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(54) **Title:** MEASUREMENT OF GAMMA-CARBOXYLATION OF PROTEINS

(57) **Abstract:** The invention relates to the measurement of the presence, quantitative level, or absence of gamma-carboxylation of glutamic acid residues in proteins (yielding Gla), and specifically to mass spectrometric methods for measuring the level of gamma-carboxylation of glutamic acid residues in specific peptides derived by proteolytic digestion of proteins.



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MEASUREMENT OF GAMMA-CARBOXYLATION OF PROTEINS

[001] The present application claims priority to U.S. Provisional Patent Application No. 61/905,398 filed November 18, 2013, which is hereby incorporated by reference in its entirety including all tables, figures, and claims.

[002] **SEQUENCE LISTING**

[003] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on November 17, 2014, is named SISPAT1PCT_SeqListing.txt and is 85 kilobytes in size.

[004] **BACKGROUND**

[005] A need exists to improve the measurement of amounts and features of clinically-important proteins, often those found in the blood, for use as biomarkers of disease, markers of treatment efficacy, indicators of disease prognosis, etc. An important example is the measurement of gamma-carboxylated glutamic acid (“Gla”) residues in certain blood proteins involved in coagulation (e.g., coagulation Factor II (prothrombin), Factor VII, Factor IX, Factor X, Protein C, Proteins S, Protein Z). Decreases in the level of gamma-carboxylation in such proteins have been found to be indicative of risk or presence of hepatocellular carcinoma (HCC;), and also indicate reductions in gamma-carboxylation that are associated with certain clinical treatments (e.g., Coumadin, warfarin) designed to inhibit blood clotting by interfering with the normal vitamin-K dependent modification of these proteins.

[006] Prothrombin occurs in blood plasma of normal adults (reference interval of 98 to 162 ug/ml), and an individual’s normal level is relatively constant, with a within-subject variability of 7.3% (Blood Coagul Fibrinolysis. 1998;9(6):525-31). Forms of prothrombin having different numbers of Gla residues have been separated by electrophoresis (Electrophoresis 12, (4) 294–297, 1991) or by liquid chromatography (J. Gastroenterol. Hepatol. 20, 1545–1552 (2005)). The preferred approach for differential measurement of various Gla-forms has however been through use of antibodies that are differentially reactive with unmodified and Gla-modified sites. Several publications have reported the development of monoclonal antibodies with such specificities (Malhotra et al, Thrombosis Research 47 (5) 501–510). The monoclonal MU-3 for example reacts strongly to DCP containing 0-1 Gla, weakly to 2-4 Gla and not at all to DCP containing

more than five Gla (Naraki, et al, BBA 1586, 287–298 (2002), and two clinical tests exist aimed at measuring non-Gla-modified aberrant prothrombin using an immunoassay (an EIA kit with a higher sensitivity (Eitest PIVKA–II, Eisai, Tokyo, Japan) and an electrochemiluminescence kit (Picolumi PIVKA–II, Eisai)). While these antibody-based tests provide some specificity for one or a range of Gla-modified or unmodified forms, they do not directly quantitate the levels of multiple different forms.

[007] Prothrombin is not the only blood protein that is Gla-modified: coagulation factors VII, IX, and X, as well as Protein C, Protein S, and Protein Z contain gamma-carboxylated glutamic acid residues. Those skilled in the art understand that many of the results and methods disclosed here can be applied to these and other Gla-modified molecules to provide important biological and clinical information.

[008] The original observation of prothrombin Gla-deficiency in hepatocellular carcinoma (Liebman, H. A. Cancer Res 49, 6493–6497 (1989) showed an average of 5 Gla residues per molecule compared to 10 Gla residues for native Prothrombin, whereas other work suggests less than 4 (Naraki, et al, BBA 1586, 287–298 (2002)). Gla-deficient Prothrombin, also referred to as des-gamma-carboxy prothrombin (DCP) or the “Protein induced by vitamin K absence, antagonist-prothrombin” (abbreviated as PIVKA–II) is measured by a widely used clinical test based on antibodies recognizing the non-Gla-modified form of prothrombin (TAKIKAWA, et al. J. Gastroenterol. Hepatol.7, 1–6 (1992).

[009] In current medical practice, the prothrombin time (PT) and its derived measures of prothrombin ratio (PR) and international normalized ratio (INR) are used as measures of coagulation through the so-called extrinsic pathway. The purpose is to provide a quantitative measure of the clotting tendency of blood, which must be neither too great (leading to unwanted clots such as cardiac infarcts) nor too little (leading to uncontrolled bleeding). The degree to which coagulation is slowed is a critical health parameter for those patients treated with warfarin, coumadin or similar drugs impacting the vitamin K-dependent processes that generate Gla modifications of proteins. Large numbers of patients are treated with such drugs to decrease the clotting tendency, and this treatment requires repeated monitoring of coagulation to adjust doses to individually effective levels.

[0010] The total amount of prothrombin (measured as prothrombin antigen in immunoassays) has been reported to be superior to the functional INR test for monitoring anticoagulant therapy (Furie, et al, Blood 75, 344–349 (1990); Kornberg, et al,

Circulation 88, 454–460 (1993)) with an INR range of 2.0 to 3.0 corresponding to a native prothrombin antigen range of 56 to 24 ug/ml (Le, et al, Ann. Intern. Med. 120, 552–558 (1994)). However versions of prothrombin are impaired as to coagulant activity by the absence of even 1 or 2 of the 10 Gla-residues normally present (Jorgensen et al J Biol Chem 262, 6729–6734 (1987)), and it is therefore of interest to assess the proportions of the various Gla-modified forms of Prothrombin as well as the total amount of immunoreactive protein. Using the PIVKA–II test developed for measuring diminished Gla-modification in liver cancer, it has been shown that Prothrombin clotting activity decreases in the series 100%, 82%, 67%, 28%, 8%, 3% are accompanied by decreases in the average number of Gla-modifications from the normal 10 to 7.9, 6.7, 6.5, 6.3, 4.5, accompanied by PIVKA–II results rising from 0 to 0.75, 1.1, 2.2, 5.6, 13.6 (Grosley, et al, J. Lab. Clin. Med. 127, 553–564 (1996)). These gradual changes across a range of physiological coagulation states indicate that accurate measurement of the distribution of Gla-modifications has value in assessing coagulation status and guiding anticoagulant therapy.

[0011] Here I provide improved methods and analytes for the measurement of gamma-carboxylation through the use of mass spectrometry, in some cases used in conjunction with dried blood spot samples.

[0012] The following publications and patents provide important background on the technology employed in the present invention.

[0013] US Patent No's US 7,632,686 entitled HIGH SENSITIVITY QUANTITATION OF PEPTIDES BY MASS SPECTROMETRY, filed Oct. 2, 2003, and 6,649,419, entitled *Method and Apparatus for Protein Manipulation*, filed 28 November 2000, are herein incorporated by reference in their entirety.

[0014] The disclosures of U.S. Patent Applications: Ser. No. 10/676,005, entitled *High Sensitivity Quantitation of Peptides by Mass Spectrometry*; filed 2 October 2003, Ser. No.11/256,946, entitled *Process For Treatment Of Protein Samples*, filed 25 October 2005, describing methods for processing protein samples imbibed within a porous membrane; Ser. No. 12/042,931, entitled *Magnetic Bead Trap and Mass Spectrometer Interface*"; Ser. No. 11/147,397, entitled *Stable Isotope Labeled Polypeptide Standards for Protein Quantitation*" describing means for production of labeled peptide internal standards as recombinant concatamer proteins filed 8 June 2005; Ser. No. PCT/US11/28569, entitled "*Improved Mass Spectrometric Assays For Peptides*", filed 15

March 2011, describing improvements in quantitation via internal standards and improved packaging of internal standards; and PCT/US13/48384, entitled “*Multipurpose Mass Spectrometric Assay Panels For Peptides*”, filed 27 June 2013, are each herein incorporated by reference in their entireties.

[0015] The disclosures of U.S. Provisional Applications; Ser. No. 60/415,499, entitled *Monitor Peptide Enrichment Using Anti-Peptide Antibodies*, filed 3 Oct 2002; Ser. No. 60/420,613, entitled *Optimization of Monitor Peptide Enrichment Using Anti-Peptide Antibodies*, filed 23 October 2002; Ser. No. 60/449,190, entitled *High Sensitivity Quantitation of Peptides by Mass Spectrometry*, filed 20 February 2003; Ser. No. 60/496,037, entitled *Improved Quantitation of Peptides by Mass Spectrometry*, filed 18 August 2003; Ser. No. 60/557,261, entitled *Selection of Antibodies and Peptides for Peptide Enrichment*, filed 29 March 2004; Ser. No. 61/314149, entitled “*MS Internal Standards at Clinical Levels*” filed on March 15, 2010 and Ser. No. 61/314154, entitled “*Stable Isotope Labeled Peptides on Carriers*” filed on March 15, 2010; the disclosures of each of which are herein incorporated by reference in their entireties.

[0016] **The following publications further described background and methods.**

[0017] Mass Spectrometric Quantitation of Peptides and Proteins Using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). Anderson, N.L., Anderson, N.G., Haines, L.R., Hardie, D.B., Olafson, R.W., and Pearson, T.W. *Journal of Proteome Research*, 3: 235-44 (2004).

[0018] Antibody-based enrichment of peptides on magnetic beads for mass-spectrometry-based quantification of serum biomarkers. J. R. Whiteaker, L. Zhao, H. Y. Zhang, L-C Feng, B. D. Piening, L. Anderson and A. G. Paulovich, *Analytical Biochemistry* 362:44–54 (2007).

[0019] Anti-peptide antibody screening: selection of high affinity monoclonal reagents by a refined surface plasmon resonance technique. Matthew E. Pope, Martin V. Soste, Brett A. Eyford, N. Leigh Anderson and Terry W. Pearson, *J Immunol Methods*, 341(1-2):86-96 (2009)

[0020] An automated and multiplexed method for high throughput peptide immunoaffinity enrichment and multiple reaction monitoring mass spectrometry-based quantification of protein biomarkers. Jeffrey R. Whiteaker, Lei Zhao, Leigh Anderson, and Amanda G. Paulovich, *Mol. Cell. Proteomics* 9:184-196 (2010)

- [0021] MALDI Immunoscreening (MiSCREEN): A Method for Selection of Anti-peptide Monoclonal Antibodies For Use in Immunoproteomics, Morteza Razavi, Matthew E. Pope, Martin V. Soste, Brett A. Eyford, N. Leigh Anderson and Terry W. Pearson, J. Immunol. Methods, 364 (2011) 50–64.
- [0022] Evaluation of Large Scale Quantitative Proteomic Assay Development Using Peptide Affinity-based Mass Spectrometry. Jeffrey R. Whiteaker, Lei Zhao, Susan E. Abbatiello, Michael Burgess, Eric Kuhn, ChenWei Lin, Matthew E. Pope, Morteza Razavi, N. Leigh Anderson, Terry W. Pearson, Steven A. Carr, and Amanda G. Paulovich, Mol Cell Proteomics (2011)
- [0023] Inter-laboratory Evaluation of Automated, Multiplexed Peptide Immunoaffinity Enrichment Multiple Reaction Monitoring Assay Performance for Quantifying Proteins in Plasma. Eric Kuhn, Jeffrey R Whiteaker, DR Mani, Angela M Jackson, Lei Zhao, Matthew Pope, Derek Smith, Keith D Rivera, N Leigh Anderson, Steven J. Skates, Terry W Pearson, Amanda G Paulovich and Steven A Carr, Submitted
- [0024] The Precision of Heavy-Light Peptide Ratios Measured by MALDI-TOF Mass Spectrometry. N. Leigh Anderson, Morteza Razavi, Terry W. Pearson, Gary Kruppa, Rainer Paape, and Detlev Suckau, J. Proteome Res (2011) DOI: 10.1021/pr201092v
- [0025] Ultra-fast quantitation of peptides from human plasma digests using SISCAPA and RapidFire high throughput mass spectrometry, Morteza Razavi, Lauren E Frick, William A LaMarr, Matthew E Pope, Christine A Miller, N Leigh Anderson and Terry W Pearson (2012), J. Proteome Res (2012) DOI: 10.1021/pr300652v
- [0026] Quantitation of a Proteotypic Peptide From Protein C Inhibitor by LC-Free SISCAPA-MALDI Mass Spectrometry Predicts Biochemical Recurrence of Prostate Cancer. Morteza Razavi, Lisa D. S. Johnson, Julian J. Lum, Gary Kruppa, N. Leigh Anderson, and Terry W. Pearson, (2013) Clin. Chem, submitted
- [0027] **MS Peptide Assays**
- [0028] A variety of methods are known in the art for the measurement of proteins and peptides by mass spectrometry. The following summary provides the steps of creating a mass spectrometric assay for a peptide of interest (usually a proteotypic peptide used as a surrogate for the protein it uniquely represents) employing specific peptide affinity enrichment (commonly referred to as “SISCAPA”). One skilled in the art will

recognize that this method is provided due to its efficiency in selected peptide measurement, while any of a range of other methods using different fractionation or enrichment schemes, and different MS instruments and methods, could have been used to obtain the desired quantitative results.

[0029] Selecting monitor peptides

[0030] Target (“monitor”) peptides to be used as analytes are selected from among the relevant peptides produced in the proteolytic digestion of the relevant biological sample. In the present case, the monitor peptides are selected from among the peptides that a) arise from proteins that play a significant role in blood coagulation, and either b) contain sites of gamma-carboxylation of glutamic acid residues (producing “Gla”) or c) provide a normalizing measurement useful in interpreting the proportion of Gla-modification in other peptides.

[0031] Creating isotope-labeled monitor peptides

[0032] An isotopically labeled version of the selected peptide (a stable isotope-labeled standard or “SIS”) can be made in which the chemical structure is maintained, but one or more atoms are substituted with an isotope such that a mass spectrometer (MS) can distinguish the labeled peptide from the normal peptide (containing the natural abundance of each element’s isotopes). Such a labeled standard is highly desirable when making quantitative measurements by MS using the well-known method referred to as stable isotope dilution MS, in which a test molecule (in this case a target peptide) is measured in relation to a known or fixed relative amount of SIS added to the peptide sample prior to introduction into the MS. In a preferred method for making SIS peptides, nitrogen-15 or carbon-13 can be introduced instead of the natural nitrogen-14 or carbon-12 at one or more positions in the synthesized peptide. The synthesized peptide will be heavier by a number of atomic mass units equal to the number of substituted nitrogens or carbons. The peptide is carefully made so that the number of added mass units is known and well-determined (*i.e.*, all of the material produced as one standard has the same mass insofar as possible – achieved by using highly enriched isotopic variants of the amino acids, for example). In one embodiment, nitrogen-15 or carbon-13 labeled amino acid precursors substituted at >98% at each substituted position are used at one or more positions in the peptide synthesis process to introduce between 4 and 10 additional mass units compared to the natural peptide. High levels of isotopic substitutions at the chosen positions are desirable: 99% isotopic purity in the

substituted positions is desirable; 97-98% is workable in many situations, while substitution levels below 95% are less useful. Such nitrogen-15 labeled amino acid precursors (or their carbon-13 labeled equivalents) are commercially available as Fmoc derivatives suitable for use directly in conventional commercial peptide synthesis machines. The resulting labeled monitor peptides can be purified using conventional LC methods (typically to >90% purity) and characterized by MS to ensure the correct sequence and mass.

[0033] SIS peptides can be introduced into the proteolytic digest in their final form (i.e., having the same sequence as the target peptide) or they can be added in the form of a longer labeled polypeptide molecule (an “extended SIS”) containing the SIS as a subsequence. An extended SIS is generally designed to be cleaved during sample proteolysis to yield the appropriate SIS molecule, and hence generally includes cleavage sites at both termini recognized by the proteolytic enzyme used (in the case of trypsin, a K or R residue is included immediately N-terminal to the SIS sequence). In one approach, an extended SIS can be composed of the SIS sequence plus peptide extensions on the N- or C-termini. In a preferred case, the extended SIS sequence is a larger subset of the sequence of the parent protein from which the SIS is released by digestion, so that the N- and C-terminal extensions provide proteolytic cleavage sites that mimic the cleavage sites in the parent protein, thus assisting in compensating for any inefficiency in proteolytic cleavage of these sites in the parent protein. An extended SIS can also be created by biosynthesis (e.g., in a bacterial, insect or mammalian cell culture system) or cell-free synthesis (e.g., in rabbit reticulocyte or wheat germ cell-free protein synthesis systems) of a polypeptide from a nucleic acid construct (or gene) coding for a protein that contains the SIS sequence. Such polypeptides can be labeled through incorporation of labeled amino acid precursors provided to the synthesis system, or through generation of labeled amino acid(s) within the system by biosynthesis from simple labeled compounds in the medium. An extended SIS can also be generated by post-synthetic labeling of a synthetic or natural polypeptide, for example by introduction of oxygen-18 into C-terminal carboxyl groups of peptides formed by trypsin cleavage.

[0034] Creating anti-peptide antibodies

[0035] Many methods used to measure peptides by MS benefit from enrichment of the peptides to be measured and depletion of other peptides (i.e., sample cleanup). Specific anti-peptide antibodies have been shown to be very effective in this application,

and can be generated using existing polyclonal and monoclonal technologies. To immunize an animal for production of anti-peptide antibodies, the target peptide (labeled or not, if the latter is, as expected, more economical) is coupled to a carrier protein (*e.g.*, keyhole limpet hemocyanin (KLH); not homologous to a human protein) and used to immunize an animal (such as a rabbit, chicken, goat or sheep) by one of the known protocols that efficiently generate anti-peptide antibodies. For convenience, the peptide used for immunization and antibody purification may contain additional c-terminal residues added to the monitor peptide sequence (here abbreviated MONITOR), *e.g.*: nterm-MONITOR-lys-gly-ser-gly-cys-cterm. The resulting extended monitor peptide can be conveniently coupled to carrier (*e.g.*, KLH) that has been previously reacted with a heterobifunctional reagent such that multiple SH-reactive groups are attached to the carrier. In classical immunization with the peptide (now as a hapten on the carrier protein), a polyclonal antiserum will be produced containing antibodies directed to the peptide, to the carrier, and to other non-specific epitopes. Alternatively, there are many methods known in the art for coupling a peptide, with or without any extensions or modifications, to a carrier for antibody production, and any of these may be used. Likewise there are known methods for producing anti-peptide antibodies by means other than immunizing an animal with the peptide on a carrier (*e.g.*, phage-display), and non-antibody reagents (*e.g.*, aptamers, or Somamers®) have been developed capable of binding peptide and protein targets to perform the enrichment function. Any of the alternatives can be used provided that a suitable specific reversible binding agent for the monitor peptide is produced.

[0036] Specific anti-peptide antibodies (*e.g.*, rabbit antibodies such as rabbit monoclonal antibodies) can be prepared from this antiserum by affinity purification on a column containing tightly-bound peptide. Such a column can be easily prepared by reacting an aliquot of the extended monitor peptide with a thiol-reactive solid support. Crude antiserum can be applied to this column, which is then washed and finally exposed to 10% acetic acid (or other elution buffer of low pH, high pH, or high chaotrope concentration) to specifically elute anti-peptide antibodies. These antibodies are neutralized or separated from the elution buffer (to prevent denaturation), and the column is recycled to physiological conditions for application of more antiserum if needed. Antibodies also may be produced by hybridoma, B-cell cloning, phage display and other techniques well known in the art.

[0037] Anti-peptide antibodies capable of binding two or more closely related structural variants of a peptide can be similarly generated. Such antibodies can bind peptides having sequence differences (generally outside the binding epitope sequence) or containing post-translational modifications (including gamma-carboxylation of glutamic acid residues). The ability of an antibody to bind such variant peptide forms is enhanced by immunization and/or selection (or affinity purification) of using multiple peptide forms. An antibody that binds multiple forms of a target peptide is desirable as it is potentially more economical than production of individual antibodies for each peptide form to be measured.

[0038] The peptide-specific antibody is finally captured or immobilized on a column, bead or other surface for use as a peptide-specific affinity capture reagent. In one embodiment, the anti-peptide antibody is immobilized on commercially available protein A-derivatized POROS chromatography media (Applied Biosystems) and covalently fixed on this support by covalent crosslinking with dimethyl pimelimidate according to the manufacturer's instructions. The resulting solid phase media can bind the monitor peptide specifically from a peptide mixture (*e.g.*, a tryptic digest of serum or plasma) and, following a wash step, release the monitor peptide under mild elution conditions (*e.g.*, 10% acetic acid). Restoring the column to neutral pH then regenerates the column for use again on another sample, a process that is well known in the art to be repeatable hundreds of times. In another embodiment, the anti-peptide antibodies are captured on magnetic beads (either before exposure to the digest or afterwards), which simplifies separation of antibody from the digest after peptide binding, washing, and peptide analyte recovery. High affinity (typical dissociation constants of 10^{-10}), high specificity antibodies are preferred, and processes of antibody generation and selection are designed to optimize these characteristics. In another embodiment, anti-peptide antibodies are bound to Protein G-coated magnetic beads (*e.g.*, Dynal Dynabeads G), and used either with or without chemical crosslinking of antibody to protein G. A range of alternative supports and binding reactions are commercially available.

[0039] Digestion of sample to peptides

[0040] A protein sample such as plasma, containing the selected protein to be measured, is digested essentially to completion with an appropriate protease (*e.g.*, trypsin) to yield peptides (including the monitor peptide selected in step 1). For a monitor peptide whose sequence appears once in the target protein sequence, this

digestion ideally generates the same number of monitor peptide molecules as there were target protein molecules in the starting sample (provided each monitor peptide sequence occurs once per protein). The digestion is carried out by first denaturing the protein sample (*e.g.*, with urea, trifluoroethanol or guanidine HCl), reducing the disulfide bonds in the proteins (*e.g.*, with dithiothreitol or mercaptoethanol), alkylating the cysteines (*e.g.*, by addition of iodoacetamide), quenching excess iodoacetamide by addition of more dithiothreitol or mercaptoethanol, and finally (after removal or dilution of the denaturant) addition of the selected proteolytic enzyme (*e.g.* trypsin), followed by incubation to allow digestion. Following incubation, the action of trypsin is terminated, either by addition of an enzyme inhibitor (*e.g.*, DFP, PMSF or aprotinin) or by denaturation (through heat or addition of denaturants, or both) or removal (if the trypsin is on a solid support) of the trypsin. The destruction of the trypsin activity is desirable in order to avoid damage to antibodies later by residual proteolytic activity in the sample.

[0041] Adding isotopically-labeled monitor peptide internal standards

[0042] A measured aliquot of isotopically-labeled synthetic monitor peptide (SIS) is added to a measured amount of the digested sample peptide mixture to provide an internally standardized mixture. The amount of SIS added is typically chosen to be close to or greater than (if the standard serves for example, as carrier for a low abundance peptide) the expected abundance of the same “natural” peptide in the sample aliquot. Following this addition the monitor peptide will be present in the sample in two forms (natural and isotopically-labeled). The concentration of the isotopically-labeled version is precisely controlled so as to be either 1) reproducible across a set of samples to be compared for calibration (*i.e.*, precise but not accurately known in terms of absolute mass) or 2) accurately determined based on the mass added and the known aliquot volumes, so as to provide an internal quantitative standard. The labeled peptide may be added separately from the antibody, although it could be added in combination with the antibody for stability and simplicity. SIS addition can occur either before or after sample digestion.

[0043] Enrichment of the monitor peptide by antibody capture and elution

[0044] The peptide mixture (sample digest with added isotopically-labeled monitor peptides) is exposed to the peptide-specific affinity capture reagent, which preferentially binds the monitor peptide but does not distinguish between labeled and unlabeled forms

of a given peptide structure (since isotopic substitutions are not expected to affect antibody binding affinity). Following one or more wash steps (*e.g.*, phosphate-buffered saline, water) the bound peptides are then eluted (*e.g.*, with 5% acetic acid, or with a mixture of water, formic acid and acetonitrile), for MS analysis. The affinity support can, if desired, be recycled in preparation for another sample. In some of the high-throughput assay applications envisioned, it will be advantageous to recycle the immobilized antibodies hundreds, if not thousands, of times when flow-through columns are used. Current evidence indicates that rabbit polyclonal antibodies can be recycled at least 200 times when antigens are eluted with 5% or 10% acetic acid and total exposure to acid is kept short (*e.g.*, less than 1 minute before regeneration with neutral pH buffer). Magnetic bead embodiments, with antibodies bound covalently or non-covalently (*e.g.*, through protein G), can also be conveniently automated in a variety of ways to achieve high throughput, and avoid the potential for carryover inherent in re-usable antibody columns.

[0045] The enrichment step is an important element of the method because it allows enrichment and concentration of low abundance peptides, derived from low abundance proteins in the sample. Ideally, this enrichment process delivers only the monitor peptide to the MS, and makes its detection a matter of absolute MS sensitivity, rather than a matter of detecting the monitor peptide against a background of many other, potentially much higher abundance peptides present in the whole sample digest. This approach effectively extends the detection sensitivity and dynamic range of the MS detector in the presence of other high abundance proteins and peptides in the sample and its digest.

[0046] In the case where a monitor peptide exists in various chemically modified forms, *e.g.*, a peptide sequence in which varying numbers of glutamic acid residues are modified by gamma-carboxylation, an antibody to one form may or may not bind most or all of the other forms. The ability to bind multiple forms, and thus for one antibody to enrich a spectrum of forms, can be improved by several methods, including 1) immunization using a mixture of the peptide forms as immunogens; 2) affinity purification of polyclonal antibodies on multiple peptide forms so as to capture a mix of antibodies capable of binding a range of forms; and 3) by selecting monoclonal clones capable of binding multiple peptide forms.

[0047] Analysis of the captured monitor peptides by MS

[0048] The monitor peptide (including natural and isotopically-labeled versions,

and where appropriate including multiple chemical forms of the peptide) enriched in the preceding step is delivered into the inlet of a mass spectrometer (*e.g.*, by MALDI or by electrospray ionization (ESI)). The mass spectrometer can be a TOF (time-of-flight), a Q-TOF, a TOF/TOF, a triple quadrupole, an ion trap, an orbitrap, an ion-cyclotron resonance machine, or any other instrument of suitable mass resolution ($>1,000$) and sensitivity, and can employ one, two, or more levels of mass selection interspersed with analyte fragmentation processes (*e.g.*, collision-induced fragmentation).

[0049] The MS typically measures the ion current or ion count (number of ions) for a specific peptide configuration. In the case of a ESI-triple quadrupole instrument fed by a liquid chromatograph, the ion current detected given specific settings of parent and daughter ion masses, collision parameters, etc., is typically integrated across a window of time when the peptide peak emerges from the LC, and the area of this peak provides a quantitative measure of peptide amount (not as yet standardized on any absolute scale). In the case of a MALDI-TOF instrument, the primary isotopic peak, or some function over the whole isotopic envelope, can be integrated to provide an area yielding an equivalent measure of peptide amount. In either case, the MS can also measure the amount of an internal standard peptide such as the SIS described above, and the unlabeled, sample-derived peptide peak area divided by the SIS peak area to yield a peak area ratio which is thereby standardized across samples, provided that the same amount of SIS was added to each (this is the conventional stable isotope dilution method applied to peptides). Alternatively the maximum peak heights of the natural and labeled peptides can be used as measures of peptide amount. In addition, the MS can also measure additional forms of a peptide (as well as SIS version of those forms) so as to obtain information on the spectrum of such forms. Further, the MS can collect equivalent data on multiple different peptide sequences, each in multiple chemically-modified forms, and each potentially accompanied by respective SIS versions. Thus it is practical to measure the amounts of several peptides and/or multiple forms of the same peptide (*e.g.*, varying in occurrence of glutamic acid gamma-carboxylation), in relation to SIS internal standards where appropriate, in a single analysis. The opportunity thus exists to develop multiple ratio and other indices standardizing and comparing these measures to deliver meaningful results.

[0050] Computation of abundance of monitor peptides and their parent proteins in the sample

[0051] Various methods are available to compute clinically-meaningful values from the quantitative peptide signals delivered by the mass spectrometer. In the simplest case, a ratio is computed between the amounts of the labeled (SIS) and unlabeled (natural) monitor peptides. Since the amount of labeled peptide added is known, either directly by analysis of the SIS sample or in relation to a different sample whose content of the target is known (either in terms of mass or in terms of constancy over time and between samples) the amount of the natural monitor peptide derived from the sample digest can then be calculated by multiplying the known or assumed concentration of labeled monitor peptide by this measured ratio. By assuming that the amount of the monitor peptide in the digest is the same as (or reproducibly related to) the amount of the parent protein from which it is derived, a measure of the protein amount in the sample can be obtained. Alternatively, ratios can be computed between the amounts (MS signals or peak areas) of different chemically distinct (post-translationally modified, e.g., by gamma carboxylation) forms of a peptide. The amounts of entered into such a ratio between forms can be made using the MS signals of the distinct forms themselves, or the normalized values obtained after ratioing these signals against the respective SIS peptides to provide improved internal standardization.

[0052] In a further alternative, MS-determined values for peptide forms (either before or after normalization by ratioing to respective SIS internal standards) can be combined through an algorithm to yield a single simple value that may better correlate with a familiar clinical variable (for example INR in the case of blood coagulation). When needed, target peptide abundances can be converted to sample protein abundances by relation to results, and resulting calibration curves or factors, derived from one or more calibration samples containing known amounts of target protein run in parallel.

[0053] The foregoing description outlines the SISCAPA method, which while one embodiment of the application of the present technology, is not the only application envisioned.

[0054] DETAILED DESCRIPTION OF THE INVENTION

[0055] In addition to those background aspects of the invention mentioned above, the invention relates to the measurement of the presence, quantitative level, or absence of gamma-carboxylation of glutamic acid residues in proteins (yielding Gla), and specifically to mass spectrometric methods for measuring the level of gamma-

carboxylation of glutamic acid residues in specific peptides derived by proteolytic digestion of proteins. Several embodiments focus on proteins involved in mammalian hemostasis, although those skilled in the art will understand that the method is applicable to other systems. Such Gla measurements are useful in the diagnosis of diseases affecting blood clotting and in the monitoring and adjustment of therapies aimed at altering clotting behavior, such as warfarin and Coumadin therapy. While there are well known methods for measuring the physical coagulation of fresh blood, these methods are time consuming, require timely access to a liquid blood sample and cannot be carried out effectively on non-liquid or preserved samples such as dried blood spots (DBS). This requirement represents a barrier to the regular use of such measurements for patients who are in remote locations, or otherwise unable to visit a clinic regularly. It is an object of the present invention to provide a means of making measurements of one or more selected peptides and/or peptide forms derived from selected blood proteins that can serve as an effective surrogate of these functional assays, and that can allow the test to be carried out on samples collected anywhere and transported to a central laboratory inexpensively (for example as dried blood spots on paper).

[0056] While gamma-carboxylation of glutamic acid residues (Gla formation) in protein clotting factors is critical to the mechanism of hemostasis, a simple measurement of Gla levels is not sufficient to replace the INR directly. The present invention can make use of a calibration step in which the level of Gla in specific peptides of protein clotting factors, together with optional normalization measurements, is experimentally related to physical clotting time (e.g., the INR value, and other measures) to produce a calibration factor or curve. This calibration can be developed on a population basis (based on the average over a group of individuals), or more preferably, the calibration relating Gla level(s) and coagulation (or INR) can be developed on a personalized basis through analysis of multiple blood samples from an individual at different times, for example prior to and during treatment with a drug such as Coumadin. INR and Gla-level results can thus be obtained on aliquots of the same samples and compared to establish a direct relationship, and this relationship (calibration factor or curve) used subsequently to predict clotting properties (that would normally be predicted from the INR) based on the Gla-level results.

[0057] Additionally, decreased levels of gamma-carboxylation of glutamic acid residues in Prothrombin have proven useful in the detection and assessment of liver

cancer, and des-gamma-carboxy prothrombin (DCP), also known as the protein induced by vitamin K absence or antagonist II (abbreviated PIVKA–II), is considered a complementary biomarker to alpha fetoprotein (AFP) and the third electrophoretic form of lentil lectin-reactive AFP% (AFP-L3%) for assessing the risk of developing HCC. It is an object of the present invention to provide an improved method of measuring this decrease in gamma-carboxylation more precisely and more conveniently in the assessment of liver cancer risk.

[0058] Prothrombin (also known as coagulation Factor II) is produced in the liver and is post-translationally modified in a vitamin K-dependent enzymatic reaction that converts ten glutamic acids on prothrombin to gamma-carboxyglutamic acid (Gla). Other proteins important in blood coagulation are also modified in this way, including Factor VII, Factor IX, Factor X, Protein C, Protein S and Protein Z. In the presence of calcium, the Gla residues promote the binding of modified proteins to phospholipid membranes. Deficiency of vitamin K or administration of the anticoagulant warfarin inhibits the production of gamma-carboxyglutamic acid residues, slowing the activation of the coagulation cascade. The existence of 10 Gla-sites in prothrombin, each of which can in principle be modified or unmodified independently, means that a total of 2^{10} , or 1,024 different Gla-modified forms of the protein can occur. It is an object of the present invention to provide a simple, clinically-interpretable summary of this complexity.

[0059] Vitamin K-dependent gamma-carboxylated human proteins include coagulation Factor II (prothrombin), Factor VII, Factor IX, Factor X, Protein C, Protein S and Protein Z. Tryptic peptides containing Gla residues from these proteins are:

No.	Peptide	ID	Protein source
1	ANTFLEEVR	SEQ ID NO:1	Factor II (prothrombin)
2	ANTFLXEVR	SEQ ID NO:2	Factor II (prothrombin)
3	ANTFLEXVR	SEQ ID NO:3	Factor II (prothrombin)
4	ANTFLXXVR	SEQ ID NO:4	Factor II (prothrombin)
5	ECVEETCSYEEAFEALLESSTATDVFWAK	SEQ ID NO:5	Factor II (prothrombin)
6	SSTATDVFWAK	SEQ ID NO:6	Factor II (prothrombin)
7	GNLER	SEQ ID NO:7	Factor II (prothrombin)
8	CSFEER	SEQ ID NO:8	Factor IX

No.	Peptide	ID	Protein source
9	ECMEEK	SEQ ID NO:9	Factor IX
10	EVFENTER	SEQ ID NO:10	Factor IX
11	LEEFVQGNLER	SEQ ID NO:11	Factor IX
12	TTEFWK	SEQ ID NO:12	Factor IX
13	ANAFLEELR	SEQ ID NO:13	Factor VII
14	DAER	SEQ ID NO:14	Factor VII
15	EEQCSFEEAR	SEQ ID NO:15	Factor VII
16	EIFK	SEQ ID NO:16	Factor VII
17	PGSLER	SEQ ID NO:17	Factor VII
18	ANSFLEEMK	SEQ ID NO:18	Factor X
19	ECMEETCSYEEAR	SEQ ID NO:19	Factor X
20	EVFEDSDK	SEQ ID NO:20	Factor X
21	GHLER	SEQ ID NO:21	Factor X
22	TNEFWNK	SEQ ID NO:22	Factor X
23	ANSFLEELR	SEQ ID NO:23	Protein C
24	ECIEEICDFEEAK	SEQ ID NO:24	Protein C
25	EIFQNVDDTLAFWSK	SEQ ID NO:25	Protein C
26	HSSLER	SEQ ID NO:26	Protein C
27	ANSLLEETK	SEQ ID NO:27	Protein S
28	ECIEELCNK	SEQ ID NO:28	Protein S
29	EEAR	SEQ ID NO:29	Protein S
30	EVFENDPETDYFYYPK	SEQ ID NO:30	Protein S
31	QGNLER	SEQ ID NO:31	Protein S
32	AGSYLLEELFEGNLEK	SEQ ID NO:32	Protein Z
33	ECYEEICVYEEAR	SEQ ID NO:33	Protein Z
34	EVFENEVVTDEFWR	SEQ ID NO:34	Protein Z
35	ETAASLLQAGYK	SEQ ID NO:35	Prothrombin (non-Gla)
36	LAACLEGNCAEGLGTNYR	SEQ ID NO:36	Prothrombin (non-Gla)
37	HQDFNSAVQLVENFCR	SEQ ID NO:37	Prothrombin (non-Gla)

No.	Peptide	ID	Protein source
38	IVEGSDAEIGMSPWQVMLFR	SEQ ID NO:38	Prothrombin (non-Gla)
39	SEGSSVNLSPPLEQCVPDR	SEQ ID NO:39	Prothrombin (non-Gla)
40	LAVTTHGLPCLAWASAQAK	SEQ ID NO:40	Prothrombin (non-Gla)
41	ECVEETCSYEEAFEALXSSTATDVFWAK	SEQ ID NO:41	Factor II (prothrombin)
42	ECVEETCSYEEAFXALESSTATDVFWAK	SEQ ID NO:42	Factor II (prothrombin)
43	ECVEETCSYEEAFXALXSSTATDVFWAK	SEQ ID NO:43	Factor II (prothrombin)
44	ECVEETCSYEXAFEALESSTATDVFWAK	SEQ ID NO:44	Factor II (prothrombin)
45	ECVEETCSYEXAFEALXSSTATDVFWAK	SEQ ID NO:45	Factor II (prothrombin)
46	ECVEETCSYEXAFXALESSTATDVFWAK	SEQ ID NO:46	Factor II (prothrombin)
47	ECVEETCSYEXAFXALXSSTATDVFWAK	SEQ ID NO:47	Factor II (prothrombin)
48	ECVEETCSYXEAFEALESSTATDVFWAK	SEQ ID NO:48	Factor II (prothrombin)
49	ECVEETCSYXEAFEALXSSTATDVFWAK	SEQ ID NO:49	Factor II (prothrombin)
50	ECVEETCSYXEAFXALESSTATDVFWAK	SEQ ID NO:50	Factor II (prothrombin)
51	ECVEETCSYXEAFXALXSSTATDVFWAK	SEQ ID NO:51	Factor II (prothrombin)
52	ECVEETCSYXXAFEALESSTATDVFWAK	SEQ ID NO:52	Factor II (prothrombin)
53	ECVEETCSYXXAFEALXSSTATDVFWAK	SEQ ID NO:53	Factor II (prothrombin)
54	ECVEETCSYXXAFXALESSTATDVFWAK	SEQ ID NO:54	Factor II (prothrombin)
55	ECVEETCSYXXAFXALXSSTATDVFWAK	SEQ ID NO:55	Factor II (prothrombin)
56	ECVEXTCSYEEAFEALESSTATDVFWAK	SEQ ID NO:56	Factor II (prothrombin)
57	ECVEXTCSYEEAFEALXSSTATDVFWAK	SEQ ID NO:57	Factor II (prothrombin)
58	ECVEXTCSYEEAFXALESSTATDVFWAK	SEQ ID NO:58	Factor II (prothrombin)
59	ECVEXTCSYEEAFXALXSSTATDVFWAK	SEQ ID NO:59	Factor II (prothrombin)
60	ECVEXTCSYEXAFEALESSTATDVFWAK	SEQ ID NO:60	Factor II (prothrombin)
61	ECVEXTCSYEXAFEALXSSTATDVFWAK	SEQ ID NO:61	Factor II (prothrombin)
62	ECVEXTCSYEXAFXALESSTATDVFWAK	SEQ ID NO:62	Factor II (prothrombin)
63	ECVEXTCSYEXAFXALXSSTATDVFWAK	SEQ ID NO:63	Factor II (prothrombin)
64	ECVEXTCSYXEAFEALESSTATDVFWAK	SEQ ID NO:64	Factor II (prothrombin)
65	ECVEXTCSYXEAFEALXSSTATDVFWAK	SEQ ID NO:65	Factor II (prothrombin)
66	ECVEXTCSYXEAFXALESSTATDVFWAK	SEQ ID NO:66	Factor II (prothrombin)

No.	Peptide	ID	Protein source
67	ECVEXTCSYXEA FXALXSSTATDVFWAK	SEQ ID NO:67	Factor II (prothrombin)
68	ECVEXTCSYXXAFEALXSSTATDVFWAK	SEQ ID NO:68	Factor II (prothrombin)
69	ECVEXTCSYXXAFEALXSSTATDVFWAK	SEQ ID NO:69	Factor II (prothrombin)
70	ECVEXTCSYXXAFXALESSTATDVFWAK	SEQ ID NO:70	Factor II (prothrombin)
71	ECVEXTCSYXXAFXALXSSTATDVFWAK	SEQ ID NO:71	Factor II (prothrombin)
72	ECVXETCSYEEAFEALXSSTATDVFWAK	SEQ ID NO:72	Factor II (prothrombin)
73	ECVXETCSYEEAFEALXSSTATDVFWAK	SEQ ID NO:73	Factor II (prothrombin)
74	ECVXETCSYEEAFXALESSTATDVFWAK	SEQ ID NO:74	Factor II (prothrombin)
75	ECVXETCSYEEAFXALXSSTATDVFWAK	SEQ ID NO:75	Factor II (prothrombin)
76	ECVXETCSYEXAFEALXSSTATDVFWAK	SEQ ID NO:76	Factor II (prothrombin)
77	ECVXETCSYEXAFEALXSSTATDVFWAK	SEQ ID NO:77	Factor II (prothrombin)
78	ECVXETCSYEXAFXALESSTATDVFWAK	SEQ ID NO:78	Factor II (prothrombin)
79	ECVXETCSYEXAFXALXSSTATDVFWAK	SEQ ID NO:79	Factor II (prothrombin)
80	ECVXETCSYXEA FEALXSSTATDVFWAK	SEQ ID NO:80	Factor II (prothrombin)
81	ECVXETCSYXEA FEALXSSTATDVFWAK	SEQ ID NO:81	Factor II (prothrombin)
82	ECVXETCSYXEA FXALESSTATDVFWAK	SEQ ID NO:82	Factor II (prothrombin)
83	ECVXETCSYXEA FXALXSSTATDVFWAK	SEQ ID NO:83	Factor II (prothrombin)
84	ECVXETCSYXXAFEALXSSTATDVFWAK	SEQ ID NO:84	Factor II (prothrombin)
85	ECVXETCSYXXAFEALXSSTATDVFWAK	SEQ ID NO:85	Factor II (prothrombin)
86	ECVXETCSYXXAFXALESSTATDVFWAK	SEQ ID NO:86	Factor II (prothrombin)
87	ECVXETCSYXXAFXALXSSTATDVFWAK	SEQ ID NO:87	Factor II (prothrombin)
88	ECVXXTCSYEEAFEALXSSTATDVFWAK	SEQ ID NO:88	Factor II (prothrombin)
89	ECVXXTCSYEEAFEALXSSTATDVFWAK	SEQ ID NO:89	Factor II (prothrombin)
90	ECVXXTCSYEEAFXALESSTATDVFWAK	SEQ ID NO:90	Factor II (prothrombin)
91	ECVXXTCSYEEAFXALXSSTATDVFWAK	SEQ ID NO:91	Factor II (prothrombin)
92	ECVXXTCSYEXAFEALXSSTATDVFWAK	SEQ ID NO:92	Factor II (prothrombin)
93	ECVXXTCSYEXAFEALXSSTATDVFWAK	SEQ ID NO:93	Factor II (prothrombin)
94	ECVXXTCSYEXAFXALESSTATDVFWAK	SEQ ID NO:94	Factor II (prothrombin)
95	ECVXXTCSYEXAFXALXSSTATDVFWAK	SEQ ID NO:95	Factor II (prothrombin)

No.	Peptide	ID	Protein source
96	ECVXXTC SYXEA FEAL ESSTATDVFWAK	SEQ ID NO:96	Factor II (prothrombin)
97	ECVXXTC SYXEA FEAL XSSTATDVFWAK	SEQ ID NO:97	Factor II (prothrombin)
98	ECVXXTC SYXEA FXAL ESSTATDVFWAK	SEQ ID NO:98	Factor II (prothrombin)
99	ECVXXTC SYXEA FXAL XSSTATDVFWAK	SEQ ID NO:99	Factor II (prothrombin)
100	ECVXXTC SYXXA FEAL ESSTATDVFWAK	SEQ ID NO:100	Factor II (prothrombin)
101	ECVXXTC SYXXA FEAL XSSTATDVFWAK	SEQ ID NO:101	Factor II (prothrombin)
102	ECVXXTC SYXXA FXAL ESSTATDVFWAK	SEQ ID NO:102	Factor II (prothrombin)
103	ECVXXTC SYXXA FXAL XSSTATDVFWAK	SEQ ID NO:103	Factor II (prothrombin)
104	XCVEETCS YEEA FEAL ESSTATDVFWAK	SEQ ID NO:104	Factor II (prothrombin)
105	XCVEETCS YEEA FEAL XSSTATDVFWAK	SEQ ID NO:105	Factor II (prothrombin)
106	XCVEETCS YEEA FXAL ESSTATDVFWAK	SEQ ID NO:106	Factor II (prothrombin)
107	XCVEETCS YEEA FXAL XSSTATDVFWAK	SEQ ID NO:107	Factor II (prothrombin)
108	XCVEETCS YEXA FEAL ESSTATDVFWAK	SEQ ID NO:108	Factor II (prothrombin)
109	XCVEETCS YEXA FEAL XSSTATDVFWAK	SEQ ID NO:109	Factor II (prothrombin)
110	XCVEETCS YEXA FXAL ESSTATDVFWAK	SEQ ID NO:110	Factor II (prothrombin)
111	XCVEETCS YEXA FXAL XSSTATDVFWAK	SEQ ID NO:111	Factor II (prothrombin)
112	XCVEETCS YXEA FEAL ESSTATDVFWAK	SEQ ID NO:112	Factor II (prothrombin)
113	XCVEETCS YXEA FEAL XSSTATDVFWAK	SEQ ID NO:113	Factor II (prothrombin)
114	XCVEETCS YXEA FXAL ESSTATDVFWAK	SEQ ID NO:114	Factor II (prothrombin)
115	XCVEETCS YXEA FXAL XSSTATDVFWAK	SEQ ID NO:115	Factor II (prothrombin)
116	XCVEETCS YXXA FEAL ESSTATDVFWAK	SEQ ID NO:116	Factor II (prothrombin)
117	XCVEETCS YXXA FEAL XSSTATDVFWAK	SEQ ID NO:117	Factor II (prothrombin)
118	XCVEETCS YXXA FXAL ESSTATDVFWAK	SEQ ID NO:118	Factor II (prothrombin)
119	XCVEETCS YXXA FXAL XSSTATDVFWAK	SEQ ID NO:119	Factor II (prothrombin)
120	XCVEETCS YEEA FEAL ESSTATDVFWAK	SEQ ID NO:120	Factor II (prothrombin)
121	XCVEETCS YEEA FEAL XSSTATDVFWAK	SEQ ID NO:121	Factor II (prothrombin)
122	XCVEETCS YEEA FXAL ESSTATDVFWAK	SEQ ID NO:122	Factor II (prothrombin)
123	XCVEETCS YEEA FXAL XSSTATDVFWAK	SEQ ID NO:123	Factor II (prothrombin)
124	XCVEETCS YEXA FEAL ESSTATDVFWAK	SEQ ID NO:124	Factor II (prothrombin)

No.	Peptide	ID	Protein source
125	XCVEXTCSYEXAFEALXSSTATDVFWAK	SEQ ID NO:125	Factor II (prothrombin)
126	XCVEXTCSYEXAFXALESSTATDVFWAK	SEQ ID NO:126	Factor II (prothrombin)
127	XCVEXTCSYEXAFXALXSSTATDVFWAK	SEQ ID NO:127	Factor II (prothrombin)
128	XCVEXTCSYXEAFEALESSTATDVFWAK	SEQ ID NO:128	Factor II (prothrombin)
129	XCVEXTCSYXEAFEALXSSTATDVFWAK	SEQ ID NO:129	Factor II (prothrombin)
130	XCVEXTCSYXEAFXALESSTATDVFWAK	SEQ ID NO:130	Factor II (prothrombin)
131	XCVEXTCSYXEAFXALXSSTATDVFWAK	SEQ ID NO:131	Factor II (prothrombin)
132	XCVEXTCSYXXAFEALESSTATDVFWAK	SEQ ID NO:132	Factor II (prothrombin)
133	XCVEXTCSYXXAFEALXSSTATDVFWAK	SEQ ID NO:133	Factor II (prothrombin)
134	XCVEXTCSYXXAFXALESSTATDVFWAK	SEQ ID NO:134	Factor II (prothrombin)
135	XCVEXTCSYXXAFXALXSSTATDVFWAK	SEQ ID NO:135	Factor II (prothrombin)
136	XCVXETCSYEEAFEALESSTATDVFWAK	SEQ ID NO:136	Factor II (prothrombin)
137	XCVXETCSYEEAFEALXSSTATDVFWAK	SEQ ID NO:137	Factor II (prothrombin)
138	XCVXETCSYEEAFXALESSTATDVFWAK	SEQ ID NO:138	Factor II (prothrombin)
139	XCVXETCSYEEAFXALXSSTATDVFWAK	SEQ ID NO:139	Factor II (prothrombin)
140	XCVXETCSYEXAFEALESSTATDVFWAK	SEQ ID NO:140	Factor II (prothrombin)
141	XCVXETCSYEXAFEALXSSTATDVFWAK	SEQ ID NO:141	Factor II (prothrombin)
142	XCVXETCSYEXAFXALESSTATDVFWAK	SEQ ID NO:142	Factor II (prothrombin)
143	XCVXETCSYEXAFXALXSSTATDVFWAK	SEQ ID NO:143	Factor II (prothrombin)
144	XCVXETCSYXEAFEALESSTATDVFWAK	SEQ ID NO:144	Factor II (prothrombin)
145	XCVXETCSYXEAFEALXSSTATDVFWAK	SEQ ID NO:145	Factor II (prothrombin)
146	XCVXETCSYXEAFXALESSTATDVFWAK	SEQ ID NO:146	Factor II (prothrombin)
147	XCVXETCSYXEAFXALXSSTATDVFWAK	SEQ ID NO:147	Factor II (prothrombin)
148	XCVXETCSYXXAFEALESSTATDVFWAK	SEQ ID NO:148	Factor II (prothrombin)
149	XCVXETCSYXXAFEALXSSTATDVFWAK	SEQ ID NO:149	Factor II (prothrombin)
150	XCVXETCSYXXAFXALESSTATDVFWAK	SEQ ID NO:150	Factor II (prothrombin)
151	XCVXETCSYXXAFXALXSSTATDVFWAK	SEQ ID NO:151	Factor II (prothrombin)
152	XCVXXTCSYEEAFEALESSTATDVFWAK	SEQ ID NO:152	Factor II (prothrombin)
153	XCVXXTCSYEEAFEALXSSTATDVFWAK	SEQ ID NO:153	Factor II (prothrombin)

No.	Peptide	ID	Protein source
154	XCVXXTCSYEEAFXALESSTATDVFWAK	SEQ ID NO:154	Factor II (prothrombin)
155	XCVXXTCSYEEAFXALXSSTATDVFWAK	SEQ ID NO:155	Factor II (prothrombin)
156	XCVXXTCSYEXAFEALESSTATDVFWAK	SEQ ID NO:156	Factor II (prothrombin)
157	XCVXXTCSYEXAFEALXSSTATDVFWAK	SEQ ID NO:157	Factor II (prothrombin)
158	XCVXXTCSYEXAFXALESSTATDVFWAK	SEQ ID NO:158	Factor II (prothrombin)
159	XCVXXTCSYEXAFXALXSSTATDVFWAK	SEQ ID NO:159	Factor II (prothrombin)
160	XCVXXTCSYXEAFALESSTATDVFWAK	SEQ ID NO:160	Factor II (prothrombin)
161	XCVXXTCSYXEAFEALXSSTATDVFWAK	SEQ ID NO:161	Factor II (prothrombin)
162	XCVXXTCSYXEAFXALESSTATDVFWAK	SEQ ID NO:162	Factor II (prothrombin)
163	XCVXXTCSYXEAFXALXSSTATDVFWAK	SEQ ID NO:163	Factor II (prothrombin)
164	XCVXXTCSYXXAFEALESSTATDVFWAK	SEQ ID NO:164	Factor II (prothrombin)
165	XCVXXTCSYXXAFEALXSSTATDVFWAK	SEQ ID NO:165	Factor II (prothrombin)
166	XCVXXTCSYXXAFXALESSTATDVFWAK	SEQ ID NO:166	Factor II (prothrombin)
167	XCVXXTCSYXXAFXALXSSTATDVFWAK	SEQ ID NO:167	Factor II (prothrombin)

[0060] A particularly favorable set of homologous peptides covering 5 Gla proteins are:

Protein	Peptide	Obs	Mass (mono)	MS1 (2H+)/2
Protein S	ANSLLEETK	***	1003.50	502.75
Factor VII	ANAFLEELR		1061.54	531.77
Factor X	ANSFLEEMK	***	1067.48	534.74
Protein C	ANSFLEELR	***	1077.53	539.77
Factor II (prothrombin)	ANTFLEEVR	***	1077.53	539.77

[0061] Of these, all but one (Factor VII SEQ ID NO:13 (ANAFLEELR)) is readily detectable in Gla and unmodified form in tryptic digests of body fluids (Obs).

[0062] The term “**amount**”, “**concentration**” or “**level**” of an analyte or internal standard means the physical quantity of the substance referred to, either in terms of mass (or equivalently moles) or in terms of concentration (the amount of mass or moles per volume of a solution or liquid sample).

[0063] The term “**analyte**” or “**ligand**” may be any of a variety of different molecules, or components, pieces, fragments or sections of different molecules that are to be measured or quantitated in a sample. An analyte may thus be a protein, a peptide derived from a protein by digestion or other fragmentation technique, a small molecule (such as a hormone, a metabolite, a drug, a drug metabolite) or nucleic acids (DNA, RNA, and fragments thereof produced by enzymatic, chemical or other fragmentation processes).

[0064] The term “**antibody**” means a monoclonal or monospecific polyclonal immunoglobulin protein such as IgG or IgM. An antibody may be a whole antibody or antigen-binding antibody fragment derived from a species (*e.g.*, rabbit or mouse) commonly employed to produce antibodies against a selected antigen, or may be derived from recombinant methods such as protein expression, and phage/virus display. See, *e.g.*, U.S. Patent Nos.: 7,732,168; 7,575,896; and 7,431,927, which describe preparation of rabbit monoclonal antibodies. Antibody fragments may be any antigen-binding fragment that can be prepared by conventional protein chemistry methods or may be engineered fragments such as scFv, diabodies, minibodies and the like. It will be understood that other classes of molecules such as DNA and RNA aptamers configured as specific and high affinity binding agents may, be used as alternatives to antibodies or antibody fragments in appropriate circumstances.

[0065] The term “**bind**” or “**react**” means any physical attachment or close association, which may be permanent or temporary. Generally, reversible binding includes aspects of charge interactions, hydrogen bonding, hydrophobic forces, van der Waals forces etc., that facilitate physical attachment between the molecule of interest and the analyte being measuring. The “binding” interaction may be brief as in the situation where binding causes a chemical reaction to occur. Reactions resulting from contact between the binding agent and the analyte are also within the definition of binding for the purposes of the present technology, provided they can be later reversed to release a monitor fragment.

[0066] The term “**binding agent**” means a molecule or substance having an affinity for one or more analytes, and includes antibodies (for example polyclonal, monoclonal, single chain, and modifications thereof), aptamers (made of DNA, RNA, modified nucleotides, peptides, and other compounds), etc. “Specific binding agents” are those with particular affinity for a specific analyte molecule.

[0067] The terms “**clinical reference range**” and “clinical reference interval” mean the range of abundance or concentration values of an analyte that are deemed to be with the “normal”

clinical range. Such ranges are frequently established by determination of analyte levels in a normal population, and the clinical reference range typically determined as the central 95% of the resulting histogram (with 2.5% of the population above and 2.5% below the resulting high and low values). As used here, these terms also refer to ranges whose bounds are defined by clinical features other than the distribution of results in normal individuals (*e.g.*, the population reference range in diabetic patients), and clinical ranges based on a patient's prior test values for the same or other analytes, alone or in combination with population test data. A variety of statistical approaches can be used to calculate such ranges from analyte measurements, and this is advantageously can be done prior to their application in the design of an assay or the determination of an amount of internal standard to use in the assay. As in the case of a single test evaluation threshold, it will be understood that a clinical reference interval for use in a specific test can be set based on results obtained using the specific test or an equivalent methodology, in order that any analytical biases inherent in the test are reflected in the threshold.

[0068] The term “**denaturant**” includes a range of chaotropic and other chemical agents that act to disrupt or loosen the 3-D structure of proteins without breaking covalent bonds, thereby rendering them more susceptible to proteolytic treatment. Examples include urea, guanidine hydrochloride, ammonium thiocyanate, trifluoroethanol and deoxycholate, as well as solvents such as acetonitrile, methanol and the like. The concept of denaturant includes non-material influences capable of causing perturbation to protein structures, such as heat, microwave irradiation, ultrasound, and pressure fluctuations.

[0069] The term “**electrospray ionization**” (ESI) refers to a method for the transfer of analyte molecules in solution into the gas and ultimately vacuum phase through use of a combination of liquid delivery to a pointed exit and high local electric field.

[0070] The terms “**particle**” or “**bead**” mean any kind of particle in the size range between 10nm and 1cm, and includes magnetic particles and beads.

[0071] The term “**MALDI**” means Matrix Assisted Laser Desorption Ionization and related techniques such as SELDI, and includes any technique that generates charged analyte ions from a solid analytecontaining material on a solid support under the influence of a laser or other means of imparting a short energy pulse.

[0072] The term “**Mass spectrometer**” (or “**MS**”) means an instrument capable of separating molecules on the basis of their mass m , or m/z where z is molecular charge, and then detecting them. In one embodiment, mass spectrometers detect molecules quantitatively. An MS may use one, two, or more stages of mass selection. In the case of multistage selection, some means of fragmenting the molecules is typically used between stages, so that later stages resolve fragments of molecules selected in earlier stages. Use of multiple stages typically affords improved overall specificity compared to a single stage device. Often, quantitation of molecules is

performed in a triple-quadrupole mass spectrometer using the method referred to as ‘Multiple Reaction Monitoring’ or “**MRM mass spectrometry**” in which measured molecules are selected first by their intact mass and secondly, after fragmentation, by the mass of a specific expected molecular fragment. However it will be understood herein that a variety of different MS configurations may be used to analyze the molecules described, and specifically MALDI instruments including MALDI-TOF, MALDI-TOF/TOF, and MALDI-TQMS and electrospray instruments including ESI-TQMS and ESI-QTOF, in which TOF means time of flight, TQMS means triple quadrupole MS, and QTOF means quadrupole TOF.

[0073] The term “**monitor fragment**” may mean any piece of an analyte up to and including the whole analyte that can be produced by a reproducible fragmentation process (or without a fragmentation if the monitor fragment is the whole analyte) and whose abundance or concentration can be used as a surrogate for the abundance or concentration of the analyte.

[0074] The term “**monitor peptide**” or “**target peptide**” means a peptide chosen as a monitor fragment of a protein or peptide.

[0075] The term “**proteolytic enzyme cleavage site**” refers to a site within an extended SIS peptide sequence at which the chosen proteolytic treatment (typically an enzyme such as trypsin) cleaves the extended SIS sequence, releasing peptides fragments (typically two) of which one is the SIS peptide sequence (identical to the analyte, or Nat, sequence for which the SIS serves as an internal standard).

[0076] The term “**proteolytic treatment**” or “**enzyme**” may refer any of a large number of different enzymes, including trypsin, chymotrypsin, lys-C, v8 and the like, as well as chemicals, such as cyanogen bromide. In this context, a proteolytic treatment acts to cleave peptide bonds in a protein or peptide in a sequence-specific manner, generating a collection of shorter peptides (a digest).

[0077] The term “**proteotypic peptide**” means a peptide whose sequence is unique to a specific protein in an organism, and therefore may be used as a stoichiometric surrogate for the protein, or at least for one or more forms of the protein in the case of a protein with splice variants.

[0078] The term “**sample**” means any complex biologically-generated sample derived from humans, other animals, plants or microorganisms, or any combinations of these sources.

“Complex digest” means a proteolytic digest of any of these samples resulting from use of a proteolytic treatment.

[0079] The terms “**SIS**”, “stable isotope standard” and “stable isotope labeled version of a peptide or protein analyte” mean a peptide or protein, such as a peptide or protein having a unique sequence that is identical or substantially identical to that of a selected peptide or protein analyte, and including a label of some kind (*e.g.*, a stable isotope) that allows its use as an internal

standard for mass spectrometric quantitation of the natural (unlabeled, typically biologically generated) version of the analyte (see US Patent No. 7,632,686 “*High Sensitivity Quantitation of Peptides by Mass Spectrometry*”). In one embodiment, a SIS peptide or protein comprises a peptide sequence that has a structure that is chemically identical to that of the molecule for which it will serve as a standard, except that it has isotopic labels at one or more positions that alter its mass. Hence a SIS is 1) recognized as equivalent to the analyte in a pre-analytical workflow, and is not appreciably differentially enriched or depleted compared to the analyte prior to mass spectrometric analysis, and 2) differs from it in a manner that can be distinguished by a mass spectrometer, either through direct measurement of molecular mass or through mass measurement of fragments (*e.g.*, through MS/MS analysis), or by another equivalent means. Stable isotope standards include peptides having non-material modifications of this sequence, such as a single amino acid substitution (as may occur in natural genetic polymorphisms), substitutions (including covalent conjugations of cysteine or other specific residues), or chemical modifications (including glycosylation, phosphorylation, and other well-known post-translational modifications) that do not materially affect enrichment or depletion compared to the analyte prior to mass spectrometric analysis. In one embodiment, SIS are those in which the level of substitution of each stable isotope (*e.g.*, C, N, O or H) at the specific sites within the peptide structure where the isotope(s) is/are incorporated (*i.e.*, those sites that depart significantly from the natural unenriched isotope distribution) is/are > 95%, > 96%, > 97%, or > 98%.

[0080] The term “**SISCAPA**” means the method described in US Patent No. 7,632,686, entitled *High Sensitivity Quantitation of Peptides by Mass Spectrometry* and in *Mass Spectrometric Quantitation of Peptides and Proteins Using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA)*. Anderson, N.L., Anderson, N.G., Haines, L.R., Hardie, D.B., Olafson, R.W., and Pearson, T.W, *Journal of Proteome Research* 3: 235-44 (2004).

[0081] The term “**stable isotope**” means an isotope of an element naturally occurring or capable of substitution in proteins or peptides that is stable (does not decay by radioactive mechanisms) over a period of a day or more. The primary examples of interest in this context are C, N, H, and O, of which the most commonly used are ¹³C and ¹⁵N.

[0082] The term “**standardized sample**” means a protein or peptide sample to which stable isotope labeled version(s) of one or more peptide or protein analytes have been added at levels corresponding to test evaluation thresholds to serve as internal standards.

[0083] The following embodiments of the present technology make use of a series of concepts described in this specification. These concepts provide background as to specific embodiments of the methods and compositions described herein.

[0084] EMBODIMENTS

[0085] In addition to the embodiments described above, additional embodiments are included as follows.

[0086] 1. In a first embodiment, the amount of the Prothrombin tryptic peptide SEQ ID NO:1 (ANTFLEEVR) having no Gla-modification on either of the glutamic acid (E) residues is determined in relation to a different Prothrombin tryptic peptide having no potential Gla-modification sites (for example the peptide SEQ ID NO:35 (ETAASLLQAGYK)). Since both E sites in peptide SEQ ID NO:1 (ANTFLEEVR) are typically highly modified in individuals who neither have liver cancer nor are being treated with anti-coagulants, the presence of a significant fraction of SEQ ID NO:1 (ANTFLEEVR) with no Gla-modification is indicative of a departure from a normal coagulation state and/or an increased likelihood of liver cancer, depending on the medical context. The amount of without Gla-modification depends on the total amount of Prothrombin in the sample as well as the fraction of Prothrombin in which SEQ ID NO:1 (ANTFLEEVR) has no Gla. Hence a separate measurement of a different peptide such as SEQ ID NO:35 (ETAASLLQAGYK) can optionally be used to normalize the measurement of unmodified SEQ ID NO:1 (ANTFLEEVR).

[0087] In a preferred method for measurement of SEQ ID NO:1 (ANTFLEEVR) without Gla-modification, a sample containing blood plasma is subjected to a digestion process to yield proteolytic peptides. The sample may be whole blood obtained by venipuncture, plasma or serum isolated from whole blood by conventional clinical laboratory means, or a sample of dried blood, serum or plasma. The sample is dissolved (if not already a liquid) and denatured prior to trypsin digestion. In the case of a dried blood spot, a sample is prepared by placing a drop of fingerprick blood on a Whatman 903 sample card and allowing it to dry in air at room temperature for 2 hours, after which the card is stored at 4C in a sealed plastic bag with a packet of desiccant until use. Immediately prior to beginning the analytical workflow, a disk 6mm in diameter is punched from the red area of dried blood using a standard office hole punch, yielding a flat red disk of paper containing the dry equivalent of about 15-20ul of whole blood. The 6mm disk is placed in the bottom of a well of a flat bottomed 96-well plate, 20ul of water is added to the well, and the plate is shaken in a circular motion on a plate shaker for 30 minutes at room temperature to redissolve the DBS proteins. In this process, most of the red color (heme) is extracted into the liquid, leaving the 903 paper a slightly dingy off-white color. Next a tablet of dry reagents is added to the well and the plate is again shaken for 30 minutes to dissolve the reagents and denature the sample proteins. This tablet is previously prepared by drying a 33.8 ul droplet of a solution of 9.13M urea, 0.5M Tris HCl pH

8.1 and 0.05M tris(2-carboxyethyl)phosphine (TECP) on a plastic sheet in air. When added to the 20 ul liquid of the redissolve DBS proteins in the well, the resulting urea concentration upon dissolution of the tablet is ~9M, ensuring good protein denaturation, and the TCEP concentration is sufficient to reduce all disulfide bonds in the proteins. Following denaturation and disulfide reduction, 20ul of a solution of iodoacetamide (7.5 mg/ml in water) is added to the well and allowed to react for 30 min at room temperature in the dark. Next the sample is diluted with 230 ul of 0.25M Tris HCl pH 8.1 in water and mixed by shaking the plate, after which 20 ul of a solution of trypsin (3.66 mg/ml trypsin, 1mM HCl in water) is added to initiate tryptic digestion of the sample proteins. The plate is placed in a 37°C incubator for 4 hours to carry out the proteolytic digestion step. Next 20 ul of a solution of 0.11 mg/ml N-a-tosyl-L-lysine chloromethyl ketone (TLCK) in 1mM HCl in water is added to the sample and mixed, inhibiting trypsin activity. The sample now consists of tryptic peptides in a solution of approximately 1M urea.

[0088] The SISCAPA method is used to enrich two target peptides to practice this embodiment of the invention. The target peptides in this case are SEQ ID NO:1 (ANTFLEEVR) and SEQ ID NO:35 (ETAASLLQAGYK). Stable isotope labeled versions of each of the target peptides (the “SIS” versions) are prepared by peptide synthesis, each incorporating a C-terminal K or R residue fully labeled with ¹⁵N and ¹³C isotopes to provide mass increments relative to the endogenous tryptic peptides of 8 or 10 amu respectively. A SIS mixture containing 50 fmol/ul of each of these SIS peptides is prepared in water containing 0.03% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) detergent, and 10ul of this mix is added to the sample and mixed by shaking to provide 500 fmol of each labeled internal standard. Next 10 ul of a solution containing, in phosphate-buffered saline, 0.1 mg/ml of each of two rabbit monoclonal antibodies, one with high affinity for SEQ ID NO:1 (ANTFLEEVR) and the other with high affinity for SEQ ID NO:35 (ETAASLLQAGYK) is added and mixed. This addition places 1 ug of each of the antibodies in the digest. Next an aliquot of 20ul of protein G-coated magnetic beads (Life Technologies 2.8 μm Protein G Dynabeads), previously washed in PBS, is added and the digest shaken for one hour while the target peptides bind to the specific antibodies and the antibodies bind to the protein G beads.

[0089] Finally the beads are moved to a 96-well plate where they are mixed with 20 μL of 1% formic acid in water for 10 minutes to elute the bound peptides, after which the eluate is moved to a clean 96-well PCR plate.

[0090] Peptide samples in the resulting eluate plate are analyzed with a system consisting of a 6490 triple quadrupole mass spectrometer coupled to a 1290 Infinity UHPLC using a JetStream interface (Agilent). A 10 μL aliquot of each sample is

separated on a 2.1 mm × 50 mm Zorbax 300 SB-C8 column with a flow rate of 0.6 mL/min. The target peptides are separated using a 3-min gradient with 0.1% formic acid in water as solvent A and 90% acetonitrile in 0.1% formic acid in water as solvent B. From initial conditions of 11% B, a gradient was developed to 16% B at 1 min, 22% B at 1.5 min, 35% B at 1.85 min, 70% B at 1.9 min, then back to 11% B from 1.95 to 3 min for column re-equilibration. Source conditions included drying gas at 200 °C, sheath gas at 250 °C, and 11 L/min flow for both drying and sheath gases. Ions are isolated in Q1 using 1.2 fwhm resolution and in Q3 using 0.7 fwhm resolution.

[0091] The following 4 MRM precursor/product ion transitions are measured during appropriate segments of the LC gradient, peptide peaks at expected retention times (previously determined for each peptide) are integrated using Agilent Mass Hunter quantitative software, and the endogenous analyte peak areas (light MRM) are divided by the corresponding labeled internal standard (heavy, SIS, where R* or K* in the table indicates the labeled amino acid) peak areas to obtain a peak area ratio. The peak area ratio is then multiplied by 500 fmol (the amount of heavy peptide added to the sample) to compute the amount of the endogenous analyte peptide in the sample. Additional transitions can optionally be measured to facilitate detection and rejection of potential interferences in MS quantitation.

<u>Protein</u>	<u>Peptide</u>	<u>Precursor Ion</u>	<u>Product Ion</u>
Prothrombin	ANTFLEEVR	539.8	645.4
Prothrombin	ANTFLEEVR*	544.8	650.4
Prothrombin	ETAASLLQAGYK	626.3	679.4
Prothrombin	ETAASLLQAGYK*	630.3	687.4

[0092] Using the peak area results of this procedure, the ratio of the MRM signals observed for SEQ ID NO:1 (ANTFLEEVR) and SEQ ID NO:35 (ETAASLLQAGYK) is calculated. Optionally this ratio can be further standardized in relation to the ratio SEQ ID NO:1 (ANTFLEEVR)* / SEQ ID NO:35 (ETAASLLQAGYK)* to normalize for differences in LC-MRM detection of the target peptides from one sample to another.

[0093] Other forms of SIS internal standards can be provided to further normalize the SEQ ID NO:1 (ANTFLEEVR)/ETAASLLQAGYK peak area ratio, for example by adding a stable isotope labeled recombinant prothrombin protein to the sample before

digestion, so that the labeled SIS versions of the two peptides are generated during digestion in parallel with the unlabeled sample peptides. This approach normalizes for differences in the digestion yield of the SEQ ID NO:1 (ANTFLEEVR) and SEQ ID NO:35 (ETAASLLQAGYK) peptides. Various chemical methods, for example methylation (Hallgren, et al, J Proteome Res 12, 2365–2374 (2013)), can be used to improve the performance of target and SIS peptides in chromatography and/or mass spectrometry.

[0094] The SEQ ID NO:1 (ANTFLEEVR)/SEQ ID NO:35 (ETAASLLQAGYK) peak area ratio, with or without the optional normalization steps described above, is then interpreted using a calibration curve relating this ratio to a conventional clinical measurement of coagulation (INR). In the general case this calibration curve is established using a series of patient samples in which both SEQ ID NO:1 (ANTFLEEVR)/ETAASLLQAGYK peak area ratio and INR have been measured. In a preferred case, the curve is patient-specific and generated using a series of samples from the same patient under different coagulation conditions (e.g., before and after treatment with anticoagulant). The use of such calibration curves is well known to those skilled in the art as a means of assessing the equivalence of two related measurements and of interpreting the significance of diagnostic measurements in a patient. Repeated measurements of SEQ ID NO:1 (ANTFLEEVR)/ SEQ ID NO:35 (ETAASLLQAGYK) peak area ratio in samples collected over time are used to monitor coagulation and to guide the adjustment of anticoagulant dose in the patient.

[0095] In a patient with normal coagulation, the SEQ ID NO:1 (ANTFLEEVR)/ SEQ ID NO:35 (ETAASLLQAGYK) peak area ratio can be interpreted against a calibration curve relating SEQ ID NO:1 (ANTFLEEVR)/ SEQ ID NO:35 (ETAASLLQAGYK) peak area ratio to the value obtained with an immunoassay for Gla-deficient prothrombin (e.g., the Fujirebio Lumipulse® G PIVKA–II test) across a suitable sample set comprising healthy patients and those with hepatocellular carcinoma. In a preferred case, the SEQ ID NO:1 (ANTFLEEVR)/ SEQ ID NO:35 (ETAASLLQAGYK) peak area ratio is measured repeatedly over time within each patient, and changes (typically increases) in this ratio used as an indicator of increasing cancer risk.

[0096] Other tryptic peptides may be used instead of SEQ ID NO:35 (ETAASLLQAGYK) to normalize the amount of SEQ ID NO:1 (ANTFLEEVR),

including SEQ ID NO:36 (LAACLEGNCAEGLGTNYR), SEQ ID NO:37 (HQDFNSAVQLVENFCR), SEQ ID NO:38 (IVEGSDAEIGMSPWQVMLFR), SEQ ID NO:39 (SEGSSVNLSPPLEQCVPDR) and SEQ ID NO:40 (LAVTTHGLPCLAWASAQAK).

[0097] The short Prothrombin peptide SEQ ID NO:7 (GNLER) also undergoes Gla-modification and could be used to provide a similar measure, though with reduced specificity.

[0098] 2. In a second embodiment, measurements of multiple modified forms of the peptide SEQ ID NO:1 (ANTFLEEVR) are used to monitor the level of Gla-modification in Prothrombin present in a sample of blood. This embodiment uses many of the same methods as the first embodiment, but expands the number of forms of SEQ ID NO:1 (ANTFLEEVR) that are measured to include those containing gamma-carboxylated glutamic acid residues (SEQ ID NO's 1-4). In this case it may be less important to measure a second non-Gla-modified peptide of Prothrombin (which is in this case considered purely optional). Of primary importance is the amounts of, and ratios among, SEQ ID NO's 1-4, which is to say the measurement of the amount(s) of the various forms of SEQ ID NO:1 (ANTFLEEVR) having a specific number of gamma-carboxylated glutamic acid residues. In contrast to the first embodiment, in which the amount of SEQ ID NO:1 (ANTFLEEVR) without Gla-modification is measured in relation to a different sequence peptide from the same protein (Prothrombin), in this embodiment, the primary object is to measure the amount of a specific peptide form taken from the group SEQ ID NO's 1-4, having a specific number of gamma-carboxylated glutamic acid residues, in relation to a different peptide from the group having a different specific number of gamma-carboxylated glutamic acid residues, where each of these peptides has the same amino acid sequence:

1. an unmodified version: SEQ ID NO:1 (ANTFLEEVR)
2. a version with the first E (glutamic acid) residue modified to gamma-carboxyglutamic acid (Gla = X in this representation): SEQ ID NO:2 (ANTFLXEVR)
3. a version with the second E (glutamic acid) residue modified to gamma-carboxyglutamic acid (Gla = X): SEQ ID NO:3 (ANTFLEXVR)
4. a version with both E (glutamic acid) residues modified to gamma-carboxyglutamic acid (Gla): SEQ ID NO:4 (ANTFLXXVR)

[0099] Important information can be obtained through measurement of fewer than all four possible forms (SEQ ID NO's 1-4). However, in a preferred embodiment all 4 forms are measured separately, or alternatively SEQ ID NO's 2 and 3 are measured together (both having one Gla-modification) while SEQ ID NO's 1 and 4 (having respectively 0 and 2 Gla-modifications) are measured individually, yielding a total of 3 measurements.

[00100] The quantitative peptide measurements can be made and interpreted directly, for example using the peak area measurements produced by a mass spectrometer, or these direct peak area measurements can be normalized for potential differences in mass spectrometer response between the peptides by using SIS internal standards (producing normalized peak area ratios of each sample-derived peptide to an added amount of a labeled version of the same peptide structure). In such a preferred embodiment, four stable isotope labeled synthetic (SIS) versions of the peptides SEQ ID NO's 1-4 are produced by conventional peptide synthesis, including a 10amu label achieved by incorporation of U-¹⁵N, U-¹³C arginine in the final position of each peptide.

[00101] Human body fluids are digested with trypsin to yield tryptic peptides using the methods described for the first embodiment. The 4 labeled peptide versions above are added to the digest to serve as internal standards, either in known amounts (for example 500 fmol) or in fixed ratios. Using one of a variety of approaches, which may include specific enrichment of these target peptides by anti-peptide antibodies (the SISCAPA method), the target peptides are separated at least partially from non-target peptides and introduced into a mass spectrometer (MS). Typically a triple-quadrupole MS capable of isolating intact peptides and specific fragments thereof for detection is used.

[00102] In a preferred case, the SISCAPA method is employed to improve the sensitivity, specificity and/or throughput of the measurement through the use of one or more antibodies to bind the target peptide(s) while unbound peptides are washed away prior to MS analysis. The bound peptides are finally eluted and delivered to the MS for measurement. An antibody directed against an epitope comprising the N-terminal portion of the SEQ ID NO:1 (ANTFLEEVR) sequence (ANTFL) captures all four forms of the peptide for enrichment (one form having no Gla, two forms having one Gla, and one form having 2 Gla). Alternatively, in the event that one or more of the 0, 1, and 2 Gla forms of the peptides fails to bind adequately to the antibody, a cocktail of antibodies may be employed that includes at least one antibody capable of binding each form.

[00103] The following table, in which X = Gla, shows the MS1 (parent peptide ion, doubly charged) and MS2 (singly-charged daughter ion fragment of the parent ion) selected for MRM measurement after collision-induced dissociation of the parent peptide in MS/MS. Different gamma-carboxylation levels of the peptide can be distinguished and separately measured using these parameters. In this approach the two versions with one Gla residue (occurring at either the first or second E position) are detected together, resulting in an aggregate measurement of forms having one Gla-modification. In a preferred embodiment, each of the endogenous target peptide peak areas is standardized by dividing it by the associated SIS peak area to yield a standardized peak area ratio. In the case of the singly-Gla-modified peptide, the indicated MRMs measure both forms together as an aggregate, although different MRM can be selected to measure these forms individually.

	Endogenous		Labeled (SIS)	
	MS1	MS2	MS1	MS2
ANTFLEEVR	539.8	645.4	544.8	655.4
ANTFLXEVR,	561.8	689.3	566.8	699.3
ANTFLEXVR				
ANTFLXXVR	583.8	733.3	588.8	743.3

[00104] A composite “Gla-Index”, measuring the average number of gamma-carboxylated glutamic acid (Gla) residues in a specific peptide (or in a set of peptides), can be computed from the resulting measurements by several methods. The purpose of the Gla-Index is to summarize a complex pattern of modifications in a simple form that can be related to clinical phenomena.

[00105] One method of computing a Gla-Index uses a simple weighted linear combination of the measured amounts peptide of the various peptide forms, modeled on the “Charge Modification Index” developed in *Carcinogenesis* **15**, 325–329 (1994) to describe chemical modifications of certain rodent liver proteins after treatment with methapyrilene. In its simplest form, this Gla-Index uses coefficients 0, 0.5, and 1.0 to weight the amounts of the peptide forms carrying 0, 1 and 2 Gla residues (in this case taking account of the fact that SEQ ID NO:2 (ANTFLXEVR) and SEQ ID NO:3

(ANTFLEXVR) are measured together using a single MRM). On this scale, a result of 0 indicates complete absence of Gla-modification on the peptide, while a result of 1.0 indicates that the peptide is fully Gla-modified at both E residues. Alternatively the weights may be adjusted to result in a Gla-Index value that more closely correlates with a relevant external measure, such as INR or PIVKA-II test result. This Gla-Index

$$= (0 * [\text{ANTFLEEVR}] + 0.5 * ([\text{ANTFLXEVR}] + [\text{ANTFLEXVR}] + *[\text{ANTFLXXVR}]) /$$

$$([\text{ANTFLEEVR}] + [\text{ANTFLXEVR}] + [\text{ANTFLEXVR}] + [\text{ANTFLXXVR}])$$

$$= [\text{ANTFLXEVR}, \text{ANTFLEXVR}] + [\text{ANTFLXXVR}] /$$

$$([\text{ANTFLEEVR}] + 2 * [\text{ANTFLXEVR}, \text{ANTFLEXVR}] + [\text{ANTFLXXVR}])$$

[00106] The Gla-Index thus equals the fraction of E residues in the peptide that have been converted to Gla. This concept can be extended to apply to peptides with any number of E residues from 1 to any number.

[00107] For example,

MRM Peak Areas						
	Target peptide	Labeled (500fmol)	Peak Area Ratio Target/ SIS	Calculated Fmol of Target	Weights for Gla-Index	Weighted Target Fmol values
ANTFLEEVR	1,204	31,045	0.04	19.39	0.00	0.00
ANTFLXEVR, ANTFLEXVR	15,034	29,566	0.51	254.24	0.50	127.12
ANTFLXXVR	30,879	27,345	1.13	564.62	1.00	564.62
	Totals			838.25		691.74
				Gla-Index	0.83	= 692/838

[00108] Alternative Gla-Index formulas can be based on the relationship between two of these measurements, for example the ratio between SEQ ID NO:1 (ANTFLEEVR) and SEQ ID NO:4 (ANTFLXXVR), or on non-linear combinations of the measurements.

Choice of the optimal Gla-Index formula may vary depending on the clinical phenotype or variable to which it is related in a specific application.

[00109] The Gla-Index result is used as an indicator of the level of gamma-carboxylation of coagulation proteins (in this case Prothrombin), and a measure of the change in this level caused by treatment with drugs such as warfarin and coumadin. By establishing the relationship of the Gla-Index to a patient's INR, or to other indices of patient coagulation and health status, the Gla-Index may be used as a surrogate for INR and other tests normally used in titration of warfarin or coumadin dose. Likewise the Gla-Index can be used as a surrogate for the measurement of PIVKA-II in the assessment of liver cancer. A preferred method of establishing a relationship, or calibration, between a Gla-Index and INR is to plot the values of Gla-Index vs INR for a series of appropriate samples (ideally human samples that yield a range of INR values covering the entire clinically-relevant range) and then to derive a simple equation summarizing the relationship which can be thereafter used to convert a Gla-Index value to an equivalent estimated INR. In the preferred case, the plotted values of Gla-Index vs INR are obtained from a series of samples provided by a specific patient, and the derived relationship is used to convert future test values from this patient in an individualized manner. Similarly, calibration relationships can be established between a Gla-index and PIVKA-II values, or between a Gla-index and clinical outcomes.

[00110] The Gla-Index can be computed on values obtained by measurements of Gla levels in peptides derived from dried blood spot samples, thereby avoiding the need for a patient to provide a liquid blood sample at the site of testing.

[00111] 3. In a third embodiment, Gla-modification of peptides derived from a plurality of proteins is measured by mass spectrometry. In this embodiment, each of five different but homologous sequences (derived from five proteins involved in coagulation) containing three (0, 1, or 2 total Gla sites) or four (--, +-, -+, or ++) different Gla forms are measured. In the preferred case, stable isotope labeled SIS versions of at least some, and preferably all, of these forms are added to the digest in known amounts to serve as internal standards in MS measurement.

[00112] In a preferred case the 5 peptides are: SEQ ID NO:27 (ANSLLEETK), SEQ ID NO:13 (ANAFLEELR), SEQ ID NO:18 (ANSFLEEMK), SEQ ID NO:23 (ANSFLEELR), and SEQ ID NO:1 (ANTFLEEVR). In a preferred case, fewer than 5 antibodies are required to bind these peptides and their Gla-modified forms in the SISCAPA method: an antibody against the N-terminal region of SEQ ID NO:18

(ANSFLEEMK) may also capture SEQ ID NO:23 (ANSFLEELR) for example, resulting in a need for fewer antibodies and thus lower reagent cost. In the most favorable case, a single antibody can be selected capable of binding all 5 sequences and modified forms.

[00113] The Gla-Indices of each of the peptides, determined as above in the second embodiment, are averaged to provide a more statistically robust and representative index of overall protein Gla-modification. Alternatively, the Gla-Indices of the 5 peptides can be used as separate data points input into a more discriminating multifactorial test algorithm. Subsets comprising two or more of the 5 peptides can also be used.

[00114] Alternatively the levels of two or more of the peptides without Gla-modification can be measured in relation to normalizing (non-Gla-modifiable) peptides derived from the same proteins, using the method of the first embodiment, and these values combined in an aggregate index for use as a surrogate measure as described.

[00115] 4. In a fourth embodiment, the general approach of the second embodiment is extended to provide a Gla-Index using the Prothrombin tryptic peptide SEQ ID NO:5 (ECVEETCSYEEAFEALSTATDVFWAK) containing 7 Gla-modifiable glutamic acid residues (resulting in a total of 128 possible versions of the peptide, with 0-7 Gla at various positions, consisting of SEQ ID NO:5 and SEQ ID NO's 41-167,). In this case, separate measurement of each of these 128 different Gla-modified forms, while possible, is inconvenient and time-consuming. Instead, the embodiment provides a simplified approach in which the amount of peptide having each of the possible aggregate levels of modification (e.g., 0, or 1 or 2, etc. Gla-modifications) is measured, providing a set of 8 values.

[00116] In the case of SEQ ID NO:5 (ECVEETCSYEEAFEALSTATDVFWAK), the absence of Gla sites from the C-terminal 11 amino acids of the peptide allows efficient SISCAPA enrichment of all Gla-modified and non-modified peptide forms by a single anti-peptide antibody directed to the C-terminal region of the peptide (i.e., SEQ ID NO:6 (SSTATDVFWAK)). Likewise the existence of a variety of efficiently produced y-series daughter ion fragments having singly-charged masses 1454.7 (+y13), 1341.6 (+y12), 1212.6 (+y11), 1125.6 (+y10), 1038.5 (+y9), 937.5 (+y8), and 866.4 (+y7) derived from the unmodified C-terminal region allows detection of all the Gla-modified versions using a single daughter ion mass in combination with 8 parent mass settings (the non-Gla-modified peptide mass of 3316.4, together with a series of 44 amu mass increments associated with incremental Gla additions). The measurements of Gla-modified and unmodified versions can be used directly (as MS peak areas) or after

normalization in relation to one or more respective SIS standards (as normalized peak area ratios). In this embodiment, the simplest weights used in a Gla-Index are 0, 0.14, 0.29, 0.43, 0.57, 0.71, 0.86, and 1.00 for the MRM peak areas corresponding to peptide forms with 0, 1, 2, 3, 4, 5, 6, and 7 Gla-modifications respectively. This approach provides an effective method of summarizing the information on 128 potential forms of a protein in 8 numbers, and further for summarizing these 8 numbers in a single Gla-Index. Alternatively a subset of the 8 Gla-content versions can be used. As described above, a Gla-Index may be directly related to INR and PIVKA-II results through a calibration curve. Alternatively an optimized set of weights can be computed to further improve the correlation of the Gla-Index with INR and PIVKA-II, or other relevant clinical variables.

[00117] 5. In a fifth embodiment, a set of two or more Gla-modifiable peptides taken from the following list are used and the results combined or compared as in the third embodiment: SEQ ID NO:1 (ANTFLEEV^R), SEQ ID NO:5 (ECVEETCSYEEAFEAL^SSTATDVFWAK), SEQ ID NO:7 (GNLER), SEQ ID NO:8 (CSFE^EEAR), SEQ ID NO:9 (ECME^EEK), SEQ ID NO:10 (EVFENTER), SEQ ID NO:11 (LEEFVQGNLER), SEQ ID NO:12 (TTEFWK), SEQ ID NO:13 (ANAFLEEL^R), SEQ ID NO:14 (DAER), SEQ ID NO:15 (EEQCSFE^EEAR), SEQ ID NO:16 (EIFK), SEQ ID NO:17 (PGSLER), SEQ ID NO:18 (ANSFLEEMK), SEQ ID NO:19 (ECMEETCSYEEAR), SEQ ID NO:20 (EVFEDSDK), SEQ ID NO:21 (GHLER), SEQ ID NO:22 (TNEFWNK), SEQ ID NO:23 (ANSFLEEL^R), SEQ ID NO:24 (ECIEEICDFEEAK), SEQ ID NO:25 (EIFQNVDDTLAFWSK), SEQ ID NO:26 (HSSLER), SEQ ID NO:27 (ANSLLEETK), SEQ ID NO:28 (ECIEELCNK), SEQ ID NO:29 (EEAR), SEQ ID NO:30 (EVFENDPETDYFY^PPK), SEQ ID NO:31 (QGNLER), SEQ ID NO:32 (AGSYLLEELFEGNLEK), SEQ ID NO:33 (ECYEEICVYEEAR), SEQ ID NO:34 (EVFENEVVTDEFWR). One skilled in the art will realize that the approaches disclosed above can be applied to quantitatively measure the levels of Gla-modification in any Gla-modified peptide or protein, including homologous peptides of different sequence from non-human species.

CLAIMS

1. A peptide selected from the group consisting of: SEQ ID NO:1 (ANTFLEEVR), SEQ ID NO:5 (ECVEETCSYEEAFEALLESSTATDVFWAK), SEQ ID NO:7 (GNLER), SEQ ID NO:8 (CSFEEAR), SEQ ID NO:9 (ECMEEK), SEQ ID NO:10 (EVFENTER), SEQ ID NO:11 (LEEFVQGNLER), SEQ ID NO:12 (TTEFWK), SEQ ID NO:13 (ANAFLEELR), SEQ ID NO:14 (DAER), SEQ ID NO:15 (EEQCSFEEAR), SEQ ID NO:16 (EIFK), SEQ ID NO:17 (PGSLER), SEQ ID NO:18 (ANSFLEEMK), SEQ ID NO:19 (ECMEETCSYEEAR), SEQ ID NO:20 (EVFEDSDK), SEQ ID NO:21 (GHLER), SEQ ID NO:22 (TNEFWNK), SEQ ID NO:23 (ANSFLEELR), SEQ ID NO:24 (ECIEEICDFEEAK), SEQ ID NO:25 (EIFQNVDDTLAFWSK), SEQ ID NO:26 (HSSLER), SEQ ID NO:27 (ANSLLEETK), SEQ ID NO:28 (ECIEELCNK), SEQ ID NO:29 (EEAR), SEQ ID NO:30 (EVFENDPETDYFYFYPK), SEQ ID NO:31 (QGNLER), SEQ ID NO:32 (AGSYLLEELFEGNLEK), SEQ ID NO:33 (ECYEEICVYEEAR), and SEQ ID NO:34 (EVFENEVVTDEFWR).
2. The peptide of claim 1, in combination with a form of said peptide wherein at least one glutamic acid residue is modified by gamma-carboxylation.
3. The combination of claim 2, wherein a stable isotope label is introduced at greater than 97% substitution and yielding a mass increment of greater than 2 amu into the peptide or into the form of the peptide.
4. A method comprising measuring the number of gamma-carboxylated glutamic acid residues in a specific peptide in a peptide digest sample, wherein said peptide digest sample is a proteolytic digest of a biological sample and wherein the peptide is selected from the group of peptides consisting of: a proteolytic digest fragment of prothrombin, a proteolytic digest fragment of Factor VII, a proteolytic digest fragment of Factor IX, a proteolytic digest fragment of Factor X, a proteolytic digest fragment of Protein C, a proteolytic digest fragment of Protein S and a proteolytic digest fragment of Protein Z, wherein each fragment contains one or more glutamic acid residues.
5. The method of claim 4, further comprising measuring the amount of (a) a specific peptide with a specific number of gamma-carboxylated glutamic acid residues and (b) the specific peptide having a different specific number of gamma-carboxylated glutamic acid residues.

6. The method of claim 4, comprising measuring the number of gamma-carboxylated glutamic acid residues in two or more different specific peptides selected from said group of peptides.
7. The method of claim 4, comprising measuring the presence or absence of a specific gamma-carboxylated glutamic acid residue in said specific peptide.
8. The method of claim 4, wherein the measurement is made using a mass spectrometer.
9. The method of claim 8, further comprising measuring a ratio of the amount of (a) a specific peptide with a specific number of gamma-carboxylated glutamic acid residues and (b) the specific peptide having a different specific number of gamma-carboxylated glutamic acid residues and wherein an internal standard for measuring (a) is used comprising a stable isotope labeled version of the specific peptide with the specific number of gamma-carboxylated glutamic acid residues and an internal standard for measuring (b) is used comprising a stable isotope labeled version of a specific peptide having the different specific number of gamma-carboxylated glutamic acid residues.
10. The method of claim 9, wherein the internal standards comprise a site at which a non-predominant stable isotope label is substituted for the predominant naturally occurring isotope of the atom appearing at that site in more than 95% of the peptide molecules.
11. The method of claim 9, wherein one or both of the internal standards are initially in an extended form that is modified to yield said labeled version.
12. The method of claim 9, wherein a) and/or b) is enriched by contacting the sample with (i) an anti-peptide antibody specific for a) and/or b), (ii) separating peptides bound by said antibody from unbound peptides, (iii) eluting said peptides bound by said antibody from said antibody, (iv) measuring the amount of a) and/or b) eluted from said antibody using a mass spectrometer, and (v) calculating the ratio of a) and b) in the biological sample.
13. The method of claim 9, wherein a) and/or b) is enriched by contacting the sample with (i) an anti-peptide antibody specific for a) and/or b), (ii) a quantity of a stable labeled version of a) and/or b), (iii) separating peptides bound by said antibody from unbound peptides, (iv) eluting said peptides bound by said antibody from said antibody, (v) measuring the amount of a) and/or b) eluted from said antibody using a mass spectrometer, and (vi) calculating the ratio of a) and b) in the biological sample.

14. The method of claim 12, wherein said antibody specifically binds a) and b) or wherein two or more antibodies are used to enrich a) and/or b).
15. The method of claim 4, wherein an antibody specifically binds two or more different peptides selected from said group of peptides and/or two or more forms of a specific peptide having different numbers of gamma-carboxylated glutamic acid residues.
16. The method of claim 4, wherein said sample is a dried blood or dried plasma.
17. The method of claim 4, wherein said measurement is used to assess blood coagulation or assess the level of liver cancer or the level of risk of liver cancer.
18. The method of claim 4 further comprising measuring the amount of a specific peptide in relation to the amount of a different peptide, wherein the specific peptide and the different peptide are fragments of the same protein.
19. The method of claim 4, wherein the specific peptide is selected from the group of peptides consisting of: SEQ ID NO:1 (ANTFLEEVVR), SEQ ID NO:5 (ECVEETCSYEEAFEALLESSTATDVFWAK), SEQ ID NO:7 (GNLER), SEQ ID NO:8 (CSFEER), SEQ ID NO:9 (ECMEEK), SEQ ID NO:10 (EVFENTER), SEQ ID NO:11 (LEEFVQGNLER), SEQ ID NO:12 (TTEFWK), SEQ ID NO:13 (ANAFLEELR), SEQ ID NO:14 (DAER), SEQ ID NO:15 (EEQCSFEER), SEQ ID NO:16 (EIFK), SEQ ID NO:17 (PGSLER), SEQ ID NO:18 (ANSFLEEMK), SEQ ID NO:19 (ECMEETCSYEEAR), SEQ ID NO:20 (EVFEDSDK), SEQ ID NO:21 (GHLER), SEQ ID NO:22 (TNEFWNK), SEQ ID NO:23 (ANSFLEELR), SEQ ID NO:24 (ECIEEICDFEEAK), SEQ ID NO:25 (EIFQNVDDTLAFWSK), SEQ ID NO:26 (HSSLER), SEQ ID NO:27 (ANSLLEETK), SEQ ID NO:28 (ECIEELCNK), SEQ ID NO:29 (EEAR), SEQ ID NO:30 (EVFENDPETDYFYFYPK), SEQ ID NO:31 (QGNLER), SEQ ID NO:32 (AGSYLLEELFEGNLEK), SEQ ID NO:33 (ECYEEICVYEEAR), SEQ ID NO:34 (EVFENEVVTDEFWR).
20. The method of claim 19, wherein the specific peptide is further selected from the group of peptides from a non-human mammal species that are homologous to the peptides of claim 19.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/066151

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - G01N 33/68 (2015.01)

CPC - G01N 33/68 (2015.01)

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(8) - G01N 33/00, 33/48, 33/50, 33/68, 30/72 (2015.01)

USPC - 435/4, 18, 23, 212; 436/86

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

CPC - G01N 33/48, 33/50, 33/68, 33/6803, 33/6848 (2015.01)

(keyword delimited)

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

Orbit, Google Patents, PubMed, Google

Search terms used: method measure gamma γ carboxylated glutamic acid glutamate residues peptide protein digest sample mass spectrometer isotope label

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2004/0180386 A1 (CARR et al) 16 September 2004 (16.09.2004) entire document	1

Y		2, 3, 19, 20
X	WO 2013/036445 A1 (MEDIMMUNE LLC) 14 March 2013 (14.03.2013) entire document	4-8, 18

Y		2, 3, 9-17, 19, 20
Y	US 2010/0311097 A1 (ANDERSON) 09 December 2010 (09.12.2010) entire document	3, 9-16
Y	WO 2011/116028 A1 (ANDERSON FORSCHUNG GROUP, INC) 22 September 2011 (22.09.2011) entire document	10-16
Y	US 6,338,821 B1 (JINA) 15 January 2002 (15.01.2002) entire document	17

☐ 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

03 March 2015

Date of mailing of the international search report

25 MAR 2015

Name and mailing address of the ISA/US

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2014/066151

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

a. (means)

☐

on paper

☒

in electronic form

b. (time)

☐

in the international application as filed

☐

together with the international application in electronic form

☒

subsequently to this Authority for the purposes of search

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs:1-34 were searched.