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**A PROPOSED INTERNATIONAL STANDARD FOR
VITAMIN B12 AND SERUM FOLATE
REPORT OF THE INTERNATIONAL COLLABORATIVE STUDY TO
EVALUATE A BATCH OF LYOPHILISED SERUM FOR B12
AND FOLATE CONTENT**

**Susan J Thorpe, Alan Heath,
National Institute for Biological Standards and Control, Potters Bar, Herts EN6 3QG, UK**

**Sheena Blackmore, Annie Lee, Malcolm Hamilton,
UK NEQAS Scheme for Haematinics, Good Hope Hospital, Sutton Coldfield, West Midlands
B75 7RR, UK**

**Bryant Nelson, National Institute of Standards and Technology, Gaithersburg, Maryland
20899, USA**

**Christine Pfeiffer, National Center for Environmental Health, Centers for Disease Control and
Prevention, Atlanta, GA 30341, USA**

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Summary

The candidate International Standard (IS) for B12 and serum folate, 03/178, was assayed using a wide range of methods in 23 laboratories in 7 countries. Methods included a range of commercial analysers and, for serum folate, candidate reference methods of isotope-dilution tandem mass spectrometry coupled to liquid chromatography (LC/MS/MS). The inclusion of three serum samples in the study, with different B12 and folate levels, demonstrated a considerable reduction in inter-laboratory variability when the B12 and folate levels in the samples were determined relative to the candidate IS (B12 and folate values assigned) rather than to the analysers' calibration.

It is recommended that preparation 03/178 is established as the 1st IS for serum folate with an assigned value of 12.08 nmol/L total folate, made up of 9.75 nmol/L 5MeTHF (CV 5.5%), 1.59 nmol/L 5FoTHF (CV 4.2%) and 0.74 nmol/L FA (CV 31.6%). The total folate content of 12.08 nmol/L is equivalent to 5.33 ng/ml, using a conventional conversion factor of 2.266.

It is recommended that preparation 03/178 is established as the 2nd IS for vitamin B12 with a consensus value of 480 ng/ml, and that the preparation is re-evaluated when a reference measurement procedure has been established.

Introduction

The assay of the vitamins B12 and folate in blood is the current routine procedure for determining deficiency of these vitamins. Deficiency can result in a number of clinical conditions including megaloblastic and pernicious anaemia. Clinical features may indicate which vitamin deficiency is the more likely, although levels of both usually need to be assessed [1].

The 1st International Reference Reagent for human serum vitamin B12 (81/563) was established by WHO in 1992. Prior to 1992, it was distributed as a British Standard. However, the preparation has since been found to be positive for anti-HCV and HCV RNA. Also, it was calibrated using a microbiological assay (Euglena assay) which has since been replaced by automated assay systems. For these reasons, it was decided to replace the 1st IRR and assign a value to the replacement preparation independently using current methodology.

Dietary folic acid is enzymatically reduced in the tissues through several steps to tetrahydrofolate, its active coenzyme form which functions as a carrier of hydroxymethyl and formyl groups in one-carbon metabolism essential for the biosynthesis of DNA. A number of different forms of folate are therefore present in whole blood although the predominant folate species in plasma or serum is 5-methyltetrahydrofolic acid (5MeTHF).

Folate deficiency symptoms include megaloblastic anaemia, arteriosclerosis, cancer, depression, impaired growth and neural tube defects, such as spina bifida, in fetuses [1,2,3,4]. The assay of blood folate is the current routine procedure for determining the patient's folate status; red cell folate (RCF) is a better index of body stores than serum folate which can be influenced by recent dietary intake [2]. In practice, the RCF concentration is determined by subtracting the serum folate concentration from the whole blood folate concentration, although the amount of folate in serum is relatively small compared to that in the red cells. However, increasingly, folate is only being measured in serum. The decrease in the use of RCF is probably a result of poor agreement of assay results, despite the availability of an IS for whole blood folate, and the time-consuming and labour intensive step of haemolysate preparation.

The traditional method for folate measurement was a microbiological assay with *Lactobacillus Casei*, although this method together with commercial kit-form competitive binding radio-dilution methodology has almost completely been replaced in clinical laboratories by the use of automated assay systems capable of measuring a range of analytes. There is a considerable lack of inter-method agreement in assays of both serum folate and whole blood folate resulting from a number of variables, including the use of different folate moieties e.g. 5MeTHF or folic acid (FA), as standards [5,6]. Although an IS for whole blood folate (95/528) was established in 1996 [6], there are as yet no internationally accepted reference materials for serum folate.

The aim of the present study was therefore to evaluate a batch of lyophilised serum for folate and B12 content using current methods, including candidate reference methods based on mass spectrometry for the specific determination of 5MeTHF and other folate forms in serum/plasma [7,8]. In view of the different folate forms in whole blood and serum/plasma, the change in assay methods in the decade since 95/528 was evaluated, the low level of usage of 95/528 for standardisation purposes and the availability of candidate reference methods for the specific determination of folate forms in serum/plasma, it was decided to assign a folate value to the proposed serum folate standard independently of 95/528.

Materials and methods

Candidate International Standard for vitamin B12 and serum folate

Pooled human serum from seven donors was kindly donated by the UK NEQAS Scheme for Haematinics. Each donor had been counseled and had signed consent forms for compliance with the Human Tissue Act. Each donor was tested and found negative for HBsAg, HCV antibody and HIV antibody. Individual donations were stored frozen before pooling. At NIBSC, the serum was thawed, pooled, dispensed into glass ampoules (~1 ml/ampoule), lyophilized, and coded 03/178. The ampoules were stored in the dark at -20°C except for a small number that were stored at -70°C, +4°C, +20°C, +37°C and +45°C for accelerated degradation studies. Three further, smaller serum pools, known to vary in their total B12/serum content, were similarly lyophilized and coded 04/116 (sample 1), 04/118 (sample 2) and 04/120 (sample 3). Full details are summarised below:

	<i>Candidate IS 03/178</i>	<i>Sample 1 04/116</i>	<i>Sample 2 04/118</i>	<i>Sample 3 04/120</i>
<i>Mean weight of the dispensed solution (number of fill weights measured)</i>	1.0062g (46)	1.0057g (6)	1.0067 (6)	1.0060
<i>Imprecision of the filling (coefficient of variation)</i>	0.08%	0.13%	0.11%	0.12%
<i>Residual moisture</i>	0.8%	0.15%	0.26%	0.19%
<i>Number of ampoules for distribution as WHO reference reagent</i>	3750	N/A	N/A	N/A

Collaborative study participants

A total of 24 laboratories in 7 countries participated in the study (Appendix 1). Each was assigned a code number, which does not reflect the order of listing. The participants included manufacturers, clinical laboratories and research laboratories. In order to ensure that all the main methods were represented, a relatively large number of UK laboratories participated as information on their methodology was readily available through the UK NEQAS scheme.

Methods

Participants were requested to perform their usual B12 and serum folate assay methodology. Most performed commercial automated competitive assays utilizing folate-binding protein and labeled folate analogues, or intrinsic factor in the case of B12 assays, and standardised by the manufacturer with PGA or 5MeTHF (for folate assays), or cyanocobalamin (for B12 assays).

Study design

Each participant was provided with 3 ampoules of each of the candidate IS, 03/178, samples 1, 2 and 3 (04/116, 04/118 and 04/120, respectively), and the 1st IRR for B12, 81/563 (optional). Participants were requested to reconstitute ampoule contents with 1ml distilled or deionised water on the day of assay. They were asked to perform two assays on each preparation on each of three days, using fresh ampoules each day, to give a total of six estimates for each of B12 and folate. Participants were requested to return their individual estimates for B12 (in pg/ml) and folate (in ng/ml) on results sheets provided.

Statistical methods

Overall means of B12 and folate concentration for each laboratory were calculated as arithmetic means across replicate assays from each day, then arithmetic means across days. Where a laboratory had indicated that a result was considered anomalous it was excluded from the calculation. No other data were excluded.

Overall means for the different preparations, and means for different assay methods, were calculated as arithmetic means of the individual laboratory means.

Variation between laboratories was assessed by calculating the coefficient of variation (% CV) between individual laboratory mean estimates.

Estimates of the concentration of B12 and folate in samples 1, 2 and 3 (04/116, 04/118 and 04/120, respectively) were also calculated relative to the candidate IS 03/178, taking the assigned unitages of 03/178 as the overall B12 mean (pg/ml) and the total folate mean (ng/ml) of the two laboratories using the candidate reference methods of LC/MS/MS.

Results

Data received

Data were received from 24 laboratories. Most laboratories used commercial analysers or microbiological assays. Two laboratories performed the candidate reference methods of isotope-dilution liquid chromatography tandem mass spectrometry (LC/MS/MS). The concentrations of the different folate forms measured (5MeTHF, FA for lab 19; 5MeTHF, 5-formyltetrahydrofolic acid (5FoTHF), FA for lab 23) are shown in Table 1. The ng/ml values for each form were combined to give a total folate estimate for each laboratory for the purpose of comparison with the other methods. Laboratory 23 also returned data from two other, different methods, which have been analysed separately. This results in a total of 26 separate data sets.

Estimates of serum folate concentration in the study preparations

The laboratory mean estimates of folate content are listed in Table 2 for all preparations. They are also shown in histogram form in Figures 1A-D. Each box represents the results from a laboratory. The boxes are labelled with the laboratory code number, and a code for the assay method used. The intra-laboratory (i.e. between assays) variability, expressed as % CV, is shown in Table 3. Many of the % CVs are below 5%.

The means of folate content in each preparation for the different assay methods are shown in Table 4, along with the overall means and the inter-laboratory (i.e. between laboratories) variability expressed as % CV.

From Figures 1A-D and Table 4, it can be seen that there is variability between the results from the different laboratories. The overall % CVs range from 17.1% - 19.5%. However, from Figures 1A-D, it can be seen that in general there is much better agreement between laboratories using the same method.

There was very good agreement between laboratories 19 and 23 on the level of 5MeTHF in the study preparations as determined using LC/MS/MS (Table 1), although there was less agreement on the FA content (see Discussion). A mean total folate concentration of 5.1 ng/ml in 03/178, as determined using LC/MS/MS (values taken from Table 2), was used for the purpose of recalculating the potencies of samples 1, 2 and 3 relative to 03/178. The folate concentrations of samples 1, 2 and 3 calculated as potencies relative to the candidate IS 03/178, using a mean LC/MS/MS value of 5.1 ng/ml, are shown in Figures 2A-C. Overall means and means obtained by the different methods are shown in Table 5.

Expressing results as potencies relative to the candidate IS considerably improves the agreement between laboratories using the different methods. This is clearly seen from Figures 1 and 2, and by the reduction in the inter-laboratory % CV from around 17-20% to 6- 9%.

There is some indication that the Bayer Centaur and ACS methods give slightly higher estimates than other methods for 04/118, and to a lesser extent 04/116, when expressed relative to 03/178 (Figures 2B and 2A, respectively). From the tables of overall means (Tables 3 and 4), the Bayer Centaur gives results close to the overall mean for 03/178, but above the overall mean for 04/118. This suggests that there may be differences in the nature of the samples, although using the candidate IS still improves overall agreement.

Estimates of B12 concentration in the study preparations

The laboratory mean estimates of B12 content are listed in Table 2 for all preparations. They are also shown in histogram form in Figures 3A-D. Each box represents the results from a laboratory. The boxes are labeled with the laboratory code number, and a code for the assay method used. The intra-laboratory (i.e. between assays) variability, expressed as % CV, is shown in Table 6. Most of the % CVs are around or below 5%, indicating good repeatability.

The means of B12 content in each preparation for the different assay methods are shown in Table 7, along with the overall means and the inter-laboratory (i.e. between laboratories) variability expressed as % CV.

From Figures 3A-D and Table 7, it can be seen that there is some variability between the results from the different laboratories. The overall % CVs range from 12.8% - 18.1%. However, from Figures 3A-D, it can be seen that in general there is much better agreement between laboratories using the same method.

The mean B12 concentration in the candidate IS 03/178 is 480 pg/ml. The overall mean B12 concentration in the existing IRR is 332 pg/ml, which is very close to its assigned value of 320 pg/ml. The B12 concentrations of samples 1, 2 and 3 calculated as potencies relative to the candidate IS, 03/178 (mean value assigned of 480 pg/ml), are shown in Figures 4A-C. Overall means and means obtained by the different methods are shown in Table 8. Expressing results as potencies relative to the candidate IS considerably improves the agreement between laboratories using the different methods. This is clearly seen from Figures 3 and 4, and by the reduction in the inter-laboratory % CVs from around 13-18% to 4-13%.

Stability

Accelerated degradation studies on the candidate IS 03/178 are underway, but data are available from an earlier trial fill of serum coded 02/242.

The B12 and folate concentrations in ampoules of 02/242 stored at -70°C , -20°C , $+37^{\circ}\text{C}$ and $+45^{\circ}\text{C}$ for 8 months were determined using microbiological assays, based on eight repeat tests for B12 and 4 repeat tests for folate. The results for the samples at higher temperatures were expressed as a proportion of those at the baseline temperature of -70°C . Weights were calculated for the ratios based on the variance of the results for the repeat tests of the samples at higher temperature and baseline.

The results (as %) are shown below.

<i>Temperature</i>	<i>B12</i>	<i>Folate</i>
-20°C	106.4%	113.6%
$+37^{\circ}\text{C}$	105%	109.1%
$+45^{\circ}\text{C}$	91%	96.7%

In both cases, the results at -20°C and $+37^{\circ}\text{C}$ are higher than at -70°C ; the results at $+45^{\circ}\text{C}$ are a little lower. However, it was noted that there was difficulty reconstituting this sample, so the results may not reflect true degradation. The Arrhenius model [9] could not be fitted, as there was insufficient observed degradation.

Initial indications therefore suggest that the B12 and folate content of lyophilized serum is stable.

Discussion

The WHO recommendations for the preparation, characterization and establishment of international and other biological reference materials have recently been revised in accordance with developments in the characterization of reference materials in other fields [10]. Thus where it is appropriate for a WHO biological reference material to be calibrated in SI units, WHO recommend that the principles outlined in ISO 17511 [11] should be followed. According to ISO 17511, which deals with the metrological traceability of values assigned to calibrators and control materials, the highest metrological level is one in which reference materials are calibrated in SI units using a primary reference measurement procedure. The recent development of candidate primary reference methods for folate measurement, based on isotope dilution mass spectrometry now make it possible to assign a folate value to the candidate IS using this methodology, and to distinguish between the different forms of folate.

There was very good agreement between laboratories 19 and 23 on the 5MeTHF content of 03/178 as determined using LC/MS/MS, but less agreement on the FA content (Table 1). This is because the analytical MS/MS sensitivity for FA in positive ion mode is poor, unlike the sensitivity for 5MeTHF or 5FoTHF. Also, the level of FA in 03/178 is close to the limit of quantification (LOQ) and the measurement imprecision at the LOQ is typically 20-25%. However, the FA content is only about 6% of the total folate.

The inclusion of three serum samples in the study demonstrated the potential improvement in inter-laboratory variability when potencies were recalculated relative to 03/178. Although for the purpose

of recalculating the folate content of samples 1, 2 and 3, a folate value of 5.1 ng/ml was assigned to 03/178 based on the mean of the folate values from laboratories 19 and 23 as listed in Table 2, it

would be more accurate to assign a mean 5MeTHF value and a mean FA value from the results of laboratories 19 and 23, and laboratory's 23 mean value for 5FoTHF, as shown below:

<i>Laboratory</i>	<i>5MeTHF</i>	<i>5FoTHF</i>	<i>FA</i>	<i>Total</i>	
	<i>ng/ml</i>	<i>ng/ml</i>	<i>ng/ml</i>	<i>nmol/L</i>	<i>ng/ml*</i>
19	4.36	0.76	0.4		
23	4.60	-	0.25		
Mean ng/ml	4.48	0.76	0.325		
%CV**	5.5%	4.2%	31.6%		
Mean nmol/L	9.75	1.59	0.74	12.08	5.33

*using a conventional conversion factor of 2.266 from nmol/L back to ng/ml

** across all assays

Conversion of ng/ml values to nmol/L allows the concentrations of the 3 different folate forms to be combined to give a total folate concentration. Using a conventional conversion factor of 2.266 (for FA) to convert the total folate content of 12.08 nmol/L back to a ng/ml value, which is the unit of measurement used by clinicians, the LC/MS/MS methods give a total folate value of 5.33 ng/ml. This value is extremely close to the overall study mean value of 5.5 ng/ml (Table 2).

The overall mean B12 content of the candidate IS was 480 pg/ml. The overall mean B12 content of the 1st IRR for Human Serum Vitamin B12, 81/563, was 332 pg/ml in the present study. This is extremely close to the value of 320 pg/ml (103.75% of the value) assigned two decades ago following a collaborative study in 7 laboratories each using the same strain of *E gracilis* as test organism in the turbidimetric *Euglena* assay [12]. The IRR was calibrated in terms of pure cyanocobalamin 'local' standards that were in routine use at individual laboratories taking part in the study (cyanocobalamin is also used to standardise current methods). Although not as many laboratories assayed 81/563 as 03/178, the results of the present study indicate reasonable consistency of the assigned unitage should the consensus value of 480 pg be assigned to 03/178. Also, the IRR is little used, so continuity of the 'unit' will not be a problem. Recalculating the B12 content of the three study samples relative to the candidate IS 03/178 demonstrated the potential improvement in inter-laboratory variability by use of a common standard. Although a value assigned from a primary reference measurement procedure would give the preparation a higher metrological status and may be more acceptable to potential users, for practical standardisation purposes of reducing inter-laboratory variability, the consensus B12 value would be satisfactory. A consensus value could therefore be assigned now, and the preparation re-evaluated when a reference method has been developed and validated, which may be in about 2 years time.

Recommendations

It is recommended that preparation 03/178 is established as the 1st IS for serum folate with an assigned value of 12.08 nmol/L total folate, made up of 9.75 nmol/L 5MeTHF (CV 5.5%), 1.59 nmol/L 5FoTHF (CV 4.2%) and 0.74 nmol/L FA (CV 31.6%). The total folate content of 12.08 nmol/L is equivalent to 5.33 ng/ml, using a conventional conversion factor of 2.266.

It is recommended that preparation 03/178 is established as the 2nd IS for vitamin B12 with a consensus value of 480 ng/ml, and that the preparation is re-evaluated when a reference measurement procedure has been established.

The opinions of the participants regarding these recommendations will be sought before the ECBS meeting in October 2005.

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Table 1 Laboratory means of folate content in the candidate IS and samples 1, 2 and 3 as determined using LC/MS/MS

Laboratory	Preparation	Folate form (%CV across all assays)						Total folate measured nmol/L
		5MeTHF		5FoTHF		FA		
		ng/ml	nmol/L ^a	ng/ml	nmol/L ^a	ng/ml	nmol/L ^a	
19	03/178	4.36 (6.7%)	9.48	-	-	0.40 (24.7%)	0.91	10.39
	04/116	4.25 (3.5%)	9.24	-	-	0.29 (13.2%)	0.65	9.89
	04/118	6.28 (2.4%) ^b	13.66	-	-	0.43 (25.2%)	0.97	14.63
	04/120	3.61 (4.1%)	7.86	-	-	0.31	0.71	8.57
23	03/178	4.60 (2.1%)	10.00	0.76 (4.2%)	1.59	0.25 (8.2%)	0.56	12.15
	04/116	4.38 (2.6%)	9.53	0.80 (7.1%)	1.69	0.16 (66.0%) ^c	0.35	11.57
	04/118	6.31 (1.9%)	13.74	0.96 (8.9%)	2.03	0.26	0.59	16.36
	04/120	3.67 (3.8%)	7.98	0.42 (12.1%)	0.88	0.16 (26.1%)	0.35	9.21

^aMolecular weights: 5MeTHF 459.46; 5FoTHF 473.4; FA 441.4

^bDay 2 values rejected from the data set according to the Q-test at the 95% confidence level

^c<limit of quantification

Table 2 Laboratory mean values for B12 and folate in the candidate IS 03/178 and study samples 1, 2 and 3 (in order of laboratory number). The B12 content of the IRR for vitamin B12, 81/563, is also shown where tested.

<i>Laboratory</i>	<i>Assay Method</i>	<i>B12 pg/ml</i>					<i>Folate ng/ml</i>			
		<i>03/178</i>	<i>Sample 1</i>	<i>Sample 2</i>	<i>Sample 3</i>	<i>81/563</i>	<i>03/178</i>	<i>Sample 1</i>	<i>Sample 2</i>	<i>Sample 3</i>
1	Beckman Access	433	405	154	257	nd	4.9	4.5	5.9	3.9
2	Bayer ACS 180	433	440	196	280	nd	6.4	6.4	9.8	5.7
3	Microbiological assay	nd	nd	Nd	nd	nd	6.6	6.1	8.7	4.9
4	Roche E170	522	518	193	305	350	6.0	5.4	7.1	5.0
5	Bayer Centaur	473	447	187	288	nd	5.4	5.5	7.6	4.6
6	Bayer Immuno 1	525	531	271	369	394	5.0	4.9	6.3	4.2
7	Beckman Access	393	376	142	232	273	4.7	4.4	5.9	3.7
8	Bayer ACS 180	490	507	231	313	nd	6.1	6.1	8.9	5.2
9	Bayer Centaur	464	480	205	314	340	5.7	5.8	8.6	5.3
10	Bayer Centaur	497	490	205	323	nd	5.6	6.2	8.3	5.0
11	DPC Immulite 2000	510	533	211	312	343	5.7	5.5	7.1	4.7
12	Abbott Architect	443	444	159	260	304	4.2	3.9	5.4	3.4
13	Bayer Centaur	506	478	200	305	nd	5.6	5.9	8.1	4.7
14	Bio-Rad RIA	437	471	167	280	nd	4.7	4.5	6.1	3.6
15	Microbiological assay	519	551	241	370	388	5.1	5.0	7.5	4.4
16	DPC Immulite 2000	505	519	188	288	nd	5.9	5.3	7.3	4.8
17	Abbott Architect	416	412	131	222	284	3.5	3.2	4.5	2.7
18	Roche-Elecsys	nd	nd	nd	nd	nd	6.4	5.6	7.9	5.5

19	LC/MS/MS ^a	nd	nd	nd	nd	nd	4.8 ^b	4.5 ^b	6.7 ^b	3.9 ^b
20	Bayer Centaur	466	461	204	291	nd	5.7	6.8	8.4	5.2
21	DPC Immulite 2000	525	545	211	316	341	5.7	5.5	7.2	4.8
22	AIA- PACK/TOSOH	680	739	225	372	nd	8.5	7.9	9.2	7.1
23	Bio-Rad RIA	440	459	157	256	298	4.6	4.5	5.9	3.5
23	LC/MS/MS ^a	nd	nd	nd	nd	nd	5.5 ^b	5.3 ^b	7.5 ^b	4.2 ^b
23	Microbiological assay	nd	nd	nd	nd	nd	5.0	4.6	6.4	4.1
24	AutoDELFI A	412	408	158	258	nd	6.0	5.7	7.4	5.1
Mean		480	486	192	296	332	5.5	5.3	7.3	4.6

^acandidate reference methods

^bsum of ng/ml values

nd = not determined

Table 3 Intra-laboratory repeatability for serum folate assays: % CV between replicate assays

<i>Laboratory</i>	<i>Assay Method</i>	<i>Intra-Lab % CV</i>			
		<i>03/178</i>	<i>Sample 1</i>	<i>Sample 2</i>	<i>Sample 3</i>
1	Beckman Access	6.7	4.2	3.1	3.1
2	Bayer ACS 180	11.2	9.0	9.2	8.7
3	Microbiological assay	5.2	7.7	4.6	4.7
4	Roche E170	4.7	4.8	2.5	3.7
5	Bayer Centaur	3.4	8.4	3.6	6.9
6	Bayer Immuno 1	2.4	1.8	1.6	8.1
7	Beckman Access	3.5	3.3	2.4	6.8
8	Bayer ACS 180	3.2	3.7	2.6	3.4
9	Bayer Centaur	12.6	9.7	14.0	11.1
10	Bayer Centaur	2.2	8.2	3.6	3.1
11	DPC Immulite 2000	3.6	3.8	5.2	3.4
12	Abbott Architect	4.4	7.1	1.2	4.3
13	Bayer Centaur	6.6	4.4	6.2	3.3
14	Bio-Rad RIA	2.2	4.7	3.8	3.1
15	Microbiological assay	3.1	6.5	4.8	4.2
16	DPC Immulite 2000	6.0	2.6	2.9	4.4
17 ^a	Abbott Architect	-	-	-	-
18	Roche-Elecsys	0.7	2.4	1.1	4.9
19	LC/MS/MS ^b	7.5	3.6	2.4	4.1
20	Bayer Centaur	4.1	2.2	3.1	2.9
21	DPC Immulite 2000	3.6	4.9	2.8	3.0
22	AIA-PACK/TOSOH	3.3	9.6	8.3	6.2
23	Bio-Rad RIA	1.4	1.1	1.4	2.3
23	LC/MS/MS ^b	1.4	4.2	2.2	2.7
23 ^c	Microbiological assay	4.3	0.0	3.3	1.7
24	AutoDELFI	1.7	1.9	2.2	3.4

^aLab 17 based on submitted overall means (fax/scan not legible for individual values)

^b candidate reference methods

^cLab 23 Microbiological assay – based on only one assay (2 replicates total).

Table 4 Means of folate content by method, and overall study means (ng/ml)

<i>Assay Method</i>	<i>Number of laboratories</i>	<i>Preparation</i>			
		<i>03/178</i>	<i>Sample 1 04/116</i>	<i>Sample 2 04/118</i>	<i>Sample 3 04/120</i>
Abbott Architect	2	3.9	3.6	4.9	3.0
AutoDELFIA	1	6.0	5.7	7.4	5.1
Bayer ACS 180	2	6.2	6.2	9.3	5.4
Bayer Centaur	5	5.6	6.0	8.2	5.0
Bayer Immuno 1	1	5.0	4.9	6.3	4.2
Beckman Access	2	4.8	4.5	5.9	3.8
Bio-Rad	2	4.7	4.5	6.0	3.6
DPC Immulite 2000	3	5.8	5.4	7.2	4.7
LC/MS/MS	2	5.1	4.9	7.1	4.1
Microbiological	3	5.6	5.2	7.5	4.5
Roche Elecsys/E170	2	6.2	5.5	7.5	5.3
Tosoh	1	8.5	7.9	9.2	7.1
Overall Mean	26	5.5	5.3	7.3	4.6
Between Lab % CV		17.1%	18.2%	17.4%	19.5%

Table 5 Means of folate content of samples 1, 2 and 3 calculated relative to 03/178 (=5.1 ng/ml) by method, and overall study means

<i>Assay Method</i>	<i>Number of laboratories</i>	<i>Preparation</i>		
		<i>Sample 1 04/116</i>	<i>Sample 2 04/118</i>	<i>Sample 3 04/120</i>
Abbott Architect	2	4.7	6.6	4.0
AutoDELFIA	1	4.9	6.4	4.4
Bayer ACS 180	2	5.2	7.7	4.5
Bayer Centaur	5	5.5	7.5	4.6
Bayer Immuno 1	1	5.1	6.5	4.3
Beckman Access	2	4.9	6.4	4.1
Bio-Rad	2	5.0	6.6	3.9
DPC Immulite 2000	3	4.8	6.4	4.2
LC/MS/MS	2	4.9	7.1	4.1
Microbiological	3	4.9	7.0	4.2
Roche Elecsys/E170	2	4.6	6.2	4.4
Tosoh	1	4.8	5.6	4.3
Overall Mean	26	5.0	6.8	4.3
Between Lab % CV		7.0%	8.8%	5.8%

Table 6 Intra-laboratory repeatability for B12 assays: % CV between replicate assays

<i>Laboratory</i>	<i>Assay Method</i>	<i>Intra-Lab % CV</i>			
		<i>03/178</i>	<i>Sample 1</i>	<i>Sample 2</i>	<i>Sample 3</i>
1	Beckman Access	2.7	2.0	2.7	1.4
2	Bayer ACS 180	4.4	5.5	7.1	8.0
4	Roche E170	2.7	1.9	1.3	2.3
5	Bayer Centaur	7.4	2.7	7.0	5.2
6	Bayer Immuno 1	1.0	1.4	5.9	7.2
7	Beckman Access	5.9	4.3	4.8	5.8
8	Bayer ACS 180	4.5	5.2	2.4	4.6
9	Bayer Centaur	4.9	4.4	9.6	3.5
10	Bayer Centaur	4.6	3.8	5.6	5.8
11	DPC Immulite 2000	4.8	4.3	8.8	6.7
12	Abbott Architect	4.7	5.0	5.9	3.0
13	Bayer Centaur	4.8	3.6	5.8	4.0
14	Bio-Rad RIA	3.1	5.2	4.0	3.5
15	Microbiological assay	5.3	3.6	8.1	7.6
16	DPC Immulite 2000	3.8	8.7	4.6	7.1
17 ^a	Abbott Architect	-	-	-	-
20	Bayer Centaur	3.0	7.0	6.4	4.1
21	DPC Immulite 2000	2.9	3.2	6.7	1.9
22	AIA-PACK/TOSOH	2.4	1.5	4.4	6.7
23	Bio-Rad	2.0	1.6	0.9	1.9
24	AutoDELFLIA	4.8	4.2	7.2	5.4

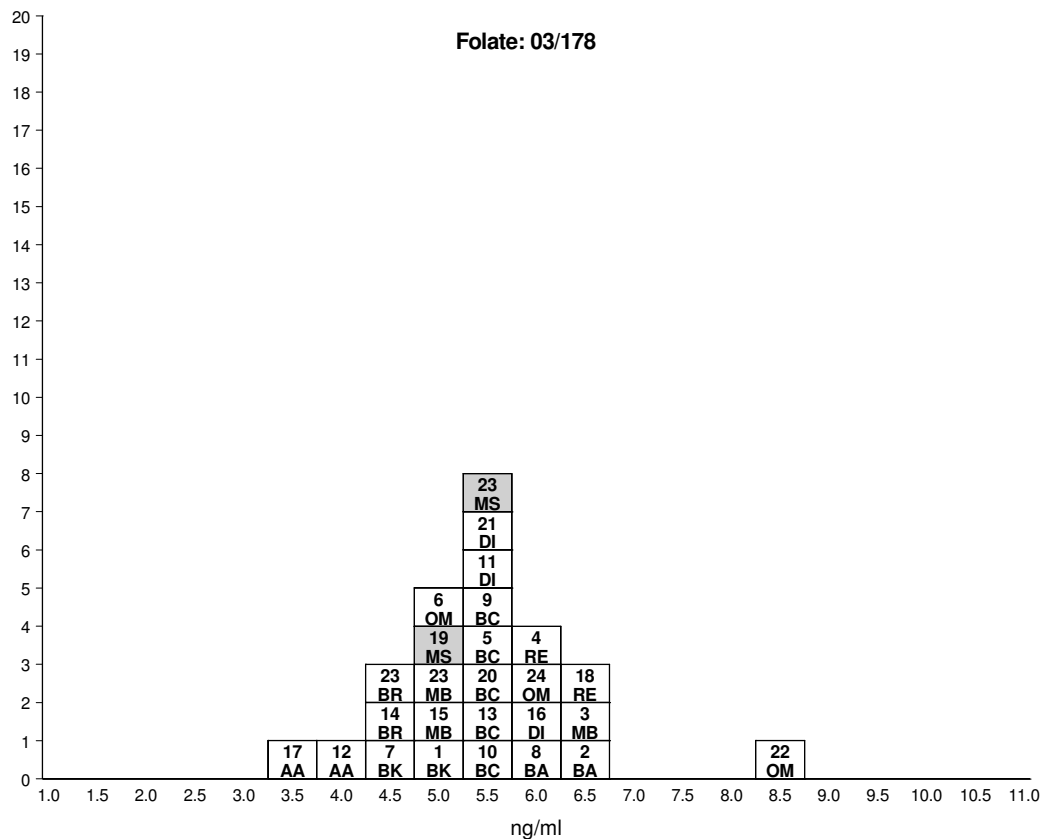
^aLab 17 based on submitted overall means (fax/scan not legible for individual values)

Table 7 Means of B12 content by method and overall study means (pg/ml)

<i>Assay Method</i>	<i>Number of laboratories</i>	<i>Preparation</i>			
		<i>03/178</i>	<i>Sample 1 04/116</i>	<i>Sample 2 04/118</i>	<i>Sample 3 04/120</i>
Abbott Architect	2	429	428	145	241
AutoDELFLIA	1	412	405	158	258
Bayer ACS 180	2	461	474	214	297
Bayer Centaur	5	481	471	200	304
Bayer Immuno 1	1	525	531	271	369
Beckman Access	2	413	390	148	244
Bio-Rad	2	439	465	162	268
DPC Immulite 2000	3	513	532	203	305
Microbiological	1	519	551	241	370
Roche Elecsys/E170	1	522	518	193	305
Tosoh	1	680	739	225	372
Overall Mean	21	480	486	192	296
Between Lab % CV		12.8%	15.7%	18.1%	14.1%

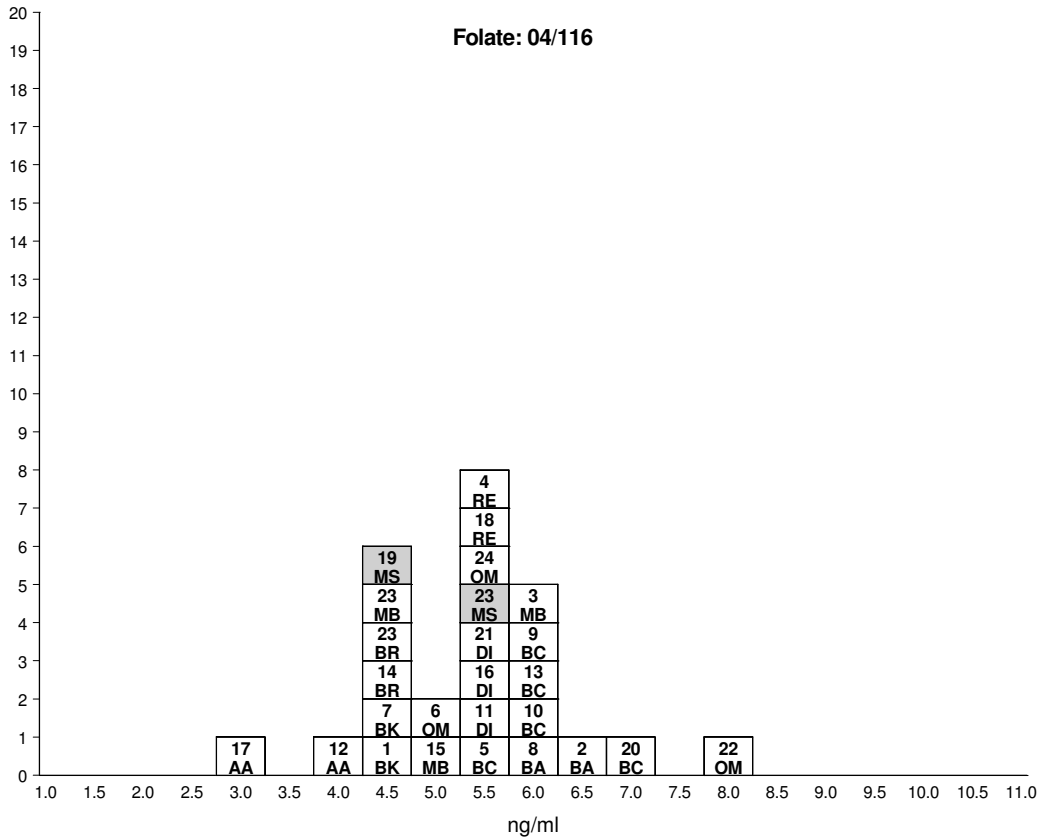
Table 8 Means of B12 content of samples 1, 2 and 3 calculated relative to 03/178 (=480 pg/ml) by method, and overall study means

<i>Assay Method</i>	<i>Number of laboratories</i>	<i>Preparation</i>		
		<i>Sample 1 04/116</i>	<i>Sample 2 04/118</i>	<i>Sample 3 04/120</i>
Abbott Architect	2	479	162	269
AutoDELFLIA	1	475	184	301
Bayer ACS 180	2	492	222	309
Bayer Centaur	5	471	200	304
Bayer Immuno 1	1	485	248	338
Beckman Access	2	454	172	284
Bio-Rad	2	509	177	293
DPC Immulite 2000	3	498	190	285
Microbiological	1	510	223	342
Roche Elecsys/E170	1	476	177	280
Tosoh	1	522	159	263
Overall Mean	21	485	192	296
Between Lab % CV		4.2%	12.6%	7.5%

Figures 1A-D Laboratory mean estimates of the folate content of the study samples**A Folate content of the candidate IS, 03/178**Assay Codes for Folate & B12 studies

AA	Abbott Architect
BA	Bayer ACS
BC	Bayer Centaur
BK	Beckman Access
BR	Bio-Rad
DI	DPC Immulite
MB	Microbiological
MS	LC/MS/MS
OM	Other Methods
RE	Roche Elecsys/170

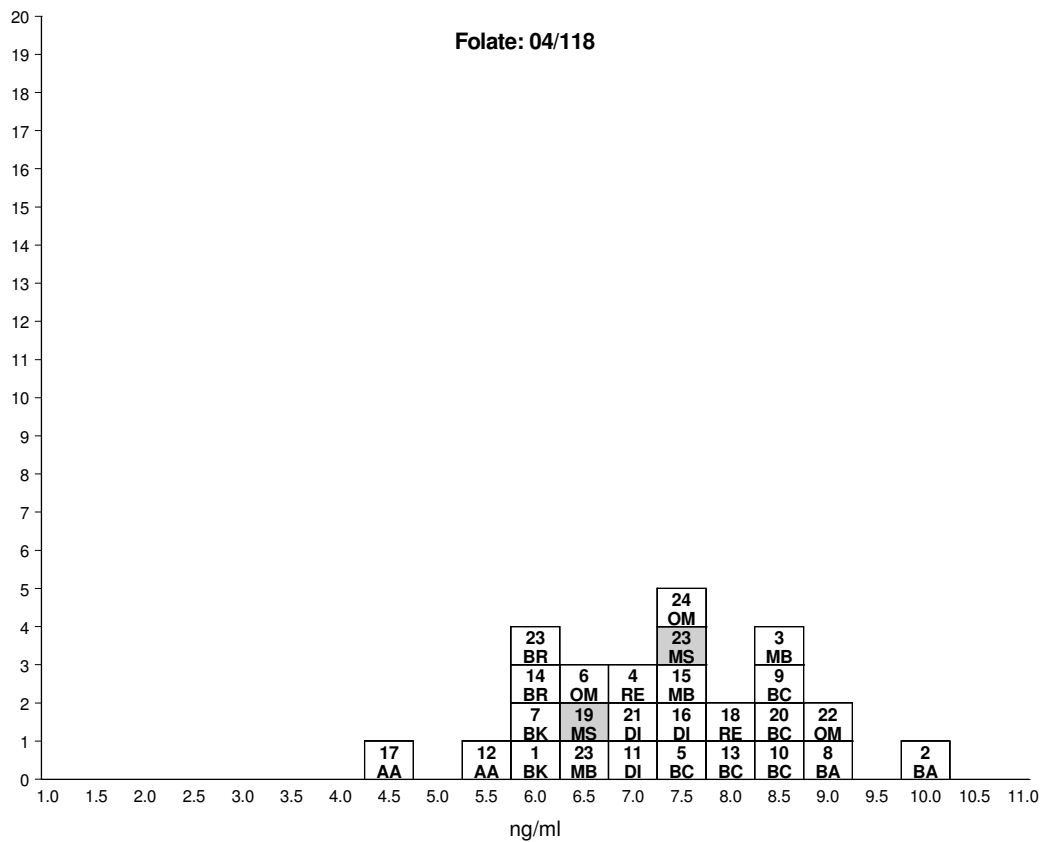
B Folate content of sample 1, 04/116



Assay Codes for Folate & B12 studies

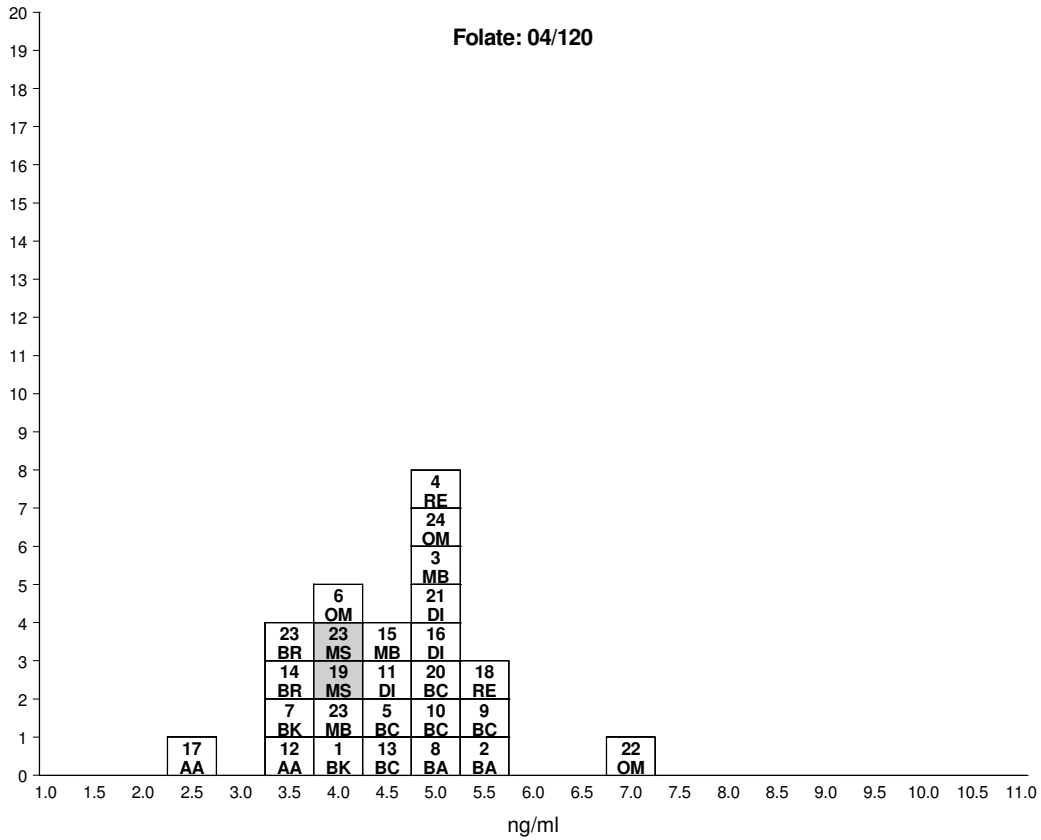
- AA Abbott Architect
- BA Bayer ACS
- BC Bayer Centaur
- BK Beckman Access
- BR Bio-Rad
- DI DPC Immulite
- MB Microbiological
- MS LC/MS/MS
- OM Other Methods
- RE Roche Elecsys/170

C Folate content of sample 1, 04/118

Assay Codes for Folate & B12 studies

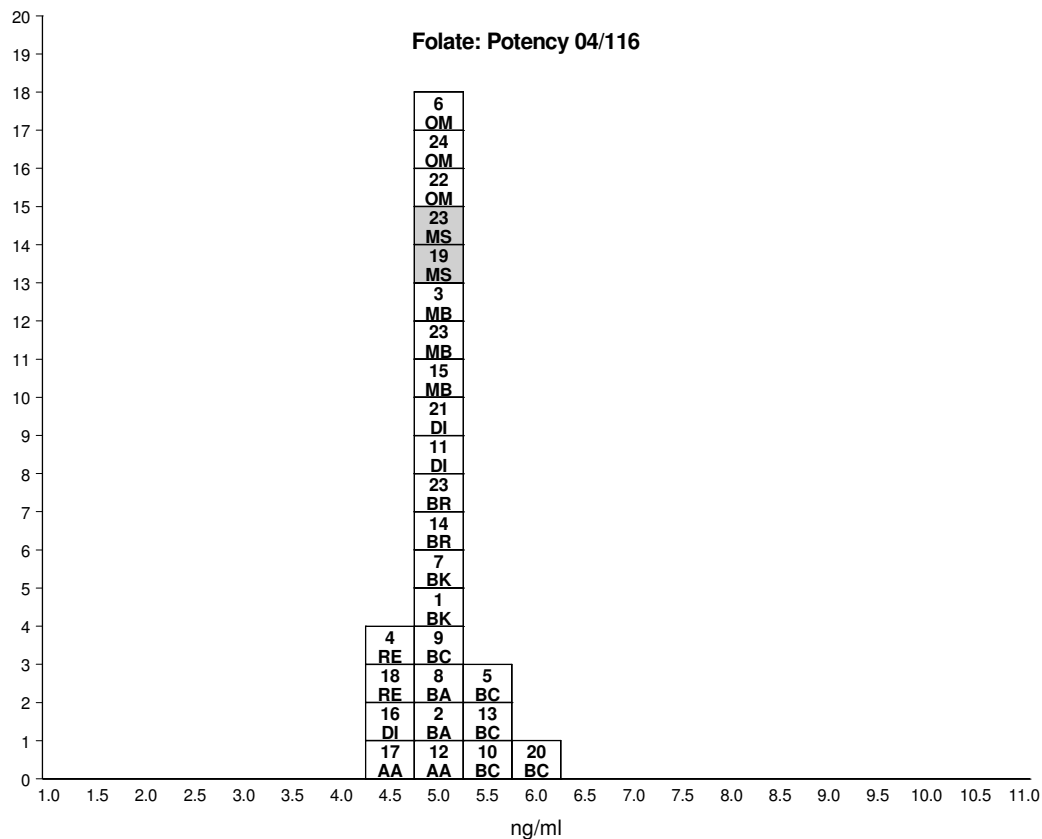
AA	Abbott Architect
BA	Bayer ACS
BC	Bayer Centaur
BK	Beckman Access
BR	Bio-Rad
DI	DPC Immulite
MB	Microbiological
MS	LC/MS/MS
OM	Other Methods
RE	Roche Elecsys/170

D Folate content of sample 1, 04/120



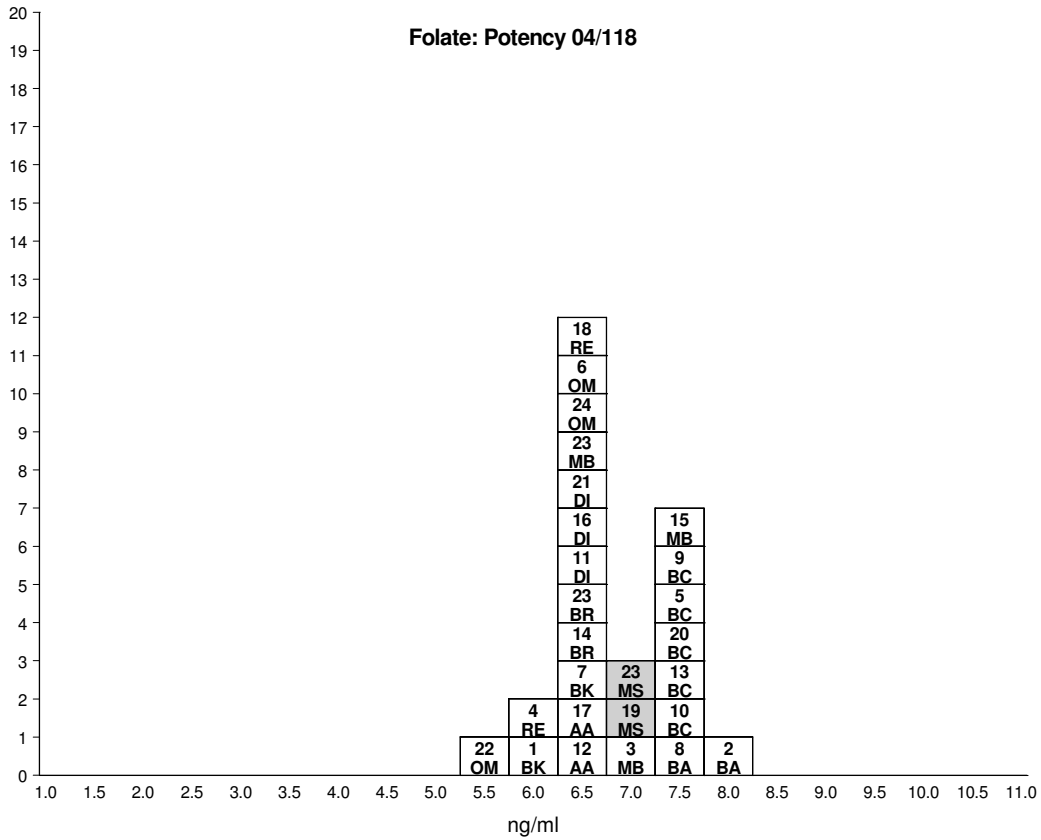
Assay Codes for Folate & B12 studies

- AA Abbott Architect
- BA Bayer ACS
- BC Bayer Centaur
- BK Beckman Access
- BR Bio-Rad
- DI DPC Immulite
- MB Microbiological
- MS LC/MS/MS
- OM Other Methods
- RE Roche Elecsys/170

Figures 2A-C Folate content of samples 1, 2 and 3 relative to the candidate IS, 03/178**A Folate content of sample 1, 04/116, relative to 03/178**Assay Codes for Folate & B12 studies

AA	Abbott Architect
BA	Bayer ACS
BC	Bayer Centaur
BK	Beckman Access
BR	Bio-Rad
DI	DPC Immulite
MB	Microbiological
MS	LC/MS/MS
OM	Other Methods
RE	Roche Elecsys/170

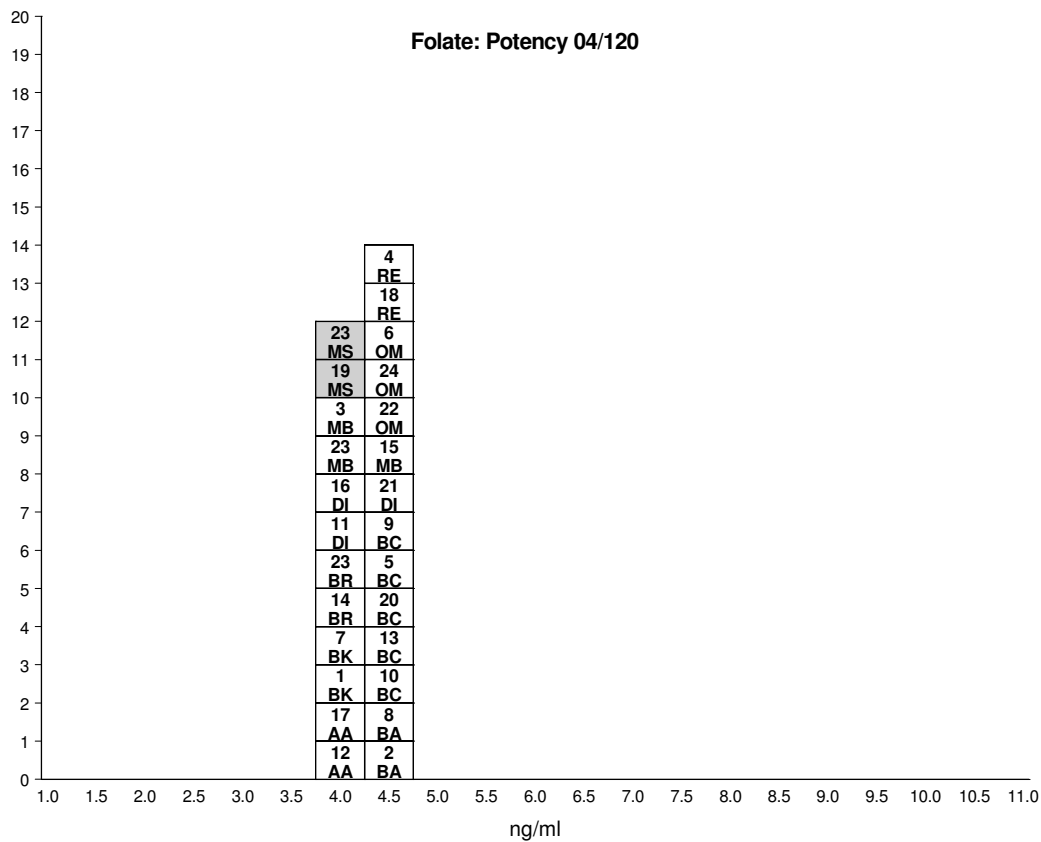
B Folate content of sample 2, 04/118, relative to 03/178



Assay Codes for Folate & B12 studies

- AA Abbott Architect
- BA Bayer ACS
- BC Bayer Centaur
- BK Beckman Access
- BR Bio-Rad
- DI DPC Immulite
- MB Microbiological
- MS LC/MS/MS
- OM Other Methods
- RE Roche Elecsys/170

C Folate content of sample 3, 04/120, relative to 03/178

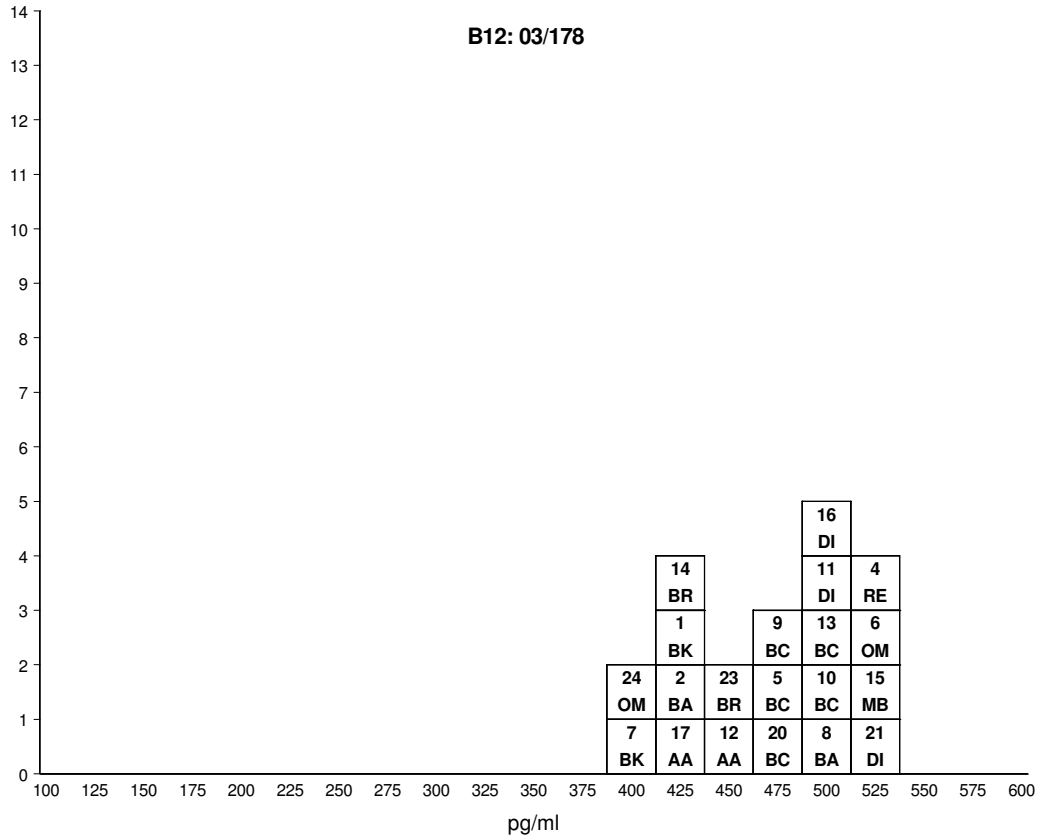


Assay Codes for Folate & B12 studies

AA	Abbott Architect
BA	Bayer ACS
BC	Bayer Centaur
BK	Beckman Access
BR	Bio-Rad
DI	DPC Immulite
MB	Microbiological
MS	LC/MS/MS
OM	Other Methods
RE	Roche Elecsys/170

Figures 3A-D Laboratory mean estimates of the B12 content of the study samples

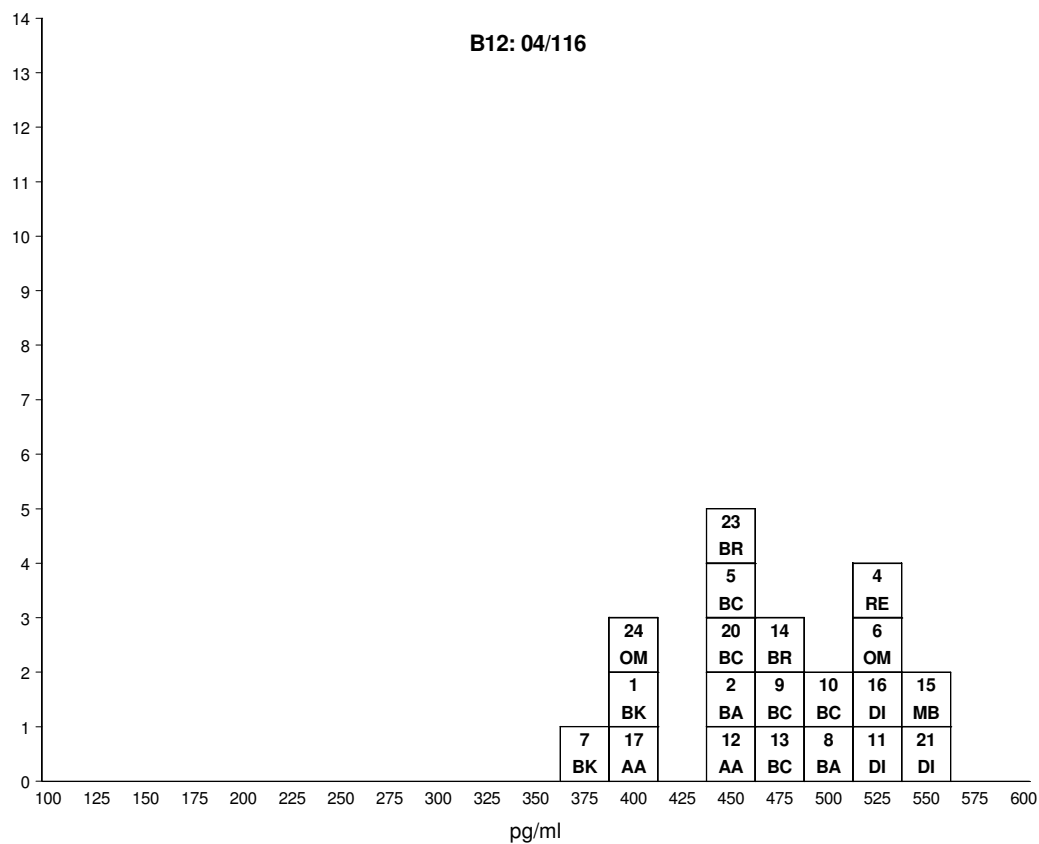
A B12 content of the candidate IS, 03/178



Assay Codes for Folate & B12 studies

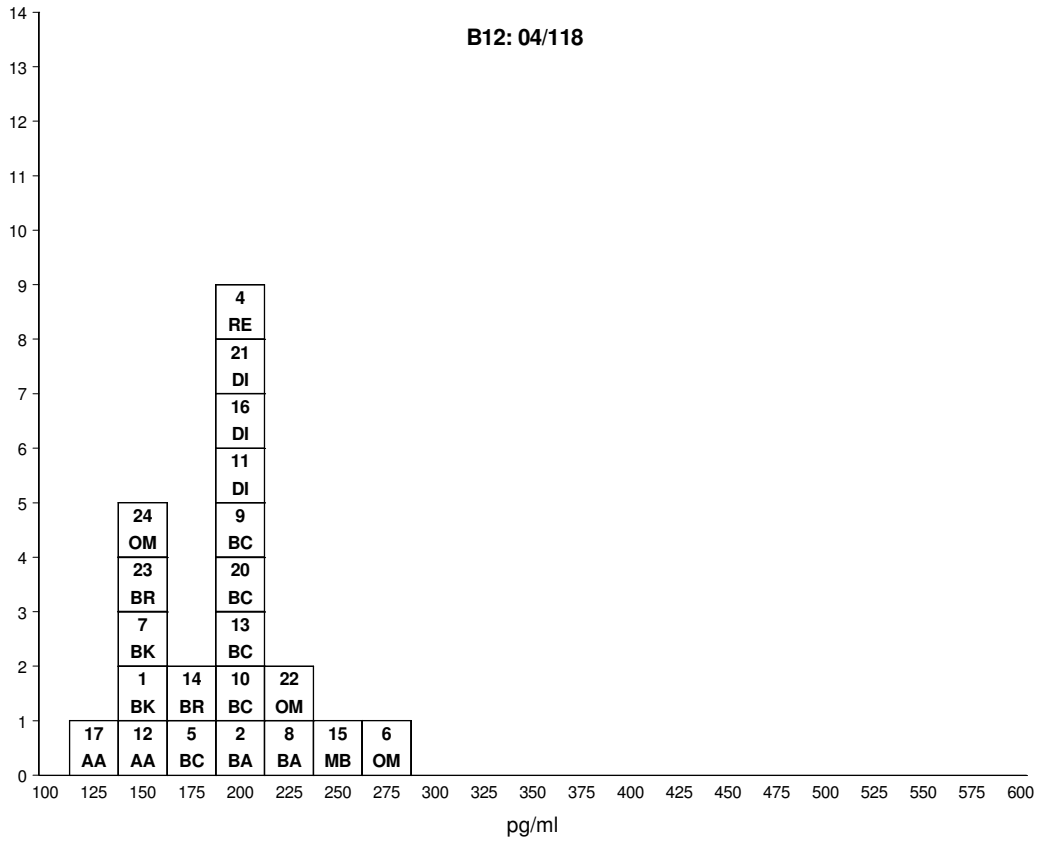
- AA Abbott Architect
- BA Bayer ACS
- BC Bayer Centaur
- BK Beckman Access
- BR Bio-Rad
- DI DPC Immulite
- MB Microbiological
- MS LC/MS/MS
- OM Other Methods
- RE Roche Elecsys/170

B B12 content of sample 1, 04/116

Assay Codes for Folate & B12 studies

AA	Abbott Architect
BA	Bayer ACS
BC	Bayer Centaur
BK	Beckman Access
BR	Bio-Rad
DI	DPC Immulite
MB	Microbiological
MS	LC/MS/MS
OM	Other Methods
RE	Roche Elecsys/170

C B12 content of sample 2, 04/118

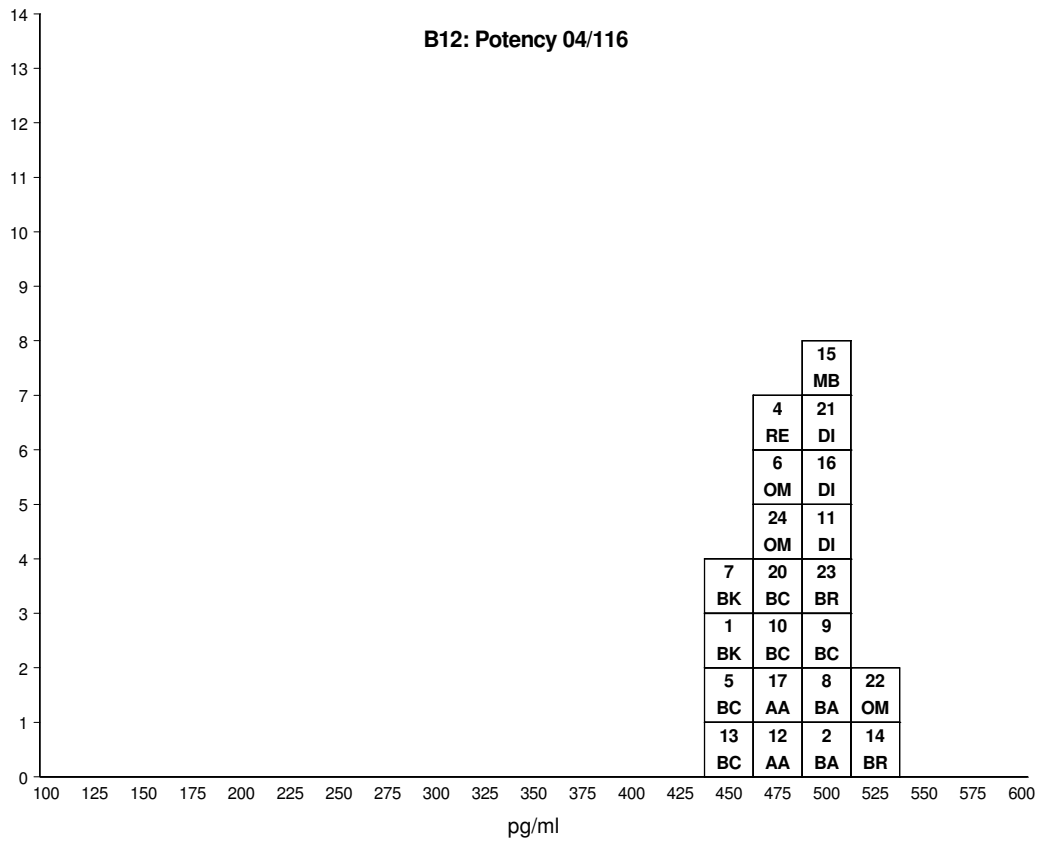


Assay Codes for Folate & B12 studies

- AA Abbott Architect
- BA Bayer ACS
- BC Bayer Centaur
- BK Beckman Access
- BR Bio-Rad
- DI DPC Immulite
- MB Microbiological
- MS LC/MS/MS
- OM Other Methods
- RE Roche Elecsys/170

Figures 4A-C B12 content of samples 1, 2 and 3 relative to the candidate IS, 03/178

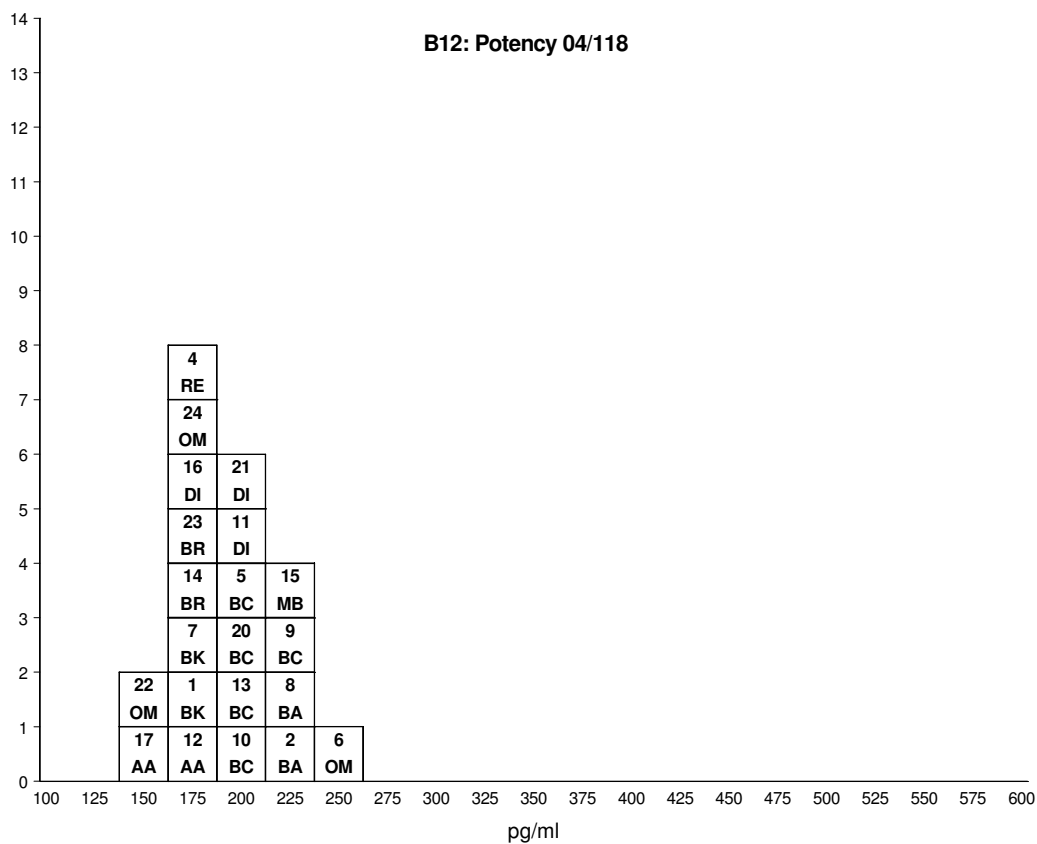
A B12 content of sample 1, 04/116, relative to 03/178



Assay Codes for Folate & B12 studies

- AA Abbott Architect
- BA Bayer ACS
- BC Bayer Centaur
- BK Beckman Access
- BR Bio-Rad
- DI DPC Immulite
- MB Microbiological
- MS LC/MS/MS
- OM Other Methods
- RE Roche Elecsys/170

B B12 content of sample 2, 04/118, relative to 03/178

Assay Codes for Folate & B12 studies

AA	Abbott Architect
BA	Bayer ACS
BC	Bayer Centaur
BK	Beckman Access
BR	Bio-Rad
DI	DPC Immulite
MB	Microbiological
MS	LC/MS/MS
OM	Other Methods
RE	Roche Elecsys/170

Appendix 1 Collaborative study participants (in alphabetical order of country)

Stefaan Marivoet and Miranda Van Hoof, Tosoh Bioscience, Tessenderlo, Belgium
Ebba Nexø and Frode Engbaek, Aarhus University Hospital, Aarhus, Denmark
Tuija Halonen and ,PerkinElmer Life and Analytical Sciences, Turku, Finland
Nicholas R Hoyle and Anja Ruschel, Roche Diagnostics GmbH, Penzberg, Germany
Sean O’Broin, St James’s Hospital, Dublin, Ireland
Dianne Bamber, Euro DPC (UK) Ltd, Caernarfon, Gwynedd UK
Kevin Knaggs, Freeman Hospital, Newcastle upon Tyne, UK
Tony Wright and Paul Finglas, Institute of Food Research, Norwich, UK
Philip Day, Leeds General Hospital, Leeds, UK
Steve Cummings, Ninewells Hospital, Dundee, UK
Alan James, Royal Devon and Exeter Hospital, Exeter, UK
Neil Porter and Paula Forrest, Royal Hallamshire Hospital, Sheffield, UK
Richard Webber, Royal Hospital for Sick Children, Glasgow, UK
Graham Thomas and Rachel Scott, Russells Hall Hospital, Dudley, UK
Sarah Brown, St Helier Hospital, Carshalton, Surrey, UK
Penny Clarke and Geoff Holder, Selly Oak Hospital, Selly Oak, Birmingham UK
Anne Lee, UKNEQAS, Good Hope Hospital, West Midlands UK
Peter Bernard, Worcestershire Royal Hospital NHS Trust, Worcester, UK
Huaiqin Wu, Abbott Laboratories, IL, USA
Lillian Mansbach and Melissa Todd, Bayer Healthcare, East Walpole, MA, USA
Angela Curtis, Bio-Rad Laboratories Inc, Hercules, CA, US
Christine Pfeiffer, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA, USA
Bryant Nelson, NIST, Gaithersburg, MD, USA
Ralph Green and Joshua W Miller, University of California, Davis, Sacramento, CA USA

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