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(54) Title: ISOLATION OF MEMBRANE VESICLES FROM BIOLOGICAL FLUIDS AND METHODS OF USING SAME

(57) Abstract: Methods of isolating membrane vesicles from a biological fluid sample are provided. In some embodiments, the methods comprise providing a biological fluid sample comprising membrane vesicles; filtering the biological fluid sample through a filtration module comprising a filter having an average pore diameter of between about 0.01 um and about 0.15 um; and collecting from the filtration module a retentate comprising the membrane vesicles, thereby isolating the membrane vesicles from the biological fluid sample.



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DESCRIPTION

ISOLATION OF MEMBRANE VESICLES FROM BIOLOGICAL FLUIDS AND METHODS OF USING SAME

RELATED APPLICATIONS

The presently disclosed subject matter claims the benefit of U.S. Provisional Patent Application Serial No. 60/795,063, filed April 26, 2006; the disclosure of which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

The presently disclosed subject matter relates to isolating membrane vesicles from biological fluids. In particular, the presently disclosed subject matter relates to isolating membrane vesicles and membrane vesicle-associated polypeptides from biological fluids for identification and/or quantitation of the polypeptides.

BACKGROUND

Analyses of the human proteome hold the promise of the ability for early identification of disease and for insights into the pathological processes involved (Berhane et al., 2005; Zhao et al., 2005; Veenstra et al., 2005). A difficult problem associated with this goal is the need to sample the proteins involved easily and to do so over a wide dynamic range (Anderson & Hunter, 2005; Vidal et al., 2005). For screening large populations, urine and plasma provide a convenient access to fluids equilibrated with total body metabolism. However, each of these sources presents unique problem sets in sample preparation.

Plasma contains high concentrations of both albumin and IgGs so that the detection and identification of non-(Alb+IgG) proteins is made difficult if the highly predominant species are not selectively removed. Affinity methods for their reduction in intact or diluted plasma have been reported (Echan et al., 2005; Greenough et al., 2004), but this removal may be accompanied by loss of peptides or other metabolites complexed to the highly abundant species (Zhou et al., 2004). Despite this problem, plasma offers a rich and relatively constant

concentration of proteins in a milieu suitable for various analytical methods used in proteomic analysis.

Urine also provides a simple access to body fluids, but the analytical difficulties are quite different than plasma (Haubitz et al., 2005). Urine has temporal variations in urine protein, peptide, and metabolomic content that must be overcome by sampling pooled collections (Lenz et al., 2005). Excluding breach of the glomerular filtration unit, protein concentrations are generally much lower than in plasma and can be accompanied by highly variable electrolyte levels so that simple concentration without electrolyte separation is not indicated. More recently, the presence of hydrophobic, membrane proteins (Thongboonkerd et al., 2002), whose source has been traced to urinary exosomes (Pisitkun et al., 2004), has opened the possibility of preliminary separation of these particles from high molecular weight, but soluble, proteins and both of these from low molecular weight proteins and peptides.

Thus, there is an unmet need in the art for improved methods of isolating polypeptides from biological fluids for proteomic analysis. Further, there is an unmet need for improved methods of isolating exosomes from biological fluids, wherein the exosomes comprise a useful subset of biomarker polypeptides having applications in proteomic analysis generally and disease diagnosis and progression in particular.

SUMMARY

This Summary lists several embodiments of the presently disclosed subject matter, and in many cases lists variations and permutations of these embodiments. This Summary is merely exemplary of the numerous and varied embodiments. Mention of one or more representative features of a given embodiment is likewise exemplary. Such an embodiment can typically exist with or without the feature(s) mentioned; likewise, those features can be applied to other embodiments of the presently disclosed subject matter, whether listed in this Summary or not. To avoid excessive repetition, this Summary does not list or suggest all possible combinations of such features.

In one embodiment of the presently disclosed matter, a method of isolating membrane vesicles from a biological fluid sample is provided. In some embodiments, the method comprises providing a biological fluid sample

comprising membrane vesicles; filtering the biological fluid sample through a filtration module comprising a filter having an average pore diameter of between about 0.01 μm and about 0.15 μm ; and collecting from the filtration module a retentate comprising the membrane vesicles, thereby isolating the membrane vesicles from the biological fluid sample.

In another embodiment of the presently disclosed subject matter, a method of identifying biomarker polypeptides and/or quantitating biomarker polypeptides in a biological fluid sample is provided. In some embodiments, the method comprises providing a biological fluid sample comprising membrane vesicles, wherein the membrane vesicles comprise biomarker polypeptides; filtering the biological fluid sample through a filtration module comprising a filter having an average pore diameter of between about 0.01 μm and about 0.15 μm ; collecting from the filtration module a retentate comprising the membrane vesicles; isolating the biomarker polypeptides from the membrane vesicles; and identifying and/or quantitating the isolated biomarker polypeptides.

In still another embodiment of the presently disclosed subject matter, a method of isolating membrane vesicle biomarker polypeptides from a biological fluid sample is provided. In some embodiments, the method comprises providing a biological fluid sample comprising membrane vesicles, wherein the membrane vesicles comprise biomarker polypeptides; filtering the biological fluid sample through a filtration module comprising a filter having an average pore diameter of between about 0.01 μm and about 0.15 μm ; collecting from the filtration module a retentate comprising the membrane vesicles; and isolating the biomarker polypeptides from the membrane vesicles. In some embodiments, the biomarker peptides are isolated by electrophoretic separation, immunoisolation, chromatography, or combinations thereof.

In still another embodiment of the presently disclosed subject matter, a method of diagnosing a disorder or measuring a disorder state in a subject is provided. In some embodiments, the method comprises providing a biological fluid sample comprising membrane vesicles, wherein the membrane vesicles comprise biomarker polypeptides; filtering the biological fluid sample through a filtration module comprising a filter having an average pore diameter of between about 0.01 μm and about 0.15 μm ; collecting from the filtration module a retentate comprising the membrane vesicles; isolating the biomarker

polypeptides from the membrane vesicles; and identifying, quantitating, or both the isolated biomarker polypeptides, wherein the identified and/or quantitated biomarker polypeptides indicates the presence of a disorder or is a measure of a disorder state in the subject. In some embodiments of the diagnostic method, the disorder is selected from the group consisting of diabetes, water-balance disorders, acute kidney injury, glomerulonephritis, drug-induced acute renal failure and allergy, acute and chronic kidney transplant rejection, inherited renal diseases, myocardial ischemia, cardiovascular risk, prostatic hypertrophy and prostatic cancer, systemic lupus erythematosus, and rheumatoid arthritis.

In some embodiments of the methods disclosed herein, the biological fluid sample provided is a clarified biological fluid sample, such as for example by low-speed centrifugation (e.g., 3,000 xg or less) and collection of a supernatant comprising the clarified biological fluid sample. In some embodiments, the biological fluid sample is selected from the group consisting of blood, blood plasma, and urine. In some embodiments, the biological fluid sample is urine, which is treated with a protease inhibitor.

In some embodiments of the methods disclosed herein, the membrane vesicles are exosomes. In some particular embodiments, the exosomes are urinary exosomes. In some embodiments, the retentate comprising the membrane vesicles is collected by washing the retentate from the filtration module. Further, in some embodiments, the collected retentate is resuspended in a buffer solution.

In some embodiments of the methods disclosed herein, the filtration module is a fiber-based filtration cartridge, which can in some embodiments include a filter comprising polypropylene hollow fibers. In some embodiments, the filtration module is a membrane filtration module, which can in some embodiments include a filter comprising a filtration disc composed of hydrophilic polyvinylidene difluoride. In some embodiments, the filter has an average pore diameter of about 0.1 μm . In some embodiments, the filter comprises a material selected from the group consisting of polypropylene, polyvinylidene difluoride, polyethylene, polyfluoroethylene, cellulose, secondary cellulose acetate, polysulfone and polyethersulfone, polyvinylalcohol and ethylenevinyl alcohol.

In some embodiments of the methods, the biomarker peptides are identified, quantitated, or both by immunoassay, mass spectrometry, or both.

In some embodiments, the mass spectrometry is matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI MS). In some embodiments, the biomarker polypeptides are separated by liquid chromatography (LC) methods. In some embodiments the biomarker polypeptides are analyzed in line with LC methods using electrospray ionization (ESI) MS methods. In some embodiments the biomarker polypeptides are analyzed directly or off line by LC methods using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI MS). Further, in some embodiments, the immunoassay is selected from the group consisting of Western blot, enzyme-linked immunoassay (ELISA), radioimmunoassay (RIA), and competitive binding assay.

Accordingly, it is an object of the presently disclosed subject matter to isolate membrane vesicles from biological fluids. This object is achieved in whole or in part by the presently disclosed subject matter.

An object of the presently disclosed subject matter having been stated hereinabove, and which is achieved in whole or in part by the presently disclosed subject matter, other objects and advantages will become evident to those of ordinary skill in the art after a study of the following description of the presently disclosed subject matter, [DRAWINGS?], and non-limiting examples.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic drawing showing sample handling and workflow.

Figure 2 is a transmission electron micrograph (TEM) of filtered urinary exosomes from fresh urine. Urine is collected by the clean catch method and 1X protease inhibitors added. Urine was spun at 3,000 xg to remove debris/casts/bacteria and subsequently filtered using a membrane fiber-based filtration cartridge as disclosed herein. Filtered vesicles are washed with 10mL PBS and then collected (back-flushed) from the cartridge with 3.5mL PBS. The PBS back-flush is spun at 200,000 xg to pellet low density vesicular bodies. The low-density vesicular pellet is cross-linked in a 4% glutaraldehyde/PBS solution and analyzed by TEM. The fiduciary mark is in units of nm. Imaged spheroids have diameters consistent with known exosomal diameters (30-80nm).

Figure 3 is a transmission electron micrograph (TEM) of filtered urinary exosomes from frozen urine. Urine is collected by the clean catch method, 1X protease inhibitors added, and frozen for >8 months at -80° C. The urine is

thawed at room temperature, spun at 3,000xg to remove cryoglobulin/debris/casts/bacteria and subsequently filtered using a disc filter membrane as disclosed herein. Filtered vesicles are washed with 10 mL PBS and then collected (by rinsing) from the filtration disc with 3.5mL PBS. The PBS rinse is spun at 200,000 xg to pellet low density vesicular bodies. The low-density vesicular pellet is cross-linked in a 4% glutaraldehyde/PBS solution and analyzed by TEM. Fiduciary mark is in units of nm. Imaged spheroids have diameters consistent with known exosomal diameters (30-80nm).

Figure 4 is an immunoblot image showing the peripheral membrane protein ezrin co-purifies with urinary exosomes. Exosomal pellets were re-solubilized with 1X LDS Laemmli buffer and separated on a 4-12% gradient gel. Proteins were transferred to nitrocellulose (0.45 μ m) by tank transfer (25V, 1h) and blotted in 5% non-fat milk TTBS. Primary rabbit anti ezrin (human) Ig was incubated with the blot at 4°C overnight. Following three 5min TTBS rinses, secondary goat anti-rabbit Ig-HRP conjugate was incubated with the blot at room temperature for 2h. Following three 5min TTBS rinses, the blot was reacted with SUPERSIGNAL WEST PICO[®] chemiluminescent substrate (Pierce Biotechnology, Inc., Rockford, Illinois, U.S.A.) for 1min and exposed to film. Ezrin MW = 80 kDa (Lane A, positive control-HEK plasma membrane; Lane B, morning void; Lane C, mid-day void 1; Lane D, mid-day void 2).

Figure 5 is a series of immunoblot images showing the integral membrane protein Na⁺/K⁺ ATPase, α -subunit copurifies with urinary exosomes. An exosomal pellet isolated by fiber filtration from fresh normal male urine was re-solubilized with 1X LDS Laemmli buffer and separated on a 4-12% gradient gel. Proteins were transferred to nitrocellulose (0.45 μ m) by tank transfer (25V, 1h) and blotted in 5% non-fat milk TTBS. After blotting, the gel was stained with Coomassie brilliant blue overnight and imaged using a LI-COR Odyssey Infrared Imaging Station. (Lane 1, residual exosomal pellet protein; Lane 2 Residual Filter fiber rinse protein; Lane 3, residual molecular weight standards). Primary mouse anti Na⁺/K⁺ ATPase α -subunit (human) Ig was incubated with the blot at 4°C overnight. Following three 5min TTBS rinses, secondary rabbit anti-mouse Ig-HRP conjugate was incubated with the blot at room temperature for 2h. Following three 5min TTBS rinses, the blot was reacted with SUPERSIGNAL WEST PICO[®] chemiluminescent substrate (Pierce Biotechnology, Inc.) for 1min

and exposed to film. Na⁺/K⁺ ATPase α -subunit MW = 90kDa (Transferred Gel: Lane 1, Exosomal pellet; Lane 2, Filter fiber rinse; Lane 3, Molecular Weight Standards; Western Blot: Lane A Exosomal pellet; Lane B, Filter fiber rinse; Lane C, Molecular Weight Standards).

Figure 6 is an immunoblot image showing the integral membrane protein neprilysin (CD10) co-purifies with urinary exosomes. An exosomal pellet isolated by disc filtration from fresh normal male urine was re-solubilized with 1X LDS Laemmli buffer and separated on a 4-12% gradient gel. Proteins were transferred to nitrocellulose (0.45 μ m) by tank transfer (25V, 1h) and blotted in 5% non-fat milk TTBS. Primary mouse anti CN10 (human) Ig was incubated with the blot at 4°C overnight. Following three 5min TTBS rinses, secondary rabbit anti-mouse Ig-HRP conjugate was incubated with the blot at room temperature for 2h. Following three 5min TTBS rinses, the blot was reacted with SUPERSIGNAL WEST PICO[®] chemiluminescent substrate (Pierce Biotechnology, Inc.) for 1min and exposed to film. Na⁺/K⁺ ATPase α -subunit MW = 90kDa (Lane A, Exosomal proteins following sucrose density spin; Lane B, Exosomal proteins following DTT reduction with subsequent sucrose density spin; Lane C, Exosomal pellet; Lane D, Filtered-urine urinary proteins; Lane E, Unfiltered-urine urinary proteins).

Figure 7 is an immunoblot image showing neprilysin (CD10) co-purifies with urinary exosomes isolated using either an exosomal filtration cartridge or a filtration disc and from either fresh or frozen urine. An exosomal pellet isolated using cartridge filtration or using disc filtration from fresh normal male urine or previously frozen normal male urine was re-solubilized with 1X LDS Laemmli buffer and separated on a 4-12% gradient gel. Proteins were transferred to nitrocellulose (0.45 μ m) by tank transfer (25V, 1h) and blotted in 5% non-fat milk TTBS. Primary mouse anti CN10 (human) Ig was incubated with the blot at 4°C overnight. Following three 5min TTBS rinses, secondary rabbit anti-mouse Ig-HRP conjugate was incubated with the blot at room temperature for 2h. Following three 5min TTBS rinses, the blot was reacted with SUPERSIGNAL WEST PICO[®] chemiluminescent substrate (Pierce Biotechnology, Inc.) for 1min and exposed to film. Na⁺/K⁺ ATPase α -subunit MW = 90kDa (Lane A, fresh urine & disc filtration; Lane B, frozen urine & disc filtration; Lane C, fresh urine & fiber filtration; Lane D, frozen urine & fiber filtration).

Figure 8 is a 1D-SDS PAGE gel image showing expected urinary

exosomal proteins are identified by MALDI-MS following 1D SDS-PAGE separation. Isolated exosome filtrate is re-solubilized with 1X LDS Laemmli buffer and separated on a 4-12% gradient gel. The sample lane is divided into 2.5mm cubes, conditioned, reduced, alkylated, and digested with trypsin. Trypsin digests are analyzed by MALDI-TOF MS and MALDI-TOF MS-MS. Representative proteins identified are annotated on the gel image; the annotations are positioned to illustrate the gel slice origin of the identified protein.

DETAILED DESCRIPTION

The details of one or more embodiments of the presently disclosed subject matter are set forth in the accompanying description below. Other features, objects, and advantages of the presently disclosed subject matter will be apparent from the detailed description, figures, and claims. All publications, patent applications, patents, and other references disclosed herein are incorporated by reference in their entirety. Some of the polypeptides disclosed herein are cross-referenced to public database accession numbers. The complete sequences cross-referenced in the database are expressly incorporated by reference as are equivalent and related sequences present in other public databases. Also expressly incorporated herein by reference are all annotations present in the database associated with the sequences disclosed herein. In case of conflict, the present specification, including definitions, will control. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the presently disclosed subject matter belongs. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the presently disclosed subject matter, representative methods, devices, and materials are now described.

Following long-standing patent law convention, the terms "a", "an", and "the" refer to "one or more" when used in this application, including the claims. Thus, for example, reference to "a cell" includes a plurality of such cells, and so forth.

Unless otherwise indicated, all numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about".

Accordingly, unless indicated to the contrary, the numerical parameters set forth in this specification and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by the presently disclosed subject matter.

As used herein, the term "about," when referring to a value or to an amount of mass, weight, time, volume, concentration or percentage is meant to encompass variations of in some embodiments $\pm 20\%$, in some embodiments $\pm 10\%$, in some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$, and in some embodiments $\pm 0.1\%$ from the specified amount, as such variations are appropriate to perform the disclosed method.

Biological fluids are valuable as indicators of a subject's well-being and can be analyzed for data indicative of the presence or absence and progression of disease. For example, urine is one biological fluid that has clinical diagnostic value (Snyder & Pendergraph, 2005). In addition to low molecular weight species like glucose, bilirubin, ketones, sodium, potassium, and nitrites, urine contains specific proteins and peptides that have significant diagnostic value. One problem with the development of diagnostic protein or peptide markers (biomarkers) is the relative (low) concentration of the species that is sensitive and specific for a given disease; especially for the detection of a disease in the pre-pathologic state.

Considerable effort has been applied toward pre-fractionation of biological fluid samples with the goal of increasing the relative concentration of all peptide species in a given sample fraction (Anderson & Hunter, 2005; Vidal et al., 2005).

Certain tissues through normal biological processes produce membrane vesicles containing a variety of polypeptides. In certain disease states, particular systems, such as for example the immune system can increase production of membrane vesicles.

The terms "polypeptide", "protein", and "peptide", which are used interchangeably herein, refer to a polymer of the 20 protein amino acids, or amino acid analogs, regardless of its size or function. Although "protein" is often used in reference to relatively large polypeptides, and "peptide" is often used in reference to small polypeptides, usage of these terms in the art overlaps and varies. The term "polypeptide" as used herein refers to peptides, polypeptides, and proteins, unless otherwise noted. The terms "protein", "polypeptide" and

“peptide” are used interchangeably herein when referring to a gene product. Thus, exemplary polypeptides include gene products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing.

The terms “polypeptide fragment” or “fragment”, when used in reference to a reference polypeptide, refers to a polypeptide in which amino acid residues are deleted as compared to the reference polypeptide itself, but where the remaining amino acid sequence is usually identical to the corresponding positions in the reference polypeptide. Such deletions can occur at the amino-terminus or carboxy-terminus of the reference polypeptide, or alternatively both. Fragments typically are at least 5, 6, 8 or 10 amino acids long, at least 14 amino acids long, at least 20, 30, 40 or 50 amino acids long, at least 75 amino acids long, or at least 100, 150, 200, 300, 500 or more amino acids long.

A fragment can retain one or more of the biological activities or diagnostic characteristics of the reference polypeptide. In some embodiments, a fragment can comprise a domain or feature, and optionally additional amino acids on one or both sides of the domain or feature, which additional amino acids can number from 5, 10, 15, 20, 30, 40, 50, or up to 100 or more residues. Further, fragments can include a sub-fragment of a specific region, which sub-fragment retains a function of the region from which it is derived.

The term “membrane vesicle” as used herein refers to essentially spherical vesicles, generally less than about 130 nm in diameter, comprising of a lipid bilayer containing a cytosolic fraction and secreted from cells. Particular membrane vesicles are more specifically produced by cells, from intracellular compartments through fusion with the plasma membrane of a cell, resulting in their release in biological fluids or in the supernatant of cells in culture. Such vesicles are generally referred to as exosomes. Exosomes can be between about 30 and about 120 nm, and more specifically between about 50 and 90 nm in diameter and, advantageously, carry membrane proteins. In addition, depending on their origin, exosomes comprise membrane proteins such as for example MHC I, MHC II, CD63, CD81 and/or HSP70 and have no endoplasmic reticulum or Golgi apparatus. Furthermore, exosomes are typically devoid of nucleic acids (e.g., DNA or RNA).

Exosome release has been demonstrated from different cell types in varied physiological contexts. For example, it has been demonstrated that B lymphocytes release exosomes carrying class II major histocompatibility complex molecules, which play a role in antigenic presentation. Similarly, it has been demonstrated that dendritic cells produce exosomes (i.e., "dexosomes" or "Dex"), with specific structural and functional characteristics and playing a role in immune response mediation, particularly in cytotoxic T lymphocyte stimulation. It has also been demonstrated that tumor cells secrete specific exosomes (i.e., "texosomes" or "Tex") in a regulated manner, carrying tumor antigens and capable of presenting these antigens or transmitting them to antigen presenting cells (see e.g., PCT International Patent Application No. WO99/03499, herein incorporated by reference in its entirety). Also, mastocyte cells accumulate molecules in intracellular vesicular compartments, which can be secreted under the effect of signals. The kidneys also produce exosomes (i.e., urinary exosomes) (Pisitkun et al., 2004).

Therefore, as a general rule, cells appear to emit signals and communicate with each other via membrane vesicles that they release, which may carry proteins or any other signal with specific structural and functional characteristics, produced in different physiological situations. The exosome in effect is the end result of a pre-fractionation process by tissues. The vesicles are then delivered to various biological fluids, including for example blood and urine. As such, disease biology might produce a diagnostic species in increased concentration localized in membrane vesicles, including for example exosomes. Therefore membrane vesicles have value as polypeptide biomarker reservoirs and efforts to simplify the purification of membrane vesicles (e.g., exosomes) from biological fluids, including blood and urine, have diagnostic and health assessment value.

Accordingly, the presently disclosed subject matter provides methods of isolating membrane vesicles from biological samples. In some embodiments, the methods comprise providing a biological fluid sample comprising membrane vesicles; filtering the biological fluid sample through a filtration module comprising a filter having an average pore diameter of between about 0.01 μm and about 0.15 μm ; and collecting from the filtration module a retentate comprising the membrane vesicles, thereby isolating the membrane vesicles

from the biological fluid sample. In some embodiments, the biological sample can be treated at some point after sample collection with one or more protease inhibitors to prevent degradation of the proteins in the biological sample prior to isolation (e.g., serine protease inhibitors, chymotrypsin inhibitors, trypsin inhibitors, etc.).

The term "isolated", when applied to a nucleic acid or polypeptide, denotes that the nucleic acid or polypeptide is essentially free of other cellular components with which it is associated in the natural state. It can be in a homogeneous state although it can be in either a dry or aqueous solution. Homogeneity and whether a molecule is isolated can be determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A polypeptide that is the predominant species present in a preparation is substantially isolated. The term "isolated" denotes that a nucleic acid or polypeptide gives rise to essentially one band in an electrophoretic gel. Particularly, it means that the nucleic acid or polypeptide is in some embodiments at least about 50% pure, in some embodiments at least about 85% pure, and in some embodiments at least about 90%, 95%, 96%, 97%, 98% or 99% pure.

As demonstrated in the present Examples, the presently disclosed methods can be used to isolate membrane vesicles that maintain the presence of peripheral and integral membrane proteins, as well as globular membrane proteins. The presence of globular membrane proteins is indicative of the maintenance of the membrane vesicle structure, and little to no loss of vesicle contents.

The term "biological sample" as used herein refers to a sample that comprises a biomolecule and/or is derived from a subject. The biological sample can be utilized for the detection of the presence and/or quantitative level of a polypeptide of interest. Representative biomolecules include, but are not limited to DNA, RNA, mRNA, and polypeptides. As such, a biological sample can comprise a cell, a group of cells, fragments of cells, or cell products, including for example membrane vesicles (e.g., exosomes). Any cell, group of cells, cell fragment, or cell product can be used with the methods of the presently claimed subject matter, although cell-types and organs that would be predicted to show differential gene and/or polypeptide expression in subjects with disorders versus

normal subjects are best suited. In some embodiments, the biological fluid can be blood, blood plasma, cerebrospinal fluid, saliva, tears, alveolar isolates, pleural fluid, pericardial fluid, bile, pancreatic exocrine fluid, ascites, cyst fluid and/or urine

In embodiments of the presently disclosed subject matter where the biological fluid is urine, the urine can be freshly collected or previously frozen urine. Additionally, the urine can be collected as a morning void/spot urine sample and/or as a mid-day void/spot urine sample. As shown in the Examples, membrane vesicles are present in urine collected at various timepoints during a day and can be isolated from both freshly collected and previously frozen urine samples. In some embodiments, the urine can also be clarified to remove, for example, casts, bacteria, and cell debris, prior to isolation of membrane vesicles by filtration. In some embodiments, the urine is clarified by low-speed centrifugation, such as for example at about 3,000 xg, 2,000 xg, 1,000 xg, or less. The supernatant can then be collected, which contains the membrane vesicles, and further processed using the methods disclosed herein to isolate the exosomes.

The major components of diagnostic interest in urine are, in decreasing size order:

- a) Urinary casts and bacteria
- b) Membrane vesicles (e.g., exosomes)
- c) Cryoglobulins
- d) Soluble, high molecular weight proteins
- e) Low molecular weight proteins and peptides
- f) Electrolytes and low molecular weight metabolites.

Each of these component categories can have diagnostic value and can each be separated from the others and either processed immediately for analysis or for storage or later analysis. Thus, the presently disclosed subject matter encompasses the separation of membrane vesicles from biological fluids as well as other components from one another. Each of these components can then be analyzed individually by techniques generally known in the art to provide data useful in diagnosing or characterizing the progression of a disorder.

The structures purified from urine by the presently disclosed methods, as demonstrated in the Examples, have the correct distribution of diameters and

morphology as provided within the scientific literature for urinary exosomes. The membrane vesicles' protein contents are demonstrated in the Examples by mass spectrometric and immunologic methods to be further consistent with literature documentation for exosomal proteins.

In some embodiments, the filtration module utilized to isolate the membrane vesicles from the biological sample is a fiber-based filtration cartridge. In some embodiments, the fibers are hollow polymeric fibers, such as for example polypropylene hollow fibers. In these embodiments, sample can be introduced into the filtration module by pumping the sample fluids into the module with a pump device, such as for example a peristaltic pump. The pump flow rate can vary, but in some embodiments, the pump flow rate is set at about 2 mL/minute.

In some embodiments, the filtration module utilized to isolate the membrane vesicles from the biological sample is a membrane filtration module. For example, in some embodiments, the membrane filtration module comprises a filter disc membrane (e.g., a hydrophilic polyvinylidene difluoride (PVDF) filter disc membrane) housed in a stirred cell apparatus (e.g., comprising a magnetic stirrer). In some embodiments, the sample moves through the filter as a result of a pressure gradient established on either side of the filter membrane.

In some embodiments, the filter within the filtration module that retains the membrane vesicles (i.e., the retentate) from the biological fluid sample (i.e., the filtrate) has an average pore diameter sufficient for exosome retention and permeation of all but the largest proteins. For example, in some embodiments, the filter has an average pore diameter of about 0.01 μm to about 0.15 μm , and in some embodiments from about .05 μm to about 0.12 μm . In some embodiments, the filter has an average pore diameter of about 0.06 μm , 0.07 μm , 0.08 μm , 0.09 μm , 0.1 μm , or 0.11 μm . In some embodiments, the filter utilized comprises a material having low hydrophobic adsorptivity and/or high hydrophilic properties. In particular embodiments, the filter has an average pore size for exosome retention and permeation of most proteins as well as a surface that is hydrophilic, thereby limiting protein adsorption. Similar filters with these properties can also be suitably used with the presently disclosed subject matter. For example, in some embodiments, the filter comprises a material selected from the group consisting of polypropylene, PVDF, polyethylene, polyfluoroethylene,

cellulose, secondary cellulose acetate, polyvinylalcohol, and ethylenevinyl alcohol (EVAL[®], Kuraray Co., Okayama, Japan). Additional materials that can be utilized in filters of certain embodiments include, but are not limited to, polysulfone and polyethersulfone.

The retentate comprising the isolated membrane vesicles is collected from the filtration module. In some embodiments, the retentate is collected by flushing the retentate from the filter. Selection of a filter composition having hydrophilic surface properties, thereby limiting protein adsorption, can facilitate easier collection of the retentate and minimize use of harsh or time-consuming collection techniques. Once collected the membrane vesicles and/or associated polypeptide biomarkers can be further purified and/or concentrated and finally suspended in a suitable buffer solution, such as for example phosphate buffered saline (PBS), depending on how the vesicles and/or polypeptides will be utilized.

Once isolated, the membrane vesicles can be analyzed to identify characteristics of the vesicles, including identification and/or quantitation of exosomal polypeptides. Identification and/or quantitation of polypeptides within the vesicle can provide information related to biomarkers expressed within a subject. The identification of biomarkers expressed in a subject can be utilized to diagnose a disorder in a subject, monitor the progress of treatment of a disorder in a subject, and generally determine the state of health of a subject as a baseline, or as compared to a previously determined biomarker analysis.

As such, the presently disclosed subject matter provides methods of identifying and/or quantitating biomarker polypeptides from a biological fluid sample using the membrane vesicle isolation methods disclosed herein. The isolated membrane vesicles can then be subjected to polypeptide separation and/or analysis procedures generally known in the art to identify and quantitate the biomarker polypeptides associated with the isolated vesicles.

The presently disclosed subject matter further provides methods of diagnosing a disorder or measuring a disorder state in a subject utilizing the membrane vesicle isolation techniques disclosed herein in combination with polypeptide isolation and quantitation techniques. For example, water channel aquaporin 2 (AQP2) is a biomarker for certain water-balance disorders and identification of peptide variants expressed by a subject can provide information related to diagnosis of the disorders. Other non-limiting examples of disorders

that can be diagnosed and/or monitored based on biomarker identification and/or quantitation include, but are not limited to diabetes, myocardial ischemia (troponin); cardiovascular risk (C-reactive protein, homocysteine); prostatic hypertrophy and prostatic cancer (PSA); systemic lupus erythematosus (ANA); and rheumatoid arthritis (Rheumatoid factor), with non-limiting exemplary biomarkers listed in parenthesis.

Further with respect to the diagnostic methods of the presently disclosed subject matter, a preferred subject is a vertebrate subject. A preferred vertebrate is warm-blooded; a preferred warm-blooded vertebrate is a mammal. A preferred mammal is most preferably a human. As used herein, the term "subject" includes both human and animal subjects. Thus, veterinary therapeutic uses are provided in accordance with the presently disclosed subject matter.

As such, the presently disclosed subject matter provides for the diagnosis of mammals such as humans, as well as those mammals of importance due to being endangered, such as Siberian tigers; of economic importance, such as animals raised on farms for consumption by humans; and/or animals of social importance to humans, such as animals kept as pets or in zoos. Examples of such animals include but are not limited to: carnivores such as cats and dogs; swine, including pigs, hogs, and wild boars; ruminants and/or ungulates such as cattle, oxen, sheep, giraffes, deer, goats, bison, and camels; and horses. Also provided is the treatment of birds, including the treatment of those kinds of birds that are endangered and/or kept in zoos, as well as fowl, and more particularly domesticated fowl, *i.e.*, poultry, such as turkeys, chickens, ducks, geese, guinea fowl, and the like, as they are also of economic importance to humans. Thus, also provided is the treatment of livestock, including, but not limited to, domesticated swine, ruminants, ungulates, horses (including race horses), poultry, and the like.

As disclosed, polypeptides from the isolated membrane vesicles can be separated and analyzed to identify and/or quantitate the polypeptides. Polypeptide separation techniques are generally known in the art and include, for example, electrophoretic and/or chromatographic techniques (*e.g.*, liquid chromatography) and immunoisolation. Polypeptide identification and quantitation techniques are also well-known in the art.

Numerous methods and devices are well known to the skilled artisan for the detection and analysis of polypeptides, which are applicable to detection and analysis of isolated biomarker peptides associated with isolated exosomes. For example, mass spectrometry and/or immunoassay devices and methods can be used, although other methods are well-known to those skilled in the art. See, e.g., U.S. Pat. Nos. 6,143,576; 6,113,855; 6,019,944; 5,985,579; 5,947,124; 5,939,272; 5,922,615; 5,885,527; 5,851,776; 5,824,799; 5,679,526; 5,525,524; and 5,480,792, each of which is hereby incorporated by reference in its entirety. These devices and methods can utilize labeled molecules in various sandwich, competitive, or non-competitive assay formats, to generate a signal that is related to the presence and/or amount of a biomarker polypeptide of interest. Additionally, certain methods and devices, such as biosensors and optical immunoassays, can be employed to determine the presence or amount of analytes without the need for a labeled molecule. See, e.g., U.S. Pat. Nos. 5,631,171; and 5,955,377, each of which is hereby incorporated by reference in its entirety.

In certain embodiments of the presently disclosed subject matter, the biomarker peptides are analyzed using an immunoassay. The presence or amount of a biomarker peptide can be determined using antibodies or fragments thereof specific for each marker and detecting specific binding. For example, in some embodiments, the antibody specifically binds a polypeptide of Table 1. In some embodiments, the antibody is a monoclonal antibody. Any suitable immunoassay can be utilized, for example, Western blots, enzyme-linked immunoassays (ELISA), radioimmunoassays (RIAs), competitive binding assays, and the like. Specific immunological binding of the antibody to the marker can be detected directly or indirectly. Direct labels include fluorescent or luminescent tags, metals, dyes, radionuclides, and the like, attached to the antibody. Indirect labels include various enzymes well known in the art, such as alkaline phosphatase, horseradish peroxidase and the like.

The use of immobilized antibodies or fragments thereof specific for the markers is also contemplated by the present subject matter. The antibodies can be immobilized onto a variety of solid supports, such as magnetic or chromatographic matrix particles, the surface of an assay plate (such as microtiter wells), pieces of a solid substrate material (such as plastic, nylon,

paper), and the like. An assay strip can be prepared by coating the antibody or a plurality of antibodies in an array on solid support. This strip can then be dipped into the test biological sample and then processed quickly through washes and detection steps to generate a measurable signal, such as for example a colored spot.

The analysis of a plurality of markers is contemplated by the presently disclosed subject matter and can be carried out separately or simultaneously with one or more test samples. Several markers can be combined into one test for efficient processing of a multiple of samples. In addition, one skilled in the art would recognize the value of testing multiple samples (for example, at successive time points) from the same subject. Such testing of serial samples provides for the identification of changes in biomarker polypeptide levels over time. Increases or decreases in marker levels, as well as the absence of change in marker levels, can provide useful information about the disease status that includes, but is not limited to identifying the approximate time from onset of the event, the presence and amount of salvageable tissue, the appropriateness of drug therapies, the effectiveness of various therapies as indicated by reperfusion or resolution of symptoms, differentiation of the various types of a disorder, identification of the severity of the event, identification of the disease severity, and identification of the subject's outcome, including risk of future events.

A panel consisting of biomarkers associated with a disorder can be constructed to provide relevant information related to the diagnosis or prognosis of the disorder and management of subjects with the disorder. Such a panel can be constructed, for example, using 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 or 20 individual biomarkers. The analysis of a single marker or subsets of markers comprising a larger panel of markers could be carried out by one skilled in the art to optimize clinical sensitivity or specificity in various clinical settings. These include, but are not limited to ambulatory, urgent care, critical care, intensive care, monitoring unit, insubject, outsubject, physician office, medical clinic, and health screening settings. The analysis of biomarker polypeptides could be carried out in a variety of physical formats as well. For example, the use of microtiter plates or automation could be used to facilitate the processing of large numbers of test samples. Alternatively, single sample formats could be developed to facilitate

immediate treatment and diagnosis in a timely fashion, for example, in ambulatory transport or emergency room settings.

In some embodiments, a kit for the isolation and analysis of biomarker polypeptides is provided that comprises a filtration module comprising a filter having an average pore diameter of between about 0.01 μm and about 0.15 μm and antibodies or fragments thereof having specificity for one or more biomarker polypeptides of interest. Such a kit can comprise devices and reagents for the analysis of at least one test sample. The kit can further comprise instructions for using the kit and conducting the analysis. Optionally the kit can contain one or more reagents or devices for converting a marker level to a diagnosis or prognosis of the subject.

Further, mass spectrometry is a useful and well-characterized tool for polypeptide identification and quantitation, alone or in combination with polypeptide separation techniques, particularly when coupled with bioinformatics analysis. Peptide molecular weights and the masses of sequencing ions can be obtained routinely using mass spectrometry to an accuracy which enables mass distinction amongst most of the 20 amino acids in the genetic code, as well as quantitation of peptides in a sample. Single or tandem mass spectrometry can be used. In tandem mass spectrometry, a peptide sample is introduced into the mass spectrometer and is subjected to analysis in two mass analyzers (denoted as MS1 and MS2). In MS1, a narrow mass-to-charge window (typically 2-4 Da), centered around the m/z ratio of the peptide to be analyzed, is selected. The ions within the selected mass window are then subjected to fragmentation via collision-induced dissociation, which typically occurs in a collision cell by applying a voltage to the cell and introducing a gas to promote fragmentation. The process produces smaller peptide fragments derived from the precursor ion (termed the 'product' or 'daughter' ions). The product ions, in addition to any remaining intact precursor ions, are then passed through to a second mass spectrometer (MS2) and detected to produce a fragmentation or tandem (MS/MS) spectrum. The MS/MS spectrum records the m/z values and the instrument-dependent detector response for all ions exiting from the collision cell. Fragmentation across the chemical bonds of the peptide backbone produces ions that are either charged on the C-terminal fragment (designated as x, y or z ions) or on the N-terminal fragment (a, b or c ions). Peptides are fragmented

using two general approaches, high and low energy collision-induced dissociation (CID) conditions. In low energy CID experiments, signals assigned to y and b ions and from losses of water and ammonia are usually the most intense. During high energy CID, peptide molecules with sufficient internal energy to cause cleavages of the amino acid side chains are produced. These side chain losses predominantly occur at the amino acid residue where the backbone cleavage occurs. The general designations for these ions are d for N-terminal and w for C-terminal charged fragments, respectively. Other useful sequencing ions occur which result from a y-type cleavage at one residue and a b type cleavage at another residue along the polypeptide backbone (internal fragment ions) (Biemann, 1990; Papayannopoulos, 1995).

In one embodiment of the presently disclosed subject matter, the polypeptides are separated and analyzed using matrix-assisted laser-desorption time-of-flight mass spectrometry (MALDI-TOF). This instrument configuration is used to generate a primary mass spectrum in order to determine the molecular weight of the polypeptide. Other mass spectrometric techniques include, without limitation, time-of-flight, Fourier transform ion cyclotron resonance, quadrupole, ion trap, and magnetic sector mass spectrometry and compatible combinations thereof. See for example U.S. Patents 6,925,389; 6,989,100; and 6,890,763 for further guidance, each of which is incorporated by reference herein in its entirety.

With regard to proteomic analysis, various computer-mediated methods are known for deducing the sequence of a peptide from an MS/MS spectrum. In one approach, 'sub-sequencing' strategies are used whereby portions of the total sequence, (i.e., sub-sequences) are tested against the mass spectrum (see Ishikawa et al., 1986; Siegel et al., 1988; Johnson et al., 1989, each of which is hereby incorporated by reference in its entirety). In this approach, sub-sequences that read or correlate to ions observed in the MS/MS spectrum are extended by a residue and the whole process is then repeated until the entire sequence is obtained. During each incremental extension of the sequence, the possibilities are reduced by comparing sub-sequences with the mass spectrum and only permitting continuation of the process for sub-sequences giving the most favorable spectral matches. Determination of amino acid composition has also been utilized to limit sequence possibilities (Zidarov et al., 1990, hereby incorporated by reference in its entirety).

Another approach utilizes computer programs for *de novo* peptide sequencing from fragmentation spectra based on graph theory (Fernandez-de-Cossjo et al., 1995; Hines et al., 1995; Knapp, 1995, which are hereby incorporated by reference in their entirety). The basic method involves mathematically transforming an MS/MS spectrum into a form where fragment ions are converted to a single fragment ion type represented by a vertex on the spectrum graph (Bartels, 1990, the contents of which is hereby incorporated by reference in its entirety). Peptide sequences are then determined by finding the longest series of these transformed ions with mass differences corresponding to the mass of an amino acid.

Other methods match spectral information with sequences in protein and translated nucleotide sequence databases. An algorithm has been described for searching protein and nucleotide databases with mass and sequence information from fragmentation spectra of tryptic peptides (MS-TAG) (Mann and Wilm, 1994; Clauser et al., 1996, which are hereby incorporated by reference in their entirety). A comparison with the fragmentation spectra of the same peptide after methylation of the carboxyl groups or enzymatic digestion in the presence of ^{18}O water to incorporate ^{18}O into the C-terminal carboxy groups (Shevchenko et al., 1997, which is hereby incorporated by reference in its entirety) can provide even more accurate results. A similar approach has been extended to the analysis of intact proteins using laser fragmentation and Fourier-transform mass spectrometry (Mortz, E. et al., 1996, which is hereby incorporated by reference in its entirety).

Another approach has been described for identifying peptide sequences from database interrogation by comparing the experimental fragmentation spectrum with theoretical spectra from a mass-constrained set of database sequences (SEQUEST) (U.S. Pat. No. 5,538,897; Yates et al., 1991, which are hereby incorporated by reference in their entirety). For each candidate sequence within the database spectrum, a theoretical fragmentation spectrum is formed according to a selected ion model of peptide fragmentation. The predicted theoretically derived mass spectra are compared to each of the experimentally derived fragmentation spectra by a cross-correlation function for scoring spectra.

EXAMPLES

The following Examples provide illustrative embodiments. In light of the present disclosure and the general level of skill in the art, those of skill will appreciate that the following Examples are intended to be exemplary only and that numerous changes, modifications, and alterations can be employed without departing from the scope of the presently claimed subject matter.

MATERIALS AND METHODS FOR EXAMPLES

Urine collection. A 50-200mL waste specimen void was collected using a) sample containers prealiquoted with protease inhibitors (PEFABLOC[®] SC (Pentapharm Ltd., Basel, Switzerland) 0.1mg/mL and aprotinin 0.01mg/mL) and bacteriostat (sodium azide; 0.5mM final concentration) and/or b) immediately centrifuged at 3000xg to sediment particulate matter (e.g. cells and casts). The clarified urine was decanted and the samples not intended for use in exosome sampling were stored at -80°C until analysis.

Exosome sampling. To avoid differential loss of exosomes during cryoprecipitate formation and collection, the freshly centrifuged, protease inhibited supernatant is filtered through a dead-end hollow fiber module containing polypropylene hollow fibers with an average pore diameter of 0.1 µm. (Membrana GmbH., Wuppertal, Germany). The urine is introduced into the exosome-filtration module using a peristaltic pump operating at a 2mL/min flow rate. The dead volume of the entire module is less than 1.5 ml, so that the exosome proteins (the retentate) can be harvested by introducing that volume of a) PBS- for exosome recovery or b) IEF equilibration buffer, fortified with trifluoroethanol for direct dissolution of exosomal proteins.

Alternatively, the clarified urine is filtered through a Millipore (Bedford, Massachusetts, U.S.A.) 45mm diameter, 0.1µm pore size type VVLP filtration disc housed in an AMICON[®] (Millipore) 50mL stirred cell apparatus with 60psi N₂ and magnetic stirrer. Filtered material is rinsed with 50mL phosphate buffered saline (PBS), pH 7.4. The retained rinse volume (2-3mL) is saved and the filter is sonicated in 4mL PBS for 10 min. The filter rinse volumes are combined and then divided between to ultracentrifuge tubes. Exosomes are pelleted as described below. Alternatively, proteins from the four (4) mL sample containing the exosomes can be isolated using precipitation (such as with trichloroacetic acid/acetone) or the exosomes can be concentrated by spin ultrafiltration

(Millipore Ultra 4 spin filters) using a bench-top centrifuge (at 4,000 x g). To evaluate the effects of storage, urine is collected and immediately frozen at -80°C. Urine is maintained for 2-6 months before thawing and processing as described above.

Exosome samples recovered in PBS were used for TEM characterization. PBS containing exosomes were pelleted by high speed ultracentrifugation at 200,000 xg. PBS was removed by pipetting and 4% glutaraldehyde in PBS was layered onto the exosomal pellet. Exosomal-protein glutaraldehyde-crosslinking was allowed to proceed for 1h. The cross linked pellet was submitted for TEM analysis. (Figure 2 & 3)

Post-Exosome Sample Handling. Samples previously frozen at -80°C were thawed at room temperature. Cryoprecipitate formed upon thawing is pelleted by centrifugation (3000 xg; 15 min). Urine was concentrated with AMICON® stirred cell concentrators (Millipore) using 10,000 MWCO YM-type (regenerated cellulose) membrane filters and N₂ gas for positive pressure. Urine samples were routinely concentrated from the original starting volume down to 1-2mL final volume. The concentrated urine samples (retentates) were transferred into 0.5-3.0mL, 10,000 Dalton MWCO SLIDE-A-LYZER® (Pierce Biotechnology, Inc., Rockford, Illinois, U.S.A.) for standardization of pH and ionic strength. Samples were dialyzed overnight at 4°C against 4L 2-5mM Tris pH 7.4. The urine filtrate containing low molecular weight species such as peptides, intermediary metabolites from carbohydrate and protein degradation, and also salts is labeled and frozen at -80°C for future studies.

The urinary protein, following removal of cellular debris, exosomes, cryoglobulins, and peptides now can be concentrated by conventional methods in order to allow high molecular weight protein a) electrophoretic separation or b) trypsinization followed by direct peptide separation using capillary HPLC. Protein concentration values for the standardized urine protein samples were determined by either a Biorad (Bradford) protein microassay or Pierce (µBCA) protein microassay against a bovine serum albumin (BSA) standard.

One-dimensional, denaturing, reducing electrophoresis and Western blotting. Exosomal protein and urinary protein is reduced and denatured by heating to 90°C for 5min in the presence of 1X LDS gel loading buffer (Invitrogen, California, U.S.A.) supplemented with dithiothreitol 20mM (DTT). Protein is

loaded into 4-12% NUPAGE[®] gels (Invitrogen, Carlsbad, California, U.S.A.) and electrophoresis at 200V until the bromophenol blue running dye migrated to the end of the gel.

Protein samples were quantified using a Pierce μ BCA protein assay. Protein samples (5-20 μ g) were resolved on 4-12% SDS- NUPAGE[®] gels (Invitrogen) using Mark12 molecular weight standards and HK2 cell lysates or total urine protein as positive controls when possible. Proteins were electroblotted onto 0.45 μ m nitrocellulose membranes for 60min at 20V. Electroblotted gels were stained with colloidal Coomassie blue stain to evaluate transfer. Blotted membranes were blocked in 5% milk proteins dissolved in a 1X Tris-Tween 20 (TTBS) solution for 2h at room temperature or overnight at 4°C. Primary antibody (0.2 μ g/mL – 1.0 μ g/mL) was dissolved in 5% albumin in TTBS and incubated on the blocked membrane for 1h at room temperature or overnight at 4°C. Secondary antibody (0.05 μ g/mL – 0.2 μ g/mL) was dissolved in 5% albumin in TTBS and incubated on the blocked membrane for 2h at room temperature or overnight at 4°C. The blotted membrane was rinsed 5-times for 5min between all steps. Secondary antibody conjugated with horseradish peroxidase (HRP) was visualized using the Pierce Femto-Super Signal Kit. Membranes were used to expose x-ray film and resulting images were developed and scanned and bands quantified.

Protein digestion, peptide mass fingerprinting and sequence tagging. The stained gel slabs were washed with 18M Ω water and each spot was punched with a clean pipette tip trimmed to produce a 1-3 mm³ punch. Gel pieces were conditioned at room temperature for 15min with 20 μ L 0.1M ammonium bicarbonate (NH₄HCO₃) followed with direct addition of 30 μ L acetonitrile (99.9%). The solution was removed after 15min and the gel pieces were dried using a Jouan Model RC 10.10 speed vacuum centrifuge. The gel pieces were rehydrated with 20 μ L of 0.02M dithiothreitol in 0.1M NH₄HCO₃ and incubated at 56°C for 45min to reduce the protein. The sample was cooled to room temperature and the solution was removed and replaced by 0.055M iodoacetamide in 0.1M NH₄HCO₃. The alkylation of the gel plugs proceeded for 30min in the dark whereupon the solution was replaced by 200 μ L 0.05M NH₄HCO₃ and incubated for 15min. The gel plugs were dehydrated by the addition of 200 μ L 99.9% acetonitrile. After 15min, the solution was removed and

the gel plugs were dried by vacuum centrifuge and re-hydrated with 5 μ L of 20ng/ μ L modified trypsin (Promega, Madison, Wisconsin, U.S.A.) in 0.05M NH₄HCO₃. Re-hydrated gel pieces were covered with 5 μ L 0.05M NH₄HCO₃ solution and incubated overnight at 37°C. The digested samples were cooled and the trypsinization reaction was stopped by the addition of 1 μ L 0.1%TFA.

MALDI matrix used throughout analysis was α -cyano-4-hydroxycinnamic acid (α -CN) containing 10mM NH₄H₂PO₄. Samples were a) spotted as 1:1 (v/v) samples of protein digest: α -CN (or b) desalted sample aliquots (0.7 μ L - 1.0 μ L, 4mg/mL α -CN, 50% acetonitrile 0.1% TFA) spotted directly onto MALDI sample targets using C18 Zip Tips. Samples were air-dried in the dark and were cleared of particulate matter with compressed gas prior to sample plate loading into the mass spectrometer.

Positive ion MALDI-TOF mass spectra were acquired using an Applied Biosystems (Foster City, CA) AB4700 protein analyzer operating in reflectron mode and with ion source pressure ~0.5 μ Torr. After a 400ns time-delayed ion extraction period, the ions were accelerated to 20kV for time-of-flight (TOF) mass spectrometric analysis. A total of 600 to 1000 laser shots (355nm Nd:YAG solid state laser operating at 200Hz) were acquired and signal averaged. Data was analyzed using Mascot (version 1.9) assuming a) monoisotopic peptides masses, b) cysteine carbamidomethylation, b) variable oxidation of methionine, c) maximum of one missed trypsin cleavage, and d) a mass accuracy of greater than 150ppm. Limitation of the original protein mass was not employed within the Mascot search.

EXAMPLE 1

URINE COLLECTION AND EXOSOME CHARACTERIZATION

Spherical membrane vesicles (e.g., exosomes) were purified from fresh and from previously-frozen urine using two independent filtration methods- 1) tangential filtration with fiber-based filtration cartridges and 2) membrane filtration with disc-membranes, as disclosed herein. See Figure 1. Exosomes were recovered from a) thawed, previously frozen urine despite formation of cryoprecipitate and b) urine collected as morning or day-time voids. Exosome collection methods allowed for desalting and rinsing of residual urine volume from the exosomes. Recovered exosomes were characterized for physical

morphology using transmission electron microscopy (TEM) (Figures 2 and 3) and for protein composition using 1-dimensional, denaturing-reducing polyacrylamide electrophoresis (1D SDS PAGE) and subsequent protein identification by mass spectrometry and bioinformatic analysis (Pisitkun et al., 2004; Caby et al., 2005; Gatti et al., 2005). Protein identification was achieved by mass spectrometric methods (peptide sequence tagging) and by antibody based methods (Western blotting).

EXAMPLE 2

ONE-DIMENSIONAL ELECTROPHORESIS AND WESTERN BLOTTING

Exosomal proteins were denatured, reduced, and resolved by mass using a 4-12% NUPAGE[®] gel (Invitrogen). Separated proteins were electro-blotted onto nitrocellulose membranes and proteins detected with antibodies specific to proteins previously identified to be present in urinary exosomes (ezrin, neprilysin, and the α -subunit of the Na^+/K^+ ATPase).

Ezrin is a peripheral membrane protein that physically interacts with integral membrane proteins such as CD43 or CD44 at the cytoplasmic face of the plasma membrane. The data demonstrate that ezrin co-purifies with urinary exosomes filtered from morning and also from mid-day void urine samples (Figure 4). The identification of ezrin co-purification is consistent with established literature (Pisitkun et al., 2004; Gatti et al., 2005; Hegmans et al., 2004).

The α -subunit of the Na^+/K^+ ATPase catalyzes the hydrolysis of ATP, coupled with the exchange of sodium and potassium ions across the plasma membrane. The proteins are located in the cell membrane and are members of the P-type cation-transporting ATPase superfamily, and as such have 10 transmembrane (TM) helices. It was demonstrated by Western blotting that the α -subunit of the Na^+/K^+ ATPase co-purifies with urinary exosomes using tangential fiber filtration (Figure 5). Furthermore, the data demonstrate that the exosome recovery is complete as the protein is absent in the filter fiber stripping solution [Laemmli buffer supplemented with an organic co-solvent (40% trifluoroethanol; TFE)] designed to maximize the recovery of hydrophobic proteins and peptides. Identification of the α -subunit of the Na^+/K^+ ATPase is

consistent with the scientific literature (Mellegol et al., 2005; van Niel et al., 2001).

Neprilysin is a Type II membrane protein and has a single transmembrane helix. Neprilysin is a major constituent of the renal brush border membrane. It was demonstrated by Western blotting that neprilysin co-purifies with exosomes using both fiber and membrane filtration methods and including both fresh and frozen urine. Additionally, using neprilysin immunoblots, it was demonstrated that the exosome filtration method depletes the urine of the neprilysin co-purifying material (exosomes) (Figures 6 and 7). The co-purification of neprilysin is consistent with the scientific literature (Pisitkun et al., 2004; Gatti et al., 2005).

The most abundant urinary protein (the Tamm-Horsfall protein; THP) is heavily glycosylated and is predisposed to the formation of aggregates. These aggregates have molecular weight distribution from ~90kDa up to >1,000 kDa. Published procedures for removal of THP aggregates include chemical denaturation via hot DTT and differential sucrose-density ultracentrifugation. By Western blotting it was demonstrated that either method can be used as a sample pre-treatment without significant loss of neprilysin.

EXAMPLE 3

PROTEIN DIGESTION, PEPTIDE MASS FINGERPRINTING AND SEQUENCE TAGGING

Isolated exosomes were dissolved into Laemmli buffer and separated on a 4-12% gradient gel (Figure 8). The length of the sample lane was cut into 2.5mm² cubes, protein content reduced and alkylated, and digested with trypsin. The trypsin digest were analyzed by MALDI-TOF MS with selective fragmentation of dominant precursor ions. Peptide sequence tagging supported by peptide mass fingerprint analysis was used to identify proteins co-purifying with urinary exosomes.

Included in the list of identified proteins are a) transmembrane (TM) proteins, b) ubiquitinated proteins, c) ligands of advance glycosylation end product (AGE) modified proteins and d) membrane associated proteolytic enzymes. The identified proteins include several proteins with documented relevance to human diseases were identified within the analyzed samples.

EXAMPLE 4
DIRECT ANALYSIS, ONE DIMENSIONAL REVERSED-PHASE LIQUID
CHROMATOGRAPHY AND ELECTROSPRAY IONIZATION MASS
SPECTROMETRY

Isolated exosomes were dispersed into a solution adjusted to contain a buffering solution of 0.1 M triethylammonium bicarbonate, pH 8.5 and a detergent solution of 0.1 % NP-40. Proteins co-isolated with the exosomes were reduced by addition of 2mm tris-carboxyethylphosphine/0.1 M triethylammonium bicarbonate, pH 8.5 with heating to 5°C for 30min. Protein samples were then cooled and reduced by 1 h incubation in the dark with addition of 20mM iodoacetamide/0.1 M triethylammonium bicarbonate, pH 8.5. Proteins were then digested by addition of 250ng mass spectrometry grade, modified trypsin, and incubation at 37°C for 1 hr with shaking. After 1 h a second aliquot (250ng) of mass spectrometry grade trypsin was added and incubation continued overnight for a total digestion time of 20h.

The trypsin digest was separated by one-dimensional reversed-phase liquid chromatography and eluting peptide ions mass quantified, fragmented and fragments mass analyzed by linear ion trap mass spectrometry. The fragmentation spectra were analyzed using SEQUEST SORCERER™ (Sage N Research, San Jose, California, U.S.A.) and PEPTIDE PROPHET & PROTEIN PROPHET™ (Institute of Systems Biology, Seattle, Washington, U.S.A.) to filter the SEQUEST data. Identified proteins included membrane proteins identified in EXAMPLE 2 and annotated on Figure 8. (See also Table 1.)

TABLE 1

No.	Accession No.	% Sequence Coverage	No. Unique Peptides	Total No. Peptides	Protein Name and Description
1	31377806	11.6	8	17	Polymeric immunoglobulin receptor [Homo sapiens]
2	4502095	12.1	10	12	Membrane alanine aminopeptidase M [Homo sapiens]
3	6042200	12.3	7	8	Membrane metallo-endopeptidase; neprilysin [Homo sapiens]

4	40217833	19.9	6	7	GPCR Family C-5-C-b; retinoic acid responsive gene protein [Homo sapiens]
5	28916691	12.1	4	5	Mucin 1; episialin [Homo sapiens]
6	4506153	7.6	2	4	Prostasin preproprotein [Homo sapiens]
7	4557849	3.8	3	3	Sodium potassium chloride cotransporter 2 [Homo sapiens]
8	7706451	3	1	1	GPCR Family C-5-B precursor; retinoic acid responsive gene protein [Homo sapiens]
9	11386147	2.9	1	1	Prosaposin [Homo sapiens]
10	21264578	5.4	1	1	Tetraspan 1; TM4SF [Homo sapiens]
11	4557503	3.3	10	15	Cubilin [Homo sapiens]
12	6806919	2.7	9	12	Megalin [Homo sapiens]
13	5174387	4	2	3	Prominin 1 [Homo sapiens]
14	17511435	3.7	2	2	Roundabout homolog 4 [Homo sapiens]
15	18765694	3.1	2	2	Dipeptidylpeptidase IV (CD26) [Homo sapiens]
16	19923603	3.5	1	2	Cytochrome b reductase 1 [Homo sapiens]
17	24497519	4.1	2	2	Mannosidase, alpha, class 1A, member 1 [Homo sapiens]
18	32313593	6.9	2	2	Olfactomedin 4 precursor [Homo sapiens]
19	4502179	7.7	1	1	Aquaporin 2 [Homo sapiens]
20	4758190	2.4	1	1	Dipeptidase 1 (renal) [Homo sapiens]
21	4759140	3.9	1	1	Solute carrier family 9 (sodium\hydrogen exchanger), isoform 3 regulator 1 [Homo sapiens]
22	13376868	2	1	1	NG22 protein; choline transporter-like protein 4 [Homo sapiens]
23	14150145	2.9	1	1	Limitrin [Homo sapiens]
24	19923362	9.3	1	1	Thy-1 T-cell antigen [Homo sapiens]
25	33598950	3.8	1	1	Podocalyxin-like precursor [Homo sapiens]

TABLE 1. Membrane proteins identified by direct analysis of trypsin digested exosomes using one-dimensional reversed phase liquid chromatography and electrospray ionization linear ion trap mass spectrometry with peptide fragmentation analysis using SEQUEST SORCERER™ and PEPTIDE

PROPHET & PROTEIN PROPHET™ to filter the SEQUEST data. Proteins identified are indicative of exosome isolation.

DISCUSSION OF EXAMPLES 1-4

As illustrated by the Examples herein, the present methods were successfully utilized to isolate exosomes from biological samples and identify proteins associated with the isolated exosomes. The present methods thus allow for isolation and enrichment of particular protein populations of interest that can provide information as to biological processes within a subject, such as for example the presence or absence of disease and responses to therapeutic treatments. For example, exosome protein compositions have been demonstrated to include transmembrane proteins such as CD10, the alpha subunit of the Na⁺/K⁺ ATPase and peripheral membrane protein such as ezrin by immunochemical methods. Further demonstration of selective enrichment for low abundant urine proteins can be achieved using the presently disclosed exosome isolation methods.

Tables 2 and 3 provide exemplary data of proteins isolated and identified from either exosome isolates using the presently disclosed methods or from human urine samples, respectively. Proteins listed in both tables were identified using computer aided data analysis (PEPTIDE PROPHET & PROTEIN PROPHET™) of exosome protein tryptic digests developed using 1D-LC-MS/MS sample analysis. A comparison between Tables 2 and 3 of the proteins identified and quantities of the proteins demonstrate selective enrichment for proteins of interest associated with urinary exosomes. Four such proteins are discussed hereinbelow as non-limiting examples demonstrating the selective enrichment of exosomal proteins from urine. Two transmembrane proteins (megalin and cubulin) are selected to demonstrate enrichment of exosomal proteins from urine. Two serum proteins (albumin and kininogen-1) are selected to demonstrate selective filtering of proteins from the exosomal protein preparation.

Megalin and cubulin are two proteins resident in the apical membrane of the renal proximal tubule. The functions of these proteins are well documented. These proteins are known to function as protein scavengers and are responsible for recycling of urinary albumin from urine. Further, presence of these proteins in

urinary exosomes is well documented. As demonstrated in Table 2, megalin represents 4.84 percent of the total share of all MS/MS spectral identifications from the LC-MS/MS analysis of tryptic exosome protein fragments. Additionally, cubulin represents 2.96 percent of the total share of all MS/MS spectral identifications from the LC-MS/MS analysis of tryptic exosome protein fragments.

In contrast, and as demonstrated in Table 3, megalin represents only 0.47 percent of the total share of all MS/MS spectral identifications from the LC-MS/MS analysis of tryptic urine protein fragments. Additionally, cubulin represents only 0.4 percent of the total share of all MS/MS spectral identifications from the LC-MS/MS analysis of tryptic urine protein fragments. Thus, use of the presently disclosed novel methods resulted in a several-fold enrichment of these two integral-membrane proteins.

Albumin and kininogen-1 are well documented plasma proteins and are known to be in the urine at low levels in normal conditions. Renal damage resulting from trauma or from genetic factors can present with increased levels of these two plasma proteins in the urine. As demonstrated in Table 2, albumin represents 4.88 percent of the total share of all MS/MS spectral identifications from the LC-MS/MS analysis of tryptic exosome protein fragments. Additionally, cubulin represents 0.66 percent of the total share of all MS/MS spectral identifications from the LC-MS/MS analysis of tryptic exosome protein fragments.

In contrast, and as demonstrated in Table 3, albumin represents 32.05 percent of the total share of all MS/MS spectral identifications from the LC-MS/MS analysis of tryptic urine protein fragments. Additionally, kininogen-1 represents 3.03 percent of the total share of all MS/MS spectral identifications from the LC-MS/MS analysis of tryptic urine protein fragments. Albumin and kininogen-1 are not selectively depleted from the urine. Thus, use of the presently disclosed novel microfiltration methods provided for depletion of otherwise very abundant plasma proteins found in the urine. The small presence of the two proteins in the exosome protein preparation is presumed to be from normal biology and the renal-urine protein recycling mechanisms.

TABLE 2

ID#	Protein gi Number	Protein Probability Score	Percent Coverage	Number of Unique Peptides	Total Number of Peptides	% Share of Spectrum ID's	Protein ID
1	21735625	0.82	4.9	1	1	0.09	14-3-3 zeta
2	13489091	1	9.1	2	2	0.22	3-Mercaptopyruvate sulfurtransferase
3	6912586	0.95	6.2	1	1	0.1	6-Phosphogluconolactonase
4	4502211	0.44	6.3	1	1	0.05	ADP-ribosylation factor 6
5	21493031	0.82	0.9	1	2	0.39	A-kinase anchor protein 13 isoform 3; guanine nucleotide exchange factor Lbc
6	4502027	1	38.3	27	47	4.88	Albumin
7	21361176	0.98	2.6	1	1	0.11	Aldehyde dehydrogenase 1A1
8	51466516	0.42	2.5	1	1	0.1	Aldo-keto reductase family 1, member B10
9	40354205	0.99	3.6	1	1	0.11	Aldolase B
10	4501881	1	15.7	3	4	0.36	Alpha 1 actin precursor
11	4504347	1.00	10.6	1	2	0.36	Alpha 1 globin
12	18641350	0.99	0.9	1	1	0.11	Alpha 1 type XV collagen precursor
13	21071030	1	8.7	4	4	0.43	Alpha 1B-glycoprotein
14	17986277	0.99	1.5	2	4	0.26	Alpha 2 type IV collagen preproprotein; canstatin
15	4501843	0.99	2.5	1	1	0.11	Alpha-1-antichymotrypsin
16	4502067	1	21.9	6	14	1.54	Alpha-1-microglobulin\bikunin
17	4502005	1	10.4	4	11	0.79	Alpha-2HS-glycoprotein
18	4505327	1	13.2	6	6	0.59	Alpha-N-acetylglicosaminidase
19	4502085	1	12.3	5	5	0.55	Amylase, pancreatic, alpha-2A
20	6912236	0.99	6.7	3	3	0.19	Angiopietin-related protein 2
21	4502107	1.00	13.4	3	3	0.57	Annexin 5
22	4557317	1	10.7	3	3	0.34	Annexin A11
23	4757756	1.00	7.7	2	2	0.38	Annexin A2 isoform 2

24	4502105	0.99	5	1	1	1	0.11	Annexin IV
25	51476111	1	14.6	3	3	3	0.32	Apolipoprotein A-I precursor (Apo-AI)
26	4502151	0.54	4.5	2	2	2	0.16	Apolipoprotein A-IV precursor
27	4502163	1	30.7	5	14	14	1.09	Apolipoprotein D precursor
28	4557325	1	17.7	4	4	4	0.43	Apolipoprotein E3
29	4502179	0.99	7.7	1	1	1	0.21	Aquaporin 2
30	4557337	0.87	3.2	1	1	1	0.1	Argininosuccinate synthetase
31	15149476	0.83	1.7	1	1	1	0.09	Arginyl-tRNA synthetase
32	4504067	0.83	1.9	1	1	1	0.09	Aspartate aminotransferase 1
33	42741659	0.96	1.5	1	1	1	0.2	ATP-binding cassette sub-family B member 1
34	21450861	0.99	1.1	1	1	1	0.11	Attractin isoform 1; mahogany protein
35	21536466	0.99	2.6	2	2	2	0.14	AXL receptor tyrosine kinase isoform 1
36	4504349	1	25.9	2	2	2	0.24	Beta globin
37	4502407	0.99	3.9	1	1	1	0.19	Betaine-homocysteine methyltransferase 1
38	13162290	0.97	2.8	1	1	1	0.24	Betaine-homocysteine methyltransferase 2
39	7706083	0.71	1.6	1	1	1	0.17	C1r-like serine protease analog
40	8923765	0.41	1.2	1	1	1	0.08	Calcium channel alpha2-delta3 subunit
41	4557395	1	14.6	2	2	2	0.15	Carbonic anhydrase B
42	51464068	0.95	2.7	1	1	1	0.1	Carboxypeptidase N 83 kDa chain
43	4503143	0.53	1.9	1	4	4	0.21	Cathepsin D preproprotein
44	4557417	1	16.8	7	9	9	0.99	CD14 antigen precursor
45	21361193	1	3	3	3	3	0.32	CD44 antigen
46	42761474	1	25	5	18	18	1.95	CD59
47	4757952	1.00	19.9	3	4	4	0.55	Cell division cycle 42 isoform 1
48	4557485	1	3.8	3	4	4	0.6	Ceruloplasmin
49	31542306	0.62	7	1	1	1	0.14	CHMP1.5 protein; C18orf2
50	4557443	0.99	3	1	1	1	0.11	Cholesteryl ester transfer protein, plasma precursor
51	40255141	0.53	3	1	4	4	0.13	Chondroitin beta1,4 N-acetylgalactosaminyltransferase

52	21361741	0.98	2.9	1	1	1	0.24	Chromosome 6 open reading frame 55, Myo12 protein
53	42716297	1	23.8	10	16	1.68	Clusterin isoform 1	
54	8922699	0.97	3.4	1	1	0.11	CNDP dipeptidase 2	
55	4503635	0.82	1.4	1	1	0.09	Coagulation factor II precursor	
56	15011913	1	6.5	4	4	0.27	Collagen, type VI, alpha 1 precursor	
57	10834974	1	2.7	2	2	0.2	Complement component (3b4b) receptor-1 isoform F precursor	
58	4557379	1	11.6	5	6	0.62	Complement component 1 inhibitor precursor	
59	4557385	1	2	2	2	0.47	Complement component 3 precursor; acylation-stimulating protein cleavage product	
60	4503015	0.41	1.7	1	1	0.1	Copine III	
61	21536286	1	7.9	2	2	0.22	Creatine kinase-B	
62	4503057	0.41	4.6	1	1	0.08	Crystallin, alpha B	
63	4557503	1	8.6	26	30	2.96	Cubilin	
64	19923603	0.62	3.5	1	1	0.07	Cytochrome b reductase 1	
65	4503355	0.45	0.7	1	1	0.05	Dedicator of cyto-kinesis 1	
66	4758190	1	14.4	4	8	1.57	Dipeptidase 1 (renal)	
67	18765694	1.00	3.1	2	2	0.38	Dipeptidylpeptidase IV (CD26, adenosine deaminase complexing protein 2)	
68	40254866	0.46	4.7	1	1	0.09	DKFZP564O123 protein	
69	4503281	0.97	2.1	1	1	0.24	Dopa decarboxylase (aromatic L-amino acid decarboxylase)	
70	9665262	0.95	1.9	1	1	0.11	EGF-containing fibulin-like extracellular matrix protein 1 isoform a precursor; fibrillin-like	
71	46195707	1	1.1	2	2	0.22	EGF-like-domain, multiple 4	
72	21264315	0.99	5.5	1	1	0.23	EH-domain containing 4	
73	4503571	1	9	3	3	0.73	Enolase 1	
74	4503491	1	16.4	26	50	5.04	Epidermal growth factor	
75	21264616	0.76	1.7	1	1	0.19	Epidermal growth factor receptor pathway substrate 8-like protein 2	

76	7657058	0.65	2.6	1	1	0.12	Eukaryotic translation initiation factor 2B, subunit 2 beta, 39kDa
77	21614499	1	17.1	7	8	0.5	Ezrin
78	45238580	0.47	3.6	1	1	0.09	F-box only protein 13
79	4503681	0.83	1.9	1	1	0.13	Fc fragment of IgG binding protein
80	47132549	1	1.9	3	3	0.32	Fibronectin 1 isoform 6 preproprotein
81	16579888	0.99	4.7	1	1	0.11	Fructose-1,6-bisphosphatase 1
82	40217833	1	13.2	6	9	0.97	G protein-coupled receptor family C, group 5, member C isoform b
83	7706451	1	6	2	2	0.22	G protein-coupled receptor, family C, group 5, member B precursor
84	10834966	1	7.8	4	4	0.43	Galactosidase, beta 1
85	5031863	1	18.1	16	39	4.13	Galectin 3 binding protein
86	4501887	1	18.9	5	9	0.84	Gamma 1 actin
87	4885271	1.00	8.3	3	3	0.47	Gamma-glutamyl transpeptidase
88	6598323	0.98	6.1	1	1	0.21	GDP dissociation inhibitor 2
89	38044288	0.76	1.1	1	2	0.24	Gelsolin isoform b
90	6912618	0.51	4.2	1	1	0.06	Glutamyl-peptide cyclotransferase precursor
91	7669492	1	12.8	3	4	0.43	Glyceraldehyde-3-phosphate dehydrogenase
92	40254926	0.98	22.2	1	1	0.11	G-protein gamma-12 subunit
93	4557617	0.85	1.3	1	1	0.09	Growth arrest-specific 6; AXL stimulatory factor
94	4504037	0.99	4.2	1	1	0.19	Guanine nucleotide binding protein, alpha 11 (Gq class)
95	11321585	1	5	2	6	0.56	Guanine nucleotide-binding protein G(l)\G(s)\G(t) beta subunit 1; beta subunit, signal-transducing proteins GS\GI ,
96	20357529	0.97	3.5	1	1	0.19	Guanine nucleotide-binding protein G(l)\G(s)\G(t) beta subunit 2
97	34419635	0.50	4.5	1	1	0.14	Heat shock 70kD protein 6 (HSP70B')
98	27436929	0.50	6.2	1	1	0.2	Heat shock 70kDa protein 1-like
99	13676857	1.00	10.1	3	3	0.46	Heat shock 70kDa protein 2

100	51461017	0.52	5.2	1	1	0.13	Heat shock cognate 71 kDa protein
101	47607492	0.86	0.5	1	1	0.16	Hemidesmosomal protein 1
102	7427517	1	0.3	1	1	0.24	Heparan sulfate proteoglycan 2 (endorepellin; perlecan)
103	51471096	0.74	2	1	1	0.08	Heterogeneous nuclear ribonucleoprotein A1
104	16418389	0.99	5.7	1	1	0.11	HGFL protein
105	24308440	0.99	5.5	1	1	0.11	Hypothetical protein BC011840
106	39930521	1	11.9	9	16	1.76	Hypothetical protein BC013767
107	51466566	0.4	0.8	1	1	0.04	Hypothetical protein CBG17606 [Poly Cystic Kidney Disease like protein 3]
108	8923271	0.60	2.8	1	1	0.12	Hypothetical protein FLJ20291
109	39752639	0.51	5.5	2	2	0.12	Hypothetical protein FLJ22374
110	33342276	0.41	1.4	1	1	0.08	Hypothetical protein FLJ22761
111	21389601	0.99	3.9	1	1	0.19	Hypothetical protein FLJ32421
112	51466862	0.72	3.1	1	1	0.08	Hypothetical protein XP_374254
113	51472926	0.98	8.9	1	2	0.22	Ig heavy chain V-III region VH26 precursor
114	51475407	1	14	2	2	0.22	Ig kappa chain
115	21489959	1	32.7	5	10	0.9	Immunoglobulin J chain
116	41150478	1	14.4	2	2	0.21	Immunoglobulin M chain
117	18874099	0.48	0.8	1	1	0.05	Importin 4
118	4826772	1	5	2	2	0.21	Insulin-like growth factor binding protein, acid labile subunit
119	31542984	1	6.6	6	9	0.96	Inter-alpha (globulin) inhibitor H4 (plasma kallikrein-sensitive glycoprotein)
120	4504875	0.99	5	1	1	0.11	Kallikrein 1 preproprotein
121	34222393	0.91	1.3	1	1	0.18	Kinase suppressor of Ras-2
122	4504893	1	12.4	5	6	0.66	Kininogen 1; alpha-2-thiol proteinase inhibitor; bradykinin
123	4557032	1	14.7	4	5	0.46	Lactate dehydrogenase B
124	5803023	0.44	2.5	1	1	0.11	Lectin, mannose-binding 2
125	16418467	1	12.7	4	6	0.5	Leucine-rich alpha-2-glycoprotein 1

126	33636750	0.43	0.7	1	1	0.08	Likely ortholog of mouse ubiquitin-conjugating enzyme E2-230K
127	14150145	1	11.8	5	5	0.36	Limitrin
128	4503849	1	9.5	7	7	0.65	Lysosomal alpha-glucosidase; acid maltase
129	4504957	1	4.9	2	2	0.2	Lysosome-associated membrane protein-2
130	385669482	0.78	1.3	1	1	0.09	Maba1
131	4758712	1	1.1	2	2	0.22	Maltase-glucoamylase
132	21264363	0.99	8.6	1	1	0.11	Mannan-binding lectin serine protease 2 isoform 1 precursor
133	24497519	1	8.6	4	7	0.37	Mannosidase, alpha, class 1A, member 1
134	5803088	0.60	0.7	1	1	0.12	MAPK/ERK kinase 4
135	6806919	1	6.1	27	40	3.84	Megalin
136	4502095	1	11.5	11	15	1.61	Membrane alanine aminopeptidase M
137	6042200	1	14.5	9	10	1.03	Membrane metallo-endopeptidase; neprilysin
138	5174551	0.42	2.1	1	2	0.08	Meprin A, beta
139	14249562	0.58	1.5	1	1	0.06	Microtubule associated serine/threonine kinase-like
140	46852147	0.72	1.3	1	1	0.14	Mitochondrial isoleucine tRNA synthetase
141	4505257	0.98	9.2	1	1	0.33	Moesin
142	28916691	1	12.5	4	6	0.59	Mucin 1, transmembrane; episialin
143	5174569	0.52	0.2	1	1	0.1	Myeloidlymphoid or mixed-lineage leukemia (trithorax (Drosophila) homolog)
144	46430642	1	2.9	2	2	0.21	Myosin IC
145	4758754	1.00	3.8	2	2	0.36	NAPSA gene product
146	13376868	0.99	2	1	1	0.11	NG22 protein; choline transporter-like protein 4
147	4505395	0.91	0.8	1	2	0.11	Nidogen (entactin)
148	5031985	0.54	11.8	1	1	0.06	Nuclear transport factor 2
149	32313593	1	9.2	4	4	0.82	Olfactomedin 4 precursor
150	21361845	0.92	2.1	1	1	0.1	Peptidoglycan recognition protein L precursor
151	33188452	0.68	7.5	1	1	0.17	Peroxiredoxin 2 isoform b
152	4505621	1	16.6	3	3	0.23	Phosphatidylethanolamine binding protein

153	4505763	0.99	4.1	1	1	1	0.11	Phosphoglycerate kinase 1
154	4505881	0.53	2	1	1	1	0.06	Plasminogen
155	33598950	1	3.8	2	2	2	0.21	Podocalyxin-like precursor
156	31377806	1	19.5	14	33	33	3.46	Polymeric immunoglobulin receptor
157	4505959	0.57	1.9	1	1	1	0.06	POU domain, class 2, transcription factor 2
158	4826898	0.75	11.4	1	1	1	0.08	Profilin-1
159	7019485	0.99	6.8	1	1	1	0.11	Programmed cell death 6; apoptosis-linked gene 2
160	22027538	1.00	7.6	6	8	8	1.18	Programmed cell death 6-interacting protein
161	5174387	1	8.6	5	6	6	0.57	Prominin 1
162	21389623	1	3.4	2	2	2	0.21	Prominin 2
163	11386147	1	4.2	1	1	1	0.12	Prosaposin (sphingolipid activator protein-1)
164	32171249	1	17.4	3	3	3	0.33	Prostaglandin-H2 D-isomerase
165	4506153	1	7.6	3	4	4	0.44	Prostasin preproprotein
166	6382064	1	21.2	6	6	6	0.65	Prostatic acid phosphatase precursor
167	4506013	0.76	5	1	1	1	0.15	Protein phosphatase 1, regulatory subunit 7
168	4506121	1	9.8	3	4	4	0.44	Protein Z, vitamin K-dependent plasma glycoprotein
169	7656922	0.44	4.1	1	1	1	0.05	Putative breast adenocarcinoma marker
170	38372933	0.69	7.2	1	1	1	0.2	Putative breast adenocarcinoma marker
171	41281489	1	6.9	3	7	7	0.52	Putative MAPK activating protein PM28
172	24431973	0.61	1.7	1	1	1	0.12	Putative NFkB activating protein
173	14165278	0.99	10.3	1	3	3	0.23	Putative nuclear protein ORF1-FL49
174	33286418	1	4	2	2	2	0.21	Pyruvate kinase 3 isoform 1; thyroid hormone-binding protein, cytosolic
175	10835049	0.69	5.7	1	1	1	0.13	Ras homolog gene family, member A; oncogene RHO H12; Aplysia ras-related homolog 12
176	34147513	0.99	6.8	1	1	1	0.11	Ras-associated protein RAB7
177	7661678	1	12	2	2	2	0.16	RAS-related protein RAP1B; K-REV
178	4506403	0.98	3.6	1	1	1	0.19	Retinoic acid induced 3

179	51474268	0.44	0.8	1	2	0.05	RIKEN cDNA 3000004C01
180	17511435	1	3.7	2	2	0.24	Roundabout homolog 4
181	5032057	0.99	15.2	1	1	0.11	S100 calcium binding protein A11 (calgizzarin)
182	9951915	0.99	3	1	1	0.19	S-adenosylhomocysteine hydrolase
183	4759166	1	18	4	8	0.88	Secreted phosphoprotein 1 (osteopontin)
184	21361198	1	6.2	3	3	0.27	Serine (or cysteine) proteinase inhibitor, clade A, member 1
185	21361195	1	28.1	13	26	2.51	Serine (or cysteine) proteinase inhibitor, clade A, member 5
186	4502133	0.99	5.8	1	2	0.22	Serum amyloid P component precursor; pentaxin-related; 9.5S alpha-1-glycoprotein
187	28827795	0.98	4.9	1	2	0.36	Snf7 homologue associated with Alix 1
188	4557849	1.00	3.8	3	3	0.58	Sodium potassium chloride cotransporter 2; Na-K-2Cl cotransporter
189	4507013	0.99	2.2	1	1	0.24	Solute carrier family 2 (facilitated glucose/fructose transporter), member 5
190	4759140	0.97	3.9	1	1	0.11	Solute carrier family 9 (sodium/hydrogen exchanger), isoform 3 regulator 1
191	18201911	1	8.4	3	3	0.33	Somatostatin B; epibolin
192	4507155	0.59	2.5	1	2	0.26	Sorbitol dehydrogenase
193	38016907	0.53	9.8	1	1	0.13	Stomatin isoform b
194	29029530	0.47	4.1	1	1	0.09	Sulfotransferase 1C1
195	4507151	1	5.4	2	2	0.22	Superoxide dismutase 3, extracellular
196	33239443	0.88	4.6	1	1	0.1	Synaptophysin-like protein isoform b
197	29568086	0.98	5.5	1	1	0.11	Syndecan 1
198	5032083	1.00	6.4	1	1	0.19	Syndecan binding protein (syntenin)
199	21264578	0.99	5.4	1	2	0.22	Tetraspan 1
200	4507745	0.97	12.4	1	1	0.19	Thioredoxin
201	40317626	0.64	1	1	1	0.07	Thrombospondin 1 precursor
202	19923362	0.99	9.3	1	2	0.19	Thy-1 T-cell antigen

203	33356179	0.66	1.5	1	1	1	0.13	Transcription termination factor, RNA polymerase I
204	4557871	1	14.8	8	10		0.84	Transferrin
205	41058276	1	9.2	2	2		0.22	Triosephosphate isomerase 1
206	51470965	0.4	1.3	1	1		0.04	Tripartite motif-containing 48
207	5729770	0.99	3.2	1	1		0.11	Tripeptidyl-peptidase I precursor
208	13376539	0.99	5.8	1	1		0.11	Tubulin, alpha 4
209	51473011	1	44.7	3	4		0.95	Ubiquitin C
210	4507833	1	23.3	32	254		23.19	Uromodulin; Tamm-Horsfall glycoprotein
211	5803215	1.00	8.7	1	1		0.19	Uroplakin 2
212	17865802	1	6.8	2	2		0.2	Vacuolar protein sorting factor 4B; suppressor of K+ transport defect 1
213	41327712	0.75	8.3	1	1		0.14	V-crk sarcoma virus CT10 oncogene homolog isoform a
214	4507879	1	7.8	2	2		0.49	Voltage-dependent anion channel 1
215	15451943	0.68	1.6	1	1		0.13	Zinc finger protein 6

TABLE 3

ID#	Protein gi Number	Protein Probability Score	Percent Coverage	Number of Unique Peptides	Total Number of Peptides	%Share of Spectrum ID's	Protein ID
1	4503849	1	8	5	7	0.61	Acid alpha-glucosidase preproprotein
2	4501987	0.55	2	2	3	0.07	Afamin precursor; alpha-albumin
3	41151826	0.41	0.6	1	1	0.04	Agtrin
4	4502027	1	54.4	65	412	32.05	Albumin precursor
5	4501881	0.99	4.3	1	1	0.11	Alpha 1 actin
6	18641350	1	2.2	3	3	0.24	Alpha 1 type XV collagen precursor
7	21071030	0.99	2.4	1	2	0.19	Alpha 1B-glycoprotein
8	4501843	1	8.8	3	5	0.41	Alpha-1-antichymotrypsin
9	4502067	1	23.9	9	28	2.78	Alpha-1-microglobulin/bikunin precursor
10	4502337	1	32.6	10	18	1.47	Alpha-2-glycoprotein, zinc
11	4502005	1	12	6	12	1.01	Alpha-2HS-glycoprotein
12	4505327	1	7	4	4	0.42	Alpha-N-acetylglucosaminidase
13	40254482	1	33.9	2	2	0.86	Amylase, alpha 1A
14	4502085	1	33.3	3	5	1.08	Amylase, pancreatic, alpha-2A
15	10280622	1	34.4	3	3	0.95	Amylase, pancreatic, alpha-2B
16	28376664	0.96	2.3	2	2	0.16	AN1, ubiquitin-like, homolog
17	6912236	1	2.3	1	1	0.11	Angiopietin-like 2 precursor
18	4502151	0.77	2.8	1	1	0.08	Apolipoprotein A-IV precursor
19	4502163	1	32.8	6	22	1.85	Apolipoprotein D precursor
20	4557325	0.72	2.8	1	1	0.07	Apolipoprotein E precursor
21	4557337	1	6.3	2	2	0.21	Argininosuccinate synthetase
22	15149476	0.61	1.7	1	1	0.06	Arginyl-tRNA synthetase
23	6005990	0.99	2.8	1	1	0.11	Arylsulfatase A precursor
24	27262647	0.53	2.3	1	2	0.06	Ataxin 2 related protein isoform A
25	10947135	0.83	1.4	1	4	0.21	ATP-binding cassette, sub-family F, member 1; ATP-binding cassette 50; ATP-binding cassette 50 (TNF-alpha stimulated)

26	21536466	0.98	1.2	1	2	0.18	AXL receptor tyrosine kinase isoform 1
27	4557327	1	4.9	2	2	0.2	Beta-2-glycoprotein I precursor
28	4757826	1	16.8	3	3	0.3	Beta-2-microglobulin precursor
29	6453813	0.98	3.3	1	1	0.1	Butyrophilin, subfamily 2, member A2 isoform a
30	4757960	1	5.3	4	4	0.31	Cadherin 1, type 1 preproprotein (epithelial)
31	16306532	0.96	1.7	1	1	0.1	Cadherin 11, type 2 isoform 1 preproprotein
32	4502719	0.99	3.8	1	2	0.15	Cadherin 13 preproprotein
33	38327526	0.97	2.3	1	1	0.1	Carboxypeptidase M precursor
34	51464068	0.99	2.7	1	1	0.1	Carboxypeptidase N 83 kDa chain (Carboxypeptidase N regulatory subunit)
35	4557417	1	22.1	5	6	0.67	CD14 antigen precursor
36	21361193	1	3	3	3	0.3	CD44 antigen
37	42761474	1	25	7	43	3.56	CD59 antigen p18-20
38	4557485	1	4.9	4	4	0.4	Ceruloplasmin
39	4557443	0.57	3	1	1	0.06	Cholesteryl ester transfer protein, plasma precursor
40	42716297	1	16.5	8	8	0.72	Clusterin isoform 1
41	4503635	0.99	4.3	1	2	0.24	Coagulation factor II precursor
42	15011913	1	10.3	9	10	0.99	Collagen, type VI, alpha 1 precursor
43	27262665	0.95	4.7	1	1	0.1	Colony stimulating factor 1 isoform c precursor
44	4557379	1	13.6	7	8	0.73	Complement component 1 inhibitor precursor
45	7706083	1	3.5	2	2	0.11	Complement component 1, r subcomponent-like precursor
46	23957690	0.97	2.7	2	2	0.13	Component of oligomeric golgi complex 7
47	4557503	1	1.8	4	4	0.4	Cubilin; intrinsic factor-cobalamin receptor
48	4503107	0.99	11	1	2	0.2	Cystatin C precursor
49	21361254	1	14.2	4	6	0.47	Deoxyribonuclease I
50	19115954	0.77	0.4	2	3	0.08	Dynein, axonemal, heavy polypeptide 5
51	9665262	1	4.9	2	3	0.3	EGF-containing fibulin-like extracellular matrix protein 1 isoform a precursor; fibrillin-like
52	13376091	0.97	1.3	1	1	0.1	Elastin microfibril interfacier 3
53	34335272	1	26.1	6	9	0.98	Endothelial protein C receptor precursor
54	4503491	1	18.2	22	38	2.97	Epidermal growth factor (beta-urogastrone)
55	29789100	0.91	2.6	1	1	0.1	Extracellular sulfatase SULF-2

56	24429586	0.96	3.8	1	1	0.11	Fc fragment of IgG, low affinity III, receptor for (CD16)
57	47132549	1	1.6	2	2	0.18	Fibronectin 1 isoform 6 preproprotein
58	19743803	0.99	2.7	1	2	0.2	Fibulin 5 precursor
59	8051586	0.99	5.1	1	1	0.1	Ficolin 2 isoform b precursor
60	4503747	0.99	0.5	1	1	0.1	Filamin B, beta (actin binding protein 278)
61	10834966	0.99	2.1	1	1	0.11	Galactosidase, beta 1
62	5031863	1	16.1	9	17	1.4	Galectin 3 binding protein
63	4501887	1	6.9	2	2	0.2	Gamma 1 Actin
64	4503987	0.88	4.1	1	1	0.09	Gamma-glutamyl hydrolase precursor
65	38044288	1	6.8	6	21	1.67	Gelsolin isoform b
66	6912618	1	16.3	6	10	0.99	Glutamyl-peptide cyclotransferase precursor
67	4504151	0.99	2.7	1	1	0.1	Granulin
68	4504349	1	15.6	2	2	0.19	Hemoglobin beta chain
69	11321561	1	4.8	2	2	0.2	Hemopexin
70	7427517	1	3	12	20	1.82	Heparan sulfate proteoglycan 2;
71	51471096	0.64	4.8	1	2	0.14	Heterogeneous nuclear ribonucleoprotein A1(Helix-destabilizing protein)
72	39930521	1	11.9	8	13	1.26	Hypothetical protein BC013767
73	51468520	0.44	2.2	1	2	0.06	Hypothetical protein DKFZp586O0120.1 - human (fragment)
74	8923092	0.81	2.9	1	1	0.08	Hypothetical protein FLJ20084
75	23503319	0.83	2.3	1	1	0.09	Hypothetical protein MGC45378
76	4504579	1	4.5	2	3	0.14	I factor (complement)
77	51472926	0.99	8.9	1	3	0.3	Ig heavy chain V-III region VH26 precursor , PREDICTED: similar to KIAA1501 protein
78	51475407	1	14	3	4	0.4	Ig kappa chain
79	51460659	0.77	6	1	1	0.08	Ig kappa variable region
80	30795212	0.47	2.4	1	1	0.05	IGF-II mRNA-binding protein 3; KH domain containing protein overexpressed in cancer
81	21489959	0.99	6.3	1	1	0.11	Immunoglobulin J chain
82	5031809	0.99	3.3	1	1	0.1	Immunoglobulin superfamily containing leucine-rich repeat
83	16445029	0.96	1.6	1	3	0.2	Immunoglobulin superfamily, member 8; CD81 partner 3
84	4504619	1	22.3	5	8	0.73	Insulin-like growth factor binding protein 7

85	31542984	1	5.6	7	11	1.08	Inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein)
86	4504875	1	10.3	2	6	0.61	Kallikrein 1 preproprotein
87	51470760	0.99	2.6	1	1	0.1	KIAA0830 protein
88	4504893	1	28.8	19	35	3.03	Kininogen 1
89	5803023	1	15.7	8	11	1.07	Lectin, mannose-binding 2
90	16418467	1	5.2	2	3	0.19	Leucine-rich alpha-2-glycoprotein 1
91	14150145	1	9.5	4	4	0.4	Limitrin
92	10835248	1	10.2	2	2	0.15	Lithostathine 1 beta
93	21264363	1	30.8	6	19	1.59	Mannan-binding lectin serine protease 2 isoform 1 precursor
94	24497519	1	4.3	2	2	0.21	Mannosidase, alpha, class 1A, member 1
95	4503001	0.48	2.4	1	1	0.05	Mast cell carboxypeptidase A3 precursor
96	13699834	0.99	3.6	1	1	0.1	Matriin 4 isoform 3 precursor
97	6806919	1	1	5	5	0.47	Megalyn
98	4502095	1	2.7	2	2	0.16	Membrane alanine aminopeptidase
99	20270317	0.49	3.4	1	1	0.05	Mitochondrial ribosome recycling factor isoform 1
100	4505395	0.98	0.8	1	3	0.2	Nidogen (enactin)
101	5031985	1	22.8	3	4	0.22	Nuclear transport factor 2; placental protein 15
102	9257232	1	22.9	4	4	0.36	Orosomucoid-1 (alpha-1-acid glycoprotein-1)
103	23943854	0.93	4.9	2	2	0.16	Pepsinogen A5
104	4827036	1	19.9	4	5	0.4	Peptidoglycan recognition protein 1; TNF superfamily, member 3 (LTB)-like (peptidoglycan recognition protein)
105	21361845	1	4.6	2	2	0.19	Peptidoglycan recognition protein L precursor
106	4505621	1	18.2	2	2	0.2	Phosphatidylethanolamine binding protein
107	7110641	0.68	2.7	1	1	0.07	Phospholipase D3
108	6006001	0.92	5.3	1	1	0.1	Plasma glutathione peroxidase 3 precursor
109	4505881	1	5.6	3	4	0.4	Plasminogen
110	4505863	0.99	2.3	2	2	0.14	Plasminogen activator, urokinase
111	19923372	0.71	4.8	2	2	0.09	Polio virus receptor; ortholog of mouse Tage4
112	31377806	1	6.8	5	7	0.43	Polymeric immunoglobulin receptor; hepatocellular carcinoma associated protein TB6
113	51473011	1	32.8	2	2	0.18	Polyubiquitin B
114	4505959	0.71	1.9	1	3	0.11	POU domain, class 2, transcription factor 2

115	4505821	1	21.2	2	2	2	0.21	Prolactin-induced protein
116	4557833	0.91	2	1	9	9	0.25	Propionyl-Coenzyme A carboxylase, alpha polypeptide precursor
117	11386147	0.98	2.9	1	1	1	0.11	Prosaposin (sphingolipid activator protein-1)
118	32171249	1	27.9	7	13	13	1.08	Prostaglandin D2 synthase 21kDa
119	4506153	0.99	4.1	1	1	1	0.1	Prostasin preproprotein
120	4502173	1	11.9	2	2	2	0.21	Prostate specific antigen isoform 1 preproprotein
121	6382064	1	19.2	7	8	8	0.79	Prostatic acid phosphatase precursor
122	4506121	1	11.8	3	5	5	0.5	Protein Z, vitamin K-dependent plasma glycoprotein
123	5803139	0.76	10.6	2	2	2	0.11	RBP4 gene product
124	9966777	0.97	10.2	1	1	1	0.11	Resistin; found in inflammatory zone 3
125	51464234	0.9	1	2	2	2	0.12	Rho GTPase activating protein 10
126	4506549	0.66	9.3	1	1	1	0.07	Ribonuclease, RNase A family, 2 (eosinophil-derived neurotoxin)
127	17511435	1	6.4	4	4	4	0.38	Roundabout homolog 4, magic roundabout
128	4506773	0.99	13.2	1	1	1	0.1	S100 calcium-binding protein A9; calgranulin B; S100 calcium-binding protein A9 (calgranulin B)
129	4506869	0.99	6.5	1	1	1	0.11	Secreted and transmembrane 1 precursor
130	4759166	1	18.3	6	14	14	1.37	Secreted phosphoprotein 1 (osteopontin)
131	32698964	1	23.3	3	7	7	0.54	Secretory protein LOC284013
132	21361198	1	24.6	11	22	22	1.67	Serine (or cysteine) proteinase inhibitor, clade A member 1
133	21361195	1	22.4	7	11	11	1.13	Serine (or cysteine) proteinase inhibitor, clade A member 2
134	22027518	0.75	2.1	1	1	1	0.08	Serine carboxypeptidase vitellogenic-like
135	13775198	0.99	16.1	1	1	1	0.1	SH3 domain binding glutamic acid-rich protein like 3
136	21687060	0.82	4.7	1	1	1	0.08	Similar to common salivary protein 1
137	41150478	0.88	7.2	1	1	1	0.09	Similar to immunoglobulin M chain
138	7019521	0.64	1.8	1	1	1	0.07	Squamous cell carcinoma antigen recognized by T cells 2
139	38202250	0.85	6.4	2	2	2	0.12	Sulfatase modifying factor 1; C-alpha-formylglycine-generating enzyme
140	4507151	1	16.7	4	4	4	0.36	Superoxide dismutase 3, extracellular
141	10092665	0.82	1.2	1	1	1	0.08	Sushi domain containing 2
142	4507557	1	19.8	5	6	6	0.58	Tetranectin (plasminogen binding protein)
143	11641299	0.56	4	1	1	1	0.06	Torsin family 3, member A; ATP-dependant interferon response protein 1

144	4557871	1	25.6	15	17	1.65	Transferrin
145	7305595	0.49	1.7	1	1	0.05	Transportin 2
146	4507725	0.99	8.8	1	1	0.11	Transthyretin
147	4885629	1	21.7	2	2	0.2	Trefoil factor 2 precursor
148	23308722	0.84	1.3	1	1	0.08	TTK protein kinase
149	9966885	1	9.4	5	7	0.63	Tumor endothelial marker 1 precursor; endosialin
150	4759246	0.85	4.7	1	3	0.18	Tumor necrosis factor receptor superfamily, member 18 isoform 1 precursor
151	4507833	1	22	25	63	5.65	Uromodulin; Tamm-Horsfall glycoprotein
152	4507809	1	12.1	2	3	0.26	Uteroglobulin
153	15619010	0.47	0.3	1	1	0.05	Vacuolar protein sorting 13A isoform A
154	4507875	0.89	1.7	1	1	0.09	Vascular cell adhesion molecule 1 isoform a precursor
155	18201911	1	5.6	2	2	0.22	Vitronectin precursor

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It will be understood that various details of the presently disclosed subject matter may be changed without departing from the scope of the disclosed subject matter. Furthermore, the foregoing description is for the purpose of illustration only, and not for the purpose of limitation.

CLAIMS

What is claimed is:

1. A method of isolating membrane vesicles from a biological fluid sample, the method comprising:
 - (a) providing a biological fluid sample comprising membrane vesicles;
 - (b) filtering the biological fluid sample through a filtration module comprising a filter having an average pore diameter of between about 0.01 μm and about 0.15 μm ; and
 - (c) collecting from the filtration module a retentate comprising the membrane vesicles, thereby isolating the membrane vesicles from the biological fluid sample.
2. The method of claim 1, wherein the biological fluid sample provided is a clarified biological fluid sample.
3. The method of claim 2, wherein the biological fluid sample is clarified by low-speed centrifugation and collection of a supernatant comprising the clarified biological fluid sample.
4. The method of claim 3, wherein the biological fluid sample is centrifuged at about 3,000 xg or less.
5. The method of claim 1, wherein the biological fluid sample is selected from the group consisting of blood, blood plasma, and urine.
6. The method of claim 5, wherein the biological fluid sample is urine.
7. The method of claim 6, wherein the urine has been treated with a protease inhibitor.
8. The method of claim 6, wherein the urine has been previously frozen.
9. The method of claim 1, wherein the membrane vesicles are exosomes.
10. The method of claim 9, wherein the exosomes are urinary exosomes.
11. The method of claim 1, wherein the filtration module is a fiber-based filtration cartridge.
12. The method of claim 11, wherein the filter comprises polypropylene hollow fibers.
13. The method of claim 1, wherein the filtration module is a membrane filtration module.

14. The method of claim 13, wherein the filter is a filtration disc comprising hydrophilic polyvinylidene difluoride.
15. The method of claim 1, wherein the filter has an average pore diameter of about 0.1 μm .
16. The method of claim 1, wherein the filter comprises a material selected from the group consisting of polypropylene, polyvinylidene difluoride, polyethylene, polyfluoroethylene, cellulose, secondary cellulose acetate, polysulfone, polyethersulfone, polyvinylalcohol and ethylenevinyl alcohol.
17. The method of claim 1, wherein the retentate comprising the membrane vesicles is collected by washing the retentate from the filtration module.
18. The method of claim 1, wherein the collected retentate comprising the membrane vesicles is resuspended in a buffer solution.
19. A method of identifying biomarker polypeptides, quantitating biomarker polypeptides, or both in a biological fluid sample, the method comprising:
 - (a) providing a biological fluid sample comprising membrane vesicles, wherein the membrane vesicles comprise biomarker polypeptides;
 - (b) filtering the biological fluid sample through a filtration module comprising a filter having an average pore diameter of between about 0.01 μm and about 0.15 μm ;
 - (c) collecting from the filtration module a retentate comprising the membrane vesicles;
 - (d) isolating the biomarker polypeptides from the membrane vesicles; and
 - (e) identifying, quantitating, or both the isolated biomarker polypeptides.
20. The method of claim 19, wherein the biological fluid sample provided is a clarified biological fluid sample.
21. The method of claim 20, wherein the biological fluid sample is clarified by low-speed centrifugation and collection of a supernatant comprising the clarified biological fluid sample.

22. The method of claim 21, wherein the biological fluid sample is centrifuged at about 3,000 xg or less.
23. The method of claim 19, wherein the biological fluid sample is selected from the group consisting of blood, blood plasma, and urine.
24. The method of claim 23, wherein the biological fluid sample is urine.
25. The method of claim 24, wherein the urine has been treated with a protease inhibitor.
26. The method of claim 24, wherein the urine has been previously frozen.
27. The method of claim 19, wherein the membrane vesicles are exosomes.
28. The method of claim 27, wherein the exosomes are urinary exosomes.
29. The method of claim 19, wherein the filtration module is a fiber-based filtration cartridge.
30. The method of claim 29, wherein the filter comprises polypropylene hollow fibers.
31. The method of claim 19, wherein the filtration module is a membrane filtration module.
32. The method of claim 31, wherein the filter is a filtration disc comprising hydrophilic polyvinylidene difluoride.
33. The method of claim 19, wherein the filter has an average pore diameter of about 0.1 μm .
34. The method of claim 19, wherein the filter comprises a material selected from the group consisting of polypropylene, polyvinylidene difluoride, polyethylene, polyfluoroethylene, cellulose, secondary cellulose acetate, polysulfone, polyethersulfone, polyvinylalcohol, and ethylenevinyl alcohol.
35. The method of claim 19, wherein the retentate comprising the membrane vesicles is collected by washing the retentate from the filtration module.
36. The method of claim 19, wherein the collected retentate comprising the membrane vesicles is resuspended in a buffer solution.
37. The method of claim 19, wherein the biomarker peptides are identified, quantitated, or both by immunoassay, mass spectrometry, or both.

38. The method of claim 37, wherein the mass spectrometry is matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI MS).
39. The method of claim 37, wherein the immunoassay is selected from the group consisting of Western blot, enzyme-linked immunoassay (ELISA), radioimmunoassay (RIA), and competitive binding assay.
40. A method of isolating membrane vesicle biomarker polypeptides from a biological fluid sample, the method comprising:
 - (a) providing a biological fluid sample comprising membrane vesicles, wherein the membrane vesicles comprise biomarker polypeptides;
 - (b) filtering the biological fluid sample through a filtration module comprising a filter having an average pore diameter of between about 0.01 μm and about 0.15 μm ;
 - (c) collecting from the filtration module a retentate comprising the membrane vesicles; and
 - (d) isolating the biomarker polypeptides from the membrane vesicles.
41. The method of claim 40, wherein the biological fluid sample provided is a clarified biological fluid sample.
42. The method of claim 41, wherein the biological fluid sample is clarified by low-speed centrifugation and collection of a supernatant comprising the clarified biological fluid sample.
43. The method of claim 42, wherein the biological fluid sample is centrifuged at about 3,000 xg or less.
44. The method of claim 40, wherein the biological fluid sample is selected from the group consisting of blood, blood plasma, and urine.
45. The method of claim 44, wherein the biological fluid sample is urine.
46. The method of claim 45, wherein the urine has been treated with a protease inhibitor.
47. The method of claim 45, wherein the urine has been previously frozen.
48. The method of claim 40, wherein the membrane vesicles are exosomes.
49. The method of claim 48, wherein the exosomes are urinary exosomes.
50. The method of claim 40, wherein the filtration module is a fiber-based filtration cartridge.

51. The method of claim 50, wherein the filter comprises polypropylene hollow fibers.
52. The method of claim 40, wherein the filtration module is a membrane filtration module.
53. The method of claim 52, wherein the filter is a filtration disc comprising hydrophilic polyvinylidene difluoride.
54. The method of claim 40, wherein the filter has an average pore diameter of about 0.1 μm .
55. The method of claim 40, wherein the filter comprises a material selected from the group consisting of polypropylene, polyvinylidene difluoride, polyethylene, polyfluoroethylene, cellulose, secondary cellulose acetate, polysulfone, polyethersulfone, polyvinylalcohol, and ethylenevinyl alcohol.
56. The method of claim 40, wherein the retentate comprising the membrane vesicles is collected by washing the retentate from the filtration module.
57. The method of claim 40, wherein the collected retentate comprising the membrane vesicles is resuspended in a buffer solution.
58. The method of claim 40, wherein the biomarker peptides are isolated by electrophoretic separation, immunoisolation, chromatography, or combinations thereof.
59. A method of diagnosing a disorder or measuring a disorder state in a subject, the method comprising:
 - (a) providing a biological fluid sample comprising membrane vesicles, wherein the membrane vesicles comprise biomarker polypeptides;
 - (b) filtering the biological fluid sample through a filtration module comprising a filter having an average pore diameter of between about 0.01 μm and about 0.15 μm ;
 - (c) collecting from the filtration module a retentate comprising the membrane vesicles;
 - (d) isolating the biomarker polypeptides from the membrane vesicles;
and
 - (e) identifying, quantitating, or both the isolated biomarker polypeptides, wherein the identified and/or quantitated biomarker

polypeptides indicates the presence of a disorder or is a measure of a disorder state in the subject.

60. The method of claim 59, wherein the biological fluid sample provided is a clarified biological fluid sample.
61. The method of claim 60, wherein the biological fluid sample is clarified by low-speed centrifugation and collection of a supernatant comprising the clarified biological fluid sample.
62. The method of claim 61, wherein the biological fluid sample is centrifuged at about 3,000 xg or less.
63. The method of claim 59, wherein the biological fluid sample is selected from the group consisting of blood, blood plasma, and urine.
64. The method of claim 63, wherein the biological fluid sample is urine.
65. The method of claim 64, wherein the urine has been treated with a protease inhibitor.
66. The method of claim 64, wherein the urine has been previously frozen.
67. The method of claim 59, wherein the membrane vesicles are exosomes.
68. The method of claim 67, wherein the exosomes are urinary exosomes.
69. The method of claim 59, wherein the filtration module is a fiber-based filtration cartridge.
70. The method of claim 69, wherein the filter comprises polypropylene hollow fibers.
71. The method of claim 59, wherein the filtration module is a membrane filtration module.
72. The method of claim 71, wherein the filter is a filtration disc comprising hydrophilic polyvinylidene difluoride.
73. The method of claim 59, wherein the filter has an average pore diameter of about 0.1 μm .
74. The method of claim 59, wherein the filter comprises a material selected from the group consisting of polypropylene, polyvinylidene difluoride, polyethylene, polyfluoroethylene, cellulose, secondary cellulose acetate, polysulfone, polyethersulfone, polyvinylalcohol, and ethylenevinyl alcohol.

75. The method of claim 59, wherein the retentate comprising the membrane vesicles is collected by washing the retentate from the filtration module.
76. The method of claim 59, wherein the collected retentate comprising the membrane vesicles is resuspended in a buffer solution.
77. The method of claim 59, wherein the biomarker peptides are identified, quantitated, or both by immunoassay, mass spectrometry, or both.
78. The method of claim 77, wherein the mass spectrometry is matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI MS).
79. The method of claim 77, wherein the immunoassay is selected from the group consisting of Western blot, enzyme-linked immunoassay (ELISA), radioimmunoassay (RIA), and competitive binding assay.
80. The method of claim 59, wherein the disorder is selected from the group consisting of diabetes, water-balance disorders, acute kidney injury, glomerulonephritis, drug-induced acute renal failure and allergy, acute and chronic kidney transplant rejection, inherited renal diseases, myocardial ischemia, cardiovascular risk, prostatic hypertrophy and prostatic cancer, systemic lupus erythematosus, and rheumatoid arthritis.
81. A kit for identifying biomarker polypeptides, quantitating biomarker polypeptides, or both in a biological sample, the kit comprising:
 - (a) a filtration module comprising a filter having an average pore diameter of between about 0.01 μm and about 0.15 μm ;
 - (b) one or more antibodies or fragments thereof that specifically recognizes a biomarker polypeptide of interest; and
 - (c) instructions for using the kit.
82. The kit of claim 81, wherein the one or more antibodies or fragments thereof are bound to a substrate.

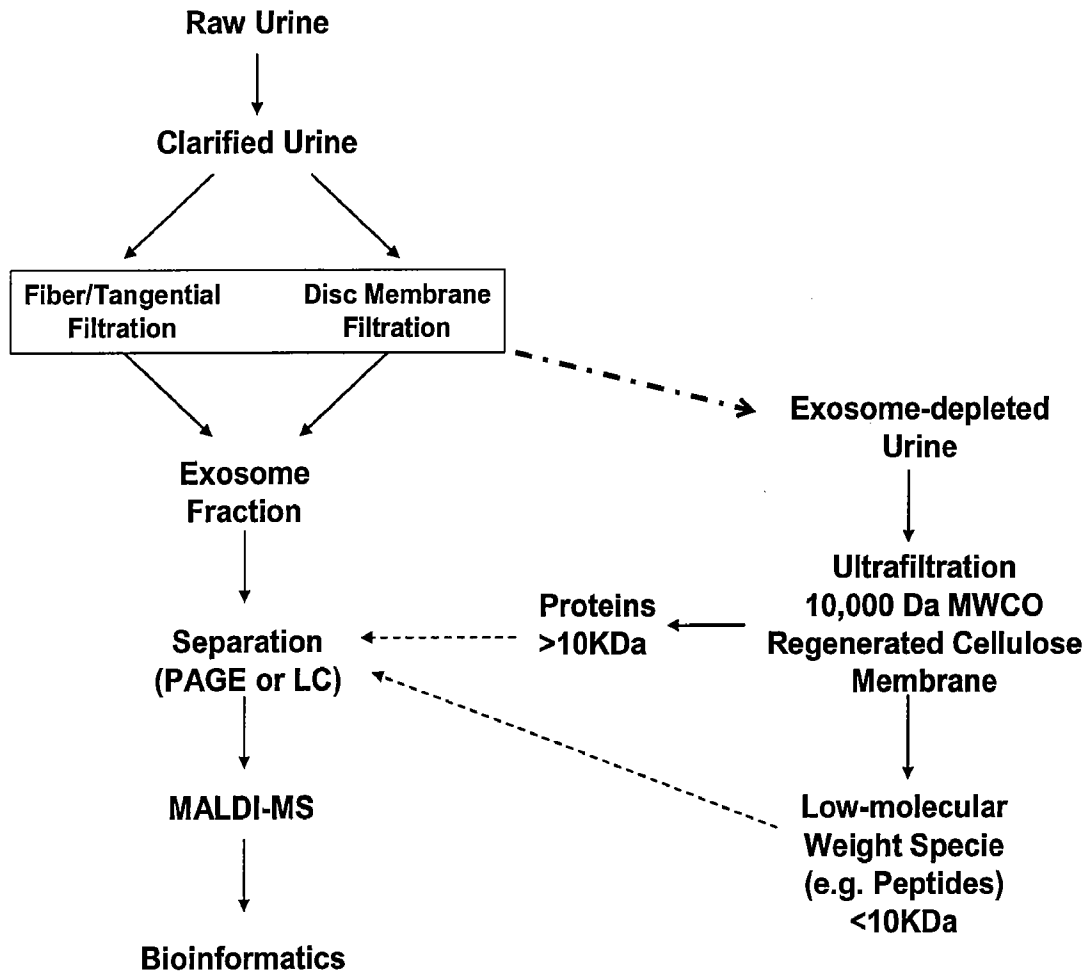


Figure 1

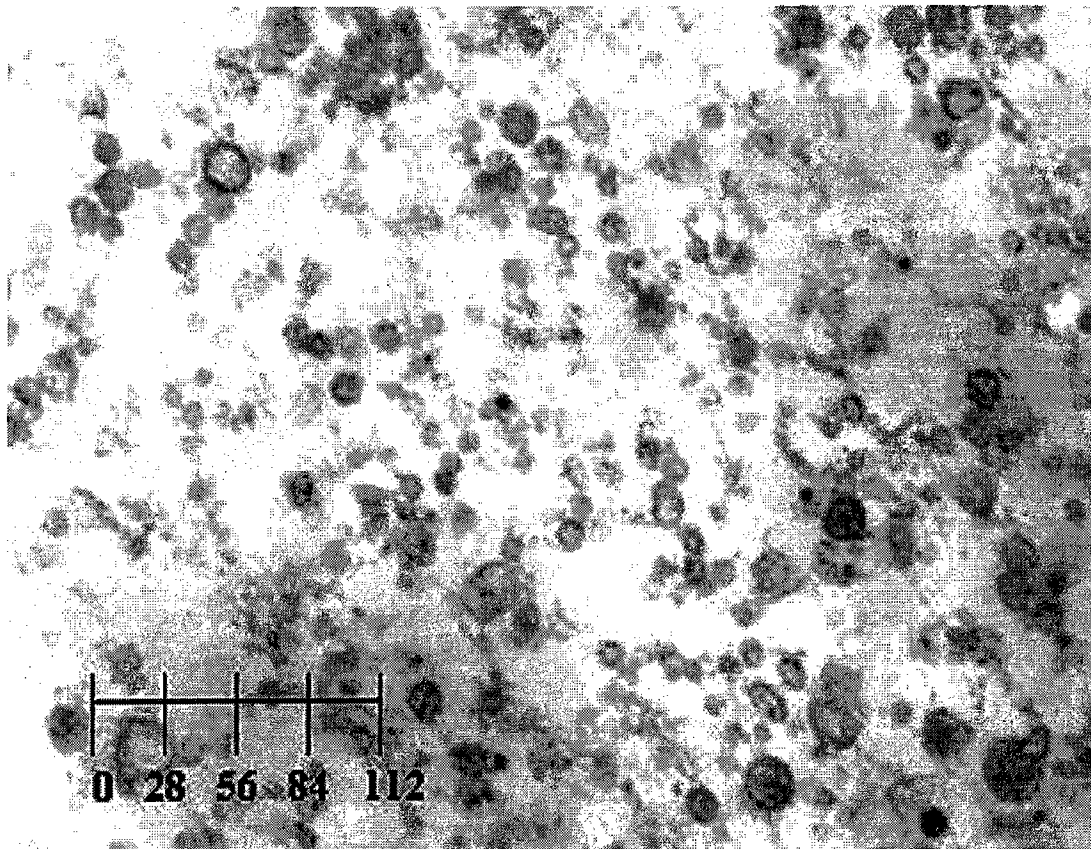


Figure 2

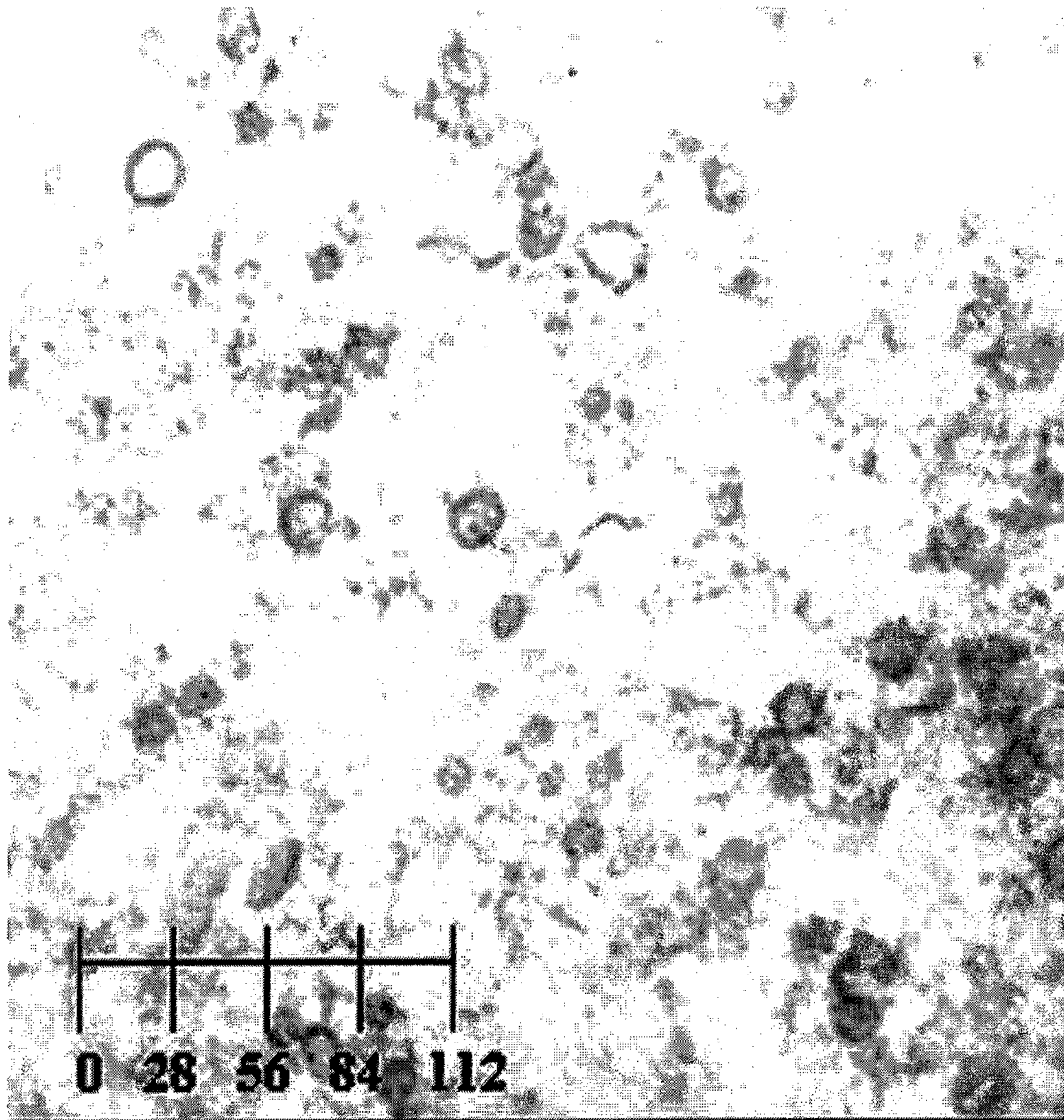


Figure 3

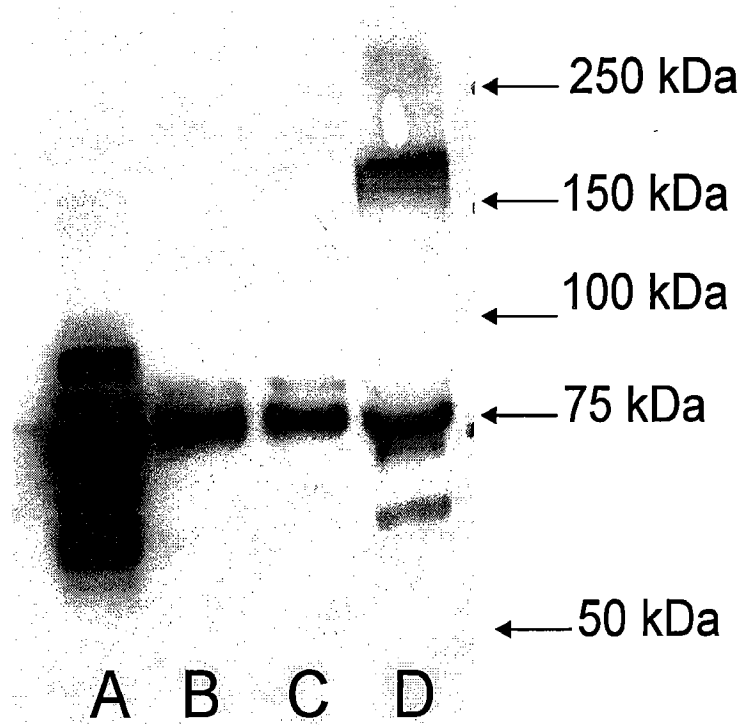


Figure 4

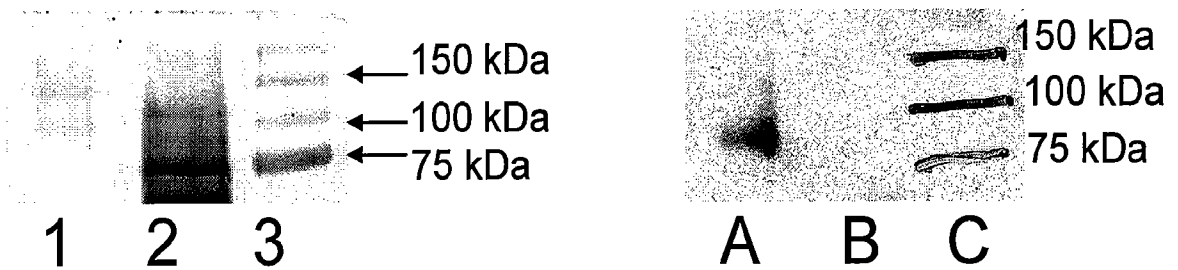


Figure 5

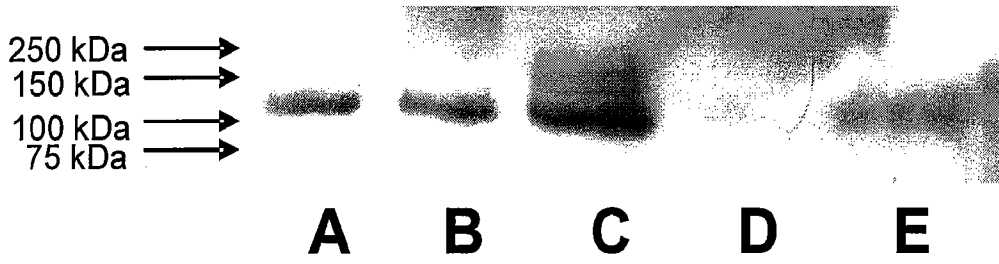


Figure 6

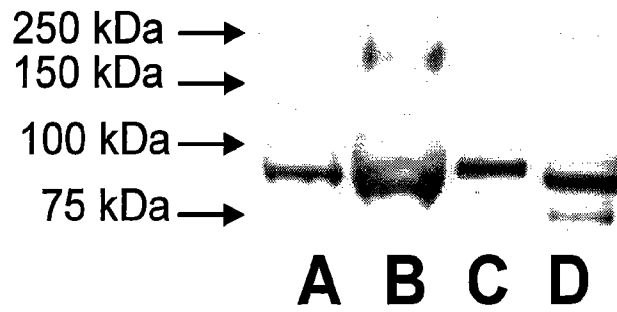


Figure 7

1D-SDS PAGE/MALDI-TOF-TOF Analysis of Proteins Co-purifying with Urinary Exosomes

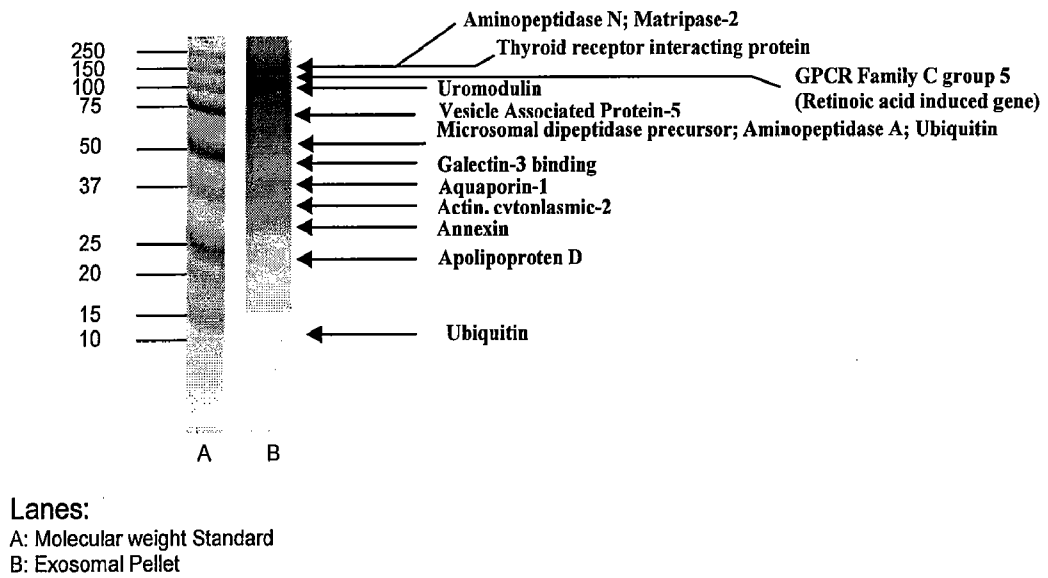


Figure 8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US07/67509

A. CLASSIFICATION OF SUBJECT MATTER
 IPC: C12Q 1/26(2006.01)

USPC: 435/25
 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)
 U.S. : 435/25

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 practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,812,023 B1 (LAMPARSKI et al) 02 November 2004 (02.11.2004), col. 15, lines 30-67; col. 16, lines 1-60; col. 17, lines 50-67; col. 18, lines 1-67; and col. 19-20	1-82
Y	Pisitkun et al. "Identification and proteomic profiling of exosomes in human urine," (PNAS), 07 September 2004, Vol. 101, No. 36, pgs. 13368-13373, published online August 23, 2004	1-82
Y	HOORN et al. "Prospects for urinary proteomics: Exosomes as a source of urinary biomarkers," (Nephrology), June 2005, Vol. 10, pgs.283-290	1-82
A	ZHOU et al, "Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery," (Kidney International), 2006, Vol. 69, pgs. 1471-1476	1-82

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	"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
"E" earlier application or patent published on or after the international filing date	"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
"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)	"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
"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	"&" document member of the same patent family

Date of the actual completion of the international search 03 September 2007 (03.09.2007)	Date of mailing of the international search report 01 OCT 2007
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for