracy.

[0182] Hierarchical and non-hierarchical clustering methods are also useful for identifying groups of genes that correlate with a subset of clinical samples such as with transplant rejection grade. Alizadeh used hierarchical clustering as the primary tool to distinguish different types of diffuse B-cell lymphomas based on gene expression profile data. See Alizadeh et al. (2000) Nature 403:503-11. Alizadeh used hierarchical clustering as the primary tool to distinguish different types of diffuse B-cell lymphomas based on gene expression profile data. A cDNA array carrying 17856 probes was used for these experiments, 96 samples were assessed on 128 arrays, and a set of 380 genes was identified as being useful for sample classification.

[0183] Perou demonstrates the use of hierarchical clustering for the molecular classification of breast tumor samples based on expression profile data. See Perou el al. (2000) Nature 406:747-52. In this work, a cDNA array carrying 8102 gene probes was used. 1753 of these genes were found to have high variation between breast tumors and were used for the analysis.

[0184] Hastie describes the use of gene shaving for discovery of expression markers. Hastie et al. (2000) Genome Biol. 1(2):RESEARCH 0003.1-0003.21. The gene shaving algorithm identifies sets of genes with similar or coherent expression patterns, but large variation across conditions (RNA samples, sample classes, patient classes). In this manner, genes with a tight expression pattern within a transplant rejection grade, but also with high variability across rejection grades are grouped together. The algorithm takes advantage of both characteristics in one grouping step. For example, gene shaving can identify useful marker genes with co-regulated expression. Sets of useful marker genes can be reduced to a smaller set, with each gene providing some non-redundant value in classification. This algorithm was used on the data set described in Alizadeh et al., supra, and the set of 380 informative gene markers was reduced to 234.

[0185] Supervised harvesting of expression trees (Hastie 2001) identifies genes or clusters that best distinguish one

class from all the others on the data set. The method is used to identify the genes/clusters that can best separate one class versus all the others for datasets that include two or more classes or all classes from each other. This algorithm can be used for discovery or testing of a diagnostic gene set.

[0186] CART is a decision tree classification algorithm (Breiman 1984). From gene expression and or other data, CART can develop a decision tree for the classification of samples. Each node on the decision tree involves a query about the expression level of one or more genes or variables. Samples that are above the threshold go down one branch of the decision tree and samples that are not go down the other branch. See example 4 for further description of its use in classification analysis and examples of its usefulness in discovering and implementing a diagnostic gene set. CART identifies surrogates for each splitter (genes that are the next best substitute for a useful gene in classification).

[0187] Multiple Additive Regression Trees (Friedman, JH 1999, MART) is similar to CART in that it is a classification algorithm that builds decision trees to distinguish groups. MART builds numerous trees for any classification problem and the resulting model involves a combination of the multiple trees. MART can select variables as it build models and thus can be used on large data sets, such as those derived from an 8000 gene microarray. Because MART uses a combination of many trees and does not take too much information from any one tree, it resists over training. MART identifies a set of genes and an algorithm for their use as a classifier.

[0188] A Nearest Shrunken Centroids Classifier can be applied to microarray or other data sets by the methods described by Tibshirani et al. 2002. This algorithms also identified gene sets for classification and determines their 10 fold cross validation error rates for each class of samples. The algorithm determines the error rates for models of any size, from one gene to all genes in the set. The error rates for either or both sample classes can are minimized when a particular number of genes are used. When this gene number is determined, the algorithm associated with the selected genes can be identified and employed as a classifier on prospective sample.

[0189] Once a set of genes and expression criteria for those genes have been established for classification, cross validation is done. There are many approaches, including a 10 fold cross validation analysis in which 10%, of the training samples are left out of the analysis and the classification algorithm is built with the remaining 90%. The 10% are then used as a test set for the algorithm. The process is repeated 10 times with 10% of the samples being left out as a test set each time. Through this analysis, one can derive a cross validation error which helps estimate the robustness of the algorithm for use on prospective (test) samples.

[0190] Clinical data are gathered for every patient sample used for expression analysis. Clinical variables can be quantitative or non-quantitative. A clinical variable that is quantitiative can be used as a variable for significance or classification analysis. Non-quantitative clinical variables, such as the sex of the patient, can also be used in a significance analysis or classification analysis with some statistical tool. It is appreciated that the most useful diagnostic gene set for a condition may be optimal when considered along with one or more predictive clinical variables. Clinical data can also be used as supervising vectors for a correlation analysis. That is to say that the clinical data associated with each sample can be used to divide the samples into meaningful diagnostic categories for analysis. For example, samples can be divided into 2 or more groups based on the presence or absence of some diagnostic criterion (a). In addition, clinical data can be utilized to select patients for a correlation analysis or to exclude them based on some undesirable characteristic, such as an ongoing infection, a medicine or some other issue. Clincial data can also be used to assess the pretest probability of an outcome. For example, patients who are female are much more likely to be diagnosed as having systemic lupus erythematosis than patients who are male.

[0191] Once a set of genes are identified that classify samples with acceptable accuracy. These genes are validated as a set using new samples that were not used to discover the gene set. These samples can be taken from frozen archieves from the discovery clinical study or can be taken from new patients prospectively. Validation using a "test set" of samples can be done using expression profiling of the gene set with microarrays or using real-time PCR for each gene on the test set samples. Alternatively, a different expression profiling technology can be used.

Immune Monitoring

[0192] Leukocyte gene expression can be used to monitor the immune system. Immune monitoring examines both the level of gene expression for a set of genes in a given cell type and for genes which are expressed in a cell type selective manner gene expression monitoring will also detect the presence or absence of new cell types, progenitor cells, differentiation of cells and the like. Gene expression patterns may be associated with activation or the resting state of cells of the immune system that are responsible for or responsive to a disease state. For example, in the process of transplant rejection, cells of the immune system are activated by the presence of the foreign tissue. Genes and gene sets that monitor and diagnose this process are providing a measure of the level and type of activation of the immune system Genes and gene sets that are useful in monitoring the immune system may be useful for diagnosis and monitoring of all diseases that involve the immune system Some examples are transplant rejection, rheumatoid arthritis, lupus, inflammatory bowel diseases, multiple sclerosis, HTV/AIDS, and viral, bacterial and fungal infection. All disorders and diseases disclosed herein are contemplated. Genes and gene sets that monitor immune activation are useful for mon-

itoring response to immunosuppressive drug therapy, which is used to decrease immune activation. Genes are found to correlate with immune activation by correlation of expression patterns to the known presence of immune activation or quiescence in a sample as determined by some other test.

Selected Diseases

[0193] In principle, diagnostic nucleotide sets of the invention may be developed and applied to essentially any disease, or disease criterion, as long as at least one subset of nucleotide sequences is differentially expressed in samples derived from one or more individuals with a disease criteria or disease and one or more individuals without the disease criteria or disease, wherein the individual may be the same individual sampled at different points in time, or the individuals may be different individuals (or populations of individuals). For example, the subset of nucleotide sequences may be differentially expressed in the sampled tissues of subjects with the disease or disease criterion (e.g., a patient with a disease or disease criteria) as compared to subjects without the disease or disease criterion (e.g., patients without a disease (control patients)). Alternatively, or in addition, the subset of nucleotide sequence(s) may be differentially expressed in different samples taken from the same patient, e.g at different points in time, at different disease stages, before and after a treatment, in the presence or absence of a risk factor, etc.

[0194] Expression profiles corresponding to sets of nucleotide sequences that correlate not with a diagnosis, but rather with a particular aspect of a disease can also be used to identify the diagnostic nucleotide sets and disease specific target nucleotide sequences of the invention. For example, such an aspect, or disease criterion, can relate to a subject's medical or family history, e.g., childhood illness, cause of death of a parent or other relative, prior surgery or other intervention, medications, symptoms (including onset and/or duration of symptoms), etc. Alternatively, the disease criterion can relate to a diagnosis, e.g., hypertension, diabetes, atherosclerosis, or prognosis (e.g., prediction of future diagnoses, events or complications), e.g., acute myocardial infarction, restenosis following angioplasty, reperfusion injury, allograft rejection, rheumatoid arthritis or systemic lupus erythematosis disease activity or the like. In other cases, the disease criterion corresponds to a therapeutic outcome, e.g., transplant rejection, bypass surgery or response to a medication, restenosis after stent implantation, collateral vessel growth due to therapeutic angiogenesis therapy, decreased angina due to revascularization, resolution of symptoms associated with a myriad of therapies, and the like. Alternatively, the disease criteria corresponds with previously identified or classic risk factors and may correspond to prognosis or future disease diagnosis. As indicated above, a disease criterion can also correspond to genotype for one or more loci. Disease criteria (including patient data) may be collected (and compared) from the same patient at different points in time, from different patients, between patients with a disease (criterion) and patients respresenting a control population, etc. Longitudinal data, i.e., data collected at different time points from an individual (or group of individuals) may be used for comparisons of samples obtained from an individual (group of individuals) at different points in time, to permit identification of differences specifically related to the disease state, and to obtain information relating to the change in expression over time, including a rate of change or trajectory of expression over time. The usefulness of longitudinal data is further discussed in the section titled "Identification of diagnostic nucleotide sets of the invention".

[0195] It is further understood that diagnostic nucleotide sets may be developed for use in diagnosing conditions for which there is no present means of diagnosis. For example, in rheumatoid arthritis, joint destruction is often well under way before a patient experience symptoms of the condition. A diagnostic nucleotide set may be developed that diagnoses rheumatic joint destruction at an earlier stage than would be possible using present means of diagnosis, which rely in part on the presentation of symptoms by a patient. Diagnostic nucleotide sets may also be developed to replace or augment current diagnostic procedures. For example, the use of a diagnostic nucleotide set to diagnose cardiac allograft rejection may replace the current diagnostic test, a graft biopsy.

[0196] It is understood that the following discussion of diseases is exemplary and non-limiting, and further that the general criteria discussed above, e.g. use of family medical history, are generally applicable to the specific diseases discussed below.

[0197] In addition to leukocytes, as described throughout, the general method is applicable to nucleotide sequences that are differentially expressed in any subject tissue or cell type, by the collection and assessment of samples of that tissue or cell type. However, in many cases, collection of such samples presents significant technical or medical problems given the current state of the art.

Organ transplant rejection and success

[0198] A frequent complication of organ transplantation is recognition of the transplanted organ as foreign by the immune system resulting in rejection. Diagnostic nucleotide sets can be identified and validated for monitoring organ transplant success, rejection and treatment. Medications currently exist that suppress the immune system, and thereby decrease the rate of and severity of rejection. However, these drugs also suppress the physiologic immune responses, leaving the patient susceptible to a wide variety of opportunistic infections and cancers. At present there is no easy,

reliable way to diagnose transplant rejection. Organ biopsy is the preferred method, but this is expensive, painful and associated with significant risk and has inadequate sensitivity for focal rejection.

[0199] Diagnostic nucleotide sets of the present disclosure can be developed and validated for use as diagnostic tests for transplant rejection and success. It is appreciated that the methods of identifying diagnostic nucleotide sets are applicable to any organ transplant population. For example, diagnostic nucleotide sets are developed for cardiac allograft rejection and success.

[0200] In some cases, disease criteria correspond to acute stage rejection diagnosis based on organ biopsy and graded using the International Society for Heart and Lung Transplantation ("ISHLT") criteria. This grading system classifies endomyocardial biopsies on the histological level as Grade 0, 1A, 1B, 2, 3A, 3B, or 4. Grade 0 biopies have no evidence of rejection, while each successive grade has increased severity of leukocyte infiltration and/or damage to the graft myocardial cells. It is appreciated that there is variability in the Grading systems between medical centers and pathologists and between repeated readings of the same pathologist at different times. When using the biopsy grade as a disease criterion for leukocyte gene expression correlation analysis, it may be desirable to have a single pathologist read all biopsy slides or have multiple pathologists read all slides to determine the variablility in this disease criterion. It is also appreciated that cardiac biopsy, in part due to variability, is not 100% sensitive or 100% specific for diagnosing acute rejection. When using the cardiac biopsy grade as a disease criterion for the discovery of diagnostic gene sets, it may be desirable to divide patient samples into diagnostic categories based on the grades. Examples of such classes are those patients with: Grade 0 vs. Grades 1A-4, Grade 0 vs. Grades 1B-4, Grade 0 vs. Grades 2-4, Grade 0-1 vs. Grade 2-4, Grade 0-1 vs. Grade 3A-4, or Grade 0 vs. Grade 3A-4.

[0201] Other disease criteria correspond to the cardiac biopsy results and other criteria, such as the results of cardiac function testing by echocardiography, hemodynamics assessment by cardiac catheterization, CMV infection, weeks post transplant, medication regimen, demographics and/or results of other diagnostic tests.

[0202] Other disease criteria correspond to information from the patient's medical history and information regarding the organ donor. Alternatively, disease criteria include the presence or absence of cytomegalovirus (CMV) infection, Epstein-Barr virus (EBV) infection, allograft dysfunction measured by physiological tests of cardiac function (e.g., hemodynamic measurements from catheterization or echocardiograph data), and symptoms of other infections. Alternatively, disease criteria correspond to therapeutic outcome, e.g. graft failure, re-transplantation, death, hospitalization, need for intravenous immunosuppression, transplant vasculopathy, response to immunosuppressive medications, etc. Disease criteria may further correspond to a rejection episode of at least moderate histologic grade, which results in treatment of the patient with additional corticosteroids, anti-T cell antibodies, or total lymphoid irradiation; a rejection with histologic grade 2 or higher; a rejection with histologic grade <2; the absence of histologic rejection and normal or unchanged allograft function (based on hemodynamic measurements from catheterization or on echocardiographic data); the presence of severe allograft dysfunction or worsening allograft dysfunction during the study period (based on hemodynamic measurements from catheterization or on echocardiographic data).; documented CMV infection by culture, histology, or PCR, and at least one clinical sign or symptom of infection; specific graft biopsy rejection grades; rejection of mild to moderate histologic severity prompting augmentation of the patient's chronic immunosuppressive regimen; rejection of mild to moderate severity with allograft dysfunction prompting plasmaphoresis or a diagnosis of "humoral" rejection; infections other than CMV, especially infection with Epstein Barr virus (EBV); lymphoproliferative disorder (also called post-transplant lymphoma); transplant vasculopathy diagnosed by increased intimal thickness on intravascular ultrasound (IVUS), angiography, or acute myocardial infarction; graft failure or retransplantation; and all cause mortality. Further specific examples of clinical data useful as disease criteria are provided in Example 3.

[0203] In another example, diagnostic nucleotide sets are developed and validated for use in diagnosis and monitoring of kidney allograft recipients. Disease criteria correspond to, e.g., results of biopsy analysis for kidney allograft rejection, serum creatine level, creatinine clearance, radiological imaging results for the kidney and urinalysis results. Another disease criterion corresponds to the need for hemodialysis, retransplantation, death or other renal replacement therapy. Diagnostic nucleotide sets are developed and validated for use in diagnosis and treatment of bone marrow transplant and liver transplantation pateints, respectively. Disease criteria for bone marrow transplant correspond to the diagnosis and monitoring of graft rejection and/or graft versus host disease, the recurrence of cancer, complications due to immunosuppression, hematologic abnormalities, infection, hospitalization and/or death. Disease criteria for liver transplant rejection include levels of serum markers for liver damage and liver function such as AST (aspartate aminotransferase), ALT (alanine aminotransferase), Alkaline phosphatase, GGT, (gamma-glutamyl transpeptidase) Bilirubin, Albumin and Prothrombin time. Further disease criteria correspond to hepatic encephalopathy, medication usage, ascites, graft failure, retransplantation, hospitalization, complications of immunosuppression, results of diagnostic tests, results of radiological testing, death and histological rejection on graft biopsy. In addition, urine can be utilized for at the target tissue for profiling in renal transplant, while biliary and intestinal secretions and feces may be used favorably for hepatic or intestinal organ allograft rejection. Diagnostic nuclotide sets can also be discovered and developed for the diagnosis and monitoring of chronic renal allograft rejection.

[0204] In the case of renal allografts, gene expression markers may be identified that are secreted proteins. These

proteins may be detected in the urine of allograft recipients using standard immunoassays. Proteins are more likely to be present in the urine if they are of low molecular weight. Lower molecular weight proteins are more likely to pass through the glomerular membrane and into the urine.

[0205] In another example, diagnostic nucleotide sets are developed and validated for use in diagnosis and treatment of xenograft recipients. This can include the transplantation of any organ from a non-human animal to a human or between non-human animals. Considerations for discovery and application of diagnostics and therapeutics and for disease criterion are substantially similar to those for allograft transplantation between humans.

[0206] In another example, diagnostic nucleotide sets are developed and validated for use in diagnosis and treatment of artificial organ recipients. This includes, but is not limited to mechanical circulatory support, artificial hearts, left ventricular assist devices, renal replacement therapies, organ prostheses and the like. Disease criteria are thrombosis (blood clots), infection, death, hospitalization, and worsening measures of organ function (e.g., hemodynamics, creatinine, liver function testing, renal function testing, functional capacity).

[0207] In another example, diagnostic nucleotide sets are developed and validated for use in matching donor organs to appropriate recipients. Diagnostic gene set can be discovered that correlate with successful matching of donor organ to recipient. Disease criteria include graft failure, acute and chronic rejection, death, hospitalization, immunosuppressive drug use, and complications of immunosuppression. Gene sets may be assayed from the donor or recipient's peripheral blood, organ tissue or some other tissue.

[0208] In another example, diagnostic nucleotide sets are developed and validated for use in diagnosis and induction of patient immune tolerance (decrease rejection of an allograft by the host immune system). Disease criteria include rejection, assays of immune activation, need for immunosupression and all disease criteria noted above for transplantation of each organ.

Viral diseases

[0209] Diagnostic leukocyte nucleotide sets may be developed and validated for use in diagnosing viral disease, as well as diagnosing and monitoring transplant rejection. In another aspect, viral nucleotide sequences may be added to a leukocyte nucleotide set for use in diagnosis of viral diseases, as well as diagnosing and monitoring transplant rejection. Alternatively, viral nucleotide sets and leukocyte nucleotides sets may be used sequentially.

Epstein-Barr virus (EBV)

[0210] EBV causes a variety of diseases such as mononucleosis, B-cell lymphoma, and pharyngeal carcinoma. It infects mononuclear cells and circulating atypical lymphocytes are a common manifestation of infection. Peripheral leukocyte gene expression is altered by infection. Transplant recipients and patients who are immunosuppressed are at increased risk for EBV-associated lymphoma.

[0211] Diagnostic nucleotide sets may be developed and validated for use in diagnosis and monitoring of EBV, as well as diagnosing and monitoring transplant rejection. In one aspect, the diagnostic nucleotide set is a leukocyte nucleotide set. Alternatively, EBV nucleotide sequences are added to a leukocyte nucleotide set, for use in diagnosing EBV. Disease criteria correspond with diagnosis of EBV, and, in patients who are EBV-sero-positive, presence (or prospective occurrence) of EBV-related illnesses such as mononucleosis, and EBV-associated lymphoma. Diagnostic nucleotide sets are useful for diagnosis of EBV, and prediction of occurrence of EBV-related illnesses.

Cytomegalovirus (CMV)

[0212] Cytomegalovirus cause inflammation and disease in almost any tissue, particularly the colon, lung, bone marrow and retina, and is a very important cause of disease in immunosuppressed patients, e.g. transplant, cancer, AIDS. Many patients are infected with or have been exposed to CMV, but not all patients develop clinical disease from the virus. Also, CMV negative recipients of allografts that come from CMV positive donors are at high risk for CMV infection. As immunosuppressive drugs are developed and used, it is increasingly important to identify patients with current or impending clinical CMV disease, because the potential benefit of immunosuppressive therapy must be balanced with the increased rate of clinical CMV infection and disease that may result from the use of immunosuppression therapy. CMV may also play a role in the occurrence of atherosclerosis or restenosis after angioplasty. CMV expression also correlates to transplant rejection, and is useful in diagnosing and monitoring transplant rejection.

[0213] Diagnostic nucleotide sets are developed for use in diagnosis and monitoring of CMV infection or re-activation of CMV infection. In one aspect, the diagnostic nucleotide set is a leukocyte nucleotide set. In another aspect, CMV nucleotide sequences are added to a leukocyte nucleotide set, for use in diagnosing CMV. Disease criteria correspond to diagnosis of CMV (e.g., sero-positive state) and presence of clinically active CMV. Disease criteria may also correspond to prospective data, e.g. the likelihood that CMV will become clinically active or impending clinical CMV infection. Antiviral

medications are available and diagnostic nucleotide sets can be used to select patients for early treatment, chronic suppression or prophylaxis of CMV activity.

Hepatitis Band C

[0214] These chronic viral infections affect about 1.25 and 2.7 million patients in the US, respectively. Many patients are infected, but suffer no clinical manifestations. Some patients with infection go on to suffer from chronic liver failure, cirrhosis and hepatic carcinoma.

[0215] Diagnostic nucleotide sets are developed for use in diagnosis and monitoring of HBV or HCV infection. In one aspect, the diagnostic nucleotide set is a leukocyte nucleotide set. In another aspect, viral nucleotide sequences are added to a leukocyte nucleotide set, for use in diagnosing the virus and monitoring progression of liver disease. Disease criteria correspond to diagnosis of the virus (e.g., sero-positive state or other disease symptoms). Alternatively, disease criteria correspond to liver damage, e.g., elevated alkaline phosphatase, ALT, AST or evidence of ongoing hepatic damage on liver biopsy. Alternatively, disease criteria correspond to serum liver tests (AST, ALT, Alkaline Phosphatase, GGT, PT, bilirubin), liver biopsy, liver ultrasound, viral load by serum PCR, cirrhosis, hepatic cancer, need for hospitalization or listing for liver transplant. Diagnostic nucleotide sets are used to diagnose HBV and HCV, and to predict likelihood of disease progression. Antiviral therapeutic usage, such as Interferon gamma and Ribavirin, can also be disease criteria.

HIV

[0216] HIV infects T cells and certainly causes alterations in leukocyte expression. Diagnostic nucleotide sets are developed for diagnosis and monitoring of HIV. In one aspect, the diagnostic nucleotide set is a leukocyte nucleotide set. In another aspect, viral nucleotide sequences are added to a leukocyte nucleotide set, for use in diagnosing the virus. Disease criteria correspond to diagnosis of the virus (e.g., sero-positive state). In addition, disease criteria correspond to viral load, CD4 T cell counts, opportunistic infection, response to antiretroviral therapy, progression to AIDS, rate of progression and the occurrence of other HIV related outcomes (e.g., malignancy, CNS disturbance). Response to antiretrovirals may also be disease criteria.

Pharmacogenomics

[0217] Pharmocogenomics is the study of the individual propensity to respond to a particular drug therapy (combination of therapies). In this context, response can mean whether a particular drug will work on a particular patient, e.g. some patients respond to one drug but not to another drug. Response can also refer to the likelihood of successful treatment or the assessment of progress in treatment. Titration of drug therapy to a particular patient is also included in this description, e.g. different patients can respond to different doses of a given medication. This aspect may be important when drugs with side-effects or interactions with other drug therapies are contemplated.

[0218] Diagnostic nucleotide sets are developed and validated for use in assessing whether a patient will respond to a particular therapy and/or monitoring response of a patient to drug therapy(therapies). Disease criteria correspond to presence or absence of clinical symptoms or clinical endpoints, presence of side-effects or interaction with other drug (s). The diagnostic nucleotide set may further comprise nucleotide sequences that are targets of drug treatment or markers of active disease.

Validation and accuracy of diagnostic nucleotide sets

[0219] Prior to widespread application of the diagnostic probe sets of the invention the predictive value of the probe set is validated. When the diagnostic probe set is discovered by microarray based expression analysis, the differential expression of the member genes may be validated by a less variable and more quantitive and accurate technology such as real time PCR. In this type of experiment the amplification product is measured during the PCR reaction. This enables the researcher to observe the amplification before any reagent becomes rate limiting for amplification. In kinetic PCR the measurement is of C_T (threshold cycle) or C_P (crossing point). This measurement (C_T=C_P) is the point at which an amplification curve crosses a threshold fluorescence value. The threshold is set to a point within the area where all of the reactions were in their linear phase of amplification. When measuring C_T, a lower C_T value is indicative of a higher amount of starting material since an earlier cycle number means the threshold was crossed more quickly.

[0220] Several fluorescence methodologies are available to measure amplification product in realtime PCR. Taqman (Applied BioSystems, Foster City, CA) uses fluorescence resonance energy transfer (FRET) to inhibit signal from a probe until the probe is degraded by the sequence specific binding and Taq 3' exonuclease activity. Molecular Beacons (Stratagene, La Jolla, CA) also use FRET technology, whereby the fluorescence is measured when a hairpin structure

is relaxed by the specific probe binding to the amplified DNA. The third commonly used chemistry is Sybr Green, a DNA-binding dye (Molecular Probes, Eugene, OR). The more amplified product that is produced, the higher the signal. The Sybr Green method is sensitive to non-specific amplification products, increasing the importance of primer design and selection. Other detection chemistries can also been used, such as ethedium bromide or other DNA-binding dyes and many modifications of the fluorescent dye/quencher dye Taqman chemistry, for example scorpions.

[0221] Real-time PCR validation can be done as described in Example 12.

[0222] Typically, the oligonucleotide sequence of each probe is confirmed, e.g. by DNA sequencing using an oligonucleotide-specific primer. Partial sequence obtained is generally sufficient to confirm the identity of the oligonucleotide probe. Alternatively, a complementary polynucleotide is fluorescently labeled and hybridized to the array, or to a different array containing a resynthesized version of the oligo nucleotide probe, and detection of the correct probe is confirmed.

[0223] Typically, validation is performed by statistically evaluating the accuracy of the correspondence between the molecular signature for a diagnostic probe set and a selected indicator. For example, the expression differential for a nucleotide sequence between two subject classes can be expressed as a simple ratio of relative expression. The expression of the nucleotide sequence in subjects with selected indicator can be compared to the expression of that nucleotide sequence in subjects without the indicator, as described in the following equations.

$\sum E_x ai/N = E_x A$ **the average expression of nucleotide sequence x in the members of group A;**

$\sum E_x bi/M = E_x B$ **the average expression of nucleotide sequence x in the members of group B;**

$E_x A / ExB = \Delta E_x AB$ **the average differential expression of nucleotide sequence x between groups A and B:**

where Σ indicates a sum; Ex is the expression of nucleotide sequence x relative to a standard; ai are the individual members of group A, group A has N members; bi are the individual members of group B, group B has M members.

[0224] The expression of at least two nucleotide sequences, e.g., nucleotide sequence X and nucleotide sequence Y are measured relative to a standard in at least one subject of group A (e.g., with a disease) and group B (e.g., without the disease). Ideally, for purposes of validation the indicator is independent from (i.e., not assigned based upon) the expression pattern. Alternatively, a minimum threshold of gene expression for nucleotide sequences X and Y, relative to the standard, are designated for assignment to group A. For nucleotide sequence x, this threshold is designated ΔEx, and for nucleotide sequence y, the threshold is designated ΔEy.

[0225] The following formulas are used in the calculations below:

Sensitivity = (true positives/true positives + false negatives)
Specificity = (true negatives/true negatives + false positives)

[0226] If, for example, expression of nucleotide sequence x above a threshold: x > ΔEx, is observed for 80/100 subjects in group A and for 10/100 subjects in group B, the sensitivity of nucleotide sequence x for the assignment to group A, at the given expression threshold ΔEx, is 80%, and the specificity is 90%.

[0227] If the expression of nucleotide sequence y is > ΔEy in 80/100 subjects in group A, and in 10/100 subjects in group B, then, similarly the sensitivity of nucleotide sequence y for the assignment to group A at the given threshold ΔEy is 80% and the specificity is 90%. If in addition, 60 of the 80 subjects in group A that meet the expression threshold for nucleotide sequence y also meet the expression threshold ΔEx and that 5 of the 10 subjects in group B that meet the expression threshold for nucleotide sequence y also meet the expression threshold ΔEx, the sensitivity of the test (x>ΔEx and y>ΔEy)for assignment of subjects to group A is 60% and the specificity is 95%.

[0228] Alternatively, if the criteria for assignment to group A are change to: Expression of x > ΔEx or expression of y > ΔEy, the sensitivity approaches 100% and the specificity is 85%.

[0229] Clearly, the predictive accuracy of any diagnostic probe set is dependent on the minimum expression threshold selected. The expression of nucleotide sequence X (relative to a standard) is measured in subjects of groups A (with disease) and B (without disease). The minimum threshold of nucleotide sequence expression for x, required for assignment to group A is designated ΔEx 1.

[0230] If 90/100 patients in group A have expression of nucleotide sequence x > ΔEx 1 and 20/100 patients in group B have expression of nucleotide sequence x > ΔEx 1, then the sensitivity of the expression of nucleotide sequence x (using ΔEx 1 as a minimum expression threshold) for assignment of patients to group A will be 90% and the specificity will be 80%.

[0231] Altering the minimum expression threshold results in an alteration in the specificity and sensitivity of the nucleotide sequences in question. For example, if the minimum expression threshold of nucleotide sequence x for assignment of subjects to group A is lowered to ΔEx 2, such that 100/100 subjects in group A and 40/100 subjects in group B meet the threshold, then the sensitivity of the test for assignment of subjects to group A will be 100% and the specificity will

be 60%.

[0232] Thus, for 2 nucleotide sequences X and Y: the expression of nucleotide sequence x and nucleotide sequence y (relative to a standard) are measured in subjects belonging to groups A (with disease) and B (without disease). Minimum thresholds of nucleotide sequence expression for nucleotide sequences X and Y (relative to common standards) are designated for assignment to group A. For nucleotide sequence x, this threshold is designated ΔEx1 and for nucleotide sequence y, this threshold is designated ΔEy1.

[0233] If in group A, 90/100 patients meet the minimum requirements of expression ΔEx1 and ΔEy1, and in group B, 10/100 subjects meet the minimum requirements of expression ΔEx1 and ΔEy1, then the sensitivity of the test for assignment of subjects to group A is 90% and the specificity is 90%.

[0234] Increasing the minimum expression thresholds for X and Y to ΔEx2 and ΔEy2, such that in group A, 70/100 subjects meet the minimum requirements of expression ΔEx2 and ΔEy2, and in group B, 3/100 subjects meet the minimum requirements of expression ΔEx2 and ΔEy2. Now the sensitivity of the test for assignment of subjects to group A is 70% and the specificity is 97%.

[0235] If the criteria for assignment to group A is that the subject in question meets either threshold, ΔEx2 or ΔEy2, and it is found that 100/100 subjects in group A meet the criteria and 20/100 subjects in group B meet the criteria, then the sensitivity of the test for assignment to group A is 100% and the specificity is 80%.

[0236] Individual components of a diagnostic probe set each have a defined sensitivity and specificity for distinguishing between subject groups. Such individual nucleotide sequences can be employed in concert as a diagnostic probe set to increase the sensitivity and specificity of the evaluation. The database of molecular signatures is queried by algorithms to identify the set of nucleotide sequences (i.e., corresponding to members of the probe set) with the highest average differential expression between subject groups. Typically, as the number of nucleotide sequences in the diagnostic probe set increases, so does the predictive value, that is, the sensitivity and specificity of the probe set. When the probe sets are defmed they may be used for diagnosis and patient monitoring as discussed below. The diagnostic sensitivity and specificity of the probe sets for the defmed use can be determined for a given probe set with specified expression levels as demonstrated above. By altering the expression threshold required for the use of each nucleotide sequence as a diagnostic, the sensitivity and specificity of the probe set can be altered by the practitioner. For example, by lowering the magnitude of the expression differential threshold for each nucleotide sequence in the set, the sensitivity of the test will increase, but the specificity will decrease. As is apparent from the foregoing discussion, sensitivity and specificity are inversely related and the predictive accuracy of the probe set is continuous and dependent on the expression threshold set for each nucleotide sequence. Although sensitivity and specificity tend to have an inverse relationship when expression thresholds are altered, both parameters can be increased as nucleotide sequences with predictive value are added to the diagnostic nucleotide set. In addition a single or a few markers may not be reliable expression markers across a population of patients. This is because of the variability in expression and measurement of expression that exists between measurements, individuals and individuals over time. Inclusion of a large number of candidate nucleotide sequences or large numbers of nucleotide sequences in a diagnostic nucleotide set allows for this variability as not all nucleotide sequences need to meet a threshold for diagnosis. Generally, more markers are better than a single marker. If many markers are used to make a diagnosis, the likelihood that all expression markers will not meet some thresholds based upon random variability is low and thus the test will give fewer false negatives.

[0237] It is appreciated that the desired diagnostic sensitivity and specificity of the diagnostic nucleotide set may vary depending on the intended use of the set. For example, in certain uses, high specificity and high sensitivity are desired. For example, a diagnostic nucleotide set for predicting which patient population may experience side effects may require high sensitivity so as to avoid treating such patients. In other settings, high sensitivity is desired, while reduced specificity may be tolerated. For example, in the case of a beneficial treatment with few side effects, it may be important to identify as many patients as possible (high sensitivity) who will respond to the drug, and treatment of some patients who will not respond is tolerated. In other settings, high specificity is desired and reduced sensitivity may be tolerated. For example, when identifying patients for an early-phase clinical trial, it is important to identify patients who may respond to the particular treatment. Lower sensitivity is tolerated in this setting as it merely results in reduced patients who enroll in the study or requires that more patients are screened for enrollment.

Methods of using diagnostic nucleotide sets.

[0238] The invention also provide methods of using the diagnostic nucleotide sets to: diagnose disease; assess severity of disease; predict future occurrence of disease; predict future complications of disease; determine disease prognosis; evaluate the patient's risk, or "stratify" a group of patients; assess response to current drug therapy; assess response to current non-pharmacological therapy; determine the most appropriate medication or treatment for the patient; predict whether a patient is likely to respond to a particular drug; and determine most appropriate additional diagnostic testing for the patient, among other clinically and epidemiologically relevant applications.

[0239] The nucleotide sets of the invention can be utilized for a variety of purposes by physicians, healthcare workers,

hospitals, laboratories, patients, companies and other institutions. As indicated previously, essentially any disease, condition, or status for which at least one nucleotide sequence is differentially expressed in leukocyte populations (or sub-populations) can be evaluated, e.g., diagnosed, monitored, etc. using the diagnostic nucleotide sets and methods of the invention. In addition to assessing health status at an individual level, the diagnostic nucleotide sets of the present invention are suitable for evaluating subjects at a "population level," e.g., for epidemiological studies, or for population screening for a condition or disease.

Collection and preparation of sample

[0240] RNA, protein and/or DNA is prepared using methods well-known in the art, as further described herein. It is appreciated that subject samples collected for use in the methods of the invention are generally collected in a clinical setting, where delays may be introduced before RNA samples are prepared from the subject samples of whole blood, e.g. the blood sample may not be promptly delivered to the clinical lab for further processing. Further delay may be introduced in the clinical lab setting where multiple samples are generally being processed at any given time. For this reason, methods which feature lengthy incubations of intact leukocytes at room temperature are not preferred, because the expression profile of the leukocytes may change during this extended time period. For example, RNA can be isolated from whole blood using a phenol/guanidine isothiocyanate reagent or another direct whole-blood lysis method, as described in, e.g., U.S. Patent Nos. 5,346,994 and 4,843,155. This method may be less preferred under certain circumstances because the large majority of the RNA recovered from whole blood RNA extraction comes from erythrocytes since these cells outnumber leukocytes 1000:1. Care must be taken to ensure that the presence of erythrocyte RNA and protein does not introduce bias in the RNA expression profile data or lead to inadequate sensitivity or specificity of probes.

[0241] Alternatively, intact leukocytes may be collected from whole blood using a lysis buffer that selectively lyses erythrocytes, but not leukocytes, as described, e.g., in (U.S. Patent Nos. 5,973,137, and 6,020,186). Intact leukocytes are then collected by centrifugation, and leukocyte RNA is isolated using standard protocols, as described herein. However, this method does not allow isolation of sub-populations of leukocytes, e.g. mononuclear cells, which may be desired. In addition, the expression profile may change during the lengthy incubation in lysis buffer, especially in a busy clinical lab where large numbers of samples are being prepared at any given time.

[0242] Alternatively, specific leukocyte cell types can be separated using density gradient reagents (Boyum, A, 1968.). For example, mononuclear cells may be separated from whole blood using density gradient centrifugation, as described, e.g., in U.S. Patents Nos. 4190535, 4350593, 4751001, 4818418, and 5053134. Blood is drawn directly into a tube containing an anticoagulant and a density reagent (such as Ficoll or Percoll). Centrifugation of this tube results in separation of blood into an erythrocyte and granulocyte layer, a mononuclear cell suspension, and a plasma layer. The mononuclear cell layer is easily removed and the cells can be collected by centrifugation, lysed, and frozen. Frozen samples are stable until RNA can be isolated. Density centrifugation, however, must be conducted at room temperature, and if processing is unduly lengthy, such as in a busy clinical lab, the expression profile may change.

[0243] Alternatively, cells can be separated using fluorescence activated cell sorting (FACS) or some other technique, which divides cells into subsets based on gene or protein expression. This may be desirable to enrich the sample for cells of interest, but it may also introduce cell manipulations and time delays, which result in alteration of gene expression profiles (Cantor et al. 1975; Galbraith et al. 1999).

[0244] The quality and quantity of each clinical RNA sample is desirably checked before amplification and labeling for array hybridization, using methods known in the art. For example, one microliter of each sample may be analyzed on a Bioanalyzer (Agilent 2100 Palo Alto, CA. USA) using an RNA 6000 nano LabChip (Caliper, Mountain View, CA. USA). Degraded RNA is identified by the reduction of the 28S to 18S ribosomal RNA ratio and/or the presence of large quantities of RNA in the 25-100 nucleotide range.

[0245] It is appreciated that the RNA sample for use with a diagnostic nucleotide set may be produced from the same or a different cell population, sub-population and/or cell type as used to identify the diagnostic nucleotide set. For example, a diagnostic nucleotide set identified using RNA extracted from mononuclear cells may be suitable for analysis of RNA extracted from whole blood or mononuclear cells, depending on the particular characteristics of the members of the diagnostic nucleotide set. Generally, diagnostic nucleotide sets must be tested and validated when used with RNA derived from a different cell population, sub-population or cell type than that used when obtaining the diagnostic gene set. Factors such as the cell-specific gene expression of diagnostic nucleotide set members, redundancy of the information provided by members of the diagnostic nucleotide set, expression level of the member of the diagnostic nucleotide set, and cell-specific alteration of expression of a member of the diagnostic nucleotide set will contribute to the usefullness of using a different RNA source than that used when identifying the members of the diagnostic nucleotide set. It is appreciated that it may be desirable to assay RNA derived from whole blood, obviating the need to isolate particular cell types from the blood.

Rapid method of RNA extraction suitable for production in a clinical setting of high quality RNA for expression profiling

[0246] In a clinical setting, obtaining high quality RNA preparations suitable for expression profiling, from a desired population of leukocytes poses certain technical challenges, including: the lack of capacity for rapid, high-throughput sample processing in the clinical setting, and the possibility that delay in processing (in a busy lab or in the clinical setting) may adversely affect RNA quality, e.g. by a permitting the expression profile of certain nucleotide sequences to shift. Also, use of toxic and expensive reagents, such as phenol, may be disfavored in the clinical setting due to the added expense associated with shipping and handling such reagent.

[0247] A useful method for RNA isolation for leukocyte expression profiling would allow the isolation of monocyte and lymphocyte RNA in a timely manner, while preserving the expression profiles of the cells, and allowing inexpensive production of reproducible high-quality RNA samples. Accordingly, the invention provides a method of adding inhibitor (s) of RNA transcription and/or inhibitor(s) of protein synthesis, such that the expression profile is "frozen" and RNA degradation is reduced. A desired leukocyte population or sub-population is then isolated, and the sample may be frozen or lysed before further processing to extract the RNA. Blood is drawn from subject population and exposed to ActinomycinD (to a final concentration of 10 μg/ml) to inhibit transcription, and cycloheximide (to a final concentration of 10 μg/ml) to inhibit protein synthesis. The inhibitor(s) can be injected into the blood collection tube in liquid form as soon as the blood is drawn, or the tube can be manufactured to contain either lyophilized inhibitors or inhibitors that are in solution with the anticoagulant. At this point, the blood sample can be stored at room temperature until the desired leukocyte population or sub-population is isolated, as described elsewhere. RNA is isolated using standard methods, e.g., as described above, or a cell pellet or extract can be frozen until further processing of RNA is convenient.

[0248] Also disclosed is a method of using a low-temperature density gradient for separation of a desired leukocyte sample as well as the invention provides the combination of use of a low-temperature density gradient and the use of transcriptional and/or protein synthesis inhibitor(s). A desired leukocyte population is separated using a density gradient solution for cell separation that maintains the required density and viscosity for cell separation at 0-4°C. Blood is drawn into a tube containing this solution and may be refrigerated before and during processing as the low temperatures slow cellular processes and minimize expression profile changes. Leukocytes are separated, and RNA is isolated using standard methods. Alternately, a cell pellet or extract is frozen until further processing of RNA is convenient. Care must be taken to avoid rewarming the sample during further processing steps.

[0249] Alternatively, a method of using low-temperature density gradient separation, combined with the use of actinomycin A and cyclohexamide, as described above can be used.

Assessing expression for diagnostic

[0250] Expression profiles for the set of diagnostic nucleotide sequences in a subject sample can be evaluated by any technique that determines the expression of each component nucleotide sequence. Methods suitable for expression analysis are known in the art, and numerous examples are discussed in the Sections titled "Methods of obtaining expression data" and "high throughput expression Assays", above.

[0251] In many cases, evaluation of expression profiles is most efficiently, and cost effectively, performed by analyzing RNA expression. Alternatively, the proteins encoded by each component of the diagnostic nucleotide set are detected for diagnostic purposes by any technique capable of determining protein expression, e.g., as described above. Expression profiles can be assessed in subject leukocyte sample using the same or different techniques as those used to identify and validate the diagnostic nucleotide set. For example, a diagnostic nucleotide set identified as a subset of sequences on a cDNA microarray can be utilized for diagnostic (or prognostic, or monitoring, etc.) purposes on the same array from which they were identified. Alternatively, the diagnostic nucleotide sets for a given disease or condition can be organized onto a dedicated sub-array for the indicated purpose. It is important to note that if diagnostic nucleotide sets are discovered using one technology, e.g. RNA expression profiling, but applied as a diagnostic using another technology, e.g. protein expression profiling, the nucleotide sets must generally be validated for diagnostic purposes with the new technology. In addition, it is appreciated that diagnostic nucleotide sets that are developed for one use, e.g. to diagnose a particular disease, may later be found to be useful for a different application, e.g. to predict the likelihood that the particular disease will occur. Generally, the diagnostic nucleotide set will need to be validated for use in the second circumstance. As discussed herein, the sequence of diagnostic nucleotide set members may be amplified from RNA or cDNA using methods known in the art providing specific amplification of the nucleotide sequences.

General Protein Methods

[0252] Protein products of the nucleotide sequences disclosed in the invention may include proteins that represent functionally equivalent gene products. Such an equivalent gene product may contain deletions, additions or substitutions of amino acid residues within the amino acid sequence encoded by the nucleotide sequences described, above, but

which result in a silent change, thus producing a functionally equivalent nucleotide sequence product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved.

[0253] For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. "Functionally equivalent", as utilized herein, refers to a protein capable of exhibiting a substantially similar in vivo activity as the endogenous gene products encoded by the nucleotide described, above.

[0254] The gene products (protein products of the nucleotide sequences) may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing the gene polypeptides and peptides of the invention by expressing nucleic acid encoding nucleotide sequences are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing nucleotide sequence protein coding sequences and appropriate transcriptional/translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. See, for example, the techniques described in Sambrook et al., 1989, supra, and Ausubel et al., 1989, supra. Alternatively, RNA capable of encoding nucleotide sequence protein sequences may be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M. J. ed., IRL Press, Oxford, which is incorporated by reference herein in its entirety

[0255] A variety of host-expression vector systems may be utilized to express the nucleotide sequence coding sequences of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the protein encoded by the nucleotide sequence of the invention in situ. These include but are not limited to microorganisms such as bacteria (e.g., E. coli, B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing nucleotide sequence protein coding sequences; yeast (e.g. Saccharomyces, Pichia) transformed with recombinant yeast expression vectors containing the nucleotide sequence protein coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the nucleotide sequence protein coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing nucleotide sequence protein coding sequences; or mammalian cell systems (e.g. COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5 K promoter).

[0256] In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the nucleotide sequence protein being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of antibodies or to screen peptide libraries, for example, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the E. coli expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the nucleotide sequence protein coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the likes of pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target nucleotide sequence protein can be released from the GST moiety. Other systems useful in the invention include use of the FLAG epitope or the 6-HIS systems.

[0257] In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign nucleotide sequences. The virus grows in Spodoptera frugiperda cells. The nucleotide sequence coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of nucleotide sequence coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect Spodoptera frugiperda cells in which the inserted nucleotide sequence is expressed. (E.g., see Smith et al., 1983, J. Virol. 46: 584; Smith, U.S. Pat. No. 4,215,051).

[0258] In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the nucleotide sequence coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric nucleotide sequence may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a

non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing nucleotide sequence encoded protein in infected hosts. (E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted nucleotide sequence coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire nucleotide sequence, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the nucleotide sequence coding sequence is inserted, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in Enzymol. 153:516-544).

[0259] In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the product of the nucleotide sequence in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Differet host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, etc.

[0260] For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the nucleotide sequence encoded protein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express nucleotide sequence encoded protein. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the nucleotide sequence encoded protein.

[0261] A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes.

[0262] An alternative fusion protein system allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88: 8972-8976). In this system, the nucleotide sequence of interest is subcloned into a vaccinia recombination plasmid such that the nucleotide sequence's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni.sup.2 +-nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

[0263] Where recombinant DNA technology is used to produce the protein encoded by the nucleotide sequence for such assay systems, it may be advantageous to engineer fusion proteins that can facilitate labeling, immobilization and/or detection.

Antibodies

[0264] Indirect labeling involves the use of a protein, such as a labeled antibody, which specifically binds to the protein encoded by the nucleotide sequence. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library.

[0265] Antibodies to the protein encoded by the nucleotide sequences disclosed are also. Described herein are methods for the production of antibodies capable of specifically recognizing one or more nucleotide sequence epitopes. Such antibodies may include, but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric

antibodies, single chain antibodies, Fab fragments, F(ab')2 fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may be used, for example, in the detection of a nucleotide sequence in a biological sample, or, alternatively, as a method for the inhibition of abnormal gene activity, for example, the inhibition of a disease target nucleotide sequence, as further described below. Thus, such antibodies may be utilized as part of cardiovascular or other disease treatment method, and/or may be used as part of diagnostic techniques whereby patients may be tested for abnormal levels of nucleotide sequence encoded proteins, or for the presence of abnormal forms of the such proteins.

[0266] For the production of antibodies to a nucleotide sequence, various host animals may be immunized by injection with a protein encoded by the nucleotide sequence, or a portion thereof. Such host animals may include but are not limited to rabbits, mice, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

[0267] Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as gene product, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as those described above, may be immunized by injection with gene product supplemented with adjuvants as also described above.

[0268] Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to the hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo.

[0269] In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

[0270] Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted to produce nucleotide sequence-single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

[0271] Antibody fragments which recognize specific epitopes may be generated by known techniques For example, such fragments include but are not limited to: the F(ab')2 fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Disease specific target nucleotide sequences

[0272] Disease specific target nucleotide sequences, and sets of disease specific target nucleotide sequences are also disclosed. The diagnostic nucleotide sets, subsets thereof, novel nucleotide sequences, and individual members of the diagnostic nucleotide sets identified as described above are also disease specific target nucleotide sequences. In particular, individual nucleotide sequences that are differentially regulated or have predictive value that is strongly correlated with a disease or disease criterion are especially favorable as disease specific target nucleotide sequences. Sets of genes that are co-regulated may also be identified as disease specific target nucleotide sets. Such nucleotide sequences and/or nucleotide sequence products are targets for modulation by a variety of agents and techniques. For example, disease specific target nucleotide sequences (or the products of such nucleotide sequences, or sets of disease specific target nucleotide sequences) can be inhibited or activated by, e.g., target specific monoclonal antibodies or small molecule inhibitors, or delivery of the nucleotide sequence or gene product of the nucleotide sequence to patients. Also, sets of genes can be inhibited or activated by a variety of agents and techniques. The specific usefulness of the target nucleotide sequence(s) depends on the subject groups from which they were discovered, and the disease or disease criterion with which they correlate.

Imaging

[0273] Further disclosed are imaging reagents. The differentially expressed leukocyte nucleotide sequences, diagnostic nucleotide sets, or portions thereof, and novel nucleotide sequences of the invention are nucleotide sequences expressed in cells with or without disease. Leukocytes expressing a nucleotide sequence(s) that is differentially expressed in a disease condition may localize within the body to sites that are of interest for imaging purposes. For example, a leukocyte expressing a nucleotide sequence(s) that are differentially expressed in an individual having atherosclerosis may localize or accumulate at the site of an atherosclerotic placque. Such leukocytes, when labeled, may provide a detection reagent for use in imaging regions of the body where labeled leukocyte accumulate or localize, for example, at the atherosclerotic plaque in the case of atherosclerosis. For example, leukocytes are collected from a subject, labeled in vitro, and reintroduced into a subject. Alternatively, the labeled reagent is introduced into the subject individual, and leukocyte labeling occurs within the patient.

[0274] Imaging agents that detect the imaging targets of the disclosure are produced by well-known molecular and immunological methods (for exemplary protocols, *see*, e.g., Ausubel, Berger, and Sambrook, as well as Harlow and Lane, *supra*).

[0275] For example, a full-length nucleic acid sequence, or alternatively, a gene fragment encoding an immunogenic peptide or polypeptide fragments, is cloned into a convenient expression vector, for example, a vector including an in-frame epitope or substrate binding tag to facilitate subsequent purification. Protein is then expressed from the cloned cDNA sequence and used to generate antibodies, or other specific binding molecules, to one or more antigens of the imaging target protein. Alternatively, a natural or synthetic polypeptide (or peptide) or small molecule that specifically binds (or is specifically bound to) the expressed imaging target can be identified through well established techniques (*see,* e.g., Mendel et al. (2000) Anticancer Drug Des 15:29-41; Wilson (2000) Curr Med Chem 7:73-98; Hamby and Showwalter (1999) Pharmacol Ther 82:169-93; and Shimazawa et al. (1998) Curr Opin Struct Biol 8:451-8). The binding molecule, e.g., antibody, small molecule ligand, etc., is labeled with a contrast agent or other detectable label, e.g., gadolinium, iodine, or a gamma-emitting source. For in-vivo imaging of a disease process that involved leukocytes, the labeled antibody is infused into a subject, e.g., a human patient or animal subject, and a sufficient period of time is passed to permit binding of the antibody to target cells. The subject is then imaged with appropriate technology such as MRI (when the label is gadolinium) or with a gamma counter (when the label is a gamma emitter).

Identification of nucleotide sequence involved in leukocyte adhesion

[0276] A method of identifying nucleotide sequences involved in leukocyte adhesion is described. The interaction between the endothelial cell and leukocyte is a fundamental mechanism of all inflammatory disorders, including the diagnosis and prognosis of allograft rejection the diseases listed in Table 1. For example, the first visible abnormality in atherosclerosis is the adhesion to the endothelium and diapedesis of mononuclear cells (e.g., T-cell and monocyte). Insults to the endothelium (for example, cytokines, tobacco, diabetes, hypertension and many more) lead to endothelial cell activation. The endothelium then expresses adhesion molecules, which have counter receptors on mononuclear cells. Once the leukocyte receptors have bound the endothelial adhesion molecules, they stick to the endothelium, roll a short distance, stop and transmigrate across the endothelium A similar set of events occurs in both acute and chronic inflammation. When the leukocyte binds the endothelial adhesion molecule, or to soluble cytokines secreted by endothelial or other cells, a program of gene expression is activated in the leukocyte. This program of expression leads to leukocyte rolling, firm adhesion and transmigration into the vessel wall or tissue parenchyma. Inhibition of this process is highly desirable goal in anti-inflammatory drug development. In addition, leukocyte nucleotide sequences and epithelial cell nucleotide sequences, that are differentially expressed during this process may be disease-specific target nucleotide sequences.

[0277] Human endothelial cells, e.g. derived from human coronary arteries, human aorta, human pulmonary artery, human umbilical vein or microvascular endothelial cells, are cultured as a confluent monolayer, using standard methods. Some of the endothelial cells are then exposed to cytokines or another activating stimuli such as oxidized LDL, hyperglycemia, shear stress, or hypoxia (Moser et al. 1992). Some endothelial cells are not exposed to such stimuli and serve as controls. For example, the endothelial cell monolayer is incubated with culture medium containing 5 U/ml of human recombinant IL-lalpha or 10 ng/ml TNF (tumor necrosis factor), for a period of minutes to overnight. The culture medium composition is changed or the flask is sealed to induce hypoxia. In addition, tissue culture plate is rotated to induce sheer stress.

[0278] Human T-cells and/or monocytes are cultured in tissue culture flasks or plates, with LGM-3 media from Clonetics. Cells are incubated at 37 degree C, 5% CO2 and 95% humidity. These leukocytes are exposed to the activated or control endothelial layer by adding a suspension of leukocytes on to the endothelial cell monolayer. The endothelial cell monolayer is cultured on a tissue culture treated plate/ flask or on a microporous membrane. After a variable duration of exposures, the endothelial cells and leukocytes are harvested separately by treating all cells with trypsin and then sorting the

endothelial cells from the leukocytes by magnetic affinity reagents to an endothelial cell specific marker such as PECAM-1 (Stem Cell Technologies). RNA is extracted from the isolated cells by standard techniques. Leukocyte RNA is labeled as described above, and hybridized to leukocyte candidate nucleotide library. Epithelial cell RNA is also labeled and hybridized to the leukocyte candidate nucleotide library. Alternatively, the epithelial cell RNA is hybridized to a epithelial cell candidate nucleotide library, prepared according to the methods described for leukocyte candidate libraries, above.

[0279] Hybridization to candidate nucleotide libraries will reveal nucleotide sequences that are upregulated or down-regulated in leukocyte and/or epithelial cells undergoing adhesion. The differentially regulated nucleotide sequences are further characterized, e.g. by isolating and sequencing the full-length sequence, analysis of the DNA and predicted protein sequence, and functional characterization of the protein product of the nucleotide sequence, as described above. Further characterization may result in the identification of leukocyte adhesion specific target nucleotide sequences, which may be candidate targets for regulation of the inflammatory process. Small molecule or antibody inhibitors can be developed to inhibit the target nucleotide sequence function. Such inhibitors are tested for their ability to inhibit leukocyte adhesion in the in vitro test described above.

Integrated systems

[0280] Integrated systems for the collection and analysis of expression profiles, and molecular signatures, as well as for the compilation, storage and access of the databases of the invention, typically include a digital computer with software including an instruction set for sequence searching and analysis, and, optionally, high-throughput liquid control software, image analysis software, data interpretation software, a robotic control armature for transferring solutions from a source to a destination (such as a detection device) operably linked to the digital computer, an input device (e.g., a computer keyboard) for entering subject data to the digital computer, or to control analysis operations or high throughput sample transfer by the robotic control armature. Optionally, the integrated system further comprises an image scanner for digitizing label signals from labeled assay components, e.g., labeled nucleic acid hybridized to a candidate library microarray. The image scanner can interface with image analysis software to provide a measurement of the presence or intensity of the hybridized label, i.e., indicative of an on/off expression pattern or an increase or decrease in expression.

[0281] Readily available computational hardware resources using standard operating systems are fully adequate, e.g., a PC (Intel x86 or Pentium chip- compatible DOS,™ OS2,™ WINDOWS,™ WINDOWS NT,™ WINDOWS95,™ WINDOWS98,™ LINUX, or even Macintosh, Sun or PCs will suffice) for use in the integrated systems of the invention. Current art in software technology is similarly adequate (i.e., there are a multitude of mature programming languages and source code suppliers) for design, e.g., of an upgradeable open-architecture object-oriented heuristic algorithm, or instruction set for expression analysis, as described herein. For example, software for aligning or otherwise manipulating , molecular signatures can be constructed by one of skill using a standard programming language such as Visual basic, Fortran, Basic, Java, or the like, according to the methods herein.

[0282] Various methods and algorithms, including genetic algorithms and neural networks, can be used to perform the data collection, correlation, and storage functions, as well as other desirable functions, as described herein. In addition, digital or analog systems such as digital or analog computer systems can control a variety of other functions such as the display and/or control of input and output files.

[0283] For example, standard desktop applications such as word processing software (e.g., Corel WordPerfect™ or Microsoft Word™) and database software (e.g., spreadsheet software such as Corel Quattro Pro™, Microsoft Excel™, or database programs such as Microsoft Access™ or Paradox™) can be adapted to the present invention by inputting one or more character string corresponding, e.g., to an expression pattern or profile, subject medical or historical data, molecular signature, or the like, into the software which is loaded into the memory of a digital system, and carrying out the operations indicated in an instruction set, e.g., as exemplified in Figure 2. For example, systems can include the foregoing software having the appropriate character string information, e.g., used in conjunction with a user interface in conjunction with a standard operating system such as a Windows, Macintosh or LINUX system For example, an instruction set for manipulating strings of characters, either by programming the required operations into the applications or with the required operations performed manually by a user (or both). For example, specialized sequence alignment programs such as PILEUP or BLAST can also be incorporated into the systems , e.g., for alignment of nucleic acids or proteins (or corresponding character strings).

[0284] Software for performing the statistical methods required for the invention, e.g., to determine correlations between expression profiles and subsets of members of the diagnostic nucleotide libraries, such as programmed embodiments of the statistical methods described above, are also included in the computer systems. Alternatively, programming elements for performing such methods as principle component analysis (PCA) or least squares analysis can also be included in the digital system to identify relationships between data. Exemplary software for such methods is provided by Partek, Inc., St. Peter, Mo; at the web site partek.com.

[0285] Any controller or computer optionally includes a monitor which can include, e.g., a flat panel display (e.g., active matrix liquid crystal display, liquid crystal display), a cathode ray tube ("CRT") display, or another display system which

serves as a user interface, e.g., to output predictive data. Computer circuitry, including numerous integrated circuit chips, such as a microprocessor, memory, interface circuits, and the like, is often placed in a casing or box which optionally also includes a hard disk drive, a floppy disk drive, a high capacity removable drive such as a writeable CD-ROM, and other common peripheral elements.

[0286] Inputting devices such as a keyboard, mouse, or touch sensitive screen, optionally provide for input from a user and for user selection, e.g., of sequences or data sets to be compared or otherwise manipulated in the relevant computer system. The computer typically includes appropriate software for receiving user instructions, either in the form of user input into a set parameter or data fields (e.g., to input relevant subject data), or in the form of preprogrammed instructions, e.g., preprogrammed for a variety of different specific operations. The software then converts these instructions to appropriate language for instructing the system to carry out any desired operation.

[0287] The integrated system may also be embodied within the circuitry of an application specific integrated circuit (ASIC) or programmable logic device (PLD). In such a case, the invention is embodied in a computer readable descriptor language that can be used to create an ASIC or PLD. The integrated system can also be embodied within the circuitry or logic processors of a variety of other digital apparatus, such as PDAs, laptop computer systems, displays, image editing equipment, etc.

[0288] The digital system can comprise a learning component where expression profiles, and relevant subject data are compiled and monitored in conjunction with physical assays, and where correlations, e.g., molecular signatures with predictive value for a disease, are established or refined. Successful and unsuccessful combinations are optionally documented in a database to provide justification/preferences for user-base or digital system based selection of diagnostic nucleotide sets with high predictive accuracy for a specified disease or condition.

[0289] The integrated systems can also include an automated workstation. For example, such a workstation can prepare and analyze leukocyte RNA samples by performing a sequence of events including: preparing RNA from a human blood sample; labeling the RNA with an isotopic or non-isotopic label; hybridizing the labeled RNA to at least one array comprising all or part of the candidate library; and detecting the hybridization pattern. The hybridization pattern is digitized and recorded in the appropriate database.

Automated RNA preparation tool

[0290] An automated RNA preparation tool for the preparation of mononuclear cells from whole blood samples, and preparation of RNA from the mononuclear cells is also disclosed. In a preferred embodiment, the use of the RNA preparation tool is fully automated, so that the cell separation and RNA isolation would require no human manipulations. Full automation is advantageous because it minimizes delay, and standardizes sample preparation across different laboratories. This standardization increases the reproducibility of the results.

[0291] Figure 2 depicts the processes performed by the RNA preparation tool. A primary component of the device is a centrifuge (A). Tubes of whole blood containing a density gradient solution, transcription/translation inhibitors, and a gel barrier that separates erythrocytes from mononuclear cells and serum after centrifugation are placed in the centrifuge (B). The barrier is permeable to erythrocytes and granulocytes during centrifugation, but does not allow mononuclear cells to pass through (or the barrier substance has a density such that mononuclear cells remain above the level of the barrier during the centrifugation). After centrifugation, the erythrocytes and granulocytes are trapped beneath the barrier, facilitating isolation of the mononuclear cell and serum layers. A mechanical arm removes the tube and inverts it to mix the mononuclear cell layer and the serum (C). The arm next pours the supernatant into a fresh tube (D), while the erythrocytes and granulocytes remained below the barrier. Alternatively, a needle is used to aspirate the supernatant and transfer it to a fresh tube. The mechanical arms of the device opens and closes lids, dispenses PBS to aid in the collection of the mononuclear cells by centrifugation, and moves the tubes in and out of the centrifuge. Following centrifugation, the supernatant is poured off or removed by a vacuum device (E), leaving an isolated mononuclear cell pellet. Purification of the RNA from the cells is performed automatically, with lysis buffer and other purification solutions (F) automatically dispensed and removed before and after centrifugation steps. The result is a purified RNA solution. In another embodiment, RNA isolation is performed using a column or filter method. In yet another embodiment, the invention includes an on-board homogenizer for use in cell lysis.

Other automated systems

[0292] Automated and/or semi-automated methods for solid and liquid phase high-throughput sample preparation and evaluation are available, and supported by commercially available devices. For example, robotic devices for preparation of nucleic acids from bacterial colonies, e.g., to facilitate production and characterization of the candidate library include, for example, an automated colony picker (e.g., the Q-bot, Genetix, U.K.) capable of identifying, sampling, and inoculating up to 10,000/4 hrs different clones into 96 well microtiter dishes. Alternatively, or in addition, robotic systems for liquid handling are available from a variety of sources, e.g., automated workstations like the automated synthesis apparatus

developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, Beckman Coulter, Inc. (Fullerton, CA)) which mimic the manual operations performed by a scientist. Any of the above devices are suitable for use with the present invention, e.g., for high-throughput analysis of library components or subject leukocyte samples. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein will be apparent to persons skilled in the relevant art.

[0293] High throughput screening systems that automate entire procedures, e.g., sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the relevant assay are commercially available. (*see, e.g.,* Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, *etc.*). These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization. Similarly, arrays and array readers are available, e.g., from Affymetrix, PE Biosystems, and others.

[0294] The manufacturers of such systems provide detailed protocols the various high throughput. Thus, for example, Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand binding, and the like.

[0295] A variety of commercially available peripheral equipment, including, e.g., optical and fluorescent detectors, optical and fluorescent microscopes, plate readers, CCD arrays, phosphorimagers, scintillation counters, phototubes, photodiodes, and the like, and software is available for digitizing, storing and analyzing a digitized video or digitized optical or other assay results, e.g., using PC (Intel x86 or pentium chip- compatible DOS™, OS2™ WINDOWS™, WINDOWS NT™ or WINDOWS95™ based machines), MACINTOSH™, or UNIX based (e.g., SUN™ work station) computers.

Embodiment in a web site.

[0296] The methods described above can be implemented in a localized or distributed computing environment. For example, if a localized computing environment is used, an array comprising a candidate nucleotide library, or diagnostic nucleotide set, is configured in proximity to a detector, which is, in turn, linked to a computational device equipped with user input and output features.

[0297] In a distributed environment, the methods can be implemented on a single computer with multiple processors or, alternatively, on multiple computers. The computers can be linked, e.g. through a shared bus, but more commonly, the computer(s) are nodes on a network. The network can be generalized or dedicated, at a local level or distributed over a wide geographic area. In certain embodiments, the computers are components of an intra-net or an internet.

[0298] The predictive data corresponding to subject molecular signatures (e.g., expression profiles, and related diagnostic, prognostic, or monitoring results) can be shared by a variety of parties. In particular, such information can be utilized by the subject, the subject's health care practitioner or provider, a company or other institution, or a scientist. An individual subject's data, a subset of the database or the entire database recorded in a computer readable medium can be accessed directly by a user by any method of communication, including, but not limited to, the internet. With appropriate computational devices, integrated systems, communications networks, users at remote locations, as well as users located in proximity to, e.g., at the same physical facility, the database can access the recorded information. Optionally, access to the database can be controlled using unique alphanumeric passwords that provide access to a subset of the data. Such provisions can be used, e.g., to ensure privacy, anonymity, etc.

[0299] Typically, a client (e.g., a patient, practitioner, provider, scientist, or the like) executes a Web browser and is linked to a server computer executing a Web server. The Web browser is, for example, a program such as IBM's Web Explorer, Internet explorer, NetScape or Mosaic, or the like. The Web server is typically, but not necessarily, a program such as IBM's HTTP Daemon or other WWW daemon (e.g., LINUX-based forms of the program). The client computer is bi-directionally coupled with the server computer over a line or via a wireless system In turn, the server computer is bi-directionally coupled with a website (server hosting the website) providing access to software implementing the methods of this invention.

[0300] A user of a client connected to the Intranet or Internet may cause the client to request resources that are part of the web site(s) hosting the application(s) providing an implementation of the methods described herein. Server program (s) then process the request to return the specified resources (assuming they are currently available). A standard naming convention has been adopted, known as a Uniform Resource Locator ("URL"). This convention encompasses several types of location names, presently including subclasses such as Hypertext Transport Protocol ("http"), File Transport Protocol ("ftp"), gopher, and Wide Area Information Service ("WAIS"). When a resource is downloaded, it may include the URLs of additional resources. Thus, the user of the client can easily learn of the existence of new resources that he or she had not specifically requested.

[0301] Methods of implementing Intranet and/or Intranet embodiments of computational and/or data access processes are well known to those of skill in the art and are documented, e.g., in ACM Press, pp. 383-392; ISO-ANSI, Working Draft, "Information Technology-Database Language SQL", Jim Melton, Editor, International Organization for Standard-

ization and American National Standards Institute, Jul. 1992; ISO Working Draft, "Database Language SQL-Part 2: Foundation (SQL/Foundation)", CD9075-2:199.chi.SQL, Sep. 11, 1997; and Cluer et al. (1992) A General Framework for the Optimization of Object-Oriented Queries, Proc SIGMOD International Conference on Management of Data, San Diego, California, Jun. 2-5, 1992, SIGMOD Record, vol. 21, Issue 2, Jun., 1992; Stonebraker, M., Editor;. Other resources are available, e.g., from Microsoft, IBM, Sun and other software development companies.

[0302] Using the tools described above, users of the reagents, methods and database as discovery or diagnostic tools can query a centrally located database with expression and subject data. Each submission of data adds to the sum of expression and subject information in the database. As data is added, a new correlation statistical analysis is automatically run that incorporates the added clinical and expression data. Accordingly, the predictive accuracy and the types of correlations of the recorded molecular signatures increases as the database grows.

[0303] For example, subjects, such as patients, can access the results of the expression analysis of their leukocyte samples and any accrued knowledge regarding the likelihood of the patient's belonging to any specified diagnostic (or prognostic, or monitoring, or risk group), i.e., their expression profiles, and/or molecular signatures. Optionally, subjects can add to the predictive accuracy of the database by providing additional information to the database regarding diagnoses, test results, clinical or other related events that have occurred since the time of the expression profiling. Such information can be provided to the database via any form of communication, including, but not limited to, the internet. Such data can be used to continually defme (and redefine) diagnostic groups. For example, if 1000 patients submit data regarding the occurrence of myocardial infarction over the 5 years since their expression profiling, and 300 of these patients report that they have experienced a myocardial infarction and 700 report that they have not, then the 300 patients define a new "group A." As the algorithm is used to continually query and revise the database, a new diagnostic nucleotide set that differentiates groups A and B (i.e., with and without myocardial infarction within a five year period) is identified. This newly defined nucleotide set is then be used (in the manner described above) as a test that predicts the occurrence of myocardial infarction over a five-year period. While submission directly by the patient is exemplified above, any individual with access and authority to submit the relevant data e.g., the patient's physician, a laboratory technician, a health care or study administrator, or the like, can do so.

[0304] As will be apparent from the above examples, transmission of information via the internet (or via an intranet) is optionally bi-directional. That is, for example, data regarding expression profiles, subject data, and the like are transmitted via a communication system to the database, while information regarding molecular signatures, predictive analysis, and the like, are transmitted from the database to the user. For example, using appropriate configurations of an integrated system including a microarray comprising a diagnostic nucleotide set, a detector linked to a computational device can directly transmit (locally or from a remote workstation at great distance, e.g., hundreds or thousands of miles distant from the database) expression profiles and a corresponding individual identifier to a central database for analysis according to the methods of the invention. According to, e.g., the algorithms described above, the individual identifier is assigned to one or more diagnostic (or prognostic, or monitoring, etc.) categories. The results of this classification are then relayed back, via, e.g., the same mode of communication, to a recipient at the same or different internet (or intranet) address.

Kits

[0305] The present disclosure is optionally provided to a user as a kit. Typically, a kit contains one or more diagnostic nucleotide sets of the disclosure. Alternatively, the kit contains the candidate nucleotide library of the disclosure. Most often, the kit contains a diagnostic nucleotide probe set, or other subset of a candidate library, e.g., as a cDNA or antibody microarray packaged in a suitable container. The kit may further comprise, one or more additional reagents, e.g., substrates, labels, primers, for labeling expression products, tubes and/or other accessories, reagents for collecting blood samples, buffers, e.g., erythrocyte lysis buffer, leukocyte lysis buffer, hybridization chambers, cover slips, etc., as well as a software package, e.g., including the statistical methods of the disclosure, e.g., as described above, and a password and/or account number for accessing the compiled database. The kit optionally further comprises an instruction set or user manual detailing preferred methods of using the diagnostic nucleotide sets in the methods of the disclosure. In one embodiment, the kit may include contents useful for the discovery of diagnostic nucleotide sets using microarrays. The kit may include sterile, endotoxin and RNAse free blood collection tubes. The kit may also include alcohol swabs, tourniquet, blood collection set, and/or PBS (phosphate buffer saline; needed when method of example 2 is used to derived mononuclear RNA). The kit may also include cell lysis buffer. The kit may include RNA isolation kit, substrates for labeling of RNA (may vary for various expression profiling techniques). The kit may also include materials for fluorescence microarray expression profiling, including one or more of the following: reverse transcriptase and 10x RT buffer, T7(dT)24 primer (primer with T7 promoter at 5' end), DTT, deoxynucleotides, optionally 100mM each, RNAse inhibitor, second strand cDNA buffer, DNA polymerase, Rnase H, T7 RNA polymerase ribonucleotides, in vitro transcription buffer, and/or Cy3 and Cy5 labeled ribonucleotides. The kit may also include microarrays containing candidate gene libraries, cover slips for slides, and/or hybridization chambers. The kit may further include software package for identification of

diagnostic gene set from data, that contains statistical methods, and/or allows alteration in desired sensitivity and specificity of gene set. The software may further facilitate access to and data analysis by centrally a located database server. The software may further include a password and account number to access central database server. In addition, the kit may include a kit user manual.

[0306] In another embodiment, the kit may include contents useful for the application of diagnostic nucleotide sets using microarrays. The kit may include sterile, endotoxin and/or RNAse free blood collection tubes. The kit may also include, alcohol swabs, tourniquet, and/or a blood collection set. The kit may further include PBS (phosphate buffer saline; needed when method of example 2 is used to derived mononuclear RNA), cell lysis buffer, and/or an RNA isolation kit. In addition, the kit may include substrates for labeling of RNA (may vary for various expression profiling techniques). For fluorescence microarray expression profiling, components may include reverse transcriptase and 10x RT buffer, T7 (dT)24 primer (primer with T7 promoter at 5' end), DTT, deoxynucleotides (optionally 100mM each), RNAse inhibitor, second strand cDNA buffer, DNA polymerase, Rnase H, T7 RNA polymerase, ribonucleotides, in vitro transcription buffer, and/or Cy3 and Cy5 labeled ribonucleotides. The kit may further include microarrays containing candidate gene libraries. The kit may also include cover slips for slides, and/or hybridization chambers. The kit may include a software package for identification of diagnostic gene set from data. The software package may contain statistical methods, allow alteration in desired sensitivity and specificity of gene set, and/or facilitate access to and data analysis by centrally located database server. The software package may include a password and account number to access central database server. In addition, the kit may include a kit user manual.

[0307] In another embodiment, the kit may include contents useful for the application of diagnostic nucleotide sets using real-time PCR. This kit may include terile, endotoxin and/or RNAse free blood collection tubes. The kit may further include alcohol swabs, tourniquet, and/or a blood collection set. The kit may also include PBS (phosphate buffer saline; needed when method of example 2 is used to derived mononuclear RNA). In addition, the kit may include cell lysis buffer and/or an RNA isolation kit. The kit may laso include substrates for real time RT-PCR, which may vary for various real-time PCR techniques, including poly dT primers, random hexamer primers, reverse Transcriptase and RT buffer, DTT, deoxynucleotides 100 mM, RNase H, primer pairs for diagnostic and control gene set, 10x PCR reaction buffer, and/or Taq DNA polymerase. The kit may also include fluorescent probes for diagnostic and control gene set (alternatively, fluorescent dye that binds to only double stranded DNA). The kit may further include reaction tubes with or without barcode for sample tracking, 96-well plates with barcode for sample identification, one barcode for entire set, or individual barcode per reaction tube in plate. The kit may also include a software package for identification of diagnostic gene set from data, and /or statistical methods. The software package may allow alteration in desired sensitivity and specificity of gene set, and/or facilitate access to and data analysis by centrally located database server. The kit may include a password and account number to access central database server. Finally, the kit may include a kit user manual.

[0308] This invention will be better understood by reference to the following non-limiting Examples:

LIST OF EXAMPLE TITLES

[0309]

Examples

Example 1: Preparation of a leukocyte cDNA array comprising a candidate gene library

[0310] Candidate genes and gene sequences for leukocyte expression profiling are identified through methods described elsewhere in this document. Candidate genes are used to obtain or design probes for peripheral leukocyte expression profiling in a variety of ways.

[0311] A cDNA microarray carrying 384 probes was constructed using sequences selected from the initial candidate library. cDNAs is selected from T-cell libraries, PBMC libraries and buffy coat libraries.

96-Well PCR

[0312] Plasmids are isolated in 96-well format and PCR was performed in 96-well format. A master mix is made that contain the reaction buffer, dNTPs, forward and reverse primer and DNA polymerase was made. 99 ul of the master mix was aliquoted into 96-well plate. 1 ul of plasmid (1-2 ng/ul) of plasmid was added to the plate. The final reaction concentration was 10 mM Tris pH 8.3, 3.5 mM MgCl2, 25 mM KCl, 0.4 mM dNTPs, 0.4 uM M13 forward primer, 0.4 M13 reverse primer, and 10 U of Taq Gold (Applied Biosystems). The PCR conditions were:

Step 1 95C for 10 min
Step 2 95C for 15 sec
Step 3 56C for 30 sec
Step 4 72C for 2 min 15 seconds
Step 5 go to Step 2 39 times
Step 6 72C for 10 minutes
Step 7 4C for ever.

PCR Purification

[0313] PCR purification is done in a 96-well format. The ArrayIt (Telechem International, Inc.) PCR purification kit is used and the provided protocol was followed without modification Before the sample is evaporated to dryness, the concentration of PCR products was determined using a spectrophotometer. After evaporation, the samples are resuspended in 1x Micro Spotting Solution (ArrayIt) so that the majority of the samples were between 0.2-1.0 ug/ul.

Array Fabrication

[0314] Spotted cDNA microarrays are then made from these PCR products by ArrayIt using their protocols, which may be found at the ArrayIt website. Each fragment was spotted 3 times onto each array. Candidate genes and gene sequences for leukocyte expression profiling are identified through methods described elsewhere in this document. Those candidate genes are used for peripheral leukocyte expression profiling. The candidate libraries can used to obtain or design probes for expression profiling in a variety of ways.

[0315] Oligonucleotide probes are prepared using the gene sequences of Table 2, Table 8, and the sequence listing. Oligo probes are designed on a contract basis by various companies (for example, Compugen, Mergen, Affymetrix, Telechem), or designed from the candidate sequences using a variety of parameters and algorithms as indicated at located at the MIT web site. Briefly, the length of the oligonucleotide to be synthesized is determined, preferably greater than 18 nucleotides, generally 18-24 nucleotides, 24-70 nucleotides and, in some circumstances, more than 70 nucleotides. The sequence analysis algorithms and tools described above are applied to the sequences to mask repetitive elements, vector sequences and low complexity sequences. Oligonucleotides are selected that are specific to the candidate nucleotide sequence (based on a Blast n search of the oligonucleotide sequence in question against gene sequences databases, such as the Human Genome Sequence, UniGene, dbEST or the non-redundant database at NCBI), and have <50% G content and 25-70% G+C content. Desired oligonucleotides are synthesized using well-known methods and apparatus, or ordered from a company (for example Sigma). Oligonucleotides are spotted onto microarrays. Alternatively, oligonucleotides are synthesized directly on the array surface, using a variety of techniques (Hughes et al. 2001, Yershov et al. 1996, Lockhart et al 1996).

Example 2: Preparation of RNA from mononuclear cells for expression profiling

[0316] Blood was isolated from the subject for leukocyte expression profiling using the following methods: Two tubes were drawn per patient. Blood was drawn from either a standard peripheral venous blood draw or directly from a large-bore intra-arterial or intravenous catheter inserted in the femoral artery, femoral vein, subclavian vein or internal jugular vein. Care was taken to avoid sample contamination with heparin from the intravascular catheters, as heparin can interfere with subsequent RNA reactions. For each tube, 8 ml of whole blood was drawn into a tube (CPT, Becton-Dickinson order #362753) containing the anticoagulant Citrate, 25°C density gradient solution (e.g. Ficoll, Percoll) and a polyester gel barrier that upon centrifugation was permeable to RBCs and granulocytes but not to mononuclear cells. The tube was inverted several times to mix the blood with the anticoagulant. The tubes were centrifuged at 1750xg in a swing-out rotor at room temperature for 20 minutes. The tubes were removed from the centrifuge and inverted 5-10 times to mix the plasma with the mononuclear cells, while trapping the RBCs and the granulocytes beneath the gel barrier. The plasma/mononuclear cell mix was decanted into a 15ml tube and 5ml of phosphate-buffered saline (PBS) is added. The 15ml tubes were spun for 5 minutes at 1750xg to pellet the cells. The supernatant was discarded and 1.8 ml of RLT lysis buffer is added to the mononuclear cell pellet. The buffer and cells were pipetted up and down to ensure complete lysis of the pellet. The cell lysate was frozen and stored until it is convenient to proceed with isolation of total RNA.

[0317] Total RNA was purified from the lysed mononuclear cells using the Qiagen Rneasy Miniprep kit, as directed by the manufacturer (10/99 version) for total RNA isolation, including homogenization (Qiashredder columns) and on-column DNase treatment. The purified RNA was eluted in 50ul of water. The further use of RNA prepared by this method is described in Examples 10 and 11. Some samples were prepared by a different protocol, as follows:

> Two 8 ml blood samples were drawn from a peripheral vein into a tube (CPT, Becton-Dickinson order #362753) containing anticoagulant (Citrate), 25°C density gradient solution (Ficoll) and a polyester gel barrier that upon centrifugation is permeable to RBCs and granulocytes but not to mononuclear cells. The mononuclear cells and plasma remained above the barrier while the RBCs and granulocytes were trapped below. The tube was inverted several times to mix the blood with the anticoagulant, and the tubes were subjected to centrifugation at 1750xg in a swing-out rotor at room temperature for 20 min. The tubes were removed from the centrifuge, and the clear plasma layer above the cloudy mononuclear cell layer was aspirated and discarded. The cloudy mononuclear cell layer was aspirated, with care taken to rinse all of the mononuclear cells from the surface of the gel barrier with PBS (phosphate buffered saline). Approximately 2 mls of mononuclear cell suspension was transferred to a 2ml microcentrifuge tube, and centrifuged for 3min. at 16,000 rpm in a microcentrifuge to pellet the cells. The supernatant was discarded and 1.8 ml of RLT lysis buffer (Qiagen) were added to the mononuclear cell pellet, which lysed the cells and inactivated Rnases. The cells and lysis buffer were pipetted up and down to ensure complete lysis of the pellet. Cell lysate was frozen and stored until it was convenient to proceed with isolation of total RNA.

[0318] RNA samples were isolated from 8 mL of whole blood. Yields ranged from 2 ug to 20ug total RNA for 8mL blood. A260/A280 spectrophotometric ratios were between 1.6 and 2.0, indicating purity of sample. 2ul of each sample were run on an agarose gel in the presence of ethidium bromide. No degradation of the RNA sample and no DNA contamination was visible.

[0319] In some cases, specific subsets of mononuclear cells were isolated from peripheral blood of human subjects. When this was done, the StemSep cell separation kits (manual version 6.0.0) were used from StemCell Technologies (Vancouver, Canada). This same protocol can be applied to the isolation of T cells, CD4 T cells, CD8 T cells, B cells, monocytes, NK cells and other cells. Isolation of cell types using negative selection with antibodies may be desirable to avoid activation of target cells by antibodies.

Example 3: Preparation of Universal Control RNA for use in leukocyte expression profiling

[0320] Control RNA was prepared using total RNA from Buffy coats and/or total RNA from enriched mononuclear cells isolated from Buffy coats, both with and without stimulation with ionomycin and PMA. The following control RNAs were prepared:

> Control 1: Buffy Coat Total RNA
> Control 2: Mononuclear cell Total RNA
> Control 3: Stimulated buffy coat Total RNA
> Control 4: Stimulated mononuclear Total RNA
> Control 5: 50% Buffy coat Total RNA /50% Stimulated buffy coat Total RNA
> Control 6: 50% Mononuclear cell Total RNA / 50% Stimulated Mononuclear Total RNA

[0321] Some samples were prepared using the following protocol: Buffy coats from 38 individuals were obtained from Stanford Blood Center. Each buffy coat is derived from ~350 mL whole blood from one individual. 10 ml buffy coat was removed from the bag, and placed into a 50 ml tube. 40 ml of Buffer EL (Qiagen) was added, the tube was mixed and placed on ice for 15 minutes, then cells were pelleted by centrifugation at 2000xg for 10 minutes at 4°C. The supernatant was decanted and the cell pellet was re-suspended in 10 ml of Qiagen Buffer EL. The tube was then centrifuged at 2000xg for 10 minutes at 4°C. The cell pellet was then re-suspended in 20 ml TRIZOL (GibcoBRL) per Buffy coat sample, the mixture was shredded using a rotary homogenizer, and the lysate was then frozen at -80°C prior to proceeding to RNA isolation.

[0322] Other control RNAs were prepared from enriched mononuclear cells prepared from Buffy coats. Buffy coats from Stanford Blood Center were obtained, as described above. 10 ml buffy coat was added to a 50 ml polypropylene tube, and 10 ml of phosphate buffer saline (PBS) was added to each tube. A polysucrose (5.7 g/dL) and sodium diatrizoate (9.0 g/dL) solution at a 1.077 +/-0.0001 g/ml density solution of equal volume to diluted sample was prepared (Histopaque 1077, Sigma cat. no 1077-1). This and all subsequent steps were performed at room temperature. 15 ml of diluted buffy coat/PBS was layered on top of 15 ml of the histopaque solution in a 50 ml tube. The tube was centrifuged at 400xg for 30 minutes at room temperature. After centrifugation, the upper layer of the solution to within 0.5 cm of the opaque interface containing the mononuclear cells was discarded. The opaque interface was transferred into a clean centrifuge tube. An equal volume of PBS was added to each tube and centrifuged at 350xg for 10 minutes at room temperature. The supernatant was discarded. 5 ml of Buffer EL (Qiagen) was used to resuspend the remaining cell pellet and the tube was centrifuged at 2000xg for 10 minutes at room temperature. The supernatant was discarded. The pellet was resuspended in 20 ml of TRIZOL (GibcoBRL) for each individual buffy coat that was processed. The sample was homogenized using a rotary homogenizer and frozen at -80C until RNA was isolated. RNA was isolated from frozen lysed Buffy coat samples as follows: frozen samples were thawed, and 4 ml of chloroform was added to each buffy coat sample. The sample was mixed by vortexing and centrifuged at 2000xg for 5 minutes. The aqueous layer was moved to new tube and then repurified by using the RNeasy Maxi RNA clean up kit, according to the manufacturer's instruction (Qiagen, PN 75162). The yield, purity and integrity were assessed by spectrophotometer and gel electrophoresis. Some samples were prepared by a different protocol, as follows. The further use of RNA prepared using this protocol is described in Example 11.

[0323] 50 whole blood samples were randomly selected from consented blood donors at the Stanford Medical School Blood Center. Each buffy coat sample was produced from ~350 mL of an individual's donated blood. The whole blood sample was centrifuged at ~4,400 x g for 8 minutes at room temperature, resulting in three distinct layers: a top layer of plasma, a second layer of buffy coat, and a third layer of red blood cells. 25 ml of the buffy coat fraction was obtained and diluted with an equal volume of PBS (phosphate buffered saline). 30 ml of diluted buffy coat was layered onto 15 ml of sodium diatrizoate solution adjusted to a density of 1.077+/-0.001 g/ml (Histopaque 1077, Sigma) in a 50mL plastic tube. The tube was spun at 800 g for 10 minutes at room temperature. The plasma layer was removed to the 30 ml mark on the tube, and the mononuclear cell layer removed into a new tube and washed with an equal volume of PBS, and collected by centrifugation at 2000 g for 10 minutes at room temperature. The cell pellet was resuspended in 10 ml of Buffer EL (Qiagen) by vortexing and incubated on ice for 10 minutes to remove any remaining erythrocytes. The mononuclear cells were spun at 2000 g for 10 minutes at 4 degrees Celsius. The cell pellet was lysed in 25 ml of a phenol/guanidinium thiocyanate solution (TRIZOL Reagent, Invitrogen). The sample was homogenized using a Power-Gene 5 rotary homogenizer (Fisher Scientific) and Omini disposable generator probes (Fisher Scientific). The Trizol lysate was frozen at -80 degrees C until the next step.

[0324] The samples were thawed out and incubated at room temperature for 5 minutes. 5 ml chloroform was added to each sample, mixed by vortexing, and incubated at room temperature for 3 minutes. The aqueous layers were transferred to new 50 ml tubes. The aqueous layer containing total RNA was further purified using the Qiagen RNeasy Maxi kit (PN 75162), per the manufacturer's protocol (October 1999). The columns were eluted twice with 1 ml Rnase-free water, with a minute incubation before each spin. Quantity and quality of RNA was assessed using standard methods. Generally, RNA was isolated from batches of 10 buffy coats at a time, with an average yield per buffy coat of 870 μg, and an estimated total yield of 43.5 mg total RNA with a 260/280 ratio of 1.56 and a 28S/18S ratio of 1.78.

[0325] Quality of the RNA was tested using the Agilent 2100 Bioanalyzer using RNA 6000 microfluidics chips. Analysis of the electrophorgrams from the Bioanalyzer for five different batches demonstrated the reproducibility in quality between the batches.

[0326] Total RNA from all five batches were combined and mixed in a 50 ml tube, then aliquoted as follows: 2 x 10 ml aliquots in 15 ml tubes, and the rest in 100 μl aliquots in 1.5 ml microcentrifuge tubes. The aliquots gave highly reproducible results with respect to RNA purity, size and integrity. The RNA was stored at -80°C.

Test hybridization of Reference RNA.

[0327] When compared with BC38 and Stimulated mononuclear reference samples, the R50 performed as well, if not

better than the other reference samples as shown in Figure 3. In an analysis of hybridizations, where the R50 targets were fluorescently labeled with Cy-5 using methods described herein and the amplified and labeled aRNA was hybridized (as in example 11) to the olignoucleotide array described in example 9. The R50 detected 97.3% of probes with a Signal to Noise ratio (S/N) of greater than three and 99.9 % of probes with S/N greater than one.

Example 4. RNA Labeling and hybridization to a leukocyte cDNA array of candidate nucleotide sequences.

Comparison of Guanine-Silica to Acid-Phenol RNA Purification (GSvsAP)

[0328] These data are from a set of 12 hybridizations designed to identify differences between the signal strength from two different RNA purification methods. The two RNA methods used were guanidine-silica (GS, Qiagen) and acid-phenol (AP, Trizol, Gibco BRL). Ten tubes of blood were drawn from each of four people. Two were used for the AP prep, the other eight were used for the GS prep. The protocols for the leukocyte RNA preps using the AP and GS techniques were completed as described here:

Guanidine-silica (GS) method:

[0329] For each tube, 8ml blood was drawn into a tube containing the anticoagulant Citrate, 25°C density gradient solution and a polyester gel barrier that upon centrifugation is permeable to RBCs and granulocytes but not to mononuclear cells. The mononuclear cells and plasma remained above the barrier while the RBCs and granulocytes were trapped below. CPT tubes from Becton-Dickinson (#362753) were used for this purpose. The tube was inverted several times to mix the blood with the anticoagulant. The tubes were immediately centrifuged @1750xg in a swinging bucket rotor at room temperature for 20 min. The tubes were removed from the centrifuge and inverted 5-10 times. This mixed the plasma with the mononuclear cells, while the RBCs and the granulocytes remained trapped beneath the gel barrier. The plasma/mononuclear cell mix was decanted into a 15ml tube and 5ml of phosphate-buffered saline (PBS) was added. The 15ml tubes are spun for 5 minutes at 1750xg to pellet the cells. The supernatant was discarded and 1.8 ml of RLT lysis buffer (guanidine isothyocyanate) was added to the mononuclear cell pellet. The buffer and cells were pipetted up and down to ensure complete lysis of the pellet. The cell lysate was then processed exactly as described in the Qiagen Rneasy Miniprep kit protocol (10/99 version) for total RNA isolation (including steps for homogenization (Qiashredder columns) and on-column DNase treatment. The purified RNA was eluted in 50ul of water.

Acid-phenol (AP) method:

[0330] For each tube, 8ml blood was drawn into a tube containing the anticoagulant Citrate, 25°C density gradient solution and a polyester gel barrier that upon centrifugation is permeable to RBCs and granulocytes but not to mononuclear cells. The mononuclear cells and plasma remained above the barrier while the RBCs and granulocytes were trapped below. CPT tubes from Becton-Dickinson (#362753) were used for this purpose. The tube was inverted several times to mix the blood with the anticoagulant. The tubes were immediately centrifuged @1750xg in a swinging bucket rotor at room temperature for 20 min. The tubes were removed from the centrifuge and inverted 5-10 times. This mixed the plasma with the mononuclear cells, while the RBCs and the granulocytes remained trapped beneath the gel barrier. The plasma/mononuclear cell mix was decanted into a 15ml tube and 5ml of phosphate-buffered saline (PBS) was added. The 15ml tubes are spun for 5 minutes @1750xg to pellet the cells. The supernatant was discarded and the cell pellet was lysed using 0.6 mL Phenol/guanidine isothyocyanate (e.g. Trizol reagent, GibcoBRL). Subsequent total RNA isolation proceeded using the manufacturers protocol.

[0331] RNA from each person was labeled with either Cy3 or Cy5, and then hybridized in pairs to the mini-array. For instance, the first array was hybridized with GS RNA from one person (Cy3) and GS RNA from a second person (Cy5).

[0332] Techniques for labeling and hybridization for all experiments discussed here were completed as detailed above. Arrays were prepared as described in example 1.

[0333] RNA isolated from subject samples, or control Buffy coat RNA, were labeled for hybridization to a cDNA array. Total RNA (up to 100 μg) was combined with 2 μl of 100 μM solution of an Oligo (dT)12-18 (GibcoBRL) and heated to 70°C for 10 minutes and place on ice. Reaction buffer was added to the tube, to a final concentration of 1xRT buffer (GibcoBRL), 10 mM DTT (GibcoBRL), 0.1 mM unlabeled dATP, dTTP, and dGTP, and 0.025 mM unlabeled dCTP, 200 pg of CAB (A. thaliana photosystem I chlorophyll a/b binding protein), 200 pg of RCA (A. thaliana RUBISCO activase), 0.25 mM of Cy-3 or Cy-5 dCTP, and 400 U Superscript II RT (GibcoBRL).

[0334] The volumes of each component of the labeling reaction were as follows: 20 μl of 5xRT buffer; 10 μl of 100 mM DTT; 1 μl of 10 mM dNTPs without dCTP; 0.5 μl of 5 mM CTP; 13 μl of H20; 0.02 μl of 10 ng/μl CAB and RCA; 1 μl of 40 Units/μl RNAseOUT Recombinatnt Ribonuclease Inhibitor (GibcoBRL); 2.5 μl of 1.0 mM Cy-3 or Cy-5 dCTP; and 2.0 μl of 200 Units/μl of Superscript II RT. The sample was vortexed and centrifuged. The sample was incubated

at 4°C for 1 hour for first strand cDNA synthesis, then heated at 70°C for 10 minutes to quench enzymatic activity. 1 μl of 10 mg/ml of Rnase A was added to degrade the RNA strand, and the sample was incubated at 37°C for 30 minutes. Next, the Cy-3 and Cy-5 cDNA samples were combined into one tube. Unincorporated nucleotides were removed using QIAquick RCR purification protocol (Qiagen), as directed by the manufacturer. The sample was evaporated to dryness and resuspended in 5 μl of water. The sample was mixed with hybridization buffer containing 5xSSC, 0.2% SDS, 2 mg/ml Cot-1 DNA (GibcoBRL), 1 mg/ml yeast tRNA (GibcoBRL), and 1.6 ng/μl poly dA40-60 (Pharmacia). This mixture was placed on the microarray surface and a glass cover slip was placed on the array (Corning). The microarray glass slide was placed into a hybridization chamber (ArrrayIt). The chamber was then submerged in a water bath overnight at 62° C. The microarray was removed from the cassette and the cover slip was removed by repeatedly submerging it to a wash buffer containing 1xSSC, and 0.1% SDS. The microarray slide was washed in 1xSSC/0.1% SDS for 5 minutes. The slide was then washed in 0.1%SSC/0.1% SDS for 5 minutes. The slide was finally washed in 0.1xSSC for 2 minutes. The slide was spun at 1000 rpm for 2 minutes to dry out the slide, then scanned on a microarray scanner (Axon Instruments, Union City, CA.).

[0335] Six hybridizations with 20 μg of RNA were performed for each type of RNA preparation (GS or AP). Since both the Cy3 and the Cy5 labeled RNA are from test preparations, there are six data points for each GS prepped, Cy3-labeled RNA and six for each GS-prepped, Cy5-labeled RNA. The mini array hybridizations were scanned on and Axon Instruments scanner using GenPix 3.0 software. The data presented were derived as follows. First, all features flagged as "not found" by the software were removed from the dataset for individual hybridizations. These features are usually due to high local background or other processing artifacts. Second, the median fluorescence intensity minus the background fluorescence intensity was used to calculate the mean background subtracted signal for each dye for each hybridization. In Figure 3, the mean of these means across all six hybridizations is graphed (n=6 for each column). The error bars are the SEM. This experiment shows that the average signal from AP prepared RNA is 47% of the average signal from GS prepared RNA for both Cy3 and Cy5.

Generation of expression data for leukocyte genes from peripheral leukocyte samples

[0336] Six hybridizations were performed with RNA purified from human blood leukocytes using the protocols given above. Four of the six were prepared using the GS method and 2 were prepared using the AP method. Each preparation of leukocyte RNA was labeled with Cy3 and 10 μg hybridized to the mini-array. A control RNA was batch labeled with Cy5 and 10 μg hybridized to each mini-array together with the Cy3-labeled experimental RNA.

[0337] The control RNA used for these experiments was Control 1: Buffy Coat RNA, as described above. The protocol for the preparation of that RNA is reproduced here:

Buffy Coat RNA Isolation:

[0338] Buffy coats were obtained from Stanford Blood Center (in total 38 individual buffy coats were used. Each buffy coat is derived from ~350 mL whole blood from one individual. 10 ml buffy coat was taken and placed into a 50 ml tube and 40 ml of a hypoclorous acid (HOCl) solution (Buffer EL from Qiagen) was added. The tube was mixed and placed on ice for 15 minutes. The tube was then centrifuged at 2000xg for 10 minutes at 4°C. The supernatant was decanted and the cell pellet was re-suspended in 10 ml of hypochlorous acid solution (Qiagen Buffer EL). The tube was then centrifuged at 2000xg for 10 minutes at 4°C. The cell pellet was then re-suspended in 20 ml phenol/guanidine thiocyanate solution (TRIZOL from GibcoBRL) for each individual buffy coat that was processed. The mixture was then shredded using a rotary homogenizer. The lysate was then frozen at -80°C prior to proceeding to RNA isolation.

[0339] The arrays were then scanned and analyzed on an Axon Instruments scanner using GenePix 3.0 software. The data presented were derived as follows. First, all features flagged as "not found" by the software were removed from the dataset for individual hybridizations. Second, control features were used to normalize the data for labeling and hybridization variability within the experiment. The control features are cDNA for genes from the plant, *Arabidopsis thaliana,* that were included when spotting the mini-array. Equal amounts of RNA complementary to two of these cDNAs were added to each of the samples before they were labeled. A third was pre-labeled and equal amounts were added to each hybridization solution before hybridization. Using the signal from these genes, we derived a normalization constant (L_j) according to the following formula:

$$L_j = \frac{\frac{\sum_{i=1}^{N} BGSS_{j,i}}{N}}{\frac{\sum_{j=1}^{K} \frac{\sum_{i=1}^{N} BGSS_{j,i}}{N}}{K}}$$

where $BGSS_i$ is the signal for a specific feature as identified in the GenePix software as the median background subtracted signal for that feature, *N* is the number of *A. thaliana* control features, *K* is the number of hybridizations, and *L* is the normalization constant for each individual hybridization. Using the formula above, the mean over all control features of a particular hybridization and dye (eg Cy3) was calculated. Then these control feature means for all Cy3 hybridizations were averaged. The control feature mean in one hybridization divided by the average of all hybridizations gives a normalization constant for that particular Cy3 hybridization.

[0340] The same normalization steps were performed for Cy3 and Cy5 values, both fluorescence and background. Once normalized, the background Cy3 fluorescence was subtracted from the Cy3 fluorescence for each feature. Values less than 100 were eliminated from further calculations since low values caused spurious results.

[0341] Figure 4 shows the average background subtracted signal for each of nine leukocyte-specific genes on the mini array. This average is for 3-6 of the above-described hybridizations for each gene. The error bars are the SEM.

[0342] The ratio of Cy3 to Cy5 signal is shown for a number of genes. This ratio corrects for variability among hybridizations and allows comparison between experiments done at different times. The ratio is calculated as the Cy3 background subtracted signal divided by the Cy5 background subtracted signal. Each bar is the average for 3-6 hybridizations. The error bars are SEM.

[0343] Together, these results show that we can measure expression levels for genes that are expressed specifically in sub-populations of leukocytes. These expression measurements were made with only 10 μg of leukocyte total RNA that was labeled directly by reverse transcription. The signal strength can be increased by improved labeling techniques that amplify either the starting RNA or the signal fluorescence. In addition, scanning techniques with higher sensitivity can be used.

Genes in Figures 4 and 5:

Gene Name/Description	GenBank Accession Number	Gene Name Abbreviation
T cell-specific tyrosine kinase Mrna	L10717	TKTCS
Interleukin 1 alpha (IL 1) mRNA, complete cds	NM_000575	IL1A
T-cell surface antigen CD2 (T11) mRNA, complete cds	M14362	CD2
Interleukin-13 (IL-13) precursor gene, complete cds	U31120	IL-13
Thymocyte antigen CD1a mRNA, complete cds	M28825	CD1a
CD6 mRNA for T cell glycoprotein CDS	NM_006725	CD6
MHC class II HLA-DQA1 mRNA, complete cds	U77589	HLA-DQA1
Granulocyte colony-stimulating factor	M28170	CD19
Homo sapiens CD69 antigen	NM_001781	CD69

Example 5: Clinical study to identify diagnostic gene sets useful in diagnosis and treatment of cardiac allograft recipients

[0344] An observational study was conducted in which a prospective cohort of cardiac transplant recipients were analyzed for associations between clinical events or rejection grades and expression of a leukocyte candidate nucleotide sequence library. Patients were identified at 4 cardiac transplantation centers while on the transplant waiting list or during their routing post-transplant care. All adult cardiac transplant recipients (new or re-transplants) who received an organ at the study center during the study period or within 3 months of the start of the study period were eligible. The first year after transplantation is the time when most acute rejection occurs and it is thus important to study patients during this

period. Patients provided informed consent prior to study procedures.

[0345] Peripheral blood leukocyte samples were obtained from all patients at the following time points: prior to transplant surgery (when able), the same day as routinely scheduled screening biopsies, upon evaluation for suspected acute rejection (urgent biopsies), on hospitalization for an acute complication of transplantation or immunosuppression, and when Cytomegalovirus (CMV) infection was suspected or confirmed. Samples were obtained through a standard peripheral vein blood draw or through a catheter placed for patient care (for example, a central venous catheter placed for endocardial biopsy). When blood was drawn from a intravenous line, care was taken to avoid obtaining heparin with the sample as it can interfere with downstream reactions involving the RNA. Mononuclear cells were prepared from whole blood samples as described in Example 2. Samples were processed within 2 hours of the blood draw and DNA and serum were saved in addition to RNA. Samples were stored at -80° C or on dry ice and sent to the site of RNA preparation in a sealed container with ample dry ice. RNA was isolated from subject samples as described in Example 2 and hybridized to a candidate library of differentially expressed leukocyte nucleotide sequences, as further described in Examples 9-10. Methods used for amplification, labeling, hybridization and scanning are described in Example 11. Analysis of human transplant patient mononuclear cell RNA hybridized to a microarray and identification of diagnostic gene sets is shown in Example 10.

[0346] From each patient, clinical information was obtained at the following time points: prior to transplant surgery (when available), the same day as routinely scheduled screening biopsies, upon evaluation for suspected acute rejection (e.g., urgent biopsies), on hospitalization for an acute complication of transplantation or immunosuppression, and when Cytomegalovirus (CMV) infection was suspected or confirmed. Data was collected directly from the patient, from the patient's medical record, from diagnostic test reports or from computerized hospital databases. It was important to collect all information pertaining to the study clinical correlates (diagnoses and patient events and states to which expression data is correlated) and confounding variables (diagnoses and patient events and states that may result in altered leukocyte gene expression. Examples of clinical data collected are: patient sex, date of birth, date of transplant, race, requirement for prospective cross match, occurrence of pretransplant diagnoses and complications, indication for transplantation, severity and type of heart disease, history of left ventricular assist devices, all known medical diagnoses, blood type, HLA type, viral serologies (including CMV, Hepatitis B and C, HIV and others), serum chemistries, white and red blood cell counts and differentials, CMV infections (clinical manifestations and methods of diagnosis), occurrence of new cancer, hemodynamic parameters measured by catheterization of the right or left heart (measures of graft function), results of echocardiography, results of coronary angiograms, results of intravascular ultrasound studies (diagnosis of transplant vasculopathy), medications, changes in medications, treatments for rejection, and medication levels. Information was also collected regarding the organ donor, including demographics, blood type, HLA type, results of screening cultures, results of viral serologies, primary cause of brain death, the need for inotropic support, and the organ cold ischemia time.

[0347] Of great importance was the collection of the results of endocardial biopsy for each of the patients at each visit. Biopsy results were all interpreted and recorded using the international society for heart and lung transplantation (ISHLT) criteria, described below. Biopsy pathological grades were determined by experienced pathologists at each center.

ISHLT Criteria

Grade	Finding	Rejection Severity
0	No lymphocytic infiltrates	None
1A	Focal (perivascular or interstitial lymphocytic infiltrates without necrosis)	Borderline mild
1B	Diffuse but sparse lymphocytic infiltrates without necrosis	Mild
2	One focus only with aggressive lymphocytic infiltrate and/or myocyte damage	Mild, focal moderate
3A	Multifocal aggressive lymphocytic infiltrates and/or myocardial damage	Moderate
3B	Diffuse inflammatory lymphocytic infiltrates with necrosis	Borderline Severe
4	Diffuse aggressive polymorphous lymphocytic infiltrates with edema hemorrhage and vasculitis, with necrosis	Severe

[0348] Because variability exists in the assignment of ISHLT grades, it was important to have a centralized and blinded reading of the biopsy slides by a single pathologist. This was arranged for all biopsy slides associated with samples in the analysis. Slides were obtained and assigned an encoded number. A single pathologist then read all slides from all centers and assigned an ISHLT grade. Grades from the single pathologist were then compared to the original grades derived from the pathologists at the study centers. For the purposes of correlation analysis of leukocyte gene expression

to biopsy grades, the centralized reading information was used in a variety of ways (see Example 10 for more detail). In some analyses, only the original reading was used as an outcome. In other analyses, the result from the centralized reader was used as an outcome. In other analyses, the highest of the 2 grades was used. For example, if the original assigned grade was 0 and the centralized reader assigned a 1A, then 1A was the grade used as an outcome. In some analyses, the highest grade was used and then samples associated with a Grade 1A reading were excluded from the analysis. In some analyses, only grades with no disagreement between the 2 readings were used as outcomes for correlation analysis.

[0349] Clinical data was entered and stored in a database. The database was queried to identify all patients and patient visits that meet desired criteria (for example, patients with > grade II biopsy results, no CMV infection and time since transplant < 12 weeks).

[0350] The collected clinical data (disease criteria) is used to define patient or sample groups for correlation of expression data. Patient groups are identified for comparison, for example, a patient group that possesses a useful or interesting clinical distinction, versus a patient group that does not possess the distinction. Examples of useful and interesting patient distinctions that can be made on the basis of collected clinical data are listed here:

1. Rejection episode of at least moderate histologic grade, which results in treatment of the patient with additional corticosteroids, anti-T cell antibodies, or total lymphoid irradiation.
2. Rejection with histologic grade 2 or higher.
3.Rejection with histologic grade <2.
4. The absence of histologic rejection and normal or unchanged allograft function (based on hemodynamic measurements from catheterization or on echocardiographic data).
5. The presence of severe allograft dysfunction or worsening allograft dysfunction during the study period (based on hemodynamic measurements from catheterization or on echocardiographic data).
6. Documented CMV infection by culture, histology, or PCR, and at least one clinical sign or symptom of infection.
7. Specific graft biopsy rejection grades
8. Rejection of mild to moderate histologic severity prompting augmentation of the patient's chronic immunosuppressive regimen
9. Rejection of mild to moderate severity with allograft dysfunction prompting plasmaphoresis or a diagnosis of "humoral" rejection
10. Infections other than CMV, esp. Epstein Barr virus (EBV)
11. Lymphoproliferative disorder (also called, post-transplant lymphoma)
12. Transplant vasculopathy diagnosed by increased intimal thickness on intravascular ultrasound (IVUS), angiography, or acute myocardial infarction.
13. Graft Failure or Retransplantation
14. All cause mortality
15. Grade 1A or higher rejection as defmed by the initial biopsy reading.
16. Grade 1B or higher rejection as defmed by the initial biopsy reading.
17. Grade 1A or higher rejection as defined by the centralized biopsy reading.
18. Grade 1B or higher rejection as defined by the centralized biopsy reading.
19. Grade 1A or higher rejection as defined by the highest of the initial and centralized biopsy reading.
20. Grade 1B or higher rejection as defined by the highest of the initial and centralized biopsy reading.
21. Any rejection > Grade 2 occurring in patient at any time in the post-transplant course.

[0351] Expression profiles of subject samples are examined to discover sets of nucleotide sequences with differential expression between patient groups, for example, by methods describes above and below. Non-limiting examples of patient leukocyte samples to obtain for discovery of various diagnostic nucleotide sets are as follows:

Leukocyte set to avoid biopsy or select for biopsy:
Samples : Grade 0 vs. Grades 1-4
Leukocyte set to monitor therapeutic response:
Examine successful vs. unsuccessful drug treatment.

Samples:

[0352] Successful: Time 1: rejection, Time 2: drug therapy Time 3: no rejection Unsuccessful: Time 1: rejection, Time 2: drug therapy; Time 3: rejection
Leukocyte set to predict subsequent acute rejection.
Biopsy may show no rejection, but the patient may develop rejection shortly thereafter. Look at profiles of patients who

subsequently do and do not develop rejection.

Samples:

[0353] Group 1 (Subsequent rejection): Time 1: Grade 0; Time 2: Grade>0
Group 2 (No subsequent rejection): Time 1: Grade 0; Time 2: Grade 0
[0354] Focal rejection may be missed by biopsy. When this occurs the patient may have a Grade 0, but actually has rejection. These patients may go on to have damage to the graft etc.

Samples:

[0355] Non-rejectors: no rejection over some period of time
Rejectors: an episode of rejection over same period
Leukocyte set to diagnose subsequent or current graft failure:

Samples:

[0356] Echocardiographic or catheterization data to define worsening function over time and correlate to profiles.
[0357] Leukocyte set to diagnose impending active CMV:

Samples:

Look at patients who are CMV IgG positive. Compare patients with subsequent (to a sample) clinical CMV infection verses no subsequent clinical CMV infection.

Leukocyte set to diagnose current active CMV:

Samples:

Analyze patients who are CMV IgG positive. Compare patients with active current clinical CMV infection vs. no active current CMV infection.

[0358] Upon identification of a nucleotide sequence or set of nucleotide sequences that distinguish patient groups with a high degree of accuracy, that nucleotide sequence or set of nucleotide sequences is validate, and implemented as a diagnostic test. The use of the test depends on the patient groups that are used to discover the nucleotide set. For example, if a set of nucleotide sequences is discovered that have collective expression behavior that reliably distinguishes patients with no histological rejection or graft dysfunction from all others, a diagnostic is developed that is used to screen patients for the need for biopsy. Patients identified as having no rejection do not need biopsy, while others are subjected to a biopsy to further define the extent of disease. In another example, a diagnostic nucleotide set that determines continuing graft rejection associated with myocyte necrosis (> grade I) is used to determine that a patient is not receiving adequate treatment under the current treatment regimen. After increased or altered immunosuppressive therapy, diagnostic profiling is conducted to determine whether continuing graft rejection is progressing. In yet another example, a diagnostic nucleotide set(s) that determine a patient's rejection status and diagnose cytomegalovirus infection is used to balance immunosuppressive and anti-viral therapy.
The methods of this example are also applicable to cardiac xenograft monitoring.

Example 6: Identification of diagnostic nucleotide sets for kidney and liver allograft rejection

[0359] Diagnostic tests for rejection are identified using patient leukocyte expression profiles to identify a molecular signature correlated with rejection of a transplanted kidney or liver. Blood, or other leukocyte source, samples are obtained from patients undergoing kidney or liver biopsy following liver or kidney transplantation, respectively. Such results reveal the histological grade, i.e., the state and severity of allograft rejection. Expression profiles are obtained from the samples as described above, and the expression profile is correlated with biopsy results. In the case of kidney rejection, clinical data is collected corresponding to urine output, level of creatine clearance, and level of serum creatine (and other markers of renal function). Clinical data collected for monitoring liver transplant rejection includes, biochemical characterization of serum markers of liver damage and function such as SGOT, SGPT, Alkaline phosphatase, GGT, Bilirubin, Albumin and Prothrombin time.
Leukocyte nucleotide sequence expression profiles are collected and correlated with important clinical states and out-

comes in renal or hepatic transplantation. Examples of useful clinical correlates are given here:

1. Rejection episode of at least moderate histologic grade, which results in treatment of the patient with additional corticosteriods, anti-T cell antibodies, or total lymphoid irradiation.
2. The absence of histologic rejection and normal or unchanged allograft function (based on tests of renal or liver function listed above).
3. The presence of severe allograft dysfunction or worsening allograft dysfunction during the study period (based on tests of renal and hepatic function listed above).
4. Documented CMV infection by culture, histology, or PCR, and at least one clinical sign or symptom of infection.
5. Specific graft biopsy rejection grades
6. Rejection of mild to moderate histologic severity prompting augmentation of the patient's chronic immunosuppressive regimen
7. Infections other than CMV, esp. Epstein Barr virus (EBV)
8. Lymphoproliferative disorder (also called, post-transplant lymphoma)
9. Graft Failure or Retransplantation
10. Need for hemodialysis or other renal replacement therapy for renal transplant patients.
11. Hepatic encephalopathy for liver transplant recipients.
12. All cause mortality

Subsets of the candidate library (or of a previously identified diagnostic nucleotide set), are identified, according to the above procedures, that have predictive and/or diagnostic value for kidney or liver allograft rejection.

Example 7: Identification of a diagnostic nucleotide set for diagnosis of cytomegalovirus

[0360] Cytomegalovirus is a very important cause of disease in immunocompromised patients, for example, transplant patients, cancer patients, and AIDS patients. The virus can cause inflammation and disease in almost any tissue (particularly the colon, lung, bone marrow and retina). It is increasingly important to identify patients with current or impending clinical CMV disease, particularly when immunosuppressive drugs are to be used in a patient, e.g. for preventing transplant rejection. Leukocytes are profiled in patients with active CMV, impending CMV, or no CMV. Expression profiles correlating with diagnosis of active or impending CMV are identified. Subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, according to the above procedures that have predictive value for the diagnosis of active or impending CMV. Diagnostic nucleotide set(s) identified with predictive value for the diagnosis of active or impending CMV may be combined, or used in conjunction with, cardiac, liver and/or kidney allograft-related diagnostic gene set(s) (described in Examples 6 and 10).
In addition, or alternatively, CMV nucleotide sequences are obtained, and a diagnostic nucleotide set is designed using CMV nucleotide sequence. The entire sequence of the organism is known and all CMV nucleotide sequences can be isolated and added to the library using the sequence information and the approach described below. Known expressed genes are preferred. Alternatively, nucleotide sequences are selected to represent groups of CMV genes that are coordinately expressed (immediate early genes, early genes, and late genes) (Spector et al. 1990, Stamminger et al. 1990).
Oligonucleotides were designed for CMV genes using the oligo design procedures of Example 8. Probes were designed using the 14 gene sequences shown here and were included on the array described in example 9:

Cytomegalovirus (CMV) Accession #X17403	HCMVTRL2 (IRL2)	1893..2240
	HCMVTRL7 (IRL7)	complement(6595..6843)
	HCMVUL21	complement(26497..27024)
	HCMVUL27	complement(32831..34657)
	HCMVUL33	43251..44423
	HCMVUL54	complement(76903..80631)
	HCMVUL75	complement(107901..110132)
	HCMVUL83	complement(119352..121037)
	HCMVUL106	complement(154947..155324)
	HCMVUL109	complement(157514..157810)
	HCMVUL113	161503..162800
	HCMVUL122	complement(169364..170599)
	HCMVUL123 (last exon at 3'-end)	complement(171006..172225)
	HCMVUS28	219200..220171

[0361] Diagnostic nucleotide set(s) for expression of CMV genes is used in combination with diagnostic leukocyte nucleotide sets for diagnosis of other conditions, e.g. organ allograft rejection.

[0362] Using the techniques described in example 2 mononuclear samples from 180 cardiac transplant recipients (enrolled in the study described in Example 5) were used for expression profiling with the leukocyte arrays. Of these samples 15 were associated with patients who had a diagnosis of primary or reactivation CMV made by culture, PCR or any specific diagnostic test.

[0363] After preparation of RNA, amplification, labeling, hybridization, scanning, feature extraction and data processing were done as described in Example 11 using the oligonucleotide microarrays described in Example 9.

[0364] The resulting log ratio of expression of Cy3 (patient sample)/ Cy5 (R50 reference RNA) was used for analysis. Significance analysis for microarrays (SAM, Tusher 2001, see Example 15) was applied to determine which genes were most significantly differentially expressed between these 15 CMV patients and the 165 non-CMV patients (Table 12). 12 genes were identified with a 0% FDR and 6 with a 0.1% FDR and are listed in Table 2. Some genes are represented by more than one oligonucleotide on the array and for 2 genes, multiple oligonucleotides from the same gene are called significant (SEQ IDs: 3061, 3064: eomesodermin and 3031, 3040, 104, 2736: small inducible cytokine A4).

[0365] Clinical variables were also included in the significance analysis. For example, the white blood cell count and the number of weeks post transplant (for the patient at the time the sample was obtained) were available for most of the 180 samples. The log of these variables was taken and the variables were then used in the significance analysis described above with the gene expression data. Both the white blood cell count (0.1% FDR) and the weeks post transplant (0% FDR) appeared to correlate with CMV status. CMV patients were more likely to have samples associated with later post transplant data and the lower white blood cell counts.

[0366] These genes and variables can be used alone or in association with other genes or variables or with other genes to build a diagnostic gene set or a classification algorithm using the approaches described herein.

[0367] Primers for real-time PCR validation were designed for some of these genes as described in Example 13 and listed in Table 2C and the sequence listing. Using the methods described in example 13, primers for Granzyme B were designed and used to validate expression findings from the arrays. 6 samples were tested (3 from patients with CMV and 3 from patients without CMV). The gene was found to be differentially expressed between the patients with and without CMV (see example 13 for full description). This same approach can be used to validate other diagnostic genes by real-time PCR. Diagnostic nucleotide sets can also be identified for a variety of other viral diseases (Table 1) using this same approach.

[0368] cDNA microarrays may be used to monitor viral expression. In addition, these methods may be used to monitor other viruses, such as Epstein-Barr virus, Herpes Simplex 1 and vesicular stomatitis virus.

Example 8- Design of oligonucleotide probes

[0369] By way of example, this section describes the design of four oligonucleotide probes using Array Designer Ver 1.1 (Premier Biosoft International, Palo Alto, CA). The major steps in the process are given first.

[0370] Obtain best possible sequence of mRNA from GenBank. If a full-length sequence reference sequence is not available, a partial sequence is used, with preference for the 3' end over the 5' end. When the sequence is known to represent the antisense strand, the reverse complement of the sequence is used for probe design. For sequences

represented in the subtracted leukocyte expression library that have no significant match in GenBank at the time of probe design, our sequence is used.

[0371] Mask low complexity regions and repetitive elements in the sequence using an algorithm such as RepeatMasker.

[0372] Use probe design software, such as Array Designer, version 1.1, to select a sequence of 50 residues with specified physical and chemical properties. The 50 residues nearest the 3' end constitute a search frame. The residues it contains are tested for suitability. If they don't meet the specified criteria, the search frame is moved one residue closer to the 5' end, and the 50 residues it now contains are tested. The process is repeated until a suitable 50-mer is found.

[0373] If no such 50-mer occurs in the sequence, the physical and chemical criteria are adjusted until a suitable 50-mer is found.

[0374] Compare the probe to dbEST, the UniGene cluster set, and the assembled human genome using the BLASTn search tool at NCBI to obtain the pertinent identifying information and to verify that the probe does not have significant similarity to more than one known gene.

Clone 40H 12

[0375] Clone 40H 12 was sequenced and compared to the nr, dbEST, and UniGene databases at NCBI using the BLAST search tool. The sequence matched accession number NM_002310, a'curated RefSeq project' sequence, see Pruitt et al. (2000) Trends Genet. 16:44-47, encoding leukemia inhibitory factor receptor (LIFR) mRNA with a reported E value of zero. An E value of zero indicates there is, for all practical purposes, no chance that the similarity was random based on the length of the sequence and the composition and size of the database. This sequence, cataloged by accession number NM_002310, is much longer than the sequence of clone 40H12 and has a poly-A tail. This indicated that the sequence cataloged by accession number NM_002310 is the sense strand and a more complete representation of the mRNA than the sequence of clone 40H12, especially at the 3' end. Accession number "NM_002310" was included in a text file of accession numbers representing sense strand mRNAs, and sequences for the sense strand mRNAs were obtained by uploading a text file containing desired accession number as an Entrez search query using the Batch Entrez web interface and saving the results locally as a FASTA file. The following sequence was obtained, and the region of alignment of clone 40H12 is outlined:

CTCTCTCCCAGAACGTGTCTCTGCTGCAAGGCACCGGGCCCTTTCGCTCTGCAGAACTGCACTTGCAAGA
CCATTATCAACTCCTAATCCCAGCTCAGAAAGGGAGCCTCTGCGACTCATTCATCGCCCTCCAGGACTGA
CTGCATTGCACAGATGATGGATATTTACGTATGTTTGAAACGACCATCCTGGATGGTGGACAATAAAAGA
ATGAGGACTGCTTCAAATTTCCAGTGGCTGTTATCAACATTTATTCTTCTATATCTAATGAATCAAGTAA
ATAGCCAGAAAAAGGGGGCTCCTCATGATTTGAAGTGTGTAACTAACAATTTGCAAGTGTGGAACTGTTC
TTGGAAAGCACCCTCTGGAACAGGCCGTGGTACTGATTATGAAGTTTGCATTGAAAACAGGTCCCGTTCT
TGTTATCAGTTGGAGAAAACCAGTATTAAAATTCCAGCTCTTTCACATGGTGATTATGAAATAACAATAA
ATTCTCTACATGATTTTGGAAGTTCTACAAGTAAATTCACACTAAATGAACAAAACGTTTCCTTAATTCC
AGATACTCCAGAGATCTTGAATTTGTCTGCTGATTTCTCAACCTCTACATTATACCTAAAGTGGAACGAC
AGGGGTTCAGTTTTTCCACACCGCTCAAATGTTATCTGGGAAATTAAAGTTCTACGTAAAGAGAGTATGG
AGCTCGTAAAATTAGTGACCCACAACACAACTCTGAATGGCAAAGATACACTTCATCACTGGAGTTGGGC
CTCAGATATGCCCTTGGAATGTGCCATTCATTTTGTGGAAATTAGATGCTACATTGACAATCTTCATTTT
TCTGGTCTCGAAGAGTGGAGTGACTGGAGCCCTGTGAAGAACATTTCTTGGATACCTGATTCTCAGACTA
AGGTTTTTCCTCAAGATAAAGTGATACTTGTAGGCTCAGACATAACATTTTGTTGTGTGAGTCAAGAAAA

AGTGTTATCAGCACTGATTGGCCATACAAACTGCCCCTTGATCCATCTTGATGGGGAAAATGTTGCAATC
AAGATTCGTAATATTTCTGTTTCTGCAAGTAGTGGAACAAATGTAGTTTTTACAACCGAAGATAACATAT
TTGGAACCGTTATTTTTGCTGGATATCCACCAGATACTCCTCAACAACTGAATTGTGAGACACATGATTT
AAAAGAAATTATATGTAGTTGGAATCCAGGAAGGGTGACAGCGTTGGTGGGCCCACGTGCTACAAGCTAC
ACTTTAGTTGAAAGTTTTTCAGGAAAATATGTTAGACTTAAAAGAGCTGAAGCACCTACAAACGAAAGCT
ATCAATTATTATTTCAAATGCTTCCAAATCAAGAAATATATAATTTTACTTTGAATGCTCACAATCCGCT
GGGTCGATCACAATCAACAATTTTAGTTAATATAACTGAAAAAGTTTATCCCCATACTCCTACTTCATTC
AAAGTGAAGGATATTAATTCAACAGCTGTTAAACTTTCTTGGCATTTACCAGGCAACTTTGCAAAGATTA
ATTTTTTATGTGAAATTGAAATTAAGAAATCTAATTCAGTACAAGAGCAGCGGAATGTCACAATCAAAGG
AGTAGAAAATTCAAGTTATCTTGTTGCTCTGGACAAGTTAAATCCATACACTCTATATACTTTTCGGATT
CGTTGTTCTACTGAAACTTTCTGGAAATGGAGCAAATGGAGCAATAAAAAACAACATTTAACAACAGAAG
CCAGTCCTTCAAAGGGGCCTGATACTTGGAGAGAGTGGAGTTCTGATGGAAAAAATTTAATAATCTATTG
GAAGCCTTTACCCATTAATGAAGCTAATGGAAAAATACTTTCCTACAATGTATCGTGTTCATCAGATGAG
GAAACACAGTCCCTTTCTGAAATCCCTGATCCTCAGCACAAAGCAGAGATACGACTTGATAAGAATGACT
ACATCATCAGCGTAGTGGCTAAAAATTCTGTGGGCTCATCACCACCTTCCAAAATAGCGAGTATGGAAAT
TCCAAATGATGATCTCAAAATAGAACAAGTTGTTGGGATGGGAAAGGGGATTCTCCTCACCTGGCATTAC
GACCCCAACATGACTTGCGACTACGTCATTAAGTGGTGTAACTCGTCTCGGTCGGAACCATGCCTTATGG
ACTGGAGAAAAGTTCCCTCAAACAGCACTGAAACTGTAATAGAATCTGATGAGTTTCGACCAGGTATAAG
ATATAATTTTTTCCTGTATGGATGCAGAAATCAAGGATATCAATTATTACGCTCCATGATTGGATATATA
GAAGAATTGGCTCCCATTGTTGCACCAAATTTTACTGTTGAGGATACTTCTGCAGATTCGATATTAGTAA
AATGGGAAGACATTCCTGTGGAAGAACTTAGAGGCTTTTTAAGAGGATATTTGTTTTACTTTGGAAAAGG
AGAAAGAGACACATCTAAGATGAGGGTTTTAGAATCAGGTCGTTCTGACATAAAAGTTAAGAATATTACT
GACATATCCCAGAAGACACTGAGAATTGCTGATCTTCAAGGTAAAACAAGTTACCACCTGGTCTTGCGAG
CCTATACAGATGGTGGAGTGGGCCCGGAGAAGAGTATGTATGTGGTGACAAAGGAAAATTCTGTGGGATT
AATTATTGCCATTCTCATCCCAGTGGCAGTGGCTGTCATTGTTGGAGTGGTGACAAGTATCCTTTGCTAT
CGGAAACGAGAATGGATTAAAGAAACCTTCTACCCTGATATTCCAAATCCAGAAAACTGTAAAGCATTAC
AGTTTCAAAAGAGTGTCTGTGAGGGAAGCAGTGCTCTTAAAACATTGGAAATGAATCCTTGTACCCCAAA
TAATGTTGAGGTTCTGGAAACTCGATCAGCATTTCCTAAAATAGAAGATACAGAAATAATTTCCCCAGTA
GCTGAGCGTCCTGAAGATCGCTCTGATGCAGAGCCTGAAAACCATGTGGTTGTGTCCTATTGTCCACCCA
TCATTGAGGAAGAAATACCAAACCCAGCCGCAGATGAAGCTGGAGGGACTGCACAGGTTATTTACATTGA
TGTTCAGTCGATGTATCAGCCTCAAGCAAAACCAGAAGAAGAACAAGAAAATGACCCTGTAGGAGGGGCA
GGCTATAAGCCACAGATGCACCTCCCCATTAATTCTACTGTGGAAGATATAGCTGCAGAAGAGGACTTAG
ATAAAACTGCGGGTTACAGACCTCAGGCCAATGTAAATACATGGAATTTAGTGTCTCCAGACTCTCCTAG
ATCCATAGACAGCAACAGTGAGATTGTCTCATTTGGAAGTCCATGCTCCATTAATTCCCGACAATTTTTG
ATTCCTCCTAAAGATGAAGACTCTCCTAAATCTAATGGAGGAGGGTGGTCCTTTACAAACTTTTTTCAGA
ACAAACCAAACGATTAACAGTGTCACCGTGTCACTTCAGTCAGCCATCTCAATAAGCTCTTACTGCTAGT
GTTGCTACATCAGCACTGGGCATTCTTGGAGGGATCCTGTGAAGTATTGTTAGGAGGTGAACTTCACTAC
ATGTTAAGTTACACTGAAAGTTCATGTGCTTTTAATGTAGTCTAAAAGCCAAAGTATAGTGACTCAGAAT
CCTCAATCCACAAAACTCAAGATTGGGAGCTCTTTGTGATCAAGCCAAAGAATTCTCATGTACTCTACCT
TCAAGAAGCATTTCAAGGCTAATACCTACTTGTACGTACATGTAAAACAAATCCCGCCGCAACTGTTTTC
TGTTCTGTTGTTTGTGGTTTTCTCATATGTATACTTGGTGGAATTGTAAGTGGATTTGCAGGCCAGGGAG
AAAATGTCCAAGTAACAGGTGAAGTTTATTTGCCTGACGTTTACTCCTTTCTAGATGAAAACCAAGCACA
GATTTTAAAACTTCTAAGATTATTCTCCTCTATCCACAGCATTCACAAAAATTAATATAATTTTTAATGT
AGTGACAGCGATTTAGTGTTTTGTTTGATAAAGTATGCTTATTTCTGTGCCTACTGTATAATGGTTATCA
AACAGTTGTCTCAGGGGTACAAACTTTGAAAACAAGTGTGACACTGACCAGCCCAAATCATAATCATGTT
TTCTTGCTGTGATAGGTTTTGCTTGCCTTTTCATTATTTTTTAGCTTTTATGCTTGCTTCCATTATTTCA
GTTGGTTGCCCTAATATTTAAAATTTACACTTCTAAGACTAGAGACCCACATTTTTTAAAAATCATTTTA
TTTTGTGATACAGTGACAGCTTTATATGAGCAAATTCAATATTATTCATAAGCATGTAATTCCAGTGACT
TACTATGTGAGATGACTACTAAGCAATATCTAGCAGCGTTAGTTCCATATAGTTCTGATTGGATTTCGTT
CCTCCTGAGGAGACCATGCCGTTGAGCTTGGCTACCCAGGCAGTGGTGATCTTTGACACCTTCTGGTGGA
TGTTCCTCCCACTCATGAGTCTTTTCATCATGCCACATTATCTGATCCAGTCCTCACATTTTTAAATATA
AAACTAAAGAGAGAATGCTTCTTACAGGAACAGTTACCCAAGGGCTGTTTCTTAGTAACTGTCATAAACT
GATCTGGATCCATGGGCATACCTGTGTTCGAGGTGCAGCAATTGCTTGGTGAGCTGTGCAGAATTGATTG
CCTTCAGCACAGCATCCTCTGCCCACCCTTGTTTCTCATAAGCGATGTCTGGAGTGATTGTGGTTCTTGG
AAAAGCAGAAGGAAAAACTAAAAAGTGTATCTTGTATTTTCCCTGCCCTCAGGTTGCCTATGTATTTTAC
CTTTTCATATTTAAGGCAAAAGTACTTGAAAATTTTAAGTGTCCGAATAAGATATGTCTTTTTTGTTTGT
TTTTTTTGGTTGGTTGTTTGTTTTTTATCATCTGAGATTCTGTAATGTATTTGCAAATAATGGATCAATT
AATTTTTTTTGAAGCTCATATTGTATCTTTTTAAAAACCATGTTGTGGAAAAAAGCCAGAGTGACAAGTG
ACAAAATCTATTTAGGAACTCTGTGTATGAATCCTGATTTTAACTGCTAGGATTCAGCTAAATTTCTGAG
CTTTATGATCTGTGGAAATTTGGAATGAAATCGAATTCATTTTGTACATACATAGTATATTAAAACTATA

TAATAGTTCATAGAAATGTTCAGTAATGAAAAAATATATCCAATCAGAGCCATCCCGAAAAAAAAAAAAA
AA (SEQ ID NO: 3101)

The FASTA file, including the sequence of NM_002310, was masked using the RepeatMasker web interface (Smit, AFA & Green, P RepeatMasker at http://ftp.genome.washmgton.edu/RM/RepeatMasker.html, Smit and Green). Specifically, during masking, the following types of sequences were replaced with "N's": SINE/MIR & LINE/L2, LINE/L1 , TR/MaLR, LTR/Retroviral , Alu, and other low informational content sequences such as simple repeats. Below is the sequence following masking:

CTCTCTCCCAGAACGTGTCTCTGCTGCAAGGCACCGGGCCCTTTCGCTCTGCAGAACTGCACTTGCAAG
ACCATTATCAACTCCTAATCCCAGCTCAGAAAGGGAGCCTCTGCGACTCATTCATCGCCCTCCAGGACT
GACTGCATTGCACAGATGATGGATATTTACGTATGTTTGAAACGACCATCCTGGATGGTGGACAATAAA
AGAATGAGGACTGCTTCAAATTTCCAGTGGCTGTTATCAACATTTATTCTTCTATATCTAATGAATCAA
GTAAATAGCCAGAAAAAGGGGGCTCCTCATGATTTGAAGTGTGTAACTAACAATTTGCAAGTGTGGAAC
TGTTCTTGGAAAGCACCCTCTGGAACAGGCCGTGGTACTGATTATGAAGTTTGCATTGAAAACAGGTCC
CGTTCTTGTTATCAGTTGGAGAAAACCAGTATTAAAATTCCAGCTCTTTCACATGGTGATTATGAAATA
ACAATAAATTCTCTACATGATTTTGGAAGTTCTACAAGTAAATTCACACTAAATGAACAAAACGTTTCC
TTAATTCCAGATACTCCAGAGATCTTGAATTTGTCTGCTGATTTCTCAACCTCTACATTATACCTAAAG
TGGAACGACAGGGGTTCAGTTTTTCCACACCGCTCAAATGTTATCTGGGAAATTAAAGTTCTACGTAAA
GAGAGTATGGAGCTCGTAAAATTAGTGACCCACAACACAACTCTGAATGGCAAAGATACACTTCATCAC
TGGAGTTGGGCCTCAGATATGCCCTTGGAATGTGCCATTCATTTTGTGGAAATTAGATGCTACATTGAC
AATCTTCATTTTTCTGGTCTCGAAGAGTGGAGTGACTGGAGCCCTGTGAAGAACATTTCTTGGATACCT
GATTCTCAGACTAAGGTTTTTCCTCAAGATAAAGTGATACTTGTAGGCTCAGACATAACATTTTGTTGT
GTGAGTCAAGAAAAAGTGTTATCAGCACTGATTGGCCATACAAACTGCCCCTTGATCCATCTTGATGGG
GAAAATGTTGCAATCAAGATTCGTAATATTTCTGTTTCTGCAAGTAGTGGAACAAATGTAGTTTTTACA
ACCGAAGATAACATATTTGGAACCGTTATTTTTGCTGGATATCCACCAGATACTCCTCAACAACTGAAT
TGTGAGACACATGATTTAAAAGAAATTATATGTAGTTGGAATCCAGGAAGGGTGACAGCGTTGGTGGGC
CCACGTGCTACAAGCTACACTTTAGTTGAAAGTTTTTCAGGAAAATATGTTAGACTTAAAAGAGCTGAA
GCACCTACAAACGAAAGCTATCAATTATTATTTCAAATGCTTCCAAATCAAGAAATATATAATTTTACT
TTGAATGCTCACAATCCGCTGGGTCGATCACAATCAACAATTTTAGTTAATATAACTGAAAAAGTTTAT
CCCCATACTCCTACTTCATTCAAAGTGAAGGATATTAATTCAACAGCTGTTAAACTTTCTTGGCATTTA
CCAGGCAACTTTGCAAAGATTAATTTTTTATGTGAAATTGAAATTAAGAAATCTAATTCAGTACAAGAG
CAGCGGAATGTCACAATCAAAGGAGTAGAAAATTCAAGTTATCTTGTTGCTCTGGACAAGTTAAATCCA
TACACTCTATATACTTTTCGGATTCGTTGTTCTACTGAAACTTTCTGGAAATGGAGCAAATGGAGCAAT
AAAAAACAACATTTAACAACAGAAGCCAGTCCTTCAAAGGGGCCTGATACTTGGAGAGAGTGGAGTTCT
GATGGAAAAAATTTAATAATCTATTGGAAGCCTTTACCCATTAATGAAGCTAATGGAAAAATACTTTCC
TACAATGTATCGTGTTCATCAGATGAGGAAACACAGTCCCTTTCTGAAATCCCTGATCCTCAGCACAAA
GCAGAGATACGACTTGATAAGAATGACTACATCATCAGCGTAGTGGCTAAAAATTCTGTGGGCTCATCA
CCACCTTCCAAAATAGCGAGTATGGAAATTCCAAATGATGATCTCAAAATAGAACAAGTTGTTGGGATG
GGAAAGGGGATTCTCCTCACCTGGCATTACGACCCCAACATGACTTGCGACTACGTCATTAAGTGGTGT
AACTCGTCTCGGTCGGAACCATGCCTTATGGACTGGAGAAAAGTTCCCTCAAACAGCACTGAAACTGTA

ATAGAATCTGATGAGTTTCGACCAGGTATAAGATATAATTTTTTCCTGTATGGATGCAGAAATCAAGGA
TATCAATTATTACGCTCCATGATTGGATATATAGAAGAATTGGCTCCCATTGTTGCACCAAATTTTACT
GTTGAGGATACTTCTGCAGATTCGATATTAGTAAAATGGGAAGACATTCCTGTGGAAGAACTTAGAGGC
TTTTTAAGAGGATATTTGTTTTACTTTGGAAAAGGAGAAAGAGACACATCTAAGATGAGGGTTTTAGAA
TCAGGTCGTTCTGACATAAAAGTTAAGAATATTACTGACATATCCCAGAAGACACTGAGAATTGCTGAT
CTTCAAGGTAAAACAAGTTACCACCTGGTCTTGCGAGCCTATACAGATGGTGGAGTGGGCCCGGAGAAG
AGTATGTATGTGGTGACAAAGGAAAATTCTGTGGGATTAATTATTGCCATTCTCATCCCAGTGGCAGTG
GCTGTCATTGTTGGAGTGGTGACAAGTATCCTTTGCTATCGGAAACGAGAATGGATTAAAGAAACCTTC
TACCCTGATATTCCAAATCCAGAAAACTGTAAAGCATTACAGTTTCAAAAGAGTGTCTGTGAGGGAAGC
AGTGCTCTTAAAACATTGGAAATGAATCCTTGTACCCCAAATAATGTTGAGGTTCTGGAAACTCGATCA
GCATTTCCTAAAATAGAAGATACAGAAATAATTTCCCCAGTAGCTGAGCGTCCTGAAGATCGCTCTGAT
GCAGAGCCTGAAAACCATGTGGTTGTGTCCTATTGTCCACCCATCATTGAGGAAGAAATACCAAACCCA
GCCGCAGATGAAGCTGGAGGGACTGCACAGGTTATTTACATTGATGTTCAGTCGATGTATCAGCCTCAA
GCAAAACCAGAAGAAGAACAAGAAAATGACCCTGTAGGAGGGGCAGGCTATAAGCCACAGATGCACCTC
CCCATTAATTCTACTGTGGAAGATATAGCTGCAGAAGAGGACTTAGATAAAACTGCGGGTTACAGACCT
CAGGCCAATGTAAATACATGGAATTTAGTGTCTCCAGACTCTCCTAGATCCATAGACAGCAACAGTGAG
ATTGTCTCATTTGGAAGTCCATGCTCCATTAATTCCCGACAATTTTTGATTCCTCCTAAAGATGAAGAC
TCTCCTAAATCTAATGGAGGAGGGTGGTCCTTTACAAACTTTTTTCAGAACAAACCAAACGATTAACAG
TGTCACCGTGTCACTTCAGTCAGCCATCTCAATAAGCTCTTACTGCTAGTGTTGCTACATCAGCACTGG
GCATTCTTGGAGGGATCCTGTGAAGTATTGTTAGGAGGTGAACTTCACTACATGTTAAGTTACACTGAA
AGTTCATGTGCTTTTAATGTAGTCTAAAAGCCAAAGTATAGTGACTCAGAATCCTCAATCCACAAAACT
CAAGATTGGGAGCTCTTTGTGATCAAGCCAAAGAATTCTCATGTACTCTACCTTCAAGAAGCATTTCAA
GGCTAATACCTACTTGTACGTACATGTAAAACAAATCCCGCCGCAACTGTTTTCTGTTCTGTTGTTTGT
GGTTTTCTCATATGTATACTTGGTGGAATTGTAAGTGGATTTGCAGGCCAGGGAGAAAATGTCCAAGTA
ACAGGTGAAGTTTATTTGCCTGACGTTTACTCCTTTCTAGATGAAAACCAAGCACAGATTTTAAAACTT
CTAAGATTATTCTCCTCTATCCACAGCATTCACNNNNNNNNNNNNNNNNNNNNNGTAGTGACAGCGAT
TTAGTGTTTTGTTTGATAAAGTATGCTTATTTCTGTGCCTACTGTATAATGGTTATCAAACAGTTGTCT
CAGGGGTACAAACTTTGAAAACAAGTGTGACACTGACCAGCCCAAATCATAATCATGTTTTCTTGCTGT
GATAGGTTTTGCTTGCCTTTTCATTATTTTTTAGCTTTTATGCTTGCTTCCATTATTTCAGTTGGTTGC
CCTAATATTTAAAATTTACACTTCTAAGACTAGAGACCCACATTTTTTAAAAATCATTTTATTTTGTGA
TACAGTGACAGCTTTATATGAGCAAATTCAATATTATTCATAAGCATGTAATTCCAGTGACTTACTATG
TGAGATGACTACTAAGCAATATCTAGCAGCGTTAGTTCCATATAGTTCTGATTGGATTTCGTTCCTCCT
GAGGAGACCATGCCGTTGAGCTTGGCTACCCAGGCAGTGGTGATCTTTGACACCTTCTGGTGGATGTTC
CTCCCACTCATGAGTCTTTTCATCATGCCACATTATCTGATCCAGTCCTCACATTTTTAAATATAAAAC
TAAAGAGAGAATGCTTCTTACAGGAACAGTTACCCAAGGGCTGTTTCTTAGTAACTGTCATAAACTGAT
CTGGATCCATGGGCATACCTGTGTTCGAGGTGCAGCAATTGCTTGGTGAGCTGTGCAGAATTGATTGCC
TTCAGCACAGCATCCTCTGCCCACCCTTGTTTCTCATAAGCGATGTCTGGAGTGATTGTGGTTCTTGGA
AAAGCAGAAGGAAAAACTAAAAAGTGTATCTTGTATTTTCCCTGCCCTCAGGTTGCCTATGTATTTTAC
CTTTTCATATTTAAGGCAAAAGTACTTGAAAATTTTAAGTGTCCGAATAAGATATGTCTTTTTTGTTTG
TTTTTTTTGGTTGGTTGTTTGTTTTTTATCATCTGAGATTCTGTAATGTATTTGCAAATAATGGATCAA

TTAATTTTTTTTGAAGCTCATATTGTATCTTTTTAAAAACCATGTTGTGGAAAAAAGCCAGAGTGACAA
GTGACAAAATCTATTTAGGAACTCTGTGTATGAATCCTGATTTTAACTGCTAGGATTCAGCTAAATTTC
TGAGCTTTATGATCTGTGGAAATTTGGAATGAAATCGAATTCATTTTGTACATACATAGTATATTAAAA
CTATATAATAGTTCATAGAAATGTTCAGTAATGAAAAAATATATCCAATCAGAGCCATCCCGAAAAAAA
AAAAAAAA (SEQ ID NO: 3102).

[0376] The length of this sequence was determined using batch, automated computational methods and the sequence, as sense strand, its length, and the desired location of the probe sequence near the 3' end of the mRNA was submitted to Array Designer Ver 1.1 (Premier Biosoft International, Palo Alto, CA). Search quality was set at 100%, number of best probes set at 1, length range set at 50 base pairs, Target Tm set at 75 C. degrees plus or minus 5 degrees, Hairpin max deltaG at 6.0 -kcal/mol., Self dimmer max deltaG at 6.0 -kcal/mol, Run/repeat (dinucleotide) max length set at 5, and Probe site minimum overlap set at 1. When none of the 49 possible probes met the criteria, the probe site would be moved 50 base pairs closer to the 5' end of the sequence and resubmitted to Array Designer for analysis. When no possible probes met the criteria, the variation on melting temperature was raised to plus and minus 8 degrees and the number of identical basepairs in a run increased to 6 so that a probe sequence was produced.

[0377] In the sequence above, using the criteria noted above, Array Designer Ver 1.1 designed a probe corresponding to oligonucleotide number 3037 and is indicated by underlining in the sequence above. It has a melting temperature of 68.4 degrees Celsius and a max run of 6 nucleotides and represents one of the cases where the criteria for probe design in Array Designer Ver 1.1 were relaxed in order to obtain an oligonucleotide near the 3' end of the mRNA (Low melting temperature was allowed).

Clone 463D12

[0378] Clone 463D 12 was sequenced and compared to the nr, dbEST, and UniGene databases at NCBI using the BLAST search tool. The sequence matched accession number AI184553, an EST sequence with the definition line "qd60a05.x1 Soares_testis_NHT Homo sapiens cDNA clone IMAGE:1733840 3' similar to gb:M29550 PROTEIN PHOSPHATASE 2B CATALYTIC SUBUNIT 1 (1HUMAN);, mRNA sequence." The E value of the alignment was 1.00×10^{-118}. The GenBank sequence begins with a poly-T region, suggesting that it is the antisense strand, read 5' to 3'. The beginning of this sequence is complementary to the 3' end of the mRNA sense strand. The accession number for this sequence was included in a text file of accession numbers representing antisense sequences. Sequences for antisense strand mRNAs were obtained by uploading a text file containing desired accession numbers as an Entrez search query using the Batch Entrez web interface and saving the results locally as a FASTA file. The following sequence was obtained, and the region of alignment of clone 463D12 is outlined:

TTTTTTTTTTTTTTCTTAAATAGCATTTATTTTCTCTCAAAAAGCCTATTATGTACTAACAAGTGTTCC
TCTAAATTAGAAAGGCATCACTACTAAAATTTTATACATATTTTTTATATAAGAGAAGGAATATTGGGT
TACAATCTGAATTTCTCTTTATGATTTCTCTTAAAGTATAGAACAGCTATTAAAATGACTAATATTGCT
AAAATGAAGGCTACTAAATTTCCCCAAGAATTTCGGTGGAATGCCCAAAAATGGTGTTAAGATATGCAG
AAGGGCCCATTTCAAGCAAAGCAATCTCTCCACCCCTTCATAAAAGATTTAAGCTAAAAAAAAAAAAAA

AAGAAGAAAATCCAACAGCTGAAGACATTGGGCTATTTATAAATCTTCTCCCAGTCCCCCAGACAGCCT

CACATGGGGGCTGTAAACAGCTAACTAAAATATCTTTGAGACTCTTATGTCCACACCCACTGACACAAG

GAGAGCTGTAACCACAGTGAAACTAGACTTTGCTTTCCTTTAGCAAGTATGTGCCTATGATAGTAAACT

GGAGTAAATGTAACAGTAATAAAACAAATTTTTTTTAAAAATAAAAATTATACCTTTTTCTCCAACAAA

CGGTAAAGACCACGTGAAGACATCCATAAAATTAGGCAACCAGTAAAGATGTGGAGAACCAGTAAACTG

TCGAAATTCATCACATTATTTTCATACTTTAATACAGCAGCTTTAATTATTGGAGAACATCAAAGTAAT

TAGGTGCCGAAAAACATTGTTATTAATGAAGGGAACCCCTGACGTTTGACCTTTTCTGTACCATCTATA

GCCCTGGACTTGA (SEQ ID NO: 3103)

The FASTA file, including the sequence of AA184553, was then masked using the RepeatMasker web interface, as shown below. The region of alignment of clone 463D12 is outlined.

TTTTTTTTTTTTTTCTTAAATAGCATTTATTTTCTCTCAAAAAGCCTATTATGTACTAACAAGTGTTCC

TCTAAATTAGAAAGGCATCACTACNNNNNNNNNNNNNNNNNNNNNNNNNNNGAGAAGGAATATTGGGT

TACAATCTGAATTTCTCTTTATGATTTCTCTTAAAGTATAGAACAGCTATTAAAATGACTAATATTGCT

AAAATGAAGGCTACTAAATTTCCCCAAGAATTTGGCTGGAATGCCCAAAAATGGTGTTAAGATATGCAG

AAGGGCCCATTTCAAGCAAAGCAATCTCTCCACCCCTTCATAAAAGATTTAAGCTAAAAAAAAAAAAAA

AAGAAGAAAATCCAACAGCTGAAGACATTGGGCTATTTATAAATCTTCTCCCAGTCCCCCAGACAGCCT

CACATGGGGGCTGTAAACAGCTAACTAAAATATCTTTGAGACTCTTATGTCCACACCCACTGACACAAG

GAGAGCTGTAACCACAGTGAAACTAGACTTTGCTTTCCTTTAGCAAGTATGTGCCTATGATAGTAAACT

GGAGTAAATGTAACAGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNCCTTTTTCTCCAACAAA

CGGTAAAGACCACGTGAAGACATCCATAAAATTAGGCAACCAGTAAAGATGTGGAGAACCAGTAAACTG

TCGAAATTCATCACATTATTTTCATACTTTAATACAGCAGCTTTAATTATTGGAGAACATCAAAGTAAT

TAGGTGCCGAAAAACATTGTTATTAATGAAGGGAACCCCTGACGTTTGACCTTTTCTGTACCATCTATA

GCCCTGGACTTGA Masked version of 463D12 sequence. (SEQ ID NO:3104)

[0379] The sequence was submitted to Array Designer as described above, however, the desired location of the probe was indicated at base pair 50 and if no probe met the criteria, moved in the 3' direction. The complementary sequence from Array Designer was used, because the original sequence was antisense. The oligonucleotide designed by Array Designer corresponds to oligonucleotide number 3054 and is complementary to the underlined sequence above. The probe has a melting temperature of 72.7 degrees centigrade and a max run of 4 nucleotides.

Clone 72D4

[0380] Clone 72D4 was sequenced and compared to the nr, dbEST, and UniGene databases at NCBI using the BLAST search tool. No significant matches were found in any of these databases. When compared to the human genome draft, significant alignments were found to three consecutive regions of the reference sequence NT_008060, as depicted below, suggesting that the insert contains three spliced exons of an unidentified gene.

Residue numbers on clone 72D4 sequence	Matching residue numbers on NT 008060
1-198	478646-478843
197 - 489	479876 - 480168
491 - 585	489271 - 489365

[0381] Because the reference sequence contains introns and may represent either the coding or noncoding strand for this gene, BioCardia's own sequence file was used to design the oligonucleotide. Two complementary probes were designed to ensure that the sense strand was represented. The sequence of the insert in clone 72D4 is shown below, with the three putative exons outlined.

CAGGTCACACAGCACATCAGTGGCTACATGTGAGCTCAGACCTGGGTCTGCTGCTGTCTGT
CTTCCCAATATCCATGACCTTGACTGATGCAGGTGTCTAGGGATACGTCCATCCCCGTCCT
GCTGGAGCCCAGAGCACGGAAGCCTGGCCCTCCGAGGAGACAGAAGGGAGTGTCGGACA
CCATGACGAGAGCTTGGCAGAATAAATAACTTCTTTAAACAATTTTACGGCATGAAGAAA
TCTGGACCAGTTTATTAAATGGGATTTCTGCCACAAACCTTGGAAGAATCACATCATCTTA
NNCCCAAGTGAAAACTGTGTTGCGTAACAAAGAACATGACTGCGCTCCACACATACATCA
TTGCCCGGCGAGGCGGGACACAAGTCAACGACGGAACACTTGAGACAGGCCTACAACTG
TGCACGGGTCAGAAGCAAGTTTAAGCCATACTTGCTGCAGTGAGACTACATTTCTGTCTAT
AGAAGATACCTGACTTGATCTGTTTTTCAGCTCCAGTTCCCAGATGTGCGTGTTGTGGTCC
CCAAGTATCACCTTCCAATTTCTGGGAGCAGTGCTCTGGCCGGATCCTTGCCGCGCGGAT
AAAAAC (SEQ ID NO: 3106)

[0382] The sequence was submitted to RepeatMasker, but no repetitive sequences were found. The sequence shown above was used to design the two 50-mer probes using Array Designer as described above. The probes are shown in bold typeface in the sequence depicted below. The probe in the sequence is oligonucleotide number 3020 (SEQ ID NO: 3020) and the complementary probe is oligonucleotide number 318 (SEQ ID NO:318). A portion of the target sequence is listed below (SEQ ID: 3106).

CAGGTCACACAGCACATCAGTGGCTACATGTGAGCTCAGACCTGGGTCTGCTGCTGTCTGTCTTCCCAA
TATCCATGACCTTGACTGATGCAGGTGTCTAGGGATACGTCCATCCCCGTCCTGCTGGAGCCCAGAGCA
CGGAAGCCTGGCCCTCCGAGGAGACAGAAGGGAGTGTCGGACACCATGACGAGAGCTTGGCAGAATAAA
TAACTTCTTTAAACAATTTTACGGCATGAAGAAATCTGGACCAGTTTATTAAATGGGATTTCTGCCACA
AACCTTGGAAGAATCACATCATCTTANNCCCAAGTGAAAACTGTGTTGCGTAACAAAGAACATGACTGC
GCTCCACACATACATCATTGCCCGGCGAGGCGGGACACAAGTCAACGACGGAACACTTGAGACAGGCCT
ACAACTGTGCACGGGTCAGAAGCAAGTTTAAGCCATACTTGCTGCAGTGAGACTACATTTCTGTCTATA
GAAGATACCTGACTTGATCTGTTTTTCAGCTCCAGTTCCCAGATGTGC
←----GTCAAGGGTCTACACG
GTGTTGTGGTCCCCAAGTATCACCTTCCAATTTCTGGGAG--→
CACAACACCAGGGGTTCATAGTGGAAGGTTAAAG-5'

CAGTGCTCTGGCCGGATCCTTGCCGCGCGGATAAAAACT---→

Confirmation of probe sequence

[0383] Following probe design, each probe sequence was confirmed by comparing the sequence against dbEST, the UniGene cluster set, and the assembled human genome using BLASTn at NCBI. Alignments, accession numbers, gi numbers, UniGene cluster numbers and names were examined and the most common sequence used for the probe.

Example 9 - Production of an array of 8000 spotted 50mer oligonucleotides

[0384] We produced an array of 8000 spotted initial candidate 50mer oligonucleotides. Example 8 exemplifies the design and selection of probes for this array.

[0385] Sigma-Genosys (The Woodlands, TX) synthesized un-modified 50-mer oligonucleotides using standard phosphoramidite chemistry, with a starting scale of synthesis of 0.05 μmole (see, e.g., R. Meyers, ed. (1995) Molecular Biology and Biotechnology: A Comprehensive Desk Reference). Briefly, to begin synthesis, a 3' hydroxyl nucleoside with a dimethoxytrityl (DMT) group at the 5' end was attached to a solid support. The DMT group was removed with trichloroacetic acid (TCA) in order to free the 5'-hydroxyl for the coupling reaction. Next, tetrazole and a phosphoramidite derivative of the next nucleotide were added. The tetrazole protonates the nitrogen of the phosphoramidite, making it susceptible to nucleophilic attack. The DMT group at the 5'-end of the hydroxyl group blocks further addition of nucleotides in excess. Next, the inter-nucleotide linkage was converted to a phosphotriester bond in an oxidation step using an oxidizing agent and water as the oxygen donor. Excess nucleotides were filtered out and the cycle for the next nucleotide was started by the removal of the DMT protecting group. Following the synthesis, the oligo was cleaved from the solid support. The oligonucleotides were desalted, resuspended in water at a concentration of 100 or 200 uM, and placed in 96-deep well format. The oligonucleotides were re-arrayed into Whatman Uniplate 384-well polyproylene V bottom plates. The oligonucleotides were diluted to a final concentration 30 μM in 1X Micro Spotting Solution Plus (Telechem/arrayit.com, Sunnyvale, CA) in a total volume of 15 μl. In total, 8,031 oligonucleotides were arrayed into twenty-one 384-well plates.

[0386] Arrays were produced on Telechem/arrayit.com Super amine glass substrates (Telechem/arrayit.com), which were manufactured in 0.1 mm filtered clean room with exact dimensions of 25x76x0.96 mm The arrays were printed using the Virtek Chipwriter with a Telechem 48 pin Micro Spotting Printhead. The Printhead was loaded with 48 Stealth SMP3B TeleChem Micro Spotting Pins, which were used to print oligonucleotides onto the slide with the spot size being 110-115 microns in diameter.

Example 10: Identification of diagnostic nucleotide sets for diagnosis of Cardiac Allograft Rejection

[0387] Genes were identified which have expression patterns useful for the diagnosis and monitoring of cardiac allograft rejection. Further, sets of genes that work together in a diagnostic algorithm for allograft rejection were identified. Patients, patient clinical data and patient samples used in the discovery of markers below were derived from a clinical study described in example 5.

[0388] The collected clinical data is used to define patient or sample groups for correlation of expression data. Patient groups are identified for comparison, for example, a patient group that possesses a useful or interesting clinical distinction, verses a patient group that does not possess the distinction. Measures of cardiac allograft rejection are derived from the clinical data described above to divide patients (and patient samples) into groups with higher and lower rejection activity over some period of time or at any one point in time. Such data are rejection grade as determined from pathologist reading of the cardiac biopsies and data measuring progression of end-organ damage, including depressed left ventricular dysfunction (decreased cardiac output, decreased ejection fraction, clinical signs of low cardiac output) and usage of inotropic agents (Kobashigawa 1998).

[0389] Expression profiles correlating with occurrence of allograft rejection are identified, including expression profiles corresponding to end-organ damage and progression of end-organ damage. Expression profiles are identified predicting allograft rejection, and response to treatment or likelihood of response to treatment. Subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, that have predictive value for the presence of allograft rejection ole prediction of allograft rejection or end organ damage.

[0390] Mononuclear RNA samples were collected from patients who had recently undergone a cardiac allograft transplantation using the protocol described in example 2. The allograft rejection status at the time of sample collection was determined by examination of cardiac biopsies as described in example 5.

[0391] 180 samples were included in the analysis. Each patient sample was associated with a biopsy and clinical data collected at the time of the sample. The cardiac biopsies were graded by a pathologist at the local center and by a centralized pathologist who read the biopsy slides from all four local centers in a blinded manner. Biopsy grades included 0,1A, 1B, 2, 3A, and 3B. No grade 4 rejection was identified. Dependent variables were developed based on these grades using either the local center pathology reading or the higher of the two readings, local or centralized. The dependent variables used for correlation of gene expression profiles with cardiac allograft rejection are shown in Table 4. Dependent variables are used to create classes of samples corresponding to the presence or absence of rejection.

[0392] Clinical data were also used to determine criteria for including samples in the analysis. The strictest inclusion criteria required that samples be from patients who did not have a bacterial or viral infection, were at least two weeks post cardiac transplant and were not currently admitted to the hospital. A second inclusion criteria (inclusion 2) reduced the post-transplant criteria to 1 week and eliminated the hospital admission criteria.

[0393] After preparation of RNA (example 2), amplification, labeling, hybridization, scanning, feature extraction and data processing were done as described in Example 11, using the oligonucleotide microarrays described in Example 9. The resulting log ratio of expression of Cy3 (patient sample)/ Cy5 (R50 reference RNA) was used for analysis. This dataset is called the "static" data. A second type of dataset, referenced, was derived from the first. These datasets compared the gene expression log ratio in each sample to a baseline sample from the same patient using the formula:

$$\text{ref log ratio} = (\text{log ratio}_{sample}) - (\text{log ratio}_{baseline})$$

Two referenced datasets were used, named "0 HG" and "Best 0". The baseline for 0 HG was a Grade 0 sample from the same patient as the sample, using the highest grade between the centralized and local pathologists. The baseline for Best 0 was a Grade 0 sample from the same patient as the sample, using both the local and centralized reader biopsy grade data. When possible a Grade 0 prior to the sample was used as the baseline in both referenced datasets.

[0394] The datasets were also divided into subsets to compare analysis between two subsets of roughly half of the data. The types of subsets constructed were as follows. First half/second half subsets were the first half of the samples and the second half of the samples from a dataset ordered by sample number. Odd/even subsets used the same source, a dataset ordered by sample number, but the odd subset consisted of every 2nd sample starting with the first and the even subset consisted of every 2nd sample starting with the second sample, Center 14/other subsets were the same datasets, divided by transplant hospital. The center 14 subset consisted of all samples from patients at center 14, while the other subset consisted of all samples from the other three centers (12,13, and 15).

[0395] Initially, significance analysis for microarrays (SAM, Tusher 2001, Example 15) was used to discover genes that were differentially expressed between the rejection and no-rejection groups. Ninety-six different combinations of dependent variables, inclusion criteria, static/referenced, and data subsets were used in SAM analysis to develop the primary lists of genes significantly differentially expressed between rejection and no-rejection. The most significant of these genes were chosen based on the following criteria. Tier 1 genes were those which appeared with an FDR of less than 20% in identical analyses in two independent subsets. Tier 2 genes were those which appeared in the top 20 genes on the list with an FDR less than 20% more than 50% of the time over all dependent variables with the inclusion criteria, and static/referenced constant. Tier 3 genes were those that appeared more than 50% of the time with an FDR less than 20% more than 50% of the time over all dependent variables with the inclusion criteria, and static/referenced constant. The genes that were identified by the analysis as statistically differentially expressed between rejection and no rejection are shown in Table 2.

[0396] SAM chooses genes as significantly different based on the magnitude of the difference between the groups and the variation among the samples within each group. An example of the difference between some Grade 0 and some Grade 3A samples for 9 genes is shown in Figure 7A.

[0397] Additionally, many of these same combinations were used in the Supervised Harvesting of Expression Trees (SHET, Hastie et al. 2001) algorithm (see example 15) to identify markers that the algorithm chose as the best to distinguish between the rejection and no rejection classes using a bias factor of 0.01. The top 20 or 30 terms were taken from the SHET output and among all comparisons in either the static or referenced data the results were grouped. Any gene found in the top 5 terms in more than 50% of the analyses was selected to be in group B1 (Table 2). The occurrences of each gene were tabulated over all SHET analysis (for either static or referenced data) and the 10 genes that occurred the most were selected to be in group B2 (Table 2).

[0398] An additional classification method used was CART (Salford Systems, San Diego, example 15). Either the static or referenced dataset was reduced to only the genes for which expression values (log ratios) were present in at least 80% of the samples. These data were used in CART with the default settings, using the Symmetric Gini algorithm. Each of the dependent variables was used with both the full sample set and the strict inclusion criteria. Two groups of genes were identified. Group C1 were those genes that were a primary splitter (1st decision node). Group C2 genes were the 10 genes that occurred as splitters the most often over all these analyses.

[0399] Two other classification models were developed and their best genes identified as markers of cardiac allograft rejection. Group D genes were identified from a set of 59 samples, referenced data, local biopsy reading grade, using logistic regression. Group E genes were identified from the primary static dataset using a K-nearest neighbor classification algorithm.

[0400] Both hierarchical clustering (Eisen et al. 1998) and CART were used to identify surrogates for each identified marker. Hierarchical clustering surrogates are genes co-expressed in these and were chosen from the nearest branches of the dendrogram. CART surrogates were identified by CART as the surrogates for those genes chosen as primary splitters at decision nodes.

[0401] Primers for real-time PCR validation were designed for each of the marker genes as described in Example 13.

[0402] CART was used to build a decision tree for classification of samples as rejection or no-rejection using the gene

expression data from the arrays. The analysis identified sets of genes that can be used together to accurately identify samples derived from cardiac allograft transplant patients. The set of genes and the identified threshold expression levels for the decision tree are referred to as a "models". This model can be used to predict the rejection state of an unknown sample. The input data were the static expression data (log ratio) and the referenced expression data (log ratio referenced to the best available grade 0 from either the centralized reader or the local reader) for 139 of our top marker genes. These two types of expression data were entered into the CART software as independent variables. The dependent variable was rejection state, defined for this model as no rejection = grade 0 and rejection = grade 3A. Samples were eliminated from consideration in the training set if they were from patients with either bacterial or viral infection or were from patients who were less than two weeks post-transplant. The method used was Symmetric Gini, allowing linear combinations of independent variables. The costs were set to 1 for both false negatives and false positives and the priors were set equal for the two states. No penalties were assessed for missing data, however the marker genes selected have strong representation across the dataset. 10-fold cross validation was used to test the model. Settings not specified remained at the default values.

[0403] The model shown in Figure 7B is based on decisions about expression values at three nodes, each a different marker gene. The cost assigned to this model is 0.292, based on the priors being equal, the costs set to 1 for each type of error, and the results from the 10-fold cross validation.

[0404] In the training set, no rejection samples were misclassified (sensitivity =100%) and only 1 no-rejection sample was misclassified (specificity = 94.4%). Following 10-fold cross validation, 2 rejection samples were misclassified (sensitivity = 87.5%) and 3 no-rejection samples were misclassified (specificity = 83.3%). The CART software assigns surrogate markers for each decision node.

[0405] These genes can be used alone or in association with other genes or variables to build a diagnostic gene set or a classification algorithm. These genes can be used in association with known gene markers for rejection (such as those identified in the prior art) to provide a diagnostic algorithm.

Example 11- Amplification, labeling, and hybridization of total RNA to an oligonucleotide microarray

Amplification, labeling, hybridization and scanning

[0406] Samples consisting of at least 0.5 to 2 μg of intact total RNA were further processed for array hybridization. When available, 2 μg of intact total RNA is used for amplification. Amplification and labeling of total RNA samples was performed in three successive enzymatic reactions. First, a single-stranded DNA copy of the RNA was made (hereinafter, "ss-cDNA"). Second, the ss-cDNA was used as a template for the complementary DNA strand, producing double-stranded cDNA (hereinafter, "ds-cDNA, or cDNA"). Third, linear amplification was performed by in vitro transcription from a bacterial T_7 promoter. During this step, fluorescent-conjugated nucleotides were incorporated into the amplified RNA (hereinafter, "aRNA").

[0407] The first strand cDNA was produced using the Invitrogen kit (Superscript II). The first strand cDNA was produced in a reaction composed of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$ (1x First Strand Buffer, Invitrogen), 0.5 mM dGTP, 0.5 mM dATP, 0.5 mM dTTP, 0.5 mM dCTP, 10 mM DTT, 200 U reverse transcriptase (Superscript II, Invitrogen, #18064014), 15 U RNase inhibitor (RNAGuard, Amersham Pharmacia, #27-0815-01), 5 μM T7T24 primer (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGTTTTTTTTTTTTTTTTTTTTT TTT-3'), (SEQ ID NO: 3105) and 0.5 to 2 μg of selected sample total RNA. Several purified, recombinant control mRNAs from the plant Arabidopsis thaliana were added to the reaction mixture: 2-20 pg of the following genes CAB, RCA, LTP4, NAC1, RCP1,XCP2, RBCL, LTP6, TIM, and PRKase (Stratagene, #252201, #252202, #252204, #252208, #252207, #252206, #252203, #252205, #252209, #252210 respectively). The control RNAs allow the estimate of copy numbers for individual mRNAs in the clinical sample because corresponding sense oligonucleotide probes for each of these plant genes are present on the microarray. The final reaction volume of 20 μl was incubated at 42°C for 90 min. For synthesis of the second cDNA strand, DNA polymerase and RNase were added to the previous reaction, bringing the final volume to 150 μl. The previous contents were diluted and new substrates were added to a final concentration of 20 mM Tris-HCl (pH 7.0) (Fisher Scientific, Pittsburgh, PA #BP1756-100), 90 mMKCl (Teknova, Half Moon Bay, CA, #0313-500), 4.6 mM $MgCl_2$ (Teknova, Half Moon Bay, CA, #0304-500), 10 mM$(NH_4)_2SO_4$ (Fisher Scientific #A702-500)(1x Second Strand buffer, Invitrogen), 0.266 mM dGTP, 0.266 mM dATP, 0.266 mM dTTP, 0.266 mM dCTP, 40 U *E. coli* DNA polymerase (Invitrogen, #18010-025), and 2 U RNaseH (Invitrogen, #18021-014). The second strand synthesis took place at 16°C for 150 minutes.

[0408] Following second-strand synthesis, the ds-cDNA was purified from the enzymes, dNTPs, and buffers before proceeding to amplification, using phenol-chloroform extraction followed by ethanol precipitation of the cDNA in the presence of glycogen.

[0409] Alternatively, a silica-gel column is used to purify the cDNA (e.g. Qiaquick PCR cleanup from Qiagen, #28104). The volume of the column purified cDNA was reduced by ethanol precipitation in the presence of glycogen in which the

cDNA was collected by centrifugation at > 10,000 xg for 30 minutes, the supernatant is aspirated, and 150 μl of 70% ethanol, 30% water was added to wash the DNA pellet. Following centrifugation, the supernatant was removed, and residual ethanol was evaporated at room temperature. Alternatively, the volume of the column purified cDNA is reduce in a vacuum evaporator where the supernatant is reduce to a final volume of 7.4 μl.

[0410] Linear amplification of the cDNA was performed by in vitro transcription of the cDNA. The cDNA pellet from the step described above was resuspended in 7.4 μl of water, and in vitro transcription reaction buffer was added to a final volume of 20 μl containing 7.5 mM GTP, 7.5 mM ATP, 7.5 mM TTP, 2.25 mM CTP, 1.025 mM Cy3-conjugated CTP (Perkin Elmer; Boston, MA, #NEL-580), 1x reaction buffer (Ambion, Megascript Kit, Austin, TX and #1334) and 1 % T_7 polymerase enzyme mix (Ambion, Megascript Kit, Austin, TX and #1334). This reaction was incubated at 37°C overnight. Following in vitro transcription, the RNA was purified from the enzyme, buffers, and excess NTPs using the RNeasy kit from Qiagen (Valencia, CA; # 74106) as described in the vendor's protocol. A second elution step was performed and the two eluates were combined for a final volume of 60 μl. RNA is quantified using an Agilent 2100 bioanalyzer with the RNA 6000 nano LabChip.

[0411] Reference RNA was prepared as described above, except Cy5-CTP was incorporated instead of Cy3CTP. Reference RNA from five reactions, each reaction started with 2 ug total RNA, was pooled together and quantitated as described above.

Hybridization to an array

[0412] RNA was prepared for hybridization as follows: for an 18mmx55mm array, 20 μg of amplified RNA (aRNA) was combined with 20 μg of reference aRNA. The combined sample and reference aRNA was concentrated by evaporating the water to 10 μl in a vacuum evaporator. The sample was fragmented by heating the sample at 95°C for 30 minutes to fragment the RNA into 50-200 bp pieces. Alternatively, the combined sample and reference aRNA was concentrated by evaporating the water to 5 μl in a vacuum evaporator. Five μl of 20 mM zinc acetate was added to the aRNA and the mix incubated at 60°C for 10 minutes. Following fragmentation, 40 μl of hybridization buffer was added to achieve final concentrations of 5×SSC and 0.20 %SDS with 0.1 μg/ul of Cot-1 DNA (Invitrogen) as a competitor DNA. The final hybridization mix was heated to 98°C, and then reduced to 50°C at 0.1°C per second.

[0413] Alternatively, formamide is included in the hybridization mixture to lower the hybridization temperature.

[0414] The hybridization mixture was applied to a pre-heated 65°C microarray, surface, covered with a glass coverslip (Corning, #2935-246), and placed on a pre-heated 65°C hybridization chamber (Telechem, AHC-10). 15 ul of 5xSSC was placed in each of the reservoir in the hybridization chamber and the chamber was sealed and placed in a water bath at 62°C for overnight (16-20 hrs). Following incubation, the slides were washed in 2xSSC, 0.1% SDS for five minutes at 30°C, then in 2xSSC for five minutes at 30°C, then in 2xSSC for another five minutes at 30°C, then in 0.2xSSC for two minutes at room temperature. The arrays were spun at 1000×g for 2 minutes to dry them. The dry microarrays are then scanned by methods described above.

[0415] The microarrays were imaged on the Agilent (Palo Alto, CA) scanner G2565AA. The scan settings using the Agilent software were as follows: for the PMT Sensitivity (100% Red and 100% Green); Scan Resolution (10 microns); red and green dye channels; used the default scan region for all slides in the carousel; using the largest scan region; scan date for Instrument ID; and barcode for Slide ID. The full image produced by the Agilent scanner was flipped, rotated, and split into two images (one for each signal channel) using TIFFSplitter (Agilent, Palo Alto, CA). The two channels are the output at 532 nm (Cy3-labeled sample) and 633 nm (Cy5-labeled R50). The individual images were loaded into GenePix 3.0 (Axon Instruments, Union City, CA) for feature extraction, each image was assigned an excitation wavelength corresponding the file opened; Red equals 633 nm and Green equals 532 nm. The setting file (gal) was opened and the grid was laid onto the image so that each spot in the grid overlaped with >50% of the feature. Then the GenePix software was used to find the features without setting minimum threshold value for a feature. For features with low signal intensity, GenePix reports "not found". For all features, the diameter setting was adjusted to include only the feature if necessary.

[0416] The GenePix software determined the median pixel intensity for each feature (F_i) and the median pixel intensity of the local background for each feature (B_i) in both channels. The standard deviation (SDF_i and SDB_i) for each is also determined. Features for which GenePix could not discriminate the feature from the background were "flagged" as described below.

[0417] Following feature extraction into a " .gpr" file, the header information of the .gpr file was changed to carry accurate information into the database. An Excel macro was written to include the following information: Name of the original .tif image file, SlideID, Version of the feature extraction software, GenePix Array List file, GenePix Settings file, ScanID, Name of person who scanned the slide, Green PMT setting, Red PMT setting, ExtractID (date .gpr file was created, formatted as yyyy.mm.dd-hh.mm.ss), Results file name (same as the .gpr file name), StorageCD, and Extraction comments.

Pre-processing with Excel Templates

[0418] Following analysis of the image and extraction of the data, the data from each hybridization was preprocessed to extract data that was entered into the database and subsequently used for analysis. The complete GPR file produced by the feature extraction in GenePix was imported into an excel file pre-processing template or processed using a AWK script. Both programs used the same processing logic and produce identical results. The same excel template or AWK script was used to process each GPR file. The template performs a series of calculations on the data to differentiate poor features from others and to combine duplicate or triplicate feature data into a single data point for each probe.

[0419] The data columns used in the pre-processing were: Oligo ID, F633 Median (median value from all the pixels in the feature for the Cy5 dye), B633 Median (the median value of all the pixels in the local background of the selected feature for Cy5), B633 SD (the standard deviation of the values for the pixels in the local background of the selected feature for Cy5), F532 Median (median value from all the pixels in the feature for the Cy3 dye), B532 Median (the median value of all the pixels in the local background of the selected feature for Cy3), B532 SD (the standard deviation of the values for the pixels in the local background of the selected feature for Cy3), and Flags. The GenePix Flags column contains the flags set during feature extraction. "-75" indicates there were no features printed on the array in that position, "-50" indicates that GenePix could not differentiate the feature signal from the local background, and "-100" indicates that the user marked the feature as bad.

[0420] Once imported, the data associated with features with -75 flags was not used. Then the median of B633 SD and B532 SD were calculated over all features with a flag value of "0". The minimum values of B633 Median and B532 Median were identified, considering only those values associated with a flag value of "0". For each feature, the signal to noise ratio (S/N) was calculated for both dyes by taking the fluorescence signal minus the local background (BGSS) and dividing it by the standard deviation of the local background:

$$S/N = \frac{F_i - B_i}{SDB_i}$$

[0421] If the S/N was less than 3, then an adjusted background-subtracted signal was calculated as the fluorescence minus the minimum local background on the slide. An adjusted S/N was then calculated as the adjusted background subtracted signal divided by the median noise over all features for that channel. If the adjusted S/N was greater than three and the original S/N were less than three, a flag of 25 was set for the Cy5 channel, a flag of 23 was set for the Cy3 channel, and if both met these criteria, then a flag of 28 was set. If both the adjusted S/N and the original S/N were less than three, then a flag of 65 was set for Cy5, 63 set for Cy3, and 68 set if both dye channels had an adjusted S/N less than three. All signal to noise calculations, adjusted background-subtracted signal, and adjusted S/N were calculated for each dye channel. If the BGSS value was greater than or equal to 64000, a flag was set to indicate saturation; 55 for Cy5, 53 for Cy3, 58 for both.

[0422] The BGSS used for further calculations was the original BGSS if the original S/N was greater than or equal to three. If the original S/N ratio was less than three and the adjusted S/N ratio was greater than or equal to three, then the adjusted BGSS was used. If the adjusted S/N ratio was less than three, then the adjusted BGSS was used, but with knowledge of the flag status.

[0423] To facilitate comparison among arrays, the Cy3 and Cy5 data were scaled. The log of the ratio of Green/Red was determined for all features. The median log ratio value for good features (Flags 0, 23, 25, 28, 63) was determined. The feature values were scaled using the following formula: Log_Scaled_Feature_Ratio = Log_Feature_Ratio-Median_Log_Ratio.

[0424] The flag setting for each feature was used to determine the expression ratio for each probe, a choice of one, two or three features. If all features had flag settings in the same category (categories=negatives, 0 to 28, 53-58, and 63-68), then the average of the three scaled, anti log feature ratios was calculated If the three features did not have flags in the same category, then the feature or features with the best quality flags were used (0>25>23>28>55>53>58>65>63>68). Features with negative flags were never used. When the best flags were two or three features in the same category, the anti log average was used. If a single feature had a better flag category than the other two then the anti log of that feature ratio was used.

[0425] Once the probe expression ratios were calculated from the one, two, or three features, the log of the scaled, averaged ratios was taken as described below and stored for use in analyzing the data. Whichever features were used to calculate the probe value, the flag from those features was carried forward and stored as the flag value for that probe. 2 different data sets can be used for analysis. Flagged data uses all values, including those with flags. Filtered data sets are created by removing flagged data from the set before analysis.

Example 12: Real-time PCR validation of array expression results

[0426] Leukocyte microarray gene expression was used to discover expression markers and diagnostic gene sets for clinical outcomes. It is desirable to validate the gene expression results for each gene using a more sensitive and quantitative technology such as real-time PCR. Further, it is possible for the diagnostic nucleotide sets to be implemented as a diagnostic test as a real-time PCR panel. Alternatively, the quantitative information provided by real-time PCR validation can be used to design a diagnostic test using any alternative quantitative or semi-quantitative gene expression technology. To validate the results of the microarray experiments we used real-time, or kinetic, PCR. In this type of experiment the amplification product is measured during the PCR reaction. This enables the researcher to observe the amplification before any reagent becomes rate limiting for amplification. In kinetic PCR the measurement is of C_T (threshold cycle) or C_P (crossing point). This measurement (C_T=C_P) is the point at which an amplification curve crosses a threshold fluorescence value. The threshold is set to a point within the area where all of the reactions were in their linear phase of amplification. When measuring C_T, a lower C_T value is indicative of a higher amount of starting material since an earlier cycle number means the threshold was crossed more quickly.

[0427] Several fluorescence methodologies are available to measure amplification product in real-time PCR. Taqman (Applied BioSystems, Foster City, CA) uses fluorescence resonance energy transfer (FRET) to inhibit signal from a probe until the probe is degraded by the sequence specific binding and Taq 3' exonuclease activity. Molecular Beacons (Stratagene, La Jolla, CA) also use FRET technology, whereby the fluorescence is measured when a hairpin structure is relaxed by the specific probe binding to the amplified DNA. The third commonly used chemistry is Sybr Green, a DNA-binding dye (Molecular Probes, Eugene, OR). The more amplified product that is produced, the higher the signal. The Sybr Green method is sensitive to non-specific amplification products, increasing the importance of primer design and selection. Other detection chemistries can also been used, such as ethedium bromide or other DNA-binding dyes and many modifications of the fluorescent dye/quencher dye Taqman chemistry.

Sample prep and cDNA synthesis

[0428] The inputs for real time PCR reaction are gene-specific primers, cDNA from specific patient samples, and standard reagents. The cDNA was produced from mononuclear RNA (prepared as in example 2) or whole blood RNA by reverse transcription using Oligo dT primers (Invitrogen, 18418-012) and random hexamers (Invitrogen, 48190-011) at a final concentration of 0.5ng/μl and 3ng/μl respectively. For the first strand reaction mix, 0.5 μg of mononuclear total RNA or 2 μg of whole blood RNA and 1 μl of the Oligo dT/ Random Hexamer Mix, were added to water to a final volume of 11.5 μl. The sample mix was then placed at 70°C for 10 minutes. Following the 70°C incubation, the samples were chilled on ice, spun down, and 88.5 μl of first strand buffer mix dispensed into the reaction tube. The final first strand buffer mix produced final concentrations of 1X first strand buffer (Invitrogen, Y00146, Carlsbad, CA), 10 mM DTT (Invitrogen, Y00147), 0.5 mM dATP (NEB, N0440S, Beverly, MA), 0.5 mM dGTP (NEB, N0442S), 0.5mM dTTP (NEB, N0443S), 0.5 mM dCTP (NEB, N0441 S), 200U of reverse transcriptase (Superscript II, Invitrogen, 18064-014), and 18U of RNase inhibitor (RNAGaurd Amersham Pharmacia, 27-0815-01, Piscataway, NJ). The reaction was incubated at 42°C for 90 minutes. After incubation the enzyme was heat inactivated at 70°C for 15 minutes, 2 U of RNAse H added to the reaction tube, and incubated at 37°C for 20 minutes.

PRIMER DESIGN

[0429] Two methods were used to design primers. The first was to use the software, Primer Express™ and recommendations for primer design that are provided with the GeneAmp® 7700 Sequence Detection System supplied by Applied BioSystems (Foster City, CA). The second method used to design primers was the PRIMER3 ver 0.9 program that is available from the Whitehead Research Institute, Cambridge, Massachusetts at the Whitehead Research web site. The program can also be accessed on the World Wide Web at the web site at the Massechusetts Institute of Technology website. Primers and Taqman/hybridization probes were designed as described below using both programs.

[0430] The Primer Express literature explains that primers should be designed with a melting temperature between 58 and 60 degrees C. while the Taqman probes should have a melting temperature of 68 to 70 under the salt conditions of the supplied reagents. The salt concentration is fixed in the software. Primers should be between 15 and 30 basepairs long. The primers should produce and amplicon in size between 50 and 150 base pairs, have a C-G content between 20% and 80%, have no more than 4 identical base pairs next to one another, and no more than 2 C's and G's in the last 5 bases of the 3' end. The probe cannot have a G on the 5' end and the strand with the fewest G's should be used for the probe.

[0431] Primer3 has a large number of parameters. The defaults were used for all except for melting temperature and the optimal size of the amplicon was set at 100 bases. One of the most critical is salt concentration as it affects the melting temperature of the probes and primers. In order to produce primers and probes with melting temperatures

equivalent to Primer Express, a number of primers and probes designed by Primer Express were examined using PRIMER3. Using a salt concentration of 50 mM these primers had an average melting temperature of 3.7 degrees higher than predicted by Primer Express. In order to design primers and probes with equivalent melting temperatures as Primer Express using PRIMER3, a melting temperature of 62.7 plus/minus 1.0 degree was used in PRIMER3 for primers and 72.7 plus/minus 1.0 degrees for probes with a salt concentration of 50 mM.

[0432] The C source code for Primer3 was downloaded and complied on a Sun Enterprise 250 server using the GCC complier. The program was then used from the command line using a input file that contained the sequence for which we wanted to design primers and probes along with the input parameters as described by help files that accompany the software. Using scripting it was possible to input a number of sequences and automatically generate a number of possible probes and primers.

[0433] Primers for β-Actin (Beta Actin, Genbank Locus: NM_001101)and β-GUS: glucuronidase, beta, (GUSB, Genbank Locus: NM_000181), two reference genes, were designed using both methods and are shown here as examples:

The first step was to mask out repetitive sequences found in the mRNA sequences using RepeatMasker program that can be accessed at: the web site University of Washington Genome Repeatmasker website. (Smit, A.F.A. & Green, P.).

[0434] The last 500 basepairs on the last 3' end of masked sequence was then submitted to PRIMER3 using the following exemplary input sequences: PRIMER_SEQUENCE_ID=>ACTB Beta Actin (SEQID 3083)

SEQUENCE=TTGGCTTGACTCAGGATTTAAAAACTGGAACGGTGAAGGTGACAGCAGTCGGTTGGACGA
GCATCCCCCAAAGTTCACAATGTGGCCGAGGACTTTGATTGCACATTGTTGTTTTTTAATAGTCATTCC
AAATATGAGATGCATTGTTACAGGAAGTCCCTTGCCATCCTAAAAGCACCCCACTTCTCTCTAAGGAGA
ATGGCCCAGTCCTCTCCCAAGTCCACACAGGGGAGGGATAGCATTGCTTTCGTGTAAATTATGTAATGC
AAAATTTTTTTAATCTTCGCCTTAATCTTTTTTATTTTGTTTTATTTTGAATGATGAGCCTTCGTGCCC
CCCCTTCCCCCTTTTTTCCCCCAACTTGAGATGTATGAAGGCTTTTGGTCTCCCTGGGAGTGGGTGGAG
GCAGCCGGGCTTACCTGTACACTGACTTGAGACCAGTTGAATAAAAGTGCACACCTTA

PRIMER_SEQUENCE_ID=>GUSB (SEQID 3084)

SEQUENCE=GAAGAGTACCAGAAAAGTCTGCTAGAGCAGTACCATCTGGGTCTGGATCAAAAACGCAGA
AAATATGTGGTTGGAGAGCTCATTTGGAATTTTGCCGATTTCATGACTGAACAGTCACCGACGAGAGTG
CTGGGGAATAAAAAGGGGATCTTCACTCGGCAGAGACAACCAAAAAGTGCAGCGTTCCTTTTGCGAGAG
AGATACTGGAAGATTGCCAATGAAACCAGGTATCCCCACTCAGTAGCCAAGTCACAATGTTTGGAAAAC
AGCCCGTTTACTTGAGCAAGACTGATACCACCTGCGTGTCCCTTCCTCCCCGAGTCAGGGCGACTTCCA
CAGCAGCAGAACAAGTGCCTCCTGGACTGTTCACGGCAGACCAGAACGTTTCTGGCCTGGGTTTTGTGG
TCATCTATTCTAGCAGGGAACACTAAAGGTGGAAATAAAAGATTTTCTATTATGGAAATAAAGAGTTGG
CATGAAAGTCGCTACTG

[0435] After running PRIMER3, 100 sets of primers and probes were generated for ACTB and GUSB. From this set, nested primers were chosen based on whether both left primers could be paired with both right primers and a single Taqman probe could be used on an insert of the correct size. With more experience we have decided not use the mix and match approach to primer selection and just use several of the top pairs of predicted primers.

[0436] For ACTB this turned out to be:

Forward 75 CACAATGTGGCCGAGGACTT(SEQID 3085),
Forward 80 TGTGGCCGAGGACTTTGATT(SEQID 3086),
Reverse 178 TGGCTTTTAGGATGGCAAGG(SEQID 3087), and
Reverse 168 GGGGGCTTAGTTTGCTTCCT(SEQID 3088).

[0437] Upon testing, the F75 and R178 pair worked best.

[0438] For GUSB the following primers were chosen:

Forward 59 AAGTGCAGCGTTCCTTTTGC(SEQID 3089),
Forward 65 AGCGTTCCTTTTGCGAGAGA (SEQID 3090),
Reverse 158 CGGGCTGTTTTCCAAACATT (SEQID 3091), and

Reverse 197 GAAGGGACACGCAGGTGGTA (SEQID 3092).
No combination of these GUSB pairs worked well.

[0439] In addition to the primer pairs above, Primer Express predicted the following primers for GUSB: Forward 178 TACCACCTGCGTGTCCCTTC (SEQID 3093) and Reverse 242 GAGGCACTTGTTCTGCTGCTG (SEQID 3094). This pair of primers worked to amplify the GUSB mRNA.

[0440] The parameters used to predict these primers in Primer Express were:

Primer Tm: min 58, Max=60, opt 59, max difference=2 degrees
Primer GC: min=20% Max =80% no 3' G/C clamp
Primer: Length: min=9 max=40 opt=20
Amplicon: min Tm=0 max Tm=85
min = 50 bp max = 150 bp
Probe: Tm 10 degrees > primers, do not begin with a G on 5' end
Other: max base pair repeat = 3
max number of ambiguous residues = 0
secondary structure: max consecutive bp = 4, max total bp = 8
Uniqueness: max consecutive match = 9
max % match = 75
max 3' consecutive match = 7

[0441] Granzyme B is a marker of transplant rejection.

[0442] For Granzyme B the following sequence (NM_004131) (SEQID 3096) was used as input for Primer3 :

GGGGACTCTGGAGGCCCTCTTGTGTGTAACAAGGTGGCCCAGGGCATTGT
CTCCTATGGACGAAACAATGGCATGCCTCCACGAGCCTGCACCAAAGTCT
CAAGCTTTGTACACTGGATAAAGAAAACCATGAAACGCTACTAACTACAG
GAAGCAAACTAAGCCCCCGCTGTAATGAAACACCTTCTCTGGAGCCAAGT
CCAGATTTACACTGGGAGAGGTGCCAGCAACTGAATAAATACCTCTCCCA
GTGTAAATCTGGAGCCAAGTCCAGATTTACACTGGGAGAGGTGCCAGCAA
CTGAATAAATACCTCTTAGCTGAGTGG

[0443] For Granzyme B the following primers were chosen for testing:

Forward 81 ACGAGCCTGCACCAAAGTCT (SEQID 3097)
Forward 63 AAACAATGGCATGCCTCCAC (SEQID 3098)
Reverse 178 TCATTACAGCGGGGGCTTAG (SEQID 3099)
Reverse 168 GGGGGCTTAGTTTGCTTCCT (SEQID 3100)

[0444] Testing demonstrated that F81 and R178 worked well.

[0445] Using this approach, primers were designed for all the genes that were shown to have expression patterns that correlated with allograft rejection. These primer pairs are shown in Table 2, Table 8, and are added to the sequence listing. Primers can be designed from any region of a target gene using this approach.

PRIMER ENDPOINT TESTING

[0446] Primers were first tested to examine whether they would produce the correct size product without non-specific amplification. The standard real-time PCR protocol was used without the Rox and Sybr green dyes. Each primer pair was tested on cDNA made from universal mononuclear leukocyte reference RNA that was produced from 50 individuals as described in Example 3 (R50).

[0447] The PCR reaction consisted of 1X RealTime PCR Buffer (Ambion, Austin, TX), 2mM MgCl2 (Applied BioSystems, B02953), 0.2mM dATP (NEB), 0.2mM dTTP (NEB), 0.2mM dCTP (NEB), 0.2mM dGTP (NEB), .625U AmpliTaq Gold (Applied BioSystems, Foster City, CA), 0.3μM of each primer to be used (Sigma Genosys, The Woodlands, TX), 5μl of the R50 reverse-transcription reaction and water to a final volume of 19μl.

[0448] Following 40 cycles of PCR, 10 microliters of each product was combined with Sybr green at a final dilution of 1:72,000. Melt curves for each PCR product were determined on an ABI 7900 (Applied BioSystems, Foster City, CA), and primer pairs yielding a product with one clean peak were chosen for further analysis. One microliter of the product

from these primer pairs was examined by agarose gel electrophoresis on an Agilent Bioanalyzer, DNA1000 chip (Palo Alto, CA). Results for 2 genes are shown in Figure 9. From the primer design and the sequence of the target gene, one can calculate the expected size of the amplified DNA product. Only primer pairs with amplification of the desired product and minimal amplification of contaminants were used for real-time PCR. Primers that produced multiple products of different sizes are likely not specific for the gene of interest and may amplify multiple genes or chromosomal loci.

PRIMER OPTIMIZATION/EFFICIENCY

[0449] Once primers passed the end-point PCR, the primers were tested to determine the efficiency of the reaction in a real-time PCR reaction. cDNA was synthesized from starting total RNA as described above. A set of 5 serial dilutions of the R50 reverse-transcribed cDNA (as described above) were made in water: 1:10, 1:20, 1:40, 1:80, and 1:160.

[0450] The Sybr Green real-time PCR reaction was performed using the Taqman PCR Reagent kit (Applied BioSystems, Foster City, CA, N808-0228). A master mix was made that consisted of all reagents except the primes and template. The final concentration of all ingredients in the reaction was 1X Taqman Buffer A (Applied BioSystems), 2mM MgCl2 (Applied BioSystems), 200μM dATP (Applied BioSystems), 200μM dCTP (Applied BioSystems), 200μM dGTP (Applied BioSystems), 400μM dUTP (Applied BioSystems), 1:400,000 diluted Sybr Green dye (Molecular Probes), 1.25U AmpliTaq Gold (Applied BioSystems). The PCR master mix was dispensed into two, light-tight tubes. Each β-Actin primer F75 and R178 (Sigma-Genosys, The Woodlands, TX), was added to one tube of PCR master mix and Each β-GUS primer F178 and R242 (Sigma-Genosys), was added to the other tube of PCR master mix to a final primer concentration of 300nM. 45μl of the β-Actin or β-GUS master mix was dispensed into wells, in a 96-well plate (Applied BioSystems). 5μl of the template dilution series was dispensed into triplicate wells for each primer. The reaction was run on an ABI 7900 Sequence Detection System (Applied BioSystems) with the following conditions: 10 min. at 95°C; 40 cycles of 95°C for 15 sec, 60°C for 1 min; followed by a disassociation curve starting at 50°C and ending at 95°C.

[0451] The Sequence Detection System v2.0 software was used to analyze the fluorescent signal from each well. The high end of the baseline was adjusted to between 8 and 20 cycles to reduce the impact on any data curves, yet be as high as possible to reduce baseline drift. A threshold value was selected that allowed the majority of the amplification curves to cross the threshold during the linear phase of amplification. The disassociation curve for each well was compared to other wells for that marker. This comparison allowed identification of "bad" wells, those that did not amplify, that amplified the wrong size product, or that amplified multiple products. The cycle number at which each amplification curve crossed the threshold (C_T) was recorded and the file transferred to MS Excel for further analysis. The C_T values for triplicate wells were averaged. The data were plotted as a function of the $\log_{10}$ of the calculated starting concentration of RNA. The starting RNA concentration for each cDNA dilution was determined based on the original amount of RNA used in the RT reaction, the dilution of the RT reaction, and the amount used (5 μl) in the real-time PCR reaction. For each gene, a linear regression line was plotted through all of the dilutions series points. The slope of the line was used to calculate the efficiency of the reaction for each primer set using the equation:

$$\mathbf{E = 10^{\left(-1/slope\right)} - 1}$$

Using this equation (Pfaffl 2001, Applied Biosystems User Bulletin #2), the efficiency for these β-actin primers is 1.28 and the efficiency for these β-GUS primers is 1.14 (Figure 10). This efficiency was used when comparing the expression levels among multiple genes and multiple samples. This same method was used to calculate reaction efficiency for primer pairs for each gene studied. A primer pair was considered successful if the efficiency was reproducibly determined to be between 0.7 and 2.4.

SYBR-GREEN ASSAYS

[0452] Once markers passed the Primer Efficiency QPCR (as stated above), they were used in real-time PCR assays. Patient RNA samples were reverse-transcribed to cDNA (as described above) and 1:10 dilutions made in water. In addition to the patient samples, a no template control (NTC) and a pooled reference RNA (see example 3) described in were included on every plate.

[0453] The Sybr Green real-time PCR reaction was performed using the Taqman Core PCR Reagent kit (Applied BioSystems, Foster City, CA, N808-0228). A master mix was made that consisted of all reagents except the primers and template. The final concentration of all ingredients in the reaction was 1X Taqman Buffer A (Applied BioSystems), 2mM MgCl2 (Applied BioSystems), 200μM dATP (Applied BioSystems), 200μM dCTP (Applied BioSystems), 200μM dGTP (Applied BioSystems), 400μM dUTP (Applied BioSystems), 1:400,000 diluted Sybr Green dye (Molecular Probes), 1.25U AmpliTaq Gold (Applied BioSystems). The PCR master mix was aliquotted into eight light-tight tubes, one for

each marker to be examined across a set of samples. The optimized primer pair for each marker was then added to the PCR master mix to a final primer concentration of 300nM. 18μl of the each marker master mix was dispensed into wells in a 384well plate (Applied BioSystems). 2μl of the 1:10 0 diluted control or patient cDNA sample was dispensed into triplicate wells for each primer pair. The reaction was run on an ABI 7900 Sequence Detection System (Applied BioSystems) using the cycling conditions described above.

[0454] The Sequence Detection System v2.0 software (Applied BioSystems) was used to analyze the fluorescent signal from each well. The high end of the baseline was adjusted to between 8 and 20 cycles to reduce the impact on any data curves, yet be as high as possible to reduce baseline drift. A threshold value was selected that allowed the majority of the amplification curves to cross the threshold during the linear phase of amplification. The disassociation curve for each well was compared to other wells for that marker. This comparison allowed identification of "bad" wells, those that did not amplify, that amplified the wrong size product, or that amplified multiple products. The cycle number at which each amplification curve crossed the threshold (C_T) was recorded and the file transferred to MS Excel for further analysis. The C_T value representing any well identified as bad by analysis of disassociation curves was deleted. The C_T values for triplicate wells were averaged. A standard deviation (Stdev) and a coefficient of variation (CV) were calculated for the triplicate wells. If the CV was greater than 2, an outlier among the three wells was identified and deleted. Then the average was re-calculated. In each plate, ΔC_T was calculated for each marker-control combination by subtracting the average C_T of the target marker from the average C_T of the control (β-Actin or β-GUS). The expression relative to the control marker was calculated by taking two to the power of the ΔC_T of the target marker. For example, expression relative to β-Actin was calculated by the equation:

$$ErA = 2^{(C_{T,Actin} - C_{T,Target})}$$

All plates were run in duplicate and analyzed in the same manner. The percent variation was determined for each sample-marker combination (relative expression) by taking the absolute value of the value of the RE for the second plate from the RE for the first plate, and dividing that by the average. If more than 25% of the variation calculations on a plate are greater than 50%, then a third plate was run.

TAQMAN PROTOCOL

[0455] Real-time PCR assays were also done using Taqman PCR chemistry.

The Taqman real-time PCR reaction was performed using the Taqman Universal PCR Master Mix (Applied BioSystems, Foster City, CA, #4324018). The master mix was aliquoted into eight, light-tight tubes, one for each marker. The optimized primer pair for each marker was then added to the correctly labeled tube of PCR master mix. A FAM/TAMRA dual-labeled Taqman probe (Biosearch Technologies, Navoto, CA, DLO-FT-2) was then added to the correctly labeled tube of PCR master mix. Alternatively, different combinations of fluorescent reporter dyes and quenchers can be used such that the absorption wavelength for the quencher matches the emission wavelength for the reporter, as shown in Table 5. 18μl of the each marker master mix was dispensed into a 384well plate (Applied BioSystems). 2μl of the template sample was dispensed into triplicate wells for each primer pair. The final concentration of each reagent was: 1X TaqMan Universal PCR Master Mix, 300nM each primer, 0.25nM probe, 2μl 1:10 diluted template. The reaction was run on an ABI 7900 Sequence Detection System (Applied Biosystems) using standard conditions (95°C for 10 min., 40 cycles of 95°C for 15 sec, 60°C for 1 min.).

[0456] The Sequence Detector v2.0 software (Applied BioSystems) was used to analyze the fluorescent signal from each well. The high end of the baseline was adjusted to between 8 and 20 cycles to reduce the impact on any data curves, yet be as high as possible to reduce baseline drift. A threshold value was selected that allowed most of the amplification curves to cross the threshold during the linear phase of amplification. The cycle number at which each amplification curve crossed the threshold (C_T) was recorded and the file transferred to MS Excel for further analysis. The C_T values for triplicate wells were averaged. The C_T values for triplicate wells were averaged. A standard deviation (Stdev) and a coefficient of variation (CV) were calculated for the triplicate wells. If the CV was greater than 2, an outlier among the three wells was identified and deleted. Then the average was re-calculated. In each plate, ΔC_T was calculated for each marker-control combination by subtracting the average C_T of the target marker from the average C_T of the control (β-Actin or β-GUS). The expression relative to the control marker was calculated by taking two to the power of the ΔC_T of the target marker. All plates were run in duplicate and analyzed in the same manner. The percent variation was determined for each sample-marker combination (relative expression) by taking the absolute value of the value of the RE for the second plate from the RE for the first plate, and dividing that by the average. If more than 25% of the variation calculations on a plate are greater than 50%, then a third plate was run.

BI-PLEXING

[0457] Variation of real-time PCR assays can arise from unequal amounts of RNA starting material between reactions. In some assays, to reduce variation, the control gene amplification was included in the same reaction well as the target gene. To differentiate the signal from the two genes, different fluorescent dyes were used for the control gene. β-Actin was used as the control gene and the TaqMan probe used was labeled with the fluorescent dye VIC and the quencher TAMRA (Biosearch Technologies, Navoto, CA, DLO-FT-2). Alternatively, other combinations of fluorescent reporter dyes and quenchers (Table 5) can be used as long as the emission wavelength of the reporter for the control gene is sufficiently different from the wavelength of the reporter dye used for the target. The control gene primers and probe were used at limiting concentrations in the reaction (150 nM primers and 0.125 nM probe) to ensure that there were enough reagents to amplify the target marker. The plates were run under the same protocol and the data are analyzed in the same way, but with a separate baseline and threshold for the VIC signal. Outliers were removed as above from both the FAM and VIC signal channels. The expression relative to control was calculated as above, using the VIC signal from the control gene.

ABSOLUTE OUANTITATION

[0458] Instead of calculating the expression relative to a reference marker, an absolute quantitation can be performed using real-time PCR. To determine the absolute quantity of each marker, a standard curve is constructed using serial dilutions from a known amount of template for each marker on the plate. The standard curve may be made using cloned genes purified from bacteria or using synthetic complimentary oligonucleotides. In either case, a dilution series that covers the expected range of expression is used as template in a series of wells in the plate. From the average C_T values for these known amounts of template a standard curve can be plotted. From this curve the C_T values for the unknowns are used to identify the starting concentration of cDNA. These absolute quantities can be compared between disease classes (i.e. rejection vs. no-rejection) or can be taken as expression relative to a control gene to correct for variation among samples in sample collection, RNA purification and quantification, cDNA synthesis, and the PCR amplification.

CELL TYPE SPECIFIC EXPRESSION

[0459] Some markers are expressed only in specific types of cells. These markers may be useful markers for differentiation of rejection samples from no-rejection samples or may be used to identify differential expression of other markers in a single cell type. A specific marker for cytotoxic T-lymphocytes (such as CD8) can be used to identify differences in cell proportions in the sample. Other markers that are known to be expressed in this cell type can be compared to the level of CD8 to indicate differential gene expression within CD8 T-cells.

Control genes for PCR

[0460] As discussed above, PCR expression measurements can be made as either absolute quantification of gene expression using a standard curve or relative expression of a gene of interest compared to a control gene. In the latter case, the gene of interest and the control gene are measured in the same sample. This can be done in separate reactions or in the same reaction (biplex format, see above). In either case, the final measurement for expression of a gene is expressed as a ratio of gene expression to control gene expression. It is important for a control gene to be constitutively expressed in the target tissue of interest and have minimal variation in expression on a per cell basis between individuals or between samples derived from an individual. If the gene has this type of expression behavior, the relative expression ratio will help correct for variability in the amount of sample RNA used in an assay. In addition, an ideal control gene has a high level of expression in the sample of interest compared to the genes being assayed. This is important if the gene of interest and control gene are used in a biplex format. The assay is set up so that the control gene reaches its threshold Ct value early and its amplification is limited by primers so that it does not compete for limiting reagents with the gene of interest.

[0461] To identify an ideal control gene for an assay, a number of genes were tested for variability between samples and expression in both mononuclear RNA samples and whole blood RNA samples using the RNA procurement and preparation methods and real-time PCR assays described above. 6 whole-blood and 6 mononuclear RNA samples from transplant recipients were tested. The intensity levels and variability of each gene in duplicate experiments on both sample types are shown in Figure 11.

[0462] Based on criteria of low variability and high expression across samples, β-actin, 18s, GAPDH, b2microglobulin were found to be good examples of control genes for the PAX samples. A single control gene may be incorporated as an internal biplex control is assays.

Controlling for variation in real time PCR

[0463] Due to differences in reagents, experimenters, and preparation methods, and the variability of pipetting steps, there is significant plate-to-plate variation in real-time PCR experiments. This variation can be reduced by automation (to reduce variability and error), reagent lot quality control, and optimal data handling. However, the results on replicate plates are still likely to be different since they are run in the machine at different times.

[0464] Variation can also enter in data extraction and analysis. Real-time PCR results are measured as the time (measured in PCR cycles) at which the fluorescence intensity (□Rn in Applied Biosystems SDS v2.1 software) crosses a user-determined threshold (CT). When performing relative quantification, the CT value for the target gene is subtracted from the CT value for a control gene. This difference, called ACT, is the value compared among experiments to determine whether there is a difference between samples. Variation in setting the threshold can introduce additional error. This is especially true in the duplexed experimental format, where both the target gene and the control gene are measured in the same reaction tube. Duplexing is performed using dyes specific to each of the two genes. Since two different fluorescent dyes are used on the plate, two different thresholds are set. Both of these thresholds contribute to each ACT. Slight differences in the each dye's threshold settings (relative to the other dye) from one plate to the next can have significant effects on the ACT.

[0465] There are several methods for setting the threshold for a PCR plate. Older versions of SDS software (Applied Biosystems) determine the average baseline fluorescence for the plate and the standard deviation of the baseline. The threshold is set to 10x the standard deviation of the baseline. In SDS 2.0 the users must set the baseline by themselves. Software from other machine manufacturers either requires the user to set the threshold themselves or uses different algorithms. The latest version of the SDS software (SDS 2.1) contains Automatic baseline and threshold setting. The software sets the baseline separately for each well on the plate using the ΔRn at cycles preceding detectable levels. Variability among plates is dependent on reproducible threshold setting. This requires a mathematical or experimental data driven threshold setting protocol. Reproducibly setting the threshold according to a standard formula will minimize variation that might be introduced in the threshold setting process. Additionally, there may be experimental variation among plates that can be reduced by setting the threshold to a component of the data. We have developed a system that uses a set of reactions on each plate that are called the threshold calibrator (TCb). The TCb wells are used to set the threshold on all plates.

1. The TCb wells contain a template, primers, and probes that are common among all plates within an experiment.
2. The threshold is set within the minimum threshold and maximum threshold determined above.
3. The threshold is set to a value in this range that results in the average CT value for the TCb wells to be the same on all plates.

[0466] These methods were used to derive the primers depicted in Table 2C.

Example 13: Real-time PCR expression markers of acute allograft rejection

[0467] In examples 14 and 16, genes were identified as useful markers of cardiac and renal allograft rejection using microarrays. Some genes identified through these studies are listed in Table 2. In order to validate these findings, obtain a more precise measurement of expression levels and develop PCR reagents for diagnostic testing, real-time PCR assays were performed on samples from allograft recipients using primers to the identified genes. Some gene specific PCR primers were developed and tested for all genes in Table 2A as described in example 12. Some primers are listed in Table 2C and the sequence listing. These primers were used to measure expression of the genes relative to β-actin or β-gus in 69 mononuclear RNA samples obtained from cardiac allograft recipients using Sybr green real-time PCR assays as described in example 12. Each sample was associated with an ISHLT cardiac rejection biopsy grade. The samples were tested in 2 phases. In phase I, 14 Grade 0, 1 Grade 1A, 3 Grade 2 and 9 Grade 3A samples were tested. In phase II, 19 Grade 2, 4 Grade 1B, 4 Grade 2 and 15 Grade 3A samples were tested. Data was analyzed for each phase individually and for the combined phase I + II sample set. These data are summarized in Table 6.

[0468] The average fold change in expression between rejection (3A) and no rejection (0) samples was calculated. A t-test was done to determine the significance with which each gene was differentially expressed between rejection and no rejection and a p-value was calculated. Genes with high average fold changes and low p-values are considered best candidates for further development as rejection markers. However, it is important to note that a gene with a low average fold change and a high p-value may still be a useful marker for rejection in some patients and may work as part of a gene expression panel to diagnose rejection. These same PCR data were used to create PCR gene expression panels for diagnosis of acute rejection as discussed in example 17.

[0469] Non-parametric tests such as the Fisher Exact Test and Mann-Whitney U test are useful for choosing useful markers. They assess the ability of markers to discrininate between different classes as well as their significance. For

example, one could use the median of all samples (including both non-rejector and rejector samples) as a threshold and apply the Fisher Exact test to the numbers of rejectors and non-rejectors above and below the threshold.

[0470] These methods were used to generate the data in Table 2D.

Example 14: Identification of diagnostic nucleotide sets for diagnosis of Cardiac Allograft Rejection using microarrays

[0471] Genes were identified which have expression patterns useful for the diagnosis and monitoring of acute cardiac allograft rejection. Further, sets of genes that work together in a diagnostic algorithm for allograft rejection were identified. Acute allograft rejection is a process that occurs in all solid organ transplantation including, heart, lung, liver, kidney, pancreas, pancreatic islet cell, intestine and others. Gene expression markers of acute cardiac rejection may be useful for diagnosis and monitoring of all allograft recipients. Patients, patient clinical data and patient samples used in the discovery of markers below were derived from a clinical study described in example 5.

[0472] The collected clinical data was used to define patient or sample groups for correlation of expression data. Patient groups were identified for comparison. For example, a patient group that possesses a useful or interesting clinical distinction, verses a patient group that does not possess the distinction. Measures of cardiac allograft rejection were derived from the clinical data to divide patients (and patient samples) into groups with higher and lower rejection activity over some period of time or at any one point in time. Such data were rejection grades as determined from histological reading of the cardiac biopsy specimens by a pathologist and data measuring progression of end-organ damage, including depressed left ventricular dysfunction (decreased cardiac output, decreased ejection fraction, clinical signs of low cardiac output) and usage of inotropic agents (Kobashigawa 1998). Mononuclear RNA samples were collected and prepared from patients who had recently undergone a cardiac allograft transplantation using the protocol described in example 2. The allograft rejection status at the time of sample collection was determined by examination of cardiac biopsies as described in example 5 and as summarized here.

[0473] 300 patient samples were included in the analysis. Each patient sample was associated with a biopsy and other clinical data collected at the time of the sample. The cardiac biopsies were graded by a pathologist at the local center and by three centralized pathologists who read the biopsy slides from all four local centers in a blinded manner. Biopsy grades included 0, 1A, 1B, 2, 3A, and 3B. No grade 4 rejection was identified. Dependent variables were developed based on these grades using the local center pathology reading, the reading of a centralized and blinded pathologist, the highest of the readings, local or centralized and a consensus grade derived from all pathological readings. Samples were classified as no rejection or rejection in the following ways: Grade 0 vs. Grades 1-4, Grades 0 and 1A vs. Grades 1B-4, Grade 0 vs. Grade 3A, Grade 0 vs. Grades 1B-4, and Grade 0 vs. Grades 1B and 3A-4. Grade 0 samples were selected such that they were not immediately followed by an episode of acute rejection in the same patient. Comparing Grade 0 samples to Grade 3A samples gives the greatest difference between the rejection and no rejection groups on average.

[0474] Taking the highest of all pathologist readings has the effect of removing any sample from the no rejection class that was not a unanimous Grade 0. It also results in an increase in the number of rejection samples used in an analysis with the assumption that if a pathologist saw features of rejection, the call was likely correct and the other pathologists may have missed the finding. Many leading cardiac pathologists and clinicians believe that ISHLT grade 2 rejection does not represent significant acute rejection. Thus, for correlation analysis, exclusion of Grade 2 samples may be warranted. Clinical data were also used to determine criteria for including samples in the analysis. For example, a patient with an active infection or in the early post-transplant period (ongoing surgical inflammation) might have immune activation unrelated to rejection and thus be difficult to identify as patients without rejection. The strictest inclusion criteria required that samples be from patients who did not have a bacterial or viral infection, were at least two weeks post cardiac transplant, were asymptomatic and were not currently admitted to the hospital.

[0475] After preparation of RNA (example 2), amplification, labeling, hybridization, scanning, feature extraction and data processing were done as described in Example 11, using the oligonucleotide microarrays described in Example 9. The resulting log ratio of expression of Cy3 (patient sample)/ Cy5 (R50 reference RNA) was used for analysis.

[0476] Significance analysis for microarrays (SAM, Tusher 2001, Example 15) was used to discover genes that were differentially expressed between the rejection and no-rejection groups. Many different combinations of dependent variables, inclusion criteria, static/referenced, and data subsets were used in SAM analysis to develop the primary lists of genes significantly differentially expressed between rejection and no-rejection. As described in example 15, SAM assigns a false detection rate to each gene identified as differentially expressed. The most significant of these genes were identified.

[0477] An exemplary analysis was the comparison of Grade 0 samples to Grade 3A-4 samples using SAM. Data from the all the pathological readings was used to identify consensus Grade 0 samples and samples with at least one reading of Grade 3A or above. Using this definition of rejection and no rejection, expression profiles from rejection samples were compared to no rejection samples using SAM. The analysis identified 7 genes with a FDR of 1%, 15 genes @ 1.4%, 35 genes @ 3.9%. Many more genes were identified at higher FDR levels.

[0478] In Table 7, a number of SAM analyses are summarized. In each case the highest grade from the 3 pathologists was taken for analysis. No rejection and rejection classes are defined. Samples are either used regardless of redundancy with respect to patients or a requirement is made that only one sample is used per patient or per patient per class. The number of samples used in the analysis is given and the lowest FDR achieved is noted.

[0479] Some of the genes identified by SAM as candidate rejection markers are noted in Table 2A and B. SAM chooses genes as significantly different based on the magnitude of the difference between the groups and the variation among the samples within each group. It is important to note that a gene which is not identified by SAM as differentially expressed between rejection and no rejection may still be a useful rejection marker because: 1. The microarray technology is not adequately sensitive to detect all genes expressed at low levels. 2. A gene might be a useful member of a gene expression panel in that it is a useful rejection marker only in a subset of patients. This gene may not be significantly differentially expressed between all rejection and no rejection samples.

[0480] For the purposes of cross-validation of the results, the datasets were also divided into subsets to compare analysis between two subsets of roughly half of the data. The types of subsets constructed were as follows. First half/second half subsets were the first half of the samples and the second half of the samples from a dataset ordered by sample number. Odd/even subsets used the same source, a dataset ordered by sample number, but the odd subset consisted of every 2^{nd} sample starting with the first and the even subset consisted of every 2^{nd} sample starting with the second sample, Center 14/other subsets were the same datasets, divided by transplant hospital. The center 14 subset consisted of all samples from patients at center 14, while the other subset consisted of all samples from the other three centers (12,13, and 15). When a gene was found to be significantly differentially expressed in both sets of data, a higher priority was put on that gene for development of a diagnostic test. This was reflected in a "Array Score" value (Table 2B) that also considered the false detection rate for the gene and the importance of the gene in classification models (see example 17).

[0481] Alternatively one can divide samples into 10 equal parts and do 10-fold cross validation of the results of SAM.

[0482] Microarray data was also used to generate classification models for diagnosis of rejection as described in example 17. Genes identified through classification models as useful in the diagnosis of rejection are noted in in Table 2B in the column "models".

[0483] As genes were identified as useful rejection markers by microarray significance analysis, classification models, PCR analysis, or through searching the prior art, a variety of approaches were employed to discover genes that had similar expression behavior (coexpression) to the gene of interest. If a gene is a useful rejection marker, then a gene that is identified as having similar expression behavior is also likely to be a useful rejection marker. Hierarchical clustering (Eisen et al. 1998, see example 15) was used to identify co-expressed genes for established rejection markers. Genes were identified from the nearest branches of the clustering dendrogram. Gene expression profiles generated from 240 samples derived from transplant recipients were generated as described above. Hierarchical clustering was performed and co-expressed genes of rejection markers were identified. An example is shown in Figure 12. SEQ ID NO:85 was shown to be significantly differentially expressed between rejection and no rejection using both microarrays and PCR. Gene SEQ ID NO:3020 was identified by hierarchical clustering as closely co-expressed with SEQ ID NO:85. In table 2B, genes identified as co-expressed with established markers are identified as such by listing the SEQ ID that they are co-expressed with in the column labeled "clusters".

[0484] Some of the primers for real-time PCR validation were designed for each of the marker genes as described in Example 12 and are listed in Table 2C and the sequence listing. PCR expression measurements using these primers were used to validate array findings, more accurately measure differential gene expression and create PCR gene expression panels for diagnosis of rejection as described in example 17.

[0485] Alternative methods of analyzing the data may involve 1) using the sample channel without normalization by the reference channel, 2) using an intensity-dependent normalization based on the reference which provides a greater correction when the signal in the reference channel is large, 3) using the data without background subtraction or subtracting an empirically derived function of the background intensity rather than the background itself.

[0486] These methods were used to identify genes listed in Table 2B.

Example 15: Correlation and Classification Analysis

[0487] After generation and processing of expression data sets from microarrays as described in Example 11, a log ratio value is used for most subsequent analysis. This is the logarithm of the expression ratio for each gene between sample and universal reference. The processing algorithm assigns a number of flags to data that are of low signal to noise, saturated signal or are in some other way of low or uncertain quality. Correlation analysis can proceed with all the data (including the flagged data) or can be done on filtered data sets where the flagged data is removed from the set. Filtered data should have less variability and noise and may result in more significant or predictive results. Flagged data contains all information available and may allow discovery of genes that are missed with the filtered data set. After filtering the data for quality as described above and in example 11, missing data are common in microarray data sets.

Some algorithms don't require complete data sets and can thus tolerate missing values. Other algorithms are optimal with or require imputed values for missing data. Analysis of data sets with missing values can proceed by filtering all genes from the analysis that have more than 5%, 10%, 20%, 40%, 50%, 60% or other % of values missing across all samples in the analysis. Imputation of data for missing values can be done by a variety of methods such as using the row mean, the column mean, the nearest neighbor or some other calculated number. Except when noted, default settings for filtering and imputation were used to prepare the data for all analytical software packages.

[0488] In addition to expression data, clinical data are included in the analysis. Continuous variables, such as the ejection fraction of the heart measured by echocardiography or the white blood cell count can be used for correlation analysis. Any piece of clinical data collected on study subjects can be used in a correlation or classification analysis. In some cases, it may be desirable to take the logarithm of the values before analysis. These variables can be included in an analysis along with gene expression values, in which case they are treated as another "gene". Sets of markers can be discovered that work to diagnose a patient condition and these can include both genes and clinical parameters. Categorical variables such as male or female can also be used as variables for correlation analysis. For example, the sex of a patient may be an important splitter for a classification tree.

[0489] Clinical data are used as supervising vectors (dependent variables) for the significance or classification analysis of expression data. In this case, clinical data associated with the samples are used to divide samples in to clinically meaningful diagnostic categories for correlation or classification analysis. For example, pathologic specimens from kidney biopsies can be used to divide lupus patients into groups with and without kidney disease. A third or more categories can also be included (for example "unknown" or "not reported"). After generation of expression data and definition of supervising vectors, correlation, significance and classification analysis are used to determine which set of genes and set of genes are most appropriate for diagnosis and classification of patients and patient samples. Two main types of expression data analyses are commonly performed on the expression data with differing results and purposes. The first is significance analyses or analyses of difference. In this case, the goal of the analysis is to identify genes that are differentially expressed between sample groups and to assign a statistical confidence to those genes that are identified. These genes may be markers of the disease process in question and are further studied and developed as diagnostic tools for the indication. The second major type of analysis is classification analysis. While significance analysis identifies individual genes that are differentially expressed between sample groups, classification analysis identifies gene sets and an algorithm for their gene expression values that best distinguish sample (patient) groups. The resulting gene expression panel and algorithm can be used to create and implement a diagnostic test. The set of genes and the algorithm for their use as a diagnostic tool are often referred to herein as a "model". Individual markers can also be used to create a gene expression diagnostic model. However, multiple genes (or gene sets) are often more useful and accurate diagnostic tools.

Significance analysis for microarrays (SAM)

[0490] Significance analysis for microarrays (SAM) (Tusher 2001) is a method through which genes with a correlation between their expression values and the response vector are statistically discovered and assigned a statistical significance. The ratio of false significant to significant genes is the False Discovery Rate (FDR). This means that for each threshold there are some number of genes that are called significant, and the FDR gives a confidence level for this claim. If a gene is called differentially expressed between two classes by SAM, with a FDR of 5%, there is a 95% chance that the gene is actually differentially expressed between the classes. SAM will identify genes that are differentially expressed between the classes. The algorithm selects genes with low variance within a class and large variance between classes. The algorithm may not identify genes that are useful in classification, but are not differentially expressed in many of the samples. For example, a gene that is a useful marker for disease in women and not men, may not be a highly significant marker in a SAM analysis, but may be useful as part of a gene set for diagnosis of a multi-gene algorithm.

[0491] After generation of data from patient samples and definition of categories using clinical data as supervising vectors, SAM is used to detect genes that are likely to be differentially expressed between the groupings. Those genes with the highest significance can be validated by real-time PCR (Example 13) or can be used to build a classification algorithm as described here.

Classification

[0492] Classification algorithms are used to identify sets of genes and formulas for the expression levels of those genes that can be applied as diagnostic and disease monitoring tests. The same classification algorithms can be applied to all types of expression and proteomic data, including microarray and PCR based expression data. Examples of classification models are given in example 17. The discussion below describes the algorithms that were used and how they were used.

[0493] Classification and Regression Trees (CART) is a decision tree classification algorithm (Breiman 1984). From

gene expression and or other data, CART can develop a decision tree for the classification of samples. Each node on the decision tree involves a query about the expression level of one or more genes or variables. Samples that are above the threshold go down one branch of the decision tree and samples that are not go down the other branch. Genes from expression data sets can be selected for classification building with CART by significant differential expression in SAM analysis (or other significance test), identification by supervised tree-harvesting analysis, high fold change between sample groups, or known relevance to classification of the target diseases. In addition, clinical data can be used as independent variables for CART that are of known importance to the clinical question or are found to be significant predictors by multivariate analysis or some other technique. CART identifies predictive variables and their associated decision rules for classification (diagnosis). CART also identifies surrogates for each splitter (genes that are the next best substitute for a useful gene in classification). Analysis is performed in CART by weighting misclassification costs to optimize desired performance of the assay. For example, it may be most important that the sensitivity of a test for a given diagnosis be > 90%. CART models can be built and tested using 10 fold cross-validation or v-fold cross validation (see below). CART works best with a smaller number of variables (5-50). Multiple Additive Regression Trees (Friedman, JH 1999, MART) is similar to CART in that it is a classification algorithm that builds decision trees to distinguish groups. MART builds numerous trees for any classification problem and the resulting model involves a combination of the multiple trees. MART can select variables as it build models and thus can be used on large data sets, such as those derived from an 8000 gene microarray. Because MART uses a combination of many trees and does not take too much information from any one tree, it resists over training. MART identifies a set of genes and an algorithm for their use as a classifier.

[0494] A Nearest Shrunken Centroids Classifier can be applied to microarray or other data sets by the methods described by Tibshirani et al. 2002. This algorithms also identified gene sets for classification and determines their 10 fold cross validation error rates for each class of samples. The algorithm determines the error rates for models of any size, from one gene to all genes in the set. The error rates for either or both sample classes can are minimized when a particular number of genes are used. When this gene number is determined, the algorithm associated with the selected genes can be identified and employed as a classifier on prospective sample.

[0495] For each classification algorithm and for significance analysis, gene sets and diagnostic algorithms that are built are tested by cross validation and prospective validation. Validation of the algorithm by these means yields an estimate of the predictive value of the algorithm on the target population. There are many approaches, including a 10 fold cross validation analysis in which 10% of the training samples are left out of the analysis and the classification algorithm is built with the remaining 90%. The 10% are then used as a test set for the algorithm. The process is repeated 10 times with 10% of the samples being left out as a test set each time. Through this analysis, one can derive a cross validation error which helps estimate the robustness of the algorithm for use on prospective (test) samples. Any % of the samples can be left out for cross validation (v-fold cross validation, LOOCV). When a gene set is established for a diagnosis with an acceptable cross validation error, this set of genes is tested using samples that were not included in the initial analysis (test samples). These samples may be taken from archives generated during the clinical study. Alternatively, a new prospective clinical study can be initiated, where samples are obtained and the gene set is used to predict patient diagnoses.

Example 16: Acute allograft rejection: biopsy tissue gene expression profiling

[0496] Acute allograft rejection involves activation of recipient leukocytes and infiltration into the rejecting organ. For example, CD8 T-cells are activated by CD4 T-cells and enter the allograft where they destroy graft tissue. These activated, graft-associated leukocytes may reside in the graft, die or exit the graft. Upon exiting, the cells can find their way into the urine or blood (in the case of renal allografts), bile or blood (liver allografts) or blood (cardiac allografts). These activated cells have specific gene expression patterns that can be measured using microarrays, PCR or other methods. These gene expression patterns can be measured in the graft tissue (graft associated leukocytes), blood leukocytes, urine leukocytes or stool/biliary leukocytes. Thus graft associated leukocyte gene expression patterns are used to discover markers of activated leukocytes that can be measured outside the graft for diagnostic testing.

[0497] Renal biopsy and cardiac biopsy tissue specimens were obtained for gene expression profiling. The specimens were obtained at the time of allograft biopsy and were preserved by flash freezing in liquid nitrogen using standard approaches or immersion in an RNA stablization reagent as per the manufacturers recommendation (RNAlater, Qiagen, Valencia, CA). Biopsy allograft pathological evaluation was also obtained and samples were classified as having a particular ISHLT rejection grade (for cardiac) or acute rejection, chronic rejection, acute tubular necrosis or no disease (for renal).

[0498] 28 renal biopsy tissue samples were transferred to RLT buffer, homogenized and RNA was prepared using RNeasy preparation kits (Qiagen, Valencia, CA). Average total RNA yield was 1.3 ug. Samples were subjected to on column DNAse digestion. 18 samples were derived from patients with ongoing acute allograft rejection and 10 were from controls with chronic rejection or acute renal failure.

[0499] RNA from the samples was used for amplification, labeling and hybridization to leukocyte arrays (example 11).

Significance analysis for microarrays (SAM, Tusher 2001, Example 15) was used to identify genes that were differentially expressed between the acute rejection samples and controls. Leukocyte markers of acute rejection that are associated with the graft should be genes that are expressed at some level in activated leukocytes. Since leukocytes appear in graft tissue with some frequency with acute rejection, leukocyte genes associate with rejection are identified by SAM as upregulated in acute rejection in this experiment. 35 genes were identified as upregulated in acute rejection by SAM with less than a 5% false detection rate and 139 were detected with < 10.0% FDR. Results of this analysis are shown in Table 8.

[0500] For each of these genes, to 50mer oligonucleotide sequence was used to search NCBI databases including Unigene and OMIM. Genes were identified by sequence analysis to be either known leukocyte specific markers, known leukocyte expressed markers, known not to be leukocyte expressed or expression unknown. This information helped selected candidate leukocyte markers from all upregulated genes. This is necessary because some of the upregulated genes may have been expressed by renal tissue. Those genes that are leukocyte specific or leukocyte expressed were selected for evaluation by PCR in urine and blood samples from patients with and without acute allograft rejection (cardiac and renal). These genes are useful expression markers of acute rejection in allograft tissue specimens and may also be useful gene expression markers for the process in circulating leukocytes, or urine leukocytes. Genes with known leukocyte expression are noted in Table 8. In addition, some of the leukocyte expressed genes from this analysis were selected for PCR validation and development for diagnosis of acute cardiac rejection and are noted in Table 2.

[0501] Five cardiac rejection markers in the peripheral blood were assayed using real-time PCR in renal biopsy specimens. The average fold change for these genes between acute rejection (n = 6) and controls (n = 6) is given below. Work is ongoing to increase the number of samples tested and the significance of the results.

[0502] PCR assays of cardiac rejection peripheral blood markers in renal allograft tissue. R = rejection, NR = No rejection.

Gene	Fold change (R/NR)
Granzyme B	2.16
CD20	1.42
NK cell receptor	1.72
T-box21	1.74
IL4	1.3

Markers of renal rejection that are secreted from cells may be measured in the urine or serum of patients as a diagnostic or screening assay for rejection. Genes with lower molecular weight are most likely to be filtered into the urine to be measured in this way. Standard immunoassays may be used to measure these proteins. In table 8, genes that are known to be secreted are noted.

Example 17: Microarray and PCR gene expression panels for diagnosis and monitoring of acute allograft rejection

Array panels / classification models

[0503] Using the methods of the invention, gene expression panels were discovered for screening and diagnosis of acute allograft rejection. Gene expression panels can be implemented for diagnostic testing using any one of a variety of technologies, including, but not limited to, microarrays and realtime PCR.

[0504] Using peripheral blood mononuclear cell RNA that was collected and prepared from cardiac allograft recipients as described in examples 2 and 5, leukocyte gene expression profiles were generated and analyzed using microarrays as described in examples 11, 13, and 15. 300 samples were analyzed. ISHLT rejection grades were used to divide patients into classes of rejection and no rejection. Multiple Additive Regression Trees (MART, Friedman, JH 1999, example 15) was used to build a gene expression panel and algorithm for the diagnosis of rejection with high sensitivity. Default settings for the implementaion of MART called TreeNet 1.0 (Salford Systems, San Diego, CA) were used except where noted.

[0505] 82 Grade 0 (rejection) samples and 76 Grade 1B-4 (no rejection) samples were divided into training (80% of each class) and testing (20% of each class) sets. A MART algorithm was then developed on the training set to distinguish rejection from no rejection samples using a cost of 1.02:1 for misclassification of rejection as no rejection. The resulting algorithm was then used to classify the test samples. The algorithm correctly classified 51 of 66 (77%) no rejection samples in the training set and 9 of 16 (56%) no rejection samples in the test set. For rejection samples 64 of 64 (100%) were correctly classified in the training set and 12 of 12 were correctly classified in the test set. The algorithm used 37

genes. MART ranks genes by order of importance to the model. In order, the 37 genes were: SEQ IDs: 3058, 3030, 3034, 3069, 3081, 3072, 3041, 3052, 3048, 3045, 3059, 3075, 3024, 279, 3023, 3053, 3022, 3067, 3020, 3047, 3033, 3068, 3060, 3063, 3028, 3032, 3025, 3046, 3065, 3080, 3039, 3055, 49, 3080, 3038, 3071.

[0506] Another MART model was built by excluding samples derived from patients in the first month post transplant and from patients with known CMV infection. 20 Grade 0 (rejection) samples and 25 Grade 1B-4 (no rejection) samples were divided into training (80% of each class) and testing (20% of each class) sets. A MART algorithm was then developed on the training set to distinguish rejection from no rejection samples using default settings. The resulting algorithm was then used to classify the test samples. The algorithm correctly classified 100% of samples of both classes in the training and testing sets. However, this model required 169 genes. The sample analysis was done a second time with the only difference being requirement that all decision trees in the algorithm be composed of two nodes (single decision, "stump model"). In this case 15/16 no rejection samples were correctly identified in the training set and 4/4 no rejection samples were correctly identified in the test set. For the rejection samples, 17/19 were correctly identified in the training set and 5/6 were correctly classified in the test set. This model required 23 genes. In order of importance, they were: SEQ IDs: 3042, 2783, 3076, 3029, 3026, 2751, 3036, 3073, 3035, 3050, 3051, 3027, 3074, 3062, 3044, 3077, 2772,3049,3043,3079,3070,3057,3078.

Real-time PCR panels / classification models

[0507] PCR primers were developed for top rejection markers and used in real-time PCR assays on transplant patient samples as described in examples 12 and 13. This data was used to build PCR gene expression panels for diagnosis of rejection. Using MART (example 15) a 10-fold cross validated model was created to diagnose rejection using 12 no rejection samples (grade 0) and 10 rejection samples (grade 3A). Default settings were used with the exception of assigning a 1.02:1 cost for misclassification of rejection as no rejection and requirement that all decision trees be limited to 2 nodes ("stump model"). 20 genes were used in the model, including: SEQ IDs:101, 3021, 102, 2781, 78, 87, 86, 36, 77, 2766, 3018, 80, 3019, 2752, 79, 99, 3016, 2790, 3020, 3056, 88. The 10-fold cross-validated sensitivity for rejection was 100% and the specificity was 85%. Some PCR primers for the genes are listed in Table 2C and the sequence listing.

[0508] A different analysis of the PCR data was performed using the nearest shrunken centroids classifier (Tibshirani et al. 2002; PAM version 1.01, see example 15). A 10-fold cross validated model was created to diagnose rejection using 13 no rejection samples (grade 0) and 10 rejection samples (grade 3A). Default settings were used with the exception of using a prior probability setting of (0.5, 0.5). The algorithm derives algorithms using any number of the genes. A 3-gene model was highly accurate with a 10 fold cross-validated sensitivity for rejection of 90%, and a specificity of 85%.

[0509] The 3 genes used in this model were: SEQ IDs 2784, 79, and 2794. Some of the PCR primers used are given in Table 2C and the sequence listing. An ROC curve was plotted for the 3-gene model and is shown in Figure 13.

Example 18: Assay sample preparation

[0510] In order to show that XDx's leukocyte-specific markers can be detected in whole blood, we collected whole blood RNA using the PAXgene whole blood collection, stabilization, and RNA isolation kit (PreAnalytix). Varying amounts of the whole blood RNA were used in the initial RT reaction (1, 2, 4, and 8ug), and varying dilutions of the different RT reactions were tested (1:5, 1:10, 1:20, 1:40, 1:80, 1:160). We did real-time PCR assays with primers specific to XDx's markers and showed that we can reliably detect these markers in whole blood.

[0511] Total RNA was prepared from 14 mononuclear samples (CPT, BD) paired with 14 whole blood samples (PAXgene, PreAnalytix) from transplant recipients. cDNA was prepared from each sample using 2ug total RNA as starting material. Resulting cDNA was diluted 1:10 and Sybr green real-time PCR assays were performed.

[0512] For real-time PCR assays, Ct values of 15-30 are desired for each gene. If a gene's Ct value is much above 30, the result may be variable and non-linear. For PAX sample, target RNA will be more dilute than in CPT samples. cDNA dilutions must be appropriate to bring Ct values to less than 30. Ct values for the first 5 genes tested in this way are shown in the table below for both whole blood RNA (PAX) and mononuclear RNA (CPT).

Gene	Ct PAX	Ct CPT
CD20	27.41512	26.70474
4761	28.45656	26.52635
3096	29.09821	27.83281

(continued)

Gene	Ct PAX	Ct CPT
GranzymeB	31.18779	30.56954
IL4	33.11774	34.8002
Actin	19.17622	18.32966
B-GUS	26.89142	26.92735

[0513] With one exception, the genes have higher Ct values in whole blood. Using this protocol, all genes can be detected with Cts <35. For genes found to have Ct values above 30 in target samples, less diluted cDNA may be needed.

Example 19: Allograft rejection diagnostic gene sequence analysis

[0514] Gene products that are secreted from cells or expressed as surface proteins have special diagnostic utility in that an assay may be developed to detect relative quantities of proteins in blood plasma or serum. Secreted proteins may also be detectable in urine, which may be a useful sample for the detection of rejection in renal allograft recipients. Cell surface markers may be detected using antigen specific antibodies in ELISA assays or using flow srting techniques such as FACS.

[0515] Each gene that is found to be differentially regulated in one population of patients has several potential applications. It may be a target for new pharmaceuticals, a diagnostic marker for a condition, a benchmark for titrating drug delivery and clearance, or used in screening small molecules for new therapeutics. Any of these applications may be improved by an understanding of the physiologic function and localization of the gene product in vivo and by relating those functions to known diseases and disorders. Identifying the basic function of each candidate gene helps identify the signaling or metabolic pathways the gene is a part of, leading us to investigate other members of those pathways as potential diagnostic markers or targets of interest to drug developers.

[0516] For each of the markers in table 2, we attempted to identify the basic function and subcellular localization of the gene. These results are summarized in Table 9. In addition to initial DNA sequencing and processing, sequence analysis, and analysis of novel clones, information was obtained from the following public resources: Online Mendelian Inheritance in Man at the NCBI, LocusLink at the NCBI, the SWISS-PROT database, and Protein Reviews on the Web. For each marker represented by a curated reference mRNA from the RefSeq project, the corresponding reference protein accession number is listed. Curated sequences are those that have been manually processed by NCBI staff to represent the best estimate of the mRNA sequence as it is transcribed, based on alignments of draft DNA sequence, predicted initiation, termination and splice sites, and submissions of EST and full-length mRNA sequences from the scientific community.

[0517] These methods were used to derive the data in Table 2E.

Example 20: Detection of proteins expressed by diagnostic gene sequences

[0518] One of ordinary skill in the art is aware of many possible methods of protein detection. The following example illustrates one possible method.

[0519] The designated coding region of the sequence is amplified by PCR with adapter sequences at either end for subcloning. An epitope or other affinity "tag" such as a "His-tag" may be added to facilitate purification and/or detection of the protein. The amplified sequence is inserted into an appropriate expression vector, most typically a shuttle vector which can replicate in either bacteria, most typically E. coli, and the organism/cell of choice for expression such as a yeast or mammalian cell. Such shuttle vectors typically contain origins of replication for bacteria and an antibiotic resistance marker for selection in bacteria, as well as the relevant replication and selection sequences for transformation/transfection into the ultimate expression cell type. In addition, the sequence of interest is inserted into the vector so that the signals necessary for transcription (a promoter) and translation operably linked to the coding region. Said expression could be accomplished in bacteria, fungi, or mammalian cells, or by in vitro translation.

[0520] The expression vector would then typically be used to transform bacteria and clones analyzed to ensure that the proper sequence had been inserted into the expression vector in the productive orientation for expression. Said verified expression vector is then transfected into a host cell and transformants selected by a variety of methods including antibiotic resistance or nutritional complementation of an auxotrophic marker. Said transformed cells are then grown under conditions conducive to expression of the protein of interest, the cells and conditioned media harvested, and the protein of interest isolated from the most enriched source, either the cell pellet or media.

[0521] The protein is then be isolated by standard of chromatographic or other methods, including immunoaffinity

chromatography using the affinity "tag" sequence or other methods, including cell fractionation, ion exchange, size exclusion chromatography, or selective precipitation. The isolated and purified protein is then be used as an antigen to generate specific antibodies. This is accomplished by standard methods including injection into heterologous species with an adjuvant, isolation of monoclonal antibodies from mice, or in vitro selection of antibodies from bacteriophage display antibody libraries. These antibodies are then used to detect the presence of the indicated protein of interest in a complex bodily fluid using standard methods such as ELISA or RIA.

Example 21: Detecting changes in the rate of hematopoiesis

[0522] Gene expression profiling of blood cells from cardiac allograft recipients was done using microarrays and real-time PCR as described in other examples herein.

[0523] Two of the genes in that were most correlated with cardiac transplant acute rejection with both microarrays and PCR were hemoglobin Beta and 2,3 DPGM. These genes are well know to be specific markers of erythrocyte lineages. This correlation was found using both purified peripheral mononuclear cells and whole blood RNA preparations.

[0524] Analysis of the five genes from the PCR data most strongly correlated with rejection showed that their expression levels were extremely highly correlated within each other (R2 > 0.85).

Gene	Hs	Acc	SEQ ID No
hemoglobin, beta (HB B)	Hs.155376	NM_000518	86
2,3-bisphosphoglycerate mut ase (B P	Hs.198365	X04327	87
cDNA FLJ20347	Hs.102669	AK000354	94
602620663F1cDNA	Hs.34549	AI123826	107
HA1247cDNA	Hs.33757	AI114652	91

[0525] This suggested that they were all elevated as part of a single response or process. When the microarray data was used to cluster these genes with each other and the other genes on the microarray, we found that these five genes clustered reasonably near each and of the other array genes which clustered tightly with them, four of the top 40 or so were platelet related genes. In addition, these a number of these genes clustered closely with CD34. CD34 is a marker of hematopoietic stem cells and is seen in the peripheral blood with increased hematopoisis.

[0526] CD34, platelet RNA and erythrocyte RNA all mark immature or progenitor blood cells and it is clear that theses marker of acute rejection are part of a coordinated hematopoietic response. A small increase in the rate of production of RBCs and platelets may result in large fold changes in RNA levels. Immune activation from acute rejection may lead to increased hamatopoiesis in the bone marrow and non-marrow sites. This leads to an increase in many lineages because of the lack of complete specificity of the marrow response. Alternatively, increased hematopoiesis may occur in a transplant recipient due to an infection (viral or other), allergy or other stimulus to the system This results in production of cells or a critical mass of immune cells that can cause rejection. In this scenario, monitoring for markers of immune activation would provide an opportunity for early diagnosis.

Table 1

Disease Classification	Disease/Patient Group
Cardiovascular Disease	Atherosclerosis Unstable angina Myocardial Infarction Restenosis after angioplasty Congestive Heart Failure Myocarditis Endocarditis Endothelial Dysfunction Cardiomyopathy Cardiovascular drug use
Infectious Disease	Hepatitis A, B, C, D, E, G Malaria

(continued)

Disease Classification	Disease/Patient Group
	Tuberculosis HIV Pneumocystis Carinii Giardia Toxoplasmosis Lyme Disease Rocky Mountain Spotted Fever Cytomegalovirus Epstein Barr Virus Herpes Simplex Virus Clostridium Dificile Colitis Meningitis (all organisms) Pneumonia (all organisms) Urinary Tract Infection (all organisms) infectious Diarrhea (all organisms) Anti-infectious drug use
Angiogenesis	Pathologic angiogenesis Physiologic angiogenesis Treatment induced angiogenesis Pro or anti-angiogenic drug use
Transplant Rejection	Heart Lung Liver Pancreas Bowel Bone Marrow Stem Cell Graft versus host disease Transplant vasculopathy Skin Cornea Islet Cells Kidney Xenotransplants Mechanical Organ Immunosupressive drug use
Hematological Disorders	Anemia - Iron Deficiency Anemia - B12, Folate deficiency Anemia - Aplastic Anemia - hemolytic Anemia - Renal failure Anemia - Chronic disease Polycythemia rubra vera Pernicious anemia Idiophic Thrrombocytopenic purpura Thrombotic Thrombocytopenic purpura Essential thrombocytosis Leukemia Cytopenias due to immunosupression

(continued)

Disease Classification	Disease/Patient Group
	Cytopenias due to Chemotherapy Myelodysplasia

Table 2A.

Gene	Gene Name	SEQ ID 50mer	HS	ACC	SEQ ID RNA/cDNA
HSRRN18S	18S ribosomal RNA	1	NA	X03205	333
ACTB	Actin, beta	2	Hs.288061	NM 001101	334
GUSB	Glucuronidase, beta	3	Hs.183868	NM 000181	335
B2M	beta 2 microglobulin	4	Hs.75415	NM 004048	336
TSN	Translin	5	Hs.75066	NM 004622	337
CCR7	1707	6	Hs.1652	NM 001838	338
IL1R2	4685-IL1R	7	Hs.25333	NM 004633	339
AIF-1	Allograft inflammatory factor 1, all variants	8	Hs.76364	NM_004847	340
ALAS2	ALAS2	9	Hs.323383	NM 000032.1	341
APELIN	APELIN	10	Hs.303084	NM 017413	342
CD80	B7-1, CD80	11	Hs.838	NM 005191	343
EPB41	Band 4.1	12	Hs.37427	NM 004437	344
CBLB	c-cbl-B	13	Hs.3144	NM 004351	345
CCR5	CCR5	14	Hs.54443	NM 000579	346
MME	CD10	15	Hs.1298	NM 000902	347
KLRC1	CD159a	16	Hs.74082	NM 002259	348
FCGR3A	CD16	17	Hs.176663	NM 000569	349
FCGR3B	CD16b	18	Hs.372679	NM 000570	350
LAG3	CD223	19	Hs.74011	NM 002286	351
PECAM1	CD31	20	Hs.78146	NM 000442	352
CD34	CD34	21	Hs.374990	NM 001773	353
FCGR1A	CD64	22	Hs.77424	NM 000566	354
TFRC	CD71 = T9, transferrin receptor	23	Hs.77356	NM 003234	355
CMA1	chymase	24	Hs.135626	NM 001836	356
KIT	c-Kit	25	Hs.81665	NM 000222	357
MPL	c-mpl	26	Hs.84171	NM 005373	358
EphB6	EphB6	27	Hs.3796	NM 004445	359
EPOR	EPO-R	28	Hs.127826	NM 000121.2	360
Foxp3	Foxp3	29	Hs.247700	NM 014009	361
GATA1	GATA1	30	Hs.765	NM 002049	362
ITGA2B	GP IIb	31	NM 000419.2	NM 000419	363

(continued)

Gene	Gene Name	SEQ ID 50mer	HS	ACC	SEQ ID RNA/cDNA
GNLY	granulysin	32	Hs.105806	NM 006433	364
GZMA	GZMA	33	Hs.90708	NM 006144	365
HBA	hemoglobin, alpha 1	34	Hs.398636	NM 000558.3	366
HBZ	hemoglobin, zeta	35	Hs.272003	NM 005332.2	367
HBB	hemoglobin, beta	36	Hs.155376	NM 000518.4	368
HBD	hemoglobin, delta	37	Hs.36977	NM 000519.2	369
HBE	hemoglobin, epsilon 1	38	Hs.117848	NM 005330	370
HBG	hemoglobin, gamma A	39	Hs.283108	NM 000559.2	371
HBQ	hemoglobin, theta 1	40	Hs.247921	NM 005331	372
HLA-DP	MH/c, class II, DP alpha 1	41	Hs.198253	NM 033554	373
HLA-DQ	MHC, class II, DQ alpha 1	42	Hs.198253	NM 002122	374
HLA-DRB	MHC, class II, DR beta 1	43	Hs.375570	NM 002124.1	375
ICOS	ICOS	44	Hs.56247	NM 012092	376
IL18	IL18	45	Hs.83077	NM 001562	377
IL3	interleukin 3 (colony-stimulating factor, multiple)	46	Hs.694	NM_000588	378
ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	47	Hs.40034	NM_000885	379
ITGAM	integrin, alpha M (complement component receptor 3, alpha; also known as CD11b(p170), macrophage antigen alpha polypeptide)	48	Hs.172631	NM_000632	380
ITGB7	integrin, beta 7	49	Hs.1741	NM 000889	381
CEBPB	LAP, CCAAT/enhancer binding protein (C/EBP), beta	50	Hs.99029	NM_005194	382
NF-E2	NF-E2	51	Hs.75643	NM 006163	383
PDCD1	programmed cell death 1, PD-1	52	Hs.158297	NM 005018	384
PF4	platelet factor 4 (chemokine (C-X-C motif) ligand 4)	53	Hs.81564	NM_002619	385
PRKCQ	protein kinase C, theta	54	Hs.211593	NM 006257.1	386
PPARGC1	PPARgamma	55	Hs.198468	NM 013261	387
RAG1	recombination activating gene 1	56	Hs.73958	NM 000448	388
RAG2	recombination activating gene 2	57	Na	NM 000536	389
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1) (SDF-1)	58	Hs.237356	NM_000609	390
TNFRSF4	tumor necrosis factor receptor superfamily, member 4	59	Hs.129780	NM_003327	391

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Gene	Gene Name	SEQ ID 50mer	HS	ACC	SEQ ID RNA/cDNA
TNFSF4	tumor necrosis factor (ligand) superfamily, member 4 (tax-transcriptionally activated glycoprotein 1, 34kDa)	60	Hs.181097	NM_003326	392
TPS1	tryptase, alpha	61	Hs.334455	NM 003293	393
ADA	ADA adenosine deaminase	62	Hs.1217	NM 000022	394
CPM	Carboxypeptidase M	63	Hs.334873	NM 001874.1	395
CSF2	colony stimulating factor, GM-CSF	64	Hs.1349	NM 000758.2	396
CSF3	colony stimulating factor 3, G-CSF	65	Hs.2233	NM 172219	397
CRP	C-reactive protein, pentraxin-related (CRP),	66	Hs.76452	NM_000567.1	398
FLT3	FMS-Related Tyrosine Kinase 3	67	Hs.385	NM 004119	399
GATA3	GATA binding protein 3	68	Hs.169946	NM 002051.1	400
IL7R	Interleukin 7 receptor	69	Hs.362807	NM 002185.1	401
KLF1	Kruppel-like factor 1 (erythroid), EKLF	70	Hs.37860	NM_006563.1	402
LCK	lymphocyte-specific protein tyrosine kinase	71	Hs.1765	NM_005356.2	403
LEF1	lymphoid enhancer-binding factor 1	72	Hs.44865	NM_016269.2	404
PLAUR	Urokinase-type Plasminogen Activator Receptor, CD87, uPAR	73	Hs.179657	NM_002659.1	405
TNFSF13B	Tumor necrosis factor (ligand) superfamily, member 13b, BlyS/TALL-1/BAFF	74	Hs.270737	NM_006573.3	406
IL8	Interleukin 8	75	Hs.624	NM 000584	407
GZMB	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	76	Hs.1051	NM_004131	408
TNFSF6	Tumor necrosis factor (ligand) superfamily, member 6	77	Hs.2007	NM_000639	409
TCIRGI	T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 protein a isoform 3	78	Hs.46465	NM_006019	410
PRF1	Perforin 1 (pore forming protein)	79	Hs.2200	NM 005041	411
IL4	Interleukin 4	80	Hs.73917	NM 000589	412
IL13	Interleukin 13	81	Hs.845	NM 002188	413
CTLA4	Cytotoxic T-lymphocyte-associated protein 4	82	Hs.247824	NM_005214	414
CD8A	CD8 antigen, alpha polypeptide (p32)	83	Hs.85258	NM_001768	415
BY55	Natural killer cell receptor, immunoglobulin superfamily member	84	Hs.81743	NM_007053	416

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Gene	Gene Name	SEQ ID 50mer	HS	ACC	SEQ ID RNA/cDNA
OID 4460	EST	85	Hs.205159	AF150295	417
HBB	Hemoglobin, beta	86	Hs.155376	NM 000518	418
BPGM	2,3-bisphosphoglycerate mutase	87	Hs.198365	NM 001724	419
MTHFD2	Methylene tetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase	88	Hs.154672	NM_006636	420
TAP1	Transporter 1, ATP-binding cassette, sub-family B (MDR1/TAP)	89	Hs.352018	NM_000593	421
KPNA6	Karyopherin alpha 6 (importin alpha 7)	90	Hs.301553	AW021037	422
OID 4365	Mitochondrial solute carrier	91	Hs.300496	AI114652	423
IGHM	Immunoglobulin heavy constant mu	92	Hs.300697	BC032249	424
OID 573	KIAA1486 protein	93	Hs.210958	AB040919	425
OID 873	KIAA1892 protein	94	Hs.102669	AK000354	426
OID 3	EST	95	Hs.104157	AW968823	427
CXCR4	Chemokine (C-X-C motif) receptor 4	96	Hs.89414	NM 003467	428
CD69	CD69 antigen (p60, early T-cell activation antigen)	97	Hs.82401	NM_001781	429
CCL5	Chemokine (C-C motif) ligand 5 (RANTES, SCYA5)	98	Hs.241392	NM_002985	430
IL6	Interleukin 6	99	Hs.93913	NM 000600	431
IL2	Interleukin 2	100	Hs.89679	NM 000586	432
KLRF1	Killer cell lectin-like receptor subfamily F, member 1	101	Hs.183125	NM_016523	433
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	102	Hs.80887	NM_002350	434
IL2RA	Interleukin 2 receptor, alpha	103	Hs.1724	NM 000417	435
CCL4	Chemokine (C-C motif) ligand 4, SCYA4	104	Hs.75703	NM_002984	436
OID 6207	EST	105	Hs.92440	D20522	437
ChGn	Chondroitin beta 1,4 N-acetylgalactosaminyltransferase	106	Hs.11260	NM_018371	438
OID 4281	EST	107	Hs.34549	AA053887	439
CXCL9	Chemokine (C-X-C motif) ligand 9 (MIG)	108	Hs.77367	NM_002416	440
CXCL10	Chemokine (C-X-C motif) ligand 10, SCYB10	109	Hs.2248	NM_001565	441
IL17	Interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8)	110	Hs.41724	NM_002190	442
IL15	Interleukin 15	111	Hs.168132	NM 000585	443

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Gene	Gene Name	SEQ ID 50mer	HS	ACC	SEQ ID RNA/cDNA
IL10	Interleukin 10	112	Hs.193717	NM 000572	444
IFNG	Interferon, gamma	113	Hs.856	NM 000619	445
HLA-DRB1	Major histocompatibility complex, class II, DR beta 1	114	Hs.308026	NM_002124	446
CD8B1	CD8 antigen, beta polypeptide 1 (p37)	115	Hs.2299	NM_004931	447
CD4	CD4 antigen (p55)	116	Hs.17483	NM 000616	448
CXCR3	Chemokine (C-X-C motif) receptor 3, GPR9	117	Hs.198252	NM_001504	449
OID 7094	XDx EST 479G12	118	NA	NA	450
OID 7605	EST	119	Hs.109302	AA808018	451
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	120	Hs.789	NM_001511	452
OID 253	EST	121	Hs.83086	AK091125	453
GPI	Glucose phosphate isomerase	122	Hs.409162	NM 000175	454
CD47	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	123	Hs.82685	NM_001777	455
HLA-F	Major histocompatibility complex, class I, F	124	Hs.377850	NM_018950	456
OID 5350	EST	125	Hs.4283	AK055687	457
TCRGC2	T cell receptor gamma constant 2	126	Hs.112259	M17323	458
OID 7016	EST	127	NA	B1018696	459
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	128	Hs.196384	NM_000963	460
OID 5847	Hypothetical protein FLJ32919	129	Hs.293224	NM 144588	461
PRDM1	PR domain containing 1, with ZNF domain	130	Hs.388346	NM_001198	462
CKB	Creatine kinase, Brain	131	Hs.173724	NM 001823	463
TNNI3	Troponin I, cardiac	132	Hs.351382	NM 000363	464
TNNT2	Troponin T2, cardiac	133	Hs.296865	NM 000364	465
MB	Myoglobin	134	Hs.118836	NM 005368	466
SLC7A11	Solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	135	Hs.6682	NM_014331	467
TNFRSF5	tumor necrosis factor receptor superfamily, member 5; CD40	136	Hs.25648	NM_001250	468
TNFRSF7	tumor necrosis factor receptor superfamily, member 7; CD27	137	Hs.355307	NM_001242	469

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Gene	Gene Name	SEQ ID 50mer	HS	ACC	SEQ ID RNA/cDNA
CD86	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)	138	Hs.27954	NM_175862	470
AIF1v2	Allograft inflammatory factor 1, splice variant 2	139	Hs.76364	NM_004847	471
EBV BCLF-1	BCLF-1 major capsid	140	NA	AJ507799	472
EBV EBV	EBNA repetitive sequence	141	NA	AJ507799	473
CMV p67	pp67	142	NA	X17403	474
CMV TRL7	c6843-6595	143	NA	X17403	475
CMV IE1e3	IE1 exon 3	144	NA	X17403	476
CMV IE1e4	IE1 exon 4 (40 variants)	145	NA	X17403	477
EBV EBNA-1	EBNA-1 coding region	146	NA	AJ507799	478
EBV BZLF-1	Zebra gene	147	NA	AJ507799	479
EBV EBN	EBNA repetitive sequence	148	NA	AJ507799	480
EBV EBNA-LP	Short EBNA leader peptide exon	149	NA	AJ507799	481
CMV IE1	IE1S	150	NA	X17403	482
CMV IE1	IE1-MC(exon3)	151	NA	X17403	483
CLC	Charot-Leyden crystal protein	152	Hs.889	NM 001828	484
TERF2IP	telomeric repeat binding factor 2, interacting protein	153	Hs.274428	NM_018975	485
HLA-A	Major histocompatibility complex, class I, A	154	Hs.181244	NM_002116	486
OID 5891	EST 3' end	155	None	AW297949	487
MSCP	mitochondrial solute carrier protein	156	Hs.283716	NM 018579	488
DUSP5	dual specificity phosphatase 5	157	Hs.2128	NM 004419	489
PR01853	Hypothetical protein PRO1853	158	Hs.433466	NM 018607	490
OID 6420	73A7, FLJ00290 protein	159	Hs.98531	AK090404	491
CDSN	Corneodesmosin	160	Hs.507	NM 001264	492
OID 4269	EST	161	Hs.44628	BM727677	493
RPS25	Ribosomal protein S25	162	Hs.409158	NM 001028	494
GAPD	Glyceraldehyde-3-phosphate dehydrogenase	163	Hs.169476	NM_002046	495
RPLP1	Ribosomal protein, large, P1	164	Hs.424299	NM 001003	496
OID_5115	qz23b07.x1 cDNA, 3' end /clone=IMAGE:2027701	165	NA	AI364926	497
SLC9A8	Solute carrier family 9 (sodium/hydrogen exchanger), isoform 8	166	Hs.380978	AB023156	498
OID 1512	IMAGE:3865861 5 clone 5'	167	Hs.381302	BE618004	499

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Gene	Gene Name	SEQ ID 50mer	HS	ACC	SEQ ID RNA/cDNA
POLR2D	Polymerase (RNA) II (DNA directed) polypeptide D	168	Hs.194638	NM_004805	500
ARPC3	Actin related protein 2/3 complex, subunit 3, 21kDa	169	Hs.293750	NM_005719	501
OID 6282	EST 3' end	170	Hs.17132	BC041913	502
PRO1073	PRO1073 protein	171	Hs.356442	AF001542	503
OID_7222	EST, weakly similar to A43932 mucin 2 precursor, intestinal	172	Hs.28310	BG260891	504
FPRL1	Formyl peptide receptor-like 1	173	Hs.99855	NM 001462	505
FKBPL	FK506 binding protein like	174	Hs.99134	NM 022110	506
PREB	Prolactin regulatory element binding	175	Hs.279784	NM_013388	507
OID 1551	Hypothetical protein LOC200227	176	Hs.250824	BE887646	508
OID 7595	DKFZP566F0546 protein	177	Hs.144505	NM 015653	509
RNF19	Ring finger protein 19	178	Hs.48320	NM 015435	510
SMCY	SMC (mouse) homolog, Y chromosome (SMCY)	179	Hs.80358	NM_004653	511
OID 4184	CMV HCMVUL109	180	NA	X17403	512
OID 7504	Hypothetical protein FLJ35207	181	Hs.86543	NM 152312	513
DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	182	Hs.9683	NM_006260	514
ARHU	Ras homolog gene family, member U	183	Hs.20252	NM_021205	515
OID 7200	Hypothetical protein FLJ22059	184	Hs.13323	NM 022752	516
SERPINB2	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	185	Hs.75716	NM_002575	517
ENO1	Enolase 1, alpha	186	Hs.254105	NM 001428	518
OID 7696	EST 3' end	187	Hs.438092	AW297325	519
OID 4173	CMV HCMVTRL2 (IRL2)	188	NA	X17403	520
CSF2RB	Upstream variant mRNA of colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	189	Hs.285401	AL540399	521
OID_7410	CM2-LT0042-281299-062-e11 LT0042 cDNA, mRNA sequence	190	Hs.375145	AW837717	522
OID 4180	CMV HCMVUS28	191	NA	X17403	523
OID 5101	EST	192	Hs.144814	BG461987	524
MOP3	MOP-3	193	Hs.380419	NM 018183	525
RPL18A	Ribosomal protein L18a	194	Hs.337766	NM 000980	526
INPP5A	Inositol polyphosphate-5-phosphatase, 40kDa	195	Hs.124029	NM_005539	527
hIAN7	Immune associated nucleotide	196	Hs.124675	BG772661	528

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Gene	Gene Name	SEQ ID 50mer	HS	ACC	SEQ ID RNA/cDNA
RPS29	Ribosomal protein S29	197	Hs.539	NM 001032	529
OID 6008	EST 3' end	198	Hs.352323	AW592876	530
OID 4186	CMVHCMVUL122	199	NA	X17403	531
VNN2	vanin 2	200	Hs.121102	NM 004665	532
OID 7703	KIAA0907 protein	201	Hs.24656	NM 014949	533
OID 7057	480F8	202	NA	480F8	534
OID 4291	EST	203	Hs.355841	BC038439	535
OID 1366	EST	204	Hs.165695	AW850041	536
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1	205	Hs.422118	NM_001402	537
PA2G4	Proliferation-associated 2G4, 38kDa	206	Hs.374491	NM 006191	538
GAPD	Glyceraldehyde-3-phosphate dehydrogenase	207	Hs.169476	NM_002046	539
CHD4	Chromodomain helicase DNA binding protein 4	208	Hs.74441	NM_001273	540
OID 7951	E2F-like protein (LOC51270)	209	Hs.142908	NM 016521	541
DAB1	Disabled homolog 1 (Drosophila)	210	Hs.344127	NM 021080	542
OID 3406	Hypothetical protein FLJ20356	211	Hs.61053	NM 018986	543
OID 6986	462H9 EST	212	Hs.434526	AK093608	544
OID 5962	EST 3' end	213	Hs.372917	AW452467	545
OID 5152	EST 3' end	214	Hs.368921	AI392805	546
S100A8	S100 calcium-binding protein A8 (calgranulin A)	215	Hs.416073	NM_002964	547
HNRPU	HNRPU Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	216	Hs.103804	BM467823	548
ERCC5	Excision repair cross-complementing rodent repair deficiency, complementation group 5 (xeroderma pigmentosum, complementation group G (Cockayne syndrome))	217	Hs.48576	NM_000123	549
RPS27	Ribosomal protein S27 (metallopanstimulin 1)	218	Hs.195453	NM_001030	550
ACRC	acidic repeat containing (ACRC),	219	Hs.135167	NM 052957	551
PSMD11	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 11	220	Hs.90744	AI684022	552
OID 1016	FLJ00048 protein	221	Hs.289034	AK024456	553
OID 1309	AV706481 cDNA	222	None	AV706481	554
OID_7582	Weakly similar to ZINC FINGER PROTEIN 142	223	Hs.16493	AK027866	555

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Gene	Gene Name	SEQ ID 50mer	HS	ACC	SEQ ID RNA/cDNA
OID_4317	ta73c09.x1 3' end /clone=IMAGE:2049712 Ribosomal Protein S 15	224	Hs.387179	AI318342	556
OID 5889	3' end /clone=IMAGE:3083913	225	Hs.255698	AW297843	557
UBL1	Ubiquitin-like 1 (sentrin)	226	Hs.81424	NM 003352	558
OID 3687	EST	227	None	W03955	559
OID 7371	EST 5'	228	Hs.290874	BE730505	560
SH3BGRL3	SH3 domain binding glutamic acid-rich protein like 3	229	Hs.109051	NM_031286	561
SEMA7A	Sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A	230	Hs.24640	NM_003612	562
OID 5708	EST 3' end	231	Hs.246494	AW081540	563
OID 5992	EST 3' end	232	Hs.257709	AW467992	564
IL21	Interleukin 21	233	Hs.302014	NM 021803	565
HERC3	Hect domain and RLD 3 (HERC3)	234	Hs.35804	NM 014606	566
OID 7799	AluJo/FLAM SINE/Alu	235		AW837717	567
P11	26 serine protease	236	Hs.997	NM 006025	568
OID 7766	EST 3' end	237	Hs.437931	AW294711	569
TIMM10	translocase of inner mitochondrial membrane 10 (yeast) homolog (TIMM10)	238	Hs.235750	NM_012456	570
EGLN1	Egl nine homolog 1 (C. elegans)	239	Hs.6523	AJ310543	571
TBCC	Tubulin-specific chaperone c	240	Hs.75064	NM 003192	572
RNF3	Ring finger protein 3	241	Hs.8834	NM 006315	573
OID_6451	170F9, hypothetical protein FLJ21439	242	Hs.288872	AL834168	574
CCNDBP1	cyclin D-type binding-protein 1 (CCNDBP1)	243	Hs.36794	NM_012142	575
OID 8063	MUC18 gene exons 1&2	244	NA	X68264	576
SUV39H1	Suppressor of variegation 3-9 homolog 1 (Drosophila)	245	Hs.37936	NM_003173	577
HSPC048	HSPC048 protein	246	Hs.278944	NM 014148	578
OID 5625	EST 3' end from T cells	247	Hs.279121	AW063780	579
WARS	Tryptophanyl-tRNA synthetase	248	Hs.82030	NM 004184	580
OID 6823	107H8	249	Hs.169610	AL832642	581
OID 7073	119F12	250	Hs.13264	AL705961	582
OID 5339	EST 3'end	251	Hs.436022	AI625119	583
OID_4263	fetal retina 937202 cDNA clone IMAGE:565899	252	Hs.70877	AA136584	584

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Gene	Gene Name	SEQ ID 50mer	HS	ACC	SEQ ID RNA/cDNA
MGC26766	Hypothetical protein MGC26766	253	Hs.288156	AK025472	585
SERPINB11	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 11	254	Hs.350958	NM_080475	586
OID 6711	58G4, IMAGE:4359351 5'	255	none	BF968628	587
RNF10	Ring finger protein 10	256	Hs.5094	NM 014868	588
MKRN1	Makorin, ring finger protein, 1	257	Hs.7838	NM 013446	589
RPS16	ribosomal protein S16	258	Hs.397609	NM 001020	590
BAZ1A	Bromodomain adjacent to zinc finger domain, 1A	259	Hs.8858	NM_013448	591
OID 5998	EST 3' end	260	Hs.330268	AW468459	592
ATP5L	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g	261	Hs.107476	NM_006476	593
OID 6393	52B9	262	NA	52B9	594
RoXaN	Ubiquitous tetratricopeptide containing protein RoXaN	263	Hs.25347	BC004857	595
NCBP2	Nuclear cap binding protein subunit 2, 20kDa	264	Hs.240770	NM_007362	596
OID 6273	EST 3' end	265	Hs.158976	AW294774	597
HZF12	zinc finger protein 12	266	Hs.164284	NM 033204	598
CCL3	Chemokine (C-C motif) ligand 3	267	Hs.73817	D90144	599
OID 4323	IMAGE:1283731 3'	268	Hs.370770	AA744774	600
OID_5181	tg93h12.x1NCI_CGAP_CLL1cDNA clone IMAGE:2116391 3' similar to contains TAR1.t1 MER22	269	NA	AI400725	601
PRDX4	Peroxiredoxin 4	270	Hs.83383	NM 006406	602
BTK	Bruton agammaglobulinemia tyrosine kinase	271	Hs.159494	NM_000061	603
OID 6298	Importin beta subunit mRNA	272	Hs.180446	AI948513	604
PGK1	Phosphoglycerate kinase 1	273	Hs.78771	NM 000291	605
TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a	274	Hs.249190	NM_003844	606
ADM	adrenomedullin	275	Hs.394	NM 001124	607
OID 357	138G5	276	NA	138G5	608
C20orf6	461A4 chromosome 20 open reading frame 6	277	Hs.88820	NM_016649	609
OID 3226	DKFZP564O0823 protein	278	Hs.105460	NM 015393	610
ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	279	Hs.75811	NM_004315	611
ATF5	Activating transcription factor 5	280	Hs.9754	NM 012068	612
OID 4887	hypothetical protein MGC14376	281	Hs.417157	NM 032895	613

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Gene	Gene Name	SEQ ID 50mer	HS	ACC	SEQ ID RNA/cDNA
OID 4239	EST	282	Hs.177376	BQ022840	614
MDM2	Mouse double minute 2, homolog of; p53-binding protein (MDM2), transcript variant MDM2,	283	Hs.170027	NM_002392	615
XRN2	5'-3' exoribonuclease 2	284	Hs.268555	AF064257	616
OID_6039	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 4 (EDG4)	285	Hs.122575	BE502246	617
OID 4210	IMAGE:4540096	286	Hs.374836	AI300700	618
OID 7698	EST 3' end	287	Hs.118899	AA243283	619
PRKRA	Protein kinase, interferon-inducible double stranded RNA dependent activator	288	Hs.18571	NM_003690	620
OID 4288	IMAGE:2091815	289	Hs.309108	AI378046	621
OID 5620	EST 3' end from T cells	290	Hs.279116	AW063678	622
OID 7384	EST 5'	291	Hs.445429	BF475239	623
OID_1209	EST Weakly similar to hypothetical protein FLJ20378	292	Hs.439346	C14379	624
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	293	Hs.238990	NM_004064	625
PLOD	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase, Ehlers-Danlos syndrome type VI)	294	Hs.75093	NM_000302	626
OID 5128	EST	295	Hs.283438	AK097845	627
OID 5877	EST 3' end	296	Hs.438118	AW297664	628
FZD4	Frizzled (Drosophila) homolog 4	297	Hs.19545	NM 012193	629
HLA-B	Major histocompatibility complex, class I, B	298	Hs.77961	NM_005514	630
OID 5624	EST 3' end from T cells	299	Hs.279120	AW063921	631
FPR1	Formyl peptide receptor 1	300	Hs.753	NM 002029	632
ODF2	Outer dense fiber of sperm tails 2	301	Hs.129055	NM 153437	633
OID_5150	tg04g01.x1 cDNA, 3' end /clone=IMAGE:2107824	302	Hs.160981	AI392793	634
OID 5639	EST 3' end from T cells	303	Hs.279139	AW064243	635
OID 6619	469A10	304	NA	469A10	636
OID 6933	463C7,4 EST hits. Aligned	305	Hs.86650	AI089520	637
OID 7049	480E2	306	NA	480E2	638
IL17C	Interleuldn 17C	307	Hs.278911	NM 013278	639
OID 5866	EST 3' end	308	Hs.255649	BM684739	640
CD44	CD44	309	Hs.169610	AA916990	641
VPS45A	Vacuolar protein sorting 45A (yeast)	310	Hs.6650	NM_007259	642

(continued)

Gene	Gene Name	SEQ ID 50mer	HS	ACC	SEQ ID RNA/cDNA
OID_4932	aa92c03.r1 Stratagene fetal retina 937202 cDNA clone IMAGE:838756	311	NA	AA457757	643
OID 7821	EST	312	NA	AA743221	644
OID_4916	zr76a03.r1 Soares_NhHMPu_S1 cDNA clone IMAGE:669292	313	NA	AA252909	645
OID 4891	Hypothetical protein LOC255488	314	Hs.294092	AL832329	646
HADHB	Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit	315	Hs.146812	NM_000183	647
FLJ22757	Hypothetical protein FLJ22757	316	Hs.236449	NM 024898	648
RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	317	Hs.173737	AK054993	649
OID 6415	72D4, FLJ00290 protein	318	Hs.98531	CA407201	650
NMES1	Normal mucosa of esophagus specific 1	319	Hs.112242	NM_032413	651
DMBT1	Deleted in malignant brain tumors 1, transcript variant 2	320	Hs.279611	NM_007329	652
RPS23	ribosomal protein S23	321	Hs.3463	NM 001025	653
ZF	HCF-binding transcription factor Zhangfei	322	Hs.29417	NM_021212	654
NFE2L3	Nuclear factor (erythroid-derived 2)-like 3	323	Hs.22900	NM_004289	655
RAD9	RAD9 homolog (S. pombe)	324	Hs.240457	NM 004584	656
OID 6295	EST 3' end	325	Hs.389327	AI880607	657
DEFCAP	Death effector filament-forming Ced-4-like apoptosis protein, transcript variant B	326	Hs.104305	NM_014922	658
RPL27A	Ribosomal protein L27a	327	Hs.76064	BF214146	659
IL22	Interleukin 22 (IL22)	328	Hs.287369	NM 020525	660
PSMA4	Proteasome (prosome, macropain) subunit, alpha type, 4, (PSMA4)	329	Hs.251531	NM_002789	661
CCNI	cyclin I (CCNI)	330	Hs.79933	NM 006835	662
THBD	Thrombomodulin	331	Hs.2030	NM 000361	663
CGR19	Cell growth regulatory with ring fmger domain	332	Hs.59106	NM_006568	664

Table 2B.

SEQ ID 50mer	Gene	Gene Name	ACC	SEQ ID RNA/cDNA	Non-Para Score	Median Rank in NR	Down Regulated
152	CLC	Charcot-Leyden crystal protein	NM 001828	484	779	4342	
153	TERF2IP	telomeric repeat binding factor 2, interacting protein	NM_018975	485	744	1775	
154	HLA-A	Major histocompatibility complex, class I, A	NM_002116	486	735	125	1
155	OID 5891	EST 3' end	AW297949	487	730	7044.5	1
156	MSCP	mitochondrial solute carrier protein	NM_018579	488	730	3465.5	
157	DUSP5	dual specificity phosphatase 5	NM 004419	489	726	3122.5	
158	PRO1853	Hypothetical protein PR01853	NM 018607	490	725	4153	
159	OID 6420	73A7, FLJ00290 protein	AK090404	491	725	7000.5	
160	CDSN	Corneodesmosin	NM 001264	492	722	2732	
161	OID 4269	EST	BM727677	493	715	5598.5	
162	RPS25	Ribosomal protein S25	NM 001028	494	710	164.5	
163	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	495	707	215.5	
164	RPLP1	Ribosomal protein, large, P1	NM 001003	496	703	157	
165	OID_5115	qz23b07.x1 cDNA, 3' end /clone=IMAGE:2027701	AI364926	497	703	6629	1
166	SLC9A8	Solute carrier family 9 (sodium/hydrogen exchanger), isoform 8	AB023156	498	702	2538.5	
167	OID 1512	IMAGE:3865861 5 clone 5'	BE618004	499	700	4008	1
168	POLR2D	Polymerase (RNA) II (DNA directed) polypeptide D	NM_004805	500	700	4190.5	
169	ARPC3	Actin related protein 2/3 complex, subunit 3, 21kDa	NM_005719	501	698	470.5	

(continued)

SEQ ID 50mer	Gene	Gene Name	ACC	SEQ ID RNA/cDNA	Non-Para Score	Median Rank in NR	Down Regulated
170	OID 6282	EST 3' end	BC041913	502	697	4371.5	
171	PRO1073	PRO1073 protein	AF001542	503	697	6754	
172	OID_7222	EST, weakly similar to A43932 mucin 2 precursor, intestinal	BG260891	504	695	6759	
173	FPRL1	Formyl peptide receptor-like 1	NM 001462	505	692	4084.5	
174	FKBPL	FK506 binding protein like	NM 022110	506	691	1780.5	
175	PREB	Prolactin regulatory element binding	NM_013388	507	690	3568	
176	OID_1551	Hypothetical protein LOC200227 BE887646		508	689	6423	1
177	OID 7595	DKFZP566F0546 protein	NM 015653	509	689	3882.5	
178	RNF19	Ring finger protein 19	NM 015435	510	689	7700.5	
179	SMCY	SMC (mouse) homolog, Y chromosome (SMCY)	NM_004653	511	687	6074.5	
180	OID 4184	CMVHCMVUL109	X17403	512	687	6810.5	
181	OID 7504	Hypothetical protein FLJ35207	NM 152312	513	686	6939	
182	DNAJC3	DnaJ (Hsp40) homolog, subfamily C, member 3	NM_006260	514	686	3932.5	
183	ARHU	Ras homolog gene family, member U	NM_021205	515	686	7584	
184	OID 7200	Hypothetical protein FLJ22059	NM 022752	516	685	2804.5	
185	SERPINB2	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	NM_002575	517	684	4690.5	
186	ENO1	Enolase 1, alpha	NM 001428	518	684	327	
187	OID 7696	EST 3' end	AW297325	519	683	4875.5	
188	OID 4173	CMV HCMVTRL2 (IRL2)	X17403	520	683	4010.5	

(continued)

SEQ ID 50mer	Gene	Gene Name	ACC	SEQ ID RNA/cDNA	Non-Para Score	Median Rank in NR	Down Regulated
189	CSF2RB	Upstream variant mRNA of colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	AL540399	521	683	3753	
190	OID_7410	CM2-LT0042-281299-062-e11 LT0042 cDNA, mRNA sequence	AW837717	522	682	7445	
191	OID 4180	CMV HCMVUS28	X17403	523	681	4359	
192	OID 5101	EST	BG461987	524	681	7272	
193	MOP3	MOP-3	NM 018183	525	681	4085.5	1
194	RPL18A	Ribosomal protein L18a	NM 000980	526	680	238	
195	INPP5A	Inositol polyphosphate-5-phosphatase, 40kDa	NM_005539	527	680	4838.5	1
196	hIAN7	Immune associated nucleotide	BG772661	528	680	4718	
197	RPS29	Ribosomal protein S29	NM 001032	529	680	107.5	
198	OID 6008	EST 3' end	AW592876	530	679	6560.5	
199	OID 4186	CMVHCMVUL122	X17403	531	677	4788.5	
200	VNN2	vanin 2	NM 004665	532	677	2620.5	
201	OID 7703	KIAA0907 protein	NM 014949	533	676	6104.5	
202	OID 7057	480F8	480F8	534	675	6862	
203	OID 4291	EST	BC038439	535	674	5618.5	
204	OID 1366	EST	AW850041	536	674	5590.5	1
205	EEF1A1	Eukaryotic translation elongation factor 1 alpha 1	NM_001402	537	672	232	
206	PA2G4	Proliferation-associated 2G4, 38kDa	NM_006191	538	672	4402	
207	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	539	671	194.5	

(continued)

SEQ ID 50mer	Gene	Gene Name	ACC	SEQ ID RNA/cDNA	Non-Para Score	Median Rank in NR	Down Regulated
208	CHD4	Chromodomain helicase DNA binding protein 4	NM_001273	540	671	2578.5	
209	OID 7951	E2F-like protein (LOC51270)	NM 016521	541	671	4467	
210	DAB1	Disabled homolog 1 (Drosophila)	NM_021080	542	670	6357.5	
211	OID 3406	Hypothetical protein FLJ20356	NM 018986	543	669	2087	
212	OID 6986	462H9 EST	AK093608	544	669	4454	1
213	OID 5962	EST 3' end	AW452467	545	668	5870.5	1
214	OID 5152	EST 3' end	AI392805	546	668	6354.5	
215	S100A8	S100 calcium-binding protein (calgranulin A)	A8 NM_002964	547	668	134	
216	HNRPU	HNRPU Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	BM467823	548	668	4108	
217	ERCC5	Excision repair cross-complementing rodent repair deficiency, complementation group 5 (xeroderma pigmentosum, complementation group G (Cockayne syndrome))	NM_000123	549	668	6430.5	
218	RPS27	Ribosomal protein S27 (metallopanstimulin 1)	NM_001030	550	668	160	
219	ACRC	acidic repeat containing	(ACRC), NM_052957	551	668	4871.5	1
220	PSMD11	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 11	AI684022	552	668	4138	
221	OID 1016	FLJ00048 protein	AK024456	553	667	5199	
222	OID 1309	AV706481 cDNA	AV706481	554	667	7279.5	

(continued)

SEQ ID 50mer	Gene	Gene Name	ACC	SEQ ID RNA/cDNA	Non-Para Score	Median Rank in NR	Down Regulated
223	OID_7582	Weakly similar to ZINC FINGER PROTEIN 142	AK027866	555	667	5003.5	1
224	OID_4317	ta73c09.x1 3' end /clone=IMAGE:2049712 Ribosomal Protein S15	AI318342	556	667	6499	
225	OID 5889	3' end /clone=IMAGE:3083913	AW297843	557	666	6837	1
226	UBL1	Ubiquitin-like 1 (sentrin)	NM 003352	558	666	1978.5	
227	OID 3687	EST	W03955	559	666	5519.5	
228	OID 7371	EST 5'	BE730505	560	665	7751.5	
229	SH3BGRL3 SH3	domain binding glutamic acid-rich protein like 3	NM_031286 561		665	310	
230	SEMA7A	Sema domain, immunoglobulin domain (Ig), and GPI membrane anchor, (semaphorin) 7A	NM_003612	562	665	3505.5	
231	OID 5708	EST 3' end	AW081540	563	665	6224.5	
232	OID 5992	EST 3' end	AW467992	564	665	5648	
233	IL21	Interleukin 21	NM 021803	565	664	5036.5	
234	HERC3	Hect domain and RLD 3 (HERC3)	NM_014606	566	664	3056.5	1
235	OID 7799	AluJo/FLAM SINE/Alu	AW837717	567	664	3544	
236	P11	26 serine protease	NM 006025	568	664	7173	
237	OID 7766	EST 3' end	AW294711	569	663	7270.5	
238	TIMM10	translocase of inner mitochondrial membrane 10 (yeast) homolog (TIMM10)	NM_012456	570	663	4779.5	
239	EGLN1	Egl nine homolog 1 (C. elegans)	AJ310543	571	662	7172.5	
240	TBCC	Tubulin-specific chaperone c	NM 003192	572	662	3384	
241	RNF3	Ring finger protein 3	NM 006315	573	661	4062	

(continued)

SEQ ID 50mer	Gene	Gene Name	ACC	SEQ ID RNA/cDNA	Non-Para Score	Median Rank in NR	Down Regulated
242	OID_6451	170F9, hypothetical protein FLJ21439	AL834168	574	661	7126	1
243	CCNDBP1	cyclin D-type binding-protein 1 (CCNDBP1)	NM_012142	575	661	1919	
244	OID 8063	MUC18 gene exons 1&2	X68264	576	661	4692.5	
245	SUV39H1	Suppressor of variegation 3-9 homolog 1 (Drosophila)	NM_003173	577	661	5103	1
246	HSPC048	HSPC048 protein	NM 014148	578	660	5981.5	
247	OID 5625	EST 3' end from T cells	AW063780	579	660	4437	1
248	WARS	Tryptophanyl-tRNA synthetase	NM_004184	580	660	905.5	
249	OID 6823	107H8	AL832642	581	659	2619	
250	OID 7073	119F12	AL705961	582	659	6837.5	
251	OID 5339	EST 3' end	AI625119	583	658	4414.5	1
252	OID_4263	fetal retina 937202 cDNA clone IMAGE:565899	AA136584	584	658	5870	
253	MGC26766	Hypothetical protein MGC26766	AK025472	585	658	1892.5	
254	SERPINB1 1	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 11	NM_080475	586	658	7535.5	1
255	OID 6711	58G4, IMAGE:4359351 5'	BF968628	587	658	7264	
256	RNF10	Ring fmger protein 10	NM 014868	588	658	3127.5	
257	MKRN1	Makorin, ring finger protein, 1	NM 013446	589	658	2228.5	
258	RPS16	ribosomal protein S16	NM 001020	590	657	165.5	
259	BAZ1A	Bromodomain adjacent to zinc fmger domain, 1A	NM_013448	591	657	2533	
260	OID 5998	EST 3' end	AW468459	592	657	6339.5	

(continued)

SEQ ID 50mer	Gene	Gene Name	ACC	SEQ ID RNA/cDNA	Non-Para Score	Median Rank in NR	Down Regulated
261	ATP5L	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g	NM_006476	593	657	1155	
262	OID 6393	52B9	52B9	594	657	7420.5	
263	RoXaN	Ubiquitous tetratricopeptide containing protein RoXaN	BC004857	595	656	7378	
264	NCBP2	Nuclear cap binding protein subunit 2, 20kDa	NM_007362	596	656	4666.5	
265	OID 6273	EST 3' end	AW294774	597	656	5498.5	
266	HZF12	zinc fmger protein 12	NM 033204	598	656	4715.5	
267	CCL3	Chemokine (C-C motif) ligand 3	D90144	599	656	4910	1
268	OID 4323	IMAGE:12837313'	AA744774	600	655	6406.5	1
269	OID_5181	tg93h12.x1 NCI_CGAP_CLL1 cDNA clone IMAGE:2116391 3' similar to contains TAR1.t1 MER22	AI400725	601	655	4838	1
270	PRDX4	Peroxiredoxin 4	NM 006406	602	655	3397.5	
271	BTK	Bruton agammaglobulinemia tyrosine kinase	NM_000061	603	655	2358	
272	OID 6298	Importin beta subunit mRNA	AI948513 604		655	2433.5	
273	PGK1	Phosphoglycerate kinase 1	NM 000291	605	655	2059.5	
274	TNFRSF10 A	Tumor necrosis factor receptor superfamily, member 10a	NM_003844 606		654	4897.5	1
275	ADM	adrenomedullin	NM 001124	607	654	4235	
276	OID 357	138G5	138G5	608	654	5427.5	1
277	C20orf6	461A4 chromosome 20 open reading frame 6	NM_016649	609	654	6343	1
278	OID 3226	DKFZP564O0823 protein	NM 015393	610	653	6187.5	

(continued)

SEQ ID 50mer	Gene	Gene Name	ACC	SEQ ID RNA/cDNA	Non-Para Score	Median Rank in NR	Down Regulated
279	ASAH1	N-acylsphingosine amidohydrolase (acid ceramidase) 1	NM_004315	611	653	1003	
280	ATF5	Activating transcription factor 5	NM_012068	612	653	4545.5	
281	OID_4887	hypothetical protein MGC14376	NM_032895	613	653	2310	1
282	OID 4239	EST	BQ022840	614	652	2774.5	
283	MDM2	Mouse double minute 2, of; p53-binding protein (MDM2), transcript variant MDM2,	homolog NM_002392	615	652	4342	
284	XRN2	5'-3' exoribonuclease 2	AF064257	616	652	6896.5	
285	OID_6039	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 4 (EDG4)	BE502246	617	652	5147	
286	OID 4210	IMAGE:4540096	AI300700	618	652	1330.5	
287	OID 7698	EST 3' end	AA243283	619	652	7432.5	1
288	PRKRA	Protein kinase, interferon-inducible double stranded RNA dependent activator	NM_003690	620	652	3512.5	
289	OID 4288	IMAGE:2091815	AI378046	621	651	6401.5	
290	OID 5620	EST 3' end from T cells	AW063678	622	651	6400	
291	OID 7384	EST 5'	BF475239	623	651	6875	
292	OID_1209	EST Weakly similar to hypothetical protein FLJ20378	C14379	624	651	1356.5	1
293	CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	NM_004064	625	650	4272.5	

(continued)

SEQ ID 50mer	Gene	Gene Name	ACC	SEQ ID RNA/cDNA	Non-Para Score	Median Rank in NR	Down Regulated
294	PLOD	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase, Ehlers-Danlos syndrome type VI)	NM_000302	626	650	3101	
295	OID 5128	EST	AK097845	627	650	6476	
296	OID 5877	EST 3' end	AW297664	628	650	6864.5	1
297	FZD4	Frizzled (Drosophila) homolog 4	NM_012193	629	650	5816	
298	HLA-B	Major histocompatibility complex, class I, B	NM_005514	630	650	229	
299	OID 5624	EST 3' end from T cells	AW063921	631	649	7812.5	
300	FPR1	Formyl peptide receptor 1	NM 002029	632	649	1156.5	
301	ODF2	Outer dense fiber of sperm tails 2	NM_153437	633	649	4982.5	
302	OID_5150	tg04g01.x1 cDNA, 3' end /clone=IMAGE:2107824	AI392793	634	649	7638	
303	OID 5639	EST 3' end from T cells	AW064243	635	648	6805	1
304	OID 6619	469A10	469A10	636	647	7110	1
305	OID 6933	463C7, 4 EST hits. Aligned	AI089520	637	647	6880.5	1
306	OID 7049	480E2	480E2	638	647	7128.5	
307	IL17C	Interleukin 17C	NM 013278	639	647	6411.5	
308	OID 5866	EST 3' end	BM684739	640	647	6532	1
309	CD44	CD44	AA916990	641	646	4758	
310	VPS45A	Vacuolar protein sorting 45A (yeast)	NM_007259	642	646	3371	
311	OID_4932	aa92c03.r1 Stratagene fetal retina 937202 cDNA clone IMAGE:838756	AA457757	643	646	6057 ,	1
312	OID 7821	EST	AA743221	644	645	7507	

(continued)

SEQ ID 50mer	Gene	Gene Name	ACC	SEQ ID RNA/cDNA	Non-Para Score	Median Rank in NR	Down Regulated
313	OID_4916	zr76a03.r1 Soares_NhHMPu_S1 cDNA clone IMAGE:669292	AA252909	645	645	6962.5	1
314	OID_4891	Hypothetical protein LOC255488 AL832329		646	645	6148.5	
315	HADHB	Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit	NM_000183	647	645	3212.5	
316	FLJ22757	Hypothetical protein FLJ22757	NM 024898	648	644	1965.5	1
317	RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	AK054993	649	644	1533	
318	OID 6415	72D4, FLJ00290 protein	CA407201	650	644	4881	
319	NMES1	Normal mucosa of esophagus specific 1	NM_032413	651	644	6217	1
320	DMBT1	Deleted in malignant brain tumors 1, transcript variant 2	NM_007329	652	644	7284	
321	RPS23	ribosomal protein S23	NM 001025	653	643	219.5	
322	ZF	HCF-binding transcription factor Zhangfei	NM_021212	654	643	4069	
323	NFE2L3	Nuclear factor (erythroid-derived 2)-like 3	NM_004289	655	643	3378	
324	RAD9	RAD9 homolog (S. pombe)	NM 004584	656	643	6453	
325	OID 6295	EST 3' end	AI880607	657	643	7493.5	
326	DEFCAP	Death effector filament-forming Ced-4-like apoptosis protein, transcript variant B	NM_014922	658	643	3059	

(continued)

SEQ ID 50mer	Gene	Gene Name	ACC	SEQ ID RNA/cDNA	Non-Para Score	Median Rank in NR	Down Regulated
327	RPL27A	Ribosomal protein L27a	BF214146	659	642	6571	1
328	IL22	Interleukin 22 (IL22)	NM 020525	660	642	3891	1
329	PSMA4	Proteasome (prosome, macropain) subunit, alpha type, 4, (PSMA4)	NM_002789	661	641	1934.5	
330	CCNI	cyclin I (CCNI)	NM 006835	662	641	980.5	
331	THBD	Thrombomodulin	NM 000361	663	640	4732.5	
332	CGR19	Cell growth regulatory with ring finger domain	NM_006568	664	640	5510	

Table 2C.

Gene	SEQ ID 50mer	SEQ ID RNA/cDNA	PCR Forward Primer 1 SEQ ID	PCR Reverse Primer 1 SEQ ID	PCR Probe 1 SEQ ID	PCR Forward Primer 2 SEQ ID	PCR Reverse Primer 2 SEQ ID	PCR Probe 2 SEQ ID
HSRRN18S	1	333	665	996	1327			
ACTB	2	334	666	997	1328			
GUSB	3	335	667	998	1329	1656	1904	2152
B2M	4	336	668	999	1330			
TSN	5	337	669	1000	1331	1657	1905	2153
CCR7	6	338	670	1001	1332			
IL1R2	7	339	671	1002	1333	1658	1906	2154
AIF-1	8	340	672	1003	1334			
ALAS2	9	341	673	1004	1335			
APELIN	10	342	674	1005	1336			
CD80	11	343	675	1006	1337	1659	1907	2145
EPB41	12	344	676	1007	1338			
CBLB	13	345	677	1008	1339	1660	1908	2156
CCR5	14	346	678	1009	1340	1661	1909	2157
MME	15	347	679	1010	1341	1662	1910	2158
KLRC1	16	348	680	1011	1342	1663	1911	2159
FCGR3A	17	349	681	1012	1343			
FCGR3B	18	350	682	1013	1344	1664	1912	2160
LAG3	19	351	683	1014	1345	1665	1913	2161
PECAM1	20	352	684	1015	1346	1666	1914	2162
CD34	21	353	685	1016	1347	1667	1915	2163
FCGR1A	22	354	686	1017	1348	1668	1916	2164
TFRC	23	355	687	1018	1349			
CMA1	24	356	688	1019	1350	1669	1917	2165
KIT	25	357	689	1020	1351			
MPL	26	358	690	1021	1352	1670	1918	2166
EphB6	27	359	691	1022	1353			
EPO-R	28	360	692	1023	1354			
Foxp3	29	361	693	1024	1355	1671	1919	2167
GATA-1	30	362	694	1025	1356			
ITGA2B	31	363	695	1026	1357	1672	1920	2168
GNLY	32	364	696	1027	1358	1673	1921	2169
GZMA	33	365	697	1028	1359	1674	1922	2170
HBA	34	366	698	1029	1360	1675	1923	2171
HBZ	35	367	699	1030	1361	1676	1924	2172

(continued)

Gene	SEQ ID 50mer	SEQ ID RNA/cDNA	PCR Forward Primer 1 SEQ ID	PCR Reverse Primer 1 SEQ ID	PCR Probe 1 SEQ ID	PCR Forward Primer 2 SEQ ID	PCR Reverse Primer 2 SEQ ID	PCR Probe 2 SEQ ID
HBB	36	368	700	1031	1362	1677	1925	2173
HBD	37	369	701	1032	1363	1678	1926	2174
HBE	38	370	702	1033	1364	1679	1927	2175
HBG	39	371	703	1034	1365	1680	1928	2176
HBQ	40	372	704	1035	1366	1681	1929	2177
HLA-DP	41	373	705	1036	1367	1682	1930	2178
HLA-DQ	42	374	706	1037	1368	1683	1931	2179
HLA-DRB	43	375	707	1038	1369	1684	1932	2180
ICOS	44	376	708	1039	1370	1685	1933	2181
IL18	45	377	709	1040	1371	1686	1934	2182
IL3	46	378	710	1041	1372	1687	1935	2183
ITGA4	47	379	711	1042	1373			
ITGAM	48	380	712	1043	1374	1688	1936	2184
ITGB7	49	381	713	1044	1375			
CEBPB	50	382	714	1045	1376	1689	1937	2185
NF-E2	51	383	715	1046	1377			
PDCD1	52	384	716	1047	1378	1690	1938	2186
PF4	53	385	717	1048	1379	1691	1939	2187
PRKCQ	54	386	718	1049	1380	1692	1940	2188
PPARGC1	55	387	719	1050	1381			
RAG1	56	388	720	1051	1382	1693	1941	2189
RAG2	57	389	721	1052	1383	1694	1942	2190
CXCL12	58	390	722	1053	1384	1695	1943	2191
TNFRSF4	59	391	723	1054	1385	1696	1944	2192
TNFSF4	60	392	724	1055	1386	1697	1945	2193
TPS1	61	393	725	1056	1387	1698	1946	2194
ADA	62	394	726	1057	1388	1699	1947	2195
CPM	63	395	727	1058	1389	1700	1948	2196
CSF2	64	396	728	1059	1390	1701	1949	2197
CSF3	65	397	729	1060	1391	1702	1950	2198
CRP	66	398	730	1061	1392	1703	1951	2199
FLT3	67	399	731	1062	1393	1704	1952	2200
GATA3	68	400	732	1063	1394	1705	1953	2201
IL7R	69	401	733	1064	1395	1706	1954	2202
KLF1	70	402	734	1065	1396	1707	1955	2203
LCK	71	403	735	1066	1397	1708	1956	2204

(continued)

Gene	SEQ ID 50mer	SEQ ID RNA/cDNA	PCR Forward Primer 1 SEQ ID	PCR Reverse Primer 1 SEQ ID	PCR Probe 1 SEQ ID	PCR Forward Primer 2 SEQ ID	PCR Reverse Primer 2 SEQ ID	PCR Probe 2 SEQ ID
LEF1	72	404	736	1067	1398	1709	1957	2205
PLAUR	73	405	737	1068	1399	1710	1958	2206
TNFSF13B	74	406	738	1069	1400	1711	1959	2207
IL8	75	407	739	1070	1401			
GZMB	76	408	740	1071	1402			
TNFSF6	77	409	741	1072	1403			
TCIRG1	78	410	742	1073	1404			
PRF1	79	411	743	1074	1405			
IL4	80	412	744	1075	1406			
IL13	81	413	745	1076	1407			
CTLA4	82	414	746	1077	1408			
CD8A	83	415	747	1078	1409			
BY55	84	416	748	1079	1410			
OID 4460	85	417	749	1080	1411			
HBB	86	418	750	1081	1412			
BPGM	87	419	751	1082	1413			
MTHFD2	88	420	752	1083	1414			
TAP1	89	421	753	1084	1415			
KPNA6	90	422	754	1085	1416			
OID 4365	91	423	755	1086	1417			
IGHM	92	424	756	1087	1418			
OID 573	93	425	757	1088	1419	1712	1960	2208
OID 873	94	426	758	1089	1420			
OID 3	95	427	759	1090	1421			
CXCR4	96	428	760	1091	1422			
CD69	97	429	761	1092	1423			
CCL5	98	430	762	1093	1424			
IL6	99	431	763	1094	1425			
IL2	100	432	764	1095	1426			
KLRF1	101	433	765	1096	1427			
LYN	102	434	766	1097	1428			
IL2RA	103	435	767	1098	1429			
CCL4	104	436	768	1099	1430			
OID 6207	105	437	769	1100	1431			
ChGn	106	438	770	1101	1432			
OID 4281	107	439	771	1102	1433			

(continued)

Gene	SEQ ID 50mer	SEQ ID RNA/cDNA	PCR Forward Primer 1 SEQ ID	PCR Reverse Primer 1 SEQ ID	PCR Probe 1 SEQ ID	PCR Forward Primer 2 SEQ ID	PCR Reverse Primer 2 SEQ ID	PCR Probe 2 SEQ ID
CXCL9	108	440	772	1103	1434			
CXCL10	109	441	773	1104	1435			
IL17	110	442	774	1105	1436			
IL15	111	443	775	1106	1437			
IL10	112	444	776	1107	1438			
IFNG	113	445	777	1108	1439	1713	1961	2209
HLA-DRB1	114	446	778	1109	1440	1714	1962	2210
CD8B1	115	447	779	1110	1441			
CD4	116	448	780	1111	1442			
CXCR3	117	449	781	1112	1443			
OID 7094	118	450	782	1113	1444			
OID 7605	119	451	783	1114	1445			
CXCL1	120	452	784	1115	1446			
OID 253	121	453	785	1116	1447			
GPI	122	454	786	1117	1448			
CD47	123	455	787	1118	1449			
HLA-F	124	456	788	1119	1450			
OID 5350	125	457	789	1120	1451			
TCRGC2	126	458	790	1121	1452			
OID 7016	127	459	791	1122				
PTGS2	128	460	792	1123	1454			
OID 5847	129	461	793	1124	1455			
PRDM1	130	462	794	1125	1456			
CKB	131	463	795	1126	1457			
TNNI3	132	464	796	1127	1458			
TNNT2	133	465	797	1128	1459			
MB	134	466	798	1129	1460			
SLC7A11	135	467	799	1130	1461			
TNFRSF5	136	468	800	1131	1462	1715	1963	2211
TNFRSF7	137	469	801	1132	1463			
CD86	138	470	802	1133	1464			
AIF1v2	139	471	803	1134	1465			
EV BCLF-1	140	472	804	1135	1466	1716	1964	2212
EV EBV	141	473	805	1136	1467	1717	1965	2213
CMV p67	142	474	806	1137	1468	1718	1966	2214
CMV TRL7	143	475	807	1138	1469	1719	1967	2215

(continued)

Gene	SEQ ID 50mer	SEQ ID RNA/cDNA	PCR Forward Primer 1 SEQ ID	PCR Reverse Primer 1 SEQ ID	PCR Probe 1 SEQ ID	PCR Forward Primer 2 SEQ ID	PCR Reverse Primer 2 SEQ ID	PCR Probe 2 SEQ ID
CMV IE1e3	144	476	808	1139	1470	1720	1968	2216
CMV IE1e4	145	477	809	1140	1471	1721	1969	2217
EV EBNA-1	146	478	810	1141	1472	1722	1970	2218
EV BZLF-1	147	479	811	1142	1473	1723	1971	2219
EV EBN	148	480	812	1143	1474	1724	1972	2220
EV EBNA-L 149		481	813	1144	1475			
CMV IE1	150	482	814	1145	1476	1725	1973	2221
CMV IE1	151	483	815	1146	1477			
CLC	152	484	816	1147	1478	1726	1974	2222
TERF2IP	153	485	817	1148	1479	1727	1975	2223
HLA-A	154	486	818	1149	1480	1728	1976	2224
OID 5891	155	487	819	1150	1481	1729	1977	2225
MSCP	156	488	820	1151	1482	1730	1978	2226
DUSP5	157	489	821	1152	1483	1731	1979	2227
PRO1853	158	490	822	1153	1484	1732	1980	2228
OID 6420	159	491	823	1154	1485	1733	1981	2229
CDSN	160	492	824	1155	1486	1734	1982	2230
OID 4269	161	493	825	1156	1487	1735	1983	2231
RPS25	162	494	826	1157	1488	1736	1984	2232
GAPD	163	495	827	1158	1489	1737	1985	2233
RPLP1	164	496	828	1159	1490	1738	1986	2234
OID 5115	165	497	829	1160	1491	1739	1987	2235
SLC9A8	166	498	830	1161	1492	1740	1988	2236
OID 1512	167	499	831	1162	1493	1741	1989	2237
POLR2D	168	500	832	1163	1494	1742	1990	2238
ARPC3	169	501	833	1164	1495	1743	1991	2239
OID 6282	170	502	834	1165	1496	1744	1992	2240
PRO1073	171	503	835	1166	1497	1745	1993	2241
OID 7222	172	504	836	1167	1498	1746	1994	2242
FPRL1	173	505	837	1168	1499	1747	1995	2243
FKBPL	174	506	838	1169	1500	1748	1996	2244
PREB	175	507	839	1170	1501	1749	1997	2245
OID 1551	176	508	840	1171	1502	1750	1998	2246
OID 7595	177	509	841	1172	1503	1751	1999	2247
RNF19	178	510	842	1173	1504	1752	2000	2248

(continued)

Gene	SEQ ID 50mer	SEQ ID RNA/cDNA	PCR Forward Primer 1 SEQ ID	PCR Reverse Primer 1 SEQ ID	PCR Probe 1 SEQ ID	PCR Forward Primer 2 SEQ ID	PCR Reverse Primer 2 SEQ ID	PCR Probe 2 SEQ ID
SMCY	179	511	843	1174	1505	1753	2001	2249
OID 4184	180	512	844	1175	1506	1754	2002	2250
OID 7504	181	513	845	1176	1507	1755	2003	2251
DNAJC3	182	514	846	1177	1508	1756	2004	2252
ARHU	183	515	847	1178	1509	1757	2005	2253
OID 7200	184	516	848	1179	1510	1758	2006	2254
SERPINB2	185	517	849	1180	1511			
ENO1	186	518	850	1181	1512	1759	2007	2255
OID 7696	187	519	851	1182	1513	1760	2008	2256
OID 4173	188	520	852	1183	1514	1761	2009	2257
CSF2RB	189	521	853	1184	1515	1762	2010	2258
OID 7410	190	522	854	1185	1516	1763	2011	2259
OID 4180	191	523	855	1186	1517	1764	2012	2260
OID 5101	192	524	856	1187	1518	1765	2013	2261
MOP3	193	525	857	1188	1519	1766	2014	2262
RPL18A	194	526	858	1189	1520	1767	2015	2263
INPP5A	195	527	859	1190	1521	1768	2016	2264
hIAN7	196	528	860	1191	1522	1769	2017	2265
RPS29	197	529	861	1192	1523	1770	2018	2266
OID 6008	198	530	862	1193	1524	1771	2019	2267
OID 4186	199	531	863	1194	1525	1772	2020	2268
VNN2	200	532	864	1195	1526	1773	2021	2269
OID 7703	201	533	865	1196	1527	1774	2022	2270
OID 7057	202	534	866	1197	1528	1775	2023	2271
OID 4291	203	535	867	1198	1529	1776	2024	2272
OID 1366	204	536	868	1199	1530	1777	2025	2273
EEF1A1	205	537	869	1200	1531	1778	2026	2274
PA2G4	206	538	870	1201	1532	1779	2027	2275
GAPD	207	539	871	1202	1533	1780	2028	2276
CHD4	208	540	872	1203	1534	1781	2029	2277
OID 7951	209	541	873	1204	1535	1782	2030	2278
DAB1	210	542	874	1205	1536	1783	2031	2279
OID 3406	211	543	875	1206	1537	1784	2032	2280
OID 6986	212	544	876	1207	1538	1785	2033	2281
OID 5962	213	545	877	1208	1539	1786	2034	2282
OID 5152	214	546	878	1209	1540	1787	2035	2283

(continued)

Gene	SEQ ID 50mer	SEQ ID RNA/cDNA	PCR Forward Primer 1 SEQ ID	PCR Reverse Primer 1 SEQ ID	PCR Probe 1 SEQ ID	PCR Forward Primer 2 SEQ ID	PCR Reverse Primer 2 SEQ ID	PCR Probe 2 SEQ ID
S100A8	215	547	879	1210	1541	1788	2036	2284
HNRPU	216	548	880	1211	1542	1789	2037	2285
ERCC5	217	549	881	1212	1543	1790	2038	2286
RPS27	218	550	882	1213	1544	1791	2039	2287
ACRC	219	551	883	1214	1545	1792	2040	2288
PSMD11	220	552	884	1215	1546	1793	2041	2289
OID 1016	221	553	885	1216	1547	1794	2042	2290
OID 1309	222	554	886	1217	1548	1795	2043	2291
OID 7582	223	555	887	1218	1549	1796	2044	2292
OID 4317	224	556	888	1219	1550	1797	2045	2293
OID 5889	225	557	889	1220	1551	1798	2046	2294
UBL1	226	558	890	1221	1552	1799	2047	2295
OID 3687	227	559	891	1222	1553	1800	2048	2296
OID 7371	228	560	892	1223	1554	1801	2049	2297
SH3BGRL3	229	561	893	1224	1555	1802	2050	2298
SEMA7A	230	562	894	1225	1556	1803	2051	2299
OID 5708	231	563	895	1226	1557	1804	2052	2300
OID 5992	232	564	896	1227	1558	1805	2053	2301
IL21	233	565	897	1228	1559	1806	2054	2302
HERC3	234	566	898	1229	1560	1807	2055	2303
OID 7799	235	567	899	1230	1561	1808	2056	2304
P11	236	568	900	1231	1562	1809	2057	2305
OID 7766	237	569	901	1232	1563	1810	2058	2306
TIMM10	238	570	902	1233	1564	1811	2059	2307
EGLN1	239	571	903	1234	1565	1812	2060	2308
TBCC	240	572	904	1235	1566	1813	2061	2309
RNF3	241	573	905	1236	1567	1814	2062	2310
OID 6451	242	574	906	1237	1568	1815	2063	2311
CCNDBP1	243	575	907	1238	1569	1816	2064	2312
OID 8063	244	576	908	1239	1570	1817	2065	2313
SUV39H1	245	577	909	1240	1571	1818	2066	2314
HSPC048	246	578	910	1241	1572	1819	2067	2315
OID 5625	247	579	911	1242	1573	1820	2068	2316
WARS	248	580	912	1243	1574	1821	2069	2317
OID 6823	249	581	913	1244	1575	1822	2070	2318
OID 7073	250	582	914	1245	1576	1823	2071	2319

(continued)

Gene	SEQ ID 50mer	SEQ ID RNA/cDNA	PCR Forward Primer 1 SEQ ID	PCR Reverse Primer 1 SEQ ID	PCR Probe 1 SEQ ID	PCR Forward Primer 2 SEQ ID	PCR Reverse Primer 2 SEQ ID	PCR Probe 2 SEQ ID
OID 5339	251	583	915	1246	1577	1824	2072	2320
OID 4263	252	584	916	1247	1578	1825	2073	2321
MGC26766	253	585	917	1248	1579	1826	2074	2322
SERPINB11	254	586	918	1249	1580	1827	2075	2323
OID 6711	255	587	919	1250	1581	1828	2076	2324
RNF10	256	588	920	1251	1582	1829	2077	2325
MKRN1	257	589	921	1252	1583	1830	2078	2326
RPS16	258	590	922	1253	1584	1831	2079	2327
BAZ1A	259	591	923	1254	1585	1832	2080	2328
OID 5998	260	592	924	1255	1586	1833	2081	2329
ATP5L	261	593	925	1256	1587	1834	2082	2330
OID 6393	262	594	926	1257	1588			
RoXaN	263	595	927	1258	1589	1835	2083	2331
NCBP2	264	596	928	1259	1590	1836	2084	2332
OID 6273	265	597	929	1260	1591	1837	2085	2333
HZF12	266	598	930	1261	1592	1838	2086	2334
CCL3	267	599	931	1262	1593	1839	2087	2335
OID 4323	268	600	932	1263	1594	1840	2088	2336
OID 5181	269	601						
PRDX4	270	602	933	1264	1595	1841	2089	2337
BTK	271	603	934	1265	1596	1842	2090	2338
OID 6298	272	604	935	1266	1597	1843	2091	2339
PGK1	273	605	936	1267	1598	1844	2092	2340
TNFRSF10A	274	606	937	1268	1599	1845	2093	2341
ADM	275	607	938	1269	1600	1846	2094	2342
OID 357	276	608	939	1270	1601	1847	2095	2343
C20orf6	277	609	940	1271	1602	1848	2096	2344
OID 3226	278	610	941	1272	1603	1849	2097	2345
ASAH1	279	611	942	1273	1604	1850	2098	2346
ATF5	280	612	943	1274	1605	1851	2099	2347
OID 4887	281	613	944	1275	1606	1852	2100	2348
OID 4239	282	614	945	1276	1607	1853	2101	2349
MDM2	283	615	946	1277	1608	1854	2102	2350
XRN2	284	616	947	1278	1609	1855	2103	2351
OID 6039	285	617	948	1279	1610	1856	2104	2352
OID 4210	286	618	949	1280	1611	1857	2105	2353

(continued)

Gene	SEQ ID 50mer	SEQ ID RNA/cDNA	PCR Forward Primer 1 SEQ ID	PCR Reverse Primer 1 SEQ ID	PCR Probe 1 SEQ ID	PCR Forward Primer 2 SEQ ID	PCR Reverse Primer 2 SEQ ID	PCR Probe 2 SEQ ID
OID 7698	287	619	950	1281	1612	1858	2106	2354
PRKRA	288	620	951	1282	1613	1859	2107	2355
OID 4288	289	621	952	1283	1614	1860	2108	2356
OID 5620	290	622	953	1284	1615	1861	2109	2357
OID 7384	291	623	954	1285	1616	1862	2110	2358
OID 1209	292	624	955	1286	1617	1863	2111	2359
CDKN1B	293	625	956	1287	1618	1864	2112	2360
PLOD	294	626	957	1288	1619	1865	2113	2361
OID 5128	295	627	958	1289	1620	1866	2114	2362
OID 5877	296	628	959	1290	1621	1867	2115	2363
FZD4	297	629	960	1291	1622	1868	2116	2364
HLA-B	298	630	961	1292	1623	1869	2117	2365
OID 5624	299	631	962	1293	1624	1870	2118	2366
FPR1	300	632	963	1294	1625	1871	2119	2367
ODF2	301	633	964	1295	1626	1872	2120	2368
OID 5150	302	634	965	1296	1627	1873	2121	2369
OID 5639	303	635	966	1297	1628	1874	2122	2370
OID 6619	304	636	967	1298	1629	1875	2123	2371
OID 6933	305	637	968	1299	1630	1876	2124	2372
OID 7049	306	638	969	1300	1631	1877	2125	2373
IL17C	307	639	970	1301	1632	1878	2126	2374,
OID 5866	308	640	971	1302	1633	1879	2127	2375
CD44	309	641	972	1303	1634	1880	2128	2376
VPS45A	310	642	973	1304	1635	1881	2129	2377
OID 4932	311	643	974	1305	1636	1882	2130	2378
OID 7821	312	644	975	1306	1637	1883	2131	2379
OID 4916	313	645	976	1307	1638	1884	2132	2380
OID 4891	314	646	977	1308	1639	1885	2133	2381
HADHB	315	647	978	1309	1640	1886	2134	2382
FLJ22757	316	648	979	1310	1641	1887	2135	2383
RAC1	317	649	980	1311	1642	1888	2136	2384
OID 6415	318	650	981	1312	1643	1889	2137	2385
NMES1	319	651	982	1313	1644	1890	2138	2386
DMBT1	320	652	983	1314	1645	1891	2139	2387
RPS23	321	653	984	1315	1646	1892	2140	2388
ZF	322	654	985	1316	1647	1893	2141	2389

(continued)

Gene	SEQ ID 50mer	SEQ ID RNA/cDNA	PCR Forward Primer 1 SEQ ID	PCR Reverse Primer 1 SEQ ID	PCR Probe 1 SEQ ID	PCR Forward Primer 2 SEQ ID	PCR Reverse Primer 2 SEQ ID	PCR Probe 2 SEQ ID
NFE2L3	323	655	986	1317	1648	1894	2142	2390
RAD9	324	656	987	1318	1649	1895	2143	2391
OID 6295	325	657	988	1319	1650	1896	2144	2392
DEFCAP	326	658	989	1320	1651	1897	2145	2393
RPL27A	327	659	990	1321	1652	1898	2146	2394
IL22	328	660	991	1322	1653	1899	2147	2395
PSMA4	329	661	992	1323	1654	1900	2148	2396
CCNI	330	662	993	1324	1655	1901	2149	2397
THBD	331	663	994	1325	1656	1902	2150	2398
CGR19	332	664	995	1326	1657	1903	2151	2399

Table 2D.

Gene	Gene Name	SEQ ID 50mer	SEQ ID RNA/cDNA	n	Non-parametric Odds ratio	Fisher p-value	t-test p-value
HBB	Hemoglobin, beta	86	418	55	8.33	0.00	0.00
OID_4365	Mitochondrial solute carrier	91	423	53	6.16	0.00	0.00
OID 873	KIAA1892 protein	94	426	55	5.09	0.01	0.01
IL4	Interleukin 4	80	412	46	4.90	0.02	0.01
OID 4281	EST	107	439	56	5.19	0.01	0.01
IGHM	Immunoglobulin heavy constant mu	92	424	52	2.89	0.09	0.01
BPGM	2,3-bisphosphoglycerate mutase	87	419	43	7.31	0.01	0.01
CTLA4	Cytotoxic T-lymphocyte-associated protein 4	82	414	52	1.84		0.02
SLC7A11	Solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	135	467	48	2.50	0.15	0.03
IL13	Interleukin 13	81	413	29	4.95	0.07	0.04
OID 6207	EST	105	437	37	3.58	0.10	0.04
PRDM1	PR domain containing 1, with ZNF domain	130	462	57	1.44		0.07
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	102	434	55	1.08		0.08
KPNA6	Karyopherin alpha 6 (importin alpha 7)	90	422	51	1.50		0.09

(continued)

Gene	Gene Name	SEQ ID 50mer	SEQ ID RNA/cDNA	n	Non-parametric Odds ratio	Fisher p-value	t-test p-value
OID 7094	XDx EST 479G12	118	450	35	1.13		0.09
IL15	Interleukin 15	111	443	51	3.78	0.05	0.09
OID 4460	EST	85	417	47	2.73	0.14	0.10
OID 7016	EST	127	459	53	2.14	0.27	0.10
MTHFD2	Methylene tetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohytirolase	88	420	43	3.50	0.07	0.11
TCIRG1	T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 protein a isoform 3	78	410	57	1.08		0.11
OID_5847	Hypothetical protein FLJ32919	129	461	45	1.08		0.12
CXCR4	Chemokine (C-X-C motif	96	428	56	1.29		0.12
CXCR3	Chemokine (C-X-C motif	117	449	54	2.10	0.27	0.12
GPI	Glucose phosphate isome	122	454	57	1.44	0.60	0.12
KLRF1	Killer cell lectin-like rece	101	433	50	1.68		0.13
CCL5	Chemokine(C-C motif)	1 98	430	34	1.96		0.13
CD47	CD47 antigen (Rh-related	123	455	55	1.45		0.13
IL10	Interleukin 10	112	444	33	1.43		0.13
OID 253	EST	121	453	26	1.93		0.15
CXCL10	Chemokine (C-X-C motif 109		441	53	1.75		0.16
IFNG	Interferon, gamma	113	445	41	1.33		0.16
PRF1	Perforin 1 (pore forming	79	411	48	1.20		0.17
IL2	Interleukin 2	100	432	33	2.00		0.17
HLA-DRB1	Major histocompatibility	114	446	42	1.50		0.18
IL6	Interleukin 6	99	431	49	1.33		0.18
IL2RA	Interleukin 2 receptor, alpha	103	435	39	2.03	0.34	0.19
OID 573	KIAA1486 protein	93	425	8	3.00		0.19
CXCL9	Chemokine (C-X-C motif) ligand 9 (MIG)	108	440	46	1.71		0.20
OID 3	EST	95	427	49	2.19		0.20
CD8B1	CD8 antigen, beta polypeptide 1 (p37)	115	447	55	1.21		0.22
CD69	CD69 antigen (p60, early T-cell activation antigen)	97	429	30	1.71		0.23
OID 7605	EST	119	451	47	3.11	0.08	0.24

(continued)

Gene	Gene Name	SEQ ID 50mer	SEQ ID RNA/cDNA	n	Non-parametric Odds ratio	Fisher p-value	t-test p-value
TNFSF6	Tumor necrosis factor (ligand) superfamily, member 6	77	409	54	1.36		0.25
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	120	452	20	2.00		0.26
OID 5350	EST	125	457	49	2.08	0.26	0.28
CD8A	CD8 antigen, alpha polypeptide (p32)	83	415	57	1.39		0.28
CD4	CD4 antigen (p55)	116	448	55	1.64		0.28
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	128	460	46	2.05	0.37	0.29
GZMB	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	76	408	40	1.81		0.33
CCL4	Chemokine (C-C motif) ligand 4, SCYA4	104	436	53	2.25		0.35
ChGn	Chondroitin beta 1,4 N-acetylgalactosaminyltransferase	106	438	31	2.57		0.36
TCRGC2	T cell receptor gamma constant 2	126	458	52	1.33		0.39
HLA-F	Major histocompatibility complex, class I, F	124	456	54	2.36	0.17	0.40
TAP1	Transporter 1, ATP-binding cassette, sub-family B (MDR1/TAP)	89	421	36	1.93		0.45
BY55	Natural killer cell receptor, immunoglobulin superfamily member	84	416	52	2.49	0.16	0.48
IL8	Interleukin 8	75	407	49	2.10	0.26	0.49

Table 2E.

Gene	ACC	SEQ ID 50mer	SEQ ID RNA/cDNA	RefSeq Peptide Accession #	SEQ ID Protein
ACTB	NM 001101	2	334	NP 001092	2400
GUSB	NM 000181	3	335	NP 000172	2401
B2M	NM 004048	4	336	NP 004039	2402
TSN	NM 004622	5	337	NP 004613	2403
CCR7	NM 001838	6	338	NP 001829	2404

(continued)

Gene	ACC	SEQ ID 50mer	SEQ ID RNA/cDNA	RefSeq Peptide Accession #	SEQ ID Protein
IL1R2	NM 004633	7	339	NP 004624	2405
AIF-1	NM 004847	8	340	NP 004838	2406
ALAS2	NM 000032.1	9	341	NP 000023	2407
APELIN	NM 017413	10	342	NP 059109	2408
CD80	NM 005191	11	343	NP 005182	2409
EPB41	NM 004437	12	344	NP 004428	2410
CBLB	NM 004351	13	345	NP 733762	2411
CCR5	NM 000579	14	346	NP 000570	2412
MME	NM 000902	15	347	NP 000893	2413
KLRC1	NM 002259	16	348	NP 002250	2414
FCGR3A	NM 000569	17	349	NP 000560	2415
FCGR3B	NM 000570	18	350	NP 000561	2416
LAG3	NM 002286	19	351	NP 002277	2417
PECAM1	NM 000442	20	352	NP 000433	2418
CD34	NM 001773	21	353	NP 001764	2419
FCGR1A	NM 000566	22	354	NP 000557	2420
TFRC	NM 003234	23	355	NP 003225	2421
CMA1	NM 001836	24	356	NP 001827	2422
KIT	NM 000222	25	357	NP 000213	2423
MPL	NM 005373	26	358	NP 005364	2424
EphB6	NM 004445	27	359	NP 004436	2425
EPO-R	NM 000121.2	28	360	NP 000112	2426
Foxp3	NM 014009	29	361	NP 054728	2427
GATA-1	NM 002049	30	362	NP 002040	2428
ITGA2B	NM 000419	31	363	NP 000410	2429
GNLY	NM 006433	32	364	NP 006424	2430
GZMA	NM 006144	33	365	NP 006135	2431
HBA	NM 000558.3	34	366	NP 000549	2432
HBZ	NM 005332.2	35	367	NP 005323	2433
HBD	NM 000519.2	37	369	NP 000510	2434
HBE	NM 005330	38	370	NP 005321	2435
HBG	NM 000559.2	39	371	NP 000550	2436
HBQ	NM 005331	40	372	NP 005322	2437
HLA-DP	NM 033554	41	373	NP 291032	2438
HLA-DQ	NM 002122	42	374	NP 002113	2439
ICOS	NM 012092	44	376	NP 036224	2440
IL18	NM 001562	45	377	NP 001553	2441

(continued)

Gene	ACC	SEQ ID 50mer	SEQ ID RNA/cDNA	RefSeq Peptide Accession #	SEQ ID Protein
IL3	NM 000588	46	378	NP 000579	2442
ITGA4	NM 000885	47	379	NP 000876	2443
ITGAM	NM 000632	48	380	NP 000623	2444
ITGB7	NM 000889	49	381	NP 000880	2445
CEBPB	NM 005194	50	382	NP 005185	2446
NF-E2	NM 006163	51	383	NP 006154	2447
PDCD1	NM 005018	52	384	NP 005009	2448
PF4	NM 002619	53	385	NP 002610	2449
PRKCQ	NM 006257.1	54	386	NP 006248	2450
PPARGC1	NM 013261	55	387	NP 037393	2451
RAG1	NM 000448	56	388	NP 000439	2452
RAG2	NM 000536	57	389	NP 000527	2453
CXCL12	NM 000609	58	390	NP 000600	2454
TNFRSF4	NM 003327	59	391	NP 003318	2455
TNFSF4	NM 003326	60	392	NP 003317	2456
TPS1	NM 003293	61	393	NP 003284	2457
ADA	NM 000022	62	394	NP 000013	2458
CPM	NM 001874.1	63	395	NP 001865	2459
CSF2	NM 000758.2	64	396	NP 000749	2460
CSF3	NM 172219	65	397	NP 757373	2461
CRP	NM 000567.1	66	398	NP 000558	2462
FLT3	NM 004119	67	399	NP 004110	2463
GATA3	NM 002051.1	68	400	NP 002042	2464
IL7R	NM 002185.1	69	401	NP 002176	2465
KLF1	NM 006563.1	70	402	NP 006554	2466
LCK	NM 005356.2	71	403	NP 005347	2467
LEF1	NM 016269.2	72	404	NP 057353	2468
PLAUR	NM 002659.1	73	405	NP 002650	2469
TNFSF13B	NM 006573.3	74	406	NP 006564	2470
IL8	NM 000584	75	407	NP 000575	2471
GZMB	NM 004131	76	408	NP 004122	2472
TNFSF6	NM 000639	77	409	NP 000630	2473
TCIRG1	NM 006019	78	410	NP 006010	2474
PRF1	NM 005041	79	411	NP 005032	2475
IL4	NM 000589	80	412	NP 000580	2476
IL13	NM 002188	81	413	NP 002179	2477
CTLA4	NM 005214	82	414	NP 005205	2478

(continued)

Gene	ACC	SEQ ID 50mer	SEQ ID RNA/cDNA	RefSeq Peptide Accession #	SEQ ID Protein
CD8A	NM 001768	83	415	NP 001759	2479
BY55	NM 007053	84	416	NP 008984	2480
HBB	NM 000518	86	418	NP 000509	2481
BPGM	NM 001724	87	419	NP 001715	2482
MTHFD2	NM 006636	88	420	NP 006627	2483
TAP1	NM 000593	89	421	NP 000584	2484
OID 873	AK000354	94	426	NP 056212	2485
CXCR4	NM 003467	96	428	NP 003458	2486
CD69	NM 001781	97	429	NP 001772	2487
CCL5	NM 002985	98	430	NP 002976	2488
IL6	NM 000600	99	431	NP 000591	2489
IL2	NM 000586	100	432	NP 000577	2490
KLRF1	NM 016523	101	433	NP 057607	2491
LYN	NM 002350	102	434	NP 002341	2492
IL2RA	NM 000417	103	435	NP 000408	2493
CCL4	NM 002984	104	436	NP 002975	2494
ChGn	NM 018371	106	438	NP 060841	2495
CXCL9	NM 002416	108	440	NP 002407	2496
CXCL10	NM 001565	109	441	NP 001556	2497
IL17	NM 002190	110	442	NP 002181	2498
IL15	NM 000585	111	443	NP 000576	2499
IL10	NM 000572	112	444	NP 000563	2500
IFNG	NM 000619	113	445 NP	000610	2501
HLA-DRB1	NM 002124	114	446	NP 002115	2502
CD8B1	NM 004931	115	447	NP 004922	2503
CD4	NM 000616	116	448	NP 000607	2504
CXCR3	NM 001504	117	449	NP 001495	2505
CXCL1	NM 001511	120	452	NP 001502	2506
GPI	NM 000175	122	454	NP 000166	2507
CD47	NM 001777	123	455	NP 001768	2508
HLA-F	NM 018950	124	456	NP 061823	2509
PTGS2	NM 000963	128	460	NP 000954	2510
OID 5847	NM 144588	129	461	NP 653189	2511
PRDM1	NM 001198	130	462	NP 001189	2512
CKB	NM 001823	131	463	NP 001814	2513
TNNI3	NM 000363	132	464	NP 000354	2514
TNNT2	NM 000364	133	465	NP 000355	2515

(continued)

Gene	ACC	SEQ ID 50mer	SEQ ID RNA/cDNA	RefSeq Peptide Accession #	SEQ ID Protein
MB	NM 005368	134	466	NP 005359	2516
SLC7A11	NM 014331	135	467	NP 055146	2517
TNFRSF5	NM 001250	136	468	NP 001241	2518
TNFRSF7	NM 001242	137	469	NP 001233	2519
CD86	NM 175862	138	470	NP 787058	2520
AIF1v2	NM 004847	139	471	NP 004838	2521
CMV IE1e3	NC 001347, compl	144	476	NP 040060	2522
CMV IE1e4	NC 001347, compl	145	477	NP 040060	2523
EV EBNA-1	NC 001345, 10795	146	478	NP 039875	2524
EV BZLF-1	NC 001345, compl	147	479	NP 039871	2525
CMV IE1	NC 001347, compl	150	482	NP 040060	2526
CMV IE1	NC 001347, compl	151	483	NP 040060	2527
CLC	NM 001828	152	484	NP 001819	2528
TERF2IP	NM 018975	153	485	NP 061848	2529
HLA-A	NM 002116	154	486	NP 002107	2530
MSCP	NM 018579	156	488	NP 061049	2531
DUSP5	NM 004419	157	489	NP 004410	2532
PRO1853	NM 018607	158	490	NP 061077	2533
CDSN	NM 001264	160	492	NP 001255	2534
RPS25	NM 001028	162	494	NP 001019	2535
GAPD	NM 002046	163	495	NP 002037	2536
RPLP1	NM 001003	164	496	NP 000994	2537
POLR2D	NM 004805	168	500	NP. 004796	2538
ARPC3	NM 005719	169	501	NP 005710	2539
FPRL1	NM 001462	173	505	NP 001453	2540
FKBPL	NM 022110	174	506	NP 071393	2541
PREB	NM 013388	175	507	NP 037520	2542
OID 7595	NM 015653	177	509	NP 056468	2543
RNF19	NM 015435	178	510	NP 056250	2544
SMCY	NM 004653	179	511	NP 004644	2545
OID 7504	NM 152312	181	513	NP 689525	2546
DNAJC3	NM 006260	182	514	NP 006251	2547
ARHU	NM 021205	183	515	NP 067028	2548
OID 7200	NM 022752	184	516	NP 073589	2549
SERPINB2	NM 002575	185	517	NP 002566	2550
ENO1	NM 001428	186	518	NP 001419	2551
MOP3	NM 018183	193	525	NP 060653	2552

(continued)

Gene	ACC	SEQ ID 50mer	SEQ ID RNA/cDNA	RefSeq Peptide Accession #	SEQ ID Protein
RPL18A	NM 000980	194	526	NP 000971	2553
INPP5A	NM 005539	195	527	NP 005530	2554
RPS29	NM 001032	197	529	NP 001023	2555
VNN2	NM 004665	200	532	NP 004656	2556
OID 7703	NM 014949	201	533	NP 055764	2557
EEF1A1	NM 001402	205	537	NP 001393	2558
PA2G4	NM 006191	206	538	NP 006182	2559
GAPD	NM 002046	207	539	NP 002037	2560
CHD4	NM 001273	208	540	NP 001264	2561
OID 7951	NM 016521	209	541	NP 057605	2562
DAB1	NM 021080	210	542	NP 066566	2563
OID 3406	NM 018986	211	543	NP 061859	2564
S100A8	NM 002964	215	547	NP 002955	2565
ERCC5	NM 000123	217	549	NP 000114	2566
RPS27	NM 001030	218	550	NP 001021	2567
ACRC	NM 052957	219	551	NP 443189	2568
UBL1	NM 003352	226	558	NP 003343	2569
SH3BGRL3	NM 031286	229	561	NP 112576	2570
SEMA7A	NM 003612	230	562	NP 003603	2571
IL21	NM 021803	233	565	NP 068575	2572
HERC3	NM 014606	234	566	NP 055421	2573
P11	NM 006025	236	568	NP 006016	2574
TIMM10	NM 012456	238	570	NP 036588	2575
EGLN1	AJ310543	239	571	NP 071334	2576
TBCC	NM 003192	240	572	NP 003183	2577
RNF3	NM 006315	241	573	NP 006306	2578
CCNDBP1	NM 012142	243	575	NP 036274	2579
SUV39H1	NM 003173	245	577	NP 003164	2580
HSPC048	NM 014148	246	578	NP 054867	2581
WARS	NM 004184	248	580	NP 004175	2582
SERPINB11	NM 080475	254	586	NP 536723	2583
RNF10	NM 014868	256	588	NP 055683	2584
MKRN1	NM 013446	257	589	NP 038474	2585
RPS16	NM 001020	258	590	NP 001011	2586
BAZ1A	NM 013448	259	591	NP 038476	2587
ATP5L ,	NM 006476	261	593	NP 006467	2588
NCBP2	NM 007362	264	596	NP 031388	2589

(continued)

Gene	ACC	SEQ ID 50mer	SEQ ID RNA/cDNA	RefSeq Peptide Accession #	SEQ ID Protein
HZF12	NM 033204	266	598	NP 149981	2590
CCL3	D90144	267	599	NP 002974	2591
PRDX4	NM 006406	270	602	NP 006397	2592
BTK	NM 000061	271	603	NP 000052	2593
PGK1	NM 000291	273	605	NP 000282	2594
TNFRSF10A	NM 003844	274	606	NP 003835	2595
ADM	NM 001124	275	607	NP 001115	2596
C20orf6	NM 016649	277	609	NP 057733	2597
OID 3226	NM 015393	278	610	NP 056208	2598
ASAH1	NM 004315	279	611	NP 004306	2599
ATF5	NM 012068	280	612	NP 036200	2600
OID 4887	NM 032895	281	613	NP 116284	2601
MDM2	NM 002392	283	615	NP 002383	2602
XRN2	AF064257	284	616	NP 036387	2603
PRKRA	NM 003690	288	620	NP 003681	2604
CDKN1B	NM 004064	293	625	NP 004055	2605
PLOD	NM 000302	294	626	NP 000293	2606
FZD4	NM 012193	297	629	NP 036325	2607
HLA-B	NM 005514	298	630	NP 005505	2608
FPR1	NM 002029	300	632	NP 002020	2609
ODF2	NM 153437	301	633	NP 702915	2610
IL17C	NM 013278	307	639	NP 037410	2611
VPS45A	NM 007259	310	642	NP 009190	2612
HADHB	NM 000183	315	647	NP 000174	2613
FLJ22757	NM 024898	316	648	NP 079174	2614
NMES1	NM 032413	319	651	NP 115789	2615
DMBT1	NM 007329	320	652	NP 015568	2616
RPS23	NM 001025	321	653	NP 001016	2617
ZF	NM 021212	322	654	NP 067035	2618
NFE2L3	NM 004289	323	655	NP 004280	2619
RAD9	NM 004584	324	656	NP 004575	2620
DEFCAP	NM 014922	326	658	NP 055737	2621
IL22	NM 020525	328	660	NP 065386	2622
PSMA4	NM 002789	329	661	NP 002780	2623
CCNI	NM 006835	330	662	NP 006826	2624
THBD	NM 000361	331	663	NP 000352	2625
CGR19	NM 006568	332	664	NP 006559	2626

(continued)

Gene	ACC	SEQ ID 50mer	SEQ ID RNA/cDNA	RefSeq Peptide Accession #	SEQ ID Protein
HSRRN18S	X03205	1	333		
HBB	NG 000007	36	368		
HLA-DRB		43	375		
OID 4460	AF150295	85	417		
KPNA6	AW021037	90	422		
OID 4365	AI114652	91	423		
IGHM	BC032249	92	424		
OID 573	AB040919	93	425		
OID 3	AW968823	95	427		
OID 6207	D20522	105	437		
OID 4281	AA053887	107	439		
OID 7094		118	450		
OID 7605	AA808018	119	451		
OID 253	AK091125	121	453		
OID 5350	AK055687	125	457		
TCRGC2	M17323	126	458		
OID 7016	BI018696	127	459		
EV EBV		141	473		
CMV p67	NC 001347	142	474		
CMV TRL7		143	475		
EV EBN		148	480		
EV EBNA-LP		149	481		
OID 5891	AW297949	155	487		
OID 6420	AK090404	159	491		
OID 4269	BM727677	161	493		
OID 5115	AI364926	165	497		
SLC9A8	AB023156	166	498		
OID 1512	BE618004	167	499		
OID 6282	BC041913	170	502		
PRO1073	AF001542	171	503		
OID 7222	BG260891	172	504		
OID 1551	BE887646	176	508		
OID 4184	X17403	180	512		
OID 7696	AW297325	187	519		
OID 4173	X17403	188	520		
CSF2RB	AL540399	189	521		
OID 7410	AW837717	190	522		

(continued)

Gene	ACC	SEQ ID 50mer	SEQ ID RNA/cDNA	RefSeq Peptide Accession #	SEQ ID Protein
OID 4180	X17403	191	523		
OID 5101	BG461987	192	524		
hIAN7	BG772661	196	528		
OID 6008	AW592876	198	530		
OID 4186	X17403	199	531		
OID 7057	480F8	202	534		
OID 4291	BC038439	203	535		
OID 1366	AW850041	204	536		
OID 6986	AK093608	212	544		
OID 5962	AW452467	213	545		
OID 5152	AI392805	214	546		
HNRPU	BM467823	216	548		
PSMD11	AI684022	220	552		
OID 1016	AK024456	221	553		
OID 1309	AV706481	222	554		
OID 7582	AK027866	223	555		
OID 4317	AI318342	224	556		
OID 5889	AW297843	225	557		
OID 3687	W03955	227	559		
OID 7371	BE730505	228	560		
OID 5708	AW081540	231	563		
OID 5992	AW467992	232	564		
OID 7799	AW837717	235	567		
OID 7766	AW294711	237	569		
OID 6451	AL834168	242	574		
OID 8063	X68264	244	576		
OID 5625	AW063780	247	579		
OID 6823	AL832642	249	581		
OID 7073	AL705961	250	582		
OID 5339	AI625119	251	583		
OID 4263	AA136584	252	584		
MGC26766	AK025472	253	585		
OID 6711	BF968628	255	587		
OID 5998	AW468459	260	592		
OID 6393	52B9	262	594		
RoXaN	BC004857	263	595		
OID 6273	AW294774	265	597		

(continued)

Gene	ACC	SEQ ID 50mer	SEQ ID RNA/cDNA	RefSeq Peptide Accession #	SEQ ID Protein
OID 4323	AA744774	268	600		
OID 5181	AI400725	269	601		
OID 6298	AI948513	272	604		
OID 357	138G5	276	608		
OID 4239	BQ022840	282	614		
OID 6039	BE502246	285	617		
OID 4210	AI300700	286	618		
OID 7698	AA243283	287	619		
OID 4288	AI378046	289	621		
OID 5620	AW063678	290	622		
OID 7384	BF475239	291	623		
OID 1209	C14379	292	624		
OID 5128	AK097845	295	627		
OID 5877	AW297664	296	628		
OID 5624	AW063921	299	631		
OID 5150	AI392793	302	634		
OID 5639	AW064243	303	635		
OID 6619	469A10	304	636		
OID 6933	AI089520	305	637		
OID 7049	480E2	306	638		
OID 5866	BM684739	308	640		
CD44	AA916990	309	641		
OID 4932	AA457757	311	643		
OID 7821	AA743221	312	644		
OID 4916	AA252909	313	645		
OID 4891	AL832329	314	646		
RAC1	AK054993	317	649		
OID 6415	CA407201	318	650		
OID 6295	AI880607	325	657		
RPL27A	BF214146	327	659		

Table 3: Viral genomes were used to design oligonucleotides for the microarrays. The accession numbers for the viral genomes used are given, along with the gene name and location of the region used for oligonucleotide design.

Virus	Gene Name	Genome Location
Adenovirus, type 2 Accession #J01917	E1a E1b_1 E2a_2 E3-1 E4 (last exon at 3'-end) IX Iva2 DNA Polymerase	1226..1542 3270...3503 complement(24089..25885) 27609..29792 complement(33193..32802) 3576..4034 complement(4081..5417) complement(5187..5418)
Cytomegalovirus (CMV) Accession #X17403	HCMVTRL2 (IRL2) HCMVTRL7 (IRL7) HCMVUL21 HCMVUL27 HCMVUL33 HCMVUL54 HCMVUL75 HCMVUL83 HCMVUL106 HCMVUL109 HCMVUL113 HCMVUL122 HCMVUL123 (last exon at 3'-end) HCMVUS28	1893..2240 complement(6595..6843) complement(26497..27024) complement(32831..34657) 43251..44423 complement(76903..80631) complement(107901..110132) complement(119352..121037) complement(154947..155324) complemmt(157514..157810) 161503..162800 complement(169364..170599) complement(171006..172225) 219200..220171
Epstein-Barr virus (EBV) Accession # NC_001345	Exon in EBNA-1 RNA Exon in EBNA-1 RNA BRLF1 BZLF1 (first of 3 exons) BMLF1 BALF2	67477..67649 98364..98730 complement(103366..105183) complement(102655..103155) complement(82743..84059) complement(161384..164770)
Human Herpesvirus 6 (HHV6) Accession #NC_001664	U16/U17 U89 U90 U86 U83 U22 DR2 (DR2L) DR7 (DR7L) U95 U94 U39 U42 U81 U91	complement(26259..27349) complement(133091..135610) complement(135664..135948) complement(125989..128136) 123528..123821 complement(33739..34347) 791..2653 5629..6720 142941..146306 complement(141394..142866) complement(59588..62080) complement(69054..70598) complement(121810..122577) 136485..136829

Table 4: Dependent variables for discovery of gene expression markers of cardiac allograft rejection.

Dependent Variable	Description	Number of Rejection Samples	Number of No-Rejection Samples
0 vs 1-4 Bx	Grade 0 vs. Grades 1-4, local biopsy reading	65	114
s0 vs 1B-4 HG	Stable Grade 0 vs Grades 1B-4, highest grade, Grade 1A not included	41	57
0-1A vs 1B-4 HG	Grades 0 and 1A vs Grades 1B-4, highest grade.	121	58
0 vs 3A HG	Grade 0 vs Grade 3A, highest grade. Grades 1A-2 and Grade 3B were not included.	56	29
0 vs 1B-4	Grade 0 vs Grades 1B-4, highest grade. Grade 1A was not included.	57	57
0 vs 1A-4	Grade 0 vs. Grades 1-4, highest grade	56	123

Table 5: Real-time PCR assay chemistries. Various combinations of reporter and quencher dyes are useful for real-time PCR assays.

Reporter	Quencher
FAM	TAMRA BHQ1
TET	TAMRA BHQ1
JOE	TAMRA BHQ1
HEX	TAMRA BHQ1
VIC	TAMRA BHQ1
ROX	BHQ2
TAMRA	BHQ2

Table 6: Real-time PCR results for rejection markers

Gene Array Probe SEQ ID	Phase 1				Phase 2				All Data			
	Fold	t-Test	NR	R	Fold	t-Test	NR	R	Fold	t-Test	NR	R
95	1.093	0.36084	10	8					0.935	0.31648	21	13
111	1.415	0.0095	12	10					1.415	0.0095	12	10
79	1.822	0.01146	6	7	0.63	0.04185	19	15	0.72	0.05632	35	26
3016	1.045	0.41017	12	10					1.001	0.49647	16	15

(continued)

Gene Array Probe SEQ ID	Phase 1				Phase 2				All Data			
	Fold	t-Test	NR	R	Fold	t-Test	NR	R	Fold	t-Test	NR	R
75	0.84	0.36674	11	8	0.595	0.15788	16	13	0.628	0.08402	34	26
2765	1.653	0.01508	10	10	0.776	0.11082	19	14	0.956	0.37421	38	29
97					0.75	0.26201	8	8	0.543	0.11489	17	12
2635	1.553	0.00533	13	10	0.834	0.16853	18	15	0.988	0.46191	36	27
96	1.495	0.06288	13	9	1.157	0.27601	18	15	1.155	0.21096	33	25
100	1.43	0.166	10	5					1.408	0.14418	12	8
2766	0.956	0.43918	12	10	0.989	0.48275	19	14	0.978	0.45101	31	24
2726	1.037	0.38205	11	9					1.037	0.38205	11	9
2768	1.211	0.02386	9	9					1.211	0.02386	9	9
94	1.601	0.02418	11	10					1.831	0.00094	17	15
2769	1.133	0.23094	12	9	1.081	0.19632	19	15	1.101	0.15032	31	24
2770	1.734	0.00017	13	10					1.381	0.01323	20	15
2647	1.557	0.04502	10	8					1.557	0.04502	10	8
2771	1.99	0.05574	13	9					1.52	0.11108	17	13
82	2.029	0.00022	8	5	1.287	0.13022	18	14	1.256	0.05356	33	23
83	1.546	0.05865	13	10	0.577	0.03934	18	14	0.795	0.11993	39	26
98					0.716	0.13	19	15	0.577	0.03352	19	14
36	1.605	0.09781	12	8	2.618	0.01227	18	11	2.808	0.00015	38	23
80	5.395	0.00049	9	6	4.404	0.05464	10	10	2.33	0.02369	29	18
89									0.295	0.02856	6	6
77	1.894	0.01602	10	10	0.537	0.01516	19	15	0.863	0.21987	35	29
2772	1.583	0.06276	10	6	0.714	0.13019	13	10	1.136	0.28841	28	17
2773	1.391	0.09236	11	6					1.391	0.09236	11	6
2774	1.59	0.00022	13	10					1.59	0.00022	13	10
102	1.245	0.05079	11	10	1.018	0.42702	17	15	1.117	0.08232	32	28
2775	0.719	0.16243	11	9					0.719	0.16243	11	9
2776	1.257	0.0516	12	9					1.257	0.0516	12	9
2667	1.343	0.03806	13	9					1.13	0.15962	20	12
115	1.199	0.26299	11	9					1.199	0.26299	11	9
2669	2.146	0.00813	12	10					1.296	0.14285	18	12
2777	1.142	0.20245	13	10					1.142	0.20245	13	10
78	1.324	0.01985	12	9	0.967	0.33851	18	14	1.007	0.46864	38	24
2670	1.388	0.11209	13	9					1.388	0.11209	13	9
88	1.282	0.14267	7	7	0.995	0.48504	17	14	1.008	0.47383	30	23
2778	1.128	0.19528	13	9					1.128	0.19528	13	9
2779	1.991	0.02513	9	5	0.642	0.05002	18	14	0.868	0.26275	32	21

(continued)

Gene Array Probe SEQ ID	Phase 1				Phase 2				All Data			
	Fold	t-Test	NR	R	Fold	t-Test	NR	R	Fold	t-Test	NR	R
2780	1.597	0.00355	13	10	0.802	0.11649	17	14	1.013	0.45521	38	26
2781					0.492	0.01344	12	12	0.819	0.25555	17	15

Table 6: Real-time PCR results for rejection markers

Gene Array Probe SEQ ID	Phase 1				Phase 2				All Data			
	Fold	t-Test	NR	R	Fold	t-Test	NR	R	Fold	t-Test	NR	R
101					0.652	0.04317	19	15	0.773	0.09274	29	22
106	1.234	0.19141	13	8					1.234	0.19141	13	8
2683	1.598	0.03723	8	8	0.633	0.03893	14	10	0.86	0.18731	28	22
2782	1.213	0.03305	12	10	0.912	0.07465	19	15	0.969	0.31955	39	27
87					4.947	0.02192	18	15	3.857	0.00389	30	23
99	0.639	0.06613	7	5	0.839	0.30304	16	8	0.694	0.04347	27	15
2692	0.801	0.21236	12	8	0.893	0.33801	18	15	0.782	0.06938	38	25
104	2.292	0.0024	11	8	0.621	0.05152	19	15	0.913	0.34506	30	23
76	1.809	0.00893	9	8	0.693	0.13027	13	8	1.274	0.11887	28	19
91	1.969	0.07789	11	8	4.047	0.00812	19	13	3.535	0.00033	37	23
92	2.859	0.05985	11	8	9.783	0.03047	18	14	8.588	0.00192	37	24
85	0.95	0.43363	12	8	0.699	0.0787	13	13	0.633	0.01486	33	24
126	1.76	0.02199	11	10					1.76	0.02199	11	10
2783	0.945	0.46023	10	5	0.852	0.26701	17	10	0.986	0.48609	29	17
2707	1.055	0.31435	13	10					1.055	0.31435	13	10
123	1.154	0.11677	11	10					1.154	0.11677	11	10
84	1.786	0.00255	9	6	0.523	0.04965	18	14	0.785	0.14976	34	22
2784	2.12	0.00022	12	10	0.498	0.01324	18	13	0.935	0.37356	37	25
2785	1.181	0.1377	10	10					1.181	0.1377	10	10
124	1.353	0.08122	11	9					1.353	0.08122	11	9
90	1.355	0.02288	13	10	0.973	0.39248	15	13	1.125	0.08671	28	23
2786	1.306	0.0773	12	10					1.306	0.0773	12	10
2787	1.086	0.32378	12	10					1.086	0.32378	12	10
3018	1.523	0.1487	12	10	0.84	0.27108	18	13	1.101	0.33276	36	26
125	1.252	0.05782	11	10					1.252	0.05782	11	10
2788	1.255	0.1221	11	10					1.255	0.1221	11	10
2789	1.152	0.31252	9	6					1.152	0.31252	9	6
3019	1.268	0.21268	6	7	0.981	0.45897	16	10	1.012	0.46612	29	19

(continued)

Gene Array Probe SEQ ID	Phase 1				Phase 2				All Data			
	Fold	t-Test	NR	R	Fold	t-Test	NR	R	Fold	t-Test	NR	R
2790	0.881	0.17766	11	8	1.22	0.04253	18	10	0.966	0.33826	40	23
2791	1.837	0.00553	13	10					1.837	0.00553	13	10
3020	1.271	0.10162	12	10	0.853	0.10567	19	13	0.965	0.36499	36	25
2792	1.504	0.05096	12	10	0.713	0.02979	19	15	0.846	0.16914	31	25
2793	1.335	0.03133	12	10	0.883	0.18577	19	15	0.916	0.23865	36	27
2794	1.936	0.00176	13	9	0.717	0.09799	19	14	0.877	0.22295	40	25
2752	1.499	0.03077	12	8	0.808	0.15363	17	13	1.004	0.48903	36	23
2795	0.815	0.24734	8	5	0.965	0.41772	19	15	0.938	0.3265	32	22
119	1.272	0.20279	10	10					1.272	0.20279	10	10

Table 7: Significance analysis for microarrays for identification of markers of acute rejection. In each case the highest grade from the 3 pathologists was taken for analysis. No rejection and rejection classes are defined. Samples are either used regardless of redundancy with respect to patients or a requirement is made that only one sample is used per patient or per patient per class. The number of samples used in the analysis is given and the lowest FDR achieved is noted.

No Rejection	Rejection	# Samples Low	FDR
All Samples			
Grade 0	Grade 3A-4	148	1
Grade 0	Grade 1B, 3A-4	158	1.5
Non-redundant within class			
Grade 0	Grade 3A-4 Grade	86	7
Grade 0	1B, 3A-4	93	16
Non-redundant (1 sample/patient)			
Grade 0	Grade 3A-4	73	11

Table 8: Renal rejection tissue gene expression SAM analysis

Array probe ID	Gene	FDR	Protein SEQ ID	Leukocyte expression	Secreted
2697	CD69 antigen (p60, early T-cell activat	1.5625	2925	+	
2645	Ras association (RalGDS/AF-6)	1.5625	2926		
2707	CD33 antigen (gp67) (CD33), mRNA	1.5625	2927	+	
2679	Ras association (RalGDS/AF-6) domain fa	1.5625	2928		
2717	EST, 5 end	1.5625			
2646	mRNA for KIAA0209 gene, partial cds /cd	1.5625	2929		

(continued)

Array probe ID	Gene	FDR	Protein SEQ ID	Leukocyte expression	Secreted
2667	leupaxin (LPXN), mRNA/cds=(93,1253)	1.5625	2930	+	
2706	c- EST 3 end /clone=IMAGE:	2.1111			
2740	c- insulin induced gene 1 (INSIG1), mRNA	2.2			
117	**chemokine (C-X-C motif) receptor 3**	2.8125	2931		
2669	IL2-inducible T-cell kinase (ITK), mRNA	2.8125	2932	+	
2674	glioma pathogenesis-related protein (RT	2.8125	2933		
2743	c- nuclear receptor subfamily 1, group I	2.8125			
326	death effector filament-forming Ced-4-1	2.8125	2934		
2716	EST cDNA, 3 end	2.8125			
2727	c- chemokine (C-X-C motif), receptor 4	3.1316	2935	+	
2721	c- EST 3 end /clone=IMAGE:	3.1316			
2641	hypothetical protein FLJ20647 (FLJ20647	3.1316	2936		
2671	tumor necrosis factor, alpha-induced pr	3.525	2937		
2752	protein tyrosine phosphatase, receptor	3.8077	2938	+	
2737	7f37g03.x1 cDNA, 3 end /clone=IMAGE:	3.8077			
2719	c- EST372075 cDNA	3.8077			
2684	molecule possessing ankyrin repeats ind	3.8077	2939		
76	granzyme B (granzyme 2, cytotoxic T-lym	3.8077	2940	+	+
2677	lectin-like NK cell receptor (LLT1), mR	3.8077	2941	+	
2748	c-107G11	3.9			
2703	c- EST, 5 end /clone=IMAGE	3.9			
2711	SAM domain, SH3 domain and nuclear	3.9	2942		
2663	phosphodiesterase 4B, cAMP-specific	3.9	2943		+

(continued)

Array probe ID	Gene	FDR	Protein SEQ ID	Leukocyte expression	Secreted
98	small inducible cytokine A5 (RANTES)	4.5645	2944	+	+
2657	tumor necrosis factor receptor superfam	4.8286	2945		
2683	B-cell lymphoma/leukaemia 11B (BCL11B)	4.8286	2946	+	
2686	phospholipase A2, group VII (platelet-a	4.8286	2947		+
2687	phosphatidylinositol 3-kinase catalytic	4.8286	2948		
2644	AV659177 cDNA, 3 end	4.9028			
2664	regulator of G-protein signalling 10 (R	5.0238	2949		
2747	c- integral membrane protein 2A (ITM2A),	5.0238	2950		
2744	c- interferon consensus sequence binding	5.0238			
2678	HSPC022 protein (HSPC022), mRNA	5.0238	2951		
2731	c- xj98c03.x1 NCI CGAP Co18 cDNA	5.0238			
2713	caspase recruitment domain protein 9 (L	5.0238	2952		
2736	c- small inducible cytokine A4 (homologo	5.1395	2953	+	+
2708	major histocompatibility complex, class	5.15	2954		
249	c-107H8	5.15			
2670	CD72 antigen (CD72), mRNA	5.15	2955	+	
2661	heat shock 70kD protein 6 (HSP70B)	5.15	2956		
2680	bridging integrator 2 (BIN2), mRNA /cds	5.15	2957		
2754	UI-H-BWO-aiy-b-10-0-UI.s1 cDNA, 3 end	5.15			
2728	c- EST380762 cDNA	5.15			
174	FKBPL	5.15	2958		
2742	c- chromobox homolog 3 (DM)	5.15			
2668	basement membrane-induced gene (ICB-1)	5.15	2959		

(continued)

Array probe ID	Gene	FDR	Protein SEQ ID	Leukocyte expression	Secreted
2750	Lysosomal-assoc. multispanning memb	5.15	2960		
2746	174D1	5.15			
2738	c- AV716627 cDNA, 5 end	5.15			
2627	solute carrier family 17 (sodium phosph	5.15	2961		
2739	c- asparaginyl-tRNA synthetase (NARS)	5.15			
124	major histocompatibility complex, class	5.15	2962		
2647	mRNA for T-cell specific protein /cds	5.15	2963	+	
2628	c-EST, 3 end	5.2295			
2638	Express cDNA library cDNA 5	5.2903			
2725	c- 601571679F1 cDNA, 5 end	5.3385	2964		
2714	qg78c05.x1 cDNA, 3 end /clone	5.3385	2965		
2635	interleukin 2 receptor gamma chain	5.3385	2966	+	
2751	7264, lectin, galactoside-binding, soluble	5.4167	2967		+
2629	8, cDNA: FLJ21559 fis, clone COL06406	5.5299	2968		
2695	mRNA; cDNA DKFZp434E0516	5.5588	2969		
2741	c- hexokinase 2 (HK2), mRNA	5.5986			
41	Similar to major histocompatibility antigen	5.5986	2970		
2691	CD5 antigen (p56-62)(CD5)	5.5986	2971		
2726	c- 602650370T1 cDNA, 3	5.6014			
2722	c- EST cDNA clone	5.6014			
	2689 interleukin-2 receptor	5.6014	2972		
2734	c- nuclear receptor subfamily 1, group I	5.6667			
2631	pre-B-cell colony-enhancing factor	5.7566	2973		+
2656	postmeiotic segregation increased	5.7756	2974		

(continued)

Array probe ID	Gene	FDR	Protein SEQ ID	Leukocyte expression	Secreted
2696	protein tyrosine phosphatase, receptor	5.7756	2975		
2676	butyrophilin, subfamily 3, member A2	5.8165	2976		
2701	c- EST 3 end	5.9048			
2730	EST 3 end /clone=IMAGE	5.9048			
2710	high affin. immunoglobulin epsilon recept.	5.9048	2977		
2632	encoding major histocompatibility comple	5.9048	2978		
2724	c- EST 3 end	5.9048			
2698	EST	6.0353			
2662	interferon regulatory factor 1 (IRF1),	6.0988	2979		
139	allograft inflammatory factor 1 (AIF1),	6.1379	2980		
2753	platelet activating receptor homolog (H	6.3182	2981		
2704	c- EST 3 end /clone=IMAGE:	7.0337			
2675	pim-2 oncogene (PIM2), mRNA	7.1222	2982		+
2700	proteoglycan 1, secretory granule (PRG1	7.375	2983		+
2640	mRNA for KIAA0870 protein, partial cds	7.375	2984		
2723	c- EST, 5 end /clone=IMAGE	7.375			
2658	FYN-binding protein (FYB-120/130) (FYB)	7.375	2985		
2688	major histocompatibility complex, class	7.375	2986		
2735	c- EST, 3 end /clone=IMAGE:	7.375			
2702	c- hypothetical protein MGC4707	7.634			
2681	hypothetical protein FLJ10652	8.1117	2987		
2755	EST, 3 end	8.1117			
2715	hypothetical protein FLJ10842	8.1117			
2732	c- EST cDNA, 3 end	8.1117			
2652	hexokinase 2 (HK2), mRNA	8.1117			

(continued)

Array probe ID	Gene	FDR	Protein SEQ ID	Leukocyte expression	Secreted
2651	colony stimulating factor 3 receptor	8.1117	2988		
2718	RNA binding motif protein, X chrom	8.2788			
2673	Src-like-adapter (SLA), mRNA	8.3048	2989		
2733	c- major histocompatibility complex	8.467			
2712	histamine receptor H2 (HRH2)	8.8583	2990		
2659	hemopoietic cell kinase (HCK)	8.8583	2991		
2654	xanthene dehydrogenase (XDH)	8.8583	2992		
2636	Arabidopsis root cap 1	8.8583	2993		
2639	fatty acid binding protein 1, liver	8.8583			
2690	adenosine deaminase (ADA)	8.8583	2994		
2705	c- EST, 3 end	8.8583	2995		
2685	hypothetical protein MGC10823	8.8583	2996		
2692	membrane-spanning 4-domains,	8.8583	2997		
2693	rearranged immunoglobulin mRNA for mu	8.8583			+
2648	protein tyrosine kinase related mRNA	8.8583			
2650	major histocompatibility complex, class	8.8583	2998		
2720	c- EST 3 end /clone=IMAGE:	8.8583			
2660	major histocompatibility complex, class	8.8583	2999		
2666	BCL2-related protein A1 (BCL2A1), mRNA	9.1446	3000		
2699	c-EST	9.4767			
2633	interleukin 4 receptor	9.4767	3001		
74	tumor necrosis factor (ligand) superfam	9.4767	3002		
2672	interferon-induced, hepatitis C-assoc.	9.4767	3003		

(continued)

Array probe ID	Gene	FDR	Protein SEQ ID	Leukocyte expression	Secreted
2642	cDNA FLJ20673 fis, clone KAIA4464	9.4767	3004		
2682	VNN3 protein (HSA238982), mRNA	9.4767	3005		
2655	cathepsin K (pycnodysostosis) (CTSK)	9.4767	3006		
2630	Integrin, alpha L (CD11A (p180), lymphoc	9.4767	3007		
2745	EST, 5 end	9.4885	3008		
2643	nuclear receptor subfamily 1, group I,	9.625			
2694	CDW52 antigen (CAMPATH-1)	9.625	3009		
2749	6977, c-178F5	9.6903	3010		
2665	small inducible cytokine subfamily A	9.6903	3011		
2649	signal transducer and activator	9.7878	3012		
2637	324,	9.7878			
2634	70 activation (Act-2) mRNA	9.7878	3013		
2709	coagulation factor VII	9.7878	3014		
2653	integrin, beta 2 (antigen CD18 (p95)	9.7878	3015		
2729	EST 3' end	9.8321			

Table 9

Array Probe SEQ ID	Gene	Gene Name	mRNA Accession #	RefSeq Peptide Accession #	Current UniGene Cluster (Build 156)	Localization	Function
111	IL15	Interleukin 15	NM_000585	NP_000576	Hs.168132	Secreted	T-cell activation and proliferation
79	PRF1	Perforin 1 (pore forming	NM_005041 protein)	NP_005032	Hs.2200	Secreted	CD8, CTL effector; channel-forming protein capable of lysing non-specifically a variety of target cells; clearance of vitally infected host cells and tumor cells; .
110	IL17	Interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8)	NM_002190	NP_002181	Hs.41724	Secreted	Induces stromal cells to produce proinflammatory and hematopoietic cytokines; enhances IL6, IL8 and ICAM-1 expression in fibroblasts; osteoclastic bone resorption in RA; expressed in only in activated CD4+T cells
75	IL8	Interleukin 8	NM_000584	NP_000575	Hs.624	Secreted	Proinflammatory cytokine
120	CXCL1	Chemokine (C-X-C motif) ligand (melanoma growth stimulating activity, alpha)	NM_001511 1	NP_001502	Hs.789	Secreted	Neurogenesis, immune system development, signaling
113	IFNG	Interferon, gamma	NM_000619	NP_000610	Hs.856	Secreted	Antiviral defense and immune activation
100	IL2	Interleukin 2	NM_000586	NP_000577	Hs.89679	Secreted	Promotes growth of B and T cells
4	B2M	beta 2 microglobulin	NM_004048	NP_004039	Hs.75415	Secreted	

(continued)

Array Probe SEQ ID	Gene	Gene Name	mRNA Accession #	RefSeq Peptide Accession #	Current UniGene Cluster (Build 156)	Localization	Function
98	CCL5	Chemokine (C-C motif) ligand (RANTES, SCYA5)	NM_002985 5	NP_002976	Hs.241392	Secreted	Chemoattractant for monocytes, memory T helper cells and eosinophils; causes release of histamine from basophils and activates eosinophils; One of the major HIV-suppressive factors produced by CD8+ cells
112	IL10	Interleukin 10	NM_000572	NP_000563	Hs.193717	Secreted	Chemotactic factor for CD8+T cells; down-regulates expression of Th1 cytokines, MHC class II Ags, and costimulatory molecules on macrophages; enhances B cell survival, proliferation, and antibody production; blocks NF kappa B, JAK-STAT regulation;
80	IL4	Interleukin 4	NM_000589	NP_000580	Hs.73917	Secreted	TH2, cytokine, stimulates CTL
2773	IL7	Interleukin 7	NM_000880	NP_000871	Hs.72927	Secreted	Proliferation of lymphoid progenitors

(continued)

Array Probe SEQ ID	Gene	Gene Name	mRNA Accession #	RefSeq Peptide Accession #	Current UniGene Cluster (Build 156)	Localization	Function
109	CXCL10	Chemokine(C-X-C motif) ligand 10, SCYB10	NM_001565	NP_001556	Hs.2248	Secreted	Stimulation of monocytes; NK and T cell migration, modulation of adhesion molecule expression
2665	CCL17	Chemokine (C-C motif) ligand	NM_002987 17	NP_002978	Hs.66742	Secreted	T cell development, trafficking and activation
101	KLRF1	Killer cell lectin-like receptor subfamily F, member 1	NM_016523	NP_057607	Hs.183125	Secreted	Induction of IgE, IgG4, CD23, CD72, surface IgM, and class II MHC antigen in B cells
99	IL6	Interleukin 6	NM_000600	NP_000591	Hs.93913	Secreted	B cell maturation
104	CCL4	Chemokine (C-C motif) ligand	NM_002984 4	NP_002975	Hs.75703	Secreted	Inflammatory and chemokinetic properties; one of the major HIV-suppressive factors produced by CD8+ T cells
76	GZMB	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	NM_004131	NP_004122	Hs.1051	Secreted	Apoptosis; CD8, CTL effector
2785	OID_4789	KIAA0963 protein	NM_014963	NP_055778	Hs.7724	Secreted	Proinflammatory; chemoattraction and activation of neutrophils
2791	XCL1	Chemokine (C motif) ligand 1 (SCYC2)	NM_002995	NP_002986	Hs.3195	Secreted	Chemotactic factor for lymphocytes but not monocytes or neutrophils

(continued)

Array Probe SEQ ID	Gene	Gene Name	mRNA Accession #	RefSeq Peptide Accession #	Current UniGene Cluster (Build 156)	Localization	Function
130	PRDM1	PR domain containing 1, ZNF domain with	NM_001198	NP_001189	Hs.388346	Nuclear	Transcription factor; promotes B cell maturation, represses human beta-IFN gene expression
2781	TBX21	T-box 21	NM_013351	NP_037483	Hs.272409	Nuclear	TH1 differentiation, transcription factor
88	MTHFD2	Methylene tetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase	NM_006636	NP_006627	Hs.154672	Mitochondrial	Folate metabolism
103	IL2RA	Interleukin 2 receptor, alpha	NM_000417	NP_000408	Hs.1724	Membrane-bound and soluble forms	T cell mediated immune response
77	TNFSF6	Tumor necrosis factor superfamily, member 6	NM_000639 (ligand)	NP_000630	Hs.2007	Membrane-bound and soluble forms	CD8, CTL effector; proapoptotic
115	CD8B1	CD8 antigen, beta polypeptide (p37)	NM_004931 1	NP_004922	Hs.2299	Membrane-bound and soluble forms	CTL mediated killing
128	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	NM_000963	NP_000954	Hs.196384	Membrane-associated	Angiogenesis, cell migration, synthesis of inflammatory prostaglandins
89	TAP I	Transporter 1, ATP-binding cassette, sub-family B (MDR1/TAP)	NM_000593	NP_000584	Hs.352018	ER membrane	Transports antigens into ER for association with MHC class I molecules
92	IGHM	Immunoglobulin heavy constant mu	BC032249		Hs.300697	Cytoplasmic and secreted forms	Antibody subunit

(continued)

Array Probe SEQ ID	Gene	Gene Name	mRNA Accession #	RefSeq Peptide Accession #	Current UniGene Cluster (Build 156)	Localization	Function
122	GPI	Glucose phosphate isomerase	NM_000175	NP_000166	Hs.409162	Cytoplasmic and secreted forms	Glycolysis and gluconeogenesis (cytoplasmic); neurotrophic factor (secreted)
2783	GSN	Gelsolin (amyloidosis, Finnish type)	NM_000177	NP_000168	Hs.290070	Cytoplasmic and secreted forms	Controls actin filament assembly/disassembly
2780	STK39	Serine threonine kinase 39 (STE20/SPS1 homolog, yeast)	NM_013233	NP_037365	Hs.199263	Cytoplasmic and nuclear	Mediator of stress-activated signals; Serine/Thr Kinase, activated p38
2770	PSMB8	Proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional protease 7)	AK092738		Hs.180062	Cytoplasmic	Processing of MHC class I antigens
2667	LPXN	Leupaxin	NM_004811	NP_004802	Hs.49587	Cytoplasmic	Signal transduction
2669	ITK	IL2-inducible T-cell kinase	L10717		Hs.211576	Cytoplasmic	Intracellular kinase, T-cell proliferation and differentiation
90	KPNA6	Karyopherin alpha 6 (importin alpha 7)	AW021037		Hs.301553	Cytoplasmic	Nucleocytoplasmic transport
2794	SH2D2A	SH2 domain protein 2A	NM_003975	NP_003966	Hs.103527	Cytoplasmic	CD8 T activation, signal transduction
2765	TNFSF5	Tumor necrosis factor (ligand) superfamily, member 5 (hyper-IgM syndrome)	NM_000074	NP_000065	Hs.652	Cellular membrane	B-cell proliferation, IgE production, immunoglobulin class switching; expressed on CD4+ and CD8+ T cells

(continued)

Array Probe SEQ ID	Gene	Gene Name	mRNA Accession #	RefSeq Peptide Accession #	Current UniGene Cluster (Build 156)	Localization	Function
97	CD69	CD69 antigen (p60, early activation antigen)	NM_001781 T-cell	NP_001772	Hs.82401	Cellular membrane	Activation of lymphocytes, monocytes, and platelets
2635	IL2RG	Interleukin 2 receptor, gamma (severe combined immunodeficiency)	NM_000206	NP_000197	Hs.84	Cellular membrane	Signalling component of many interleukin receptors (IL2, IL4, IL7, IL9, and IL15),
96	CXCR4	Chemokine (C-X-C motif) receptor 4	NM_003467	NP_003458	Hs.89414	Cellular membrane	B-cell lymphopoiesis, leukocyte migration, angiogenesis; mediates intracellular calcium flux
2766	CD19	CD19 antigen	NM_001770	NP_001761	Hs.96023	Cellular membrane	Signal transduction; B lymphocyte development, activation, and differentiation
2769	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	NM_002211	NP_002202	Hs.287797	Cellular membrane	Cell-cell and cell-matrix interactions
2647	TRB	T cell receptor beta, constant region	K02885		Hs.300697	Cellular membrane	Antigen recognition
82	CTLA4	Cytotoxic T-lymphocyte-associated protein 4	NM_005214	NP_005205	Hs.247824	Cellular membrane	Negative regulation of T cell activation, expressed by activated T cells
83	CD8A	CD8 antigen, alpha polypeptide (p32)	NM_001768	NP_001759	Hs.85258	Cellular membrane	CD8 T-cell specific marker and class I MHC receptor
114	HLA-DRB1	Major histocompatibility complex, class II, DR beta 1	NM_002124	NP_002115	Hs.308026	Cellular membrane	Antigen presentation

(continued)

Array Probe SEQ ID	Gene	Gene Name	mRNA Accession #	RefSeq Peptide Accession #	Current UniGene Cluster (Build 156)	Localization	Function
2772	CD3Z	CD3Z antigen, zeta polypeptide (TiT3 complex)	NM_000734	NP_000725	Hs.97087	Cellular membrane	T-cell marker; couples antigen recognition to several intracellular signal-transduction pathways
2	ACTB	Actin, beta	NM_001101	NP_001092	Hs.288061	Cellular membrane	Cell adhesion and recognition
2774	ITGAL	Integrin, alpha L (antigen CD11 (p180), lymphocyte function-associated antigen 1; alpha polypeptide)	NM_002209 A	NP_002200	Hs.174103	Cellular membrane	All leukocytes; cell-cell adhesion, signaling
78	TCIRG1	T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 protein a isoform 3	NM_006019	NP_006010	Hs.46465	Cellular membrane	T cell activation
2670	CD72	CD72 antigen	NM_001782	NP_001773	Hs.116481	Cellular membrane	B cell proliferation
2779	D12S2489E	DNA segment on chromosome (unique) 2489 expressed sequence	NM_007360 12	NP_031386	Hs.74085	Cellular membrane	NK cells marker
2692	MS4A1	Membrane-spanning 4-domains, subfamily A, member 1, CD20	NM_152866	NP_690605	Hs.89751	Cellular membrane	B-cell activation, plasma cell development
126	TCRGC2	T cell receptor gamma constant 2	M17323		Hs.112259	Cellular membrane	
116	CD4	CD4 antigen (p55)	NM_000616	NP_000607	Hs.17483	Cellular membrane	T cell activation, signal transduction, T-B cell adhesion

(continued)

Array Probe SEQ ID	Gene	Gene Name	mRNA Accession #	RefSeq Peptide Accession #	Current UniGene Cluster (Build 156)	Localization	Function
117	CXCR3	Chemokine (C-X-C motif) receptor 3, GPR9	NM_001504	NP_001495	Hs.198252	Cellular membrane	Integrin activation, cytoskeletal changes and chemotactic migration of leukocytes
2707	CD33	CD33 antigen (gp67)	NM_001772	NP_001763	Hs.83731	Cellular membrane	Cell adhesion; receptor that inhibits the proliferation of normal and leukemic myeloid cells
123	CD47	CD47 antigen related antigen, integrin-associated signal transducer)	(Rh-NM_001777	NP_001768	Hs.82685	Cellular membrane	Cell adhesion, membrane transport, signaling transduction, permeability
84	BY55	Natural killer cell receptor, immunoglobulin superfamily member	NM_007053	NP_008984	Hs.81743	Cellular membrane	NK cells and CTLs, costim with MHC I
2784	KLRD1	Killer cell lectin-like receptor subfamily D, member I	NM_002262	NP_002253	Hs.41682	Cellular membrane	NK cell regulation
124	HLA-F	Major histocompatibility complex, class I, F	NM_018950	NP_061823	Hs.377850	Cellular membrane	Antigen presentation
2752	PTPRCAP	Protein tyrosine phosphatase, receptor type, C-associated protein	NM_005608	NP_005599	Hs.155975	Cellular membrane	T cell activation

Table 12: Markers for CMV Infection

New								SAM
SEQID	Source	Unigene	Acc	GI	Name	Strand	Probe Sequence	FDR
408	cDNA	Hs.1051	NM_004131	7262379	granzyme B TACACTGGGAGAGGTGC	1	GGAGCCAAGTCCAGATT CAGCAACTGAATAAAT	0%
3108	db mining	Hs.169824	NM_002258	4504878	like receptor	killer cell lectin- 1 TGGATCTGCCAAAAAGA	ACTAACACCTGTGAGAA ATAAAGTGTATCCTGA	0%
3109	cDNA	Hs.170019	NM_004350	4757917	runt-related transcription factor 3	1	GCTGGGTGGAAACTGCT TTGCACTATCGTTTGCT TGGTGTTTGTTTTTAA	0%
433	cDNA	Hs.183125	NM_016523	7705573	like receptor F	killer cell lectin- 1 TTCCAGGCTTTTGCTAC	TCTTCACTCAGCTACAA TAAACATCCTGAATGT	0%
3110	db mining	Hs.2014	X06557	37003	T-cell receptor-delta	1	GGGGTTTATGTCCTAAC TGCTTTGTATGCTGTTT TATAAAGGGATAGAAG	0.10%
3111	cDNA	Hs.211535	A1823649	5444320	EST IMAGE:240014 8	-1	GAAGCCTTTTCTTTTCT GTTCACCCTCACCAAGA GCACAACTTAAATAGG	0.10%
3112	cDNA	Hs.301704	AW002985	5849991	eomesodermin (Xenopus laevis)	-1	AACAAGCCATGTTTGCC CTAGTCCAGGATTGCCT CACTTGAGACTTGCTA	0%
3112	Table 3B	Hs.301704	AW002985	5849991	eomesodermin (Xenopus laevis)	-1	AACAAGCCATGTTTGCC CTAGTCCAGGATTGCCT CACTTGAGACTTGCTA	0%
3113	cDNA	Hs.318885	NM_000636	10835186	superoxide dismutase 2	1	TACTTTGGGGACTTGTA GGGATGCCTTTCTAGTC CTATTCTATTGCAGTT	0.10%
3114	literature	Hs.41682	NM_007334	7669498	like receptor D	killer cell lectin- 1 GGGCAGAGAAGGTGGAG	AGTAAAGACCCAACATT ACTAACAATGATACAG	0%
3115	cDNA	Hs.71245	AI954499	5746809	EST IMAGE:502221	-1	TGGTAATAGTGTTTGAC TCCAGGGAAGAACAGAT GGGTGCCAGAGTGAAA	0%

(continued)

New								SAM
SEQID	**Source**	**Unigene**	**Acc**	**GI**	**Name**	**Strand**	**Probe Sequence**	**FDR**
3116	cDNA	Hs.75596	NM_000878	4504664	interleukin 2 receptor, beta	1	ATGGAAATTGTATTTGC CTTCTCCACTTTGGGAG GCTCCCACTTCTTGGG	0%
436	cDNA	Hs.75703	NM_002984	4506844	small inducible cytokine A4	1	CCACTGTCACTGTTTCT CTGCTGTTGCAAATACA TGGATAACACATTTGA	0%
436	cDNA	Hs.75703	NM_002984	4506844	small inducible cytokine A4	1	CCACTGTCACTGTTTCT CTGCTGTTGCAAATACA TGGATAACACATTTGA	0.10%
436	cDNA	Hs.75703	NM_002984	4506844	small inducible cytokine A4	1	GTCCACTGTCACTGTTT CTCTGCTGTTGCAAATA CATGGATAACACATTT	0%
436	cDNA	Hs.75703	NM_002984	4506844	small inducible cytokines A4	-1	TGGTCCACTGTCACTGT TTCTCTGCTGTTGCAAA TACATGGATAACACAT	0.10%
415	cDNA	Hs.85258	BC025715	19344021	CD8 antigen	1	CTGAGAGCCCAAACTGC TGTCCCAAACATGCACT TCCTTGCTTAAGGTAT	0.10%
3117	cDNA	Hs.111554	AA806222	2874972	cDNA196D7	-1	TGATTTCTGTAATGTTT GACCTAATAATAGCCCT TTTCGTCTCTGACCCA	0%
WBC	N/A	N/A	N/A	N/A		N/A	N/A	0.10%
WPT	N/A	N/A	N/A	N/A		N/A	N/A	0%

Claims

1. A method of diagnosing or monitoring cardiac allograft rejection in a patient, comprising detecting, in a blood sample from said patient, the expression level of a nucleic acid in said patient to assess cardiac allograft rejection versus non-rejection and wherein said nucleic acid comprises the nucleotide sequence SEQ ID NO: 67 and wherein said expression level is detected by measuring the RNA level expressed by said nucleic acid.

2. The method according to claim 1, wherein the expression level is detected in peripheral blood leukocytes.

3. The method according to any one of claims 1-2, further including isolating RNA from said patient prior to detecting said RNA level expressed by said nucleic acid.

4. The method according to claim 3, wherein said RNA level is detected by PCR.

5. The method according to claim 3, wherein said RNA level is detected by hybridization.

6. The method according to claim 3, wherein said RNA level is detected by hybridization to an oligonucleotide.

7. The method according to claim 6, wherein said oligonucleotide comprises DNA, RNA, cDNA, PNA, genomic DNA, or synthetic oligonucleotides.

8. Use of a nucleic acid for assessing cardiac allograft rejection in a patient, wherein the expression level of said nucleic acid in said patient is detected in a blood sample from said patient to assess cardiac allograft rejection versus non-rejection, wherein said nucleic acid comprises the nucleotide sequence SEQ ID NO: 67 and wherein said expression level is detected by measuring the RNA level expressed by said nucleic acid.

9. The use according to claim 8, wherein the expression level is detected in peripheral blood leukocytes.

10. The use according to any one of claims 8-9, further including isolating RNA from said patient prior to detecting said RNA level expressed by said nucleic acid.

11. The use according to claim 10, wherein said RNA level is detected by PCR.

12. The use according to claim 10, wherein said RNA level is detected by hybridization.

13. The use according to claim 10, wherein said RNA level is detected by hybridization to an oligonucleotide.

14. The use according to claim 13, wherein said oligonucleotide comprises DNA, RNA, cDNA, PNA, genomic DNA, or synthetic oligonucleotides.

Patentansprüche

1. Verfahren zum Diagnostizieren oder Überwachen der Herz-Allotransplantatabstoßung bei einem Patienten, welches das Feststellen des Expressionsgrades einer Nukleinsäure in dem Patienten in einer Blutprobe des Patienten zum Beurteilen der Herz-Allotransplantatabstoßung im Vergleich zur Nicht-Abstoßung umfasst und wobei die Nukleinsäure die Nukleotidsequenz SEQ ID Nr. 67 umfasst und wobei der Expressionsgrad durch Messen der Menge der von der Nukleinsäure exprimierten RNA festgestellt wird.

2. Verfahren nach Anspruch 1, wobei der Expressionsgrad in Leukozyten des peripheren Bluts festgestellt wird.

3. Verfahren nach einem der Ansprüche 1-2, welches vor dem Feststellen der Menge der von der Nukleinsäure exprimierten DNA des Weiteren das Isolieren von DNA aus dem Patienten umfasst.

4. Verfahren nach Anspruch 3, wobei die RNA-Menge mittels PCR festgestellt wird.

5. Verfahren nach Anspruch 3, wobei die RNA-Menge mittels Hybridisierung festgestellt wird.

6. Verfahren nach Anspruch 3, wobei die RNA-Menge mittels Hybridisierung an ein Oligonukleotid festgestellt wird.

7. Verfahren nach Anspruch 6, wobei das Oligonukleotid DNA, RNA, cDNA, PNA, genomische DNA oder synthetische Oligonukleotide umfasst.

8. Verwendung einer Nukleinsäure zum Beurteilen der Herz-Allotransplantatabstoßung bei einem Patienten, wobei der Expressionsgrad der Nukleinsäure in dem Patienten in einer Blutprobe des Patienten festgestellt wird, um Herz-Allotransplantatabstoßung im Vergleich zur Nicht-Abstoßung festzustellen, wobei die Nukleinsäure die Nukleotidsequenz SEQ ID Nr. 67 umfasst und wobei der Expressionsgrad durch Messen der Menge der von der Nukleinsäure exprimierten RNA festgestellt wird.

9. Verwendung nach Anspruch 8, wobei der Expressionsgrad in Leukozyten des peripheren Bluts festgestellt wird.

10. Verwendung nach einem der Ansprüche 8-9, welches vor dem Feststellen der Menge der von der Nukleinsäure exprimierten RNA des Weiteren das Isolieren von RNA aus dem Patienten umfasst.

11. Verwendung nach Anspruch 10, wobei die RNA-Menge mittels PCR festgestellt wird.

12. Verwendung nach Anspruch 10, wobei die RNA-Menge mittels Hybridisierung festgestellt wird.

13. Verwendung nach Anspruch 10, wobei die RNA-Menge mittels Hybridisierung an ein Oligonukleotid festgestellt wird.

14. Verwendung nach Anspruch 13, wobei das Oligonukleotid DNA, RNA, cDNA, PNA, genomische DNA oder synthetische Oligonukleotide umfasst.

Revendications

1. Procédé de diagnostic ou de surveillance du rejet d'une allogreffe cardiaque chez un patient, comprenant la détection, dans un échantillon de sang prélevé chez ledit patient, du niveau d'expression d'un acide nucléique chez ledit patient afin d'évaluer le rejet de l'allogreffe cardiaque par rapport à l'absence de rejet, dans lequel l'acide nucléique comprend la séquence nucléotidique SEQ ID N° 67 et ledit niveau d'expression est détecté en mesurant le niveau d'ARN exprimé par ledit acide nucléique.

2. Procédé selon la revendication 1, dans lequel le niveau d'expression est détecté dans les leucocytes du sang périphérique.

3. Procédé selon l'une quelconque des revendications 1 et 2, comprenant en outre l'isolation de l'ARN provenant dudit patient avant la détection dudit niveau d'ARN exprimé par ledit acide nucléique.

4. Procédé selon la revendication 3, dans lequel ledit niveau d'ARN est détecté par PCR.

5. Procédé selon la revendication 3, dans lequel ledit niveau d'ARN est détecté par hybridation.

6. Procédé selon la revendication 3, dans lequel ledit niveau d'ARN est détecté par hybridation à un oligonucléotide.

7. Procédé selon la revendication 6, dans lequel ledit oligonucléotide comprend de l'ADN, de l'ARN, de l'ADNc, un APN, de l'ADN génomique ou des oligonucléotides synthétiques.

8. Utilisation d'un acide nucléique pour évaluer le rejet d'une allogreffe cardiaque chez un patient, dans laquelle le niveau d'expression dudit acide nucléique chez ledit patient est détecté dans un échantillon de sang provenant dudit patient afin d'évaluer le rejet de l'allogreffe cardiaque par rapport à l'absence de rejet, ledit acide nucléique comprenant la séquence nucléotidique SEQ ID N° 67 et ledit niveau d'expression étant détecté en mesurant le niveau d'ARN exprimé par ledit acide nucléique.

9. Utilisation selon la revendication 8, dans laquelle le niveau d'expression est détecté dans les leucocytes du sang périphérique.

10. Utilisation selon l'une quelconque des revendications 8 et 9, comprenant en outre l'isolation de l'ARN provenant dudit patient avant la détection dudit niveau d'ARN exprimé par ledit acide nucléique.

11. Utilisation selon la revendication 10, dans laquelle ledit niveau d'ARN est détecté par PCR.

12. Utilisation selon la revendication 10, dans laquelle ledit niveau d'ARN est détecté par hybridation.

13. Utilisation selon la revendication 10, dans laquelle ledit niveau d'ARN est détecté par hybridation à un oligonucléotide.

14. Utilisation selon la revendication 13, dans laquelle ledit oligonucléotide comprend de l'ADN, de l'ARN, de l'ADNc, un APN, de l'ADN génomique ou des oligonucléotides synthétiques.

Figure 1: Novel Gene Sequence Analysis

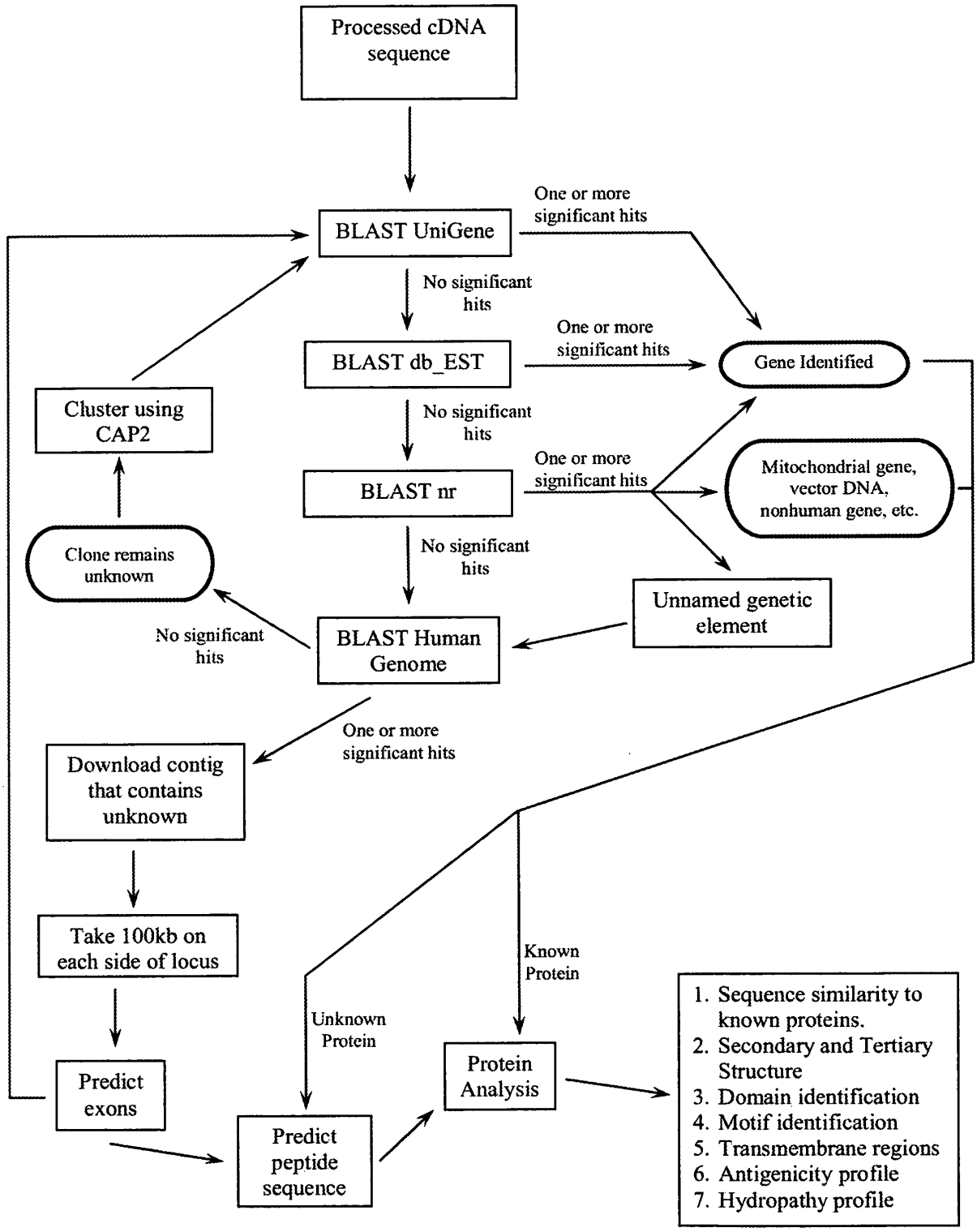


Figure 2. Automated Mononuclear Cell RNA Isolation Device

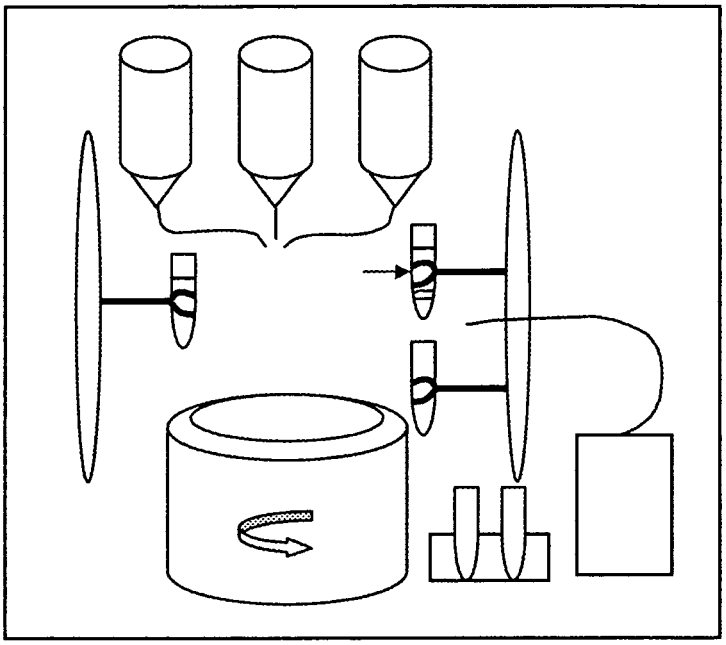


Figure 3

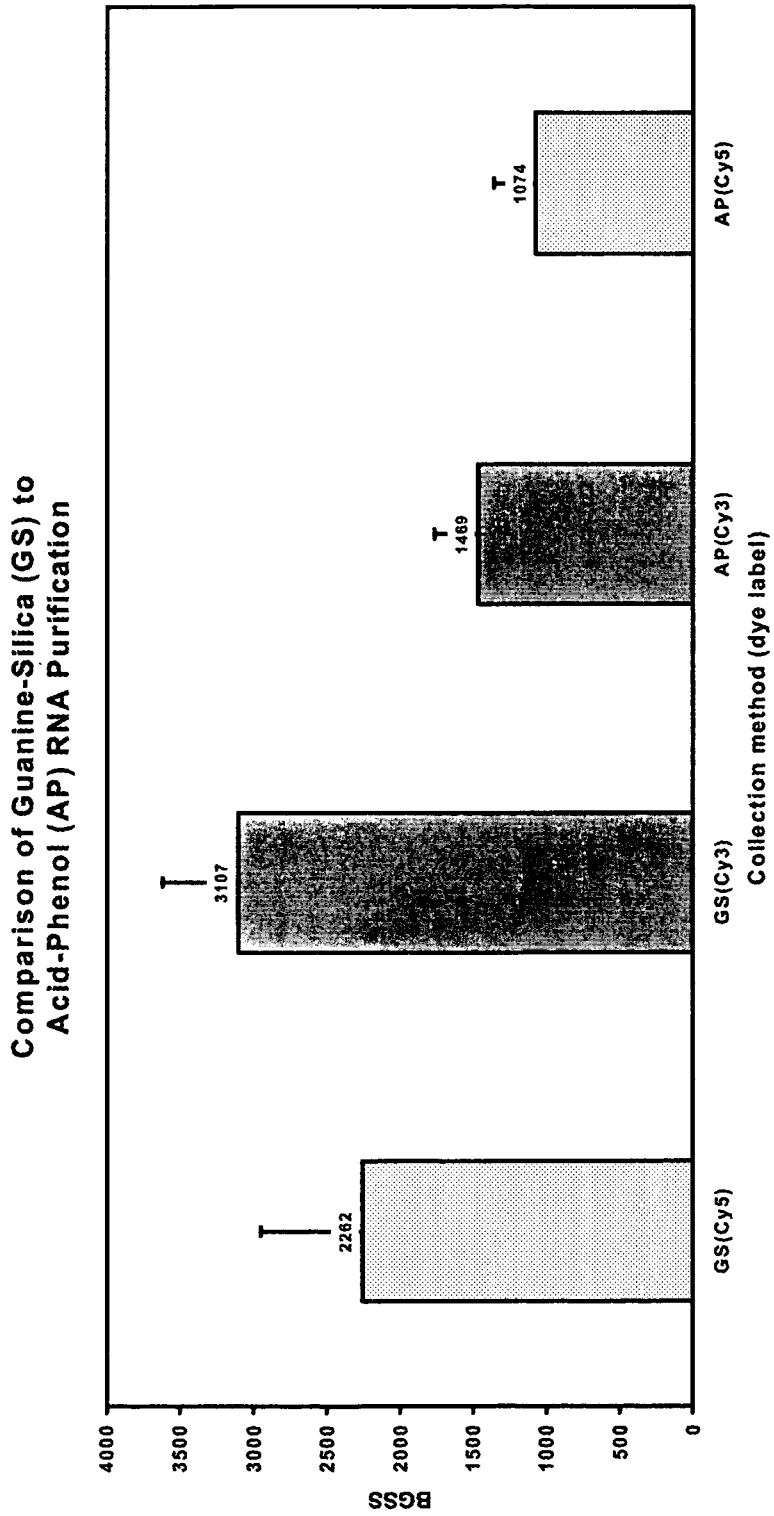

Comparison of Guanine-Silica (GS) to
Acid-Phenol (AP) RNA Purification
BGSS
4000
3500
3000
2500
2000
1500
1000
500
0
2262
3107
1469
1074
GS(Cy5)
GS(Cy3)
AP(Cy3)
AP(Cy5)
Collection method (dye label)

Figure 4

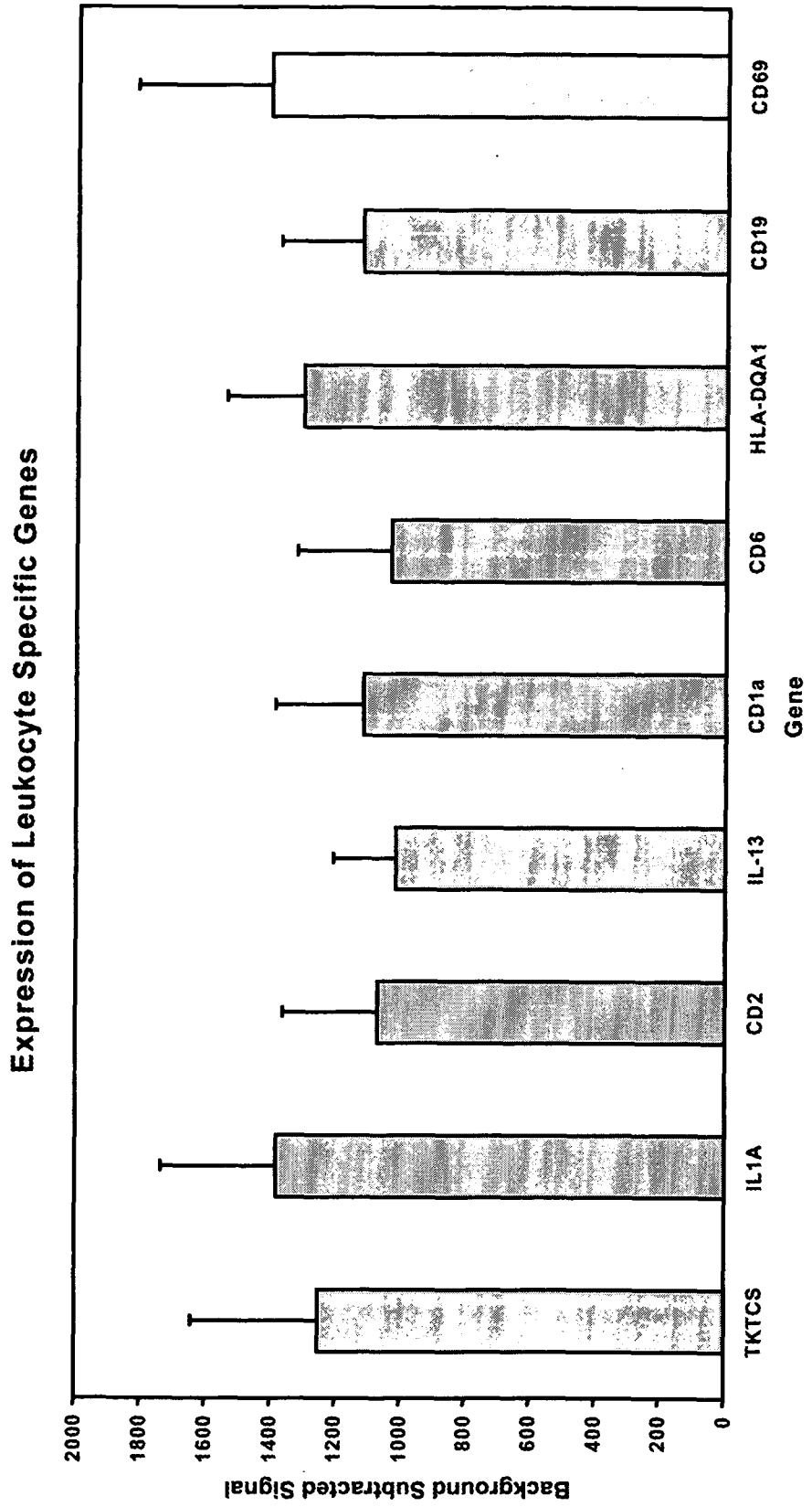

Expression of Leukocyte Specific Genes
Background Subtracted Signal
2000
1800
1600
1400
1200
1000
800
600
400
200
0
TKTCS
IL1A
CD2
IL-13
CD1a
CD6
HLA-DQA1
CD19
CD69
Gene

Figure 5

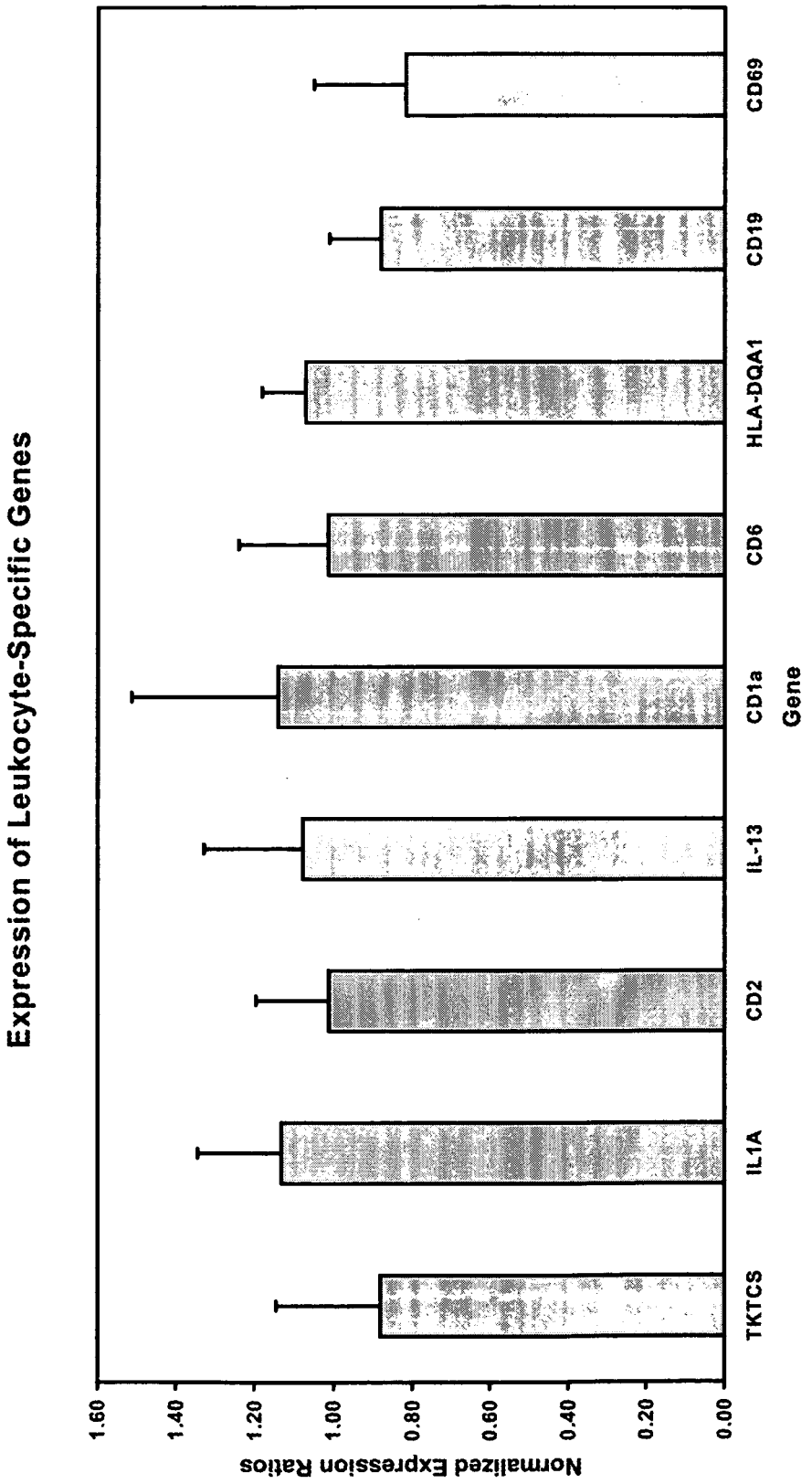

Expression of Leukocyte-Specific Genes
Normalized Expression Ratios
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
TKTCS
IL1A
CD2
IL-13
CD1a
CD6
HLA-DQA1
CD19
CD69
Gene

FIGURE 6

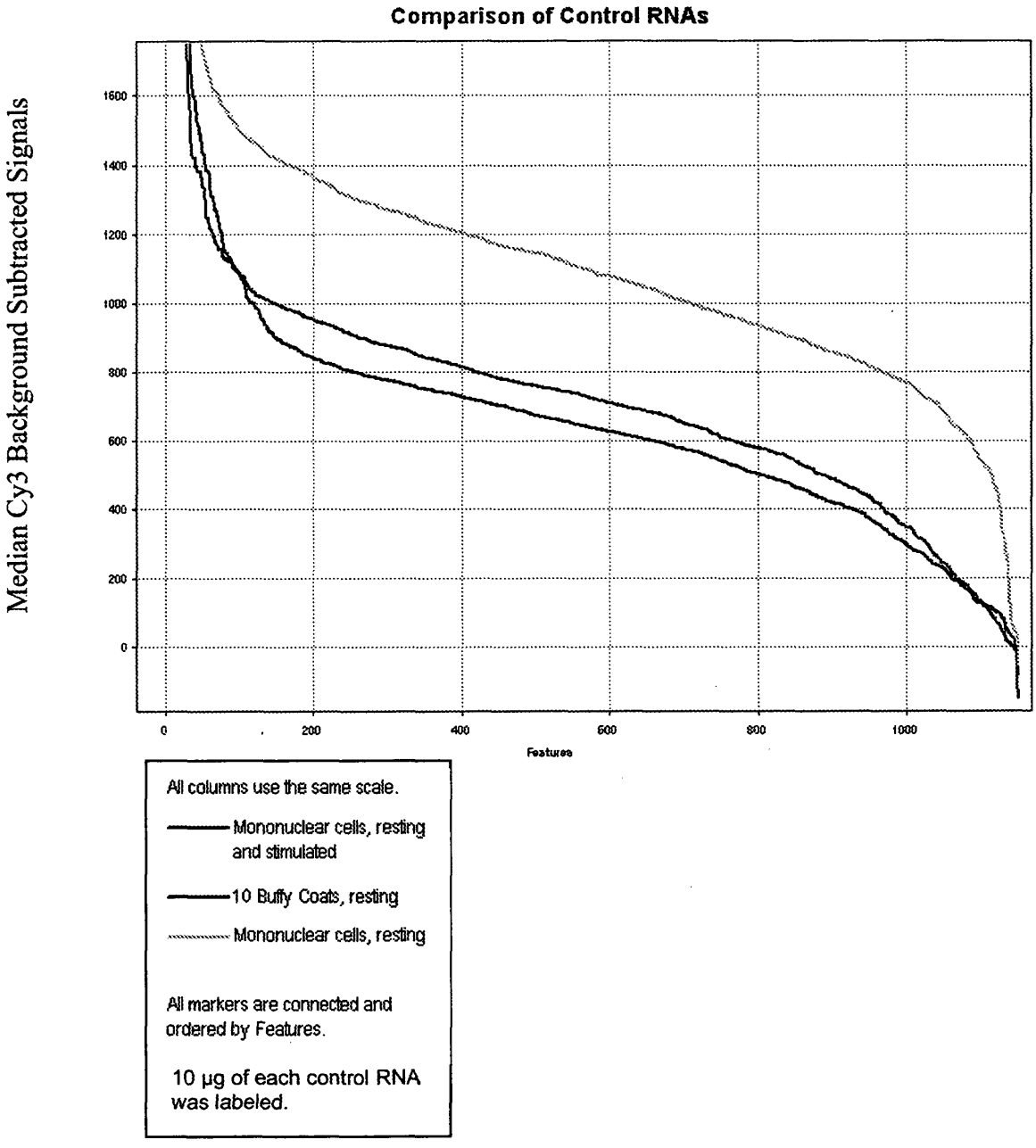
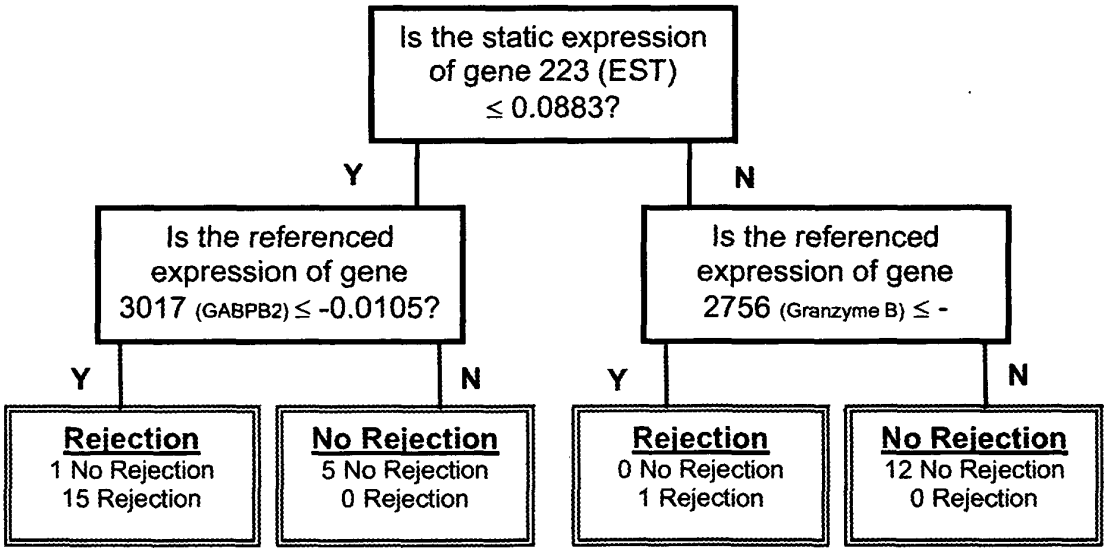

Comparison of Control RNAs
Median Cy3 Background Subtracted Signals
1600
1400
1200
1000
800
600
400
200
0
0
200
400
600
800
1000
Features
All columns use the same scale.
Mononuclear cells, resting and stimulated
10 Buffy Coats, resting
Mononuclear cells, resting
All markers are connected and ordered by Features.
10 µg of each control RNA was labeled.

Figure 7: Cardiac Allograft rejection diagnostic genes.

A.

		Marker Gene Expression Ratios				
Sample	Grade	3020	3019	2760	3018	85
12-0025-02	0	3.90	3.69	5.49	3.24	3.34
12-0024-04	0	3.66	4.05	5.89	3.75	3.03
15-0024-01	0	3.55	4.01	5.61	2.90	3.23
12-0029-03	0	3.44	3.12	4.25	3.55	3.07
12-0024-03	0	2.88	2.54	2.56	2.20	2.38
14-0021-05	0	1.31	1.03	1.07	0.91	0.99
14-0005-06	3A	0.42	0.27	0.51	0.22	0.26
14-0012-07	3A	0.60	0.62	0.70	0.42	0.61
14-0001-06	3A	0.93	0.71	0.58	0.37	0.44
14-0009-01	3A	0.71	0.63	0.68	0.61	0.66
12-0012-02	3A	0.86	0.85	0.73	0.41	0.72
12-0001-01	3A	1.08	0.97	1.01	0.40	1.06
Average Grade 0:		3.13	3.07	4.14	2.76	2.67
Average Grade 3A:		0.77	0.68	0.70	0.40	0.62
Fold Difference:		**4.08**	**4.55**	**5.91**	**6.82**	**4.28**

B. **CART classification model.**



C. Surrogates for the CART classification model.

Primary Splitter	static 223	ref 3017	ref 4
Surrogate 1	ref 167	ref 102	ref 2761
Surrogate 2	ref 3016	static 36	ref 2762
Surrogate 3	ref 1760	ref 2764	ref 3016
Surrogate 4	ref 85	ref 2759	ref 2757
Surrogate 5	ref 2763	ref 2761	ref 2758

Figure 8A: Validation of differential expression of Granzyme B in CMV patients using Real-time PCR

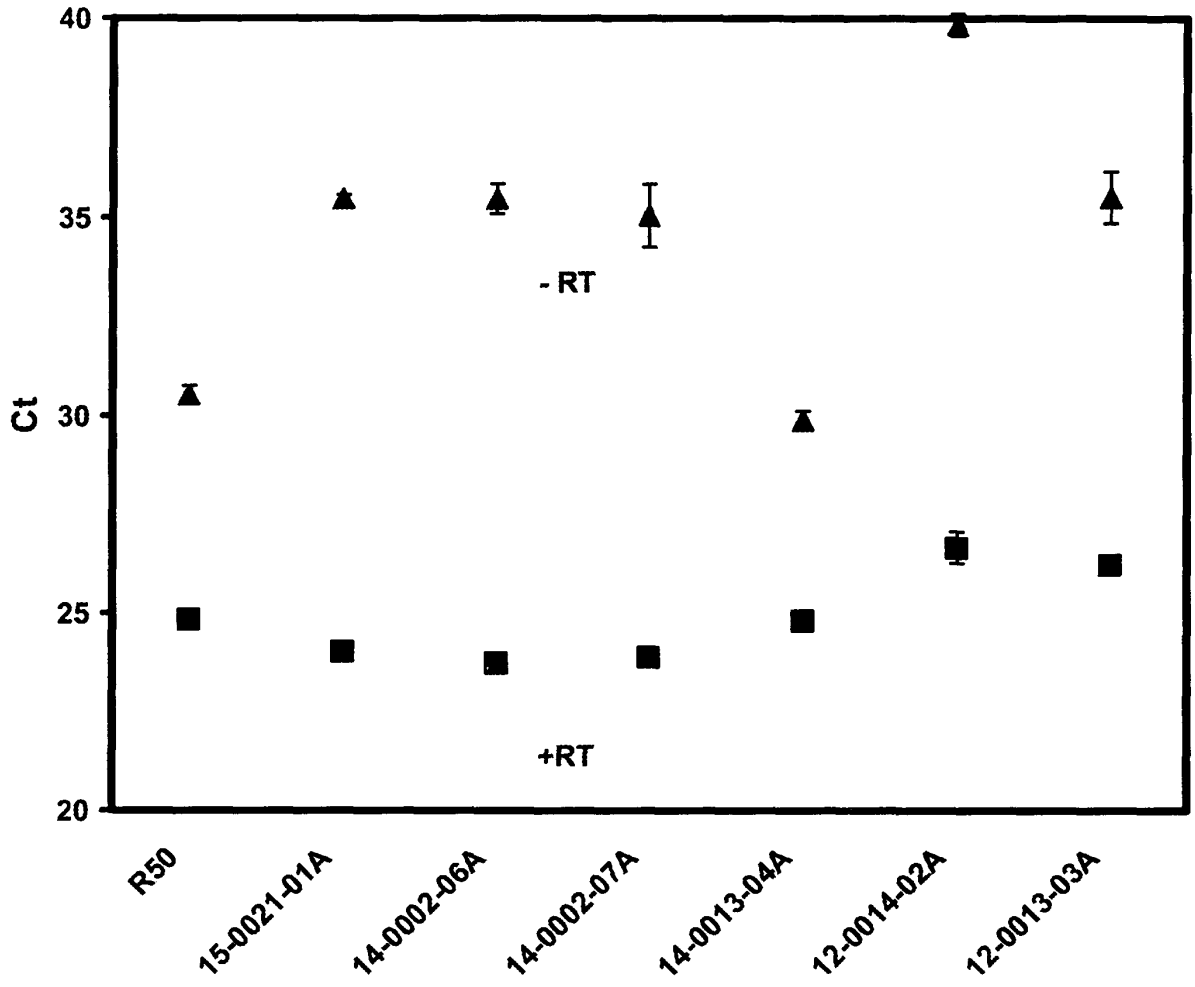


Figure 8B.

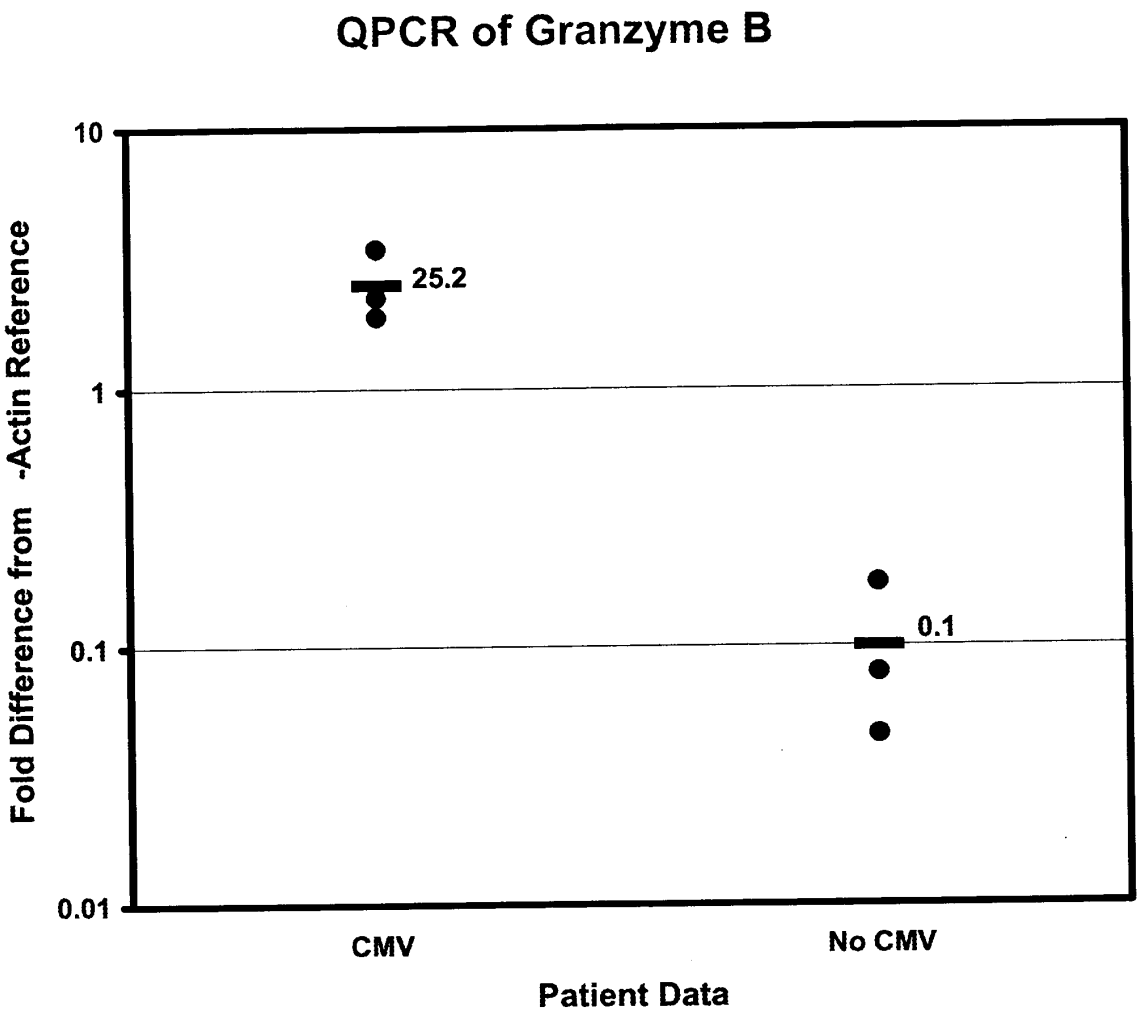
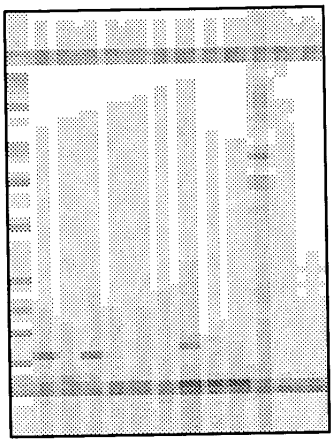

QPCR of Granzyme B
10
1
0.1
0.01
Fold Difference from -Actin Reference
25.2
0.1
CMV
No CMV
Patient Data

Figure 9

A.



B.

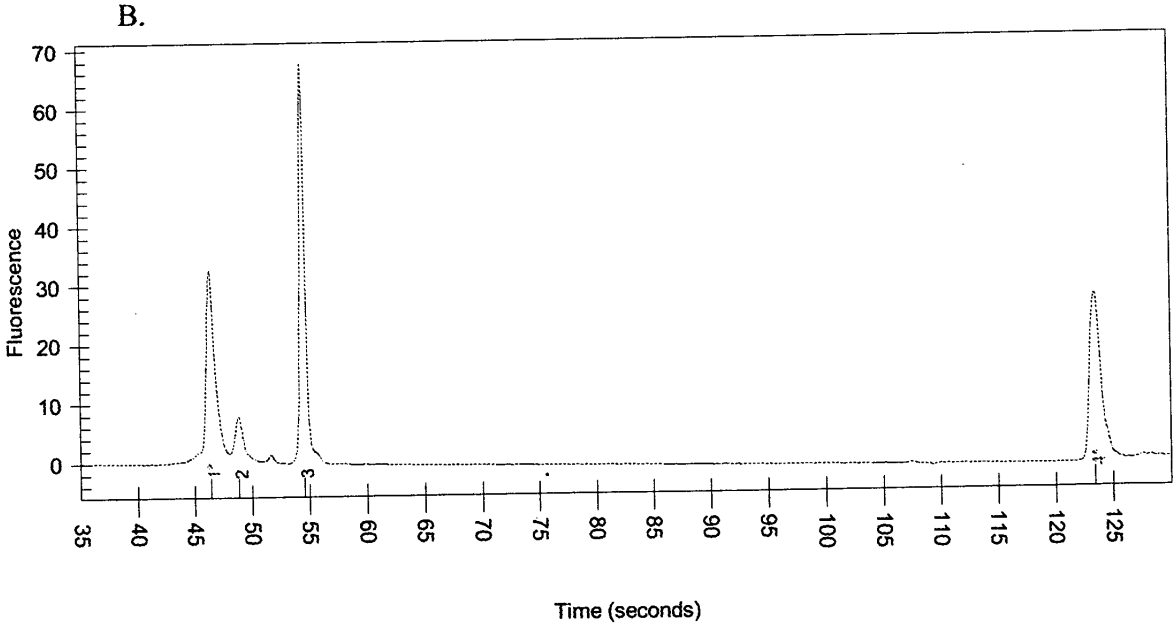
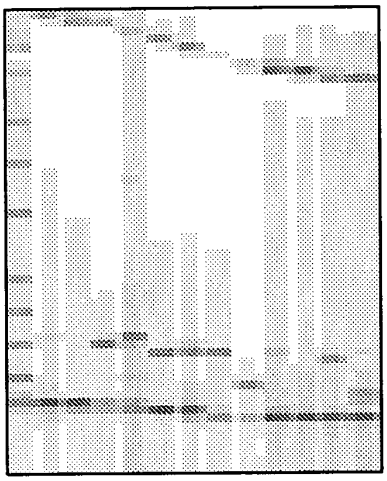

Fluorescence
70
60
50
40
30
20
10
0
1
2
3
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
125
Time (seconds)

Figure 9

C.



D.

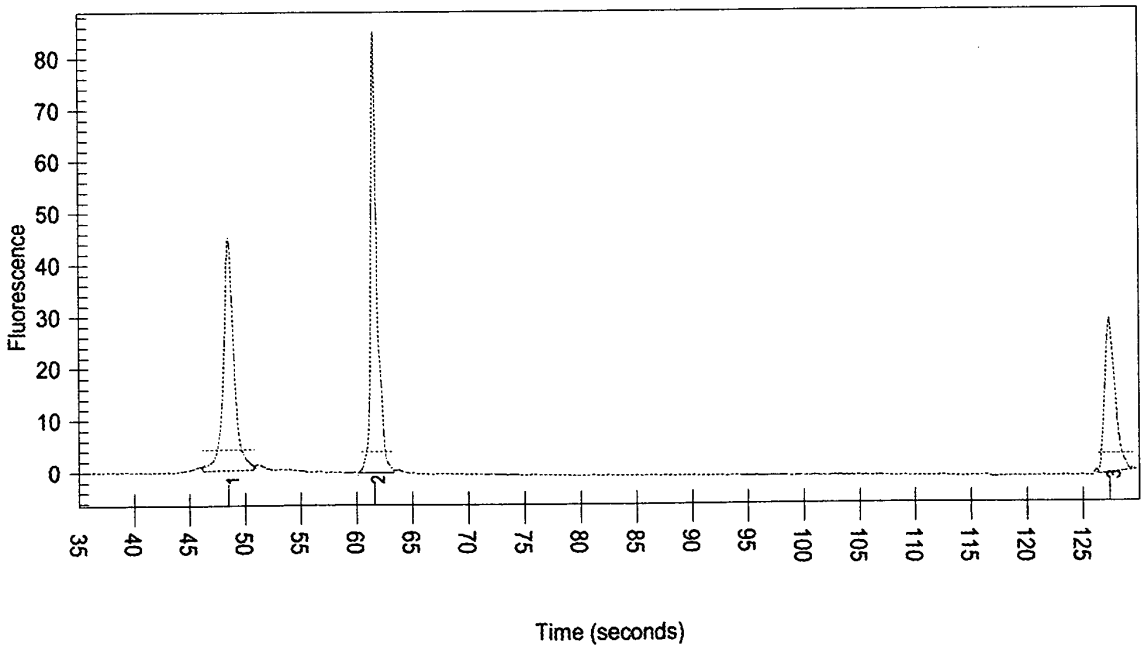

Fluorescence
80
70
60
50
40
30
20
10
0
1
2
3
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
125
Time (seconds)

Figure 10

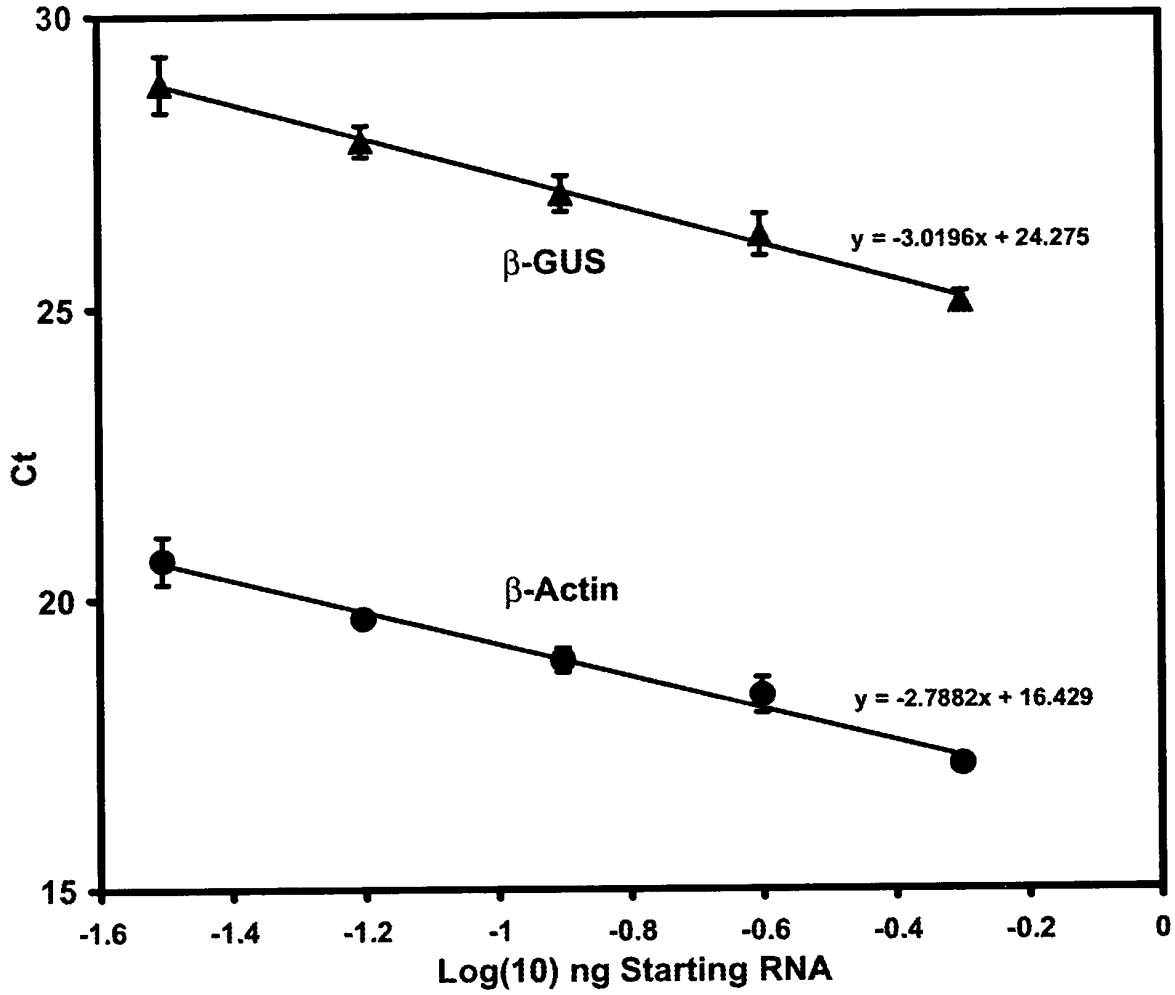
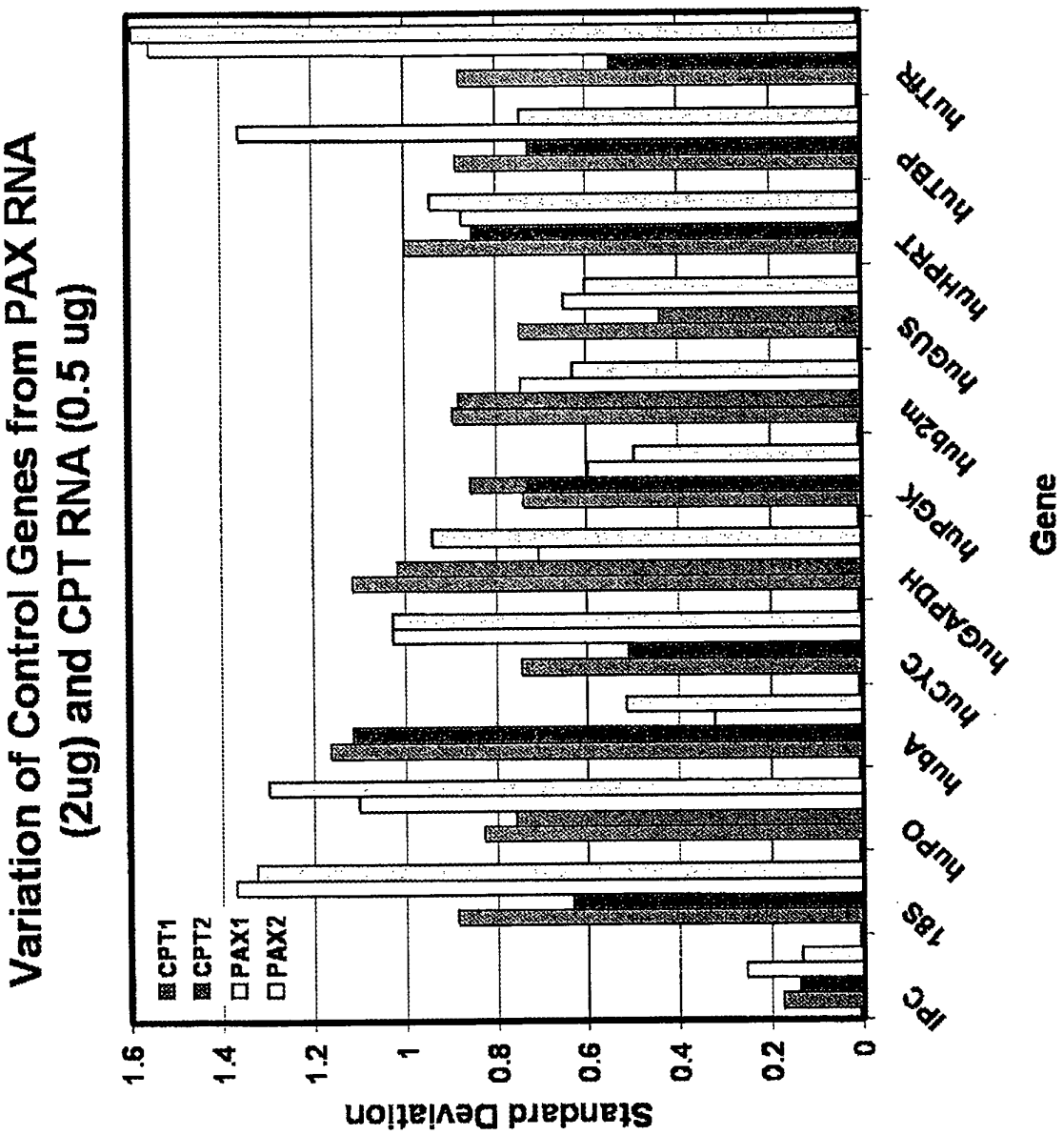

30
25
20
15
Ct
β-GUS
y = -3.0196x + 24.275
β-Actin
y = -2.7882x + 16.429
-1.6
-1.4
-1.2
-1
-0.8
-0.6
-0.4
-0.2
0
Log(10) ng Starting RNA

Figure 11


Variation of Control Genes from PAX RNA (2ug) and CPT RNA (0.5 ug)
CPT1
CPT2
PAX1
PAX2
Standard Deviation
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
IPC
18S
huPO
hubA
huCYC
huGAPDH
huPGK
hub2m
huGUS
huHPRT
huTBP
huTfR
Gene

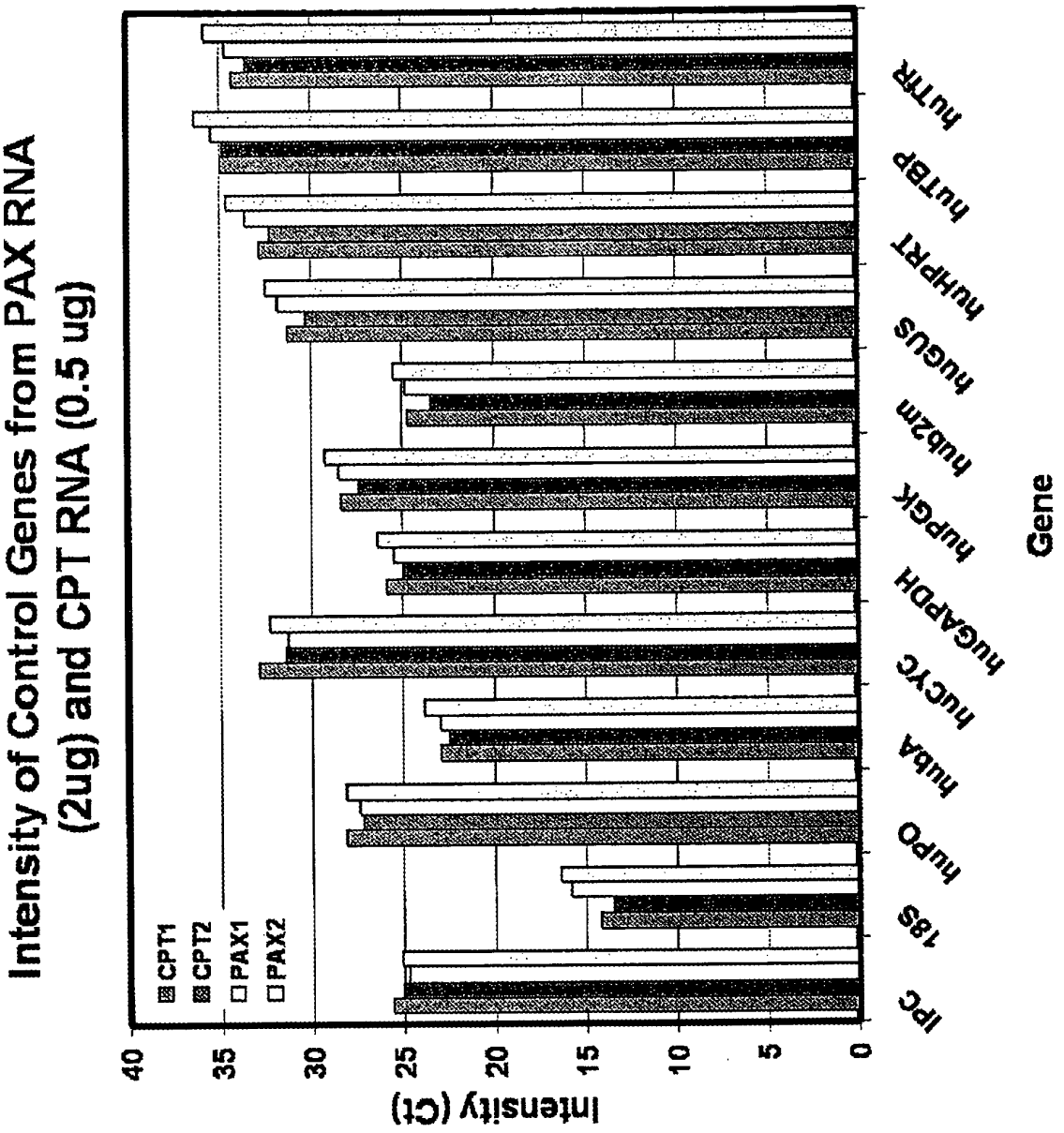

Intensity of Control Genes from PAX RNA
(2ug) and CPT RNA (0.5 ug)
CPT1
CPT2
PAX1
PAX2
Intensity (Ct)
0
5
10
15
20
25
30
35
40
Gene
IPC
18S
huPO
hubA
huCYC
huGAPDH
huPGK
hub2m
huGUS
huHPRT
huTBP
huTFR

Figure 12

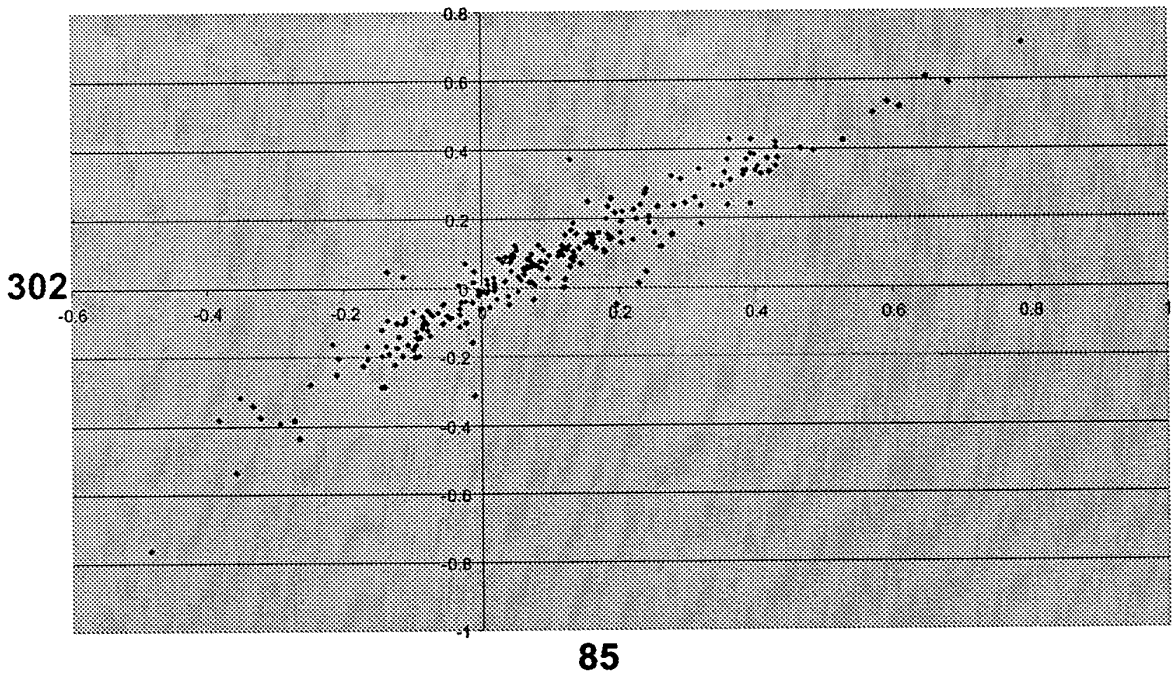
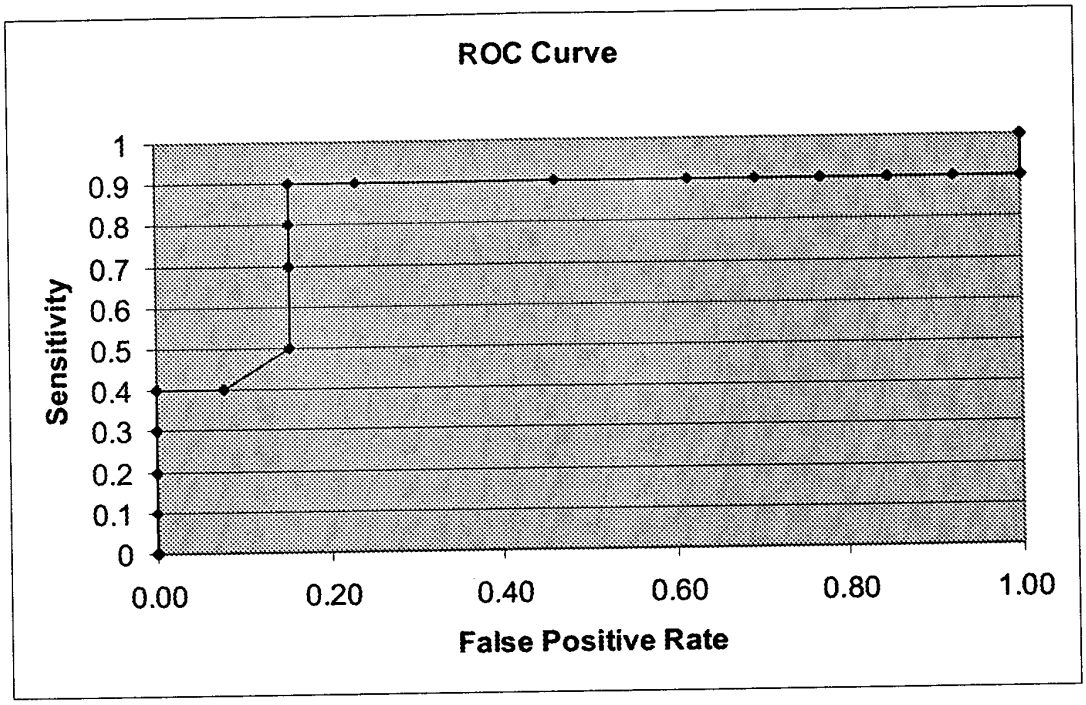


Figure 13


ROC Curve
Sensitivity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.00
0.20
0.40
0.60
0.80
1.00
False Positive Rate

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专利名称(译)	基于标志物表达水平诊断和监测心脏同种异体移植物排斥		
公开(公告)号	EP2253719B1	公开(公告)日	2013-03-20
申请号	EP2010157687	申请日	2003-04-24
申请(专利权)人(译)	XDX INC.		
当前申请(专利权)人(译)	XDX INC.		
[标]发明人	WOHLGEMUTH JAY FRY KIRK WOODWARD ROBERT LY NGOC PRENTICE JAMES MORRIS MACDONALD ROSENBERG STEVEN		
发明人	WOHLGEMUTH, JAY FRY, KIRK WOODWARD, ROBERT LY, NGOC PRENTICE, JAMES MORRIS, MACDONALD ROSENBERG, STEVEN		
IPC分类号	C12Q1/68 G01N33/567 G01N33/564 G01N33/68 C12N15/09 C07H21/00 C12N15/12 C12P19/34 C12Q C12Q1/00 C12Q1/04 G01N G01N1/00 G01N24/08 G01N33/53		
CPC分类号	C12Q1/6883 C12Q1/6881 C12Q1/6888 C12Q2600/158 G01N33/564 G01N33/6863 G01N2800/245		
优先权	10/131831 2002-04-24 US 10/325899 2002-12-20 US		
其他公开文献	EP2253719A3 EP2253719A2		
外部链接	Espacenet		

摘要(译)

描述了通过检测患者中一种或多种基因的表达水平来诊断或监测患者中的移植排斥，特别是心脏移植排斥的方法。还描述了用于诊断或监测移植排斥，特别是心脏移植排斥的诊断寡核苷酸和包含其的试剂盒或系统。