Appendix Q Methods of blood and urinary analysis, quality control (QC) and quality assessment (QA) in the National Diet and Nutrition Survey 2019 to 2023

Q.1 Introduction

This appendix details the analytical methods, internal quality control (QC) and external quality assessment (EQA) performance for each analyte measured as part of NDNS 2019 to 2023. The majority of assays were performed at the Nutritional Biomarker Laboratory (NBL), MRC Epidemiology Unit, University of Cambridge. Other assays were performed either at other laboratories on the Cambridge Biomedical Campus (Addenbrooke`s Laboratory Research Services (LRS) and Core Biochemical Assay Laboratory (CBAL)) or at specialist external laboratories (Trace Element Laboratory, University Hospitals Southampton, UK (UHS) and Centers for Disease Control and Prevention, Atlanta, Georgia, USA (CDC). Details are provided in table Q.1.

Table Q.1 Summary of analytes and methods of analysis during NDNS years 12 to 15

Analyte	Appendix section	Analytical method or platform	Responsible laboratory
Full blood count	Q.3.1	Siemens ADVIA 2120	LRS
C-reactive protein	Q.3.2	Siemens Dimension EXL200	CBAL
Ferritin	Q.3.3	Siemens Dimension EXL200	CBAL
Serum cholesterol (total, HDL, LDL)	Q.3.4	Siemens Dimension EXL200	CBAL
Triglycerides	Q.3.5	Siemens Dimension EXL200	CBAL
ETKAC (thiamin)	Q.3.6	Enzymatic method	NBL
EGRAC (riboflavin)	Q.3.7	Enzymatic method	NBL
Vitamin B6 (PLP and 4PA)	Q.3.8	HPLC	NBL
Total folate (serum)	Q.3.9	LC-MS/MS	NBL
Whole blood folate (red blood cell folate)	Q.3.10	Microbiological method	CDC
Vitamin B12	Q.3.11	Siemens ADVIA Centaur	LRS
Holotranscobalamin	Q.3.12	ELISA	NBL
Vitamin C	Q.3.13	Fluorescence method	NBL
Retinol (vitamin A), α- and γ-tocopherol (vitamin E) and carotenoids	Q.3.14	HPLC	NBL
25-hydroxyvitamin $D_{2/3}$	Q.3.15	LC-MS/MS	NBL
Selenium and zinc	Q.3.16	ICP-MS	UHS

Iodine	Q.3.17	ICP-MS	UHS

Abbreviations: EGRAC, Erythrocyte glutathione reductase activation coefficient; ELISA, enzyme-linked immunosorbent assay; ETKAC, Erythrocyte transketolase activation coefficient; HPLC, high performance liquid chromatography; ICP-MS, inductively coupled plasma mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; PA, pyridoxic acid; PLP, pyridoxal-5-phosphate.

The collection, transport and processing of blood and urine samples are described in appendices M and P. A complete list of analytes measured and the priority order of analyses are provided in the tables that accompany appendix P. There were no changes to the laboratory analytical methods from the previous NDNS report for years 9 to 11 (2016 to 2019) and the methods described in this appendix are identical to those described previously. As detailed elsewhere in the report, fieldwork years 12 to 15 were disrupted by the COVID-19 pandemic. Consequently, field blood collections and laboratory analysis were not performed continuously as during earlier years of NDNS. Year 12 blood samples were collected between October 2019 and March 2020 and analysed during 2020. Blood samples for years 13 to 15 were collected between November 2021 and June 2023 with overlap between fieldwork years, and samples were analysed between November 2021 and September 2023. Therefore, rather than reporting quality control data for separate NDNS fieldwork years as in earlier reports (where fieldwork years were distinct), data is instead presented for year 12 and for years 13 to 15 combined.

Q.2 Notes on interpretation of external quality assessment (EQA) schemes

Different schemes within the UK National External Quality Assessment Services (NEQAS) and other EQA schemes (described relevant to analyte in the respective sections) have varying approaches to reporting and presenting performance data. In this report, the results of the performance in EQA schemes are presented as the per cent bias and SD of per cent bias from the method mean or the all-laboratory trimmed mean (ALTM) calculated in-house or using the scheme-specific scores; the notes below provide a summary of information to interpret the scheme-specific scores.

EQA data points are missing for EQA schemes and EQA scores undertaken in 2020 (fieldwork year 12) due to the COVID-19 pandemic and closure of the NBL. In contrast, CBAL and LRS remained open and enrolled in EQA schemes throughout 'lock-down' periods and have EQA data available continuously from 2020 to 2023. Consequently, CBAL EQA data are available for and are presented by calendar year rather than NDNS fieldwork year(s). Details are provided under each analyte in the sections below.

Q.2.1 Performance scores for fat-soluble vitamins, vitamin D, vitamin B12, holoTC, serum folate and ferritin

NEQAS scores for these schemes are reported as A, B and C scores:

Accuracy (A) score has been transformed and uses a 'degree of difficulty' factor. A scores are broadly comparable across analytes and provide an overall performance accuracy score which assesses bias, consistency of bias and clinical acceptability of performance over approximately 6 months. A score of 100 indicates median performance for that assay.

Bias (B) score has been calculated as the mean bias over last 6 months (per cent). Bias is calculated as specimen per cent bias = [(result - target) ÷ target] × 100

Consistency (C) score of bias represented by the SD of bias over last 6 months (per cent)

Q.3 Methods

Details of the analysis method and the associated QC procedures for each analyte or set of analytes are provided in section Q.3.1 onwards. For all analytes, internal QC samples were run in every batch to assess assay imprecision. Accuracy was assessed by comparison with certified reference materials and target values from external QC materials. Laboratories participated in EQA schemes where such schemes were available. Analyses were performed at NBL unless otherwise stated.

Q.3.1 Full blood count including haemoglobin and haematocrit

Full blood count was measured at LRS using a Siemens Advia 2120, which uses the Coulter Principle to count red blood cells (RBC), mean cell volume (MCV), white blood cells (WBC) and platelet counts. Haemoglobin was measured by photometric measurement. Other parameters such as the mean cell haemoglobin (MCH), haematocrit and red cell distribution width (RDW) were calculated from the above measured parameters.

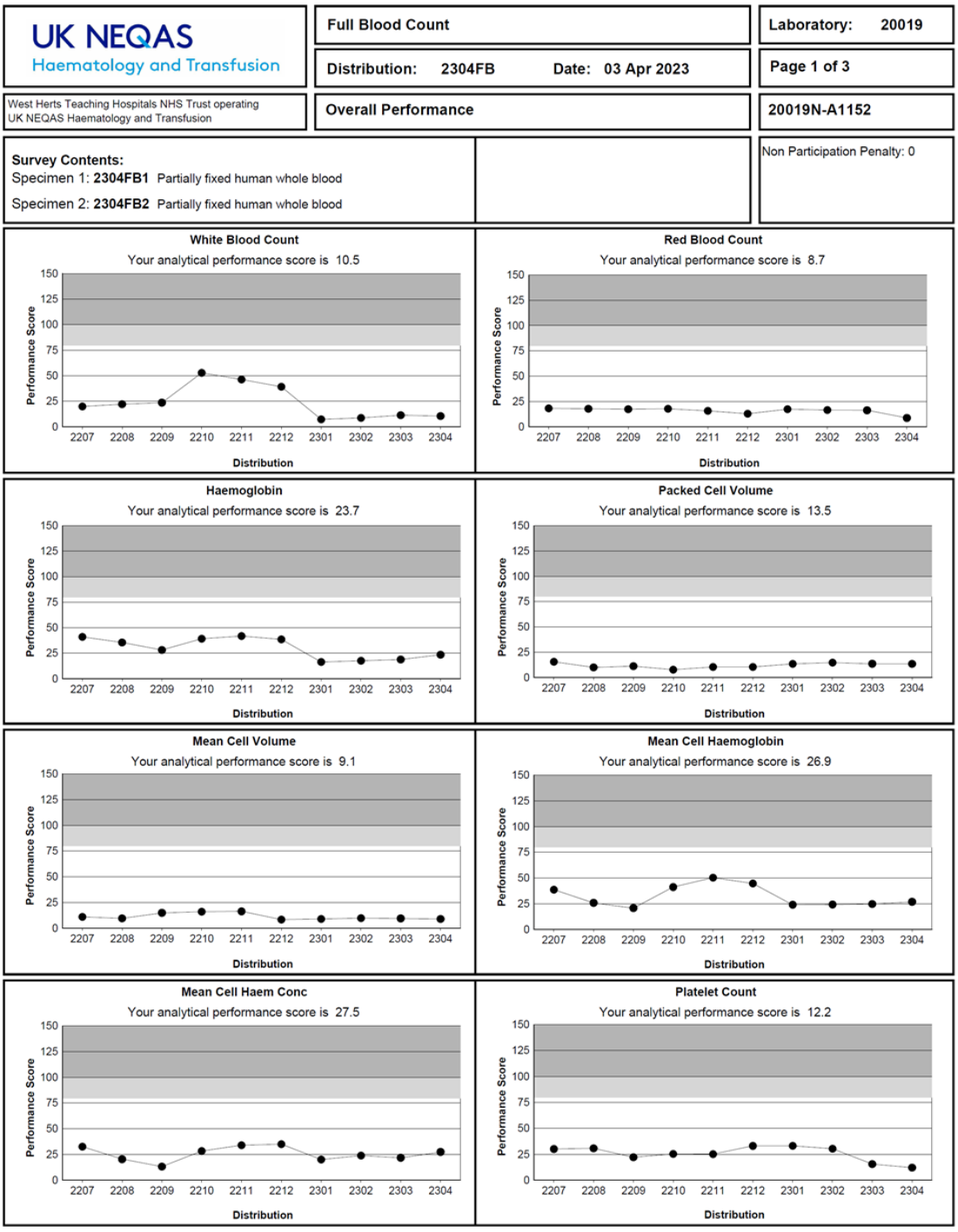
Q.3.1.1 Internal QC for full blood count

QC was performed as part of LRS standard practice. The QC results of the instruments measuring full blood count are monitored continually, and analysis is stopped if the results are not satisfactory, ensuring that results are only reported if the analysis is within the QC parameters set by the relevant Pathology Department Laboratory Manager. However, it is not possible to extract these results for reporting for each specific time period relevant to the NDNS analyses. Typical LRS assay per cent CVs are 3.1% (RBC), 1.8% (MCV), 2.8% (WBC), 3.9% (platelet count), 2.2% (MCH), 2.4% (RDW) 1.8% (haemoglobin) and 1.9% (haematocrit).

Q.3.1.2 EQA for full blood count

Quality of results was assessed externally through NEQAS. In figure Q.1 NEQAS results over the year to April 2023 are presented as a performance score. This score shows how the laboratory and specific instrument performed against a trimmed (excluding a proportion of the highest and lowest values) average for each analyte. The lower the performance score, the closer the laboratory or instrument value is to the consensus value. A performance score of between 0 and 75 is considered satisfactory.

Figure Q.1 EQA data for full blood count



Q.3.2 C-reactive protein (CRP)

At CBAL, serum C-reactive protein (CRP) was assayed using a high-sensitivity (extended range) assay (RCRP, DF34) on a Siemens Dimension EXL200 system clinical chemistry analyser. The CRP method is based on a particle enhanced turbidimetric immunoassay (PETIA) technique, giving high sensitivity by extending the detection range from 0.5mg/L to 250mg/L. Latex particles coated with anti-CRP antibody aggregate in the presence of CRP in the sample. The increase in turbidity that accompanies aggregation is proportional to the CRP concentration.

Consistent with reporting in previous NDNS RP years, values less than 1.1mg/mL were given an assigned value of 0.7 mg/L (calculated as LOQ ÷ √2) to reflect the high imprecision at values less than 1.1mg/L.

Q 3.2.1 Internal QC for CRP

Internal QC material for CRP (Bio-Rad Laboratories Ltd, Hertfordshire, UK) consisted of Liquichek Immunology Control, levels 1 and 3 (product code 594) and Liquichek Cardiac Markers Plus Control, level 2 (product code 180). Tables Q.3 and Q.4 show imprecision data for year 12 and years 13 to 15, respectively.

Table Q.2 Internal QC for CRP (NDNS year 12)

CRP QC	Year 12 QC1		Year 12 QC2		Year 12 QC3
Lot no.	664021	68911	660403	68913	29882
Mean (mg/L)	10.1	9.4	47.2	46.6	2.9
SD (mg/L)	0.15	0.24	1.38	1.13	0.22
Per cent CV	1.49	2.56	2.93	2.42	7.57
n	9	17	9	17	26

Table Q.3 Internal QC for CRP (NDNS years 13 to 15)

CRP QC	Years 13 to 15 QC1			Years 13 to 15 QC2			Years 13 to 15 QC3		
Lot no.	68911	68951	68991	68913	68953	68993	29882	87812	87842
Mean (mg/L)	8.8	8.4	9.8	44.1	42.7	46.1	2.3	1.8	1.9
SD (mg/L)	0.26	0.35	0.23	1.05	1.78	1.68	0.26	0.28	0.13
Per cent CV	2.95	4.19	2.34	2.38	4.17	3.64	11.25	16.00	6.98
n	6	46	16	6	47	17	16	32	17

Q 3.2.2 External QA for CRP

External QA was achieved through the NEQAS CRP and ultra-sensitive CRP (usCRP) schemes (table Q.4). Performance criteria for NEQAS are based on the following scores: MRBIS (mean running bias) and MRVIS (mean running variance index score). Performance of a laboratory over the given time period is characterised as follows:

Ideal: MRVIS < 50 Good:
MRVIS 50 to 100
Adequate: MRVIS 101 to 200
Poor: MRVIS > 200 or SDBIS > 200

Target values are based on all laboratory trimmed means and include a large number of different analysers. The population of analysers and numbers of laboratories enrolled in the scheme has changed dramatically over the course of years 12 to 15. In 2020, 276 participants were enrolled in the scheme for CRP and 44 in the usCRP scheme. In 2023, this had risen to 606 and 102, respectively. It should also be noted that the method used in NDNS is not designed as a high-sensitivity assay as generally used for the usCRP scheme. This, together with the changes in scheme participants over the NDNS fieldwork year will partly explain the variation observed in the external QC scores.

Table Q.4 EQA for CRP (NDNS years 12 to 15)

CRP NEQAS	Year 12 CRP	Year 12 usCRP	2021 CRP	2021 usCRP	2022 CRP	2022 usCRP	2023 CRP	2023 usCRP
MRVIS	+34	+158	+60	+124	+72	+125	+107	+177
MRBIS	+5	+49	+6	-11	+52	+15	+106	+155
SDBIS	43	202	79	167	70	165	69	143

Q.3.3 Ferritin

Serum ferritin was measured on a Siemens Dimension EXL200 analyser at CBAL. The assay uses chromium dioxide particles coated with specific antibodies to human ferritin. The assay is coupled to a colour reaction and the concentration of ferritin in the sample is proportional to the intensity of colour produced in the reaction. Ferritin is quantified by comparison to calibrants of known concentration.

Q.3.3.1 Internal QC for ferritin

QC material was Bio-Rad Lyphochek Immunoassay Plus Control - level 1 and 3 (product code 370) and Bio-Rad Lyphochek Anemia Control (product code 500). Summaries of internal QC data are shown in tables Q.5 and Q.6 for NDNS year 12 and years 13 to 15, respectively.

Table Q.5 Internal QC for ferritin (NDNS year 12)

Ferritin QC	Year 12 QC1	Year 12 QC2	Year 12 QC3
Lot no.	40361	40363	43280
Mean (µg/L)	66.8	381	6.4
SD (µg/L)	1.19	8.5	0.51
Per cent CV	1.78	2.23	7.99
n	14	14	14

Table Q.6 Internal QC for ferritin (NDNS years 13 to 15)

Ferritin QC	Year 13 to 15 QC1			Year 13 to 15 QC2			Year 13 to 15 QC3	
Lot no.	40361	40391	40411	40363	40393	40413	43280	43320
Mean (µg/L)	67.5	81.9	80.7	404	385	479	6.7	7.6
SD (µg/L)	1.22	3.56	2.02	24.0	14.3	11.9	0.66	0.71
Per cent CV	1.81	4.35	2.50	5.95	3.71	2.48	9.81	9.34
n	6	34	15	6	34	15	20	30

Q.3.3.2 External QA for ferritin

External QA was through the NEQAS Haematinics scheme. Table Q.7 shows the per cent bias relative to the target concentration in ferritin results during years 12 to 15.

Table Q.7 External QA for ferritin (NDNS years 12 to 15)

Ferritin NEQAS	Year 12	2021	2022	2023
Mean per cent bias	2.1	6.5	13.9	11.3
SD of per cent bias	4.78	10.46	6.19	7.08
n	30	33	33	24

Q.3.4 Total, high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol

Total cholesterol and high-density lipoprotein (HDL) cholesterol were measured in serum by Siemens Dimension EXL200 analyser at CBAL.

The total cholesterol method (Siemens CHOL DF27) uses cholesterol esterase to catalyse the hydrolysis of cholesterol esters to produce free cholesterol which, along with pre-existing free cholesterol, is oxidised in a reaction catalysed by cholesterol oxidase to form cholest-4-ene-3-one and hydrogen peroxide. In the presence of horseradish peroxidase, the hydrogen peroxide thus formed is used to oxidize N,N-diethylaniline-HCl/4-aminoantipyrine to produce a chromophore that absorbs at 540nm.

The AHDL cholesterol assay (Siemens DF48B) is a method for directly measuring HDL cholesterol concentrations. The method is based on accelerating the reaction of cholesterol oxidase with non-HDL unesterified cholesterol and dissolving HDL selectively using a specific detergent. In the first reaction, non-HDL unesterified cholesterol is subject to a cholesterol oxidase reaction and the peroxide generated is consumed by a peroxidase reaction with DSBmT yielding a colourless product. The second reagent consists of a detergent capable of solubilising HDL specifically, cholesterol esterase and chromagenic coupler to develop colour for the quantitative determination of HDL-C.

Low-density lipoprotein (LDL) concentration was calculated by CBAL using the Friedmann calculation: LDL = (total cholesterol – triglycerides) ÷ (2.2 - HDL cholesterol). If a triglyceride result was more than 4.5mmol/L then LDL cholesterol was not calculated and no LDL cholesterol result was recorded.

Q.3.4.1 Internal QC for total cholesterol and HDL cholesterol

Internal QC material was Bio-Rad Liquid Unassayed Multiqual Levels 1 (QC1) and 3 (QC2) (product code 697). Results are shown in tables Q.8 to Q.9 for total and HDL cholesterol for Years 12 to 15, respectively.

Table Q.8 Internal QC for total cholesterol (NDNS years 12 to 15)

Total cholesterol QC	Year 12 QC1	Year 12 QC2	Years 13 to 15 QC1			Years 13 to 15 QC2		
Lot no.	47991	47993	47991	56671	56701	47993	56673	56703

Mean (mmol/L)	2.8	7.1	2.8	2.7	2.7	7.3	6.9	6.9
SD (mmol/L)	0.10	0.15	0.08	0.09	0.13	0.17	0.11	0.14
Per cent CV	3.42	2.13	2.86	3.44	4.82	2.34	1.60	2.07
n	69	69	30	93	69	28	91	68

Table Q.9 Internal QC for HDL cholesterol (NDNS years 12 to 15)

HDL cholesterol QC	Year 12 QC1	Year 12 QC2	Years 13 to 15 QC1			Years 13 to 15 QC2		
Lot no.	47991	47993	47991	56671	56701	47993	56673	56703
Mean (mmol/L)	0.6	1.4	0.6	0.7	0.7	1.4	1.5	1.5
SD (mmol/L)	0.03	0.05	0.02	0.02	0.03	0.05	0.07	0.06
Per cent CV	4.38	3.44	2.55	3.59	3.56	3.79	4.43	3.73
n	46	48	18	47	41	17	46	41

Q.3.4.2 External QA for total and HDL cholesterol

External QA was achieved through NEQAS (for lipid investigations) and the Randox International QA Scheme (RIQAS) (lipid programme); NEQAS pooled samples are unsuitable for the total cholesterol method used by the Siemens instruments. Tables Q.10 and Q.11 provide EQA results from RIQAS for total and HDL cholesterol, respectively, and table Q.12 NEQAS results for HDL cholesterol.

Table Q.10 External RIQAS QA results for total cholesterol (NDNS years 12 to 15)

Total cholesterol RIQAS	Year 12 Cycle 42	Year 12 Cycle 43	2021 Cycle 44	2021 Cycle 45	2022 Cycle 46	2022 Cycle 47	2023 Cycle 48
Mean per cent bias	-1.6	-0.5	0.1	-1.3	-1.0	-2.5	-1.6
SD of per cent bias	1.73	1.49	2.75	1.23	1.59	0.91	2.76
n	12	12	11	11	12	12	12

Table Q.11 External RIQAS QA results for HDL cholesterol (NDNS years 12 to 15)

HDL cholesterol RIQAS	Year 12 Cycle 42	Year 12 Cycle 43	2021 Cycle 44	2021 Cycle 45	2022 Cycle 46	2022 Cycle 47	2023 Cycle 48

Mean per cent bias	0.7	0.2	-0.6	-0.8	-1.0	-0.8	-1.4
SD of per cent bias	1.83	2.46	2.98	1.66	2.30	1.21	1.33
n	12	12	11	11	12	12	12

Table Q.12 External NEQAS QA results for HDL cholesterol (NDNS years 12 to 15)

HDL cholesterol NEQAS	Year 12	2021	2022	2023
Mean per cent bias	-12.0	-4.0	+1.1	-1.9
SD of per cent bias	12.54	12.51	8.74	7.15
n	33	30	33	24

Q.3.5 Triglycerides (triacylglycerols)

Serum triglycerides were measured on a Siemens Dimension EXL200 analyser at CBAL. The triglycerides (triacylglycerols) method is based on an enzymatic procedure in which a combination of enzymes are employed for the measurement of serum or plasma triglycerides. The sample is incubated with lipoprotein lipase (LPL) enzyme reagent that converts triglycerides into free glycerol and fatty acids. Glycerol kinase (GK) catalyses the phosphorylation of glycerol by adenosine-5-triphosphate (ATP) to glycerol-3-phosphate. Glycerol-3-phosphate-oxidase oxidises glycerol-3-phosphate to dihydroxyacetone phosphate and hydrogen peroxide (H_2O_2). The catalytic action of peroxidase (POD) forms quinoneimine from H_2O_2, aminoantipyrine and 4-chlorophenol. The change in absorbance due to the formation of quinoneimine is directly proportional to the total amount of glycerol and its precursors in the sample and is measured using a bichromatic (510nm, 700nm) endpoint technique.

Q.3.5.1 Internal QC for triglycerides

Internal QC material was Bio-Rad Liquid Unassayed Multiqual Levels 1 (QC 1) and 3 (QC 2) (product code 697). Data are reported for years 12 to 15 (table Q.13).

Table Q.13 Internal QC for triglycerides (NDNS years 12 to 15)

Triglycerides QC	Year 12 QC1	Year 12 QC2	Years 13 to 15 QC1			Years 13 to 15 QC2		
Lot no.	47991	47993	47991	56671	56701	47993	56673	56703
Mean (mmol/L)	1.0	2.4	1.0	0.96	0.94	2.4	2.4	2.4
SD (mmol/L)	0.04	0.08	0.02	0.04	0.05	0.10	0.07	0.09
Per cent CV	3.69	3.33	2.24	3.78	4.91	4.30	2.98	3.79
n	76	76	33	94	66	32	93	68

Q.3.5.2 External QA for triglycerides

External QA was achieved through RIQAS (lipids programme) and NEQAS (for lipid

investigations). Table Q.14 indicates the percentage deviation of results obtained from the target concentration.

Table Q.14 External RIQAS QA results for HDL cholesterol (NDNS years 12 to 15)

Triglycerides RIQAS	Year 12 Cycle 42	Year 12 Cycle 43	2021 Cycle 44	2021 Cycle 45	2022 Cycle 46	2022 Cycle 47	2023 Cycle 48
Mean per cent bias	0.2	0.6	2.7	-0.9	-2.3	-1.1	0.0
SD of per cent bias	1.52	1.89	6.55	2.47	1.99	2.09	1.88
n	12	12	11	11	12	12	12

Table Q.15 External NEQAS QA results for HDL cholesterol (NDNS years 12 to 15)

Triglycerides NEQAS	Year 12	2021	2022	2023
Mean per cent bias	-3.9	-4.2	0.5	-5.3
SD of per cent bias	3.85	3.78	17.03	10.33
n	33	33	36	24

Q.3.6 Erythrocyte transketolase activation coefficient (ETKAC) for thiamin (vitamin B1) status

Thiamin diphosphate (ThDP) is a cofactor for the erythrocyte transketolase enzyme. Measurement of transketolase activity in washed red blood cells before and after the addition of exogenous ThDP, and calculation of the activity coefficient, provides a measure of the saturation of the transketolase enzyme and consequently, thiamine status. Erythrocyte transketolase activation coefficient (ETKAC) is a sensitive and specific marker of thiamin status. Analysis was performed on a Thermo Multiskan FC plate reader. The method details as performed for NDNS are published (Jones and others, 2021).

In thiamin sufficiency, addition of exogenous ThDP will make little difference to the enzyme activity and an ETKAC close to 1 will be obtained. In thiamin insufficiency or deficiency, the addition of exogenous ThDP has a progressively greater effect on ETKAC, with higher values indicating a higher risk of thiamin deficiency. Although there is no international consensus on cutoffs, the commonly used threshold for risk of deficiency is an ETKAC of >1.25 (EFSA, 2016).

There are no available sources of erythrocytes with known ETKAC; therefore, QC material was prepared in-house. Erythrocytes obtained from the National Blood Transfusion Service (NBTS) or commercial sources were washed to remove the buffy coat and lysed by threefold dilution with normal saline. This lysate was stored at -70˚C in single-use aliquots. The lysate was stored and assayed both neat and further diluted 2 times with water. No source of thiamin deficient erythrocytes has been identified in sufficient quantity with which to prepare a lysate giving high ETKAC.

QC material `S` and LR160223 are dilute lysates in which the reaction rates are very low and are included to assess assay performance in similarly dilute samples.

Q.3.6.1 QC for ETKAC

Descriptive statistics in table Q.16 for internal QCs indicate good batch-to-batch consistency of ETKAC results during years 12 to 15.

There are no external QA schemes available for ETKAC.

Table Q.16 Internal QC for ETKAC (NDNS years 12 to 15)

ETKAC QC	Year 12 QC C	Year 12 QC S	Year 12 QC Q	Year 13 to 15 QC C	Years 13 to 15 QC S	Year 13 to 15 QC Q	Year 13 to 15 LR160223	Year 13 to 15 Q160223
Mean	1.08	1.18	1.22	1.08	1.18	1.19	1.13	1.16
SD	0.03	0.05	0.02	0.03	0.04	0.03	0.02	0.02
Per cent CV	2.9	4.1	1.6	2.4	3.7	2.8	2.1	2.1
n	8	8	8	49	28	37	19	19

Q.3.7 Erythrocyte glutathione reductase activation coefficient (EGRAC) for riboflavin (vitamin B2) status

Riboflavin (vitamin B2) is a component of the co-enzyme, flavin adenine dinucleotide (FAD). As for thiamin and ETKAC in section Q.3.6 the activity coefficient of erythrocyte glutathione reductase (EGRAC), a FAD-dependent enzyme, is a biomarker of riboflavin status. The EGRAC is calculated from the ratio of unstimulated ('basal') EGR to stimulated ('activated') EGR with exogenous FAD. The preincubation with FAD is carried out for a relatively long period, 30 minutes at 37°C, in order to ensure full reactivation of apo-enzyme, and, at a low final concentration of FAD (1.5µM), to eliminate activation coefficients (ratios) <1.0; this can result from enzyme inhibition by FAD, or its breakdown products, which may occur if the final concentration of FAD is too high. The generally accepted threshold for riboflavin adequacy is EGRAC below 1.30 (EEFSA, 2017). Analysis was performed on a Thermo Multiskan FC plate reader following a published method (Parkington and others, 2023).

Q.3.7.1 QC for EGRAC

There is no QC material available with known EGRAC, therefore washed erythrocytes were prepared in-house. Erythrocytes obtained from the National Blood Transfusion Service (NBTS) or commercial sources were washed to remove the buffy coat and lysed by threefold dilution with normal saline. This lysate was stored at -70˚C in single-use aliquots. In addition to the native samples a saturated control was made by incubation with FAD before aliquoting (Control X). These 3 controls were run on each assay plate. There is no external QA scheme available for EGRAC.

Descriptive statistics in table Q.17 for internal QCs indicate good batch-to-batch consistency of EGRAC results during years 12 to 15.

There are no external QA schemes available for EGRAC.

Table Q.17 Internal QC for EGRAC (NDNS years 12 to 15)

EGRAC QC	Year 12 QC X	Year 12 QC C	Year 12 QC A	Year 13 to 15 QC X	Year 13 to 15 QC C	Year 13 to 15 QC A
Mean (ratio)	0.99	1.49	2.17	0.98	1.57	2.34
SD	0.01	0.06	0.10	0.02	0.05	0.10
Per cent CV	0.7	4.1	4.4	2.3	3.2	4.1
n	9	9	9	66	66	65

Q.3.8 Plasma vitamin B6 (Pyridoxal-5-phosphate and 4-pyridoxic acid)

The assay for vitamin B6, based on that published by Rybak and Pfeiffer (2004), uses a metaphosphoric acid protein precipitation followed by filtration of the supernatant to prepare plasma samples for analysis. Detection of pyridoxal-5-phosphate (PLP) and 4-pyridoxic acid (PA) is accomplished by HPLC with fluorescence detection employing a post-column derivatisation step with sodium chlorite.

Analytes are resolved using reversed phase HPLC on a BDS hypersil, C_{18} 3 × 150 mm 5 µm column at 35°C with a gradient mobile phase combining 50mM sodium dihydrogen phosphate buffer at pH 2.7 plus 0.2% acetonitrile and methanol at 0.7mL/min.

Q.3.8.1 Internal QC for vitamin B6

In years 12 to 15 three in-house plasma controls containing endogenous vitamin B6 across the assay range for both PLP and PA and a spiked plasma sample with a 50nmol/L nominal concentration were analysed at the beginning and at the end of each batch of samples. Years 12 to 15 QC data are summarised for PLP and PA in tables Q.18 and Q.19.

Table Q.18 Internal QC for PLP (NDNS years 12 to 15)

PLP QC	Year 12 A02	Year 12 A07	Year 12 PA Old	Year 12 Spike QC	Years 13 to 15 A02	Years 13 to 15 A07	Years 13 to 15 PA Old	Years 13 to 15 Spike
Mean (nmol/L)	48.0	26.6	14.0	52.5	48.5	25.0	11.7	53.2

SD (nmol/L)	2.6	1.5	1.3	2.5	2.1	1.7	1.5	3.1
Per cent CV	5.3	5.5	9.5	4.7	4.4	6.9	12.5	5.8
n	10	10	10	10	51	51	49	51

Table Q.19 Internal QC for PA (NDNS years 12 to 15)

PA QC	Year 12 A02	Year 12 A07	Year 12 PA Old	Year 12 Spike QC	Years 13 to 15 A02	Years 13 to 15 A07	Years 13 to 15 PA Old	Years 13 to 15 Spike
Mean (nmol/L)	120.1	12.6	36.6	51.8	128.2	13.4	37.4	53.5
SD (nmol/L)	8.8	0.7	2.2	2.9	5.0	1.2	1.1	2.7
Per cent CV	7.3	5.7	6.0	5.5	3.9	9.2	3.0	5.1
n	10	10	10	10	51	51	49	51

Q.3.8.2 Accuracy for vitamin B6

Standard Reference Material (SRM) 1950 from the National Institute of Standards and Technology (NIST) was assayed once with every run to assess the accuracy of the assay (table Q.20).

Table Q.20 Accuracy for PLP and PA (NDNS years 12 to 15)

Vitamin B6 SRM 1950	Year 12 PLP	Year 12 PA	Years 13 to 15 PLP	Years 13 to 15 PA
Mean (nmol/L)	34.2	26.9	34.9	28.2
SD (nmol/L)	2.2	0.3	2.7	1.3
Per cent CV	6.4	1.1	7.8	4.8
n	3	3	12	12
Target value (nmol/L)	33.17 ± 1.86	28.7[1]	33.17 ± 1.86	28.7[2]

Q.3.8.3 External QA for PLP

The NBL participated in the Royal College of Pathologists of Australasia Quality Assurance Program (RCPAQAP). There are only 6 participants in the scheme, the majority of whom use the Chromsystems complete HPLC kit (Chromsystems, Germany) reporting PLP. There is no external QA scheme for PA. Table Q.21 provides a summary of EQA data for years 12 to 15.

[1] Values do not have an associated measurement uncertainty
[2] Values do not have an associated measurement uncertainty

Due to laboratory closures associated with the COVID-19 pandemic, no EQA material was analysed for year 12, however accuracy of the method was validated through inclusion of NIST SRMs as described above.

Table Q.21 External QA for vitamin B6 (RCPQAP) (NDNS years 12 to 15)

Vitamin B6 (PLP) RCPAQAP	Year 12	Years 13 to 15
Mean bias (per cent)[3]	-	-10.8
SD of bias	-	9.9
Mean Z-score[4]	-	-0.72
n	-	8

Q.3.9 Serum folate

The assay for serum total folate uses solid phase extraction with phenyl columns to isolate the folate vitamers from serum samples (Meadows. 2017). Analyte matched stable isotope labelled internal standards are added during the extraction step and undergo processing identical to the analytes thereby normalizing for sample preparation and instrument variability. Highly specific detection of the 6 vitamers; methyltetrahydrofolate (MTHF), tetrahydrofolate (THF), formyltetrahydrofolate (FTHF), folic acid, 5,10-methenyltetrahydrofolate (CH+THF) and pyrazino-s-triazine derivative of 4α-hydroxy-5-methyl tetrahydrofolate (MeFox) and internal standards is accomplished by UPLC-MS/MS analysis and the ratio of analyte to internal standard signal is compared to that of a calibration curve to determine analyte concentration.

Analytes are separated using reversed phase UPLC on a Waters ACQUITY UPLC® HSS T3 C8 2.1 × 100 mm 1.7 µm column at 30°C with a 49.5:40:10:0.5 water:methanol:acetonitrile:acetic acid isocratic mobile phase prior to mass spectrometry analysis. In NDNS, serum total folate is calculated from the sum of the 6 folate vitamers, including MeFox.

Q.3.9.1 Internal QC for serum folate

In-house plasma controls containing endogenous folates across the assay range for MTHF, folic acid and MeFox and a spiked serum sample with a 5 nmol/L nominal concentration for FTHF, THF and CH+THF with endogenous levels of MTHF, folic acid and MeFox were analysed at the beginning and at the end of each batch of samples for assessment of assay precision. Tables Q.22 to Q.25 provide a summary of QC data for years 12 to 15.

Table Q.22 Internal QC for MTHF (NDNS years 12 to 15)

MTHF QC	Year 12 QC 1 100118	Year 12 QC 3 100118	Year 12 Spike QC 170418	Year 12 QC 4 070220	Year 13 to 15 Spike QC 170418	Year 13 to 15 QC 4 070220	Year 13 to 15 QC 6 151020

[3] Z score = (lab result - all results mean) ÷ all results SD
[4] Z score = (lab result - all results mean) ÷ all results SD

Mean (nmol/L)	27.2	17.2	62.0	8.9	62.7	9.4	26.3
SD (nmol/L)	1.38	1.24	4.85	0.44	3.38	0.50	1.33
Per cent CV	5.1	7.2	7.8	4.1	5.38	5.31	5.06
n	4	4	6	6	27	27	27

Table Q.23 Internal QC for folic acid (NDNS years 12 to 15)

Folic acid QC	Year 12 QC 1 100118	Year 12 QC 3 100118	Year 12 Spike QC 170418	Year 12 QC 4 070220	Year 13 to 15 Spike QC 170418	Year 13 to 15 QC 4 070220	Year 13 to 15 QC 6 151020
Mean (nmol/L)	7.6	0.6	5.0	1.6	5.1	1.6	0.75
SD (nmol/L)	0.20	0.14	0.28	0.23	0.38	0.16	0.11
Per cent CV	2.6	23.5	5.7	14.3	7.5	9.9	15.0
n	4	4	6	6	27	27	27

Table Q.24 Internal QC for MeFox (NDNS years 12 to 15)

MeFox QC	Year 12 QC 1 100118	Year 12 QC 3 100118	Year 12 Spike QC 170418	Year 12 QC 4 070220	Year 13 to 15 Spike QC 170418	Year 13 to 15 QC 4 070220	Year 13 to 15 QC 6 151020
Mean (nmol/L)	6.3	1.2	8.2	6.4	8.3	6.5	6.4
SD (nmol/L)	0.65	0.09	0.37	0.47	0.46	0.29	0.29
Per cent CV	10.4	7.7	4.5	7.3	5.5	4.4	4.5
n	4	4	6	6	27	27	27

Table Q.25 Internal QC for other folates (NDNS years 12 to 15)

Other folates QC	Year 12 QC 170418 CH+THF	Year 12 QC 170418 THF	Year 12 QC 170418 FTHF	Year 13 to 15 QC 170418 CH+THF	Year 13 to 15 QC 170418 THF	Year 13 to 15 QC 170418 FTHF
Mean (nmol/L)	5.2	5.5	5.7	5.3	5.4	6.2
SD (nmol/L)	0.30	0.43	0.30	0.44	0.45	0.58
Per cent CV	5.7	7.9	5.3	8.4	9.32	0.58
n	6	6	6	27	27	27

Q.3.9.2 Accuracy of serum folate

NIST SRM 1950 was assayed with every run to assess and monitor the accuracy of the assay for MTHF and folic acid. Results are presented in table Q.26.

Table Q.26 Accuracy for serum folate (NDNS years 12 to 15)

Serum folate SRM 1950	Year 12 MTHF	Years 13 to 15 MTHF	Year 12 Folic acid	Years 13 to 15 Folic acid

Mean (nmol/L)	27.1	27.6	3.1	3.0
SD (nmol/L)	0.93	1.22	0.19	0.19
Per cent CV	3.4	4.4	6.2	6.5
n	3	14	3	14
SRM target values (nmol/L)	26.91 ± 0.7	26.91 ± 0.7	3.42 ± 1.02	3.42 ± 1.02

Q.3.9.3 External QA of serum folate

The NBL participated in NEQAS and VITAL quality assurance schemes. Table Q.27 shows the NEQAS scores for year 12 and years 13 to 15. Due to laboratory closures associated with the COVID-19 pandemic, no VITAL EQA material was analysed for year 12. However, accuracy of the method was validated through inclusion of NIST SRMs as described above. VITAL-EQA samples were received twice a year and had total folate target concentrations assigned using the 'gold standard' microbiological method run by CDC. Rounds 38, 39 and 40 were run during years 13 to 15 (table Q.28).

Table Q.27 External QA (NEQAS) for serum folate (NDNS years 12 to 15)

Total folate NEQAS	Year 12	Year 13 to 15	Limits
A	71	100	200
B (per cent)	-5.4	-3.8	±25
C (per cent)	19.6	26.1	25

Table Q.28 External QA (VITAL) for serum folate (NDNS years 13 to 15)

Total folate VITAL	Round 38 S1	Round 38 S2	Round 38 S3	Round 39 S1	Round 39 S2	Round 39 S3	Round 40 S1	Round 40 S2	Round 40 S3
Mean (ng/mL)	6.8	8.3	22.6	5.5	6.4	8.0	3.0	9.3	12.3
Imprecision (per cent)	5.7	6.3	3.8	5.1	7.8	1.8	9.4	5.4	1.1
Bias (per cent)	-9.8	-17.4	5.7	-15.6	-17.4	-12.4	-1.4	1.9	4.1
Target value (ng/mL)	7.5	10.0	21.4	6.5	7.7	9.1	3.0	9.1	11.8

Q.3.10 Whole blood (WB) folate

Whole blood (WB) haemolysate specimens (WB diluted and stabilised with ascorbic acid, 1/11) were analysed for total folate at CDC in years 12 to 15 using the *Lactobacillus rhamnosus* microbiologic growth assay by an adaptation of O'Broin and Kelleher (1992) and Molloy and Scott (1997) as described by Pfeiffer and others (2011).

Aliquots of whole blood (WB) samples were preserved with 1% ascorbic acid (appendix P) and frozen at -70 °C before sending on dry ice to CDC.

Diluted specimen (4 replicates, duplicates at 2 dilutions) was added to an assay medium containing the microorganism and all of the nutrients, except for folate, necessary for the

growth of the microorganism. Since the growth of *L. rhamnosus* is proportional to the amount of total folate present in the specimen, the total folate level was assessed by measuring the turbidity of the inoculated medium at 590nm in a microplate reader. The assay was calibrated with 5-methyl-tetrahydrofolate (MTHF), using an 11-point calibration curve (0–1.0 nmol/L; 8 replicates/point) with a 3rd degree polynomial curve fit.

Sample dilutions with a concentration below the lowest calibrator or above the highest calibrator were repeated for confirmation, at lower or higher dilution. The standard dilution used for WB haemolysate specimens in this study was 1/70.

Results from the 4 replicates were averaged to generate the final result and the CV from the 4 replicates had to be ≤15% (≤10% if only 3 replicates were used). No result was reported from less than 3 replicates. Assays were repeated where necessary.

Samples with a WB folate concentration <127nmol/L (corresponding to a RBC folate concentration of <305nmol/L RBC if a haematocrit of 0.4L/L is assumed) were considered to represent potential folate deficiency and samples were repeated for confirmation. This is the standard practice in the CDC laboratory.

Accuracy has been established by spiked recovery and by periodic assaying of the International Standard for Whole Blood Folate (NIBSC World Health Organization (WHO) standard (95/528))

Q.3.10.1 Internal QC for WB folate

QC limits for WB folate were established by duplicate analysis of 3 in-house quality control pools. The 3 WB bench QC pools were analysed in duplicate in each run (n=5 in year 12 and n=10 in years 13 to 15). The between-run imprecision for WB folate and the target concentration are shown in table Q.29.

Table Q.29 Internal QC for WB folate (NDNS years 12 to 15)

WB folate QC	LB14810_MA Year 12	LB14810_MA Year 13 to 15 A	LB14810_MA Year 13 to 15 B	MB14811_MA Year 12	MB14811_MA Year 13 to 15 A	MB14811_MA Year 13 to 15 B	HB14812_MA Year 12	HB14812_MA Year 13 to 15 A	HB14812_MA Year 13 to 15 B
Mean (nmol/L)	264	256	249	438	433	418	729	725	702
SD (nmol/L)	21	12	23	18	19	25	35	34	31
Per cent CV	7.9	4.7	9.3	4.1	4.5	5.9	4.8	4.6	4.4
Target (nmol/L)	254	254	254	443	443	443	745	745	745
Difference from target, per cent	3.8	1.0	-2.1	-1.1	-2.2	-5.6	-2.2	-2.7	-5.8

Four additional WB QC pools were analysed `blind` (that is target concentration unknown to analyst) as part of this study at a rate of 1 blind QC sample in every 20 unknown samples. The between-run imprecision and target concentration are shown in table Q.30.

Table Q.30 WB folate blind QC pool analysis (NDNS years 12 to 15)

WB folate blind QC	936 Year 12	936 Year 13 to 15 A	936 Year 13 to 15 B	937 Year 12	937 Year 13 to 15 A	937 Year 13 to 15 B	938 Year 12	938 Year 13 to 15 A	938 Year 13 to 15 B	939 Year 12	939 Year 13 to 15 A	939 Year 13 to 15 B
Mean (nmol/L)	529	493	512	301	260	249	416	385	367	200	214	178
SD (nmol/L)	20	32	20	-	23	17	18	27	19	12	-	10
Per cent CV	3.7	6.5	4.0	-	8.9	6.7	4.3	6.9	5.2	6.0	-	5.5
n	4	7	5	1	4	4	4	5	3	3	1	8
Target (nmol/L)	527	527	527	256	256	256	354	354	354	187	187	187
Difference from target, per cent	0.4	6.3	-2.7	17.5	1.7	-2.8	17.5	8.7	3.5	7.3	14.7	-4.5

Q.3.10.2 Calculation of red blood cell (RBC) folate

Red blood cell (RBC) folate is calculated from WB folate concentration, serum folate concentration and haematocrit (quantified as part of the full blood count) using the equation:

RBC folate = (WB folate - (serum folate × (1 - haematocrit))) ÷ haematocrit

Where a serum folate concentration was not available a surrogate of 18nmol/L was used in the calculation. Where haematocrit was not available, a surrogate of 0.4L/L was used. If neither serum folate or haematocrit were available then no RBC folate was calculated.

For the calculation of RBC folate, if the WB folate concentration was less than the limit of detection (44nmol/L) then a value of 31nmol/L (calculated as the LOD divided by the sqrt(2)) was assigned to that sample for the calculation of RBC folate. Where there was no result for WB folate then the calculation of RBC was not performed.

Q.3.11 Vitamin B_{12}

Serum vitamin B12 was measured at LRS. The ADVIA Centaur B_{12} assay is a competitive immunoassay using direct chemiluminescence. Vitamin B_{12} from a participant's sample competes with vitamin B_{12} labelled with acridinium ester for a limited amount of labelled intrinsic factor. The intrinsic factor is covalently bound to paramagnetic particles. The assay uses a releasing agent (sodium hydroxide) and dithiothreitol (DTT) to release the B_{12} from the endogenous binding proteins in the sample.

Q.3.11.1 Internal QC for vitamin B_{12}

The QC results of the instrument measuring Vitamin B_{12} at LRS are monitored continually, and analysis is stopped if the results are not satisfactory, ensuring that results are only reported if the analysis is within the QC parameters set by the Laboratory Manager. However, it is not possible to extract these results for NDNS reporting. Therefore in order to monitor between-batch precision, control material was aliquoted and included in the regular delivery of NDNS samples to LRS. Control material was purchased from Bio-Rad and was chosen to reflect the full range of vitamin B_{12} concentrations. Different batches of control material (indicated by underscore) are used across NDNS years as batches expire or run out which allows monitoring of continuity between NDNS years. QC data are summarised in table Q.31.

Table Q.31 Internal QC for vitamin B_{12} (NDNS years 12 to 15)

Vitamin B12 QC	QC1_1 Y12	QC1_1 Y13 to 15	QC2 Y12	QC2 Y13 to 15	QC3 Y12	QC3 Y13 to 15	QC4 Y12	QC4 Y13 to 15	QC5 Y12	QC5 Y13 to15	QC1_2 Y12	QC1_2 Y13to 15
Mean (ng/L)	155	164	176	193	124	141	612	602	1017	1052	-	181
SD (ng/L)	23	20	11	27	17	25	33	49	52	91	-	24
Per cent CV	15	12	6	14	13	18	5	8	5	9	-	13
n	7	19	7	38	7	39	7	39	7	39	-	25

Abbreviations: QC1: Lyphochek Anemia Control, single level QC2: Liquid Assayed Multiqual, Level 2 QC3: Lyphochek Assayed Chemistry Control, Level 2 QC4: Lyphochek Immunoassay Plus Control, Level 2 QC5: Lyphochek Immunoassay Plus Control, Level 3

Q.3.11.2 External QA for vitamin B_{12}

External QA was achieved through the UK NEQAS Haematinics scheme (table Q.32).

Table Q.32 Vitamin B12 NEQAS (NDNS years 12 to 15)

Vitamin B12 NEQAS	Year 12	Years 13 to 15	Limits
A	52	81	200
B (per cent)	+3.0	+4.0	± 20
C (per cent)	7.3	10.8	15.0

Q.3.12 Holotranscobalamin (holoTC); `active` B_{12}`

Vitamin B_{12} (cobalamin) is transported in the circulation bound to transcobalamin (TC) (10 to 30%) and to haptocorrin (HC) (70 to 90%). When TC and HC bind vitamin B_{12} the resulting complexes are known as holotranscobalamin (holoTC) and holohaptocorrin (holoHC) to distinguish them from the proteins carrying no vitamin.

The serum holoTC assay is an enzyme-linked immunosorbent assay (ELISA) manufactured by Axis Shield. It is conducted in 96-well microplates. HoloTC reacts with a specific antibody immobilised on the plate surface; a second, labelled antibody then reacts to form a `sandwich`. The enzyme label is quantitated using a coloured substrate and the absorbance read in a microplate spectrophotometer.

Q.3.12.1 Internal QC for holoTC

Two sets of QC samples were measured. Those supplied by the manufacturer were measured in duplicate with every assay. Measured values were always with the limits set by the manufacturer for different lot numbers and per cent CV within lots was less than 5%. Three serum QC samples spanning the normal range were prepared in-house were used to monitor lot to lot variation. Additional serum QC samples (QC D, E and F) were introduced in years 13 to 15 due to limited remaining aliquots of QC A, B and C. Results are provided in Table Q.33. In year 12, analysis was performed on a single ELISA lot. In years 13 to 15, QCs A, B and C were measured on 2 ELISA lots and QCs D, E and F on 4 ELISA lots. Between lot variation was <10%.

Table Q.33 Internal QC for holoTC (NDNS year 12 to 15).

HoloTC QC	Year 12 QC A	Year 12 QC B	Year 12 QC C	Year 13 to 15 QC A	Year 13 to 15 QC B	Year 13 to 15 QC C	Year 13 to 15 QC D	Year 13 to 15 QC E	Year 13 to 15 QC F
Mean (pmol/L)	33.7	55.0	78.6	47.8	60.1	101.1	42.2	66.1	93.5
SD (pmol/L)	1.3	1.5	1.8	3.4	6.0	6.5	7.9	7.6	9.1
Per cent CV	3.7	2.7	2.2	7.2	10.0	6.4	18.6	11.5	9.7
n	6	6	6	24	25	25	23	23	23

Q.3.12.2 External QA for holoTC

In each survey year, analysis of samples for holoTC was performed as batches over a short time scale and therefore did not allow for `live` participation in the EQA scheme. Three samples are received per NEQAS distribution (4 distributions per year). NEQAS samples were stored at -70°C and assayed retrospectively alongside respective samples for each year, the results being compared with the NEQAS ALTM for results obtained by all participating laboratories (table Q.34), most of whom used automated methods.

Table Q.34 External QA for holoTC (NDNS years 12 to 15)

HoloTC NEQAS[5]	Year 12	Years 13 to 15
Bias against ALTM (per cent)	-17 (10)	6 (27)
Bias against method mean (per cent)	-24 (11)	15 (27)
n	18	65

Q.3.12.3 Accuracy for holoTC

In year 13, NIBSC World Health Organization (WHO) international standard (03-178) was also run regularly. The standard 03/178 has an assigned consensus holoTC value of 107pmol/L. Performance of the assay against the consensus value for holoTC is reported in table Q.35.

Table Q.35 Performance against WHO International Standard for holoTC (NDNS years 13 to 15)

HoloTC NIBSC (03-178) Accuracy[6]	Years 13 to 15
Consensus (target) value, pmol/L	107
Mean (SD), pmol/L	109 (6)
Per cent CV	6
n	19
Mean (SD) bias, per cent	2 (6)

Q.3.13 Vitamin C (ascorbic acid)

This assay is based on the procedure described by Vuilleumier and Keck (1989). Samples are stabilised immediately after separation using an equal volume of 10% metaphosphoric acid and plasma (see appendix P).

[5] Bias was calculated against the target value as % bias = (result - target)/target) × 100
[6] Bias was calculated against the target value as % bias = (result - target)/target) × 100

Vitamin C (ascorbic acid) in the sample is converted to dehydroascorbic acid by ascorbate oxidase, followed by coupling of the resulting dehydroascorbate with o-phenylene diamine to form a fluorescent derivative quinoxaline. The formation of quinoxaline is linearly related to the amount of vitamin C in the sample. The assay was performed on the BMG Labtech FLUOstar Omega plate reader.

Q.3.13.1 Internal QC for vitamin C

QC samples were made in-house by spiking vitamin C-depleted plasma. Results are provided in table Q.36.

Table Q.36 Internal QC for vitamin C (NDNS years 12 to 15)

Vitamin C QC	Year 12 QC1	Year 12 QC2	Year 12 QC3	Years 13 to 15 QC1	Years 13 to 15 QC2	Years 13 to 15 QC3	Years 13 to 15 QC1	Years 13 to 15 QC2	Years 13 to 15 QC3
Mean (µmol/L)	10.5	34.1	50.9	10.8	30.8	47.8	14.1	26.8	55.1
SD (µmol/L)	0.9	3.7	1.5	0.7	1.7	2.8	1.2	2.0	3.8
Per cent CV	8	11	3	6	6	6	9	7	7
n	12	12	12	7	77	7	64	64	64

Q.3.13.2 External QA for vitamin C

NBL participated in the Royal College of Pathologists of Australasia Quality Assurance Program (RCPAQAP). The bias is calculated from the ALTM. The majority of the participating laboratories use the Chromsystems HPLC method and calibrator, which are different from the fluorescence method and calibrator used in the NDNS; results from NBL show a bias relative to the Chromsystems method (table Q.37). The NBL calibrators were prepared gravimetrically from the AnalaR ascorbic acid, the purest grade available. This methodological difference shows as a deviation of NBL results from the Chromsystems consensus. The consistency of the bias indicates consistency in NBL results, as does the low 'per cent imprecision' result.

Table Q.37 External QA for vitamin C (NDNS years 12 to 15)

Vitamin C RCPAQAP	Year 12 Cycle 41	Year 12 Cycle 42	Years 13 to 15 Cycle 22	Years 13 to 15 Cycle 23
Mean Bias (per cent)[7]	-23	-19	-29	-27
Mean bias imprecision (SD)	3.1	8.7	4.0	4.1
n	8	14	17	16

[7] Bias was calculated against target value as % bias = (result - target)/target) × 100 when measured value was greater than the assay limit of quantitation. The reported target values were obtained from the end of cycle reports and represent the median of all assay results submitted to the scheme

Q.3.14 Retinol, α– and γ–tocopherol, and individual carotenoids (fat-soluble vitamins (FSVs)

The assay for FSVs is based on that published by Sowell and others (1994) and uses a methanol protein precipitation step followed by liquid-liquid extraction in hexane to separate fat soluble vitamins from other analytes in plasma samples. Internal standards are added during the extraction step and undergo processing identical to the analytes thereby normalising for sample preparation and instrument variability. Detection of the 9 analytes; retinol, α-tocopherol, γ-tocopherol, lutein and zeaxanthin (combined due to co-elution), lycopene, α-cryptoxanthin, β-cryptoxanthin, α-carotene, β-carotene and internal standards; tocopherol acetate and apo-8'-carotenal is accomplished by HPLC analysis with PDA (UV, photo diode array) detection. The ratio of analyte to internal standard signal is compared to that of a calibration curve to determine analyte concentration.

Analytes are resolved using reversed phase HPLC on a YMC-pack pro C_{18} 4.6 x 150 mm 3 µm column at 40°C with a 25:75 ethanol + 0.1% triethylamine: acetonitrile + 0.1% triethylamine isocratic mobile phase at 1.2mL/min.

Q.3.14.1 Internal QC for plasma retinol, α– and γ–tocopherol and individual carotenoids

Serum internal quality controls contained physiologically relevant concentrations of each analyte; these were aliquoted for use in each analytical run. Between-batch precision was calculated from these values, as for all other analytes measured in NDNS. Some QC material used in previous NDNS years had limited remaining aliquots and therefore during a review of FSV QCs, the use of some QC materials was discontinued, whilst new QCs were introduced prepared from commercial sources of plasma. In order to provide sufficient concentration range for the analytes in this assay, a larger number of QC materials are required than for standard assays. Assay LOD is 0.09µmol/L for all analytes except α-cryptoxanthin (0.05µmol/L). Tables Q.38 and Q.39 provide a summary of QC data for year 12 and years 13 to 15, respectively.

Table Q.38 Internal QC for fat soluble vitamins (NDNS year 12)[8]

Fat-soluble vitamins QC	µmol/L	QC1	QC068	QC071	QC636775	QC (high)
Retinol	Mean SD Per cent CV	2.64 (0.17) 3%	0.97 (0.04) 4%	1.60 (0.08) 3%	2.35 (0.10) 4%	2.73 (0.08) 3%
α-tocopherol	Mean SD Per cent CV	34.81 (1.33) 4%	18.26 (0.62) 3%	17.01 (0.55) 3%	21.76 (0.43) 2%	32.49 (1.11) 3%
γ-tocopherol	Mean SD Per cent CV	2.32 (0.13) 6%	5.87 (0.27) 5%	3.88 (0.17) 4%	6.93 (0.18) 3%	2.01 (0.09) 5%
Lutein & zeaxanthin	Mean SD Per cent CV	0.50 (0.02) 4%	0.16 (0.01) 5%	0.19 (0.005) 2%	0.21 (0.01) 4%	0.40 (0.02) 6%
α-cryptoxanthin	Mean SD Per cent CV	0.07 (0.01) 9%	0.03 (0.004) 12%	0.03 (0.004) 14%	0.03 (0.002) 8%	0.03 (0.005) 18%
β-cryptoxanthin	Mean SD Per cent CV	0.31 (0.02) 6%	0.14 (0.01) 8%	0.04 (0.004) 10%	0.07 (0.01) 8%	0.16 (0.01) 9%
Lycopene	Mean SD Per cent CV	0.49 (0.03) 6%	1.26 (0.11) 9%	1.23 (0.10) 8%	1.02 (0.07) 7%	0.75 (0.05) 6%
α-carotene	Mean SD Per cent CV	0.13 (0.01) 6%	Not detected	0.05 (0.02) 44%	Not detected	0.17 (0.01) 4%
β-carotene	Mean SD Per cent CV	0.77 (0.04) 5%	0.23 (0.01) 6%	0.28 (0.02) 8.4%	0.15 (0.01) 6%	1.25 (0.04) 04%

[8] Data generated from 6 replicates

Table Q.39 Internal QC for retinol (NDNS years 13 to 15)

Fat-soluble vitamins QC	µmol/L	QC068 (n 27)	QC071 (n 27)	QC636775 (n 26)	QC (high) (n 23)	QC11237645 (n 27)	QC11237830 (n 27)
Retinol	Mean	0.90	1.54	2.30	2.68	1.56	2.30
	SD	0.09	0.10	0.19	0.21	0.13	0.16
	Per cent CV	11	6	8	8	8	7
α-tocopherol	Mean	19.4	17.9	22.3	33.5	25.4	37.8
	SD	1.75	0.54	0.95	0.84	1.13	4.1
	Per cent CV	9	3	4	3	4	11
γ-tocopherol	Mean	5.30	3.52	6.33	1.73	0.82	1.23
	SD	0.48	0.18	0.31	0.10	0.12	0.20
	Per cent CV	20	3	5	6	15	16
Lutein & zeaxanthin	Mean	0.20	0.29	0.29	0.53	0.23	0.37
	SD	0.04	0.06	0.06	0.12	0.04	0.07
	Per cent CV	21	23	21	23	19	19
α-cryptoxanthin	Mean	0.05	0.04	0.04	0.04	0.04	0.08
	SD	0.01	0.01	0.01	0.01	0.01	0.01
	Per cent CV	21	23	23	27	22	12
β-cryptoxanthin	Mean	0.19	0.05	0.10	0.21	0.21	2.21
	SD	0.03	0.02	0.02	0.03	0.03	0.28
	Per cent CV	17.8	28	17	15	13	13
Lycopene	Mean	1.40	1.38	1.13	0.81	1.23	0.65
	SD	0.17	0.12	0.11	0.07	0.11	0.07
	Per cent CV	12	9	10	8	9	10
α-carotene	Mean	Not	0.11	Not	0.23	0.25	0.13
	SD	detected	0.03	detected	0.05	0.05	0.03
	Per cent CV		27		21	19	22
β-carotene	Mean	0.25	0.30	0.17	1.27	1.10	1.30
	SD	0.03	0.02	0.01	0.11	0.11	0.13
	Per cent CV	11	6	5	9	10	10

Q.3.14.2 Accuracy for plasma retinol, α– and γ–tocopherol and individual carotenoids

NIST SRM 1950 was assayed with every run (n= 12) during years 13 to 15 to assess the accuracy of the assay. Results are presented in table Q.40.

Table Q.40 Accuracy for FSVs assessed with NIST SRM 1950[9]

Fat soluble vitamins SRM 1950	Retinol	α-tocopherol	γ-tocopherol	Lutein and zeaxanthin	Lycopene	β-carotene
Mean (µmol/L)	1.50	19.9	3.98	0.19	0.66	0.17
SD (µmol/L)	0.09	0.74	0.21	0.2	0.08	0.01
Per cent CV	6	4	5	12	11	9
n	12	12	12	12	12	12
NIST targets (µmol/L)	1.41 ± 0.122	19.0 ± 0.511	4.1 ± 0.408	0.16 ± 0.049	0.615 ± 0.037	0.15 ± 0.07

Q.3.14.3 EQA for plasma retinol, α– and γ–tocopherol and individual carotenoids

NBL participated in the NEQAS and VITAL (retinol only) EQA schemes during NDNS RP Years 12 to 15, although participation was affected by the break in laboratory work during the COVID-19 period.

Table Q.41 shows NEQAS scores for year 12 and years 13 to 15. It should be noted that there were 14 laboratories returning results for β-carotene and 4 laboratories returning results for lutein + zeaxanthin and lycopene. NBL returned results for α-carotene and α- and β-cryptoxanthins when possible but these were frequently below the limit of detection so there are no cumulative scores consistently available for these assays. Table Q.42 shows the VITAL EQA scheme results for retinol.

[9] Lutein and zeaxanthin are not separated in the method used for NDNS and consequently the reported result is for the combined concentration. For this comparison, we have therefore summed the target concentrations for lutein and zeaxanthin reported for SRM 1950

Table Q.41 External QA (NEQAS) for FSVs (NDNS years 12 to 15)

FSVs NEQAS	Retinol Y12	Retinol Y13 to 15	α-tocopherol Y12	α-tocopherol Y13 to 15	Lutein and zeaxanthin Y12	Lutein and zeaxanthin Y13 to 15	Lycopene Y12	Lycopene Y13 to 15	β-carotene Y12	β-carotene Y13 to 15	Limits
A	131	93	55	101	92	113	34	29	124	70	200
B (per	9	-7.6	3.2	-4.6	-17.1	20.8	-1.3	9	20.9	8.4	±15
C (per	10.9	5.5	3.5	6.7	5.3	10.4	17.8	10	13.2	11.3	20

Table Q.42 VITAL EQA performance for retinol (NDNS years 13 to 15)

VITAL EQA - retinol	Round 38 S1	Round 38 S2	Round 38 S3	Round 39 S1	Round 39 S2	Round 39 S3	Round 40 S1	Round 40 S2	Round 40 S3
Mean (µg/dL)	39.3	45.4	52	29.5	43.9	45.4	43.6	49.9	48.8
Impression (per cent)	13.5	10.3	0.3	6.7	5.6	1.2	16.7	3.3	2.9
Bias (per cent)	0.0	4.3	9.4	5.4	5.1	-2.8	1.7	6.4	0.0
Target (µg/L)	39.3	43.5	47.5	28	41.7	46.7	42.8	46.9	48.8

Q.3.15 25-hydroxyvitamin D (25OHD)

The assay for serum 25(OH)D uses a methanol protein precipitation step followed by liquid-liquid extraction in hexane to separate 25OHD from other analytes in serum samples. Stable isotope labelled internal standards are added during the extraction step and undergo processing identical to the analytes thereby normalizing for sample preparation and instrument variability. Highly specific detection of the analytes and internal standards is accomplished by UPLC-MS/MS analysis using a Waters ACQUITY UPLC system coupled to an AB Sciex QTrap mass spectrometer and the ratio of analyte to internal standard signal is compared to that of a calibration curve to determine analyte concentration.

Analytes are resolved using reversed phase UPLC on a Waters HSS PFP 2.1 × 100 mm 1.8 µm column at 40°C with a 72% methanol +0.1% formic acid isocratic mobile phase at 0.3L/min prior to mass spectrometry analysis.

Total 25(OH)D is calculated from the sum of 25(OH)D_2 and 25(OH)D_3. The epimer of 25 hydroxyvitamin D_3 is not reported in NDNS but is chromatographically separated using this method and so is not included in the total 25OHD.

Q.3.15.1 Internal QC for total 25(OH)D

QC material was Bio-Rad Lyphochek Specialist Immunoassay Control. The concentrations of 25(OH)D2 and 25(OH)D3 controls covered the concentration range observed in NDNS; QC material was analysed at the beginning and at the end of each batch of samples. Tables Q.43 and Q.44 provide a summary of QC data for 25(OH)D2 and 25(OH)D3. An in-house serum QC was also analysed and included C3-epimer 25(OH)D3 (table Q.45). In NDNS, vitamin D status is reported as total 25(OH)D calculated from the sum of 25(OH)2 and 25(OH)D3. Where 25(OH)D2 concentration was below the assay LOQ (6nmol/L) and above from the LOD, a concentration was recorded as LOQ/√2 (4.2nmol/L) to calculate total 25(OH)D.

Table Q.43 Internal QC for $25OHD_2$ (NDNS years 12 to 15)

$25(OH)D_2$	**Bio-Rad QC Year 12**	**Bio-Rad QC Year 12**	**Bio-Rad QC Year 12**	**Bio-Rad QC Year 13 to 15**	**Bio-Rad QC Year 13 to 15**	**Bio-Rad QC Year 13 to 15**	**Bio-Rad QC Year 13 to 15**	**Bio-Rad QC Year 13 to 15**	**Bio-Rad QC Year 13 to 15**
Lot	25251	25252	25283	88701	88702	88703	88721	88722	88723
Mean (nmol/L)	11.9	19.6	53.2	7.1	17.9	49.2	5.2	16.5	48.0
SD (nmol/L)	1.10	0.71	2.20	0.91	1.30	2.31	0.94	2.36	4.86
Per cent CV	9	4	4	13	7	5	18	14	10
n	8	8	7	20	20	18	14	16	16

Table Q.44 Internal QC for $25OHD_3$ (NDNS years 12 to 15)

$25(OH)D_3$	**Bio-Rad QC Year 12**	**Bio-Rad QC Year 12**	**Bio-Rad QC Year 12**	**Bio-Rad QC Years 13 to 15**	**Bio-Rad QC Years 13 to 15**	**Bio-Rad QC Years 13 to 15**	**Bio-Rad QC Years 13 to 15**	**Bio-Rad QC Years 13 to 15**	**Bio-Rad QC Years 13 to 15**
Lot	**25251**	**25252**	**25283**	**88701**	**88702**	**88703**	**88721**	**88722**	**88723**
Mean (nmol/L)	16.0	26.0	77.2	11.3	22.4	69.1	12.4	21.5	48.0
SD (nmol/L)	0.71	0.61	2.7	0.87	1.39	3.85	1.49	1.67	4.86
Per cent CV	5	2	4	8	6	6	12	8	10
n	8	8	7	20	20	18	16	16	16

Table Q.45 In-house serum QC (NDNS years 12 to 15)

25(OH)D QC	Year 12 25(OH)D_2	Year 12 25(OH)D_3	Year 12 C3-epimer 25(OH)D_3	Years 13 to 15 25(OH)D_2	Years 13 to 15 25(OH)D_3	Years 13 to 15 C3-epimer 25(OH)D_3
Mean (nmol/L)	7.9	58.6	9.4	7.56	65.42	8.91
SD (nmol/L)	0.7	1.5	1.2	1.14	3.31	0.80
Per cent CV	9	3	13	15	5	9
n	8	8	8	33	34	33

Q.3.15.2 Accuracy for 25(OH)D

Samples from the Vitamin D EQA Scheme (DEQAS), which have concentrations assigned by the reference measurement procedure at CDC, were analysed every quarter. Table Q.46 summarises NBL performance in the DEQAS scheme.

Table Q.46 DEQAS performance (NDNS years 12 to 15)

25(OH)D DEQAS	Year 12	Years 13 to 15
Mean bias (per cent)	-7.1	-6.8
SD of per cent bias	4.7	9.9
n	5	23

Q.3.15.3 External QA for 25(OH)D

NBL participated in NEQAS and VITAL EQA schemes for vitamin D. Table Q.47 shows the NEQAS scores. NEQAS performance is assessed against the mass spectrometry mean. Table Q.48 summarises performance in the CDC's VITAL EQA scheme against their reference method.

Table Q.47 External EQA (NEQAS) performance for 25(OH)D (NDNS years 12 to 15)[10]

25(OH)D NEQAS	Year 12	Year 13 to 15	Limits
A	Not applicable	Not applicable	Not applicable
B (per cent)	-7	-2.2	±20
C (per cent)	3.7	8.9	25

[10] No A-score is currently reported by NEQAS for this EQA scheme

Table Q.48 VITAL EQA performance for 25(OH)D (NDNS years 12 to 15)

VITAL EQA – vitamin D	Round 38 S1	Round 38 S2	Round 38 S3	Round 39 S1	Round 39 S2	Round 39 S3	Round 40 S1	Round 40 S2	Round 40 S3
Mean (µg/L)	19.5	39.3	107	27.6	71.0	81.1	26.9	24.6	27.6
Impression (per cent)	0.4	4.1	3.3	1.5	16.8	4.7	1.8	2.9	1.5
Bias (per cent)	-11.5	6.2	-2.1	-7.9	11.2	3	0.8	-8.3	1.7
Target (µg/L)	22	37	108.8	30	63.8	78.8	26.6	26.8	27.1

Q.3.16 Serum selenium and serum zinc

Analysis of selenium and zinc concentrations was sub-contracted to the Trace Element Laboratory, UHS.

Selenium (Se) and zinc (Zn) concentrations in serum were determined by inductively coupled plasma mass spectrometry (ICP-MS) using a Perkin Elmer NEXION 300D, equipped with a dynamic reaction cell (DRC).

Prior to analysis the ICP-MS instrument was tuned for optimum signal sensitivity and minimum oxide species and doubly charged ion formation.

Serum was diluted 1 in 50 in 0.01% nitric acid for Zn and 0.5% butanol for Se with rhodium (Rh) as internal standard and run against matrix matched calibration solutions prepared with bovine serum (Sigma-Aldrich). ^{66}Zn and ^{78}Se were determined and the analyte isotope signals were compared against the internal standard, to compensate for matrix related effects and any signal fluctuation due to instrument drift. Se analysis employed dynamic reaction cell (DRC) suppression to overcome argon-based interferences, with methane as the DRC gas.

Trial samples, blanks, calibration standards and QCs were analysed in each batch and the signal data generated was converted to concentrations via the calibration plot.

Q.3.16.1 Internal QC for selenium and zinc

Internal QC material was analysed in conjunction with the blanks, calibration standards and samples. Tables Q.49 and Q.50 summarise QC data for selenium and zinc, respectively.

Table Q.49 Internal QC for selenium analysis (NDNS years 12 to 15)

Selenium	Year 12 AS 1	Year 12 AS 2	Year 12 AS 3	Years 13 to 15 AS 1	Years 13 to 15 AS 2	Years 13 to 15 AS 3
Target (µmol/L)	0.65	1.15	2.10	0.30	0.90	2.10
Acceptable range (µmol/L)	0.50 to 0.74	0.95 to 1.35	1.85 to 2.35	0.15 to 0.45	0.70 to 1.10	1.80 to 2.40
Mean (µmol/L)	0.60	1.11	1.97	0.29	1.02	2.20
SD (µmol/L)	0.04	0.06	0.14	0.03	0.13	0.26
Per cent CV	7	6	7	12	13	12
n	18	14	15	60	56	53

Table Q.50 Internal QC for zinc analysis (NDNS years 12 to 15)

Zinc	Year 12 171	Year 12 172	Year 12 173	Years 13 to 15 171	Years 13 to 15 172	Years 13 to 15 173	Years 13 to 15 181	Years 13 to 15 182	Years 13 to 15 183
Target (µmol/L)	5.0	11.0	21.0	5.0	11.0	21.0	4.1	12.7	19.8
Acceptable range (µmol/L)	3.0 to 7.0	9.0 to 13.0	17.0 to 25.0	3.0 to 7.0	9.0 to 13.0	17.0 to 25.0	3.2 to 5.0	10.2 to 15.3	16.2 to 23.4
Mean (µmol/L)	5.27	10.41	20.85	5.38	11.41	20.61	4.50	13.08	19.76
SD	0.57	0.88	1.56	0.80	0.90	1.27	0.44	1.09	2.04
Per cent CV	11	9	8	15	8	6	10	8	10
n	19	16	15	12	11	10	51	46	46

Q.3.16.2 EQA for selenium and zinc in serum

Southampton Trace Elements participates in TEQAS (NEQAS for Trace Elements, Guildford). Performance in TEQAS scheme is shown in figures Q.2 and Q.3 for selenium and zinc, respectively. Closed circles indicate the rolling average score and open circles observed results for each round.

Figure Q.2 TEQAS serum selenium performance March 2022 to March 2023

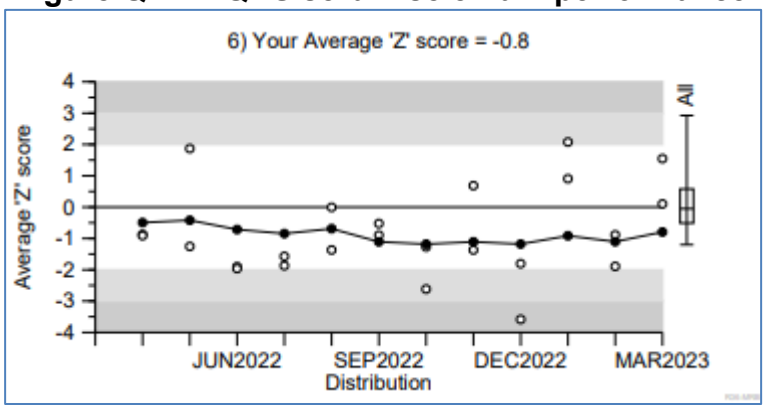
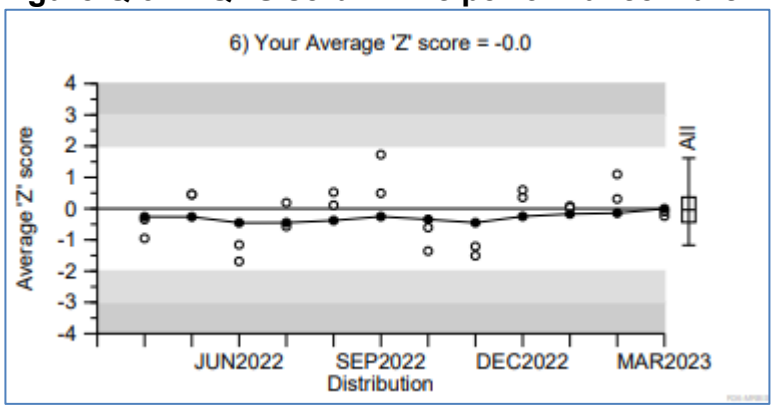


Figure Q.3 TEQAS serum zinc performance March 2022 to March 2023



Q.3.17 Urinary iodine

Analysis of urinary iodine concentration was sub-contracted to the Trace Element Laboratory, UHS. Urine iodine concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS), using a Perkin Elmer NEXION 300D.

Urine samples and QC materials were diluted 1 in 25 in 0.33% blood diluent (ammonia dihydrogen orthophosphate, EDTA and ammonia solution) with rhodium (Rh) as internal standard. ^{127}I isotope signals were measured and compared against the internal standard, to compensate for matrix related effects and any signal fluctuation due to instrument drift. Matrix matched external calibration standards were prepared in human urine for each analytical batch.

Prior to analysis the ICP-MS instrument was tuned for optimum signal sensitivity and minimum oxide species and doubly charged ion formation. Trial samples, blanks, calibration standards and QCs were analysed in each batch and the signal data generated converted to concentrations via the calibration plot.

Q.3.17.1 Internal QC for urinary iodine

In order to establish quality assurance of each analytical batch and inter-batch variation, QC material (Seronorm trace elements urine was analysed in conjunction with blanks, calibration standards and samples (table Q.51).

Table Q.51 Internal QC analysis urinary iodine (NDNS years 12 to 15)

Urinary iodine (Seronorm urine) QC	Year 12 Level 1	Year 12 Level 2	Years 13 to 15 Level 1	Years 13 to 15 Level 2
Target concentration / range (µmol/L)	0.83 (0.66 to 0.99)	2.30 (1.9 to 2.8)	1.34 (1.07 to 1.61)	4.50 (3.6 to 5.4)
Mean measured concentration (µmol/L)	0.76	2.21	1.18	3.88
SD (µmol/L)	0.05	0.13	0.04	0.11
Per cent CV	7	6	3	3
n	54	51	32	33

Q.3.17.2 External QC for urinary iodine

During year 12, the laboratory participated in the Interlaboratory Comparison Program for Metals in Biological Matrices (PCI). Results are provided in table Q. 52. Participation in the PCI scheme was later discontinued and EQA subsequently provided by participation in the Multi-Element External Quality Assurance Scheme (QMEQAS) operated by Centre de Toxicologie du Québec at the Institut national de santé publique du Québec (INSPQ) (table Q.53).

Table Q.52 EQA performance (PCI EQA scheme) for urinary iodine analysis (NDNS year 12)

Urinary iodine TEQAS	Sample I-2013	Sample I-2014	Sample I-2015
Result (µmol/L)	0.91	1.55	0.69
Target (µmol/L)	0.86	1.69	0.71
Per cent bias	5.5	-8.3	-3.2
Z-score	0.8	-1.2	-0.5

Table Q. 53 EQA performance (QMEQAS) for urinary iodine analysis (NDNS years 13 to 15)

Urinary iodine QMEQAS	2204	2205	2206	2213	2214	2215	2304	2305	2306	2313	2314	2315
Result (µg/L)	104	246	192	75	240	155	83	121	245	96	327	123
Z-score	2.2	1.8	2.6	-1.1	-1.0	-0.8	-0.1	-0.3	-0.6	-1.3	-1.1	-0.5

Acknowledgements

We would like to thank personnel at the following institutions for their assistance in local sample processing during NDNS years 12 and 13 prior to and during the evaluation of the postal processing model:

Aberdeen Royal Infirmary, Aberystwyth University, Basildon Hospital, Bristol Royal Infirmary, Countess of Chester Hospital, University Hospital Coventry , Great Western Hospital (Swindon), Hull Royal Infirmary, James Cook Hospital (Middleborough), Kings Mill Hospital (Mansfield), Leicester Royal Infirmary, Middlesex University, Midland Pathology Services, Nationwide Laboratories Poulton, Nuffield Hospital Glasgow, Nuffield Hospital Newcastle, Queen Elizabeth Hospital Woolwich, Royal Bournemouth Hospital, Royal Cornwall Hospital, Royal Devon & Exeter Hospital, Royal Oldham Hospital, Royal Shrewsbury Hospital, Royal Sussex Hospital (Brighton), Sheffield Hallam University, St James Hospital (Leeds), UK Biocentre (Milton Keynes), University of East Anglia (Norwich), University of Dundee, University of North Midlands, University of Surrey, Wellcome Trust Clinical Research Facility Edinburgh, Wellcome Trust Clinical Research Facility Manchester

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