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(54) **Title**: BIOMARKERS FOR DETECTION OF TUBERCULOSIS RISK

(57) **Abstract**: The present application relates generally to biomarkers for tuberculosis (TB) infection and disease and methods of detection thereof. In various embodiments, the invention relates to one or more biomarkers, biomarker panels, methods, devices, reagents, systems, and kits for detecting and/or characterizing TB infection and/or disease.

BIOMARKERS FOR DETECTION OF TUBERCULOSIS RISK

This application claims the benefit of priority of US Provisional Application No. 62/108,424, filed January 27, 2015; US Provisional Application No. 62/108,431, filed January 27, 2015; and US Provisional Application No. 62/158,930, filed May 8, 2015; each of which is incorporated by reference herein in its entirety for any purpose.

FIELD

The present application relates generally to biomarkers for determining the risk of a subject with latent tuberculosis (TB) infection developing active TB disease, and methods of use thereof. In various embodiments, the invention relates to one or more biomarkers, biomarker panels, methods, devices, reagents, systems, and/or kits for detecting and/or characterizing the risk of a subject with a latent TB infection developing active TB disease.

BACKGROUND

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* and other disease causing mycobacteria. The bacteria usually attack the lungs, but TB bacteria can attack any part of the body such as the kidney, spine, and brain. If not treated properly, TB disease can be fatal. Not everyone infected with TB bacteria becomes sick. As a result, two TB-related conditions exist: latent TB infection and active TB disease. Both latent TB infection and active TB disease can be treated.

SUMMARY

In some embodiments, methods of determining the risk of a subject with latent tuberculosis (TB) infection developing active TB disease are provided.

In some embodiments, a method comprises detecting the presence or level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble) in a sample (e.g., plasma, serum, urine, saliva, etc.) from the subject. In some embodiments, the subject is identified as having a latent TB infection that is likely to transition into active TB disease if the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, or eight of the biomarkers is higher than a control level of the respective biomarker. In some embodiments, a method comprises detecting the presence or level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or ten biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16

(soluble), and C9 in a sample (e.g., plasma, serum, urine, saliva, etc.) from the subject. In some embodiments, the subject is identified as having a latent TB infection that is likely to transition into active TB disease if the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine of the biomarkers is higher than a control level of the respective biomarker.

In some embodiments, a method comprises detecting the level of C9 and optionally one or more of AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble) in a sample from the subject. In some embodiments, a method comprises detecting the level of AMBN and optionally one or more of C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a method comprises detecting the level of C5 and optionally one or more of AMBN, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a method comprises detecting the level of MMP-1 and optionally one or more of AMBN, C5, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a method comprises detecting the level of D-dimer and optionally one or more of AMBN, C5, MMP-1, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a method comprises detecting the level of SG1C1 and optionally one or more of AMBN, C5, MMP-1, D-dimer, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a method comprises detecting the level of 2DMA and optionally one or more of AMBN, C5, MMP-1, D-dimer, SG1C1, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a method comprises detecting the level of IP-10 and optionally one or more of AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a method comprises detecting the level of CXCL16 (soluble) and optionally one or more of AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and C9 in a sample from the subject. In some embodiments, a method comprises detecting the level of KCNE2 and optionally one or more of AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, CXCL16 (soluble), and C9 in a sample from the subject.

In some embodiments, detection of a particular level of AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and/or C9 in a sample (e.g., plasma, serum, urine, saliva, etc.) from the subject that is higher than a control level of the respective biomarker is indicative of and/or diagnostic for a latent TB infection that is likely to develop into active TB disease within 30 days, 45 days, 60 days, 90 days, 120 days, 180

days, 270 days, 360 days, 450 days, or 540 days transitioning to active TB infection. In some embodiments, a level of at least one biomarker selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and/or C9 that is higher than the level of the respective biomarker in a control sample indicates that a subject with latent TB infection is likely to develop active TB disease.

In some embodiments, provided herein are methods of determining a likelihood of a latent tuberculosis (TB) infection in a subject transitioning to active TB disease, comprising detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, or at least eight, biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble) in a sample from the subject, wherein the subject is identified as having a latent TB infection that is likely to develop into active TB disease within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days if the level of the respective biomarker is higher relative to a control level of the respective biomarker. In some embodiments, methods further comprise detecting the level of one or more biomarkers that are indicative of one or more of: the presence of latent TB infection, the presence of active TB disease, the strain of TB, the antibiotic resistance/sensitivity of TB, and/or the presence of other diseases. In some embodiments, methods comprise detecting the levels of 2 to 20 biomarkers, or 2 to 10 biomarkers, or 2 to 9 biomarkers, or 3 to 20 biomarkers, or 3 to 10 biomarkers, or 3 to 9 biomarkers, or 4 to 20 biomarkers, or 4 to 10 biomarkers, or 4 to 9 biomarkers, or 5 to 20 biomarkers, or 5 to 10 biomarkers, or 5 to 9 biomarkers.

In some embodiments, provided herein are methods of determining a likelihood of a latent TB infection in a subject transitioning to active TB disease, comprising detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or ten biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject, wherein the subject is identified as having a latent TB infection that is likely to develop into active TB disease within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days if the level of the respective biomarker is higher relative to a control level of the respective biomarker. In some embodiments, methods further comprise detecting the level of one or more biomarkers that are indicative of one or more of: the presence of latent TB infection, the presence of active TB disease, the strain of TB, the antibiotic resistance/sensitivity of TB, and/or the presence of other diseases. In some embodiments, methods comprise detecting the levels of 2 to 20 biomarkers, or 2 to 10

biomarkers, or 2 to 9 biomarkers, or 3 to 20 biomarkers, or 3 to 10 biomarkers, or 3 to 9 biomarkers, or 4 to 20 biomarkers, or 4 to 10 biomarkers, or 4 to 9 biomarkers, or 5 to 20 biomarkers, or 5 to 10 biomarkers, or 5 to 9 biomarkers.

In some embodiments, provided herein are methods of monitoring a latent TB infection in a subject for the likelihood of the latent TB infection transitioning to active TB disease, comprising detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble) in a sample from the patient at a first time point, and measuring the level of the at least one, at least two, at least three, at least four, at least five, at least six, at least seven, or eight biomarkers at a second time point. In some embodiments, if the level of the biomarkers is further from a control level at the second time point than the first time point, the likelihood of the latent TB infection transitioning to active TB disease has increased. In some embodiments, if the level of the biomarkers is higher at the second time point than at the first time point, the likelihood of the latent TB infection transitioning to active TB disease has increased. In some embodiments, if the level of the biomarkers is lower at the second time point than at the first time point, the likelihood of the latent TB infection transitioning to active TB disease has decreased.

In some embodiments, provided herein are methods of monitoring a latent TB infection in a subject for the likelihood of the latent TB infection transitioning to active TB disease, comprising detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or ten biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the patient at a first time point, and measuring the level of the at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine biomarkers at a second time point. In some embodiments, if the level of the biomarkers is further from a control level at the second time point than the first time point, the likelihood of the latent TB infection transitioning to active TB disease within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days has increased. In some embodiments, if the level of the biomarkers is higher at the second time point than at the first time point, the likelihood of the latent TB infection transitioning to active TB disease has increased. In some embodiments, if the level of the biomarkers is nearer to a control level at the second time point than the first time point, the likelihood of the latent TB infection transitioning to active TB disease has decreased. In some embodiments,

if the level of the biomarkers is lower at the second time point than at the first time point, the likelihood of the latent TB infection transitioning to active TB disease has decreased.

In some embodiments, provided herein are methods of monitoring treatment of a latent TB infection, comprising detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble) in a sample from the patient at a first time point, administering at least one treatment for TB infection to the patient, and detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble) in a sample from the patient at a second time point, wherein the treatment is effective at reducing the likelihood of the latent TB infection transitioning to active TB disease if the level of the biomarkers is nearer to a control level at the second time point compared to the first time point. In some embodiments, provided herein are methods of monitoring treatment of a latent TB infection, comprising detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble) in a sample from the patient at a first time point, administering at least one treatment for TB infection to the patient, and detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or ten biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the patient at a second time point. In some embodiments, the treatment is effective at reducing the likelihood of the latent TB infection transitioning to active TB disease if the level of the biomarkers is nearer to a control level, or is not further from a control level than, at the second time point compared to the first time point. In some embodiments, the treatment is effective at reducing the likelihood of the latent TB infection transitioning to active TB disease if the level of the biomarkers is lower at the second time point compared to the first time point. In some embodiments, the at least one treatment for TB infection is selected from the group consisting of isoniazid (INH), rifampin (RIF), rifapentine (RPT), ethambutol (EMB), pyrazinamide (PZA), and/or another approved TB therapeutic to the subject.

In some embodiments, a control level is the level of the respective biomarker in a subject or population of subjects with latent TB infection who are known not to have developed active TB within a particular time period. In some embodiments, a control level is

the level of the respective biomarker in a subject or population of subjects with latent TB infection who are known not to have developed active TB within 540 days of sample collection. In some embodiments, a control level is the level of the respective biomarker in a subject or population of subjects with latent TB infection who are known not to have developed active TB within 2 years of sample collection.

In some embodiments, methods further comprise performing one or more additional tests for TB infection. In some embodiments, additional tests for TB infection comprise chest x-ray.

In some methods described herein, each biomarker is a protein biomarker. In some embodiments, methods comprise contacting biomarkers of the sample from the subject or patient with a set of biomarker capture reagents, wherein each biomarker capture reagent of the set of biomarker capture reagents specifically binds to a different biomarker being detected. In some embodiments, each biomarker capture reagent is an antibody or an aptamer. In some embodiments, at least one aptamer is a slow off-rate aptamer. In some embodiments, at least one slow off-rate aptamer comprises at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least 10 nucleotides with modifications. In some embodiments, each slow off-rate aptamer binds to its target protein with an off rate ($t_{1/2}$) of $\geq$ 30 minutes, $\geq$ 60 minutes, $\geq$ 90 minutes, $\geq$ 120 minutes, $\geq$ 150 minutes, $\geq$ 180 minutes, $\geq$ 210 minutes, or $\geq$ 240 minutes.

In some embodiments, the sample is a blood sample. In some embodiments, the sample is a serum sample.

In some embodiments, a method for determining whether a a latent TB infection is likely to advance into active TB disease in a subject within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days comprises (a) forming a biomarker panel having N biomarker proteins selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, CXCL16 (soluble), and C9; or AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble); and (b) detecting the level of each of the N biomarker proteins of the panel in a sample from the subject. In some embodiments, N is 1 to 9. In some embodiments, N is 2 to 9. In some embodiments, N is 3 to 9. In some embodiments, N is 4 to 9. In some embodiments, N is 5 to 9. In some embodiments, N is 6 to 9. In some embodiments, N is 7 to 9. In some embodiments, N is 8 to 9. In some embodiments, N is 9. In some embodiments, N is 2 to 8. In some embodiments, N is 3 to 7. In some embodiments, N is 4 to 6. In some embodiments, N is 1 to 8. In some embodiments, N is 2 to 8. In some embodiments, N is 3 to 8. In some embodiments, N is 4 to 8. In some

embodiments, N is 5 to 8. In some embodiments, N is 6 to 8. In some embodiments, N is 7 to 8. In some embodiments, N is 8. In some embodiments, a method comprises forming a biomarker panel having X biomarker proteins, wherein N of the X biomarker proteins are selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9; or from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, and CXCL16 (soluble); and detecting the level of each of the X biomarker proteins of the panel in a sample from the subject. In some embodiments, X is 100 or fewer (e.g., <90 biomarkers, <80 biomarkers, <70 biomarkers, <60 biomarkers, <50 biomarkers, <40 biomarkers, <30 biomarkers, <20 biomarkers, <15 biomarkers). In some embodiments, X is 10 or greater (e.g., >11 biomarkers, >12 biomarkers, >13 biomarkers, >14 biomarkers, >15 biomarkers, >20 biomarkers, >30 biomarkers, >40 biomarkers, >50 biomarkers). In some embodiments, X is between 10 and 100, between 10 and 90, between 10 and 80, between 10 and 70, between 10 and 60, between 10 and 50, between 10 and 40, between 10 and 30, between 10 and 20, or between 10 and 15. In some embodiments, N is between 1 and 9 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9) or between 1 and 8 (e.g., 1, 2, 3, 4, 5, 6, 7, 8).

In some embodiments, a set of biomarker proteins with a sensitivity + specificity value of 1.3 or greater, 1.35 or greater, 1.4 or greater, 1.45 or greater, 1.5 or greater, is selected that comprises one or more biomarkers selected from: AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9; or from: AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble); or from: AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, KCNE2, and CXCL16 (soluble); or from AMBN, C5, MMP-1, C9, SG1C1, 2DMA, KCNE2, and CXCL16 (soluble).

In some embodiments, one or more additional steps are taken upon identifying a subject as having a latent TB infection that is likely to transition into active TB disease within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days. In some embodiments, methods further comprise a subsequent step of treating said subject or patient for latent TB. In some embodiments, methods further comprise a subsequent step of treating said subject or patient for active TB disease. In some embodiments, methods further comprise a subsequent step of additional TB-diagnostic steps. In some embodiments, said additional TB-diagnostic steps comprise a chest x-ray. In some embodiments, methods further comprise generating a report indicating that said subject is likely to develop active TB disease within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days. In some embodiments, a subject with such a risk is treated for active TB disease before developing symptoms of active TB disease.

In any of the embodiments described herein, the each biomarker may be a protein biomarker. In any of the embodiments described herein, the method may comprise contacting biomarkers of the sample from the subject or patient with a set of biomarker detection reagents. In any of the embodiments described herein, the method may comprise contacting biomarkers of the sample from the subject or patient with a set of biomarker capture reagents, wherein each biomarker capture reagent of the set of biomarker capture reagents specifically binds to a biomarker being detected. In some embodiments, each biomarker capture reagent of the set of biomarker capture reagents specifically binds to a different biomarker being detected. In any of the embodiments described herein, each biomarker capture reagent may be an antibody or an aptamer. In any of the embodiments described herein, each biomarker capture reagent may be an aptamer. In any of the embodiments described herein, at least one aptamer may be a slow off-rate aptamer. In any of the embodiments described herein, at least one slow off-rate aptamer may comprise at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least 10 nucleotides with modifications. In some embodiments, the modifications are hydrophobic modifications. In some embodiments, the modifications are hydrophobic base modifications. In some embodiments, one or more of the modifications may be selected from the modifications shown in Figure 52. In some embodiments, each slow off-rate aptamer binds to its target protein with an off rate ($t_{½}$) of $\geq$ 30 minutes, $\geq$ 60 minutes, $\geq$ 90 minutes, $\geq$ 120 minutes, $\geq$ 150 minutes, $\geq$ 180 minutes, $\geq$ 210 minutes, or $\geq$ 240 minutes.

In any of the embodiments described herein, the sample may be a blood sample. In some embodiments, the blood sample is selected from a serum sample and a plasma sample. In some embodiments, the sample is a body fluid selected from tracheal aspirate fluid, bronchoalveolar fluid, bronchoalveolar lavage sample, blood or portion thereof, serum, plasma, urine, semen, saliva, tears, etc.

In any of the embodiments described herein, a method may further comprise treating the subject or patient for TB infection or TB disease. In some embodiments, treating the subject or patient for TB infection or TB disease comprises a treatment regimen of administering one or more of: isoniazid (INH), rifampin (RIF), rifapentine (RPT), ethambutol (EMB), pyrazinamide (PZA), and/or another approved TB therapeutic to the subject or patient.

In some embodiments, kits are provided. In some embodiments, a kit comprises at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at

least eight, at least nine, or ten aptamers, wherein each aptamer specifically binds to a target protein selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9; or at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine aptamers, wherein each aptamer specifically binds to a target protein selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble). In some embodiments, a kit comprises an aptamer that specifically binds C9 and optionally one or more aptamers that specifically bind one or more of AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble) in a sample from the subject. In some embodiments, a kit comprises an aptamer that specifically binds AMBN and optionally one or more aptamers that specifically bind one or more of C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a kit comprises an aptamer that specifically binds C5 and optionally one or more aptamers that specifically bind one or more of AMBN, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a kit comprises an aptamer that specifically binds MMP- and optionally one or more aptamers that specifically bind one or more of AMBN, C5, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a kit comprises an aptamer that specifically binds D-dimer and optionally one or more aptamers that specifically bind one or more of AMBN, C5, MMP-1, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a kit comprises an aptamer that specifically binds SG1C1 and optionally one or more aptamers that specifically bind one or more of AMBN, C5, MMP-1, D-dimer, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a kit comprises an aptamer that specifically binds 2DMA and optionally one or more aptamers that specifically bind one or more of AMBN, C5, MMP-1, D-dimer, SG1C1, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a kit comprises an aptamer that specifically binds IP-10 and optionally one or more aptamers that specifically bind one or more of AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments a kit comprises an aptamer that specifically binds CXCL16 (soluble) and optionally one or more aptamers that specifically bind one or more of AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and C9 in a sample from the subject. In some embodiments a kit comprises an aptamer that specifically binds KCNE2 and optionally one or more aptamers that specifically bind one or

more of AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, CXCL16 (soluble), and C9 in a sample from the subject.

In some embodiments, the kit comprises a total of 2 to 20 aptamers, or 2 to 10 aptamers, or 2 to 9 aptamers, or 3 to 20 aptamers, or 3 to 10 aptamers, or 3 to 9 aptamers, or 4 to 20 aptamers, or 4 to 10 aptamers, or 4 to 9 aptamers, or 5 to 20 aptamers, or 5 to 10 aptamers, or 5 to 9 aptamers. In some embodiments, a kit comprises X aptamers, wherein N aptamers specifically bind to a biomarker protein selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, CXCL16 (soluble), and C9; or AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble). In some embodiments, X is less than 100 (e.g., <90, <80, <70, <60, <50, <40, <30, <20, <15). In some embodiments, X is 10 or more (e.g., >10, >11, >12, >13, >14, >15, >20, >30, >40, >50). In some embodiments, X is between 10 and 100, between 10 and 90, between 10 and 80, between 10 and 70, between 10 and 60, between 10 and 50, between 10 and 40, between 10 and 30, between 10 and 20, or between 10 and 15. In some embodiments, N is 1 to 9 (1, 2, 3, 4, 5, 6, 7, 8, 9). In some embodiments, N is 1 to 8 (1, 2, 3, 4, 5, 6, 7, 8).

In some embodiments, compositions are provided comprising proteins of a sample from a subject or patient and at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine aptamers, wherein each aptamer specifically binds to a different target protein selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9. In some embodiments, compositions are provided comprising proteins of a sample from a subject or patient and at least one, at least two, at least three, at least four, at least five, at least six, at least seven, or eight aptamers, wherein each aptamer specifically binds to a different target protein selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble).

In any of the embodiments described herein, a kit or composition may comprise at least one aptamer that is a slow off-rate aptamer. In any of the embodiments described herein, each aptamer of a kit or composition may be a slow off-rate aptamer. In some embodiments, at least one slow off-rate aptamer comprises at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least 10 nucleotides with modifications. In some embodiments, at least one nucleotide with a modification is a nucleotide with a hydrophobic base modification. In some embodiments, each nucleotide with a modification is a nucleotide with a hydrophobic base modification. In some embodiments, each hydrophobic base modification is independently selected from the modification in Figure 52. In some embodiments, each slow off-rate aptamer in a kit binds to

its target protein with an off rate ($t_{½}$) of ≥ 30 minutes, ≥ 60 minutes, ≥ 90 minutes, ≥ 120 minutes, ≥ 150 minutes, ≥ 180 minutes, ≥ 210 minutes, or ≥ 240 minutes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows (left) beeswarm plots of sampling time for all cases in the discovery and verification sets for all time points, and (right) empirical cumulative distribution functions of time to diagnosis in discovery and verification.

Figure 2 shows boxplots of log2 transformed hybridization normalization scale factors for each plate (left), and cumulative distribution functions of raw normalization scale factors for each plate (right).

Figure 3 shows boxplots of Log2 transformed median normalization scale factors.

Figure 4 shows a subspace projection for each sample from a PCA performed using the top 50 ranked proteins which were observed to differentiate gender.

Figure 5 shows a plot of the empirical CDF of age (left) and a demographic table (right) for all TB Cases and Controls, 0 to 950 to beginning of treatment.

Figure 6 shows empirical CDFs for the top 9 ranked proteins comparing all TB case and all Control samples.

Figure 7 shows a plot of KS distances with class randomization statistics for the top 100 features.

Figure 8 shows a volcano plot of 3040 proteins from a univariate KS analysis comparing all TB Cases and all Controls..

Figure 9 shows RFU trajectories of individual TB cases overlaid onto a 'control band' created by interpolating the median, IQR and range of the control data. The top axis corresponds to the controls and the bottom the TB cases. Time moves to the right.

Figure 10 shows a heat map of t-statistics arranged by hierarchical clustering for the top 200 t-statistics ranked by the median across all bins.

Figure 11 shows a heat map of t-statistics (left) and corresponding CDFs of cases and controls for subcluster A, which was selected based on inconsistencies in the 3TB/8Controls bin.

Figure 12 shows a heat map of t-statistics (left) and corresponding CDFs of cases and controls for subcluster B, which was selected based on inconsistencies in the 4TB/6Controls bin.

Figure 13 shows a heat map of t-statistics (left) and corresponding CDFs of cases and controls for subcluster C, which was selected because most proteins seemed to be homogenously higher in the TB cases.

Figure 14 shows a heat map of t-statistics (left) and corresponding CDFs of cases and controls for subcluster D, which was selected based on most proteins being homogenously lower in the TB Cases.

Figure 15 shows a heat map of t-statistics (left) and corresponding CDFs of cases and controls for subcluster E, which was selected based on inconsistencies in several bins.

Figure 16 shows Linear fits for all TB cases are shown as a function of time to treatment. The dark band corresponds to the interquartile range (IQR), while the lighter shaded region corresponds to the whiskers, or the nearest data point that's within the upper/lower quartile + 1.5*IQR. Data outside this range is considered an outlier.

Figure 17 shows sample times for all TB subjects as a function of time to the beginning of treatment. Negative values are days on treatment.

Figure 18 shows a plot of the empirical CDF of age (left) and a demographic table (right) for TB Cases 0-180 days to beginning of treatment, and matched Controls.

Figure 19 shows empirical CDFs for the top 9 ranked proteins comparing non-TB vs. TB 0-180 days before treatment.

Figure 20 shows a KS Plot of KS distances with class randomization statistics for the top 50 features comparing non-TB vs. TB 0-180 days before treatment (pvalue threshold=1.12e-3).

Figure 21 shows a volcano plot of 3040 proteins from a univariate KS analysis comparing TB Cases 0-180 days pre-Rx to matched controls.

Figure 22 shows RFU trajectories for the top markers found to distinguish non-TB vs. TB 0-180 days before treatment. Individual TB cases were overlaid onto a 'control band' created by interpolating the median, IQR and range of the control data. The top axis corresponds to the controls and the bottom to the TB cases. Time moves to the right.

Figure 23 shows a plot of the empirical CDF of age (left) and a demographic table (right) for TB Cases 180-360 days to beginning of treatment and matched Controls

Figure 24 shows empirical CDFs for the top 9 ranked proteins comparing non-TB vs. TB 180-360 days before treatment.

Figure 25 shows a KS Plot of KS distances with class randomization statistics for the top 50 features comparing non-TB vs. TB 180-360 days before treatment (pvalue threshold=8.55e-3).

Figure 26 shows a volcano plot of 3040 proteins from a univariate KS analysis comparing TB Cases 180-360 days pre-Rx to matched controls.

Figure 27 shows RFU trajectories for the top markers found to distinguish non-TB vs. TB 180-360 days before treatment. Individual TB cases were overlaid onto a 'control band' created by interpolating the median, IQR and range of the control data. The top axis corresponds to the controls and the bottom the TB cases. Time moves to the right.

Figure 28 shows a plot of the empirical CDF of age (left) and a demographic table (right) for TB Cases 360-540 days to beginning of treatment and matched Controls

Figure 29 shows empirical CDFs for the top 9 ranked proteins comparing non-TB vs. TB 360-540 days before treatment.

Figure 30 shows plot of KS distances with class randomization statistics for the top 50 features comparing non-TB vs. TB 360-540 days before treatment (pvalue threshold=3.84-3).

Figure 31 shows a volcano plot of 3040 proteins from a univariate KS analysis comparing TB Cases 360-540 days pre-Rx to matched controls.

Figure 32 shows RFU trajectories for the top markers found to distinguish non-TB vs. TB 360-540 days before treatment. Individual TB cases were overlaid onto a 'control band' created by interpolating the median, IQR and range of the control data. The top axis corresponds to the controls and the bottom the TB cases. Time moves to the right.

Figure 33 shows a plot of the empirical CDF of age (left) and a demographic table (right) for TB Cases 540-700 days to beginning of treatment and matched Controls.

Figure 34 shows empirical CDFs for the top 6 ranked proteins comparing non-TB vs. TB 540-700 days before treatment.

Figure 35 shows a plot of KS distances with class randomization statistics for the top 50 features comparing non-TB vs. TB 540-700 days before treatment (pvalue threshold=2.5e-2).

Figure 36 shows a volcano plot of 3040 proteins from a univariate KS analysis comparing TB Cases 540-700 days pre-Rx to matched controls .

Figure 37 shows RFU trajectories for the top markers found to distinguish non-TB vs. TB 540-700 days before treatment. Individual TB cases were overlaid onto a 'control band' created by interpolating the median, IQR and range of the control data. The top axis corresponds to the controls and the bottom the TB cases. Time moves to the right.

Figure 38 shows stability paths (top) and regularization paths (bottom) for non-TB Controls vs. TB Cases 0-180 days pre-Rx.

Figure 39 shows empirical CDFs for the top 11 proteins whose maximum selection probability exceeded 50%.

Figure 40 shows CDFs of the 17 proteins included in model buildings comparing all 57 TB case samples to all 197 control samples.

Figure 41 shows box plots of cross-validated model performance (sensitivity+specificity) as a function of model size for Naive Bayes models using forward selection (top) and backward selection (bottom).

Figure 42 shows a bar graph of the frequency that each protein was included in an optimal model using forward and backward selection.

Figure 43 shows ROC with 95% bootstrap confidence intervals and decision boundary plot of the log-odds for all subjects colored by diagnosis. Blue dots are Control samples, red dots are TB cases, and hollow data points indicate a misclassification.

Figure 44 shows log-odds trajectories of individual TB cases overlaid onto a 'control band' created by the median, IQR and range of the control data (left) and responsiveness versus performance of the model (right).

Figure 45 shows a boxplot of time binned log-odds generated from another TB biomarker panel (9 proteins) and the 8-protein model described herein.

Figure 46 shows a seriated correlation matrix for all 17 proteins used in model building (left), and the scatter plots for a 3 protein cluster identified using a Spearman threshold of 0.7.

Figure 47 shows scatter plots for a 6 protein cluster identified using a Spearman threshold of 0.7.

Figure 48 shows a plot showing sorted log odds for the 8 marker model (solid data points) and the change when adding C9 to the model (hollow data points).

Figure 49 shows ROC with 95% bootstrap confidence intervals and decision boundary plot of the log-odds for all subjects colored by diagnosis. Control samples are above (left plot) and left (right plot) of the mean; TB cases are below (left plot) and right (right plot) of the mean; and hollow data points indicate a misclassification.

Figure 50 shows: log-odds trajectories of individual TB cases overlaid onto a 'control band' created by the median, IQR and range of the control data; Time moves to the right (right); and responsiveness of the model to time to diagnosis versus model performance (left).

Figure 51 shows a boxplot of time binned log-odds generated from another TB biomarker panel (9 proteins) and the 9-protein model described herein.

Figure 52 shows certain exemplary modified pyrimidines that may be incorporated into aptamers, such as slow off-rate aptamers.

Figure 53 illustrates a non-limiting exemplary computer system for use with various computer-implemented methods described herein.

Figure 54 illustrates a non-limiting exemplary aptamer assay that can be used to detect one or more biomarkers in a biological sample.

Figure 55 shows the univariate CDF for protein KCNE2.

Figure 56 shows box plots of cross-validated model performance (sensitivity+specificity) as a function of model size for Naive Bayes models using forward selection (top) and backward selection (bottom).

Figure 57 shows a bar graph of the frequency that each protein was included in an optimal model using forward and backward selection.

Figure 58 shows ROC with 95% bootstrap confidence intervals and decision boundary plot of the log-odds for all subjects colored by diagnosis. Blue dots are Control samples (in the right panel, blue dots are to the left of the center vertical line), red dots are TB cases (in the right panel, red dots are to the right of the center vertical line), and hollow data points indicate a misclassification.

Figure 59 shows log-odds trajectories of individual TB cases overlaid onto a 'control band' created by the median, IQR and range of the control data. Time moves to the right. Responsiveness of the model to time to diagnosis versus model performance.

Figure 60 shows a boxplot of time binned log-odds generated from the HR9 and 8 protein models.

Figure 61 shows a seriated correlation matrix for all 18 proteins used in model building.

Figure 62 shows the scatter plots for a 3 protein cluster identified using a Spearman threshold of 0.7 (left) and for a 6 protein cluster identified using a Spearman threshold of 0.7 (right).

Figure 63 is a plot showing sorted log odds for the 8 protein model (solid data points) and the change when adding C9 to the model (hollow data points).

Figure 64 shows a ROC plot with 95% bootstrap confidence intervals (right) and decision boundary plot of the log-odds for all subjects colored by diagnosis (left). Blue dots are Control samples (in the left panel, blue dots are to the left of the center vertical line), red dots are TB cases (in the left panel, red dots are to the right of the center vertical line), and hollow data points indicate a misclassification.

Figure 65 shows a histogram of features selected in the optimal model during 18 runs of 5-fold double cross validation.

Figure 66 shows histograms of model performance metrics across 18 runs of 5-fold double cross validation.

DETAILED DESCRIPTION

While the invention will be described in conjunction with certain representative embodiments, it will be understood that the invention is defined by the claims, and is not limited to those embodiments.

One skilled in the art will recognize many methods and materials similar or equivalent to those described herein may be used in the practice of the present invention. The present invention is in no way limited to the methods and materials described.

Unless defined otherwise, technical and scientific terms used herein have the meaning commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice of the invention, certain methods, devices, and materials are described herein.

All publications, published patent documents, and patent applications cited herein are hereby incorporated by reference to the same extent as though each individual publication, published patent document, or patent application was specifically and individually indicated as being incorporated by reference.

As used in this application, including the appended claims, the singular forms "a," "an," and "the" include the plural, unless the context clearly dictates otherwise, and may be used interchangeably with "at least one" and "one or more." Thus, reference to "an aptamer" includes mixtures of aptamers; reference to "a probe" includes mixtures of probes, and the like.

As used herein, the terms "comprises," "comprising," "includes," "including," "contains," "containing," and any variations thereof, are intended to cover a non-exclusive inclusion, such that a process, method, product-by-process, or composition of matter that comprises, includes, or contains an element or list of elements may include other elements not expressly listed.

The present application includes biomarkers, methods, devices, reagents, systems, and kits for detecting, characterizing, monitoring progression, and/or monitoring treatment of TB infection and/or TB disease.

As used herein, "tuberculosis infection" or "TB infection" refers to the infection of an individual with any of a variety of TB disease-causing mycobacteria (e.g., *Mycobacterium tuberculosis*). TB infection encompasses both "latent TB infection" (non-transmissible and without symptoms) and "active TB infection" (transmissible and symptomatic). Observable signs of active TB infection include, but are not limited to, chronic cough with blood-tinged sputum, fever, night sweats, and weight loss. As used herein, "individual" and "subject" and "patient" are used interchangeably to refer to a test subject or patient. The individual can be a mammal or a non-mammal. In various embodiments, the individual is a mammal. A mammalian individual can be a human or non-human. In various embodiments, the individual is a human. A "non-infected" individual is one which has not been infected with a TB disease-causing mycobacterium (e.g., *Mycobacterium tuberculosis*), does not have either latent TB infection or active TB disease, and/or for whom TB infection is not detectable by conventional diagnostic methods.

As used herein, a "subject at risk of TB infection" refers to a subject with or exposed to one or more risk factors for TB infection. Such risk factors include HIV infection, poverty, geographic location, chronic lung disease, poverty, diabetes, genetic susceptibility, imprisonment, etc.

In one aspect, one or more biomarkers are provided for use either alone or in various combinations to detect TB infection and/or disease, to differentiate latent TB infection from active TB disease, to identify subjects at risk of transition from latent to active TB infection, etc. Biomarkers and biomarker panels provided herein are particularly useful for distinguishing samples obtained from individuals with latent TB infection that will advance to active TB disease (or are at high risk of advancing to TB disease) from samples from individuals with latent TB infection that will not advance to active TB disease (or are at low risk of advancing to TB disease).

As described in detail herein, exemplary embodiments include one or more biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble); or from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9; or from: AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, KCNE2, and CXCL16 (soluble); or from AMBN, C5, MMP-1, C9, SG1C1, 2DMA, KCNE2, and CXCL16 (soluble).

Methods and kits are also described herein for grouping the above biomarkers with additional biomarkers described herein and/or with additional biomarkers not listed herein (e.g., biomarkers for diagnosis of TB infection/disease, biomarkers for identification of the

strain of infection, biomarkers for identifying antibiotic resistant TB, etc.). In some embodiments, panels of at least two, at least three, at least four, at least five, or at least 6 biomarkers, at least 7 biomarkers, at least 8 biomarkers, at least 9 biomarkers, at least 10 biomarkers, at least 11 biomarkers, at least 12 biomarkers, at least 13 biomarkers, at least 14 biomarkers, at least 15 biomarkers, at least 16 biomarkers, at least 17 biomarkers, at least 18 biomarkers, at least 19 biomarkers, at least 20 biomarkers are provided.

In some embodiments, the number and identity of biomarkers in a panel are selected based on the sensitivity and specificity for the particular combination of biomarker values. The terms "sensitivity" and "specificity" are used herein with respect to the ability to correctly classify an individual, based on one or more biomarker levels detected in a biological sample. "Sensitivity" indicates the performance of the biomarker(s) with respect to correctly classifying individuals as, for example at risk (e.g., high risk or likely) of transitioning from latent TB infection to active TB disease. "Specificity" indicates the performance of the biomarker(s) with respect to correctly classifying individuals who have latent TB infection and are not at risk (e.g., low risk) of transitioning from latent TB infection to active TB disease. For example, 85% specificity and 90% sensitivity for a panel of markers used to test a set of control samples (such as samples from individuals with latent TB infections that did not advance to active TB disease) and test samples (such as samples from TB-infected individuals that developed active TB disease) indicates that 85% of the control samples were correctly classified as control samples by the panel, and 90% of the test samples were correctly classified as test samples by the panel.

In some embodiments, overall performance of a panel of one or more biomarkers is represented by the area-under-the-curve (AUC) value. The AUC value is derived from receiver operating characteristic (ROC) plots, which are exemplified herein. The ROC curve is the plot of the true positive rate (sensitivity) of a test against the false positive rate (1-specificity) of the test. The term "area under the curve" or "AUC" refers to the area under the curve of a receiver operating characteristic (ROC) curve, both of which are well known in the art. AUC measures are useful for comparing the accuracy of a classifier across the complete data range. Classifiers with a greater AUC have a greater capacity to classify unknowns correctly between two groups of interest (e.g., low-risk vs. high risk individuals). ROC curves are useful for plotting the performance of a particular feature (e.g., any of the biomarkers described herein and/or any item of additional biomedical information) in distinguishing between two populations (e.g., cases in which subjects transitioned from latent to active TB vs. controls in which TB infection remained latent). Typically, the feature data

across the entire population (e.g., all tested subject) are sorted in ascending order based on the value of a single feature. Then, for each value for that feature, the true positive and false positive rates for the data are calculated. The true positive rate is determined by counting the number of cases above the value for that feature and then dividing by the total number of cases. The false positive rate is determined by counting the number of controls above the value for that feature and then dividing by the total number of controls. Although this definition refers to scenarios in which a feature is elevated in cases compared to controls, this definition also applies to scenarios in which a feature is lower in cases compared to the controls (in such a scenario, samples below the value for that feature would be counted). ROC curves can be generated for a single feature as well as for other single outputs, for example, a combination of two or more features can be mathematically combined (e.g., added, subtracted, multiplied, etc.) to provide a single sum value, and this single sum value can be plotted in a ROC curve. Additionally, any combination of multiple features, in which the combination derives a single output value, can be plotted in a ROC curve.

In some embodiments, methods comprise contacting a sample or a portion of a sample from a subject with at least one capture reagent, wherein each capture reagent specifically binds a biomarker the levels of which are being detected. In some embodiments, the method comprises contacting the sample, or proteins from the sample, with at least one aptamer, wherein each aptamer specifically binds a biomarker, the levels of which are being detected.

In some embodiments, a method comprises detecting the level of at least one biomarker from at least a first panel of biomarkers, the first panel comprising biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble); or from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9; or from: AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, KCNE2, and CXCL16 (soluble); or from AMBN, C5, MMP-1, C9, SG1C1, 2DMA, KCNE2, and CXCL16 (soluble). In some embodiments, if the level of one or more biomarkers from the first panel are higher than a control level, outside a control range, and/or beyond a threshold value, the subject is identified as at-risk of transitioning from latent TB infection to active TB disease. In some embodiments, methods further comprise detecting at least one biomarker from at least a second panel of biomarkers, the second panel comprising biomarkers for detection of TB infection, detection of active TB disease, characterization of the type, strain, and/or resistance/sensitivity of the TB infection, etc. In some embodiments, if the level of one or more biomarkers from the second panel are altered (e.g., higher or lower) from a control

level, outside a control range, and/or beyond a threshold value, the subject and/or the infection are characterized according to the particular second panel being analyzed.

The biomarkers identified herein provide a number of choices for subsets or panels of biomarkers that can be used to effectively characterize TB infection (e.g., characterize the risk of transition from latent to active). Selection of the appropriate number of such biomarkers may depend on the specific combination of biomarkers chosen. In addition, in any of the methods described herein, except where explicitly indicated, a panel of biomarkers may comprise additional biomarkers not listed herein. In some embodiments, a method comprises detecting the level of at least one biomarker, at least two biomarkers, at least three biomarkers, at least four biomarkers, at least five biomarkers, at least six biomarkers, at least seven biomarkers, or at least eight biomarkers, selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble) in a sample from the subject. In some embodiments, a method comprises detecting the level of at least one biomarker, at least two biomarkers, at least three biomarkers, at least four biomarkers, at least five biomarkers, at least six biomarkers, at least seven biomarkers, at least eight biomarkers, or nine biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, CXCL16 (soluble), and C9 in a sample from the subject. In some embodiments, a method comprises detecting the level of any number or combination of AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble); or any number or combination of AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9.

"Biological sample", "sample", and "test sample" are used interchangeably herein to refer to any material, biological fluid, tissue, or cell obtained or otherwise derived from an individual. This includes blood (including whole blood, leukocytes, peripheral blood mononuclear cells, buffy coat, plasma, and serum), sputum, tears, mucus, nasal washes, nasal aspirate, breath, urine, semen, saliva, peritoneal washings, ascites, cystic fluid, meningeal fluid, amniotic fluid, glandular fluid, lymph fluid, nipple aspirate, bronchial aspirate (e.g., bronchoalveolar lavage), bronchial brushing, synovial fluid, joint aspirate, organ secretions, cells, a cellular extract, and cerebrospinal fluid. This also includes experimentally separated fractions of all of the preceding. For example, a blood sample can be fractionated into serum, plasma, or into fractions containing particular types of blood cells, such as red blood cells or white blood cells (leukocytes). In some embodiments, a sample can be a combination of samples from an individual, such as a combination of a tissue and fluid sample. The term "biological sample" also includes materials containing homogenized solid material, such as from a stool sample, a tissue sample, or a tissue biopsy, for example. The term "biological

sample" also includes materials derived from a tissue culture or a cell culture. Any suitable methods for obtaining a biological sample can be employed; exemplary methods include, e.g., phlebotomy, swab (e.g., buccal swab), and a fine needle aspirate biopsy procedure. Exemplary tissues susceptible to fine needle aspiration include lymph node, lung, lung washes, BAL (bronchoalveolar lavage), thyroid, breast, pancreas, and liver. Samples can also be collected, e.g., by micro dissection (e.g., laser capture micro dissection (LCM) or laser micro dissection (LMD)), bladder wash, smear (e.g., a PAP smear), or ductal lavage. A "biological sample" obtained or derived from an individual includes any such sample that has been processed in any suitable manner after being obtained from the individual.

Further, in some embodiments, a biological sample may be derived by taking biological samples from a number of individuals and pooling them, or pooling an aliquot of each individual's biological sample. The pooled sample may be treated as described herein for a sample from a single individual, and, for example, if high-risk TB infection is detected in the pooled sample, then each individual biological sample can be re-tested to identify the individual(s) with latent TB infection that is likely to transition into active TB disease within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days.

"Target", "target molecule", and "analyte" are used interchangeably herein to refer to any molecule of interest that may be present in a biological sample. A "molecule of interest" includes any minor variation of a particular molecule, such as, in the case of a protein, for example, minor variations in amino acid sequence, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component, which does not substantially alter the identity of the molecule. A "target molecule", "target", or "analyte" refers to a set of copies of one type or species of molecule or multi-molecular structure. "Target molecules", "targets", and "analytes" refer to more than one type or species of molecule or multi-molecular structure. Exemplary target molecules include proteins, polypeptides, nucleic acids, carbohydrates, lipids, polysaccharides, glycoproteins, hormones, receptors, antigens, antibodies, affybodies, antibody mimics, viruses, pathogens, toxic substances, substrates, metabolites, transition state analogs, cofactors, inhibitors, drugs, dyes, nutrients, growth factors, cells, tissues, and any fragment or portion of any of the foregoing. In some embodiments, a target molecule is a protein, in which case the target molecule may be referred to as a "target protein."

As used herein, a "capture agent' or "capture reagent" refers to a molecule that is capable of binding specifically to a biomarker. A "target protein capture reagent" refers to a

molecule that is capable of binding specifically to a target protein. Nonlimiting exemplary capture reagents include aptamers, antibodies, adnectins, ankyrins, other antibody mimetics and other protein scaffolds, autoantibodies, chimeras, small molecules, nucleic acids, lectins, ligand-binding receptors, imprinted polymers, avimers, peptidomimetics, hormone receptors, cytokine receptors, synthetic receptors, and modifications and fragments of any of the aforementioned capture reagents. In some embodiments, a capture reagent is selected from an aptamer and an antibody.

The term "antibody" refers to full-length antibodies of any species and fragments and derivatives of such antibodies, including Fab fragments, $F(ab')_2$ fragments, single chain antibodies, Fv fragments, and single chain Fv fragments. The term "antibody" also refers to synthetically-derived antibodies, such as phage display-derived antibodies and fragments, affybodies, nanobodies, etc.

As used herein, "marker" and "biomarker" are used interchangeably to refer to a target molecule that indicates or is a sign of a normal or abnormal process in an individual or of a disease or other condition in an individual. More specifically, a "marker" or "biomarker" is an anatomic, physiologic, biochemical, or molecular parameter associated with the presence of a specific physiological state or process, whether normal or abnormal, and, if abnormal, whether chronic or acute. Biomarkers are detectable and measurable by a variety of methods including laboratory assays and medical imaging. In some embodiments, a biomarker is a target protein.

As used herein, "biomarker level" and "level" refer to a measurement that is made using any analytical method for detecting the biomarker in a biological sample and that indicates the presence, absence, absolute amount or concentration, relative amount or concentration, titer, a level, an expression level, a ratio of measured levels, or the like, of, for, or corresponding to the biomarker in the biological sample. The exact nature of the "level" depends on the specific design and components of the particular analytical method employed to detect the biomarker.

A "control level" of a target molecule refers to the level of the target molecule in the same sample type from an individual that does not exhibit the characteristic being assayed for (e.g., TB infection, risk of transition from latent TB infection to active TB disease, etc.). A "control level" of a target molecule need not be determined each time the present methods are carried out, and may be a previously determined level that is used as a reference or threshold to determine whether the level in a particular sample is higher or lower than a normal level. In some embodiments, a control level in a method described herein is the level that has been

observed in one or more subjects whose latent TB infection did not advance to active TB disease within a particular time period, such as within 540 days or 2 years of sample collection. In some embodiments, a control level in a method described herein is the average or mean level, optionally plus or minus a statistical variation, which has been observed in a plurality of subjects with latent TB infection that did not advance to active TB disease within the particular time period. In some embodiments, a control level in a method described herein is a level that is indicative of chronic latent TB infection.

A "threshold level" of a target molecule refers to the level beyond which (e.g., above or below, depending upon the biomarker) is indicative of or diagnostic for a particular infection, disease, condition, or characteristic thereof. For example, a threshold level of for the likelihood of latent TB infection transitioning into active TB disease is a level of a target molecule beyond which (e.g., above or below, depending upon the biomarker) is indicative of a latnet TB infection that is likely to transition into active TB disease within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days. A "threshold level" of a target molecule need not be determined each time the present methods are carried out, and may be a previously determined level that is used as a reference or threshold to determine whether the level in a particular sample is higher or lower than a normal level. In some embodiments, a subject with a biomarker level beyond (e.g., above or below, depending upon the biomarker) a threshold level has a statistically significant likelihood (e.g., 80% confidence, 85% confidence, 90% confidence, 95% confidence, 98% confidence, 99% confidence, 99.9% confidence, etc.) of having a latent TB infection transition into active TB disease.

"Diagnose", "diagnosing", "diagnosis", and variations thereof refer to the detection, determination, or recognition of a health status or condition of an individual on the basis of one or more signs, symptoms, data, or other information pertaining to that individual. The health status of an individual can be diagnosed as healthy/normal (e.g., a diagnosis of the absence of a disease or condition), diagnosed as ill/abnormal (e.g., a diagnosis of the presence, or an assessment of the characteristics, of a disease or condition), and/or high-risk/low-risk (e.g., of developing a disease or condition, of transitioning from a latent infection to an active disease state). The terms "diagnose", "diagnosing", "diagnosis", etc., encompass, with respect to a particular disease or condition: the initial detection of the disease; the characterization or classification of the disease; the characterization of likelihood of advancement of the disease (e.g., from latent to active); the detection of the progression,

remission, or recurrence of the disease; and/or the detection of disease response after the administration of a treatment or therapy to the individual.

"Prognose", "prognosing", "prognosis", and variations thereof refer to the prediction of a future course of a disease or condition in an individual who has the disease or condition (e.g., predicting patient survival, predicting likelihood of transition from latent infection to active disease, etc.), and such terms encompass the evaluation of disease response after the administration of a treatment or therapy to the individual.

"Evaluate", "evaluating", "evaluation", and variations thereof encompass both "diagnose" and "prognose" and also encompass determinations or predictions about the future course of a disease or condition in an individual who does not have the disease as well as determinations or predictions regarding the likelihood that a disease or condition will recur in an individual who apparently has been cured of the disease. The term "evaluate" also encompasses assessing an individual's response to a therapy, such as, for example, predicting whether an individual is likely to respond favorably to a therapeutic agent or is unlikely to respond to a therapeutic agent (or will experience toxic or other undesirable side effects, for example), selecting a therapeutic agent for administration to an individual, or monitoring or determining an individual's response to a therapy that has been administered to the individual. Thus, "evaluating" TB can include, for example, any of the following: diagnosing a subject with TB infection, diagnosing a subject as suffering from TB disease, determining a subject should undergo further testing (e.g., chest x-ray for TB); prognosing the future course of TB infection/disease in an individual; prognosing a the likelihood of TB transitioning from latent to active; determining whether a TB treatment being administered is effective in the individual; or determining or predicting an individual's response to a TB treatment; or selecting a TB treatment to administer to an individual based upon a determination of the biomarker levels derived from the individual's biological sample.

As used herein, "detecting" or "determining" with respect to a biomarker level includes the use of both the instrument used to observe and record a signal corresponding to a biomarker level and the material/s required to generate that signal. In various embodiments, the level is detected using any suitable method, including fluorescence, chemiluminescence, surface plasmon resonance, surface acoustic waves, mass spectrometry, infrared spectroscopy, Raman spectroscopy, atomic force microscopy, scanning tunneling microscopy, electrochemical detection methods, nuclear magnetic resonance, quantum dots, and the like.

As used herein "host biomarkers" are biological molecules (e.g., proteins) that are endogenous to an individual, the expression or level of which is altered (e.g., increased or decreased) upon infection by a pathogenic agent (e.g., *Mycobacterium tuberculosis*). Detection and/or quantification of host biomarkers allows for characterization of a pathogenic infection.

As used herein "pathogen biomarkers" are molecules (e.g., proteins) that are not endogenous to an infected individual, but produced by a pathogen (e.g., *Mycobacterium tuberculosis*) that has infected the individual. Detection and/or quantification of pathogen biomarkers (e.g., Mtb biomarkers) allows for characterization of pathogenic infection.

Embodiments described herein include biomarkers, panels of biomarkers, methods, devices, reagents, systems, and kits for detecting, identifying, characterizing, and/or diagnosing infection of a subject (e.g., human subject) with Mycobacterium tuberculosis (Mtb). In particular, embodiments relate to characterizing a latent TB infection: (1) as one is likely to advancing or transition into active TB disease within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days; or (2) as one that is unlikely to advance or transition into active TB disease within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days. Such embodiments involve the quantification of one or more biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, and CXCL16 (soluble); or from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, CXCL16 (soluble), and C9; or from: AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, KCNE2, and CXCL16 (soluble); or from AMBN, C5, MMP-1, C9, SG1C1, 2DMA, KCNE2, and CXCL16 (soluble).

"Solid support" refers herein to any substrate having a surface to which molecules may be attached, directly or indirectly, through either covalent or non-covalent bonds. A "solid support" can have a variety of physical formats, which can include, for example, a membrane; a chip (e.g., a protein chip); a slide (e.g., a glass slide or coverslip); a column; a hollow, solid, semi-solid, pore- or cavity- containing particle, such as, for example, a bead; a gel; a fiber, including a fiber optic material; a matrix; and a sample receptacle. Exemplary sample receptacles include sample wells, tubes, capillaries, vials, and any other vessel, groove or indentation capable of holding a sample. A sample receptacle can be contained on a multi-sample platform, such as a microtiter plate, slide, microfluidics device, and the like. A support can be composed of a natural or synthetic material, an organic or inorganic material. The composition of the solid support on which capture reagents are attached generally depends on the method of attachment (e.g., covalent attachment). Other exemplary

receptacles include microdroplets and microfluidic controlled or bulk oil/aqueous emulsions within which assays and related manipulations can occur. Suitable solid supports include, for example, plastics, resins, polysaccharides, silica or silica-based materials, functionalized glass, modified silicon, carbon, metals, inorganic glasses, membranes, nylon, natural fibers (such as, for example, silk, wool and cotton), polymers, and the like. The material composing the solid support can include reactive groups such as, for example, carboxy, amino, or hydroxyl groups, which are used for attachment of the capture reagents. Polymeric solid supports can include, e.g., polystyrene, polyethylene glycol tetraphthalate, polyvinyl acetate, polyvinyl chloride, polyvinyl pyrrolidone, polyacrylonitrile, polymethyl methacrylate, polytetrafluoroethylene, butyl rubber, styrenebutadiene rubber, natural rubber, polyethylene, polypropylene, (poly)tetrafluoroethylene, (poly)vinylidenefluoride, polycarbonate, and polymethylpentene. Suitable solid support particles that can be used include, e.g., encoded particles, such as Luminex®-type encoded particles, magnetic particles, and glass particles.

Exemplary Uses of Biomarkers

In various exemplary embodiments, methods are provided for determining the likelihood or risk of a subject infected with *Mycobacterium tuberculosis* (e.g., a subject with latent TB infection) transitioning into active TB disease within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days.

In some embodiments, a finding that a TB-infected subject is unlikely to transition into active TB disease indicates that the subject is not presently at significant risk of active TB disease.

In certain exemplary embodiments, methods are provided for determining the likelihood or risk that a non-infected subject would transition from latent infection to active TB disease within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days, should they become infected by *Mycobacterium tuberculosis* (or another agent causative of TB).

In some embodiments, methods comprise testing a subject for TB infection, for example, by skin test, sputum culture, blood test, tissue culture, body fluid culture, chest x-ray, and/or using the methods described in U.S. Prov. Pat. App. 61/987,888, which is herein incorporated by reference in its entirety. Following a determination that a subject is infected with TB (e.g. latent infection), and a determination (e.g., by monitoring symptoms, by chest x-ray, etc.) that a subject does not have active TB disease, methods described herein are

employed to determine the likelihood that such an infection may progress into active TB disease.

In addition to testing biomarker levels (e.g., one or more of the TB biomarkers identified in experiments conducted during development of embodiments of the present invention (e.g., one or more biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble); or from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9) as a stand-alone diagnostic test, in some embodiments, biomarker levels are tested in conjunction with other markers or assays for characterizing TB (e.g., skin test, sputum culture, blood test, tissue culture, body fluid culture, chest x-ray, methods described in U.S. Prov. Pat. App. 61/987,888 (herein incorporated by reference in its entirety), etc.). In addition to testing biomarker levels in conjunction with other TB diagnostic methods, information regarding the biomarkers can also be evaluated in conjunction with other types of data, particularly data that indicates an individual's risk for TB (e.g., lifestyle, location, age, etc.). These various data can be assessed by automated methods, such as a computer program/software, which can be embodied in a computer or other apparatus/device.

Detection and Determination of Biomarkers and Biomarker Levels

A biomarker level for the biomarkers described herein can be detected using any of a variety of known analytical methods. In one embodiment, a biomarker level is detected using a capture reagent. In various embodiments, the capture reagent can be exposed to the biomarker in solution or can be exposed to the biomarker while the capture reagent is immobilized on a solid support. In other embodiments, the capture reagent contains a feature that is reactive with a secondary feature on a solid support. In these embodiments, the capture reagent can be exposed to the biomarker in solution, and then the feature on the capture reagent can be used in conjunction with the secondary feature on the solid support to immobilize the biomarker on the solid support. The capture reagent is selected based on the type of analysis to be conducted. Capture reagents include but are not limited to aptamers, antibodies, adnectins, ankyrins, other antibody mimetics and other protein scaffolds, autoantibodies, chimeras, small molecules, $F(ab')_2$ fragments, single chain antibody fragments, Fv fragments, single chain Fv fragments, nucleic acids, lectins, ligand-binding receptors, affybodies, nanobodies, imprinted polymers, avimers, peptidomimetics, hormone receptors, cytokine receptors, and synthetic receptors, and modifications and fragments of these.

In some embodiments, biomarker presence or level is detected using a biomarker/capture reagent complex.

In some embodiments, the biomarker presence or level is derived from the biomarker/capture reagent complex and is detected indirectly, such as, for example, as a result of a reaction that is subsequent to the biomarker/capture reagent interaction, but is dependent on the formation of the biomarker/capture reagent complex.

In some embodiments, biomarker presence or level is detected directly from the biomarker in a biological sample.

In some embodiments, biomarkers are detected using a multiplexed format that allows for the simultaneous detection of two or more biomarkers in a biological sample. In some embodiments of the multiplexed format, capture reagents are immobilized, directly or indirectly, covalently or non-covalently, in discrete locations on a solid support. In some embodiments, a multiplexed format uses discrete solid supports where each solid support has a unique capture reagent associated with that solid support, such as, for example quantum dots. In some embodiments, an individual device is used for the detection of each one of multiple biomarkers to be detected in a biological sample. Individual devices can be configured to permit each biomarker in the biological sample to be processed simultaneously. For example, a microtiter plate can be used such that each well in the plate is used to analyze one or more of multiple biomarkers to be detected in a biological sample.

In one or more of the foregoing embodiments, a fluorescent tag can be used to label a component of the biomarker/capture reagent complex to enable the detection of the biomarker level. In various embodiments, the fluorescent label can be conjugated to a capture reagent specific to any of the biomarkers described herein using known techniques, and the fluorescent label can then be used to detect the corresponding biomarker level. Suitable fluorescent labels include rare earth chelates, fluorescein and its derivatives, rhodamine and its derivatives, dansyl, allophycocyanin, PBXL-3, Qdot 605, Lissamine, phycoerythrin, Texas Red, and other such compounds.

In some embodiments, the fluorescent label is a fluorescent dye molecule. In some embodiments, the fluorescent dye molecule includes at least one substituted indolium ring system in which the substituent on the 3-carbon of the indolium ring contains a chemically reactive group or a conjugated substance. In some embodiments, the dye molecule includes an AlexFluor molecule, such as, for example, AlexaFluor 488, AlexaFluor 532, AlexaFluor 647, AlexaFluor 680, or AlexaFluor 700. In some embodiments, the dye molecule includes a first type and a second type of dye molecule, such as, e.g., two different AlexaFluor

molecules. In some embodiments, the dye molecule includes a first type and a second type of dye molecule, and the two dye molecules have different emission spectra.

Fluorescence can be measured with a variety of instrumentation compatible with a wide range of assay formats. For example, spectrofluorimeters have been designed to analyze microtiter plates, microscope slides, printed arrays, cuvettes, etc. See Principles of Fluorescence Spectroscopy, by J.R. Lakowicz, Springer Science + Business Media, Inc., 2004. See Bioluminescence & Chemiluminescence: Progress & Current Applications; Philip E. Stanley and Larry J. Kricka editors, World Scientific Publishing Company, January 2002.

In one or more embodiments, a chemiluminescence tag can optionally be used to label a component of the biomarker/capture complex to enable the detection of a biomarker level. Suitable chemiluminescent materials include any of oxalyl chloride, Rodamin 6G, Ru(bipy)32+ , TMAE (tetrakis(dimethylamino)ethylene), Pyrogallol (1,2,3-trihydroxibenzene), Lucigenin, peroxyoxalates, Aryl oxalates, Acridinium esters, dioxetanes, and others.

In some embodiments, the detection method includes an enzyme/substrate combination that generates a detectable signal that corresponds to the biomarker level. Generally, the enzyme catalyzes a chemical alteration of the chromogenic substrate which can be measured using various techniques, including spectrophotometry, fluorescence, and chemiluminescence. Suitable enzymes include, for example, luciferases, luciferin, malate dehydrogenase, urease, horseradish peroxidase (HRPO), alkaline phosphatase, beta-galactosidase, glucoamylase, lysozyme, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, uricase, xanthine oxidase, lactoperoxidase, microperoxidase, and the like.

In some embodiments, the detection method can be a combination of fluorescence, chemiluminescence, radionuclide or enzyme/substrate combinations that generate a measurable signal. In some embodiments, multimodal signaling could have unique and advantageous characteristics in biomarker assay formats.

In some embodiments, the biomarker levels for the biomarkers described herein can be detected using any analytical methods including, singleplex aptamer assays, multiplexed aptamer assays, singleplex or multiplexed immunoassays, mRNA expression profiling, miRNA expression profiling, mass spectrometric analysis, histological/cytological methods, etc. as discussed below.

Determination of Biomarker Levels using Aptamer-Based Assays

Assays directed to the detection and quantification of physiologically significant molecules in biological samples and other samples are important tools in scientific research and in the health care field. One class of such assays involves the use of a microarray that includes one or more aptamers immobilized on a solid support. The aptamers are each capable of binding to a target molecule in a highly specific manner and with very high affinity. See, e.g., U.S. Patent No. 5,475,096 entitled "Nucleic Acid Ligands"; see also, e.g., U.S. Patent No. 6,242,246, U.S. Patent No. 6,458,543, and U.S. Patent No. 6,503,715, each of which is entitled "Nucleic Acid Ligand Diagnostic Biochip". Once the microarray is contacted with a sample, the aptamers bind to their respective target molecules present in the sample and thereby enable a determination of a biomarker level corresponding to a biomarker.

As used herein, an "aptamer" refers to a nucleic acid that has a specific binding affinity for a target molecule. It is recognized that affinity interactions are a matter of degree; however, in this context, the "specific binding affinity" of an aptamer for its target means that the aptamer binds to its target generally with a much higher degree of affinity than it binds to other components in a test sample. An "aptamer" is a set of copies of one type or species of nucleic acid molecule that has a particular nucleotide sequence. An aptamer can include any suitable number of nucleotides, including any number of chemically modified nucleotides. "Aptamers" refers to more than one such set of molecules. Different aptamers can have either the same or different numbers of nucleotides. Aptamers can be DNA or RNA or chemically modified nucleic acids and can be single stranded, double stranded, or contain double stranded regions, and can include higher ordered structures. An aptamer can also be a photoaptamer, where a photoreactive or chemically reactive functional group is included in the aptamer to allow it to be covalently linked to its corresponding target. Any of the aptamer methods disclosed herein can include the use of two or more aptamers that specifically bind the same target molecule. As further described below, an aptamer may include a tag. If an aptamer includes a tag, all copies of the aptamer need not have the same tag. Moreover, if different aptamers each include a tag, these different aptamers can have either the same tag or a different tag.

An aptamer can be identified using any known method, including the SELEX process. Once identified, an aptamer can be prepared or synthesized in accordance with any known method, including chemical synthetic methods and enzymatic synthetic methods.

The terms "SELEX" and "SELEX process" are used interchangeably herein to refer generally to a combination of (1) the selection of aptamers that interact with a target molecule in a desirable manner, for example binding with high affinity to a protein, with (2) the amplification of those selected nucleic acids. The SELEX process can be used to identify aptamers with high affinity to a specific target or biomarker.

SELEX generally includes preparing a candidate mixture of nucleic acids, binding of the candidate mixture to the desired target molecule to form an affinity complex, separating the affinity complexes from the unbound candidate nucleic acids, separating and isolating the nucleic acid from the affinity complex, purifying the nucleic acid, and identifying a specific aptamer sequence. The process may include multiple rounds to further refine the affinity of the selected aptamer. The process can include amplification steps at one or more points in the process. See, e.g., U.S. Patent No. 5,475,096, entitled "Nucleic Acid Ligands". The SELEX process can be used to generate an aptamer that covalently binds its target as well as an aptamer that non-covalently binds its target. See, e.g., U.S. Patent No. 5,705,337 entitled "Systematic Evolution of Nucleic Acid Ligands by Exponential Enrichment: Chemi-SELEX."

The SELEX process can be used to identify high-affinity aptamers containing modified nucleotides that confer improved characteristics on the aptamer, such as, for example, improved in vivo stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX process-identified aptamers containing modified nucleotides are described in U.S. Patent No. 5,660,985, entitled "High Affinity Nucleic Acid Ligands Containing Modified Nucleotides", which describes oligonucleotides containing nucleotide derivatives chemically modified at the 5'- and 2'-positions of pyrimidines. U.S. Patent No. 5,580,737, see supra, describes highly specific aptamers containing one or more nucleotides modified with 2'-amino (2'-NH_2), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). See also, U.S. Patent Application Publication No. 2009/0098549, entitled "SELEX and PHOTOSELEX", which describes nucleic acid libraries having expanded physical and chemical properties and their use in SELEX and photoSELEX.

SELEX can also be used to identify aptamers that have desirable off-rate characteristics. See U.S. Publication No. US 2009/0004667, entitled "Method for Generating Aptamers with Improved Off-Rates", which describes improved SELEX methods for generating aptamers that can bind to target molecules. Methods for producing aptamers and photoaptamers having slower rates of dissociation from their respective target molecules are

described. The methods involve contacting the candidate mixture with the target molecule, allowing the formation of nucleic acid-target complexes to occur, and performing a slow off-rate enrichment process wherein nucleic acid-target complexes with fast dissociation rates will dissociate and not reform, while complexes with slow dissociation rates will remain intact. Additionally, the methods include the use of modified nucleotides in the production of candidate nucleic acid mixtures to generate aptamers with improved off-rate performance. Nonlimiting exemplary modified nucleotides include, for example, the modified pyrimidines shown in Figure 52. In some embodiments, an aptamer comprises at least one nucleotide with a modification, such as a base modification. In some embodiments, an aptamer comprises at least one nucleotide with a hydrophobic modification, such as a hydrophobic base modification, allowing for hydrophobic contacts with a target protein. Such hydrophobic contacts, in some embodiments, contribute to greater affinity and/or slower off-rate binding by the aptamer. Nonlimiting exemplary nucleotides with hydrophobic modifications are shown in Figure 52. In some embodiments, an aptamer comprises at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least 10 nucleotides with hydrophobic modifications, where each hydrophobic modification may be the same or different from the others. In some embodiments, at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least 10 hydrophobic modifications in an aptamer may be independently selected from the hydrophobic modifications shown in Figure 52.

In some embodiments, a slow off-rate aptamer (including an aptamers comprising at least one nucleotide with a hydrophobic modification) has an off-rate ($t_{1/2}$) of $\geq$ 30 minutes, $\geq$ 60 minutes, $\geq$ 90 minutes, $\geq$ 120 minutes, $\geq$ 150 minutes, $\geq$ 180 minutes, $\geq$ 210 minutes, or $\geq$ 240 minutes.

As used herein, a "SOMAmer" or "Slow Off-Rate Aptamer" refers to an aptamer having improved off-rate characteristics. Slow off-rate aptamers can be generated using the modified SELEX methods described in U.S. Publication No. 20090004667; herein incorporated by reference in its entirety. The methods disclosed herein are in no way limited to slow off-rate aptamers, however, use of the slow off-rate process described in U.S. Pat. No. 7,964,356 and U.S. Publication No. 2012/0115752 (herein incorporated by reference in their entireties), may provide improved results.

In some embodiments, an assay employs aptamers that include photoreactive functional groups that enable the aptamers to covalently bind or "photocrosslink" their target molecules. See, e.g., U.S. Patent No. 6,544,776 entitled "Nucleic Acid Ligand Diagnostic

Biochip". These photoreactive aptamers are also referred to as photoaptamers. See, e.g., U.S. Patent No. 5,763,177, U.S. Patent No. 6,001,577, and U.S. Patent No. 6,291,184, each of which is entitled "Systematic Evolution of Nucleic Acid Ligands by Exponential Enrichment: Photoselection of Nucleic Acid Ligands and Solution SELEX"; see also, e.g., U.S. Patent No. 6,458,539, entitled "Photoselection of Nucleic Acid Ligands". After the microarray is contacted with the sample and the photoaptamers have had an opportunity to bind to their target molecules, the photoaptamers are photoactivated, and the solid support is washed to remove any non-specifically bound molecules. Harsh wash conditions may be used, since target molecules that are bound to the photoaptamers are generally not removed, due to the covalent bonds created by the photoactivated functional group(s) on the photoaptamers. In this manner, the assay enables the detection of a biomarker level corresponding to a biomarker in the test sample.

In some assay formats, the aptamers are immobilized on the solid support prior to being contacted with the sample. Under certain circumstances, however, immobilization of the aptamers prior to contact with the sample may not provide an optimal assay. For example, pre-immobilization of the aptamers may result in inefficient mixing of the aptamers with the target molecules on the surface of the solid support, perhaps leading to lengthy reaction times and, therefore, extended incubation periods to permit efficient binding of the aptamers to their target molecules. Further, when photoaptamers are employed in the assay and depending upon the material utilized as a solid support, the solid support may tend to scatter or absorb the light used to effect the formation of covalent bonds between the photoaptamers and their target molecules. Moreover, depending upon the method employed, detection of target molecules bound to their aptamers can be subject to imprecision, since the surface of the solid support may also be exposed to and affected by any labeling agents that are used. Finally, immobilization of the aptamers on the solid support generally involves an aptamer-preparation step (i.e., the immobilization) prior to exposure of the aptamers to the sample, and this preparation step may affect the activity or functionality of the aptamers.

Aptamer assays that permit an aptamer to capture its target in solution and then employ separation steps that are designed to remove specific components of the aptamer-target mixture prior to detection have also been described (see U.S. Publication No. 2009/0042206, entitled "Multiplexed Analyses of Test Samples"). The described aptamer assay methods enable the detection and quantification of a non-nucleic acid target (e.g., a protein target) in a test sample by detecting and quantifying a nucleic acid (i.e., an aptamer). The described methods create a nucleic acid surrogate (i.e., the aptamer) for detecting and

quantifying a non-nucleic acid target, thus allowing the wide variety of nucleic acid technologies, including amplification, to be applied to a broader range of desired targets, including protein targets.

Aptamers can be constructed to facilitate the separation of the assay components from an aptamer biomarker complex (or photoaptamer biomarker covalent complex) and permit isolation of the aptamer for detection and/or quantification. In one embodiment, these constructs can include a cleavable or releasable element within the aptamer sequence. In other embodiments, additional functionality can be introduced into the aptamer, for example, a labeled or detectable component, a spacer component, or a specific binding tag or immobilization element. For example, the aptamer can include a tag connected to the aptamer via a cleavable moiety, a label, a spacer component separating the label, and the cleavable moiety. In one embodiment, a cleavable element is a photocleavable linker. The photocleavable linker can be attached to a biotin moiety and a spacer section, can include an NHS group for derivatization of amines, and can be used to introduce a biotin group to an aptamer, thereby allowing for the release of the aptamer later in an assay method.

Homogenous assays, done with all assay components in solution, do not require separation of sample and reagents prior to the detection of signal. These methods are rapid and easy to use. These methods generate signal based on a molecular capture or binding reagent that reacts with its specific target. In some embodiments of the methods described herein, the molecular capture reagents comprise an aptamer or an antibody or the like and the specific target may be a biomarker described herein (e.g., AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), C9, etc.).

In some embodiments, a method for signal generation takes advantage of anisotropy signal change due to the interaction of a fluorophore-labeled capture reagent with its specific biomarker target. When the labeled capture reacts with its target, the increased molecular weight causes the rotational motion of the fluorophore attached to the complex to become much slower changing the anisotropy value. By monitoring the anisotropy change, binding events may be used to quantitatively measure the biomarkers in solutions. Other methods include fluorescence polarization assays, molecular beacon methods, time resolved fluorescence quenching, chemiluminescence, fluorescence resonance energy transfer, and the like.

An exemplary solution-based aptamer assay that can be used to detect a biomarker level in a biological sample includes the following: (a) preparing a mixture by contacting the biological sample with an aptamer that includes a first tag and has a specific affinity for the

biomarker, wherein an aptamer affinity complex is formed when the biomarker is present in the sample; (b) exposing the mixture to a first solid support including a first capture element, and allowing the first tag to associate with the first capture element; (c) removing any components of the mixture not associated with the first solid support; (d) attaching a second tag to the biomarker component of the aptamer affinity complex; (e) releasing the aptamer affinity complex from the first solid support; (f) exposing the released aptamer affinity complex to a second solid support that includes a second capture element and allowing the second tag to associate with the second capture element; (g) removing any non-complexed aptamer from the mixture by partitioning the non-complexed aptamer from the aptamer affinity complex; (h) eluting the aptamer from the solid support; and (i) detecting the biomarker by detecting the aptamer component of the aptamer affinity complex.

A non-limiting exemplary method of detecting biomarkers in a biological sample using aptamers is described, for example, in Kraemer et al., 2011, PLoS One 6(10): e26332; herein incorporated by reference in its entirety.

Determination of Biomarker Levels using Immunoassays

Immunoassay methods are based on the reaction of an antibody to its corresponding target or analyte and can detect the analyte in a sample depending on the specific assay format. To improve specificity and sensitivity of an assay method based on immuno-reactivity, monoclonal antibodies and fragments thereof are often used because of their specific epitope recognition. Polyclonal antibodies have also been successfully used in various immunoassays because of their increased affinity for the target as compared to monoclonal antibodies. Immunoassays have been designed for use with a wide range of biological sample matrices. Immunoassay formats have been designed to provide qualitative, semi-quantitative, and quantitative results.

Quantitative results are generated through the use of a standard curve created with known concentrations of the specific analyte to be detected. The response or signal from an unknown sample is plotted onto the standard curve, and a quantity or level corresponding to the target in the unknown sample is established.

Numerous immunoassay formats have been designed. ELISA or EIA can be quantitative for the detection of an analyte. This method relies on attachment of a label to either the analyte or the antibody and the label component includes, either directly or indirectly, an enzyme. ELISA tests may be formatted for direct, indirect, competitive, or sandwich detection of the analyte. Other methods rely on labels such as, for example,

radioisotopes (I125) or fluorescence. Additional techniques include, for example, agglutination, nephelometry, turbidimetry, Western blot, immunoprecipitation, immunocytochemistry, immunohistochemistry, flow cytometry, Luminex assay, and others (see ImmunoAssay: A Practical Guide, edited by Brian Law, published by Taylor & Francis, Ltd., 2005 edition).

Exemplary assay formats include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, fluorescent, chemiluminescence, and fluorescence resonance energy transfer (FRET) or time resolved-FRET (TR-FRET) immunoassays. Examples of procedures for detecting biomarkers include biomarker immunoprecipitation followed by quantitative methods that allow size and peptide level discrimination, such as gel electrophoresis, capillary electrophoresis, planar electrochromatography, and the like.

Methods of detecting and/or for quantifying a detectable label or signal generating material depend on the nature of the label. The products of reactions catalyzed by appropriate enzymes (where the detectable label is an enzyme; see above) can be, without limitation, fluorescent, luminescent, or radioactive or they may absorb visible or ultraviolet light. Examples of detectors suitable for detecting such detectable labels include, without limitation, x-ray film, radioactivity counters, scintillation counters, spectrophotometers, colorimeters, fluorometers, luminometers, and densitometers.

Any of the methods for detection can be performed in any format that allows for any suitable preparation, processing, and analysis of the reactions. This can be, for example, in multi-well assay plates (e.g., 96 wells or 386 wells) or using any suitable array or microarray. Stock solutions for various agents can be made manually or robotically, and all subsequent pipetting, diluting, mixing, distribution, washing, incubating, sample readout, data collection and analysis can be done robotically using commercially available analysis software, robotics, and detection instrumentation capable of detecting a detectable label.

Determination of Biomarker Levels using Gene Expression Profiling

Measuring mRNA in a biological sample may, in some embodiments, be used as a surrogate for detection of the level of the corresponding protein in the biological sample. Thus, in some embodiments, a biomarker or biomarker panel described herein can be detected by detecting the appropriate RNA.

In some embodiments, mRNA expression levels are measured by reverse transcription quantitative polymerase chain reaction (RT-PCR followed with qPCR). RT-PCR is used to create a cDNA from the mRNA. The cDNA may be used in a qPCR assay to produce

fluorescence as the DNA amplification process progresses. By comparison to a standard curve, qPCR can produce an absolute measurement such as number of copies of mRNA per cell. Northern blots, microarrays, Invader assays, and RT-PCR combined with capillary electrophoresis have all been used to measure expression levels of mRNA in a sample. See Gene Expression Profiling: Methods and Protocols, Richard A. Shimkets, editor, Humana Press, 2004.

Detection of Biomarkers Using In Vivo Molecular Imaging Technologies

In some embodiments, a biomarker described herein may be used in molecular imaging tests. For example, an imaging agent can be coupled to a capture reagent, which can be used to detect the biomarker in vivo.

In vivo imaging technologies provide non-invasive methods for determining the state of a particular disease in the body of an individual. For example, entire portions of the body, or even the entire body, may be viewed as a three dimensional image, thereby providing valuable information concerning morphology and structures in the body. Such technologies may be combined with the detection of the biomarkers described herein to provide information concerning the biomarker in vivo.

The use of in vivo molecular imaging technologies is expanding due to various advances in technology. These advances include the development of new contrast agents or labels, such as radiolabels and/or fluorescent labels, which can provide strong signals within the body; and the development of powerful new imaging technology, which can detect and analyze these signals from outside the body, with sufficient sensitivity and accuracy to provide useful information. The contrast agent can be visualized in an appropriate imaging system, thereby providing an image of the portion or portions of the body in which the contrast agent is located. The contrast agent may be bound to or associated with a capture reagent, such as an aptamer or an antibody, for example, and/or with a peptide or protein, or an oligonucleotide (for example, for the detection of gene expression), or a complex containing any of these with one or more macromolecules and/or other particulate forms.

The contrast agent may also feature a radioactive atom that is useful in imaging. Suitable radioactive atoms include technetium-99m or iodine-123 for scintigraphic studies. Other readily detectable moieties include, for example, spin labels for magnetic resonance imaging (MRI) such as, for example, iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron. Such labels are well known in the art and could easily be selected by one of ordinary skill in the art.

Standard imaging techniques include but are not limited to magnetic resonance imaging, computed tomography scanning, positron emission tomography (PET), single photon emission computed tomography (SPECT), and the like. For diagnostic in vivo imaging, the type of detection instrument available is a major factor in selecting a given contrast agent, such as a given radionuclide and the particular biomarker that it is used to target (protein, mRNA, and the like). The radionuclide chosen typically has a type of decay that is detectable by a given type of instrument. Also, when selecting a radionuclide for in vivo diagnosis, its half-life should be long enough to enable detection at the time of maximum uptake by the target tissue but short enough that deleterious radiation of the host is minimized.

Exemplary imaging techniques include but are not limited to PET and SPECT, which are imaging techniques in which a radionuclide is synthetically or locally administered to an individual. The subsequent uptake of the radiotracer is measured over time and used to obtain information about the targeted tissue and the biomarker. Because of the high-energy (gamma-ray) emissions of the specific isotopes employed and the sensitivity and sophistication of the instruments used to detect them, the two-dimensional distribution of radioactivity may be inferred from outside of the body.

Commonly used positron-emitting nuclides in PET include, for example, carbon-11, nitrogen-13, oxygen-15, and fluorine-18. Isotopes that decay by electron capture and/or gamma-emission are used in SPECT and include, for example iodine-123 and technetium-99m. An exemplary method for labeling amino acids with technetium-99m is the reduction of pertechnetate ion in the presence of a chelating precursor to form the labile technetium-99m-precursor complex, which, in turn, reacts with the metal binding group of a bifunctionally modified chemotactic peptide to form a technetium-99m-chemotactic peptide conjugate.

Antibodies are frequently used for such in vivo imaging diagnostic methods. The preparation and use of antibodies for in vivo diagnosis is well known in the art. Similarly, aptamers may be used for such in vivo imaging diagnostic methods. For example, an aptamer that was used to identify a particular biomarker described herein may be appropriately labeled and injected into an individual to detect the biomarker in vivo. The label used will be selected in accordance with the imaging modality to be used, as previously described. Aptamer-directed imaging agents could have unique and advantageous characteristics relating to tissue penetration, tissue distribution, kinetics, elimination, potency, and selectivity as compared to other imaging agents.

Such techniques may also optionally be performed with labeled oligonucleotides, for example, for detection of gene expression through imaging with antisense oligonucleotides. These methods are used for in situ hybridization, for example, with fluorescent molecules or radionuclides as the label. Other methods for detection of gene expression include, for example, detection of the activity of a reporter gene.

Another general type of imaging technology is optical imaging, in which fluorescent signals within the subject are detected by an optical device that is external to the subject. These signals may be due to actual fluorescence and/or to bioluminescence. Improvements in the sensitivity of optical detection devices have increased the usefulness of optical imaging for in vivo diagnostic assays.

Other techniques are review, for example, in N. Blow, Nature Methods, 6, 465-469, 2009; herein incorporated by reference in its entirety.

Determination of Biomarkers using Histology/Cytology Methods

In some embodiments, the biomarkers described herein may be detected in a variety of tissue samples using histological or cytological methods. For example, endo- and trans-bronchial biopsies, fine needle aspirates, cutting needles, and core biopsies can be used for histology. Bronchial washing and brushing, pleural aspiration, and sputum, can be used for cyotology. Any of the biomarkers identified herein can be used to stain a specimen as an indication of disease.

In some embodiments, one or more capture reagent/s specific to the corresponding biomarker/s are used in a cytological evaluation of a sample and may include one or more of the following: collecting a cell sample, fixing the cell sample, dehydrating, clearing, immobilizing the cell sample on a microscope slide, permeabilizing the cell sample, treating for analyte retrieval, staining, destaining, washing, blocking, and reacting with one or more capture reagent/s in a buffered solution. In another embodiment, the cell sample is produced from a cell block.

In some embodiments, one or more capture reagent/s specific to the corresponding biomarkers are used in a histological evaluation of a tissue sample and may include one or more of the following: collecting a tissue specimen, fixing the tissue sample, dehydrating, clearing, immobilizing the tissue sample on a microscope slide, permeabilizing the tissue sample, treating for analyte retrieval, staining, destaining, washing, blocking, rehydrating, and reacting with capture reagent/s in a buffered solution. In another embodiment, fixing and dehydrating are replaced with freezing.

In another embodiment, the one or more aptamer/s specific to the corresponding biomarker/s are reacted with the histological or cytological sample and can serve as the nucleic acid target in a nucleic acid amplification method. Suitable nucleic acid amplification methods include, for example, PCR, q-beta replicase, rolling circle amplification, strand displacement, helicase dependent amplification, loop mediated isothermal amplification, ligase chain reaction, and restriction and circularization aided rolling circle amplification.

In one embodiment, the one or more capture reagent/s specific to the corresponding biomarkers for use in the histological or cytological evaluation are mixed in a buffered solution that can include any of the following: blocking materials, competitors, detergents, stabilizers, carrier nucleic acid, polyanionic materials, etc.

A "cytology protocol" generally includes sample collection, sample fixation, sample immobilization, and staining. "Cell preparation" can include several processing steps after sample collection, including the use of one or more aptamers for the staining of the prepared cells.

Determination of Biomarker Levels using Mass Spectrometry Methods

A variety of configurations of mass spectrometers can be used to detect biomarker levels. Several types of mass spectrometers are available or can be produced with various configurations. In general, a mass spectrometer has the following major components: a sample inlet, an ion source, a mass analyzer, a detector, a vacuum system, and instrument-control system, and a data system. Difference in the sample inlet, ion source, and mass analyzer generally define the type of instrument and its capabilities. For example, an inlet can be a capillary-column liquid chromatography source or can be a direct probe or stage such as used in matrix-assisted laser desorption. Common ion sources are, for example, electrospray, including nanospray and microspray or matrix-assisted laser desorption. Common mass analyzers include a quadrupole mass filter, ion trap mass analyzer and time-of-flight mass analyzer. Additional mass spectrometry methods are well known in the art (see Burlingame et al. Anal. Chem. 70:647 R-716R (1998); Kinter and Sherman, New York (2000)).

Protein biomarkers and biomarker levels can be detected and measured by any of the following: electrospray ionization mass spectrometry (ESI-MS), ESI-MS/MS, ESI-MS/(MS)n, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), desorption/ionization on silicon (DIOS), secondary ion

mass spectrometry (SIMS), quadrupole time-of-flight (Q-TOF), tandem time-of-flight (TOF/TOF) technology, called ultraflex III TOF/TOF, atmospheric pressure chemical ionization mass spectrometry (APCI-MS), APCI-MS/MS, APCI-(MS)N, atmospheric pressure photoionization mass spectrometry (APPI-MS), APPI-MS/MS, and APPI-(MS)N, quadrupole mass spectrometry, Fourier transform mass spectrometry (FTMS), quantitative mass spectrometry, and ion trap mass spectrometry.

Sample preparation strategies are used to label and enrich samples before mass spectroscopic characterization of protein biomarkers and determination biomarker levels. Labeling methods include but are not limited to isobaric tag for relative and absolute quantitation (iTRAQ) and stable isotope labeling with amino acids in cell culture (SILAC). Capture reagents used to selectively enrich samples for candidate biomarker proteins prior to mass spectroscopic analysis include but are not limited to aptamers, antibodies, nucleic acid probes, chimeras, small molecules, an $F(ab')_2$ fragment, a single chain antibody fragment, an Fv fragment, a single chain Fv fragment, a nucleic acid, a lectin, a ligand-binding receptor, affybodies, nanobodies, ankyrins, domain antibodies, alternative antibody scaffolds (e.g. diabodies etc) imprinted polymers, avimers, peptidomimetics, peptoids, peptide nucleic acids, threose nucleic acid, a hormone receptor, a cytokine receptor, and synthetic receptors, and modifications and fragments of these.

The foregoing assays enable the detection of biomarker levels that are useful in the methods described herein, where the methods comprise detecting, in a biological sample from an individual, at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or at least nine biomarkers selected from the described herein. Thus, while some of the described biomarkers may be useful alone for detecting TB infection, methods are also described herein for the grouping of multiple biomarkers and subsets of the biomarkers to form panels of two or more biomarkers. In accordance with any of the methods described herein, biomarker levels can be detected and classified individually or they can be detected and classified collectively, as for example in a multiplex assay format.

Classification of Biomarkers and Calculation of Disease Scores

In some embodiments, a biomarker "signature" for a given diagnostic test contains a set of markers, each marker having characteristic levels in the populations of interest. Characteristic levels, in some embodiments, may refer to the mean or average of the biomarker levels for the individuals in a particular group. In some embodiments, a diagnostic

method described herein can be used to assign an unknown sample from an individual into one of two groups: TB infected or non-infected, active TB or no active TB, latent TB or no TB infection, etc. The assignment of a sample into one of two or more groups (e.g.,, TB infection, latent infection, active infection, non-infected, etc.) is known as classification, and the procedure used to accomplish this assignment is known as a classifier or a classification method. Classification methods may also be referred to as scoring methods. There are many classification methods that can be used to construct a diagnostic classifier from a set of biomarker levels. In some instances, classification methods are performed using supervised learning techniques in which a data set is collected using samples obtained from individuals within two (or more, for multiple classification states) distinct groups one wishes to distinguish. Since the class (group or population) to which each sample belongs is known in advance for each sample, the classification method can be trained to give the desired classification response. It is also possible to use unsupervised learning techniques to produce a diagnostic classifier.

Common approaches for developing diagnostic classifiers include decision trees; bagging + boosting + forests; rule inference based learning; Parzen Windows; linear models; logistic; neural network methods; unsupervised clustering; K-means; hierarchical ascending/ descending; semi-supervised learning; prototype methods; nearest neighbor; kernel density estimation; support vector machines; hidden Markov models; Boltzmann Learning; and classifiers may be combined either simply or in ways which minimize particular objective functions. For a review, see, e.g., Pattern Classification, R.O. Duda, et al., editors, John Wiley & Sons, 2nd edition, 2001; see also, The Elements of Statistical Learning - Data Mining, Inference, and Prediction, T. Hastie, et al., editors, Springer Science+Business Media, LLC, 2nd edition, 2009.

To produce a classifier using supervised learning techniques, a set of samples called training data are obtained. In the context of diagnostic tests, training data includes samples from the distinct groups (classes) to which unknown samples will later be assigned. For example, samples collected from individuals in a control population and individuals in a particular disease population can constitute training data to develop a classifier that can classify unknown samples (or, more particularly, the individuals from whom the samples were obtained) as either having the disease or being free from the disease. The development of the classifier from the training data is known as training the classifier. Specific details on classifier training depend on the nature of the supervised learning technique. Training a naïve Bayesian classifier is an example of such a supervised learning technique (see, e.g.,

Pattern Classification, R.O. Duda, et al., editors, John Wiley & Sons, 2nd edition, 2001; see also, The Elements of Statistical Learning - Data Mining, Inference, and Prediction, T. Hastie, et al., editors, Springer Science+Business Media, LLC, 2nd edition, 2009). Training of a naïve Bayesian classifier is described, e.g., in U.S. Publication Nos: 2012/0101002 and 2012/0077695.

Since typically there are many more potential biomarker levels than samples in a training set, care must be used to avoid over-fitting. Over-fitting occurs when a statistical model describes random error or noise instead of the underlying relationship. Over-fitting can be avoided in a variety of way, including, for example, by limiting the number of markers used in developing the classifier, by assuming that the marker responses are independent of one another, by limiting the complexity of the underlying statistical model employed, and by ensuring that the underlying statistical model conforms to the data.

An illustrative example of the development of a diagnostic test using a set of biomarkers includes the application of a naïve Bayes classifier, a simple probabilistic classifier based on Bayes theorem with strict independent treatment of the biomarkers. Each biomarker is described by a class-dependent probability density function (PDF) for the measured RFU values or log RFU (relative fluorescence units) values in each class. The joint PDFs for the set of markers in one class is assumed to be the product of the individual class-dependent PDFs for each biomarker. Training a naïve Bayes classifier in this context amounts to assigning parameters ("parameterization") to characterize the class dependent PDFs. Any underlying model for the class-dependent PDFs may be used, but the model should generally conform to the data observed in the training set.

The performance of the naïve Bayes classifier is dependent upon the number and quality of the biomarkers used to construct and train the classifier. A single biomarker will perform in accordance with its KS-distance (Kolmogorov-Smirnov). The addition of subsequent markers with good KS distances (>0.3, for example) will, in general, improve the classification performance if the subsequently added markers are independent of the first marker. Using the sensitivity plus specificity as a classifier score, many high scoring classifiers can be generated with a variation of a greedy algorithm. (A greedy algorithm is any algorithm that follows the problem solving metaheuristic of making the locally optimal choice at each stage with the hope of finding the global optimum.)

Another way to depict classifier performance is through a receiver operating characteristic (ROC), or simply ROC curve or ROC plot. The ROC is a graphical plot of the sensitivity, or true positive rate, vs. false positive rate (1 − specificity or 1 − true negative

rate), for a binary classifier system as its discrimination threshold is varied. The ROC can also be represented equivalently by plotting the fraction of true positives out of the positives (TPR = true positive rate) vs. the fraction of false positives out of the negatives (FPR = false positive rate). Also known as a Relative Operating Characteristic curve, because it is a comparison of two operating characteristics (TPR & FPR) as the criterion changes. The area under the ROC curve (AUC) is commonly used as a summary measure of diagnostic accuracy. It can take values from 0.0 to 1.0. The AUC has an important statistical property: the AUC of a classifier is equivalent to the probability that the classifier will rank a randomly chosen positive instance higher than a randomly chosen negative instance (Fawcett T, 2006. An introduction to ROC analysis. Pattern Recognition Letters .27: 861–874). This is equivalent to the Wilcoxon test of ranks (Hanley, J.A., McNeil, B.J., 1982. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 143, 29–36.).

Exemplary embodiments use any number of the biomarkers provided herein in various combinations to produce diagnostic tests for detecting TB infection in a sample from an individual. The markers provided herein can be combined in many ways to produce classifiers. For example, a classifier may comprise AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and/or C9; or any suitable combinations or sub-combinations thereof.

In some embodiments, once a panel is defined to include a particular set of biomarkers and a classifier is constructed from a set of training data, the diagnostic test parameters are complete. In some embodiments, a biological sample is run in one or more assays to produce the relevant quantitative biomarker levels used for classification. The measured biomarker levels are used as input for the classification method that outputs a classification and an optional score for the sample that reflects the confidence of the class assignment.

In some embodiments, a biological sample is optionally diluted and run in a multiplexed aptamer assay, and data is assessed as follows. First, the data from the assay are optionally normalized and calibrated, and the resulting biomarker levels are used as input to a Bayes classification scheme. Second, the log-likelihood ratio is computed for each measured biomarker individually and then summed to produce a final classification score, which is also referred to as a diagnostic score. The resulting assignment as well as the overall classification score can be reported. In some embodiments, the individual log-likelihood risk factors computed for each biomarker level can be reported as well.

Kits

Any combination of the biomarkers described herein can be detected using a suitable kit, such as for use in performing the methods disclosed herein. The biomarkers described herein may be combined in any suitable combination, or may be combined with other markers not described herein. Furthermore, any kit can contain one or more detectable labels as described herein, such as a fluorescent moiety, etc.

In some embodiments, a kit includes (a) one or more capture reagents (such as, for example, at least one aptamer or antibody) for detecting one or more biomarkers in a biological sample, and optionally (b) one or more software or computer program products for predicting whether the individual from whom the biological sample was obtained is TB infected. Alternatively, rather than one or more computer program products, one or more instructions for manually performing the above steps by a human can be provided.

In some embodiments, a kit comprises a solid support, a capture reagent, and a signal generating material. The kit can also include instructions for using the devices and reagents, handling the sample, and analyzing the data. Further the kit may be used with a computer system or software to analyze and report the result of the analysis of the biological sample.

The kits can also contain one or more reagents (e.g., solubilization buffers, detergents, washes, or buffers) for processing a biological sample. Any of the kits described herein can also include, e.g., buffers, blocking agents, mass spectrometry matrix materials, antibody capture agents, positive control samples, negative control samples, software and information such as protocols, guidance and reference data.

In some embodiments, kits are provided for the analysis of TB infection, wherein the kits comprise PCR primers for one or more biomarkers described herein. In some embodiments, a kit may further include instructions for use and correlation of the biomarkers with TB infection. In some embodiments, a kit may include a DNA array containing the complement of one or more of the biomarkers described herein, reagents, and/or enzymes for amplifying or isolating sample DNA. The kits may include reagents for real-time PCR, for example, TaqMan probes and/or primers, and enzymes.

For example, a kit can comprise (a) reagents comprising at least one capture reagent for determining the level of one or more biomarkers in a test sample, and optionally (b) one or more algorithms or computer programs for performing the steps of comparing the amount of each biomarker quantified in the test sample to one or more predetermined cutoffs. In some embodiments, an algorithm or computer program assigns a score for each biomarker

quantified based on said comparison and, in some embodiments, combines the assigned scores for each biomarker quantified to obtain a total score. Further, in some embodiments, an algorithm or computer program compares the total score with a predetermined score, and uses the comparison to determine, for example, likelihood of latent TB infection advancing into active TB disease. Alternatively, rather than one or more algorithms or computer programs, one or more instructions for manually performing the above steps by a human can be provided.

Computer Methods and Software

Once a biomarker or biomarker panel is selected, a method may comprise the following: 1) collect or otherwise obtain a biological sample; 2) perform an analytical method to detect and measure the biomarker or biomarkers in the panel in the biological sample; and 3) report the results of the biomarker levels. In some embodiments, the results of the biomarker levels are reported qualitatively rather than quantitatively, such as, for example, a proposed diagnosis or numeric result indicating the percent likelihood (e.g., within a margin of error) of a latent infection transitioning to active TB. In some embodiments, a qualitative or quantitative risk of developing active TB disease within a particular time period is provided (e.g., within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days). In some embodiments, a method comprises the following: 1) collect or otherwise obtain a biological sample; 2) perform an analytical method to detect and measure the biomarker or biomarkers in the panel in the biological sample; 3) perform any data normalization or standardization; 4) calculate each biomarker level; and 5) report the results of the biomarker levels. In some embodiments, the biomarker levels are combined in some way and a single value for the combined biomarker levels is reported. In this approach, in some embodiments, the reported value may be a single number determined from the sum of all the marker calculations that is compared to a pre – set threshold value that is an indication of the presence or absence of disease. Or the diagnostic score may be a series of bars that each represent a biomarker value and the pattern of the responses may be compared to a pre-set pattern for determination of the presence or absence of disease.

At least some embodiments of the methods described herein can be implemented with the use of a computer. An example of a computer system 100 is shown in Figure 53. With reference to Figure 53, system 100 is shown comprised of hardware elements that are electrically coupled via bus 108, including a processor 101, input device 102, output device

103, storage device 104, computer-readable storage media reader 105a, communications system 106 processing acceleration (e.g., DSP or special-purpose processors) 107 and memory 109. Computer-readable storage media reader 105a is further coupled to computer-readable storage media 105b, the combination comprehensively representing remote, local, fixed and/or removable storage devices plus storage media, memory, etc. for temporarily and/or more permanently containing computer-readable information, which can include storage device 104, memory 109 and/or any other such accessible system 100 resource. System 100 also comprises software elements (shown as being currently located within working memory 191) including an operating system 192 and other code 193, such as programs, data and the like.

With respect to Figure 53, system 100 has extensive flexibility and configurability. Thus, for example, a single architecture might be utilized to implement one or more servers that can be further configured in accordance with currently desirable protocols, protocol variations, extensions, etc. However, it will be apparent to those skilled in the art that embodiments may well be utilized in accordance with more specific application requirements. For example, one or more system elements might be implemented as sub-elements within a system 100 component (e.g., within communications system 106). Customized hardware might also be utilized and/or particular elements might be implemented in hardware, software or both. Further, while connection to other computing devices such as network input/output devices (not shown) may be employed, it is to be understood that wired, wireless, modem, and/or other connection or connections to other computing devices might also be utilized.

In one aspect, the system can comprise a database containing features of biomarkers characteristic of TB infection. The biomarker data (or biomarker information) can be utilized as an input to the computer for use as part of a computer implemented method. The biomarker data can include the data as described herein.

In one aspect, the system further comprises one or more devices for providing input data to the one or more processors.

In some embodiments, the system further comprises a memory for storing a data set of ranked data elements.

In another aspect, the device for providing input data comprises a detector for detecting the characteristic of the data element, e.g., such as a mass spectrometer or gene chip reader.

The system additionally may comprise a database management system. User requests or queries can be formatted in an appropriate language understood by the database management system that processes the query to extract the relevant information from the database of training sets.

The system may be connectable to a network to which a network server and one or more clients are connected. The network may be a local area network (LAN) or a wide area network (WAN), as is known in the art. Preferably, the server includes the hardware necessary for running computer program products (e.g., software) to access database data for processing user requests.

The system may include an operating system (e.g., UNIX® or Linux) for executing instructions from a database management system. In one aspect, the operating system can operate on a global communications network, such as the internet, and utilize a global communications network server to connect to such a network.

The system may include one or more devices that comprise a graphical display interface comprising interface elements such as buttons, pull down menus, scroll bars, fields for entering text, and the like as are routinely found in graphical user interfaces known in the art. Requests entered on a user interface can be transmitted to an application program in the system for formatting to search for relevant information in one or more of the system databases. Requests or queries entered by a user may be constructed in any suitable database language.

The graphical user interface may be generated by a graphical user interface code as part of the operating system and can be used to input data and/or to display inputted data. The result of processed data can be displayed in the interface, printed on a printer in communication with the system, saved in a memory device, and/or transmitted over the network or can be provided in the form of the computer readable medium.

The system can be in communication with an input device for providing data regarding data elements to the system (e.g., expression values). In one aspect, the input device can include a gene expression profiling system including, e.g., a mass spectrometer, gene chip or array reader, and the like.

The methods and apparatus for analyzing biomarker information according to various embodiments may be implemented in any suitable manner, for example, using a computer program operating on a computer system. A conventional computer system comprising a processor and a random access memory, such as a remotely-accessible application server, network server, personal computer or workstation may be used. Additional computer system

components may include memory devices or information storage systems, such as a mass storage system and a user interface, for example a conventional monitor, keyboard and tracking device. The computer system may be a stand-alone system or part of a network of computers including a server and one or more databases.

The biomarker analysis system can provide functions and operations to complete data analysis, such as data gathering, processing, analysis, reporting and/or diagnosis. For example, in one embodiment, the computer system can execute the computer program that may receive, store, search, analyze, and report information relating to the biomarkers. The computer program may comprise multiple modules performing various functions or operations, such as a processing module for processing raw data and generating supplemental data and an analysis module for analyzing raw data and supplemental data to generate a disease status and/or diagnosis. Methods may comprise generating or collecting any other information, including additional biomedical information, regarding the condition of the individual relative to the disease, identifying whether further tests may be desirable, or otherwise evaluating the health status of the individual.

Some embodiments described herein can be implemented so as to include a computer program product. A computer program product may include a computer readable medium having computer readable program code embodied in the medium for causing an application program to execute on a computer with a database.

As used herein, a "computer program product" refers to an organized set of instructions in the form of natural or programming language statements that are contained on a physical media of any nature (e.g., written, electronic, magnetic, optical or otherwise) and that may be used with a computer or other automated data processing system. Such programming language statements, when executed by a computer or data processing system, cause the computer or data processing system to act in accordance with the particular content of the statements. Computer program products include without limitation: programs in source and object code and/or test or data libraries embedded in a computer readable medium. Furthermore, the computer program product that enables a computer system or data processing equipment device to act in pre-selected ways may be provided in a number of forms, including, but not limited to, original source code, assembly code, object code, machine language, encrypted or compressed versions of the foregoing and any and all equivalents.

In one aspect, a computer program product is provided for characterizing the TB-infection status (e.g., likelihood of advancement to active TB) of a subject. The computer

program product includes a computer readable medium embodying program code executable by a processor of a computing device or system, the program code comprising: code that retrieves data attributed to a biological sample from an individual, wherein the data comprises biomarker levels that correspond to one or more of the biomarkers described herein, and code that executes a classification method that indicates the TB-infection status of the individual as a function of the biomarker levels.

While various embodiments have been described as methods or apparatuses, it should be understood that embodiments can be implemented through code coupled with a computer, e.g., code resident on a computer or accessible by the computer. For example, software and databases could be utilized to implement many of the methods discussed above. Thus, in addition to embodiments accomplished by hardware, it is also noted that these embodiments can be accomplished through the use of an article of manufacture comprised of a computer usable medium having a computer readable program code embodied therein, which causes the enablement of the functions disclosed in this description. Therefore, it is desired that embodiments also be considered protected by this patent in their program code means as well. Furthermore, the embodiments may be embodied as code stored in a computer-readable memory of virtually any kind including, without limitation, RAM, ROM, magnetic media, optical media, or magneto-optical media. Even more generally, the embodiments could be implemented in software, or in hardware, or any combination thereof including, but not limited to, software running on a general purpose processor, microcode, programmable logic arrays (PLAs), or application-specific integrated circuits (ASICs).

It is also envisioned that embodiments could be accomplished as computer signals embodied in a carrier wave, as well as signals (e.g., electrical and optical) propagated through a transmission medium. Thus, the various types of information discussed above could be formatted in a structure, such as a data structure, and transmitted as an electrical signal through a transmission medium or stored on a computer readable medium.

Methods of Treatment

In some embodiments, following characterization of a subject's TB status (e.g., no infection; latent infection not likely to advance to active TB; latent infection – likely to advance to active TB within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days; active TB disease; etc.), the subject is treated for TB infection. In some embodiments, medications used to treat latent TB infection include: isoniazid (INH), rifampin (RIF), and rifapentine (RPT). In some embodiments, TB disease is

treated by taking several drugs for 6 to 9 months. There are 10 drugs currently approved by the U.S. Food and Drug Administration (FDA) for treating TB. Of the approved drugs, the first-line anti-TB agents that form the core of treatment regimens include: isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA). Regimens for treating TB disease have an initial phase of 2 months, followed by a choice of several options for the continuation phase of either 4 or 7 months (total of 6 to 9 months for treatment).

In some embodiments, methods of monitoring TB infection/disease and/or treatment of TB infection/disease are provided. In some embodiments, the present methods of detecting TB infection are carried out at a time 0. In some embodiments, the method is carried out again at a time 1, and optionally, a time 2, and optionally, a time 3, etc., in order to monitor the progression of TB infection or to monitor the effectiveness of one or more treatments of TB. Time points for detection may be separated by, for example at least 1 day, at least 2 days, at least 4 days, at least 1 week, at least 2 weeks, at least 1 month, at least 2 months, at least 3 months, at least 4 months, at least 6 months, or by 1 year or more. In some embodiments, a treatment regimen is altered based upon the results of monitoring (e.g., upon determining that a first treatment is ineffective).

EXAMPLES

Example 1: Samples and subjects

Normalization and Calibration

Samples were obtained from a study of TB risk conducted by the South African Tuberculosis Vaccine Initiative (SATVI) in collaboration with the University of Cape Town (UCT). The TB Risk study enrolled 6,363 adolescents (12-18 years of age) prospectively at several high schools in an area ~100km from Cape Town with a high burden of TB. Blood was collected at mobile collection centers from participants at 6 month intervals between 2006 and 2008 and during this time some participants developed active TB. TB diagnosis was determined by bacteriological testing, though subjects had positive Quantiferon Gold In-Tube (QFT) and tuberculin skin tests (TST) at time of enrollment as immunological evidence of Mtb infection. The samples used for the SOMAscan V3+ 3000plex were CPT heparin plasma. TB case samples were placed into 'bins' along with matched control samples based on gender, age, ethnicity, high school and history of TB. A tabulation of the resulting patient demographics is summarized in Table 1 and Table 2 below.

Table 1: Participant demographics for the case cohort in discovery (training) and verification (test) sets, as well as a p-value for the two group comparison using a t-test

	Participants	Age	Male	Black	Coloured	Prior TB
		Mean, (min-max)	n, (%)	n, (%)	n, (%)	
Discovery	29	15.79 (12-18)	9 (31%)	2 (7%)	27 (93%)	3 (10%)
Verification	15	15.13 (12-18)	4 (28%)	1 (7%)	14 (93%)	2 (7%)
p		0.3272	1	1	1	1

Table 2: Number of study participants, samples, and sample locations for the TB case and non-TB control cohorts. * discovery samples only listed (verification samples are blinded)

	Participants		Samples			
	Discovery	Verification	Discovery	Verification	SATVI	Seattle
Cases	29	15	84	40	24*	60*
Controls	68	38	199	93	97*	102*

All Cases were diagnosed with active TB disease by 2 positive sputum smears and/or positive sputum culture. All TB cases have an exact date of treatment initiation with day zero being defined as the date of treatment initiation. Figure 1 shows plots of sample time distributions for the discovery and verification sets.

All samples were normalized and calibrated using standard hybridization and median normalization procedures. Hybridization normalization was performed using elution probes and is performed on a per sample basis. Hybridization scale factors are expected to be within the range 0.4-2.5, and all samples passed. As shown in Figure 3, the median hybridization scale factor in each run is within 10% of unity except for plate B, which is slightly brighter compared to the other plates.

Example 2: Biomarker discovery

Two Group Diagnostic Comparison

All Data were log10 transformed to control for heteroscedasticity, thereby stabilizing the variance. The non-parametric Kolmogorov-Smirnov (KS) test was used to first compare TB Case and non-TB Control samples across all time points, then Case and matched Control groups were compared within the intervals 0-3, 6-12, and 12-18 months before the initiation of treatment. Hierarchical clustering was performed on all proteins by bin to determine which proteins have bin-dependent expression levels. This was further investigated using a generalized linear mixed model (GLMM) using the bin as a random effect. Linear regression

was also performed on all TB cases to determine which proteins move linearly with time to diagnosis/treatment.

For each TB case within a time interval, controls were selected by matching bin number and study day. All samples were put into bins selected to control for various factors such as age, location, Mycobacterium tuberculosis exposure, etc. Stability selection using L1-regularized logistic regression was used to identify stable features in the presence of the available clinical covariates.

Prior to stratifying the samples by time to treatment, a cross-sectional comparison of all pre-treatment TB cases and all Control samples was performed using univariate KS. Figure 5 shows demographic information for 57 TB Case samples (pre-treatment) and 197 Control samples used in the analysis. Comparing all 197 Control samples to all 57 TB case samples, 500 proteins were found to be significant at a 5% Benjamini-Hochberg False Discovery Rate (bhFDR). Of these, 348 proteins were higher in the TB cases and 152 were lower. Also significant at a 5% FDR were the HR9 proteins C9 (p=1.62e-6, rank 19), SERPINA4 (p=1.35e-4, rank 102), FCGR3B (p=1.4e4, rank 104) and SPOCK2 (p=7.22e-3, rank 467), as well as the TB-specific SOMAmers ESXA (p=1e-4, rank94), CH10 (p=7.36e-3, rank 471) and DNAK (p=2.3e-2, rank 739). Table 3 below shows the KS statistics for the top 25 ranked proteins.

Table 3: Top 25 ranked proteins differentiating all TB Cases from all non-TB Controls. Proteins with positive KS distances are lower in TB cases.

Rank	Target Name	UniProt ID	KS Dist.	p-value	p_{emp}	bhFDR
1	GALT1	Q9NP70	-0.492	6.60E-07	4.06E-10	0.0002
2	AMBN	Q93038	-0.454	6.60E-07	1.15E-08	0.0002
3	C5	Q10472	-0.447	6.60E-07	1.97E-08	0.0002
4	DR3	Q8IXJ6	-0.436	6.60E-07	4.85E-08	0.0002
5	MMP-1	Q86YW7	-0.435	6.60E-07	5.21E-08	0.0002
6	SAP	P01031	-0.434	6.60E-07	5.84E-08	0.0002
7	Angiopoietin-1	Q6H9L7	-0.432	6.60E-07	6.68E-08	0.0002
8	Plasminogen	Q8TD33	-0.419	6.60E-07	1.85E-07	0.0002
9	NID2	P03956	-0.412	6.60E-07	3.15E-07	0.0002
10	D-dimer	P28067	-0.409	6.60E-07	4.19E-07	0.0002
11	GPHB5	O14896	-0.409	6.60E-07	4.19E-07	0.0002
12	SG1C1	P02743	-0.405	1.30E-06	5.69E-07	0.0003
13	TGF-b3	P00747	-0.402	1.30E-06	6.76E-07	0.0003
14	2DMA	Q01459	-0.402	1.30E-06	7.03E-07	0.0003
15	Coagulation Factor X	P10600	-0.401	1.30E-06	7.51E-07	0.0003
16	C5b, 6 Complex	Q14112	-0.399	2.00E-06	8.90E-07	0.0003
17	IRF6	P01031,P13671 P02671 P02675	0.397	2.00E-06	1.01E-06	0.0003
18	Factor I	P02679	-0.394	2.00E-06	1.25E-06	0.0003
19	C9	Q15389	-0.39	2.60E-06	1.62E-06	0.0004

20	SIRT2	P02748	0.387	3.90E-06	1.98E-06	0.0005
21	IP-10	P05231	-0.387	3.90E-06	2.11E-06	0.0005
22	CLC6A	Q92575	-0.387	3.90E-06	2.11E-06	0.0005
23	PIM1	Q6EIG7	-0.386	3.90E-06	2.25E-06	0.0005
24	DIAC	Q9Y6J6	-0.385	4.60E-06	2.33E-06	0.0005
25	IL-6	P52943	-0.385	4.60E-06	2.42E-06	0.0005

Figure 6 shows the cumulative distribution functions (CDFs) for the top 9 proteins listed in the table above. P-values were calculated using a standard distribution (p-value) as well as an empirical null distribution created through class scrambling (Pemp).

Figure 7 shows the KS distances with class randomization statistics for the top 150 ranked proteins, which corresponded to a p-value cutoff of 1.35e-4. The total height of each bar represents the KS distance for the TB Case vs. Control comparison, with the top being green for proteins that are higher in TB Cases and red if they are lower. The height of the orange portion of each bar represents the median KS distance achieved through class randomization for that feature, and the error bars represent 95% confidence intervals.

Figure 8 shows a volcano plot of the negative log10-transformed p-values versus the log2 of the median TB RFU value over the median Control RFU value. A value of 1 on the horizontal axis corresponds to a 2-fold change in RFU.

Figure 9 shows the longitudinal RFU measurements for the 16 TB subjects with >1 time points overlaid on to a 'control band' created by interpolating the median, inter-quartile range and range of the control data. The control band is analogous to an interpolated boxplot of the RFU values of the Controls between days in study. The top axis (Days in Study) corresponds to the controls and the bottom axis (Days to Rx) corresponds to the TB cases. Time moves to the right in both groups.

Hierarchical Clustering of Top Proteins within Each Bin

All samples were placed into 19 'bins' which were matched according to age, high school, etc. Of these, 7 were observed to have > 1 TB Case and >3 Controls. A t-test was used to find the magnitude of the intra-bin differences between TB Cases and Controls, and proteins were then ranked according to the median t-statistic across the 7 bins. Hierarchical clustering was performed on the top 200. A heat map of clustered t-statistics is shown in Figure 10. The five regions in Figure 10 marked A-E correspond to areas visually identified to have inconsistencies across the Bins.

Hierarchical clustering arranges proteins according to similarities in expression. In Figures 11-15 (left), each row corresponds to a bin and each column a protein. Therefore, the coloring of each column represents the magnitude of the t-statistics for a particular protein

across the 7 bins. Dendrograms (Figurea 11-15, right) show the hierarchical grouping structure for regions marked A-E, with the height of each branch corresponding to the similarity between the underlying groups.

Generalized Linear Mixed Effects Model

A generalized linear mixed effects model was used to determine the ability of each protein to classify subjects based on diagnosis while controlling for the bin number, as well as to determine which proteins have differences between the two groups which are dependent on the bin itself. Table 4 below shows statistics for the top 50 ranked proteins. A single p-value was generated for each protein (p_{fixed}) and was corrected for 3040 multiple comparisons (q_{fixed}). For each protein, 21 random effects p-value (p_{random}) were generate (one for each bin), and the minimum value is shown in the table. Overall, only proteins PDGFRA and SIA7A were found to have at least one bin that had a significant bin/random effect after correcting for multiple comparisons. At a 1% ($p_{random}<0.01$) uncorrected level, 327 proteins were found to have significant random effects. In the top 50 ranked proteins, none were found to have $p_{random}<0.01$. In the top 100 ranked proteins, only IL-12, STAT6 and Alpha-amylase 2B were found to have $p_{random}<0.01$.

Table 4: Generalized linear mixed model statistics comparing all 57 TB cases to 197 Controls.

Rank	Target	p_{fixed}	q_{fixed}	min(p_{random})	LogLikelihood	r^2
1	IRF6	1.64E-07	1.25E-04	1.000	-632.9	0.28
2	C9	3.20E-07	1.95E-04	1.000	-621.8	0.13
3	MMP-2	4.18E-07	2.12E-04	0.321	-610.0	0.13
4	Factor I	6.15E-07	2.67E-04	1.000	-617.4	0.13
5	D-dimer	1.08E-06	4.11E-04	0.255	-639.1	0.22
6	C5	2.69E-06	9.08E-04	0.067	-648.3	0.29
7	CK-MB	3.05E-06	9.26E-04	0.327	-614.8	0.19
8	Albumin	4.53E-06	1.25E-03	1.000	-613.1	0.12
9	Fibrinogen g-chain dimer	5.01E-06	1.27E-03	0.422	-618.8	0.20
10	CRP	7.67E-06	1.79E-03	0.349	-606.2	0.12
11	SAP	8.85E-06	1.92E-03	0.017	-618.9	0.25
12	C9	1.10E-05	2.23E-03	0.498	-603.7	0.18
13	SET	1.35E-05	2.57E-03	1.000	-602.6	0.09
14	CK-MM	1.47E-05	2.63E-03	1.000	-624.3	0.17
15	MDC	2.68E-05	4.53E-03	0.444	-601.5	0.12
16	MDHC	3.04E-05	4.86E-03	1.000	-612.1	0.10
17	FGL1	3.54E-05	5.39E-03	1.000	-597.2	0.07
18	TGF-b3	4.11E-05	5.95E-03	1.000	-625.2	0.81
19	Dynactin subunit 2	5.48E-05	7.57E-03	1.000	-603.1	0.07
20	Plasminogen	5.78E-05	7.64E-03	0.350	-601.4	0.11
21	LRRT3	6.83E-05	8.65E-03	0.177	-604.3	0.26

22	PIP	7.20E-05	8.76E-03	1.000	-600.6	0.09
23	MMP-1	7.81E-05	9.13E-03	0.110	-605.4	0.18
24	Factor B	8.41E-05	9.15E-03	1.000	-600.7	0.07
25	FCN1	8.43E-05	9.15E-03	1.000	-594.5	0.06

The top 100 proteins ranked by KS distance and stability selection were also investigated for proteins with significant bin effects. 15 proteins in the KS ranked list and 24 in the stability selection ranked list were found to have $p_{random}<0.01$, and none were significant after correcting for multiple comparisons.

Linear Regression

In order to find proteins which respond to the progression of TB pathogenesis, a linear regression was run on all TB case samples taken <300 before the beginning of treatment. This time window was chosen because protein signals were observed to generally stabilize >300 days pre-Rx, which would confound regression statistics. Table 5 below shows regression statistics for RFU level as a function of days to initiation of treatment.

Table 5: Linear regression statistics for all TB Case samples <300 days before treatment initiation.

Rank	Target	Beta	R^2	p-value	q-value
1	IMPA3	0.001	0.428	6.62E-05	0.142
2	B4GT6	0.001	0.413	9.72E-05	0.142
3	NLGN2	0.001	0.399	1.40E-04	0.142
4	C9	-0.001	0.351	4.46E-04	0.306
5	C9	-0.001	0.345	5.09E-04	0.306
6	BOC	0.001	0.338	6.04E-04	0.306
7	PTPRD	0.001	0.312	1.08E-03	0.410
8	CRP	-0.002	0.308	1.19E-03	0.410
9	CA2D3	0.001	0.304	1.29E-03	0.410
10	IL-11 RA	0.001	0.303	1.35E-03	0.410
11	OMD	0.001	0.293	1.68E-03	0.462
12	CA226	-0.001	0.289	1.82E-03	0.462
13	Cathelicidin peptide	-0.001	0.276	2.42E-03	0.543
14	F150B	0	0.273	2.54E-03	0.543
15	Lymphotoxin a1/b2	0	0.267	2.94E-03	0.543
16	BMP-6	0.001	0.264	3.10E-03	0.543
17	Periostin	0.001	0.261	3.32E-03	0.543
18	NDST1	0	0.256	3.66E-03	0.543
19	C3b	-0.002	0.249	4.23E-03	0.543
20	IL-19	0.001	0.247	4.42E-03	0.543
21	EMIL3	0.001	0.245	4.68E-03	0.543
22	PolyUbiquitin K48	0	0.24	5.18E-03	0.543
23	PKB a/b/g	0	0.239	5.26E-03	0.543
24	TSP4	0.001	0.238	5.36E-03	0.543

25	Factor B	0	0.237	5.47E-03	0.543

Figure 16 shows scatter plots for the top 9 ranked proteins from a linear regression. Linear fits for all TB cases are shown as a function of time to treatment, with time moving to the left. To provide a notion for how well these proteins distinguish the TB from non-TB cohorts, this information is overlaid onto data representing a boxplot for all control RFU data. The dark band corresponds to the interquartile range (IQR), while the lighter shaded region corresponds to the whiskers, or the nearest data point that's within the upper/lower quartile + 1.5*IQR.

Time Point Stratification

In order to break the serial sampling structure in the data which leads to a decreased estimate of the true variance, the data was stratified into time bins and a univariate analysis was repeated. Time bins were created based on the distribution of non-repeated subjects within each time interval. Figure 17 shows the Time to treatment (Rx) for each TB case. Based on these criteria, intervals of 0 to 180 days (n=12), 180 to 360 days (n=20), 360 to 540 days (n=12) and 540-700 were chosen. For all TB cases within a given time range, a control population was constructed based on the bin and study day. For a given case, the Control samples from its bin with a matching study day were selected. There were typically 1-3 matched controls for each case. However, since the controls within a bin are typically serial samples from control subjects, if TB cases are from multiple study days within a given bin then serial sampling of the controls is not broken. This will underestimate the true variance of the control population. Figure 17 shows sample times for all TB subjects as a function of time to the beginning of treatment. Figure 18 shows demographics for TB Cases 0 to 180 days pre-treatment and matched controls.

Comparing 12 TB cases 0 to 180 days before diagnosis and treatment with all 60 matched non-TB Controls, a KS test identified 30 proteins to be differentially expressed between TB and non-TB subjects at 5% Benjamini-Hochberg False Discovery Rate (bhFDR). This includes the protein C9 (p=3.233-3, rank 5), which has previously been shown to be diagnostic for TB infection and/or disease. Nine of the 30 proteins were higher in the TB. At a 10% bhFDR 82 proteins were significant with 29 being higher in the TB group, and at a 20% FDR 303 proteins were significant with 128 of those being higher in the TB group. Table 6 below shows KS statistics for the top 25 ranked proteins.

Table 6: Top 25 ranked proteins differentiating TB Cases 0-180 days pre-Rx from matched non-TB Controls. Proteins with positive KS distances are lower in TB cases.

Rank	Target Name	UniProt ID	KS Dist.	p-value	p_{emp}	bhFDR
1	MMP-2	P08253	0.8	6.60E-07	1.53E-06	0.002
2	CLFB_STAAE	O86476	0.717	1.80E-05	2.47E-05	0.026
3	IL-6	P05231	-0.7	3.00E-05	4.15E-05	0.028
4	C1QT3	Q9BXJ4	0.667	6.90E-05	1.13E-04	0.037
5	C9	P02748	-0.65	1.50E-04	1.83E-04	0.037
6	EDA	Q92838	0.65	1.50E-04	1.83E-04	0.037
7	D-dimer	P02671 P02675 P02679	-0.65	1.50E-04	1.83E-04	0.037
8	CA2D3	Q8IZS8	0.65	1.50E-04	1.83E-04	0.037
9	RAD51	Q06609	0.65	1.70E-04	1.83E-04	0.037
10	RSPO4	Q2I0M5	-0.65	1.70E-04	1.83E-04	0.037
11	MRP6	O95255	0.65	1.70E-04	1.83E-04	0.037
12	AMBN	Q9NP70	-0.633	1.80E-04	2.94E-04	0.037
13	B4GT6	Q9UBX8	0.633	2.80E-04	2.94E-04	0.037
14	PGCB	Q96GW7	0.633	2.80E-04	2.94E-04	0.037
15	IgG	P01857	-0.633	2.80E-04	2.94E-04	0.037
16	Fibrinogen g-chain dimer	P02679	-0.633	2.80E-04	2.94E-04	0.037
17	Nr-CAM	Q92823	0.633	2.80E-04	2.94E-04	0.037
18	CBPE	P16870	0.633	2.80E-04	2.94E-04	0.037
19	MED-1	Q15648	0.633	2.80E-04	2.94E-04	0.037
20	NPS	P0C0P6	0.633	2.80E-04	2.94E-04	0.037
21	NLGN2	Q8NFZ4	0.633	2.80E-04	2.94E-04	0.037
22	WFKN2	Q8TEU8	0.617	4.50E-04	4.64E-04	0.042
23	SIRT2	Q8IXJ6	0.617	4.50E-04	4.64E-04	0.042
24	PKB beta	P31751	0.617	4.50E-04	4.64E-04	0.042
25	Ephrin-A3	P52797	0.617	4.50E-04	4.64E-04	0.042

The second ranked protein CLFB_STAAE is a Staphylococcus aureus protein. Figure 19 shows CDFs for the top 9 ranked proteins, including CLFB_STAAE, and Figure 20 shows KS distances with class randomization statistics for the top 100 ranked proteins, which corresponded to a p-value cutoff of 1.12e-3.

Figure 21 shows a volcano plot of the negative log10-transformed p-values versus the log2 of the median TB RFU value over the median Control RFU value. A value of 1 on the horizontal axis corresponds to a 2-fold change in RFU. Figure 22 shows control band plots for the top 6 ranked proteins.

Figure 23 shows demographics for the controls at all time-points as well as the TB cases 180 to 360 days pre-treatment.

Comparing 21 TB cases to 94 matched non-TB Controls, a KS test identified 0 proteins to be differentially expressed between TB and non-TB subjects at 5% FDR. 16

proteins were found to be significant at a 20% FDR with 14 having higher expression in the TB group. None of the known TB-specific were observed to have an FDR < 40%.

Table 7: Top 25 ranked proteins differentiating TB Cases 180-360 days pre-Rx from matched non-TB Controls. Proteins with positive KS distances are lower in TB cases.

Rank	Target Name	UniProt ID	KS Dist.	p-value	p_{emp}	bhFDR
1	ISM2	Q6H9L7	-0.539	3.20E-05	4.64E-05	0.084
2	AMBN	Q9NP70	-0.523	6.90E-05	8.56E-05	0.084
3	SG1C1	Q8TD33	-0.517	8.60E-05	1.06E-04	0.084
4	DIAC	Q01459	-0.486	2.80E-04	3.38E-04	0.185
5	RNAS7	Q9H1E1	-0.475	4.20E-04	4.92E-04	0.185
6	MMP-1	P03956	-0.475	4.40E-04	5.01E-04	0.185
7	DR3	Q93038	-0.47	4.90E-04	5.87E-04	0.185
8	IRF6	O14896	0.469	5.40E-04	6.08E-04	0.185
9	RMD3	Q96TC7	-0.465	6.00E-04	7.11E-04	0.185
10	DAG1	Q14118	-0.458	8.20E-04	8.74E-04	0.185
11	GPHB5	Q86YW7	-0.454	8.60E-04	1.00E-03	0.185
12	TGF-b3	P10600	-0.453	9.30E-04	1.04E-03	0.185
13	sCD163	Q86VB7	-0.453	9.30E-04	1.04E-03	0.185
14	TXD11	Q6PKC3	-0.453	9.30E-04	1.04E-03	0.185
15	SCGF-alpha	Q9Y240	-0.453	1.00E-03	1.05E-03	0.185
16	Aminopeptidase	Q9Y646	0.453	1.00E-03	1.05E-03	0.185
17	NID2	Q14112	-0.438	1.60E-03	1.73E-03	0.255
18	Periostin	Q15063	-0.438	1.60E-03	1.73E-03	0.255
19	USE1	Q9NZ43	0.432	2.10E-03	2.11E-03	0.307
20	Angiopoietin-1	Q15389	-0.431	2.10E-03	2.14E-03	0.307
21	CRIP2	P52943	-0.423	2.50E-03	2.76E-03	0.351
22	DLC8	P63167	-0.422	2.60E-03	2.85E-03	0.351
23	SCGF-beta	Q9Y240	-0.421	2.90E-03	2.94E-03	0.351
24	RETST	Q6NUM9	-0.421	2.90E-03	2.94E-03	0.351
25	SIRT2	Q8IXJ6	0.411	3.80E-03	3.95E-03	0.381

Figure 24 shows CDFs for the top 9 ranked proteins.

Figure 28 shows demographics for TB Cases 360 to 540 days pre-treatment along with their matched controls. Comparing 13 TB Cases to 66 matched non-TB Controls, 2 proteins were found to be significant at a 5% FDR. At a 20% FDR 40 proteins were significant with 39 proteins having higher expression in TB cases. Only SIRT2 (p=2e-4, rank 6) was found to be lower. No TB-specific or HR9 proteins were significant at a 20% FDR. Even though the case and control sample sizes were both approximately 1/3 of the 180-360 day time point the magnitude of the differences was noticeably higher. 11 proteins have an FDR less than 10% compared to 3 proteins in the previous time point. Only one protein, RNAS7, was found to be significant in both groups at a 20%, suggesting that the differences between groups may be driven by different biological processes.

Table 8: Top 25 ranked proteins differentiating TB Cases 360-540 days pre-Rx from matched non-TB Controls. Proteins with positive KS distances are lower in TB cases.

Rank	Target Name	UniProt ID	KS Dist.	p-value	p_{emp}	bhFDR
1	Cadherin-2	P19022	-0.679	1.60E-05	3.39E-05	0.048
2	FA20A	Q96MK3	-0.664	3.40E-05	5.51E-05	0.050
3	PIM1	P11309	-0.648	6.60E-05	9.17E-05	0.065
4	RNAS7	Q9H1E1	-0.634	1.10E-04	1.40E-04	0.066
5	STX1B	P61266	-0.634	1.10E-04	1.40E-04	0.066
6	SIRT2	Q8IXJ6	0.619	2.00E-04	2.21E-04	0.083
7	MA2B2	Q9Y2E5	-0.619	2.00E-04	2.21E-04	0.083
8	DSC3	Q14574	-0.605	2.70E-04	3.32E-04	0.089
9	CLC6A	Q6EIG7	-0.605	2.70E-04	3.32E-04	0.089
10	SLUG	O43623	-0.604	3.10E-04	3.43E-04	0.091
11	sperm acrosome associated 5	Q96QH8	-0.603	3.40E-04	3.55E-04	0.091
12	ELA2A	P08217	-0.589	5.10E-04	5.27E-04	0.124
13	NRN1	Q9NPD7	-0.587	5.60E-04	5.44E-04	0.126
14	GALT1	Q10472	-0.575	7.00E-04	7.76E-04	0.146
15	TPA_MOUSE	P11214	-0.573	8.00E-04	8.01E-04	0.155
16	EMIL3	Q9NT22	-0.572	8.60E-04	8.27E-04	0.157
17	transcription factor MLR1, ...	Q8N3X6	-0.559	1.10E-03	1.17E-03	0.183
18	SC61B	P60468	-0.558	1.10E-03	1.20E-03	0.183
19	C5b, 6 Complex	P01031,P13671	-0.557	1.30E-03	1.24E-03	0.183
20	Galectin-7	P47929	-0.545	1.40E-03	1.69E-03	0.183
21	C5	P01031	-0.544	1.60E-03	1.74E-03	0.183
22	sCD4	P01730	-0.544	1.60E-03	1.74E-03	0.183
23	IL-23	P29460, Q9NPF7	-0.544	1.60E-03	1.74E-03	0.183
24	NLGN2	Q8NFZ4	-0.544	1.60E-03	1.74E-03	0.183
25	GLIP1	P48060	-0.544	1.60E-03	1.74E-03	0.183

Figures 29-32 show CDFs of the top 9, the top 100 features with class randomization statistics, a volcano plot and control band plots for the top 6, respectively.

Figure 33 shows demographics for TB cases 540 to 700 days pre-treatment, as well as their matched controls. Comparing 8 TB Cases with 33 matched non-TB Controls, 0.92 was the smallest FDR attained. However, the KS distances were relatively large with an absolute range of [0.842 0.699] for the top 10 ranked proteins. Table 9 below shows KS statistics for the top 25 proteins. Plasminogen(#6), I-TAC (#17), Fibronectin (#22), D-dimer(#24). IgG (#22) and D-dimer (#7) were also found to be a top 20 markers in the 0-180 time point. Also, 2DMA (#5) is a major histo-compatibility antigen which has implications in infection. Figures 34-37 shows biomarker data for 540 to 700 days pre-treatment.

Table 9: Top 25 ranked proteins differentiating TB Cases 540-700 days pre-Rx from matched non-TB Controls. Proteins with positive KS distances are lower in TB cases.

Rank	Target Name	UniProt ID	KS Dist.	p-value	p_{emp}	bhFDR
1	LRRT4	Q86VH4	0.842	4.30E-04	4.60E-04	0.922
2	SELPL	Q14242	0.789	1.30E-03	1.27E-03	0.922
3	CDY1	Q9Y6F8	0.737	2.90E-03	3.28E-03	0.922
4	UB2J2	Q8N2K1	-0.714	4.00E-03	4.82E-03	0.922
5	2DMA	P28067	-0.699	6.30E-03	6.20E-03	0.922
6	Plasminogen	P00747	-0.699	6.30E-03	6.20E-03	0.922
7	F19A5	Q7Z5A7	-0.699	6.30E-03	6.20E-03	0.922
8	IGFL4	Q6B9Z1	-0.699	6.30E-03	6.20E-03	0.922
9	ITA5	P08648	-0.699	6.30E-03	6.20E-03	0.922
10	LRTM1	Q9HBL6	-0.699	6.30E-03	6.20E-03	0.922
11	FGF23	Q9GZV9	-0.684	8.80E-03	7.93E-03	0.922
12	CLFA_STAAW	Q8NXJ1	0.684	8.80E-03	7.93E-03	0.922
13	PPIF_MOUSE	Q99KR7	-0.684	8.80E-03	7.93E-03	0.922
14	NALD2	Q9Y3Q0	-0.684	8.80E-03	7.93E-03	0.922
15	RCN1	Q15293	0.684	8.80E-03	7.93E-03	0.922
16	PABP3	Q9H361	0.662	1.10E-02	1.13E-02	0.922
17	I-TAC	O14625	-0.662	1.10E-02	1.13E-02	0.922
18	kallikrein 9	Q9UKQ9	0.662	1.10E-02	1.13E-02	0.922
19	MP64_MYCTU	P0A5Q4	-0.662	1.10E-02	1.13E-02	0.922
20	B3GN2	Q9NY97	-0.662	1.10E-02	1.13E-02	0.922
21	Secretagogin	O76038	-0.647	1.30E-02	1.43E-02	0.922
22	FN1.3	P02751	-0.647	1.30E-02	1.43E-02	0.922
23	IgG	P01857	-0.647	1.30E-02	1.43E-02	0.922
24	D-dimer	P02671 P02675 P02679	-0.647	1.30E-02	1.43E-02	0.922
25	MCCD1	P59942	-0.647	1.30E-02	1.43E-02	0.922

Stability Selection using Logistic Regression

Metadata for GENDER, SITE_ID, BMI, and AGE were included along with all 3040 human, non-human, and TB-specific proteins when performing stability selection using an L1-regularized logistic regression model. As with the univariate KS analysis this was done using all TB cases and matched controls, then within each time point.

Outlying values in logistic regression affect the hyperplane and can greatly influence the learning procedure. Initial runs of stability selection found that the L1-regularized model was very sensitive to a small subset of outliers (1-3 TB cases) and overly selecting for these proteins. Outliers were defined as being >4 median absolute deviations (MADs) from the median and were replaced with values taken from the 90% percentile of a simulated distribution.

Table 10 below shows stability selection statistics for proteins with maximum selection probabilities exceeding 50% when comparing all TB Case samples with their matched Control samples.

Table 3: Stability selection statistics for proteins with a maximum selection probability >50%.

Rank	Target	max(Pr{Selection})	Area
1)	SIRT2	0.858	0.10
2)	AMBN	0.795	0.10
3)	C5	0.777	0.11
4)	B3GN8	0.748	0.06
5)	LD78-beta	0.700	0.05
6)	MMP-1	0.683	0.05
7)	KI2LA	0.608	0.03
8)	PCD10	0.592	0.04
9)	IL-7	0.570	0.03
10)	CXCL16, soluble	0.538	0.05
11)	DR3	0.522	0.07

AMBN, C5, MMP-1, DR3, SIRT2, and C9 were also found to be in the top 25 proteins ranked by KS distance comparing all TB Case to all Control samples. Figure 38 shows the stability paths for all proteins in the upper panel and the regularization paths in the bottom. Stability paths are labeled by total area under the path in each figure, while tables are ranked by maximum selection probability.

Figure 39 shows CDFs for the top 6 proteins ranked by selection probability. Logistic regression was performed on standardized RFU values $|(X-\mu)/\sigma|$ where μ is the global mean, which are the units of the CDFs and can be interpreted as number of standard deviations from the mean. Although values $|(X-\mu)/\sigma|>4$ were replaced with simulated values from the 90th percentile of a simulated distribution, the CDF plots show the actual standardized scores without replacement. No proteins in the top 11 were observed to have values $|(X-\mu)/\sigma|>4$, suggesting outliers were indeed driving the selection of the proteins when not removed.

Example 3: Model Building

A subset of proteins for model building was first selected using the ranked lists from stability selection and univariate KS tests. From the stability selection list only proteins with a maximum selection probability >50% were included, and a KS distance of 0.4 was used as a threshold for the univariate KS list. These proteins were then selected against poor analytical performance by investigating the % coefficient of variation (CV) in a healthy

normal population and the overall signal quality/strength. The CDFs for each protein were also checked for abnormalities such as bimodal distributions or multiple outliers which have a lower probability of reproducing in the general population. Table 11 and Table 12 below show the ranked protein lists from stability selection and univariate KS with relevant performance measures.

Table 11: Ranked proteins by maximum probability of selection by stability selection with L1-regularized logistic regression with measures of signal strength and assay performance.

Rank	Target	Δ(medians)	max(Pr{Selection})	Dilution
1)	SIRT2	1892.40	0.858	40%
2)	AMBN	6459.60	0.795	0.005%
3)	C5	1394.30	0.777	0.005%
6)	MMP-1	398.50	0.683	1%
10)	CXCL16, soluble	939.70	0.538	1%
11)	DR3	6610.60	0.522	0.005%

Table 12: Ranked proteins by KS distance with measures of signal strength and assay performance.

Rank	Target	Δ(medians)	KS Distance	q-value	Dilution
2)	AMBN	6095.7	-0.454	9.74E-05	0.005%
3)	C5	1521.9	-0.447	9.74E-05	0.005%
4)	DR3	6006.5	-0.436	9.74E-05	0.005%
5)	MMP-1	431.4	-0.435	9.74E-05	1%
8)	Plasminogen	546.6	-0.419	9.74E-05	0.005%
10)	D-dimer	3553.5	-0.409	9.74E-05	0.005%
11)	GPHB5	3020.9	-0.409	9.74E-05	0.005%
12)	SG1C1	4511.4	-0.405	9.74E-05	0.005%
14)	2DMA	231.6	-0.402	9.74E-05	1%
15)	Coagulation Factor X	637.5	-0.401	9.74E-05	0.005%
17)	IRF6	814	0.397	9.74E-05	40%
18)	Factor I	1959.6	-0.394	9.74E-05	0.005%
19)	C9	3989.8	-0.39	9.74E-05	0.005%
20)	SIRT2	1675.8	0.387	9.74E-05	40%
21)	IP-10	380.2	-0.387	1.50E-04	40%
24)	DIAC	3233	-0.385	1.50E-04	1%

Protein SAP was excluded due to its implications in general inflammatory processes, making it a risk for false positives. A KS threshold of 0.4 was used; however, since several proteins with high biological significance and good analytical performance were found to have a KS distance within 0.015 of 0.4, ranked proteins 17-21 as well as 24 were included. This resulted in a final list of 17 proteins for model building, which are listed in Table 13 below. For each

of these proteins a single model cross-validation was performed. Figure 40 shows their univariate CDFs.

Table 43: Table of proteins used for model building

AMBN	DR3	GPHB5	C5	2DMA	SIRT2	Coagulation Factor X	MMP-1	DIAC
D-dimer	IP-10	C9	Factor I	IRF6	SG1C1	CXCL16, soluble	Plasminogen	

Repeated three-fold stratified cross validation was used to select the number of proteins in each model to balance of complexity (number of proteins) and performance (AUC, sensitivity+specificity, etc.). This process involved splitting the data into n subsets, or 'folds', and recursively generating a model from n-1 subsets of data and testing it on the other nth fold. Forward selection is a greedy procedure in that it selects the best single protein model first, then selects the best two protein model containing the single best protein. In contrast, backward selection finds the best n-1 protein model and removes the worst performing protein. This process was repeated until a specified maximum complexity was reached. 50-125 repeated runs of 3-fold validation were used to maintain ~19 case samples in each fold.

Forward and backward selection was repeated 10 times utilizing 10 different random number seeds. Figure 41 shows boxplots of model performance as a function of model size for forward and backward selection using one of the same seed. The dark dot indicates the highest median performance achieved, and the light dot indicates the lowest complexity model with equal performance within error, which would be the optimal model using this seed.

Figure 42 is a bar graph showing the frequencies each protein was included in the model from the 10 different seeds using forward and backward selection. Although the selection frequencies differ slightly, both methods repeatedly selected the same 8 protein model.

A Naïve Bayes model was fit to all 57 TB cases with 145 controls matched according to bin and study day. Performance was then quantified using all data, which includes an additional 52 controls which were not matched to any of the TB cases and therefore represent a single-class 'test set'. Figure 43 shows the overall performance of the model on the training and single class test set. The left panel shows the ROC with the operating point and bootstrap 95% confidence bounds. The model correctly classified 42/52 of the 52 hold out controls that were not used for model fitting (AUC from the data set without these controls was 0.88). The right panel shows the log-odds for all subjects colored by diagnosis; the blue dots are control

samples and the red dots are TB samples. Hollow data points indicate a misclassification based on the operating point.

Due to the longitudinal design of some experiments conducted during development of embodiments described herein, model performance as a function of time is an important factor. The odds of developing active TB should increase as the time of diagnosis (here start of treatment) draws nearer. Figure 44 shows a control band plot of the odds of developing active TB as a function of time to treatment initiation. Responsiveness was quantified by first fitting a locally weighted least squares (LWLS) estimate to the log odds of all TB cases as a function of time. These functions fit the estimated mean of the data and are represented as the bold dotted line in Figure 44 left. The area between the dotted line and the top of the light gray region of the control data in was used as a responsiveness index. The area within 200 days of diagnosis was weighted twice to select for models which show large increases in log-odds of the TB cases within 6 months of diagnosis. The responsiveness indices were normalized to the maximum area observed for any model.

Figure 45 shows a boxplot of the log-odds from another TB-diagnostic 9-marker model ("HR9") re-fit to the training data, and the 8 marker model described herein, on samples binned by intervals of 180 day intervals. Time moves to the right. Although the median log odds for the control population is higher for the 8-marker model compared to HR9, the range of the scores is smaller. The HR9 log-odds only became positive within 180 days of treatment. In contrast, the log-odds for the 8-marker model are positive 540+ days from treatment.

A Kruskall-Wallis test with Tukey's HSD (honest significant difference) for individual comparisons was used to determine which time points were different in the context of the overall error generated by the model predictions. Only the controls were found to be significantly different from the others time points for the 8 protein model ($p<1e-4$), with all TB case time points not being different from one another ($p>0.05$). In the HR9 model, the control population was not different from the 540+, 360-540 or 180-360 time points ($p>0.05$), but was significantly different from the 0-180 time point ($p=2.2e-5$). Table 14 shows univariate KS statistics for the proteins in the 8 marker model.

Table 14: Univariate KS statistics for all proteins in the 8 marker model

KS Rank	Target	Feature	Signed KS	p_{emp}	p-value	q-value
2	AMBN	AMBN.6522.57.3	-0.454	6.60E-07	1.15E-08	1.02E-04
3	C5	C5.2381.52.4	-0.447	6.60E-07	1.97E-08	1.02E-04
5	MMP-1	MMP1.4924.32.1	-0.435	6.60E-07	5.21E-08	1.02E-04
10	D-dimer	FGA.FGB.FGG.4907.56.1	-0.409	6.60E-07	4.19E-07	1.02E-04

12	SG1C1	SCGB1C1.5960.49.3	-0.405	6.60E-07	5.69E-07	1.02E-04
14	2DMA	HLA.DMA.10639.1.3	-0.402	6.60E-07	7.03E-07	1.02E-04
21	IP-10	CXCL10.4141.79.1	-0.387	1.30E-06	2.11E-06	1.62E-04
153	CXCL16, soluble	CXCL16.2436.49.4	-0.304	3.60E-04	4.04E-04	6.97E-03

The correlation matrix in the left panel of Figure 46 shows Spearman correlations of all samples for the 17 proteins that entered model building with the proteins in the 8-marker model highlighted. The ordering is based on a greedy seriation procedure to identify correlation structure. Two clusters are evident with Spearman $\rho>0.6$; a 3 protein cluster composed of C5, C9 and Factor I, as well as a 6 protein cluster of 2DMA, DR3, DIAC, SG1C1, AMBN and GPHB5 (scatter plot is shown in Figure 47).

The only HR9 marker selected for model building was C9. Although it was not selected for the 8 protein model, linear regression and a Cox Model found it to have a high level of responsiveness to time. C9 was added to the 8-marker model and the resulting model's performance is shown in Figure 48. Although it does not seem to affect overall performance, adding C9 decreases the log-odds of developing TB in the control samples and increases the log odds in the TB samples.

Based on this small increase in performance, a model was refit to the training data using the 8 protein model plus C9. Figure 49 shows performance plots for the 9-protein predictive model. The overall AUC of the model is the same as the 9-protein predictive model (AUC=0.87). The 9-protein predictive model misclassifying 11/52 compared to 10/52 for the 8-protein model without C9.

Figure 50 shows responsiveness plots for the 9-protein predictive model. The addition of C9 to the 8-protein model increased responsiveness by ~10%.

The 8-protein model was then compared against HR9, which Figure 51 shows. A Kruskal-Wallis test again found all pre-treatment time points to be different from the Control log-odds, but no difference was found between the individual time points for TB cases. Although the IQR of the 180-360 day time point appears to become more negative, this corresponds to the reduction in log-odds for a single data point.

Example 4: Additional Model Building

A further subset of proteins for model building was selected using the ranked lists from stability selection and univariate KS tests. From the stability selection list only proteins with a maximum selection probability >50% were included, and a KS distance of 0.4 was

used as a threshold for the univariate KS list. These proteins were then selected against poor analytical performance by investigating the % coefficient of variation (CV) in a healthy normal population and the overall signal quality/strength. The CDFs for each protein were also checked for abnormalities such as bimodal distributions or multiple outliers which have a lower probability of reproducing in the general population. Table 15 and Table 16 below show the ranked protein lists from stability selection and univariate KS with relevant performance measures.

Table 15: Ranked proteins by maximum probability of selection by stability selection with L1-regularized logistic along with shift in median signal level between groups and sample dilution for associated SOMAmer.

Rank	Target	Δ(medians)	max(Pr{Selection})	Dilution
1)	SIRT2	1892.40	0.858	40%
2)	AMBN	6459.60	0.795	0.005%
3)	C5	1394.30	0.777	0.005%
6)	MMP-1	398.50	0.683	1%
10)	CXCL16, soluble	939.70	0.538	1%
11)	DR3	6610.60	0.522	0.005%
13)	KCNE2	1074.90	0.462	40%

Table 16: Ranked proteins by KS distance with measures of signal strength and assay performance.

Rank	Target	Δ(medians)	KS Distance	q-value	Dilution
2)	AMBN	6095.7	-0.454	9.74E-05	0.005%
3)	C5	1521.9	-0.447	9.74E-05	0.005%
4)	DR3	6006.5	-0.436	9.74E-05	0.005%
5)	MMP-1	431.4	-0.435	9.74E-05	1%
8)	Plasminogen	546.6	-0.419	9.74E-05	0.005%
10)	D-dimer	3553.5	-0.409	9.74E-05	0.005%
11)	GPHB5	3020.9	-0.409	9.74E-05	0.005%
12)	SG1C1	4511.4	-0.405	9.74E-05	0.005%
14)	2DMA	231.6	-0.402	9.74E-05	1%
15)	Coagulation Factor X	637.5	-0.401	9.74E-05	0.005%
17)	IRF6	814	0.397	9.74E-05	40%
18)	Factor I	1959.6	-0.394	9.74E-05	0.005%
19)	C9	3989.8	-0.39	9.74E-05	0.005%
20)	SIRT2	1675.8	0.387	9.74E-05	40%
21)	IP-10	380.2	-0.387	1.50E-04	40%
24)	DIAC	3233	-0.385	1.50E-04	1%

As before, protein SAP was excluded due to its implications in general inflammatory processes, making it a risk for false positives. A KS threshold of 0.4 was used; however, since several proteins with high biological significance and good analytical performance were

found to have a KS distance within 0.015 of 0.4, ranked proteins 17-21 as well as 24 were included. This resulted in a final list of 18 proteins for model building, which are listed in Table 17 below. For each of these proteins a single model cross-validation was performed, as discussed above and shown in Figure 40. Figure 55 shows the univariate CDF for protein KCNE2. Table 17 shows the names for all proteins included in model building, and Figures 40 and 55 shows their univariate CDFs.

Table 57: Table of proteins used for model building

AMBN	DR3	GPHB5	C5	2DMA	SIRT2	Coagulation Factor X	MMP-1	DIAC
D-dimer	IP-10	C9	Factor I	IRF6	SG1C1	CXCL16, soluble	Plasminogen	KCNE2

Repeated three-fold stratified cross validation was used to select the number of proteins in each model to balance of complexity (number of proteins) and performance (AUC, sensitivity+specificity, etc.). This process involved splitting the data into n subsets, or 'folds', and recursively generating a model from n-1 subsets of data and testing it on the other nth fold. Forward selection is a greedy procedure in that it selects the best single protein model first, then selects the best two protein model containing the single best protein. In contrast, backward selection finds the best n-1 protein model and removes the worst performing protein. This process was repeated until a specified maximum complexity was reached. 50-125 repeated runs of 3-fold validation were used to maintain ~19 case samples in each fold.

Forward and backward selection was repeated 10 times utilizing 10 different random number seeds. Figure 56 shows boxplots of model performance as a function of model size for forward and backward selection using one of the same seed. The dark dot indicates the highest median performance achieved, and the light dot indicates the lowest complexity model with equal performance within error, which would be the optimal model using this seed.

Figure 57 is a bar graph showing the frequencies each protein was included in the model from the 10 different seeds using forward and backward selection. Although the selection frequencies differ slightly, both methods repeatedly selected the same 8 protein model.

A Naïve Bayes model using the 8 proteins repeatedly selected was fit to all 57 TB cases with 145 controls matched according to bin and study day. Performance was then quantified using all data, which includes an additional 52 controls which were not matched to any of the TB cases and therefore represent a single-class 'test set'. Figure 58 shows the

overall performance of the model on the training and single class test set. The left panel shows the ROC with the operating point and bootstrap 95% confidence bounds. The model correctly classified 42/52 of the 52 hold out controls that were not used for model fitting (AUC from the data set without these controls was 0.88). The right panel shows the log-odds for all subjects colored by diagnosis; the blue dots are control samples and the red dots are TB samples. Hollow data points indicate a misclassification based on the operating point.

Due to the longitudinal design of some experiments conducted during development of embodiments described herein, model performance as a function of time is an important factor. The odds of developing active TB should increase as the time of diagnosis (here start of treatment) draws nearer. To quantify the "responsiveness" to proximity of treatment initiation the area under the "average" response as a function of time was estimated using an increased weight for the ~6 month interval immediately preceeding the time of treatment initiation.

Figure 59 (left frame) shows a control band plot of the odds of developing active TB as a function of time to treatment initiation. The bold dotted line is a locally weighted least squares (LWLS) estimate of logOdds as function of time for TB cases. The area between between the dotted line and the top of the light gray region of the control data in was used as a responsiveness index with the area within 200 days of treatment activation weighted twice to select for models which show large increases in log-odds of the TB cases within 6 months of diagnosis. The subsequent weighted area was normalized to the maximum area observed for any model to create a "responsiveness index". Figure 59 (right frame) shows the responsive index of each model versus performance (AUC) for models of increasing complexity. Model performance saturates at D-dimer, but substantial gains in responsiveness are observed with the additions of IP-10, MMP-1 and SG1C1.

Figure 60 shows a boxplot of the log-odds from another TB-diagnostic 9-marker model ("HR9") re-fit to the training data, and the 8 marker model shown in Figure 59, on samples binned by intervals of 180 day intervals. Time moves to the right. Although the median log odds for the control population is higher for TBR8 compared to HR9, the robust range (region between the whiskers) of the scores is smaller. The HR9 log-odds only become positive within 180 days of treatment and then decrease again after treatment in response to changes in "host response" associated with the development and resolution of active disease. In contrast, the log-odds for the TBR9 model are positive 540+ days from treatment, although they appear to oscillate somewhat with a marked decrease at the 180-360 day time point.

A Kruskall-Wallis test with Tukey's HSD (honest significant difference) for individual comparisons was used to determine which time points were different in the context of the overall error generated by the model predictions. Only the controls were found to be significantly different from the others time points for the 8 protein model ($p<1e-4$), with all TB case time points not being different from one another ($p>0.05$). In the HR9 model, the control population was not different from the 540+, 360-540 or 180-360 time points ($p>0.05$), but was significantly different from the 0-180 time point ($p=2.2e-5$). Table 18 shows univariate KS statistics for the proteins in the 8 marker model.

Table 18: Univariate KS statistics for all proteins in the 8 marker model

KS Rank	Target	Feature	Signed KS	p_{emp}	p-value	q-value
2	AMBN	AMBN.6522.57.3	-0.454	6.60E-07	1.15E-08	1.02E-04
3	C5	C5.2381.52.4	-0.447	6.60E-07	1.97E-08	1.02E-04
5	MMP-1	MMP1.4924.32.1	-0.435	6.60E-07	5.21E-08	1.02E-04
10	D-dimer	FGA.FGB.FGG.4907.56.1	-0.409	6.60E-07	4.19E-07	1.02E-04
12	SG1C1	SCGB1C1.5960.49.3	-0.405	6.60E-07	5.69E-07	1.02E-04
14	2DMA	HLA.DMA.10639.1.3	-0.402	6.60E-07	7.03E-07	1.02E-04
27	KCNE2	KCNE2.10427.2.3	-0.381	3.24E-06	5.90E-06	6.50E-04
153	CXCL16, soluble	CXCL16.2436.49.4	-0.304	3.60E-04	4.04E-04	6.97E-03

The correlation matrix in the left panel of Figure 61 shows Spearman correlations of all samples for the 18 proteins that entered model building with the proteins in the 8-marker model highlighted. The ordering is based on a greedy seriation procedure to identify correlation structure. Two clusters are evident with Spearman $\rho>0.6$; a 3 protein cluster composed of C5, C9 and Factor I, as well as a 6 protein cluster of 2DMA, DR3, DIAC, SG1C1, AMBN and GPHB5 (scatter plots are shown in Figure 62).

Example 5: Substitution of D-dimer in 8 Protein Model

Of the 8 proteins in the Naïve Bayes model discussed in Example 4, D-dimer was observed to have poor concordance between serum and plasma. This may be due to D-dimer being part of the clotting cascade and therefore not being present in serum.

C9 was found to have the highest Spearman correlation with D-dimer, and was therefore evaluated as a replacement marker. Although it was not selected for the 8 protein model, linear regression and a Cox Model found C9 to have a high level of responsiveness to time and to be a robust "host-response" marker.

A Naïve Bayes model was fit for the TBR8 model (the model described in Example 4, which includes KCNE2), and then for a separate 8 protein model in which D-dimer was replaced by C9. Figure 63 shows the change in log-odds for each model.

Figure 64 shows performance plots for the 7 protein model + C9. The AUC is the same compared to TBR8 with mildly larger confidence intervals.

Example 6: Cross-Validation

The performance estimates from the ROC curve in Figure 64 were obtained by bootstrapping, or repeatedly sampling the training population and estimating the class labels without re-fitting the model. A more conservative estimate of the performance of the model is to take bias in the model fit into account through cross-validation. A cross-validated performance estimate was generated by fitting the model to n-1 folds and predicting on the nth fold.

By using the training data to select features and estimate model performance, it is possible to have a selection bias which inflates the performance estimates compared to the true values. In some instances, this bias may be overcome by adding an external cross validation loop to the feature selection/model building process, where the non-biased performance of the model can be quantified on the hold-out fold of the data. Given n folds, the feature selection/model build are run n times giving n models. None of these models are used in the end; the final model is obtained by running the entire feature selection/build on all of the data (as shown, e.g., in Example 4), using the double cross validated performance estimates.

Cross validation may be considered to assess the performance of a procedure for fitting a model, rather than the final model itself. If the feature set varies greatly from one fold of the cross validation to another, it is an indication that the selection strategy is unstable.

The entire feature selection and model building process was put into an external 5-fold validation loop. Each fold was balanced for diagnosis and Progressors were stratified by time to treatment. Univariate KS distance and L_1-regularized logistic regression using Lasso were used to select a subset of features for the model building procedure. Features were excluded if they had a calibrator CV >15% or a median class difference <300 RFU.

Five runs of forward and backward selection were the run on the resulting feature list using 3-fold cross validation and a unique random number seed for each run. Features with inclusion probabilities >50% were then included in the final model for that particular fold, and model coefficients were fit using the training data. Performance estimates were then

generated by applying the resulting model to the test fold. This process was repeated 18 times for a total of 90 optimal models.

Figure 65 shows the frequency that each protein was included in the optimal model, with TBR8 proteins highlighted in blue. Seven of the TBR8 proteins are within the top 10 most selected features, with SG1C1 being selected less frequently. Within the double cross validation folds, these 7 TBR8 proteins were chosen in >30% of the optimal models, indicating very reasonable stability in the model building process.

Figure 66 shows a histogram of AUC estimates on the test set for each of the 90 optimal models generated. The average performance estimate was 0.8 with a standard deviation of 0.08 and a 95% confidence interval of [0.63 0.94].

Example 7: Four and Five Protein Models

The foregoing embodiments and examples are intended only as examples. No particular embodiment, example, or element of a particular embodiment or example is to be construed as a critical, required, or essential element or feature of any of the claims. Various alterations, modifications, substitutions, and other variations can be made to the disclosed embodiments without departing from the scope of the present application, which is defined by the appended claims. The specification, including the figures and examples, is to be regarded in an illustrative manner, rather than a restrictive one, and all such modifications and substitutions are intended to be included within the scope of the application. Steps recited in any of the method or process claims may be executed in any feasible order and are not limited to an order presented in any of the embodiments, the examples, or the claims. Further, in any of the aforementioned methods, one or more specifically listed biomarkers can be specifically excluded either as an individual biomarker or as a biomarker from any panel.

REFERENCES

The following references are herein incorporated by reference in their entireties.

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CLAIMS

1. A method of determining a likelihood of a latent tuberculosis (TB) infection in a subject transitioning to active TB disease, comprising detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble) in a sample from the subject, wherein the subject is identified as having a latent TB infection that is likely to transition into active TB disease if the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, or eight of the biomarkers is higher than a control level of the respective biomarker.

2. The method of claim 1, further comprising detecting the level of one or more biomarkers that are indicative of one or more of: the presence of latent TB infection, the presence of active TB disease, the strain of TB, the antibiotic resistance/sensitivity of TB, and/or the presence of other diseases.

3. The method of one of claims 1 or 2, comprising detecting the levels of 2 to 20 biomarkers, or 2 to 10 biomarkers, or 2 to 9 biomarkers, or 3 to 20 biomarkers, or 3 to 10 biomarkers, or 3 to 9 biomarkers, or 4 to 20 biomarkers, or 4 to 10 biomarkers, or 4 to 9 biomarkers, or 5 to 20 biomarkers, or 5 to 10 biomarkers, or 5 to 9 biomarkers.

4. A method of determining a likelihood of a latent TB infection in a subject transitioning to active TB disease, comprising detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or ten biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the subject, wherein the subject is identified as being likely to transition to active TB disease if the level of at least one, at least two ,at least three, at least four, at least five, at least six, at least seven, at least eight, or nine of the biomarkers is higher than a control level of the respective biomarker.

5. The method of claim 4, further comprising detecting the level of one or more biomarkers that are indicative of one or more of: the presence of latent TB infection, the presence of active TB disease, the strain of TB, the antibiotic resistance/sensitivity of TB, and/or the presence of other diseases.

6. The method of one of claims 4 or 5, comprising detecting the levels of 2 to 20 biomarkers, or 2 to 10 biomarkers, or 2 to 9 biomarkers, or 3 to 20 biomarkers, or 3 to 10 biomarkers, or 3 to 9 biomarkers, or 4 to 20 biomarkers, or 4 to 10 biomarkers, or 4 to 9 biomarkers, or 5 to 20 biomarkers, or 5 to 10 biomarkers, or 5 to 9 biomarkers.

7. The method of any one of the preceding claims, wherein the subject is identified as being likely to transition to active TB disease within 30 days, 45 days, 60 days, 90 days, 120 days, 180 days, 270 days, 360 days, 450 days, or 540 days if the level of at least one, at least two ,at least three, at least four, at least five, at least six, at least seven, at least eight, or nine of the biomarkers is higher than a control level of the respective biomarker.

8. A method of monitoring a latent TB infection in a subject for the likelihood of the latent TB infection transitioning to active TB disease, comprising detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble) in a sample from the patient at a first time point, and measuring the level of the at least one, at least two, at least three, at least four, at least five, at least six, at least seven, or eight biomarkers at a second time point, wherein if the level of the biomarkers is higher at the second time point than at the first time point, the likelihood of the latent TB infection transitioning to active TB disease has increased.

9. A method of monitoring a latent TB infection in a subject for the likelihood of the latent TB infection transitioning to active TB disease, comprising detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or ten biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the patient at a first time point, and measuring the level of the at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine biomarkers at a second time point, wherein if the level of the biomarkers is higher at the second time point than the first time point, the likelihood of the latent TB infection transitioning to active TB disease has increased.

10. The method of claim 8 or claim 9, wherein if the level of the biomarkers lower at the second time point than at the first time point, the likelihood of the latent TB infection transitioning to active TB disease has decreased.

11. A method of monitoring treatment of a latent TB infection, comprising detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble) in a sample from the patient at a first time point, administering at least one treatment for TB infection to the patient, and detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine biomarkers selected from AMBN, C5, MMP-1, D-

dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble) in a sample from the patient at a second time point, wherein the treatment is effective at reducing the likelihood of the latent TB infection transitioning to active TB disease if the level of the biomarkers is lower at the second time point compared to the first time point.

12. A method of monitoring treatment of a latent TB infection, comprising detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or ten biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the patient at a first time point, administering at least one treatment for TB infection to the patient, and detecting the level of at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least 9, or ten biomarkers selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9 in a sample from the patient at a second time point, wherein the treatment is effective at reducing the likelihood of the latent TB infection transitioning to active TB disease if the level of the biomarkers is lower at the second time point compared to the first time point.

13. The method of any one of claims 10 to 12, wherein at least one treatment for TB infection is selected from the group consisting of isoniazid (INH), rifampin (RIF), rifapentine (RPT), ethambutol (EMB), pyrazinamide (PZA), and/or another approved TB therapeutic to the subject.

14. The method of any one of the preceding claims, wherein the method further comprises performing one or more additional tests for TB infection.

15. The method of claim 14, wherein said one or more additional tests for TB infection comprises chest x-ray.

16. The method of any one of the preceding claims, wherein each biomarker is a protein biomarker.

17. The method of any one of the preceding claims, wherein the method comprises contacting biomarkers of the sample from the subject or patient with a set of biomarker capture reagents, wherein each biomarker capture reagent of the set of biomarker capture reagents specifically binds to a different biomarker being detected.

18. The method of claim 17, wherein each biomarker capture reagent is an antibody or an aptamer.

19. The method of claim 18, wherein each biomarker capture reagent is an aptamer.

20. The method of claim 19, wherein at least one aptamer is a slow off-rate aptamer.

21. The method of claim 20, wherein at least one slow off-rate aptamer comprises at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least 10 nucleotides with modifications.

22. The method of claim 20 or claim 21, wherein each slow off-rate aptamer binds to its target protein with an off rate ($t_{½}$) of ≥ 30 minutes, ≥ 60 minutes, ≥ 90 minutes, ≥ 120 minutes, ≥ 150 minutes, ≥ 180 minutes, ≥ 210 minutes, or ≥ 240 minutes.

23. The method of any one of the preceding claims, wherein the sample is a blood sample.

24. The method of claim 23, wherein the sample is a plasma sample or a serum sample.

25. The method of any one of the preceding claims, wherein the method comprises detecting the levels of a set of biomarkers selected from:

a) AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, and CXCL16 (soluble);

b) AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, CXCL16 (soluble), and C9;

c) AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, KCNE2, and CXCL16 (soluble);

d) AMBN, C5, MMP-1, SG1C1, 2DMA, IP-10, CXCL16 (soluble), and C9; and

e) AMBN, C5, MMP-1, SG1C1, 2DMA, KCNE2, CXCL16 (soluble), and C9.

26. A kit comprising at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or ten aptamers, wherein each aptamer specifically binds to a target protein selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9.

27. A kit comprising at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine aptamers, wherein each aptamer specifically binds to a target protein selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble).

28. The kit of claim 26 or claim 27, wherein the kit comprises a total of 2 to 20 aptamers, or 2 to 10 aptamers, or 2 to 9 aptamers, or 3 to 20 aptamers, or 3 to 10 aptamers, or 3 to 9 aptamers, or 4 to 20 aptamers, or 4 to 10 aptamers, or 4 to 9 aptamers, or 5 to 20 aptamers, or 5 to 10 aptamers, or 5 to 9 aptamers.

29. A composition comprising proteins of a sample from a subject and at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or ten aptamers, wherein each aptamer specifically binds to a target protein selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, CXCL16 (soluble), and C9.

30. A composition comprising proteins of a sample from a subject and at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, or nine aptamers, wherein each aptamer specifically binds to a target protein selected from AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, KCNE2, and CXCL16 (soluble).

31. The composition of claim 29 or claim 30, wherein each aptamer specifically binds to a different target protein.

32. The composition of any one of claims 29 to 31, wherein the sample is a blood sample.

33. The composition of any one of claims 29 to 31, wherein the sample is a serum sample.

34. The kit or composition of any one of claims 26 to 33, wherein at least one aptamer is a slow off-rate aptamer.

35. The kit or composition of claim 34, wherein each aptamer is a slow off-rate aptamer.

36. The kit or composition of claim 34 or claim 35, wherein at least one slow off-rate aptamer comprises at least one, at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least 10 nucleotides with hydrophobic modifications.

37. The kit or composition of any one of claims 34 to 36, wherein each slow off-rate aptamer binds to its target protein with an off rate ($t_{½}$) of $\geq$ 30 minutes, $\geq$ 60 minutes, $\geq$ 90 minutes, $\geq$ 120 minutes, $\geq$ 150 minutes, $\geq$ 180 minutes, $\geq$ 210 minutes, or $\geq$ 240 minutes.

38. The kit or composition of any one of claims 26 to 37, comprising a set of aptamers, wherein each aptamer specifically binds to a target protein of a set of target proteins selected from:

a) AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, and CXCL16 (soluble);

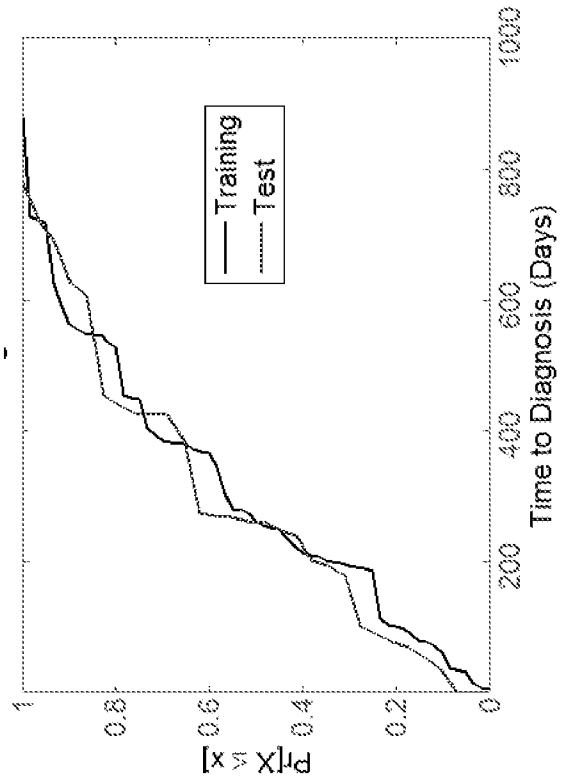
b) AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, IP-10, CXCL16 (soluble), and C9;

c) AMBN, C5, MMP-1, D-dimer, SG1C1, 2DMA, KCNE2, and CXCL16 (soluble);

d) AMBN, C5, MMP-1, SG1C1, 2DMA, IP-10, CXCL16 (soluble), and C9; and

e) AMBN, C5, MMP-1, SG1C1, 2DMA, KCNE2, CXCL16 (soluble), and C9.

FIG. 1


Training
Test
Time to Diagnosis (Days)
Pr[X ≤ x]
0
0.2
0.4
0.6
0.8
1
200
400
600
800
1000

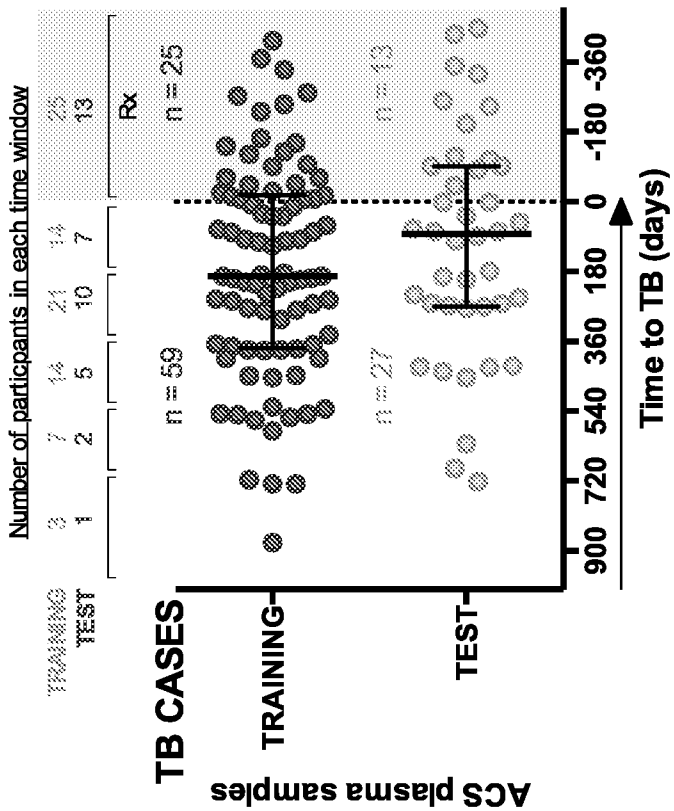

ACS plasma samples
TB CASES
TRAINING
TEST
Number of participants in each time window
TRAINING
TEST
n = 59
n = 27
n = 25
n = 13
Rx
Time to TB (days)
900 720 540 360 180 0 -180 -360

FIG. 2

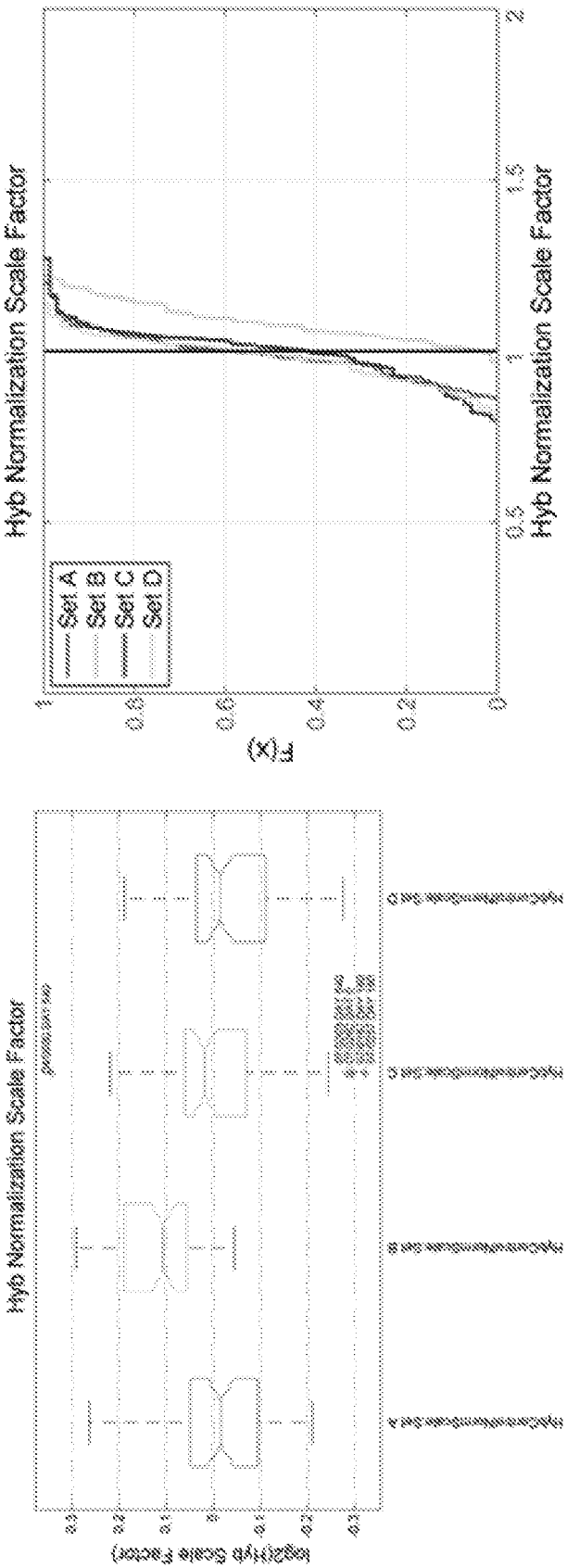

Hyb Normalization Scale Factor
Set A
Set B
Set C
Set D
F(x)
Hyb Normalization Scale Factor
Hyb Normalization Scale Factor
log2(Hyb Scale Factor)

FIG 3.

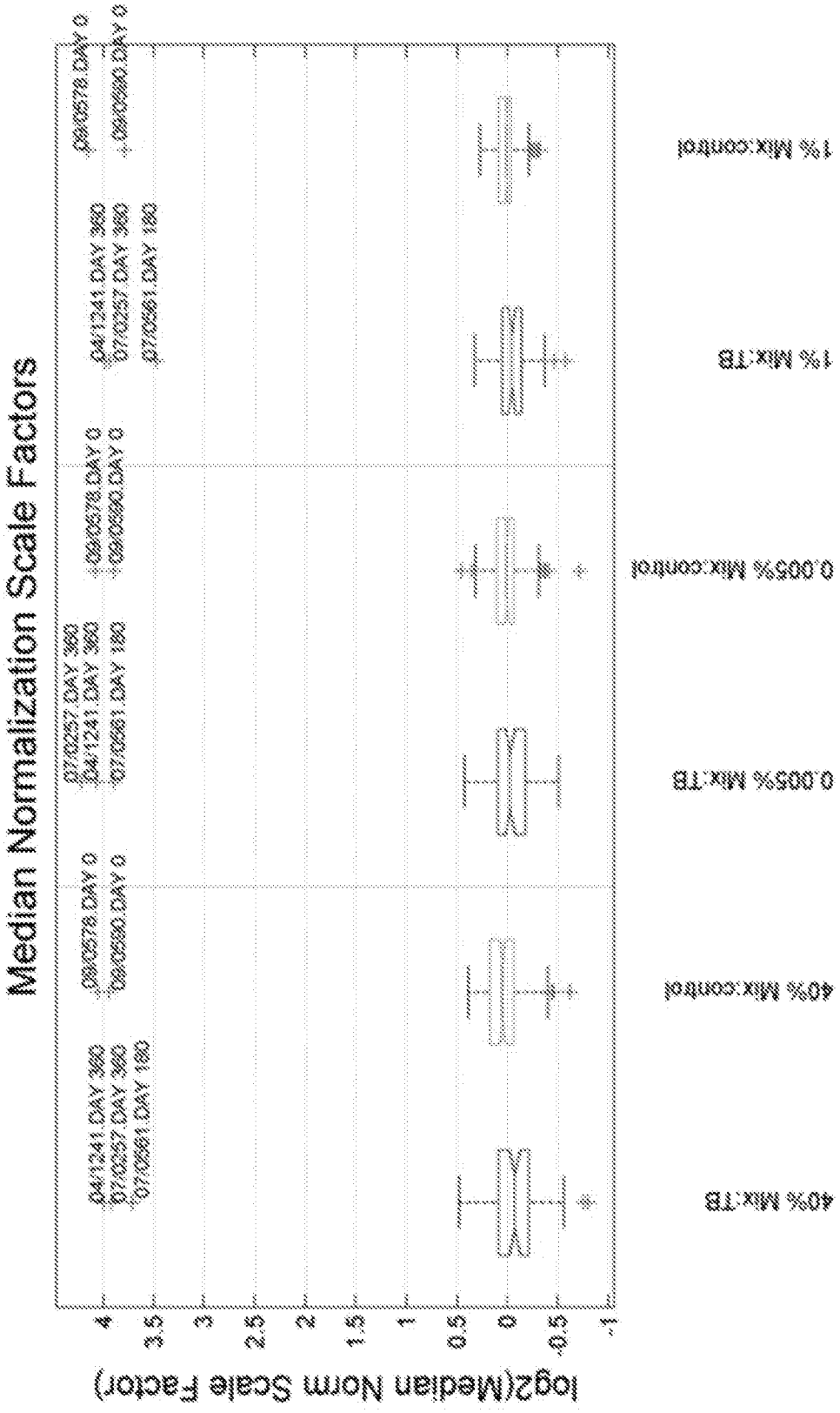

Median Normalization Scale Factors
log2(Median Norm Scale Factor)
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
-1
07/0241.DAY 360
07/0257.DAY 360
07/0561.DAY 180
09/0578.DAY 0
09/0590.DAY 0
07/0257.DAY 360
04/1241.DAY 360
07/0561.DAY 180
09/0578.DAY 0
09/0590.DAY 0
04/1241.DAY 360
07/0257.DAY 360
07/0561.DAY 180
09/0578.DAY 0
09/0590.DAY 0
40% Mix:TB
40% Mix:control
0.005% Mix:TB
0.005% Mix:control
1% Mix:TB
1% Mix:control

FIG. 4

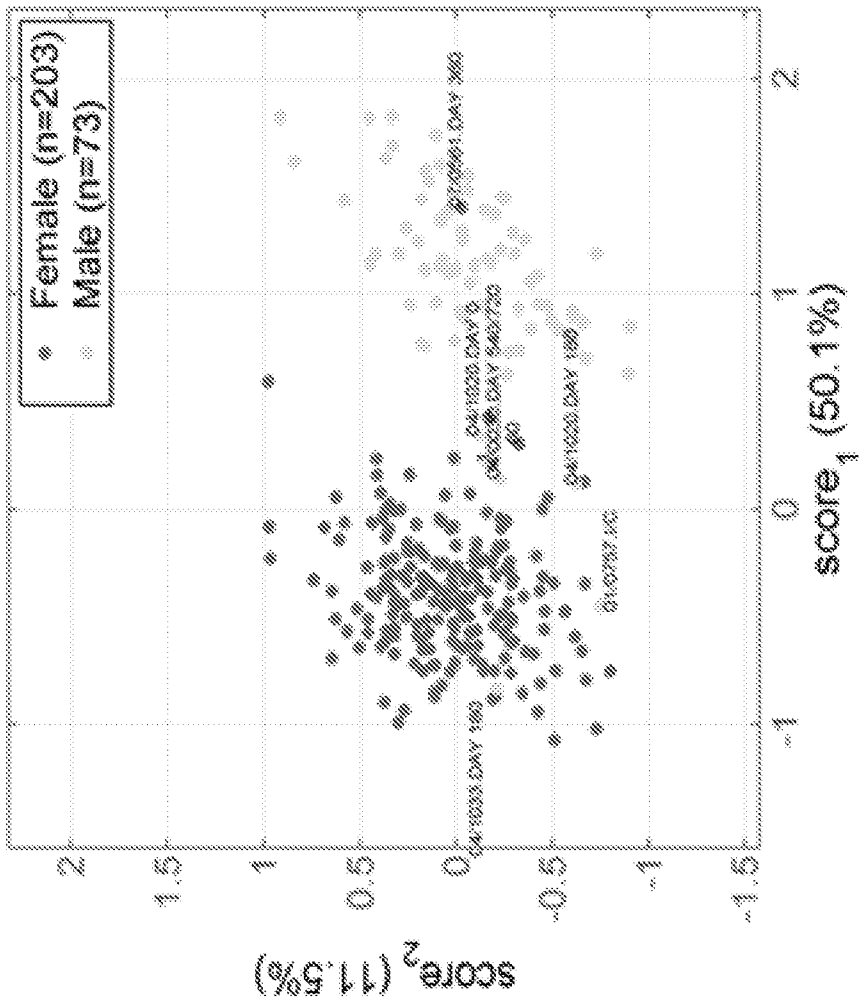
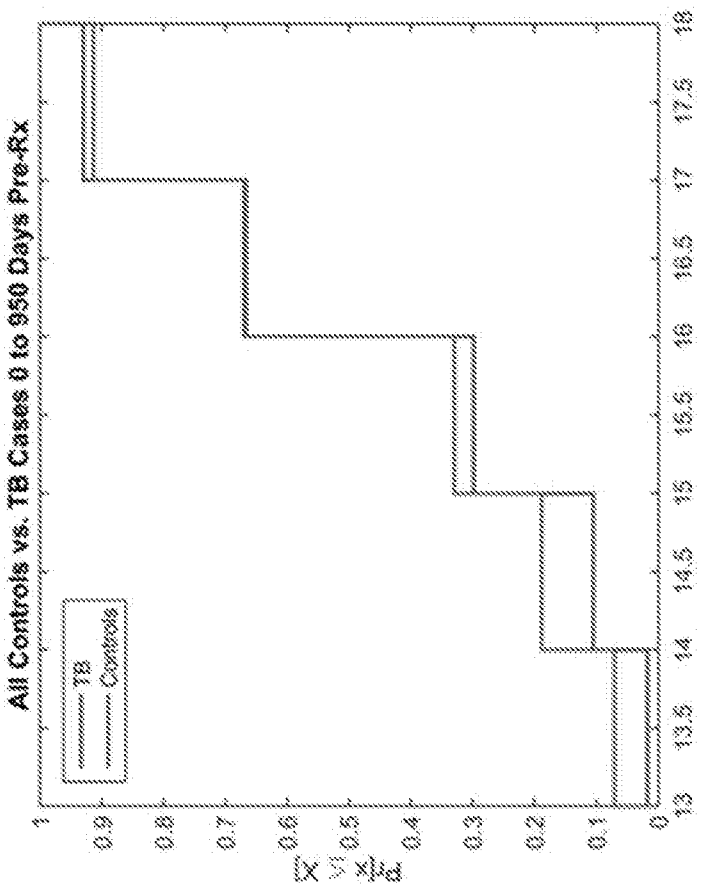

Female (n=203)
Male (n=73)
score2 (11.5%)
score1 (50.1%)
2
1.5
1
0.5
0
-0.5
-1
-1.5
-1
0
1
2

FIG. 5



	Samples	Males	Females	Pregnant (?)
Cases	57	12 (21%)	45 (79%)	4 (7%)
Controls	197	54 (27%)	153 (78%)	8 (4%)

FIG. 6

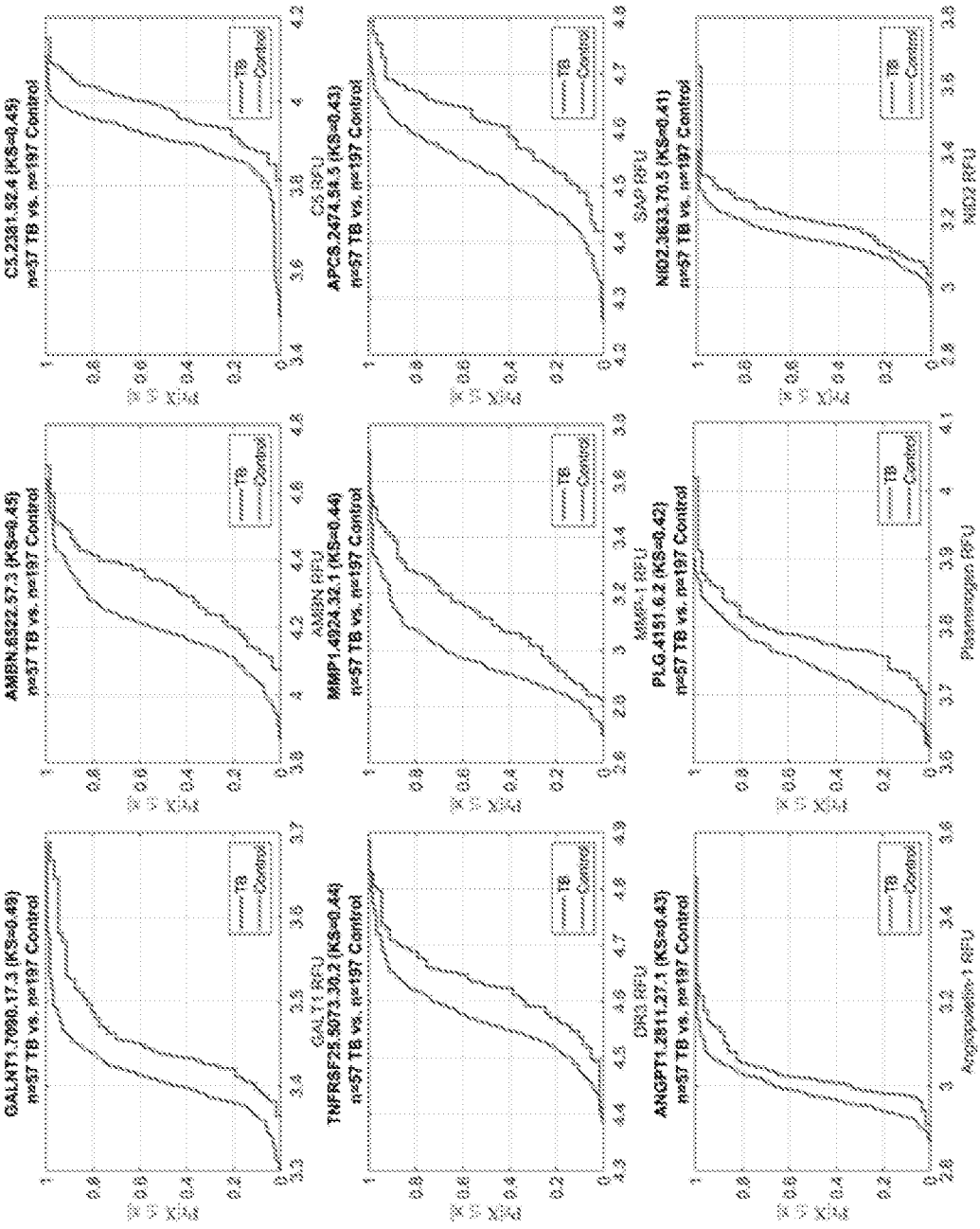

C5.2381.52.4 (KS=0.45)
n=57 TB vs. n=197 Control
AMBN.6522.57.3 (KS=0.45)
n=57 TB vs. n=197 Control
APCS.2474.84.5 (KS=0.43)
n=57 TB vs. n=197 Control
MB2.3833.79.5 (KS=0.41)
n=57 TB vs. n=197 Control
GALNT1.7090.17.3 (KS=0.45)
n=57 TB vs. n=197 Control
MMP1.4924.32.1 (KS=0.44)
n=57 TB vs. n=197 Control
PLG.4151.6.2 (KS=0.42)
n=57 TB vs. n=197 Control
TNFRSF25.5073.30.2 (KS=0.44)
n=57 TB vs. n=197 Control
ANGPT1.2811.27.1 (KS=0.43)
n=57 TB vs. n=197 Control
TB
Control
Percentage
C5 RFU
AMBN RFU
SAP RFU
MB2 RFU
MMP1 RFU
Plasminogen RFU
GALNT1 RFU
DR3 RFU
Angiopoietin-1 RFU

FIG. 7

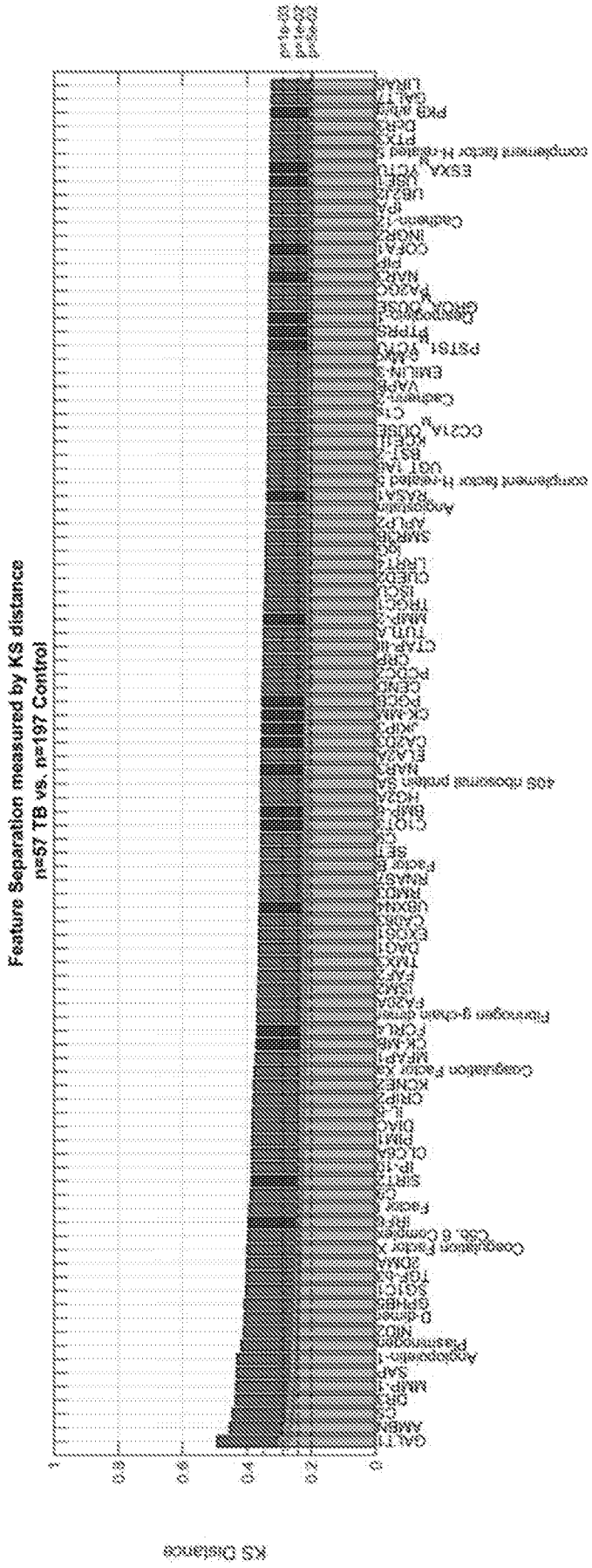

Feature Separation measured by KS distance
n=57 TB vs. n=197 Control
KS Distance
1
0.8
0.6
0.4
0.2
0
complement factor H-related protein
complement factor H-related protein
40S ribosomal protein
Coagulation
Fibrinogen

FIG. 8

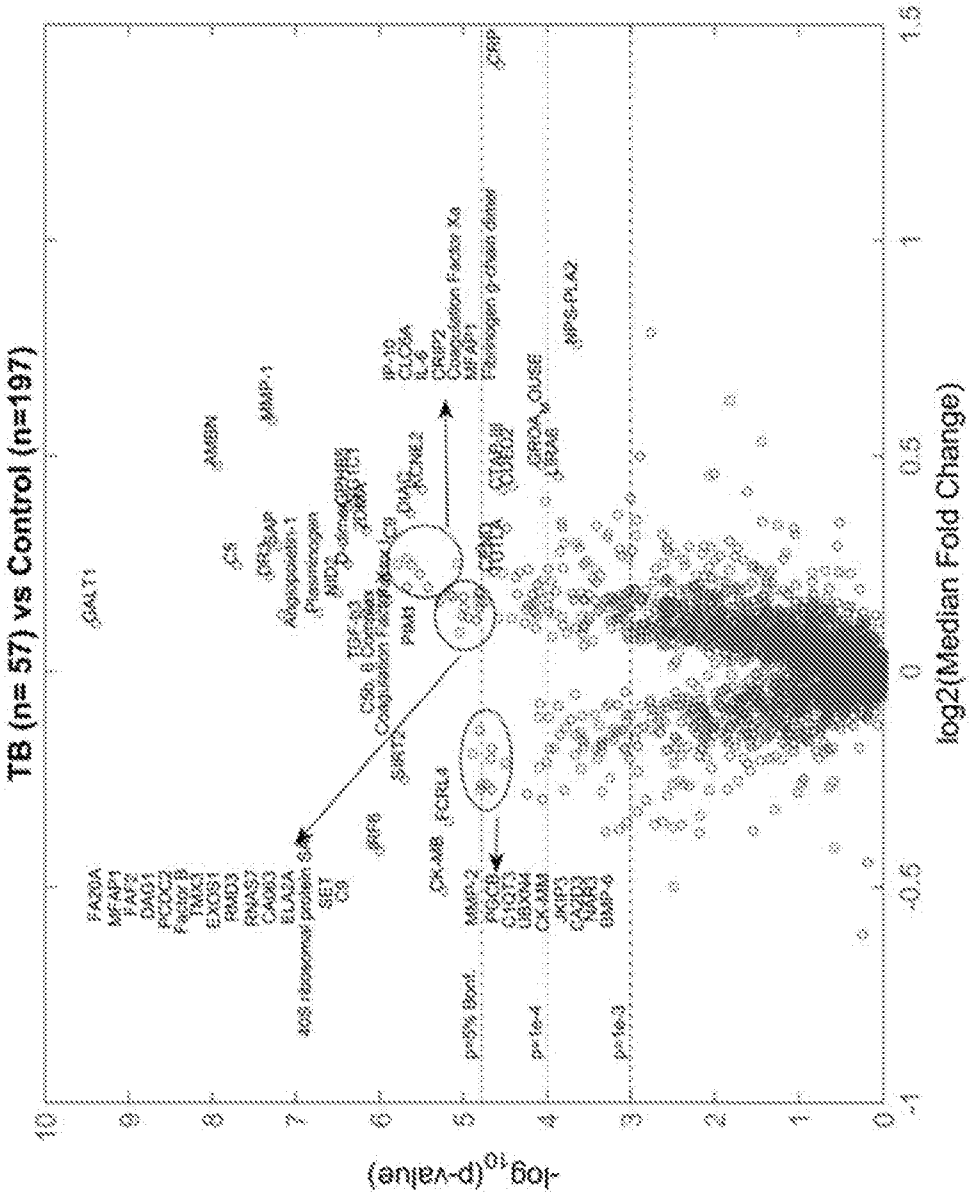

TB (n= 57) vs Control (n=197)
-log10(p-value)
log2(Median Fold Change)
10
9
8
7
6
5
4
3
2
1
0
1.5
1
0.5
0
-0.5
-1
p=1e-4
p=1e-3

FIG. 9

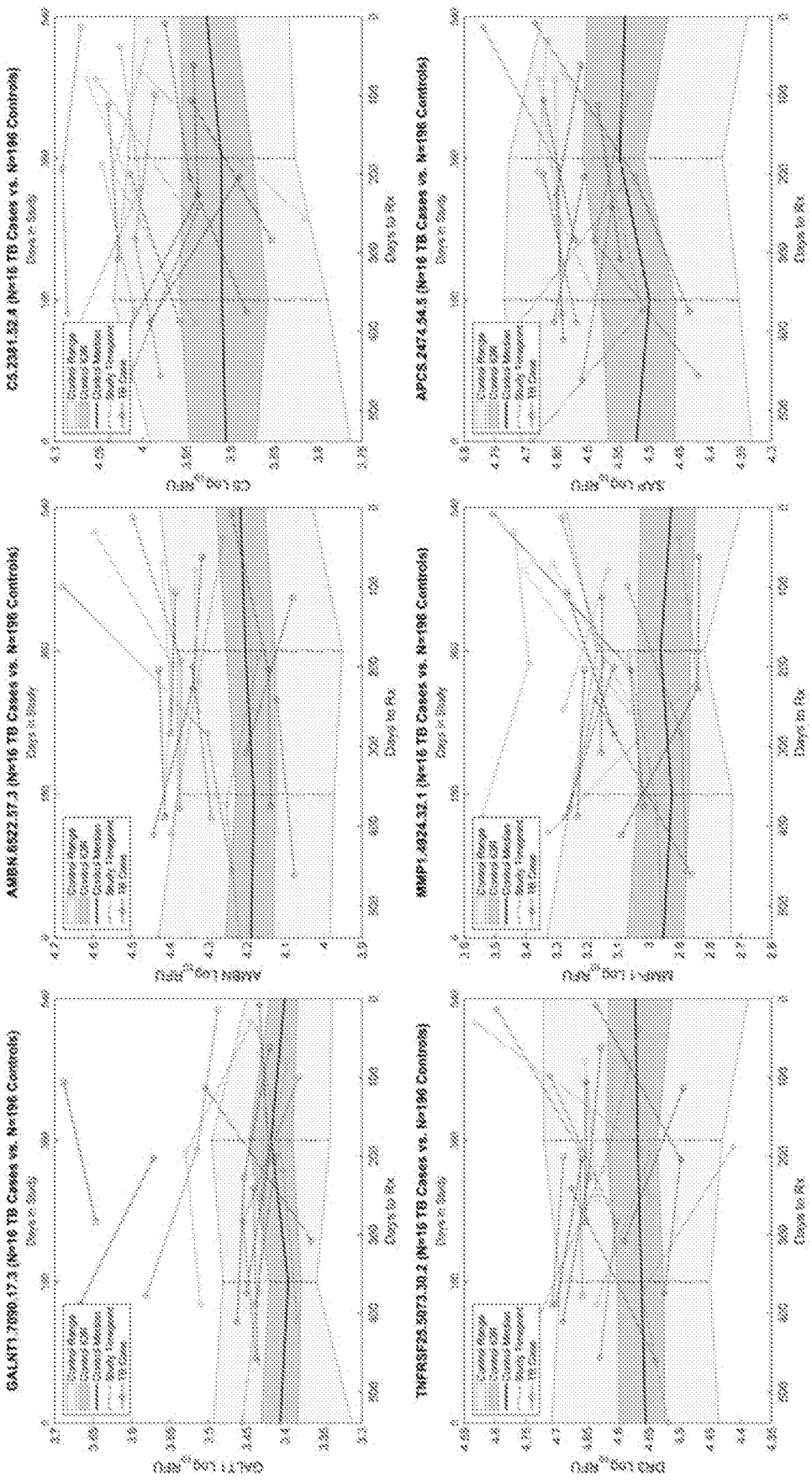


FIG. 10

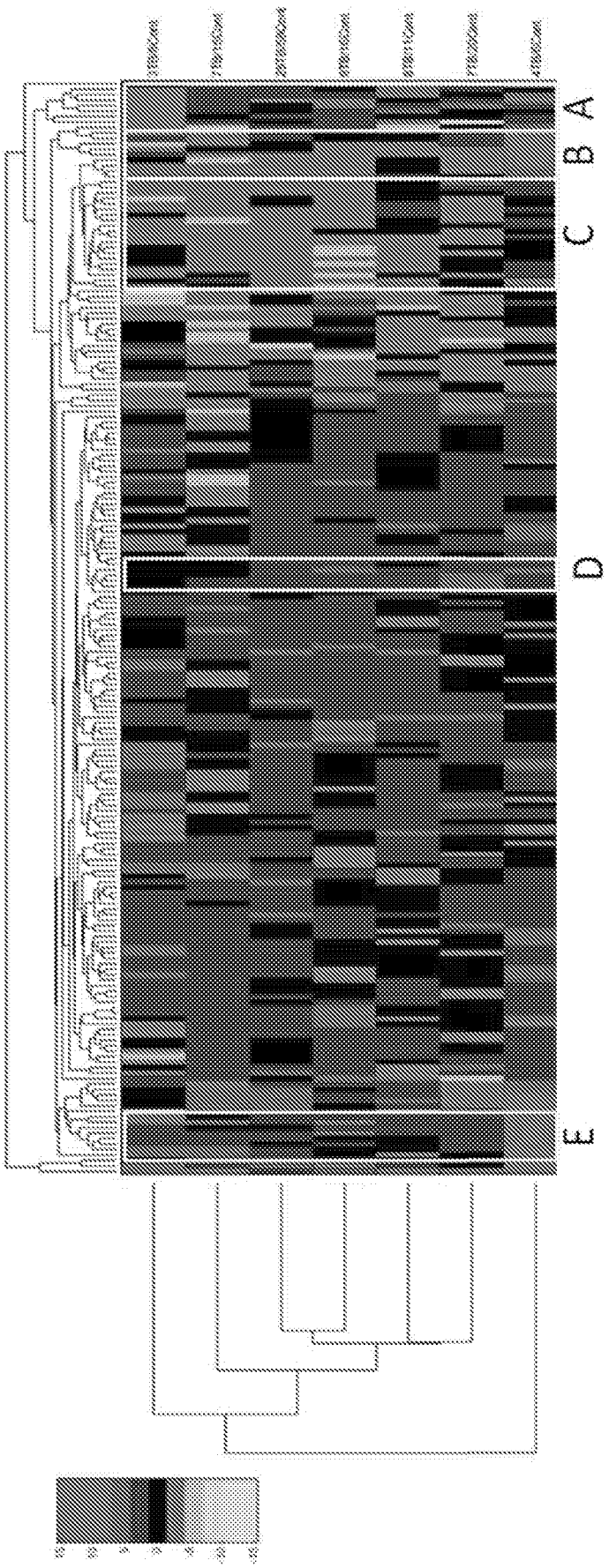
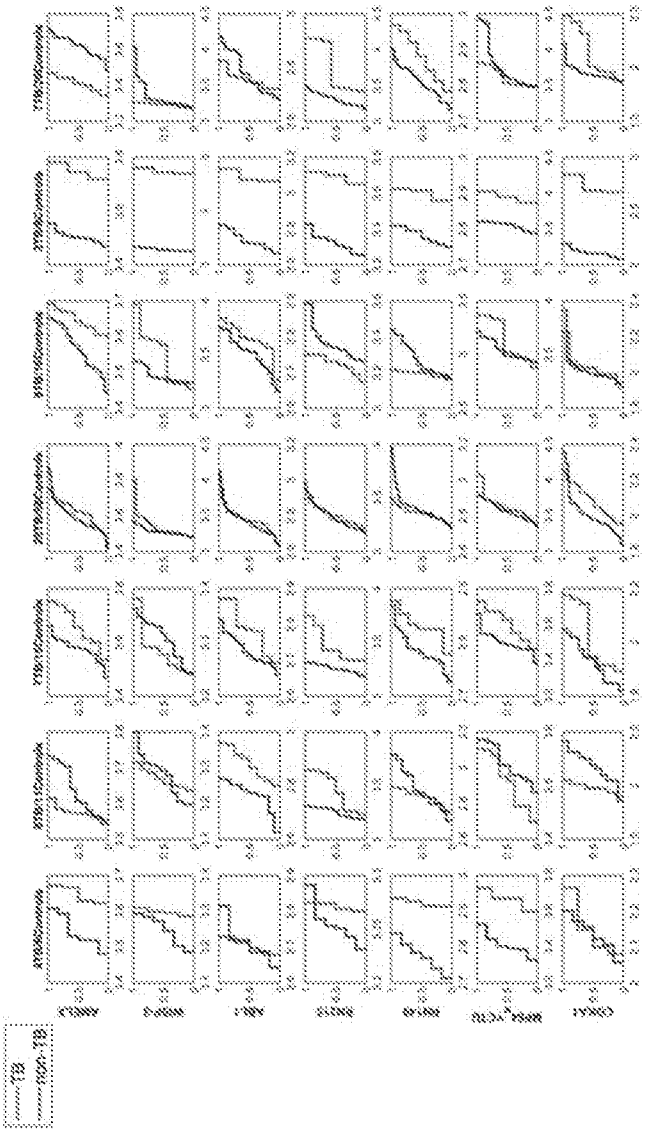


FIG. 11



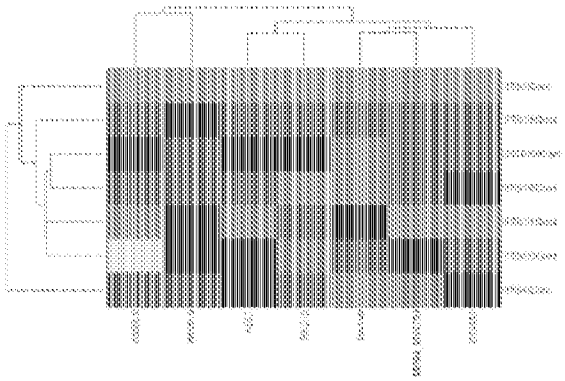
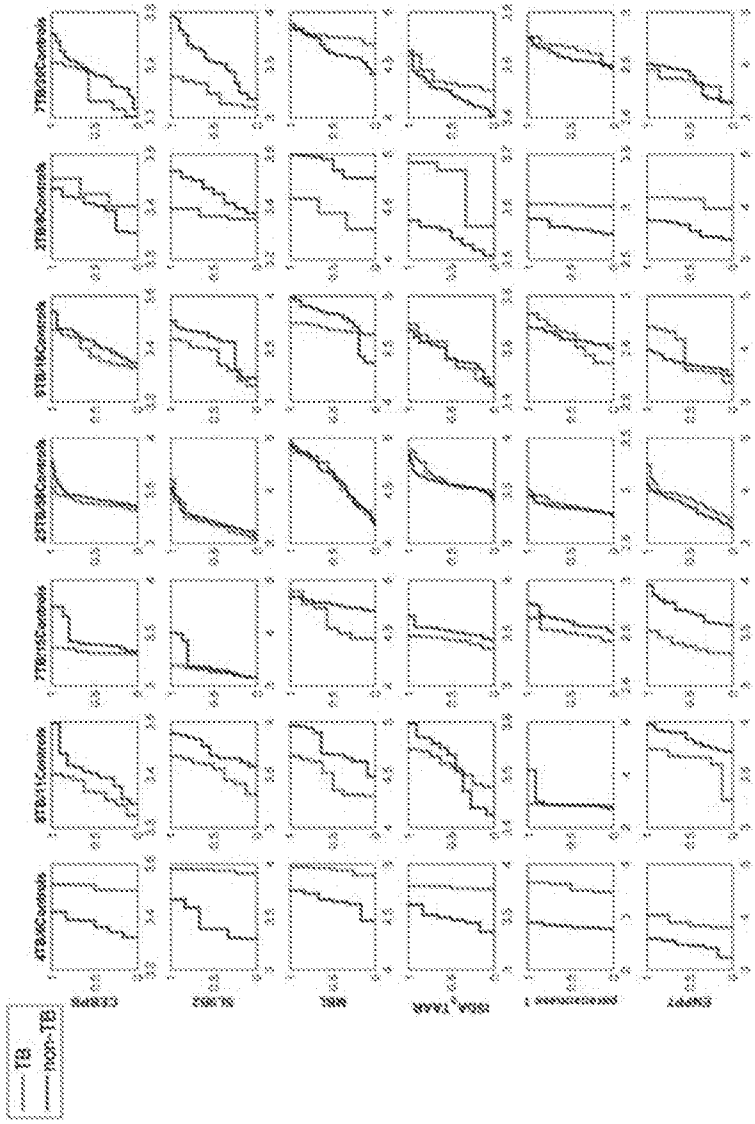


FIG. 12



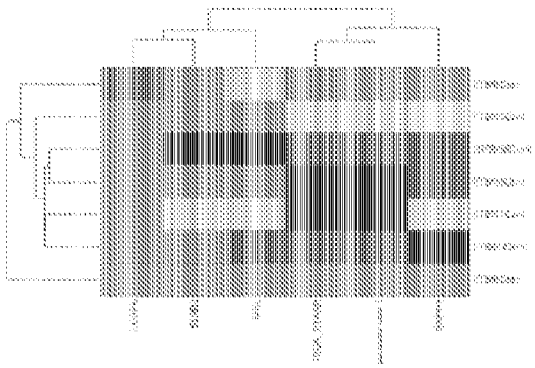
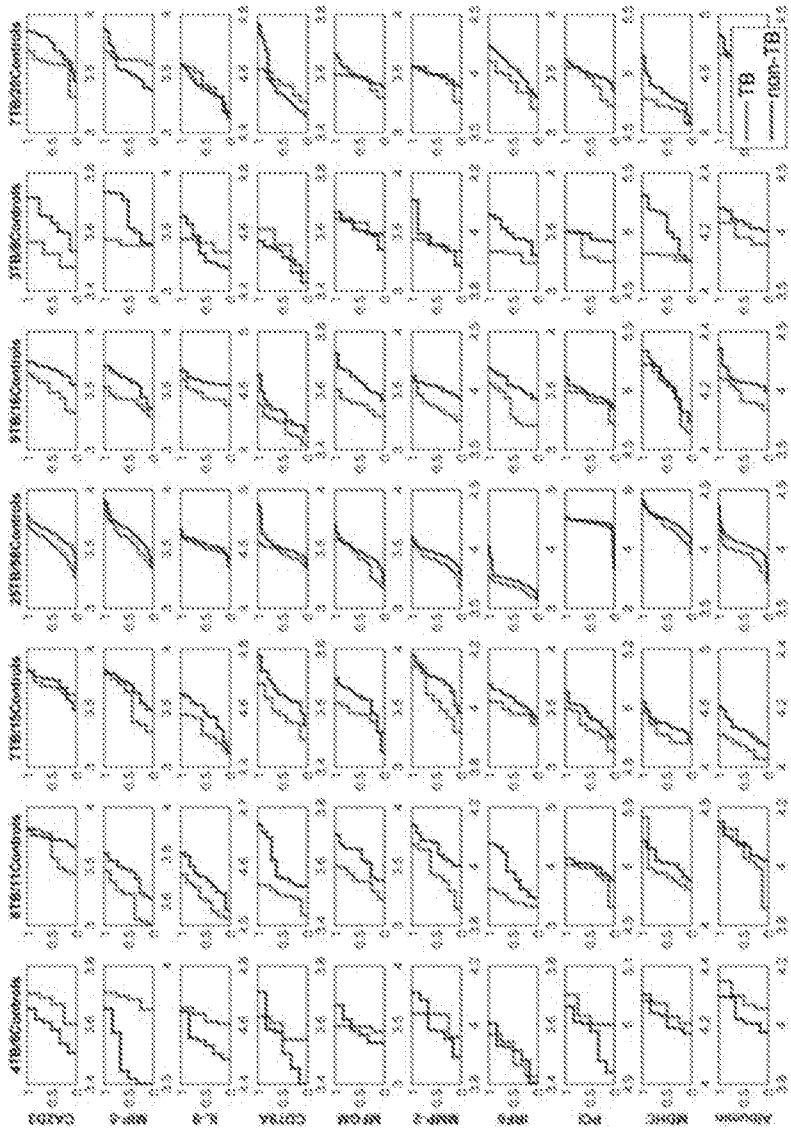


FIG. 13



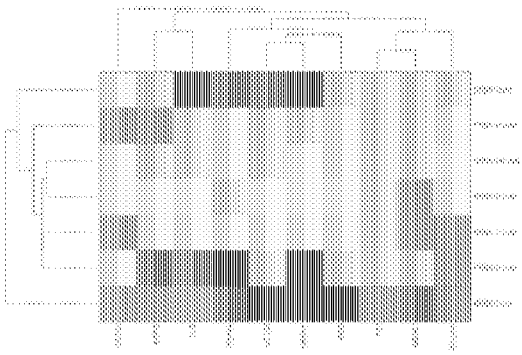


FIG. 14

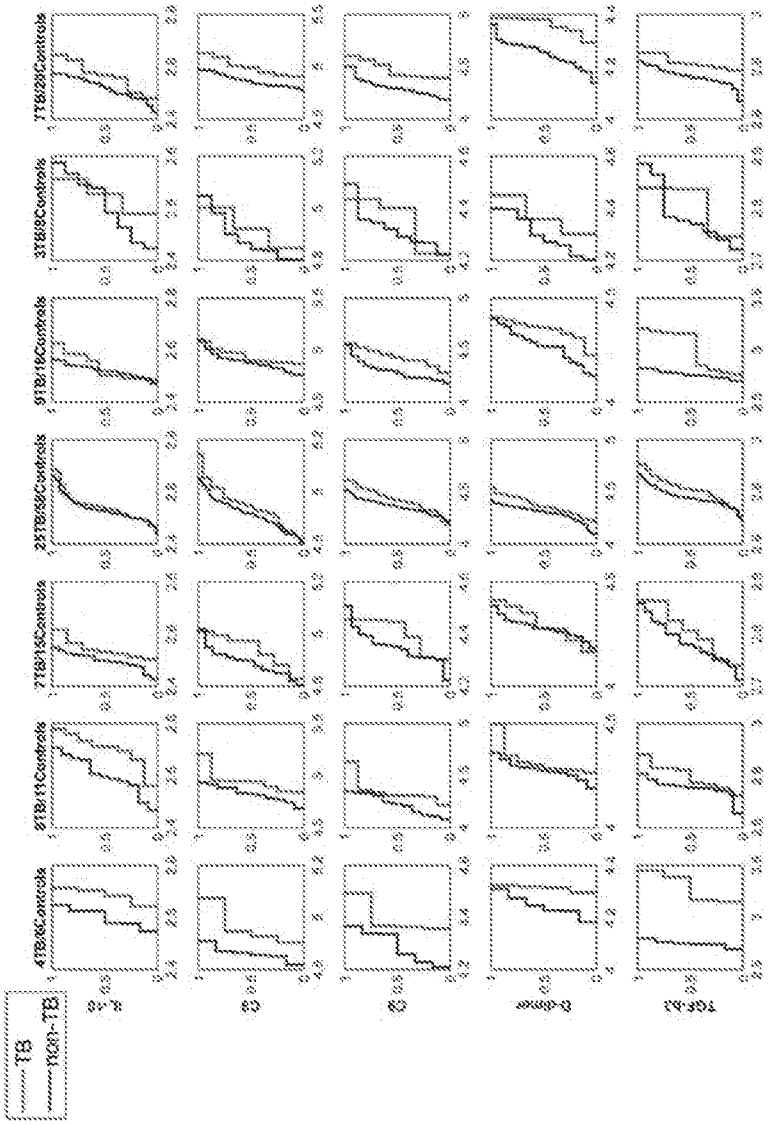
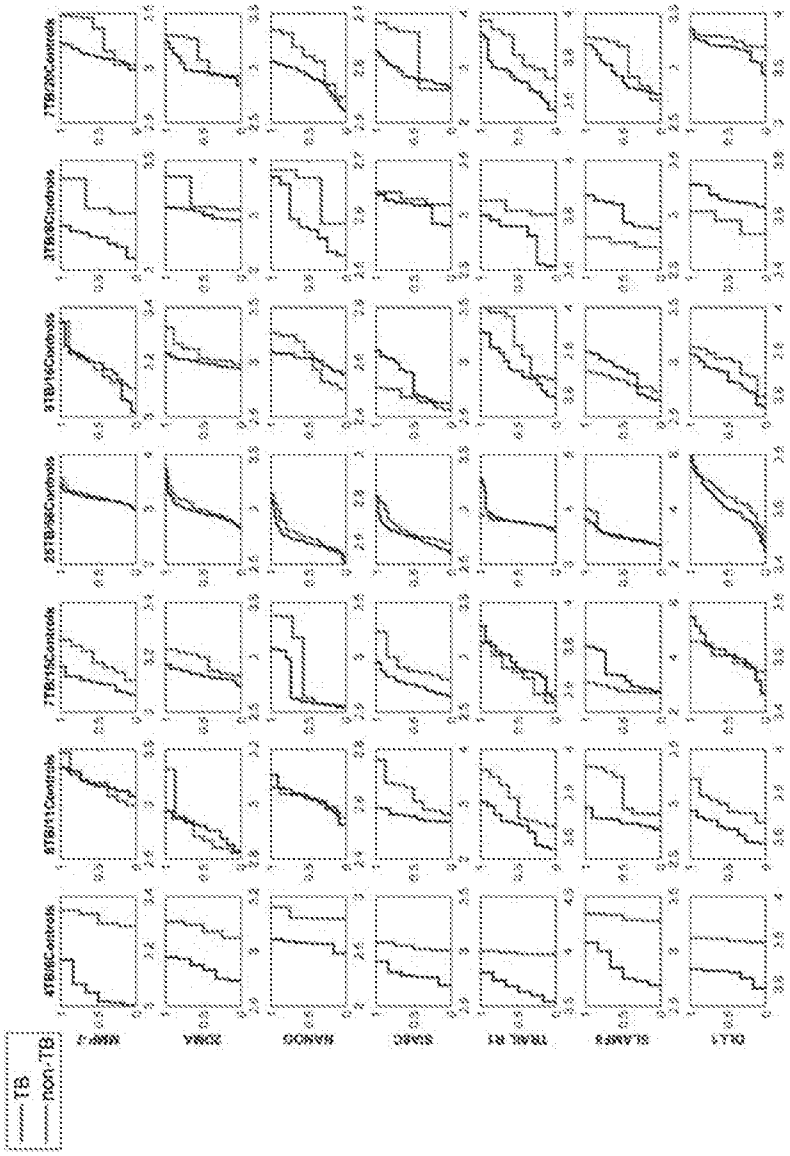

TB
non-TB
4TB/4Controls
5TB/11Controls
7TB/15Controls
21TB/58Controls
9TB/18Controls
3TB/8Controls
7TB/28Controls
IL-2
IL-6
IL-8
IL-10
G-CSF
TGF-b1

FIG. 15



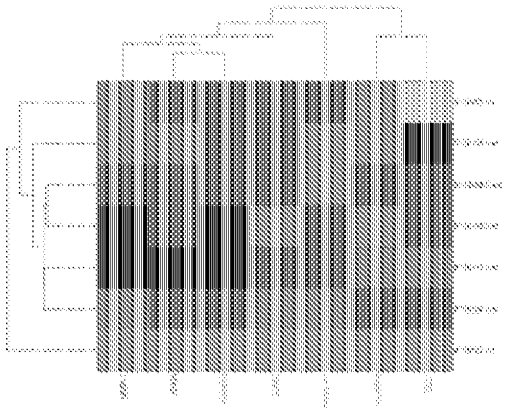


FIG. 16

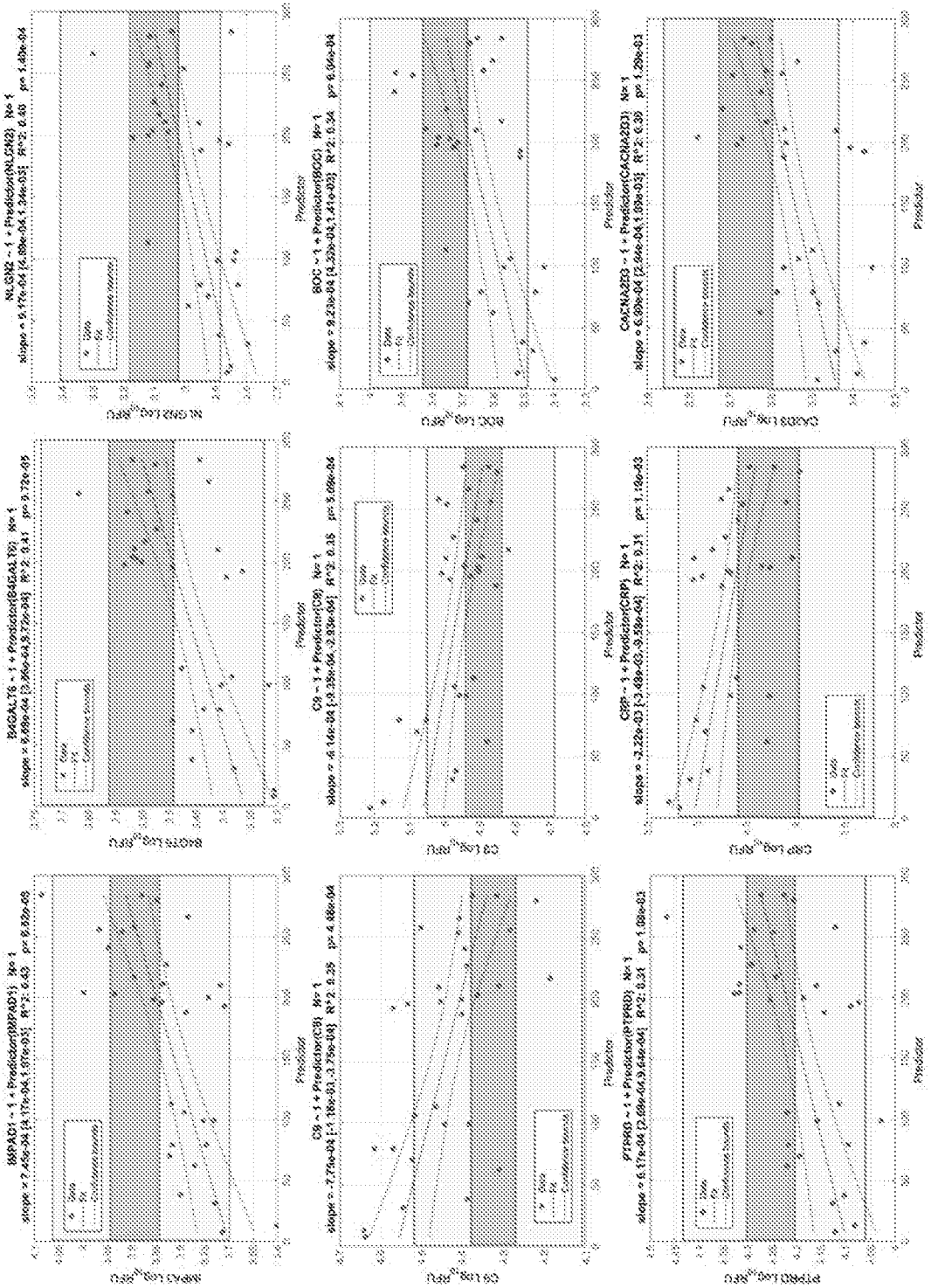


FIG. 17

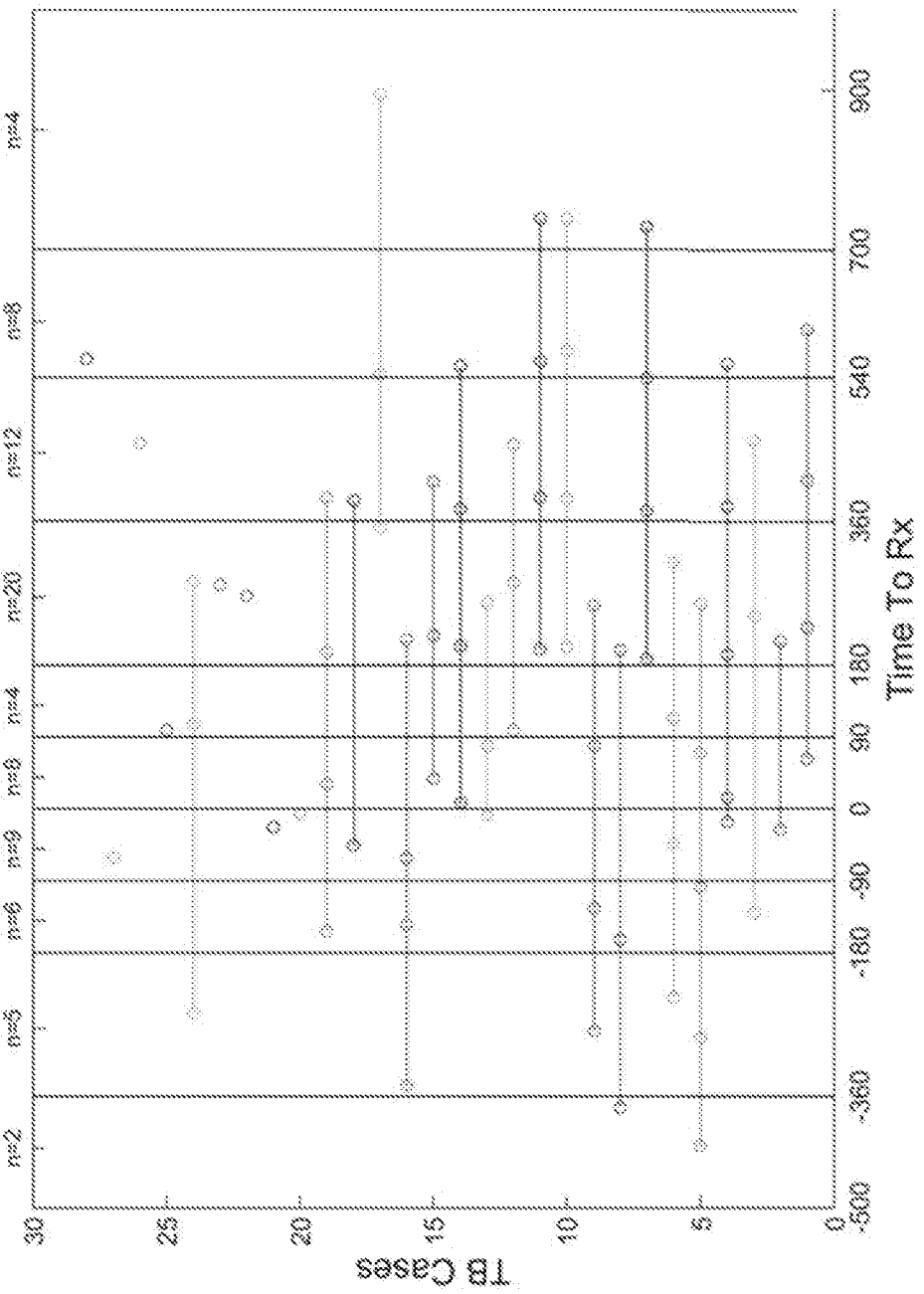
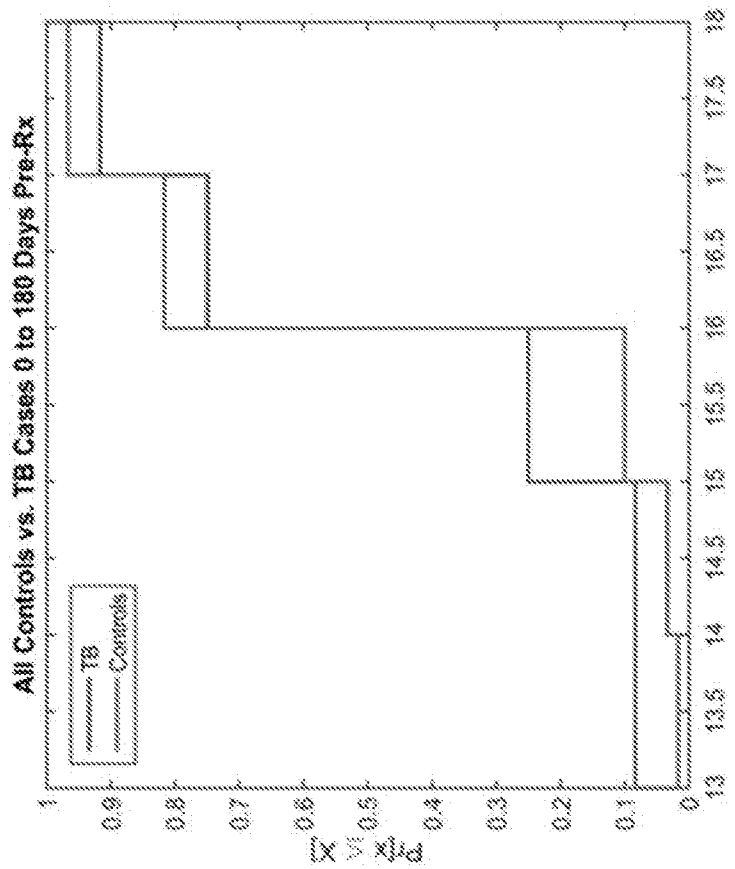

TB Cases
Time To Rx
n=2
n=5
n=6
n=9
n=0
n=4
n=20
n=12
n=8
n=4
30
25
20
15
10
5
0
-500
-360
-180
-90
0
90
180
360
540
700
900

FIG. 18



	Samples	Males	Females	Pregnant (?)
Cases	12	2 (17%)	10 (83%)	2 (17%)
Controls	60	8 (13%)	52 (87%)	5 (8%)

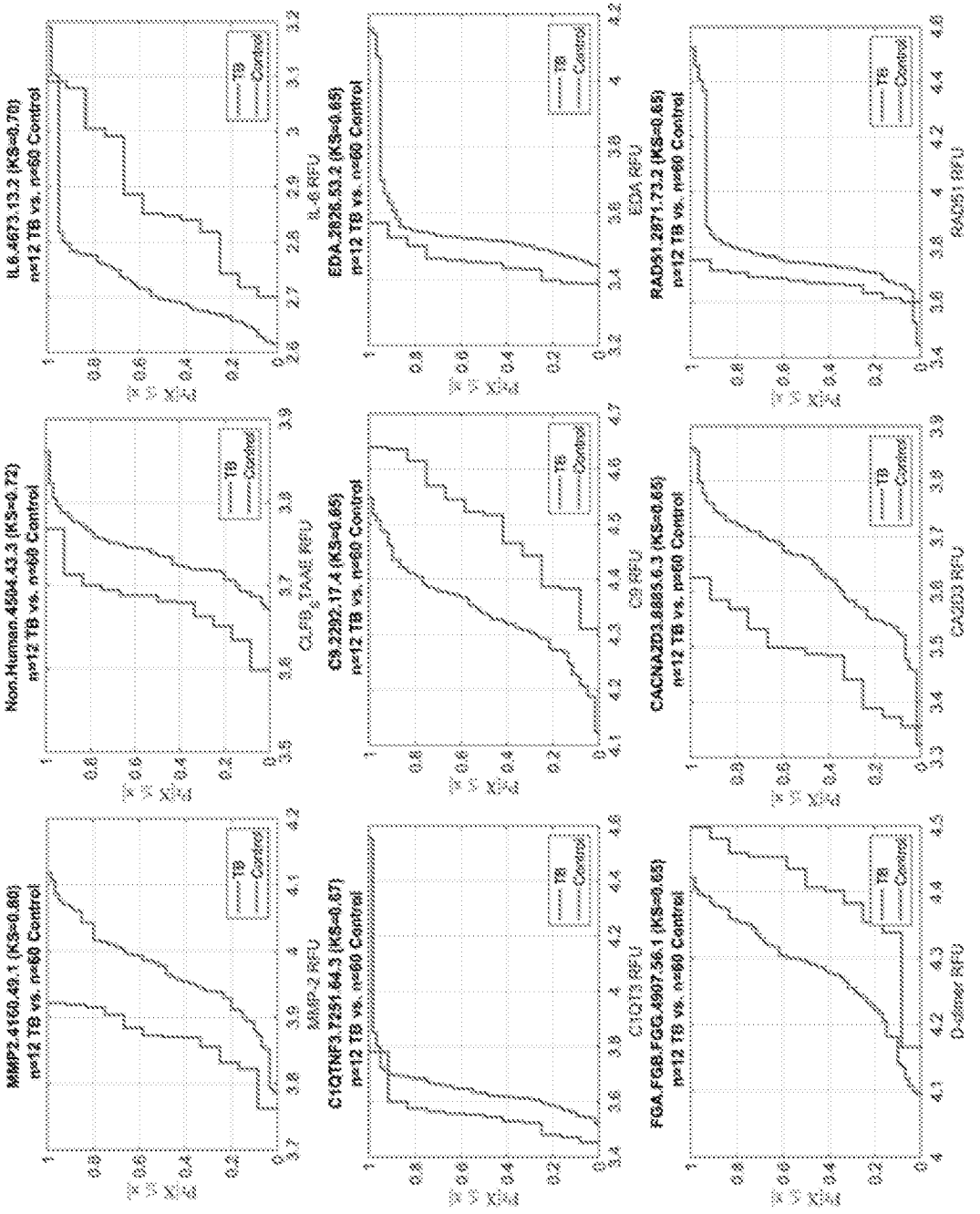

MMP2.4160.49.1 (KS=0.60)
n=12 TB vs. n=60 Control
MMP-2 RFU
Kon.Human.4584.43.3 (KS=0.72)
n=12 TB vs. n=60 Control
CLEC5A/MAFAP RFU
IL6.4673.13.2 (KS=0.70)
n=12 TB vs. n=60 Control
IL-6 RFU
C1QTNF3.7251.64.3 (KS=0.67)
n=12 TB vs. n=60 Control
C1QTL3 RFU
C9.2292.17.4 (KS=0.65)
n=12 TB vs. n=60 Control
C9 RFU
EDA.2826.53.2 (KS=0.65)
n=12 TB vs. n=60 Control
EDA RFU
FGA.FGB.FGG.4997.36.1 (KS=0.65)
n=12 TB vs. n=60 Control
D-dimer RFU
CACNA2D3.6885.6.3 (KS=0.65)
n=12 TB vs. n=60 Control
CACD3 RFU
RAD51.2871.73.2 (KS=0.65)
n=12 TB vs. n=60 Control
RAD51 RFU
TB
Control

FIG. 19

FIG. 20

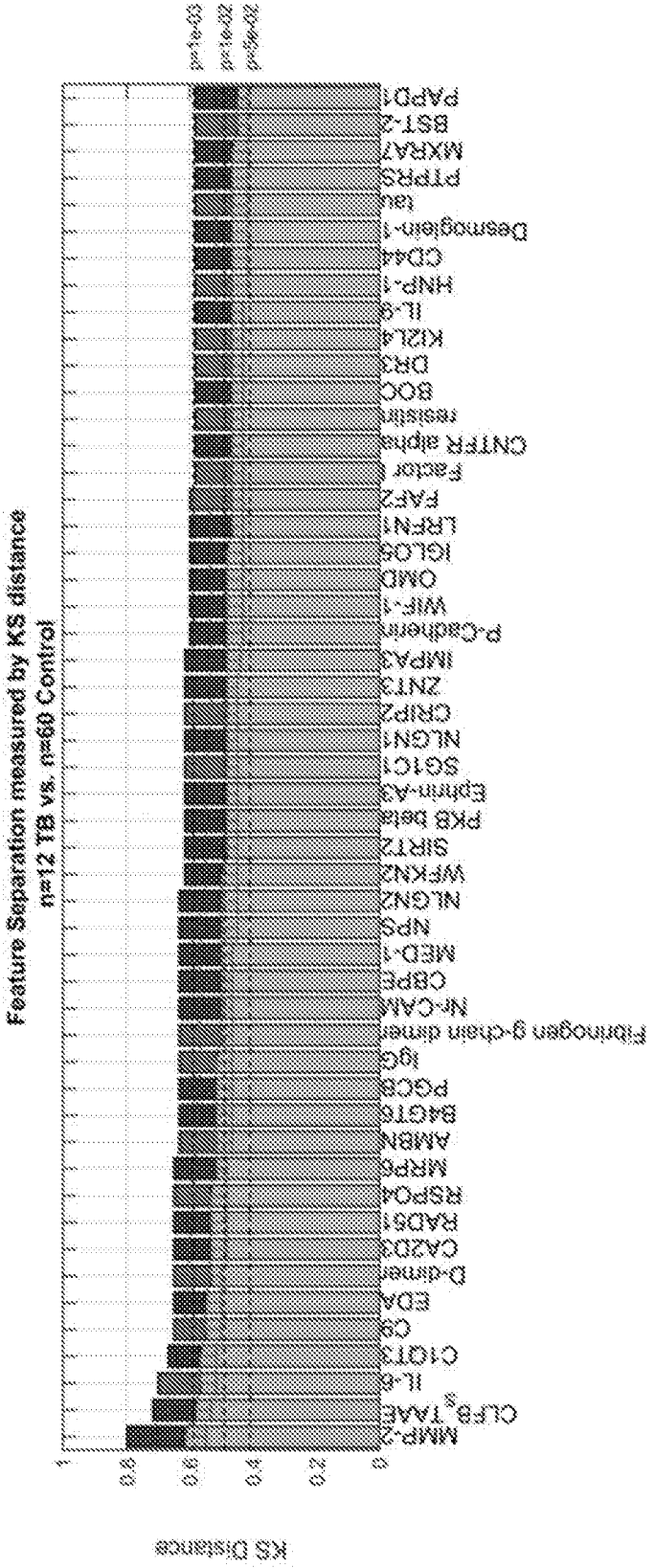

Feature Separation measured by KS distance
n=12 TB vs. n=60 Control
p=1e-03
p=1e-02
p=5e-02
MMP-2
CLFB_TAAE
IL-6
C1QT3
C9
EDA
D-dimer
CA2D3
RAD51
RSPO4
MRPC
AMBN
B4GTC
PGCB
IgG
Fibrinogen g-chain dimer
N-CAM
CBPE
MED-1
NPS
NLGN2
WFKN2
SIRT2
PKB beta
Ephrin-A3
SG1C1
NLGN1
CRIP2
ZNT3
IMPA3
P-Cadherin
WIF-1
OMD
IGLC5
LRFN1
FAF2
Factor
CNTFR alpha
resistin
BOC
DR3
KI2L4
IL-9
HNP-1
CD44
Desmoglein-1
tau
PTPRS
MXRA7
BST-2
PAPD1
1
0.8
0.6
0.4
0.2
0
KS Distance

FIG. 21

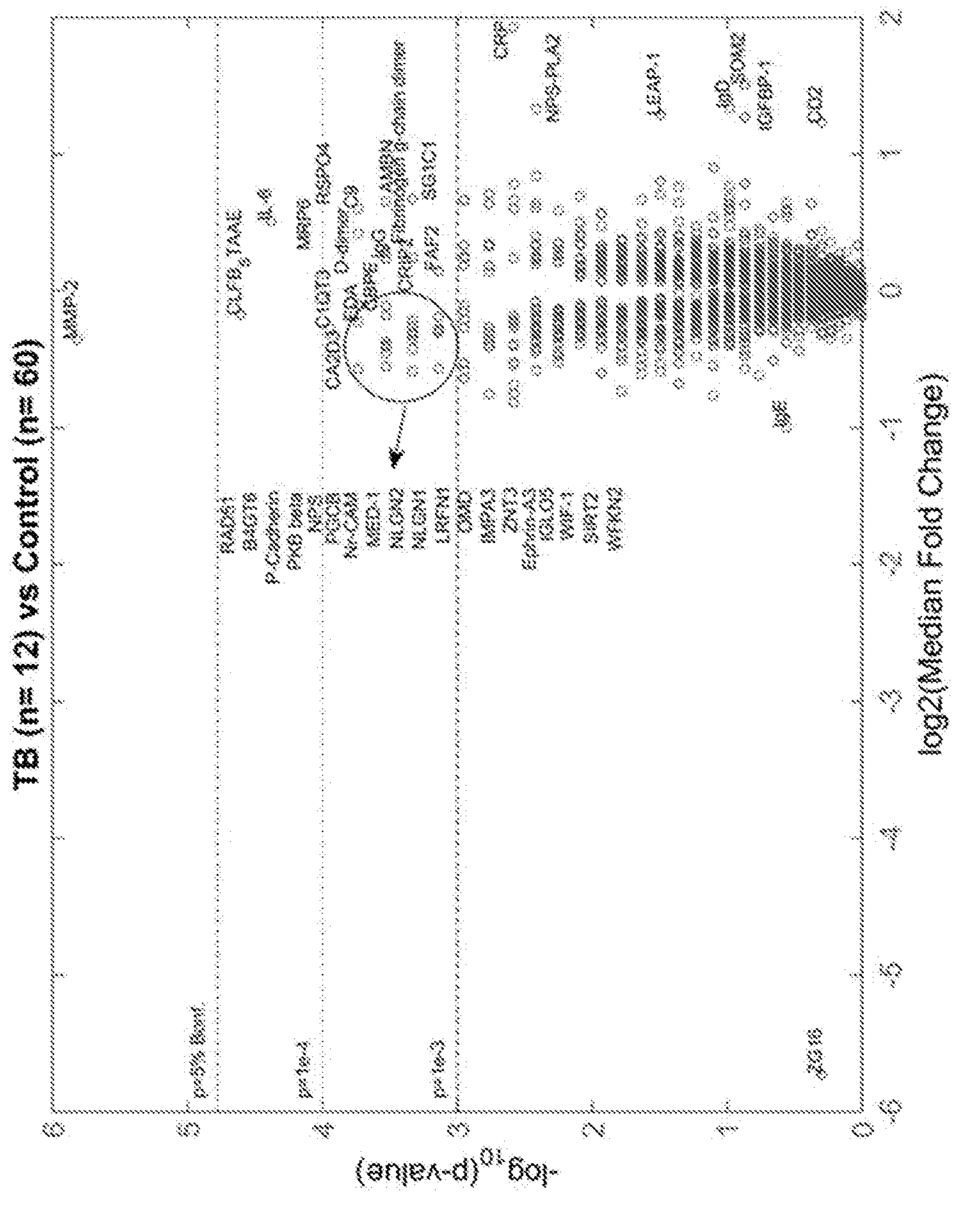

TB (n= 12) vs Control (n= 60)
log2(Median Fold Change)
-log10(p-value)
p=5% Bonf.
p=1e-4
p=1e-3

FIG. 22

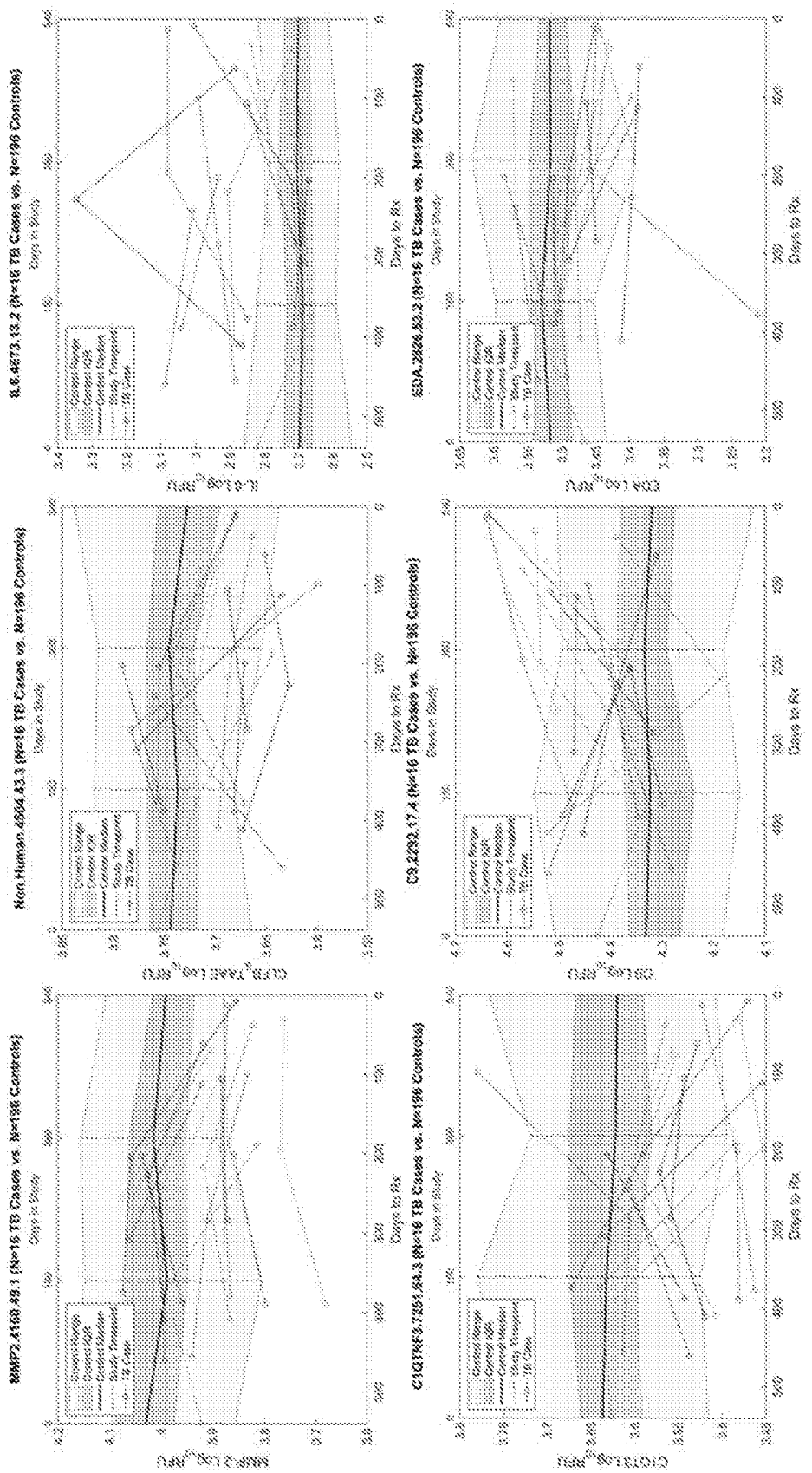
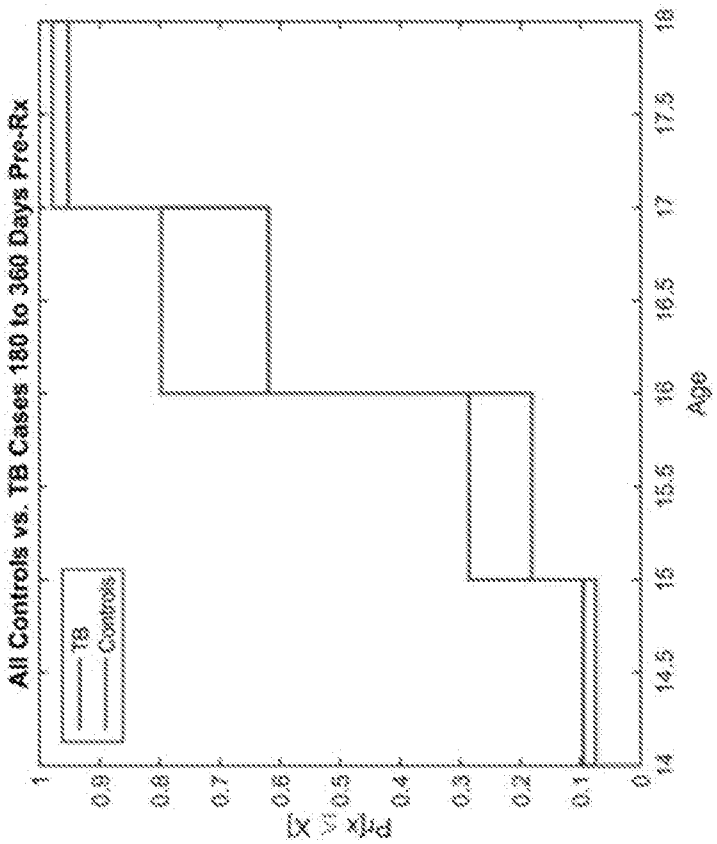

IL6.4873.13.2 (N=16 TB Cases vs. N=196 Controls)
Non.Human.A504.43.3 (N=16 TB Cases vs. N=196 Controls)
EDA.2228.53.2 (N=16 TB Cases vs. N=196 Controls)
MMP2.4169.49.1 (N=16 TB Cases vs. N=196 Controls)
CQ.2292.17.4 (N=16 TB Cases vs. N=196 Controls)
C1QTNF3.7251.84.3 (N=16 TB Cases vs. N=196 Controls)
Days in Study
Days to Rx
TB Case

FIG. 23



	Samples	Males	Females	Pregnant
Cases	21	5 (24%)	16 (76%)	2 (10%)
Controls	94	15 (16%)	79 (84%)	7 (7.5%)

FIG. 24

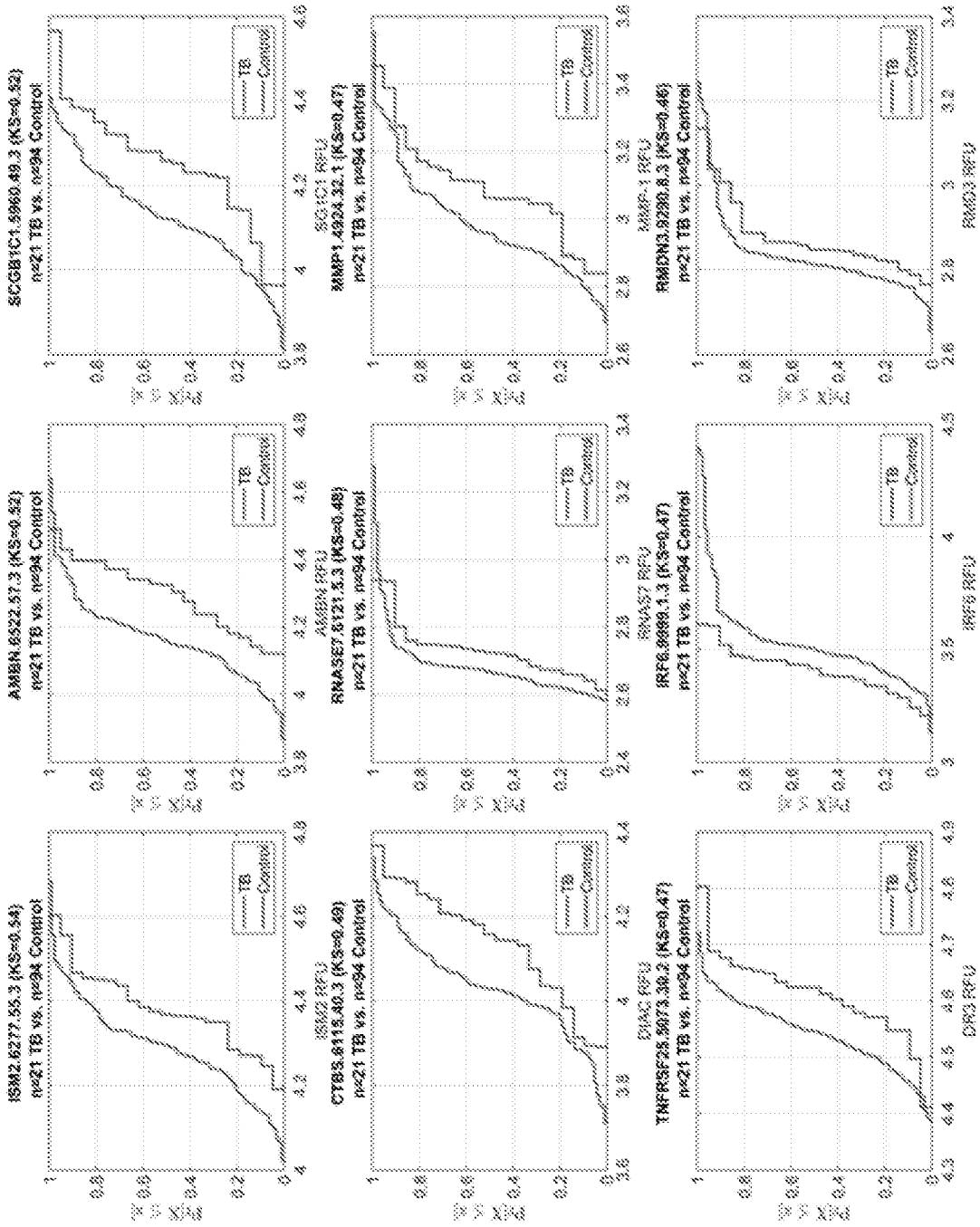

SCGB1C1.5960.49.3 (KS=0.52)
n=21 TB vs. n=94 Control
SCGB1C1 RFU
AMBN.6522.57.3 (KS=0.52)
n=21 TB vs. n=94 Control
AMBN RFU
MMP1.4824.32.1 (KS=0.47)
n=21 TB vs. n=94 Control
MMP-1 RFU
ISM2.6277.55.3 (KS=0.54)
n=21 TB vs. n=94 Control
ISM2 RFU
RNASE7.6121.5.3 (KS=0.48)
n=21 TB vs. n=94 Control
RNASE7 RFU
RMDN3.8280.8.3 (KS=0.46)
n=21 TB vs. n=94 Control
RMDN3 RFU
CTB3.6118.40.3 (KS=0.49)
n=21 TB vs. n=94 Control
CTB3 RFU
IRF6.6660.1.3 (KS=0.47)
n=21 TB vs. n=94 Control
IRF6 RFU
TNFRSF25.5073.38.2 (KS=0.47)
n=21 TB vs. n=94 Control
DR3 RFU
TB
Control
Pr(X ≤ x)

FIG. 25

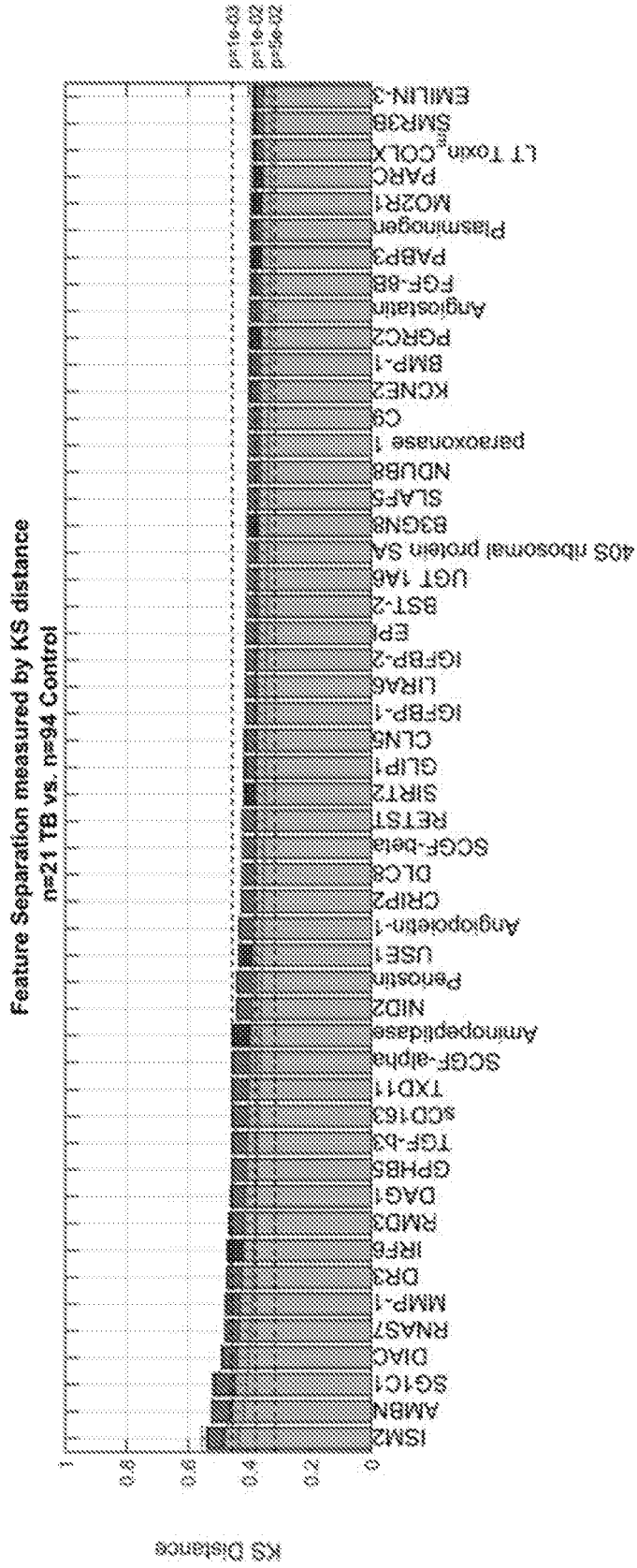

Feature Separation measured by KS distance
n=21 TB vs. n=94 Control
p<1e-03
p<1e-02
p<1e-01
ISM2
AMBN
SG1C1
DIAC
RNAS7
MMP-1
DR3
IRF6
RMD3
DAG1
GPHBS
TGF-b3
sCD163
TXD11
SCGF-alpha
Aminopeptidase
NID2
Periostin
USE1
Angiopoietin-1
CRIP2
DLC8
SCGF-beta
RETST
SIRT2
GLIP1
CLNS
IGFBP-1
LIRA6
IGFBP-2
EPI
BST-2
UGT 1A6
40S ribosomal protein SA
B3GN8
SLAF5
NDUB8
paraoxonase 1
C9
KCNE2
BMP-1
PGRC2
Angiostatin
FGF-8B
PAEP3
Plasminogen
MO2R1
PARC
LT Toxin B COLX
SMR3B
EMILIN-3
1
0.8
0.6
0.4
0.2
0
KS Distance

FIG. 26

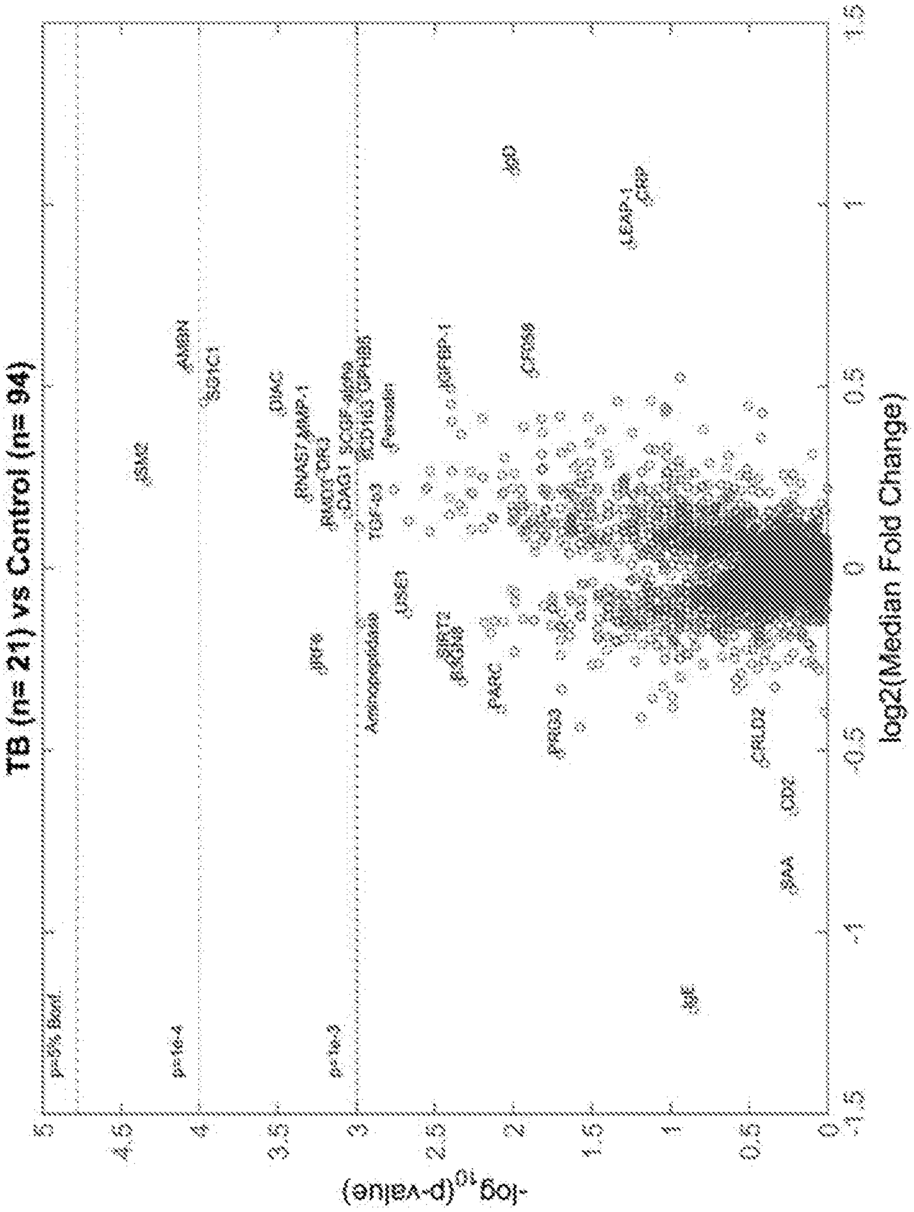

TB (n= 21) vs Control (n= 94)
log2(Median Fold Change)
-log10(p-value)
p=1e-3
p=1e-4

FIG. 27

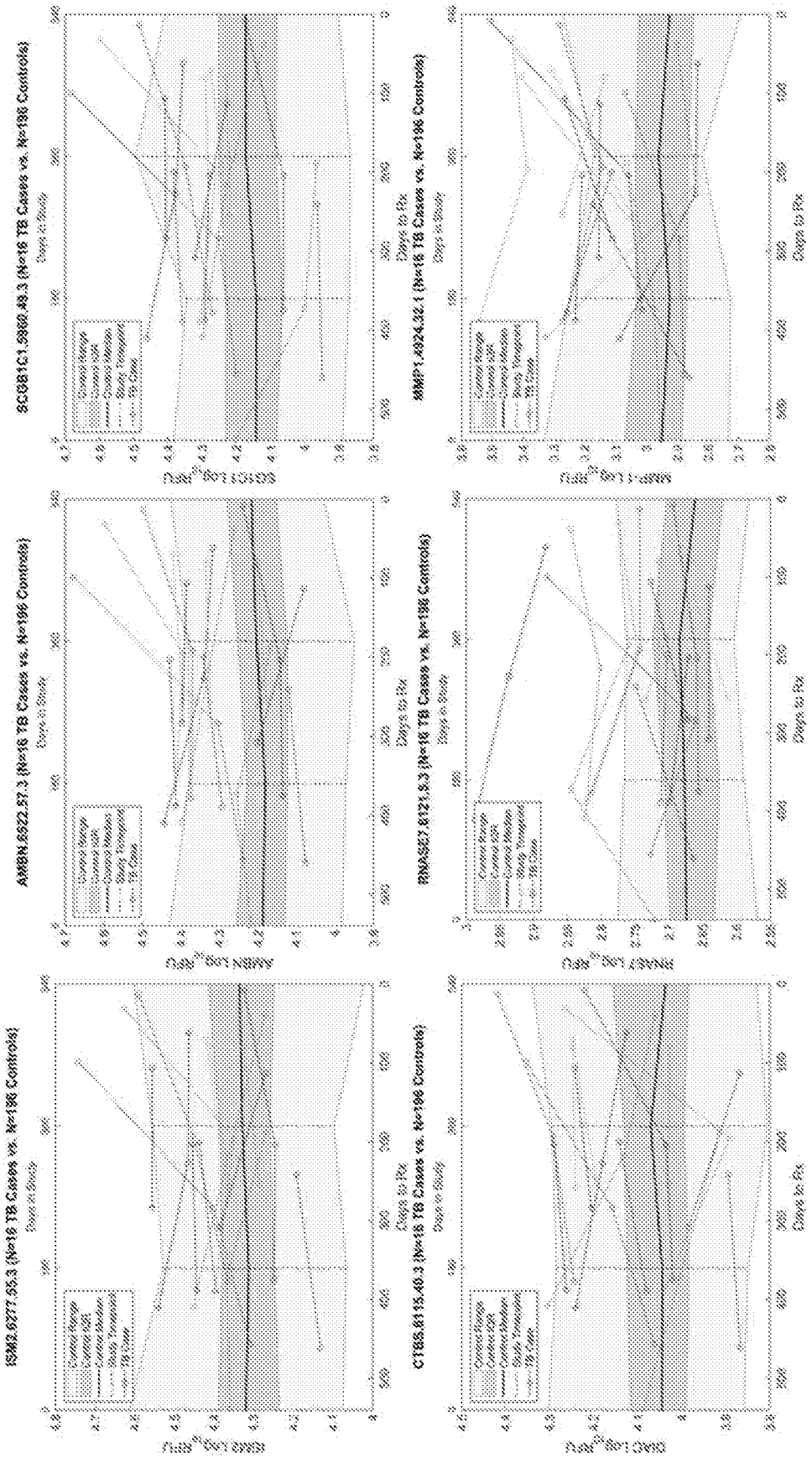
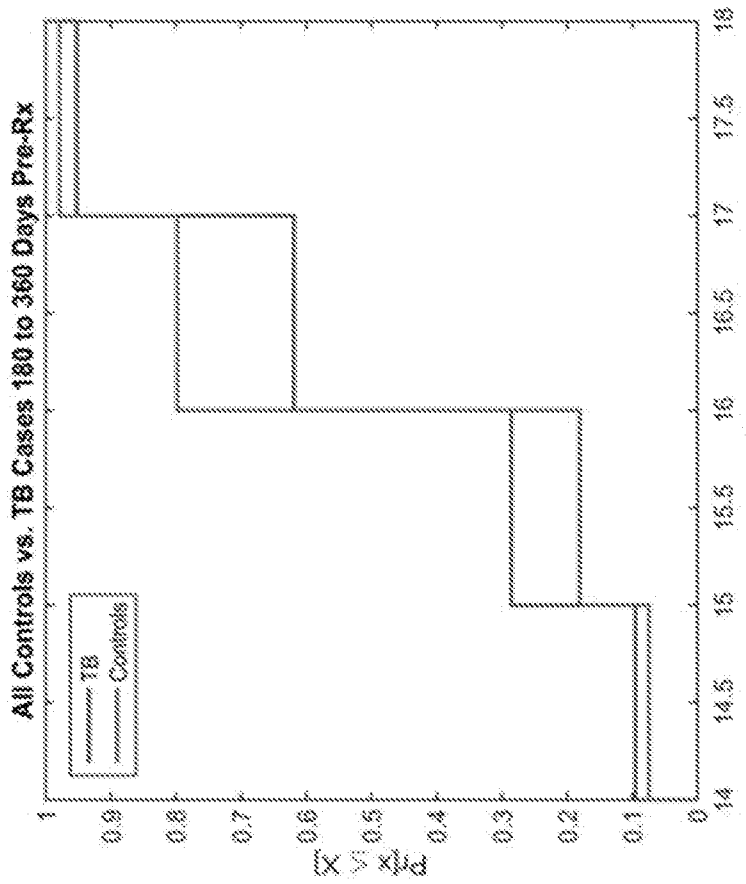

SC03C1.5360.49.3 (N=16 TB Cases vs. N=196 Controls)
MMP1.4924.22.1 (N=16 TB Cases vs. N=196 Controls)
AMBN.8522.57.3 (N=16 TB Cases vs. N=196 Controls)
RNASE7.6121.5.3 (N=16 TB Cases vs. N=196 Controls)
ISM2.6277.55.3 (N=16 TB Cases vs. N=196 Controls)
CTB5.6115.46.3 (N=16 TB Cases vs. N=196 Controls)
Days in Study
Days to Rx
Control Range
Control IQR
Control Median
Study Timepoint
TB Case

FIG. 28



	Samples	Males	Females	Pregnant
Cases	21	5 (24%)	16 (76%)	2 (10%)
Controls	94	15 (16%)	79 (84%)	7 (7.5%)

FIG. 29

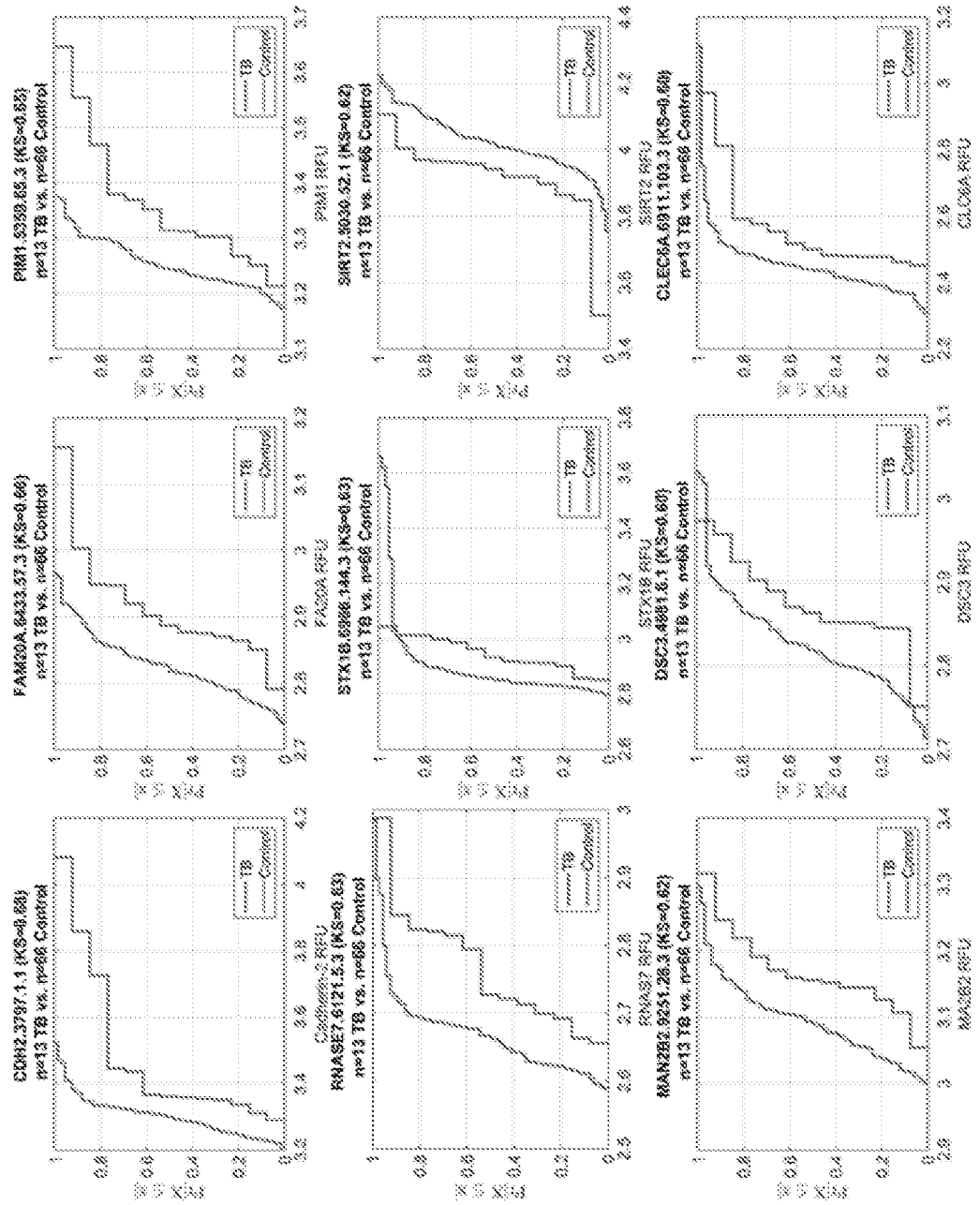

PIM1.5358.65.3 (KS=0.65)
n=13 TB vs. n=66 Control
PIM1 RFU
FAM20A.6423.57.3 (KS=0.66)
n=13 TB vs. n=66 Control
FAM20A RFU
SIRT2.9030.52.1 (KS=0.62)
n=13 TB vs. n=66 Control
SIRT2 RFU
STX18.6366.144.3 (KS=0.63)
n=13 TB vs. n=66 Control
STX18 RFU
CLEC6A.6911.103.3 (KS=0.66)
n=13 TB vs. n=66 Control
CLEC6A RFU
CDH2.3797.1.1 (KS=0.66)
n=13 TB vs. n=66 Control
Cadherin-2 RFU
DSC3.4881.6.1 (KS=0.66)
n=13 TB vs. n=66 Control
DSC3 RFU
RNASE7.6121.5.3 (KS=0.63)
n=13 TB vs. n=66 Control
RNASE7 RFU
MAN2B2.9251.28.3 (KS=0.62)
n=13 TB vs. n=66 Control
MAN2B2 RFU
TB
Control
1 - Specificity
Sensitivity

FIG. 30

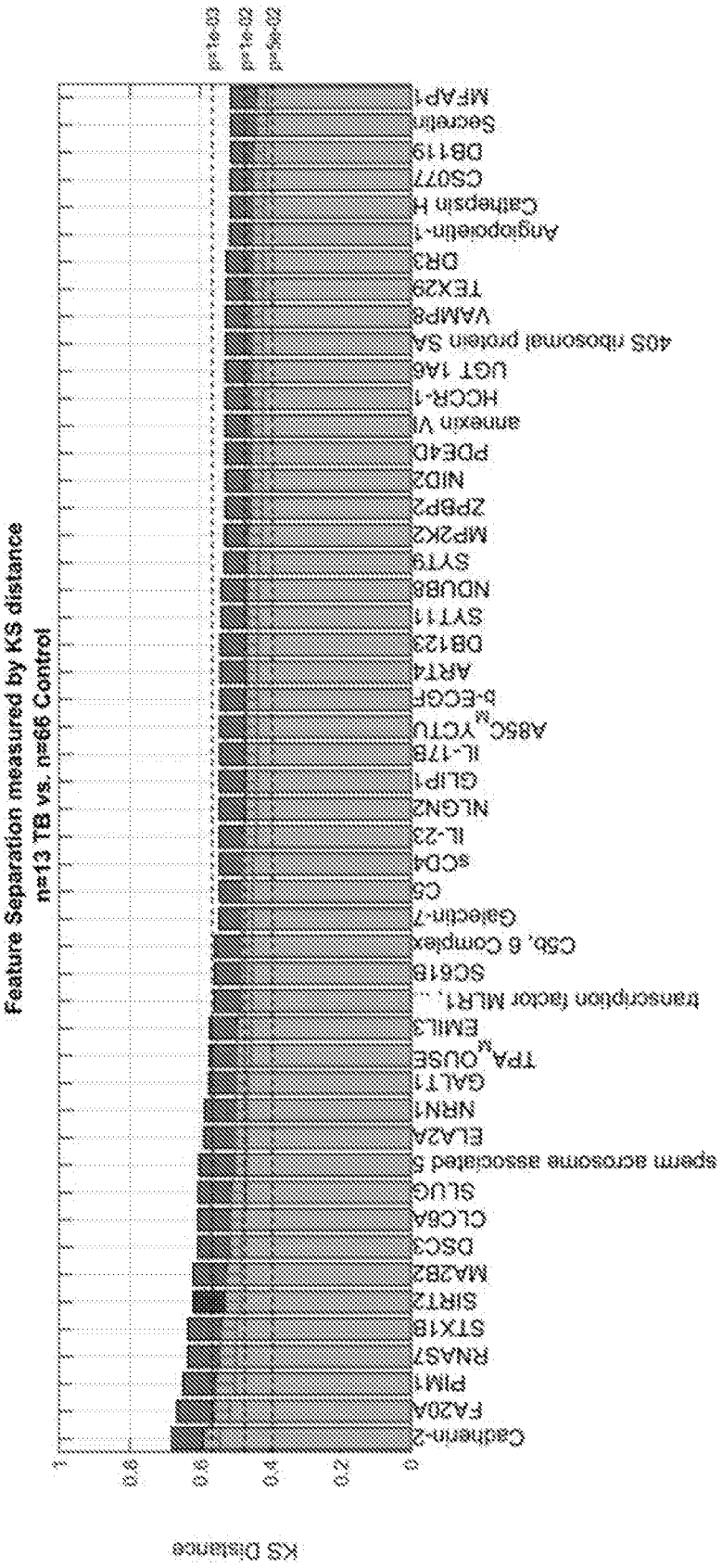

Feature Separation measured by KS distance
n=13 TB vs. n=66 Control
KS Distance
1
0.8
0.6
0.4
0.2
0
Cadherin-2
FA20A
PIM1
RNAS7
STX1B
SIRT2
MA2B2
DSC3
CLC8A
SLUG
sperm acrosome associated 5
ELA2A
NRN1
GALT1
TPA_OUSE
ENIL3
transcription factor ML.R1, ...
SC61B
CSb, 6 complex
Galectin-7
C3
sCD4
IL-23
NLGN2
GLIP1
IL-17B
A85C_YCTU
b-ECGF
ART4
DB123
SYT11
NDUB5
SYTB
MP2K2
ZPBP2
NID2
PDE4D
annexin V
HCCR-1
UGT 1A6
40S ribosomal protein SA
VAMP8
TEX29
DR3
Angiopoietin-1
Caltrepsin H
CS077
DB119
Secretin
MFAP-1

FIG. 31

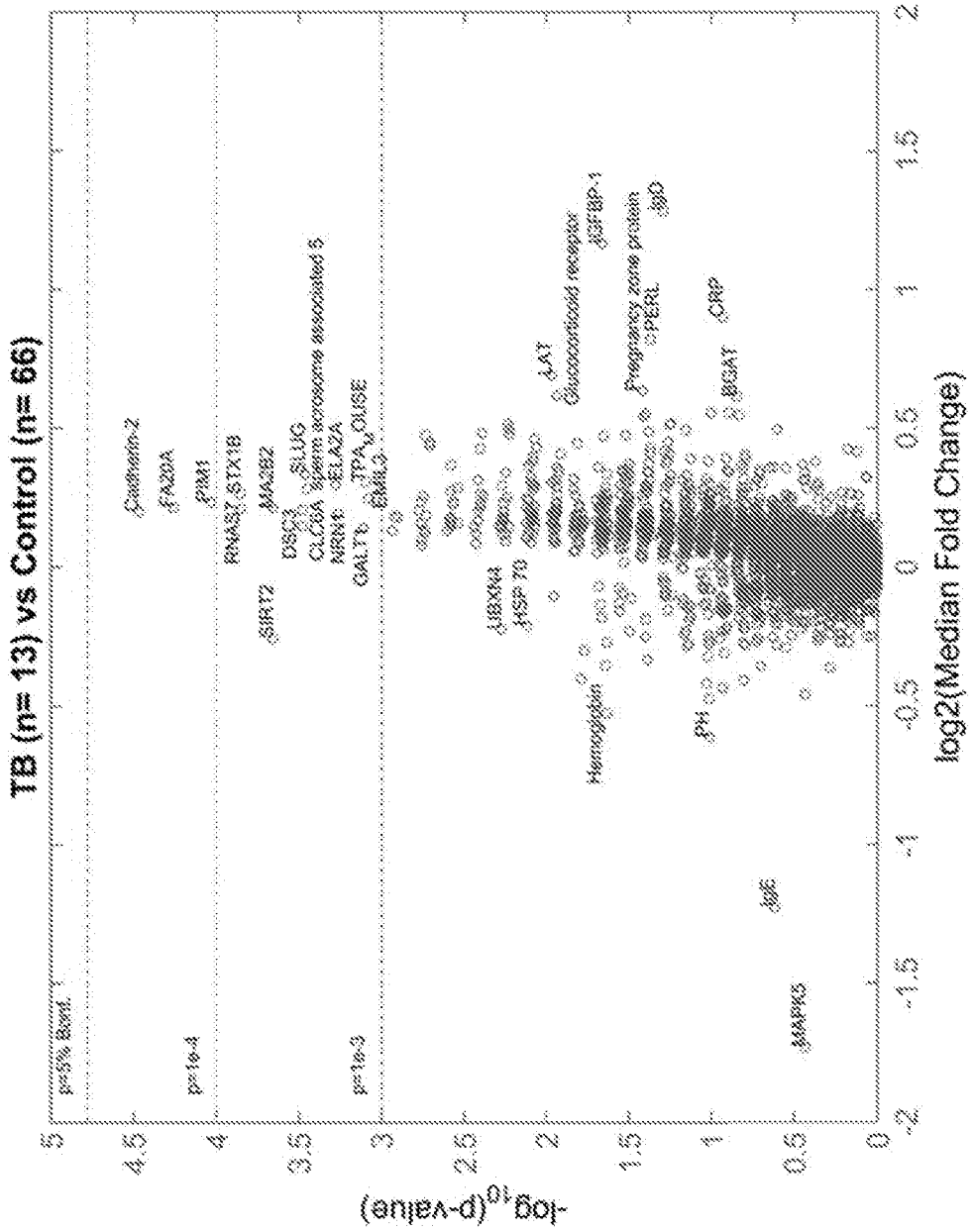

TB (n= 13) vs Control (n= 66)
p=5% fdr
p=1e-4
p=1e-3
Cadherin-2
FAZDA
PIM1
RNASE 6/TX1B
SIRT2
MAJIE2
DSC3
SLUG
CLCBA
sperm acrosome associated 6
MRN1e
ELAZA
GALT6
TPA_K OLIGE
EML3
HBXIA
HSP 70
Hemoglobin
LAT
Glucocorticoid receptor
IGFBP-1
Pregnancy zone protein
PERL
SID
PH
IBSAT
CRP
MAPK6
-log10(p-value)
log2(Median Fold Change)

FIG. 32

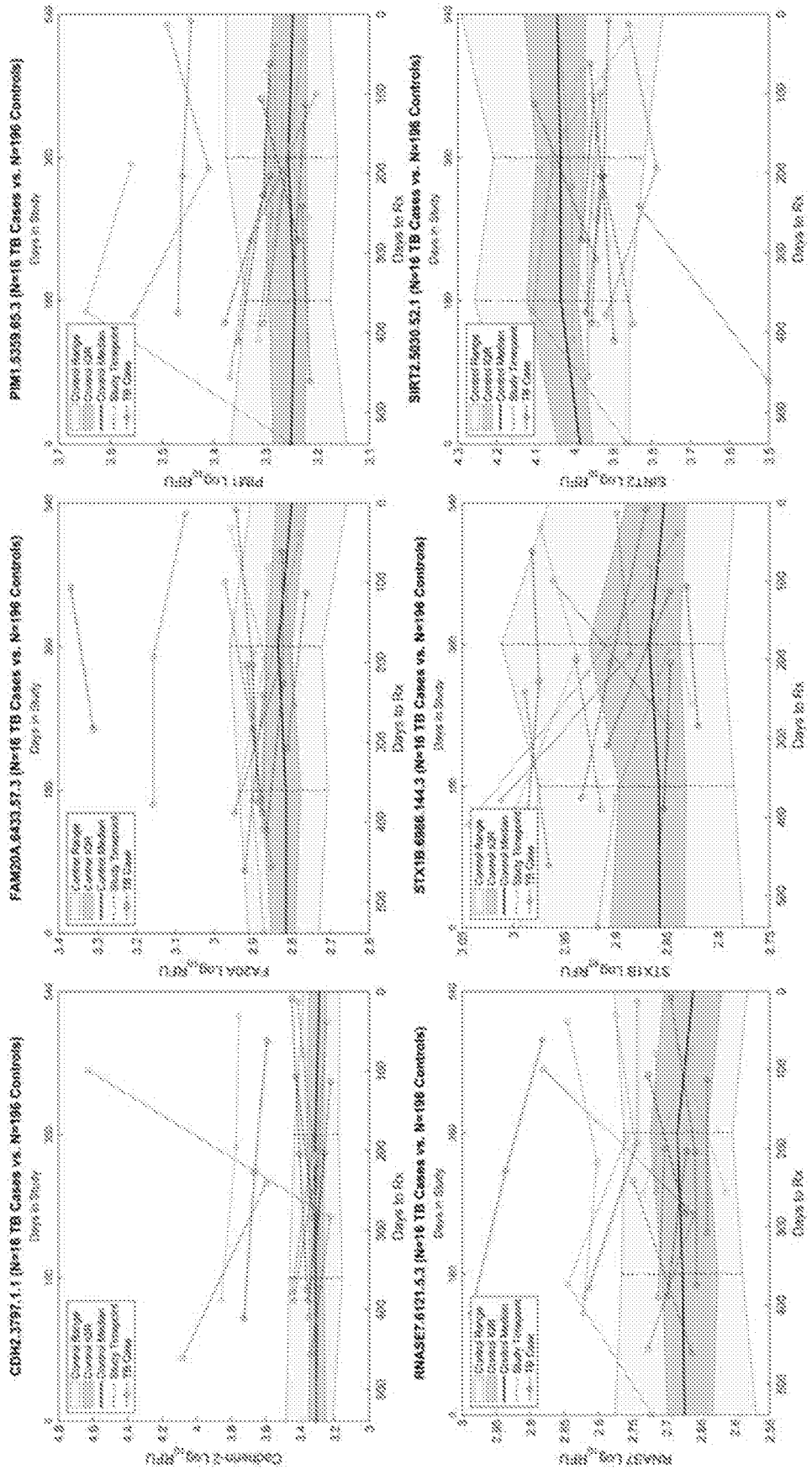
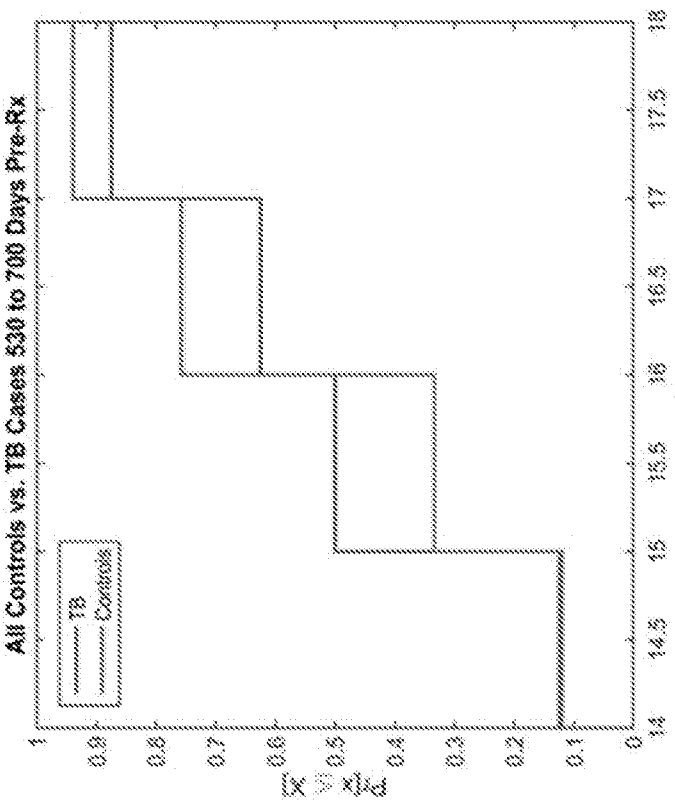

CDH2.2797.1.1 (N=16 TB Cases vs. N=196 Controls)
FAM20A.6433.87.3 (N=16 TB Cases vs. N=196 Controls)
PIM1.5339.65.3 (N=16 TB Cases vs. N=196 Controls)
RNASET2.6131.5.3 (N=16 TB Cases vs. N=196 Controls)
STX18.6988.144.3 (N=16 TB Cases vs. N=196 Controls)
SIRT2.5830.52.1 (N=16 TB Cases vs. N=196 Controls)
Days in Study
Days to Rx
Control Range
Control IQR
Control Median
Study Timepoint
TB Case

FIG. 33



	Samples	Males	Females	Pregnant
Cases	8	2 (25%)	6 (75%)	0 (0%)
Controls	33	6 (18%)	27 (82%)	1 (3%)

FIG. 34

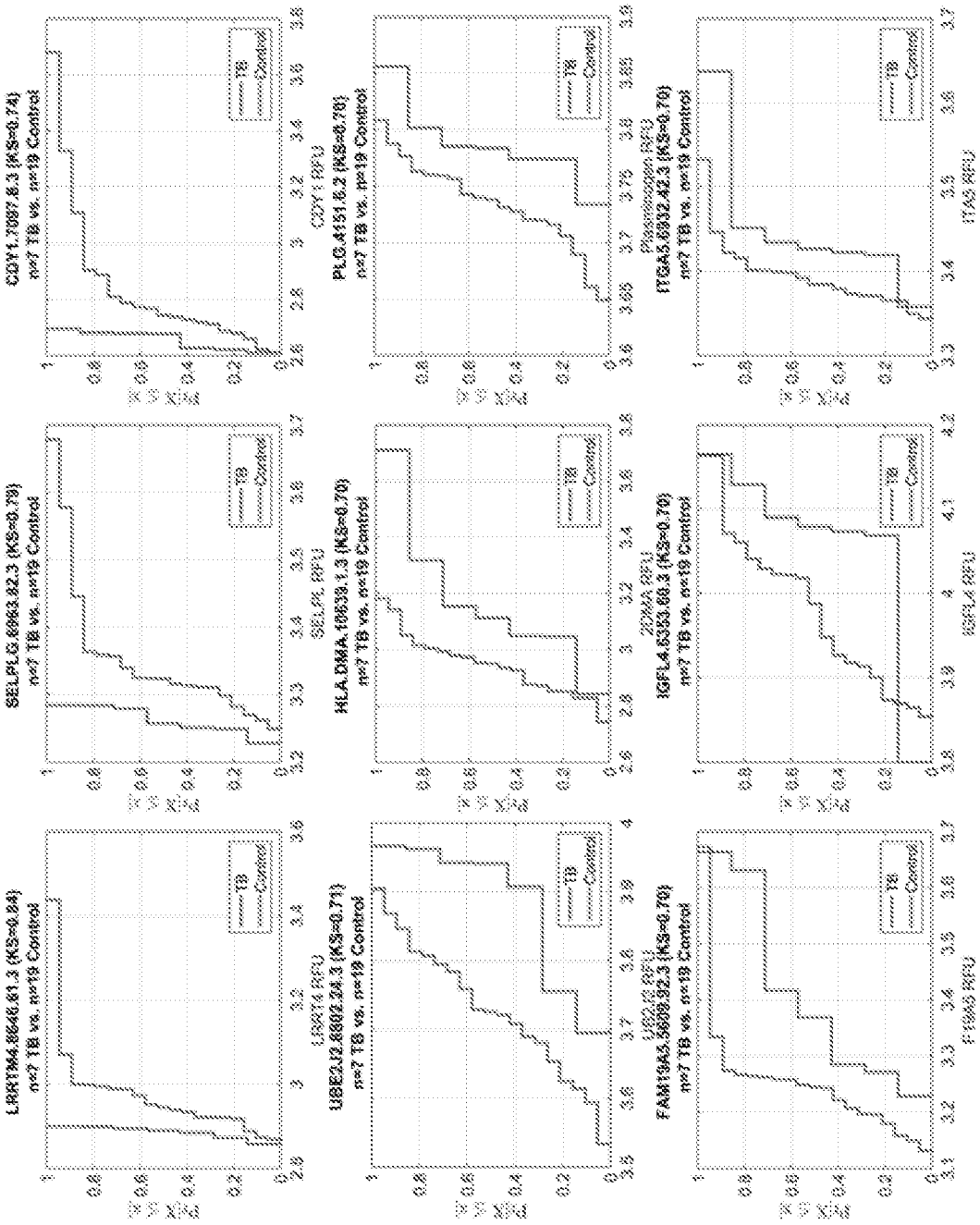

LRRTM4.86646.61.3 (KS=0.84)
n=7 TB vs. n=19 Control
SELPLG.6963.82.3 (KS=0.79)
n=7 TB vs. n=19 Control
CDY1.7097.8.3 (KS=0.74)
n=7 TB vs. n=19 Control
U8E2J2.8602.24.3 (KS=0.71)
n=7 TB vs. n=19 Control
HLA.DMA.10633.1.3 (KS=0.76)
n=7 TB vs. n=19 Control
PLG.4151.6.2 (KS=0.76)
n=7 TB vs. n=19 Control
FAM19A5.5609.82.3 (KS=0.76)
n=7 TB vs. n=19 Control
IGFL4.6353.68.3 (KS=0.76)
n=7 TB vs. n=19 Control
ITGA5.6932.42.3 (KS=0.76)
n=7 TB vs. n=19 Control
TB
Control

FIG. 35

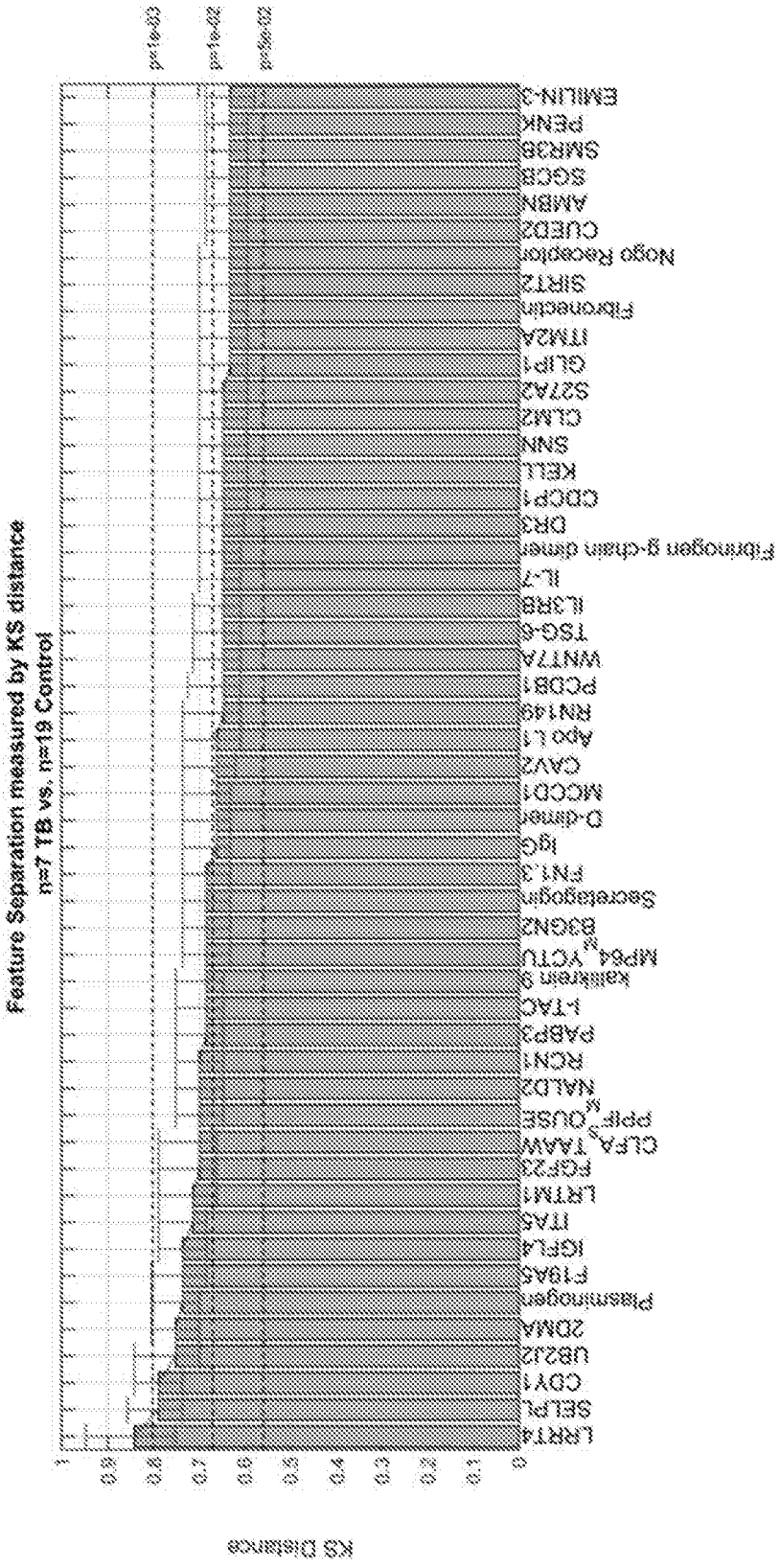

Feature Separation measured by KS distance
n=7 TB vs. n=19 Control
p=1e-03
p=1e-02
p=5e-02
EMILIN-3
PENK
SMR3B
SGCB
AMBN
CUEDC2
Nogo Receptor
SIRT2
Fibronectin
ITM2A
GLP1
S27A2
CLM2
SNN
KELL
CDCP1
DR3
Fibrinogen g-chain dimer
IL-7
IL3RB
TSG-6
WNT7A
PCDB1
RN149
Apo L1
CAV2
MCCD1
D-dimer
IgG
FN1.3
Secretagogin
B3GN2
MP64
kallikrein 9
L-TAC
PABP3
RCN1
NALD2
PPEF
CLEA
FGF23
LRTM1
ITA5
IGFL4
F19A5
Plasminogen
2DMA
UB2L2
CDY1
SELPL
LRRT4
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
KS Distance

FIG. 36

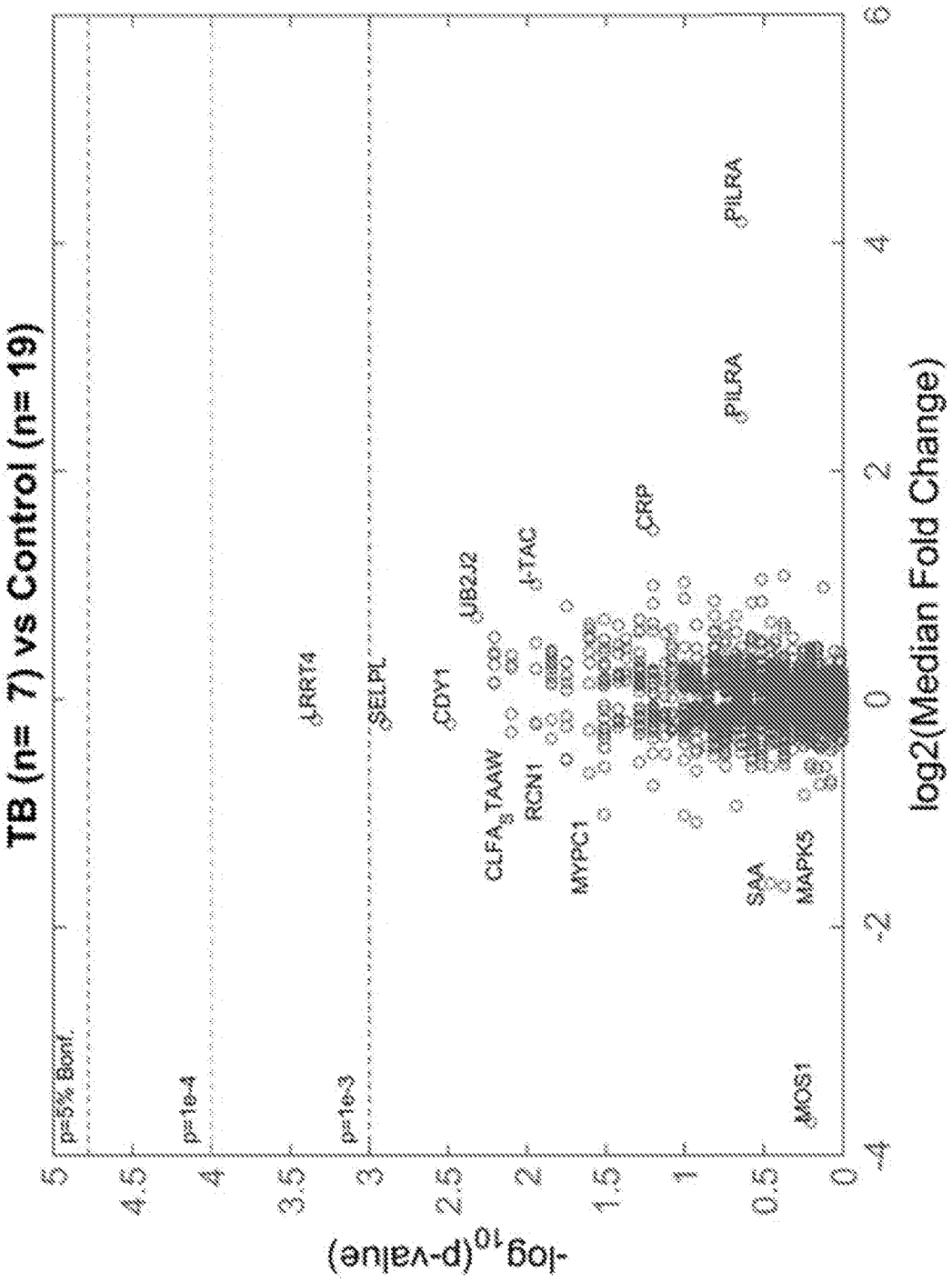

TB (n= 7) vs Control (n= 19)
p=5% Bonf.
p=1e-4
p=1e-3
LRRT4
SELPL
CDY1
UBE2J2
CSPG
I-TAC
CLFA_TAAW
RCN1
MYPC1
CRP
PILRA
PILRA
SAA
MAPK5
MOS1
-log10(p-value)
log2(Median Fold Change)

FIG. 37

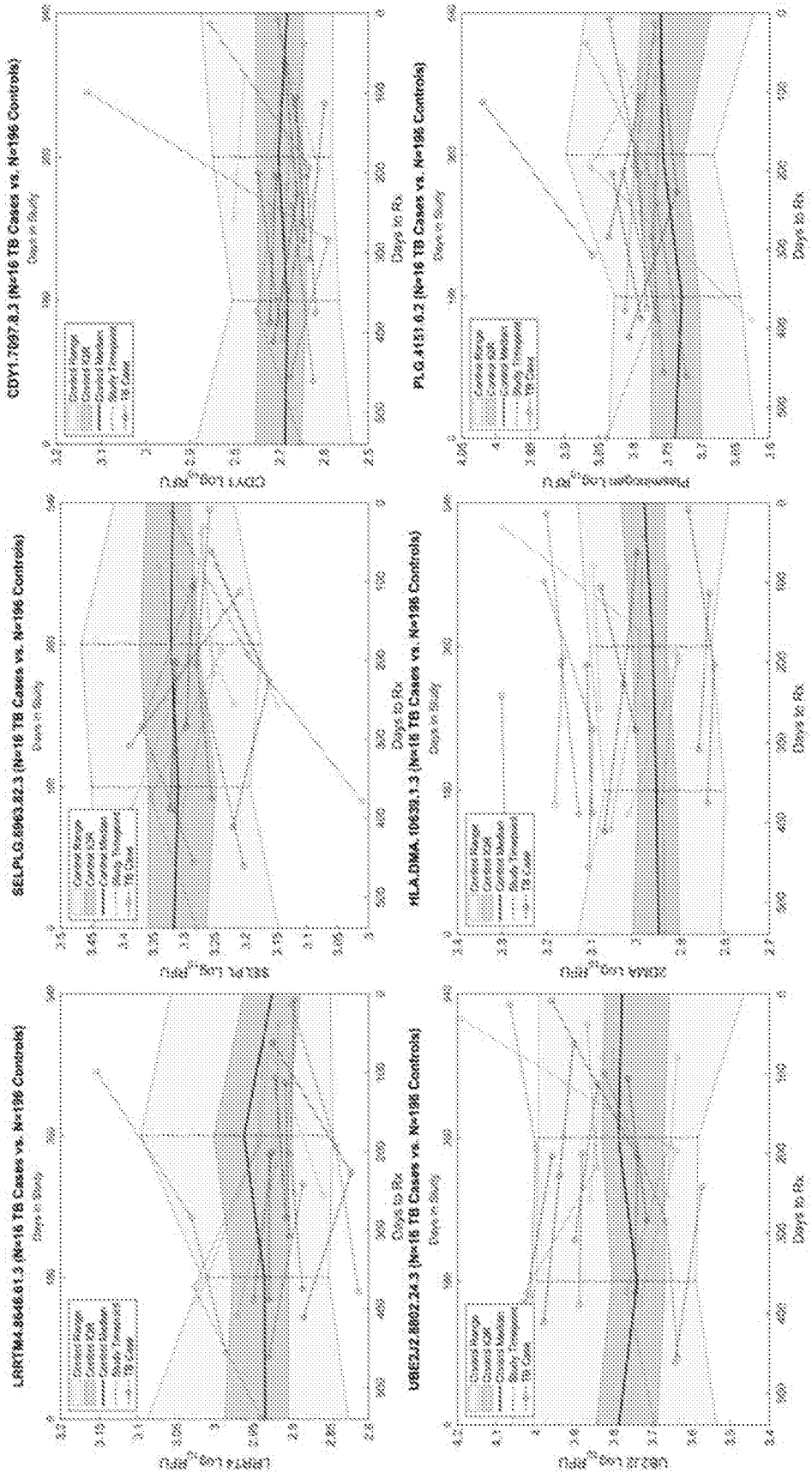

CD1.7697.8.3 (N=16 TB Cases vs. N=196 Controls)
PLG.3153.6.2 (N=16 TB Cases vs. N=196 Controls)
SELPLG.5963.22.3 (N=16 TB Cases vs. N=196 Controls)
HLA.DMA.10633.1.3 (N=16 TB Cases vs. N=196 Controls)
LRRTM4.8646.61.3 (N=16 TB Cases vs. N=196 Controls)
UBE3JJ2.8802.24.3 (N=16 TB Cases vs. N=196 Controls)
Days in Study
Days to Rx
Control Range
Control IQR
Control Median
Study Threshold
TB Case

FIG. 38

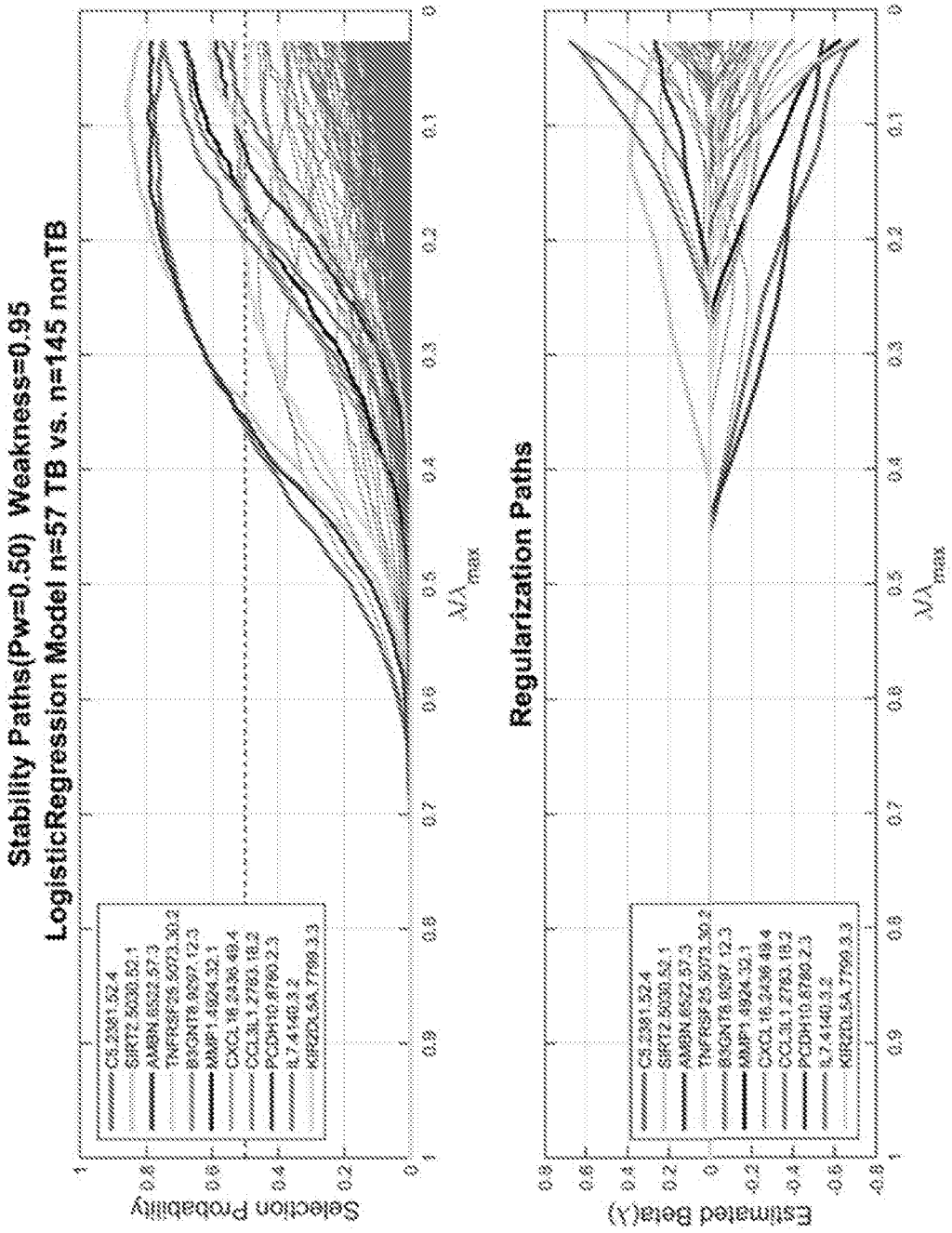

Stability Paths(Pw=0.50) Weakness=0.95
LogisticRegression Model n=57 TB vs. n=145 nonTB
Selection Probability
λ/Λ_{max}
C5.2381.52.4
SIRT2.5030.52.1
AMBN.6522.57.3
TNFRSF25.8073.30.2
BICDNT6.6090.12.3
MMP1.4924.32.1
CXCL16.2436.49.4
CCL3L1.2783.18.2
PCDH10.8760.2.3
IL7.4146.3.2
KIRREL3A.7799.3.3
Regularization Paths
Estimated Beta(λ)
λ/Λ_{max}

FIG. 39

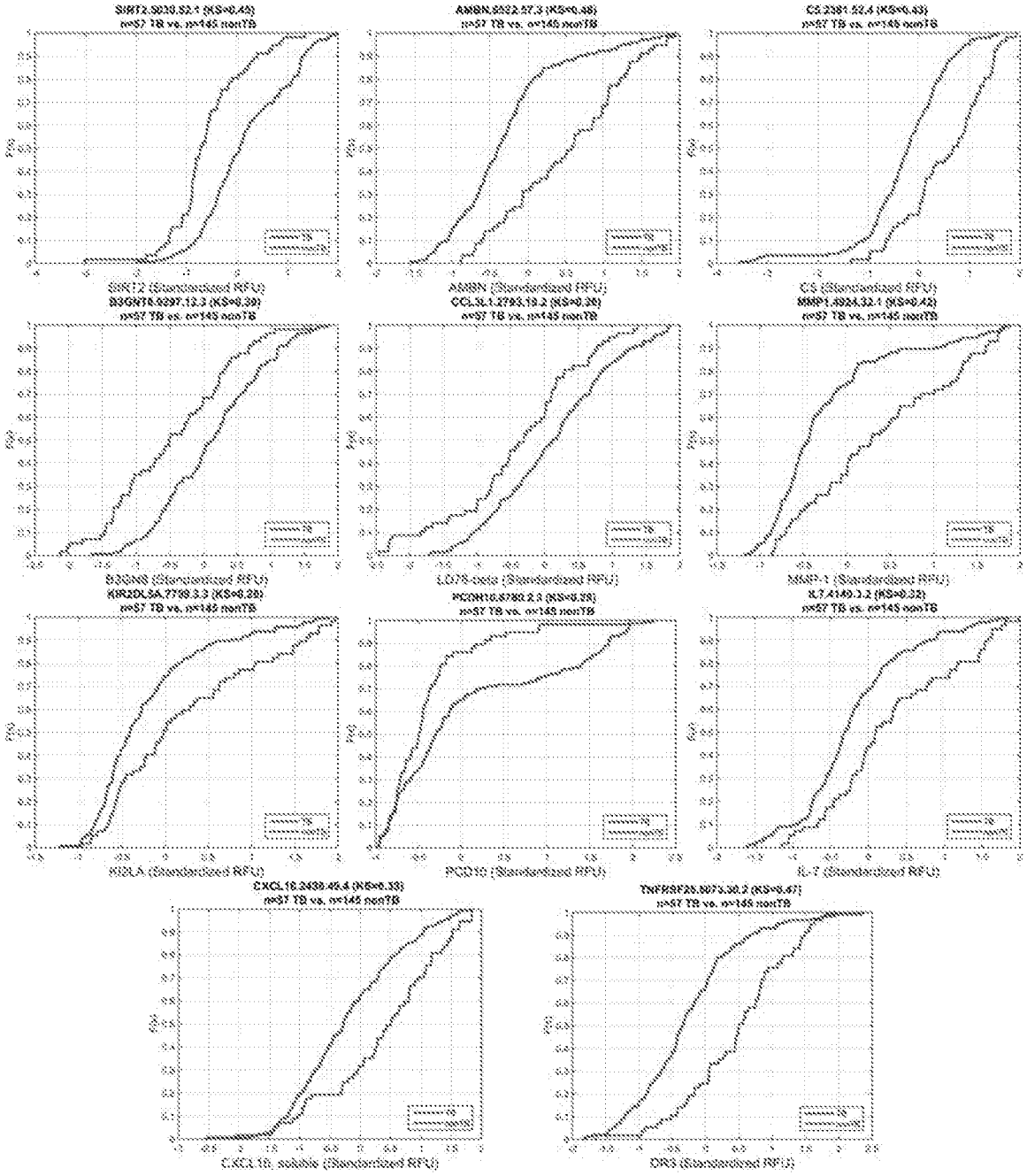

SIRT2.9239.52.1 (KS=0.42)
n=57 TB vs. n=145 nonTB
AMBN.6922.27.3 (KS=0.48)
n=57 TB vs. n=145 nonTB
CS.2381.52.4 (KS=0.43)
n=57 TB vs. n=145 nonTB
SIRT2 (Standardized RFU)
B3GNT8.9397.12.3 (KS=0.39)
n=57 TB vs. n=145 nonTB
AMBN (Standardized RFU)
CCL3L1.2793.13.2 (KS=0.39)
n=57 TB vs. n=145 nonTB
CS (Standardized RFU)
MMP1.4924.32.1 (KS=0.42)
n=57 TB vs. n=145 nonTB
B3GNT8 (Standardized RFU)
KIR2DL3A.7798.3.3 (KS=0.39)
n=57 TB vs. n=145 nonTB
LG75 beta (Standardized RFU)
PCDH10.6788.2.3 (KS=0.38)
n=57 TB vs. n=145 nonTB
MMP-1 (Standardized RFU)
IL7.4149.3.2 (KS=0.32)
n=57 TB vs. n=145 nonTB
KIR3LA (Standardized RFU)
PCD10 (Standardized RFU)
IL-7 (Standardized RFU)
CXCL10.2438.46.4 (KS=0.33)
n=57 TB vs. n=145 nonTB
TNFRSF25.8073.30.2 (KS=0.47)
n=57 TB vs. n=145 nonTB
CXCL10, soluble (Standardized RFU)
DR3 (Standardized RFU)

FIG. 40

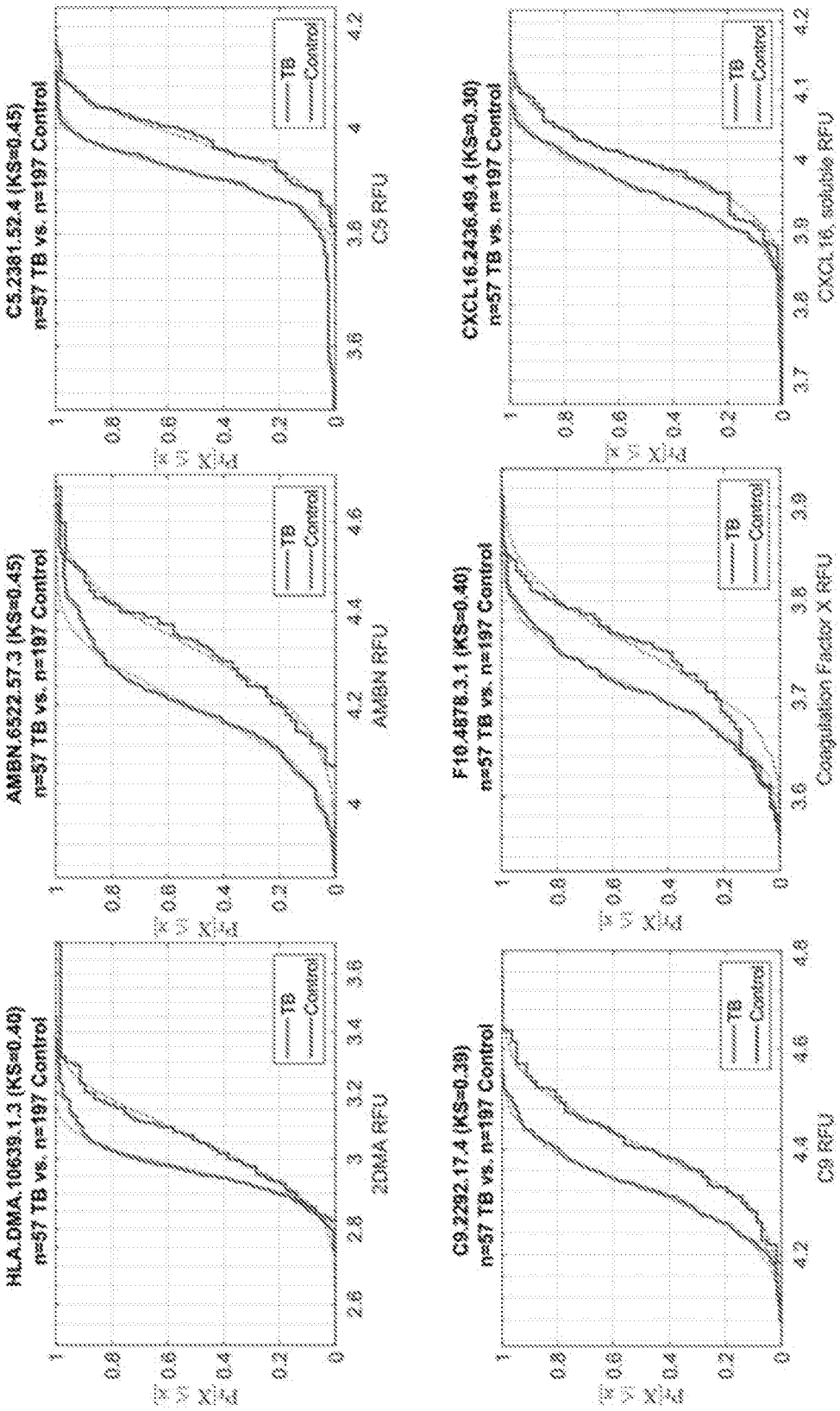

HLA.DMA.10638.1.3 (KS=0.40)
n=57 TB vs. n=197 Control
2DMA RFU
AMBN.6522.57.3 (KS=0.45)
n=57 TB vs. n=197 Control
AMBN RFU
C5.2381.52.4 (KS=0.45)
n=57 TB vs. n=197 Control
C5 RFU
F10.4878.3.1 (KS=0.40)
n=57 TB vs. n=197 Control
Coagulation Factor X RFU
CXCL16.2436.49.4 (KS=0.30)
n=57 TB vs. n=197 Control
CXCL16, soluble RFU
C9.2292.17.4 (KS=0.39)
n=57 TB vs. n=197 Control
C9 RFU
TB
Control
P[X ≤ x]

FIG. 40 (cont.)

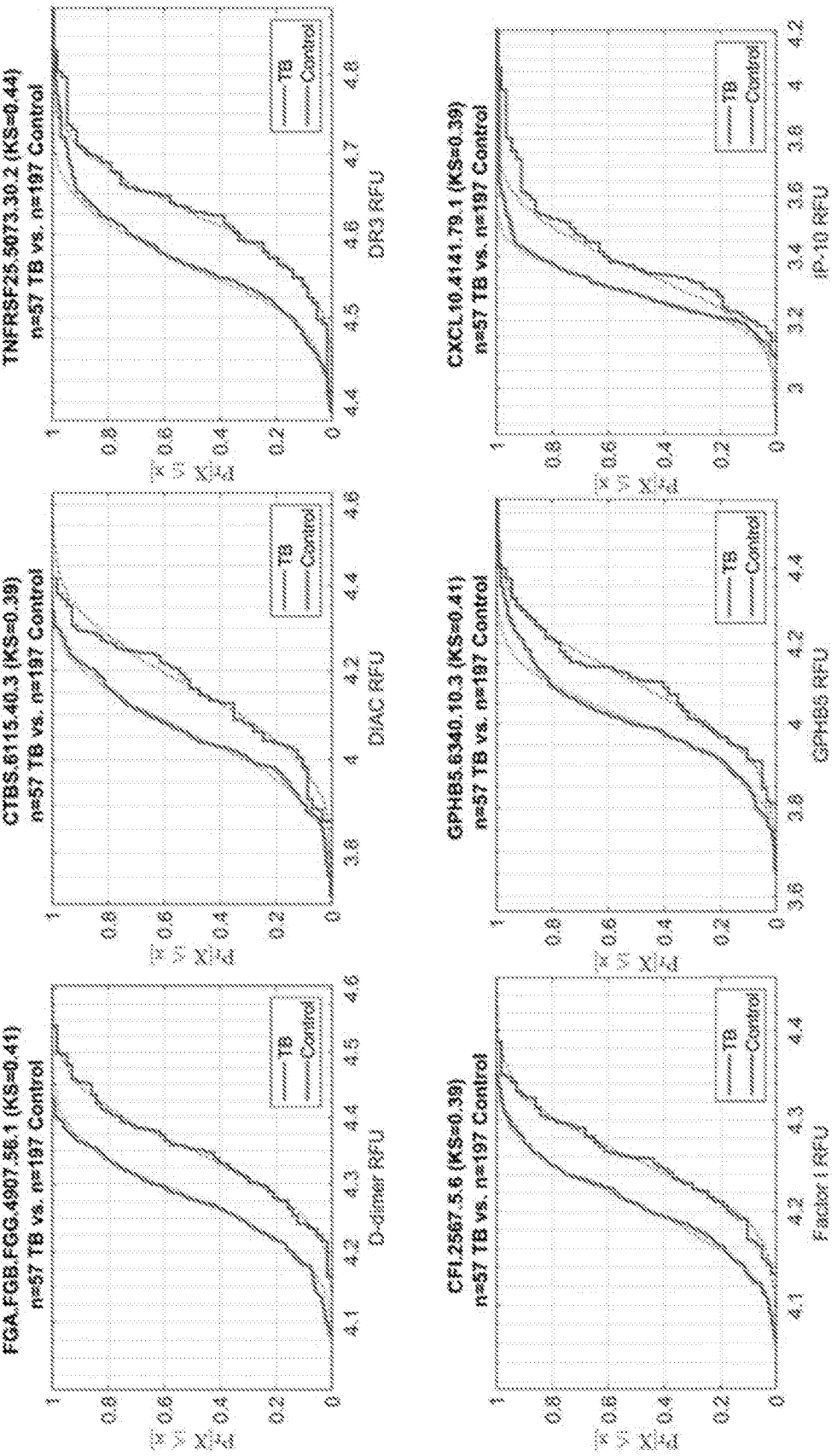

TNFRSF25.5073.30.2 (KS=0.44)
n=57 TB vs. n=187 Control
DR3 RFU
CTBS.6115.40.3 (KS=0.39)
n=57 TB vs. n=187 Control
DIAC RFU
GPHB5.6340.10.3 (KS=0.41)
n=57 TB vs. n=187 Control
GPHB5 RFU
CXCL10.4141.79.1 (KS=0.39)
n=57 TB vs. n=197 Control
IP-10 RFU
FGA.FGB.FGG.4007.56.1 (KS=0.41)
n=57 TB vs. n=187 Control
D-dimer RFU
CFI.2567.5.6 (KS=0.39)
n=57 TB vs. n=197 Control
Factor I RFU
TB
Control
P(X ≤ x)

FIG. 40 (cont.)

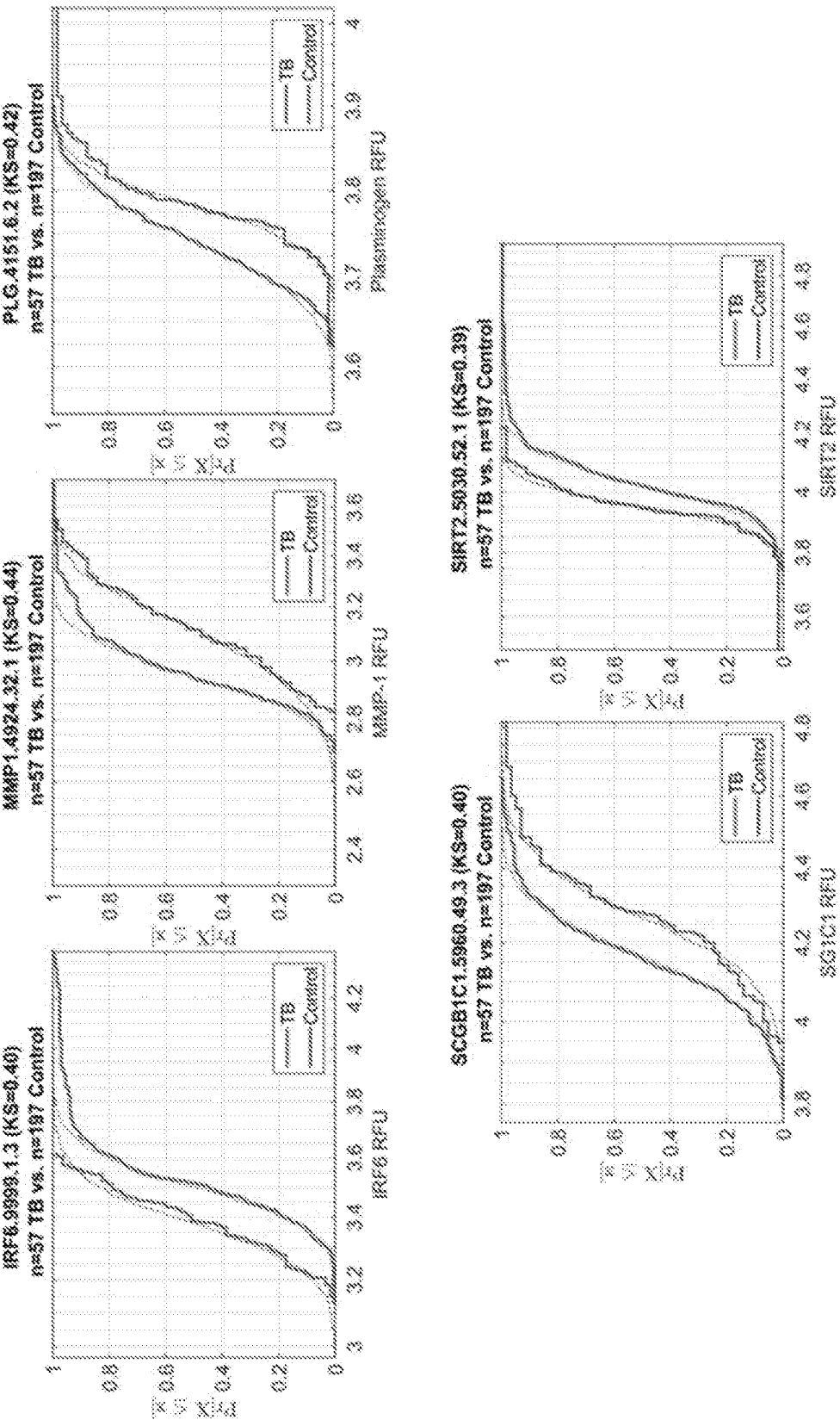

IRF6.9999.1.3 (KS=0.40)
n=57 TB vs. n=197 Control
IRF6 RFU
MMP1.4924.32.1 (KS=0.44)
n=57 TB vs. n=197 Control
MMP-1 RFU
PLG.4151.6.2 (KS=0.42)
n=57 TB vs. n=197 Control
Plasminogen RFU
SCCB1C1.5960.49.3 (KS=0.40)
n=57 TB vs. n=197 Control
SG1C1 RFU
SIRT2.5030.52.1 (KS=0.39)
n=57 TB vs. n=197 Control
SIRT2 RFU
TB
Control
P(X ≤ x)

FIG. 41

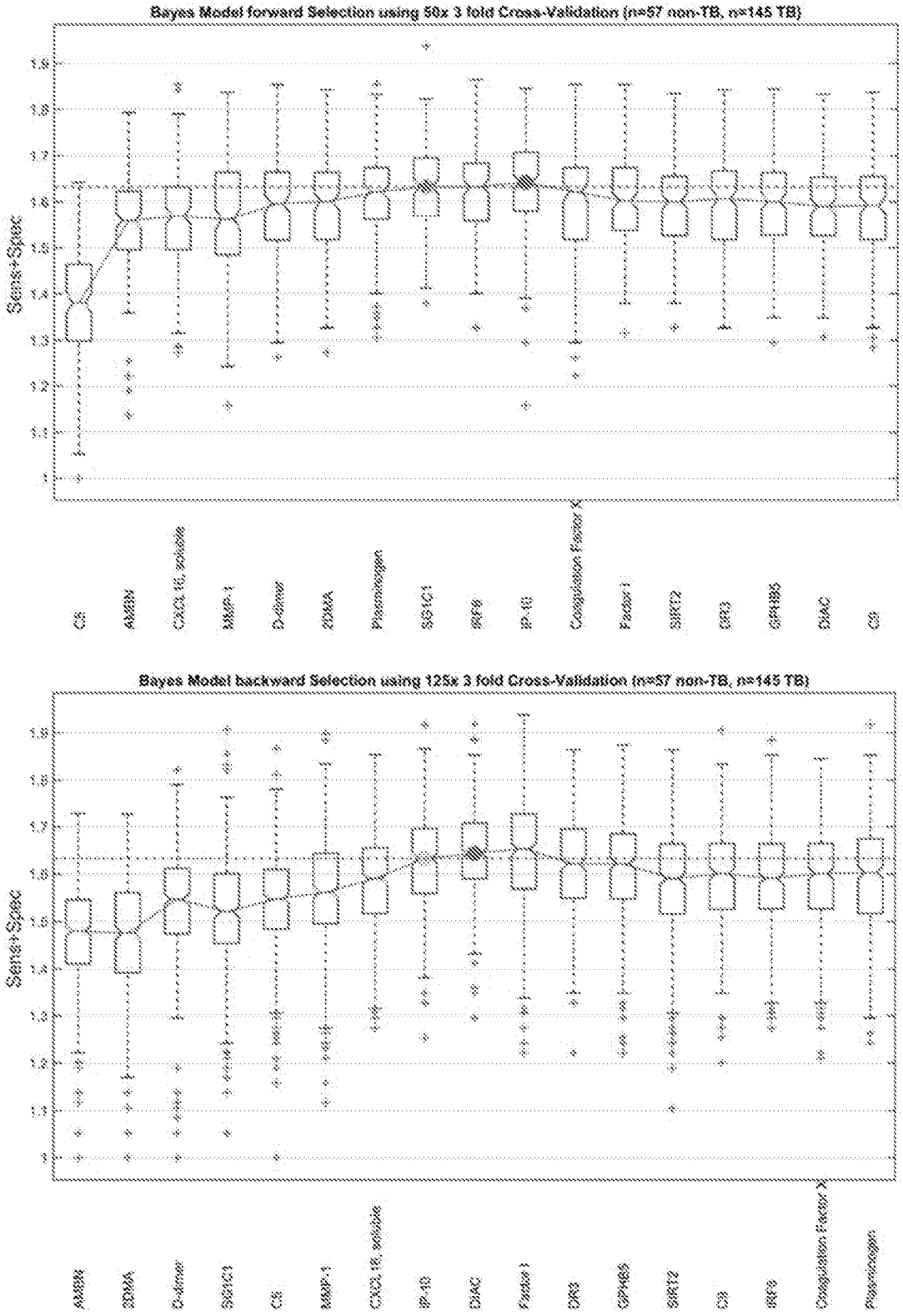

Bayes Model forward Selection using 50x 3 fold Cross-Validation (n=57 non-TB, n=145 TB)
Sens+Spec
C8
AMBN
CXCL16, soluble
MMP-1
D-dimer
2DMA
Fibrinogen
SB1C1
IRF8
IP-10
Coagulation Factor X
Factor I
SIRT2
GH3
GPHB5
DMC
C9
Bayes Model backward Selection using 125x 3 fold Cross-Validation (n=57 non-TB, n=145 TB)
Sens+Spec
AMBN
2DMA
D-dimer
SB1C1
C8
MMP-1
CXCL16, soluble
IP-10
DMC
Factor I
GH3
GPHB5
SIRT2
C9
IRF8
Coagulation Factor X
Fibrinogen

FIG. 42

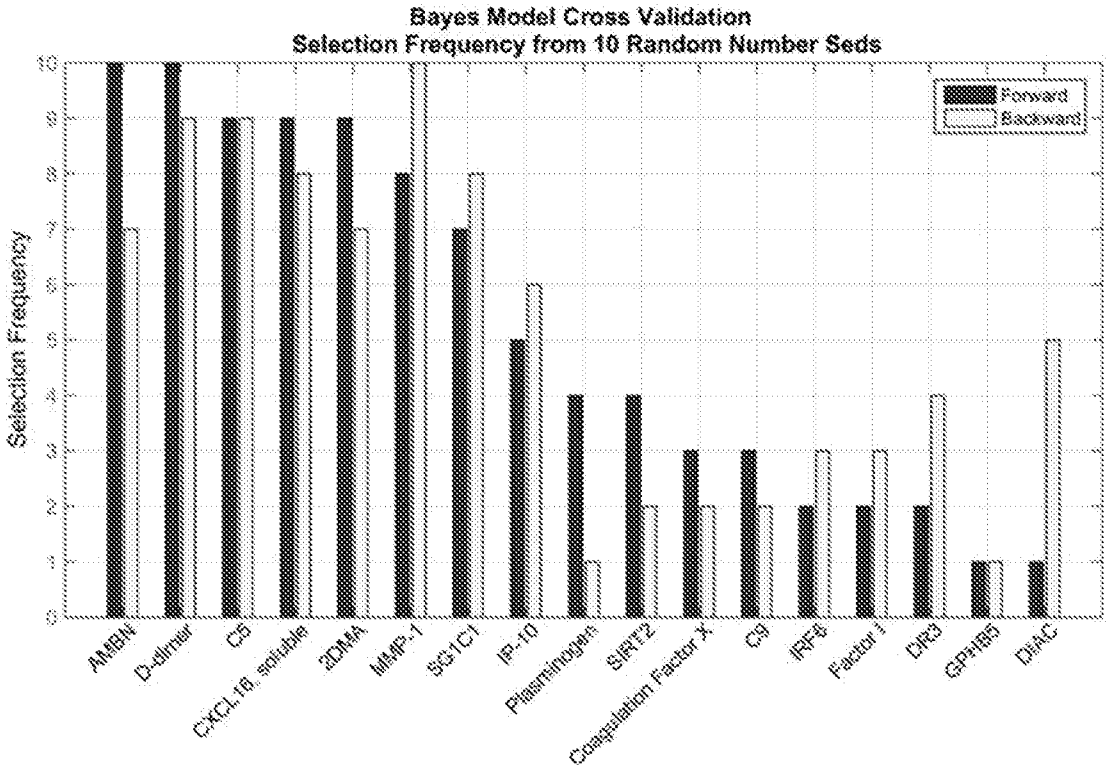

Bayes Model Cross Validation
Selection Frequency from 10 Random Number Seeds
Selection Frequency
Forward
Backward
AMBN
D-dimer
C5
CXCL16, soluble
2DMA
MMP-1
SG1C1
IP-10
Plasminogen
SIRT2
Coagulation Factor X
C9
IRF6
Factor 1
DR3
GPnB5
DEAC

FIG. 43

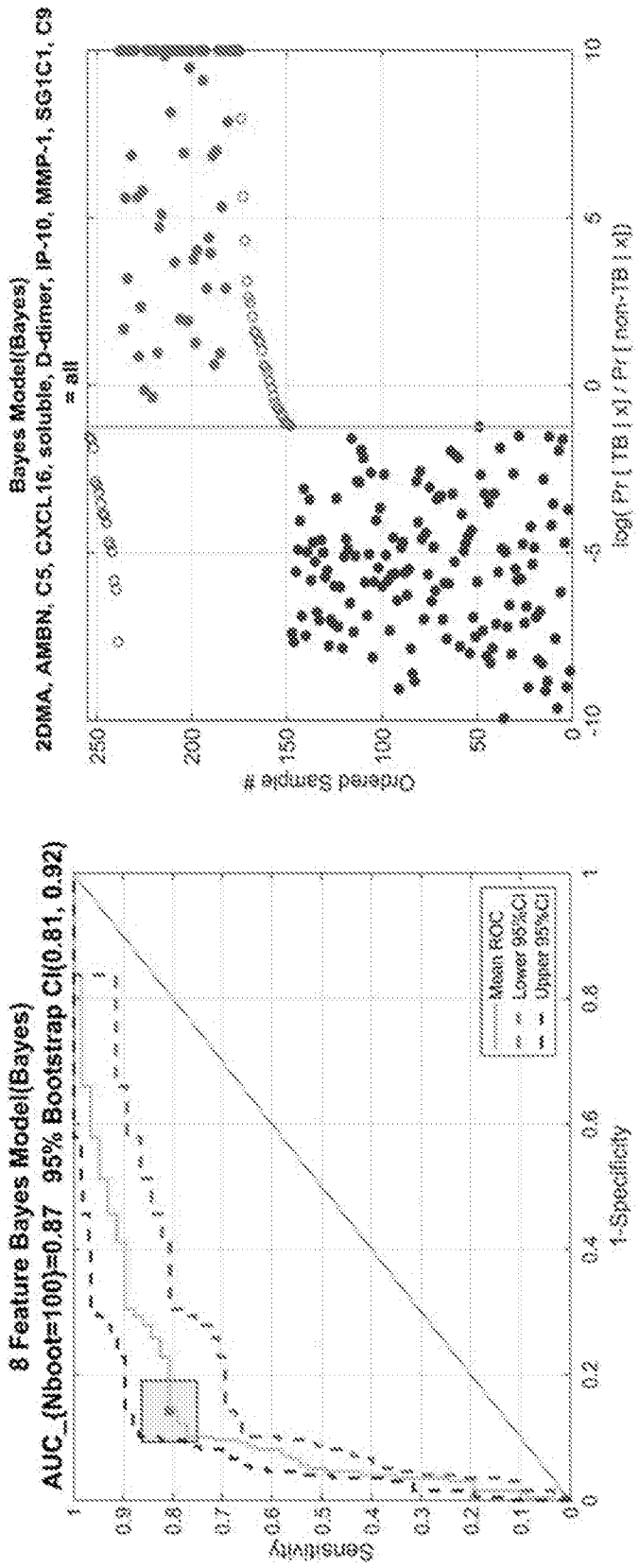

Bayes Model(Bayes)
2DMA, AMBN, C5, CXCL16, soluble, D-dimer, IP-10, MMP-1, SG1C1, C9
= all
Ordered Sample #
log(Pr [TB | x] / Pr [non-TB | x])
8 Feature Bayes Model(Bayes)
AUC_(Nboot=100)=0.87 95% Bootstrap CI(0.81, 0.92)
Sensitivity
1-Specificity
Mean ROC
Lower 95%CI
Upper 95%CI

FIG. 44

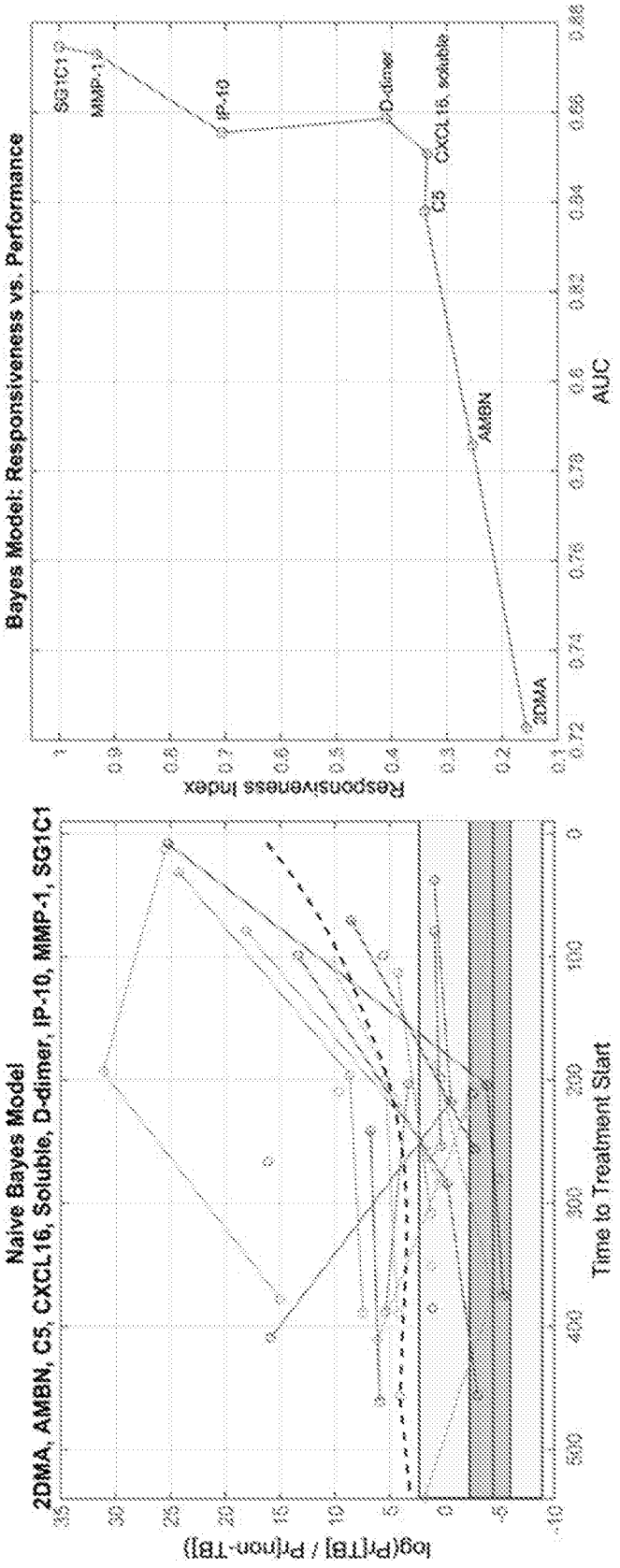

Bayes Model: Responsiveness vs. Performance
Responsiveness Index
AUC
SG1C1 P
MMP-1
IP-10
D-dimer
CXCL16, soluble
C5
AMBN
2DMA
Naive Bayes Model
2DMA, AMBN, C5, CXCL16, Soluble, D-dimer, IP-10, MMP-1, SG1C1
log(P[TB] / P[non-TB])
Time to Treatment Start

FIG. 45

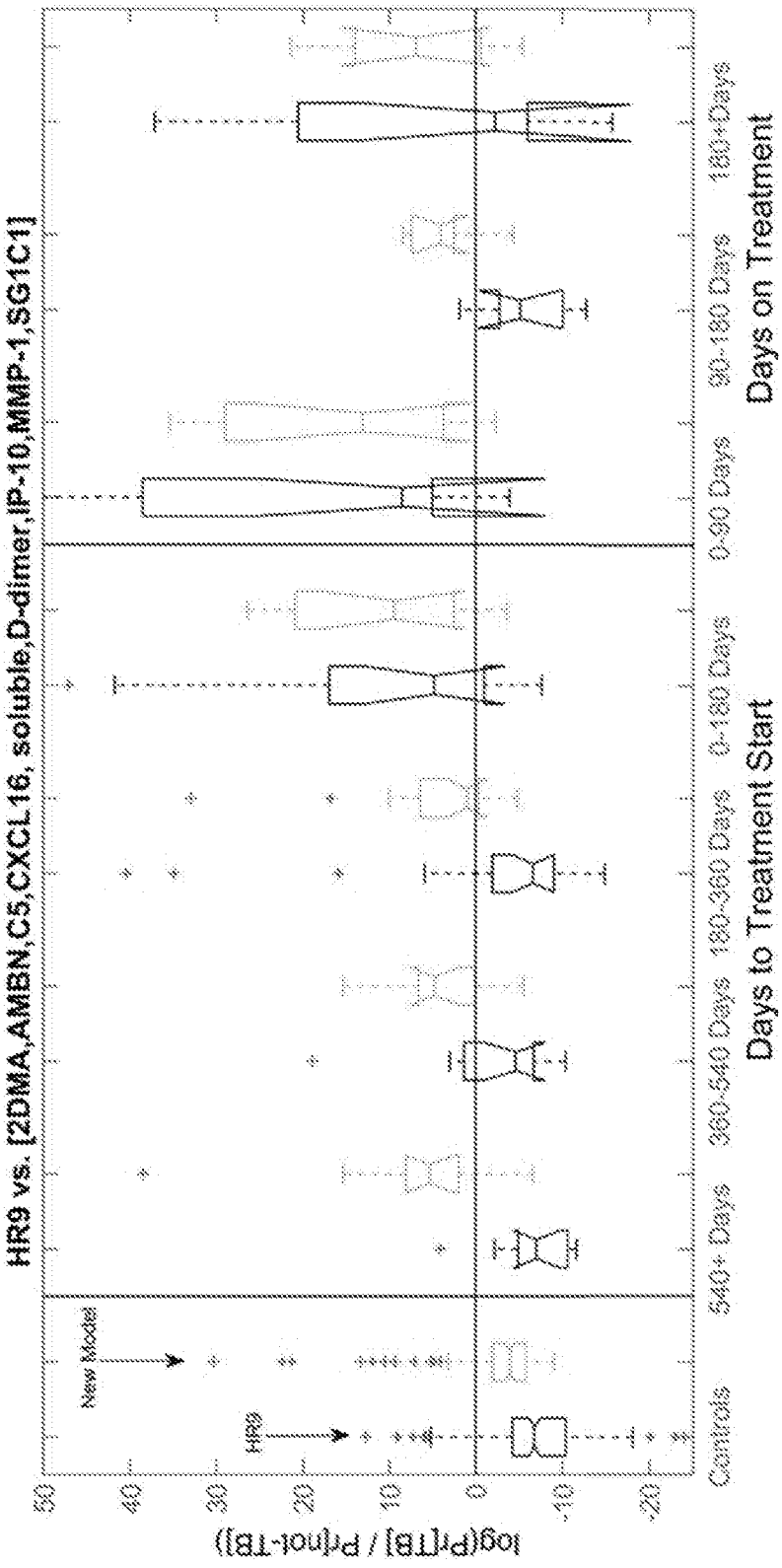

HR9 vs. [2DMA,AMBN,C5,CXCL16, soluble,D-dimer,IP-10,MMP-1,SG1C1]
log(Pr[TB] / Pr[non-TB])
60
40
30
20
10
0
-10
-20
New Model
HR9
Controls
540+ Days
360-540 Days
180-360 Days
0-180 Days
0-90 Days
90-180 Days
180+Days
Days to Treatment Start
Days on Treatment

FIG. 46

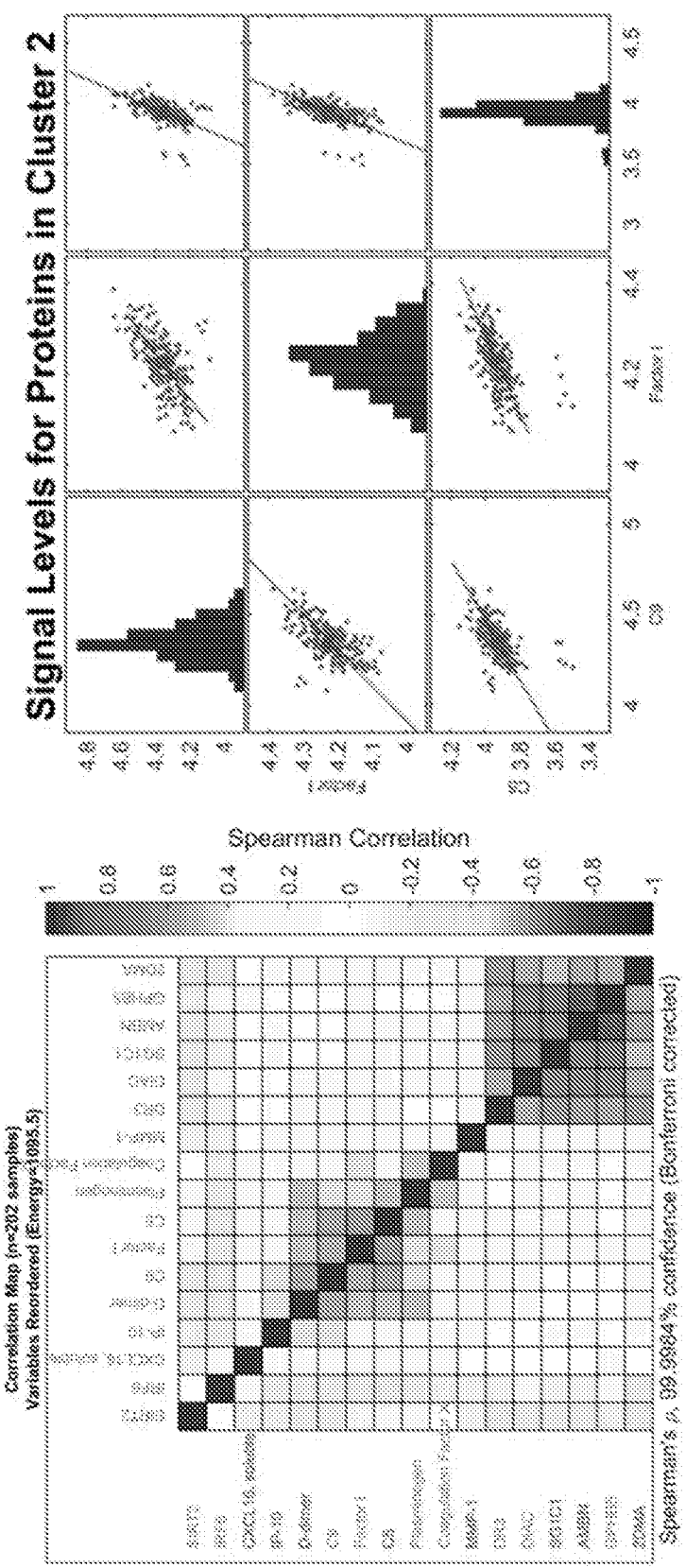

Signal Levels for Proteins in Cluster 2
Spearman Correlation
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
Correlation Map (n=202 samples)
Variables Reordered (Energy=1095.5)
Spearman's ρ, 99.9984% confidence (Bonferroni corrected)

FIG. 47

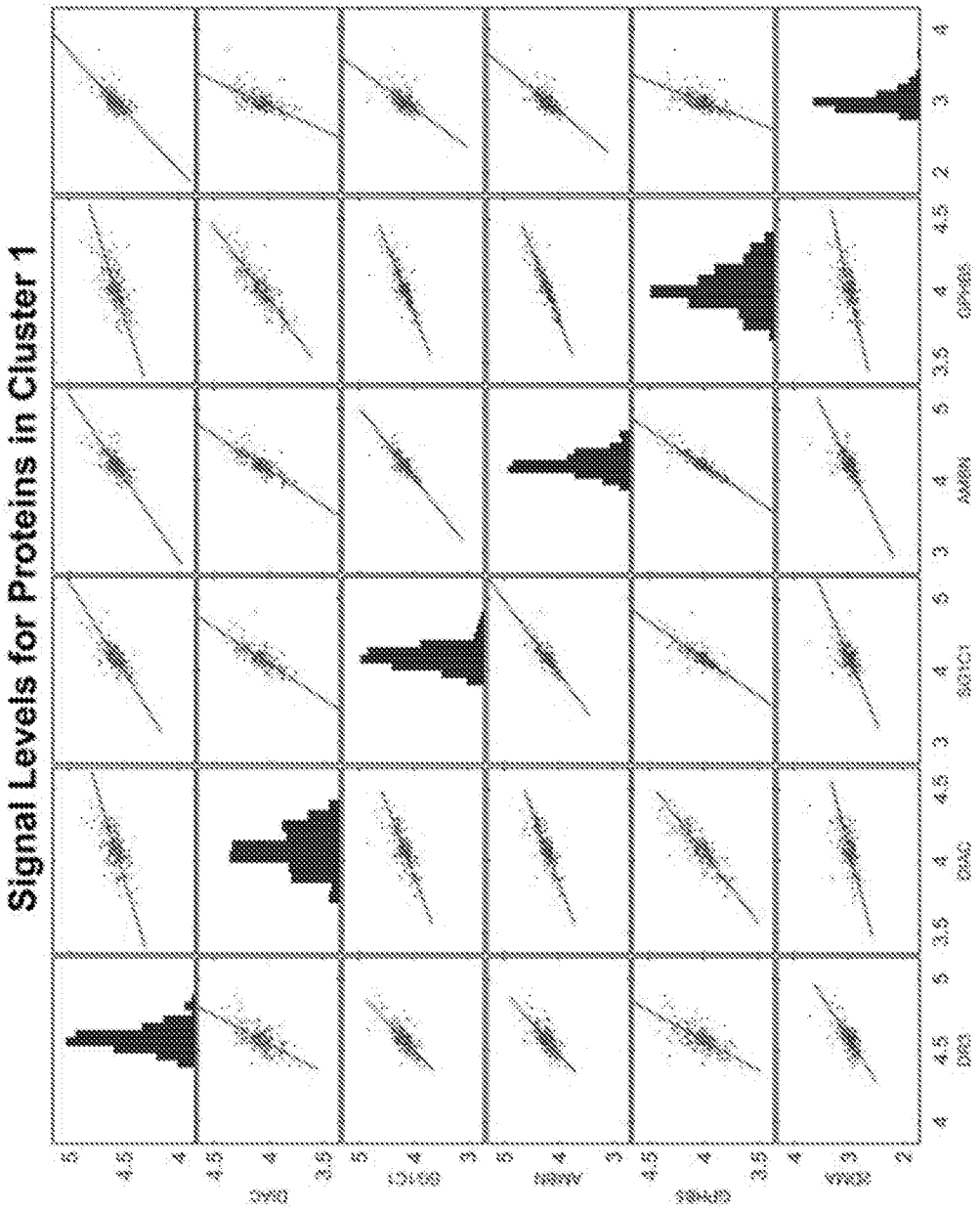

Signal Levels for Proteins in Cluster 1
DR3
DAG
SIGLEC1
AMBN
GPNMB
DR3
DAG
SIGLEC1
AMBN
GPNMB

FIG. 48

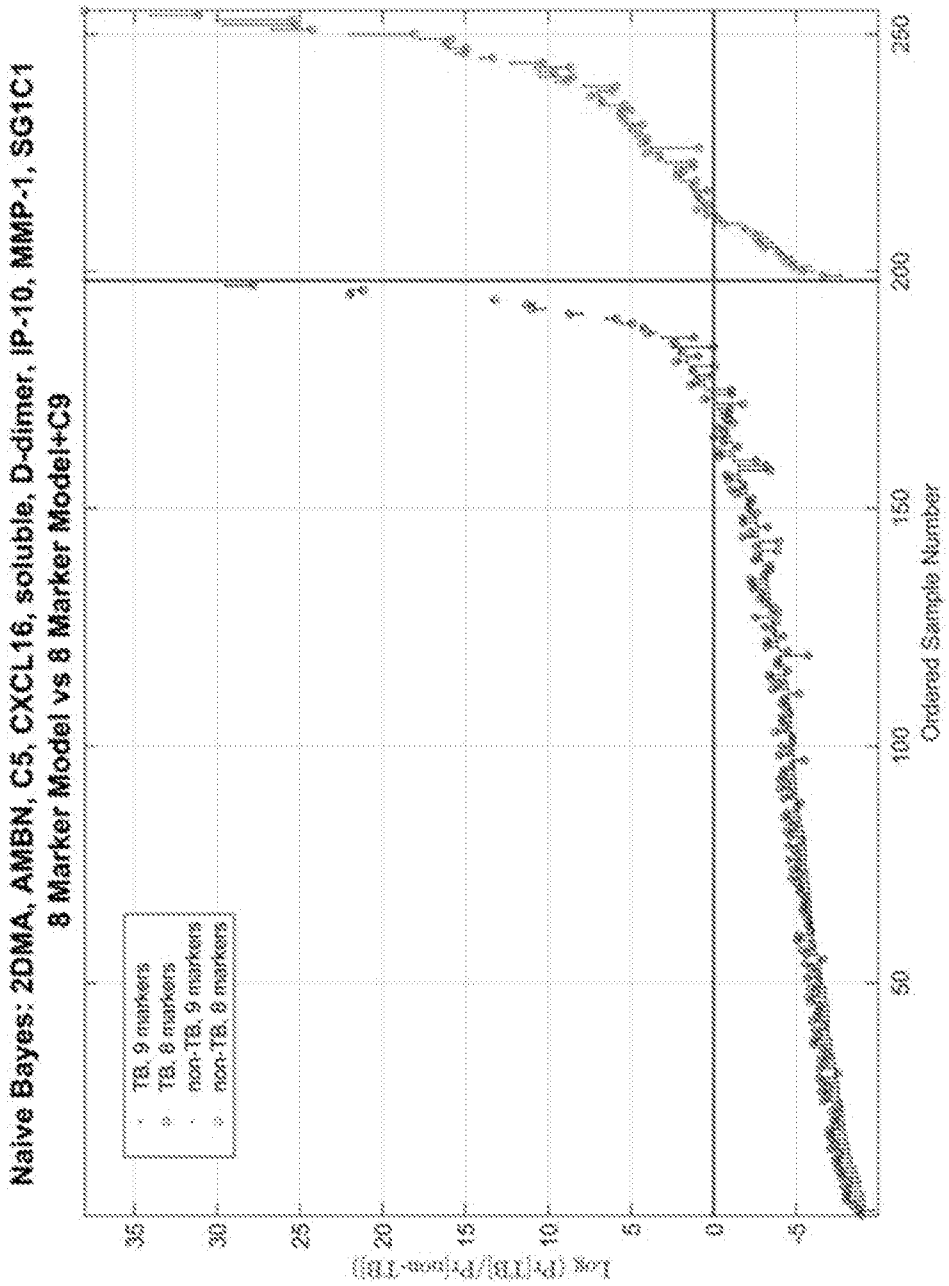

Naive Bayes: 2DMA, AMBN, C5, CXCL16, soluble, D-dimer, IP-10, MMP-1, SG1C1
8 Marker Model vs 8 Marker Model+C9
TB, 9 markers
TB, 8 markers
non-TB, 9 markers
non-TB, 8 markers
Log (Pr(TB)/Pr(non-TB))
Ordered Sample Number
35
30
25
20
15
10
5
0
-5
50
100
150
200
250

FIG. 49

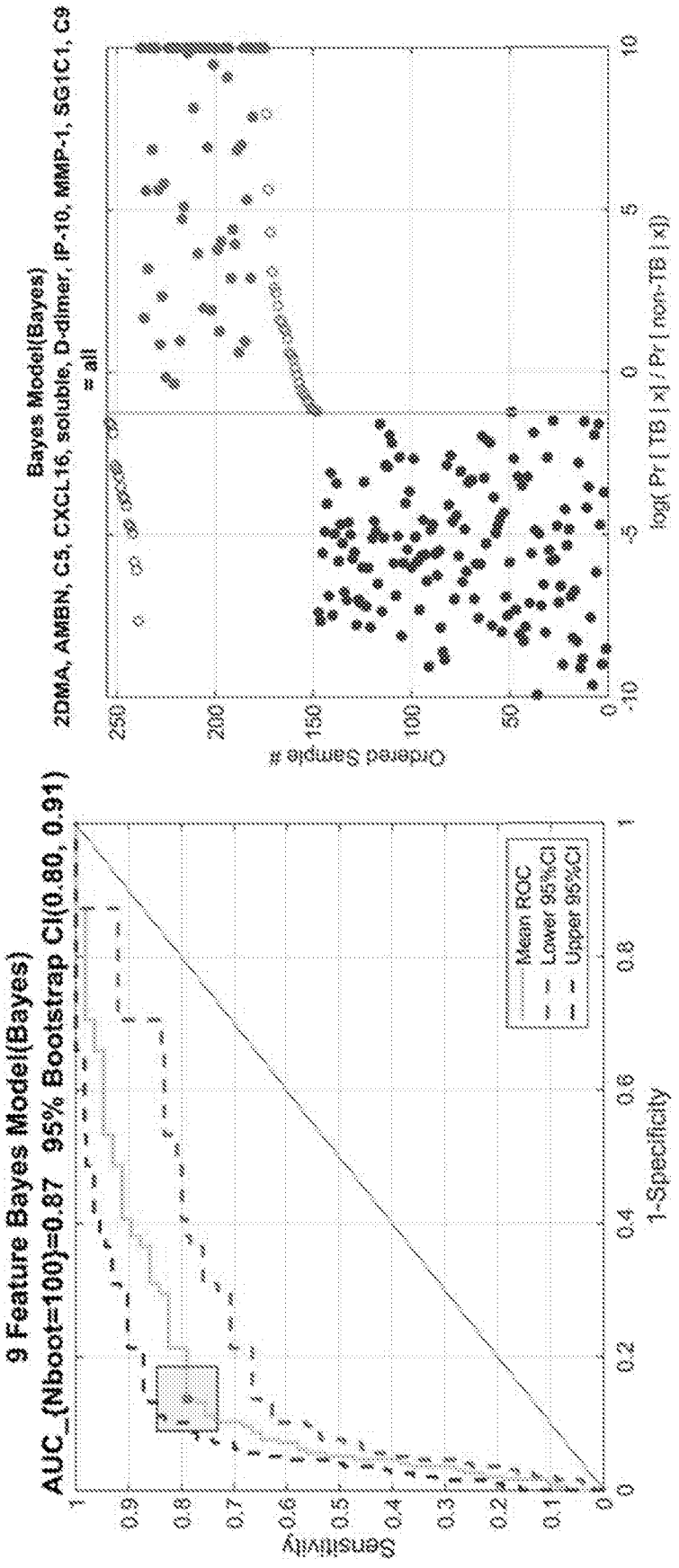

9 Feature Bayes Model(Bayes)
AUC_(Nboot=100)=0.87 95% Bootstrap CI(0.80, 0.91)
Sensitivity
1-Specificity
Mean ROC
Lower 95%CI
Upper 95%CI
Bayes Model(Bayes)
2DMA, AMBN, C5, CXCL16, soluble, D-dimer, IP-10, MMP-1, SG1C1, C9
= all
Ordered Sample #
log(Pr [TB | x] / Pr [non-TB | x])

FIG. 50

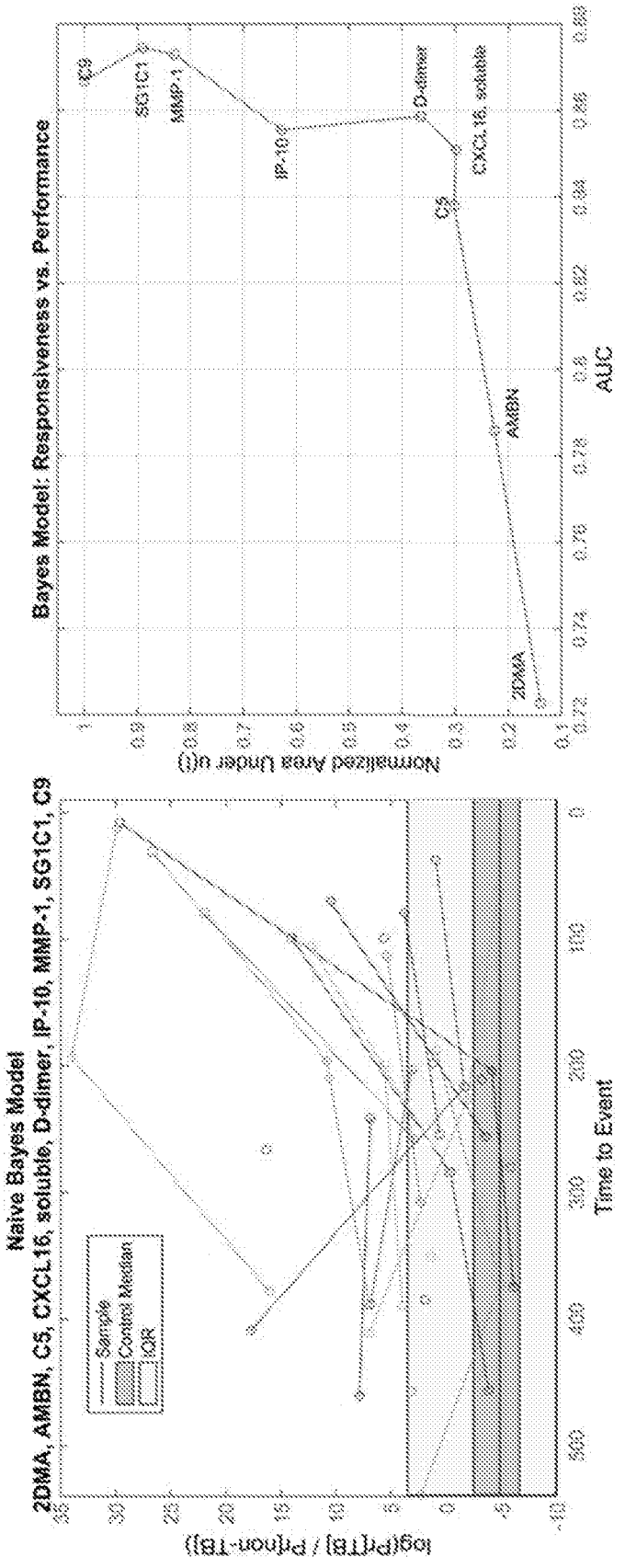

Bayes Model: Responsiveness vs. Performance
Normalized Area Under u(t)
AUC
C9
SG1C1
MMP-1
IP-10
D-dimer
C5
CXCL16, soluble
AMBN
2DMA
Naive Bayes Model
2DMA, AMBN, C5, CXCL16, soluble, D-dimer, IP-10, MMP-1, SG1C1, C9
log(P(TB) / P(non-TB))
Time to Event
Sample
Control Median
IQR

FIG. 51

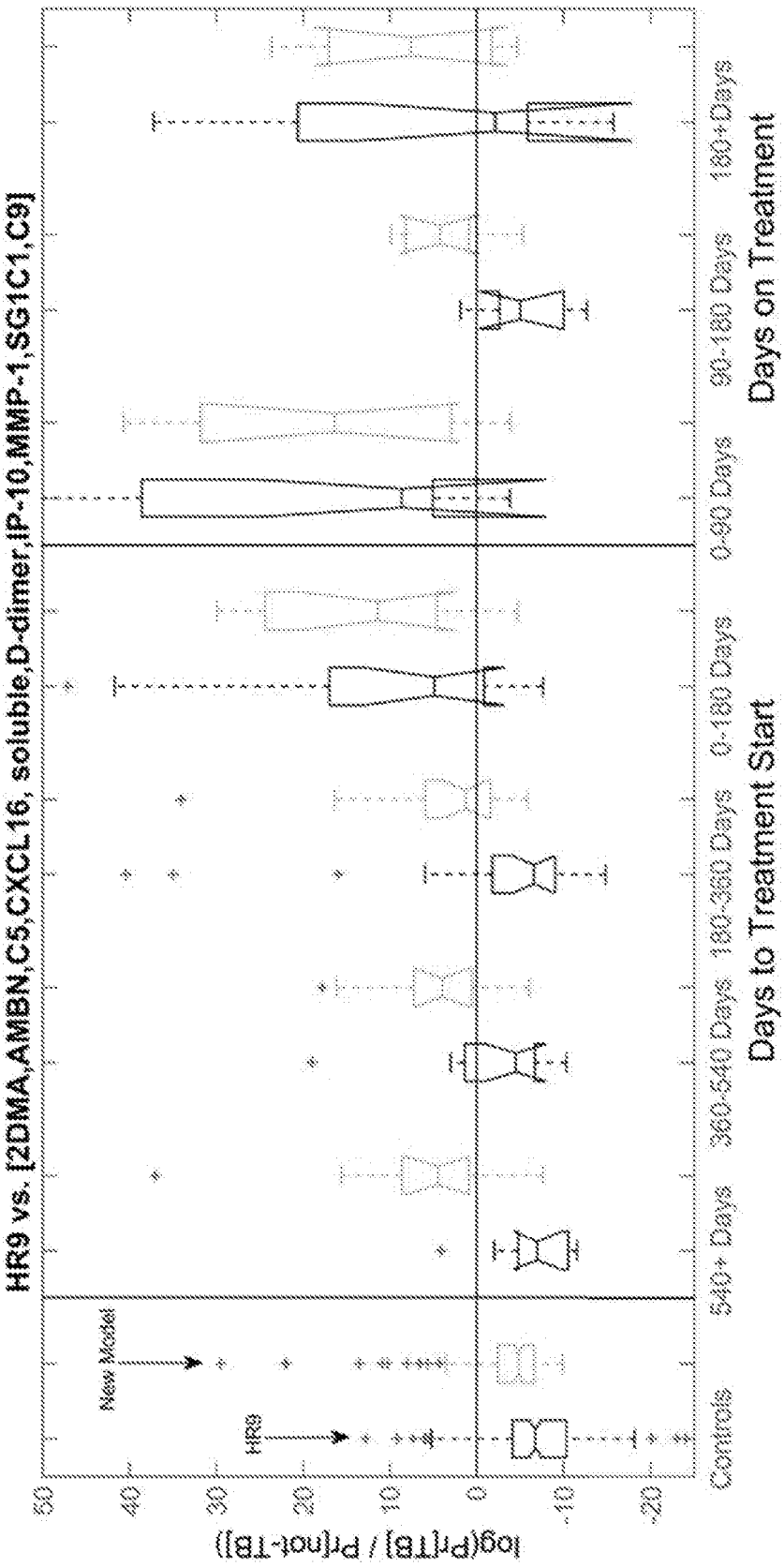
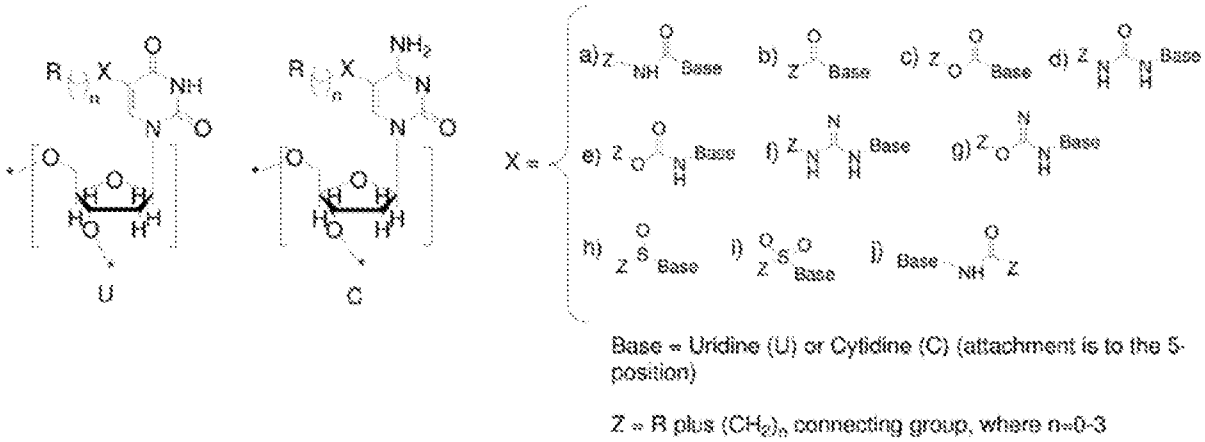

HR9 vs. [2DMA,AMBN,C5,CXCL16, soluble,D-dimer,IP-10,MMP-1,SG1C1,C9]
log(Pr[TB] / Pr[not-TB])
50
40
30
20
10
0
-10
-20
New Model
HR9
Controls
540+ Days
360-540 Days
180-360 Days
0-180 Days
0-90 Days
90-180 Days
180+Days
Days to Treatment Start
Days on Treatment

FIG. 52



Base = Uridine (U) or Cytidine (C) (attachment is to the 5-position)

Z = R plus $(CH_2)_n$ connecting group, where n=0-3

FIG. 52 (cont.)

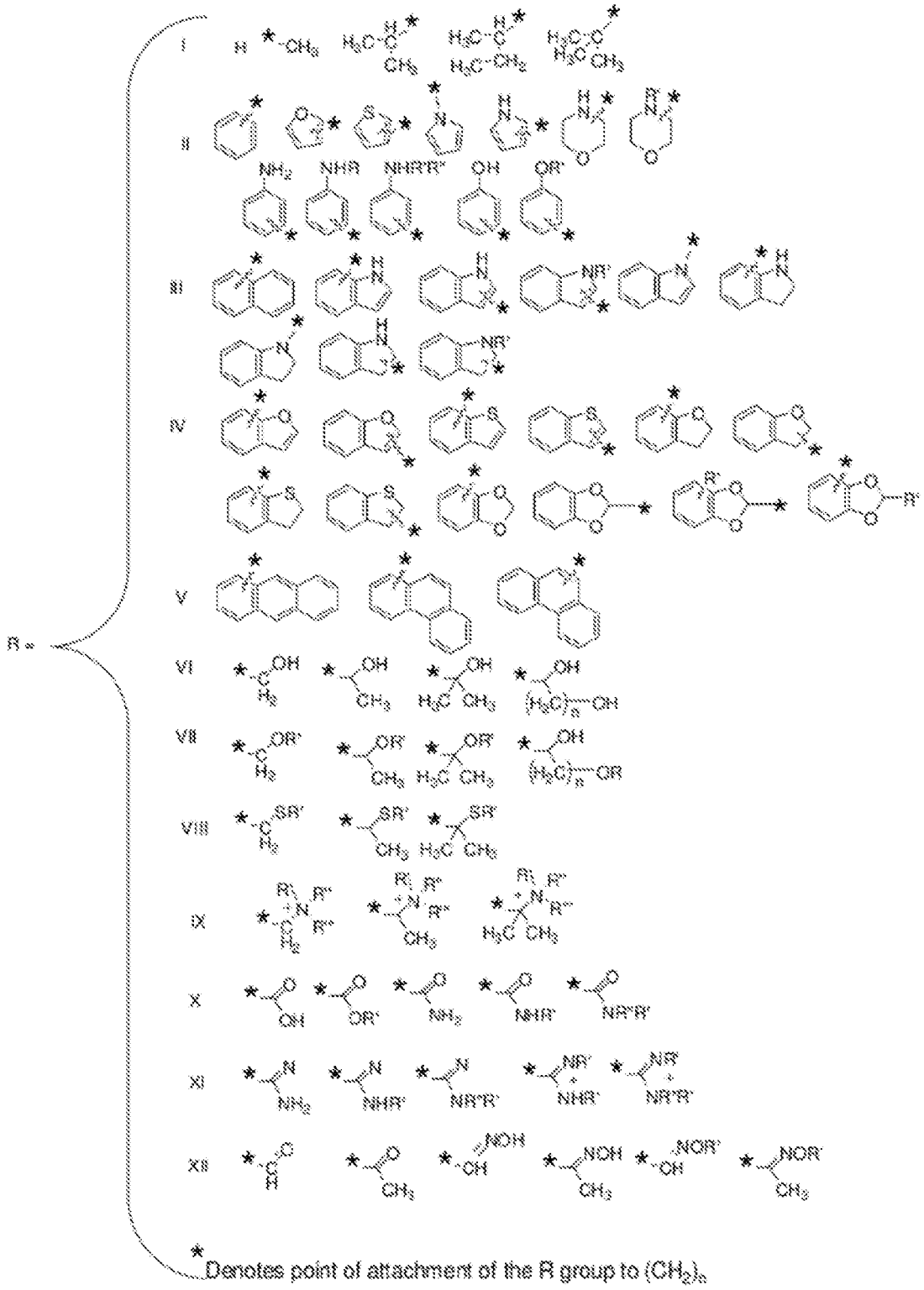

R =
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
* Denotes point of attachment of the R group to (CH2)n

FIG. 53

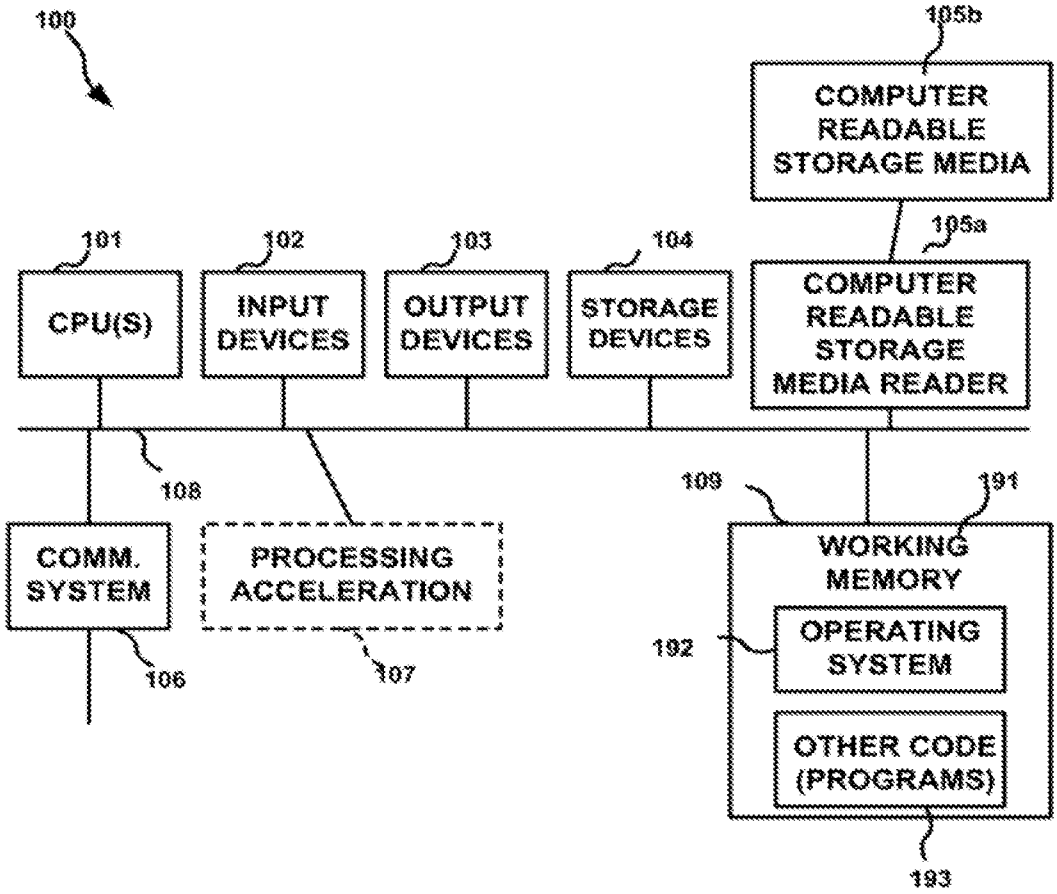

100
105b
COMPUTER READABLE STORAGE MEDIA
105a
101
102
103
104
CPU(S)
INPUT DEVICES
OUTPUT DEVICES
STORAGE DEVICES
COMPUTER READABLE STORAGE MEDIA READER
108
109
191
COMM. SYSTEM
PROCESSING ACCELERATION
WORKING MEMORY
192
OPERATING SYSTEM
OTHER CODE (PROGRAMS)
106
107
193

FIG. 54

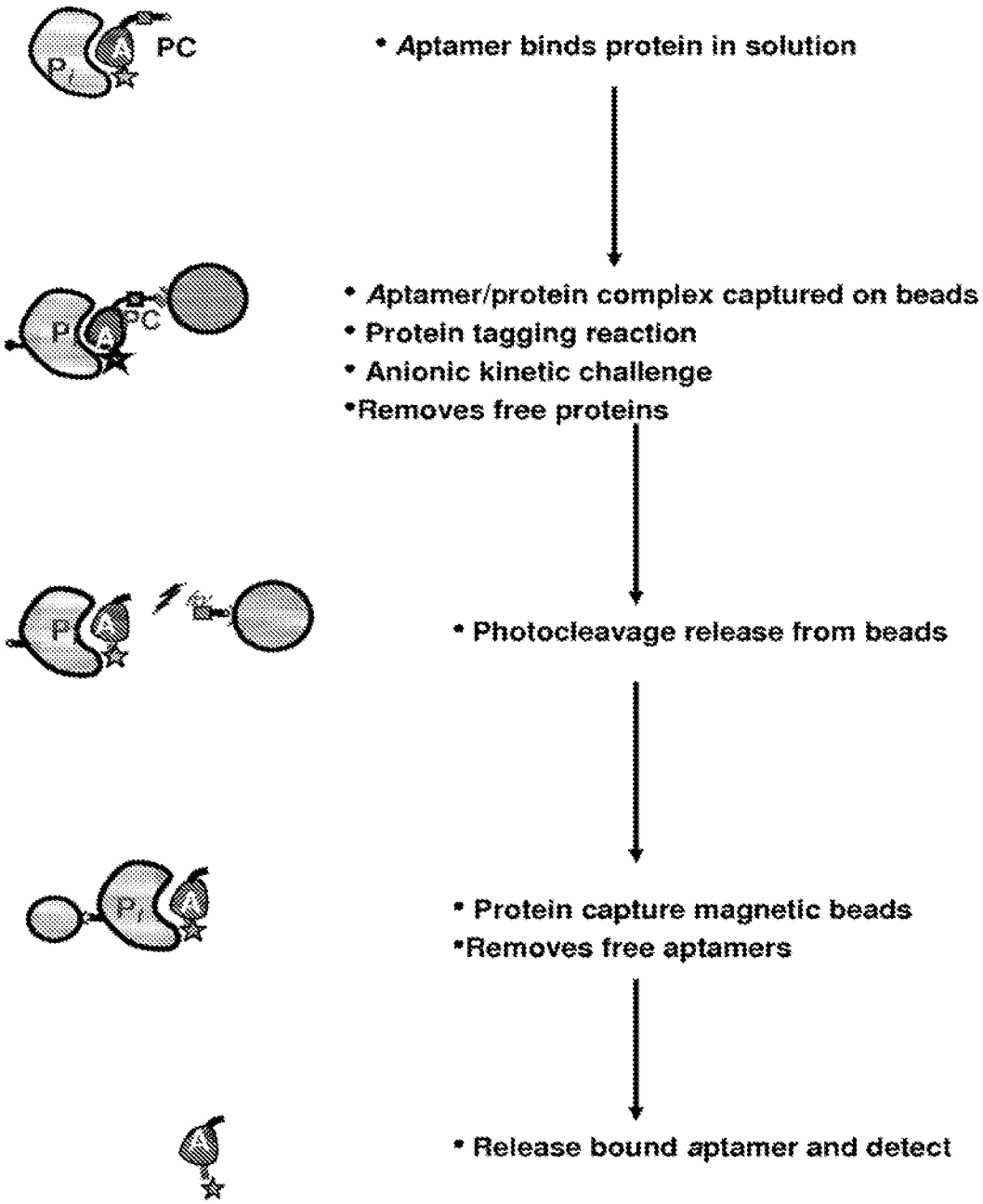

PC
• Aptamer binds protein in solution
• Aptamer/protein complex captured on beads
• Protein tagging reaction
• Anionic kinetic challenge
•Removes free proteins
• Photocleavage release from beads
• Protein capture magnetic beads
•Removes free aptamers
• Release bound aptamer and detect

FIG. 55

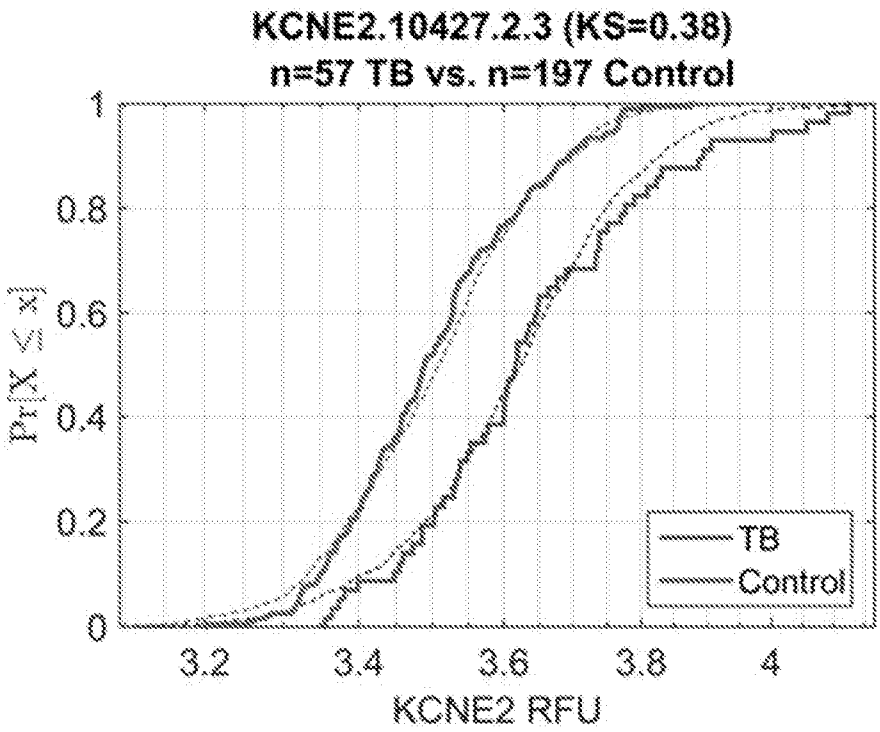

KCNE2.10427.2.3 (KS=0.38)
n=57 TB vs. n=197 Control
1
0.8
0.6
0.4
0.2
0
3.2
3.4
3.6
3.8
4
KCNE2 RFU
TB
Control

FIG. 56

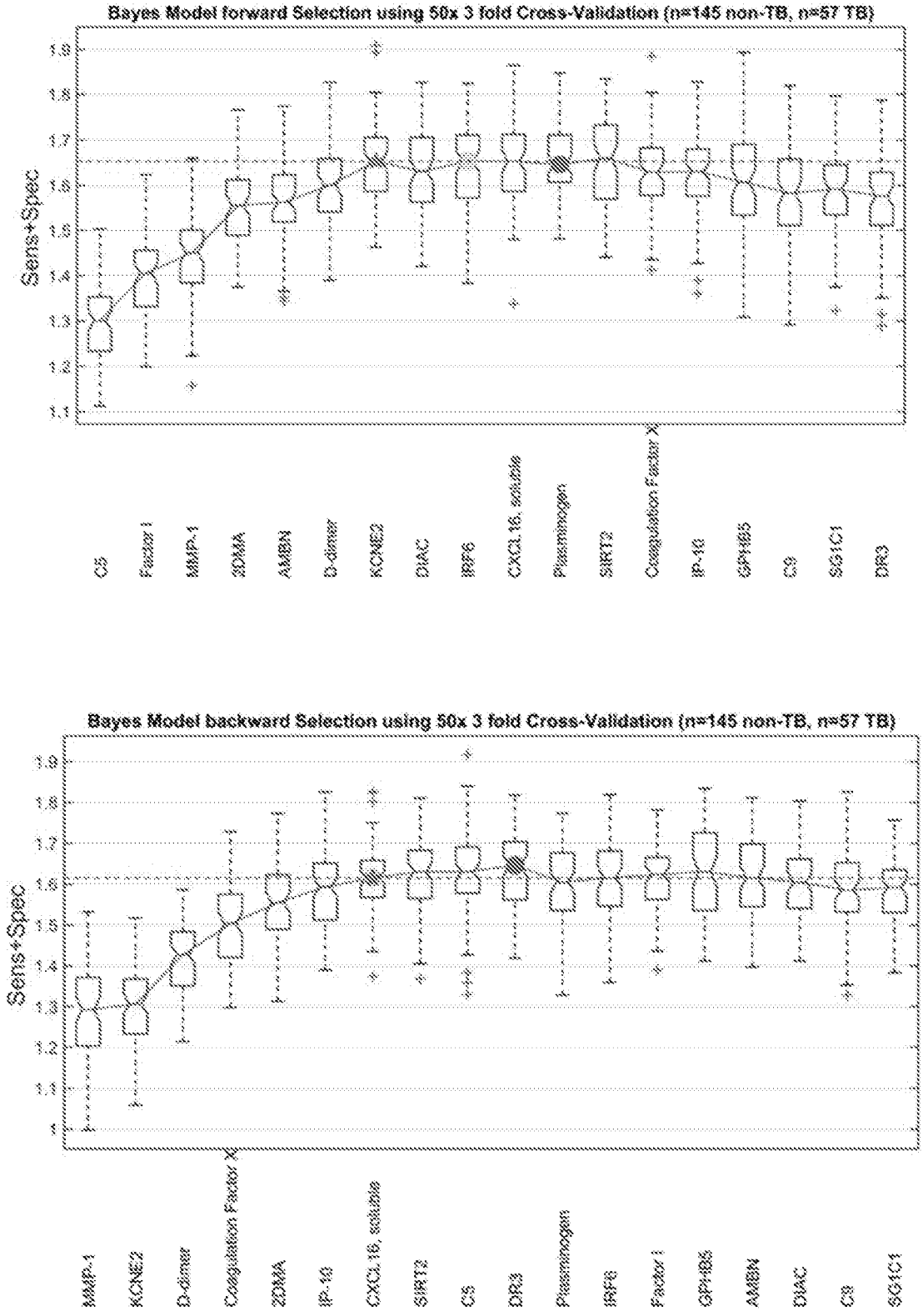

Bayes Model forward Selection using 50x 3 fold Cross-Validation (n=145 non-TB, n=57 TB)
Sens+Spec
C5
Factor I
MMP-1
2DMA
AMBN
D-dimer
KONE2
DIAC
IRF6
CXCL16, soluble
Plasminogen
SIRT2
Coagulation Factor X
IP-10
GPHB5
C9
SG1C1
DR3
Bayes Model backward Selection using 50x 3 fold Cross-Validation (n=145 non-TB, n=57 TB)
Sens+Spec
MMP-1
KONE2
D-dimer
Coagulation Factor X
2DMA
IP-10
CXCL16, soluble
SIRT2
C5
DR3
Plasminogen
IRF6
Factor I
GPHB5
AMBN
DIAC
C9
SG1C1

FIG. 57

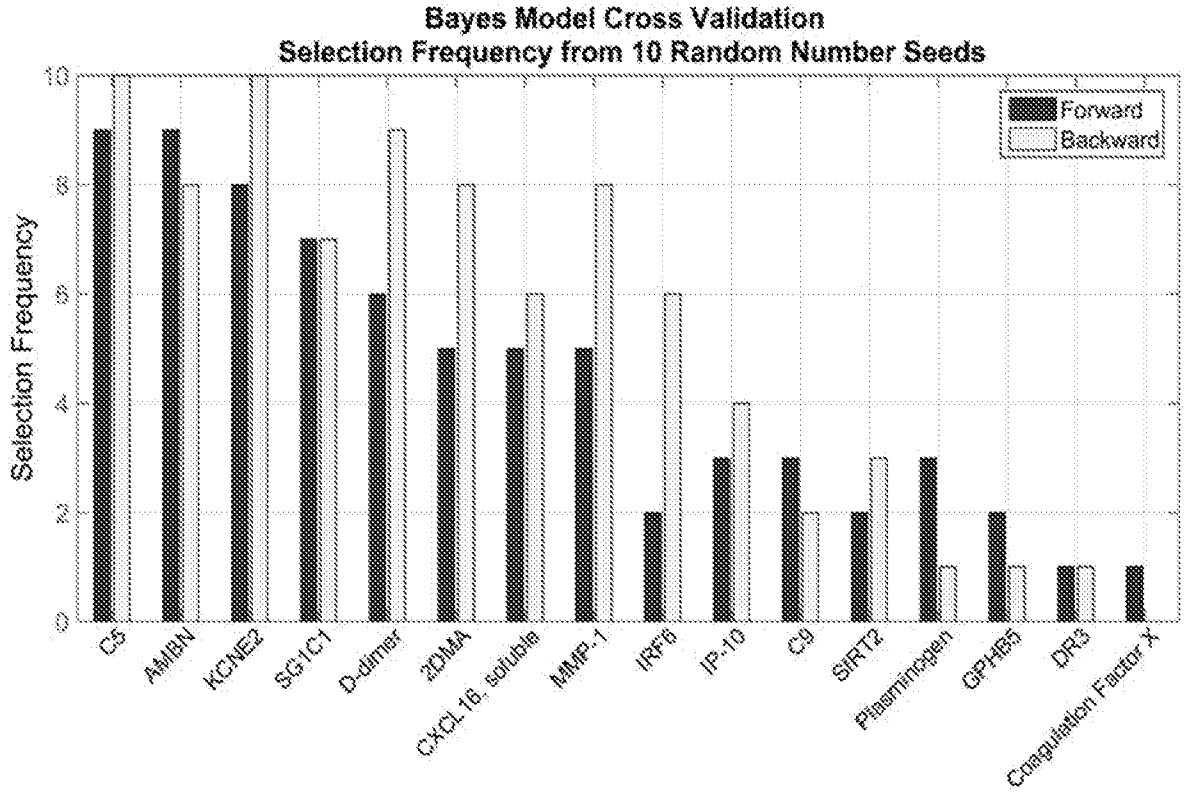

Bayes Model Cross Validation
Selection Frequency from 10 Random Number Seeds
Selection Frequency
0
2
4
6
8
10
Forward
Backward
C5
AMBN
KCNE2
SG1C1
D-dimer
2DMA
CXCL16, soluble
MMP-1
IRF6
IP-10
C9
SIRT2
Plasminogen
GPHB5
DR3
Coagulation Factor X

FIG. 58

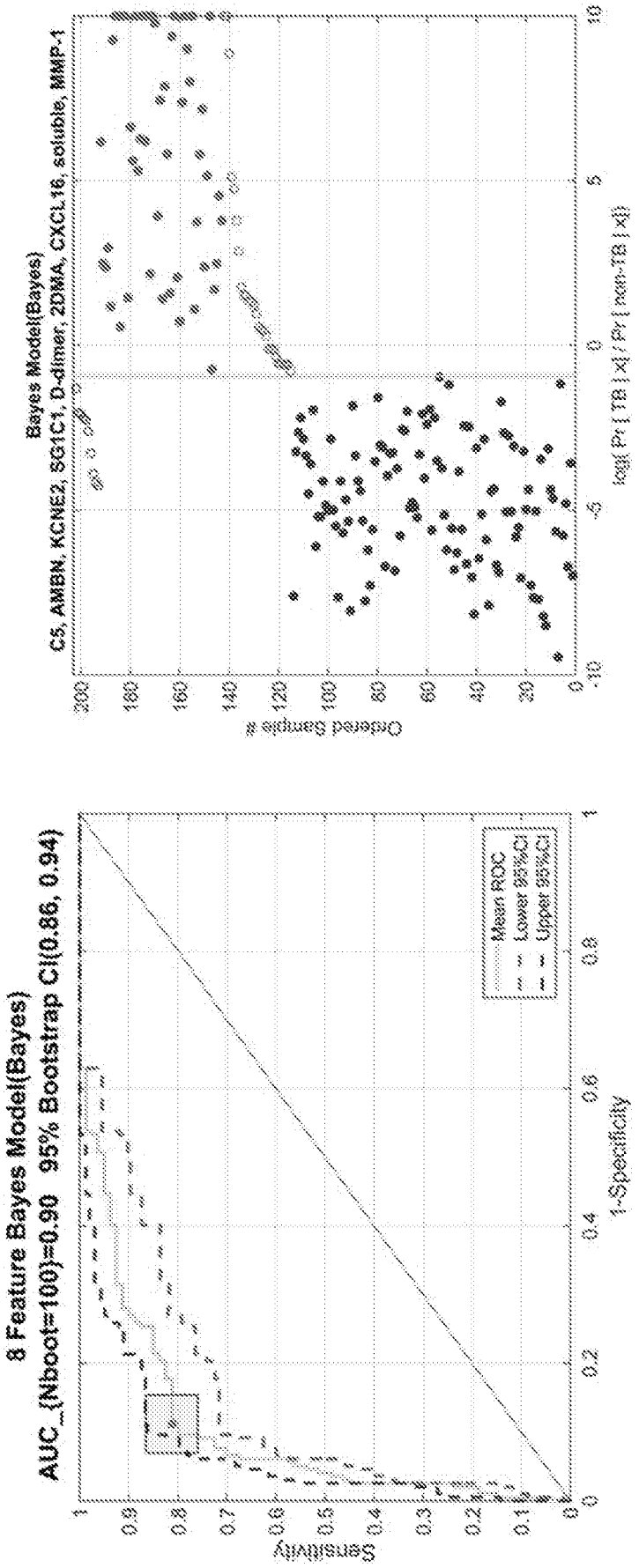

8 Feature Bayes Model(Bayes)
AUC_{Nboot=100}=0.90 95% Bootstrap CI(0.86, 0.94)
Sensitivity
1-Specificity
Mean ROC
Lower 95%CI
Upper 95%CI
Bayes Model(Bayes)
C5, AMBN, KCNE2, SG1C1, D-dimer, 2DMA, CXCL16, soluble, MMP-1
Ordered Sample #
log[Pr [TB | x] / Pr [non-TB | x]]

FIG. 59

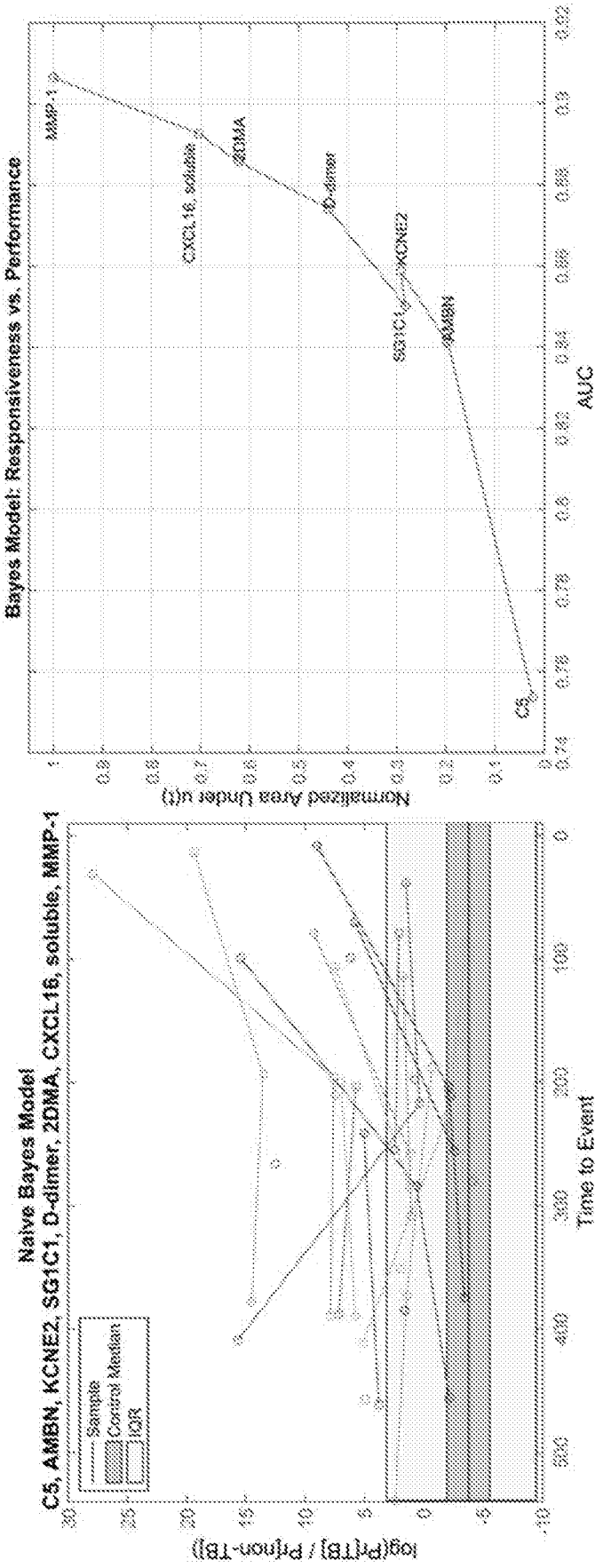

Bayes Model: Responsiveness vs. Performance
Normalized Area Under u(t)
AUC
MMP-1
CXCL16, soluble
2DMA
D-dimer
KCNE2
SG1C1
AMBN
C5
Naive Bayes Model
C5, AMBN, KCNE2, SG1C1, D-dimer, 2DMA, CXCL16, soluble, MMP-1
Sample
Control Median
IQR
log(Pr[TB] / Pr[non-TB])
Time to Event

FIG. 60

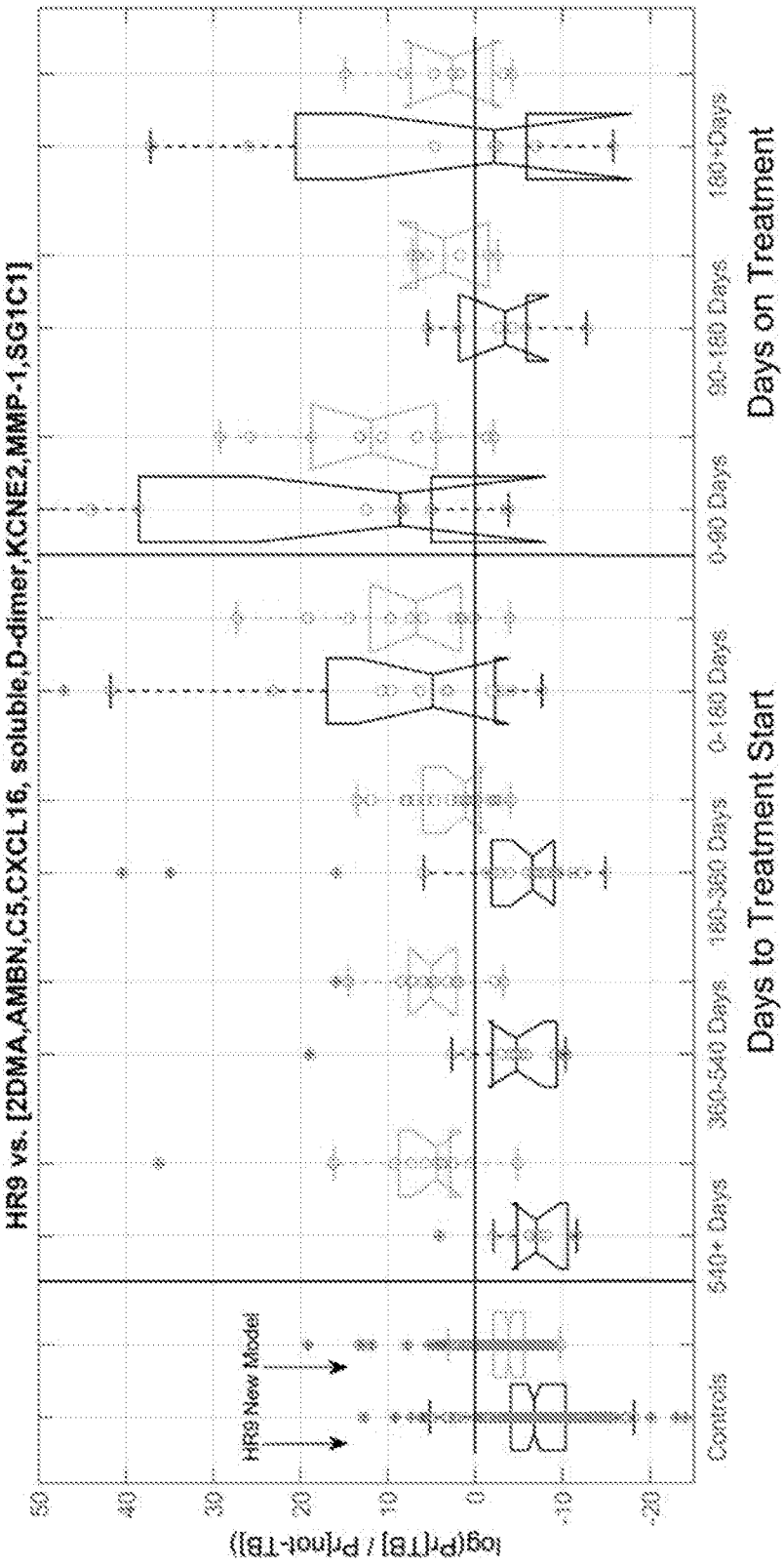

HR9 vs. [2DMA,AMBN,C5,CXCL16, soluble,D-dimer,KCNE2,MMP-1,SG1C1]
log(Pr[TB] / Pr[non-TB])
50
40
30
20
10
0
-10
-20
HR9 New Model
Controls
540+ Days
360-540 Days
180-360 Days
0-180 Days
0-90 Days
90-180 Days
180+Days
Days to Treatment Start
Days on Treatment

FIG. 61

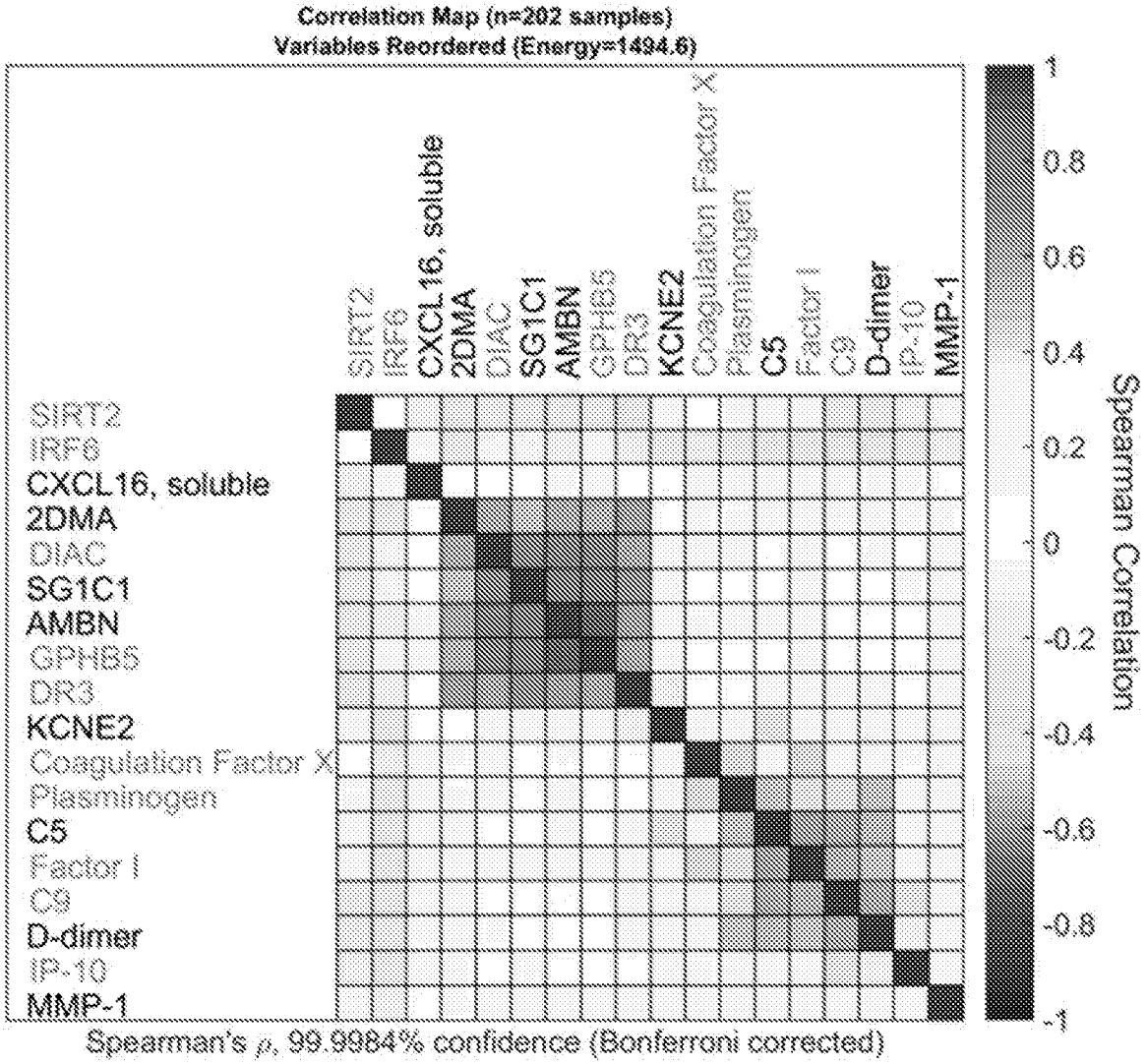

Correlation Map (n=202 samples)
Variables Reordered (Energy=1494.6)
SIRT2
IRF6
CXCL16, soluble
2DMA
DIAC
SG1C1
AMBN
GPHB5
DR3
KCNE2
Coagulation Factor X
Plasminogen
C5
Factor I
C9
D-dimer
IP-10
MMP-1
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
Spearman Correlation
Spearman's ρ, 99.9984% confidence (Bonferroni corrected)

FIG. 62

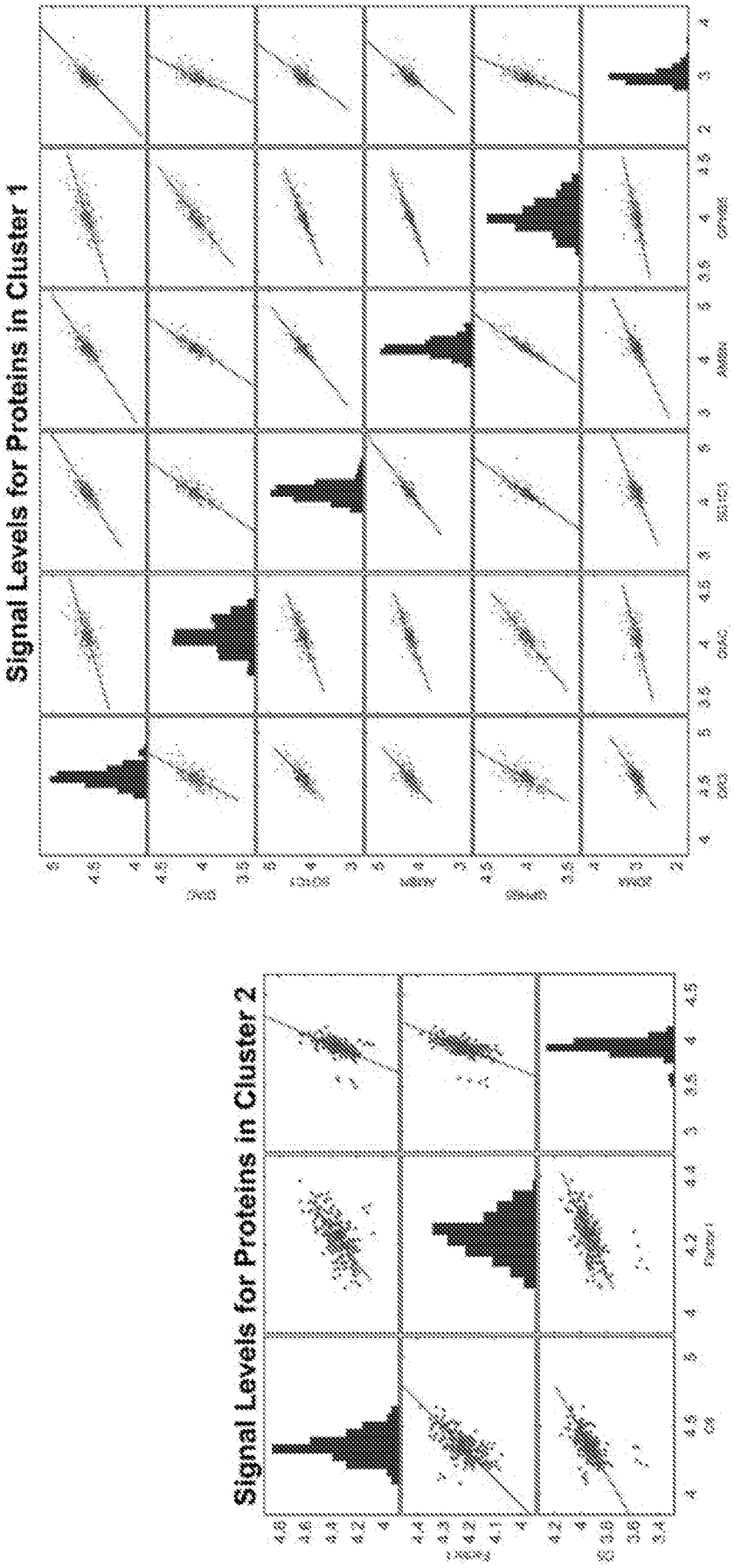

Signal Levels for Proteins in Cluster 1
Signal Levels for Proteins in Cluster 2

FIG. 63

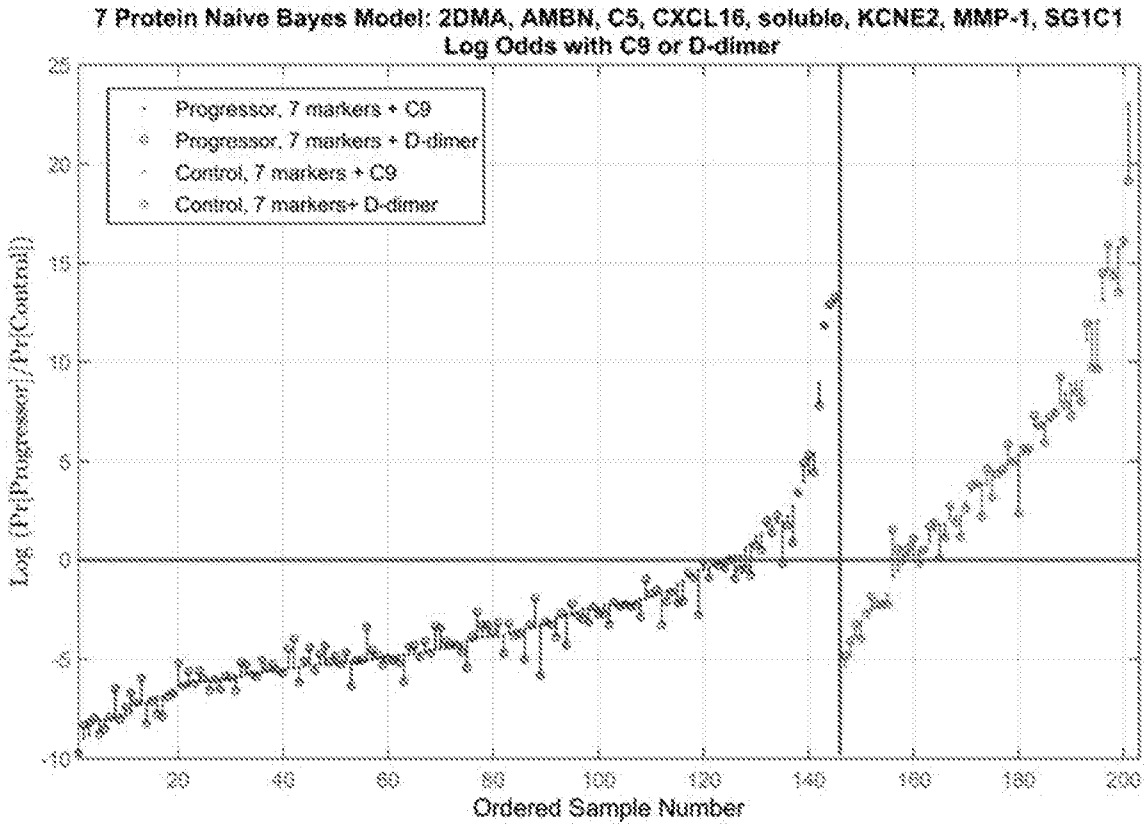

7 Protein Naive Bayes Model: 2DMA, AMBN, C5, CXCL16, soluble, KCNE2, MMP-1, SG1C1
Log Odds with C9 or D-dimer
Progressor, 7 markers + C9
Progressor, 7 markers + D-dimer
Control, 7 markers + C9
Control, 7 markers+ D-dimer
Log (Pr(Progressor)/Pr(Control))
Ordered Sample Number
25
20
15
10
5
0
-5
-10
20
40
60
80
100
120
140
160
180
200

FIG. 64

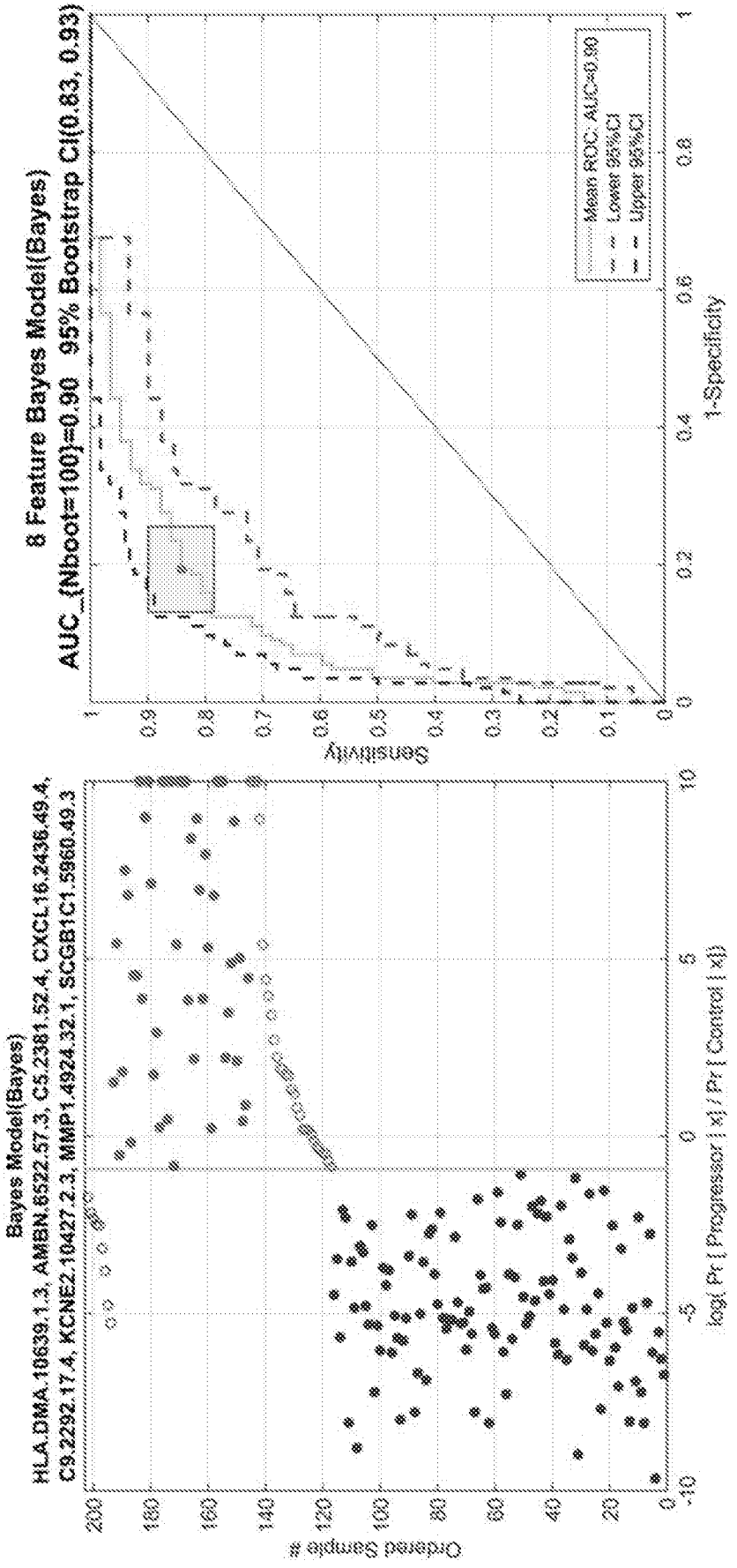

8 Feature Bayes Model(Bayes)
AUC_{Nboot=100}=0.90 95% Bootstrap CI(0.83, 0.93)
Sensitivity
1-Specificity
Mean ROC: AUC=0.90
Lower 95%CI
Upper 95%CI
Bayes Model(Bayes)
HLA.DMA.10639.1.3, AMBN.6522.57.3, C5.2381.52.4, CXCL16.2436.49.4,
C9.2292.17.4, KCNE2.10427.2.3, MMP1.4924.32.1, SCGB1C1.5960.49.3
Ordered Sample #
log(Pr [Progressor | x] / Pr [Control | x])

FIG. 65

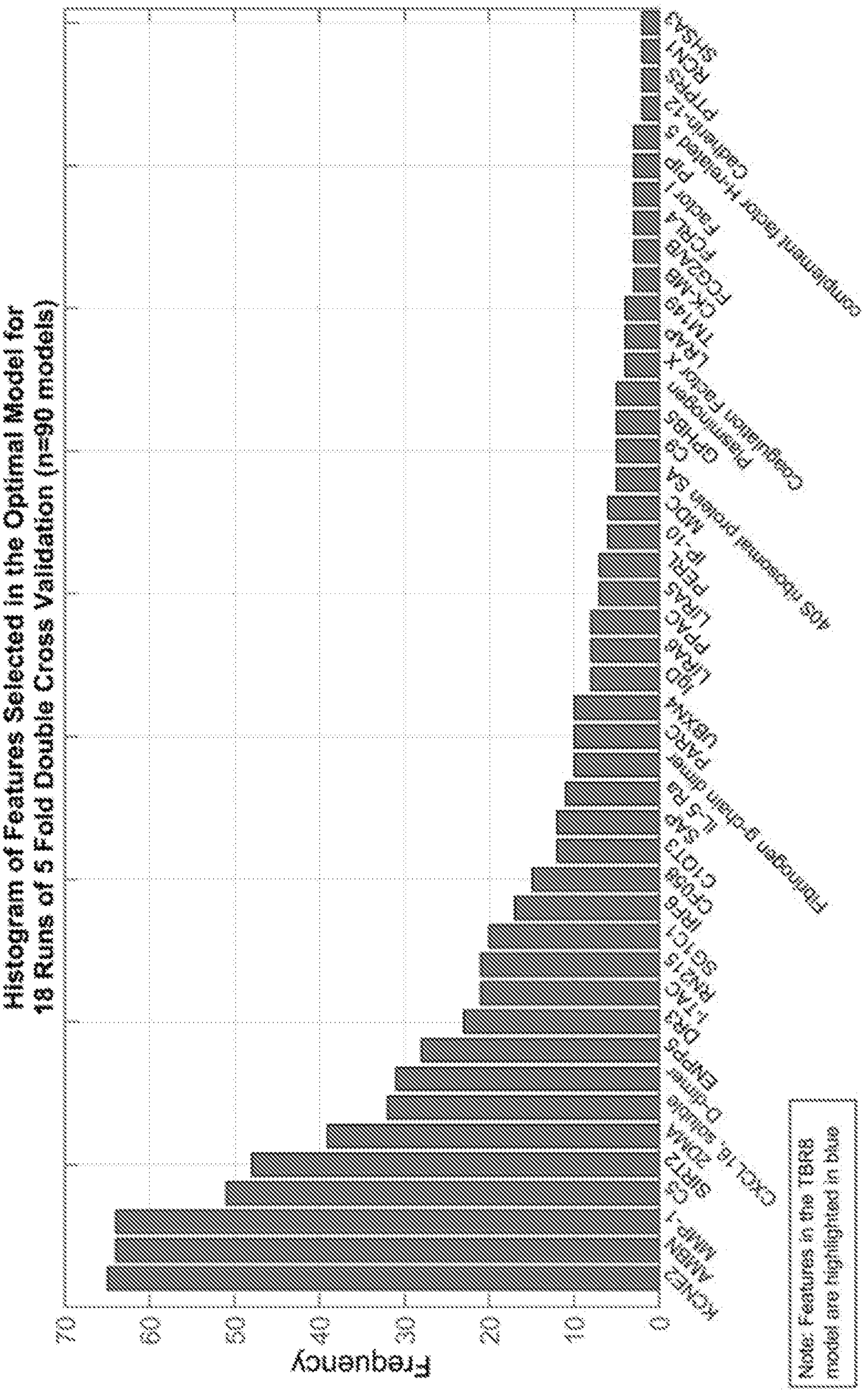
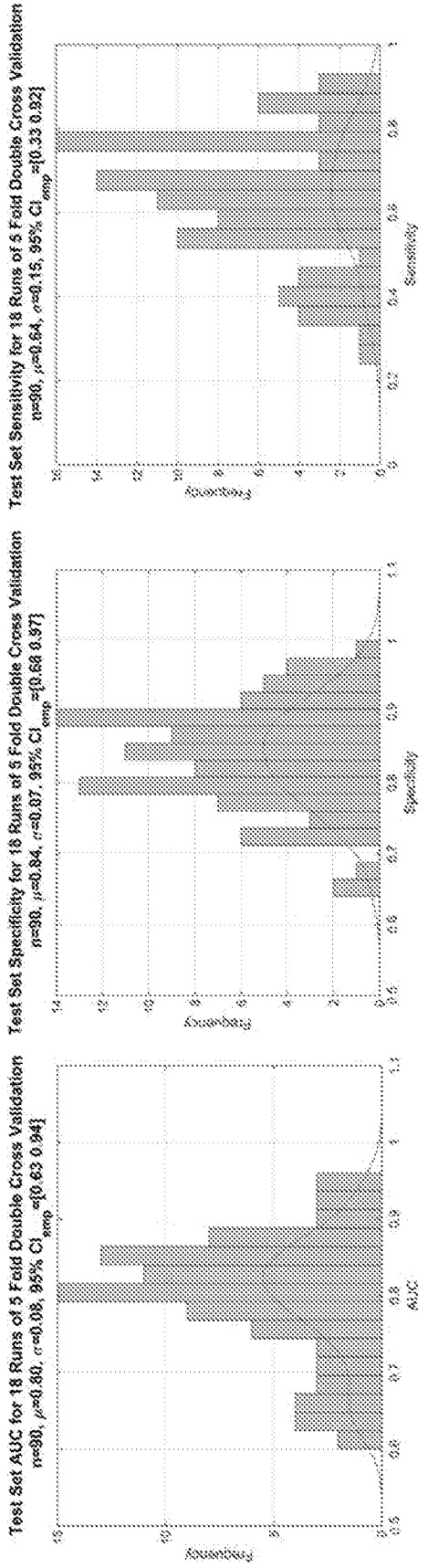

Histogram of Features Selected in the Optimal Model for
18 Runs of 5 Fold Double Cross Validation (n=90 models)
Frequency
0
10
20
30
40
50
60
70
KCNE2
AMBN
MMP-1
C5
SIRT2
2DMA
CXCL16, soluble
D-dimer
ENPP5
DR3
LTAC
RN215
SG1C1
IRF6
CF058
C1QT3
SAP
IL-5 Ra
Fibrinogen g-chain dimer
PARC
UBXN4
IgD
LIRA6
PPAC
LIRA5
PERL
IP-10
MDC
40S ribosomal protein SA
C9
GPHB5
Plasminogen
Coagulation Factor X
LRAP
TM149
CK-MB
FCG2A/B
FCRL4
Factor I
PIP
complement factor H-related 5
Cadherin-12
PTPRS
RCN1
SHSA3
Note: Features in the TBR8
model are highlighted in blue

FIG. 66


Test Set AUC for 18 Runs of 5 Fold Double Cross Validation
n=90, μ=0.80, σ=0.08, 95% CI emp =[0.63 0.94]
Frequency
AUC
Test Set Specificity for 18 Runs of 5 Fold Double Cross Validation
n=90, μ=0.84, σ=0.07, 95% CI emp =[0.68 0.97]
Frequency
Specificity
Test Set Sensitivity for 18 Runs of 5 Fold Double Cross Validation
n=90, μ=0.64, σ=0.15, 95% CI emp =[0.33 0.82]
Frequency
Sensitivity

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/014840

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/569
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

EPO-Internal, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROHIT MISTRY ET AL: "Gene-Expression Patterns in Whole Blood Identify Subjects at Risk for Recurrent Tuberculosis", JID, vol. 195, 1 February 2007 (2007-02-01), pages 357-365, XP055259542, abstract; figure 2	1-38

-/--

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 March 2016	17/06/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Rosin, Oliver

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/014840

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

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

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

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

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

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

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

see additional sheet

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

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

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

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

1-38(partially)

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Bridget Lollo: "SOMAscan TM : A Quantitative Multiplex Proteomic Platform that Measures 1129 Analytes in Complex Matrices", , 1 January 2012 (2012-01-01), XP055259693, Retrieved from the Internet: URL:http://www.somalogic.com/somalogic/media/Assets/Posters/Lollo-et-al-HUPO-poster-v6.pdf [retrieved on 2016-03-18] the whole document -----	26-38

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-38(partially)

Methods, kit and compositions encompassing the biomarker AMBN.

2-9. claims: 1-38(partially)

Methods, kit and compositions encompassing the biomarker C5 (invention 2), MMP-1 (invention 3), D-dimer (invention 4), SG1C1 (invention 5), 2DMA (invention 6), IP-10 (invention 7), KCNE2 (invention 8), CXCL16 (invention 9).
