Appendix P Methods of blood analysis and quality control

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P.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 following assays were conducted at Addenbrooke's:

- full blood count including haemoglobin and haematocrit (see section P.2.1)
- serum C-reactive protein, using a high-sensitivity assay (see section P.2.2)
- serum vitamin B_{12} (see section P.2.3)
- serum Total, HDL and LDL cholesterol (see section P.2.4)
- serum triglycerides (triacylglycerols) (see section P.2.5)

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 described in sections P.2.6 to P.2.16 (and listed below) were conducted at HNR:

- plasma ferritin (see section P.2.6)
- plasma soluble transferrin receptors (see section P.2.7)
- plasma vitamin C (see section P.2.8)
- ETKAC for thiamin status (see section P.2.9)
- EGRAC for riboflavin status (see section P.2.10)
- plasma vitamin B_6 (PLP and PA) (see section P.2.11)
- plasma total homocysteine (see section P.2.12)
- plasma retinol (see section P.2.13)
- plasma α-tocopherol (see section P.2.13)
- plasma γ-tocopherol (see section P.2.13)
- plasma individual carotenoids; α–carotene, β–carotene, α–cryptoxanthin, β–cryptoxanthin, lycopene, lutein and zeaxanthin (see section P.2.13)
- plasma 25-hydroxyvitamin D (see section P.2.14)
- plasma creatinine (see section P.2.15)
- plasma selenium (see section P.2.16)
- plasma zinc (see section P.2.16)

Serum and whole blood folate concentrations in NDNS Rolling Programme (NDNS RP) blood samples were measured at the Centre for Disease Control (CDC) in Atlanta, USA. Folate results for Wales Years 2 to 5 have been published in a separate folate report along with results for Years 1 to 4 for the UK as a whole, Scotland and Northern Ireland.[1] Details of analytical methods for the analysis of folate and quality control data will be published alongside the folate results. Appendix W provides details for analytes that were measured in the NDNS RP blood samples 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.

P.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 P.2.1 to P.2.16. 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.

P.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[2,3] 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.

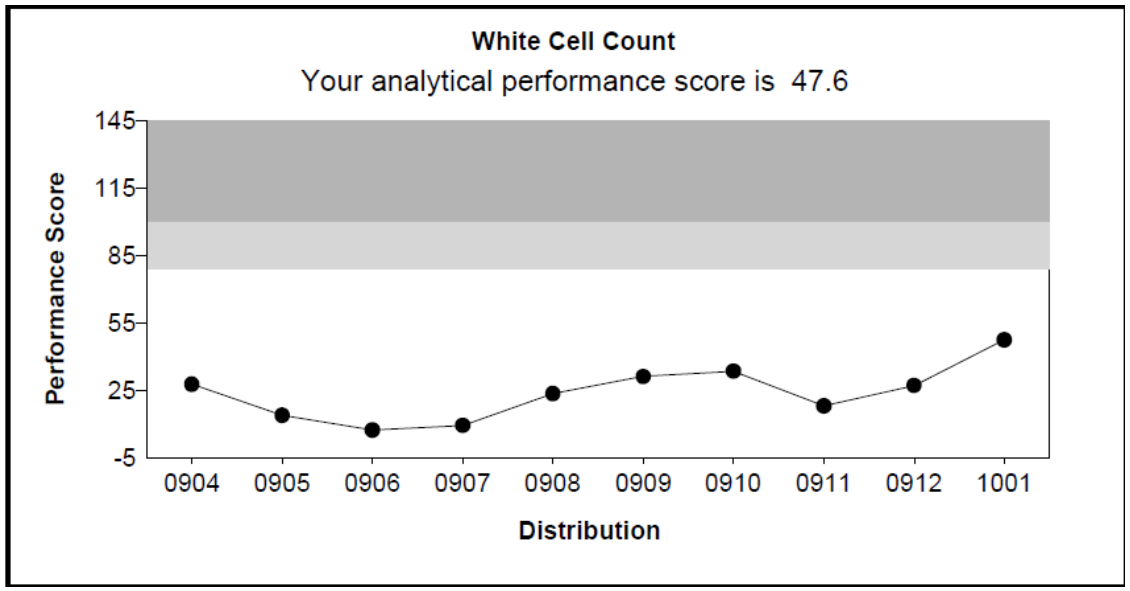
Haemoglobin was measured spectrophotometrically at 525nm by a photocell in a sample that was diluted 1:256 (final) with isotonic diluent and lysing solution. The red cells were destroyed with a lysing agent releasing the haemoglobin into solution, which enabled the white blood cell count to be estimated using the Coulter Principle (impedance counting of the white blood cells)[2,3] without interference by red cells. The same lysing reagent also converted the haemoglobin to cyanmethaemoglobin.

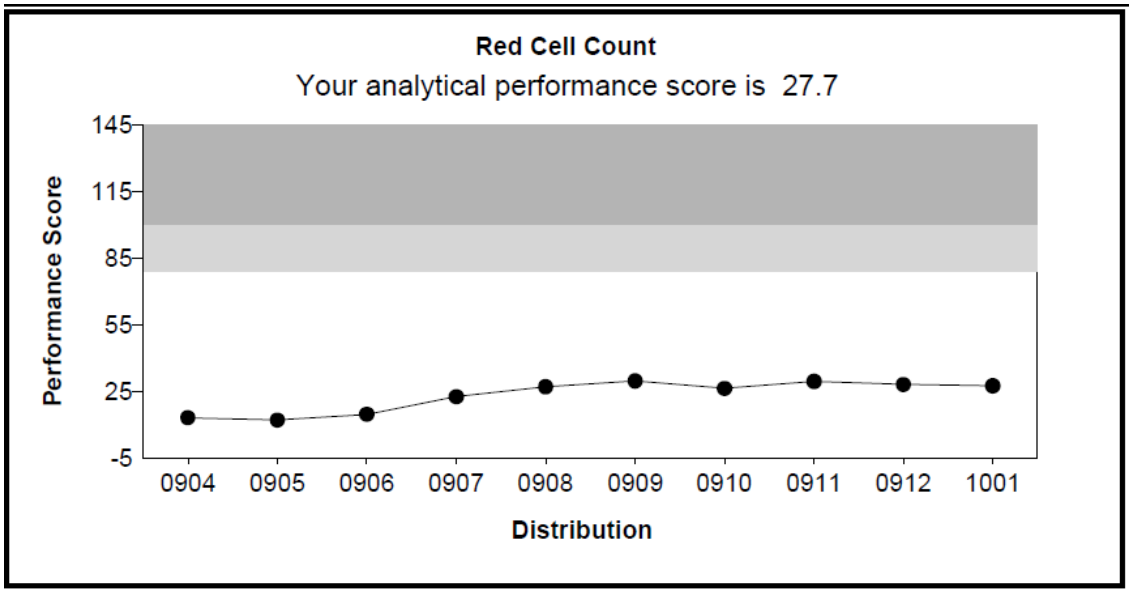
P.2.1.1 Quality controls for full blood count including haemoglobin and haematocrit

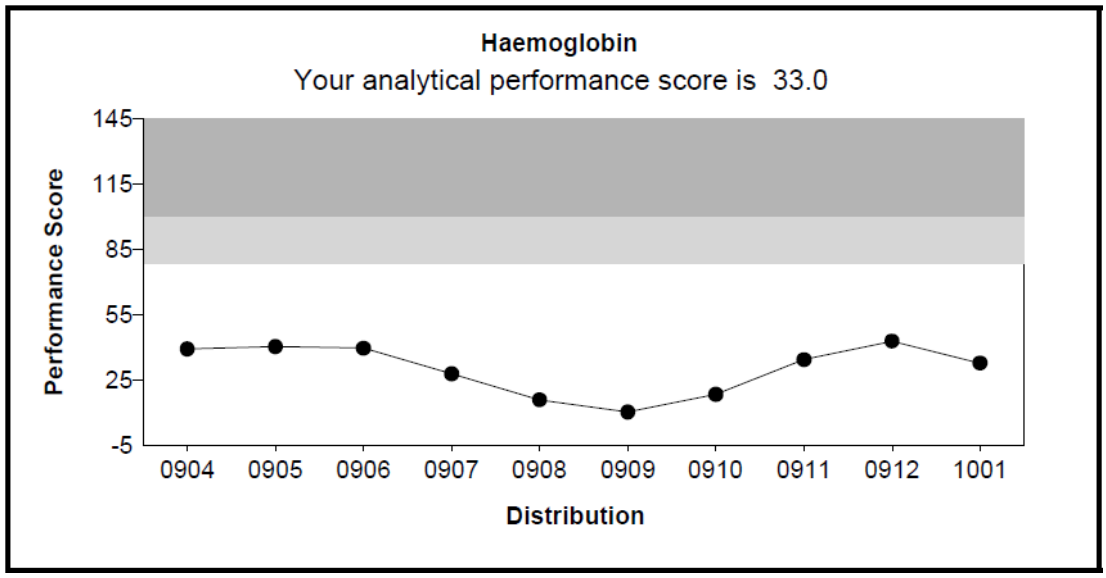
Quality of results was checked using Addenbrooke's internal controls at regular intervals and also assessed externally through UKNEQAS. NEQAS results are compared against the All Laboratories Trimmed Mean (ALTM) calculated for laboratories in the NEQAS scheme using the same analyser and method as that used by Addenbrooke's. Figures P.1 to P.4 show illustrative UKNEQAS overall performance results for full blood counts including haemoglobin and haematocrit for the periods covering analysis of samples for Years 2 to 5 of the NDNS RP. Results within the white area of the charts indicate acceptable performance as determined by

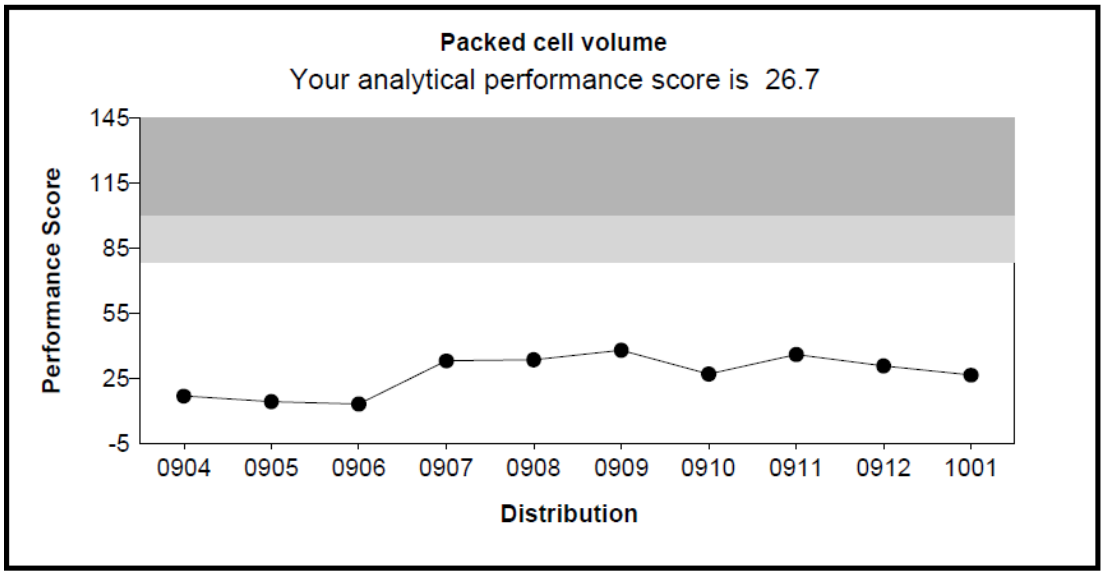
UKNEQAS. “Performance index” is derived by the NEQAS administrators as a function of the deviation of the laboratory from the consensus mean. The dark shaded area indicates unacceptable performance and the paler area indicates a borderline situation.

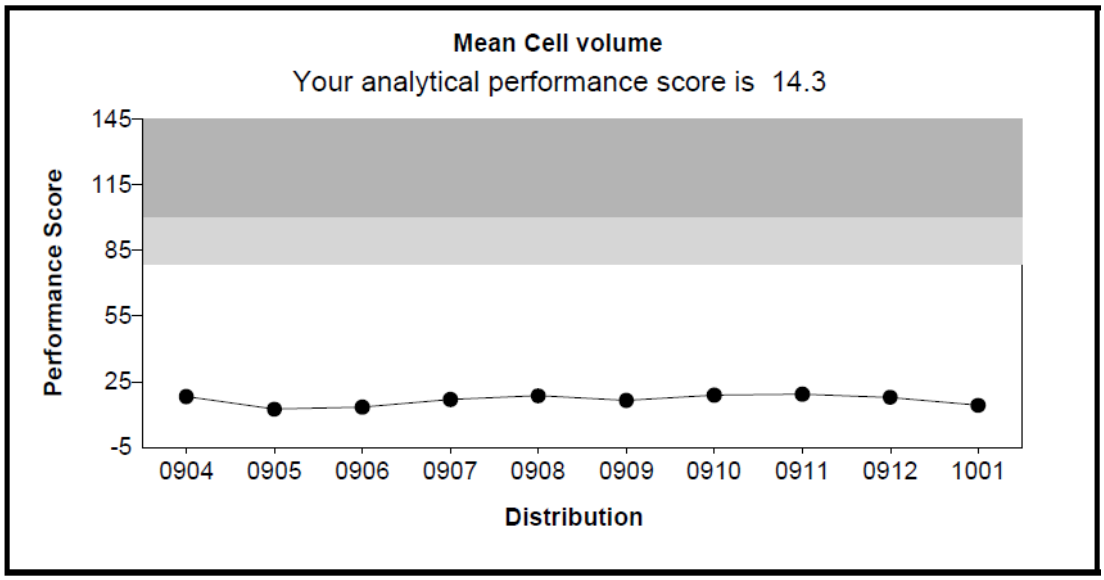
Figure P.1 Illustrative overall performance charts for UKNEQAS for Year 2 of the NDNS RP

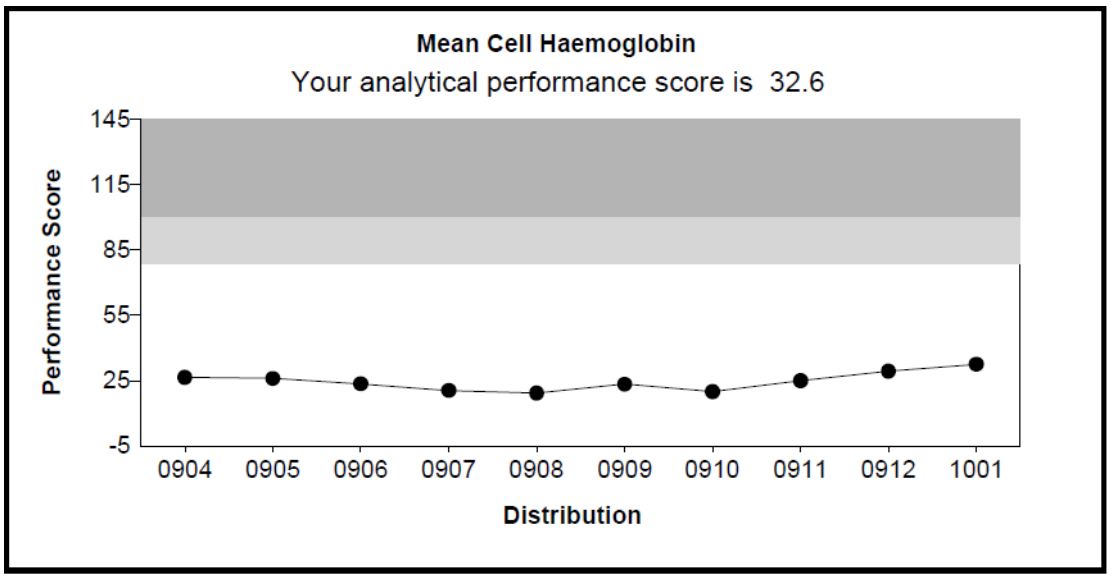


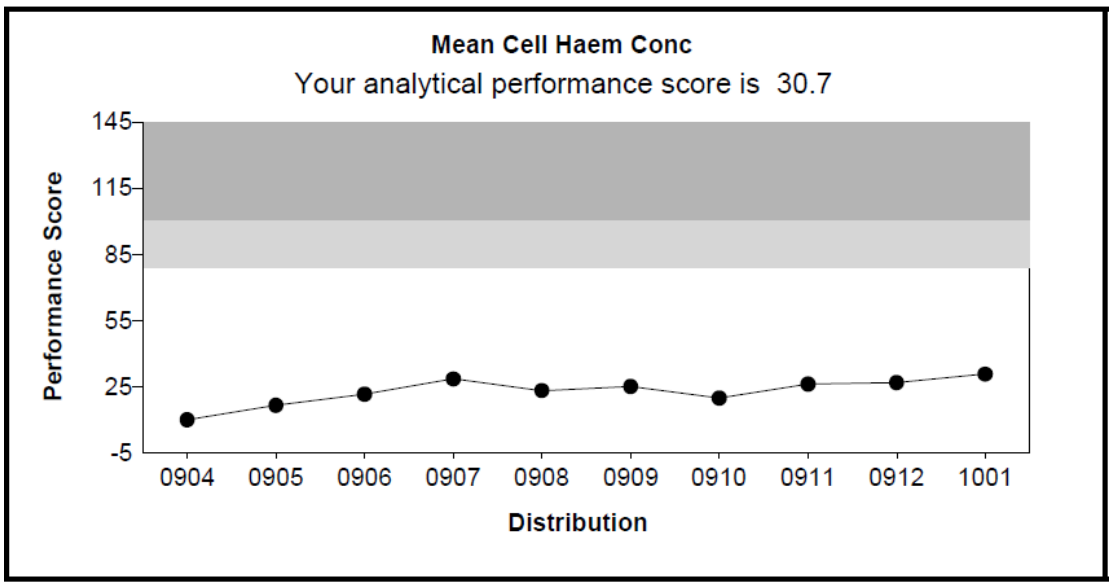



Haemoglobin
Your analytical performance score is 33.0
Performance Score
145
115
85
55
25
-5
0904
0905
0906
0907
0908
0909
0910
0911
0912
1001
Distribution


Packed cell volume
Your analytical performance score is 26.7
Performance Score
145
115
85
55
25
-5
0904
0905
0906
0907
0908
0909
0910
0911
0912
1001
Distribution


Mean Cell volume
Your analytical performance score is 14.3
Performance Score
145
115
85
55
25
-5
0904
0905
0906
0907
0908
0909
0910
0911
0912
1001
Distribution


Mean Cell Haemoglobin
Your analytical performance score is 32.6
Performance Score
145
115
85
55
25
-5
0904
0905
0906
0907
0908
0909
0910
0911
0912
1001
Distribution


Mean Cell Haem Conc
Your analytical performance score is 30.7
Performance Score
145
115
85
55
25
-5
0904
0905
0906
0907
0908
0909
0910
0911
0912
1001
Distribution

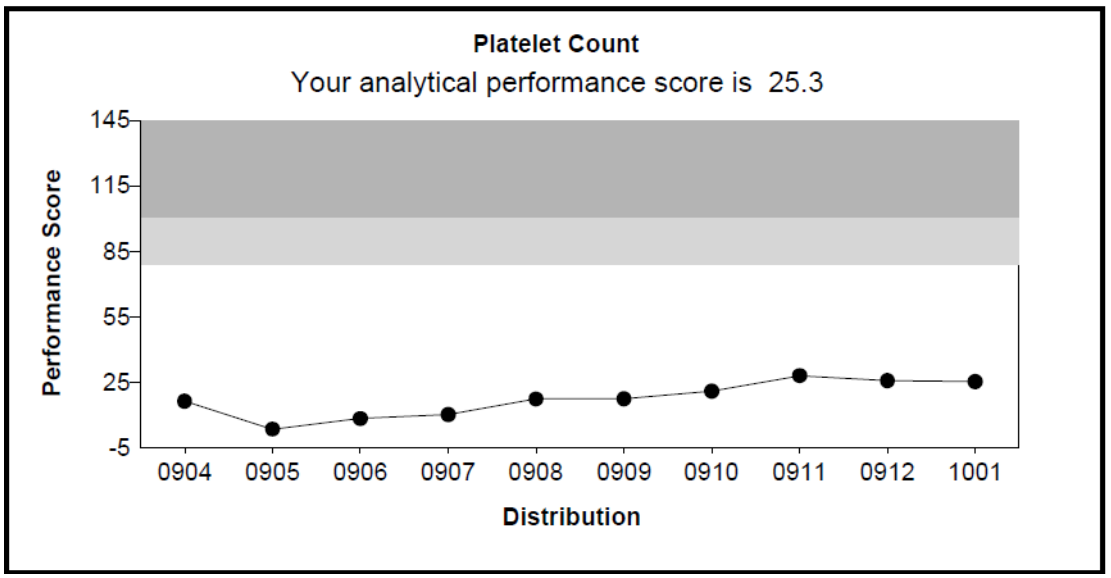
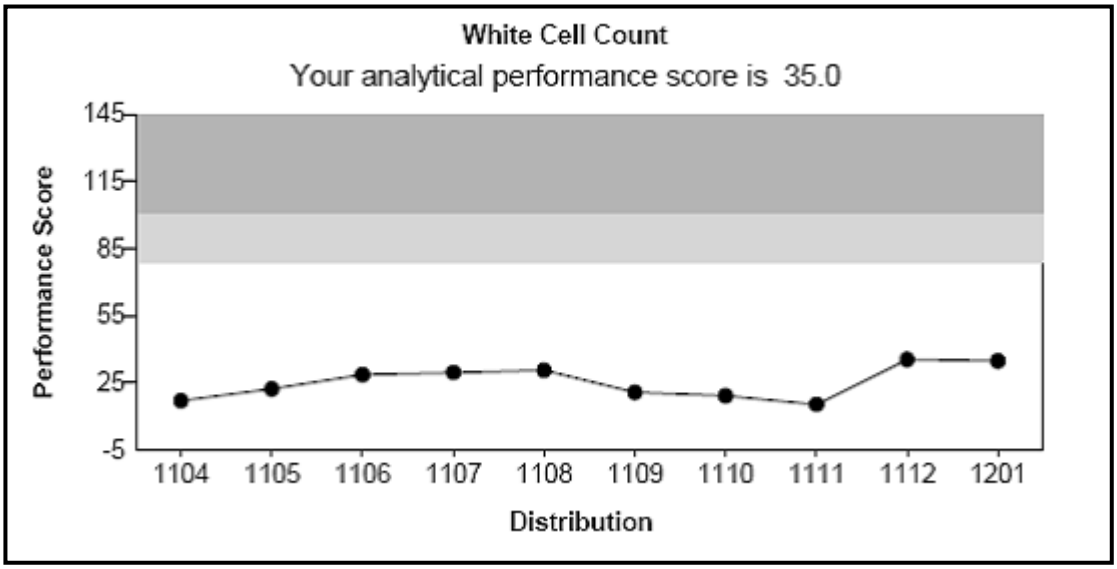
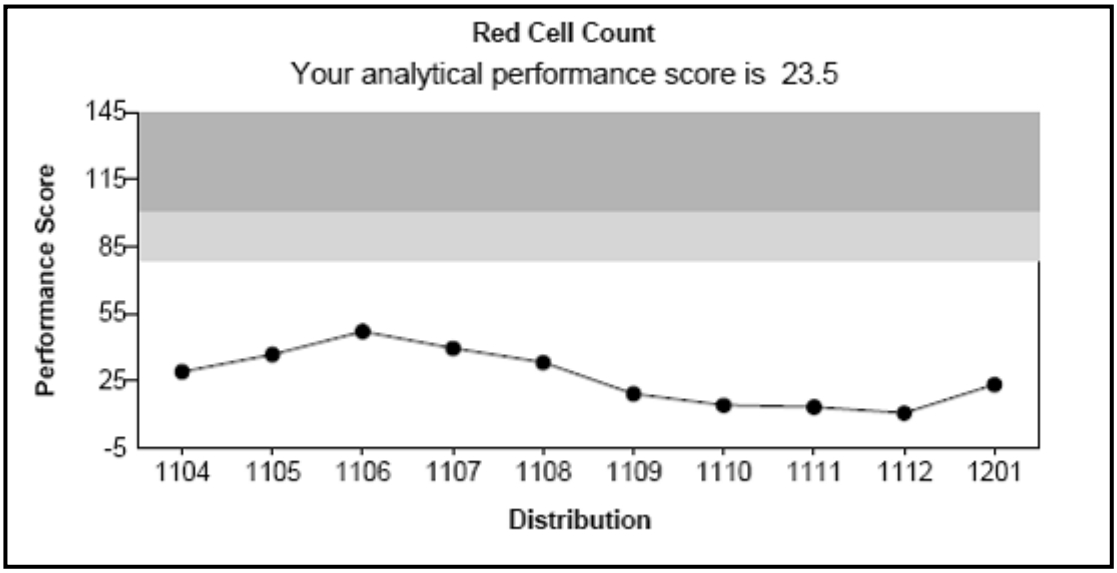
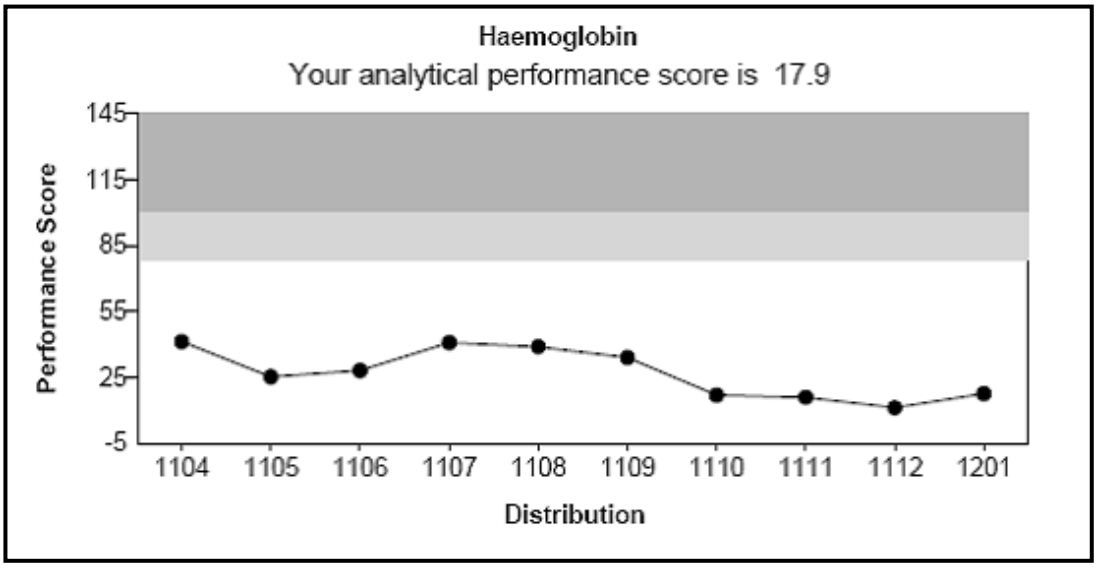
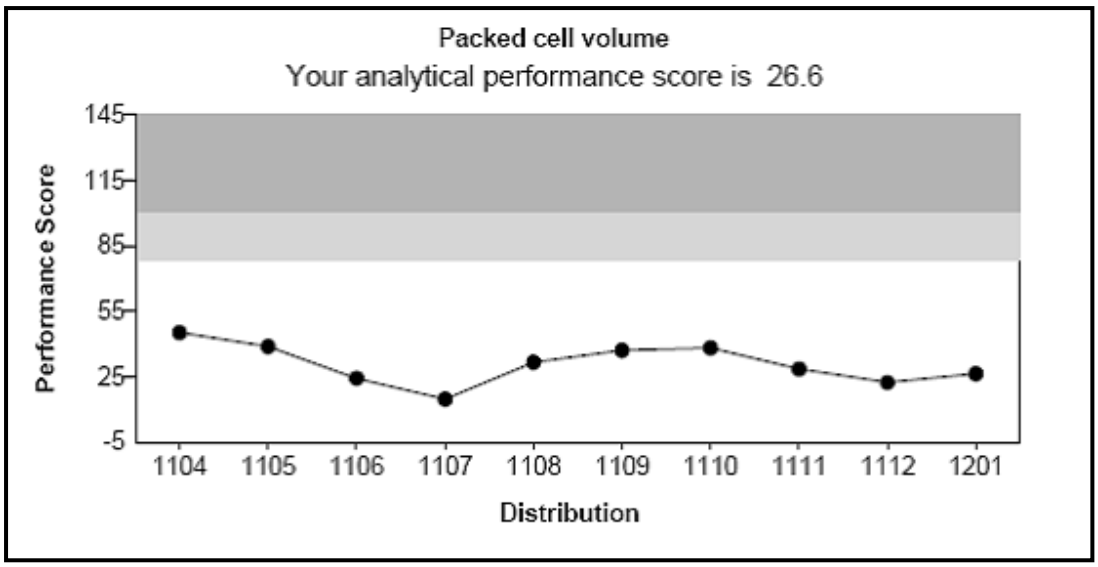

Platelet Count
Your analytical performance score is 25.3
Performance Score
145
115
85
55
25
-5
0904
0905
0906
0907
0908
0909
0910
0911
0912
1001
Distribution

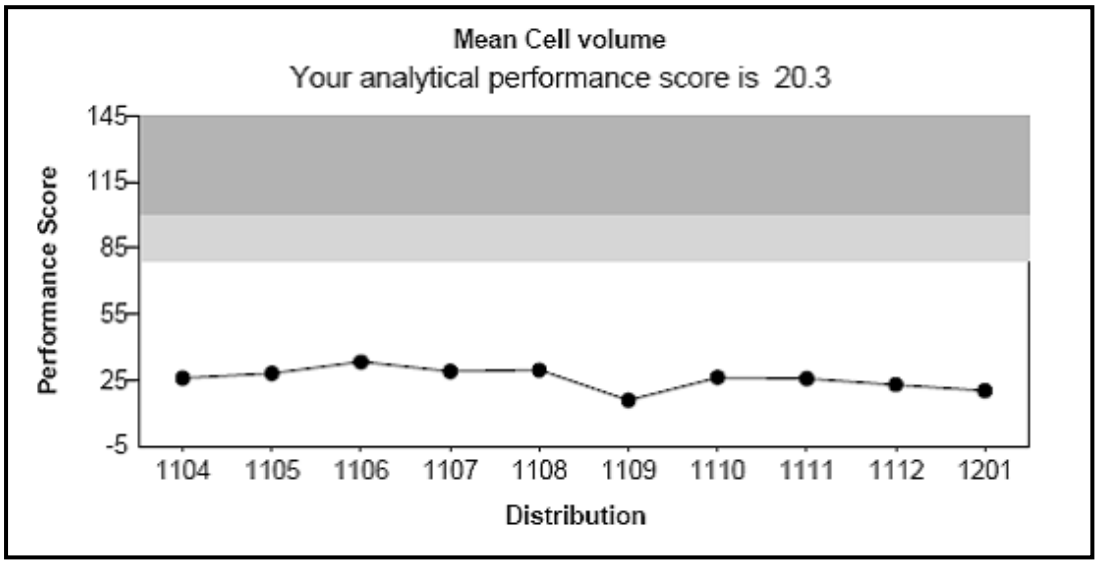
Figure P.2 Illustrative overall performance charts for UKNEQAS for Year 3 of the NDNS RP

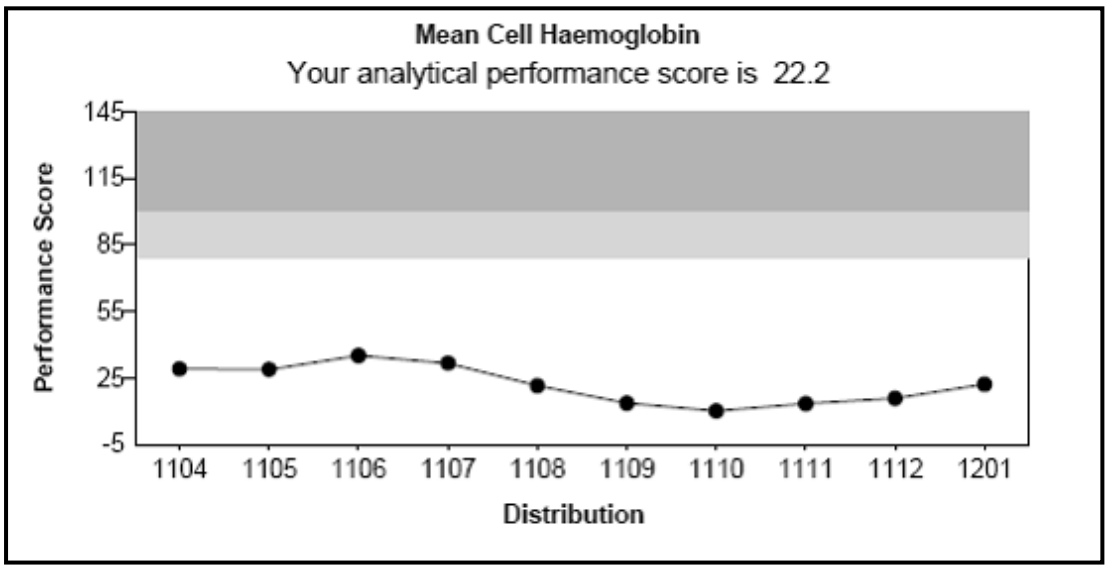


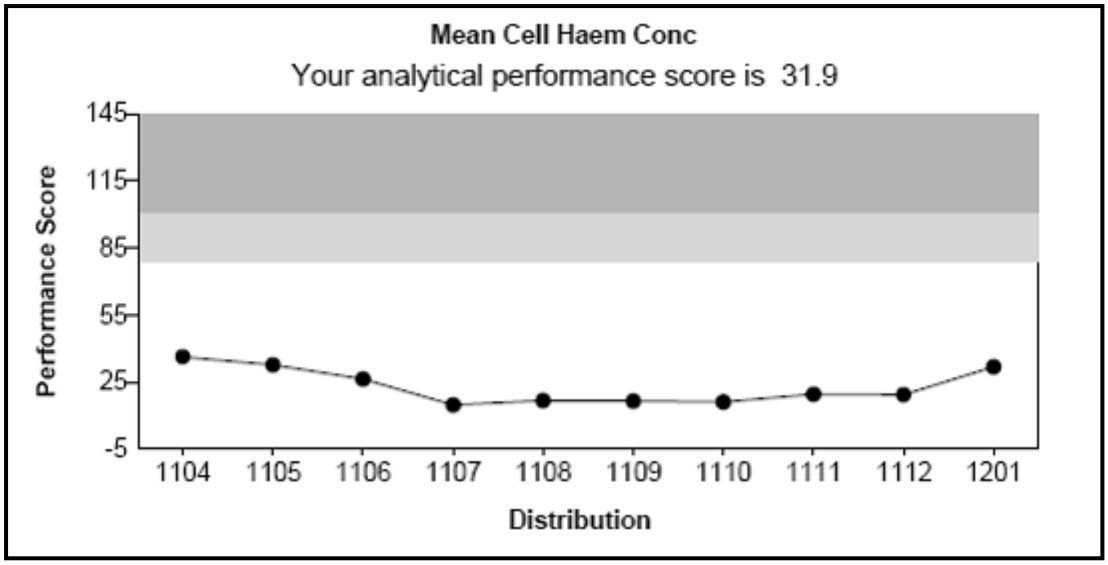





Packed cell volume
Your analytical performance score is 26.6
Performance Score
145
115
85
55
25
-5
1104 1105 1106 1107 1108 1109 1110 1111 1112 1201
Distribution


Mean Cell volume
Your analytical performance score is 20.3
Performance Score
145
115
85
55
25
-5
1104 1105 1106 1107 1108 1109 1110 1111 1112 1201
Distribution


Mean Cell Haemoglobin
Your analytical performance score is 22.2
Performance Score
145
115
85
55
25
-5
1104 1105 1106 1107 1108 1109 1110 1111 1112 1201
Distribution


Mean Cell Haem Conc
Your analytical performance score is 31.9
Performance Score
145
115
85
55
25
-5
1104
1105
1106
1107
1108
1109
1110
1111
1112
1201
Distribution

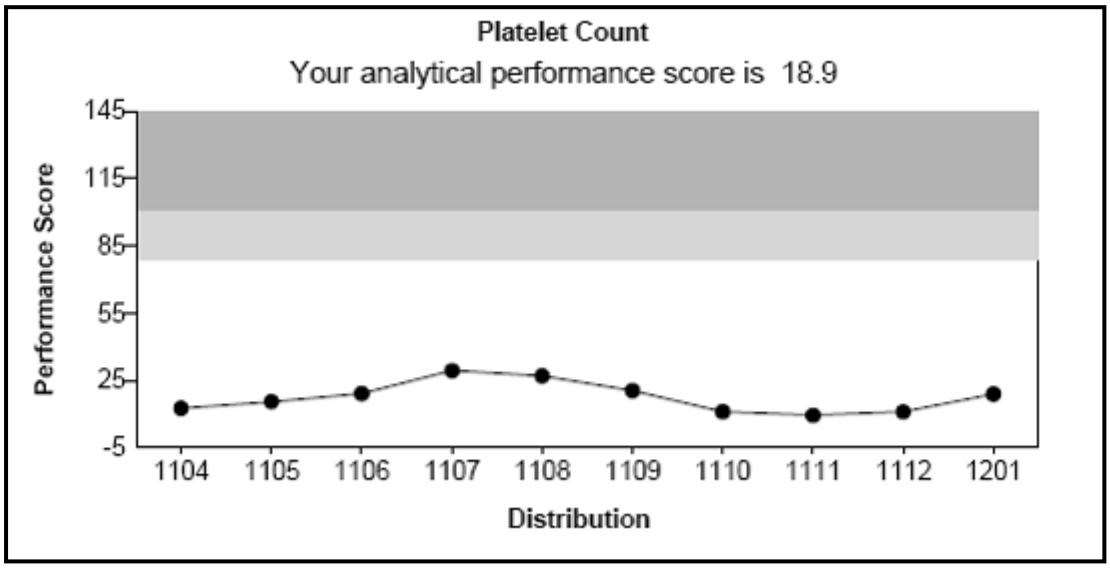
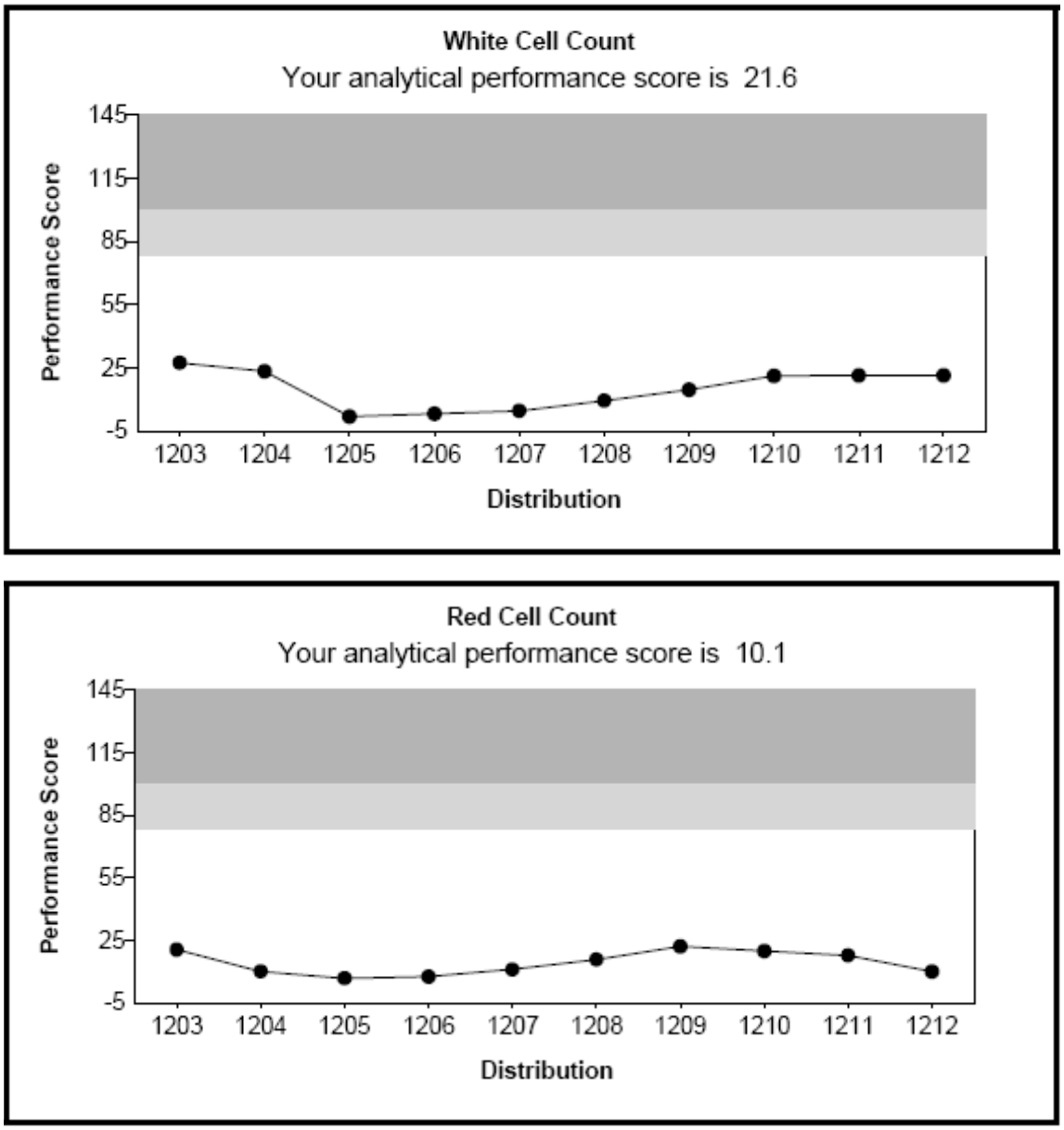
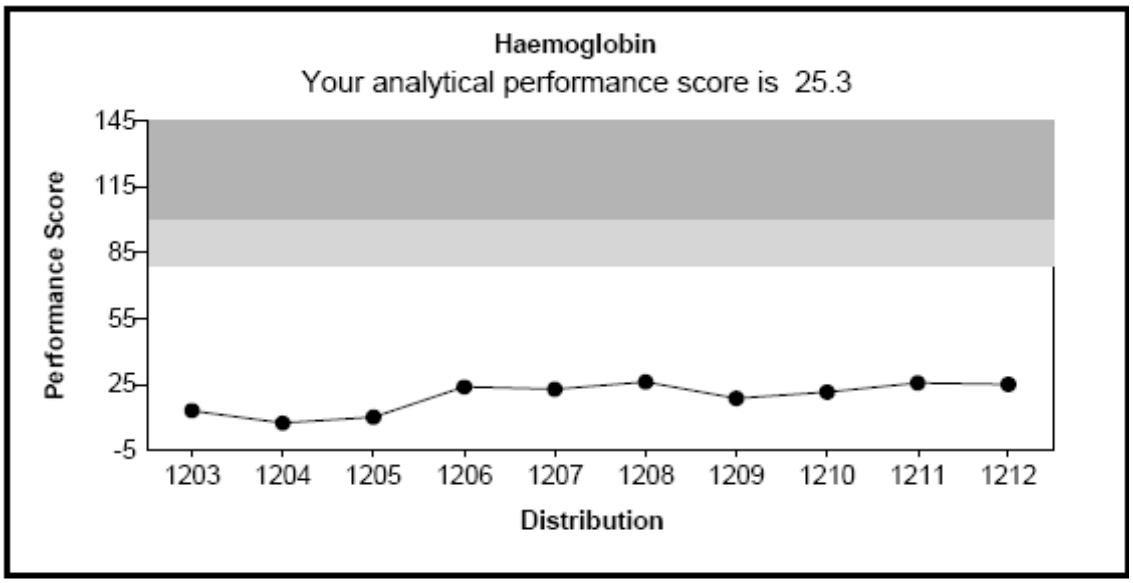
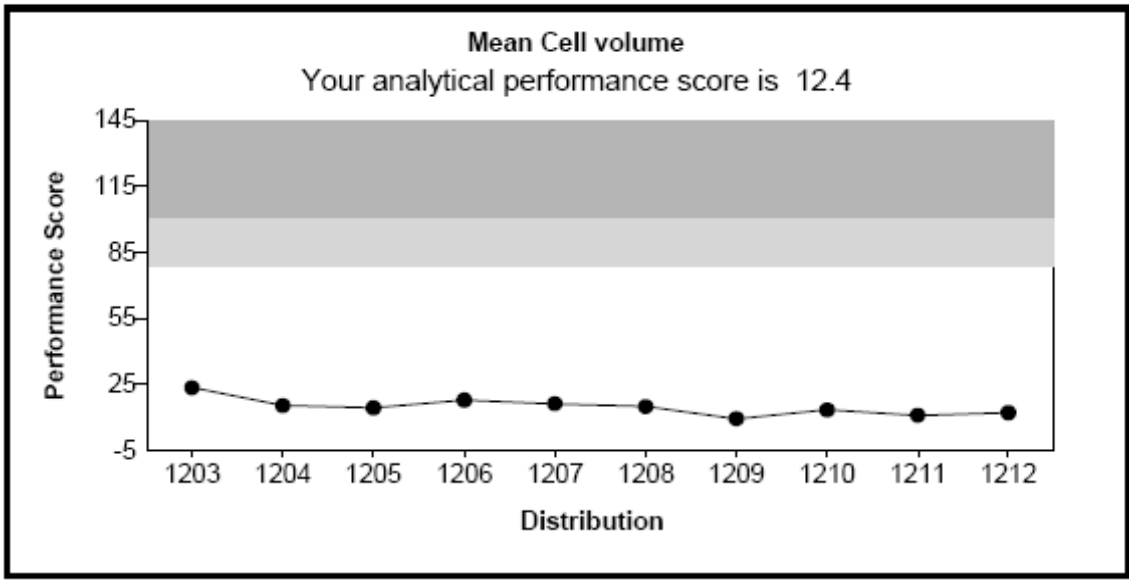
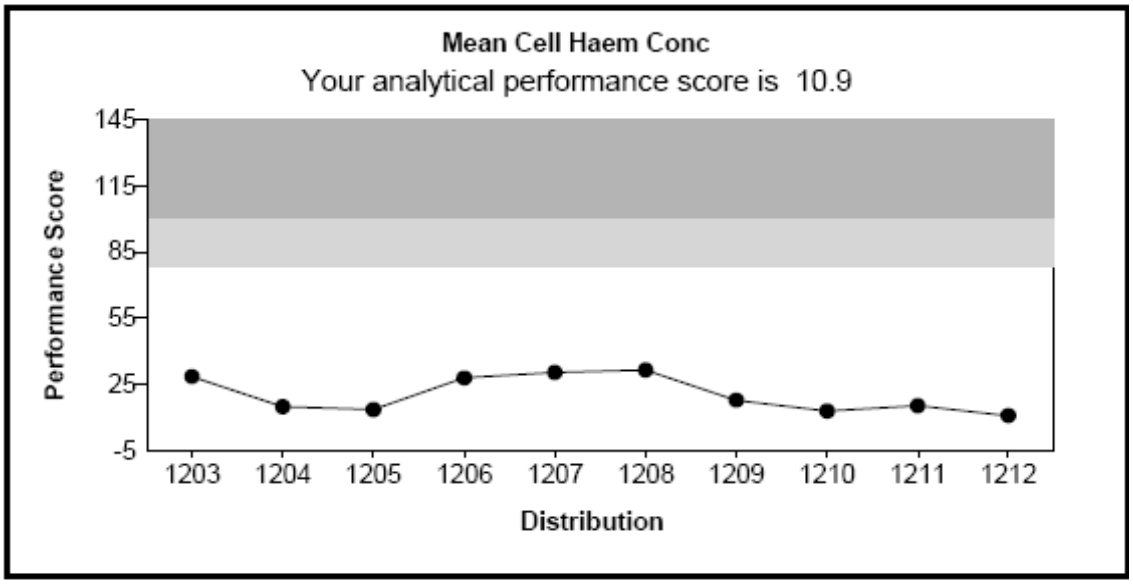

Platelet Count
Your analytical performance score is 18.9
Performance Score
145
115
85
55
25
-5
1104
1105
1106
1107
1108
1109
1110
1111
1112
1201
Distribution

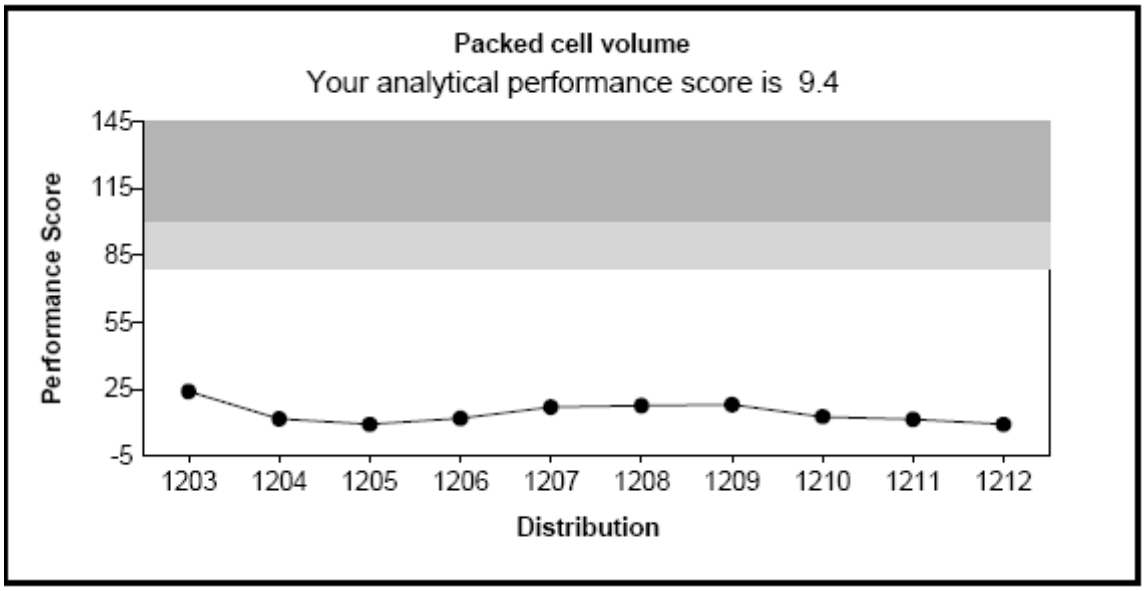
Figure P.3 Illustrative overall performance charts for UKNEQAS for Year 4 of the NDNS RP

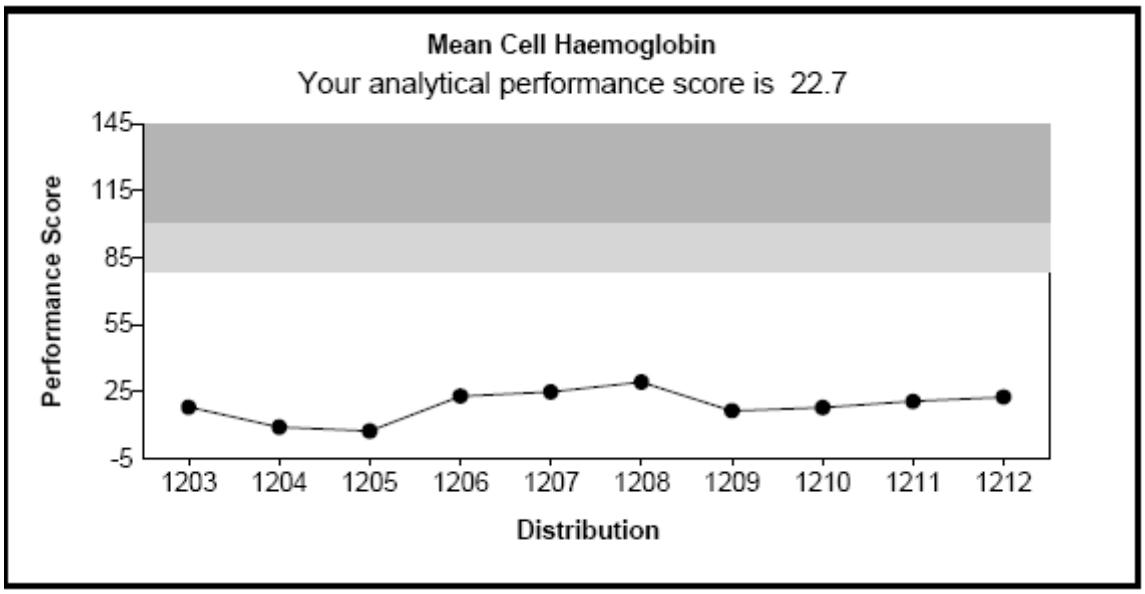



Haemoglobin
Your analytical performance score is 25.3
Performance Score
145
115
85
55
25
-5
1203 1204 1205 1206 1207 1208 1209 1210 1211 1212
Distribution


Mean Cell volume
Your analytical performance score is 12.4
Performance Score
145
115
85
55
25
-5
1203 1204 1205 1206 1207 1208 1209 1210 1211 1212
Distribution


Mean Cell Haem Conc
Your analytical performance score is 10.9
Performance Score
145
115
85
55
25
-5
1203 1204 1205 1206 1207 1208 1209 1210 1211 1212
Distribution


Packed cell volume
Your analytical performance score is 9.4
Performance Score
145
115
85
55
25
-5
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
Distribution


Mean Cell Haemoglobin
Your analytical performance score is 22.7
Performance Score
145
115
85
55
25
-5
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
Distribution

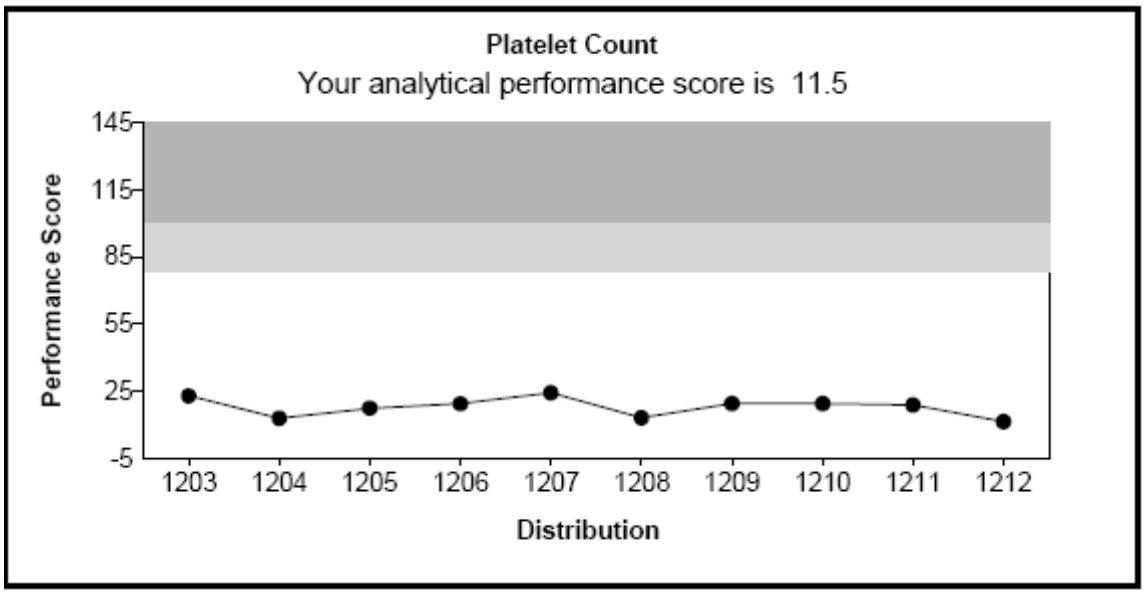
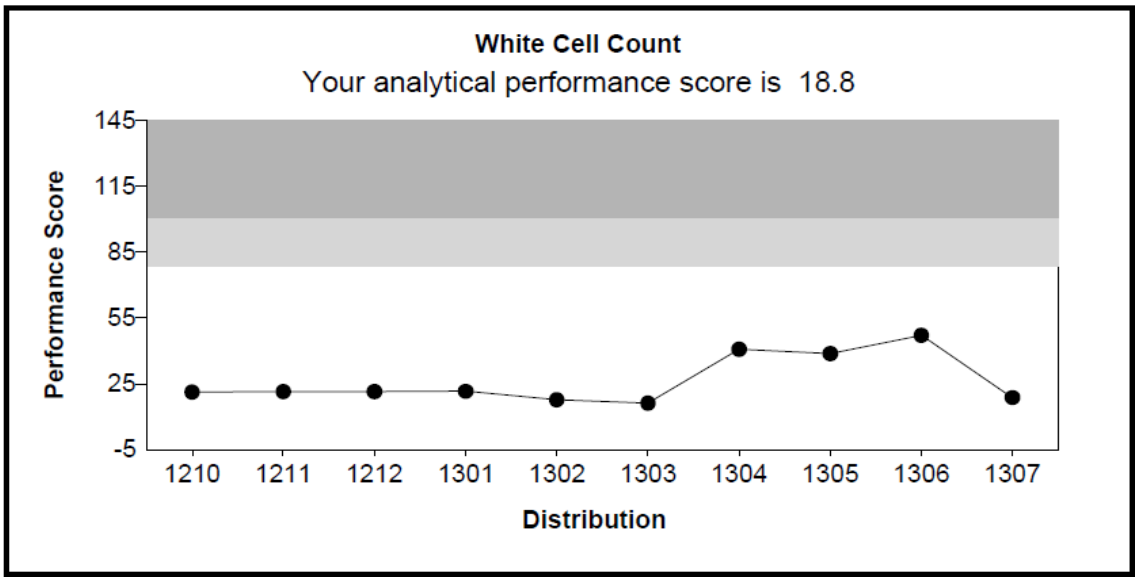
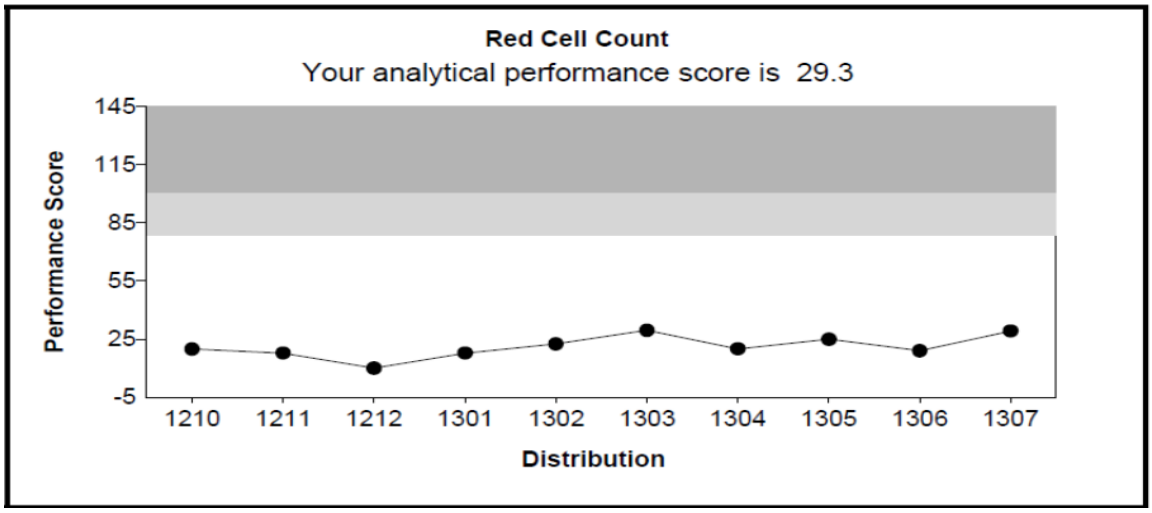
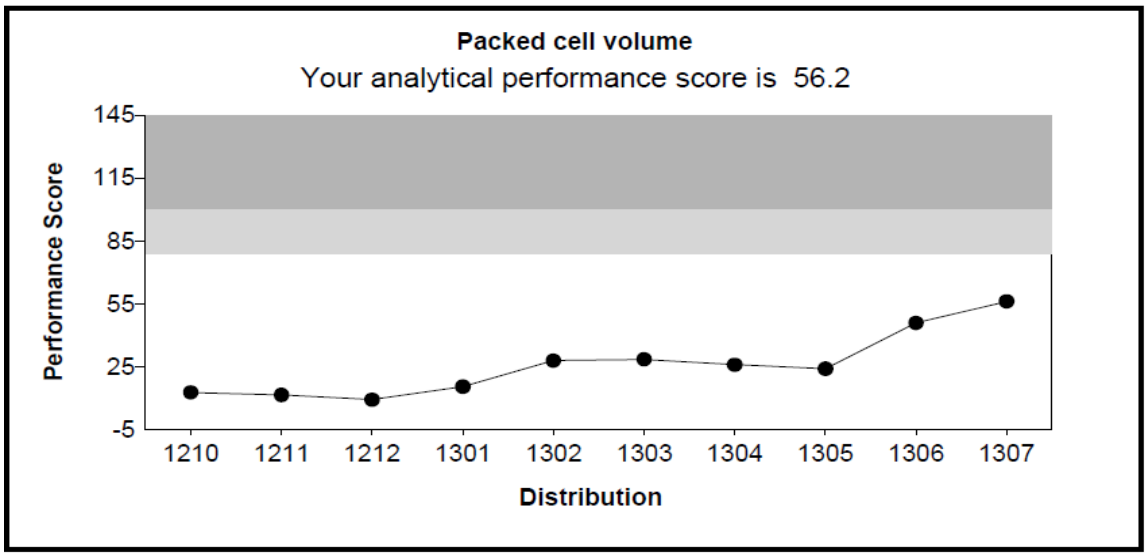
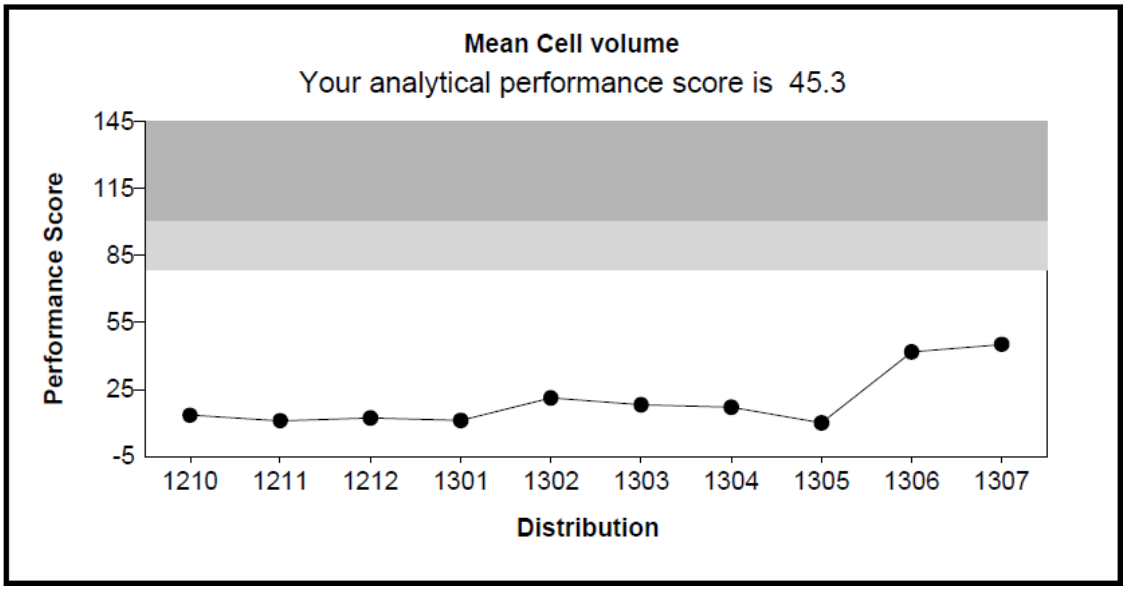

Platelet Count
Your analytical performance score is 11.5
Performance Score
145
115
85
55
25
-5
1203
1204
1205
1206
1207
1208
1209
1210
1211
1212
Distribution

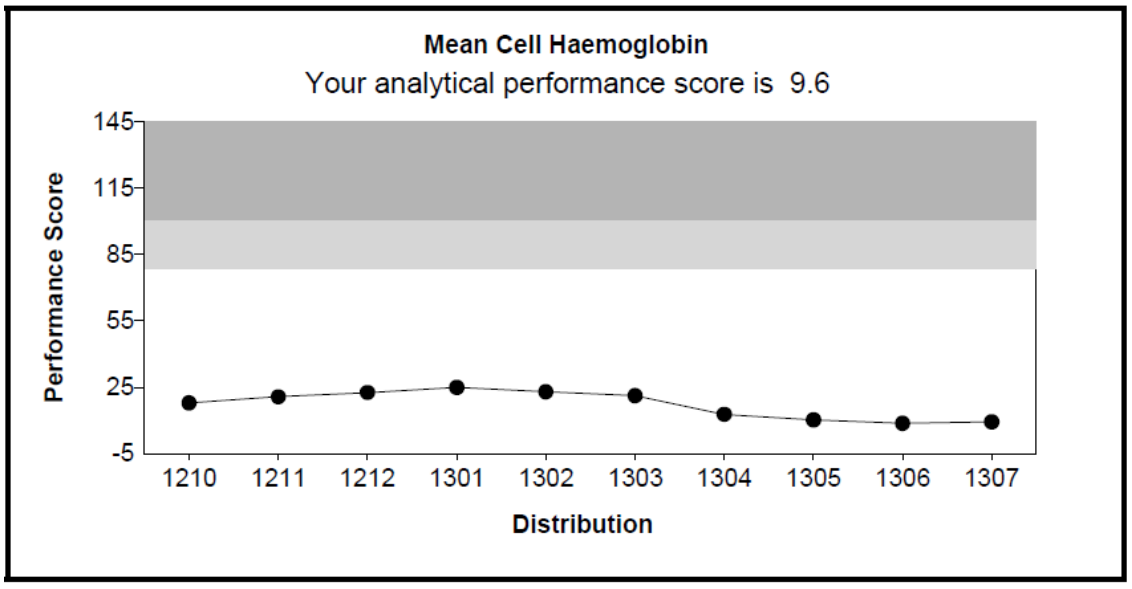
Figure P.4 Illustrative overall performance charts for UKNEQAS for Year 5 of the NDNS RP

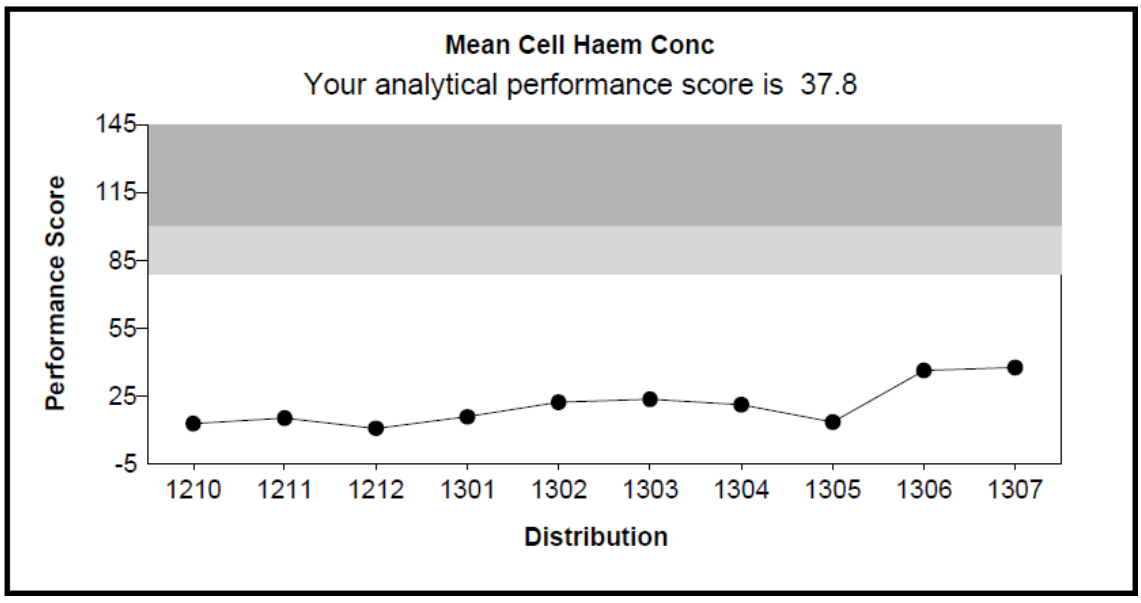







Mean Cell volume
Your analytical performance score is 45.3
Performance Score
145
115
85
55
25
-5
1210
1211
1212
1301
1302
1303
1304
1305
1306
1307
Distribution


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


Mean Cell Haem Conc
Your analytical performance score is 37.8
Performance Score
145
115
85
55
25
-5
1210
1211
1212
1301
1302
1303
1304
1305
1306
1307
Distribution

P.2.2 Serum C-reactive protein (CRP)

C-reactive protein (CRP) was assayed using a high-sensitivity assay on a Dade Behring Dimension RXL 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.

P.2.2.1 Internal quality controls for CRP

The performance statistics in tables P.1-P.4 were calculated using data from two or three different reagent lots in order to include batch to batch variation. Table P.1 shows imprecision data produced from a typical month in Year 2 (January 2010). Table P.2 shows imprecision data produced from three typical months in Year 3 (01/09/2010 – 30/11/2010). Table P.3 shows imprecision data produced from a period covering analysis of Year 4 samples. Table P.4 shows imprecision data produced in Year 5.

Table P.1 Internal quality controls for C-reactive protein (CRP) for Year 2 of the NDNS RP

	QC Lot No 29743	QC Lot No 991179
Mean (mg/L)	7.55	87.30
SD	0.40	3.50
% CV	5.30	4.01
Data points included	360	341

Table P.2 Internal quality controls for C-reactive protein (CRP) for Year 3 of the NDNS RP

	QC Lot No 29753	QC Lot No 35282
Mean (mg/L)	7.88	81.80
SD	0.46	2.87
% CV	5.80	3.51
Data points included	1098	1120

Table P.3 Internal quality controls for C-reactive protein (CRP) for Year 4 of the NDNS RP

	QC Lot No 29773	QC Lot No 29783
Mean (mg/L)	7.59	7.14
SD	0.537	0.250
% CV	7.1	3.5
Data points included	3839	1040

	QC Lot No 35312	QC Lot No 35322	QC Lot No 35332
Mean (mg/L)	88.18	81.47	85.96
SD	3.943	2.318	3.828
% CV	4.5	2.8	4.5
Data points included	1798	669	2297

Table P.4 Internal quality controls for C-reactive protein (CRP) for Year 5 of the NDNS RP

	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

P.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 P.5-P.7 show the laboratory's bias relative to the All Laboratory Trimmed Mean CRP concentration measured in the sample; these graphs are included by kind permission of Addenbrooke's and UKNEQAS for Years 3 to 5 of the NDNS RP. Equivalent graphs are not available for Year 2.
A score of <50 is "ideal"; 50-100 is "good" and 100-200 "adequate" for clinical purposes.

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

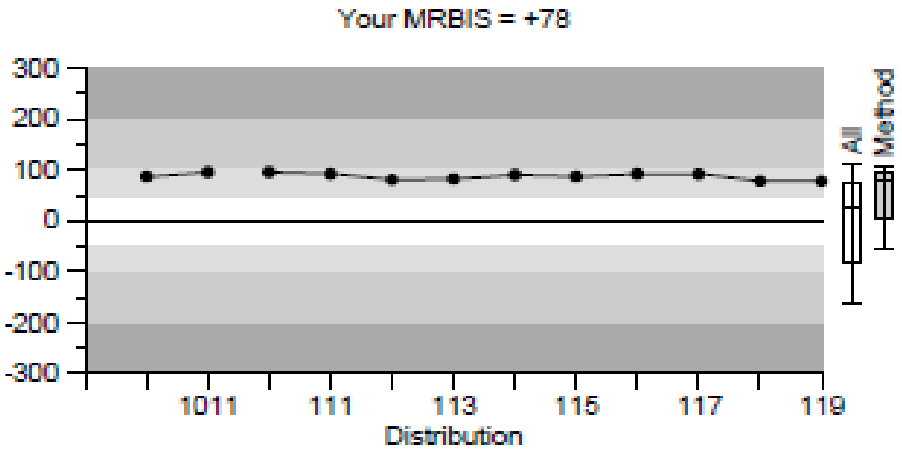


Figure P.6 NEQAS performance for CRP assay for Year 4 of the NDNS RP

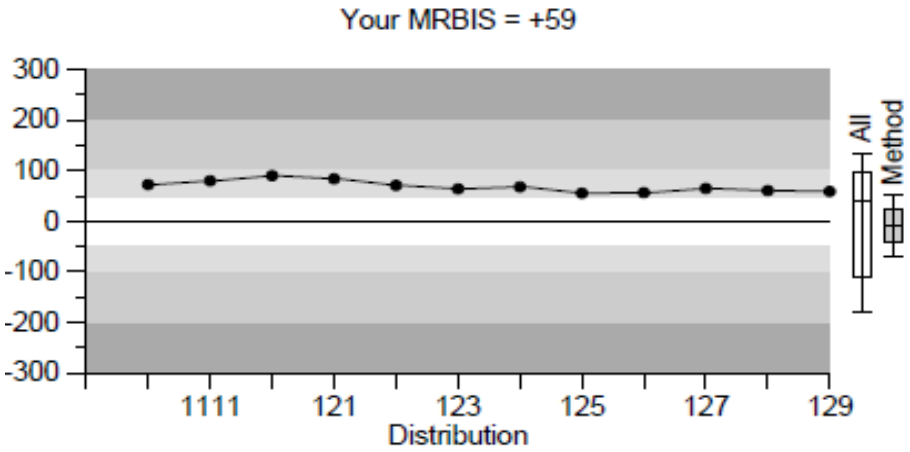
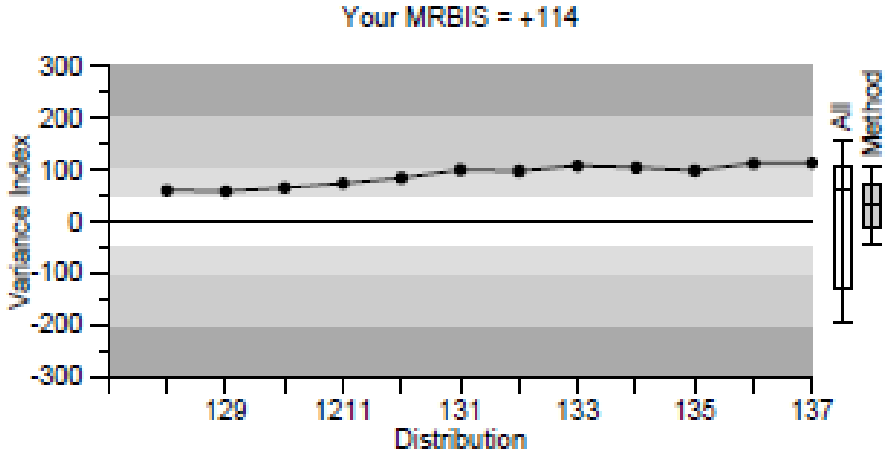


Figure P.7 NEQAS performance for CRP assay for Year 5 of the NDNS RP



P.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.

P.2.3.1 Internal quality controls for vitamin B_{12}

The performance statistics in tables P.5-P.8 were calculated using data from two or three different reagent lots in order to include batch to batch variation. Table P.5 shows two lots of Lyphochek QC data produced for Year 2. Data in the upper section of the table is for the period 08/07/2009-01/05/2010 and data in the lower section of the table is for the period 01/05/2010-09/08/2010. Table P.6 shows imprecision data produced from a combination of five typical months in Year 3 (01/08/2010-31/12/2010). Table P.7 shows imprecision data for a period covering the analysis of Year 4 samples. Table P.8 shows imprecision data for a period covering the analysis of Year 5 samples.

Table P.5 Internal quality controls for vitamin B_{12} for Year 2 of the NDNS RP

	QC Lot No 40201	QC Lot No 40202	QC Lot No 40203
Mean	396	658	1120
SD	35.2	48.1	82.9
% CV	8.9	7.3	7.4
Data points included	559	555	556
	QC Lot No 40231	**QC Lot No 40232**	**QC Lot No 40233**
Mean	354	651	1373
SD	30.0	46.9	82.9
% CV	8.4	7.2	6.0
Data points included	149	156	125

Table P.6 Internal quality controls for vitamin B_{12} for Year 3 of the NDNS RP

	QC Lot No 40231	QC Lot No 40232	QC Lot No 40233	QC Lot No 43170
Mean (µg/L)	345	677	1403	131
SD	24.4	38.0	105.4	19.9
% CV	7.06	5.86	7.51	15.17
Data points included	347	338	336	291

Table P.7 Internal quality controls for vitamin B_{12} for Year 4 of the NDNS RP

	QC Lot No 40241	QC Lot No 40242	QC Lot No 40243	QC Lot No 43170
Mean (µg/L)	401	562	1307	138
SD	30.9	38.6	86.1	23.2
% CV	7.70	6.87	6.59	16.77
Data points included	1137	547	647	481

	QC Lot No 40261	QC Lot No 40262	QC Lot No 40263	QC Lot No 43180
Mean (µg/L)	400	567	1114	130
SD	27.3	37.5	85.3	24.6
% CV	6.83	6.61	7.66	18.99
Data points included	94	243	267	314

	QC Lot No 40252	QC Lot No 40253	QC Lot No 45200
Mean (µg/L)	557	1120	130
SD	34.9	60.6	18.8
% CV	6.26	5.41	14.44
Data points included	452	269	366

Table P.8 Internal quality controls for vitamin B_{12} for Year 5 of the NDNS RP

	QC Lot No 40241	QC Lot No 40261	QC Lot No 40262	QC Lot No 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

P.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 2 to 5 are reproduced below with permission of Addenbrooke's and the NEQAS Haematinics Scheme organisers.

Figures P.8-P.11 show the bias relative to the target concentration during the years when NDNS 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 All Laboratory Trimmed Mean. 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 0.05 (Year 2), -0.06 (Year 3), 0.36 (Year 4) and -0.56 (Year 5) indicate that there was good overall agreement between Addenbrooke's results and the target concentrations.

Figure P.8 Illustrative overall performance charts for UKNEQAS for Year 2 of the NDNS RP for vitamin B_{12}

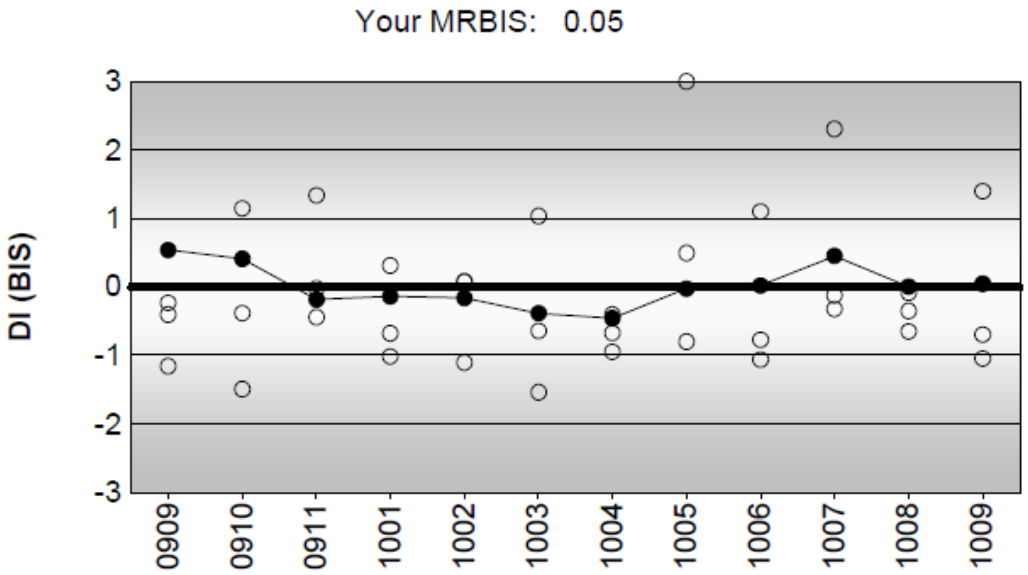


Figure P.9 Illustrative overall performance charts for UKNEQAS for Year 3 of the NDNS RP for vitamin B_{12}

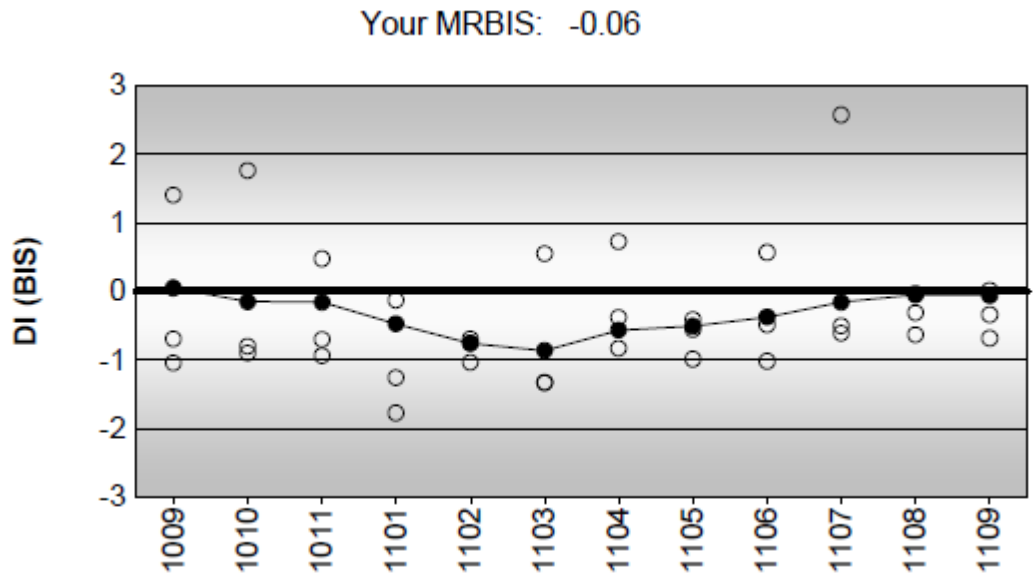


Figure P.10 Illustrative overall performance charts for UKNEQAS for Year 4 of the NDNS RP for vitamin B_{12}

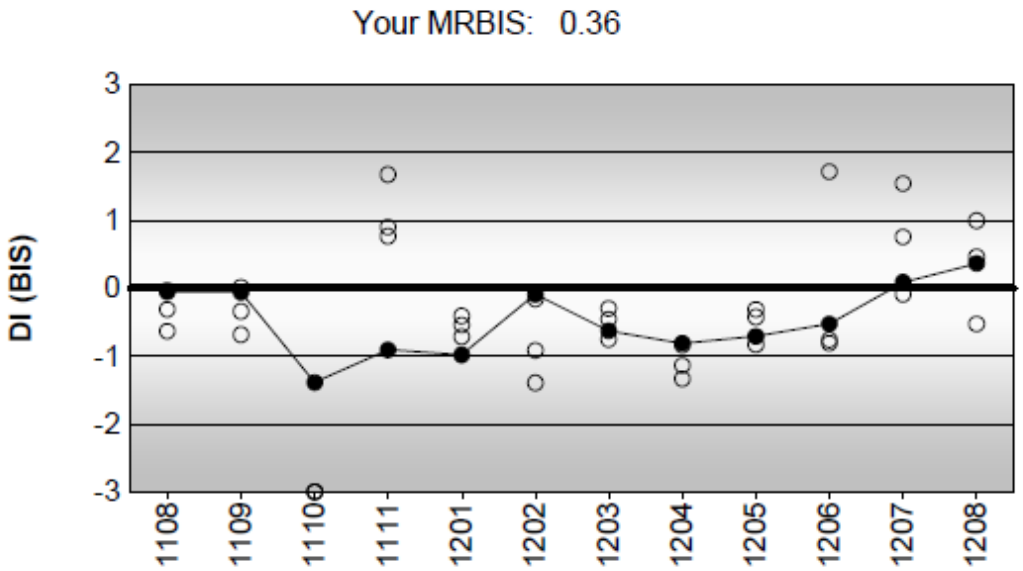
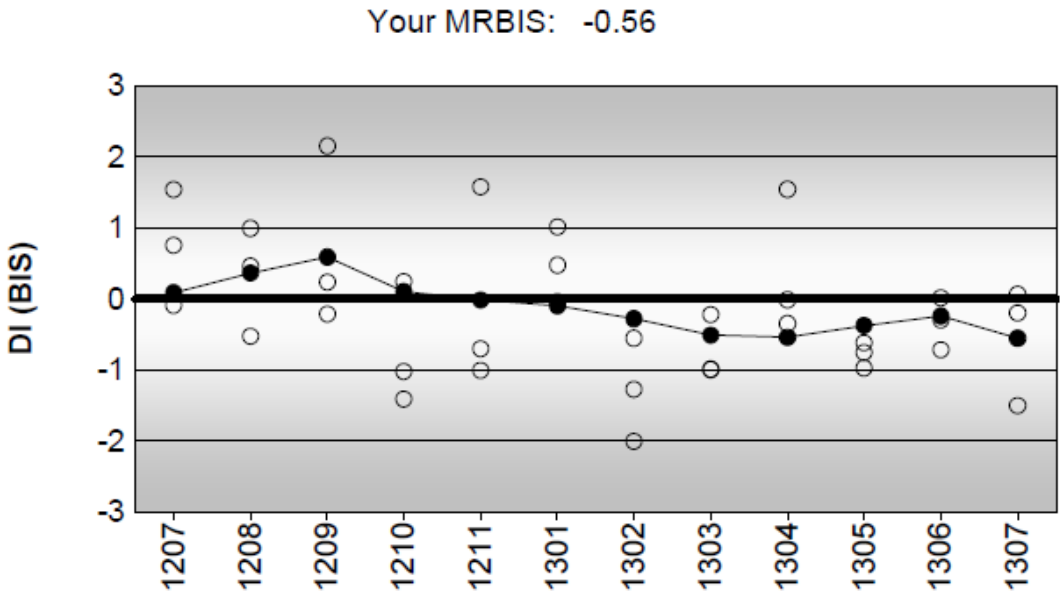


Figure P.11 Illustrative overall performance charts for UKNEQAS for Year 5 of the NDNS RP for vitamin B_{12}



P.2.4 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[4] and later adapted by other workers, including Rautela and Liedtke.[5] 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.

P.2.4.1 Internal quality controls for total cholesterol

The performance statistics in tables P.9-P.12 were calculated using data from two or three different reagent lots in order to include batch to batch variation. Table P.9 shows imprecision data produced from a typical month in Year 2 (January 2010). Table P.10 shows imprecision data produced from a combination of three typical months in Year 3 (01/09/2010-30/11/2010). Table P.11 shows imprecision data for a period covering the analysis of Year 4 samples. Table P.12 shows imprecision data for a period covering the analysis of Year 5 samples.

Table P.9 Internal quality controls for total cholesterol for Year 2 of the NDNS RP

	QC Lot No 46381	QC Lot No 46383
Mean (mmol/L)	2.35	6.53
SD	0.105	0.126
% CV	4.5	1.9
Data points included	389	147

Table P.10 Internal quality controls for total cholesterol for Year 3 of the NDNS RP

	QC Lot No 46401	QC Lot No 46403
Mean (mmol/L)	2.63	6.75
SD	0.088	0.159
% CV	3.3	2.4
Data points included	1115	1209

Table P.11 Internal quality controls for total cholesterol for Year 4 of the NDNS RP

	QC Lot No 46433
Mean (mmol/L)	6.48
SD	0.104
% CV	1.6
Data points included	141

	QC Lot No 46441	QC Lot No 46443
Mean (mmol/L)	2.65	6.69
SD	0.090	0.158
% CV	3.4	2.4
Data points included	4693	3447

	QC Lot No 46461	QC Lot No 46463
Mean (mmol/L)	2.71	6.66
SD	0.081	0.144
% CV	3.0	2.2
Data points included	732	1575

Table P.12 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
Data points included	3240	2457	471	1155

P.2.4.2 Internal quality controls for HDL cholesterol

The performance statistics in tables P.13-P.16 were calculated using data from two or three different reagent lots in order to include batch to batch variation. Table P.13 shows imprecision data produced from a typical month in Year 2 (January 2010). Table P.14 shows imprecision data produced from a combination of three typical months in Year 3 (01/09/2010-30/11/2010). Table P.15 shows imprecision data for a period covering the analysis of Year 4 samples. Table P.16 shows imprecision data for a period covering the analysis of Year 5 samples.

Table P.13 Internal quality controls for HDL cholesterol for Year 2 of the NDNS RP

	QC Lot No 46381	QC Lot No 46383
Mean (mmol/L)	0.93	2.15
SD	0.03	0.09
% CV	3.2	4.2
Data points included	232	237

Table P.14 Internal quality controls for HDL cholesterol for Year 3 of the NDNS RP

	QC Lot No 46401	QC Lot No 46403
Mean (mmol/L)	0.95	1.91
SD	0.05	0.08
% CV	4.9	4.0
Data points included	738	862

Table P.15 Internal quality controls for high density lipoprotein cholesterol for Year 4 of the NDNS RP

	QC Lot No 46433
Mean (mmol/L)	1.90
SD	0.074
% CV	3.9
Data points included	96

	QC Lot No 46441	QC Lot No 46443*
Mean (mmol/L)	0.69	1.78
SD	0.045	0.140
% CV	6.4	7.9
Data points included	2921	2328

	QC Lot No 46461	QC Lot No 46463*
Mean (mmol/L)	0.76	1.65
SD	0.029	0.101
% CV	3.8	6.1
Data points included	456	1006

*46443 and 46463 contains data obtained on material whose integrity could not be guaranteed.

Table P.16 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
Data points included	2009	276	1594	685

P.2.4.3 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 P.17 indicates the percentage deviation of results obtained by Addenbrooke's from the target concentration. These have been calculated at HNR from raw data kindly supplied by

RIQAS for Years 2 to 5 of the NDNS RP and are included with the permission of the laboratory and the Scheme organisers.

Table P.17 RIQAS results for lipid analyses - deviation from target concentration

Analyte		Year 2	Year 3	Year 4	Year 5
Cholesterol	mean % deviation	-1.65	-1.71	-2.39	-1.56
	SD	3.49	2.00	2.07	2.06
HDL-Cholesterol	mean % deviation	-4.91	0.20	-1.34	-4.31
	SD	4.11	14.48	4.89	8.63

P.2.5 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.

P.2.5.1 Internal quality controls for serum triglycerides (triacylglycerols)

The performance statistics in tables P.18-P.21 were calculated using data from two or three different reagent lots in order to include batch to batch variation. Table P.18 shows imprecision data produced from a typical month in Year 2 (January 2010). Table P.19 shows imprecision data produced from a combination of three typical months in Year 3 (01/09/2010-30/11/2010). Table P.20 shows imprecision data in Year 4. Table P.21 shows imprecision data in Year 5.

Table P.18 Internal quality controls for serum triglycerides (triacylglycerols) for Year 2 of the NDNS RP

	QC Lot No 46381	QC Lot No 46383
Mean (mmol/L)	0.73	2.28
SD	0.05	0.07
% CV	6.8	3.1
Data points included	304	297

Table P.19 Internal quality controls for serum triglycerides (triacylglycerols) for Year 3 of the NDNS RP

	QC Lot No 46401	QC Lot No 46403
Mean (mmol/L)	0.88	2.42
SD	0.04	0.06
% CV	4.3	2.6
Data points included	807	820

Table P.20 Internal quality controls for triglyceride for Year 4 of the NDNS RP

	QC Lot No 46433
Mean (mmol/L)	2.38
SD	0.050
% CV	2.1
Data points included	100
Used	01/07/2011 to 12/07/2011

	QC Lot No 46441	QC Lot No 46443
Mean (mmol/L)	1.00	2.40
SD	0.067	0.076
% CV	6.7	3.2
Data points included	3227	2289
Used	01/07/2011 to 03/07/2012	12/07/2011 to 19/04/2012

	QC Lot No 46461	QC Lot No 46463
Mean (mmol/L)	0.88	2.35
SD	0.053	0.075
% CV	6.0	3.2
Data points included	459	1046
Used	01/07/2012 to 31/08/2012	19/04/2012 to 31/08/2012

Table P.21 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

P.2.5.2 External quality controls for serum triglycerides (triacylglycerols)

External quality control was achieved through RIQAS. Table P.22 indicates the percentage deviation of results obtained by Addenbrooke's from the target concentration. These have been calculated at HNR from raw data kindly supplied by RIQAS for Years 2 to 5 of the NDNS RP and are included with the permission of the laboratory and the Scheme organisers.

Table P.22 RIQAS results for Triglycerides: deviation from target concentration

	Year 2	Year 3	Year 4	Year 5
mean % deviation	-2.90	-2.55	-2.83	-1.49
sd	5.61	5.90	4.93	7.35

P.2.6 Plasma ferritin

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.

P.2.6.1 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 P.23-P.26 indicate good between-batch consistency for ferritin results during Years 2 to 5.

Table P.23 Internal quality controls for ferritin for Year 2 of the NDNS RP

Year 2	Low	Medium	High
Mean(µg/l)	37.5	114.1	153.7
SD (µg/l)	3.0	10.2	12.3
% CV	8.1	8.9	8.0
N	15	14	15

Table P.24 Internal quality controls for ferritin for Year 3 of the NDNS RP

Year 3	Low		Medium		High	
Mean (µg/l)	37.6	38.9	98.6	92.6	155.2	140.9
SD (µg/l)	3.7	3.9	9.1	6.6	15.0	12.6
CV (%)	9.8	10.1	9.2	7.2	9.6	8.9
N	11	17	15	14	13	17

Table P.25 Internal quality controls for ferritin for Year 4 of the NDNS RP

Year 4	Low		Medium		High	
Mean (µg/l)	42.0	39.3	98.3	96.0	143.9	141.4
SD (µg/l)	3.4	7.1	6.5	3.8	6.1	6.0
CV (%)	8.1	7.1	6.6	4.0	4.3	4.2
N	11	17	17	10	17	11

Table P.26 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

P.2.6.2 External quality controls for plasma ferritin

External quality assessment was through the UK NEQAS Haematinics scheme. Figures P.12-P.15 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 All Laboratory Trimmed Mean. 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 0.22 (Year 2), -0.44 (Year 3), 0.10 (Year 4) and 0.55 (Year 5) indicate that there was good overall agreement between HNR results and the target concentrations.

Figure P.12 External quality controls for ferritin for Year 2 of the NDNS RP (filled circles represent NDNS RP results; open circles represent results obtained by other users of the same analytical method)

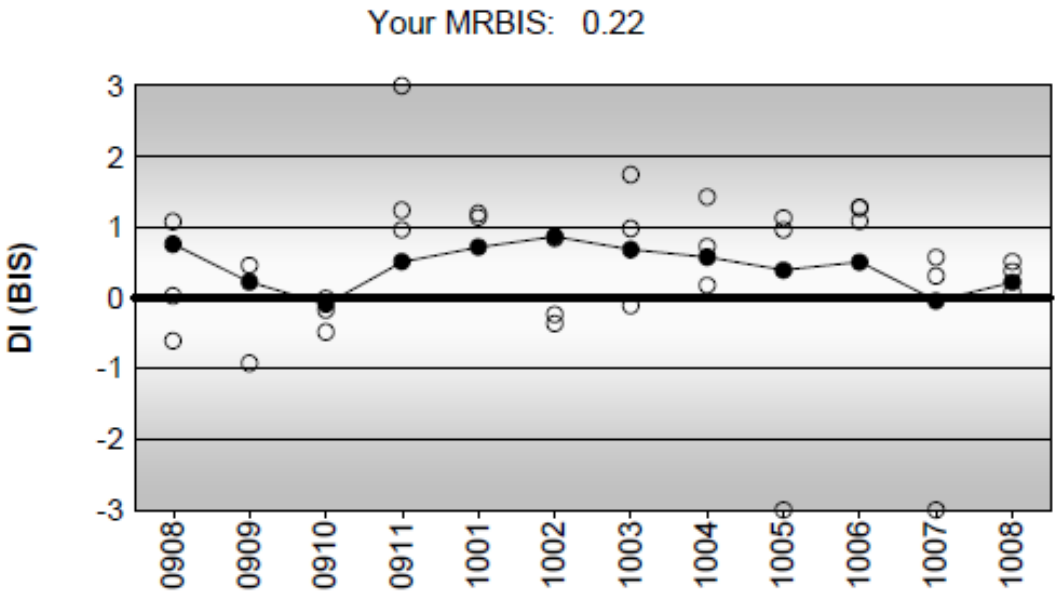


Figure P.13 External quality controls for ferritin for Year 3 of the NDNS RP (filled circles represent NDNS RP results; open circles represent results obtained by other users of the same analytical method).

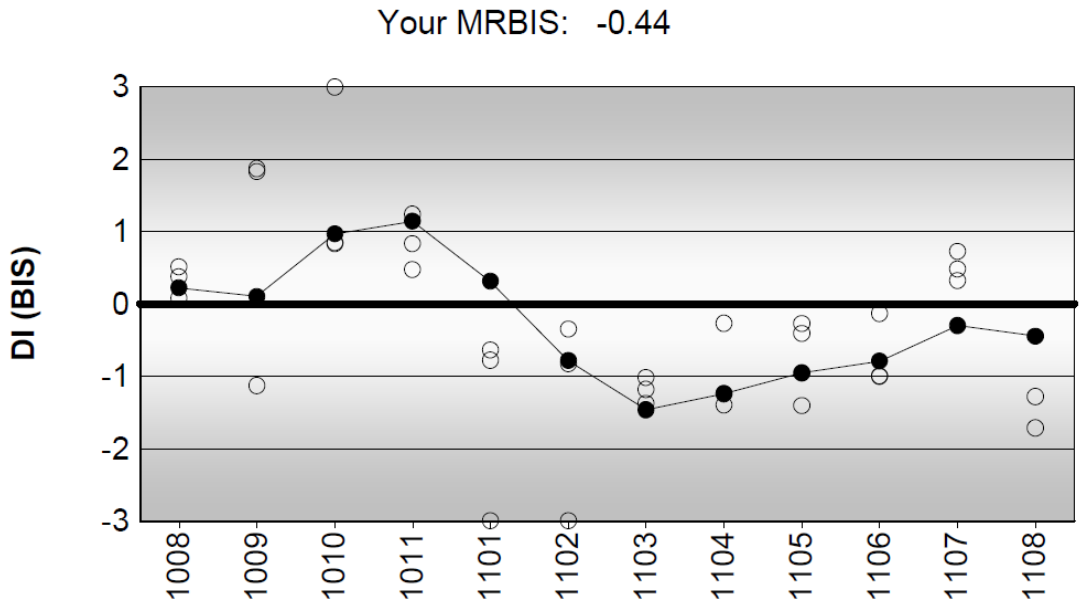


Figure P.14 External quality controls for ferritin for Year 4 of the NDNS RP (filled circles represent NDNS RP results; open circles represent results obtained by other users of the same analytical method).

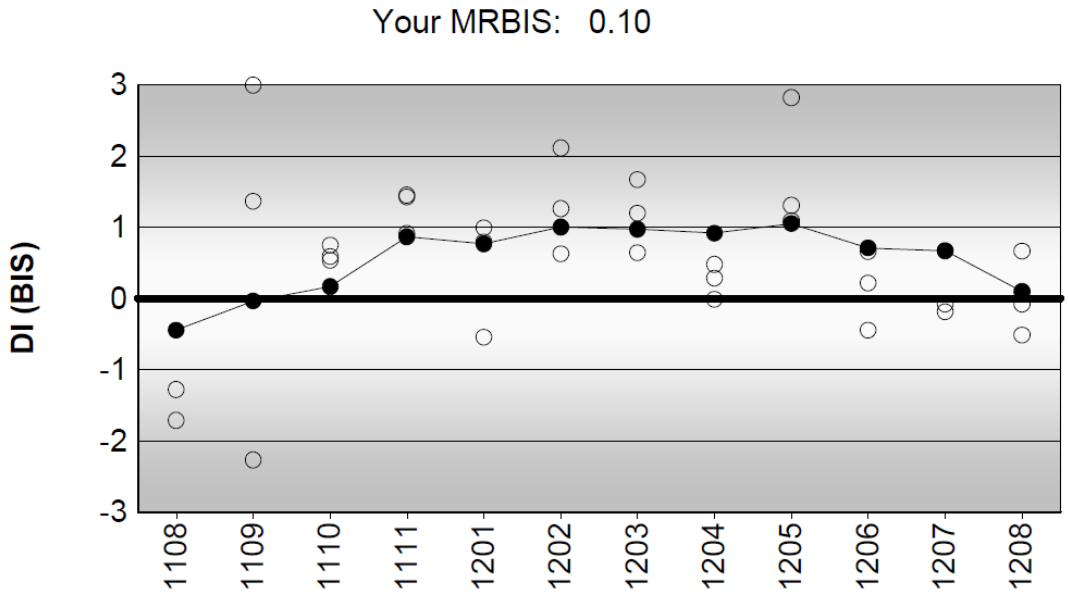
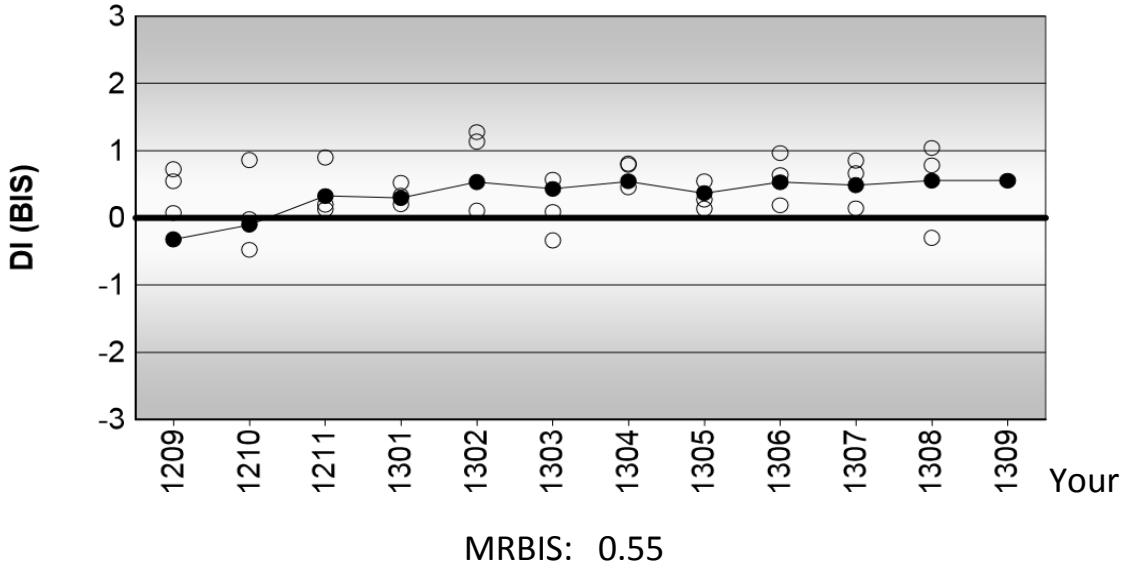


Figure P.15 External quality controls for ferritin for Year 5 of the NDNS RP (filled circles represent NDNS RP results; open circles represent results obtained by other users of the same analytical method).



P.2.7 Plasma soluble transferrin receptors (sTfR)

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.

P.2.7.1 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 tables P.27-P.30 indicate good between-batch consistency for sTfR results during Years 2 to 5.

There is no external quality assessment scheme for sTfR measurement.

Table P.27 Internal quality controls for sTfR for Year 2 of the NDNS RP

	Kit low control	Kit high control	Dade low control	Dade medium control	Dade high control	Unassayed serum
Mean (µg/ml)	5.0	15.9	2.80	4.41	5.52	8.7
SD (µg/ml)	0.22	1.11	0.16	0.21	0.27	0.42
% CV	4.4	7.0	5.7	4.9	4.9	4.8
N	35	35	30	30	31	35

Table P.28 Internal quality controls for sTfR for Year 3 of the NDNS RP

	Kit low control	Kit high control	Kit low control	Kit high control	Kit low control	Kit high control	In-house QA
Mean (µg/ml)	5.03	16.68	4.50	18.26	4.59	20.70	8.05
SD (µg/ml)	0.26	1.42	0.40	1.45	0.49	2.96	0.56
% CV	5.12	8.52	8.81	7.95	10.63	14.31	6.95
N	27	28	26	26	18	18	76

Dade controls were discontinued during the year.

Table P.29 Internal quality controls for sTfR for Year 4 of the NDNS RP

	Kit low control	Kit high control	In-house QA
Mean (µg/ml)	4.51	17.22	7.41
SD (µg/ml)	0.42	1.58	0.53
% CV	9.4	9.2	7.2
N	50	50	50

Table P.30 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

P.2.8 Plasma vitamin C

This assay is based on the procedure described by Vuilleumier and Keck.[6] 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.

*P.2.8.1 Internal quality controls for plasma vitamin C*QC samples were made in-house by spiking ascorbic acid-depleted plasma. The results in tables P.31-P.34 indicate good between-batch consistency for vitamin C (ascorbic acid) measurements during Years 2 to 5.

Table P.31 Internal quality controls for vitamin C for Year 2 of the NDNS RP

Vitamin C	µmol/L	
	QC 2	QC 3
Mean	28.0	51.0
SD	3.8	6.2
% CV	13.4	12.0
N	80	80

Table P.32 Internal quality controls for vitamin C for Year 3 of the NDNS RP

Vitamin C	µmol/L	
	QC2	QC3
mean (µmol/L)	28.1	52.6
sd (µmol/L)	2.5	2.9
cv (%)	9.0	5.6
N	68	68

Table P.33 Internal quality controls for vitamin C for Year 4 of the NDNS RP

Vitamin C	µmol/L	
	QC2	QC3
mean (µmol/L)	24.9	51.8
sd (µmol/L)	1.6	2.3
cv (%)	6.6	4.5
N	78	78

Table P.34 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

P.2.8.2 External quality controls for vitamin C

HNR subscribes to the NIST 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 P.16 External quality controls for vitamin C for Year 2 of the NDNS RP. Comparison of Measurements to "Interlaboratory Consensus Values"

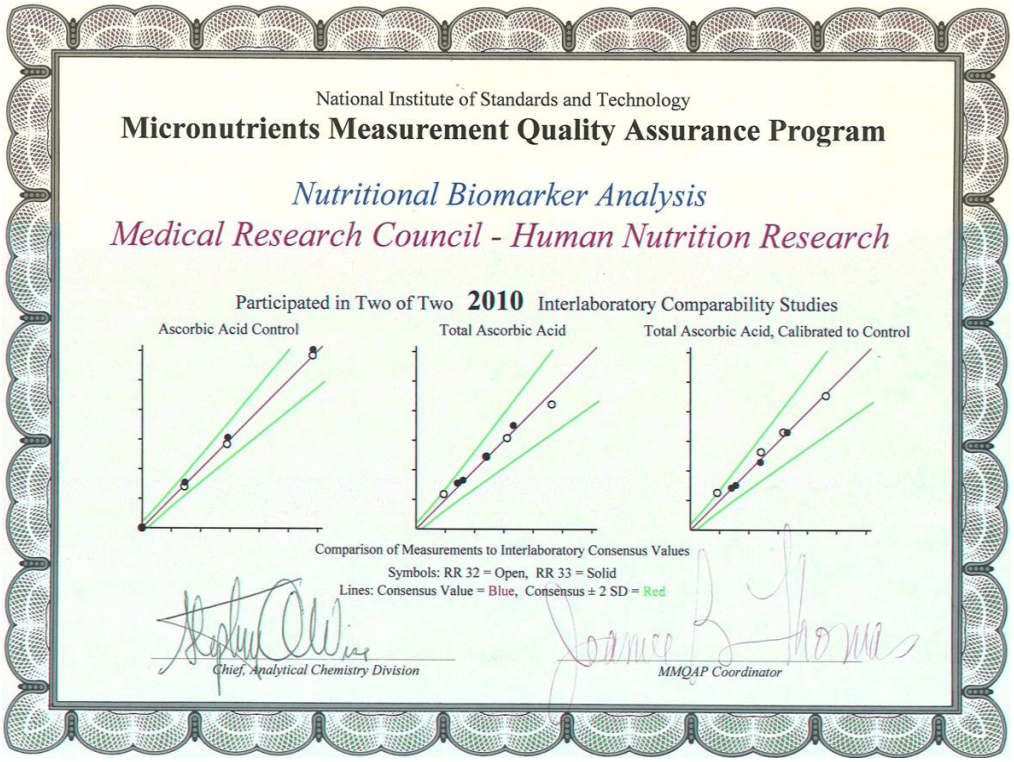


Figure P.17 External quality controls for vitamin C for Year 3 of the NDNS RP. Comparison of Measurements to "Interlaboratory Consensus"

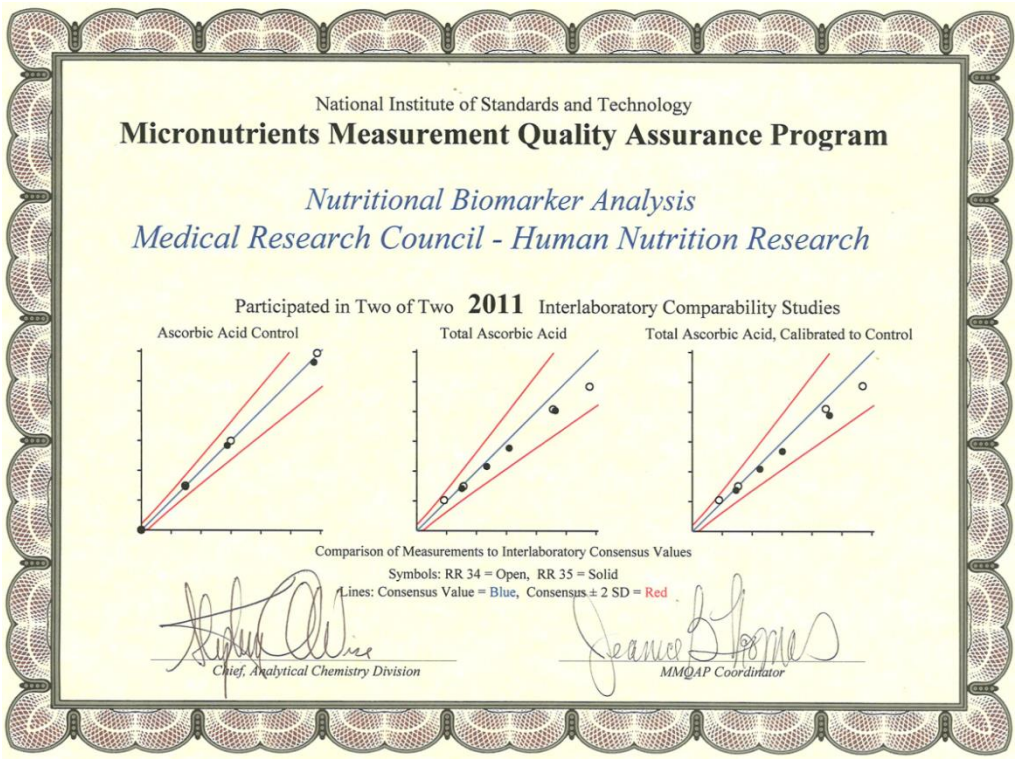
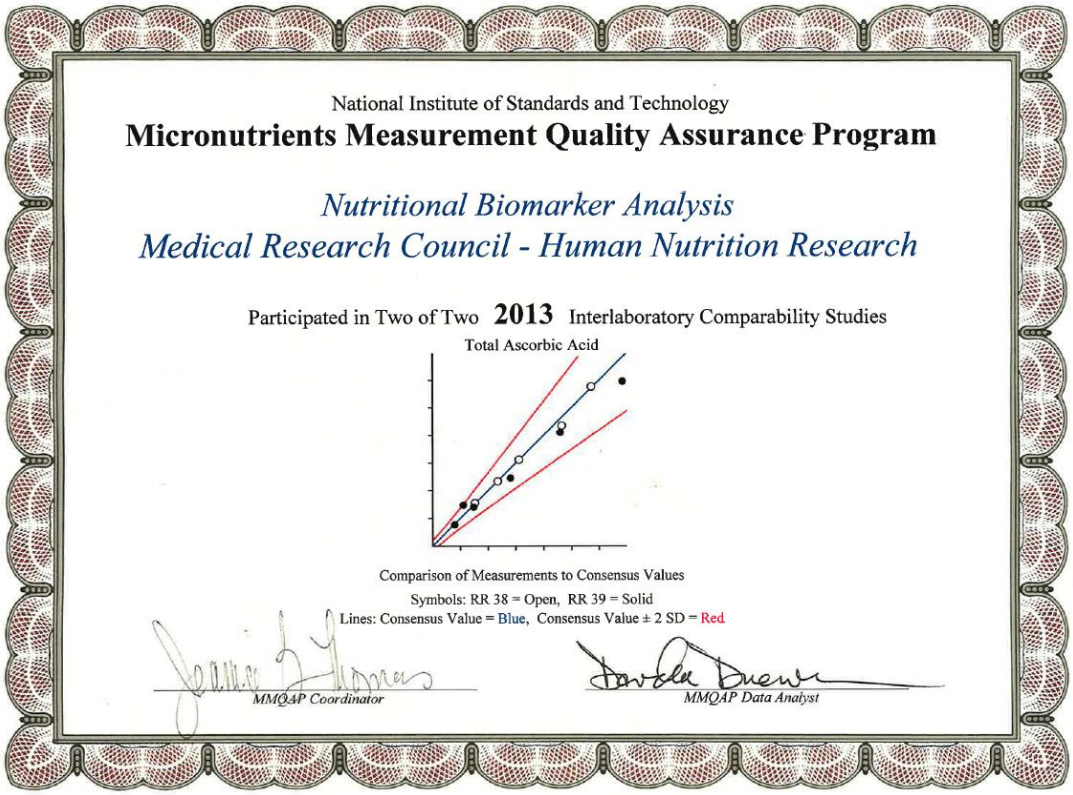


Figure P.18 External quality controls for vitamin C for Year 4 of the NDNS RP. Comparison of Measurements to "Interlaboratory Consensus"

Figure P.19 External quality controls for vitamin C for Year 5 of the NDNS RP. Comparison of Measurements to "Interlaboratory Consensus"



P.2.9 Erythrocyte transketolase activation coefficient (ETKAC) for thiamin status

This assay is based on that of Vuilleumier *et al*[7] 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).

P.2.9.1 Quality control results for ETKAC

Descriptive statistics in tables P.35-P.38 for internal QCs indicate good batch-to-batch consistency of ETKAC results during Years 2 to 5.

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

Table P.35 Internal quality controls for ETKAC for Year 2 of the NDNS RP

Control ID	Scipac B	UK NBTS neat	UK NBTS, diluted x 2
Mean	1.07	1.02	1.03
;SD	0.06	0.07	0.04
% CV	5.4	6.8	3.6
N	29	29	29

Table P.36 Internal quality controls for ETKAC for Year 3 of the NDNS RP

Control ID	Scipac B	UK NBTS neat	UK NBTS, diluted x 2	Scipac A	Scipac C	Scipac A diluted x2
Mean	1.05	1.03	1.06	1.11	1.08	1.11
SD	0.04	0.03	0.03	0.07	0.03	0.07
% CV	3.66	2.86	2.37	6.20	3.12	6.12
N	22	30	30	26	26	25

Table P.37 Internal quality controls for ETKAC for Year 4 of the NDNS RP

Control ID	Scipac A	Scipac C	Scipac A diluted x2	Scipac P	Scipac Q
mean	1.08	1.09	1.11	1.05	1.15
sd	0.06	0.04	0.05	0.04	0.05
cv	5.79	3.30	4.86	3.71	4.44
n	37	38	30	26	26

Table P.38 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

P.2.10 Erythrocyte glutathione reductase activation coefficient (EGRAC) for riboflavin status

This assay was developed from the original manual technique developed by Glatzle *et al*[8] 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.

P.2.10.1 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.

P.2.10.1.1 Internal quality control results during Years 2 to 5

Descriptive statistics in tables P.39-P.42 for internal QCs indicate good batch-to-batch consistency of EGRAC results during Years 2 to 5.

Table P.39 Internal quality controls for EGRAC for Year 2 of the NDNS RP

Control ID	A	C	X
Mean	2.28	1.60	0.99
SD	0.15	0.06	0.02
% CV	6.4	3.6	2.3
N	33	34	34

Table P.40 Internal quality controls for EGRAC for Year 3 of the NDNS RP

Control ID	A	C	X
Mean	2.27	1.63	1.00
SD	0.10	0.03	0.02
% CV	4.23	2.14	1.95
N	71	69	72

Table P.41 Internal quality controls for EGRAC for Year 4 of the NDNS RP

Control ID	A	C	X
Mean	2.30	1.61	0.99
SD	0.08	0.03	0.01
% CV	3.60	2.16	1.44
N	37	40	40

Table P.42 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

P.2.11 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.[9]

P.2.11.1 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.

P.2.11.1.1 Internal quality controls for vitamin B_6

The basal and spiked plasma used to calculate analytical recovery for each assay were also used to determine assay precision (Years 3 and 4 only), where concentrations were appropriate. However for Year 2 and the beginning of Year 3 the unspiked plasma contained a concentration of PLP and PA close to the limit of quantitation and too low for determining precision; any samples containing similarly low concentrations of PLP and PA were re-assayed at double volume to permit quantitation. Therefore this unspiked plasma cannot be used to determine precision and only the spiked QC results are available for this time period.

QC results for unspiked plasma have been corrected for analytical recovery where this correction was also performed for the unknown samples in that batch.
QC results for spiked plasma have not been so corrected; as the spike recovery was used to define the correction factor (if any) for that batch, using this factor to correct the concentration measured in the same spiked control would be inappropriate. Therefore the imprecision shown for spiked controls is likely to overestimate the imprecision applicable to measured concentrations of unknown samples.
From Year 5 new quality procedures were implemented. 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 survey. 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 P.43-P.44) and the expected values indicate a quantitative recovery of vitamin B_6 in this assay.

Table P.43 Internal quality controls for PLP and PA (unspiked plasma) for Years 3 (part), 4 and 5 of the NDNS RP[1]

PLP	Year 3 (part)	Year 4	Year 5	
Plasma	K4082310	K4082310	A07	A02
Mean (nmol/L)	11.0	10.5	23.9	49.3
SD	2.0	0.9	2.0	2.0
% CV	18.4	8.9	8.1	4.0
N	24	48	27	28

[1] *no data available for earlier assays, see above*

PA	Year 3 (part)	Year 4	Year 5	
Plasma	K4082310	K4082310	K4082310	A02
Mean (nmol/L)	34.8	34.3	36.7	123.7
SD	1.8	2.0	1.3	4.1
% CV	5.0	5.9	3.4	3.3
N	24	48	28	27

Table P.44 Internal quality controls for PLP and PA (spiked plasma) for Years 2 to 5 of the NDNS RP

PLP	Year 2	Year 3	Year 4	Year 5
Mean (nmol/L)	44.1	45.9	44.9	47.9
SD	3.0	4.3	2.1	1.7
% CV	6.8	9.4	4.8	3.5
n	36	36	48	28

PA	Year 2	Year 3	Year 4	Year 5
Mean (nmol/L)	52.3	53.8	54.9	58.0
SD	1.9	3.1	3.3	2.2
% CV	3.7	5.8	6.1	3.9
n	35	36	48	28

Note that for Years 2 to 4 the expected PLP concentration of the spiked plasma was 43nmol/L (the sum of the basal level in the plasma plus the spike concentration). The expected PA concentration of the spiked plasma was 51nmol/L (the sum of the basal level in the plasma plus the spike concentration).

For Year 5 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.

P.2.12 Plasma total homocysteine

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.

P.2.12.1 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.

P.2.12.1.1 Internal quality controls for plasma total homocysteine

The results in tables P.45-P.48 indicate good between-batch consistency for homocysteine results during Years 2 to 5.

Table P.45 Internal quality controls for homocysteine for Year 2 of the NDNS RP

Year 2	Low	Medium	High
Mean (µmol/l)	7.6	11.9	24.3
SD (µmol/l)	0.5	1.0	1.5
% CV	6.9	8.8	6.0
N	11	12	13

Table P.46 Internal quality controls for homocysteine for Year 3 of the NDNS RP

Year 3	Low			Medium		High		
Mean (µmol/l)	7.2	6.5	7.0	10.8	12.0	25.2	23.8	24.1
SD (µmol/l)	0.8	0.5	0.7	1.1	1.1	2.0	1.7	2.6
% CV	11.0	7.5	9.6	9.7	8.8	7.8	7.2	10.7
N	11	7	12	9	10	11	7	12

Table P.47 Internal quality controls for homocysteine for Year 4 of the NDNS RP

Year 4	Low			Medium			High		
Mean (µmol/l)	7.7	8.5	7.0	12.1	11.6	12.9	26.1	25.0	24.1
SD (µmol/l)	0.8	0.8	0.6	1.6	1.6	1.2	2.4	3.4	1.7
% CV	10.0	8.9	8.5	13.5	13.6	9.0	9.1	13.7	6.9
N	3	8	16	3	13	11	3	13	11

Table P.48 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

P.2.13 Plasma retinol, α– and γ–tocopherol, and individual carotenoids: Years 2 to 4

The methodology for these analytes was changed between Years 4 and 5. Conversion factors were determined for each analyte in order that data generated in Years 2 to 5 could be combined for analysis and to facilitate comparisons of population statistics between years. Data from Years 2, 3, 4 were converted using these factors to the equivalent result which would have been given by the Year 5 method (see section P.2.13.2 for detail of the Year 5 methodology). Internal and external quality control data below have not been so converted.

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 Thurnham *et al.*[10] 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. An internal standard of tocopherol acetate was used to monitor losses during the extraction process and to account for any changes in volumes.

The analytical methods used in the current RP were essentially the same as that used in all previous NDNS surveys. However it should be noted that differences in calibration techniques and in the application of extraction efficiency corrections may mean that it is not possible to directly compare the results of this survey with previous NDNS surveys.

P.2.13.1 Quality controls for plasma retinol, α– and γ–tocopherol and individual carotenoids

The nature of these assays, which measure ten analytes in a single HPLC run, means that achieving good accuracy and precision are a greater challenge than in a single-analyte assay. Therefore in order to get the best results possible more quality processes are needed than for the other assays.

Heparinised human plasma from a commercial source (e.g. Seralab International, UK) was run in duplicate for every batch of samples. To check the extraction efficiency of the method a sample of this plasma was spiked with a known concentration of each fat soluble vitamin analyte. This spiking was performed freshly, immediately before sample preparation for each analytical batch.

Following extraction, the concentration of each fat soluble vitamin component was determined (duplicates) in both unspiked and fortified plasma and the percentage recovery for each spike determined from the mean concentrations, by subtraction. Participant results in any batch where the extraction efficiency was different from 100% were corrected to allow for this.

Acceptable extraction efficiency was based on ranges established in this laboratory.

Table P.49 Extraction efficiencies for fat soluble vitamins (FSV) assays for Years 2 to 5 of the NDNS RP

Extraction Efficiencies - FSV assays (%)				
Analyte		**Year 2 n=16**	**Year 3 n=15**	**Year 4 n=18**
retinol	Mean	93	108	95
	sd	16	13	10
α- tocopherol	Mean	96	92	102
	sd	19	8	12
γ-tocopherol	Mean	90	93	94
	sd	14	9	12
Lutein + zeaxanthin	Mean	88	104	96
	sd	19	14	9
α-cryptoxanthin	Mean	83	84	78
	sd	16	6	10
β-cryptoxanthin	Mean	84	84	91
	sd	7	5	10
lycopene	Mean	99	78	83
	sd	21	23	8
α-carotene	Mean	83	69	63
	sd	20	11	5
β-carotene	Mean	78	69	66
	sd	12	10	6

The measured concentrations in the unspiked and spiked samples for each assay have retrospectively been compared to give an approximate indication of assay imprecision for each year, where the concentrations are high enough for this to be valid. The difficulties inherent in spiking plasma with analytes which are not water-soluble precluded spiking in bulk and freezing aliquots for multiple assays, and therefore conventional assay imprecision data are not available .

Participation in studies conducted by NIST, CDC VITAL-External Quality Assurance (CDC VITAL EQA) and UKNEQAS allowed inter-laboratory comparison of results. HNR also participated in a twice yearly “round robin” with both NIST and VITAL

EQA. At the start of the NDNS RP NEQAS samples were received bi-monthly; the frequency was later increased to monthly samples. 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. In such cases the charts are not presented in this report.

P.2.13.1.1 Internal quality controls for plasma retinol, α– and γ–tocopherol and individual carotenoids

The FSV results for Years 2 to 4 were reported as plasma retinol, α– and γ–tocopherol and individual carotenoids (Lutein and zeaxanthin co-elute and therefore are measured as a sum).

Tables P.50-P.54 show, for each analyte, the precision of measurement of unspiked plasma and of the individually spiked plasma samples used for recovery efficiency calculation for each batch. These are presented in addition to the precision of spike recovery (tables P.55-P.58) which has been included in previous NDNS RP reports.

The unspiked and spiked controls were selected and included in each batch for accuracy assessment and have retrospectively been tabulated for the purposes of precision monitoring. In some cases the concentrations of unspiked plasma are extremely low and are not suitable for this purpose.

Control concentrations are "as measured"; they have not been corrected for extraction efficiency. They therefore represent an overestimate of the imprecision of FSV concentrations in NDNS RP samples. Also, as mentioned above, the *de novo* preparation of the spiked samples individually for each assay will inevitably have contributed to the overall imprecision.

Table P.50 Unspiked plasma results for plasma retinol, α– and γ– tocopherol and individual carotenoids for Year 2 of the NDNS RP (new QC plasma batch)

	Retinol (µmol/L)	α-tocopherol (µmol/L)	γ-tocopherol (µmol/L)	α-carotene (µmol/L)
Mean	1.44	20.57	3.62	0.08*
SD	0.14	1.69	0.76	0.02
% CV	9.7	8.2	20.9	23.3
N	9	8	8	7

**concentration similar to the 2.5th percentile of NDNS RP results*

	β-carotene (µmol/L)	α-cryptoxanthin (µmol/L) [1]	β-cryptoxanthin (µmol/L)	Lycopene (µmol/L)	Lutein + zeaxanthin(µmol/L)
Mean	0.27*	-	0.09	0.46	0.30
SD	0.09	-	0.02	0.07	0.03
% CV	33.1	-	17.8	16.1	8.6
N	7	-	7	7	7

[1] *No value is given for α-cryptoxanthin in unspiked plasma because the concentrations are too low for reliable quantitation, and are lower than the 2.5th percentile of NDNS RP results.*

**concentration similar to the 2.5th percentile of NDNS RP results*

Table P.51 Unspiked Plasma results for plasma retinol, α– and γ– tocopherol and individual carotenoids for Year 3 of the NDNS RP

	Retinol (µmol/L)	α-tocopherol (µmol/L)	γ-tocopherol (µmol/L)	α-carotene (µmol/L)
Mean	1.57	17.1	4.82	0.05
SD	0.19	2.25	1.42	0.02
% CV	12.2	13.1	29.4	47.4
N	15	15	15	15

	β-carotene (µmol/L)	α-cryptoxanthin (µmol/L) [1]	β-cryptoxanthin (µmol/L)	Lycopene (µmol/L)	Lutein + zeaxanthin (µmol/L)
Mean	-	-	0.06	0.22*	0.23*
SD	-	-	0.01	0.12	0.07
% CV	-	-	21.0	53.4	32.3
N	-	-	15	15	15

[1] No value is given for α-cryptoxanthin or β-carotene in unspiked plasma because the concentrations are too low for reliable quantitation, and are lower than the 2.5th percentile of NDNS RP results.

** concentration similar to the 2.5th percentile of NDNS RP results*

Table P.52 Unspiked Plasma results for plasma retinol, α– and γ– tocopherol and individual carotenoids for Year 4 of the NDNS RP

	Retinol (µmol/L)	α-tocopherol (µmol/L)	γ-tocopherol (µmol/L)	α-carotene (µmol/L)
Mean	1.36	19.1	3.31	
SD	0.11	1.31	0.29	
% CV	8.1	6.9	8.6	
N	18	18	18	

	β-carotene (µmol/L)	α-cryptoxanthin (µmol/L) [1]	β-cryptoxanthin (µmol/L)	Lycopene (µmol/L)	Lutein + zeaxanthin (µmol/L)
Mean	-	-	0.034	0.18*	0.15*
SD	-	-	0.02	0.08	0.03
% CV	-	-	49.6	44.9	21.0
N	-	-	18	18	18

[1] No value is given for β-carotene or α-cryptoxanthin in unspiked plasma because the concentrations are too low for reliable quantitation, and are lower than the 2.5th percentile of NDNS RP results.
**concentration similar to the 2.5th percentile of NDNS RP results*

Table P.53 Precision of concentration measurement in individually-spiked recovery samples - plasma retinol, α– and γ– tocopherol and individual carotenoids for Year 2 of the NDNS RP

	Retinol (µmol/L)	α-tocopherol (µmol/L)	γ-tocopherol (µmol/L)	α-carotene (µmol/L)
Mean	3.95	37.2	7.84	0.245
SD	0.28	1.85	0.64	0.048
% CV	7.2	5.0	8.2	19.7
N	18	15	16	14

	β-carotene (µmol/L)	α-cryptoxanthin (µmol/L)	β-cryptoxanthin (µmol/L)	Lycopene (µmol/L)	Lutein + zeaxanthin (µmol/L)
Mean	0.781781	0.158	0.208	0.85	0.604
SD	0.139	0.028	0.019	0.15	0.069
% CV	17.8	17.7	9.1	17.6	11.3
N	14	14	14	14	14

Table P.54 Precision of concentration measurement in individually-spiked recovery samples - plasma retinol, α– and γ– tocopherol and individual carotenoids for Year 3 of the NDNS RP

	Retinol (µmol/L)	α-tocopherol (µmol/L)	γ-tocopherol (µmol/L)	α-carotene (µmol/L)
Mean	4.92	34.4	9.19	0.177
SD	0.80	3.18	1.43	0.039
% CV	16.2	9.2	15.5	22
N	25	28	28	27

	β-carotene (µmol/L)	α-cryptoxanthin (µmol/L)	β-cryptoxanthin (µmol/L)	Lycopene (µmol/L)	Lutein (µmol/L)
Mean	0.676	0.143	0.184	0.505	0.626
SD	0.132	0.02	0.019	0.177	0.104
% CV	19.6	13.8	10.2	35.1	16.7
N	30	30	30	30	30

Table P.55 Precision of concentration measurement in individually-spiked recovery samples - plasma retinol, α– and γ– tocopherol and individual carotenoids for Year 4 of the NDNS RP

	Retinol (µmol/L)	α-tocopherol (µmol/L)	γ-tocopherol (µmol/L)	α-carotene (µmol/L)
Mean	4.28	36.7	7.81	0.133
SD	0.53	3.09	0.754	0.019
% CV	12.5	8.4	9.7	13.9
N	36	36	36	36

	β-carotene (µmol/L)	α-cryptoxanthin (µmol/L)	β-cryptoxanthin (µmol/L)	Lycopene (µmol/L)	Lutein + zeaxanthin (µmol/L)
Mean	0.604	0.118	0.166	0.495	0.491
SD	0.088	0.022	0.025	0.084	0.058
% CV	14.5	18.2	14.8	16.9	11.9
N	36	35	36	36	36

Table P.56 Precision of recovered spike for plasma retinol, α– and γ– tocopherol and individual carotenoids for Year 2 of the NDNS RP

	Retinol (µmol/L)	α-tocopherol (µmol/L)	γ-tocopherol (µmol/L)	α-carotene (µmol/L)
Mean	2.50	16.64	4.11	0.15
SD	0.05	0.85	0.17	0.04
% CV	2.0	5.1	4.2	23.7
N	8	6	6	15

	β-carotene (µmol/L)	α-cryptoxanthin (µmol/L)	β-cryptoxanthin (µmol/L)	Lycopene (µmol/L)	Lutein + zeaxanthin (µmol/L)
Mean	0.59	0.12	0.12	0.37	0.31
SD	0.07	0.03	0.01	0.07	0.05
% CV	12.4	22.9	8.9	17.9	16.1
N	15	13	15	15	15

Table P.57 Precision of recovered spike for plasma retinol, α– and γ– tocopherol and individual carotenoids for Year 3 of the NDNS RP

	Retinol (µmol/L)	α-tocopherol (µmol/L)	γ-tocopherol (µmol/L)	α-carotene (µmol/L)
Mean	3.48	16.74	4.6	0.13
SD	0.49	1.66	0.45	0.02
% CV	14.1	9.9	9.7	17.8
N	12	13	13	14

	β-carotene (µmol/L)	α-cryptoxanthin (µmol/L)	β-cryptoxanthin (µmol/L)	Lycopene (µmol/L)	Lutein + zeaxanthin (µmol/L)
Mean	0.5	0.12	0.12	0.28	0.39
SD	0.07	0.01	0.01	0.08	0.10
% CV	14.1	11.0	6.8	28.7	25.4
N	14	14	14	14	14

Table P.58 Precision of recovered spike for plasma retinol, α– and γ– tocopherol and individual carotenoids for Year 4 of the NDNS RP

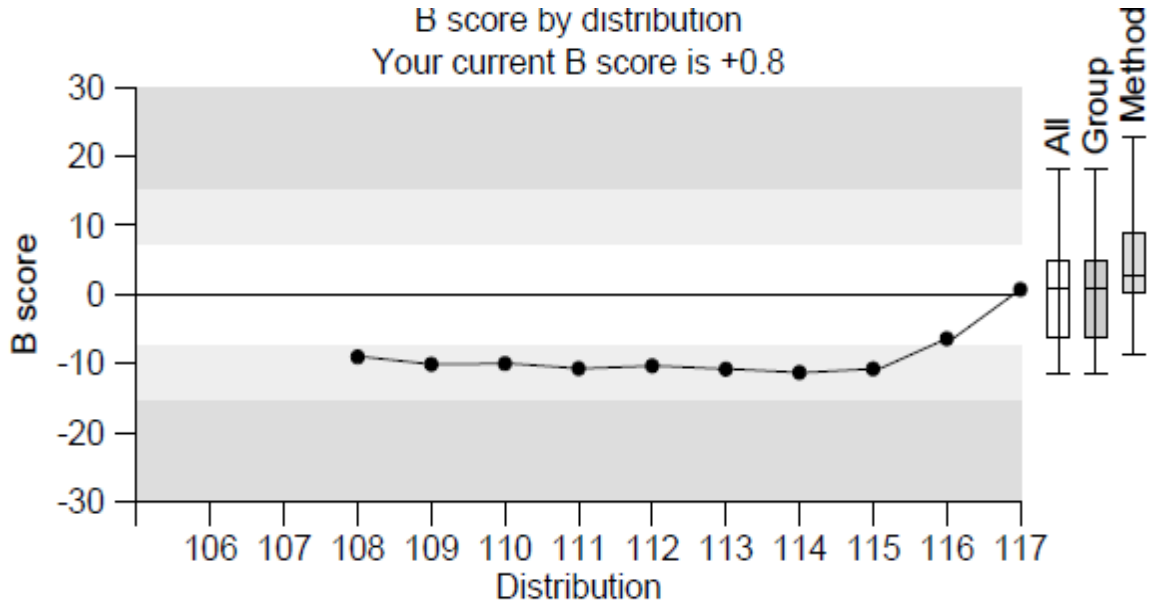
	Retinol (µmol/L)	α-tocopherol (µmol/L)	γ-tocopherol (µmol/L)	α-carotene (µmol/L)
Mean	2.92	17.60	4.51	0.12
SD	0.38	2.12	0.55	0.01
% CV	13.1	12.0	12.2	8.0
n	18	18	18	18

	β-carotene (µmol/L)	α-cryptoxanthin (µmol/L)	β-cryptoxanthin (µmol/L)	Lycopene (µmol/L)	Lutein + zeaxanthin (µmol/L)
Mean	0.50	0.11	0.13	0.31	0.34
SD	0.05	0.01	0.01	0.04	0.03
% CV	9.6	12.9	10.5	12.1	9.6
n	18	18	18	18	18

P.2.13.1.2 External quality controls for plasma retinol, α– and γ–tocopherol and individual carotenoids

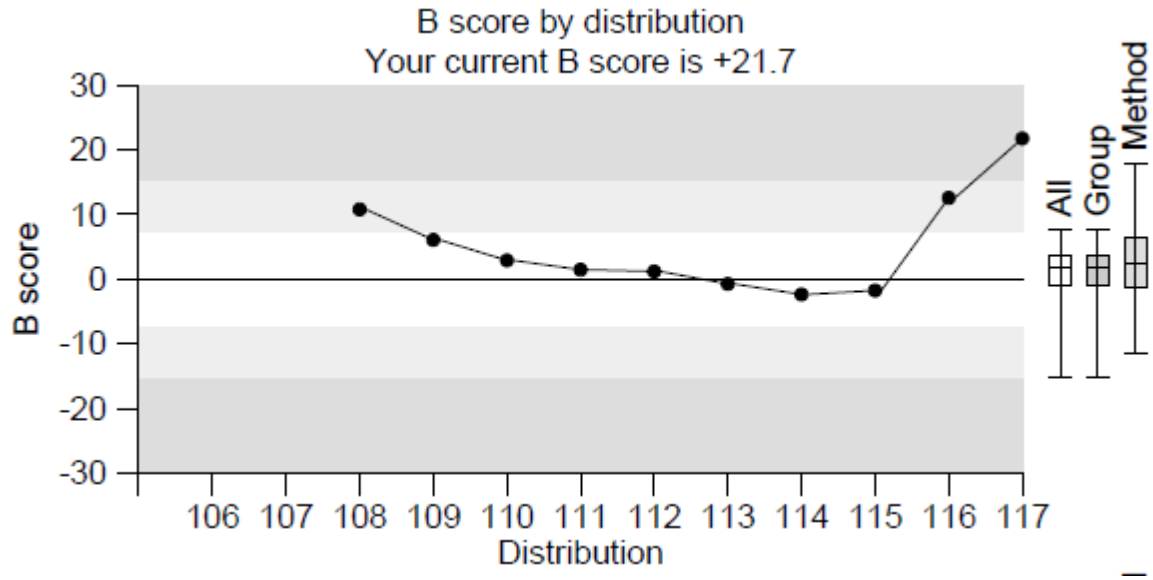
Figures P.20-P.25 shows bias scores for UKNEQAS returns during the period covering Years 2 to 4 of the NDNS RP. Percentage bias was calculated as (result-target)/target*100 and was calculated as a rolling average by UKNEQAS. The results from the external quality schemes shown in Figures P.21-P.25 suggest that HNR is typically within the range for each FSV analyte. For the purpose of this report retinol and α-tocopherol are represented by the vitamin A and vitamin E UKNEQAS returns respectively. Data are only presented where the number of participating laboratories exceeds 10; this excludes carotenoids other than β-carotene.

Figure P.20 NDNS RP Year 2 NEQAS bias score data for retinol (40 labs in scheme)



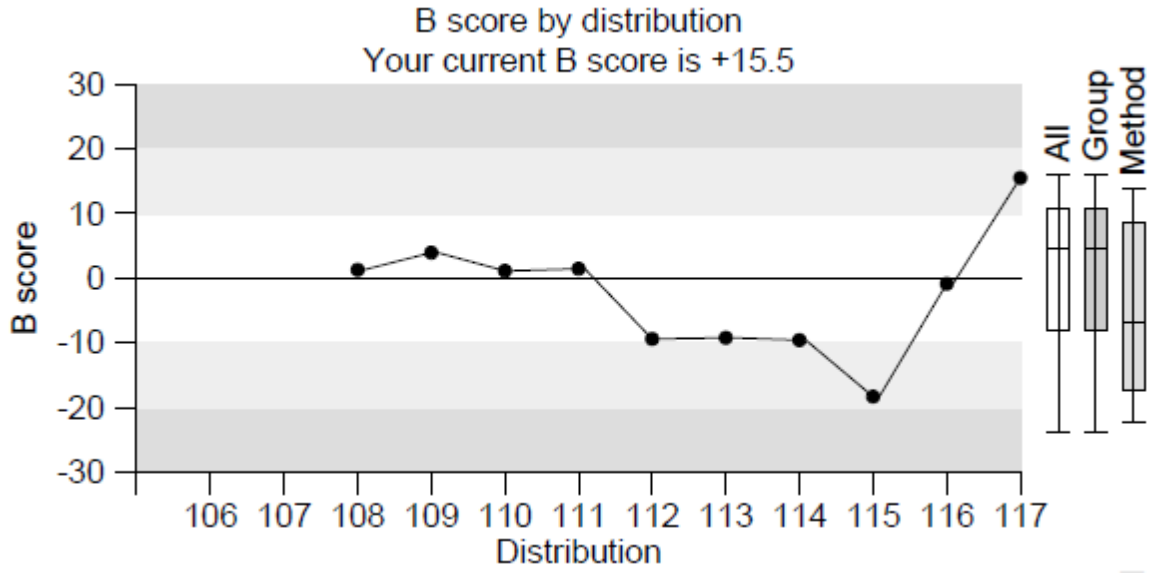
Distribution 112 to 116 covers NDNS Year 2

Figure P.21 NDNS RP Year 2 NEQAS bias score data for vitamin E (alpha-tocopherol) (40 labs in scheme)



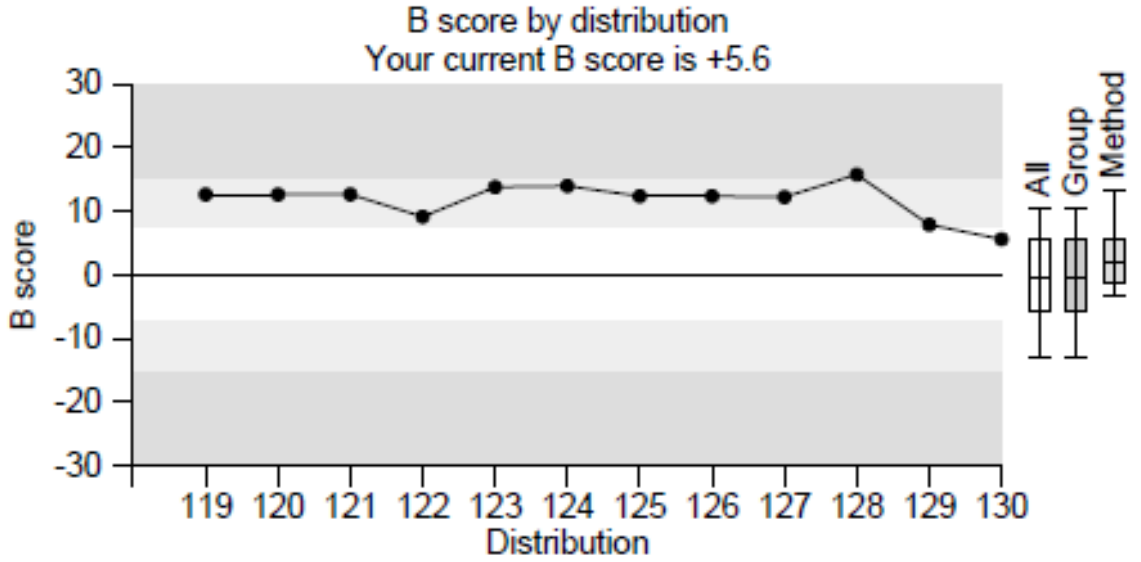
Distribution 112 to 116 covers NDNS RP Year 2

Figure P.22 NDNS RP Year 2 NEQAS bias score data for beta-carotene (19 labs in scheme)



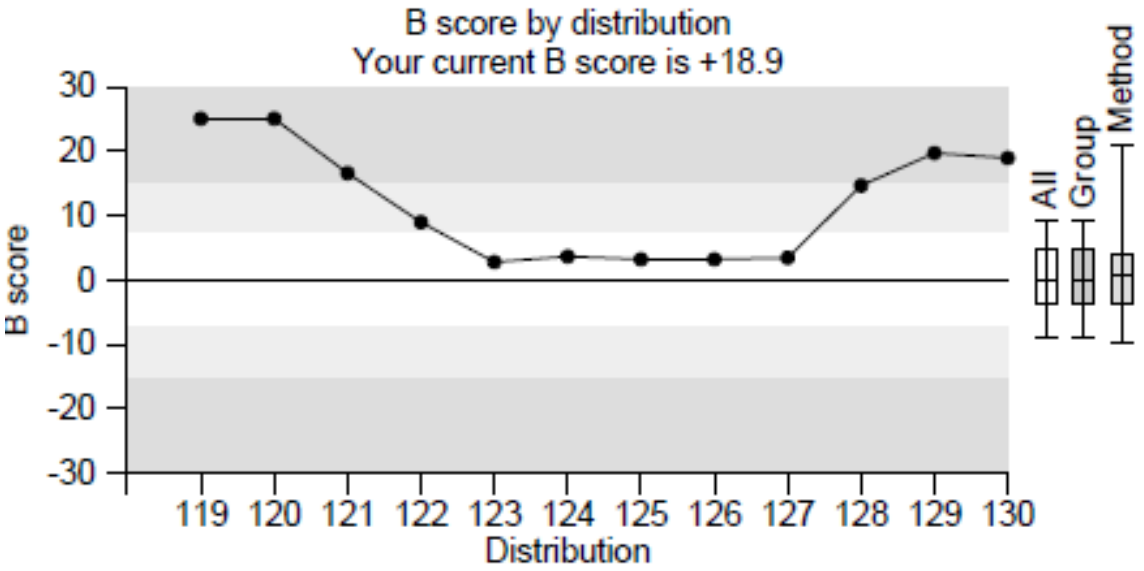
Distribution 112 to 116 covers NDNS year 2

Figure P.23 NDNS RP Years 3 and 4 NEQAS bias score data for retinol (45 labs in scheme)



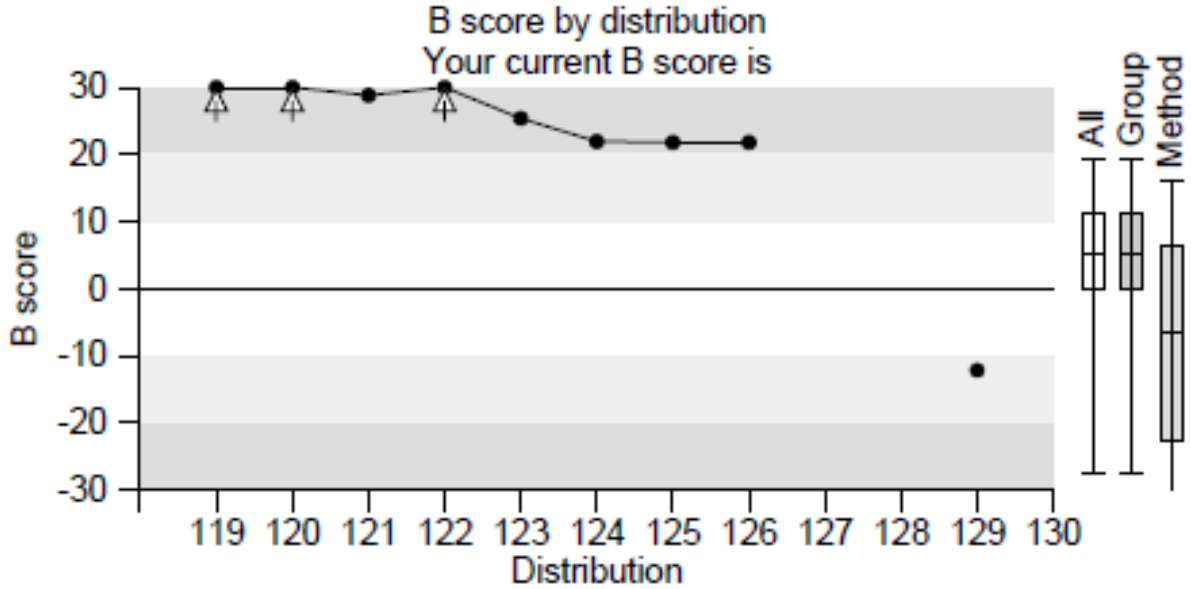
Distribution 119 to 121 covers NDNS Year 3; distribution 128 to 130 covers NDNS Year 4

Figure P.24 NDNS RP Years 3 and 4 NEQAS bias score data for vitamin E (alpha-tocopherol) (45 labs in scheme)



Distribution 119 to 121 covers NDNS Year 3; distribution 128 to 130 covers NDNS Year 4

Figure P.25 NDNS RP Years 3 and 4 NEQAS bias score data for beta-carotene (18 labs in scheme)



Distribution 119 to 121 covers NDNS Year 3; distribution 128 to 130 covers NDNS Year 4

P.2.13.2 Plasma retinol, α– and γ–tocopherol and individual carotenoids: Year 5

Internal development work undertaken after the Year 4 samples were analysed but before starting the Year 5 analysis has improved the robustness, accuracy and precision of the assay.

The method used in Year 5 is similar in principle to that used in previous years of the RP, see section P.2.13.1. The main changes are that phase separation was improved by snap-freezing, the reconstitution procedure was modified to ensure complete re-solubilisation of all analytes, and a secondary internal standard (Apo-8-carotenal) was included in the extraction procedure.

Because of the achievement of extraction efficiency close to 100% for all analytes the correction of results for recovery was no longer required, and assessment of this using spiked plasma was discontinued. Between-batch precision controls were introduced. Conversion factors were determined for each analyte in order that data from Years 2 to 5 can be combined. Data from Years 2 to 4 were converted using these factors to the equivalent result which would have been given by the Year 5 method.

P.2.13.2.1 Quality controls for plasma retinol, α– and γ–tocopherol and individual carotenoids

P.2.13.2.1.1 Internal quality controls for plasma retinol, α– and γ–tocopherol and individual carotenoids

The FSV results for Year 5 were reported as plasma retinol,α– and γ–tocopherol and individual carotenoids (Lutein and zeaxanthin co-elute and therefore are measured as a sum).

Following method revision to remove the causes of poor extraction efficiency, there was no necessity to assess spike recovery on each occasion. Instead, 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 NDNS RP. Accuracy was determined using the external quality assessment scheme led by NIST with UKNEQAS returns as corroboration, see section 2.13.2.1.2.

The Year 5 data presented in the Tables P.59-P.67 comes from the NDNS RP UK sample. The Wales samples were analysed separately at the beginning of this process.

Table P.59 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	0.17	0.15	0.18	0.08	0.09
% CV	8.5	6.3	7.1	4.7	6.1
n	13	15	15	14	14

Table P.60 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	1.39	1.41	1.88	0.79
% CV	3.9	4.4	5.2	4.8
n	13	15	15	14

Table P.61 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	0.27
% CV	10.6	4.8	8.8	11.5	8.5
n	13	15	15	14	14

Table P.62 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	5.4	7.0
n	13	15

Table P.63 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	4.4	5.2	5.2	5.0
n	13	15	15	14

Table P.64 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	6.9	8.3
n	13	15

Table P.65 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	0.00	0.01	0.01
% CV	3.1	3.0	5.8	3.6
n	13	15	15	14

Table P.66 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	0.04	0.10	0.02	0.02
% CV	5.4	5.0	5.1	3.4	7.3
n	13	15	15	14	14

Table P.67 Precision of internal QC for plasma lutein + zeaxanthin for Year 5 of the NDNS RP

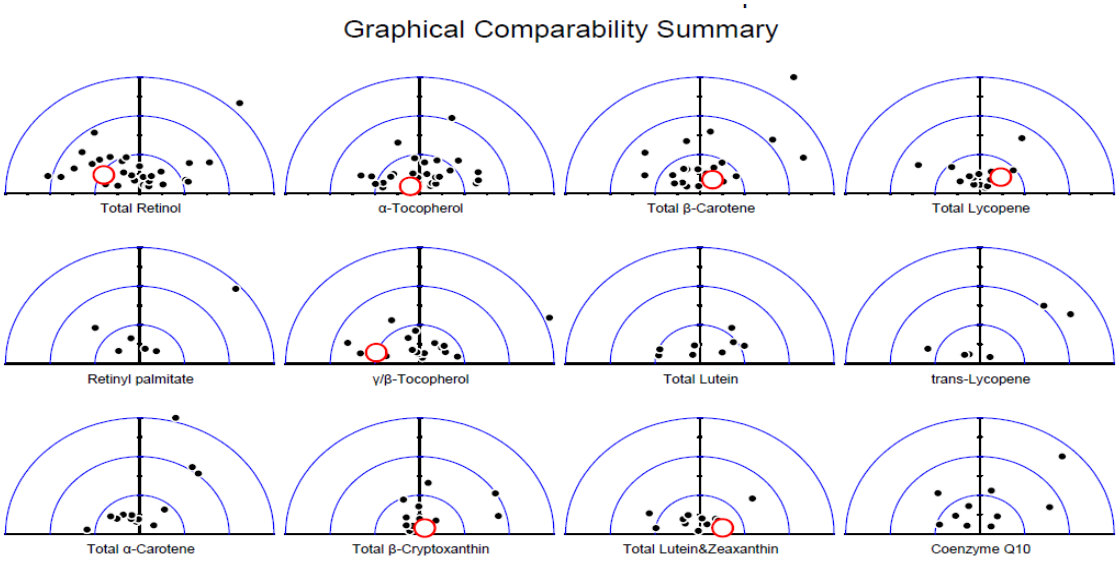
	QC2	QC6	QC7	QC11
Mean (µmol/L)	0.65	0.22	0.46	0.21
SD	0.04	0.02	0.03	0.01
% CV	6.8	9.3	7.0	6.3
n	13	15	15	14

P.2.13.2.1.2 External quality controls for plasma retinol, α– and γ–tocopherol and individual carotenoids

In order to validate the method prior to routine use 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.

One NIST EQA return (LXXV) was submitted around the period of the Wales Year 5 sample analysis; an extract of the report for the single set of samples is shown below, indicating very close agreement with the target concentration.

Figure P.26 NDNS RP Year 5 NIST return LXXV



In the graphical representation 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 in this return and the 'result' above is a summary of all five.

P.2.13.2.2 Conversion factors

In order to understand the overall impact of the method change for 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 Wales Years 2 to 4) with data generated in Year 5 and *vice versa*.

Table P.68 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 with Years 2 to 4:*
Multiply Year 2-4 results x conversion factor or
Divide Year 5 results by conversion factor

P.2.14 Plasma 25-hydroxyvitamin D (25-OHD)

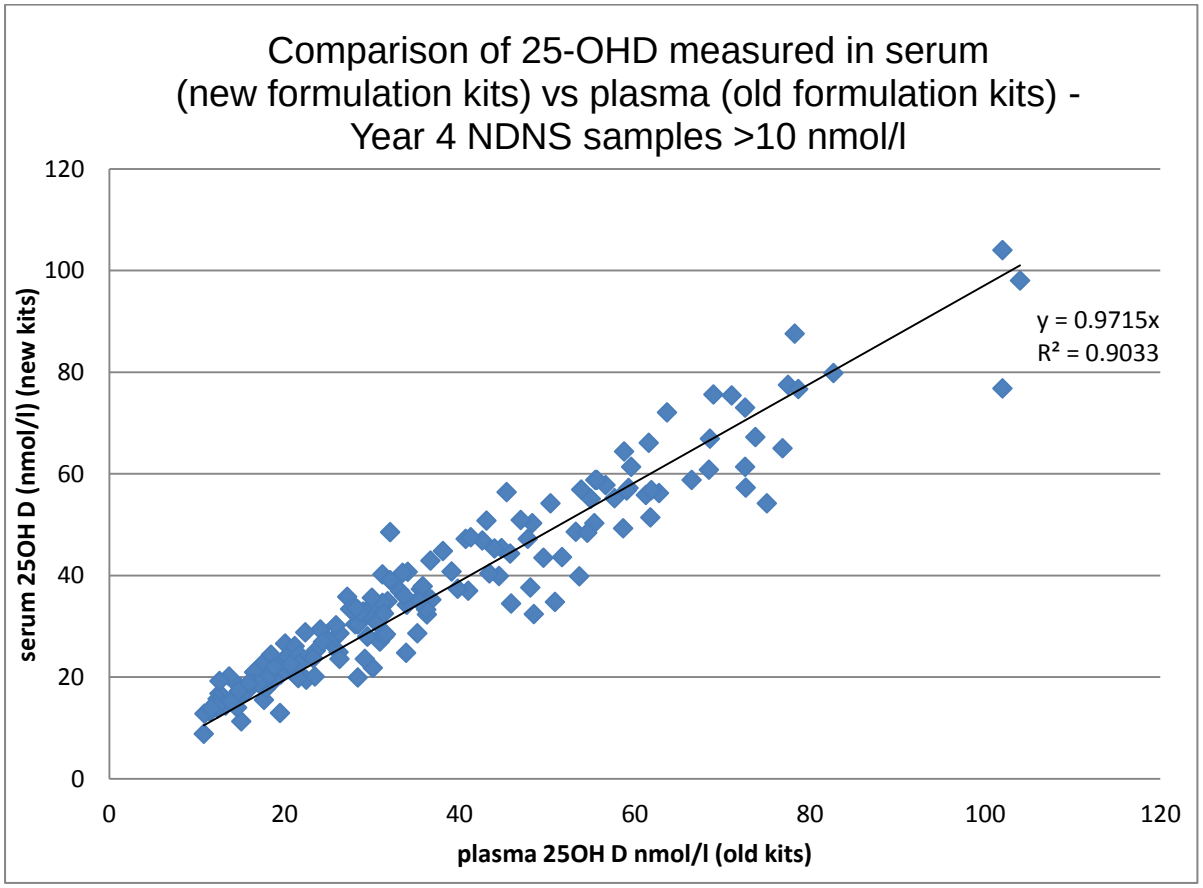
The DiaSorin Liaison method for quantitative determination of 25-OHD is a direct, competitive chemiluminescence immunoassay (CLIA). A specific antibody to vitamin D is used for coating magnetic particles (solid phase), and vitamin D is linked to an isoluminol derivative. During the incubation, 25-OHD is dissociated from its binding protein, and competes with labeled 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-OHD present in calibrators, controls, or samples.

The DiaSorin radio-immunoassay method was used in previous NDNS[11,12,13,14] to measure 25-OHD in plasma. A comparison study was carried out between the DiaSorin RIA method and the DiaSorin Liaison method; this showed good agreement.

Samples for Year 2, 3 and early months of Year 4 were analysed in lithium heparin plasma, using DiaSorin Liaison reagents prior to the company's reformulation of the reagent kit during late 2011. The remainder of the Year 4 and Year 5 samples were

analysed in serum using the reformulated Diasorin kits which are accredited for serum only, not plasma. A comparison of over 100 samples conducted at HNR indicated that there is no bias between the results given by the two versions of the Diasorin Liaison method, as shown in figure P.27.

Figure P.27 Comparison of 25-OHD measured in serum (new formulation kits) vs plasma (old formulation kits)



P.2.14.1 Quality controls for 25-OHD

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

P.2.14.1.1 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 P.69 Internal quality controls for 25-OHD for Year 2 of the NDNS RP

	Control 1 (123521D) (nmol/L)	Control 2 (123521D) (nmol/L)	Lyphochek (nmol/L)
Mean	40.1	134	51.4
SD	3.8	10.3	6.0
% CV	9.4	7.7	11.7
N	23	22	21

Table P.70 Internal quality controls for 25-OHD for Year 3 of the NDNS RP

	Manufacturer's controls: (nmol/L)						Lyphochek (nmol/L)
Mean	41.6	146.2	41.0	133.2	40.6	130.4	55.9
SD	3.4	7.8	3.2	9.3	4.4	8.8	6.6
% CV	8.3	5.3	7.7	7.0	10.9	6.7	11.9
N	16	16	23	23	18	18	42

Table P.71 Internal quality controls for 25-OHD for Year 4 of the NDNS RP

	Manufacturer's controls: (nmol/L)								Lyphochek (nmol/L)
Mean	40.9	127	31.1	106.3	46	147	41.7	130.4	54.1
SD	4.2	8.6	3.0	4.9	2.7	5.9	1.9	3.6	3.9
% CV	10.1	8.5	9.6	4.6	6	4.0	4.6	2.8	7.2
N	10	10	9	9	9	9	11	11	26

Table P.72 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

P.2.14.1.2 External quality controls 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 All Laboratory Trimmed Mean).DEQAS do not issue cumulative performance data as do NEQAS.

Figures P.28 and P.29 show the relationship 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), and the relationship between the results obtained in the NBA lab at HNR and the international All Laboratory Trimmed Mean (ALTM, n=approx.1100 laboratories).

Figure P.28 External quality controls for plasma 25OH vitamin D – Years 2 to 5 of the NDNS RP (DEQAS; HNR results versus Liaison method mean)

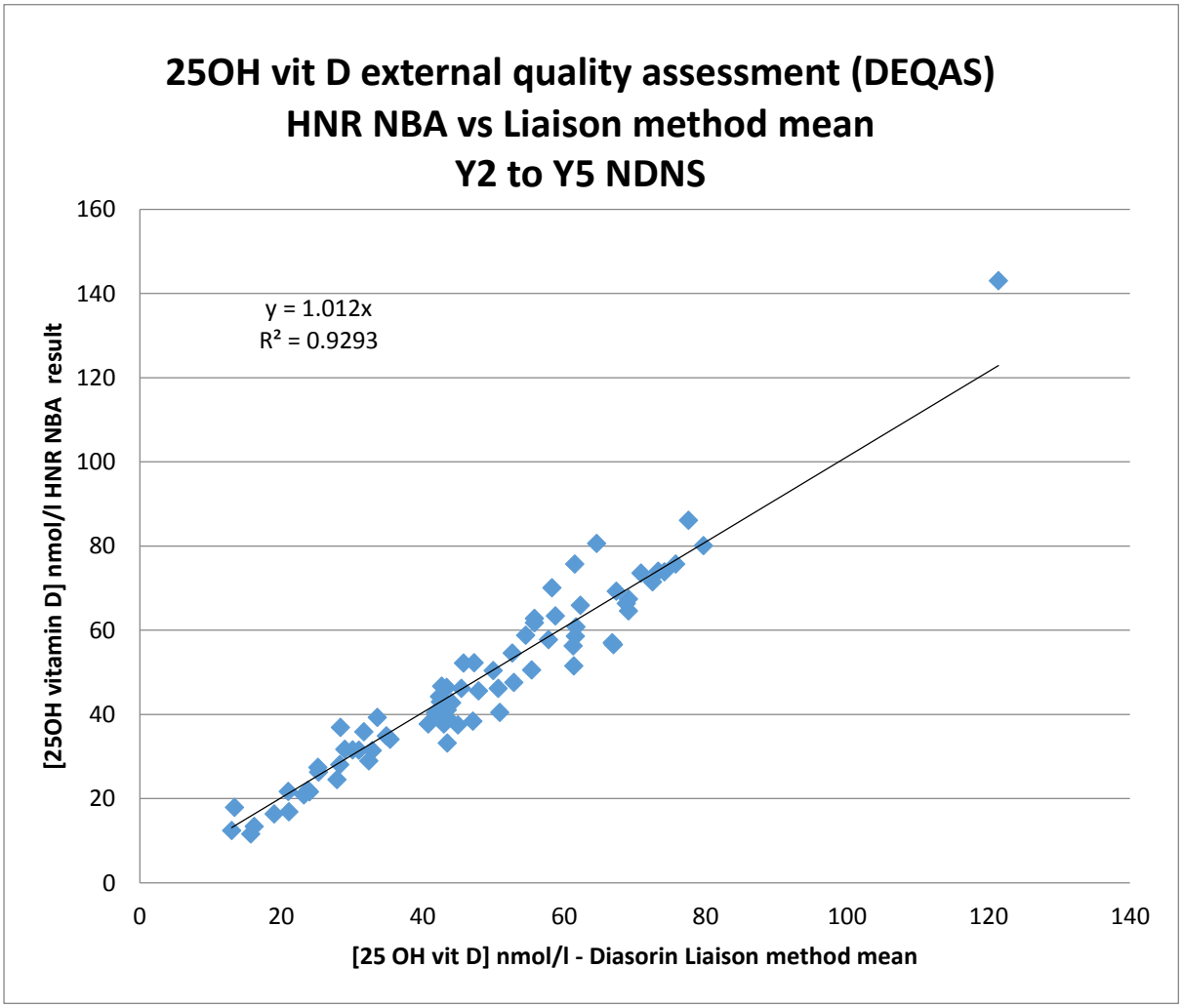
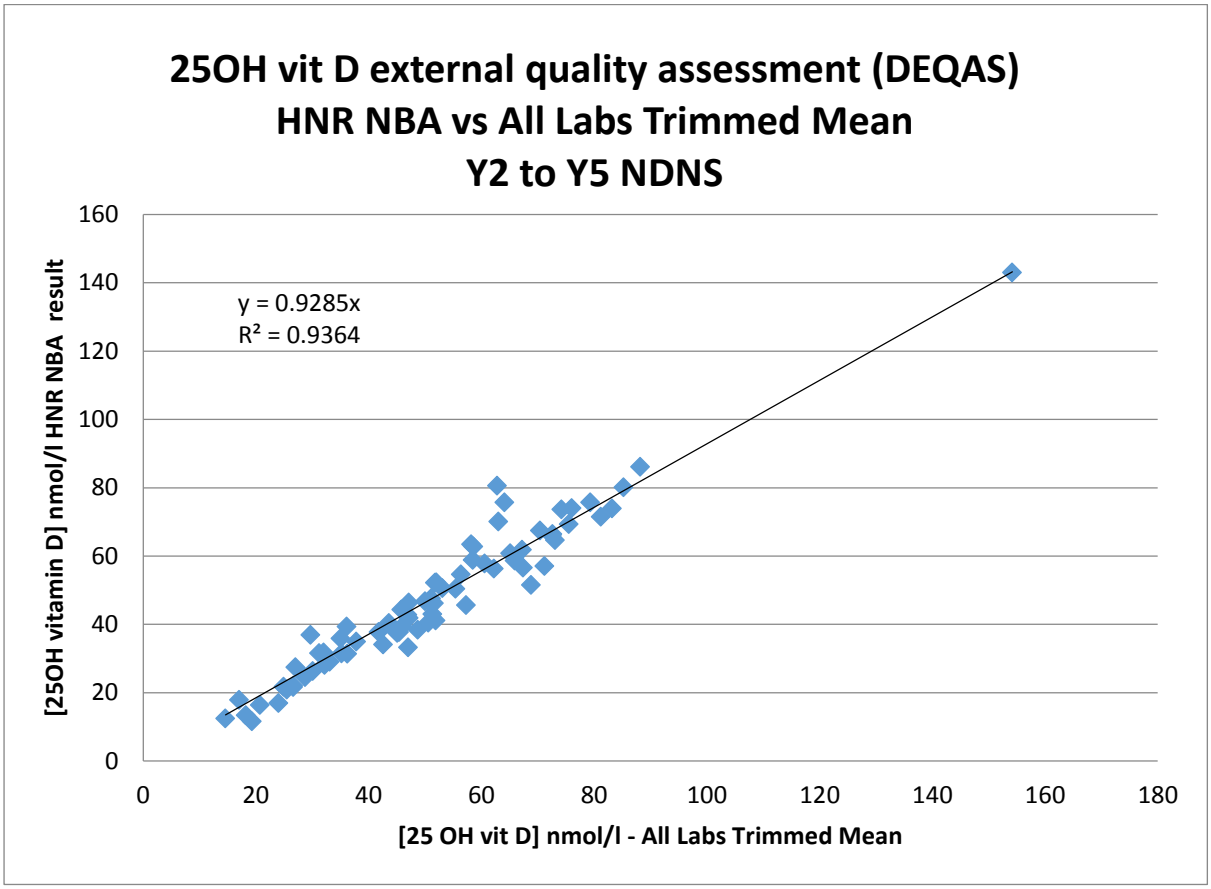


Figure P.29 External quality controls for plasma 25OH vitamin D – Years 2 to 5 of the NDNS RP (DEQAS; HNR results versus ALTM)



P.2.15 Plasma creatinine

The creatinine method used in the NDNS RP 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.

P.2.15.1 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 P.73-76 show internal QC results for creatinine, covering the period when NDNS RP Years 2 to 5 samples were analysed, respectively.

Table P.73 Internal quality controls for plasma creatinine for Year 2 of the NDNS RP

	Low	Medium	High
Mean creatinine µmol/L	62.9	170.7	607.1
SD µmol/L	4.2	3.0	13.0
CV %	6.7	1.7	2.1
N	36	36	33

Table P.74 Internal quality controls for plasma creatinine for Year 3 of the NDNS RP

	Low		Medium		High	
Mean creatinine µmol/L	69.2	65.3	169.4	166.2	578.6	588.7
SD µmol/L	4.0	3.3	4.9	4.8	13.0	18.1
CV %	5.8	5.1	2.9	2.9	2.2	3.1
N	34	41	36	31	32	45

Table P.75 Internal quality controls for plasma creatinine for Year 4 of the NDNS RP

	Low	Medium	High
Mean creatinine µmol/L	63.2	164.5	575.2
SD µmol/L	4.1	4.7	9.7
CV %	6.5	2.9	1.7
N	43	43	42

Table P.76 Internal quality controls for plasma creatinine for Year 5 of the NDNS RP

	Low	Medium	High
Mean creatinine µmol/L	68.2	170.5	582.7
SD µmol/L	3.2	4.0	8.1
CV %	4.8	2.4	1.4
N	24	22	24

P.2.15.2 External quality controls for plasma creatinine

HNR subscribes to the UKNEQAS clinical chemistry. Figure P.28 is the UKNEQAS results for creatinine distributions 858 (19 April 2010) to 869 (4 October 2010). This covers the period when Year 2 creatinine retrospective reassay was performed and include an estimate of bias with respect to the All Laboratory Trimmed Mean (B score) and the consistency of that bias (C score). Results within the white area of the resulting chart indicate acceptable performance as determined by UKNEQAS. Similarly, Figures P.30 to P.33 are the UKNEQAS results for creatinine distributions 867 (August 2010) to 890 (August 2011), 889 (July 2011) to 911 (August 2102) and 912 (Sept 2012) to 935 (Aug 2013); they cover the periods when Years 3, 4 and 5 creatinine analysis was performed, respectively. The bars to the right of the charts indicate the performance of the kinetic Jaffe method. The charts below show that HNR's assay is very well controlled in terms both of bias relative to the consensus mean/method mean, and consistency of that bias.

Figure P. 30 External quality controls for plasma creatinine –Year 2 of the NDNS RP (Bias and Consistency)

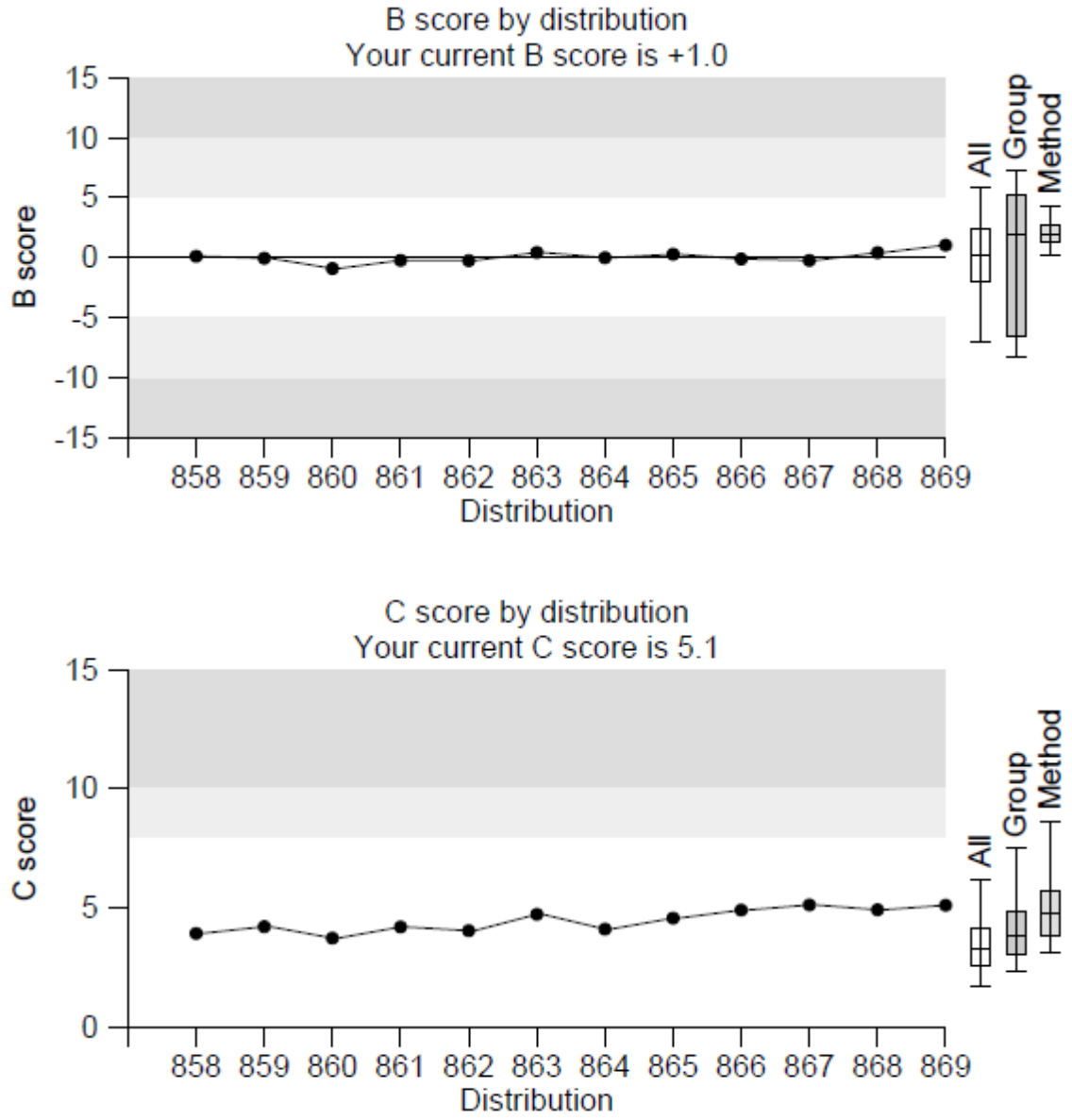
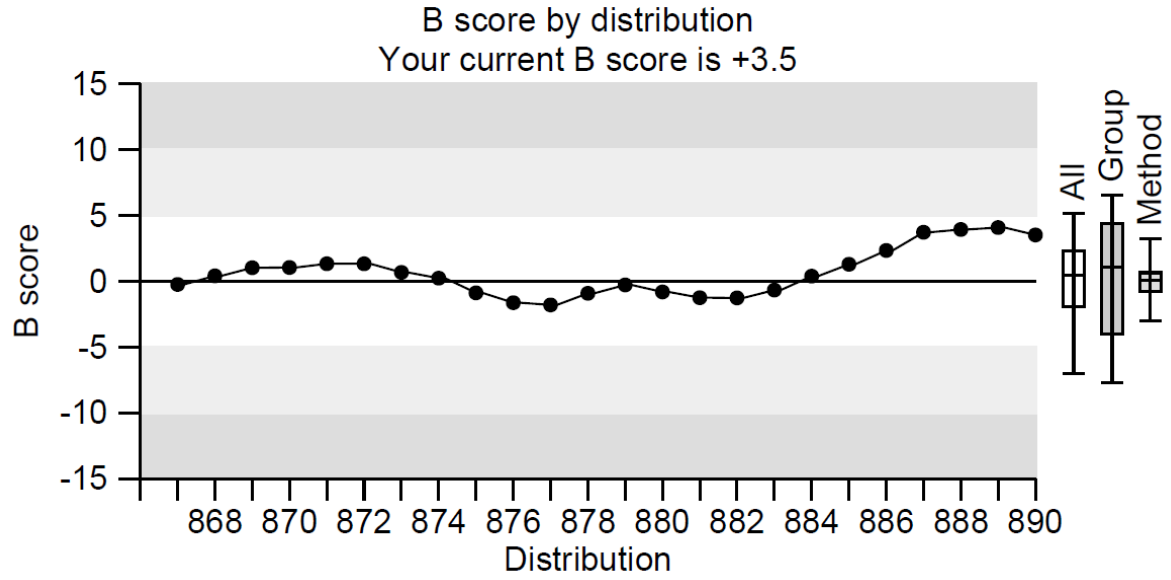


Figure P.31 External QC for creatinine – performance during Year 3 of the NDNS RP (Bias and Consistency)



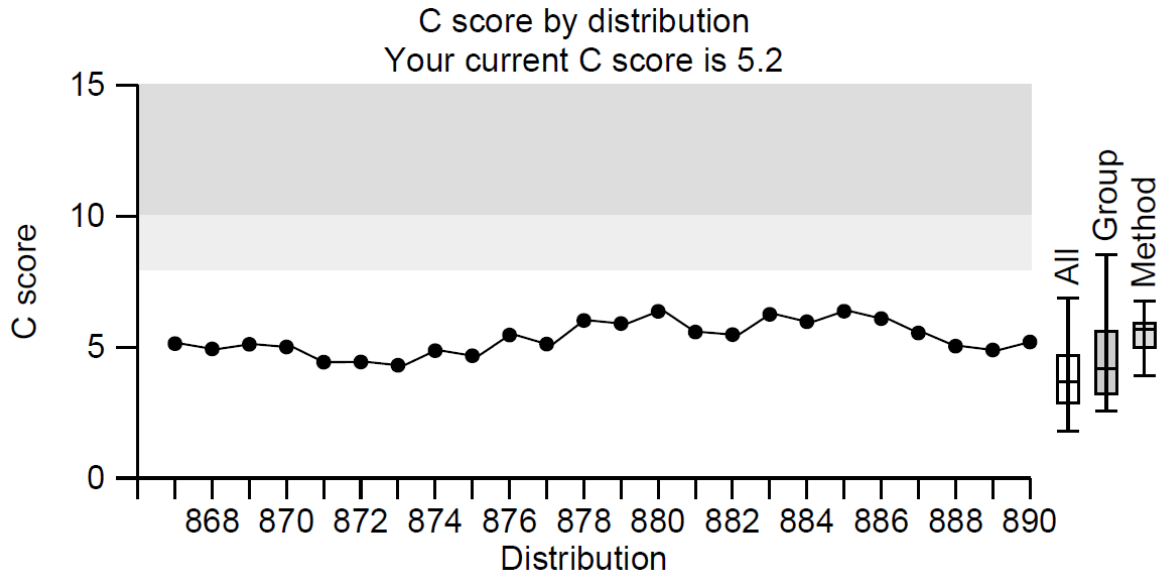
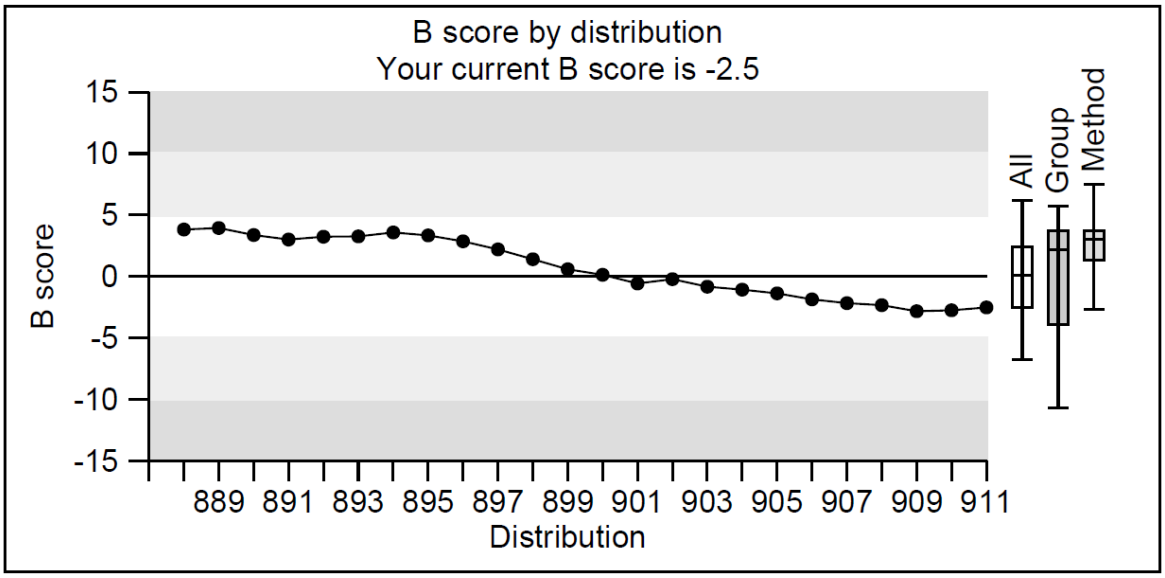


Figure P.32 External QC for creatinine – performance during Year 4 of the NDNS RP (Bias and Consistency)



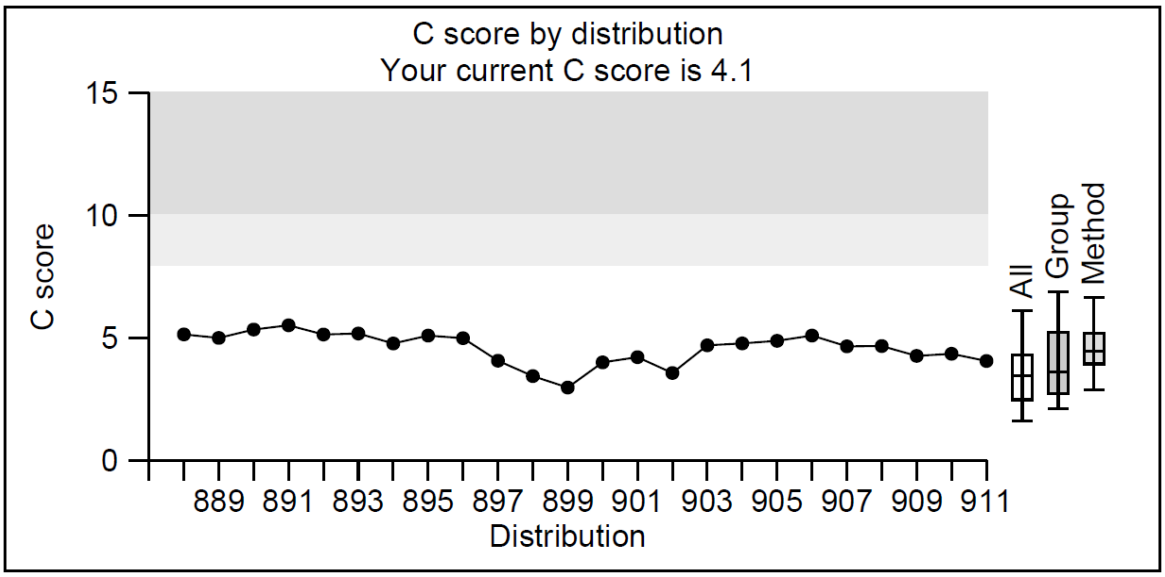
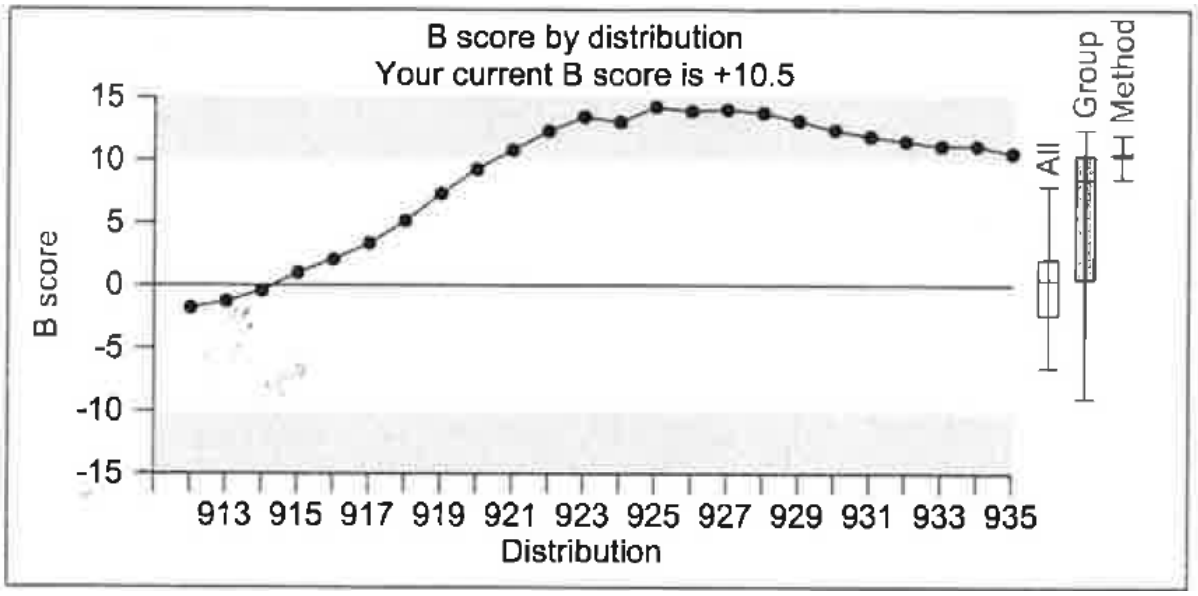


Figure P.33 External QC for creatinine – performance during Year 5 of the NDNS RP (Bias and Consistency)



P.2.16 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 which specifically affect Se measurement. Samples were introduced to the ICP-MS via a V-groove nebuliser and cyclonic spray chamber arrangement.

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

Matrix matched external calibration standards were prepared in commercially prepared calf serum (Sigma Aldrich) for each analytical batch.
Prior to analysis the ICP-MS instrument was tuned for optimum signal sensitivity and minimum oxide species and doubly charged ion formation. 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.

P.2.16.1 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.

P.2.16.1.1 Inter-batch variability

Tables P.77 to P.80 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 P.77 QC analysis for Year 2 of the NDNS RP

	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)	84.1	1552	121	2023
N (QC samples)	56	62	56	62
SD	4	100	5	201
%CV	5	6	4	10

Table P.78 QC analysis for Year 3 of the NDNS RP

	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)	85.4	1356	122	1762
N (QC samples)	41	41	46	45
SD	4.7	137	6	144
%CV	6	10	5	8

Table P.79 QC analysis for Year 4 of the NDNS RP

	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)	81.8	1444	119	1888
N (QC samples)	42	42	42	42
SD	5.6	79	9	111
%CV	7	5	7	6

Table P.80 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

P.2.16.1.2 Inter-year variability

Assessment of inter-year variability is achieved by comparing the annual mean QC sample results over Years 2 to 5 of the NDNS RP.

Table P.81 shows the mean measured concentration data for Se and Zn analysis of the same QC material for each year of the programme and that inter-year variability is low with CV being 2% for selenium and 6% for zinc.

Table P.81 Quality control analysis over Years 2 to 5 of the NDNS RP

	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)			
Year 2	84.1	1552	121	2023
Year 3	85.4	1356	122	1762
Year 4	81.8	1444	119	1888
Year 5*	82.6	1384	119	809
Mean (Years 2-5)*	**83.5**	**1434**	**120**	**1871**
SD	**1.4**	**75**	**1.3**	**99**
%CV	**2**	**5**	**1**	**5**

**Y5 from lot number 906 only (see table P.73)*

P.2.16.2 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: 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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