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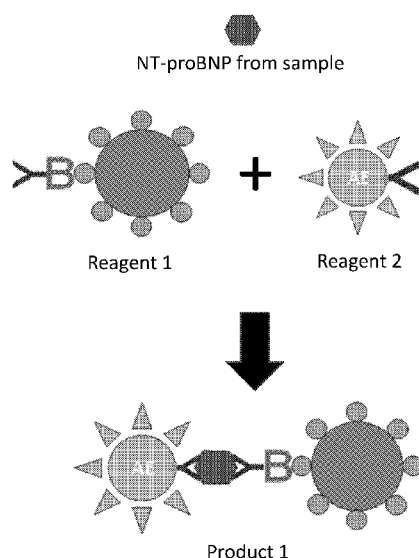


FIG. 3

(57) Abstract: Methods, assays, systems, and kits for determining the presence, severity, and/or predisposition of heart failure (HF) in an individual are disclosed. The methods utilize a solid substrate, wherein streptavidin-coated particles are pre-incubated with biotinylated antibody to form the solid phase reagent. Because the solid phase reagent is now presented with the biotinylated antibody in a pre-complexed form, there are fewer opportunities for biotin from the sample to interfere in the assay. The methods, systems, assays, and kits of the present disclosure allow the NT-proBNP assay to demonstrate no biotin interference up to a concentration of 3510 ng/mL biotin.

METHODS, REAGENTS, AND KITS FOR TUNING AN IMMUNOASSAY SIGNAL**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims the benefit of US Provisional Application No. 63/514,108, filed July 17, 2023. The entire contents of the above-referenced patent application(s) are hereby expressly incorporated herein by reference.

FIELD

[0002] The present disclosure is in the field of immunoassays and assay formats suitable for tuning a signal by altering the signal to noise ratio thereof. Further the present disclosure relates to stabilizing reagents and eliminating interference of free biotin in a biological sample. In certain non-limiting embodiments, the present disclosure tunes the signal to noise ratio of an NT-proBNP sandwich immunoassay suitable for use in a high-throughput analyzer.

BACKGROUND

[0003] Left ventricular dysfunction can occur as part of coronary heart disease, arterial hypertension, valvular disease, and primary myocardial disease. If the left ventricular dysfunction remains untreated and is progressive, the potential for mortality is high, for example, due to sudden cardiac death. Chronic cardiac insufficiency is a clinical syndrome caused by impairment of the cardiac pumping function. Based on the symptoms, the presence and severity of cardiac insufficiency may be classified in stages I–IV such as by the New York Heart Association (NYHA). Clinical tests and imaging procedures are used to diagnose left ventricular dysfunction.

[0004] Natriuretic peptides reported in the literature include atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). The significance of natriuretic peptides in the control of cardiovascular system function has been established. Brain natriuretic peptide biomarkers are commonly used to aid in diagnosis and assess severity in patients with heart failure (HF).

[0005] ANP and BNP have natriuretic and diuretic properties. As antagonists of the renin-angiotensin-aldosterone system, ANP and BNP influence the electrolyte and fluid balance in the body. In subjects with left ventricular dysfunction, serum and plasma concentrations of BNP increase, as do the concentrations of the biologically inactive prohormone, proBNP. ProBNP includes 108 amino acids. It is secreted mainly by the left ventricle of the heart and, in this process, is cleaved into physiologically active BNP (Amino

Acid 77–108), and the N-terminal fragment NT-proBNP (Amino Acid 1–76) (See e.g., Nadar SK, Shaikh MM. Biomarkers in Routine Heart Failure Clinical Care. *Card Fail Rev.* 2019;5(1):50-56. doi: 10.15420/cfr.2018.27.2).

[0006] Biomarkers such as NT-proBNP act as surrogates for clinically meaningful outcomes and may or may not reflect the pathogenesis underlying a disease. Examples of clinical utility include diagnosis, the prediction of disease progression or regression, and prognostication of mortality. A biomarker should be easily acquired, reliably measured, and available for serial monitoring. Ideally, it would also provide an advantage of currently used clinical measures in ease, timeframe, and/or expense.

[0007] Certain substances in a biological sample problematically interfere with an assay format for measuring a biomarker. It is well known that biotin present in a biological sample may bias assay results and/or interfere with an assay, for example, in assays where biotinylated antibody is a separate reagent from a solid phase that includes a biotin binding molecule, such as (but not limited to) unconjugated streptavidin-coated particles. The assay format problematically allows time for any biotin naturally present in a biological sample to complex with the streptavidin-coated particles, resulting in interference in which the biotinylated antibody is blocked from binding to the streptavidin-coated particles.

[0008] Prior art of interest includes U.S. Patent No. 8,252,605, entitled *Method and Composition For Stabilizing Liquid Reagents* to Janzen et al. (herein incorporated entirely by reference). However, the prior art is deficient in that it does not show improvement to reducing biotin interference as shown and described herein, especially to an NT-proBNP detecting immunoassay in accordance with the present disclosure.

[0009] Therefore, there is a continuing need in the art for improved assays such as NT-proBNP assays having improved performance where problematic free or natural biotin is or may be present in a biological sample at a concentration sufficient to cause interference. It is to such biomarker assay formats, as well as compositions/reagents/kits containing reagents for measuring a biomarker, along with methods of using same, that the present disclosure is directed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Embodiments of the present disclosure, briefly summarized above and discussed in greater detail below, can be understood by reference to the illustrative embodiments of the disclosure depicted in the appended drawings. However, the appended drawings

illustrate only typical embodiments of the disclosure and are therefore not to be considered limiting of scope, for the disclosure may admit to other equally effective embodiments.

[0011] FIG. 1 depicts a prior art immunoassay format for NT-proBNP. The prior art immunoassay utilizes three reagents: a streptavidin-coated particle (Reagent 1), a biotinylated antibody (Reagent 2), and an acridinium ester-labeled antibody (Reagent 3). The two antibodies bind to non-overlapping epitopes of NT-proBNP so that a sandwich complex can be formed (Product 1).

[0012] FIG. 2 depicts the effects of biotin interference on the prior art immunoassay of FIG. 1.

[0013] FIG. 3 depicts one non-limiting embodiment of an NT-proBNP immunoassay constructed in accordance with the present disclosure. In this embodiment, a solid phase reagent is produced before addition of the sample by pre-incubating streptavidin-coated particles with biotinylated antibody (or binding fragment thereof). Because the solid phase reagent is presented with the biotinylated antibody in a pre-complexed form, biotin interference from free or natural biotin in the sample is eliminated or substantially eliminated in the immunoassay.

[0014] FIG. 4 depicts an exemplary block diagram of a computer system 1100 suitable for executing the methods of the present disclosure on a chemical analyzer.

[0015] FIG. 5 graphically illustrates a receiver operating characteristic (ROC) curve demonstrating the clinical sensitivity and specificity of the immunoassay constructed in accordance with the present disclosure.

[0016] To facilitate understanding, identical reference numerals have been used, where possible, to designate identical elements that are common to the figures. The figures are not drawn to scale and may be simplified for clarity. Elements and features of one embodiment may be beneficially incorporated in other embodiments without further recitation.

DETAILED DESCRIPTION

[0017] Before explaining at least one embodiment of the present disclosure in detail by way of exemplary language and results, it is to be understood that the present disclosure is not limited in its application to the details of construction and the arrangement of the components set forth in the following description. The present disclosure is capable of other embodiments or of being practiced or carried out in various ways. As such, the language used

herein is intended to be given the broadest possible scope and meaning; and the embodiments are meant to be exemplary - not exhaustive. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

[0018] 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. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The foregoing techniques and procedures are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. The nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art.

[0019] All patents, published patent applications, and non-patent publications mentioned in the specification are indicative of the level of skill of those skilled in the art to which the present disclosure pertains. All patents, published patent applications, and non-patent publications referenced in any portion of this application are herein expressly incorporated by reference in their entirety to the same extent as if each individual patent or publication was specifically and individually indicated to be incorporated by reference.

[0020] All of the compositions, devices, kits, and/or methods disclosed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions, devices, kits, and/or methods have been described in terms of particular embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions, devices, kits, and/or methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit, and scope of the present disclosure. All such similar substitutions and modifications apparent to those skilled in the art are deemed to be within the spirit, scope, and concept of the present disclosure as defined by the appended claims.

Definitions

[0021] As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0022] The use of the term “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.” As such, the terms “a,” “an,” and “the” include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to “a compound” may refer to one or more compounds, two or more compounds, three or more compounds, four or more compounds, or greater numbers of compounds. The term “plurality” refers to “two or more.”

[0023] The use of the term “at least one” will be understood to include one as well as any quantity more than one, including but not limited to, 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, 100, etc. The term “at least one” may extend up to 100 or 1000 or more, depending on the term to which it is attached; in addition, the quantities of 100/1000 are not to be considered limiting, as higher limits may also produce satisfactory results. In addition, the use of the term “at least one of X, Y, and Z” will be understood to include X alone, Y alone, and Z alone, as well as any combination of X, Y, and Z.

[0024] The use of ordinal number terminology (i.e., “first,” “second,” “third,” “fourth,” etc.) is solely for the purpose of differentiating between two or more items and, unless explicitly stated otherwise, is not meant to imply any sequence or order or importance to one item over another or any order of addition, for example.

[0025] The use of the term “or” in the claims is used to mean an inclusive “and/or” unless explicitly indicated to refer to alternatives only or unless the alternatives are mutually exclusive. For example, a condition “A or B” is satisfied by any of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A and B are true (or present).

[0026] As used herein, any reference to “one embodiment,” “an embodiment,” “some embodiments,” “one example,” “for example,” or “an example” means that a particular element, feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment. The appearance of the phrase “in some embodiments” or “one example” in various places in the specification is not necessarily all referring to the same embodiment, for example. Further, all references to one or more embodiments or examples are to be construed as non-limiting to the claims.

[0027] Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for a composition/apparatus/ device, the method

being employed to determine the value, or the variation that exists among the study subjects. For example, but not by way of limitation, when the term “about” is utilized, the designated value may vary by plus or minus twenty percent, or fifteen percent, or twelve percent, or eleven percent, or ten percent, or nine percent, or eight percent, or seven percent, or six percent, or five percent, or four percent, or three percent, or two percent, or one percent from the specified value, as such variations are appropriate to perform the disclosed methods and as understood by persons having ordinary skill in the art.

[0028] The term “antibody” is used herein in the broadest sense and refers to, for example, intact monoclonal antibodies and polyclonal antibodies, multi-specific antibodies (e.g., bispecific antibodies), antibody fragments and conjugates thereof that exhibit the desired biological activity of analyte binding (such as, but not limited to, Fab, Fab', F(ab')₂, Fv, scFv, Fd, diabodies, single-chain antibodies, and other antibody fragments and conjugates thereof that retain at least a portion of the variable region of an intact antibody), antibody substitute proteins or peptides (i.e., engineered binding proteins/peptides), and combinations or derivatives thereof. The antibody can be of any type or class (e.g., IgG, IgE, IgM, IgD, and IgA) or sub-class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2).

[0029] The term “biomarker” or “biological marker” is used herein, consistent with its use in the art, to refer to an entity whose presence, level, or form correlates with a particular biological event or state of interest, so that it is considered to be a “marker” of that event or state. To give but a few examples, in some non-limiting embodiments, a biomarker may be or include a marker for a particular disease state, or for likelihood that a particular disease, disorder, or condition may develop, occur, or reoccur. In some non-limiting embodiments, a biomarker may be or include a marker for a particular disease or therapeutic outcome or likelihood thereof. Thus, in some non-limiting embodiments, a biomarker is predictive of the relevant biological event or state of interest; in some non-limiting embodiments, a biomarker is prognostic of the relevant biological event or state of interest; and in some non-limiting embodiments, a biomarker is diagnostic of the relevant biological event or state of interest. In some non-limiting embodiments, a biomarker is a possible biomarker of the relevant biological event or state of interest. A biomarker may be an entity of any chemical class. For example, in some non-limiting embodiments, a biomarker may be or include a nucleic acid, a polypeptide, a small molecule, or a combination thereof. In some non-limiting embodiments, a biomarker is a cell surface marker. In some non-limiting embodiments, a biomarker is

intracellular. In some non-limiting embodiments, a biomarker is found in a particular tissue (e.g., lung tissue). In some non-limiting embodiments, a biomarker is found outside of cells (e.g., is secreted or is otherwise generated or present outside of cells, e.g., in a body fluid such as blood, urine, tears, saliva, cerebrospinal fluid, etc.).

[0030] As described herein, in some non-limiting embodiments, a biomarker is an NT-proBNP Biomarker. An “NT-proBNP Biomarker” as used herein refers to a biological marker for Heart Failure (HF). In some non-limiting embodiments, one or more NT-proBNP Biomarkers include the N-terminal fragment NT-proBNP (Amino Acid 1–76) of proBNP. (See e.g., Nadar SK, Shaikh MM. Biomarkers in Routine Heart Failure Clinical Care. *Card Fail Rev.* 2019;5(1):50-56. doi: 10.15420/cfr.2018.27.2).

[0031] The term “characteristic fragment” refers to a fragment of a biomarker (e.g., NT-proBNP Biomarker) that is sufficient to identify the biomarker from which the fragment was derived. For example, in some non-limiting embodiments, a “characteristic fragment” of a biomarker is one that contains an amino acid sequence, or a collection of amino acid sequences, that together allow for the biomarker from which the fragment was derived to be distinguished from other possible biomarkers, proteins, or polypeptides. In some non-limiting embodiments, a characteristic fragment includes at least 10, at least 20, at least 30, at least 40, or at least 50 amino acids. In certain non-limiting embodiments, a characteristic fragment refers to a fragment of a biomarker that has at least 90%, at least 95%, at least 99% sequence identity to the biomarker from which the characteristic fragment was derived.

[0032] The term “hybridization” refers to the physical property of single-stranded nucleic acid molecules (e.g., DNA or RNA) to anneal to complementary nucleic acid molecules. Hybridization can typically be assessed in a variety of contexts – including where interacting nucleic acid molecules are studied in isolation or in the context of more complex systems (e.g., while covalently or otherwise associated with a carrier entity and/or in a biological system or cell). In some non-limiting embodiments, hybridization can be detected by a hybridization technique, such as a technique selected from the group consisting of *in situ* hybridization (ISH), microarray, Northern blot, Southern blot, and the like. In some non-limiting embodiments, hybridization refers to 100% annealing between the single-stranded nucleic acid molecules and the complementary nucleic acid molecule. In some non-limiting embodiments, annealing is less than 100% (e.g., at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70% of a single-stranded nucleic acid molecule anneals to a

complementary nucleic acid molecule). Hybridization techniques, and methods for evaluating hybridization, are well known in the art. See, e.g., Sambrook, et al., 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Plainview, N.Y. Those skilled in the art understand how to estimate and adjust the stringency of hybridization conditions such that sequences having at least a desired level of complementarity will stably hybridize, while those having lower complementarity will not. For examples of hybridization conditions and parameters, see, e.g., Sambrook, et al., 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Press, Plainview, N.Y.; Ausubel, F. M. et al. 1994, *Current Protocols in Molecular Biology*. John Wiley & Sons, Secaucus, N.J.

[0033] The term “detection agent” as used herein refers to any element, molecule, functional group, compound, fragment, or moiety that is detectable. In some non-limiting embodiments, a detection agent is provided or utilized alone. In some non-limiting embodiments, a detection agent is provided and/or utilized in association with (e.g., joined to) another agent. Examples of detection agents include, but are not limited to: various ligands, radionuclides (e.g., ^3H , ^{14}C , ^{18}F , ^{19}F , ^{32}P , ^{35}S , ^{135}I , ^{125}I , ^{123}I , ^{64}Cu , ^{187}Re , ^{111}In , ^{90}Y , $^{99\text{m}}\text{Tc}$, ^{177}Lu , ^{89}Zr etc.), fluorescent dyes, chemiluminescent agents (such as, for example, acridinum esters, stabilized dioxetanes, and the like), bioluminescent agents, spectrally resolvable inorganic fluorescent semiconductors, nanocrystals (i.e., quantum dots), metal nanoparticles (e.g., gold, silver, copper, platinum, etc.), nanoclusters, paramagnetic metal ions, enzymes, colorimetric labels (such as, for example, dyes, colloidal gold, and the like), biotin, dioxigenin, haptens, and proteins for which antisera or monoclonal antibodies are available.

[0034] As used herein, the term “diagnostic test” refers to a step or series of steps that is or has been performed to attain information that is useful in determining whether a patient has a disease, disorder, or condition and/or in classifying a disease, disorder, or condition into a phenotypic category or any category having significance with regard to prognosis of a disease, disorder, or condition, or likely response to treatment (either treatment in general or any particular treatment) of a disease, disorder, or condition. Similarly, the term “diagnosis” refers to providing any type of diagnostic information, including, but not limited to, whether a subject is likely to have or develop a disease, disorder, or condition; state, staging, or characteristic of a disease, disorder, or condition as manifested in the subject; information related to the nature or classification of a condition such as a tumor or a heart condition; information related to prognosis; and/or information useful in selecting an

appropriate treatment or additional diagnostic testing. Selection of treatment may include the choice of a particular therapeutic agent or other treatment modality such as surgery, radiation, etc., a choice about whether to withhold or deliver therapy, a choice relating to dosing regimen (e.g., frequency or level of one or more doses of a particular therapeutic agent or combination of therapeutic agents), etc. Selection of additional diagnostic testing may include more specific testing for a given disease, disorder, or condition.

[0035] As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”), or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps. For example, a process, method, article, or apparatus that comprises a list of elements is not necessarily limited to only those elements but may include other elements not expressly listed or inherently present therein.

[0036] The term “or combinations thereof” as used herein refers to all permutations and combinations of the listed items preceding the term. For example, “A, B, C, or combinations thereof” is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, AAB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

[0037] As used herein, the term “substantially” means that the subsequently described event or circumstance completely occurs or that the subsequently described event or circumstance occurs to a great extent or degree. For example, when associated with a particular event or circumstance, the term “substantially” means that the subsequently described event or circumstance occurs at least 80% of the time, or at least 85% of the time, or at least 90% of the time, or at least 95% of the time. The term “substantially adjacent” may mean that two items are 100% adjacent to one another, or that the two items are within close proximity to one another but not 100% adjacent to one another, or that a portion of one of the two items is not 100% adjacent to the other item but is within close proximity to the other item.

[0038] As used herein, the phrase “associated with” includes both direct association of two moieties to one another as well as indirect association of two moieties to one another. Non-limiting examples of associations include covalent binding of one moiety to another moiety either by a direct bond or through a spacer group, non-covalent binding of one moiety to another moiety either directly or by means of specific binding pair members bound to the moieties, incorporation of one moiety into another moiety such as by dissolving one moiety in another moiety or by synthesis, and coating one moiety on another moiety.

[0039] The term “biological fluid sample” as used herein will be understood to include any liquid test sample that may be obtained from a patient and utilized in accordance with the present disclosure. Examples of biological fluid samples that may be utilized include, but are not limited to, whole blood or any portion thereof (i.e., plasma or serum), serum, EDTA plasma, lithium heparin plasma, combinations thereof, and the like.

[0040] As used herein, the term “volume” as it relates to the liquid test samples utilized in accordance with the present disclosure typically refers to a volume of liquid test sample in a range of from about 0.1 μ l to about 100 μ l, or a range of from about 1 μ l to about 75 μ l, or a range of from about 2 μ l to about 60 μ l, or a value less than or equal to about 50 μ l, or the like.

[0041] The term “patient” as utilized herein includes human and veterinary subjects. In certain non-limiting embodiments, a patient is a mammal. In certain other non-limiting embodiments, the patient is a human. The term “mammal” for purposes of diagnosis/treatment refers to any animal classified as a mammal, including human, domestic and farm animals, nonhuman primates, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc.

[0042] A “health care provider” or “health care decision maker” includes any individual authorized to diagnose or treat a patient, or to assist in the diagnosis or treatment of a patient. In the context of identifying useful new drugs to treat a particular disease, disorder, or condition, a health care provider can be an individual who is not authorized to diagnose or treat a patient, or to assist in the diagnosis or treatment of a patient.

[0043] “Point of care testing” refers to real time diagnostic testing that can be done in a rapid time frame so that the resulting test is performed faster than comparable tests that do not employ this system. Point of care testing can be performed rapidly and on site, such as in a doctor's office, at a bedside, in a stat laboratory, emergency room, or other such locales,

particularly where rapid and accurate results are required. The patient can be present, but such presence is not required. Point of care includes, but is not limited to: emergency rooms, operating rooms, hospital laboratories and other clinical laboratories, doctor's offices, in the field, or in any situation in which a rapid and accurate result is desired.

[0044] The term “specific binding partner,” as used in particular (but not by way of limitation) herein in the terms “target analyte-specific binding partner” or “biotin-specific binding partner,” will be understood to refer to any molecule capable of specifically associating with the target analyte. For example, but not by way of limitation, the binding partner may be an antibody, a receptor, a ligand, aptamers, molecular imprinted polymers (i.e., inorganic matrices), combinations or derivatives thereof, as well as any other molecules capable of specific binding to the target analyte.

[0045] The term “immunoassay” as utilized herein refers to an assay to determine the presence of a diagnostic biomarker in a biological sample by reacting the sample with an antibody (or fragment thereof) that specifically binds to the diagnostic biomarker or characteristic fragment thereof, wherein the reaction is carried out for a time and under conditions that allow for the formation of an immunocomplex between the antibody (or fragment thereof) and the diagnostic biomarker. The quantitative determination of such an immunocomplex is then performed.

[0046] Sample: As used herein, the term “sample” refers to a biological sample obtained or derived from a human subject, as described herein. In some embodiments, a biological sample includes biological tissue or fluid. In some embodiments, a biological sample may include blood; blood cells; tissue or fine needle biopsy samples; cell-containing body fluids; free floating nucleic acids; cerebrospinal fluid; lymph; tissue biopsy specimens; surgical specimens; other body fluids, secretions, and/or excretions; and/or cells therefrom. In some embodiments, a biological sample includes cells obtained from an individual, e.g., from a human or animal subject. In some embodiments, obtained cells are or include cells from an individual from whom the sample is obtained. In some embodiments, a sample is a “primary sample” obtained directly from a source of interest by any appropriate means. For example, in some embodiments, a primary biological sample is obtained by methods selected from the group consisting of biopsy (e.g., fine needle aspiration or tissue biopsy), surgery, collection of body fluid (e.g., blood). In some embodiments, a sample is cardiac tissue obtained from the subject. In some embodiments, as will be clear from context, the term “sample” refers to a

preparation that is obtained by processing (e.g., by removing one or more components of and/or by adding one or more agents to) a primary sample. For example, filtering using a semi-permeable membrane. As another example of sample processing, the sample may be a plasma sample that is treated with an anticoagulant selected from the group consisting of EDTA, heparin, and citrate. As another example of sample processing, the sample may be processed to isolate one or more proteins (e.g., by capturing proteins with one or more antibodies). A “processed sample” may include, for example, nucleic acids or polypeptides extracted from a sample or obtained by subjecting a primary sample to techniques such as amplification or reverse transcription of mRNA, isolation and/or purification of certain components.

[0047] Subject: As used herein, the term “subject” refers to an organism, for example, a mammal (e.g., a human). In some embodiments a human subject is an adult, adolescent, or pediatric subject. In some embodiments, a subject is at least 50, at least 55, at least 60, at least 65, at least 70, at least 75, or at least 80 years of age. In some embodiments, a subject is suffering from a disease, disorder or condition, *e.g.*, a disease, disorder or condition that can be treated as provided herein. In some embodiments, a subject is susceptible to a disease, disorder, or condition; in some embodiments, a susceptible subject is predisposed to and/or shows an increased risk (as compared to the average risk observed in a reference subject or population) of developing the disease, disorder or condition. In some embodiments, a subject displays one or more symptoms of a disease, disorder or condition. In some embodiments, a subject does not display a particular symptom (e.g., clinical manifestation of disease) or characteristic of a disease, disorder, or condition. In some embodiments, a subject does not display any symptom or characteristic of a disease, disorder, or condition such as heart failure. In some embodiments, a subject is a patient. In some embodiments, a subject is an individual to whom diagnosis and/or therapy is and/or has been administered.

[0048] Threshold value: As used herein, the term “threshold value” refers to a value (or values) that are used as a reference to attain information on and/or classify the results of a measurement, for example, the results of a measurement attained in an assay. A threshold value can be determined based on one or more control samples. A threshold value can be determined prior to, concurrently with, or after the measurement of interest is taken. In some embodiments, a threshold value can be a range of values. In some embodiments, a

threshold value can be a value (or range of values) reported in the relevant field (e.g., a value found in a standard table).

[0049] The term “stratification” refers to the division of potential patient groups or patient groups into subgroups such as strata or blocks. In embodiments, “strata” refers to a particular section of a patient population.

[0050] Turning now to various non-limiting embodiments of the present disclosure, improved biomarker assay formats, as well as compositions/reagents/kits containing reagents for measuring a biomarker, along with methods of using same, are described herein. In certain non-limiting embodiments, the present disclosure provides improved assays, such as (but not limited to) improved NT-proBNP assays, having improved performance where problematic free or natural biotin is or may be present in a biological sample and can cause interference in existing assay formats. In embodiments, biomarker assay formats are formulated to tune signal from a signal moiety.

[0051] In certain non-limiting embodiments, the assay format includes one or more streptavidin-coated solid supports (such as but not limited to one or more bead substrates or particles) that have been pre-incubated with one or more biotinylated antibodies (or binding fragments thereof) to form one or more solid phase reagents suitable for use in an immunoassay while eliminating or substantially eliminating interference of free biotin in a biological sample. In certain non-limiting embodiments, the present disclosure tunes the signal to noise ratio of an NT-proBNP sandwich immunoassay suitable for use in a high-throughput analyzer. In certain non-limiting embodiments, because the solid phase reagent is presented with the biotinylated antibody in a pre-complexed form, there are substantially no opportunities for free or natural biotin from the sample to interfere in the assay. In certain non-limiting embodiments, such as for an NT-proBNP analyte, “no interference to biotin” is defined as a percent bias of $\leq 10\%$. In certain non-limiting embodiments, the present disclosure allows the NT-proBNP assay to claim no interference up to 3510 ng/mL biotin.

[0052] In certain non-limiting embodiments, the present immunoassay may be run on a high-throughput chemical analyzer capable of running more than 20, 30, 40, 50, 75, 100, or more tests per hour. In certain non-limiting embodiments, the present disclosure also includes non-transient computer readable media suitable for executing the methods of the present disclosure on a chemical analyzer.

[0053] In certain particular (but non-limiting) embodiments, the immunoassays may detect a complex between a serum marker or characteristic fragment thereof and a serum marker-binding antibody using a second antibody that is labeled and also binds to the serum marker or fragment thereof. In certain non-limiting embodiments, sandwich immunoassays are used where a serum marker-binding antibody can be a capture antibody attached to an insoluble material (such as, but not limited to, a magnetic bead), and the second antibody can be a labeling antibody. The above-described sandwich immunoassay procedures can be used with the antibodies described hereinafter.

[0054] Turning now to particular non-limiting embodiments of the present disclosure, embodiments of the present disclosure include an assay format suitable for detecting individual biomarkers such as NT-proBNP or a characteristic fragment thereof, as well as compositions/devices/kits containing same, methods of producing and using same, kits, and diagnostic tests related thereto. Certain non-limiting embodiments of the present disclosure include a preselected assay format for detecting NT-proBNP in serum. Certain non-limiting embodiments of the present disclosure advantageously reduce, eliminate, or substantially eliminate biotin interference in a reaction. In certain non-limiting embodiments, a preselected assay format advantageously limits biotin from being associated with reagents of the assay.

[0055] Certain non-limiting embodiments of the present disclosure are directed to a method of determining the presence, severity, and/or predisposition of heart failure in an individual using a single biomarker. In certain non-limiting embodiments, the present disclosure includes a method having a process sequence including: (a) forming a solid phase immunoassay reagent by contacting one or more streptavidin-coated particles with one or more biotinylated antibodies to form a complex; and (b) separating the solid phase immunoassay reagent from an immunoassay lite reagent and storing or packaging the solid phase reagent separate from the lite reagent. In certain non-limiting embodiments, because a solid phase reagent is presented with the biotinylated antibody in a pre-complexed form, there are substantially no opportunities for biotin from the biological fluid sample to interfere in the assay. For NT-proBNP analyte, “no interference to biotin” is defined as a percent bias of $\leq 10\%$. In certain non-limiting embodiments of the present disclosure, the term “no biotin interference” includes no interference up to a concentration of 3510 ng/mL biotin.

[0056] The present disclosure facilitates diagnosis, such as point of care or remote diagnoses, of HF and assists health care providers in monitoring the status or progress of HF at two or more time points. Embodiments of the present disclosure are suitable for use in an outpatient setting, such as where a patient in need thereof provides a biological sample from , or utilizes one or more embodiments of the present disclosure as part of an ambulatory care strategy, such as where medical service embodiments of the present disclosure do not require hospitalization, or wherein patients are free to leave a medical facility once a service or procedure of the present disclosure is complete. Non-limiting examples of outpatient care include home health services, annual check-ups at a physician's office or non-admitting clinic settings such as medical offices, clinics, ambulatory surgery centers, hospital outpatient departments and other non-hospital centers. In some embodiments, embodiment of the present disclosure are suitable for use by a user in home ambulatory care to promote patient independence, permit a transition from hospital to home care, or as part of a home monitoring strategy e.g., cardiac monitoring. In embodiments, the assays of the present disclosure can be applied in a single occurrence in an outpatient care setting such as an outpatient facility where the patient does not need to be present for more than 1-24 hours, 24 hours, or 12 hours.

[0057] In embodiments, a patient in need thereof will provide a biological sample such as blood or serum from an outpatient setting. The biological sample may be shared with a facility suitable for performing one or more assays or embodiments of the present disclosure in order to analyze the patient health information such as cardiac information. In embodiments, after sample collection, chemical reactions between an analyte in a patient's biological sample and reagents are contacted under conditions suitable for performing the assays of the present disclosure and resulting in the generation of various signals, or tuned signals that measured by an analyzer.

[0058] Also provided by the present disclosure are kits including one or more anti-HF Biomarker agents and instructions for use (e.g., treatment, prophylactic, or diagnostic use). In some non-limiting embodiments, the kit is used for an in vitro diagnostic assay to diagnose HF. In some non-limiting embodiments, the one or more anti-HF Biomarker agents include antibody agents. In some non-limiting embodiments, one or more of the antibody agents are labeled with a detectable moiety. In some non-limiting embodiments, the kit further includes a detection agent (e.g., one or more acridinium ester molecules). In some non-limiting

embodiments, one or more of the antibody agents are labeled with one or more of the acridinium ester molecules. In some non-limiting embodiments, the kit further includes one or more secondary antibody agents that specifically bind to one or more of the anti-HF Biomarker antibody agents. Additional kit embodiments are described in the Examples below.

[0059] In certain non-limiting embodiments, the instructions for use provide a method for use suitable for an ATELLICA® CI Analyzer (Siemens Healthineers USA, Malvern, PA), e.g., 1900 model.

[0060] In some non-limiting embodiments, the kit further includes one or more control samples. In some non-limiting embodiments, the control samples include one or more HF Biomarker standards.

[0061] In addition to the above, a kit can include other ingredients, such as (but not limited to) a solvent or buffer, a stabilizer or a preservative, and/or an agent for treating a condition or disorder described herein. Alternatively, other ingredients can be included in a kit, but in different compositions or containers than the anti-HF Biomarker agents. In such embodiments, a kit can include instructions for admixing the anti-HF Biomarker agents and the other ingredients, or for using the anti-HF Biomarker together with the other ingredients. In certain non-limiting embodiments, the instructions provided eliminate time for any biotin in the sample to complex with the streptavidin-coated particles and/or interfere with the biotin labeled antibodies from becoming associated with the streptavidin-coated particles. In certain non-limiting embodiments, the instructions include using the streptavidin-coated particles characterized as pre-incubated with the biotinylated antibody and are in the form a solid phase reagent.

[0062] In certain non-limiting embodiments, kits for use in accordance with the present disclosure may include a reference or control sample(s), instructions for processing samples, instructions for performing tests on samples, and/or instructions for interpreting the results, as well as buffers and/or other reagents necessary for performing tests.

[0063] Methods and kits provided herein detect NT-proBNP in a sample with a sensitivity and a specificity that renders the outcome of the test reliable enough to be medically actionable. Methods and kits described herein for detection and/or diagnosis of HF in a subject detects NT-proBNP with a sensitivity greater than 75%, greater than 80%, greater than 85%, greater than 90%, greater than 95%, greater than 96%, greater than 97%, greater than 98%, greater than 99%, or about 100%. In some non-limiting embodiments, methods and kits

provided herein can detect NT-proBNP with a sensitivity that is between about 70%-100%, between about 80%-100%, or between about 90-100%. In some non-limiting embodiments, methods and kits provided herein can detect NT-proBNP with a sensitivity and a specificity that is between about 50%-100%, between about 60%-100%, between about 70%-100%, between about 80%-100%, or between about 90-100%.

[0064] Also provided herein are compositions. In some non-limiting embodiments, a composition includes one or more biotinylated antibodies associated with one or more streptavidin-coated solid supports (such as, but not limited to, streptavidin-coated beads or particles). In certain non-limiting embodiments, the assay format includes one or more streptavidin-coated particles such as one or more bead substrates having been pre-incubated with one or more biotinylated antibodies to form one or more solid phase reagents suitable for use in eliminating or substantially eliminating interference of free biotin in a biological fluid sample. In certain non-limiting embodiments, the present disclosure tunes the signal to noise ratio of an NT-proBNP sandwich immunoassay suitable for use in a high-throughput analyzer. In certain non-limiting embodiments, because the solid phase reagent is presented with the biotinylated antibody in a pre-complexed form, there are substantially no opportunities for free or natural biotin from the sample to interfere in the assay when the reagents are mixed with sample. In certain non-limiting embodiments, such as for an NT-proBNP analyte, “no interference to biotin” is defined as a percent bias of $\leq 10\%$. In certain non-limiting embodiments, the present disclosure allows the NT-proBNP assay to claim no interference up to 3510 ng/mL biotin.

[0065] In certain non-limiting embodiments, the present immunoassay may be run on a high-throughput chemical analyzer capable of running more than 20, 30, 40, 50, 75, or 100 tests per hour. In certain non-limiting embodiments, the present immunoassay may be run, for example, on an ATELLICA® CI Analyzer (Siemens Healthineers USA, Malvern, PA), e.g., 1900 model.

[0066] In one form of a diagnostic method, an immunoassay, one or more specific binding species are used. Typical examples are the sandwich immunoassay, where two specific binding species (antibody or antigen) bind to non-overlapping epitopes of the analyte of interest. One of the specific binding species is commonly attached to a so-called label or tag, which may be an atom (e.g., radioactive), molecule (e.g., an enzyme, fluorescent, or

luminescent compound) or particle (magnetic or latex). This label allows for detection of the analyte of interest through a variety of detection methods corresponding to the label utilized.

[0067] The other specific binding species is frequently associated with a solid or suspendable substrate ("the solid phase") covalently or through adsorption. Alternatively, it may be linked to a first member of a second binding pair (e.g., biotin), while the second member of the second binding pair (e.g., streptavidin) is attached to the solid phase. This allows the specific binding species to bind to the solid phase via the second binding pair interaction (e.g., biotin-streptavidin).

[0068] Solid phases may be macroscopic solid phases, such as (but not limited to) microtiter well, tube and ball in tube devices, or suspendable solid phases, such as (but not limited to) beads, latex beads, magnetic latex beads, and the like, as well as other paramagnetic materials. A secondary binding species is commonly labeled through the use of a tag. The interaction between the tag and the solid phase allows for detection and quantification of the analyte of interest through a variety of detection methods corresponding to the label utilized.

[0069] FIGS. 1-2 demonstrate a prior art sandwich immunoassay for an analyte such as (but not limited to) NT-proBNP, along with the effects of biotin interference thereon. As seen in FIG. 1, the prior art assay utilizes three reagents. Reagent 1 is a streptavidin-coated particle (such as, but not limited to, a streptavidin-coated magnetic bead); Reagent 2 is a biotinylated first antibody; and Reagent 3 is an acridinium-labeled second antibody. The two antibodies of Reagents 2 and 3 bind to non-overlapping epitopes of NT-proBNP so that both antibodies can bind to a single molecule of NP-proBNP. When the three reagents are mixed with a biological sample suspected of containing NT-proBNP (such as, but not limited to, plasma or serum), the two antibodies bind to NP-proBNP, and the biotinylated antibody binds to the streptavidin of the particles to form Reaction Product 1, in which the acridinium ester is indirectly associated with the solid phase particle via the binding of the two antibodies to NT-proBNP. Thus, the Reaction Product 1 can be detected via the signal generated by the acridinium ester associated with the solid phase.

[0070] However, when free biotin is present in the biological sample, as shown in FIG. 2, the free biotin competes with the biotinylated antibody for binding to the streptavidin-coated particles. As such, three reaction products are produced: the detectable Reaction Product 1, along with Reaction Product 2 that contains a NP-proBNP molecule bound by both antibodies

(and thus contains the acridinium ester), and a Reaction Product 3 that contains the free biotin bound to the streptavidin-coated particles. Therefore, a falsely low (or false negative) measurement is determined, as a portion of the NT-proBNP present in the sample is not present in Product 1 and therefore is not detected, because the acridinium ester label associated with the two antibodies and NT-proBNP analyte (Product 2) cannot associate with the streptavidin-coated particle (Product 3) for detection of the label.

[0071] In contrast, FIG. 3 illustrates a non-limiting embodiment of a kit, system, and method for performing an immunoassay for a target analyte (such as, but not limited to, NT-proBNP) in accordance with the present disclosure. In this embodiment, only two reagents are utilized: Reagent 1 is the streptavidin-coated particle having the biotinylated first antibody pre-bound thereto, and Reagent 2 is the acridinium-labeled second antibody. Use of these two reagents ensures that free biotin present in the sample does not interfere or substantially interfere with the formation of Product 1, in which the binding of NT-proBNP by the two antibodies associates the acridinium ester label with the solid phase particles.

[0072] In certain particular (but non-limiting) embodiments, the use of streptavidin-coated magnetic beads provides a fast magnetic response time and is suitable for use in high throughput and multiplexed assays. The streptavidin coating is contacted with one or more biotinylated antibodies under conditions suitable to associate the streptavidin coating and biotinylated antibodies. Therefore, the immunoassay of the present disclosure can be a fully automated 2-site sandwich immunoassay using direct chemiluminescent technology, which uses constant or substantially constant amounts of two monoclonal antibodies. The Solid Phase contains a bound biotinylated monoclonal sheep anti-human antibody specific to NT-proBNP and conjugated to streptavidin magnetic particles. The Lite Reagent contains an acridinium-ester-labeled monoclonal sheep anti-human NT-proBNP F(ab')₂ fragment specific to NT-proBNP. In certain non-limiting embodiments, a direct relationship exists between the amount of NT-proBNP present in the patient sample and the amount of relative light units (RLUs) detected by the system.

[0073] Methods described herein can be implemented in a computer system having a processor that executes specific instructions in a computer program. In some non-limiting embodiments, a computer system may be arranged to output an HF Biomarker score based on receiving an HF Biomarker profile and/or a level of NT-proBNP Biomarkers. Particularly, a

computer program may include instructions for the system to select appropriate next steps, including additional medication, a treatment, and/or additional testing for a subject.

[0074] In some non-limiting embodiments, the computer program may be configured such that the computer system can identify a subject for further testing (e.g., heart tests), identify a subject as being at risk of having or having HF, and/or identify a subject to receive medication based on received data (e.g., an NT-proBNP Biomarker profile) and use the data to calculate an NT-proBNP Biomarker score.

[0075] FIG. 4 is a block diagram of a computer system 1100 that can be used in the operations described above, according to one non-limiting embodiment. The system 1100 includes a processor 1110, a memory 1120, a storage device 1130, and an input/output device 1140. Each of the components 1110, 1120, 1130, and 1140 are interconnected using a system bus 1150. The system may optionally further include analyzing equipment 1160 for determining a level of one or more biomarkers of the present disclosure in a sample.

[0076] In certain non-limiting embodiments, the processor 1110 is capable of processing instructions for execution within the system 1100. In one non-limiting embodiment, the processor 1110 is a single-threaded processor. In another non-limiting embodiment, the processor 1110 is a multi-threaded processor. The processor 1110 is capable of processing instructions stored in the memory 1120 or on the storage device 1130, including for receiving or sending information through the input/output device 1140.

[0077] In certain non-limiting embodiments, the memory 1120 stores information within the system 1100. In one non-limiting embodiment, the memory 1120 is a computer-readable medium. In one non-limiting embodiment, the memory 1120 is a volatile memory unit. In another embodiment, the memory 1120 is a non-volatile memory unit.

[0078] The storage device 1130 is capable of providing mass storage for the system 1100. In one non-limiting embodiment, the storage device 1130 is a computer-readable medium.

[0079] The input/output device 1140 provides input/output operations for the system 1100. In one non-limiting embodiment, the input/output device 1140 includes a keyboard and/or pointing device. In one non-limiting embodiment, the input/output device 1140 includes a display unit for displaying graphical user interfaces.

[0080] The system 1100 can be used to build a database. In certain non-limiting embodiments, a method of the present disclosure is performed in the system 1100 disposed within a chemical analyzer. For example, a computer program product can include

instructions that cause the processor 1110 to perform the steps of any of the methods disclosed or otherwise contemplated herein.

[0081] Additionally, non-transitory computer readable media containing executable instructions that when executed cause a processor to perform operations including a method as provided herein are provided. For example, a non-transitory computer readable medium containing executable instructions that when executed cause a processor to perform operations including any of the methods disclosed or otherwise contemplated herein. In certain non-limiting embodiments, a non-transitory computer readable medium includes a hard drive, external hard drive, discs, CDs, DVDs, and/or the like that stores data. In certain non-limiting embodiments, software disposed within a physical medium is suitable for use herein.

[0082] In some non-limiting embodiments, a non-transitory computer readable media is disclosed that contains executable instructions that when executed cause a processor to perform operations including a method of determining the presence, severity, and/or predisposition of heart failure (HF) in an individual, wherein the method includes the steps of: incubating the assay components of the present disclosure with a biological sample, and determining the amount of NT-proBNP in a sample.

EXAMPLES

[0083] Examples are provided herein below. However, the present disclosure is to be understood to not be limited in its application to the specific experimentation, results, and laboratory procedures disclosed hereinafter. Rather, the Examples are simply provided as one of various embodiments and is meant to be exemplary, not exhaustive.

Example 1

[0084] The present disclosure includes in a non-limiting embodiment an NT-proBNP assay suitable for *in vitro* diagnostic use in the quantitative determination of N-terminal pro-brain natriuretic peptide (NT-proBNP) in human serum and plasma (EDTA and lithium heparin) using a chemical analyzer, such as (but not limited to) the ADVIA Centaur® XP system. In the Emergency Department (ED) and Outpatient (OP) populations, measurements of NT-proBNP are used as an aid in the diagnosis of heart failure (HF) in patients with clinical suspicion of new onset or worsening HF, and for assessment of HF severity.

[0085] In one non-limiting embodiment, the Materials present in the kit include:

- Lite reagent (7.5 ml/reagent pack) containing monoclonal sheep anti-human NT-proBNP F(ab')₂ fragment antibody (~0.36 µg/ml) labeled with acridinium ester in buffer; bovine serum albumin (BSA); bovine gamma globulin; and preservatives.
- Solid Phase reagent (20.0 ml/reagent pack) containing monoclonal sheep anti-human NT-proBNP antibody (~2µg/ml) labeled with biotin bound to streptavidin magnetic particles (~220 mg/L) in buffer; BSA; bovine gamma globulin; sheep gamma globulin; and preservatives.
- Optional Ancillary Well Reagent (7.5 ml/reagent pack) containing Buffer; BSA; bovine gamma globulin; sheep gamma globulin; and preservatives.

[0086] In certain non-limiting embodiments, the assay of the present disclosure is a fully automated 2-site sandwich immunoassay using direct chemiluminescent technology, which uses constant amounts of 2 monoclonal antibodies. The Solid Phase contains a bound biotinylated monoclonal sheep anti-human antibody specific to NT-proBNP and conjugated to streptavidin magnetic particles. The Lite Reagent contains an acridinium-ester-labeled monoclonal sheep anti-human NT-proBNP F(ab')₂ fragment specific to NT-proBNP. A direct relationship exists between the amount of NT-proBNP present in the patient sample and the amount of relative light units (RLUs) detected by the system.

[0087] Non-limiting procedures for collecting the specimen or sample may involve one or more of the following optional steps: observe universal precautions when collecting specimens; handle all specimens as if they are capable of transmitting disease; follow recommended procedures for collection of diagnostic blood specimens by venipuncture; follow the instructions provided with your specimen collection device for use and processing; allow blood specimens to clot completely before centrifugation; and/or keep tubes capped at all times.

[0088] Non-limiting procedures for storing the specimen or sample may involve one or more of the following optional steps: after centrifugation, serum specimens stored on the clot are stable for up to 24 hours at 2–8°C; separated samples are stable for up to 3 days at room temperature, and for up to 4 days at 2–8°C; separated samples are stable at ≤ -20°C for up to 12 months; avoid more than 1 freeze-thaw cycle; do not store in a frost-free freezer; and/or thoroughly mix thawed samples and centrifuge them before using.

[0089] Non-limiting procedures for transporting the specimen or sample may involve one or more of the following optional steps: package and label specimens for shipment in

compliance with applicable federal and international regulations covering the transport of clinical specimens and etiological agents; and/or if during shipment, specimens may be subjected to temperatures $> 25^{\circ}\text{C}$, then ship specimens frozen.

[0090] Non-limiting procedures for preparing the samples may involve one or more of the following optional steps: this assay requires 20 μL of sample for a single determination. This volume does not include the unusable volume in the sample container, or the additional volume required when performing duplicates or other tests on the same sample; the sample volume required to perform onboard dilution differs from the sample volume required to perform a single determination on an undiluted sample; do not use samples with apparent contamination; before placing samples on the system, ensure that samples are free of: Bubbles or foam, fibrin or other particulate matter; and/or remove particulates by centrifugation according to CLSI guidance and the collection device manufacturer's recommendations.

[0091] Non-limiting assay procedures may involve one or more of the following optional steps. The system (including a high throughput chemical analyzer) automatically performs the following steps: dispenses 20 μL of sample into a cuvette; dispenses 200 μL of Solid Phase and 75 μL of Ancillary Well Reagent, then incubates for 3 minutes at 37°C ; dispenses 75 μL of Lite Reagent, then incubates for 6 minutes at 37°C ; performs a wash sequence using a wash reagent (such as, but not limited to, ADVIA Centaur Wash 1); dispenses 300 μL each of acid and base reagent (such as, but not limited to, ADVIA Centaur Acid Reagent and ADVIA Centaur Base Reagent) into initiate the chemiluminescent reaction; and/or reports results.

[0092] Non-limiting instructions for preparing the reagents for use on an automated system include: all reagents are liquid and ready to use. Before loading the packs onto the system, reagents require mixing. For information about mixing the reagents, refer to the system online help.

[0093] Non-limiting instructions for preparing the automated system to perform the assays of the present disclosure include: ensure that sufficient materials are loaded on the system. Refer to Materials Provided and Materials Required but Not Provided for guidance about required reagents.

[0094] Non-limiting instructions for setting Master Curve Definition include: before initiating calibration on each new lot of reagent, enter the assay master curve values by scanning the master curve card.

[0095] Non-limiting instructions for performing calibration include: For calibration of one certain non-limiting embodiments of the assay, use the calibrators provided with each kit. Calibrators provided in an assay kit must only be used with the reagent lot provided in the same kit.

[0096] Non-limiting instructions for Calibration Frequency include: Perform a calibration if one or more of the following conditions exist: At the end of the 36-day calibration interval; When changing lot numbers of primary reagent packs; When indicated by quality control results; and after major maintenance or service, if indicated by quality control results. Follow government regulations or accreditation requirements for calibration frequency. Individual laboratory quality control programs and procedures may require more frequent calibration.

[0097] Non-limiting instructions for preparing the calibrators include one or more of the following optional steps: add 2.0 mL of reagent water into each vial. Replace cap. Let the vials stand for 30 minutes at room temperature to allow the lyophilized material to dissolve. Gently mix and invert the vials to ensure homogeneity of the material. For extended storage, aliquot and seal tightly. Store reconstituted material according to stability limits specified in *Reagents*. Do not store in a frost-free freezer. Before using frozen calibrators, allow the material to thaw completely. Gently mix and invert the vials to ensure homogeneity of the material. Use calibrators within the stability limits specified in *Reagents* and discard any remaining material.

[0098] Non-limiting instructions for performing the calibration procedure include: ensuring that the appropriate master curve and calibrator assigned values are entered on the system. For information about defining the master curve and entering calibrator values, refer to the system online help. Load the required reagents for the assay. Schedule the calibrators. Label two sample containers with barcode labels: one container for the low calibrator and one container for the high calibrator. Place the barcode labels on the sample containers with the readable characters oriented vertically. Barcode labels are lot-specific. Do not use barcode labels from one lot of calibrators with any other lot of calibrators. Gently mix the product and dispense a sufficient volume of each calibrator into the appropriate sample containers. Avoid bubbles. The required sample volume for testing depends on several factors. For information about sample volume requirements, refer to the system online help. Load the samples according to the system online help. Dispose of any calibrator that remains in the sample

container after 6 hours. Do not refill or reuse sample containers. Do not return any calibrator material back into the original container.

[0099] Non-limiting instructions for performing quality control include: for quality control of certain non-limiting embodiments of the assays of the present disclosure, use an appropriate quality control material of known analyte concentration with a minimum of two levels (low and high) at least once during each day that samples are analyzed. Use the quality control material in accordance with the quality control instructions for use. Additional quality control material can be used at the discretion of the laboratory. Use the quality control material in accordance with the quality control instructions for use. In addition, perform quality control: following a valid calibration; with use of a new lot of reagent; when troubleshooting test results that do not match clinical conditions or symptoms. Follow government regulations or accreditation requirements for quality control frequency. Individual laboratory quality control programs and procedures may require more frequent quality control testing. Acceptable performance is achieved when the analyte values obtained are within the expected control interval for the system, as indicated by the manufacturer of the control material or within the interval determined by an internal laboratory quality control procedure. Follow your laboratory's quality control procedures if the results obtained do not fall within the acceptable limits. For information about entering quality control definitions, refer to the system online help.

[0100] Non-limiting instructions for Taking Corrective Action include: If the quality control results do not fall within the expected control interval, do not report results. Perform corrective actions in accordance with established laboratory protocol. For suggested protocol, refer to the system online help.

Results

Calculation of Results

[0101] The system determines the result using the calculation procedure described in the system online help. The system reports results in pg/mL (common units) or pmol/L (SI units), depending on the units defined when setting up the assay. Conversion formula: 1 pg/mL = 0.118 pmol/L For information about results outside the specified measuring interval, refer to *Measuring Interval*.

[0102] Dilutions

[0103] The measuring interval is 35–35,000 pg/mL (4.13–4130 pmol/L). For information about dilution options, refer to the system online help. Dilute and retest samples with NT-proBNP levels > 35,000 pg/mL (4130 pmol/L) to obtain accurate results. For automated dilutions, perform the following activities: Load a multi-diluent (such as, but not limited to, ADVIA Centaur Multi-Diluent 1). Ensure that sufficient sample volume is available. Refer to the table below. Select the appropriate dilution factor. For automatic dilutions enter a Dilution Point ≤ 35,000 pg/mL (4130 pmol/L).

TABLE 1

Sample	Dilution	Sample Volume (μL)
Serum and plasma	1:5	40
Serum and plasma	1:10	30

[0104] If patient results exceed the measuring interval of the assay when using automated dilution, or if laboratory protocol requires manual dilution, manually dilute the patient sample. For manual dilutions, perform the following actions: Use a multi-diluent (such as but not limited to, ADVIA Centaur Multi-Diluent 1; vial) to prepare a manual dilution. In embodiments one may refer to an operation manual and discussion related to optional materials. For information about ordering tests for manually diluted samples, refer to the system online help. Ensure that results are mathematically corrected for dilution. If a dilution factor is entered when scheduling the test, the system automatically calculates the result.

Interpretation of Results

[0105] Results from assays of the present disclosure should be interpreted with the appropriate clinical guidelines and in conjunction with the patient’s medical history, clinical presentation, and other findings. Guidelines recommend using natriuretic peptides in both Emergency Department (ED) and Outpatient (OP) settings for diagnosis or exclusion of heart failure. The performance of the assays of the present disclosure was evaluated separately in each of these settings using published age-independent and age-dependent cut-off values.

Emergency Department (ED) Population

[0106] For patients presenting to ED settings with clinical suspicion of HF, the NT-proBNP assay results should be interpreted as indicated in Table 2.

TABLE 2

Age Group (Years)	Assay Results (pg/mL)	Interpretation
All	< 300	Negative: Heart Failure Unlikely
< 50	≥ 300 – ≤ 450	Gray Zone: Result indeterminate - Consider other reasons for NT-proBNP elevation
50–75	≥ 300 – ≤ 900	
> 75	≥ 300 – ≤ 1800	
< 50	> 450	Positive: Heart failure Likely
50–75	> 900	
> 75	> 1800	

[0107] Elevated or depressed natriuretic peptides values can be caused by conditions which may confound the diagnosis of heart failure. In the ED, conditions such as chronic heart failure, acute coronary syndrome, atrial fibrillation, pulmonary embolism, valvular heart disease, myocarditis, pulmonary hypertension, renal deficiency, stroke, and sepsis can elevate NT- proBNP levels in the absence of acute heart failure. In addition, obesity, flash pulmonary edema, pericarditis, and cardiac tamponade are associated with reduced NT-proBNP levels.

Outpatient Population

[0108] In outpatient settings, the optimal use of natriuretic peptide assays excludes the HF diagnosis. Therefore, a lower rule-out cut-off value which increases sensitivity and negative predictive value is needed, as these patients can present with limited, less acute HF symptoms. For ambulatory patients presenting to outpatient facilities with clinical suspicion of HF not previously diagnosed, the assay results should be interpreted as indicated in Table 3.

TABLE 3

Age Group (Years)	Assay Results (pg/mL)	Interpretation
All	< 125	Negative: Heart Failure Unlikely
All	≥ 125	Consider Heart Failure as well as other causes of NT-proBNP elevation ^a

[0109] Clinical conditions such as acute coronary syndrome, atrial fibrillation, pulmonary embolism, valvular heart disease, myocarditis, pulmonary hypertension, renal deficiency, stroke, and sepsis can elevate NT-proBNP levels in the absence of heart failure.

Limitations

[0110] Patient samples may contain heterophilic antibodies that could react in immunoassays and cause falsely elevated or depressed results. This assay is designed to

minimize interference from heterophilic antibodies. Additional information may be required for diagnosis.

[0111] Expected Values in a Healthy Population (Table 4)

[0112] Expected values were established non-parametrically using the ADVIA Centaur® XP system in accordance with CLSI Document EP28-A3c on a population of 723 apparently healthy subjects (362 females and 361 males) without HF.

TABLE 4

Sex	Age	N	Mean pg/mL (pmol/L)	SD pg/mL (pmol/L)	Median pg/mL (pmol/L)	95th Percentile pg/mL (pmol/L)	% < 125 pg/mL	% < 300 pg/mL
Male	< 50 years	120	56 (6.61)	95.0 (21.2)	< 35 (4.13)	124 (14.6)	N/A ^a	97.5
	50–75 years	121	78 (9.20)	136.4 (16.1)	< 35 (4.13)	322 (38.0)	N/A	94.2
	> 75 years	120	56 (6.61)	50.6 (5.97)	< 35 (4.13)	154 (18.2)	N/A	98.3
Female	< 50 years	122	57 (6.73)	33.1 (3.91)	37 (4.37)	133 (15.7)	N/A	100
	50–75 years	120	77 (9.09)	124.0 (14.6)	40 (4.72)	192 (22.7)	N/A	97.5
	> 75 years	120	89 (10.5)	216.4 (25.5)	43 (5.07)	178 (21.0)	N/A	97.5
Overall	Overall	723	69 (8.14)	124.9 (14.7)	< 35 (4.13)	163 (19.2)	92.0	97.5

[0113] As with all in vitro diagnostic assays, each laboratory should determine its own reference interval for the diagnostic evaluation of patient results. Consider these values as guidance only.

[0114] Disease Study Group

[0115] ED Population: A total of 3128 subject who presented to the ED with signs and symptoms of HF were enrolled. Of these, 1148 subjects (476 females and 672 males) were diagnosed with acute HF and included in the disease study group. NT-proBNP values are summarized in Tables 5-6 for subjects with acute HF by subgroups based on age group (Table 5) and NYHA Functional Class (Table 6). The males with Acute HF are summarized in Table 7, while the females with acute HF are summarized in Table 8.

TABLE 5: ED Population – All Subjects with Acute HF

Age Group	< 50 years	50–75 years	> 75 years
Mean pg/mL (pmol/L)	6004 (708)	6820 (805)	8591 (1014)
SD pg/mL (pmol/L)	7601 (897)	8182 (965)	8984 (1060)
Median pg/mL (pmol/L)	3552 (419)	3677 (434)	5295 (625)
95th Percentile pg/mL (pmol/L)	25,401 (2997)	26,473 (3124)	34,451 (4065)
% > 300 pg/mL	94.3	95.0	98.4

TABLE 6

Age Group	< 50 years	50–75 years	> 75 years
% > 450 pg/mL	90.9	N/A ^a	N/A
% > 900 pg/mL	N/A	83.8	N/A
% > 1800 pg/mL	N/A	N/A	85.8
N	230	481	437

TABLE 7

Age Group	< 50 years	50–75 years	> 75 years
Mean pg/mL (pmol/L)	6715 (792)	6849 (808)	9229 (1089)
SD pg/mL (pmol/L)	8088 (954)	7698 (908)	9761 (1152)
Median pg/mL (pmol/L)	3992 (471)	4085 (482)	5717 (675)
95th Percentile pg/mL (pmol/L)	27,720 (3271)	24,251 (2862)	> 35,000 (4130)
% > 300 pg/mL	97.3	96.5	98.8
% > 450 pg/mL	94.5	N/A ^a	N/A
% > 900 pg/mL	N/A	87.3	N/A
% > 1800 pg/mL	N/A	N/A	84.7
N	146	284	242

^a The cut-off value is not applicable for the age group indicated.

TABLE 8

Age Group	< 50 years	50–75 years	> 75 years
Mean pg/mL (pmol/L)	4768 (563)	6778 (800)	7798 (920)
SD pg/mL (pmol/L)	6532 (771)	8853 (1045)	7867 (928)
Median pg/mL (pmol/L)	2701 (319)	2889 (341)	4826 (569)
95th Percentile pg/mL (pmol/L)	17,118 (2020)	> 35,000 (4130)	26,736 (3155)
% > 300 pg/mL	89.3	92.9	97.9
% > 450 pg/mL	84.5	N/A ^a	N/A
% > 900 pg/mL	N/A	78.7	N/A
% > 1800 pg/mL	N/A	N/A	87.2
N	84	197	195

[0116] ED Population - all subjects with Acute HF (e.g., such as NYHA Functional Class). The NYHA classifications for the ED population with acute HF are provided in Table 9. Males with Acute HF (NYHA Functional Class) are shown in Table 10, while females with acute HF are shown in Tables 11-12.

TABLE 9

NYHA Functional Class	All Subjects	NYHA I	NYHA II	NYHA III	NYHA IV
Mean pg/mL (pmol/L)	7311 (863)	1520 (179)	5467 (645)	7510 (886)	7506 (886)
SD pg/mL (pmol/L)	8422 (994)	1544 (182)	7529 (888)	8542 (1008)	8312 (981)
Median pg/mL (pmol/L)	4232 (499)	1520 (179)	2673 (315)	4362 (515)	4427 (522)
5th Percentile pg/mL (pmol/L)	385 (45.4)	N/A ^a	406 (47.9)	338 (39.9)	449 (53.0)
95th Percentile pg/mL (pmol/L)	30,816 (3636)	N/A	25,448 (3003)	31,341 (3698)	30,968 (3654)
% > cut-off (300 pg/mL)	96.2	100	97.7	95.7	96.6
Minimum pg/mL (pmol/L)	< 35 (4.13)	428 (50.5)	74 (8.73)	< 35 (4.13)	< 35 (4.13)
Maximum pg/mL (pmol/L)	> 35,000 (4130)	2612 (308)	> 35,000 (4130)	> 35,000 (4130)	> 35,000 (4130)
N	1147 ^b	2 ^c	128	533	468

^a Not applicable: insufficient samples to calculate percentiles.¹²

^b Unable to determine NYHA classification for 16 subjects.

^c One male subject, identified as an outlier with an abnormally high PBNPII value, was excluded from the NYHA I classification summary.

TABLE 10

NYHA Functional Class	All Subjects	NYHA I	NYHA II	NYHA III	NYHA IV
Mean pg/mL (pmol/L)	7645 (902)	-	6478 (764)	7304(862)	8248 (973)
SD pg/mL (pmol/L)	8609 (1016)	-	8951 (1056)	7992 (943)	9032 (1066)
Median pg/mL (pmol/L)	4408 (520)	-	3063 (361)	4476 (528)	4835 (571)
5th Percentile pg/mL (pmol/L)	453 (53.5)	-	420 (49.6)	344 (40.6)	762 (89.9)
95th Percentile pg/mL (pmol/L)	31,457 (3712)	-	31,507 (3718)	27,160 (3205)	> 35,000 (4130)
% > cut-off (300 pg/mL)	97.5	-	100	96.0	98.5
Minimum pg/mL (pmol/L)	53 (6.25)	-	321 (37.9)	53 (6.25)	65 (7.67)
Maximum pg/mL (pmol/L)	> 35,000 (4130)	-	> 35,000 (4130)	> 35,000 (4130)	> 35,000 (4130)
N	671 ^a	0	76	321	264

^a Unable to determine NYHA classification for 10 subjects.

TABLE 11

NYHA Functional Class	All Subjects	NYHA I	NYHA II	NYHA III	NYHA IV
Mean pg/mL (pmol/L)	6841 (807)	1520 (179)	3989 (471)	7822 (923)	6547 (773)
SD pg/mL (pmol/L)	8137 (960)	1544 (182)	4425 (522)	9323 (1100)	7182 (847)
Median pg/mL (pmol/L)	3668 (433)	1520 (179)	2306 (272)	4190 (494)	4053 (478)

TABLE 12

NYHA Functional Class	All Subjects	NYHA I	NYHA II	NYHA III	NYHA IV
5th Percentile pg/mL (pmol/L)	229 (27.0)	N/A ^a	173 (20.4)	322 (38.0)	225 (26.6)
95th Percentile pg/mL (pmol/L)	26,562 (3134)	N/A	13,628 (1608)	> 35,000 (4130)	22,407 (2644)
% > cut-off (300 pg/mL)	94.3	100	94.2	95.3	94.1
Minimum pg/mL (pmol/L)	< 35 (4.13)	428 (50.5)	74 (8.73)	< 35 (4.13)	< 35 (4.13)
Maximum pg/mL (pmol/L)	> 35,000 (4130)	2612 (308)	22,489 (2654)	> 35,000 (4130)	> 35,000 (4130)
N	476 ^b	2	52	212	204

^a Not applicable: insufficient samples to calculate percentiles.^{1,2}

[0117] Outpatient (OP) Population

[0118] A total of 1033 subjects who presented to the OP setting with signs and symptoms of HF were enrolled. Of these, 185 subjects (102 females and 83 males) were diagnosed with new onset HF and included in the disease study group. NT-proBNP values are summarized for subjects with new onset HF based on age group (Tables 13-15) and NYHA Functional Class (Tables 16-18). Table 13 summarizes OP population – all subjects with new onset HF. Males with new onset HF are summarized in Table 14, while females with new onset HF are summarized in Table 15.

[0119] The NYHA classifications for the OP population with new onset HF are provided in Table 16. Males with new onset HF (NYHA Functional Class) are shown in Table 17, while females with new onset HF are shown in Table 18.

TABLE 13

Age Group	≤ 75 years	> 75 years
Mean pg/mL (pmol/L)	1313 (155)	2418 (285)
SD pg/mL (pmol/L)	3756 (443)	5536 (653)
Median pg/mL (pmol/L)	449 (53.0)	661 (78.0)
95th Percentile pg/mL (pmol/L)	4547 (537)	13,518 (1595)
% > 125 pg/mL	82.1	96.1
N	134	51

TABLE 14

Age Group	≤ 75 years	> 75 years
Mean pg/mL (pmol/L)	2248 (265)	1880 (222)
SD pg/mL (pmol/L)	5368 (633)	2671 (315)
Median pg/mL (pmol/L)	607 (71.6)	638 (75.3)
95th Percentile pg/mL (pmol/L)	11,609 (1370)	10,017 (1182)
% > 125 pg/mL	85.5	90.5
N	62	21

TABLE 15

Age Group	≤ 75 years	> 75 years
Mean pg/mL (pmol/L)	508 (59.9)	2794 (330)
SD pg/mL (pmol/L)	500 (59.0)	6897 (814)
Median pg/mL (pmol/L)	284 (33.5)	775 (91.5)
95th Percentile pg/mL (pmol/L)	1641 (194)	25,710 (3034)
% > 125 pg/mL	79.2	100
N	72	30

TABLE 16

NYHA Functional Class	All Subjects	NYHA I	NYHA II	NYHA III	NYHA IV
Mean pg/mL (pmol/L)	1618 (191)	240 (28.3)	1352 (160)	2195 (259)	4284 (506)
SD pg/mL (pmol/L)	4332 (511)	148 (17.5)	3868 (456)	5696 (672)	4115 (486)
Median pg/mL (pmol/L)	504 (59.5)	240 (28.3)	477 (56.3)	651 (76.8)	2939 (347)
5th Percentile pg/mL (pmol/L)	48.2 (5.69)	N/A ^a	44.7 (5.27)	109 (12.9)	N/A
95th Percentile pg/mL (pmol/L)	6537 (771)	N/A	3870 (457)	10,271 (1212)	N/A
% > cut-off (125 pg/mL)	85.9	100	82.5	95.0	100
Minimum pg/mL (pmol/L)	< 35 (4.13)	135 (15.9)	< 35 (4.13)	92 (10.9)	574 (67.7)
Maximum pg/mL (pmol/L)	> 35,000 (4130)	345 (40.7)	> 35,000 (4130)	> 35,000 (4130)	11,235 (1326)
N	185	2	137	40	6

^a Not applicable; insufficient samples to calculate percentiles.¹²

TABLE

17

NYHA Functional Class	All Subjects	NYHA I	NYHA II	NYHA III	NYHA IV
Mean pg/mL (pmol/L)	2155 (254)	-	1918 (226)	2313 (273)	5364 (633)
SD pg/mL (pmol/L)	4817 (568)	-	5134 (606)	3060 (361)	4685 (553)
Median pg/mL (pmol/L)	621 (73.3)	-	555 (65.5)	661 (78.0)	4595 (542)
5th Percentile pg/mL (pmol/L)	35.6 (4.2)	-	< 35 (4.13)	268 (31.6)	N/A ^a
95th Percentile pg/mL (pmol/L)	11,079 (1307)	-	10,272 (1212)	10,457 (1234)	N/A
% > cut-off (125 pg/mL)	86.7	-	82.8	100	100
Minimum pg/mL (pmol/L)	< 35 (4.13)	-	< 35 (4.13)	268 (31.6)	1032 (122)

Maximum pg/mL (pmol/L)	> 35,000 (4130)	-	> 35,000 (4130)	10,457 (1234)	11,235 (1326)
N	83	0	64	15	4

^a Not applicable: insufficient samples to calculate percentiles.¹²

TABLE 18

NYHA Functional Class	All Subjects	NYHA I	NYHA II	NYHA III	NYHA IV
Mean pg/mL (pmol/L)	1180 (139)	240 (28.3)	857 (101)	2124 (251)	2124 (251)
SD pg/mL (pmol/L)	3864 (456)	148 (17.5)	2159 (255)	6873 (811)	2191 (259)
Median pg/mL (pmol/L)	466 (55.0)	240 (28.3)	447 (52.7)	641 (75.6)	2124 (251)
5th Percentile pg/mL (pmol/L)	53.1 (6.27)	N/A ^a	46.4 (5.48)	96.8 (11.4)	N/A
95th Percentile pg/mL (pmol/L)	2238 (264)	N/A	2192 (259)	25,072 (2958)	N/A
% > cut-off (125 pg/mL)	85.3	100	82.2	92.0	100
Minimum pg/mL (pmol/L)	< 35 (4.13)	135 (15.9)	< 35 (4.13)	92 (10.9)	574 (67.7)
Maximum pg/mL (pmol/L)	> 35,000 (4130)	345 (40.7)	18,109 (2137)	> 35,000 (4130)	3673 (433)
N	102	2	73	25	2

^a Not applicable: insufficient samples to calculate percentiles.¹²

[0120] OP Population – Receiver Operating Characteristic Curve

[0121] The Receiver Operating Characteristic Curve (ROC) shown in FIG. 5 presents the clinical sensitivity and specificity for the enrolled 185 subjects diagnosed with new onset HF and 848 subjects without HF. The area under the ROC curve for the NT-proBNP assay is 0.839 with a 95% confidence interval of 0.804 to 0.868.

[0122] Performance Characteristics

[0123] Measuring Interval: 35 – 35,000 pg/mL (4.13 – 4130 pmol/L). The lower limit of the measuring interval is defined by the limit of quantitation (LoQ). Report results below the measuring interval as < 35 pg/mL (4.13 pmol/L).

[0124] Detection Capability:

Limit of Blank (LoB), 13 pg/mL (1.53 pmol/L)

Limit of Detection (LoD), 20 pg/mL (2.36 pmol/L)

Limit of Quantitation (LoQ), 35 pg/mL (4.13 pmol/L)

LoB and LoD values are representative data. The LoB corresponds to the highest measurement result likely to be observed for a blank sample with a probability of 95%. The LoD corresponds to the lowest concentration of NT-proBNP that can be detected with a probability of 95%. The LoQ corresponds to the lowest amount of NT-proBNP in a sample at

which the within laboratory CV is 20%. Detection capability was determined in accordance with CLSI Document EP17-A2.

Clinical Performance

[0125] A total of 3128 subjects with signs and symptoms of acute HF who presented to the ED, were prospectively enrolled in a multi-site clinical evaluation of one non-limiting embodiment of the NT-proBNP assay of the present disclosure. Diagnosis and severity of HF were determined by an independent central adjudication panel of expert clinicians (cardiologists). 1148 subjects were adjudicated as acute HF, and 1980 subjects were adjudicated as without HF.

[0126] The descriptive statistics for the NT-proBNP test results (pg/ml) were determined for the ED Population at enrollment.

[0127] Table 19 summarizes the baseline characteristics for all of the ED population, while Table 20 summarizes the baseline characteristics of the ED population by age group.

TABLE 19

	Adjudicated Diagnosis	
	Positive	Negative
N	1148	1980
Mean pg/mL (pmol/L)	7330 (865)	1226 (145)
SD pg/mL (pmol/L)	8444 (996)	3097 (365)
Median pg/mL (pmol/L)	4237 (500)	206 (24.3)
Quartile 1 ^a pg/mL (pmol/L)	1999 (236)	57 (6.73)
Quartile 3 ^b pg/mL (pmol/L)	8882 (1048)	1001 (118)
Minimum pg/mL (pmol/L)	< 35 (4.13)	< 35 (4.13)
Maximum pg/mL (pmol/L)	> 35,000 (4130)	> 35,000 (4130)

^a 25th Percentile.

^b 75th Percentile.

TABLE 20

	Adjudicated Diagnosis	
	Positive	Negative
Age	< 50 years	
N	230	678
Mean pg/mL (pmol/L)	6004 (708)	577 (68.1)
SD pg/mL (pmol/L)	7601 (897)	2435 (287)
Median pg/mL (pmol/L)	3552 (419)	72 (8.5)
Quartile 1 ^a pg/mL (pmol/L)	1410 (166)	< 35 (4.13)
Quartile 3 ^b pg/mL (pmol/L)	7083 (836)	235 (27.7)
Minimum pg/mL (pmol/L)	< 35 (4.13)	< 35 (4.13)
Maximum pg/mL (pmol/L)	> 35,000 (4130)	> 35,000 (4130)
Age	50–75 years	
N	481	820
Mean pg/mL (pmol/L)	6820 (805)	1040 (123)
SD pg/mL (pmol/L)	8182 (965)	3057 (361)
Median pg/mL (pmol/L)	3677 (434)	205 (24.2)
Quartile 1 pg/mL (pmol/L)	1654 (195)	66 (7.79)
Quartile 3 pg/mL (pmol/L)	8602 (1015)	786 (92.7)
Minimum pg/mL (pmol/L)	< 35 (4.13)	< 35 (4.13)

	Adjudicated Diagnosis	
	Positive	Negative
Maximum pg/mL (pmol/L)	> 35,000 (4130)	> 35,000 (4130)
Age		> 75 years
N	437	482
Mean pg/mL (pmol/L)	8591 (1014)	2455 (290)
SD pg/mL (pmol/L)	8984 (1060)	3613 (426)
Median pg/mL (pmol/L)	5295 (625)	1069 (126)
Quartile 1 pg/mL (pmol/L)	2764 (326)	287 (33.9)
Quartile 3 pg/mL (pmol/L)	10,309 (1216)	3021 (356)
Minimum pg/mL (pmol/L)	79 (9.32)	< 35 (4.13)
Maximum pg/mL (pmol/L)	> 35,000 (4130)	26,256 (3098)

^a 25th Percentile.

^b 75th Percentile.

[0128] Likelihood Ratio

[0129] The NT-proBNP results were stratified into three determinations based on an overall rule-out cut-off value of 300 pg/ml and age-specific rule-in cut-off values, and compared to the adjudicated diagnosis for acute HF. The pre-test risk (or prevalence) of HF within this study was 36.7%. Relative to this pre-test risk, the risk of HF increased to 67.2% for subjects with a positive NT-proBNP determination (post-test risk). The likelihood ratio compares the probability of a NT-proBNP test result given acute heart failure to the probability of a NT-proBNP test result given heart failure. For a positive NT-proBNP test result, a likelihood ratio of 3.53 indicates a person with acute HF is 3.5 times more likely to have a positive test result than a person with no acute HF. Subjects with an indeterminate result had a reduction in post-test risk of HF to 24.3% and were nearly half (LR 0.55) as likely to be in the HF population than in the negative diagnosis population. For a negative NT-proBNP test result, a likelihood ratio of 0.07 indicates a person with acute HF is 0.07 (less than 1/10th) times likely to have a negative test than a person with no acute HF.

[0130] The analysis of clinical performance between the non-limiting embodiment of the NT-proBNP assay of the present disclosure and the adjudicated diagnosis employs a 2-by-3 contingency table format. Tables 21-32 summarize the NT-proBNP results versus the adjudicated diagnosis for acute HF (positive or negative) using the following determinations:

- greater than the age-specific rule-in cut-off value (positive)
- between the rule-in and rule-out cut-off values (indeterminate)
- below the rule-out cut-off value (negative)

The LR should only be used for the ED population.

[0131] ED population – Clinical agreement between the NT-proBNP results of the non-limiting embodiment of the assay of the present disclosure and Acute HF Diagnosis. Table 21 summarizes the results for all sex and age groups combined; Table 22 summarizes the results by age group; Table 23 summarizes the male subjects by age group; and Table 24 summarizes the female subjects by age group. Analyses were also performed for relevant ED population clinical subgroups as shown in Tables 25-32.

TABLE 21

NT-proBNP Determination	Cut-off Value	Adjudicated Diagnosis			Post-Test Risk (%)	LR+	95% Confidence Interval (CI)
		Positive	Negative	Total			
Positive	> ASC ^a	987	482	1469	67.2% (987/1469) (64.7%, 69.6%) ^b	3.53	(3.26, 3.83)
Indeterminate	300 pg/mL – ≤ ASC	117	365	482	24.3% (117/482) (20.5%, 28.3%)	0.55	(0.45, 0.67)
Negative	< 300 pg/mL	44	1133	1177	3.7% (44/1177) (2.7%, 4.8%)	0.07	(0.05, 0.09)
Total		1148	1980	3128	Pre-Test Risk = 35.7% (1148/3128) 95% CI: 35.1%, 38.5%		

^a Age specific rule-in cut-off value.

^b 95% Confidence Interval.

$$LR+ = \frac{(\# \text{ HF Positive for NT-proBNP Determination} / \# \text{ HF Positive Diagnosis Total})}{(\# \text{ HF Negative for NT-proBNP Determination} / \# \text{ HF Negative Diagnosis Total})}$$

Example

$$\text{Positive NT-proBNP Determination} = \frac{(987 / 1148)}{(482 / 1980)} = 3.53$$

The analysis was conducted by pooling all age groups and by subgroups based on age group and sex.

TABLE 22

Age (years)	NT-proBNP	Cut-off pg/mL	Adjudicated Diagnosis			Post-Test Risk (%)	LR+	95% CI
			Positive	Negative	Total			
< 50	Positive	> 450	209	123	332	63.0% (209/332) (57.5%, 68.2%)*	5.03	(4.25, 5.91)
	Indeterminate	300 – ≤ 450	8	19	27	29.6% (8/27) (13.8%, 50.2%)	1.24	(0.55, 2.80)
	Negative	< 300	13	535	549	2.4% (13/549) (1.3%, 4.0%)	0.07	(0.04, 0.12)
	Total		230	678	908	Pre-Test Risk = 25.3% (230/908)		
50–75	Positive	> 900	403	185	588	68.5% (403/588) (64.6%, 72.3%)	3.71	(3.25, 4.24)
	Indeterminate	300 – ≤ 900	54	160	214	25.2% (54/214) (19.6%, 31.6%)	0.58	(0.43, 0.77)
	Negative	< 300	24	475	499	4.8% (24/499) (3.1%, 7.1%)	0.09	(0.06, 0.13)
	Total		481	820	1301	Pre-Test Risk = 37.0% (481/1301)		
> 75	Positive	> 1800	375	174	549	68.3% (375/549) (64.2%, 72.2%)	2.38	(2.10, 2.69)
	Indeterminate	300 – ≤ 1800	55	186	241	22.8% (55/241) (17.7%, 27.7%)	0.33	(0.25, 0.43)
	Negative	< 300	7	122	129	5.4% (7/129) (2.2%, 10.9%)	0.06	(0.03, 0.13)
	Total		437	482	919	Pre-Test Risk = 47.6% (437/919)		

* 95% Confidence Interval.

TABLE 23

Age (years)	NT-proBNP	Cut-off pg/ml	Adjudicated Diagnosis			Post-Test Risk (%)	LR+	95% CI
			Positive	Negative	Total			
< 50	Positive	> 450	138	69	207	66.7% (138/207) (59.8%, 73.1%) ^a	4.29	(3.47, 5.30)
	Indeterminate	300 -- ≤ 450	4	7	11	36.4% (4/11) (10.9%, 69.2%)	1.23	(0.36, 4.12)
	Negative	< 300	4	237	241	1.7% (4/241) (0.5%, 4.2%)	0.04	(0.01, 0.10)
	Total		146	313	459	Pre-Test Risk = 31.8% (146/459)		
50-75	Positive	> 900	248	104	352	70.5% (248/352) (65.4%, 75.2%)	3.45	(2.91, 4.10)
	Indeterminate	300 -- ≤ 900	26	91	117	22.2% (26/117) (15.1%, 30.8%)	0.41	(0.27, 0.62)
	Negative	< 300	10	216	226	4.4% (10/226) (2.1%, 8.0%)	0.07	(0.04, 0.12)
	Total		284	411	695	Pre-Test Risk = 40.9% (284/695)		
> 75	Positive	> 1800	205	83	288	71.2% (205/288) (65.6%, 76.3%)	2.22	(1.86, 2.66)
	Indeterminate	300 -- ≤ 1800	34	89	123	27.6% (34/123) (20.0%, 36.4%)	0.34	(0.24, 0.49)
	Negative	< 300	3	46	49	6.1% (3/49) (1.3%, 16.9%)	0.06	(0.02, 0.19)
	Total		242	218	460	Pre-Test Risk = 52.6% (242/460)		

^a 95% Confidence Interval.

TABLE 24

Age (years)	NT-proBNP	Cut-off pg/mL	Adjudicated Diagnosis			Post-Test Risk (%)	LR+	95% CI
			Positive	Negative	Total			
< 50	Positive	> 450	71	54	125	56.8% (71/125) (47.6%, 65.6%) ^a	5.71	(4.39, 7.43)
	Indeterminate	300 – ≤ 450	4	12	16	25.0% (4/16) (7.3%, 52.4%)	1.45	(0.46, 4.38)
	Negative	< 300	9	299	308	2.9% (9/308) (1.3%, 5.5%)	0.13	(0.07, 0.24)
	Total		84	365	449	Pre-Test Risk = 18.7% (84/449)		
50–75	Positive	> 900	155	81	236	65.7% (155/236) (59.2%, 71.7%)	3.97	(3.23, 4.89)
	Indeterminate	300 – ≤ 900	28	69	97	28.9% (28/97) (20.1%, 39.0%)	0.84	(0.56, 1.26)
	Negative	< 300	14	259	273	5.1% (14/273) (2.8%, 8.5%)	0.11	(0.07, 0.19)
	Total		197	409	606	Pre-Test Risk = 32.5% (197/606)		
> 75	Positive	> 1800	170	91	261	65.1% (170/261) (59.0%, 70.9%)	2.53	(2.12, 3.01)
	Indeterminate	300 – ≤ 1800	21	97	118	17.8% (21/118) (11.4%, 25.9%)	0.29	(0.19, 0.45)
	Negative	< 300	4	76	80	5.0% (4/80) (1.4%, 12.3%)	0.67	(0.03, 0.19)
	Total		195	264	459	Pre-Test Risk = 42.5% (195/459)		

^a 95% Confidence Interval.

TABLE 25

ED population – Clinical Agreement Between

NT-proBNP Results and Acute HF Diagnosis in Patients with BMI <30 kg/m².

Age (years)	NT-proBNP	Cut-off pg/mL	Adjudicated Diagnosis			Post-Test Risk (%)	LR+	95% CI
			Positive	Negative	Total			
< 50	Positive	> 450	52	52	104	50.0% (52/104) (40.0%, 60.0%) ^a	4.77	(3.75, 6.07)
	Indeterminate	300 – ≤ 450	0	8	8	0.0% (0/8)	N/A ^b	N/A
	Negative	< 300	0	188	188	0.0% (0/188)	N/A	N/A
	Total		52	248	300	Pre-Test Risk = 17.3% (52/300)		

50-75	Positive	> 900	168	101	269	62.5% (168/269) (56.4%, 68.3%)	3.45	(2.92, 4.08)
	Indeterminate	300 ~ ≤ 900	6	71	77	7.8% (6/77) (2.9%, 16.2%)	0.18	(0.08, 0.40)
	Negative	< 300	0	189	189	0.0% (0/189)	N/A	N/A
	Total ^b		174	361	535	Pre-Test Risk = 32.5% (174/535)		
> 75	Positive	> 1800	240	131	371	64.7% (240/371) (59.6%, 69.6%)	2.12	(1.85, 2.42)
	Indeterminate	300 ~ ≤ 1800	21	107	128	16.4% (21/128) (10.5%, 24.0%)	0.23	(0.15, 0.35)
	Negative	< 300	1	65	66	1.5% (1/66) (0.0%, 8.2%)	0.02	(0.00, 0.13)
	Total ^b		262	303	565	Pre-Test Risk = 46.4% (262/565)		

^a 95% Confidence Interval.

^b Not applicable.

TABLE 26

ED population – Clinical Agreement Between

NT-proBNP Results and Acute HF Diagnosis in Patients with BMI ≤30 kg/m².

Age (years)	NT-proBNP	Cut-off pg/mL	Adjudicated Diagnosis			Post-Test Risk (%)	LR+	95% CI
			Positive	Negative	Total			
< 50	Positive	> 450	147	69	216	68.1% (147/216) (61.4%, 74.2%) ^a	5.13	(4.11, 6.40)
	Indeterminate	300 ~ ≤ 450	7	10	17	41.2% (7/17) (18.4%, 67.1%)	1.69	(0.65, 4.35)
	Negative	< 300	13	323	336	3.9% (13/336) (2.1%, 6.5%)	0.10	(0.06, 0.16)
	Total		167	402	569	Pre-Test Risk = 29.3% (167/569)		
50-75	Positive	> 900	217	67	284	76.4% (217/284) (71.0%, 81.2%)	4.81	(3.83, 6.05)
	Indeterminate	300 ~ ≤ 900	46	86	132	34.8% (46/132) (26.8%, 43.6%)	0.79	(0.57, 1.10)
	Negative	< 300	23	272	295	7.8% (23/295) (5.0%, 11.5%)	0.13	(0.08, 0.19)
	Total		286	425	711	Pre-Test Risk = 40.2% (286/711)		
> 75	Positive	> 1800	111	38	149	74.5% (111/149) (66.7%, 81.3%)	3.02	(2.26, 4.04)
	Indeterminate	300 ~ ≤ 1800	30	61	91	33.0% (30/91) (23.5%, 43.6%)	0.51	(0.35, 0.74)
	Negative	< 300	5	52	57	8.8% (5/57) (2.9%, 19.3%)	0.10	(0.04, 0.24)
	Total		146	151	297	Pre-Test Risk = 49.2% (146/297)		

^a 95% Confidence Interval.

TABLE 27

ED population – Clinical Agreement Between NT-proBNP Results
and Acute HF Diagnosis in Patients with eGFR < 60 mL/Min/1.73 m².

Age (years)	NT-proBNP	Cut-off pg/mL	Adjudicated Diagnosis			Post-Test Risk (%)	LR+	95% CI
			Positive	Negative	Total			
< 50	Positive	> 450	72	39	111	64.9% (72/111) (55.2%, 73.7%) ^a	1.68	(1.35, 2.08)
	Indeterminate	300 ~ ≤ 450	3	3	6	50.0% (3/6) (11.8%, 88.2%)	0.91	(0.19, 4.35)
	Negative	< 300	1	27	28	3.6% (1/28) (0.1%, 18.3%)	0.03	(0.00, 0.24)
	Total		76	69	145	Pre-Test Risk = 52.4% (76/145)		
50-75	Positive	> 900	176	82	258	68.2% (176/258) (62.2%, 73.9%)	2.08	(1.75, 2.48)
	Indeterminate	300 ~ ≤ 900	19	36	55	34.5% (19/55) (22.2%, 48.6%)	0.51	(0.30, 0.86)
	Negative	< 300	7	78	85	8.2% (7/85) (3.4%, 16.2%)	0.09	(0.04, 0.18)
	Total		202	196	398	Pre-Test Risk = 50.8% (202/398)		
> 75	Positive	> 1800	260	105	365	71.2% (260/365) (66.3%, 75.8%)	1.99	(0.73, 2.30)
	Indeterminate	300 ~ ≤ 1800	22	88	110	20.0% (22/110) (13.0%, 28.7%)	0.20	(0.13, 0.31)
	Negative	< 300	0	34	34	0.0% (0/34)	N/A ^b	N/A
	Total		282	227	509	Pre-Test Risk = 55.4% (282/509)		

^a 95% Confidence Interval.

^b Not applicable.

TABLE 28

ED population – Clinical Agreement Between NT-proBNP Results and
Acute HF Diagnosis in Patients with eGFR ≥ 60 mL/Min/1.73 m².

Age (years)	NT-proBNP	Cut-off $\mu\text{g/mL}$	Adjudicated Diagnosis			Post-Test Risk (%)	LR+	95% CI
			Positive	Negative	Total			
< 50	Positive	> 450	137	83	220	62.3% (137/220) (55.5%, 68.7%) ^a	6.22	(5.07, 7.64)
	Indeterminate	300 – $\leq$ 450	4	15	19	21.1% (4/19) (6.1%, 45.6%)	1.01	(0.34, 2.99)
	Negative	< 300	11	475	486	2.3% (11/486) (1.1%, 4.0%)	0.09	(0.05, 0.15)
	Total		152	573	725	Pre-Test Risk = 21.0% (152/725)		
50–75	Positive	> 900	223	101	324	68.8% (223/324) (63.5%, 73.8%)	4.87	(4.04, 5.87)
	Indeterminate	300 – $\leq$ 900	34	122	156	21.8% (34/156) (15.6%, 29.1%)	0.61	(0.43, 0.87)
	Negative	< 300	17	381	398	4.3% (17/398) (2.5%, 6.8%)	0.10	(0.06, 0.16)
	Total		274	604	878	Pre-Test Risk = 31.2% (274/878)		
> 75	Positive	> 1800	113	67	180	62.8% (113/180) (55.3%, 69.9%)	2.77	(2.21, 3.47)
	Indeterminate	300 – $\leq$ 1800	32	97	129	24.8% (32/129) (17.6%, 33.2%)	0.54	(0.38, 0.77)
	Negative	< 300	7	86	93	7.5% (7/93) (3.1%, 14.9%)	0.13	(0.06, 0.28)
	Total		152	250	402	Pre-Test Risk = 37.8% (152/402)		

^a 95% Confidence Interval.

TABLE 29

ED Population – Clinical Agreement between NT-proBNP Results and
 Adjudicated Acute Heart Failure Diagnosis in Patients with History of Heart Failure

Age (years)	NT-proBNP	Cut-off pg/mL	Adjudicated Diagnosis			Post-Test Risk (%)	LR±	95% CI
			Positive	Negative	Total			
< 50	Positive	> 450	157	63	220	71.4% (157/220) (64.9%, 77.2%)*	1.72	(1.44, 2.05)
	Indeterminate	300 ~ ≤ 450	5	8	13	38.5% (5/13) (13.9%, 68.4%)	0.43	(0.14, 1.29)
	Negative	< 300	12	49	61	19.7% (12/61) (10.6%, 31.8%)	0.17	(0.09, 0.30)
	Total		174	120	294	Pre-Test Risk = 59.2% (174/294)		
50–75	Positive	≥ 900	312	94	406	76.8% (312/406) (72.4%, 80.9%)	2.15	(1.83, 2.54)
	Indeterminate	300 ~ ≤ 900	44	49	93	47.3% (44/93) (35.9%, 57.9%)	0.58	(0.40, 0.85)
	Negative	< 300	20	101	121	16.5% (20/121) (10.4%, 24.4%)	0.13	(0.08, 0.20)
	Total		376	244	620	Pre-Test Risk = 60.6% (376/620)		
> 75	Positive	> 1800	265	86	351	75.5% (265/351) (70.7%, 79.9%)	1.66	(1.44, 1.95)
	Indeterminate	300 ~ ≤ 1800	30	63	93	32.3% (30/93) (22.9%, 42.7%)	0.26	(0.18, 0.38)
	Negative	< 300	4	14	18	22.2% (4/18) (6.4%, 47.6%)	0.16	(0.05, 0.47)
	Total		299	163	462	Pre-Test Risk = 64.7% (299/462)		

* 95% Confidence Interval.

TABLE 30

ED Population – Clinical Agreement between NT-proBNP Results and Adjudicated Acute Heart Failure Diagnosis in Patients with No History of Heart Failure

Age (years)	NT-proBNP	Cut-off pg/ml	Adjudicated Diagnosis			Post-Test Risk (%)	LR+	95% CI
			Positive	Negative	Total			
< 50	Positive	> 450	47	53	100	47.0% (47/100) (36.3%, 57.2%) ^a	8.94	(6.84, 11.68)
	Indeterminate	300 -- ≤ 450	3	10	13	23.1% (3/13) (5.0%, 53.8%)	3.02	(0.86, 10.64)
	Negative	< 300	1	451	452	0.2% (1/452) (0.0%, 1.2%)	0.02	(0.00, 0.16)
	Total		51	514	565	Pre-Test Risk = 9.0% (51/565)		
50-75	Positive	> 900	87	80	167	52.1% (87/167) (44.2%, 59.9%)	5.73	(4.62, 7.11)
	Indeterminate	300 -- ≤ 900	10	102	112	8.9% (10/112) (4.4%, 15.8%)	0.52	(0.28, 0.95)
	Negative	< 300	3	345	348	0.9% (3/348) (0.2%, 2.5%)	0.05	(0.02, 0.14)
	Total		100	527	627	Pre-Test Risk = 15.9% (100/627)		
> 75	Positive	> 1800	106	83	189	56.1% (106/189) (48.7%, 63.3%)	2.83	(2.31, 3.47)
	Indeterminate	300 -- ≤ 1800	25	118	143	17.5% (25/143) (11.6%, 24.7%)	0.47	(0.32, 0.69)
	Negative	< 300	3	96	99	3.0% (3/99) (0.6%, 8.6%)	0.07	(0.02, 0.21)
	Total		134	297	431	Pre-Test Risk = 31.1% (134/431)		

^a 95% Confidence Interval.

TABLE 31

ED Population – Clinical Agreement between NT-proBNP Results and
Adjudicated Acute Heart Failure Diagnosis in Patients with Comorbidities

Age (years)	NT-proBNP	Cut-off pg/ml	Adjudicated Diagnosis			Post-Test Risk (%)	LR+	95% CI
			Positive	Negative	Total			
< 50	Positive	> 450	173	89	262	66.0% (173/262) {59.9%, 71.7%} ^a	3.99	(3.30, 4.82)
	Indeterminate	300 – ≤ 450	7	13	20	35.0% (7/20) {15.4%, 59.2%}	1.11	(0.45, 2.72)
	Negative	< 300	11	290	301	3.7% (11/301) {1.8%, 6.4%}	0.08	(0.04, 0.14)
	Total ^b		191	392	583	Pre-Test Risk = 32.8% (191/583)		
50–75	Positive	> 900	374	160	534	70.0% (374/534) {66.0%, 73.9%}	3.67	(3.18, 4.23)
	Indeterminate	300 – ≤ 900	52	141	193	26.9% (52/193) {20.8%, 33.8%}	0.58	(0.43, 0.78)
	Negative	< 300	24	405	429	5.6% (24/429) {3.6%, 8.2%}	0.09	(0.06, 0.14)
	Total ^b		450	706	1156	Pre-Test Risk = 38.9% (450/1156)		
> 75	Positive	> 1800	351	155	506	69.4% (351/506) {65.1%, 73.4%}	2.41	(2.11, 2.76)
	Indeterminate	300 – ≤ 1800	52	168	220	23.6% (52/220) {18.2%, 29.8%}	0.33	(0.25, 0.44)
	Negative	< 300	7	114	121	5.8% (7/121) {2.4%, 11.6%}	0.07	(0.03, 0.14)
	Total ^b		410	437	847	Pre-Test Risk = 48.4% (410/847)		

^a 95% Confidence Interval.

^b Includes subjects with at least one of the following comorbidities: diabetes, renal insufficiency (eGFR values < 60 mL/min/1.73 m²), and/or hypertension.

TABLE 32

ED Population – Clinical Agreement between NT-proBNP Results and
 Adjudicated Acute Heart Failure Diagnosis in Patients without Comorbidities

Age (years)	NT-proBNP	Cut-off pg/mL	Adjudicated Diagnosis			Post-Test Risk (%)	LR+	95% CI
			Positive	Negative	Total			
< 50	Positive	> 450	29	31	60	48.3% (29/60) (35.2%, 61.6%) ^a	7.72	(5.44, 10.94)
	Indeterminate	300 – ≤ 450	1	6	7	14.3% (1/7) (0.4%, 57.9%)	1.38	(0.17, 11.06)
	Negative	< 300	2	227	229	0.9% (2/229) (0.1%, 3.1%)	0.07	(0.02, 0.28)
	Total ^b		32	264	296	Pre-Test Risk = 10.8% (32/296)		
50–75	Positive	> 900	25	23	48	52.1% (25/48) (37.2%, 66.7%)	4.23	(2.00, 6.16)
	Indeterminate	300 – ≤ 900	2	18	20	10.0% (2/20) (1.2%, 31.7%)	0.43	(0.11, 1.75)
	Negative	< 300	0	64	64	0.0% (0/64)	N/A ^c	N/A
	Total ^b		27	105	132	Pre-Test Risk = 20.5% (27/132)		
> 75	Positive	> 1800	22	18	40	55.0% (22/40) (38.5%, 70.7%)	2.10	(1.44, 3.08)
	Indeterminate	300 – ≤ 1800	3	17	20	15.0% (3/20) (3.2%, 37.9%)	0.30	(0.10, 0.93)
	Negative	< 300	0	8	8	0.0% (0/8)	N/A	N/A
	Total ^b		25	43	68	Pre-Test Risk = 36.82% (25/68)		

^a 95% Confidence Interval.

^b Includes subjects with at least one of the following comorbidities: diabetes, renal insufficiency (eGFR values < 60 mL/min/1.73 m²), and/or hypertension.

^c Not applicable.

[0132] The following statements are based on performance in the clinical study:

[0133] Patients with BMI ≥ 30 kg/m² had a higher rate of false negatives compared to those patients with BMI < 30 kg/m². Of the total false negatives (44/1148), 41 (93%) came from patients with BMI ≥ 30 kg/m², 1 (2.2%) came from a patient with a BMI < 30 kg/m², and 2 (4.4%) were from patients with unknown BMI.

[0134] Patients with eGFR < 60 mL/min/1.73 m² had a higher rate of false positives compared to those patients with eGFR ≥ 60 mL/min/1.73 m². Of the 492 total patients with eGFR < 60 mL/min/1.73 m² adjudicated as no acute HF, 226 (45.9%) had NT-proBNP concentrations ≥ ASC. Of the 1427 total patients with eGFR ≥ 60 mL/min/1.73 m² adjudicated as no acute HF, 251 (17.6%) had NT-proBNP concentrations ≥ ASC. Sixty-one (61) patients adjudicated as no acute HF (3.1%) had unknown eGFR.

[0135] Patients with a history of HF had a higher rate of false positives compared to those patients with no history of HF. Of the 527 total patients with a history of HF adjudicated as no acute HF, 243 (46.1%) had NT-proBNP concentrations $\geq$ ASC. Of the 1338 total patients with no history of HF adjudicated as no HF, 216 (16.1%) had NT-proBNP concentrations of $\geq$ ASC. Of the 1980 patients adjudicated as no acute HF, 115 (5.8%) had unknown history of HF.

[0136] Variation in NT-proBNP concentrations due to high BMI, low GFR, and history of HF are extensively supported by literature.

Clinical Performance in the OP Population

[0137] A total of 1033 OP subjects with signs and symptoms of new onset HF were prospectively enrolled in a multi-site clinical evaluation of one non-limiting embodiment of the NT-proBNP assay of the present disclosure. The clinical performance was assessed using a single cut-off value of 125 pg/ml.

[0138] Diagnosis and severity of HF were determined by an independent central adjudication panel of expert clinicians (cardiologists). One hundred eighty-five (185) subjects were adjudicated as new onset HF, and 848 subjects were adjudicated as without HF.

TABLE 33

OP Population – Baseline Characteristics

	Subjects with new onset HF	Subjects without HF
N	185	848
Mean pg/mL (pmol/L)	1618 (191)	200 (23.6)
SD pg/mL (pmol/L)	4332 (511)	364 (43)
Median pg/mL (pmol/L)	504 (59.5)	79 (9.32)
Quartile 1 ^a pg/mL (pmol/L)	222 (26.2)	38 (4.48)
Quartile 3 ^b pg/mL (pmol/L)	1152 (136)	189 (22.3)
Minimum pg/mL (pmol/L)	< 35 (4.13)	< 35 (4.13)
Maximum pg/mL (pmol/L)	> 35,000 (4130)	3943 (465)

^a 25th Percentile.

^b 75th Percentile.

Clinical Sensitivity and Specificity

[0139] Clinical sensitivity and specificity were determined for the OP population and relevant OP clinical subgroups by comparing the performance of the non-limiting embodiment of the NT-proBNP assay of the present disclosure to the adjudicated diagnosis. Using a single cut-off of 126 pg/mL, relative to the adjudicated diagnosis of new onset HF, the analysis consisted of calculations of clinical sensitivity, clinical specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV). The results are summarized in Tables 34-38.

[0140] Clinical sensitivity and specificity were determined in accordance with CLSI Document EP12-A2.

TABLE 34

OP Population Clinical Performance – All Subjects

Cut-off Value	Sensitivity	Specificity	PPV	NPV
125 pg/mL	86.0 % (159/185) (80.1%, 90.6%) ^a	64.3% (545/848) (60.9%, 67.5%)	34.4% (159/462) (30.1%, 39.0%)	95.5% (545/571) (93.4%, 97.0%)

^a 95% Confidence Interval.

TABLE 35

OP Population Clinical Performance by sex and age group

Group	Sensitivity	Specificity	PPV	NPV
Males ≤ 75 years	85.5% (53/62) (74.2%, 93.1%) ^a	68.9% (179/260) (62.8%, 74.4%)	39.6% (53/134) (31.2%, 48.4%)	95.2% (179/188) (91.1%, 97.8%)
Females ≤ 75 years	79.2% (57/72) (67.5%, 87.8%)	70.8% (335/473) (67.0%, 74.9%)	29.2% (57/195) (23.0%, 36.2%)	95.7% (335/350) (93.0%, 97.6%)
Males > 75 years	90.5% (19/21) (69.6%, 98.8%)	37.2% (16/43) (22.8%, 51.7%)	41.3% (19/46) (27.0%, 56.8%)	88.9% (16/18) (65.3%, 98.6%)
Females > 75 years	100% (30/30) (88.4%, 100%)	20.8% (15/72) (12.2%, 32.0%)	34.5% (30/87) (24.6%, 45.4%)	100% (15/15) (78.2%, 100%)

^a 95% Confidence Interval.

TABLE 36

OP Population Clinical Performance by BMI

BMI ^a	Sensitivity	Specificity	PPV	NPV
BMI < 30 kg/m ² (N = 469)	88.2% (67/76) (87.7%, 99.6%) ^b	60.6% (238/393) (63.5%, 75.0%)	30.2% (67/222) (31.9%, 49.1%)	96.4% (238/247) (95.1%, 99.9%)
BMI ≥ 30 kg/m ² (N = 558)	84.4 (92/109) (76.2%, 90.6%)	67.5% (303/449) (62.9%, 71.8%)	38.7% (92/238) (32.4%, 45.2%)	94.7% (303/320) (91.6%, 96.9%)

^a BMI status not available (N = 6).

^b 95% Confidence Interval.

TABLE 37

OP Population Clinical Performance by eGFR

eGFR ^a	Sensitivity	Specificity	PPV	NPV
eGFR < 60 mL/min/1.73 m ² (N = 88)	100.0% (33/33) (89.4%, 100.0%) ^b	32.7% (18/55) (20.7%, 46.7%)	47.1% (33/70) (35.1%, 59.5%)	100.0% (18/18) (81.5%, 100.0%)
eGFR ≥ 60 mL/min/1.73 m ² (N = 318)	96.4% (54/56) (87.7%, 99.6%)	69.5% (182/262) (63.5%, 75.0%)	40.3% (54/134) (31.9%, 49.1%)	98.9% (182/184) (96.1%, 99.9%)

^a eGFR status not available (N = 627).^b 95% Confidence Interval.

TABLE 38

OP Population Clinical Performance by Comorbidities

Comorbidities ^a	Sensitivity	Specificity	PPV	NPV
Comorbidities present (N = 829)	86.4% (153/177) (80.5%, 91.1%) ^b	60.7% (396/652) (56.9%, 64.5%)	37.4% (153/409) (32.7%, 42.3%)	94.3% (396/420) (91.6%, 96.3%)
Comorbidities not present (N = 187)	75.0% (6/8) (34.9, 96.8%)	76.0% (136/179) (69.0%, 82.0%)	12.2% (6/49) (4.6%, 24.8%)	98.6% (136/138) (94.9%, 99.8%)

^a Includes subjects with at least one of the following comorbidities: diabetes, renal insufficiency (eGFR values < 60), and/or hypertension. Comorbidity status not available (N = 17).^b 95% Confidence Interval.**[0141]** Precision

[0142] Precision was determined in accordance with CLSI Document EP05-A3. Samples were assayed in duplicate in 2 runs per day for 20 days. The results in Table 39 are representative of the performance of the assay.

TABLE 39

Sample	N ^a	Mean pg/mL (pmol/L)	Repeatability		Within-Laboratory Precision	
			SD ^b pg/mL (pmol/L)	CV ^c (%)	SD pg/mL (pmol/L)	CV (%)
Serum A	80	116 (13.7)	6.1 (0.720)	5.3	7.8 (0.920)	6.7
Serum B	80	271 (32.0)	9.6 (1.13)	3.5	10.8 (1.27)	4.0
Serum C	80	380 (44.8)	5.4 (0.637)	1.4	8.9 (1.05)	2.3
Serum D	80	806 (95.1)	15.3 (1.81)	1.9	16.7 (1.97)	2.1
Serum E	80	1597 (188)	25.7 (3.03)	1.6	31.6 (3.73)	2.0
Serum F	80	25,073 (2959)	416.4 (49.1)	1.7	690.1 (81.4)	2.8

Sample	N ^a	Mean pg/mL (pmol/L)	Repeatability		Within-Laboratory Precision	
			SD ^b pg/mL (pmol/L)	CV ^c (%)	SD pg/mL (pmol/L)	CV (%)
Control 1	80	144 (17.0)	5.6 (0.661)	3.9	6.1 (0.720)	4.2
Control 2	80	418 (49.3)	8.7 (1.03)	2.1	12.1 (1.43)	2.9
Control 3	80	4778 (564)	93.0 (11.0)	1.9	134.7 (15.9)	2.8

^a Number of measurements.

^b Standard deviation.

^c Coefficient of variation.

The assay was designed to have the following precision.

Concentration Interval	Precision	
	Repeatability (Within-Run)	Within-Laboratory (Total Precision)
100–500 pg/mL (11.8–59.0 pmol/L)	≤ 5.5% CV	≤ 8.0% CV
> 500 pg/mL (59.0 pmol/L)	≤ 7.0% CV	≤ 10.0% CV

[0143] Reproducibility

[0144] Reproducibility was determined in accordance with CLSI Document EP05-A3. Samples (N = 90) were assayed in triplicate in 2 runs per day for 5 days at 3 sites. The results shown in Table 40 are representative of the performance of the assay.

TABLE 40

Sample	Mean pg/mL (pmol/L)	Repeatability		Between Run		Between Day		Between Site		Reproducibility	
		SD ^a pg/mL (pmol/L)	CV ^b (%)	SD pg/mL (pmol/L)	CV (%)	SD pg/mL (pmol/L)	CV (%)	SD pg/mL (pmol/L)	CV (%)	SD pg/mL (pmol/L)	CV (%)
Serum A	141 (16.6)	4.1 (0.484)	2.9	0 (0.000)	0.0	0.8 (0.094)	0.6	5.7 (0.673)	4.0	7 (0.826)	5.0
Serum B	324 (38.2)	7.6 (0.897)	2.4	0 (0.000)	0.0	2.6 (0.307)	0.8	6.7 (0.791)	2.1	10.5 (1.24)	3.2
Serum C	489 (57.7)	8.5 (1.00)	1.7	0 (0.000)	0.0	4.9 (0.578)	1.0	15 (1.77)	3.1	18 (2.12)	3.7
Serum D	824 (97.2)	14.7 (1.73)	1.8	0 (0.000)	0.0	9.8 (1.16)	1.2	18.5 (2.18)	2.2	25.5 (3.01)	3.1
Serum E	1641 (194)	29.4 (3.47)	1.8	13.9 (1.64)	0.8	9.6 (1.13)	0.6	35 (4.13)	2.1	48.7 (5.75)	3.0
Serum F	10,428 (1231)	184.6 (21.8)	1.8	49.7 (5.86)	0.5	95.7 (11.3)	0.9	374.3 (44.2)	3.6	431.1 (50.9)	4.1
Serum G	19,469 (2297)	349.1 (41.2)	1.8	147.4 (17.4)	0.8	0 (0.000)	0.0	643.1 (75.9)	3.3	746.5 (88.1)	3.8
Serum H	28,860 (3405)	696.8 (82.2)	2.4	0 (0.000)	0.0	127.4 (15.0)	0.4	736.1 (86.9)	2.6	1021.6 (121)	3.5
Control 1	146 (17.2)	4 (0.472)	2.8	1.2 (0.142)	0.8	1.7 (0.201)	1.2	4.8 (0.566)	3.3	6.6 (0.779)	4.5
Control 2	430 (50.7)	9.3 (1.10)	2.2	3.8 (0.448)	0.9	1.9 (0.224)	0.4	12.9 (1.52)	3.0	16.5 (1.95)	3.8
Control 3	4965 (586)	89 (10.5)	1.8	42.9 (5.06)	0.9	32.4 (3.82)	0.7	129 (15.2)	2.6	165.7 (19.6)	3.3

^a Standard deviation.^b Coefficient of variation.**[0145]** Specimen Equivalency

[0146] Specimen equivalency was determined with the Passing-Bablok regression model in accordance with CLSI Document EP09c-ed3. Agreement of the specimen types may vary depending on the study design and population tested.

TABLE 41

Tube (y) vs. Serum (x)	Regression Equation	Sample Interval	N ^a	r ^b
Plasma, dipotassium EDTA	$y = 1.00x + 1 \text{ pg/mL}$ ($y = 1.00x + 0.118 \text{ pmol/L}$)	50–30,134 pg/mL (5.90–3556 pmol/L)	50	1.000
Plasma, lithium heparin	$y = 1.00x + 4 \text{ pg/mL}$ ($y = 1.00x + 0.472 \text{ pmol/L}$)	57–30,390 pg/mL (6.73–3586 pmol/L)	50	0.999
Gel-barrier tube 1 (serum)	$y = 1.00x - 2 \text{ pg/mL}$ ($y = 1.00x - 0.236 \text{ pmol/L}$)	54–30,122 pg/mL (6.37–3554 pmol/L)	50	1.000
Gel-barrier tube 2 (serum)	$y = 1.00x + 4 \text{ pg/mL}$ ($y = 1.00x + 0.472 \text{ pmol/L}$)	53–30,237 pg/mL (6.25–3568 pmol/L)	50	1.000

^a Number of samples tested.^b Correlation coefficient.

[0147] The assay is designed to have a correlation coefficient of 0.950 – 1.000, a slope of 1.00 ± 0.05 , and an intercept of $\leq 6 \text{ pg/mL}$ (0.708 pmol/L).

[0148] Interferences

[0149] Hemolysis, Icterus, Lipemia (HIL)

[0150] Interference testing was performed in accordance with CLSI Document EP07-ed3. The substances listed in Table 42 do not interfere with the assay when present in serum at the concentrations indicated. Bias due to these substances does not exceed 10% at NT-proBNP concentrations of 123 - 137 pg/mL (14.5 – 16.2 pmol/L) and 1612 - 1813 pg/mL (190 - 214 pmol/L).

TABLE 42

Substance	Substance Test Concentration
Hemoglobin	1000 mg/dL
Bilirubin, conjugated	60.0 mg/dL
Bilirubin, unconjugated	60.0 mg/dL
Lipemia (Intralipid)	3000 mg/dL

[0151] Other substances

[0152] Interference testing was performed in accordance with CLSI Document EP07-ed3. The substances listed in Table 43 do not interfere with the assay when present in serum at the concentrations indicated. Bias due to these substances does not exceed 10% at NT-proBNP concentrations of 116 – 163 pg/mL (13.7 – 19.2 pmol/L) and 1478 - 2225 pg/mL (174 - 263 pmol/L).

TABLE 43

Substance	Substance Test Concentration	Substance	Substance Test Concentration
Abciximab	21.0 µg/mL	L-dopa (Levodopa)	0.750 mg/dL
Acetaminophen	20.0 mg/dL	Lidocaine	8.00 mg/dL
Acetylcysteine	15.0 mg/dL	Lisinopryl	16.0 µg/mL
Allopurinol	2.50 mg/dL	Lovastatin	16.0 µg/mL
Amiodarone	42.0 µg/mL	L-Thyroxine	60.0 µg/dL
Amlodipine Besylate	4.00 µg/mL	Methyldopa	2.50 mg/dL
Ampicillin	7.50 mg/dL	Methylprednisolone	0.783 mg/dL
Ascorbic Acid	5.00 mg/dL	Metoprolol tartrate	15.0 mg/dL
Atenolol	1.00 mg/dL	Metronidazole	12.3 mg/dL
Atorvastatin	32.0 mg/dL	Milrinone lactate	2.40 µg/mL
Biotin	3510 ng/mL	Molsidomin	0.018 mg/dL
Bisoprolol	0.258 µg/mL	Nicotine	0.100 mg/dL
Caffeine	10.8 mg/dL	Nifedipine	6.00 mg/dL
Calcium dobesilate	6.00 mg/dL	Nitrofurantoin	40.0 µg/mL
Captopril	5.00 mg/dL	Nitroglycerine	0.160 µg/mL
Carvedilol	4.32 mg/dL	Oxazepam	12.0 µg/mL
Cefoxitin	660 mg/dL	Oxytetracycline	100 µg/mL
Chloramphenicol	7.80 mg/dL	Phenobarbital	69.0 mg/dL
Chlordiazepoxide	1.00 mg/dL	Phenprocoumon	1.50 mg/dL
Cholesterol	500 mg/dL	Phenylbutazone	32.1 mg/dL
Cinnarizine	3.00 mg/dL	Phenytoin	6.00 mg/dL
Clopidogrel bisulfate	30.0 µg/mL	Pravastatin	0.021 mg/dL
Creatinine	30.0 mg/dL	Probenecid	200 µg/mL
Cyclosporine	4000 ng/mL	Propafenone HCL	30.0 mg/dL
Diclofenac	60.0 µg/mL	Propanolol	0.150 mg/dL
Digitoxin	75.0 ng/mL	Protein: Albumin	6.00 g/dL
Digoxin	390 ng/mL	Protein: Total	11.1 g/dL
Diltiazem	120 µg/mL	Quinidine	20.0 µg/mL
Dipotassium EDTA	9.00 mg/mL	Retavase (reteplase)	3.33 mg/dL
Dipyridamole	30.0 µg/mL	Rheumatoid Factor	1500 IU/mL

Substance	Substance Test Concentration	Substance	Substance Test Concentration
Disopyramide	40.0 µg/mL	Rifampicin (Rifampin)	4.80 mg/dL
Dopamine	16.0 mg/dL	Salicylic Acid	60.0 mg/dL
Doxycycline hyclate	1.80 mg/dL	Simvastatin	32.0 µg/mL
Enalapril maleate	16.0 µg/mL	Sotalol hydrochloride	0.510 mg/dL
Epinephrine	0.050 mg/dL	Spironolactone	7.50 mg/dL
Erythromycin	13.8 mg/dL	Streptokinase	150,000 U/L
Furosemide	6.00 mg/dL	Sulfamethoxazole	320 µg/mL
Gentamycin sulfate	3.51 mg/dL	Theophylline	4.00 mg/dL
HAMA (Human Anti-Mouse Antibody)	800 µg/L	Tolbutamide	150 mg/dL
Heparin	330 U/dL	Torsemide	1.50 mg/dL
Hydralazine	20.0 µg/mL	Triglycerides	1000 mg/dL
Hydrochlorothiazide	20.0 µg/mL	Trimethoprim	64.0 µg/mL
Immunoglobulin G (IgG)	5.00 g/dL	Urokinase	150,000 U/L
Indomethacin	16.0 µg/mL	Verapamil	96.0 µg/mL
Insulin	0.160 mg/dL	Warfarin	8.00 mg/dL
Isosorbide dinitrate	6.00 mg/dL		

[0153] Cross-Reactivity

[0154] Cross-reactivity was determined in accordance with CLSI Document EP07-ed3. Cross-reactants listed in Table 44 were tested at NT-proBNP concentrations of 0 pg/mL (0 pmol/L) and 126 – 158 pg/mL (14.9 – 18.6 pmol/L).

TABLE 44

Cross-reactant	Cross-reactant Concentration	Cross-reactivity (%)
Adrenomedullin	1.00 ng/mL	Not Detectable < 1.0%
Aldosterone	0.600 ng/mL	Not Detectable < 1.0%
Angiotensin I	0.600 ng/mL	Not Detectable < 1.0%
Angiotensin II	0.600 ng/mL	Not Detectable < 1.0%
Angiotensin III	1.00 ng/mL	Not Detectable < 1.0%
ANP28	3.10 µg/mL	Not Detectable < 1.0%
Arg-Vasopressin	1.00 ng/mL	Not Detectable < 1.0%
BNP32	3.50 µg/mL	Not Detectable < 1.0%
CNP32	2.20 µg/mL	Not Detectable < 1.0%
DNP	1.00 ng/mL	Not Detectable < 1.0%
Endothelin	20.0 pg/mL	Not Detectable -5.0%
preproANP26–55	3.50 µg/mL	Not Detectable < 1.0%
preproANP56–92	1.00 ng/mL	Not Detectable < 1.0%
preproANP104–123	1.00 ng/mL	Not Detectable < 1.0%
proBNP (glycosylated)	3000 pg/mL	1.0% 19.0%
proBNP (non-glycosylated)	3000 pg/mL	13.0% 30.0%
Renin (human)	50.0 ng/mL	Not Detectable < 1.0%
Urodilatin	3.50 µg/mL	Not Detectable < 1.0%
VNP	1.00 ng/mL	Not Detectable < 1.0%

- [0155] Linearity
- [0156] Linearity testing was performed in accordance with CLSI Document EP06-ed2. The assay is linear for the measuring interval of 35 – 35,000 pg/mL (4.13 – 4130 pmol/L).
- [0157] Onboard Dilution Recovery
- [0158] Serum samples were diluted onboard the ADVIA Centaur XP System with Multi-Diluent 1. The results in Table 45 are representative of the performance of the assay.

TABLE 45

Sample	Dilution	Observed (pg/mL)	Expected (pg/mL)	Observed (pmol/L)	Expected (pmol/L)	Recovery (%)
1	1:5	32,764	37,555	3866	4431	87.2
2	1:5	40,188	45,052	4742	5316	89.2
3	1:5	40,312	44,967	4757	5306	89.6
4	1:5	62,285	62,430	7350	7367	99.8
5	1:5	47,984	48,968	5662	5778	98.0
Mean						92.8
1	1:10	33,979	37,555	4010	4431	90.5
2	1:10	40,914	45,052	4828	5316	90.8
3	1:10	40,944	44,967	4831	5306	91.1
4	1:10	60,496	62,430	7139	7367	96.9
5	1:10	44,582	48,968	5261	5778	91.0
Mean						92.1

- [0159] High-Dose Hook Effect
- [0160] High NT-proBNP concentrations can cause a paradoxical decrease in the RLUs (high-dose hook effect). In this assay, patient samples with NT-proBNP concentrations above the measuring interval and as high as 300,000 pg/mL (35,400 pmol/L) will report > 35,000 pg/mL (4130 pmol/L).
- [0161] Standardization
- [0162] The assay is traceable to an internal standard manufactured using highly purified material. Assigned values for calibrators are traceable to this standardization. Currently no reference standard is available for this assay.

Example 2

[0163] Tables 46-47 outline the characteristics and reagents of one non-limiting embodiment of a kit constructed in accordance with the present disclosure. The non-assay related reagents listed (i.e., wash reagents, multidiluents, etc.) are available, for example but not by way of limitation, from Siemens Healthineers (Malvern, PA).

TABLE 46

Assay	NT-proBNP
Materials Required but Not Provided	Wash Reagents
Optional Materials	Multi-Diluent Master Curve Material
Specimen Types	Serum, EDTA plasma, lithium heparin plasma
Sample Volume	20 μ L
Measuring Interval	35 – 35,000 pg/mL (4.13 – 4130 pmol/L)

TABLE 47

Material Description
Lite Reagent: Monoclonal sheep anti-human NT-proBNP F(ab') ₂ fragment antibody labeled with acridinium ester in buffer; bovine serum albumin (BSA); bovine gamma globulin; preservatives
Solid Phase Reagent: Monoclonal sheep anti-human NT-proBNP labeled with biotin bound to streptavidin magnetic particles in buffer; BSA; bovine gamma globulin; sheep gamma globulin; preservatives
Ancillary Well Reagent: Buffer; BSA; bovine gamma globulin; sheep gamma globulin; preservatives
NT-proBNP Calibrator: After reconstitution, low or high levels of NT-proBNP antigen; buffer; BSA; preservatives
Multi-Diluent:

Equine serum; sodium azide (0.1%); preservatives
Wash Reagent: Phosphate-buffered saline; sodium azide (<0.1%); surfactant

[0164] Note that in some non-limiting embodiments, the lite reagent, solid phase reagent, ancillary well reagent, calibrator, multi-diluent, and/or wash reagent can be stored unopened at 2-8°C. In addition, in some non-limiting embodiments, the lite reagent, solid phase reagent, and/or ancillary well reagent are substantially stable for at least 36 days once onboard.

Example 3

[0165] This Example contains a comparison of the biotin interference observed between the prior art 3-reagent NT-proBNP assay and one non-limiting embodiment of the 2-reagent NT-proBNP assay of the present disclosure. As can be seen, biotin interference of -28% to -23% was observed at biotin concentrations of 200 ng/mL in the prior art 3-reagent NT-proBNP assay. In contrast, substantially no biotin interference (defined as a percent bias of $\leq 10\%$) was observed in the assay of the present disclosure, even at a biotin concentration that is over 17x higher than the biotin concentration at which substantial biotin interference was observed in the prior art assay.

TABLE 48

Assay	Biotin Test Concentration	Control pg/mL	Test pg/mL	% Bias
Prior Art 3-reagent Assay	75 ng/mL	123	116	-6%
Prior Art 3-reagent Assay	75 ng/mL	1783	1751	-2%
Prior Art 3-reagent Assay	200 ng/mL	138	99	-28%
Prior Art 3-reagent Assay	200 ng/mL	2553	1978	-23%
New 2-reagent Assay	3510 ng/mL	121	127	5.0%
New 2-reagent Assay	3510 ng/mL	1709	1651	-3.4%

Example 4

[0166] This Example contains a comparison of biotin interference observed between the prior art 3-reagent NT-proBNP assay and one non-limiting embodiment of the 2-reagent NT-

proBNP assay of the present disclosure. As can be seen, the acceptable percent bias observed for the assay of the present concentration occurs at a biotin concentration that is at least about 50x higher than the biotin concentration at which an acceptable percent bias was observed in the prior art assay.

TABLE 49

Assay	Biotin Test Concentration	Control pg/mL	Test pg/mL	% Bias
Prior art 3-reagent assay	75 ng/mL	123	116	-6%
Prior art 3-reagent assay	75 ng/mL	1783	1751	-2%
New 2-reagent assay	3510 ng/mL	121	127	5.0%
New 2-reagent assay	3510 ng/mL	1709	1651	-3.4%

NON-LIMITING ILLUSTRATIVE EMBODIMENTS

[0167] Illustrative embodiment 1. A kit for use in determining an amount of NT-proBNP in a biological sample, the kit comprising: (a) a solid phase reagent comprising a solid support having a first anti-NT-proBNP antibody or binding fragment thereof directly or indirectly attached thereto; and (b) a lite reagent comprising a second anti-NT-proBNP antibody or binding fragment thereof labeled with an acridinium ester; and wherein the first and second anti-NT-proBNP antibodies or binding fragments thereof bind to non-overlapping epitopes of NT-proBNP and thereby form an immunocomplex comprising (a), (b), and NT-proBNP.

[0168] Illustrative embodiment 2. The kit of Illustrative embodiment 1, wherein the first anti-NT-proBNP antibody or binding fragment thereof is biotinylated, and wherein at least a portion of a surface of the solid support is coated with a biotin-specific binding molecule, whereby the first anti-NT-proBNP antibody or binding fragment thereof is indirectly attached to the solid support.

[0169] Illustrative embodiment 3. The kit of Illustrative embodiment 2, wherein the biotin-specific binding molecule is selected from the group consisting of streptavidin, traptavidin, and avidin.

[0170] Illustrative embodiment 4. The kit of Illustrative embodiment 3, wherein the biotin-specific binding molecule is streptavidin.

[0171] Illustrative embodiment 5. The kit of any of Illustrative embodiments 1-4, wherein the solid support comprises magnetic and/or latex particles.

[0172] Illustrative embodiment 6. The kit of any of Illustrative embodiments 1-5, wherein the solid phase reagent of (a) comprises a biotinylated monoclonal sheep anti-human antibody specific to NT-proBNP conjugated to a magnetic particles having streptavidin coated on at least a portion of a surface thereof.

[0173] Illustrative embodiment 7. The kit of any of Illustrative embodiments 1-6, wherein the lite reagent of (b) comprises an acridinium-ester-labeled monoclonal sheep anti-human NT-proBNP F(ab')₂ fragment specific to NT-proBNP.

[0174] Illustrative embodiment 8. The kit of any of Illustrative embodiments 1-7, wherein (a) is packaged separately from (b).

[0175] Illustrative embodiment 8A. The kit of any of illustrative embodiments 1-8, wherein the kit is configured for use in an emergency department setting.

[0176] Illustrative embodiment 8B. The kit of any of illustrative embodiments 1-8A, wherein the kit is configured for use in an outpatient setting.

[0177] Illustrative embodiment 8C. The kit of any of illustrative embodiments 1-8B, wherein the kit is configured for use in an emergency department and/or outpatient setting.

[0178] Illustrative embodiment 9. A method of determining the presence, severity, and/or predisposition of Heart Failure (HF) in an individual, the method including the steps of: (a) combining, either simultaneously or wholly or partially sequentially, to form a reaction mixture: (i) a biological fluid sample; (ii) a solid phase reagent comprising a solid support having a first anti-NT-proBNP antibody or binding fragment thereof attached thereto; and (iii) a lite reagent comprising a second anti-NT-proBNP antibody or binding fragment thereof labeled with an acridinium ester, wherein the first and second anti-NT-proBNP antibodies or binding fragments thereof bind to non-overlapping epitopes of NT-proBNP; (b) incubating the reaction mixture under conditions that allow for binding of (ii) and (iii) to NT-proBNP present in the biological fluid sample to form an immunocomplex; and (c) measuring an amount of immunocomplex formed to obtain a measured value for NT-proBNP in the sample.

[0179] Illustrative embodiment 10. The method of Illustrative embodiment 9, further comprising the step of: (d) using a mathematical algorithm to obtain an HF score based on the measured value of NT-proBNP in the sample.

[0180] Illustrative embodiment 11. The method of Illustrative embodiment 9 or 10, wherein the biological fluid sample is selected from the group consisting of blood, serum, plasma, and combinations thereof.

[0181] Illustrative embodiment 12. The method of any of Illustrative embodiments 9-11, wherein the first anti-NT-proBNP antibody or binding fragment thereof is biotinylated, and wherein at least a portion of a surface of the solid support is coated with a biotin-specific binding molecule, whereby the first anti-NT-proBNP antibody or binding fragment thereof is indirectly attached to the solid support.

[0182] Illustrative embodiment 13. The method of Illustrative embodiment 12, wherein the biotin-specific binding molecule is selected from the group consisting of streptavidin, traptavidin, and avidin.

[0183] Illustrative embodiment 13A. The method of illustrative embodiment 13, wherein the biotin-specific binding molecule is streptavidin.

[0184] Illustrative embodiment 14. The method of any of Illustrative embodiments 9-13A, wherein the solid support comprises magnetic and/or latex particles.

[0185] Illustrative embodiment 15. The method of any of Illustrative embodiments 9-14, wherein the solid phase reagent of (ii) comprises a biotinylated monoclonal sheep anti-human antibody specific to NT-proBNP conjugated to a magnetic particles having streptavidin coated on at least a portion of a surface thereof.

[0186] Illustrative embodiment 16. The method of any of Illustrative embodiments 9-15, wherein the lite reagent of (iii) comprises an acridinium-ester-labeled monoclonal sheep anti-human NT-proBNP F(ab')₂ fragment specific to NT-proBNP.

[0187] Illustrative embodiment 17. The method of any of Illustrative embodiments 9-16, wherein substantially no interference from biotin present in the biological fluid sample is observed.

[0188] Illustrative embodiment 17A. The method of Illustrative embodiment 17, wherein substantially no biotin interference is defined as a percent bias of $\leq 10\%$.

[0189] Illustrative embodiment 17B. The method of Illustrative embodiment 17 or 17A, wherein substantially no biotin interference is observed at a biotin concentration in the biological fluid sample of up to about 3510 ng/ml biotin

[0190] Illustrative embodiment 18. The method of any of Illustrative embodiments 9-17, wherein at least one of steps (b), (c), and (d) is performed on an automated analyzer.

[0191] Illustrative embodiment 19. The method of Illustrative embodiment 18, wherein step (c) is further defined as measuring an amount of relative light units (RLUs), wherein the amount of RLUs is directly proportional to an amount of NT-proBNP in the sample.

[0192] Illustrative embodiment 19A. The method of any of Illustrative embodiments 9-19, wherein the method is performed in an emergency department setting.

[0193] Illustrative embodiment 19B. The method of any of Illustrative embodiments 9-19A, wherein the method or a portion thereof is performed in an outpatient setting. For example, a sample may be obtained in the out-patient setting or from an out-patient strata and shared with a facility suitable for performing the methods of the present disclosure or using kits of the present disclosure. In embodiments, the sharing may be done by sending sample to the facility under conditions suitable for shipping biological materials.

[0194] Illustrative embodiment 19C. The method of any of Illustrative embodiments 9-19B, wherein the method is performed in an emergency department, point-of-care, and/or outpatient setting.

[0195] Illustrative embodiment 20. A non-transitory computer readable medium containing executable instructions that when executed cause a processor to perform operations including the method of any of Illustrative embodiments 9-19C.

[0196] Illustrative embodiment 21. A kit for use in determining an amount of NT-proBNP in a biological sample, the kit comprising: (a) a solid phase reagent comprising a biotinylated monoclonal sheep anti-human antibody specific to NT-proBNP conjugated to a magnetic particle having streptavidin coated on at least a portion of a surface thereof; (b) a lite reagent comprising acridinium-ester-labeled monoclonal sheep anti-human NT-proBNP F(ab')₂ fragment specific to NT-proBNP; and wherein the antibody of (a) and F(ab')₂ fragment of (b) bind to non-overlapping epitopes of NT-proBNP and thereby form an immunocomplex comprising (a), (b), and NT-proBNP.

[0197] Illustrative embodiment 22. The kit of Illustrative embodiment 21, wherein (a) is packaged separately from (b).

[0198] Illustrative embodiment 22A. The kit of illustrative embodiment 21 or 22, wherein the kit is configured for use in an emergency department setting.

[0199] Illustrative embodiment 22B. The kit of any of illustrative embodiments 21-22A, wherein the kit is configured for use in an outpatient setting.

[0200] Illustrative embodiment 23. A method of determining the presence, severity, and/or predisposition of Heart Failure (HF) in an individual, the method including the steps of: (a) combining, either simultaneously or wholly or partially sequentially, to form a reaction mixture: (i) a biological fluid sample; (ii) a solid phase reagent comprising a biotinylated

monoclonal sheep anti-human antibody specific to NT-proBNP conjugated to a magnetic particle having streptavidin coated on at least a portion of a surface thereof; (iii) a lute reagent comprising acridinium-ester-labeled monoclonal sheep anti-human NT-proBNP F(ab')₂ fragment specific to NT-proBNP, wherein the antibody of (ii) and F(ab')₂ fragment of (iii) bind to non-overlapping epitopes of NT-proBNP; and (b) incubating the reaction mixture under conditions that allow for binding of (ii) and (iii) to NT-proBNP present in the biological fluid sample to form an immunocomplex; and (c) measuring an amount of immunocomplex formed to obtain a measured value for NT-proBNP in the sample.

[0201] Illustrative embodiment 24. The method of Illustrative embodiment 23, further comprising the step of: (d) using a mathematical algorithm to obtain an HF score based on the measured value of NT-proBNP in the sample.

[0202] Illustrative embodiment 25. The method of Illustrative embodiment 23 or 24, wherein the biological fluid sample is selected from the group consisting of blood, serum, plasma, and combinations thereof.

[0203] Illustrative embodiment 26. The method of any of Illustrative embodiments 23-25, wherein substantially no interference from biotin present in the biological fluid sample is observed.

[0204] Illustrative embodiment 26A. The method of Illustrative embodiment 26, wherein substantially no biotin interference is defined as a percent bias of $\leq 10\%$.

[0205] Illustrative embodiment 26B. The method of Illustrative embodiment 26 or 26A, wherein substantially no biotin interference is observed at a biotin concentration in the biological fluid sample of up to about 3510 ng/ml biotin.

[0206] Illustrative embodiment 27. The method of any of Illustrative embodiments 23-26, wherein at least one of steps (b), (c), and (d) is performed on an automated analyzer.

[0207] Illustrative embodiment 28. The method of Illustrative embodiment 27, wherein step (c) is further defined as measuring an amount of relative light units (RLUs), wherein the amount of RLUs is directly proportional to an amount of NT-proBNP in the sample.

[0208] Illustrative embodiment 28A. The method of any of Illustrative embodiments 23-28, wherein the method is performed in an emergency department setting.

[0209] Illustrative embodiment 28B. The method of any of Illustrative embodiments 23-28A, wherein the method is performed in an outpatient setting.

[0210] Illustrative embodiment 28C. The method of any of illustrative embodiments 23-28, wherein the method is performed in an emergency department and/or or outpatient setting.

[0211] Illustrative embodiment 29. A non-transitory computer readable medium containing executable instructions that when executed cause a processor to perform operations including the method of any of illustrative embodiments 23-28C.

[0212] Illustrative embodiment 30. A method of determining the presence, severity, and/or predisposition of Heart Failure (HF) in an individual, the method including the steps of:

- (a) combining, either simultaneously or wholly or partially sequentially, to form a reaction mixture:
 - (i) a biological fluid sample;
 - (ii) a solid phase reagent comprising a solid support having a first anti-NT-proBNP antibody or binding fragment thereof attached thereto; and
 - (iii) a lite reagent comprising a second anti-NT-proBNP antibody or binding fragment thereof labeled with an acridinium ester, wherein the first and second anti-NT-proBNP antibodies or binding fragments thereof bind to non-overlapping epitopes of NT-proBNP;
- (b) incubating the reaction mixture under conditions that allow for binding of (ii) and (iii) to NT-proBNP present in the biological fluid sample to form an immunocomplex; and
- (c) measuring an amount of immunocomplex formed to obtain a measured value for NT-proBNP in the sample, wherein the method optionally includes the step of:
- (d) using a mathematical algorithm to obtain an HF score based on the measured value of NT-proBNP in the sample, wherein the biological fluid sample is selected from the group consisting of blood, serum, plasma, and combinations thereof.

[0213] Illustrative embodiment 31. Embodiment 30 including wherein the method is performed in an emergency department, point-of-care and/or outpatient setting.

[0214] Illustrative embodiment 32, Embodiment 30-31, wherein the biological fluid sample is obtained in the outpatient setting, and subsequently provided to the reaction mixture.

[0215] Illustrative embodiment 33, Embodiment 30-32, wherein the individual is characterized as from an out-patient strata.

[0216] Illustrative embodiment 34, Embodiment 30-33, wherein the first anti-NT-proBNP antibody or binding fragment thereof is biotinylated, and wherein at least a portion of a surface of the solid support is coated with streptavidin, whereby the first anti-NT-proBNP antibody or binding fragment thereof is indirectly attached to the solid support, wherein the solid support comprises magnetic and/or latex particles, wherein: the solid phase reagent of (ii) comprises a biotinylated monoclonal sheep anti-human antibody specific to NT-proBNP conjugated to a magnetic particles having streptavidin coated on at least a portion of a surface thereof; and the lite reagent of (iii) comprises an acridinium-ester-labeled monoclonal sheep anti-human NT-proBNP F(ab')₂ fragment specific to NT-proBNP, wherein no biotin interference is observed at a biotin concentration in the biological fluid sample of up to about 3510 ng/ml, and wherein at least one of steps (b), (c), and (d) is performed on an automated analyzer.

[0217] Thus, in accordance with the present disclosure, there have been provided compositions, kits, systems, and/or methods which fully satisfy the objectives and advantages set forth hereinabove. Although the present disclosure has been described in conjunction with the specific drawings, experimentation, results, and language set forth hereinabove, it is evident that many alternatives, modifications, and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications, and variations that fall within the spirit and broad scope of the present disclosure.

What is claimed is:

1. A kit for use in determining an amount of NT-proBNP in a biological sample, the kit comprising:
 - (a) a solid phase reagent comprising a solid support having a first anti-NT-proBNP antibody or binding fragment thereof directly or indirectly attached thereto; and
 - (b) a lite reagent comprising a second anti-NT-proBNP antibody or binding fragment thereof labeled with an acridinium ester; andwherein the first and second anti-NT-proBNP antibodies or binding fragments thereof bind to non-overlapping epitopes of NT-proBNP and thereby form an immunocomplex comprising (a), (b), and NT-proBNP.
2. The kit of claim 1, wherein the first anti-NT-proBNP antibody or binding fragment thereof is biotinylated, and wherein at least a portion of a surface of the solid support is coated with streptavidin, whereby the first anti-NT-proBNP antibody or binding fragment thereof is indirectly attached to the solid support.
3. The kit of claim 1, wherein the solid support comprises magnetic and/or latex particles.
4. The kit of claim 1, wherein:
 - the solid phase reagent of (a) comprises a biotinylated monoclonal sheep anti-human antibody specific to NT-proBNP conjugated to a magnetic particles having streptavidin coated on at least a portion of a surface thereof; and
 - the lite reagent of (b) comprises an acridinium-ester-labeled monoclonal sheep anti-human NT-proBNP F(ab')₂ fragment specific to NT-proBNP.
5. The kit of claim 1, wherein (a) is packaged separately from (b).
6. The kit of claim 1, wherein the kit is configured for use in an emergency department and/or outpatient setting.

7. A method of determining the presence, severity, and/or predisposition of Heart Failure (HF) in an individual, the method including the steps of:
- (a) combining, either simultaneously or wholly or partially sequentially, to form a reaction mixture:
 - (i) a biological fluid sample;
 - (ii) a solid phase reagent comprising a solid support having a first anti-NT-proBNP antibody or binding fragment thereof attached thereto; and
 - (iii) a lite reagent comprising a second anti-NT-proBNP antibody or binding fragment thereof labeled with an acridinium ester, wherein the first and second anti-NT-proBNP antibodies or binding fragments thereof bind to non-overlapping epitopes of NT-proBNP;
 - (b) incubating the reaction mixture under conditions that allow for binding of (ii) and (iii) to NT-proBNP present in the biological fluid sample to form an immunocomplex; and
 - (c) measuring an amount of immunocomplex formed to obtain a measured value for NT-proBNP in the sample.
8. The method of claim 7, further comprising the step of:
- (d) using a mathematical algorithm to obtain an HF score based on the measured value of NT-proBNP in the sample.
9. The method of claim 7, wherein the biological fluid sample is selected from the group consisting of blood, serum, plasma, and combinations thereof.
10. The method of claim 7, wherein the first anti-NT-proBNP antibody or binding fragment thereof is biotinylated, and wherein at least a portion of a surface of the solid support is coated with streptavidin, whereby the first anti-NT-proBNP antibody or binding fragment thereof is indirectly attached to the solid support.
11. The method of claim 7, wherein the solid support comprises magnetic and/or latex particles.

12. The method of claim 7, wherein:
- the solid phase reagent of (ii) comprises a biotinylated monoclonal sheep anti-human antibody specific to NT-proBNP conjugated to a magnetic particles having streptavidin coated on at least a portion of a surface thereof; and
 - the lite reagent of (iii) comprises an acridinium-ester-labeled monoclonal sheep anti-human NT-proBNP F(ab')₂ fragment specific to NT-proBNP.
13. The method of claim 7, wherein no biotin interference is observed at a biotin concentration in the biological fluid sample of up to about 3510 ng/ml.
14. The method of claim 7, wherein at least one of steps (b), (c), and (d) is performed on an automated analyzer.
15. The method of claim 14, wherein step (c) is further defined as measuring an amount of relative light units (RLUs), wherein the amount of RLUs is directly proportional to an amount of NT-proBNP in the sample.
16. The method of claim 7, wherein the method is performed in an emergency department, point-of-care and/or outpatient setting.
17. The method of claim 7, wherein the biological fluid sample is obtained in the outpatient setting, and subsequently provided to the reaction mixture.
18. The method of claim 7, wherein the individual is characterized as from an outpatient strata.
17. A non-transitory computer readable medium containing executable instructions that when executed cause a processor to perform operations including the method of claim 7.

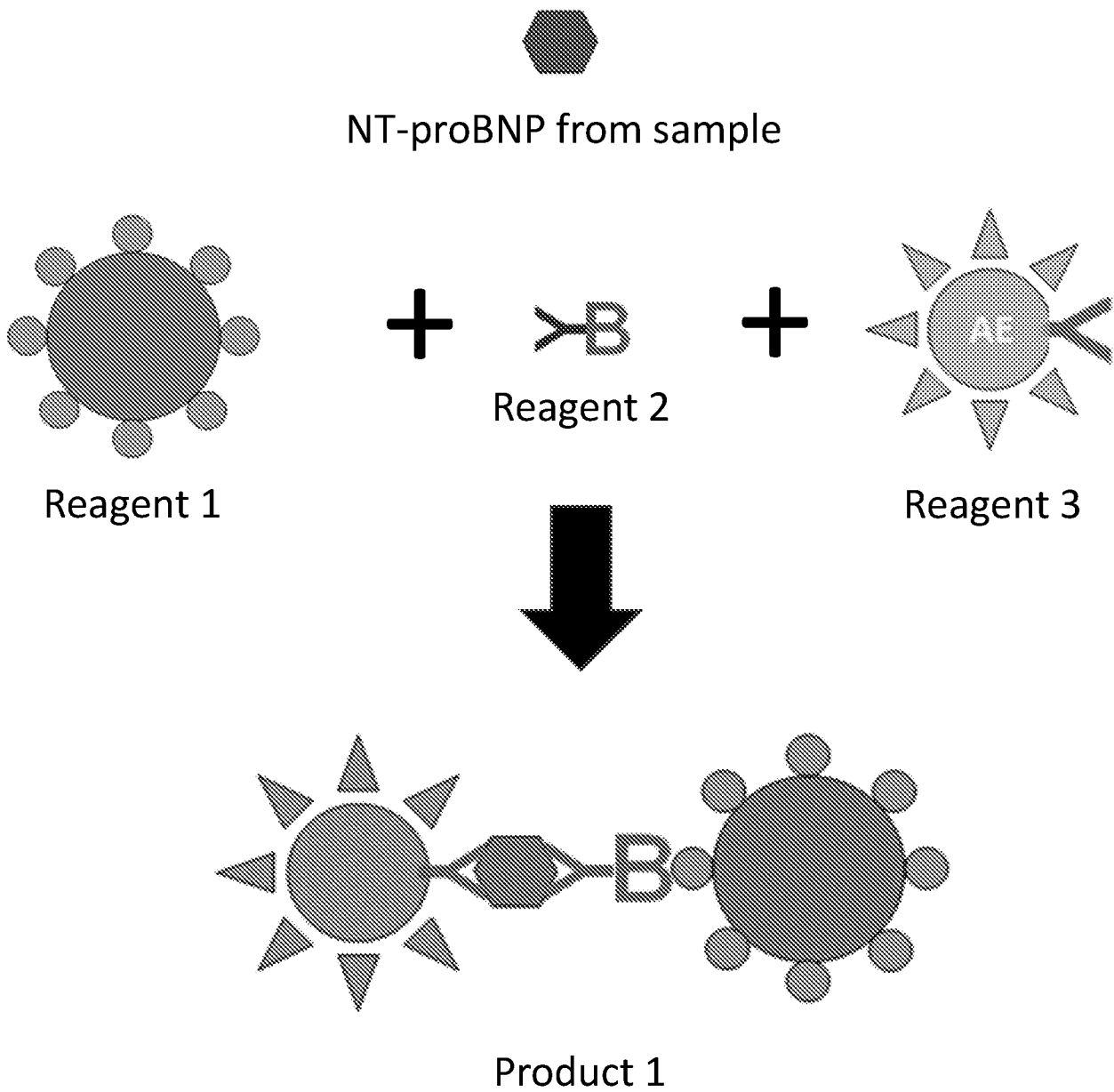


FIG. 1
PRIOR ART

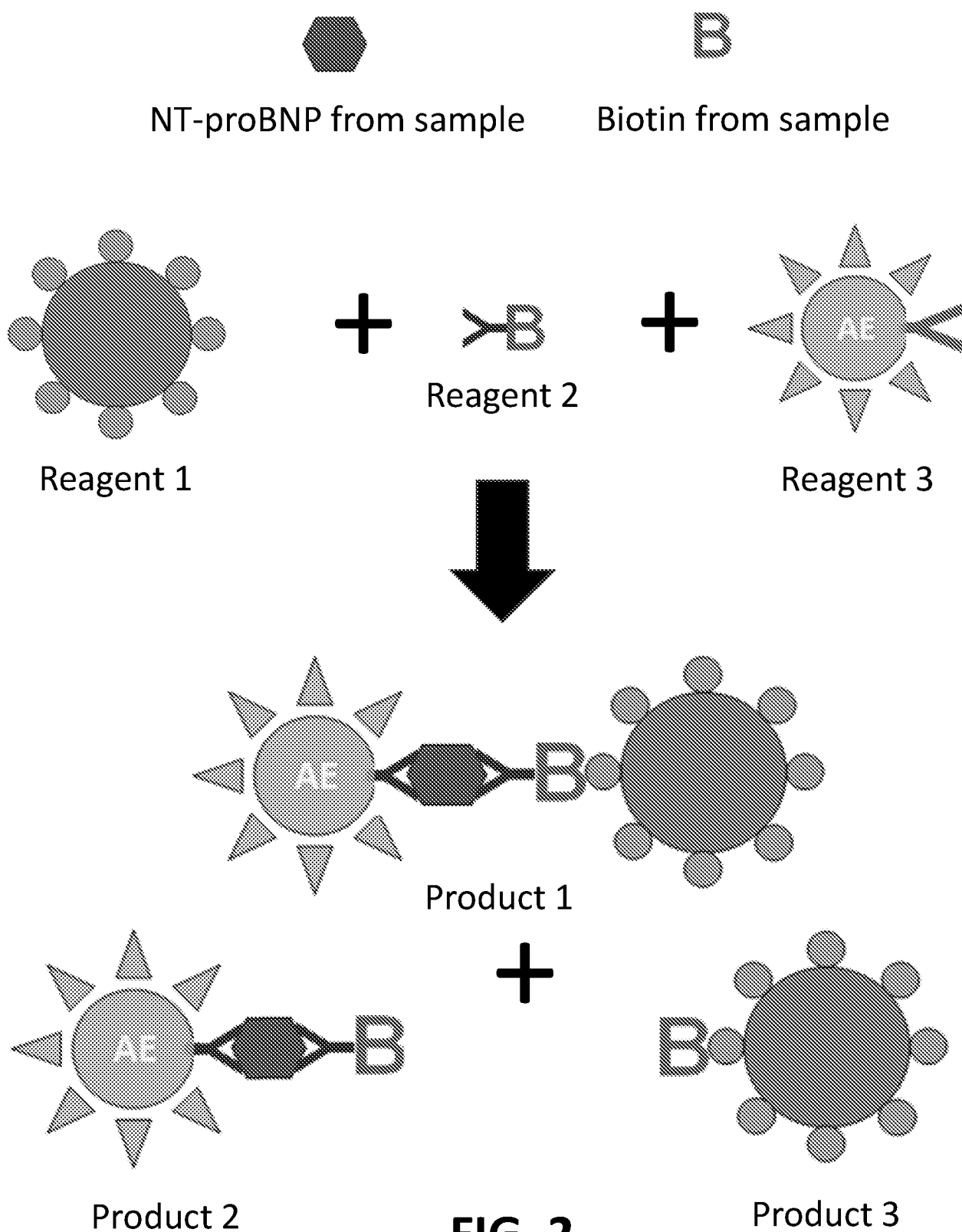
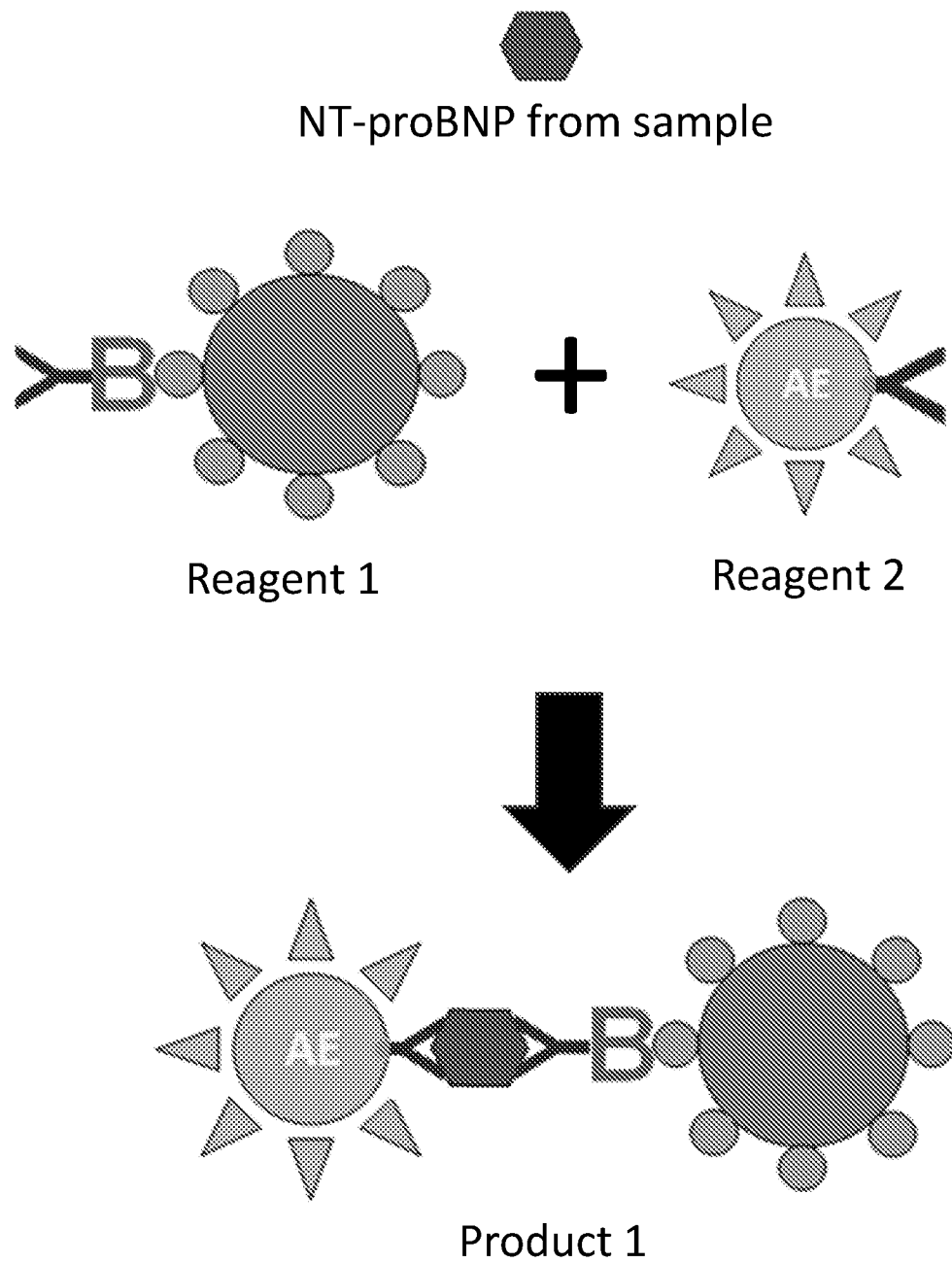
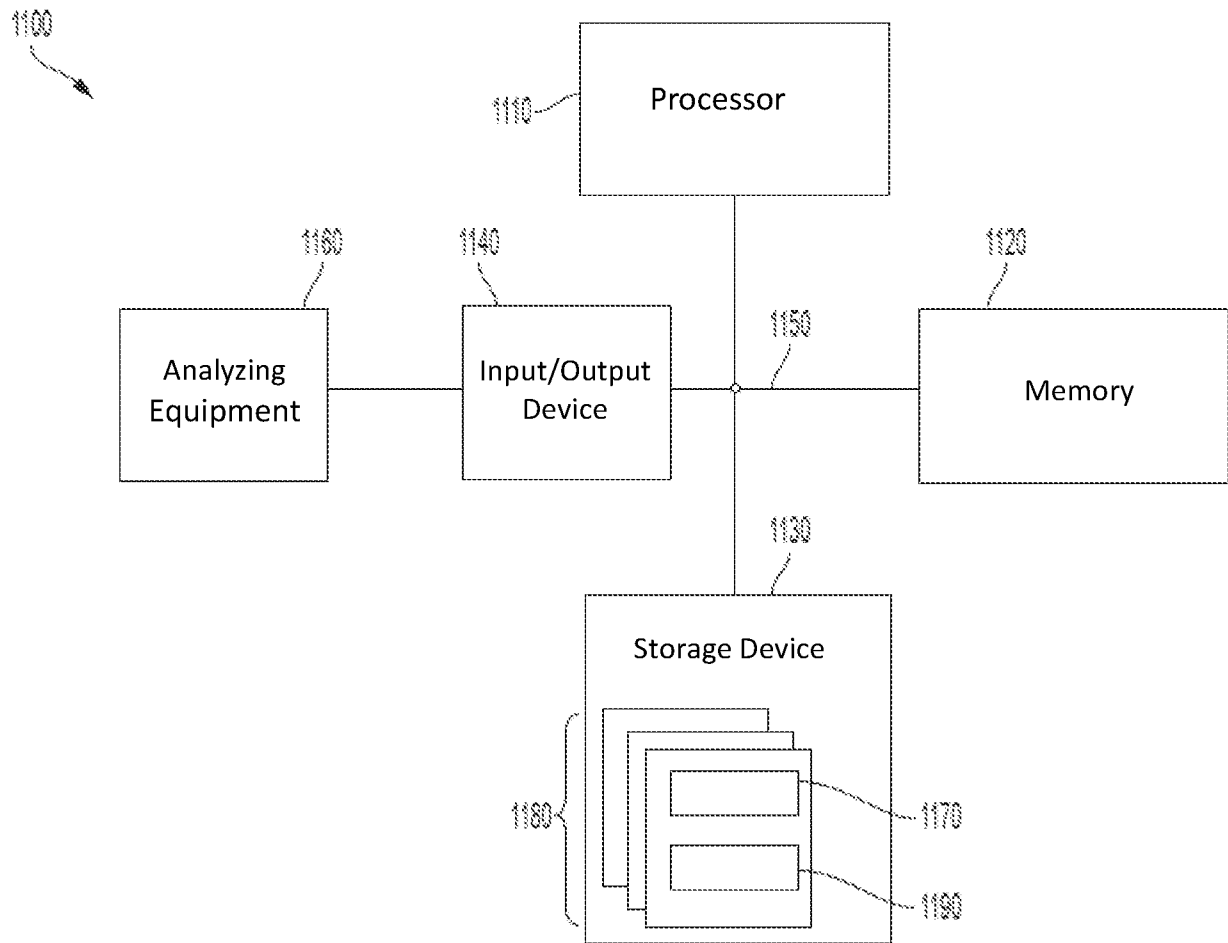
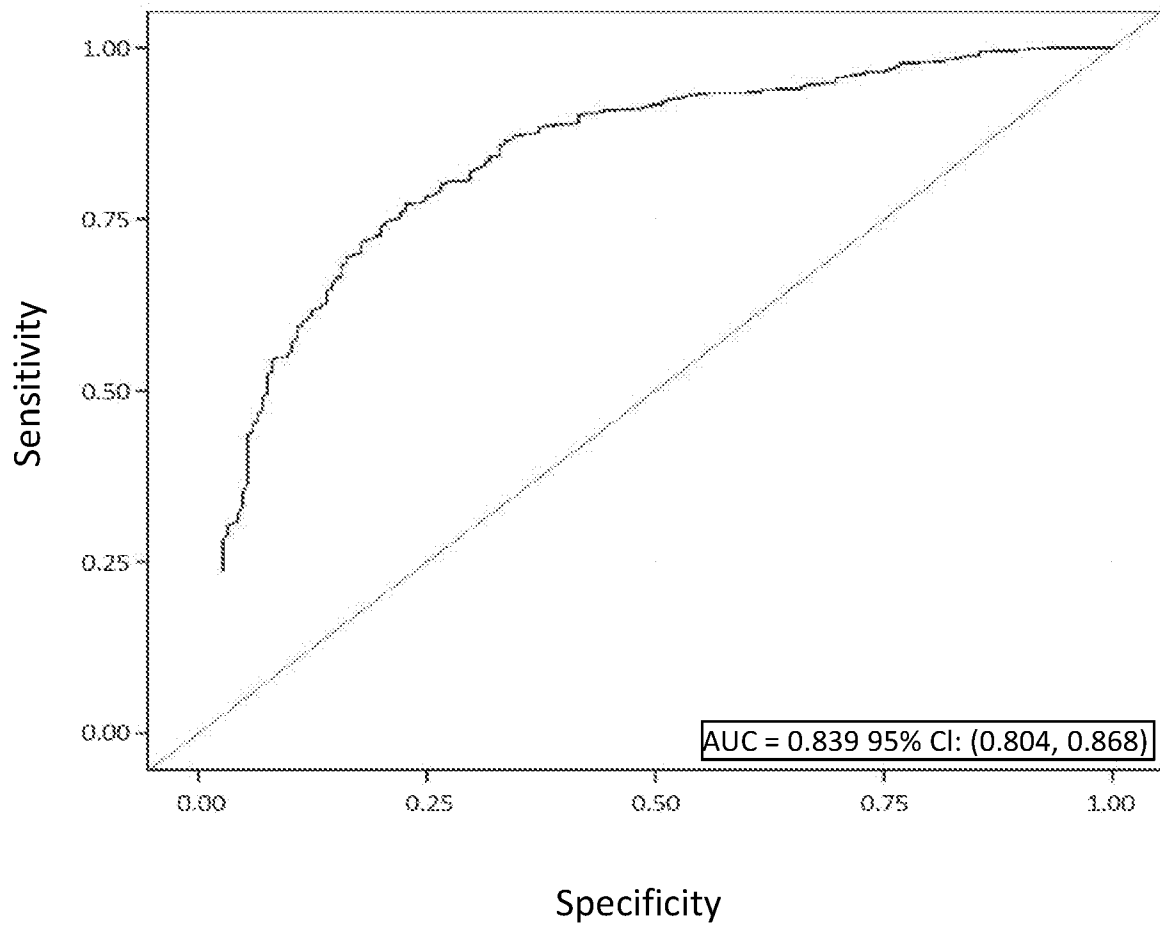


FIG. 2
PRIOR ART

**FIG. 3**

**FIG. 4**

**FIG. 5**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/038174

A. CLASSIFICATION OF SUBJECT MATTERIPC: **G01N 33/74** (2024.01); **C07K 14/58** (2024.01); **C07K 16/26** (2024.01); **G01N 33/577** (2024.01)CPC: **G01N 33/6887**; **G01N 33/74**; **C07K 14/58**; **C07K 16/26**; **G01N 33/577**; **G01N 2333/58**; **G01N 2800/325**

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)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2017/0269100 A1 (ABBOTT LABORATORIES) 21 September 2017 (21.09.2017)	
Y	entire document	1, 3, 5, 6
Y	entire document	2, 4
Y	US 2005/0239138 A1 (HESS et al.) 27 October 2005 (27.10.2005)	
Y	entire document	4
Y	CN 109678958 B (CHONGQING TIANSHENG TECHNOLOGY) 18 March 2022 (18.03.2022)	
Y	entire document	4
A	WO 2022/126055 A1 (SIEMENS HEALTHCARE DIAGNOSTICS INC) 16 June 2022 (16.06.2022)	
A	entire document	1-6



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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

“D” document cited by the applicant in the international application

“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

02 September 2024 (02.09.2024)

Date of mailing of the international search report

21 October 2024 (21.10.2024)

Name and mailing address of the ISA/US

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Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

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

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

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I: claims 1-6 are drawn to kits for use in determining an amount of NT-proBNP in a biological sample.

Group II: claims 7-19 are drawn to methods of determining the presence, severity, and/or predisposition of Heart Failure (HF) in an individual.

The inventions listed in Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The special technical features of Group I, kits for use in determining an amount of NT-proBNP in a biological sample, are not present in Group II; and the special technical features of Group II methods of determining the presence, severity, and/or predisposition of Heart Failure (HF) in an individual, are not present in Group I.

Additionally, even if Groups I and II were considered to share the technical features of determining an amount of NT-proBNP in a biological sample, comprising: (a) a solid phase reagent comprising a solid support having a first anti-NTproBNP antibody or binding fragment thereof directly or indirectly attached thereto; and (b) a lite reagent comprising a second anti-NT-proBNP antibody or binding fragment thereof labeled with an acridinium ester; and wherein the first and second anti-NT-proBNP antibodies or binding fragments thereof bind to non-overlapping epitopes of NT-proBNP and thereby form an immunocomplex comprising (a), (b), and NT-proBNP, these shared technical features do not represent a contribution over the prior art as disclosed by WO 2022/126055 A1 to Siemens Healthcare Diagnostics Inc (hereinafter, "Siemens").

Siemens teaches determining an amount of NT-proBNP in a biological sample (The analyte-specific binder may bind to any target analyte for which detection is desired. In certain non-limiting embodiments, the target analyte may be... a cardiac marker.... Non-limiting examples of cardiac markers include... pro B-type natriuretic peptide; such as, but not limited to, N-terminal pro B-type natriuretic peptide (NT-proBNP), Para. [0074]; a sample is combined, either simultaneously or wholly or partially sequentially, with the first and second reagents of the immunoassay kit, Para. [00105]; The conjugates were added in step 2 at a concentration of 0.4 pg/mL in a DIPSO buffer at pH 7.6, followed by incubation, magnetic separation, and quantitative measurement of the chemiluminescent signal, Para. [0134]), comprising: (a) a solid phase reagent comprising a solid support having a first anti-NTproBNP antibody or binding fragment thereof directly or indirectly attached thereto (a first reagent comprising a monoclonal antibody or fragment thereof having at least one DISFPD-AE directly or indirectly conjugated thereto and a second reagent comprising a solid phase labeled with a monoclonal antibody or fragment thereof, Para. [00105]); and (b) a lite reagent comprising a second anti-NT-proBNP antibody or binding fragment thereof labeled with an acridinium ester (The immunoassay employs a solid phase reagent that includes paramagnetic particles (PMP) covalently labeled with a monoclonal anti-CA 19-9 antibody and a lite reagent that includes an anti-CA 19-9 monoclonal antibody-acridinium ester (CA 19-9- MAb-AE) conjugate, Para. [0009]); and wherein the first and second anti-NT-proBNP antibodies or binding fragments thereof bind to non-overlapping epitopes of NT-proBNP and thereby form an immunocomplex comprising (a), (b), and NT-proBNP (The mixture is then incubated under conditions that will allow for binding of the first reagent and the second reagent to any target analyte present in the sample, thereby forming a complex, Para. [00105]; each of the analyte-specific binders of the first and second reagents specifically binds to an epitope of the target analyte, and the epitopes do not substantially overlap, such that the two analyte-specific binders can bind to the same target analyte molecule, Para. [00105]).

The inventions listed in Groups I and II therefore lack unity under Rule 13 because they do not share a same or corresponding special technical feature.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/038174

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-6**

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/038174

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LEE et al., Optimization of biotin labeling of antibodies using mouse IgG and goat anti-mouse IgG-conjugated fluorescent beads and their application as capture probes on protein chip, Journal of Immunological Methods, Vol. 362, 08 September 2010, Pgs. 38-42, [retrieved on 02.09.2024]. Retrieved from the internet: <URL: https://doi.org/10.1016/j.jim.2010.08.006 >. entire document	2