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Evaluation of the technical performance of novel holotranscobalamin (holoTC) assays in a multicenter European demonstration project

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Abstract

A commercially available holotranscobalamin (holoTC) radioimmunoassay (RIA) (Axis-Shield, Dundee, Scotland) was evaluated in four laboratories and compared with a holoTC ELISA run in one laboratory. The performance of the holoTC RIA assay was comparable in three of the four participating laboratories. The results from these three laboratories, involving at least 20 initial runs of ''low'', ''medium'' and ''high'' serum-based controls (mean holoTC concentrations 34, 60 and 110 pmol/L, respectively) yielded an intra-laboratory imprecision of 6–10%. No systematic inter-laboratory deviations were observed on runs involving 72 patient samples (holoTC concentration range 10–160 pmol/L). A fourth laboratory demonstrated higher assay imprecision for control samples and systematic deviation of results for the patient samples. Measurement of holoTC by ELISA showed an imprecision of 4–5%, and slightly higher mean values for the controls (mean holoTC concentrations 40, 70 and 114 pmol/L, respectively). Comparable results were obtained for the patient samples. The long-term intra-laboratory imprecision was 12% for the holoTC RIA and 6% for the ELISA. In conclusion, it would be prudent to check the calibration and precision prior to starting to use these holoTC assays in research or clinical practice. The results obtained using the holoTC RIA were similar to those obtained using the holoTC ELISA assay.

Keywords: ELISA; holotranscobalamin (holoTC); radioimmunoassay (RIA); validation.

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Introduction

There is no agreement on how best to diagnose vitamin B_{12} deficiency due to limitations in the currently available laboratory tests. Measurement of total cobalamin levels in blood does not appear to identify all those in need of vitamin B_{12} supplementation (1–5). Measurement of the metabolites methylmalonic acid (MMA) and plasma total homocysteine (tHcy), which are known to accumulate in the setting of vitamin B_{12} deficiency, both have some serious limitations to their wider use. Measurement of MMA is analytically difficult and expensive to perform (6) and tHcy analysis requires rigorous sample handling and is a non-specific marker of vitamin B_{12} deficiency (7).

Thus, there is a need for more reliable tests to replace or supplement the more than 20 million total cobalamin assays performed world-wide every year to identify patients requiring vitamin B_{12} treatment.

On theoretical grounds, holotranscobalamin (holoTC) concentrations may represent an ideal marker of vitamin B_{12} status (8). HoloTC comprises approximately 20% of the total plasma cobalamin and is the part of plasma cobalamin bound to transcobalamin (TC), a protein that delivers the vitamin to all cells in the body. The remainder of plasma cobalamin (80%) is believed to be biologically unavailable for most cells. The very low plasma concentrations of holoTC have hindered the development of reliable holoTC assays. However, the recent cloning and expression of human TC has provided the opportunity to develop the tools necessary (recombinant human TC, monoclonal and polyclonal antibodies) for the development of holoTC assays. Recently, two new holoTC assays have been developed and one is now commercially available (9, 10). The principles of the two assays are summarized in Figure 1. In the commercially available assay [holoTC radioimmunoassay (holoTC RIA) from Axis-Shield, Dundee, Scotland], all the TC in the sample is precipitated by monoclonal antibodies that are coupled to magnetic beads, and the amount of cobalamin present in the precipitate is measured using a conventional competitive protein-binding assay for cobalamin. In the holoTC enzyme-linked immunosorbent assay (holoTC ELISA), all the unsaturated cobalamin-binding proteins are precipitated by vitamin B_{12} coupled to magnetic beads prior to measurement of the protein moiety of holoTC remaining in the supernatant by ELISA.

This paper describes an evaluation of the technical performance of the novel RIA and ELISA for the determination of holoTC concentrations in four laborato-

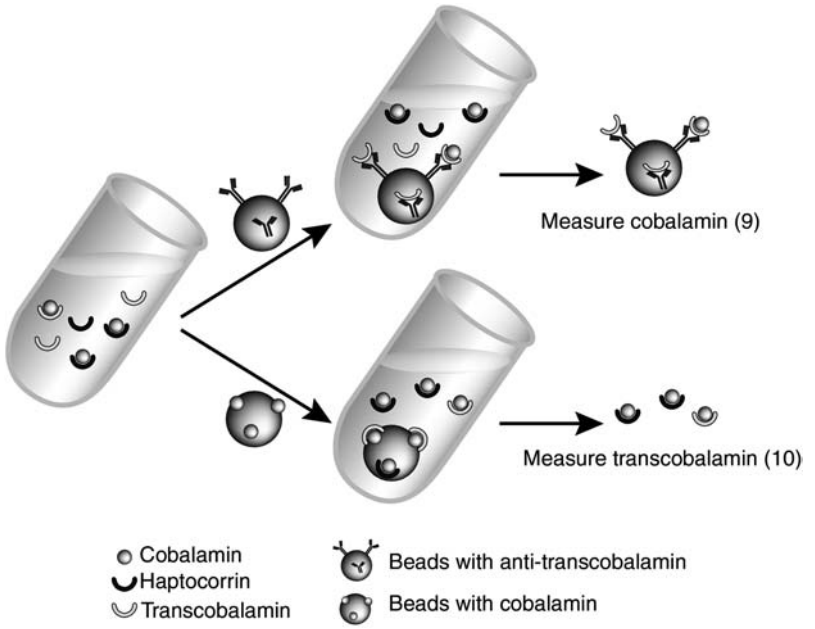

Figure 1 Principles of the assays used to measure holoTC concentrations. Upper panel: holoTC-RIA. Transcobalamin is precipitated by monoclonal antibodies coupled to magnetic beads and the amount of cobalamin in the precipitate is measured. Lower panel: holoTC-ELISA. Unsaturated cobalamin-binding proteins are precipitated by magnetic beads coated with vitamin B_{12} prior to measurement of the protein moiety of holoTC present in the supernatant. References are indicated in parentheses.

ries in four European countries as part of a European Union BIOMED demonstration project.

Materials and methods

Reagents and equipment

The holoTC RIA (9) was supplied by Axis-Shield. All samples were used undiluted. The assay was run according to the manufacturer's instructions and using standard gamma-counters to estimate the ^{57}Co-labeled vitamin B_{12}. The calibration curve covered the range 10–160 pmol/L. The holoTC ELISA was carried out as previously described (10, 11) on a BEP2000 analyzer (Dade Behring, Marburg, Germany). The calibration curve covered the range 1.6–100 pmol/L, but since all samples were diluted 1:5 prior to analysis, the effective range of the calibration curve was 8–500 pmol/L.

Samples

In addition to the kit controls supplied by the manufacturer of the holoTC RIA and internal controls used for the holoTC ELISA, three control samples were prepared from pooled serum containing ''low'', ''intermediate'' and ''high'' concentrations of holoTC to assess imprecision. Patient samples were selected from 72 individuals who had a holoTC concentration ranging from 10 to 160 pmol/L and were used to compare the performance of the assays. All samples were prepared in one laboratory and stored frozen at –80°C and shipped on dry ice to the other laboratories.

Study design

Four laboratories from four European countries participated as part of a European Union BIOMED demonstration project. All four performed the holoTC RIA and one laboratory performed the holoTC ELISA. The study adopted a similar design to the one used to evaluate the diagnostic utility of tHcy assays in a previous European Demonstration project (12). The first phase included a familiarization phase with the holoTC RIA. This involved running the holoTC RIA according to the manufacturer's instructions and verifying that the kit control results were within the predefined limits for run acceptability. The second phase included a comparison for both the holoTC RIA and the holoTC ELISA that involved at least 20 runs including ''low'', ''medium'' and ''high'' controls, in addition to 72 patient samples that were to be analyzed twice as single estimates in each run. One laboratory running the holoTC RIA analyzed only 61 of the 72 patient samples, because replacement samples for a batch that was lost during shipment were available for only 61 samples.

The holoTC RIA runs were accepted or rejected based on the kit control values according to kit control rules. The ELISA was accepted or rejected according to internal controls. The third phase performed by two laboratories for holoTC RIA and one laboratory for holoTC ELISA involved running samples from clinical studies including the ''low'' and ''high'' controls in single estimates to assess the long-term imprecision.

Statistical methods

Imprecision was expressed using coefficients of variation. The intra-laboratory imprecision was calculated as the variation between the results obtained from controls run in the same laboratory. The intra-run imprecision was calculated from controls analyzed twice in the same run. For the holoTC RIA, the total imprecision was calculated using all available control values obtained in the different laboratories, and the inter-laboratory imprecision was estimated by subtracting the intra-laboratory imprecision from the total imprecision.

Results

Four laboratories evaluated the holoTC RIA by familiarization, followed by running the three control sam-

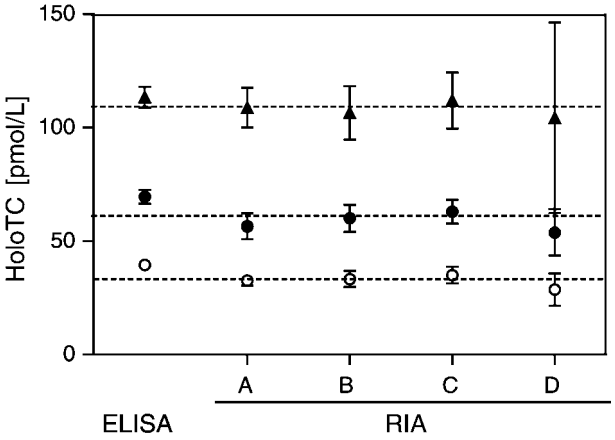

Figure 2 Imprecision (mean and SD) of the holoTC ELISA and holoTC RIA performed in four laboratories (A–D). Low (○), intermediate (●) and high (▲) control. Broken lines indicate the mean of the low, intermediate and high controls obtained with the holoTC RIA in the three laboratories with similar performance (A, B and C).

ples in at least 20 runs and by analyzing 72 patient samples. Subsequently, two laboratories used the assay for clinical studies. One laboratory ran the three control samples through 20 runs, in addition to the 72 patient samples using the holoTC ELISA.

Familiarization

Each technician performed three accepted runs on the holoTC RIA based on kit control rules prior to continuation of the study. Major problems were encountered in achieving absorption of TC to the antibody-covered beads in the initial step (Figure 1). The bias was corrected using capped tubes that were rotated on a roller to avoid trapping of the beads in the caps.

Imprecision

Three laboratories excluded none of their runs and achieved comparable performance with the holoTC RIA. One center had to exclude 7 out of 23 holoTC RIA runs and performed poorly on the runs accepted in terms of precision and accuracy (Figure 2). Table 1 shows that for the three laboratories, the total imprecision for the holoTC RIA was 9–10%, intra-laboratory imprecision was 6–10% and intra-run imprecision was 5–8% for holoTC values of 34, 60 and 110 pmol/L. One laboratory ran the three controls using a holoTC ELISA. The intra-laboratory imprecision was 4–5% and the intra-run imprecision 3–4%. The holoTC concentrations measured using the ELISA were 6, 10 and 4 pmol/L higher than those obtained for the controls using the holoTC RIA.

Patient samples

Analysis of patient samples selected to represent a range of values from 10 to 160 pmol/L using the holoTC RIA and ELISA allowed comparison of results obtained between the four laboratories running the holoTC RIA and between results obtained using the two methods. In the absence of a reference method to measure holoTC concentrations, we used the average of the holoTC RIA results from the three laboratories with similar performance in the imprecision study to indicate the ''true'' concentration. As indicated in Figure 3A–C, the three laboratories obtained comparable results, with no systematic deviation. Figure 3D shows that the fourth laboratory demonstrated an unacceptably large variation and systematic deviation in the results obtained. Figure 3E shows that the results obtained with the holoTC ELISA were quite comparable to those obtained with the holoTC RIA, although there was a small, but significant, deviation.

Long-term performance

The holoTC RIA was used to assess a large number of samples in the two laboratories. The imprecision calculated for the ''low'' (n = 110) and ''high'' controls (n = 100) that were carried out over 20 months was 12% (intra-laboratory) and this included batch variation of the kit reagents and calibrators. The imprecision calculated from data (n = 42) using the same batch over a 4.5-month period in the same laboratory was 10%. The mean holoTC concentration for the controls assessed in two laboratories over a prolonged period demonstrated good agreement between laboratories, indicating comparable accuracy for the holoTC RIA over time. The long-term intra-laboratory imprecision for the holoTC ELISA over a period of 3 months was 6% for the ''low'' and ''high'' controls

Table 1 Imprecision for the holoTC RIA and ELISA estimated using results obtained for control serum samples run during the initial phase and during a prolonged follow-up period.

	Low		Intermediate		High	
	RIA	ELISA	RIA	ELISA	RIA	ELISA
Initial phase						
Mean holoTC, pmol/L	34	40	60	70	110	114
CV_{total}, % (n)	10 (148)	–	9 (138)	–	10 (148)	–
$CV_{intra\text{-}laboratory}$, % (n)	9 (148)	4 (40)	6 (138)	5 (40)	10 (148)	5 (40)
$CV_{intra\text{-}run}$, % (n)	7 (69)	4 (20)	5 (69)	3 (20)	8 (69)	4 (20)
Long-term						
$CV_{intra\text{-}laboratory}$, % (n)	12 (110)	6 (23)	–	–	12 (100)	6 (23)

For the initial phase, the results were based on runs performed in three laboratories for holoTC RIA (number of runs, 79) and in one laboratory for holoTC ELISA (number of runs, 20). Long-term imprecision was based on the ''low'' and ''high'' controls run in two laboratories for holoTC RIA and in one laboratory for holoTC ELISA.

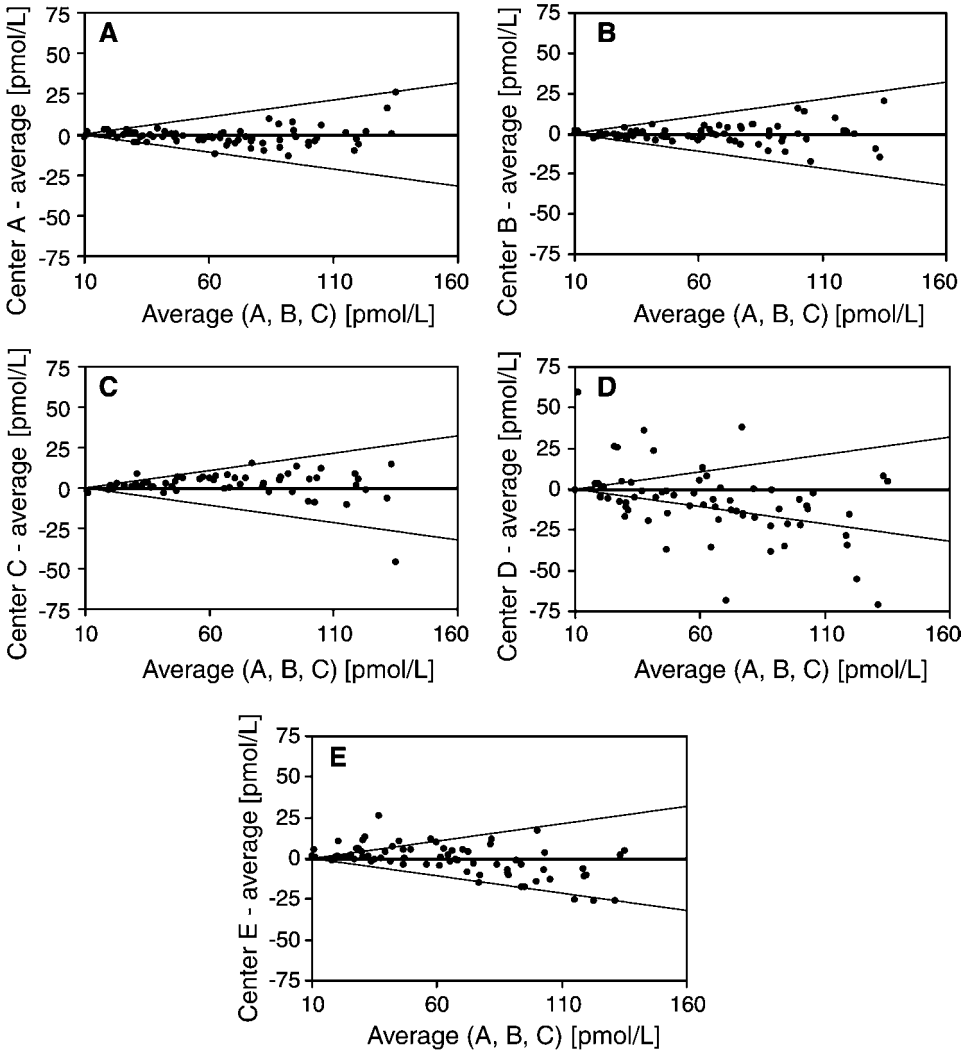

Figure 3 HoloTC in 72 patient samples. HoloTC was measured using the holoTC RIA in four laboratories (A–D) and the holoTC ELISA (E). X-axis: average (centers A, B and C). Y-axis: difference between the result obtained at the center in question and the "true value" defined as the average (centers A, B, and C). Thick lines indicate the expected mean and thin lines (±10%) indicate an estimate of the minimum expected variation due to analytical imprecision.

(n=23). The mean control concentrations did not differ over time, suggesting good assay accuracy.

Discussion

The RIA used to measure holoTC involves several steps and is not easy to perform. The present study demonstrates that careful evaluation of analytical performance is required to ensure optimum performance of this assay in research or routine laboratory practice. One out of four laboratories failed to achieve adequate performance with the holoTC RIA, despite a successful familiarization period. The other three laboratories performed alike, indicating that it was possible to obtain comparable results between laboratories. The major problems involved in the holoTC RIA include the multiple manual step procedures and the requirement to use a gamma-counter. The holoTC RIA is time-consuming and requires a skilled technician. Furthermore, the RIA requires a large sample volume of 400 μL. The lowest concentration of holoTC that can be measured is relatively high and the measurement range of the assay is relatively narrow, spanning from 10 to 160 pmol/L. The holoTC ELISA is time-consuming and it requires skill to pre-treat the samples, while the remaining part of the assay can be adapted to an automatic platform. The ELISA only requires a sample volume of approximately 100 μL. The detection limit is low and the measurement range is high, with a calibration curve spanning values from 1.6 to 100 pmol/L. Since samples are prediluted 1:5, this corresponds to a measurement range of 8–500 pmol/L. Both assays show acceptable long-term imprecision of 12% for the holoTC RIA and half this value for the holoTC ELISA.

The principle of the holoTC RIA is to measure the cobalamin attached to TC after absorption of TC on magnetic beads. The results for the holoTC RIA compared reasonably well with the results obtained using the holoTC ELISA. In the latter assay, unsaturated cobalamin-binding proteins are removed prior to measuring the protein moiety of holoTC by ELISA. The reasonable agreement between results obtained

Table 2 Reference intervals for serum holoTC concentrations from apparently healthy European populations.

Reference interval, pmol/L	HoloTC assay	Population	n	Ref.
54 (17–114)	RIA	Healthy subjects	93	17
53 (11–196)	RIA	Healthy subjects (no vitamin supplements)	74	26
54 (16–122)	RIA	Healthy subjects (omnivores)	79	27
61 (29–113)	RIA	Healthy subjects	65	28
79 (37–171)	RIA	Healthy subjects (no elderly with biochemical B_{12} deficiency)	303	23
59 (24–157)	RIA	Healthy subjects	105	9
70 (40–159)	ELISA	Donors	137	10
80 (41–204)	ELISA	Donors	148	–[a]

Reference intervals are presented as median (95% interval). [a] Unpublished data (Morkbak and Nexo) on a healthy donor population [women (men) $\leq$50 years, n=36 (37); women (men) $>$50 years, n=35 (40)].

with the two methods allows comparison of studies in which holoTC has been measured by either method.

Although neither the holoTC RIA nor the holoTC ELISA seems suitable for routine use, they may be used in the research setting and thereby allow studies to be performed to judge the clinical utility of a potential routine assay for holoTC. A number of studies have already been performed and various questions of relevance for a future clinical use of the holoTC assay have been addressed as summarized in the following sections.

Patient preparation and sample requirement

No special preparation of the patient is required and there is no need for collection of fasting samples, since the diurnal variation in holoTC is minimal on a normal diet (13). The long-term within-person variability in holoTC concentrations is 10–20% (14–16).

Both serum and EDTA plasma may be used; however, values obtained for plasma are approximately 6–8% higher than for serum (9, 10). Apparently, serum samples can be kept frozen for at least up to 20 months, based on results obtained for serum control samples included in the present study, but so far, no data are available on the effects of repeated freeze-thaw cycles.

Biological determinants of holoTC

Several studies have reported a strong relationship between serum creatinine and holoTC (17, 18), and from the largest study performed to date, it is estimated that holoTC increases by approximately 50 pmol/L for a creatinine increment of 100 μmol/L (18).

Genetic polymorphisms for holoTC may be of some importance. The plasma and cerebrospinal fluid levels of holoTC are 10–30% lower in the RR-genotype (259 arginine) compared to the PP-genotype (259 proline) of TC (19–22).

No major differences in holoTC concentrations between males and females have been observed (9, 10, 23). However, there may be hormonal regulation of holoTC concentrations, as current users of oral contraceptives have 25% higher holoTC concentrations compared with non-users (24).

Lower holoTC concentrations have been reported in Syrian and Indian populations compared to European populations (17, 25). However, the latter differences may be caused by lower vitamin B_{12} status rather than by racial differences in holoTC concentrations.

Reference intervals

There is no consensus concerning the choice of reference intervals for holoTC. We compared data on holoTC from eight studies, each including more than 50 apparently healthy European individuals (9, 10, 17, 23, 26–28) (Table 2). A wide range of holoTC spanning from 11 to 41 pmol/L has been reported for the lower limit of the reference interval in these reports. We find it most likely that the best estimate is $\sim$40 pmol/L, since the chance of overestimating the lower limit is far less than the chance of an underestimate induced by including persons with early vitamin B_{12} deficiency in the reference population. In accordance, a large Finnish study excluding elderly individuals with biochemical signs of vitamin B_{12} deficiency reported 37 pmol/L as the lower level for the reference interval (23). The importance of an upper limit for the reference interval for holoTC is not known. Based on the studies reported, the upper limit also shows considerable variation, but a reasonable estimate, again considering the Finnish study, seems to be 170 pmol/L (23).

Conditions with low levels of holoTC

As expected, holoTC levels are low in patients with biochemical signs of vitamin B_{12} deficiency, with mean values of approximately 25 pmol/L (29). Notably low values have been reported in vegetarians (17, 27), vegans (27, 30) and in populations with a low intake of vitamin B_{12} (25). Interestingly, low serum holoTC was reported in patients with Alzheimer's disease compared to a healthy control group (28), most likely indicating a high prevalence of vitamin B_{12} deficiency in this patient population.

Low levels of holoTC are also expected in children with inherited TC deficiency (31, 32), but so far, the holoTC RIA or ELISA has not been used for this group of patients.

Conditions with high levels of holoTC

No systematic studies have addressed the question of high levels of holoTC, but an increased level is expected in conditions with increased levels of total TC. This includes conditions with increased macrophage activity, notably histocytosis (33), and conditions with autoantibodies against TC, as reported in patients treated with vitamin B_{12} injections (34). Upon analyzing holoTC in 937 patients suspected for vitamin B_{12} deficiency (18), we observed 13 patients with unexpected high levels of holoTC (above 500 pmol/L). In addition, ten of these patients had high levels of total TC (2200–10,000 pmol/L, reference interval, 600–1500 pmol/L) (11).

Use of holoTC in the clinical setting

Most studies published so far suggest holoTC as a sensitive marker of vitamin B_{12} deficiency, but questions about specificity have been raised. The largest study carried out to date evaluated holoTC concentrations in patients with suspected vitamin B_{12} deficiency based on a previous measurement of an increased level of MMA (18). The study reported comparable area under the curves (AUC) upon ROC analysis for cobalamin (AUC = 0.85) compared to holoTC (AUC = 0.90) to detect patients with MMA $\geq$ 0.75 μmol/L and tHcy $\geq$ 15 μmol/L. Several additional studies supported the usefulness of holoTC measurements (17, 30), while another questioned whether additional information could be gained from holoTC compared to other markers (35).

HoloTC shows an average increase of 40% 24 h after ingestion of a high physiological dose (3 × 9 μg of vitamin B_{12} at 6 h intervals) (15). Based on these results, measurement of holoTC may prove helpful in evaluating the absorption of vitamin B_{12} either in its free form or attached to intrinsic factor.

Concluding remarks

Assays suitable for the measurement of holoTC are now available, but rigorous evaluation in each laboratory is required before their introduction into research or routine clinical practice. Current assays are suitable for use in the research setting, but are too complicated for use in a routine laboratory. Data indicating the usefulness of measuring holoTC in the clinical setting are accumulating, but it is unclear whether measurement of holoTC may prove superior to measurement of cobalamin for the diagnosis of vitamin B_{12} deficiency or whether it should be used as a supplementary test. Additional clinical studies are required to clarify the relevance of holoTC. Importantly, widespread use of this assay will require a holoTC assay that can be adapted to an automated platform suitable for use in a routine laboratory setting.

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