Appendix Q Methods of blood analysis and quality control

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 two 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 -20ºC (typically at -40°C) at field laboratories for a period of six to eight 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 B_{12}"; see section Q.2.4)
- serum Total, high-density lipoprotein (HDL) 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 (PLP and PA) (see section Q.2.13)
- 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)

Serum and whole blood folate concentrations in the NDNS RP blood samples were measured at the Centre 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. However, their data will be deposited at the UK Data Archive together with data for the other analytes presented in this report.

Q.2 Analysis of blood samples

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 schemes) for those analytes where such schemes were available.

Q.2.1 Full blood count including haemoglobin and haematocrit

Full Blood Count was analysed from the start of Year 7 (July 2014) until January 2016 on a Beckman Coulter LH750 analyser, and from January 2016 to July 2016 using a Siemens Advia 2120. Both instruments use 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.

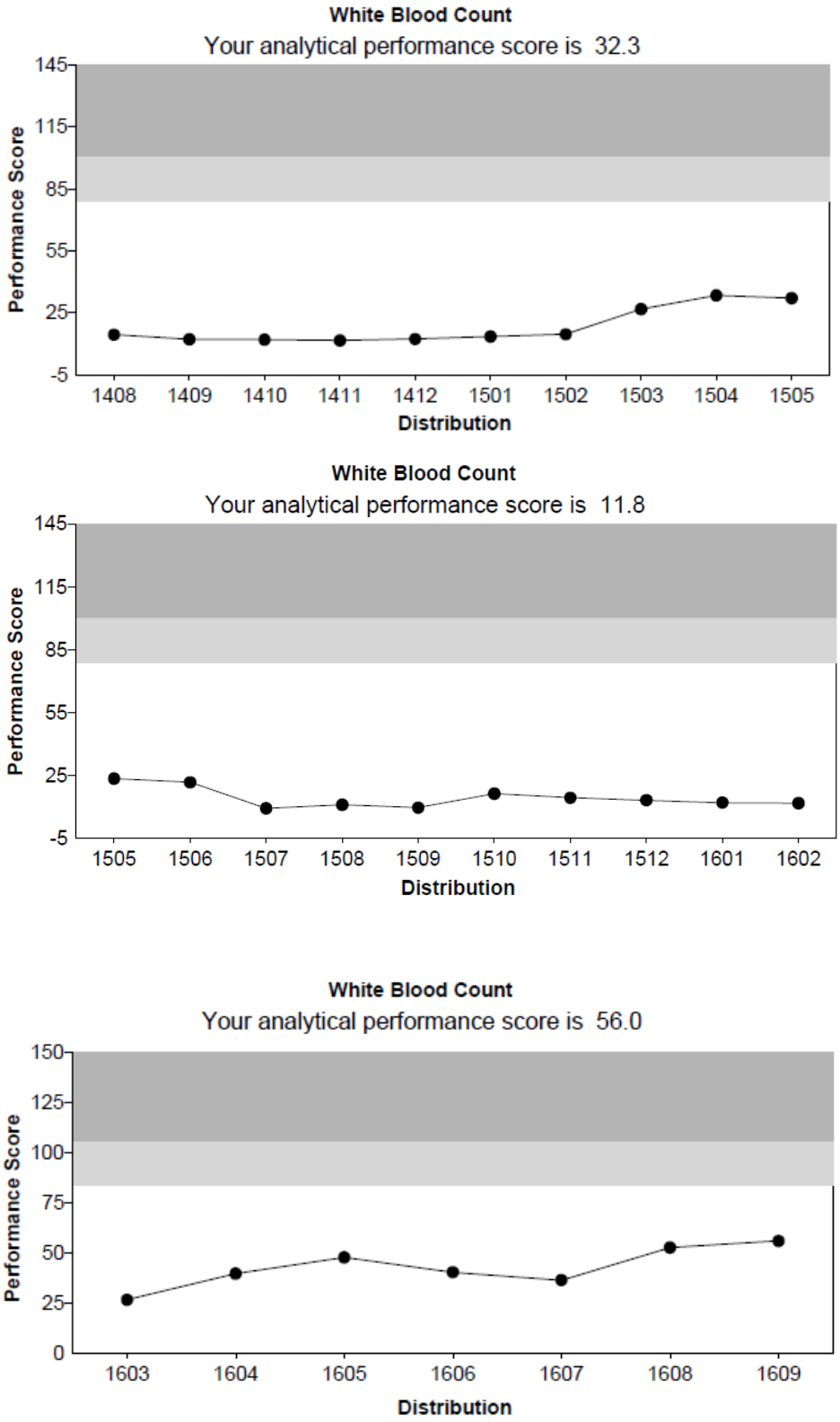
Q.2.1.1 Quality controls for full blood count including haemoglobin and haematocrit

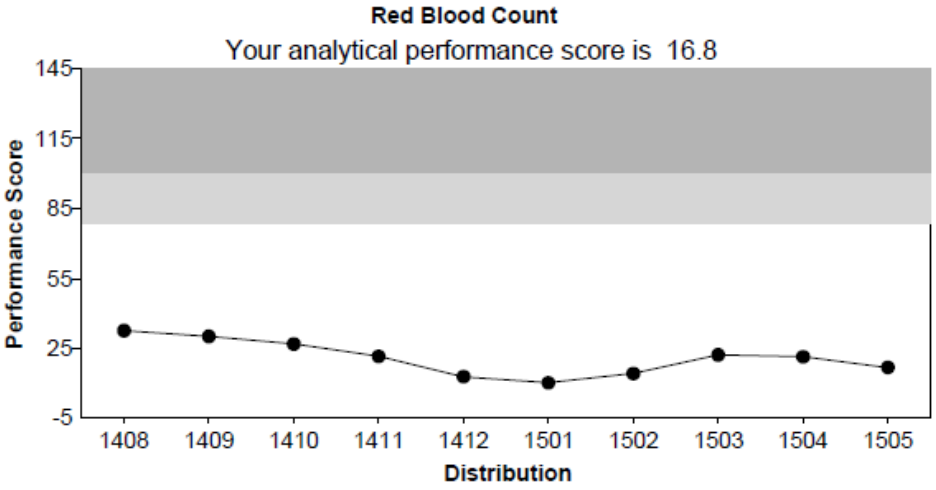
The quality control results of the instruments measuring full blood count at Addenbrooke's Hospital are monitored continually, and analysis is stopped if the results are not satisfactory, ensuring that results are only reported if the analysis is within the QC parameters set by the Laboratory Manager. However, it is not possible

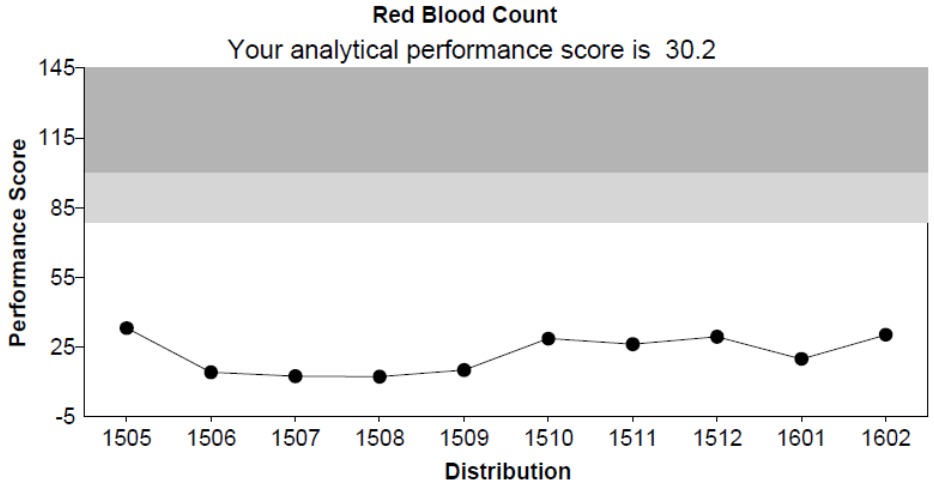
to extract these results for reporting. Therefore no internal QC data can be provided but validation of results is achieved automatically.

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 show the performance charts from UK NEQAS returns for August 2014 to April 2015 (Year 7; Coulter 750), for May 2015 to February 2016 (Years 7 and 8, Coulter 750) and for March 2016 to September 2016 (Year 8 and start of Year 9, Siemens Advia 2120). 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 dark shaded area indicates unacceptable performance and the paler area indicates a borderline situation.

Figure Q.1 Illustrative overall performance charts for UKNEQAS for Years 7 and 8 of the NDNS RP




Red Blood Count
Your analytical performance score is 16.8
Performance Score
145
115
85
55
25
-5
1408 1409 1410 1411 1412 1501 1502 1503 1504 1505
Distribution


Red Blood Count
Your analytical performance score is 30.2
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1505 1506 1507 1508 1509 1510 1511 1512 1601 1602
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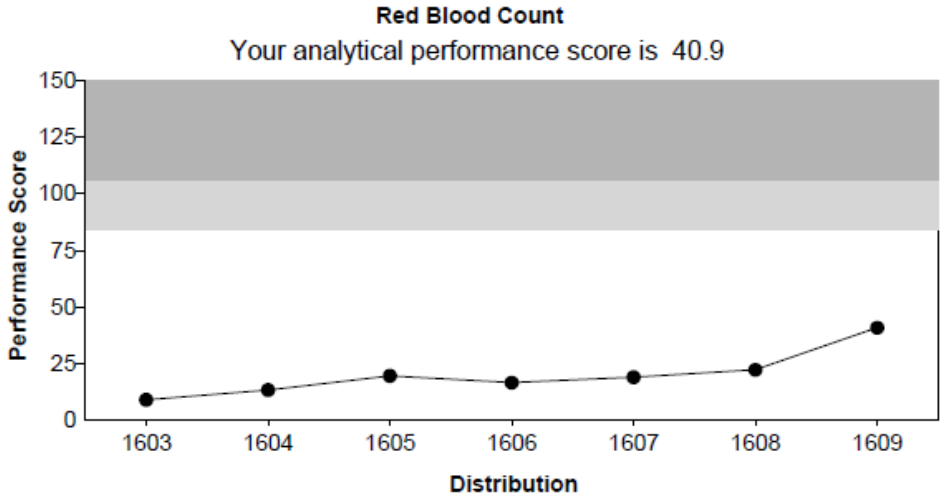
Red Blood Count
Your analytical performance score is 40.9
Performance Score
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50
25
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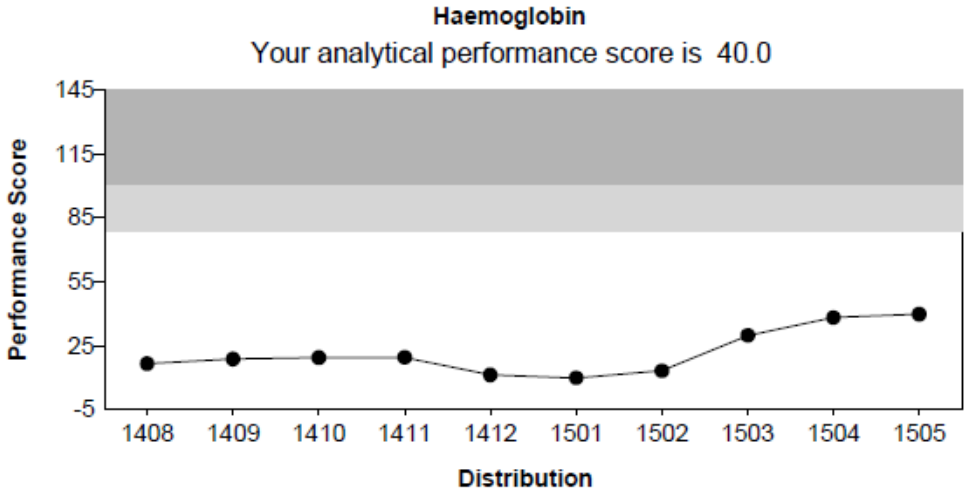
Distribution


Haemoglobin
Your analytical performance score is 40.0
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85
55
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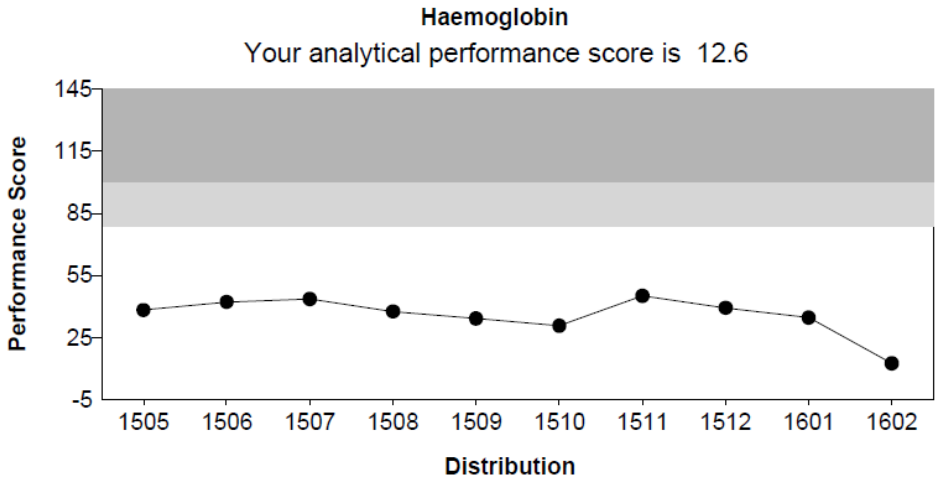
Distribution


Haemoglobin
Your analytical performance score is 12.6
Performance Score
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85
55
25
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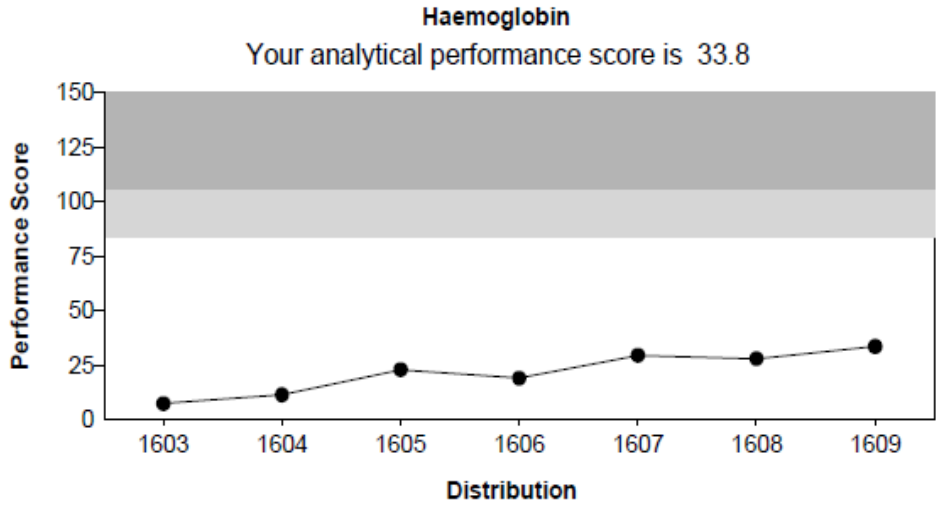
Distribution


Haemoglobin
Your analytical performance score is 33.8
Performance Score
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125
100
75
50
25
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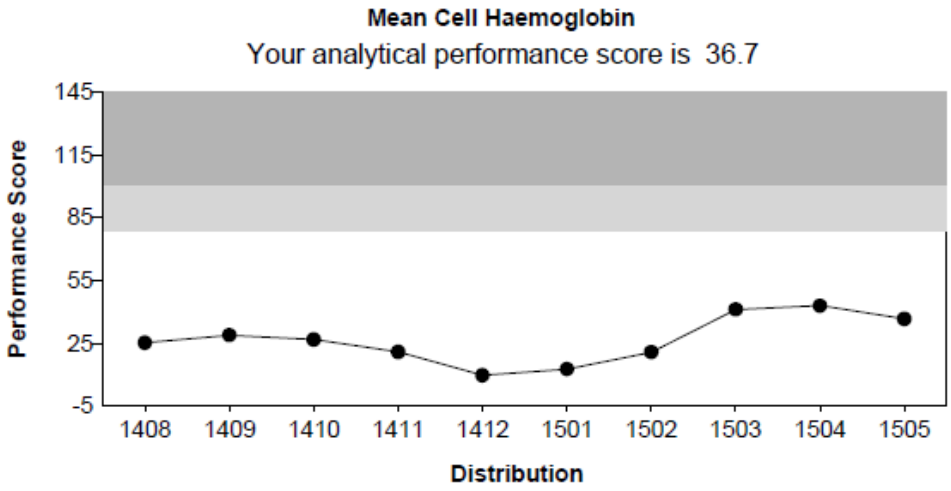
1603 1604 1605 1606 1607 1608 1609
Distribution


Mean Cell Haemoglobin
Your analytical performance score is 36.7
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25
-5
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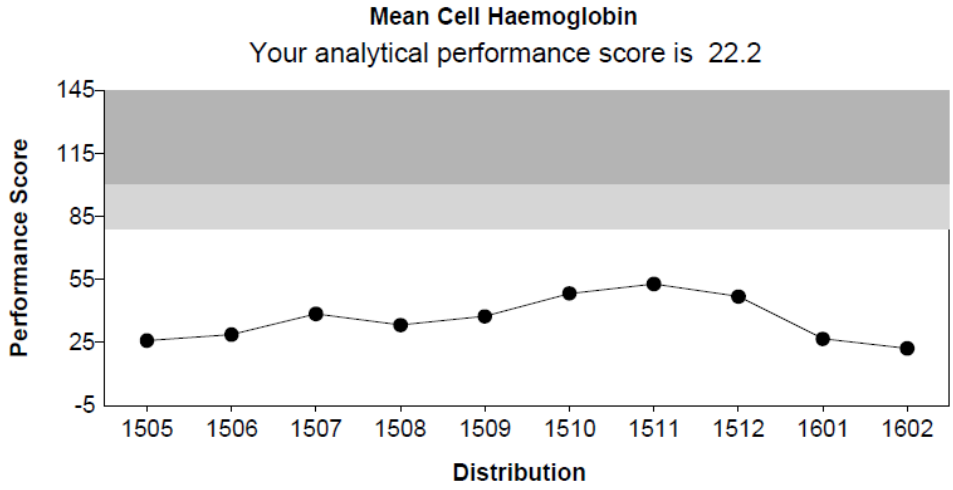
Distribution


Mean Cell Haemoglobin
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Performance Score
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85
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25
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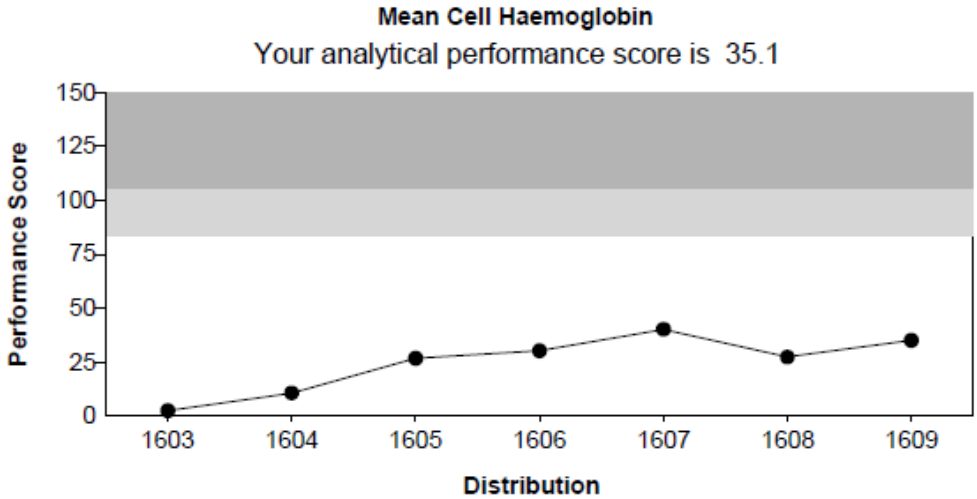
Distribution


Mean Cell Haemoglobin
Your analytical performance score is 35.1
Performance Score
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75
50
25
0
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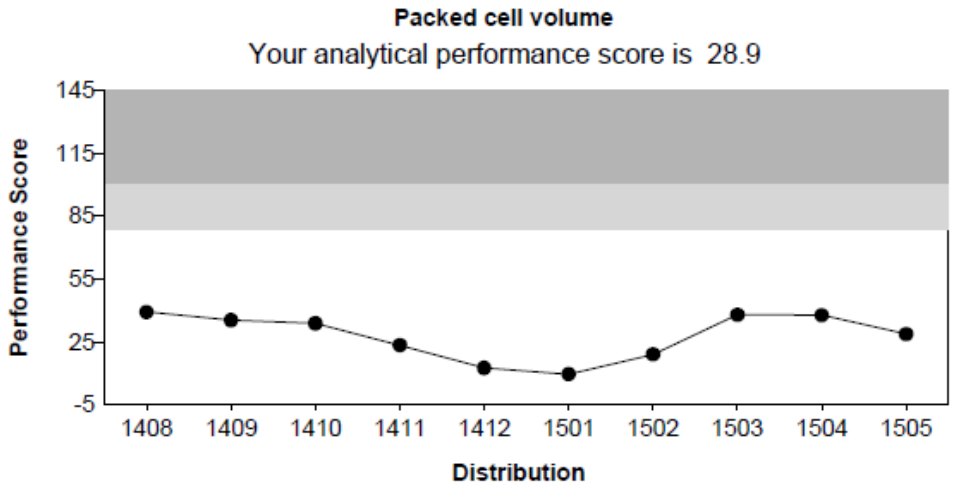
Distribution


Packed cell volume
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55
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-5
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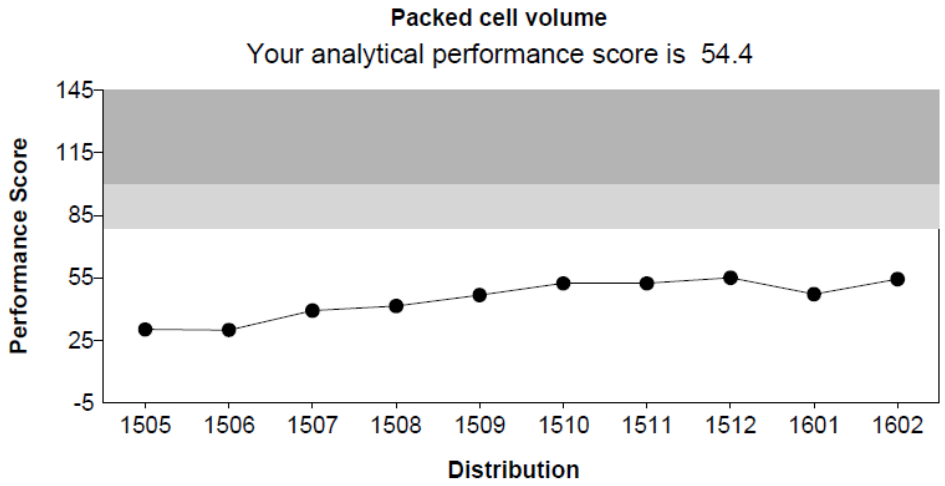
Distribution


Packed cell volume
Your analytical performance score is 54.4
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85
55
25
-5
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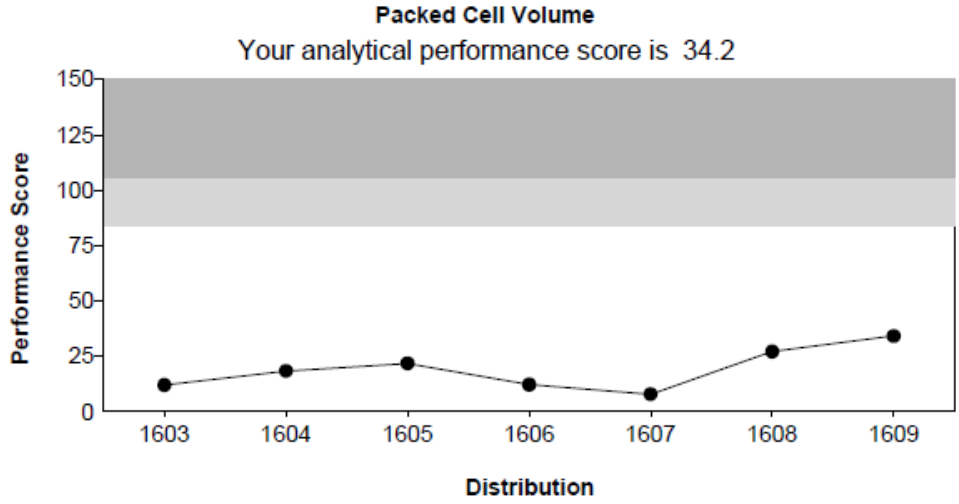
Distribution


Packed Cell Volume
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Performance Score
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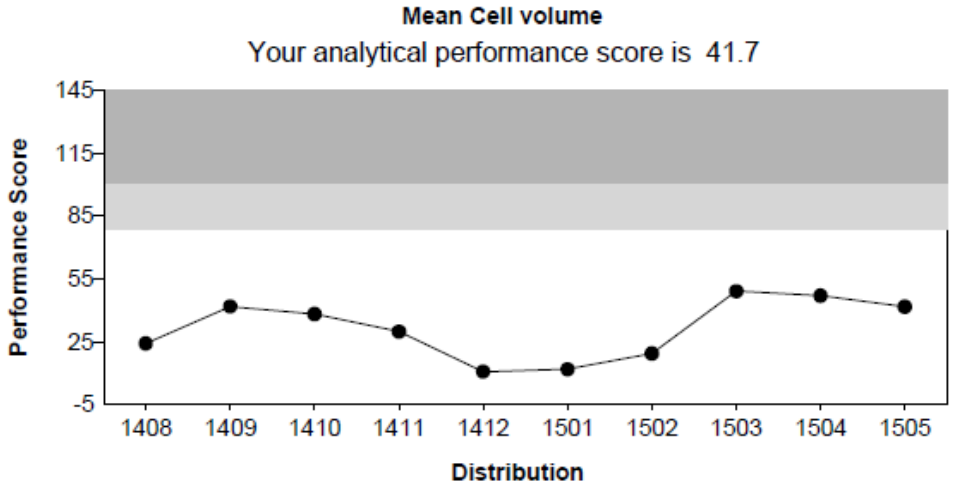
125
100
75
50
25
0
1603 1604 1605 1606 1607 1608 1609
Distribution


Mean Cell volume
Your analytical performance score is 41.7
Performance Score
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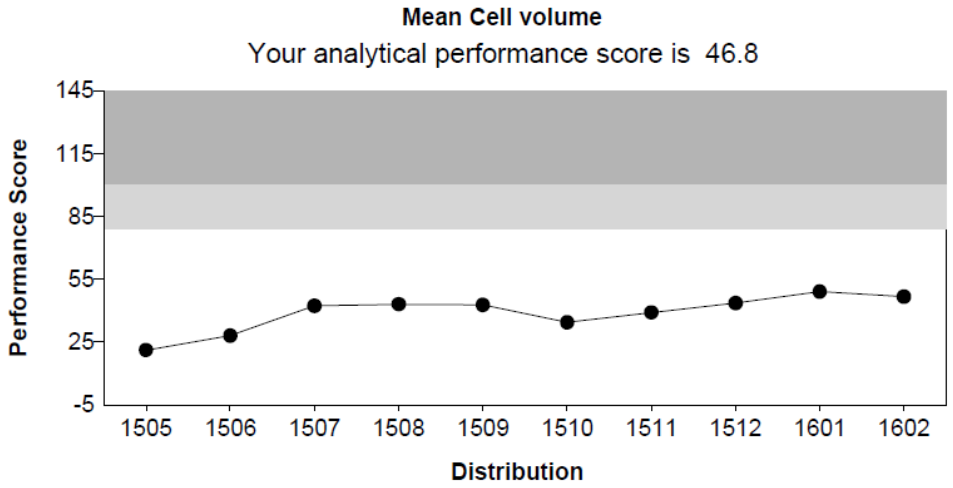
115
85
55
25
-5
1408
1409
1410
1411
1412
1501
1502
1503
1504
1505
Distribution


Mean Cell volume
Your analytical performance score is 46.8
Performance Score
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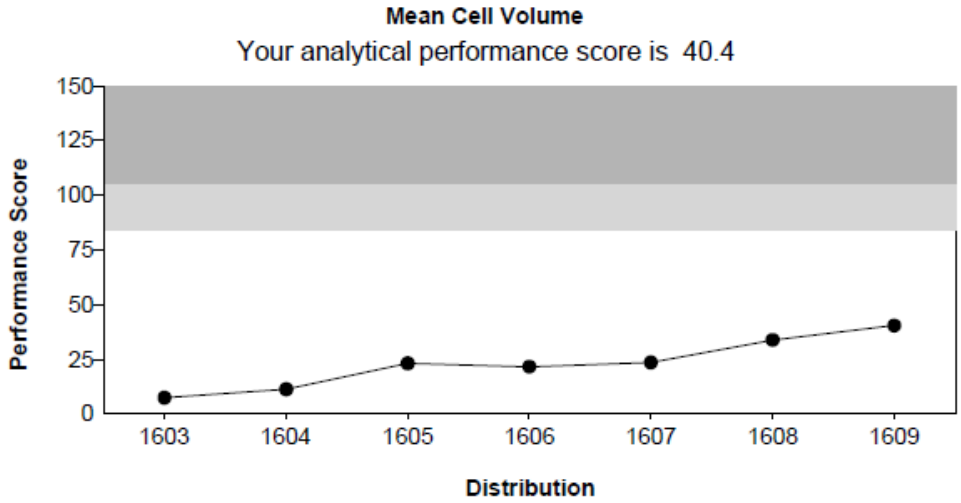
115
85
55
25
-5
1505
1506
1507
1508
1509
1510
1511
1512
1601
1602
Distribution


Mean Cell Volume
Your analytical performance score is 40.4
Performance Score
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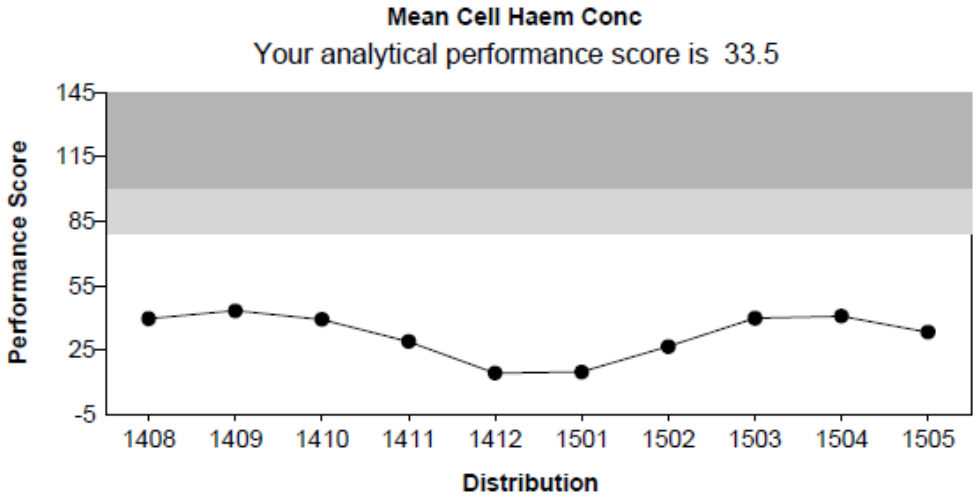
125
100
75
50
25
0
1603
1604
1605
1606
1607
1608
1609
Distribution


Mean Cell Haem Conc
Your analytical performance score is 33.5
Performance Score
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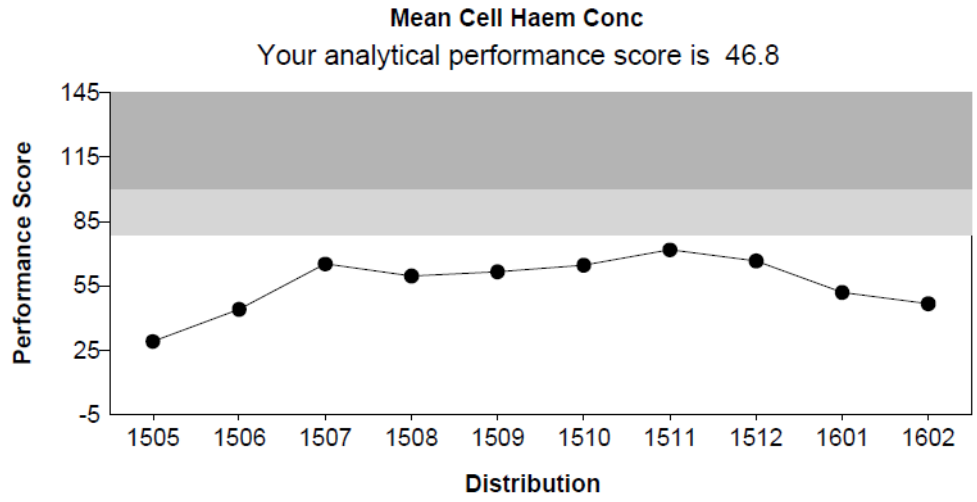
85
55
25
-5
1408 1409 1410 1411 1412 1501 1502 1503 1504 1505
Distribution


Mean Cell Haem Conc
Your analytical performance score is 46.8
Performance Score
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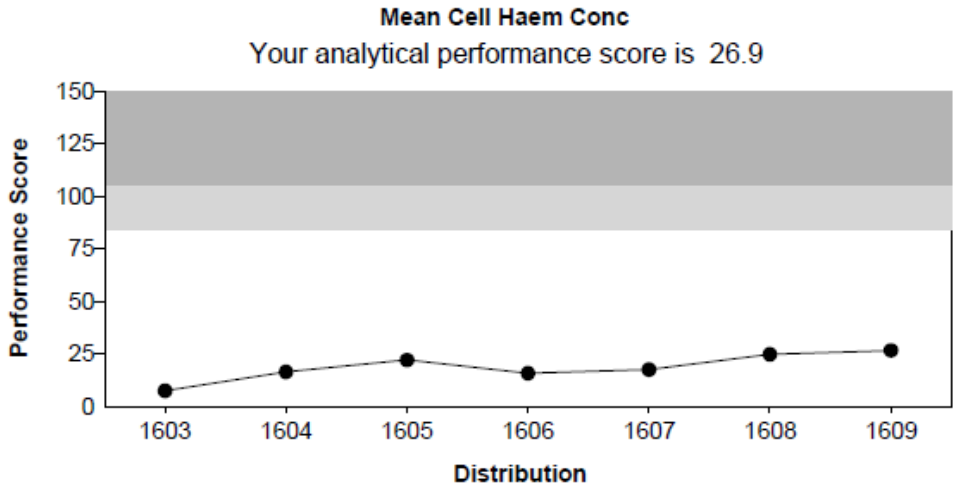
115
85
55
25
-5
1505 1506 1507 1508 1509 1510 1511 1512 1601 1602
Distribution


Mean Cell Haem Conc
Your analytical performance score is 26.9
Performance Score
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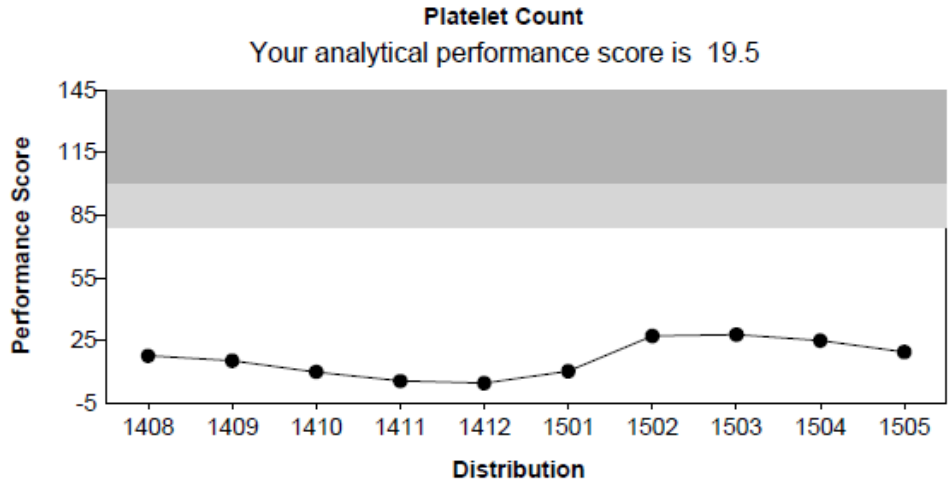
125
100
75
50
25
0
1603 1604 1605 1606 1607 1608 1609
Distribution


Platelet Count
Your analytical performance score is 19.5
Performance Score
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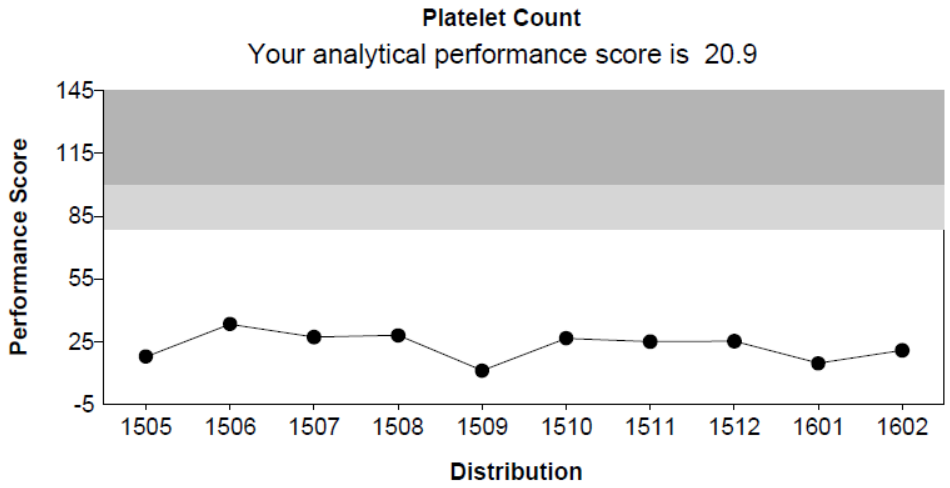
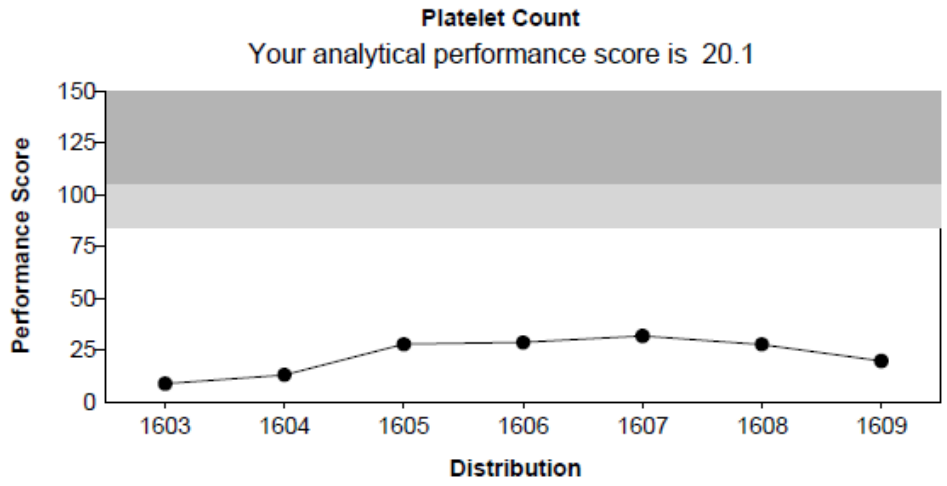
115
85
55
25
-5
1408 1409 1410 1411 1412 1501 1502 1503 1504 1505
Distribution


Platelet Count
Your analytical performance score is 20.9
Performance Score
145
115
85
55
25
-5
1505 1506 1507 1508 1509 1510 1511 1512 1601 1602
Distribution


Platelet Count
Your analytical performance score is 20.1
Performance Score
150
125
100
75
50
25
0
1603 1604 1605 1606 1607 1608 1609
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 quality controls for CRP

Tables Q.1 and Q.2 show imprecision data in Year 7 and Year 8 respectively.

Table Q.1 Internal quality controls for C-reactive protein (CRP) for Year 7 of the NDNS RP

	153UL	**833UN**	**619UE**
CRP (mg/L)	1.21 (0.91 - 1.50)	21.01 (20.63 - 21.39)	52.49 (50.78 - 54.20)
Mean (mg/L)	1.27	20.84	52.08
SD	0.30	0.18	0.71
% CV	23.65	0.84	1.37
Data points included	36	37	37

Table Q.2 Internal quality controls for C-reactive protein (CRP) for Year 8 of the NDNS RP

	199UL	1015UN	762UE
CRP (mg/L)	1.33 (0.60 - 2.07)	19.81 (18.76 - 20.85)	51.57 (48.58 - 54.56)
Mean (mg/L)	1.24	19.51	50.43
SD	0.23	0.33	0.91
% CV	18.44	1.71	1.80
Data points included	44	46	43

Q.2.2.2 External quality controls for CRP

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

Table Q.3 External quality controls for C-reactive protein (CRP) for Years 7 and 8 of the NDNS RP

CRP (low concentrations)	Year 7	Year 8
mean % bias	2.5	7
sd of % bias	5.3	9
n	22	24
CRP (all concentrations)	**Year 7**	**Year 8**
mean % bias	0.3	0.7
sd of % bias	3.9	3.3
n	17	24

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 quality controls for vitamin B_{12}

The quality control results of the instrument measuring Vitamin B12 at Addenbrooke's Hospital 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 no internal QC data can be provided but validation of results is achieved automatically.

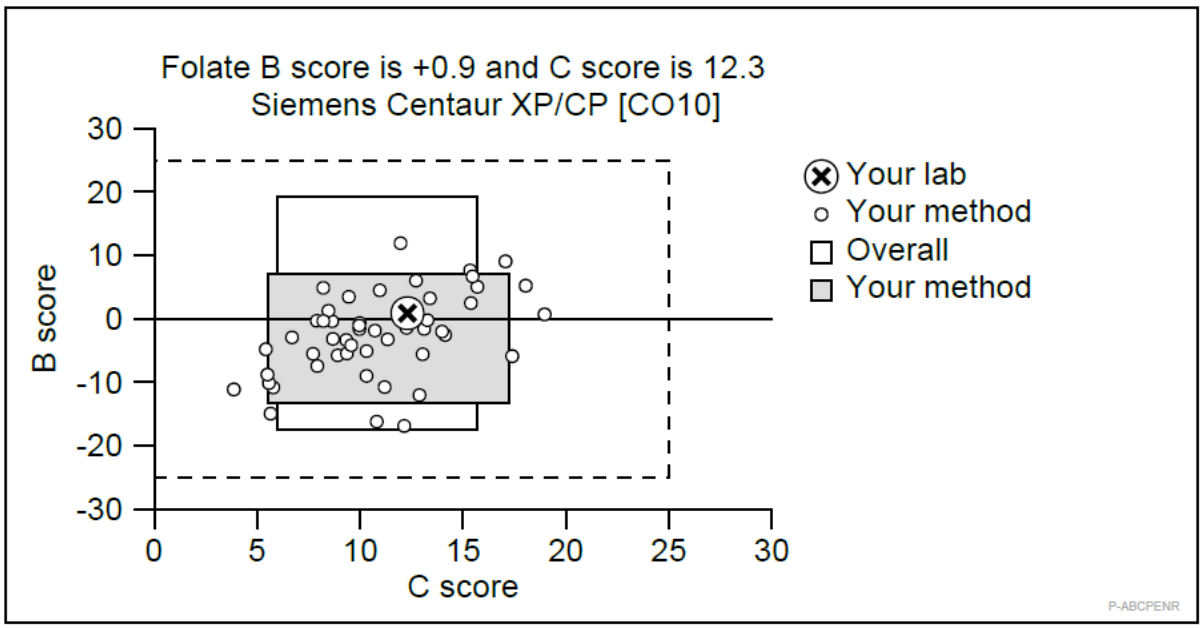
Q.2.3.2 External quality controls for vitamin B_{12}

Quality control was achieved through the UK NEQAS Haematinics scheme. Charts relating to performance during Years 7 and 8 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 cumulative "b score" (bias) and "c score" (consistency of bias) over approximately 6 months for each graph. Bias is calculated to the All Laboratories Trimmed Mean (ALTM). Small numbers indicate close agreement with the ALTM.

Figure Q.2 Illustrative overall performance charts for UKNEQAS for Year 7 of the NDNS RP for vitamin B_{12}

March 2015



August 2015

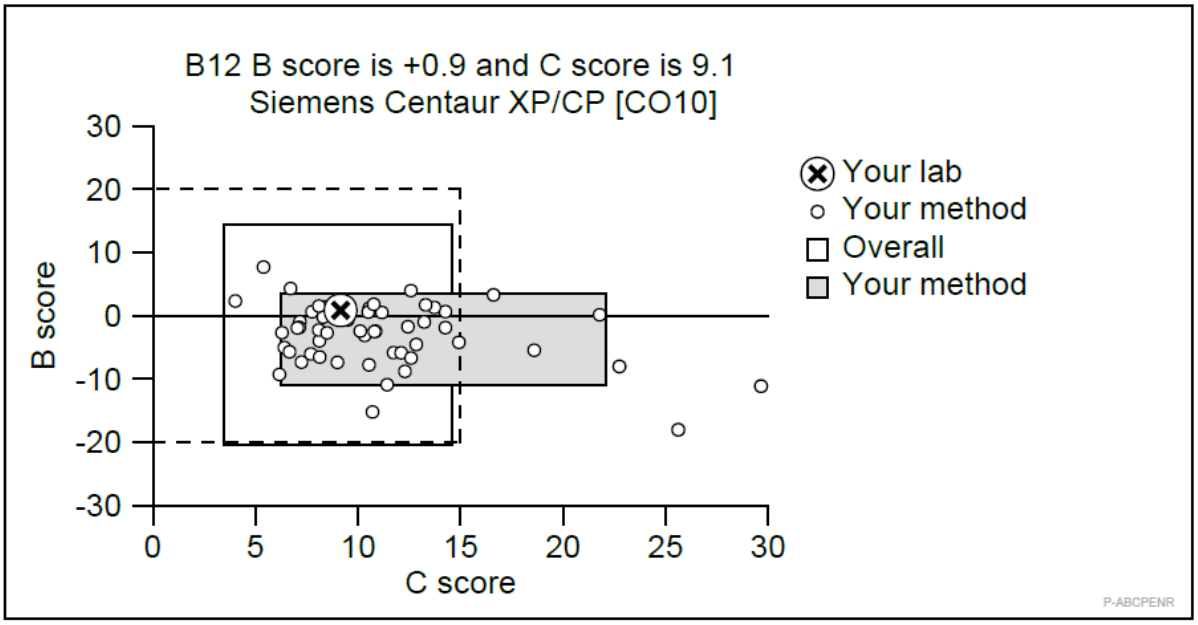
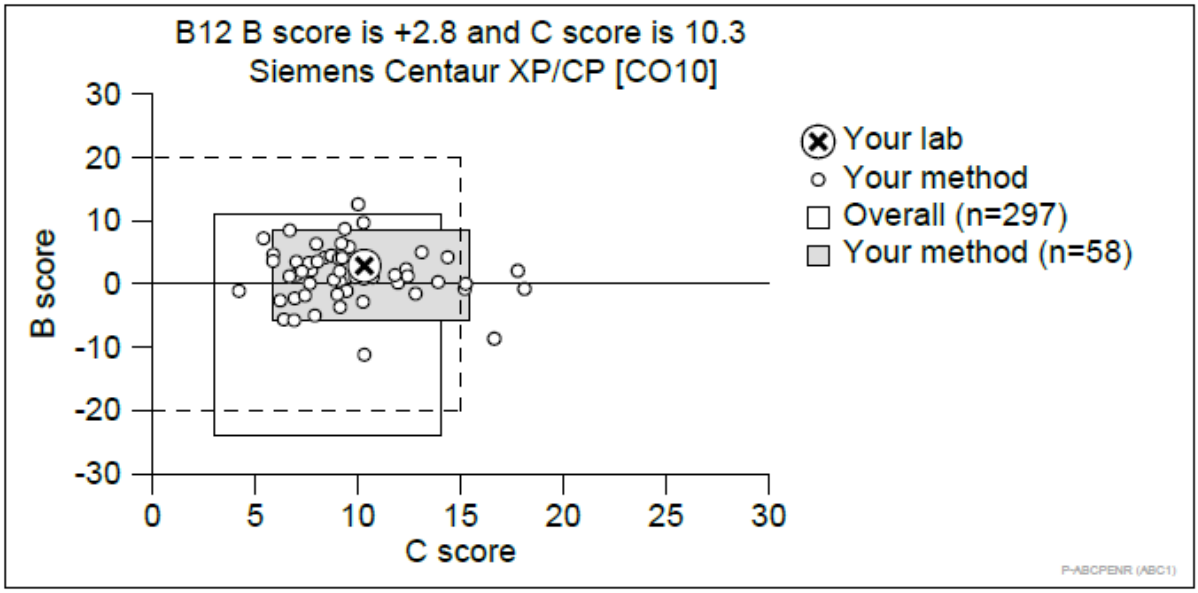
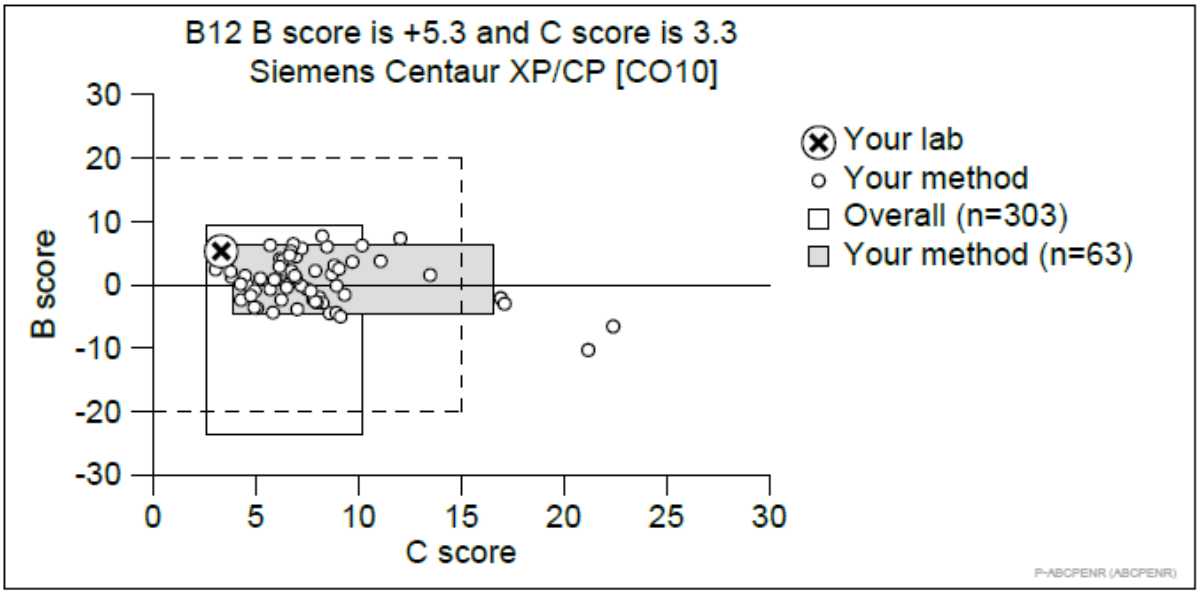


Figure Q.3 Illustrative overall performance charts for UKNEQAS for Year 8 of the NDNS RP for vitamin B_{12}

March 2016



September 2016



Q.2.4 Holotranscobalamin (holoTC); "active B12"

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 Years 7 and 8 were conducted at MRC EWL, automated on a DS2 ELISA processor (Launch Diagnostics Ltd).

Q.2.4.1 Internal quality controls 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 Years 7 and 8 samples.

Table Q.4 Internal quality controls for holoTC for Year 7-8 of the NDNS RP

HoloTC	Year 7 Low Control 25pmol/L (15-35)	Year 7 High Control 60pmol/L (36-84)	Year 8 Low Control 25pmol/L (15-35)	Year 8 High Control 60pmol/L (36-84)	Year 8 QA 74pmol/L (52-96)[a]
Mean	24.3	64.5	23.6	60.4	71.7
SD	2.0	4.4	1.2	4.4	5.5
% CV	8.1	6.8	5.2	7.4	7.7
n	26	26	37	37	28

[a] There was no equivalent QA in Year 7

Q.2.4.2 External quality assessment for holoTC

NEQAS are conducting a pilot study for external assessment of holoTC results. The short time-scale over which the Years 7 and 8 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 NDNS RP Years 7 and 8 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 Years 7 and 8 results.

Table Q.5 External Quality Assessment for holoTC for Years 7 and 8 of the NDNS RP

holoTC pilot scheme	Year 7	Year 8
mean % bias	3.8	6.9
sd of % bias	7.1	7.1
n	15	3

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 quality controls for total cholesterol

Tables Q.6 and Q.7 show imprecision data for a period covering the analysis of Years 7 and 8 samples.

Table Q.6 Internal quality controls for total cholesterol for Year 7 of the NDNS RP

QC	153UL	833UN	619UE
total cholesterol (mmol/L)	3.44 (3.39 - 3.49)	4.44 (4.23 - 4.64)	7.04 (6.84 - 7.23)
mean	3.50	4.43	7.01
sd	0.07	0.08	0.07
cv	2.02	1.72	1.06
n	53	54	54

Table Q.7 Internal quality controls for total cholesterol for Year 8 of the NDNS RP

QC	199UL	1015UN	762UE
total cholesterol (mmol/L)	3.35 (3.20 - 3.49)	4.48 (4.27 - 4.68)	7.01 (6.77 - 7.24)
mean	3.36	4.50	7.01
sd	0.05	0.06	0.08
cv	1.54	1.43	1.17
n	67	68	67

Q.2.5.2 Internal quality controls for HDL cholesterol

Tables Q.8 and Q.9 show imprecision data for a period covering the analysis of Years 7 and 8 samples.

Table Q.8 Internal quality controls for HDL cholesterol for Year 7

QC	153UL	833UN	619UE
HDL (mmol/L)	1.77 (1.69 - 1.84)	2.67 (2.18 - 3.16)	4.55 (4.23 - 4.86)
Mean (mmol/L)	1.80	2.78	4.54
SD	0.06	0.20	0.18
% CV	3.26	7.22	3.87
n	54	55	55

Table Q.9 Internal quality controls for HDL cholesterol for Year 8 of the NDNS RP

QC	199UL	1015UN	762UE
HDL (mmol/L)	1.46 (1.38 - 1.54)	1.41 (1.29 - 1.54)	2.62 (2.38 - 2.86)
mean	1.46	1.38	2.57
sd	0.03	0.03	0.06
cv	1.76	2.34	2.53
n	65	66	63

Q.2.5.3 External quality controls for total and HDL cholesterol

External quality control was achieved through UKNEQAS and also the Randox International Quality Assessment Scheme (RIQAS); NEQAS pooled samples are unsuitable for the total cholesterol method used by the Siemens Dimension instruments. Table Q.10 indicates the percentage deviation of results obtained by MRC EWL from the target concentration for Year 7 and Year 8. These have been calculated at MRC EWL.

Table Q.10 NEQAS and RIQAS results for lipid analyses - deviation from target concentration

	NEQAS	Year 7	Year 8
HDL - cholesterol	mean % bias	-3.8	-3.9
	sd of % bias	3.2	4.9
	n	36	36

	RIQAS	Year 7		Year 8	
Total cholesterol	cycle	32	33	34	35
	mean % bias	-2.44	-1	0.44	-0.91
	sd % bias	0.82	0.36	0.16	0.33

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 quality controls for serum triglycerides (triacylglycerols)

Tables Q.11 and Q.12 show imprecision data in Years 7 and 8.

Table Q.11 Internal quality controls for serum triglycerides (triacylglycerols) for Year 7 of the NDNS RP

	QC1 153UL	QC2 833UN	QC3 619UE
Target (mmol/L)	1.44 (1.41 - 1.48)	2.46 (2.24 - 2.69)	3.99 (3.83 - 4.16)
Mean (mmol/L)	1.46	2.54	4.04
SD	0.03	0.11	0.08
% CV	2.30	4.27	2.06
n	53	53	54

Table Q.12 Internal quality controls for serum triglycerides (triacylglycerols) for Year 8 of the NDNS RP

	QC1 199UL	QC2 1015UN	QC3 762UE
Target (mmol/L)	1.42 (1.37 - 1.46)	2.54 (2.46 - 2.62)	3.83 (3.75 - 3.91)
Mean (mmol/L)	1.42	2.54	3.83
SD	0.02	0.03	0.03
% CV	1.39	1.29	0.83
n	67	66	64

Q.2.6.2 External quality assessment for serum triglycerides (triacylglycerols)

External quality assessment was achieved through RIQAS. Table Q.13 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.13 RIQAS results for triglycerides (triacylglycerols): deviation from target concentration

	NEQAS	Year 7	Year 8
Triglycerides	mean % bias	0.6	-4.1
	sd of % bias	4.4	3.3
	n	36	36

	RIQAS	Year 7		Year 8	
Triglycerides	cycle	32	33	34	35
	mean % bias	-6.22	-1.63	-2	0.63
	sd % bias	1.5	0.4	0.48	0.16

Q.2.7 HbA1c

Haemoglobin A1c (HbA1c) was measured by HPLC at Addenbrooke's Hospital, Cambridge, 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.14 show good precision over Year 7 and Year 8.

Table Q.14 Internal quality controls for HbA1c for Year 7 of NDNS RP

	Year 7		Year 8	
HbA1c mmol/mol	Biorad Low	Biorad High	Biorad Low	Biorad High
mean	36	81	35	83
sd	0.7	1.0	0.8	1.3
cv %	1.9	1.3	2.3	1.6
n	183	183	136	136

Q.2.7.2 External quality assessment for HbA1c

The HbA1c external assessment scheme calculates “SDI” for each distribution – this is “total error” and includes both inaccuracy and imprecision. Figures Q.4 and Q.5 show the SDI calculated for each distribution in Year 7 and Year 8. The solid line shows the performance of the Addenbrooke’s lab and the dotted lines are the national median and 97.5 percentile performance. These graphs demonstrate that the Addenbrooke’s lab performs close to the median UK performance for this assay.

Figure Q.4 External quality Assessment for HbA1c during Year 7

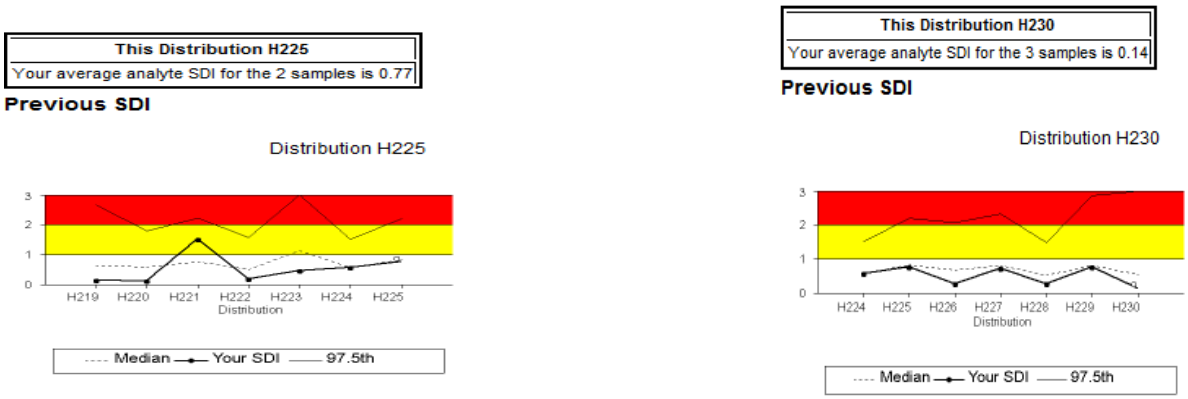
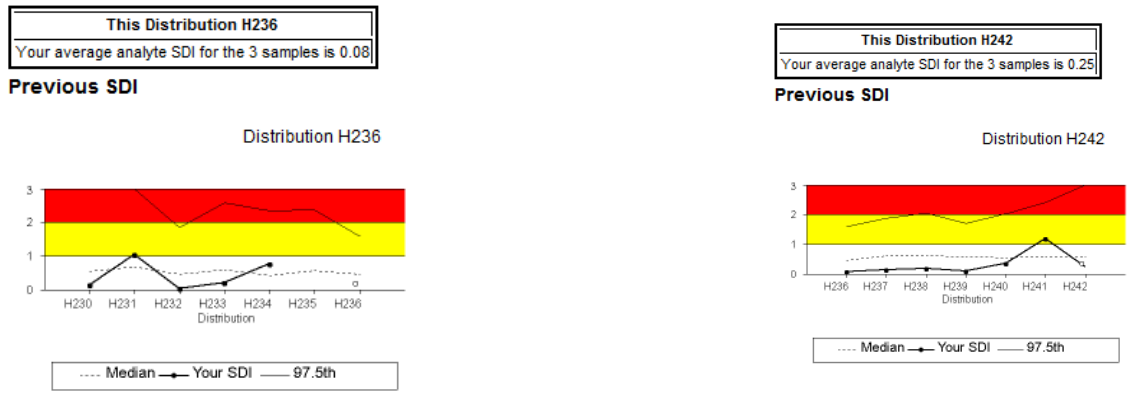


Figure Q.5 External quality Assessment for HbA1c during Year 8



Q.2.8 Plasma glucose

Glucose was measured at the Elsie Widdowson Laboratory 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 tables Q.15 and Q.16 indicate good between-batch consistency for glucose results during Year 7 and Year 8.

Table Q.15 Internal quality controls for glucose for Year 7 of the NDNS RP

Glucose mmol/L	153UL	833UN	619UE
Target	3.05 (2.94 - 3.16)	7.28 (6.97 - 7.59)	19.94 (19.62 - 20.28)
mean	3.28	7.47	20.09
sd	0.17	0.19	0.22
cv	5.08	2.59	1.09
n	48	48	49

Table Q.16 Internal quality controls for glucose for Year 8 of the NDNS RP

Glucose mmol/L	199UL	1015UN	762UE
Target	3.36 (3.22 - 3.51)	7.87 (7.62 - 8.19)	21.41 (20.41 - 22.42)
mean	3.38	7.86	21.13
sd	0.08	0.13	0.28
cv	2.48	1.69	1.34
n	54	56	64

Q.2.8.2 External Quality assessment for plasma glucose

External quality assessment was through the UK NEQAS scheme.

Table Q.17 shows the percentage bias relative to the target concentration in glucose results from MRC EWL during Year 7 and Year 8.

Table Q.17 External quality assessment (UKNEQAS) results for glucose for Year 7 and Year 8

	Year 7	Year 8
mean % bias	2.2	5.8
sd % bias	4.6	4.0
n	48	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 quality controls 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 tables Q.18 and Q.19 indicate good between-batch consistency for ferritin results during Year 7 and Year 8.

Table Q.18 Internal quality controls for ferritin for Year 7 of the NDNS RP

QC	153UL	833UN	619UE
Target (µg/L)	30 (28.9 - 31.9)	63.7 (58.2 - 69.2)	78.0(69.7 - 86.2)
Mean (µg/L)	31.58	65.0	79.0
SD (µg/L)	0.98	1.95	2.58
CV (%)	3.12	2.99	3.27
n	32	33	33

Table Q.19 Internal quality controls for ferritin for Year 8 of the NDNS RP

QC	199UL	1015UN	762UE
Target (µg/L)	29.2 (26.7 - 31.8)	54.8 (50.0 - 59.7)	73.7 (66.7 - 80.7)
Mean (µg/L)	29.77	55.3	73.7
SD (µg/L)	1.02	1.94	3.07
CV (%)	3.42	3.51	4.17
n	18	19	19

Q.2.9.2 External quality assessment for plasma ferritin

External quality assessment was through the UK NEQAS Haematinics scheme. Table Q.20 shows the percentage bias relative to the target concentration in ferritin results from MRC EWL during Year 7 and Year 8.

Table Q.20 Summary of NEQAS bias assessment in Years 7 and 8 of the NDNS RP

Ferritin NEQAS	Year 7	Year 8
Mean % bias from ALTM	5.2	2.9
SD % bias	6	0.3
Number of NEQAS samples	36	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 quality controls for plasma vitamin C

QC samples were made in-house by spiking ascorbic acid-depleted plasma. The results in tables Q.21 and Q.22 indicate good between-batch consistency for vitamin C (ascorbic acid) measurements during Years 7 and 8.

Table Q.21 Internal quality controls for vitamin C for Year 7 of the NDNS RP

Vitamin C	QC1	QC2	QC3
Year 7			
mean (µmol/L)	11.8	34.0	58.0
sd (µmol/L)	1.7	2.8	4.5
cv (%)	14.6	8.2	7.7
N	79	79	79

Table Q.22 Internal quality controls for vitamin C for Year 8 of the NDNS RP

Vitamin C	QC1	QC2	QC3
Year 8			
mean (µmol/L)	11.3	33.1	52.6
sd (µmol/L)	1.2	2.0	3.3
cv (%)	10.2	6.1	6.2
N	116	116	116

Q.2.10.2 External quality controls for vitamin C

MRC EWL subscribed to the NIST (National institute for Standardisation and Control, US) EQAS for vitamin C until its cessation at the end of 2015. Samples were distributed quarterly and results were always within the target range. Results are quoted by NIST in terms of agreement with the "interlaboratory consensus" which is validated against Standard Reference Material 970. MRC EWL results are in close agreement with this validated "interlaboratory consensus" indicating accuracy with respect to the Standard Reference Material.

Figure Q.6 External quality controls for vitamin C for Year 7 of the NDNS RP. Comparison of Measurements to "Inter-laboratory Consensus"

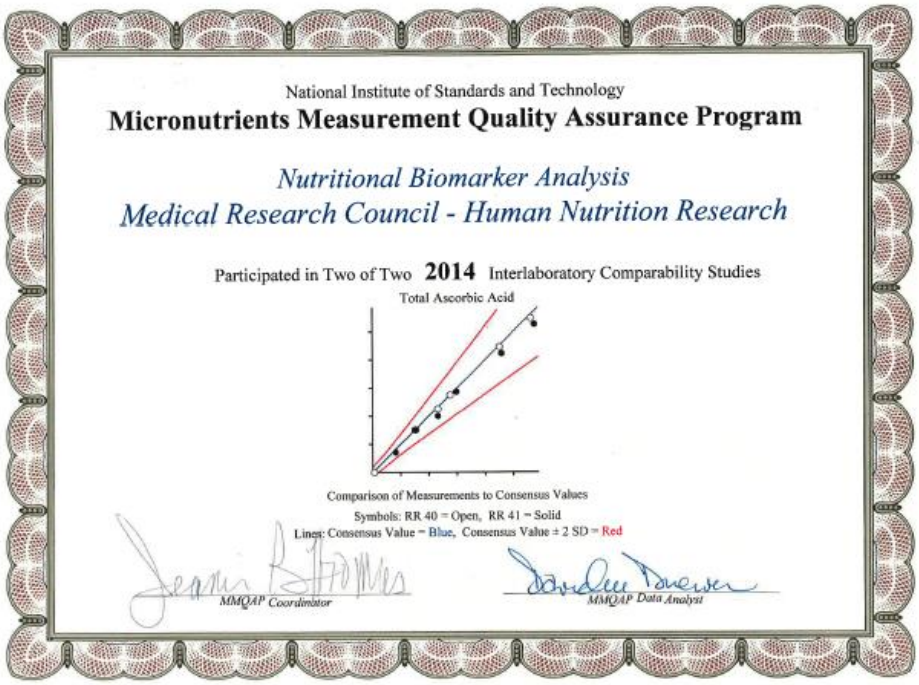
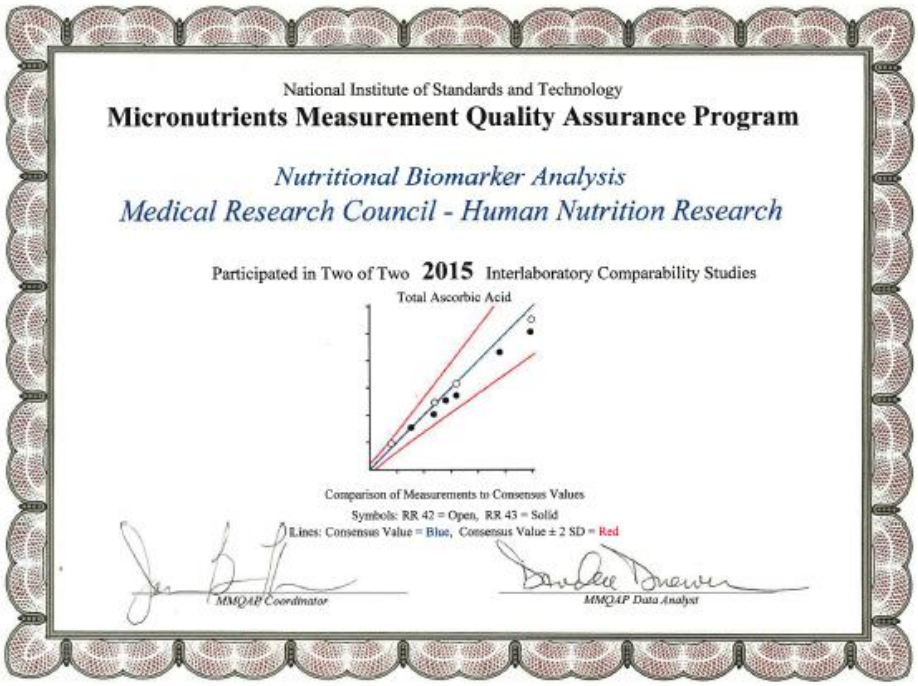


Figure Q.7 External quality controls for vitamin C for Year 8 of the NDNS RP. Comparison of Measurements to "Inter-laboratory Consensus"



Q.2.11 Erythrocyte transketolase activation coefficient (ETKAC) for thiamin 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 two 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 tables Q.23 and Q.24 for internal QCs indicate good batch-to-batch consistency of ETKAC results during Years 7 and 8.

There are no external Quality Assurance or QC schemes available for ETKAC.

Table Q.23 Internal quality controls for ETKAC for Year 7 of the NDNS RP

Control ID	C	K *	P
mean	1.08	1.10	1.06
sd	0.04	0.06	0.04
cv	3.42	5.64	3.57
n	25	22	24

Table Q.24 Internal quality controls for ETKAC for Year 8 of the NDNS RP

Control ID	C	K *	P
mean	1.10	1.08	1.07
sd	0.03	0.06	0.03
cv	2.92	5.26	2.55
n	29	23	30

* 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.

Q.2.12.1 Quality controls for EGRAC

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

Q.2.12.1.1 Internal quality control results during Years 7 and 8

Descriptive statistics in tables Q.25 and Q.26 for internal QCs indicate good batch-to-batch consistency of EGRAC results during Years 7 and 8.

Table Q.25 Internal quality controls for EGRAC for Year 7 of the NDNS RP

Control ID	A	C	X
Mean	2.14	1.53	0.97
SD	0.09	0.03	0.01
% CV	3.98	2.25	1.06
N	24	24	24

Table Q.26 Internal quality controls for EGRAC for Year 8 of the NDNS RP

Control ID	**A**	**C**	**X**
Mean	2.18	1.55	0.98
SD	0.06	0.04	0.02
% CV	2.76	2.45	1.56
N	41	43	40

Q.2.13 Plasma vitamin B_6 (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.[17]

Q.2.13.1 Internal quality controls for vitamin B_6

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.

There were no external quality schemes for the vitamin B_6 HPLC method.

From Year 5 plasma from two 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.

The good agreement between the obtained values for PLP and PA in the quality control (tables Q.27 and Q.28) and the expected values indicate a quantitative recovery of vitamin B_6 in this assay.

Table Q.27 Internal quality controls for PLP and PA (unspiked plasma) for Years 7 and 8 of the NDNS RP

PLP	Year 7		Year 8	
Plasma	A07	A02	A07	A02
Mean (nmol/L)	26.0	50.3	25.7	50.4
SD	2.2	1.8	1.2	2.1
% CV	8.6	3.6	4.5	4.2
N	25	26	28	28

PA	Year 7		Year 8	
Plasma	K4082310	A02	K4082310	A02
Mean (nmol/L)	35.9	126.1	36.5	127.7
SD	1.8	5.0	1.8	3.3
% CV	5.0	4.0	4.9	2.6
N	26	26	28	28

Table Q.28 Internal quality controls for PLP and PA (spiked plasma) for Years 7 and 8 of the NDNS RP

PLP	Year 7	Year 8 part 1	Year 8 part 2 (new spiked controls start)
Mean (nmol/L)	52.2	52.0	55.5
SD	1.8	1.6	3.2
% CV	3.4	3.1	5.8
n	26	18	10

PA	Year 7	Year 8 part 1	Year 8 part 2 (new spiked controls start)
Mean (nmol/L)	58.7	58.6	54.4
SD	2.8	2.2	3.0
% CV	4.8	3.8	5.4
n	26	18	10

For Year 7 and part 1 of Year 8 the expected PLP concentration of the spiked plasma was 51.7nmol/L (the sum of the basal level in the plasma plus the spike concentration). The expected PA concentration of the spiked plasma was 58.1nmol/L (the sum of the basal level in the plasma plus the spike concentration). For part 2 of Year 8 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 55nmol/L (the sum of the basal level in the plasma plus the spike concentration). Table Q.28 above indicates consistent accuracy for both spiked controls.

Q.2.14 Serum and whole blood folate

Q.2.14.1 Serum folate

Serum folate for Years 7 and 8. Serum folate was measured at MRC EWL by 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 normalizing for sample preparation and instrument variability. Highly specific detection of the six vitamers: methyltetrahydrofolate, tetrahydrofolate, formyltetrahydrofolate, folic acid, 5,10 methenyltetrahydrofolate and MeFox and internal standards is accomplished by LC-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 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 is calculated from the sum of the six folate vitamers.

Q.2.14.1.1 Internal quality control for serum folate

Three controls containing folic acid as well as naturally occurring vitamers were analysed with each batch of samples for assessment of assay precision. Total folate was calculated by summing the individual folate vitamers (including folic acid).

Because of the limitations of external quality assessment schemes for serum folate, accuracy was continually monitored in-house by including Standard Reference Material as an accuracy control. Standard Reference Material (SRM) 1950 was stored in single-use aliquots and run with alternate batches of samples in order to assess assay accuracy. Results are presented in tables Q.29 and Q.30.

Table Q.29 Precision assessment

Year 7	**QC1**		**QC2**		**QC3**	
Serum folate, nmol/L	folic acid	total folate	folic acid	total folate	folic acid	total folate
Mean	8.76	34.6	5.02	91.0	1.09	18.4
SD	0.50	1.37	0.29	3.43	0.09	1.12
%cv	5.66	3.97	5.74	3.77	8.03	6.07
n	14	14	14	14	14	14

Year 8	**QC1**		**QC2**		**QC3**	
serum folate, nmol/L	folic acid	total folate	folic acid	total folate	folic acid	total folate
mean	9.11	35.4	5.25	92.0	1.11	19.4
sd	0.57	1.81	0.42	4.92	0.07	0.86
%cv	6.26	5.12	7.93	5.35	5.87	4.44
n	15	15	16	16	16	16

Table Q.30 Accuracy assessment (Standard Reference Material 1950)

	Year 7		Year 8	
	MTHF	Folic Acid	MTHF	folic acid
Target	26.91 +/- 0.7	3.42 +/- 1.02	26.91 +/- 0.7	3.42 +/- 1.02
Mean	27.94	3.08	28.91	3.11
SD	0.83	0.07	1.32	0.07
%cv	2.96	2.40	4.55	2.09
n	7	7	8	8

Q.2.14.1.2 External quality assessment of serum folate

The laboratory participated in the VITAL-EQA program organised by CDC; round 26 during Year 7 and Round 8 during Year 8. Three samples are sent for each round. Comparison is against a target concentration determined using the Bio-Tek Power Wave microbiological method. In Round 26 performance was classified as “optimum”/”desirable” in terms of precision and “acceptable”/”desirable” in terms of bias; in round 7 both bias and imprecision were classified as “optimum”/”desirable”. 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, comparison with the ALTM is not a valid measure of accuracy.

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, a surrogate of 0.4L/L was used.

Q.2.14.2.1 Whole blood folate – analytical method (information from CDC)

Whole blood haemolysate specimens (whole blood diluted and stabilized with ascorbic acid) were analysed for total folate using the *Lactobacillus rhamnosus* microbiologic growth assay by an adaptation of O'Broin *et al.*[18] and Molloy *et al.*,[19] as described by Pfeiffer *et al.*[20] Diluted specimen (four replicates at two 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 four replicates at two different dilutions were averaged to generate the final result and the CV from the four replicates had to be ≤15% (≤10% if only three replicates were used). No result was reported from less than three 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.

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. Any possible deterioration in folate concentration during overnight postage is likely to be less than 10%.[21]

Q.2.14.2.2 Internal QC for whole blood folate (information supplied by CDC)

Three whole blood bench QC pools were analysed in duplicate in every 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.31.

Table Q.31 Whole blood total folate concentration (nmol/L)

Year 7	Bench QC Pool		
Parameter	**LB14810a_MA**	**MB14811a_MA**	**HB14812a_MA**
Mean, nmol/L	246	427	724
SD, nmol/L	13.0	27.0	50.3
CV	5.3%	6.3%	6.9%
Target, nmol/L	232	429	722
Difference from target	5.8%	-0.4%	0.2%

Year 8	Bench QC Pool		
Parameter	**LB14810a_MA**	**MB14811a_MA**	**HB14812a_MA**
Mean, nmol/L	240	440	734
SD, nmol/L	28	46	69
CV	12%	10%	9%
Target, nmol/L	232	429	722
Difference from target	3.3%	2.7%	1.5%

Four additional whole blood QC pools were analysed “blind” (ie target concentration unknown to analyst) as part of this study at a rate of one blind QC sample in every 20 unknown samples. The between-run imprecision and target concentration are shown in table Q.32.

Table Q.32 Whole blood total folate concentration (nmol/L)

Year 7	Blind QC Pool			
Parameter	**936**	**937**	**938**	**939**
Mean, nmol/L	525	258	371	177
SD, nmol/L	53	20	32	24
CV	10.1%	7.8%	8.5%	13.5%
n	4	10	12	4
Target, nmol/L	527	256	354	187
Difference from target	-1.9%	0.4%	10.1%	-4.3%

Year 8	Blind QC Pool			
Parameter	**936**	**937**	**938**	**939**
Mean, nmol/L	499	245	373	172
SD, nmol/L	42	21	25	24
CV	8%	9%	7%	9%
n	14	8	6	6
Target, nmol/L	527	256	354	187
Difference from target	-5%	-4%	5%	-8%

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.2.15 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.*[22] Samples were assayed as singletons. Plasma concentrations of vitamin A (retinol), α-, and γ-tocopherol, and six 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 and 6; these differ from the methods used for Years 1-4. Details of the conversion factors needed for comparison with data collected during Years 1 to 4 are detailed in the report for Years 5 and 6.

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

The FSV results for Years 7 and 8 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 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 quality assessment scheme led by NIST with UKNEQAS returns as corroboration, see section Q.2.15.2.

Table Q.33 Precision of internal QC for plasma retinol for Years 7 and 8 of the NDNS RP

Retinol	Year 7	Year 8
	QC11	QC11
Mean (µmol/L)	1.56	1.55
SD	0.13	0.14
%CV	8.4	8.8
n	25	27

Table Q.34 Precision of internal QC for plasma α-tocopherol for Years 7 and 8 of the NDNS RP

ATC	Year 7			Year 8		
	QC2	QC11	Serum 183	QC2	QC11	Serum 183
Mean (µmol/L)	34.05	15.68	25.77	34.54	16.33	26.97
SD	2.71	1.02	2.04	2.65	1.09	1.75
%CV	7.9	6.5	7.9	7.7	6.7	6.5
n	25	25	26	30	30	29

Table Q.35 Precision of internal QC for plasma γ-tocopherol for Years 7 and 8 of the NDNS RP

GTC	Year 7	Year 8
	QC11	QC11
Mean (µmol/L)	1.01	0.98
SD	0.18	0.17
%CV	17.7	17.5
n	25	34

Table Q.36 Precision of internal QC for plasma α-carotene for Year 7 and Year 8 of the NDNS RP

Alpha carotene	Year 7	Year 8
	QC2	QC2
Mean (µmol/L)	0.11	0.11
SD	0.01	0.01
%CV	8.9	6.8
n	24	29

Table Q.37 Precision of internal QC for plasma β-carotene for Year 7 and Year 8 of the NDNS RP

Beta carotene	Year 7		Year 8	
	QC11	Serum 183	QC11	Serum 183
Mean (µmol/L)	0.15	0.72	0.16	0.76
SD	0.01	0.04	0.01	0.05
%CV	8.0	5.1	7.6	6.0
n	26	26	30	29

Table Q.38 Precision of internal QC for plasma α-cryptoxanthin for Year 7 and Year 8 of the NDNS RP

Alpha cryptoxanthin	Year 7	Year 8
	QC2	QC2
Mean (µmol/L)	0.09	0.08
SD	0.01	0.01
%CV	9.0	12.5
n	25	27

Table Q.39 Precision of internal QC for plasma β-cryptoxanthin for Year 7 and Year 8 of the NDNS RP

Beta cryptoxanthin	Year 7	Year 8
	QC11	**QC11**
Mean (µmol/L)	0.17	0.17
SD	0.01	0.01
%CV	8.0	8.1
n	26	30

Table Q.40 Precision of internal QC for plasma lycopene for Year 7 and Year 8 of the NDNS RP

lycopene	Year 7	Year 8
	QC11	**QC11**
Mean (µmol/L)	0.64	0.63
SD	0.04	0.05
%CV	6.2	8.8
n	26	30

Table Q.41 Precision of internal QC for plasma lutein + zeaxanthin for Year 7 and Year 8 of the NDNS RP

Lutein/zeaxanthin	Year 7	Year 8
	QC11	**QC11**
Mean (μmol/L)	0.19	0.20
SD	0.02	0.01
%CV	11.9	7.1
n	26	30

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

Participation in studies conducted by NIST, CDC VITAL-External Quality Assurance (CDC VITAL EQA) and UKNEQAS allowed inter-laboratory comparison of results. MRC EWL participated in a twice yearly "round robin" with both NIST and VITAL EQA (Years 7 and 8 only). 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 six 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 returns LXXVIII (Year 7), LXXIX and LXXX (Year 8) were submitted during their respective sample analysis periods; extracts of the reports are shown in figures Q.8-Q.10, indicating very close agreement with the target concentration.

Figure Q.8 NDNS RP Year 7 NIST return LXXVIII

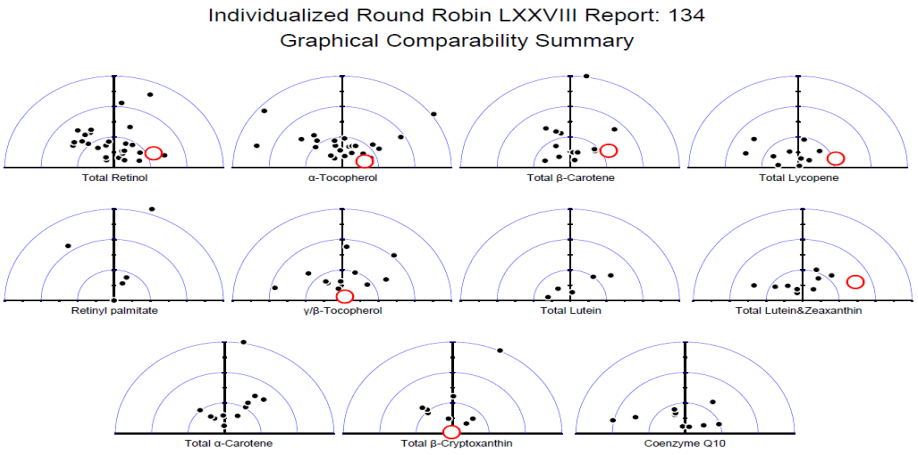


Figure Q.9 NDNS RP Year 8 NIST return LXXIX

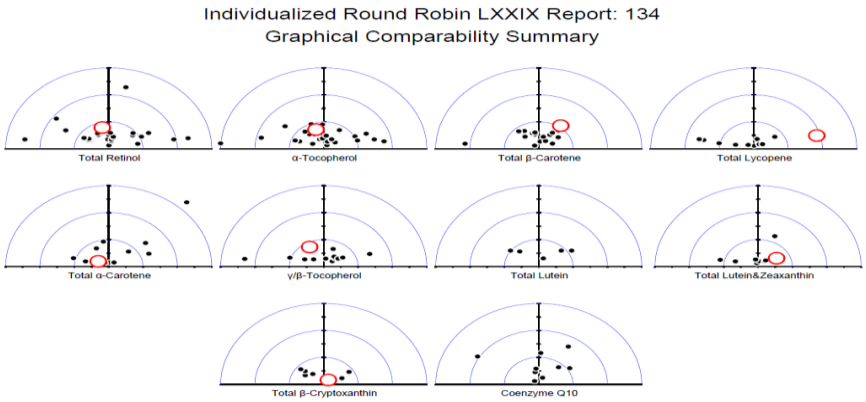
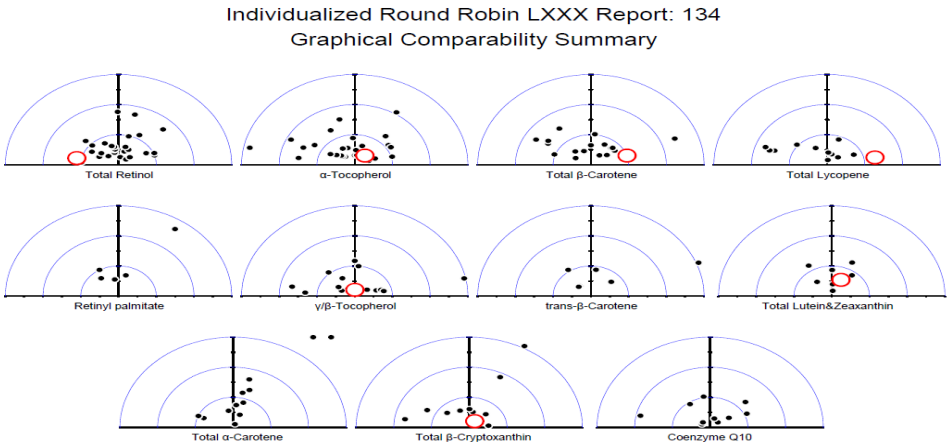


Figure Q.10 NDNS RP Year 8 NIST return LXXX



In the graphical representations in figures Q.8-Q.10 our result for each analyte measured is represented as an open larger circle with other labs 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. There are five samples analysed for every NIST return and the analyte 'result' above is a summary of all five.

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

For Years 7 and 8, 25-OHD was measured in serum by LCMS-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.2.16.1 Quality control for 25-OHD

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

Q.2.16.1.1 Internal quality controls 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 tables Q.42-Q.43.

Table Q.42 Internal quality controls for 25-OHD for Year 7 of the NDNS RP

Manufacturer's controls: (nmol/L)					Lyphochek (nmol/L)	
Year 7	Lot 3613					
	level 1		level 2		in-house	
	D2	D3	D2	D3	D2	D3
mean	37.2	44.6	81.2	112.0	12.2	19.5
SD	3.3	2.0	5.5	5.3	1.1	1.0
%cv	9.0	4.5	6.8	4.7	9.4	4.9
n	26	26	26	26	27	27

Table Q.43 Internal quality controls for 25-OHD for Year 8 of the NDNS RP

Manufacturer's controls: (nmol/L)					Lyphochek (nmol/L)	
Year 8	Lot 2714					
	level 1		level 2		in-house	
	D2	D3	D2	D3	D2	D3
mean	42.3	37.5	97.5	86.0	11.8	19.3
SD	2.2	2.3	7.2	5.0	1.4	0.9
%cv	5.2	6.1	7.4	5.8	12.1	4.7
n	24	24	24	24	24	24

Q.2.16.1.2 External quality assessment for 25-OHD

MRC EWL subscribed to the DEQAS external quality assessment 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 ALTM). DEQAS do not issue cumulative performance data as do NEQAS.

Table Q.44 shows the relationships between 25-OHD as reported on individual DEQAS samples by EWL and the target concentration as provided by NIST.

Table Q.44 Relationships between 25-OHD as reported on individual DEQAS samples by EWL

	Year 7	Year 8
mean % deviation from Target Value	-0.4	-0.2
sd	5.0	5.0
n	27	28

Q.2.17 Plasma creatinine

The creatinine method used in the NDNS RP Years 7 and 8 employs a modification of the kinetic Jaffe reaction reported by Larsen.

Under alkaline conditions, creatinine reacts with picrate to form a red chromophore. The rate of increasing absorbance at 510nm due to the formation of this chromophore is directly proportional to the creatinine concentration in the sample and is measured using a bichromatic (510nm, 600nm) rate technique. Bilirubin is oxidised by potassium ferricyanide to prevent interference. This method has been reported to be less susceptible than conventional methods to interference from non-creatinine, Jaffe-positive compounds; however, plasma which has been in contact with blood cells for more than eight hours before separation is not suitable for analysis.

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 quality controls 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. Tables Q.45 and Q.46 show internal QC results for creatinine, covering the period when Years 7 and 8 samples were analysed, respectively.

Table Q.45 Internal quality controls for plasma creatinine for Year 7 of the NDNS RP

QC	153UL	833UN	619UE
Creatinine (Jaffe) µmol/L	68.8 (61.4 - 75.02)	145.03 (109.32 - 180.75)	413.15 (373.68 - 452.63)
mean	82.89	150.24	415.11
sd	8.20	8.99	13.82
cv	9.90	5.99	3.33
n	59	60	59

Table Q.46 Internal quality controls for plasma creatinine for Year 8 of the NDNS RP

QC	199UL	1015UN	762UE
Creatinine (enzymatic) µmol/L	48.96 (46.16 - 51.77)	135.85 (130.57 - 141.13)	389.78 (376.46 - 403.09)
mean	48.45	134.22	386.40
sd	1.16	2.26	5.90
cv	2.40	1.69	1.53
n	43	41	43

Q.2.17.2 External quality assessment for plasma creatinine

MRC EWL subscribes to the UKNEQAS clinical chemistry. Table Q.47 shows that during Years 7 and 8 the Dimension assay at MRC EWL gave results acceptably close to the consensus of all laboratories using the same method.

Table Q.47 External quality assurance for creatinine performance during Year 7 (kinetic Jaffe method) and Year 8 relative to method mean (enzymatic method)

creatinine	Year 7 (Jaffe)	Year 8 (enzymatic)
mean % bias	-1.1	4.9
sd of % bias	9.4	4.3
n	75	54

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 quality control (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 Quality controls 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

Tables Q.48 to Q.49 summarise 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.48 QC analysis for Year 7 of the NDNS RP

	ClinChek L1		ClinChek L2	
	Selenium	**Zinc**	**Selenium**	**Zinc**
Lot number	423		423	
Target Concentration /Range (µg/L)	81.4 (65.1 – 97.7)	1160 (928 – 1390)	120 (96 – 144)	1540 (1230 – 1850)
Mean Measured Concentration (µg/L)	73.2	1127.0	110.0	1561.6
N (QC samples)	31	29	28	26
SD	3.7	64.5	4.1	48.0
%CV	5.1	5.7	3.7	3.1

Table Q.49 QC analysis for Year 8 of the NDNS RP

	ClinChek L1		ClinChek L2	
	Selenium	**zinc**	**Selenium**	**Zinc**
Lot number	423		423	
Target Concentration /Range (µg/L)	81.4 (65.1 – 97.7)	1160 (928 – 1390)	120 (96 – 144)	1540 (1230 – 1850)
Mean Measured Concentration (µg/L)	73.9	1109.1	112.7	1542.6
N (QC samples)	31	31	32	32
SD	2.8	69.2	3.7	72.2
%CV	3.8	6.2	3.3	4.7

Q.2.18.1.2 External Quality Controls 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 quality controls is valid.

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Brighton and Sussex University Hospitals NHS Trust
Calderdale and Huddersfield NHS Foundation Trust
Cancer Research UK Clinical Centre
St James's University Hospital
Cardiff and Vale University Health Board
Clinicheck (London)
Colchester Hospital University NHS Foundation Trust
Countess of Chester NHS Foundation Trust
Coventry and Warwickshire University Hospitals NHS Trust
Cwm Taf Health Board
Dartford and Gravesham NHS Trust
Derby Hospitals NHS Foundation Trust
East and North Hertfordshire NHS Trust
East Cheshire NHS Trust
East Sussex Healthcare NHS Trust
Great Western Hospitals NHS Foundation Trust
Harrogate and District NHS Foundation Trust
Homerton University Hospital NHS Foundation Trust
Hull and East Yorkshire Hospitals NHS Trust
Hywel Dda Health Board
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NHS Arran and Ayrshire

NHS Borders

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NHS Fife

NHS Highland

NHS Isle of Wight

NHS Lanarkshire

NHS Lothian

NHS Shetland

NHS Western Isles

Norfolk and Norwich University Hospital NHS Trust

North Cumbria University Hospitals NHS Trust

North Devon Healthcare NHS Trust

Northern Lincolnshire and Goole Hospitals NHS Foundation Trust

Nuffield Health Exeter Hospital

Nuffield Health Glasgow Hospital

Nuffield Health Newcastle Upon Tyne Hospital

Nuffield Health Tunbridge Wells Hospital

Nuffield Health Wessex Hospital

Nuffield Health Wolverhampton Hospital

Oxford Radcliffe Hospitals NHS Trust

Peterborough and Stamford Hospitals NHS Foundation Trust

Royal Cornwall Hospitals NHS Trust

Salisbury NHS Foundation Trust

Sheffield Hallam University

Source BioScience (Nottingham)

South Devon Healthcare NHS Foundation Trust

South London Healthcare NHS Trust

South Tees NHS Foundation Trust

Spire Bristol Hospital

Spire Bushey Hospital

Spire Gatwick Park Hospital

Spire Southampton Hospital

Spire Portsmouth Hospital

Taunton and Somerset NHS Foundation Trust

The Dudley Group of Hospitals NHS Foundation Trust

The Hillingdon Hospitals NHS Foundation Trust

The Mid Yorkshire Hospitals NHS Trust

The Pennine Acute Hospitals NHS Trust

The Princess Alexandra Hospital NHS Trust

The Royal Bournemouth and Christchurch Hospitals NHS Foundation Trust

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University Hospital of North Staffordshire NHS Trust

University Hospitals of Leicester NHS Trust

University of Aberdeen Rowett Institute of Nutrition and Health

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