VitaFast®
Vitamin Test Kits

Reliable microbiological vitamin determination in food, feed and pharmaceutical products.

- READY TO USE
  All reagents required for the microbiological assay are contained in the test kit (i.e. microtiter plate coated with microorganisms, sterile water, assay-medium, standard material.

- FLEXIBLE
  The reagents are portioned in 3 independent sets and the microtiter plate is in 12 x 8 well format, which is suitable for small or large laboratories. The assay can be carried out with sterile disposable lab equipment.

- FAST
  Compared to traditional microbiological methods which take 5 days, results are available within 24 - 48 h.

- TIME AND COST REDUCTION
  The colony forming microorganisms in the wells are adapted and optimised to the respective vitamins.

- INTEGRATED PROCEDURES
  The VitaFast® test kits feature uniform procedures across the product line.
VitaFast® Vitamin Test Kits

Reliable microbiological vitamin determination in food, feed and pharmaceutical products.

The test kits contains a microtiter plate which is coated with the specific microorganism which metabolises the vitamin to be determined. After extraction of the vitamin from the sample, the assay medium, standards or sample are pipetted into the wells of the microtiter plate. Thereafter, the test is incubated at 37°C for 24 - 44 h in the dark and evaluated in a microtiter plate reader at 610 - 630 nm (alternatively 540 - 550 nm). Sterile working techniques are recommended during the assay.

- **Reliable**
  Kits developed according to reference methods (AOAC, EU, § 64 LFGB). High accuracy and precision (CV < 10 %), excellent recoveries for certified reference samples.

- **Ready to use**
  All reagents required for the microbiological assay are contained in the test kit (i.e. microtiter plate coated with microorganisms, sterile water, assay-medium, standard material, adhesive foils and an additional holder for microtiter strips).

- **Flexible**
  The reagents are portioned in 3 independent sets and the microtiter plate is in 12 x 8 well format, which is suitable for small or large laboratories.
  The assay can be carried out with sterile disposable lab equipment in order to avoid any contamination.
  Therefore, sterile glass ware is not needed.

- **Fast**
  Compared to traditional microbiological methods which take 5 days, results are available within 24 - 48 h.

- **Time and cost reduction**
  The colony forming microorganisms in the wells are adapted and optimised to the respective vitamins.
  The cost intensive production and storage of microorganisms are no longer required.

- **Integrated procedures**
  The VitaFast® test kits feature uniform procedures across the product line.
VitaFast® Vitamin Test Kits

Please return this fax to:

I am interested in:

- A demonstration of the products in my laboratory
- A visit of a sales representative
- Product information referring:

VitaFast®

- Folic Acid (Vitamin B9)
- Cyanocobalmin (Vitamin B12)
- Biotin (Vitamin B7)
- Niacin (Vitamin B3)
- Pantothenic acid (Vitamin B5)
- Thiamin (Vitamin B1)
- Riboflavin (Vitamin B2)
- Pyridoxine (Vitamin B6)
- Choline
- Inositol
- Methionine
- Lysine
- Cystine

__________________________
Company:

__________________________
Name:

__________________________
Address:

__________________________
Phone/Fax:

__________________________
E-mail:

__________________________
Date:  Signature:
VitaFast® Vitamin Test Kits

The traditional vitamin analysis easy and ready-to-use!

VitaFast® Vitamin test kits are based on the microbiological vitamin detection procedure in microtiter plate format. The wells of a microtiter plate are coated with the specific microorganisms, which metabolises the vitamin to be determined. The cost intensive production and storage of microorganisms are no longer required. The amount of microorganisms in the wells are adapted and optimised to the respective vitamin. Only the double-strength assay medium, the vitamin in standard concentrations and the diluted sample extract must be added to the wells of the microtiter plate.

The test is incubated in an incubator and evaluated in a microtiter plate reader. The test comprises everything, the microorganisms ready to use in the wells, the assay medium and a defined standard, which is diluted for the standard range in simple steps.

## VitaFast® Vitamin Test Kits

Microbiological microtiter plate tests for the detection of vitamins in food, feed and pharmaceutical products.

<table>
<thead>
<tr>
<th>Traditional Vitamin Method AOAC, EN, DIN (§ 64 LFGB)</th>
<th>VitaFast® Vitamin Test Kits</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1</strong> Preparation of microorganisms on an agar plate Incubation 24 h</td>
<td>Extraction of the samples, preparation of assay medium and standards, test set up Incubation 44 - 48 h</td>
</tr>
<tr>
<td><strong>Day 2</strong> Stock culture by stab inoculation Incubation 24 h</td>
<td>Results</td>
</tr>
<tr>
<td><strong>Day 3</strong> Preparation of inoculation Incubation 24 h</td>
<td></td>
</tr>
<tr>
<td><strong>Day 4</strong> Extraction of the samples, preparation of assay medium and standards Incubation 24 - 48 h</td>
<td></td>
</tr>
<tr>
<td><strong>Day 5-6</strong> Results</td>
<td></td>
</tr>
</tbody>
</table>

### Features
- method developed according to the reference methods: AOAC, EN, DIN (§ 64 LFGB)
- ready-to-use test kits
- medium and standard included
- available for all water soluble vitamins and certain amino acids
- results available within 24 - 48 h
- high accuracy and precision data below 5%
- recovery between 95 - 105%
- easy test performance
- reduces hands on time by 60 - 70%
- cost reduction
- reduction of material of about 70%
- high degree of automation

### The VitaFast® Product Line:

- **Vitamins**
  - Folsäure / Folic Acid P1001
  - Vitamin B12 (Cyanocobalamin) P1002
  - Vitamin B7 (Biotin) P1003
  - Vitamin B3 (Niacin) P1004
  - Pantothenäure / Pantothenic Acid P1005
  - Vitamin B1 (Thiamin) P1006
  - Vitamin B2 (Riboflavin) P1007
  - Vitamin B6 (Pyridoxin) P1008

- **Related products**
  - Choline
  - Inositol
  - Methionine
  - Lysine
  - Cystine

For further information please contact:
VitaFast® Vitamin Test Kits
Flowchart for VitaFast® Folsäure (Folic Acid)

The kit contains three sets of reagents (48 determinations per set). Working under sterile conditions is recommended.

1. Extract sample

Vitaminized samples solid samples: Take 1 g of homogenized sample and extract with redist. water at 100 °C (212 °F); after sterile filtration dilute with sterile water.

Liquid samples: After sterile filtration dilute with sterile water.

Natural folate: Carry out an enzymatic treatment, then sterile filtration and dilute with sterile water.

2. Take kit reagents

Take 1 set of bottles and the microtiter plate. Insert a sufficient number of strips in the additional holder, return the unused strips into the bag and close it. Return the remaining kit components in the refrigerator.

3. Prepare assay medium

Add 10 ml of sterile water and 1 ml of the folic acid buffer to the assay medium, heat 5 min at 95 °C (200°F). Quickly chill down to below 30 °C (86 °F). Filtrate the medium in a sterile centrifuge vial.

4. Prepare standards

Add x ml of sterile water (see Quality Assurance Certificate) to the standard bottle. Pipette from the dissolved standard concentrate the different diluted standards.

5. Carry-out assay

Pipette into the wells 150 μl assay medium, 150 μl standard or sample, then cover the strips carefully with adhesive foil.

6. Incubate

44 - 48 h in the dark at 37 °C (98 °F)

7. Measure

Place the microtiter plate upside down on the table and dissolve the microorganisms by shaking the plate on the surface of the table. Invert the plate in the regular position and remove adhesive foil. Measure in an ELISA reader at 610 - 630 nm (alternatively 540 - 550 nm).

8. Evaluate

Use a four point software for the evaluation of the data, multiply the result with the respective dilution factor.
Test Validation

VitaFast® Folsäure / Folic Acid
Art. No. P1001

1. Test specification

Format: 96 well microtiter plate
(12 strips, 8 single wells)

Standard range: 0.16 - 1.28 µg / 100 g /ml

Incubation time: 44 - 48 h

2. Sample preparation

The sample preparation depends on whether native or supplemented folates are analysed. Supplemented folates are extracted for 30 min at 100 °C (212°F) in hot water. Native folates have to be treated enzymatically.

3. Reproducibility of standard curve

Table 1  Intra-assay variance (n=3) of the standard curve

<table>
<thead>
<tr>
<th>Standard conc. in µg / 100 ml</th>
<th>Mean absorbance</th>
<th>Coefficient of variation in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.051</td>
<td>2.8</td>
</tr>
<tr>
<td>0.16</td>
<td>0.249</td>
<td>2.6</td>
</tr>
<tr>
<td>0.32</td>
<td>0.427</td>
<td>0.5</td>
</tr>
<tr>
<td>0.64</td>
<td>0.713</td>
<td>2.6</td>
</tr>
<tr>
<td>0.96</td>
<td>0.912</td>
<td>1.6</td>
</tr>
<tr>
<td>1.28</td>
<td>1.081</td>
<td>1.5</td>
</tr>
</tbody>
</table>

4. Accuracy for reference materials

Table 2  Accuracy for the NIST reference material which is fortified with folate

<table>
<thead>
<tr>
<th>Reference material</th>
<th>Target concentration in µg / 100 g</th>
<th>Concentration measured in µg / 100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIST 1846 Infant formula</td>
<td>129 (101 – 157 )</td>
<td>120 (dilution 200)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>127 (dilution 300)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>124 (dilution 400)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>137 (dilution 500)</td>
</tr>
</tbody>
</table>

The standard reference material NIST1846 was manufactured by preparing a spray-dried formula base and then combining with a dry-blend premix that supplied all of the vitamins.
Test Validation

VitaFast® Folsäure / Folic Acid  
Art. No. P1001

Table 3  Accuracy for a reference material containing native and supplemented folate

<table>
<thead>
<tr>
<th>Reference material</th>
<th>Target concentration in μg / 100 mg</th>
<th>Concentration measured in μg / 100 mg</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRM 421 Milk powder</td>
<td>144 (128 – 156)</td>
<td>144 (n=4) 132 (n=4) 132 (n=1) 129 (n=1)</td>
<td>Extraction 24h / pancreatin</td>
</tr>
</tbody>
</table>

CRM 421 is a reference material which is folate enriched and also contains natural folate, therefore an enzymatic sample treatment is necessary. If only a hot water extraction is carried out, then the measured concentration is 91 μg / 100 g (n=4). This results compares well with HPLC combined with a microbiological assay (diss. Kehlenbach, Hannover 2004) according to which the CRM contains 92 μg folic acid / 100 g and 40 μg 5-methyl-tetrahydrofolate / 100 g.

Table 4  Accuracy for reference material containing only native folates

<table>
<thead>
<tr>
<th>Reference material</th>
<th>Target concentration in μg / 100 mg</th>
<th>Concentration measured in μg / 100 mg</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRM 121 Whole wheat flour</td>
<td>50 (43 – 57)</td>
<td>45 (n=2)</td>
<td>Extraction 12h / Pancreatin</td>
</tr>
</tbody>
</table>

5. Accuracy for representative food matrices

Table 5  Intra-assay variance of food samples  
(triplicate analysis per sample dilution)

<table>
<thead>
<tr>
<th>Sample description (conc. indicated on label in μg / 100 mg)</th>
<th>Dilution factor</th>
<th>Mean result in μg / 100 mg</th>
<th>Mean result of dilutions in μg / 100 mg</th>
<th>CV in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cereal flakes with fruits (261)</td>
<td>2000</td>
<td>254.6</td>
<td>275.7</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>1500</td>
<td>296.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>276.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet potato soup (100)</td>
<td>600</td>
<td>117.4</td>
<td>123.2</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>132.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>119.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tomato soup (200)</td>
<td>1200</td>
<td>108.0</td>
<td>111.8</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>103.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>123.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant formula (15)</td>
<td>100</td>
<td>29.1</td>
<td>28.2</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>28.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>26.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Four different kinds of folate enriched food products from the market have been tested. The mean results of the different dilutions have a coefficient of variation of less than 10 % and indicate that no bacteria inhibiting substances are present in the food extracts.
Test Validation

VitaFast® Folsäure / Folic Acid
Art. No. P1001

Table 6  Intra-assay variance of food samples

<table>
<thead>
<tr>
<th>Sample description</th>
<th>Conc. indicated on label in µg / 100 g</th>
<th>Mean result of dilutions in µg / 100 g</th>
<th>CV in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose powder RM – Vit001 intern</td>
<td>200</td>
<td>204 (n=4) 211 (n=3) 205 (n=4) 204 (n=4)</td>
<td>6.8 8.4 6.2 6.6</td>
</tr>
<tr>
<td>Ham sausage</td>
<td>200</td>
<td>415 (n=3)</td>
<td>2.1</td>
</tr>
<tr>
<td>Cake</td>
<td>150</td>
<td>119 (n=4)</td>
<td>6.0</td>
</tr>
</tbody>
</table>

The “true” concentration of the respective food products is not known. The manufacturers information on the food label is indicated in the table as well. Very often the concentration in food samples is higher than indicated on the label, because the manufacturers take the vitamin degradation during storage into account.

6. Comparison with other analytical methods

As far as vitamin analysis is concerned different analytical methods such as ELISA and HPLC yield different results than microbiological analysis. Often this is due to different sample extractions but the specificity of the antibody in the ELISA and the HPLC conditions play also an important role.

The microbiological method is the only method which is officially validated by international organisations. The test kit VitaFast® Folsäure / Folic Acid yields comparable results to the traditional microbiological method. When analysing a cereal based product both methods found a concentration of 1400 µg / 100g

7. Conclusion

The validation report for VitaFast® Folsäure / Folic Acid shows excellent accuracy and precision which gives the user a high level of security in food analysis. The test system is very robust and well suited for routine analysis.
Vitamins

Background information

Food supplements are concentrated sources of nutrients whose purpose is to supplement the normal diet. They are marketed 'in dose' form i.e. as pills, tablets and capsules. Common fortified foods are infant formula, cereal products and juices. The European Directive 2002/46/EC establishes harmonised rules for the labeling of food supplements and introduces specific rules on vitamins in food supplements.

Requirements for Analytical Testing

The addition of vitamins to foods must be carefully controlled, because vitamins are biologically active compounds that are required in small amounts, and are added to foods in very small quantities. Since vitamins are usually sold as premixes, these should come with certificates of analysis, and if stored, should be re-analysed at intervals to verify their potency.

It is proposed that manufacturers will be required to establish procedures for verifying the content of the vitamins in the final product. To accomplish this, the manufacturer should conduct tests to determine:

- the uniformity of distribution of the vitamin in the food
- the stability of the vitamin in the food
- the minimum amount of overage required to maintain the level of the vitamin in the food throughout its shelf-life.

In order to evaluate the accuracy of nutrition label information for compliance purposes, FDA regulations define that vitamins added in fortified or fabricated foods must be present at 100 % or more of the value declared on the label. Vitamins occurring naturally in a food product must be present at 80 % or more of the value declared on the label. Reasonable excesses of vitamins above labelled amounts are usually considered acceptable by the agency within good manufacturing practices.

In order to take account of natural and technological variations, the Association of German Chemists (GdCh) recommends a compliance of +/- 30 % for folic acid, vitamin B12 and biotin. In case of a necessary overdosage the labelled content should not be exceeded by 50 %.

Methods to determine vitamins

It is important to note, that as far as folic acid is concerned the microbiological method is the only method which is officially validated by international organisations. However, many different methods such as microbiology, ELSIA, HPLC and LC-MS are used for the determination of water soluble vitamins. Discrepancies between analysed vitamin content and declared values are not uncommon. Often the extraction procedure are the cause of these differences but also the specificity of the antibody in an ELISA etc. should be considered.
It is also important to consider whether the concentration for the “active vitamin” or the “vitamin compound” is indicated (see table for further info).

<table>
<thead>
<tr>
<th>Vitamin compound standard material</th>
<th>Conversion factor for active vitamin</th>
<th>Conversion factor for vitamin compound</th>
<th>Active vitamin label declaration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca-D-Pantothenate</td>
<td>0.9</td>
<td>1.11</td>
<td>Pantothenic acid</td>
</tr>
<tr>
<td>Thiamine-HCl B1</td>
<td>0.787</td>
<td>1.27</td>
<td>Thiamine B1</td>
</tr>
<tr>
<td>Pyridoxine-HCl B6</td>
<td>0.822</td>
<td>1.22</td>
<td>Pyridoxine B6</td>
</tr>
</tbody>
</table>

**Natural and added vitamins**

Naturally occurring vitamins are present in a variety of isomeric forms (from 100 % biologically active to totally inactive). Naturally occurring folate, for example, comprises a group of mono- and polyglutamate derivatives of pteroic acid. Total folate in foods is determined after extraction by homogenisation, deconjugation of polyglutamate forms to monoglутamate using conjugase and microbiological assay determination.

In the USA grain products are fortified with folic acid to reduce birth defects. The major synthetic forms of folate used by food processors are folic acid and 5MTHF (e.g. Metafolin™). Folate in fortified food can be easily determined by a hot water extraction followed by a microbiological assay.

When using reference materials it is important to know whether native or added vitamins are present. CRM 421, for example, contains native and supplemented folates; a hot water extraction will only measure the supplemented folates. NIST 1846 contains predominately added vitamins; a hot water extraction will measure all added vitamins.

**VitaFast® Folic Acid can be used to determine:**

- added folate in enriched food (hot water extraction to prepare sample)
- total folate in non-enriched food (enzyme treatment to prepare sample)
- total folate in enriched food (enzyme treatment to prepare sample).

VitaFast® Folic Acid is well suited as a routine method capable of determining added folic acid for enforcement purpose according to regulations.
Overview on vitamin methods (not complete) December 2005

Vitamin B1 (Thiamine Mononitrate)
- AOAC 953.17 Thiamine (Vitamin B1) in Grain Products, Flourimetric (Rapid) Method (1953)

Vitamin B2 (Riboflavin)
- AOAC 970.65 Riboflavin (Vitamin B2) in Foods and Vitamin Preparations, Flourimetric Method (1970)
- AOAC 940.33 Riboflavin (Vitamin B2) in Vitamin Preparations, Microbiological Method (1960)

Vitamin B3 (Niacinamid)
- AOAC 944.13 Niacin and Niacinamide in Vitamin Preparations, Microbiological Method (1960)
- AOAC 961.14 Niacin and Niacinamide in Drugs, Foods and Feeds, Colorimetric Method (1962)
- AOAC 968.32 Niacinamide in Multivitamin Preparations, Spectrophotometric Method (1969)
- AOAC 985.34 Niacin and Niacinamide in Ready-to-Feed Milk-Based Infant Formula, Microbiological Turbidimetric Method (1988) (Applicable to baby foods (meat based), beverages, and juices, cereal products, cheese, dairy products, fruits and potato products)

Vitamin B5 (D-Calcium Pantothenate)
- AOAC 945.73 Calcium Pantothenate in Vitamin Preparations, Spectrophotometric Method (1945)
- AOAC 945.74 Pantothenic Acid in Vitamin Preparations, Microbiological Method (1960)
- AOAC 992.07 Pantothenic Acid in Milk-Based Infant Formula, Microbiological Turbidimetric Method (1995)

Vitamin B6 (Pyridoxine)
- (Prenorm) DIN V ENV 14164, Edition:2002-05, Determination of Vitamin B6 with HPLC
- (Prenorm) DIN V ENV 14166, Edition:2002-02, Food – Microbiological Determination of Vitamin B6
- XP V03-136, Edition:2002-09-01 (France), Determination of Vitamin B6 with HPLC
- AOAC 961.15 Vitamin B6 (Pyridoxine, Pyridoxal, Pyridoxamine) in Food Extracts, Microbiological Methods (1975)
- AOAC 985.32 Vitamin B6 (Pyridoxine, Pyridoxal, Pyridoxamine) in Ready-to-Feed Milk Based Infant Formula, Microbiological Method (1988)
- AOAC 2004.07 Vitamin B6 in Reconstituted Infant Formula, Liquid Chromatographic method (2004) (Applicable to the determination of vitamin B6 in milk- and soy based liquid infant formula at 0 – 1 mg/ 100g)

Vitamin B12 (Cyanocobalamide)
- AOAC 952.20 Cobalamin (Vitamin B12 Activity) in Vitamin Preparations, Microbiological Methods (1960)

Biotin (Vitamin H) (Raw)
- no official methods known
Folic Acid (Raw)
- DIN EN 14131, Edition:2003-09, Food – Microbiological Determination of Folate
- AOAC 944.12 Folic Acid in Vitamin Preparations, Microbiological Method (1960)
- AOAC 992.05 Folic Acid in Infant Formula, Microbiological Method (1995)
- AOAC 2004.05 Total Folates in Cereals and Cereal Foods, Microbiological Assay Trienzyme Procedure (2004) (Applicable to cereal grains and cereal grain foods containing added folate or natural occurring folates with levels from 7.6 µg /100 g to 100 % folate)
- USP Method 411 Folic Acid Assay, HPLC
Trouble Shooting VitaFast®

Complete absence of growth in all cavities
- Inhibitory substance present in e.g. glassware or pipette tips
- Overnight change in the temperature of the incubator
- Assay medium not pipetted into cavities

Absence of growth in samples only
- Sample contains antibiotics or other inhibitor
- Wrong dilution chosen
- Sample at incorrect pH

Absence of growth in the standard only
- Deterioration of the standard such as exposure from light
- Standard kept too long
- Inhibitory material introduced into the standard solution

Excessive growth in all cavities, including the blanks
- Non sterile working conditions during assay
- Water contaminated
- The assayed vitamin or a substitute is present in the glassware or diluent

Irregular growth
- Insufficient trained staff setting up the assay
- Pipette not operating properly
- Assay medium bottle or sample vials not tightly closed in the waterbath
- Dirty glassware leading to contamination with other bacteria
- Microtiter plate allowed to stand in bright sunlight concerns esp. folic acid
- Adhesive foil is not sealing the cavities properly which leads to evaporation and yields higher absorptions
- Different temperatures in different areas of incubator
- Air bubbles in the wells during photometer reading
- Faulty spectrophotometer

Excessively high or low results
- Miscalculation
- Deterioration of standard
- Sample potency different than expected
- Omission or addition of a dilution step