COMPOSITIONS AND METHODS RELATED TO DIAGNOSIS OF PROSTATE CANCER

RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to U.S. provisional patent application, U.S.S.N. 61/972,099 filed March 28, 2014, the entire contents of which is incorporated herein by reference.

BACKGROUND OF INVENTION

Elevated blood levels of total prostate-specific antigen (PSA) are associated with prostate-related disorders, including prostate cancer. There is considerable evidence that measuring levels of isoforms of PSA separately, rather than combining them together in a single measure of total PSA, leads to improved predictions relating to the presence of prostate cancer in a subject. There is also evidence that measurements of hK2, a molecule that converts PSA from its pro- to active form, are informative to such predictions. Moreover, multimarker panels based on such measurements have been proposed for assessing prostate cancer status in subject. However, there remains a need for improved methods for assessing prostate cancer, particularly for evaluating the need for invasive prostate tissue biopsies.

SUMMARY OF INVENTION

Aspects of the disclosure relate to improved methods for predicting whether a prostate tissue biopsy obtained from a subject will contain detectable prostate cancer. In some embodiments, the methods involve using a blood sample obtained from a subject to conduct one or more immunoassays that measure levels of prostate specific antigens. In some embodiments, it has been found that measuring levels of prostate specific antigens in plasma preparations leads to better predictive results than are obtainable by measuring levels in other blood preparations, such as serum preparations. In some embodiments, it has been found that performing certain immunoassays in low pH buffer leads to more sensitive antigen detection and thus better predictive results. Moreover, in some embodiments, it has been found that improved predictive results can be obtained by combining information regarding measured prostate specific antigen levels with information concerning one or more of a subject's age, past digital rectal exam results and prior biopsy status. Improved methods disclosed herein are useful for predicting whether or not an invasive prostate tissue biopsy is merited for purposes of determining whether

the subject has prostate cancer, particularly a prostate cancer of high grade (*e.g.*, Gleason score of greater than or equal to 7.0). Furthermore, methods disclosed herein are advantageous because they produce results that are informative of the likelihood that invasive and relatively risky diagnostic procedures, such as prostate tissue biopsies, will be informative and worthwhile to perform. Accordingly, the methods are useful because they enable healthcare providers to make more informed decisions regarding the care of subjects.

Aspects of the disclosure relate to methods of determining the probability that a prostate tissue biopsy obtained from a subject would contain detectable prostate cancer. In some embodiments, the methods comprise i) subjecting a blood plasma sample of the subject to an immunoassay that measures a level of total prostate specific antigen (tPSA) in the blood plasma sample; ii) if the tPSA level is above a threshold level, determining the probability that the prostate tissue biopsy would contain detectable prostate cancer by weighting the measured level of tPSA and a parameter indicative of whether the subject has had a prior biopsy of prostate tissue; and iii) if the tPSA level is at or below the threshold level, subjecting the blood plasma sample to an immunoassay that measures levels of free prostate specific antigen (fPSA), intact prostate specific antigen (iPSA), and human kallikrein 2 (hK2) in the blood plasma sample, and determining the probability that the prostate tissue biopsy would contain detectable prostate cancer by weighting the measured levels of tPSA, fPSA, iPSA, and hK2 and a parameter indicative of whether the subject has had a prior biopsy of prostate tissue. In some embodiments, the methods comprise i) subjecting a blood plasma sample of the subject to immunoassays that measure levels of free prostate specific antigen (fPSA), intact prostate specific antigen (iPSA), total prostate specific antigen (tPSA) and human kallikrein 2 (hK2); and ii) determining the probability that the prostate tissue biopsy would contain detectable prostate cancer by weighting the measured levels of fPSA, iPSA, tPSA, and hK2 and a parameter indicative of whether the subject had a prior biopsy of prostate tissue.

Further aspects of the disclosure relate to methods of determining whether a subject is a candidate for a prostate tissue biopsy. In some embodiments, the methods comprise i) obtaining a blood sample from the subject; ii) determining the probability that the prostate tissue biopsy would contain detectable prostate cancer using the blood sample obtained in step i), wherein a) if a tPSA level measured using the blood sample is above a threshold level, the probability is based on weighting the tPSA level and a parameter indicative of whether the subject had a prior biopsy of prostate tissue, otherwise, b) if the tPSA level is at or below the threshold level, the

probability is based on weighting levels of tPSA, fPSA, iPSA, and hK2 measured using the blood sample and a parameter indicative of whether the subject had a prior biopsy of prostate tissue; and iv) determining whether the subject is a candidate for the prostate tissue biopsy based on the probability that the prostate tissue biopsy will contain detectable prostate cancer as determined in step ii).

Further aspects of the disclosure relate to methods of assessing whether a subject has prostate cancer. In some embodiments, the methods comprise i) obtaining a blood sample from the subject; ii) determining the probability that a prostate tissue biopsy obtained from the subject would contain detectable prostate cancer, wherein a) if a tPSA level measured using the blood sample is above a threshold level, the probability is based on weighting the tPSA level and a parameter indicative of whether the subject had a prior biopsy of prostate tissue, otherwise, b) if the tPSA level is at or below the threshold level, the probability is based on weighting levels of tPSA, fPSA, iPSA, and hK2 measured using the blood sample and a parameter indicative of whether the subject had a prior biopsy of prostate tissue; iii) determining whether the subject is a candidate for the prostate tissue biopsy based on the results of step ii); and iv) if the subject is a candidate for the prostate tissue biopsy based on the results of step ii), obtaining the prostate tissue biopsy from the subject and determining whether the subject has prostate cancer based on an analysis of the prostate tissue biopsy.

Further aspects of the disclosure relate to methods of determining whether a subject has prostate cancer. In some embodiments, the methods comprise i) obtaining a prostate tissue biopsy from the subject, wherein the subject is indicated for the prostate tissue biopsy based on a probability that the prostate tissue biopsy would contain detectable prostate cancer, wherein a) if a tPSA level measured using a blood sample obtained from the subject is above a threshold level, the probability is based on weighting the tPSA level and a parameter indicative of whether the subject had a prior biopsy of prostate tissue, otherwise, b) if the tPSA level is at or below the threshold level, the probability is based on weighting levels of tPSA, fPSA, iPSA, and hK2 measured using the blood sample and a parameter indicative of whether the subject had a prior biopsy of prostate tissue; and ii) determining whether the subject has prostate cancer based on an analysis of the prostate tissue biopsy.

Further aspects of the disclosure relate to immunoassay methods that measure the level of iPSA in a sample. In some embodiments, the methods comprise contacting iPSA present in the sample with a capture antibody specific for iPSA and nicked PSA under conditions in which

the capture antibody binds at least to iPSA, thereby producing a capture-antibody- iPSA complex; combining the capture-antibody-iPSA complex with an appropriate tracer in a buffer having a pH in a range of 6.5 to less than 7.75 such that the tracer binds to the capture-antibody-iPSA complex; and detecting tracer bound to the capture-antibody- iPSA complex. In some embodiments, the methods comprise contacting iPSA present in the sample with a capture antibody specific for iPSA and nicked PSA under conditions in which the capture antibody binds at least to iPSA, thereby producing a capture-antibody- iPSA complex, wherein the capture antibody is a Fab; combining the capture-antibody- iPSA complex with an appropriate tracer under conditions in which the tracer binds to the capture-antibody- iPSA complex; and detecting the tracer bound to the capture-antibody- iPSA complex. In some embodiments, the Fab is a 5A10 Fab.

Further aspects of the disclosure relate to immunoassay methods that measure the level of hK2 in a sample. In some embodiments, the methods comprise contacting hK2 present in the sample with a capture antibody specific for hK2 and nicked PSA under conditions in which the capture antibody binds at least to hK2, thereby producing a capture-antibody-hK2 complex, wherein the capture antibody is a Fab; combining the capture-antibody-hK2 complex with an appropriate tracer; and detecting the tracer bound to the capture-antibody-hK2 complex. In some embodiments, the Fab is a F(ab)2. In some embodiments, the F(ab)2 is 6H10 F(ab)2.

Further aspects of the disclosure relate to methods for evaluating a sample (e.g., a blood plasma sample). In some embodiments, the methods comprise (a) subjecting a sample to immunoassays that measure levels of fPSA, iPSA, tPSA and hK2, wherein the immunoassay that measures the level of fPSA comprises contacting fPSA present in the sample with a H117 capture antibody to produce a capture-antibody-fPSA complex, and detecting the capture-antibody-fPSA complex using a 5A10 tracer antibody, wherein the immunoassay that measures the level of iPSA comprises contacting iPSA present in the sample with a 5A10 Fab capture antibody to produce a capture-antibody- iPSA complex, and detecting the capture-antibody-iPSA complex using a 4D4 tracer antibody, wherein the immunoassay that measures the level of tPSA comprises contacting tPSA present in the sample with a H117 capture antibody to produce a capture-antibody-tPSA complex, and detecting the capture-antibody-tPSA complex with a H50 tracer antibody, wherein the immunoassay that measures the level of hK2 comprises contacting PSA in the plasma blood sample with blocking antibodies, contacting hK2 present in the sample with a 6H10 F(ab)2 capture antibody to produce a capture-antibody-hK2 complex, and detecting

the capture-antibody-hK2 complex with a 7G1 tracer antibody; and (b) evaluating the sample based on the measured levels of fPSA, iPSA, tPSA and hK2.

Further aspects of the disclosure relate to methods for determining a probability of an event associated with prostate cancer. In some embodiments, the methods comprise receiving, via an input interface, information indicative of the level of tPSA present in a blood plasma sample of a subject; receiving, via an input interface, information about whether the subject had a prior biopsy of prostate tissue; evaluating, using at least one processor, a logistic regression model based, at least in part, on the received information to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises: determining the probability of the event associated with prostate cancer based, at least in part, on the tPSA value and the information about whether the subject had a prior biopsy of prostate tissue; and outputting an indication of the probability of the event associated with prostate cancer.

In some embodiments, the methods comprise receiving, via an input interface, information indicative of levels of tPSA, fPSA, iPSA, and hK2 present in a blood plasma sample of a subject; receiving, via an input interface, information about whether the subject had a prior biopsy of prostate tissue; evaluating, using at least one processor, a logistic regression model based, at least in part, on the received information to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises: determining the probability of the event associated with prostate cancer based, at least in part, on the information indicative of levels of tPSA, fPSA, iPSA, and hK2 and the information about whether the subject had a prior biopsy of prostate tissue; and outputting an indication of the probability of the event associated with prostate cancer.

In some aspects of the disclosure a computer is provided for determining a probability of an event associated with prostate cancer. In some embodiments, the computer comprises an input interface configured to receive information indicative of the level of tPSA present in a blood plasma sample of a subject and information about whether the subject had a prior biopsy of prostate tissue; at least one processor programmed to evaluate a logistic regression model based, at least in part, on the received information to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises: determining the probability of the event associated with prostate cancer based, at least in part, on the tPSA value and the information about whether the subject had a prior biopsy

of prostate tissue; and an output interface configured to output an indication of the probability of the event associated with prostate cancer.

In some embodiments, the computer comprises an input interface configured to receive information indicative of levels of tPSA, fPSA, iPSA, and hK2 present in a blood plasma sample of a subject and information about whether the subject had a prior biopsy of prostate tissue; at least one processor programmed to evaluate a logistic regression model based, at least in part, on the received information to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises: determining the probability of the event associated with prostate cancer based, at least in part, on the information indicative of levels of tPSA, fPSA, iPSA, and hK2 and the information about whether the subject had a prior biopsy of prostate tissue; and an output interface configured to output an indication of the probability of the event associated with prostate cancer.

In other aspects of the disclosure, a system is provided for determining a probability of an event associated with prostate cancer. In some embodiments, the system comprises a) a detector configured to measure a level of tPSA present in a blood plasma sample of a subject; and b) a computer in electronic communication with the detector, wherein the computer comprises: i) an input interface configured to receive information from the detector indicative of the measured level of tPSA, and to receive information about whether the subject had a prior biopsy of prostate tissue; ii) at least one processor programmed to evaluate a logistic regression model based, at least in part, on the received information to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises: determining the probability of the event associated with prostate cancer based, at least in part, on the information indicative of the level of tPSA and the information about whether the subject had a prior biopsy of prostate tissue; and iii) an output interface configured to output an indication of the probability of the event associated with prostate cancer. In some embodiments, the system comprises a) a detector configured to measure levels of tPSA, fPSA, iPSA, and hK2 present in a blood plasma sample of a subject; and b) a computer in electronic communication with the detector, wherein the computer comprises: i) an input interface configured to receive information from the detector indicative of the measured levels of tPSA, fPSA, iPSA, and hK2, and to receive information about whether the subject had a prior biopsy of prostate tissue; ii) at least one processor programmed to evaluate a logistic regression model based, at least in part, on the received information to determine a probability of an event

associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises: determining the probability of the event associated with prostate cancer based, at least in part, on the information indicative of levels of tPSA, fPSA, iPSA, and hK2 and the information about whether the subject had a prior biopsy of prostate tissue; and iii) an output interface configured to output an indication of the probability of the event associated with prostate cancer.

In further aspects of the disclosure, a computer-readable storage medium is provided that is encoded with a plurality of instructions that, when executed by a computer, perform a method for determining a probability of an event associated with prostate cancer. In some embodiments, the method comprises: evaluating a logistic regression model based, at least in part, on information indicative of the level of tPSA present in a blood plasma sample of a subject and information about whether the subject had a prior biopsy of prostate tissue to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises: determining the probability of the event associated with prostate cancer based, at least in part, on the tPSA value and the information about whether the subject had a prior biopsy of prostate tissue; and outputting an indication of the probability of the event associated with prostate cancer. In some embodiments, the method comprises evaluating a logistic regression model based, at least in part, on information indicative of levels of tPSA, fPSA, iPSA, and hK2 present in a blood plasma sample of a subject and information about whether the subject had a prior biopsy of prostate tissue to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises: determining the probability of the event associated with prostate cancer based, at least in part, on the information indicative of levels of tPSA, fPSA, iPSA, and hK2 and the information about whether the subject had a prior biopsy of prostate tissue; and outputting an indication of the probability of the event associated with prostate cancer.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1A is a non-limiting schematic showing a process for determining the probability that a biopsy will contain detectable prostate cancer;

FIG. 1B is a non-limiting schematic of a computer configured for implementing a process for determining the probability that a biopsy will contain detectable prostate cancer;

FIG. 1C is a non-limiting schematic of a computer network configured for implementing a process for determining the probability that a biopsy will contain detectable prostate cancer;

FIG. 2 is a non-limiting example of a graph comparing actual risk versus predicted risk of high grade cancer;

FIG. 3 is a non-limiting example of a graph comparing actual risk versus predicted risk of any grade of cancer;

FIG. 4 is a non-limiting example of a graph showing a decision curve analysis for high grade cancer;

FIG. 5 is a non-limiting example of a graph showing a decision curve analysis for any grade cancer;

FIG. 6 is a non-limiting example of a graph of a Receiver Operating Curve (ROC) for high grade cancer;

FIG. 7 is a non-limiting example of a graph of a Receiver Operating Curve (ROC) for any grade cancer;

FIG. 8A is a non-limiting example of a graph of a positive predictive value by biopsy threshold for high grade cancer;

FIG. 8B is a non-limiting example of a graph of a negative predictive value by biopsy threshold for high grade cancer;

FIG. 9A is a non-limiting example of a graph of a positive predictive value by biopsy threshold for any grade cancer;

FIG. 9B is a non-limiting example of a graph of a negative predictive value by biopsy threshold for any grade cancer;

FIG. 10 shows a non-limiting example of a plot showing the proportion of men who harbored high grade disease by age at biopsy;

FIGs. 11A and 11B show a non-limiting example of plots showing predicted versus actual probabilities of detecting high grade cancer in all patients of a validation study;

FIG 11C shows a non-limiting example of a plot showing predicted versus actual probabilities of detecting any grade of cancer in all patients of a validation study;

FIGs. 12A and 12B show a non-limiting example of plots showing predicted versus actual probabilities of detecting high grade cancer in patients aged 50-75 of a validation study;

FIG 12C shows a non-limiting example of a plot showing predicted versus actual probabilities of detecting any grade of cancer in all patients aged 50-75 of a validation study;

FIGs. 13A and 13B show a non-limiting example of plots showing predicted versus actual probabilities of detecting high grade cancer in patients aged less than 71 of a validation study;

FIG. 13C shows a non-limiting example of a plot showing predicted versus actual probabilities of detecting any grade cancer in patients aged less than 71;

FIGs. 14A and 14B show a non-limiting example of plots showing net benefit versus threshold probability levels for all patients of a validation study;

FIGs. 15A and 15B show a non-limiting example of plots showing net benefit versus threshold probability levels for patients aged 50-75 of a validation study; and

FIGs. 16A and 16B show a non-limiting example of plots showing net benefit versus threshold probability levels for all patients aged less than 71 of a validation study.

DETAILED DESCRIPTION OF INVENTION

Aspects of the disclosure relate to improved methods for predicting whether a prostate tissue biopsy obtained from a subject will contain detectable prostate cancer, including high grade prostate cancer (Gleason 7 or greater). Thus, methods disclosed herein may be employed by a healthcare provider for purposes of determining whether a prostate tissue biopsy is merited. In some embodiments, the methods involve using a blood sample obtained from a subject to conduct one or more immunoassays that measure levels of prostate specific antigens, such as total prostate-specific antigen (tPSA), free prostate specific antigen (fPSA), intact prostate specific antigen (iPSA) and human Kallikrein 2 (hK2). In some embodiments, it has been found that measuring one or more levels of these antigens in plasma preparations leads to better predictive results than are obtainable by measuring levels in other blood preparations, such as serum. In some embodiments, a predictive model (*e.g.*, a logistic regression model) is provided that incorporates plasma levels of tPSA, fPSA, iPSA and/or hK2 to determine the probability that a prostate tissue biopsy will contain detectable cancer. Moreover, in some embodiments, it has been found that improved predictive results can be obtained by combining information regarding measured prostate specific antigen levels with patient information, particularly information concerning whether or not a subject has had a prior biopsy to detect the presence of prostate cancer. Accordingly, improved methods are provided that are useful for determining whether a subject should undergo an invasive prostate tissue biopsy.

Aspects of the disclosure provide methods of determining the probability that a prostate tissue biopsy obtained from a subject would contain detectable prostate cancer. Such methods may involve subjecting a blood plasma sample of a subject to an immunoassay that measures at least a level of total prostate specific antigen (tPSA) in the blood plasma sample. If the tPSA level is above a threshold level, then the probability that a prostate tissue biopsy would contain detectable prostate cancer may be determined by weighting the measured level of tPSA and a parameter indicative of whether the subject has had a prior biopsy of prostate tissue. On the other hand, if the tPSA level is at or below the threshold level, then the probability that a prostate tissue biopsy would contain detectable prostate cancer may be determined by weighting measured levels of tPSA, fPSA, iPSA, and hK2 and a parameter indicative of whether the subject has had a prior biopsy of prostate tissue. Accordingly, in some embodiments, methods provided herein may involve subjecting the blood plasma sample to an immunoassay that measures levels of free prostate specific antigen (fPSA), intact prostate specific antigen (iPSA), and human kallikrein 2 (hK2) in the blood plasma sample. In some embodiments, the probability is further determined by weighting a parameter indicative of the subject's age. In some embodiments, the probability is further determined by weighting one or more parameters indicative of the outcome of a digital rectal examination performed on the subject.

In some embodiments, the threshold level of tPSA used for model selection is a level that indicates whether using tPSA alone, or together with certain patient specific information (*e.g.*, prior biopsy status), would be sufficient for purposes of establishing a probability that a prostate tissue biopsy would contain detectable prostate cancer. In some embodiments, the threshold level is 5 ng/mL, 10 ng/mL, 15 ng/mL, 20 ng/mL, 25 ng/mL, 30 ng/mL, 35 ng/mL or 40 ng/mL. Because tPSA levels combined with certain patient specification information, particularly prior biopsy status, may be enough to make informative predictions, in some embodiments, it may be cost effective not to perform immunoassays to detect other antigens before first determining levels of tPSA. However, in some embodiments, levels of tPSA may be determined in parallel or together with other marker levels, *e.g.*, fPSA, iPSA, or hK2.

In some embodiments, antigen levels (*e.g.*, levels of two or more of tPSA, fPSA, iPSA, and hK2) are determined in parallel in the same assay. In other embodiments, such antigen levels are determined in separate assays. In some embodiments, antigen levels are determined from the same original blood draw (*e.g.*, a venous blood draw) from a subject. In some embodiments, antigen levels are determined from different blood draws. In some embodiments,

antigen levels are determined using plasma preparations from the same or different blood draws. In some embodiments, one or more antigen levels are determined using a plasma preparation and one or more other antigens are determined using a different type of blood preparation, *e.g.*, serum. Blood plasma is a pale-yellow liquid component of blood. In some embodiments, blood plasma may be prepared by spinning a tube of blood containing an anticoagulant (*e.g.*, Heparin, EDTA, *etc.*) in a centrifuge until blood cells and debris move to the bottom of the tube, after which the blood plasma may be poured or drawn off.

Methods are provided herein for determining whether a subject is a candidate for a prostate tissue biopsy. Such methods may involve a physician or health care provider obtaining a blood sample from a subject and determining the probability that the prostate tissue biopsy would contain detectable prostate cancer based, at least in part, on measured levels of antigens determined using the blood sample. The blood sample may be processed locally (*e.g.*, within the same health care facility or business that the subject is being evaluated) or may send it out to an external or third-party laboratory or facility for processing and analysis. If a tPSA level measured using the blood sample is above a threshold level, the probability is determined based on weighting the tPSA level. Otherwise, if the tPSA level is at or below the threshold level, the probability is based on weighting levels of tPSA, fPSA, iPSA, and hK2 measured using the blood sample. In either case, the probability is typically also based on weighting a parameter indicative of whether the subject had a prior biopsy of prostate tissue. The physician or healthcare provider may determine whether the subject is a candidate for the prostate tissue biopsy based on the probability that the prostate tissue biopsy will contain detectable prostate cancer.

In some embodiments, a physician or healthcare provider may set a probability cut-off in which a biopsy will be indicated if a probability is at or above the cut-off. For example, if the probably is greater than 5 %, 7.5% 10 %, 12.5 %, 15 %, 20 %, 25 %, .30 %, 35 %, 40 %, 45 %, 50 %, 55 %, 60 %, 65 %, 70 %, 75 %, 80 %, 85 %, 90 %, 95 %, 99 %, or more, then the physician or healthcare provider may determine that the subject is a candidate for the prostate tissue biopsy. In some embodiments, a cut-off based on a probability that a prostate tissue biopsy will contain detectable prostate cancer of high grade (e.g., a Gleason score of 7 or greater) is 5 %, 7.5%, 10 %, 12.5 %, or 15 %. In some embodiments, a cut-off based on a probability that a prostate tissue biopsy will contain detectable prostate cancer of any grade is 10 %, 12.5 %, 15 %, 20 %, 25 %, or 30 %. In some embodiments, if a probability is below a cut-

off then a physician or healthcare provider will not order a biopsy but will continue to monitor the subject, e.g., for increases in probability levels or changes in other risk factors indicative of prostate cancer.

In some embodiments, if a subject is determined to be a candidate for a prostate tissue biopsy, then the physician or health care provider may obtain or order to be obtained a prostate tissue biopsy from the subject and determine whether the subject has prostate cancer based on an analysis of the prostate tissue biopsy. The prostate tissue biopsy may be analyzed using any appropriate method including, for example, a cytological or histological analysis. The tissue sample may be characterized based on its clinical stage of cancer. The sample may be characterized based on a Gleason grade. Gleason 3+3 (6.0) corresponds to a tumor of low grade and a favorable prognosis. Gleason 3+4 (7.0) and 3+5 (8.0) typically correspond to tumors that have tissue of primarily low grade transformation with some high grade transformation. Gleason 4+3 (7.0) and 5+3 (8.0) typically correspond to tumor that have tissue of primarily high grade transformation with some low grade transformation. Gleason 4+4 (8.0), 4+5 (9.0), (9.0), and 5+5 (10.0) corresponds to high grade tumors. Accordingly, in some embodiments, the prostate cancer comprises high grade cancer (*e.g.*, Gleason $\geq$ 7.0).

Immunoassays

Levels of prostate specific antigens (*e.g.*, tPSA, iPSA, fPSA, and hK2) can be assessed by any appropriate method. In some embodiments, antibodies or antigen-binding fragments are provided that are suited for use in immunoassays. Immunoassays utilizing such antibody or antigen-binding fragments may competitive and non-competitive immunoassays in either a direct or indirect formats. Non-limiting examples of such immunoassays are Enzyme Linked Immunoassay (ELISA), radioimmunoassay (RIA), sandwich assay (immunometric assay), flow cytometry, western blot assay, immunoprecipitation assays, immunohistochemistry, immuno-microscopy, lateral flow immuno-chromatographic assays, and proteomics arrays. Antigens or antibodies or antigen-binding fragments that bind to them can be immobilized, *e.g.*, by binding to solid supports (*e.g.*, carriers, membrane, columns, proteomics array, etc.). Examples of solid support materials include glass, polystyrene, polyvinyl chloride, polyvinylidene difluoride, polypropylene, polyethylene, polycarbonate, dextran, nylon, amyloses, natural and modified celluloses, such as nitrocellulose, polyacrylamides, agaroses, and magnetite. The nature of the support can be either fixed or suspended in a solution (*e.g.*, beads).

In some embodiments, labeled antibodies or antigen binding fragments may be used as tracers to detect antigen bound antibody complexes. Examples of the types of labels which can be used to generate tracers include enzymes, radioisotopes, colloidal metals, fluorescent compounds, magnetic, chemiluminescent compounds, and bioluminescent compounds. Radiolabeled antibodies are prepared in known ways by coupling a radioactive isotope such as ^{153}Eu, ^{3}H, ^{32}P, ^{35}S, ^{59}Fe, or ^{125}I, which can then be detected by gamma counter, scintillation counter or by autoradiography. As discussed herein, antibodies and antigen-binding fragments may alternatively be labeled with enzymes such as yeast alcohol dehydrogenase, horseradish peroxidase, alkaline phosphatase, and the like, then developed and detected spectrophotometrically or visually. Suitable fluorescent labels include fluorescein isothiocyanate, fluorescamine, rhodamine, and the like. Suitable chemiluminescent labels include luminol, imidazole, oxalate ester, luciferin, and others.

An immunoassay may comprise contacting the sample, *e.g.*, a plasma sample, containing an antigen with an antibody, or antigen-binding fragment (*e.g.*, F(ab), $F(ab)_2$), under conditions enabling the formation of binding complexes between antibody or antigen-binding fragment and antigen. In some embodiments, a plasma sample is contacted with an antibody or antigen-binding fragment under conditions suitable for binding of the antibody or antigen-binding fragment to a target antigen, if the antigen is present in the sample. This may be performed in a suitable reaction chamber, such as a tube, plate well, membrane bath, cell culture dish, microscope slide, and other chamber. In some embodiments, an antibody or antigen-binding fragment is immobilized on a solid support. An antibody or antigen binding fragments that binds to an antigen in a sample may be referred to as a capture antibody. In some embodiments, the capture antibody comprises a tag (*e.g.*, a biotin label) that facilitates its immobilization to a solid support by an interaction involving the tag (*e.g.*, a biotin-streptavidin interaction in which the streptavidin is immobilized to a solid support). In some embodiments, the solid support is the surface of reaction chamber. In some embodiments, the solid support is of a polymeric membrane (*e.g.*, nitrocellulose strip, Polyvinylidene Difluoride (PVDF) membrane, etc.). In other embodiments, the solid support is a biological structure (*e.g.*, bacterial cell surface). Other exemplary solid supports are disclosed herein and will be apparent to one of ordinary skill in the art.

In some embodiments, the antibody and antigen-binding fragment is immobilized on the solid support prior to contacting with the antigen. In other embodiments, immobilization of the

antibody and antigen-binding fragment is performed after formation of binding complexes. In still other embodiments, antigen is immobilized on a solid support prior to formation of binding complexes. In some embodiments, a tracer may be added to the reaction chamber to detect immobilized binding complexes. In some embodiments, the tracer comprises a detectably labeled secondary antibody directed against the antigen. In some embodiments, the tracer comprises a detectably labeled secondary antibody directed against the capture antibody. In some embodiments, the primary antibody or antigen-binding fragment is itself detectable labeled.

In one embodiment, immunoassay methods disclosed herein comprise immobilizing antibodies or antigen-binding fragments to a solid support; applying a sample (*e.g.*, a plasma sample) to the solid support under conditions that permit binding of antigen to the antibodies or antigen-binding fragment, if present in the sample; removing the excess sample from the solid support; applying a tracer (*e.g.*, detectably labeled antibodies or antigen-binding fragments) under conditions that permit binding of the tracer to the antigen-bound immobilized antibodies or antigen-binding fragments; washing the solid support and assaying for the presence tracer.

In some embodiments, the antibody and antigen-binding fragment is immobilized on the solid support after contacting with the antigen in a reaction chamber. In some embodiments, the antibody and antigen-binding fragment is immobilized on the solid support prior to contacting with the antigen in a reaction chamber. In either case, a tracer may be added to the reaction chamber to detect immobilized binding complexes. In some embodiments, a tracer comprises a detectably labeled secondary antibody directed against the antigen. In some embodiments, the tracer comprises a detectably labeled secondary antibody directed against the primary antibody or antigen-binding fragment. As disclosed herein, the detectable label may be, for example, a radioisotope, a fluorophore, a luminescent molecule, an enzyme, a biotin-moiety, an epitope tag, or a dye molecule. Suitable detectable labels are described herein.

In some embodiments, it has been found that performing certain immunoassays in low pH buffer leads to more sensitive antigen detection. Accordingly, in some embodiments, a tracer antibody is contacted with a capture antibody in a buffer having a pH in a range of 6.5 to less than 7.75 such that the tracer binds to the capture-antibody- antigen complex. In some embodiments, the buffer pH is about 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, or 7.6.

It should be appreciated that in any of the assays disclosed herein capture antibodies may be swapped with tracer antibodies.

In some embodiments, an immunoassay that measures the level of fPSA involves contacting fPSA present in the plasma blood sample with a capture antibody specific for fPSA under conditions in which the first capture antibody binds to fPSA, thereby producing a capture-antibody-fPSA complex; and detecting the capture-antibody-fPSA complex using a tracer. The capture antibody may be a H117 antibody. In some embodiments, the tracer comprises a 5A10 antibody or fragment thereof (*e.g.*, a F(ab) fragment).

The heavy and light chain sequences of 5A10 antibody, which may be incorporated into fragments, are shown below:

5A10 Heavy chain

EVQLVESGPGILQPSQTLSLTCSFSGFSLSTTGMGVSWIRQPSGKGLEWLAHLYWDED KRYNPSLKSRLTISEDSSRNQVFLKITSVGPADSATYYCARKGYYGYFDYWGQGTALT VSS (SEQ ID NO: 1)

5A10 Light chain

DIVMTQSQKFMSTSVGDRVSVTCKASQNVNTDVAWYQQKPGQSPKALIFSTSY RSSGVPDRFTGSGSGTDFTLTITNVQSEDLAEYFCQQYSNYPLTFGAGTKVDLN (SEQ ID NO: 2)

In some embodiments, an immunoassay that measures the level of iPSA involves contacting iPSA present in the plasma blood sample with a capture antibody specific for free PSA, which includes iPSA and nicked PSA, under conditions in which the second capture antibody binds at least to iPSA, thereby producing a capture-antibody-iPSA complex and detecting the capture-antibody- iPSA complex using a second tracer. In some embodiments, the tracer comprises a 4D4 antibody. In some embodiments, the capture antibody is a 5A10 antibody or fragment thereof (*e.g.*, a F(ab) fragment).

In some embodiments, an immunoassay that measures the level of tPSA involves contacting tPSA present in the plasma blood sample with a capture antibody specific for tPSA under conditions in which the third capture antibody binds to tPSA, thereby producing a capture-antibody-tPSA complex; and detecting the capture-antibody-tPSA complex using a third tracer. In some embodiments, the tracer comprises a H50 antibody. In some embodiments, the capture antibody is a H117 antibody.

In some embodiments, an immunoassay that measures the level of hK2 involves contacting PSA in the plasma blood sample with blocking antibodies specific for PSA; contacting hK2 present in the plasma blood sample with a fourth capture antibody specific for

hK2 under conditions in which the fourth capture antibody binds to hK2, thereby producing a capture-antibody-hK2 complex; and detecting the capture-antibody-hK2 complex using a fourth tracer. In some embodiments, the tracer comprises a 7G1 antibody. In some embodiments, the capture antibody is a 6H10 $F(ab)_2$. In some embodiments, the blocking antibodies comprise a 5H7 antibody, a 5H6 antibody, and a 2E9 antibody.

Table 0 below lists antibodies and antigen-binding fragments that may be used in the methods disclosed herein and their corresponding epitopes.

Table 0: Antibodies and Epitopes/Sources of Antibodies

Antibody Name	**Epitope**	**Reference or Source**
$F(ab)_2$ 6H10		Becker *et al.* 2000. Sensitive and Specific Immunodetection of Human Glandular Kallikrein 2 in Serum. Clin Chem. 46(2), 198-206.
2E9	amino acids 79-93 and/or 80-91 of PSA protein (SEQ ID NO: 3)	Lilja *et al.* 1991. Prostate-Specific Antigen in Serum Occurs Predominantly in Complex with alpha-1-Antichymotrypsin. Clin Chem. 37(9), 1618-1625. Piironen, *et al.* Determination and analysis of antigenic epitopes of prostate specific antigen (PSA) and human glandular kallikrein 2 (hK2) using synthetic peptides and computer modeling. Protein Science (1998), 7:259-269

5F7		Nurmikko *et al.* 2000. Production and Characterization of Novel Anti-Prostate-specific Antigen (PSA) Monoclonal Antibodies That Do Not Detect Internally Cleaved Lys145-Lys146 Inactive PSA. Clin Chem. 46(10):1610-1618.
5H6	amino acids 225-237 of PSA protein (SEQ ID NO: 3)	Nurmikko *et al.* 2000. *Supra*
7G1		Nurmikko *et al.* 2000. *Supra*
Fab 5A10	amino acids 75-89, 80-94 and/or 82-39 of PSA protein (SEQ ID NO: 3)	Eriksson *et al.* 2000. Dual-label time-resolved immunofluorometric assay of free and total Prostate-specific Antigen Based on Recombinant Fab Fragments. Clin Chem 46(5), 658-666. Piironen *et al. Supra*
4D4	amino acids 130-144 of PSA protein (SEQ ID NO: 3)	U.S. Patent No. 7872104
H117		U.S. Patent No. 5672480
H50		U.S. Patent No. 5672480
5A10	amino acids 75-89, 80-94 and/or 82-39 of PSA protein	U.S. Patent No. 5939533, European Collection of Animal Cell Cultures

	(SEQ ID NO: 3)	Accession number 93091201. Piironen *et al. Supra*

Microfluidic Sample Analyzers

It should be appreciated that any of the immunoassay methods disclosed herein may be performed or implemented using a microfluidic device (e.g., a microfluidic sample analyzer). For example, a microfluidic device may be used to determine one or more characteristics of markers (e.g., levels of tPSA, fPSA, iPSA, or hK2). In some embodiments, a device is a microfluidic sample analyzer, which for example, may be configured to analyze a sample provided in a cassette having one or more microfluidic channels for containing and/or directing flow of a sample that comprises immunoassay components (e.g., antigen-antibody complexes, tracers, *etc.*). In some embodiments, a device further comprises an optical system including one or more light sources and/or one or more detectors configured for measuring levels of antigen-antibody complexes and/or tracers present in one or more microfluidic channels. Furthermore, in some embodiments, systems are provided, which may include a processor or computer programmed to evaluate a predictive model (e.g., a logistic regression model) in electronic communication with a microfluidic device (e.g., a microfluidic sample analyzer) or other device for determining a probability of an event associated with prostate cancer based on levels of markers (e.g., levels of tPSA, fPSA, iPSA, or hK2).

Non-limiting examples of suitable microfluidic devices are disclosed in US Patent Application Publication Number US 2013/0273643, entitled "METHODS AND APPARATUSES FOR PREDICTING RISK OF PROSTATE CANCER AND PROSTATE GLAND VOLUME," which published on October 17, 2013, the contents of which are incorporated herein by reference in their entirety for all purposes. It should be appreciated, however, that other types of device may also be used (e.g., plate readers, analyzers for microwell ELISA-type assays, *etc.*) as the disclosure is not limited in this respect.

Predictive Models and Computer Implemented Methods

Aspects of the disclosure provide computer implemented methods for determining a probability of an event associated with prostate cancer, such as the probability that a prostate tissue biopsy would contain detectable cancer. Such methods may involve receiving, via an

input interface, information indicative of the level of tPSA present in a blood plasma sample of a subject and receiving, via an input interface, information about whether the subject had a prior biopsy of prostate tissue. In some embodiments, the methods further involve evaluating, using at least one processor, a suitable predictive model (*e.g.*, a logistic regression model) based, at least in part, on the received information to determine a probability of the event associated with prostate cancer in the subject. The predictive model may generate the probability of the event associated with prostate cancer based, at least in part, on measured levels of tPSA and information about whether the subject had a prior biopsy of prostate tissue. The predictive model may generate the probability of the event associated with prostate cancer based, at least in part, on measured levels of tPSA, fPSA, iPSA, and hK2 and information about whether the subject had a prior biopsy of prostate tissue.

FIG. 1 illustrates a flowchart of a process 100 in accordance with some embodiments of the disclosure. In step 101, one or more values representing patient data corresponding to age, digital examination status and/or prior biopsy status are received by at least one processor for processing using one or more of the techniques described herein. In step 102 one or more values representing marker data for tPSA, fPSA, iPSA, and/or hK2 are received by the at least one processor. The values may be received in any suitable way including, but not limited to, through a local input interface such as a keyboard, touch screen, microphone, or other input device, from a network-connected interface that receives the value(s) from a device located remote from the processor(s), or directly from one or more detectors that measure the blood marker value(s) (*e.g.*, in an implementation where the processor(s) are integrated with a measurement device that includes the one or more detectors).

In step 103, after receiving the value(s) for tPSA, the process proceeds such that if levels of tPSA are above a threshold (*e.g.*, 25 ng/mL), then a first predictive model is selected and, if levels of tPSA are at or below the threshold, then a second predictive model is selected. Accordingly, at step 104, if the levels of tPSA are above the threshold level then a predictive model is selected that is based DRE status, prior biopsy status and tPSA levels. Alternatively, at step 105, if the levels of tPSA are at or below the threshold level, then a predictive model is selected based on DRE status, prior biopsy status and tPSA, fPSA, iPSA and hK2 levels. The predictive model of step 104, 105 is used to determine the probability that a subject has a prostate cancer. The prediction may be for a cancer of any grade or for a cancer of high grade, depending on the model used.

After determining a probability of a cancer, the process proceeds to step 106, where the probability is output to a user (*e.g.*, a physician, a patient) to guide a further diagnostic procedure and/or treatment decisions. The probability may be output in any suitable way. For example, in some embodiments, the probability may be output by displaying a numeric value representing the probability on a display screen of a device. In other embodiments, the probability may be output using one or more lights or other visual indicators on a device. In yet other embodiments, the probability may be provided using audio output, tactile output, or some combination of one or more of audio, tactile, and visual output. In some embodiments, outputting the probability comprises sending information to a network-connected device to inform a user about the determined probability. For example, the probability may be determined by one or more processors located at a remote site, and an indication of the probability may be sent to an electronic device of a user (*e.g.*, a physician) using one or more networks, in response to determining the probability at the remote site. The electronic device that provides output to a user in accordance with the techniques described herein may be any suitable device including, but not limited to, a laptop, desktop, or tablet computer, a smartphone, a pager, a personal digital assistant, and an electronic display.

In some embodiments, the probability of the prostate cancer is determined in accordance with equation (I), reproduced below:

$$\text{Probability} = \frac{e^L}{1+e^L} \quad (1)$$

where the logit (L) is determined using any of a plurality of logistic regression models. Non-limiting examples of different types of logistic regression models that may be used in accordance with the techniques described herein include:

1. Simple Model (tPSA only)

$$L = \beta_0 + \beta_1(Age) + \beta_2(tPSA) + \beta_3(priorbx) \quad (2)$$

or

$$L = \beta_0 + \beta_1 tpsa + \beta_2 dre_{neg} + \beta_3 dre_{pos} + \beta_4 priorbx \quad (3)$$

2. Four assay model using free/total ratio

In this model, the ratio of free PSA to total PSA is substituted for the free PSA term.

$$L = \beta_0 + \beta_1(Age) + \beta_2(tPSA) + \beta_3\left(\frac{fPSA}{tPSA}\right) + \beta_4(iPSA) + \beta_5(hK2) + \beta_6(priorbx) \quad (4)$$

3. Four assay model using log(tPSA) and free/total ratio

In this model, the log of tPSA is substituted for the tPSA term to account for the increased contribution of this predictive factor.

$$L = \beta_0 + \beta_1(Age) + \beta_2(\log[tPSA]) + \beta_3\left(\frac{fPSA}{tPSA}\right) + \beta_4(iPSA) + \beta_5(hK2) + \beta_6(priorbx) \quad (5)$$

4. Polynomial Model

In this model, additional non-linear terms for tPSA and fPSA are included. In the example equation provided below, the square of tPSA is used to emphasize the direct relationship between this term and risk of prostate cancer, and the square root of the free/total PSA term is used to reflect the inverse association of this term with risk. It should be appreciated however, that polynomial terms of higher order (*e.g.*, cubic) may also be included in some embodiments.

$$L = \beta_0 + \beta_1(Age) + \beta_2(tPSA) + \beta_3(fPSA) + \beta_4(iPSA) + \beta_5(hK2) + \beta_6(tPSA^2) + \beta_7\left(\sqrt{\frac{fPSA}{tPSA}}\right) + \beta_8(priorbx) \quad (6)$$

5. Linear Splines for all four assays

In this model, linear splines are added, with a single knot at the median value. The splines may be determined using the following equations:

$$\begin{aligned} sp1(x) &= x \text{ if } x < knot \\ sp1(x) &= knot \text{ if } x \geq knot \\ sp2(x) &= 0 \text{ if } x < knot \\ sp2(x) &= x - knot \text{ if } x \geq knot \end{aligned} \quad (7)$$

with the model being represented as:

$$L = \beta_0 + \beta_1(Age) + \beta_2(tPSA) + \beta_3(fPSA) + \beta_4(iPSA) + \beta_5(hK2) + \beta_6(sp1[tPSA])$$
$$+\beta_7(sp2[tPSA]) + \beta_8(sp1[fPSA]) + \beta_9(sp2[fPSA]) + \beta_{10}(sp1[iPSA]) + \beta_{11}(sp2[iPSA]) +$$
$$+\beta_{12}(sp1[hK2]) + \beta_{13}(sp2[hK2]) + \beta_{14}(priorbx) \quad (8)$$

6. Linear Splines for tPSA and fPSA

In this model, linear splines are included only for tPSA and fPSA to reduce the number of variables and simplify the model.

$$L = \beta_0 + \beta_1(Age) + \beta_2(tPSA) + \beta_3(fPSA) + \beta_4(iPSA) + \beta_5(hK2) + \beta_6(sp1[tPSA])$$
$$+\beta_7(sp2[tPSA]) + \beta_8(sp1[fPSA]) + \beta_9(sp2[fPSA]) + \beta_{10}(priorbx) \quad (9)$$

In the equations above "priorbx" is a binary value indicate of whether a subject had a prior biopsy to detect prostate cancer. A value of 1 indicates that a prior biopsy occurred and a value of 0 indicates that the prior biopsy did not occur.

7. Cubic Splines for all four assays

In this model, cubic splines are included for each term. In the example provided below, a cubic spline with four knots is described. It should be appreciated, however, that a cubic spline using any suitable number of knots including, but not limited to, five knots, six knots, seven knots, and eight knots, may alternatively be used. The splines may be determined using the following equations:

$$sp[x]1 = \max([x] - knot1, 0)^3 - \max([x] - knot3, 0)^3 \frac{knot4 - knot1}{knot4 - knot3}$$
$$+\max([x] - knot4, 0)^3 \frac{knot3 - knot1}{knot4 - knot3} \quad (10)$$

$$sp[x]2 = \max([x] - knot2, 0)^3 - \max([x] - knot3, 0)^3 \frac{knot4 - knot2}{knot4 - knot3}$$
$$+\max([x] - knot2, 0)^3 \frac{knot3 - knot2}{knot4 - knot3} \quad (11)$$

where *knot1* and *knot4* are external knots for the cubic spline, and *knot2* and *knot3* are internal knots for the cubic spline. The external knots may be set as the minimum and maximum levels of tPSA, fPSA, iPSA, or hK2 in a population. An internal knot (*e.g.*, knot2)

may be set as the 33.3 percentile value of tPSA, fPSA, iPSA, or hK2 levels in a population. Another internal knot (*e.g.*, knot3) may be set as the 66.6 percentile value of tPSA, fPSA, iPSA, or hK2 levels in a population.

In some embodiments, the internal knots are specified within the range of between about 2 to about 8 and between about 3 to about 6 for tPSA, between about 0.25 to about 2 and between about 0.5 to about 1.5 for fPSA, between about 0.2 to about 0.5 and between about 0.4 to about 0.8 for iPSA, and between about 0.02 to about 0.04 and between about 0.04 to about 0.08 for hK2. For example, in one implementation, values of 3.92 and 5.61 are used for the internal knots for tPSA, values of 0.82 and1.21 are used for the internal knots for fPSA, values of 0.3 and 0.51 are used for the internal knots of iPSA, and values of 0.036 and 0.056 are used for the internal knots of hK2.

In certain embodiments, one or more internal knots for tPSA may independently be in the range of between about 3 to about 5, between about 3 to about 6, between about 2.5 to about 6, between about 2.5 to about 6.5, between about 5 to about 8, between about 5.5 to about 8, between about 5 to about 9, between about 5 to about 10, between about 1 to about 5, between about 1 to about 4, and between about 1 to about 3. Other ranges are also possible.

In certain embodiments, one or more internal knots for fPSA may independently be in the range of between about 0.1 to about 1.0, between about 0.1 to about 1.2, between about 0.3 to about 0.8, between about 0.4 to about 0.9, between about 0.5 to about 1.2, between about 0.7 to about 1.4, between about 0.7 to about 0.9, between about 1.1 to about 1.6, between about 1.1 to about 1.2, and between about 1.1 to about 2. Other ranges are also possible.

In certain embodiments, one or more internal knots for iPSA may independently be in the range of between about 0.05 to about 0.5, between about 0.1 to about 0.5, between about 0.2 to about 0.5, between about 0.1 to about 0.8, between about 0.2 to about 0.8, between about 0.4 to about 0.8, between about 0.4 to about 1.0, between about 0.3 to about 0.6, between about 0.5 to about 1.0, and between about 0.6 to about 0.8. Other ranges are also possible.

In certain embodiments, one or more internal knots for hK2 may independently be in the range of between about 0.01 to about 0.03, between about 0.01 to about 0.04, between about 0.01 to about 0.05, between about 0.02 to about 0.05, between about 0.02 to about 0.06, between about 0.03 to about 0.05, between about 0.4 to about 0.07, between about 0.04 to about 1.0, between about 0.5 to about 1.0, and between about 0.6 to about 1.0. Other ranges are also possible.

As discussed above, cubic splines incorporating any suitable number of internal knots (*e.g.*, three, four, five, six internal knots) may be used, and the example of a cubic spline including two internal knots is provided merely for illustration and not limitation. In embodiments that include more than two internal knots, the knots may be placed within one or more of the ranges discussed above, or in some other suitable range. For example, in some embodiments, the knots may be specified such that the length of the segments of the spline between each of the pairs of neighboring knots is essentially equal.

The model may be represented as:

$$L = \beta_0 + \beta_1(Age) + \beta_2(tPSA) + \beta_3(fPSA) + \beta_4(iPSA) + \beta_5(hK2) + \beta_6(sp1[tPSA]) + \beta_7(sp2[tPSA]) + \beta_8(sp1[fPSA]) + \beta_9(sp2[fPSA]) + \beta_{10}(sp1[iPSA]) + \beta_{11}(sp2[iPSA]) + \beta_{12}(sp1[hK2]) + \beta_{13}(sp2[hK2]) + \beta_{14}(priorbx) \quad (12)$$

8. tPSA Threshold Model

In some embodiments, the model selected may depend on the whether or not a threshold level of tPSA is detected in sample. In some embodiments, if the level of tPSA is above a threshold in a sample, then the predictive model is as follows:

$$L = \beta_0 + \beta_1(tPSA) + \beta_2(DRE)_{neg} + \beta_3(DRE)_{pos} + \beta_4(priorbx) \quad (13)$$

In some embodiments, the range of values of the weighting coefficients in this model are as set forth in Table 1 below. Coefficients suitable for determining the probability that a prostate tissue biopsy will have a cancer of any grade are shown in the second and third columns; whereas coefficients suitable for determining the probability that a prostate tissue biopsy will have a cancer of high grade are shown in the fourth and fifth columns.

Table 1: Weighting Coefficients to be used when level of tPSA is greater than Threshold

Weighting Coefficient Ranges	Cancer of Any Grade		Cancer of High Grade (Gleason Score >= 7.0)	
	Low	High	Low	High
β_0	-1.22E+00	-9.07E-01	7.83E-01	9.31E-01
β_1	1.04E-01	1.22E-01	1.24E-02	1.59E-02

β_2	-6.62E-02	-4.99E-02	-2.19E-01	-1.72E-01
β_3	1.34E-01	1.71E-01	5.23E-01	6.44E-01
β_4	-1.30E+00	-8.91E-01	-1.94E+00	-1.68E+00

In some embodiments, if the level of tPSA detected in a sample is less than or equal to a threshold level, then the predictive model is as follows:

$$L = \beta_0 + \beta_1(Age) + \beta_2(tPSA) + \beta_3 sp1(tPSA) + \beta_4 sp2(tPSA) + \beta_5(fPSA) + \beta_6 sp1(fPSA) + \beta_7 sp2(fPSA) + \beta_8(iPSA) + \beta_9(hK2) + \beta_{10}(DRE_{neg}) + \beta_{11}(DRE_{pos}) + \beta_{12}(priorbx) \quad (14)$$

In some embodiments, the range of values of the weighting coefficients in this model are as set forth in Table 2 below. Coefficients suitable for determining the probability that a prostate tissue biopsy will have a cancer of any grade are shown in the second and third columns; whereas coefficients suitable for determining the probability that a prostate tissue biopsy will have a cancer of high grade are shown in the fourth and fifth columns.

Table 2: Weighting Coefficients to be used when level of tPSA is less than or equal to a threshold

Weighting Coefficient Ranges	Cancer of Any Grade		Cancer of High Grade (Gleason Score > 7.0)	
	Low	High	Low	High
β_0	-2.86E+00	-1.97E+00	-7.35E+00	-6.00E+00
β_1	2.88E-01	4.03E-01	4.79E-02	6.38E-02
β_2	3.76E-01	4.72E-01	7.44E-01	9.19E-01
β_3	-2.18E-04	-1.78E-04	-6.43E-03	-4.32E-03
β_4	-1.22E-03	-9.46E-04	1.20E-02	1.66E-02
β_5	-3.63E+00	-3.18E+00	-6.27E+00	-4.43E+00
β_6	5.07E-01	7.07E-01	7.63E-01	1.04E+00
β_7	-2.02E+00	-1.55E+00	-2.76E+00	-2.17E+00
β_8	4.16E-02	5.45E-02	1.96E+00	2.40E+00
β_9	7.87E+00	1.11E+01	6.62E+00	7.59E+00
β_{10}	-6.62E-02	-4.65E-02	-2.44E-01	-1.74E-01
β_{11}	1.28E-01	1.85E-01	4.57E-01	5.89E-01
β_{12}	-1.45E+00	-1.01E+00	-1.97E+00	-1.53E+00

The spline terms of sp1(tPSA), sp2(tPSA), sp1(fPSA), and sp2(fPSA) in the model above may be determined according to the cubic spline formula presented above under model #7 above

(Equations (10 and 11)). In some embodiments, the values of internal knots 2 and 3 and external knots 1 and 4 are within the ranges set forth in Table 3 below for tPSA and fPSA.

Table 3: Knot value ranges

Knot value ranges	Total PSA		Free PSA	
	Low	High	Low	High
Knot 1	0	2	0	0.5
Knot 2	3.72E+00	4.16E+00	7.38E-01	9.43E-01
Knot 3	4.71E+00	6.56E+00	1.10E+00	1.43E+00
Knot 4	2.33E+02	3.13E+02	2.04E+01	2.78E+01

Computer Implementation

An illustrative implementation of a computer system 106 on which some or all of the techniques and/or user interactions described herein may be implemented is shown in FIG. 1B. The computer system 106 may include one or more processors 107 and one or more computer-readable non-transitory storage media (*e.g.*, memory 108 and one or more non-volatile storage media 110). The processor(s) 107 may control writing data to and reading data from the memory 108 and the non-volatile storage device 110 in any suitable manner, as the aspects of the present invention described herein are not limited in this respect.

To perform any of the functionality described herein, the processor(s) 107 may execute one or more instructions, such as program modules, stored in one or more computer-readable storage media (*e.g.*, the memory 108), which may serve as non-transitory computer-readable storage media storing instructions for execution by the processor 107. Generally, program modules include routines, programs, objects, components, data structures, etc. that perform particular tasks or implement particular abstract data types. Embodiments may also be implemented in distributed computing environments where tasks are performed by remote processing devices that are linked through a communications network. In a distributed computing environment, program modules may be located in both local and remote computer storage media including memory storage devices. Data inputs and program commands may be received by the computer 106 through a input interface 109. The input interface 109 may comprise a keyboard, touchscreen, USB port, CD drive, DVD drive, or other input interface.

Computer 106 may operate in a networked environment using logical connections to one or more remote computers. The one or more remote computers may include a personal computer, a server, a router, a network PC, a peer device or other common network node, and typically include many or all of the elements described above relative to the computer 106. Logical connections between computer 106 and the one or more remote computers may include, but are not limited to, a local area network (LAN) and a wide area network (WAN), but may also include other networks. Such networks may be based on any suitable technology and may operate according to any suitable protocol and may include wireless networks, wired networks or fiber optic networks. Such networking environments are commonplace in offices, enterprise-wide computer networks, intranets and the Internet.

When used in a LAN networking environment, the computer 106 may be connected to the LAN through a network interface or adapter. When used in a WAN networking environment, the computer 106 typically includes a modem or other means for establishing communications over the WAN, such as the Internet. In a networked environment, program modules, or portions thereof, may be stored in the remote memory storage device.

Various inputs described herein for assessing a risk of prostate cancer and/or determining a prostate gland volume may be received by computer 106 via a network (*e.g.*, a LAN, a WAN, or some other network) from one or more remote computers or devices that stores data associated with the inputs. One or more of the remote computers/devices may perform analysis on remotely-stored data prior to sending analysis results as the input data to computer 300. Alternatively, the remotely stored data may be sent to computer 106 as it was stored remotely without any remote analysis. Additionally, inputs may be received directly by a user of computer 106 using any of a number of input interfaces (*e.g.*, input interface 109) that may be incorporated as components of computer 106.

Various outputs described herein, including output of a probability of prostate cancer risk and/or prostate gland volume, may be provided visually on an output device (*e.g.*, a display) connected directly to computer 106 or the output(s) may be provided to a remotely-located output device connected to computer 106 via one or more wired or wireless networks, as embodiments of the invention are not limited in this respect. Outputs described herein may additionally or alternatively be provided other than using visual presentation. For example, computer 300 or a remote computer to which an output is provided may include one or more

output interfaces including, but not limited to speakers, and vibratory output interfaces, for providing an indication of the output.

It should be appreciated that although computer 106 is illustrated in FIG. 1 as being a single device, in some embodiments, computer 106 may comprise a plurality of devices communicatively coupled to perform some or all of the functionality described herein, and computer 106 is only one illustrative implementation of a computer that may be used in accordance with embodiments of the invention. For example, in some embodiments, computer 106 may be integrated into and/or in electronic communication with the system. As described above, in some embodiments, computer 106 may be included in a networked environment, where information about one or more blood markers, used to determine a probability of prostate cancer and/or prostate gland volume, is sent from an external source to computer 106 for analysis using one or more of the techniques described herein. An illustrative networked environment 111 in accordance with some embodiments of the invention is shown in FIG. 1C. In networked environment 111, computer 106 is connected to an assay system 112 via network 114. As discussed above, network 114 may be any suitable type of wired or wireless network, and may include one or more local area networks (LANs) or wide area networks (WANs), such as the Internet.

The calculation methods, steps, simulations, algorithms, systems, and system elements described herein may be implemented using a computer system, such as the various embodiments of computer systems described below. The methods, steps, systems, and system elements described herein are not limited in their implementation to any specific computer system described herein, as many other different machines may be used.

The computer system may include a processor, for example, a commercially available processor such as one of the series x86, Celeron and Pentium processors, available from Intel, similar devices from AMD and Cyrix, the 680X0 series microprocessors available from Motorola, the PowerPC microprocessor from IBM, and ARM processors. Many other processors are available, and the computer system is not limited to a particular processor.

A processor typically executes a program called an operating system, of which Windows 7, Windows 8, UNIX, Linux, DOS, VMS, MacOS and OSX, and iOS are examples, which controls the execution of other computer programs and provides scheduling, debugging, input/output control, accounting, compilation, storage assignment, data management and memory management, communication control and related services. The processor and operating

system together define a computer platform for which application programs in high-level programming languages are written. The computer system is not limited to a particular computer platform.

The computer system may include a memory system, which typically includes a computer readable and writeable non-volatile recording medium, of which a magnetic disk, optical disk, a flash memory and tape are examples. Such a recording medium may be removable, for example, a floppy disk, read/write CD or memory stick, or may be permanent, for example, a hard drive.

Such a recording medium stores signals, typically in binary form (i.e., a form interpreted as a sequence of one and zeros). A disk (e.g., magnetic or optical) has a number of tracks, on which such signals may be stored, typically in binary form, i.e., a form interpreted as a sequence of ones and zeros. Such signals may define a software program, e.g., an application program, to be executed by the microprocessor, or information to be processed by the application program.

The memory system of the computer system also may include an integrated circuit memory element, which typically is a volatile, random access memory such as a dynamic random access memory (DRAM) or static memory (SRAM). Typically, in operation, the processor causes programs and data to be read from the non-volatile recording medium into the integrated circuit memory element, which typically allows for faster access to the program instructions and data by the processor than does the non-volatile recording medium.

The processor generally manipulates the data within the integrated circuit memory element in accordance with the program instructions and then copies the manipulated data to the non-volatile recording medium after processing is completed. A variety of mechanisms are known for managing data movement between the non-volatile recording medium and the integrated circuit memory element, and the computer system that implements the methods, steps, systems and system elements described above is not limited thereto. The computer system is not limited to a particular memory system.

At least part of such a memory system described above may be used to store one or more data structures (e.g., look-up tables) or equations described above. For example, at least part of the non-volatile recording medium may store at least part of a database that includes one or more of such data structures. Such a database may be any of a variety of types of databases, for example, a file system including one or more flat-file data structures where data is organized into data units separated by delimiters, a relational database where data is organized into data

units stored in tables, an object-oriented database where data is organized into data units stored as objects, another type of database, or any combination thereof.

The computer system may include a video and audio data I/O subsystem. An audio portion of the subsystem may include an analog-to-digital (A/D) converter, which receives analog audio information and converts it to digital information. The digital information may be compressed using known compression systems for storage on the hard disk to use at another time. A typical video portion of the I/O subsystem may include a video image compressor/decompressor of which many are known in the art. Such compressor/decompressors convert analog video information into compressed digital information, and vice-versa. The compressed digital information may be stored on hard disk for use at a later time.

The computer system may include one or more output devices. Example output devices include a cathode ray tube (CRT) display, liquid crystal displays (LCD) and other video output devices, printers, communication devices such as a modem or network interface, storage devices such as disk or tape, and audio output devices such as a speaker.

The computer system also may include one or more input devices. Example input devices include a keyboard, keypad, track ball, mouse, pen and tablet, communication devices such as described above, and data input devices such as audio and video capture devices and sensors. The computer system is not limited to the particular input or output devices described herein.

It should be appreciated that one or more of any type of computer system may be used to implement various embodiments described herein. Aspects of the disclosure may be implemented in software, hardware or firmware, or any combination thereof. The computer system may include specially programmed, special purpose hardware, for example, an application-specific integrated circuit (ASIC). Such special-purpose hardware may be configured to implement one or more of the methods, steps, simulations, algorithms, systems, and system elements described above as part of the computer system described above or as an independent component.

The computer system and components thereof may be programmable using any of a variety of one or more suitable computer programming languages. Such languages may include procedural programming languages, for example, C, Pascal, Fortran and BASIC, object-oriented languages, for example, C++, Java and Eiffel and other languages, such as a scripting language or even assembly language.

The methods, steps, simulations, algorithms, systems, and system elements may be implemented using any of a variety of suitable programming languages, including procedural programming languages, object-oriented programming languages, other languages and combinations thereof, which may be executed by such a computer system. Such methods, steps, simulations, algorithms, systems, and system elements can be implemented as separate modules of a computer program, or can be implemented individually as separate computer programs. Such modules and programs can be executed on separate computers.

Such methods, steps, simulations, algorithms, systems, and system elements, either individually or in combination, may be implemented as a computer program product tangibly embodied as computer-readable signals on a computer-readable medium, for example, a non-volatile recording medium, an integrated circuit memory element, or a combination thereof. For each such method, step, simulation, algorithm, system, or system element, such a computer program product may comprise computer-readable signals tangibly embodied on the computer-readable medium that define instructions, for example, as part of one or more programs, that, as a result of being executed by a computer, instruct the computer to perform the method, step, simulation, algorithm, system, or system element.

It should be appreciated that various embodiments may be formed with one or more of the above-described features. The above aspects and features may be employed in any suitable combination as the present invention is not limited in this respect. It should also be appreciated that the drawings illustrate various components and features which may be incorporated into various embodiments. For simplification, some of the drawings may illustrate more than one optional feature or component. However, the invention is not limited to the specific embodiments disclosed in the drawings. It should be recognized that the disclosure encompasses embodiments which may include only a portion of the components illustrated in any one drawing figure, and/or may also encompass embodiments combining components illustrated in multiple different drawing figures.

EXAMPLES

Example 1- Assay and Predictive Model

Described herein is an assay based on a panel of four kallikrein markers that include total prostate specific antigen (tPSA), free PSA (fPSA), intact PSA (iPSA), and human Kallikrein 2

(hK2) linked to patient specific information via a multivariate algorithm. This algorithm returns two calibrated probabilities: one for the risk of cancer of any grade and another for the risk of high grade cancer (Gleason 7 or greater) prior to biopsy.

The four kallikrein markers have been studied individually and in various combinations for prostate cancer detection applications. A logistic regression algorithm incorporating the blood plasma levels of these four markers as well as patient-specific information such as age, result from a digital rectal exam (DRE) and existence of prior negative prostate biopy(-ies) demonstrated a higher positive predictive value for prostate cancer than the PSA test alone.

Three hundred patients were included in an initial calibration study. This included the first 5 patients enrolled at each study site, then sequentially enrolled patients. Exclusions were made for samples that were not optimally stored and/or shipped, or where the sample produced abnormal results during measurement of the kallikrein markers.

Logistic regression algorithm for calculating risk of cancer on biopsy

A formula for a predictive model for calculating risk of cancer on biopsy was established through the calibration study and is presented below. As noted, a different formula is used depending on the total PSA levels. Moreover, different weighting coefficients are used depending on whether the model is being used to determine the probability of a biopsy containing a detectable cancer of any grade versus a detectable cancer of high grade (e.g., Gleason score of 7.0 or greater). Weighting coefficients are within the ranges specified in Tables 1 and 2 herein. The variables of the formulae are described in Table 4.

If Total PSA≤25 ng/mL

$$X\beta = \beta_0 + \beta_1 age + \beta_2 tpsa + \beta_3 sptpsa1 + \beta_4 sptpsa2 + \beta_5 fpsa + \beta_6 spfpsa1 + \beta_7 spfpsa2 + \beta_8 ipsa + \beta_9 hK2 + \beta_{10} dre_{neg} + \beta_{11} dre_{pos} + \beta_{12} priorbx \quad (14)$$

If Total PSA>25 ng/mL

$$X\beta = \beta_0 + \beta_1 tpsa + \beta_2 dre_{neg} + \beta_3 dre_{pos} + \beta_4 priorbx \quad (13)$$

$$Risk\ of\ Cancer\ on\ Biopsy = \frac{e^{X\beta}}{1 + e^{X\beta}} \quad (15)$$

Restricted Cubic Spline Terms:

For some variables in the models (total PSA and free PSA), restricted cubic spline terms were included, meaning that two additional terms are added to each of the models for each splined term. The formulas for calculating the two spline terms are below.

$$sp[var]1 = \max([var] - knot1, 0)^3 - \max([var] - knot3, 0)^3 \frac{knot4 - knot1}{knot4 - knot3} + \max([var] - knot4, 0)^3 \frac{knot3 - knot1}{knot4 - knot3} \quad (10)$$

$$sp[var]2 = \max([var] - knot2, 0)^3 - \max([var] - knot3, 0)^3 \frac{knot4 - knot2}{knot4 - knot3} + \max([var] - knot4, 0)^3 \frac{knot3 - knot2}{knot4 - knot3} \quad (11)$$

Sp[var]1 and sp[var]2 are computed for total and free PSA using the formulae above. The spline term for total PSA was calculated using knot values within the ranges specified in Table 3.

Table 4. Variables for formula for calculating risk of cancer on biopsy

Variable Name	Description
age	Age at Blood Draw
tpsa	Total PSA in ng/ml
fpsa	Free PSA in ng/ml
ipsa	Intact PSA in ng/ml
hk2	hK2 in ng/ml
sptpsa1	First spline term for total PSA
sptpsa2	Second spline term for total PSA
spfpsa1	First spline term for free PSA
spfpsa2	Second spline term for free PSA
priorbx	Prior Biopsy; 0 if no prior biopsy, 1 if had prior biopsy
dreneg	Value is equal to 1 if the DRE has been confirmed as negative, 0 otherwise
drepos	Value is equal to 1 if the DRE has been confirmed as positive, 0 otherwise

Results from the Calibration

The characteristics of patients enrolled in the calibration phase of the study are shown in Table 5.

Table 5. Characteristics of patients in the calibration phase

Characteristic	Negative Biopsy (N=173)	Positive Biopsy (N=127)	p-value
Age at Blood Draw, average age in years (25 and 75 percentiles)	63 (59, 69)	65 (60, 70)	0.046
Abnormal DRE, n	43 (25%)	39 (31%)	0.3
Prior Prostate Biopsy, n	37 (21%)	15 (12%)	0.030
Total PSA, ng/mL (25 and 75 percentiles)	4.5 (3.4, 5.8)	5.4 (4.3, 7.5)	<0.0001
Free PSA, ng/mL (25 and 75 percentiles)	0.9 (0.6, 1.2)	0.7 (0.5, 1.2)	0.2
Intact PSA, ng/mL (25 and 75 percentiles)	0.4 (0.3, 0.6)	0.5 (0.3, 0.7)	0.4
hK2, ng/mL (25 and 75 percentiles)	0.1 (0.0, 0.1)	0.1 (0.1, 0.1)	0.034
Clinical T Stage			
T1C		57 (45%)	
T2A		33 (26%)	
T2B		15 (12%)	
T2C		21 (17%)	
T3A		1 (0.8%)	
Biopsy Gleason Grade (Detail)			
3+3		67 (53%)	
3+4		31 (24%)	
3+5		1 (0.8%)	
4+3		14 (11%)	
4+4		8 (6.3%)	
4+5		3 (2.4%)	
5+4		1 (0.8%)	
5+5		2 (1.6%)	

Model Calibration

A model was developed based on a European cohort. Logistic regression recalibration was used with both slope and intercept coefficients to test for miscalibration on an American cohort.

$$\beta_0 + \beta_1 \log\left(\frac{L}{1-L}\right) \quad (16)$$

If there was evidence that $\beta_0 \neq 0$ or $\beta_1 \neq 1$, this would indicate that it would be useful to recalibrate the model.

The model predicting high grade cancer exhibited near perfect calibration for predictions below 0.2 (or 20%), while there appeared to be some underestimation of actual risk for predictions greater than 0.2 (or 20%) (FIG. 2). It is noted that the decision to refer patients for biopsy would occur at thresholds below 0.2 (or 20%), where the model appears to accurately predict the true risk of high grade cancer. For this reason, no recalibration was performed for the high grade model. The model predicting any grade of cancer did not exhibit significant miscalibration, and was therefore not recalibrated (FIG. 3). Data points in FIGs. 2 and 3 shows the relationship between predicted and actual probabilities and the dotted line is a line fitted to the data. Bars indicating the extent of variation in actual probability are shown. The solid line reflects perfect calibration where actual probabilities equal predicted probabilities.

Model Performance

Below is the report of the performance of the predictive model. All statistics were corrected for overfit using repeated 10-fold cross validation.

Table 6. *Discrimination of the Predictive Model*

	AUC (95% CI)
Any Grade Cancer	0.769 (0.715, 0.824)
High Grade Cancer	0.857 (0.805, 0.909)

Table 7. *Brier Score for the Predictive Model*

	Brier Score
Any Grade Cancer	0.1967
High Grade Cancer	0.1144

Biopsies Avoided Under Varying Biopsy Schemes

The number of High Grade Cancers (Table 5) and Any Grade Cancers (Table 6) found and missed via different biopsying schemes per 1000 patients were determined.

Table 8. High Grade Cancers Found/Missed

Label	Biopsies	Biopsies Avoided	High Grade Cancers Found	High Grade Cancers Missed
Prob of High Grade PCa>5%	686	314	190	10
>7.5%	538	462	181	19
>10%	445	555	173	27
>12.5%	370	630	167	33

Table 9. Any Grade Cancers Found/Missed

Label	Biopsies	Biopsies Avoided	Cancers Found	Cancers Missed
Prob of High Grade PCa>5%	686	314	371	52
>7.5%	538	462	329	95
>10%	445	555	297	126
>12.5%	370	630	270	154

Decision Curve Analysis

The decision curve analysis for High Grade Cancer is shown in FIG. 4. The decision curve analysis for Any Grade Cancer is shown in FIG. 5.

Receiver Operating Curves (ROC)

The ROC for High Grade Cancer is shown in FIG. 6. The ROC for Any Grade Cancer is shown in FIG. 7.

Negative Predictive Value and Positive Predictive Value by Biopsy Threshold

The Positive Predictive Value and Negative Predictive Value by Biopsy Threshold for High Grade Cancer are shown in FIG. 8A and 8B, respectively. The Positive Predictive Value and Negative Predictive Value by Biopsy Threshold for Any Grade Cancer are shown in FIG. 9A and 9B, respectively.

Example 2: Validation Study:

An assessment of the performance of the model presented in Example 1 and as set forth in Equations (10, 11, 13, 14), which is referred to in this example as the "test model", was performed based on 663 patients enrolled in the validation phase of the study. Results are presented separately for the entire cohort, men with a prior biopsy, men with no prior biopsy, and men aged 50-75. The FIG. 10 shows the proportion of men who harbored high grade disease by age at biopsy. Older men had much higher rates of high grade disease.

One possibility for the observed increase in risk at higher ages is more selective biopsy. In other words, urologists may only biopsy a man over the age of 70 – the upper limit for PSA screening in many guidelines – if there is a compelling reason to do so. To assess whether the increase in is the proportion of high grade cancers among older men was due to biopsy selection we utilized the PCPT risk calculator (*See* Thompson IM, Ankerst DP, Chi C, Goodman PJ, Tangen CM, Lucia MS, Feng Z, Parnes HL, Coltman CA Jr. Assessing prostate cancer risk: Results from the Prostate Cancer Prevention Trial, Journal of the National Cancer Institute 98: 529-534, 2006.). The PCPT risk calculator was built on a cohort of men where biopsy was offered to all men regardless of age. In a logistic regression model with high grade disease as the outcome and PCPT risk and older age as covariate, if the age coefficient is significant it suggests that the effect of age we are observing is due to the selection, rather than a biologic increase in risk. These results indicate that risk in older men is higher than expected (p=0.072), suggesting a selection effect. A subgroup analysis was performed of men aged 50 – 75. Since there were 20 patients of an age less than 50, an additional subgroup analysis was conducted that excluded patients aged over 70.

Two separate models were compared: the "test model" and a base model that was based on total PSA, age, prior biopsy, and DRE. Table 10 is an outline of differences in patient characteristics between the calibration phase and the validation phase cohorts.

Table 10. Patient Characteristics

	Validation phase Cohort (N=663; 69%)	Calibration Phase (N=300; 31%)	p-value
Age at Blood Draw, years (25 and 75 percentiles)	64 (58, 69)	64 (59, 69)	0.7

<50	24 (3.6%)	19 (6.3%)	
50-75	587 (89%)	261 (87%)	
>75	52 (7.8%)	20 (6.7%)	
Abnormal DRE	152 (23%)	82 (27%)	0.14
Prior Prostate Biopsy	128 (19%)	52 (17%)	0.5
Total PSA, ng/m l(25 and 75 percentiles)	4.7 (3.3, 6.8)	4.8 (3.6, 6.5)	0.4
Free PSA, ng/ml (25 and 75 percentiles)	0.9 (0.6, 1.4)	1.0 (0.6, 1.4)	0.7
Intact PSA, ng/ml (25 and 75 percentiles)	0.4 (0.3, 0.7)	0.4 (0.3, 0.6)	0.7
hK2, ng/ml (25 and 75 percentiles)	0.1 (0.1, 0.1)	0.1 (0.0, 0.1)	0.4
Positive Biopsy	297 (45%)	127 (42%)	0.5
High Grade Cancer	146 (22%)	60 (20%)	0.5

Table 11 below provides patient characteristics of the validation phase cohort separated by cancer status.

It was found that the test model has higher discrimination for high grade disease that the base model, with an increase in AUC by about 0.06. This difference is relatively stable across conditions. It is slightly greater for patients with prior biopsy (0.09) and in the diagnostic "grey zone" (0.07 – 0.09). Differences between base and test models for the endpoint of positive biopsy are smaller, clearly demonstrating the selectivity of the test models for high grade disease.

Tables 14 and 15 below outline the number of high grade found and missed for all patients and those under 70 years of age via different biopsy schemes per 1000 patients. In an analysis of clinical consequences, it was found that using a cut-point of 7.5% reduces the number of biopsies by about 50%. This does lead to missing some high grade cancers, an effect that is reduced when the analysis is restricted to men aged less than 71. Of younger patients with a risk <7.5%, 5.5% had Gleason score 7 or 8, meaning that 18 biopsies would need to be conducted to find one high grade cancer in this group. Of the missed high grade cancers, 53% were 3 + 4, 40% were 4 + 3 and 7% 4 + 4.

Table 11. Validation Study Cohort by Cancer Status

	No Cancer (N=366; 55%)	Cancer (N=297; 45%)	p-value
Age at Blood Draw, years (25 and 75 percentiles)	63 (58, 68)	65 (59, 71)	0.0004
<50	19 (5.2%)	5 (1.7%)	
50-75	324 (89%)	263 (89%)	
>75	23 (6.3%)	29 (10%)	
Abnormal DRE	77 (21%)	75 (25%)	0.2
Prior Prostate Biopsy	90 (25%)	38 (13%)	0.0001
Total PSA, ng/ml (25 and 75 percentiles)	4.3 (2.8, 5.8)	5.3 (4.0, 8.1)	<0.0001
Free PSA, ng/ml (25 and 75 percentiles)	0.9 (0.6, 1.4)	1.0 (0.7, 1.4)	0.085
Intact PSA, ng/ml (25 and 75 percentiles)	0.4 (0.3, 0.6)	0.5 (0.3, 0.7)	0.0003
hK2, ng/ml (25 and 75 percentiles)	0.1 (0.0, 0.1)	0.1 (0.1, 0.1)	<0.0001
Clinical T Stage			
T1A		1 (0.3%)	
T1B		1 (0.3%)	
T1C		194 (65%)	
T2A		53 (18%)	
T2B		22 (7.4%)	
T2C		23 (7.7%)	
T3A		2 (0.7%)	
T4		1 (0.3%)	
Biopsy Gleason Grade			
6		151 (51%)	
7		102 (34%)	
8		25 (8.4%)	
9		17 (5.7%)	
10		2 (0.7%)	

Table 12. Model Differences

All Patients	Test model	Base Model (tPSA, Age, DRE, and Prior Biopsy)	PCPT
High Grade Cancer (Bx GGS>6)	0.824 (0.784, 0.864)	0.763 (0.719, 0.806)	0.760 (0.718, 0.802)
Positive Biopsy	0.729 (0.691, 0.768)	0.704 (0.665, 0.744)	0.680 (0.639, 0.720)
Age 50-75			
High Grade Cancer (Bx GGS>6)	0.816 (0.771, 0.860)	0.747 (0.699, 0.796)	0.741 (0.693, 0.788)
Positive Biopsy	0.730 (0.690, 0.771)	0.694 (0.651, 0.736)	0.662 (0.619, 0.706)

Prior Biopsy			
High Grade Cancer (Bx GGS>6)	0.775 (0.654, 0.896)	0.687 (0.557, 0.817)	0.671 (0.524, 0.818)
Positive Biopsy	0.702 (0.596, 0.808)	0.654 (0.548, 0.759)	0.639 (0.535, 0.743)
Biopsy Naive			
High Grade Cancer (Bx GGS>6)	0.835 (0.795, 0.875)	0.766 (0.720, 0.813)	0.791 (0.749, 0.834)
Positive Biopsy	0.715 (0.672, 0.758)	0.692 (0.648, 0.737)	0.684 (0.639, 0.729)
Age less than 71			
High Grade Cancer (Bx GGS>6)	0.822 (0.773, 0.870)	0.757 (0.705, 0.810)	0.757 (0.707, 0.806)
Positive Biopsy	0.737 (0.694, 0.780)	0.709 (0.665, 0.753)	0.684 (0.639, 0.729)
PSA 2 – 10 ng / mL ("Grey zone")			
High Grade Cancer (Bx GGS>6)	0.768 (0.708, 0.829)	0.700 (0.634, 0.766)	0.677 (0.612, 0.741)
Positive Biopsy	0.707 (0.657, 0.757)	0.666 (0.614, 0.718)	0.622 (0.568, 0.675)

Table 13. Brier Score

All Patients	Test model	Base Model (tPSA, Age, DRE, and Prior Biopsy)	PCPT
High Grade Cancer (Bx GGS>6)	0.1255	0.1432	0.1680
Positive Biopsy	0.2060	0.2178	0.2577
Age 50-75			
High Grade Cancer (Bx GGS>6)	0.1222	0.1410	0.1615
Positive Biopsy	0.2054	0.2210	0.2609
Prior Biopsy			
High Grade Cancer (Bx GGS>6)	0.1111	0.1156	0.1166
Positive Biopsy	0.1787	0.1921	0.2009
Biopsy Naive			
High Grade Cancer (Bx GGS>6)	0.1289	0.1498	0.1802
Positive Biopsy	0.2126	0.2239	0.2712
Age less than 71			
High Grade Cancer (Bx	0.1116	0.1308	0.1471

GGS>6)			
Positive Biopsy	0.1990	0.2143	0.2495

All Patients

Table 14. High Grade Cancer

Label	Biopsies	Biopsies Avoided	High Grade Cancers Found	High Grade Cancers Missed
Prob of High Grade PCa>2.5%	805	195	210	11
>5%	664	336	204	17
>7.5%	534	466	193	27
>10%	454	546	181	39
>12.5%	386	614	169	51

Age less than 71

Table 15. High Grade Cancer

threshold	Biopsies	Biopsies Avoided	High Grade Cancers Found	High Grade Cancers Missed
Prob of High Grade PCa>2.5%	779	221	178	11
>5%	624	376	170	19
>7.5%	490	510	161	28
>10%	406	594	151	37
>12.5%	340	660	140	49

FIGs. 11A and 11B show predicted versus actual probabilities of detecting high grade cancer in all patients (n=663). FIG 11C shows predicted versus actual probabilities of detecting any grade cancer in all patients (n=663). FIGs. 12A and 12B show predicted versus actual probabilities of detecting high grade cancer in patients aged 50-75 (n=587). FIG 12C shows predicted versus actual probabilities of detecting any grade cancer in all patients aged 50-75

(n=587). FIGs. 13A and 13B show predicted versus actual probabilities of detecting high grade cancer in patients aged less than 71 (n=535). FIG 13C shows predicted versus actual probabilities of detecting any grade cancer in all patients aged less than 71 (n=535). The foregoing results show that there is a degree of underprediction of risk, an effect that is reduced by restricting the sample to patients aged less than 71. For FIGs 11 to 13, data points show the relationship between predicted and actual probabilities and the dotted line is a line fitted to the data. Bars indicating the extent of variation in actual probabilities are shown. The solid lines reflect perfect calibration where actual probabilities equal predicted probabilities.

FIGs. 14A and 14B show net benefit versus threshold probability levels for all patients (n = 663). FIGs. 15A and 15B show net benefit versus threshold probability levels for patients aged 50-75 (n=587). FIGs. 16A and 16B show net benefit versus threshold probability levels for all patients aged less than 71 (n=535). The data indicate that use of the predictive model is associated with clear net benefit for detecting high grade cancer. This effect is strengthened for the patients aged less than 71. Net benefit is evaluated as described in Vickers A.J. *et al.*, Net benefit and threshold probability were established using methods disclosed in *Med Decis Making*. 2006 ; 26(6): 565–574, the entire contents of which is incorporated herein by reference.

Example 3 – Immunoassay Methods:

The following assay methods are useful for determining levels (*e.g.*, levels in ng/mL) of tPSA, fPSA, iPSA, and hK2 present in human plasma samples using the AutoDELFIA automatic immunoassay system and were used in connection with Examples 1 and 2. The averaged amount of each marker is calculated from the duplicate tests for each marker and is used in a predictive model to determine a risk score for a given human plasma sample as presented in Example 2. tPSA and fPSA may also be determined using an Elecsys immunoassay analyzer (Roche Diagnostics).

Each run uses at least one set of three plates – one plate for f/tPSA, one plate for iPSA and one plate for hK2. A complete run at full capacity involves two sets of these three plates. The whole procedure involves approximately 3 to 5 hours from the initiation to obtaining the test results depending on the number of plates being run.

Reagents:

- hK2 Assay Buffer

- iPSA Standards (A – G)
- hK2 Standards (A – G)
- Enhancement Solution (Perkin Elmer Product # 1380 0753)
- Wash Concentrate (Perkin Elmer Product # B117-100)
- iPSA Assay Controls (Low, Middle, and High)
- hK2 Assay Controls (Low, Middle, and High)
- 96 well, yellow Streptavidin plate (Perkin Elmer Product # AAAND-0005)
- Reagent Grade Water
- PROSTATUS Kit for t/f PSA (Perkin Elmer Product # B073-301)
- iPSA Biotinylated Capture Solution (100x)
- iPSA Tracer Solution (100x)
- hK2 Biotinylated Capture Solution (100x)
- hK2 Blocker Solution (50x)
- hK2 Tracer Solution (100x)

The details of certain antibodies and reagents are shown in the tables below.

Table 16: Regents

Reagent Name	Biotinylated Intact (iPSA) PSA Capture 100x Solution
Raw Material Description	The Fab-5A10-Cys-His6 protein is expressed in E. coli RV308 cells that carry a pKK tac vector encoding the 5A10-Fab protein fused at its N-terminus to a periplasmic targeting sequence and at its C-terminus to a Ser-Cys-Pro-His6 (SEQ ID NO: 5) sequence. After it is expressed to the periplasmic space, cells are lysed by osmotic shock. The His-tagged protein is captured from the periplasmic fraction on Ni-NTA resin (under native conditions), washed, and biotinylated with EZ-Link PEO-maleimide-activated biotin. After elution with imidazole, the protein is buffer-exchanged into storage buffer using desalting column.
Cell Type	E. coli RV308
Vector	pKK tac-Fab-4A10-Cys-His6 derived from the cDNA sequence cloned from cell line producing 5A10 monoclonal antibody as described in Eriksson *et al.* 2000
Protein Concentration	150 µg/ml, as calculated from dilution from stock of raw material
Buffer	50 mM Tris HCl, pH 7.75, 0.9% NaCl, 0.05% azide with 0.1% BSA

Reagent Name	Intact PSA (iPSA) Tracer 100X Solution
Raw Material Description	The 4D4 monoclonal antibody (IgG) from SP2/0-derived hybridoma cell culture supernatant is purified by Protein G column, eluted in 0.1M glycine, pH 2.7, immediately neutralized in 1/10th volume 1 M Tris HCl, pH 9, and exchanged by desalting column into 0.9% NaCl. The labeling reaction contains antibody in 0.9% NaCl, 50 mM NaHCO3, pH 9.8 and 50-200-fold molar excess of N1 europium(III) chelate (N1-(4-isothiocyanatobenzyl) diethylenetriamine-N1,N2,N3,N4-tetraacetic acid-Eu(III)) overnight at +2…+8 °C followed by buffer exchange into TSA, pH 7.75, by SEC using Superdex 200 HP or other equivalent column.
Cell type	SP2/0-derived hybridoma
Clone	4D4C10C12; described in Nurmikko *et al.* 2000
Antibody Concentration	100 µg/mL, as calculated from dilution from stock of raw material (A280)
Purity	Clone 4D4 with >95% purity by SDS PAGE (reducing and non-reducing conditions) before conjugation with Eu chelate
Buffer	50 mM Tris HCl, pH 7.75, 0.9% NaCl, 0.05% azide with 0.1% BSA

Reagent Name	Biotinylated hK2 Capture 100X Solution
Raw Material Description	The 6H10 monoclonal antibody (IgG) from SP2/0-derived hybridroma cell culture supernatant is digested with bromelain and purified over a HiTrap Protein G column. The purified F(ab)2-6H10 fragment is biotinylated with biotin isothiocyanate at alkaline pH (50 mM NaHCO3, pH 9.8) and then buffer-exchanged into storage buffer by size exclusion using desalting column purification).
Cell type	SP2/0-derived hybridoma
Clone	6H10G3D8; described in Becker *et al.* 2000
Monoclonal Antibody Conc.	250 µg/mL, as calculated from dilution from stock of raw material (A280)
Buffer	50 mM Tris HCl, pH 7.75, 0.9% NaCl, 0.05% azide with 0.1% BSA

Reagent Name	hK2 Tracer 100x Solution
Product Description	A mixture of qualified 7G1-Eu-labeled antibody and unlabeled 5H6 blocker antibody diluted into buffer to 100x their concentration in the hK2 assay.
Europium-labeled 7G1 Monoclonal Antibody	50 µg/mL

5H6 Monoclonal Antibody Concentration	250 µg/mL
Buffer	50 mM Tris HCl, pH 7.75, 0.9% NaCl, 0.05% azide with 0.1% BSA

Reagent Name	hK2 Blocker 50x Solution
Product Description	A 50X solution containing a mixture of qualified 2E9, 5F7, and 5H6 monoclonal antibodies diluted in buffer to 50X their concentration required for the hK2 immunoassay.
2E9 Antibody Concentration	500 µg/mL
5F7 Antibody Concentration	500 µg/mL
5H6 Antibody Concentration	250 µg/mL
Buffer	50 mM Tris HCl, pH 7.75, 0.9% NaCl, 0.05% azide with 0.1% BSA

Reagent Name	Intact PSA (iPSA) Antigen
Product Description	Recombinant pre-pro PSA is expressed in Trichoplusia ni (High Five™) cells carrying baculoviral particles that include the coding sequence for pre-pro PSA (amplified from a human prostate cDNA library). Intact PSA (iPSA) antigen is affinity-purified from the lysate using biotinylated monoclonal antibody 5A10 coupled Pierce High Capacity Streptavidin Chromatography Cartridge and eluted in 0.2 M glycine, pH 2.5, then neutralized in 1/10th volume 1M Tris HCl, pH 9. The pooled fractions were changed into 100 mM sodium phosphate buffer pH 7.0, 150 mM NaCl using desalting column and frozen at -70 °C. When the protein in taken out of the freezer, a stabilizing buffer (containing 50 mM TSA, pH 7.75 with 0.5% BSA) is immediately added.
Cell type	Trichoplusia ni (High Five™)
Vector	AcFAST-Bac-PSA (described in Rajakoski *et al.* 1997)
Protein Concentration	at least 1 µg/mL by Prostatus free/total PSA assay
Buffer	50 mM sodium phosphate buffer, 75mM NaCl, 25 mM TRIS-HCl, 0.25% BSA , pH 7.5

Reagent Name	hK2 Antigen

Product Description	Recombinant ekhK2 is expressed in Trichoplusia ni (High Five™) cells carrying baculoviral particles that include the coding sequence for ekhK2 (preprohK2 gene amplified from a human prostate cDNA library and then mutated to change the hK2 consensus site at -1 to an enterokinase site). ekhK2 is affinity-purified from the lysate using biotinylated monoclonal antibody 11B6 coupled to Pierce High Capacity Streptavidin Chromatography Cartridge and eluted in 0.2 M glycine, pH 2.5, then neutralized in 1/10th volume 1M Tris HCl, pH 9. The pooled fractions are frozen at -70 °C. When the protein in taken out of the freezer, a stabilizing buffer (containing 50 mM TSA, pH 7.75 with 0.5% BSA) is immediately added.
Cell type	Trichoplusia ni (High Five™)
Vector	AcekhK2 (described in Rajakoski *et al.* 1997, Lovgren *et al.* 1999)
Protein Concentration	at least 1 µg/mL by (Prostatus t-PSA assay)
Buffer	30 mM Glycine, 72 mM TRIS-HCl, 83 mM NaCl, 0.008% sodium azide (w/v) with 0.42% BSA, pH 7.0

Supplies:

- Wallac Pipette tips, 1.25 mL (Perkin Elmer Product # 1235-402)
- Dilution Vessels (Perkin Elmer Product # 1235-411)
- 15 mL tubes
- Permanent Marker

Equipment:

- Pipette 101-1000 µL capacity
- Pipette tips
- AutoDELFIA plate processor (Perkin Elmer: 1235-001)
- AutoDELFIA sample processor (Perkin Elmer: 1297-014)
- AutoDELFIA PC (Perkin Elmer: 1235-8060)

(includes software, workstation, and Multicalc)

Sample:

- Plasma

Procedure:

Load aliquot of patient specimen in the immunoassay system (e.g., Roche instrument) to determine tPSA and fPSA. For determination of iPSA, hK2 and (and optionally fPSA and tPSA) with AutoDELFIA instrument follow the procedure below: all reagents are equilibrated to room temperature, including Assay Specific Standards (7 levels), Assay Specific Controls (3 levels). Dilute the iPSA Biotinylated Capture Solution (100x) 100-fold with pH 6.8 iPSA Assay Buffer, and dispense capture solutions in each well of the plates for iPSA. Dilute the hK2 Biotinylated Capture Solution (100x) 100-fold with hK2 pH 7.8 Assay Buffer, and dispense capture solutions in each well of the plates for hK2. Incubate at room temperature for 30 to 60 min. If using the Prostatus kit to determine tPSA and fPSA, follow kit instruction to determine tPSA and fPSA. Load the assay reagent and patient specimen into the AutoDELFIA instrument. Run the instrument protocols iPSA assay and hK2 assay, through completion.

Specimen Acquisition and processing

Blood is drawn into the K_2EDTA tube, and stored at 2-8 ^{0}C until it is shipped overnight to the laboratory with frozen ice packs. Upon arrival in the laboratory, specimen are inspected and (if acceptable) accessioned in the laboratory tracking system and stored in the K_2EDTA tube at 2- 8^{0}C refrigerator. The blood is spun as quickly as possible and the plasma i pipetted into a transfer tube. For periods of 24 hours or less from the time of receipt, the plasma specimen is stored at 2-8 ^{0}C, but for periods greater than 24 hours, the plasma is stored at -70 ^{0}C to -80 ^{0}C.

Example 4: Sequences for PSA and human kallikrein 2

PSA protein (SEQ ID NO: 3)
IVGGWECEKHSQPWQVLVASRGRAVCGGVLVHPQWVLTAAHCIRNKSVILLGRHSLF
HPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPGDDSSHDLMLLRLSEPAELTDAVKVM
DLPTQEPALGTTCYASGWGSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKFMLC
AGRWTGGKSTCSGDSGGPLVCNGVLQGITSWGSEPCALPERPSLYTKVVHYRKWIKDT
IVANP

hK2 protein (SEQ ID NO: 4)
IVGGWECEKHSQPWQVAVYSHGWAHCGGVLVHPQWVLTAAHCLKKNSQVWLGRHN
LFEPEDTGQRVPVSHSFPHPLYNMSLLKHQSLRPDEDSSHDLMLLRLSEPAKITDVVKV

LGLPTQEPALGTTCYASGWGSIEPEEFLRPRSLQCVSLHLLSNDMCARAYSEKVTEFML
CAGLWTGGKDTCGGDSGGPLVCNGVLQGITSWGPEPCALPEKPAVYTKVVHYRKWIK
DTIAANP

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CLAIMS

1. A method of determining the probability that a prostate tissue biopsy obtained from a subject would contain detectable high-grade prostate cancer, the method comprising:

i) subjecting a blood plasma sample of the subject to an immunoassay that measures a level of total prostate specific antigen (tPSA) in the blood plasma sample;

ii) if the tPSA level is above a threshold level, determining the probability that the prostate tissue biopsy would contain detectable high-grade prostate cancer by weighting the measured level of tPSA and a parameter indicative of whether the subject has had a prior biopsy of prostate tissue; and

iii) if the tPSA level is at or below the threshold level, subjecting the blood plasma sample to an immunoassay that measures levels of free prostate specific antigen (fPSA), intact prostate specific antigen (iPSA), and human kallikrein 2 (hK2) in the blood plasma sample, and determining the probability that the prostate tissue biopsy would contain detectable high-grade prostate cancer by weighting the measured levels of tPSA, fPSA, iPSA, and hK2 and a parameter indicative of whether the subject has had a prior biopsy of prostate tissue.

2. A method of determining the probability that a prostate tissue biopsy obtained from a subject would contain detectable high-grade prostate cancer, the method comprising:

i) subjecting a blood plasma sample of the subject to immunoassays that measure levels of free prostate specific antigen (fPSA), intact prostate specific antigen (iPSA), total prostate specific antigen (tPSA) and human kallikrein 2 (hK2); and

ii) determining the probability that the prostate tissue biopsy would contain detectable high-grade prostate cancer by weighting the measured levels of fPSA, iPSA, tPSA, and hK2 and a parameter indicative of whether the subject had a prior biopsy of prostate tissue.

3. The method of any one of claims 1-2, wherein the probability that the prostate tissue biopsy would contain detectable high-grade prostate cancer is determined by also

weighting a parameter indicative of the subject's age.

4. The method of any one of claims 1-3, wherein the probability that the prostate tissue biopsy would contain detectable high-grade prostate cancer is determined by also weighting a parameter indicative of the outcome of a digital rectal examination performed on the subject.

5. The method of any one of claims 1-4, wherein the probability that the prostate tissue biopsy would contain detectable high-grade prostate cancer is determined by also weighting a cubic spline term based on the measured tPSA level.

6. The method of any one of claims 1-5, wherein the probability that the prostate tissue biopsy would contain detectable high-grade prostate cancer is determined by also weighting a cubic spline term based on the measured fPSA level.

7. The method of any one of claims 1-6, wherein the immunoassay that measures the level of fPSA comprises:

contacting fPSA present in the blood plasma sample with a first capture antibody specific for fPSA under conditions in which the first capture antibody binds to fPSA, thereby producing a capture-antibody-fPSA complex; and

detecting the capture-antibody-fPSA complex using a first tracer.

8. The method of claim 7, wherein the first tracer is a 5A10 antibody.

9. The method of any one of claims 7-8, wherein the first capture antibody is a H117 antibody.

10. The method of any one of claims 1-9, wherein the immunoassay that measures the level of iPSA comprises:

contacting iPSA present in the blood plasma sample with a second capture antibody specific for iPSA and nicked PSA, under conditions in which the second

capture antibody binds at least to iPSA, thereby producing a capture-antibody- iPSA complex; and

detecting the capture-antibody- iPSA complex using a second tracer.

11. The method of claim 10, wherein the second tracer comprises a 4D4 antibody.

12. The method of any one of claims 10-11, wherein the second capture antibody is a 5A10 Fab.

13. The method of any one of claims 1-12, wherein the immunoassay that measures the level of tPSA comprises:

contacting tPSA present in the blood plasma sample with a third capture antibody specific for tPSA under conditions in which the third capture antibody binds to tPSA, thereby producing a capture-antibody-tPSA complex; and

detecting the capture-antibody-tPSA complex using a third tracer.

14. The method of claim 13, wherein the third tracer comprises a H50 antibody.

15. The method of any one of claims 13-14, wherein the third capture antibody is a H117 antibody.

16. The method of any one of claims 1-15, wherein the immunoassay that measures the level of hK2 comprises:

contacting PSA in the blood plasma sample with blocking antibodies specific for PSA;

contacting hK2 present in the blood plasma sample with a fourth capture antibody specific for hK2 under conditions in which the fourth capture antibody binds to hK2, thereby producing a capture-antibody-hK2 complex; and

detecting the capture-antibody-hK2 complex using a fourth tracer.

17. The method of claim 16, wherein the fourth tracer comprises a 7G1 antibody.

18. The method of any one of claims 16-17, wherein the fourth capture antibody is a 6H10 $F(ab)_2$.

19. The method of any one of claims 16-18, wherein the blocking antibodies comprise a 5F7 antibody, a 5H6 antibody, and a 2E9 antibody.

20. The method of any one of claims 7-19, wherein each or any capture antibody is bound to a solid support.

21. The method of any one of claims 7-20, wherein each or any tracer comprises a Europium label.

22. A method for determining a probability of an event associated with prostate cancer, the method comprising:

receiving, via an input interface, information indicative of the level of tPSA present in a blood plasma sample of a subject;

receiving, via an input interface, information about whether the subject had a prior biopsy of prostate tissue;

evaluating, using at least one processor, a logistic regression model based, at least in part, on the received information to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises:

determining the probability of the event associated with prostate cancer based, at least in part, on the tPSA value and the information about whether the subject had a prior biopsy of prostate tissue; and

outputting an indication of the probability of the event associated with prostate cancer;

wherein the event associated with prostate cancer is a prostate tissue biopsy containing detectable high-grade prostate cancer.

23. A method for determining a probability of an event associated with prostate

cancer, the method comprising:

receiving, via an input interface, information indicative of levels of tPSA, fPSA, iPSA, and hK2 present in a blood plasma sample of a subject;

receiving, via an input interface, information about whether the subject had a prior biopsy of prostate tissue;

evaluating, using at least one processor, a logistic regression model based, at least in part, on the received information to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises:

determining the probability of the event associated with prostate cancer based, at least in part, on the information indicative of levels of tPSA, fPSA, iPSA, and hK2 and the information about whether the subject had a prior biopsy of prostate tissue; and

outputting an indication of the probability of the event associated with prostate cancer;

wherein the event associated with prostate cancer is a prostate tissue biopsy containing detectable high-grade prostate cancer.

24. A computer for determining a probability of an event associated with prostate cancer, the computer comprising:

an input interface configured to receive information indicative of the level of tPSA present in a blood plasma sample of a subject and information about whether the subject had a prior biopsy of prostate tissue;

at least one processor programmed to evaluate a logistic regression model based, at least in part, on the received information to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises:

determining the probability of the event associated with prostate cancer based, at least in part, on the tPSA value and the information about whether the subject had a prior biopsy of prostate tissue; and

an output interface configured to output an indication of the probability of the event associated with prostate cancer;

wherein the event associated with prostate cancer is a prostate tissue biopsy containing detectable high-grade prostate cancer.

25. A computer for determining a probability of an event associated with prostate cancer, the computer comprising:

an input interface configured to receive information indicative of levels of tPSA, fPSA, iPSA, and hK2 present in a blood plasma sample of a subject and information about whether the subject had a prior biopsy of prostate tissue;

at least one processor programmed to evaluate a logistic regression model based, at least in part, on the received information to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises:

determining the probability of the event associated with prostate cancer based, at least in part, on the information indicative of levels of tPSA, fPSA, iPSA, and hK2 and the information about whether the subject had a prior biopsy of prostate tissue; and

an output interface configured to output an indication of the probability of the event associated with prostate cancer;

wherein the event associated with prostate cancer is a prostate tissue biopsy containing detectable high-grade prostate cancer.

26. A system for determining a probability of an event associated with prostate cancer, the system comprising:

a) a detector configured to measure a level of tPSA present in a blood plasma sample of a subject; and

b) a computer in electronic communication with the detector, wherein the computer comprises:

i) an input interface configured to receive information from the detector indicative of the measured level of tPSA, and to receive information about whether the subject had a prior biopsy of prostate tissue;

ii) at least one processor programmed to evaluate a logistic regression model based, at least in part, on the received information to determine a

probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises:

determining the probability of the event associated with prostate cancer based, at least in part, on the information indicative of the level of tPSA and the information about whether the subject had a prior biopsy of prostate tissue; and

iii) an output interface configured to output an indication of the probability of the event associated with prostate cancer;

wherein the event associated with prostate cancer is a prostate tissue biopsy containing detectable high-grade prostate cancer.

27. A system for determining a probability of an event associated with prostate cancer, the system comprising:

a) a detector configured to measure levels of tPSA, fPSA, iPSA, and hK2 present in a blood plasma sample of a subject; and

b) a computer in electronic communication with the detector, wherein the computer comprises:

i) an input interface configured to receive information from the detector indicative of the measured levels of tPSA, fPSA, iPSA, and hK2, and to receive information about whether the subject had a prior biopsy of prostate tissue;

ii) at least one processor programmed to evaluate a logistic regression model based, at least in part, on the received information to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises:

determining the probability of the event associated with prostate cancer based, at least in part, on the information indicative of levels of tPSA, fPSA, iPSA, and hK2 and the information about whether the subject had a prior biopsy of prostate tissue; and

iii) an output interface configured to output an indication of the probability of the event associated with prostate cancer;

wherein the event associated with prostate cancer is a prostate tissue biopsy containing detectable high-grade prostate cancer.

28. A computer-readable storage medium encoded with a plurality of instructions that, when executed by a computer, perform a method for determining a probability of an event associated with prostate cancer, wherein the method comprises:

evaluating a logistic regression model based, at least in part, on information indicative of the level of tPSA present in a blood plasma sample of a subject and information about whether the subject had a prior biopsy of prostate tissue to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises:

determining the probability of the event associated with prostate cancer based, at least in part, on the tPSA value and the information about whether the subject had a prior biopsy of prostate tissue; and

outputting an indication of the probability of the event associated with prostate cancer;

wherein the event associated with prostate cancer is a prostate tissue biopsy containing detectable high-grade prostate cancer.

29. A computer-readable storage medium encoded with a plurality of instructions that, when executed by a computer, perform a method for determining a probability of an event associated with prostate cancer, wherein the method comprises:

evaluating a logistic regression model based, at least in part, on information indicative of levels of tPSA, fPSA, iPSA, and hK2 present in a blood plasma sample of a subject and information about whether the subject had a prior biopsy of prostate tissue to determine a probability of an event associated with prostate cancer in the subject, wherein evaluating the logistic regression model comprises:

determining the probability of the event associated with prostate cancer based, at least in part, on the information indicative of levels of tPSA, fPSA, iPSA, and hK2 and the information about whether the subject had a prior biopsy of prostate tissue; and

outputting an indication of the probability of the event associated with prostate cancer;

wherein the event associated with prostate cancer is a prostate tissue biopsy containing detectable high-grade prostate cancer.

30. The method of any one of claims 1-21, wherein each or any immunoassay is performed using a microfluidic device.

31. A method of determining whether a subject is a candidate for a prostate tissue biopsy, the method comprising:

i) obtaining a blood sample from the subject;

ii) determining the probability that the prostate tissue biopsy would contain detectable high-grade prostate cancer using the blood sample obtained in step i), wherein

a) if a tPSA level measured using the blood sample is above a threshold level, the probability is based on weighting the tPSA level and a parameter indicative of whether the subject had a prior biopsy of prostate tissue, otherwise,

b) if the tPSA level is at or below the threshold level, the probability is based on weighting levels of tPSA, fPSA, iPSA, and hK2 measured using the blood sample and a parameter indicative of whether the subject had a prior biopsy of prostate tissue; and

iii) determining whether the subject is a candidate for the prostate tissue biopsy based on the probability that the prostate tissue biopsy will contain detectable high-grade prostate cancer as determined in step ii).