Appendix Q: Methods of blood analysis and quality control

Original authors: Sonja Nicholson, Lorna Cox, Stephen Young, Peter Winship, Chris Bates and Ann Prentice
Updated by: Sonja Nicholson, Lorna Cox, Stephen Young, Amanda McKillion

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 during Year 5:

- full blood count including haemoglobin and haematocrit (see section Q.2.1)
- serum C-reactive protein, using a high-sensitivity assay (see section Q.2.2)
- serum vitamin B_{12} (see section Q.2.3)
- serum Total, HDL and LDL cholesterol (see section Q.2.5)
- serum triglycerides (triacylglycerols) (see section Q.2.6)

The assays listed below were conducted at Addenbrooke's during Year 6:

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

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 HNR and delivered by courier on dry ice to each field laboratory. Blood sample sub-fractions were stored frozen at a maximum of -20^{o}C (typically at -40°C) at field laboratories for a period of six to eight weeks, before the samples were transported to HNR on dry ice, where they were stored frozen, at -80°C, until further subdivided and analysed.

The assays listed below were conducted at HNR during Year 5:

- plasma ferritin (see section Q.2.7)
- plasma soluble transferrin receptors (see section Q.2.8)

- plasma vitamin C (see section Q.2.9)
- ETKAC for thiamin status (see section Q.2.10)
- EGRAC for riboflavin status (see section Q.2.11)
- plasma vitamin B_6 (pyridoxal-5-phosphate (PLP) and 4-pyridoxic acid (PA)) (see section Q.2.12)
- plasma total homocysteine (see section Q.2.13)
- plasma retinol (see section Q.2.14)
- plasma α-tocopherol (see section Q.2.14)
- plasma γ-tocopherol (see section Q.2.14)
- plasma individual carotenoids; α–carotene, β–carotene, α–cryptoxanthin, β–cryptoxanthin, lycopene, lutein and zeaxanthin (see section Q.2.143)
- plasma 25-hydroxyvitamin D (see section Q.2.15)
- plasma creatinine (see section Q.2.16)
- plasma selenium (see section Q.2.17)
- plasma zinc (see section Q.2.17)

The assays listed below were conducted at HNR during Year 6:

- serum C-reactive protein, using a high-sensitivity assay (see section Q.2.2)
- holotranscobalamin (holoTC; “active B_{12}”; see section Q.2.4)
- serum Total, HDL and LDL cholesterol (see section Q.2.5)
- serum triglycerides (triacylglycerols) (see section Q.2.6)
- plasma ferritin (see section Q.2.7)
- plasma vitamin C (see section Q.2.9)
- ETKAC for thiamin status (see section Q.2.10)
- EGRAC for riboflavin status (see section Q.2.11)
- plasma vitamin B_6 (PLP and PA) (see section Q.2.12)
- plasma retinol (see section Q.2.14)
- plasma α-tocopherol (see section Q.2.14)
- plasma γ-tocopherol (see section Q.2.14)
- plasma individual carotenoids; α–carotene, β–carotene, α–cryptoxanthin, β–cryptoxanthin, lycopene, lutein and zeaxanthin (see section Q.2.14)
- plasma 25-hydroxyvitamin D (see section Q.2.15)
- plasma creatinine (see section Q.2.16)
- plasma selenium (see section Q.2.17)
- plasma zinc (see section Q.2.17)

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. Details of analytical methods for the analysis of folate and quality control data will be published alongside the folate results at a later date.

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.2.17. 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 on a Beckman Coulter LH700 series analyser which mainly uses the Coulter Principle[i,ii] 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.

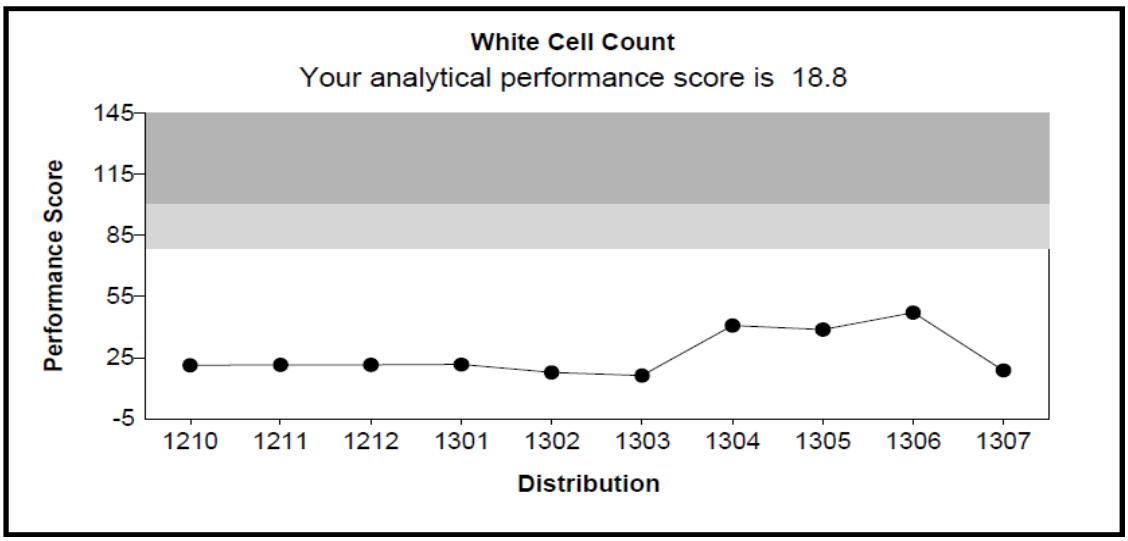
Haemoglobin was measured spectrophotometrically at 525nm by a photocell in a sample that was diluted 1:256 (final) with isotonic diluent and lysing solution. The red cells were destroyed with a lysing agent releasing the haemoglobin into solution, which enabled the white blood cell count to be estimated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

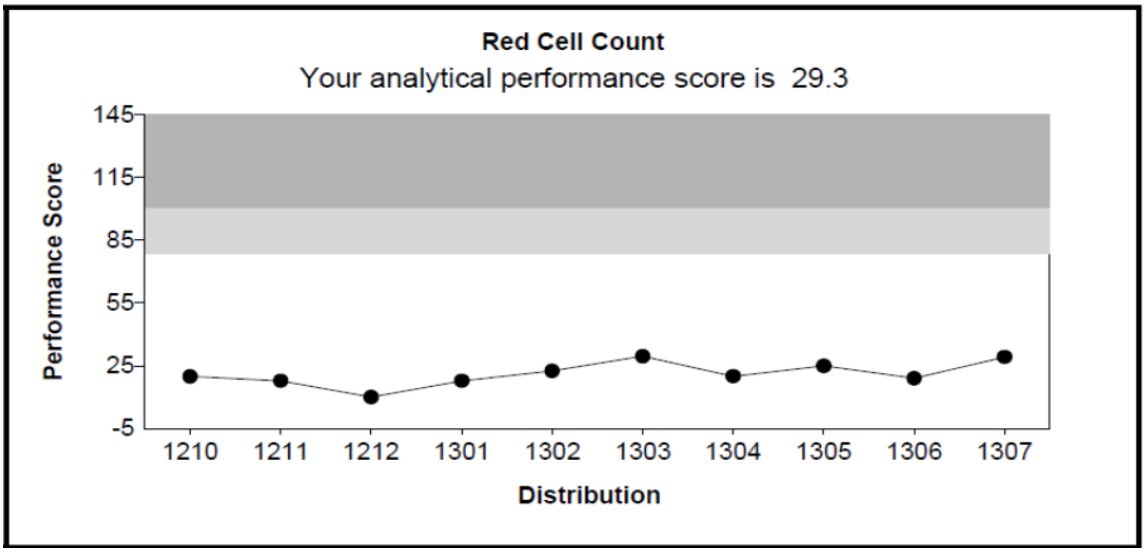
Q.2.1.1 Quality controls for full blood count including haemoglobin and haematocrit

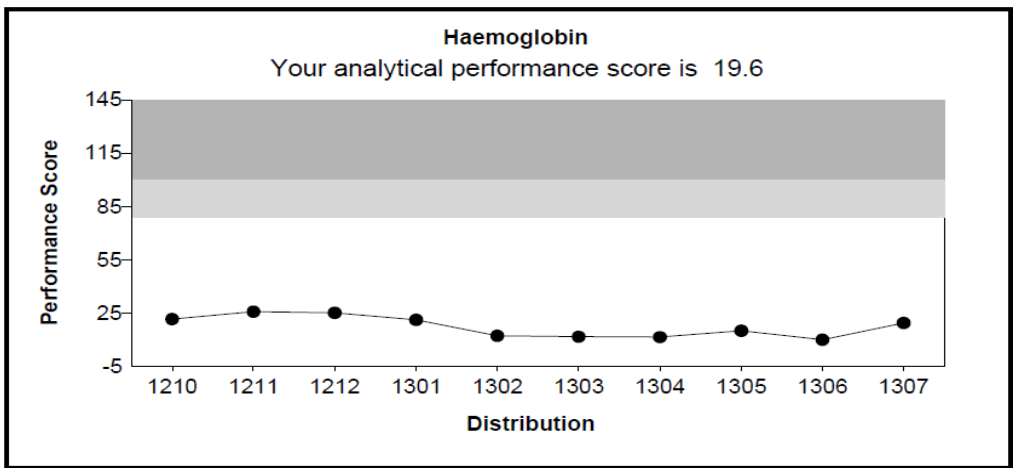
Quality of results was checked using Addenbrooke's internal controls at regular intervals and also assessed externally through UKNEQAS. NEQAS results are compared against the All Laboratories Trimmed Mean (ALTM) calculated for laboratories in the NEQAS scheme using the same analyser and method as that used by Addenbrooke's. Figures Q.1 and Q.2 show illustrative UKNEQAS overall performance results for full blood counts including haemoglobin and haematocrit for the periods covering analysis of samples for Years 5 and 6. 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

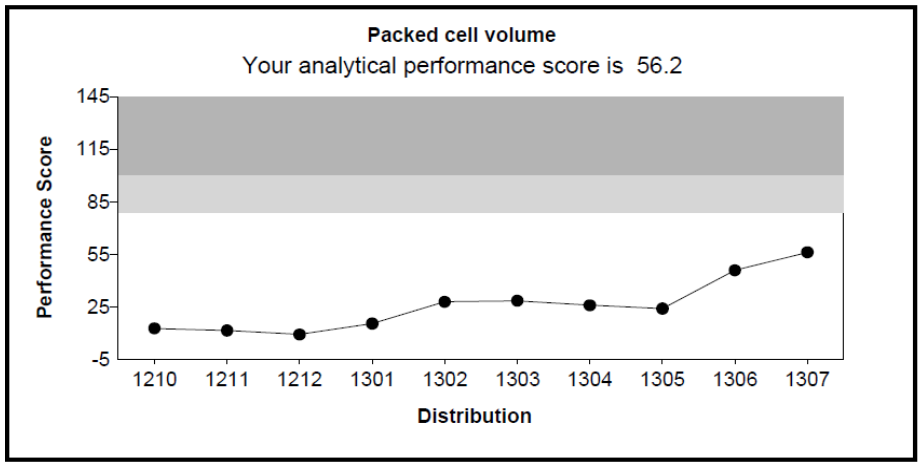
consensus mean. The dark shaded area indicates unacceptable performance and the paler area indicates a borderline situation.

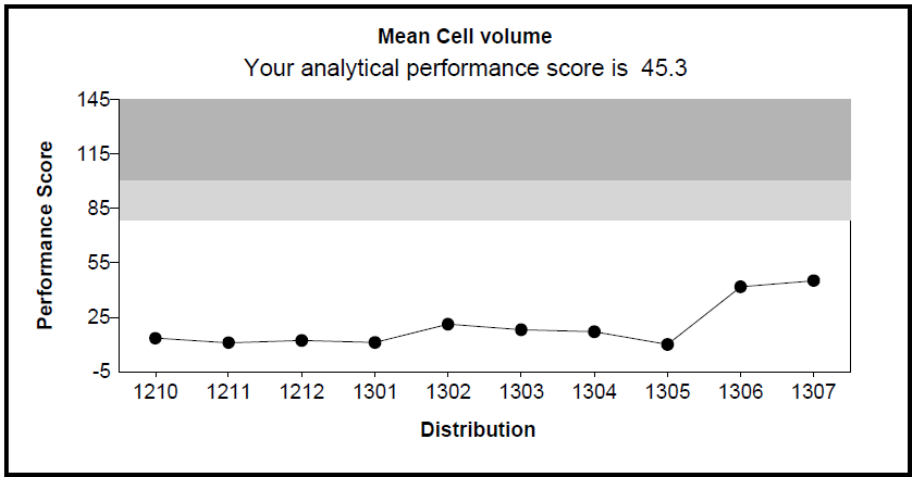
Figure Q.1 Illustrative overall performance charts for UKNEQAS for Year 5 of the NDNS RP

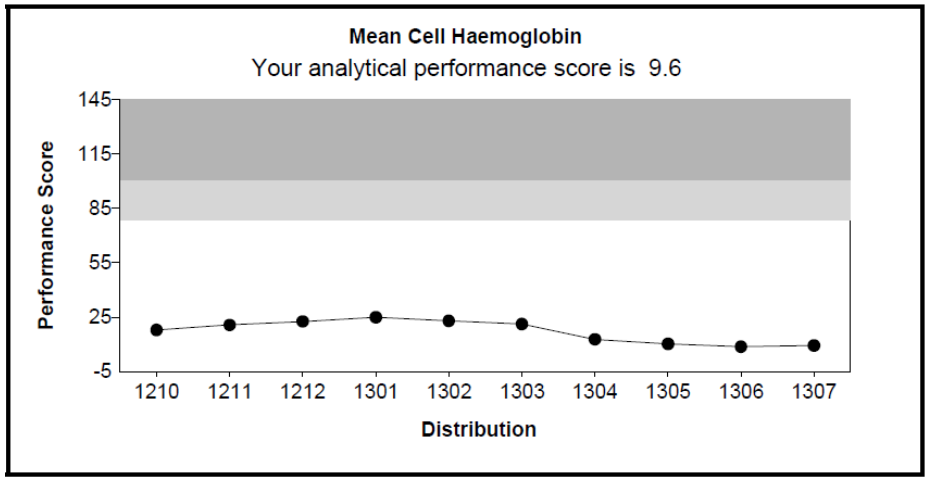







Packed cell volume
Your analytical performance score is 56.2
Performance Score
145
115
85
55
25
-5
1210 1211 1212 1301 1302 1303 1304 1305 1306 1307
Distribution


Mean Cell volume
Your analytical performance score is 45.3
Performance Score
145
115
85
55
25
-5
1210 1211 1212 1301 1302 1303 1304 1305 1306 1307
Distribution


Mean Cell Haemoglobin
Your analytical performance score is 9.6
Performance Score
145
115
85
55
25
-5
1210 1211 1212 1301 1302 1303 1304 1305 1306 1307
Distribution

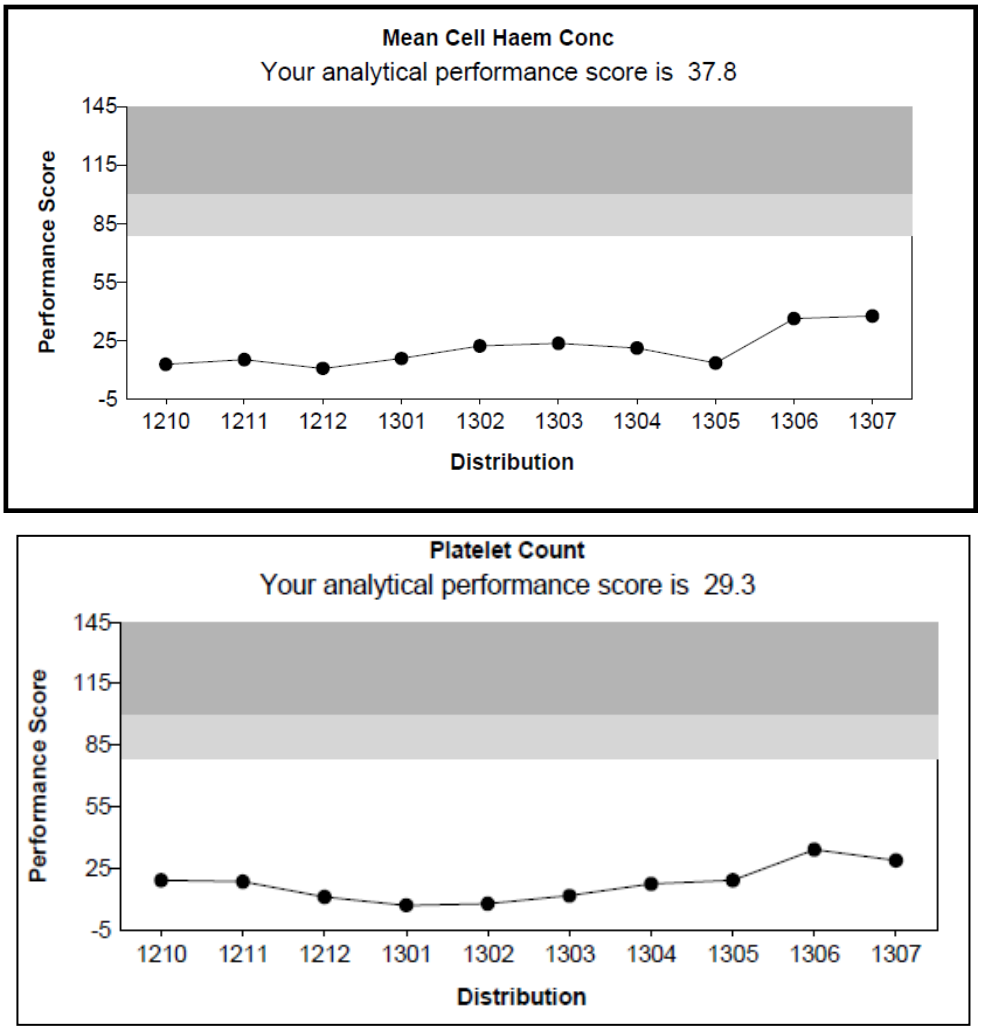
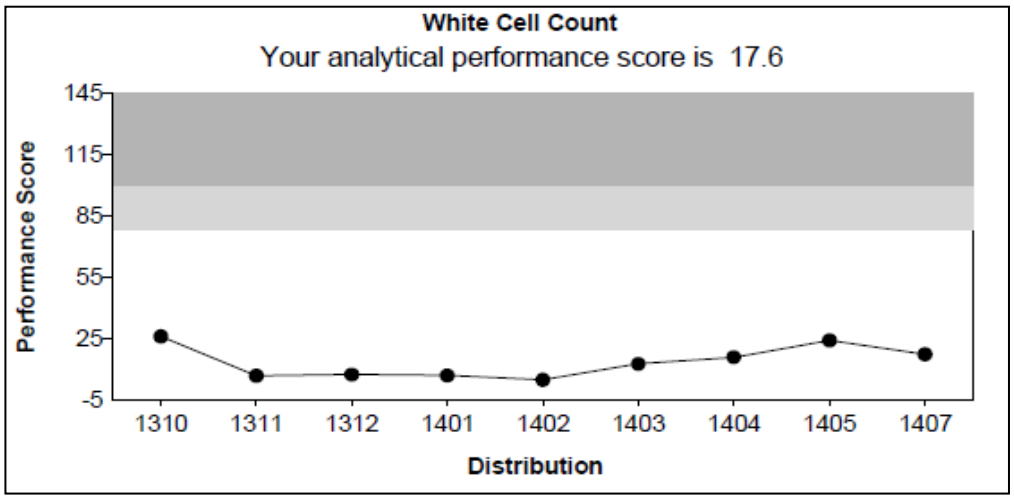
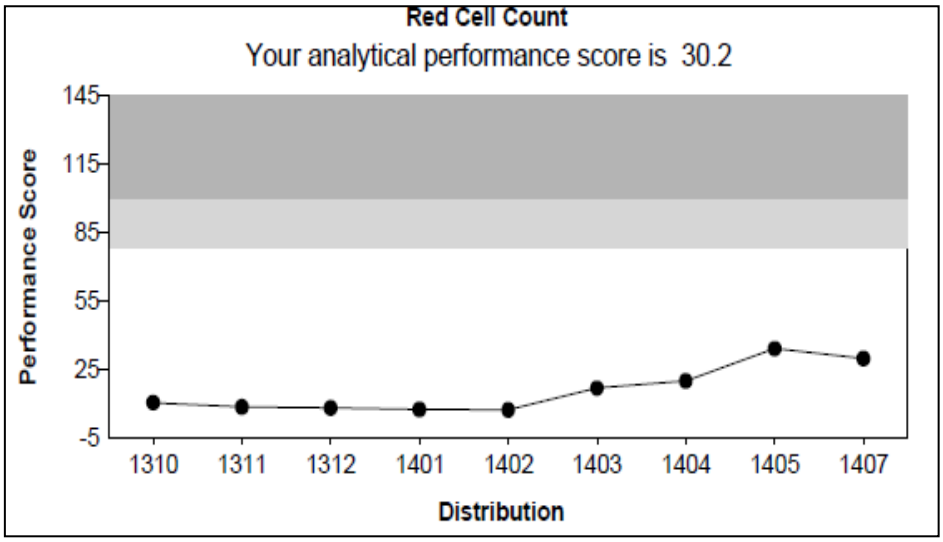
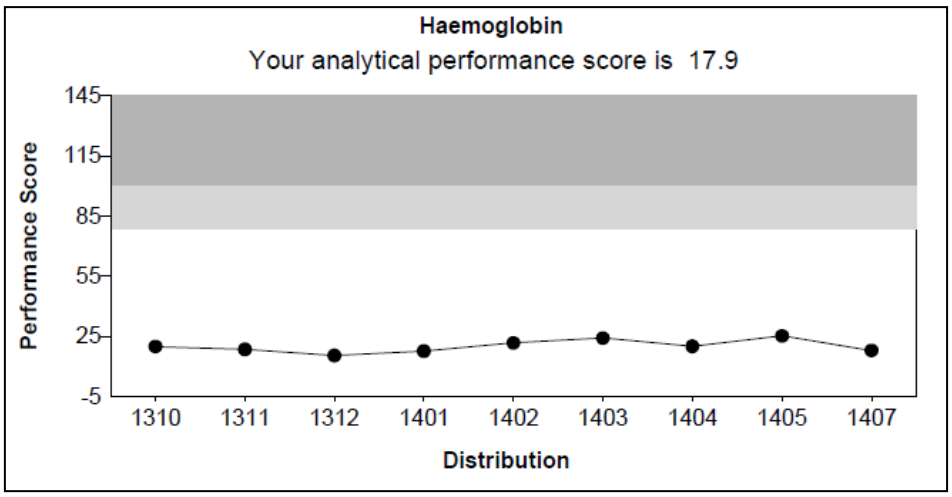
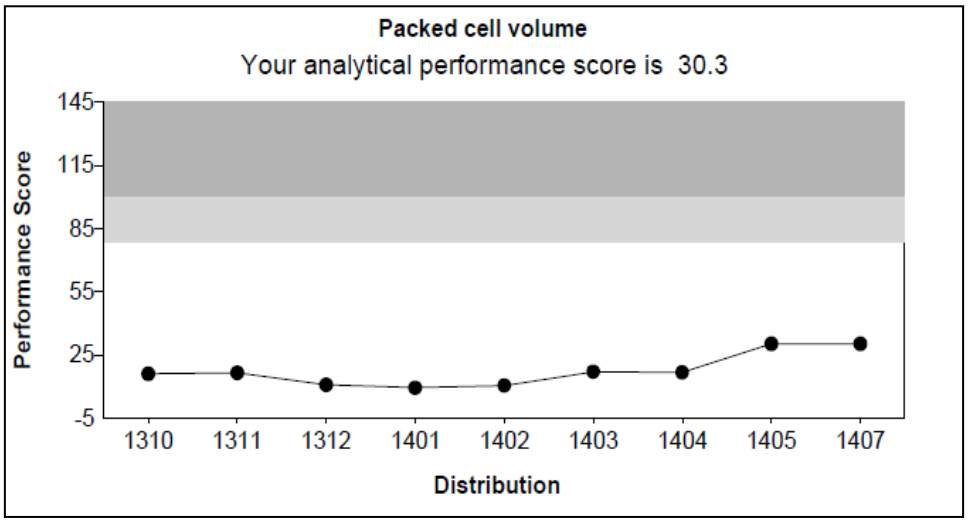


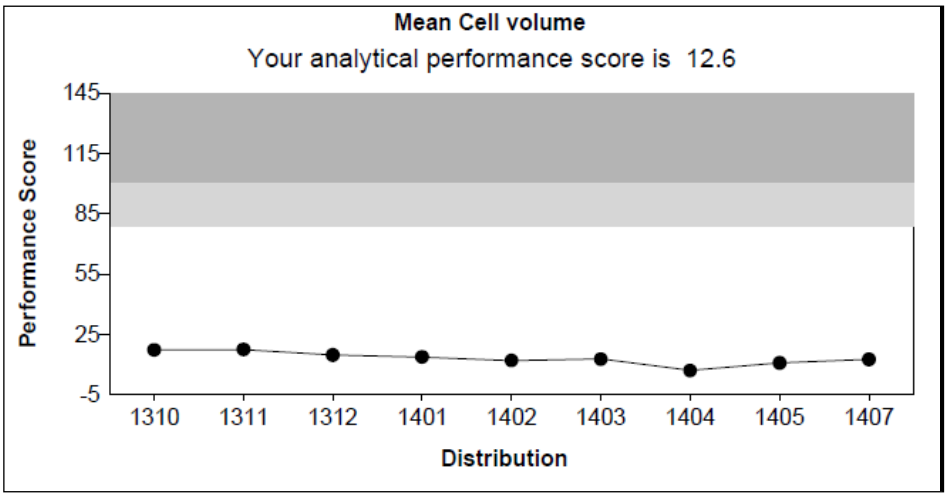
Figure Q.2 Illustrative overall performance charts for UKNEQAS for Year 6 of the NDNS RP

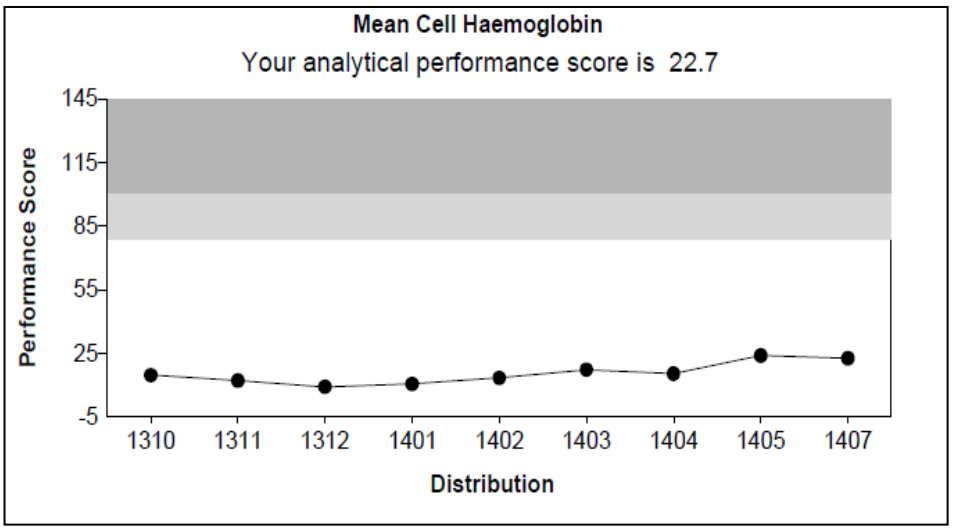


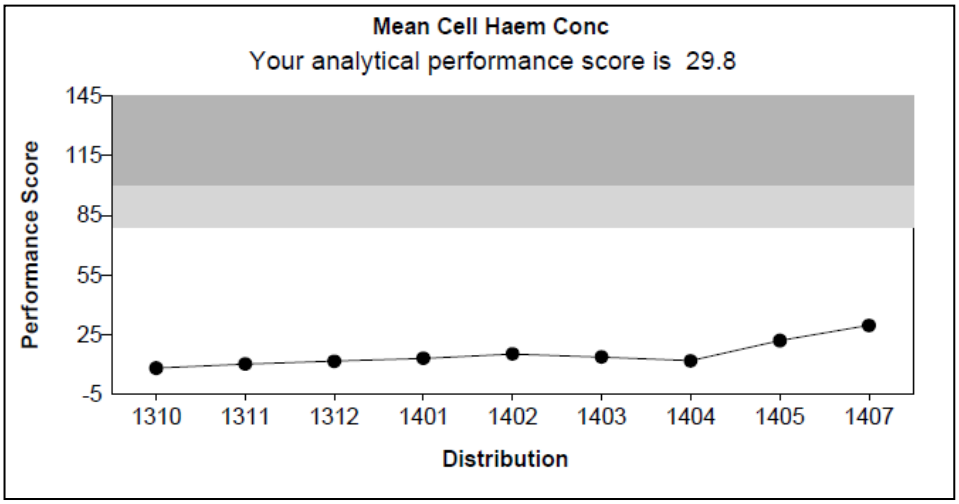

Red Cell Count
Your analytical performance score is 30.2
Performance Score
145
115
85
55
25
-5
1310
1311
1312
1401
1402
1403
1404
1405
1407
Distribution

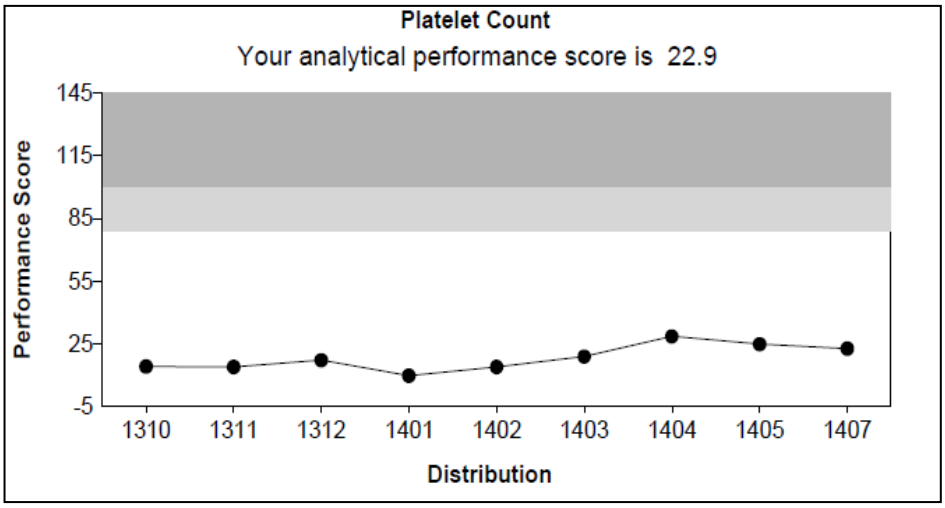

Haemoglobin
Your analytical performance score is 17.9
Performance Score
145
115
85
55
25
-5
1310
1311
1312
1401
1402
1403
1404
1405
1407
Distribution


Packed cell volume
Your analytical performance score is 30.3
Performance Score
145
115
85
55
25
-5
1310
1311
1312
1401
1402
1403
1404
1405
1407
Distribution


Mean Cell volume
Your analytical performance score is 12.6
Performance Score
145
115
85
55
25
-5
1310
1311
1312
1401
1402
1403
1404
1405
1407
Distribution


Mean Cell Haemoglobin
Your analytical performance score is 22.7
Performance Score
145
115
85
55
25
-5
1310
1311
1312
1401
1402
1403
1404
1405
1407
Distribution


Mean Cell Haem Conc
Your analytical performance score is 29.8
Performance Score
145
115
85
55
25
-5
1310
1311
1312
1401
1402
1403
1404
1405
1407
Distribution



Q.2.2 Serum C-reactive protein (CRP)

C-reactive protein (CRP) was assayed using a high-sensitivity (extended range) assay on a Dade Behring Dimension RXL Clinical Chemistry analyser in Year 5 and a Dimension Xpand clinical Chemistry analyser in Year 6. 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 5 (Addenbrooke's) and Year 6, (HNR) respectively.

Table Q.1 Internal quality controls for C-reactive protein (CRP) for Year 5 of the NDNS RP (Addenbrooke's)

	QC Lot No 35332	QC Lot No 35342	QC Lot No 29783	QC Lot No 29793
Mean (mg/L)	83.6	82.3	7.3	7.3
SD	3.09	2.81	0.32	0.3
% CV	3.7	3.4	4.4	4.1
Data points included	2235	1004	2194	1032

Table Q.2 Internal quality controls for C-reactive protein (CRP) for Year 6 of the NDNS RP (HNR)

	QC1 158uL	QC3 646UE	QC2 831UN
Mean (mg/l)	1.81	47.0	21.0
SD	0.41	0.84	0.42
%CV	22.5	1.8	2.0
n	73	60	61

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.

The graphs of bias index and overall MRBIS (Mean Rolling Bias Index Score) versus distribution reproduced in figures Q.3 and Q.4 show the laboratory's bias relative to the All Laboratory Trimmed Mean (ALTM) CRP concentration measured in the sample; these graphs are included by kind permission of Addenbrooke's and UKNEQAS for Years 5 and 6. A score of <50 is "ideal"; 50-100 is "good" and 100-200 "adequate" for clinical purposes.

Figure Q.3 NEQAS performance for CRP assay for Year 5 of the NDNS RP

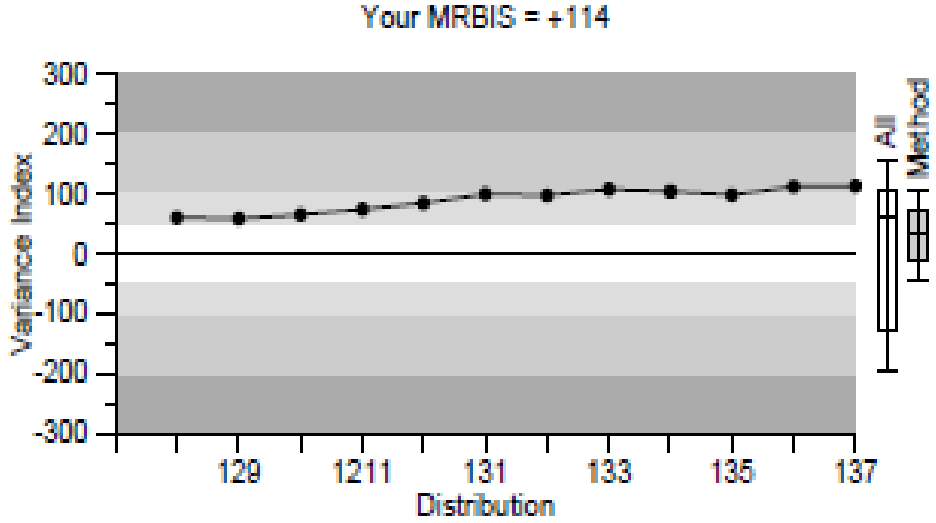
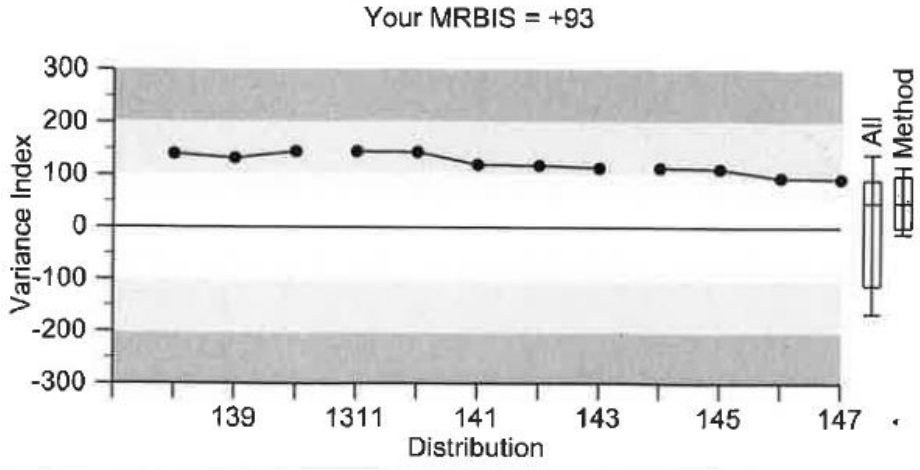


Figure Q.4 NEQAS performance for CRP assay for Year 6 of the NDNS RP



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 performance statistics in tables Q.3 and Q.4 were calculated using data from two or three different reagent lots in order to include batch to batch variation. The large number of QC lots used results from the heavy workload of this hospital laboratory. Tables Q.3 and Q.4 (in two sections) show imprecision data for a period covering the analysis of Years 5 and 6 samples.

Table Q.3 Internal quality controls for vitamin B_{12} for Year 5 of the NDNS RP

QC lot no:	40241	40261	40262	40263
Mean (µg/L)	405	396	560	1089
SD	31.4	31.8	38.4	82.2
% CV	7.8	8.0	6.9	7.5
Data points included	95	890	995	905

Table Q.4 Internal quality controls for vitamin B_{12} for Year 6 of the NDNS RP

QC lot no:	43210	43220	43230	40261	40281	40291
Mean (µg/L)	150	158	174	404	423	356
SD	25	27	29	30	37	32
% CV	16.5%	17.0%	16.5%	7.3%	8.8%	8.9%
Data points included	192	318	359	432	253	348

QC lot no:	40262	40282	40292	40263	40283	40293
Mean (µg/L)	549	567	555	1028	812	716
SD	34	43	42	47	57	47
% CV	6.2%	7.6%	7.6%	4.6%	7.0%	6.6%
Data points included	434	354	467	356	223	317

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 5 and 6 are reproduced below with permission of Addenbrooke's and the NEQAS Haematinics Scheme organisers.

Figures Q.5 and Q.6 show the bias relative to the target concentration during the years when NDNS RP samples were being analysed. Filled circles represent Addenbrooke's results; open circles represent results from other laboratories which use the same method. DI (Deviation Index) relates to the distribution of results from all laboratories and indicates by how many standard deviations a result differs from the ALTM. Better performance is indicated by lighter shading.

A small DI (+ or -) indicates close agreement. MRBIS is the mean of the 10 most recent bias estimates; results of -0.56 (Year 5) and -0.28 (Year 6) indicate that there was good overall agreement between Addenbrooke's results and the target concentrations.

Figure Q.5 Illustrative overall performance charts for UKNEQAS for Year 5 of the NDNS RP for vitamin B_{12}

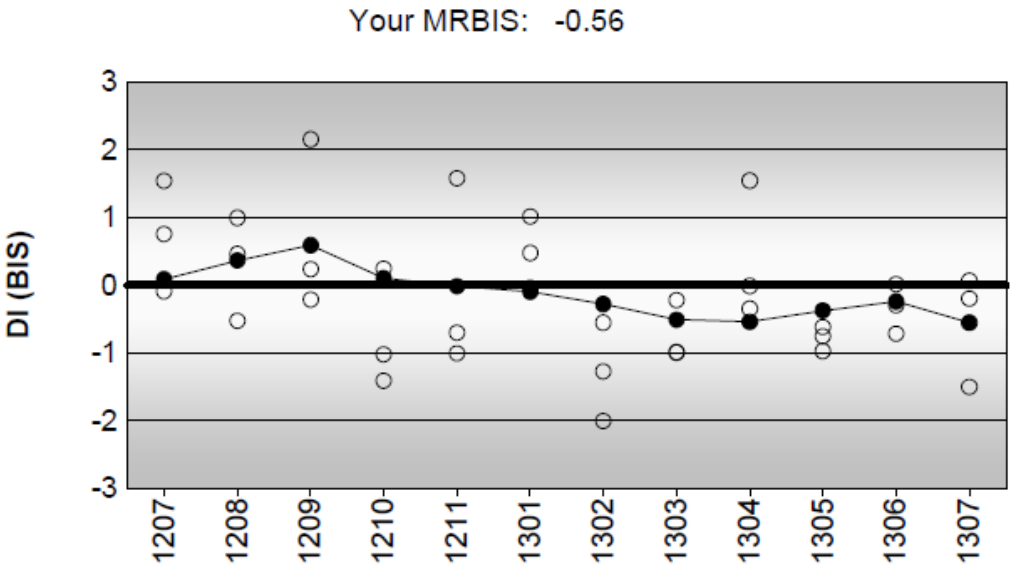
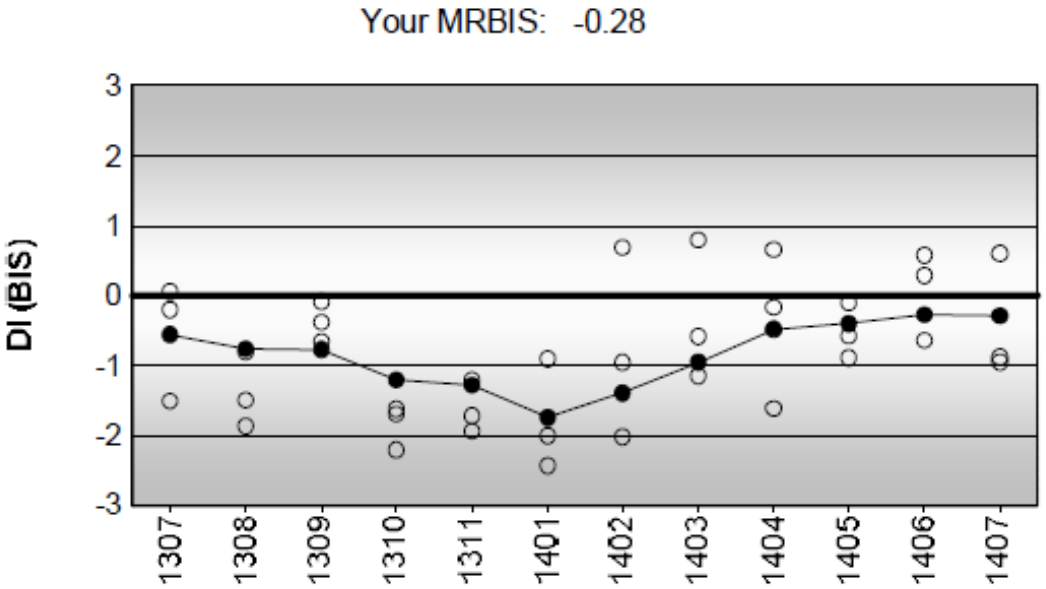


Figure Q.6 Illustrative overall performance charts for UKNEQAS for Year 6 of the NDNS RP for vitamin B_{12}



Q.2.4 Holotranscobalamin

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.[iii] Several studies have been published which conclude that HoloTC would be a better indicator of vitamin B_{12} status than total Serum B_{12}.[iv,v] As expected, HoloTC concentrations are low in patients with biochemical signs of vitamin B_{12} deficiency.[vi] Low values have been reported in vegetarians,[vii] vegans[viii] and in populations with a low intake of vitamin B_{12}.[ix] 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.[x] HoloTC concentrations are said to reflect vitamin B_{12} status, independent of recent absorption of the vitamin.[xi]

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 6 were conducted at HNR, automated on a DS2 ELISA processor (Launch Diagnostics Ltd).

P.2.4.1 Internal quality controls for HoloTC

QC samples were supplied by the manufacturer and included in every assay. Table Q.5 shows QC data for a period covering the analysis of Year 6 samples.

Table Q.5 Internal quality controls for holoTC for Year 6 of the NDNS RP

	Low QC (pmol/l)	High QC (pmol/l)
Manufacturers quoted range	19.5-28.3	46.4-67.2
Mean	24.1	57.7
SD	2.5	6.1
%CV	10.5	10.6
N	30	30

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 Year 6 assays were conducted (batched at the end of Year 6, assayed in October 2014) 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 Year 6 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 6 results.

Table Q.6 External Quality Assessment for holoTC for Year 6 of the NDNS RP

Mean bias from ALTM of NEQAS pilot scheme	0.4%
SD of bias from ALTM	8.3
Number of NEQAS samples assayed retrospectively	9

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[xii] and later adapted by other workers, including

Rautela and Liedtke.[xiii] 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 540 nm.

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.

Year 5 samples were assayed at Addenbrooke's on a Dimension RXL and Year 6 samples at HNR on a Dimension Xpand, using the same analytical method.

Q.2.5.1 Internal quality controls for total cholesterol

Tables Q.7 and Q.8 show imprecision data for a period covering the analysis of Years 5 and 6 samples.

Table Q.7 Internal quality controls for total cholesterol for Year 5 of the NDNS RP

	QC Lot No 46461	QC Lot No 46463	QC Lot No 46491	QC Lot No 46493
Mean (mmol/L)	2.68	6.65	2.28	6.3
SD	0.09	0.159	0.093	0.154
% CV	3.4	2.4	4.1	2.4
n	3240	2457	471	1155

Table Q.8 Internal quality controls for total cholesterol for Year 6 of the NDNS RP

	QC1 158UL	QC2 831UN	QC3 646UE
Mean (mmol/L)	2.54	4.52	6.39
SD	0.07	0.11	0.17
% CV	2.91	2.35	2.64
n	80	78	79

Q.2.5.2 Internal quality controls for HDL cholesterol

Tables Q.9 and Q.10 show imprecision data for a period covering the analysis of Years 5 and 6 samples.

Table Q.9 Internal quality controls for HDL cholesterol for Year 5 of the NDNS RP

	QC Lot No 46461	QC Lot No 46491	QC Lot No 46463	QC Lot No 46493
Mean (mmol/L)	0.76	0.65	1.6	1.79
SD	0.037	0.026	0.12	0.1
% CV	4.9	4.0	7.5	5.6
n	2009	276	1594	685

Table Q.10 Internal quality controls for HDL cholesterol for Year 6 of the NDNS RP

	QC1 158UL	QC2 831UN	QC3 646UE
Mean (mmol/L)	0.96	2.74	2.32
SD	0.03	0.19	0.10
% CV	3.0	7.1	4.4
n	73	71	71

Q.2.5.3 External quality controls for total and HDL cholesterol External quality control was achieved through the Randox International Quality Assessment Scheme (RIQAS); NEQAS pooled samples are unsuitable for the methods used by the Siemens Dimension instruments. Table Q.11 indicates the percentage deviation of results obtained by Addenbrooke's from the target concentration for Year 5 and by HNR for Year 6. These have been calculated at HNR and are included with the permission of the laboratory and the Scheme organisers.

Table Q.11 RIQAS results for lipid analyses - deviation from target concentration

Analyte		Year 5 (Addenbrooke's)	Year 6 (HNR)
Cholesterol	mean % deviation	-1.56	-1.66
	SD	2.06	1.14
HDL-Cholesterol	mean % deviation	-4.31	-5.40
	SD	8.63	4.09

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. Year 5 samples were assayed at Addenbrooke's on a Dimension RXL and Year 6 samples at HNR on a Dimension Xpand, using the same analytical method.

Q.2.6.1 Internal quality controls for serum triglycerides (triacylglycerols)

Tables Q.12 and Q.13 show imprecision data in Years 5 and 6. The performance statistics were calculated using data from two or three different reagent lots in order to include batch to batch variation.

Table Q.12 Internal quality controls for serum triglycerides (triacylglycerols) for Year 5 of the NDNS RP

	QC Lot No 46461	QC Lot No 46491	QC Lot No 46463	QC Lot No 46493
Mean (mmol/L)	0.87	0.81	2.36	2.37
SD	0.046	0.047	0.071	0.078
% CV	5.3	5.8	3.0	3.3
Data points included	2171	322	1602	698

Table Q.13 Internal quality controls for serum triglycerides (triacylglycerols) for Year 6 of the NDNS RP

	QC1 158UL	QC2 831UN	QC3 646UE
Mean (mmol/L)	1.44	2.54	3.82
SD	0.07	0.10	0.10
% CV	4.7	4.1	2.6
n	79	78	79

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

External quality assessment was achieved through RIQAS. Table Q.14 indicates the percentage deviation of results obtained (by Addenbrooke's in Year 5 and HNR in Year 6) from the target concentration. These have been calculated at HNR and are included with the permission of the laboratory and the Scheme organisers.

Table Q.14 RIQAS results for triglycerides (triacylglycerols): deviation from target concentration

	Year 5	Year 6
mean % deviation	-1.49	-5.51
SD	7.35	2.03

Q.2.7 Plasma ferritin

During Year 5, this assay was performed using the Siemens BN ProSpec® system which uses particle-enhanced immunonephelometry for the quantitative determination of ferritin in heparinised human plasma. Polystyrene particles coated with specific antibodies to human ferritin are agglutinated when mixed with samples containing human ferritin. The intensity of the scattered light in the nephelometer is proportional to the ferritin content of the sample; therefore, the ferritin concentration can be quantitated by comparison to dilutions of a calibrant of known concentration.

From the beginning of Year 6 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.

A detailed comparison study conducted in the HNR laboratories concluded that overall results given by the two methods are directly comparable and no conversion factor between the two is necessary. This is corroborated by the similar mean NEQAS bias score. The Dimension method has the advantages of decreased sample-specific interference (demonstrated by decreased NEQAS standard deviation of bias) and secure sample identification.

Q.2.7.1 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.15 and Q.16 indicate good between-batch consistency for ferritin results during Year 5 and Year 6.

Table Q.15 Internal quality controls for ferritin for Year 5 of the NDNS RP

Year 5	Low	Medium	High
Mean (µg/L)	70.0	149	354
SD (µg/L)	5.25	17	25
CV (%)	7.5	11.6	7.0
n	49	49	49

Table Q.16 Internal quality controls for ferritin for Year 6 of the NDNS RP

	Low control QC1 158UL	Medium control QC2 831UN
Mean (µg/L)	27.9	62.9
SD (µg/L)	0.72	1.55
CV (%)	2.60	2.47
n	56	58

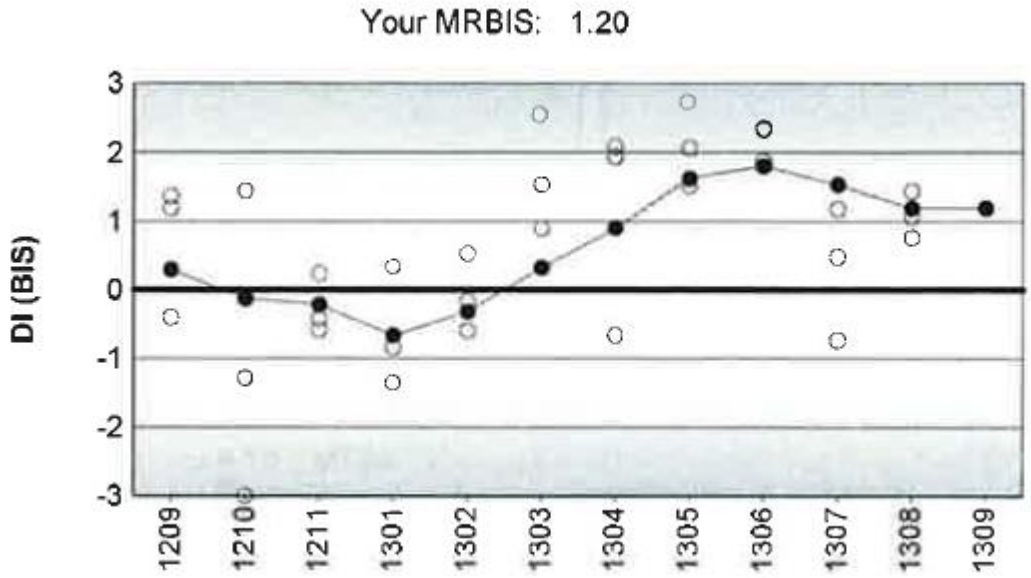
Q.2.7.2 External quality assessment for plasma ferritin

External quality assessment was through the UK NEQAS Haematinics scheme. Figures Q.7-Q.8 show the bias relative to the target concentration during the years when NDNS RP samples were being analysed. Filled circles represent HNR results; open circles represent results from other laboratories which use the same method as HNR. DI (Deviation Index) relates to the distribution of results from all laboratories and indicates by how many standard deviations a HNR result differs from the ALTM. Better performance is indicated by lighter shading.

A small DI (+ or -) indicates close agreement. MRBIS is the mean of the 10 most recent bias estimates; results of 1.20 (BN ProSpec; Year 5) and 0.70 (Siemens Dimension; Year 6) indicate that there was good overall agreement between HNR results and the

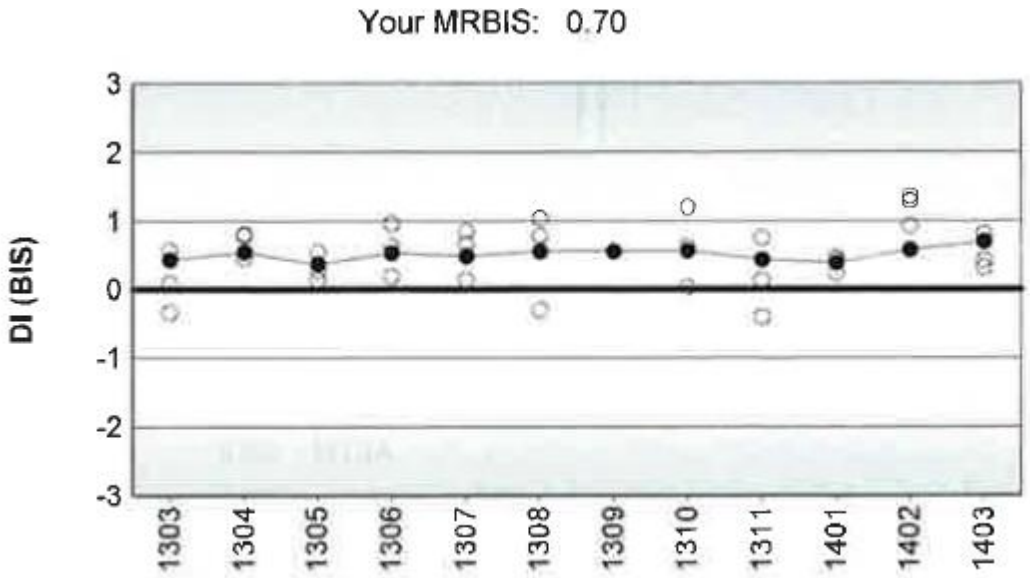
target concentrations. The Scheme provider changed in April 2014 and cumulative results across the scheme change are not available.

Figure Q.7 External quality assessment for ferritin for Year 5 of the NDNS RP (ProSpec)[1]



[1] filled circles represent NDNS RP results; open circles represent results obtained by other users of the same analytical method

Figure Q.8 External quality assessment (Dimension Xpand) for ferritin for Year 6 of the NDNS RP[1]



[1] until scheme change in April 2014

Table Q.17 Summary of NEQAS bias assessment in Years 5 and 6 of the NDNS RP

	Year 5 *BN ProSpec*	*Year 5 Siemens Dimension (for information; results not reported)*	Year 6 *Siemens Dimension*
Mean % bias from ALTM	5.6	4.5	8.0
SD % bias	14.4	4.5	6.0
Number of NEQAS samples	30	30	27

Q.2.8 Plasma soluble transferrin receptors (sTfR) (Year 5 only)

The soluble transferrin receptor (sTfR) assay is an enzyme immunoassay (EIA) based upon the double antibody sandwich method (Ramco Laboratories Inc, Texas, USA). Plasma samples are diluted in buffer and pipetted into microwells pre-coated with a polyclonal antibody to sTfR. Horseradish peroxidase (HRP) conjugated murine monoclonal antibody specific for sTfR is added to the wells and incubated for two hours at room temperature. During this incubation, the sTfR binds to the polyclonal antibodies adsorbed to the wells and the HRP-conjugated second antibodies bind to the captured sTfR. Any unbound sTfR and excess HRP conjugate are removed from the wells by washing. Enzyme substrate (tetramethylbenzidine, TMB) is added to the wells and through the action of HRP forms a blue product. Upon the addition of an acid stop solution the blue product is converted to a yellow colour, the intensity of which is measured in a plate reader set at 450nm. A standard curve is generated by plotting the absorbance versus concentration of the sTfR standards provided in the kit. The concentration of the sTfR in the sample is then determined by comparing the sample's absorbance with the standard curve.

The manufacturers' estimate of limit of detection is 0.07µg/mL. In order to optimise sample ID tracking and to minimise analyst-to analyst variation HNR has automated this assay using the BEST 2000 liquid handling platform (Launch Diagnostics). Results are not compromised by haemolysis.

Q.2.8.1 Quality controls for plasma soluble transferrin receptors

QC samples (low, high) supplied by the kit manufacturer were run in each batch. Because batch changes for these controls will preclude comparisons over the RP, unassayed human plasma was also included. The controls were assayed at the beginning and end of each batch and therefore these statistics represent a combination of intra- and inter-assay precision.

Results for each run were checked to ensure they fell within the manufacturer's target range. The results in table Q.18 indicate good between-batch consistency for sTfR results during Years 5. The measurement of sTfR was discontinued after Year 5. There is no external quality assessment scheme for sTfR measurement.

Table Q.18 Internal quality controls for sTfR for Year 5 of the NDNS RP

	Kit low control	Kit high control	In-house QA
Mean (µg/ml)	4.46	15.31	4.10
SD (µg/ml)	0.46	1.00	0.25
% CV	10.37	6.56	6.19
N	34	34	30

Q.2.9 Plasma vitamin C

This assay is based on the procedure described by Vuilleumier and Keck.[xiv] 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.2.9.1 Internal quality controls for plasma vitamin C

QC samples were made in-house by spiking ascorbic acid-depleted plasma. The results in tables Q.20 and Q.21 indicate good between-batch consistency for vitamin C (ascorbic acid) measurements during Years 5 and 6.

Table Q.20 Internal quality controls for vitamin C for Year 5 of the NDNS RP

Vitamin C	µmol/L	
	QC2	QC3
mean (µmol/L)	26.7	52.9
sd (µmol/L)	1.6	2.6
cv (%)	6.0	4.9
N	30	30

Table Q.21 Internal quality controls for vitamin C for Year 6 of the NDNS RP

Vitamin C	µmol/L	
	QC2	QC3
mean (µmol/L)	26.7	53.1
sd (µmol/L)	1.6	2.3
cv (%)	6.1	4.3
N	50	50

Q.2.9.2 External quality controls for vitamin C

HNR subscribes to the NIST (National institute for Standardisation and Control, US) EQAS for vitamin C. 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. HNR results are in close agreement with this validated "interlaboratory consensus" indicating accuracy with respect to the Standard Reference Material.

Figure Q.9 External quality controls for vitamin C for Year 5 of the NDNS RP. Comparison of Measurements to "Inter-laboratory Consensus"


National Institute of Standards and Technology

Micronutrients Measurement Quality Assurance Program

Nutritional Biomarker Analysis

Medical Research Council - Human Nutrition Research

Participated in Two of Two **2013** Interlaboratory Comparability Studies

Total Ascorbic Acid

Comparison of Measurements to Consensus Values

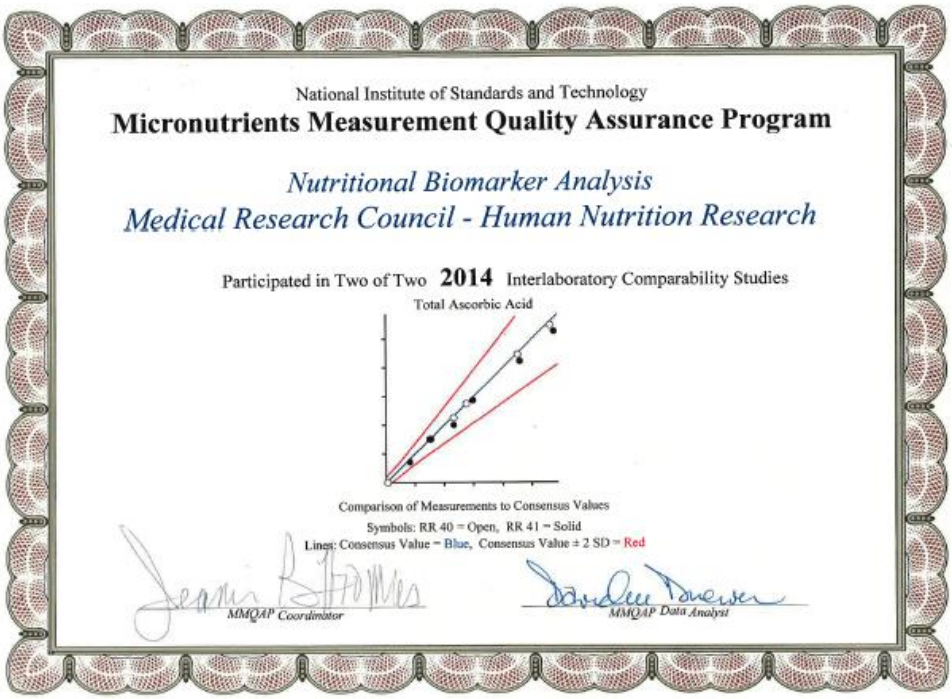
Symbols: RR 38 = Open, RR 39 = Solid

Lines: Consensus Value = Blue, Consensus Value ± 2 SD = Red

MMQAP Coordinator

MMQAP Data Analyst

Figure Q.10 External quality controls for vitamin C for Year 6 of the NDNS RP. Comparison of Measurements to "Inter-laboratory Consensus"



Q.2.10 Erythrocyte transketolase activation coefficient (ETKAC) for thiamin status

This assay is based on that of Vuilleumier *et al*[xv] 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).

Q.2.10.1 Quality control results for ETKAC

Descriptive statistics in tables Q.22 and Q.23 for internal QCs indicate good batch-to-batch consistency of ETKAC results during Years 5 and 6.

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

Table Q.22 Internal quality controls for ETKAC for Year 5 of the NDNS RP

Control ID	Control K	Scipac P	Scipac Q
mean	1.10	1.07	1.18
sd	0.04	0.04	0.05
cv	3.99	3.41	4.12
n	17	22	18

Table Q.23 Internal quality controls for ETKAC for Year 6 of the NDNS RP

Control ID	Control K	Scipac P	Scipac Q	Scipac S
mean	1.10	1.06	1.19	1.14
sd	0.06	0.03	0.05	0.05
cv	5.68	2.68	4.40	4.26
n	11	26	26	14

Q.2.11 Erythrocyte glutathione reductase activation coefficient (EGRAC) for riboflavin status

This assay was developed from the original manual technique developed by Glatzle *et al*[xvi] 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.2.11.1 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 -80^{o}C. 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.2.11.1.1 Internal quality control results during Years 5 and 6

Descriptive statistics in tables Q.24 and Q.25 for internal QCs indicate good batch-to-batch consistency of EGRAC results during Years 5 and 6.

Table Q.24 Internal quality controls for EGRAC for Year 5 of the NDNS RP

Control ID	A	C	X
Mean	2.25	1.61	0.97
SD	0.15	0.04	0.02
% CV	6.59	2.56	2.54
N	28	27	29

Table Q.25 Internal quality controls for EGRAC for Year 6 of the NDNS RP

Control ID	**A**	**C**	**X**
Mean	2.17	1.56	0.97
SD	0.06	0.03	0.02
% CV	2.73	2.13	2.13
N	25	26	26

Q.2.12 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.[xvii]

Q.2.12.1 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.26 and Q.27) and the expected values indicate a quantitative recovery of vitamin B_6 in this assay.

Table Q.26 Internal quality controls for PLP and PA (unspiked plasma) for Years 5 and 6 of the NDNS RP

PLP	Year 5		Year 6	
Plasma	A07	A02	A07	A02
Mean (nmol/L)	23.8	49.3	24.8	49.2
SD	2.0	2.0	1.5	2.5
% CV	8.3	4.1	6.1	5.0
N	27	28	30	30

PA	Year 5		Year 6	
Plasma	K4082310	A02	K4082310	A02
Mean (nmol/L)	36.7	123.7	36.5	124.7
SD	1.3	4.2	1.5	3.4
% CV	3.5	3.4	4.0	2.7
N	28	27	30	30

Table Q.27 Internal quality controls for PLP and PA (spiked plasma) for Years 5 and 6 of the NDNS RP

PLP	Year 5	Year 6
Mean (nmol/L)	47.9	50.3
SD	1.7	1.8
% CV	3.5	3.5
n	28	30

PA	Year 5	Year 6
Mean (nmol/L)	58.0	57.9
SD	2.2	1.5
% CV	3.9	2.5
n	28	30

For Year 5 and 6 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). The table above indicates consistent accuracy.

Q.2.13 Plasma total homocysteine (Year 5 only)

This assay was performed using the Siemens BN ProSpec® system which uses particle-enhanced immunonephelometry for the quantitative determination of homocysteine in heparinised human plasma. In the competitive assay, bound homocysteine in the sample is reduced to free homocysteine by the action of dithiothreitol, and then converted enzymatically to S-adenosyl-homocysteine (SAH) in the next step. Conjugated S-adenosylcysteine (SAC), added at the onset of the reaction, competes with the SAH in the sample for bonding by anti-SAH antibodies bound to polystyrene particles. In the presence of SAH, there is either no aggregation or a weaker aggregation of particles. In the absence of SAH in the sample, an aggregation of the polystyrene particles by the conjugated SAC occurs. The higher the SAH content of the reaction mixture, the smaller the scattered light signal. The concentration is determined by comparison with a calibrant of known concentration.

Q.2.13.1 Quality controls for plasma total homocysteine

QC was achieved through internal and external procedures. Control serum was obtained commercially containing low medium and high concentrations of homocysteine and was included in each run. Results were checked to ensure they fell within the manufacturer's target range. Because the manufacturer's "acceptable" results range was very wide, HNR determined more stringent site-specific precision requirements within the manufacturer's published range and QC results for each analytical run were judged against these more demanding criteria, using JMPIN QC software.

In order to confirm analytical accuracy, HNR participated in an international external quality assessment scheme for homocysteine. Four samples were distributed by the scheme per year. HNR results were rated "within consensus" relative to the overall spread of results, indicating acceptable conformity of HNR's reported homocysteine results to the inter-laboratory consensus concentration.

Q.2.13.1.1 Internal quality controls for plasma total homocysteine

The results in table Q.28 indicate good between-batch consistency for homocysteine results during Year 5.

Table Q.28 Internal quality controls for homocysteine for Year 5 of the NDNS RP

Year 5	Low		Medium		High	
Mean (µmol/l)	6.8	6.8	11.5	11.5	24.2	24.6
SD (µmol/l)	0.6	0.8	1.3	1.4	2.2	1.7
% CV	9.4	11.4	11.1	11.8	9.2	7.0
N	6	6	7	6	8	8

Q.2.14 Plasma retinol, α– and γ–tocopherol, and individual carotenoids:

Years 5 and 6

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.*[xviii] 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 method used for Years 5 and 6 differs from that used during the previous years of the NDNS RP. In order to compare these data to that presented in the NDNS RP Years 1 to 4 reports conversion factors need to be applied; these are outlined in section Q.2.14.3.

Q.2.14.1 Internal quality controls for plasma retinol, α– and γ–tocopherol and individual carotenoids

The FSV results for Years 5 and 6 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 low, medium and high 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.2.14.2.

Table Q.29 Precision of internal QC for plasma retinol for Year 5 of the NDNS RP

	QC2	QC6	QC7	QC11	K4082310
Mean (µmol/L)	2.04	2.46	2.58	1.61	1.43
SD	0.18	0.16	0.19	0.08	0.09
% CV	8.9	6.5	7.3	4.9	6.3
n	13	15	15	14	14

Table Q.30 Precision of internal QC for plasma retinol for Year 6 of the NDNS RP

	QC2	QC6	QC7	QC11	K4082310
Mean (µmol/L)	2.03	2.48	2.56	1.61	1.42
SD	0.14	0.18	0.26	0.15	0.07
% CV	6.9	7.4	10.2	9.1	5.0
n	14	14	14	14	14

Table Q.31 Precision of internal QC for plasma α-tocopherol for Year 5 of the NDNS RP

	QC2	QC6	QC7	QC11
Mean (µmol/L)	35.95	31.73	36.41	16.60
SD	1.45	1.46	1.95	0.82
% CV	4.0	4.6	5.4	4.9
n	13	15	15	14

Table Q.32 Precision of internal QC for plasma α-tocopherol for Year 6 of the NDNS RP

	QC2	QC6	QC7	QC11
Mean (µmol/L)	35.17	31.60	35.66	16.65
SD	1.36	1.60	2.03	0.92
% CV	3.9	5.1	5.7	5.5
n	14	14	14	14

Table Q.33 Precision of internal QC for plasma γ-tocopherol for Year 5 of the NDNS RP

	QC2	QC6	QC7	QC11	K4082310
Mean (µmol/L)	1.03	2.64	1.69	1.03	3.12
SD	0.11	0.13	0.15	0.12	0.28
% CV	11.1	5.0	9.1	12.0	8.9
n	13	15	15	14	14

Table Q.34 Precision of internal QC for plasma γ-tocopherol for Year 6 of the NDNS RP

	QC2	QC6	QC7	QC11	K4082310
Mean (µmol/L)	1.19	2.74	1.78	1.13	3.22
SD	0.05	0.11	0.09	0.08	0.15
% CV	4.0	3.9	5.1	7.0	4.5
n	14	14	14	14	14

Table Q.35 Precision of internal QC for plasma α-carotene for Year 5 of the NDNS RP

	QC2	QC7
Mean (µmol/L)	0.12	0.11
SD	0.01	0.01
% CV	5.6	7.2
n	13	15

Table Q.36 Precision of internal QC for plasma α-carotene for Year 6 of the NDNS RP

	QC2	QC7
Mean (µmol/L)	0.12	0.12
SD	0.02	0.01
% CV	12.8	9.3
n	14	13

Table Q.37 Precision of internal QC for plasma β-carotene for Year 5 of the NDNS RP

	QC2	QC6	QC7	QC11
Mean (µmol/L)	1.77	0.17	0.30	0.15
SD	0.08	0.01	0.02	0.01
% CV	4.6	5.4	5.4	5.2
n	13	15	15	14

Table Q.38 Precision of internal QC for plasma β-carotene for Year 6 of the NDNS RP

	QC2	QC6	QC7	QC11
Mean (µmol/L)	1.74	0.16	0.29	0.14
SD	0.08	0.01	0.01	0.01
% CV	4.4	8.5	3.5	8.7
n	14	14	14	14

Table Q.39 Precision of internal QC for plasma α-cryptoxanthin for Year 5 of the NDNS RP

	QC2	QC7
Mean (µmol/L)	0.09	0.06
SD	0.01	0.00
% CV	7.2	8.6
n	13	15

Table Q.40 Precision of internal QC for plasma α-cryptoxanthin for Year 6 of the NDNS RP

	QC2	QC7
Mean (µmol/L)	0.07	0.06
SD	0.01	0.01
% CV	17.4	12.5
n	14	10

Table Q.41 Precision of internal QC for plasma β-cryptoxanthin for Year 5 of the NDNS RP

	QC2	QC6	QC7	QC11
Mean (µmol/L)	0.68	0.17	0.15	0.18
SD	0.02	0.01	0.01	0.01
% CV	3.3	3.1	6.0	3.8
n	13	15	15	14

Table Q.42 Precision of internal QC for plasma β-cryptoxanthin for Year 6 of the NDNS RP

	QC2	QC6	QC7	QC11
Mean (µmol/L)	0.63	0.15	0.12	0.16
SD	0.04	0.01	0.02	0.01
% CV	5.8	8.8	16.8	6.2
n	14	13	13	14

Table Q.43 Precision of internal QC for plasma lycopene for Year 5 of the NDNS RP

	QC2	QC6	QC7	QC11	K4082310
Mean (µmol/L)	1.41	0.89	1.89	0.65	0.22
SD	0.08	0.05	0.10	0.02	0.02
% CV	5.7	5.2	5.3	3.5	7.6
n	13	15	15	14	14

Table Q.44 Precision of internal QC for plasma lycopene for Year 6 of the NDNS RP

	QC2	QC6	QC7	QC11	K4082310
Mean (µmol/L)	1.48	0.92	1.91	0.67	0.23
SD	0.08	0.03	0.08	0.04	0.03
% CV	5.1	3.8	4.0	5.2	14.7
n	14	14	14	14	14

Table Q.45 Precision of internal QC for plasma lutein + zeaxanthin for Year 5 of the NDNS RP

	QC2	QC6	QC7	QC11
Mean (µmol/L)	0.65	0.22	0.46	0.21
SD	0.05	0.02	0.03	0.01
% CV	7.1	9.6	7.2	6.5
n	13	15	15	14

Table Q.46 Precision of internal QC for plasma lutein + zeaxanthin for Year 6 of the NDNS RP

	QC2	QC6	QC7	QC11
Mean (µmol/L)	0.64	0.22	0.43	0.20
SD	0.04	0.01	0.03	0.02
% CV	5.5	6.5	7.1	8.2
n	14	14	14	14

Q.2.14.2 External quality controls for plasma retinol, α– and γ–tocopherol and individual carotenoids

In order to validate the new method prior to routine use in Year 5 a specific validation study was set up using ninety six blinded samples sent from NIST. NIST analysed the results generated at HNR then supplied HNR with graphical data indicating close correlation between our results and target results as defined by NIST (results generated by many labs over many occasions). The NIST comparison indicates an agreement within 5% for retinol and α-tocopherol, an agreement within 10% for γ-tocopherol and five of the carotenoids and an agreement within 20% for lycopene.

Participation in studies conducted by NIST, CDC VITAL-External Quality Assurance (CDC VITAL EQA) and UKNEQAS allowed inter-laboratory comparison of results. HNR participated in a twice yearly “round robin” with both NIST and VITAL EQA. HNR 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 five 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 LXXV and LXXVI were submitted during the Years 5 and 6 sample analysis periods respectively; extracts of the reports are shown below, indicating very close agreement with the target concentration.

Figure Q.11 NDNS RP Year 5 NIST return LXXV

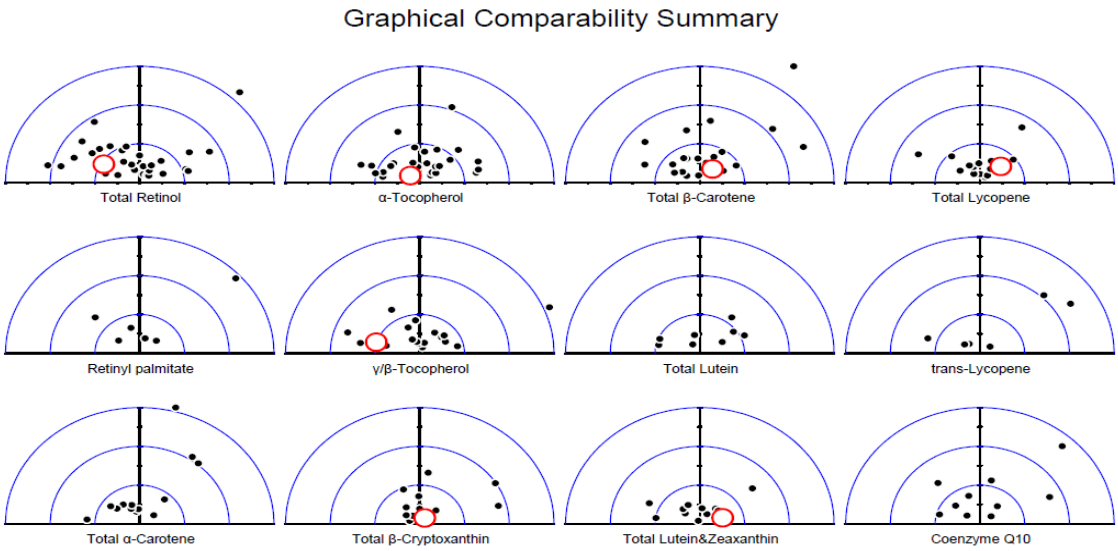
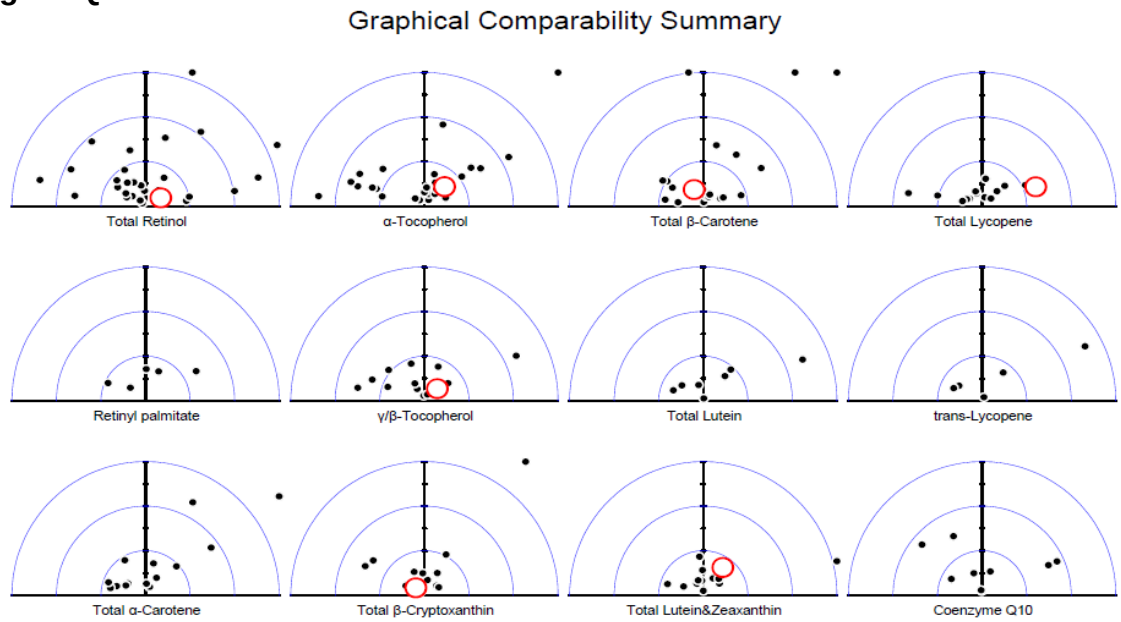


Figure Q.12 NDNS RP Year 6 NIST return LXXVI



In the graphical representations above 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.2.14.3 Conversion factors

In order to understand the overall impact of the method improvement undertaken between Years 4 and 5 of the NDNS RP, and to identify any step-changes in population distributions, comparative analysis was carried out on a subset of the Year 4 samples (n=80). Regression analysis of the results generated by the two methods indicated good linear correlation however the results by the two methods are not equivalent. Conversion factors have therefore been calculated for each analyte to allow conversion of data generated under the old method (as produced for NDNS RP UK Years 1 to 4) with data generated in Year 5 and 6 and *vice versa*.

Table Q.47 Conversion factors

Analyte	Conversion factor*
Retinol	0.84
α-tocopherol	0.82
γ-tocopherol	0.64
Lutein+zeaxanthin	0.70
α-cryptoxanthin	0.69
β-cryptoxanthin	0.83
Lycopene	1.25
α-carotene	0.83
β-carotene	0.88

**To compare results for Year 5&6 with Years 1 to 4:*
Multiply Year 1-4 results by conversion factor or divide Year 5&6 results by conversion factor

Q.2.15 Plasma 25-hydroxyvitamin D (25-OH D)

The DiaSorin Liaison method for quantitative determination of 25-OH D is a direct, competitive chemiluminescence immunoassay (CLIA). A specific antibody to vitamin D is used for coating magnetic particles (solid phase), and vitamin D is linked to an isoluminol derivative. During the incubation, 25-OH D is dissociated from its binding protein, and competes with labelled vitamin D for binding sites on the antibody. After the incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as relative light units (RLU) and is inversely proportional to the concentration of 25-OH D present in calibrators, controls, or samples. Year 5 and Year 6 samples were analysed in serum using the Diasorin kits reformulated by the manufacturer during 2011.

Q.2.15.1 International standardisation of 25-OHD concentrations

The HNR laboratory has taken part in two international initiatives to standardise measurements of 25-OHD concentration to international reference methods, the Vitamin D Standardisation Program (VDSP) and EU project ODiN.[xix] These have shown that the Liaison assays used for NDNS gave results which are on average marginally lower than the international standard. An LC-MS/MS method has been developed at HNR and has been accepted as giving results very close to the international "gold standard" assays conducted by NIST and the University of Ghent. Accordingly, 25-OHD concentrations in this report have been standardised to the HNR LC-MS/MS assay, giving direct comparability of results in this report with results for Year 7 onwards, which will be generated using the HNR LC-MS/MS assay.

The equation which has been applied to analytical results for Years 5 and 6 (reformulated Liaison assay) is:

Standardised 25-OHD = 0.908 *(reformulated Liaison 25-OHD)+ 6.2 (nmol/L)

Q.2.15.2 Quality control for 25-OHD

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

Q.2.15.2.1 Internal quality controls for 25-OHD

Manufacturer's controls were run with each kit. These allow an instant assessment of whether the results obtained for respondents' samples are within limits. However as each batch of these is only issued for a relatively short period they do not assess longer-term stability of the assay and therefore Lyphochek control was also included to assess longer-term assay stability and consistency.

Table Q.48 Internal quality controls for 25-OHD for Year 5 of the NDNS RP

	Manufacturer's controls: (nmol/L)				Lyphochek (nmol/L)
Mean	38.6	127.2	38.5	138.9	54.5
SD	1.5	5.5	2.9	10.1	4.5
% CV	4.0	4.4	7.7	7.3	8.2
N	10	10	34	34	34

Table Q.49 Internal quality controls for 25-OHD for Year 6 of the NDNS RP

	Manufacturer's controls: (nmol/L)				Lyphochek (nmol/L)
Mean	39.1	129.4	38.8	129.4	52.8
SD	3.8	10.6	4.7	6.9	6.4
% CV	9.8	8.2	12.2	5.4	12.1
N	14	14	6	8	18

Q.2.15.2.2 External quality assessment for 25-OHD

HNR 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.50 shows the relationships between 25-OHD as reported on individual DEQAS samples by HNR and the mean value obtained internationally using the Diasorin Liaison (n = approx. 400 laboratories), the ALTM (approximately.1,100 laboratories) and the target concentration as provided by NIST.

Table Q.50 Relationships between 25-OHD as reported on individual DEQAS samples by HNR

	Year 5			Year 6		
	% bias vs method mean (Diasorin Liaison)	% bias vs ALTM	% bias vs target concn	% bias vs method mean (Diasorin Liaison)	% bias vs ALTM	% bias vs target concn
Mean	1.6	-10.4	-15.1	-0.7	-12.2	-9.0
SD	11.7	10.9	11.5	6.6	8.1	10.5
n	20	20	10	20	20	20

Q.2.16 Plasma creatinine

The creatinine method used in the NDNS RP Years 5 and 6 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.

Q.2.16.1 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.51 and Q.52 show internal QC results for creatinine, covering the period when Years 5 and 6 samples were analysed, respectively.

Table Q.51 Internal quality controls for plasma creatinine for Year 5 of the NDNS RP

	Low	Medium	High
Mean creatinine µmol/L	68.2	171	583
SD µmol/L	3.2	4.0	8.1
CV %	4.8	2.4	1.4
N	24	22	24

Table Q.52 Internal quality controls for plasma creatinine for Year 6 of the NDNS RP

	QC1 158uL	QC2 831UN	QC3 646UE
Mean creatinine µmol/L	83.3	152	421
SD µmol/L	7.7	8.3	9.8
CV %	9.2	5.4	2.3
N	84	83	83

Q.2.16.2 External quality assessment for plasma creatinine

HNR subscribes to the UKNEQAS clinical chemistry. The table below shows that during Years 5 and 6 the Dimension assay at HNR gave results acceptably close to the consensus of all laboratories using the same method.

Table Q.53 External quality assurance for creatinine performance during Year 5 and Year 6 relative to method mean (kinetic Jaffe method)

	Year 5	Year 6
Mean bias from method mean %	-0.6	3.6
SD bias %	3.8	6.9
n	69	60

Q.2.17 Selenium and 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 via a V-groove nebuliser and cyclonic spray chamber arrangement for Year 5 samples while a more efficient system, namely a flow injection system combined with the Sea spray nebulizer and cyclonic spray chamber arrangement, was used for Year 6.

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 calf serum (Sigma Aldrich) for each analytical batch of Year 5 and in commercially prepared human serum (Seralabs) for each analytical batch of Year 6.

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.2.17.1 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.2.17.2 Inter-batch variability

Tables Q.54 and Q.55 summarise the measured concentration of selenium and zinc following analysis of these QC samples for Years 5 and 6. 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.54 QC analysis for Year 5 of the NDNS RP

	ClinChek L1		ClinChek L2	
	Selenium	**Zinc**	**Selenium**	**Zinc**
Lot number	129		129	
Target Concentration /Range (µg/L)	80 (64-96)	925 (740-1110)	118 (94.4-142)	1363 (1090-1636)
Mean Measured Concentration (µg/L)	83.1	959	118	1359
n (QC samples)	35	35	35	35
SD	3.9	57	4.1	72
%CV	5	6	4	5

	ClinChek L1		ClinChek L2	
	Selenium	**Zinc**	**Selenium**	**Zinc**
Lot number	906		906	
Target Concentration /Range (µg/L)	81.0 (64.8-97.2)	1417 (1134-1700)	118 (94.4-142)	1826 (1552-2100)

Mean Measured Concentration (µg/L)	82.6	1384	119	1809
n (QC samples)	6	6	13	13
SD	5.4	112	4.4	128
%CV	7	8	4	7

Table Q.55 QC analysis for Year 6 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)	78.5	1098	117.3	1508
n (QC samples)	24	24	24	24
SD	6.2	81	7.4	126
%CV	8	7	6	8

Q.2.17.3 External Quality Controls for Selenium and Zinc in Serum

HNR 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 that 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.

Q.3 Acknowledgements

We are indebted to the personnel at the following institutions for their assistance in local sample processing and storage:

Abertawe Bro Morgannwg University Health Board; Aintree University Hospitals NHS Foundation Trust; Basildon and Thurrock General Hospitals NHS Trust; Belfast Health and Social Care Trust; Betsi Cadwaldr University Health Board; BMI The Chaucer Hospital (Canterbury); 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; Kingston Hospital NHS Trust; Midland Pathology Services (Birmingham); Milton Keynes Hospital NHS Foundation Trust; MRC Human Nutrition Research; NationWide Laboratories (Poulton-le-Fylde); NHS Arran and Ayrshire; NHS Borders; NHS Dumfries and Galloway; 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; The Yorkshire Clinic (Bingley); University Hospital of North Staffordshire NHS Trust; University Hospitals of Leicester NHS Trust; University of Aberdeen Rowett Institute of Nutrition and Health; University of Dundee; University of Surrey; Wellcome Trust Clinical Research Facility (Manchester); Western Health and Social Care Trust; Worcestershire Hospitals NHS Trust; Wye Valley NHS Trust and York Teaching Hospital NHS Foundation Trust.

[i] Coulter WH. High speed automatic blood cell counter and cell size analyzer. Paper presented at National Electronics Conference, Chicago, IL, 1956; October 3.

[ii] Brecher G, Schneiderman M and Williams GZ. Evaluation of electronic red blood cell counter. American Journal of Clinical Pathology, 1956; 26:1439-1449.

[iii] Nexo E. Hvas A-M. Bleie Ø et al. Holo-transcobalamin is an early marker of changes in cobalamin homeostasis. A randomized placebo-controlled study. Clin Chem 2002;48(10):1768-71

[iv] Valente E. Scott JM. Ueland PM et al. Diagnostic accuracy of holotranscobalamin, methylmalonic acid, serum cobalamin and other indicators of tissue vitamin B12 status in the elderly. Clin Chem 2011;57(6):856-863.

[v] Nexo E. Hoffmann-Lucke E. Holotranscobalamin, a marker of vitamin B12 status: analytical aspects and clinical utility. Am J Clin Nutr 2011;94(1):359S-365S.

[vi] Obeid R. Jouma M. Hermann W. Cobalamin status (holotranscobalamin. methylmalonic acid) and folate as determinants of homocysteine concentration. Clin Chem 2002;48(11):2064-5.

[vii] Herrmann W. Schorr H. Obeid R et al. Vitamin B12 status, particularly holotranscobalamin II and methylmalonic acid concentrations and hyperhomocysteinemia in vegetarians. Am J Clin Nutr 2003;78:131-6.

[viii] Lloyd-Wright Z. Hvas A-M. Moller J et al. Holotranscobalamin as an indicator of dietary vitamin B12 deficiency. Clin Chem 2003;49(12):2076-8.

[ix] Refsum H. Yajnik CS. Gadkari M et al. Hyperhomocysteinemia and elevated methylmalonic acid indicate a high prevalence of cobalamin deficiency in Asian Indians. Am J Clin Nutr 2001;74:233-41

[x] Refsum H. Smith AD. Low Vitamin B12 status in confirmed Alzheimer's disease as revealed by serum holotranscobalamin. J Neurol Neurosurg Psychiatry 2003;74:959-61

[xi] Chen X. Remacha AF. Sarda MP et al. Influence of cobalamin deficiency compared with that of cobalamin absorption on serum holo-transcobalamin II. Am J Clin Nutr 2005;81:110-14.

[xii] Stadtman TC. In: Colowick SP, Caplan SO, eds. *Methods in enzymology*, vol III. New York, Academy Press, 1957:392–4 & 67–81.

[xiii] Rautela GS, Liedtke RJ. Automated enzymatic measurement of total cholesterol in serum. *Clinical chemistry*, 1978, 24:108–14.

[xiv] Vuilleumier JP, Keck E. Fluorometric assay of vitamin C in biological materials using a centrifugal analyser with fluorescence attachment. Journal of Micronutrient Analysis, 1989; 5: 25-34.

[xv] Vuilleumier JP, Keller HE, Keck E. Clinical chemical methods for the routine assessment of the vitamin Status in human populations. Part III: The apoenzyme stimulation tests for vitamin B1, B2 and B6. adapted to the Cobas Bio. International Journal of Vitamin Research, 1990; 60:126-135.

[xvi] Glatzle D, Körner WF, christeller S, Wiss O. Method for the detection of a biochemical riboflavin deficiency stimulation of NADPH2-dependent glutathione reductase from human erythrocytes by FAD *in vitro*: Investigations on the vitamin B2 status in healthy people and geriatric patients. International Journal of Vitamin Research, 1970; 40:166-183.

[xvii] Rybak ME, Pfeiffer CM. Clinical analysis of vitamin B6: Determination of pyridoxal 5-phosphate and 4-pyridoxic acid in human serum by reversed-phase high-performance liquid chromatography with chlorite postcolumn derivatization. Analytical Biochemistry 2004; 333:336-344.

[xviii] Sowell AL, Huff DL, Yeager PR, Caudill SP, Gunter EW., 'Retinol, alpha-tocopherol, lutein/zeaxanthin, beta-cryptoxanthin, lycopene, alpha-carotene, trans-beta-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multiwavelength detection.' Clin Chem. 1994 Mar; 40(3):411-6.

[xix] Food-based solutions for optimal vitamin D nutrition and health through the life cycle; Project No.: FP7-613977-ODIN.