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(71) Applicant: **ABBOTT LABORATORIES** [US/US]; 100 Abbott Park Road, Abbott Park, Illinois 60064 (US).

(72) Inventors: **BELIGERE, Gangamani S.**; c/o Abbott Laboratories, 100 Abbott Park Road, Abbott Park, Illinois 60064 (US). **BRENNAN, Melissa B.**; c/o Abbott Laboratories, 100 Abbott Park Road, Abbott Park, Illinois 60064 (US). **GRIESHABER, Jessica**; c/o Abbott Laboratories, 100 Abbott Park Road, Abbott Park, Illinois 60064 (US). **PACENTI, David**; c/o Abbott Laboratories, 100 Abbott Park Road, Abbott Park, Illinois 60064 (US). **DATWYLER, Saul A.**; c/o Abbott Laboratories, 100 Abbott Park Road, Abbott Park, Illinois 60064 (US). **RAMP, John**; c/o Abbott Laboratories, 100 Abbott Park Road, Abbott Park, Illinois 60064 (US).

(74) Agent: **MUELLER, Lisa V.**; Casimir Jones, S.C., 2275 Deming Way, Suite 310, Middleton, Wisconsin 53562 (US).

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(54) Title: IMPROVED METHODS FOR MEASURING UBIQUITIN CARBOXY-TERMINAL HYDROLASE L1 LEVELS IN BLOOD

(57) Abstract: Disclosed herein are improved methods of processing, measuring, and detecting levels of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) in blood samples taken from a human subject at time points within about 8 hours (or about 8 hours or less) after obtaining the sample from the subject. UCH-L1 is an early biomarker for traumatic brain injury (TBI), and there is a need for improved methods for assessing UCH-L1 in blood can aid in the diagnosis and evaluation of a human subject who has sustained or may have sustained a head injury.

# IMPROVED METHODS FOR MEASURING UBIQUITIN CARBOXY-TERMINAL HYDROLASE L1 LEVELS IN BLOOD

## RELATED APPLICATION INFORMATION

**[0001]** This application claims priority to U.S. 62/528,187 filed on July 3, 2017, the contents of which are herein incorporated by reference.

## TECHNICAL FIELD

**[0002]** The present disclosure relates to improved methods of processing, measuring, and detecting levels of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) in blood samples taken from a human subject at time points within about 8 hours (or about 8 hours or less) after obtaining the sample from the subject. UCH-L1 is an early biomarker for traumatic brain injury (TBI); therefore, improved methods for assessing UCH-L1 in blood can aid in the diagnosis and evaluation of a human subject who has sustained or may have sustained a head injury.

## BACKGROUND

**[0003]** More than 5 million mild traumatic brain injuries (TBIs) occur each year in the United States alone. Clinical tools such as physical exams and questionnaires are subjective and lack sensitivity and specificity. Computerized tomography (CT) scan or magnetic resonance imaging (MRI) are costly, not available in all parts of the world and are routinely done only if the clinical tools and/or questionnaire suggests that there is a need to perform such imaging. Therefore, high false negative rates have been associated with the diagnosis and evaluation of TBI. Clinicians and patients need objective, reliable information to accurately evaluate this condition to promote appropriate triage and recovery.

**[0004]** In some cases, biomarkers for TBI have been identified, including ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), lactate dehydrogenase (LDH), glial fibrillary acid protein (GFAP), neuron specific enolase (NSE), and S-100β. Although the use of biomarker data is promising for providing the objective and reliable diagnostic information sought by clinicians and patients, limited data have been available for the use of TBI biomarkers in the acute care setting to aid in patient evaluation and management.

**[0005]** Moreover, it has been challenging to determine the most advantageous clinical parameters and conditions for obtaining and processing patient samples, so that the specificity and accuracy provided by biomarker assessment can be established and maintained after obtaining samples from a patient. For example, it is well known in the art that preanalytical errors are quite frequent in the area of blood testing, including point-of-care testing ((See, Heyer, et al., Effectiveness of practices to reduce blood sample hemolysis in EDs: A laboratory medicine best practices systemic review and meta-analysis, *Clin. Biochem.*, 45(0):1012-1032 (September 2012), the contents of which are herein incorporated by reference)). Unfortunately, approximately 60-80% of laboratory errors occur outside of the analytical phase and most often outside of the laboratory (e.g., namely, during the preanalytical and post-analytical phase). Moreover, it is the preanalytical phase that is most vulnerable to errors. Some of the most common preanalytical errors include:

**[0006]** 1. Patient preparation errors such as sampling at the incorrect time. Sampling time is important because it is known that many analytes and blood parameters vary with time during the day, month or even seasons.

**[0007]** 2. Sampling techniques such as "milking" during capillary sample and venous sampling using a syringe. Excessive massage and squeezing around the puncture site (commonly called "milking") is often done when capillary blood flow is not adequate to obtain the necessary blood volume to fill the tube. Excessive massage can cause falsely decreased concentrations (up to 10%) of some analysis due to the dilution of the blood samples with tissue fluid and falsely increased concentration of potassium due to sample hemolysis. The use of syringes generally increases the risk of hemolysis and clot formation.

**[0008]** 3. Underfilled or incorrectly filled collection tubes. When collection tubes with additives are used, care must be taken during sampling to fill the tubes until the vacuum is exhausted and blood flow ceases. It is important to ensure the proper blood-to-additive ratio. Underfilled tubes are one of the most common preanalytical errors. The quantity of the additive (e.g., anticoagulant) in tubes is such to ensure the proper ratio of blood and anticoagulant if the tube is completely filled.

**[0009]** 4. Hemolysis caused by blood lysis. Hemolysis is the most frequent preanalytical error. It is also the most common cause of sample rejection by a central laboratory.

Hemolysis cases interference with many assays due to the release of the blood-cell components from the lysed blood cells. When blood samples are hemolyzed, a new clinical sample is often required.

**[0010]** Thus, there is a need for improved methods of obtaining and processing samples that will reduce preanalytical errors thereby allowing for a more accurate measurement of biomarkers of TBI that can be used to diagnose and treat patients, especially in the context of acute care.

## SUMMARY

**[0011]** The present disclosure is directed to an improvement of a method of measuring an amount of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) in a blood sample obtained from a subject. In one embodiment, the method includes processing the sample from the subject within no more than about eight hours (e.g., eight hours or less) after the sample is obtained from the subject, in order to avoid a rise in UCH-L1 level that results from storage of the sample. For example, in one aspect, the sample is processed within a period of time after the sample is obtained from the subject of from about zero hours to about 6 hours. For example, in another aspect, the sample is processed within a period of time after the sample is obtained from the subject of from about zero hours to about 2 hours. In yet another example, the sample is processed within a period of time after the sample is obtained from the subject of from about from about zero hours to about 1 hour. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of from about 1 hour to about 8 hours. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of from about 1 hour to about 6 hours. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of from about 1 hour to about 2 hours. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of about 8 hours or less. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of about 6 hours or less. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of about 4 hours or less. In still yet another

example, the sample is processed within a period of time after the sample is obtained from the subject of about 2 hours or less. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of about 1 hour or less.

**[0012]** In another aspect, the above described method further comprises subsequently performing an assay or test to measure the amount of UCH-L1 in the sample.

**[0013]** In yet another aspect, the above described method further comprises subsequently performing an assay or test to detect UCH-L1 in the sample.

**[0014]** In yet another aspect, in the above described method, the processing of the sample comprises separating plasma from blood cells in the sample and subsequently performing an assay using the plasma that measures the amount of UCH-L1 in the sample.

**[0015]** In yet another aspect, in the above described method, the sample is obtained from the subject using a container comprising an anticoagulant selected from the group consisting of heparin and EDTA.

**[0016]** In yet another aspect, in the above described method, the processing of the sample comprises separating serum from any clots that arise in the sample and, subsequently performing an assay using the serum that measures the amount of UCH-L1 in the sample.

**[0017]** In the above described method, the sample is collected using a serum collection tube.

**[0018]** In the above described method, the assay comprises any method by which UCH-L1 amount can be assessed (e.g, UCH-L1 is detected and/or measured). For example, the assay can be selected from the group consisting of an immunoassay, chemical analysis, SDS PAGE and Western blot analysis, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, chromatography, and spectrophotometry. In one aspect, the assay employed is in a clinical chemistry format. In another aspect, the assay employed is an immunoassay comprising:

a) contacting the sample, either simultaneously or sequentially, in any order with:
(1) at least one capture antibody, which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a capture antibody-UCH-L1 antigen complex, and
(2) at least one detection antibody which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the capture antibody, to form a UCH-L1 antigen-detection antibody complex,

such that a capture antibody-UCH-L1 antigen-detection antibody complex is formed, and

b) measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the capture antibody-UCH-L1 antigen-detection antibody complex.

**[0019]** In yet another aspect, in the above described method, the sample is maintained at room temperature for some period of time between the time point that the sample is obtained from the subject and the time point when the assay is performed.

**[0020]** In yet another aspect, in the above described method, the sample is maintained at a temperature from about 2 °C to about 8 °C for some period of time between the time point that the sample is obtained from the subject and the time point when the assay is performed.

**[0021]** In yet another aspect, in the above described method, the sample is not mixed during or between the period of time between the time point that the sample is obtained from the subject and the time point when the assay is performed.

**[0022]** In yet another aspect, in the above described method, the UCH-L1 amount in the sample from the subject is assessed as a measure of traumatic brain injury, wherein the subject has sustained or may have sustained an injury to the head.

**[0023]** In another embodiment, the present invention is directed to a method of avoiding or preventing an increase or rise in UCH-L1 levels between the period of time a whole blood sample is obtained from a subject and prior to performing an assay on the sample (such as, for example to detected and/or measure the amount of UCH-L1 in the sample). Specifically, the method avoids or prevents an erroneously high level of UCH-L1 in the sample between the period of time the sample is obtained from the subject and prior to performing the assay, which can lead to false positives, erroneous or other mistaken results. The method involves the step of processing the sample within no more than about eight hours (such as, for example, 8 hours or less) after the sample is obtained from the subject to avoid an increase or rise in UCH-L1 level that results from storage of the sample prior to performing the assay. For example, in one aspect, the sample is processed within a period of time after the sample is obtained from the subject of from about zero hours to about 6 hours. For example, in another aspect, the sample is processed within a period of time after the sample is obtained

from the subject of from about zero hours to about 2 hours. In yet another example, the sample is processed within a period of time after the sample is obtained from the subject of from about from about zero hours to about 1 hour. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of from about 1 hour to about 8 hours. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of from about 1 hour to about 6 hours. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of from about 1 hour to about 2 hours. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of about 8 hours or less. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of about 6 hours or less. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of about 4 hours or less. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of about 2 hours or less. In still yet another example, the sample is processed within a period of time after the sample is obtained from the subject of about 1 hour or less.

**[0024]** In another aspect, the above described method further comprises subsequently performing an assay or test to measure the amount of UCH-L1 in the sample.

**[0025]** In yet another aspect, the above described method further comprises subsequently performing an assay or test to detect UCH-L1 in the sample.

**[0026]** In yet another aspect, in the above described method, the processing of the sample comprises separating plasma from blood cells in the sample and subsequently performing an assay using the plasma that measures the amount of UCH-L1 in the sample.

**[0027]** In yet another aspect, in the above described method, the sample is obtained from the subject using a container comprising an anticoagulant selected from the group consisting of heparin and EDTA.

**[0028]** In yet another aspect, in the above described method, the processing of the sample comprises separating serum from any clots that arise in the sample and, subsequently performing an assay using the serum that measures the amount of UCH-L1 in the sample.

**[0029]** In the above described method, the sample is collected using a serum collection tube.

**[0030]** In the above described method, the assay comprises any method by which UCH-L1 amount can be assessed (e.g, UCH-L1 is detected and/or measured). For example, the assay can be selected from the group consisting of an immunoassay, chemical analysis, SDS PAGE and Western blot analysis, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, chromatography, and spectrophotometry. In one aspect, the assay employed is in a clinical chemistry format. In another aspect, the assay employed is an immunoassay comprising:

a) contacting the sample, either simultaneously or sequentially, in any order with:
(1) at least one capture antibody, which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a capture antibody-UCH-L1 antigen complex, and
(2) at least one detection antibody which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the capture antibody, to form a UCH-L1 antigen-detection antibody complex,
such that a capture antibody-UCH-L1 antigen-detection antibody complex is formed, and

b) measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the capture antibody-UCH-L1 antigen-detection antibody complex.

**[0031]** In yet another aspect, in the above described method, the sample is maintained at room temperature for some period of time between the time point that the sample is obtained from the subject and the time point when the assay is performed.

**[0032]** In yet another aspect, in the above described method, the sample is maintained at a temperature from about 2 °C to about 8 °C for some period of time between the time point that the sample is obtained from the subject and the time point when the assay is performed.

**[0033]** In yet another aspect, in the above described method, the sample is not mixed during or between the period of time between the time point that the sample is obtained from the subject and the time point when the assay is performed.

**[0034]** In yet another aspect, in the above described method, the UCH-L1 amount in the sample from the subject is assessed as a measure of traumatic brain injury, wherein the subject has sustained or may have sustained an injury to the head.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0035]** FIGS. 1A-1B show representative bar graphs of UCH-L1 levels in whole blood samples.

**[0036]** FIGS. 2A-2B show representative bar graphs of UCH-L1 levels in plasma samples.

**[0037]** FIGS. 3A-3B show representative bar graphs of UCH-L1 levels in whole blood samples obtained using lithium heparin (FIG. 3A) or EDTA (FIG. 3B) collection tubes.

**[0038]** FIGS. 4A-4B show representative bar graphs of UCH-L1 levels in plasma samples obtained using with EDTA collection tubes at room temperature (FIG. 4A) or 2-8 °C (FIG. 4B).

**[0039]** FIGS. 5A-5B show representative bar graphs of UCH-L1 levels in plasma samples obtained using with lithium heparin collection tubes at room temperature (FIG. 5A) or 2-8 °C (FIG. 5B).

**[0040]** FIGS. 6A-6B show representative bar graphs of UCH-L1 levels in serum samples at room temperature (FIG. 6A) or 2-8 °C (FIG. 6B).

**[0041]** FIG. 7 shows a representative bar graph of UCH-L1 levels in serum samples with or without exposure to a blood clot.

**[0042]** FIG. 8 shows a representative line graph comparing hemoglobin (Hgb) levels to UCH-L1 levels in hemolysate samples spiked with plasma from EDTA collection tubes.

**[0043]** FIG. 9 shows a representative bar graph of UCH-L1 levels in whole blood samples obtained using EDTA collection tubes spiked with UCH-L1 recombinant analyte.

## DETAILED DESCRIPTION

**[0044]** The present disclosure relates to improved methods of processing, measuring, and detecting levels of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) in blood samples taken from a human subject at time points within about 8 hours or about 8 hours or less after obtaining the sample from the subject. The methods disclosed herein can be useful for assessing UCH-L1 (generally considered a biomarker of TBI) in blood to aid in the diagnosis and evaluation of a human subject who has sustained or may have sustained a head injury. Additionally, studies have reported inconsistent UCH-L1 levels in blood over the first 24 hour period after the blood samples have been obtained from a subject, and at least part of

this increase may be due to preanalytical processing conditions or factors that take place or occur prior to the time that the samples are tested or an assay is performed (e.g., to detect and/or measure UCH-L1 levels). For example, it has been reported that certain standard processing conditions that cause hemolysis have little to no effect on UCH-L1 levels. However, the present disclosure has surprisingly found that certain preanalytical processing conditions and methods can cause an increase (namely, an erroneous increase) in UCH-L1 levels in whole blood, thus resulting in false positives; such false positives can undermine thge accuracy of using UCH-L1 as a diagnostic marker for aiding in the detection, determination and/or assessment of disorders such as TBI.

**[0045]** Thus, the present disclosure provides methods to improve the manner in which UCH-L1 can be obtained and measured in samples, such as in blood samples (e.g., whole blood or plasma). Specifically, the present disclosure provides methods of avoiding or preventing an increase or rise in ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) level in a blood sample (e.g., whole blood sample or a plasma sample) obtained from a subject prior to performing a test or an assay. More specifically, methods described herein avoid or prevent an erroneously high level of UCH-L1 in the sample between the period of time the sample is obtained from the subject and prior to performing the assay. Such methods involve processing a blood sample within no more than about eight hours after the sample is obtained from the subject to avoid an increase or rise in UCH-L1 level that results from storing the sample prior to performing the assay. Such methods are effective to stabilize the levels of UCH-L1 in the blood sample until such time as a test or assay is performed to assess (e.g., detect and/or measure) the UCH-L1 in the sample.

**[0046]** Section headings as used in this section and the entire disclosure herein are merely for organizational purposes and are not intended to be limiting.

## 1. Definitions

**[0047]** Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. In case of conflict, the present document, including definitions, will control. Preferred methods and materials are described below, although methods and materials similar or equivalent to those described

herein can be used in practice or testing of the present disclosure. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples disclosed herein are illustrative only and not intended to be limiting.

**[0048]** The terms "comprise(s)," "include(s)," "having," "has," "can," "contain(s)," and variants thereof, as used herein, are intended to be open-ended transitional phrases, terms, or words that do not preclude the possibility of additional acts or structures. The singular forms "a," "an" and "the" include plural references unless the context clearly dictates otherwise. The present disclosure also contemplates other embodiments "comprising," "consisting of" and "consisting essentially of," the embodiments or elements presented herein, whether explicitly set forth or not.

**[0049]** For the recitation of numeric ranges herein, each intervening number there between with the same degree of precision is explicitly contemplated. For example, for the range of 6-9, the numbers 7 and 8 are contemplated in addition to 6 and 9, and for the range 6.0-7.0, the number 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, and 7.0 are explicitly contemplated.

**[0050]** "Antibody" and "antibodies" as used herein refers to monoclonal antibodies, multispecific antibodies, human antibodies, humanized antibodies (fully or partially humanized), animal antibodies such as, but not limited to, a bird (for example, a duck or a goose), a shark, a whale, and a mammal, including a non-primate (for example, a cow, a pig, a camel, a llama, a horse, a goat, a rabbit, a sheep, a hamster, a guinea pig, a cat, a dog, a rat, a mouse, etc.) or a non-human primate (for example, a monkey, a chimpanzee, etc.), recombinant antibodies, chimeric antibodies, single-chain Fvs ("scFv"), single chain antibodies, single domain antibodies, Fab fragments, F(ab') fragments, $F(ab')_2$ fragments, disulfide-linked Fvs ("sdFv"), and anti-idiotypic ("anti-Id") antibodies, dual-domain antibodies, dual variable domain (DVD) or triple variable domain (TVD) antibodies (dual-variable domain immunoglobulins and methods for making them are described in Wu, C., et al., *Nature Biotechnology*, 25(11):1290-1297 (2007) and PCT International Application WO 2001/058956, the contents of each of which are herein incorporated by reference), and functionally active epitope-binding fragments of any of the above. In particular, antibodies include immunoglobulin molecules and immunologically active fragments of

immunoglobulin molecules, namely, molecules that contain an analyte-binding site. Immunoglobulin molecules can be of any type (for example, IgG, IgE, IgM, IgD, IgA, and IgY), class (for example, IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2), or subclass. For simplicity sake, an antibody against an analyte is frequently referred to herein as being either an "anti-analyte antibody" or merely an "analyte antibody" (e.g., an anti-UCH-L1 antibody or a UCH-L1 antibody).

**[0051]** "Affinity matured antibody" is used herein to refer to an antibody with one or more alterations in one or more CDRs, which result in an improvement in the affinity (*i.e.*, $K_D$, $k_d$ or $k_a$) of the antibody for a target antigen compared to a parent antibody, which does not possess the alteration(s). Exemplary affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. A variety of procedures for producing affinity matured antibodies is known in the art, including the screening of a combinatory antibody library that has been prepared using bio-display. For example, Marks *et al.*, *BioTechnology*, 10: 779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by Barbas *et al.*, *Proc. Nat. Acad. Sci. USA*, 91: 3809-3813 (1994); Schier *et al.*, *Gene*, 169: 147-155 (1995); Yelton *et al.*, *J. Immunol.*, 155: 1994-2004 (1995); Jackson *et al.*, *J. Immunol.*, 154(7): 3310-3319 (1995); and Hawkins *et al*, J. Mol. Biol., 226: 889-896 (1992). Selective mutation at selective mutagenesis positions and at contact or hypermutation positions with an activity-enhancing amino acid residue is described in U.S. Patent No. 6,914,128 B1.

**[0052]** "Antibody fragment" as used herein refers to a portion of an intact antibody comprising the antigen-binding site or variable region. The portion does not include the constant heavy chain domains (i.e., CH2, CH3, or CH4, depending on the antibody isotype) of the Fc region of the intact antibody. Examples of antibody fragments include, but are not limited to, Fab fragments, Fab' fragments, Fab'-SH fragments, $F(ab')_2$ fragments, Fd fragments, Fv fragments, diabodies, single-chain Fv (scFv) molecules, single-chain polypeptides containing only one light chain variable domain, single-chain polypeptides containing the three CDRs of the light-chain variable domain, single-chain polypeptides containing only one heavy chain variable region, and single-chain polypeptides containing the three CDRs of the heavy chain variable region.

**[0053]** The "area under curve" or "AUC" refers to area under a ROC curve. AUC under a ROC curve is a measure of accuracy. An AUC of 1 represents a perfect test, whereas an AUC of 0.5 represents an insignificant test. A preferred AUC may be at least approximately 0.700, at least approximately 0.750, at least approximately 0.800, at least approximately 0.850, at least approximately 0.900, at least approximately 0.910, at least approximately 0.920, at least approximately 0.930, at least approximately 0.940, at least approximately 0.950, at least approximately 0.960, at least approximately 0.970, at least approximately 0.980, at least approximately 0.990, or at least approximately 0.995.

**[0054]** "Bead" and "particle" are used herein interchangeably and refer to a substantially spherical solid support. One example of a bead or particle is a microparticle. Microparticles that can be used herein can be any type known in the art. For example, the bead or particle can be a magnetic bead or magnetic particle. Magnetic beads/particles may be ferromagnetic, ferrimagnetic, paramagnetic, superparamagnetic or ferrofluidic. Exemplary ferromagnetic materials include Fe, Co, Ni, Gd, Dy, $CrO_2$, MnAs, MnBi, EuO, and NiO/Fe. Examples of ferrimagnetic materials include $NiFe_2O_4$, $CoFe_2O_4$, $Fe_3O_4$ (or $FeO{\cdot}Fe_2O_3$). Beads can have a solid core portion that is magnetic and is surrounded by one or more non-magnetic layers. Alternately, the magnetic portion can be a layer around a non-magnetic core. The microparticles can be of any size that would work in the methods described herein, *e.g.,* from about 0.75 to about 5 nm, or from about 1 to about 5 nm, or from about 1 to about 3 nm.

**[0055]** "Binding protein" is used herein to refer to a monomeric or multimeric protein that binds to and forms a complex with a binding partner, such as, for example, a polypeptide, an antigen, a chemical compound or other molecule, or a substrate of any kind. A binding protein specifically binds a binding partner. Binding proteins include antibodies, as well as antigen-binding fragments thereof and other various forms and derivatives thereof as are known in the art and described herein below, and other molecules comprising one or more antigen-binding domains that bind to an antigen molecule or a particular site (epitope) on the antigen molecule. Accordingly, a binding protein includes, but is not limited to, an antibody a tetrameric immunoglobulin, an IgG molecule, an IgG1 molecule, a monoclonal antibody, a

chimeric antibody, a CDR-grafted antibody, a humanized antibody, an affinity matured antibody, and fragments of any such antibodies that retain the ability to bind to an antigen.

**[0056]** "Bispecific antibody" is used herein to refer to a full-length antibody that is generated by quadroma technology (see Milstein *et al.*, *Nature*, 305(5934): 537-540 (1983)), by chemical conjugation of two different monoclonal antibodies (see, Staerz *et al.*, *Nature,* 314(6012): 628-631 (1985)), or by knob-into-hole or similar approaches, which introduce mutations in the Fc region (see Holliger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90(14): 6444-6448 (1993)), resulting in multiple different immunoglobulin species of which only one is the functional bispecific antibody. A bispecific antibody binds one antigen (or epitope) on one of its two binding arms (one pair of HC/LC), and binds a different antigen (or epitope) on its second arm (a different pair of HC/LC). By this definition, a bispecific antibody has two distinct antigen-binding arms (in both specificity and CDR sequences), and is monovalent for each antigen to which it binds to.

**[0057]** "CDR" is used herein to refer to the "complementarity determining region" within an antibody variable sequence. There are three CDRs in each of the variable regions of the heavy chain and the light chain. Proceeding from the N-terminus of a heavy or light chain, these regions are denoted "CDR1", "CDR2", and "CDR3", for each of the variable regions. The term "CDR set" as used herein refers to a group of three CDRs that occur in a single variable region that binds the antigen. An antigen-binding site, therefore, may include six CDRs, comprising the CDR set from each of a heavy and a light chain variable region. A polypeptide comprising a single CDR, (*e.g.,* a CDR1, CDR2, or CDR3) may be referred to as a "molecular recognition unit." Crystallographic analyses of antigen-antibody complexes have demonstrated that the amino acid residues of CDRs form extensive contact with bound antigen, wherein the most extensive antigen contact is with the heavy chain CDR3. Thus, the molecular recognition units may be primarily responsible for the specificity of an antigen-binding site. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

**[0058]** The exact boundaries of these CDRs have been defined differently according to different systems. The system described by Kabat (Kabat *et al.*, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987) and (1991)) not

only provides an unambiguous residue numbering system applicable to any variable region of an antibody, but also provides precise residue boundaries defining the three CDRs. These CDRs may be referred to as "Kabat CDRs". Chothia and coworkers (Chothia and Lesk, *J. Mol. Biol.,* 196: 901-917 (1987); and Chothia *et al.*, *Nature,* 342: 877-883 (1989)) found that certain sub-portions within Kabat CDRs adopt nearly identical peptide backbone conformations, despite having great diversity at the level of amino acid sequence. These sub-portions were designated as "L1", "L2", and "L3", or "H1", "H2", and "H3", where the "L" and the "H" designate the light chain and the heavy chain regions, respectively. These regions may be referred to as "Chothia CDRs", which have boundaries that overlap with Kabat CDRs. Other boundaries defining CDRs overlapping with the Kabat CDRs have been described by Padlan, *FASEB J.*, 9: 133-139 (1995), and MacCallum, *J. Mol. Biol.*, 262(5): 732-745 (1996). Still other CDR boundary definitions may not strictly follow one of the herein systems, but will nonetheless overlap with the Kabat CDRs, although they may be shortened or lengthened in light of prediction or experimental findings that particular residues or groups of residues or even entire CDRs do not significantly impact antigen binding. The methods used herein may utilize CDRs defined according to any of these systems, although certain embodiments use Kabat- or Chothia-defined CDRs.

**[0059]** "Component," "components," or "at least one component," refer generally to a capture antibody, a detection or conjugate a calibrator, a control, a sensitivity panel, a container, a buffer, a diluent, a salt, an enzyme, a co-factor for an enzyme, a detection reagent, a pretreatment reagent/solution, a substrate (e.g., as a solution), a stop solution, and the like that can be included in a kit for assay of a test sample, such as a patient urine, whole blood, serum or plasma sample, in accordance with the methods described herein and other methods known in the art. Some components can be in solution or lyophilized for reconstitution for use in an assay.

**[0060]** "Correlated to" as used herein refers to compared to.

**[0061]** "CT scan" as used herein refers to a computerized tomography (CT) scan. A CT scan combines a series of X-ray images taken from different angles and uses computer processing to create cross-sectional images, or slices, of the bones, blood vessels and soft tissues inside your body. The CT scan may use X-ray CT, positron emission tomography (PET), single-

photon emission computed tomography (SPECT), computed axial tomography (CAT scan), or computer aided tomography. The CT scan may be a conventional CT scan or a spiral/helical CT scan. In a conventional CT scan, the scan is taken slice by slice and after each slice the scan stops and moves down to the next slice, e.g., from the top of the abdomen down to the pelvis. The conventional CT scan requires patients to hold their breath to avoid movement artefact. The spiral/helical CT scan is a continuous scan which is taken in a spiral fashion and is a much quicker process where the scanned images are contiguous.

**[0062]** "Determined by an assay" is used herein to refer to the determination of a reference level by any appropriate assay. The determination of a reference level may, in some embodiments, be achieved by an assay of the same type as the assay that is to be applied to the sample from the subject (for example, by an immunoassay, clinical chemistry assay, a single molecule detection assay, protein immunoprecipitation, immunoelectrophoresis, chemical analysis, SDS-PAGE and Western blot analysis, or protein immunostaining, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, or chromatography or spectrometry methods, such as high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC/MS)). The determination of a reference level may, in some embodiments, be achieved by an assay of the same type and under the same assay conditions as the assay that is to be applied to the sample from the subject. As noted herein, this disclosure provides exemplary reference levels (e.g., calculated by comparing reference levels at different time points). It is well within the ordinary skill of one in the art to adapt the disclosure herein for other assays to obtain assay-specific reference levels for those other assays based on the description provided by this disclosure. For example, a set of training samples comprising samples obtained from human subjects known to have sustained an injury to the head (and more particularly, samples obtained from human subjects known to have sustained a (i) mild TBI; and/or (ii) moderate, severe, or moderate to severe TBI and samples obtained from human subjects known not to have sustained an injury to the head may be used to obtain assay-specific reference levels. It will be understood that a reference level "determined by an assay" and having a recited level of "sensitivity" and/or "specificity" is used herein to refer to a reference level which has been determined to provide a method of the recited sensitivity and/or specificity when said

reference level is adopted in the methods of the invention. It is well within the ordinary skill of one in the art to determine the sensitivity and specificity associated with a given reference level in the methods of the invention, for example by repeated statistical analysis of assay data using a plurality of different possible reference levels.

**[0063]** Practically, when discriminating between a subject as having a traumatic brain injury or not having a traumatic brain injury or a subject as having a a mild versus a moderate, severe, or moderate to severe traumatic brain injury, the skilled person will balance the effect of raising a cutoff on sensitivity and specificity. Raising or lowering a cutoff will have a well-defined and predictable impact on sensitivity and specificity, and other standard statistical measures. It is well known that raising a cutoff will improve specificity but is likely to worsen sensitivity (proportion of those with disease who test positive). In contrast, lowering a cutoff will improve sensitivity but will worsen specificity (proportion of those without disease who test negative). The ramifications for detecting traumatic brain injury or determining a mild versus moderate, severe, or moderate to severe traumatic brain injury will be readily apparent to those skilled in the art. In discriminating whether a subject has or does not have a traumatic brain injury or a mild versus a moderate, severe, or moderate to severe traumatic brain injury, the higher the cutoff, specificity improves as more true negatives (i.e., subjects not having a traumatic brain injury, not having a mild traumatic brain injury, not have a moderate traumatic brain injury, not having a severe traumatic brain injury or not having a moderate to severe traumatic brain injury) are distinguished from those having a traumatic brain injury, a mild traumatic brain injury, a moderate traumatic brain injury, a severe traumatic brain injury or a moderate to severe traumatic brain injury. But at the same time, raising the cutoff decreases the number of cases identified as positive overall, as well as the number of true positives, so the sensitivity must decrease. Conversely, the lower the cutoff, sensitivity improves as more true positives (i.e., subjects having a traumatic brain injury, having a mild traumatic brain injury, having a moderate traumatic brain injury, having a severe traumatic brain injury or having a moderate to severe traumatic brain injury) are distinguished from those who do not have a traumatic brain injury, a mild traumatic brain injury, a moderate traumatic brain injury, a severe traumatic brain injury or a moderate to severe traumatic brain injury. But at the same time, lowering the cutoff increases the number

of cases identified as positive overall, as well as the number of false positives, so the specificity must decrease.

**[0064]** Generally, a high sensitivity value helps one of skill rule out disease or condition (such as a traumatic brain injury, mild traumatic brain injury, moderate traumatic brain injury, severe traumatic brain injury or moderate to severe traumatic brain injury), and a high specificity value helps one of skill rule in disease or condition. Whether one of skill desires to rule out or rule in disease depends on what the consequences are for the patient for each type of error. Accordingly, one cannot know or predict the precise balancing employed to derive a test cutoff without full disclosure of the underlying information on how the value was selected. The balancing of sensitivity against specificity and other factors will differ on a case-by-case basis. This is why it is sometimes preferable to provide alternate cutoff (e.g., reference) values so a physician or practitioner can choose.

**[0065]** "Derivative" of an antibody as used herein may refer to an antibody having one or more modifications to its amino acid sequence when compared to a genuine or parent antibody and exhibit a modified domain structure. The derivative may still be able to adopt the typical domain configuration found in native antibodies, as well as an amino acid sequence, which is able to bind to targets (antigens) with specificity. Typical examples of antibody derivatives are antibodies coupled to other polypeptides, rearranged antibody domains, or fragments of antibodies. The derivative may also comprise at least one further compound, e.g., a protein domain, said protein domain being linked by covalent or non-covalent bonds. The linkage can be based on genetic fusion according to the methods known in the art. The additional domain present in the fusion protein comprising the antibody may preferably be linked by a flexible linker, advantageously a peptide linker, wherein said peptide linker comprises plural, hydrophilic, peptide-bonded amino acids of a length sufficient to span the distance between the C-terminal end of the further protein domain and the N-terminal end of the antibody or vice versa. The antibody may be linked to an effector molecule having a conformation suitable for biological activity or selective binding to a solid support, a biologically active substance (e.g., a cytokine or growth hormone), a chemical agent, a peptide, a protein, or a drug, for example.

**[0066]** "Drugs of abuse" is used herein to refer to one or more additive substances (such as a drug) taken for non-medical reasons (such as for, example, recreational and/or mind-altering effects). Excessive overindulgence, use or dependence of such drugs of abuse is often referred to as "substance abuse". Examples of drugs of abuse include alcohol, barbiturates, benzodiazepines, cannabis, cocaine, hallucinogens (such as ketamine, mescaline (peyote), PCP, psilocybin, DMT and/or LSD), methaqualone, opioids, amphetamines (including methamphetamines), anabolic steroids, inhalants (namely, substances which contain volatile substances that contain psychoactive properties such as, for example, nitrites, spray paints, cleaning fluids, markers, glues, etc.) and combinations thereof.

**[0067]** "Dual-specific antibody" is used herein to refer to a full-length antibody that can bind two different antigens (or epitopes) in each of its two binding arms (a pair of HC/LC) (see PCT publication WO 02/02773). Accordingly, a dual-specific binding protein has two identical antigen binding arms, with identical specificity and identical CDR sequences, and is bivalent for each antigen to which it binds.

**[0068]** "Dual variable domain" is used herein to refer to two or more antigen binding sites on a binding protein, which may be divalent (two antigen binding sites), tetravalent (four antigen binding sites), or multivalent binding proteins. DVDs may be monospecific, *i.e.*, capable of binding one antigen (or one specific epitope), or multispecific, *i.e.*, capable of binding two or more antigens (*i.e.*, two or more epitopes of the same target antigen molecule or two or more epitopes of different target antigens). A preferred DVD binding protein comprises two heavy chain DVD polypeptides and two light chain DVD polypeptides and is referred to as a "DVD immunoglobulin" or "DVD-Ig." Such a DVD-Ig binding protein is thus tetrameric and reminiscent of an IgG molecule, but provides more antigen binding sites than an IgG molecule. Thus, each half of a tetrameric DVD-Ig molecule is reminiscent of one half of an IgG molecule and comprises a heavy chain DVD polypeptide and a light chain DVD polypeptide, but unlike a pair of heavy and light chains of an IgG molecule that provides a single antigen binding domain, a pair of heavy and light chains of a DVD-Ig provide two or more antigen binding sites.

**[0069]** Each antigen binding site of a DVD-Ig binding protein may be derived from a donor ("parental") monoclonal antibody and thus comprises a heavy chain variable domain (VH)

and a light chain variable domain (VL) with a total of six CDRs involved in antigen binding per antigen binding site. Accordingly, a DVD-Ig binding protein that binds two different epitopes (*i.e.,* two different epitopes of two different antigen molecules or two different epitopes of the same antigen molecule) comprises an antigen binding site derived from a first parental monoclonal antibody and an antigen binding site of a second parental monoclonal antibody.

**[0070]** A description of the design, expression, and characterization of DVD-Ig binding molecules is provided in PCT Publication No. WO 2007/024715, U.S. Patent No. 7,612,181, and Wu *et al.*, *Nature Biotech.*, 25: 1290-1297 (2007). A preferred example of such DVD-Ig molecules comprises a heavy chain that comprises the structural formula VD1-(X1)n-VD2-C-(X2)n, wherein VD1 is a first heavy chain variable domain, VD2 is a second heavy chain variable domain, C is a heavy chain constant domain, X1 is a linker with the proviso that it is not CH1, X2 is an Fc region, and n is 0 or 1, but preferably 1; and a light chain that comprises the structural formula VD1-(X1)n-VD2-C-(X2)n, wherein VD1 is a first light chain variable domain, VD2 is a second light chain variable domain, C is a light chain constant domain, X1 is a linker with the proviso that it is not CH1, and X2 does not comprise an Fc region; and n is 0 or 1, but preferably 1. Such a DVD-Ig may comprise two such heavy chains and two such light chains, wherein each chain comprises variable domains linked in tandem without an intervening constant region between variable regions, wherein a heavy chain and a light chain associate to form tandem functional antigen binding sites, and a pair of heavy and light chains may associate with another pair of heavy and light chains to form a tetrameric binding protein with four functional antigen binding sites. In another example, a DVD-Ig molecule may comprise heavy and light chains that each comprise three variable domains (VD1, VD2, VD3) linked in tandem without an intervening constant region between variable domains, wherein a pair of heavy and light chains may associate to form three antigen binding sites, and wherein a pair of heavy and light chains may associate with another pair of heavy and light chains to form a tetrameric binding protein with six antigen binding sites.

**[0071]** In a preferred embodiment, a DVD-Ig binding protein not only binds the same target molecules bound by its parental monoclonal antibodies, but also possesses one or more

desirable properties of one or more of its parental monoclonal antibodies. Preferably, such an additional property is an antibody parameter of one or more of the parental monoclonal antibodies. Antibody parameters that may be contributed to a DVD-Ig binding protein from one or more of its parental monoclonal antibodies include, but are not limited to, antigen specificity, antigen affinity, potency, biological function, epitope recognition, protein stability, protein solubility, production efficiency, immunogenicity, pharmacokinetics, bioavailability, tissue cross reactivity, and orthologous antigen binding.

**[0072]** A DVD-Ig binding protein binds at least one epitope of UCH-L1. Non-limiting examples of a DVD-Ig binding protein include a DVD-Ig binding protein that binds one or more epitopes of UCH-L1, a DVD-Ig binding protein that binds an epitope of a human UCH-L1 and an epitope of UCH-L1 of another species (for example, mouse), and a DVD-Ig binding protein that binds an epitope of a human UCH-L1 and an epitope of another target molecule.

**[0073]** "Dynamic range" as used herein refers to a range over which an assay readout is proportional to the amount of target molecule or analyte in the sample being analyzed.

**[0074]** "Epitope," or "epitopes," or "epitopes of interest" refer to a site(s) on any molecule that is recognized and can bind to a complementary site(s) on its specific binding partner. The molecule and specific binding partner are part of a specific binding pair. For example, an epitope can be on a polypeptide, a protein, a hapten, a carbohydrate antigen (such as, but not limited to, glycolipids, glycoproteins or lipopolysaccharides), or a polysaccharide. Its specific binding partner can be, but is not limited to, an antibody.

**[0075]** "Expanded window of detection" as used herein refers to the fact that the described and/or claimed improved methods can be carried out in or under a variety of clinical scenarios when compared to other UCH-L1 assays. For example, the methods of the present disclosure can be carried out on any subject without regard to factors selected from the group consisting of the subject's clinical condition (e.g., whether or not there are comorbid conditions in addition to the reason for checking on UCH-L1), the subject's laboratory values (e.g., laboratory values other than UCH-L1 levels, including but not limited to values on standard laboratory tests that are run to assess a patient's overall health, and values on more particularized tests that are run when a subject is suspected of having been in an accident or

exposed to some sort of trauma including but not limited to those that may result in head injury), the subject's classification as suffering from mild, moderate, severe, moderate or severe or moderate to severe TBI, the subject's exhibition (e.g., demonstration or possession) of low or high levels of UCH-L1, and the timing of any event (e.g., relative to testing) where the subject may have sustained an injury to the head. Additionally, by way of a further example, the methods of the present disclosure differ from other methods known in the prior art which may or require dilution, or alternately, may lack one or more of the benefits of the improved assays as described herein (namely, measure up to 25,000 pg/mL, dynamic range of 5 log, assay linearity over the dynamic range, measure of UCH-L1 in a volume less than 20 microliters of sample, expanded window of detection, etc.).

**[0076]** "Fragment antigen-binding fragment" or "Fab fragment" as used herein refers to a fragment of an antibody that binds to antigens and that contains one antigen-binding site, one complete light chain, and part of one heavy chain. Fab is a monovalent fragment consisting of the VL, VH, CL and CH1 domains. Fab is composed of one constant and one variable domain of each of the heavy and the light chain. The variable domain contains the paratope (the antigen-binding site), comprising a set of complementarity determining regions, at the amino terminal end of the monomer. Each arm of the Y thus binds an epitope on the antigen. Fab fragments can be generated such as has been described in the art, e.g., using the enzyme papain, which can be used to cleave an immunoglobulin monomer into two Fab fragments and an Fc fragment, or can be produced by recombinant means.

**[0077]** "$F(ab')_2$ fragment" as used herein refers to antibodies generated by pepsin digestion of whole IgG antibodies to remove most of the Fc region while leaving intact some of the hinge region. $F(ab')_2$ fragments have two antigen-binding F(ab) portions linked together by disulfide bonds, and therefore are divalent with a molecular weight of about 110 kDa. Divalent antibody fragments ($F(ab')_2$ fragments) are smaller than whole IgG molecules and enable a better penetration into tissue thus facilitating better antigen recognition in immunohistochemistry. The use of $F(ab')_2$ fragments also avoids unspecific binding to Fc receptor on live cells or to Protein A/G. $F(ab')_2$ fragments can both bind and precipitate antigens.

**[0078]** "Framework" (FR) or "Framework sequence" as used herein may mean the remaining sequences of a variable region minus the CDRs. Because the exact definition of a CDR sequence can be determined by different systems (for example, see above), the meaning of a framework sequence is subject to correspondingly different interpretations. The six CDRs (CDR-L1, -L2, and -L3 of light chain and CDR-H1, -H2, and -H3 of heavy chain) also divide the framework regions on the light chain and the heavy chain into four sub-regions (FR1, FR2, FR3, and FR4) on each chain, in which CDR1 is positioned between FR1 and FR2, CDR2 between FR2 and FR3, and CDR3 between FR3 and FR4. Without specifying the particular sub-regions as FR1, FR2, FR3, or FR4, a framework region, as referred by others, represents the combined FRs within the variable region of a single, naturally occurring immunoglobulin chain. As used herein, a FR represents one of the four sub-regions, and FRs represents two or more of the four sub-regions constituting a framework region.

**[0079]** Human heavy chain and light chain FR sequences are known in the art that can be used as heavy chain and light chain "acceptor" framework sequences (or simply, "acceptor" sequences) to humanize a non-human antibody using techniques known in the art. In one embodiment, human heavy chain and light chain acceptor sequences are selected from the framework sequences listed in publicly available databases such as V-base (hypertext transfer protocol://vbase.mrc-cpe.cam.ac.uk/; phone number: +49 - 531 - 6181 687) or in the international ImMunoGeneTics® (IMGT®) information system (hypertext transfer protocol://imgt.cines.fr/texts/IMGTrepertoire/LocusGenes/; phone number: +33 (0)4 34 35 99 65).

**[0080]** "Functional antigen binding site" as used herein may mean a site on a binding protein (*e.g.,* an antibody) that is capable of binding a target antigen. The antigen binding affinity of the antigen binding site may not be as strong as the parent binding protein, *e.g.,* parent antibody, from which the antigen binding site is derived, but the ability to bind antigen must be measurable using any one of a variety of methods known for evaluating protein, *e.g.,* antibody, binding to an antigen. Moreover, the antigen binding affinity of each of the antigen binding sites of a multivalent protein, *e.g.,* multivalent antibody, herein need not be quantitatively the same.

**[0081]** "Glasgow Coma Scale" or "GCS" as used herein refers to a 15 point scale for estimating and categorizing the outcomes of brain injury on the basis of overall social capability or dependence on others. The test measures the motor response, verbal response and eye opening response with these values: I. Motor Response (6 – Obeys commands fully; 5 – Localizes to noxious stimuli; 4 – Withdraws from noxious stimuli; 3 – Abnormal flexion, i.e., decorticate posturing; 2 – Extensor response, i.e., decerebrate posturing; and 1 – No response); II. Verbal Response (5 – Alert and Oriented; 4 – Confused, yet coherent, speech; 3 – Inappropriate words and jumbled phrases consisting of words; 2 – Incomprehensible sounds; and 1 – No sounds); and III. Eye Opening (4 – Spontaneous eye opening; 3 – Eyes open to speech; 2 – Eyes open to pain; and 1 – No eye opening). The final score is determined by adding the values of I+II+III. The final score can be categorized into four possible levels for survival, with a lower number indicating a more severe injury and a poorer prognosis: Mild (13-15); Moderate Disability (9-12) (Loss of consciousness greater than 30 minutes; Physical or cognitive impairments which may or may resolve: and Benefit from Rehabilitation); Severe Disability (3-8) (Coma: unconscious state. No meaningful response, no voluntary activities); and Vegetative State (Less Than 3) (Sleep wake cycles; Arousal, but no interaction with environment; No localized response to pain). Moderate brain injury is defined as a brain injury resulting in a loss of consciousness from 20 minutes to 6 hours and a Glasgow Coma Scale of 9 to 12. Severe brain injury is defined as a brain injury resulting in a loss of consciousness of greater than 6 hours and a Glasgow Coma Scale of 3 to 8.

**[0082]** "Glasgow Outcome Scale" as used herein refers to a global scale for functional outcome that rates patient status into one of five categories: Dead, Vegetative State, Severe Disability, Moderate Disability or Good Recovery.

**[0083]** "Extended Glasgow Outcome Scale" or "GOSE" as used interchangeably herein provides more detailed categorization into eight categories by subdividing the categories of severe disability, moderate disability and good recovery into a lower and upper category as shown in Table 1.

**Table 1**

| | | | |
|---|---|---|---|
| 1 | Death | D | |
| 2 | Vegetative state | VX | Condition of unawareness with only reflex responses but with periods of spontaneous |

| | | | |
|---|---|---|---|
| | | | eye opening |
| 3 | Lower severe disability | SD - | Patient who is dependent for daily support for mental or physical disability, usually a combination of both. If the patient can be left alone for more than 8 hours at home it is upper level of SD, if not then it is low level of SD. |
| 4 | Upper severe disability | SD + | |
| 5 | Lower moderate disability | MD - | Patients have some disability such as aphasia, hemiparesis or epilepsy and/or deficits of memory or personality but are able to look after themselves. They are independent at home but dependent outside. If they are able to return to work even with special arrangement it is upper level of MD, if not then it is low level of MD. |
| 6 | Upper moderate disability | MD + | |
| 7 | Lower good recovery | GR - | Resumption of normal life with the capacity to work even if pre-injury status has not been achieved. Some patients have minor neurological or psychological deficits. If these deficits are not disabling then it is upper level of GR, if disabling then it is lower level of GR. |
| 8 | Upper good recovery | GR + | |

**[0084]** "Humanized antibody" is used herein to describe an antibody that comprises heavy and light chain variable region sequences from a non-human species (*e.g.,* a mouse) but in which at least a portion of the VH and/or VL sequence has been altered to be more "human-like," *i.e.,* more similar to human germline variable sequences. A "humanized antibody" is an antibody or a variant, derivative, analog, or fragment thereof, which immunospecifically binds to an antigen of interest and which comprises a framework (FR) region having substantially the amino acid sequence of a human antibody and a complementary determining region (CDR) having substantially the amino acid sequence of a non-human antibody. As used herein, the term "substantially" in the context of a CDR refers to a CDR having an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to the amino acid sequence of a non-human antibody CDR. A humanized antibody comprises substantially all of at least one, and typically two, variable domains (Fab, Fab', $F(ab')_2$, FabC, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin (*i.e.,* donor antibody) and all or

substantially all of the framework regions are those of a human immunoglobulin consensus sequence. In an embodiment, a humanized antibody also comprises at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. In some embodiments, a humanized antibody contains the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain. In some embodiments, a humanized antibody only contains a humanized light chain. In some embodiments, a humanized antibody only contains a humanized heavy chain. In specific embodiments, a humanized antibody only contains a humanized variable domain of a light chain and/or humanized heavy chain.

**[0085]** A humanized antibody can be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA, and IgE, and any isotype, including without limitation IgG1, IgG2, IgG3, and IgG4. A humanized antibody may comprise sequences from more than one class or isotype, and particular constant domains may be selected to optimize desired effector functions using techniques well-known in the art.

**[0086]** The framework regions and CDRs of a humanized antibody need not correspond precisely to the parental sequences, *e.g.,* the donor antibody CDR or the consensus framework may be mutagenized by substitution, insertion, and/or deletion of at least one amino acid residue so that the CDR or framework residue at that site does not correspond to either the donor antibody or the consensus framework. In a preferred embodiment, such mutations, however, will not be extensive. Usually, at least 80%, preferably at least 85%, more preferably at least 90%, and most preferably at least 95% of the humanized antibody residues will correspond to those of the parental FR and CDR sequences. As used herein, the term "consensus framework" refers to the framework region in the consensus immunoglobulin sequence. As used herein, the term "consensus immunoglobulin sequence" refers to the sequence formed from the most frequently occurring amino acids (or nucleotides) in a family of related immunoglobulin sequences (see, *e.g.,* Winnaker, *From Genes to Clones* (Verlagsgesellschaft, Weinheim, 1987)). A "consensus immunoglobulin sequence" may thus comprise a "consensus framework region(s)" and/or a "consensus CDR(s)". In a family of immunoglobulins, each position in the consensus sequence is

occupied by the amino acid occurring most frequently at that position in the family. If two amino acids occur equally frequently, either can be included in the consensus sequence.

**[0087]** "Identical" or "identity," as used herein in the context of two or more polypeptide or polynucleotide sequences, can mean that the sequences have a specified percentage of residues that are the same over a specified region. The percentage can be calculated by optimally aligning the two sequences, comparing the two sequences over the specified region, determining the number of positions at which the identical residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the specified region, and multiplying the result by 100 to yield the percentage of sequence identity. In cases where the two sequences are of different lengths or the alignment produces one or more staggered ends and the specified region of comparison includes only a single sequence, the residues of the single sequence are included in the denominator but not the numerator of the calculation.

**[0088]** "Injury to the head" or "head injury" as used interchangeably herein, refers to any trauma to the scalp, skull, or brain. Such injuries may include only a minor bump on the skull or may be a serious brain injury. Such injuries include primary injuries to the brain and/or secondary injuries to the brain. Primary brain injuries occur during the initial insult and result from displacement of the physical structures of the brain. More specifically, a primary brain injury is the physical damage to parenchyma (tissue, vessels) that occurs during the traumatic event, resulting in shearing and compression of the surrounding brain tissue. Secondary brain injuries occur subsequent to the primary injury and may involve an array of cellular processes. More specifically, a secondary brain injury refers to the changes that evolve over a period of time (from hours to days) after the primary brain injury. It includes an entire cascade of cellular, chemical, tissue, or blood vessel changes in the brain that contribute to further destruction of brain tissue.

**[0089]** An injury to the head can be either closed or open (penetrating). A closed head injury refers to a trauma to the scalp, skull or brain where there is no penetration of the skull by a striking object. An open head injury refers a trauma to the scalp, skull or brain where there is penetration of the skull by a striking object. An injury to the head may be caused by physical shaking of a person, by blunt impact by an external mechanical or other force that results in a

closed or open head trauma (e.g., vehicle accident such as with an automobile, plane, train, etc.; blow to the head such as with a baseball bat, or from a firearm), a cerebral vascular accident (e.g., stroke), one or more falls (e.g., as in sports or other activities), explosions or blasts (collectively, "blast injuries") and by other types of blunt force trauma. Alternatively, an injury to the head may be caused by the ingestion and/or exposure to a chemical, toxin or a combination of a chemical and toxin. Examples of such chemicals and/or toxins include fires, molds, asbestos, pesticides and insecticides, organic solvents, paints, glues, gases (such as carbon monoxide, hydrogen sulfide, and cyanide), organic metals (such as methyl mercury, tetraethyl lead and organic tin) and/or one or more drugs of abuse. Alternatively, an injury to the head may be caused as a result of a subject suffering from an autoimmune disease, a metabolic disorder, a brain tumor, one or more viruses, meningitis, hydrocephalus, hypoxia or any combinations thereof. In some cases, it is not possible to be certain whether any such event or injury has occurred or taken place. For example, there may be no history on a patient or subject, the subject may be unable to speak, the subject may be aware of what events they were exposed to, etc. Such circumstances are described herein as the subject "may have sustained an injury to the head." In certain embodiments herein, the closed head injury does not include and specifically excludes a cerebral vascular accident, such as stroke.

**[0090]** "Isolated polynucleotide" as used herein may mean a polynucleotide (e.g., of genomic, cDNA, or synthetic origin, or a combination thereof) that, by virtue of its origin, the isolated polynucleotide is not associated with all or a portion of a polynucleotide with which the "isolated polynucleotide" is found in nature; is operably linked to a polynucleotide that it is not linked to in nature; or does not occur in nature as part of a larger sequence.

**[0091]** "Label" and "detectable label" as used herein refer to a moiety attached to an antibody or an analyte to render the reaction between the antibody and the analyte detectable, and the antibody or analyte so labeled is referred to as "detectably labeled." A label can produce a signal that is detectable by visual or instrumental means. Various labels include signal-producing substances, such as chromagens, fluorescent compounds, chemiluminescent compounds, radioactive compounds, and the like. Representative examples of labels include moieties that produce light, e.g., acridinium compounds, and moieties that produce fluorescence, e.g., fluorescein. Other labels are described herein. In this regard, the moiety,

itself, may not be detectable but may become detectable upon reaction with yet another moiety. Use of the term "detectably labeled" is intended to encompass such labeling.

**[0092]** "Limit of Detection (LOD)" as used herein refers to the lowest concentration of the measured object (i.e., a quantity intended to be measured) that can be detected at a specified level of confidence. The level of confidence is typically 95%, with a 5% likelihood of a false negative measurement. The LoD term used herein is based on the definition from Clinical and Laboratory Standards Institute (CLSI) protocol EP17-A2 ("Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline - Second Edition," EP17A2E, by James F. Pierson-Perry et al., Clinical and Laboratory Standards Institute, June 1, 2012).

**[0093]** "Linear" means that there is less than or equal to about 20%, about 19%, about 18%, about 17%, about 16%, about 15%, about 14%, about 13%, about 12%, about 11%, about 10%, about 9%, or about 8% variation for or over an exemplary range or value recited.

**[0094]** Any suitable detectable label as is known in the art can be used. For example, the detectable label can be a radioactive label (such as 3H, 14C, 32P, 33P, 35S, 90Y, 99Tc, 111In, 125I, 131I, 177Lu, 166Ho, and 153Sm), an enzymatic label (such as horseradish peroxidase, alkaline peroxidase, glucose 6-phosphate dehydrogenase, and the like), a chemiluminescent label (such as acridinium esters, thioesters, or sulfonamides; luminol, isoluminol, phenanthridinium esters, and the like), a fluorescent label (such as fluorescein (*e.g.,* 5-fluorescein, 6-carboxyfluorescein, 3'6-carboxyfluorescein, 5(6)-carboxyfluorescein, 6-hexachloro-fluorescein, 6-tetrachlorofluorescein, fluorescein isothiocyanate, and the like)), rhodamine, phycobiliproteins, R-phycoerythrin, quantum dots (*e.g.,* zinc sulfide-capped cadmium selenide), a thermometric label, or an immuno-polymerase chain reaction label. An introduction to labels, labeling procedures and detection of labels is found in Polak and Van Noorden, *Introduction to Immunocytochemistry*, 2nd ed., Springer Verlag, N.Y. (1997), and in Haugland, *Handbook of Fluorescent Probes and Research Chemicals* (1996), which is a combined handbook and catalogue published by Molecular Probes, Inc., Eugene, Oregon. A fluorescent label can be used in FPIA (see, *e.g.,* U.S. Patent Nos. 5,593,896, 5,573,904, 5,496,925, 5,359,093, and 5,352,803, which are hereby incorporated by reference in their entireties). An acridinium compound can be used as a detectable label in a homogeneous

chemiluminescent assay (see, *e.g.,* Adamczyk *et al.*, Bioorg. *Med. Chem. Lett.* 16: 1324-1328 (2006); Adamczyk *et al.*, *Bioorg. Med. Chem. Lett.* 4: 2313-2317 (2004); Adamczyk *et al.*, *Biorg. Med. Chem. Lett.* 14: 3917-3921 (2004); and Adamczyk *et al.*, *Org. Lett.* 5: 3779-3782 (2003)).

**[0095]** In one aspect, the acridinium compound is an acridinium-9-carboxamide. Methods for preparing acridinium 9-carboxamides are described in Mattingly, *J. Biolumin. Chemilumin.* 6: 107-114 (1991); Adamczyk *et al.*, *J. Org. Chem.* 63: 5636-5639 (1998); Adamczyk *et al.*, *Tetrahedron* 55: 10899-10914 (1999); Adamczyk *et al.*, *Org. Lett.* 1: 779-781 (1999); Adamczyk *et al.*, *Bioconjugate Chem.* 11: 714-724 (2000); Mattingly *et al.*, *In Luminescence Biotechnology: Instruments and Applications*; Dyke, K. V. Ed.; CRC Press: Boca Raton, pp. 77–105 (2002); Adamczyk *et al.*, *Org. Lett.* 5: 3779-3782 (2003); and U.S. Patent Nos. 5,468,646, 5,543,524 and 5,783,699 (each of which is incorporated herein by reference in its entirety for its teachings regarding same).

**[0096]** Another example of an acridinium compound is an acridinium-9-carboxylate aryl ester. An example of an acridinium-9-carboxylate aryl ester of formula II is 10-methyl-9-(phenoxycarbonyl)acridinium fluorosulfonate (available from Cayman Chemical, Ann Arbor, MI). Methods for preparing acridinium 9-carboxylate aryl esters are described in McCapra *et al.*, Photochem. Photobiol. 4: 1111-21 (1965); Razavi *et al.*, *Luminescence* 15: 245-249 (2000); Razavi *et al.*, *Luminescence* 15: 239-244 (2000); and U.S. Patent No. 5,241,070 (each of which is incorporated herein by reference in its entirety for its teachings regarding same). Such acridinium-9-carboxylate aryl esters are efficient chemiluminescent indicators for hydrogen peroxide produced in the oxidation of an analyte by at least one oxidase in terms of the intensity of the signal and/or the rapidity of the signal. The course of the chemiluminescent emission for the acridinium-9-carboxylate aryl ester is completed rapidly, *i.e.,* in under 1 second, while the acridinium-9-carboxamide chemiluminescent emission extends over 2 seconds. Acridinium-9-carboxylate aryl ester, however, loses its chemiluminescent properties in the presence of protein. Therefore, its use requires the absence of protein during signal generation and detection. Methods for separating or removing proteins in the sample are well-known to those skilled in the art and include, but are not limited to, ultrafiltration, extraction, precipitation, dialysis, chromatography, and/or

digestion (see, *e.g.,* Wells, *High Throughput Bioanalytical Sample Preparation. Methods and Automation Strategies*, Elsevier (2003)). The amount of protein removed or separated from the test sample can be about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. Further details regarding acridinium-9-carboxylate aryl ester and its use are set forth in U.S. Patent App. No. 11/697,835, filed April 9, 2007. Acridinium-9-carboxylate aryl esters can be dissolved in any suitable solvent, such as degassed anhydrous N,N-dimethylformamide (DMF) or aqueous sodium cholate.

**[0097]** "Linking sequence" or "linking peptide sequence" refers to a natural or artificial polypeptide sequence that is connected to one or more polypeptide sequences of interest (*e.g.,* full-length, fragments, etc.). The term "connected" refers to the joining of the linking sequence to the polypeptide sequence of interest. Such polypeptide sequences are preferably joined by one or more peptide bonds. Linking sequences can have a length of from about 4 to about 50 amino acids. Preferably, the length of the linking sequence is from about 6 to about 30 amino acids. Natural linking sequences can be modified by amino acid substitutions, additions, or deletions to create artificial linking sequences. Linking sequences can be used for many purposes, including in recombinant Fabs. Exemplary linking sequences include, but are not limited to: (i) Histidine (His) tags, such as a 6X His tag, which has an amino acid sequence of HHHHHH (SEQ ID NO:2), are useful as linking sequences to facilitate the isolation and purification of polypeptides and antibodies of interest; (ii) Enterokinase cleavage sites, like His tags, are used in the isolation and purification of proteins and antibodies of interest. Often, enterokinase cleavage sites are used together with His tags in the isolation and purification of proteins and antibodies of interest. Various enterokinase cleavage sites are known in the art. Examples of enterokinase cleavage sites include, but are not limited to, the amino acid sequence of DDDDK (SEQ ID NO:3) and derivatives thereof (*e.g.,* ADDDDK (SEQ ID NO:4), etc.); (iii) Miscellaneous sequences can be used to link or connect the light and/or heavy chain variable regions of single chain variable region fragments. Examples of other linking sequences can be found in Bird *et al.*, *Science* 242: 423-426 (1988); Huston *et al.*, *PNAS USA* 85: 5879-5883 (1988); and McCafferty *et al.*, *Nature* 348: 552-554 (1990). Linking sequences also can be modified for

additional functions, such as attachment of drugs or attachment to solid supports. In the context of the present disclosure, the monoclonal antibody, for example, can contain a linking sequence, such as a His tag, an enterokinase cleavage site, or both.

**[0098]** "Monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.,* the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigen. Furthermore, in contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological.

**[0099]** "Magnetic resonance imaging" or "MRI" as used interchangeably herein refers to magnetic resonance imaging, which is a medical imaging technique used in radiology to form pictures of the anatomy and the physiological processes of the body in both health and disease. MRI is a form of medical imaging that measures the response of the atomic nuclei of body tissues to high-frequency radio waves when placed in a strong magnetic field, and that produces images of the internal organs. MRI scanners, which is based on the science of nuclear magnetic resonance (NMR), use strong magnetic fields, radio waves, and field gradients to generate images of the inside of the body.

**[0100]** "Multivalent binding protein" is used herein to refer to a binding protein comprising two or more antigen binding sites (also referred to herein as "antigen binding domains"). A multivalent binding protein is preferably engineered to have three or more antigen binding sites, and is generally not a naturally occurring antibody. The term "multispecific binding protein" refers to a binding protein that can bind two or more related or unrelated targets,

including a binding protein capable of binding two or more different epitopes of the same target molecule.

**[0101]** "Negative predictive value" or "NPV" as used interchangeably herein refers to the probability that a subject has a negative outcome given that they have a negative test result.

**[0102]** "Preanalytical processing conditions" as used interchangeably herein refers to one or more handling, temperature, chemical and/or environmental conditions that are applied to or conducted on a sample (such as a whole blood sample) in the period of time between the time the sample is obtained from the subject and the time point when an assay or test is conducted or performed on the sample. In one embodiment, a preanalytical processing condition can be processing a sample within no more than about eight hours after the sample is obtained from a subject. In another embodiment, a preanalytical processing condition can be storing a sample at room temperature in the period of time between the time the sample is obtained from the subject and the assay is performed. In another embodiment, a preanalytical processing condition can be storing a sample at 2-8°C in the period of time between the time the sample is obtained from the subject and the assay is performed. In another embodiment, a preanalytical processing condition can be not mixing the sample in the period of time between the time the sample is obtained from the subject and the time point when the assay or test is performed.

**[0103]** "Reference level" as used herein refers to an assay cutoff value that is used to assess diagnostic, prognostic, or therapeutic efficacy and that has been linked or is associated with various clinical parameters (*e.g.,* presence of disease, stage of disease, severity of disease, progression, non-progression, or improvement of disease, etc.). This disclosure provides exemplary reference levels. However, it is well-known that reference levels may vary depending on the nature of the immunoassay (*e.g.,* antibodies employed, reaction conditions, sample purity, etc.) and that assays can be compared and standardized. It further is well within the ordinary skill of one in the art to adapt the disclosure herein for other immunoassays to obtain immunoassay-specific reference levels for those other immunoassays based on the description provided by this disclosure. Whereas the precise value of the reference level may vary between assays, the findings as described herein should be generally applicable and capable of being extrapolated to other assays.

**[0104]** "Point-of-care device" refers to a device used to provide medical diagnostic testing at or near the point-of-care (namely, outside of a laboratory), at the time and place of patient care (such as in a hospital, physician's office, urgent or other medical care facility, a patient's home, a nursing home and/or a long term care and/or hospice facility). Examples of point-of-care devices include those produced by Abbott Laboratories (Abbott Park, IL) (e.g., i-STAT® and i-STAT® Alinity, Universal Biosensors (Rowville, Australia) (see US 2006/0134713), Axis-Shield PoC AS (Oslo, Norway) and Clinical Lab Products (Los Angeles, USA).

**[0105]** "Positive predictive value" or "PPV" as used interchangeably herein refers to the probability that a subject has a positive outcome given that they have a positive test result.

**[0106]** "Quality control reagents" in the context of immunoassays and kits described herein, include, but are not limited to, calibrators, controls, and sensitivity panels. A "calibrator" or "standard" typically is used (e.g., one or more, such as a plurality) in order to establish calibration (standard) curves for interpolation of the concentration of an analyte, such as an antibody or an analyte. Alternatively, a single calibrator, which is near a reference level or control level (e.g., "low," "medium," or "high" levels), can be used. Multiple calibrators (i.e., more than one calibrator or a varying amount of calibrator(s)) can be used in conjunction to comprise a "sensitivity panel."

**[0107]** A "receiver operating characteristic" curve or "ROC" curve refers to a graphical plot that illustrates the performance of a binary classifier system as its discrimination threshold is varied. For example, an ROC curve can be a plot of the true positive rate against the false positive rate for the different possible cutoff points of a diagnostic test. It is created by plotting the fraction of true positives out of the positives (TPR = true positive rate) vs. the fraction of false positives out of the negatives (FPR = false positive rate), at various threshold settings. TPR is also known as sensitivity, and FPR is one minus the specificity or true negative rate. The ROC curve demonstrates the tradeoff between sensitivity and specificity (any increase in sensitivity will be accompanied by a decrease in specificity); the closer the curve follows the left-hand border and then the top border of the ROC space, the more accurate the test; the closer the curve comes to the 45-degree diagonal of the ROC space, the

less accurate the test; the slope of the tangent line at a cutoff point gives the likelihood ratio (LR) for that value of the test; and the area under the curve is a measure of test accuracy.

**[0108]** "Recombinant antibody" and "recombinant antibodies" refer to antibodies prepared by one or more steps, including cloning nucleic acid sequences encoding all or a part of one or more monoclonal antibodies into an appropriate expression vector by recombinant techniques and subsequently expressing the antibody in an appropriate host cell. The terms include, but are not limited to, recombinantly produced monoclonal antibodies, chimeric antibodies, humanized antibodies (fully or partially humanized), multi-specific or multi-valent structures formed from antibody fragments, bifunctional antibodies, heteroconjugate Abs, DVD-Ig®s, and other antibodies as described in (i) herein. (Dual-variable domain immunoglobulins and methods for making them are described in Wu, C., *et al.*, *Nature Biotechnology*, 25:1290-1297 (2007)). The term "bifunctional antibody," as used herein, refers to an antibody that comprises a first arm having a specificity for one antigenic site and a second arm having a specificity for a different antigenic site, *i.e.*, the bifunctional antibodies have a dual specificity.

**[0109]** "Risk assessment," "risk classification," "risk identification," or "risk stratification" of subjects (e.g., patients) as used herein refers to the evaluation of factors including biomarkers, to predict the risk of occurrence of future events including disease onset or disease progression, so that treatment decisions regarding the subject may be made on a more informed basis.

**[0110]** "Sample," "test sample," "specimen," "biological sample", "sample from a subject," and "patient sample" as used herein may be used interchangeable and may be a sample of blood, such as whole blood, tissue, urine, serum, plasma, amniotic fluid, cerebrospinal fluid, placental cells or tissue, endothelial cells, leukocytes, or monocytes. In some embodiments, the sample is a whole blood sample. In some embodiments, the sample is a serum sample. In yet other embodiments, the sample is a plasma sample. The sample can be used directly as obtained from a patient or can be pre-treated, such as by filtration, distillation, extraction, concentration, centrifugation, inactivation of interfering components, addition of reagents, and the like, to modify the character of the sample in some manner as discussed herein or otherwise as is known in the art.

**[0111]** A variety of cell types, tissue, or bodily fluid may be utilized to obtain a sample. Such cell types, tissues, and fluid may include sections of tissues such as biopsy and autopsy samples, frozen sections taken for histologic purposes, blood (such as whole blood), plasma, serum, red blood cells, platelets, interstitial fluid, cerebral spinal fluid, etc. Cell types and tissues may also include lymph fluid, cerebrospinal fluid, a fluid collected by A tissue or cell type may be provided by removing a sample of cells from a human and a non-human animal, but can also be accomplished by using previously isolated cells (e.g., isolated by another person, at another time, and/or for another purpose). Archival tissues, such as those having treatment or outcome history, may also be used. Protein or nucleotide isolation and/or purification may not be necessary.

**[0112]** "Sensitivity" of an assay as used herein refers to the proportion of subjects for whom the outcome is positive that are correctly identified as positive (e.g., correctly identifying those subjects with a disease or medical condition for which they are being tested). For example, this might include correctly identifying subjects as having a TBI from those who do not have a TBI, correctly identifying subjects having a moderate, severe, or moderate to severe TBI from those having a mild TBI, correctly identifying subjects as having a mild TBI from those having a moderate, severe, or moderate to severe TBI, correctly identifying subjects as having a moderate, severe, or moderate to severe TBI from those having no TBI or correctly identifying subjects as having a mild TBI from those having no TBI, correctly identifying subjects as likely to benefit from imaging or a head CT scan or a MRI from those who are not likely to benefit from a head imaging or a CT scan or MRI, etc.).

**[0113]** "Specificity" of an assay as used herein refers to the proportion of subjects for whom the outcome is negative that are correctly identified as negative (e.g., correctly identifying those subjects who do not have a disease or medical condition for which they are being tested). For example, this might include correctly identifying subjects having an TBI from those who do not have a TBI, correctly identifying subjects not having a moderate, severe, or moderate to severe TBI from those having a mild TBI, correctly identifying subjects as not having a mild TBI from those having a moderate, severe, or moderate to severe TBI or correctly identifying subjects as not having any TBI, or correctly identifying subjects as having a mild TBI from those having no TBI, etc.).

**[0114]** "Series of calibrating compositions" refers to a plurality of compositions comprising a known concentration of UCH-L1, wherein each of the compositions differs from the other compositions in the series by the concentration of UCH-L1.

**[0115]** "Specific binding" or "specifically binding" as used herein may refer to the interaction of an antibody, a protein, or a peptide with a second chemical species, wherein the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope "A", the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled "A" and the antibody, will reduce the amount of labeled A bound to the antibody.

**[0116]** "Solid phase" or "solid support" as used interchangeably herein, refers to any material that can be used to attach and/or attract and immobilize (1) one or more capture agents or capture specific binding partners, or (2) one or more detection agents or detection specific binding partners. The solid phase can be chosen for its intrinsic ability to attract and immobilize a capture agent. Alternatively, the solid phase can have affixed thereto a linking agent that has the ability to attract and immobilize the (1) capture agent or capture specific binding partner, or (2) detection agent or detection specific binding partner. For example, the linking agent can include a charged substance that is oppositely charged with respect to the capture agent (*e.g.,* capture specific binding partner) or detection agent (*e.g.,* detection specific binding partner) itself or to a charged substance conjugated to the (1) capture agent or capture specific binding partner or (2) detection agent or detection specific binding partner. In general, the linking agent can be any binding partner (preferably specific) that is immobilized on (attached to) the solid phase and that has the ability to immobilize the (1) capture agent or capture specific binding partner, or (2) detection agent or detection specific binding partner through a binding reaction. The linking agent enables the indirect binding of the capture agent to a solid phase material before the performance of the assay or during the performance of the assay. For examples, the solid phase can be plastic, derivatized plastic, magnetic, or non-magnetic metal, glass or silicon, including, for example, a test tube,

microtiter well, sheet, bead, microparticle, chip, and other configurations known to those of ordinary skill in the art.

**[0117]** "Specific binding partner" is a member of a specific binding pair. A specific binding pair comprises two different molecules, which specifically bind to each other through chemical or physical means. Therefore, in addition to antigen and antibody specific binding pairs of common immunoassays, other specific binding pairs can include biotin and avidin (or streptavidin), carbohydrates and lectins, complementary nucleotide sequences, effector and receptor molecules, cofactors and enzymes, enzymes and enzyme inhibitors, and the like. Furthermore, specific binding pairs can include members that are analogs of the original specific binding members, for example, an analyte-analog. Immunoreactive specific binding members include antigens, antigen fragments, and antibodies, including monoclonal and polyclonal antibodies as well as complexes and fragments thereof, whether isolated or recombinantly produced.

**[0118]** "Stability" as used herein refers to the change in concentration of an analyte, biomarker (e.g., such as UCH-L1) or target substance over time (such as in a sample). Stability can be determined by calculating the difference between a first test value and one or more subsequent test values (e.g., second test value, third test value, fourth test value, fifth test value, sixth test value, seventh test value, eighth test value, ninth test value, tenth test value, etc.). If the difference between the first test value and one or more subsequent test values is less than 10% (plus or minus), then the analyte, biomarker or target substance in the sample is considered to be stable. If the difference between the first test value and one or more subsequent test values is greater than 10% (plus or minus 10%), then the analyte, biomarker or target substance in the sample is not considered to be stable.

**[0119]** "Statistically significant" as used herein refers to the likelihood that a relationship between two or more variables is caused by something other than random chance. Statistical hypothesis testing is used to determine whether the result of a data set is statistically significant. In statistical hypothesis testing, a statistical significant result is attained whenever the observed $p$-value of a test statistic is less than the significance level defined of the study. The $p$-value is the probability of obtaining results at least as extreme as those observed, given that the null hypothesis is true. Examples of statistical hypothesis analysis

include Wilcoxon signed-rank test, t-test, Chi-Square or Fisher's exact test. "Significant" as used herein refers to a change that has not been determined to be statistically significant (e.g., it may not have been subject to statistical hypothesis testing).

**[0120]** "Subject" and "patient" as used herein interchangeably refers to any vertebrate, including, but not limited to, a mammal (e.g., cow, pig, camel, llama, horse, goat, rabbit, sheep, hamsters, guinea pig, cat, dog, rat, and mouse, a non-human primate (for example, a monkey, such as a cynomolgous or rhesus monkey, chimpanzee, etc.) and a human). In some embodiments, the subject may be a human or a non-human. In some embodiments, the subject is a human. The subject or patient may be undergoing other forms of treatment. In some embodiments, when the subject is a human, the subject does not include any humans who have suffered a cerebrovascular accident (e.g., a stroke).

**[0121]** "Treat," "treating" or "treatment" are each used interchangeably herein to describe reversing, alleviating, or inhibiting the progress of a disease and/or injury, or one or more symptoms of such disease, to which such term applies. Depending on the condition of the subject, the term also refers to preventing a disease, and includes preventing the onset of a disease, or preventing the symptoms associated with a disease. A treatment may be either performed in an acute or chronic way. The term also refers to reducing the severity of a disease or symptoms associated with such disease prior to affliction with the disease. Such prevention or reduction of the severity of a disease prior to affliction refers to administration of a pharmaceutical composition to a subject that is not at the time of administration afflicted with the disease. "Preventing" also refers to preventing the recurrence of a disease or of one or more symptoms associated with such disease. "Treatment" and "therapeutically," refer to the act of treating, as "treating" is defined above. "Traumatic Brain Injury" or "TBI" as used interchangeably herein refers to a complex injury with a broad spectrum of symptoms and disabilities. TBI is most often an acute event similar to other injuries. TBI can be classified as "mild," "moderate," or "severe." The causes of TBI are diverse and include, for example, physical shaking by a person, a car accident, injuries from firearms, cerebral vascular accidents (e.g., strokes), falls, explosions or blasts and other types of blunt force trauma. Other causes of TBI include the ingestion and/or exposure to one or more chemicals or toxins (such as fires, molds, asbestos, pesticides and insecticides, organic solvents, paints, glues,

gases (such as carbon monoxide, hydrogen sulfide, and cyanide), organic metals (such as methyl mercury, tetraethyl lead and organic tin), one or more drugs of abuse or combinations thereof). Alternatively, TBI can occur in subjects suffering from an autoimmune disease, a metabolic disorder, a brain tumor, hypoxia, one or more viruses, meningitis, hydrocephalus or combinations thereof. Young adults and the elderly are the age groups at highest risk for TBI. In certain embodiments herein, traumatic brain injury or TBI does not include and specifically excludes cerebral vascular accidents such as strokes.

**[0122]** "Mild TBI" as used herein refers to a brain injury where loss of consciousness is brief and usually a few seconds or minutes and/or confusion and disorientation is shorter than 1 hour. Mild TBI is also referred to as a concussion, minor head trauma, minor TBI, minor brain injury, and minor head injury. While MRI and CT scans are often normal, the individual with mild TBI may have cognitive problems such as headache, difficulty thinking, memory problems, attention deficits, mood swings and frustration.

**[0123]** Mild TBI is the most prevalent TBI and is often missed at time of initial injury. Typically, a subject has a Glasgow Coma scale number of between 13-15 (such as 13-15 or 14-15). Fifteen percent (15%) of people with mild TBI have symptoms that last 3 months or more. Mild TBI is defined as the result of the forceful motion of the head or impact causing a brief change in mental status (confusion, disorientation or loss of memory) or loss of consciousness for less than 30 minutes. Common symptoms of mild TBI include fatigue, headaches, visual disturbances, memory loss, poor attention/concentration, sleep disturbances, dizziness/loss of balance, irritability-emotional disturbances, feelings of depression, and seizures. Other symptoms associated with mild TBI include nausea, loss of smell, sensitivity to light and sounds, mood changes, getting lost or confused, and/or slowness in thinking.

**[0124]** "Moderate TBI" as used herein refers to a brain injury where loss of consciousness and/or confusion and disorientation is between 1 and 24 hours and the subject has a Glasgow Coma scale number of between 9-13 (such as 9-12 or 9-13). The individual with moderate TBI have abnormal brain imaging results. "Severe TBI" as used herein refers to a brain injury where loss of consciousness is more than 24 hours and memory loss after the injury or penetrating skull injury longer than 24 hours and the subject has a Glasgow Coma scale

number between 3-8. The deficits range from impairment of higher level cognitive functions to comatose states. Survivors may have limited function of arms or legs, abnormal speech or language, loss of thinking ability or emotional problems. Individuals with severe injuries can be left in long-term unresponsive states. For many people with severe TBI, long-term rehabilitation is often necessary to maximize function and independence.

**[0125]** "Moderate to severe" TBI as used herein refers to a spectrum of brain injury that includes moderate to severe and thus encompasses moderate TBI alone, severe TBI alone and moderate to severe TBI combined. Subjects suffering from a moderate to severe TBI can have a Glasgow Coma scale number of between 3-13 (such as 3-12 or 3-13). For example, in some clinical situations, a subject may initially be diagnosed as having a moderate TBI but who, over the course of time (minutes, hours or days), progress to having a severe TBI (such, as for example, in situations when there is a brain bleed). Such subjects would be examples of patients that could be classified as "moderate to severe". Common symptoms of moderate to severe TBI include cognitive deficits including difficulties with attention, concentration, distractibility, memory, speed of processing, confusion, perseveration, impulsiveness, language processing, and/or "executive functions", not understanding the spoken word (receptive aphasia), difficulty speaking and being understood (expressive aphasia), slurred speech, speaking very fast or very slow, problems reading, problems writing, difficulties with interpretation of touch, temperature, movement, limb position and fine discrimination, the integration or patterning of sensory impressions into psychologically meaningful data, partial or total loss of vision, weakness of eye muscles and double vision (diplopia), blurred vision, problems judging distance, involuntary eye movements (nystagmus), intolerance of light (photophobia), hearing, such as decrease or loss of hearing, ringing in the ears (tinnitus), increased sensitivity to sounds, loss or diminished sense of smell (anosmia), loss or diminished sense of taste, the convulsions associated with epilepsy that can be several types and can involve disruption in consciousness, sensory perception, or motor movements, control of bowel and bladder, sleep disorders, loss of stamina, appetite changes, regulation of body temperature, menstrual difficulties, dependent behaviors, emotional ability, lack of motivation, irritability, aggression, depression, disinhibition, or denial/lack of awareness.

**[0126]** "Ubiquitin carboxy-terminal hydrolase L1" or "UCH-L1" as used interchangeably herein refers to a deubiquitinating enzyme that is encoded by the *UCH-L1* gene in humans, and which can be produced (e.g., by recombinant means, in other species). UCH-L1, also known as ubiquitin carboxyl-terminal esterase L1 and ubiquitin thiolesterase, is a member of a gene family whose products hydrolyze small C-terminal adducts of ubiquitin to generate the ubiquitin monomer.

**[0127]** "UCH-L1 status" can mean either the level or amount of UCH-L1 at a point in time (such as with a single measure of UCH-L1), the level or amount of UCH-L1 associated with monitoring (such as with a repeat test on a subject to identify an increase or decrease in UCH-L1 amount), the level or amount of UCH-L1 associated with treatment for traumatic brain injury (whether a primary brain injury and/or a secondary brain injury) or combinations thereof.

**[0128]** "Variant" is used herein to describe a peptide or polypeptide that differs in amino acid sequence by the insertion, deletion, or conservative substitution of amino acids, but retain at least one biological activity. Representative examples of "biological activity" include the ability to be bound by a specific antibody or to promote an immune response. Variant is also used herein to describe a protein with an amino acid sequence that is substantially identical to a referenced protein with an amino acid sequence that retains at least one biological activity. A conservative substitution of an amino acid, *i.e.,* replacing an amino acid with a different amino acid of similar properties (*e.g.,* hydrophilicity, degree, and distribution of charged regions) is recognized in the art as typically involving a minor change. These minor changes can be identified, in part, by considering the hydropathic index of amino acids, as understood in the art. Kyte *et al.*, *J. Mol. Biol.* 157:105-132 (1982). The hydropathic index of an amino acid is based on a consideration of its hydrophobicity and charge. It is known in the art that amino acids of similar hydropathic indexes can be substituted and still retain protein function. In one aspect, amino acids having hydropathic indexes of ±2 are substituted. The hydrophilicity of amino acids can also be used to reveal substitutions that would result in proteins retaining biological function. A consideration of the hydrophilicity of amino acids in the context of a peptide permits calculation of the greatest local average hydrophilicity of that peptide, a useful measure that has been reported to correlate well with antigenicity and

immunogenicity. U.S. Patent No. 4,554,101, incorporated fully herein by reference. Substitution of amino acids having similar hydrophilicity values can result in peptides retaining biological activity, for example immunogenicity, as is understood in the art. Substitutions may be performed with amino acids having hydrophilicity values within ±2 of each other. Both the hydrophobicity index and the hydrophilicity value of amino acids are influenced by the particular side chain of that amino acid. Consistent with that observation, amino acid substitutions that are compatible with biological function are understood to depend on the relative similarity of the amino acids, and particularly the side chains of those amino acids, as revealed by the hydrophobicity, hydrophilicity, charge, size, and other properties. "Variant" also can be used to refer to an antigenically reactive fragment of an anti-UCH-L1 antibody that differs from the corresponding fragment of anti- UCH-L1 antibody in amino acid sequence but is still antigenically reactive and can compete with the corresponding fragment of anti- UCH-L1 antibody for binding with UCH-L1. "Variant" also can be used to describe a polypeptide or a fragment thereof that has been differentially processed, such as by proteolysis, phosphorylation, or other post-translational modification, yet retains its antigen reactivity.

**[0129]** "Vector" is used herein to describe a nucleic acid molecule that can transport another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double-stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors can replicate autonomously in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. "Plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, other forms of expression vectors, such as viral

vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions, can be used. In this regard, RNA versions of vectors (including RNA viral vectors) may also find use in the context of the present disclosure.

**[0130]** Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. For example, any nomenclatures used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those that are well known and commonly used in the art. The meaning and scope of the terms should be clear; in the event, however of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular.

## 2. Preanalytical Processing Conditions Affecting Ubiquitin Carboxy-Terminal Hydrolase L1 (UCH-L1) Levels in Samples and Methods of Measuring Ubiquitin Carboxy-Terminal Hydrolase L1 (UCH-L1) Levels

**[0131]** The present disclosure is directed to an improvement of a method of measuring an amount of UCH-L1 in a sample (e.g., blood sample (such as a whole blood, serum or plasma sample)) obtained from a subject. As described herein, certain preanalytical processing conditions or factors can influence UCH-L1 levels or concentrations, and in some cases, lead to increases in UCH-L1 levels or concentrations during or between the period of time during which the blood sample is being maintained or stored until a test or assay is performed (such as to detect and/or measure the amount of UCH-L1 in the sample). Such increases in UCH-L1 levels or concentrations are erroneous (namely, an erroneous increase or erroneously high) and may not be an accurate reflection of a disease state (such as TBI) and may lead to false positive results. Thus, the present disclosure is also directed to methods of avoiding or preventing an erroneous increase or rise in UCH-L1 levels in a blood sample obtained from a subject prior to performing a test or assay to detect and/or measure UCH-L1 levels by performing the methods described herein. Finally, the present disclosure is also directed to

methods of stabilizing UCH-L1 levels in a blood sample to prevent or avoid an increase or rise in such levels during storage prior to performing a test or assay to detect and/or measure UCH-L1 levels in the blood sample.

**[0132]** In one embodiment, a preanalytical processing condition or factor discovered to influence UCH-L1 levels or concentration is the processing time of the blood sample, namely, how quickly the blood sample is processed after it is obtained from a subject (namely, prior to testing or performing an assay). In some embodiments, it is advantageous to process a blood sample (e.g., a whole blood sample) obtained from a subject within about 24 hours from the time point when the blood sample was drawn or obtained from the subject (but prior to testing or performance of an assay). In some embodiments, it is advantageous to process a blood sample obtained from a subject within about 12 hours, within about 10 hours, within about 8 hours, within about 6 hours, within about 4 hours, within about 2 hours, or within about 1 hour from the time point when the blood sample was drawn from the subject. In other embodiments, it is advantageous to process a blood sample within a time period after the sample is obtained from a subject, including from about 1 hour to about 12 hours, from about 1 hour to about 10 hours, from about 1 hour to about 8 hours, from about 1 hour to about 6 hours, from about 1 hour to about 4 hours, or from about 1 hour to about 2 hours, after the sample has been obtained from the subject. In some embodiments, it is advantageous to process a blood sample obtained from a subject within about 12 hours or less from the time point when the blood sample was drawn from the subject. In some embodiments, it is advantageous to process a blood sample obtained from a subject within about 10 hours or less from the time point when the blood sample was drawn from the subject. In some embodiments, it is advantageous to process a blood sample obtained from a subject within about 8 hours or less from the time point when the blood sample was drawn from the subject. In some embodiments, it is advantageous to process a blood sample obtained from a subject within about 6 hours or less from the time point when the blood sample was drawn from the subject. In some embodiments, it is advantageous to process a blood sample obtained from a subject within about 4 hours or less from the time point when the blood sample was drawn from the subject. In some embodiments, it is advantageous to process a blood sample obtained from a subject within about 2 hours or less from the time

point when the blood sample was drawn from the subject. In some embodiments, it is advantageous to process a blood sample obtained from a subject within about 1 hour or less from the time point when the blood sample was drawn from the subject.

**[0133]** In accordance with these embodiments, it has been found that during processing that mechanical agitation (such as mixing) of the blood sample should be avoided during or between the period of time after the sample is obtained from the subject to the time when the test or assayed is performed (e.g., to detect and/or measure UCH-L1). In other words, continuous mixing up until testing or performance of an assay should be avoided. In some cases, mixing the blood samples during processing can cause hemolysis of the blood samples and release UCH-L1 into the sample, which can cause increased UCH-L1 levels to be obtained. However, one skilled in the art that would recognize that minor mechanical agitation or mixing may be necessary when processing blood samples; however, mixing to a degree that would lead to hemolysis should generally be avoided. For example, a blood sample should not be mixed for longer than is necessary to mix the blood sample with any anti-coagulant that may be present in a collection tube (e.g, such as in a heparin tube, ethylene diamine tetra acetic acid (EDTA) tube, etc.). In another aspect, the blood sample should not be mixed for longer than 5 seconds. In yet another aspect, the blood sample should not be mixed for longer than 10 seconds. In yet another aspect, the blood sample should not be mixed for longer than 15 seconds. In yet another aspect, the blood sample should not be mixed for longer than 20 seconds. In yet another aspect, the blood sample should not be mixed for longer than 25 seconds. In yet another aspect, the blood sample should not be mixed for longer than 30 seconds. In yet another aspect, the blood sample should not be mixed for longer than 40 seconds. In yet another aspect, the blood sample should not be mixed for longer than 50 seconds. In yet another aspect, the blood sample should not be mixed for longer than 60 seconds. In yet another aspect, the blood sample should not be mixed for longer than 2 minutes. In yet another aspect, the blood sample should not be mixed for longer than 3 minutes. In yet another aspect, the blood sample should not be mixed for longer than 4 minutes. In yet another aspect, the blood sample should not be mixed for longer than 5 minutes. In yet another aspect, the blood sample should not be mixed for longer than 5 minutes. In yet another aspect, the blood sample

should not be mixed for longer than 2 minutes. In yet another aspect, the blood sample should not be mixed for longer than 3 minutes. In yet another aspect, the blood sample should not be mixed for longer than 4 minutes. In yet another aspect, the blood sample should not be mixed for longer than 5 minutes. In yet another aspect, the blood sample should not be mixed for longer than 6 minutes. In yet another aspect, the blood sample should not be mixed for longer than 7 minutes. In yet another aspect, the blood sample should not be mixed for longer than 8 minutes. In yet another aspect, the blood sample should not be mixed for longer than 9 minutes. In yet another aspect, the blood sample should not be mixed for longer than 10 minutes. In yet another aspect, the blood sample should not be mixed for longer than 11 minutes. In yet another aspect, the blood sample should not be mixed for longer than 12 minutes. In yet another aspect, the blood sample should not be mixed for longer than 13 minutes. In yet another aspect, the blood sample should not be mixed for longer than 14 minutes. In yet another aspect, the blood sample should not be mixed for longer than 15 minutes. In yet another aspect, the blood sample should not be mixed for longer than 16 minutes. In yet another aspect, the blood sample should not be mixed for longer than 17 minutes. In yet another aspect, the blood sample should not be mixed for longer than 18 minutes. In yet another aspect, the blood sample should not be mixed for longer than 19 minutes. In yet another aspect, the blood sample should not be mixed for longer than 20 minutes. In yet another aspect, the blood sample should not be mixed for longer than 25 minutes. In yet another aspect, the blood sample should not be mixed for longer than 30 minutes.

**[0134]**

**[0135]** In accordance with these embodiments, in another aspect, the blood samples can be maintained or adjusted at various temperatures during processing, including, for example, at temperatures ranging from about 2°C to about 8°C to about room temperature (e.g., about 20°C to about 25°C). Blood samples can be maintained within these temperature ranges for any period of time prior to performing a test or assay to assess (e.g., detect and/or measure) UCH-L1 levels, including for up to about 24 hours after the sample has been obtained from a subject. In some cases, it is advantageous to maintain blood samples at a temperature ranging from about 2°C to about 8 °C for no more than about 8 hours after the sample has

been obtained from a subject, and up until the sample is assessed for UCH-L1 concentrations. In some cases, it is advantageous to maintain blood samples at about room temperature (namely, from about 20°C to about 25°C) for no more than about 8 hours after the sample has been obtained from a subject, and up until the sample is assessed for UCH-L1 concentrations. In some embodiments, whole blood assays (e.g., i-STAT® and i-STAT® Alinity) require working with samples in a manner that may include mixing or prolonged exposure to room temperature, which may have an effect on the assessment of UCH-L1 concentrations, as described herein. In some embodiments, the blood samples can be maintained at a temperature from about 2°C to about 8°C. For example, if the blood sample is a whole sample, the sample can be maintained at a temperature of from about 2°C to about 8°C. By way of another example, if the blood sample is a plasma sample, the sample can be maintained at a temperature of from about 2°C to about 8°C. In yet another example, if the blood sample is a serum sample, the sample can be maintained at a temperature of from about 2°C to about 8°C. In another embodiment, the blood samples can be maintained at room temperature (e.g., about 20°C to about 25°C). For example, if the blood sample is a plasma sample, the sample can be maintained at room temperature. In yet another example, if the blood sample is a serum sample, the sample can be maintained at room temperature.

**[0136]** Processing whole blood sample can include any steps or methods used to obtain the blood sample from the subject, as well as any steps or methods used to separate out various blood components into serum, plasma, and red blood cell (RBC) fractions. Processing can also include any steps or methods involved with testing, detecting, measuring, or assessing blood samples for various components, such as UCH-L1 and other biomarkers of TBI. In cases where assessing UCH-L1 levels are not included as part of processing the whole blood samples (e.g., UCH-L1 assessments are performed in a laboratory separate from the site where the blood samples were obtained from a subject), testing, detecting, or measuring UCH-L1 samples can occur shortly after or immediately after the whole blood sample has been processed. In some embodiments, methods of the present disclosure include processing a whole blood sample from a subject within no more than about 8 hours after the sample is obtained from the subject, in order to avoid a rise in UCH-L1 levels that occurs due to improper storage conditions and/or processing methods.

**[0137]** In some embodiments, processing includes separating serum and/or plasma from blood cells in the sample in order to assess UCH-L1 levels in the plasma and/or serum. This process is generally referred to as blood fractionation and may be done by subjecting the blood samples to routine techniques known in the art, such as, for example, centrifugation, chromatography, ethanol fractionation, lab-on-a-chip cellular sorting (e.g., magnetic cellular sorting), size exclusion in cross-flow filtration, dielectrophoretic techniques, pinched-flow fractionation, inertial microfluidics, separation utilizing electro-hydrodynamic forces, and combinations thereof.

**[0138]** In some embodiments, blood fractionation may be done by centrifugation, which results in the separation of the sample into a plasma component, a buffy coat (leukocytes and platelets), and erythrocytes (red blood cells). Collection tubes, which are used to collect and store blood samples obtained from subjects, can also be used to separate plasma from blood cells, such as during centrifugation. A variety of collection tubes can be used in the methods described herein. For example, collection tubes can contain or be coated to contain various components to facilitate the fractionation of blood components. In some embodiments, collection tubes can contain one or more anti-coagulants, such as EDTA) or salts of EDTA (such as $K_2$EDTA or $K_3$EDTA), which is an anti-coagulant that can be used to prevent blood clotting by chelating calcium, an essential component of coagulation. Moreover, plasma isolated from blood samples taken with EDTA collection tubes can also be used to measure or detect a variety of proteins found in the plasma, such as UCH-L1 and other biomarkers of TBI, and genetic material can easily be stored from buffy coats from EDTA collection tubes. Examples of other anti-coagulants that can be used include without limitation heparin, such as lithium or sodium heparin, ethylene glycol tetra acetic acid (EGTA), salts of EGTA, hirudin, heparin, citric acid, salts of citric acid, oxalic acid, salts of oxalic acid (e.g., potassium oxalate, sodium oxalate, or the like), and combinations thereof.

**[0139]** In some embodiments, processing includes separating serum from plasma and blood cells in the sample in order to assess UCH-L1 levels in the serum. Serum generally refers to the blood component that remains after clotting occurs; serum is the cell-free liquid that is also depleted of coagulation factors. Serum can be separated from plasma and blood cells in the sample using the methods described previously herein. Specifically, serum separation

tubes (SSTs) can be used to separate serum from blood cells (such as during centrifugation). Specifically, SSTs are tubes that may contain a gel, such as a silicone gel. When centrifuged, the silicone gel forms a layer on top of the buffy coat, allowing the blood serum to be removed more effectively for testing and related purposes. SSTs are sometimes called "marble-top tubes," "target-tops," or "gold-topped tubes," referring to the stoppers which are gold, red with a gold ring on top, or marbled red and grey. Marble-top tubes are also referred to as "tiger-tops" in some clinical settings.

**[0140]** The present disclosure is directed to an improvement of a method of measuring an amount of UCH-L1 in a blood sample, in which processing the sample includes performing a test that measures the amount of UCH-L1 in the sample. In some embodiments, the test for detecting, measuring, quantifying, or assessing UCH-L1 includes any available methods for assaying proteins, including, but not limited to, an immunoassay, chemical analysis, SDS PAGE and Western blot analysis, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, chromatography, spectrophotometry, and the like. In some embodiments, immunoassays, such as the types described herein (e.g., i-STAT® and i-STAT® Alinity) are advantageous for measuring UCH-L1 levels in whole blood samples.

**[0141]** In some embodiments, the present disclosure is directed to an improvement of a method of measuring an amount of UCH-L1 in a blood sample, which includes performing an immunoassay-based test for measuring UCH-L1. The immunoassay test can include contacting the sample, either simultaneously or sequentially, in any order with at least one capture antibody, which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a capture antibody-UCH-L1 antigen complex. Additionally, the immunoassay can include at least one detection antibody, which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the capture antibody, to form a UCH-L1 antigen-detection antibody complex, such that a capture antibody-UCH-L1 antigen-detection antibody complex is formed. In some embodiments, measuring the amount or concentration of UCH-L1 in a sample is based on the signal generated by the detectable label in the capture antibody-UCH-L1 antigen-detection antibody complex.

### 3. Methods of Aiding in the Diagnosis and Evaluation of Whether a Human Subject has Sustained an Injury to the Head

**[0142]** In another embodiment, the methods described herein can be used to provide an aid in the diagnosis and evaluation of whether a human subject has sustained or may have sustained an injury to the head by determining the levels of UCH-L1 in a subject. The method can aid in determining the extent of traumatic brain injury in a human subject with a suspected injury to the head, *e.g.*, determining whether the subject has mild traumatic brain injury or moderate to severe traumatic brain injury. As used here, "determining whether the subject has mild traumatic brain injury or moderate to severe traumatic brain injury" refers to the fact that the aforementioned method can be used, e.g., with other information (e.g., clinical assessment data), to determine that the subject is more likely than not to have mild traumatic brain injury or moderate to severe traumatic brain injury. The method may be used to detect or assess traumatic brain injury in a subject using the anti-UCH-L1 antibodies described below, or antibody fragments thereof. The method can include the steps of (a) obtaining a sample from a subject, (b) determining the level of UCH-L1 in the sample using one or more anti-UCH-L1 antibodies, or antibody fragments thereof, and (c) determining whether the subject has sustained a mild or a moderate to severe traumatic brain injury (TBI) based on the UCH-L1 levels. In some embodiments, the subject is determined as having (1) a moderate, severe or moderate to severe TBI when the level of the early biomarker in the sample is higher than a reference level of the early biomarker, or (2) a mild TBI when the level of the early biomarker in the sample is lower than a reference level of the early biomarker. The sample can be a blood sample.

**[0143]** Alternatively, the method can include the steps of (a) determining the level of UCH-L1 in the sample obtained from a subject using one or more anti-UCH-L1 antibodies, or antibody fragments thereof, and (b) determining whether the subject has sustained a mild or a moderate to severe traumatic brain injury (TBI) based on the UCH-L1 levels. In some embodiments, the subject is determined as having (1) a moderate, severe or moderate to severe TBI when the level of the early biomarker in the sample is higher than a reference level of the early biomarker, or (2) a mild TBI when the level of the early biomarker in the

sample is lower than a reference level of the early biomarker. The sample can be a blood sample.

**[0144]** By measuring and assessing UCH-L1, the method allows for more diseases to be more accurately diagnosed and subsequently treated more successfully, compared to other commercially available UCH-L1 immunoassays. The method can be adapted for use in an automated system or a semi-automated system.

**[0145]** Generally, a reference level of UCH-L1 can be employed as a benchmark against which to assess results obtained upon assaying a test sample for UCH-L1. Generally, in making such a comparison, the reference level of UCH-L1 is obtained by running a particular assay a sufficient number of times and under appropriate conditions such that a linkage or association of analyte presence, amount or concentration with a particular stage or endpoint of TBI or with particular indicia can be made. Typically, the reference level of UCH-L1 is obtained with assays of reference subjects (or populations of subjects). The UCH-L1 measured can include UCH-L1 fragments thereof, degradation products thereof, and/or enzymatic cleavage products thereof. In certain embodiments, the reference level of UCH-L1 may be correlated with control subjects that have not sustained a head injury. Generally, the same assay used to determine the reference level is used to assay or test the sample.

**[0146]** The reference level in this method can be the level of UCH-L1 in a subject having or suspected of having traumatic brain injury. In some embodiments, levels higher than or equal to 5 pg/mL, 10 pg/mL, 20 pg/mL, 30 pg/mL, 40 pg/mL, 50 pg/mL, 60 pg/mL, 70 pg/mL, 80 pg/mL, 90 pg/mL, 100 pg/mL, 500 pg/mL, 1000 pg/mL, 5000 pg/mL, 10000 pg/mL, or 50000 pg/mL in serum of UCH-L1 identify the subject as having traumatic brain injury. Optionally, in some cases, levels higher than or equal to 100000 pg/mL, 500000 pg/mL, 1000000 pg/mL, 150000 pg/mL, 200000 pg/mL, or 500000 pg/mL in serum of UCH-L1 identify the subject as having traumatic brain injury. It is known that, absent standardization, reference levels can vary between methods and assays. Accordingly, the method described herein is intended to apply to any appropriate reference level.

**[0147]** In some embodiments, the method further includes treating the human subject predicted as having moderate to severe traumatic brain injury with a traumatic brain injury treatment, as described below. In some embodiments, the method further includes

monitoring the human subject predicted as having mild traumatic brain injury, as described below.

## 4. Methods for Aiding in Determining the Risk of a Subject of Developing Traumatic Brain Injury

**[0148]** In yet another embodiment, the methods described herein also can be used as an aid to determine whether or not a subject has a traumatic brain injury or is at risk of developing a traumatic brain injury (such as a severe traumatic brain injury from a moderate traumatic brain injury) by determining the levels of UCH-L1 in a subject using the anti-UCH-L1 antibodies described below, or antibody fragments thereof. Thus, in particular embodiments, the disclosure also provides a method for aiding in determining whether a subject having, or at risk for, traumatic brain injuries, discussed herein and known in the art, is a candidate for therapy or treatment. As used here, "determining whether the subject has, or is at risk for, traumatic brain injuries refers to the fact that the aforementioned method can be used, e.g., with other information (e.g., clinical assessment data), to determine that the subject is more likely than not to have traumatic brain injuries. Generally, the subject is at least one who: (i) has experienced, or is suspected of having experienced, an injury to the head; (ii) ingested and/or been exposed to one or more chemicals and/or toxins; (iii) suffers from an autoimmune disease, a metabolic disorder, a brain tumor, hypoxia, one or more viruses, meningitis, hydrocephalus or suffers from any combinations thereof; or (iv) any combinations of (i)-(iii); or, (v) who has actually been diagnosed as having, or being at risk for TBI (such as, for example, subjects suffering from an autoimmune disease, a metabolic disorder, a brain tumor, hypoxia, one or more viruses, meningitis, hydrocephalus or combinations thereof), and/or who demonstrates an unfavorable (i.e., clinically undesirable) concentration or amount of UCH-L1 or UCH-L1 fragment, as described herein.

**[0149]** Specifically, such a method can comprise the steps of: (a) determining the concentration or amount in a test sample from a subject of UCH-L1 using the methods described herein, or methods known in the art); and (b) comparing the concentration or amount of UCH-L1 determined in step (a) with a reference level, wherein, if the concentration or amount of UCH-L1 determined in step (a) is favorable with respect to a

reference level, then the subject is determined not to have or be at risk for traumatic brain injury as discussed herein and known in the art. However, if the concentration or amount of UCH-L1 determined in step (a) is unfavorable with respect to the reference level, then the subject is determined to have or be at risk for traumatic brain injury as discussed herein and known in the art.

**[0150]** The reference level in this method can be the level of UCH-L1 in a subject having or suspected of having traumatic brain injury. In some embodiments, levels higher than or equal to 5 pg/mL, 10 pg/mL, 20 pg/mL, 30 pg/mL, 40 pg/mL, 50 pg/mL, 60 pg/mL, 70 pg/mL, 80 pg/mL, 90 pg/mL, 100 pg/mL, 500 pg/mL, 1000 pg/mL, 5000 pg/mL, 10000 pg/mL, or 50000 pg/mL in serum of UCH-L1 identify the subject as having traumatic brain injury. Optionally, in some cases, levels higher than or equal to 100000 pg/mL, 500000 pg/mL, 1000000 pg/mL, 150000 pg/mL, 200000 pg/mL, or 500000 pg/mL in serum of UCH-L1 identify the subject as having traumatic brain injury. It is known that, absent standardization, reference levels can vary between methods and assays. Accordingly, the method described herein is intended to apply to any appropriate reference level.

**[0151]** In some embodiments, the method further includes treating the human subject with a traumatic brain injury with a traumatic brain injury treatment and/or monitoring the human subject, as described below.

## 5. Method of Aiding in the Determination of Whether to Perform a CT scan on a Human Subject Who has Sustained an Injury to the Head

**[0152]** The present disclosure relates, among other methods, to a method of aiding in determining whether to perform a computerized tomography (CT) scan on a human subject who has sustained or may have sustained a suspected injury to the head. As used here, "determination of whether to perform a CT scan on a human subject" refers to the fact that the aforementioned method can be used, e.g., with other information (e.g., clinical assessment data), to determine that the subject is more likely than not to have a positive head CT scan. Specifically, such a method can comprise the steps of: (a) performing an assay on a sample obtained from the subject within about 24 hours after a suspected injury to the head to measure or detect a level of UCH-L1 in the sample; and (b) performing a CT scan on the

subject when the level of UCH-L1 in the sample is higher than a reference level of UCH-L1 and not performing a CT scan on the subject when the level of UCH-L1 in the sample is lower than a reference level of UCH-L1. The sample can be a sample.

**[0153]** In this aspect of the invention, one skilled in the art would appreciate the blood samples obtained from such subjects would be processed using the methods as described previously herein as quickly as possible (such as, for example, in about 8 hours or less) to allow a test or assay to be performed within about 24 hours after a suspected injury to the head as described herein.

**[0154]** By measuring and assessing UCH-L1, the method allows for the need to perform a CT scan on a subject more accurately, and subsequently treated more successfully, compared to other commercially available UCH-L1 immunoassays. The method can be adapted for use in an automated system or a semi-automated system.

**[0155]** Generally, a reference level of UCH-L1 can be employed as a benchmark against which to assess results obtained upon assaying a test sample for UCH-L1. Generally, in making such a comparison, the reference level of UCH-L1 is obtained by running a particular assay a sufficient number of times and under appropriate conditions such that a linkage or association of analyte presence, amount or concentration with a particular stage or endpoint of TBI or with particular indicia can be made. Typically, the reference level of UCH-L1 is obtained with assays of reference subjects (or populations of subjects). The UCH-L1 measured can include UCH-L1 fragments thereof, degradation products thereof, and/or enzymatic cleavage products thereof. In certain embodiments, the reference level of UCH-L1 may be correlated with control subjects that have not sustained a head injury.

**[0156]** The reference level in this method can be the level of UCH-L1 in a subject having or suspected of having traumatic brain injury. In some embodiments, levels higher than or equal to 5 pg/mL, 10 pg/mL, 20 pg/mL, 30 pg/mL, 40 pg/mL, 50 pg/mL, 60 pg/mL, 70 pg/mL, 80 pg/mL, 90 pg/mL, 100 pg/mL, 500 pg/mL, 1000 pg/mL, 5000 pg/mL, 10000 pg/mL, or 50000 pg/mL in serum of UCH-L1 identify the subject as having traumatic brain injury. Optionally, in some cases, levels higher than or equal to 100000 pg/mL, 500000 pg/mL, 1000000 pg/mL, 150000 pg/mL, 200000 pg/mL, or 500000 pg/mL in serum of UCH-L1 identify the subject as having traumatic brain injury. It is known that, absent

standardization, reference levels can vary between methods and assays. Accordingly, the method described herein is intended to apply to any appropriate reference level.

**[0157]** In some embodiments, the method further includes treating the human subject with a traumatic brain injury with a traumatic brain injury treatment and/or monitoring the human subject, as described below.

## 6. Methods of Aiding in Determining the Extent of Traumatic Brain Injury in a Human Subject Who May have Sustained an Injury to the Head

**[0158]** The present disclosure relates to a method of aiding in determining the extent of traumatic brain injury in a human subject with a suspected injury to the head, *e.g.*, determining whether the subject has mild traumatic brain injury or moderate to severe traumatic brain injury. As used here, "determining the extent of traumatic brain injury in a human subject with a suspected injury to the head" refers to the fact that the aforementioned method can be used, e.g., with other information (e.g., clinical assessment data), to determine that the subject is more likely than not to have mild traumatic brain injury or moderate to severe traumatic brain injury. The method includes performing an assay on at least two samples obtained from the subject and detecting UCH-L1 in the at least two samples. The first sample is taken from the human subject within 24 hours of injury and the second sample is taken from the human subject about 3 to about 6 hours after the first sample is taken. The UCH-L1 appears within about 2 to about 24 hours after the onset of injury to the head. The onset of the presence of UCH-L1 appears within about 0 to about 6 hours after the onset of the suspected injury. Levels of UCH-L1 are determined for each of the first sample and second sample. The level of UCH-L1 is determined to decrease or increase. The extent of the traumatic brain injury is determined in the subject based on whether the level of UCH-L1 decreases, increases, or remains the same from the first sample to the second sample. The UCH-L1 increases within about 0 to about 6 hours after the suspected injury and then decreases or increases thereafter in subjects with traumatic brain injury. In some embodiments, the onset of the presence of UCH-L1 appears within about 0, about 0.5 hours, about 1 hour, about 2 hours, about 3 hours, about 4 hours, about 5 hours, or about 6 hours after injury to the head.

**[0159]** In this aspect of the invention, one skilled in the art would appreciate the blood samples obtained from such subjects would be processed using the methods as described previously herein as quickly as possible (such as, for example, in about 8 hours or less) to allow a test or assay to be performed within about 24 hours after a suspected injury to the head as described herein.

**[0160]** In some embodiments, the first sample is taken from the subject at a first time point within 24 hours of the suspected injury and the second sample is taken from the subject at a second time point after the first time point and the subject is determined to have mild or mild to severe traumatic brain injury when the level of UCH-L1 decreases from the first sample to the second sample. In some embodiments, the UCH-L1 decreases at least about 5% from the increased levels. For example, the UCH-L1 levels may decrease about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 100%, about 200%, about 300%, about 400%, about 500%, about 600%, about 700%, about 800%, about 900%, or about 1000% from the increased levels. In some embodiments, the UCH-L1 decreases at least about 0.1-fold, at least about 0.2-fold, at least about 0.3-fold, at least about 0.4-fold, at least about 0.5-fold, at least about 0.55-fold, at least about 0.6-fold, at least about 0.7-fold, at least about 0.73-fold, at least about 0.8-fold, at least about 0.9-fold, at least about 1-fold, at least about 1.5-fold, at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least 8-fold, at least 9-fold, at least 10-fold, at least 11-fold, at least 12-fold, at least 13-fold, at least 14-fold, at least 15-fold, at least 16-fold, at least 17-fold, at least 18-fold, at least 19-fold, or at least 20-fold from the increased levels. In some embodiments, the UCH-L1 decreases less than about 0.1-fold, less than about 0.2-fold, less than about 0.3-fold, less than about 0.4-fold, less than about 0.5-fold, less than about 0.55-fold, at least about 0.6-fold, at least about 0.7-fold, at least about 0.73-fold, at least about 0.8-fold, at least about 0.9-fold, less than about 1-fold, less than about 1.5-fold, less than about 2-fold, less than about 3-fold, less than about 4-fold, less than about 5-fold, less than about 6-fold, less than about 7-fold, less than 8-fold, less than 9-fold, less than 10-fold, less than 11-fold, less than 12-fold, less than 13-fold, less than 14-fold, less than 15-fold, less than 16-fold, less than 17-fold, less than 18-fold, less than 19-fold, or less than 20-fold from the increased levels.

**[0161]** In some embodiments, the second sample is obtained within about 1 hour to about 10 hours after the first sample is obtained, such as about 3 hours to about 6 hours after the first sample is obtained. In some embodiments, the second time sample is obtained within about 1 hour, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, or about 10 hours after the first sample is obtained.

**[0162]** In some embodiments, the method further includes treating a human subject assessed as having moderate to severe traumatic brain injury with a traumatic brain injury treatment, as described below. In some embodiments, the method further includes monitoring a human subject assessed as having mild traumatic brain injury, as described below.

## 7. Methods of Monitoring the Progression of Traumatic Brain Injury in a Subject

**[0163]** In yet another embodiment, the methods described herein also can be used as an aid to monitor the progression of disease and/or injury, such as traumatic brain injury, in a subject by determining the levels of UCH-L1 in a subject using the anti-UCH-L1 antibodies described below, or antibody fragments thereof. As used here, "monitoring the progression of disease and/or injury" refers to the fact that the aforementioned method can be used, e.g., with other information (e.g., clinical assessment data), to determine if the disease in the subject has more likely than not continued, progressed or worsened. Optimally, the method includes the steps of (a) determining the concentration or amount of UCH-L1 in a test sample from a subject using the anti-UCH-L1 antibodies described below, or antibody fragments thereof, (b) determining the concentration or amount of UCH-L1 in a later test sample from a subject using the anti-UCH-L1 antibodies described below, or antibody fragments thereof, and (c) comparing the concentration or amount of UCH-L1 as determined in step (b) with the concentration or amount of UCH-L1 determined in step (a), wherein if the concentration or amount determined in step (b) is unchanged or is unfavorable when compared to the concentration or amount of UCH-L1 determined in step (a), then the disease in the subject is determined to have continued, progressed or worsened. By comparison, if the concentration or amount of UCH-L1 as determined in step (b) is favorable when compared to the concentration or amount of UCH-L1 as determined in step (a), then the disease in the subject is determined to have discontinued, regressed or improved.

**[0164]** Optionally, the method further comprises comparing the concentration or amount of UCH-L1 as determined in step (b), for example, with a reference level. Further, optionally the method comprises treating the subject with one or more pharmaceutical compositions for a period of time if the comparison shows that the concentration or amount of UCH-L1 as determined in step (b), for example, is unfavorably altered with respect to the reference level.

**[0165]** Still further, the methods can be used to monitor treatment in a subject receiving treatment with one or more pharmaceutical compositions or other traumatic brain injury treatment, as described below. Specifically, such methods involve providing a first test sample from a subject before the subject has been administered one or more pharmaceutical compositions. Next, the concentration or amount in a first test sample from a subject of UCH-L1 is determined (e.g., using the methods described herein or as known in the art). After the concentration or amount of UCH-L1 is determined, optionally the concentration or amount of UCH-L1 is then compared with a reference level. If the concentration or amount of UCH-L1 as determined in the first test sample is lower than the reference level, then the subject is not treated with one or more pharmaceutical compositions or alternatively, the subject may be treated with one or more pharmaceutical compositions. If the concentration or amount of UCH-L1 as determined in the first test sample is higher than the reference level, then the subject is treated with one or more pharmaceutical compositions for a period of time or alternatively, the subject is not treated with one or more pharmaceutical compositions. The period of time that the subject is treated with the one or more pharmaceutical compositions can be determined by one skilled in the art (for example, the period of time can be from about seven (7) days to about two years, preferably from about fourteen (14) days to about one (1) year).

**[0166]** During the course of treatment with the one or more pharmaceutical compositions, second and subsequent test samples are then obtained from the subject. The number of test samples and the time in which said test samples are obtained from the subject are not critical. For example, a second test sample could be obtained seven (7) days after the subject is first administered the one or more pharmaceutical compositions, a third test sample could be obtained two (2) weeks after the subject is first administered the one or more pharmaceutical compositions, a fourth test sample could be obtained three (3) weeks after the subject is first

administered the one or more pharmaceutical compositions, a fifth test sample could be obtained four (4) weeks after the subject is first administered the one or more pharmaceutical compositions, etc.

**[0167]** After each second or subsequent test sample is obtained from the subject, the concentration or amount of UCH-L1 is determined in the second or subsequent test sample is determined (e.g., using the methods described herein or as known in the art). The concentration or amount of UCH-L1 as determined in each of the second and subsequent test samples is then compared with the concentration or amount of UCH-L1 as determined in the first test sample (e.g., the test sample that was originally optionally compared to the reference level). If the concentration or amount of UCH-L1 as determined in step (c) is favorable when compared to the concentration or amount of UCH-L1 as determined in step (a), then the disease in the subject is determined to have discontinued, regressed, or improved, and the subject can continue to be administered the one or pharmaceutical compositions of step (b). However, if the concentration or amount determined in step (c) is unchanged or is unfavorable when compared to the concentration or amount of UCH-L1 as determined in step (a), then the disease in the subject is determined to have continued, progressed or worsened, and the subject can be treated with a higher concentration of the one or more pharmaceutical compositions administered to the subject in step (b) or the subject can be treated with one or more pharmaceutical compositions that are different from the one or more pharmaceutical compositions administered to the subject in step (b). Specifically, the subject can be treated with one or more pharmaceutical compositions that are different from the one or more pharmaceutical compositions that the subject had previously received to decrease or lower said subject's UCH-L1 level.

**[0168]** Generally, for assays in which repeat testing may be done (e.g., monitoring disease progression and/or response to treatment), a second or subsequent test sample is obtained at a period in time after the first test sample has been obtained from the subject. Specifically, a second test sample from the subject can be obtained minutes, hours, days, weeks or years after the first test sample has been obtained from the subject. For example, the second test sample can be obtained from the subject at a time period of about 1 minute, about 5 minutes, about 10 minutes, about 15 minutes, about 30 minutes, about 45 minutes, about 60 minutes,

about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, about 10 hours, about 11 hours, about 12 hours, about 13 hours, about 14 hours, about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, about 24 hours, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 9 weeks, about 10 weeks, about 11 weeks, about 12 weeks, about 13 weeks, about 14 weeks, about 15 weeks, about 16 weeks, about 17 weeks, about 18 weeks, about 19 weeks, about 20 weeks, about 21 weeks, about 22 weeks, about 23 weeks, about 24 weeks, about 25 weeks, about 26 weeks, about 27 weeks, about 28 weeks, about 29 weeks, about 30 weeks, about 31 weeks, about 32 weeks, about 33 weeks, about 34 weeks, about 35 weeks, about 36 weeks, about 37 weeks, about 38 weeks, about 39 weeks, about 40 weeks, about 41 weeks, about 42 weeks, about 43 weeks, about 44 weeks, about 45 weeks, about 46 weeks, about 47 weeks, about 48 weeks, about 49 weeks, about 50 weeks, about 51 weeks, about 52 weeks, about 1.5 years, about 2 years, about 2.5 years, about 3.0 years, about 3.5 years, about 4.0 years, about 4.5 years, about 5.0 years, about 5.5. years, about 6.0 years, about 6.5 years, about 7.0 years, about 7.5 years, about 8.0 years, about 8.5 years, about 9.0 years, about 9.5 years, or about 10.0 years after the first test sample from the subject is obtained.

**[0169]** When used to monitor disease progression, the above assay can be used to monitor the progression of disease in subjects suffering from acute conditions. Acute conditions, also known as critical care conditions, refer to acute, life-threatening diseases or other critical medical conditions involving, for example, the cardiovascular system or excretory system. Typically, critical care conditions refer to those conditions requiring acute medical intervention in a hospital-based setting (including, but not limited to, the emergency room, intensive care unit, trauma center, or other emergent care setting) or administration by a paramedic or other field-based medical personnel. For critical care conditions, repeat monitoring is generally done within a shorter time frame, namely, minutes, hours or days (e.g., about 1 minute, about 5 minutes, about 10 minutes, about 15 minutes, about 30 minutes, about 45 minutes, about 60 minutes, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, about 10 hours,

about 11 hours, about 12 hours, about 13 hours, about 14 hours, about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, about 24 hours, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days or about 7 days), and the initial assay likewise is generally done within a shorter timeframe, e.g., about minutes, hours or days of the onset of the disease or condition.

**[0170]** The assays also can be used to monitor the progression of disease in subjects suffering from chronic or non-acute conditions. Non-critical care conditions or non-acute conditions, refers to conditions other than acute, life-threatening disease or other critical medical conditions involving, for example, the cardiovascular system and/or excretory system. Typically, non-acute conditions include those of longer-term or chronic duration. For non-acute conditions, repeat monitoring generally is done with a longer timeframe, such as hours, days, weeks, months or years (e.g., about 1 hour, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, about 10 hours, about 11 hours, about 12 hours, about 13 hours, about 14 hours, about 15 hours, about 16 hours, about 17 hours, about 18 hours, about 19 hours, about 20 hours, about 21 hours, about 22 hours, about 23 hours, about 24 hours, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 9 weeks, about 10 weeks, about 11 weeks, about 12 weeks, about 13 weeks, about 14 weeks, about 15 weeks, about 16 weeks, about 17 weeks, about 18 weeks, about 19 weeks, about 20 weeks, about 21 weeks, about 22 weeks, about 23 weeks, about 24 weeks, about 25 weeks, about 26 weeks, about 27 weeks, about 28 weeks, about 29 weeks, about 30 weeks, about 31 weeks, about 32 weeks, about 33 weeks, about 34 weeks, about 35 weeks, about 36 weeks, about 37 weeks, about 38 weeks, about 39 weeks, about 40 weeks, about 41 weeks, about 42 weeks, about 43 weeks, about 44 weeks, about 45 weeks, about 46 weeks, about 47 weeks, about 48 weeks, about 49 weeks, about 50 weeks, about 51 weeks, about 52 weeks, about 1.5 years, about 2 years, about 2.5 years, about 3.0 years, about 3.5 years, about 4.0 years, about 4.5 years, about 5.0 years, about 5.5. years, about 6.0 years, about 6.5 years, about 7.0 years, about 7.5 years, about 8.0 years, about 8.5 years, about 9.0 years, about 9.5 years or about 10.0 years), and the

initial assay likewise generally is done within a longer time frame, e.g., about hours, days, months or years of the onset of the disease or condition.

**[0171]** Furthermore, the above assays can be performed using a first test sample obtained from a subject where the first test sample is obtained from one source, such as whole blood, serum, or plasma. Optionally the above assays can then be repeated using a second test sample obtained from the subject where the second test sample is obtained from another source. For example, if the first test sample was obtained from whole blood, the second test sample can be obtained from serum or plasma. The results obtained from the assays using the first test sample and the second test sample can be compared. The comparison can be used to assess the status of a disease or condition in the subject.

**[0172]** With respect to a reference level as employed for monitoring disease progression and/or treatment or for determining the risk of a subject of developing traumatic brain injury, the amount or concentration of UCH-L1 or UCH-L1 fragment may be "unchanged," "favorable" (or "favorably altered"), or "unfavorable" (or "unfavorably altered"). "Elevated" or "increased" refers to an amount or a concentration in a test sample that is higher or greater than a typical or normal level or range (e.g., reference level), or is higher or greater than another reference level or range (e.g., earlier or baseline sample). The term "lowered" or "reduced" refers to an amount or a concentration in a test sample that is lower or less than a typical or normal level or range (e.g., reference level), or is lower or less than another reference level or range (e.g., earlier or baseline sample). The term "altered" refers to an amount or a concentration in a sample that is altered (increased or decreased) over a typical or normal level or range (e.g., reference level), or over another reference level or range (e.g., earlier or baseline sample).

**[0173]** The typical or normal level or range for UCH-L1 is defined in accordance with standard practice. A so-called altered level or alteration can be considered to have occurred when there is any net change as compared to the typical or normal level or range, or reference level or range that cannot be explained by experimental error or sample variation. Thus, the level measured in a particular sample will be compared with the level or range of levels determined in similar samples from a so-called normal subject. In this context, a "normal subject" is an individual with no detectable disease or disorder, and a "normal"

(sometimes termed "control") patient or population is/are one(s) that exhibit(s) no detectable disease or disorder, respectively, for example. An "apparently normal subject" is one in which UCH-L1 has not been or is being assessed. The level of an analyte is said to be "elevated" when the analyte is normally undetectable (e.g., the normal level is zero, or within a range of from about 25 to about 75 percentiles of normal populations), but is detected in a test sample, as well as when the analyte is present in the test sample at a higher than normal level. Thus, inter alia, the disclosure provides a method of screening for a subject having, or at risk of having, traumatic brain injury.

## 8. Methods of Diagnosing or Assessing Involving Other Factors

**[0174]** The methods of diagnosing, prognosticating, and/or assessing, as described above, can further include using other factors for the diagnosis, prognostication, and assessment. In some embodiments, traumatic brain injury may be diagnosed using the Glasgow Coma Scale or the outcome of the traumatic brain injury may be predicted using the Extended Glasgow Outcome Scale (GOSE). Other tests, scales or indices can also be used either alone or in combination with the Glasgow Coma Scale. An example is the Ranchos Los Amigos Scale. The Ranchos Los Amigos Scale measures the levels of awareness, cognition, behavior and interaction with the environment. The Ranchos Los Amigos Scale includes: Level I: No Response; Level II: Generalized Response; Level III: Localized Response; Level IV: Confused-agitated; Level V: Confused-inappropriate; Level VI: Confused-appropriate; Level VII: Automatic-appropriate; and Level VIII: Purposeful-appropriate.

**[0175]** Other classification systems based on CT scan results can be used to predict outcome in patients, such as any classification systems known in the art. An example is the Marshall classification of traumatic brain injury, which places patients into one of six categories (I to VI) of increasing severity on the basis of findings on non-contrast CT scan of the brain. Higher categories have worse prognosis and survival. The Marshall classification is primarily concerned with two features: 1) degree of swelling, as determined by midline shift and/or compression of basal cisterns, and 2) presence and size of contusions/hemorrhages referred to "high or mixed density lesions." Another example is the Rotterdam score, which incorporates additional variables (e.g. subarachnoid hemorrhage) and attempts to address

some of the recognized limitations of the Marshall system, such as struggling to classifying patients who have injuries of multiple types. The Rotterdam classification includes four independently scored elements. Similar to the Marshall system, the Rotterdam classification includes 1) degree of basal cistern compression and 2) degree of midline shift. The Rotterdam does not, however, include contusions, but rather restricts mass lesions to 3) epidural hematomas, and adds 4) intraventricular and/or subarachnoid blood. Each of these is given a score, and these scores are tallied, with the addition of 1 to the total. Higher scores worse prognosis and survival.

**[0176]** Some instruments (such as, for example the Abbott Laboratories instrument ARCHITECT®, and other core laboratory instruments) other than a point-of-care device may be capable of measuring levels of UCH-L1 in a sample higher or greater than 25,000 pg/mL. Use of the methods as described herein may provide one or more of the benefits described herein on those devices (e.g., measure up to 25,000 pg/mL, dynamic range of 5 log, assay linearity over the dynamic range, measure of UCH-L1 in a volume less than 20 microliters of sample, expanded window of detection, etc.).

**[0177]** Other methods of detection include the use of or can be adapted for use on a nanopore device or nanowell device. Examples of nanopore devices are described in International Patent Publication No. WO 2016/161402, which is hereby incorporated by reference in its entirety. Examples of nanowell device are described in International Patent Publication No. WO 2016/161400, which is hereby incorporated by reference in its entirety.

**9. Combinations of UCH-L1 with other Biomarkers**

**[0178]** The antibodies described herein can be used in a variety of methods to detect and measure levels and concentrations of UCH-L1 in combination with one or more biomarkers or immunoassays specific for disease. The present disclosure contemplates that the combination of UCH-L1 with one or more biomarkers or immunoassays specific for disease may provide a greater discrimination between healthy controls and individuals with disease compared to measuring UCH-L1 alone. For example, measuring a panel of UCH-L1 and additional traumatic brain injury biomarkers may provide a greater discrimination between healthy controls and individuals with disease compared to a panel of UCH-L1 alone. The

combination of UCH-L1 with at least one or more biomarkers may provide greater discrimination between healthy controls and individuals who have traumatic brain injury.

**[0179]** Examples of the one or more biomarkers include glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100b), brain lipid binding protein (BLBP), aldolase C (ALDOC), astrocytic phosphoprotein 15 (PEA15), glutamine synthetase (GS), and crystallin B chain (CRYAB).

**10. Treatment and Monitoring of Subjects Suffering from Traumatic Brain Injury**

**[0180]** The subject identified or assessed in the methods described above as having traumatic brain injury, such as mild traumatic brain injury or moderate to severe traumatic brain injury, may be treated or monitored. In some embodiments, the method further includes treating the human subject assessed as having traumatic brain injury with a traumatic brain injury treatment, such as any treatments known in the art. For example, treatment of traumatic brain injury can take a variety of forms depending on the severity of the injury to the head. For example, for subjects suffering from mild TBI, the treatment may include one or more of rest, abstaining from physical activities, such as sports, avoiding light or wearing sunglasses when out in the light, medication for relief of a headache or migraine, anti-nausea medication, etc. Treatment for patients suffering from severe TBI might include administration of one or more appropriate medications (such as, for example, diuretics, anti-convulsant medications, medications to sedate and put an individual in a drug-induced coma, or other pharmaceutical or biopharmaceutical medications (either known or developed in the future for treatment of TBI), one or more surgical procedures (such as, for example, removal of a hematoma, repairing a skull fracture, decompressive craniectomy, etc.) and one or more therapies (such as, for example one or more rehabilitation, cognitive behavioral therapy, anger management, counseling psychology, etc.). In some embodiments, the method further includes monitoring the human subject assessed as having traumatic brain injury (*e.g.,* mild or moderate to severe traumatic). In some embodiments, a subject identified as having traumatic brain injury, such as mild traumatic brain injury or severe traumatic brain injury, may be monitored with CT scan or MRI.

## 11. Methods of Measuring UCH-L1

**[0181]** In the methods described above, UCH-L1 levels can be measured by any means, such as antibody dependent methods, such as immunoassays, protein immunoprecipitation, immunoelectrophoresis, chemical analysis, SDS-PAGE and Western blot analysis, protein immunostaining, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, or chromatography or spectrometry methods, such as high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC/MS). Also, the assay can be employed in clinical chemistry format such as would be known by one skilled in the art. For example, a clinical chemistry format can include an assay that involves one antibody or no antibody. Examples of analyzers that can be used for the clinical chemistry format are described in U.S. Patent publication Nos. 2016/0320422 and 2015/0112630.

**[0182]** In some embodiments, measuring the level of UCH-L1 includes contacting the sample with a first specific binding member and second specific binding member. In some embodiments the first specific binding member is a capture antibody and the second specific binding member is a detection antibody. In some embodiments, measuring the level of UCH-L1 includes contacting the sample, either simultaneously or sequentially, in any order: (1) a capture antibody (e.g., UCH-L1-capture antibody), which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a capture antibody-UCH-L1 antigen complex (e.g., UCH-L1-capture antibody-UCH-L1 antigen complex), and (2) a detection antibody (e.g., UCH-L1-detection antibody), which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the capture antibody, to form a UCH-L1 antigen-detection antibody complex (e.g., UCH-L1 antigen-UCH-L1-detection antibody complex), such that a capture antibody-UCH-L1 antigen-detection antibody complex (e.g., UCH-L1-capture antibody-UCH-L1 antigen-UCH-L1-detection antibody complex) is formed, and measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the capture antibody-UCH-L1 antigen-detection antibody complex.

**[0183]** In some embodiments, the first specific binding member is immobilized on a solid support. In some embodiments, the second specific binding member is immobilized on a

solid support. In some embodiments, the first specific binding member is a UCH-L1 antibody as described below.

**[0184]** In some embodiments, the sample is diluted or undiluted. The sample can be from about 1 to about 25 microliters, about 1 to about 24 microliters, about 1 to about 23 microliters, about 1 to about 22 microliters, about 1 to about 21 microliters, about 1 to about 20 microliters, about 1 to about 18 microliters, about 1 to about 17 microliters, about 1 to about 16 microliters, about 15 microliters or about 1 microliter, about 2 microliters, about 3 microliters, about 4 microliters, about 5 microliters, about 6 microliters, about 7 microliters, about 8 microliters, about 9 microliters, about 10 microliters, about 11 microliters, about 12 microliters, about 13 microliters, about 14 microliters, about 15 microliters, about 16 microliters, about 17 microliters, about 18 microliters, about 19 microliters, about 20 microliters, about 21 microliters, about 22 microliters, about 23 microliters, about 24 microliters or about 25 microliters. In some embodiments, the sample is from about 1 to about 150 microliters or less or from about 1 to about 25 microliters or less.

**[0185]** Some instruments (such as, for example the Abbott Laboratories instrument ARCHITECT®, and other core laboratory instruments) other than a point-of-care device may be capable of measuring levels of UCH-L1 in a sample higher or greater than 25,000 pg/mL.

**[0186]** Other methods of detection include the use of or can be adapted for use on a nanopore device or nanowell device. Examples of nanopore devices are described in International Patent Publication No. WO 2016/161402, which is hereby incorporated by reference in its entirety. Examples of nanowell device are described in International Patent Publication No. WO 2016/161400, which is hereby incorporated by reference in its entirety.

**12. Ubiquitin Carboxy-Terminal Hydrolase L1 (UCH-L1) Antibodies**

**[0187]** The methods described herein may use an isolated antibody that specifically binds to ubiquitin carboxy-terminal hydrolase L1 ("UCH-L1") (or fragments thereof), referred to as "UCH-L1 antibody." The UCH-L1 antibodies can be used to assess the UCH-L1 status as a measure of traumatic brain injury, detect the presence of UCH-L1 in a sample, quantify the amount of UCH-L1 present in a sample, or detect the presence of and quantify the amount of UCH-L1 in a sample.

### a) Ubiquitin Carboxy-Terminal Hydrolase L1 (UCH-L1)

**[0188]** Ubiquitin carboxy-terminal hydrolase L1 ("UCH-L1"), which is also known as "ubiquitin C-terminal hydrolase," is a deubiquitinating enzyme. UCH-L1 is a member of a gene family whose products hydrolyze small C-terminal adducts of ubiquitin to generate the ubiquitin monomer. Expression of UCH-L1 is highly specific to neurons and to cells of the diffuse neuroendocrine system and their tumors. It is abundantly present in all neurons (accounts for 1-2% of total brain protein), expressed specifically in neurons and testis/ovary. The catalytic triad of UCH-L1 contains a cysteine at position 90, an aspartate at position 176, and a histidine at position 161 that are responsible for its hydrolase activity.

**[0189]** Human UCH-L1 may have the following amino acid sequence: MQLKPMEINPEMLNKVLSRLGVAGQWRFVDVLGLEEESLGSVPAPACALLLLFPLT AQHENFRKKQIEELKGQEVSPKVYFMKQTIGNSCGTIGLIHAVANNQDKLGFEDGSV LKQFLSETEKMSPEDRAKCFEKNEAIQAAHDAVAQEGQCRVDDKVNFHFILFNNVD GHLYELDGRMPFPVNHGASSEDTLLKDAAKVCREFTEREQGEVRFSAVALCKAA (SEQ ID NO: 1). The human UCH-L1 may be a fragment or variant of SEQ ID NO: 1. The fragment of UCH-L1 may be between 5 and 225 amino acids, between 10 and 225 amino acids, between 50 and 225 amino acids, between 60 and 225 amino acids, between 65 and 225 amino acids, between 100 and 225 amino acids, between 150 and 225 amino acids, between 100 and 175 amino acids, or between 175 and 225 amino acids in length. The fragment may comprise a contiguous number of amino acids from SEQ ID NO: 1.

### b) UCH-L1-Recognizing Antibody

**[0190]** The antibody is an antibody that binds to UCH-L1, a fragment thereof, an epitope of UCH-L1, or a variant thereof. The antibody may be a fragment of the anti-UCH-L1 antibody or a variant or a derivative thereof. The antibody may be a polyclonal or monoclonal antibody. The antibody may be a chimeric antibody, a single chain antibody, an affinity matured antibody, a human antibody, a humanized antibody, a fully human antibody or an antibody fragment, such as a Fab fragment, or a mixture thereof. Antibody fragments or derivatives may comprise $F(ab')_2$, Fv or scFv fragments. The antibody derivatives can be

produced by peptidomimetics. Further, techniques described for the production of single chain antibodies can be adapted to produce single chain antibodies.

**[0191]** The anti-UCH-L1 antibodies may be a chimeric anti-UCH-L1 or humanized anti-UCH-L1 antibody. In one embodiment, both the humanized antibody and chimeric antibody are monovalent. In one embodiment, both the humanized antibody and chimeric antibody comprise a single Fab region linked to an Fc region.

**[0192]** Human antibodies may be derived from phage-display technology or from transgenic mice that express human immunoglobulin genes. The human antibody may be generated as a result of a human in vivo immune response and isolated. See, for example, Funaro *et al.*, *BMC Biotechnology*, 2008(8):85. Therefore, the antibody may be a product of the human and not animal repertoire. Because it is of human origin, the risks of reactivity against self-antigens may be minimized. Alternatively, standard yeast display libraries and display technologies may be used to select and isolate human anti-UCH-L1 antibodies. For example, libraries of naïve human single chain variable fragments (scFv) may be used to select human anti-UCH-L1 antibodies. Transgenic animals may be used to express human antibodies.

**[0193]** Humanized antibodies may be antibody molecules from non-human species antibody that binds the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and framework regions from a human immunoglobulin molecule.

**[0194]** The antibody is distinguishable from known antibodies in that it possesses different biological function(s) than those known in the art.

**(1) Epitope**

**[0195]** The antibody may immunospecifically bind to UCH-L1 (SEQ ID NO: 1), a fragment thereof, or a variant thereof. The antibody may immunospecifically recognize and bind at least three amino acids, at least four amino acids, at least five amino acids, at least six amino acids, at least seven amino acids, at least eight amino acids, at least nine amino acids, or at least ten amino acids within an epitope region. The antibody may immunospecifically recognize and bind to an epitope that has at least three contiguous amino acids, at least four contiguous amino acids, at least five contiguous amino acids, at least six contiguous amino

acids, at least seven contiguous amino acids, at least eight contiguous amino acids, at least nine contiguous amino acids, or at least ten contiguous amino acids of an epitope region.

**c) Antibody Preparation/Production**

**[0196]** Antibodies may be prepared by any of a variety of techniques, including those well known to those skilled in the art. In general, antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies via conventional techniques, or via transfection of antibody genes, heavy chains, and/or light chains into suitable bacterial or mammalian cell hosts, in order to allow for the production of antibodies, wherein the antibodies may be recombinant. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is possible to express the antibodies in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells is preferable, and most preferable in mammalian host cells, because such eukaryotic cells (and in particular mammalian cells) are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody.

**[0197]** Exemplary mammalian host cells for expressing the recombinant antibodies include Chinese Hamster Ovary (CHO cells) (including dhfr-CHO cells, described in Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77: 4216-4220 (1980)), used with a DHFR selectable marker, *e.g.*, as described in Kaufman and Sharp, *J. Mol. Biol.*, 159: 601-621 (1982), NS0 myeloma cells, COS cells, and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

**[0198]** Host cells can also be used to produce functional antibody fragments, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure may be performed. For example, it may be desirable to transfect a host cell with DNA

encoding functional fragments of either the light chain and/or the heavy chain of an antibody. Recombinant DNA technology may also be used to remove some, or all, of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to the antigens of interest. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody (*i.e.,* binds human UCH-L1) and the other heavy and light chain are specific for an antigen other than human UCH-L1 by crosslinking an antibody to a second antibody by standard chemical crosslinking methods.

**[0199]** In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are cultured to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells, and recover the antibody from the culture medium. Still further, the method of synthesizing a recombinant antibody may be by culturing a host cell in a suitable culture medium until a recombinant antibody is synthesized. The method can further comprise isolating the recombinant antibody from the culture medium.

**[0200]** Methods of preparing monoclonal antibodies involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity. Such cell lines may be produced from spleen cells obtained from an immunized animal. The animal may be immunized with UCH-L1 or a fragment and/or variant thereof. The peptide used to immunize the animal may comprise amino acids encoding human Fc, for example the fragment crystallizable region or tail region of human antibody. The spleen cells may then be immortalized by, for example, fusion with a myeloma cell fusion partner. A variety of

fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports that growth of hybrid cells, but not myeloma cells. One such technique uses hypoxanthine, aminopterin, thymidine (HAT) selection. Another technique includes electrofusion. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and their culture supernatants tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity may be used.

**[0201]** Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. Affinity chromatography is an example of a method that can be used in a process to purify the antibodies.

**[0202]** The proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the F(ab) fragments) each comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the $F(ab')_2$ fragment, which comprises both antigen-binding sites.

**[0203]** The Fv fragment can be produced by preferential proteolytic cleavage of an IgM, and on rare occasions IgG or IgA immunoglobulin molecules. The Fv fragment may be derived using recombinant techniques. The Fv fragment includes a non-covalent VH::VL heterodimer including an antigen-binding site that retains much of the antigen recognition and binding capabilities of the native antibody molecule.

**[0204]** The antibody, antibody fragment, or derivative may comprise a heavy chain and a light chain complementarity determining region ("CDR") set, respectively interposed between a heavy chain and a light chain framework ("FR") set which provide support to the CDRs and define the spatial relationship of the CDRs relative to each other. The CDR set may contain three hypervariable regions of a heavy or light chain V region.

**[0205]** Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, but not limited to, methods that select recombinant antibody from a peptide or protein library (*e.g.,* but not limited to, a bacteriophage, ribosome, oligonucleotide, RNA, cDNA, yeast or the like, display library); *e.g.,* as available from various commercial vendors such as Cambridge Antibody Technologies (Cambridgeshire, UK), MorphoSys (Martinsreid/Planegg, Del.), Biovation (Aberdeen, Scotland, UK) BioInvent (Lund, Sweden), using methods known in the art. See U.S. Patent Nos. 4,704,692; 5,723,323; 5,763,192; 5,814,476; 5,817,483; 5,824,514; 5,976,862. Alternative methods rely upon immunization of transgenic animals (*e.g.,* SCID mice, Nguyen *et al.* (1997) *Microbiol. Immunol.* 41:901-907; Sandhu *et al.* (1996) *Crit. Rev. Biotechnol.* 16:95-118; Eren *et al.* (1998) *Immunol.* 93:154-161) that are capable of producing a repertoire of human antibodies, as known in the art and/or as described herein. Such techniques, include, but are not limited to, ribosome display (Hanes *et al.* (1997) *Proc. Natl. Acad. Sci. USA*, 94:4937-4942; Hanes *et al.* (1998) *Proc. Natl. Acad. Sci. USA*, 95:14130-14135); single cell antibody producing technologies (*e.g.,* selected lymphocyte antibody method ("SLAM") (U.S. Patent No. 5,627,052, Wen *et al.* (1987) *J. Immunol.* 17:887-892; Babcook *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:7843-7848); gel microdroplet and flow cytometry (Powell *et al.* (1990) Biotechnol. 8:333-337; One Cell Systems, (Cambridge, Mass).; Gray *et al.* (1995) J. Imm. Meth. 182:155-163; Kenny *et al.* (1995) Bio/Technol. 13:787-790); B-cell selection (Steenbakkers *et al.* (1994) *Molec. Biol. Reports* 19:125-134 (1994)).

**[0206]** An affinity matured antibody may be produced by any one of a number of procedures that are known in the art. For example, see Marks *et al.*, *BioTechnology*, 10: 779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by Barbas *et al.*, *Proc. Nat. Acad. Sci. USA*, 91: 3809-3813 (1994); Schier *et al.*, Gene, 169: 147-155 (1995); Yelton *et al.*, *J. Immunol.*, 155: 1994-2004 (1995); Jackson *et al.*, *J. Immunol.*, 154(7): 3310-3319 (1995); Hawkins *et al*, *J. Mol. Biol.*, 226: 889-896 (1992). Selective mutation at selective mutagenesis positions and at contact or hypermutation positions with an activity enhancing amino acid residue is described in U.S. Patent No. 6,914,128 B1.

**[0207]** Antibody variants can also be prepared using delivering a polynucleotide encoding an antibody to a suitable host such as to provide transgenic animals or mammals, such as goats, cows, horses, sheep, and the like, that produce such antibodies in their milk. These methods are known in the art and are described for example in U.S. Patent Nos. 5,827,690; 5,849,992; 4,873,316; 5,849,992; 5,994,616; 5,565,362; and 5,304,489.

**[0208]** Antibody variants also can be prepared by delivering a polynucleotide to provide transgenic plants and cultured plant cells (*e.g.,* but not limited to tobacco, maize, and duckweed) that produce such antibodies, specified portions or variants in the plant parts or in cells cultured therefrom. For example, Cramer *et al.* (1999) *Curr. Top. Microbiol. Immunol.* 240:95-118 and references cited therein, describe the production of transgenic tobacco leaves expressing large amounts of recombinant proteins, *e.g.,* using an inducible promoter. Transgenic maize have been used to express mammalian proteins at commercial production levels, with biological activities equivalent to those produced in other recombinant systems or purified from natural sources. See, *e.g.,* Hood *et al.*, *Adv. Exp. Med. Biol.* (1999) 464:127-147 and references cited therein. Antibody variants have also been produced in large amounts from transgenic plant seeds including antibody fragments, such as single chain antibodies (scFv's), including tobacco seeds and potato tubers. See, *e.g.,* Conrad *et al.* (1998) *Plant Mol. Biol.* 38:101-109 and reference cited therein. Thus, antibodies can also be produced using transgenic plants, according to known methods.

**[0209]** Antibody derivatives can be produced, for example, by adding exogenous sequences to modify immunogenicity or reduce, enhance or modify binding, affinity, on-rate, off-rate, avidity, specificity, half-life, or any other suitable characteristic. Generally, part or all of the non-human or human CDR sequences are maintained while the non-human sequences of the variable and constant regions are replaced with human or other amino acids.

**[0210]** Small antibody fragments may be diabodies having two antigen-binding sites, wherein fragments comprise a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (VH VL). See for example, EP 404,097; WO 93/11161; and Hollinger *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448. By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two

antigen-binding sites. See also, U.S. Patent No. 6,632,926 to Chen *et al.* which is hereby incorporated by reference in its entirety and discloses antibody variants that have one or more amino acids inserted into a hypervariable region of the parent antibody and a binding affinity for a target antigen which is at least about two fold stronger than the binding affinity of the parent antibody for the antigen.

**[0211]** The antibody may be a linear antibody. The procedure for making a linear antibody is known in the art and described in Zapata *et al.*, (1995) *Protein Eng.* 8(10):1057-1062. Briefly, these antibodies comprise a pair of tandem Fd segments (VH-CH1-VH-CH1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

**[0212]** The antibodies may be recovered and purified from recombinant cell cultures by known methods including, but not limited to, protein A purification, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. High performance liquid chromatography ("HPLC") can also be used for purification.

**[0213]** It may be useful to detectably label the antibody. Methods for conjugating antibodies to these agents are known in the art. For the purpose of illustration only, antibodies can be labeled with a detectable moiety such as a radioactive atom, a chromophore, a fluorophore, or the like. Such labeled antibodies can be used for diagnostic techniques, either in vivo, or in an isolated test sample. They can be linked to a cytokine, to a ligand, to another antibody. Suitable agents for coupling to antibodies to achieve an anti-tumor effect include cytokines, such as interleukin 2 (IL-2) and Tumor Necrosis Factor (TNF); photosensitizers, for use in photodynamic therapy, including aluminum (III) phthalocyanine tetrasulfonate, hematoporphyrin, and phthalocyanine; radionuclides, such as iodine-131 (131I), yttrium-90 (90Y), bismuth-212 (212Bi), bismuth-213 (213Bi), technetium-99m (99mTc), rhenium-186 (186Re), and rhenium-188 (188Re); antibiotics, such as doxorubicin, adriamycin, daunorubicin, methotrexate, daunomycin, neocarzinostatin, and carboplatin; bacterial, plant, and other toxins, such as diphtheria toxin, pseudomonas exotoxin A, staphylococcal enterotoxin A, abrin-A toxin, ricin A (deglycosylated ricin A and native ricin A), TGF-alpha toxin, cytotoxin from chinese cobra (naja naja atra), and gelonin (a plant toxin); ribosome

inactivating proteins from plants, bacteria and fungi, such as restrictocin (a ribosome inactivating protein produced by Aspergillus restrictus), saporin (a ribosome inactivating protein from Saponaria officinalis), and RNase; tyrosine kinase inhibitors; ly207702 (a difluorinated purine nucleoside); liposomes containing anti cystic agents (*e.g.*, antisense oligonucleotides, plasmids which encode for toxins, methotrexate, etc.); and other antibodies or antibody fragments, such as F(ab).

**[0214]** Antibody production via the use of hybridoma technology, the selected lymphocyte antibody method (SLAM), transgenic animals, and recombinant antibody libraries is described in more detail below.

**(1) Anti-UCH-L1 Monoclonal Antibodies Using Hybridoma Technology**

**[0215]** Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow *et al.*, Antibodies: *A Laboratory Manual*, second edition, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1988); Hammerling, *et al.*, *In Monoclonal Antibodies and T-Cell Hybridomas*, (Elsevier, N.Y., 1981). It is also noted that the term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

**[0216]** Methods of generating monoclonal antibodies as well as antibodies produced by the method may comprise culturing a hybridoma cell secreting an antibody of the invention wherein, preferably, the hybridoma is generated by fusing splenocytes isolated from an animal, *e.g.*, a rat or a mouse, immunized with UCH-L1 with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind a polypeptide of the invention. Briefly, rats can be immunized with a UCH-L1 antigen. In a preferred embodiment, the UCH-L1 antigen is administered with an adjuvant to stimulate the immune response. Such adjuvants include complete or incomplete Freund's adjuvant, RIBI (muramyl dipeptides) or ISCOM (immunostimulating complexes). Such adjuvants may protect the polypeptide from rapid dispersal by sequestering it in a local

deposit, or they may contain substances that stimulate the host to secrete factors that are chemotactic for macrophages and other components of the immune system. Preferably, if a polypeptide is being administered, the immunization schedule will involve two or more administrations of the polypeptide, spread out over several weeks; however, a single administration of the polypeptide may also be used.

**[0217]** After immunization of an animal with a UCH-L1 antigen, antibodies and/or antibody-producing cells may be obtained from the animal. An anti-UCH-L1 antibody-containing serum is obtained from the animal by bleeding or sacrificing the animal. The serum may be used as it is obtained from the animal, an immunoglobulin fraction may be obtained from the serum, or the anti-UCH-L1 antibodies may be purified from the serum. Serum or immunoglobulins obtained in this manner are polyclonal, thus having a heterogeneous array of properties.

**[0218]** Once an immune response is detected, *e.g.,* antibodies specific for the antigen UCH-L1 are detected in the rat serum, the rat spleen is harvested and splenocytes isolated. The splenocytes are then fused by well-known techniques to any suitable myeloma cells, for example, cells from cell line SP20 available from the American Type Culture Collection (ATCC, Manassas, Va., US). Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding UCH-L1. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing rats with positive hybridoma clones.

**[0219]** In another embodiment, antibody-producing immortalized hybridomas may be prepared from the immunized animal. After immunization, the animal is sacrificed and the splenic B cells are fused to immortalized myeloma cells as is well known in the art. See, *e.g.,* Harlow and Lane, supra. In a preferred embodiment, the myeloma cells do not secrete immunoglobulin polypeptides (a non-secretory cell line). After fusion and antibiotic selection, the hybridomas are screened using UCH-L1, or a portion thereof, or a cell expressing UCH-L1. In a preferred embodiment, the initial screening is performed using an enzyme-linked immunosorbent assay (ELISA) or a radioimmunoassay (RIA), preferably an ELISA. An example of ELISA screening is provided in PCT Publication No. WO 00/37504.

**[0220]** Anti-UCH-L1 antibody-producing hybridomas are selected, cloned, and further screened for desirable characteristics, including robust hybridoma growth, high antibody production, and desirable antibody characteristics. Hybridomas may be cultured and expanded in vivo in syngeneic animals, in animals that lack an immune system, *e.g.,* nude mice, or in cell culture in vitro. Methods of selecting, cloning and expanding hybridomas are well known to those of ordinary skill in the art.

**[0221]** In a preferred embodiment, hybridomas are rat hybridomas. In another embodiment, hybridomas are produced in a non-human, non-rat species such as mice, sheep, pigs, goats, cattle, or horses. In yet another preferred embodiment, the hybridomas are human hybridomas, in which a human non-secretory myeloma is fused with a human cell expressing an anti-UCH-L1 antibody.

**[0222]** Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, Fab and $F(ab')_2$ fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce two identical Fab fragments) or pepsin (to produce an $F(ab')_2$ fragment). A $F(ab')_2$ fragment of an IgG molecule retains the two antigen-binding sites of the larger ("parent") IgG molecule, including both light chains (containing the variable light chain and constant light chain regions), the CH1 domains of the heavy chains, and a disulfide-forming hinge region of the parent IgG molecule. Accordingly, an $F(ab')_2$ fragment is still capable of crosslinking antigen molecules like the parent IgG molecule.

**(2) Anti-UCH-L1 Monoclonal Antibodies Using SLAM**

**[0223]** In another aspect of the invention, recombinant antibodies are generated from single, isolated lymphocytes using a procedure referred to in the art as the selected lymphocyte antibody method (SLAM), as described in U.S. Patent No. 5,627,052; PCT Publication No. WO 92/02551; and Babcook *et al.*, *Proc. Natl. Acad. Sci. USA*, 93: 7843-7848 (1996). In this method, single cells secreting antibodies of interest, *e.g.,* lymphocytes derived from any one of the immunized animals are screened using an antigen-specific hemolytic plaque assay, wherein the antigen UCH-L1, a subunit of UCH-L1, or a fragment thereof, is coupled to sheep red blood cells using a linker, such as biotin, and used to identify single cells that secrete antibodies with specificity for UCH-L1. Following identification of antibody-

secreting cells of interest, heavy- and light-chain variable region cDNAs are rescued from the cells by reverse transcriptase-PCR (RT-PCR) and these variable regions can then be expressed, in the context of appropriate immunoglobulin constant regions (*e.g.,* human constant regions), in mammalian host cells, such as COS or CHO cells. The host cells transfected with the amplified immunoglobulin sequences, derived from in vivo selected lymphocytes, can then undergo further analysis and selection in vitro, for example, by panning the transfected cells to isolate cells expressing antibodies to UCH-L1. The amplified immunoglobulin sequences further can be manipulated in vitro, such as by in vitro affinity maturation method. See, for example, PCT Publication No. WO 97/29131 and PCT Publication No. WO 00/56772.

**(3) Anti-UCH-L1 Monoclonal Antibodies Using Transgenic Animals**

**[0224]** In another embodiment of the invention, antibodies are produced by immunizing a non-human animal comprising some, or all, of the human immunoglobulin locus with a UCH-L1 antigen. In an embodiment, the non-human animal is a XENOMOUSE® transgenic mouse, an engineered mouse strain that comprises large fragments of the human immunoglobulin loci and is deficient in mouse antibody production. See, *e.g.,* Green *et al.*, *Nature Genetics*, 7: 13-21 (1994) and U.S. Patent Nos. 5,916,771; 5,939,598; 5,985,615; 5,998,209; 6,075,181; 6,091,001; 6,114,598; and 6,130,364. See also PCT Publication Nos. WO 91/10741; WO 94/02602; WO 96/34096; WO 96/33735; WO 98/16654; WO 98/24893; WO 98/50433; WO 99/45031; WO 99/53049; WO 00/09560; and WO 00/37504. The XENOMOUSE® transgenic mouse produces an adult-like human repertoire of fully human antibodies, and generates antigen-specific human monoclonal antibodies. The XENOMOUSE® transgenic mouse contains approximately 80% of the human antibody repertoire through introduction of megabase sized, germline configuration YAC fragments of the human heavy chain loci and x light chain loci. See Mendez *et al.*, *Nature Genetics*, 15: 146-156 (1997), Green and Jakobovits, *J. Exp. Med.*, 188: 483-495 (1998), the disclosures of which are hereby incorporated by reference.

### (4) Anti-UCH-L1 Monoclonal Antibodies Using Recombinant Antibody Libraries

**[0225]** In vitro methods also can be used to make the antibodies of the invention, wherein an antibody library is screened to identify an antibody having the desired UCH-L1 -binding specificity. Methods for such screening of recombinant antibody libraries are well known in the art and include methods described in, for example, U.S. Patent No. 5,223,409 (Ladner *et al.*); PCT Publication No. WO 92/18619 (Kang *et al.*); PCT Publication No. WO 91/17271 (Dower *et al.*); PCT Publication No. WO 92/20791 (Winter *et al.*); PCT Publication No. WO 92/15679 (Markland *et al.*); PCT Publication No. WO 93/01288 (Breitling *et al.*); PCT Publication No. WO 92/01047 (McCafferty *et al.*); PCT Publication No. WO 92/09690 (Garrard *et al.*); Fuchs *et al.*, Bio/Technology, 9: 1369-1372 (1991); Hay *et al.*, *Hum. Antibod. Hybridomas*, 3: 81-85 (1992); Huse *et al.*, *Science*, 246: 1275-1281 (1989); McCafferty *et al.*, *Nature*, 348: 552-554 (1990); Griffiths *et al.*, *EMBO J.*, 12: 725-734 (1993); Hawkins *et al.*, J. Mol. Biol., 226: 889-896 (1992); Clackson *et al.*, Nature, 352: 624-628 (1991); Gram *et al.*, *Proc. Natl. Acad. Sci. USA*, 89: 3576-3580 (1992); Garrard *et al.*, *Bio/Technology*, 9: 1373-1377 (1991); Hoogenboom *et al.*, Nucl. Acids Res., 19: 4133-4137 (1991); Barbas *et al.*, *Proc. Natl. Acad. Sci. USA*, 88: 7978-7982 (1991); U.S. Patent Application Publication No. 2003/0186374; and PCT Publication No. WO 97/29131, the contents of each of which are incorporated herein by reference.

**[0226]** The recombinant antibody library may be from a subject immunized with UCH-L1, or a portion of UCH-L1. Alternatively, the recombinant antibody library may be from a naive subject, *i.e.,* one who has not been immunized with UCH-L1, such as a human antibody library from a human subject who has not been immunized with human UCH-L1. Antibodies of the invention are selected by screening the recombinant antibody library with the peptide comprising human UCH-L1 to thereby select those antibodies that recognize UCH-L1. Methods for conducting such screening and selection are well known in the art, such as described in the references in the preceding paragraph. To select antibodies of the invention having particular binding affinities for UCH-L1, such as those that dissociate from human UCH-L1 with a particular $K_{off}$ rate constant, the art-known method of surface plasmon resonance can be used to select antibodies having the desired $K_{off}$ rate constant. To select

antibodies of the invention having a particular neutralizing activity for hUCH-L1, such as those with a particular $IC_{50}$, standard methods known in the art for assessing the inhibition of UCH-L1 activity may be used.

**[0227]** In one aspect, the invention pertains to an isolated antibody, or an antigen-binding portion thereof, that binds human UCH-L1. Preferably, the antibody is a neutralizing antibody. In various embodiments, the antibody is a recombinant antibody or a monoclonal antibody.

**[0228]** For example, antibodies can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. Such phage can be utilized to display antigen-binding domains expressed from a repertoire or combinatorial antibody library (*e.g.,* human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, *e.g.,* using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv, or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies include those disclosed in Brinkmann *et al.*, *J. Immunol. Methods*, 182: 41-50 (1995); Ames *et al.*, J. Immunol. Methods, 184:177-186 (1995); Kettleborough *et al.*, *Eur. J. Immunol.*, 24: 952-958 (1994); Persic *et al.*, Gene, 187: 9-18 (1997); Burton *et al.*, Advances in *Immunology*, 57: 191-280 (1994); PCT Publication No. WO 92/01047; PCT Publication Nos. WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743; and 5,969,108.

**[0229]** As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies including human antibodies or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, *e.g.,* as described in detail below. For example, techniques to recombinantly produce Fab, Fab', and

$F(ab')_2$ fragments can also be employed using methods known in the art such as those disclosed in PCT publication No. WO 92/22324; Mullinax *et al.*, *BioTechniques*, 12(6): 864-869 (1992); Sawai *et al.*, *Am. J. Reprod. Immunol.*, 34: 26-34 (1995); and Better *et al.*, *Science*, 240: 1041-1043 (1988). Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patent Nos. 4,946,778 and 5,258,498; Huston *et al.*, Methods in Enzymology, 203: 46-88 (1991); Shu *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 7995-7999 (1993); and Skerra *et al.*, Science, 240: 1038-1041 (1988).

**[0230]** Alternative to screening of recombinant antibody libraries by phage display, other methodologies known in the art for screening large combinatorial libraries can be applied to the identification of antibodies of the invention. One type of alternative expression system is one in which the recombinant antibody library is expressed as RNA-protein fusions, as described in PCT Publication No. WO 98/31700 (Szostak and Roberts), and in Roberts and Szostak, *Proc. Natl. Acad. Sci. USA*, 94: 12297-12302 (1997). In this system, a covalent fusion is created between an mRNA and the peptide or protein that it encodes by in vitro translation of synthetic mRNAs that carry puromycin, a peptidyl acceptor antibiotic, at their 3' end. Thus, a specific mRNA can be enriched from a complex mixture of mRNAs (*e.g.*, a combinatorial library) based on the properties of the encoded peptide or protein, *e.g.*, antibody, or portion thereof, such as binding of the antibody, or portion thereof, to the dual specificity antigen. Nucleic acid sequences encoding antibodies, or portions thereof, recovered from screening of such libraries can be expressed by recombinant means as described above (*e.g.*, in mammalian host cells) and, moreover, can be subjected to further affinity maturation by either additional rounds of screening of mRNA-peptide fusions in which mutations have been introduced into the originally selected sequence(s), or by other methods for affinity maturation in vitro of recombinant antibodies, as described above. A preferred example of this methodology is PROfusion display technology.

**[0231]** In another approach, the antibodies can also be generated using yeast display methods known in the art. In yeast display methods, genetic methods are used to tether antibody domains to the yeast cell wall and display them on the surface of yeast. In particular, such yeast can be utilized to display antigen-binding domains expressed from a repertoire or combinatorial antibody library (*e.g.*, human or murine). Examples of yeast display methods

that can be used to make the antibodies include those disclosed in U.S. Patent No. 6,699,658 (Wittrup *et al.*) incorporated herein by reference.

**d) Production of Recombinant UCH-L1 Antibodies**

**[0232]** Antibodies may be produced by any of a number of techniques known in the art. For example, expression from host cells, wherein expression vector(s) encoding the heavy and light chains is (are) transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection, and the like. Although it is possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells is preferable, and most preferable in mammalian host cells, because such eukaryotic cells (and in particular mammalian cells) are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody.

**[0233]** Exemplary mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr-CHO cells, described in Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77: 4216-4220 (1980), used with a DHFR selectable marker, *e.g.*, as described in Kaufman and Sharp, *J. Mol. Biol.*, 159: 601-621 (1982), NS0 myeloma cells, COS cells, and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

**[0234]** Host cells can also be used to produce functional antibody fragments, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure may be performed. For example, it may be desirable to transfect a host cell with DNA encoding functional fragments of either the light chain and/or the heavy chain of an antibody of this invention. Recombinant DNA technology may also be used to remove some, or all, of

the DNA encoding either or both of the light and heavy chains that is not necessary for binding to the antigens of interest. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention (*i.e.,* binds human UCH-L1) and the other heavy and light chain are specific for an antigen other than human UCH-L1 by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

**[0235]** In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are cultured to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells, and recover the antibody from the culture medium. Still further, the invention provides a method of synthesizing a recombinant antibody of the invention by culturing a host cell of the invention in a suitable culture medium until a recombinant antibody of the invention is synthesized. The method can further comprise isolating the recombinant antibody from the culture medium.

**(1) Humanized Antibody**

**[0236]** The humanized antibody may be an antibody or a variant, derivative, analog or portion thereof which immunospecifically binds to an antigen of interest and which comprises a framework (FR) region having substantially the amino acid sequence of a human antibody and a complementary determining region (CDR) having substantially the amino acid sequence of a non-human antibody. The humanized antibody may be from a non-human

species antibody that binds the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and framework regions from a human immunoglobulin molecule.

**[0237]** As used herein, the term "substantially" in the context of a CDR refers to a CDR having an amino acid sequence at least 90%, at least 95%, at least 98% or at least 99% identical to the amino acid sequence of a non-human antibody CDR. A humanized antibody comprises substantially all of at least one, and typically two, variable domains (Fab, Fab', $F(ab')_2$, FabC, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin (*i.e.,* donor antibody) and all or substantially all of the framework regions are those of a human immunoglobulin consensus sequence. According to one aspect, a humanized antibody also comprises at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. In some embodiments, a humanized antibody contains both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain. In some embodiments, a humanized antibody only contains a humanized light chain. In some embodiments, a humanized antibody only contains a humanized heavy chain. In specific embodiments, a humanized antibody only contains a humanized variable domain of a light chain and/or of a heavy chain.

**[0238]** The humanized antibody can be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including without limitation IgG 1, IgG2, IgG3, and IgG4. The humanized antibody may comprise sequences from more than one class or isotype, and particular constant domains may be selected to optimize desired effector functions using techniques well-known in the art.

**[0239]** The framework and CDR regions of a humanized antibody need not correspond precisely to the parental sequences, *e.g.,* the donor antibody CDR or the consensus framework may be mutagenized by substitution, insertion and/or deletion of at least one amino acid residue so that the CDR or framework residue at that site does not correspond to either the donor antibody or the consensus framework. In one embodiment, such mutations, however, will not be extensive. Usually, at least 90%, at least 95%, at least 98%, or at least 99% of the humanized antibody residues will correspond to those of the parental FR and

CDR sequences. As used herein, the term "consensus framework" refers to the framework region in the consensus immunoglobulin sequence. As used herein, the term "consensus immunoglobulin sequence" refers to the sequence formed from the most frequently occurring amino acids (or nucleotides) in a family of related immunoglobulin sequences (See *e.g.*, Winnaker, *From Genes to Clones* (Verlagsgesellschaft, Weinheim, Germany 1987)). In a family of immunoglobulins, each position in the consensus sequence is occupied by the amino acid occurring most frequently at that position in the family. If two amino acids occur equally frequently, either can be included in the consensus sequence.

**[0240]** The humanized antibody may be designed to minimize unwanted immunological response toward rodent anti-human antibodies, which limits the duration and effectiveness of therapeutic applications of those moieties in human recipients. The humanized antibody may have one or more amino acid residues introduced into it from a source that is non-human. These non-human residues are often referred to as “import” residues, which are typically taken from a variable domain. Humanization may be performed by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such “humanized” antibodies are chimeric antibodies wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. For example, see U.S. Patent No. 4,816,567, the contents of which are herein incorporated by reference. The humanized antibody may be a human antibody in which some hypervariable region residues, and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies. Humanization or engineering of antibodies of the present disclosure can be performed using any known method, such as but not limited to those described in U.S. Patent Nos. 5,723,323; 5,976,862; 5,824,514; 5,817,483; 5,814,476; 5,763,192; 5,723,323; 5,766,886; 5,714,352; 6,204,023; 6,180,370; 5,693,762; 5,530,101; 5,585,089; 5,225,539; and 4,816,567.

**[0241]** The humanized antibody may retain high affinity for UCH-L1 and other favorable biological properties. The humanized antibody may be prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available. Computer programs are available that illustrate and display

probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.,* the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristics, such as increased affinity for UCH-L1, is achieved. In general, the hypervariable region residues may be directly and most substantially involved in influencing antigen binding.

**[0242]** As an alternative to humanization, human antibodies (also referred to herein as "fully human antibodies") can be generated. For example, it is possible to isolate human antibodies from libraries via PROfusion and/or yeast related technologies. It is also possible to produce transgenic animals (*e.g.,* mice that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, the homozygous deletion of the antibody heavy-chain joining region ($J_H$) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. The humanized or fully human antibodies may be prepared according to the methods described in U.S. Patent Nos. 5,770,429; 5,833,985; 5,837,243; 5,922,845; 6,017,517; 6,096,311; 6,111,166; 6,270,765; 6,303,755; 6,365,116; 6,410,690; 6,682,928; and 6,984,720, the contents each of which are herein incorporated by reference.

**e) Anti-UCH-L1 antibodies**

**[0243]** Anti-UCH-L1 antibodies may be generated using the techniques described above as well as using routine techniques known in the art. In some embodiments, the anti-UCH-L1 antibody may be an unconjugated UCH-L1 antibody, such as UCH-L1 antibodies available from United State Biological (Catalog Number: 031320), Cell Signaling Technology (Catalog Number: 3524), Sigma-Aldrich (Catalog Number: HPA005993), Santa Cruz Biotechnology, Inc. (Catalog Numbers: sc-58593 or sc-58594), R&D Systems (Catalog Number: MAB6007), Novus Biologicals (Catalog Number: NB600-1160), Biorbyt (Catalog

Number: orb33715), Enzo Life Sciences, Inc. (Catalog Number: ADI-905-520-1), Bio-Rad (Catalog Number: VMA00004), BioVision (Catalog Number: 6130-50), Abcam (Catalog Numbers: ab75275 or ab104938), Invitrogen Antibodies (Catalog Numbers: 480012), ThermoFisher Scientific (Catalog Numbers: MA1-46079, MA5-17235, MA1-90008, or MA1-83428), EMD Millipore (Catalog Number: MABN48), or Sino Biological Inc. (Catalog Number: 50690-R011). The anti-UCH-L1 antibody may be conjugated to a fluorophore, such as conjugated UCH-L1 antibodies available from BioVision (Catalog Number: 6960-25) or Aviva Systems Biology (Cat. Nos. OAAF01904-FITC).

**13. Variations on Methods**

**[0244]** The disclosed methods of determining the presence or amount of UCH-L1 present in a sample may be as described herein. The methods may also be adapted in view of other methods for analyzing analytes. Examples of well-known variations include, but are not limited to, immunoassay, such as sandwich immunoassay (*e.g.,* monoclonal-monoclonal sandwich immunoassays, monoclonal-polyclonal sandwich immunoassays, including enzyme detection (enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA), competitive inhibition immunoassay (*e.g.,* forward and reverse), enzyme multiplied immunoassay technique (EMIT), a competitive binding assay, bioluminescence resonance energy transfer (BRET), one-step antibody detection assay, homogeneous assay, heterogeneous assay, capture on the fly assay, etc.

**a) Immunoassays**

**[0245]** The analyte of interest, and/or peptides of fragments thereof (e.g., UCH-L1, and/or peptides or fragments thereof, i.e., UCH-L1 fragments), may be analyzed using UCH-L1 antibodies in an immunoassay. The presence or amount of analyte (e.g., UCH-L1) can be determined using antibodies and detecting specific binding to the analyte (e.g., UCH-L1). For example, the antibody, or antibody fragment thereof, may specifically bind to the analyte (e.g., UCH-L1). If desired, one or more of the antibodies can be used in combination with one or more commercially available monoclonal/polyclonal antibodies. Such antibodies are available from companies such as R&D Systems, Inc. (Minneapolis, MN) and Enzo Life Sciences International, Inc. (Plymouth Meeting, PA).

**[0246]** The presence or amount of analyte (e.g., UCH-L1) present in a body sample may be readily determined using an immunoassay, such as sandwich immunoassay (e.g., monoclonal-monoclonal sandwich immunoassays, monoclonal-polyclonal sandwich immunoassays, including radioisotope detection (radioimmunoassay (RIA)) and enzyme detection (enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA) (e.g., Quantikine ELISA assays, R&D Systems, Minneapolis, MN)). An example of a point-of-care device that can be used is i-STAT® (Abbott, Laboratories, Abbott Park, IL). Other methods that can be used include a chemiluminescent microparticle immunoassay, in particular one employing the ARCHITECT® automated analyzer (Abbott Laboratories, Abbott Park, IL), is an example. Other methods include, for example, mass spectrometry, and immunohistochemistry (e.g., with sections from tissue biopsies), using anti-analyte (e.g., anti-UCH-L1) antibodies (monoclonal, polyclonal, chimeric, humanized, human, etc.) or antibody fragments thereof against analyte (e.g., UCH-L1). Other methods of detection include those described in, for example, U.S. Patent 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. Specific immunological binding of the antibody to the analyte (e.g., UCH-L1) can be detected via direct labels, such as fluorescent or luminescent tags, metals and radionuclides attached to the antibody or via indirect labels, such as alkaline phosphatase or horseradish peroxidase.

**[0247]** The use of immobilized antibodies or antibody fragments thereof may be incorporated into the immunoassay. The antibodies may be immobilized onto a variety of supports, such as magnetic or chromatographic matrix particles, the surface of an assay plate (such as microtiter wells), pieces of a solid substrate material, and the like. An assay strip can be prepared by coating the antibody or plurality of antibodies in an array on a solid support. This strip can then be dipped into the test sample and processed quickly through washes and detection steps to generate a measurable signal, such as a colored spot.

**[0248]** A homogeneous format may be used. For example, after the test sample is obtained from a subject, a mixture is prepared. The mixture contains the test sample being assessed for analyte (*e.g.,* UCH-L1), a first specific binding partner, and a second specific binding

partner. The order in which the test sample, the first specific binding partner, and the second specific binding partner are added to form the mixture is not critical. The test sample is simultaneously contacted with the first specific binding partner and the second specific binding partner. In some embodiments, the first specific binding partner and any UCH-L1 contained in the test sample may form a first specific binding partner-analyte (*e.g.*, UCH-L1)-antigen complex and the second specific binding partner may form a first specific binding partner-analyte of interest (*e.g.*, UCH-L1)-second specific binding partner complex. In some embodiments, the second specific binding partner and any UCH-L1 contained in the test sample may form a second specific binding partner-analyte (*e.g.*, UCH-L1)-antigen complex and the first specific binding partner may form a first specific binding partner-analyte of interest (*e.g.*, UCH-L1)-second specific binding partner complex. The first specific binding partner may be an anti-analyte antibody (*e.g.*, anti-UCH-L1 antibody that binds to an epitope having an amino acid sequence comprising at least three contiguous (3) amino acids of SEQ ID NO: 1). The second specific binding partner may be an anti-analyte antibody (*e.g.*, anti-UCH-L1 antibody that binds to an epitope having an amino acid sequence comprising at least three contiguous (3) amino acids of SEQ ID NO: 1). Moreover, the second specific binding partner is labeled with or contains a detectable label as described above.

**[0249]** A heterogeneous format may be used. For example, after the test sample is obtained from a subject, a first mixture is prepared. The mixture contains the test sample being assessed for analyte (*e.g.*, UCH-L1) and a first specific binding partner, wherein the first specific binding partner and any UCH-L1 contained in the test sample form a first specific binding partner-analyte (*e.g.*, UCH-L1)-antigen complex. The first specific binding partner may be an anti-analyte antibody (*e.g.*, anti-UCH-L1 antibody that binds to an epitope having an amino acid sequence comprising at least three contiguous (3) amino acids of SEQ ID NO: 1). The order in which the test sample and the first specific binding partner are added to form the mixture is not critical.

**[0250]** The first specific binding partner may be immobilized on a solid phase. The solid phase used in the immunoassay (for the first specific binding partner and, optionally, the second specific binding partner) can be any solid phase known in the art, such as, but not

limited to, a magnetic particle, a bead, a test tube, a microtiter plate, a cuvette, a membrane, a scaffolding molecule, a film, a filter paper, a disc, and a chip. In those embodiments where the solid phase is a bead, the bead may be a magnetic bead or a magnetic particle. Magnetic beads/particles may be ferromagnetic, ferrimagnetic, paramagnetic, superparamagnetic or ferrofluidic. Exemplary ferromagnetic materials include Fe, Co, Ni, Gd, Dy, $CrO_2$, MnAs, MnBi, EuO, and NiO/Fe. Examples of ferrimagnetic materials include $NiFe_2O_4$, $CoFe_2O_4$, $Fe_3O_4$ (or $FeO{\cdot}Fe_2O_3$). Beads can have a solid core portion that is magnetic and is surrounded by one or more non-magnetic layers. Alternately, the magnetic portion can be a layer around a non-magnetic core. The solid support on which the first specific binding member is immobilized may be stored in dry form or in a liquid. The magnetic beads may be subjected to a magnetic field prior to or after contacting with the sample with a magnetic bead on which the first specific binding member is immobilized.

**[0251]** After the mixture containing the first specific binding partner-analyte (*e.g.*, UCH-L1) antigen complex is formed, any unbound analyte (*e.g.*, UCH-L1) is removed from the complex using any technique known in the art. For example, the unbound analyte (*e.g.*, UCH-L1) can be removed by washing. Desirably, however, the first specific binding partner is present in excess of any analyte (*e.g.*, UCH-L1) present in the test sample, such that all analyte (*e.g.*, UCH-L1) that is present in the test sample is bound by the first specific binding partner.

**[0252]** After any unbound analyte (*e.g.*, UCH-L1) is removed, a second specific binding partner is added to the mixture to form a first specific binding partner-analyte of interest (*e.g.*, UCH-L1)-second specific binding partner complex. The second specific binding partner may be an anti-analyte antibody (*e.g.*, anti-UCH-L1 antibody that binds to an epitope having an amino acid sequence comprising at least three contiguous (3) amino acids of SEQ ID NO: 1). Moreover, the second specific binding partner is labeled with or contains a detectable label as described above.

**[0253]** The use of immobilized antibodies or antibody fragments thereof may be incorporated into the immunoassay. The antibodies may be immobilized onto a variety of supports, such as magnetic or chromatographic matrix particles (such as a magnetic bead), latex particles or modified surface latex particles, polymer or polymer film, plastic or plastic film, planar

substrate, the surface of an assay plate (such as microtiter wells), pieces of a solid substrate material, and the like. An assay strip can be prepared by coating the antibody or plurality of antibodies in an array on a solid support. This strip can then be dipped into the test sample and processed quickly through washes and detection steps to generate a measurable signal, such as a colored spot.

**(1) Sandwich immunoassay**

**[0254]** A sandwich immunoassay measures the amount of antigen between two layers of antibodies (*i.e.*, at least one capture antibody) and a detection antibody (*i.e.*, at least one detection antibody). The capture antibody and the detection antibody bind to different epitopes on the antigen, *e.g.*, analyte of interest such as UCH-L1. Desirably, binding of the capture antibody to an epitope does not interfere with binding of the detection antibody to an epitope. Either monoclonal or polyclonal antibodies may be used as the capture and detection antibodies in the sandwich immunoassay.

**[0255]** Generally, at least two antibodies are employed to separate and quantify analyte (*e.g.*, UCH-L1) in a test sample. More specifically, the at least two antibodies bind to certain epitopes of analyte (*e.g.*, UCH-L1) forming an immune complex which is referred to as a "sandwich". One or more antibodies can be used to capture the analyte (*e.g.*, UCH-L1) in the test sample (these antibodies are frequently referred to as a "capture" antibody or "capture" antibodies) and one or more antibodies is used to bind a detectable (namely, quantifiable) label to the sandwich (these antibodies are frequently referred to as the "detection" antibody or "detection" antibodies). In a sandwich assay, the binding of an antibody to its epitope desirably is not diminished by the binding of any other antibody in the assay to its respective epitope. Antibodies are selected so that the one or more first antibodies brought into contact with a test sample suspected of containing analyte (*e.g.*, UCH-L1) do not bind to all or part of an epitope recognized by the second or subsequent antibodies, thereby interfering with the ability of the one or more second detection antibodies to bind to the analyte (*e.g.*, UCH-L1).

**[0256]** The antibodies may be used as a first antibody in said immunoassay. The antibody immunospecifically binds to epitopes on analyte (*e.g.*, UCH-L1). In addition to the antibodies of the present disclosure, said immunoassay may comprise a second antibody that immunospecifically binds to epitopes that are not recognized or bound by the first antibody.

**[0257]** A test sample suspected of containing analyte (*e.g.*, UCH-L1) can be contacted with at least one first capture antibody (or antibodies) and at least one second detection antibodies either simultaneously or sequentially. In the sandwich assay format, a test sample suspected of containing analyte (*e.g.*, UCH-L1) is first brought into contact with the at least one first capture antibody that specifically binds to a particular epitope under conditions which allow the formation of a first antibody-analyte (*e.g.*, UCH-L1) antigen complex. If more than one capture antibody is used, a first multiple capture antibody-UCH-L1 antigen complex is formed. In a sandwich assay, the antibodies, preferably, the at least one capture antibody, are used in molar excess amounts of the maximum amount of analyte (*e.g.*, UCH-L1) expected in the test sample. For example, from about 5 µg/ml to about 1 mg/ml of antibody per ml of microparticle coating buffer may be used.

*i. Anti-UCH-L1 Capture Antibody*

**[0258]** Optionally, prior to contacting the test sample with the at least one first capture antibody, the at least one first capture antibody can be bound to a solid support which facilitates the separation the first antibody-analyte (*e.g.*, UCH-L1) complex from the test sample. Any solid support known in the art can be used, including but not limited to, solid supports made out of polymeric materials in the forms of wells, tubes, or beads (such as a microparticle). The antibody (or antibodies) can be bound to the solid support by adsorption, by covalent bonding using a chemical coupling agent or by other means known in the art, provided that such binding does not interfere with the ability of the antibody to bind analyte (*e.g.*, UCH-L1). Moreover, if necessary, the solid support can be derivatized to allow reactivity with various functional groups on the antibody. Such derivatization requires the use of certain coupling agents such as, but not limited to, maleic anhydride, N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

**[0259]** After the test sample suspected of containing analyte (*e.g.*, UCH-L1) is incubated in order to allow for the formation of a first capture antibody (or multiple antibody)-analyte (*e.g.*, UCH-L1) complex. The incubation can be carried out at a pH of from about 4.5 to about 10.0, at a temperature of from about 2°C to about 45°C, and for a period from at least about one (1) minute to about eighteen (18) hours, from about 2-6 minutes, from about 7 -12 minutes, from about 5-15 minutes, or from about 3-4 minutes.

*ii. Detection Antibody*

**[0260]** After formation of the first/multiple capture antibody-analyte (*e.g.*, UCH-L1) complex, the complex is then contacted with at least one second detection antibody (under conditions that allow for the formation of a first/multiple antibody-analyte (*e.g.*, UCH-L1) antigen-second antibody complex). In some embodiments, the test sample is contacted with the detection antibody simultaneously with the capture antibody. If the first antibody-analyte (*e.g.*, UCH-L1) complex is contacted with more than one detection antibody, then a first/multiple capture antibody-analyte (*e.g.*, UCH-L1)-multiple antibody detection complex is formed. As with first antibody, when the at least second (and subsequent) antibody is brought into contact with the first antibody-analyte (*e.g.*, UCH-L1) complex, a period of incubation under conditions similar to those described above is required for the formation of the first/multiple antibody-analyte (*e.g.*, UCH-L1)-second/multiple antibody complex. Preferably, at least one second antibody contains a detectable label. The detectable label can be bound to the at least one second antibody prior to, simultaneously with or after the formation of the first/multiple antibody-analyte (*e.g.*, UCH-L1)-second/multiple antibody complex. Any detectable label known in the art can be used.

**[0261]** Chemiluminescent assays can be performed in accordance with the methods described in Adamczyk *et al.*, *Anal. Chim. Acta* 579(1): 61-67 (2006). While any suitable assay format can be used, a microplate chemiluminometer (Mithras LB-940, Berthold Technologies U.S.A., LLC, Oak Ridge, TN) enables the assay of multiple samples of small volumes rapidly. The chemiluminometer can be equipped with multiple reagent injectors using 96-well black polystyrene microplates (Costar #3792). Each sample can be added into a separate well, followed by the simultaneous/sequential addition of other reagents as determined by the type of assay employed. Desirably, the formation of pseudobases in neutral or basic solutions employing an acridinium aryl ester is avoided, such as by acidification. The chemiluminescent response is then recorded well-by-well. In this regard, the time for recording the chemiluminescent response will depend, in part, on the delay between the addition of the reagents and the particular acridinium employed.

**[0262]** The order in which the test sample and the specific binding partner(s) are added to form the mixture for chemiluminescent assay is not critical. If the first specific binding

partner is detectably labeled with an acridinium compound, detectably labeled first specific binding partner-UCH-L1 antigen complexes form. Alternatively, if a second specific binding partner is used and the second specific binding partner is detectably labeled with an acridinium compound, detectably labeled first specific binding partner-analyte (*e.g.*, UCH-L1)-second specific binding partner complexes form. Any unbound specific binding partner, whether labeled or unlabeled, can be removed from the mixture using any technique known in the art, such as washing.

**[0263]** Hydrogen peroxide can be generated in situ in the mixture or provided or supplied to the mixture before, simultaneously with, or after the addition of an above-described acridinium compound. Hydrogen peroxide can be generated in situ in a number of ways such as would be apparent to one skilled in the art.

**[0264]** Alternatively, a source of hydrogen peroxide can be simply added to the mixture. For example, the source of the hydrogen peroxide can be one or more buffers or other solutions that are known to contain hydrogen peroxide. In this regard, a solution of hydrogen peroxide can simply be added.

**[0265]** Upon the simultaneous or subsequent addition of at least one basic solution to the sample, a detectable signal, namely, a chemiluminescent signal, indicative of the presence of UCH-L1 is generated. The basic solution contains at least one base and has a pH greater than or equal to 10, preferably, greater than or equal to 12. Examples of basic solutions include, but are not limited to, sodium hydroxide, potassium hydroxide, calcium hydroxide, ammonium hydroxide, magnesium hydroxide, sodium carbonate, sodium bicarbonate, calcium hydroxide, calcium carbonate, and calcium bicarbonate. The amount of basic solution added to the sample depends on the concentration of the basic solution. Based on the concentration of the basic solution used, one skilled in the art can easily determine the amount of basic solution to add to the sample. Other labels other than chemiluminescent labels can be employed. For instance, enzymatic labels (including but not limited to alkaline phosphatase) can be employed.

**[0266]** The chemiluminescent signal, or other signal, that is generated can be detected using routine techniques known to those skilled in the art. Based on the intensity of the signal generated, the amount of analyte of interest (*e.g.*, UCH-L1) in the sample can be quantified.

Specifically, the amount of analyte (*e.g.,* UCH-L1) in the sample is proportional to the intensity of the signal generated. The amount of analyte (*e.g.,* UCH-L1) present can be quantified by comparing the amount of light generated to a standard curve for analyte (*e.g.,* UCH-L1) or by comparison to a reference standard. The standard curve can be generated using serial dilutions or solutions of known concentrations of analyte (*e.g.,* UCH-L1) by mass spectroscopy, gravimetric methods, and other techniques known in the art.

**(2) Forward Competitive Inhibition Assay**

**[0267]** In a forward competitive format, an aliquot of labeled analyte of interest (*e.g.,* analyte (*e.g.,* UCH-L1) having a fluorescent label, a tag attached with a cleavable linker, etc.) of a known concentration is used to compete with analyte of interest (*e.g.,* UCH-L1) in a test sample for binding to analyte of interest antibody (*e.g.,* UCH-L1 antibody).

**[0268]** In a forward competition assay, an immobilized specific binding partner (such as an antibody) can either be sequentially or simultaneously contacted with the test sample and a labeled analyte of interest, analyte of interest fragment or analyte of interest variant thereof. The analyte of interest peptide, analyte of interest fragment or analyte of interest variant can be labeled with any detectable label, including a detectable label comprised of tag attached with a cleavable linker. In this assay, the antibody can be immobilized on to a solid support. Alternatively, the antibody can be coupled to an antibody, such as an antispecies antibody, that has been immobilized on a solid support, such as a microparticle or planar substrate.

**[0269]** The labeled analyte of interest, the test sample and the antibody are incubated under conditions similar to those described above in connection with the sandwich assay format. Two different species of antibody-analyte of interest complexes may then be generated. Specifically, one of the antibody-analyte of interest complexes generated contains a detectable label (*e.g.,* a fluorescent label, etc.) while the other antibody-analyte of interest complex does not contain a detectable label. The antibody-analyte of interest complex can be, but does not have to be, separated from the remainder of the test sample prior to quantification of the detectable label. Regardless of whether the antibody-analyte of interest complex is separated from the remainder of the test sample, the amount of detectable label in the antibody-analyte of interest complex is then quantified. The concentration of analyte of interest (such as membrane-associated analyte of interest, soluble analyte of interest,

fragments of soluble analyte of interest, variants of analyte of interest (membrane-associated or soluble analyte of interest) or any combinations thereof) in the test sample can then be determined, *e.g.,* as described above.

**(3) Reverse Competitive Inhibition Assay**

**[0270]** In a reverse competition assay, an immobilized analyte of interest (*e.g.,* UCH-L1) can either be sequentially or simultaneously contacted with a test sample and at least one labeled antibody.

**[0271]** The analyte of interest can be bound to a solid support, such as the solid supports discussed above in connection with the sandwich assay format.

**[0272]** The immobilized analyte of interest, test sample and at least one labeled antibody are incubated under conditions similar to those described above in connection with the sandwich assay format. Two different species analyte of interest-antibody complexes are then generated. Specifically, one of the analyte of interest-antibody complexes generated is immobilized and contains a detectable label (*e.g.,* a fluorescent label, etc.) while the other analyte of interest-antibody complex is not immobilized and contains a detectable label. The non-immobilized analyte of interest-antibody complex and the remainder of the test sample are removed from the presence of the immobilized analyte of interest-antibody complex through techniques known in the art, such as washing. Once the non-immobilized analyte of interest antibody complex is removed, the amount of detectable label in the immobilized analyte of interest-antibody complex is then quantified following cleavage of the tag. The concentration of analyte of interest in the test sample can then be determined by comparing the quantity of detectable label as described above.

**(4) One-Step Immunoassay or "Capture on the Fly" Assay**

**[0273]** In a capture on the fly immunoassay, a solid substrate is pre-coated with an immobilization agent. The capture agent, the analyte (*e.g.,* UCH-L1) and the detection agent are added to the solid substrate together, followed by a wash step prior to detection. The capture agent can bind the analyte (*e.g.,* UCH-L1) and comprises a ligand for an immobilization agent. The capture agent and the detection agents may be antibodies or any other moiety capable of capture or detection as described herein or known in the art. The ligand may comprise a peptide tag and an immobilization agent may comprise an anti-

peptide tag antibody. Alternately, the ligand and the immobilization agent may be any pair of agents capable of binding together so as to be employed for a capture on the fly assay (*e.g.,* specific binding pair, and others such as are known in the art). More than one analyte may be measured. In some embodiments, the solid substrate may be coated with an antigen and the analyte to be analyzed is an antibody.

**[0274]** In certain other embodiments, in a one-step immunoassay or "capture on the fly", a solid support (such as a microparticle) pre-coated with an immobilization agent (such as biotin, streptavidin, etc.) and at least a first specific binding member and a second specific binding member (which function as capture and detection reagents, respectively) are used. The first specific binding member comprises a ligand for the immobilization agent (for example, if the immobilization agent on the solid support is streptavidin, the ligand on the first specific binding member may be biotin) and also binds to the analyte of interest (*e.g.,* UCH-L1). The second specific binding member comprises a detectable label and binds to an analyte of interest (*e.g.,* UCH-L1). The solid support and the first and second specific binding members may be added to a test sample (either sequentially or simultaneously). The ligand on the first specific binding member binds to the immobilization agent on the solid support to form a solid support/first specific binding member complex. Any analyte of interest present in the sample binds to the solid support/first specific binding member complex to form a solid support/first specific binding member/analyte complex. The second specific binding member binds to the solid support/first specific binding member/analyte complex and the detectable label is detected. An optional wash step may be employed before the detection. In certain embodiments, in a one-step assay more than one analyte may be measured. In certain other embodiments, more than two specific binding members can be employed. In certain other embodiments, multiple detectable labels can be added. In certain other embodiments, multiple analytes of interest can be detected, or their amounts, levels or concentrations, measured, determined or assessed.

**[0275]** The use of a capture on the fly assay can be done in a variety of formats as described herein, and known in the art. For example, the format can be a sandwich assay such as described above, but alternately can be a competition assay, can employ a single specific binding member, or use other variations such as are known.

**14. Kits**

**[0276]** Provided herein is a kit, which may be used for obtaining a blood test sample from a subject and assaying or assessing the test sample for levels of UCH-L1 or UCH-L1 fragment. The kits of the present disclosure can include a package of components for drawing a blood sample. Without limitation, kits can be used for obtaining blood samples from, for example, an adult patient, a juvenile patient, a neonatal patient, and a patient that may have suffered a traumatic brain injury. Components of the kit can include a means for sterilizing the subject's skin in the area of intended puncture. A typical and conventional sterilizing means is a piece of fabric commonly referred to as a gauze. The gauze preferably has a sterilant occluded therein and optionally may be attached to a grasping portion. A preferred sterilant is alcohol, although other sterilants such as antibacterial agents may be used. Exemplary of suitable antibacterials are the bisbiguanides, of which chlorhexidine is the best known member. The chlorhexidine may preferably be provided as a soluble salt in aqueous or alcoholic solution.

**[0277]** The skin penetrating means for initiation of blood flow may be a conventional lancet or may be any of a variety of conventional devices which includes a needle. The needle may be single or double ended and may be of any gauge, preferably 21 or 23 gauge. The needle may include a safety sleeve, may be attached to a needle hub, and can be used with a conventional tube holder. The needle may also be part of a conventional syringe assembly including barrel and plunger. Also, as known in the art, the penetrating means may be part of a conventional blood collection set in which a penetrating needle having a grasping means, such as wings, is connected via a hub and tubing to a delivery needle for puncture of a septum of an evacuated tube.

**[0278]** The holding means of the kit may be any type of container for receiving the sample, such as, for example, a syringe barrel. Preferred holding means are conventional tubes or vials having a closed end and an open end. Such tubes may have an internal volume of 100 μl to 10 mL. Smaller tubes are generally used with a lancet for collection of very small quantities of blood by gravity flow. Representative of such tubes are Microtainer™ brand tubes supplied by Becton, Dickinson and Company. The tube of the kit may also be an evacuated tube in which the open end is covered by a puncturable septum or stopper, such as Vacutainer™ brand tubes supplied by Becton Dickinson and Co. Evacuated tubes are

generally used with a conventional tube holder and blood collection set for collection of multiple larger blood samples, and may contain any of a variety of conventional blood analysis additives, such as anticoagulants, blood clotting agents and serum separating gels. Exemplary anticoagulants can include lithium heparin and ethylenediaminetetra acetic acid (EDTA), and can include a siliceous particle. The holding means may also be a test strip in which the sample is allowed to flow directly onto a glass or plastic strip containing reagents for analysis, such as by immunoassay.

**[0279]** When a subject's skin is punctured in order to take a blood sample, the puncture wound is usually covered to stanch bleeding and protect the wound during healing. Accordingly, the kit of the invention may include additional gauze to cover the wound and a means, such as adhesive, to affix the covering gauze over the wound. In accordance with current medical practice, the kit can include a disposable glove to avoid any contact between the phlebotomist and the sample. To promote blood flow, the kit may also include a tourniquet.

**[0280]** All components of the kit may be supplied in packaging, such as a compartmentalized plastic enclosure, preferably with a hermetically sealable cover so that the contents of the kit can be sterilized and sealed for storage.

**[0281]** The kit can include at least one component for assaying the test sample for UCH-L1 instructions for assaying the test sample for UCH-L1. For example, the kit can comprise instructions for assaying the test sample for UCH-L1 by immunoassay, e.g., chemiluminescent microparticle immunoassay. Instructions included in kits can be affixed to packaging material or can be included as a package insert. While the instructions are typically written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this disclosure. Such media include, but are not limited to, electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. As used herein, the term "instructions" can include the address of an internet site that provides the instructions. The at least one component may include at least one composition comprising one or more isolated antibodies or antibody fragments thereof that specifically bind to UCH-L1. The antibody may be an UCH-L1 capture antibody and/or a UCH-L1 detection antibody.

**[0282]** Alternatively or additionally, the kit can comprise a calibrator or control, e.g., purified, and optionally lyophilized, UCH-L1, and/or at least one container (e.g., tube, microtiter plates or strips, which can be already coated with an anti-UCH-L1 monoclonal antibody) for conducting the assay, and/or a buffer, such as an assay buffer or a wash buffer, either one of which can be provided as a concentrated solution, a substrate solution for the detectable label (e.g., an enzymatic label), or a stop solution. Preferably, the kit comprises all components, i.e., reagents, standards, buffers, diluents, etc., which are necessary to perform the assay. The instructions also can include instructions for generating a standard curve.

**[0283]** The kit may further comprise reference standards for quantifying UCH-L1. The reference standards may be employed to establish standard curves for interpolation and/or extrapolation of UCH-L1 concentrations. The reference standards may include a high UCH-L1 concentration level, for example, about 100000 pg/mL, about 125000 pg/mL, about 150000 pg/mL, about 175000 pg/mL, about 200000 pg/mL, about 225000 pg/mL, about 250000 pg/mL, about 275000 pg/mL, or about 300000 pg/mL; a medium UCH-L1 concentration level, for example, about 25000 pg/mL, about 40000 pg/mL, about 45000 pg/mL, about 50000 pg/mL, about 55000 pg/mL, about 60000 pg/mL, about 75000 pg/mL or about 100000 pg/mL; and/or a low UCH-L1 concentration level, for example, about 1 pg/mL, about 5 pg/mL, about 10 pg/mL, about 12.5 pg/mL, about 15 pg/mL, about 20 pg/mL, about 25 pg/mL, about 30 pg/mL, about 35 pg/mL, about 40 pg/mL, about 45 pg/mL, about 50 pg/mL, about 55 pg/mL, about 60 pg/mL, about 65 pg/mL, about 70 pg/mL, about 75 pg/mL, about 80 pg/mL, about 85 pg/mL, about 90 pg/mL, about 95 pg/mL, or about 100 pg/mL.

**[0284]** Any antibodies, which are provided in the kit, such as recombinant antibodies specific for UCH-L1, can incorporate a detectable label, such as a fluorophore, radioactive moiety, enzyme, biotin/avidin label, chromophore, chemiluminescent label, or the like, or the kit can include reagents for labeling the antibodies or reagents for detecting the antibodies (e.g., detection antibodies) and/or for labeling the analytes (*e.g.*, UCH-L1) or reagents for detecting the analyte (*e.g.*, UCH-L1). The antibodies, calibrators, and/or controls can be provided in separate containers or pre-dispensed into an appropriate assay format, for example, into microtiter plates.

**[0285]** Optionally, the kit includes quality control components (for example, sensitivity panels, calibrators, and positive controls). Preparation of quality control reagents is well-known in the art and is described on insert sheets for a variety of immunodiagnostic products. Sensitivity panel members optionally are used to establish assay performance characteristics, and further optionally are useful indicators of the integrity of the immunoassay kit reagents, and the standardization of assays,

**[0286]** The kit can also optionally include other reagents required to conduct a diagnostic assay or facilitate quality control evaluations, such as buffers, salts, enzymes, enzyme co-factors, substrates, detection reagents, and the like. Other components, such as buffers and solutions for the isolation and/or treatment of a test sample (e.g., pretreatment reagents), also can be included in the kit. The kit can additionally include one or more other controls. One or more of the components of the kit can be lyophilized, in which case the kit can further comprise reagents suitable for the reconstitution of the lyophilized components.

**[0287]** The various components of the kit optionally are provided in suitable containers as necessary, e.g., a microtiter plate. The kit can further include containers for holding or storing a sample (e.g., a container or cartridge for a urine, whole blood, plasma, or serum sample). Where appropriate, the kit optionally also can contain reaction vessels, mixing vessels, and other components that facilitate the preparation of reagents or the test sample. The kit can also include one or more instrument for assisting with obtaining a test sample, such as a syringe, pipette, forceps, measured spoon, or the like.

**[0288]** If the detectable label is at least one acridinium compound, the kit can comprise at least one acridinium-9-carboxamide, at least one acridinium-9-carboxylate aryl ester, or any combination thereof. If the detectable label is at least one acridinium compound, the kit also can comprise a source of hydrogen peroxide, such as a buffer, solution, and/or at least one basic solution. If desired, the kit can contain a solid phase, such as a magnetic particle, bead, test tube, microtiter plate, cuvette, membrane, scaffolding molecule, film, filter paper, disc, or chip.

**[0289]** If desired, the kit can further comprise one or more components, alone or in further combination with instructions, for assaying the test sample for another analyte, which can be a biomarker, such as a biomarker of traumatic brain injury or disorder.

**[0290]** The kit (or components thereof), as well as the method for assessing or determining the concentration of UCH-L1 in a test sample by an immunoassay as described herein, can be adapted for use in a variety of automated and semi-automated systems (including those wherein the solid phase comprises a microparticle), as described, e.g., U.S. Patent No. 5,063,081, U.S. Patent Application Publication Nos. 2003/0170881, 2004/0018577, 2005/0054078, and 2006/0160164 and as commercially marketed e.g., by Abbott Laboratories (Abbott Park, IL) as Abbott Point of Care (i-STAT® or i-STAT Alinity, Abbott Laboratories) as well as those described in U.S. Patent Nos. 5,089,424 and 5,006,309, and as commercially marketed, e.g., by Abbott Laboratories (Abbott Park, IL) as ARCHITECT® or the series of Abbott Alinity devices.

**[0291]** Some of the differences between an automated or semi-automated system as compared to a non-automated system (e.g., ELISA) include the substrate to which the first specific binding partner (e.g., analyte antibody or capture antibody) is attached (which can affect sandwich formation and analyte reactivity), and the length and timing of the capture, detection, and/or any optional wash steps. Whereas a non-automated format such as an ELISA may require a relatively longer incubation time with sample and capture reagent (e.g., about 2 hours), an automated or semi-automated format (e.g., ARCHITECT® and any successor platform, Abbott Laboratories) may have a relatively shorter incubation time (e.g., approximately 18 minutes for ARCHITECT®). Similarly, whereas a non-automated format such as an ELISA may incubate a detection antibody such as the conjugate reagent for a relatively longer incubation time (e.g., about 2 hours), an automated or semi-automated format (e.g., ARCHITECT® and any successor platform) may have a relatively shorter incubation time (e.g., approximately 4 minutes for the ARCHITECT® and any successor platform).

**[0292]** Other platforms available from Abbott Laboratories include, but are not limited to, AxSYM®, IMx® (see, e.g., U.S. Patent No. 5,294,404, which is hereby incorporated by reference in its entirety), PRISM®, EIA (bead), and Quantum™ II, as well as other platforms. Additionally, the assays, kits, and kit components can be employed in other formats, for example, on electrochemical or other hand-held or point-of-care assay systems. As mentioned previously, the present disclosure is, for example, applicable to the

commercial Abbott Point of Care (i-STAT®, Abbott Laboratories) electrochemical immunoassay system that performs sandwich immunoassays. Immunosensors and their methods of manufacture and operation in single-use test devices are described, for example in, U.S. Patent No. 5,063,081, U.S. Patent App. Publication Nos. 2003/0170881, 2004/0018577, 2005/0054078, and 2006/0160164, which are incorporated in their entireties by reference for their teachings regarding same.

**[0293]** In particular, with regard to the adaptation of an assay to the i-STAT® system, the following configuration is preferred. A microfabricated silicon chip is manufactured with a pair of gold amperometric working electrodes and a silver-silver chloride reference electrode. On one of the working electrodes, polystyrene beads (0.2 μm diameter) with immobilized capture antibody are adhered to a polymer coating of patterned polyvinyl alcohol over the electrode. This chip is assembled into an i-STAT® cartridge with a fluidics format suitable for immunoassay. On a portion of the silicon chip, there is a specific binding partner for UCH-L1, such as one or more UCH-L1 antibodies (one or more monoclonal/polyclonal antibody or a fragment thereof, a variant thereof, or a fragment of a variant thereof that can bind UCH-L1) or one or more anti-UCH-L1 DVD-Igs (or a fragment thereof, a variant thereof, or a fragment of a variant thereof that can bind UCH-L1), either of which can be detectably labeled. Within the fluid pouch of the cartridge is an aqueous reagent that includes p-aminophenol phosphate.

**[0294]** In operation, a sample from a subject suspected of suffering from TBI is added to the holding chamber of the test cartridge, and the cartridge is inserted into the i-STAT® reader. A pump element within the cartridge pushes the sample into a conduit containing the chip. The sample is brought into contact with the sensors allowing the enzyme conjugate to dissolve into the sample. The sample is oscillated across the sensors to promote formation of the sandwich of approximately 2-12 minutes. In the penultimate step of the assay, the sample is pushed into a waste chamber and wash fluid, containing a substrate for the alkaline phosphatase enzyme, is used to wash excess enzyme conjugate and sample off the sensor chip. In the final step of the assay, the alkaline phosphatase label reacts with p-aminophenol phosphate to cleave the phosphate group and permit the liberated p-aminophenol to be electrochemically oxidized at the working electrode. Based on the measured current, the

reader is able to calculate the amount of UCH-L1 in the sample by means of an embedded algorithm and factory-determined calibration curve.

**[0295]** The methods and kits as described herein necessarily encompass other reagents and methods for carrying out the immunoassay. For instance, encompassed are various buffers such as are known in the art and/or which can be readily prepared or optimized to be employed, e.g., for washing, as a conjugate diluent, and/or as a calibrator diluent. An exemplary conjugate diluent is ARCHITECT® conjugate diluent employed in certain kits (Abbott Laboratories, Abbott Park, IL) and containing 2-(N-morpholino)ethanesulfonic acid (MES), a salt, a protein blocker, an antimicrobial agent, and a detergent. An exemplary calibrator diluent is ARCHITECT® human calibrator diluent employed in certain kits (Abbott Laboratories, Abbott Park, IL), which comprises a buffer containing MES, other salt, a protein blocker, and an antimicrobial agent. Additionally, as described in U.S. Patent Application No. 61/142,048 filed December 31, 2008, improved signal generation may be obtained, e.g., in an i-STAT® cartridge format, using a nucleic acid sequence linked to the signal antibody as a signal amplifier.

**[0296]** While certain embodiments herein are advantageous when employed to assess disease, such as traumatic brain injury, the assays and kits also optionally can be employed to assess UCH-L1 in other diseases, disorders, and conditions as appropriate.

**[0297]** The method of assay also can be used to identify a compound that ameliorates diseases, such as traumatic brain injury. For example, a cell that expresses UCH-L1 can be contacted with a candidate compound. The level of expression of UCH-L1 in the cell contacted with the compound can be compared to that in a control cell using the method of assay described herein.

## 15. Examples

**[0298]** It will be readily apparent to those skilled in the art that other suitable modifications and adaptations of the methods of the present disclosure described herein are readily applicable and appreciable, and may be made using suitable equivalents without departing from the scope of the present disclosure or the aspects and embodiments disclosed herein. Having now described the present disclosure in detail, the same will be more clearly

understood by reference to the following examples, which are merely intended only to illustrate some aspects and embodiments of the disclosure, and should not be viewed as limiting to the scope of the disclosure. The disclosures of all journal references, U.S. patents, and publications referred to herein are hereby incorporated by reference in their entireties.

**[0299]** The present disclosure has multiple aspects, illustrated by the following non-limiting examples.

## Example 1
## i-STAT® UCH-L1 Assay

**[0300]** An i-STAT UCH-L1 assay in development was used in the examples described herein. However, it is understood that any UCH-L1 assay using the same or different antibodies can be improved using the present disclosure described herein. Monoclonal antibody pairs, such as Antibody A as a capture monoclonal antibody and Antibody B and C as a detection monoclonal antibody, were used. Antibody A is an exemplary anti-UCH-L1 antibody that was internally developed at Abbott Laboratories (Abbott Park, IL). Antibody B and C, developed by Banyan Biomarkers (Alachua, Florida), recognize different epitopes of UCH-L1 and enhance the detection of antigen in the sample. Other antibodies that were internally developed at Abbott Laboratories (Abbott Park, IL) or which have been described in the literature also show or are expected to show similar enhancement of signal when used together as capture antibodies or detection antibodies, in various combinations. The UCH-L1 assay design was evaluated against key performance attributes. The cartridge configuration was Antibody Configuration: Antibody A (Capture Antibody)/Antibody B+C (Detection Antibody); Reagent conditions: 0.8% solids, 125 µg /mL Fab Alkaline Phosphatase cluster conjugate; and Sample Inlet Print: UCH-L1 standard. The assay time was 10-15 min (with 7-12 min sample capture time).

## Example 2
## Stability of UCH-L1 in Whole Blood

**[0301]** The stability of UCH-L1 in blood samples was evaluated due to the observation that subjects that have not experienced an injury to the head, or have not been diagnosed as

having a disease or disorder relating to UCH-L1 ("normal" subjects), have measurable levels of UCH-L1 in whole blood samples. The i-STAT® platform was used to measure UCH-L1 in whole blood without prior separation of the cells from the plasma portion, and the results are shown in FIGS. 1A-1B.

**[0302]** The initial experiment was designed to understand the stability of the endogenous UCH-L1 antigen in whole blood from normal subjects. Blood was drawn from 5 normal donors into EDTA collection tubes following an approved Institutional Review Board study protocol; each participant gave written informed consent. The blood was continuously mixed at room temperature for 24 hours and tested at 1 hour, 4 hour, 8 hour and 24 hour time points after collection/mixing. A small amount of the blood was transferred into an Eppendorf™ LoBind tube at each time point and centrifuged to test the corresponding plasma at each time point.

**[0303]** As demonstrated in FIGS. 1A-1B (UCH-L1 levels and UCH-L1 levels as a percent of baseline, respectively), the concentration of UCH-L1 for each of the whole blood samples increased at each time point and over 24 hours. Additionally, there was a significant rise in UCH-L1 concentration with each of the whole blood samples that were continuously mixed for the full 24 hours (e.g., meaning during the entire time during the testing period). The difference from the point when the experiment began for each donor (e.g., baseline (which was the first testing time point once the whole blood was obtained) is shown in FIG. 1B.

## Example 3
## Stability of UCH-L1 in Plasma

**[0304]** The stability of UCH-L1 in plasma samples was evaluated in normal subjects (FIGS. 2A-2B). UCH-L1 concentration of the corresponding plasma separated at each of these time points discussed above in Example 1 (FIGS. 1A-1B) showed similar results. Blood was drawn from 5 normal donors into EDTA collection tubes following an approved Institutional Review Board study protocol; each participant gave written informed consent. The blood was continuously mixed at room temperature for 24 hours and tested at 1 hour, 4 hour, 8 hour and 24 hour time points after collection/mixing. A small amount of the blood was transferred into an Eppendorf ™ LoBind tube at each time point and centrifuged to test the corresponding

plasma at each time point. As shown in FIG. 2A, there was a 2 to 5 fold increase in the plasma values over 24 hours when the plasma was not separated from the whole blood until the sample was removed from the mixing device and processed for UCH-L1 assessment. The continuous mixing used in these experiments indicates that hemolysis may be an important factor influencing UCH-L1 measurements.

**[0305]** As demonstrated in FIGS. 2A-2B (UCH-L1 levels and UCH-L1 levels as a percent of baseline, respectively), the concentration of UCH-L1 for each of the plasma samples increased at each time point and over 24 hours when stored as whole blood during the time period. Additionally, there was a significant rise in UCH-L1 concentration with each of the whole blood samples that were continuously mixed for the full 24 hours. The difference from the point when the experiment began for each donor (baseline) is shown in FIG. 2B.

## Example 4
## Effects of Anti-coagulants on UCH-L1 Stability

**[0306]** Experiments were conducted to investigate the effects of anti-coagulants on the stability of UCH-L1 in whole blood samples from normal subjects. Blood was collected in Li heparin, EDTA and serum tubes from 4 donors. The EDTA and lithium (Li) heparin whole blood samples were quickly aliquoted into 2 mL tubes, and these samples were held stationary at room temperature and 2-8 °C for 2 hour, 6 hour, 24 hours, and 48 hours before processing and testing for UCH-L1 levels. At each time point, whole blood samples (FIGS. 3A-3B) and the corresponding plasma samples (Table 2) were assessed for UCH-L1 concentrations. The whole blood samples were thoroughly mixed by inversion (approximately ten times (which takes less 60 seconds)) prior to testing.

**[0307]** Stability of UCH-L1 from whole blood samples processed in Li heparin tubes at room temperature compared to 2-8 °C is shown in FIG. 3A. UCH-L1 concentrations were stable (<10% change) in whole blood samples at room temperature for at least the initial 24 hours after the samples were obtained, when stored stationary. However, UCH-L1 concentrations from whole blood samples from the same donors were only stable at 2-8 °C until about the first 6 hours; there was at least about a 10% difference (increase in UCH-L1 levels) in three of the four samples at 24 hours and 48 hours (FIG. 3A). See Table 2 below.

**[0308]** Stability of UCH-L1 from whole blood samples processed in EDTA tubes at room temperature compared to 2-8 °C is shown in FIG. 3B. UCH-L1 concentrations were stable (<15% change) in whole blood samples at room temperature for at least the initial 6 hours after the samples were obtained, when stored stationary. However, UCH-L1 concentrations from whole blood samples from the same donors tested at 24 hours and 48 hours did show that storage at 2-8 °C improved UCH-L1 stability compared to storage at room temperature. There was less than about a 20% difference in the concentrations of samples stored at 2-8 °C, whereas the whole blood stored at room temperature showed an increase in UCH-L1 concentrations up to about 200% higher than the initial concentrations (FIG. 3B). See Table 2 below.

**Table 2 Concentrations of UCH-L1 (pg/mL) for whole blood sample stability testing.**

| Time After Draw | Sample | | Tube Type | Donor 1 | Donor 2 | Donor 3 | Donor 4 |
|---|---|---|---|---|---|---|---|
| **Baseline** | **Whole Blood** | | **Li Hep** | 154.7 | 55.0 | 143.8 | 80.6 |
| | | | **EDTA** | 154.7 | 66.9 | 149.6 | 89.2 |
| **2 hours** | **Whole Blood** | **RT** | **Li Hep** | 156.8 | 51.0 | 144.6 | 80.5 |
| | | | **EDTA** | 163.3 | 65.8 | 163.6 | 97.1 |
| | | **2-8°C** | **Li Hep** | 152.5 | 56.0 | 142.2 | 77.7 |
| | | | **EDTA** | 161.3 | 68.4 | 153.0 | 100.4 |
| **6 hours** | **Whole Blood** | **RT** | **Li Hep** | 161.5 | 56.8 | 144.1 | 81.6 |
| | | | **EDTA** | 164.5 | 71.9 | 163.6 | 98.2 |
| | | **2-8°C** | **Li Hep** | 162.0 | 53.5 | 151.8 | 84.4 |
| | | | **EDTA** | 166.2 | 66.4 | 162.1 | 100.5 |
| **24 hours** | **Whole Blood** | **RT** | **Li Hep** | 156.4 | 53.1 | 155.8 | 86.9 |
| | | | **EDTA** | 208.5 | 128.5 | 291.3 | 209.3 |
| | | **2-8°C** | **Li Hep** | 158.1 | 63.4 | 158.9 | 95.7 |
| | | | **EDTA** | 182.5 | 66.9 | 173.5 | 95.1 |
| **48 hours** | **Whole Blood** | **RT** | **Li Hep** | 161.1 | 100.2 | 241.4 | 106.1 |
| | | | **EDTA** | 302.3 | 177.3 | 458.1 | 273.1 |
| | | **2-8°C** | **Li Hep** | 161.9 | 83.4 | 219.4 | 99.6 |
| | | | **EDTA** | 181.8 | 60.8 | 180.2 | 90.9 |

**[0309]** Stability of UCH-L1 from corresponding plasma samples processed in EDTA and Li heparin tubes at room temperature compared to 2-8 °C is shown in Table 3 below. At each time point for each of the whole blood samples tested, the corresponding plasma samples

were isolated and immediately tested to evaluate the variability in the plasma samples. The UCH-L1 concentration values and the differences observed in the plasma samples were very similar to the results in whole blood (FIGS. 3A-3B), thus indicating that the factors contributing to the increased concentrations of UCH-L1 at 24 hours and 48 hours likely involve the pre-analytical phases of sample processing (i.e., after obtaining the blood samples and/or during storage) and did not vary with the reagents.

**Table 3 Concentrations of UCH-L1 (pg/mL) for plasma sample stability testing.**

| Time After Draw | Sample | | Tube Type | Donor 1 | Donor 2 | Donor 3 | Donor 4 |
|---|---|---|---|---|---|---|---|
| **Baseline** | **Plasma** | | **Li Hep** | 113.1 | 28.2 | 80.2 | 56.8 |
| | | | **EDTA** | 117.1 | 42.2 | 79.8 | 58.5 |
| **2 hours** | **Plasma** | **RT** | **Li Hep** | 114.6 | 33.0 | 88.0 | 58.1 |
| | | | **EDTA** | 121.8 | 39.4 | 79.8 | 62.5 |
| | | **2-8°C** | **Li Hep** | 124.3 | 43.0 | 94.5 | 59.6 |
| | | | **EDTA** | 121.4 | 40.0 | 80.1 | 58.4 |
| **6 hours** | **Plasma** | **RT** | **Li Hep** | 106.7 | 33.5 | 81.7 | 52.3 |
| | | | **EDTA** | 112.5 | 38.0 | 77.0 | 57.6 |
| | | **2-8°C** | **Li Hep** | 123.9 | 36.4 | 106.0 | 67.8 |
| | | | **EDTA** | 120.6 | 41.3 | 91.3 | 64.3 |
| **24 hours** | **Plasma** | **RT** | **Li Hep** | 113.0 | 32.8 | 83.5 | 50.3 |
| | | | **EDTA** | 154.2 | 73.6 | 178.9 | 155.5 |
| | | **2-8°C** | **Li Hep** | 117.4 | 44.6 | 101.6 | 73.7 |
| | | | **EDTA** | 143.1 | 39.5 | 90.2 | 58.3 |
| **48 hours** | **Plasma** | **RT** | **Li Hep** | 116.0 | 62.0 | 139.7 | 67.3 |
| | | | **EDTA** | 253.2 | 118.3 | 341.2 | 230.5 |
| | | **2-8°C** | **Li Hep** | 122.3 | 69.6 | 146.9 | 81.5 |
| | | | **EDTA** | 139.8 | 37.5 | 101.4 | 57.6 |

**[0310]** The results in Tables 2 and 3 show that Li heparin and EDTA tubes show similar effect as there is some donor-to-donor variability in both sample types (whole blood and plasma). However, the results in Tables 2 and 3 demonstrate an improvement in the stability of UCH-L1 levels in the sample when the amount of mixing is reduced by holding the sample stationary (namely, not continuously mixing as shown in Example 2) during storage regardless of the temperature (namely, room temperature or at 2-8°C). Further improvement in the stability of UCH-L1 levels in the sample is shown when such samples (e.g., those that

are not continuously mixed) are stored at a temperature of 2-8°C (such samples are stable up to 24 to 48 hours after being obtained from a subject).

## Example 5
## Stability of UCH-L1 in Plasma and Serum

**[0311]** Experiments were conducted to assess the stability of UCH-L1 in plasma and serum samples that were isolated shortly after whole blood samples were obtained from normal donors, in contrast to being isolated after the whole blood samples were subjected to mixing and/or stored at room temperature or 2-8 °C. Fresh serum and plasma was separated prior to initiation of the experiment (time point 0). The samples were then aliquoted and stored at room temperature or 2-8 °C. At each time point (0 hours, 2 hours, 8 hours, 24 hours, and 48 hours after draw), an aliquot of plasma was mixed and tested for UCH-L1 levels.

**[0312]** As shown in FIGS. 4A (room temperature) and 4B (2-8 °C), UCH-L1 was stable for just the initial few hours after obtaining the blood samples and separating the plasma using EDTA tubes, and there was no significant difference in UCH-L1 stability in plasma at room temperature compared to 2-8 °C. In FIGS. 5A (room temperature) and 5B (2-8 °C), the use of Li heparin tubes did not produce significantly different results as compared to EDTA tubes. Additionally, as shown in FIGS. 6A (room temperature) and 6B (2-8 °C), the use of serum tubes to isolate serum and test for UCH-L1 levels did not produce significantly different results as compared to plasma samples.

**[0313]** These results contrast with the results above for whole blood samples, in which UCH-L1 concentrations increased at 24 and 48 hours, thus indicating that whole blood samples reacted differently than plasma and serum samples to the same processing conditions. Specifically, these results show that sample handling is important with respect to whole blood samples (e.g., mixing should be avoided with these samples and these samples should be stored at 2-8°C).

## Example 6

## Effects of Clot Formation on UCH-L1 Stability in Serum

**[0314]** Serum is the liquid portion of the whole blood that is isolated after a clot and clotting factors are formed (i.e., plasma without the clotting factors). Generally, it takes about 30-60 min for a clot to form in a whole blood sample at room temperature before the samples can be centrifuged to separate the serum. It is possible for cells to lyse during the clotting process, which may result in the release of cellular components in the serum. To evaluate the effects a clot may have on the components of serum when serum is exposed to a clot, two tubes of blood from 4 normal donors were collected in standard serum tubes. The serum was isolated from one tube and transferred to a secondary tube for each of the donors. The secondary tube was held stationary at room temperature for 8 hours, and serum from the second tube with the clot was separated after 8 hours and tested.

**[0315]** As shown in FIG. 7, UCH-L1 concentrations were higher when the serum was not separated from the clot. UCH-L1 concentrations were at least 30% higher for each of the samples, thus indicating the possibility of release of UCH-L1 from the clot into the serum.

**[0316]** In another experiment, fresh serum and plasma were isolated from whole blood within 1 hour of collection and aliquoted into separate Eppendorf™ LoBind tubes, and each of the aliquots were stored at RT or 2-8 °C. The plasma and serum aliquots were tested at 2 hours, 8 hours, 24 hours, and 48 hours to evaluate the stability of the neat plasma and serum that was isolated from the whole blood samples. Table 4 below shows the concentrations of the plasma sample for each of the 4 donors over time. UCH-L1 levels in the samples were relatively stable at 2-8 °C for up to 8 hours, and at RT for up to 2 hours. However, unlike the whole blood stability results, the concentration of UCH-L1 decreased with time at both temperatures for all sample types, suggesting that the increase in UCH-L1 levels in the whole blood samples was due to presence of the cellular components of the blood. In summary, these results show that sample handling is extremely important with respect to whole blood samples (e.g., mixing should be avoided with these samples and these samples should be stored at 2-8°C). In contrast, once plasma and serum are separated from cells/clots, these samples demonstrate good stability up to 8 hours.

**Table 4 Concentrations of UCH-L1 (pg/mL) for sample stability testing.**

Storage at RT

| Donor | sample type | Concentration | | | | |
|---|---|---|---|---|---|---|
| | | Time 0 | 2 hour | 8 hour | 24 hour | 48 hour |
| 1 | serum | 160.0 | 145.5 | 125.5 | 101.5 | 80.5 |
| 2 | serum | 46.8 | 43.9 | 38.2 | 25.7 | 33.4 |
| 3 | serum | 130.2 | 133.1 | 115.2 | 96.2 | 83.8 |
| 4 | serum | 80.6 | 72.3 | 65.6 | 49.6 | 40.3 |
| 1 | K2 EDTA | 153.4 | 135.9 | 126.1 | 118.4 | 117.0 |
| 2 | K2 EDTA | 45.1 | 43.9 | 47.6 | 31.0 | 45.0 |
| 3 | K2 EDTA | 95.1 | 89.3 | 87.5 | 79.7 | 76.7 |
| 4 | K2 EDTA | 68.4 | 65.1 | 63.6 | 52.3 | 51.0 |
| 1 | Li-Hep | 155.1 | 139.5 | 134.6 | 91.3 | 95.7 |
| 2 | Li-Hep | 40.2 | 41.7 | 30.7 | 21.6 | 24.3 |
| 3 | Li-Hep | 106.6 | 104.2 | 102.0 | 76.0 | 75.3 |
| 4 | Li-Hep | 70.5 | 66.5 | 64.8 | 42.6 | 40.4 |

Storage at 2 to 8 °C

| Donor | sample type | Concentration | | | | |
|---|---|---|---|---|---|---|
| | | Time 0 | 2 hour | 8 hour | 24 hour | 48 hour |
| 1 | serum | 160.0 | 155.8 | 139.5 | 104.2 | 111.5 |
| 2 | serum | 46.8 | 50.7 | 49.9 | 33.1 | 34.5 |
| 3 | serum | 130.2 | 135.3 | 132.1 | 99.1 | 95.5 |
| 4 | serum | 80.6 | 77.9 | 81.2 | 55.3 | 51.2 |
| 1 | K2 EDTA | 153.4 | 150.6 | 142.2 | 120.3 | 116.3 |
| 2 | K2 EDTA | 45.1 | 44.8 | 46.3 | 38.0 | 33.8 |
| 3 | K2 EDTA | 95.1 | 101.0 | 90.9 | 83.5 | 79.1 |
| 4 | K2 EDTA | 68.4 | 69.2 | 64.6 | 57.8 | 47.4 |
| 1 | Li-Hep | 155.1 | 146.7 | 149.4 | 94.6 | 99.6 |
| 2 | Li-Hep | 40.2 | 38.3 | 41.5 | 29.4 | 31.4 |
| 3 | Li-Hep | 106.6 | 102.5 | 103.3 | 80.1 | 81.4 |
| 4 | Li-Hep | 70.5 | 63.8 | 63.2 | 48.7 | 52.4 |

## Example 7
## Contribution of Hemolysis for UCH-L1 Stability

**[0317]** Evaluation of interference studies indicated the possibility of a component present in hemolysate as the cause of the increase in UCH-L1 readings, when hemolysate was spiked into a UCH-L1 low panel (data not shown). It was also shown that this UCH-L1 "interference" signal could be specifically reduced by addition of the anti-UCH-L1 capture antibody used in the immunoassay; however, non-specific mouse IgG showed no difference when it was added as a control.

**[0318]** To test the possibility of hemolysate interference, four hemolysates were prepared using the packed red blood cells (RBC's) from the bottom of the tube from four normal donor subjects. Removing cells from the bottom was an attempt to minimize the amount of buffy coat in these hemolysates. The packed RBCs were washed with saline repeatedly and lysed by freezing to generate the hemolysate solution. A small portion of this hemolysate solution was added to heat treated EDTA plasma (containing approximately 10 pg/mL UCH-L1), and tested to evaluate the change in signal.

**[0319]** Each hemolysate was spiked into an EDTA plasma pool targeting three concentrations of hemoglobin (0.75, 0.50, and 0.25 g/dL) and the samples were tested using UCH-L1 assay. The hemoglobin level for each of these samples was measured on a hematology analyzer. During the period of spiking and measurement, the sample was mixed for 20 to 30 minutes as known in the art. FIG. 8 shows the UCH-L1 concentration differences as a function of hemoglobin concentration. Additionally, FIG. 9 shows that whole blood samples from 3 normal donors spiked with recombinant UCH-L1 did not exhibit the same increase in UCH-L1 over time. These data indicate that the increase in UCH-L1 levels in whole blood described above is more apparent in subjects with relatively low baseline UCH-L1 concentrations.

**[0320]** It is understood that the foregoing detailed description and accompanying examples are merely illustrative and are not to be taken as limitations upon the scope of the invention, which is defined solely by the appended claims and their equivalents.

**[0321]** Various changes and modifications to the disclosed embodiments will be apparent to those skilled in the art. Such changes and modifications, including without limitation those

relating to the chemical structures, substituents, derivatives, intermediates, syntheses, compositions, formulations, or methods of use of the invention, may be made without departing from the spirit and scope thereof.

**[0322]** For reasons of completeness, various aspects of the invention are set out in the following numbered clauses:

**[0323]** Clause 1. In an improvement of a method of measuring an amount of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) in a whole blood sample obtained from a subject, wherein said improvement comprises processing the sample within no more than about eight hours after the sample is obtained from the subject to avoid a rise in UCH-L1 level that results from storage of the sample.

**[0324]** Clause 2. The improvement of clause 1, wherein the sample is processed within a period of time after the sample is obtained from the subject selected from the group consisting of: (a) from about zero hours to about 6 hours; (b) from about zero hours to about 4 hours; (c) from about zero hours to about 2 hours; and (d) from about zero hours to about 1 hour.

**[0325]** Clause 3. The improvement of clause 1, wherein the sample is processed within a period of time after the sample is obtained from the subject selected from the group consisting of: (a) from about 1 hour to about 8 hours; (b) from about 1 hour to about 6 hours; (c) from about 1 hour to about 4 hours; and (d) from about 1 hour to about 2 hours.

**[0326]** Clause 4. The improvement of any one of clauses 1-3, wherein processing the sample comprises separating plasma from blood cells in the sample; and, subsequently performing a test using the plasma that measures the amount of UCH-L1.

**[0327]** Clause 5. The improvement of any one of clauses 1-4, wherein the sample is obtained from the subject using a container comprising an anticoagulant selected from the group consisting of heparin and EDTA.

**[0328]** Clause 6. The improvement of any one of clauses 1-4, wherein processing the sample comprises separating serum from any clots that arise in the sample; and, subsequently performing a test using the serum that measures the amount of UCH-L1.

**[0329]** Clause 7. The improvement of clause 6, wherein the sample is collected using a serum collection tube.

**[0330]** Clause 8. The improvement of any one of clauses 1-3, wherein processing the sample comprises performing a test that measures the amount of UCH-L1 in the sample.

**[0331]** Clause 9. The improvement of any one of clauses 4-8, wherein the test comprises any method by which UCH-L1 amount can be assessed.

**[0332]** Clause 10. The improvement of clause 9, wherein the test is selected from the group consisting of an immunoassay, chemical analysis, SDS PAGE and Western blot analysis, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, chromatography, and spectrophotometry.

**[0333]** Clause 11. The improvement of any one of clauses 4-10, wherein the test is employed in a clinical chemistry format.

**[0334]** Clause 12. The improvement of clause 10 or 11, wherein the test is an immunoassay comprising: contacting the sample, either simultaneously or sequentially, in any order with: (1) at least one capture antibody, which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a capture antibody-UCH-L1 antigen complex, and (2) at least one detection antibody which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the capture antibody, to form a UCH-L1 antigen-detection antibody complex, such that a capture antibody-UCH-L1 antigen-detection antibody complex is formed, and measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the capture antibody-UCH-L1 antigen-detection antibody complex.

**[0335]** Clause 13. The improvement of any one of clauses 4-12, wherein the sample is maintained at room temperature for some period of time during the time point that the sample is obtained from the subject to a time point when the test is performed.

**[0336]** Clause 14. The improvement of any one of clauses 4-12, wherein the sample is maintained at a temperature from about 2 °C to about 8 °C for some period of time during the time point that the sample is obtained from the subject to the time point when the test is performed.

**[0337]** Clause 15. The improvement of any one of clauses 8-12, wherein the sample is not mixed between the period of time after the sample is obtained from the subject to the time point when the test is performed.

**[0338]** Clause 16. The improvement of any one of clauses 1-15, wherein the UCH-L1 amount in the sample from the subject is assessed as a measure of traumatic brain injury, wherein the subject may have sustained an injury to the head.

**[0339]** Clause 17. A method of avoiding or preventing an increase or rise in ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) levels between the period of time a whole blood sample is obtained from a subject and prior to performing an assay on the sample, the method comprising the step of: processing the sample within no more than about eight hours after the sample is obtained from the subject to avoid an increase or rise in UCH-L1 level that results from storage of the sample prior to performing the assay.

**[0340]** Clause 18. The method of clause 17, wherein the method avoids or prevents an erroneously high level of UCH-L1 in the sample between the period of time the sample is obtained from the subject and prior to performing the assay

**[0341]** Clause 19. The method of clause 17, wherein the sample is processed within a period of time after the sample is obtained from the subject selected from the group consisting of: (a) from about zero hours to about 6 hours; (b) from about zero hours to about 4 hours; (c) from about zero hours to about 2 hours; and (d) from about zero hours to about 1 hour.

**[0342]** Clause 20. The method of clause 17, wherein the sample is processed within a period of time after the sample is obtained from the subject selected from the group consisting of: (a) from about 1 hour to about 8 hours; (b) from about 1 hour to about 6 hours; (c) from about 1 hour to about 4 hours; and (d) from about 1 hour to about 2 hours.

**[0343]** Clause 21. The method of any one of clauses 17-20, further comprising subsequently performing an assay to measure the amount of UCH-L1 in the sample.

**[0344]** Clause 22. The method of any one of clauses 17-20, wherein processing the sample comprises separating plasma from blood cells in the sample; and, subsequently performing an assay using the plasma that measures the amount of UCH-L1 in the sample.

**[0345]** Clause 23. The method of any one of clauses 17-22, wherein the sample is obtained from the subject using a container comprising an anticoagulant selected from the group consisting of heparin and EDTA.

**[0346]** Clause 24. The method of any one of clauses 17-22, wherein processing the sample comprises separating serum from any clots that arise in the sample; and, subsequently performing an assay using the serum that measures the amount of UCH-L1 in the sample

**[0347]** Clause 25. The method of clause 24, wherein the sample is collected using a serum collection tube.

**[0348]** Clause 26. The method of any one of clauses 17-25, wherein the assay comprises any method by which UCH-L1 amount can be assessed.

**[0349]** Clause 27. The method of clause 26, wherein the assay is selected from the group consisting of an immunoassay, chemical analysis, SDS PAGE and Western blot analysis, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, chromatography, and spectrophotometry.

**[0350]** Clause 28. The method of any one of clauses 21-26, wherein the assay is employed in a clinical chemistry format.

**[0351]** Clause 29. The method of clause 27, wherein the assay is an immunoassay comprising:

a) contacting the sample, either simultaneously or sequentially, in any order with:
(1) at least one capture antibody, which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a capture antibody-UCH-L1 antigen complex, and
(2) at least one detection antibody which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the capture antibody, to form a UCH-L1 antigen-detection antibody complex,
such that a capture antibody-UCH-L1 antigen-detection antibody complex is formed, and

b) measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the capture antibody-UCH-L1 antigen-detection antibody complex.

**[0352]** Clause 30. The method of any one of clauses 21-29, wherein the sample is maintained at room temperature for some period of time between the time point that the sample is obtained from the subject and the time point when the assay is performed.

**[0353]** Clause 31. The method of any one of clauses 21-29, wherein the sample is maintained at a temperature from about 2 °C to about 8 °C for some period of time between the time point that the sample is obtained from the subject and the time point when the assay is performed.

**[0354]** Clause 32. The method of any one of clauses 25-29, wherein the sample is not mixed between the period of time between the time point that the sample is obtained from the subject and the time point when the test is performed.

**[0355]** Clause 33. The method of any one of clauses 17-32, wherein the UCH-L1 amount in the sample from the subject is assessed as a measure of traumatic brain injury, wherein the subject may have sustained an injury to the head.

**[0356]** Clause 34. In an improvement of a method of measuring an amount of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) in a whole blood sample obtained from a subject, wherein said improvement comprises reducing errors in UCH-L1 measurements by controlling preanalytical processing conditions.

**[0357]** Clause 35. The improvement of clause 34, wherein controlling preanalytical processing conditions comprise processing the sample within on more than about eight hours after the sample is obtained from the subject.

**[0358]** Clause 36. The improvement of clause 35, wherein the sample is processed within a period of time after the sample is obtained from the subject selected from the group consisting of: (a) from about zero hours to about 6 hours; (b) from about zero hours to about 4 hours; (c) from about zero hours to about 2 hours; and (d) from about zero hours to about 1 hour.

**[0359]** Clause 37. The improvement of clause 35, wherein the sample is processed within a period of time after the sample is obtained from the subject selected from the group consisting of: (a) from about 1 hour to about 8 hours; (b) from about 1 hour to about 6 hours; (c) from about 1 hour to about 4 hours; and (d) from about 1 hour to about 2 hours.

**[0360]** Clause 38. The improvement of any one of clauses 35-37, wherein processing the sample comprises separating plasma from blood cells in the sample; and, subsequently performing a test using the plasma that measures the amount of UCH-L1.

**[0361]** Clause 39. The improvement of any one of clauses 34-38, wherein the sample is obtained from the subject using a container comprising an anticoagulant selected from the group consisting of heparin and EDTA.

**[0362]** Clause 40. The improvement of any one of clauses 35-39, wherein processing the sample comprises separating serum from any clots that arise in the sample; and, subsequently performing a test using the serum that measures the amount of UCH-L1.

**[0363]** Clause 41. The improvement of clause 40, wherein the sample is collected using a serum collection tube.

**[0364]** Clause 42. The improvement of any one of clauses 35-40, wherein processing the sample comprises performing a test that measures the amount of UCH-L1 in the sample.

**[0365]** Clause 43. The improvement of any one of clauses 38-41, wherein the test comprises any method by which UCH-L1 amount can be assessed.

**[0366]** Clause 44. The improvement of clause 43, wherein the test is selected from the group consisting of an immunoassay, chemical analysis, SDS PAGE and Western blot analysis, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, chromatography, and spectrophotometry.

**[0367]** Clause 45. The improvement of any one of clauses 38-44, wherein the test is employed in a clinical chemistry format.

**[0368]** Clause 46. The improvement of clause 44 or 45, wherein the test is an immunoassay comprising: contacting the sample, either simultaneously or sequentially, in any order with: (1) at least one capture antibody, which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a capture antibody-UCH-L1 antigen complex, and (2) at least one detection antibody which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the capture antibody, to form a UCH-L1 antigen-detection antibody complex, such that a capture antibody-UCH-L1 antigen-detection antibody complex is formed, and measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the capture antibody-UCH-L1 antigen-detection antibody complex.

**[0369]** Clause 47. The improvement of any one of clauses 38-46, wherein the sample is maintained at room temperature for some period of time during the time point that the sample is obtained from the subject to a time point when the test is performed.

**[0370]** Clause 48. The improvement of any one of clauses 38-46, wherein the sample is maintained at a temperature from about 2 °C to about 8 °C for some period of time during the time point that the sample is obtained from the subject to the time point when the test is performed.

**[0371]** Clause 49. The improvement of any one of clauses 42-46, wherein the sample is not mixed between the period of time after the sample is obtained from the subject to the time point when the test is performed.

**[0372]** Clause 50. The improvement of any one of clauses 34-49, wherein the UCH-L1 amount in the sample from the subject is assessed as a measure of traumatic brain injury, wherein the subject may have sustained an injury to the head.

## CLAIMS

What is claimed is:

1. In an improvement of a method of measuring an amount of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) in a whole blood sample obtained from a subject, wherein said improvement comprises processing the sample within no more than about eight hours after the sample is obtained from the subject to avoid a rise in UCH-L1 level that results from storage of the sample.

2. The improvement of claim 1, wherein the sample is processed within a period of time after the sample is obtained from the subject selected from the group consisting of: (a) from about zero hours to about 6 hours; (b) from about zero hours to about 4 hours; (c) from about zero hours to about 2 hours; and (d) from about zero hours to about 1 hour.

3. The improvement of claim 1, wherein the sample is processed within a period of time after the sample is obtained from the subject selected from the group consisting of: (a) from about 1 hour to about 8 hours; (b) from about 1 hour to about 6 hours; (c) from about 1 hour to about 4 hours; and (d) from about 1 hour to about 2 hours.

4. The improvement of any one of claims 1-3, wherein processing the sample comprises separating plasma from blood cells in the sample; and, subsequently performing a test using the plasma that measures the amount of UCH-L1.

5. The improvement of any one of claims 1-4, wherein the sample is obtained from the subject using a container comprising an anticoagulant selected from the group consisting of heparin and EDTA.

6. The improvement of any one of claims 1-4, wherein processing the sample comprises separating serum from any clots that arise in the sample; and, subsequently performing a test using the serum that measures the amount of UCH-L1.

7. The improvement of claim 6, wherein the sample is collected using a serum collection tube.

8. The improvement of any one of claims 1-3, wherein processing the sample comprises performing a test that measures the amount of UCH-L1 in the sample.

9. The improvement of any one of claims 4-8, wherein the test comprises any method by which UCH-L1 amount can be assessed.

10. The improvement of claim 9, wherein the test is selected from the group consisting of an immunoassay, chemical analysis, SDS PAGE and Western blot analysis, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, chromatography, and spectrophotometry.

11. The improvement of any one of claims 4-10, wherein the test is employed in a clinical chemistry format.

12. The improvement of claim 10 or 11, wherein the test is an immunoassay comprising:

a) contacting the sample, either simultaneously or sequentially, in any order with:
(1) at least one capture antibody, which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a capture antibody-UCH-L1 antigen complex, and
(2) at least one detection antibody which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the capture antibody, to form a UCH-L1 antigen-detection antibody complex,
such that a capture antibody-UCH-L1 antigen-detection antibody complex is formed, and

b) measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the capture antibody-UCH-L1 antigen-detection antibody complex.

13. The improvement of any one of claims 4-12, wherein the sample is maintained at room temperature for some period of time during the time point that the sample is obtained from the subject to a time point when the test is performed.

14. The improvement of any one of claims 4-12, wherein the sample is maintained at a temperature from about 2 °C to about 8 °C for some period of time during the time point that the sample is obtained from the subject to the time point when the test is performed.

15. The improvement of any one of claims 8-12, wherein the sample is not mixed during the period of time after the sample is obtained from the subject to the time point when the test is performed.

16. The improvement of any one of claims 1-15, wherein the UCH-L1 amount in the sample from the subject is assessed as a measure of traumatic brain injury, wherein the subject may have sustained an injury to the head.

17. A method of avoiding or preventing an increase or rise in ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) levels between the period of time a whole blood sample is obtained from a subject and prior to performing an assay on the sample, the method comprising the step of: processing the sample within no more than about eight hours after the sample is obtained from the subject to avoid an increase or rise in UCH-L1 level that results from storage of the sample prior to performing the assay.

18. The method of claim 17, wherein the method avoids or prevents an erroneously high level of UCH-L1 in the sample between the period of time the sample is obtained from the subject and prior to performing the assay.

19. The method of claim 17, wherein the sample is processed within a period of time after the sample is obtained from the subject selected from the group consisting of: (a) from about zero hours to about 6 hours; (b) from about zero hours to about 4 hours; (c) from about zero hours to about 2 hours; and (d) from about zero hours to about 1 hour.

20. The method of claim 17, wherein the sample is processed within a period of time after the sample is obtained from the subject selected from the group consisting of: (a) from about 1 hour to about 8 hours; (b) from about 1 hour to about 6 hours; (c) from about 1 hour to about 4 hours; and (d) from about 1 hour to about 2 hours.

21. The method of any one of claims 17-20, further comprising subsequently performing an assay to measure the amount of UCH-L1 in the sample.

22. The method of any one of claims 17-20, wherein processing the sample comprises separating plasma from blood cells in the sample; and, subsequently performing an assay using the plasma that measures the amount of UCH-L1 in the sample.

23. The method of any one of claims 17-22, wherein the sample is obtained from the subject using a container comprising an anticoagulant selected from the group consisting of heparin and EDTA.

24. The method of any one of claims 17-22, wherein processing the sample comprises separating serum from any clots that arise in the sample; and, subsequently performing an assay using the serum that measures the amount of UCH-L1 in the sample

25. The method of claim 24, wherein the sample is collected using a serum collection tube.

26. The method of any one of claims 17-25, wherein the assay comprises any method by which UCH-L1 amount can be assessed.

27. The method of claim 26, wherein the assay is selected from the group consisting of an immunoassay, chemical analysis, SDS PAGE and Western blot analysis, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, chromatography, and spectrophotometry.

28. The method of any one of claims 21-26, wherein the assay is employed in a clinical chemistry format.

29. The method of claim 27, wherein the assay is an immunoassay comprising:

a) contacting the sample, either simultaneously or sequentially, in any order with:
(1) at least one capture antibody, which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a capture antibody-UCH-L1 antigen complex, and
(2) at least one detection antibody which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the capture antibody, to form a UCH-L1 antigen-detection antibody complex,
such that a capture antibody-UCH-L1 antigen-detection antibody complex is formed, and

b) measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the capture antibody-UCH-L1 antigen-detection antibody complex.

30. The method of any one of claims 21-29, wherein the sample is maintained at room temperature for some period of time between the time point that the sample is obtained from the subject and the time point when the assay is performed.

31. The method of any one of claims 21-29, wherein the sample is maintained at a temperature from about 2 °C to about 8 °C for some period of time between the time point that the sample is obtained from the subject and the time point when the assay is performed.

32. The method of any one of claims 25-29, wherein the sample is not mixed between the period of time between the time point that the sample is obtained from the subject and the time point when the test is performed.

33. The method of any one of claims 17-32, wherein the UCH-L1 amount in the sample from the subject is assessed as a measure of traumatic brain injury, wherein the subject may have sustained an injury to the head.

34. In an improvement of a method of measuring an amount of ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) in a whole blood sample obtained from a subject, wherein said improvement comprises reducing errors in UCH-L1 measurements by controlling preanalytical processing conditions.

35. The improvement of claim 34, wherein controlling preanalytical processing conditions comprise processing the sample within on more than about eight hours after the sample is obtained from the subject.

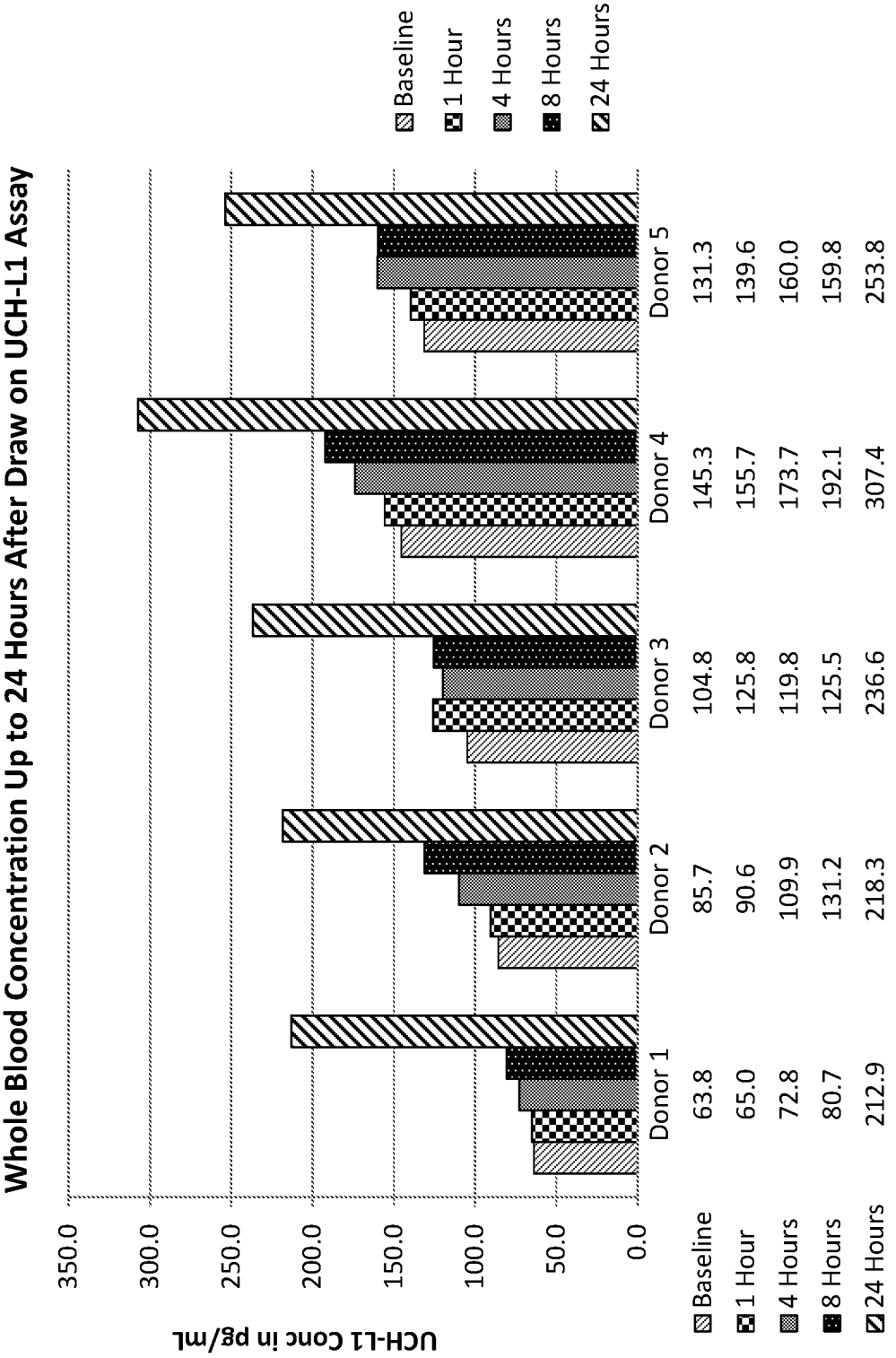
Whole Blood Concentration Up to 24 Hours After Draw on UCH-L1 Assay
UCH-L1 Conc in pg/mL
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
Baseline
1 Hour
4 Hours
8 Hours
24 Hours
Donor 1
Donor 2
Donor 3
Donor 4
Donor 5
Donor
Baseline 63.8 85.7 104.8 145.3 131.3
1 Hour 65.0 90.6 125.8 155.7 139.6
4 Hours 72.8 109.9 119.8 173.7 160.0
8 Hours 80.7 131.2 125.5 192.1 159.8
24 Hours 212.9 218.3 236.6 307.4 253.8

FIG. 1A

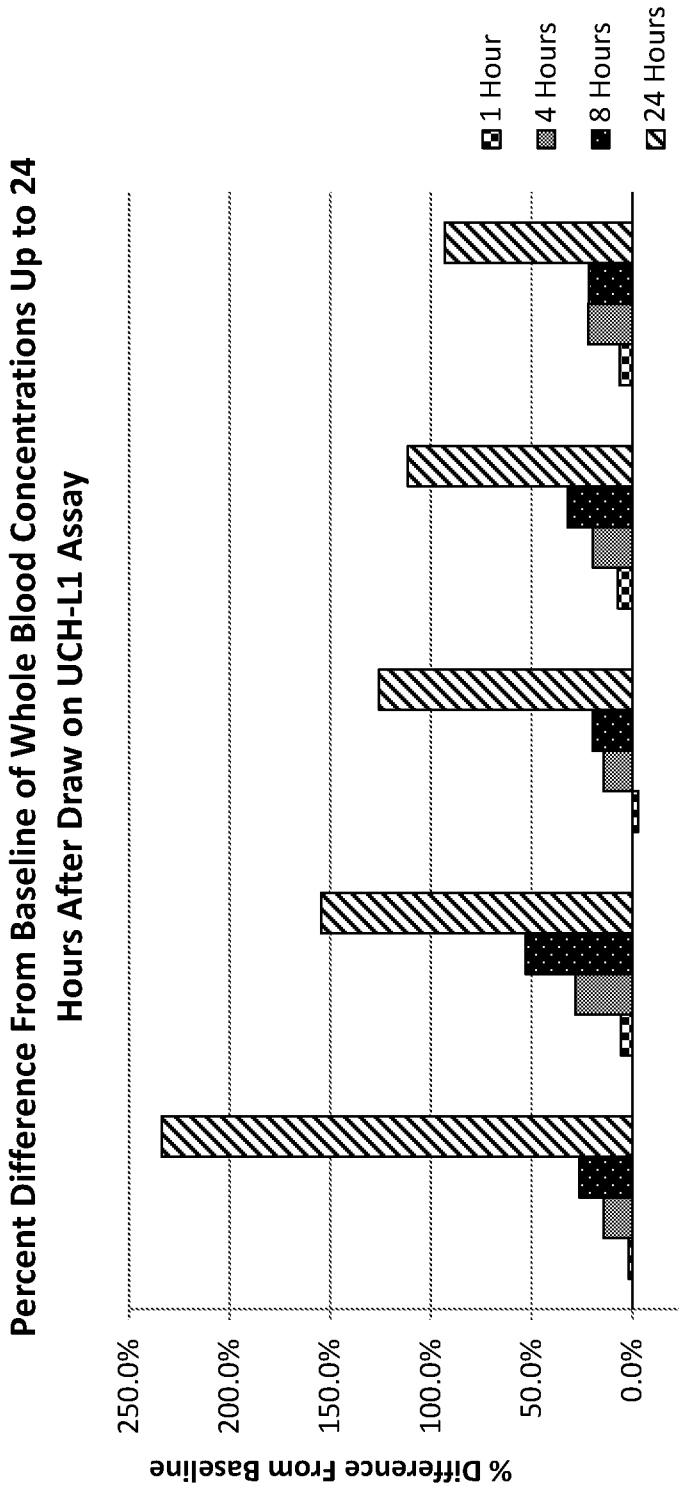
Percent Difference From Baseline of Whole Blood Concentrations Up to 24 Hours After Draw on UCH-L1 Assay
% Difference From Baseline
250.0%
200.0%
150.0%
100.0%
50.0%
0.0%
-50.0%
1 Hour
4 Hours
8 Hours
24 Hours
Donor
Donor 1 Donor 2 Donor 3 Donor 4 Donor 5
1 Hour 1.9% 5.7% -3.1% 7.1% 6.3%
4 Hours 14.2% 28.2% 14.4% 19.5% 21.9%
8 Hours 26.5% 53.0% 19.7% 32.2% 21.7%
24 Hours 233.8% 154.6% 125.7% 111.5% 93.3%

FIG. 1B

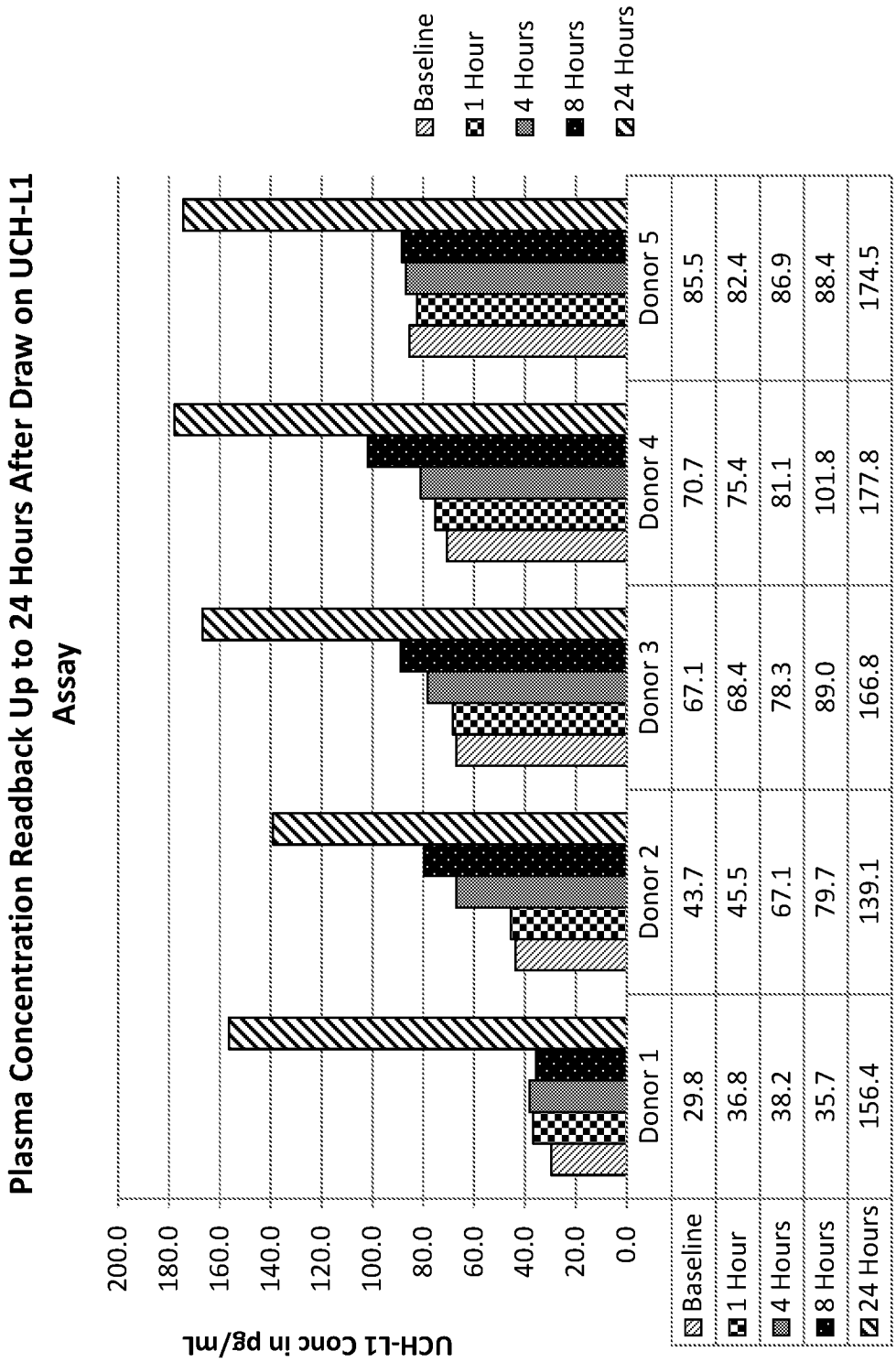
Plasma Concentration Readback Up to 24 Hours After Draw on UCH-L1 Assay
UCH-L1 Conc in pg/mL
200.0
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Baseline
1 Hour
4 Hours
8 Hours
24 Hours
Donor 1
Donor 2
Donor 3
Donor 4
Donor 5
Donor
29.8
43.7
67.1
70.7
85.5
36.8
45.5
68.4
75.4
82.4
38.2
67.1
78.3
81.1
86.9
35.7
79.7
89.0
101.8
88.4
156.4
139.1
166.8
177.8
174.5

FIG. 2A

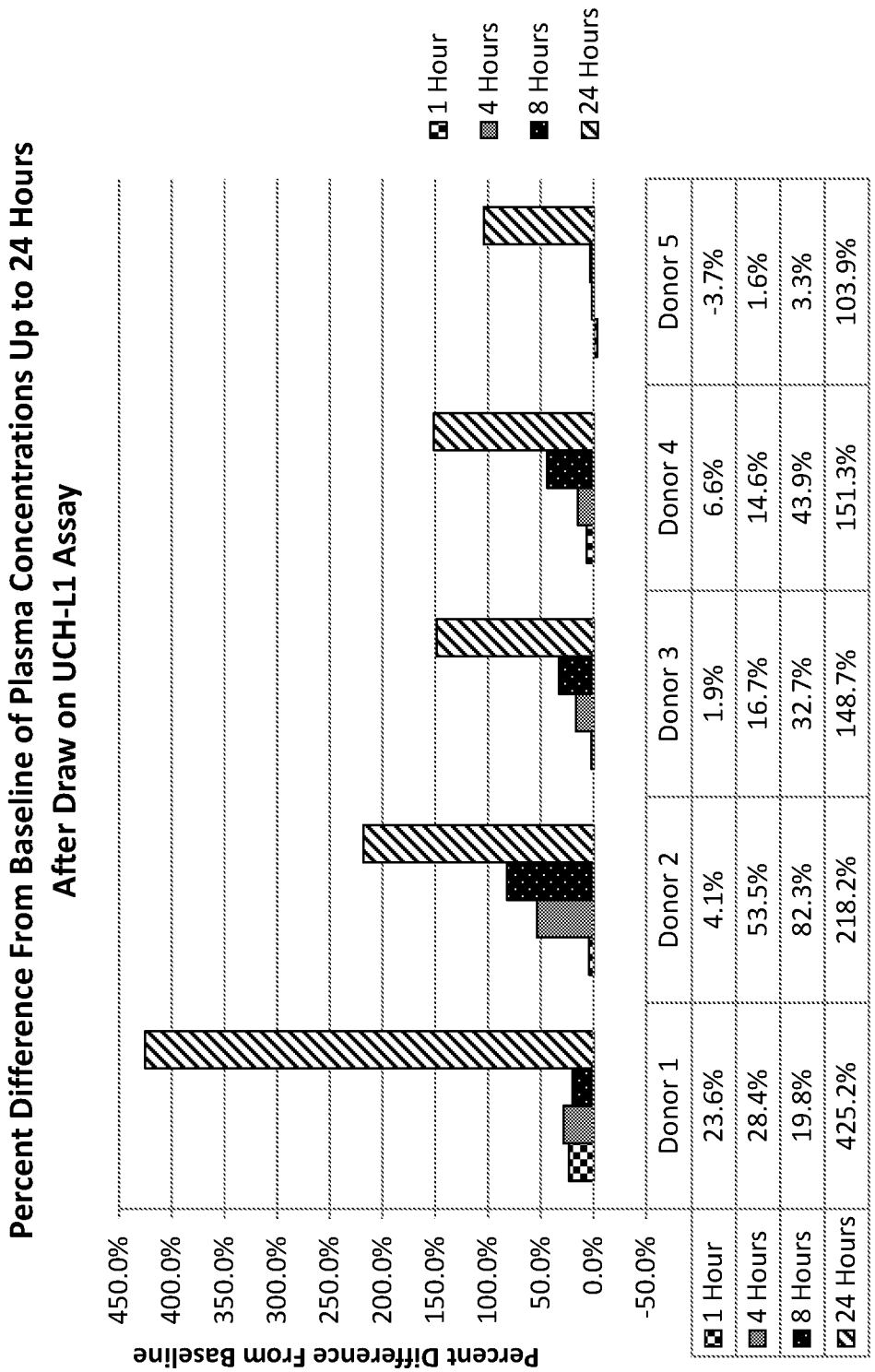
Percent Difference From Baseline of Plasma Concentrations Up to 24 Hours After Draw on UCH-L1 Assay
Percent Difference From Baseline
450.0%
400.0%
350.0%
300.0%
250.0%
200.0%
150.0%
100.0%
50.0%
0.0%
-50.0%
1 Hour
4 Hours
8 Hours
24 Hours
Donor
Donor 1
Donor 2
Donor 3
Donor 4
Donor 5
1 Hour 23.6% 4.1% 1.9% 6.6% -3.7%
4 Hours 28.4% 53.5% 16.7% 14.6% 1.6%
8 Hours 19.8% 82.3% 32.7% 43.9% 3.3%
24 Hours 425.2% 218.2% 148.7% 151.3% 103.9%

FIG. 2B

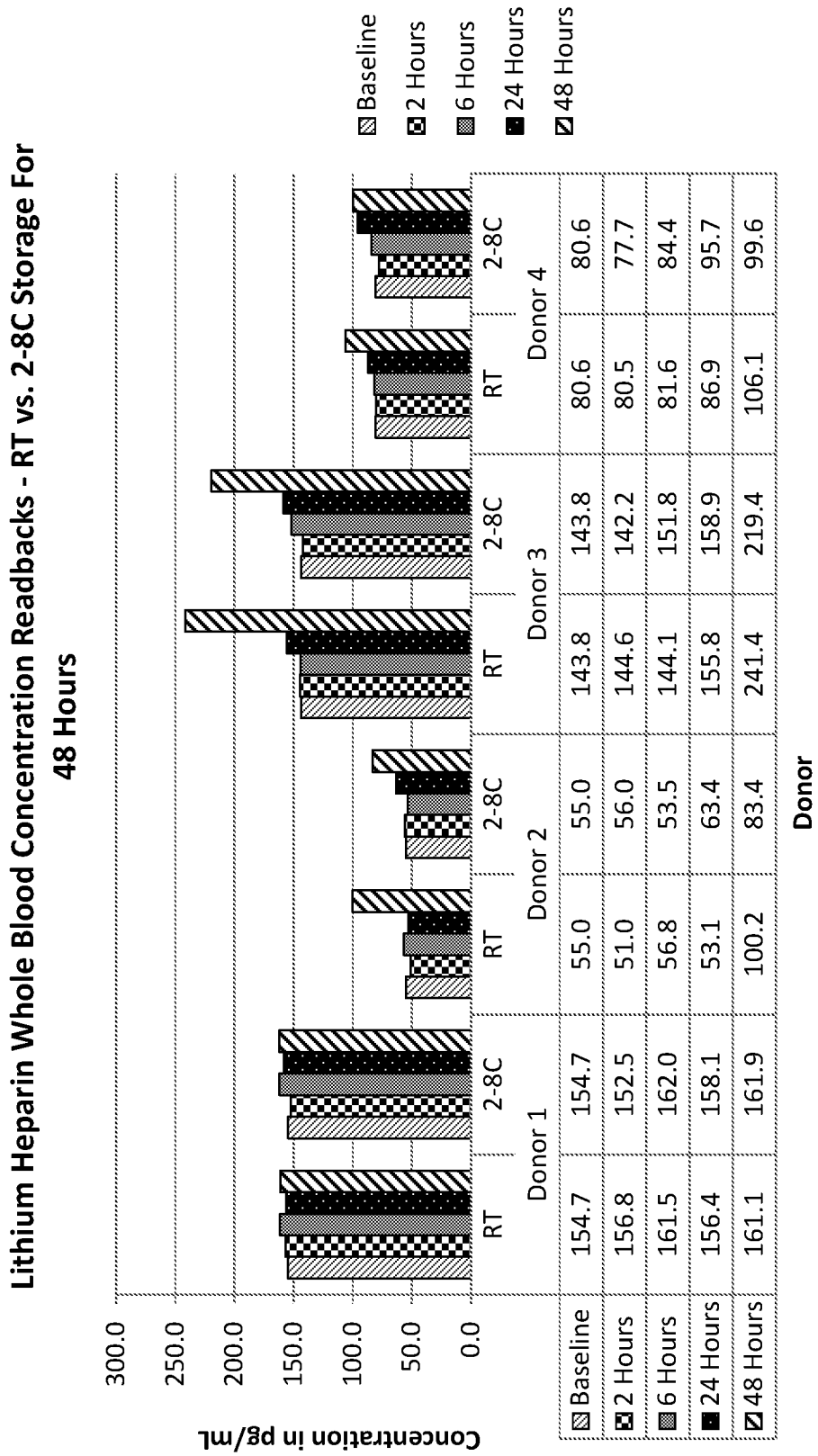
Lithium Heparin Whole Blood Concentration Readbacks - RT vs. 2-8C Storage For 48 Hours
Concentration in pg/mL
300.0
250.0
200.0
150.0
100.0
50.0
0.0
Baseline
2 Hours
6 Hours
24 Hours
48 Hours
RT
2-8C
Donor 1
Donor 2
Donor 3
Donor 4
Donor
154.7 55.0 143.8 80.6
156.8 51.0 144.6 80.5
161.5 56.8 144.1 81.6
156.4 53.1 155.8 86.9
161.1 100.2 241.4 106.1
154.7 55.0 143.8 80.6
152.5 51.0 142.2 80.5
162.0 56.8 151.8 81.6
158.1 53.1 158.9 86.9
161.9 100.2 219.4 106.1
154.7 55.0 143.8 80.6
152.5 56.0 142.2 77.7
162.0 53.5 151.8 84.4
158.1 63.4 158.9 95.7
161.9 83.4 219.4 99.6

FIG. 3A

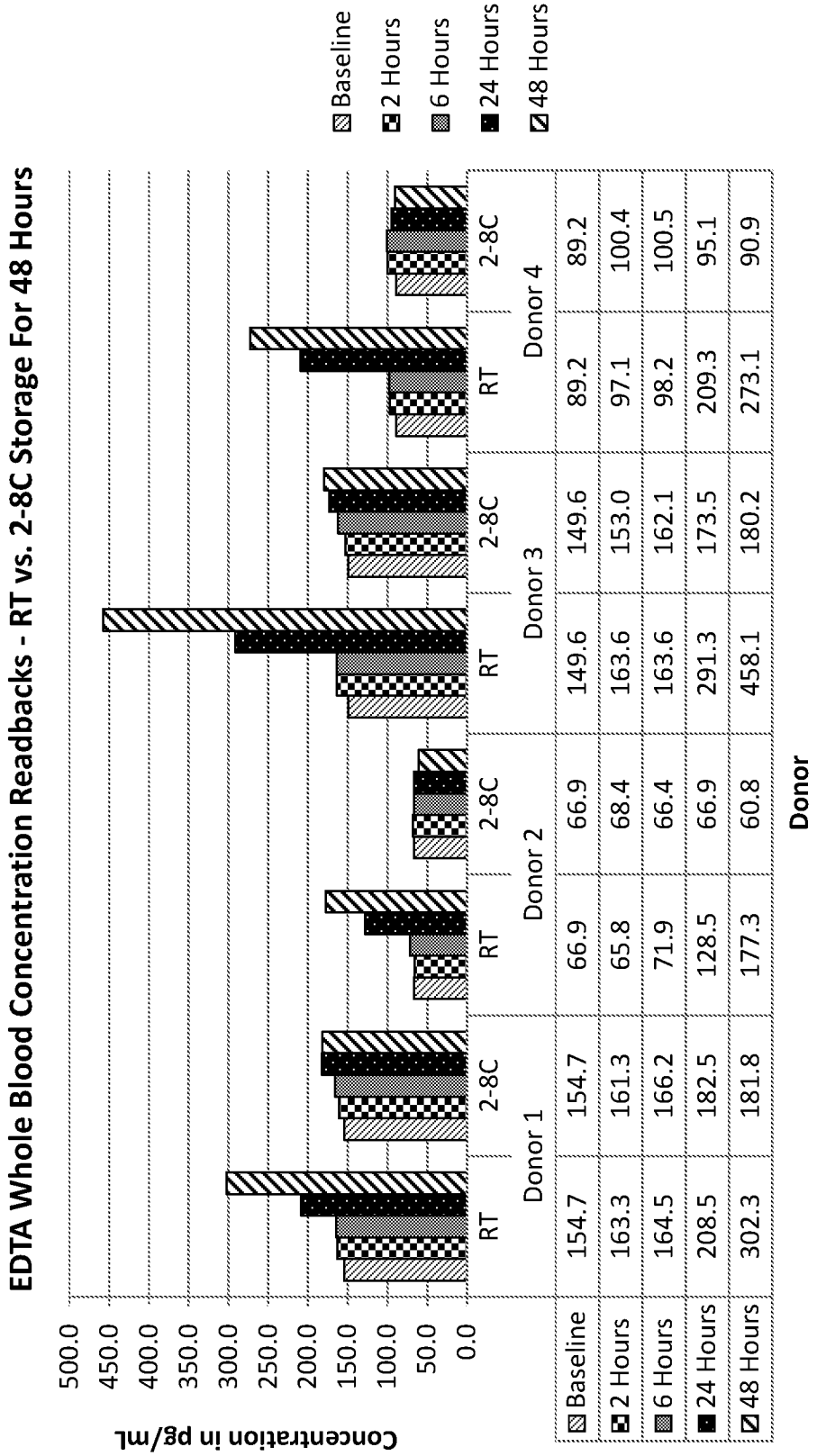
EDTA Whole Blood Concentration Readbacks - RT vs. 2-8C Storage For 48 Hours
Concentration in pg/mL
500.0
450.0
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
Baseline
2 Hours
6 Hours
24 Hours
48 Hours
RT
2-8C
Donor 1
Donor 2
Donor 3
Donor 4
Donor
Baseline 154.7 154.7 66.9 66.9 149.6 149.6 89.2 89.2
2 Hours 163.3 161.3 65.8 68.4 163.6 153.0 97.1 100.4
6 Hours 164.5 166.2 71.9 66.4 163.6 162.1 98.2 100.5
24 Hours 208.5 182.5 128.5 66.9 291.3 173.5 209.3 95.1
48 Hours 302.3 181.8 177.3 60.8 458.1 180.2 273.1 90.9

FIG. 3B

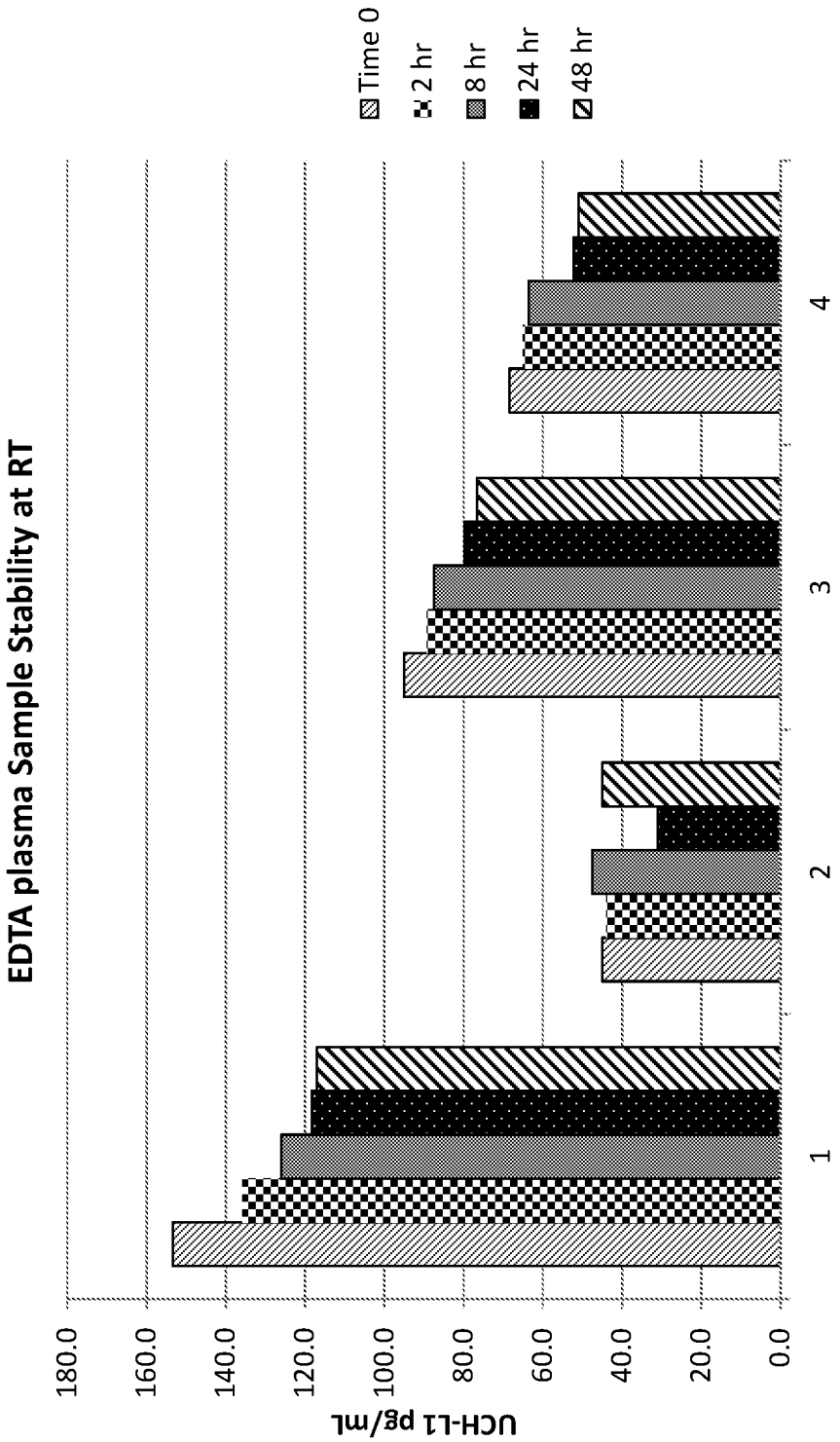
EDTA plasma Sample Stability at RT
Time 0
2 hr
8 hr
24 hr
48 hr
UCH-L1 pg/mL
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1
2
3
4
Donor

FIG. 4A

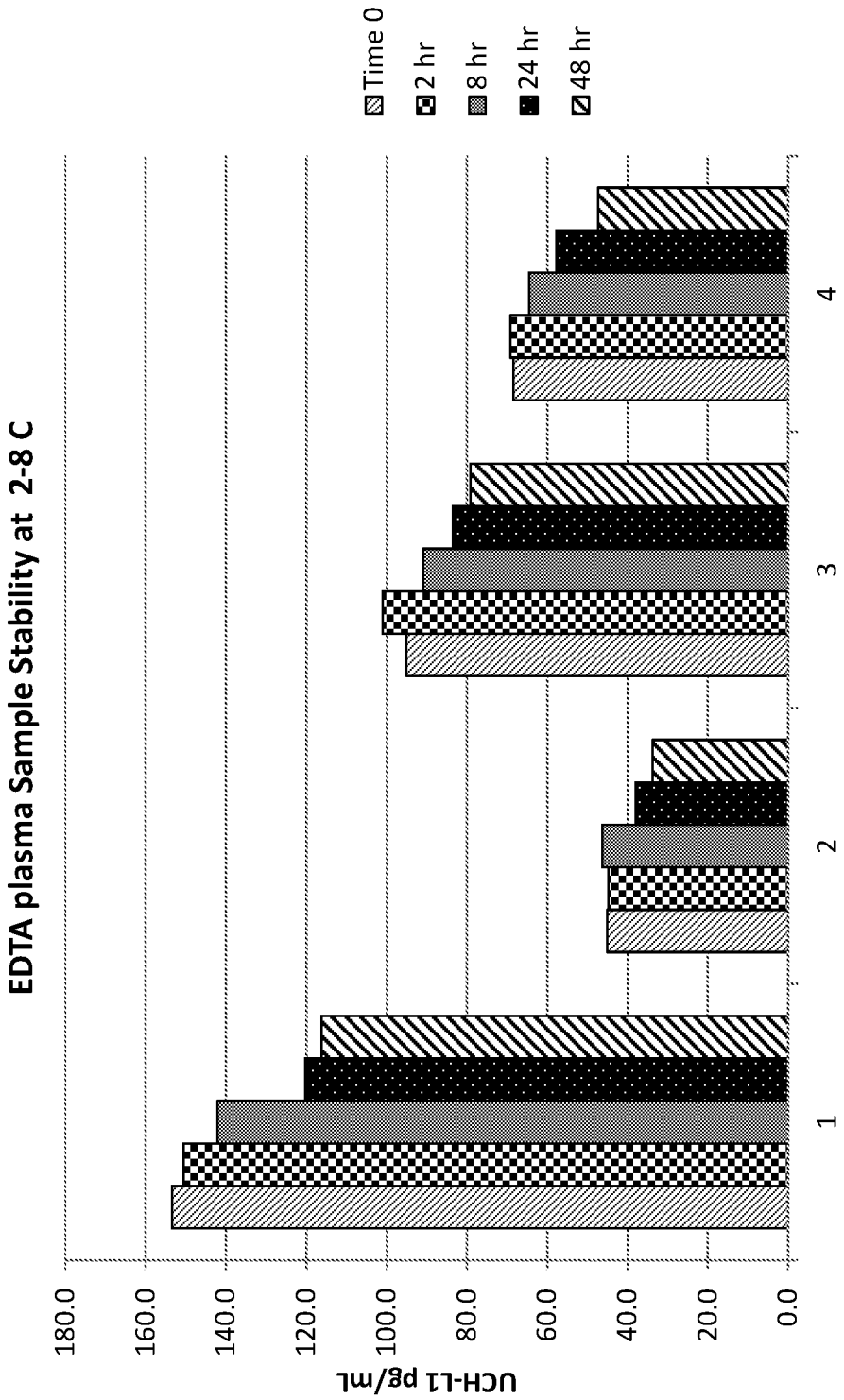
EDTA plasma Sample Stability at 2-8 C
Time 0
2 hr
8 hr
24 hr
48 hr
UCH-L1 pg/mL
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1
2
3
4
Donor

FIG. 4B

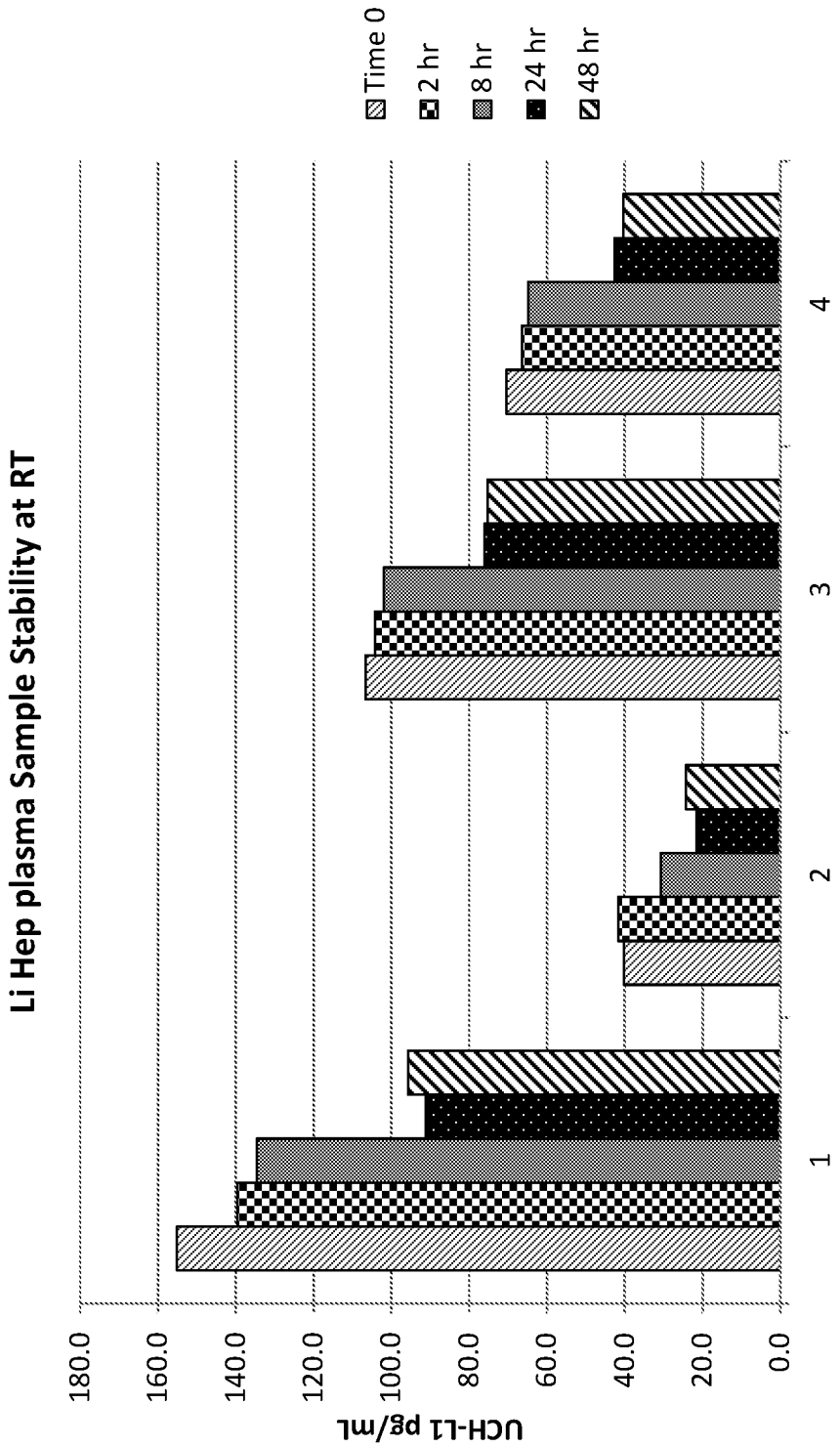
Li Hep plasma Sample Stability at RT
Time 0
2 hr
8 hr
24 hr
48 hr
UCH-L1 pg/mL
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1
2
3
4
Donor

FIG. 5A

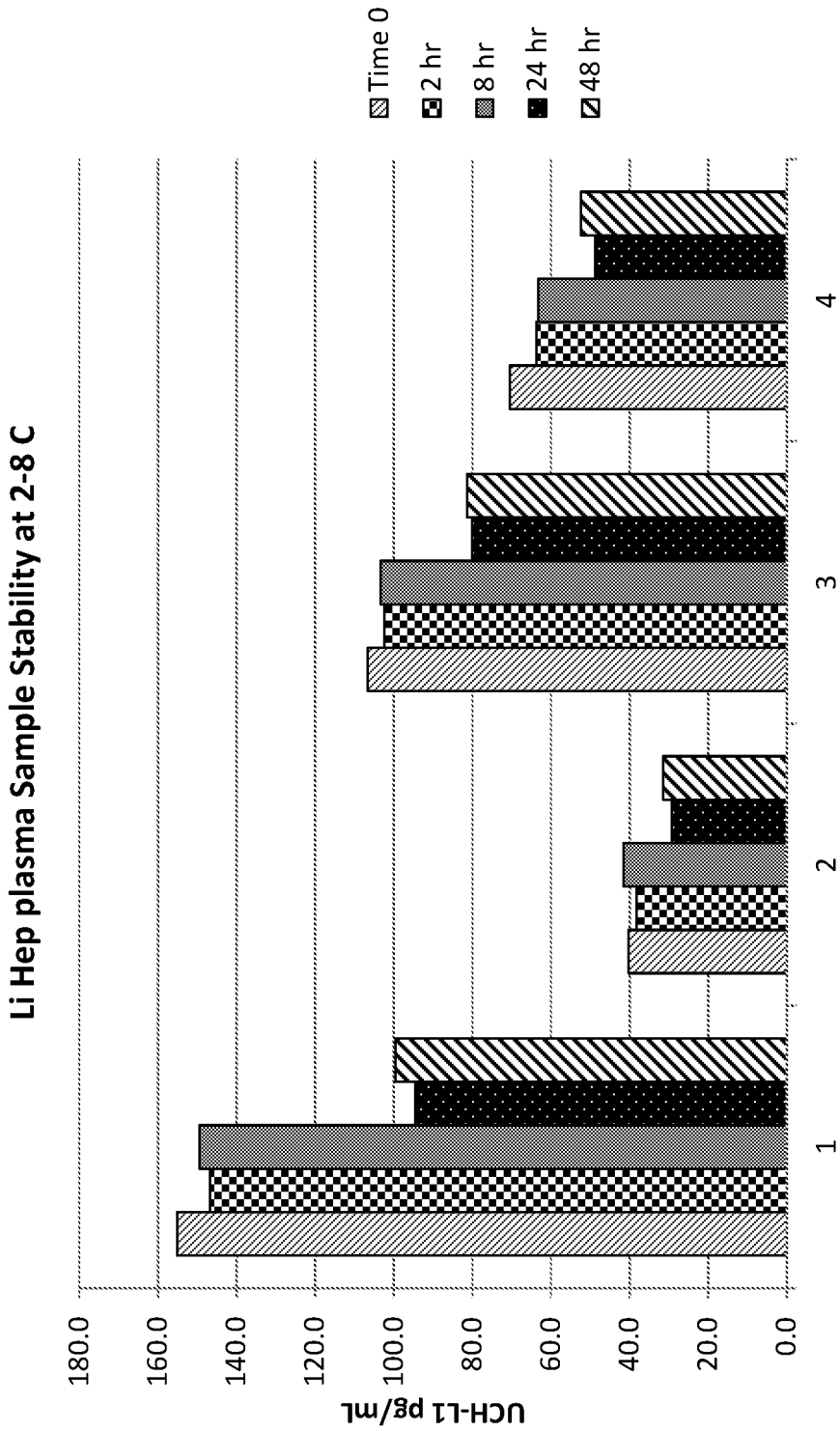
Li Hep plasma Sample Stability at 2-8 C
Time 0
2 hr
8 hr
24 hr
48 hr
UCH-L1 pg/mL
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1
2
3
4
Donor

FIG. 5B


Serum Sample Stability at RT
Time 0
2 hr
8 hr
24 hr
48 hr
UCH-L1 pg/mL
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1
2
3
4
Donor

FIG. 6A

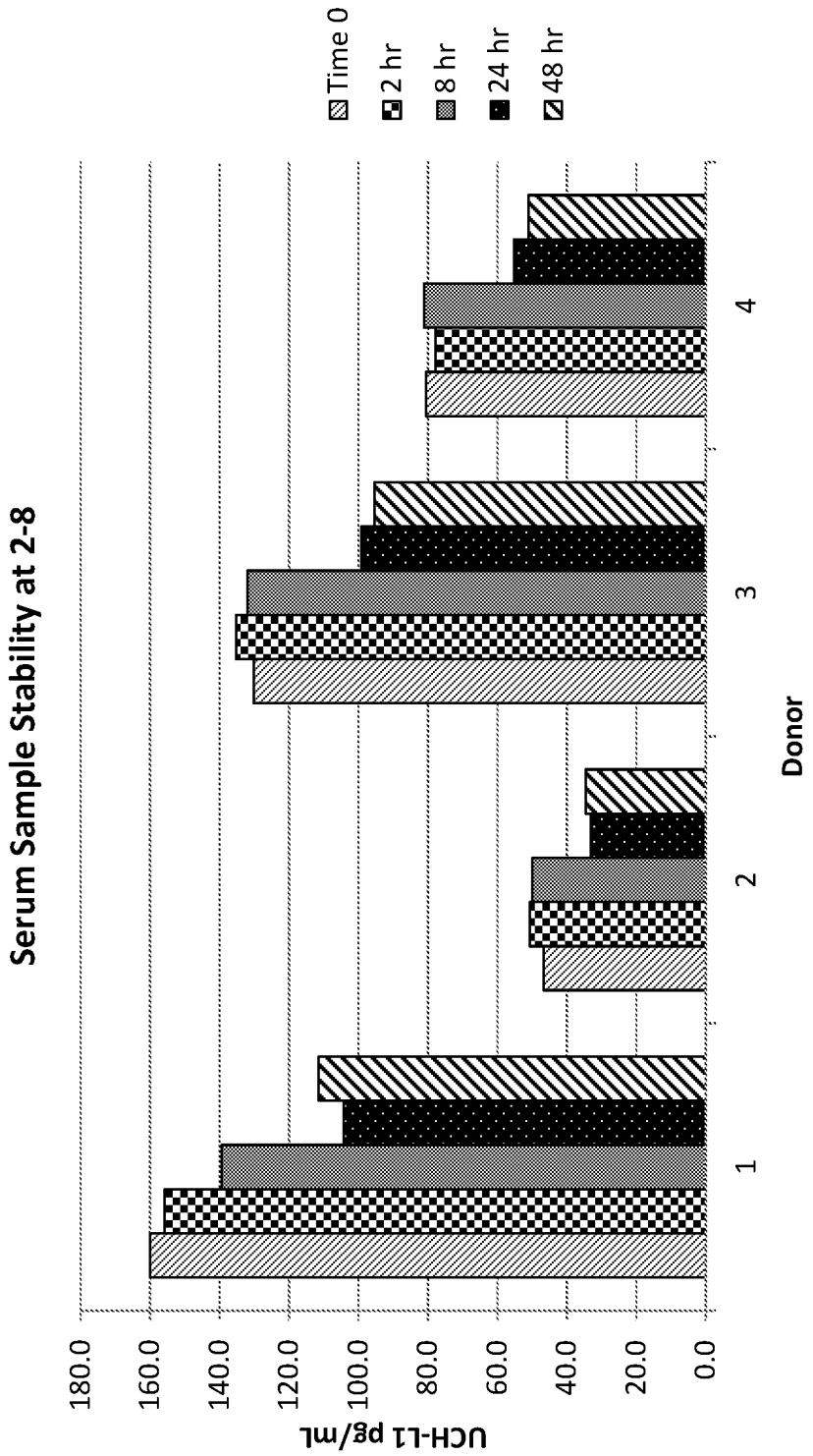
Serum Sample Stability at 2-8
Time 0
2 hr
8 hr
24 hr
48 hr
UCH-L1 pg/mL
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1
2
3
4
Donor

FIG. 6B

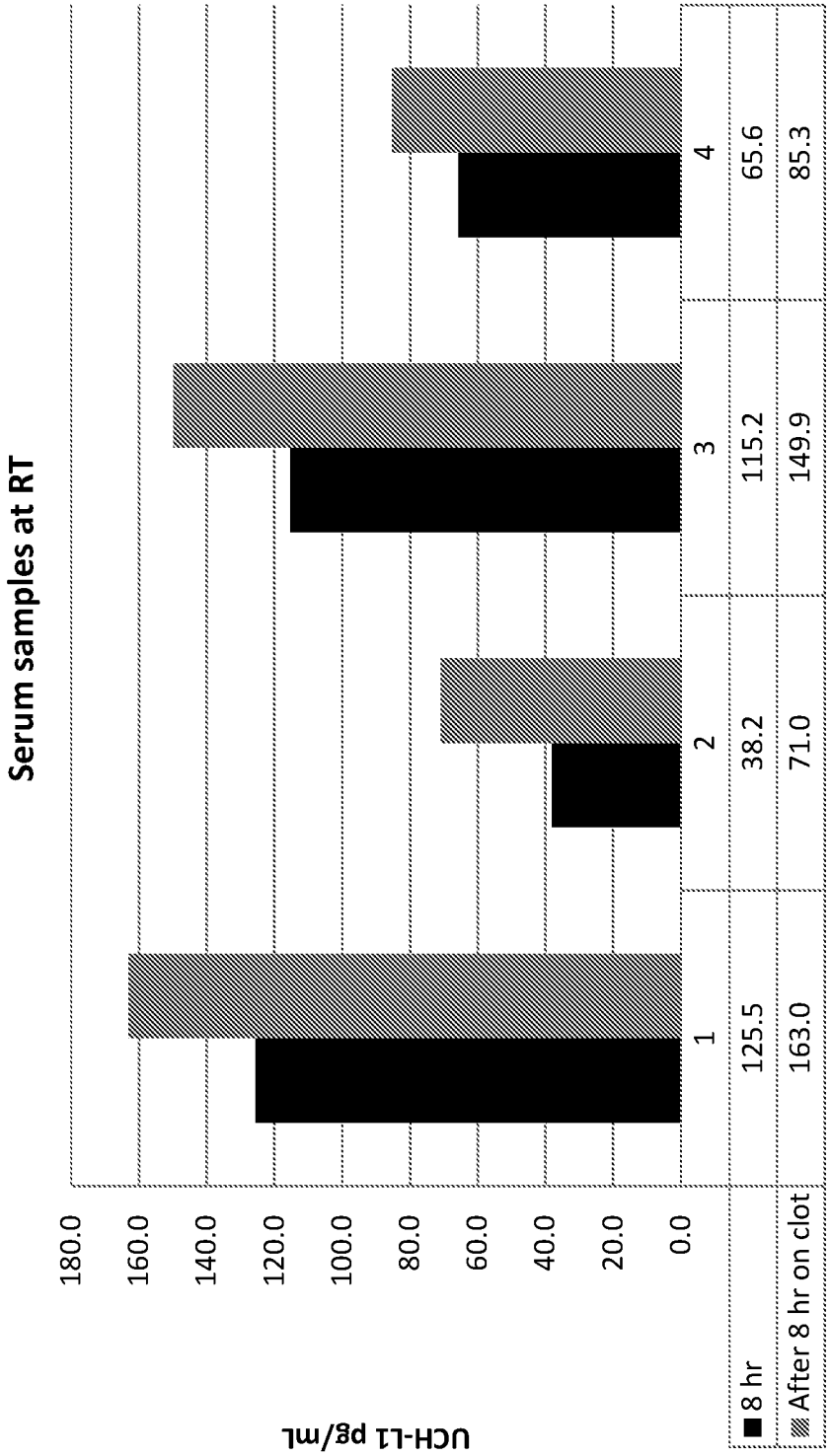
Serum samples at RT
UCH-L1 pg/mL
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
1
2
3
4
8 hr
125.5
38.2
115.2
65.6
After 8 hr on clot
163.0
71.0
149.9
85.3

FIG. 7

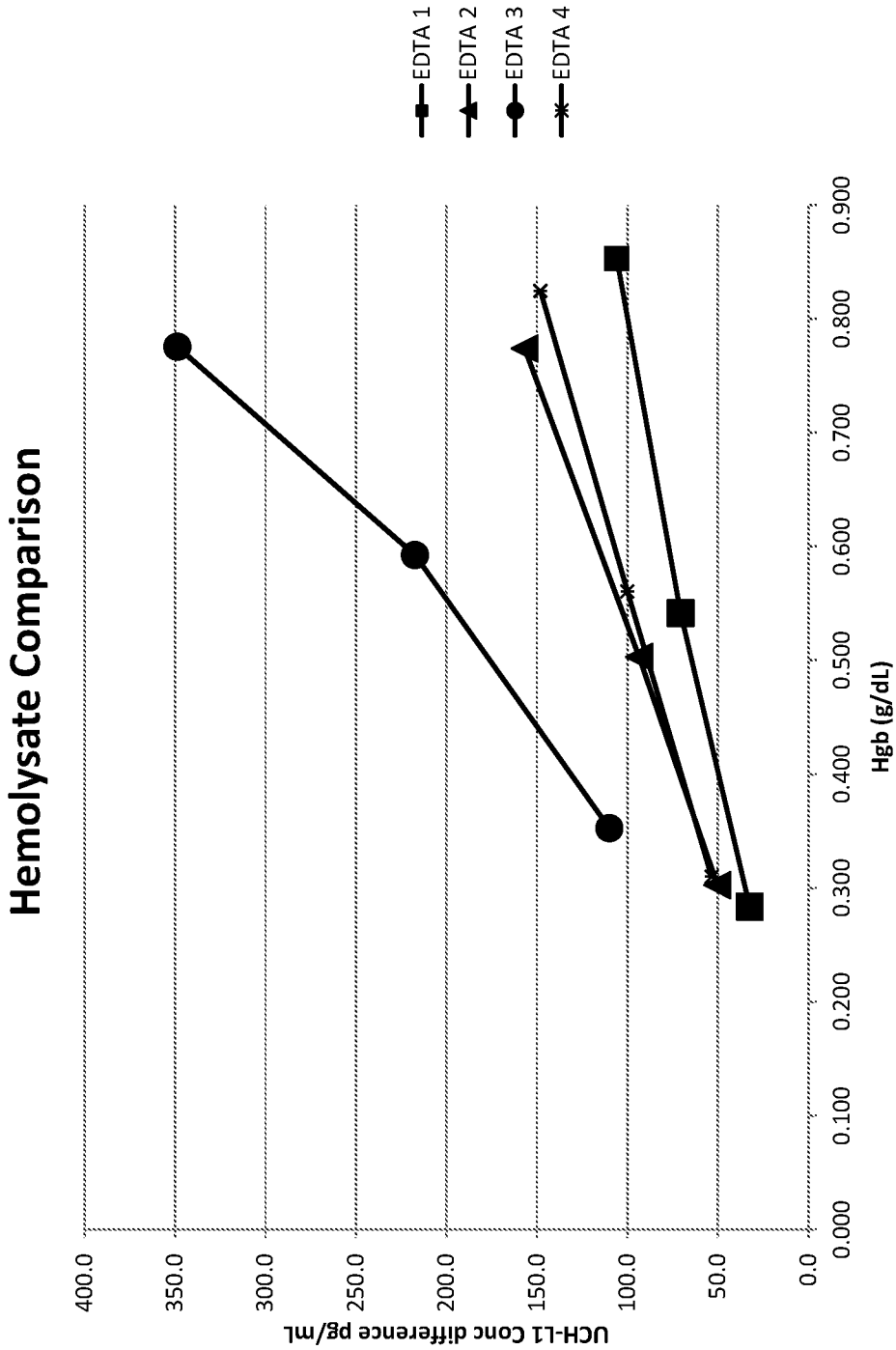
Hemolysate Comparison
EDTA 1
EDTA 2
EDTA 3
EDTA 4
UCH-L1 Conc difference pg/mL
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
0.000
0.100
0.200
0.300
0.400
0.500
0.600
0.700
0.800
0.900
Hgb (g/dL)

FIG. 8

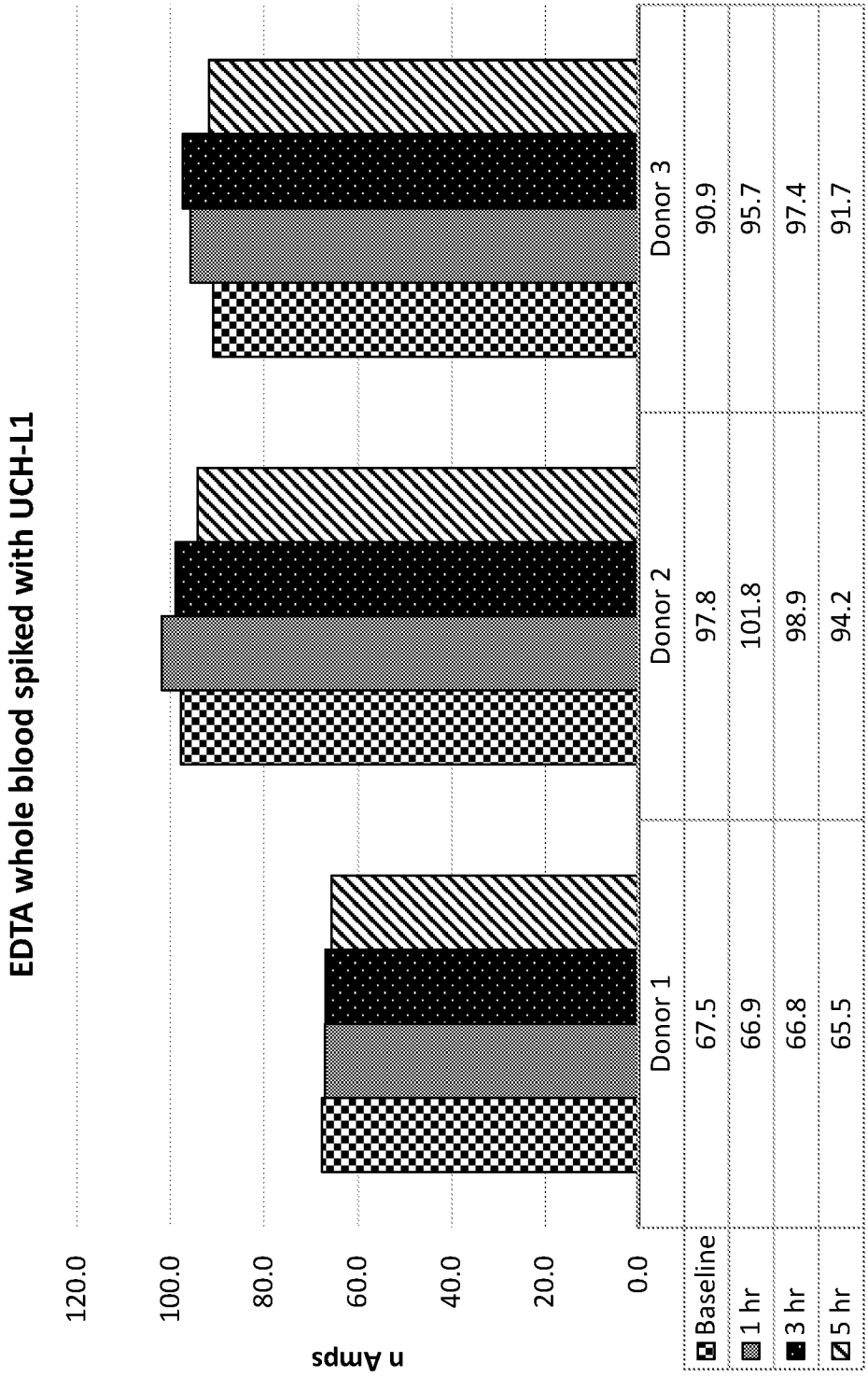
EDTA whole blood spiked with UCH-L1
n Amps
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Donor 1
Donor 2
Donor 3
Baseline
1 hr
3 hr
5 hr
67.5
66.9
66.8
65.5
97.8
101.8
98.9
94.2
90.9
95.7
97.4
91.7

FIG. 9

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/040612

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

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)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | TAKALA RIIKKA S K ET AL: "Glial Fibrillary Acidic Protein and Ubiquitin C-Terminal Hydrolase-L1 as Outcome Predictors in Traumatic Brain Injury", WORLD NEUROSURGERY, vol. 87, 8 March 2016 (2016-03-08), pages 8-20, XP029455539, ISSN: 1878-8750, DOI: 10.1016/J.WNEU.2015.10.066 page 9, column 2, paragraph 1 ----- -/-- | 1-35 |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 5 September 2018 | 17/09/2018 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Hinchliffe, Philippe |

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/040612

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | STEFANIA MONDELLO ET AL: "Serum Concentrations of Ubiquitin C-Terminal Hydrolase-L1 and Glial Fibrillary Acidic Protein after Pediatric Traumatic Brain Injury", SCIENTIFIC REPORTS, vol. 6, no. 1, 20 June 2016 (2016-06-20), XP055493324, DOI: 10.1038/srep28203 see materials and methods, biomarker measurement | 1-35 |
| X | GRETCHEN M. BROPHY ET AL: "Biokinetic Analysis of Ubiquitin C-Terminal Hydrolase-L1 (UCH-L1) in Severe Traumatic Brain Injury Patient Biofluids", JOURNAL OF NEUROTRAUMA., vol. 28, no. 6, 1 June 2011 (2011-06-01), pages 861-870, XP055486226, US ISSN: 0897-7151, DOI: 10.1089/neu.2010.1564 see section entitled "sample collection" | 1-35 |
| X | KIISKI HEIKKI ET AL: "Increased plasma UCH-L1 after aneurysmal subarachnoid hemorrhage is associated with unfavorable neurological outcome", JOURNAL OF NEUROLOGICAL SCIENCES, ELSEVIER SCIENTIFIC PUBLISHING CO, AMSTERDAM, NL, vol. 361, 29 December 2015 (2015-12-29), pages 144-149, XP029393288, ISSN: 0022-510X, DOI: 10.1016/J.JNS.2015.12.046 page 145, column 1, paragraph 5 | 1-35 |
| X | TANYA BOGOSLOVSKY ET AL: "Fluid Biomarkers of Traumatic Brain Injury and Intended Context of Use", DIAGNOSTICS, vol. 6, no. 4, 18 October 2016 (2016-10-18), page 37, XP055504281, DOI: 10.3390/diagnostics6040037 page 5/22, paragraph 2 | 1-35 |
| A | WO 2015/066211 A1 (BANYAN BIOMARKERS INC [US]) 7 May 2015 (2015-05-07) paragraph [0008] - paragraph [0009] | 1-35 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2018/040612

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 2015066211 A1 | 07-05-2015 | NONE | |