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First ISNS Reference Preparation for Neonatal Screening for thyrotropin, phenylalanine and 17α-hydroxyprogesterone in blood spots

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Rapport in het kort

Het eerste ISNS Referentie Preparaat voor Neonatale Screening op thyrotropine, fenylalanine and 17α-hydroxyprogesteron in bloedvlekken

Bij screening van pasgeborenen voor aangeboren stofwisselingsziekten wordt in een bloedmonster, verkregen via de hielprik en opgevangen op filtreerpapier, een aantal bloedcomponenten gemeten. Veelal wordt gebruik gemaakt van commercieel verkrijgbare reagentiasets. De fabrikanten kalibreren hun reagentiasets vaak met hun eigen kalibratoren. Bovendien zijn er in de wereld meerdere filtreerpapiersoorten in gebruik, met verschillen in specificaties. Door deze beide oorzaken zijn de uitslagen van verschillende screeningslaboratoria vaak niet goed vergelijkbaar. Dit bemoeilijkt de evaluatie van zulke screeningsprogramma's.
De International Society for Neonatal Screening (ISNS) heeft eerder goede ervaringen opgedaan met het laten bereiden van referentiematerialen in filtreerpapierbloed en het overreden van fabrikanten om deze materialen te gebruiken als maatstaf. In 2004 heeft de ISNS aan het RIVM verzocht om een gecombineerd referentiemateriaal te maken voor filtreerpapierbloed met bekende concentraties aan thyrotropine, 17α-hydroxyprogesteron, en phenylalanine, merkstoffen die van belang zijn voor de screening op stoornissen in de schildklier, bijnier, respectievelijk eiwitmetabolisme.
In dit rapport is de bereiding en evaluatie van dit referentiemateriaal beschreven.

Trefwoorden: pasgeborenen; hielprik; screening; ags; pku; cht; referentie preparaat

Abstract

First ISNS Reference Preparation for Neonatal Screening for thyrotropin, phenylalanine and 17α-hydroxyprogesterone in blood spots.

Many countries have a screening programme for newborns for congenital metabolic disorders. For this screening several components are measured in dried blood spots collected by a heel stick on filter paper. Most laboratories use commercially available reagent sets for the measurements. Manufacturers of these reagents often calibrate their reagent sets against own calibrators. Moreover several types of filter paper, each with different specifications are in use all over the world. These factors make it difficult to compare results from different screening laboratories and to evaluate such screening programmes.
In the past the International Society for Neonatal Screening (ISNS) has been able to persuade manufacturers to use previously prepared dried blood spot reference materials as calibration standards. Having run out of stock, in 2004 the ISNS asked the RIVM to produce a combined reference preparation in filter paper blood with known concentrations of thyrotropine, 17α-hydroxyprogesterone and phenylalanine, being the markers of interest for screening on disorders of the thyroid gland, the adrenal gland and amino acid metabolism, respectively.
This report describes the production and evaluation of this reference preparation.

Keywords: newborn; screening; cah; pku; cht; reference preparation

Summary

Introduction
Neonatal screening for congenital disorders is generally performed in dried blood spots on filter paper. The analytes of interest for phenylketonuria (PKU), congenital hypothyroidism (CH) and congenital adrenal hyperplasia (CAH) are phenylalanine, thyroid stimulating hormone (TSH) and 17α-hydroxyprogesterone (17OHP), respectively. Analyses are usually carried out with commercial kits containing blood spot calibrators on filter paper. Differences in the way these materials are produced and calibrated lead to differences in results for neonatal samples between and among screening laboratories. Another variable is the type of filter paper: Schleicher & Schuell #903, Whatman BFC180 or Toyo Roshi 545.
Existing reference materials for blood spots on filter paper are available for phenylalanine and also for TSH, but this material has expired recently. There is no reference material for 17OHP. The International Society for Neonatal Screening (ISNS) recognised the need for a combined reference material for the three analytes on the three types of filter paper. This '1st ISNS Reference Preparation for Neonatal Screening for TSH, phenylalanine and 17OHP in blood spots' (1st ISNS-RPNS) has been prepared by the RIVM (Bilthoven).
Results
The number of filter paper cards with blood spot calibrators prepared was 480, 42 and 69 for Schleicher & Schuell #903, Whatman BFC180 and Toyo Roshi 545, respectively. Each filter paper card has two sets of six calibrators; the volume of blood dispensed was 50 µL. The range of concentrations is for TSH: 1 – 121 mIU/L blood, for phenylalanine: 65 – 865 µmol/L blood and for 17OHP: 2.2 – 302 nmol/L blood. The linearity of the blood spot calibrators and the homogeneity of the batch (only tested for Schleicher & Schuell) were good. The differences between the three filter papers were small: the potency of the ISNS-RPNS on Whatman and Toyo Roshi in terms of Schleicher & Schuell varied between 0.98 and 1.09 for the three analytes.
The calibrators of the 1st ISNS-RPNS were analysed as 'routine neonatal samples' in 22 neonatal screening laboratories using different methods. The overall recovery (mean ± SD) was 96 ± 15%, 100 ± 10% and 92 ± 11% for TSH, phenylalanine and 17OHP, respectively
Conclusion
The 1st ISNS-RPNS for TSH, phenylalanine and 17OHP is suitable as formal reference preparation and serves as a source for (re)calibrating kit calibrators.

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1. Introduction

Neonatal screening for congenital disorders like phenylketonuria (PKU), congenital hypothyroidism (CH) and congenital hyperplasia (CAH) is generally performed in dried blood spots on filter paper. For use in neonatal screening special filter papers are on the market and the ones most often used are Schleicher & Schuell, type #903, Whatman type BFC180 and Toyo Roshi, type 545. These filter papers have to fulfil certain criteria, one of the most important being serum absorption volume, which has been formulated by the NCCLS (now: CLSI) [1]. Kits intended for use in neonatal screening usually have blood spot calibrators on filter paper. Ideally, these calibrators are made in such a way that they match with neonatal samples as far as possible, i.e. same matrix (human blood), spot size (11-12 mm) and filter paper. In practice, manufacturers of kits use different matrices, while there is also a variety in spot size. Other sources of variation are the 'standard' material which is used for preparation of the calibrators, and the way the concentration of the calibrators is assigned. All these factors lead to differences between sets of calibrators and finally in results for neonatal samples obtained with these calibrators. Differences between kits and even between lots of calibrators from the same kit are clearly demonstrated by results from external quality assessment programmes.
For the determination of analytes in neonatal screening no standard methods are available. So manufacturers of kits can not revert to such methods for a proper calibration of their calibrators. An alternative for manufacturers of kits is the availability of 'reference materials for blood spots on filter paper'. In 1996 the 'first European Working Standard for phenylalanine in blood spots' had been prepared by the RIVM [2,3]. This EWS-PHE-01 was available for manufacturers of kits and for organizers of quality control programmes and was followed by the EWS-PHE-02 in 1999 and the EWS-PHE-03 in 2003. Also in 1996 the 'Amino Acid Reference Material' (AARM) had been prepared by the CDC [4]. In 1999 a certified reference material for thyrotropin (CRM-TSH) was prepared by CDC on request of the Standard Committee on Quality Assurance (SCQA) of the International Society for Neonatal Screening (ISNS). For 17α-hydroxyprogesterone, which is a parameter for screening on congenital hyperplasia (CAH), no such material is available yet. For this reason and due to the fact that CRM-TSH expired in December 2004, the ISNS decided to make a combined reference material for thyrotropin, phenylalanine and 17α-hydroxyprogesterone (17OHP) in blood spots. This 'First ISNS Reference Preparation for Neonatal Screening for thyrotropin, phenylalanine and 17α-hydroxyprogesteron in blood spots' has been prepared by the RIVM in November 2004. The 1st ISNS-RPNS, prepared on Schleicher & Schuell #903, Whatman BFC180 and Toyo Roshi 545, is available for manufacturers of kits for neonatal screening and for organisers of quality assessment schemes. This report describes in detail the production and the results of the evaluation of this 1st ISNS-RPNS.

2. Materials and methods

The procedure for the preparation of the 1st ISNS-RPNS is described in detail in Appendix 1. Here we confine ourselves to the procedure in general.[1]

2.1 Preparation of the 1st ISNS-RPNS

Blood was collected from a healthy donor, adjusted to 50% hematocrit and divided into six portions. These portions were enriched with TSH 81/565 (proposed 3rd IRP for TSH, NIBSC, London; range: 0 – 120 mIU/L blood), phenylalanine (product no 78019, Fluka, Switzerland; range: 0 – 800 µmol/L blood) and 17α-hydroxyprogesterone (product no 56240, Fluka, Switzerland; range: 0 – 300 nmol/L blood). The portions were homogenised on a roller bank for five hours. Blood spots were prepared on filter paper Schleicher & Schuell type #903, Whatman type BFC180 and Toyo Roshi type No 545. On each filter paper card two 50 µL spots from each calibrator A – F were spotted; for a limited number filter paper cards S&S #903 the volume was 100 µL. After overnight drying in the dark at room temperature, the cards were packed individually in a laminated aluminium bag with dessicant and stored at -20 ^{o}C.

2.2 Assessment of TSH, phenylalanine and 17OHP in basal and enriched plasma

Plasma and a 5-sulfosalicylic acid extract were prepared from basal and enriched blood and frozen immediately. For each analyte 0.6 mL portions of the plasmas were sent to three laboratories for the assessment of TSH, phenylalanine and 17OHP. One of these laboratories measured phenylalanine in plasma and in 5-sulfosalicylic extracts. The laboratories selected for measurement of phenylalanine were: Hôpital d'Enfants, Laboratoire de Biochimie Spécialisée, Dyon, France (Dr. S. Ewing); Hôpital Necker-Enfants maladies, Department Laboratoire de Biochemie B, Paris, France (Dr D. Rabier); Hôpital Universitaire des Enfants, ULB-Laboratoire de Pédiatrie, Brussels, Belgium (Prof. P. Bourdoux). The laboratories for TSH and 17OHP were: UMC-Utrecht, Laboratory for Endocrinology, Utrecht, The Netherlands (Dr. E. Lentjes); UMC-St Radboud, Laboratoy for Experimental Endocrinology, Nijmegen, The Netherlands (M.F.G. Segers); AMC, Laboratory for Endocrinology, Amsterdam, The Netherlands (Dr. E. Endert).

2.3 Assignment of TSH, phenylalanine and 17OHP in basal and enriched blood

The concentration of TSH, phenylalanine and 17OHP in basal blood was based on the determinations in basal plasma (see 2.2). The concentration of the analytes in the enriched blood was based on the concentration in basal blood and the amount of the analytes added to the blood.

2.4 Evaluation of the 1st ISNS-RPNS in neonatal screening laboratories

The 1st ISNS-RPNS was sent to 18 laboratories participating in the RIVM QA-scheme for TSH, phenylalanine and 17OHP. These laboratories analysed the calibrators A-F with their routine neonatal screening methods. The 1st ISNS-RPNS was also analysed by all members of the Standard Committee on Quality Assurance of the ISNS.

[1] Note: use of trade names is for identification only and does not imply endorsement by the National Institute for Public Health and the Environment (RIVM) or the International Society for Neonatal Screening.

2.5 Homogeneity of the batch on filter paper S&S #903

The homogeneity of the ISNS-RPNS on filter paper S&S #903 was tested. Filter paper cards coming from 1%, 25%, 50%, 75% and 100% of the batch were analysed in quadruplicate for TSH and 17OHP and in triplicate for phenylalanine. A second experiment was carried out for TSH and phenylalanine, but only for calibrator F. The homogeneity for the batches on Whatman BFC180 and Toyo Roshi was not tested, because these batches were relative small.

2.6 Comparison of the 1st ISNS-RPNS on three different filter papers

The batches of the 1st ISNS-RPNS on S&S #903, Whatman and Toyo Roshi were compared by determination of TSH, phenylalanine and 17OHP in 3-5 laboratories (all members of the Standard Committee on Quality Assurance of the ISNS).

2.7 Stability

Forty cards from S&S#903, each with five 50 µL spots from calibrator D, were prepared for a stability study. These cards were placed at -80 °C, -20 °C, 4 °C, 22 °C and 37 °C. After 1, 2, 4, 8, 12, 18 and 24 months cards from the temperatures -20 °C, 4 °C, 22 °C and 37 °C have been or will be transferred to -80 °C. At the end of the period all samples will be analysed in quadruplicate for TSH, phenylalanine and 17OHP.

3. Results

3.1 Number of sets calibrators prepared

The following numbers of filter paper cards, each with two series of calibrators, were prepared: S&S #903: N=480; Whatman BFC180: N=42; Toyo Roshi 545: N=69. For these cards the volume of blood dispensed was 50 µL. A total of 20 cards, all S&S #903 were withdrawn from the batch because of irregular spot size, or blood migrated partly under the label. Fourteen cards of S&S #903 were prepared with two series of calibrators and 'spot size' 100 µL. These cards were intended for comparison with the CRM-TSH preparation (1999) with the same spot size. Figure 1 shows the final layout of the ISNS-RPNS on filter paper S&S #903.

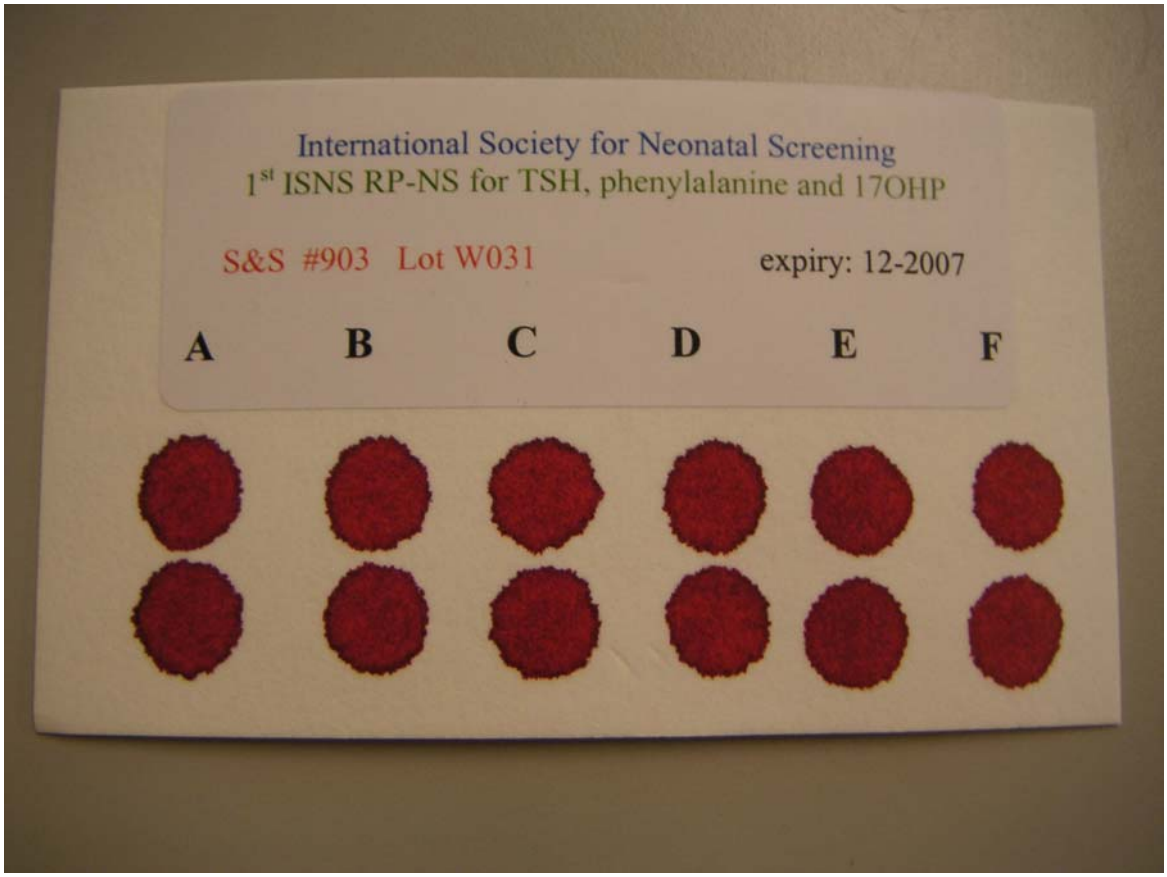

Figure 1. 1st ISNS RP-NS for TSH, phenylalanine and 17OHP on S&S #903.

3.2 Concentration of TSH, phenylalanine and 17OHP in basal and enriched plasma

TSH, phenylalanine and 17OHP were determined in plasma, prepared from basal and enriched blood, by three laboratories. These laboratories were asked to determine the analytes in triplicate. This appeared not always to be possible due to the amount of plasma available and the fact that samples had to be re-analysed after dilution. The mean measured concentration per laboratory and per analyte is summarised in Table 1a-c. See Appendix 2, Table 1 for the individually measured concentrations.

The variation between laboratories in the measured concentration for plasma A was for 17OHP and TSH very well within the limits which may be expected for the methods used.

For phenylalanine the concentration in plasma A as measured by laboratory C was relatively high compared to the other laboratories; there was also a larger difference between the 'measured' and 'calculated' concentration for this laboratory (see below).
The basal concentration of TSH, phenylalanine and 17OHP in plasma A was also assessed as the intercept of the regression line for plasma A-F for each laboratory. Table 2 shows the parameters for the formula of the regression lines and the concentration for plasma A derived from both methods: 'measured' and 'calculated from the intercept'. For TSH and phenylalanine the differences between the results of both methods were small relative to the additions. For 17OHP the regression line could be calculated for only two laboratories; the intercept for both lines was quite different; no explanation was found.
It was decided to use the 'mean measured' concentration for plasma A for TSH and 17OHP and the 'median measured' concentration for phenylalanine as basal concentration for plasma A and finally for blood A. Based on the 'basal' concentration for plasma A, the expected concentrations for plasma B-F were assessed (basal + added). The mean recovery for each analyte for plasma B-F is given in Table 1a-c. The overall recovery in plasma was 99% for TSH, 110% for phenylalanine and 87% for 17OHP.

Table 1a. Mean concentration (mIU/L plasma) and recovery (%) of TSH in basal and enriched plasma as measured by three laboratories. Methods: Lab A: Architect (Abbott); Lab B: Delfia (PerkinElmer) and Lab C: Centaur (Bayer).

	TSH mIU/L plasma observed				**mIU/L plasma**		**%recovery**
	Lab A	*Lab B*	*Lab C*	*mean*	*added*	*expected*	*(mean/expected)*
A	2.3	1.9	2.0	**2.0**	0	**2.0**	---
B	14.8	12.3	13.7	13.3	12.5	14.5	93.4
C	28.6	24.8	26.9	27.2	25	27	99.1
D	55.4	51.3	54.2	53.3	50	52	103.1
E	113.0	102.3	104.0	106.7	100	102	104.3
F	253.5	231.0	238.5	241.3	240	242	99.6
						mean→	**99.9**

Table 1b. Median concentration (µmol/L plasma) and recovery (%) of phenylalanine in basal and enriched plasma as measured by three laboratories. Method: Lab A (amino acid analyser); Lab B (amino acid analyser) and Lab C (MS/MS). Laboratory B analysed both plasma and 5-sulfosalicylic acid extract.

	phenylalanine µmol/L plasma observed					**µmol/L plasma**		**%recovery**
	Lab A	*Lab B*	*LabB**	*Lab C*	*median*	*added*	*expected*	*(median/expected)*
A	64.7	64.3	65.6	75.1	**65.2**	0	**65.2**	---
B	118.3	123.3	125.3	119.9	121.6	50	115	105.6
C	175.0	184.7	173.7	170.7	174.3	100	165	105.6
D	277.0	297.7	295.7	330.5	296.7	200	265	111.9
E	505.3	521.0	520.7	541.2	520.8	400	465	112.0
F	957.0	1008.0	1001.7	1026.6	1004.8	800	865	116.1
							mean→	**110.2**

* results in 5-sulfosalicylicacid extract

Table 1c. Mean concentration (nmol/L plasma) and recovery (%) of 17OHP in basal and enriched plasma as measured by three laboratories. Method: Lab A: RIA (DPC), Lab B: RIA (in house) and Lab C: paper chromatography → RIA (in house). Lab C analysed only basal plasma A.

	17OHP nmol/L plasma observed				**nmol/L plasma**		**%recovery**
	Lab A	*Lab B*	*Lab C*	*mean*	*added*	*expected*	*(mean/expected)*
A	5.0	4.1	3.8	**4.3**	0	**4.3**	---
B	27.9	22.7	---	25.3	25	29.3	86.3
C	49.0	45.7	---	47.3	50	54.3	87.2
D	101.0	103.5	---	102.3	100	104.3	98.0
E	170.3	>	---	170.3	200	204.3	83.4
F	481.3	>	---	481.3	600	604.3	79.7
						mean→	**86.9**

Table 2. Parameters for the formula of the regression line (Y=aX+b) and coefficient of correlation (r) for TSH, phenylalanine and 17OHP in plasma as determined by three laboratories. Phenylalanine was determined by Lab B in plasma as well as in a 5-sulfosalicylic acid extract.

			basal concentration plasma A as determined by:	
TSH	**regression line**	**r**	**regression**	**measured**
Lab A	y = 1.05x + 3.02	0.999	3.02	2.30
Lab B	y = 0.96x + 2.16	0.999	2.16	1.87
Lab C	y = 0.99x + 2.89	0.999	2.89	2.05
average			**2.69**	**2.07**
median			**2.89**	**2.05**
phenylalanine	**regression line**	**r**	**regression**	**measured**
Lab A	y = 1.12x + 61.1	0.999	61.1	64.7
Lab B	y = 1.17x + 63.1	0.999	63.1	64.3
Lab B (5-SS)	y = 1.17x + 61.8	0.999	61.8	65.6
Lab C	y = 1.20x + 67.6	0.999	67.6	75.1
average			**63.4**	**67.4**
median			**62.4**	**65.2**
17OHP	**regression line**	**r**	**regression**	**measured**
Lab A[1]	y = 0.96x + 3.8	0.999	3.8	5.0
Lab B	y = 1.01x - 0.02	0.995	-0.02	4.1
Lab C[2]	---	---	---	3.8
average			**1.9**	**4.3**
median			**1.9**	**4.1**

[1] calculated for range 0 -100 nmol/L plasma
[2] laboratory C measured only basal plasma A

3.3 Assignment of the concentration in basal and enriched blood

The assignment of the concentrations of the calibrators A-F was based on i) the **basal** concentration in blood spot A and ii) the TSH, phenylalanine and 17OHP **added** to the blood. The results in units/L blood and units/L serum (for TSH and 17OHP) and some conversion factors are summarised in Table 3a-c.

*Table 3a. TSH, phenylalanine and 17OHP-concentration for calibrator A – F of the ISNS-RPNS expressed as units/L **blood**.*

Analyte	A	B	C	D	E	F	Units	
TSH	1.0	7.25	13.5	26.0	51.0	121	mIU/L	**blood**
Phenylalanine	65	115	165	265	465	865	µmol/L	**blood**
17OHP	2.2	14.6	27.2	52.2	102	302	nmol/L	**blood**

*Table 3b. TSH and 17OHP concentration for calibrator A – F of the ISNS-RPNS expressed as units/L **serum.***

Analyte	A	B	C	D	E	F	Units	
TSH	2.0	14.5	27.0	52.0	102	242	mIU/L	**serum**
17OHP	4.3	29.3	54.3	104.3	204.3	604.3	nmol/L	**serum**

Table 3c. Conversionfactors for phenylalanine and 17OHP

Analyte	**Conversion factors**
Phenylalanine (MW=165.2)	1 µmol/L blood = 0.0167 mg/dL blood
OHP (MW=330.5)	1 nmol/L blood = 0.33 ng/mL blood 1 nmol/L blood = 0.66 ng/mL serum

3.4 ISNS-RPNS in QC-programme for neonatal screening

The RIVM organizes a neonatal screening QC-programme for screening laboratories in The Netherlands, Belgium and Luxembourg. Furthermore some individual laboratories in other countries and manufacturers of kits for neonatal screening participate. In RIVM survey 2004-4 the ISNS-RPNS was sent to all participating laboratories (TSH: N=17; phenylalanine: N=14; 17OHP: N=13). These laboratories analysed the calibrators A – F according to their routine methods in singlicate (N=12) or in duplicate (N=5). The ISNS-RPNS was also analysed by the members of the SCQA of the ISNS, some of them using two different methods per analyte. The number of replicates for these laboratories varied from 2 – 8. The mean measured concentration and recovery (%) for each laboratory are summarised in Table 4a-c. For 17OHP and TSH the recovery was calculated only for calibrator B-F, because the concentration of calibrator A was often reported to be lower then the limit of detection.

TSH: the overall recovery for all laboratories was 95.5 ± 14.3% (mean ± SD) and 97.9 ± 9.0% without laboratory 16, having a recovery of only 45%; the mean recovery was between 85-115% for 18 out of 22 laboratories

Phenylalanine: the overall recovery for all laboratories was 100.1 ± 10.0% (mean ± SD) and 101.5 ± 7.4% without laboratory 11, having a recovery of only 69%; the mean recovery was between 85-115% for 21 out of 23 laboratories.

OHP: the overall recovery for all laboratories was 91.5 ± 11.1% (mean ± SD) and 93.2 ± 10.1% without laboratory 10, having a recovery of only 69%; the mean recovery was between 85-115% for 13 out of 18 laboratories.

Tabel 4a. TSH concentration (mIU/L blood and % recovery) for the ISNS-RPNS as measured by participants in RIVM-survey 2004-4 and members of the Standard Committee on Quality Assurance of the ISNS (N=22).

			calibrator	**A**	**B**	**C**	**D**	**E**	**F**	**A**	**B**	**C**	**D**	**E**	**F**	**mean B-F**
			added	0	6.25	12.5	25	50	120							(1)
			expected	1	7.25	13.5	26	51	121							
labnr	**method**	**kit lot**	**cal. lot**	**observed (mIU/L blood)**						**%recovery (observed/expected*100%)**						
5	AutoDelfia	241898	235611	1.0	5.5	10.0	23.0	41.0	95.5	---	76	74	88	80	79	80
4	AutoDelfia	244408	239185	<1	6.0	11.5	26.0	54.0	123	---	83	85	100	106	102	95
8	AutoDelfia	247993	242805	1.0	6.3	14.6	29.5	50.2	126	---	86	108	113	98	104	102
2	AutoDelfia	248504	242805	<1	6.0	12.0	24.5	44.5	120	---	83	89	94	87	99	90
1	AutoDelfia	251535	242805	1.0	5.5	12.5	29.0	50.5	130	---	76	93	112	99	107	97
13	AutoDelfia	252839	242805	0.9	6.8	13.9	27.4	55	129	---	94	103	105	108	106	103
6	AutoDelfia	252839	249055	1.3	6.8	14.5	28.5	52.5	130	---	93	107	110	103	107	104
11	AutoDelfia	257304	253548	1.0	5.8	11.8	25.9	54.5	135	---	80	87	99	107	111	97
7	AutoDelfia	257305	253548	1.0	6.6	13.9	27.6	47.5	114	---	90	103	106	93	94	97
18	AutoDelfia	260469	257180	1.0	7.0	15.0	25.0	50.0	120	---	97	111	96	98	99	100
20	AutoDelfia	265398	263349	0.8	5.3	11.2	24.5	47.2	114	---	73	83	94	92	95	87
9a	AutoDelfia	---	---	1.9	7.4	14.5	27.6	56.2	127	---	101	108	106	110	105	106
15	Bio-Rad	109	---	0.0	6.6	14.9	36.0	73.6	122	---	91	110	138	144	101	117
3	Brahms	401610	37915	1.5	7.0	14.5	31.5	63.5	123	---	97	107	121	125	102	110
12	Delfia	225926	in-house	0.9	6.3	11.7	24.9	56.6	128	---	87	87	96	111	105	97
21	Delfia	225926	in-house	0.4	6.2	12.2	26.3	54.4	123	---	85	90	101	107	102	97
16	ELISA	---	---	2.2	3.9	5.4	9.0	18.5	72.7	---	54	40	35	36	60	45
14	ELISA	---	---	0.8	7.8	13.8	26.1	46.2	>100	---	107	103	100	91	---	100
19	ELISA; Eiken	46001	---	0.3	5.8	11.6	22.4	42.4	>90	---	79	86	86	83	---	84
10	In house	---	38279	2.6	6.7	13.7	25.1	42.4	62.0	---	92	101	97	83	51	85
17	RIA	---	---	0.0	7.0	14.0	27.0	62.0	116.0	---	97	104	104	122	96	104
9b	RIA; CisBio	---	---	0.2	6.6	13.0	30.2	51.4	133	---	92	96	116	101	110	103
mean				1.0	6.3	12.7	26.2	50.6	117.0	---	86.9	94.3	100.8	99.3	96.7	95.5
SD				0.7	0.8	2.2	4.9	10.4	19.1	---	11.4	16.1	18.8	20.5	15.8	14.3
%VC				69.4	13.1	17.0	18.6	20.6	16.3	---	13.1	17.0	18.6	20.6	16.3	15.0

(1) without laboratory 16: 97.9 ± 9.0 (mean ± SD)

Tabel 4b. Phenylalanine concentration (µmol/L blood and % recovery) for the ISNS-RPNS as measured by participants in RIVM-survey 2004-4 and members of the Standard Committee on Quality Assurance of the ISNS. Note: some laboratories used two different methods.

			calibrator	**A**	**B**	**C**	**D**	**E**	**F**	**A**	**B**	**C**	**D**	**E**	**F**	**mean B-F**
			added	0	50	100	200	400	800							
			expected	65	115	165	265	465	865							
labnr	**method**	**kit lot**	**cal. lot**	**observed (µmol/L blood)**						**% recovery (observed/expected*100%)**						
1	Quantase	148	335	100	150	200	300	490	1000	154	130	121	113	105	116	117
2	Quantase	148	385	79	116	153	246	440	852	122	101	93	93	95	98	96
3	Quantase	148	472	50	110	150	220	570	1030	77	96	91	83	123	119	102
4	Quantase	148	472	90	119	168	240	421	781	138	103	102	91	91	90	95
5	Quantase	148	472	50	130	160	240	400	780	77	113	97	91	86	90	95
6	Quantase	148	472	70	120	160	280	450	860	108	104	97	106	97	99	101
7	Quantase	148	472	80	110	170	290	470	860	123	96	103	109	101	99	102
8	Quantase	148	472	73	109	179	275	484	890	112	95	108	104	104	103	103
9	Quantase	---	---	74	117	157	241	473	880	113	102	95	91	102	102	98
10	MS/MS	---	---	71	122	173	284	496	902	109	106	105	107	107	104	106
11	MS/MS	in house	in house	48	80	106	189	321	599	74	70	64	71	69	69	69
12	MS/MS	---	---	64	108	149	240	434	820	99	94	90	91	93	95	93
13	MS/MS	---	248467	70	120	180	310	560	910	108	104	109	117	120	105	111
19	MS/MS	---	244161	68	130	180	250	510	890	105	113	109	94	110	103	106
20	MS/MS	---	---	62	111	162	240	456	756	95	96	98	91	98	87	94
21	MS/MS	---	---	78	112	181	268	498	839	120	98	110	101	107	97	103
8	HPLC	---	---	67	116	176	264	480	949	103	101	107	100	103	110	104
14	HPLC	---	---	64	113	151	261	421	825	98	98	91	99	91	95	95
16	HPLC	---	---	51	---	159	276	518	991	78	---	96	104	111	115	107
9	fluorometric	---	---	83	129	179	273	474	921	128	112	109	103	102	106	106
14	fluorometric	---	---	65	118	165	264	482	894	100	103	100	99	104	103	102
15	Bio-Rad	153	585	71	99	129	194	457	759	109	86	78	73	98	88	85
18	AutoDelfia	238046	232615	50	130	210	270	540	960	77	113	127	102	116	111	114
mean				68	117	164	257	470	869	105.5	101.5	100.0	97.1	101.4	100.3	100.1
SD				13.4	13.4	21.6	30.0	54.2	95.1	20.6	11.7	13.1	11.3	11.7	11.0	10.0
%VC				20	11	13	12	12	11	20	11	13	12	12	11	10

Tabel 4c. 17OHP concentration (nmol/L blood and % recovery) for the ISNS-RPNS as measured by participants in RIVM-survey 2004-4 and members of the Standard Committee on Quality Assurance of the ISNS.

			calibrator	**A**	**B**	**C**	**D**	**E**	**F**	**A**	**B**	**C**	**D**	**E**	**F**	**mean B-F**
			added	0	12.5	25	50	100	300							
			expected	2.2	14.7	27.2	52.2	102.2	302.2							
labnr	**method**	**kit lot**	**cal. lot**	**observed (nmol/L blood)**						**%recovery (observed/expected*100%)**						
4	AutoDelfia	201524	189437	<1	17.0	32.5	56.5	102.5	320	---	116	120	108	100	106	110
18	AutoDelfia	231068	224559	0	18	30	50	99	260	---	123	110	96	97	86	102
2	AutoDelfia	248015	239183	5.0	13.5	24.5	58.5	91.5	283	---	92	90	112	90	94	96
1	AutoDelfia	248854	239183	4.5	16.0	29.0	54.5	97.5	300	---	109	107	105	95	99	103
7	AutoDelfia	246424	239183	4.5	15.5	28.0	47.0	95.5	306	---	106	103	90	93	101	99
5	AutoDelfia	253229	246380	6.0	14.5	24.0	49.5	87.5	275	---	99	88	95	86	91	92
6	AutoDelfia	253229	246380	2.8	13.0	27.0	50.0	83.0	282	---	89	99	96	81	93	92
8	AutoDelfia	251378	246380	3.5	14.0	26.8	55.0	97.5	316	---	96	99	105	95	105	100
13	AutoDelfia	262130	251129	2.3	12.3	27.4	51.0	91.0	292	---	84	101	98	89	97	94
3	AutoDelfia	262418	255395	<1	10.0	27.0	55.5	130.0	>300	---	68	99	106	127	---	100
11	AutoDelfia	262418	255395	3.2	15.8	28.9	50.0	92.0	303	---	108	106	96	90	100	100
20	AutoDelfia	262418	255395	2.4	11.0	21.5	51.1	97.0	275	---	75	79	98	95	91	88
9	AutoDelfia	---	---	1.4	10.0	19.9	36.8	81.1	254	---	68	73	71	79	84	75
15	Bio-Rad	105	522	2.8	13.0	25.8	48.6	89.1	177	---	88	95	93	87	59	84
12	Delfia	---	---	0.8	9.3	19.7	40.0	82.9	250	---	64	73	77	81	83	75
16	ELISA	---	---	9.8	19.2	19.4	38.3	70.8	198	---	131	71	73	69	66	82
14	ELISA	---	---	3.4	14.4	25.1	40.4	86.3	>120		98	93	78	84	---	88
10	Schering	---	38171	<1	9.0	19.0	38.5	76.0	194	---	61	70	74	74	64	69
mean				3.5	13.6	25.3	48.4	91.7	267.8	---	93.1	93.2	92.8	89.7	88.6	91.5
SD				2.4	3.0	4.0	6.8	12.7	43.9	---	20.5	14.8	13.1	12.5	14.5	11.1
%VC				67.6	22.0	15.8	14.1	13.9	16.4	---	22.0	15.8	14.1	13.9	16.4	12.2

3.5 Homogeneity of the batch on S&S #903

Homogeneity of the batch was tested for S&S #903 by analysing card numbers: 2 and 4, 113 and 124, 224 and 236, 360 and 366, 458 and 470 coming from respectively 1%, 25%, 50%, 75% and 100% of the batch. The cards were analysed in quadruplicate with the AutoDelfia method for TSH and 17OHP and in triplicate with the Quantase method (BioRad) for phenylalanine. The data were analysed in a 'linear mixed effect model; S-plus; Dr. A.L.M. Dekkers, RIVM)'. Tables 5a-c show the mean concentration and percentage recovery in terms of the 'mean concentration' per calibrator and per card for the three analytes.

TSH

The batch was homogeneous for all calibrators except for calibrator F. For calibrator F a small but significant ($p<0.01$) decrease was observed at the end of the batch. The variation between replicates was higher for calibrator F as compared with the other calibrators. This was possibly due to the fact that the level of this calibrator was at the end of the calibration curve. In a separate experiment homogeneity was tested only for spot F. In this experiment extra cards were taken from the beginning and the end of the batch; the following card numbers were used 25, 31, 32, 95, 185, 327, 458, 459 and 460. In this experiment there was still a small decline ($p=0.01$) but this was due to the last samples (card 458, 459 and 460) of the batch. After combination of both experiments the batch was homogeneous ($p=0.045$) for calibrator A-F when the last cards were omitted. This conclusion applies for cards 1-366. Figure 2 shows the combined results of both experiments.

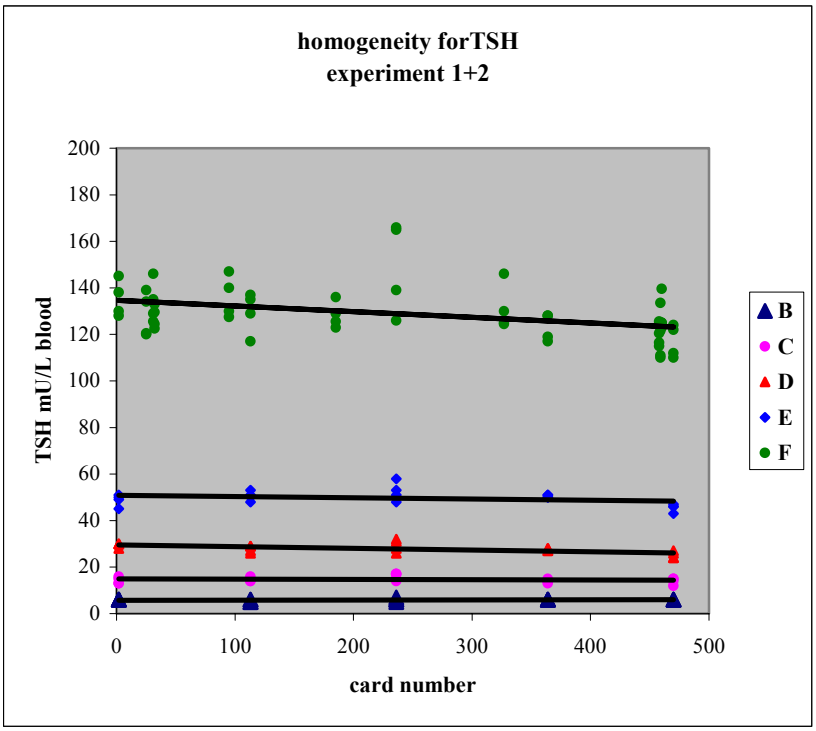

Figure 2. Homogeneity for TSH; combined results from experiment 1 and 2.

Phenylalanine

The batch was homogeneous for calibrator A-E ($p=0.28$). For calibrator F a small increase phenylalanine was observed at the end of the batch. In a separate experiment homogeneity was tested for only calibrator F. Extra cards were taken from the beginning and from the end of the batch; the following card numbers were used 25, 31, 32, 95, 185, 327, 458, 459

and 460. In this second experiment the batch was found to be homogeneous (p=0.62) and this was also the case when the results from both experiments were combined (p=0.29). See figure 3.

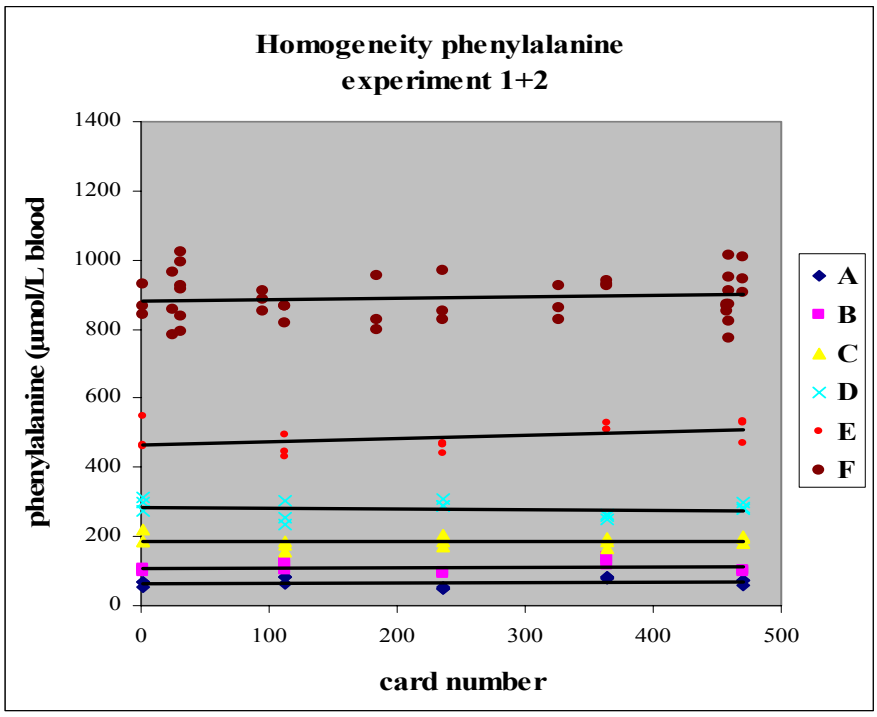

Figure 3. Homogeneity for phenylalanine.

17OHP

The batch was homogenous for 17OHP (p=0.86). See figure 4.

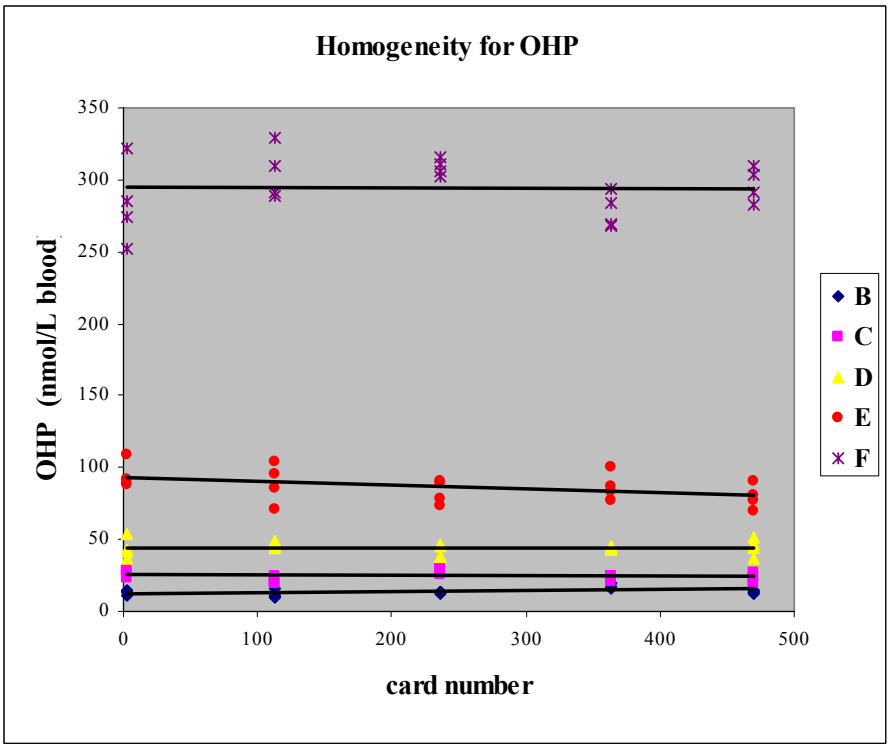

Figure 4. Homogeneity for 17OHP.

Table 5a. Homogeneity for TSH for card numbers 2, 113, 236, 364 and 470 of the batch on filter paper S&S #903. Each concentration is the mean of a quadruplicate.

mIU/L blood						
spot	**#2**	**#113**	**#236**	**#364**	**#470**	**mean**
A	<1	<1	<1	<1	<1	**<1**
B	6.0	5.8	6.0	6.0	6.0	**5.9**
C	14.3	14.5	16.3	14.0	14.0	**14.7**
D	29.5	28.8	29.3	27.5	25.3	**27.9**
E	48.8	51.0	52.5	50.5	45.5	**49.6**
F	135.3	129.8	132.5	123.0	117.0	**126.9**

% relative to 'mean'					
spot	**#2**	**#113**	**#236**	**#364**	**#470**
A	---	---	---	---	---
B	102	97	102	102	102
C	97	99	111	96	96
D	106	103	105	99	91
E	98	103	106	102	92
F	107	102	104	97	92
mean	**102**	**101**	**106**	**99**	**94**

Table 5b. Homogeneity for phenylalanine for cardnumbers 4, 124, 224, 366 and 458 of the batch on filter paper S&S #903. Each concentration is the mean of a quadruplicate.

µmol/L blood						
spot	**#4**	**#124**	**#224**	**#366**	**#458**	**mean**
A	62	75	50	80	67	**67**
B	105	118	96	132	103	**111**
C	196	173	187	182	192	**186**
D	296	265	295	260	288	**281**
E	491	456	458	517	511	**487**
F	880	850	883	933	954	**900**

% relative to 'mean'					
spot	**#4**	**#124**	**#224**	**#366**	**#458**
A	93	112	74	119	101
B	95	106	86	119	93
C	105	93	100	98	103
D	105	94	105	92	103
E	101	94	94	106	105
F	98	94	98	104	106
mean	**100**	**99**	**93**	**106**	**102**

Table 5c. Homogeneity for 17OHP for card numbers 2, 113, 236, 364 and 470 of the batch on filter paper S&S #903. Each concentration is the mean of a quadruplicate.

nmol/L blood						
spot	**#2**	**#113**	**#236**	**#364**	**#470**	**mean**
A	<<	<<	<<	<<	<<	<<
B	13.1	11.5	13.3	18	13.6	**13.9**
C	26.9	21.4	28.5	23.8	23.5	**24.8**
D	43.8	46	42.1	43.8	44.9	**44.1**
E	94.4	89	83.3	87	79.6	**86.7**
F	283.3	305	308	279	297	**294.3**

% relative to 'mean'					
spot	**#2**	**#113**	**#236**	**#364**	**#470**
A	---	---	---	---	---
B	94	83	95	129	98
C	108	86	115	96	95
D	99	104	96	99	102
E	109	103	96	100	92
F	96	103	105	95	101
mean	**101**	**96**	**101**	**104**	**98**

3.6 Comparison filter papers

The ISNS-RPNS on the three filter papers S&S903, Whatman BFC180 and Toyo Roshi 545 were compared for each analyte by 3-5 laboratories. One of the laboratories also analysed the 100 µL spots on S&S#903. The number of replicates per calibrator and per analyte varied for the laboratories (laboratory 9, 14 and 20: duplicates; laboratory 4: quadruplicate; laboratory 12: four runs in duplicate). The mean concentration per laboratory, per calibrator and per filter paper is summarised in Table 6a-c for TSH, phenylalanine and 17OHP, respectively. These tables also show the measured concentration for Whatman BFC180, Toyo Roshi 545 and S&S-100µL, expressed as percentage recovery against the measured concentration for S&S #903. Data were statistically evaluated in a 'linear mixed effect model'(S-plus) (Dr. A.L.M. Dekkers, RIVM). Table 7 shows the potency (95% confidence limits and p-value) for the three analytes for Whatman BFC180 and Toyo Roshi 545 in terms of S&S #903. The differences between the filterpapers were very small; nevertheless there was a significant difference ($p<0.05$) between Whatman BFC180 and S&S #903 for phenylalanine, between Toyo Roshi 545 and S&S #903 for TSH and 17OHP and between 50µL and 100 µL spots on S&S #903 for phenylalanine.

Tabel 7. The potency (95%-range; p-value) per analyte in terms of S&S #903 for Whatman BFC180, Toyo Roshi 545 and S&S #903-100µL spots.

	Whatman BFC180	Toyo Roshi 545	S&S 100µL
TSH	1.02 (0.99-1.05; 0.12)	1.04 (1.00-1.07; 0.03)	0.97 (0.93-1.02; 0.27)
Phenylalanine	1.04 (1.02-1.06; <0.01)	0.99 (0.97-1.01; 0.18)	1.05 (1.02-1.08;<0.01)
17OHP	0.98 (0.94-1.03; 0.47)	1.09 (1.04-1.14;<0.01)	1.00 (0.93-1.08; 0.68)

Table 6a. Mean TSH concentration (mIU/L blood) per spot and per filter paper as measured by 3 laboratories using 3 different methods. Lab 12 also compared 50 µL and 100 µL spots on S&S #903.For Whatman BFC180, Toyo Roshi 545 and S&S-100µL the measured concentration was also expressed as a percentage of the measured concentration in S&S #903 (50 µL spots).

			mIU/L observed						% in terms of S&S #903						
lab	**method**	**filter paper**	**A**	**B**	**C**	**D**	**E**	**F**	**A**	**B**	**C**	**D**	**E**	**F**	**mean B-F**
4	AutoDelfia	S&S#903	<<	5.9	14.7	27.9	49.6	127							
		Whatman	<<	6.8	14.5	29.5	52.0	135	---	114.4	99.0	105.9	104.9	106.4	**106.1**
		Toyo	<<	6.0	14.3	27.5	50.8	138	---	101.7	97.3	98.7	102.4	109.0	**101.8**
14	Enzaplate	S&S#903	0.8	7.8	13.8	26.1	46.2	>100							
		Whatman	0.4	7.0	15.1	29.7	49.8	>100	---	89.9	109.3	113.7	107.8	---	**105.2**
		Toyo	0.5	7.7	14.8	27.5	52.7	>100	---	99.0	106.9	105.4	114.0	---	**106.3**
12	Delfia	S&S#903	0.4	6.2	12.2	26.3	54.4	123							
		Whatman	0.4	6.4	12.7	26.0	52.5	123	---	102.9	104.5	99.0	96.5	99.7	**100.5**
		Toyo	1.2	6.7	13.3	28.1	52.6	125	---	108.9	109.5	107.2	96.7	101.4	**104.7**
		S&S100µL	0.3	5.8	12.3	24.3	50.5	114	---	93.8	101.2	92.6	92.9	92.5	**94.6**
20	AutoDelfia	S&S#903	0.4	6.2	12.2	26.3	54.4	123							
		Whatman	0.4	6.4	12.7	26.0	52.5	123	---	102.9	104.5	99.0	96.5	99.7	**100.5**

Table 6b. Mean phenylalanine concentration (µmol/L blood) per spot and per filter paper as measured by 4 laboratories (lab 14 used two different methods). Lab 12 also compared 50 µL and 100 µL spots on S&S #903. For Whatman BFC180, Toyo Roshi 545 and S&S-100 µL spots the measured concentration was also expressed as a percentage of the measured concentration in S&S #903 (50 µL spots).

			µmol/L observed						% in terms of S&S #903						
lab	**method**	**filter paper**	**A**	**B**	**C**	**D**	**E**	**F**	**A**	**B**	**C**	**D**	**E**	**F**	**mean A-F**
4	Quantase	S&S#903	81.7	128	166	264	446	873							
		Whatman	79.0	132	165	264	459	808	96.7	103.4	99.2	100.1	102.9	92.6	**99.2**
		Toyo	83.3	132	170	262	463	808	102.0	103.4	102.4	99.5	103.9	92.6	**100.6**
14	HPLC	S&S#903	63.6	113	151	261	421	825							
		Whatman	65.1	115	151	246	429	737	102.4	101.6	100.4	94.3	101.9	89.4	**98.3**
		Toyo	65.7	104	156	236	416	765	103.3	92.2	103.5	90.3	98.6	92.7	**96.8**
14	Enzaplate	S&S#903	65.1	118	165	264	482	894							
		Whatman	66.6	127	167	286	481	929	102.3	107.2	101.1	108.6	99.8	103.9	**103.8**
		Toyo	71.7	116	165	257	508	810	110.1	98.0	99.8	97.4	105.4	90.6	**100.2**
12	MS/MS	S&S#903	64.3	108	149	240	434	820							
		Whatman	68.4	115	160	253	473	890	106.4	106.4	107.5	105.5	109.0	108.5	**107.2**
		Toyo	65.0	105	154	235	409	782	101.1	97.1	103.6	97.7	94.3	95.3	**98.2**
		S&S100µL	65.8	113	164	253	443	855	102.4	105.0	110.1	105.5	102.0	104.3	**104.9**
20	MS/MS	S&S#903	62.0	111	162	240	456	756							
		Whatman	76.3	113	161	271	434	804	123.0	102.3	99.0	112.5	95.2	106.4	**106.4**

Table 6c. Mean 17OHP concentration (nmol/L blood) per spot and per filter paper as measured by 3 laboratories using 3 different methods. Lab 12 also compared 50 µL and 100 µL spots on S&S #903. For Whatman BFC180, Toyo Roshi 545 and S&S-100 µL the measured concentration was also expressed as a percentage of the measured concentration in S&S #903 (50 µL spots).

			nmol/L observed						recovery (%) in terms of S&S #903						
lab	**method**	**filter paper**	**A**	**B**	**C**	**D**	**E**	**F**	**A**	**B**	**C**	**D**	**E**	**F**	**mean B-F**
4	AutoDelfia	S&S#903	3.9	14.0	27.4	47.8	100.0	340							
		Whatman	3.4	15.0	25.8	52.1	105.0	370	---	107.1	94.1	109.2	105.0	108.7	**104.8**
		Toyo	3.8	14.6	30.3	55.9	117.6	376	---	104.5	110.5	117.0	117.6	110.5	**112.0**
14	Enzaplate	S&S#903	3.4	14.4	25.1	40.4	86.3	>120							
		Whatman	3.9	14.3	22.3	41.1	77.8	>120	---	99.2	88.8	101.7	90.1	---	**94.9**
		Toyo	4.1	14.3	25.5	43.7	83.8	>120	---	99.4	101.4	108.1	97.1	---	**101.5**
12	Delfia	S&S#903	0.8	9.3	19.7	40.0	82.9	250							
		Whatman	1.0	8.7	19.4	41.6	73.5	237	---	93.0	98.3	104.1	88.7	94.7	**95.8**
		Toyo	5.8	22.5	22.8	41.3	79.3	242	---	241.0	115.5	103.4	95.6	96.5	**102.8**[1]
		S&S100µL	0.4	10.6	19.2	35.0	84.9	277	---	113.8	97.1	87.6	102.4	110.6	**102.3**
20	AutoDelfia	S&S#903	2.4	11.0	21.5	51.1	97.0	275							
		Whatman	1.3	11.2	23.5	53.7	106.1	298	---	101.4	109.6	105.2	109.4	108.4	**106.8**

[1] without calibrator B

3.7 Comparison ISNS-RPNS for TSH with CRM-TSH (1999)

On request of the ISNS a certified reference material for TSH (CRM-TSH) had been prepared in 1999 by the CDC on different filter papers and 50µL and 100µL 'spot size'. ISNS-RPNS on S&S #903 was compared with CRM-TSH on S&S #903 (50 µL 'spot size') using the AutoDelfia Neonatal TSH method. Table 8 gives the results for CRM-TSH in terms of the ISNS-RPNS. The formula for the linear regression line for calibrator B-F was: y = 0.96x -0.73; r = 0.999 (x= ISNS-RPNS; y=CRM-TSH). The mean ± SD recovery for CRM-TSH was 93 ± 4.5%. Figure 5 shows both reference preparations as read on the calibrators of the kit.

Table 8. CRM-TSH (mIU/L blood; %recovery) in terms of ISNS-RPNS.

	expected	observed			
	mIU/L blood	mIU/L blood		recovery (%)	
		x1	*x2*	*x1*	*x2*
A	0	<1	<1	---	---
B	7.5	7	7	93	93
C	15	12	14	80	93
D	25	24	25	96	100
E	50	45	46	90	92
F	75	71	74	95	99
				mean: 93 ± 4.5%	

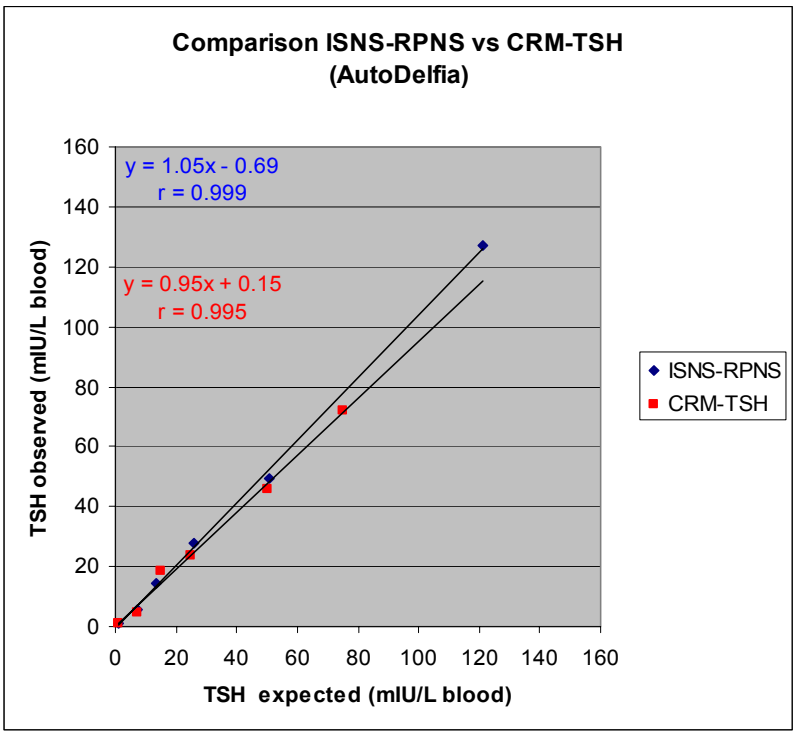

Figure 5. ISNS-RPNS and CRM-TSH in AutoDelfia Neonatal TSH assay.

3.8 Comparison ISNS-RPNS for phenylalanine with EWS-PHE-03 and AARM

On request of the European branch of the ISNS the European Working Standard for phenylalanine in blood spots had been prepared by RIVM. The first batch was produced in 1996 [3] and the latest batch EWS-PHE-03 was produced in 2003. CDC prepared in 1996 the 'Amino Acids Reference Materials' (AARM), a reference material for, amongst others, phenylalanine [4]. The EWS-PHE-03 and AARM were compared with the ISNS-RPNS in an enzymatic/colorimetric method in triplicate (Quantase; laboratory 4). Figure 6 shows the mean absorbance (570/690 nm) for the three preparations. The regression lines for the three preparations nearly coincided. Table 9 gives the mean concentration and recovery in terms of the ISNS-RPNS. The mean recovery for EWS-PHE-03 and AARM against ISNS-RPNS was 111 ± 8% and 108 ± 6% respectively.

Table 9. Phenylalanine (µmol/L; %recovery) for EWS-PHE-03 and AARM in Quantase with ISNS_RPNS as calibrators.

	EWS-PHE-03			AARM		
	expected	*observed*	*recovery (%)*	*expected*	*observed*	*recovery (%)*
A	50	61	123	73	71	98
B	170	192	113	315	333	106
C	290	326	112	557	630	113
D	530	558	105	799	852	107
E	770	793	103	1041	1172	113
F				1283	1430	111
Mean			111 ± 8%			108 ± 6%

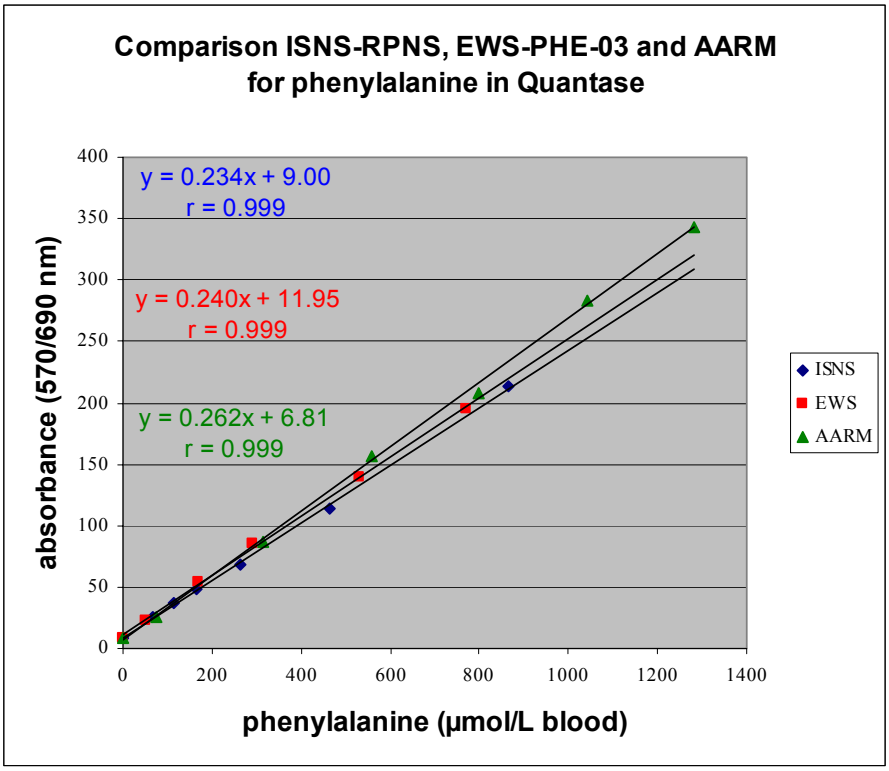

Figure 6. ISNS-RPNS, EWS-PHE-03 and AARM in Quantase for phenylalanine.

The ISNS-RPNS and EWS-PHE-03 were also analysed in one run in triplicate by MS/MS by two laboratories (laboratory 10 and 21). The mean concentration and recovery are

summarised in Table 10. Figure 7 shows the regression lines for both preparations and both laboratories. Although the recovery for laboratory 10 is rather high for both preparations, it can be concluded that for both laboratories ISNS-RPNS and EWS-PHE-03 do not differ significantly.

Table 10. Phenylalanine (µmol/L; %recovery) in ISNS-RPNS and EWS-PHE-03, analysed with MS/MS in laboratory 10 and 21.

calibrator	expected (µmol/L)	observed (µmol/L)		recovery (%)	
		lab-10	lab-21	lab-10	lab-21
ISNS-A	65.2	82.3	78.0	126	120
-B	115	142	112	123	98
-C	165	201	181	122	110
-D	265	302	268	114	101
-E	465	577	498	124	107
-F	865	1027	839	119	97
			mean	121	105
			SD	4.4	8.7
EWS-A	50	75.7	51.7	151	103
-B	170	185	170	109	100
-C	290	356	292	123	101
-D	530	726	488	137	92
-E	770	993	712	129	93
			mean	130	98
			SD	16	5

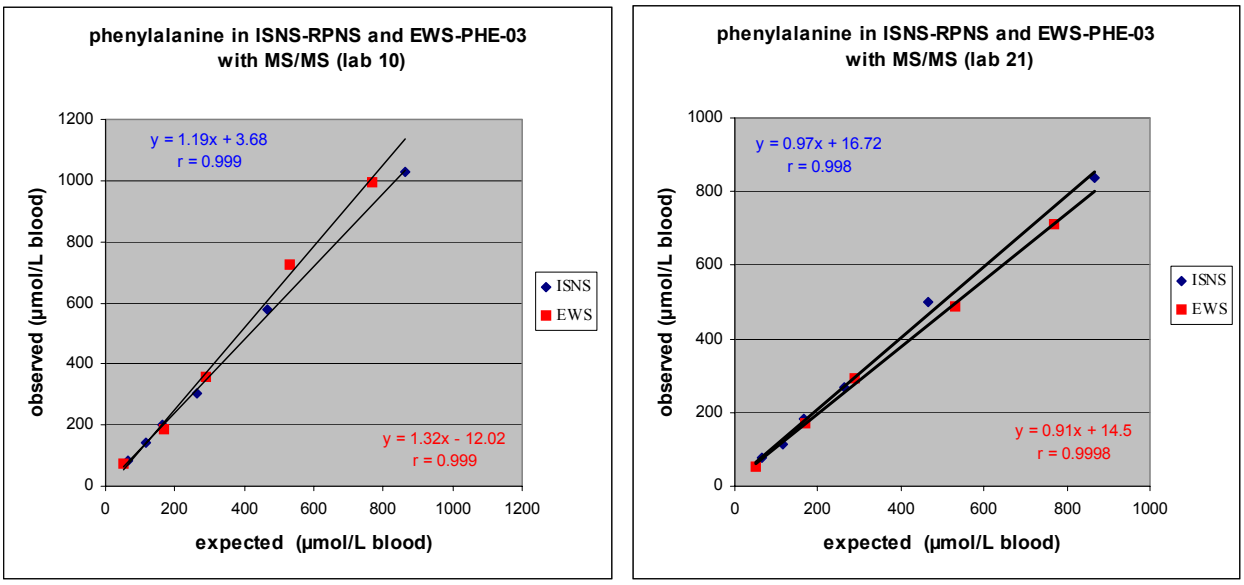

Figure 7. Phenylalanine in ISNS-RPNS and EWS-PHE-03 analysed in laboratory 10 and 21 with MS/MS.

3.9 Stability study

A stability study is started with extra blood spots from calibrator D on filter paper cards S&S #903. These cards will be stored at -80, -20, +4, +20, and +37 ^{o}C for 0, 1, 2, 4, 8, 12, 18 and 24 months. At the end of this period all cards will be analysed in quadruplicate for the three analytes. The expiry date for ISNS-RPNS is stated on December 2007, i.e. 3 years after the preparation. This period was based on experience with EWS-PHE-01 [3] and CRM-TSH (results not published).

4. Discussion

In planning the production of the 1st ISNS-RPNS decisions had to be made about amongst others: types of filter paper to be used, the number and layout of the cards to be produced, matrix, spot size, analytes to be included in this combined preparation, range of concentrations for the diverse analytes and finally how to assess the concentrations for the calibrators both in basal and spiked blood.

Filter paper

Until the end of 1999 Schleicher & Schuell marketed two types of filter paper for neonatal screening: #2992 mainly used in Europe and #903 used in Australia, Canada and USA. The most important difference between both papers was the absorption capacity per unit area: #903 absorbed 20% more serum then #2992. In October 1999, Schleicher & Schuell decided only to promote #903 for neonatal screening. To date, most European countries switched to #903. Besides this filter paper, Whatman BFC180 is used in a few countries just like Toyo Roshi type 545, the last one being used only in Japan. It was therefore decided to prepare the majority of the cards with the 1st ISNS-RPNS on Schleicher & Schuell #903 and a limited number on Whatman and Toyo Roshi.

Number of cards

Since 1996 three reference materials for neonatal screening in blood spots had been prepared: i) the European Working Standard for Phenylalanine in blood spots (EWS-PHE-01 in 1996; EWS-PHE-02 in 1999; EWS-PHE-03 in 2003; prepared by RIVM), ii) Amino Acid Reference Material (AARM in 1996; prepared by CDC) and the ISNS Certified Reference Material for TSH (CRM-TSH in 1999; prepared by CDC). The number of cards of the 1st ISNS-RPNS to be prepared was based on an inventory of shipments of CRM-TSH and EWS-PHE-03 (see Appendix 3). Furthermore we decided that the number of cards had to be prepared with blood from only one donor. This limited the maximum numbers of cards. Finally, the following numbers of filter paper cards, each with two series of calibrators, were prepared: S&S #903: N=480; Whatman BFC180: N=42; Toyo Roshi: N=69.

Spot size

During the collection of the blood on a filter paper card there is piling of blood cells in the centre of the blood spot and migration of serum to the outer side of the spot. Due to this 'chromatography effect' a punch taken from the centre of the spot contains less serum and thus analyte, as compared to a punch taken from the outer side of the spot. This difference increases with an increasing size of the blood spot and is more pronounced for analytes which are mainly in the serum-compartment of the blood. It is therefore important that calibrators have the same spot size as routine neonatal samples. On average the spot size for neonatal samples on S&S #903 is 11-12 mm, which corresponds with 45-50 µL. For this reason we decided to use 50 µL for the 1st ISNS-RPNS

Matrix

The matrix of blood spot calibrators preferably resembles the matrix of neonatal samples as much as possible. This is particularly important for a 'reference material' which is intended for use in all types of assays (ELISA, IRMA, colorimetric assays, fluorimetric assays, MS/MS etc). We therefore decided to use fresh heparinised human blood from one healthy donor and to manipulate it as few as possible. That meant that besides the addition of analytes the only manipulation would be the adjustment to 50% hematocrit, if necessary. It is well known that EDTA and citrate blood might affect some assays often used for neonatal screening. We therefore decided to use heparin as anti-coagulant.

Analytes
In most so called developed countries of the world neonates are screened for phenylketonuria (PKU) and congenital hypothyroidism (CH) and often also for congenital adrenal hyperplasia (CAH). The analytes of interest for these diseases are phenylalanine for PKU and 17α-hydroxyprogesterone (17OHP) for CAH. For CH however the situation is more complicated. Most countries use thyrotropin (TSH) as the primary analyte of interest. In some states of the USA and in The Netherlands the screening is primarily based on the measurement of thyroxin (T4). From this point of view it would be practical to include T4 in the combined preparation. Inclusion of T4 would have meant however considerable manipulation of blood to remove the endogenous T4 (treatment of plasma with charcoal or resin) and it is likely that this would have had also implications for the other analytes. Another analyte of interest is immunoreactive trypsinogen (IRT) which is used for neonatal screening for cystic fibrosis (CF). Although there is a rising interest in screening on CF and therefore for a 'reference material', we decided not to include IRT in the 1st ISNS-RPNS, because at this moment, we had insufficient experience with IRT to be sure to make a reliable reference material. So, we finally decided to produce a combined reference material for TSH, phenylalanine and 17OHP.

Range of concentrations
The intended concentration range for the three analytes was assessed after consultation of experts in the field of neonatal screening and manufacturers of kits for neonatal screening. Furthermore an inventory of cut off values by CDC [5] was taken into account. We decided to use SI-units, for example for phenylalanine 'µmol/L blood' instead of 'mg/dL blood'.

Assignment of the concentration of the calibrators
The concentration of the analytes in plasma, prepared from basal and spiked blood, was measured by three laboratories (see table 1a-c). Linear regression lines were calculated between the amounts of analyte added and the amounts of analyte observed. The linearity appeared to be excellent, the coefficient of correlation being ≥ 0.999 for nearly all laboratories. The basal concentration in plasma A was calculated as the mean measured concentration and also as the intercept of the regression lines for the plasma A-F. For TSH and phenylalanine there was a close agreement between the 'measured' and the 'calculated' concentration of the basal plasma. For 17OHP the regression line could be calculated for only two laboratories and the intercept for both lines differed too much to combine them. The observed differences for the measured concentration in basal plasma between the laboratories were very well within limits which might be expected for the type of methods used. It was therefore decided to base the assignment of the concentration of the calibrators on the measured basal concentration in unspiked blood and the amounts of the analytes added to the spiked blood.

Comparison with other 'reference' materials
For 17OHP no reference material is available. With respect to phenylalanine the 1st ISNS-RPNS was compared with i) the European Working Standard for phenylalanine in blood spots (EWS-PHE-03) in three laboratories and ii) the Amino Acid Reference Material (AARM) in one laboratory. The difference between the preparations within one laboratory was always <10%. For TSH the 1st ISNS-RPNS was compared with the 'Certified Reference Material for TSH' (CRM-TSH). The mean recovery for CRM-TSH in terms of the 1st ISNS-RPNS was 93%. This relative low recovery is no problem taken into account that the CRM-TSH was prepared in 1999 and that 10% difference between two batches of blood spot calibrators is quite normal.
It was concluded that the observed differences between these reference materials are quite acceptable.

Homogeneity of the batch
Two experiments were carried out to check whether the batch on filter paper S&S #903 was homogeneous. From the statistical analysis of the data from the first experiment it was concluded that the batch was homogeneous for 17OHP and also for calibrators A – E for TSH and phenylalanine. For calibrator F a slight decrease was observed for TSH and a slight increase for phenylalanine. From the results of the second experiment, which was focused on calibrator F only, it could be concluded that the batch was homogeneous for phenylalanine and this was also the case for calibrator A – F when the results of both experiments were combined. For TSH, again a slight but significant decrease was observed, although the batch was homogeneous if the last cards were omitted from the evaluation. We have no explanation for this discrepancy. The portions blood for calibrator A – F were treated all identical, while the time between start and end of the actual spotting of calibrators A – F was also equal. There is no reason why calibrator F would behave different from the other calibrators. Furthermore, a decrease was found for TSH, an increase for phenylalanine and no difference for 17OHP. The variation between replicates was larger for calibrator F in comparison with the other calibrators. This is probably due to the fact that the concentration of calibrator F is at or below the highest calibrator of the methods used. It was therefore decided to disregard the small changes in calibrator F and to consider the batch to be homogeneous.

Comparison filter papers
The results from the comparison of the three filter papers show that the differences between the filter papers are small. With the exception of 17OHP on Toyo Roshi (1.09) all other potencies in terms of S&S #903 were between 0.95 – 1.05. Differences of this magnitude can also been found for different batches of filter paper from the same manufacturer.

The1st ISNS-RPNS in routine neonatal screening
The calibrators of the 1st ISNS-RPNS were analysed as 'routine neonatal samples' in 22 neonatal screening laboratories using different methods. The overall recovery (mean ± SD) for the three analytes was 96 ± 15%, 100 ± 10% and 92 ± 11% for TSH, phenylalanine and 17OHP respectively. For most laboratories the overall recovery is between 85 – 115%. The recovery for calibrator A - F was for most laboratories fairly constant, which involves parallelism between kit calibrators and the ISNS-RPNS.

In conclusion
The 1st ISNS-RPNS for TSH, phenylalanine and 17OHP is suitable as formal reference preparation and serves as a source for (re)calibrating kit calibrators. It is available to manufacturers for a small handling charge and to EQAS organizers for free.

Acknowledgements

We thank Schleicher & Schuell, Whatman and Toyo Roshi for making available the filter paper cards. We thank Guido Diependaal, Idder Belmouden and Mark Jonker (RIVM; LIS) for their assistance by the preparation of the 1st ISNS-RPNS and Dr. A.L.M. Dekkers (RIVM; IMA) for the statistical analyses. We thank the following laboratories for the assessment of TSH, phenylalanine and 17OHP in basal and enriched plasma: Dr. S. Ewing (Hôpital d'Enfants, Laboratoire de Biochimie Spécialisée, Dyon, France); Dr D. Rabier (Hôpital Necker-Enfants maladies, Department Laboratoire de Biochemie B, Paris, France); Prof. P. Bourdoux (Hôpital Universitaire des Enfants, ULB-Laboratoire de Pédiatrie, Brussels, Belgium; Dr. E. Lentjes (UMC-Utrecht, Laboratory for Endocrinology, Utrecht, The Netherlands; Ing. M.F.G. Segers (UMC-St Radboud, Laboratoy for Experimental Endocrinology, Nijmegen, The Netherlands); Dr. E. Endert (AMC, Laboratory for Endocrinology, Amsterdam, The Netherlands).

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Appendix 1

Detailed description of the preparation of the 1st ISNS-RPNS

Note: use of trade names is for identification only and does not imply endorsement by the National Institute for Public Health and the Environment (RIVM) or the International Society for Neonatal Screening.

Materials and methods

1. Materials

1.1 Filter paper cards, dimension 7.5 x 12.5 cm, were obtained free of charge from a) Schleicher & Schuell, type #903, lot W031, 600 cards, b) Whatman, type BFC180, lot 145475; 40 cards and c) Toyo Roshi Kaisha Ltd, type 545, lot 40921511, 100 cards

1.2 Thyroid stimulating hormone, proposed 3rd IRP for TSH, code 81/565; 11.5 mIU/ampoule; NIBSC, London

1.3 L-phenylalanine, M=165.2, product no 78019, purity ≥ 99%, lot 438863/2 11104103; Fluka, Switzerland

1.4 17α-hydroxyprogesterone, M=330.5, product no 56240, lot 2006994; Fluka, Switzerland

1.5 Saline (sterile), product no p250.65.0100, lot 20040200631, exp date 2006-02-25; Tritium Microbiologie BV, Veldhoven, The Netherlands

1.6 Ethanol abs., product no 1.00983, lot K31218583; Merck, Darmstadt, Germany

1.7 Blood pack unit without anticoagulant, code R0002; Baxter

1.8 Sodium Heparine, 5000 IU/mL, Leo BV, Weesp, The Netherlands

1.9 5-Sulfosalicylic acid, product no 5021; VEL BV, The Netherlands

1.10 Minipax sorbent (2 gram); Multisorb Technologies, Buffalo, New York

1.11 Lamigrip, aluminium bags, 120x180 mm, code 352, Minigrip BV, the Netherlands

1.12 QC-materials: TSH lot 411-413; 17OHP lot 351-353; phenylalanine lot 421-424 and AminoAcidsReferenceMaterial (AARM); CDC, Atlanta, USA

2. Stock solutions

Before use, the analytical balance was calibrated with a certified weight (Mettler; 200 mg) On 27-10-2004 the following stock solutions were prepared:

2.1 TSH 81/565; 10 mIU/mL

Needed for enrichment: 0.08*(6.25+12.5+50+120)+0.09*25 = 17.35 mIU. The contents of 2 ampoules was reconstituted in 1.15 mL saline per ampoule. The contents of both ampoules was combined → 10 mIU/mL **code: TSH-0**

2.2 Phenylalanine; 0.10 mol/L

1.652 gram phenylalanine was weighed on an analytical balance and dissolved by warming ~40 °C in a volumetric flask in 100.0 mL saline → 0.10 mol/L **code: PHE-0**

2.3 OHP; 33.33 nmol/mL
330.5 mg 17OHP was weighed on an analytical balance and dissolved in 100 mL ethanol in a volumetric flask → 10 mmol/L **code OHP-0**
0.333 mL 17OHP-0 + 99.67 mL saline → 33.33 nmol/mL **code OHP-1**

2.4 5-sulfosalicylic acid, 5%
2 gram 5-sulfosalicylicacid was dissolved in 20 mL distilled water

3. Methods

3.1 Preparation of the filter paper cards

Figure 1 shows the intended layout for filter paper card for S&S #903); dimensions filter paper card 12.5 x 7.5 cm; 2 series of calibrators per card; spot size: 50 µL. A label (Avery 5261; 10.16x 2.54 cm) was attached to the filter paper card, showing type and lot of filter paper. All cards were numbered uniquely on the back.

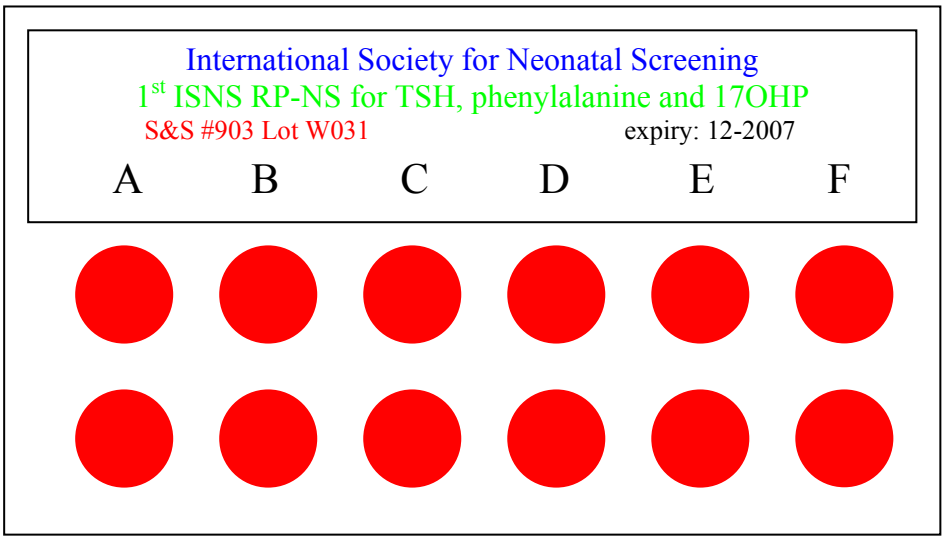

Figure 1. Proposed layout 1st ISNS RP-NS.

3.2 Basal blood (49.5-50.5%HT) code: B-0

On 28-10-2004 1.5 mL LEO heparine (5000 IU/mL) was injected via the middle inlet of a blood pack unit; 540 mL blood from an healthy male volunteer (JGL) was collected. Hematocrit was determined in duplicate by the microcentrifugal method (Standard Operation Procedure TOX/286, Laboratory for Toxicology, Pathology and Genetics, RIVM). %HT was 49.5% and needed no further adjustment. The blood was tested and found to be negative for hepatitis B surface antigen, anti-hepatitis C and anti-HIV 1 and 2 antibodies.

3.3 Enrichment of blood with TSH, 17OHP and phenylalanine.

100 mL blood was weighed in a volumetric flask: 105.21 gram. Based on this, six portions blood B-0 were prepared in glass bottles by weighing: **code: A – F**

calibrator A, B, C, E and F : 84.17 gram = 80 mL
calibrator D : 94.69 gram = 90 mL (extra blood for stability study)
The portions blood were mildly centrifuged for 10 minutes, 1400 rpm (800 g) at room temperature (Beckman centrifuge). Plasma was removed and saline, TSH, 17OHP and phenylalanine were added, using calibrated pipetting devices, according to table 1.

Table 1. Preparation of calibrators A-F.

Cal.	Blood	Remove serum	TSH-0 (10 mIU/mL)	Phe-0 (0.10 mmol/mL)	OHP-1 (33.33 nmol/mL)	Saline
	mL	µL	µL	µL	µL	µL
A	80	2320	0	0	0	2320
B	80	2320	50	40	30	2200
C	80	2320	100	80	60	2080
D *	90	2610	225	180	135	2070
E	80	2320	400	320	240	1360
F	80	2320	960	640	720	0

* extra blood D for stability study

The following Eppendorf pipets were used:
25-100 µL pipette # 2501 for: 30µL OHP-1 and 40 µL Phe-0
50-200 µL pipette #1912 for: 50, 100, 225, 400 µL TSH-0; 80, 180, 320 µL Phe-0; 60, 135, 240 µL OHP-1
100-1000 µL pipette #1930 for: 960 µL TSH-0; 640 µL Phe-0; 720 µL 17OHP
500-2500 µL pipette #2362 for: all volumes >1000 µL

3.4 Basal/enriched plasma for analysis of TSH, 17OHP and phenylalanine

The enriched blood was homogenised on a roller bank for 5 hours at room temperature.
For each calibrator 16 mL blood was centrifuged for 10 minutes, 2400 rpm (1200 g) at room temperature. Plasma was divided in 10 portions of 600 µL for determination of TSH, 17OHP and phenylalanine in plasma and stored until shipment at -20 ^{o}C.

3.5 5-Sulfosalicylic acid extract for analysis of phenylalanine

A 5% (w/v) 5-sulfosalicylicacid solution (5-SSA) was prepared by dissolving 2 gram 5-sulfosalicylic acid in 40 mL distilled water. Two 600 µl portions of basal and enriched plasma were treated with an equal volume of 5-SSA, and vortex-mixed for 10 seconds. After 2 minutes the tubes were centrifuged for 10 minutes and 1600 g at room temperature. The clear supernates were transferred to polypropylene tubes, frozen immediately and stored until shipment at -20 ^{o}C.

3.6 Preparation of blood spots

Five hours after the enrichment of the blood, blood spots were prepared using a Matrix Impact^2 pipette (Matrix Technologies Corp; Hudson, NH, USA). Two 50 µL blood spots (~ 12 mm spot size) per calibrator were pipeted on the filter paper cards. On 14 cards two 100 µL blood spots were pipeted for comparison with an earlier prepared ISNS-TSH reference preparation. On 40 filter paper cards from S&S #903 five 50 µL spots from calibrator D were pipeted for a stability study. The total time needed for the preparation of the blood spot cards was 75 minutes. During the preparation of the cards the remainder of the blood was homogenised on a roller bank. The cards were prepared in the following ranking: Whatman 1-42, S&S #903 (50 µL) 1-100, S&S #903 (100 µL) 1-14, S&S #903 (50 µL) 101-400, Toyo Roshi 1- 69 and S&S #903 (50 µL) 401-480.

3.7 Storage of the filter paper cards

After overnight drying at room temperature in the dark the filter paper cards were sealed in PE-laminated aluminium sachets with dessicant. For S&S #903 the batch was divided in 10 series, coded 001 – 010 (series 001: cards 001-048, series 002: cards 049-096 etc.) A label was attached to each sachet showing the same information as was on the label on the filter paper card and for S&S #903 also the series number.

3.8 Effect of storage conditions on TSH, phenylalanine and 17OHP

To study the effect of storage conditions 36 filter paper cards were prepared on S&S #903, each with five 50 µL spots of calibrator D. After overnight drying the cards were sealed in aluminium sachets with dessicant and a humidity indicator (MS20003-2). Cards will be stored at different temperatures: -80 ^{o}C, -20 ^{o}C, +4 ^{o}C, +22 ^{o}C and +37 ^{o}C for 1, 2, 4, 8, 12, 18 and 24 months. Determination of TSH, phenylalanine and 17OHP in quadruplicate is planned after one year and after two years.

3.9 Analysis in basal and enriched plasma

Basal end enriched plasma was sent by ordinary mail to three laboratories in The Netherlands for analysis of TSH and 17OHP. Plasma and/or 5SS-extracts were sent on dry-ice to three laboratories in France and Belgium for analysis of phenylalanine.

Appendix 2

Individual results in plasma A-F per laboratory for TSH, phenylalanine and 17OHP.

TSH (mIU/L plasma)

laboratory	**laboratory A**			**laboratory B**			**laboratory C**			**mean**
method	Architect; Abbott			Delfia; Perkin Elmer			Centaur; Bayer			
plasma	*x1*	*x2*	*x3*	*x1*	*x2*	*x3*	*x1*	*x2*	*x3*	
A	2.3	2.3	---	1.9	1.9	1.8	2.0	2.1	---	2.03
B	14.9	14.6	---	12.1	12.6	12.2	13.7	---	---	13.3
C	28.9	28.4	---	[17.0]	24.8	[17.5]	27.0	26.9	---	27.2
D	56.1	54.7	---	52.0	50.0	52.0	53.1	55.2	---	53.3
E	112.3	114.6	112.0	100.0	105.0	102.0	105.0	102.9	---	106.7
F	255.7	252.3	252.4	225.0	233.0	235.0	236.0	241.0	---	241.3

Phenylalanine (µmol/L plasma) in plasma and 5%-Sulfosalicylicacid extract.

laboratory	**laboratory A**			**laboratory B**			**laboratory C**			**median**[1]
method	aminoacid analyses			aminoacid analyses			MS/MS			
plasma	*x1*	*x2*	*x3*	*x1*	*x2*	*x3*	*x1*	*x2*	*x3*	
A	64	66	64	65	66	62	82	69	74	65
B	119	123	113	123	124	123	120	122	117	122
C	168	177	180	185	187	182	171	189	153	174
D	273	278	280	297	298	298	240	433	318	297
E	492	510	514	531	524	508	593	517	513	521
F	997	954	920	1057	1044	923	1082	994	1004	1005
5% SS										
A				65	66	66				
B				124	126	126				
C				170	167	184				
D				293	296	298				
E				520	522	520				
F				1016	1006	983				

[1] median values for plasma and 5%-SS extract

OHP (nmol/L plasma)

laboratory	**laboratory A**			**laboratory B**			**laboratory C**			**mean**
method	RIA; DPC			in house RIA + extraction			paper chromatography + RIA			
plasma	*x1*	*x2*	*x3*	*x1*	*x2*	*x3*	*x1*	*x2*	*x3*	
A	5.1	5.1	4.8	4.5	3.8	4.1	3.6	3.8	3.9	4.3
B	24.4	29.4	30.0	21.0	24.0	23.0	---	---	---	25.3
C	48.0	50.0	49.0	45.0	45.0	47.0	---	---	---	47.3
D	106.0	93.0	104.0	103.0	104.0	---	---	---	---	102.3
E	163.0	175.0	173.0	>	>	---	---	---	---	170.3
F	406.0	516.0	522.0	>	>	---	---	---	---	481.3

Appendix 3

Distribution of CRM-TSH and EWS-PHE-03

For the discussion about the number of cards to be prepared, an inventory was made of the distribution of CRM-TSH and EWS-PHE-03.

CRM-TSH

- Distributed by RIVM, Bilthoven, The Netherlands (L.H. Elvers/ Dr. J.G. Loeber)

The RIVM was in charge of the distribution of all 50 µL spots on S&S #2992 and S&S #903, as well as all Whatman BFC-spots. The number of shipments of sheets (15 spots per concentration) in the period 10-07-2001 untill 12-7-2004 (3 years) was as follows:

	S&S #2992 (50 µL)	S&S #903 (50 µL)	Whatman BFC180
number of sheets (15 spots/calibrator)	5	11	3
number of shipments	5	13	3
number of 'different' customers	5	9	3
series of calibrators (=15 * sheets)	75	165	45
last shipment date	16-10-2002	12-07-2004	16-10-2002

- Distributed by CDC, Atlanta, USA (Dr. W.H. Hannon):

CDC distributed about 25 sheets of the S&S 903 preparation /100 µL spotsize and a couple of sets of Whatman.

- Distributed by the Institute of Public Health, Sapporo City, (Masaru Fukushi):

CRM-TSH on filter paper Toyo Roshi was provided to 3 companies and 4 main screening laboratories in Japan, 90 sheets was sent in all. Last shipment date: Nov. 2003

EWS-PHE-03

The EWS-PHE-03 is distributed by RIVM, Bilthoven, The Netherlands
The EWS-PHE-03 was prepared on S&S #903 and Whatman; 3 series of calibrators/card. The number of shipments and cards distributed was as follows:

	S&S #903 (45 µL)	Whatman BFC180
number of cards (3 series calibrators/card)	90	6
number of shipments	17	2
number of 'different' customers	12	2
series of calibrators (=3 * cards)	270	18
last shipment date	13-07-2004	24-03-2003

Summary

Total number of sheets/series of calibrators for CRM-TSH and EWS-PHE-03

	CRM–TSH		**EWS-PHE-03**	
	sheets	**calibrators ***	**sheets**	**calibrators**
S&S #903	**36**	**540**	**90**	**270**
Whatman	**±6**	**90**	**6**	**18**
Toyo	**90**	**1350**	**---**	**---**

Remark: the need for 'series of calibrators' is probably lower; however the relative high number is caused by the fact that the 'unit' was '15 spots/sheet'