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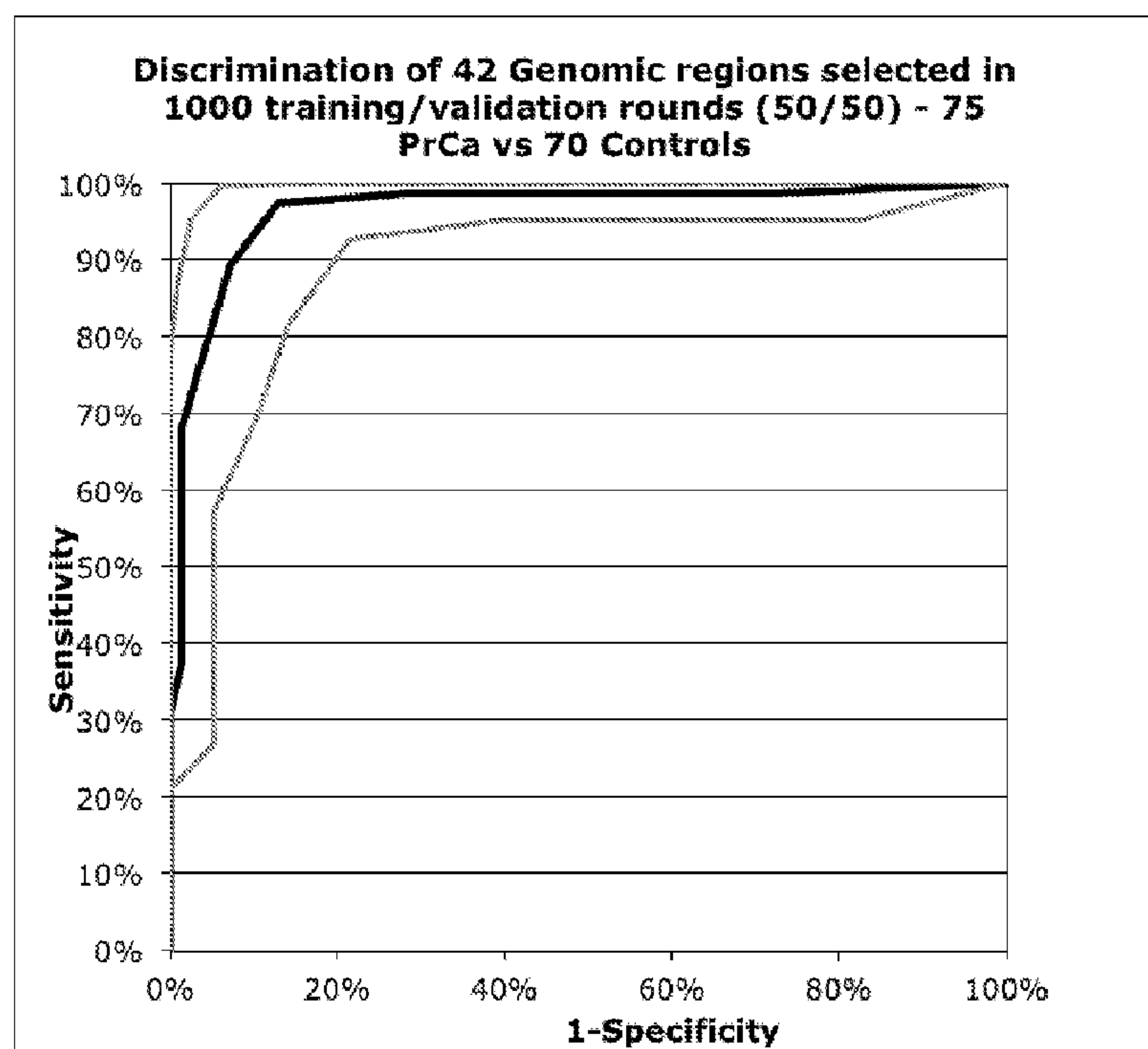
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(54) Titre : BIOMARQUEURS D'ACIDES NUCLEIQUES CIRCULANTS ASSOCIES AU CANCER DE LA PROSTATE
(54) Title: PROSTATE CANCER ASSOCIATED CIRCULATING NUCLEIC ACID BIOMARKERS

Figure 1



(57) **Abrégé/Abstract:**

Methods and reagents for diagnosing prostate cancer are disclosed, wherein said methods comprise detection of circulating nucleic acid biomarkers from a patient to be evaluated.

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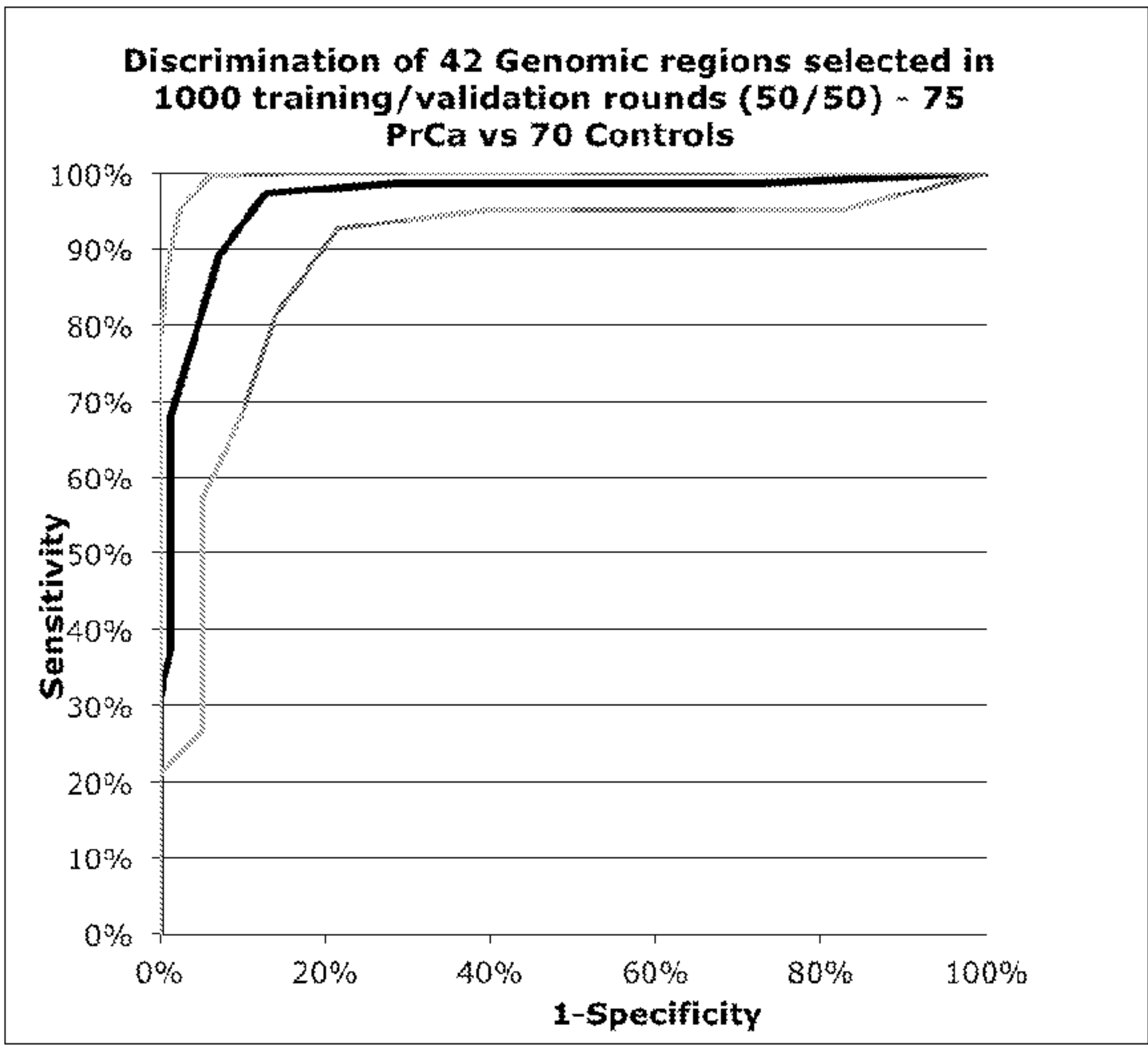
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Figure 1



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CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 146

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JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 146

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NOTE POUR LE TOME / VOLUME NOTE:

Prostate Cancer Associated Circulating Nucleic Acid Biomarkers

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of U.S. provisional application no. 61/351,708, filed
5 June 4, 2010, which application is herein incorporated by reference.

BACKGROUND OF THE INVENTION

[0002] Methods to detect prostate cancer, including PSA tests, are extremely unreliable
(see, *e.g.*, Wever *et al.*, *J Natl Cancer Inst* 2010;102:352–355, 2010; Schröder *et al.*, *N. Engl.*
J. Med 360:1320-1328, 2009). There is a need for effective detection methods. This
10 invention addresses that need.

BRIEF SUMMARY OF THE INVENTION

[0003] The invention is based, in part, on the discovery of circulating nucleic acids (CNA)
biomarkers associated with prostate cancer. In some embodiments, the CNA biomarkers are
nucleic acid sequences, in the current invention DNA sequences, that are present in the blood,
15 *e.g.*, in a serum or plasma sample, of a prostate cancer patient, but are rarely present, if at all,
in the blood, *e.g.*, a serum or plasma sample, obtained from a normal individual, *i.e.*, in the
context of this invention, an individual that does not have prostate cancer. In some
embodiments, the CNA biomarkers are nucleic acid sequences, in the current invention DNA
sequences, *i.e.*, DNA fragments, that are present in the blood, *e.g.*, in a serum or plasma
20 sample, of a normal individual, but are rarely present, if at all, in the blood, *e.g.*, a serum or
plasma sample, obtained from a prostate cancer patient.

[0004] Accordingly, in one aspect, the invention provides a method of analyzing CNA in a
sample (blood, serum or plasma) from a patient comprising detecting the presence of at least
one cell-free DNA having a nucleotide sequence falling within a chromosomal region set
25 forth in Tables 2-5 (or having a nucleotide sequence that is part of one of the sequences set
forth in Tables A-D) in the sample. In some embodiments, detecting the presence of, or the
amount of, the at least one biomarker comprises detecting a cell-free DNA molecule having
between 50 and 400 consecutive nucleotides of a unique sequence within a chromosomal
region as set forth in Tables 2-5 (or of a unique sequence set forth in Tables A-D).

[0005] In one embodiment, a method of analyzing circulating free DNA in a patient sample is provided, comprising determining, in a sample that is blood, serum or plasma, the presence or absence, or the amount of, at least 2, 3, 4, 5, 7, 8, 9, 10, 15, 20, 30, 40, or at least 50 cell-free DNA molecules each having a sequence falling within a different chromosomal region set forth in Table 2, 3, 4, or 5, and preferably the sequences of the cell-free DNA molecules are free of repetitive element. In preferred embodiments, the cell-free DNA molecules have sequences falling within different chromosomal regions in the same table selected from Tables 2-5.

[0006] In another aspect, the present invention provides a kit including two or more (*e.g.*, at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 25, 30, 40, or at least 50, but less than 115) sets of oligonucleotides. Each set comprises one or more oligonucleotides with a nucleotide sequence falling within one single chromosomal region that is set forth in Tables 2-5. Preferably, different oligonucleotide sets correspond to different chromosomal regions within the same table selected from Tables 2-5. Also, preferably the oligonucleotides are free of repetitive element. Optionally, the oligonucleotides are attached to one or more solid substrates such as microchips and beads.

[0007] In another aspect, the present invention provides a method of diagnosing or screening for prostate cancer in a patient. The method includes the steps of: (a) determining, in a sample that is blood, serum or plasma from a patient, the presence or absence or the amount of, at least 2, 3, 4, 5, 7, 8, 9, 10, 15, 20, 30, 40, or at least 50 cell-free DNA molecules each having a sequence falling within a different chromosomal region set forth in Table 2, 3, or 4; and (b) correlating the presence of, or an increased amount of, said first and second cell-free DNAs with an increased likelihood that the patient has prostate cancer. Preferably, the sequences of the cell-free DNA molecules are free of repetitive element. In preferred embodiments, the cell-free DNA molecules have sequences falling within different chromosomal regions in the same table chosen from Tables 2-4.

[0008] In one aspect, the invention provides a method of identifying a patient that has a CNA biomarker associated with prostate cancer, the method comprising detecting the presence of at least one biomarker set forth in Table 2, Table 3, or Table 4 in a CNA sample obtained from serum or plasma from the patient. A biomarker can be identified using any number of methods, including sequencing of CNA as well as use of a probe or probe set to detect the presence of the biomarker.

[0009] In some embodiments, the invention provides a method of identifying a patient that has a CNA biomarker that is associated with the absence of prostate cancer, the method comprising detecting the presence of at least one biomarker set forth in Table 5 in a CNA sample from serum or plasma from the patient. A biomarker can be identified using any
5 number of methods, including sequencing of CNA as well as use of a probe or probe set to detect the presence of the biomarker.

[0010] In a further aspect, the invention provides a kit for identifying a patient that has a biomarker for prostate cancer and/or that has a biomarker associated with a normal individual that does not have prostate cancer, wherein the kit comprises at least one polynucleotide
10 probe to a biomarker set forth in Table 2, 3, 4, or 5. Preferably, such a kit comprises probes to multiple biomarkers, e.g., at least 2, 3, 4, 5, 10, 20, 30, 40, 50, or more, of the biomarkers set forth in Tables 2-5. In some embodiments, the kit also includes an electronic device or computer software to compare the hybridization patterns of the CNA in the patient sample to a prostate cancer data set comprising a listing of biomarkers that are present in prostate
15 cancer patient CNA, but not CNA samples from normal individuals.

[0011] In some embodiments, the presence of the at least one biomarker in CNA is determined by sequencing. In some embodiments, the presence of the at least one biomarker in CNA is determined using an array. In some embodiments, the presence of the at least one biomarker in CNA is determined using an assay that comprises an amplification reaction,
20 such as a polymerase chain reaction (PCR). In some embodiments, a nucleic acid array forming a probe set comprising probes to two or more chromosomal regions set forth in Tables 2-5 is employed. In some embodiments, a nucleic acid array forming a probe set comprising 2, 3, 4, 5, 6, 7, 8, 9, 10 or more chromosomal regions, or all of the chromosomal regions, set forth in Table 2 is employed. In some embodiments, a nucleic acid array
25 forming a probe set comprising 2, 3, 4, 5, 6, 7, 8, 9, 10, or more chromosomal regions, or all of the chromosomal regions, set forth in Table 3 is employed. In some embodiments, a nucleic acid array forming a probe set comprising 2, 3, 4, 5, 6, 7, 8, 9, 10 or more chromosomal regions, or all of the chromosomal regions, set forth in Table 4 is employed. In some embodiments, a nucleic acid array forming a probe set comprising two or more
30 chromosomal regions, or all of the chromosomal regions set forth in Table 5 is employed.

[0012] In an additional aspect, the invention provides a method of detecting prostate cancer in a patient that has, or is suspected of having, prostate cancer, the method comprising contacting DNA from the serum or plasma sample with a probe that selectively hybridizes to a sequence present on a chromosomal region described herein, e.g., a sequence set forth in

Tables A-D under conditions in which the probe selectively hybridizes to the sequence; and detecting the presence or absence of hybridization of the probe, wherein the presence of hybridization to a sequence set forth in Table A, B, or C is indicative of prostate cancer.

[0013] The Tables of Sequences A, B, C, and D provide examples of sequences corresponding to the chromosome regions set forth in Table 2, Table 3, Table 4, and Table 5, respectively. The designation (N)x in Tables A-D refer to repetitive element sequences.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figure 1 provides an example of a ROC curve using the 42 highest ranking regions of the chromosomal regions identified in Tables 4 and 5, collectively. The actual ROC curve - build from score sums is given together with the 95% confidence limits.

DETAILED DESCRIPTION OF THE INVENTION

[0015] As used herein, a “biomarker” as used herein refers to a nucleic acid sequence that corresponds to a chromosomal region, where the presence of the nucleic acid in CNA is associated with prostate cancer.

[0016] In the current invention, a “chromosomal region” listed in any one of Tables 1 to 5 refers to the region of the chromosome that corresponds to the nucleotide positions indicated in the tables. The nucleotide positions on the chromosomes are numbered according to *Homo sapiens* (human) genome, build 37.1 as of June 2010 (Tables 1-3) and *Homo sapiens* (human) genome, build 36 as of March 2006 (Table 4, 5). As understood in the art, there are naturally occurring polymorphisms in the genome of individuals. Thus, each chromosome region listed in the tables encompasses allelic variants as well as the particular sequence in the database, *e.g.*, the sequences in Tables A-D corresponds to the chromosomal regions noted. An allelic variant typically has at least 95% identity, often at least 96%, at least 97%, at least 98%, or at least 99% identity to the sequence of a chromosomal region noted in the Tables that is present in a particular database, *e.g.*, the National Center for Biotechnology Information (*Homo sapiens* Build 37.1 at the website <http://www.ncbi.nlm.nih.gov/mapview/>.) Percent identity can be determined using well known algorithms, including the BLAST algorithm, *e.g.*, set to the default parameters. Further, it is understood that the nucleotide sequences of the chromosomes may be improved upon as errors in the current database are discovered and corrected. The term “chromosomal region” encompasses any variant or corrected version of the same region as defined in Tables 1-5. Given the information provided in the tables in the present disclosure, especially in view of

the sequences listed in Tables A-D, a skilled person in the art will be able to understand the chromosomal regions used for the present invention even after new variants are discovered or errors are corrected.

[0017] “Detecting the presence of a chromosomal region” in CNA in the context of this invention refers to detecting any sequence from a chromosomal region shown in Table 2, 3, 4, or 5, where the sequence detected can be assigned unambiguously to that chromosomal region. Thus, this term refers to the detection of unique sequences from the chromosomal regions. Methods of removing repetitive sequences from the analysis are known in the art and include use of blocking DNA, *e.g.*, when the target nucleic acids are identified by hybridization. In some embodiments, typically where the presence of a prostate cancer biomarker is determined by sequencing the CNA from a patient, well known computer programs and manipulations can be used to remove repetitive sequences from the analysis (see, *e.g.*, the EXAMPLES section). In addition, sequences that have multiple equally fitting alignment to the reference database are typically omitted from further analyses.

[0018] The term “detecting a biomarker” as used herein refers to detecting a sequence from a chromosomal region listed in Table 2, Table 3, Table 4, or Table 5. A biomarker is considered to be present if any nucleic acid sequence present in the CNA is unambiguously assigned to the chromosomal region.

[0019] The term “unambiguously assigned” in the context of this invention refers to determining that a DNA detected in the CNA of a patient is from a particular chromosomal region. Thus, in detection methods that employ hybridization, the probe hybridizes specifically to that region. In detection methods that employ amplification, the primer(s) hybridizes specifically to that region. In detection methods that employ sequencing, the sequence is assigned to that region based on well-known algorithms for identity, such as the BLAST algorithm using high stringent parameters, such as $e < 0.0001$. In addition, such a sequence does not have a further equally fitting hit on the used database.

[0020] The term “circulating nucleic acids” refers to acellular nucleic acids that are present in the blood.

[0021] The term “circulating cell-free DNA” as used herein means free DNA molecules of 25 nucleotides or longer that are not contained within any intact cells in human blood, and can be obtained from human serum or plasma.

[0022] The term "hybridization" refers to the formation of a duplex structure by two single stranded nucleic acids due to complementary base pairing. Hybridization can occur between exactly complementary nucleic acid strands or between nucleic acid strands that contain minor regions of mismatch. As used herein, the term "substantially complementary" refers to sequences that are complementary except for minor regions of mismatch. Typically, the total number of mismatched nucleotides over a hybridizing region is not more than 3 nucleotides for sequences about 15 nucleotides in length. Conditions under which only exactly complementary nucleic acid strands will hybridize are referred to as "stringent" or "sequence-specific" hybridization conditions. Stable duplexes of substantially complementary nucleic acids can be achieved under less stringent hybridization conditions. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length and base pair concentration of the oligonucleotides, ionic strength, and incidence of mismatched base pairs. For example, computer software for calculating duplex stability is commercially available from National Biosciences, Inc. (Plymouth, Minn.); *e.g.*, OLIGO version 5, or from DNA Software (Ann Arbor, Michigan), *e.g.*, Visual OMP 6.

[0023] Stringent, sequence-specific hybridization conditions, under which an oligonucleotide will hybridize only to the target sequence, are well known in the art (see, *e.g.*, the general references provided in the section on detecting polymorphisms in nucleic acid sequences). Stringent conditions are sequence-dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C lower to 5°C higher than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the duplex strands have dissociated. Relaxing the stringency of the hybridizing conditions will allow sequence mismatches to be tolerated; the degree of mismatch tolerated can be controlled by suitable adjustment of the hybridization conditions.

[0024] The term "primer" refers to an oligonucleotide that acts as a point of initiation of DNA synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced, *i.e.*, in the presence of four different nucleoside triphosphates and an agent for polymerization (*i.e.*, DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. A primer is preferably a single-stranded oligodeoxyribonucleotide. The primer includes a "hybridizing region" exactly or substantially complementary to the target sequence, preferably about 15 to about 35 nucleotides in length. A primer oligonucleotide can either consist entirely of the

hybridizing region or can contain additional features which allow for the detection, immobilization, or manipulation of the amplified product, but which do not alter the ability of the primer to serve as a starting reagent for DNA synthesis. For example, a nucleic acid sequence tail can be included at the 5' end of the primer that hybridizes to a capture
5 oligonucleotide.

[0025] The term "probe" refers to an oligonucleotide that selectively hybridizes to a target nucleic acid under suitable conditions. A probe for detection of the biomarker sequences described herein can be any length, *e.g.*, from 15-500 bp in length. Typically, in probe-based assays, hybridization probes that are less than 50 bp are preferred.

10 [0026] The term "target sequence" or "target region" refers to a region of a nucleic acid that is to be analyzed and comprises the sequence of interest.

[0027] As used herein, the terms "nucleic acid," "polynucleotide" and "oligonucleotide" refer to primers, probes, and oligomer fragments. The terms are not limited by length and are generic to linear polymers of polydeoxyribonucleotides (containing 2-deoxy-D-ribose),
15 polyribonucleotides (containing D-ribose), and any other N-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine bases. These terms include double- and single-stranded DNA, as well as double- and single-stranded RNA. Oligonucleotides for use in the invention may be used as primers and/or probes.

[0028] A nucleic acid, polynucleotide or oligonucleotide can comprise phosphodiester
20 linkages or modified linkages including, but not limited to phosphotriester, phosphoramidate, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, phosphorothioate, methylphosphonate, phosphorodithioate, bridged phosphorothioate or sulfone linkages, and combinations of such linkages.

25 [0029] A nucleic acid, polynucleotide or oligonucleotide can comprise the five biologically occurring bases (adenine, guanine, thymine, cytosine and uracil) and/or bases other than the five biologically occurring bases. These bases may serve a number of purposes, *e.g.*, to stabilize or destabilize hybridization; to promote or inhibit probe degradation; or as attachment points for detectable moieties or quencher moieties. For example, a
30 polynucleotide of the invention can contain one or more modified, non-standard, or derivatized base moieties, including, but not limited to, N6-methyl-adenine, N6-tert-butyl-benzyl-adenine, imidazole, substituted imidazoles, 5-fluorouracil, 5 bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5

(carboxyhydroxymethyl)uracil, 5 carboxymethylaminomethyl-2-thiouridine, 5
 carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6
 isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-
 methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-
 5 methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D
 mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-
 isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2
 thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-
 oxyacetic acidmethylester, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, 2,6-
 10 diaminopurine, and 5-propynyl pyrimidine. Other examples of modified, non-standard, or
 derivatized base moieties may be found in U.S. Patent Nos. 6,001,611; 5,955,589; 5,844,106;
 5,789,562; 5,750,343; 5,728,525; and 5,679,785, each of which is incorporated herein by
 reference in its entirety. Furthermore, a nucleic acid, polynucleotide or oligonucleotide can
 comprise one or more modified sugar moieties including, but not limited to, arabinose, 2-
 15 fluoroarabinose, xylulose, and a hexose.

[0030] The term "repetitive element" as used herein refers to a stretch of DNA sequence of
 at least 25 nucleotides in length that is present in the human genome in at least 50 copies.

[0031] The terms "arrays," "microarrays," and "DNA chips" are used herein
 interchangeably to refer to an array of distinct polynucleotides affixed to a substrate, such as
 20 glass, plastic, paper, nylon or other type of membrane, filter, chip, bead, or any other suitable
 solid support. The polynucleotides can be synthesized directly on the substrate, or
 synthesized separate from the substrate and then affixed to the substrate. The arrays are
 prepared using known methods.

Introduction

[0032] The invention is based, at least in part, on the identification CNA sequences from
 particular chromosomal regions that are present or at an increased amount in the blood of
 patients that have prostate cancer, but are rarely, if ever, present, or at a lower amount, in the
 blood of normal patients that do not have prostate cancer. The invention is also based, in
 part, on the identification of biomarkers in the CNA in normal individuals, *i.e.*, in the context
 30 of this invention, individuals not diagnosed with prostate cancer, that are rarely, if ever,
 present in patients with prostate cancer. Thus, the invention provides methods and devices
 for analyzing for the presence of sequences from a chromosomal region corresponding to at
 least one of the chromosomal regions set forth in Table 2, Table, 3, Table 4, or Table 5.

[0033] Accordingly, in one aspect, the invention provides a method of analyzing CNA in a sample (blood, serum or plasma) from a patient comprising detecting the presence of, or an amount of, at least one circulating cell-free DNA having a nucleotide sequence of at least 25 nucleotides falling within a chromosomal region set forth in Table 2, 3, 4, or 5 (or having a nucleotide sequence that is a part of one of the sequences set forth in Table A, B, C, or D). Preferably, the circulating cell-free DNA is free of repetitive element. In one embodiment, the patient is an individual suspected of or diagnosed with cancer, *e.g.*, prostate cancer.

[0034] By “falling within” it is meant herein that the nucleotide sequence of a circulating cell-free DNA is substantially identical (*e.g.*, greater than 95% identical) to a part of the nucleotide sequence of a chromosome region. In other words, the circulating cell-free DNA can hybridize to under stringent conditions, or be derived from, the chromosomal region.

[0035] In one embodiment, a method of analyzing circulating cell-free DNA in a patient sample is provided, comprising determining, in a sample that is blood, serum or plasma, the presence or the amount of, a plurality of circulating cell-free DNA molecules each having a sequence of at least 25 nucleotides in length falling within the same one single chromosomal region set forth in Table 2, Table 3, Table 4, or Table 5 (or each having a nucleotide sequence of at least 25 consecutive nucleotides in length that is a part of the same sequence set forth in Table A, Table B, Table C, or Table D). There may be two or more or any number of different circulating cell-free DNA molecules that are all derived from the same one chromosomal region set forth in Table 2, 3, 4, or 5, and in some embodiments, all such circulating cell-free DNA molecules are detected and/or the amounts thereof are determined.

[0036] Preferably the sequences of the circulating cell-free DNA molecules are free of repetitive elements.

[0037] In one embodiment, a method of analyzing circulating cell-free DNA in a patient sample is provided, comprising determining, in a sample that is blood, serum or plasma, the presence or absence or the amount of, at least 2, 3, 4, 5, 7, 8, 9, 10, 15, 20, 30, 40, or at least 50 circulating cell-free DNA molecules each having a sequence of at least 25 base pairs falling within a different chromosomal region set forth in Table 2, Table 3, Table 4, or Table 5 (or having a nucleotide sequence of at least 25, 40, 50, 60, 75 or 100 consecutive nucleotides in length that is a part of one of the sequences set forth in Table A, Table B, Table C, or Table D). Preferably the sequences of the circulating cell-free DNA molecules are free of repetitive elements. In preferred embodiments, the cell free DNA molecules have sequences falling within different chromosomal regions in the same table that is chosen from

Tables 2-5. In one specific embodiment, the presence or absence or the amounts of, at least 2, 3, 4, 5, 7, 8, 9, 10, 15, 20, or at least 25, or of 30, 31, or 32, circulating cell-free DNA molecules are determined, the sequence of each falling within a different chromosomal region set forth in Table 2 (or having a nucleotide sequence of at least 25, 40, 50, 60, 75 or 100 consecutive nucleotides in length that is a part of one of the sequences set forth in Table A). In another specific embodiment, the presence or absence or the amounts of, at least 2, 3, 4, 5, 7, 8, 9, 10, or at least 15, or of 20, 21, or 22, circulating cell-free DNA molecules are determined, the sequence of each falling within a different chromosomal region set forth in Table 3 (or having a nucleotide sequence of at least 25, 40, 50, 60, 75 or 100 consecutive nucleotides in length that is a part of one of the sequences set forth in Table B). In one specific embodiment, the presence or absence or the amounts of, at least 2, 3, 4, 5, 7, 8, 9, 10, 15, 20, 25, 30, 35, 50, 45, or 50, or of 52, circulating cell-free DNA molecules are determined, the sequence of each falling within a different chromosomal region set forth in Table 4 (or having a nucleotide sequence of at least 25, 40, 50, 60, 75 or 100 consecutive nucleotides in length that is a part of one of the sequences set forth in Table C). In yet another specific embodiment, the presence or absence or the amounts of, at 1, 2, 3, or 4 circulating cell-free DNA molecules are determined each having a sequence falling within a different chromosomal region set forth in Table 5 (or having a nucleotide sequence of at least 25, 40, 50, 60, 75 or 100 consecutive nucleotides in length that is a part of one of the sequences set forth in Table D).

[0038] In a specific embodiment, the method of analyzing circulating cell-free DNA includes the steps of: isolating, from blood, serum or plasma sample of a patient, substantially all circulating cell-free DNA molecules having a length of at least 20, 25, 30, 40, 50, 75 or 100 consecutive nucleotides in length, or between 50 and 400 nucleotides in length, obtaining the sequence of each of the circulating cell-free DNA molecules, and comparing the sequence to one or more of the sequences set forth in Tables A-D to determine whether the sequence falls within a chromosomal region set forth in Table 2, 3, 4, or 5.

[0039] In another specific embodiment, the method of analyzing circulating cell-free DNA includes the steps of: isolating, from blood, serum or plasma sample of a patient, substantially all circulating cell-free DNA molecules having a length of at least 20, 25, 30, 40, 50, 75 or 100 consecutive nucleotides in length, or between 50 and 400 nucleotides in length, and contacting the circulating cell-free DNA molecules to a plurality of oligonucleotides (e.g., on a DNA chip or microarray) to determine if one of the circulating cell-free DNA molecules hybridizes to any one of the plurality of oligonucleotide probes under stringent conditions.

Each of the oligonucleotide probes has a nucleotide sequence identical to a part of the sequence of a chromosomal region chosen from Tables 2-5 (or a sequence set forth in Tables A-D). Thus, if a circulating DNA molecule hybridizes under stringent conditions to one of the oligonucleotide probes, it indicates that the circulating DNA molecule has a nucleotide sequence falling within a chromosomal region set forth in Table 2, Table 3, Table 4, or Table 5.

[0040] In the above various embodiments, preferably the circulating cell-free DNA molecules have at least 25 consecutive nucleotides in length (preferably at least 50, 70, 80, 100, 120 or 200 consecutive nucleotides in length). More preferably, the circulating cell-free DNA molecules have between about 50 and about 300 or 400, preferably from about 75 and about 300 or 400, more preferably from about 100 to about 200 consecutive nucleotides of a unique sequence within a chromosomal region as set forth in Table 2, Table 3, Table 4, or Table 5 (or of a unique sequence set forth in Table A, Table B, Table C, or Table D).

[0041] In another aspect, the present invention provides a method of diagnosing or screening for prostate cancer in a patient. The method includes the steps of: (a) determining, in a sample that is blood, serum or plasma from a patient, the presence or absence or the amount of, at least 1, 2, 3, 4, 5, 7, 8, 9, 10, 15, 20, 30, 40, or at least 50 circulating cell-free DNA molecules each having a sequence of at least 25 nucleotides in length falling within a different chromosomal region set forth in Table 2, Table 3, or Table 4 (or having a nucleotide sequence of at least 25 consecutive nucleotides in length that is a part of one of the sequences set forth in Table A, Table B, or Table C); and (b) correlating the presence of or an increased amount of the circulating cell-free DNAs with an increased likelihood that the patient has prostate cancer.

[0042] Alternatively, the method of invention includes the steps of: (a) determining, in a sample that is blood, serum or plasma from a patient, the presence or absence or the amount of 1, 2, 3, or 4 circulating cell-free DNA molecules each having a sequence of at least 25 nucleotides in length falling within a different chromosomal region set forth in Table 5 (or having a nucleotide sequence of at least 25 consecutive nucleotides in length that is a part of one of the sequences set forth in Table D); and (b) correlating the presence of or an increased amount of the circulating cell-free DNAs with a decreased likelihood that the patient has prostate cancer.

[0043] When the steps of the above methods are applied to a patient diagnosed of cancer, the patient may be monitored for the status of prostate cancer, or for determining the treatment effect of a particular treatment regimen, or detecting cancer recurrence or relapse.

[0044] In the diagnosis/monitoring method of the present invention, preferably the sequences of the circulating cell-free DNA molecules are free of repetitive elements. In preferred embodiments, the cell free DNA molecules have sequences falling within different chromosomal regions in the same table chosen from Tables 2-5.

[0045] In one embodiment, a method of diagnosing prostate cancer in an individual is provided, comprising (a) determining the presence or absence or the amounts of, at least 2, 3, 4, 5, 7, 8, 9, 10, 15, 20, or at least 25, or of 30, 31, or 32, circulating cell-free DNA molecules the sequence of each falling within a different chromosomal region set forth in Table 2 (or having a nucleotide sequence of at least 25 consecutive nucleotides in length that is a part of one of the sequences set forth in Table A); and (b) correlating the presence of or an increased amount of, one or more of the circulating cell-free DNA molecules with an increased likelihood that the individual has prostate cancer or a recurrence of prostate cancer or a failure of treatment for prostate cancer.

[0046] In another embodiment, a method of diagnosing prostate cancer in an individual is provided, comprising (a) determining the presence or absence or the amounts of, at least 2, 3, 4, 5, 7, 8, 9, 10, or at least 15, or of 20, 21, or 22, circulating cell-free DNA molecules the sequence of each falling within a different chromosomal region set forth in Table 3 (or having a nucleotide sequence of at least 25 consecutive nucleotides in length that is a part of one of the sequences set forth in Table B); and (b) correlating the presence of, or an increased amount of, one or more of the circulating cell-free DNA molecules with an increased likelihood that the individual has prostate cancer or a recurrence of prostate cancer or a failure of treatment for prostate cancer.

[0047] In one embodiment, a method of diagnosing prostate cancer in an individual is provided, comprising (a) determining the presence or absence or the amounts of, at least 2, 3, 4, 5, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50, or of 52, circulating cell-free DNA molecules the sequence of each falling within a different chromosomal region set forth in Table 4 (or having a nucleotide sequence of at least 25 consecutive nucleotides in length that is a part of one of the sequences set forth in Table C); and (b) correlating the presence of or an increased amount of, one or more of the circulating cell-free DNA molecules with an

increased likelihood that the individual has prostate cancer or a recurrence of prostate cancer or a failure of treatment for prostate cancer.

[0048] In one embodiment, a method of diagnosing/monitoring prostate cancer in an individual is provided, comprising (a) determining the presence or absence or the amounts of, 5 1, 2, 3, or 4 circulating cell-free DNA molecules the sequence of each falling within a different chromosomal region set forth in Table 5 (or having a nucleotide sequence of at least 25 consecutive nucleotides in length that is a part of one of the sequences set forth in Table D); and (b) correlating the presence of or an increased amount of, one or more of the circulating cell-free DNA molecules with an increased likelihood that the individual does not 10 have prostate cancer or a recurrence of prostate cancer or a failure of treatment for prostate cancer.

[0049] In yet another embodiment, the method of diagnosing, monitoring or screening for prostate cancer in a patient, includes determining, in a sample that is blood, serum or plasma from the patient, the presence or absence or the total amount of, each and all circulating cell-free DNAs each having a sequence falling within the same one single chromosomal region 15 set forth in Table 2, Table 3, or Table 4; and correlating the presence of one of said circulating cell-free DNAs or an increased total amount of said circulating cell-free DNAs, with an increased likelihood that said patient has prostate cancer, or recurrence of prostate cancer. In other words, there can be any number of, and typically many, different circulating 20 cell-free DNA molecules derived from one single same chromosomal region set forth in Table 2, Table 3, or Table 4, and all of such different circulating cell-free DNA molecules are detected and/or the amount each or all is determined, and correlation with the status of prostate cancer is made.

[0050] In a specific embodiment, substantially all circulating cell-free DNA molecules 25 having a length of at least 20, 25, 30, 40, 50, 75 or 100 consecutive nucleotides in length, or between 50 and 400 nucleotides in length, are isolated from a blood, serum or plasma sample of a patient. The sequence of each of the circulating cell-free DNA molecules is determined, and compared with one or more of the sequences set forth in Tables A-D to determine whether the sequence of a circulating cell-free DNA falls within a chromosomal region set 30 forth in Table 2, Table 3, or Table 4. If so, a diagnosis of prostate cancer is made. In the case of a patient treated with a therapy for prostate cancer, recurrence is indicated if a circulating cell-free DNA falls within a chromosomal region set forth in Table 2, Table 3, or Table 4 is detected. In preferred embodiments, a diagnosis of prostate cancer or prostate cancer treatment failure or recurrence is indicated if two or more circulating cell-free DNA

molecules fall within 2, 3, 4 or more chromosomal regions set forth in Tables 2-4, preferably all such chromosomal regions being in the same table.

[0051] In another specific embodiment, substantially all circulating cell-free DNA molecules having a length of at least 20, 25, 30, 40, 50, 75 or 100 consecutive nucleotides in length, or between 50 and 400 nucleotides in length, are isolated from a blood, serum or plasma sample of a patient. These circulating cell-free DNA molecules are hybridized to a microarray that is described above in the context of the kit invention to determine if one of the circulating cell-free DNA molecules hybridizes to any one of a plurality of oligonucleotide probes under stringent conditions. Each of the oligonucleotide probes has a nucleotide sequence identical to a part of the sequence of a chromosomal region chosen from Tables 2-5 or a sequence set forth in Tables A-D). Thus, if a circulating DNA molecule hybridizes under stringent conditions to one of the oligonucleotide probes, it indicates that the circulating DNA molecule has a nucleotide sequence falling within a chromosomal region set forth in Table 2, Table 3, or Table 4. If so, a diagnosis of prostate cancer is made. In the case of a patient treated with a therapy for prostate cancer, recurrence is indicated if a circulating cell-free DNA falls within a chromosomal region set forth in Table 2, Table 3, or Table 4 is detected. In preferred embodiments, a diagnosis of prostate cancer or prostate cancer treatment failure or recurrence is indicated if two or more circulating cell-free DNA molecules fall within 2, 3, 4 or more chromosomal regions set forth in Tables 2-4, preferably all such chromosomal regions being in the same table, *e.g.*, Table 2, Table 3, or Table 4.

[0052] In the above various embodiments, preferably the circulating cell-free DNA molecules have at least 25 consecutive nucleotides in length (preferably at least 50, 70, 80, 100, 120 or 200 consecutive nucleotides in length). More preferably, the circulating cell-free DNA molecules have between about 50 and about 300 or 400, preferably from about 75 and about 300 or 400, more preferably from about 100 to about 200 consecutive nucleotides of a unique sequence within a chromosomal region as set forth in Tables 2-5 (or of a unique sequence set forth in Tables A-D).

Detection of circulating nucleic acids in the blood

[0053] In order to detect the presence of circulating nucleic acids in the blood of patients that may have, or are suspected of having, prostate cancer, a blood sample is obtained from the patient. Serum or plasma from the blood sample is then analyzed for the presence of a circulating cell-free DNA or biomarker as described herein. Nucleic acids can be isolated from serum or plasma using well known techniques, see, *e.g.*, the example sections. In the

context of the current invention, the nucleic acid sequences that are analyzed are DNA sequences. Thus, in this section, methods described as evaluating “nucleic acids” refers to the evaluation of DNA.

[0054] Detection techniques for evaluating nucleic acids for the presence of a biomarker involve procedures well known in the field of molecular genetics. Further, many of the methods involve amplification of nucleic acids. Ample guidance for performing is provided in the art. Exemplary references include manuals such as PCR Technology: Principles and Applications for DNA Amplification (ed. H. A. Erlich, Freeman Press, NY, N.Y., 1992); PCR Protocols: A Guide to Methods and Applications (eds. Innis, et al., Academic Press, San Diego, Calif., 1990); Current Protocols in Molecular Biology, Ausubel, 1994-1999, including supplemental updates through April 2004; Sambrook & Russell, *Molecular Cloning, A Laboratory Manual* (3rd Ed, 2001).

[0055] Although the methods may employ PCR steps, other amplification protocols may also be used. Suitable amplification methods include ligase chain reaction (*see, e.g.*, Wu & Wallace, *Genomics* 4:560-569, 1988); strand displacement assay (*see, e.g.*, Walker *et al.*, *Proc. Natl. Acad. Sci. USA* 89:392-396, 1992; U.S. Pat. No. 5,455,166); and several transcription-based amplification systems, including the methods described in U.S. Pat. Nos. 5,437,990; 5,409,818; and 5,399,491; the transcription amplification system (TAS) (Kwoh *et al.*, *Proc. Natl. Acad. Sci. USA* 86:1173-1177, 1989); and self-sustained sequence replication (3SR) (Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA* 87:1874-1878, 1990; WO 92/08800). Alternatively, methods that amplify the probe to detectable levels can be used, such as Q β -replicase amplification (Kramer & Lizardi, *Nature* 339:401-402, 1989; Lomeli *et al.*, *Clin. Chem.* 35:1826-1831, 1989). A review of known amplification methods is provided, for example, by Abramson and Myers in *Current Opinion in Biotechnology* 4:41-47, 1993.

[0056] In some embodiments, the detection of biomarker in the CNA of a patient is performed using oligonucleotide primers and/or probes to detect a target sequence, wherein the target sequence is present in (*e.g.*, comprises some unambiguously assigned portion of) any of the chromosomal regions listed in Table 2, Table 3, Table 4, or Table 5).

Oligonucleotides can be prepared by any suitable method, usually chemical synthesis, and can also be purchased through commercial sources. Oligonucleotides can include modified phosphodiester linkages (*e.g.*, phosphorothioate, methylphosphonates, phosphoramidate, or boranophosphate) or linkages other than a phosphorous acid derivative into an oligonucleotide may be used to prevent cleavage at a selected site. In addition, the use of 2'-amino modified sugars tends to favor displacement over digestion of the oligonucleotide

when hybridized to a nucleic acid that is also the template for synthesis of a new nucleic acid strand.

[0057] In one embodiment, the biomarker is identified by hybridization under sequence-specific hybridization conditions with a probe that targets a chromosomal region described herein. The probe used for this analysis can be a long probe or sets for short oligonucleotide probes, *e.g.*, from about 20 to about 150 nucleotides in length may be employed.

[0058] Suitable hybridization formats are well known in the art, including but not limited to, solution phase, solid phase, oligonucleotide array formats, mixed phase, or in situ hybridization assays. In solution (or liquid) phase hybridizations, both the target nucleic acid and the probe or primers are free to interact in the reaction mixture. Techniques such as real-time PCR systems have also been developed that permit analysis, *e.g.*, quantification, of amplified products during a PCR reaction. In this type of reaction, hybridization with a specific oligonucleotide probe occurs during the amplification program to identify the presence of a target nucleic acid. Hybridization of oligonucleotide probes ensure the highest specificity due to thermodynamically controlled two state transition. Examples for this assay formats are fluorescence resonance energy transfer hybridization probes, molecular beacons, molecular scorpions, and exonuclease hybridization probes (*e.g.*, reviewed in Bustin, *J. Mol. Endocrin.* 25:169-93, 2000).

[0059] Suitable assay formats include array-based formats, described in greater detail below in the “**Device**” section, where probe is typically immobilized. Alternatively, the target may be immobilized.

[0060] In a format where the target is immobilized, amplified target DNA is immobilized on a solid support and the target complex is incubated with the probe under suitable hybridization conditions, unhybridized probe is removed by washing under suitably stringent conditions, and the solid support is monitored for the presence of bound probe. In formats where the probes are immobilized on a solid support, the target DNA is typically labeled, usually during amplification. The immobilized probe is incubated with the amplified target DNA under suitable hybridization conditions, unhybridized target DNA is removed by washing under suitably stringent conditions, and the solid support/probe is monitored for the presence of bound target DNA.

[0061] In typical embodiments, multiple probes are immobilized on a solid support and the target chromosomal regions in the CNA from a patient are analyzed using the multiple probes simultaneously. Examples of nucleic acid arrays are described by WO 95/11995.

[0062] In an alternative probe-less method, amplified nucleic acid corresponding to a target nucleic acid present in a chromosomal region is performed using nucleic acid primers to the chromosomal region and is detected by monitoring the increase in the total amount of double-stranded DNA in the reaction mixture, is described, *e.g.*, in U.S. Pat. No. 5,994,056; and
5 European Patent Publication Nos. 487,218 and 512,334. The detection of double-stranded target DNA relies on the increased fluorescence various DNA-binding dyes, *e.g.*, SYBR Green, exhibit when bound to double-stranded DNA.

[0063] As appreciated by one in the art, specific amplification methods can be performed in reaction that employ multiple primers to target the chromosomal regions such that the
10 biomarker can be adequately covered.

DNA sequencing

[0064] In preferred embodiments, the presence of a sequence from a chromosomal region set forth in Table 2, Table 3, Table 4 or Table 5 in the CNA from a patient undergoing evaluation is detected by direct sequencing. Such sequencing, especially using the Roche
15 454, Illumina, and Applied Biosystems sequencing systems mentioned below or similar advanced sequencing systems, can include quantitation (*i.e.*, determining the level) of nucleic acids having a particular sequence. Such quantitation can be used in the embodiments of the invention that involve determining the level of a biomarker (some embodiments of which involve correlating a particular level to the presence or absence of cancer). Methods include
20 *e.g.*, dideoxy sequencing-based methods although other methods such as Maxam and Gilbert sequencing are also known (*see, e.g.*, Sambrook and Russell, *supra*). In typical embodiments, CNA from a patient is sequenced using a large-scale sequencing method that provides the ability to obtain sequence information from many reads. Such sequencing platforms includes those commercialized by Roche 454 Life Sciences (GS systems), Illumina
25 (*e.g.*, HiSeq, MiSeq) and Applied Biosystems (*e.g.*, SOLiD systems).

[0065] The Roche 454 Life Sciences sequencing platform involves using emulsion PCR and immobilizing DNA fragments onto bead. Incorporation of nucleotides during synthesis is detected by measuring light that is generated when a nucleotide is incorporated.

[0066] The Illumina technology involves the attachment of randomly fragmented genomic
30 DNA to a planar, optically transparent surface. Attached DNA fragments are extended and bridge amplified to create an ultra-high density sequencing flow cell with clusters containing copies of the same template. These templates are sequenced using a sequencing-by-synthesis technology that employs reversible terminators with removable fluorescent dyes.

[0067] Methods that employ sequencing by hybridization may also be used. Such methods, *e.g.*, used in the ABI SOLiD4+ technology uses a pool of all possible oligonucleotides of a fixed length, labeled according to the sequenced position. Oligonucleotides are annealed and ligated; the preferential ligation by DNA ligase for matching sequences results in a signal
5 informative of the nucleotide at that position.

[0068] The sequence can be determined using any other DNA sequencing method including, *e.g.*, methods that use semiconductor technology to detect nucleotides that are incorporated into an extended primer by measuring changes in current that occur when a nucleotide is incorporated (see, *e.g.*, U.S. Patent Application Publication Nos. 20090127589
10 and 20100035252). Other techniques include direct label-free exonuclease sequencing in which nucleotides cleaved from the nucleic acid are detected by passing through a nanopore (Oxford Nanopore) (Clark *et al.*, *Nature Nanotechnology* 4: 265 – 270, 2009); and Single Molecule Real Time (SMRT™) DNA sequencing technology (Pacific Biosciences), which is a sequencing-by synthesis technique.

15 **Devices and Kits**

[0069] In a further aspect, the invention provides diagnostic devices and kits useful for identifying one or more prostate cancer-associated biomarkers in the CNA from a patient where the one or more biomarkers is a sequence corresponding to any of the chromosomal regions set forth in Table 2, Table 3, Table 4, or Table 5. As will be apparent to skilled
20 artisans, the kit of the present invention is useful in the above-discussed method for analyzing circulating cell-free DNA in a patient sample and in diagnosing, screening or monitoring prostate cancer as described above.

[0070] Thus, in one aspect, the present invention provides the use of at least one oligonucleotide for the manufacture of a diagnostic kit useful in diagnosing, screening or
25 monitoring prostate cancer. The nucleotide sequence of the oligonucleotide falls within a chromosomal region set forth in Table 2, Table 3, Table 4, or Table 5 (or matches a part of a sequence set forth in Table A, Table B, Table C, or Table D).

[0071] Preferably, the kit of the present invention includes one, two or more (*e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 20, 25, 30, 40 or at least 50, but preferably less than 111,
30 preferably from one to about 50, more preferably from 2 to about 50, or from 3 to about 50 sets of oligonucleotides. Each set comprises one or more oligonucleotides (*e.g.*, from about one to about 10,000, preferably from 50, 100, 200 or 300 to about 10,000). All of the nucleotide sequences of such one or more oligonucleotides in each set fall within the same

one single chromosomal region that is set forth in Table 2, Table 3, Table 4, or Table 5 (or match a part of the same one single sequence set forth in Table A, Table B, Table C, or Table D). Each oligonucleotide should have from about 18 to 100 nucleotides, or from 20 to about 50 nucleotides, and is capable of hybridizing, under stringent hybridization conditions, to the chromosomal region in which its sequence falls. The oligonucleotides are useful as probes for detecting circulating cell-free DNA molecules derived from the chromosomal regions. Preferably, each set includes a sufficient number of oligonucleotides with sequences mapped to one chromosomal region such that any circulating cell-free DNA molecules derived from the chromosomal region can be detected with the oligonucleotide set. Thus, the number of oligonucleotides required in each set is determined by the total length of unique nucleotide sequence of a particular chromosomal region, as will be apparent to skilled artisans. Such total lengths are indicated in Tables 2-5 and should also be apparent from Tables A-D.

[0072] Preferably, in the kit of the present invention, different oligonucleotide sets correspond to different chromosomal regions within the same table. Preferably, the oligonucleotides are free of repetitive element. Optionally, the oligonucleotides are attached to one or more solid substrates such as microchips and beads. In preferred embodiments, the kit is a microarray with the above oligonucleotides.

[0073] In one embodiment, the kit of the present invention includes a plurality of oligonucleotide sets capable of hybridizing to the chromosomal regions set forth in Table 2, Table 3, Table 4, and Table 5, respectively. That is, the kit includes oligonucleotide probes corresponding to each and every chromosomal regions set forth in Tables 2, 3, 4, and 5 (or matching each and every sequence set forth in Tables A, B, C, and D) such that all circulating cell-free DNA derived from any chromosomal region set forth in Tables 2-5 can be detected using the kit.

[0074] Use of the oligonucleotides included in the kit described for the manufacture of the kit useful for diagnosing, screening or monitoring prostate cancer is also contemplated. The manufacturing of such kit should be apparent to a skilled artisan.

[0075] In some embodiments, a diagnostic device comprises probes to detect at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, 60, 75, or 100, or all 110 chromosomal regions set forth in Tables 2-5. In some embodiments, the present invention provides probes attached to a solid support, such as an array slide or chip, *e.g.*, as described in DNA Microarrays: A Molecular Cloning Manual, 2003, Eds. Bowtell and Sambrook, Cold Spring Harbor Laboratory Press. Construction of such devices are well known in the art, for example as

described in US Patents and Patent Publications U.S. Patent No. 5,837,832; PCT application W095/11995; U.S. Patent No. 5,807,522; US Patent Nos. 7,157,229, 7,083,975, 6,444,175, 6,375,903, 6,315,958, 6,295,153, and 5,143,854, 2007/0037274, 2007/0140906, 2004/0126757, 2004/0110212, 2004/0110211, 2003/0143550, 2003/0003032, and
5 2002/0041420. Nucleic acid arrays are also reviewed in the following references: *Biotechnol Annu Rev* 8:85-101 (2002); Sosnowski *et al*, *Psychiatr Genet* 12(4):181-92 (Dec. 2002); Heller, *Annu Rev Biomed Eng* 4: 129-53 (2002); Kolchinsky *et al*, *Hum. Mutat* 19(4):343-60 (Apr. 2002); and McGail *et al*, *Adv Biochem Eng Biotechnol* 77:21-42 (2002).

[0076] Any number of probes may be implemented in an array. A probe set that hybridizes
10 to different, preferably unique, segments of a chromosomal region may be used where the probe set detects any part of the chromosomal region. Alternatively, a single probe to a chromosomal region may be immobilized to a solid surface. Polynucleotide probe can be synthesized at designated areas (or synthesized separately and then affixed to designated areas) on a substrate, *e.g.*, using a light-directed chemical process. Typical synthetic
15 polynucleotides can be about 15-200 nucleotides in length.

[0077] The kit can include multiple biomarker detection reagents, or one or more biomarker detection reagents in combination with one or more other types of elements or components (*e.g.*, other types of biochemical reagents, containers, packages such as packaging intended for commercial sale, substrates to which biomarker detection reagents are
20 attached, electronic hardware components, etc.). Accordingly, the present invention further provides biomarker detection kits and systems, including but not limited to arrays/microarrays of nucleic acid molecules, and beads that contain one or more probes or other detection reagents for detecting one or more biomarkers of the present invention. The kits can optionally include various electronic hardware components; for example, arrays
25 ("DNA chips") and microfluidic systems ("lab-on-a-chip" systems) provided by various manufacturers typically comprise hardware components. Other kits may not include electronic hardware components, but may be comprised of, for example, one or more biomarker detection reagents (along with, optionally, other biochemical reagents) packaged in one or more containers.

30 [0078] Biomarker detection kits/systems may contain, for example, one or more probes, or sets of probes, that hybridize to a nucleic acid molecule present in a chromosomal region set forth in Tables 2-5.

[0079] A biomarker detection kit of the present invention may include components that are used to prepare CNA from a blood sample from a patient for the subsequent amplification and/or detection of a biomarker.

Correlating the presence of biomarkers with prostate cancer

5 [0080] The present invention provides methods and reagents for detecting the presence of a biomarker in CNA from a patient that has prostate cancer or that is being evaluated to determine if the patient may have prostate cancer. In the context of the invention, "detection" or "identification" or "identifying the presence" or "detecting the presence" of a biomarker associated with prostate cancer in a CNA sample from a patient refers to determining any
10 level of the biomarker in the CNA of the patient where the level is greater than a threshold value that distinguishes between prostate cancer and non-prostate cancer CNA samples for a given assay.

[0081] In the current invention, for example, the presence of any one of the chromosomal regions (i.e., biomarkers) listed in Tables 2-4 is indicative of prostate cancer. As appreciated
15 by one of skill in the art, biomarkers may be employed in analyzing a patient sample where the biomarker has also been observed infrequently in a normal patient in order to increase the sensitivity of the detection. For example, the biomarkers indicated by bold font in Tables 2 and 3 have been observed to be present infrequently in CNA obtained from normal individuals; however, given the low frequency of occurrence in normal samples relative to
20 the higher frequency of occurrence in prostate cancer, the presence of the biomarker in a patient indicates that the patient has a 95% or greater likelihood of having prostate cancer. Thus, for example, arrays used to detect the chromosomal regions can include those that identify the chromosomal regions in Tables 2 and 3 that are indicated in bold font.

[0082] The biomarkers set forth in Tables 2-4 are associated with prostate cancer, *i.e.*, they
25 are over-represented in prostate cancer patients compared to individuals not diagnosed with prostate cancer. Thus, the detection of one or more of the biomarkers set forth in Tables 2-4 is indicative of prostate cancer, *i.e.*, the patient has an increased probability of having prostate cancer compared to a patient that does not have the biomarker. In some embodiments, the detection of two or more biomarkers set forth in Tables 2-4 in the CNA of a patient is
30 indicative of a greater probability for prostate cancer. As understood in the art, other criteria, *e.g.*, clinical criteria, etc., are also employed to diagnose prostate cancer in the patient. Accordingly, patients that have a biomarker associated with prostate cancer also undergo other diagnostic procedures.

[0083] In some embodiments, one or more biomarkers that are under-represented in prostate cancer may be detected in the CNA of a patient. Thus, for example, a biomarker listed in Table 5 may be detected in a CNA sample from a patient where the detection of the biomarker is indicative of a normal diagnosis, *i.e.*, that the patient does not have prostate cancer.

[0084] “Over-represented” or “increased amount” means that the level of one or more circulating cell-free DNAs is higher than normal levels. Generally this means an increase in the level as compared to an index value. Conversely “under-represented” or “decreased amount” means that the level of one or more particular circulating cell-free DNA molecules is lower than normal levels. Generally this means a decrease in the level as compared to an index value.

[0085] In preferred embodiments, the test value representing the amount of a particular circulating cell-free DNA is compared to one or more reference values (or index values), and optionally correlated to prostate cancer or cancer recurrence. Optionally, an increased likelihood of prostate cancer is indicated if the test value is greater than the reference value.

[0086] Those skilled in the art are familiar with various ways of deriving and using index values. For example, the index value may represent the copy number or concentration of a particular cell-free DNA according to the present invention in a blood sample from a patient of interest in healthy state, in which case a copy number or concentration in a sample from the patient at a different time or state significantly higher (e.g., 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 100-fold or more higher) than this index value would indicate, e.g., prostate cancer or increased likelihood of cancer recurrence.

[0087] Alternatively, the index value may represent the average concentration or copy number of a particular circulating cell-free DNA for a set of individuals from a diverse cancer population or a subset of the population. For example, one may determine the average copy number or concentration of a circulating cell-free DNA in a random sampling of patients with prostate cancer. Thus, patients having a copy number or concentration (test value) comparable to or higher than, this value identified as having an increased likelihood of having prostate cancer or prostate cancer recurrence than those having a test value lower than this value.

[0088] Often the copy number or amount (e.g., concentration) will be considered “increased” only if it differs significantly from the index value, e.g., at least 1.5 fold

difference in absolute or relative (e.g., as reflected by hybridization signal) copy number or concentration.

[0089] A useful index value may represent the copy number or concentration of a particular circulating cell-free DNA or of a combination (weighted or straight addition) of two or more circulating cell-free DNAs corresponding to the same chromosomal region or different chromosomal regions. When two or more biomarkers or circulating cell-free DNA molecules are used in the diagnosis/monitoring method, the presence or absence of, or the amount of, each biomarker or circulating cell-free DNA can be weighted and combined. Thus, a test value may be provided by (a) weighting the determined status or amount of each circulating cell-free DNA molecule with a predefined coefficient, and (b) combining the weighted status or amount to provide a test value. The combining step can be either by straight addition or averaging (i.e., weighted equally) or by a different predefined coefficient.

[0090] The information obtained from the biomarker analysis may be stored in a computer readable form. Such a computer system typically comprises major subsystems such as a central processor, a system memory (typically RAM), an input/output (I/O) controller, an external device such as a display screen via a display adapter, serial ports, a keyboard, a fixed disk drive via a storage interface and a floppy disk drive operative to receive a floppy disc, and a CD-ROM (or DVD-ROM) device operative to receive a CD-ROM. Many other devices can be connected, such as a network interface connected via a serial port.

[0091] The computer system may also be linked to a network, comprising a plurality of computing devices linked via a data link, such as an Ethernet cable (coax or 10BaseT), telephone line, ISDN line, wireless network, optical fiber, or other suitable signal transmission medium, whereby at least one network device (e.g., computer, disk array, etc.) comprises a pattern of magnetic domains (e.g., magnetic disk) and/or charge domains (e.g., an array of DRAM cells) composing a bit pattern encoding data acquired from an assay of the invention.

[0092] The computer system can comprise code for interpreting the results of a study evaluating the presence of one or more of the biomarkers. Thus in an exemplary embodiment, the biomarker analysis results are provided to a computer where a central processor executes a computer program for determining the likelihood of a patient that has prostate cancer.

[0093] The invention also provides the use of a computer system, such as that described above, which comprises: (1) a computer; (2) a stored bit pattern encoding the biomarker

testing results obtained by the methods of the invention, which may be stored in the computer; (3) and, optionally, (4) a program for determining the likelihood of a patient having prostate cancer.

[0094] The invention further provides methods of generating a report based on the detection of one or more biomarkers set forth in Tables 2-5.

[0095] Thus, the present invention provides systems related to the above methods of the invention. In one embodiment the invention provides a system for analyzing circulating cell-free DNA, comprising: (1) a sample analyzer for executing the method of analyzing circulating cell-free DNA in a patient's blood, serum or plasma as described in the various embodiments above (incorporated herein by reference); (2) a computer system for automatically receiving and analyzing data obtained in step (1) to provide a test value representing the status (presence or absence or amount, i.e., concentration or copy number) of one or more circulating cell-free DNA molecules having a nucleotide sequence of at least 25 nucleotides falling within a chromosomal region set forth in Table 2, Table 3, Table 4, or Table 5 (or having a nucleotide sequence that is a part of one of the sequences set forth in Table A, Table B, Table C, or Table D), and optionally for comparing the test value to one or more reference values each associated with a predetermined status of prostate cancer. In some embodiments, the system further comprises a display module displaying the comparison between the test value and the one or more reference values, or displaying a result of the comparing step.

[0096] Thus, as will be apparent to skilled artisans, the sample analyzer may be, e.g., a sequencing machine (e.g., Illumina HiSeqTM, Ion Torrent PGM, ABI SOLiDTM sequencer, PacBio RS, Helicos HeliscopeTM, etc.), a PCR machine (e.g., ABI 7900, Fluidigm BioMarkTM, etc.), a microarray instrument, etc.

[0097] In one embodiment, the sample analyzer is a sequencing instrument, e.g., a next-generation sequencing instrument such as Roche's GS systems, Illumina's HiSeq and MiSeq, and Life Technologies' SOLiD systems. Circulating cell-free DNA molecules are isolated from a patient's blood or serum or plasma, and the sequences of all of the circulating cell-free DNA molecules are obtained using the sample analyzer. The sequencing instrument is used in sequencing the circulating cell-free DNA molecules, and obtaining the sequences of these molecules. A computer system is then employed for automatically analyzing the sequences to determine the presence or absence or amount of a circulating cell-free DNA molecule having a nucleotide sequence of at least 25 nucleotides falling within a chromosomal region

set forth in Table 2, Table 3, Table 4, or Table 5 (or having a nucleotide sequence that is a part of one of the sequences set forth in Table A, Table B, Table C, or Table D) in the sample. For example, the computer system may compare the sequence of each circulating cell-free DNA molecule in the sample to each sequence in Tables A, B, C, and D to

5 determine if there is a match, i.e., if the sequence of a circulating cell-free DNA molecule falls within a sequence in Table A, Table B, Table C, or Table 2 or within a chromosomal region set forth in Table 2, Table 3, Table 4, or Table 5. The copy number of a particular circulating cell-free DNA molecule is also automatically determined by the computer system. Optionally the computer system automatically correlates the sequence analysis result with a
10 diagnosis regarding prostate cancer. For example, if one, two or more circulating cell-free DNA molecules are identified to be derived from a chromosomal region in Table 2, Table 3, or Table 4 and preferably two or more circulating cell-free DNA molecules with sequences falling within different chromosomal regions from one single table chosen from Tables 2-4, then the computer system automatically correlates this analysis result with a diagnosis of
15 prostate cancer. Optionally, the computer system further comprises a display module displaying the results of sequence analysis and/or the result of the correlating step. The display module may be for example, a display screen, such as a computer monitor, TV monitor, or the touch screen, a printer, and audio speakers.

[0098] The computer-based analysis function can be implemented in any suitable language
20 and/or browsers. For example, it may be implemented with C language and preferably using object-oriented high-level programming languages such as Visual Basic, SmallTalk, C++, and the like. The application can be written to suit environments such as the Microsoft WindowsTM environment including WindowsTM 98, WindowsTM 2000, WindowsTM NT, and the like. In addition, the application can also be written for the MacIntoshTM, SUNTM, UNIX
25 or LINUX environment. In addition, the functional steps can also be implemented using a universal or platform-independent programming language. Examples of such multi-platform programming languages include, but are not limited to, hypertext markup language (HTML), JAVATM, JavaScriptTM, Flash programming language, common gateway interface/structured query language (CGI/SQL), practical extraction report language (PERL), AppleScriptTM and
30 other system script languages, programming language/structured query language (PL/SQL), and the like. JavaTM- or JavaScriptTM-enabled browsers such as HotJavaTM, MicrosoftTM ExplorerTM, or NetscapeTM can be used. When active content web pages are used, they may include JavaTM applets or ActiveXTM controls or other active content technologies.

[0099] The analysis function can also be embodied in computer program products and used in the systems described above or other computer- or internet-based systems. Accordingly, another aspect of the present invention relates to a computer program product comprising a computer-usable medium having computer-readable program codes or instructions embodied thereon for enabling a processor to carry out the analysis and correlating functions as described above. These computer program instructions may be loaded onto a computer or other programmable apparatus to produce a machine, such that the instructions which execute on the computer or other programmable apparatus create means for implementing the functions or steps described above. These computer program instructions may also be stored in a computer-readable memory or medium that can direct a computer or other programmable apparatus to function in a particular manner, such that the instructions stored in the computer-readable memory or medium produce an article of manufacture including instruction means which implement the analysis. The computer program instructions may also be loaded onto a computer or other programmable apparatus to cause a series of operational steps to be performed on the computer or other programmable apparatus to produce a computer implemented process such that the instructions which execute on the computer or other programmable apparatus provide steps for implementing the functions or steps described above.

[0100] The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of non-critical parameters that could be changed or modified to yield essentially similar results.

EXAMPLES

Example 1. Identification of prostate cancer-associate CNA

Sequencing of CNA:

[0101] Sera from 197 patients having biopsy-proven prostate cancer with a Gleason Score of 3 to 10 were used. The data were compared to samples obtained from 92 male apparently healthy blood donors (normals).

[0102] After extraction of DNA from serum or plasma, using standard silica based methods, a whole genome amplification was performed in duplicate. The products were pooled and used for further analysis.

Long sequencing runs:

[0103] The primer sequences for 454 sequencing were added to the product using fusion primers in not more than 20 cycles of PCR. The resulting product was treated according to the 454 sequencer manual and used for direct sequence detection.

5 Computational analysis:

[0104] The sequence reads were appointed to the sample source by reading the identifier sequence string and all non source parts were cut out (e.g. primers).

[0105] The origin of the circulating DNA was investigated by local alignment analyses using the BLAST program using high stringent parameters (30). Repetitive elements were
10 detected and masked by using a local install of the Repeatmasker software package (31) using the rebase (version 12.09) that was obtained from the Genetic Information Research Institute (32). After masking of the repetitive elements and region of low sequence complexity each sequence was subjected to sequential BLAST analyses querying databases of bacterial, viral and fungal genomes and the human genome (reference genome build 37.1).
15 Bacterial, viral, fungal and human genomes were obtained from the National Center for Biotechnology Information (NCBI, (<ftp.ncbi.nih.gov>)). After each of the sequential database searches all parts of a queried sequence that produced significant hits ($e < 0.0001$) were masked and masked sequences were subsequently used to query the next database. Masked nucleotides were counted and subtracted from the total nucleotide counts resulting in the
20 amounts of unidentified nucleotides.

[0106] For each query fragment and each database search, the highest scoring BLAST hit with a length of more than 50% of the query sequence was recorded in a SQL database. The highest scoring BLAST hit was defined as the longest hit with the highest percent identity (maximum of hitlength x identity). For each of the sequences, the start and stop positions for
25 query and database were recorded.

- Disease association analysis:

[0107] To investigate which sequences/sequence tags are disease associated, all alignments that could be unambiguously placed on the human genomic database (Homo sapiens Build 37.1 <http://www.ncbi.nlm.nih.gov/mapview/>) were used. Those were categorized into 4060
30 region of 750,000 bp intervals and selected for differences between normal controls and cancer patients on the basis of a group comparison using the non-parametric median test.

Five hundred chromosomal regions having the lowest p-values were used for further analyses.

[0108] In 347 rounds of bootstrap random selected samples linear multivariate regressions modeling was performed using the above selected 750k regions as independent parameters.

5 The 347 rounds of bootstrap yielded 8.9×10^7 models. From these, the best 42 independent variables (750k pieces, based on $\Sigma AIC\omega_i$) were chosen and are shown in Table 1.

Selection of clusters in the pre-selected regions

[0109] As a second step, a cluster search within the 42 regions was performed and clusters of cancer samples were found that did not contain any normal samples. Additional clusters

10 were searched where only one or fewer normal samples were found in a cluster of at least 10 cancer samples. The cluster regions were restricted to those that had a distance to the next normal hit of at least 200bp. Given these limits, 88 clusters were found, of which 25 contained one hit in the normal group. A sample is called positive if at least one hit is found in any of the cluster regions (see Table 2). A selection of 30 clusters without false positives

15 covering 441 kbp was sufficient to provide a 94% sensitivity and 100% specificity, which can be increased to 96% when two more clusters with a hit in one normal was included, achieving a specificity of 99%.

Selection of genomic clusters

[0110] A search for genomic clusters was also performed over the whole non-repetitive

20 genome. The limit was set to have more than 18 independent patients hits per cluster, where over 600 clusters with not more than 1 hit in samples from normals was found. A selection of 22 clusters covering about 475 kbp was sufficient to give a rate of 99.5% true positives at a 99% true negative level (see Table 3). Excluding those two regions that had one hit in the group of normal, non-prostate cancer patients, the true positive rate was 96% and

25 consequently 100% specificity.

Example 2. Identification of additional biomarkers that are over- or under-represented in prostate cancer.

[0111] In a further analysis to identify biomarkers, a subset of 77 Prostate Cancer samples and 70 Controls were sequenced on a Life Technologies SOLiD4+ System, which is a high-

30 throughput sequencer that typically can run hundreds of millions of sequences of lengths of about 30 to about 75 bases in parallel. (Another example of such a sequencing technology is the Genome Analyzer (Illumina)). Briefly, samples were prepared as described for the 454

sequencing, where adaptors specific to SOLiD sequencing are used. For each sample about 5 million reads of 40 bp were achieved and aligned to the human genome (Build Hg18) using the Lifetechnologies “Bioscope” software suite with default stringency values. All uniquely aligned reads were used for further analysis.

- 5 **[0112]** In order to define the chromosomal regions, where samples of either group cluster in comparison the second group, all hits are ranked in order of their appearance on the chromosomes. Biomarker regions were initially defined as those regions where at least 15 hits covering at least 10 samples of a group were counted, but not more than one sample of the other group demonstrated a hit.
- 10 **[0113]** In 1000 round of random resampling the samples were divided into a training and validation set of 50% of each group. For each region, a sample in the region was assigned a score of 1 if the cluster was a prostate cancer (PrCa) cluster and a score of -1 for region where normal samples clustered. The average AUC for the validation set was found to be 0.92 if the score sums of 42 cluster regions are used. The regions used in the 1000 round of
- 15 re-sampling were ranked according to the number of round in which either region participated. Tables 4 and 5 shows the 56 highest ranking regions. Table 4 summarizes those clusters identified in this analysis that were overrepresented in prostate cancer serum samples. Table 5 summarizes clusters identified in this analysis that were underrepresented in prostate cancer serum samples; the ROC curve using the 42 highest ranking regions is
- 20 shown in Figure 1. The AUC below the ROC curve was calculated as 0.96 (0.886 - 0.996) with and accuracy of 92% (86% - 97%).

- 25 **[0114]** All patents, patent applications, and other published reference materials cited in this specification are hereby incorporated herein by reference in their entirety for their disclosures of the subject matter in whose connection they are cited herein.

Table 1

Chromosome	Region Start position-End position	Cyto banding	Rank (AIC)	Chromosome Length
Hs1	98250001-99000000	1p21.3	41	226.9 Mb
	215250001-216000000	1q41	26	
	225000001-225750000	1q42.12/13	31	
Hs2	49500001-50250000	2p16.3	13	238.4 Mb
	61500001-62250000	2p15	6	
	98250001-99000000	2q11.2	14	
	132750001-133500000	2q21.2	1	
	162000001-162750000	2q24.2	37	
	180750001-181500000	2q31.3	5	
	1500001-2250000	3p26.3	44	
Hs3	105000001-105750000	3q13.11	8	198 Mb
	150000001-150750000	3q24-25.1	39	
	164250001-165000000	3q26.1	16	
	6000001-6750000	4p16.1	45	
Hs4	1500001-2250000	5p15.33	20	188.1 Mb
Hs5	40500001-41250000	5p13.1	28	
	81000001-81750000	5q14.1/2	30	
	159750001-160500000	5q33.3-34	15	
	167250001-168000000	5q34-35.1	9	
Hs6	31500001-32250000	6p21.33/32	7	171 Mb
Hs7	3750001-4500000	7p22.2	38	156 Mb
	124500001-125250000	7q31.33	12	
	156000001-156750000	7q36.3	34	
Hs8	12000001-12750000	8p23.1-22	42	143 Mb
	19500001-20250000	8p21.3	10	
	24750001-25500000	8p21.2	35	
	129000001-129750000	8q24.21	29	
Hs9	750001-1500000	9p24.3	18	121.5 Mb
	8250001-9000000	9p24.1	19	
	141000001-141213431	9qter	21	
Hs10	134250001-135000000	10q26.3	48	131.7 Mb
Hs11	87750001-88500000	11q14.2/3	49	135 Mb
Hs12	31500001-32250000	12p11.21	11	130.5 Mb
	84000001-84750000	12q21.31	40	
Hs13	21000001-21750000	13q12.11	4	95.6 Mb
	48000001-48750000	13q14.2	25	
	114750001-115169878	13q34-qter	46	
Hs14	63000001-63750000	14q23.2	17	88.3 Mb
	103500001-104250000	14q32.33	27	
Hs15	34500001-35250000	15q14	36	82.2 Mb
	45750001-46500000	15q21.1	33	
	57000001-57750000	15q22.1/2	43	
Hs16	23250001-24000000	16p12.1	22	78.9 Mb
	33750001-34500000	16p11.2/1	24	
	68250001-69000000	16q22.1	23	
Hs17	18000001-18750000	17p11.2	32	78.2 Mb
Hs19	53250001-54000000	19q13.32/33	50	56.1 Mb
	57000001-57750000	19q13.33-41	2	
Hs20	24750001-25500000	20p11.21	3	63 Mb
Hs21	21000001-21750000	21q21.1	47	48 Mb

Table 2

Chromosome	Start Position	End Position	Length	Unique Length	#Ind.	Line # In Table1
Hs9	1203203	1208552	5349	4100	10	28
Hs13	21589501	21597794	8293	4825	15	35
Hs9	141121140	141129705	8565	4477	11	30
Hs17	18509184	18526779	17595	9089	15	46
Hs14	63439924	63453744	13820	7671	10	38
Hs5	1792424	1811001	18577	14985	13	15
Hs9	8896121	8911530	15409	8912	10	29
Hs5	40665598	40693396	27798	22274	18	16
Hs13	48226162	48242250	16088	9178	10	36
Hs2	162242923	162262521	19598	11865	12	8
Hs12	31814898	31835553	20655	7945	12	33
Hs8	12276056	12297091	21035	14663	12	24
Hs5	167303952	167325309	21357	13170	12	19
Hs15	34692781	34717541	24760	17408	14	40
Hs15	45945913	45974928	29015	16061	15	41
Hs8	129491613	129511269	19656	12165	10	27
Hs14	63572445	63603375	30930	11049	15	38
Hs2	61907413	61956856	49443	19877	23	5
Hs2	181047163	181068902	21739	14079	10	9
Hs2	49920547	49991893	71346	35434	31	4
Hs3	105168575	105194180	25605	16362	11	11
Hs5	81091424	81128988	37564	17433	16	17
Hs16	68707139	68733885	26746	14395	11	45
Hs5	81651300	81683959	32659	20395	12	17
Hs5	1851815	1879589	27774	22549	10	15
Hs5	167930740	167967508	36768	16984	18	19
Hs12	31553223	31586248	33025	15905	11	33
Hs14	63337644	63376856	39212	19982	13	38
Hs15	35174423	35207743	33320	20692	11	40
Hs1	225305228	225352841	47613	16886	14	3
Hs12	31981016	32000913	19897	6010	14	33
Hs12	84147927	84232206	84279	40700	24	34

# of Clusters	Sens	Spec	Length	Unique Length
30	93.9%	100.0%	801314	440810
31	94.9%	98.9%	821211	446820
32	95.9%	98.9%	905490	487520

Table 3

#	Chromosome	Start Position	End Position	Length	Unique Length	#Ind.
1	Hs14	70096330	70127234	30904	20903	27
2	Hs6	39345271	39370386	25115	16483	21
3	Hs15	53064016	53089483	25467	21509	21
4	Hs12	116499466	116530409	30943	20444	24
5	Hs2	157539155	157566634	27479	22048	21
6	Hs14	80304012	80330694	26682	19106	20
7	Hs10	114581158	114608169	27011	18506	20
8	Hs1	101763313	101791825	28512	18018	21
9	Hs7	50308799	50337414	28615	19398	21
10	Hs1	149307024	149338559	31535	17715	24
11	Hs5	171222089	171253456	31367	14683	21
12	Hs6	51313581	51352528	38947	28500	26
13	Hs5	137264839	137297311	32472	20322	20
14	Hs5	66278164	66313008	34844	27403	21
15	Hs3	170530159	170567250	37091	19172	22
16	Hs10	120162288	120199466	37178	26822	23
17	Hs12	126284041	126322408	38367	17006	21
18	Hs9	24039045	24078308	39263	23866	21
19	Hs2	216752448	216796426	43978	23279	21
20	Hs15	61191103	61235085	43982	30792	20
21	Hs9	35794560	35821014	26454	22335	19
22	Hs4	158937672	158995447	57775	26461	21

# of Clusters	Sens	Spec	Length	Unique Length
20	96.4%	100.0%	659752	425975
21	97.5%	98.9%	686206	448310
22	99.5%	98.9%	743981	474771

Table 4

Rank	Chromosome	Position	Region	Group
1	Hs12	30.6Mb	30570547 - 30570743	PrCa
2	Hs12	21.5Mb	21486871 - 21487052	PrCa
3	Hs4	86.2Mb	86221239 - 86221507	PrCa
4	Hs12	25.3Mb	25327892 - 25328036	PrCa
5	Hs10	113.1Mb	113115517 - 113115668	PrCa
6	Hs14	38.9Mb	38862061 - 38862327	PrCa
7	Hs16	25.3Mb	25252221 - 25252481	PrCa
8	Hs22	46.1Mb	46118701 - 46118844	PrCa
9	Hs3	157.7Mb	157749009 - 157749191	PrCa
10	Hs10	78Mb	77959547 - 77959711	PrCa
11	Hs11	44.6Mb	44630448 - 44630727	PrCa
12	Hs6	123.9Mb	123900311 - 123900449	PrCa
13	Hs22	30.4Mb	30448964 - 30449173	PrCa
14	Hs16	77.8Mb	77757034 - 77757233	PrCa
15	Hs2	114.8Mb	114825678 - 114825874	PrCa
16	Hs21	34.9Mb	34883267 - 34883616	PrCa
17	Hs4	20.7Mb	20747267 - 20747398	PrCa
18	Hs5	6Mb	5955117 - 5955276	PrCa
19	Hs5	25.8Mb	25838138 - 25838267	PrCa
21	Hs6	35.4Mb	35436306 - 35436437	PrCa
22	Hs8	145.3Mb	145289592 - 145289753	PrCa
23	Hs2	49.9Mb	49901877 - 49901945	PrCa
24	Hs15	83.7Mb	83697580 - 83697795	PrCa
26	Hs8	15.4Mb	15424921 - 15425143	PrCa
27	Hs4	15.1Mb	15054643 - 15054930	PrCa
28	Hs19	39.4Mb	39368301 - 39368596	PrCa
29	Hs7	49.7Mb	49689619 - 49689826	PrCa
30	Hs20	36.2Mb	36231639 - 36231810	PrCa
31	Hs9	4.1Mb	4124816 - 4125132	PrCa
32	Hs1	24.2Mb	24210790 - 24210949	PrCa
33	Hs3	24.3Mb	24265368 - 24265621	PrCa
34	Hs10	127.5Mb	127536020 - 127536197	PrCa
35	Hs10	133Mb	132973900 - 132974068	PrCa
36	Hs5	9.5Mb	9484677 - 9484862	PrCa
37	Hs1	214.7Mb	214667412 - 214667588	PrCa
38	Hs12	69.9Mb	69877688 - 69877868	PrCa
39	Hs3	29.4Mb	29448099 - 29448452	PrCa
40	Hs4	16.5Mb	16544904 - 16545167	PrCa
41	Hs8	64.7Mb	64704166 - 64704321	PrCa
42	Hs9	119.3Mb	119339188 - 119339363	PrCa
43	Hs7	19.8Mb	19816439 - 19816659	PrCa
44	Hs1	86.4Mb	86350713 - 86350904	PrCa
45	Hs2	62.4Mb	62369682 - 62369921	PrCa
46	Hs3	65.7Mb	65747782 - 65747986	PrCa
48	Hs9	109.8Mb	109841019 - 109841101	PrCa
49	Hs3	25.8Mb	25806856 - 25807015	PrCa
50	Hs8	33.6Mb	33615921 - 33616142	PrCa
51	Hs2	44.1Mb	44129332 - 44129600	PrCa
52	Hs19	36Mb	35994062 - 35994387	PrCa
53	Hs3	109Mb	108989445 - 108989612	PrCa
54	Hs19	35.5Mb	35468803 - 35469027	PrCa
56	Hs17	40.7Mb	40658673 - 40658927	PrCa

Table 5

20	Hs1	81.6Mb	81624931 - 81625149	normal
25	Hs6	42.2Mb	42228631 - 42228778	normal
47	Hs11	42.4Mb	42370286 - 42370710	normal
55	Hs8	24.6Mb	24591709 - 24591956	normal

Table A

>Hs1_225305228-225352841

5 ACTTTTCATTAAGCATGGAATGTTATTTTAACTGATACTACTATATATACCATAACTAATATTGTGGCTTTTGT
 GTAAGTATAGGTCAAAATGAGGATGTAATTCATATCATAATTAAGCATATATTCTGAATTGCATTTCTTTTGATT
 CTGGACAGGATTACCTTGAAGTTAAAAGATTAATTTTCCCAAGATTTTACTTTCTTAGCAATGCCGAGCTTCTTG
 ATATTCTAGCTGATAGCAGAAATCCTGAGTCTGTACAGGTAATAACATCTTTCTCTGCTCAGCAATATTATATTA
 GCGATATTTCAGATCACAATTCAATAATATTACTGACATTTGTTTCTGGTTTACAGCCTCATCTTGTGAAATGCTT
 TGAAAATATAAAACAATTATTGATATGGAAACAAGACATTGGCCCTCCTGCTGTAAAAATGCTAATATCTGCTGA
 10 AGGGGAAGGTCTCGTGCTGCCAAAGTATGATAAATGTTACAACTGTTTAGATTCTGTACAACGTCATTATTTAA
 AATCTCTGCTTTGAAATTTTGGATATATTATAAAAGTGCGCTAAGCAAACCTGTTTCTTCAAAGATTATATCCTTA
 CGTAAATATACTTTTGCCTTTCCATATTCCCTACAATACATAATCTCACCTTTGCTCTAATTAAAAGTTGAGAAA
 GCAGTCTTCAAACCTCCTGGGTGCATTCTAATTTGCACTTACATTTTGTCTGTCAATTAATTTCTGCCTTAAGTTT
 CCTACTCTCTTTTGTAGTGCTTTTCCCTTTTGGTTTTAGTATATGAATATAGATAAGAAGAAAATTGGCTATTT
 15 GGAGTTGTTGATGCTACTCCCACTACTGCTTCTGCTGATCTCTGGTTTCACTGTGGTTTGTCTCCAGGCAATAAT
 GACAACTAGGTCCTTCCATTACACTGGTTCAATTTGCCAGCCCCAGCAAGGGCTCTTAACCTTTGGCAAGTGG
 TCCTGATGTAAGTTGATTTTAAAAAAATGCTGTGCCCAACCCTTCTTATCTACCAAATCCCTATTCCAACCCT
 GCTGTTTAAAAGATCCAGTGAGGAA (N) xTTCCAGTGAGGAATCCAAAACCTGTTTTATAACACAATTTGTTTATC
 TTGTAAATACCATTTTACATGAACTTATTTATTGTGTAAATTCATGATTTTGTATATCAGAATTTCTTAAAGCAT
 20 GTTCATATGTTTTCTGCATAGTTTTCAAATTAAGATTTAGAAAAACCAAAGGCAGTAGTAGTCCTTTGGCCCA
 TGAATATTATTAAAGTATGTAGAAAATTTAGTCTACAGGCTTGTCAAAGGACTGAAACATGAATTAAAAATAAT
 TCTTCATGATATTATTTTGAACATAATTTTAGGAAAATTCGTGTAAGAAGTGCTGTAGAACAGTGGCTGGTAAA
 TGTAGAAAAAAGCATGTTTCGATGTGCTAAAAAAGTAAGTACAATTTTCAAATCCTAAAATTATATATTT (N) xTT
 TTTAAACTCACTTATCTACTAATCTAAGTGAGAAATATCCAGAAAAGAATATGGAGTCAAATCTCTTATGAAAT
 25 GGGTTTTAATGGAACATGATGACACATGATGACTAAATGGAAAATAATCACTTTTACAGTGTAAGTGTGAGTTTG
 AGAGTGTTTACATGCACAACCTTCAGATGGCTTTTGTGTACGTTGAGATTCAGCTGTATCAGAAAACCTGTTCCAGG
 ATCTGAACTCTGCTCAGTAGGGCTCCCGTTTGGAGCACACCTCACTACTTTGCATTTTGGTTCTACTTTTACTTCC
 TAGGATTGTTTACTTGGCTTACTCTTTCTAACGTTCTCCTTTTTCATATTTAAGTCTATCATTGCATCTGTTTGA
 ATGAAGAAAAAACCTCAGAGCGTGAACCTTCAAACCTAAGTGTGACTAGAACTGTTATATTGTATTTCTAATTAT
 30 TCATTGTTGGTAATAGAAAGGCTATTGACTT (N) xTTTTTTTCTAAAGAGAATGTACAAGTACATTCATCAAATG
 TAACAGATCGTTGGCAGTACTTTATCTTACCAGAAAACATCAATAGAAGATTTTATACTC (N) xTACTGGTGAG
 TCCCTCTTCTAAAAGCAAATACTAGTTTCTAACTACAACAACCTATATGCCGTGGTTGCAACTGACAAGCAGGGC
 ATGTGGAAGGACCTAGTCTGTGAGTAACTTGCCTTTTGTGAGTGAAGTTCCTTCCCATTCTCCTGGAGGTCAC
 AAACCTACTTGTGACACTCTACTGTGCCTCTAGGTCAGCACGTTTTAAGTATTCCATATACTTATAGTATATCAAT
 35 GCGAGTGTGCAGTAAGATCTTCATTAAGATTTTGTCAAAGAGAAATGTAAGTATATGTTGTTTGGAGAGATGT
 ATAGCAATGTGACAAAAAGTTATGAATAAAAGTCATAAGGACTTTTCATGGAACTGCCAACAAAAAGTGTTTAA
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62

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134

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Table B

>Hs1_101763313-101791825

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DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 146

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 146

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

WHAT IS CLAIMED IS:

- 1 1. A method of analyzing circulating free DNA in a patient sample,
2 comprising determining, in a sample that is blood, serum or plasma, the presence or absence
3 or the amount of,
4 a first cell-free DNA having a sequence falling within a first chromosomal
5 region set forth in Table 2, Table 3, Table 4, or Table 5, and
6 a second cell-free DNA having a sequence falling within a second
7 chromosomal region set forth in Table 2, Table 3, Table 4, or Table 5,
8 wherein the sequences of said first and second cell-free DNAs are free of
9 repetitive elements.
- 1 2. The method of claim 1, wherein said first and second chromosomal
2 regions are different.
- 1 3. The method of claim 1 or 2, wherein said patient is suspected of having
2 prostate cancer.
- 1 4. The method of claim 1 or 2 or 3, further comprising determining in
2 said sample a third cell free DNA having a sequence falling within a third chromosomal
3 region set forth in Table 2, Table 3, Table 4, or Table 5, wherein said third chromosomal
4 region is different from said first and second chromosomal regions, and the sequence of said
5 third cell free DNA is free of repetitive elements.
- 1 5. The method of any one of claims 1 to 4, further comprising
2 determining in said sample at least 5, 8, 10, 20, 30, or 40 additional different cell free DNAs
3 each falling within a different chromosomal region set forth in Tables 2 and 3.
- 1 6. The method of any one of claims 1 to 4, wherein said chromosomal
2 regions are all set forth within the same table.
- 1 7. A kit comprising a plurality of oligonucleotides each having a
2 nucleotide sequence falling within one same chromosomal region set forth in a table selected
3 from the group consisting of Table 2, Table 3, Table 4, and Table 5, wherein said plurality
4 includes a number of said oligonucleotides sufficient to detect all circulating cell-free DNA
5 molecules derived from said chromosomal region.
- 1 8. A kit comprising:

2 a first plurality of oligonucleotides wherein each oligonucleotide within each
3 of said plurality has a nucleotide sequence falling within the same first chromosomal region
4 that is set forth in a table selected from the group consisting of Table 2, Table 3, Table 4, and
5 Table 5; and

6 a second plurality of oligonucleotides each having a nucleotide sequence
7 falling within the same second chromosomal region that is chosen from those set forth in said
8 table, wherein said first and second chromosomal regions are different and wherein said
9 oligonucleotides are free of repetitive element.

1 9. The kit according to claim 7 or 8, wherein said oligonucleotides are
2 attached to a solid substrate.

1 10. A method of diagnosing or screening for prostate cancer in a patient,
2 comprising:

3 determining, in a sample that is blood, serum or plasma from said patient, the
4 presence or absence or the amount of, a circulating cell-free DNA having a sequence falling
5 within a chromosomal region set forth in a table selected from the group consisting of Table
6 2, Table 3, and Table 4; and

7 correlating the presence of or an increased amount of said circulating cell-free
8 DNA with an increased likelihood that said patient has prostate cancer.

1 11. A method of diagnosing or screening for prostate cancer in a patient,
2 comprising:

3 determining, in a sample that is blood, serum or plasma from said patient, the
4 total amount of all circulating cell-free DNAs each having a sequence falling within the same
5 one single chromosomal region set forth in a table selected from the group consisting of
6 Table 2, Table 3, and Table 4; and

7 correlating an increased total amount with an increased likelihood that said
8 patient has prostate cancer.

1 12. A method of diagnosing or screening for prostate cancer in a patient,
2 comprising:

3 determining, in a sample that is blood, serum or plasma from said patient, the
4 presence or absence or the amount of, a first circulating cell-free DNA having a sequence
5 falling within a first chromosomal region set forth in a table selected from the group
6 consisting of Table 2, Table 3, and Table 4, and a second circulating cell-free DNA having a

7 sequence falling within a second chromosomal region set forth in said table, wherein said
8 first and second chromosomal regions are different, and wherein the sequences of said first
9 and second cell free DNAs are free of repetitive element, and
10 correlating the presence of or an increased amount of said first and second cell
11 free DNAs with an increased likelihood that said patient has prostate cancer.

1 13. Use of at least one oligonucleotide for the manufacture of a diagnostic
2 kit useful in diagnosing, screening or monitoring prostate cancer, said oligonucleotide having
3 a nucleotide sequence falling within a chromosomal region set forth in Table 2, Table 3,
4 Table 4, or Table 5 (or identical to a part of a sequence set forth in Table A, Table B, Table
5 C, or Table D).

1 14. A system for analyzing circulating cell-free DNA, comprising:
2 a sample analyzer for determining the presence or absence, or the amount of, a
3 circulating cell-free DNA having a nucleotide sequence of at least 25 nucleotides falling
4 within a chromosomal region set forth in a table selected from the group consisting of Table
5 2, Table 3, or Table 4;
6 a computer system for automatically receiving and analyzing data obtained in
7 step (1), and for correlating the presence of, or an increased amount of, said circulating cell-
8 free DNA with a diagnosis of prostate cancer.

1 15. The system of claim 14, further comprising a display module
2 displaying the result of the correlating step.

1 16. A method of detecting a biomarker that is associated with prostate
2 cancer in a patient, the method comprising identifying at least one biomarker that corresponds
3 to a chromosomal region set forth in Table 2, Table 3, Table 4, or Table 5 in circulating
4 nucleic acid in a serum or plasma sample from the patient, wherein the identification of a
5 biomarker from Table 2, Table 3, or Table 4 is indicative of prostate cancer; and the
6 identification of a biomarker from Table 5 is indicative of the absence of prostate cancer.

1 17. The method of claim 16, further comprising detecting a second
2 biomarker that corresponds to a chromosomal region set forth in Table 2, Table 3, Table 4, or
3 Table 5 in circulating nucleic acid in a serum or plasma sample from the patient.

1 18. The method of claim 17, further comprising detecting a third
2 biomarker that corresponds to a chromosomal region set forth in Table 2, Table 3, Table 4, or
3 Table 5 in circulating nucleic acid in a serum or plasma sample from the patient.

1 19. The method of claim 16, 17, or 18, wherein the step of identifying the
2 presence of the at least one biomarker comprises sequencing the CNA from the serum or
3 plasma sample.

1 20. The method of claim 16, wherein the step of identifying the presence
2 of the at least one biomarker comprises contacting at least one probe that is selective for a
3 chromosome region set forth in Table 2, Table 3, Table 4, or Table 5 with a DNA sample
4 obtained from the serum or plasma sample from the patient under conditions in which the
5 probe selectively hybridizes to a target sequence present on the chromosome region; and
6 detecting hybridization of the probe.

1 21. The method of claim 20, wherein the probe is attached to a solid
2 surface.

1 22. The method of claim 20 or 21, further comprising contacting the DNA
2 sample with at least 20, 25, 30, 50, or 52 probes, wherein each probe is selective for a
3 chromosome region set forth in Table 2, Table 3, Table 4, or Table 5.

1 23. A method of detecting prostate cancer in a patient that has, or is
2 suspected of having prostate cancer, the method comprising contacting DNA from the serum
3 or plasma sample with a probe that selectively hybridizes to a sequence set forth in Table A,
4 Table B, Table C, or Table D under conditions in which the probe selectively hybridizes to
5 the sequence; and detecting the presence or absence of hybridization of the probe, wherein
6 the presence of hybridization to a sequence set forth in Table A, Table B, or Table C is
7 indicative of prostate cancer; and the presence of hybridization to a sequence set forth in
8 Table D is indicative of the absence of prostate cancer.

1/1

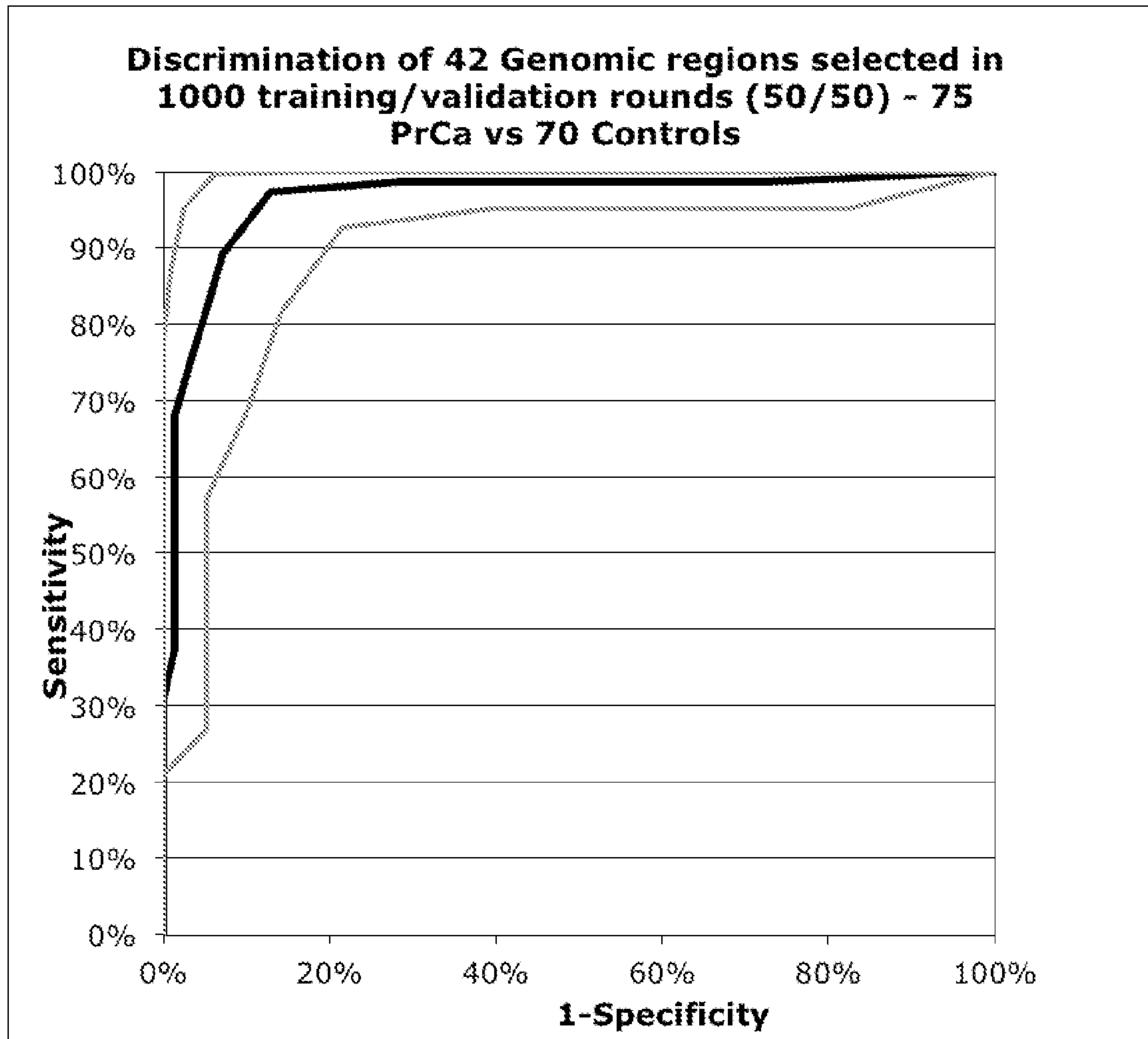
Figure 1

Figure 1

**Discrimination of 42 Genomic regions selected in
1000 training/validation rounds (50/50) - 75
PrCa vs 70 Controls**

