CERTIFICATION

AOAC® *Performance Tested*SM

Certificate No.

101002

The AOAC Research Institute hereby certifies the performance of the test kit known as:

VitaFast® B12 Microbiological Microtiter Plate Test for the Determination of Vitamin B12

manufactured by
Institut fur Produktqualitaet GmbH
Wagner-Régeny-Str. 8
12489 Berlin
Germany

distributed by
R-Biopharm AG
An der neuen Bergstraβe 17
64297 Darmstadt
Germany

This method has been evaluated in the AOAC® *Performance Tested Methods*SM Program, and found to perform as stated by the manufacturer contingent to the comments contained in the manuscript. This certificate means that an AOAC® Certification Mark License Agreement has been executed which authorizes the manufacturer to display the AOAC *Performance Tested*SM certification mark along with the statement - "THIS METHOD'S PERFORMANCE WAS REVIEWED BY AOAC RESEARCH INSTITUTE AND WAS FOUND TO PERFORM TO THE MANUFACTURER'S SPECIFICATIONS" - on the above mentioned method for a period of one calendar year from the date of this certificate (November 06, 2019 – December 31, 2020). Renewal may be granted at the end of one year under the rules stated in the licensing agreement.

Scott Coates

Scott Coates, Senior Director
Signature for AOAC Research Institute

November 06, 2019
Date

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KIT NAME(S)
VitaFast® B12 Microbiological Microtiter Plate Test for the Determination of Vitamin B12

CATALOG NUMBERS
P1002

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APPLICABILITY OF METHOD
Target analyte – B vitamin cyanocobalmin (B12)

Matrices – (1 g) - Cereals, multivitamin pills, powders, beverages like fruit juice & milk

Performance claims - The performance characteristics of VitaFast® Vitamin B12 meet the following specifications:
1) Time required for completion of the sample extraction was 2 hours and less than 48 hours for the test implementation.
2) The test kit components are stable as indicated on the test kit labels (shelf life is 12 months).
3) Analytical Sensitivity was found at LOD 0.021 µg / 100 g as measured by 100 blank samples from 10 different lots. LOQ was set at 0.03 µg Vitamin B12 / 100 g sample, which corresponds to standard 1 of the curve.
4) Accuracy was investigated by analysis of reference materials from five proficiency programs, and also by commercial product analysis and spike recovery studies. In general recovery was within acceptable limits.
5) The VitaFast test kit was shown to have a high degree of precision, with inter-assay variances below 10 % for all matrices.
6) The VitaFast plate test is not sensitive to temperature changes between 36 °C and 38 °C, incubation time between 44 and 52 hours, or assay medium volumes between 145 and 155 µl.

ORIGINAL CERTIFICATION DATE
October 14, 2010

CERTIFICATION RENEWAL RECORD
Renewed Annually through December 2020

METHOD MODIFICATION RECORD
1. **March 2017 Level 2**

SUMMARY OF MODIFICATION
1. **Location Change to Wagner-Régeny-Str. 8, Berlin**

Under this AOAC® *Performance Tested*SM License Number, 101002 this method is distributed by:
R-Biopharm AG

Under this AOAC® *Performance Tested*SM License Number, 101002 this method is distributed as:
VitaFast® B12 Microbiological Microtiter Plate Test for the Determination of Vitamin B12

PRINCIPLE OF THE METHOD (1)
Vitamin B12 is extracted from a homogenized sample and the extract is diluted. A Vitamin B12 assay medium and standards or the diluted sample extract are pipetted to the wells of a microtiter plate coated with *Lactobacillus delbrueckii* subsp. *lactis* (*leichmannii*). The growth of the organism is dependent on the vitamin content of the sample, or standard. Following the addition of Vitamin B12 as a standard or as a compound of the sample, the bacteria grow until the vitamin is consumed. The incubation is done in the dark at 37 °C (98.6 °F) for 44 - 48 h. The intensity of growth in relation to the extracted vitamin B12 is measured as turbidity and compared to the standard curve. After the incubation period, absorbances of each well are measured using a microtiter plate reader at 610 - 630 nm (alternatively at 540 - 550 nm). Results are interpolated from the standard calibration curve which covers a range from 0.03 - 0.18 µg / 100 g.

DISCUSSION OF THE VALIDATION STUDY (1)

The VitaFast® Vitamin B12 test kit is calibrated according to a standard curve of five standard concentrations, using 4-parameter fitting software. The curve shown in figure 1 is typical. Variation within the curve is consistently minor, at a level of variance below 10 %. Stability is also demonstrated over the entire shelf life of the product, and regular quality tests ensure this is true for all lots produced.

Lot-to-lot tests show a high degree of repeatability across lots, throughout the entire shelf life. Not only was the result for the AACC reference material consistent across the four lots with a CV of less than 10 %, but CVs for the raw absorbance data of the standards across the four lots indicate little variation in the calibration of individual tests. This demonstrates the excellent uniformity of the kits.

Accuracy was established using recognized and reliable reference materials, as well as spike recovery data and analysis of various food products available on the market. It was shown that small variations in test implementation did not significantly affect the performance of the test kit. The assay was sufficiently rugged across varying incubation times and temperatures, and reagent volumes which may be introduced non-purposefully by the operating technician. These ruggedness studies show that the test kit will still reliably produce high quality results under minor fluctuations in conditions.

The Test kit components showed excellent stability over a period of 12 months without any loss of analytical capacity. Furthermore the test was not influenced by small changes above and below environmental and operating parameters such as temperature, incubation time and volume.The independent laboratory study confirmed the accuracy and repeatability of the VitaFast® method. Although the VitaFast® method did seem to produce results that were higher than those generated by the reference method, results of both reference materials were well within the range of acceptance. Analysis of the autoclaved extract from the reference method on the VitaFast microtiter plate produced a result above the upper limit of the NIST sample. Nevertheless, the VitaFast® method when implemented as outlined in the product insert performed as expected for both sample types.

Table 6. Intra-assay variance of food samples (triplicate analysis per sample dilution) (1)

Sample description (conc. indicated on label in µg / 100 g (ml))	Dilution factor	Mean result in µg / 100 g (ml)	Mean result of dilutions in µg / 100 g (ml)	Coefficient of variation in %
Vitamin pills (4.3 - 5.8)	30	1.37	1.41	4.5
	40	1.37		
	60	1.48		
Yoghourt strawberry, cranberry, raspberry (0.2)	2	0.24	0.25	4.6
	4	0.26		
	8	0.26		
Multivitamin bonbon (3.5)	20	3.4	3.7	7.2
	40	3.8		
	80	3.9		
Vitamin premix (3500)	20000	3597	3398	5.6
	40000	3220		
	80000	3377		
Ham sausage (2.0)	30	1.89	1.99	7.1
	40	2.09		
Children yoghourt (0.2)	8	0.25	0.25	2.9
	32	0.24		
Sirup (none)	2	0.34	0.36	4.3
	4	0.37		
	8	0.36		

Table 7. Intra-assay variance of food samples (1)

Sample description	Concentration indicated on label in µg / 100g (ml)	Mean result of dilutions in µg / 100 g (ml)	Coefficient of variation in %
Dextrose powder RM – Vit001 internal Reference material	1.0	0.98 (n=9) 0.93 (n=9) 1.08 (n=9)	7.7
Cherry bonbon	3.5	3.1 (n=4)	4.6
Multi vitamin pills	65	73 (n=6)	5.7
Energy drink	2	2.0 (n=4)	1.9
Multivitamin juice drink 1	1	1.35 (n=4)	4.3
Multivitamin juice drink 2	0.15	0.21 (n=4)	5.5

Table 8 . Comparison of results from various analytical methods performed internally and externally (1)

Sample description	Concentration indicated on label in µg / 100g (ml)	VitaFast	Other Methods
		Mean result of dilutions in µg / 100 g (ml)	
Drink food without lactose	0.70	0.74 (n=4)	0.71 internal HPLC
Drink food extra	0.47	0.29 (n=6)	0.29 internal HPLC 0.34 external HPLC
Hospital milk drink standard	0.21	0.21 (n=4)	0.22 internal HPLC 0.20 external HPLC
Hospital milk drink extra	0.21	0.32 (n=6)	0.26 intern HPLC 0.31 extern HPLC
Cereals	1.0	1.29 (n=6)	1.33 internal HPLC
Fruit mix	1.2	1.61 (n=6)	1.56 internal HPLC
Juice milk drink	0.20	0.20 (n=4)	0.17 external VitaFast
Banana milk pudding	0.65	0.82 (n=6)	0.90 (external Lab 1) 0.76 (external Lab 2) 0.89 (external Lab 3) classic microbiological assay AOAC 960.46

REFERENCES CITED

1. Kerr, Jessica, and Johnson, Kurt., Evaluation of the VitaFast® B12 (Cyanocobalamin): Microbiological Microtiter Plate Test for the Determination of B12, AOAC® *Performance Tested*℠ certification number 101002.
2. AOAC Research Institute Validation Outline for VitaFast® B12 (Cyanocobalamin): Microbiological Microtiter Plate Test for the Determination of B12, Approved – October 2010.
3. Higdon, J. Linus Pauling Institute, Oregon State University. 2008. Micronutrient Information Center: Vitamin B12. [Accessed online January 25, 2008: http://lpi.oregonstate.edu/infocenter/vitamins/pa/]
4. Food and Nutrition Board, Institute of Medicine. 1998. Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin and Choline. [Accessed online April 20, 2009: http://www.nap.edu/catalog.php?record_id=6015#toc]
5. European Food Information Council. 2006. Vitamins: What they do and where to Find them. [Accessed online april 15, 2009: http://www.eufic.org/article/en/page/MARCHIVE/expid/miniguide-vitamins/#9]
6. Health Canada. 2005. Addition of Vitamins and Minerals to Foods. [Accessed online January 25, 2008: http://www.hc-sc.gc.ca/fn-an/nutrition/vitamin/fortification_final_doc_1-eng.php]
7. U.S. Food and Drug Administration. 2009. Fortify Your Knowledge about Vitamins. [Accessed online April 15,2009: http://www.fda.gov/consumer/updates/vitamins111907.html]
8. Ball, G.F.M. 1998. Bioavailability and Analysis of Vitamins in Foods. Chapman & Hall.
9. Allen, L.H. 2003. B Vitamins: Proposed Fortification Levels for Complementary Foods for Young Children. J. Nutr. 133: 3000S-3007S
10. Weber, W., Evaluation of Modification Report for Location Change, AOAC® *Performance Tested*℠ certification number 101002. Approved March 2017.