Abstract:
The present invention relates to newborn screening kits, methods, stable isotopically-labeled internal standards or internal standard solution for high throughput screening and analysis of metabolic disorders using liquid chromatography mass spectrometry (LC-MS) are provided. The metabolic disorders can be amino acid, organic acid or fatty acid oxidation disorders, and particularly urea cycle disorders or deficiencies, hyperammonemia, Hyperornithinemia- hyperammonemia-homocitrullinuria (HHH), and/or argininosuccinic aciduria. The newborn screening kits, methods, stable isotopically-labeled internal standards or internal standard solution are particularly useful for newborn screening (NBS) of metabolic disorders.
NOVEL METHODS AND KITS FOR DETECTING UREA CYCLE DISORDERS USING MASS SPECTROMETRY

FIELD OF THE INVENTION

[0001] Methods, reagents, internal standard solutions, and kits for high throughput screening and analysis of metabolic disorders using liquid chromatography mass spectrometry (LC-MS) are provided. The metabolic disorders can be amino acid, organic acid or fatty acid oxidation disorders, and particularly urea cycle disorders or deficiencies, hyperammonemia, argininosuccinic aciduria, and/or Hyperomithinemia-hyperammonemia-homocitrullinuria (HHH).

[0002] The methods, reagents, internal standard solutions, and kits are particularly useful for conducting a plurality of in vitro screening tests in newborns and detecting a panel of metabolic disorders at high speeds, for confirmation and/or follow up of the same diseases.

BACKGROUND OF THE INVENTION

[0002] Screening for biological disorders, in particular newborn screening (NBS) for these disorders, is currently performed using a variety of methods depending on the particular disorder screened. Amino acid and acylcarnitine analysis for NBS is currently performed by many parties using electrospray ionization coupled with tandem mass spectrometry (ESI-MS/MS). Liquid chromatography (LC) coupled with mass spectrometry (MS) has also been used for NBS.

[0003] Particular newborn metabolic deficiencies to be detected in newborns are urea cycle deficiencies. The urea cycle is a metabolic pathway for disposal of the toxic metabolite ammonia and surplus nitrogen as urea in mammals. The urea cycle plays an important role in prevention of the accumulation of toxic nitrogenous compounds and further contains several of the biochemical reactions required for the de novo biosynthesis and degradation of arginine. As shown in Figure 1, the urea cycle is catalyzed by five enzymes: carbamoyl phosphate synthetase 1 (CPS1), ornithine transcarbamoylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARG1). The first two enzymes of the urea cycle are located within the mitochondrial matrix and the remaining three enzymes are cytosolic. CPS1 promotes the formation of carbamoyl phosphate from ammonium ions and carbon dioxide in the first step. The next reaction is catalyzed by OTC which transfers a carbamoyl group from carbamoyl phosphate to ornithine to form citrulline. ASS is involved in the formation of argininosuccinate from citrulline and aspartate in the third step and ASL facilitates the formation of arginine and fumarate.
from argininosuccinate in the fourth step. ARG1 catalyzes the formation of ornithine and urea from arginine in the fifth step. In addition, N-acetyl glutamate synthetase (NAGS) catalyses the formation of N-acetyl glutamate which activates the CPS1. Therefore, interruptions in the metabolic pathway for urea synthesis may be caused by the deficiency or inactivity of any one of several enzymes involved in specific steps in the cascade. A defect in the ureagenic pathway has two consequences: arginine becomes an essential amino acid (except in arginase deficiency, where the enzyme defect results in a failure of degradation of arginine) and nitrogen atoms accumulate in a variety of molecules, the pattern of which varies according to the specific enzymatic defects, although plasma levels of ammonia and glutamine are increased in all urea cycle disorders not under metabolic control.

Urea cycle disorders (UCD) include: (a) carbamyl phosphate synthetase deficiency (CPSD or CPS1), (b) N-acetyl glutamate synthetase deficiency (NAGS), (c) ornithine transcarbamylase deficiency (OTCD), (d) argininosuccinic acid synthetase deficiency (ASD), (e) argininosuccinate lyase deficiency or citrullinemia type 1 (ALD or ASS1), and (f) arginase deficiency (ARG1). Except for OTCD, which is an X-linked generic disorder, urea cycle disorders are inherited in an autosomal recessive way. Each of these diseases represents a defect in the biosynthesis of one of the normally expressed enzymes of the urea cycle and is characterized by signs and symptoms induced by the accumulation of precursors of urea, primarily ammonia and secondary glutamine.

The common pathologic sequelae of these clinical disorders are the extreme elevation of the plasma ammonia level (hyperammonemia). Severe urea cycle disorders are characterized by plasma ammonia level of about 2000 to 2500 micrograms/dL. Detection of hyperammonemia is most important for early diagnosis and effective treatment. Hyperammonemia is typically associated with an increase in ammonia buildup are acute episodes of vomiting, abnormal liver enzyme levels, lethargy, convulsions and coma. Even treated, protracted severe hyperammonemia leads to mental and physical retardation. There is however an existing clinical situation that challenges the introduction of universal neonatal screening for UCDs. At the mild end of the spectrum, there are patients described with a late-onset of disease with only a single, few or even absence of symptom(s) and only a biochemical phenotype. These patients were, for instance in the case of ASS deficiency, described as suffering from mild citrullinemia type 1, a condition allelic to classical citrullinemia type 1, but nevertheless much milder and with less, if any need, medical intervention. Such patients were often identified in neonatal screening programs and it
has been discussed whether the mild course would result from early detection and initiation of
treatment, or from a relevant residual enzyme or transporter function. It has been suggested that
metabolites and/or mutation analysis may help to identify attenuated patients in an attempt to
avoid stigmatisation of non-diseases, potentially unnecessary treatment and unnecessary anxiety
to parents but no definitive solution have been offered so far.

[0006] In the case of ASS deficiency (citrullinemia) and ARG1 deficiency (hyperargininemia),
there is a significant accumulation of the respective substrates (citrulline and arginine) in both
blood and urine. In ASL deficiency, the substrate argininosuccinate does not accumulate in blood
in any appreciable amount because of the low renal threshold. Some citrulline, however, does
accumulate in blood and argininosuccinate is excreted in large quantities in the urine. To this
regard, a recent UCD guideline concluded that NBS for proximal disorders cannot currently be
recommended, but it may be considered for the distal UCDs. In effect, attempts to develop UCD
screening tests which are based on direct measurements of enzymes or accumulated substrates in
blood or urine are limited to the last steps in the cycle, since the enzymes are restricted to the
mitochondria, for the first steps, e.g., NAGS, CPS1 and OTC, and there is thus no substrate
accumulation (Figure 1). However, technically, the direct measurement of ammonia in a dried
blood spot is nearly impossible.

[0007] Using arginine and citrulline as markers, ASSD and ASLD have been included in the
expanded newborn screening programs in some states in the US (California, Massachusetts,
Michigan, New York, Newark, Wisconsin) since 2001. Using citrulline as a marker, ASSD and
ASLD have been screened for as part of the 'Recommended Uniform Screening Panel' in all of
the United States since 2006. Published data from 6,077,736 births (covering years from 2001 to
2012 for different states) resulted in a cumulative incidence of 1 in 117,000 newborns for the two
disorders.

[0008] One of the most common and severe defect of the urea cycle, OTC deficiency, was
considered for inclusion in the panel, but it did not meet the assigned evaluation criteria, due to
the lack of a screening test that had been validated in the general newborn population. OTC
deficiency, an X-linked disorder, has a wide range of clinical variability and can present in a
severe neonatal-onset form that is life threatening within the first few days after birth or as a late-
onset, typically milder, form of the disease. Female carriers can also experience symptoms related
to increased ammonia levels. The laboratory indications for OTC deficiency are elevated
concentrations of glutamine and ammonia, low citrulline, and elevated excretion of orotic acid in
the urine. NBS laboratories have thus attempted to use low citrulline and several ratios to identify infants at risk for OTC deficiency, but have had so far limited success.

[0009] Highlighting the difficulty to use low citrulline as a marker for the detection of proximal UCDs, a study in Tuscany applying LC-MS/MS was performed between 2001 and 2008. The authors concluded that hypocitrullinemia was not a reliable marker for OTCD newborn screening, especially for late-onset forms that may exhibit normal citrulline levels. Low citrulline concentrations may also be found in other metabolic disorders further challenging its use as screening marker. In a recent UCD guideline, it was therefore concluded that NBS for proximal disorders cannot currently be recommended, but it may be considered for the distal UCDs.

[0010] Orotic acid though often elevated in the urine of patients with OTC deficiency cannot be used reliably as a marker in blood. Despite the fact that all UCDs affect the function of the urea cycle and therefore lead to hyperammonemia, their biochemical profile is very different.

[0011] Several other caveats regarding newborn screening for urea cycle defects are that CPS1 deficiency, OTC deficiency, and NAGS deficiency currently cannot be reliably detected. Furthermore, although hyperargininemia or ARG1 deficiency has been detected by these methods, newborn screening cannot be expected to reliably detect all cases. Even in UCDs detectable by newborn screening, neonates are often symptomatic prior to availability of the screening results; thus a high level of clinical suspicion on the part of healthcare providers is necessary. With the currently available techniques for detecting UCDs, the sensitivity and specificity of such screening is not absolute.

[0012] The common factor to all five enzyme deficiencies in this pathway and that is the extreme elevation of the plasma ammonia level (hyperammonemia). Detection of hyperammonemia would thus be most important for early diagnosis and effective treatment. Since the direct measurement of ammonia is not feasible in NBS, glutamine would be another metabolite that is generally elevated in UCDs. However, numbers of difficulties have also been reported for using glutamine as a marker for UCDs. Indeed, glutamine is highly unstable in plasma and serum, and spontaneously converts into glutamate and pyroglutamate, which formations lead to false low glutamine levels, rendering glutamine currently not a suitable screening parameter for NBS using LC-MS.

[0013] The use of mass spectrometry (MS) in clinical laboratories has very much increased with time. This development is obviously due to great advances in mass spectrometry applications in the last fifteen years. Mass spectrometry permits a very rapid measurement of different
metabolites in different biological specimens using filter paper spots or directly in different biological fluids. Because of its high sensitivity, this technique can be used for qualitative and quantitative analysis of many analytes or metabolites such as amino acids and acylcarnitines, homocysteine, orotic acid, succinylacetone, etc… with appropriate internal standards.

Particularly, MS is extensively used for analysis of metabolites from dried blood spots taken at birth (Guthrie-cards) but among the detected metabolites those due to UCDs are not effectively detected because the defects discussed in the earlier paragraphs.

[0014] While the inclusion of UCD screening into newborn screening (NBS) is highly desirable, it is however hampered by the fact that there is not a specific marker for every single UCD, by the fact that so far the common feature of UCDs, e.g., hyperammonemia, is not directly detectable in dried blood spots (DBS), and by the fact that the detection of secondary elevations of glutamine seemed not feasible, because of the proposed instability of glutamine in DBS.

[0015] Hence, the aim of the present invention is to provide an analytical method, kits, reagents, and internal standard solutions, which allow the determination of metabolites correctly indicative of UCDs, particularly of glutamine, along with the determination of other metabolites that are commonly determined for metabolites screening, especially those screening performed on dried blood spots taken at birth. The present invention thus relates to a newborn screening kit, methods, reagents and internal standard solution allowing for a fast and reliable determination and detection of a plurality of UCDs as well other metabolic deficiencies using tandem mass spectrometry (tandem-MS NBS).

[0016] The present invention thus provides reliable methods, kits, reagents, and internal standard solution for the simultaneous detection of lysine and glutamine from a sample in multiple reaction monitoring (MRM) with a second-tier UPLC method for the separation and a specific quantitation of glutamine. The present invention thus makes it feasible to detect UCDs using novel markers or novel combination of markers, which were not achievable by the methods reported in prior art.

[0017] Such methods, kits, reagents, and internal standard solution may be advantageously combined with the measurement of all specific amino acids (arginine, argininosuccinic acid, citrulline, ornithine, and proline), N-acetyl-glutamate, and orotic acid. This novel combination, method, kits, reagent kit and internal standard solutions allow for very fast and reliable determination and detection of a plurality of UCDs in newborns using tandem mass spectrometry (tandem-MS NBS).
The present invention is particularly useful for detection of proximal markers like glutamine, which serve as diagnostic marker for proximal urea cycle disorders defects. Detecting markers like glutamine allows discriminating between the proximal and distal urea cycle defects, since such markers could be products of the proximal enzymes and a substrate for the distal enzymes. Prompt replacement of glutamine and other amino acids is then possible once it is determined whether the defect is in a proximal or distal urea cycle disorder. Dosing of intravenous glutamine in proximal urea cycle disorders may then be carried out for fast recovery of the subject.

The present invention thus provides reliable and sensitive methods and reagent kits to evaluate the predisposition, presence and severity of a broader number of UCDs including OTC deficiency, argininosuccinate synthetase deficiency (citrullinemia), argininosuccinate lyase deficiency (argininosuccinicaciduria), arginase deficiency and hyperammonemia-hyperornithinemia-homocitrullinemia syndrome (HHH), at an improved detection and/or precision level than the methods typically practiced at the present time, by the determination and quantification of a combination of various indicator metabolites in a biological sample.

SUMMARY OF THE INVENTION

The present invention thus relates to screening methods, kits, reagents, and internal standard solutions for the detection and assessment of the levels of glutamine metabolite glutamine in a sample obtained from a subject, such as a dried blood spot obtained from the newborns. The kits, internal standard solutions, and methods are useful for newborn screening (NBS) and particularly for detecting urea cycle disorders or deficiencies (UCDs), OTC deficiency, and/or hyperammonemia, and/or argininosuccinic aciduria in newborns. Such newborn screening methods, kits, reagents, and internal standard solutions are also useful for detecting and/or quantifying further metabolites including amino acids, organic acids, a plurality of carnitines, and/or succinyl acetone in said sample.

The present invention also relates to newborn screening methods, kits, reagents, and internal standard solution for the diagnosis of metabolic disorders such as UCDs, hyperammonemia, HHH, and/or argininosuccinic aciduria in newborns, comprising detecting and/or measuring the levels of metabolites, particularly glutamine in a sample, such as a dried blood spot obtained from the newborns.
[0022] The present invention further provides newborn screening kits, comprising a testing tray which itself comprises a plurality of cells, and individually stored internal standards, comprising at least lysine stable isotopically-labeled internal standard, which are placed in the plurality of cells of the testing tray, and optionally a solvent dispenser such as micropipette or any other means to dispense the solvent. Such individually stored standards may be preferably dried.

[0023] The present invention finally provides a novel set of stable isotopically-labeled standards or internal standard solution which may be used in the newborn screening method kits of the present invention, comprising at least a lysine stable isotopically-labeled internal standard, and further comprising one or more additional stable isotopically-labeled internal standard corresponding to additional metabolites to be tested in said newborn screening kit. The novel set of stable isotopically-labeled standards or the internal standard solution may be present in dried form.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0024] Figure 1: shows a schematic of the urea cycle.

[0025] Figure 2: is a table showing pathological values for the main enzymatic deficiencies within the urea cycle.

[0026] Figure 3: is a table providing some control levels of endogenous amino acids.

[0027] Figure 4: is a graph showing the linearity of the measurement of glutamine, lysine, argininosuccinic acid, and orotic acid and standard solutions of the respective analyte using tandem mass spectrometry.

[0028] Figure 5: is a graph showing the linearity of the measurement of glutamine, lysine, argininosuccinic acid, and orotic acid and standard solutions of the respective analyte using tandem mass spectrometry.

[0029] Figure 6: is a graph showing the linearity of the measurement of glutamine, lysine, argininosuccinic acid, and orotic acid and standard solutions of the respective analyte using tandem mass spectrometry.

[0030] Figure 7: is a graph showing the linearity of the measurement the sum of lysine and glutamine in dried blood spots (DBS) using ion exchange chromatography.

[0031] Figure 8: shows the proposed results of the measurement of the sum of glycine and lysine, as well as measured values from 180 DBS of healthy newborns, measured values from 2 samples of a patient with proven OTC deficiency, and expected range of patients with urea cycle
defects (UCDs). In Figure 8, the legends are as follows: Lysin = Reference Range from Literature, Glutamine = Reference Range from Literature, Sum(Gln + Lys) = Combined Reference Range, Normal = 180 normal NBS samples

[0032] OTC = 2 DBS from a patient with proven OTC deficiency, UCD = expected range for newborns with UCD.

[0033] Figure 9: is a table showing the physiological levels of amino acids in plasma (μmol/L) in men, women, adolescents and children.

[0034] Figure 10: is a flowchart showing the decision tree starting from the detection of elevated sum of glutamine and lysine as early markers to subsequent diagnosis steps for detecting more precisely the type of UCD in the subject.

DETAILED DESCRIPTION OF THE INVENTION

[0035] While the present invention is described in conjunction with various embodiments, it is not intended that the present invention be limited to such embodiments. On the contrary, the present invention encompasses various alternatives, modifications, and equivalents, as will be appreciated by those of skill in the art.

[0036] The present invention is directed a newborn screening kit, method and novel sets of internal standards or internal standard solutions, for detecting the presence and/or measuring the levels of metabolites in a sample obtained from a subject, by tandem mass spectrometry, said metabolites comprising metabolites having similar mass structure, one of the metabolites being instable, comprising the following steps:

(i) extracting said metabolites from the sample with an extraction solution;

(ii) providing, before or after the step (i), a solution comprising a known amount of one or more stable isotopically-labeled internal standards corresponding to said metabolites to be detected in the sample,

(iii) ionizing said metabolites and said one or more stable isotopically-labeled internal standards to generate ions,

(iv) acquiring the mass to charge (m/z) ratio of said one or more ions in the mass spectrum in the Multiple Reaction Monitoring (MRM) mode,

(v) determining the sum of said metabolites having similar mass structure in MRM mode, and
(vi) detecting and quantifying the amount of said metabolites with stable isotopically-labeled or internal standard corresponding to one of said metabolites which is stable, and deducing the amount of the other metabolite which is stable.

[0037] In vitro method newborn screening kit, and novel sets of internal standards or internal standard solutions of the present invention allow quantifying target metabolites having same isobaric properties, and the quantitative concentration of one or more isobars in a sample can be determined by using the sum or ratios.

[0038] In the first embodiment of the present invention, the newborn screening methods, reagent, kits and internal standards allow quantifying glutamine and lysine, and more particularly glutamine.

[0039] According to this embodiment, the present invention also relates to newborn screening kits, methods and novel sets of internal standards or internal standard solutions, for detecting the presence and/or measuring the levels of metabolites in a sample obtained from a subject, by tandem mass spectrometry, said metabolites comprising at least glutamine, and/or sum of lysine and glutamine, comprising the following steps:

(i) extracting said metabolites from the sample with an extraction solution;

(ii) providing, before or after the step (i), a solution comprising a known amount of one or more stable isotopically-labeled internal standards corresponding to said metabolites to be detected in the sample,

(iii) ionizing said metabolites and said one or more stable isotopically-labeled internal standards to generate ions,

(iv) acquiring the mass to charge (m/z) ratio of said one or more ions in the mass spectrum in the Multiple Reaction Monitoring (MRM) mode,

(v) determining the sum of glutamine and lysine in MRM mode, and

(vi) detecting and quantifying the amount of glutamine and/or glutamine and lysine with stable isotopically-labeled internal standard corresponding to lysine.

[0040] The methods thus provides for high throughput analysis of metabolites in complex mixtures for unresolved chromatographic peaks and or unstable metabolites. Such methods allow for deconvoluting contributions of a plurality of metabolites in a sample from a mass spectrometer signal, preferably a tandem mass spectrometry signal. Quantitative concentrations of metabolites, such as, for example, isobars or structural isomers may be obtained. Because of the isobaric natures of glutamine and lysine, sum and or ratios of such isobars can be assessed from
the chromatographic signal, subsequently the levels of glutamine may then be deduced by comparing with the stable isotopically-labeled internal standard corresponding to lysine or to alternatives to lysine as mentioned above.

[0041] According to the present invention, lysine is thus used to quantify the amount of glutamine. It is however understood that the present methods, kits and internal standards are not limited to lysine. Any metabolite having a similar mass structure as that of lysine and being isobaric to glutamine may also be used as an alternative to lysine in the methods and reagent kits of the present invention. The quantitative concentration of one or more isobars in a sample can be determined by using the sum or ratios, and to quantify the amount of glutamine in the sample of subjects. By way of examples of alternatives for lysine, we can cite glutamate, methionine, methyl-, dimethyl-, trimethyl-, hydroxy-, acetyl-, sumoyl-, glocisalted- lysine, as well as precursors of lysine synthesis, such as aspartic acid, etc...

[0042] The present invention thus relates to a newborn screening kit, in vitro diagnosis method and novel sets of stable isotopically-labeled or a novel internal standard solutions for the diagnosis and/or the detection of metabolic disorders such as UCDs, hyperammonemia, argininosuccinic aciduria, and/or Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome, comprising detecting the presence and/or measuring the levels of metabolites in a sample obtained from a newborn, by tandem mass spectrometry, said metabolites comprising at least glutamine, and comprising the following steps:

(i) extracting the metabolites from the sample with an extraction solution;
(ii) providing, before or after the step (i), a solution comprising a known amount of one or more stable isotopically-labeled internal standards corresponding to the metabolites to be detected in the sample,
(iii) ionizing said metabolites and said one or more stable isotopically-labeled internal standards to generate ions,
(iv) acquiring the mass to charge (m/z) ratio of said one or more ions in the mass spectrum in the Multiple Reaction Monitoring (MRM) mode,
(v) determining the sum of glutamine and lysine in MRM mode, and
(vi) detecting and quantifying the amount of glutamine with stable isotopically-labeled internal standard corresponding to lysine, and optionally
(vii) determining whether the level of glutamine as obtained in step (vi) is elevated compared to physiological levels of glutamine.
Expected plasmatic physiological levels of glutamine for patients having such deficiencies are well known in the art (See for example Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases, Editors: Nenad Blau, Marinus Duran, Milan E. Blaskovics, K. Michael Gibson, Springer-Verlag Berlin Heidelberg, 2nd Edition 2003, which is incorporated herein by reference). Such levels of glutamine in plasma of healthy subjects are provided in Figures 2 and 3. For example, physiological levels of glutamine in children may range from around 200 to 900 µmol/L, or from 250 to 850 µmol/L, or from around 330 to 809 µmol/L.

As shown in Figure 1, Urea Cycle Disorders (UCDs) include a variety of genetic defects, which lead to inefficient urea synthesis. UCDs may include one or more enzyme deficiencies within the urea cycle: (a) carbamyl phosphate synthetase deficiency (CPSD or CPS1), (b) N-acetyl glutamate synthetase deficiency (NAGS), (c) ornithine transcarbamylase deficiency (OTCD), (d) argininosuccinic acid synthetase deficiency (ASD), (e) argininosuccinate lyase deficiency or Citrullinemia type 1 (ALD or ASS1), and (f) arginase deficiency (ARG1). Except for OTCD, which is an X-linked genetic disorder, urea cycle disorders are inherited in an autosomal recessive fashion. Each of these diseases represents a defect in the biosynthesis of one of the normally expressed enzymes of the urea cycle and is characterized by signs and symptoms induced by the accumulation of precursors of urea, principally ammonium and glutamine.

The present invention is thus particularly useful for detection of proximal markers like glutamine, which serve as diagnostic marker for proximal urea cycle disorders defects. The current invention allows detecting additional markers like glutamine and therefore discriminating between the proximal and distal urea cycle defects, as such markers could be products of the proximal enzymes and a substrate for the distal enzymes. Prompt replacement of glutamine and other amino acids is then possible once it is determined whether the defect is in a proximal or distal urea cycle disorder.

Determining the presence and quantifying the amount of glutamine correlates with the presence or absence of urea cycle disorders and/or hyperammonemia, and/or argininosuccinic aciduria, and/or Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome.

Hyperammonemia refers to a clinical condition associated with elevated ammonia levels manifested by a variety of symptoms and signs, including significant central nervous system (CNS) abnormalities. Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH) syndrome is characterized by high plasma concentrations of glutamine and ammonia.
It is crucial to include diagnostic method for early assessment of neonatal predisposition to UCDs, hyperammonemia, and/or argininosuccinic aciduria in a human neonatal patient, before the physiologic and behavior symptoms become apparent.

Indeed, characteristic clinical biochemical abnormalities in these cases are significant elevations in plasma glutamine and ammonia concentrations. However, as plasma ammonia concentrations return to normal, plasma glutamine concentrations may remain mildly elevated (1.5- to twofold the upper limits of control values). Generally ammonia formed is trapped by glutamate to form glutamine by the enzyme, Glutamine Synthetase. Any elevation of glutamine would thus lead to a higher trapping of ammonia. Hence, glutamine would serve as a useful marker for detection and for planning further treatment of HHH by administration of pharmacological agents that can act as glutamine trap in particular and amino acid trap in general, hence diverting nitrogen from urea synthesis to alternatives routes of excretion.

According to a second embodiment, newborn screening kit, in vitro diagnosis method and novel sets of stable isotopically-labeled or a novel internal standard solutions are useful for further detecting the presence and/or measuring the levels of additional metabolites alongside to glutamine in a blood sample obtained from a newborn, by tandem mass spectrometry, and is thus particularly useful to detect all UCDs, including proximal abnormalities along with classical distal deficiencies.

Said additional metabolites may comprise arginine, citrulline, argininosuccinate, ornithine, lysine, and/or orotic acid. Indeed as showed in the diagnosis decision tree in Figure 10, once an elevated level of glutamine has been detected according to the present invention, a precise determination of the type of UCDs may be further diagnosed, such as in particular, new proximal urea cycle deficiencies including N-acetylglutamate synthase (NAGS) deficiency, carbamyl phosphate synthetase (CPS) deficiency, ornithine transcarbamylase (OTC) deficiency, and/or ornithine translocase deficiency (HHH).

According to this embodiment, newborn screening kit, in vitro diagnosis method and novel sets of stable isotopically-labeled standards or a novel internal standard solutions are useful for further detecting the presence and/or measuring the levels of additional metabolites alongside to glutamine, selected among arginine, citrulline, argininosuccinate, ornithine, lysine, and/or orotic acid, in a blood sample obtained from a newborn, by tandem mass spectrometry, and is thus particularly useful for conducting a plurality of in vitro tests to assay further metabolites in said blood sample, comprising the following steps:
(i) extracting said metabolites from the sample with an extraction solution;
(ii) providing, before or after the step (i), a solution comprising a known amount of one or more stable isotopically-labeled internal standards corresponding to the metabolites to be detected in the sample;
(iii) ionizing the extracted metabolites and said one or more stable isotopically-labeled internal standards to generate ions;
(iv) acquiring the mass to charge (m/z) ratio of said one or more ions in the mass spectrum in the Multiple Reaction Monitoring (MRM) mode;
(v) determining the sum of glutamine and lysine in MRM mode,
(vi) detecting and quantifying the amount of glutamine with stable isotopically-labeled internal standard corresponding to lysine; and
(vii) determining the presence and/or the levels of said one or more metabolites by analyzing the mass MRM spectrum as obtained in step (iv).

[0053] The present invention thus provides reliable and sensitive newborn screening kits and methods to evaluate the predisposition, presence and severity of a broader number of UCDs including OTC deficiency, argininosuccinate synthetase deficiency (citrullinemia), argininosuccinate lyase deficiency (argininosuccinicaciduria), arginase deficiency and hyperammonemia-hyperornithinemia-homocitrullinemia syndrome (HHH), at an improved detection and/or precision level than the methods typically practiced at the present time, by the determination and quantification of a combination of various indicator metabolites in a biological sample.

[0054] According to a first aspect of this embodiment, the present invention relates to newborn screening kits, newborn screening in vitro methods, and novel set of stable isotopically-labeled internal standards or internal standard solution for detecting or diagnosing in newborns a suspicion of arginase deficiency (ARG1), comprising detecting the presence and/or measuring the levels of metabolites in a sample obtained from a newborn, by tandem mass spectrometry, said metabolites comprising at least glutamine and arginine, and said kit and method comprising the following steps:
(i) extracting the metabolites from the sample with an extraction solution;
(ii) providing, before or after the step (i), a solution comprising a known amount of one or more stable isotopically-labeled internal standards corresponding to the metabolites to be detected in the sample,
(iii) ionizing said metabolites and said one or more stable isotopically-labeled internal standards to generate ions,
(iv) acquiring the mass to charge \((m/z)\) ratio of said one or more ions in the mass spectrum in the Multiple Reaction Monitoring (MRM) mode,
(v) determining the sum of glutamine and lysine in MRM mode, and
(vi) detecting and quantifying the amount of glutamine with stable isotopically-labeled internal standard corresponding to lysine,
(vii) determining whether the amount of glutamine as obtained in step (vi) is elevated compared to physiological levels of glutamine, and
(viii) if the level of glutamine as obtained in step (vii) is elevated, further determining the level of arginine and whether the level of arginine is elevated compared to physiological levels of arginine, thereby allowing to diagnose a suspicion of arginase deficiency.

According to a second aspect of this embodiment, the present invention relates to newborn screening kits, newborn screening \textit{in vitro} methods, and novel set of stable isotopically-labeled internal standards or internal standard solution for detecting or diagnosing in newborns a suspicion of N-acetylglutamate synthetase (NAGS) deficiency, comprising detecting the presence and/or measuring the levels of metabolites in a sample obtained from a newborn, by tandem mass spectrometry, said metabolites comprising at least glutamine, arginine, citrulline, said kit and method comprising the following steps:

(i) extracting the metabolites from the sample with an extraction solution;
(ii) providing, before or after the step (i), a solution comprising a known amount of one or more stable isotopically-labeled internal standards corresponding to the metabolites to be detected in the sample,
(iii) ionizing said metabolites and said one or more stable isotopically-labeled internal standards to generate ions,
(iv) acquiring the mass to charge \((m/z)\) ratio of said one or more ions in the mass spectrum in the Multiple Reaction Monitoring (MRM) mode,
(v) determining the sum of glutamine and lysine in MRM mode, and
(vi) detecting and quantifying the elevated level of glutamine with stable isotopically-labeled internal standard corresponding to lysine,
(vii) detecting the level of arginine and citrulline with stable isotopically-labeled internal standards corresponding to arginine and citrulline, respectively, and
(viii) if the level of glutamine as obtained in step (vi) is elevated, and the level of arginine is decreased compared to physiological levels of arginine, further determining whether the level of citrulline is decreased compared to physiological levels of citrulline, thereby suspecting a deficiency of the N-acetyl glutamate synthetase (NAGS).

[0056] According to third aspect of this embodiment, the present invention relates to newborn screening kits and newborn screening in vitro methods, and novel set of stable isotopically-labeled internal standards or internal standard solution for detecting or diagnosing in newborns of argininosuccinate synthase (ASS) deficiency, argininosuccinate aciduria (ASA) and/or citrullinemia, comprising detecting the presence and/or measuring the levels of metabolites in a sample obtained from a newborn, by tandem mass spectrometry, said metabolites comprising at least glutamine, arginine, citrulline, argininosuccinate, and said kit and method comprising the following steps:

(i) extracting the metabolites from the sample with an extraction solution;
(ii) providing, before or after the step (i), a solution comprising a known amount of one or more stable isotopically-labeled internal standards corresponding to the metabolites to be detected in the sample,
(iii) ionizing said metabolites and said one or more stable isotopically-labeled internal standards to generate ions,
(iv) acquiring the mass to charge (m/z) ratio of said one or more ions in the mass spectrum in the Multiple Reaction Monitoring (MRM) mode,
(v) determining the sum of glutamine and lysine in MRM mode, and
(vi) detecting and quantifying the elevated level of glutamine with stable isotopically-labeled internal standard corresponding to lysine,
(vii) detecting the level of arginine, citrulline, and argininosuccinate with an internal standard solution corresponding to stable isotopically-labeled internal standards corresponding to arginine, citrulline, and argininosuccinate, and
(viii) if the level of glutamine as obtained in step (vi) is elevated and the level of arginine is decreased compared to physiological levels of arginine, and further determining whether the level of citrulline is increased compared to physiological levels of citrulline, thereby diagnosing a suspicion of citrullinemia, and further determining whether the level of argininosuccinate is elevated compared to physiological levels of argininosuccinate, thereby suspecting a deficiency of the argininosuccinate aciduria (ASA).
According to fourth aspect of this embodiment, the present invention relates to newborn screening kits, newborn screening in vitro methods, and novel set of stable isotopically-labeled internal standards or internal standard solution for detecting or diagnosing in newborns a deficiency of ornithine transcarbamylase deficiency (OTCD) and/or of carbamyl phosphate synthetase (CPS) deficiency, comprising detecting the presence and/or measuring the levels of metabolites in a sample obtained from a newborn, by tandem mass spectrometry, said metabolites comprising at least glutamine, arginine, citrulline, and orotic acid, and said kit and method comprising the following steps:

(i) extracting the metabolites from the sample with an extraction solution;

(ii) providing, before or after the step (i), a solution comprising a known amount of one or more stable isotopically-labeled internal standards corresponding to the metabolites to be detected in the sample,

(iii) ionizing said metabolites and said one or more stable isotopically-labeled internal standards to generate ions,

(iv) acquiring the mass to charge (m/z) ratio of said one or more ions in the mass spectrum in the Multiple Reaction Monitoring (MRM) mode,

(v) determining the sum of glutamine and lysine in MRM mode, and

(vi) detecting and quantifying the elevated level of glutamine with stable isotopically-labeled internal standard corresponding to lysine,

(vii) detecting the level of arginine, citrulline, and orotic acid with an internal standard solution comprising stable isotopically-labeled internal standards corresponding to arginine, citrulline, and orotic acid, and

(viii) if the level of glutamine as obtained in step (vi) is elevated and the levels of arginine and citrulline are decreased compared to physiological levels of arginine and citrulline, further determining whether the level of orotic acid is increased compared to physiological levels of orotic acid, thereby diagnosing a suspicion of deficiency of the ornithine transcarbamylase (OTCD), or whether the level of orotic acid is normal, thereby diagnosing a suspicion of carbamoyl phosphate synthetase (CPS) deficiency.

According to fifth aspect of this embodiment, the present invention relates to newborn screening kits, newborn screening in vitro methods, and novel set of stable isotopically-labeled internal standards or internal standard solution for screening or diagnosis in newborns a suspicion of Lysinuric protein intolerance (LPI) or Hyperornithinemia-hyperammonemia-homocitrullinuria...
(HHH), comprising detecting the presence and/or measuring the levels of metabolites in a sample obtained from a newborn, by tandem mass spectrometry, said metabolites comprising at least glutamine, lysine, and ornithine, and said kit and method comprising the following steps:

(i) extracting the metabolites from the sample with an extraction solution;

(ii) providing, before or after the step (i), a solution comprising a known amount of one or more stable isotopically-labeled internal standards corresponding to the metabolites to be detected in the sample,

(iii) ionizing said metabolites and said one or more stable isotopically-labeled internal standards to generate ions,

(iv) acquiring the mass to charge (m/z) ratio of said one or more ions in the mass spectrum in the Multiple Reaction Monitoring (MRM) mode,

(v) determining the sum of glutamine and lysine in MRM mode, and

(vi) detecting and quantifying the amount of glutamine and lysine with stable isotopically-labeled internal standard corresponding to lysine,

(vii) determining whether the amount of glutamine as obtained in step (vi) is elevated and whether the level of lysine is decreased as compared to physiological levels of glutamine and lysine;

(viii) if the level of glutamine as obtained in step (vii) is elevated and level of lysine is decreased compared to physiological levels, further determining whether the level of ornithine is elevated compared to physiological levels of ornithine, thereby allowing to diagnose a suspicion of Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH), or whether the level of ornithine is normal, thereby allowing to diagnose a suspicion of a Lysinuric protein intolerance (LPI).

[0059] Physiological levels of arginine, citrulline, arginosuccinate, orotic acid, and ornithine are well known in the art. Those are inter alia described in Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases, Editors: Nenad Blau, Marinus Duran, Milan E. Blaskovics, K. Michael Gibson, Springer-Verlag Berlin Heidelberg, 2nd Edition 2003, ISBN 978-3-642-62709-5, herein incorporated by reference). Levels of amino acids in plasma of healthy subjects are provided in Figure 9.

[0060] Additional metabolites which may be detected using newborn screening kits, methods and internal standard solutions of the present invention comprise for example N-acetyl glutamate, proline, serine, aspartate, and/or homocitrulline, thereby allowing a more precise diagnosis of the
UCD. Indeed, it has been reported so far that a low level of N-acetyl glutamic acid correlated with a deficiency of the NAGS enzyme, or that low level of proline correlated with a deficiency of the pyrroline-5-carboxylase synthase (P5CS), or again that homocitrulline detection in blood correlate with the hyperornithinemia-hyperammonemia-homocitrulluria (HHH) syndrome.

[0061] Therefore, according to this second embodiment, newborn screening kits and methods, as well as novel set of stable isotopically-labeled internal standards or internal standard solution, advantageously provide a more complete metabolic profile allowing for a more reliable and efficient in vitro diagnosis or prediction of UCDs, hyperammonemia, HHH, and/or argininosuccinic aciduria in a subject, since the accumulation of glutamine, which is an early marker of the urea cycle is now detected in a biological sample of a newborn subject alongside to further metabolites indicative of UCDs, hyperammonemia, and/or argininosuccinic aciduria. As shown in the table of Figure 2, glutamine is one of the most important markers as it is indicative of a wide range of UCDs. Therefore, detecting and measuring glutamine in combination with other marker metabolites is crucial for the screening and detection of UCDs.

[0062] A metabolic profile as described herein can be useful for monitoring the metabolism of a subject (e.g., a mammal such as a human), such as neonate. As a non-limiting example, the methods can be used for determining therapeutic efficacy of a particular treatment. Based on this determination, the subject can be offered additional or alternative therapeutic options. The metabolic profile can also be useful for assessing patient compliance with a particular treatment modality, such as dietary restriction. Therefore, the technology described herein is applicable to screening, diagnosis, prognosis, monitoring therapy and compliance, and any other application in which determining the presence or amount of panels of two or more biomolecules is useful.

[0063] According to a third embodiment, newborn screening kits and in vitro methods as well as novel set of stable isotopically-labeled internal standards or internal standard solution is used to determine the presence and/or levels of further additional metabolites which are commonly used as markers of metabolic diseases, especially in newborns, thereby ensuring a much broader detection of disease conditions in newborns via a single screening.

[0064] Said further additional metabolites may include without any limitations, amino acids, organic acids, carnitines or a plurality of carnitines, as well as succinylacetone (SUAC). Elevated amino acids, free carnitine and acylcarnitine levels are examples of metabolites that can be indicative of one or more of several metabolic disorders. Free carnitine and acylcarnitines are markers for disorders that are classified as fatty acid oxidation (FAO) disorders and organic
acidurias (OA). Similarly, amino acids are used as markers for several metabolic disorders collectively known as amino acidopathies.

[0065] Therefore according to this third embodiment, the newborn screening kits, in vitro methods, and novel set of stable isotopically-labeled internal standards or internal standard solution may be used to identify, detect and/or quantitate clinically relevant amino acids which are commonly used as markers of metabolic diseases in newborns.

[0066] The identity and amount of amino acids in a patient's body fluid is important in a patient's health for a number of reasons. Aberrant amino acid levels can be used to diagnose disease or illness. The presence, absence, identity, amount or modification of an endogenous amino acid as well as its presence and amount in comparison to other amino acids (i.e., the overall profile of free amino acids) are important parameters in assessing a subject's metabolic state. Figure 3 provides some control levels of endogenous amino acids. Aberrant amino acid levels or increased/decreased levels of certain amino acids in comparison to other amino acids can indicate a metabolic disturbance. The qualitative and quantitative analysis of free amino acids in blood is central to the diagnosis and management of a wide variety of metabolic disturbances including primary amino acid enzymopathies (e.g., phenylketonuria, maple syrup urine disease) and disorders of amino acid transport (e.g., cystinuria).

[0067] By way of examples, these metabolites may be selected within proteinogenic amino acids plus non-proteinogenic amino acids. More precisely, they may be chosen among alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, γ-aminobutyric acid (GABA), L-Dopa, hydroxyproline, selenomethionine, phosphoserine, α-aminoacidic acid, phosphoethanolamine, sarcosine, β-alanine, taurine, β-aminoisobutyric acid, carnosine, methyl histidine, alpha-aminobutyric acid, anserine, ethanolamine, cystathionine, hydroxylysine, ornithine, argininosuccinate, s-sulfocysteine, homocitrulline, hawkinsin. Amino acid metabolites are preferably chosen among valine, alanine, leucine, lysine, ornithine, phenylalanine, tyrosine, glycine, aspartate, glutamate, citrulline, arginine, proline, methionine, serine, homocitrulline, asparagine, and/or 5-oxoproline.

[0068] Other further additional metabolites may also comprise carnitines such as free carnitine, acetyl carnitine, propionylcarnitine, butyrylcarnitine, isovalerylcarinitine, glutarylcarnitine, hexanoylcarnitine, octanoylcarnitine, decanoylcarnitine, lauroylcarnitine, myristoylcarnitine, palmitoylcarnitine, and/or stearyl carnitine.
Acylcarnitine profile (ACP) analysis is performed for the biochemical screening of disorders of fatty acid oxidation (FAO) and organic acid metabolism. Inherited FAO disorders are inborn errors of metabolism (IEM) of relatively recent discovery. They may present at any age, from birth to adulthood, frequently leading to life-threatening episodes of metabolic decompensation. Typical manifestations are hypoketotic hypoglycemia, liver disease, skeletal and cardiomyopathy, and sudden unexpected death. Organic acidemias are a more heterogeneous group of IEM. They typically present with recurrent episodes of acute life-threatening illness, hypo- or hypertonia, failure to thrive, and developmental delay. Common acute manifestations include vomiting, lethargy, coma, and seizures.

In Organic Acidurias (OA), the metabolic pathways of organic acids are disrupted and thus accumulation of the acids in blood and urine alters the acid-base balance of the body. Resulting modifications or adaptations to intermediary metabolic pathways may cause numerous clinical symptoms, including metabolic acidosis, ketosis, hyperammonemia, failure to thrive, sepsis or coma. In particular, determination of succinylacetone (SUAC) from a sample is also particularly useful. Newborn screening for tyrosinemia type I (Tyr-I) is mandatory to identify infants at risk before life-threatening symptoms occur. The analysis of tyrosine alone is limited, and might lead to false-negative results. Consequently, the analysis of SUAC is needed. According to the present invention, there is no need for a separate derivatization step for SUAC.

Therefore, further additional metabolites are preferably be detected and quantified according to the third embodiment of present invention are chosen among comprise arginosuccinate, succinylacetone, valine, alanine, leucine, lysine, ornithine, phenylalanine, tyrosine, glycine, aspartate, glutamate, citrulline, arginine, proline, methionine, serine, free carnitine, acetyl carnitine, propionylcarnitine, butyrylcarnitine, isovalerylcarinitine, glutarylcarinitine, hexanoylcarinitine, octanoylcarinitine, decanoylcarinitine, lauroylcarinitine, myristoylcarinitine, palmitoylcarinitine, and/or stearoylcarinitine.

Newborn screening kits, in vitro methods, and novel set of stable isotopically-labeled internal standards or internal standard solution of the present invention are thus useful for the diagnosis of all UCDs, as well as routine metabolic newborn screening for metabolic disorders which are generally performed in laboratories early on in life in newborns, thereby providing an expanded but single neonatal screening. Metabolic disorders are generally inborn errors of metabolism in a newborn.
In particular, newborn screening kits, in vitro methods, and novel set of internal standards or internal standard solution of the present invention allow for early assessment of neonatal predispositions to UCDs, hyperammonemia, HHH, and/or argininosuccinic aciduria, as well as additional metabolic deficiencies which are generally tested in human newborns, such as, but not limited to, amino acid disorders, fatty acid oxidation (FAO) disorders, and organic acidurias (OA).

Amino acid disorders can include, for example but are not limited to, phenylketonuria (PKU) and other hyperphenylalaninemas, maple syrup urine disease (MSUD), homocysteinemia, citrullinemia (types I and II), argininemia, tyrosinemia (types I and II), methionine adenosyltransferase (MAT) deficiency, biopterin deficiencies, hyperprolinemia, hypermethioninemia, and gyrate atrophy of choroid and retina. Screening for amino acid disorders by MS/MS usually involves making quantitative or semi-quantitative measurements of amino acids.

FAO disorders can include, for example but are not limited to, medium chain acyl-CoA dehydrogenase (MCAD) deficiency, very long chain acyl-CoA dehydrogenase (VLCAD) deficiency, short chain acyl-CoA dehydrogenase (SCAD) deficiency, multiple acyl-CoA dehydrogenase (MAD) deficiency or glutaric academia type II (GA-II), long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency, medium/short chain L-3-hydroxyacyl-CoA dehydrogenase (M/SCHAD) deficiency, trifunctional protein deficiency (TFP), carnitine palmitoyltransferase deficiencies of types I and II (CPT-I, CPT-II), carnitine-acylcarnitine translocase (CACT) deficiency, carnitine transporter deficiency/carnitine uptake defect, short chain 3-ketoacyl-CoA thiolase (SKAT) deficiency, medium chain 3-ketoacyl-CoA thiolase (MCKAT) deficiency, and 2,4-dienoyl-CoA reductase deficiency. Screening for fatty acid disorders by MS/MS usually involves making quantitative or semi-quantitative measurements on acylcarnitines. Free carnitine is not an acylcarnitine, but as those skilled in the art will understand, use of the term "acylcarnitines", in the context of making measurements for the purpose of NBS, often includes free carnitine along with true acylcarnitines.

Furthermore, organic acid disorders can include, for example but are not limited to, 3-methylcrotonyl-CoA carboxylase (3-MCC) deficiency, glutaric academia type I (GA-I), methylmalonic acidemias (MMA), propionic acidemia (PA), isovaleric acidemia (IVA), malonic aciduria (MA), multiple carboxylase deficiency (MCD), 2-methyl-3-hydroxybutyrl-CoA Dehydrogenase (MHBD) deficiency, 3-hydroxy-3-methylglutaryl-CoA lyase (HMG) deficiency,
2-methylbutyryl-CoA dehydrogenase (2 MBCD) deficiency, 3-methylglutaconic acidurias (MGA), isobutyryl-CoA dehydrogenase (IBD) deficiency, beta-ketothiolase deficiency (BKT), and ethylmalonic encephalopathy (EE).

[0077] The present invention thus provides improved in vitro method using tandem mass spectrometry to analyze biological samples, assuring a broader detection of disease conditions of a subject, specifically a subject. Subject suspected of having a metabolic disorder may be a human being, a child, a neonate, a newborn, or a child, but is preferably a newborn and said diagnosed metabolic disorder is an inborn error of metabolism. Preferably, such diagnosis is performed in neonates is between 1 to 5 days of life.

[0078] Biological samples, such as body fluid sample, blood sample and may be blood samples of newborns, most preferably in the form of a dried blood spot (DBS). The dried blood samples may be obtained from a subject or patient by any means. For example, samples may be obtained from newborns for example, by pricking the patient's skin (e.g., a heel prick) and depositing whole blood on filter paper, or filter card (or Guthrie cards) as one or more spots. The design of sampling cards varies, but typically the sampling cards contain from 2 to 10 spots per card. The spot or spots may then be punched (e.g., with a diameter in the range of about 3/16 inches to 2/16 inches) and placed into a container. For example, different spots may be placed within different wells of a microtiter plate. Alternatively, the blood sample may be provided in any other form appropriate to the desired test(s) and/or application, such as in the form of hemosylate, stored liquid blood or blood products or freeze dried samples, etc.

[0079] A biological sample containing preferably dried blood, is subjected to mass spectrometry to yield a plurality of mass spectrometry peaks, at least one of which is analyzed. Optionally, prior to mass spectrometric analysis, the sample is rapidly preprocessed, for example by chromatography, ultrafiltration, electrophoresis or dialysis. Examples of chromatography include ion exchange chromatography, affinity chromatography, hydrophobic chromatography, hydrophilic chromatography and reverse phase chromatography.

[0080] Newborn screening kits according to the previous embodiments may comprise at least one plate or microplate, dried blood spot controls, stored stable isotopically-labeled internal standards of known concentrations, extraction solution, eluent solution, and covers.

[0081] Preferably, newborn screening kits comprise one or more of the following elements:

- extraction plates, such as for example U-bottomed microplates, or any appropriate microplates for extraction;
- analysis plates, such as for example V-bottomed microplates, or any appropriate microplates for analysis;
- dried blood spot Controls;
- stored stable isotopically-labeled internal standards of known concentrations, comprising labeled amino acids, and/or labeled acylcarnitines, and/or labeled succinyl acetone, and/or labeled argininosuccinic acid;
- extraction solution;
- eluent solution;
- adhesive plastic covers; and
- optionally aluminium foil covers.

[0082] Most preferably, the newborn screening kits comprise the following elements:
(a) extraction plates (U-bottomed microplates),
(b) analysis plates (V-bottomed microplates),
(c) dried blood spot Controls,
(d) individually stored stable isotopically-labeled internal standards of known concentrations (labeled amino acids);
(e) individually stored stable isotopically-labeled internal standards of known concentrations (labeled acylcarnitines);
(f) individually stored stable isotopically-labeled internal standards of known concentrations (labeled succinyl acetone);
(g) individually stored stable isotopically-labeled internal standards of known concentrations (labeled argininosuccinic acid);
(h) extraction solution;
(i) eluent solution;
(j) adhesive plastic covers; and
(k) aluminium foil covers.

[0083] Said internal standard solutions comprises internal stable isotopically-labeled standards may be stored individually in separate vials or in a common vial. The stable isotopically-labeled internal standards are as described above in the various embodiments and aspects of the present invention. They are preferably stored in dried form.

[0084] The extraction microplates and analysis microplates may be made of any appropriate material, such as plastic or metal or combinations, and may contain cells of varying size and
number. Also, test kit cover pieces may be aseptic. Extraction and eluent solutions contained in
the kit can be stored in for example silanized glass vials, or any appropriate containers, vials or
flasks.

[0085] The newborn screening kit may optionally comprises a solvent or extraction solution and
a dispenser such as micropipette, multi-channel micropipettes and robotic dispensers or any other
mean to dispense the extraction and eluent solutions. One or more components of the kit can be
stored in a container that prevents or minimizes loss of material or evaporation of a solvent.

[0086] Said diagnostic newborn screening kits and in vitro methods may comprise a technical
information sheet and/or instruction protocols describing that the diagnosis steps which may
include i) obtaining a blood sample of said neonatal patient immediately, in particular 1 minute to
6 hours after birth, (ii) measuring the levels of endogenous metabolites in a dried blood sample
obtained from the newborn, via tandem mass spectrometry, said metabolites comprising at least
glutamine and optionally any other endogenous metabolites of interest for detecting a plurality of
additional metabolic deficiencies in the metabolic pathways of amino acids, acylcarnitine, and or
organic acids of the newborn as described above, in order to produce a metabolic profile of the
endogenous metabolites in the newborn, (iii) comparing the measured levels of the endogenous
metabolites with the corresponding levels of the endogenous metabolites in a biological sample
obtained from a control, in order to assess the predisposition and/or the presence or absence of
UCD, hyperammonemia, HHH, and/or argininosuccinic aciduria, and optionally of the presence
the plurality of additional metabolic deficiencies which are generally tested in newborns.

[0087] A volume of the extraction solution may then be added to each container that includes a
dried blood sample. This may be done manually or preferably using automated sample handling
equipment. Once the extraction solution had been added to each sample well, the samples are
eluted, for example on a shaker table using gentle shaking action. The supernatant is then
removed from each container and the remnants of the blood samples are discarded. The solvent in
the supernatant is finally removed, for example, by evaporation using heated nitrogen flow.

[0088] Said technical information sheet and/or instruction protocols may preferably describe in
details the steps for using the screening kits, wherein said steps comprise a step of preparation of
a working solution comprising said one or more reconstituted labeled internal standards with the
extraction solution, and a step of sample extraction, wherein said steps may be performed in any
order.
[0089] Preferably, the newborn screening kit according to the present invention comprises instructions for the following steps:

- a step of preparation of internal standards (sable isotopically-labeled amino acids, labeled acylcarnitines standards, etc.),

- a step of preparation of a working solution comprising reconstituted sable isotopically-labeled internal standards with the extraction solution, and/or

- a step of sample extraction.

[0090] The newborn screening kit may further comprise instructions for instrument settings according to the instrument manufacturer, and/or for the calculation of the results according to the instrument manufacturer.

[0091] Most preferably, the newborn screening kit comprises instructions for the following steps:

Step 1: preparation of internal standard solution comprising the stable isotopically-labeled standards, such as stable isotopically-labeled amino acids, stable isotopically-labeled acylcarnitines, etc., are dissolved in 1 ml extraction solution.

Step 2: preparation of a "working solution"

A working solution may be prepared in any appropriate container, for example a 10 ml volumetric flask. The working solution is prepared by

a. adding 100 µl reconstituted labeled amino acids, 100 µl reconstituted labeled acyl carnitines, etc.

b. filling up to 10 ml with extraction solution, and

c. mixing thoroughly.

Step 3: Sample extraction.

A blood sample of a newborn is obtained and deposited on a Dried Blood Spot (for example a Guthrie card or similar). Blood spot punches, each of them bearing aliquots of newborn dried blood sample.

[0092] Sample extraction may be conducted by:

i punching 3 mm disks of dried blood controls into the U-bottomed microplate (a),

ii punching 3 mm disks from patient samples into the U-bottomed microplate (a),

iii adding 100 µl of "working solution" as prepared in step 2,

iv ensuring that disks are properly soaked,

v covering the plate with adhesive plastic cover (j),

vi shaking at room temperature (RT) for 20 min (+/- 4 min) at 650 rpm
vii removing plastic cover (j).
viii transferring 70 µl of the content of each well to a V-bottomed microplate (b),
ix covering the plate with aluminium foil cover (k),
x placing the covered microplate to the autosampler of the tandem mass spectrometer and

injection the sample.

[0093] Screening kit may further contain instructions regarding:
- the instrument settings according to the instrument manufacturer, and optionally
- the calculation of the results according to the instrument manufacturer.

[0094] The set of individual standards comprising a known amount of one or more stable isotopically-labeled internal standards corresponding to the metabolites to be detected is then added to the sample before or after the extraction step, so that the test sample that is eventually analyzed by mass spectrometry comprises stable isotopically-labeled internal standards. Dried blood samples may thus be treated with an extraction and eluent solutions, before or after the addition of the stable isotopically-stable internal standards.

[0095] Newborn screening kits according to the present invention may thus comprise a solvent for addition to the cells already containing dried stable isotopically-labeled internal standards. The sample of the newborn's blood may thus be extracted from the punched blood spot with a solvent or an extraction solution which also contains stable isotopically-labeled standards of known concentrations. As described above, such solvent comprises a lysine stable isotopically-labeled internal standard and any appropriate additional stable isotopically-labeled internal standards which may be further added in the kits to perform the desired and/or required further additional plurality of tests of the blood sample from the newborns.

[0096] The blood spot extracts, containing the isotopically-labeled standards may be subject to chemical modification or analyzed directly. All conditions to which the sample is exposed also apply to the internal standards. Kits and methods may or may not require derivatization of the metabolites. The derivatization step is performed by covalently modifying, i.e., by methylation or ester formation, one or more metabolites of the sample, in order to detect and measure said metabolites. Typically, a derivatizing agent such as butanol-n-HCl is used for this purpose, or other appropriate derivatizing agent. The samples must then be dried again to remove excess derivatizing agent. The methods and kits according to the present invention preferably do not require any derivatization.
Alternatively, the test tray having cells may already provide with one or more dried stable isotopically-labeled internal standards. In both cases, said stable isotopically-labeled internal standards comprise at least a lysine stable isotopically-labeled internal standard, and may further comprise one or more stable isotopically-labeled internal standards as appropriate for determining the amount of any further metabolites of interest which are also tested with the newborn screening kits according to the present invention. The amount of material measured in the blood spot extract by tandem mass spectrometry is inferred by observation of the ratio of instrument response of the known standard to the unknown substance undergoing analysis.

Preferred newborn screening kits and methods of the present invention utilizes dried stable isotopically-labeled standards for newborn screening which are dissolved and used on a sample-by-sample basis. The standards may be dried according to known methods including heat drying and freeze drying (lyophilization).

According to a fourth embodiment, the present invention comprises an internal standard solution comprising a novel set of stable isotopically-labeled internal standards for detection of UCDs, hyperammonemia, HHH, and/or argininosuccinic aciduria, comprising at least a stable isotopically-labeled internal standard for lysine, and may further comprise one or more stable isotopically-labeled internal standards corresponding to one or more metabolites to be further detected in the biological sample of a subject.

Preferably, the set of stable isotopically-labeled internal standards allows the detection of all UCDs as well as additional routine neonatal screening as described above, and thus comprises a lysine dried stable isotopically-labeled internal standard, and additional dried stable isotopically-labeled internal standards corresponding to one or more metabolites of interest to be further detected in the newborn screening kit.

According to one aspect of this embodiment, said one or more stable isotopically-labeled internal standards corresponding to one or more metabolites are selected among arginine, citrulline, argininosuccinate, orotic acid, and/or ornithine.

According to a second aspect of this embodiment, such internal standard solution may thus comprise at least a lysine stable isotopically-labeled internal standard, optionally a glutamine stable isotopically-labeled internal standard, and one or more stable isotopically-labeled internal standards corresponding to one or more metabolites selected among argininosuccinate, succinylacetone, orotic acid, ornithine, valine, alanine, leucine, phenylalanine, tyrosine, glycine, aspartate, glutamate, citrulline, arginine, proline, methionine, serine, free carnitine, acetyl
carnitine, propionylcarnitine, butyrylcarnitine, isovalerylcarbnitine, glutarylcarbnitine, hexanoylcarbnitine, octanoylcarbnitine, decanoylcarbnitine, lauroylcarbnitine, myristoylcarbnitine, palmitylcarbnitine, and/or stearoylcarbnitine.

[0103] Said stable isotopically-labeled internal standards are preferably present in dried form.

[0104] Preferably, internal standard solution may comprise a lysine dried stable isotopically-labeled internal standard in combination with a stable isotopically-labeled internal standard corresponding to citrulline, argininosuccinate, orotic acid and ornithine, thereby allowing for the detection of all UCDs as well as NAGS deficiency, CPS deficiency, OTC deficiency, HHH, citrullinemia as described above.

[0105] The lysine dried stable isotopically-labeled internal standard may be used in combination with a stable isotopically-labeled internal standard corresponding to leucine and/or valine for detecting all UCDs and at the same time of MSUD disorder, and/or with phenylalanine for the detection of PKU and hyperphenylalaninemia as well.

[0106] Other combinations according to this fourth embodiment of the present invention are lysine stable isotopically-labeled internal standard with a stable isotopically-labeled internal standard corresponding to tyrosine for the detections of all UCDs and tyrosinemia, and/or in addition with a stable isotopically-labeled internal standard corresponding to argininosuccinic acid for further detecting argininesucciny-CoA-lyase (ASAL) deficiency.

[0107] Said internal standard solution may also comprise a lysine dried stable isotopically-labeled internal standard in combination with a stable isotopically-labeled internal standard corresponding to citrulline, argininosuccinate, orotic acid, ornithine, leucine, phenylalanine, valine and/or alanine.

[0108] To any of the above combinations may be added a stable isotopically-labeled internal standard corresponding to free carnitine, acetyl carnitine, propionylcarnitine, butyrylcarnitine, isovalerylcarbnitine, glutarylcarbnitine, hexanoylcarbnitine, octanoylcarbnitine, decanoylcarbnitine, lauroylcarbnitine, myristoylcarbnitine, palmitylcarbnitine, and/or stearoylcarbnitine, for further detecting fatty acid oxidation disorders.

[0109] A stable isotopically-labeled internal standard is separately detectable from the molecule based on unique physical characteristics, such as a unique mass or mass-to-charge ratio. Stable isotopically labeled form of the metabolite of interest represents commonly used internal standards. For example, if the analyte is MPP, the internal standard can be an isotopically-labeled MPP. Stable isotope labeled analogs can be used to quantitate the corresponding metabolite of
interest using the technique known as isotope dilution mass spectrometry wherein the metabolite and internal standards are processed in the same sample.

[0110] As used herein, isotopic labeled, isotopically-labeled, and other similar terms are used as is understood in the art. Specifically, an isotopically-labelled compound is a compound in which at least one atom of known position is enriched with an isotope other than the most abundant naturally-occurring isotope for that element. For example, methane may be $^{13}$C-isotopically-labelled, and have the structure $^{13}$CH$_4$, or deuterium-labelled. Deuterium-labelled methane may refer to a compound in which one or more of the four hydrogen atom positions associated with methane are enriched with $^2$H (D). Common deuterium-labeled methane structures include CDH$_3$ and CD$_4$. Isotopically-labelling refers to isotopic enrichment above natural abundance. Preferably, the isotopic purity at the enriched position is greater than 50%, or greater than 80%, greater than 90%, greater than 95%, greater than 97%, greater 98%, or greater than 99%.

[0111] Internal standards can be designed such that (1) the labeling causes a shift in mass of at least 1 mass unit and (2) that none of the stable isotope labels are located in labile sites to prevent exchange. The actual location of the labels on the molecule can vary provided the prerequisite (2) above is satisfied. Moreover, the position of the labels and the potential change in the mass of the fragment ions can also be used to confirm separation of the internal standard and metabolites.

[0112] According to the current invention for the internal standards, one or more isotopic labels can be used, and when more than one is used, multiple of the same label (e.g., deuterium) or different labels (e.g., deuterium and $^{13}$C) can be present. Exemplary isotopically-labeled internal standards are those derivatives that can be clearly differentiated from the isotope peaks of the metabolite of interest.

[0113] Any appropriate isotopic labels can be used including, for example, $^2$H, $^{13}$C, $^{15}$N, and $^{18}$O or combination thereof. While not being bound by any theory, the physicochemical behavior of such stable isotopically-labeled derivative with respect to sample preparation and signal generation would be expected to be identical to that of the unlabeled analyte, but clearly differentiable on the mass spectrometer.

[0114] Amino acid standards which are labeled with one or more of stable isotopes preferably have three or more of mass difference relative to unlabeled amino acid. By labeling to an unlabeled amino acid with stable isotope so as to bring about 3 or more of mass differences, the influence due to natural existence ratio of the isotope in the unlabeled object may be reduced, and highly precise analysis can be performed. For example, distribution of the natural isotopes in
alanine (molecular formula: C₃H₇NO₂, molecular mass 88) are molecular weight 88 (95.8%), 89 (3.74%), 90 (0.34%), and 91 (0.02%), and by making the mass difference into 3 or more, the influence of natural isotopes is avoided.

[0115] Therefore, according to this fourth embodiment, internal standard solution useful in the newborn screening kits and methods may comprise at least lysine-[^12]C⁶-[^13]N₂, and one or more of the stable isotopically- labeled amino acid standards chosen among [²H₄]-alanine, [²H₄]-[^13]C-arginine, [²H₂]-citrulline, [¹⁵N]-[^13]C-glycine, [²H₃]-leucine, [²H₃]-methionine, [²H₆]-ornithine, [¹³]C-phenylalanine, [²H₅]-valine, [²H₃]-aspartic acid, [²H₅]-glutamic acid, [¹³]C₃-serine, [¹³]C₆-tyrosine, [²H₅]-proline, [¹³]C₅-succinylacetone, and/or [¹⁵N₄]-[^13]C₆-argininosuccinate, and/or one or more labeled carnitine standards chosen among [²H₉]-carnitine, [²H₃]-acetylcarnitine, [²H₅]-propionylcarnitine, [²H₃]-butyrylcarnitine, [²H₉]-isovaleryl carnitine, [²H₃]-glutaryl carnitine, [²H₃]-hexanoylcarnitine, [²H₃]-octanoylcarnitine, [²H₃]-decanoylcarnitine, [²H₅]-lauroyl carnitine, [²H₉]-myristoylcarnitine, [²H₅]-palmitoylcarnitine, [²H₃]-stearoylcarnitine. Preferred concentrations of amino acids range from 0.2 to 50 μmol/L. Preferred concentrations of (acyl) carnitines in the kit reagents of the present invention range from 0.001 to 1 μmol/L. Said internal standards are preferably present in dried form.

[0116] Preferably, an internal standard solution which may be used according to the present invention comprises at least one agent selected from components A to D wherein component A comprises of valine, glycine, alanine, arginine, and glutamate, component B comprises of serine, proline, tyrosine, leucine, isoleucine, lysine, methionine, ornithine and citrulline, component C comprises of carnitine, acetylcarnitine, propionylcarnitine, butyrylcarnitine, isovaleryl carnitine, glutaryl carnitine, hexanoylcarnitine, octanoylcarnitine, decanoylcarnitine lauroyl carnitine, myristoyl carnitine, palmitoyl carnitine, stearoyl carnitine, component D comprises of succinylacetone, orotic acid and argininosuccinate, wherein one or more atoms of each compound are labeled with a stable isotope.

[0117] Another preferred internal standard solution comprises glycine, valine, succinylacetone, argininosuccinate and at least one of leucine, phenylalanine, tyrosine, and citrulline.

[0118] The methods and kits described herein involve detecting the ratios or sum or weighted sum two or more amino acids, say for example of lysine and glutamine and one or more additional biological metabolites (e.g., amino acids, free carnitine, or acylcarnitines, SUAC, organic acids) where the presence or amount of each biomolecule correlates the presence or absence of a metabolic disorder. The methods described herein can be used quantitatively, if
desired, to allow comparison of test sample results with known or a pre-determined standard amount of a particular metabolite (e.g., by using an internal standard as described above). The methods and kits may also be used qualitatively when a test sample is compared with a reference sample, which can be either a normal reference or metabolic disorder reference. In this format, the relative amount of biomolecules can be indicative of a metabolic disorder. A reference sample, for example, can be from a subject having, not suspected of having, or not at risk of developing a disorder such as a metabolic disorder such as urea cycle disorder.

[0119] Generally, a cut-off value for a given biomolecule can vary and would be known in the art for commonly tested metabolites and enzymes. Routine, obvious adaptations of methods known in the art can be used to establish cut-off values for uncommonly tested metabolites. A cut-off value is typically a biomolecule amount, or ratio with another biomolecule, above or below which is considered indicative of a metabolic disorder or cause for retest. Thus, in accordance with the invention described herein a reference level of at least one biomolecule in a particular sample type is identified as a cut-off value, which there is a significant correlation between the presence (or absence) of the at least one biomolecule and presence (or absence) of a metabolic disorder. It is understood that biomolecule panels can be interpreted as a whole, in parts or on an analyte-by-analyte basis.

[0120] Those of skill in the art will recognize that some cut-off values are not absolute in that clinical correlations are still significant over a range of values on either side of the cutoff; however, it is possible to select an optimal cut-off value (e.g., varying H-scores, and the like) of biomolecule for a particular sample type. Cut-off values determined for use in the methods described herein generally are compared with published ranges but can be individualized to the methodology used and patient population. It is understood that improvements in optimal cut-off values could be determined depending on the sophistication of statistical methods used and on the number and source of samples used to determine reference level values for the different biomolecules and sample types. Therefore, established cut-off values can be adjusted up or down, on the basis of periodic re-evaluations or changes in methodology or population distribution. In addition, instrument-specific cut-off values can be used, if desired, for example such as when inter-instrument performance comparability is >10%.

[0121] The reference level can be determined by a variety of methods, provided that the resulting reference level accurately provides an amount of each biomolecule above which exists a first group of subjects (e.g., humans) having a different probability of metabolic disorder than that
of a second group of subjects having metabolite or enzyme activity amount below the reference level. The reference level can be determined by comparison of biomolecule amount in, e.g., populations of subjects (e.g., patients) having the same metabolic disorder. This can be accomplished, for example, by histogram analysis, in which an entire cohort of patients are graphically presented, wherein a first axis represents the amount of biomolecule and a second axis represents the number of subjects in the cohort whose sample contain one or more biomolecules at a given amount.

[0122] Therefore the kits and in vitro method for conducting newborn screening are conducted by analyzing contributions of a plurality of metabolites utilizing a tandem mass spectrometry signal, said method comprising: obtaining a tandem mass spectrometry signal of a sample comprising a plurality of peak intensities, wherein more than one of said metabolites in said sample contributes to at least one of said peak intensities; providing a model relating said peak intensities to a contribution intensity for each of said metabolites; wherein said model comprises representing said signal as a weighted sum of reference signals which includes measuring the sum of lysine and glutamine, where each reference signal corresponding to one of said metabolites; calculating said contribution intensities of each of said metabolites using said model; providing a calibration curve for at least one of said metabolites relating contribution intensity to concentration; and determining, based on said calibration curve, a concentration of at least one metabolite and using the results to interpret urea cycle disorders as well as further metabolic disorders as described above.

[0123] According to the methods and kits of the present invention, ionizing step (iii) is performed by delivering said metabolites extracted from DBS samples and dried stable isotope internal standards to an ion source of the mass spectrometer. The extracted metabolites and dried stable isotopically-labelled internal standards used as controls are sprayed through a small tube into a strong electric field in the presence of a flow of warm nitrogen gas to assist desolvation and formation of ions. The ionization process typically involves transfer of a charge to the solvent droplets, evaporation of the solvent and, finally, production of positively and negatively charged ions. Such ion formation process is a starting point for mass spectrum analysis.

[0124] Several ionization methods are available and the choice of ionization method depends on the sample to be analyzed. For example, for the analysis of polypeptides, a relatively gentle ionization procedure such as electrospray ionization (ESI) may be used. For ESI, a solution containing the sample is passed through a fine needle at high potential which creates a strong
electrical field resulting in a fine spray of highly charged droplets that is directed into the mass spectrometer. Other ionization procedures include, for example, fast-atom bombardment (FAB) which uses a high-energy beam of neutral atoms to strike a solid sample causing desorption and ionization. Matrix-assisted laser desorption ionization (MALDI) is a method in which a laser pulse is used to strike a sample that has been crystallized in an UV-absorbing compound matrix. Other ionization procedures known in the art include, for example, plasma and glow discharge, plasma desorption ionization, resonance ionization, and secondary ionization.

In the preferred embodiment of the invention, the solvated test sample/standards are directly introduced into a tandem mass spectrometer that is adapted to process the test sample/standards using an electrospray. The tandem mass spectrometer may be any appropriate spectrometer commercially available.

Electrospray ionization (ESI) has several properties that are useful for the invention described herein. For example, ESI can be used for biological molecules such as polypeptides that are difficult to ionize or vaporize. In addition, the efficiency of ESI can be very high which provides the basis for highly sensitive measurements. Furthermore, ESI produces charged molecules from solution, which is convenient for analyzing reporters that are in solution. In contrast, ionization procedures, such as MALDI, require crystallization of the sample prior to ionization. Since ESI can produce charged molecules directly from solution, it is compatible with samples from liquid chromatography systems. For example, a mass spectrometer can have an inlet for a liquid chromatography system, such as an HPLC, so that fractions flow from the chromatography column into the mass spectrometer. This in-line arrangement of a liquid chromatography system and mass spectrometer is sometimes referred to as LC-MS. A LC-MS system can be used, for example, to separate un-cleaved or partially cleaved samples from cleaved tag reporters before mass spectrometry analysis. In addition, chromatography can be used to remove salts or other buffer components from the tag reporter sample before mass spectrometry analysis. For example, desalting of a sample using a reversed-phase HPLC column, in-line or off-line, can be used to increase the efficiency of the ionization process and thus improve sensitivity of detection by mass spectrometry. Preferably, the ionizing step (iii) is performed by delivering said extracted metabolites and standards to an ion source of the mass spectrometer by liquid chromatography system.

This vaporized and ionized mixture enters the first MS which functions as a separating device and allows only the ion(s) of interest to pass through. Ions passing through the first MS are
called precursors or parent ions which enter the collision cell where the fragmentation takes place. Fragmentation is achieved by putting an inert collision gas such as nitrogen or argon into the collision cell. The fragments generated in the collision cell are called products or daughter ions. The mass of these fragments are measured in the second MS. Fragments in the second MS are correlated with the intact molecules produced in the first MS. This process enables unique MS such as precursor ion scans and neutral loss scans.

[0128] According to the present invention, the acquisition step (iv) of the mass to charge (m/z) ratio is performed in Multiple Reaction Monitoring (MRM) mode and refers to a tandem spectrometry (MS-MS) experiment where one or more specific products of a selected precursor ion, i.e. a parent ion, a molecular ion or a daughter ion, is monitored.

[0129] A variety of configurations of mass spectrometers can be used in a method of the invention. In general, a mass spectrometer has the following major components: a sample inlet, an ion source, a mass analyzer, a detector, a vacuum system, and instrument-control system, and a data system. Differences in the sample inlet, ion source, and mass analyzer generally define the type of instrument and its capabilities. For example, an inlet can be a capillary-column liquid chromatography source or can be a direct probe or stage such as used in matrix-assisted laser desorption. Common ion sources are, for example, electrospray, including nanospray and microspray or matrix-assisted laser desorption. Common mass analyzers include a quadrupole mass filter, ion trap mass analyzer and time-of-flight mass analyzer. Preferably, such data acquisition step (iv) may be performed with a triple-quadrupole mass spectrometer or high-resolution mass spectrometer.

[0130] The test sample is scanned using a mass spectrometer to produce one or more mass spectra. Any mass spectrometer that can detect a signal from the extracted metabolites and the standards can be used in the inventive methods. A tandem mass spectrometer is preferably used according to the present invention as it identifies different molecular entities that produce a common fragment when subjected to collision-induced dissociation (CID). In tandem mass spectrometry, two mass analyzers are linked in series via a collision cell. The first mass analyzer (first quadrupole) is used to select an ion of interest (e.g., an ion of a particular mass-to-charge ratio (m/z)). The selected ions are then transferred to a collision cell where they are fragmented by collision with an inert gas (e.g., nitrogen or helium or argon). This process is called collisionally activated dissociation (CAD) and is performed in the collision cell of the mass spectrometer. Once the precursor ions have been fragmented, the second mass analyzer (third quadrupole) is
used to either scan or detect all of the produced product ions, or to select and detect particular fragment ions.

[0131] Tandem mass spectrometry is used to ionize lysine and/or glutamine as well as any additional metabolites to be detected in the newborn screening kits and methods of the present invention. Such tandem mass spectrometry allows ion fragmentation and detection specific peaks that are indicative of the presence of these molecules in the sample.

[0132] The tandem mass spectrometry detection can be accomplished in a number of ways. In one type of tandem mass spectrometry (commonly performed on triple quadrupole tandem mass spectrometers), ions that fragment to produce common product (fragment) ions can be detected as a class by performing a "precursor ion scan", where by selecting the appropriate mass for the common fragmentation in the collision cell, all ions that produce the common fragment ions are detected. In a different form of tandem mass spectrometry, ions that fragment to produce a common neutral loss can be detected as a class by performing a so called neutral loss scan where by setting an appropriate mass offset equal to the common neutral loss between first and third quadrupoles all ions that fragment to produce the specified neutral loss are detected. In yet another type of tandem mass spectrometry known as multiple reaction monitoring (MRM), a precursor ion of interest is selected in the first quadrupole, fragmented in the collision cell and a specific fragment ion resulting from the collisional activation is selected in the third quadrupole and finally detected.

[0133] First and third quadrupoles are fixed to respectively select the corresponding precursor and fragment ion pairs of interest for a predetermined amount of time (a few milliseconds). If additional analytes or metabolites need to be detected, additional detection transitions can be introduced in the experiment. The data from all selected mass transitions can be acquired sequentially to obtain the desired information.

[0134] A quadrupole mass analyzer, as well as the other mass analyzers described herein, can be programmed to analyze a defined m/z or mass range. Since the mass range of cleaved tag reporters will be known prior to an assay, a mass spectrometer can be programmed to transmit ions of the projected correct mass range while excluding ions of a higher or lower mass range. The ability to select a mass range can decrease the background noise in the assay and thus increase the signal-to-noise ratio. In addition, a defined mass range can be used to exclude analysis of molecules. Therefore, the mass spectrometer can accomplish an inherent separation step as well as detection and identification of metabolites.
Ion trap mass spectrometry utilizes an ion trap mass analyzer. In these mass analyzers, fields are applied so that ions of all m/z are initially trapped and oscillate in the mass analyzer. Ions enter the ion trap from the ion source through a focusing device such as an octapole lens system. Ion trapping takes place in the trapping region before excitation and ejection through an electrode to the detector. Mass analysis is accomplished by sequentially applying voltages that increase the amplitude of the oscillations in a way that ejects ions of increasing m/z out of the trap and into the detector. In contrast to quadrupole mass spectrometry, all ions are retained in the fields of the mass analyzer except those with the selected m/z. One advantage to ion traps is that they have very high sensitivity, as long as one is careful to limit the number of ions being trapped at one time. Control of the number of ions can be accomplished by varying the time over which ions are injected into the trap. The mass resolution of ion traps is similar to that of quadrupole mass filters, although ion traps do have low m/z limitations.

Time-of-flight mass spectrometry utilizes a time-of-flight mass analyzer. For this method of m/z analysis, an ion is first given a fixed amount of kinetic energy by acceleration in an electric field (generated by high voltage). Following acceleration, the ion enters a field-free or "drift" region where it travels at a velocity that is inversely proportional to its m/z. Therefore, ions with low m/z travel more rapidly than ions with high m/z. The time required for ions to travel the length of the field-free region is measured and used to calculate the m/z of the ion.

One consideration in this type of mass analysis is that the set of ions being studied be introduced into the analyzer at the same time. For example, this type of mass analysis is well suited to ionization techniques like MALDI which produces ions in short well-defined pulses. Another consideration is to control velocity spread produced by ions that have variations in their amounts of kinetic energy. The use of longer flight tubes, ion reflectors, or higher accelerating voltages can help minimize the effects of velocity spread. Time-of-flight mass analyzers have a high level of sensitivity and a wider m/z range than quadrupole or ion trap mass analyzers. Also data can be acquired quickly with this type of mass analyzer because no scanning of the mass analyzer is necessary.

Tandem mass spectrometry can utilize combinations of the mass analyzers described above. Tandem mass spectrometers can use a first mass analyzer to separate ions according to their m/z in order to isolate an ion of interest for further analysis. The isolated ion of interest is then broken into fragment ions (called coUisionally activated dissociation or collision induced dissociation) and the fragment ions are analyzed by the second mass analyzer. These types of
tandem mass spectrometer systems are called tandem in space systems because the two mass analyzers are separated in space, usually by a collision cell. Tandem mass spectrometer systems also include tandem in time systems where one mass analyzer is used, however the mass analyzer is used sequentially to isolate an ion, induce fragmentation, and then perform mass analysis.

[0139] Mass spectrometers in the tandem in space category have more than one mass analyzer. For example, a tandem quadrupole mass spectrometer system can have a first quadrupole mass filter, followed by a collision cell, followed by a second quadrupole mass filter and then the detector. Another arrangement is to use a quadrupole mass filter for the first mass analyzer and a time-of-flight mass analyzer for the second mass analyzer with a collision cell separating the two mass analyzers. Other tandem systems are known in the art including reflectron-time-of-flight, tandem sector and sector-quadrupole mass spectrometry.

[0140] Mass spectrometers in the tandem in time category have one mass analyzer that performs different functions at different times. For example, an ion trap mass spectrometer can be used to trap ions of all m/z. A series of rf scan functions are applied which ejects ions of all m/z from the trap except the m/z of ions of interest. After the m/z of interest has been isolated, an rf pulse is applied to produce collisions with gas molecules in the trap to induce fragmentation of the ions. Then the m/z values of the fragmented ions are measured by the mass analyzer. Ion cyclotron resonance instruments, also known as Fourier transform mass spectrometers, are an example of tandem-in-time systems.

[0141] Several types of tandem mass spectrometry experiments can be performed by controlling the ions that are selected in each stage of the experiment. The different types of experiments utilize different modes of operation, sometimes called "scans," of the mass analyzers. In a first example, called a mass spectrum scan, the first mass analyzer and the collision cell transmit all ions for mass analysis into the second mass analyzer. In a second example, called a product ion scan, the ions of interest are mass-selected in the first mass analyzer and then fragmented in the collision cell. The ions formed are then mass analyzed by scanning the second mass analyzer. In a third example, called a precursor ion scan, the first mass analyzer is scanned to sequentially transmit the mass analyzed ions into the collision cell for fragmentation. The second mass analyzer mass-selects the product ion of interest for transmission to the detector. Therefore, the detector signal is the result of all precursor ions that can be fragmented into a common product ion. Other experimental formats include neutral loss scans where a constant mass difference is accounted for in the mass scans. The use of these different tandem mass spectrometry scan
procedures can be advantageous when large sets of metabolites are measured in a single experiment as with multiplex experiments.

[0142] These techniques are well-known to skilled person in the art who can readily recognize that different mass spectrometry methods, for example, quadrupole mass spectrometry, ion trap mass spectrometry, time-of-flight mass spectrometry and tandem mass spectrometry, can use various combinations of ion sources and mass analyzers which allows for flexibility in designing customized detection protocols. In addition, mass spectrometers can be programmed to transmit all ions from the ion source into the mass spectrometer either sequentially or at the same time. Furthermore, a mass spectrometer can be programmed to select ions of a particular mass for transmission into the mass spectrometer while blocking other ions. The ability to precisely control the movement of ions in a mass spectrometer allows for greater options in detection protocols which can be advantageous when a large number of metabolites, for example, from a multiplex experiment, are being analyzed.

[0143] Different mass spectrometers have different levels of resolution, that is, the ability to resolve peaks between ions closely related in mass. The resolution is defined as $R = \frac{m}{\Delta m}$, where $m$ is the ion mass and $\Delta m$ is the difference in mass between two peaks in a mass spectrum. For example, a mass spectrometer with a resolution of 1000 can resolve an ion with m/z of 100.0 from an ion with m/z of 100.1. Those skilled in the art will therefore select a mass spectrometer having a resolution appropriate for the metabolites to be detected.

[0144] Mass spectrometers can resolve ions with small mass differences and measure the mass of ions with a high degree of accuracy. Therefore, metabolites of similar masses can be used together in the same experiment since the mass spectrometer can differentiate the mass of even closely related molecules. The high degree of resolution and mass accuracy achieved using mass spectrometry methods allow the use of large sets of metabolites because they can be distinguished from each other.

[0145] The level of metabolites in the test sample is then determined by comparing a peak in the one or more mass spectra that corresponds to the metabolites with a peak in the one or more mass spectra that corresponds to the isotopically enriched standard. In one embodiment, relative peak heights are used. In another embodiment, the areas under the peaks are integrated and compared.

[0146] Once the metabolite level in a test sample or dried blood sample has been determined it can be compared with a range of normal levels. If the level is outside this normal range then the dried blood sample or the patient from whom it was obtained may be referred for further analysis.
For example, the test could be repeated with one or more additional blood spots to obtain an average level. Additionally or alternatively the metabolite level could be measured using an alternative method known in the art, e.g., an immunochemical method or a mass spectral method using a serum sample.

[0147] In a preferred embodiment the system includes the MRM mode of operation of a triple quadrupole (tandem) mass analyzer. In MRM mode of operation, the first mass analyzing quadrupole is set to select a specific "precursor" ion from the ions passing through the first mass analyzing quadrupole, a second non-mass analyzing quadrupole is used to cause controlled dissociation of the precursor ion, and the third quadrupole (the second mass analyzing quadrupole) is set to select only a specific fragment, or "product", ion of the precursor ion. The precursor and product combination is referred to as the MRM ion pair.

[0148] According to some embodiments of the present invention, MRM data can be acquired in several different manners, with the differences being in how the laser is allowed to interact with the sample on the target plate, for example, depending on the ablation mode that is used. A "raster" ablation mode involves the laser beam cutting a straight line swath across one or more samples. To accomplish this "rastering" the laser beam is fired at a non-sample location prior to a sample spot of interest, and the target plate can then be moved continuously to present new samples to the laser impingement point. This means that the laser desorption point will encounter an alternating series of sample/no sample sections of the target plate. The resulting MRM data contains a series of ion signal "peaks", indicated by ion counts per second as a function of mass spectrometer analysis time, with non-zero signal where sample was encountered, interspersed with regions of zero-signal baseline in between sample spots. Depending on laser power, speed of target plate movement under the laser beam, number of metabolites being monitored and sample composition, it can be typical to acquire the data for a single sample (monitoring one, or several, MRM ion pairs) in approximately 0.25 to 5 seconds for this mode of ablation.

[0149] In a "discrete" mode of operation, with the laser in a non-firing state the target plate can be positioned under the laser impingement point and the laser can then be turned on for some specific period of time. While the laser is not firing, the mass spectrometer records a signal of zero (since no ions are generated). When the laser commences firing the laser beam desorbs material from this specific location, creating an MRM signal "peak". The MRM signal intensity rises from zero and quickly maximizes, and it then decreases as the sample is depleted from that specific location on the target plate. The MRM signal level returns to the zero baseline level either
by turning off the laser, or by permitting the laser to fire until the sample is completely consumed from the particular location and there is no more sample from which to generate ions. After the laser firing is stopped, the sample plate can then be moved so as to present a new sample location for the next ablation. Depending on laser power, sample composition, and the length of time the laser is fired, it can be typical to acquire the data for a single sample in well under one second for this mode of ablation.

[0150] Other ablation modes involve the movement of a sample spot according to some pattern, such that the continually firing laser generates a pattern across the sample spot. This "pattern raster" mode generates a steady stream of ions from a few seconds to several minutes, depending on the form and speed of the pattern within the sample spot. One or more sample spots can be included in such a mode, with proper accommodation when moving from one sample spot to the next. The mass spectrometer method can be constructed to perform a number of different measurements, even including the mixing of mass spectrometer scan types. Pattern rasters are useful for measurements which require ablation times longer than those typically used for MRM analyses with this technique (e.g., precursor or neutral loss scans).

[0151] In the preferred reagent kits and methods of the present invention, the extraction step (ii) is performed by using an extraction buffer comprising one or more organic solvents, ionizing step (iii) is performed by delivering said extracted metabolites and internal standard solution to an ion source of the mass spectrometer by liquid chromatography system, data acquisition step (iv) is performed with a triple-quadrupole mass spectrometer or high-resolution mass spectrometer, the step (v) is performed by selecting at least one accurate mass-to-charge (m/z) ratio ions corresponding to an at least one calculated mass-to-charge (m/z) ratio of said metabolites present in said sample. Also, a direct tandem mass spectrometric analysis of metabolites in dried blood spots is performed without chemical derivatization for neonatal screening. Most preferably, multiple reaction monitoring (MRM) technique IS applied for tandem mass spectrometric measurements, such that each metabolite has its individual precursor and product ion settings.

[0152] In the most preferred reagent kits and methods of the present invention, comprise a step of separating the one or more metabolites by liquid chromatography (LC) prior to proceeding to the mass spectrometry (MS) analysis as described above. Therefore most preferred kits and methods are liquid chromatography-mass spectrometry methods and kits.

[0153] High pressure liquid chromatography (HPLC) is a form of column-based chromatography that is routinely used in analytical chemistry to separate, identify or detect and quantify
molecules. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that detects the abundance of the molecules and shows their retention on the chromatographic column in relation to the elapsed time (retention time). Retention times vary depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used. A sample containing the metabolites is injected into the mobile phase manually or by an automated autosampler. The polarity of the metabolites, the stationary phase of the column(s) used and the mobile phase(s) determine the retention time of the metabolite as well as its separation from interferences and extent of quantifiability. Amino acid separation using HPLC may be performed with any commercially available LC apparatus using automated or manual sample injection and adjustable, consistent and reproducible solvent flow rates.

[0154] Columns suitable for liquid chromatography contain packing materials that include very small and usually spherical particles, e.g., silica particles, having a diameter of 3-50 microns and a pore size of 100-1000 angstroms. Commonly, HPLC is performed with a stationary phase attached to the outside surface of such small particles; such stationary phase may provide that surface hydrophobic properties or enable ion change or ion pairing. A chromatographic column typically includes two ports, one inlet port for receiving a sample and one outlet port for discharging an effluent that may or may not include the sample.

[0155] In some embodiments of the present invention, the one or more amino acids in a sample enter a column from the inlet port, are then eluted with a solvent or solvent mixture, and eventually discharged at the outlet port. In preferred embodiments, one or more amino acids in a sample enter a column from the inlet port where after the flow across said column is reversed and one or more amino acids are then eluted with a solvent or solvent mixture, and eventually discharged back at the inlet port (flow-reverse). Using a chromatographic column flow-reverse, and specifically using the first chromatographic column of two successive chromatographic columns flow-reverse, proved beneficial in delaying the elution of hydrophilic amino acids and in improving their ionization in the mass detector, leading to increased analytical sensitivity.

[0156] Different solvents or solvent mixtures may be selected for eluting the amino acids. For example, liquid chromatography may be performed using a gradient mode with differing amounts of solvents in the mixture, an isocratic mode with continuously fixed amounts of solvents in the mixture or a partially isocratic, partially gradient mixed mode. Suitable solvents and solvent
mixtures include sodium or lithium buffers (for cation exchange HPLC) or acetonitrile (for reverse phase HPLC).

[0157] The internal diameter of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity. Column dimensions in preferred embodiments of the present invention include a column internal diameter of 2.1-3.0 mm and a column length of 3-10 cm.

[0158] Liquid chromatography is based on the principle that a metabolite is adsorbed to a stationary phase and eventually desorbed and eluted with the mobile phase into a detection unit for proper detection and/or quantitation. The choice of both stationary and mobile phase greatly influences the success of chromatographic separation.

[0159] Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is treated silica. With these stationary phases, retention time is longer for molecules which are more non-polar, while polar molecules elute more rapidly. The mobile phase is generally a binary mixture of water and a miscible polar organic solvent like methanol, acetonitrile or tetrahydrofuran (THF). Reversed phase chromatography is based on partition and is typically used for separations by non-polar differences.

[0160] In contrast to reversed phase HPLC, normal phase HPLC (NP-HPLC) uses a polar stationary phase and a non-aqueous, non-polar mobile phase, and works effectively for separating metabolites readily soluble in non-polar solvents. The metabolites associate with and are retained by the polar stationary phase until final elution. Typical stationary phases for normal phase chromatography are silica or organic moieties with cyano- and/or amino-functional groups. In NP-HPLC, the most nonpolar molecules elute first and the most polar molecules elute last. The mobile phase consists of a very nonpolar solvent like hexane or heptane mixed with a slightly more polar solvent like isopropanol, ethyl acetate or chloroform. Retention increases, as the amount of nonpolar solvent in the mobile phase increases. NP-HPLC is based on adsorption and is typically employed for the analysis of solutes readily soluble in organic solvents, based on their polar differences such as amines, acids, metal complexes, etc…

[0161] Use of ion exchange chromatography may also be within the scope of the invention, since amino acids, by definition, contain at least one amino-group and one carboxyl acid group, they are ionizable and consequently carry a positive or negative charge, when the pH of the mobile phase differs from the amino acid’s pKa. Below the neutral pH of 7.0, amino acids with
primarily basic groups (e.g., amino groups) are positively charged, whereas above the neutral pH of 7.0, amino acids with primarily acidic groups (e.g., carboxylic acid groups) are negatively charged. The 20 proteinogenic amino acids that represent the building blocks of proteins differ in their side-chain groups, which influence the amino acids' chemical reactivity, ionic charge, relative hydrophilicity or hydrophobicity and polarity. Ion-exchange chromatography is a process that allows the separation of ions and polar molecules based on the charge properties of the metabolites. Charged amino acids may be either acidic or basic. The stationary phase surface displays ionic functional groups (R--X) that interact with analyte ions of opposite charge. This type of chromatography is further subdivided into cation exchange chromatography and anion exchange chromatography. The target metabolites (anions or cations) are retained on the stationary phase but can be eluted by increasing concentrations of similarly charged species that will displace the analyte ions from the stationary phase. For example, in cation exchange chromatography, the positively charged analyte could be displaced by the addition of positively charged sodium ions.

[0162] Ion-pairing chromatography may also be contemplated for use within the scope of this invention. Similar to ion-exchange chromatography, ion pairing chromatography utilizes the ionizability and charge properties of the metabolites for the chromatographic separation. However, instead of exchanging ions, ion-pairing systems are established using perfluorinated carboxylic acids such as tridecafluoroheptanoic acid (TDFHA) or trifluoroacetic acid (TFA) as mobile phase constituents and a stationary phase that can accept or donate electrons or both. Specifically, ion pairing chromatography can be used to separate ionic metabolites on a reversed-phase column in order to suppress the ionic characteristic of charged organic compounds. Ion pair reagents have a charge opposite of the metabolites and a hydrophobic region to interact with the stationary phase. The charge of the absorbed ion pair reagent interacts electrostatically with the charge of the metabolites. As an example, amines can produce a serious tailing chromatographic peak on a reversed phase column, while addition of an ion pair agent such as trifluoroacetic acid curtails tailing. With advances in column phases and a better selection of ion pair reagents, ion pair chromatography not only sharpens chromatographic peaks but also modulates the retention of ionic metabolites on reverse-phase columns. Typical ion pair reagents include tetra-alkylammonium ions and perfluorinated organic acids. The type of ion pair reagent, the concentration of ion pair reagent, the type of organic modifier in the mobile phase, the concentration (gradient) of the organic modifier, and the proper selection of the columns are
critical to a successful ion pair chromatography experiment. In preferred embodiments, ion pair chromatography was used.

[0163] According to a fifth embodiment, newborn screening kits and methods according to the present invention may also be extended to include detection of variant proteins and polypeptides implicated in serious diseases. For example, many variant or mutant forms of the polypeptide sub-units of hemoglobin are known to result in various forms of anemia, and many such mutations are of only one amino acid.

[0164] Preferred kits and methods would be targeting specific selected ionised species. Kits and methods may be applied with advantage to detect not only variants containing amino acids which differ from the norm, as in the many possible known hemoglobinopathies described hereinafter, but also to detect variants in glycosylation patterns of polypeptides, as well as deletions and/or additions of one or more amino acid residues from the expected polypeptide sequence.

[0165] According to this fifth embodiment, kits and methods are particularly useful for testing a sample by mass spectrometry, where the ionisation technique produces a multiply-charged spectrum, to further detect (a) the presence or absence of a known polypeptide or derivative of a polypeptide, or (b) the presence or absence of a variant of a known polypeptide or derivative of a polypeptide, in which scanning of the sample is targeted to selected ionised species of known mass/charge ratio, the absence of the expected value of mass/charge ratio being indicative of the absence of the known polypeptide or derivative thereof, or the presence of the variant polypeptide or derivative thereof being indicated by a shift in mass/charge ratio from the expected value. Such method thus allows concentrating data acquisition on a restricted mass/charge range (mass window) to include the normal polypeptide and the variant polypeptide or polypeptides of interest. Targeting in this manner is more reproducible and reveals peaks corresponding to the normal mass/charge ratio and any shifts from the norm due to variants present in samples taken from patients who are either homozygous or heterozygous in this respect. Thus the method may frequently require only one restricted mass window to be targeted. The use of separate windows for the normal and for the variant polypeptide is also possible. Additional mass windows may be used to target other variants or other polypeptides.

[0166] According to the current method ionisation technique is used to produce multiply-charged spectrum, to detect (a) the presence or absence of a known polypeptide or derivative of a polypeptide, or (b) the presence or absence of a variant of a known polypeptide or derivative of a polypeptide, in which scanning of the sample is targeted to selected ionised species of known
mass/charge ratio, the absence of the expected value of mass/charge ratio being indicative of the absence of the known polypeptide or derivative thereof, or the presence of the variant polypeptide or derivative thereof being indicated by a shift in mass/charge ratio from the expected value.

[0167] Kits and methods according to this embodiment of the present invention may thus be used to detect any protein variant such as a protein mutation or an abnormal concentration of a wild-type protein. Any inherited disorder leading to variant protein production may therefore be detected using kits and method according to this embodiment. Preferably hemoglobin variants, albumin, myoglobin, cytochromes, and variant proteins associated with various congenital disorders like for example disorders of glycosylation are detected.

[0168] Preferred kits and methods of the present invention are used to detect clinically important hemoglobin variants such as Hb S, Hb C, Hb D\textsuperscript{\textalpha}; Hb O\textgamma, Hb Lepore, Hb E, delta beta thalassemia, hereditary persistence of fetal hemoglobin trait (HPFH) and alpha zero thalassemia trait. It is further preferred that the method of the present invention is used to detect the following clinically important hemoglobin variants: S, C, E, D\textsuperscript{\textalpha}; and C\textgamma. The protein variant to be detected may be any protein including a glycoprotein. In particular, specific glycoproteins indicative of a metabolic disorder may be detected using the method of the present invention.

[0169] Hemoglobin variants analysis may be conducted simultaneously or sequentially with the analysis of metabolites described above, \textit{Lc}, glutamine, and further amino acids, carnitines, acylcarnitines, SUAC, ASA either from the same sample like dried blood spot or from a different sample like different lot of dried blood spot.

[0170] The method or kit according to another embodiment can further comprise the step of (v) assaying the enzymatic activity of the polypeptide in the sample, and/or (vi) assaying the amino acid composition of said polypeptide. The step of assaying enzymatic activity may comprise (a) adding a substrate for said enzymatic activity to said sample, and (b) analyzing the sample for the presence or the absence of said substrate and/or for the presence or absence of a product resulting from said enzymatic activity on said substrate. The step of assaying the amino acid composition may comprise (i) adding an endopeptidase like trypsin (ii) analyzing the polypeptides in said sample after the endopeptidase treatment, and (iii) inferring the amino acid composition of said polypeptide.
EXAMPLES

Example 1: Determination of the levels of glutamine, lysine, arginino succinic acid, and orotic acid

To determine the linearity of the measurement of glutamine, lysine, arginino succinic acid, and orotic acid, standard solutions of the respective analyte up to 20 mM were prepared and 10 μL injected into the tandem mass spectrometer and the respective ions/transition were measured. (Fig. 6 - 8)

Example 2: Determination of the levels of glutamine and lysine in dried blood samples

To determine the linearity of the measurement of the sum of lysine and glutamine in dried blood spots (DBS), blood of a healthy donor was spiked with lysine. Endogenous concentrations of lysine and glutamine were determined from an aliquot by standard amino acid determination using ion exchange chromatography. (Fig. 9)

Example 3: Determination of the sum of glutamine and lysine in dried blood samples

Figure 10 shows the proposed results of the measurement of the sum of glycine and lysine. For lysine and glutamine the reference range of lysine and glycine is plotted, together with the calculated sum of those 2 reference ranges (sum of lower - sum of upper reference range), measured values from 180 DBS of healthy newborns, measured values from 2 samples of a patient with proven OTC deficiency, expected range of patients with urea cycle defects (UCD).
CLAIMS

1. A method of detecting the presence and/or measuring the levels of one or more metabolites in a sample obtained from a subject, by tandem mass spectrometry, said metabolites comprising at least glutamine and said method comprising the following steps:

5. (i) extracting said metabolites from the sample with an extraction solution;

(ii) providing, before or after the step (i), a solution comprising a known amount of one or more a stable isotopically-labeled internal standard corresponding to said metabolites to be detected in the sample,

(iii) ionizing said metabolites and said one or more stable isotopically-labeled internal standards to generate ions,

(iv) acquiring the mass to charge (m/z) ratio of said one or more ions in the mass spectrum in the Multiple Reaction Monitoring (MRM) mode,

(v) determining the sum of glutamine and lysine in MRM mode, and

(vi) detecting and quantifying the amount of glutamine with stable isotopically-labeled internal standard corresponding to lysine.

2. The method according to claim 1 for diagnosis of one or more metabolic deficiencies selected among urea cycle disorders, and/or hyperammonemia, Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH), and/or argininosuccinic aciduria, further comprising a step (vii) wherein elevated level of glutamine as obtained in step (vi) is detected compared to physiological level of glutamine.

3. The method according to claim 1 or 2, wherein one or more metabolites are selected among amino acids, organic acids, acylcarnitines, a plurality of carnitines, and/or succinylacetone are further detected and quantified in said sample, and wherein said method further comprises a step (v) for determining the presence and/or the amounts of said one or more metabolites by analyzing the mass MRM spectrum as obtained in step (iv).

4. The method according to claim 3, wherein said amino acids comprise aspartate, serine, proline, phenylalanine, tyrosine, methionine, valine, leucine, citrulline, homocitrulline, ornithine, arginine, alanine, N-acetyl glutamate, glycine, argininosuccinate and/or oxoproline, and/or wherein said organic acid comprises orotic acid, or said acyl carnitines or plurality of carnitines comprise free carnitines, acetyl carnitine, propionylcarnitine, butyrylcarnitine, isovalerylcarnitine, glutaryl carnitine, hexanoylcarnitine, octanoylcarnitine, decanoylcarnitine, lauroylcarnitine, myristoylcarnitine, palmitoylcarnitine, and/or stearoylcarnitine.
5. The method according to any one of claims 1 to 4, wherein the stable isotopically-labeled internal standard solution comprises at least a lysine stable isotopically-labeled internal standard.

6. The method according to any one of claims 1 to 5, wherein the stable isotopically-labeled internal standard solution further comprises one or more stable isotopically-labeled internal standards corresponding to alanine, arginine, citrulline, glycine, leucine, methionine, ornithine, phenylalanine, glutamine valine, lysine, aspartic acid, glutamic acid, tyrosine, serine, oxoproline, proline, argininosuccinate, succinylacetone, orotic acid, free carnitine, acetyl carnitine, propionylcarnitine, butyrylcarnitine, isovalerylcarnitinite, glutaryl carnitine, hexanoylcarnitine, octanoylcarnitine, decanoylcarnitine, lauroyl carnitine, myristoylcarnitine, palmitoylcarnitine, and/or stearoylcarnitine.

7. The method according to any one of claims 1 to 6, wherein said one or more stable isotopically-labeled internal standards are dried.

8. The method according to any one of claims 1 to 7, wherein said sample is a body fluid sample, or a blood sample, or a dried blood sample.

9. The method according to any one of claims 1 to 8, wherein said subject is a subject suspected of having a metabolic disorder, such as a neonate, a newborn, or a child, or a newborn.

10. The method according to any one of claims 1 to 9, wherein said ionizing step (iii) is performed by delivering said extracted metabolites and standards to an ion source of the mass spectrometer by liquid chromatography system.

11. The method according to any one of claims 1 to 10, wherein said data acquisition step (iv) is performed with a triple-quadrupole mass spectrometer or high-resolution mass spectrometer.

12. The method according to any one of claims 1 to 11, wherein the step (v) is performed by selecting at least one accurate mass-to-charge (m/z) ratio ions corresponding to an at least one calculated mass-to-charge (m/z) ratio of said one or more metabolites present in said sample.

13. The method according any one of claims 1 to 12, wherein the determined amount or presence of glutamine correlates with the presence or absence of urea cycle disorder, and/or hyperammonemia, and/or Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH), and/or argininosuccinic aciduria.

14. A method according for diagnosis of one or more metabolic deficiencies selected among urea cycle disorders, and/or hyperammonemia, Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH), and/or argininosuccinic aciduria, by tandem mass spectrometry, in a newborn subject, comprising the following steps:
(i) extracting glutamine metabolite from the sample of said newborn subject with an extraction solution;
(ii) providing, before or after the step (i), a solution comprising a known amount of lysine stable isotopically-labeled internal standard corresponding to be detected in the sample,
(iii) ionizing said metabolites and said one or more stable isotopically-labeled internal standards to generate ions,
(iv) acquiring the mass to charge (m/z) ratio of said one or more ions in the mass spectrum in the Multiple Reaction Monitoring (MRM) mode,
(v) determining the sum of glutamine and lysine in MRM mode, and
(vi) detecting and quantifying the amount of glutamine with stable isotopically-labeled internal standard corresponding to lysine, and
(vii) wherein elevated level of glutamine as obtained in step (vi) is detected compared to physiological level of glutamine.

15. A newborn screening kit for detecting the presence and/or measuring the levels of glutamine in a blood sample of a newborn, by tandem mass spectrometry, according to the method as defined in any one of claims 1 to 14, comprising an internal standard solution comprising at least a stable isotopically-labeled internal standard corresponding to lysine.

16. Newborn screening kit according to claim 15, for the diagnosis of one or more metabolic deficiencies selected among urea cycle disorders, and/or hyperammonemia, Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH), and/or argininosuccinic aciduria.

17. Newborn screening kit according to claim 15 or 16, wherein said internal standard solution further comprises one or more stable isotopically-labeled internal standards corresponding to one or more metabolites selected among amino acids, organic acids, acylcarnitines, a plurality of carnitines, and/or succinylacetone.

18. Newborn screening kit according to any one of claims 15 to 17, wherein said internal standard solution further comprises one or more stable isotopically-labeled internal standards corresponding to alanine, arginine, citrulline, homocitrulline, glycine, leucine, methionine, ornithine, phenylalanine, valine, aspartic acid, glutamic acid, tyrosine, serine, oxoproline, proline, glutamic acid, argininosuccinate, succinylacetone, orotic acid, free carnitine, acetyl carnitine, propionylcarnitine, butyrylcarnitine, isovaleryl carnitine, glutaryl carnitine, hexanoyl carnitine, octanoyl carnitine, decanoyl carnitine, lauroyl carnitine, myristoyl carnitine, palmitoyl carnitine, and/or stearoyl carnitine, wherein said standards are optionally dried, and wherein said kit allows
for the diagnosis in said newborns or of one or more metabolic deficiencies selected among urea cycle disorders, and/or hyperammonemia, Hyperornithinemia-hyperammonemia-homocitrullinuria (HHH), and/or argininosuccinic aciduria, as well as further metabolic newborn disorders, selected among amino acid disorders, fatty acid oxidation (FAO) disorders, and organic acidurias (OA).

19. Newborn screening kit according to any one of claims 15 to 18, wherein said sample is a body fluid sample, preferably a blood sample, and more preferably a dried blood sample, and/or wherein said newborn is suspected of having a metabolic disorder.

20. Newborn screening kit according to any one of claims 15 to 19, further comprising at least one plate or microplate, dried blood spot controls, extraction solution, eluent solution, and/or one or more covers.

21. Newborn screening kit according to any one of claims 15 to 20, which comprises one or more of the following elements:
   - one or more extraction plates;
   - one or more analysis plates;
   - dried blood spot controls;
   - stored stable isotopically-labeled internal standards of known concentrations;
   - extraction solution;
   - eluent solution;
   - adhesive plastic covers; and/or
   - optionally one or more aluminium foil covers.

22. Newborn screening kit according to any one of claims 15 to 21, wherein said stored stable isotopically-labeled internal standards of known concentrations comprise labeled amino acids, and/or labeled acylcarnitines, and/or labeled succinyl acetone; and/or labeled argininosuccinic acid, wherein said internal standards are stored in separate vials or in a common vial, and wherein said internal standards are optionally dried.

23. Newborn screening kit according to any one of claims 15 to 22, further comprising a technical sheet or instructions or protocol steps for the screening, wherein said steps comprise of preparation of a working solution comprising said one or more reconstituted labeled internal standards with the extraction solution, and a step of sample extraction, wherein said steps may be performed in any order.
24. Newborn screening kit according to any one of claims 15 to 23, further comprising instructions for the following steps:
- a step of preparation of internal standards (labeled amino acids, labeled acylcarnitines, etc.),
- a step 2 of preparation of a working solution comprising reconstituted labeled internal standards with the extraction solution, and/or
- a step of sample extraction.

25. Newborn screening kit according to any one of claims 15 to 24, optionally further comprising instructions for instrument settings according to the instrument manufacturer, and/or for calculation of the results according to the instrument manufacturer.

26. An internal standard solution as defined in the method of any one of claims 1 to 14, comprising at least a lysine stable isotopically-labeled internal standard, and further comprising one or more stable isotopically-labeled internal standards corresponding to alanine, citrulline, homocitrulline glycine, leucine, methionine, ornithine, glutamine, phenylalanine, valine, glutamic acid, aspartic acid, glutamic acid, tyrosine, serine, oxoproline, proline, argininosuccinate, succinylacetone, orotic acid, free carnitine, acetylcarnitine, propionylcarnitine, butyrylcarnitine, isovalerylcarntinite, glutarylcarntinite, hexanoylcarnitine, octanoylcarnitine, decanoylcarnitine, lauroylcarntinite, myristoylcarnitine, palmitoylcarnitine, and/or stearoylcarntinite, wherein said standards are optionally dried.

27. Internal standard solution of claim 26, wherein said lysine stable isotopically-labeled internal standard is $^{13}$C$_{6}$-$^{15}$N$_2$-lysine, and said further stable isotopically-labeled internal standards are $^2$H$_4$-alanine, $^2$H$_2$-citrulline, $^{15}$N-$^{13}$C-glycine, $^2$H$_3$-leucine, $^2$H$_3$-methionine, $^2$H$_6$-ornithine, $^{13}$C$_6$-phenylalanine, $^2$H$_g$-valine, $^2$H$_3$-aspartic acid, $^2$H$_3$-glutamic acid, $^{13}$C$_3$-serine, $^{13}$C$_6$-tyrosine, $^2$H$_5$-proline, $^{13}$Cs-succinylacetone, $^{15}$N$_4$-$^{13}$C$_6$-argininosuccinate, $^2$H$_g$-carnitine, $^2$H$_3$-acetylcarnitine, $^2$H$_3$-propionylcarnitine, $^2$H$_3$-butyrylcarnitine, $^2$H$_g$-isovalerylcarntinite, $^2$H$_3$-glutarylcarntinite, $^2$H$_3$-hexanoylcarnitine, $^2$H$_3$-octanoylcarnitine, $^2$H$_3$-decanoylecarnitine, $^2$H$_3$-lauroylcarnitine, $^{13}$H$_4$-myristoylcarnitine, $^2$H$_3$-palmitoylcarntinite, and/or $^2$H$_3$-stearoylcarntinite, wherein said standards are optionally dried.
FIGURE 2

<table>
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<tr>
<th></th>
<th>CPS</th>
<th>OTC</th>
<th>CITR</th>
<th>ASA</th>
<th>ARG</th>
<th>NAGS</th>
<th>LPI</th>
<th>HHH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>↑-↑↑</td>
<td>↑-↑↑</td>
<td>↑-↑↑</td>
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</tr>
<tr>
<td>Alanine</td>
<td>n-↑</td>
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<td>n-↑</td>
<td>n-↑</td>
<td>n-↑</td>
<td>n-↑</td>
<td>n-↑</td>
<td>n-↑</td>
</tr>
<tr>
<td>Asparagine</td>
<td>n-↑</td>
<td>n-↑</td>
<td>n-↑</td>
<td>n-↑</td>
<td>n-↑</td>
<td>n-↑</td>
<td>n-↑</td>
<td>n-↑</td>
</tr>
<tr>
<td>Citrulline</td>
<td>↓-n</td>
<td>↓-n</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>Ornithine</td>
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<td>n</td>
<td>n</td>
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<td>n</td>
<td>↓-n</td>
<td>↓-n</td>
<td>↑↑</td>
</tr>
<tr>
<td>Lysine</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>↓-n</td>
<td>↓-n</td>
<td>n</td>
</tr>
<tr>
<td>Arginine</td>
<td>↓-n</td>
<td>↓-n</td>
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<td>↓-n</td>
<td>↑↑↑</td>
<td>↓-n</td>
<td>↓-n</td>
<td>n</td>
</tr>
<tr>
<td>Urine</td>
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<td></td>
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<td>Lysine, ornithine</td>
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<td>n</td>
<td>n</td>
<td>↑(Arg)</td>
<td>n</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑(Orn)</td>
</tr>
<tr>
<td>Homocitrulline</td>
<td>n</td>
<td>↑</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>↑↑</td>
<td>↑</td>
</tr>
<tr>
<td>Orotate, orotidine, uracil</td>
<td>n ↑↑↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>n</td>
<td>→</td>
<td>→</td>
<td>→</td>
</tr>
</tbody>
</table>

CPS, OTC and NAGS deficiencies need to be confirmed by enzymatic assays on liver biopsies (OTC deficiency could be diagnosed also on small bowel biopsy). The remaining disorders except LPI can be confirmed by enzymatic assays on easily accessible tissue (RBC or cultured skin fibroblasts).
<table>
<thead>
<tr>
<th></th>
<th>Control 1 Endogen. Only (μM)</th>
<th>Control 2 Endogen. +spiked (μM)</th>
<th>Control 3 Endogen. +spiked (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe</td>
<td>~30 μM</td>
<td>90,5</td>
<td>332,7</td>
</tr>
<tr>
<td>Tyr</td>
<td>~30 μM</td>
<td>85,0</td>
<td>196,0</td>
</tr>
<tr>
<td>Met*</td>
<td>0 μM</td>
<td>34,0</td>
<td>101,0</td>
</tr>
<tr>
<td>Val</td>
<td>~70 μM</td>
<td>155,0</td>
<td>582,0</td>
</tr>
<tr>
<td>Leu</td>
<td>~60 μM</td>
<td>136,0</td>
<td>517,0</td>
</tr>
<tr>
<td>Cit</td>
<td>~10 μM</td>
<td>67,00</td>
<td>295,00</td>
</tr>
<tr>
<td>Glu</td>
<td>~110 μM</td>
<td>450,00</td>
<td>790,00</td>
</tr>
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<td>484,0</td>
</tr>
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<td>Ala</td>
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<td>1199,0</td>
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<td>Pro</td>
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<td>387,00</td>
<td>1082,00</td>
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<tr>
<td>Lys</td>
<td>~50 μM</td>
<td>458,00</td>
<td>1069,00</td>
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</table>
### FIGURE 9

**Amino acids in plasma (μmol/l)**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Men ((n = 50)^a)</th>
<th>Women ((n = 15)^a)</th>
<th>Adolescents ((n = 80)^b)</th>
<th>Children ((n = 52)^b)</th>
<th>Infants &lt;3mo ((n = 17)^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taurine</td>
<td>27–95</td>
<td>18–66</td>
<td>2–90</td>
<td>20–120</td>
<td>10–167</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>2–9</td>
<td>3–6</td>
<td>3–15</td>
<td>1–17</td>
<td>0–31</td>
</tr>
<tr>
<td>Threonine</td>
<td>92–180</td>
<td>93–197</td>
<td>102–246</td>
<td>40–204</td>
<td>46–222</td>
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<tr>
<td>Serine</td>
<td>89–165</td>
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<td>Glutamic acid</td>
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<td>17–69</td>
<td>14–78</td>
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<td>Proline</td>
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<td>58–324 (^d)</td>
<td>40–332 (^c)</td>
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<td>Alanine</td>
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<td>Citrulline</td>
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<td>19–52 (^d)</td>
<td>8–47 (^c)</td>
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<td>a-Aminobutyrate</td>
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<td>12–43 (^c)</td>
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<td>Cystine</td>
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<td>36–58 (^d)</td>
<td>23–68 (^c)</td>
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<td>Methionine</td>
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<td>–</td>
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<td>Arginine</td>
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<td>1–81</td>
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Range = mean ± 2SD

\(^a\) Modified from ref. [1].

\(^b\) Modified from ref. [2].

\(^c\) Modified from ref. [5].

\(^d\) Modified from ref. [6].

\(^e\) Shih, unpublished data.
INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/07Q724

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68 G01N33/58

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

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
G01N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category* Citation of document, with indication, where appropriate, of the relevant passages Relevant to claim No.

A

Anonymous: "Cell-Free Protein Expression for Generating Stable Isotope-Labeled Protein ns \ Thermo Fisher Scientifi c",
10 November 2012 (2012-11-10) XP055275448,
Retrieved from the Internet:
[retrieved on 2016-05-25]
the whole document

Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"Z" document member of the same patent family

Date of the actual completion of the international search

7 October 2016

Date of mailing of the international search report

12/12/2016

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

Authorized officer

Lunter, Pim

Form PCT/ISA/210 (second sheet) (April 2005)
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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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### INTERNATIONAL SEARCH REPORT

**Box No. II** Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **☐** Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. **☐** Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. **☐** Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**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:

see additional 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 an 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 - 14

**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.
This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-14
   Method of detecting the presence and/or measuring the levels of one or more metabolites comprising at least glutamine, using stable-isotope labeled internal standards corresponding to said metabolites.
   ---

2. claims: 15-25
   Newborn screening kit comprising an internal standard solution comprising at least a stable-isotopically-labeled internal standard corresponding to lysine.
   ---

3. claims: 26, 27
   Internal standard solution comprising at least a lysine stable-isotope labeled internal standard and further comprising one or more other stable-isotope labeled internal standards.
   ---
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<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
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