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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

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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 Also, it was calibrated using a microbiological assay (Euglena assay) which has since been replaced by automated assay systems.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 hydroxylmethyl 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^{o}C except for a small number that were stored at -70^{o}C, +4^{o}C, +20^{o}C, +37^{o}C and +45^{o}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). Fill details are summarised below:

	Candidate IS 03/178	***Sample 1 04/116***	***Sample 2 04/118***	***Sample 3 04/120***
Mean weight of the dispensed solution (number of fill weights measured)	1.0062g (46)	1.0057g (6)	1.0067 (6)	1.0060
Imprecision of the filling (coefficient of variation)	0.08%	0.13%	0.11%	0.12%
Residual moisture	0.8%	0.15%	0.26%	0.19%
Number of ampoules for distribution as WHO reference reagent	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°C and +45°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.

Temperature	***B12***	***Folate***
-20°C	106.4%	113.6%
+37°C	105%	109.1%
+45°C	91%	96.7%

In both cases, the results at -20°C and +37°C are higher than at -70°C; the results at +45°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:

Laboratory	***5MeTHF***	***5FoTHF***	***FA***	***Total***	
	ng/ml	***ng/ml***	***ng/ml***	***nmol/L***	***ng/ml****
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

[a]Molecular weights: 5MeTHF 459.46; 5FoTHF 473.4; FA 441.4
[b]Day 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.

Laboratory	*Assay Method*	*B12 pg/ml*					*Folate ng/ml*			
		03/178	*Sample 1*	*Sample 2*	*Sample 3*	*81/563*	*03/178*	*Sample 1*	*Sample 2*	*Sample 3*
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	AutoDELFIA	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**

[a]candidate reference methods
[b]sum of ng/ml values
nd = not determined

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

Laboratory	*Assay Method*	*Intra-Lab % CV*			
		03/178	*Sample 1*	*Sample 2*	*Sample 3*
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	AutoDELFIA	1.7	1.9	2.2	3.4

[a]Lab 17 based on submitted overall means (fax/scan not legible for individual values)
[b] candidate reference methods
[c]Lab 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)

Assay Method	*Number of laboratories*	*Preparation*			
		03/178	*Sample 1 04/116*	*Sample 2 04/118*	*Sample 3 04/120*
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

Assay Method	*Number of laboratories*	*Preparation*		
		Sample 1 04/116	*Sample 2 04/118*	*Sample 3 04/120*
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

Laboratory	*Assay Method*	*Intra-Lab % CV*			
		03/178	*Sample 1*	*Sample 2*	*Sample 3*
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	AutoDELFIA	4.8	4.2	7.2	5.4

[a]Lab 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)

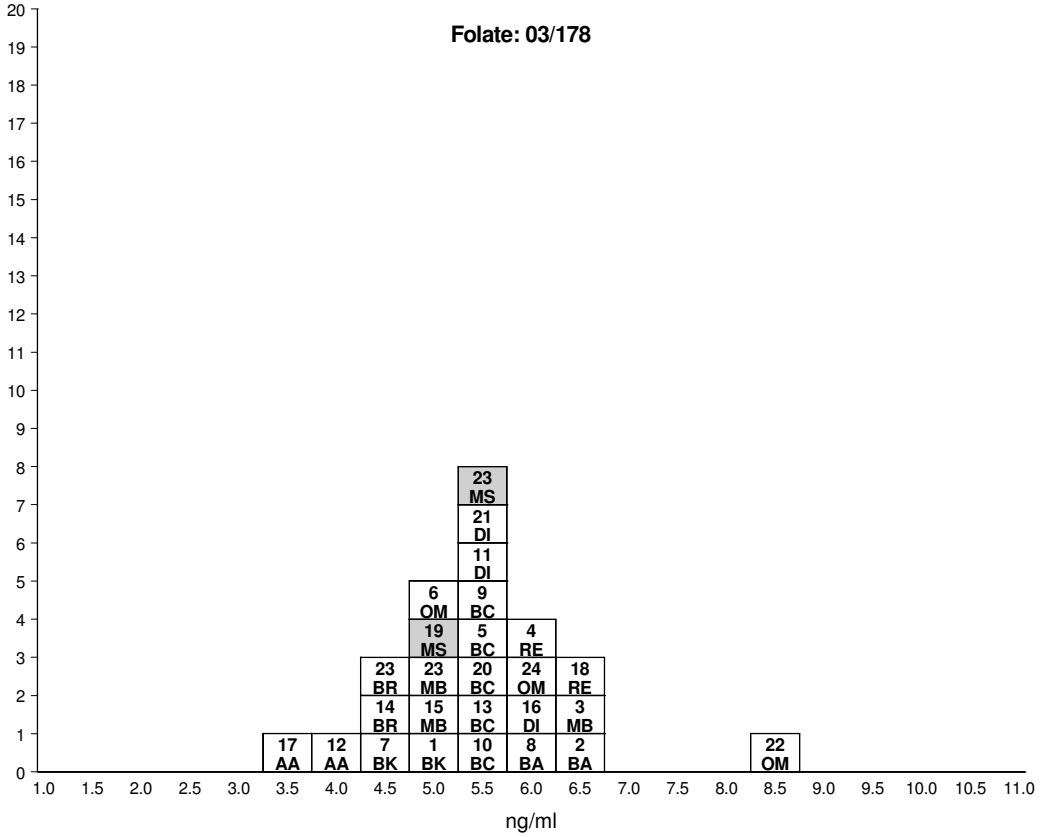
Assay Method	*Number of laboratories*	*Preparation*			
		03/178	*Sample 1 04/116*	*Sample 2 04/118*	*Sample 3 04/120*
Abbott Architect	2	429	428	145	241
AutoDELFIA	1	412	405	158	258
Bayer ACS 180	2	461	474	214	297
Bayer Centaur	5	481	471	200	304
Bayer Immuno 1	1	525	531	271	369
Beckman Access	2	413	390	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

Assay Method	*Number of laboratories*	*Preparation*		
		Sample 1 04/116	*Sample 2 04/118*	*Sample 3 04/120*
Abbott Architect	2	479	162	269
AutoDELFIA	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

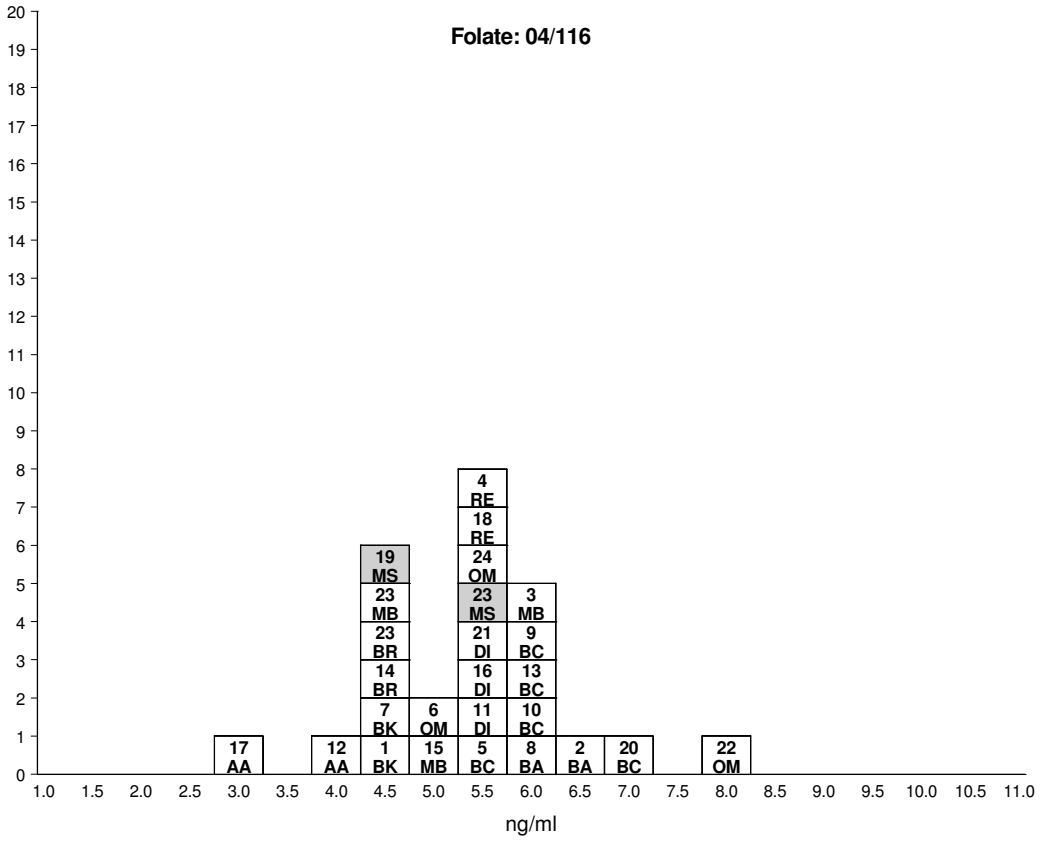
A Folate content of the candidate IS, 03/178



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

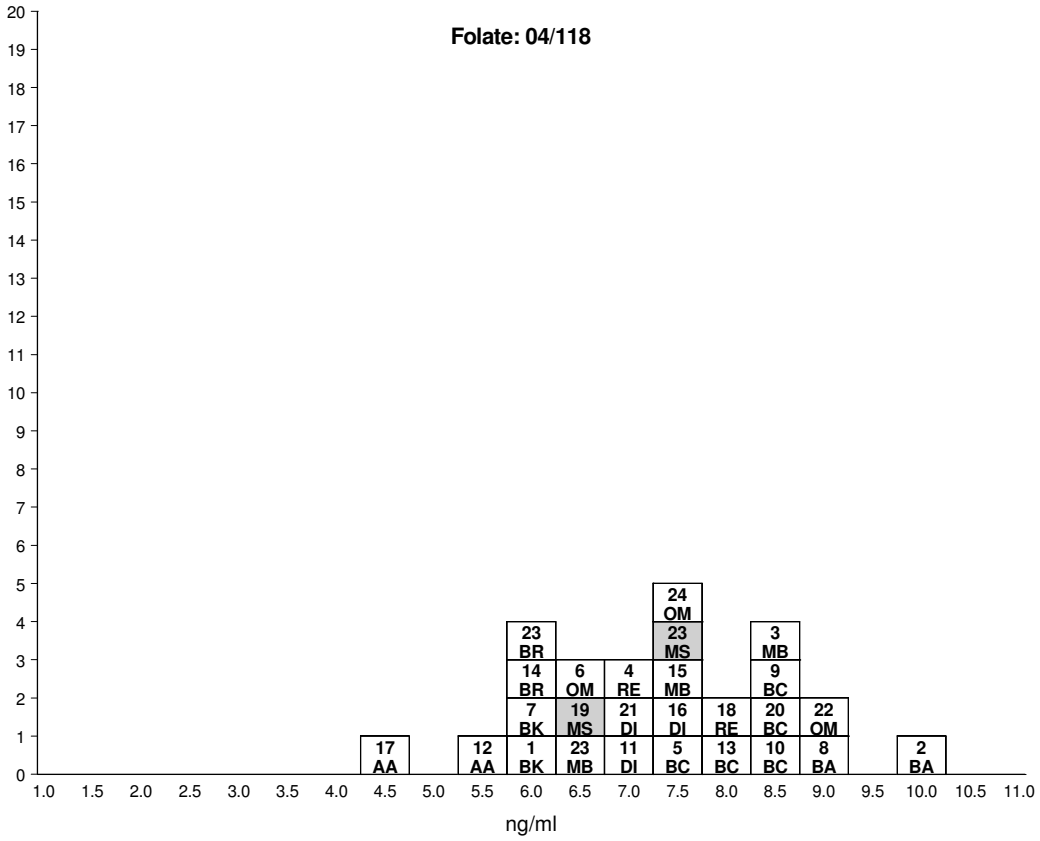
B Folate content of sample 1, 04/116



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

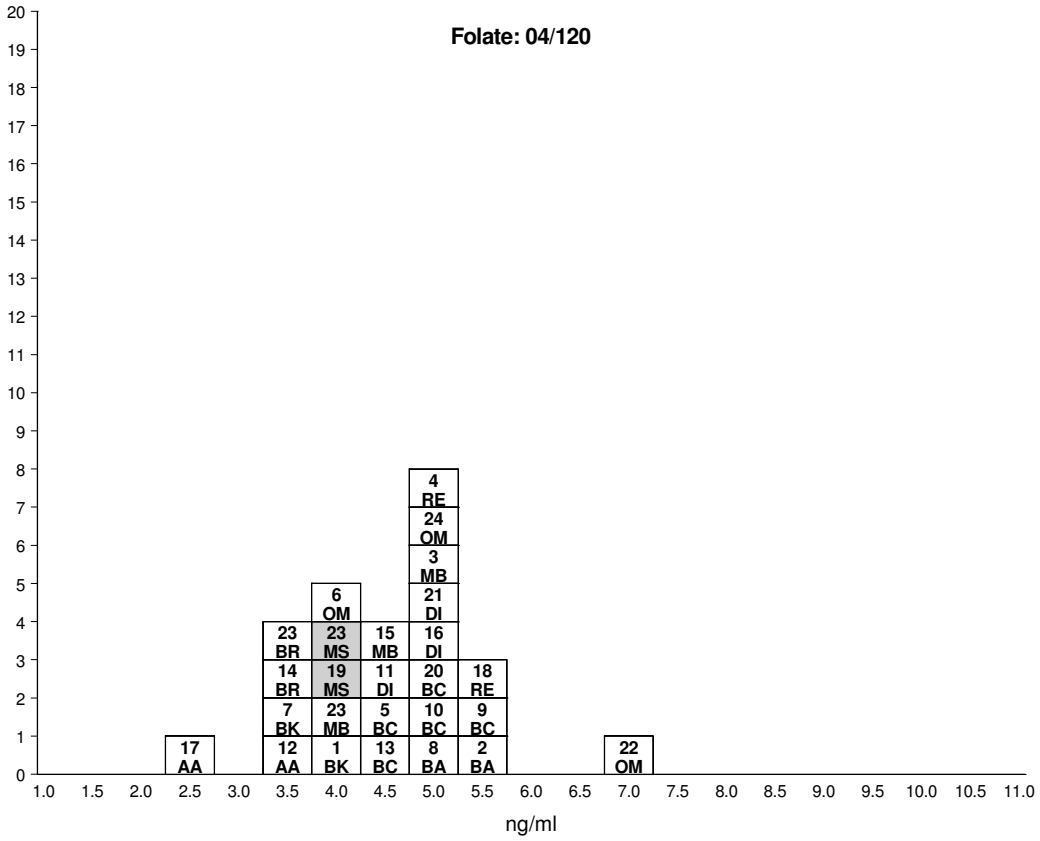
C Folate content of sample 1, 04/118



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

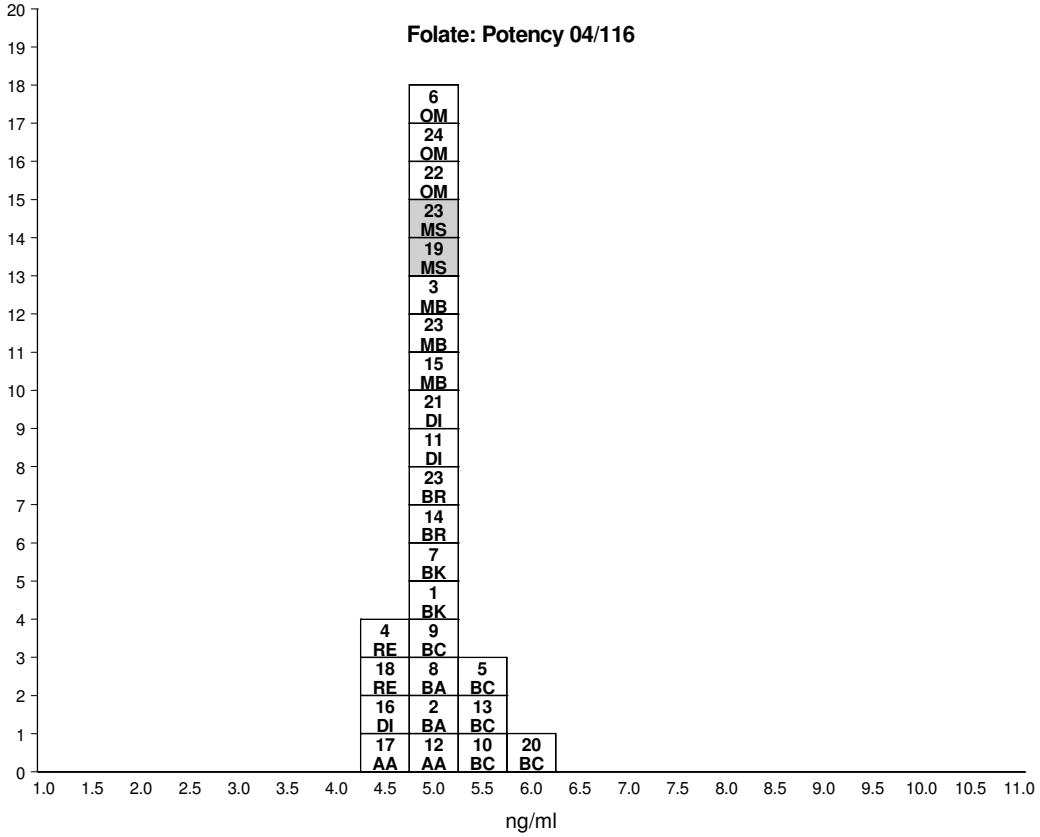


Assay Codes for Folate & B12 studies

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

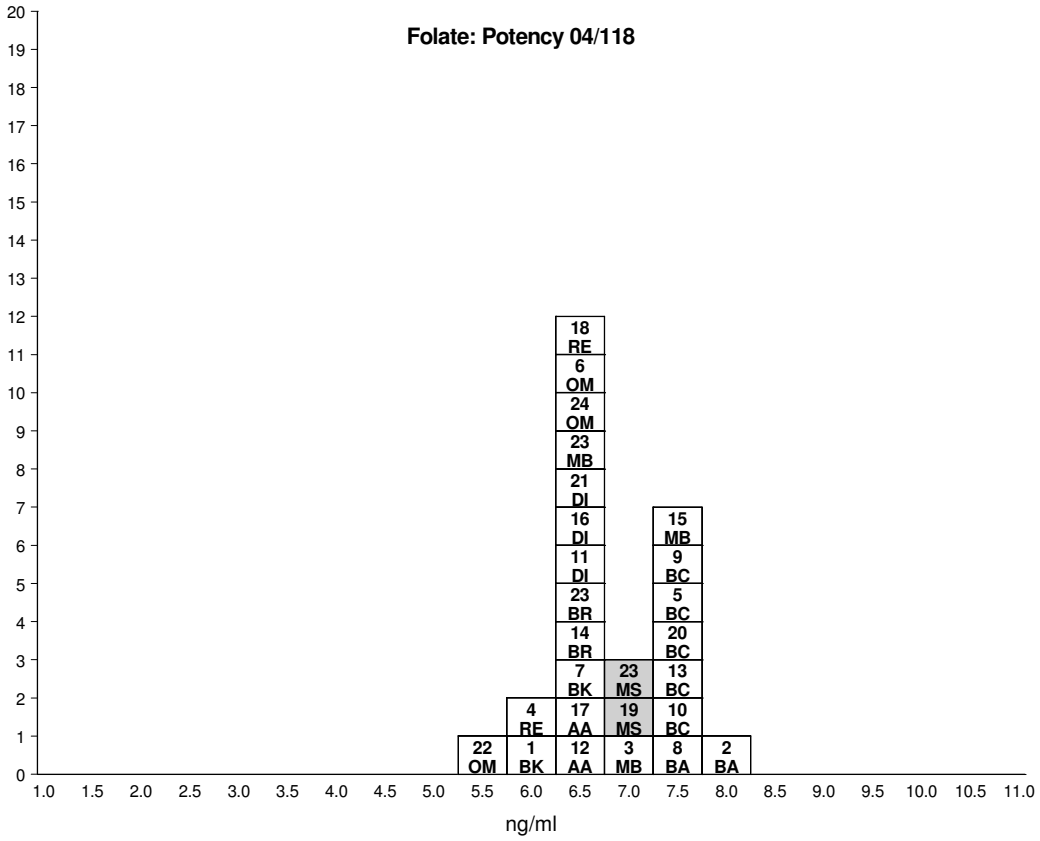
A Folate content of sample 1, 04/116, relative to 03/178



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

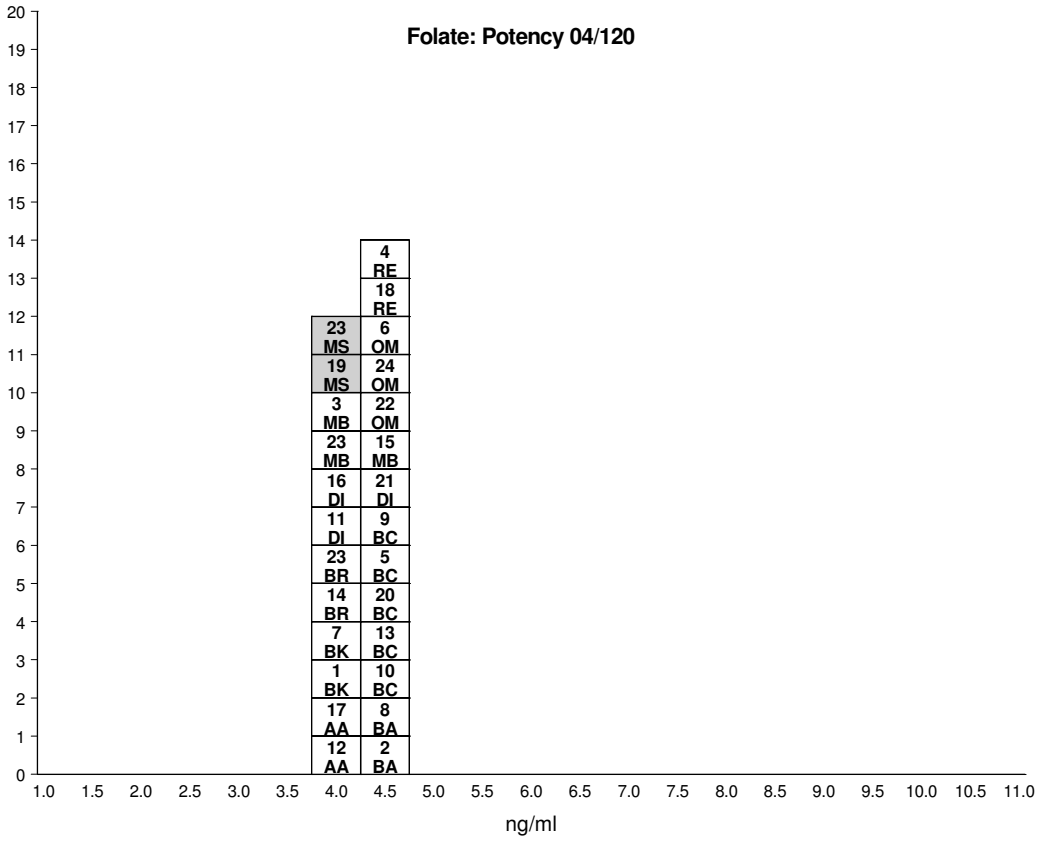
B Folate content of sample 2, 04/118, relative to 03/178



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

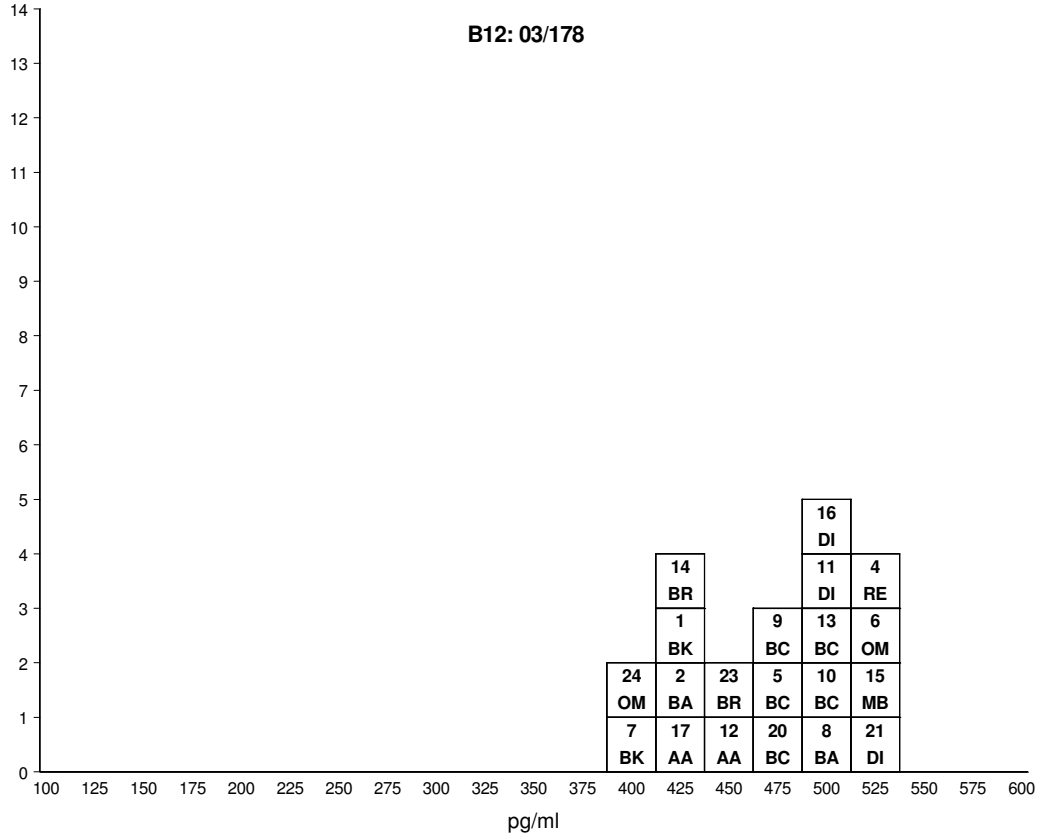


Assay Codes for Folate & B12 studies

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

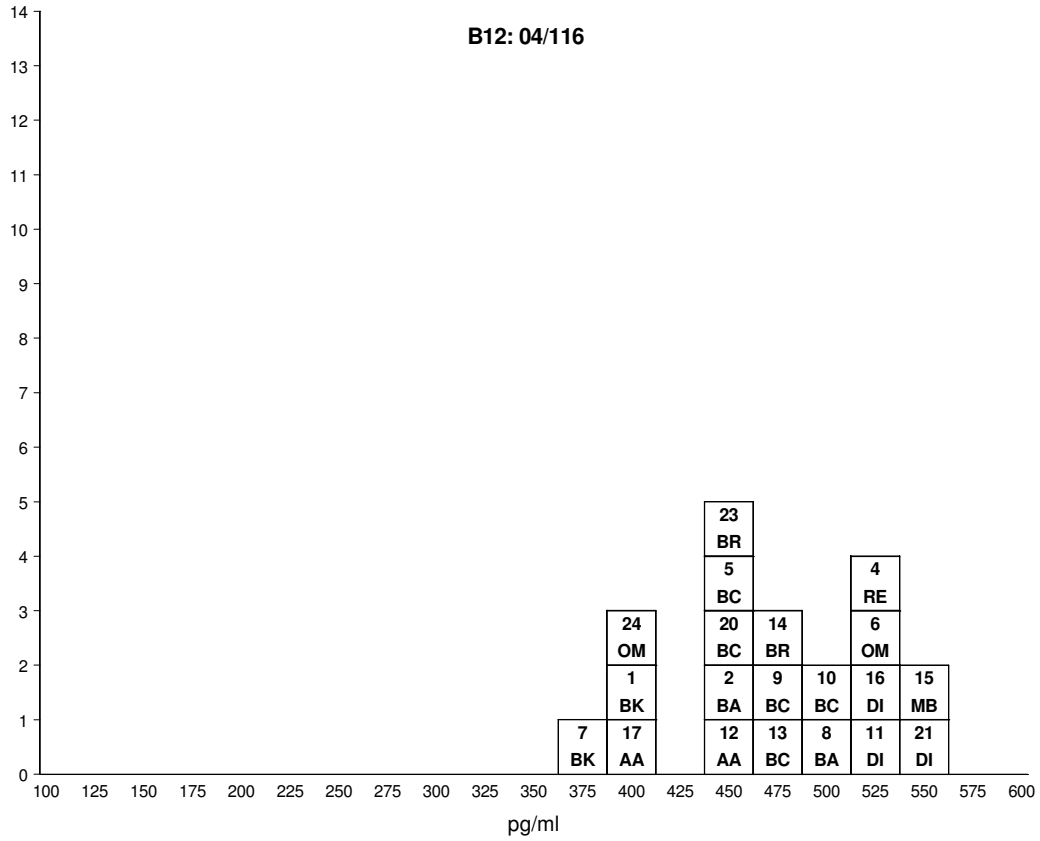
A B12 content of the candidate IS, 03/178



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

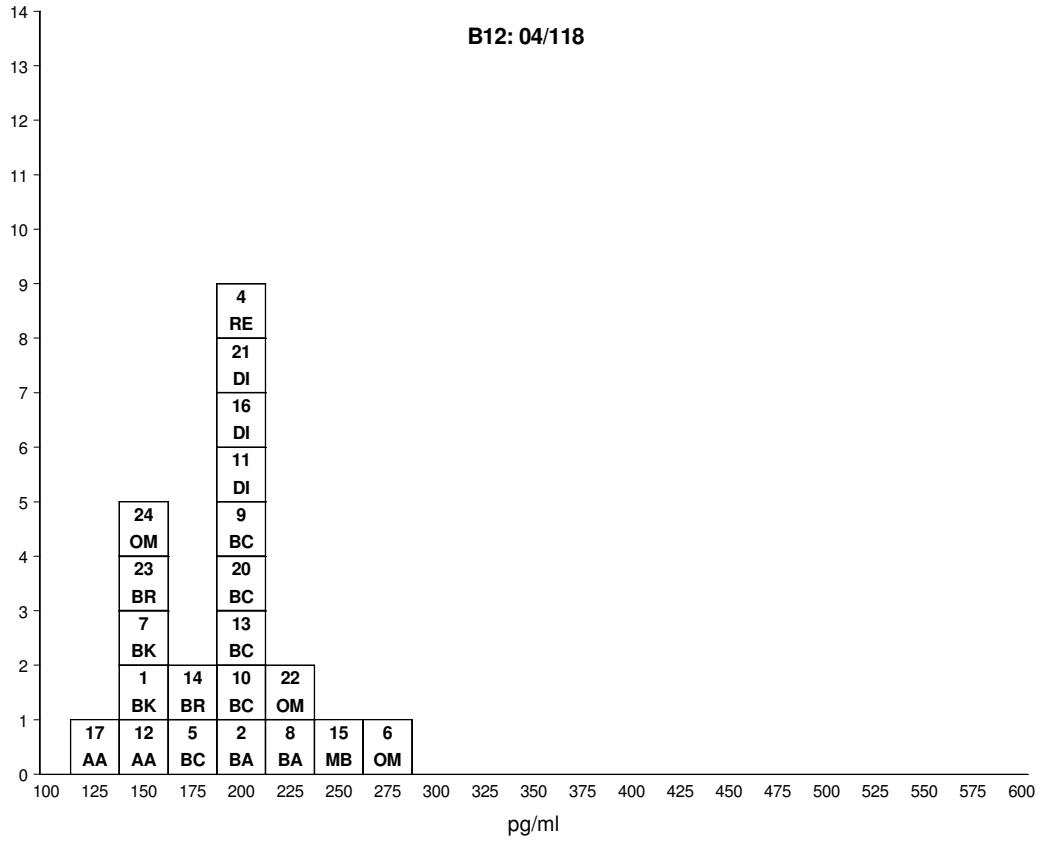
B B12 content of sample 1, 04/116



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

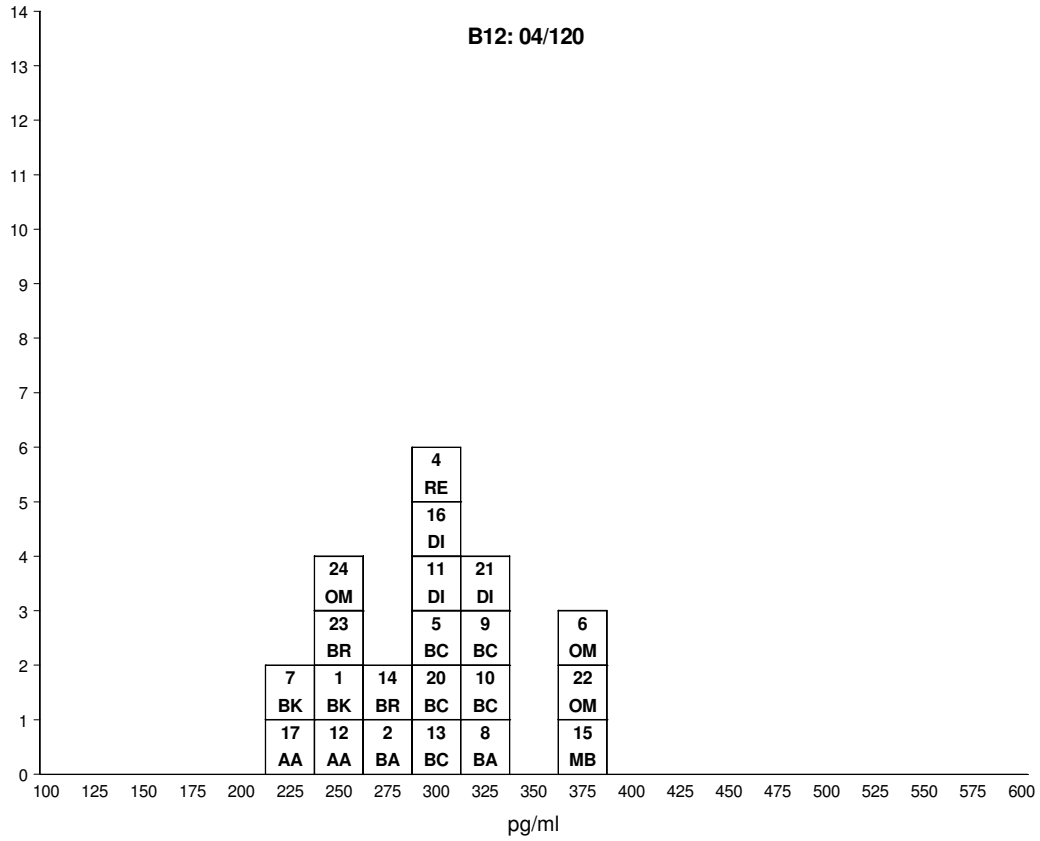
C B12 content of sample 2, 04/118



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

D B12 content of sample 3, 04/120

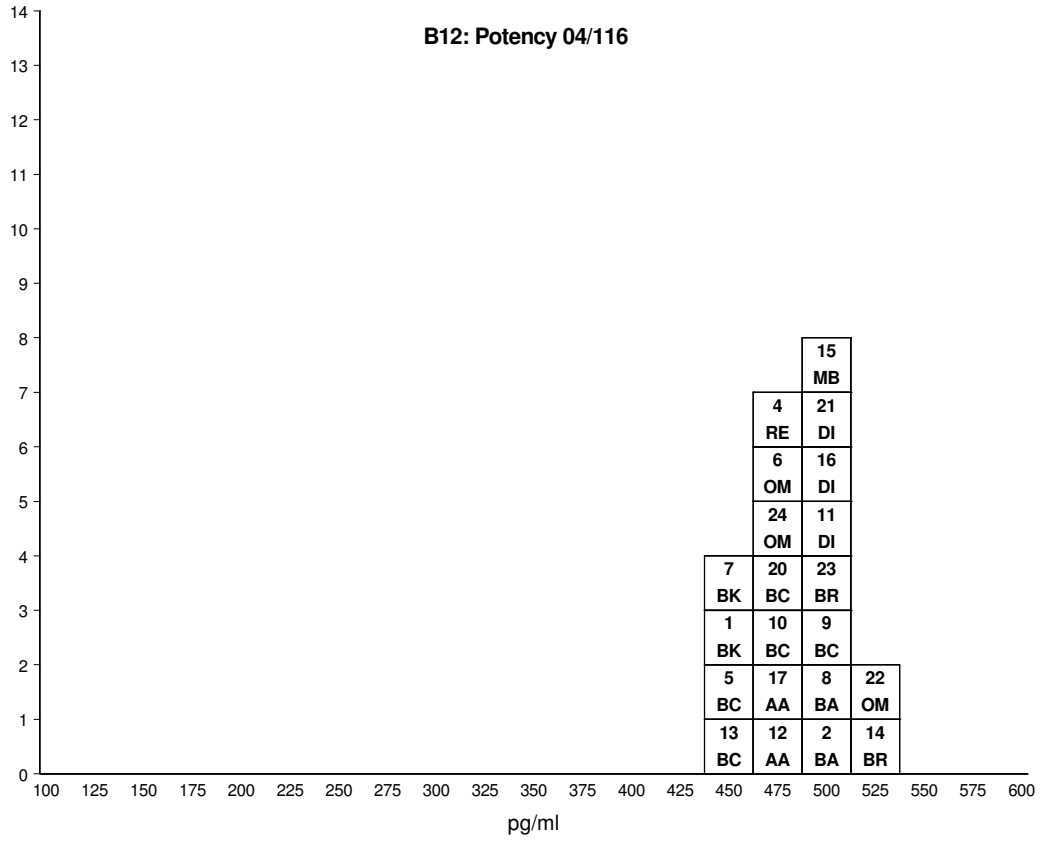


Assay Codes for Folate & B12 studies

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

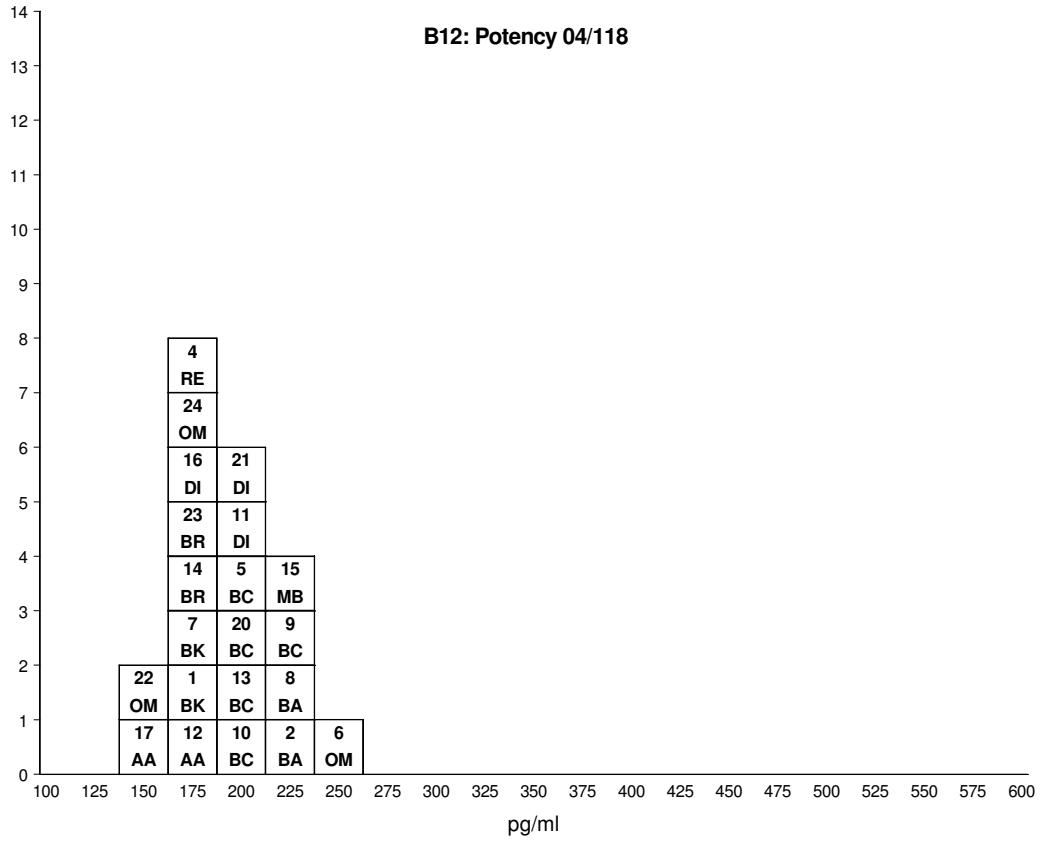
A B12 content of sample 1, 04/116, relative to 03/178



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

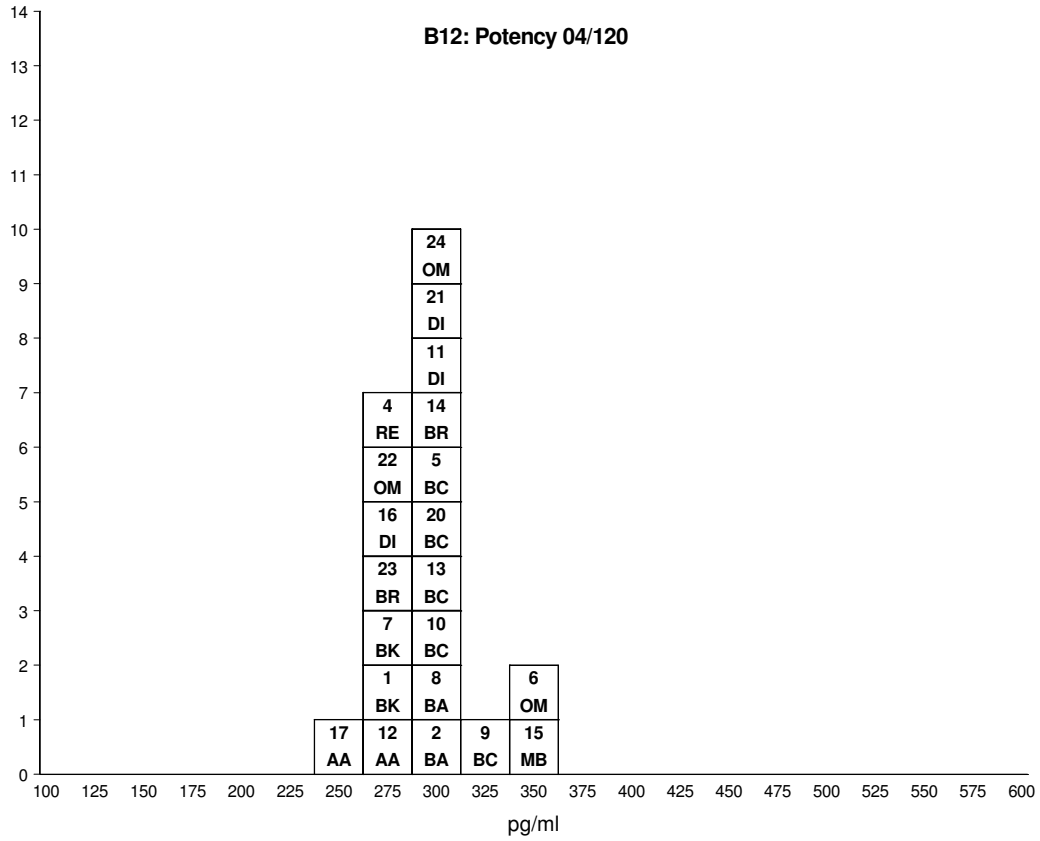
B B12 content of sample 2, 04/118, relative to 03/178



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

C B12 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

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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World Health Organization

WHO/BS/ 2015.2263
ENGLISH ONLY

EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 12 to 16 October 2015

International Standard for holotranscobalamin (holoTC)
Report of the international collaborative study to assign a holoTC value to the International Standard for folate and B12 (03/178)

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Note:

This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **14 September 2015** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr M Nübling** at email: nueblingc@who.int

Summary
There is evidence that holotranscobalamin (holoTC), the active portion of B12, is a better marker of early B12 deficiency than total B12. The recent availability of commercial assays for holoTC prompted an international collaborative study to assign a holoTC value to the 1st International Standard (IS) for serum folate and B12, 03/178.
The IS, 03/178, was assayed by 12 laboratories in 8 countries using manual and automated immunoassays for holoTC; one laboratory additionally performed an in-house assay. Fourteen sets of data were analysed.
An overall geometric mean value of 106.8 pmol/L was obtained, with an inter-laboratory GCV of 10.5%. The inclusion of three serum samples in the study, with different holoTC levels, demonstrated a reduction in inter-laboratory variability when the holoTC levels in the samples were determined relative to the IS (holoTC value of 106.8 pmol/L assigned) rather than to the assays' calibration.
It is recommended that a holoTC value of 107 pmol/L is assigned to 03/178 for use as the 1st International Standard for folate, B12 and holoTC.

Background
Vitamin B12 is a porphyrin-like molecule containing cobalt (termed cobalamin) which is necessary for the transformation of methyltetrahydrofolate to tetrahydrofolate for DNA synthesis. B12 deficiency can therefore lead to functional folate deficiency and impaired DNA synthesis, resulting in pernicious (megaloblastic) anaemia [reviewed in 1].
Approximately 20% of circulating B12 is bound to the protein carrier transcobalamin. This complex, holotranscobalamin (holoTC), is the active portion of B12 available to cells. There is evidence that holoTC is a better marker of early B12 deficiency than total B12 [2, 3]. Furthermore, holoTC measurement has additional advantages over B12 measurement: it may give fewer indeterminate results, particularly for patients over 65; holoTC assays give a better indication of B12 status in pregnancy and for patients taking oral contraceptives [3, 4].
The WHO International Standard for serum folate and vitamin B12 (03/178) was established in 2005 with an assigned folate value of 12.1 nmol/L (5.33 ng/mL) and an assigned B12 value of 480 pg/mL [5]. Assays for the measurement of holoTC are now commercially available [6, 7] and it is therefore proposed to assign a holoTC value to 03/178 following an international collaborative study. The availability of an international reference material for holoTC will facilitate further development of assay methodologies, and ensure continuity of unitage and comparability of results across methods.

Materials and methods
International Standard for vitamin B12 and serum folate (03/178) and serum samples
The IS for vitamin B12 and serum folate, 03/178, consists of a pool of human serum from seven donors, kindly donated by the UK NEQAS Scheme for Haematinics, lyophilised in glass ampoules (~1 mL/ampoule; CV 0.08%) [5]. Three serum samples, known to vary in their holoTC content, were similarly lyophilised for use in the collaborative study. Fill details of 03/178 are available on FileDirector at NIBSC.

Collaborative study participants

A total of 12 laboratories in 8 countries participated in the study (Appendix 1), returning 14 sets of data. Each laboratory was assigned a code number, which does not reflect the order of listing. The participants included manufacturers, clinical laboratories and research laboratories.

Methods

Laboratories performed commercial immunoassays (Axis-Shield Diagnostics) utilising holoTC-specific antibodies, in manual and/or automated (Abbott Architect) formats, and standardised by the manufacturer with recombinant holoTC. One laboratory additionally performed an in-house method [9]. The methods used by participating laboratories are shown in Appendix 2.

Study Design

Each participant was provided with 3 ampoules of each of the IS, 03/178, and serum samples 1, 2 and 3. Participants were requested to reconstitute ampoule contents with 1 mL distilled or deionised water on the day of assay. They were asked to assay two independent doubling dilution series of each preparation on each of three days, using fresh ampoules each day, to give a total of 3 estimates for each of 03/178 and samples 1, 2 and 3. Based on the known approximate levels of holoTC in the study samples, participants were requested to assay 03/178 and serum sample 3 from neat to 1/16 dilution, serum sample 2 from neat to 1/8 dilution, and serum sample 1 neat and diluted 1/2 only. Participants were requested to return their results (as raw data or holoTC concentrations in pmol/L for each dilution) on results sheets provided. The study protocol is given in Appendix 3.

Statistical Analysis

Results in pmol/L reported by the participants for 03/178 and serum samples 1, 2 and 3 were corrected for dilution factor and used for further analysis. On day 3, laboratory 12 analysed each replicate dilution series using a different reagent kit lot; these were treated as individual assays. All mean results shown in this report are unweighted geometric means (GM). Variability between assays within laboratories and between laboratories was expressed using geometric coefficients of variation (GCV = $\{10^{s}-1\}\times 100\%$ where s is the standard deviation of the $\log_{10}$ transformed estimates). Comparison of $\log_{10}$ pmol/L results was carried out in Minitab 17 (Minitab. Inc., State College, PA, USA) by fitting a general linear model with laboratory and dilution as factors, with post-hoc Tukey's test being used to compare results obtained using different dilutions. Grubbs' test on log potencies was used to identify any outlier laboratory mean results.

Acquisition of bulk material for a replacement standard

Serum for a replacement for 03/178 would most likely be obtained in another collaboration with UK NEQAS for Haematinics.

Results

Estimated holoTC content of 03/178

An overall analysis to compare results obtained using different dilutions, as described above, showed no significant differences between the 1/2, 1/4 and 1/8 dilutions. Final results were calculated using these dilutions only. The holoTC values (in pmol/L) for individual assays along with the laboratory geometric means and intra-laboratory variability, expressed as %GCV, are

shown in Table 1 and Figure 1. Intra-laboratory repeatability was good with all laboratories having GCVs below 10%, with the exception of laboratory 11. An overall geometric mean value of 106.8 pmol/L was obtained, with an inter-laboratory GCV of 10.5% (Table 1).

Parallelism of serum samples and 03/178

Parallelism of the dose-response lines for 03/178 with serum samples 2 and 3 was assessed using the slope ratios shown in Table 2. Serum sample 1 was not considered as only two dilutions of this sample were tested. Taking 0.80 – 1.25 as a typical range of slope ratios that can be used to conclude parallelism, the data demonstrated an acceptable level of parallelism. Slope ratios less than 0.80 and greater than 1.25 were noted in only 6 (7%) and 4 cases (5%), respectively, i.e., similar numbers above and below the range illustrating no overall 'trend' in the slope ratios.

Estimated holoTC content of serum samples 1, 2 and 3

As for 03/178, final results were calculated after exclusion of some dilutions for serum sample 2 (1/8 excluded) and serum sample 3 ('neat' and 1/16 excluded). The holoTC values (in pmol/L) for individual assays, relative to the assay calibrants, along with the laboratory geometric means and intra-laboratory variability, expressed as %GCV, are shown in Tables 3, 4 and 5, and Figure 1. The estimated holoTC content of the serum samples relative to 03/178 is also shown in Tables 3, 4 and 5, and Figure 1, where the pmol/L values were calculated by taking the content of 03/178 to be 106.8 pmol/L.

For all serum samples, inter-laboratory %GCV values were lower for the relative potencies (12.3%, 14.9% and 9.9% for serum samples 1, 2 and 3, respectively) when compared to those for directly estimated pmol/L contents (17.3%, 19.6% and 15.6%, respectively). For serum samples 2 and 3, laboratory 4a was noted as an outlier ($p<0.05$) and the exclusion of this laboratory further reduced inter-laboratory %GCV values for the relative potencies of these samples to 8.9% and 6.6%, respectively.

Thus, despite the already good agreement between laboratories on the estimated holoTC values, there was a further reduction in inter-laboratory variability when the holoTC content of the samples was determined relative to the IS.

Stability

Ampoules of 03/178 that had been stored at a range of temperatures (-70^{o}C, -20^{o}C, +4^{o}C, +20^{o}C, +37^{o}C) for 9 years and 8 months were assayed using a manual ELISA kit. Replicate dilution series were assayed in two independent assay runs using a fresh set of ampoules per run.

Temperature ^{o}C	holoTC content expressed as a % of that of ampoules stored at -70^{o}C		
	Assay 1	**Assay 2**	**Mean**
-70	100	100	100
-20	100.9	97.2	99.1
+4	95.8	100.9	98.4
+20	77.3	97.4	87.4
+37	58.4	66.5	62.5
+45	Would not reconstitute		

These data give a predicted loss of 0.035% potency per year when stored at -20^{o}C.

Instructions for Use

The revised Instructions for Use to accompany IS 03/178 are provided in Appendix 4.

Participant feedback

All of the participants accepted the recommendation.

Discussion

There was overall good agreement between laboratories on the holoTC content of 03/178 (%GCV 10.5%). Although the number of different methods was limited, it is essential that an IS is available *before* the development of further assays for holoTC. Introducing a new IS after the development of a number of assays for diagnostic analytes can be too late to ensure effective standardisation. This was clearly demonstrated by the difficulty in persuading manufacturers to recalibrate against the WHO Reference Reagent for sTfR, 07/202, once it had been established, and where assay results were not even being reported in the same units [9].

The candidate IS for holoTC, 03/178, demonstrated overall parallelism to the study patient samples included in the study, demonstrating 'like against like' properties. The inclusion of these samples, with different holoTC levels, demonstrated a reduction in inter-laboratory variability when the holoTC levels in the samples were determined relative to the IS (holoTC value of 106.8 pmol/L assigned) rather than to the assays' calibration. Thus 03/178 shows commutability, albeit with a limited number of patient samples, and the data indicate that its use as a standard would minimise inter-method variability.

The collaborative study data presented in this report thus show that the IS, 03/178, is fit for purpose i.e., fit for use as a WHO International Standard to standardise assays for serum holoTC.

Proposal

It is proposed to assign a holoTC value of 107 pmol/L to the IS for serum folate and B12, 03/178.

Implementation plan

- Manufacturers will be informed about the additional assignment.
- Users will be informed about the additional assignment. UK NEQAS for Haematinics can promote use of the new IS (scheme members are international).
- The new IS will appear in the NIBSC product catalogue and in the newsletter.
- The report of the collaborative study will be published in an international scientific journal.

Acknowledgements

The study organisers thank the staff of the Centre for Biological Reference Materials, NIBSC, for sample despatch.
We are extremely grateful to the study participants for contributing data.

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Table 1 Estimates of holoTC (pmol/L) and assay variability (%GCV) for 03/178

Lab	Assay 1	2	3	4	GM*	GCV†
1	100.7	91.4	95.1		95.7	5.0%
2	111.7	112.9	106.3		110.3	3.2%
3a	120.7	122.6	124.1		122.5	1.4%
3b	113.9	113.2	109.3		112.1	2.3%
4a	99.6	103.0	101.3		101.3	1.7%
4b	89.8	78.1	80.9		82.8	7.5%
5	102.2	98.9	104.3		101.8	2.7%
6	104.2	107.0	102.3		104.5	2.2%
7	110.7	110.1	110.3		110.4	0.3%
8	111.3	110.1	108.0		109.8	1.5%
9	129.4	127.4	111.3		122.5	8.6%
10	109.6	110.9	112.4		111.0	1.3%
11	103.1	92.9	151.5		113.2	29.4%
12	103.3	104.3	101.2	110.2	104.7	3.7%
Overall GM					106.8	
Inter-laboratory GCV					10.5%	
95% Confidence Limits					100.8	113.2

*GM = geometric mean; †GCV = geometric coefficient of variation

Table 2 Fitted slopes and slope ratios relative to 03/178

Sample	Lab	Fitted slopes				Slope ratio (relative to 03/178)			
		Assay				Assay			
		1	2	3	4	1	2	3	4
03/178	1	0.659	0.676	0.679					
	2	1.061	1.078	1.121					
	3a	0.999	0.950	0.956					
	3b	0.901	0.752	0.944					
	4a	0.993	0.964	1.015					
	4b	1.008	1.005	1.092					
	5	1.144	1.153	1.160					
	6	1.046	1.059	1.078					
	7	1.173	1.173	1.132					
	8	1.138	1.099	1.077					
	9	0.804	0.832	0.598					
	10	2.082	2.099	2.126					
	11	0.626	0.782	0.730					
	12	1.144	1.138	1.120	1.067				
Serum 2	1	0.591	0.474	0.518		0.899	0.701	0.763	
	2	0.901	0.912	0.885		0.850	0.846	0.790	
	3a	0.851	0.843	0.840		0.852	0.888	0.879	
	3b	1.154	0.862	1.044		1.281	1.146	1.105	
	4a	1.176	1.082	1.243		1.184	1.122	1.225	
	4b	1.047	1.181	1.520		1.038	1.175	1.392	
	5	0.949	0.942	1.020		0.829	0.817	0.880	
	6	0.872	0.867	0.860		0.833	0.818	0.798	
	7	0.957	0.886	0.913		0.816	0.756	0.807	
	8	0.880	0.898	0.953		0.773	0.817	0.885	
	9	0.779	0.793	0.824		0.970	0.954	1.378	
	10	1.995	2.047	2.002		0.958	0.976	0.942	
	11	0.671	0.684	0.661		1.072	0.874	0.905	
	12	0.968	0.992	1.021	0.945	0.846	0.872	0.911	0.885
Serum 3	1	0.672	0.670	0.657		1.020	0.991	0.968	
	2	1.057	1.006	0.998		0.996	0.933	0.890	
	3a	0.899	0.876	0.911		0.900	0.922	0.953	
	3b	1.020	0.916	1.007		1.132	1.218	1.067	
	4a	1.069	0.988	1.042		1.076	1.025	1.027	
	4b	1.044	1.152	1.359		1.036	1.147	1.244	
	5	1.133	1.165	1.134		0.990	1.010	0.977	
	6	1.017	0.998	1.015		0.973	0.942	0.942	
	7	1.111	1.022	1.121		0.948	0.872	0.990	
	8	0.974	1.055	1.067		0.856	0.960	0.991	
	9	0.771	0.757	0.961		0.959	0.910	1.608	
	10	2.089	2.104	2.095		1.004	1.003	0.985	
	11	0.684	0.488	0.658		1.092	0.624	0.901	
	12	1.080	1.086	1.127	1.092	0.943	0.954	1.006	1.024

Table 3 Estimates of holoTC (pmol/L) and assay variability (%GCV) for serum sample 1

	pmol/L						Potency relative to 03/178					
	Assay						Assay					
Lab	1	2	3	4	GM	GCV	1	2	3	4	GM	GCV
1	12.9	9.0	10.6		10.7	19.8%	0.128	0.098	0.141		0.112	14.1%
2	15.3	14.9	15.1		15.1	1.6%	0.137	0.132	0.142		0.137	3.7%
3a	15.1	15.1	15.8		15.3	2.6%	0.125	0.123	0.127		0.125	1.7%
3b	12.3	13.7	13.7		13.2	6.4%	0.108	0.121	0.125		0.118	8.0%
4a	15.9	19.1	17.7		17.5	9.5%	0.160	0.185	0.175		0.173	7.7%
4b	11.8	12.8	8.0		10.7	28.1%	0.131	0.164	0.099		0.129	28.5%
5	14.9	14.4	14.6		14.6	1.6%	0.145	0.145	0.140		0.144	2.1%
6	15.0	15.0	15.1		15.0	0.4%	0.144	0.140	0.148		0.144	2.7%
7	15.7	16.2	15.4		15.8	2.4%	0.142	0.147	0.140		0.143	2.5%
8	13.2	13.4	13.2		13.3	0.8%	0.119	0.121	0.122		0.121	1.7%
9	19.9	21.8	13.9		18.2	26.6%	0.154	0.171	0.125		0.149	17.1%
10	16.8	16.7	17.1		16.9	1.2%	0.154	0.151	0.152		0.152	0.9%
11	14.2	13.3	16.8		14.7	12.7%	0.138	0.143	0.111		0.130	14.9%
12	13.1	12.5	12.3	16.4	13.5	14.2%	0.127	0.120	0.122	0.149	0.129	10.4%
Overall GM					14.4		Overall GM				0.135	
Inter-laboratory GCV					17.3%		Inter-laboratory GCV				12.3%	
95% Confidence Limits					13.2	15.8	95% Confidence Limits				0.126	0.145

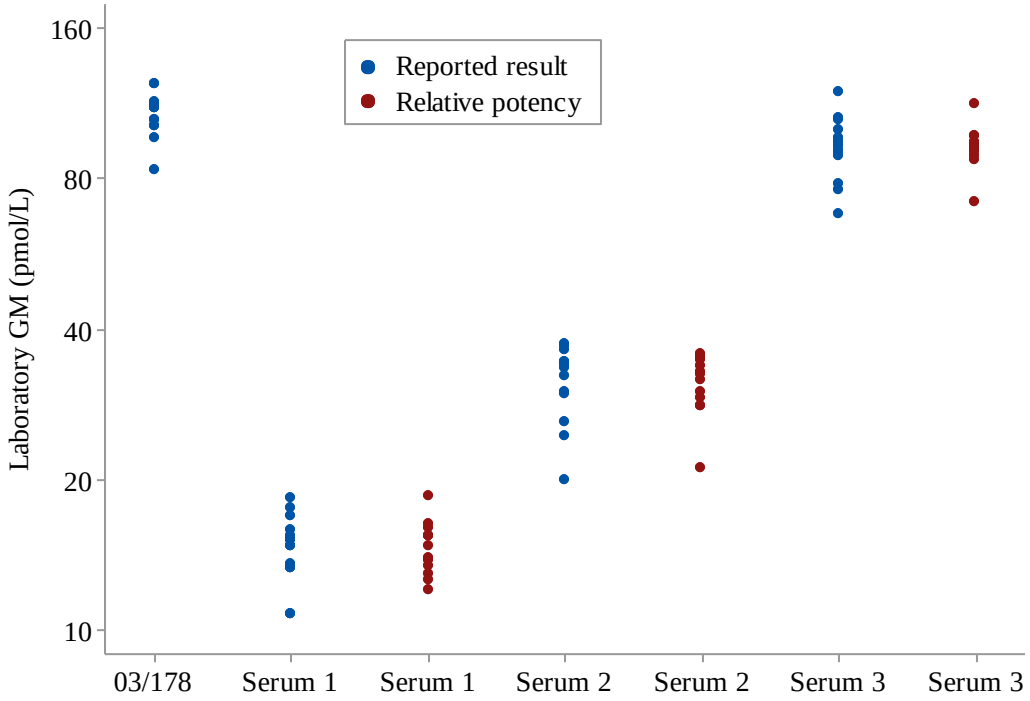
Table 4 Estimates of holoTC (pmol/L) and assay variability (%GCV) for serum sample 2

	pmol/L						Potency relative to 03/178					
	Assay						Assay					
Lab	1	2	3	4	GM	GCV	1	2	3	4	GM	GCV
1	27.1	23.5	27.3		25.9	8.8%	0.269	0.257	0.287		0.271	5.7%
2	33.9	33.3	34.3		33.8	1.4%	0.303	0.295	0.322		0.307	4.6%
3a	33.5	33.5	35.6		34.2	3.6%	0.278	0.273	0.287		0.279	2.5%
3b	28.2	30.4	29.7		29.4	3.9%	0.248	0.269	0.272		0.262	5.1%
4a	18.7	21.5	19.6		19.9	7.2%	0.188	0.209	0.194		0.197	5.5%
4b	27.9	25.6	20.2		24.3	18.0%	0.311	0.327	0.250		0.294	15.4%
5	32.7	31.1	32.5		32.1	2.9%	0.320	0.314	0.311		0.315	1.4%
6	34.5	34.3	34.1		34.3	0.5%	0.331	0.321	0.333		0.328	2.1%
7	36.6	36.4	35.2		36.1	2.0%	0.330	0.330	0.320		0.327	1.9%
8	33.5	33.7	32.8		33.3	1.4%	0.301	0.306	0.304		0.304	0.7%
9	39.8	41.6	30.6		37.0	17.9%	0.307	0.326	0.275		0.302	9.0%
10	35.6	36.7	38.5		36.9	4.0%	0.325	0.331	0.342		0.333	2.7%
11	25.1	25.7	40.5		29.7	30.9%	0.243	0.277	0.267		0.262	6.9%
12	32.7	32.1	32.9	38.0	33.9	8.1%	0.317	0.308	0.325	0.345	0.323	5.0%
Overall GM					31.1		Overall GM				0.291	
Inter-laboratory GCV					19.6%		Inter-laboratory GCV				14.9%	
95% Confidence Limits					28.0	34.4	95% Confidence Limits				0.268	0.315

Table 5 Estimates of holoTC (pmol/L) and assay variability (%GCV) for serum sample 3

	pmol/L						Potency relative to 03/178					
	Assay						Assay					
Lab	1	2	3	4	GM	GCV	1	2	3	4	GM	GCV
1	84.0	70.8	78.8		77.7	9.0%	0.834	0.775	0.829		0.812	4.2%
2	85.6	92.7	95.6		91.2	5.9%	0.766	0.821	0.899		0.827	8.4%
3a	101.3	103.7	106.8		103.9	2.7%	0.839	0.846	0.861		0.849	1.3%
3b	92.0	93.4	96.8		94.1	2.7%	0.808	0.825	0.886		0.839	5.0%
4a	64.5	72.3	66.6		67.8	6.0%	0.648	0.702	0.658		0.669	4.4%
4b	77.3	82.0	67.1		75.2	10.8%	0.861	1.050	0.829		0.908	13.5%
5	88.5	88.1	89.5		88.7	0.8%	0.866	0.891	0.859		0.872	2.0%
6	90.6	90.7	88.4		89.9	1.5%	0.869	0.848	0.863		0.860	1.3%
7	101.1	96.2	91.8		96.3	5.0%	0.913	0.874	0.832		0.872	4.8%
8	100.7	99.6	98.8		99.7	0.9%	0.904	0.905	0.915		0.908	0.7%
9	119.7	111.6	88.2		105.6	17.3%	0.924	0.876	0.792		0.862	8.1%
10	94.6	91.7	96.3		94.2	2.5%	0.863	0.827	0.856		0.849	2.3%
11	148.8	94.5	119.2		118.8	25.5%	1.444	1.017	0.787		1.050	35.6%
12	93.8	89.6	93.6	93.5	92.6	2.2%	0.908	0.859	0.924	0.848	0.884	4.3%
Overall GM					91.7		Overall GM				0.858	
Inter-laboratory GCV					15.6%		Inter-laboratory GCV				9.9%	
95% Confidence Limits					84.3	99.7	95% Confidence Limits				0.813	0.906

Figure 1 High-low plots showing mean laboratory holoTC estimates of IS 03/178, and samples 1, 2 and 3 relative to assay calibrants (reported result), and holoTC estimates of samples 1, 2 and 3 relative to IS 03/178 with an assigned potency of 106.8 pmol/L (relative potency)



Appendix 1 Participants of the collaborative study (in alphabetical order of country)

Benjamin Teis, Sullivan Nicolaides Pathology, Australia
Ebba Nexø and Cindy Søndersø Knudsen, Aarhus University Hospital, Denmark
Eija Stenholm and Annukka Paju, HUSLAB, Meilahti Hospital, Finland
Liisa Ahola, United Medix Laboratoriot Oy, Finland
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Arjan de Mare and A Knuif, Medlon and Medisch Spectrum Twente, The Netherlands
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Kate Guberg, Nutritional Biochemistry Laboratory, MRC, UK
Joshua W Miller and Melissa Murphy, Miller Research Laboratory at Rutgers University, USA

Appendix 2 Methods used by participating laboratories

Laboratory number	**Method** (as reported by the laboratory)
1	Manual ELISA
2	Automated ELISA
3a	Automated ELISA
3b	Manual ELISA
4a	In-house method
4b	Manual ELISA
5	Automated ELISA
6	Automated ELISA
7	Automated ELISA
8	Automated ELISA
9	Manual ELISA
10	Automated ELISA
11	Manual ELISA
12	Automated ELISA

Appendix 3 Collaborative study protocol

COLLABORATIVE STUDY PROTOCOL TO ASSIGN A HOLOTC VALUE TO THE INTERNATIONAL STANDARD FOR FOLATE AND B12

Co-ordinator*

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David Craig, Axis Shield

1. AIM

To assign a HoloTC value to the International Standard for folate and B12 (03/178).

2. MATERIALS PROVIDED

A) Lyophilised samples

- 3 ampoules of the WHO International Standard for folate and B12 (03/178). The HoloTC value is APPROXIMATELY 90 pmol/L.
- 3 ampoules of serum sample 1, approximate value 15 pmol/L
- 3 ampoules of serum sample 2, approximate value 35 pmol/L
- 3 ampoules of serum sample 3, approximate value 80 pmol/L

Store all ampoules at –20^{o}C until reconstitution and use. Do not reconstitute until the day of use.

Accurately reconstitute the contents of each ampoule with 1.0 mL distilled/deionized H_2O using a calibrated pipette according to the 'Instructions for Use' sheets supplied with the materials. Vortex gently and inspect contents to ensure complete dissolution. Transfer the reconstituted contents to a capped tube.

Additional ampoules/vials can be provided on request in the case of breakages or errors.

B) Active-B12 (Holotranscobalamin) kits

Architect and manual ELISA kits will be provided separately to participants of the collaborative study so participation should not incur additional costs to the participants.

C) SeraSub diluent

This diluent is to be used in the preparation of doubling dilution series of the study samples.

3. STUDY DESIGN and PROTOCOL

The study requires the accurate production of replicate series of doubling dilutions of the materials provided, using calibrated pipettes e.g., Socorex or Biohit. Assaying dilution series of each of the preparations is essential as it allows assessment of linearity and parallelism of responses between the preparations.

Please ensure you provide details of all calibrations performed and record which calibration was used for each set of results.

DAY 1:

Reconstitute one ampoule of each of 03/178, sample 1, sample 2 and sample 3.

Assay 2 **independent** doubling dilution series e.g., neat, 1/2, 1/4, 1/8, 1/16, prepared using the SeraSub diluent provided, of each of the reconstituted preparations. Each dilution series should be prepared separately from the neat, reconstituted ampoule contents; do not assay duplicates of

a single dilution series. Include your manual ELISA calibrators, if applicable, **in the same assay run**.
The dilution series should be prepared bearing in mind the approximate HoloTC values of each of the preparations so that responses are in the linear part of the dose-response curve of your assay - see Appendix 1 for dilution protocol.

DAY 2:
Reconstitute a second ampoule of each of 03/178, sample 1, sample 2 and sample 3.
Assay 2 independent doubling dilution series, prepared using the SeraSub diluent provided, of each of the reconstituted preparations. Include your manual ELISA calibrators, if applicable, **in the same assay run**.

DAY 3:
Reconstitute a third ampoule of each of 03/178, sample 1, sample 2 and sample 3.
Assay 2 independent doubling dilution series, prepared using the SeraSub diluent provided, of each of the reconstituted preparations. Include your manual ELISA calibrators, if applicable, **in the same assay run**.

4. RECORDING RESULTS

A) Calibration data

Please enter the raw data of the calibration curve on the day of assay i.e., calibrator value and absorbance for ELISA method or calibrator and relative light units (RLU) for Architect/Axsym automated tests, on the results sheets provided.
These details are available from the calibration curve details on the Abbott Architect. See Appendix 2 for information on how to print these details.

B) Sample results

Please enter the HoloTC concentrations (in pmol/L) for automated assays or the raw data (absorbances) for manual ELISA of the neat and dilution series of 03/178, sample 1, sample 2 and sample 3 on the results sheet provided, along with any comments.

Return electronic copies of the results to susan.thorpe@nibsc.org (preferred). Alternatively, results may be faxed to +44 (0)1707 641057, FAO Dr Susan Thorpe.

PLEASE RETURN YOUR RESULTS BY 6th MARCH 2015

Appendix 1 Suggested dilution scheme based on the approximate HoloTC content of the study samples

03/178 approx 90 pmol/L	**Sample 1** approx 15 pmol/L	**Sample 2** approx 35 pmol/L	**Sample 3** approx 80 pmol/L
Prepare 2 separate sets of the dilutions below from each ampoule for testing			
Neat	**Neat**	**Neat**	**Neat**
1/2	**1/2**	**1/2**	**1/2**
1/4		**1/4**	**1/4**
1/8		**1/8**	**1/8**
1/16			**1/16**

Appendix 2 Obtaining calibration curve details on Architect

- Select Overview/Snapshot to obtain the Home page screen
- Select QC-Cal and calibration status from the drop down menu
- Select HoloTC and highlight
- Select Print Cal Curve details report
- Done

Scan this print-out and return with the results of the HoloTC dilutions

International Collaborative Study to assign a HoloTC value to the International Standard for B12 and folate 03/178

RESULTS SHEET FOR DAY 1 (1st set of ampoules)

Name: Date:

Laboratory

Reagent lot used: Calibrator lot used:

Calibration curve reference:

Preparation	**Dilution or concentration**	**HoloTC concentration (pmol/L) or RLU or absorbance depending on assay readout**	
		Replicate 1	**Replicate 2**
Assay calibrator	A: B: C: D: E: F:		
IS 03/178	Neat 1/2 1/4 1/8 1/16		
Sample 1	Neat 1/2		
Sample 2	Neat 1/2 1/4 1/8		

Sample 3	Neat 1/2 1/4 1/8 1/16		

International Collaborative Study to assign a HoloTC value to the International Standard for B12 and folate 03/178

RESULTS SHEET FOR DAY 2 (2nd set of ampoules)

Name: Date:

Laboratory

Reagent lot used: Calibrator lot used:

Calibration curve reference:

Preparation	**Dilution or concentration**	**HoloTC concentration (pmol/L) or RLU or absorbance depending on assay readout**	
		Replicate 1	**Replicate 2**
Assay calibrator	A: B: C: D: E: F:		
IS 03/178	Neat 1/2 1/4 1/8 1/16		
Sample 1	Neat 1/2		
Sample 2	Neat 1/2 1/4 1/8		

Sample 3	Neat 1/2 1/4 1/8 1/16		

International Collaborative Study to assign a HoloTC value to the International Standard for B12 and folate 03/178

RESULTS SHEET FOR DAY 3 (3rd set of ampoules)

Name: Date:

Laboratory

Reagent lot used: Calibrator lot used:

Calibration curve reference:

Preparation	**Dilution or concentration**	**HoloTC concentration (pmol/L) or RLU or absorbance depending on assay readout**	
		Replicate 1	**Replicate 2**
Assay calibrator	A: B: C: D: E: F:		
IS 03/178	Neat 1/2 1/4 1/8 1/16		
Sample 1	Neat 1/2		
Sample 2	Neat 1/2 1/4 1/8		

Sample 3	Neat 1/2 1/4 1/8 1/16		

Please state any deviations from the protocol:

Additional comments:

Appendix 4 Draft Instructions for Use

WHO International Standard
Serum folate, vitamin B12 and holoTC
NIBSC code: 03/178
Instructions for use
(Version 1.00, Dated)

Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

1. INTENDED USE

The assay of blood levels of the vitamins B12 and folate 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.

The International Standard (IS) for serum B12 and serum folate, 03/178, was assayed using a wide range of methods in 24 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 IS with assigned B12 and folate values rather than to the analysers' calibration.

Since the IS, 03/178, was first established, commercial assays for holotranscobalamin (holoTC), the active portion of B12, have become available. There is evidence that holoTC is a better marker of early B12 deficiency than total B12. This prompted another international collaborative study to assign a holoTC value to the 1st International Standard (IS) for serum folate and B12, 03/178.

2. CAUTION

This preparation is not for administration to humans.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg, anti-HIV and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The IS 03/178 has an assigned value of 12.1 nmol/L total folate, made up of 9.75 nmol/L 5MeTHF (5-methyltetrahydrofolate; coefficient of variation (CV) 5.5%), 1.59 nmol/L 5FoTHF (5-formyltetrahydrofolate; CV 4.2%) and 0.74 nmol/L FA (folic acid; CV 31.6%), when reconstituted with 1.0 mL distilled/deionised water, as determined using LC/MS/MS. The total folate content of 12.1 nmol/L is equivalent to 5.33 ng/mL, using a conventional conversion factor of 2.266.

The IS 03/178 has an assigned consensus value of 480 pg vitamin B12 (480 pg/mL when reconstituted with 1.0 mL distilled/deionised water). The preparation will be reevaluated when a reference measurement procedure has been established.

The IS 03/178 has an assigned consensus value of 107 pmol/L holoTC when reconstituted with 1.0 mL distilled/deionised water (0.107 pmol/mL).

The variance in the ampoule contents was determined to be 0.08%.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Each ampoule contains the lyophilized residue of ~1 mL human serum.

5. STORAGE

Unopened ampoules should be stored at -20°C or below.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.

Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.

Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution

Reconstitute the contents with 1.0 mL distilled/deionised water on the day of assay.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

NIBSC follows the policy of WHO with respect to its reference materials.

Accelerated degradation studies on 03/178 indicate that the lyophilized material will be adequately stable at -20°C with respect to folate, B12, and holoTC content. Once reconstituted, users should determine the stability of the material according to their own conditions of storage and use.

Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact NIBSC.

9. REFERENCES

SJ Thorpe, A Heath, S Blackmore, A Lee, M Hamilton, S O'Broin, BC Nelson and C Pfeiffer. An international standard for serum vitamin B12 and serum folate: international collaborative study to evaluate a batch of lyophilized serum for B12 and folate content. Clin Chem Lab Med 45, 380-386 (2007).

10. ACKNOWLEDGEMENTS

We thank the participants of the collaborative studies.

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:

National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG
WHO International Laboratory for Biological Standards, UK Official Medicines Control Laboratory
T +44 (0)1707 641000
nibsc.org

http://www.nibsc.org/products/ordering_information/frequently_asked_questions.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Physical and Chemical properties			
Physical appearance: Lyophilizate		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	Unknown
Flammable:	No	Handling:	See caution, Section 2
Other (specify):	Contains material of human origin		
Toxicological properties			
Effects of inhalation:	Not established, avoid inhalation		
Effects of ingestion:	Not established, avoid ingestion		
Effects of skin absorption:	Not established, avoid contact with skin		
Suggested First Aid			
Inhalation:	Seek medical advice		
Ingestion:	Seek medical advice		
Contact with eyes:	Wash with copious amounts of water. Seek medical advice		
Contact with skin:	Wash thoroughly with water.		
Action on Spillage and Method of Disposal			
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.			

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 0.08g
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached:** No

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biol efstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.