



**NATIONAL STANDARD OF THE PEOPLE'S REPUBLIC OF  
CHINA**

GB 5413.14—2010

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**National food safety standard  
Determination of vitamin B12 in foods for  
infants and young children, milk and milk  
products**

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## Foreword

Instead of the standard GB / T 5413.14-1997 "determination of vitamin B12 in foods and dairy for infants"

The major changes with GB / T 5413.14-1997 are as follows:

- The standard name "Determination of vitamin B12 in foods for infants and young children, milk and milk products"

Appendix A is normative appendix.

The replaced standard versions are:

- GB 5413-1985, GB / T 5413.14-1997.

# Determination of vitamin B12 in foods for infants and young children, milk and milk products

## 1. Range:

This standard is formulated a microbial method for determination of vitamin B12 in foods for infants and young children, milk and milk products.

This standard is applicable to determination of vitamin B12 in foods for infants and young children, milk and milk products.

## 2. Referenced normative documents

The following standards contain provisions, which through reference in this text, are indispensable part of this Standard. For dated references, subsequent amendments (exclude correction) to or revisions of any of these publications shall not apply to this Standard. For undated references the latest edition (include correction) of the publication referred to applies.

## 3. Principle:

Take advantage of *Lactobacillus leichmannii*'s specificity and sensitivity to vitamin B12, determine concentration of vitamin B12 in sample. The determination medium supply all nutrients except vitamin B12, so this bacteria growth condition depend on the concentration of vitamin B12 in sample and standards. Draw standard curve with the different concentration of vitamin B12 standard according to different transmittance value, then determine the content of vitamin B12 in the sample.

## 4. Reagent, strain and culture medium

All reagents, if no special specification, refers to analytic reagent; All experiment water, if no special order, refers to GB/T 6682 level 2 water.

4.1. Strain: *Lactobacillus leichmannii* ATCC 7830

4.2. vitamin B12 standard:  $C_{63}H_{88}CoN_{14}O_{14}P$ ; purity  $\geq 99\%$

4.3. culture medium

4.3.1. *Lactobacillus* agar culture medium: see appendix A

4.3.2. *Lactobacillus* broth culture medium: see appendix A

4.3.3. Vitamin B12 determine medium: see appendix A

(note: the commercial synthetic medium is better. preparation according to commercial medium label instruction)

4.4. Normal saline: 9g/L

Weigh 9g NaCl into 1000ml water, mix thoroughly. Dispense each tube 10ml, and plug the caps, sterilize 15min at 121 °C..

4.5. ethanol: 25%(v/v)

4.6. Disodium hydrogen phosphate anhydrous ( $Na_2HPO_4$ )

4.7. Anhydrous sodium sulfite ( $Na_2S_2O_5$ )

4.8. Citric Acid(with a hydrate)

4.9. Preparation of standard solution

4.9.1. Vitamin B12 stock solution: 10  $\mu\text{g}/\text{mL}$

Accurately weigh vitamin B12 standard and dilute with ethanol(4.5) to make concentration exactly 10  $\mu\text{g}/\text{mL}$ .

4.9.2. Vitamin B12 intermediate solution: 100ng/mL.

Dilute 5 mL stock solution(4.9.1) with ethanol(4.5) to total 500mL

4.9.3. Vitamin B12 work solution:1ng/ml.

Dilute 5 mL intermediate solution(4.9.2) with ethanol(4.5) to total 500mL

4.9.4. Work standard solution: two different concentrations of vitamin B12.

High concentration: 0.02ng/mL.

Dilute 5ml work solution(4.9.3) with distilled water to total 250mL.

Low concentration: 0.01ng/ml.

Dilute 5ml work solution(4.9.3) with distilled water to total 500mL.

Note: All standard solutions should be stored in the refrigerator. 4.9.1,4.9.2 and 4.9.3 save of three months. 4.9.4 prepared just before use.

## 5. Apparatus

Common laboratory equipment and:

5.1. Analytical Balance: resolution 0.1mg.

5.2. pH meter: accuracy  $\leq 0.01$

5.3. Spectrophotometer.

5.4. vortex mixer

5.5. centrifuge:  $\geq 2000\text{r}/\text{min}$

5.6. Biochemical Incubator:  $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$

5.7. refrigerator:  $2^{\circ}\text{C} \sim 5^{\circ}\text{C}$

5.8. sterile pipette: 10 mL (with 0.1 mL scale) or micro-pipette and tips.

5.9. Eppendorf Varispenser: 0 mL ~ 10 mL

5.10. conical flask: 200mL

5.11. volumetric flask (A class): 100 mL, 250 mL, 500 mL

5.12. Single-scale pipette (A class): capacity 5 mL

5.13. Hopper: diameter 90 mm.

5.14. filter paper: diameter 90 mm

5.15. tube: 18 mm  $\times$  180 mm

## 6. analytical procedure

6.1. preparation of strain

6.1.1 Transfer Lactobacillus (Lactobacillus leichmannii) ATCC 7830 freeze-dried powder into Lactobacillus broth culture medium (4.3.1) tube, incubate 24 h at  $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . Subculture the strain for 2-3 generations. Store the strain at  $2^{\circ}\text{C} \sim 5^{\circ}\text{C}$  refrigerator. Subculture this strain every 15d.

6.1.2 Inoculate the subcultued Lactobacillus leichmannii strain to Lactobacillus broth culture medium (4.3.2). Incubate 18h-24h at  $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . Centrifuge culture for 10minutes at 2000r/min, and then decant supernate. Resuspend cells by 10mL normal saline (4.4) and

centrifuge it again. Repeat above steps again. Then resuspend cells by 10mL normal saline (4.4), and transfer 1ml suspension into 10mL normal saline (4.4), mix thoroughly.

6.1.3 Using normal saline (4.4) as blank reference, the transmittance of the suspension (6.1.2) at 550nm spectrophotometer should between 60%-80%.

## 6.2 preparation of sample:

6.2.1 Dissolve Disodium hydrogen phosphate anhydrous (4.6) 1.3g, Anhydrous sodium sulfite (4.7) 1.0g, Citric Acid(with a hydrate)(4.8) 1.2g with 100ml distilled water.

6.2.2 Weight a amount of samples(accurate to 0.1mg),equivalent contain 50-100ng vitamin B12, mixed with 10mL above solution(6.2.1), then add 150ml distilled water. Hydrolyze for 10min at 121°C, then cool-off and adjust to PH4.5± 0.2. Constant volume with water to 250 mL, then filter. Pipet 5 mL filtrate into 20 mL ~ 30 mL water, adjust pH to 6.8 ± 0.2, volume by water to 100 mL. In final solution the concentration of vitamin B12 is between 0.01-0.02ng/ml and Sodium Metabisulfite is less than 0.03mg./mL.

## 6.3 preparation of solution of standard curve

Add distilled water, standard solution(4.9.4) and vitamin B12 determine medium(4.3.3) into tubes according to the table1, making triplicate.

Table1:

Tube No:	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Distilled water:(ml)	5	5	4	3	2	1	0	2	1	0
0.01ng/mL Standard solution:(ml)	0	0	1	2	3	4	5	0	0	0
0.02ng/mL Standard solution:(ml)	0	0	0	0	0	0	0	3	4	5
Medium:(ml)	5	5	5	5	5	5	5	5	5	5

## 6.4 assay solution:

Add distilled water, sample solution(6.2.2) and vitamin B12 determine medium(4.3.3) into tubes according to the table2, making triplicate.

Table2:

Tube No:	1	2	3	4
Distilled water :(ml)	4	3	2	1
Sample solution: (ml)	1	2	3	4
Medium:(ml)	5	5	5	5

## 6.5 sterilization

Cap all tubes (6.2 and 6.3) and sterilize for 5mins at 121°C, (for commercial medium sterilization according to the label instruction)

## 6.6 inoculation

Cool-off these tubes rapidly to beneath 30°C. Drip a drop(about 50 µL) of suspension(6.1.2) into each above tube by pipette(except standard S1).

## 6.7 incubate

Incubate for 19-20h at 36°C±1°C.

## 6.8 assay

Predict the growth situation through visually inspect each tubes: unincubation tube should clear. If the unincubation tube is turbid, the result is invalid.

6.8.1 Assay the T of the maximum concentration tube transmittance with using a blank inoculation tube as reference. And assay this tube again after 2h. If the difference between this two results are  $\leq 2\%$ , that mean you can take out all the tubes and assay their transmittance.

6.8.2 Using uninoculation tube(S1) as blank, adjust the transmittance of spectrophotometer to 100% (or absorbance to 0), to assay the inoculation tube (S2). Then using inoculation tube(S2) as blank, adjust the transmittance of spectrophotometer to 100% (or absorbance to 0), to assay transmittance of other tubes.

6.8.3 with a vortex mixer(5.4) well-mixed each test tube (or add a drop of antifoaming agent), transfer the culture immediately into the cuvette, read the transmittance after 30s at 550nm. Each tube should have the same settling time. With the amount of vitamin B12 in standard solution as X-axis, the data of T as Y-axis, draw standard curve.

6.8.4 Quantitative determine the value of concentration of vitamin B12 from the standard curve in accordance with test value. Then calculate the content of vitamin B12 in the sample according to the dilution factor and the mass of the sample. Abandon the value less than S3 or high than S10.

6.8.5 Calculate each tube's concentration of vitamin B12 according to its transmittance. Calculate the average value of the 3 same serial number tubes, compare each value with the average value and abandon the one exceeding  $\pm 15\%$ . If calculable value you received is less than 2 / 3 of total tubes, must be redone; If calculable value is more than 2 / 3 of total tubes, recalculate the average value with calculable value. This average value can calculate the total average value of all serial number tubes  $C_x$ .

Note: can use the transmittance (T%), or the absorbance (A) as Y-axis draw standard curve.

## 7. Indication

The content of vitamin B12 in sample according to this formula:

$$X = C_x / m \times F / 1000 \times 100$$

X: Content of vitamin B12 in sample ,  $\mu\text{g}/100\text{g}$ ;

$C_x$ : the average value of vitamin B12 checked from the working curve(6.8.5), ng;

F: dilution factor;

m: mass of the sample, g;

## 8. Allowable error

The difference between the values of the twice tests to the same sample should  $\leq 10\%$ .

## 9. Limitation

The limitation of this standard is  $0.1\mu\text{g}/100\text{g}$ .

# Appendix A

(Normative Annex)

Medium and reagent

## A.1 Lactobacillus agar culture medium

### A.1.1 ingredient

Tomato juice 100 mL, on the 3rd peptone 7.5 g, yeast extract 7.5 g, glucose 10.0 g, potassium dihydrogen phosphate 2.0 g, poly sorbitol monostearate 1.0 g, agar 14.0 g, water 1000 mL, pH 6.8  $\pm$  0.1 (25 °C  $\pm$  5 °C).

### A.1.2 preparation

Dissolve all ingredients except agar into water, adjust PH and then add agar. Boil the medium to dissolved thoroughly. Dispense each tube 10ml, sterilize 15min at 121°C..

## A.2 Lactobacillus broth culture medium

### A.1.1 ingredient

Tomato juice 100 mL, on the 3rd peptone 7.5 g, yeast extract 7.5 g, glucose 10.0 g, potassium dihydrogen phosphate 2.0 g, poly sorbitol monostearate 1.0 g, water 1000 mL, pH 6.8  $\pm$  0.1 (25 °C  $\pm$  5 °C).

### A.1.2 preparation

Dissolve all ingredients(A.2.1) into water, adjust PH and boil the medium to dissolved thoroughly. Dispense each tube 10ml, sterilize 15min at 121°C..

## A.3 Vitamin B12 determine medium

### A.3.1 ingredient

Acid hydrolysis of vitamin-free casein 15.0 g, glucose 40.0 g, asparagine 0.2 g, sodium acetate, 20.0 g, ascorbate acid 4.0 g, L-cystine 0.4 g, DL-tryptophan 0.4 g, adenine sulfate 20.0 mg, guanine hydrochloride 20.0 mg, uracil 20.0 mg, xanthine 20.0 mg, riboflavin 1.0 mg, thiamine hydrochloride 1.0 mg, biotin 10.0  $\mu$ g, niacin 2.0 mg, p-amino benzoic acid 2.0 mg, calcium pantothenate 1.0 mg, Pyridoxine hydrochloride 4.0 mg, pyridoxal hydrochloride 4.0 mg, hydrochloric acid pyridoxamine 800.0  $\mu$ g, folic acid 200.0  $\mu$ g, potassium dihydrogen phosphate 1.0 g, potassium hydrogen phosphate 1.0 g, magnesium sulfate 0.4 g, chlorine Sodium 20.0 mg, ferrous sulfate 20.0 mg, manganese sulfate 20.0 mg, polyethylene sorbitol monostearate (Tween 80) 2.0 g, water 1000 mL, pH 6.0  $\pm$  0.1 (25 °C  $\pm$  5 °C).

### A.3.2 preparation

Dissolve the ingredients into water, adjust PH and sterilization.