Appendix Q Methods of blood analysis and quality control (QC)/quality assessment (QA)

Q.1 Introduction

Samples of coagulated and ethylenediaminetetraacetate (EDTA) anticoagulated blood were sent directly by post to the Department of Haematology and Department of Clinical Biochemistry and Immunology, Addenbrooke's Hospital, Cambridge (Addenbrooke's) after their collection. Serum samples were obtained by centrifugation of the coagulated blood sample.

The assays listed below were conducted at Addenbrooke's:

- full blood count including haemoglobin and haematocrit (see section Q.2.1)
- serum vitamin B_{12} (see section Q.2.3)
- HbA1c (see section Q.2.7)

Samples of coagulated, EDTA anticoagulated and lithium heparin anticoagulated blood were collected and stored in a cool box, at approximately 4°C, and delivered to a local processing field laboratory within 2 hours of collection. The field laboratories processed blood samples into whole blood, red cells, plasma, serum and metaphosphoric acid stabilised plasma portions. The metaphosphoric acid had been previously prepared and aliquotted at the Medical Research Council Elsie Widdowson Laboratory (MRC EWL) and delivered by courier on dry ice to each field laboratory. Blood sample sub-fractions were stored frozen at a maximum of -20°C (typically at -40°C) at field laboratories for a period of 6 to 8 weeks, before the samples were transported to MRC EWL on dry ice, where they were stored frozen, at -80°C, until further subdivided and analysed.

The assays listed below were conducted at MRC EWL:

- serum C-reactive protein(CRP), using a high-sensitivity assay (see section Q.2.2)
- holotranscobalamin (holoTC; "active B12"; see section Q.2.4)
- serum total cholesterol, high-density lipoprotein (HDL)

cholesterol and low-density lipoprotein (LDL) cholesterol (see section Q.2.5)
- serum triglycerides (triacylglycerols) (see section Q.2.6)
- plasma glucose (see section Q.2.8)
- plasma ferritin (see section Q.2.9)
- plasma vitamin C (see section Q.2.10)
- erythrocyte transketolase activation coefficient (ETKAC) for thiamin status (see section Q.2.11)
- erythrocyte glutathione reductase activation coefficient (EGRAC) for riboflavin status (see section Q.2.12)
- plasma vitamin B_6 (pyridoxal-5-phosphate (PLP) and 4-pyridoxic acid (PA)) (see section Q.2.13)
- serum folate (see section Q.2.14)
- plasma retinol (see section Q.2.15)
- plasma α-tocopherol (see section Q.2.15)
- plasma γ-tocopherol (see section Q.2.15)
- plasma individual carotenoids; α–carotene, β–carotene, α–cryptoxanthin, β–cryptoxanthin, lycopene, lutein and zeaxanthin (see section Q.2.15)
- plasma 25-hydroxyvitamin D (25-OHD) (see section Q.2.16)
- plasma creatinine (see section Q.2.17)
- plasma selenium (see section Q.2.18)
- plasma zinc (see section Q.2.18)

Whole blood folate concentrations in the NDNS RP blood samples were measured at the Centers for Disease Control and Prevention (CDC) in Atlanta, USA (see section Q.2.14).

Appendix T provides details for analytes that were measured but are not included in the present report. Data for all analytes measured in fieldwork years 1-9 will be deposited at the UK Data Service.

During the 9-year period there were a few changes in analytical procedures where the analytical portfolio was amended or where methods were superseded. All changes in analytical method or platform were monitored by rigorous comparison studies to ensure that data obtained during the RP were comparable over time. These changes are shown in Table A which, for completeness, lists all analytical changes irrespective of whether the analytes are included in this report.

Some analytes were not measured over the whole 9 years covered by this report:

Analytes measured only during Years 1 to 5:

- Homocysteine
- Serum transferrin receptors

Analytes measured only during Years 6 to 9:

- Holotranscobalamin (”active vitamin B_{12}”)
- Urinary iodine (spot urine sample)

Table A: Changes in laboratory analytical methodology introduced during Years 1 to 9 of the NDNS RP

Analyte	Method at start of RP	Method changed to:	Comment	Change reported:
Ferritin	Immunonephelometry on Dade Behring ProSpec Year 1 to 5	Immunoturbidimetry on Siemens Dimension Xpand Years 6 to 9	No difference to data.	Years 5 and 6 (combined) report. Appendix Q, section Q.2.7
25-OHD	Diasorin Liaison method, plasma: Years 1,2,3 and part of Year 4	Reformulated Diasorin Liaison method, serum: part of Year 4 and Years 5 and 6	All standardised through Vitamin D Standardisation Program	Years 1 to 4 (combined) report, Appendix P, section P.2.14 and Years 7-8 report, Appendix Q, section Q.2.16
Retinol, tocopherol and carotenoids ("fat-soluble vitamins"	HPLC, Thurnham method Years 1 to 4	HPLC, Sowell method Years 5 to 9	Conversion factors provided	Years 5 and 6 (combined) report. Appendix Q section Q.2.14
Creatinine	Jaffe on Siemens Dimension Years	Enzymatic on Siemens Dimension	Manufacturer withdrew Jaffe reagent. Enzymatic	Years 7 to 8 (combined) report. Appendix Q

Analyte	Method at start of RP	Method changed to:	Comment	Change reported:
	1 to 7	Years 8 to 9	method more specific.	section Q.2.17
CRP, total cholesterol. HDL cholesterol, triglycerides	Dimension RXL at Addenbrooke's Years 1 to 5	Dimension Xpand at MRC EWL Years 6 to 9	Same manufacturer and analytical methods; change of location of analysis	Years 5 and 6 (combined) report. Appendix Q, section Q.2.2 (CRP) and Q.2.5 (lipids)
Serum folate	LC-MS/MS[1] at CDC Years 1 to 6	LC-MS/MS[1] at EWL for Years 7 to 9	CDC results for Years 1 to 4 revised and reissued Nov 2017 CDC method introduced to EWL; no difference in results between revised CDC and EWL methods.	Folate report Years 1 to 4 (combined), version 2.0 published November 2017 Years 7 to 8 (combined) report. Appendix Q, section Q.14.1

[1] Liquid chromatography-tandem mass spectrometry

Q.2 Method

Details of the method of analysis and the associated quality control (QC) procedures for each analyte are given in sections Q.2.1 to Q.2.18. Where appropriate, the results of these procedures are also shown. Internal quality control samples were run in every batch to assess assay precision for each analyte; results are tabulated below. Accuracy was assessed by comparisons with target values (determined by the manufacturer using appropriate reference materials) and/or results obtained by other laboratories by taking part in EQAS (external quality assessment (QA) schemes) for those analytes where such schemes were available.

Q.2.1 Full blood count including haemoglobin and haematocrit

Full Blood Count was analysed using a Siemens Advia 2120, which uses the Coulter Principle[1,2] to count the red blood cells, mean cell volume (MCV), white blood cells and platelet counts. Haemoglobin was measured by photometric measurement. Other parameters such as the mean cell haemoglobin (MCH), haematocrit (Hct) and red cell distribution width (RDW) were calculated from the above measured parameters.

Haemoglobin was measured spectrophotometrically at 525nm by a photocell in a sample that was diluted 1:256 (final) with isotonic diluent and lysing solution. The red cells were destroyed with a lysing agent releasing the haemoglobin into solution, which enabled the white blood cell count to be estimated using the Coulter Principle (impedance counting of the white blood cells)[1,2] without interference by red cells. The same lysing reagent also converted the haemoglobin to cyanmethaemoglobin.

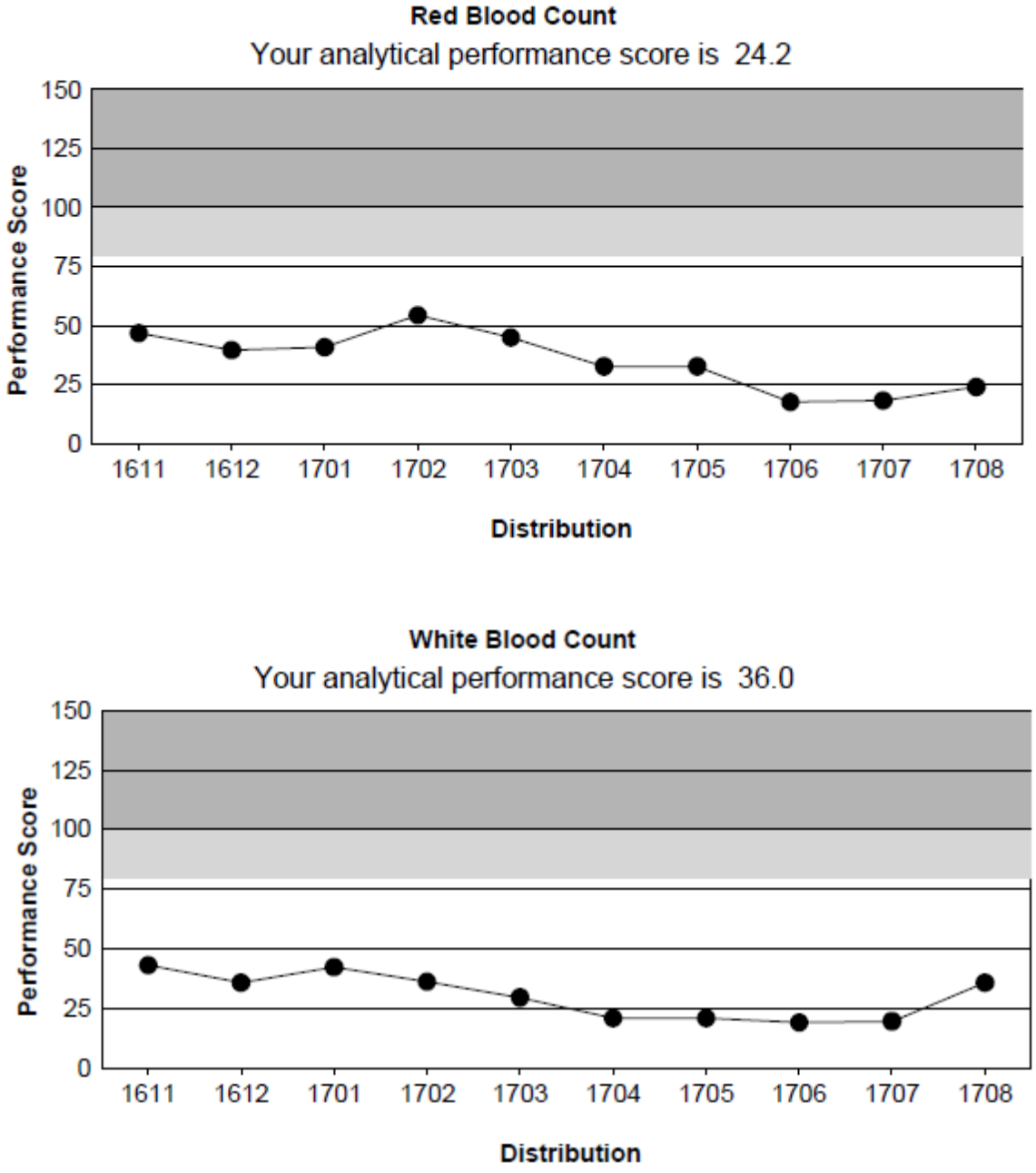
Q.2.1.1 QCs for full blood count including haemoglobin and haematocrit

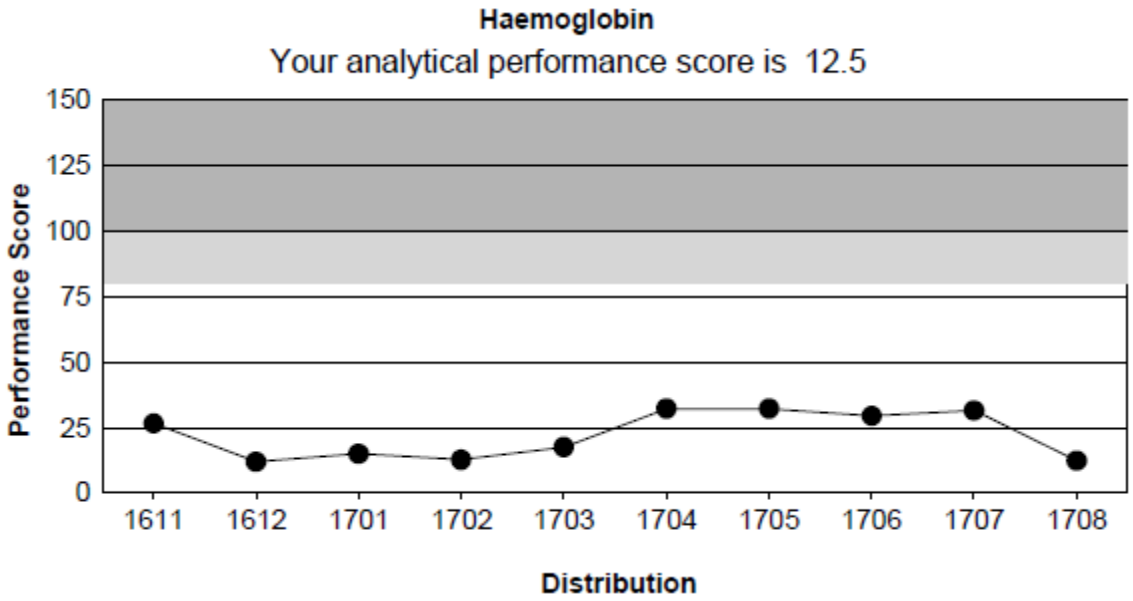
The QC results of the instruments measuring full blood count at Addenbrooke's are monitored continually, and analysis is stopped if the results are not satisfactory, ensuring that results are only reported if the analysis is within the QC parameters set by the Laboratory Manager. However it is not possible to extract these results for reporting. Therefore no internal QC data can be provided but validation of results is achieved automatically.

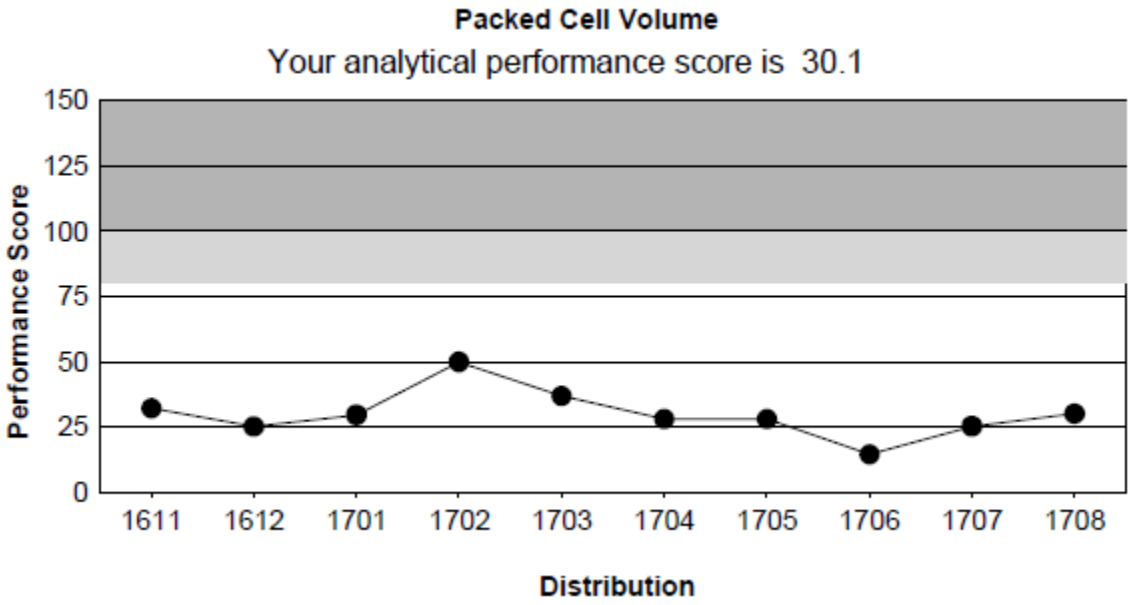
Quality of results was also assessed externally through UKNEQAS. NEQAS results are compared against the All Laboratories Trimmed Mean (ALTM) calculated for laboratories in the NEQAS scheme using the same analyser and method as that used by Addenbrooke's. Figure Q.1 shows, as an example, the cumulative performance charts from UK NEQAS returns for August 2017 of 1 of the 4 Siemens Advia 2120 instruments in use in the laboratory). The "distribution" axis indicates the year and month of the UKNEQAS return. Results within the white area of the charts

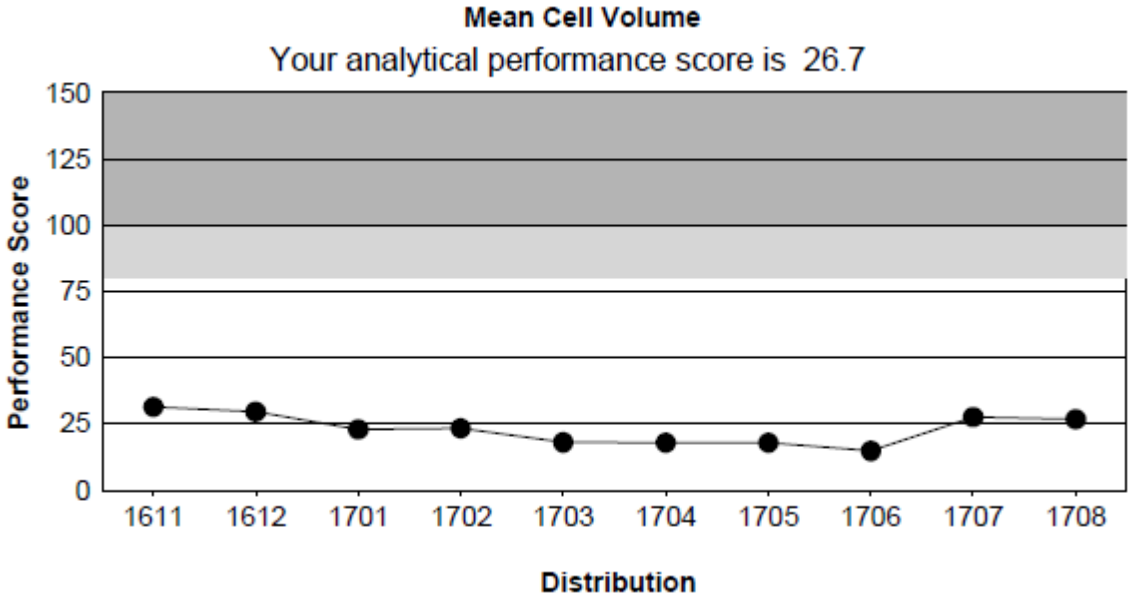
indicate acceptable performance as determined by UKNEQAS. “Performance index” is derived by the NEQAS administrators as a function of the deviation of the laboratory from the consensus mean; the number given is for the most recent return and the plot shows the scores obtained from November 2016 to August 2017. The dark shaded area indicates unacceptable performance and the paler area indicates a borderline situation.

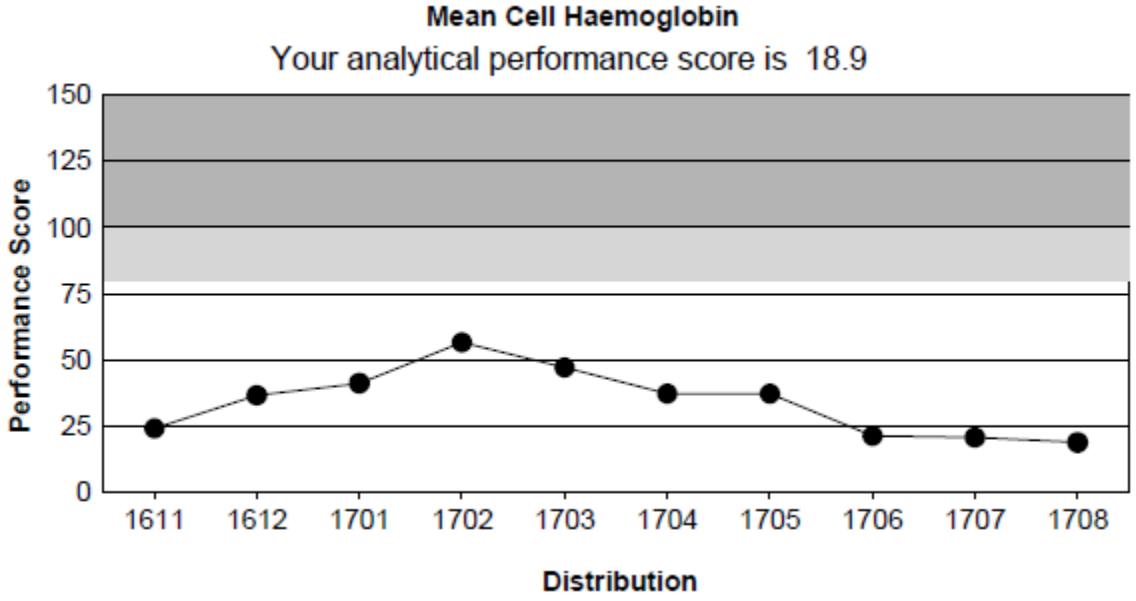
Figure Q.1 Illustrative overall performance charts for UKNEQAS (NDNS RP Year 9)

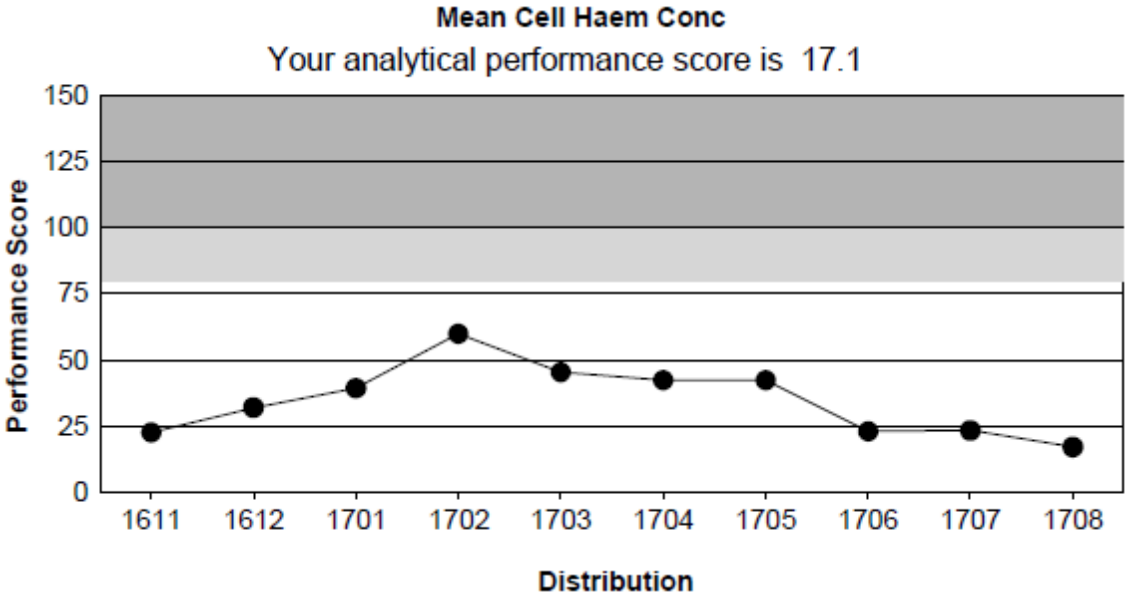


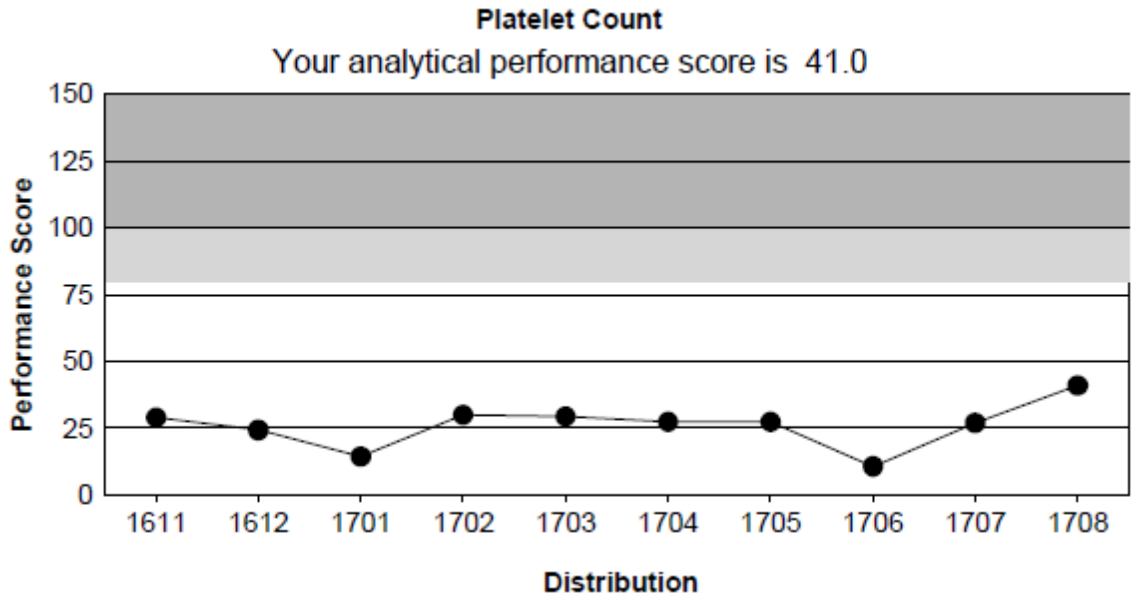

Haemoglobin
Your analytical performance score is 12.5
150
125
100
75
50
25
0
Performance Score
1611
1612
1701
1702
1703
1704
1705
1706
1707
1708
Distribution


Packed Cell Volume
Your analytical performance score is 30.1
150
125
100
75
50
25
0
Performance Score
1611
1612
1701
1702
1703
1704
1705
1706
1707
1708
Distribution


Mean Cell Volume
Your analytical performance score is 26.7
Performance Score
150
125
100
75
50
25
0
1611
1612
1701
1702
1703
1704
1705
1706
1707
1708
Distribution


Mean Cell Haemoglobin
Your analytical performance score is 18.9
Performance Score
150
125
100
75
50
25
0
1611
1612
1701
1702
1703
1704
1705
1706
1707
1708
Distribution





Q.2.2 Serum C-reactive protein (CRP) using a high-sensitivity assay

C-reactive protein (CRP) was assayed using a high-sensitivity (extended range) assay on a Dimension Xpand clinical Chemistry analyser. The CRP method is based on a particle enhanced turbidimetric immunoassay (PETIA) technique, giving high sensitivity by extending the detection range down to 1.0mg/L. Latex particles coated with antibody to CRP aggregate in the presence of CRP in the sample. The increase in turbidity that accompanies aggregation is proportional to the CRP concentration.

Q.2.2.1 Internal QCs for CRP

Table Q.1 shows imprecision data in Year 9.

Table Q.1 Internal QCs for CRP (NDNS RP Year 9)

	QC1	QC2	QC3
Mean (mg/L)	1.22	17.15	49.28
SD (mg/L)	0.33	0.38	1.02
% cv	27.15	2.21	2.08
n	37	38	38

Q.2.2.2 External QCs for CRP

External QC was achieved through the UKNEQAS CRP scheme which distributes samples to a large number of laboratories for comparison of the results obtained.

Table Q.2 External QCs for CRP (NDNS RP Year 9)

CRP	NEQAS	Year 9
	mean % bias	3.0
	SD % bias	5.7
	n	22

"Ultra-sensitive" CRP	NEQAS	Year 9

	mean % bias	15.2
	SD % bias	17.6
	n	22

Q.2.3 Serum vitamin B_{12}

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.2.3.1 Internal QCs for vitamin B_{12}

The QC results of the instrument measuring Vitamin B_{12} at Addenbrooke's 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 no longer possible to extract these results for reporting. Therefore in order to monitor between-batch precision, control material was aliquoted at MRC EWL laboratories and included in the monthly delivery of NDNS RP samples to Addenbrooke's.

Table Q.3 Internal QCs for vitamin B_{12} (NDNS RP Year 9)

	QC 1	QC 2	QC 3	QC 4	QC 5
Mean (ng/L)	**124**	**201**	**127**	**565**	**799**
SD (ng/L)	**13.1**	**15.9**	**14.6**	**20.1**	**36.9**
% cv	**10.6**	**7.9**	**11.5**	**3.6**	**4.6**
n	**11**	**11**	**11**	**9**	**9**

Q.2.3.2 External QCs for vitamin B_{12}

QC was achieved through the UK NEQAS Haematinics scheme.

Charts relating to performance during Year 9 are reproduced below with permission of Addenbrooke's and the NEQAS Haematinics Scheme organisers.

Figures Q.2 and Q.3 show the performance of the assay as demonstrated by the overall performance "A" score which assesses bias, consistency of bias and clinical acceptability of performance over approximately 6 months for each graph. Bias is calculated to the All Laboratories Trimmed Mean (ALTM). Small numbers (area shaded white) indicate close agreement with the ALTM.

Figure Q.2 Illustrative overall performance chart for UKNEQAS for vitamin B_{12} (NDNS RP Year 9; October 2016 to April 2017)

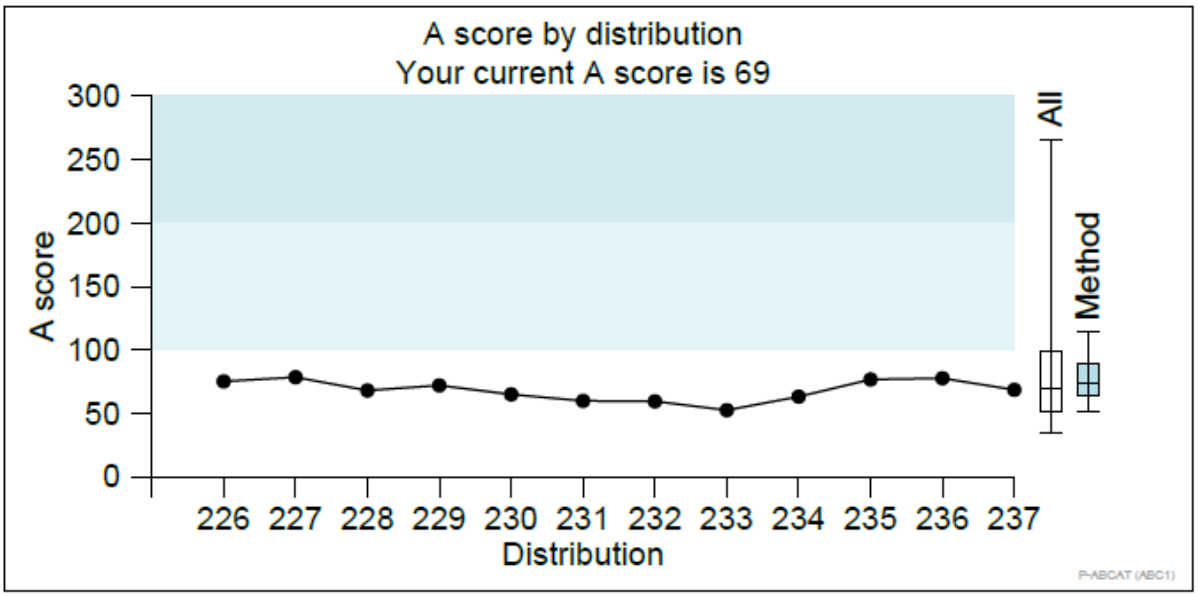
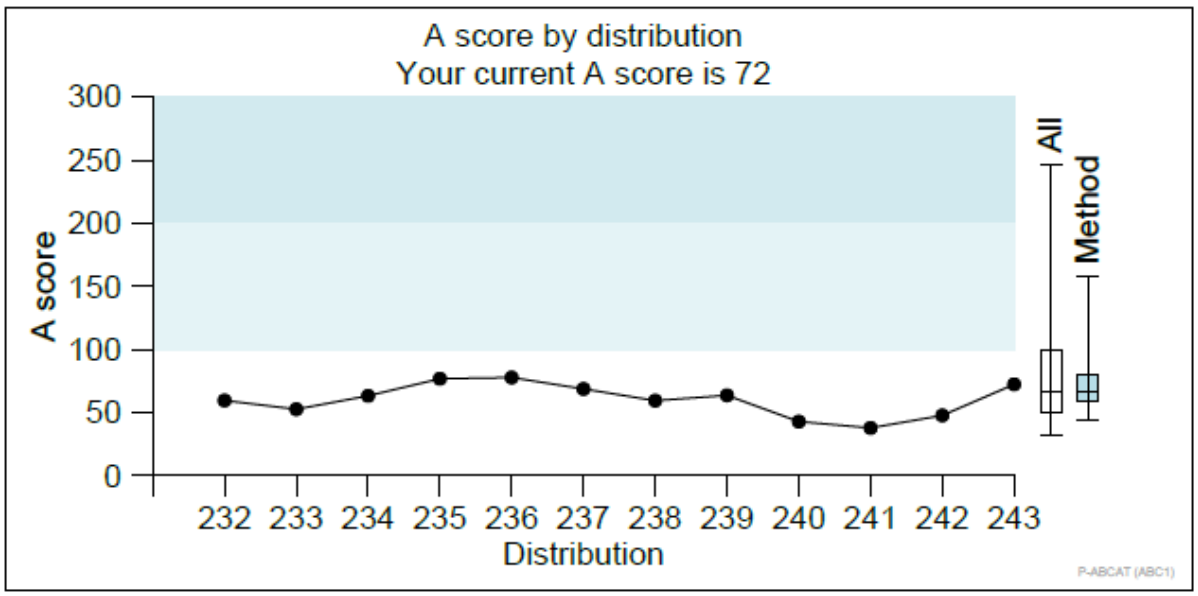


Figure Q.3 Illustrative overall performance chart for UKNEQAS for vitamin B_{12} (NDNS RP Year 9; May 2017 to October 2017)



Q.2.4 Holotranscobalamin (holoTC); "active B_{12}"

Vitamin B_{12} (cobalamin) is transported in the circulation bound to transcobalamin (TC) (10-30%) and to haptocorrin (HC) (70-90%). 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.

HoloTC is the only form of vitamin B_{12} that can be taken up by cells in the body; holoHC is biologically inert. The TC protein alone transports vitamin B_{12} from its site of absorption in the ileum to tissues and cells where it is used as a co-enzyme for essential cellular functions such as DNA synthesis. It has been suggested that as holoTC has a shorter circulating half-life than holoHC the earliest change that occurs on entering negative vitamin B_{12} balance is very likely to be a decrease in serum holoTC concentration.[3] Several studies have been published which conclude that holoTC would be a better indicator of vitamin B_{12} status than total serum B_{12}.[4,5] As expected, holoTC concentrations are low in patients with biochemical signs of vitamin B_{12} deficiency.[6] Low values have been reported in vegetarians,[7] vegans[8] and in populations with a low intake of vitamin B_{12}.[9] Low levels of holoTC but not total B_{12} in serum were reported in patients with Alzheimer's disease compared to levels in a healthy control group.[10] HoloTC concentrations are said to reflect vitamin B_{12} status, independent of recent absorption of the vitamin.[11]

The 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 react to form a "sandwich". The enzyme label is quantitated using a coloured substrate and the absorbance read in a microplate spectrophotometer. Concentration is interpolated from a calibration curve. The assays for Year 9 were conducted manually at MRC EWL.

Q.2.4.1 Internal QCs for holoTC

QC samples were supplied by the manufacturer and included in every assay. Table Q.4 shows QC data for a period covering the analysis of Year 9 samples.

Table Q.4 Internal QCs for holoTC (NDNS RP Year 9)

	Low Control 25 pmol/L (15-35)	High Control 60 pmol/L (36-84)	QA 73.8 pmol/L (52.2-95.5)

Mean (pmol/L)	25.2	61.1	77.1
SD (pmol/L)	2.19	6.42	8.96
% cv	8.69	10.52	11.62
n	22	22	22

Q.2.4.2 External QA for holoTC

NEQAS are conducting a pilot study for external assessment of holoTC results. The short time-scale over which the NDNS RP Year 9 assays were conducted did not allow for "live" participation in this pilot scheme. NEQAS samples received during the year were stored at -80^{o}C and assayed retrospectively alongside the Year 9 samples, the results being compared with the NEQAS ALTM for results obtained by other laboratories, most of whom used the method automated on the Abbot Architect analyser. This validates the accuracy of Year 9 results.

Table Q.5 External QA for holoTC (NDNS RP Year 9)

	UK NEQAS pilot	Year 9
Holotranscobalamin (holoTC)	mean % bias*	0.25
	SD of % bias	5.4
	n	9

* relative to method mean i.e. ELISA

Q.2.5 Serum total, high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol

The total cholesterol method on the Siemens Dimension analyser is based on the principle first described by Stadtman[12] and later adapted by other workers, including

Rautela and Liedtke.[13] Cholesterol esterase (CE) catalyses 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 (CO) to form cholest-4-ene-3-one and hydrogen peroxide. In the presence of horseradish peroxidase (HPO), the hydrogen peroxide thus formed is used to oxidize N,N-diethylaniline-HCl/4-aminoantipyrine (DEA-HCl/AAP) to produce a chromophore that absorbs at 540nm.

The AHDL cholesterol assay is a homogeneous method for directly measuring HDL cholesterol concentrations.

The method is based on accelerating the reaction of cholesterol oxidase (CO) 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 (CE) and chromagenic coupler to develop colour for the quantitative determination of HDL-C. The assays were conducted on a Siemens Dimension Xpand analyser.

Q.2.5.1 Internal QCs for total cholesterol

Table Q.6 Internal QCs for total cholesterol (NDNS RP Year 9)

	QC 1	QC 2	QC 3
Mean	3.30	4.46	7.18
SD	0.07	0.06	0.09
% CV	2.03	1.45	1.26
n	58	58	57

Q.2.5.2 Internal QCs for HDL cholesterol

Table Q.7 Internal QCs for HDL cholesterol (NDNS RP Year 9)

	QC 1	QC 2	QC 3
Mean	1.60	1.58	3.07
SD	0.06	0.06	0.09
% CV	3.58	3.89	3.07
n	61	61	60

Q.2.5.3 External QCs for total and HDL cholesterol

External QC was achieved through UKNEQAS and also the Randox International QA Scheme (RIQAS); NEQAS pooled samples are unsuitable for the total cholesterol method used by the Siemens Dimension instruments. Table Q.8 indicates the percentage deviation of results obtained by MRC EWL from the target concentration for Year 9. These have been calculated at MRC EWL.

Table Q.8 NEQAS and RIQAS results for lipid analyses - deviation from target Concentration (NDNS RP Year 9)

	RIQAS	Cycle 35	Cycle 36
total cholesterol	Mean % bias	-0.91	-1.09
	SD of bias	0.33	0.35
	n	11	11

	UKNEQAS	Year 9
HDL cholesterol	Mean % bias	0.5
	SD of % bias	5.8
	n	37

Q.2.6 Serum triglycerides (triacylglycerols)

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 (triacylglycerols). The sample is incubated with lipoprotein lipase (LPL) enzyme reagent that converts triglycerides (triacylglycerols) 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. The assays were conducted on a Siemens Dimension Xpand.

Q.2.6.1 Internal QCs for serum triglycerides (triacylglycerols)

Table Q.9 Internal QCs for serum triglycerides (triacylglycerols) (NDNS RP Year 9)

	QC 1	QC 2	QC 3
Mean	1.49	2.45	3.95

SD	0.03	0.04	0.06
% CV	2.02	1.83	1.48
n	59	58	58

Q.2.6.2 External QA for serum triglycerides (triacylglycerols)

External QA was achieved through UKNEQAS and RIQAS. Table Q.10 indicates the percentage deviation of results obtained from the target concentration. These have been calculated at MRC EWL and are included with the permission of the Scheme organisers.

Table Q.10 UKNEQAS and RIQAS results for triglycerides (triacylglycerols) (NDNS RP Year 9)

	UKNEQAS	Year 9
Triglycerides	mean % bias	-4.6
	SD of % bias	4.5
	n	37

	RIQAS	Cycle 35	Cycle 36
Triglycerides	mean % bias	-2.55	-5.08
	SD of bias	0.65	1.18

	n	11	11

Q.2.7 Haemoglobin A1c (HbA1c)

Haemoglobin A1c (HbA1c) was measured by HPLC at Addenbrooke's, using the Tosoh Automated Glycohemoglobin Analyser. Results are traceable to the US National Glycohemoglobin Standardization Program and to the International Federation of Clinical Chemistry.

Q.2.7.1 Internal Quality Control for HbA1c

The internal QC results in table Q.11 show good precision over Year 9.

Table Q.11 Internal QCs for HbA1c (NDNS RP Year 9) *

HbA1c	TOSOH low (G8L) LOT AB5040	TOSOH high (G8L) LOT AB5040	Biorad Low (G8L) LOT 33911	Biorad High (G8L) LOT 33912
Mean mol/mol	28.9	82.9	33.9	82.1
SD	0.6	0.9	0.5	0.9
%CV	2.2	1.1	1.5	1.1
n	192	192	192	192

*Example – 1 of 3 instruments, QCs used between Aug 2016 and April 2017

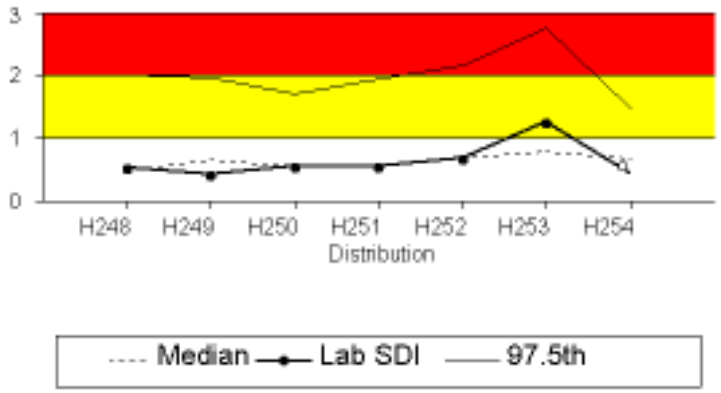
Q.2.7.2 External QA for HbA1c

The HbA1c external assessment scheme calculates standard deviation index (SDI) for each distribution – this is "total error" and includes both inaccuracy and imprecision. Figure Q.4 shows the SDI calculated for each distribution in NDNS RP Year 9. Figure Q.4 is an example of an external QA plot for the instruments at Addenbrooke's and a summary of the scores for each of their instruments in one of the quarterly distributions. Scores <1 indicate good performance. The solid line shows the performance of Addenbrooke's and the dotted lines are the national median and 97.5 percentile performance. These graphs demonstrate that Addenbrooke's performs close to the median UK performance for this assay.

Figure Q.4 External QA for HbA1c (NDNS RP Year 9) compared with all users of Siemens Dimension EXL

Lab: HV . Scheme: Glycated Haemoglobin. Distribution Code: H254.
Distribution Date: 27/06/17. Final. Report Issued: 17/07/17

This Distribution	
Overall Lab SDI:	0.43
Median All Laboratory:	0.68
97.5th centile:	1.49



All SDI Ranges	
< 1	Good
1 - 2	Acceptable
> 2	Poor

Section SDI scores for this distribution

Section	EXL	G8 Left-10173304	G8 Right -12842102	WDEC/WESTON G8
Overall	**0.51**	**0.45**	**0.46**	**0.30**
HbA1c IFCC	0.51	0.45	0.46	0.30

Q.2.8 Plasma glucose

Glucose was measured at MRC EWL on the Siemens Dimension Xpand using hexokinase coupled to glucose-6 phosphate dehydrogenase. Quantitation was by measurement of NADH at the endpoint using bichromatic spectrophotometry at 340nm and 383nm.

Q.2.8.1 Internal QC for plasma glucose

Control serum was obtained commercially containing low, medium and high concentrations of glucose and was included in each run. Results were checked to ensure they fell within the manufacturer's target range. The results in table Q.12 indicate good between-batch consistency for glucose results during Year 9.

Table Q.12 Internal QCs for glucose (NDNS RP Year 9)

	QC 1	QC 2	QC 3
Mean mmol/L	3.19	7.56	20.65
SD mmol/L	0.11	0.15	0.26
CV %	3.38	1.99	1.28
n	47	45	44

Q.2.8.2 External QA for plasma glucose

External QA was through the UK NEQAS scheme. Table Q.13 shows the percentage bias relative to the target concentration in glucose results from MRC EWL during Year 9.

Table Q.13 External QA (UKNEQAS) results for glucose (NDNS RP Year 9)

	UK NEQAS	Year 9
Glucose mmol/L	Mean % bias from ALTM	4.2
	SD of bias	4.1
	n	63

Q.2.9 Plasma ferritin

This assay was performed on the Siemens Dimension Xpand analyser which uses chromium dioxide particles coated with specific antibodies to human ferritin. The assay is coupled to a colour reaction and the intensity of colour is determined by the concentration of ferritin in the sample. Ferritin is quantitated by comparison to calibrants of known concentration.

Q.2.9.1 Internal QCs for plasma ferritin

Control serum was obtained commercially containing low, medium and high concentrations of ferritin and was included in each run. Results were checked to ensure they fell within the manufacturer's target range. The results in table Q.14 indicate good between-batch consistency for ferritin results during Year 9.

Table Q.14 Internal QCs for ferritin (NDNS RP Year 9)

	Lyphocheck Immunoassay		
	1	2	3
Target (µg/L)	59.8	124	311
Range (µg/L)	44-76	95-152	250-372
Mean (µg/L)	58.8	114.7	292.6

SD (µg/L)	5.5	11.0	14.2
% CV	9.3	9.6	4.8
n	31	31	31

Q.2.9.2 External QA for plasma ferritin

External QA was through the UK NEQAS Haematinics scheme.

Table Q.15 shows the percentage bias relative to the target concentration in ferritin results from MRC EWL during Year 9.

Table Q.15 Summary of NEQAS bias assessment (NDNS RP Year 9)

	UK NEQAS	Year 9
Ferritin µg/L	mean % bias	-1.7
	SD of % bias	8.2
	n	36

Q.2.10 Plasma vitamin C

This assay is based on the procedure described by Vuilleumier and Keck.[14] Samples are stabilised immediately after separation using an equal volume of 10% metaphosphoric acid.

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 OPTIMA plate reader, which measures the fluorescence.

Q.2.10.1 Internal QCs for plasma vitamin C

QC samples were made in-house by spiking ascorbic acid-depleted plasma. The results in table Q.16 indicates good between-batch consistency for vitamin C (ascorbic acid) measurements during Year 9.

Table Q.16 Internal QCs for vitamin C (NDNS RP Year 9)

	QC1 11.9 (7.6-16.2)	QC2 35.7 (23.0-48.4)	QC3 59.0 (45.3-72.7)
Mean (µmol/L)	11.0	32.3	50.3
SD (µmol/L)	1.5	1.6	1.7
% CV	13.3	4.8	3.4
n	82	82	82

Q.2.10.2 External QCs for vitamin C

Table Q.17 External QCs for vitamin C (NDNS RP Year 9)*

RCPAQAP	cycle 35	cycle 36	cycle 37
Mean imprecision (%)	3.4	4.7	6.4
%CV of imprecision	4.7	6.5	9.2

Average Bias (%)	-14.6	-15.5	-13.4

* Bias and imprecision as assessed by Royal College of Pathologists of Australasia QA Program (RCPAQAP).

The bias is calculated from the ALTM. The great majority of the participating laboratories use the Chromsystems HPLC method and calibrator, which are different from the MRC EWL fluorescence method and calibrator; MRC EWL results show a bias relative to the Chromsystems methods. The MRC EWL calibrators are made up gravimetrically from the AnalaR ascorbic acid, the purest grade available. This methodological difference shows as a deviation of our results from the Chromsystems consensus. The consistency of the bias indicates consistency in MRC EWL results, as does the low "% imprecision" result. The MRC EWL method shows excellent back compatibility with previous studies which used the same methodology.

Q.2.11 Erythrocyte transketolase activation coefficient (ETKAC) for thiamine status

This assay is based on that of Vuilleumier *et al*[15] and depends on the coupling of pyridine nucleotide oxidation to glycerol phosphate dehydrogenase (GDH) (NADH linked), which produces glycerol-3-phosphate after the transketolase-catalysed conversion of ribose-5-phosphate. The rate of oxidation of NADH is monitored at 340nm, on the Multiskan FC plate-reader, in which instrument temperature equivalence across the plate can be achieved. Thiamin status is assessed using the activation coefficient, which is the ratio of cofactor-stimulated activity to the basal activity without any added cofactor.

This method is identical in principle with its predecessor on the Cobas Fara platform. An analysis of bias between the results determined on the 2 platforms was performed ahead of the NDNS RP.

There are no available sources of erythrocytes with known ETKAC; therefore unassayed material was prepared in-house. Erythrocytes from the National Blood Transfusion Service (NBTS) or commercial sources were washed to remove the buffy coat and lysed by threefold dilution with water. This lysate was stored at -80^{o}C in single-use aliquots. The lysate was stored and assayed both neat and further diluted x2 with water. No source of thiamin deficient erythrocytes has been identified with which to prepare a lysate giving high ETKAC; similarly none of the participant's samples had resulted in an ETKAC in the deficient range (greater than 1.25).

QC material "K" is a dilute lysate in which the reaction rates are very low, included to assess assay performance in similarly dilute samples.

Q.2.11.1 Quality control results for ETKAC

Descriptive statistics in table Q.18 for internal QCs indicate good batch-to-batch consistency of ETKAC results during Year 9.

There are no external QA or QC schemes available for ETKAC.

Table Q.18 Internal QCs for ETKAC (NDNS RP Year 9)

Internal control	C	K*	P
Mean	1.07	1.07	1.08
SD	0.04	0.08	0.03
% CV	3.86	7.54	3.01
n	25	20	24

* selected to have very low reaction rates

Q.2.12 Erythrocyte glutathione reductase activation coefficient (EGRAC) for riboflavin status

This assay was developed from the original manual technique developed by Glatzle *et al*[16] and was adapted to the 'in-house' method using a Cobas Fara centrifugal analyser, which in turn has been modified to an assay carried out on microplates and read on a Thermo iEMS plate reader. The ratio of flavin adenine dinucleotide (FAD) stimulated to unstimulated activity is the EGRAC and is a measure of riboflavin status. The method is a kinetic test with decreasing absorbance and 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. The assay is conducted at a low final concentration of FAD (1.5µM), which is necessary 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 assay is in principle identical to its predecessor which used the Cobas Fara. A comparison of results obtained on the two platforms was performed using NDNS RP

Year 1 quarter 1 samples, which showed good agreement. The accepted threshold for riboflavin adequacy is EGRAC below 1.30.

Recent research has indicated that the 1.30 threshold may be set too low, so giving an overestimate of the prevalence of functionally-significant low riboflavin status. It has been recommended that the EGRAC threshold should be raised to a level above 1.30 to better recognise riboflavin inadequacy; this requires further consideration.[17]

Q.2.12.1 QCs for EGRAC

There is no control with known EGRAC available, therefore washed erythrocytes were prepared in-house, aliquoted for single use and stored at -80^{o}C. In addition to the native samples a saturated control was made by incubation with FAD before aliquoting. These 3 controls were run on each assay plate. There is no external QA scheme available for EGRAC.

Q.2.12.1.1 Internal QC results during Year 9

Descriptive statistics in table Q.19 for internal QCs indicate good batch-to-batch consistency of EGRAC results during Year 9.

Table Q.19 Internal QCs for EGRAC (NDNS RP Year 9)

Internal control	A	C	X
Mean	2.20	1.54	0.97
SD	0.04	0.03	0.01
% CV	1.98	2.14	1.12
n	20	19	20

Q.2.13 Plasma vitamin B6 (PLP and PA)

A reverse-phase high performance liquid chromatography (HPLC) method with post column derivatisation and fluorimetric detection was used to determine pyridoxal-5-phosphate (PLP) and 4-pyridoxic acid (PA) in plasma.[18]

Q.2.13.1 Internal QCs for vitamin B6

QC was achieved through internal procedures. QC material was produced by spiking human plasma with aqueous solutions of PLP and PA. The final QC concentration was designed to match typical mid-range human samples. The QC material was spiked so that the additional aqueous content represented only 0.02% of the total medium. Duplicate analysis of the QC material was performed with each analytical run, and the mean recovery of added PLP and PA was calculated for each run. When the mean percentage recovery was outside of the range 95 to 105% of nominal the analytical results for that run were corrected accordingly.

From Year 5 plasma from 2 individuals (designated as A02 and A07) was used as additional quality assurance. These samples, analysed in duplicate in each run, plus the existing unspiked plasma (K4082310) allowed drift monitoring over the range of PLP and PA concentrations seen in the NDNS RP. The spiked QC plasma was used to monitor accuracy and to adjust the concentration of samples and unspiked QC controls if required.

Table Q.20 Internal QCs for PLP and PA (unspiked plasma) (NDNS RP Year 9)

	PLP A02	PA A02	PLP A07	PA K4082310
mean	49.7	127.8	25.5	36.7
SD	3.6	7.5	2.0	1.9
% CV	7.2	5.9	7.8	5.1
n	26	26	24	26

Table Q.21 Internal QCs for PLP and PA (spiked plasma) (NDNS RP

Year 9)

	PLP	PA
target	55.9	55.0
mean	55.9	54.4
SD	2.8	2.1
% CV	5.0	3.9
n	25	25

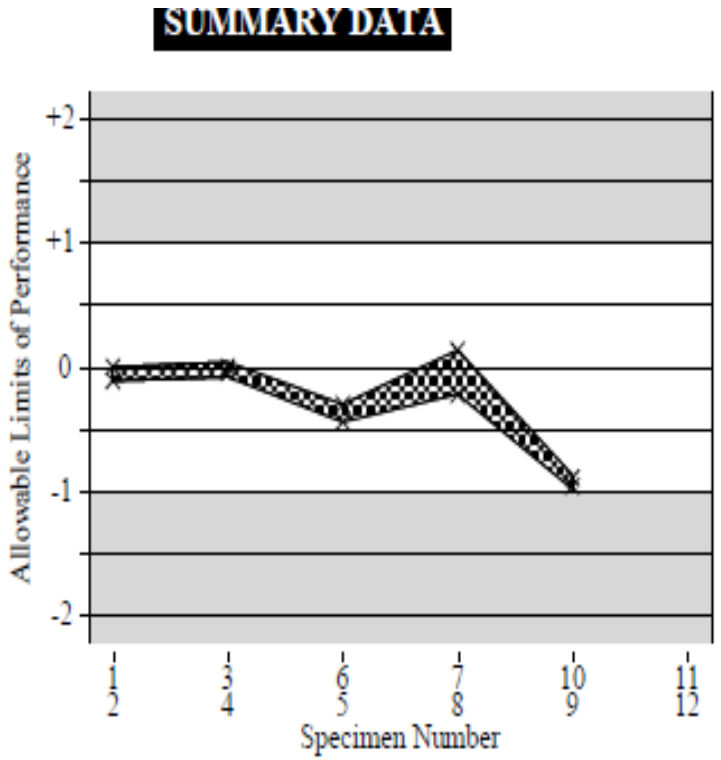
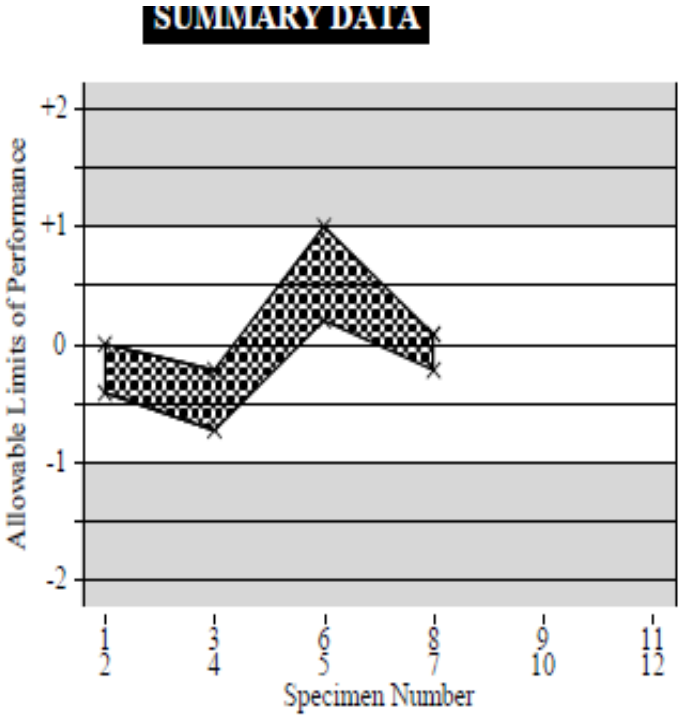
During Year 9 the expected PLP concentration of the spiked plasma was 55.9nmol/L (the sum of the basal level in the plasma plus the spike concentration). The expected PA concentration of the spiked plasma was 55.0nmol/L (the sum of the basal level in the plasma plus the spike concentration). Table Q21 indicates consistent accuracy for both spiked controls, i.e. a quantitative recovery of vitamin B_6 from plasma samples.

Q.2.13.2 External QCs for PLP

Participation in studies conducted by the Royal College of Pathologists of Australasia Quality Assurance Program (RCPAQAP) during Year 9 allowed inter-laboratory comparison of results. MRC HNR/MRC EWL participated in monthly 'round robin' studies using lyophilised samples received from RCPAQAP on an annual basis. Internationally, a small number of laboratories (5 to 7) are involved in the scheme. Therefore the returns from this scheme are only useful for indicating whether each laboratory's results are broadly similar to those obtained by other participating laboratories. There are no other participating laboratories in the UK.

For each return in figure Q.5, 2 points are plotted on the graph representing 2 supplied specimens at different concentrations. The zero line represents the median value from all the participating laboratories. The vertical axis represent the ratio of the given result to the median compared to the allowable limit of performance at that concentration level. Results within +/- 1 are within the allowable limits. This suggests that MRC EWL results agree favourably with other laboratories in the scheme.

Figure Q.5 MRC HNR/MRC EWL participating in monthly 'round robin' studies using lyophilised samples received from RCPAQAP during the period of analysis of NDNS RP Year 9 samples

January to June 2017 (NDNS Yr9)	July to October 2017 (NDNS Yr9)
	

Q.2.14 Serum and whole blood folate

Q.2.14.1 Serum folate

Serum folate was measured at MRC EWL by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The assay uses solid phase extraction with phenyl columns to isolate the folate forms in serum samples. Stable isotope labelled internal standards are added during the extraction step and undergo processing identical to the analytes thereby normalising for sample preparation and instrument variability. Highly specific detection of the six vitamers: methyltetrahydrofolate, tetrahydrofolate, formyltetrahydrofolate, folic acid, 5,10 methenyltetrahydrofolate and an oxidation product of 5-methyltetrahydrofolate (MeFox) is accomplished by LC-MS/MS analysis. The ratio of analyte to internal standard signal is compared to that of a calibration curve to determine analyte concentration. Analytes are analysed using reversed phase ultra-performance liquid chromatography (UPLC) on a Waters ACQUITY UPLC® HSS T3 C8 1.7µ 2.1 x 100mm column at 300C with a 49.5:40:10:0.5 Water:Methanol:Acetonitrile:Acetic Acid isocratic mobile phase prior to mass spectrometry analysis. The retention times for all the analytes are very similar and the internal standards are identical to their corresponding analytes but due to their differing masses, there is clear distinction between them in the assay. Formyltetrahydrofolate and MeFox have the same molecular weights and cannot be chromatographically separated so transitions unique to each form have to be used.

Total folate for reporting is calculated from the sum of the 6 folate vitamers.

Q.2.14.1.1 Internal QC for serum folate

Three controls containing folic acid as well as naturally-occuring vitamers were analysed at the beginning and at the end of each batch of samples for assessment of assay precision.

Because of the limitations of external QA schemes for serum folate, accuracy was continually monitored in-house by including Standard Reference Material (SRM) as an accuracy control. SRM 1950 was stored in single-use aliquots and run once with each batch of samples in order to assess assay accuracy. Results are presented in tables Q.22 and Q.23.

Table Q.22 Precision assessment (NDNS RP Year 9)

	QC1	QC 2	QC 3

	Total folate	Folic Acid	Total folate	Folic Acid	Total folate	Folic Acid
Mean (nmol/L)	35.15	9.03	94.37	6.55	19.11	1.10
SD (nmol/L)	1.40	0.56	4.11	0.40	0.81	0.09
% CV	3.99	6.23	4.36	6.17	4.25	8.57
n	21	20	21	20	21	20

Table Q.23 Accuracy assessment (SRM 1950) (NDNS RP Year 9)

	SRM 1950 (nmol/L)	
	MTHF	Folic Acid
Mean	28.51	3.20
SD	1.29	0.19
% CV	4.54	5.84
n	10	10
SRM target values	*26.91 +/- 0.7*	*3.42 +/- 1.02*

Q.2.14.1.2 External QA of serum folate

The laboratory participated in the VITAL-EQA program organised by CDC; Round 28 was sent in Spring 2017, during Year 9; three samples are sent for each round.

Comparison is against a target concentration determined using the Bio-Tek Power Wave microbiological method. Bias and precision were classified as “optimum” at the lowest and middle concentration and “desirable” at the highest concentration.

The laboratory is a member of UKNEQAS for serum folate but because all other participating laboratories use clinical competitive protein binding methods rather than LC-MS/MS, which is more accurate when compared to the “gold-standard” microbiological method, comparison with the ALTM is not a valid measure of accuracy.

Table Q.24 Serum folate VITAL-EQA data, Round 28 (all concentrations are in ng/mL) (NDNS RP Year 9)

Your Overall Mean	***4.75***	***6.07***	***11.9***
Your Within-Lab SD	0.23	0.30	0.76
Your Within-Lab Imprecision (CV)	**4.9%**	**4.9%**	**6.4%**
Your Imprecision Evaluation	***Optimum***	***Optimum***	***Desirable***
Your Deviation from Target (%)	**5.2%**	**-0.7%**	**9.7%**
Your Bias Evaluation	***Optimum***	***Optimum***	***Desirable***

Q.2.14.2 Whole blood folate and red blood cell (RBC) folate quantitation

RBC folate is calculated from whole blood folate concentration (see below), serum folate concentration and Hct (as quantitated as part of the full blood count) using the equation:

RBC folate = (whole blood folate - (serum folate x (1-Hct))) / Hct

Where a serum folate concentration was not available a surrogate of 18nmol/L was used in the calculation. Where Hct was not available, a surrogate of 0.4L/L was used.

Q.2.14.2.1 Whole blood folate – analytical method[a]

Whole blood haemolysate specimens (whole blood diluted and stabilized with ascorbic acid) were analysed for total folate at the Centers for Disease Control, Atlanta, Georgia, USA, (CDC) using the *Lactobacillus rhamnosus* microbiologic

[a] QC information provided by CDC.

growth assay by an adaptation of O'Broin *et al.*[19] and Molloy *et al.*,[20] as described by Pfeiffer *et al.*[21] Diluted specimen (4 replicates at 2 dilutions) was added to an assay medium containing the microorganism and all of the nutrients necessary for the growth of the microorganism except for folate. 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 (5-methylTHF), using an 11-point calibration curve (0–1.0nmol/L; 8 replicates/point) with a third 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 whole blood haemolysate specimens in this study was 1/94.

Results from 4 replicates at 2 different dilutions 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 whole blood folate concentration <127nmol/L (corresponding to a RBC folate concentration of <317nmol/L RBC if a Hct of 0.4L/L is assumed) were considered to represent potential folate deficiency and assays were repeated for confirmation. This is the standard practice in the CDC laboratory.

All blood samples were posted at ambient temperature to the laboratory at Addenbrooke's where aliquots of the blood were preserved with 1% ascorbic acid and frozen at -80^{o}C for storage, before sending on dry ice to CDC. Any possible deterioration in folate concentration during overnight postage is likely to be less than 10%.[22]

Q.2.14.2.2 Internal QC for whole blood folate**Error! Bookmark not defined.**

Three whole blood bench QC pools were analysed in duplicate in each run, bracketing the unknown samples (n = 32 runs). The between-run imprecision for whole blood folate and the target concentration are shown in table Q.25.

Table Q.25 Whole blood total folate concentration (nmol/L) (NDNS RP Year 9)

		Bench QC Pool		
Analyte	**Parameter**	**LB14810a_MA**	**MB14811a_MA**	**HB14812a_MA**
WBF	Mean, nmol/L	256	440	742
WBF	SD, nmol/L	20	30	42
WBF	% CV	8%	7%	6%
WBF	Target nmol/L	232	429	722
WBF	Difference from target	10.5%	2.7%	2.8%

Four additional whole blood QC pools were analysed “blind” (i.e. 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.26.

Table Q.26 Whole blood total folate concentration (nmol/L) (NDNS RP Year 9)

		Blind QC Pool			
Analyte	**Parameter**	**936**	**937**	**938**	**939**
WBF	Mean, nmol/L	527	257	391	186
WBF	SD, nmol/L	9	23	19	11

WBF	% CV	1.7%	9.1%	4.9%	5.9%
WBF	*n*	7	11	8	6
WBF	Target nmol/L	527	256	354	187
WBF	Difference from target	0.1%	0.3%	10.3%	-0.1%

Accuracy has been established by spiking recovery, by periodic assaying of the 1st International Standard for Whole Blood Folate 95/528, and by successful participation in UK NEQAS Haematinics programme (http://www.ukneqas-haematinics.org.uk).

Q.2.14.2.3 Note regarding folate status assessment

Our data demonstrate a decrease over time in geometric mean folate concentration for all age/sex groups in the population. This finding is significant in terms of public health and therefore we regard it as relevant to

consider the possibility that the interpretation could be confounded by analytical or pre-analytical causes.

The laboratories who performed these assays have good quality control evidence for long-term consistency of their results. Crucially, the analytical matrix, the pre-analytical procedures and the analytical methodology for RBC folate and serum folate were completely independent of each other. The serum folate concentration was measured using LC-MS/MS on a serum sample separated promptly at the Field Laboratory and kept frozen until analysis. The RBC folate concentration was derived principally from whole blood folate measured using the gold-standard reference microbiological assay and from haematocrit; both of these were measured in a whole blood sample posted by the nurse to Addenbrooke's where the haematocrit was measured and an aliquot of the blood stabilised and frozen for folate assay. Because these processes were so different for the 2 analytes we do not consider that the decrease in folate status recorded by the NDNS RP over the 9-year period can be ascribed to analytical or pre-analytical causes. The agreement between the time trends in these 2 measures of folate status is evidence that the data represent a genuine decline in UK population folate status between 2007/08 to 2016/17.

Q.2.15 Plasma retinol, α– and γ–tocopherol, and individual carotenoids

Fat soluble micronutrients were determined by HPLC coupled to a photodiode array detector, capable of multi-wavelength detection. The analytical method used was derived from Sowell et al.[23] Samples were assayed as singletons. Plasma concentrations of vitamin A (retinol), α–, and γ–tocopherol, and 6 carotenoids (α– and β–carotene, α– and β–cryptoxanthin, lycopene and the sum of co-eluting lutein and zeaxanthin [xanthophyll]) were determined. Internal standards of tocopherol acetate and apo-8-carotenal were used to monitor losses during the extraction process and to account for any changes in volumes. The analytical methods used were the same as during Years 5 to 8; these differ from the methods used for Years 1 to 4. Details of the conversion factors needed for comparison with data collected during Years 1 to 4 are detailed in NDNS RP Years 5 and 6 (combined) report.[24]

Q.2.15.1 Internal QCs for plasma retinol, α– and γ–tocopherol and individual carotenoids

The FSV results for Year 9 were reported as plasma retinol, α– and γ–tocopherol and individual carotenoids (Lutein and zeaxanthin co-elute and therefore are measured as a sum). Internal controls were selected containing appropriate 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 the NDNS RP.

Accuracy was determined using the external QA scheme led by NIST with UKNEQAS returns as corroboration, see section Q.2.15.2.

Table Q.27 Precision of internal QC for plasma retinol (NDNS RP Year 9)

Retinol Year 9			
	QC 11	**NIST 257**	**QC7**
mean	1.45	1.80	2.35
SD	0.06	0.09	0.11

% CV	4.1	5.1	4.5
n	18	22	22

Table Q.28 Precision of internal QC for plasma α-tocopherol

(NDNS RP Year 9)

Alpha tocopherol Year 9				
	NIST 170	**QC7**	**QC 2**	**QC 11**
mean	14.36	34.72	33.36	15.64
SD	0.81	1.56	0.82	0.71
% CV	5.7	4.5	2.5	4.5
n	22	22	18	18

Table Q.29 Precision of internal QC for plasma γ-tocopherol

(NDNS RP Year 9)

	Gamma tocopherol Year 9	
	QC 7	**QC 11**
mean	1.76	1.08

SD	0.20	0.23
% CV	11.1	21.1
n	22	17

Table Q.30 Precision of internal QC for plasma α-carotene

(NDNS RP Year 9)

	Alpha carotene Year 9		
	QC7	**NIST 265**	**QC2**
mean	0.10	0.16	0.11
SD	0.01	0.01	0.01
% CV	5.8	4.3	5.4
n	18	22	18

Table Q.31 Precision of internal QC for plasma β-carotene

(NDNS RP Year 9)

	Beta carotene Year 9		
	QC 11	**NIST 265**	**NIST 170**

mean	0.15	0.56	1.08
SD	0.01	0.02	0.03
% CV	5.0	4.4	3.2
n	18	22	22

Table Q.32 Precision of internal QC for plasma α-cryptoxanthin

(NDNS RP Year 9)

Note: Lower sensitivity for alpha-cryptoxanthin in Year 9 meant the limit of quantitation was raised to 0.10 µmol/L. The internal QC concentrations are below the limit of quantitation established for Year 9 and should be seen as indicative only.

	Alpha cryptoxanthin Year 9	
	QC2	**NIST 265**
mean	0.06	0.05
SD	0.01	0.003
% CV	19.9	6.5
n	16	9

Table Q.33 Precision of internal QC for plasma β-cryptoxanthin

(NDNS RP Year 9)

	Beta cryptoxanthin Year 9	
	QC11	**QC7**
mean	0.16	0.12
SD	0.01	0.01
% CV	7.1	7.0

n	18	22

Table Q.34 Precision of internal QC for plasma lycopene (NDNS RP Year 9)

	Lycopene Year 9			
	NIST 170	**NIST 265**	**QC7**	**QC11**
mean	0.45	0.68	1.85	0.62
SD	0.02	0.03	0.09	0.03
% CV	4.5	5.0	4.6	4.7
n	22	22	22	18

Table Q.35 Precision of internal QC for plasma lutein + zeaxanthin (NDNS RP Year 9)

	Lutein plus zeaxanthin Year 9			
	NIST 257	**NIST 170**	**QC7**	**QC11**
mean	0.11	0.24	0.37	0.17
SD	0.01	0.01	0.02	0.01
% CV	7.9	4.7	5.2	5.9

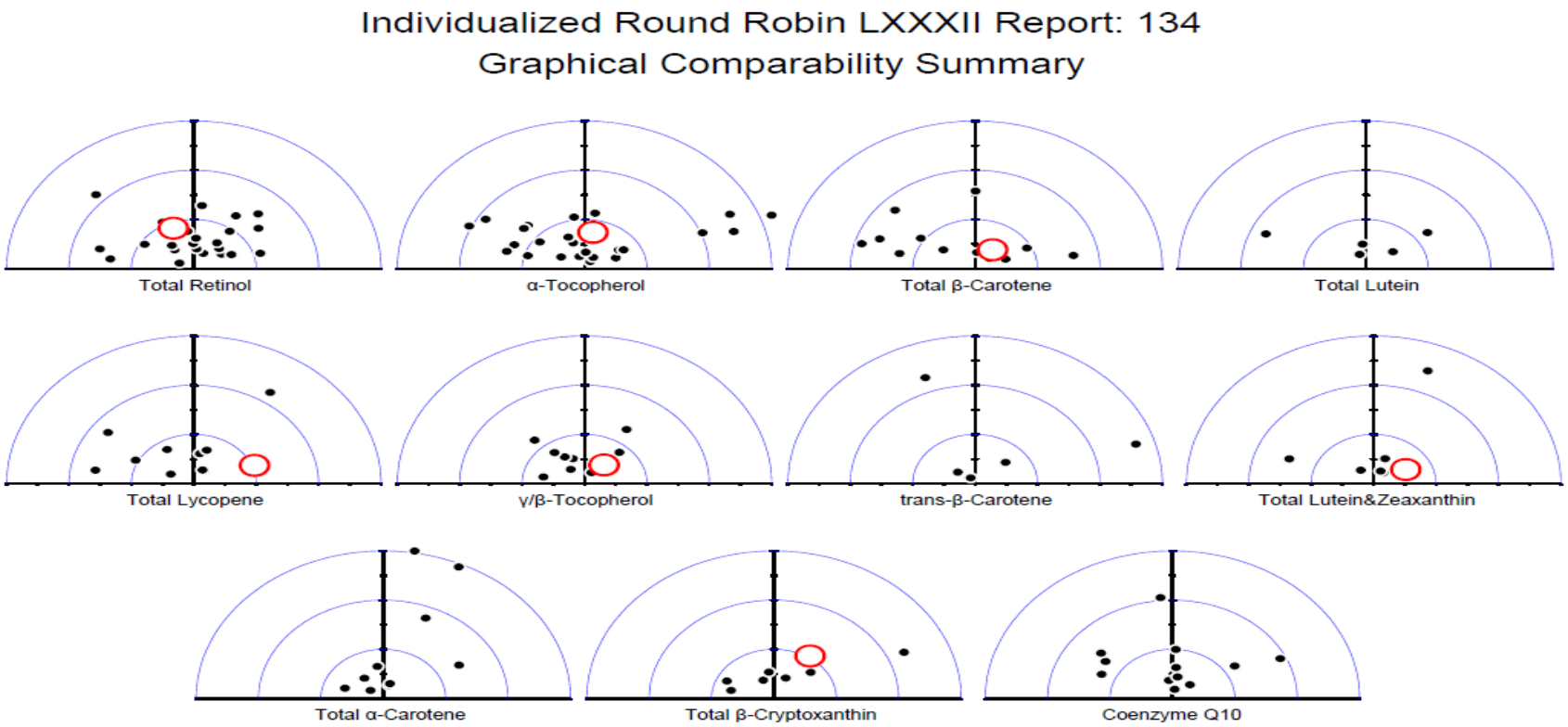
n	22	22	22	18

Q.2.15.2 External QCs for plasma retinol, α– and γ–tocopherol and individual carotenoids

Participation in studies conducted by NIST and UKNEQAS allowed inter-laboratory comparison of results. MRC EWL also received samples from UKNEQAS on a monthly basis. For UKNEQAS the following carotenoids: α-carotene, β-cryptoxanthin, lutein/zeaxanthin and lycopene are measured by 6 laboratories or fewer and therefore the returns from these schemes are only useful for indicating whether each laboratory's results are broadly similar to those obtained by other participating laboratories.

NIST EQA return LXXXII was submitted during Year 9 and the extract below in figure Q.6 shows that agreement with the target concentration was very good.

Figure Q.6 NDNS RP Year 9 NIST return LXXXII



In the graphical representations in figures Q.6 our result for each analyte measured is represented as an open larger circle with other laboratories in the scheme represented as closed black circles. The vertical axis indicates average deviation from the mean; the horizontal axis indicates the variability of that deviation. The closer to the origin the better the result, in terms of precision and accuracy. Five samples are analysed for every NIST return; the analyte 'result' above is a summary of all 5.

Q.2.16 Serum 25-hydroxyvitamin D (25-OHD)

Q.2.16.1 Method changes during the NDNS Rolling Programme

At the beginning of the NDNS RP, plasma 25-hydroxyvitamin D (25-OHD) was measured using the Diasorin Liaison. During Year 4 the manufacturers reformulated the assay and the matrix was changed to serum. From the beginning of Year 7 the Liaison assay was discontinued and serum 25-OHD was measured using an in-house LC-MS/MS method. The LC-MS/MS and Liaison assays have been standardised against the international reference methods under the Vitamin D Standardisation Program. The LC-MS/MS method has been shown to produce results equivalent to the international reference methods and a standardisation factor has been determined for the Diasorin Liaison; standardised results are used in the data analysis.

Q.2.16.2 Serum 25-hydroxyvitamin D (25-OHD) Year 9

During Year 9, 25-OHD was measured in serum by LC-MS/MS at MRC EWL

The assay uses a methanol protein precipitation step followed by liquid-liquid extraction in hexane to separate 25-Hydroxyvitamin D 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 LC-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 Thermo Scientific Hypersil GOLD PFP 2.1 x 100mm 1.9µm column at 400C with a 72% Methanol +0.1% formic acid isocratic mobile phase at 0.25mL/min prior to mass spectrometry analysis.

Total 25-OHD is calculated from the sum of 25-OHD2 and 25-OHD3.

This assay has been calibrated against International Reference Methods at NIST and the University of Ghent under the auspices of the Vitamin D Standardisation Program.

Q.2.16.3 Quality control for 25-OHD

Internal QCs were run with every batch and MRC EWL also subscribed to the DEQAS external QA scheme.

Q.2.16.3.1 Internal QCs for 25-OHD

Controls (Chromsystems Level 1 and Level 2 and an in-house control) were run with every assay batch. 25-OHD2 and 25-OHD3 are quantitated separately and presented in table Q.36, alongside in-house controls.

Table Q.36 Internal QCs for 25-OHD (NDNS RP Year 9)

Year 9	lot 2714				lot 0716				QC151014	
	level 1		level 2		level 1		level 2		in-house	
	D2	D3	D2	D3	D2	D3	D2	D3	D2	D3
mean	44.0	37.6	101.8	85.1	37.4	39.6	84.7	88.8	12.8	19.0
SD	2.5	1.3	6.0	3.7	2.8	2.3	5.9	3.6	1.0	1.4
% CV	5.8	3.6	5.9	4.4	7.5	5.9	7.0	4.0	8.0	7.6
n	12	12	14	14	13	13	13	13	24	24

Q.2.16.3.2 External QA for 25-OHD

MRC EWL subscribed to the DEQAS external QA scheme and performance was assessed by the scheme organisers as meeting the performance target set by the

DEQAS Advisory Panel (i.e. 80% or more of results were within +/- 30% of the target. The target is measured by the National Institute of Science and Technology, Maryland USA using their isotope-dilution LC-MS/MS Reference Method for 25-OHD2 and 25-OHD3; the two are added together to provide the Target Concentration for total 25-OHD). DEQAS do not issue cumulative performance data as do NEQAS.

Table Q.37 shows the relationships between 25-OHD as reported on individual DEQAS samples by EWL and the target concentration as provided by NIST.

Table Q.37 Relationships between 25-OHD as reported on individual DEQAS samples by MRC EWL between April 2016 and October 2017

25 (OH)D nmol/L	Year 9
Mean bias from target concentration	0.3
SD of bias	4.9
n	30

Q.2.17 Plasma creatinine

At the start of Year 8 the creatinine assay was changed to an enzymatic method which uses creatininase coupled to creatinase, sarcosine oxidase and peroxidase. The coloured end product is measured bichromatically (540nm, 700nm) at the endpoint of the reaction. Enzymatic creatinine methods are reported to be less susceptible to non-creatinine interfering substances.

Q.2.17.1 Internal QCs for plasma creatinine

Multiqual QCs containing low, moderate and high concentrations of creatinine are run with each sample set. If the results obtained are not within manufacturer's range and also within the range determined within our laboratory, the run is rejected. Table Q.38 shows internal QC results for creatinine, covering the period when Year 9 samples were analysed.

Table Q.38 Internal QCs for plasma creatinine (NDNS RP Year 9)

	qc 1	qc2	qc3
Mean	52.89	127.97	401.65
SD	3.16	3.04	8.02
% cv	5.98	2.38	2.00
n	43	44	42

Q.2.17.2 External QA for plasma creatinine

MRC EWL subscribes to the UKNEQAS clinical chemistry. Table Q.39 shows that during Year 9 the Dimension assay at MRC EWL gave results acceptably close to the consensus of all laboratories using the same method.

Table Q.39 External quality assurance for creatinine performance relative to method mean (enzymatic method) (NDNS RP Year 9)

	UK NEQAS	Year 9
creatinine	Mean % bias	2.5
	SD % bias	4.5
	n	57

Q.2.18 Plasma selenium and plasma zinc

Total selenium (Se) and zinc (Zn) concentrations of human blood plasma were determined by measuring the ^{78}Se and ^{68}Zn isotopes using an inductively coupled plasma mass spectrometer (ICP-MS) equipped with a dynamic reaction cell (DRC). Methane (CH_4) was used as a DRC gas to overcome Argon based interferences.

Samples were introduced to the ICP-MS through a flow injection system combined with the Sea spray nebulizer and cyclonic spray chamber arrangement.

Human blood plasma samples and QC materials were prepared in diluent which included rhodium (Rh) as internal standard. The Se and Zn isotope signals were compared against the internal standard, enabling any signal fluctuation due to instrument drift to be accounted for.

Matrix matched external calibration standards were prepared in commercially prepared human serum or plasma (Seralabs).

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

Q.2.18.1 QCs for selenium and zinc

In order to establish quality assurance of each analytical batch and inter-batch variation across the year's cohort as a whole, ClinChek Plasma Control Lyophilised for Trace Elements Level 1 and 2 (Recipe Chemicals and Instruments GmbH) QC samples were analysed in conjunction with the blanks, calibration standards and samples.

Q.2.18.1.1 Inter-batch variability

Table Q.40 summarises the measured concentration of selenium and zinc following analysis of these QC samples for each individual year of the NDNS RP. For each year the mean measured concentration of the QC was within the target concentration range defined by the manufacturer and CV was ≤10% for each of the years described, showing that for each year there was acceptable analytical accuracy and precision.

Table Q.40 QC analysis (NDNS RP Year 9)

Selenium	L1 423	L1 1286	L2 423	L2 1286
Target (µg/L)	81.4	73.8	120	120
Acceptable range (µg/L)	65.1-97.7	59.0-88.5	96-144	96-144
Mean Measured (µg/L)	76.4	70.6	113	114
Standard deviation (SD)	4.2	1.7	5.8	2.2
% CV	5.6	2.4	5.2	1.9
Agreement with Target (%)	93.8	95.6	94.2	95.6
n	23	7	21	6

Zinc	L1 423	L1 1286	L2 423	L2 1286
Target (µg/L)	1160	1760	1540	2130
Acceptable range (µg/L)	928-1390	1500-2020	1230-1850	1810-2450
Mean Measured (µg/L)	1177	1773	1591	2158
Standard deviation (SD)	51.5	28.3	58.6	43.1
% CV	4.4	1.6	3.7	2.0

Agreement with Target (%)	101.5	100.8	103.3	101.3
n	23	7	21	6

Q.2.18.1.2 External QCs for Selenium and Zinc in Serum

MRC EWL participates in the Interlaboratory Comparison Program for Metals in Biological Matrices (PCI), operated by Centre de toxicologie du Québec at the Institut national de santé publique du Québec (INSPQ). Selenium and zinc analysis of serum samples gives values which are within the criteria defined in this multi-laboratory programme.

Note: at the method dilution used there is no significant difference between serum and plasma as a biological matrix and use of these external QCs is valid.

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