



(86) Date de dépôt PCT/PCT Filing Date: 2005/02/17
(87) Date publication PCT/PCT Publication Date: 2005/09/09
(45) Date de délivrance/Issue Date: 2015/11/24
(85) Entrée phase nationale/National Entry: 2006/08/17
(86) N° demande PCT/PCT Application No.: US 2005/005263
(87) N° publication PCT/PCT Publication No.: 2005/081867
(30) Priorités/Priorities: 2004/02/20 (US60/546,507);
2004/02/21 (US60/546,521)

(51) Cl.Int./Int.Cl. *C12Q 1/68* (2006.01),
C07H 21/04 (2006.01)
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(54) Titre : PROFILAGE DE L'ARNM SALIVAIRE, BIOMARQUEURS ET METHODES ET TROUSSES DE PARTIES
(54) Title: SALIVARY MRNA PROFILING, BIOMARKERS, AND RELATED METHODS AND KITS OF PARTS

(57) **Abrégé/Abstract:**

A method to detect a biomarker in saliva wherein the biomarker is an extracellular mRNA, comprises detecting the extracellular mRNA in the cell-free saliva; transcriptome analysis of saliva comprises detecting a transcriptome pattern in the cell-free saliva; a method to detect genetic alterations in an organ or in a gene in the organ by analyzing saliva, comprises detecting a transcriptome pattern and/or the mRNA profiling of the gene in cell-free saliva; a method to diagnose an oral or systemic pathology disease or disorder in a subject, comprises: detecting profile of a biomarker associated with the pathology disease or disorder, in particular mRNA and/or protein, in cell-free saliva and/or serum; kits comprising identifier for at least one biomarker for performing at least one of the methods; and use of salivary biomarker salivary and/or serum mRNAs as biomarkers for oral and/or systemic pathology, disease or disorder.



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
9 September 2005 (09.09.2005)

PCT

(10) International Publication Number
WO 2005/081867 A3

(51) International Patent Classification:

C12Q 1/68 (2006.01) *C07H 21/04* (2006.01)

(21) International Application Number:

PCT/US2005/005263

(22) International Filing Date:

17 February 2005 (17.02.2005)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

60/546,507 20 February 2004 (20.02.2004) US
60/546,521 21 February 2004 (21.02.2004) US

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(81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

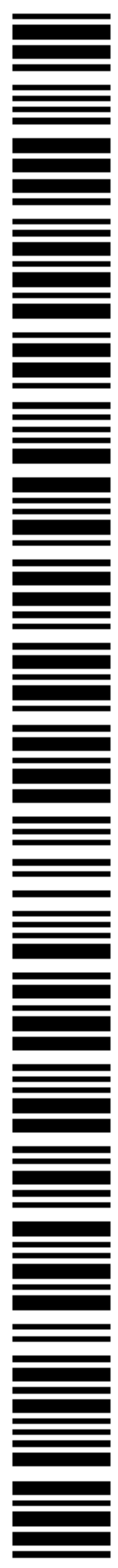
Published:— *with international search report*

(88) **Date of publication of the international search report:**
6 July 2006

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** SALIVARY MRNA PROFILING, BIOMARKERS, AND RELATED METHODS AND KITS OF PARTS

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DEMANDES OU BREVETS VOLUMINEUX

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JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
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THIS IS VOLUME __1__ OF __2__

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

SALIVARY MRNA PROFILING, BIOMARKERS AND RELATED METHODS AND KITS OF PARTS

[0001] This invention was made with Government support of grant U01-
5 DE15018 awarded by the NIH. The Government has certain rights on this
invention

FIELD OF THE DISCLOSURE

[0002] The present disclosure relates to profiling of biomarkers and to
method and kits using said biomarkers. In particular, the present disclosure
10 related to biomarkers for detection of cancer and in particular of Oral Cavity
and Oropharyngeal squamous Cell Carcinoma (OSCC).

BACKGROUND OF THE DISCLOSURE

[0003] Biomarkers are molecular indicators of a specific biological property,
a biochemical feature or facet that can be used to measure the progress of
15 disease or the effects of treatment.

[0004] Proteins and nucleic acids are exemplary biomarkers. In particular, it
has been widely accepted that genomic messengers detected extracellularly
can serve as biomarkers for diseases [6]. In particular, nucleic acids have
been identified in most bodily fluids including blood, urine and cerebrospinal
20 fluid, and have been successfully adopted for using as diagnostic biomarkers
for diseases [28, 42, 49].

[0005] Saliva is not a passive "ultrafiltrate" of serum [41], but contains a
distinctive composition of enzymes, hormones, antibodies, and other
molecules. In the past 10 years, the use of saliva as a diagnostic fluid has
25 been successfully applied in diagnostics and predicting populations at risk for
a variety of conditions [47].

[0006] Specific and informative biomarkers in saliva are desirable to serve
for diagnosing disease and monitoring human health [30, 47, 6]. For example
biomarkers have been identified in saliva for monitoring caries, periodontitis,
30 oral cancer, salivary gland diseases, and systemic disorders, e.g., hepatitis

and HIV [35]. Also previous studies show that human DNA biomarkers can be identified in saliva and used for oral cancer detection [30, 36]. RNA is more labile than DNA and is presumed to be highly susceptible to degradation by RNases. Furthermore, RNase activity, is reported to be elevated in saliva, which constitutes an inexpensive, non-invasive and accessible bodily fluid suitable to act as an ideal diagnostic medium. In particular, RNAase activity is reported to be elevated in saliva of cancer patients [83]. It has, thus, been commonly presumed that human mRNA could not survive extracellularly in saliva. OSCC is the sixth most common cancer in the world, and affects 50,000 Americans annually. Worldwide, cancers of the oral cavity and oropharynx represent a great public health problem. OSCC accounts for nearly 50% of all newly diagnosed cancers in India and is a leading cause of death in France [1].

[0007] Despite improvements in locoregional control, morbidity and mortality rates have improved little in the past 30 years [2]. Therefore, early detection or prevention of this disease is likely to be most effective. Detecting OSCC at an early stage is believed to be the most effective means to reduce death and disfigurement from this disease. The absence of definite early warning signs for most head and neck cancers suggests that sensitive and specific biomarkers are likely to be important in screening high risk patients.

SUMMARY OF THE DISCLOSURE

[0008] According to a first aspect, a method to detect a biomarker in a bodily fluid including a cell phase and a fluid phase, wherein the biomarker is an extracellular mRNA and bodily fluid is saliva, preferably unstimulated saliva, is disclosed. The method comprises: providing a cell-free fluid phase portion of the bodily fluid; and detecting the extracellular mRNA in the cell-free fluid phase portion of the bodily fluid.

[0009] In particular, detecting the extracellular mRNA can comprise: isolating the extracellular mRNA from the cell-free fluid phase portion of the bodily fluid, and amplifying the extracellular mRNA.

[0010] According to a second aspect, transcriptome analysis of a bodily fluid, including a cell phase and a fluid phase, wherein the bodily fluid is saliva, is disclosed. The method comprises: providing a cell-free fluid phase portion of the bodily fluid; and detecting a transcriptome pattern in the cell-free fluid phase portion of the bodily fluid. The bodily fluid is preferably unstimulated saliva.

[0011] In particular, detecting transcriptome pattern in the saliva supernatant is preferably performed by microarray assay, most preferably by high-density oligonucleotide microarray assay. Detecting transcriptome pattern in the saliva supernatant can also performed by quantitative PCR analysis or RT-PCR analysis.

[0012] According to a third aspect, a method to detect genetic alterations in an organ by analyzing a bodily fluid draining from the organ and including a cell phase and a fluid phase, is disclosed. The bodily fluid is in particular saliva, preferably unstimulated saliva and method comprises: providing cell-free fluid phase portion of the bodily fluid; detecting a transcriptome pattern in the cell-free fluid phase portion of the bodily fluid; and comparing the transcriptome pattern with a predetermined pattern, the predetermined pattern being indicative of a common transcriptome pattern of normal cell-free fluid phase portion of the bodily fluid.

[0013] According to a fourth aspect, a method to detect genetic alteration of a gene in an organ by analyzing a bodily fluid draining from the organ and including a cell phase and a fluid phase, is disclosed. The bodily fluid is in particular saliva and the method comprises: providing a cell-free fluid phase portion of the bodily fluid; detecting an mRNA profile of the gene in the cell-free fluid phase portion of the bodily fluid; and comparing the mRNA profile of the gene with a predetermined mRNA profile of the gene, the predetermined mRNA profile of the gene being indicative of the mRNA profile of the gene in normal cell-free fluid phase portion of the bodily fluid,.

[0014] According to a fifth aspect, a method to diagnose an oral or systemic pathology disease or disorder in a subject, is disclosed. The method

comprises: providing a cell-free fluid phase portion of the saliva of the subject, detecting in the provided cell-free saliva fluid phase portion an mRNA profile of a gene associated with the pathology, disease or disorder; and comparing the RNA profile of the gene with a predetermined mRNA profile of the gene,
 5 the predetermined mRNA profile of the gene being indicative of the presence of the pathology, disease, or disorder in the subject.

[0015] In a first embodiment the pathology, disease or disorder is a cancer of the oral cavity and/or of oropharynx, the bodily fluid is saliva and the gene is selected from the group consisting of the gene coding for IL8 (Interleukin 8),
 10 IL1B (Interleukin 1, beta), DUSP1 (Dual specificity phosphatase 1), H3F3A (H3 histone, family 3A), OAZ1 (Ornithine decarboxylase antizyme 1), S100P (S100 calcium binding protein P) and SAT (Spermidine/spermine N1-acetyltransferase).

[0016] In a second embodiment, the pathology, disease or disorder is a
 15 cancer of the oral cavity and/or of oropharynx, the bodily fluid is blood serum and the gene is selected from IL6 (interleukin 6), H3F3A, TPT1 (Tumor protein translationally controlled 1), FTH1 (Ferritin heavy polypeptide 1), NCOA4 (Nuclear receptor coactivator 4) and ARCR (Ras homolog gene family, member A).

20 **[0017]** Diseases that can be diagnosed include oropharyngeal squamous cell carcinoma and possibly other systemic diseases.

[0018] According to a sixth aspect, a method to diagnose an oral or systemic pathology, disease or disorder in a subject is disclosed. The method comprises: providing a cell-free fluid phase portion of the saliva of the subject;
 25 detecting in the provided cell-free fluid phase portion a transcriptome pattern associated with the pathology, disease or disorder; and comparing the transcriptome pattern with a predetermined pattern, recognition in the transcriptome pattern of characteristics of the predetermined pattern being diagnostic for the pathology, disease or disorder in the subject.

[0019] In an embodiment, the pathology, disease or disorder is a cancer of the oral cavity and/or of oropharynx, and transcriptome include transcript is selected from the group consisting of transcripts for IL8, IL1B, DUSP1, H3F3A, OAZ1, S100P, SAT from saliva.

5 [0020] According to a seventh aspect, a method to diagnose an oral or systemic pathology, disease or disorder in a subject is disclosed, the method comprising: providing serum of the subject; detecting in the provided serum a transcriptome pattern associated with the pathology, disease or disorder; and
10 comparing the transcriptome pattern with a predetermined pattern, recognition in the transcriptome pattern of characteristics of the predetermined pattern being diagnostic for the pathology, disease or disorder in the subject.

[0021] In an embodiment, the pathology, disease or disorder is a cancer of the oral cavity and/or of oropharynx, and transcriptome include transcript is selected from the group consisting of transcripts for IL6, H3F3A, TPT1, FTH1,
15 NCOA4 and ARCR from serum.

[0022] Diseases that can be diagnosed include oropharyngeal squamous cell carcinoma possibly other systemic diseases.

[0023] According to a eight aspect, a method for diagnosing a cancer, in a subject is disclosed. The method comprises: providing a bodily fluid of the
20 subject; detecting in the bodily fluid a profile of a biomarker, comparing the profile of the biomarker with a predetermined profile of the biomarker, recognition in the profile of the biomarker of characteristics of the predetermined profile of the biomarker being diagnostic for the cancer.

[0024] Pathologies, diseases or disorders that can be diagnosed include
25 oropharyngeal squamous cell carcinoma and possibly other systemic diseases. Biomarkers include IL8, IL1B, DUSP1, H3F3A, OAZ1, S100P, SAT, IL6, H3F3A, TPT1, FTH1, NCOA4 and ARCR.

[0025] In a first embodiment, the pathology, disease or disorder is oropharyngeal squamous cell carcinoma, the biomarker is selected from the
30 group consisting of IL8 IL1B, DUSP1, H3F3A, OAZ1, S100P, SAT, the bodily

fluid is saliva and detecting a profile of a biomarker is performed by detecting the mRNA profile of the biomarker.

[0026] In a second embodiment, the pathology, disease or disorder is oropharyngeal squamous cell carcinoma, the biomarker is selected from the group consisting of IL6, H3F3A, TPT1, FTH1, NCOA4 and ARCR the bodily fluid is serum and detecting a profile of a biomarker is performed by detecting the mRNA profile of the biomarker.

[0027] In a third embodiment, the pathology, disease or disorder is oropharyngeal squamous cell carcinoma, the biomarker is IL6, the bodily fluid is blood serum and detecting a profile of a biomarker is performed by detecting the protein profile of the biomarker

[0028] According to an eighth aspect, a kit for the diagnosis of an oral and/or systemic pathology, disease or disorder is disclosed, the kit comprising: an identifier of at least one biomarker in a bodily fluid, the biomarker selected from the group consisting of IL8, IL1B, DUSP1, H3F3A, OAZ1, S100P, SAT, IL6, H3F3A, TPT1, FTH1, NCOA4 and ARCR; and a detector for the identifier.

[0029] Pathologies, diseases or disorders that can be diagnosed include oropharyngeal squamous cell carcinoma, and possibly the other systemic diseases.

[0030] The identifier and the detector are to be used in detecting the bodily fluid profile of the biomarker according to the methods herein disclosed. In particular, the identifier is associated to the biomarker in the bodily fluid, and the detector is used to detect the identifier, the identifier and the detector thereby enables the detection of the bodily fluid profile of the biomarker.

[0031] According to a ninth aspect, a method to diagnose an oral and/or systemic pathology disease or disorder, is disclosed. The method comprising: using salivary and/or serum mRNAs as biomarkers for oral and/or systemic pathology, disease or disorder.

[0032] In a preferred embodiment the mRNA codifies for at least one of the biomarker selected from the group consisting of IL8, IL1B, DUSP1, H3F3A, OAZ1, S100P, SAT, IL6, H3F3A, TPT1, FTH1, NCOA4 and ARCR.

[0033] Diseases that can be diagnosed include oropharyngeal squamous cell carcinoma, and possibly other systemic diseases.

[0034] According to a tenth aspect, a method to diagnose an oral and/or system pathology, is disclosed. The method comprising: using salivary or serum proteins as biomarkers for oral and/or systemic pathology, disease or disorder, in particular IL6 protein in serum and IL8 protein in saliva.

[0035] The methods and kits of the disclosure will be exemplified with the aid of the enclosed figures.

[0035A] Various embodiments of the invention provide a method to detect mRNA in saliva, the saliva including a cell phase and a cell-free supernatant, the method comprising: providing a saliva supernatant; isolating mRNA from the saliva supernatant; and amplifying mRNA thereby detecting mRNA in the saliva supernatant.

[0035B] Various embodiments of the invention provide a method to diagnose oropharyngeal squamous cell carcinoma (OSCC) in a test subject, the method comprising: providing a saliva supernatant from the test subject, detecting in the saliva supernatant an mRNA profile of a gene; comparing the mRNA profile of the gene with a predetermined mRNA profile of a gene, wherein the predetermined mRNA profile is an OSCC mRNA profile, and wherein the gene is selected from the group consisting of the gene coding for Interleukin 8 (IL8), Interleukin-1 beta (IL-1 β), Dual specificity phosphatase 1 (DUSP1), Histone H3, family 3A (H3F3A), Ornithine decarboxylase antizyme 1 (OAZ 1), S100 calcium binding protein P (S 100P) and Spermidine/spermine N 1-acetyltransferase (SAT), wherein the expression of any one of these genes is elevated in a subject with OSCC relative to a control and wherein a statistically significant correlation between the mRNA profile and the predetermined mRNA profile is indicative of the presence of OSCC.

[0035C] Various embodiments of the invention provide a kit for the diagnosis of oropharyngeal squamous cell carcinoma (OSCC), the kit comprising: a

biomarker control profile, wherein the control profile is derived from a subject with OSCC and at least one binding reagent, wherein the binding reagent is a polynucleotide and the binding reagent binds a biomarker, wherein the biomarker is selected from the group consisting of: Interleukin 8 (IL8), Interleukin-1 beta (IL1 B), Dual specificity phosphatase 1 (DUSP1), Histone H3, family 3A (H3F3A), Ornithine decarboxylase antizyme I (OAZ I), S100 calcium binding protein P (S100P) and Spermidine/spermine N1-acetyltransferase (SAT), and a detector for the binding reagent.

[0035D] Various embodiments of the invention provide a method to diagnose oropharyngeal squamous cell carcinoma (OSCC) in a test subject, the method comprising: providing a saliva supernatant from the test subject; detecting in the provided saliva supernatant a transcriptome pattern, comparing the transcriptome pattern with a predetermined pattern, wherein the predetermined pattern is an mRNA pattern detected in subjects with OSCC, wherein the predetermined pattern comprises a plurality of mRNA profiles, wherein the plurality of mRNA profiles comprises a mRNA profile of each of Interleukin 8 (IL8), Interleukin-1 beta (IL 1B), Ornithine decarboxylase antizyme I (OAZ I), and Spermidine/spermine N1-acetyltransferase (SAT), wherein the expression of these genes is elevated in a subject with OSCC relative to a control and wherein a statistically significant correlation between the transcriptome pattern and the predetermined pattern is indicative of the presence of OSCC.

DESCRIPTION OF THE FIGURES

[0036] Figure 1A shows results of a RT-PCR typing for ACTB performed on RNA isolated from cell-free saliva supernatant from human beings after storage for 1 month (lane 2), 3 months (lane 3) and 6 months (lane 4), with a 100bp ladder molecular weight marker (lane 1) and a negative control (omitting templates) (lane 5). A molecular size marker is indicated on the left side of the Figure by arrows.

[0037] Figure 1B shows results of a RT-PCR performed on RNA isolated from cell-free saliva supernatant from human beings (lane 1) and typing GAPDH (B1), RPS9 (B2) and ACTB (B3), with positive control (human total RNA, BD Biosciences Clontech, Palo Alto,

CA, USA) (lane 2) and negative controls (omitting templates) (lane 3). A molecular size marker is indicated on the left side of the Figure by arrows.

[0038] Figure 2A shows results of a capillary electrophoresis performed to monitor RNA amplification from RNA isolated from cell-free saliva supernatant from human beings. Lanes 1 to 5 show 1kb DNA ladder (lane 1), 5µl saliva after RNA isolation (undetectable) (lane 2), 1 µl two round amplified cRNA (range from 200 bp to ~4kb) (lane 3), 1 µl cRNA after fragmentation (around

100bp) (lane 4) and Ambion RNA Century Marker (lane 5). A molecular size marker is indicated on the left side and right side of the Figure by arrows.

[0039] Figure 2B shows results of a PCR performed on RNA isolated from cell-free saliva supernatant from human beings at various stage of amplification and typing for ACTB. Lane 1 to 8 shows 100bp DNA ladder (lane 1), total RNA isolated from cell-free saliva (lane 2), 1st round cDNA (lane 3), 1st round cRNA after RT (lane 4), 2nd round cDNA (lane 5), 2nd round cRNA after RT (lane 6), positive control (human total RNA, BD Biosciences Clontech, Palo Alto, CA, USA) (lane 7) and negative control (omitting templates) (lane 8). A molecular size marker is indicated on the left side of the Figure by arrows.

[0040] Figure 2C shows a diagram reporting results of the analysis of target cRNA performed by Agilent 2100 bioanalyzer before hybridization on microarray. On x axis, the molecular weight (bp) of the fragmented cRNA with reference to the marker RNA, is indicated. On y axis, the quantity of the fragmented cRNA (ug/ml) measurable by a Bioanalyzer, is indicated.

[0041] Figure 3 shows results of a RT-PCR performed on RNA isolated from cell-free saliva supernatant from human beings (saliva) together with a ladder (Mrkr) positive controls (Ctrl(+)) and negative controls (Ctrl(-)) and typing for IL6 (IL6), IL8 (IL8) and β -Actin (β -Actin).

[0042] Figure 4 shows results of a PCR performed for the housekeeping β -actin on whole saliva, serum samples, and samples that had been centrifuged at 0 $\times$ g (0 $\times$ g), 1,000 $\times$ g (1,000 $\times$ g), 2,600 $\times$ g (2,600 $\times$ g), 5,000 $\times$ g (5,000 $\times$ g) and 10,000 $\times$ g (10,000 $\times$ g) using genomic DNA as marker (Mrkr) for cell lysis and spillage of intracellular compounds.

[0043] Figure 5A shows a diagram reporting the mean concentrations of mRNA for IL8 detected in replicate samples by qRT-PCR in saliva from patients with OSCC (Cancer) and normal subjects (Control). On x axis the sample groups are reported. On y axis the number of copies detected is reported .

- [0044] Figure 5B shows a diagram reporting the mean concentrations of IL8 detected in replicate samples by ELISA in saliva from patients with OSCC (Cancer) and normal subjects (Control). On x axis the sample groups are reported. On y axis the concentration expressed in pg/ml, is reported.
- 5 [0045] Figure 6A shows a diagram reporting the mean concentrations of mRNA for IL6 detected in replicate samples by qRT-PCR in serum from patients with OSCC (Cancer) and normal subjects (Control). On x axis the sample groups are reported. On y axis the number of copies detected is reported.
- 10 [0046] Figure 6B shows a diagram reporting the mean concentrations of IL6 detected in replicate samples by ELISA in serum from patients with OSCC (Cancer) and normal subjects (Control). On x axis, the sample groups are reported. On y axis the concentration expressed in pg/ml, is reported
- 15 [0047] Figure 7A shows a diagram reporting the Receiver Operating Characteristic (ROC) curve calculated for IL8 in Saliva. On the x axis 1-specificity is reported. On y axis the sensitivity is reported.
- [0048] Figure 7B shows a diagram reporting the ROC curve calculated for IL6 in serum. On the x axis 1-specificity is reported. On y axis the sensitivity is reported.
- 20 [0049] Figure 7C shows a diagram reporting the ROC curve calculated for a combination of IL8 in saliva and IL6 in serum. On the x axis 1-specificity is reported. On y axis the sensitivity is reported.
- 25 [0050] Figure 8 shows results of a PCR reaction performed on serum human mRNA phenotyping of salivary mRNAs for RPS9 (Lane 2, 3 and 4); GAPDH (Lane 5, 6 and 7); B2M (Lane 8, 9 and 10) and ACTB (Lane 11, 12 and 13), together with DNA ladder, as a control (Lane 1).
- [0051] Figure 9 shows a diagram reporting a ROC curve of the logistic regression model for the circulating mRNA in serum. On the x axis 1-specificity is reported. On y axis the sensitivity is reported.

[0052] Figure 10 shows a diagram reporting the classification and regression trees (CART) model assessing the serum mRNA predictors for OSCC.

5 [0053] Figure 11 shows a diagram reporting a ROC curve of the logistic regression model for the predictive power of combined salivary mRNA biomarkers. On the x axis 1-specificity is reported. On y axis the sensitivity is reported.

10 [0054] Figure 12 shows a diagram reporting the classification and regression trees (CART) model assessing the salivary mRNA predictors for OSCC.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

15 [0055] A method to detect an extracellular mRNA in a bodily fluid, is disclosed wherein the bodily fluid is saliva and the extracellular mRNA is detected in a cell-free fluid phase portion of saliva. Presence of RNAs in the cell-free fluid phase portion of saliva was confirmed by the procedures extensively described in the Examples, the quality of the detected mRNA meeting the demand for techniques such as PCR, qPCR, and microarray assays.

20 [0056] In the method, detecting extracellular mRNAs herein also informative mRNAs, is performed in a bodily fluid, saliva, that meets the demands of an inexpensive, non-invasive and accessible bodily fluid to act as an ideal medium for investigative analysis.

25 [0057] Detecting informative mRNAs is in particular performed in a portion of saliva (cell-free fluid phase) wherein presence of microorganisms and the extraneous substances such as food debris is minimized, which allows analyzing the molecules in simple and accurate fashion. Preferably, the cell-free fluid phase portion of derived from unstimulated saliva.

30 [0058] In the method, the saliva can be collected according to procedures known in the art and then processed to derive the cell-free fluid phase thereof, for example by centrifugation of the collected saliva, which results in a

pelleted saliva cell phase and a cell-free saliva fluid phase supernatant. (see procedures extensively described in Examples 1, 5 and 13)

5 [0059] According to the present disclosure, the conditions for separating the cell-phase and the fluid phase of saliva are optimized to avoid mechanical rupture of cellular elements which would contribute to the RNA detected in the fluid cell-free phase.

[0060] In embodiments wherein the separation is performed by centrifugation, optimization can be performed by testing housekeeping genes on samples centrifuged at various speed and on whole saliva samples, using
10 DNA as a marker of cell lysis and spillage, to derive the optimized centrifugation speed. (See procedure described in Example 5).

[0061] Detection of the extracellular mRNA in the cell-free saliva fluid phase portion (salivary mRNA) can then be performed by techniques known in the art allowing mRNA qualitative and/or a quantitative analysis, such as RT-
15 PCR, Q-PCR and Microarray. The detection can in particular be performed according to procedures that can include isolation and an amplification of the salivary mRNA and that are exemplified in the Examples.

[0062] Detection of the salivary mRNA in the method can be performed for the purpose of profiling the salivary mRNA.

20 [0063] In a first series of embodiments, the expression of predetermined genes, can be profiled in a cell-free fluid phase portion of saliva. In those embodiments, detection of the mRNA profile can be performed by RT-PCR or any techniques allowing identification of a predetermined target mRNA. Quantitative analysis can then be performed with techniques such as
25 Quantitative PCR (Q-PCR) to confirm the presence of mRNA identified by the RT-PCR. A reference database can then be generated based on the mRNA profiles so obtained. Exemplary procedures to perform such qualitative and quantitative analyses of salivary mRNA are described in details in Examples 1, 4 and 9.

[0064] In a second series of embodiments, a transcriptome analysis of saliva can be performed by detecting a transcriptome pattern in the cell-free fluid phase portion of saliva. Detection of the transcriptome pattern can be performed by isolating and linearly amplifying salivary mRNA, which can then
5 be profiled with techniques such as high-density oligonucleotide microarrays. Quantitative analysis can then be performed with techniques such as Q-PCR to confirm the presence of mRNA in the pattern identified by the microarray. A reference database can then be generated based on the mRNA profiles so obtained. Exemplary procedures to perform such qualitative and quantitative
10 analyses of salivary mRNA are described in details in Examples 2-3, 9-10 and 14-15.

[0065] Profiling salivary RNA can be performed to detect and/or monitor human health and disease or to investigate biological questions, such as for example, the origin, release and clearance of mRNA in saliva. The salivary
15 mRNA provides actual or potential biomarkers to identify populations and patients at high risk for oral and systemic pathologies, diseases or disorders.

[0066] Alterations of the salivary mRNA profiles and transcriptome patterns characterizing the cell-free fluid phase portion of saliva or normal subjects can be indicative of pathologies, diseases or disorders of various origin. Examples
20 of those pathologies, diseases or disorders are provided by the inflammatory conditions of the oral cavity, OSCC or other conditions such as diabetes, breast cancer and HIV.

[0067] Also comparison between the mRNA profiles and transcriptome patterns of subject affected with a determined pathology, disease or disorder,
25 can result in the identification of informative biomarkers for the determined pathology disease or disorder. In particular, salivary mRNA can be used as diagnostic biomarkers for oral and systemic pathologies, diseases or disorders that may be manifested in the oral cavity.

[0068] In particular, salivary mRNA can be used as diagnostic biomarkers
30 for cancer that may be manifested and/or affect the oral cavity. Saliva-based

mRNA assays have the needed specificity and sensitivity for reliable diagnostics.

[0069] In case of various forms of cancer, alterations of the normal salivary mRNA and transcriptome patterns can also reflect the genetic alterations in one or more portions of the oral cavity which are associated with presence of the tumor. For oral cancer patients, the detected cancer-associated RNA signature is likely to originate from the matched tumor and/or a systemic response (local or distal) that further reflects itself in the whole saliva coming from each of the three major sources (salivary glands, gingival crevicular fluid, and oral mucosal cells). It is conceivable that disease-associated RNA can find its way into the oral cavity via the salivary gland or circulation through the gingival crevicular fluid. A good example is the elevated presence of HER-2 proteins in saliva of breast cancer patients [87].

[0070] A common transcriptome of normal cell-free saliva, including approximately 185 different human mRNAs, also defined as Normal Salivary Core Transcriptome (NSCT) was identified in outcome of a transcriptome analysis performed on cell-free fluid phase of saliva from normal subject (see Example 2, Table 2).

[0071] Since the NSCT was identified using the probe sets on HG U133A microarray representing only ~19,000 human genes, and the human genome composed of more than 30,000 genes [48], it is expected that more human mRNAs will be identified in saliva by other methodologies and additional salivary patterns are identifiable by the method herein disclosed.

[0072] The NSCT and/or other salivary transcriptome patterns in cell-free saliva from normal populations can serve in a Salivary Transcriptome Diagnostics (SlvTD), for potential applications in disease diagnostics as well as normal health surveillance.

[0073] Accordingly, in a first embodiment of the SlvTD, a method to diagnose an oral or systemic pathology disease or disorder in a subject, is disclosed. The method comprises: providing a cell-free fluid phase portion of

the saliva of the subject; detecting in the provided cell-free saliva fluid phase portion an mRNA profile of a gene associated with the disease; and comparing the RNA profile of the gene with a predetermined mRNA profile of the gene, the predetermined mRNA profile of the gene being indicative of the presence of the disease in the subject.

[0074] In a second embodiment of the SlvTD, a method to diagnose an oral or systemic pathology disease or disorder in a subject, is disclosed. The method comprises: providing cell-free saliva supernatant of the subject; detecting in the cell-free saliva supernatant a transcriptome pattern associated with the pathology disease or disorder; and comparing the transcriptome pattern with a predetermined pattern, recognition in the transcriptome pattern of characteristics of the predetermined pattern being diagnostic for the pathology disease or disorder in the subject.

[0075] In a third embodiment of the SlvTD, a method to identify a biomarker associated with a predetermined pathology disease or disorder is disclosed. The method comprises: detecting a first mRNA profiling of a predetermined gene in cell-free fluid phase portion of saliva of a subject affected by the pathology disease or disorder; detecting a second mRNA profiling of the predetermined gene in cell-free fluid phase portion of saliva of a normal subject; comparing the first mRNA profiling with the second mRNA profiling, recognition of differences between the first mRNA profiling and the second mRNA profiling, the differences validated by statistical analysis, being indicative of the identification of the predetermined gene as a biomarker for the predetermined pathology disease or disorder.

[0076] In particular the difference between the RNA profiling from one disease category to one healthy category is analyzed by microarray statistical methodologies. The algorithms used include MAS 5.0, DNA-Chip analyzer 1.3 and RMA 3.0. Preferably, the analysis is performed by a combination of these methods to provide more powerful and accurate markers to test. The markers identified by microarray will then be tested by conventional techniques such as Q-PCR.

[0077] In a fourth embodiment of the SlvTD a diagnostic method can be performed, wherein the cell-free saliva is contacted with an identifier for the presence or expression of the biomarker, and the presence of the identifier associated to presence or expression of the biomarker is detected, preferably
5 by means of a detector.

[0078] The SlvTD allow detection of diseases such as tumors at a stage early enough that treatment is likely to be successful, with screening tools exhibiting the combined features of high sensitivity and high specificity. Moreover, the screening tool are sufficiently noninvasive and inexpensive to
10 allow widespread applicability.

[0079] The results of the above methods of the SlvTD can be integrated with a corresponding analysis performed at an mRNA and/or protein level and/or in other bodily fluid, such as blood serum.

[0080] Biomarkers, such as protein or transcriptome patterns detected in
15 serum can also serve in a Serum Transcriptome Diagnostics (SrmTD), for potential applications in disease diagnostics as well as normal health surveillance. Embodiments of the SrmTD include methods corresponding to the ones reported above for the SlvTD, wherein the bodily fluid analyzed is serum instead of cell-free saliva.

20 [0081] In particular, the results obtained following the SlvTD can be combined with results obtained with the SrmTD, in a combined Salivary and Serum Transicriptome approach (SSTD).

[0082] According to the SSTD a diagnostic method can be performed, wherein the bodily fluid, serum and/or saliva is contacted with an identifier for
25 the presence or expression of the biomarker, wherein the biomarker can be a protein or an mRNA and the presence of the identifier associated to presence or expression of the biomarker is detected, preferably by means of a detector.

[0083] Examples of the SlvTD, SrmTD and SSTD are herein provided with reference to the OSCC. The person skilled in the art can derive the

appropriate modifications of the SID herein exemplified for diseases different than OSCC upon reading of the present disclosure.

[0084] Profiling of two specific cytokines, IL6 and IL8, was measured in the cell-free fluid phase portion of saliva and serum of patients with OSCC according to procedures extensively disclosed in Examples 4-8. IL8 was detected at higher concentrations in the saliva of patients with OSCC ($P < 0.01$) and IL6 was detected at higher concentrations in the serum of patients with OSCC ($P < 0.01$). These results were confirmed at both the mRNA and the protein levels, and the results were concordant. The concentration of IL8 in saliva and IL6 in serum did not appear to be associated with gender, age, or alcohol or tobacco use ($P > 0.75$). The data were subjected to statistical analysis, in particular to ROC analysis, and were able to determine the threshold value, sensitivity, and specificity of each biomarker for detecting OSCC (see Example 8, Table 3). Furthermore, the inventors were able to measure mRNA in salivary specimens.

[0085] A transcriptome analysis of unstimulated saliva collected from patients with OSCC and normal subjects was performed as disclosed in Examples 9-12 and in Examples 13-16.

[0086] RNA isolation was performed from the saliva supernatant, followed by two-round linear amplification with T7 RNA polymerase. Human Genome U133A microarrays were applied for profiling human salivary transcriptome. The different gene expression patterns were analyzed by combining a t test comparison and a fold-change analysis on 10 matched cancer patients and controls. Quantitative polymerase chain reaction (qPCR) was used to validate the selected genes that showed significant difference ($P < 0.01$) by microarray. The predictive power of these salivary mRNA biomarkers was analyzed by receiver operating characteristic curve and classification models.

[0087] The results of a first set of microarray analysis showed that there are 1,679 genes exhibited significantly different expression level in saliva between cancer patients and controls ($P < 0.05$). Seven cancer-related mRNA biomarkers that exhibited at least a 3.5-fold elevation in OSCC saliva ($P <$

U.01) were consistently validated by qPCR on saliva samples from OSCC patients ($n = 32$) and controls ($n = 32$). These salivary RNA biomarkers are transcripts of IL8, IL1B, DUSP1, H3F3A, OAZ1, S100P, and SAT. The combinations of these biomarkers yielded sensitivity (91%) and specificity (91%) in distinguishing OSCC from the controls. (see Examples 13-16)

[0088] The results of a second set of microarray analysis showed five of ten up-regulated genes selected based on their reported cancer-association, showed significantly elevated transcripts in serum of OSCC patient. These RNA biomarkers are transcripts of H3F3A, TPT1, FTH1, NCOA4 and ARCR. The results validated by qPCR confirmed that transcripts of these five genes were significantly elevated in the serum of OSCC patient (Wilcoxon Signed Rank test, $P < 0.05$). (See Examples 9 to 12)

[0089] Using the described collection and processing protocols, the presence of ACTB, B2W, GAPDH and RPS9 mRNAs (controls mRNA) were confirmed in all serum (patients and controls) by RT-PCR.

[0090] Accordingly, a method for diagnosing a cancer, in particular OSCC in a subject, is disclosed. The method comprises: providing a bodily fluids of the subject; detecting in the bodily fluid a profile of a biomarker, the biomarker selected from the group consisting of IL8 IL1B, DUSP1, H3F3A, OAZ1, S100P, SAT, IL6, H3F3A, TPT1, FTH1, NCOA4 and ARCR, comparing the profile of the biomarker with a predetermined profile of the biomarker, recognition in the profile of the biomarker of characteristics of the predetermined profile of the biomarker being diagnostic for the cancer.

[0091] Also method to diagnose oral and/or systemic pathology, disease or disorder, in particular OSCC, is disclosed. The method comprises using salivary mRNAs as biomarkers for oral and/or systemic diseases, in particular salivary mRNAs of selected from the group consisting of IL8 IL1B, DUSP1, H3F3A, OAZ1, S100P and SAT.

[0092] Additionally a method to diagnose oral and/or systemic pathology, disease or disorder, in particular OSCC, is disclosed. The method comprises:

using serum mRNAs and/or protein as biomarkers for oral and/or systemic diseases, in particular serum mRNAs of selected from the group consisting of IL6, H3F3A, TPT1, FTH1, NCOA4 and ARCR, and serum IL6 protein.

[0093] Given the multifactorial nature of oncogenesis and the heterogeneity
5 in oncogenic pathways use of combinations of salivary and/or serum
biomarkers, ensuring higher specificity and sensitivity, to detect the disease,
is preferred. Multiple statistical strategies reported and risk models described
in the examples can be used to identify combinations of biomarkers that can
identify OSCC patients samples and to facilitate assigning the appropriate
10 serum transcriptome-based diagnosis for patients' specific cancer risk.

[0094] Monitoring of profile of salivary mRNA in cell-free fluid phase portion
of saliva and/or in other bodily fluid such as blood serum, can be used in the
postoperative management of OSCC patients. It could potentially be used for
monitoring the efficacy of treatment, or disease recurrence after therapy has
15 concluded. Salivary mRNAs and in particular IL8 may also serve as
prognostic indicators to direct the treatment of patients with oral cavity cancer.
In perspective, high-risk patients can be directed to more aggressive or
adjuvant treatment regimens.

[0095] The use of these biomarkers may also improve the staging of the
20 tumor. With traditional techniques, the presence of microscopic distant
disease is often under recognized. In recent years, there has been a shift
from locoregional failure to distant failure for patients treated for presumed
locoregional disease.[18] This in part is a reflection of subclinical distant
disease present prior to the initiation of therapy. Testing for the presence of
25 biomarkers may allow the detection of small amounts of tumor cells in a
background of normal tissue. Salivary mRNAs as biomarkers specific for
head and neck tumors or a panel of such biomarkers may allow the detection
of distant microscopic disease. For oral cancer, one of the most important
applications of the STD approach in this respect is to detect the cancer
30 conversion of oral premalignant lesions.

[0096] Profiling of salivary mRNAs can also be used to investigate the role of genes in the development of cancer, in particular whether the aberrant expressions of these genes functionally contribute to the development of human OSCC. The biological significance of differential expression of these genes in head and neck/oral cancer should be determined. Identification of cancer-associated genes that are consistently changed in cancer patients will provide us not only with diagnostic markers but also with insights about molecular profiles involved in head and neck cancer development. Understanding the profile of molecular changes in any particular cancer will be extremely useful because it will become possible to correlate the resulting phenotype of that cancer with molecular events.

[0097] Kits of parts associated with the methods herein disclosed are also disclosed. In an exemplary embodiment, a kit comprises: a identifier of a biomarker in a bodily fluid, such as a salivary mRNA or protein, and serum mRNA or protein, the biomarker selected from the group consisting of IL8, IL1B, DUSP1, H3F3A, OAZ1, S100P, SAT, IL6, H3F3A, TPT1, FTH1, NCOA4 and ARCR; and a detector for the identifier, the identifier and the detector to be used in detecting the bodily fluid profile of the biomarker of one the methods herein disclosed, wherein the identifier is associated to the biomarker in the bodily fluid, and the detector is used to detect the identifier, the identifier and the detector thereby enabling the detection of the bodily fluid profile of the biomarker.

[0098] The bodily fluid can be saliva, with the detection performed in the cell-free fluid phase portion thereof, or another bodily fluid such as blood serum.

[0099] The identifier and the detector able to detect the identifier, are identifiable by a person skilled in the art. Other compositions and/or components that may be suitably included in the kit and are also identifiable by a person skilled in the art.

[00100] The identifier and the reagent can be included in one or more compositions where the identifier and/or the reagent are included with a suitable vehicle, carrier or auxiliary agent.

5 [00101] In the diagnostic kits herein disclosed, the agents and identifier reagents can be provided in the kits, with suitable instructions and other necessary reagents, in order to perform the methods here disclosed. The kit will normally contain the compositions in separate containers. Instructions, for example written or audio instructions, on paper or electronic support such as tapes or CD-ROMs, for carrying out the assay, will usually be included in the
10 kit. The kit can also contain, depending on the particular method used, other packaged reagents and materials (i.e. wash buffers and the like).

[00102] Further details concerning the identification of the suitable carrier agent or auxiliary agent of the compositions, and generally manufacturing and packaging of the kit, can be identified by the person skilled in the art upon
15 reading of the present disclosure.

[00103] The kit of parts herein disclosed can be used in particular for diagnostic purpose. As a result a non-invasive diagnostic detection of pathologies, diseases or disorder and in particular of oral cavity and oropharyngeal cancer in patients, is disclosed.

20 [00104] The use of the fluid phase of saliva has unique advantages over the use of exfoliated cells. Depending on the location of the tumor, one may not be able to easily access and swab the tumor bed. Although salivary biomarkers could not identify the site from which the tumor originated, they could identify patients at risk. Such a saliva test could be administered by
25 nonspecialists in remote locations as a screening tool to select patients for referral for careful evaluation of the upper aerodigestive tract. Finding early stage, previously undetected disease may ultimately save lives. Moreover, the use of easily accessible biomarkers may prove highly beneficial in large populations or chemoprevention trials. This could be envisioned during routine
30 dental visits or targeted screening of individuals at high risk of development of the disease. A home test kit can also be envisioned.

[00105] Also the use of blood test is envisioned in particular for cancer early detection. Recovering the cell-free circulating mRNA or protein biomarkers in the serum of cancer patients representing characteristics of tumor genetic alteration, such as IL6 mRNA and protein, H3F3A, mRNA TPT1 mRNA ,
5 FTH1 mRNA , NCOA4 mRNA and ARCR mRNA diagnostic for OSCC, could be envisioned as a screening test for presence of occult OSCC during routine physician's visit with blood work or targeted screening of individuals at high risk for oral cancer development. A home test kit can also be envisioned, including preferably

10 **[00106]** In particular, peripheral blood can be obtained from subjects using routine clinical procedures, and mRNA and proteins can be isolated, preferably with an optimized procedures herein disclosed. Real time quantitative PCR and ELISA for the respective cytokine will be performed for one or biomarkers, such as IL6.

15 **[00107]** A perspective embodiments of the methods herein disclosed are directed towards the eventual creation of micro-/nano-electrical mechanical systems (MEMS/NEMS) for the ultrasensitive detection of molecular biomarkers in oral fluid. RNA and protein expression for the validated OSCC biomarkers will be selected as targets for cancer detection. The integration of
20 these detection systems for the concurrent detection of mRNA and protein for multiple OSCC biomarkers will result in an efficient, automated, affordable system for oral fluid based cancer diagnostics.

[00108] Further details concerning reagents, conditions, compositions techniques to be used in the method and kits of the disclosure are identifiable
25 by a person skilled in the art upon reading of the present disclosure.

[00109] Also appropriate modifications of the STD methods and kits herein disclosed and exemplified as associated to OSCC and/or HSNCC, for the mRNA profiling and transcriptome analysis associated with investigation and diagnosis of other pathology diseases and disorders can be made by a
30 person skilled in the art upon reading of the present disclosure.

[00110] The following examples are provided to describe the invention in further detail. These examples, which set forth a preferred mode presently contemplated for carrying out the invention, are intended to illustrate and not to limit the invention

5 EXAMPLES

EXAMPLE 1: RNA ISOLATION, AMPLIFICATION AND GENE EXPRESSION PROFILING FROM CELL-FREE SALIVA OF NORMAL DONORS

Normal subjects

[00111] Saliva samples were obtained from ten normal donors from the
10 Division of Otolaryngology, Head and Neck Surgery, at the Medical Center, University of California, Los Angeles (UCLA), CA, in accordance with a protocol approved by the UCLA Institutional Review Board. The following inclusion criteria were used: age 30 years; no history of malignancy, immunodeficiency, autoimmune disorders, hepatitis, HIV infection or smoking.
15 The study population was composed of 6 males and 4 females, with an average age of 42 years (range from 32 to 55 years).

Saliva collection and processing to obtain the relevant fluid phase

[00112] *Unstimulated saliva* were collected between 9 am and 10 am in accordance with published protocols [38]. Subjects were asked to refrain from
20 eating, drinking, smoking or oral hygiene procedures for at least one hour prior to saliva collection. Saliva samples were centrifuged at 2,600 x g for 15 min at 4 °C. Saliva supernatant was separated from the cellular phase. RNase inhibitor (Superase-In, Ambion Inc., Austin, TX, USA) and protease inhibitor (Aprotinin, Sigma, St. Louis, MO, USA) were then added into the cell-free
25 saliva supernatant.

RNA isolation from cell-free saliva

[00113] RNA was isolated from cell-free saliva supernatant using the modified protocol from the manufacturer (QIAamp Viral RNA kit, Qiagen,

Valencia, CA, USA). Saliva (560 μ L), mixed well with AVL buffer (2,240 μ L), was incubated at room temperature for 10 min. Absolute ethanol (2,240 μ L) was added and the solution passed through silica columns by centrifugation at 6,000 x g for 1 min. The columns were then washed twice, centrifuged at 20,000 x g for 2 min, and eluted with 30 μ L RNase free water at 9,000 x g for 2 min. Aliquots of RNA were treated with RNase-free DNase (DNase I-DNA-free, Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions.

[00114] The stability of the isolated RNA was examined by RT-PCR typing for actin- β (ACTB) after storage for 1, 3, and 6 months. The results reported on Figure 1A show that the mRNA isolated could be preserved without significant degradation for more than 6 month at -80 °C.

[00115] The quality of isolated RNA was examined by RT-PCR for three house-keeping gene transcripts: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin- β (ACTB) and ribosomal protein S9 (RPS9). Primers were designed using PRIMER3 software and were synthesized commercially (Fisher Scientific, Tustin, CA, USA) as follows: the primers having the sequence reported in the attached sequence listing as SEQ ID NO: 1 and SEQ ID NO: 2 for GAPDH; the primers having the sequence reported in the attached sequence listing as SEQ ID NO: 3 and SEQ ID NO: 4 for ACTB; the primers having the sequence reported in the attached sequence listing as SEQ ID NO: 5 and SEQ ID NO: 6 for RPS9. The quantity of RNA was estimated using Ribogreen® RNA Quantitation Kit (Molecular Probes, Eugene, OR, USA). The results are shown in Figure 1B, wherein GAPDH (B1), RPS9 (B2) and ACTB (B3) were detected consistently in all 10 cases tested, demonstrating that all 10 saliva samples contain mRNAs that encode for house keeping genes: GAPDH, ACTB and RPS9.

[00116] The mRNA of these genes could be preserved without significant degradation for more than 6 months at -80 °C, (see results for ACTB reported on Fig. 1A).

Target cRNA preparation

[00117] Isolated RNA was then subjected to linear amplification according to published method from our laboratory (Ohyama H, Zhang X, Kohno Y, Alevizos I, Posner M, Wong DT, Todd R. Laser capture microdissection-generated target sample for high-density oligonucleotide array hybridization. *Biotechniques*, 2000 Sep;29(3):530-6.). In brief reverse transcription using T7-oligo-(dT)₂₄ (SEQ ID NO:53) as the primer was performed to synthesize the first strand cDNA. The first round of *in vitro* transcription (IVT) was carried out using T7 RNA polymerase (Ambion Inc., Austin, TX, USA). The BioArray™ High Yield RNA Transcript Labeling System (Enzo Life Sciences, Farmingdale, NY, USA) was used for the second round IVT to biotinylate the cRNA product; the labeled cRNA was purified using GeneChip® Sample Cleanup Module (Affymetrix, Santa Clara, CA, USA).

[00118] The quantity and quality of cRNA were determined by spectrophotometry and gel electrophoresis. Exemplary results of agarose gel electrophoresis test reported on Figure 2A show different quantities of amplified cRNA at the different stages of the RNA amplification.

[00119] Also small aliquots from each of the isolation and amplification steps were used to assess the quality by RT-PCR. Exemplary results reported in Figure 2B show PCR typing ACTB performed at the various stages of RNA amplification, wherein the expected single band (153bp) can be detected in every main step of the salivary RNA amplification process.

[00120] The quality of the fragmented cRNA (prepared as described by Kelly JJ, Chernov BK, Tovstanovsky I, Mirzabekov AD, Bavykin SG. Radical-generating coordination complexes as tools for rapid and effective fragmentation and fluorescent labeling of nucleic acids for microchip hybridization. *Anal Biochem.*, 2002 Dec. 15;311(2):103-18.) was also assessed by capillary electrophoresis using the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Exemplary results reported in Figure 2C show one single peak in a narrow range (50-200bp) demonstrating proper fragmentation.

Gene expression profiling in the targeted cRNA preparation

[00121] Gene expression profiling was performed in cell free-saliva obtained from ten normal donors, wherein on average, 60.5 ± 13.1 ng (n=10) of total RNA was obtained from 560 μ L cell-free saliva samples. The results are reported on Table 1.

Table 1.

Subject	Gender	Age	RNA (ng)a	cRNA (~tg)'~	Present Probes	Probe ~%"
1	F	53	60.4	44.3	3172	14.24
2	M	42	51.6	40.8	2591	11.62
3	M	55	43.2	34.8	2385	10.70
4	M	42	48.2	38.0	2701	12.12
5	M	46	60.6	42.7	3644	16.35
6	M	48	64.8	41.8	2972	13.34
7	F	40	75.0	44.3	2815	12.63
8	M	33	77.8	49.3	4159	18.66
9	F	32	48.8	41.4	2711	12.17
10	F	32	79.8	44.4	4282	19.22
Mean±SD		42±8.3	60.5±13.12	42.2±3.94	3143±665.0	14.11±2.98

[00122] The total RNA quantity is the RNA in 560p.L cell-free saliva supernatant; the cRNA quantity is after two rounds of T7 amplification. Number of probes showing present call on HG U133A microarray (detection p<0.04). Present percentage (P%) = Number of probes assigned present call / Number of total probes (22,283 for HG U133A microarray).

[00123] After two rounds of T7 RNA linear amplification, the average yield of biotinylated cRNA was $42.2 \pm 3.9 \mu\text{g}$ with $A_{260}/A_{280} = 2.067 \pm 0.082$ (Table1). The cRNA ranged from 200 bp to 4 kb before fragmentation; and was concentrated to approximately 100bp after fragmentation. The quality of cRNA probe was confirmed by capillary electrophoresis before the hybridizations. ACTB mRNA was detectable using PCR/RT-PCR on original sample and products from each amplification steps: first cDNA, first In Vitro Transcription (IVT), second cDNA and second IVT, with a resulting agarose electrophoresis pattern comparable to the one shown in Fig. 2B.

EXAMPLE 2: MICROARRAY PROFILING OF MRNA FROM CELL-FREE SALIVA OF NORMAL DONORS

[00124] Saliva was collected processed and the RNA isolated as reported in Example 1. Also, stability, quality and quantity of the RNA was assessed are reported in Example 1.

HG-U1331A Microarray analysis

[00125] The Affymetrix Human Genome U133A Array, which contains 22,215 human gene cDNA probe sets representing ~19,000 genes (*i.e.*, each gene may be represented by more than one probe sets), was applied for gene expression profiling. The array data were normalized and analyzed using Microarray Suite (MAS) software (Affymetrix). A detection p -value was obtained for each probe set. Any probe sets with $p < 0.04$ was assigned "present", indicating the matching gene transcript is reliably detected (Affymetrix, 2001). The total number of present probe sets on each array was obtained and the present percentage (P%) of present genes was calculated. Functional classification was performed on selected genes (present on all ten arrays, $p < 0.01$) by using the Gene Ontology Mining Tool.

[00126] Salivary mRNA profiles of ten normal subjects were obtained using HG U133A array contains 22,283 cDNA probes. An average of $3,143 \pm 665.0$ probe sets ($p < 0.04$) was found on each array ($n=10$) with assigned present calls. These probe sets represent approximately 3,000 different mRNAs. The average present call percentage was $14.11 \pm 2.98\%$ ($n=10$). A reference database which includes data from the ten arrays was generated. The probe sets representing GAPDH, ACTB and RPS9 assigned present calls on all 10 arrays. There were totally 207 probe sets representing 185 genes assigned present calls on all 10 arrays with detection $p < 0.01$. These 10 genes were categorized on the basis of their known roles in biological processes and molecular functions. Biological processes and molecular functions of 185 genes in cell-free saliva from ten normal donors (data obtained by using Gene Ontology Mining Tool) are reported on Table 2.

Table 2.

Biological process ^a	Genes,nb	Molecular function ^a	Genes,nb
Cell growth and/or maintenance	119	Binding	118
Metabolism	93	Nucleic acid binding	89
Biosynthesis	70	RNA binding	73
Protein metabolism	76	Calcium ion binding	12
Nucleotide metabolism	10	Other binding	23
Other metabolisms	18	Structural molecule	95
Cell organization and biogenesis	2	Ribosomal constituent	73
Homeostasis	3	Cytoskeleton constituent	17
Cell cycle	5	Muscle constituent	2
Cell proliferation	11	Obsolete	15
Transport	5	Transporter	4
Cell motility	8	Enzyme	20
Cell communication	34	Signal transduction	10
Response to external stimulus	19	Transcription regulator	7
Cell adhesion	3	Translation regulator	5
Cell-cell signaling	5	Enzyme regulator	9
Signal transduction	17	Cell adhesion molecule	1
Obsolete	8	Molecular function unknown	6
Development	18		
Death	2		
Biological process unknown	11		

[00127] One gene may have multiple molecular functions or participate in different biological processes. Number of genes classified into a certain group/subgroup. The major functions of the 185 genes are related to cell growth/maintenance (119 genes), molecular binding (118 genes) and cellular structure composition (95 genes). We termed these 185 genes as “Normal Salivary Core Transcriptome (NSCT)”.

EXAMPLE 3: Q-PCR VALIDATION AND QUANTITATION ANALYSIS OF MICROARRAY PROFILING FROM CELL-FREE SALIVA OF NORMAL DONORS

[00128] The Microarray analysis performed in Example 2 was validated through a quantitative gene expression analysis by Q-PCR

Quantitative gene expression analysis by Q-PCR

[00129] Real time quantitative PCR (Q-PCR) was used to validate the presence of human mRNA in saliva by quantifying selected genes from the 185 "Normal Salivary Core Transcriptome" genes detected by the Microarray
5 profiling reported in Example 2. Genes IL1B, SFN and K-ALPHA-1, which were assigned present calls on all 10 arrays, were randomly selected for validation.

[00130] Q-PCR was performed using iCycler™ thermal Cycler (Bio-Rad, Hercules, CA, USA). A 2 µL aliquot of the isolated salivary RNA (without
10 amplification) was reverse transcribed into cDNA using MuLV Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). The resulting cDNA (3 µL) was used for PCR amplification using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The primers were synthesized by Sigma-Genosys (Woodlands, TX, USA) as follows: the primers having the sequence reported in
15 the attached sequence listing as SEQ ID NO: 7 and SEQ ID NO: 8 for interleukin 1, beta (IL1B); the primers having the sequence reported in the attached sequence listing as SEQ ID NO: 9 and SEQ ID NO: 10 for stratifin (SFN); the primers having the sequence reported in the attached sequence listing as SEQ ID NO: 11 and SEQ ID NO: 12 for tubulin, alpha, ubiquitous (K-ALPHA-1). All
20 reactions were performed in triplicate with conditions customized for the specific PCR products. The initial amount of cDNA of a particular template was extrapolated from a standard curve using the LightCycler™ software 3.0 (Bio-Rad, Hercules, CA, USA). The detailed procedure for quantification by standard curve has been previously described (Ginzinger DG. Gene quantification using real-time quantitative
25 PCR: an emerging technology hits the mainstream. Exp Hematol., 2002 Jun;30(6):503-12.).

[00131] Q-PCR results showed that mRNA of IL1B, SFN and K-ALPHA-1 were detectable in all 10 original, unamplified, cell-free saliva. The relative amounts (in copy number) of these transcripts (n=10) are: $8.68 \times 10^3 \pm 4.15 \times 10^3$ for IL1B; $1.29 \times 10^5 \pm 1.08 \times 10^5$ for SFN; and $4.71 \times 10^6 \pm 8.37 \times 10^5$ for K-
30 ALPHA-1. The relative RNA expression levels of these genes measured by Q-PCR were similar to those measured by the microarrays (data not shown).

EXAMPLE 4: IL6 AND IL8 MRNA ISOLATION AMPLIFICATION AND ANALYSIS OF THE EXPRESSION IN CELL-FREE SALIVA OF OSCC PATIENTS

Patients selection

5 [00132] Patients were recruited from the Division of Head and Neck Surgery at the University of California, Los Angeles (UCLA) Medical Center, Los Angeles, CA; the University of Southern California (USC) Medical Center, Los Angeles, CA; and the University of California San Francisco (UCSF) Medical Center, San Francisco, CA, over a 6 -month period.

10 [00133] Thirty-two patients with documented primary T1 or T2 squamous cell carcinoma of the oral cavity (OC) or oropharynx (OP) were included in this study. All patients had recently been diagnosed with primary disease, and had not received any prior treatment in the form of chemotherapy, radiotherapy, surgery, or alternative remedies. An equal number of age and sex matched subjects with comparable smoking histories were selected as a
15 control comparison group.

[00134] Among the two subject groups, there were no significant differences in terms of mean age (standard deviation, SD): OSCC patients, 49.3 (7.5) years; normal subjects, 48.8 (5.7) years (Student's *t* test $P > 0.80$); gender (Student's *t* test $P > 0.90$); or smoking history (Student's *t* test $P > 0.75$). No
20 subjects had a history of prior malignancy, immunodeficiency, autoimmune disorders, hepatitis, or HIV infection. Each of the individuals in the control group underwent a physical examination by a head and neck surgeon, to ensure that no suspicious mucosal lesion was present.

Saliva Collection And Processing

25 [00135] Informed consent had been given by all patients. Saliva and serum procurement procedures were approved by the institutional review board at each institution: the University of California, Los Angeles (UCLA); the University of Southern California (USC); and the University of California San Francisco (UCSF).

[00136] Saliva from 32 patients with OC or OP SCCA, and 32 unarrected age- and gender-matched control subjects were obtained for a prospective comparison of cytokine concentration.

5 [00137] The subjects were required to abstain from eating, drinking, smoking, or using oral hygiene products for at least one hour prior to saliva collection. Saliva collection was performed using the "draining (drooling)" method of Navazesh and Christensen,[7] for a total donation of 5 cc saliva. Saliva samples were subjected to centrifugation at 3500 rpm (2600xg) for 15 minutes at 4°C by a Sorvall RT6000D centrifuge (DuPont, Wilmington, DE). The fluid-
10 phase was then removed, and RNase (Superase-In, RNase Inhibitor, Ambion Inc., Austin, TX) and protease (Aprotinin, Sigma, St. Louis, MO; Phenylmethylsulfonylfluoride, Sigma, St. Louis, MO; Sodium Orthovanadate, Sigma, St. Louis, MO) inhibitors were then added promptly on ice. The conditions for the separation of the cellular and fluid phases of saliva were
15 optimized to ensure no mechanical rupture of cellular elements which would contribute to the mRNA detected in the fluid phase. All samples were subsequently treated with DNase (DNaseI-DNA-free, Ambion Inc., Austin, TX). The cell pellet was retained and stored at -80°C.

RNA Isolation from cell-free saliva

20 [00138] 560 µL of saliva supernatant were then processed using the QIAamp Viral RNA mini kit (QIAGEN, Chatsworth, CA) kit. RNA was extracted according to the manufacturer's instructions. Samples were air-dried and resuspended in water treated with diethyl pyrocarbonate and were kept on ice for immediate usage or stored at -80°C. Aliquots of RNA were treated with
25 RNase-free DNase (DNaseI-DNA-free, Ambion Inc., Austin, TX) according to the manufacturer's instructions. Concentrations of RNA were determined spectrophotometrically, and the integrity was checked by electrophoresis in agarose gels containing formaldehyde.

Reverse Transcriptase-Polymerase Chain Reaction

[00139] Presence of IL6 and IL8 mRNA transcripts in the fluid phase in saliva was tested by using reverse transcriptase-polymerase chain reaction (RT-PCR).

5 **[00140]** RNA from each sample was reverse-transcribed in 40 μ L of reaction mixture containing 2.5 U of Moloney murine leukemia virus reverse transcriptase (Applied Biosystems Inc.(ABI, Foster City, CA) and 50 pmol of random hexanucleotides (ABI, Foster City, CA) at 42°C for 45 minutes. Based on the published sequences, oligonucleotide primers were synthesized
10 commercially at Fisher Scientific (Tustin, CA) for PCR as follows: the primers having the sequence reported attached sequence listing as SEQ ID NO: 13 and SEQ ID NO: 14 for β -actin; the primers having the sequence reported attached sequence listing as SEQ ID NO: 15 and SEQ ID NO: 16 for IL8; and the primers having the sequence reported attached sequence listing as SEQ
15 ID NO: 17 and SEQ ID NO: 18 for IL6.

[00141] Amplification of the complementary DNA (cDNA) was carried out using 50 cycles at 95 °C for 20 seconds, 60 °C for 30 seconds, and 72°C for 30 seconds; followed by a final extension cycle of 72 °C for 7 minutes. Specificity of the PCR products was verified by the predicted size and by
20 restriction digestion. To establish the specificity of the responses, negative controls were used in which input RNA was omitted or in which RNA was used but reverse transcriptase omitted. As a positive control, mRNA was extracted from total salivary gland RNA (Human Salivary Gland Total RNA, Clontech, Palo Alto, CA). To ensure RNA quality, all preparations were
25 subjected to analysis of expression.

[00142] The RT-PCR studies so performed showed that saliva and serum contained mRNA encoding for IL6 and IL8. Exemplary results reported in Figure 3, show PCR products of the sizes (95 bp for IL6 and 88 bp for IL8) that were expected from the selected primers. The same-sized products were
30 expressed in the positive control.

[00143] In order to ensure that the RNA and protein analyzed were from the fluid phase of saliva only and to ensure the lack of contamination by intracellular components, the centrifugation speed for the saliva samples was optimized. PCR for the housekeeping genes β -actin and ubiquitin on whole saliva samples, and samples that had been centrifuged at various speeds using DNA as a marker of cell lysis and spillage of intracellular components. The results support an optimal centrifugation speed for saliva samples of $2,600 \pm 52 \times g$, with a preferred speed of $2,600 \times g$ (see exemplary results reported on Figure 4)

10 **EXAMPLE 5: IL6 AND IL8 MRNA ISOLATION, AMPLIFICATION AND ANALYSIS OF THE EXPRESSION IN SERUM OF OSCC PATIENTS**

[00144] Patients recruited as reported in Example 4, where subjected to analysis of presence of IL6 and IL8 mRNA in blood serum.

Serum collection and processing

15 [00145] Serum from 19 patients with OC or OP SCCA, and 32 unaffected age- and gender-matched control subjects were obtained for a prospective comparison of cytokine concentration. Among the subject groups, there were no significant differences in terms of age, gender, alcohol consumption, or smoking history ($P > 0.75$).

20 [00146] Blood was drawn from control subjects and patients prior to treatment. Sera were collected by centrifuging whole blood at 3000 rpm (1000xg) for 10 minutes at 15°C by a Sorvall RT6000D centrifuge (DuPont, Wilmington, DE). Serum was then separated, and RNase (Superase-In, RNase Inhibitor, Ambion Inc., Austin, TX) and protease (Aprotinin, Sigma, St. Louis, MO; Phenylmethylsulfonylfluoride, Sigma, St. Louis, MO; Sodium Orthovanadate, Sigma, St. Louis, MO) inhibitors were then added promptly on ice. All samples were subsequently treated with DNase (DNaseI-DNA-free, 25 Ambion Inc., Austin, TX). The aliquots were stored at -80°C until further use.

Reverse Transcriptase-Polymerase Chain Reaction

[00147] Presence of IL6 and IL8 mRNA transcripts in the serum was tested by using reverse transcriptase-polymerase chain reaction (RT-PCR) performed as described in Example 4 above.

- 5 [00148] The RT-PCR studies so performed showed that serum contained mRNA encoding for IL6 and IL8, with electrophoresis gel pattern comparable to the one shown in Figure 3.

[00149] In order to ensure that the RNA and protein analyzed were from the fluid phase of serum only and to ensure the lack of contamination by
10 intracellular components, the centrifugation speed for the serum samples was optimized following the same approach described in Example 4 for saliva samples. The results support an optimal centrifugation speed for saliva samples of $1,000 \pm 20 \times g$ with a preferred speed of $1,000 \times g$.

15 **EXAMPLE 6: IL6 AND IL8 CYTOKINE LEVELS ANALYSIS IN SALIVA FROM OSCC PATIENTS**

[00150] On demonstrating that IL6 and IL8 mRNA transcripts were present in the fluid phase in saliva, we prospectively examined and compared the levels of IL6 and IL8 in the saliva of unaffected subjects and patients with OSCC using quantitative real time PCR (qRT-PCR) and ELISA.

- 20 [00151] Saliva from 32 patients with OSCC, and 32 age- and gender-matched control subjects were obtained. Among the subject groups, there were no significant differences in terms of age, gender, alcohol consumption, or smoking history ($P > 0.75$).

25 Real Time PCR for Quantification of IL6 and IL8 mRNA Concentrations in Saliva from Patients and Normal Subjects

[00152] To analyze quantitatively the result of RT-PCR, quantitative real-time PCR (Bio-Rad iCycler, Thermal Cycler, Bio-Rad Laboratories, Hercules, CA) was used. Each sample was tested in triplicate. The amplification reactions were carried out in a 20 μ L mixture, using iQ SYBR Green Supermix (Bio-Rad

Laboratories, Hercules, CA). After initial denaturation at 95°C for 3 minutes, 50 PCR cycles were performed at 60°C for 20 seconds, then 20 seconds at 72°C, then 20 seconds at 83°C, followed by 1 minute at 95°C, then followed by a final 1 minute extension at 55°C. Aliquots were taken from each well and
5 checked by electrophoresis in agarose gels in order to ensure the specificity of the products.

[00153] The RT-PCR results are illustrated by the diagram shown in Figure 5 A. Such results show that IL8 at both the mRNA and protein levels, was detected in higher concentrations in the saliva of patients with OSCC when
10 compared with control subjects (*t* test, $P < 0.01$). There was a significant difference in the amount of IL8 mRNA expression between saliva from OSCC patients and disease-free controls. The mean copy number was 1.1×10^3 for the OSCC group, and 2.6×10^1 for the control group. The difference between the two groups was highly statistically significant ($P < 0.0008$).

15 [00154] No significant differences were instead found in the salivary concentration of IL6 at the mRNA level. Within the sample size studies, the inventors were also unable to detect differences between smoking and nonsmoking subjects.

20 ELISA for Quantification of IL6 and IL8 Protein Concentrations in Saliva from Patients and Normal Subjects

[00155] ELISA kits for IL6 and IL8 were used (Pierce Endogen, Rockford, IL) according to the manufacturer's protocol. Each sample was tested in duplicate in each of two replicate experiments. After development of the colorimetric reaction, the absorbance at 450 nm was quantitated by an eight
25 channel spectrophotometer (EL800 Universal Microplate Reader, BIO-TEK Instruments Inc., Winooski, VT), and the absorbance readings were converted to pg/ml based upon standard curves obtained with recombinant cytokine in each assay. If the absorbance readings exceeded the linear range of the standard curves, ELISA assay was repeated after serial dilution of the
30 supernatants. Each sample was tested in at least two ELISA experiments, and the data were calculated from the mean of tests for each sample.

[00156] The ELISA findings are illustrated by the diagram shown in Figure 5B. The levels of IL8 in the saliva of OSCC patients were significantly higher (720 pg/dL) than those in the saliva of the control group (250 pg/dL) ($P < 0.0001$). To ensure that the elevated levels of IL8 protein in saliva were not due to an elevation of total protein levels in the saliva of OSCC patients, we compared the total protein concentrations in saliva among the two groups. No significant differences were found ($P > 0.05$).

[00157] No significant differences were found in the salivary concentration of IL6 at the protein level. Also in the ELISA analysis, no differences were detected within the sample size studies between smoking and nonsmoking subjects.

EXAMPLE 7: IL6 AND IL8 CYTOKINE LEVELS ANALYSIS IN SERUM FROM OSCC PATIENTS

[00158] We also examined and compared the levels of IL6 and IL8 in the serum of unaffected subjects and patients with OSCC using qRT-PCR and ELISA. The patients were selected as described in Example 4 and the serum processed as described in Example 5.

Real Time PCR for Quantification of IL6 and IL8 mRNA Concentrations in Serum from Patients and Normal Subjects

[00159] To analyze quantitatively the result of RT-PCR, quantitative real-time PCR was performed as described in Example 6.

[00160] The RT-PCR results are illustrated by the diagram shown in Figure 6A. Such results show that IL6 at mRNA level was detected in higher concentrations in the serum of patients with OSCC when compared with control subjects (t test, $P < 0.001$). We noted a significant difference in the amount of IL6 mRNA expression between serum from OSCC patients and disease-free controls. The mean copy number was 5.2×10^4 for the OSCC group, and 3.3×10^3 for the control group. The difference between the two groups was highly statistically significant ($P < 0.0004$).

[00161] No significant differences were instead found in the serum concentration of IL8 at the mRNA level. Within the sample size studies, the inventors were also unable to detect differences between smoking and nonsmoking subjects.

5 ELISA for Quantification of IL6 and IL8 Protein Concentrations in Serum from Patients and Normal Subjects

[00162] ELISA tests for quantification of IL6 and IL8 protein concentrations in serum were performed as described in Example 6.

10 [00163] The relevant ELISA findings are illustrated by the diagram shown in Figure 6 B. The mean levels of IL6 in the serum of OSCC patients were significantly higher (87 pg/dL) than those in the serum of the control group (0 pg/dL) ($P < 0.0001$).

15 [00164] No significant differences were found in the serum concentration of IL8 at the protein level. Also in the ELISA analysis, no differences were detected within the sample size studies between smoking and nonsmoking subjects.

EXAMPLE 8: ROC AND SENSITIVITY/SPECIFICITY ANALYSIS

20 [00165] Statistical analysis of the data collected in outcome of the experiments reported on Examples 1 to 7 above demonstrates the specificity and sensitivity of these biomarkers for HNSCC, and their predictive value.

Statistical Analysis

25 [00166] The distributions of patient demographics were calculated overall and separately for OSCC cases and controls, and were compared between the two arms with either the Student's *t*-test for continuous measures or two-by-two Chi-square tables for categorical measures. The distributions of IL6 and IL8 levels in saliva and serum were computed and compared between the OSCC cases and controls using two independent group *t*-tests. Differences were considered significant for *P* values less than 0.01. Due to the range of the IL6 and IL8 levels, log transformations of these measures were also used

in the analyses. Data were expressed as the mean $\pm$ SD. Age, gender, and smoking history were controlled at the group level in the experimental design; these patient factors were also adjusted in the analyses when comparing IL6 and IL8 through regression modeling.

5 [00167] Using the binary outcome of the disease (OSCC cases) and non-disease (controls) as dependent variables, logistic regression models were fitted to estimate the probability of developing OSCC as a function of each of the potential biomarkers (IL6 or IL8), controlling for patient age, gender, and smoking history. Using the fitted logistic models, receiver operating
10 characteristic (ROC) curve analyses were conducted to evaluate the predictive power of each of the biomarkers[8][9][10]. Through the ROC analyses, we calculated sensitivities and specificities by varying the criterion of positivity from the least (cut at probability of 0) to the most stringent (cut at probability of 1). The optimal sensitivity and specificity was determined for
15 each of the biomarkers, and the corresponding cutoff/threshold value of each of the biomarkers was identified. The biomarker that has the largest area under the ROC curve was identified as having the strongest predictive power for detecting OSCC.

Clinical Data

20 [00168] The mean (SD) age of the patients with OSCC was 49.3 (7.5) years (range, 42-67 years) vs. 48.8 (5.7) years (range, 40-65 years) in the control group; (Student's *t* test $P > 0.80$). Among the two subject groups, there were no significant differences in terms of age (mean age): OSCC patients, 49.3 years; normal subjects, 48.8 years (Student's *t* test $P > 0.80$); gender
25 (Student's *t* test $P > 0.90$); or smoking history (Student's *t* test $P > 0.75$).

[00169] ROC (Receiver Operating Characteristic) curves, plots of sensitivities versus 1-specificities, were generated for each of the potential biomarkers. Age, gender, and smoking history were controlled as described above. The areas under the ROC curves were calculated, as measures of the utility of
30 each biomarker for detecting OSCC.

[00170] Figure 7A and Figure 7B show the ROC curves for IL8 in saliva and IL6 in serum, respectively. The calculated ROC values (for predicting OSCC) were 0.978 for IL8 in saliva; and 0.824 for IL6 in serum. Based on the distribution of sensitivities and specificities, thresholds of biomarkers were chosen for detecting OSCC. Based upon our data, for IL8 in saliva, a threshold value of 600 pg/dL yields a sensitivity of 86% and a specificity of 97%. Similarly, for IL6 in serum, a threshold value of greater than 0 pg/dL yields a sensitivity of 64% and a specificity of 81%.

[00171] The combination of biomarkers: IL-8 in saliva and IL-6 in serum holds great potential for OSCC diagnostics as ROC analysis yields a sensitivity of 99% and a specificity of 90% as shown in Figure 7C.

[00172] The detailed statistics of the area under the ROC curves, the threshold values, and the corresponding sensitivities and specificities for each of the potential biomarkers in saliva and in serum are listed in Table 3.

[00173] The detailed statistics of the area under the ROC curves, the threshold values, and the corresponding sensitivities and specificities for each of the potential biomarkers in saliva and in serum are listed in table 3 below.

Table 3

Biomarker	Area under ROC	Threshold/Cutoff	Sensitivity	Specificity
IL8 saliva protein	0.978	600 pg/mL	86%	97%
IL6 serum protein	0.824	> 0 pg/mL	57%	100%
IL8 saliva protein & IL6 serum protein	0.994	> 600 pg/ml > 0 p/ml	99%	90%

20 **EXAMPLE 9: RNA ISOLATION, AMPLIFICATION AND GENE EXPRESSION PROFILING FROM SERUM OF OSCC PATIENTS**

Subject selection

[00174] Thirty-two OSCC patients were recruited from Medical Centers at University of California, Los Angeles (UCLA) and University of Southern

California (USC), Los Angeles, CA. All patients had recently been diagnosed with primary T1/T2 OSCC, and had not received any prior treatment in the form of chemotherapy, radiotherapy, surgery, or alternative remedies. Thirty-five normal donors were recruited as controls from the general population at School of Dentistry, UCLA. No subjects had a history of prior malignancy, immunodeficiency, autoimmune disorders, hepatitis, or HIV infection. All subjects signed the Institutional Review Board approved consent form agreeing to serve as blood donors for this study.

[00175] Totally sixty-seven subjects were recruited, including 32 OSCC patients and 35 normal subjects. Among the two subject groups, there were no significant differences in terms of mean age (standard deviation, SD): OSCC patients, 49.3 (7.5) years; normal subjects, 47.8 (6.4) years (Student's *t* test $P = 0.84$). The gender distribution in OSCC group was 10:22 (female number/male number) and in control group was 14:21 (Chi-square test $P \approx 1$). We matched the smoking history of these two groups by determining the follows. All subjects were asked: (1) For how many years had they smoked? (2) How many packs per day had they smoked? (3) How many years had elapsed since they had quit smoking (if they had indeed quit)? (4) Did they only smoke cigarettes, or did they also use cigars, pipes, chewing tobacco, or marijuana? We then optimized the match between patients and controls in terms of the above: (1) similar pack-year history (2) similar time lapse since they had quit smoking (3) use of cigarettes exclusively. There was no significant difference between two groups in the smoking history (Student's *t* test $P = 0.77$).

25 Blood collection and processing.

[00176] Blood procurement procedure was approved by the institutional review board at UCLA and USC. Blood was drawn from control subjects and patients prior to treatment. The whole blood then underwent a centrifugation by 1,000 x *g* for 10 minutes at 15°C by a Sorvall RT6000D centrifuge (DuPont, Wilmington, DE). Serum was then separated, and 100U/mL RNase inhibitor

(Suprase-In, Ambion Inc., Austin, TX) was added promptly to the serum. The aliquots were stored at -80°C until further use.

RNA isolation from serum.

[00177] RNA was isolated from 560 μl serum using QIAamp Viral RNA kit (Qiagen, Valencia, CA). Aliquots of isolated RNA were treated with RNase-free DNase (DNaseI-DNA-free, Ambion Inc., Austin, TX) according to the manufacturer's instructions. The quality of isolated RNA was examined by RT-PCR for four housekeeping gene transcripts: β -actin (ACTB), β -2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ribosomal protein S9 (RPS9). Based on the published sequences, oligonucleotide primers were designed and then synthesized (Sigma Genosis, Woodlands, TX) for PCR. RT-PCR was performed to amplify the mRNAs' coding region phenotyped in 3 segments using a common upstream primer and three different downstream primers selected from the four housekeeping gene transcripts for RT-PCR shown in Table 4.

Table 4

Name	Accession no. (NCBI)	Full length (bp)	Primer sequences	Amplicon (bp)
ACTB	X00351	1761	F: SEQ ID NO: 19	
			R1: SEQ ID NO: 20	195
			R2: SEQ ID NO: 21	705
			R3: SEQ ID NO: 22	1000
B2M	NM_004048	987	F: SEQ ID NO: 23	
			R1: SEQ ID NO: 24	216
			R2: SEQ ID NO: 25	591
			R3: SEQ ID NO: 26	848
GAPDH	M33197	1268	F: SEQ ID NO: 27	
			R1: SEQ ID NO: 28	140
			R2: SEQ ID NO: 29	755
			R3: SEQ ID NO: 30	1184
RPS9	NM_001013	692	F: SEQ ID NO: 31	
			R1: SEQ ID NO: 32	188
			R2: SEQ ID NO: 33	426
			R3: SEQ ID NO: 34	614

[00178] In particular four serum human mRNAs were selected and coding region phenotyped in 3 segments using a common upstream primer and three different downstream primers dividing the coding region approximately into three parts. 10 µl of each PCR reaction was electrophoresed on a 2% agarose gel and stained with EtBr.

[00179] Specificity of all the PCR products was verified by the predicted size comparing the positive control (Human Salivary Gland Total RNA, Clontech, Palo Alto, CA). Negative controls were used in which input RNA was omitted or in which RNA was used but reverse transcriptase omitted.

[00180] The serum phenotype of mRNA product from human was evaluated by RT-PCR and electrophoresis. Exemplary results reported in Figure 8, showed transcripts from four housekeeping genes (ACTB, B2M, GAPDH, and RPS9) could be detected. In particular, amplicons for RPS9 with sizes of 188, 426 and 614bp were detected (see Figure 8 lane 2, 3 and 4 respectively); amplicons for GAPDH with sizes of 140,755 and 1,184bp were detected (see Figure 8 lane 5, 6 and 7 respectively); amplicons for B2M with sizes of 216,591 and 848bp were detected (see Figure 8 lane 8, 9 and 10 respectively); and amplicons for ACTB with sizes of 195,705 and 1,000bp were detected (see Figure 8 lane 11, 12 and 13 respectively). Controls were performed even if controls data are not shown in the Figure.

[00181] The longest PCR products we amplified covered 56.8% (ACTB), 85.9% (B2M), 93.4% (GAPDH) and 88.9% (RPS9) of the full length of the corresponding mRNAs, according to the NCBI GenBank database. This result also indicated there could be intact human mRNA circulating in blood in a cell-free form.

EXAMPLE 10: MICROARRAY PROFILING OF MRNA OF SERUM FROM OSCC PATIENTS

[00182] Serum from ten OSCC patients (8 male, 2 female, age=51 ± 9.0) and from ten gender and age matched normal donors (age=49 ± 5.6) was

collected and processed as reported in Example 9 for use in microarray analysis.

Microarray analysis

- [00183] Isolated RNA from serum was subjected to linear amplification by RiboAmpTM RNA Amplification kit (Arcturus, Mountain View, CA). Following previously reported protocols [55], the Affymetrix Human Genome U133A Array, which contains 22,215 human gene cDNA probe sets representing ~19,000 genes (*i.e.*, each gene may be represented by more than one probe sets), was applied for gene expression profiling.
- [00184] The raw data were imported into DNA-Chip Analyzer 1.3 (dChip) software for normalization and model-based analysis [60]. dChip gives the expression index which represents the amount of mRNA/Gene expression and another parameter, called the present call of, whether or not the mRNA transcript was actually present in the sample (14). S-plusTM 6.0 (Insightful, Seattle, WA) was used for all statistical tests.
- [00185] Three criteria were used to determine differentially expressed genes between OSCCs and controls. First, genes that were assigned as "absent" call in all samples were excluded. Second, a two-tailed student's *t* test was used for comparison of average gene expression levels among the OSCCs (*n*=10) and controls (*n*=10). The critical alpha level of 0.05 was defined for statistical significance. Third, fold ratios were calculated for those genes that showed statistically significant difference ($P < 0.05$). Only those genes that exhibit at least 2-fold change will be included for further analysis.
- [00186] The HG U133A microarrays were used to identify the difference in salivary RNA profiles between cancer patients and matched normal subjects. Among the 14,268 genes included by the previously described criteria, we identified 335 genes with *P* value less than 0.05 and a fold change ≥ 2 . Among these genes, there are 223 up-regulated genes and 112 down-regulated genes in the OSCC group. According to Affymetrix, a gene that was assigned with a present call indicates this gene is reliably detected in the

original sample. The number of genes that were assigned present and the present percentage on each array were shown in Table 5 reporting the human mRNA expression profiling in serum.

Table 5

Subject	Normal				OSCC			
	Gender	Age	Present Probes ^a	Probe P% ^b	Gender	Age	Present Probes ^a	Probe P% ^b
1	F	53	1564	7.02	F	55	1990	8.93
2	M	55	1600	7.18	M	61	2924	13.12
3	M	42	1600	7.18	M	42	2126	9.54
4	M	46	1716	7.7	M	46	3316	14.88
5	M	42	1845	8.28	M	42	2937	13.18
6	M	54	1854	8.32	M	52	1794	8.05
7	F	51	1903	8.54	F	67	2119	9.51
8	M	48	2032	9.12	M	46	2019	9.06
9	M	56	1823	8.18	M	61	4646	20.85
10	M	42	1979	8.88	M	44	2362	10.6
Mean±SD		49±5.6	1792±165	8.04±0.74		51±9.0	2623±868*	11.8±3.90

5

[00187] (a) Number of probes showing present call on HG U133A microarray (detection $P < 0.04$).

[00188] (b) Present percentage (P%) = Number of probes assigned present call / Number of total probes (22,283 for HG U133A microarray).

10 **[00189]** * The arrays for OSCC have significant more probes assigned with present call than those for control group ($P \leq 0.002$, Wilcoxon test).

[00190] On average, there are 2623±868 probes in OSCC arrays and 1792±165 probes in control arrays that were assigned with present calls. OSCC group have significant more present probes than control group ($P \leq$
15 0.002, Wilcoxon test).

[00191] Using a more stringent criterion that, for a certain gene, the present call was assigned consistently to all arrays among all cancers (n=10) or all controls (n=10), we identified 62 genes to be the candidates for further

analysis. We noted that these 62 genes are all up-regulated in OSCC serum, whereas there are no genes found down-regulated using the same filtering criteria.

**EXAMPLE 11: Q-PCR VALIDATION AND QUANTITATION ANALYSIS OF
5 MICROARRAY PROFILING FROM CELL-FREE SALIVA OF OSCC PATIENTS**

[00192] qPCR was performed to quantify a subset of differently expressed transcripts in saliva and to validate the microarray findings of Example 10, on an enlarged sample size including saliva from 32 OSCC patients and 35 controls.

10 Quantitative PCR (qPCR) assay.

[00193] Primer sets were designed by using PRIMER3 software (Table 2). Using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) and random hexamers as primer (ABI, Foster City, CA), cDNA was synthesized from the original and un-amplified serum RNA. The qPCR reactions were
15 performed in an iCycler™ iQ real-time PCR detection system (Bio-Rad, Hercules, CA, USA), using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). All reactions were performed in triplicate with customized conditions for specific products. The relative amount of cDNA/RNA of a particular template was extrapolated from the standard curve using the LightCycler software 3.0
20 (Bio-Rad, Hercules, CA, USA). A two-tailed student's *t* test was used for statistical analysis.

[00194] Ten significant up-regulated genes: H3F3A, TPT1, FTH1, NCOA4, ARCR, THSMB (Thymosin beta 10), PRKCB1 (Protein Kinase C, beta 1), FTL1 (Ferritin Light polypeptide), COX4I1 (Cytochrome c oxidase subunit IV isoform 1)
25 and SERP1 (stress associated endoplasmic reticulum protein 1; ribosome associated membrane protein 4) were selected based on their reported cancer-association as shown in Table 6, reporting ten genes selected for qPCR validation.

Table 6

Probe set ID (HG U133A)	Gene name	Symbol	Accession No. (NCBI)	qPCR <i>P</i> (<i>t</i> test)
211940_x_at	H3 histone, family 3A	H3F3A	BE869922	0.003
211943_x_at	Tumor protein, translationally-controlled 1	TPT1	AL565449	0.005
200748_s_at	Ferritin, heavy polypeptide 1	FTH1	NM_002032	0.008
210774_s_at	Nuclear receptor coactivator 4	NCOA4	AL162047	0.021
200059_s_at	Ras homolog gene family, member A	ARCR	BC001360	0.048
217733_s_at	Thymosin, beta 10	THSMB	NM_021103	0.318
209685_s_at	Protein kinase C, beta 1	PRKCB1	M13975	0.615
208755_x_at	Ferritin, light polypeptide	FTL1	BF312331	0.651
200086_s_at	Cytochrome c oxidase subunit IV isoform 1	COX4I1	AA854966	0.688
200971_s_at	Stress-associated endoplasmic reticulum protein 1; ribosome associated membrane protein 4	SERP1	NM_014445	0.868

[00195] Table 6 presents their quantitative alterations in serum from OSCC patients, determined by qPCR. The results confirmed that transcripts of H3F3A, TPT1, FTH1, NCOA4 and ARCR were significantly elevated in the saliva of OSCC patient (Wilcoxon Signed Rank test, $P < 0.05$). We did not detect the statistically significant differences in the amount of the other five transcripts by qPCR.

EXAMPLE 12: ROC AND SENSITIVITY/SPECIFICITY ANALYSIS

[00196] Statistical analysis of the data collected in outcome of the experiments reported on Examples 9 to 11 above demonstrates the specificity and sensitivity of these biomarkers for HNSCC, and their predictive value.

5 Receiver Operating Characteristic Curve Analysis and Prediction Models.

[00197] Utilizing the qPCR results, multivariate classification models were constructed to determine the best combination of the selected serum transcripts for cancer prediction. Firstly, using the binary outcome of the disease (OSCC) and non-disease (normal) as dependent variables, a logistic regression model was constructed [61]. Age, gender and smoking history are controlled in the data collection procedure.

[00198] Leave-one out cross validation was used to validate the logistic regression model. The cross validation strategy first removes one observation and then fits a logistic regression model from the remaining cases using all markers. Stepwise model selection is used for each of these models to remove variables that do not improve the model. Subsequently, the observing values for the case that was left out were used to compute a predicted class for that observation. The cross validation error rate is then the number of samples predicted incorrectly divided by the number of samples.

20 [00199] The Receiver operating characteristic (ROC) curve analysis was then computed for the best final logistic model (S-plus 6.0), using the fitted probabilities from the model as possible cut-points for computation of sensitivity and specificity. Area under the curve was computed via numerical integration of the ROC curve.

25 [00200] To demonstrate the utility of circulating mRNAs in serum for OSCC discrimination, two classification/prediction models were observed. Using the qPCR data, a logistic regression model was built compose of six serum transcripts previously examined, ARHA, FTH1, H3F3A, TPT1, COX4I1 and FTL1. Those six transcripts in combination provided the best prediction, which was then validated by the leaving-one-out validation. Out of 67 leaving-one-

30

out trial, 54 (81%) of the best logistic models was found to be the same model as the one from the whole data and the validation error rate was 31.3% (21/67).

[00201] Results are reported in Figure 9, wherein the ROC curve computed for this logistic regression model is shown.

- 5 [00202] Using a cut-off probability of 44% a sensitivity of 84% and a specificity of 83% were obtained. The final model predicts correctly for 56 (83.5%) subjects out of 67 with 0.84 (27/32) sensitivity and 0.83 (29/35) specificity and it misclassifies 6 subjects for control and 5 for OSCC. The calculated area under the ROC curve was 0.88 for this logistic regression
10 model.

Tree-based classification model, classification and regression tree (CART).

- [00203] Secondly, another prediction model utilizing the qPCR results was built by a tree-based classification method. The classification and regression trees (CART), was constructed by S-plus 6.0 using the serum transcripts as
15 predictors from qPCR result. CART fits the classification model by binary recursive partitioning, where each step involves searching for the predictor variable that results in the best split of the cancer versus the normal groups [62]. CART used the entropy function with splitting criteria determined by default settings for S-plus. By this approach, the parent group containing the
20 entire samples (n=67) was subsequently divided into cancer groups and normal groups. Our initial tree was pruned to remove all splits that did not result in sub-branches with different classifications.

[00204] A second model, the "classification and regression trees (CART) model", was generated according to the diagram reported in Figure 10.

- 25 [00205] Our fitted CART model used the serum mRNA concentrations of THSMB and FTH1 as predictor variables for OSCC. THSMB, chosen as the initial split, with a threshold of $4.59\text{E-}17$ M, produced two child groups from the parent group containing the total 67 samples. 47 samples with the THSMB concentration $< 4.59\text{E-}17$ M were assigned into "Normal-1", while 20 with
30 THSMB concentration $\geq 4.59\text{E-}17$ M were assigned into "Cancer-1". The

“Normal-1” group was further partitioned by FTH1 with a threshold of 8.44×10^{-16} M. The resulting subgroups, “Normal-2” contained 28 samples with FTH1 concentration $< 8.44 \times 10^{-16}$ M, and “Cancer-2” contained 19 samples with FTH1 concentration $\geq 8.44 \times 10^{-16}$ M. Consequently, the 67 serum samples involved in our study were classified into the “Normal” group and the “Cancer” group by CART analysis.

[00206] The “Normal” group was composed of the samples from “Normal-2” which included a total of 28 samples, 25 from normal subjects and 3 from cancer patients. Thus, by using the combination of THSMB and FTH1 for OSCC prediction, the overall specificity is 78% (25/35). The “Cancer” group was composed of the samples from “Cancer-1” and “Cancer-2”. There are a total of 39 samples assigned in the final “Cancer” group, 29 from cancer patients and 10 from normal subjects. Therefore, by using the combination of these two serum mRNA for OSCC prediction, the overall sensitivity is 91% (29/32, in cancer group) and specificity is 78% (25/35, in normal group).

EXAMPLE 13: RNA ISOLATION, AMPLIFICATION AND GENE EXPRESSION PROFILING FROM SALIVA OF OSCC PATIENTS

Patient Selection.

[00207] OSCC patients were recruited from Medical Centers at University of California, Los Angeles (UCLA); University of Southern California (USC), Los Angeles, CA; and University of California San Francisco, San Francisco, CA.

[00208] Thirty-two patients with documented primary T1 or T2 OSCC were included. All of the patients had recently received diagnoses of primary disease and had not received any prior treatment in the form of chemotherapy, radiotherapy, surgery, or alternative remedies.

[00209] An equal number of age- and sex-matched subjects with comparable smoking histories were selected as a control group. Among the two subject groups, there were no significant differences in terms of mean age: OSCC patients, 49.8 ± 7.6 years; normal subjects, 49.1 ± 5.9 years (Student's *t* test,

$P > 0.80$); gender ($P > 0.90$); or smoking history ($P > 0.75$). No subjects had a history of prior malignancy, immunodeficiency, autoimmune disorders, hepatitis, or HIV infection. All of the subjects signed the institutional review board-approved consent form agreeing to serve as saliva donors for the experiments.

Saliva Collection and RNA Isolation.

[00210] Unstimulated saliva samples were collected between 9 a.m. and 10 a.m. with previously established protocols [38]. Subjects were asked to refrain from eating, drinking, smoking, or oral hygiene procedures for at least 1 hour before the collection. Saliva samples were centrifuged at $2,600 \times g$ for 15 minutes at 4°C .

[00211] The supernatant was removed from the pellet and treated with RNase inhibitor (Superase-In, Ambion Inc., Austin, TX). RNA was isolated from $560 \mu\text{L}$ of saliva supernatant with QIAamp Viral RNA kit (Qiagen, Valencia, CA). Aliquots of isolated RNA were treated with RNase-free DNase (DNaseI-DNA-free, Ambion Inc.) according to the manufacturer's instructions. The quality of isolated RNA was examined by RT-PCR for three cellular maintenance gene transcripts: glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), actin- β (*ACTB*), and ribosomal protein S9 (*RPS9*). Only those samples exhibiting PCR products for all three mRNAs were used for subsequent analysis.

[00212] On average, $54.2 \pm 20.1 \text{ ng}$ ($n = 64$) of total RNA was obtained from $560 \mu\text{L}$ of saliva supernatant. There was no significant difference in total RNA quantity between the OSCC and matched controls (t test, $P = 0.29$, $n = 64$). RT-PCR results demonstrated that all of the saliva samples ($n = 64$) contained transcripts from three genes (*GAPDH*, *ACTB*, and *RPS9*), which were used as quality controls for human salivary RNAs [55]. A consistent amplifying magnitude (658 ± 47.2 , $n = 5$) could be obtained after two rounds of RNA amplification. On average, the yield of biotinylated cRNA was $39.3 \pm 6.0 \mu\text{g}$ (n

= 20). There were no significant differences of the cRNA quantity yielded between the OSCC and the controls (t test, $P = 0.31, n = 20$).

EXAMPLE 14: MICROARRAY PROFILING OF MRNA OF SALIVA FROM OSCC PATIENTS

5 [00213] Saliva from 10 OSCC patients (7 male, 3 female; age, 52 ± 9.0 years) and from 10 gender- and age-matched normal donors (age, 49 ± 5.6 years) was used for a microarray study. Isolated RNA from saliva was subjected to linear amplification by RiboAmp RNA Amplification kit (Arcturus, Mountain View, CA). The RNA amplification efficiency was measured by using
10 control RNA of known quantity ($0.1 \mu\text{g}$) running in parallel with the 20 samples in five independent runs.

Microarray Analysis.

[00214] Following previously reported protocols [55], the Human Genome U133A Array (HG U133A, Affymetrix, Santa Clara, CA) was applied for gene
15 expression analysis. The arrays were scanned and the fluorescence intensity was measured by Microarray Suit 5.0 software (Affymetrix, Santa Clara, CA); the arrays were then imported into DNA-Chip Analyzer software (<http://www.dchp.org>) for normalization and model-based analysis [60]. S-plus 6.0 (Insightful, Seattle, WA) was used to carry out all statistical tests.

20 [00215] Three criteria were used to determine differentially expressed gene transcripts. First, probe sets on the array that were assigned as "absent" call in all samples were excluded. Second, a two-tailed Student's t test was used for comparison of average gene expression signal intensity between the OSCCs ($n = 10$) and controls ($n = 10$). The critical level of 0.05 was defined
25 for statistical significance. Third, fold ratios were calculated for those gene transcripts that showed statistically significant difference ($P < 0.05$). Only those gene transcripts that exhibited at least 2-fold change were included for further analysis.

[00216] The HG U133A microarrays were used to identify the difference in
30 salivary RNA profiles between cancer patients and matched normal subjects.

Among the 10,316 transcripts included by the previously described criteria, we identified 1,679 transcripts with P value less than 0.05. Among these transcripts, 836 were up-regulated and 843 were down-regulated in the OSCC group. These transcripts observed were unlikely to be attributable to chance alone (2 test, $P < 0.0001$), considering the false positives with $P < 0.05$. Using a predefined criteria of a change in regulation >3 -fold in all 10 OSCC saliva specimens and a cutoff of P value < 0.01 , 17 mRNA, were identified showing significant up-regulation in OSCC saliva. 17 transcripts showed a change in regulation >3 -fold in all 10 OSCC saliva specimens, and a more stringent cutoff of P value < 0.01 . It should be noted that these 17 salivary mRNA are all up-regulated in OSCC saliva, whereas there are no mRNAs found down-regulated with the same filtering criteria. The biological functions of these genes and their products are presented in Table 7 showing Salivary mRNA up-regulated (>3 -fold, $P < 0.01$) in OSCC identified by microarray

Table 7

Gene symbol	Gene name	GenBank accession No.	Locus	Gene functions
B2M _	β -2-microglobulin	NM_04048	15q21-q22.2	Antiapoptosis; antigen presentation
DUSP1	Dual specificity phosphatase 1	NM_04417	5q34	Protein modification; signal transduction oxidative stress;
FTH1	Ferritin, heavy polypeptide 1	NM_02032	11q13	Iron ion transport; cell proliferation
G0S2	Putative lymphocyte G0-G1 switch gene	NM_015714	1q32.2-q41	Cell growth and/or maintenance; regulation of cell cycle
GADD45 B	Growth arrest and DNA-damage-inducible β	NM_015675	19p13.3	Kinase cascade; apoptosis
H3F3A	H3 histone, family 3A	BE869922	1q41	DNA binding activity
HSPC016	Hypothetical protein HSPC016	BG167522	3p21.31	Unknown
IER3	Immediate early response 3	NM_003897	6p21.3	Embryogenesis; morphogenesis; apoptosis; cell growth and maintenance
IL1B	Interleukin 1 β	M15330	2q14	Signal transduction; proliferation; inflammation apoptosis
IL8	Interleukin 8	NM_000584	4q13-q21	Angiogenesis; replication; calcium-mediated signaling pathway; cell adhesion; chemotaxis cell cycle arrest; immune response
MAP2K3	Mitogen-activated protein kinase kinase 3	AA780381	17q11.2	Signal transduction; protein modification
OAZ1	Ornithine decarboxylase antizyme 1	D87914	19p13.3	Polyamine biosynthesis
PRG1	Proteoglycan 1, secretory granule	NM_002727	10q22.1	Proteoglycan
RGS2	Regulator of G-protein signaling 2, 24 kda	NM_002923	1q31	Oncogenesis; G-protein signal transduction
S100P	S100 calcium binding protein P	NM_005980	4p16	Protein binding; calcium ion binding
SAT	Spermidine/spermine N1-acetyltransferase	NM_002970	Xp22.1	Enzyme, transferase activity
EST	highly similar ferritin light chain	BG537190		Iron ion homeostasis, ferritin complex

[00217] The Human Genome U133A microarrays were used to identify the difference in RNA expression patterns in saliva from 10 cancer patients and 10 matched normal subjects. Using a criteria of a change in regulation >3-fold in all 10 OSCC saliva specimens and a cutoff of P value < 0.01, we identified 17 mRNA, showing significant up-regulation in OSCC saliva.

EXAMPLE 15: Q-PCR VALIDATION AND QUANTITATION ANALYSIS OF MICROARRAY PROFILING FROM CELL-FREE SALIVA OF OSCC PATIENTS

[00218] Quantitative polymerase chain reaction (qPCR) was performed to validate a subset of differently expressed transcripts identified by the microarray analysis of Example 14.

Quantitative Polymerase Chain Reaction Validation

[00219] cDNA from the original and unamplified salivary RNA. was synthesized using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) and random hexamers as primer (Applied Biosystems). The qPCR reactions were performed in an iCycler PCR system' with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Primer sets were designed by using PRIMER3 software.

[00220] All of the reactions were performed in triplicate with customized conditions for specific products. The initial amount of cDNA/RNA of a particular template was extrapolated from the standard curve as described previously [32]. This validation completed by testing all of the samples ($n= 64$) including those 20 previously used for microarray study. Wilcoxon Signed Rank test was used for statistical analysis.

[00221] Quantitative PCR was performed to validate the microarray findings on an enlarged sample size including saliva from 32 OSCC patients and 32 matched controls. Nine candidates of salivary mRNA biomarkers: *DUSP1*, *GADD45B*, *H3F3A*, *IL1B*, *IL8*, *OAZ1*, *RGS2*, *SIOOP*, and *SAT* were selected based on their reported cancer association reported in Table 7. Table 8 presents the

quantitative alterations of the above nine candidates in saliva from OSCC patients, determined by qPCR.

Table 8

Gene symbol	Primer sequence (5' to 3')	Validated *	P value	Mean fold increase
DUSP1	F: SEQ ID NO: 35	Yes	0.039	2.60
	R: SEQ ID NO: 36			
H3F3A	F: SEQ ID NO: 37	Yes	0.011	5.61
	R: SEQ ID NO: 38			
IL1B	F: SEQ ID NO: 39	Yes	0.005	5.48
	R: SEQ ID NO: 40			
IL8	F: SEQ ID NO: 41	Yes	0.000	24.3
	R: SEQ ID NO: 42			
OAZ1	F: SEQ ID NO: 43	Yes	0.009	2.82
	R: SEQ ID NO: 44			
S100P	F: SEQ ID NO: 45	Yes	0.003	4.88
	R: SEQ ID NO: 46			
SAT	F: SEQ ID NO: 47	Yes	0.005	2.98
	R: SEQ ID NO: 48			
GADD45B	F: SEQ ID NO: 49	No	0.116	
	R: SEQ ID NO: 50			
RGS2	F: SEQ ID NO: 51	No	0.149	
	R: SEQ ID NO: 52			

Seven of the nine potential candidate were validated by qPCR ($P < 0.05$). *

- 5 Wilcoxon's Signed Rank test: if $P < 0.05$, validated (Yes); if $P \geq 0.05$, not validated (No)

[00222] The results confirmed that transcripts of 7 of the 9 candidate mRNA (78%), DUSP1, H3F3A, IL1B, IL8, OAZ1, S100P, and SAT, were significantly elevated in the saliva of OSCC patient (Wilcoxon Signed-Rank test, $P < 0.05$).

- 10 We did not detect the statistically significant differences in the amount of RGS2 ($P = 0.149$) and GADD45B ($P = 0.116$) by qPCR. The validated seven genes could be classified in three ranks by the magnitude of increase: high up-regulated mRNA including IL8 (24.3-fold); moderate up-regulated mRNAs

including H3F3A (5.67-fold), IL1B (5.48), and STUP (4.88-fold); and low up-regulated mRNAs including DUSP1 (2.60-fold), OAZ1 (2.82-fold), and SAT (2.98-fold).

5 EXAMPLE 16: ROC AND SENSITIVITY/SPECIFICITY ANALYSIS

[00223] Using the qPCR results, Receiver Operating Characteristic (ROC) curve analyses was performed [82] by S-plus 6.0 to evaluate the predictive power of each of the biomarkers identified in the Example 15.

Receiver Operating Characteristic Curve Analysis and Prediction Models.

10 [00224] The optimal cutpoint was determined for each biomarker by searching for those that yielded the maximum corresponding sensitivity and specificity. ROC curves were then plotted on the basis of the set of optimal sensitivity and specificity values. Area under the curve was computed via numerical integration of the ROC curves. The biomarker that has the largest
15 area under the ROC curve was identified as having the strongest predictive power for detecting OSCC.

[00225] Next, multivariate classification models were constructed to determine the best combination of salivary markers for cancer prediction. Firstly, using the binary outcome of the disease (OSCC) and nondisease
20 (normal) as dependent variables, we constructed a logistic regression model controlling for patient age, gender, and smoking history. The backward stepwise regression [61] was used to find the best final model.

[00226] Leave-one-out cross-validation was used to validate the logistic regression model. The cross-validation strategy first removes one observation
25 and then fits a logistic regression model from the remaining cases with all of the markers. Stepwise model selection is used for each of these models to remove variables that do not improve the model. Subsequently, the marker values were used for the case that was left out to compute a predicted class for that observation. The cross-validation error rate is then the number of
30 samples predicted incorrectly divided by the number of samples.

[00227] The ROC curve, illustrated in Figure 11, was then computed for the logistic model by a similar procedure, with the fitted probabilities from the model as possible cutpoints for computation of sensitivity and specificity.

[00228] The detailed statistics of the area under the receiver operator characteristics (ROC) curves, the threshold values, and the corresponding sensitivities and specificities for each of the seven potential salivary mRNA biomarkers for OSCC are listed in Table 9 showing the ROC curve analysis of OSCC-associated salivary mRNA biomarkers

10

Table 9

Biomarker	Area under ROC curve	Threshold/cutoff (M)	Sensitivity (%)	Specificity (%)
<i>DUSP1</i>	0.65	8.35E-17	59	75
<i>H3F3A</i>	0.68	1.58E-15	53	81
<i>IL1B</i>	0.70	4.34E-16	63	72
<i>IL8</i>	0.85	3.19E-18	88	81
<i>OAZ1</i>	0.69	7.42E-17	100	38
<i>S100P</i>	0.71	2.11E-15	72	63
<i>SAT</i>	0.70	1.56E-15	81	56

[00229] Utilizing the qPCR results, we conducted ROC curve analyses to evaluate the predictive power of each of the biomarkers. The optimal cutpoint was determined yielding the maximum corresponding sensitivity and specificity. The biomarker that has the largest area under the ROC curve was identified as having the strongest predictive power for detecting OSCC.

[00230] The data showed IL8 mRNA performed the best among the seven potential biomarkers for predicting the presence of OSCC. The calculated area under the ROC curve for IL8 was 0.85. With a threshold value of 3.19E - 18 mol/L, *IL8* mRNA in saliva yields a sensitivity of 88% and a specificity of 81% to distinguish OSCC from the normal.

[00231] To demonstrate the utility of salivary mRNAs for disease discrimination, two classification/prediction models were examined. A logistic regression model was built based on the four of the seven validated

biomarkers, IL1B, OAZ1, SAT, and IL8, which in combination provided the best prediction (Table 10). Table 10 shows salivary for OSCC selected by logistic regression model

5

Table 10

Biomarker	Coefficient value	SE	P value
Intercept	-4.79	1.51	0.001
<i>IL1B</i>	5.10E +19	2.68E+19	0.062
<i>OAZ1</i>	2.18E+20	1.08E+20	0.048
<i>SAT</i>	2.63E+19	1.10E+19	0.020
<i>IL8</i>	1.36E+17	4.75E+16	0.006

[00232] The logistic regression model was built based on the four of seven validated biomarkers (IL1B, OAZ1, SAT, and IL8) that, in combination, provided the best prediction. The coefficient values are positive for these four markers, indicating that the synchronized increase in their concentrations in saliva increased the probability that the sample was obtained from an OSCC subject.

[00233] The coefficient values are positive for these four markers, indicating that the synchronized rise in their concentrations in saliva increased the probability that the sample was obtained from an OSCC subject. The leave-one-out cross-validation error rate based on logistic regression models was 19% (12 of 64). All but one (of the 64) of the models generated in the leave-one-out analysis used the same set of four markers found to be significant in the full data model specified in Table 10.

[00234] The ROC curve was computed for the logistic regression model. Using a cutoff probability of 50%, we obtained a sensitivity of 91% and a specificity of 91%. The calculated area under the ROC curve was 0.95 for the logistic regression model (Fig. 11).

Tree-based classification model, classification and regression tree (CART).

[00235] A second model, a tree-based classification model, classification and regression tree (CART) model," was generated. The CART model was

constructed by S-plus 6.0 with the validated mRNA biomarkers as predictors. CART fits the classification model by binary recursive partitioning, in which each step involves searching for the predictor variable that results in the best split of the cancer *versus* the normal groups [62]. CART used the entropy function with splitting criteria determined by default settings for S-plus. By this approach, the parent group containing the entire samples ($n = 64$) was subsequently divided into cancer groups and normal groups. Our initial tree was pruned to remove all splits that did not result in sub-branches with different classifications.

[00236] Results are shown in the diagram of Fig. 12. Our fitted CART model used the salivary mRNA concentrations of IL8, H3F3A, and SAT as predictor variables for OSCC. IL8, chosen as the initial split, with a threshold of $3.14E - 18$ mol/L, produced two child groups from the parent group containing the total 64 samples. 30 samples with the IL8 concentration $< 3.14E - 18$ mol/L were assigned into "Normal-1," whereas 34 with IL8 concentration $\geq 3.14E - 18$ were assigned into "Cancer-1". The "Normal-1" group was further partitioned by SAT with a threshold of $1.13E - 14$ mol/L.

[00237] The resulting subgroups, "Normal-2" contained 25 samples with SAT concentration $< 1.13E - 14$ mol/L, and "Cancer-2" contained 5 samples with SAT concentration $\geq 1.13E - 14$ mol/L. Similarly, the "Cancer-1" group was further partitioned by H3F3A with a threshold of $2.07E - 16$ mol/L. The resulting subgroups, "Cancer-3" contained 27 samples with H3F3A concentration $\geq 2.07E - 16$ mol/L, and "Normal-3" group contained 7 samples with H3F3A concentration $< 2.07E - 16$ mol/L.

[00238] Consequently, the 64 saliva samples involved in our study were classified into the "Cancer" group and the "Normal" group by CART analysis. The "Normal" group was composed of the samples from "Normal-2" and those from "Normal-3". There are a total of 32 samples assigned in the "Normal" group, 29 from normal subjects and 3 from cancer patients.

[00239] Thus, by using the combination of IL8, SAT, and H3F3A for OSCC prediction, the overall sensitivity is 90.6% (29 of 32). The "Cancer" group was

composed of the samples from "Cancer-2" and "Cancer-3". There are a total of 32 samples assigned in the final "Cancer" group, 29 from cancer patients and 3 from normal subjects. Therefore, by using the combination of these three salivary mRNA biomarkers for OSCC prediction, the overall specificity is
 5 90.6% (29 of 32).

[00240] In summary the present disclosure refers to a method to detect a biomarker in saliva wherein the biomarker is an extracellular mRNA, comprises detecting the extracellular mRNA in the cell-free saliva; transcriptome analysis of saliva comprises detecting a transcriptome pattern
 10 in the cell-free saliva; a method to detect genetic alterations in an organ or in a gene in the organ by analyzing saliva, comprises detecting a transcriptome pattern and/or the mRNA profiling of the gene in cell-free saliva; a method to diagnose an oral or systemic pathology disease or disorder in a subject, comprises: detecting profile of a biomarker associated with the pathology
 15 disease or disorder, in particular mRNA and/or protein, in cell-free saliva and/or serum; kits comprising identifier for at least one biomarker for performing at least one of the methods; and use of salivary biomarker salivary and/or serum mRNAs as biomarkers for oral and/or systemic pathology, disease or disorder.

20 **[00241]**

[00242] The present disclosure has been explained with reference to specific embodiments. Other embodiments will be apparent to those of ordinary skill in the art in view of the foregoing description. The scope of protection of the
 25 present disclosure is defined by the appended claims.

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DEMANDES OU BREVETS VOLUMINEUX

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

CECI EST LE TOME __1__ DE __2__

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__

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

Claims:

1. A method to detect mRNA in saliva, the saliva including a cell phase and a cell-free supernatant, the method comprising:
 providing a saliva supernatant;
 isolating mRNA from the saliva supernatant;
 and amplifying mRNA thereby detecting mRNA in the saliva supernatant.
2. The method of claim 1, wherein the saliva is unstimulated saliva.
3. A method to perform transcriptome analysis of saliva, the saliva including a cell phase and a cell-free supernatant phase, the method comprising
 providing a saliva supernatant;
 isolating mRNA from the saliva supernatant; and amplifying mRNA thereby detecting a transcriptome pattern in the saliva supernatant.
4. The method of claim 3, wherein detecting the transcriptome pattern is performed by microarray assay.
5. The method of claim 4, wherein detecting the transcriptome pattern is performed by high-density oligonucleotide microarray assay.
6. The method of claim 3, wherein detecting the transcriptome pattern is performed by quantitative polymerase chain reaction (qPCR) analysis or reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.
7. A method to diagnose oropharyngeal squamous cell carcinoma (OSCC) in a test subject, the method comprising:
 providing a saliva supernatant from the test subject, detecting in the saliva supernatant an mRNA profile of a gene; comparing the mRNA profile of the gene with a predetermined mRNA profile of a gene, wherein the predetermined mRNA profile is an OSCC mRNA profile, and wherein the gene is selected from the group consisting of the gene coding for Interleukin 8 (IL8), Interleukin-1 beta (IL-1 β), Dual specificity phosphatase I (DUSP1), Histone H3, family 3A (H3F3A), Ornithine

decarboxylase antizyme I (OAZ I), S100 calcium binding protein P (S 100P) and Spermidine/spermine N I-acetyltransferase (SAT), wherein the expression of any one of these genes is elevated in a subject with OSCC relative to a control and wherein a statistically significant correlation between the mRNA profile and the predetermined mRNA profile is indicative of the presence of OSCC.

8. The method of claim 7, wherein the gene is the gene coding for IL8.

9. A method to diagnose oropharyngeal squamous cell carcinoma (OSCC) in a test subject, the method comprising:

providing a saliva supernatant from the test subject;

detecting in the provided saliva supernatant a transcriptome pattern, comparing the transcriptome pattern with a predetermined pattern, wherein the predetermined pattern is an mRNA pattern detected in subjects with OSCC, wherein the predetermined pattern comprises a plurality of mRNA profiles, wherein the plurality of mRNA profiles comprises a mRNA profile of each of Interleukin 8 (IL8), Interleukin-1 beta (IL1 β), Dual specificity phosphatase I (DUSP1), Histone H3, family 3A (H3F3A), Ornithine decarboxylase antizyme I (OAZ I), S100 calcium binding protein P (S 100P) and Spermidine/spermine N I-acetyltransferase (SAT), wherein the expression of these genes is elevated in a subject with OSCC relative to a control and wherein a statistically significant correlation between the transcriptome pattern and the predetermined pattern is indicative of the presence of OSCC.

10. A method to diagnose oropharyngeal squamous cell carcinoma (OSCC) in a test subject, the method comprising

providing a saliva supernatant from the test subject, isolating mRNA from the saliva supernatant and amplifying mRNA and thereby detecting in the saliva an mRNA profile of a biomarker, the biomarker selected from the group consisting of Interleukin 8 (IL8), Interleukin-1 beta (IL 1 β), Dual specificity phosphatase I (DUSP1), Histone H3, family 3A (H3F3A), Ornithine decarboxylase antizyme (OAZ I), S100 calcium binding protein P (S100P) and Spermidine/spermine NI-acetyltransferase (SAT), and comparing the profile of the detected biomarker profile with a

predetermined profile of the biomarker, wherein the expression of any one of these biomarkers is elevated in a subject with OSCC relative to a control and wherein a statistical correlation between the predetermined profile in the detected biomarker profile is diagnostic of oropharyngeal squamous cell carcinoma.

11. The method of claim 10, wherein the biomarker is selected from the group consisting of IL8, IL 1 β , OAZ1, and SAT.

12. A method for detecting oropharyngeal squamous cell carcinoma (OSCC) in a test subject the method comprising measuring the levels of Interleukin-8 (IL8) in saliva supernatant and Interleukin-6 (IL6) in blood serum, wherein the presence of IL6 and at least 600 pg/mL of IL8 is indicative of OSCC.

13. A kit for the diagnosis of oropharyngeal squamous cell carcinoma (OSCC), the kit comprising:

a biomarker control profile, wherein the control profile is derived from a subject with OSCC and at least one binding reagent, wherein the binding reagent is a polynucleotide and the binding reagent binds a biomarker, wherein the biomarker is selected from the group consisting of: Interleukin 8 (IL8), Interleukin-1 beta (IL1 B), Dual specificity phosphatase 1 (DUSP1), Histone H3, family 3A (H3F3A), Ornithine decarboxylase antizyme I (OAZ I), S100 calcium binding protein P (S100P) and Spermidine/spermine N1-acetyltransferase (SAT), and a detector for the binding reagent.

14. A method to diagnose oropharyngeal squamous cell carcinoma (OSCC) in a test subject, the method comprising:

providing a saliva supernatant from the test subject;

detecting in the provided saliva supernatant a transcriptome pattern, comparing the transcriptome pattern with a predetermined pattern, wherein the predetermined pattern is an mRNA pattern detected in subjects with OSCC, wherein the predetermined pattern comprises a plurality of mRNA profiles, wherein the plurality of mRNA profiles comprises a mRNA profile of each of Interleukin 8 (IL8), Interleukin-1 beta (IL 1B), Ornithine decarboxylase antizyme I (OAZ I), and Spermidine/spermine N I-

acetyltransferase (SAT), wherein the expression of these genes is elevated in a subject with OSCC relative to a control and wherein a statistically significant correlation between the transcriptome pattern and the predetermined pattern is indicative of the presence of OSCC.

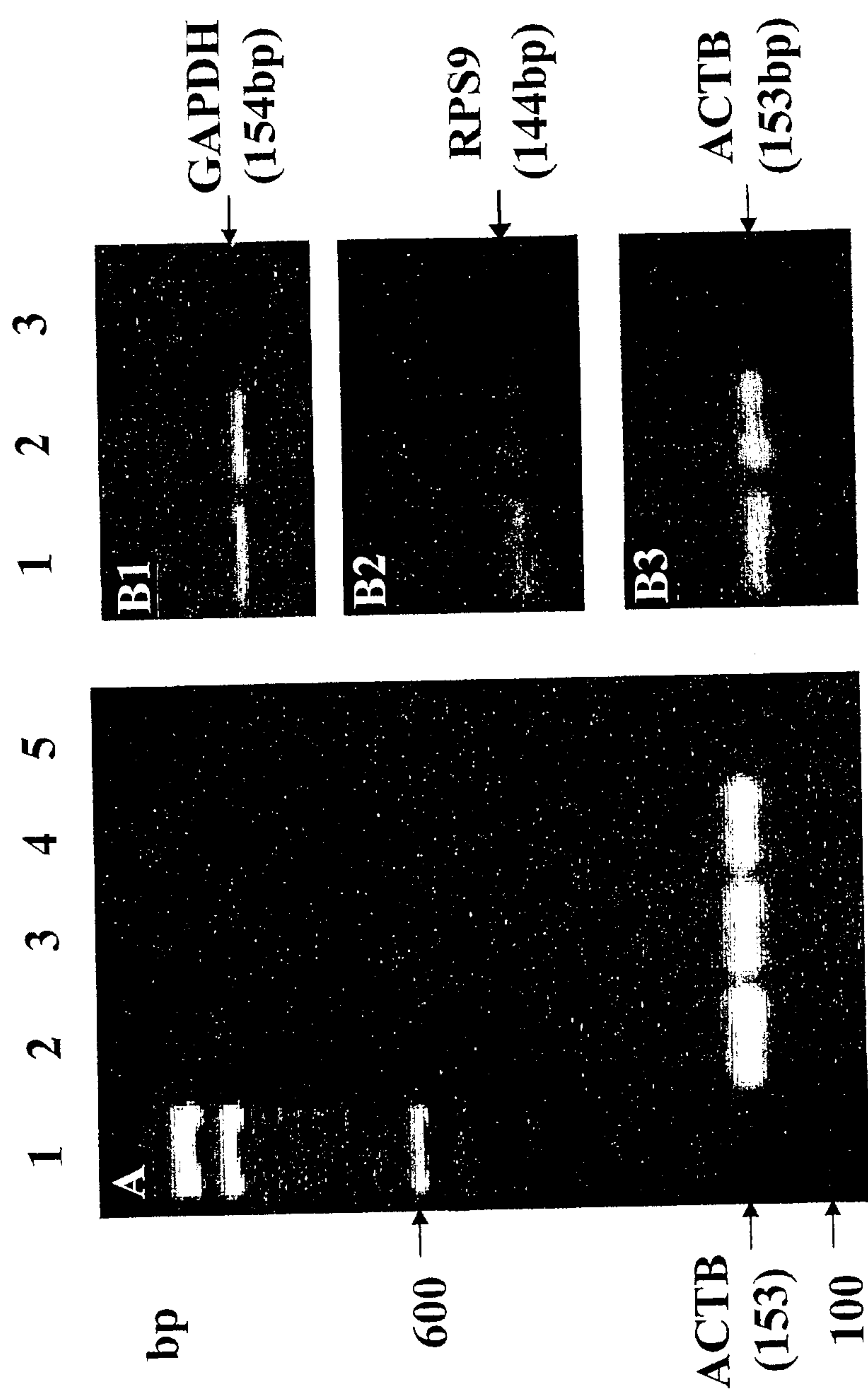


FIG. 1

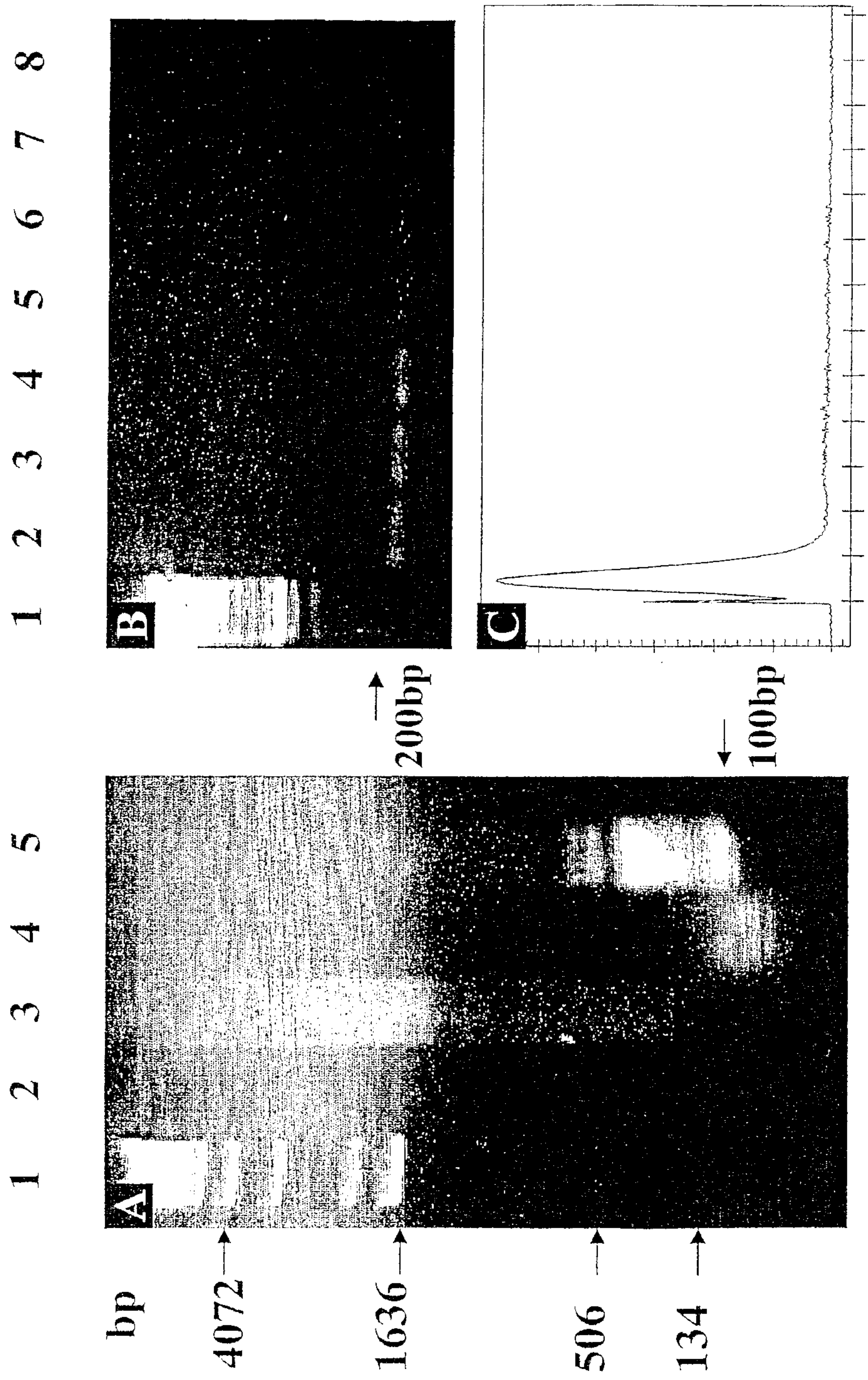


FIG. 2

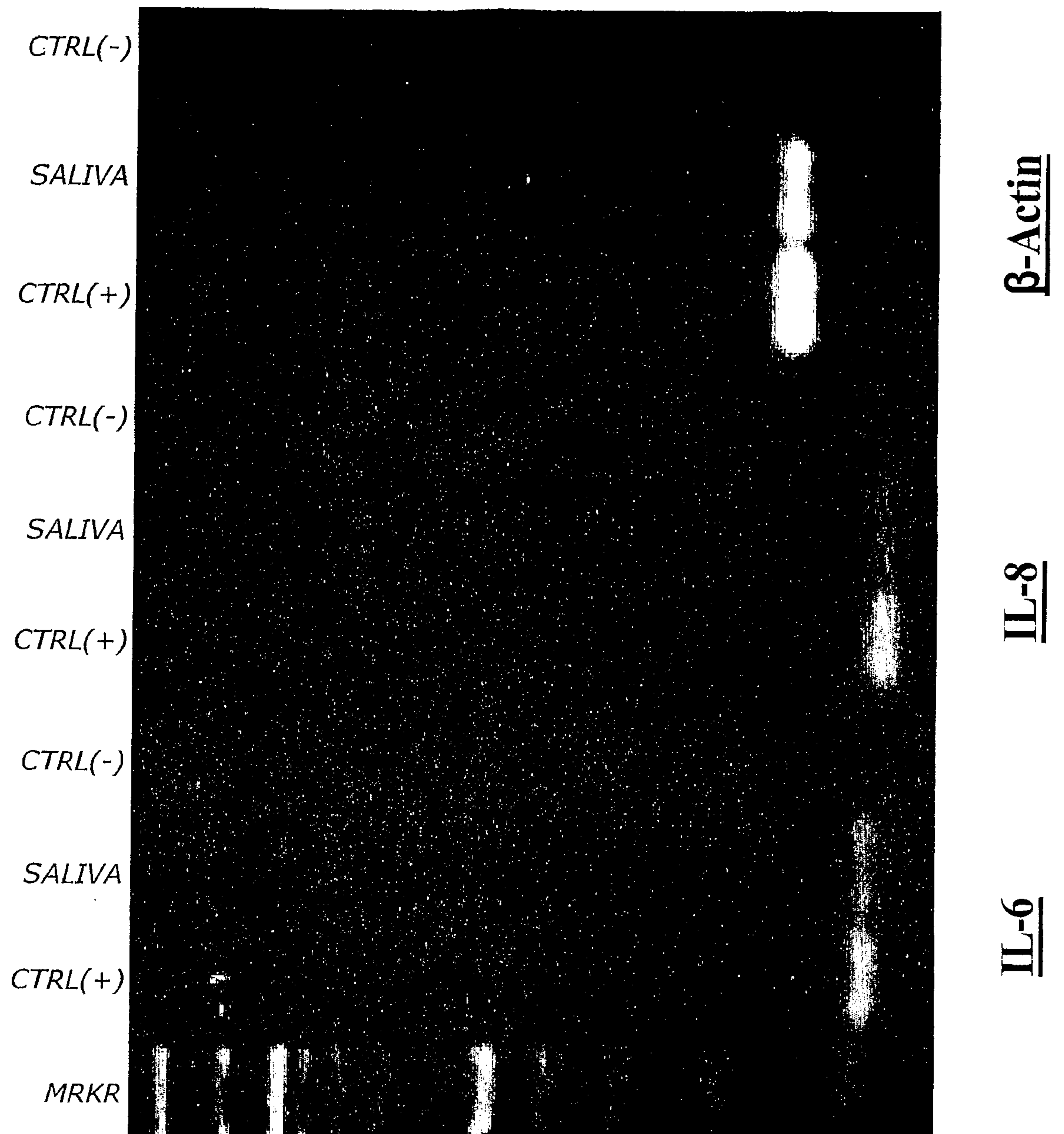


FIG. 3

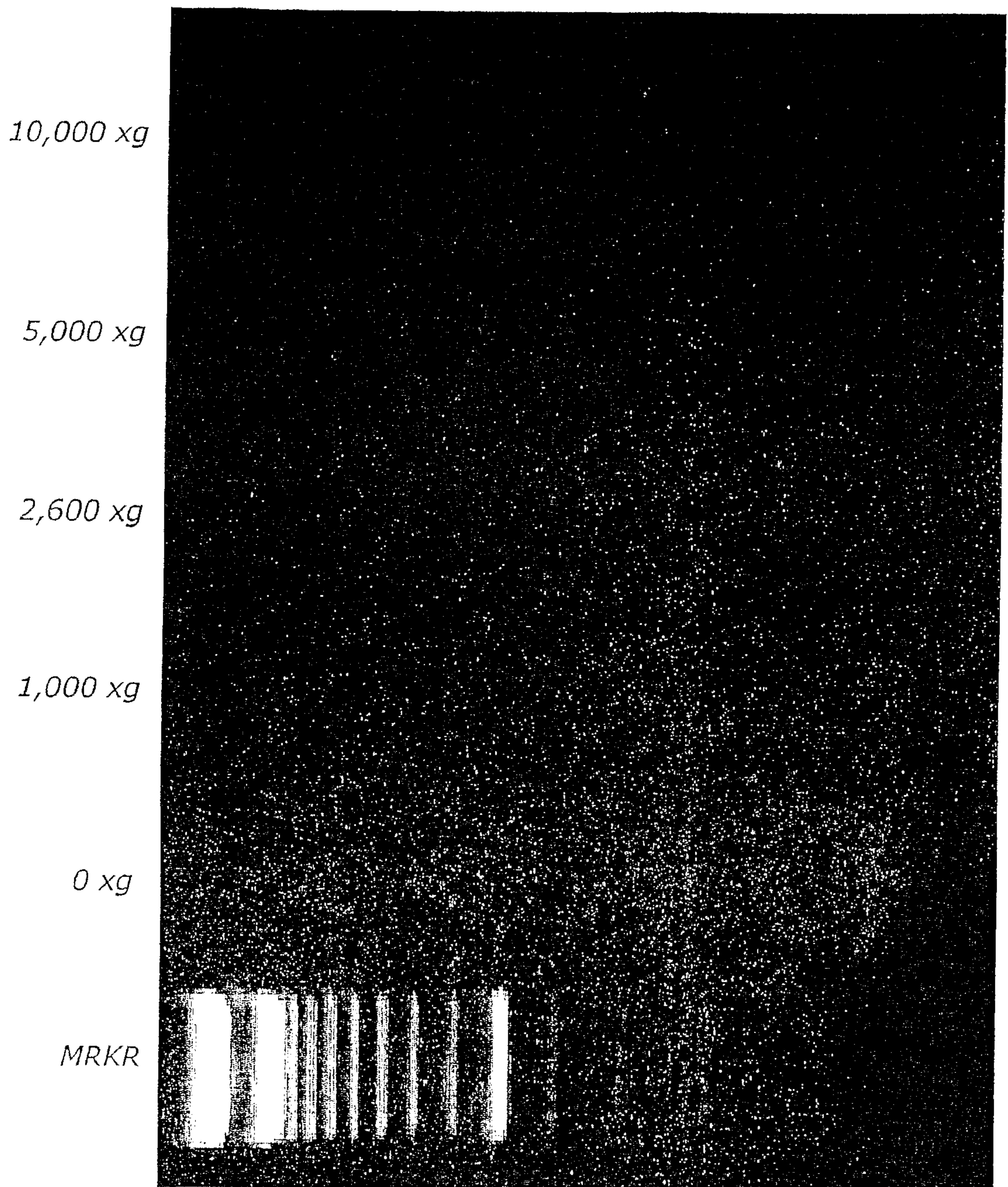


FIG. 4

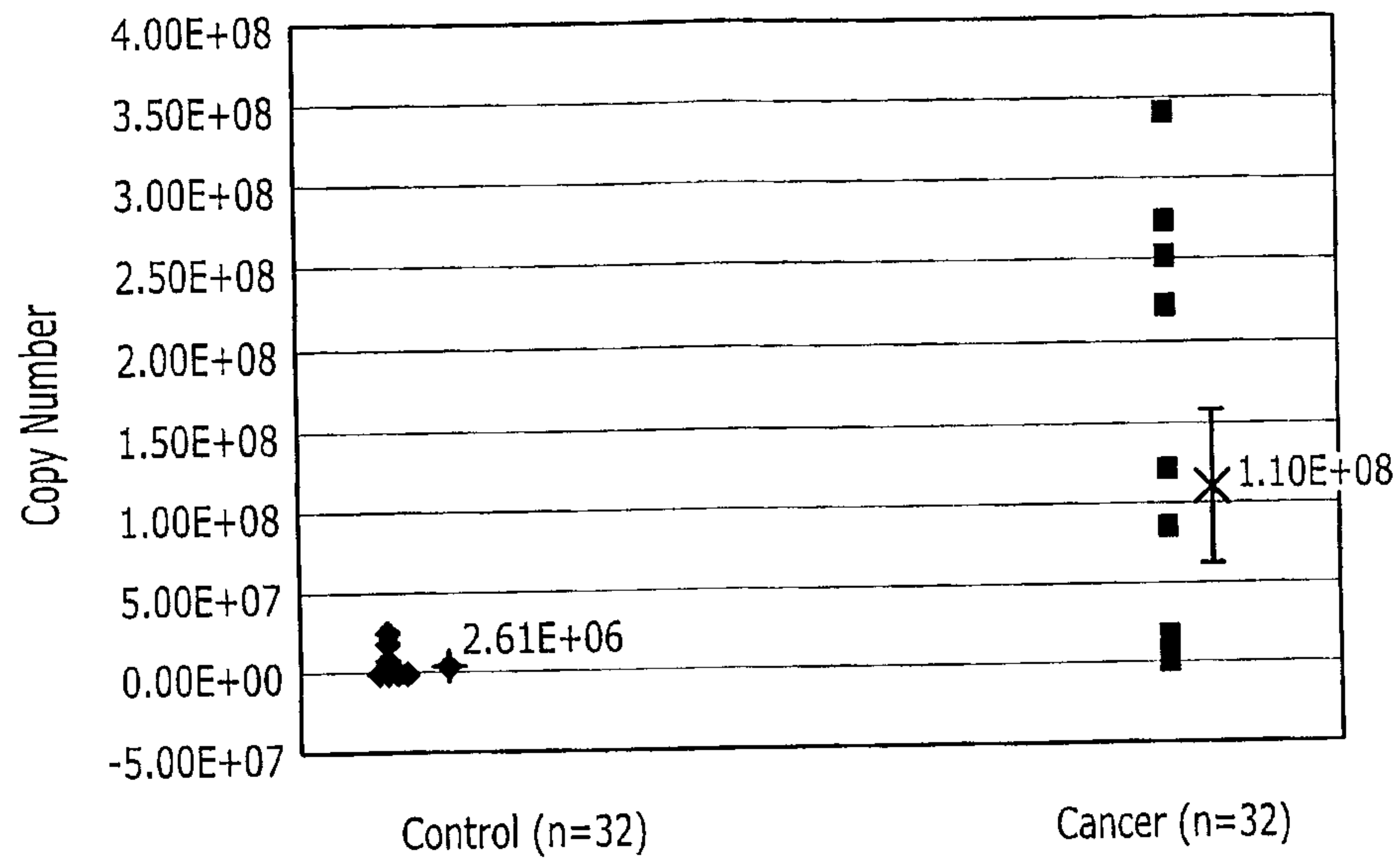
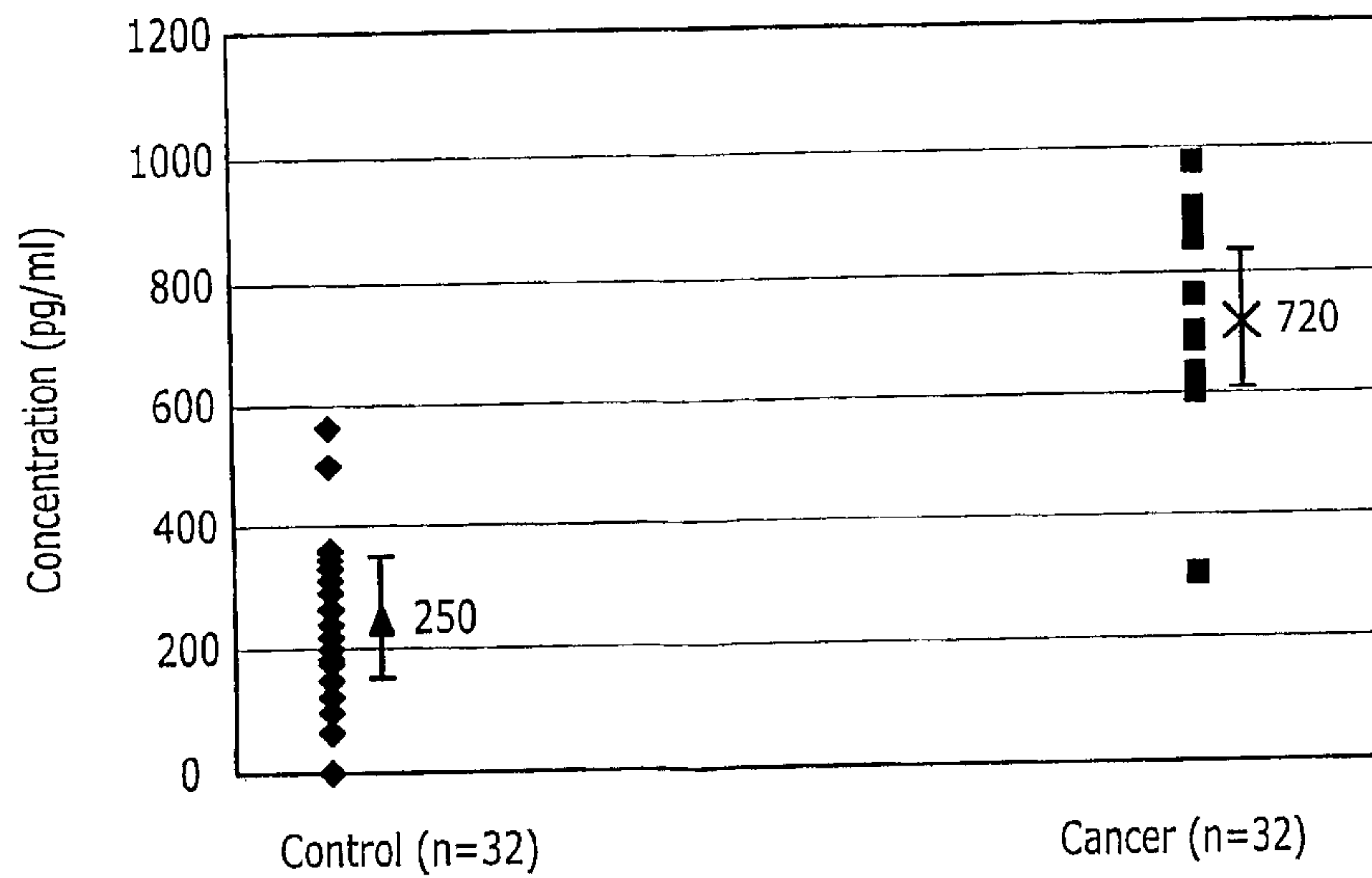
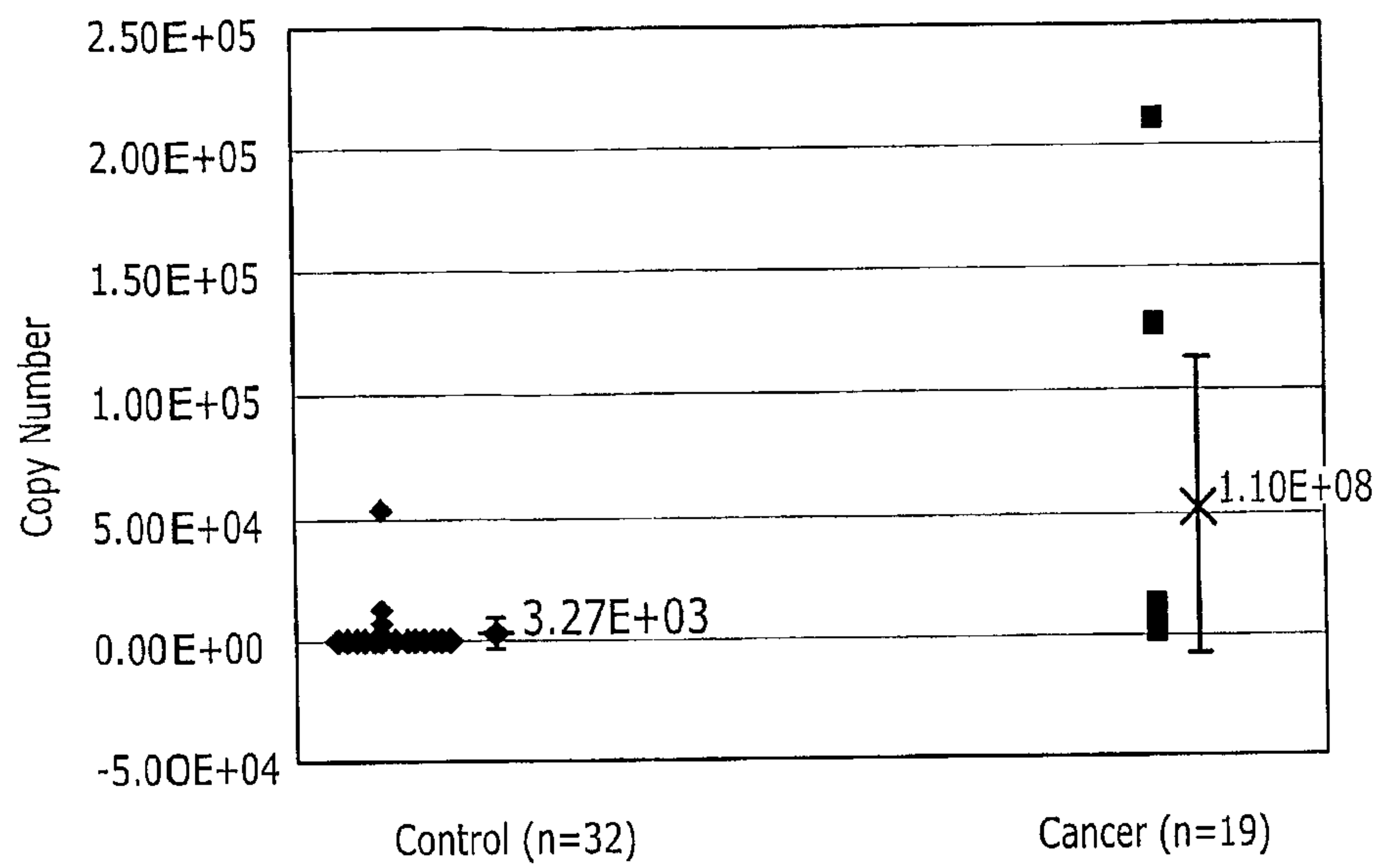
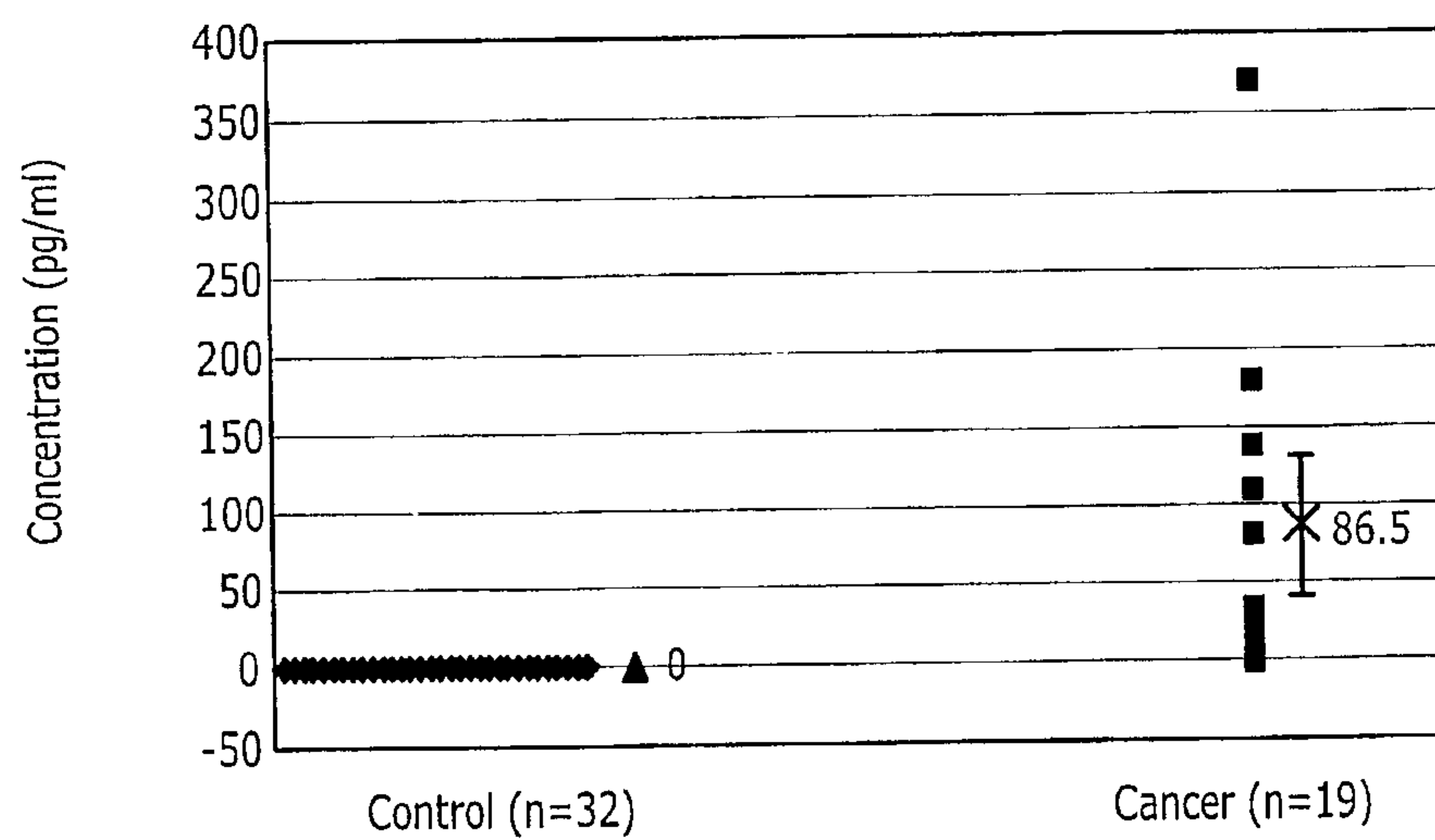
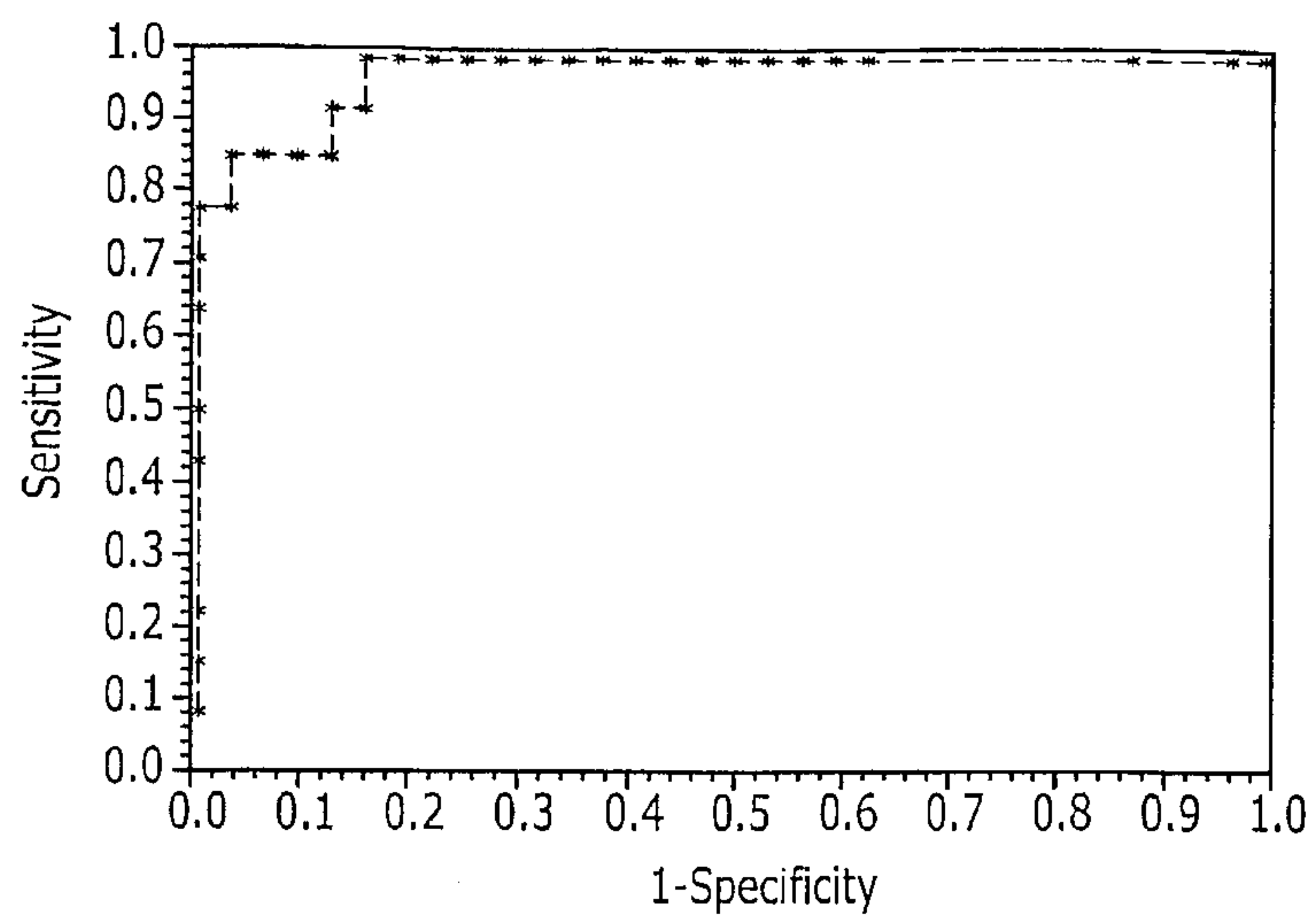
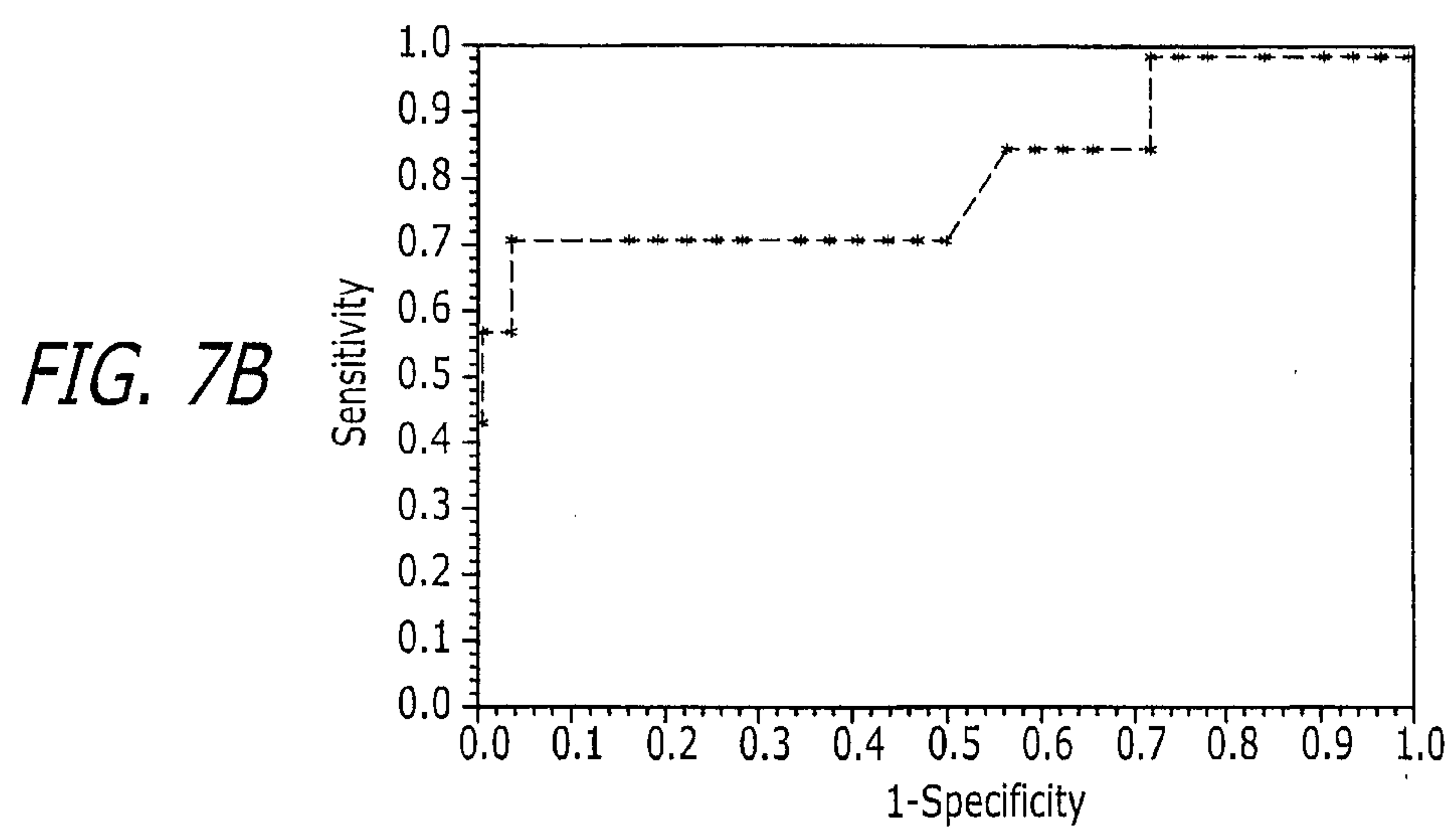
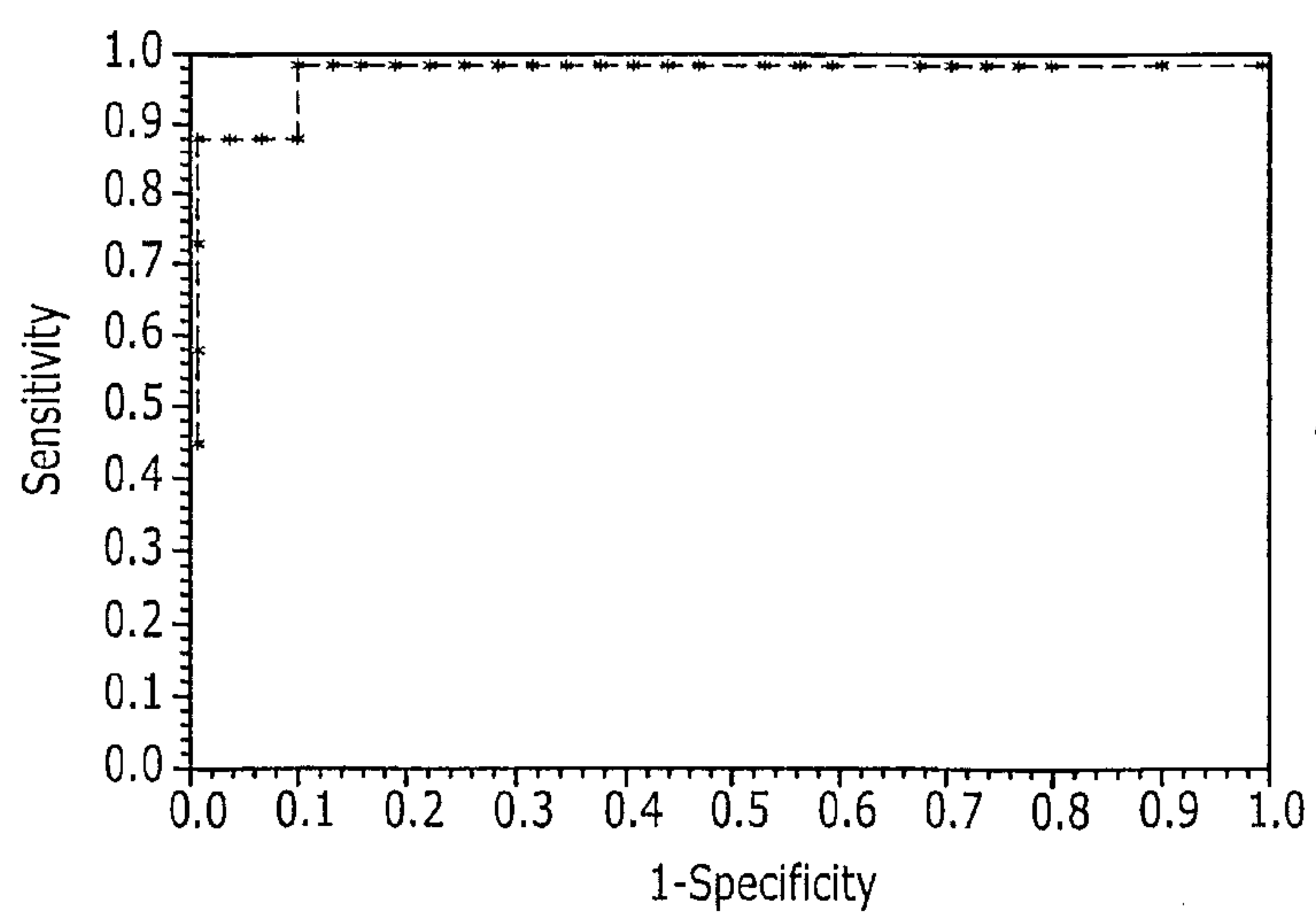
FIG. 5A*FIG. 5B*

FIG. 6A*FIG. 6B*

*FIG. 7A**FIG. 7B**FIG. 7C*

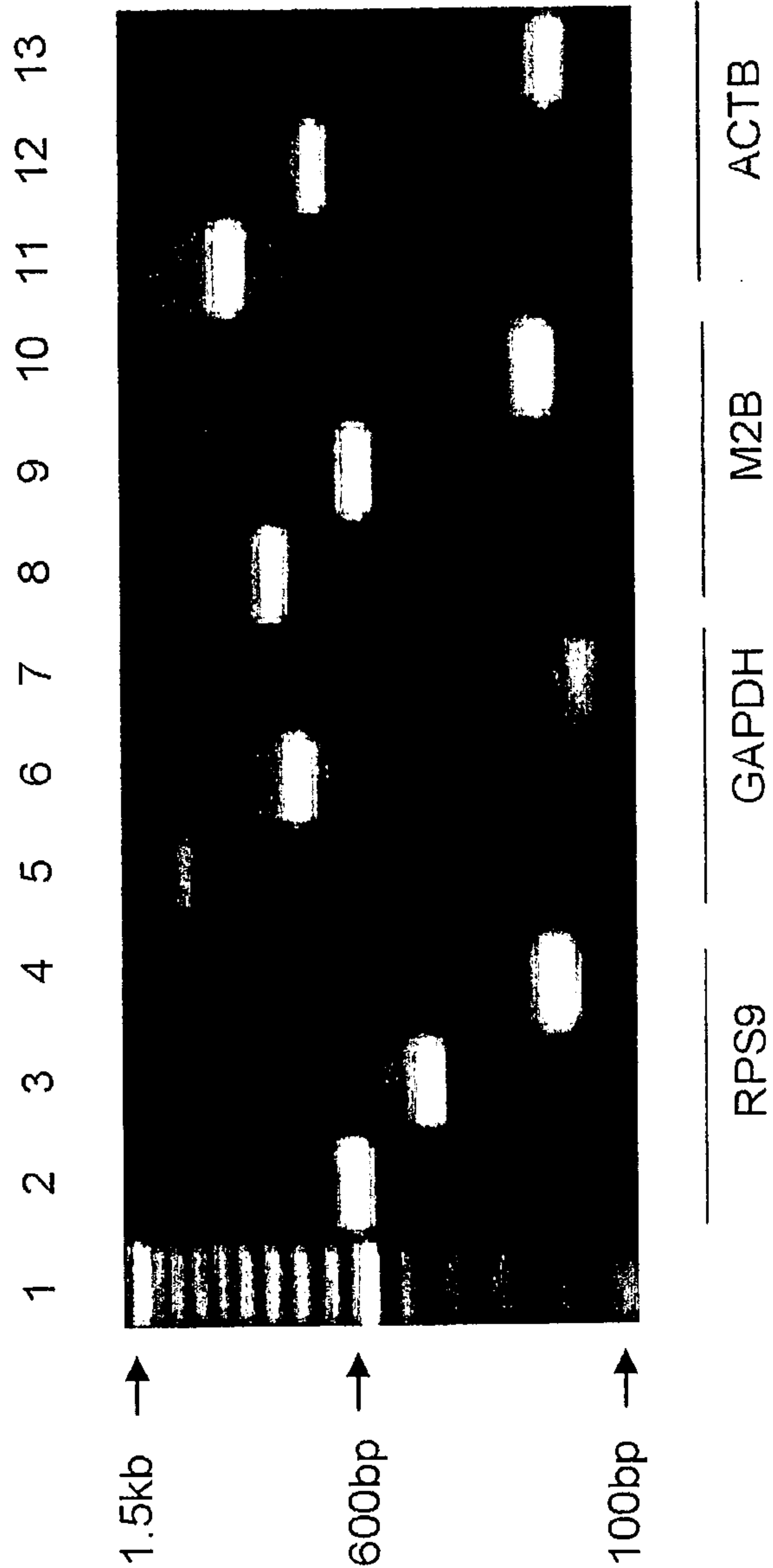


FIG. 8

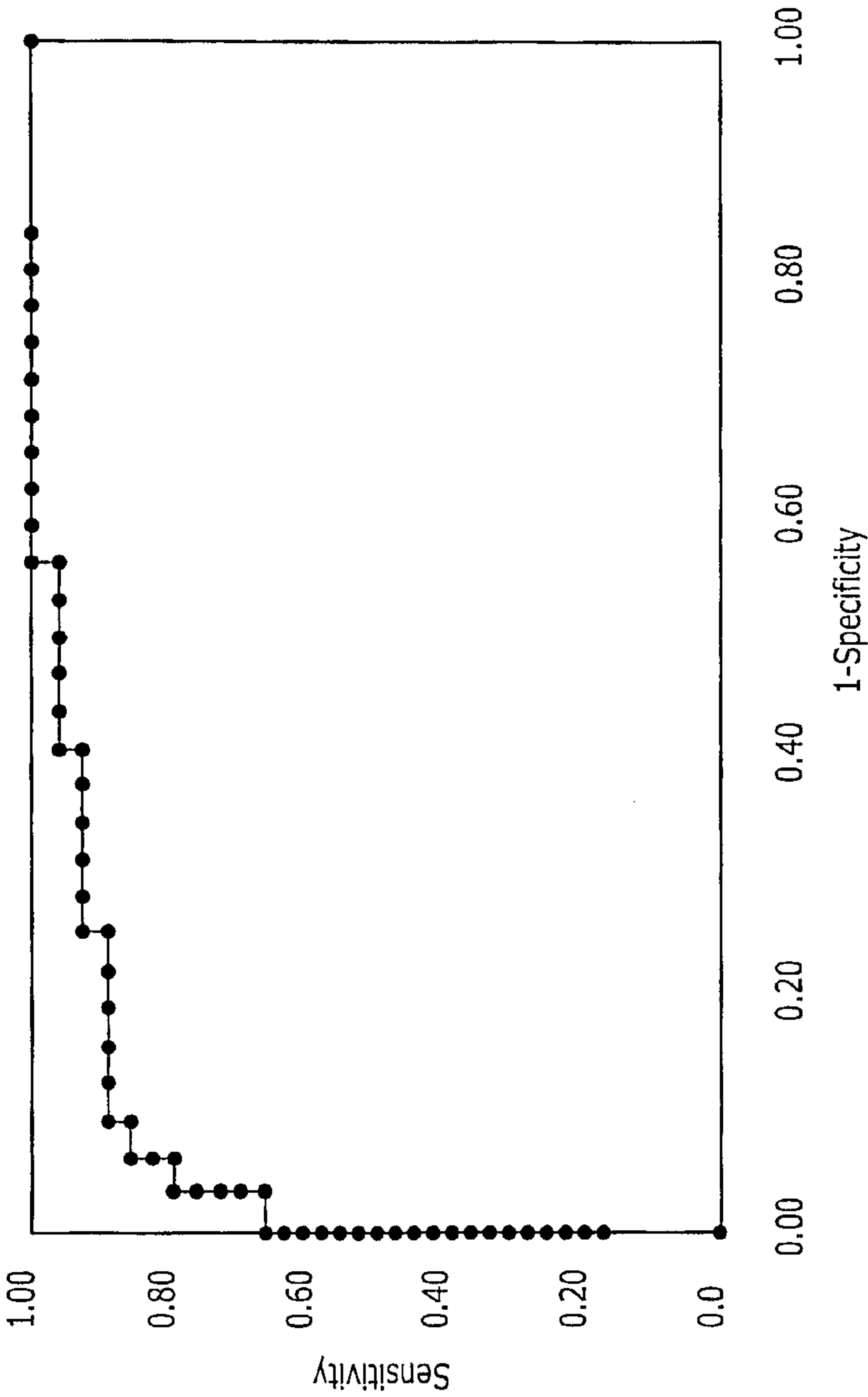


FIG. 11

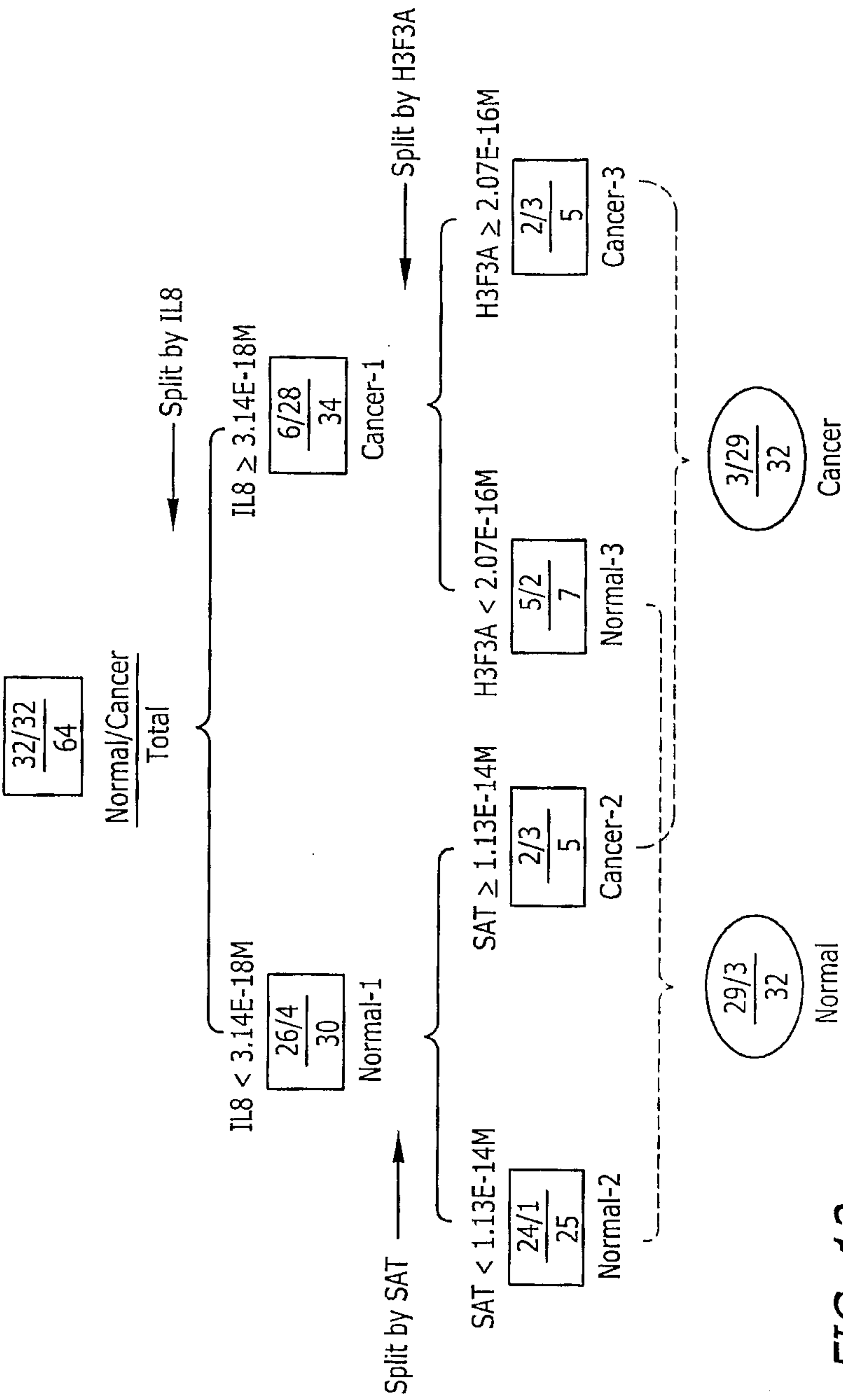


FIG. 12