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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 July 2011 (21.07.2011)

PCT

(10) International Publication Number
WO 2011/087803 A1

(51) International Patent Classification:
C07K 14/635 (2006.01) *C12Q 1/68* (2006.01)

(21) International Application Number:
PCT/US2010/061656

(22) International Filing Date:
21 December 2010 (21.12.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
12/644,284 22 December 2009 (22.12.2009) US

(71) Applicant *(for all designated States except US)*: **THERMO FINNIGAN LLC** [US/US]; 355 River Oaks Parkway, San Jose, CA 95134 (US).

(72) Inventor; and
(71) Applicant : **ORAN, Paul** [US/US]; 12007 N. 138th Street, Scottsdale, AZ 85259 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **LOPEZ, Mary, Frances** [US/US]; 21 Worcester Sq., Apt 5, Boston, MA 02118 (US). **SARRACINO, David, A.** [US/US]; 17 Harvard Road, Andover, MA 01810 (US). **PRAKASH, Amol** [IN/US]; 790 Memorial Drive, Suite 201, Cambridge, MA 02139 (US). **NELSON, Randall, W.** [US/GB]; 1607 W. Pearce Road, Phoenix, AZ 85041 (US). **KRASTINS, Bryan, E.** [US/US]; 67 Hudson Street, Somerville, MA 02143 (US). **REZAI, Taha** [US/US]; 812 Memorial Drive, Apt.1204, Cambridge, MA 02139 (US).

(74) Agent: **KATZ, Charles, B.**; Thermo Fisher Scientific Inc., 355 River Oaks Parkway, San Jose, CA 95134 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD,

[Continued on next page]

(54) Title: ASSAY FOR MONITORING PARATHYROID HORMONE (PTH) VARIANTS BY TANDEM MASS SPECTROMETRY

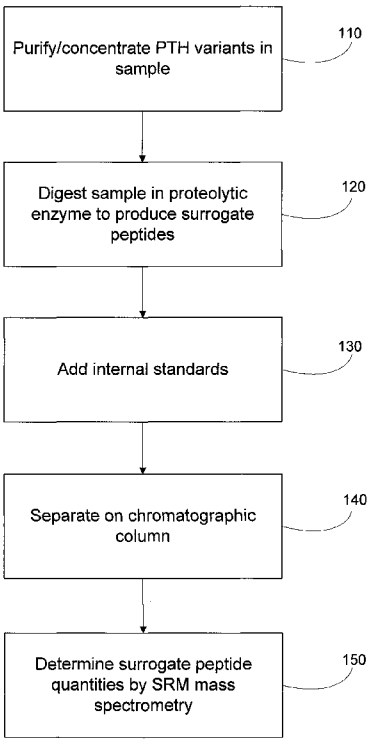


FIG. 1

(57) Abstract: Methods are described for monitoring the amounts of PTH variants in a biological sample by digesting the sample to produce surrogate peptides specific to the targeted PTH variants, and detecting and quantifying the surrogate peptides by selective reaction monitoring (SRM) mass spectrometry, using a set of precursor-to-product ion transitions optimized for sensitivity and selectivity. The PTH variants, or a portion thereof, may be concentrated in the sample by means of immunoaffmity capture or other suitable technique. The mass spectrometric method described herein enables the concurrent measurement of peptides representative of a plurality of targeted PTH variants in a single assay.

WO 2011/087803 A1

ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

ASSAY FOR MONITORING PARATHYROID HORMONE (PTH) VARIANTS BY TANDEM MASS SPECTROMETRY

FIELD OF THE INVENTION

[0001] The present invention relates generally to monitoring parathyroid hormone (PTH) in biological samples, and more particularly to a tandem mass spectrometry-based method for concurrently measuring peptides characteristic of two or more PTH variants.

BACKGROUND OF THE INVENTION

[0002] Parathyroid hormone (PTH) is produced in the parathyroid glands through the two-step conversion of pre-pro-PTH (115-amino acids) to pro-PTH (90-amino acids) to the 84-amino acid peptide PTH(1-84). The hormone is secreted into the circulatory system to produce basal (healthy) concentrations of ~15-65 pg/mL, and is assayed to assist in the diagnosis of hypo/hyperparathyroidism, hypercalcemia and in monitoring for renal osteodystrophy in patients with end-stage renal failure. Conventional PTH assays typically rely on two-antibody recognition systems coupled to a variety of detection modalities (e.g., enzymatic amplification, electrochemiluminescence, and fluorescence). Notably, assays of greatest specificity are able to differentiate between different truncated forms of PTH, and are referred to as "second" or "third" generation PTH assays.

[0003] Of particular importance to these later-generation assays is their ability to selectively monitor different PTH forms of known biological consequence. Notably, two variants, full-length PTH(1-84) and PTH missing the six N-terminal amino acids (PTH(7-84)), are the subject of increased clinical investigation and potential diagnostic capability. Due to confounding microheterogeneity, these variants were historically considered as a single PTH value (i.e., by the "first" generation assays). Classification of each as its own molecular entity, and the ability to analyze and study each independently, suggests an antagonistic relationship between the two different forms in relationship to calcium homeostasis. As such, there is mounting clinical evidence that the ratio between PTH(1-84) and PTH(7-84) may differentiate between hyperparathyroid bone turnover and adynamic bone disease.

[0004] This PTH(1-84)-to-PTH(7-84) paradigm is a most recent example of describing the biological and clinical utility of microheterogeneity within the PTH protein. Indeed, other PTH variants have been defined as far back as ~40 years ago. Perhaps the most mature of these, PTH(1-34), has been identified as an endogenous variant that exhibits biochemical activity comparable to the full-length protein. Consequently, it represents a classic example of a peptide-based bioactive variant that has transitioned through drug development to the point of FDA-approval for the treatment of osteoporosis (rPTH(1-34) (teriparatide)). Collectively the routine monitoring of these clinically-relevant PTH variants – PTH(1-84), PTH(7-84) and PTH(1-34) – is achieved through three separate, high-specificity immunometric assays. However, there are indications that even greater microheterogeneity exists within PTH, which has yet to be fully characterized to determine clinical utility and/or confounding effects on present-day assays. The accurate examination of known PTH variants, while simultaneously evaluating other possible variants, requires a degree of analytical freedom of which conventional assays are generally incapable.

SUMMARY

[0005] Roughly described, the present invention provides methods for concurrent monitoring of a plurality of PTH variants of interest by selective reaction monitoring (SRM) tandem mass spectrometry. According to one illustrative embodiment, a biological sample, such as blood serum or plasma, is prepared for analysis by proteolytically (e.g., tryptically) digesting the sample to yield peptide fragments, certain ones of which are specific to a particular PTH variant (referred to herein as surrogate peptides). Immunoaffinity capture may be employed either prior to or following digestion as a means for concentrating the PTH or peptide fragments derived therefrom. The prepared sample, containing the surrogate peptides, is separated and purified by liquid chromatography (LC) or other suitable technique and subsequently introduced into a tandem mass spectrometer, for example a triple quadrupole mass spectrometer, capable of monitoring multiple transitions in SRM mode. By selection of an appropriate set of precursor-to-product ion transitions corresponding to ions produced by ionizations of the surrogate peptides, the amounts of two or more surrogate peptides present in the sample may be determined concurrently with high sensitivity and specificity.

[0006] In a specific implementation, the PTH variants of interest include PTH(1-84), otherwise referred to as intact PTH, and one or more truncated forms of PTH, such as PTH(34-84) and PTH(7-84). To monitor PTH(1-84), the mass spectrometer is operated to measure mass spectral peaks appearing at least two precursor-to-product ion transitions corresponding to the SVSEIQLMHNLGK peptide, which may include *m/z* 486 → 636 and 486→684. To monitor PTH(34-84), the mass spectrometer is operated to measure peaks appearing at least two precursor-to-product ion transitions corresponding to the FVALGAPLAPR peptide, which may include *m/z* 556→343, 556→456, 556→553, 556→624, 556→681, 556→794 and 556→865. Finally, to monitor PTH(7-84), the mass spectrometer is operated to measure signals appearing at least two precursor-to-product ion transitions corresponding to the LMHNLGK peptide, which may include 407→317, 407→431, and 407→568.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] In the accompanying drawings:

[0008] FIG. 1 is a flowchart depicting steps of a method for concurrently monitoring a plurality of PTH variants present in a biological sample;

[0009] FIG. 2 is a graph showing the linear variation in peak intensity with increasing PTH concentration for transitions characteristic of the SVSEIQLMHNLGK surrogate peptide;

[0010] FIGS. 3A and 3B are screen captures of mass spectrometry data acquired for two surrogate peptides: SVSEIQLMHNLGK and LMHNLGK; and

[0011] FIGS. 4A-4E are receiver operating characteristic (ROC) curves showing the relationship between sensitivity and 1-selectivity in the diagnosis of renal failure by measurements of various surrogate peptides.

DETAILED DESCRIPTION OF EMBODIMENTS

[0012] FIG. 1 is a flowchart depicting a method for monitoring PTH variants in a biological sample, in accordance with an illustrative embodiment of the present invention. In

general, the method includes steps for preparing a biological sample, and then conducting selective reaction monitoring (SRM) mass spectrometric analysis on the prepared sample to detect and quantify precursor/product ion pairs formed from surrogate peptides, with each surrogate peptide being specific to a corresponding PTH variant. As used herein, the term "PTH variant" embraces intact PTH as well as any endogenously modified forms of PTH, including forms truncated at the N-terminus and/or C-terminus. The biological sample will preferably take the form of blood serum or plasma, although alternative implementations of the present assay may test other biological fluids or tissue extracts.

[0013] In step 110, the PTH present in the biological sample is purified and concentrated to remove potentially interfering species and improve the overall limits of detection and quantitation. Purification and concentration of the PTH may be accomplished by utilization of immunoaffinity capture, the pertinent details of which are discussed in the example set forth below. As is known in the art, immunoaffinity capture entails exposing the sample to antibody binding agents immobilized on a support, such that a targeted protein present in the sample are selectively captured by the binding agents. The targeted proteins are subsequently released from the support by elution with a small volume of solvent. In the present example, a single, high-affinity polyclonal antibody is employed to simultaneously capture PTH variants having a conserved C-terminus. Other implementations of the assay may utilize a different binding agent to extract PTH variants having a truncated C-terminus. It should be understood that the scope of the present invention should not be construed as being limited to methods that utilize immunoaffinity capture, and that other protein purification/concentration techniques known in the art may be employed in addition to or in place of immunoaffinity capture.

[0014] In step 120, the purified sample is proteolytically (e.g., tryptically) digested to produce peptide fragments. A portion of the proteolytic peptide fragments (also referred to herein as PTH peptides) will be specific to corresponding PTH variants, and thus may be employed as surrogate peptides for monitoring the quantities of the corresponding PTH variants by mass spectrometry. In the example discussed below, the sample is digested by the addition of trypsin, and three targeted PTH variants are monitored by mass spectrometry: PTH(1-84) (intact PTH) and two N-terminally truncated forms, PTH(7-84) and PTH(34-84). The tryptic fragment SVSEIQLMHNLGK, constituting the first to thirteenth amino acid of

the full PTH sequence (aa1-13), is specific to PTH(1-84) and serves as its surrogate peptide. The tryptic fragments LMHNLGK (aa7-13) and FVALGAPLAPR (aa34-44) are specific to PTH(7-84) and PTH(34-84), respectively, and serve as their surrogate peptides.

[0015] It will be recognized by those skilled in the art that a surrogate peptide does not have to uniquely correspond to (i.e., be solely derived from) a single PTH variant, but need only be specific to one PTH variant of the group of targeted PTH variants monitored in a mass spectrometric assay. For example, the tryptic fragment SVSEIQLMHNLGK may be produced by PTH variants other than PTH(1-84), specifically those variants having a conserved N-terminus, but is not produced by the other targeted PTH variants PTH(7-84) and PTH(34-84), and hence SVSEIQLMHNLGK may serve as a surrogate for PTH(1-84) in the example described above.

[0016] According to an alternative embodiment of the assay, the biological sample may first be proteolytically digested, followed by concentration of at least the surrogate peptides. If the immunoaffinity capture technique is employed for purification, then capture antibodies for each of the purified peptide fragments will need to be identified or developed, since peptide fragments present in the digest that are not captured will not be detectable in the mass spectrometric analysis.

[0017] To facilitate accurate quantitation of the surrogate peptides by mass spectrometry, a set of isotopically-labeled synthetic versions of the surrogate peptides may be added in known amounts to the sample for use as internal standards, step 130. Since the isotopically-labeled peptides have physical and chemical properties substantially identical to the corresponding surrogate peptide, they co-elute from the chromatographic column and are easily identifiable on the resultant mass spectrum. In other implementations of the invention, labeled peptides may be added to the sample prior to the purification and/or digestion steps.

[0018] Next, the purified and digested sample containing the surrogate peptides (and associated internal standards) is chromatographically separated by using a suitable HPLC column and elution protocol in order to temporally isolate different compounds in the prepared sample and simplify the process of identifying and quantifying substances in the mass spectra, step 140. In a preferred mode of the invention, chromatographic separation is performed on-line, such that the column eluate is directly introduced into the inlet of the ion

source of the mass spectrometer, for example through an electrospray ionization probe. Alternatively, fractions of the eluate may be collected and stored for later mass analysis. Other suitable separation techniques known in the art, such as electrophoresis, may be utilized in place of or in addition to HPLC.

[0019] In step 150, the prepared sample is ionized, for example by electrospray ionization, and the resultant ions are analyzed by mass spectrometry to detect and quantify two or more surrogate peptides present in the sample. Mass spectrometric analysis of the prepared sample may be carried out in any instrument capable of monitoring multiple transitions in SRM mode. In a preferred implementation, the prepared sample is analyzed in a triple quadrupole mass spectrometer, such as the Thermo Scientific Vantage TSQ instrument. Triple quadrupole mass spectrometers utilize two resolving quadrupoles, also referred to as quadrupole mass filters, each of which is operable to selectively transmit only ions having a mass-to-charge ratio (m/z) of interest. An RF-only quadrupole filled with a collision gas (a collision cell) is interposed in the ion path between the two resolving quadrupoles. Precursor ions selected in the first resolving quadrupole undergo energetic collisions in the collision cell to yield fragment ions. The fragment ions pass into the second resolving quadrupole, and the selectively transmitted product ions strike a detector, which generates a signal representative of the quantity of transmitted ions. A specified pair of precursor/product ion m/z's to which the first and second resolving quadrupoles are tuned is referred to as a transition. The instrument controller, which controls the RF and resolving DC voltages individually applied to the first and second resolving quadrupoles, is configured to rapidly cycle among individual transitions in a stored list such that a large number of different transitions may be concurrently monitored. The number of transitions that may be concurrently monitored in a given time period will depend on various operational factors, including the settling time of the resolving quadrupoles and the rate at which product ions are removed from the collision cell; commercially available instruments are typically capable of monitoring a few hundred transitions per second.

[0020] Due to the multiplicity of charge states and fragmentation that exist for each peptide fragment, monitoring all possible precursor-to-product ion transitions for the group of surrogate peptides is generally impractical. Instead, the mass spectrometer is operated to monitor a subset of transitions selected to optimize sensitivity and selectivity for each surrogate peptide. In a typical assay, a set of 4-8 transitions are monitored for each targeted

peptide fragment, comprising 2-4 transitions for the wild-type peptide (i.e., the peptide fragment derived from digestion of the PTH variant in the sample) and an equivalent number of transition for the corresponding isotopically-labeled synthetic peptide. The selection of optimized transitions for particular surrogate peptides may be performed in an automated or semi-automated fashion using Thermo Scientific Pinpoint software, which identifies and refines an optimized set of transitions based on the expected transition intensity (determined using fragmentation rules and/or previously acquired data) and potential interferences (arising for example, from co-eluting peptide fragments derived from the sample matrix or other surrogate peptide fragments). The process of optimizing a set of transitions for a targeted peptide fragment is discussed in greater detail in U.S. Patent Application Serial No.12/163,928 for "Optimizing Selection of SRM Transitions for Analysis of Biomolecules by Tandem Mass Spectrometry", the disclosure of which is incorporated herein by reference.

[0021] The following sets of transitions have been found to provide high degrees of sensitivity and selectivity for detection/quantitation of the associated surrogate peptides. The peptide SVSEIQLMHNLGK, which is specific to the PTH(1-84) variant, may be identified by operating the mass spectrometer to detect ions at the following transitions: *m/z* 486 → 636 and 486→684. The peptide FVALGAPLAPR, which is specific to the PTH(34-84) variant, 556→343, 556→ 456, 556→553, 556→624, 556→681, 556→794 and 556→866. Finally, the peptide LMHNLGK, which is specific to the PTH(7-84) variant, may be identified by operating the mass spectrometer to detect ions at the following transitions: 407→317, 407→ 431, and 407→568. It will be recognized that the m/z values specified above are nominal (rounded integer) values and are therefore approximate, and that the preferred embodiments of the invention include the use of transitions that have precursor/product ion m/z's that are within ±0.5 of the foregoing values. Examples of more precise transition values are listed in the appended tables, which are discussed below. Alternative implementations of the assay may use a greater or lesser number of transitions for each surrogate peptide; however, it is generally advisable to use at least two transitions for each peptide to ensure an acceptable degree of specificity and avoid misidentification of targeted substances. It will be further recognized that when isotopically-labeled synthetic peptides are employed as internal standards, the isotopically-labeled peptides may be identified by monitoring a set of transitions that correspond to those used for identification of the associated surrogate peptide, adjusted for the increased or decreased mass arising from the isotope labels.

[0022] Various data acquisition strategies have been developed and have been implemented in control software for increasing instrument efficiency by avoiding the need to monitor all of the specified transitions at all time. These strategies, which have been referred to as "intelligent SRM", may involve time-scheduled monitoring of primary transitions corresponding to molecules of interest based on expected elution times, as well as data-dependent monitoring of secondary transitions of the molecules of interest (to provide greater specificity) triggered by detecting intensities at the primary transitions that exceed a threshold. Utilization of intelligent SRM techniques may be particularly advantageous in implementations where a large number of PTH variants are monitored, or where the analyzed sample contains a complex mixture of PTH-derived peptides and components produced by digestion of compounds in the sample matrix.

[0023] Once the mass spectrometric analysis of the prepared sample has been completed, the quantities of the surrogate peptides in the sample may be determined by integration of the relevant peak areas, as known in the prior art. When isotopically-labeled internal standards are used, as described above, the quantities of the surrogate peptides of interest are established via an empirically-derived or predicted relationship between surrogate peptide quantity (which may be expressed as concentration) and the area ratio of the surrogate peptide and internal standard peaks at specified transitions. Other implementations of the assay may utilize external standards or other expedients for peptide quantification. The absolute or relative quantities of the surrogate peptides, representative of the respective PTH variants from which they are formed, may provide useful information for a variety of clinical purposes, including (without limitation), the diagnosis of renal or skeletal diseases, and monitoring the effectiveness of treatments for such diseases.

[0024] It will be appreciated that the SRM mass spectrometry technique practiced in accordance with the present invention enables the concurrent monitoring of a plurality of PTH variants in a biological sample, via detection and quantitation of their corresponding surrogate peptides. The term "concurrent', as used herein, does not denote or require simultaneous or near-simultaneous detection of the different surrogate peptides, but instead denotes that the two or more surrogate peptides may be measured in a single assay, e.g., within a single chromatographic run. This approach stands in contradistinction to

immunoassays and other prior art techniques, in which separate variant-specific assays need to be conducted to monitor selected PTH variants.

[0025] A detailed example of an implementation of the PTH assay of the invention is set forth below. The following example is intended to illustrate rather than limit the scope of the present invention. In particular, the specific PTH variants discussed above and in the following example represent a small subset of possible PTH variants, and the SRM mass spectrometry technique of the invention should not be construed as being limited to the disclosed PTH variants. Those skilled in the art will recognize that there are thousands of possible PTH variants that arise from truncation from intact PTH(1-84), and that this number is expanded exponentially when post-translation modifications of the truncated PTH variants are considered. The technique of the present invention, in which SRM mass spectrometry is employed to concurrently determine the amounts of surrogate peptides derived from two or more PTH variants, may be advantageously utilized in connection with any combination of the possible PTH variants for which monitoring is desired.

Example

Reagents

[0026] Goat polyclonal Anti PTH39-84 antibody was purchased from Immutopics International (San Clemente, CA). Recombinant human PTH (rhPTH) was obtained from Bachem (Torrance, CA). Premixed MES-buffered saline powder packets were from Pierce (Rockford, IL). Extraction of PTH from plasma was carried out with proprietary MSIA pipette tips (MSIA-Tips) from Intrinsic Bioprobes (Tempe, AZ) derivatized with the PTH antibodies via 1,1' carbonyldiimidazole (CDI) chemistry as described below. Premade 10X 0.1M Hepes-buffered saline (HBS-N) with 30 mM EDTA and 0.2% (v/v) surfactant P20 (HBS-EP) was purchased from BIACORE (Piscataway, NJ). Synthetic heavy labeled peptides were obtained from Thermo Fisher Scientific (Ulm, Germany). All other chemicals were obtained from Sigma-Aldrich (St.Louis, MO).

Samples

[0027] All samples used in this study were acquired under Institutional Review Board (IRB) approved protocols and with informed consent. Twenty four serum samples representing twelve clinically diagnosed individuals with renal failure (10 males and 2

female; mean age 66.7) and twelve healthy individuals (10 males and 2 female; mean age 65) were used in the population study. Standard curves were prepared in pooled human plasma by step-wise, 2-fold serial dilution from an initial sample containing rhPTH at a concentration of 150 pg/mL (7-steps: range; 150 pg/mL to1.2 pg/mL).

Sample Preparation and Immunocapture

[0028] MSIA-Tips were provided by Intrinsic Bioprobes pre-exposed to 15 kDa carboxymethyldextran and CDI creating a highly functionalized carboxylic acid surface, as previously described in Niederkofler et al., *Novel mass spectrometric immunoassays for the rapid structural characterization of plasma apolipoproteins*, J. Lipid Res. 44:3, 630-9 (2003), the contents of which are incorporated herein by reference. The MSIA-Tips were rinsed with 0.2 M hydrochloric acid and acetone to generate free acid carboxyl groups by washing off any remaining sodium/potassium salts. MSIA-tips were subsequently activated with CDI in 1-methyl-2-pyrrolidone (NMP) by continuously pipetting the solution up and down (150 µL/well; 50 µL vol; 450 cycles) with a Beckman Multimek 96 pipetting robot (Beckman Coulter, Fullerton, CA, USA). Tips were then blotted to remove excess NMP, rinsed twice with NMP (400 µL/well; 100 µL vol; 10 cycles), and finally blotted once more to remove any remaining excess NMP. While still slightly moist with NMP, MSIA-Tips were then immediately exposed to anti-PTH antibodies in MES buffered saline, pH 4.7 (0.25 g/L; 30 µL/well; 10 µL volume; 50 cycles; 15 repetitions/loops, approximately 45 minute exposure time) to facilitate covalent attachment to the MSIA-Tips. Finally, MSIA-Tips were rinsed with 1M ethanolamine, pH 8.5 (400 µL/well; 100 µL vol; 50 cycles) to quench any remaining CDI-preactivated sites, followed by two rinses of HBS-N (400 µL/well; 100 µL vol; 50 cycles). MSIA-Tips were stored in HBS-N at 4°C and used within one-week of preparation. For all samples in this study, one milliliter of human serum (or plasma) was diluted with 750 µL HBSEP to result in a total analytical volume of 1.75 mL. PTH was then extracted with the aid of a Beckman Multimek 96 pipetting robot by repeatedly (1,500 repetitions) drawing and expelling (back into the analytical volume) 125 µL aliquots of the analytical volume through the antibody prelinked MSIA-Tip. After extraction, the pipettes were rinsed using HBS-EP and H2O, (in this order, each rinse = 15 repetitions of 150 µL), after which PTH was analyzed using MRM mass spectrometry as described below.

Sample Elution and Trypsin Digestion

[0029] Bound proteins were eluted from the tips into a 96 well plate (Abgene, AB-1300) by pipetting 100μL of 30% acetonitrile/0.5% formic acid up and down for a total of 15 cycles. Samples were lyophilized to dryness, resuspended in 30μL of 30% n-propanol/100mM ammonium bicarbonate pH 8.0, diluted with 100μL of 25mM acetic acid containing 100ng of trypsin. Samples were allowed to digest for 4 hours at 37°C. Post digestion, samples were lyophilized and resuspended in 30μL of 3% (v/v) acetonitrile/0.2% (v/v) formic acid/glucagon/PTH heavy peptides.

Multiple Reaction Monitoring Assays

[0030] SRM assays were developed on a Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer, Accela pump, CTC PAL Autosampler and an IonMax electrospray ionization source equipped with a high flow metal needle (Thermo Fisher Scientific). Reverse phase separations were carried out on a 1mm X 100mm Hypersil Gold 1.9μm C18 particle column (Thermo Fisher Scientific). Solvent A was LC-MS grade water with 0.2% (v/v) formic acid, and solvent B was LC-MS grade 30% (v/v) acetonitrile with 0.2% (v/v) formic acid (Optima grade reagents, Thermo Fisher Scientific). Pinpoint software (Thermo Fisher Scientific) was used for targeted protein quantitation. The recently developed software algorithm automates the prediction of candidate peptides and the choice of multiple fragment ions for SRM assay design. In addition, Pinpoint creates the instrument method and sequence file, and automates peptide identity confirmation and quantitative data processing. For the workflow described herein, the intact PTH sequence was imported into Pinpoint, digested with trypsin *in silico*, and transitions for each peptide were predicted using the batch option in the software. This initial list of peptides and transitions (which are presented in the appended Table I) was tested with recombinant PTH digest to determine those peptides and transitions delivering optimal signal. After several iterations, a subset of 6 peptides, including 2 peptides specific for the truncated variants PTH(7-84) and PTH(34-84), were chosen, along with multiple corresponding transitions. Table II lists the targeted peptides and associated transitions selected for the assay. Further assays were conducted with this optimized method. Once the target peptides were identified, synthetic heavy versions were synthesized (Thermo Fisher Scientific, Ulm, Germany) to be used as internal quantitative standards.

[0031] Target peptides were subsequently identified and quantified by co-eluting light and heavy-labeled transitions in the chromatographic separation. Time alignment and relative quantification of the transitions was performed with Pinpoint. All samples were assayed in triplicate.

Generation of Standard Profile and Evaluation using Intact PTH

[0032] Standard curves were created for all target tryptic peptides through serial dilution using recombinant human PTH (rhPTH) spiked into stock human blood plasma. As illustrated in the FIG. 2 example for peptide SVSEIQLMHNLGK (specific to PTH(1-84)), SRM transitions exhibited linear responses (with an R^2 value of about 0.93) relative to rhPTH concentration, with a limit of detection for intact PTH of approximately 8 pg/ml. The standard error of analysis for triplicate measurements ranged from 3-12% for all peptides with less than < 5% chromatographic drift between replicates, indicating high accuracy and reproducibility between measurements. Monitoring of variant SRM transitions showed no inflections relative to rhPTH concentration, as may be expected due to the absence of truncated variants in the stock rhPTH.

Evaluation of Clinical Samples Using Standard Profile and Variant Transitions.

[0033] FIGS. 3A and 3B respectively represent Pinpoint screen captures of SRM data for two peptides, SVSEIQLMHNLGK and LMHNLGK (aa7-13) acquired from replicate normal clinical samples. The topmost panels indicate the peptides and transitions monitored with associated information such as retention time, internal standards and total peak areas. The leftmost bottom panels illustrate the total signal per sample and the center bottom panels illustrate the chromatographic peptide peak shapes and transition ratios for each sample. The rightmost bottom panels illustrate the peak integration areas and individual fragment transitions for both light and heavy peptides.

[0034] FIGS. 4A-4E are receiver operating characteristic (ROC) curves constructed from the acquired mass spectrometry data showing the relationship between sensitivity (true positive rate) and 1-specificity (false positive rate) for diagnosis of renal failure based measurement of the amounts of, respectively, the LQDVHNFVALGAPLAPR, SVSEIQLMHNLGK, HLNSMER, FVALGAPLAPR and ADVNVLTK peptides. Each figure includes as an inset the raw signal intensities for samples in the renal failure group

(indicated as diamonds) and control group (indicated as squares), as well as the computed AUC (area under the curve). The degree to which the AUC approaches unity is a measure of the performance of a diagnostic test since it reflects the test performance at all possible cut-off levels. In the present example, the ROC for LQDVHNFVALGAPLAPR has an AUC of 0.987, the ROC for SVSEIQLMHNLGK has an AUC of 0.92, the ROC of HLNSMER has an AUC of 0.888, the ROC of FVALGAPLAPR has an AUC of 0.775, and the ROC of ADVNVLTK has an AUC of 0.996. These values indicate that each of the aforementioned peptides, and in particular LQDVHNFVALGAPLAPR, SVSEIQLMHNLGK and ADVNVLTK, possess significant diagnostic value in discriminating between normal and renal failure conditions. The data also suggest that diagnostic tests of very high reliability may be conducted using a panel of two or more of the aforementioned peptides.

TABLE I

Precursor	Product	Sequence	Sequence variant target	Precursor charge state	Ion type
443.714	435.202	HLNSMER		2	y3
296.145	435.202	HLNSMER		3	y3
448.718	445.21	HLNSMER[HeavyR]		2	y3
299.481	445.21	HLNSMER[HeavyR]		3	y3
443.714	522.234	HLNSMER		2	y4
296.145	522.234	HLNSMER		3	y4
448.718	532.242	HLNSMER[HeavyR]		2	y4
299.481	532.242	HLNSMER[HeavyR]		3	y4
443.714	636.276	HLNSMER		2	y5
296.145	636.276	HLNSMER		3	y5
448.718	646.285	HLNSMER[HeavyR]		2	y5
299.481	646.285	HLNSMER[HeavyR]		3	y5
341.205	343.208	GAPLAPR	38-84	2	y3
341.205	456.292	GAPLAPR	38-84	2	y4
341.205	553.345	GAPLAPR		2	y5
406.726	317.218	LMHNLGK	3-84	2	y3
271.486	317.218	LMHNLGK	3-84	3	y3
410.733	325.232	LMHNLGK[HeavyK]	3-84	2	y3
274.158	325.232	LMHNLGK[HeavyK]	3-84	3	y3
406.726	431.261	LMHNLGK	3-84	2	y4
271.486	431.261	LMHNLGK	3-84	3	y4
410.733	439.275	LMHNLGK[HeavyK]	3-84	2	y4
274.158	439.275	LMHNLGK[HeavyK]	3-84	3	y4
406.726	568.32	LMHNLGK	3-84	2	y5
271.486	568.32	LMHNLGK	3-84	3	y5
410.733	576.334	LMHNLGK[HeavyK]	3-84	2	y5
274.158	576.334	LMHNLGK[HeavyK]	3-84	3	y5
397.747	343.208	LGAPLAPR	37-84	2	y3
397.747	456.292	LGAPLAPR	37-84	2	y4
397.747	553.345	LGAPLAPR	37-84	2	y5
397.747	624.382	LGAPLAPR	37-84	2	y6
430.248	361.244	ADVNVLTK		2	y3
287.168	361.244	ADVNVLTK		3	y3
434.255	369.258	ADVNVLTK[HeavyK]		2	y3
289.839	369.258	ADVNVLTK[HeavyK]		3	y3
430.248	460.312	ADVNVLTK		2	y4
287.168	460.312	ADVNVLTK		3	y4
434.255	468.327	ADVNVLTK[HeavyK]		2	y4
289.839	468.327	ADVNVLTK[HeavyK]		3	y4

430.248	574.355	ADVNVLTK		2	y5
287.168	574.355	ADVNVLTK		3	y5
434.255	582.37	ADVNVLTK[HeavyK]		2	y5
289.839	582.37	ADVNVLTK[HeavyK]		3	y5
430.248	673.424	ADVNVLTK		2	y6
287.168	673.424	ADVNVLTK		3	y6
434.255	681.438	ADVNVLTK[HeavyK]		2	y6
289.839	681.438	ADVNVLTK[HeavyK]		3	y6
556.334	343.208	FVALGAPLAPR	34-84	2	y3
556.334	456.292	FVALGAPLAPR	34-84	2	y4
556.334	553.345	FVALGAPLAPR	34-84	2	y5
556.334	624.382	FVALGAPLAPR	34-84	2	y6
556.334	681.404	FVALGAPLAPR	34-84	2	y7
556.334	794.488	FVALGAPLAPR	34-84	2	y8
556.334	865.525	FVALGAPLAPR	34-84	2	y9
728.385	317.218	SVSEIQLMHNLGK	1-84	2	y3
485.926	317.218	SVSEIQLMHNLGK	1-84	3	y3
732.392	325.232	SVSEIQLMHNLGK[HeavyK]	1-84	2	y3
488.597	325.232	SVSEIQLMHNLGK[HeavyK]	1-84	3	y3
728.385	431.261	SVSEIQLMHNLGK	1-84	2	y4
485.926	431.261	SVSEIQLMHNLGK	1-84	3	y4
732.392	439.275	SVSEIQLMHNLGK[HeavyK]	1-84	2	y4
488.597	439.275	SVSEIQLMHNLGK[HeavyK]	1-84	3	y4
728.385	568.32	SVSEIQLMHNLGK	1-84	2	y5
485.926	568.32	SVSEIQLMHNLGK	1-84	3	y5
732.392	576.334	SVSEIQLMHNLGK[HeavyK]	1-84	2	y5
488.597	576.334	SVSEIQLMHNLGK[HeavyK]	1-84	3	y5
728.385	699.36	SVSEIQLMHNLGK	1-84	2	y6
485.926	699.36	SVSEIQLMHNLGK	1-84	3	y6
732.392	707.374	SVSEIQLMHNLGK[HeavyK]	1-84	2	y6
488.597	707.374	SVSEIQLMHNLGK[HeavyK]	1-84	3	y6
728.385	812.444	SVSEIQLMHNLGK	1-84	2	y7
485.926	812.444	SVSEIQLMHNLGK	1-84	3	y7
732.392	820.458	SVSEIQLMHNLGK[HeavyK]	1-84	2	y7
488.597	820.458	SVSEIQLMHNLGK[HeavyK]	1-84	3	y7
728.385	940.503	SVSEIQLMHNLGK	1-84	2	y8
485.926	940.503	SVSEIQLMHNLGK	1-84	3	y8
732.392	948.517	SVSEIQLMHNLGK[HeavyK]	1-84	2	y8
488.597	948.517	SVSEIQLMHNLGK[HeavyK]	1-84	3	y8
728.385	1053.587	SVSEIQLMHNLGK	1-84	2	y9
485.926	1053.587	SVSEIQLMHNLGK	1-84	3	y9
732.392	1061.601	SVSEIQLMHNLGK[HeavyK]	1-84	2	y9
488.597	1061.601	SVSEIQLMHNLGK[HeavyK]	1-84	3	y9
728.385	1182.629	SVSEIQLMHNLGK	1-84	2	y10
485.926	1182.629	SVSEIQLMHNLGK	1-84	3	y10
732.392	1190.644	SVSEIQLMHNLGK[HeavyK]	1-84	2	y10
488.597	1190.644	SVSEIQLMHNLGK[HeavyK]	1-84	3	y10

728.385	1269.661	SVSEIQLMHNLGK	1-84	2	y11
485.926	1269.661	SVSEIQLMHNLGK	1-84	3	y11
732.392	1277.676	SVSEIQLMHNLGK[HeavyK]	1-84	2	y11
488.597	1277.676	SVSEIQLMHNLGK[HeavyK]	1-84	3	y11
909.505	343.208	LQDVHNFVALGAPLAPR		2	y3
606.672	343.208	LQDVHNFVALGAPLAPR		3	y3
914.509	353.217	LQDVHNFVALGAPLAPR[HeavyR]		2	y3
610.008	353.217	LQDVHNFVALGAPLAPR[HeavyR]		3	y3
909.505	456.292	LQDVHNFVALGAPLAPR		2	y4
606.672	456.292	LQDVHNFVALGAPLAPR		3	y4
914.509	466.301	LQDVHNFVALGAPLAPR[HeavyR]		2	y4
610.008	466.301	LQDVHNFVALGAPLAPR[HeavyR]		3	y4
909.505	553.345	LQDVHNFVALGAPLAPR		2	y5
606.672	553.345	LQDVHNFVALGAPLAPR		3	y5
914.509	563.353	LQDVHNFVALGAPLAPR[HeavyR]		2	y5
610.008	563.353	LQDVHNFVALGAPLAPR[HeavyR]		3	y5
909.505	624.382	LQDVHNFVALGAPLAPR		2	y6
606.672	624.382	LQDVHNFVALGAPLAPR		3	y6
914.509	634.391	LQDVHNFVALGAPLAPR[HeavyR]		2	y6
610.008	634.391	LQDVHNFVALGAPLAPR[HeavyR]		3	y6
909.505	681.404	LQDVHNFVALGAPLAPR		2	y7
606.672	681.404	LQDVHNFVALGAPLAPR		3	y7
914.509	691.412	LQDVHNFVALGAPLAPR[HeavyR]		2	y7
610.008	691.412	LQDVHNFVALGAPLAPR[HeavyR]		3	y7
909.505	794.488	LQDVHNFVALGAPLAPR		2	y8
606.672	794.488	LQDVHNFVALGAPLAPR		3	y8
914.509	804.496	LQDVHNFVALGAPLAPR[HeavyR]		2	y8
610.008	804.496	LQDVHNFVALGAPLAPR[HeavyR]		3	y8
909.505	865.525	LQDVHNFVALGAPLAPR		2	y9
606.672	865.525	LQDVHNFVALGAPLAPR		3	y9
914.509	875.533	LQDVHNFVALGAPLAPR[HeavyR]		2	y9
610.008	875.533	LQDVHNFVALGAPLAPR[HeavyR]		3	y9
909.505	964.593	LQDVHNFVALGAPLAPR		2	y10
606.672	964.593	LQDVHNFVALGAPLAPR		3	y10
914.509	974.602	LQDVHNFVALGAPLAPR[HeavyR]		2	y10
610.008	974.602	LQDVHNFVALGAPLAPR[HeavyR]		3	y10
909.505	1111.662	LQDVHNFVALGAPLAPR		2	y11
606.672	1111.662	LQDVHNFVALGAPLAPR		3	y11
914.509	1121.67	LQDVHNFVALGAPLAPR[HeavyR]		2	y11
610.008	1121.67	LQDVHNFVALGAPLAPR[HeavyR]		3	y11
909.505	1225.705	LQDVHNFVALGAPLAPR		2	y12
606.672	1225.705	LQDVHNFVALGAPLAPR		3	y12
914.509	1235.713	LQDVHNFVALGAPLAPR[HeavyR]		2	y12
610.008	1235.713	LQDVHNFVALGAPLAPR[HeavyR]		3	y12
909.505	1362.764	LQDVHNFVALGAPLAPR		2	y13
606.672	1362.764	LQDVHNFVALGAPLAPR		3	y13
914.509	1372.772	LQDVHNFVALGAPLAPR[HeavyR]		2	y13

610.008	1372.772	LQDVHNFVALGAPLAPR[HeavyR]	3	y13
909.505	1461.832	LQDVHNFVALGAPLAPR	2	y14
606.672	1461.832	LQDVHNFVALGAPLAPR	3	y14
914.509	1471.84	LQDVHNFVALGAPLAPR[HeavyR]	2	y14
610.008	1471.84	LQDVHNFVALGAPLAPR[HeavyR]	3	y14

TABLE II

Precursor	Product	Sequence	Precursor charge state	Ion type	Sequence variant target	aa
486.292	635.655	SVSEIQLMHNLGK	3	y3	1-84	1-13
486.292	684.469	SVSEIQLMHNLGK	3	y4	1-84	1-13
488.655	707.6	SVSEIQLMHNLGK[HeavyK]	3	y3	1-84	1-13
488.655	639.456	SVSEIQLMHNLGK[HeavyK]	3	y4	1-84	1-13
406.726	317.218	LMHNLGK	2	y3	7-84	7-13
406.726	431.261	LMHNLGK	2	y4	7-84	7-13
406.726	568.32	LMHNLGK	2	y5	7-84	7-13
410.733	325.232	LMHNLGK[HeavyK]	2	y3	7-84	7-13
410.733	439.275	LMHNLGK[HeavyK]	2	y4	7-84	7-13
410.733	576.334	LMHNLGK[HeavyK]	2	y5	7-84	7-13
443.702	636.379	HLNSMER	2	y3	Monitoring	14-20
443.714	619.31	HLNSMER	2	y5	Monitoring	14-20
443.702	749.572	HLNSMER	2	y4	Monitoring	14-20
448.762	646.43	HLNSMER[HeavyR]	2	y4	Monitoring	14-20
448.762	759.501	HLNSMER[HeavyR]	2	y3	Monitoring	14-20
606.687	681.289	LQDVHNFVALGAPLAPR	3	y3	Monitoring	28-44
606.687	553.177	LQDVHNFVALGAPLAPR	3	y4	Monitoring	28-44
606.687	569.259	LQDVHNFVALGAPLAPR	3	y5	Monitoring	28-44
610.008	691.412	LQDVHNFVALGAPLAPR[HeavyR]	3	y3	Monitoring	28-44
610.008	563.353	LQDVHNFVALGAPLAPR[HeavyR]	3	y4	Monitoring	28-44
610.008	569.306	LQDVHNFVALGAPLAPR[HeavyR]	3	y5	Monitoring	28-44
556.334	343.208	FVALGAPLAPR	2	y3	34-84	34-44
556.334	456.292	FVALGAPLAPR	2	y4	34-84	34-44
556.334	553.345	FVALGAPLAPR	2	y5	34-84	34-44
556.334	624.382	FVALGAPLAPR	2	y6	34-84	34-44
556.334	681.404	FVALGAPLAPR	2	y7	34-84	34-44
556.334	794.488	FVALGAPLAPR	2	y8	34-84	34-44
556.334	865.525	FVALGAPLAPR	2	y9	34-84	34-44
430.248	337.216	ADVNVLTK	2	y3	Monitoring	73-80
430.248	460.312	ADVNVLTK	2	y4	Monitoring	73-80
430.248	574.355	ADVNVLTK	2	y5	Monitoring	73-80
430.248	673.424	ADVNVLTK	2	y6	Monitoring	73-80

What is claimed is:

1. A method for concurrently monitoring parathyroid hormone (PTH) variants in a biological sample, comprising steps of:

preparing a sample containing PTH peptides from the biological sample; and

subjecting the prepared sample to selective reaction monitoring (SRM) tandem mass spectrometry to concurrently determine the amounts of at least a first and a second PTH peptide, wherein the first PTH peptide is specific to a first PTH variant and the second peptide is specific to a second PTH variant.

2. The method of claim 1, wherein the step of subjecting the prepared sample to SRM tandem mass spectrometry includes monitoring at least two precursor-to-product ion transitions characteristic of the first PTH peptide and at least two precursor-to-product ion transitions characteristic of the second PTH peptide.

3. The method of claim 1 or 2, wherein the step of preparing test sample includes proteolytically digesting at least a portion of the biological sample to produce the PTH peptides.

4. The method of any of claims 1-3, wherein the first PTH variant is intact PTH.

5. The method of any of the preceding claims, wherein the second PTH variant is a truncated form of PTH selected from a group consisting of PTH (34-84) and PTH (7-84).

6. The method of claim 4, wherein the first PTH peptide is SVSEIQLMHNLGK.

7. The method of claim 6, wherein the at least two precursor-to-product ion transitions characteristic of the first PTH peptide are *m/z* 486 →636 and 486 →684.

8. The method of claim 5, wherein the second PTH variant is PTH (7-84) and the second PTH peptide is LMHNLGK.

9. The method of claim 8, wherein the at least two precursor-to-product ion transitions characteristic of the second PTH peptide are selected from the group consisting of *m/z* 407 →317, 407→431, and *m/z* 407 →568.

10. The method of claim 5, wherein the second PTH variant is PTH (34-84), and the second PTH peptide is FVALGAPLAPR.

11. The method of claim 10, wherein the at least two precursor-to-product ion transitions characteristic of the second PTH peptide are selected from the group consisting of *m/z* 556 →343, 556 →456, 556 →553, 556 →624, *m/z* 556 →681, 556 →794 and *m/z* 556 →866.

12. The method of claim 1, wherein the at least a first and a second PTH peptide includes a third PTH peptide derived from a third PTH variant.

13. The method of claim 1, wherein the biological sample is one of blood serum and blood plasma.

14. The method of claim 1, wherein the step of preparing the sample includes chromatographically separating the biological sample.

15. The method of claim 1, wherein the step of preparing the sample includes adding at least one internal reference standard, the reference standard being an isotopically-labeled version of one of the first or second PTH peptides.

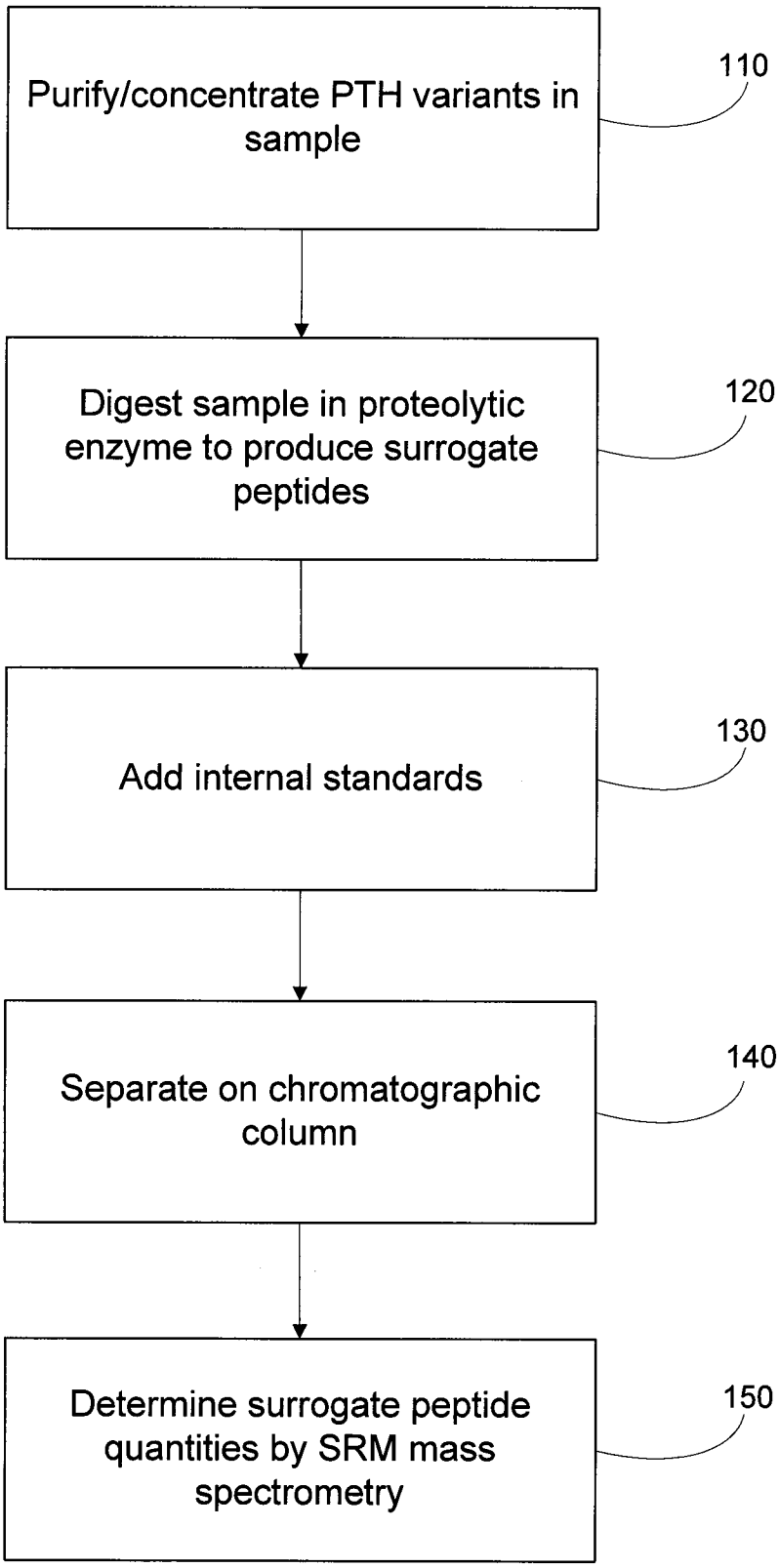

Purify/concentrate PTH variants in sample
110
Digest sample in proteolytic enzyme to produce surrogate peptides
120
Add internal standards
130
Separate on chromatographic column
140
Determine surrogate peptide quantities by SRM mass spectrometry
150

FIG. 1

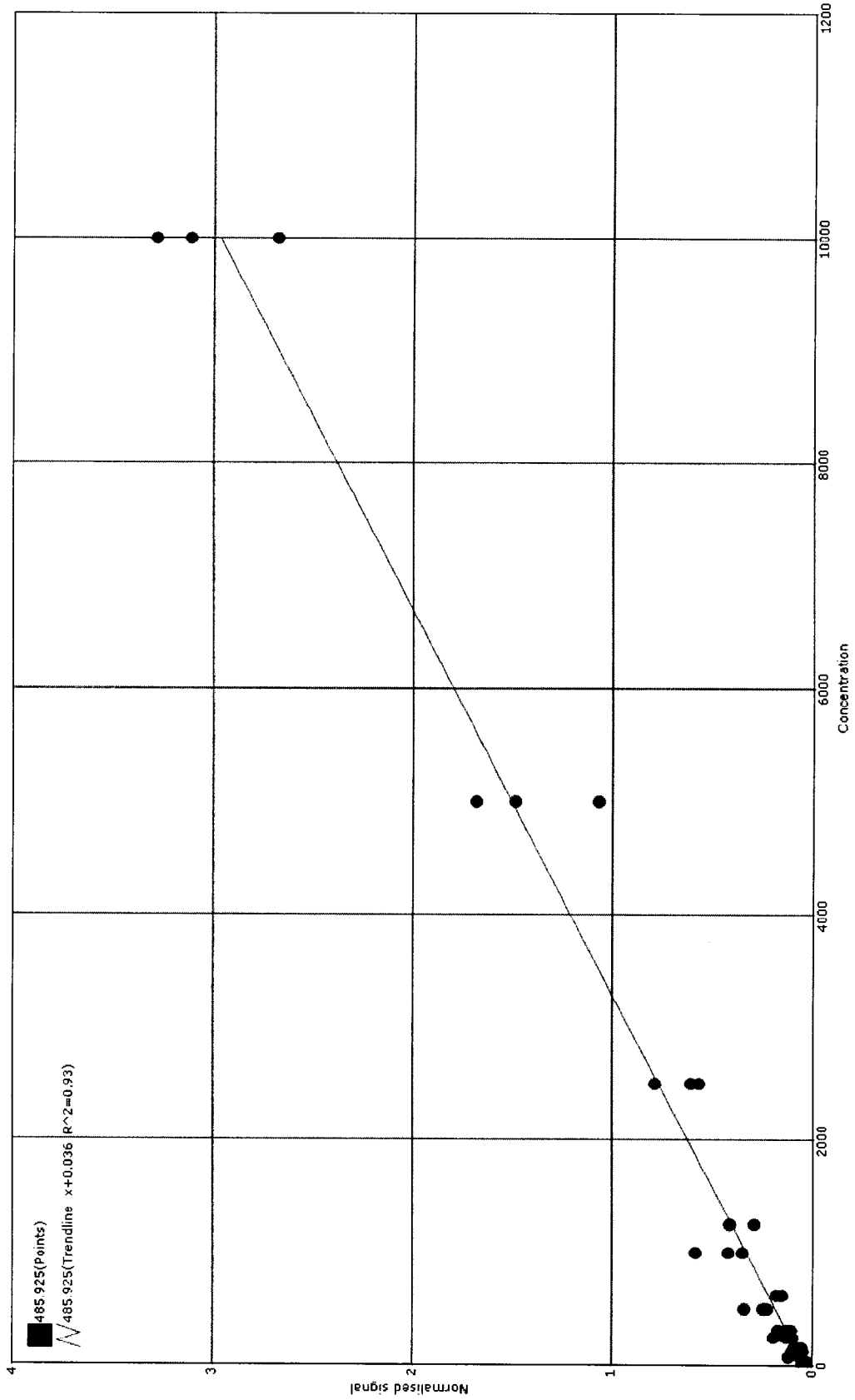

485.925(Points)
485.925(Trendline x+0.036 R^2=0.93)
Normalised signal
Concentration
0
1
2
3
4
2000
4000
6000
8000
10000
12000

FIG. 2

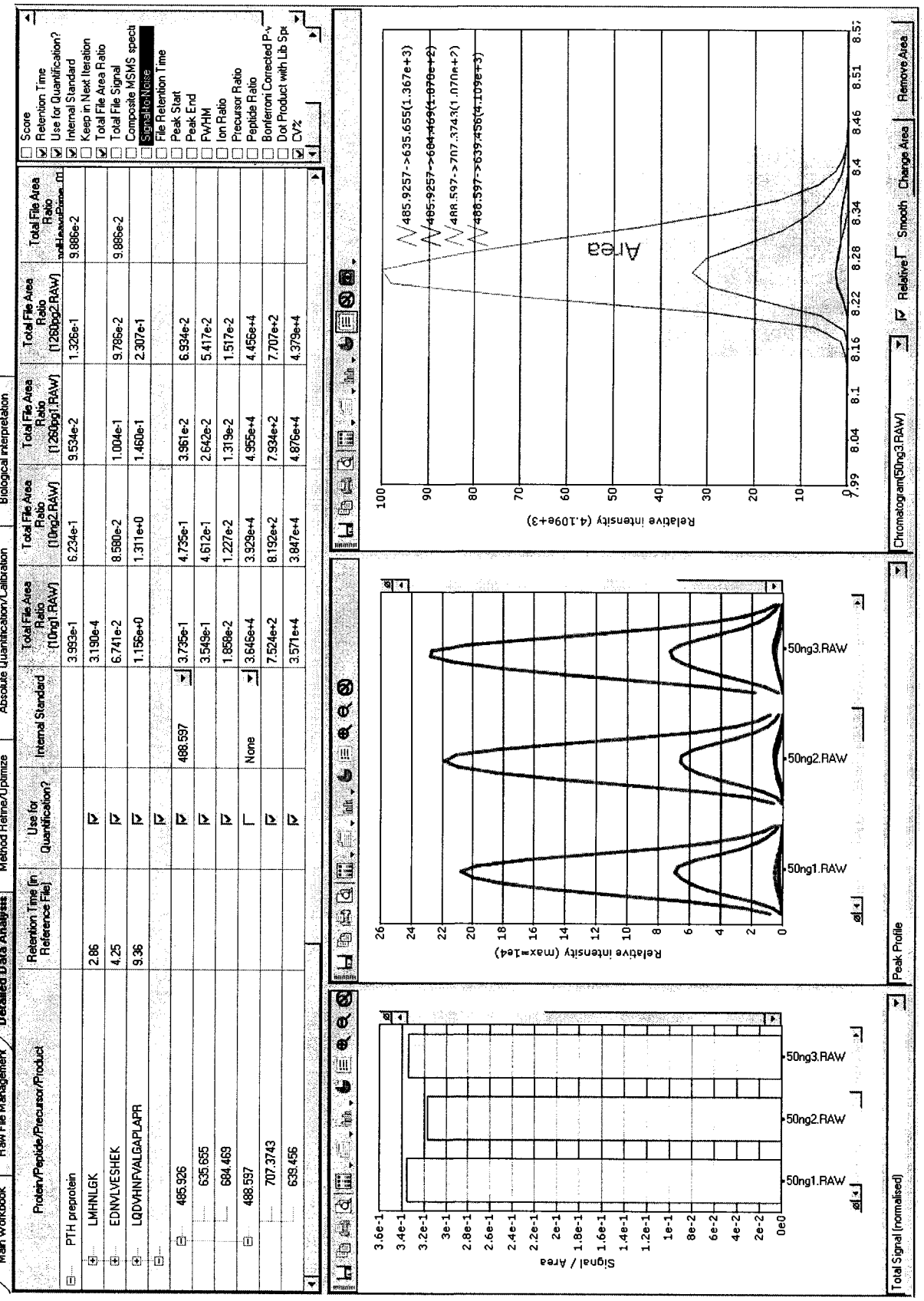

Main Workbook
Raw File Management
Detailed Data Analysis
Method Refine/Optimize
Absolute Quantification/Calibration
Biological interpretation
Protein/Peptide/Precursor/Product
Retention Time (in Reference File)
Use for Quantification?
Internal Standard
Total File Area Ratio (10ng1.RAW)
Total File Area Ratio (10ng2.RAW)
Total File Area Ratio (125bg1.RAW)
Total File Area Ratio (126bg2.RAW)
Total File Area Ratio
PTH preprotein
LMHNLGK
EDNVLVESHEK
LQDVHNFVALGAPLAPR
485.926
635.655
684.469
488.597
707.3743
639.456
Score
Retention Time
Use for Quantification?
Internal Standard
Keep in Next Iteration
Total File Area Ratio
Total File Signal
Composite MSMS spectr
Signal:to:Noise
File Retention Time
Peak Start
Peak End
FWHM
Ion Ratio
Precursor Ratio
Peptide Ratio
Bonferroni Corrected P-v
Dot Product with Lib Spe
CV%
Total Signal (normalised)
Signal / Area
50ng1.RAW
50ng2.RAW
50ng3.RAW
Peak Profile
Relative Intensity (max=1e4)
Chromatogram(50ng3.RAW)
Relative Intensity (4.1096+3)
Area
485.9257->635.655(1.367e+3)
Relative
Smooth
Change Area
Remove Area

FIG. 3A

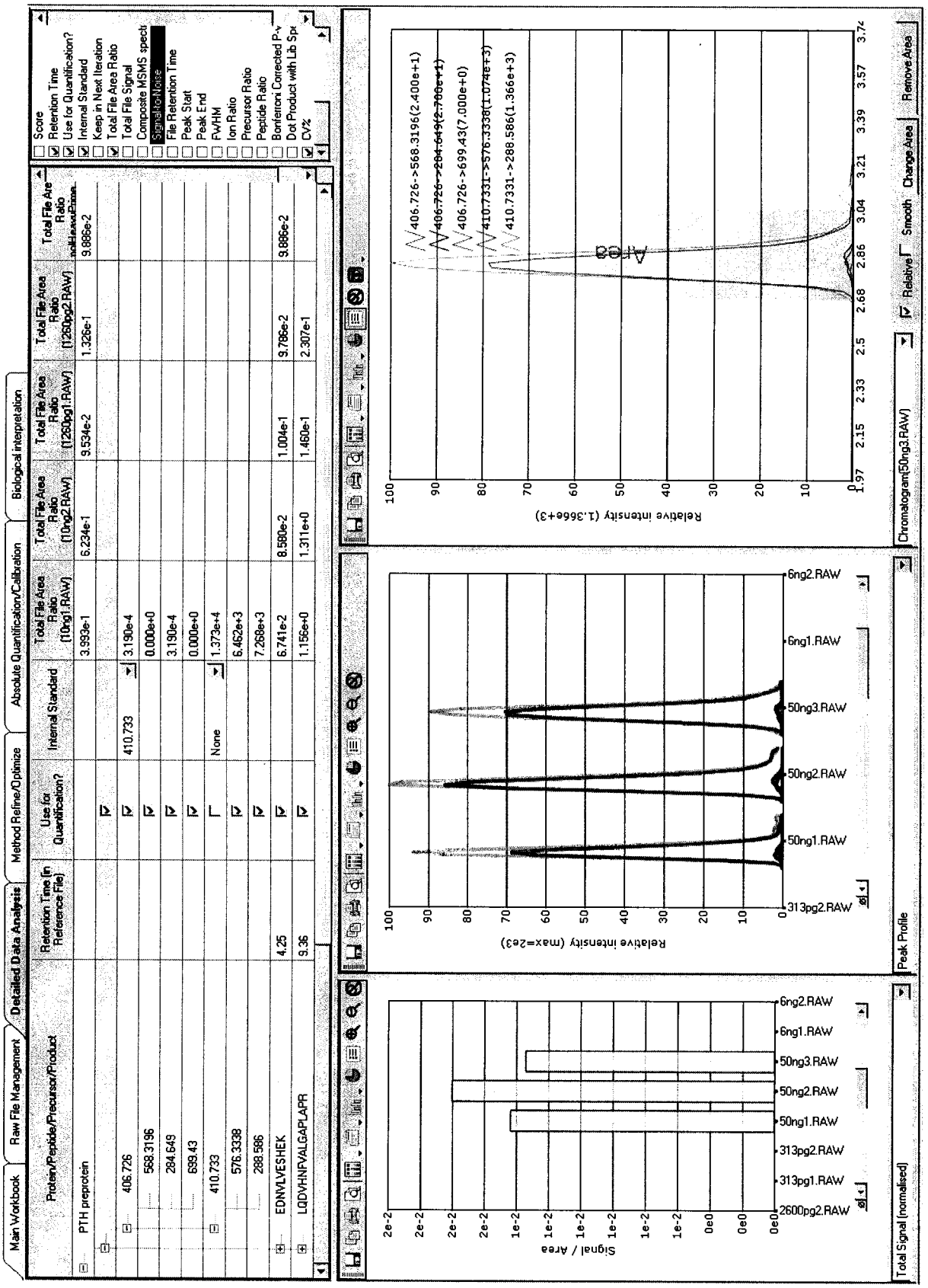

Main Workbook
Raw File Management
Detailed Data Analysis
Method Refine/Optimize
Absolute Quantification/Calibration
Biological interpretation
Protein/Peptide/Precursor/Product
Retention Time (in Reference File)
Use for Quantification?
Internal Standard
Total File Area Ratio (10ng1.RAW)
Total File Area Ratio (10ng2.RAW)
Total File Area Ratio (1260pg1.RAW)
Total File Area Ratio (1260pg2.RAW)
Total File Are Ratio
PTH preprotein
406.726
568.3196
284.649
699.43
410.733
576.3338
288.586
EDNVLVESHEK
LQDVHNFVALGAPLAPR
Score
Retention Time
Use for Quantification?
Internal Standard
Keep in Next Iteration
Total File Area Ratio
Total File Signal
Composite MSMS spectu
Signal-to-Noise
File Retention Time
Peak Start
Peak End
FWHM
Ion Ratio
Precursor Ratio
Peptide Ratio
Bonferoni Corrected P-v
Dot Product with Lib Spr
CV%
Total Signal (normalised)
Peak Profile
Signal / Area
Relative Intensity (max=2e3)
6ng2.RAW
6ng1.RAW
50ng3.RAW
50ng2.RAW
50ng1.RAW
313pg2.RAW
313pg1.RAW
2600pg2.RAW
313pg2.RAW
Chromatogram[50ng3.RAW]
Relative
Smooth
Change Area
Remove Area
Relative Intensity (1.3666+3)
406.726->568.3196(2.400e+1)
406.726->284.649(2.700e+1)
406.726->699.43(7.000e+0)
410.7331->576.3338(1.074e+3)
410.7331->288.586(1.366e+3)
Area

FIG. 3B

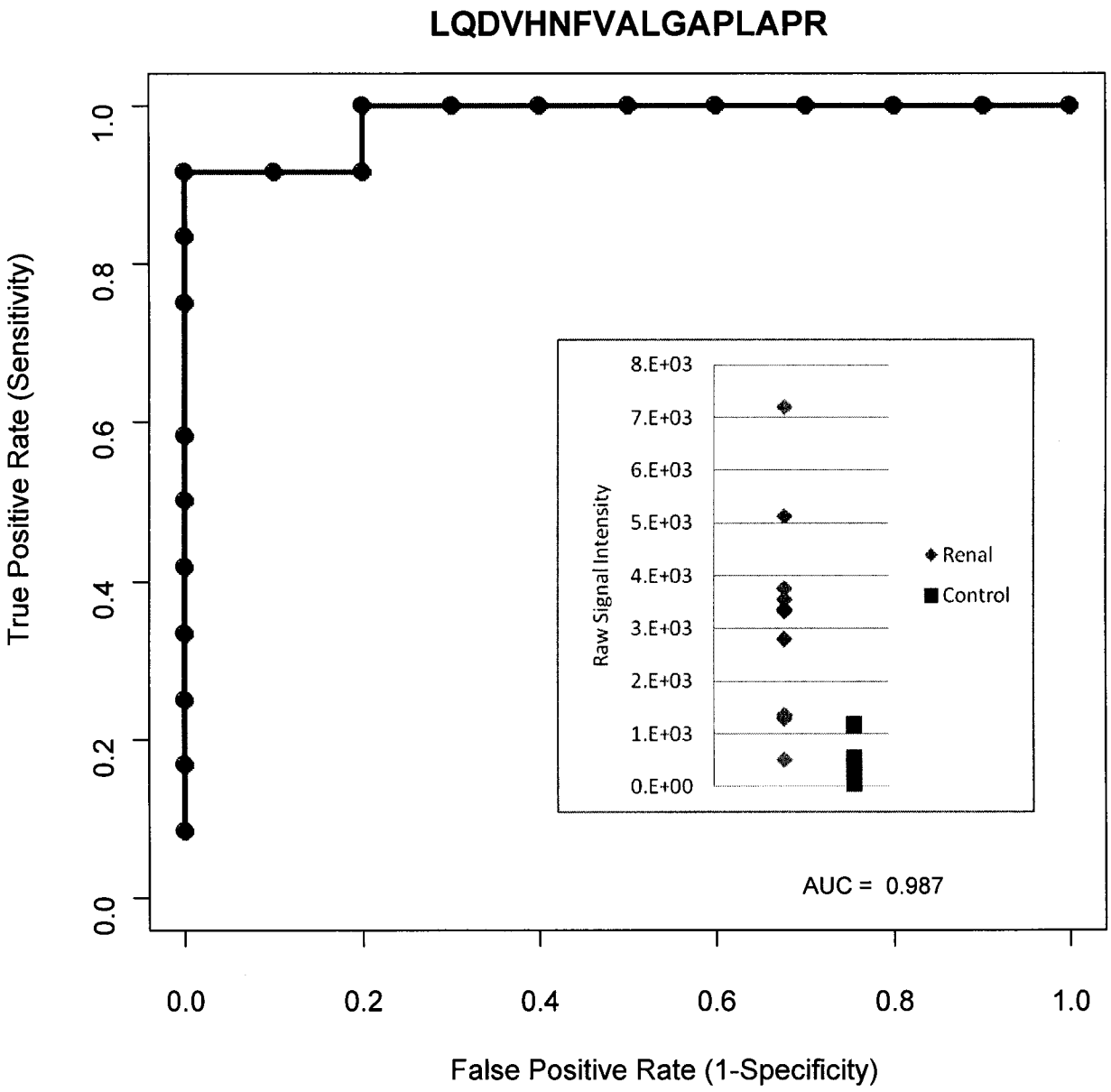

LQDVHNFVALGAPLAPR
True Positive Rate (Sensitivity)
0.0
0.2
0.4
0.6
0.8
1.0
False Positive Rate (1-Specificity)
0.0
0.2
0.4
0.6
0.8
1.0
Raw Signal Intensity
8.E+03
7.E+03
6.E+03
5.E+03
4.E+03
3.E+03
2.E+03
1.E+03
0.E+00
Renal
Control
AUC = 0.987

FIG. 4A

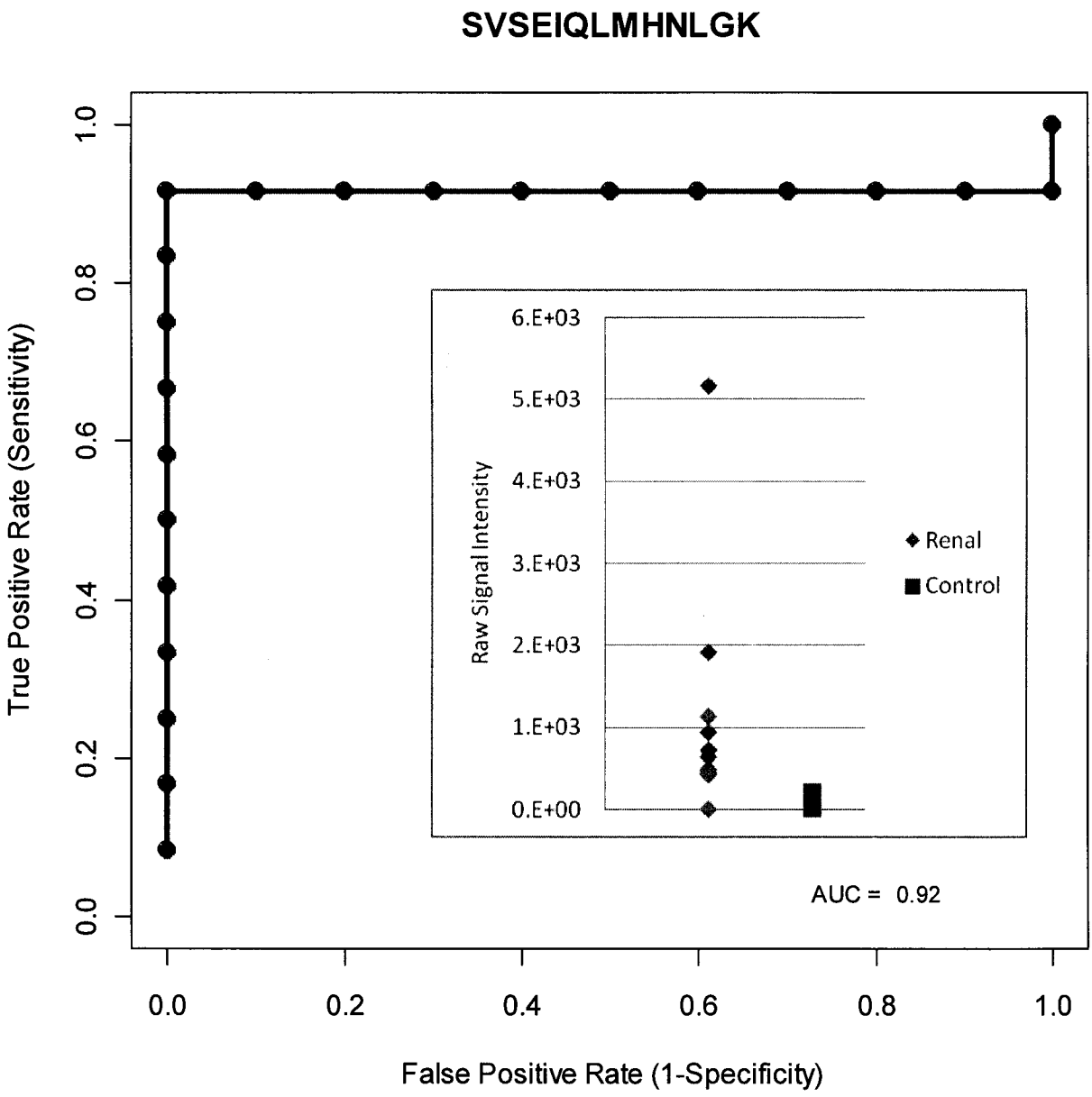

SVSEIQLMHNLGK
True Positive Rate (Sensitivity)
0.0
0.2
0.4
0.6
0.8
1.0
False Positive Rate (1-Specificity)
Raw Signal Intensity
6.E+03
5.E+03
4.E+03
3.E+03
2.E+03
1.E+03
0.E+00
Renal
Control
AUC = 0.92

FIG. 4B

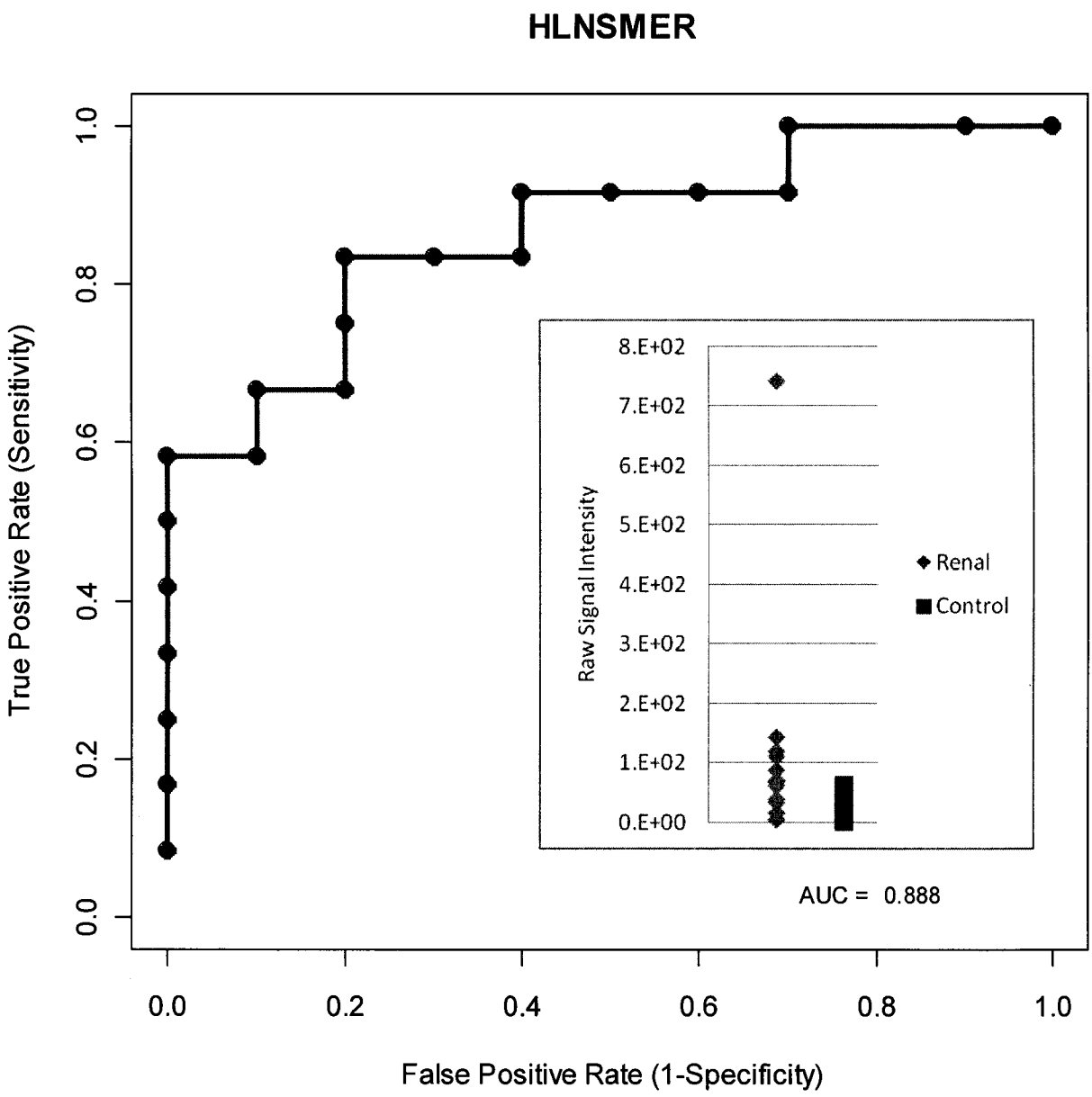

HLNSMER
True Positive Rate (Sensitivity)
0.0
0.2
0.4
0.6
0.8
1.0
False Positive Rate (1-Specificity)
Raw Signal Intensity
8.E+02
7.E+02
6.E+02
5.E+02
4.E+02
3.E+02
2.E+02
1.E+02
0.E+00
Renal
Control
AUC = 0.888

FIG. 4C

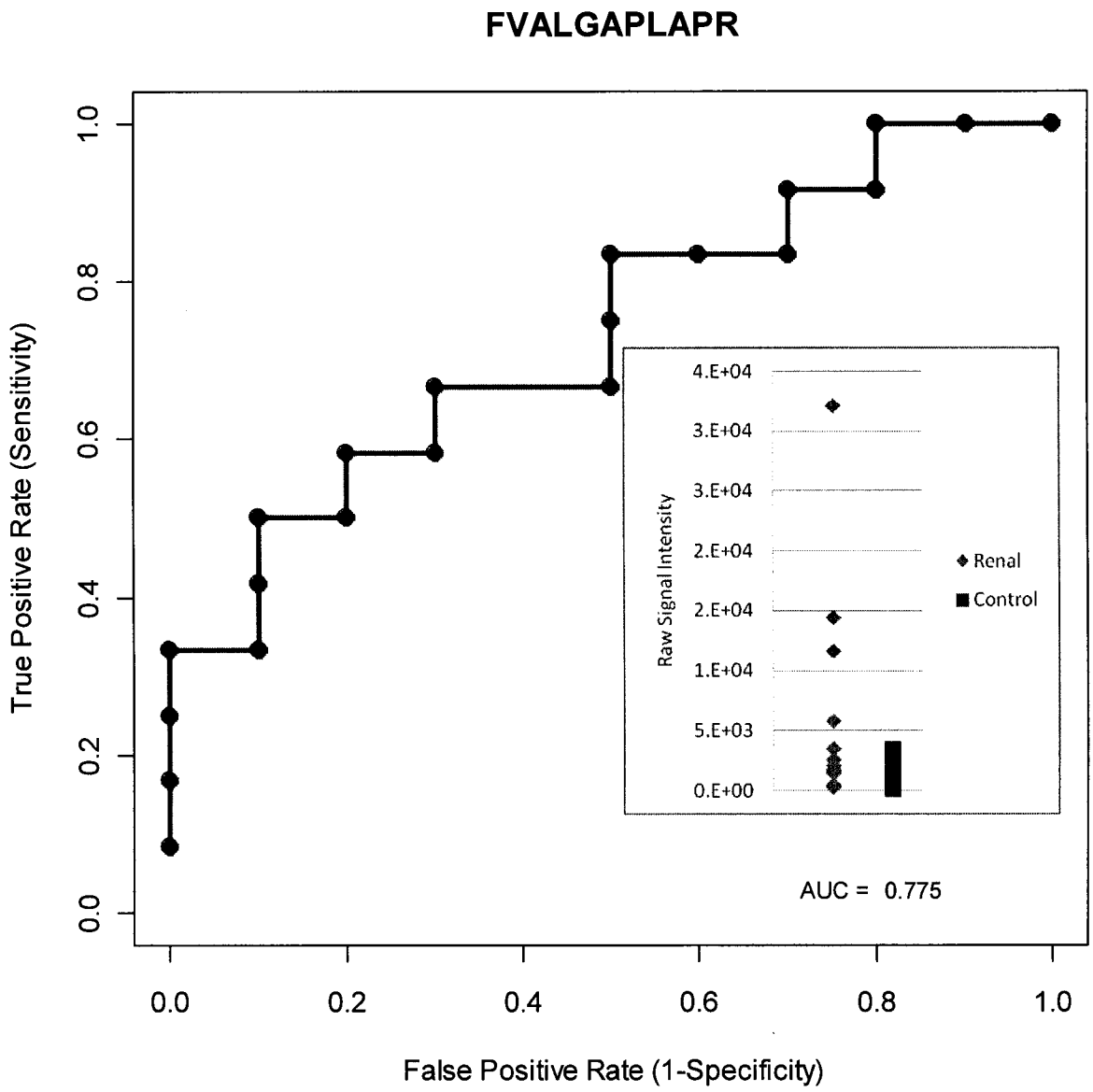

FVALGAPLAPR
True Positive Rate (Sensitivity)
0.0
0.2
0.4
0.6
0.8
1.0
False Positive Rate (1-Specificity)
0.0
0.2
0.4
0.6
0.8
1.0
Raw Signal Intensity
4.E+04
3.E+04
3.E+04
2.E+04
2.E+04
1.E+04
5.E+03
0.E+00
Renal
Control
AUC = 0.775

FIG. 4D

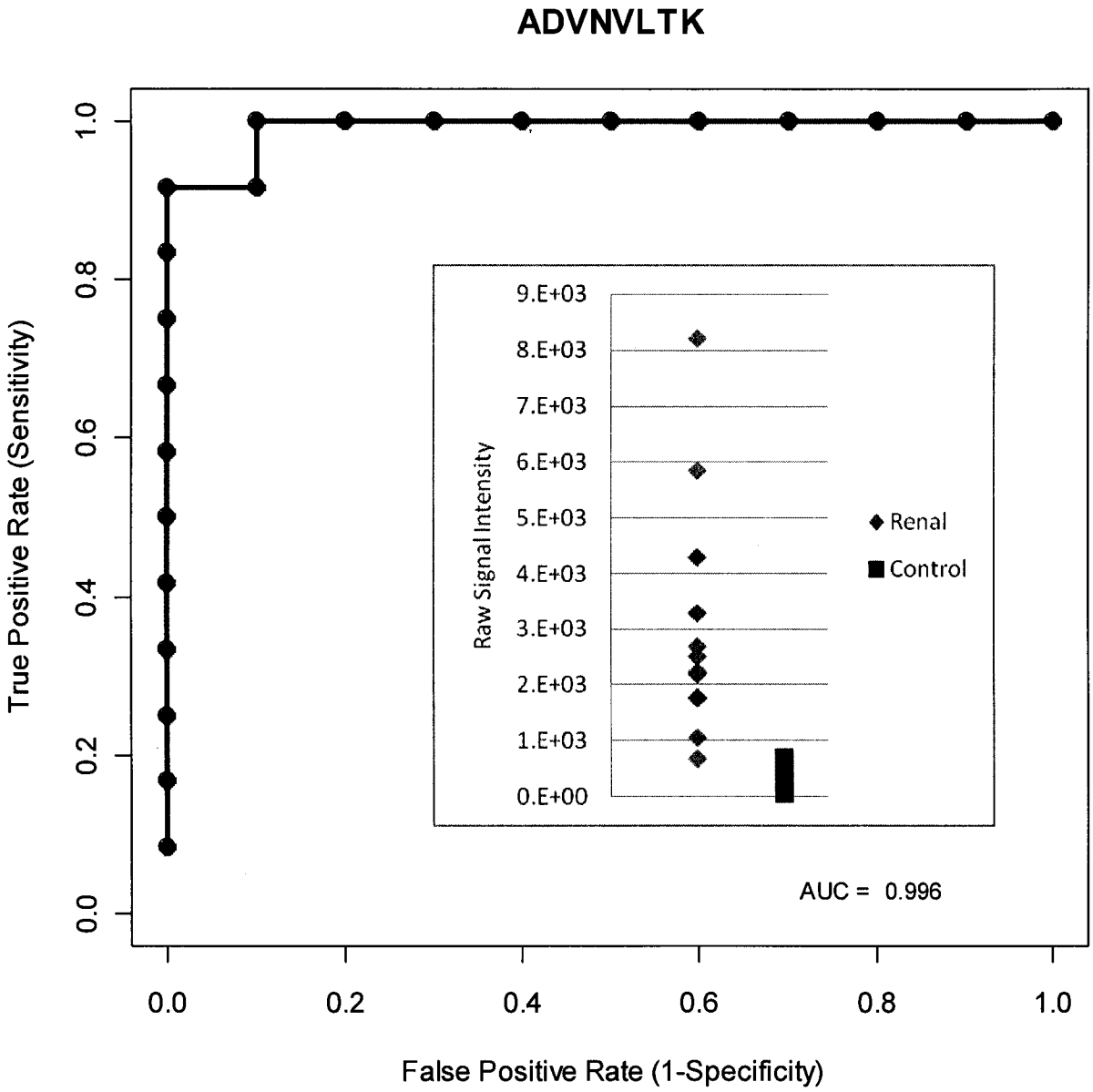

ADVNVLTK
True Positive Rate (Sensitivity)
False Positive Rate (1-Specificity)
0.0
0.2
0.4
0.6
0.8
1.0
Raw Signal Intensity
9.E+03
8.E+03
7.E+03
6.E+03
5.E+03
4.E+03
3.E+03
2.E+03
1.E+03
0.E+00
Renal
Control
AUC = 0.996

FIG. 4E

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/61656

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07K 14/635; C12Q 1/68 (2011.01)
USPC - 530/324 ; 525/54.11; 436/86 ; 250/288
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: C07K 14/635; C12Q 1/68 (2011.01)
USPC: 530/324 ; 525/54.11; 436/86 ; 250/288

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC: C07K 14/635; C12Q 1/68 (2011.01) (text search only)
USPC: 530/324; 525/54.11; 436/86; 250/288 (text search only)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
(USPT, PGPB, EPAB, JPAB); Google Patent, Google Scholar
Search terms on extra sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Lopez et al., "Selected Reaction Monitoring-Mass Spectrometric Immunoassay Responsive to Parathyroid Hormone and Related Variants." Clinical Chemistry, 56: 281-290, 18 December 2009 (18.12.2009), Abstract, p. 1-7 [online] url=<http://www.clinchem.org/cgi/content/full/56/2/281> First published December 18, 2009; 10.1373/clinchem.2009.137323	1-3, 12-15
X ----- Y	US 2009/0090856 A1 (Grant et al.) 30 May 2008 (30.05.2008), Abstract, para [0072], [0073], [0074], [0078], [0079], [0086], [0087], [0095], [0099], [0100], [0106], [0143], [0144], [0149], [0162], [0172], [0176]	1, 2, 12-15 ---------- 3
Y	US 2005/0124010 A1 (Short et al.) 09 Jun 2005 (09.06.2005) (para [0564], [0565], [0622], [0625], [0628], [1735])	3
Y	US 2003/0092076 A1 (Regnier et al.) 15 May 2003 (15.05.2003), para [0096], [0118], [0023]	1-3, 12-15

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 Feb 2011 (09.02.2011)	14 MAR 2011
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/61656

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

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

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

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

3. ☒ Claims Nos.: 4-11
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

continued from box B

Search terms:
PTH, parathyroid hormone, tandem mass spectrometry, chromatography, sample, tandem MS, MS, peptide, SRM, selective reaction monitoring, blood, serum, plasma, internal standard, isotopically labeled, ion, parent, proteolytically digesting, precursor, product ion