Folic Acid ELISA Test
Enzyme Immunoassay for the quantitative determination
of Folic acid in food
Cat. No. COR-1736

Folic Acid
Enzyme Immunoassay for the quantitative determination of folic acid in food

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>2 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery rate</td>
<td>100 ± 10 %</td>
</tr>
<tr>
<td>Incubation time</td>
<td>140 minutes</td>
</tr>
<tr>
<td>Cross-reactivity relative to Folic acid (= 100 %):</td>
<td></td>
</tr>
<tr>
<td>Dihydrofolic acid</td>
<td>18 %</td>
</tr>
<tr>
<td>Tetrahydrofolic acid</td>
<td>5 %</td>
</tr>
<tr>
<td>5-Formyltetrahydrofolic acid</td>
<td>0.1 %</td>
</tr>
</tbody>
</table>

Introduction
Folates do play an important role in the synthesis of nucleic acids and some amino acids and gained recently due to their belonging to the group of antioxidative working Vitamins increased interest. In the last years increasingly the influence of Folic acid supplementation to avoid abortion and dysraphism was a topic of research. Folic acid as most stable representative of the group of Folates is added to a broad range of food.

Traditional methods are mostly microbiological methods, but also TLC and HPLC, where all the described methods are linked to a big time consumption and need a lot of equipment.

This test kit allows the quicker detection (2.5 to 4 hrs. incl. sample preparation) of folic acid in supplemented food compared to traditional techniques (24-48 hrs).

Principle of the test
Folic acid is bound to the surface of the microtitre plate. Samples, standards and a mouse-anti-folic acid antibody are added to the wells. During following 1 hr incubation at room temperature competition takes place between folic acid fixed on the plate and folic acid in the solution. Unbound material is washed away and an anti-mouse-IgG-HRP antibody is added, which binds only to mouse antibody bound on the plate. This technique offers the advantage that the HRP enzyme is not affected by interfering substances. After 1 hr incubation at room temperature any unbound material is washed away carefully and a
colourless substrate is added. Blue colour is formed during following 20 min incubation which is stopped after 20 min by acid addition. Solution turns from blue to yellow. Optical density is measured at 450 nm with a plate reader, and the folic acid concentration is calculated against the standard curve. The colour intensity is indirect proportional to the concentration of Folic Acid in the sample, i.e. the less Folic Acid was in the sample the more conjugate could be bound and this causes a deeper colour.

Precautions

- Before performing the test all kit components should have reached ambient temperature.
- All reagents should be mixed carefully (without forming foam) before using them.
- After having started the test, all steps should be performed without time losses.
- After having transferred reagents from the vials the vials must be closed again with their stoppers.
- For each sample use a separate pipette tip to avoid cross contamination.
- For each series of samples a new calibration with the standard solutions of the kit is strongly recommended.
- Components of different kits may not be mixed together.
- Don’t use kit components after expiry date printed on the label.
- The precision and accuracy of the lab equipment should be controlled routinely (ELISA reader, pipettes,...)
- The test should be performed in duplicate.

Health and safety instructions

- Do not smoke, eat or drink in the lab. Do not pipette reagents using the mouth.
- In case of contact of the reagents with skin wash the parts immediately with water.
- The handling and the discard of the reagents should happen according to GLP.

Reagents

The reagents are sufficient for 96 wells; they should be stored at 4 to 8 °C. The expiry date of each component is labelled on the vials.

1. Microplate 96 wells (12 strips with each 8 wells), coated with Folic Acid.
2. 6 Bottles Folic Acid-standards, each 1 mL (0; 4; 10; 40; 100; 400 ng/mL), red coloured, ready to use.
3. Mouse-anti-folic acid antibody, 6 mL, red coloured, ready to use.
4. Conjugate (Anti-mouse-IgG-Peroxidasis), 11 mL, red coloured, ready to use.
5. Substrate (tetramethylbenzidin, TMB), 11 mL, ready to use.
6. Stopping-solution (1 M sulphuric acid), 11 mL, ready to use.
7. Sample diluent (PBS), 2 × 60 mL, ready to use.
8. Wash buffer concentrate (PBS + Tween 20), 60 mL as 10-fold-concentrate.
9. 2 × sealing film for covering plates during incubation.
10. Reusable plastic bag for storage unused wells.
Additional Reagents and Instrumentation (not provided)

Equipment
- micropipettes 5 µL-, 50 µL- and 1000 µL
- measuring pipettes
- mortar or mill
- centrifuge
- microplate reader (450 nm), optional 620 nm as reference

Reagents
- Potassium hexacyanoferrat(II)-3-hydrat (Carrez I), 150 g/L distilled water
- Zincesulphate-7-hydrat (Carrez II), 300 g/L distilled water
- distilled water, double distilled water
- Sodium hydroxide 1M
- Hydrochloric acid 1M

Preparation of the kit reagents
- Microplate: Before opening the foil bag let all components of the kit reach ambient temperature. Take of the necessary number of wells, and put back the not needed wells into the bag with the desiccant and close the bag.

- Wash buffer: Dilute the wash buffer concentrate 1:10 with distilled. water (1 part concentrate + 9 parts distilled water). In case, crystals have formed during storage, they can be dissolved by heating the concentrate to 37°C for 15 minutes and cooling down to ambient temperature again.

Sample preparation
The vitamin is extracted from matrix with double distilled water. pH is adjusted by NaOH 1M or HCl 1M to pH 6-7. After addition of Carrez-I-solution and Carrez-II-solution distilled water is added to a defined volume. The formed precipitate is removed by centrifugation. In cold water insoluble samples might be dissolved under slight warming.

The clear supernatant is diluted to an approximate concentration of 4-100 ng Folic Acid/mL with sample diluent. Dilution factor should be minimum 10. At higher concentrations matrix effects might cause interference. 100 µL of the final solution are used in the kit.

Cereal samples contain small amounts of folic acid. To avoid high dilution factors, the sample may be extracted with sample diluent (not wash buffer!). Additional sample diluent can be ordered separately from Diagnostic Automation, Inc.
Vitamin tablets

- The ground vitamin tablets are dissolved in double distilled water. If necessary heat to 35-40°C.
- Adjust pH to 6-7 (NaOH or HCl) and add 0.5 mL Carrez I solution and then 0.5mL Carrez II solution.
- Make up to a certain volume with double distilled water.
- Centrifuge the solution and dilute 100 µL of the clear supernatant with sample diluent to end up in a concentration of 4-100 ng Folic Acid/mL. Dilution factor should not be less than 10.
- This solution is used in step 9 (procedure)

Fruit juices

- Adjust pH to 6-7 (NaOH or HCl) and add 0.5 mL Carrez I solution and then 0.5 mL Carrez II solution.
- Make up to a certain volume with double distilled water.
- Centrifuge the solution and dilute 100 µL of the clear supernatant with sample diluent to end up in a concentration of 4-100 ng Folic Acid/mL. Dilution factor should not be less than 10.
- This solution is used in step 9 (procedure)

Marmalade

- Dilute app. 8 g marmalade with double distilled water to a certain volume and mix thoroughly.
- Adjust pH to 6-7 (NaOH or HCl) and add 0.5 mL Carrez I solution and then 0.5mL Carrez II solution.
- Make up to a certain volume with double distilled water.
- Centrifuge the solution and dilute 100 µL of the clear supernatant with sample diluent to end up in a concentration of 4-100 ng Folic Acid/mL. Dilution factor should not be less than 10.
- This solution is used in step 9 (procedure)

Cereals

- Homogenise 3-5 g sample with a mortar or a mill and add double distilled water and mix thoroughly. In case the sample is known to contain very small amounts of Folic Acid instead of water the use of the sample diluent is recommended.
- Adjust pH to 6-7 (NaOH or HCl) and add 0.5 mL Carrez I solution and then 0.5 mL Carrez II solution.
- Make up to a certain volume with double distilled water.
- Centrifuge the solution and dilute 100 µL of the clear supernatant with sample diluent to end up in a concentration of 4-100 ng Folic Acid/mL. Dilution factor should not be less than 10.
- This solution is used in step 9 (procedure).
Drops

- The ground drops are dissolved in double distilled water. If necessary heat to 35-40°C.
- Adjust pH to 6-7 (NaOH or HCl) and add 0.5 mL Carrez I solution and then 0.5 mL Carrez II solution.
- Make up to a certain volume with double distilled water.
- Centrifuge the solution and dilute 100 µL of the clear supernatant with sample diluent to end up in a concentration of 1-10 ng Folic Acid/mL. Dilution factor should not be less than 5.
- This solution is used in step 9 (procedure).

Milk powder and milk products

- Dissolve 20 g sample in 100 mL distilled water at 35°C.
- Centrifuge sample.
- Take of clear supernatant and dilute 1:5 with sample diluent.
- This solution is used in step 9 (procedure).

Procedure

1. Mark the wells as standard or sample wells.
2. Pipette in the appropriate wells in duplicate 100 µL of the standard solutions or the sample solutions.
3. Pipette 50 µL of the mouse-anti-folic acid antibody and mix the wells shortly without loosing liquid.
4. Seal the wells with adhesive film (supplied with the kit) and incubate 60 minutes at ambient temperature on a shaker.
5. Discard the film and invert wells and shake out liquid into the sink. Wash the wells 3 times with diluted wash buffer and empty them again. Ensure that every well is filled completely (300 µL). After final wash tap wells onto tissue paper until completely dry.
6. Immediately after the final wash add 100 µL anti-mouse IgG HRP to each well. Seal wells with new adhesive film, mix shortly and incubate the wells at ambient temperature for 60 minutes.
7. Discard the film and invert wells and shake out liquid into the sink. Wash the wells 3 times with diluted wash buffer and empty them again. Ensure that every well is filled completely (300 µL). After final wash tap wells onto tissue paper until completely dry.
8. Add 100 µL substrate to each well, mix carefully and incubate for 20 min in the dark at room temperature. Colour development from colourless to blue.
9. Stop colour reaction by addition of 100 µL stop solution (Attention: 1 M sulphuric acid), and mix the wells carefully. Colour change blue to yellow.
10. Mix wells before measuring absorbance at 450 nm within 30 minutes, best using a plate reader (or equivalent photometer). As reference wavelength 620 nm can be used. Colour is stable for 30 min.

Standard curve and interpretation of results

- Calculate for each standard and sample the mean absorbance.
- Calculate the $B_0$ value by dividing the absorbances through the absorbance of the 0 ng/mL standard.
Plot B$_0$-absorbance of the standards against $\log_{10}$ standard concentration.
Read sample concentrations from the standard curve and multiply the value with the factor (2 dilution steps!) resulting of the sample preparation.

**Example**

A vitamin tablet was extracted according the described method. After Carrez precipitation the solution was made up to 50 mL and the supernatant diluted 1:500 with sample diluent. In the test a concentration of 40 ng/mL was measured. The resulting factor "F" is calculates as follows:

$$F = A \times B$$

A: Dilution factor 1 (in this case 50, for the tablet was dissolved in 50 mL)
B: Dilution factor 2 (in this case 500, for the supernatant was diluted after centrifugation 1:500)

The dilution factor has got the dimension mL/tablet. The measured concentration is multiplied with the factor to get the real concentration.
Real concentration = 40 ng/mL $\times$ 25,000 mL/tablet = 1,000,000 ng/tablet = 1 mg/tablet
For milk samples the result must be multiplied by 25.

**Typical standard curve**

The following table is an example for a standard curve. Real values can differ. This values may not be used as real standard curve, which has to be measured for each measuring series.

<table>
<thead>
<tr>
<th>Folic acid-concentration (ng/mL)</th>
<th>Absorption (% of 0 ng/mL-Standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>40</td>
<td>25</td>
</tr>
<tr>
<td>100</td>
<td>11</td>
</tr>
<tr>
<td>400</td>
<td>4</td>
</tr>
</tbody>
</table>

**Sensitivity**

The sensitivity of the DIAGNOSTIC AUTOMATION, INC. Folic Acid test is 2 ng/mL (based on the standard curve).

**Recovery**

Recovery with spiked samples was determined at 100 ± 10 %.
Intraassay-Precision

Intraassay-CV value was determined in 20 determinations at 3 %.

Specificity

The specificity of the DIAGNOSTIC AUTOMATION, INC. Folic acid test was assessed by the determination of the cross-reactivity against the following substances:

Cross-reactivity relative to Folic acid (= 100 %):

<table>
<thead>
<tr>
<th>Substance</th>
<th>%</th>
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<tr>
<td>Dihydrofolic acid</td>
<td>18</td>
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<td>5-Formyltetrahydrofolic acid</td>
<td>0.1</td>
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Literature


ISO 13485-2003