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(54) Title: METHOD FOR THE DIRECT DETECTION AND QUANTITATION OF ASPARAGINE SYNTHETASE IN BIOLOGICAL SAMPLES

(57) Abstract: The invention provides a method for direct detection and quantitation of asparagine synthetase using stable isotope labeled standard peptides and mass spectrometry.



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METHOD FOR THE DIRECT DETECTION AND QUANTITATION OF ASPARAGINE SYNTHETASE IN BIOLOGICAL SAMPLES

The contents of all patents, patent applications, and references cited throughout
5 this specification are hereby incorporated by reference in their entireties.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

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BACKGROUND OF THE INVENTION

Quantitative measurement of biochemical markers for disease is the basis for the
15 design of effective drug regimens. Detection and quantitation of a signature protein
during the course of a disease and its treatment may provide researchers and clinicians
with a sensitive way to modify patient drug therapy, as well as to monitor the
progression of an illness. Chemoresistant cancers are an attractive target for
understanding what signature markers develop during the course of disease and
20 treatment. Acute lymphoblastic leukemia (ALL) is one type of cancer that shows
chemoresistance associated with increased expression of the protein asparagine
synthetase (ASNS) by leukemic cells.¹⁻⁵

Presently, methods to detect and quantitate ASNS protein or activity in a sample
remain limited. There is an urgent need in the art to develop a method to monitor either
25 the ASNS protein or its activity in a sample to allow for a better understanding of the
role of ASNS in the ASNase resistance of cancer and to design and modify patient drug
therapy.

SUMMARY OF THE INVENTION

30 As described below, the present invention generally features a method for direct
detection and quantitation of a protein expressed in a sample. In particular, the
invention provides methods for direct detection and quantitation of asparagine
synthetase (ASNS), as an indication of an asparaginase resistant phenotype, and methods
of diagnosis and monitoring asparagine synthetase in subjects having cancer, for

example. Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

In a particular aspect, the invention is directed to a method for detecting and quantifying asparagine synthetase (ASNS) in a sample, the method comprising the steps
5 of substantially purifying an ASNS protein from a sample, adding standards to the protein, and determining by mass spectrometry, the amount of protein in the sample.

In one embodiment of the method, the substantial purification is by SDS-PAGE separation, size exclusion chromatography, cation or anion exchange chromatography, or antibody-mediated enrichment. In a particular embodiment, the antibody specifically
10 binds an ASNS protein. In a further embodiment, the antibody specifically binds the standards. In another embodiment of the method, the sample is a biological sample from a patient. In a particular embodiment, the sample is derived from blood, serum, bone marrow, or tissue. In a further embodiment, the sample is a peripheral blood sample. In yet another embodiment of the method, the peripheral blood sample is from a subject
15 suspected of having cancer selected from the group consisting of: hematogenous cancer, leukemia, and ovarian cancer. In a further embodiment, the peripheral blood sample is from a subject suspected of having leukemia. In another further embodiment, the peripheral blood sample is from a subject being treated with a chemotherapeutic.

In one particular embodiment, the chemotherapeutic is an L-asparaginase
20 compound. In a particular embodiment of the method, the standards are added as pairs. In another embodiment, the method detects between 30 amol and 200 fmol of asparagine synthetase. In one embodiment, the method further comprises comparing the level of ASNS in the sample and correlating the level with a disease state. In a particular embodiment, the disease state is cancer. In a further embodiment, an increase of at least
25 about 10-fold identifies a chemoresistant cancer. In another further embodiment, an increase of at least about 40-fold identifies a chemoresistant cancer. In one embodiment of the method, the mass spectrometry is reversed phase dual mass spectrometry.

In another particular aspect, the invention is directed to a method for identifying a subject having a chemoresistant neoplasia, the method comprising substantially
30 purifying an ASNS protein from a sample, adding standards to the protein, and determining by mass spectrometry, the amount of protein in the sample, wherein an increased level of asparagine synthetase in the sample relative to a reference identifies the subject as having a chemoresistant neoplasia.

In another aspect, the invention is directed to a method of diagnosing a subject as having, or having a propensity to develop, cancer, the method comprising substantially purifying an ASNS protein from a sample, adding standards to the protein, and determining by mass spectrometry, the amount of protein in the sample, wherein an increased level of asparagine synthetase relative to a reference indicates that the subject has or has a propensity to develop cancer.

In one embodiment, the method further comprises identifying the subject as having cancer. In a particular embodiment, the cancer is leukemia.

In another particular aspect, the invention is directed to a method of diagnosing a subject as having, or having a propensity to develop, chemoresistant cancer, comprising substantially purifying an ASNS protein from a sample, adding standards to the protein, and determining by mass spectrometry, the amount of protein in the sample, wherein an increased level of asparagine synthetase relative to a reference indicates that the subject has or has a propensity to develop chemoresistant cancer. In one embodiment of the method, the subject is identified as having chemoresistant cancer. In another particular embodiment, the chemoresistant cancer is leukemia.

In one aspect, the invention is directed to a method of monitoring a subject diagnosed as having cancer for disease severity, comprising substantially purifying an ASNS protein from a sample, adding standards to the protein, and determining by mass spectrometry, the amount of protein in the sample, wherein an alteration in the level of ASNS in the subject sample relative to the level in a reference indicates the severity of cancer in the subject. In one embodiment of the method, the alteration is an increase that indicates an increased severity of cancer.

In a further aspect, the invention is directed to a method of monitoring a subject diagnosed as having cancer for chemoresistance, comprising substantially purifying an ASNS protein from a sample, adding standards to the protein, and determining by mass spectrometry, the amount of protein in the sample, wherein an alteration in the level of ASNS in the subject sample relative to the level in a reference indicates the level of chemoresistance of the cancer in the subject.

In one particular embodiment, the alteration is an increase, and an increase indicates an increased chemoresistance of the cancer. In a further embodiment the alteration is a decrease, and a decrease indicates a decreased chemoresistance of the cancer. In one embodiment of any of the methods of the invention, the sample is a total cell lysate. In a further embodiment of any of the methods of the invention, the sample

is a bone marrow sample. In another embodiment, the sample is a peripheral blood sample. In still a further embodiment of the methods, the peripheral blood sample is from a subject having or suspected of having a cancer selected from the group consisting of: hematogenous cancer, leukemia, and ovarian cancer. In another embodiment, the peripheral blood sample is from a subject having leukemia. In a particular embodiment, the peripheral blood sample is from a subject being treated with an L-asparaginase compound. In another embodiment of any of the methods of the aspects of the invention, the standards are added as pairs. In still another embodiment of the methods of the aspects of the invention, the subject is being treated for cancer selected from the group consisting of: hematogenous cancer, leukemia, and ovarian cancer. In another particular embodiment of the method of the aspects of the invention, the subject is being treated with an L-asparaginase compound. In another particular embodiment of the aspects of the invention, the reference is a control subject sample. In a further embodiment, the reference is a subject sample obtained at an earlier time point.

In another embodiment of the invention, the method of any of the aspects of the invention is used to determine the treatment regimen for a subject having cancer or chemoresistant cancer. In a particular embodiment, the method of any of the aspects of the invention is used to monitor the condition of a subject being treated for cancer or chemoresistant cancer. In another embodiment, the method of any of the aspects of the invention is used to determine the prognosis of a subject having cancer or chemoresistant cancer. In one embodiment, a poor prognosis determines an aggressive treatment regimen for the subject. In another embodiment of any of the aspects of the invention, the cancer the cancer is acute lymphoblastic leukemia.

In one embodiment of any of the aspects of the invention, the standard is a peptide comprising a fragment of asparagine synthetase. In a particular embodiment, the standard is a peptide consisting essentially of a fragment of asparagine synthetase. In a further embodiment, the standards are peptides selected from the group consisting of: 467 ETFEDSNLIPK 477, 540 WINATDPSAR 549, and 49 LAVVDPLFGMQPIR 62. In a particular embodiment, the fragment is selected from the group consisting of: 467 ET*FEDSNLIPK 477, 540 WINATD*PSAR 549, and 49 LAVVDPLFGMQPIR 62. In another particular embodiment of the aspect of the invention the standard comprises an amino acid sequence selected from the group consisting of: 467 ETFEDSNLIPK 477, 540 WINATDPSAR 549, and 49 LAVVDPLFGMQPIR 62.

In one aspect, the invention is directed to a polypeptide standard for measuring asparagine synthetase in a sample comprising at least a fragment of ASNS.

In another aspect, the invention is directed to a polypeptide standard for measuring asparagine synthetase in a sample consisting essentially of a fragment of ASNS. In one
5 embodiment of the aspect, the fragment is selected from the group consisting of: 467 ET*FEDSNLIPK 477, 540 WINATD*PSAR 549, and 49 LAVVDPLFGMQPIR 62. In another embodiment, the polypeptide comprises the amino acid sequence 467 ETFEDSNLIPK 477 or 540 WINATDPSAR 549. In another embodiment of the aspect, the polypeptide consists essentially of the amino acid sequence 467 ETFEDSNLIPK 477
10 or 540 WINATDPSAR 549. In a further embodiment, the polypeptide standard consists essentially of the amino acid sequence 467 ETFEDSNLIPK 477, wherein F is a heavy isotope. In another embodiment, the polypeptide standard consists essentially of the amino acid sequence 540 WINATDPSAR 549, wherein P is a heavy isotope. IN another further embodiment of the aspect, the heavy isotope comprises carbon or nitrogen.

15 In a further aspect, the invention is directed to a polypeptide standard for use as a tracer in measuring asparagine synthetase in a sample comprising at least a fragment of ASNS.

In another aspect, the invention is directed to a polypeptide standard for use as a tracer in measuring asparagine synthetase in a sample consisting essentially of the amino
20 acid sequence 49 LAVVDPLFGMQPIR 62.

In one aspect, the invention is directed to a method of identifying a compound that inhibits cancer, the method comprising contacting a cell that expresses asparagine synthetase with a candidate compound, and comparing level of polypeptide in the cell contacted by the candidate compound with the level present in a control cell not
25 contacted by the candidate compound, wherein the level of asparagine synthetase is measured using the method of the aspects of the invention, and wherein a compound that decreases ASNS level is identified as a compound that inhibits cancer. In one embodiment of the aspect, the compound inhibits a chemoresistant cancer. In another embodiment, the chemoresistant cancer is leukemia

30 In one particular aspect, the invention is directed to an isolated polypeptide comprising the amino acid sequence 467 ETFEDSNLIPK 477, wherein F is a heavy isotope.

In another particular aspect, the invention is directed to an isolated polypeptide comprising the amino acid sequence 540 WINATDPSAR 549, wherein P is a heavy isotope.

5 In still another particular aspect, the invention is directed to an isolated polypeptide comprising the amino acid sequence 49 LAVVDPLFGMQPIR 62 that is used as a tracer peptide. In one embodiment of the aspects, the heavy isotope comprises carbon or nitrogen.

In a further aspect, the invention is directed to a kit for the detection of asparagine synthetase using mass spectrometry, the kit comprising peptide standards, and written
10 instructions for use of the kit in mass spectrometry. In one particular embodiment of the aspect, the peptide standard comprises an amino acid sequence selected from the group consisting of: 467 ETFEDSNLIPK 477, 540 WINATDPSAR 549 , and 49 LAVVDPLFGMQPIR 62. In another embodiment, the peptide standard comprises the amino acid sequence 467 ETFEDSNLIPK 477. In a particular embodiment, F is a heavy
15 isotope. In another embodiment peptide standard comprises the amino acid sequence 540 WINATDPSAR 549, wherein P is a heavy isotope. In another particular embodiment, the heavy isotope comprises carbon or nitrogen. In still a further embodiment, the 49 LAVVDPLFGMQPIR 62 peptide standard is used as a tracer peptide.

20

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many
25 of the terms used in this invention: Singleton et al., Dictionary of Microbiology and Molecular Biology (2nd ed. 1994); The Cambridge Dictionary of Science and Technology (Walker ed., 1988); The Glossary of Genetics, 5th Ed., R. Rieger et al. (eds.), Springer Verlag (1991); and Hale & Marham, The Harper Collins Dictionary of Biology (1991). As used herein, the following terms have the meanings ascribed to
30 them below, unless specified otherwise.

The term "asparagine synthetase" (ASNS) is meant to refer to a protein or protein variant, or fragment thereof, that is substantially identical to at least a portion of GenBank Accession No. P08243, and that has an asparagine synthetase biological

activity (e.g. biosynthesis of cellular asparagine), or is useful as a marker in a mass spectrometry assay.

The term "sample" refers to any biological or chemical mixture for use in the method of the invention. The sample can be a biological sample. The biological samples are generally derived from a patient, preferably as a bodily fluid (such as blood, bone marrow, cerebrospinal fluid, phlegm, saliva, or urine) or cell lysate. The cell lysate can be prepared from a tissue sample (e.g. a tissue sample obtained by biopsy), for example, a tissue sample (e.g. a tissue sample obtained by biopsy), blood, cerebrospinal fluid, phlegm, saliva, urine, or the sample can be cell lysate.

The terms "standard" and "standards" refers to the peptide fragments of ASNS.

The term "reference" refers to a control sample or state. In one embodiment, the level of ASNS in a sample is compared to the level of ASNS present in a control sample obtained from a normal subject. In another embodiment, the level of ASNS in the sample is compared to the level present in a non-chemoresistant cancer. Where the method monitors ASNS in a patient, the level of ASNS present in a patient sample is compared to the level present in a patient sample prior to treatment or at an earlier time point in treatment. The term "direct quantitation" or "directly quantitating" refers to measurement of ASNS levels that does not depend on a secondary signal or measurement.

The term "tracer peptide" is intended to include peptides that can be used as biomarkers in the methods of the invention. In certain embodiments of the invention, a tracer peptide can be used to indicate the presence of a protein of interest.

The term "subject" is intended to include vertebrates, preferably a mammal. Mammals include, but are not limited to, humans.

The term "peripheral blood sample" includes at least blast cells, leukocytes and platelets.

The phrase "asparaginase resistant" or "Asparaginase resistance" refers to resistance or lack of response to the chemotherapeutic agent L-asparaginase. Resistance or lack of response is defined at levels of 5%, 10%, 15%, 20% or greater of the total sample.

The terms "treat," "treating," "treatment," and the like are meant to refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not

require that the disorder, condition or symptoms associated therewith be completely eliminated.

The terms "prevent," "preventing," "prevention," "prophylactic treatment" and the like are meant to refer to reducing the probability of developing a disorder or
5 condition in a subject, who does not have, but is at risk of or susceptible to developing a disorder or condition.

The term "heavy isotope" is meant to include the element with a heavier atomic weight than the lighter atomic weight counterpart. Carbon and nitrogen are useful heavy isotopes. For example, the heavy isotope amino acids of the invention incorporate a L-
10 phenylalanine with six ^{13}C isotopes.

The term "mass spectrometry" refers to an analytical technique that is used to identify unknown compounds, to quantify known compounds, and to elucidate the structure and chemical properties of molecules. Detection of compounds can be accomplished with very minute quantities (as little as 10^{-12}g , 10^{-15} moles for a compound
15 of mass 1000 Daltons), such that compounds can be identified at very low concentrations (one part in 10^{12}) in chemically complex mixtures. Types of mass spectrometry (MS) include: tandem mass spectrometry, triple quadrupole mass spectrometer (TQMS); Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS), electrospray ionization Fourier transform ion cyclotron resonance mass
20 spectrometry (ESI-FTICR MS), liquid chromatograph dual phase mass spectrometry (LC/MS/MS), quadrupole mass spectrometry, quadrupole ion trap mass spectrometry.

The term "chemoresistance resistance" as used herein means that an increased amount of chemotherapeutic is required to achieve a desired therapeutic effect relative to the level required to achieve that therapeutic effect in a non-chemoresistant t disease
25 state. Cancer cells or a cancer can be chemoresistant, and cancer cells or a cancer can acquire chemoresistance over time.

The term "L-asparaginase compound" is intended to any agent that depletes asparagine. Examples include chemotherapeutic agents such as L-asparaginase.

In this disclosure, "comprises," "comprising," "containing" and "having" and the
30 like can have the meaning ascribed to them in U.S. Patent law and can mean "includes," "including," and the like; "consisting essentially of" or "consists essentially" likewise has the meaning ascribed in U.S. Patent law and the term is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of

that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

The term "neoplasia" is meant any disease that is caused by or results in inappropriately high levels of cell division, inappropriately low levels of apoptosis, or both. For example, cancer is an example of a neoplasia. Examples of cancers include, without limitation, leukemias (e.g., acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, acute myeloblastic leukemia, acute promyelocytic leukemia, acute myelomonocytic leukemia, acute monocytic leukemia, acute erythroleukemia, chronic leukemia, chronic myelocytic leukemia, chronic lymphocytic leukemia), polycythemia vera, lymphoma (Hodgkin's disease, non-Hodgkin's disease), Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors such as sarcomas and carcinomas (e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, uterine cancer, testicular cancer, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, schwannoma, meningioma, melanoma, neuroblastoma, and retinoblastoma). Lymphoproliferative disorders are also considered to be proliferative diseases.

mass spectrometry

The term "increased" means a positive alteration. Exemplary increases include 2-fold, 5-fold, 10-fold, 20-fold, 40-fold, or even 100-fold.

The term "substantially pure" refers to material that is free to varying degrees from components which normally accompany it as found in its native state. A "substantially purified" protein is at least 75%, 85%, 90%, or 95% free of other materials that would interfere with its measurement by mass spectrometry. Purity and homogeneity are typically determined using analytical chemistry techniques, for

example, polyacrylamide gel electrophoresis or high performance liquid chromatography. Various levels of purity may be applied as needed according to this invention in the different methodologies set forth herein; the customary purity standards known in the art may be used if no standard is otherwise specified.

5 The term “aggressive treatment regimen” is intended to mean reducing or ameliorating a disorder and/or symptoms associated therewith with a method of treatment (e.g. combination of chemotherapeutic agents) more intensive or comprehensive than usual, for instance in dosage or extent. It will be appreciated that, although not precluded, aggressively treating a disorder or condition does not require
10 that the disorder, condition or symptoms associated therewith be completely eliminated. The term “inhibits” means decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease. The term “hematogenous cancer” is intended to include any cancer originating in the blood or spread through the blood stream.

15

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Figure 1 shows an electrospray ionization Fourier transform ion cyclotron resonance mass spectrometer (ESI-FTICR MS) spectrum of trypsin digested recombinant human asparagine synthetase (rhASNS). Spectrum was obtained by direct
20 infusion of digested rhASNS at 300 nL/min with 128K data collection, and is an accumulation of 100 scans. Peptides labeled with an asterisk (W540-R549, E395-R403, E449-K459) were chosen as strongly ionizing, non-labile peptides that were composed of fewer than 15 amino acids and were also identified in μ LC-ESI-MS/MS analysis of MOLT-4 R samples. Peptide E395-R403 was not used as a standard based on a
25 potential site of degradation in its sequence.

Figure 2. Figure 2 is two panels of graphs showing full scan dual mass spectrometry (MS/MS) spectra of peptides 540 WINATDPSAR 549 (top panel) and 540 WINATD*PSAR 549 (bottom panel). Note the 6 m/z unit shift in many of the fragment
30 ions, reflecting the position of the heavy-isotope labeled proline (*P). Each MS/MS spectrum represents 37.5 fmol of the peptide.

Figure 3. Figure 3 is two panels of graphs showing full scan MS/MS spectra of peptides 467 ETFEDSNLIPK 477 (top panel) and 467 ET*FEDSNLIPK 477 (bottom panel).

Fragment ions matching the theoretical fragment ions for the proposed peptide sequence are indicated on the graphs with lettered labels (e.g. y2, y3, b4, b8). Note the 6 m/z unit shift in many of the fragment ions, reflecting the position of the heavy-isotope labeled phenylalanine (*F). Each MS/MS spectrum represents 37.5 fmol of the peptide.

5

Figures 4(A – C). Figure 4A – C show three ion chromatograms for 1:1 ratio of light and heavy isotope peptides. Panel A is the base peak chromatogram. Panel B is a mass chromatogram for the 3 fragment ions of peptide 540 WINATDPSAR 549, resulting from fragmentation of m/z = 566.12. Panel C is a mass chromatogram for the 3
10 fragment ions of 540 WINATD*PSAR 549, resulting from fragmentation of m/z = 569.12. Both peptides were present at approximately 37.5 fmol.

Figures 5(A – C). Figure 5A – C show ion chromatograms for a 1:1 ratio of light and heavy isotope peptides. Panel A is the base peak chromatogram. Panel B is a mass
15 chromatogram for the 3 fragment ions of peptide 460 ETFEDSNLIPK 467, resulting from fragmentation of m/z = 647.0. Panel C is a mass chromatogram for the 3 fragment ions of 460 ETFEDSNLI*PK 467, resulting from fragmentation of m/z = 650.0. Both peptides were present at approximately 37.5 fmol. The MS method was programmed to only fragment the light and heavy peptide ions during its elution time (23-28 minutes).
20 The absence of ion signal in other areas of chromatograms B and C is due to a lack of MS/MS data for those parent ions.

Figure 6. Figure 6 is a graphical representation of the ratio of MS response of light and heavy (L:H) 540 WINATDPSAR 549 peptide versus ratio of peptide amount (L:H).
25 The data are an average of 3 separate dilutions, and the error bars represent the standard deviation. The line equation and errors in slope and y-intercept are shown. The standard curve shown represents an analyte range of 37.5 amol to 375 fmol for the light peptide with a fixed analyte amount of 37.5 fmol for the heavy peptide standard.

30 **Figure 7.** Figure 7 is a graphical representation of the ratio of MS response of light and heavy (L:H) 467 ETFEDSNLIPK 477 peptide versus ratio of peptide amount (L:H). The data are an average of 3 separate dilutions, and the error bars represent the standard deviation. The line equation and errors in slope and y-intercept are shown. The

standard curve shown represents an analyte range of 37.5 amol to 187.5 fmol for the light peptide with a fixed analyte amount of 37.5 fmol for the heavy peptide standard.

Figures 8(A and B). Figure 8A shows a gel and a schematic of sample preparation for MS analysis. Figure 8B is an SDS-PAGE gel of total protein lysate from 7 different cancer cell lines, before (panel 1, left) and after (panel 2, right) excision of the band where ASNS migrates. Lane 1: purified rhAS, 1 µg; Lane 2: blank; Lane 3: 50 µg MOLT-4 S; Lane 4: 50 µg MOLT-4 R, Lane 5: 50 µg K562; Lane 6: 50 µg Jurkat; Lane 7: 50 µg RCH-ACV; Lane 8: 50 µg Nalm6; Lane 9: 50 µg REH.

10

Figure 9. Figure 9 is a graphical representation of MS quantitation of ASNS in 7 different cancer cell lines. The detected amount of ASNS is plotted on the y-axis, with error bars representing a 95% confidence interval. The inset provides a closer look at the cell lines containing lower levels of ASNS.

15

Figure 10. Figure 10 is a Western blot of an SDS-PAGE gel similar to that shown in Figure 8. Lane 1: rhAS, 5 ng; Lane 2: blank; Lane 3: MOLT-4 S, 50 µg (total protein load); Lane 4: MOLT-4 R, 50 µg; Lane 5: K562, 50 µg; Lane 6: Jurkat, 50 µg; Lane 7: RCH-ACV, 50 µg; Lane 8: Nalm6, 50 µg; Lane 9: REH, 50 µg.

20

Figure 11. Figure 11 is a graphical comparison of Western blot and MS quantitation data for the detection of ASNS in cancer cell lines. Both Western blot and MS data were normalized to the MOLT-4 R sample data, which was determined to have the largest amount of ASNS by both methods. Error bars in the Western blot data represent the standard deviation of two measurements for each cell line. Error bars in the MS data represent the relative standard deviation of the normalized data.

25

Figure 12. Figure 12 is a comparison of Western blot and MS quantitation data with the omission of MOLT-4 R and K562 samples so that the lower level samples can be visualized.

30

Figures 13(A and B). Figures 13 (A and B) show SDS-PAGE and Western Blot of MOLT-4 S and R samples, respectively. Panel A: SDS-PAGE of 50 µg each MOLT-4 S

(lanes 1-3) and MOLT-4 R (lanes 4-6) proteins, stained with Coomassie blue. Lanes 7, 8, and 9 contain 10, 28, and 100 ng of purified rhAS, respectively. Panel B: Western blot for the detection of ASNS. Lane assignments are the same as for the gel (panel A). No ASNS is detected in the MOLT-4 S samples by Western blot analysis.

5

Figures 14(A and B). Figures 14 (A and B) show SDS-PAGE and Western Blot of ALL Patient Samples, respectively. Panel A is a Coomassie stained SDS-PAGE gel, while Panel B is the corresponding Western blot, probed for the presence of ASNS. Lane assignments for each are identical: Lane 1: M082246; Lane 2: M079837; Lane 3: M080788; Lane 4: M079880, Lane 5: blank; Lane 6: MOLT-4 S; Lane 7: molecular weight markers; Lane 8: MOLT-4 R; Lane 9: blank; Lane 10: 5 ng rhAS.

10

DETAILED DESCRIPTION OF THE INVENTION

The invention features a method for detecting and quantitating a protein in a sample. In particular, the invention provides sensitive methods for the direct detection and quantitation of asparagine synthetase (ASNS) even when present at low levels in a sample. The invention further provides methods for diagnosing cancer or chemoresistant cancer and monitoring ASNS in subjects having cancer. In particular, the invention provides a means for directly detecting ASNS at very low levels in chemoresistant subjects.

20

Detection and quantitation of a signature protein during the course of a disease and its treatment provides researchers and clinicians with a sensitive way to modify patient drug therapy, as well as to monitor the progression of the illness. For example, in acute lymphoblastic leukemia (ALL), chemoresistance is often accompanied by an increase in the expression of asparagine synthetase (ASNS) by leukemic cells.¹⁻⁵ This increase in ASNS is induced by either deprivation of asparagine from the cell medium, or by introduction of the chemotherapeutic agent L-asparaginase (ASNase).^{1,2} Results of these studies indicate that expression of ASNS by leukemia cells is sufficient to induce resistance to L-asparaginase. This conclusion is valid because ASNase depletes extracellular asparagine, and the leukemic cells are able to produce the necessary levels of asparagine for survival through increased ASNS expression. In addition, *in vitro* studies with U937 cells have shown that ASNase sensitive cells can be made resistant by repeated exposure to increasing levels of sub-lethal doses of the drug.⁴

30

A number of methods have been developed for the detection and quantitation of ASNS in both *in vitro* and *in vivo* studies. Most recently, a real-time quantitative polymerase chain reaction (RQ-PCR) method was described as an effective means for the quantitation of ASNS expression in both leukemia cell lines and samples from human patients.¹² This method relies on isolation of mRNA from the sample, and through amplification of the ASNS mRNA and a control mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the resultant quantitative value is a normalized expression value of the ASNS signal divided by that of the GAPDH signal. While this provides an adequate normalized value for ASNS expression within a sample, it does not measure expression of the ASNS protein, but instead the amount of ASNS mRNA that is present. In addition, the RQ-PCR assay does not provide absolute quantitation of how much ASNS mRNA has been expressed, but instead a relative value, based on the internal control protein. There has yet to be established a strong correlation between mRNA levels and protein expression,¹³ thus, the mere presence of mRNA for ASNS does not definitively prove that the protein is present.

Alternative methods for detection of ASNS in cells rely on immunostaining and Western blotting (antibody-based assays), and RNA microarrays. While antibody-based experiments can be highly selective in the detection of ASNS, the observed signal is not from the protein itself, but from a secondary antibody that is specific for the primary antibody, which is specific for ASNS. Detection of a signal does not necessarily prove the presence of ASNS, but instead a molecule to which the primary (or secondary) antibody can bind. While most antibodies are highly selective for single antigens, alternate methods of sample identification are beneficial.

25 **Asparagine Synthetase**

Asparagine Synthetase (ASNS) is an enzyme involved in amino acid biosynthesis. Particularly, ASNS is responsible for the production of cellular asparagine. ASNS is found in a range of organisms, including human mammals, non-human mammals, bacteria, yeast, and plants. An example of human ASNS corresponds to Gen Bank Accession No. P08243.

Types of Samples

The direct detection and quantitation of a protein expressed in a sample mixture can be measured. In certain embodiments, the direct detection and quantitation of a

protein expressed in a sample mixture can be from a patient with cancer or chemoresistance, and levels of ASNS can be determined in different types of sample mixtures. In other embodiments, the direct detection and quantitation of a protein expressed in a sample mixture from a patient at risk for developing cancer or chemoresistance can be determined in different types of sample mixtures. In other certain embodiments, the direct detection and quantitation of a protein expressed in a sample mixture can be from a patient being treated with L-asparaginase compounds, such as compounds that deplete asparagine in cells, such as the chemotherapeutic agent L-asparaginase. The proteins detected include ASNS. The biological samples are generally derived from a patient, preferably as a bodily fluid, such as blood, serum, bone marrow, tissue, cerebrospinal fluid, or cell lysate. The cell lysate can be prepared from a tissue sample (e.g. a tissue sample obtained by biopsy).

Method for Detection and Quantitation

In one aspect, the invention provides a method for the direct detection and quantitation of asparagine synthetase ASNS in cell lines using liquid chromatography dual mass spectrometry (LC/MS/MS) detection.

In an exemplary embodiment of the method for the direct detection and quantitation of asparagine synthetase (ASNS), two tryptic peptides of asparagine synthetase were selected and synthesized with normal and heavy-isotope amino acids to serve as internal standards for quantitation. The linear range of quantitative detection and limits of detection (LOD) and quantitation (LOQ) were evaluated by analyzing the peptides by nanoflow reversed-phase liquid chromatography (nanoRPLC) coupled online with tandem mass spectrometry (MS/MS). Protein extracts were substantially purified from several cancer cell lines. Substantial purification includes separation of the protein. Protein extracts can be separated, for example, by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE is used to separate the proteins within the gel, to minimize extraneous proteins, and to allow for in-gel digestion. Other methods that can be considered for separation and isolation of the protein extracts that would be useful in the method of the invention include size exclusion chromatography, cation or anion exchange chromatography of the proteins and/or peptides from the digested material, and antibody-mediated enrichment, using an antibody specific for the protein of interest, or the specific target peptides in the protein (e.g., the two peptide standards defined above). Following the purification step, the

target protein was excised and, following addition of the stable isotope peptide standards, in-gel digested with trypsin. Quantitation was accomplished by the comparison of peak areas generated from reconstructed ion chromatograms from full-scan tandem mass spectra resulting from fragmentation of the selected peptide ions.

5 The method for direct detection and quantitation of ASNS polypeptide expressed in a complex sample mixture can be applied to the analysis of samples as described above, for example biological samples obtained from cell lysate or peripheral blood, to provide a reasonable estimate of the amount of ASNS recovered from each sample.

10 In a particular embodiment, the method of detection and quantitation of the invention correlates the level of ASNS expression with an asparaginase resistant phenotype. The lower limit of detection for this method was established to be in the range of 25 – 30 amol, preferably 30 amol. The lower limit of quantitation was for this method was thus established to be about 100 amol.

15 In certain embodiments of the invention, a tracer peptide can be used as a standard to indicate the presence of a protein of interest. In a preferred embodiment of the invention, the tracer peptide is 49 LAVVDPLFGMQPIR 62. The tracer peptide useful in the invention is not labeled with a heavy-isotope because the methionine in the tracer peptide would likely oxidize over time, and the actual concentration of the un-oxidized peptide would change over time. A tracer peptide is a peptide that provides
20 unique proof of identity and presence of a protein of interest, here ASNS, without specific quantitative values calculated. The tracer peptide is a normal portion of the ASNS protein and is produced after trypsin digestion of the enzyme. Detection of this peptide, which is unsuitable for use as a quantitative marker, indicates the presence of
25 this protein in the sample.

Diagnostics

Asparagine Synthetase (ASNS) is an enzyme that is responsible for the production of cellular asparagine. Expression of ASNS has been correlated with chemoresistance in certain types of cancer. For example, development of
30 chemoresistant acute lymphoblastic leukemia (ALL) is often accompanied by an increase in expression of asparagine synthetase (ASNS) by leukemic cells. Neoplastic cells or tissues usually have very low or no expression of ASNS. Further, neoplastic cells or tissues may express higher levels of ASNS polypeptide than corresponding chemo-sensitive cells or tissues. Thus, an alteration in the expression level of ASNS can

be used to diagnose a subject as having cancer, or to identify a chemoresistant cancer. In leukemia cancer cell lines, an increase in ASNS of between 40 and 100 fold has been detected between normal and chemotherapeutic resistant lines using patient using dual mass spectrometry (MS/MS). Accordingly, increased levels of ASNS in a sample from
5 a patient is indicative of chemoresistance or is indicative of a propensity to develop chemoresistance. For example, in some embodiments, increases in ASNS between 5 and 40-fold (e.g., 5, 10, 15, 20, 25, 30, 35, 40-fold) indicate that the subject has or is at risk of developing a chemoresistant cancer. In other embodiments, increases in ASNS between 40 and 200-fold (e.g., 40, 50, 75, 100, 125, 150, 200-fold) are indicative of
10 chemoresistance or a risk of developing chemoresistance.

In one embodiment, subjects may be diagnosed for a propensity to develop cancer. The method involves determining the level of expression of ASNS polypeptide in a subject sample, wherein an increased level of expression of asparagine synthetase relative to a reference indicates that the subject has or has a propensity to develop
15 cancer. In a further embodiment, subjects may be diagnosed for a propensity to develop chemotherapeutic resistant cancer, the method involves determining the level of expression of ASNS polypeptide in a subject sample, wherein an increased level of expression of asparagine synthetase relative to a reference indicates that the subject has or has a propensity to develop chemoresistance.

20 Increased expression of ASNS polypeptide is associated with increased biosynthesis of asparagine, and can be correlated with cancer, and is further correlated with chemoresistance. Increased expression of ASNS has been specifically correlated with L-asparaginase resistance in leukemia cells. Further, expression of ASNS by leukemia cells is sufficient to induce resistance to L-asparaginase. L-asparaginase
25 depletes extracellular asparagine, and the leukemic cells are able to produce the necessary levels of asparagine for survival through increased ASNS expression. Accordingly, the invention provides methods for identifying cancer or a chemoresistant cancer in a subject by determining the level of expression of ASNS polypeptide in a subject sample. Alterations in ASNS protein are detected using methods of direct
30 detection and quantitation as described herein. Such information can be used to diagnose cancer or a chemoresistant cancer. In certain embodiments, the subject may be undergoing treatment with a compound that depletes cellular asparaginase, such as L-asparaginase. Accordingly, the invention provides methods for identifying cancer or a

chemoresistant cancer in a subject undergoing treatment with a compound that depletes cellular asparaginase.

Patient Monitoring

5 The disease state or treatment of a patient having cancer can be monitored using the methods and compositions of the invention. In one embodiment, the chemoresistance of a patient can be monitored by determining an alteration in the level of asparagine synthetase using dual mass spectrometry (MS/MS). Such monitoring may be useful, for example, in assessing the efficacy of a particular drug in a patient. For
10 example, the resistance of a patient to the chemotherapeutic agent L-asparaginase can be monitored through detection of ASNS levels. In certain leukemia cell lines, a fold difference of ASNS levels between 40, 50, 55, 60, 70, 80, 85, 90, 95, and 100 indicates resistance to chemotherapeutic agents. Higher ASNS levels are found in chemoresistant cells. Likewise, the method of the invention can be used to look for sensitivity to
15 chemotherapeutic agents, i.e. ASNS levels may decrease as a cell becomes sensitive to chemotherapy. Thus, therapeutics that alter the expression of ASNS polypeptide are taken as particularly useful in the invention. Such monitoring can also be useful in, for example, determining the treatment regimen for a subject. Determining an alteration in the level of asparagine synthetase using dual mass spectrometry (MS/MS) is
20 accomplished at much lower protein levels using the method of the invention. For example, the level of ASNS can be used to indicate the severity of the cancer in a patient, or to indicate chemoresistance. The method is useful for detecting chemoresistance earlier and tailoring treatment accordingly. Chemotherapy regimen can be tailored based on the severity of the condition.

25 In a particular use of the method for detecting ASNS, a subject can be monitored for chemoresistance. The subject may be diagnosed as already having cancer. An alteration in the level of ASNS in the subject sample relative to the level in a reference can indicate the level of chemoresistance of the cancer in the subject. In certain embodiments the alteration in ASNS level is an increase, and the increase indicates an
30 increased chemoresistance of the cancer. In other embodiments, the alteration is a decrease, and the decrease indicates a decreased chemoresistance of the cancer. Detection of ASNS at low levels using a mass spectrometry method may provide a more sensitive means for determining the alteration in protein levels. In other embodiments of the invention, subjects that are being treated with agents that deplete cellular asparagine,

for instance asparaginase compounds, for example L-asparaginase, can be monitored for chemoresistance methods and compositions of the invention, for instance a mass spectrometry based detection method.

5 Screening Assays

One embodiment of the invention encompasses a method of identifying an agent that inhibits cancer or chemoresistance. Accordingly, compounds that modulate the expression or activity an ASNS polypeptide, variant, or portion thereof are useful in the methods of the invention for the treatment or prevention of cancer (e.g., leukemia) or chemoresistance. The method of the invention may measure an alteration in ASNS polypeptide levels using, for example, dual mass spectrometry (MS/MS). Many methods are available for carrying out screening assays to identify such compounds. In one approach, the method comprises contacting a cell that expresses ASNS with a candidate compound, and comparing level of expression of the polypeptide in the cell contacted by the candidate compound with the level of expression in a control cell not contacted by the candidate compound, wherein an alteration in the level of expression of ASNS identifies the candidate compound as a candidate compound that inhibits chemoresistance. In another approach, candidate compounds are identified that specifically bind to and alter the levels of ASNS. The efficacy of such a candidate compound is dependent upon its ability to interact with an ASNS polypeptide, a variant, or portion. Such an interaction can be readily assayed using any number of standard binding techniques and functional assays (e.g., those described in Ausubel et al., *supra*).

Compounds that are identified as binding to a polypeptide of the invention with an affinity constant less than or equal to 10 mM are considered particularly useful in the invention. Alternatively, any *in vivo* protein interaction detection system, for example, any two-hybrid assay may be utilized to identify compounds that interact with ASNS nucleic acid or polypeptide. Interacting compounds isolated by this method (or any other appropriate method) may, if desired, be further purified (e.g., by high performance liquid chromatography). Compounds isolated by any approach described herein may be used as therapeutics to treat a neoplasia in a human patient.

In one approach, the effect of candidate compounds can be measured at the level of polypeptide production to identify those that promote an alteration in an ASNS polypeptide level. The level of ASNS polypeptide can be assayed using any standard method. Standard immunological techniques include Western blotting or

immunoprecipitation with an antibody specific for an ASNS polypeptide. For example, immunoassays may be used to detect or monitor the expression of at least one of the polypeptides of the invention in an organism. Polyclonal or monoclonal antibodies (produced as described above) that are capable of binding to such a polypeptide may be used in any standard immunoassay format (e.g., ELISA, Western blot, or RIA assay) to measure the level of the polypeptide. In some embodiments, a compound that promotes a decrease in the expression or biological activity of the polypeptide is considered particularly useful. Again, such a molecule may be used, for example, as a therapeutic to delay, ameliorate, or treat a neoplasia or chemoresistance in a human patient.

The invention also includes novel compounds identified by the above-described screening assays. Optionally, such compounds are characterized in one or more appropriate animal models to determine the efficacy of the compound for the treatment of a neoplasia. Desirably, characterization in an animal model can also be used to determine the toxicity, side effects, or mechanism of action of treatment with such a compound. Furthermore, novel compounds identified in any of the above-described screening assays may be used for the treatment of cancer or chemoresistance or multi-drug resistance in a subject. Such compounds are useful alone or in combination with other conventional therapies known in the art.

Test Compounds and Extracts

In general, compounds capable of inhibiting cancer or chemoresistance by altering, in preferred embodiments decreasing, level of ASNS polypeptide are identified from large libraries of either natural product or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Methods for making siRNAs are known in the art and are described in the Examples. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available from Brandon Associates (Merrimack, N.H.) and Aldrich Chemical (Milwaukee, Wis.). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceanographics Institute (Ft. Pierce, Fla.), and PharmaMar, U.S.A. (Cambridge, Mass.).

Test compounds that inhibit cancer or chemoresistance of the invention are present in any combinatorial library known in the art, including: biological libraries; peptide libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone which are resistant to enzymatic degradation but which nevertheless remain bioactive; see, e.g., Zuckermann, R.N. *et al.*, *J. Med. Chem.* 37:2678-85, 1994); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library and peptoid library approaches are limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, *Anticancer Drug Des.* 12:145, 1997).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 90:6909, 1993; Erb *et al.*, *Proc. Natl. Acad. Sci. USA* 91:11422, 1994; Zuckermann *et al.*, *J. Med. Chem.* 37:2678, 1994; Cho *et al.*, *Science* 261:1303, 1993; Carrell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33:2059, 1994; Carrell *et al.*, *Angew. Chem. Int. Ed. Engl.* 33:2061, 1994; and Gallop *et al.*, *J. Med. Chem.* 37:1233, 1994.

Libraries of compounds may be presented in solution (e.g., Houghten, *Biotechniques* 13:412-421, 1992), or on beads (Lam, *Nature* 354:82-84, 1991), chips (Fodor, *Nature* 364:555-556, 1993), bacteria (Ladner, U.S. Patent No. 5,223,409), spores (Ladner U.S. Patent No. 5,223,409), plasmids (Cull *et al.*, *Proc Natl Acad Sci USA* 89:1865-1869, 1992) or on phage (Scott and Smith, *Science* 249:386-390, 1990; Devlin, *Science* 249:404-406, 1990; Cwirla *et al.* *Proc. Natl. Acad. Sci.* 87:6378-6382, 1990; Felici, *J. Mol. Biol.* 222:301-310, 1991; Ladner *supra.*).

In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their anti-neoplastic activity should be employed whenever possible.

In an embodiment of the invention, a high throughput approach can be used, in combination with the method for detection and quantitation of the invention, to screen different chemicals for their potency to affect ASNS polypeptide expression levels.

Those skilled in the field of drug discovery and development will understand that the precise source of a compound or test extract is not critical to the screening

procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as
5 modification of existing compounds.

When a crude extract is found to alter the expression level of ASNS polypeptide, variant, or fragment thereof, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and
10 identification of a chemical entity within the crude extract having anti-neoplastic activity. Methods of fractionation and purification of such heterogeneous extracts are known in the art. If desired, compounds shown to be useful agents for the treatment of a neoplasm are chemically modified according to methods known in the art.

15 Kits

The invention provides kits for the diagnosis or monitoring of cancer or chemoresistance. In particular, the kits provide reagents for measuring alteration in the level of asparagine synthetase using dual mass spectrometry (MS/MS). The kit detects an alteration in the expression of ASNS polypeptide relative to a reference level of
20 expression. In related embodiments, the kit includes reagents that include peptides comprising heavy isotope amino acid standards. Standards included in the kit include the peptide fragments of asparagine synthetase. In preferred embodiments, the standards include 467 ET*FEDSNLIPK 477, 540 WINATD*PSAR 549, and 49 LAVVDPLFGMQPIR 62. The peptide 49 LAVVDPLFGMQPIR 62 is used as a tracer
25 peptide according to the instructions of the kit. The tracer peptide is a normal portion of the ASNS protein and is produced after trypsin digestion of the enzyme. Detection of this peptide, which is unsuitable for use as a quantitative marker, indicates the presence of this protein in the sample.

Optionally, the kit includes directions for monitoring the polypeptide levels of
30 ASNS in a biological sample derived from a subject. Preferably, the kit further comprises any one or more of the reagents described in the diagnostic assays described herein. In other embodiments, the instructions include at least one of the following: description of the standards; methods for using the enclosed materials for the diagnosis of cancer or chemoresistance; precautions; warnings; indications; clinical or research

studies; and/or references. The instructions may be printed directly on the container (when present), or as a label applied to the container, or as a separate sheet, pamphlet, card, or folder supplied in or with the container.

- 5 The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the assay, screening, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

10 **Exemplification**

Abbreviations are as follows: acute lymphoblastic leukemia (ALL); asparagine synthetase (ASNS); recombinant human asparagine synthetase (rhASNS); L-asparaginase (ASNase); real-time quantitative polymerase chain reaction (RQ-PCR); nano reversed-phase liquid chromatography (nRPLC); nano reversed phase liquid
15 chromatography electrospray ionization dual mass spectrometry (nRPLC-ESI-MS/MS); Stable isotope standard (SIS); isotope dilution mass spectrometry (IDMS); triple quadrupole mass spectrometer (TQMS); Fourier transform ion cyclotron resonance mass spectrometer (FTICR MS); electrospray ionization (ESI); electrospray ionization Fourier transform ion cyclotron resonance mass spectrometer (ESI-FTICR MS); liquid
20 chromatograph dual phase mass spectrometry (LC/MS/MS); lower limit of detection (LLOD); selected reaction monitoring (SRM); multiple reaction monitoring (MRM); Fluorenylmethoxycarbonyl (Fmoc).

Example 1: Selection of Peptides for Use as Heavy-Isotope Standards

- 25 The use of heavy-isotope internal standards has long been recognized as a suitable method for quantitation by mass spectrometry (MS) detection.¹⁹⁻²² Over the past decade, isotope dilution mass spectrometry (IDMS) has been explored for the quantitation of peptides and proteins from complex sample matrices.²³⁻²⁸ In the majority of these experiments, a peptide sequence is selected from the target protein of interest,
30 and a heavy-isotope version of the peptide, through enrichment of ¹³C or ¹⁵N, is synthesized. The heavy-isotope peptide is spiked into the sample in a known amount and digested with trypsin. The resultant mixture is then analyzed by liquid chromatograph dual phase mass spectrometry (LC/MS/MS) and the parent ion mass of

the natural-abundance peptide and the heavy-isotope standards are specifically targeted for fragmentation.

In some cases, either due to the complexity of the sample or the very low abundance of the target protein, the samples are enriched prior to digestion by SDS-PAGE,²⁶ size-exclusion chromatography²⁷ or antibody-mediated isolation.²⁸ While sample recovery during these steps may be diminished,²⁸ the reduction in overall sample complexity usually results in enhanced analyte detection.

Most IDMS studies employ a triple quadrupole mass spectrometer (TQMS) for fragmentation and detection of the target parent and fragment ions. The TQMS is capable of selected reaction monitoring (SRM) and multiple reaction monitoring (MRM), methods by which a selected parent ion is exclusively isolated for fragmentation in the second quadrupole, and only selected fragment ions are passed into the third quadrupole for mass analysis.²⁹ The TQMS is known for its selectivity and sensitivity, related to the efficiency of transferring ions from one quadrupole to the next and its ability to efficiently contain ions during the fragmentation step.^{29, 30}

Recently, a linear ion trap (LIT) mass spectrometer was introduced that exhibited increased ion storage capacity and altered detector configuration to allow for improved ion detection.³¹ This mass spectrometer was used for quantitative nRPLC-MS/MS analysis of ASNS from the cellular lysate of cancer cell lines. The results herein demonstrate the reproducibility, selectivity and sensitivity of the measurements of the LIT MS for quantitative analyses of peptides using the IDMS methodology

Purified recombinant human asparagine synthetase (rhASNS) was digested with trypsin and analyzed by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometer (ESI-FTICR MS) for selection of suitable peptides for use as internal standards. The desalted protein digest was directly analyzed without chromatographic separation of peptides in order to create a sample mixture of some complexity, as shown in **Figure 1**. The ESI-FTICR MS spectrum shown in Figure 1 was obtained by direct infusion of digested rhASNS at 300 nL/min with 128K data collection, and is an accumulation of 100 scans. The peptides labeled with an asterisk (W540-R549, E395-R403, E449-K459) were chosen as strongly ionizing, non-labile peptides that were composed of fewer than 15 amino acids. Additionally, a sample of MOLT-4 R cytosolic proteins was analyzed by SDS-PAGE and the region around the 62 kDa molecular weight marker was excised, in-gel digested, and analyzed by nano reverse phase liquid chromatography electrospray ionization dual mass spectrometry

(nRPLC-ESI-MS/MS). The in-gel digest data were searched against the human protein database using SEQUEST,³² and ASNS was identified by a number of peptides, three of which corresponded to strongly-ionizing peptides in the digest sample analyzed by ESI-FTICR MS. The peptides selected for use as heavy-isotope standards were 540

- 5 WINATDPSAR 549 and 467 ETFEDSNLIPK 477 based on the amino acid sequence of human ASNS (ASNS_HUMAN, accession number P08243). These peptides were chosen because of their adequate size (between 10 and 15 amino acids), their ability to ionize and be detected by MS in a somewhat complex mixture, and the absence of labile amino acids such as methionine and cysteine. The stable isotope-labeled amino acids
- 10 proline and phenylalanine were selected based on the incorporation of six heavy-isotope atoms (¹³C and ¹⁵N) per amino acid, and the availability of an N-terminal Fluorenylmethoxycarbonyl (Fmoc) group for protection during peptide synthesis.

Example 2: Generation of the Response Curve for Standard Peptides of ASNS by

15 **LC/MS/MS**

- A standard curve was prepared for each of the peptides selected for use as a heavy-isotope standard. Fragmentation conditions were optimized to provide consistent fragmentation patterns of each of the SIS and natural abundance isotope peptide pairs. Three of the most abundant fragment ions from the dual mass spectrometry (MS/MS)
- 20 spectrum for each peptide were selected for quantitation using mass chromatograms constructed from the MS/MS fragment data. Fragment ions chosen for the 540 WINATDPSAR 549 peptide pair were y₄, y₆ and y₈, as shown in **Figure 2**. Note the 6 m/z unit shift in many of the fragment ions, reflecting the position of the heavy-isotope labeled proline (*P). Similarly, fragment ions chosen for the 467 ETFEDSNLIPK 477
- 25 peptides were y₂, b₈-H₂O, and b₉-H₂O, as shown in **Figure 3**. Again, note the 6 m/z unit shift in many of the fragment ions, reflecting the position of the heavy-isotope labeled phenylalanine (*F). In both experiments, each dual mass spectrometry (MS/MS) spectrum represents 37.5 fmol of the peptide.

- Twelve solutions containing 12.5 nM each of 540 WINATD*PSAR 549 and 467
- 30 ET*FEDSNLIPK477 SIS peptides and varying concentrations of the natural-abundance form of each peptide (12.5 pM to 125 nM) were analyzed by nano reversed phase liquid chromatography electrospray ionization dual mass spectrometry (nRPLC-ESI-MS/MS) to determine the lower limit of detection, lower limit of quantitation, and the linear response range for each peptide. Standard curves were prepared in triplicate from three

independent sample dilutions and analyzed on separate days. Full-scan MS/MS of each target peptide ion pair during their elution times provided 15-25 data points across each peak for generation of a mass chromatogram based on three major fragment ions, as demonstrated in the ion chromatograms shown in **Figure 4** and **Figure 5**. Figure 4, panel A is the base peak chromatogram, and panel B is a mass chromatogram for the 3 fragment ions of peptide 540 WINATDPSAR 549, resulting from fragmentation of $m/z = 566.12$. Panel C is a mass chromatogram for the 3 fragment ions of 540 WINATD*PSAR 549, resulting from fragmentation of $m/z = 569.12$. Both peptides were present at approximately 37.5 fmol. Here, the use of mass chromatograms enhances selectivity and clearly indicates retention times of peptides. Figure 5, panel A is again the base peak chromatogram, and panel B is a mass chromatogram for the 3 fragment ions of peptide 467 ETFEDSNLIPK 477, resulting from fragmentation of $m/z = 647.0$. Panel C is a mass chromatogram for the 3 fragment ions of 467 ETFEDSNLI*PK 477, resulting from fragmentation of $m/z = 650.0$. In the experiments shown in Figures 4 and 5, both peptides were present at approximately 37.5 fmol, and the MS method was programmed to only fragment the light and heavy peptide ions during its elution time (23-28 minutes). Further, in both experiments, the absence of ion signal in other areas of chromatograms B and C is due to a lack of dual mass spectrometry (MS/MS) data for those parent ions.

Next, the ratio of the peak area from the mass chromatograms for each natural abundance isotope and SIS peptide pair was plotted against the ratio of natural abundance isotope vs SIS peptide amount analyzed for each run, as shown in **Figure 6** and **Figure 7**, using a linear fit. Both Figure 6 and 7 are graphical representation of the ratio of MS response of light and heavy (L:H) 540 WINATDPSAR 549 peptide or 467 ETFEDSNLIPK 477 peptide versus ratio of peptide amount (L:H). Regression analysis was carried out to determine the error in the slope of the curves and the y-intercepts, and reflects the error in the measured MS signal. In order to determine the lower limit of detection (LLOD) for each peptide, peak area values for the natural abundance isoforms of each peptide were recorded from 12 blank analyses. The average blank signal (S_{bl}) and its standard deviation (σ_{bl}) were calculated and incorporated into equation 1 as shown below:

$$S_{LLOD} = S_{bl} + 3(\sigma_{bl}) \quad (1)$$

where S_{LLOD} is the signal required to satisfy the lower limit of detection (LLOD). The constant 3 in equation 1 represents an 89% confidence level of detection, and is the value recommended in literature.^{33, 34} S_{LLOD} is then incorporated into equation 2, in which the value for m was experimentally determined from the calibration curve for each peptide (MS response versus peptide amount, data not shown). The slope of each calibration curve, m , represents the sensitivity of the detection method and C_{LLOD} represents the concentration of the LLOD.

$$C_{LLOD} = (S_{LLOD} - S_{bl})/m \quad (2)$$

The LLOD for 467 ETFEDSNLIPK 477 was found to be 30 amol, while the LLOD for 540 WINATDPSAR 549 was found to be 100 amol.

Values for the lower limit of quantitation (C_{LLOQ}) were calculated as ten times the standard deviation of the blank measurement, σ_{bl} (equation 3).

$$C_{LLOQ} = (10 \times \sigma_{bl})/m \quad (3)$$

The lower limit of quantitation for peptide 467 ETFEDSNLIPK 477 was determined to be 100 amol, while for 540 WINATDPSAR 549 is was 350 amol. These results are summarized in **Table 1**, as follows. These results were established with purified synthetic peptides dissolved in water. The addition of a complex sample matrix may ultimately alter the lower limits of detection and quantitation with the possibility of contaminant analytes. However, the linear ion trap mass detector is capable of three orders of magnitude linearity, and sub-femtomole limits of detection in this study.

Table 1. Results for Peptides.

	540 WINATDPSAR 549	467 ETFEDSNLIPK 477
Linear Range	350 amol – 400 fmol	100 amol – 200 fmol
Limit of Detection	100 amol	30 amol
Lower Limit of Quantitation	350 amol	100 amol

Example 3: Investigation of Protein Extracts from Cancer Cell Lines

The nano reversed phase liquid chromatography electrospray ionization dual mass spectrometry (nRPLC-ESI-MS/MS) method was applied to several protein samples isolated from cancer cell lines. **Figure 8a** shows a gel and a schematic of the process of sample preparation for MS analysis. Briefly, the process entails cell lysis, SDS- PAGE separation of the soluble proteins, excision of the molecular weight region of interest, destaining and digestion with trypsin, with simultaneous addition of heavy-isotope standards. Post-digestion, peptides were extracted from the gel and analyzed by nRPLC-ESI-MS/MS. Fifty micrograms of total protein extract from MOLT-4 S, MOLT-4 R, K562, REH, Jurkat, Nalm6, and RCH-ACV were resolved by SDS-PAGE, and compared to a standard of purified rhAS, as shown in **Figure 8b**.

Analysis of the gel slices using the described method resulted in detection and quantitation of ASNS, as summarized in **Table 2**, below. The uncertainty in these values represents the 95% confidence interval.

Table 2. Quantitation of asparagine synthetase (ASNS) from 7 Cancer Cell Lines

Gel Lane	Cell Line	Moles Detected	Grams of AS in Sample	% of AS in Sample
3	MOLT-4 S	1.1 ± 0.7 fmol	150 ± 100 pg	$0.0003 \pm 0.0002\%$
4	MOLT-4 R	170 ± 100 fmol	20 ± 10 ng	$0.05 \pm 0.03\%$
5	K562	40 ± 15 fmol	5 ± 2 ng	$0.010 \pm 0.004\%$
6	Jurkat	6 ± 3 fmol	800 ± 400 pg	$0.0020 \pm 0.0006\%$
7	RCH-ACV	4 ± 3 fmol	600 ± 400 pg	$0.0012 \pm 0.0006\%$
8	Nalm6	900 ± 400 amol	130 ± 60 pg	$0.0003 \pm 0.0001\%$
9	REH	4.3 ± 3.2 fmol	599 ± 440 pg	$0.0012 \pm 0.0008\%$

The values shown in Table 2 demonstrate that each of the cell lines analyzed was shown to have ASNS present within the limits of detection and quantitation of the method (30 and 100 amol, respectively, for peptide 467 ETFEDSNLIPK 477). **Figure 9** graphically illustrates the moles of ASNS present in each seven different cancer cell lines, based on analysis of 50 µg of total protein for each sample. Error bars represent 95% confidence intervals.

Taken together, these data from the seven cell lines shown in Table 2 and Figure 9 were compared using the Student's t-test to determine if each cell line contained significantly different levels of ASNS. The results indicate that all but the REH cell line can be divided into 4 distinct groups listed in order of decreasing ASNS measured: MOLT-4 R, K562, Jurkat/RCH, and MOLT-4S/Nalm6. The error in replicate measurements of the REH sample caused it to significantly overlap with the measurements from the Jurkat/RCH and the MOLT-4 S/Nalm6 groups, which were found to be significantly different from each other.

Western blot analysis were carried out on similarly prepared gels as that previously shown in Figure 8, with the exception that the amount of purified rhASNS loaded as a control was 5 ng instead of 1 µg. The results are shown in **Figure 10**. The Western blot was probed with the antibody for ASNS, and the darkened bands correspond to the presence of the protein. The membrane was probed with mouse-α-ASNS primary antibody (dilution of 1:100), then with goat -α-mouse secondary antibody, coupled to horseradish peroxidase. After introduction to ECL reagents, the chemiluminescent signal was detected by exposure to x-ray film for 30 seconds. In Figure 10, Lane 1, the rhASNS sample is shifted upward from the endogenous ASNS detected in the cell line samples because it contains C-terminal *c-myc* and multi-His tags, causing its molecular weight to be about 2 kD higher than that of the endogenous protein.¹⁷ The Western blot was analyzed to determine the relative pixel intensity of the darkened band for each cell line, and the data were normalized to the pixel intensity of the MOLT-4 R sample.

When the results of the MS quantitation assay are compared to the Western blot data, the overall trends appear to be in agreement. MS data were normalized to the sample containing the largest amount of ASNS, MOLT-4 R, allowing for the direct comparison of the normalized data for each method. Normalized Western blot data (two data sets) and MS data are plotted and shown in **Figure 11**. Error bars represent the

standard deviation in the measurement of the Western blot intensities, and the relative standard deviation of the normalized MS data. The MOLT-4 R and K562 samples were detected as having the largest amount of ASNS by both MS and Western methods. In order to observe the trends in the samples containing lower amounts of ASNS, the data were plotted without the MOLT-4 R and K562 data, and the results are shown in **Figure 12**.

Taken together, these results indicate that the MS data have a similar trend to that of the Western blot data, when normalized to the MOLT-4 R sample. These data indicate that the MS method of quantitation is at least as good as data obtained by Western blotting. Further, the MS method provides an estimate of how much ASNS is present in each sample, which is not possible with Western blotting.

Example 4: Analysis of additional MOLT-4 S and R protein samples

A separate set of MOLT-4 S and R proteins were analyzed by Western blotting analysis and the quantitative MS method. The results of the SDS-PAGE gel and Western blot are shown in **Figures 13 A and B**.

The Western blot analysis of the samples was unable to detect ASNS in the MOLT-4 S sample, although ASNS was detected in the MOLT-4 R and purified recombinant human asparagine synthetase (rhASNS) samples. The purified rhASNS samples were loaded in such a low amount that they were below the level of detection for Coomassie stain, as shown in Figure 13 A.

Example 5: Investigation of peptide internal standards

During the analysis of the MOLT-4 S and R samples, there appeared to be discrepancy in values of ASNS calculated from one peptide standard to the other, where the 540 WINATDPSAR 549 peptide was consistently 5-fold higher in the cell line samples than the 467 ETFEDSLIPK 477 peptide. In order to determine what was causing this disagreement, the data were re-analyzed. Instead of constructing one mass chromatogram for the three fragment ions of each peptide, a separate mass chromatogram was constructed for each fragment ion for each peptide and its corresponding internal standard. Additionally, the data from the standard curves, as previously shown in Figures 6 and 7, were re-analyzed in this manner. The peak areas of the three fragment ions for each peptide were normalized to the ion with the smallest peak area for each data point: the y_4 , y_6 , and y_8 ions of 540WINATDPSAR549 were all

divided by the y_6 peak area for each point; the y_2 , b_8 -H₂O, and b_9 -H₂O ions were divided by the b_8 -H₂O peak area for each point. The results for the 467 ETFEDSNLIPK 477 peptide showed a y_2 : b_8 -H₂O: b_9 -H₂O ratio of 1.4:1:1.7, which was consistent with only 5% RSD for both the natural abundance isotope and SIS peptide, as long as the data were gathered in the linear range established for the method (see Table 1). The 540 WINATDPSAR 549 peptide's y_4 : y_6 : y_8 ratio was found to be 2.2:1:3.85 for the natural abundance isotope and SIS peptides analyzed in the standard curve. However, in the samples analyzed from cell lines, the ratio of the natural abundance isotope peptide, arising from endogenous ASNS in the sample was found to significantly deviate from the established fragment ratio, and the y_6 ion was consistently the fragment ion with the largest peak area, instead of the smallest, as shown in **Table 3**, below. This change in fragmentation pattern of the peptide is most likely caused by co-isolation of a precursor ion with similar m/z as the doubly-charged parent ion of the natural abundance peptide, 566.12 m/z . The likely cause of the elevation in the peak area of fragment ion y_6 is a contaminant ion that produces a fragment ion at the same m/z , or close enough to the targeted fragment ion that the contribution from the isotope distribution of that ion artificially inflates the intensity of the target fragment ion. There is no elevation in the y_6 fragment ion of the stable isotope standard (SIS) peptide, thus it is likely not directly related to fragmentation of the peptide itself. The quantitative data generated from the 540 WINATDPSAR 549 peptide for the cell lines was therefore not used in calculating the amount of ASNS present in the samples.

Table 3. Fragment Ion Ratios for Natural Abundance Isotope and stable isotope standard (SIS) Peptide 540 WINATDPSAR 549

Fragment ions (* = heavy isotope standard ions)	y₄	y₆	y₈	y₄[*]	y₆[*]	y₈[*]
Standard curve determination of fragment ion ratios	2.1	1	3.85	2.1	1	3.85
MOLT-4 S, n = 9	0.065	1	0.10	1.9	1	3.85
MOLT-4 R n = 9	0.64	1	1.16	1.98	1	3.98

The amount of asparagine synthetase (ASNS) was then calculated using only the data from the 467 ETFEDSNLIPK 477 peptide, which retained its fragment ion ratio throughout all analyses. The data are summarized in **Table 4** as follows.

5 The values shown in Table 4 for the MOLT-4 S and R proteins differ slightly from the values shown previously in Table 2. Nearly all of these values fall within the calculated 95% confidence interval. The composition of the samples was also different in that the samples reported in Table 2 were from a total protein fraction, and the samples reported in Table 4 were a cytosolic protein fraction. The cellular location of asparagine synthetase (ASNS) has not been reported. During the protein fractionation
10 process of cell lysis, the nuclear pellets are removed from the soluble cytosolic proteins. It is possible that some cytosolic proteins might have been lost during this process. Alternatively, a population of ASNS may be present in the nucleus of the MOLT-4 cells, and removal of these proteins might decrease the overall quantity of ASNS recovered.

Table 4. Amounts of ASNS present in MOLT-4 S and R Cytosolic Protein Samples
Detected by MS

Sample	Moles Detected	Grams in Sample	% of ASNS in Sample
MOLT-4 S, n = 9	1.0 ± 0.2 fmol	$140 \text{ pg} \pm 30 \text{ pg}$	$0.00010 \pm 0.00006\%$
MOLT-4 R, n = 9	90 ± 20 fmol	12 ± 3 ng	$0.024 \pm 0.007\%$

Example 6: Analysis of ALL Patient Samples

The composition of the patient samples analyzed in this study was nearly pure (100%) blast cells isolated from peripheral blood. An alternative source of sampling
5 from an acute lymphoblastic leukemia (ALL) patient is the bone marrow. The sample identification code, cell count and total protein recovery from ALL patient samples after lysis are shown in **Table 5**, below.

Table 5. Sample Code, Cell Count and Total Protein Recovery from acute lymphoblastic leukemia (ALL) Patient Samples

Sample Code	Cell Count (cells/vial)	Recovered Protein
M079880	24.4 million	2.9 mg
M079837	49.5 million	3.0 mg
M080788	35.6 million	2.2 mg
M082246	46.6 million	2.6 mg

Two gels were prepared with the ALL patient samples. Total protein fractions of MOLT-4 S and MOLT-4 R were used as controls, as well as a sample of rhASNS. Fifty micrograms of each sample were loaded on the gel, except rhASNS, in which only 5 ng were loaded. After electrophoresis, one gel was stained to visualize the protein bands, while the other was electroblotted to a PVDF membrane for Western analysis. Once the Western blot results were obtained, the gel was overlaid on the blot film to specifically target the regions of the gel that had a corresponding signal in the Western blot. The SDS-PAGE gel and Western blot results are shown in **Figure 14, A and B**.

Based on results of the Western Blot shown in Figure 14, the levels of ASNS present in the patient samples are low, and vary among the samples. Additional bands appeared at lower molecular weight in the Western blot, possibly indicating a smaller or truncated form of asparagine synthetase ASNS is present in the samples that reacts with the antibodies. These lower molecular weight bands were also excised and analyzed using the nano reversed phase liquid chromatography electrospray ionization dual mass spectrometry (nRPLC-ESI-MS/MS) method for quantitation of ASNS. Five additional samples for each patient were prepared on 4 separate SDS-PAGE gels, one set of 5 replicates per gel with MOLT-4 S and R and 5 ng rhASNS control lanes and the ~62 kDa band was excised for in-gel digestion and quantitative analysis. These results are summarized in **Table 6** and **Table 7** as follows.

Table 6. Results of ASNS Quantitation Using the 540 WINATDPSAR 549 Peptide

Gel Lane	Sample Code	Moles ASNS Detected	Moles ASNS in Sample	Copies of ASNS per Cell
1	M082246	300 ± 100 amol	700 ± 200 amol	450 ± 120
2	M079837	1.0 ± 0.3 fmol	2.2 ± 0.6 fmol	1200 ± 300
3	M080788	260 ± 70 amol	600 ± 200 amol	400 ± 100
4	M079880	170 ± 50 amol	370 ± 120 amol	500 ± 200
6	MOLT-4 S	620 ± 200 amol	1.3 ± 0.4 fmol	Not determined
8	MOLT-4 R	10 ± 2 fmol	20 ± 5 fmol	Not determined

Table 7. Results of ASNS Quantitation Using the 467 ETFEDSNLIPK 477 Peptide

Gel Lane	Sample Code	Moles AS Detected	Moles AS in Sample	Copies of AS per Cell
1	M082246	25 ± 8 amol	54 ± 17 amol	40 ± 10
2	M079837	170 ± 40 amol	360 ± 80 amol	200 ± 40
3	M080788	60 ± 20 amol	130 ± 50 amol	100 ± 30
4	M079880	30 ± 20 amol	60 ± 30 amol	100 ± 50
6	MOLT-4 S	240 ± 50 amol	500 ± 100 amol	Not determined
8	MOLT-4 R	6 ± 2 fmol	12 ± 4 fmol	Not determined

5

Table 8. Fragment Ion Ratios for 540 WINATDPSAR 549 Peptide in ALL Patient Samples

Sample	y4	y6	y8	y4*	y6*	y8*
M079837	3.6 ± 1.0	1	3.3 ± 1.5	2.1 ± 0.1	1	3.8 ± 0.1
M080788	3 ± 2	1	3 ± 2	2.6 ± 1.0	1	4.8 ± 2.0
M082246	4 ± 3	1	1.2 ± 0.7	2.2 ± 0.1	1	3.9 ± 0.1
M079880	5 ± 5	1	1.2 ± 0.8	2.0 ± 0.1	1	3.9 ± 0.2

10

Table 9. Fragment Ion Ratios for 467 ETFEDSNLIPK 477 Peptide in ALL Patient Samples

Sample	y2	b8 - H ₂ O	b9 - H ₂ O	y2*	b8 - H ₂ O*	b9 - H ₂ O*
M079837	1.3 ± 0.4	1	2.1 ± 0.8	1.4 ± 0.1	1	1.9 ± 0.4
M080788	1.4 ± 0.5	1	2.0 ± 1.4	1.4 ± 0.1	1	1.9 ± 0.1
M082246	7.3 ± 6.8	1	4.3 ± 6.1	1.4 ± 0.1	1	1.8 ± 0.1
M079880	1.1 ± 0.6	1	1.6 ± 1.2	1.4 ± 0.1	1	1.9 ± 0.1

To properly interpret the data several aspects of the method should be noted. Two peptides from asparagine synthetase (ASNS) were used for quantitation, but the data from each peptide provided different quantitative results, with the 540 WINATDPSAR 549 peptide presenting about a 5-fold higher estimation of protein.

- 5 These results are similar to what was found upon initial analyses of the MOLT-4 S and R cell lines; however, in the acute lymphoblastic leukemia (ALL) patient samples, the fragmentation ion peak ratios for the 540 WINATDPSAR 549 fragment ions are more consistent with that of the heavy-isotope control (**Tables 8 and 9**, shown above), and appear to be less of a contributing factor to the discrepancy in calculation of ASNS from
- 10 each heavy-isotope peptide standard. These data do not appear to be affected by a contaminant ion as shown previously in Table 3. The difference in calculation of asparagine synthetase (ASNS) between the two standard peptides may be an effect of cleavage, where the 467 ETFEDSNLIPK 477 peptide may not be produced at the same rate as the 540 WINATDPSAR 549 peptide from in-gel digestion with trypsin.
- 15 Degradation of the peptide post-digestion may be ruled out because the heavy-isotope standard would likely undergo the same degradation under the same conditions in the sample. The 467 ETFEDSNLIPK 477 peptide has less variation in fragment ion measurement from the ALL patient samples, and may be the more reliable standard used for quantitation.
- 20 The standard deviation in the fragment ion ratios shown in Table 8 and Table 9 is larger in most cases for the natural abundance peptides than it is for the heavy-isotope peptides. Further, the ratios are closer to those of the heavy-isotope values in samples in which more ASNS was detected. This may indicate that the fragment ions used for detection and quantitation of ASNS are at or below the lower limit of the linear range in
- 25 these complex samples. Therefore, monitoring the individual contributions of the fragment ions to the peak area in the mass chromatograms will provide useful criteria to determine if the samples analyzed contain ASNS within the linear range and lower limit of detection of the method.

Also of note is sample recovery from the gel. While the efficiency of extraction of

30 ASNS from the gel pieces is unknown, the extraction efficiency is comparable for similar samples. The calculation of "copies of ASNS per cell" as shown in Tables 6 and 7 considers 100% percent efficiency in extraction for all samples. The exact number of ASNS molecules per cell may deviate from these calculated values, but using this nano reversed phase liquid chromatography electrospray ionization dual mass spectrometry

(nRPLC-ESI-MS/MS) method, the amount of ASNS detected in each analysis can be determined. Further, this method provides data from which an absolute quantity of ASNS may be calculated. While the error in measurement of ASNS using this method may be considered significant in terms of analytical figures of merit, it provides a suitable starting point for determining the biological relevance of this protein in human ALL samples.

Taken together, the results from these analyses show that asparagine synthetase (ASNS) has been detected in ALL patient samples using a mass spectrometry-based method. The data from the Western blot analyses imply that the amount of ASNS present in the patient samples is much less than that present in the MOLT-4 S cell line, however the response of the signal from Western blot analyses is not linear over large changes in protein amount.³⁵ The results from the MS quantitative method also demonstrate the ever increasing sensitivity and selectivity of MS-based methods for detection of targeted analyte ions.

Methods of the Invention

The results reported herein were obtained using the following Materials and Methods:

Evaluation of Recombinant Human Asparagine Synthetase for Robust Peptide Standards

Purified recombinant human asparagine synthetase (rhASNS) was previously expressed and purified in relatively large quantities for the use as a control protein model (Ciustea, 2005). One hundred micrograms were diluted in digestion buffer (50 mM NH_4HCO_3 , pH 8.4) to approximately 1 mg/mL, and trypsin (Sigma) added such that the ASNS:trypsin ratio was 1:20 (w/w) (total volume 120 μL). Proteolysis was carried out at 30 °C for 18 h before the reaction was terminated by flash freezing in liquid N_2 . Desalting of the tryptic digest prior to MS detection was accomplished using C18 PepClean spin columns (Pierce Biotechnology, Inc., Rockford, IL), and the peptide mixture was eluted in one fraction using 50% aqueous acetonitrile containing 0.1% formic acid. The sample was analyzed by direct infusion on a 4.7 Tesla Bruker Apex 47e Fourier transform ion cyclotron resonance mass spectrometer (FTICR MS), with an electrospray ionization (ESI) source (Analytica of Branford) at a flow rate of 0.3 $\mu\text{L}/\text{min}$. Data acquisition was carried out using a MIDAS data acquisition system.¹⁶

Peptide masses were calculated based on the mono-isotopic peak and charge state of each ion cluster and compared to those expected for tryptic peptides derived from recombinant, C-terminally tagged, human ASNS.¹⁷ Peptides providing reproducible ionization and few labile amino acids were selected for use as heavy-isotope internal standards.

Heavy-isotope Peptide Preparation and Addition

Heavy-isotope amino acids were purchased from Cambridge Isotopes, Inc (Andover, MA). Fluorenylmethoxycarbonyl (Fmoc)-derivatized L-phenylalanine containing six ¹³C (99%) isotopes was incorporated into the following peptide: 467ET*FEDSNLIPK477. Fmoc-derivatized L-proline containing five ¹³C (98%) and one ¹⁵N (98%) isotopes was incorporated into the following peptide: 540 WINATD*PSAR 549. Stable isotope standard (SIS) peptides and natural abundance isotope peptides (light) were synthesized by Mr. Alfred Chung at the ICBR Protein Core, University of Florida, Gainesville, Florida. Synthesized peptides were purified by HPLC and the masses verified by MALDI-TOF MS and ESI-FTICR MS. Lyophilized peptides were prepared in ~1 mg/mL (w/v) solutions in water and the concentrations verified by amino acid analysis. Purity was also assessed by LC-ESI-MS/MS of the peptide solutions.

Generation of the Response Curve for Each Peptide

All peptides were serially diluted from stock solutions to 125 μ M, separately in water. The SIS peptide solutions were combined in a 1:1 molar ratio to a concentration of 1.25 μ M for each peptide (5 μ L each 125 μ M SIS peptide stock and 490 μ L water). This was repeated for the natural abundance isotope peptides. Combined peptide stock solutions were diluted to 500 nM, then 25 nM in water. Twelve solutions with a fixed concentration of SIS peptides at 12.5 nM and varying concentrations of natural abundance isotope peptides between 12.5 pM and 125 nM were prepared to generate a standard curve for LC/MS/MS analysis. All solutions were prepared with a total volume of 50 μ L: 25 μ L of 25 nM SIS peptide stock were combined with 500 nM, 25 nM, 2.5 nM or 0.25 nM stock solutions of natural abundance isotope peptides and water. Three standard curves were prepared on separate days and analyzed by LC-ESI-MS/MS as described below. Three microliters of each sample were analyzed, providing 37.5 fmol

of heavy-isotope peptide and between 37.5 amol and 375 fmol light-isotope peptide on-column.

LC-ESI-MS/MS analysis

5 Samples were separated by on-line nano reversed-phase liquid chromatography (nRPLC) ESI MS/MS. The end of a length of fused silica capillary, 75 μm ID x 360 μm OD (Polymicro Technologies, Phoenix, AZ) was pulled to a fine tip (5-7 μm) using a butane torch. The capillary was slurry packed with Jupiter C18 reversed phase resin, with 5 μm bead diameter and 200 $\text{\AA}$ pore size (Phenomenex, Torrance, CA) to a bed
10 length of 10 cm, as previously described.¹⁸ Solvent flow was supplied by an Agilent 1100 capillary LC system (Agilent Technologies, Palo Alto, CA). Samples were loaded using an Agilent HPLC autosampler, maintained at 4°C. After loading the sample, the column was washed with 2% B for 30 minutes at a flow rate of 0.5 $\mu\text{L}/\text{min}$, then the flow rate was decreased to 0.250 $\mu\text{L}/\text{min}$ before initiation of the gradient. The gradient
15 was as follows: 2-40% B over 40 minutes, 40-98% B over 30 minutes, where mobile phase A was 0.1% formic acid in water (v/v) and mobile phase B was 0.1% formic acid in acetonitrile (v/v). High voltage contact for electrospray ionization was provided through a metal union connecting the microcapillary column to the LC pump. The Thermo LTQ mass spectrometer method was created for full-scan MS then full-scan
20 MS/MS of 5 most intense ions (data dependent mode), except during the retention times of the target peptides. During method times 23-28 minutes, the LTQ method was programmed for full-scan MS, then full-scan MS/MS (150 – 900 m/z) of 566.12 m/z and 569.12 m/z, sequentially, the natural abundance isotope and SIS doubly-charged ions of peptide 540 WINATDPSAR 549. During method times 28-33 minutes, the LTQ
25 method was programmed for full-scan MS, then full-scan MS/MS (150 – 1100 m/z) of 647.00 m/z and 650.00 m/z, sequentially, the natural abundance isotope and SIS doubly-charged ions of peptide 467 ETFEDSNLIPK 477. The retention time windows were based on an initial injection of the SIS peptides to determine their elution times. The molecular ion isolation width in full-scan MS/MS mode was set for 2.5 m/z with
30 collision energy at 35%, and q = 0.250.

MS Data Analysis

A mass chromatogram for each peptide was generated using Xcaliber software (Thermo Electron, San Jose, CA). The three most abundant and consistent fragment ions for each doubly-charged peptide ion were chosen based on MS/MS fragmentation data of each purified peptide. Mass chromatograms for each peptide were initially generated by plotting the sum of the three selected fragment ions versus time using an MS/MS filter for the parent ion, resulting in a peak area for each natural abundance isotope and SIS peptide, and all peak areas were recorded manually in a spreadsheet file. Additional mass chromatograms were generated for each separate fragment ion, and those peak areas were also recorded. The ratio of the MS peak area for each natural abundance isotope and SIS peptide pair was plotted versus the ratio of the amount of natural abundance isotope and SIS peptides analyzed. The data were fit using a line function, and regression analysis of the data was carried out to determine the error in the slope of the line and the y-intercept.

Cell Lysis and Desalt

Human acute lymphoblastic leukemia cell line MOLT-4 (ATCC CRL 1582) was propagated in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 10 mL/L ABAM (100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B) (GIBCO, Gaithersburg, MD) and 30 µg/mL gentamycin (Sigma, St. Louis, MO) as previously described.² All suspension cultures were maintained at 37 °C in a 5% CO₂ incubator (Nuair, Plymouth MN). Twenty-four hours before all experiments, cells were collected by centrifugation for 5 min at 288 x g, rinsed once with phosphate buffered saline PBS (0.15 M sodium, chloride, 10 mM sodium phosphate, pH 7.4), and resuspended at a density of approximately 5 x 10⁵ cells/mL in fresh medium. MOLT-4 parental (MOLT-4 S) cells were maintained in RPMI-1640 medium without any ASNase. MOLT-4 chemoresistant (MOLT-4 R) cells were maintained in RPMI-1640 medium containing 1 Unit/mL ASNase (Merck, West Point, PA). MOLT-4 S and MOLT-4 R cells were collected by centrifugation for 5 minutes at 288x g, rinsed twice with PBS, and re-suspended in 1 mL lysis buffer (10 mM HEPES, pH 7.9, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20%glycerol, 1 mM DTT, 1 mM PMSF, Protease Inhibitor Cocktail (Roche)) by gently pipetting up and down several

times. Samples were transferred to 2 mL microcentrifuge tubes and incubated on ice for 15 min. Triton-X 100 was added to a final concentration of 1% (100 μ L of 10% solution), and samples were vortexed for 5 seconds. Centrifugation at 500 x g for 5 min at 4 °C provided the cytosolic protein fraction in the supernatant. These protein samples were provided by Nan Su and Dr. Michael Kilberg, Department of Biochemistry, University of Florida (Gainesville, FL). Additional MOLT-4 S and R cells were lysed as described, except the original lysis buffer contained 1% Triton X-100, and the soluble protein fraction collected after centrifugation was considered the total soluble protein fraction.

Total protein extracts from the following cell lines were provided by Dr. Mi Zhou and Dr. Stephen Hunger, University of Florida, College of Medicine (Gainesville, FL): K562, Jurkat, Nalm6, REH, RCH-ACV, MOLT-4 S and R. Protein samples were precipitated using 20% trichloroacetic acid and cold acetone to remove salts and detergents. Proteins were re-dissolved in 50 mM NH_4HCO_3 , pH 8.3. Protein concentration was determined using the bicinchoninic (BCA) assay (Pierce Biotechnologies, Inc, Rockford, IL). Samples were diluted 1:5 (5 μ L sample, 25 μ L water) with water and combined with the BCA working reagent in a 1:1 ratio (10 μ L each sample and working reagent). A standard curve was prepared as described using BSA. The color was allowed to develop at 37°C for 30 minutes. Absorbance was monitored at 562 nm using a ND-1000 nanodrop diode array spectrophotometer (NanoDrop Technologies, Wilmington, DE). Sample concentration was calculated based on the calibration curve of BSA.

Preparation of ALL Patient Samples

Four samples of peripheral blood from patients diagnosed with ALL were provided by Dr. Alan Wayne, clinical director of the Pediatric Oncology Branch at the National Cancer Institute, Bethesda, MD. These samples were designated M079880, M079837, M080788, and M082246, and were previously treated to recover the mononuclear cells using a ficoll-hypaque gradient, with the recovered blast cells counted and frozen for storage. In preparation for cell lysis, the frozen cell samples were warmed in a 37°C water bath until almost completely thawed, then washed twice in cold PBS. The PBS was removed using a pipette and replaced with 500 μ L lysis buffer (20 mM NH_4HCO_3 , pH 8.3, 0.5% sodium dodecyl-sulfate (SDS), 5 mM tris-carboxyethyl

phosphine (TCEP), 5 mM sodium orthovanadate (NaVO_4), 10 mM sodium fluoride (NaF), 1 mM EDTA, 25 mM glycerophosphate, and Protease Inhibitor Cocktail (Sigma-Aldrich)) and incubated on ice for 15 minutes. The cell samples were lysed by 5 rounds of sonication, 15 seconds each at 12% amplitude, until homogenous. The samples were
5 boiled for 10 minutes, and a small aliquot (10 μL) was removed for protein concentration determination, as described previously.

SDS-PAGE Separation

Fifty micrograms of each sample were combined with SDS-reducing sample
10 loading buffer and boiled for 10 minutes. After vortexing and centrifugation, samples were loaded in 20 μL volumes onto NuPage 4-12% Bis-Tris SDS-PAGE gels (Invitrogen, Carlsbad, CA). Gels were run at 60-100V per gel for over an hour, or until the dye-front reached the bottom of the gel. After electrophoresis, the gels were washed three times for 5 minutes each in de-ionized water. Gels were stained with Simply Blue
15 (Invitrogen) Coomassie stain until the protein lanes were visible. The gels were then washed in water to remove the background stain.

Western Blot Analysis

In the case of western blotting analysis, two identical gels were prepared for SDS-
20 PAGE. After SDS-PAGE separation, the gel was applied to a PVDF (polyvinyl difluoride) membrane (Invitrogen) and electrotransferred for 1 hour at 80 V using a mini Protean 3 Trans-Blot electrophoretic transfer apparatus (Bio-Rad, Hercules, CA). Non-specific binding to the membrane was blocked by incubation in 5% non-fat dried milk in TBST (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% Tween 20) for 1 hour. The
25 membrane was probed for one hour with α -AS monoclonal antibody (University of Florida Hybridoma Core, Gainesville, FL) at a dilution of 1:100 in 5% non-fat dried milk in TBST. The membrane was rinsed 3 times for 5 minutes each in TBST prior to incubation with the secondary, horseradish peroxidase-conjugated goat- α -mouse antibody (Pierce Biotechnologies, Rockford, IL) at a dilution of 1:7,500 for one hour.
30 The membrane was rinsed again three times for 5 minutes each in TBST prior to treatment with enhanced chemiluminescence reagent (SuperSignal West Pico Substrate, Pierce, Rockford, IL). The peroxidase-conjugated antibody was visualized with x-ray film (CL-XPosure film, Pierce Biotechnologies, Inc). The x-ray film was photographed

and the image was analyzed by UN-SCAN-IT (Silk Scientific Corporation) to determine the pixel intensity of each of the bands.

In the case of the ALL patient samples, the antibody concentration and incubation times were increased to improve detection of ASNS: the α -ASNS monoclonal antibody was prepared at a dilution of 1:100 and the membrane was incubated overnight (12-14 hours). The secondary antibody was diluted 1:5,000 and incubated with the membrane for 1 hour. After treatment with chemiluminescent substrate, the x-ray film was exposed to the membrane for 5 minutes. Longer exposure times did not appear to affect signal intensity. In order to verify equal sample loading, the membrane was probed with α -actin at a dilution of 1:10,000 for 1 hour and re-exposed to the secondary antibody (1:20,000 dilution) for 1 hour. Chemiluminescent signal was detected by film exposure to the blot for 1-2 seconds.

In-gel Digestion and Heavy-Isotope Peptide Addition

Protein bands were excised using a scalpel and placed in separate 600 μ L microcentrifuge tubes. Location of the areas of excision was based upon the molecular weight markers and a sample of purified recombinant human ASNS (rhASNS) run in a separate lane, and was normally near the 62 kDa molecular weight marker. The rhASNS sequence contains an extended C-terminal *c-myc* and multi-histidine tag and normally migrates to a higher position on the SDS-PAGE gels than endogenous human ASNS. Therefore a generous region of the gel was excised between the 49 kDa and just above the 62 kDa molecular weight markers (SeeBlue Plus2 markers, Invitrogen). Gel bands were destained with two volumes of 500 μ L of 25 mM NH_4HCO_3 , pH 8.3, 50% acetonitrile. The excised gel bands were chopped into smaller pieces using a scalpel. Gel pieces were dehydrated by vortexing for 10 minutes in 100% acetonitrile. Bulk acetonitrile was removed and samples were lyophilized for 20 minutes in a vacuum centrifuge. Trypsin was prepared to a final concentration of 20 ng/ μ L in 25 mM NH_4HCO_3 , pH 8.3 (Promega) and added in 40-50 μ L volumes to the dehydrated gel pieces, then incubated on ice for 1 hour until the gel pieces were fully rehydrated. Excess trypsin solution was removed and replaced with 40 μ L of combined heavy-isotope peptide standards (2.5 nM of 540 WINATD*PSAR 549 and 467 ET*FEDSNLIPK 477) diluted in 25 mM NH_4HCO_3 , pH 8.3, resulting in 100 fmol of each heavy-isotope peptide. Samples were incubated overnight at 37°C. Alternatively,

the heavy-isotope peptide standards were spiked in at higher or lower amounts (50 fmol or 150 fmol, each). Peptide solutions were prepared fresh for each experiment, starting from the 500 nM combined peptide stock, and diluted serially to 2.5 nM with 25 mM NH_4HCO_3 , pH 8.3.

5 In the case of the human ALL patient samples, whenever possible, the SDS-PAGE gel was aligned with a corresponding western blot to target the excision of the ASNS band, which was not visible by Coomassie stain, and to minimize excess gel in the sample.

10 **Extraction and Analysis of Digested Peptides**

Trypsin digestion was quenched by addition of 50 μL of 70% acetonitrile, 5% formic acid. Gel samples were sonicated for 10 minutes, and the solution removed to fresh tubes. Extraction was repeated twice more. Peptide extract was lyophilized by vacuum centrifugation. Samples were rehydrated with 20 μL 0.1% TFA and vortexed
15 for 15 minutes. Samples were desalted using C18 ZipTips (Millipore). After initial desalt, the unbound portion of samples was re-subjected to fresh C18 ZipTips. The eluates were combined and lyophilized, then re-dissolved in 15 μL of 0.1% TFA in preparation for nano reversed phase liquid chromatography electrospray ionization dual mass spectrometry (nRPLC-ESI-MS/MS) analysis (as described above). Seven
20 microliters of each sample were analyzed twice by nRPLC-ESI-MS/MS. Data interpretation was carried out as described above.

Other Embodiments

From the foregoing description, it will be apparent that variations and
25 modifications may be made to the invention described herein to adopt it to various usages and conditions. Such embodiments are also within the scope of the following claims.

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or
30 subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

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10 All patents and publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent patent and publication was specifically and individually indicated to be incorporated by reference.

What is claimed is:

1. A method for detecting and quantifying asparagine synthetase (ASNS) in a sample, the method comprising
 - (i) substantially purifying an ASNS protein from a sample;
 - (ii) adding standards to the protein; and
 - (iii) determining by mass spectrometry, the amount of protein in the sample.
2. The method of claim 1, wherein the substantial purification is by SDS-PAGE separation, size exclusion chromatography, cation or anion exchange chromatography, or antibody-mediated enrichment.
3. The method of claim 2, wherein the antibody specifically binds an ASNS protein.
4. The method of claim 2, wherein the antibody specifically binds the standards.
5. The method of claim 1, wherein the sample is a biological sample from a patient.
6. The method of claim 1, wherein the sample is derived from blood, serum, bone marrow, or tissue.
7. The method of claim 1, wherein the sample is a peripheral blood sample.
8. The method of claim 7, wherein the peripheral blood sample is from a subject suspected of having cancer selected from the group consisting of: hematogenous cancer, leukemia, and ovarian cancer.
9. The method of claim 7, wherein the peripheral blood sample is from a subject suspected of having leukemia.
10. The method of claim 7, wherein the peripheral blood sample is from a subject being treated with a chemotherapeutic.

11. The method of claim 10, wherein the chemotherapeutic is an L-asparaginase compound.
- 5 12. The method of claim 1, wherein the standards are added as pairs.
13. The method of claim 1, wherein the method detects between 30 amol and 200 fmol of asparagine synthetase.
- 10 14. The method of claim 1, further comprising comparing the level of ASNS in the sample and correlating the level with a disease state.
15. The method of claim 14, wherein the disease state is cancer.
- 15 16. The method of claim 15, wherein an increase of at least about 10-fold identifies a chemoresistant cancer.
17. The method of claim 16, wherein an increase of at least about 40-fold identifies a chemoresistant cancer.
- 20 18. The method of claim 1, wherein the mass spectrometry is reversed phase dual mass spectrometry.
19. A method for identifying a subject having a chemoresistant neoplasia, the
25 method comprising
- (i) substantially purifying an ASNS protein from a sample;
 - (ii) adding standards to the protein; and
 - (iii) determining by mass spectrometry, the amount of protein in the sample,
- 30 wherein an increased level of asparagine synthetase in the sample relative to a reference identifies the subject as having a chemoresistant neoplasia.

20. A method of diagnosing a subject as having, or having a propensity to develop, cancer, the method comprising:

- (i) substantially purifying an ASNS protein from a sample;
- 5 (ii) adding standards to the protein; and
- (iii) determining by mass spectrometry, the amount of protein in the sample,

wherein an increased level of asparagine synthetase relative to a reference indicates that the subject has or has a propensity to develop cancer.

10

21. The method of claim 20, further comprising identifying the subject as having cancer.

22. The method of claim 21, wherein the cancer is leukemia.

15

23. A method of diagnosing a subject as having, or having a propensity to develop, chemoresistant cancer, comprising

- (i) substantially purifying an ASNS protein from a sample;
- 20 (ii) adding standards to the protein; and
- (iii) determining by mass spectrometry, the amount of protein in the sample,

wherein an increased level of asparagine synthetase relative to a reference indicates that the subject has or has a propensity to develop chemoresistant cancer.

25

24. The method of claim 23, further comprising identifying the subject as having chemoresistant cancer.

25. The method of claim 24, wherein the chemoresistant cancer is leukemia.

30

26. A method of monitoring a subject diagnosed as having cancer for disease severity, comprising

- (i) substantially purifying an ASNS protein from a sample;

- (ii) adding standards to the protein; and
- (iii) determining by mass spectrometry, the amount of protein in the sample,

wherein an alteration in the level of ASNS in the subject sample relative to the level in a
5 reference indicates the severity of cancer in the subject.

27. The method of claim 26, wherein the alteration is an increase that indicates an increased severity of cancer.

10 28. A method of monitoring a subject diagnosed as having cancer for chemoresistance, comprising

- (i) substantially purifying an ASNS protein from a sample;
- (ii) adding standards to the protein; and
- 15 (iii) determining by mass spectrometry, the amount of protein in the sample,

wherein an alteration in the level of ASNS in the subject sample relative to the level in a reference indicates the level of chemoresistance of the cancer in the subject.

20 29. The method of claim 18, wherein the alteration is an increase, and wherein an increase indicates an increased chemoresistance of the cancer.

30. The method of claim 18, wherein the alteration is a decrease, and wherein a decrease indicates a decreased chemoresistance of the cancer.

25

31. The method of any one of claims 19 - 30, wherein the sample is a total cell lysate.

32. The method of any one of claims 19 - 30, wherein the sample is a bone marrow
30 sample.

33. The method of any one of claims 19 - 30, wherein the sample is a peripheral blood sample.

34. The method of claim 33, wherein the peripheral blood sample is from a subject having or suspected of having a cancer selected from the group consisting of: hematogenous cancer, leukemia, and ovarian cancer.
- 5 35. The method of claim 34, wherein the peripheral blood sample is from a subject having leukemia.
36. The method of claim 22, wherein the peripheral blood sample is from a subject being treated with an L-asparaginase compound.
- 10 37. The method of any one of claims 19 - 30, wherein the standards are added as pairs.
38. The method of any one of claims 19 - 30, wherein the subject is being treated for
15 cancer selected from the group consisting of: hematogenous cancer, leukemia, ovarian cancer.
39. The method of any one of claims 19, 20, 23, or 28, wherein the subject is being treated with an L-asparaginase compound.
- 20 40. The method of any one of claims 19 - 30, wherein the reference is a control subject sample.
41. The method of any one of claims 19 - 30, wherein the reference is a subject
25 sample obtained at an earlier time point.
42. The method of any one of claims 19 - 30, wherein the method is used to determine the treatment regimen for a subject having cancer or chemoresistant cancer.
- 30 43. The method of any one of claims 19 - 30, wherein the method is used to monitor the condition of a subject being treated for cancer or chemoresistant cancer.
44. The method of any one of claims 19 - 30, wherein the method is used to determine the prognosis of a subject having cancer or chemoresistant cancer.

45. The method of claim 44, wherein a poor prognosis determines an aggressive treatment regimen for the subject.
- 5 46. The method of any of claims 1 - 45, wherein the cancer is acute lymphoblastic leukemia.
47. The method of any of claims 1 - 46, wherein the standard is a peptide comprising a fragment of asparagine synthetase.
- 10 48. The method of any of claims 1 - 46, wherein the standard is a peptide consisting essentially of a fragment of asparagine synthetase.
49. The method of any of claims 13 - 46, wherein the standards are peptides selected from the group consisting of: 467 ETFEDSNLIPK 477, 540 WINATDPSAR 549, and 49 LAVVDPLFGMQPIR 62.
- 15 50. The method of claims 47 or 48, wherein the fragment is selected from the group consisting of: 467 ET*FEDSNLIPK 477, 540 WINATD*PSAR 549, and 49 LAVVDPLFGMQPIR 62.
- 20 51. The method of claim 1, wherein the standard comprises an amino acid sequence selected from the group consisting of: 467 ETFEDSNLIPK 477, 540 WINATDPSAR 549, and 49 LAVVDPLFGMQPIR 62.
- 25 52. A polypeptide standard for measuring asparagine synthetase in a sample comprising at least a fragment of ASNS.
53. A polypeptide standard for measuring asparagine synthetase in a sample consisting essentially of a fragment of ASNS.
- 30 54. The polypeptide standard of claim 52, wherein the fragment is selected from the group consisting of: 467 ET*FEDSNLIPK 477, 540 WINATD*PSAR 549, and 49 LAVVDPLFGMQPIR 62.

55. The polypeptide standard of claim 52, wherein the polypeptide comprises the amino acid sequence 467 ETFEDSNLIPK 477 or 540 WINATDPSAR 549.
- 5 56. The polypeptide standard of claim 52, wherein the polypeptide consists essentially of the amino acid sequence 467 ETFEDSNLIPK 477 or 540 WINATDPSAR 549.
57. The polypeptide standard of claim 55 or claim 56 consisting essentially of the
10 amino acid sequence 467 ETFEDSNLIPK 477, wherein F is a heavy isotope.
58. The polypeptide standard of claim 55 or claim 56 consisting essentially of the amino acid sequence 540 WINATDPSAR 549, wherein P is a heavy isotope.
- 15 59. The polypeptide standard of claim 57 or claim 58, wherein the heavy isotope comprises carbon or nitrogen.
60. A polypeptide standard for use as a tracer in measuring asparagine synthetase in a sample comprising at least a fragment of ASNS.
- 20 61. A polypeptide standard for use as a tracer in measuring asparagine synthetase in a sample consisting essentially of the amino acid sequence 49 LAVVDPLFGMQPIR 62.
62. A method of identifying a compound that inhibits cancer, the method
25 comprising:
- (i) contacting a cell that expresses asparagine synthetase with a candidate compound; and
- (ii) comparing level of polypeptide in the cell contacted by the candidate compound
30 with the level present in a control cell not contacted by the candidate compound,
- wherein the level of asparagine synthetase is measured using the method of claim 1, wherein a compound that decreases ASNS level is identified as a compound that inhibits cancer.

63. The method of claim 62, wherein compound inhibits a chemoresistant cancer.
64. The method of claim 63, wherein the chemoresistant cancer is leukemia
- 5 65. An isolated polypeptide comprising the amino acid sequence 467
ETFEDSNLIPK 477, wherein F is a heavy isotope.
66. An isolated polypeptide comprising the amino acid sequence 540
- 10 WINATDPSAR 549, wherein P is a heavy isotope.
67. An isolated polypeptide comprising the amino acid sequence
49 LAVVDPLFGMQPIR 62 that is used as a tracer peptide.
- 15 68. The polypeptide standard of claim 66 or claim 67, wherein the heavy isotope
comprises carbon or nitrogen.
69. A kit for the detection of asparagine synthetase using mass spectrometry, the kit
comprising: peptide standards, and written instructions for use of the kit in mass
- 20 spectrometry.
70. The kit of claim 69, wherein the peptide standard comprises an amino acid
sequence selected from the group consisting of: 467 ETFEDSNLIPK 477, 540
WINATDPSAR 549, and 49 LAVVDPLFGMQPIR 62.
- 25 71. The kit of claim 70, wherein the peptide standard comprises the amino acid
sequence 467 ETFEDSNLIPK 477.
72. The peptide standard of claim 71, wherein F is a heavy isotope.
- 30 73. The kit of claim 69, wherein the peptide standard comprises the amino acid
sequence 540 WINATDPSAR 549.
74. The peptide standard of claim 73, wherein P is a heavy isotope.

75. The peptide standards of claims 72 or 74, wherein the heavy isotope comprises carbon or nitrogen.
- 5 76. The kit of claim 69, wherein the 49 LAVVDPLFGMQPIR 62 peptide standard is used as a tracer peptide.

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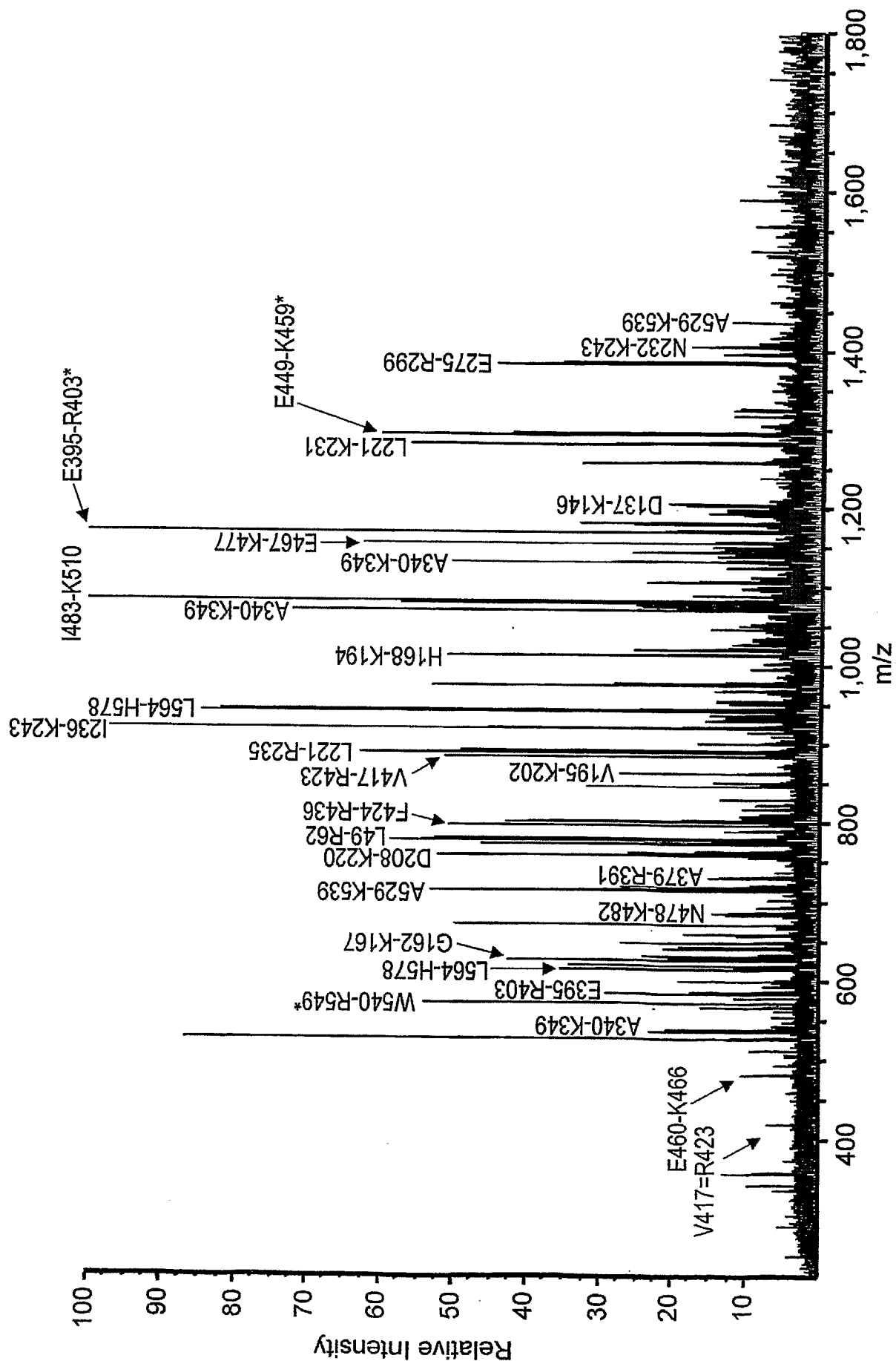


FIG. 1

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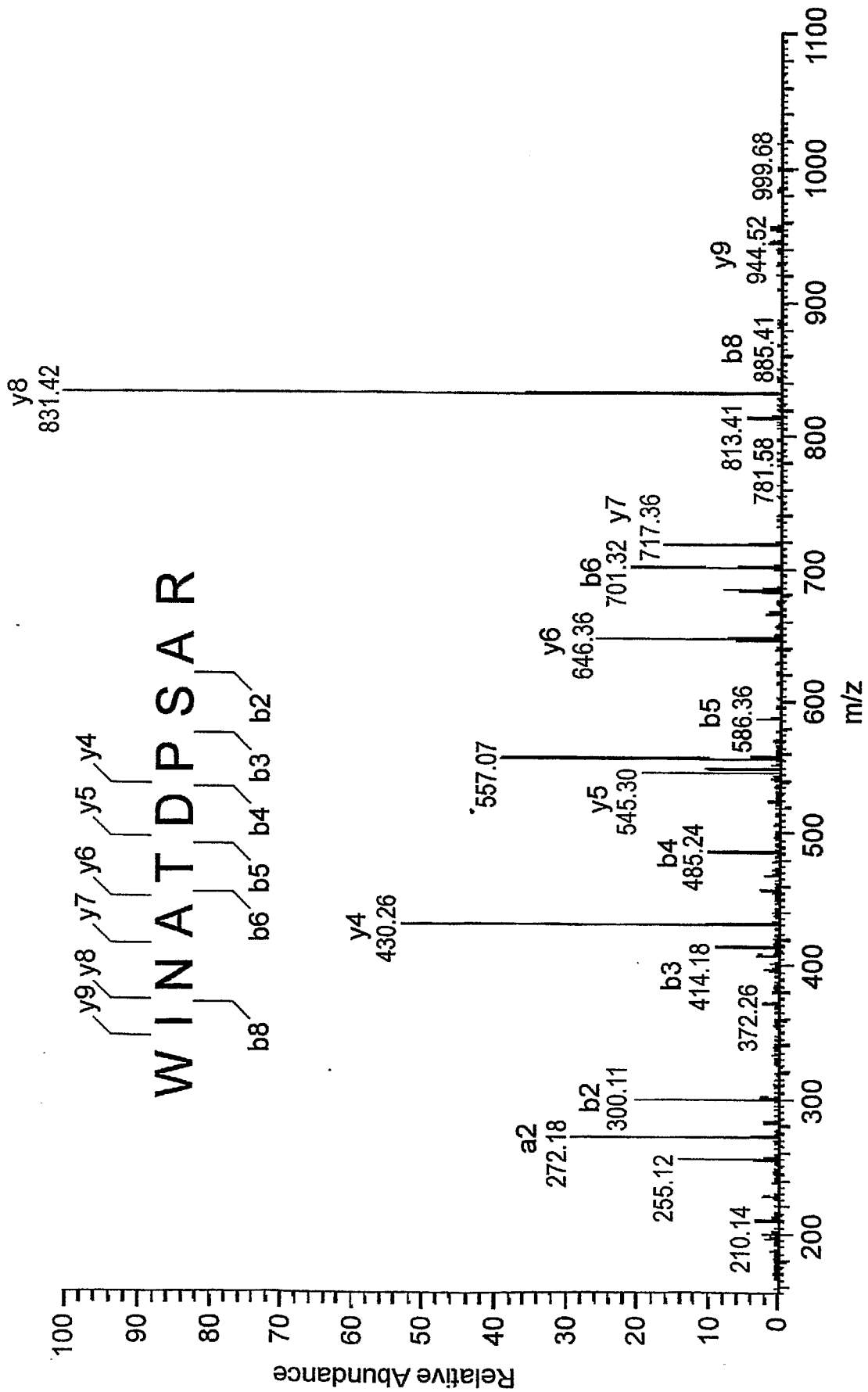


FIG. 2A

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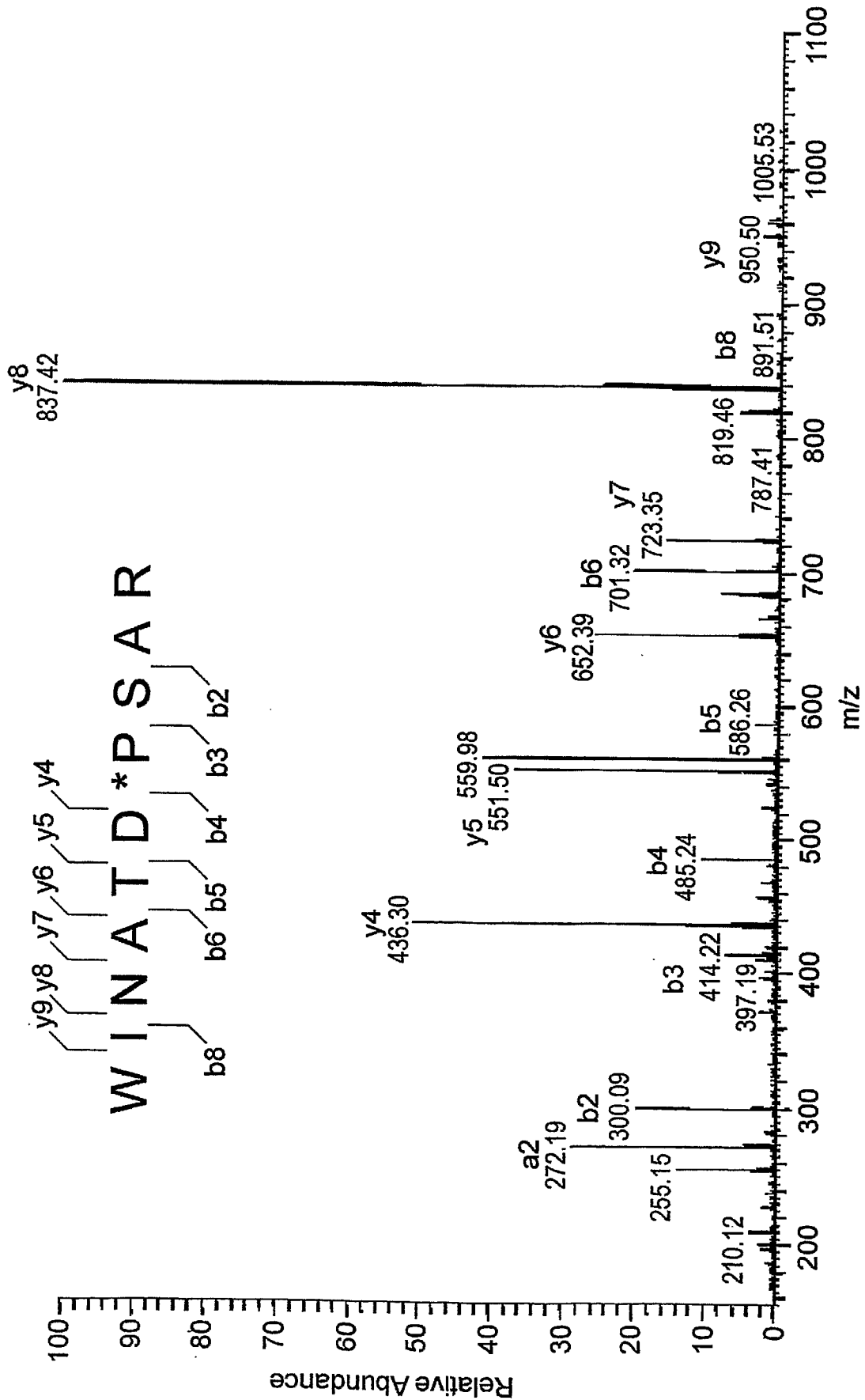


FIG. 2B

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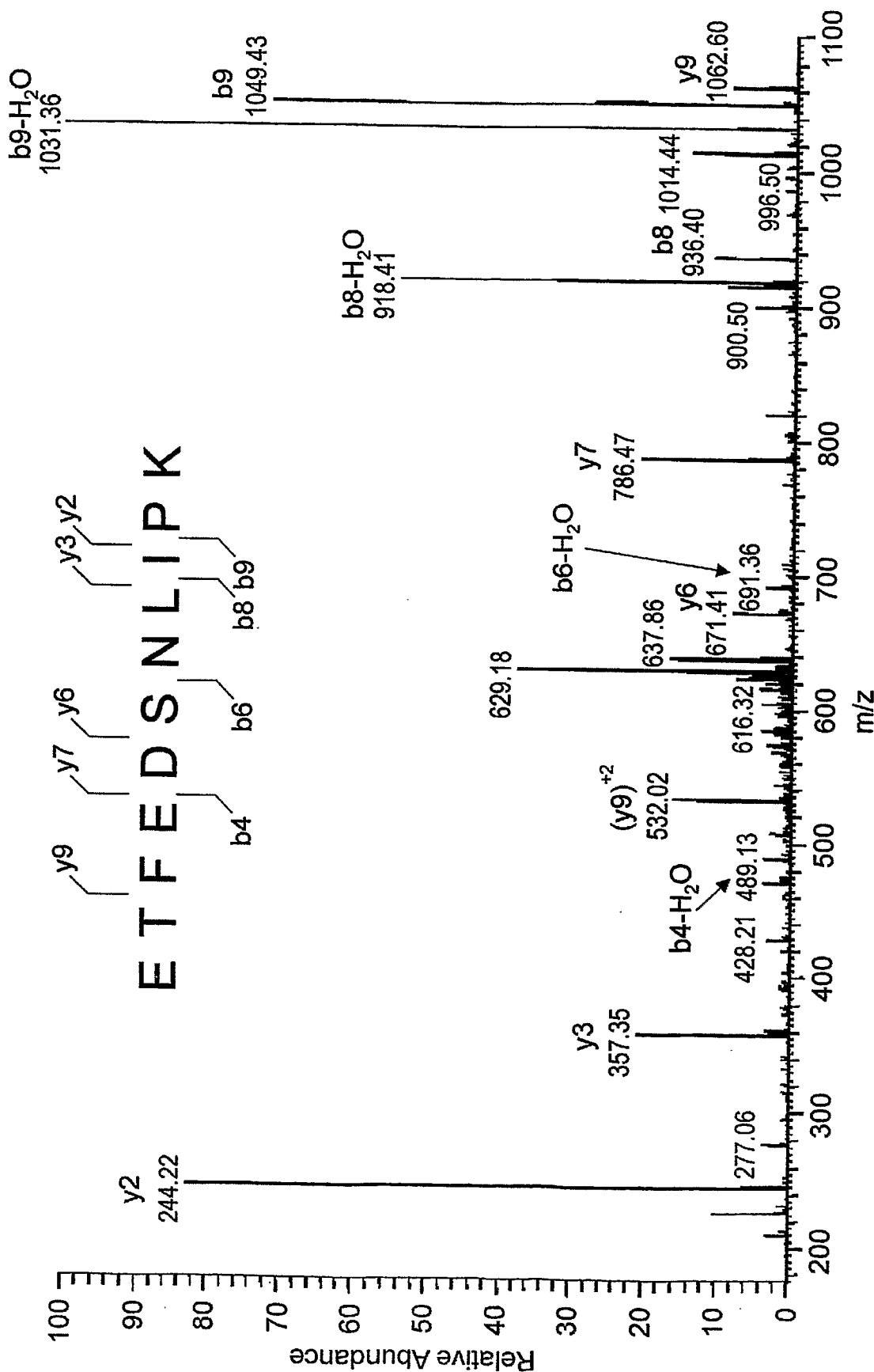


FIG. 3A

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FIG. 3B

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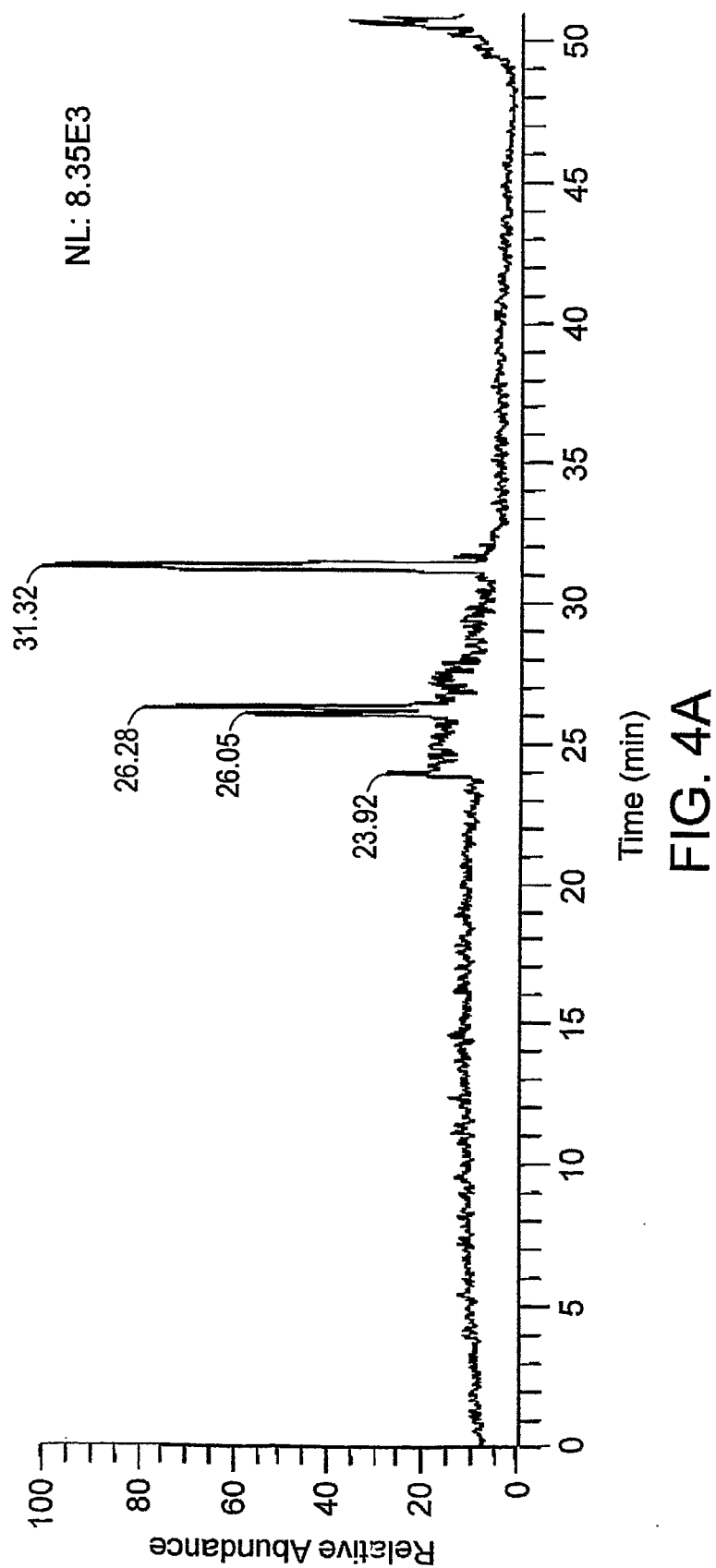


FIG. 4A

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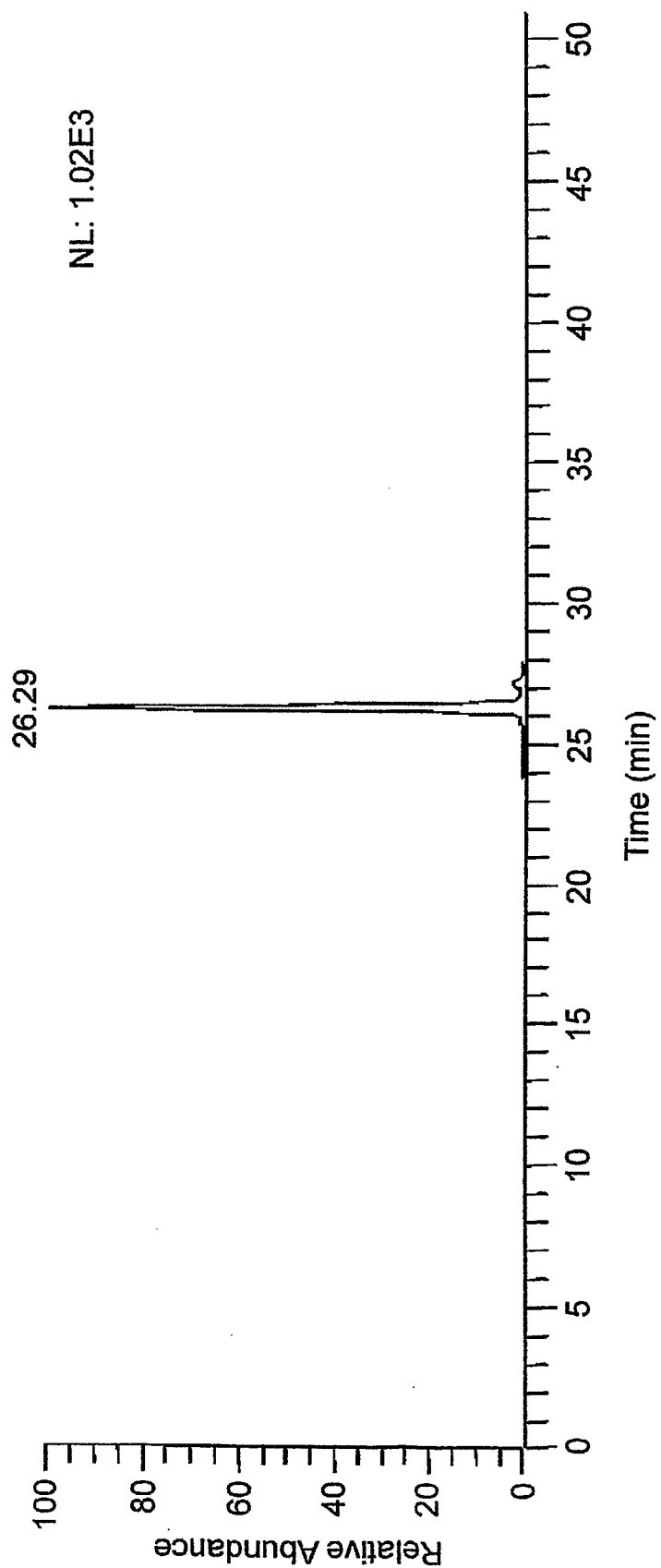


FIG. 4B

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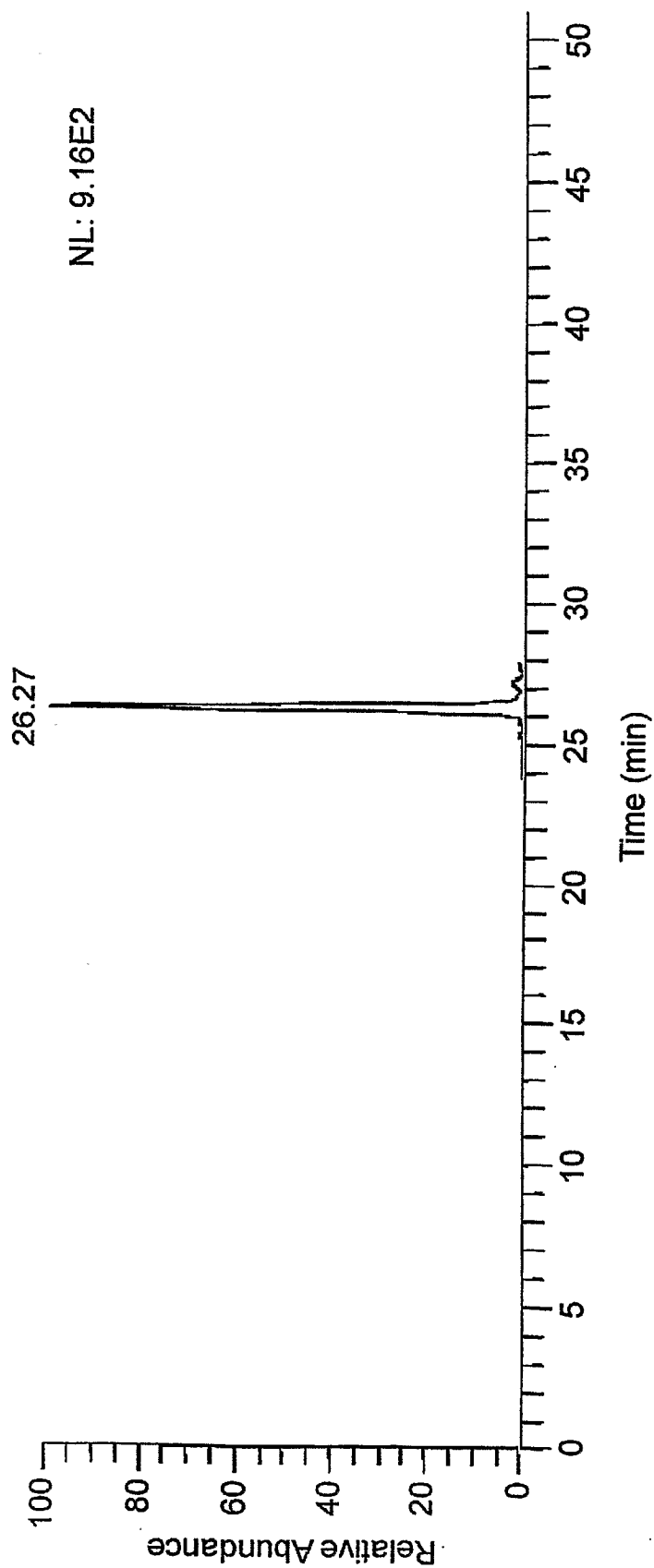


FIG. 4C

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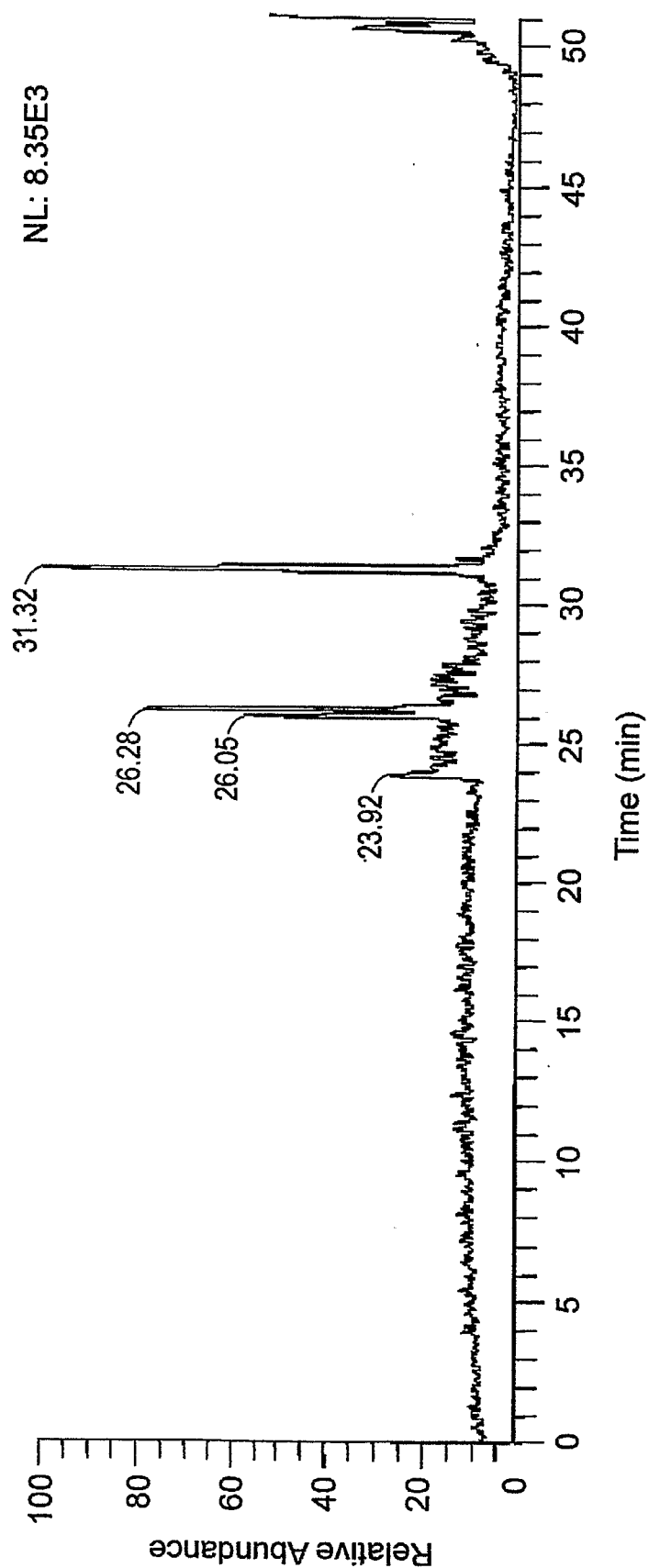


FIG. 5A

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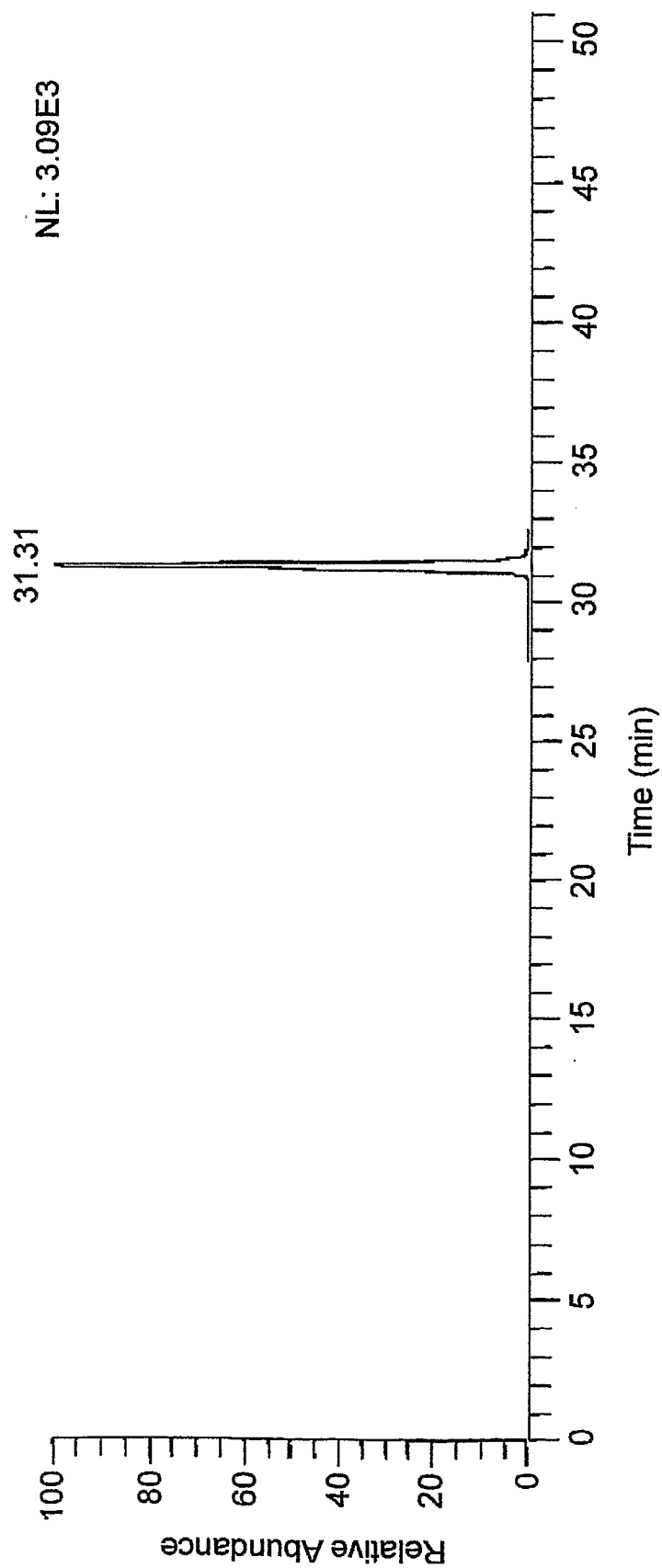


FIG. 5B

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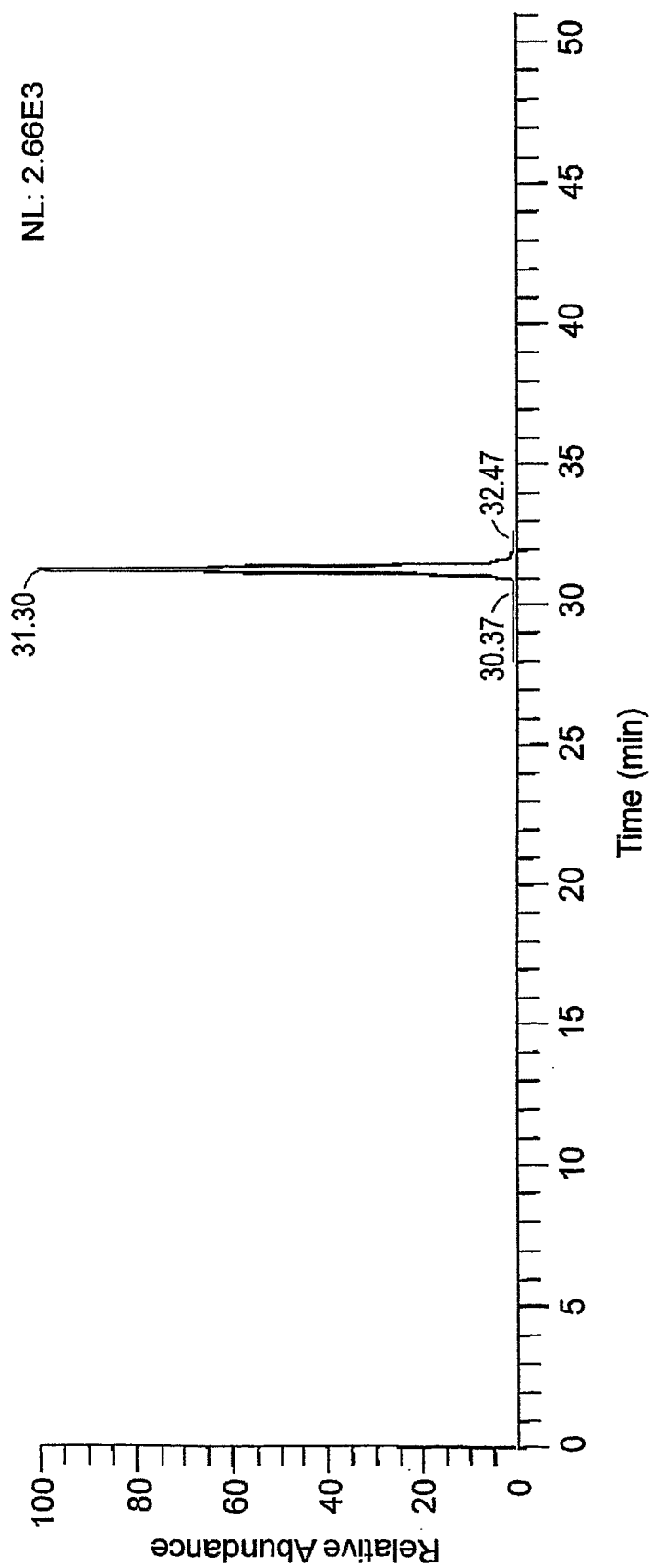


FIG. 5C

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Average MS Response Ratio vs Peptide Amount Ratio, WINA TDPSAR

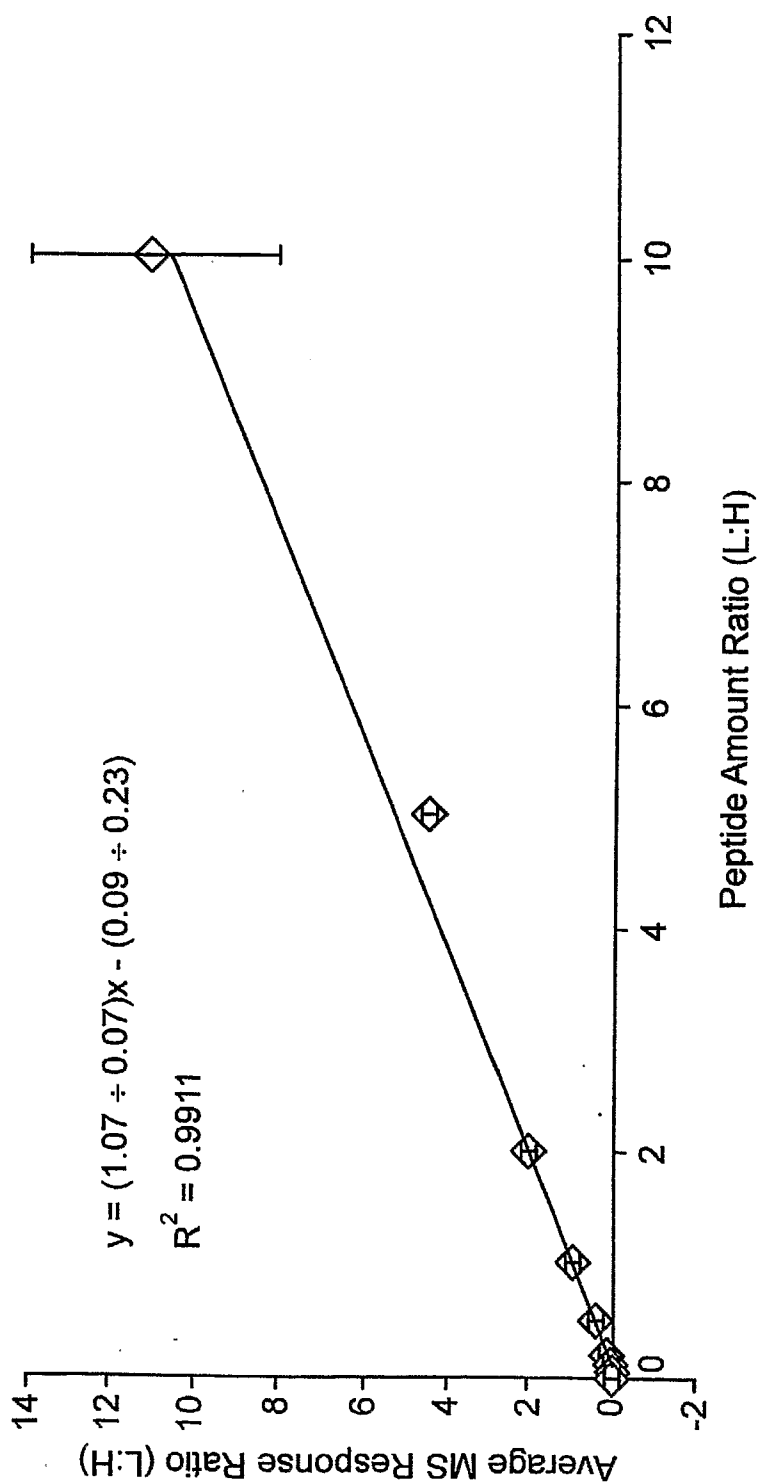


FIG. 6

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Average MS Response Ratio vs Peptide Amount Ratio, ETFEDSNLIPK

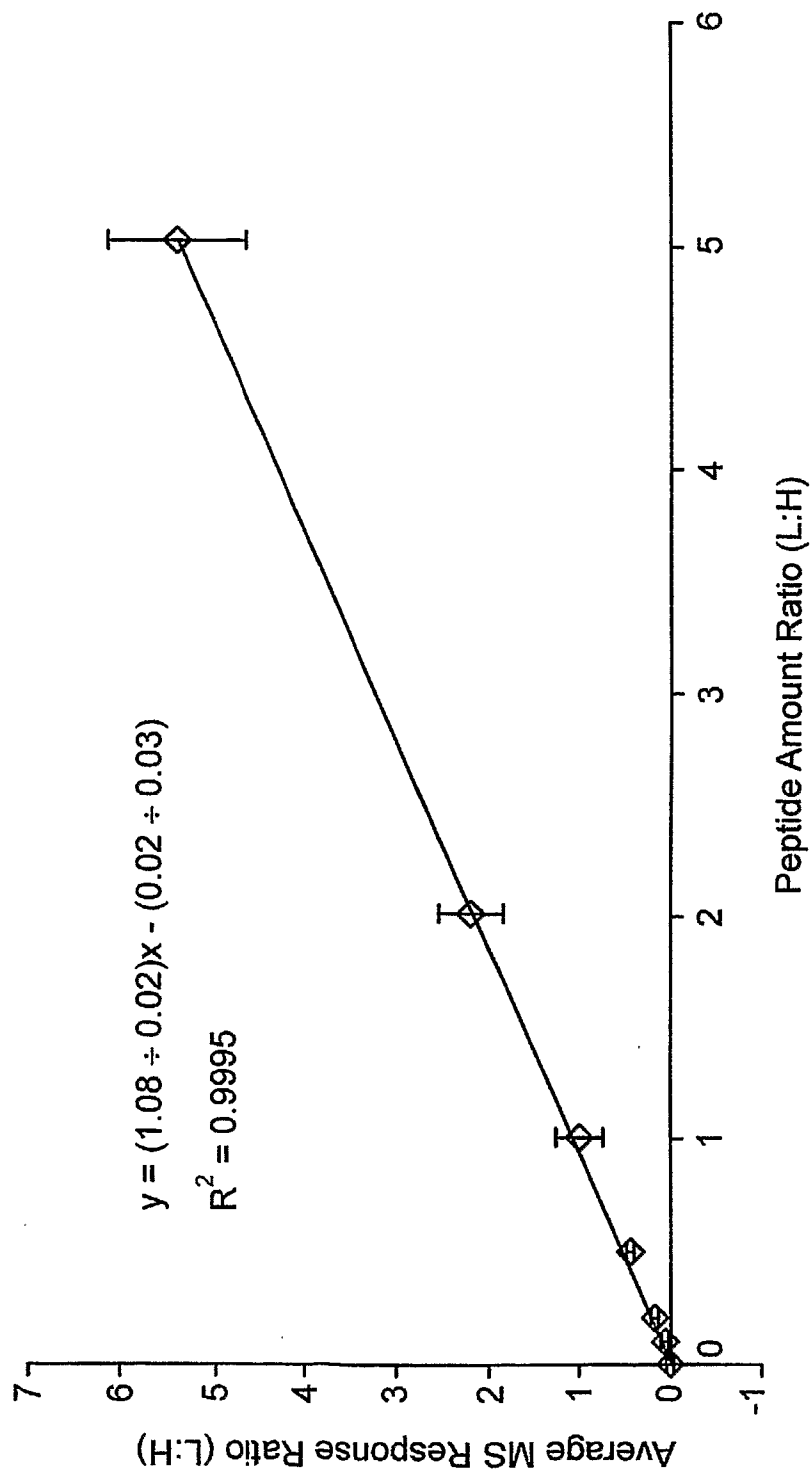


FIG. 7

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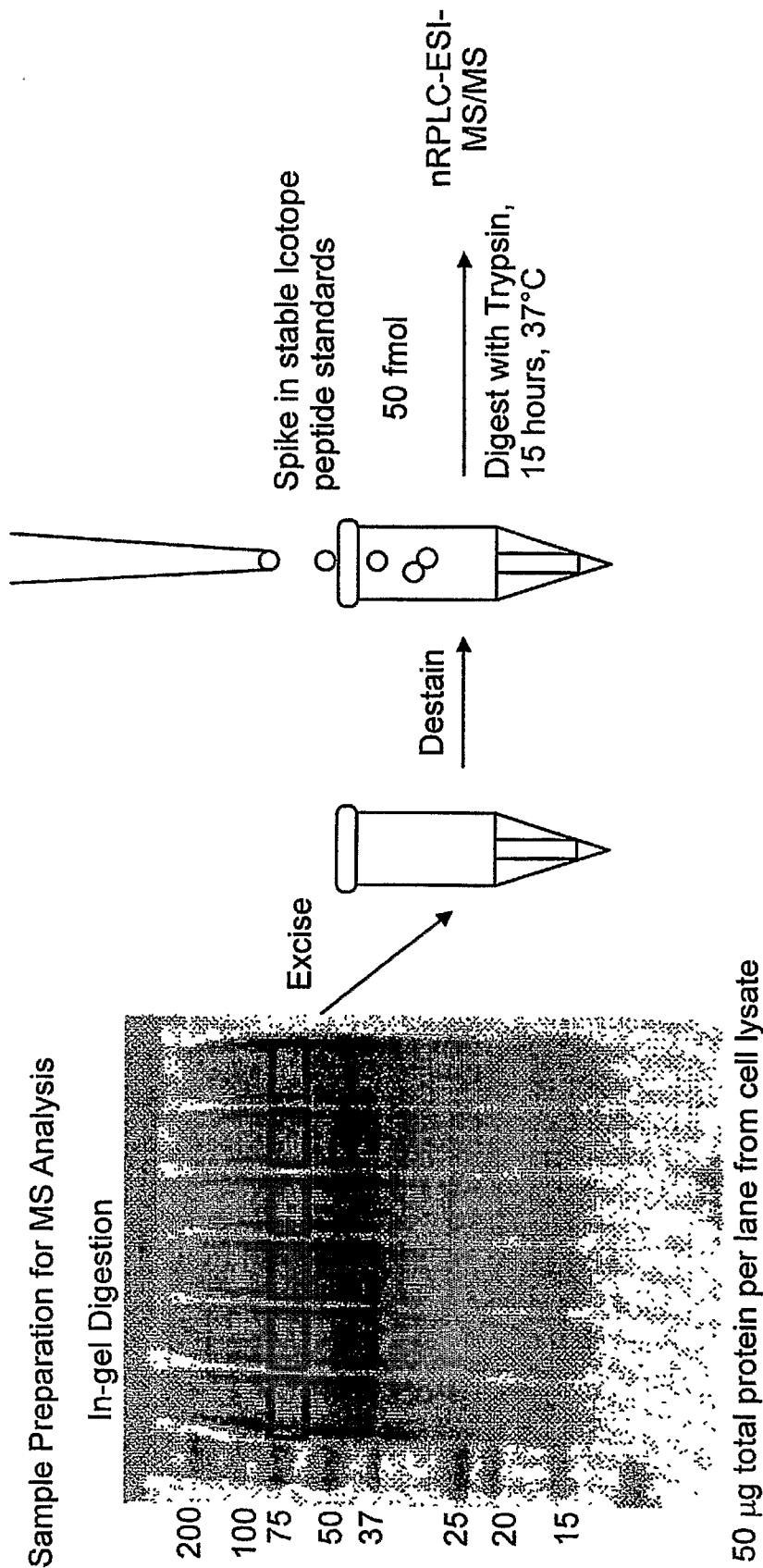


FIG. 8A-1

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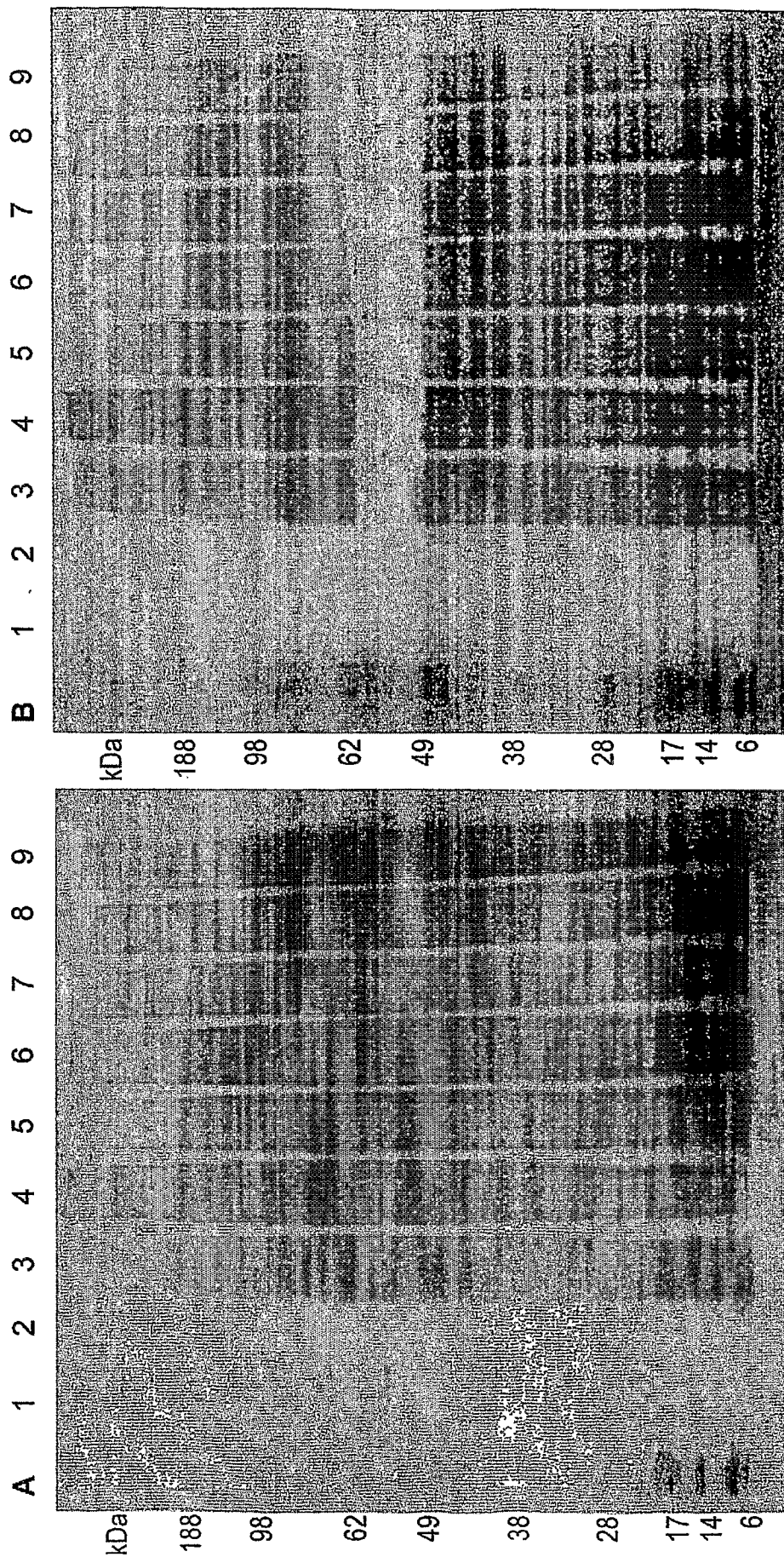


FIG. 8B

FIG. 8A

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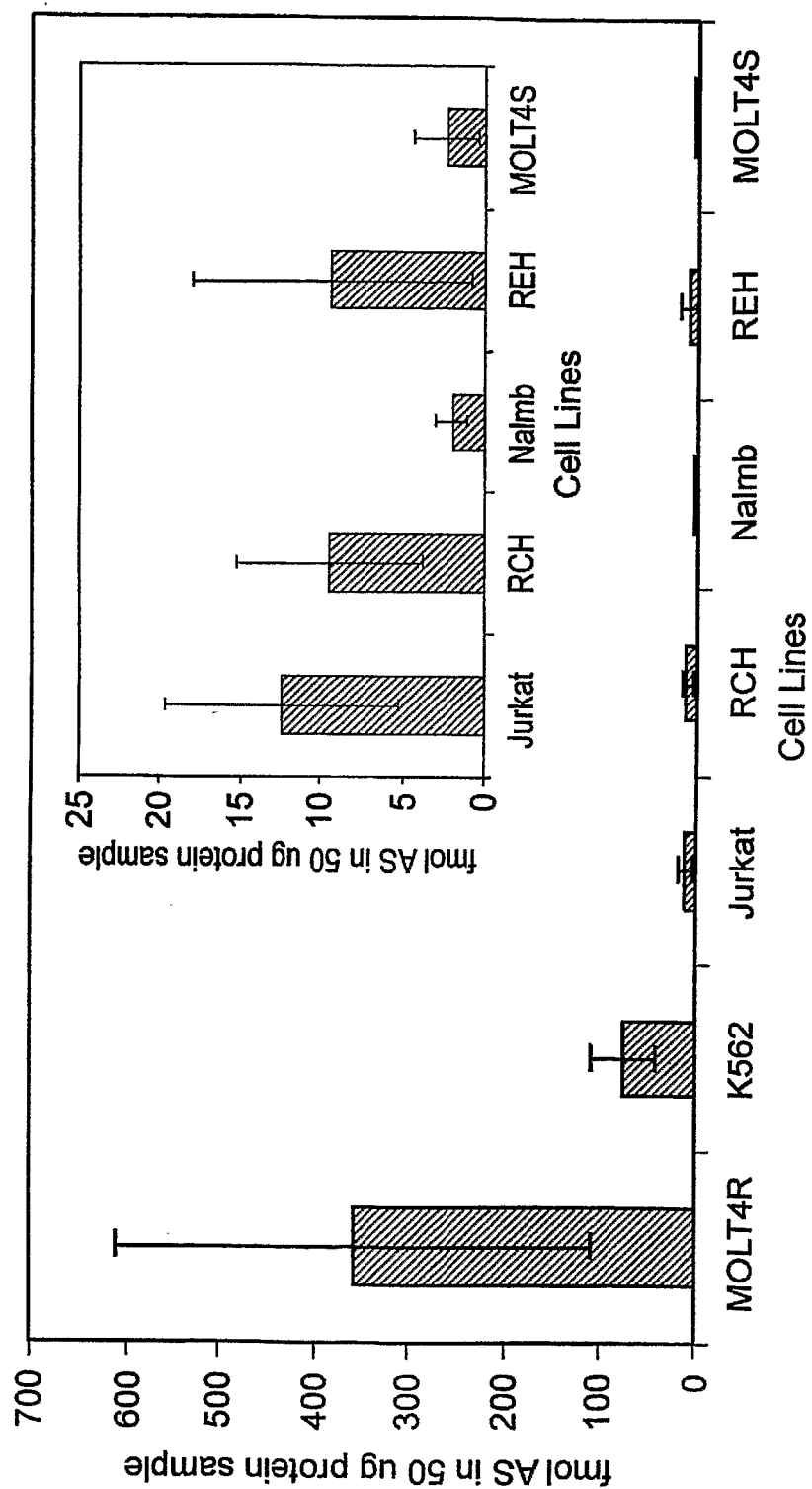


FIG. 9

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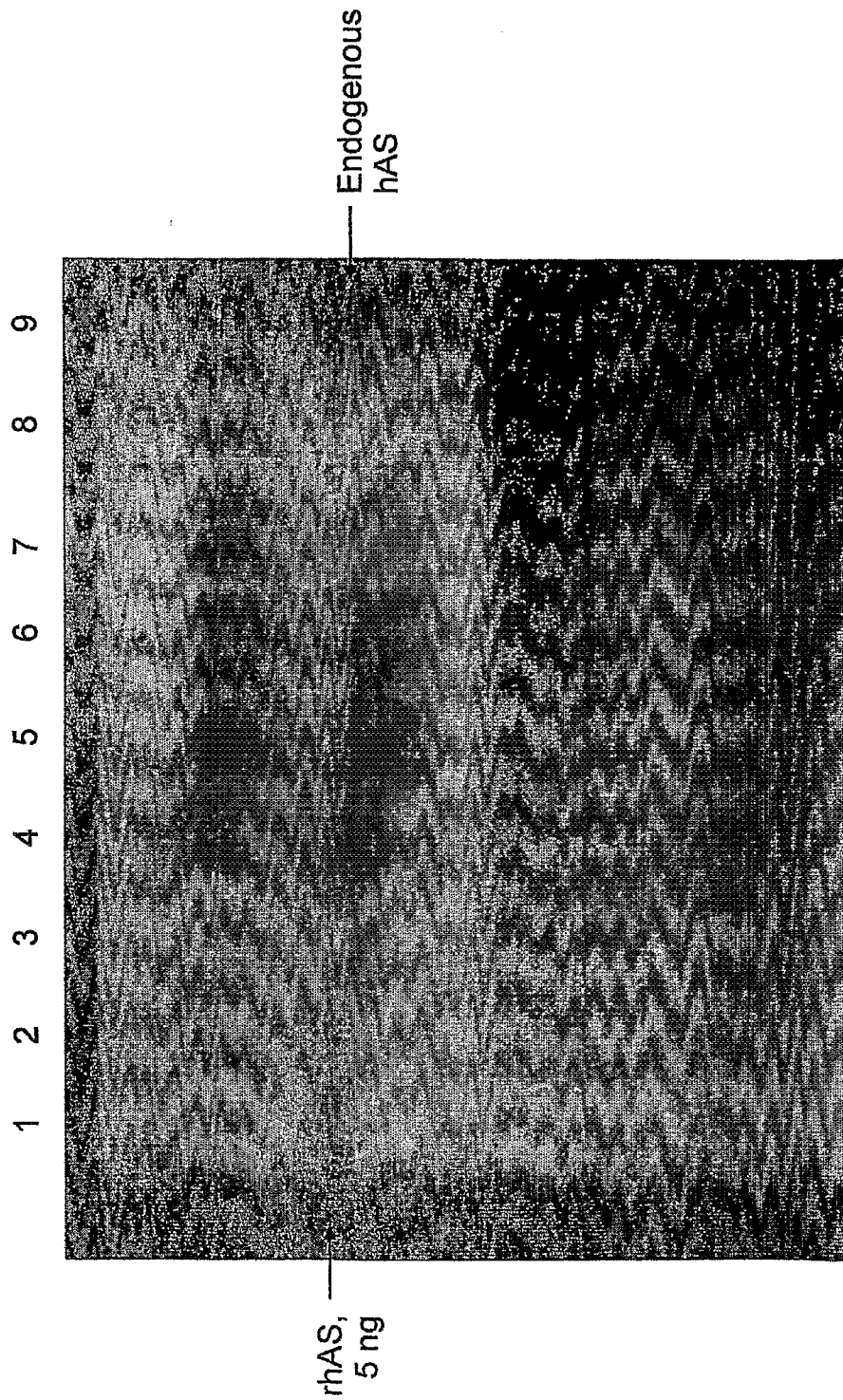


FIG. 10

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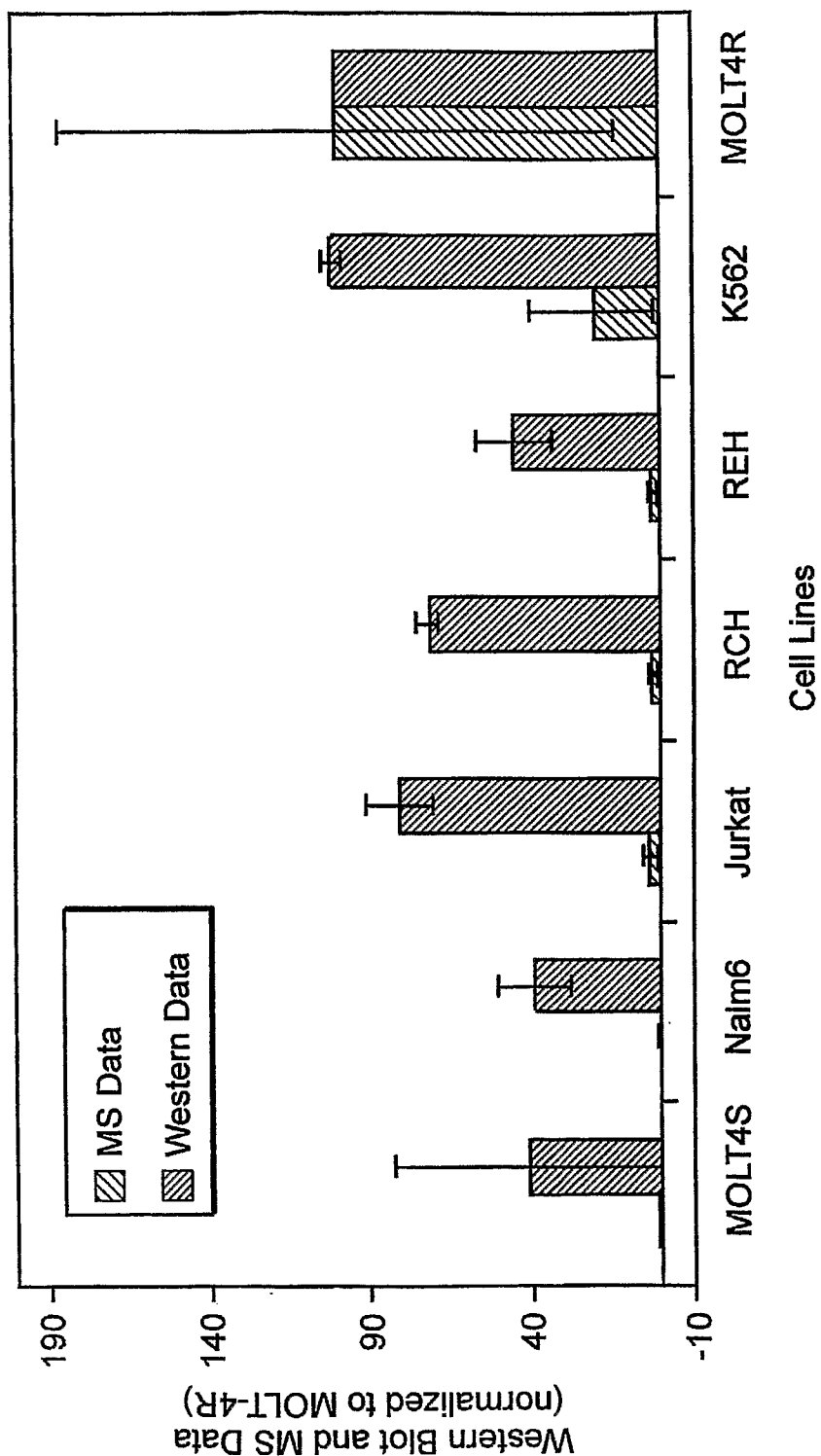


FIG. 11

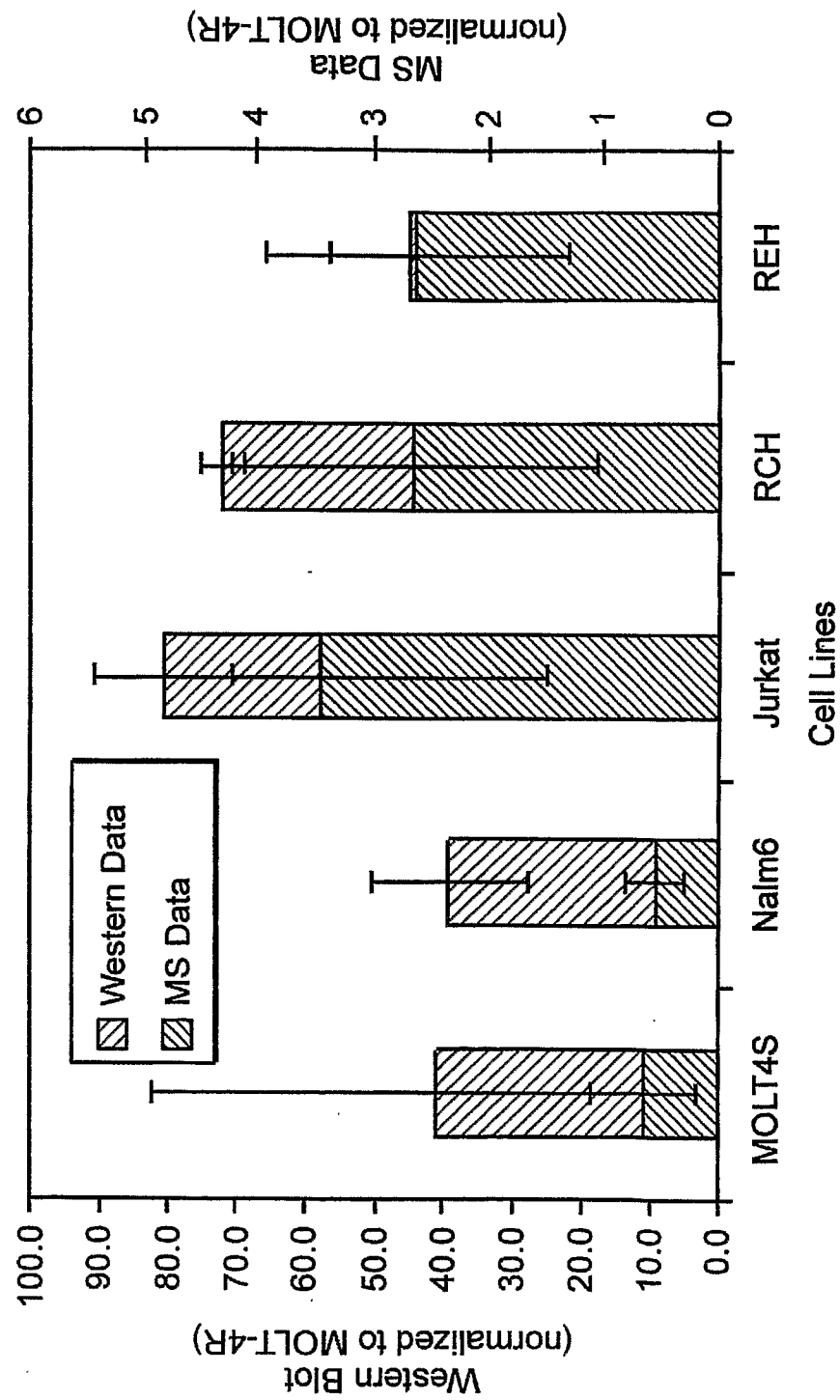


FIG. 12

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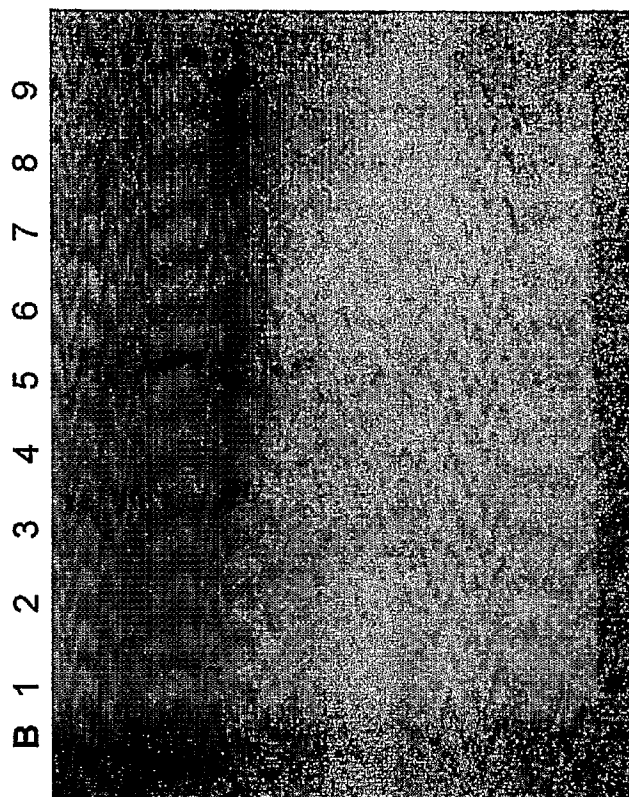


FIG. 13B

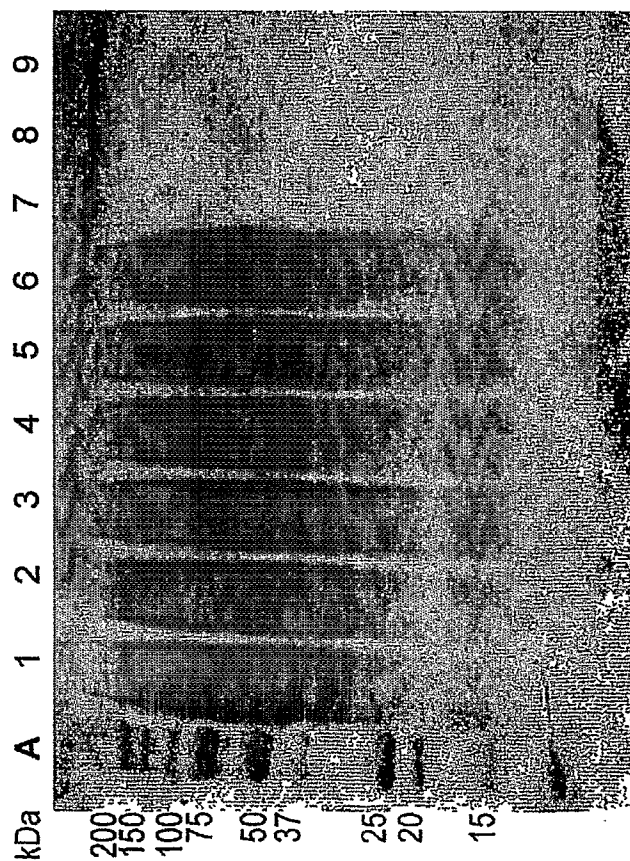


FIG. 13A

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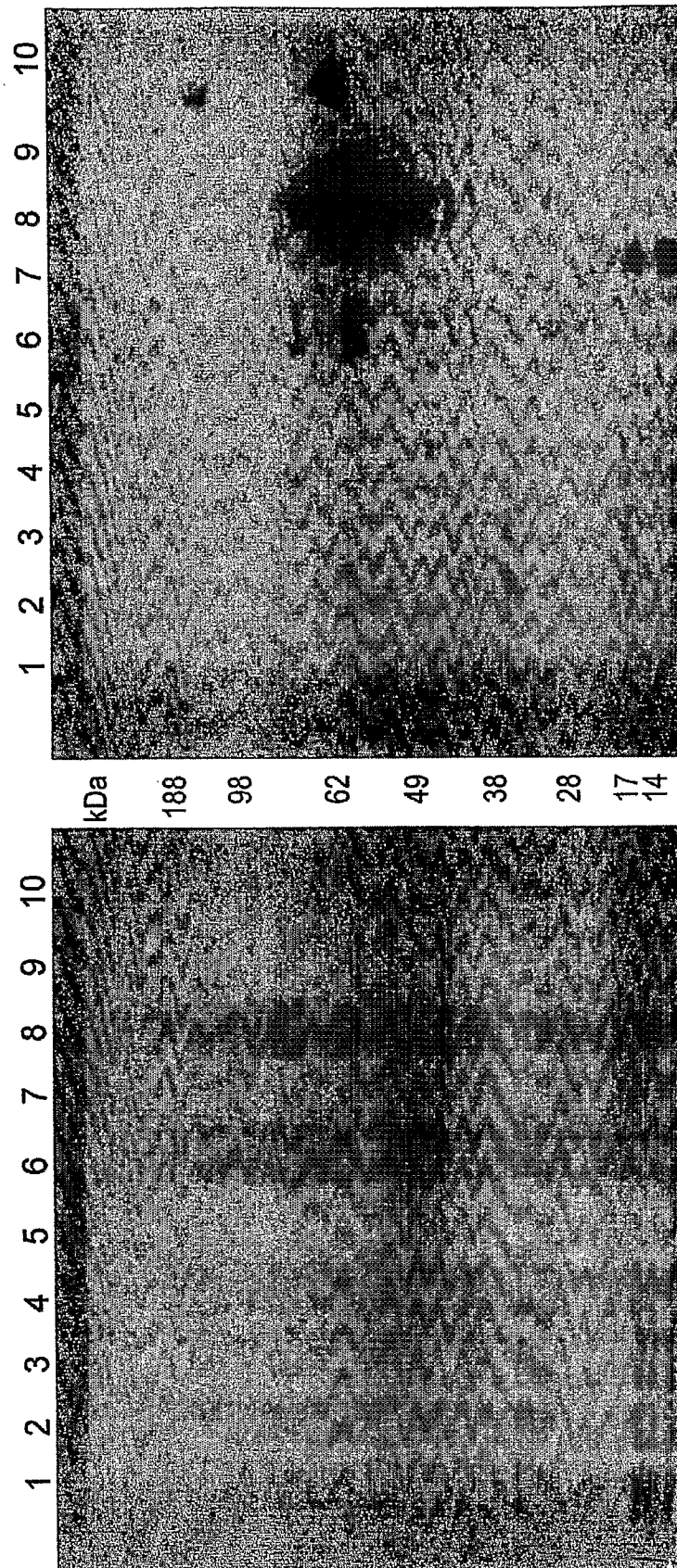


FIG. 14B

FIG. 14A

Box No. VIII (v) DECLARATION: NON-PREJUDICIAL DISCLOSURES OR EXCEPTIONS TO LACK OF NOVELTY

The declaration must conform to the standardized wording provided for in Section 215; see Notes to Boxes Nos. VIII, VIII(i) to (v) (in general) and the specific Notes to Box No. VIII (v). If this Box is not used, this sheet should not be included in the request.

Declaration as to non-prejudicial disclosures or exceptions to lack of novelty (Rules 4.17(v) and 51bis.1(a)(v)):

In relation to this international application, the undersigned declare that the subject matter relating to this international application was disclosed as follows:

(i) Kind of disclosure:

(b) Poster Presentation and on-line publication of abstract, www.asms.org.

(ii) Date of Disclosure: May 2006

(iii) Title of Disclosure: Method Development for Quantitation of Asparagine Synthetase in Leukemia Cells

(iv) Place of Disclosure: American Society for Mass Spectrometry (ASMS) Conference, Seattle, WA and corresponding website, www.asms.org.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2006/028965

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/573 G01N33/68 C12N9/00

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)

G01N C12N

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

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

EPO-Internal, BIOSIS, Sequence Search, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CIUSTEA M ET AL: "Efficient expression, purification, and characterization of C-terminally tagged, recombinant human asparagine synthetase" ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, NEW YORK, US, US, vol. 440, no. 1, 1 August 2005 (2005-08-01), pages 18-27, XP004992194 ISSN: 0003-9861 the whole document ----- -/-	52-56, 60,61, 67, 69-71, 73,76

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

☐ See patent family annex.

* 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 document 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

22 June 2007

Date of mailing of the international search report

10/07/2007

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INTERNATIONAL SEARCH REPORT

International application No

PCT/US2006/028965

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAN HEEKE G ET AL: "EXPRESSION OF HUMAN ASPARAGINE SYNTHETASE IN ESCHERICHIA-COLI" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 264, no. 10, 1989, pages 5503-5509, XP002438811 ISSN: 0021-9258 abstract, p. 5505, col. 1, 2nd paragraph, Fig. 3 and 4	67,69, 71,73
T	ABBATIello SUSAN E ET AL: "QUANTITATIVE DETECTION OF ASPARAGINE SYNTHETASE FROM LEUKEMIA CELL LINES AND PATIENT BLAST CELLS USING SELECTED REACTION MONITORING MASS SPECTROMETRY" PROCEEDINGS OF THE ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, NEW YORK, NY, US, vol. 48, 18 April 2007 (2007-04-18), page 641, XP001536512 ISSN: 0197-016X abstract	1-76
A	STAMS WENDY A G ET AL: "Asparagine synthetase expression is linked with L-asparaginase resistance in TEL-AML1-negative but not TEL-AML1-positive pediatric acute lymphoblastic leukemia" BLOOD, vol. 105, no. 11, June 2005 (2005-06), pages 4223-4225, XP002438812 ISSN: 0006-4971 abstract	1-76
P,A	LORENZI PHILIP L ET AL: "Asparagine synthetase as a causal, predictive biomarker for L-asparaginase activity in ovarian cancer cells." MOLECULAR CANCER THERAPEUTICS NOV 2006, vol. 5, no. 11, November 2006 (2006-11), pages 2613-2623, XP002438813 ISSN: 1535-7163 abstract	1-76
A	ANDERSON LEIGH ET AL: "Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins" MOLECULAR & CELLULAR PROTEOMICS, vol. 5, no. 4, April 2006 (2006-04), pages 573-588, XP002438814 ISSN: 1535-9476 p. 573,p. 574, col. 1	1-76