PH TECH
Paya Hamsan Technologies

Instruction Manual for Newborn Screening Complete Kit

PH NBS

PH NBS/ PH NBS-D
Amino Acids and Acylcarnitines
From Dried Blood Spots

IVD For in Vitro Diagnostic Use

REF PH 2001, PH 2002

ISO 13485:2016


PHI
NBS

PH NBS

Amino Acids and Acylcarnitines From Dried Blood Spots

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Contents:

1. IVD Symbols

Symbols according to the ISO 15223-1:2012 (Symbols to be used with medical device labels, labelling and information to be supplied), are used on the product labels and in the instruction manual.

	In vitro diagnostic medical device
	Consult instructions for use
	Upper limit of temperature
	Temperature limit
	Use-by date
	Catalogue number
	Batch code
	Packing Number
	Contains sufficient for n tests
	Manufacturer

2. Intended Use

The PH NBS/PH NBS-D Complete kits are intended for the semi-quantitative measurement and evaluation of amino acids, free carnitine and acylcarnitines concentrations in dried blood samples on the filter paper for newborns screening.
The evaluated values of these analytes (Table 1) and their relationship with each other is intended to provide analyte concentration profiles that interpret metabolic disorder in newborn screening.

Table 1: List of measurable analytes using the PH NBS/PH NBS-D complete kits

Analyte	Abbreviation
Amino acids	
Alanine	Ala
Arginine	Arg
Aspartic Acid	Asp
Citrulline	Cit
Glutamic Acid	Glu
Glycine	Gly
Leucine/ Isoleucine/ Hydroxyproline	Leu/Ile/Pro-OH
Methionine	Met
Ornithine	Orn
Phenylalanine	Phe
Proline	Pro
Tyrosine	Tyr
Valine	Val
Acylcarnitines and free Carnitine	
Carnitine	C0
Acetylcarnitine	C2
Propionylcarnitine	C3
Malonylcarnitine/3-Hydroxybutyrylcarnitine	C3DC/C4OH
Butyrylcarnitine	C4
Methylmalonylcarnitine/3-Hydroxyisovalerylcarnitine	C4DC/C5OH
Isovalerylcarnitine	C5
Tiglylcarnitine	C5:1
Glutarylcarnitine/3-Hydroxyhexanoylcarnitine	C5DC/C6OH
Hexanoylcarnitine	C6
Adipylcarnitine	C6DC
Octanoylcarnitine	C8
Octenoylcarnitine	C8:1
Decanoylcarnitine	C10
Decenoylcarnitine	C10:1
Decadienoylcarnitine	C10:2
Dodecanoylcarnitine	C12
Dodecenoylcarnitine	C12:1
Tetradecanoylcarnitine	C14
Tetradecenoylcarnitine	C14:1
Tetradecadienoylcarnitine	C14:2
3-Hydroxy-Tetradecanoylcarnitine	C14OH
Hexadecanoylcarnitine	C16
Hexadecenoylcarnitine	C16:1
3-Hydroxy-Hexadecanoylcarnitine	C16OH
3-Hydroxy-Hexadecenoylcarnitine	C16:1OH
Octadecanoylcarnitine	C18
Octadecenoylcarnitine	C18:1
Octadecadienoylcarnitine	C18:2
3-Hydroxy-Octadecanoylcarnitine	C18OH
3-Hydroxy-Octadecenoylcarnitine	C18:1OH

The components of the PH NBS/PH NBS-D complete kits are intended to be used according to this instruction manual.

3.Clinical Background

Newborn screening is a preventive measure to detect the genetic metabolic deficiency. Free carnitine and acylcarnitines are markers for fatty acid oxidation (FAO) disorders or organic aciduria (OA) and amino acids are marker for amino acidopathies.

3.1. Fatty acid oxidation (FAO) disorders

Fatty acid oxidation disorders, are a group of about 20 defects in fatty acid transport and mitochondrial β-oxidation that are inherited as autosomal recessive disorders.
FAO disorders are caused by a lack or deficiency of the enzymes needed to break down or oxidation of fatty acids, resulting in delayed mental and physical development.

3.2. Organic aciduria (OA) disorders

Organic acidurias (OA) are an important class of inherited metabolic disorders arising due to defect in intermediary metabolic pathways of carbohydrate, amino acids and fatty acid oxidation.
It leads to accumulation of organic acids in tissues and their subsequent excretion in urine, that resulting in numerous clinical symptoms, including metabolic acidosis, ketosis, hyperammonemia, failure to thrive, sepsis or coma.

3.3. Amino acidopathies

In this inherited defect is reflected downstream as a lack or a partial biological activity of enzymes involved in amino acids metabolism. As a result, the concentration of the affected amino acids and their metabolites, increases in the infant's body. These excesses can have severe deleterious effects on the infant's health including death.
Amino acid disorders are managed by medical support and nutritional restrictions, supplements and medical foods that limit consumption of an offending amino acid or in some cases protein consumption.

4.Target diseases

The range and demands on the newborn screening program vary in different countries. In IRAN the screening of currently 53 target diseases is planned by Ministry of Health and Medical Education according to Table 2.

Table 2. Target diseases in IRAN

Uniform Screening Panel 1 (20 Core Conditions)		
Amino Acid Disorders	**Fatty Acid Oxidation Disorders**	**Organic Acid Disorders**
Argininosuccinic Aciduria	Primary Carnitine Deficiency / Carnitine Transporter Defect	Propionic Acidemia
Citrullinemia Type 1	Medium Chain Acyl-CoA Dehydrogenase Deficiency	Methylmalonic Acidemia: Methylmalonyl-CoA mutase
Maple Syrup Urine Disease	Very Long Chain Acyl-CoA Dehydrogenase Deficiency	Methylmalonic Acidemia: Cobalamin Disorders
Homocystinuria	Long Chain 3-Hydroxyacyl-CoA Dehydrogenase Deficiency	Isovaleric Acidemia
Phenylketonuria Classic	Trifunctional Protein Deficiency	3-Methylcrotonyl-CoA Carboxylase Deficiency
Tyrosinemia Type 1		3-Hydroxy-3-Methylglutaric Aciduria
		Holocarboxylase Synthetase Deficiency
		β-Ketothiolase Deficiency
		Glutaric Acidemia Type 1
Uniform Screening Panel 2 (33 Secondary Conditions)		
Amino Acid Disorders	**Fatty Acid Oxidation Disorders**	**Organic Acid Disorders**
Argininemia	Short Chain Acyl-CoA Dehydrogenase Deficiency	Methylmalonic acidemia, cblC form
Citrullinemia Type 2	Medium/Short Chain Acyl-CoA Dehydrogenase Deficiency	Methylmalonic acidemia, cblD form
Hypermethioninemia	Glutaric Acidemia Type 2	Malonic Acidemia
Benign Hyperphenylalaninemia	Medium Chain ketoacyl-CoA Thiolase Deficiency	Isobutyrylglycinuria
Biopterin Biosynthesis Defect (2 conditions)	2,4-Dienoyl-CoA Reductase Deficiency	2-Methylbutyrylglycinuria
Biopterin Regeneration Defect (2 conditions)	Carnitine Palmitoyltransferase 1 Deficiency	3-Methylglutaconic Aciduria
Non-Ketotic Hyperglycinemia	Carnitine Palmitoyltransferase 2 Deficiency	2-Methyl-3-hydroxybutyric Aciduria
Ornithine Transcarbamylase Deficiency	Carnitine Acylcarnitine Translocase Deficiency	Ethylmalonic Encephalopathy
Carbamoyl Phosphate Synthetase 1 Deficiency		
HHH Syndrome		
Tyrosinemia Types 2,3 (2 conditions)		
Glycine N-methyltransferase (GNMR) deficiency		
Adenosylhomocysteine hydrolase deficiency		
Pyruvate carboxylase deficiency		

5.General description of the assay

The PH NBS/PH NBS-D kits are supplied with 26 internal standards and controls for the measurement of carnitines and amino acids. Therefore, the PH NBS/PH NBS-D kits have capability of measuring 13 amino acids and 31 carnitine species (Table 3), because of acylcarnitines with same chain length have similar performance characteristics. In this way, C16 internal standard can be used to determine the concentrations of the acylcarnitine series C16, C16:1, C16OH and C16:1OH. Additionally, unlabeled C16 can be used as an external control for all C16 acylcarnitine series.

Table 3: Measurable analytes using PH NBS/PH NBS-D kits and their corresponding internal standards and controls

Analyte	Internal Standard	Controls
Amino acids		
Alanine	Alanine-D4	Alanine
Arginine	Arginine-D7	Arginine
Aspartic Acid	Aspartic acid-D3	Aspartic Acid
Citrulline	Citrulline-D2	Citrulline
Glutamic Acid	Glutamic acid-D5	Glutamic Acid
Glycine	Glycine-$^{13}C_2$, ^{15}N	Glycine
Leucine/ Isoleucine/ Hydroxyproline	Leucine-D3	Leucine
Methionine	Methionine-D3	Methionine
Ornithine	Ornithine-D6	Ornithine
Phenylalanine	Phenylalanine-D5	Phenylalanine
Proline	Proline-D7	Proline
Tyrosine	Tyrosine-D4	Tyrosine
Valine	Valine-D8	Valine
Acylcarnitines and free Carnitine		
C0	C0-Carnitine-D9	C0
C2	C2-Carnitine-D3	C2
C3	C3-Carnitine-D3	C3
C3DC/C4OH	C4-Carnitine-D3	C4
C4	C4-Carnitine-D3	C4
C4DC/C5OH	C5-Carnitine-D9	C5
C5	C5-Carnitine-D9	C5
C5:1	C5-Carnitine-D9	C5
C5DC/C6OH	C5DC-Carnitine-D9	C5DC
C6	C6-Carnitine-D3	C6
C6DC	C5DC-Carnitine-D9	C5DC
C8	C8-Carnitine-D3	C8
C8:1	C8-Carnitine-D3	C8
C10	C10-Carnitine-D3	C10
C10:1	C10-Carnitine-D3	C10
C10:2	C10-Carnitine-D3	C10
C12	C12-Carnitine-D3	C12
C12:1	C12-Carnitine-D3	C12
C14	C14-Carnitine-D3	C14
C14:1	C14-Carnitine-D3	C14
C14:2	C14-Carnitine-D3	C14
C14OH	C14-Carnitine-D3	C14
C16	C16-Carnitine-D3	C16
C16:1	C16-Carnitine-D3	C16
C16OH	C16-Carnitine-D3	C16
C16:1OH	C16-Carnitine-D3	C16
C18	C18-Carnitine-D3	C18
C18:1	C18-Carnitine-D3	C18
C18:2	C18-Carnitine-D3	C18
C18OH	C18-Carnitine-D3	C18
C18:1OH	C18-Carnitine-D3	C18

The analysis is done semi-quantitatively from dried blood spots and sample is spiked using the internal standard, the analytes are extracted from the dried blood matrix and analyzed with and without derivatization step using a tandem mass spectrometry technique. The response of each analyte relative to their corresponding stable-isotope labelled internal standard is proportional to analyte concentration.

In the derivatization step the analytes are converted to the corresponding n-Buthylester (Figues 1 and 2).

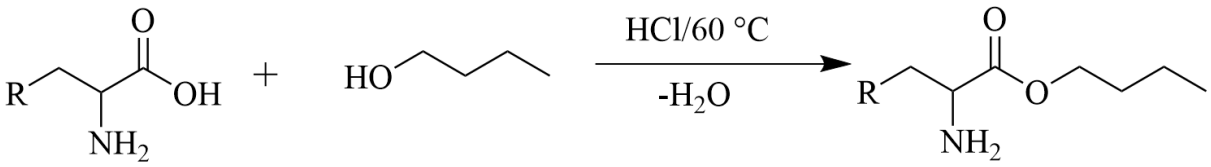


Figure 1. Derivatization of the amino acids (R is side chain of the amino acids)

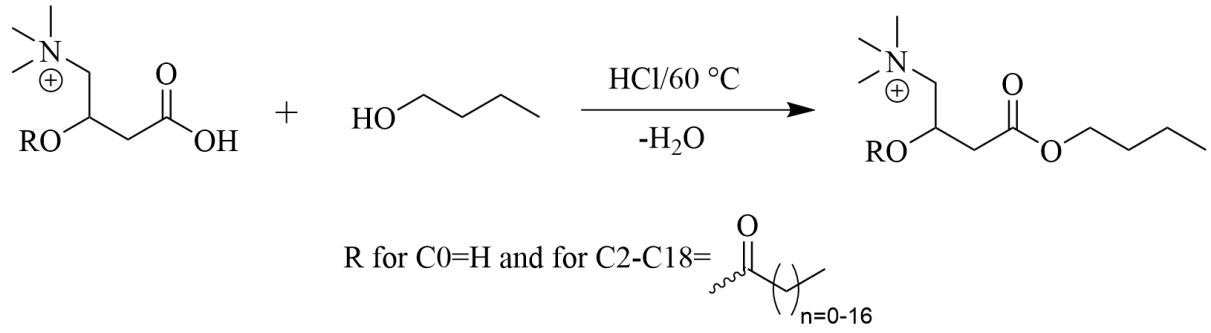


Figure 2. Derivatization of the acylcarnitines

After sample preparation, the sample is introduced to the tandem mass spectrometer, and it is delivered to the ion source. During the electrospray ionization, the sample components are ionized and then transferred into the gas phase. The ions pass through a vacuum interface and non-charged molecules are pumped away and the focused ion beam is introduced into the first mass filtering quadrupole and ions are separated by their m/z ratio. Ions traveling through the collision cell collide with the gas molecules and fragment to the smaller ions. The smaller ions or daughter ions are separated by their m/z values in the second stage mass analyzer and finally are sent to the detector for signal recording. Therefore, the measurement of the analytes is carried out in MRM mode. Measurement in MRM mode ensures identification and quantification with high selectivity and sensitivity, with the analyte determination based on its characteristic mass transitions.

6.Components of the complete kits

6.1. Ordering information

Table 4. Ordering information for PH NBS complete kit, order no. PH 2001

Order No.	Description	Quantity
PH 2001	PH NBS complete kit, for Amino Acids and Acylcarnitines, From Dried Blood spot for 960 assays	1 pc
	Internal Standard	4 vials
	Mobile Phase	1 bottle
	Rinsing Solution	1 bottle
	Extraction Buffer	2 bottles
	Reconstitution Buffer	2 bottles
	Protective Sheets for 96 Well Plates, aluminum foil	10 pcs
	96 Well Plates, flat bottom	10 pcs
	96 Well Plates, conical bottom	10 pcs
	Dried Blood Spot Control Level I , II	1 pc
	Manual	

Table 5. Ordering information for PH NBS-D complete kit, order no. PH 2002

Order No.	Description	Quantity
PH 2002	PH NBS-D complete kit, for Amino Acids and Acylcarnitines, From Dried Blood spot for 960 assays	1 pc
	Internal Standard	4 vials
	Mobile Phase	1 bottle
	Rinsing Solution	1 bottle
	Extraction Buffer	2 bottles
	Derivatization Reagent	3 bottles
	Reconstitution Buffer	2 bottles
	Protective Sheets for 96 Well Plates, aluminum foil	10 pcs
	96 Well Plates, flat bottom	10 pcs
	96 Well Plates, conical bottom	10 pcs
	Dried Blood Spot Control Level I , II	1 pc
	Manual	

The expiry date of all components of kit is stated on the labels. Store each component as indicated on the corresponding labels.

6.1.1. Internal Standard Concentration

The lot specified quality control certificate included in the kit states the concentration of the stable isotope labelled amino acids, free carnitine and acylcarnitines internal standards as their concentration in each vial after reconstitution (Table 6).

Table 6. Amino Acids / Acylcarnitines concentrations

Substance	Unit	Concentration
Alanine-D4	mg/l	1.15
	µmol/l	12.3
Arginine-D7	mg/l	0.56
	µmol/l	3.08
Aspartic acid-D3	mg/l	1.93
	µmol/l	14.2
Citrulline-D2	mg/l	0.55
	µmol/l	3.11
Glutamic acid-D5	mg/l	1.94
	µmol/l	12.7
Glycine-$^{13}C_2$, ^{15}N	mg/l	1.69
	µmol/l	21.7
Leucine-D3	mg/l	1.73
	µmol/l	12.9
Methionine-D3	mg/l	0.74
	µmol/l	4.84
Ornithine-D6	mg/l	1.02
	µmol/l	7.4
Phenylalanine-D5	mg/l	1.04
	µmol/l	6.1
Proline-D7	mg/l	1.07
	µmol/l	8.76
Tyrosine-D4	mg/l	1.61
	µmol/l	8.67
Valine-D8	mg/l	1.32
	µmol/l	10.5
C0-Carnitine-D9	mg/l	0.129
	µmol/l	0.76
C2-Carnitine-D3	mg/l	0.066
	µmol/l	0.322
C3-Carnitine-D3	mg/l	0.017
	µmol/l	0.078
C4-Carnitine-D3	mg/l	0.009
	µmol/l	0.039

Substance	Unit	Concentration
C5-Carnitine-D9	mg/l	0.011
	µmol/l	0.042
C5DC-Carnitine-D9	mg/l	0.011
	µmol/l	0.042
C6-Carnitine-D3	mg/l	0.009
	µmol/l	0.036
C8-Carnitine-D3	mg/l	0.012
	µmol/l	0.04
C10-Carnitine-D3	mg/l	0.013
	µmol/l	0.042
C12-Carnitine-D3	mg/l	0.014
	µmol/l	0.041
C14-Carnitine-D3	mg/l	0.015
	µmol/l	0.041
C16-Carnitine-D3	mg/l	0.032
	µmol/l	0.079
C18-Carnitine-D3	mg/l	0.033
	µmol/l	0.076

6.2. Safety Information

Some components of the PH NBS/ NBS-D kit are chemical solvents and reagents and may be contained hazardous materials. For safety information of any components of the kit, please consult the noted information on the labels or the respective Material Safety Data Sheets (MSDS).

6.3. Disposal of laboratory waste

Laboratory waste should be collected separately in the basis of different chemical properties. Recommendations for the disposal of product and packaging are indicated in the respective of Material Safety Data Sheets (MSDS).

7. Required instruments

The use of PH NBS/NBS-D kits require a liquid chromatograph coupled with tandem mass spectrometer with appropriate sensitivity and a suitable software for data evaluation.

Required liquid chromatograph modules:

- Autosampler
- Isocratic HPLC pump
- Degasser

Laboratory instruments and equipment:

- Hand or automated pipette
- Pipette tips
- 25 ml volumetric flask
- Reagent reservoirs
- Chemical fume hood
- Punching device (capable of punching filter paper disks with a diameter of 3.2 mm or 1/8 inch)
- Incubator
- Shaker for well plates

8. Implementation of the analytical procedure

8.1. Specimen collection and storage

Acylcarnitines and amino acids are determined in dried blood spots (DBS). Blood specimens should be taken directly from a heel prick onto filter paper. Blood from a neonatal heel prick is usually collected in the first 36-72 hours after birth. A method based on dried blood specimens requires skillful collecting, handling and transport of specimens. The collection technique is according to CLSI document NBS01-A6 in details, and the main points are as follow:

- Clean the skin with an alcohol swap and let to air-dry.
- Puncture the infant's heel with a sterile lancet device.
- Wipe away the first drop of blood with a dry and sterile gauze pad.
- Allow a new drop of blood to form and gently touch the filter paper against a drop of blood and allow to completely fill a preprinted circle on the filter paper. Representative samples regarding the sample quality are available in the appendix of the above mentioned CLSI standard.
- Put the blood specimen to air dry in a horizontal position for at least three hours at the ambient temperature of 18 °C-25 °C. Keep the specimens from direct sunlight, heat, stack and cross contamination.
- Be sure that the required information on the specimen collection card has been completed.
- Package properly the specimen for transportation and analysis the same day. So that, collection cards be separated by a physical barrier or rotated 180° from the blood spot on the cards in the stack.
- Follow local postal and transport regulation for specimen packing and transport.
- Transport the specimen to the laboratory within 24 hours after collection, unless otherwise directed by the screening laboratory.

8.2. Storage of the dried blood samples during and after analysis

Samples can be stored at room temperature (18 °C-25 °C) for 24-48 h or refrigerated (2 °C-8 °C) for 21 days. Most amino acids and acylcarnitines are stable if dried blood specimens are stored at (2 °C-8 °C) and protected from humidity and sunlight.

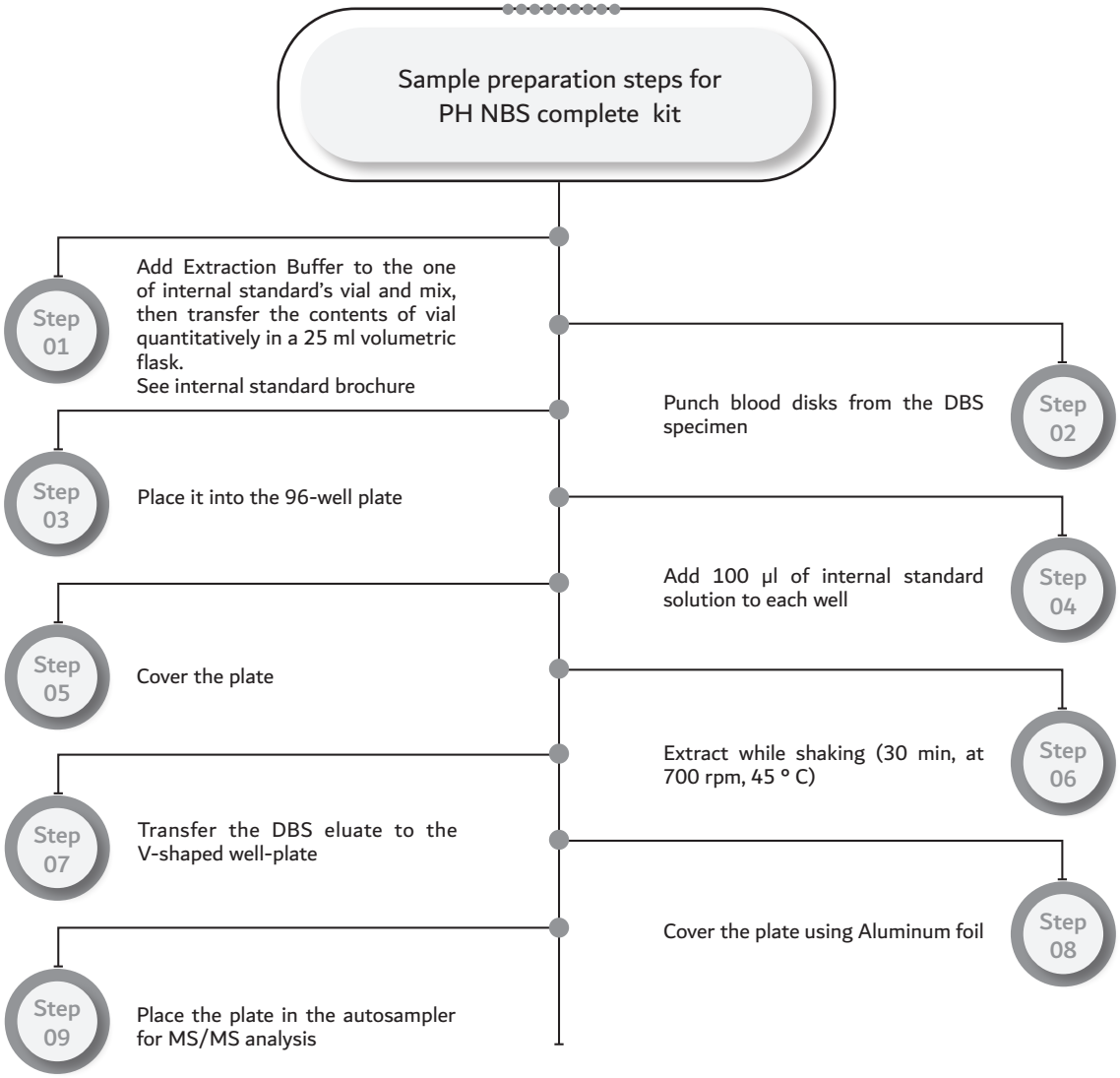
Residual samples need to be stored at temperatures below -18 °C and should be protected from humidity for longer period of storage.

8.3. Sample preparation

Sample preparation steps are described for both PH NBS and PH NBS-D kits.
Before running the assay, qualified personnel should be optimized your particular instrument and complete the method adjustment.
Use only the concentrations indicated in the lot specified quality control certificate for the stable isotope labelled internal standards.

8.3.1. Sample preparation steps for PH NBS complete kit

Work flow for sample preparation steps is as follow:



8.3.1.1. Reconstitute the lyophilized internal standard

The content of internal standard vial has to be extracted and pooled to a final volume of exact 25 ml. for this purpose proceed as follow:
Add extraction buffer to the internal standards vial and mix occasionally and gently for about 15 min. Then transfer the contents of vial quantitatively in a 25 ml volumetric flask, flush vial two times with 3 ml of extraction buffer and transfer these volumes in the volumetric flask. Finally, fill the volumetric flask with extraction buffer exactly to 25 ml and stir for homogeneity.

8.3.1.2. Extraction/Spiking with internal standard

Punch out a 3.2 mm dried blood disc of the filter paper (control or patient sample) and transfer it into the defined well of flat bottom well plate. Then pipette 100 µl internal standard (reconstituted with extraction buffer) on the sample.
For the sample extraction cover the well plate with the protective sheet and extract while shaking for 30 min at 700 rpm, 45 ° C (shaker for well-plate).

8.3.1.3. Transfer

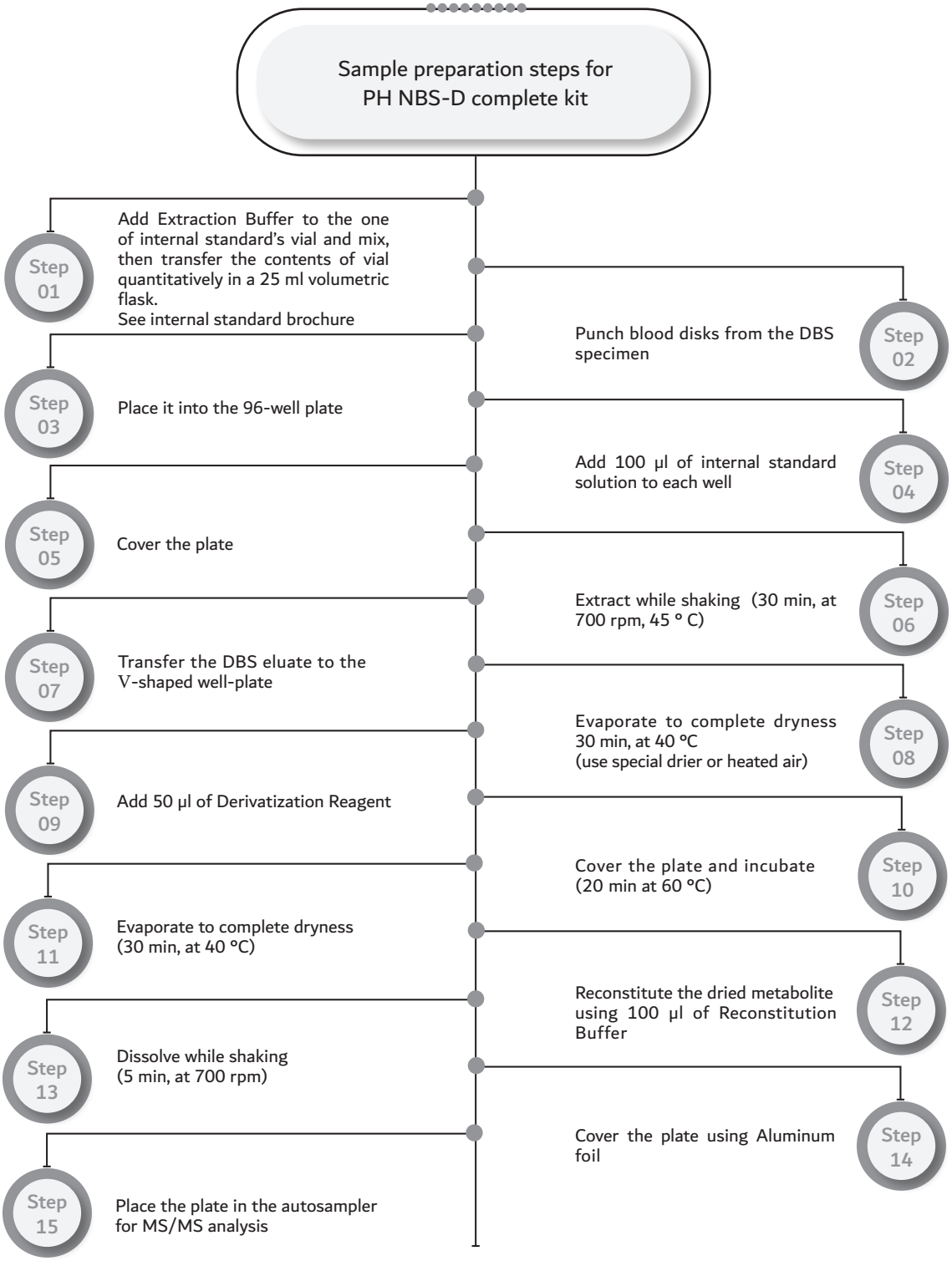
Remove the protective sheet and transfer the sample supernatant into a conical bottom well plate. Cover the plate with the protective sheet of aluminum foil to minimize evaporation of solution.

8.3.1.4. LC-MS-MS analysis

Place the covered well plate into the autosampler. Inject 10-20 µl of the supernatant into the LC-MS-MS system. The injection volume should be selected with respect to the sensitivity of the tandem mass spectrometer in use.

8.3.2. Sample preparation steps for PH NBS-D complete kit

Work flow for sample preparation steps is as follow:


Sample preparation steps for PH NBS-D complete kit
Step 01
Add Extraction Buffer to the one of internal standard's vial and mix, then transfer the contents of vial quantitatively in a 25 ml volumetric flask. See internal standard brochure
Step 02
Punch blood disks from the DBS specimen
Step 03
Place it into the 96-well plate
Step 04
Add 100 µl of internal standard solution to each well
Step 05
Cover the plate
Step 06
Extract while shaking (30 min, at 700 rpm, 45 ° C)
Step 07
Transfer the DBS eluate to the V-shaped well-plate
Step 08
Evaporate to complete dryness 30 min, at 40 °C (use special drier or heated air)
Step 09
Add 50 µl of Derivatization Reagent
Step 10
Cover the plate and incubate (20 min at 60 °C)
Step 11
Evaporate to complete dryness (30 min, at 40 °C)
Step 12
Reconstitute the dried metabolite using 100 µl of Reconstitution Buffer
Step 13
Dissolve while shaking (5 min, at 700 rpm)
Step 14
Cover the plate using Aluminum foil
Step 15
Place the plate in the autosampler for MS/MS analysis

8.3.2.1. Reconstitute the lyophilized internal standard

The content of internal standard vial has to be extracted and pooled to a final volume of exact 25 ml. for this purpose proceed as follow:
Add extraction buffer to the internal standards vial and mix occasionally and gently for about 15 min. Then transfer the contents of vial quantitatively in a 25 ml volumetric flask, flush vial two times with 3 ml of extraction buffer and transfer these volumes in the volumetric flask. Finally, fill the volumetric flask with extraction buffer exactly to 25 ml and stir for homogeneity.

8.3.2.2. Extraction/Spiking with internal standard

Punch out a 3.2 mm dried blood disc of the filter paper (control or patient sample) and transfer it into the defined well of flat bottom well plate. Then pipette 100 µl internal standard (reconstituted with extraction buffer) on the sample.
For the sample extraction cover the well plate with the protective sheet and extract while shaking for 30 min at 700 rpm, 45 ° C (shaker for well-plate).

8.3.2.3. Transfer

Remove the protective sheet and transfer the sample supernatant into a conical bottom well plate. Evaporate to complete dryness (30 min, at 40 °C).

8.3.2.4. Derivatization

Pipette 50 µl of derivatization reagent on the sample. Cover the well plate with a protective sheet and incubate it for 20 min at 60 °C.
After incubation, please remove the protective sheet and evaporate to complete dryness by blowing off with air (30 min, at 40 °C).

8.3.2.5. Reconstitution

Pipette 100 µl reconstitution buffer on the sample. Cover the well plate with an aluminum foil protective sheet and dissolve while shaking for 5 min at 700 rpm (shaker for well plate).

8.3.2.6. LC-MS-MS analysis

Place the covered well plate into the autosampler. Inject 10-20 µl of the supernatant into the LC-MS-MS system. The injection volume should be selected with respect to the sensitivity of the tandem mass spectrometer in use.

8.4. LC-MS-MS Instrument

It is recommended to regularly check the mass accuracy of the instrument. If necessary, please recalibrate the spectrometer.

8.4.1. Optimization of the tandem mass spectrometer and test run

Optimization of the operation parameters of ion source and the compound mass transitions must be done in the instrument optimization procedure. The commercial optimization mix solutions and Dried Blood Spot control samples can be used for optimization.

8.4.2. Equilibration of the analytical system

Allow to equilibrate the analytical system compartments for at last 30 minutes prior to sample injection.
It is recommended that, three blank injections carried out at the beginning of each analytical series to facilitate the reproducibility of analytical results. For equilibration of test run, please inject the prepared Dried Blood Spots Control Samples level I and II for several times until two consecutive comparable chromatograms are obtained from retention time and peak area viewpoints.

8.4.3. Measurement and evaluation of the sample

Sample is spiked with the internal standard solution and the analyte concentrations are calculated using known concentrations of internal standard according to 6.1.1 section and internal standard brochure.
The analyte concentration is calculated according to this formula:

$$C\,(Analyte)[\mu mol/l] = \frac{A\,(analyte) \times C(IS)[\mu mol/l]}{A\,(IS)} \times \frac{V\,(IS)[\mu l]}{V\,(A)[\mu l]}$$

Where :
C (Analyte) is analyte concentration in the sample
A (Analyte) is analyte peak area in the sample
C (IS) is concentration of the isotope-labelled analytes in the internal standard (IS)
A (IS) is peak area of the isotope-labelled analytes in the internal standard (IS)
V (IS) is the volume of the internal standard (IS)
V (A) is the volume of the sample
The basis for this calculation is a volume of 100 µl internal standard and a sample disk with 3.2 mm diameter.

The real volume of sample may vary from case to case, but a medium sample volume is accepted for certified filter papers. Therefore, this method cannot apply for quantification of amino acids and acylcarnitines, exactly. This method is a semiquantitative determination method for newborn screening.

9. Analytical Performance

Analytical performance for complete kit was studied with the following products:

Order no.:	PH 2001
Product name:	PH NBS (Complete Kit for Amino Acids and Acylcarnitines from Dried Blood Spots)
Order no.:	PH 2002
Product name:	PH NBS-D (Complete Kit for Amino Acids and Acylcarnitines from Dried Blood Spots)

9.1 Accuracy of measurement

9.1.1. Trueness of measurement

9.1.1.1. Recovery

The recovery was evaluated on Dried Blood Spot Control by three preparations and measurement of the samples in 5 different runs using AB SCIEX API3200 system.

Table 7: Recoveries of the analytes [%]

Analyte	PH NBS-D (PH 2002)		PH NBS (PH 2001)	
	Level I	Level II	Level I	Level II
Amino Acids				
Ornithine	107.91	97.04	103.21	95.36
Proline	108.46	112.16	103.89	104.26
Methionine	92.45	109.16	105.59	109.42
Leucine	110.84	114.93	109.54	112.77
Glutamic acid	99.61	95.66	109.35	117.23
Citrulline	106.21	109.62	95.23	87.40
Arginine	94.89	91.06	112.65	115.66
Tyrosine	98.61	99.33	110.61	110.87
Aspartic acid	104.38	105.36	93.16	99.46
Valine	106.97	112.12	109.68	119.83
Glycine	103.19	95.23	98.38	96.69
Phenylalanine	109.97	109.02	109.86	120.10
Alanine	96.67	99.52	107.03	91.19
Acylcarnitines and free Carnitine				
C0	93.92	96.07	117.26	120.49
C2	106.70	94.20	102.99	98.01
C3	90.74	90.46	102.90	96.09
C4	94.38	90.76	101.53	108.89
C5	98.52	91.35	110.79	102.59
C5DC	99.15	96.44	90.79	44.72
C6	97.07	111.87	91.44	89.11
C8	101.01	102.62	84.00	83.64
C10	98.71	99.74	74.97	75.43
C12	102.03	106.19	112.25	108.50
C14	111.03	111.25	92.24	88.53
C16	104.35	94.14	94.81	88.82
C18	101.28	115.22	104.72	105.02

9.1.1.2. Method Comparison

Dried Blood Spot Control samples were measured after sample preparation with PH NBS kit and PH NBS-D kit on AB SCIEX 3200 instrument.

Table 8: Mean values of 15 QC samples (Level I) [µmol/L] and Deviation٪ of PH NBS / PH NBS-D kit

Analyte	AB SCIEX 3200		
	PH NBS-D kit	PH-NBS kit	Deviation ٪
Amino Acids			
Ornithine	183.44	237	23
Proline	295.02	311	5
Methionine	47.15	52	9
Leucine	271.55	305	11
Glutamic acid	464.16	507	9
Citrulline	69.04	65	-7
Arginine	111.97	109	-2
Tyrosine	200.17	212	6
Aspartic acid	100.20	102	2
Valine	264.22	268	1
Glycine	312.67	253	-24
Phenylalanine	112.17	133	16
Alanine	320.94	379	15
Acylcarnitines and free Carnitine			
C0	40.52	58.28	29
C2	23.90	21.63	-11
C3	4.32	4.50	4
C4	0.76	0.84	9
C5	0.44	0.54	18
C5DC	0.50	0.54	9
C6	0.40	0.41	3
C8	0.41	0.41	-1
C10	0.33	0.36	9
C12	0.48	0.52	7
C14	0.51	0.44	-15
C16	5.25	4.47	-17
C18	2.31	2.59	11

Table 9: Mean values of 15 QC samples (Level II) [µmol/L] and Deviation٪ of PH NBS / PH NBS-D kit

Analyte	PH NBS-D kit	PH-NBS kit	Deviation ٪
Amino Acids			
Ornithine	409.52	522	21
Proline	817.68	807	-1
Methionine	220.50	209	-6
Leucine	580.38	568	-2
Glutamic acid	797.77	856	7
Citrulline	286.10	262	-9
Arginine	288.67	260	-11
Tyrosine	581.11	626	7
Aspartic acid	268.68	260	-4
Valine	519.12	508	-2
Glycine	814.24	628	-28
Phenylalanine	439.36	524	16
Alanine	641.91	671	4
Acylcarnitines and free Carnitine			
C0	88.38	121.69	27
C2	59.35	58.90	-1
C3	12.30	12.49	2
C4	3.23	3.58	10
C5	1.86	2.23	16
C5DC	1.95	1.16	-
C6	1.97	1.89	-4
C8	1.95	1.82	-7
C10	1.44	1.48	3
C12	2.16	2.27	5
C14	2.19	1.85	-18
C16	14.21	11.65	-22
C18	8.38	8.70	4

9.1.2. Precision of measurement

9.1.2.1. Repeatability (intra assay precision)

The intraassay precision was evaluated on AB SCIEX 3200 instrument by 20-fold preparations and measurement of the Dried Blood Spot Control samples in a single run.

Table 10: Intraassay-CV [٪]

Analyte	PH NBS-D kit (PH 2002)		PH NBS kit (PH 2001)	
	Level I	Level II	Level I	Level II
Amino Acids				
Ornithine	6.66	4.52	9.42	7.43
Proline	4.81	4.88	6.00	4.36
Methionine	5.91	10.45	4.24	4.46
Leucine	5.03	4.84	5.27	3.74
Glutamic acid	5.44	5.13	7.30	5.29
Citrulline	3.95	4.08	6.25	6.37
Arginine	13.17	6.16	8.83	4.68
Tyrosine	5.20	4.27	4.10	2.99
Aspartic acid	4.36	3.92	15.49	7.75
Valine	5.29	4.79	6.74	3.87
Glycine	4.60	4.53	12.65	5.67
Phenylalanine	4.97	4.91	4.39	3.41
Alanine	4.52	4.85	4.11	4.24
Acylcarnitines and free Carnitine				
C0	7.20	5.49	5.14	6.97
C2	4.36	4.81	9.29	4.55
C3	7.89	5.95	5.32	4.69
C4	4.31	5.83	5.83	4.34
C5	6.50	5.11	5.83	4.47
C5DC	3.96	5.00	14.31	9.82
C6	5.92	5.37	5.57	6.09
C8	4.93	5.66	4.62	4.68
C10	5.78	4.76	5.26	4.68
C12	9.37	5.09	5.03	4.50
C14	6.21	5.98	8.09	3.98
C16	9.68	5.11	2.73	3.40
C18	3.86	4.50	3.45	3.41

9.1.2.2. Reproducibility (inter assay precision)

The intraassay precision was evaluated on AB SCIEX 3200 instrument by 2-fold preparations and measurement of the Dried Blood Spot Control samples in 5 different runs.

Table 11: Intraassay-CV [%] for Amino Acids

Analyte	CV	PH NBS-D kit (PH 2002)		PH NBS kit (PH 2001)	
		Level I	Level II	Level I	Level II
Amino Acids					
Alanine	Total	5.64	9.85	4.21	8.22
	Repeatability	3.51	9.85	3.94	6.16
	Between Day	4.42	0.00	1.49	5.45
Arginine	Total	9.06	15.11	9.25	12.40
	Repeatability	4.53	5.06	5.20	1.81
	Between Day	7.84	14.24	7.64	12.27
Aspartic acid	Total	5.62	9.78	9.32	10.37
	Repeatability	3.56	7.48	5.66	4.42
	Between Day	4.34	6.31	7.41	9.38
Ornithine	Total	11.97	17.31	16.16	7.91
	Repeatability	7.10	5.89	6.81	2.63
	Between Day	9.63	16.27	14.66	7.46
Proline	Total	5.34	10.11	4.81	8.87
	Repeatability	3.50	10.11	2.61	5.06
	Between Day	4.04	0.00	4.04	7.28
Methionine	Total	15.15	13.58	7.22	9.91
	Repeatability	11.86	13.58	5.48	6.01
	Between Day	9.42	0.00	4.71	7.88
Glutamic acid	Total	10.79	13.65	5.11	8.74
	Repeatability	4.10	9.55	3.05	5.49
	Between Day	9.98	9.75	4.10	6.80
Leucine	Total	12.60	17.38	5.46	9.76
	Repeatability	4.97	9.31	4.07	5.95
	Between Day	11.58	14.67	3.64	7.74
Citrulline	Total	5.92	9.35	5.60	6.40
	Repeatability	2.98	7.42	5.60	4.34
	Between Day	5.11	5.69	0.00	4.70
Tyrosine	Total	4.01	8.48	4.62	8.59
	Repeatability	3.40	7.38	3.81	5.15
	Between Day	2.13	4.19	2.62	6.88
Valine	Total	8.30	11.60	6.37	9.81
	Repeatability	3.67	10.01	3.24	5.71
	Between Day	7.45	5.87	5.48	7.97
Glycine	Total	8.56	10.94	13.45	7.84
	Repeatability	2.32	8.21	13.45	6.18
	Between Day	8.24	7.24	0.00	4.83
Phenylalanine	Total	10.08	13.26	5.02	8.85
	Repeatability	2.70	8.91	3.50	5.98
	Between Day	9.71	9.81	3.60	6.53

Table 12: Intraassay-CV [%] for Acylcarnitines and free Carnitines

Analyte	CV	PH NBS-D kit (PH 2002)		PH NBS kit (PH 2001)	
		Level I	Level II	Level I	Level II
Acylcarnitines and free Carnitine					
C0	Total	8.05	11.03	6.42	12.50
	Repeatability	6.67	9.09	5.97	6.50
	Between Day	4.49	6.25	2.35	10.67
C2	Total	8.46	8.47	5.92	8.14
	Repeatability	5.38	8.47	3.19	4.47
	Between Day	6.53	0.00	4.99	6.80
C3	Total	9.43	9.21	3.92	8.62
	Repeatability	7.45	9.21	3.43	6.04
	Between Day	5.78	0.00	1.89	6.16
C4	Total	5.84	9.09	5.48	6.60
	Repeatability	5.80	8.66	3.93	5.53
	Between Day	0.62	2.75	3.82	3.60
C5	Total	8.97	13.63	5.20	7.89
	Repeatability	5.15	10.37	5.20	5.75
	Between Day	7.34	8.84	0.00	5.39
C5DC	Total	10.74	12.89	7.87	17.83
	Repeatability	9.45	8.06	4.44	7.29
	Between Day	5.09	10.06	6.50	16.27
C6	Total	12.39	12.71	5.32	6.76
	Repeatability	7.65	10.90	4.89	5.83
	Between Day	9.74	6.53	2.08	3.43
C8	Total	12.50	13.18	5.18	10.94
	Repeatability	11.37	12.64	4.71	7.36
	Between Day	5.19	3.73	2.16	8.09
C10	Total	9.49	14.83	4.17	10.81
	Repeatability	5.03	13.74	4.10	7.00
	Between Day	8.05	5.58	0.78	8.23
C12	Total	13.00	14.75	3.13	9.59
	Repeatability	7.11	12.14	2.45	7.72
	Between Day	10.88	8.38	1.95	5.69
C14	Total	9.25	13.11	6.34	9.59
	Repeatability	8.17	11.70	4.44	6.06
	Between Day	4.33	5.91	4.52	7.43
C16	Total	12.56	11.43	3.52	6.74
	Repeatability	8.48	9.15	3.39	5.78
	Between Day	9.26	6.85	0.97	3.46
C18	Total	10.17	11.70	4.11	5.54
	Repeatability	7.49	9.68	3.47	4.80
	Between Day	6.87	6.57	2.22	2.77

9.2. Analytical sensitivity (LOD, LLOQ)

The Dried Blood Spot Control samples were diluted with “Extraction Buffer +IS”:
1:10; 1:20; 1:50; 1:100; 1:200; 1:500; 1:1000
The diluted extracts were injected into AB SCIEX 3200 instrument. Single determination was done for Level I and 2 fold preparations for Level II on 5 different days.

Table 13: Results of LOD, LLOQ [µmol/L]

Amino Acids				
Analytes	**PH NBS-D kit (PH 2002)**		**PH NBS kit (PH 2001)**	
	LOD	**LLOQ**	**LOD**	**LLOQ**
Ornithine	4.10	12.30	4.16	12.48
Proline	1.30	3.90	2.69	8.06
Methionine	2.10	6.30	0.77	2.30
Leucine	1.30	3.90	1.32	3.97
Glutamic acid	4.60	13.80	2.97	8.91
Citrulline	1.20	3.60	2.39	7.17
Arginine	1.10	3.30	1.11	3.33
Tyrosine	1.10	3.30	1.12	3.35
Aspartic acid	2.20	6.60	2.14	6.42
Valine	4.40	13.20	2.23	6.69
Glycine	6.90	20.70	6.91	20.72
Phenylalanine	1.20	3.60	1.17	3.51
Alanine	3.50	10.50	3.54	10.61
Acylcarnitines and free Carnitine				
C0	0.20	0.60	0.41	1.22
C2	0.10	0.30	0.29	0.88
C3	0.03	0.09	0.03	0.09
C4	0.04	0.12	0.04	0.12
C5	0.01	0.03	0.01	0.03
C5DC	0.01	0.03	0.02	0.06
C6	0.02	0.06	0.01	0.02
C8	0.03	0.09	0.00	0.01
C10	0.01	0.03	0.01	0.03
C12	0.02	0.06	0.02	0.07
C14	0.01	0.03	0.01	0.02
C16	0.01	0.03	0.03	0.08
C18	0.01	0.03	0.01	0.03

9.3. Analytical specificity

9.3.1. Interferences

9.3.1.1. Interference: Asparagine (Asn) and Ornithine (Orn) (M=132 Da)

The Dried Blood Spot Control samples were analyzed before and after spiking with Asparagine (Asn) up to 2500 µmol/L by monitoring the mass transition of Ornithine. On average, lower than 20% of Asparagine will be detected as Ornithine.

9.3.1.2. Interference: Glutamic Acid (Glu) and Acetyl-Carnitine (C2) (M=260 Da)

The Dried Blood Spot Control samples were analyzed before and after spiking with Glutamic acid (Glu) up to 2500 µmol/L by monitoring the mass transition of acetylcarnitine (C2). No significant interference of Glu on C2 (< 2%) could be observed.

9.3.1.3. Interference: Leucine (Leu) and Isoleucine (Ile) (M=132 Da)

Isoleucine and leucine are isobar substances with identical fragmentation and are detected together. The concentration measured in the sample therefore is the sum of both compounds.

9.3.1.4. Interference: Leucine (Leu) and Hydroxyproline (Hpr) (M=132 Da)

Hydroxyproline and leucine are isobar substances with identical fragmentation and are detected together. However even in the rare cases of Hydroxyprolinemia no false-positive MSUD results are to be expected.

9.3.2. Blank Test

Blank filter papers were prepared like patient sample and were measured on AB SCIEX 3200 instrument. The measured values of blank filter paper samples are below the LLOQ.

9.3.3. Carry-Over

Blank filter paper samples were injected directly after 2-fold injections of the Dried Blood Spot Control samples (Level II) on AB SCIEX 3200 instrument. The measured values of blank filter paper samples are below the LLOQ.

9.4. Measuring range of the assay (Linearity)

The Dried Blood Spot Control samples were enriched to increase analyte concentration that covered the entire clinical range. The linearity was evaluated on AB SCIEX 3200 instrument by 2-fold preparations.

Table 14: Results of the linearity study [µmol/L] for PH NBS-D kit (PH 2002)

Analyte	Repeatability [CV%]	Nonlinearity [%]	Linear Range [µmol/L]
Amino Acids			
Orn	16.82	-14.48-4.31	15.94-1001.25
Pro	11.6	-6.60-3.23	37.76-1208.25
Met	27.86	-2.51-7.58	27.7-886.5
Leu	19.43	-10.17-5.28	46.48-1487.25
Glu	15.48	-6.56-3.17	64.125-2052
Cit	10.61	-8.02-3.93	16.8-537.75
Arg	2.54	-14.32-5.08	14.86-475
Tyr	12.05	-11.49-1.72	19.55-1251
Asp	8.99	-12.35-3.45	7.52-481.5
Val	19.59	-8.02-4.09	38.67-1237.5
Gly	14.51	-14.69-1.94	24.27-3107.25
Phe	17.51	-10.58-5.65	52.38-1676.25
Ala	12.82	-14.16-2.88	50.5-3235.5
Acylcarnitines and free Carnitine			
C0	11.23	-14.28-2.38	5.3-337.5
C2	9.20	-13.70-7.70	4.11-131.4
C3	15.31	-13.39-2.68	0.358-22.95
C4	11.12	-6.18-2.82	0.55-17.48
C5	14.76	-14.78-3.02	0.078-4.973
C5DC	8.79	-3.93-14.74	0.478-7.65
C6	6.71	-5.49-4.36	0.165-2.64
C8	11.76	-8.04-3.69	0.13-4.16
C10	12.99	-10.41-5.28	0.08-2.56
C12	11.52	-9.03-4.23	0.426-13.63
C14	13.09	-11.88-5.58	0.223-7.15
C16	14.07	-11.53-0.73	0.206-26.325
C18	11.66	-8.14-1.31	0.127-8.145

Table 15: Results of the linearity study [µmol/L] for PH NBS kit (PH 2001)

Analyte	Repeatability [CV%]	Nonlinearity [%]	Linear Range [µmol/L]
Amino Acids			
Orn	10.15	-10.66-6.89	31.29-1001.25
Pro	6.62	-9.03-4.78	37.76-1208.25
Met	10.77	-5.76-2.46	27.7-886.5
Leu	7.00	-8.55-1.61	23.24-1487.25
Glu	10.42	-9.65-5.45	64.125-2052
Cit	8.02	-7.05-3.36	16.8-537.75
Arg	8.91	-3.12-12.19	15.61-249.75
Tyr	6.99	-5.95-1.92	39.1-1251
Asp	7.74	-3.17-14.85	30.1-481.5
Val	8.57	-2.76-1.26	38.67-1237.5
Gly	13.92	-1.72-7.91	24.27-3107.25
Phe	9.13	-7.5-3.49	52.38-1676.25
Ala	7.02	-11.75-2.62	50.55-3235.5
Acylcarnitines and free Carnitine			
C0	8.36	-9.15-0.77	2.837-337.5
C2	9.93	-0.46-11.52	0.513-131.4
C3	6.92	-12.94-0.95	0.179-22.95
C4	7.77	-1.10-7.64	0.273-17.48
C5	9.11	-11.32-0.57	0.019-4.973
C5DC	13.21	-4.01-12.56	0.119-0.956
C6	9.01	-11.95-6.47	0.072-2.295
C8	7.80	-6.76-1.17	0.065-4.1625
C10	8.51	-1.33-8.43	0.04-2.565
C12	7.64	-5.78-2.59	0.426-13.635
C14	9.79	-0.38-10.71	0.028-7.155
C16	7.13	-13.41-2.20	0.411-26.325
C18	7.65	-13.76-2.23	0.127-8.145

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