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Annual report on neonatal screening for 2020.

The Newborn Screening Center of the Slovak Republic (SCN SR) at the Children's University Hospital with a polyclinic in Banská Bystrica is a central laboratory for neonatal screening for congenital hypothyroidism (KH), congenital adrenal hyperplasia (CAH), cystic fibrosis (CF) and selected inherited metabolic disorders (DMP). It examines dry blood samples from all newborns, taken at 72-96 hours of life. Neonatal screening of KH, CAH, CF and selected DMPs is legally based on the 42nd Expert Guidelines of the Bulletin of the Ministry of Health of the Slovak Republic, amount 39 - 60 of 27.12.2012. The standardized procedures of SCN SR meet the individual Articles of the Professional Guideline of the Ministry of Health of the Slovak Republic.

A. Neonatal screening for congenital hypothyroidism.

In KH screening, thyrotropic hormone (TSH) in a dry blood sample is examined by the GSP TSH method, which is an immunofluorescence quantification of TSH with a Perkin Elmer kit. The recommended and verified value of the cut-off limit is 7 mIU / L for the 4th day of life and 6 mIu / L for the 10th -14. day of life. Values in the range of 7 (6) -20 mIU / L are considered "slightly" suspicious (gray zone), values above 20 mIU / L are considered "hot" recall. SCN SR is included in the external quality control (Referenzinstitute fur Bioanalytik DGKL, Bonn) and has a valid Certificate, monitored 4 times a year. The laboratory also has internal quality control for the accuracy and correctness of the method.

Region	Number of examined newborns	Recall	%	Capture KH
West	23 457 + 115*	182	0,78	12
Centre	14 362 + 29*	100	0,70	5
East	18 772 + 21*	91	0,48	14
Total	**56 591 + 165* = 56 756**	**373**	**0,65**	**31**

***Births at home**

In 2020, 56,756 newborns were examined and 31 cases of KH were detected, the incidence being 1: 1 831 liveborn.

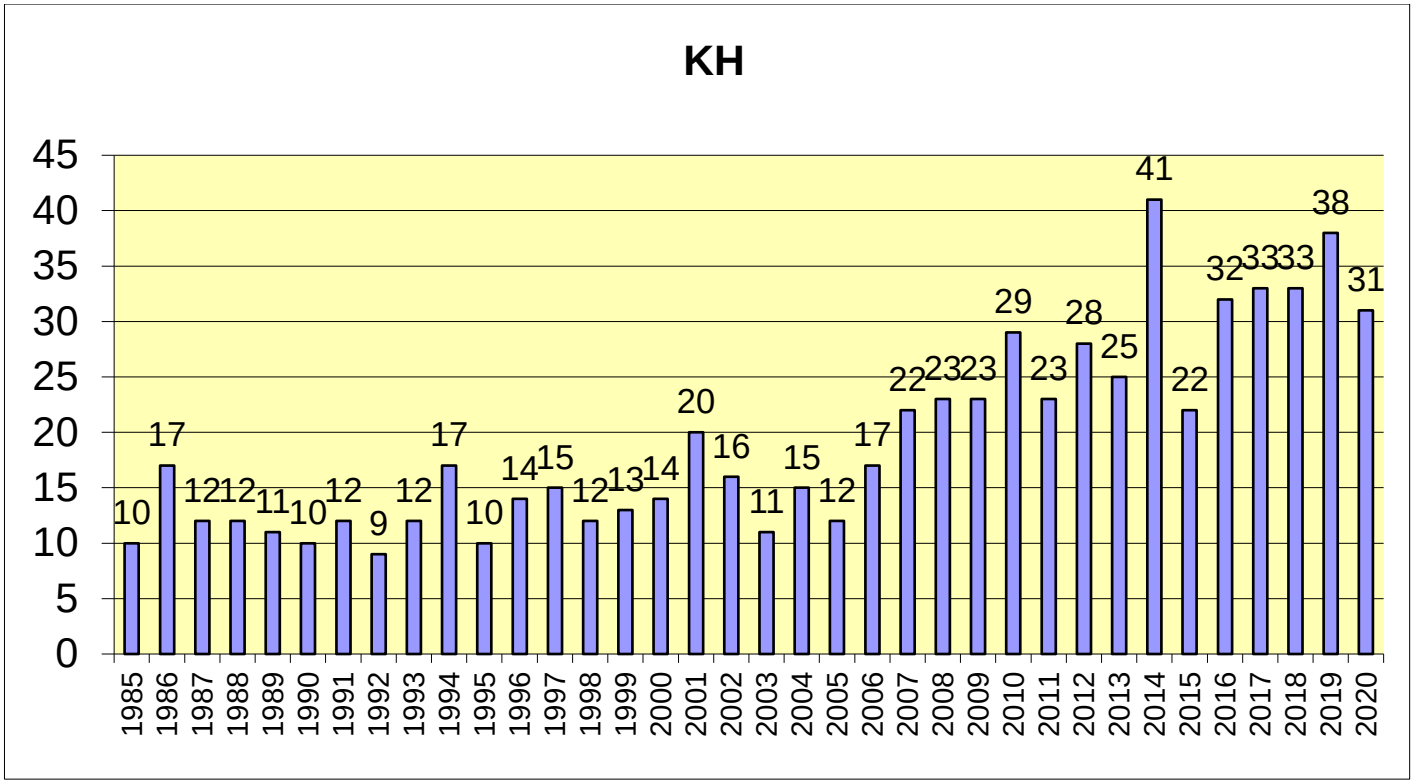
Breakdown of cases caught:
Hypoplasia.....2
Ectopia.....2
Dyshormogenesis.....7
ATYREOSIS.....1
KH not specified.....9
KH subclinical.....6
Delay - The late rise of TSH.....4

Out of the number of 373 suspected recalls, 9 transient hypothyroidisms were detected in addition to classic congenital hypothyroidism, they remain in the monitoring of recall centers.
Delayed elevated TSH levels were detected in 4 cases. The increase in TSH was captured in the rescreening.
Since 2004, when rescraining was introduced, there have been a total of 89 cases of hypothyroidism detected.
Most of them are children with NPH and immature.
Of the 30 seizures, 18 are girls and 13 are boys.
In the period 1985 - 2020, 2,213,465 newborns were examined in the screening of KH and 696 thyroid disorders were detected, the incidence of the disease was 1: 3,180 live births.

	Positive CH	**Negative CH**	
Positive	**TP = 31**	**FP = 343**	**→PPV = TP/ (TP + FP) = 8,04 %**
Negative	**FN = 0**	**TN = 56 726**	**→NPV = TN/ (FN + TN) = 100%**
	↓ Sensitivity = TP/ (TP + FN) = 100%	**↓ Specificity = TN/ (FP + TN) = 99,40%**	

Table of statistical parameters of neonatal screening KH for the year 2020

Test validity = TP + TN / TP + FP + FN + TN = 99.40%



Graphic representation of confirmed cases of KH 1985-2020

B. Neonatal screening of phenylketonuria.

FKU screening from 1.1.2013, together with screening for selected DMPs, is performed using LC-MS / MS-tandem mass spectrometry. The principle of determination is quantitatively the concentrations of the relevant amino acids and acylcarnitines using tandem mass spectrometry (MS / MS) without chromatographic separation - the so-called flow injection analysis (FIA) after their extraction from a dry drop of blood.
We use the certified ClinSpot LC-MS / MS Complete Kit from RECIPE for sample preparation. The determination of phenylalanine is subject to the external quality control of the CDC in Atlanta 4 times a year, the results of the external quality control are confirmed by the Certificate.
For FKU screening, a cut - off limit of 110 [mu] mol / L and a Phe / Tyr (phenylalanine / tyrosine) ratio of 1.5 are set. The ratio is important to distinguish the effect of parenteral neonatal nutrition in the amino acid profile. Phe values above 240 μmol / L and at the same time Phe / Tyr ratio above 1.5 are reported as hot recall.

Region	Number of examined newborns	Recall	%	Capture FKU
West	23 457 + 115*	35	0,15	2
Centre	14 362 + 29*	15	0,10	1
East	18 772 + 21*	38	0,20	4
Total	**56 591 + 165*= 56 756**	**88**	**0,15**	**7**

***Births at home**

In 2020, 56,756 neonates were examined and 7 cases of phenylketonuria / hyperphenylalaninemia were detected. Incidence 1: 8 108 live births.
Breakdown of cases caught:
Hyperphenylalaninemia 2
Phenylketonuria 5

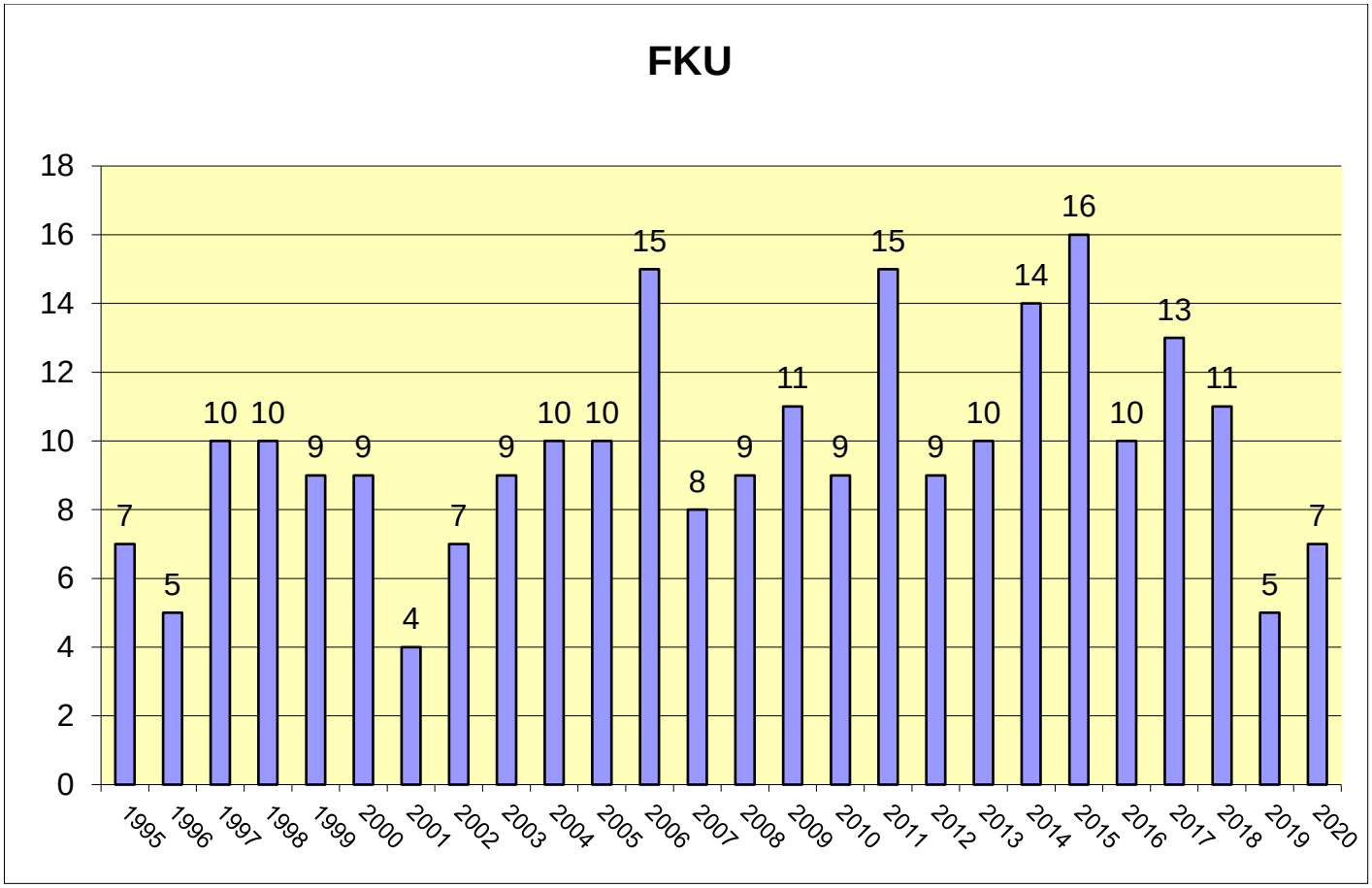
The mean value of phenylalanine in the detected cases was 722 µmol / L (180 - 1201).
The average day of reporting the diagnosis to recall centers was 7.5 days (5 - 9). Of the detected cases, there are 4 girls and 3 boys.
By determining phenylalanine from a dry blood sample, we help monitor the accuracy of phenylketonuria treatment in patients with phenylketonuria. We monitor 300 patients, we examined 1801 samples. Among them are adult pregnant patients with phenylketonuria, where control is extremely important in terms of fetal development and maternal condition.

Since 1995, 1,436,184 newborns and 252 children with phenylketonuria have been examined, and the incidence of the disease is 1: 5,699 live births.

	Positive PKU	**Negative PKU**	
Positive	**TP = 7**	**FP = 81**	**→PPV = TP/ (TP + FP) = 7,95 %**
Negative	**FN = 0**	**TN = 56 749**	**→NPV = TN/ (FN + TN) = 100 %**
	↓ Sensitivity = TP/ (TP + FN) = 100%	**↓ Specificity = TN/ (FP + TN) = 99,80%**	

Table of statistical parameters of FKU neonatal screening for 2020

Test validity = TP + TN / TP + FP + FN + TN = 99.8%



Graphic representation of confirmed cases of FKU 1995 - 2020

C. Neonatal screening for congenital adrenal hyperplasia.

CAH screening is based on measuring the level of 17 hydroxyprogesterone (17OHP) in the dry drop of neonatal blood by the GSP TSH method, which is an immunofluorescence quantification of 17OHP with a Perkin Elmer kit. The level of 17 OHP depends on the birth weight and maturity of the newborn, to which the cut off limit is adjusted, in the range from 14 - 50 nmol / L. We are included in the external quality control with a valid certificate, monitored 4 times a year together with TSH in DGKL Bonn.

Región	**Number of examined newborns**	**Recall**	**%**	**Capture CAH**
West	23 457 + 115*	61	0,26	0
Centre	14 362 + 29*	59	0,41	1
East	18 772 + 21	76	0,40	3
Total	**56 591 + 165* = 56 756**	**196**	**0,35**	**4**

***Births at home**

In 2020, 56,756 newborns were examined and 4 cases of CAH were detected.
Incidence 1: 14 189 live births.

Division of captured cases: CAH, classical form with salt disorder ..... 4 (3 boys and 1 girl)

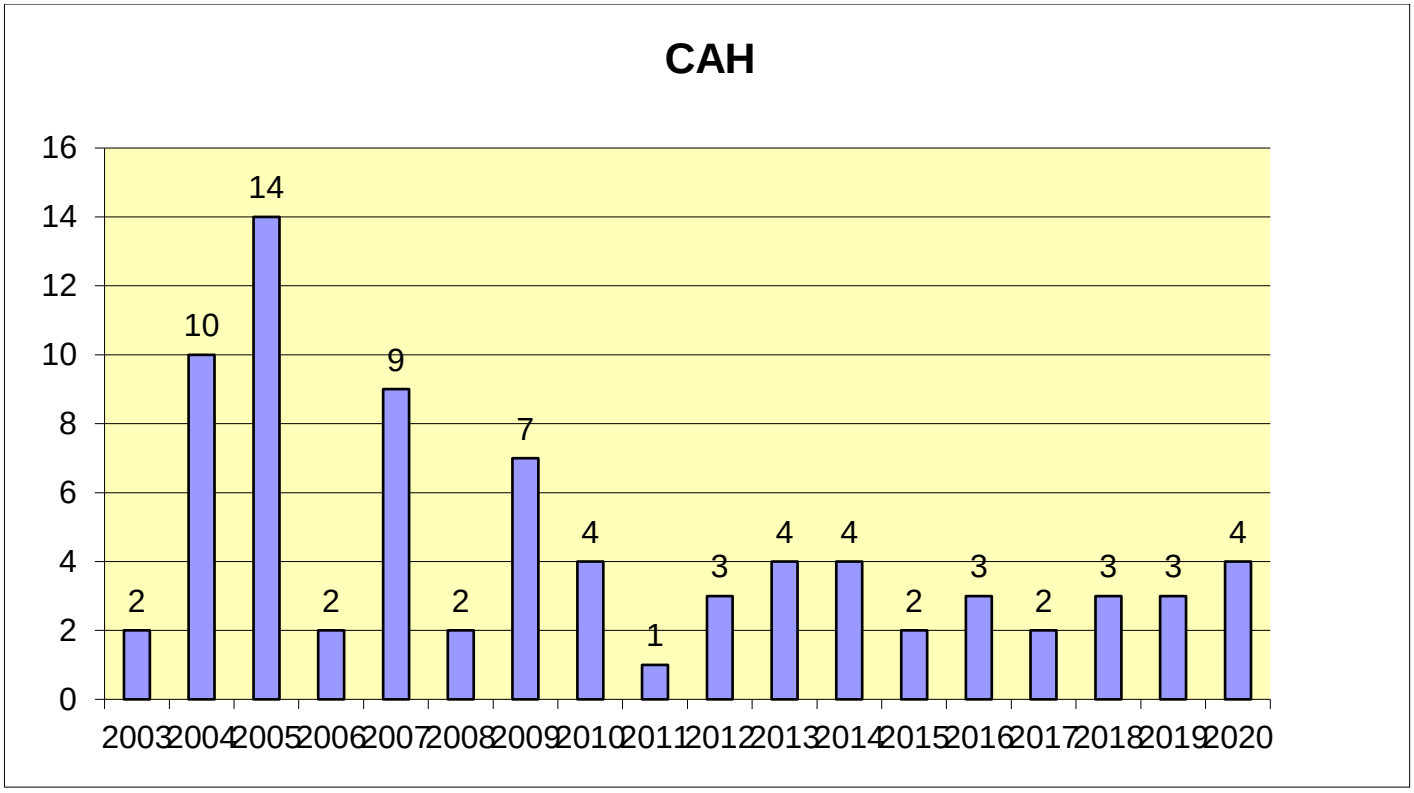
The mean 17OHP for the salt form of the disease was 220 ng / mL and the mean day of diagnosis is day 13 of life.

Since 2003, 951,200 newborns have been examined and 80 cases of CAH have been detected. Incidence 1: 11,890 live births. The monitor for CAH was on 95 samples.

	Positive CAH	**Negative CAH**	
Positive	**TP = 4**	**FP = 196**	**→PPV = TP/ (TP + FP) = 2,02 %**
Negative	**FN = 0**	**TN = 56 752**	**→NPV = TN/ (FN + TN) = 100 %**
	↓ Sensitivity = TP/ (TP + FN) = 100%	**↓ Specificity = TN/ (FP + TN) = 99,65%**	

Table of statistical parameters of neonatal CAH screening for 2020

Test validity = TP + TN / TP + FP + FN + TN = 99.65%



Graphical representation of confirmed cases of CAH 2003 - 2020

D. Neonatal screening for cystic fibrosis.

CF screening has been examined at SCN SR since February 1, 2009. in a dry blood sample by the GSP IRT method, which is an immunofluorescence quantification assay with an IRT kit from Perkin Elmer. The recommended and verified cut-off limit value is 60 ng / mL for day 4 of life and 55 ng / mL for day 14-30 of life. Romani neonates have a cut off of 75 ng / mL for the first intake and 70 ng / mL for the second intake, which takes into account the biochemically higher IRT of the Romani ethnic group. An IRT / IRT algorithm is introduced in CF screening, which means measuring IRT in two samples from suspected patients. The introduction of the ethnic parameter made it possible to statistically differentiate IRT concentrations in the Caucasian and Roma ethnic groups.
We have external quality control in two laboratories - CDC (Centers for Disease Control and Prevention), Atlanta, Newborn Screening Quality Assurance Program and RfB (Referenzinstitute fur Bioanalytik DGKL, Bonn). The certificate is monitored 4 times a year. The laboratory also has internal quality control for the accuracy and correctness of the method.

Region	Number of examined newborns	Recall	%	Capture–
West	23 457 + 115*	208	0,87	2 +1
Centre	14 362 + 29*	121	0,84	
East	18 772 + 21*	246	1,31	2
Total	**56 591 + 165*= 56 756**	**575**	**1,0**	**5**

Table of the first Recall IRT / 1, second sample requested

Region	Number of examined newborns	Recall	%	Capture CF
West	23 457 + 115*	8		2 +1
Centre	14 362 + 29*	7		
East	18 772 + 21*	21		2
Total	**56 591 + 165*= 56 756**	**36**		**5**

Recall table with two positive values IRT = IRT1 / IRT2, reported to CF Centers in Bratislava, Banská Bystrica and Košice.

***Births at home**

In 2020, 56,756 neonates were examined and 4 cases of cystic fibrosis were detected, one case was evaluated in NS as negative, but both genetics and clinics are positive for CF with dg, CF + Pseudo-Barter syndrome. Incidence of disease 1: 11 351 live births.

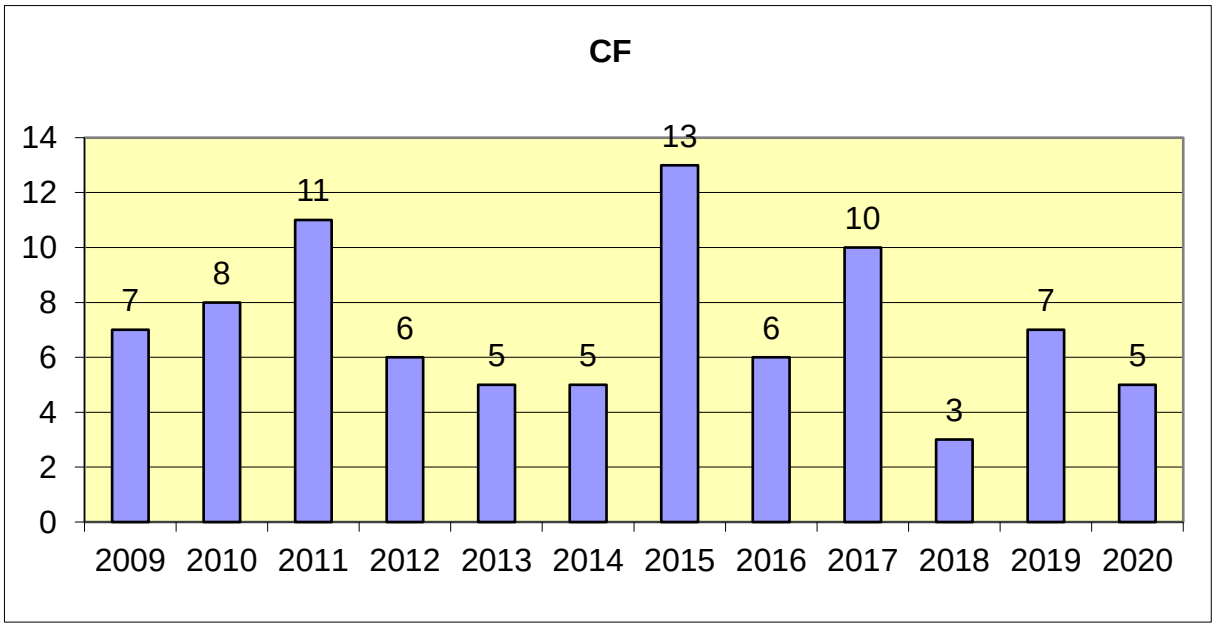
The mean IRT / 1 was 224 ng / mL (92-292), the IRT / 2 was 136 ng / mL (63-213). The average day of diagnosis was 18 days of age (13-24). Of the detected cases, 1 girl and 3 boys, the boy is not detected by the screening. Not a single positive patient with CF is Roma in 2020.
Genetic mutations where genetics have already been tested are as follows:
Δ F508 / Δ F508 5

As of February 1, 2009, 672,859 newborns and 87 children with cystic fibrosis were examined, and the incidence of the disease was 1: 7,734 live births.

	Positive CF	Negative CF	
Positive	**TP = 4**	**FP = 571**	**→PPV = TP/ (TP + FP) = 0,69 %**
Negative	**FN = 1**	**TN = 56 181**	**→NPV = TN/ (FN + TN) = 99,99 %**
	↓ Sensitivity = TP/ (TP + FN) = 80%	**↓ Specificity = TN/ (FP + TN) = 99,01%**	

Table of statistical parameters of neonatal CF screening for 2020

Test validity = TP + TN / TP + FP + FN + TN = 99, 01%



Graphic representation of confirmed cases CF 2009 - 2020

Ethnic parameters in neonatal screening in Slovakia and the aspect of cystic fibrosis in 2020

Region	Number of newborns	European ethnicity	Roma ethnic group
ZSK	**23 572**	**23 068**	**504**
SSK	**14 391**	**12 936**	**1 455**
VSK	**18 793**	**12 269**	**6 524**
Total **%**	**56 756** **100%**	**48 273** **85,05 %**	**8 483** **14,95%**

Total	Number of newborns.	Recall	%
European ethnicity	**48 273**	27	**0,06**
Roma ethnic group	**8 483**	9	**0,11**

Table of percentages of Recalls from 2020 by ethnicity

In the statistical analysis comparing the average IRT concentration by ethnicity, higher IRT values were found in the Roma ethnic group - by 34% - compared to the Caucasian ethnic group. The results were presented at the international ISNS conference in 2012 and 2013. In CF screening, we use IRT / 1 cut off 75 ng / mL and IRT / 2 cut off 70 ng / mL for the Roma ethnic group, taking into account the statistical difference between the average IRT values of the Roma and Caucasian ethnic groups. . Despite the higher cut off limit, the recall percentage of Roma children is higher. In contrast, no classic case of cystic fibrosis in the Roma ethnic group in neonatal screening has been confirmed so far by a diagnostic test since 2009. In 2019, a non-classical type of CF was confirmed, chlorides were negative.

CF screening 2009 - 2020	Number of newborns	Number of captured CF	Incidence in the region
ZSK	**273 793**	**54**	**1 : 5 070**
SSK	**177 071**	**19**	**1 : 9 319**
VSK	**221 995**	**14**	**1 : 15 856**
Total in Slovakia	**672 859**	**87**	**1 : 7 734**

Table of aggregate CF capture by regions of Slovakia for the years 2009 - 2020

Statistics of the Roma ethnic group in CF screening 2010 - 2020 (official monitoring of ethnicity since 2010)

ZSK 2010 – 2020……..8 866 Roma newborns
SSK 2010 – 2020.....16 505 Roma newborns
VSK 2010 – 2020.....65 132 Roma newborns
Total 2010 –2020.....90 503 Roma newborns

In NS CF, a positive case of classical CF disease was not confirmed in the Roma ethnic group, in the years 2010 - 2020 we have registered 90,503 newborns of the Roma ethnic group, screening prevalence> 1: 90 503.

E. Neonatal screening of selected inherited metabolic disorders

Since January 1, 2013 in Slovakia, we are investigating the following diseases in the screening of inherited metabolic disorders by tandem mass spectrometry - LC-MS / MS: FKU / HPA, MSUD - leucinosis, MCAD - medium chain acyl CoA dehydrogenase deficiency MK, VLCAD - acyl CoA deficiency very long chain MK dehydrogenases, LCHAD - 3OH acyl CoA long chain MK dehydrogenase, CPT I. - carnitine palmitoyltransferase I deficiency, CPT II / CACT - deficicarnitine palmitoyltransferase II deficiency / carnitine acylcarnitine transferase deficiency, GA.I. - glutaric aciduria I., IVA - isovaleric aciduria. In a pilot study since 2015, we have been investigating other DMPs - methylmalonic aciduria - MMA, propionic aciduria - PA, carnitine transporter deficiency - CUD of mother and child, urea cycle disorders - citrulinaemia I., arginine succinate lyase deficiency, tyrosinemia type I. and II., disorders of metabolism of sulfur amino acids methionine and homocysteine - isolated hypermethioninemia, classical homocystinuria, short-chain MK dehydrogenase deficiency - SCADD, mother-child 3-methylcrotonyl CoA carboxylase deficiency - 3-MCC, beta ketothiolase deficiency.
Markers for each disease were processed by the derivatized dry blood drop method and analyzed on an Agilent 1260/6420 mass spectrometer. The sensing of 2 ions that are characteristic of the substance - MRM mode - is suitable for polar to medium polar metabolites - in our case they are amino acids and acylcarnitines. In one examination, we quantified 73 analytes, which were evaluated by an online system, specially modified software. The cut off for each marker was determined by comparison with Region 4 Genetics and other sites and statistically adjusted according to laboratory results at the 99% percentile level. The last adjustment of the cut off limits is from August 2020. In the evaluation of the disease we use the so-called Multiparametric evaluation - the primary marker of the disease and the secondary marker are chosen, which are the mathematical ratios of selected amino acids and acylcranitins. Multiparametric evaluation will allow us to better distinguish true positivity from false positivity of neonatal samples, especially in cases of JIS and parenteral nutrition or the impact of treatment in neonates. External quality control is provided by the American CDC, where we regularly send the results of dry blood samples for amino acids and acylcarnitines 4 times a year. The result of external quality is an annual Certificate.

Region	Number of examined newborns	Recall (without FKU)	%	DMP capture (without FKU)
West	23 457 + 115*	19	0,08	5
Centre	14 362 + 29*	21	0,15	8
East	18 772 + 21*	110	0,58	9+91
Total	**56 591 + 165*= 56 756**	**150**	**0,27**	**27+91**

In 2020, 56,756 neonates were examined and 111 cases of inherited metabolic disorders were detected, together with FKU, their number is 118. Screening prevalence of DMP diseases is 1: 481 live births.

From **aminoacidopathies** we confirmed 7 cases of **hyperphenylalaninemia**, divided into 5 classic FKU and 2 HPA **(see chapter C),** we detected **1 Citrulinaemia, tyrosinemias** were transient - 9, they are not classic seizures, they are monitored by outpatient clinics.
From organic acidemias we detected one MMA, 6 disorders of leucine metabolism - 3 - methylcrotonyl Co - A carboxylase (3 - MCC) deficiency, one beta ketothiolase deficiency, very rare disease, occurrence of 1: 100,000. From fatty acid oxidation disorders we detected 3 cases of MCADD, 97 cases of SCADD, one case of TMEM 70 disease indirectly through positivity for SCADD, 3 cases of carnitine deficiency - CUD (also maternal forms). A total of 103 cases of fatty acid oxidation disorders.
The incidence for MCAD is 1: 5,716 live births. Several seizures in NS are monitored by Recall centers for transient states, heads of Tyrosine, Citrulline, free carnitine in immature newborns, resp. other diseases are confirmed where the elevated analyte is secondary.

Since 2015, we have been monitoring and reporting in neonatal screening positive neonates with suspected mitochondrial disorder - MK dehydrohenase deficiency with a short SCADD chain, where the primary marker is butyryl carnitine C4 - isoform. Secondary markers are the ratios of acylcarnitines C4 / C2, C4 / C3 and C4 / C8. Newborns are reported to recall centers after two positive samples. The positivity of C4, confirmed by the finding of ethylmalonic acid in urine and the genetic finding, predominates for SCADD. It is almost exclusively in the Roma ethnic group and the increase in the number of seizures of SCADD results in a surprisingly high incidence for DMP in Slovakia, especially among the Roma ethnic group..

DMP	Majority	Roma	Total
Aminoacidopathy **FKU / HPA**	**7**	**0**	**7**
Citrullinemia		**1**	**1**
Organic acidemia - MMA	**1**	**0**	**1**
Oxidation disorders MK - **MCADD**	**0**	**3**	**3**
Oxidation disorders MK - **SCADD**	**2**	**95**	**97**
CUD carnitine transporter deficiency (also maternal form)	**0**	**3**	**3**
3 -MCC - also maternal form	**5**	**1**	**6**
Total DMP captures	**15**	**103**	**118**

	Positive DMP	Negative DMP	
Positive	**TP = 118**	**FP = 43**	**→PPV = TP/ (TP + FP) = 73,29 %**
Negative	**FN = 0**	**TN = 56 756**	**→NPV = TN/ (FN + TN) = 100 %**
	↓ Sensitivity = TP/ (TP + FN) = 100%	**↓ Specificity = TN/ (FP + TN) = 99,92%**	

Table of statistical parameters of neonatal screening of DMP for the year 2020
Test validity = TP + TN / TP + FP + FN + TN = 99.92%

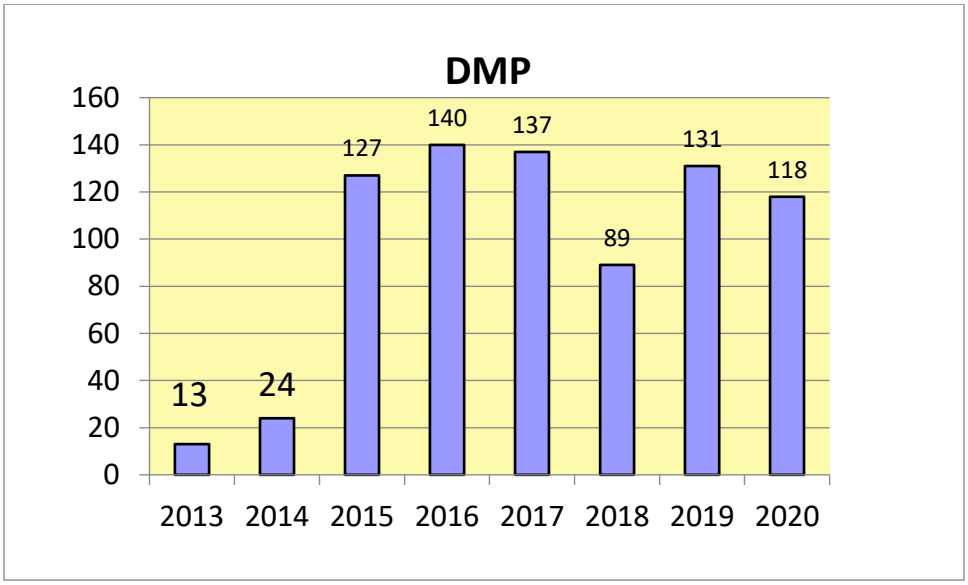
Since 1 January 2013, we have examined 453,075 newborns in DMP screening and detected 841 metabolic disorders.
In the MCADD screening, we captured 47 newborns during the period, the incidence of MCADD in Slovakia in the period 2013-2020 is 1: 9,640 live births.
The overall screening prevalence, including FKU, is 1: 539 live births.

Ethnic parameters in DMP screening for the period 2013 - 2020.

Total newborns **453 073**	**Caucasian ethnicity** **383 956**	**Roma ethnic group** **69 117**
Captures	**191**	**664**
Incidence	**1 : 2010**	**1 : 104**

Table of ethnic distribution of seizures in DMP screening

In the period 2013 - 2020, out of the number of 453,073, we examined 383,956 newborns of the Caucasian ethnic group and
69 117 newborns of Roma ethnicity.
For the Caucasian ethnic group, the total number of seizures in the DMP is 191, the screening prevalence of the Caucasian ethnic group is 1: 2010. For the Roma ethnic group, the total number of seizures in the DMP is 664, and the screening prevalence of the Roma ethnic group is 1: 104.



Graphical representation of confirmed cases of DMP 2013-2020

The **Covid pandemic** affected neonatal DMP screening, less acute seizures in screening - mainly SCADD - were reported to the Metabolic Outpatient Clinic by place of residence, as well as to the first contact physician. Not all of them were further examined directly in the Metabolic Outpatient Clinic, 69.2% of children with SCADD are not diagnostically confirmed by 2020, only monitored by a first contact doctor. Gradually, patients are invited to a metabolic clinic to refine the diagnosis in 2021.
In 2020, we also used the LC-MS / MS method to examine patients with suspected DMP, siblings and mothers of detected positive patients, where our profile can help monitor patients with diseases as well as detect new cases. They examined **2,713 dry blood drop samples**.

F. Effectiveness of screening newborns in the Slovak Republic

In addition to the first samples, we also examine repeated dry blood samples in indicated newborns (rescreening), collection according to the Professional Guideline on the 10th - 14th day of life.
In 2020, we examined 5,562 rescrings, which represents 9.8% of the number of children born.
From rescrings, we detected 4 cases of congenital hypothyroidism (listed above).

Number of live births in Slovakia in 2020 (Statistical Office of the Slovak Republic):		**56 650**
Real number of children for screening	:	**56 650**
Number of children examined in the SCN	:	**56 756**
Difference between the number of children born (real) and the number of examined children**:**		**+106**
Population capture by screening		**100%**
Total number of seizures in 2020	**:**	**157**
Number of children examined in the SCN, the first sample	:	**57 756**
Number of rescreening examinations (immature children, medication, etc.)	:	**5 562**
Total number of screening examinations 2020	**:**	**63 318**

We would like to thank all participants in the neonatal screening program for the above results - employees of the SCN SR laboratory, Neonatology Departments, pediatricians - VLDD, employees in Recall Centers for individual diseases in Bratislava, Banská Bystrica and Košice. Their systematic and often not easy work keeps our screening program at a high level.

In Banská Bystrica, May 13, 2020

RNDr. Mária Knapková, PhD., Head of the SCN SR laboratory
Prof. MUDr. Svetozár Dluholucký, CSc., Odb. guarantor SCN SR

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Contents

	Name, Function	Date, Signature
Prepared by:	Lukas Brunner, Associate scientist R&D	29.10.2021 Lukas Brunner
Reviewed and approved by:	Dr. Gernot Wolfram, CEO	02.11.2021 G. Wolfram

1. Introduction

1.1 Overview and purpose of this document

This document refers to the following product:

Order no.:	MS10000
Product name:	ClinSpot® Complete Kit for Amino Acids and Acylcarnitines in Dried Blood Spots (DBS)

This document is intended to provide information about a clinical performance study conducted at Clinical Hospital Centre Zagreb.

1.2 Document History (Change Control)

Changes of the previous version are indicated in green. Not applicable (first version).

Version no.	Date of release	Description of changes	Documentation
1	29.10.2021	n.a.	-

1.3 Clinical background

Neonatal screening is an important population-wide preventive measure to detect congenital metabolism disorders at a very early stage. The range and demands on the screening program vary in the different European countries, usually regulated within the respective national legislation.
All diseases are caused by genetic enzyme defects. The enzyme defect leads to extreme metabolism disorders and an accumulation of toxic metabolites, which may cause irreversible organ damage already in the first days of life.
For the neonatal screening dried blood samples are tested by LC-MS/MS. Due to the high analytical sensitivity and selectivity of the tandem mass spectrometry the samples can be tested in a time-saving way without HPLC separation. The samples however are determined semi-quantitatively only as the blood volume varies depending on the used filter paper and the hematocrit level of the dried blood sample.
The screening on its own therefore does not allow to establish a diagnosis, it just gives reason to suspect the presence of a disease. Thus, any positive screening result needs to be verified by additional tests such as molecular-genetic tests, the quantitative analysis of amino acids and acylcarnitines in plasma as well as organic acids in urine.

1.4 Scope

This report summarizes data from the Clinical Hospital Centre Zagreb in order to demonstrate clinical performance as required in IVDR Annex XIII, Section 2 and according to ISO 20916 for a risk class C screening device. Clinical performance will be demonstrated by performance parameters such as sensitivity and specificity, which were determined in a study at Clinical Hospital Centre Zagreb. The values obtained were provided to RECIPE and are presented in the following report. RECIPE was not involved in the process of planning and execution of the study. All relevant declarations were obtained from the Clinical Hospital Centre Zagreb as part of their neonatal screening program.
RECIPE provided required Kit materials to the laboratory at the Clinical Hospital Centre Zagreb. All procedures, sample preparation and analytical determinations were performed at the Clinical Hospital Centre Zagreb.

2. Clinical Performance Study

2.1 Study Device

2.1.1 General description of the principle of the assay method

The complete kit MS10000 is used for the semi-quantitative determination of the following amino acids and acylcarnitines, after derivatization, in dried blood spot specimen (DBS):

Table 1: Amino acids and acylcarnitines in MS10000

Amino acids		**Acylcarnitines**	
Ala	Alanine	C0	Free Carnitine
Arg	Arginine	C2	Acetylcarnitine
Asp	Aspartic acid	C3	Propionylcarnitine
Cit	Citrulline	C4	Butyrylcarnitine
Glu	Glutamic acid	C5	Isovalerylcarnitine
Gly	Glycine	C5DC	Glutarylcarnitine
Leu	Leucine	C6	Hexanoylcarnitine
Met	Methionine	C8	Octanoylcarnitine
Orn	Ornithine	C10	Decanoylcarnitin
Phe	Phenylalanine	C12	Dodecanoylcarnitine
Pro	Proline	C14	Tetradecanoylcarnitine (Myristoylcarnitine)
Tyr	Tyrosine	C16	Hexadecanoylcarnitine (Palmitoylcarnitine)
Val	Valine	C18	Octadecanoylcarnitine (Stearoylcarnitine)

Procedures were executed according to the IFU.
Prior to LC-MS/MS analysis a sample preparation needs to be performed. Therefore, the sample is mixed with the internal standard, analytes are extracted from the dried blood matrix and are subsequently derivatized. Sample preparation is performed with well plates only. Analysis should be performed on qualified LC-MS/MS systems by qualified staff only.

2.1.2 Components of the kit

Table 2 contains the list of components required for the analytical assay.

Table 2: List of Components order no. MS10000

Order No.	Description
MS10100	ClinSpot® Complete Kit for Amino Acids and Acylcarnitines
	in Dried Blood Spots (DBS) with Filter-Plates
	for 960 assays
	Content:
	Autosampler Washing Solution
	Mobile Phase
	Internal Standard IS, lyophil.
	Reagent A
	Reagent B
	Reagent C
	96-Well-Plates (370 µl)
	Covers for 96-Well-Plates
	Dried Blood Spot Control, Level I, II
	Manual
	Separately available components:
MS10005	Autosampler Washing Solution
MS10010	Mobile Phase
MS10012	Internal Standard IS, lyophil.
MS10012A	Internal Standard IS, lyophil.
MS10014	Optimisation Mix, lyophil.
MS10021	Reagent A
MS10022	Reagent B
MS10023	Reagent C
MS10040	96-Well-Plates (370 µl)
MS10041	Covers for 96-Well-Plates
	Accessories:
MS10042	Protective Sheets for 96-Well-Plates (PE/PP foil, 80 x 140 mm)
MS10045	Backpressure regulator, PEEK version, 34 bar
MS10046	Replacement cartridge gold coat, 34 bar
FK7400	Inline-Filter (stainless steel sieve, free of dead volume)
FK7340	Sealings and sieves for order no. FK7400
	ClinChek® Controls:
MS10182	Dried Blood Spot Control,
	Level I, II

2.1.3 Working instructions

LC-Conditions:

Pump (Flowrate): 0.09 ml/min (isocratic)
Autosampler: Injection interval: 1.6 min

MS/MS-Conditions:

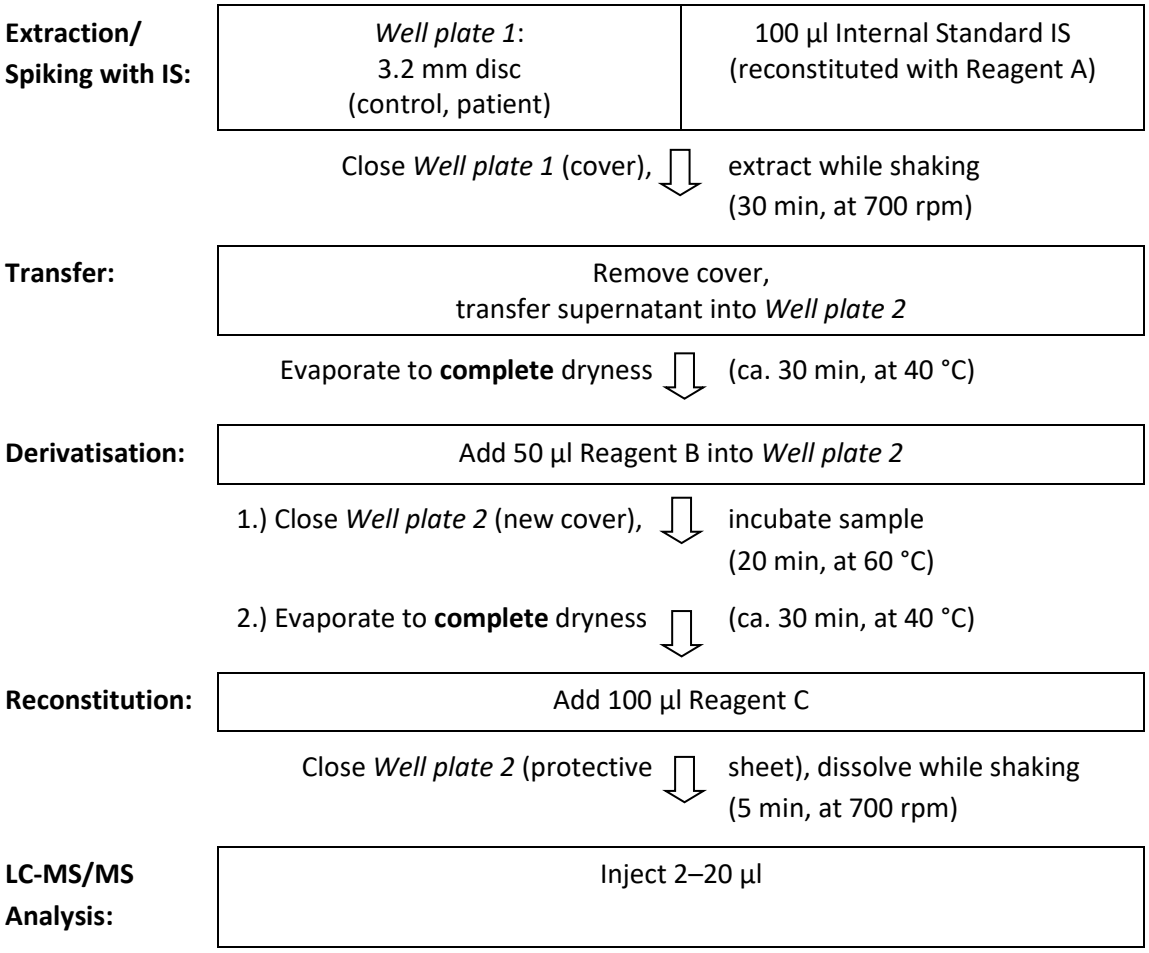
Table 4: Mass transitions for amino acids and respective internal standards

Analyt	Precursor [amu]	Product [amu]	Analyt	Precursor [amu]	Product [amu]
Glycine	132	76	^{15}N, 2-^{13}C-Glycine	134	78
Alanine	146	44	$^{13}C_3$, ^{15}N-Alanine	150	47
Proline	172	116	$^{13}C_5$-Proline	177	121
Valine	174	72	$^{2}H_8$-Valine	182	80
Leucine	188	86	$^{2}H_3$-Leucine	191	89
Ornithine	189	70	$^{2}H_6$-Ornithine	195	76
Methionine	206	104	$^{2}H_3$-Methionine	209	107
Phenylalanine	222	120	$^{13}C_6$-Phenylalanine	228	126
Arginine	231	70	$^{13}C_6$-Arginine	237	74
Citrulline	232	113	$^{2}H_7$-Citrulline	239	120
Tyrosine	238	136	$^{13}C_6$-Tyrosine	244	142
Aspartic acid	246	134	$^{13}C_4$-Aspartic acid	250	138
Glutamic acid	260	158	$^{13}C_5$-Glutamic acid	265	162

Table 5: Mass transitions for acylcarnitines and respective internal standards

Analyt	Precursor [amu]	Product [amu]	Analyt	Precursor [amu]	Product [amu]
Carnitin	218	85	Carnitin-$^{2}H_9$	227	85
C2-Carnitine	260	85	C2-Carnitine-$^{2}H_3$	263	85
C3-Carnitine	274	85	C3-Carnitine-$^{2}H_3$	277	85
C4-Carnitine	288	85	C4-Carnitine-$^{2}H_3$	291	85
C5-Carnitine	302	85	C5-Carnitine-$^{2}H_9$	311	85
C5DC-Carnitine	388	85	C5DC-Carnitine-$^{2}H_9$	397	85
C6-Carnitine	316	85	C6-Carnitine-$^{2}H_3$	319	85
C8-Carnitine	344	85	C8-Carnitine-$^{2}H_3$	347	85
C10-Carnitine	372	85	C10-Carnitine-d_3	375	85
C12-Carnitine	400	85	C12-Carnitine-$^{2}H_3$	403	85
C14-Carnitine	428	85	C14-Carnitine-$^{2}H_3$	431	85
C16-Carnitine	456	85	C16-Carnitine-$^{2}H_3$	459	85
C18-Carnitine	484	85	C18-Carnitine-$^{2}H_3$	487	85

Workflow:


Extraction/ Spiking with IS:
Well plate 1: 3.2 mm disc (control, patient)
100 µl Internal Standard IS (reconstituted with Reagent A)
Close Well plate 1 (cover), extract while shaking (30 min, at 700 rpm)
Transfer:
Remove cover, transfer supernatant into Well plate 2
Evaporate to complete dryness (ca. 30 min, at 40 °C)
Derivatisation:
Add 50 µl Reagent B into Well plate 2
1.) Close Well plate 2 (new cover), incubate sample (20 min, at 60 °C)
2.) Evaporate to complete dryness (ca. 30 min, at 40 °C)
Reconstitution:
Add 100 µl Reagent C
Close Well plate 2 (protective sheet), dissolve while shaking (5 min, at 700 rpm)
LC-MS/MS Analysis:
Inject 2–20 µl

2.2 Clinical Study Overview

2.2.1 Study Objective

Evaluation of clinical performance for the RECIPE Complete Kit with order no. MS10000 in a clinical routine environment.

2.2.2 Study device – Intended Use

The ClinSpot® Complete Kit with order no. MS10000 is intended for the semi-quantitative determination of amino acids and acylcarnitines for neonatal screening. The determination is performed with LC-MS/MS from dried blood spot specimen.
The components of the complete kit are intended to be used according to the IFU.

2.2.3 Study design

The clinical study was conducted at the Clinical Hospital Centre Zagreb under the direction of Prof. Dr. Sc. Fumic. Laboratory data were acquired with Recipe Complete Kit MS10000, from a total of 72973 newborns in the period from 01/10/2017 to 01/10/2019. Statistical analyses were performed by the Clinical Hospital Centre Zagreb

2.2.4 Study Population and Sampling

72973 dried blood samples from the routine newborn screening program in Croatia were analyzed. Blood samples were collected between the 48th and 72nd hour after birth.

2.2.5 LC-MS/MS systems

The clinical performance study was performed on the analytical system specified in Table 4.

Table 4: Instrumentation used for Clinical performance study

System1:	
- HPLC Components:	Degasser: Shimadzu DGU-20A$_{5R}$
	Pump: Shimadzu Nexera X2 LC-30AD
	Autosampler: Nexera X2 SIL-30AC
	Column Oven: Prominence CTO-20AC
- MS/MS Detector:	Shimadzu 8050

2.2.6 Performance Evaluation

Evaluation of the measurement data and statistical evaluation was carried out by the principle investigator. Values were provided to Recipe Chemicals + Instruments GmbH.

2.3 Study Results

For the evaluation of PKU, cut-offs of 120 µmol/ for phenylalanine concentration and a Phe/Tyr-ratio of 1 were used. Thus the following results were obtained:

	Positive PKU	Negative PKU
Positive	True Positive TP = 18	False Positive FP = 48
Negative	False Negative FN = 0	True Negative TN = 72955
	Sensitivity = TP / (TP + FN) = **100 %**	**Specificity** = TN / (FP + FN) = **99.93 %**

3. Conclusion

A cut-off value of 120 µmol/l for phenylalanine was used to reliably identify PKU in clinical routine NBS programs ate the Clinical Hospital Centre Zagreb using the RECIPE Complete Kit (order no. MS10000). Clinical Sensitivity was 100% and clinical Specificity 99.93% in a population of 72973 newborns.