National Standard of the People's Republic of China

GB 4789.3-2016

National Food Safety Standard

Food Microbiological Examination-Enumeration of Coliforms

食品安全国家标准

食品微生物学检验 大肠菌群计数

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- Issued by NHFPC & CFDA

Foreword

This standard replaces GB 4789.3-2010 “National Food Safety Standard Food Microbiological Examination Enumeration of Coliforms”, GB/T 4789.32-2002 “Food Hygiene Microbiological Examination Rapid Detection of Coliform” and content concerning enumeration of coliforms in SN/T 0169-2010 “Detection Method of Coliform, Fecal Coliform and Coli bacillus in Import and Export Food”.

Compared with GB 4789.3-2010, major changes of this standard are as follows:

— Examination principle has been added;

— The application scope has been revised;

— Morphological description of typical bacterial colony has been revised;

— Number of colony chosen on the plate has been revised in Method II

— Confirmatory test of method II has been revised

— The report of plate count in method II has been revised.

National Food Safety Standard
Food Microbiological Examination-Enumeration of Coliforms

1. Scope

This standard sets out the method for the enumeration of coliforms in foods.
Method I in this standard applies to the enumeration of coliforms in foods which contain lower level of coliforms; Method II applies to the enumeration of coliforms in foods which contain higher level of coliforms

2. Terms and definitions

2.1 Coliforms
A cluster of concurrently aerobic and anaerobic gram negative sporeless bacilli which can ferment lactose and generate acid and gas under certain culture conditions.
2.2 Most probable number, MPN
A Poisson distribution-based indirect count method.

3. Examination principle

3.1 MPN method
MPN method is such a quantitative detection method that combines statistics and microbiology. After the samples were diluted and cultured, the maximum possible number of coliforms in samples could be estimated through probability theory based on minimum dilution degree to which colony does not grow and maximum dilution degree to which colony grows.
3.2 Plate count
Coliforms can make acid while fermenting the lactose in solid culture medium. With the help of indicator, countable red or purple bacterial colonies which could be formed with or without precipitation ring.

4. Apparatus and materials

Apart from the conventional apparatus for sterilization and incubation in microbiological laboratory, other apparatus and materials are as below:

4.1 Constant temperature incubator: 36℃±1℃.

4.2 Refrigerator: 2℃~5℃.

4.3 Constant temperature water bath: 46℃±1℃.

4.4 Balance: with sensitivity of 0.1g.
4.5 Homogenizer.
4.6 Shaker.
4.7 Sterile pipette: with nominal capacities of 1mL (graduated in 0.01mL division) and 10mL (graduated in 0.1mL division), or micropipette and pipette tips.
4.8 Sterile conical flask: with nominal capacity of 500mL.
4.9 Sterile culture dish: with diameter of 90mm.
4.10 pH meter or pH colorimetric tube or precise pH test paper.
4.11 Colony counter.

5. Culture mediums and reagents

5.1 Lauryl sulfate tryptose (LST) broth: See A.1
5.2 Brilliant green lactose bile (BGLB) broth: See A.2.
5.3 Violet red bile agar (VRBA): See A.3.
5.4 Sterile phosphate buffer solution: See A.4.

5.5 Sterile physiological saline solution: See A.5.
5.6 1mol/L NaOH: See A.6.
5.7 1mol/L HCL: See Section A.7.

Method I Coliforms MPN Count

6. Examination procedures

See Figure 1 for the examination procedures of Coliforms MPN count.

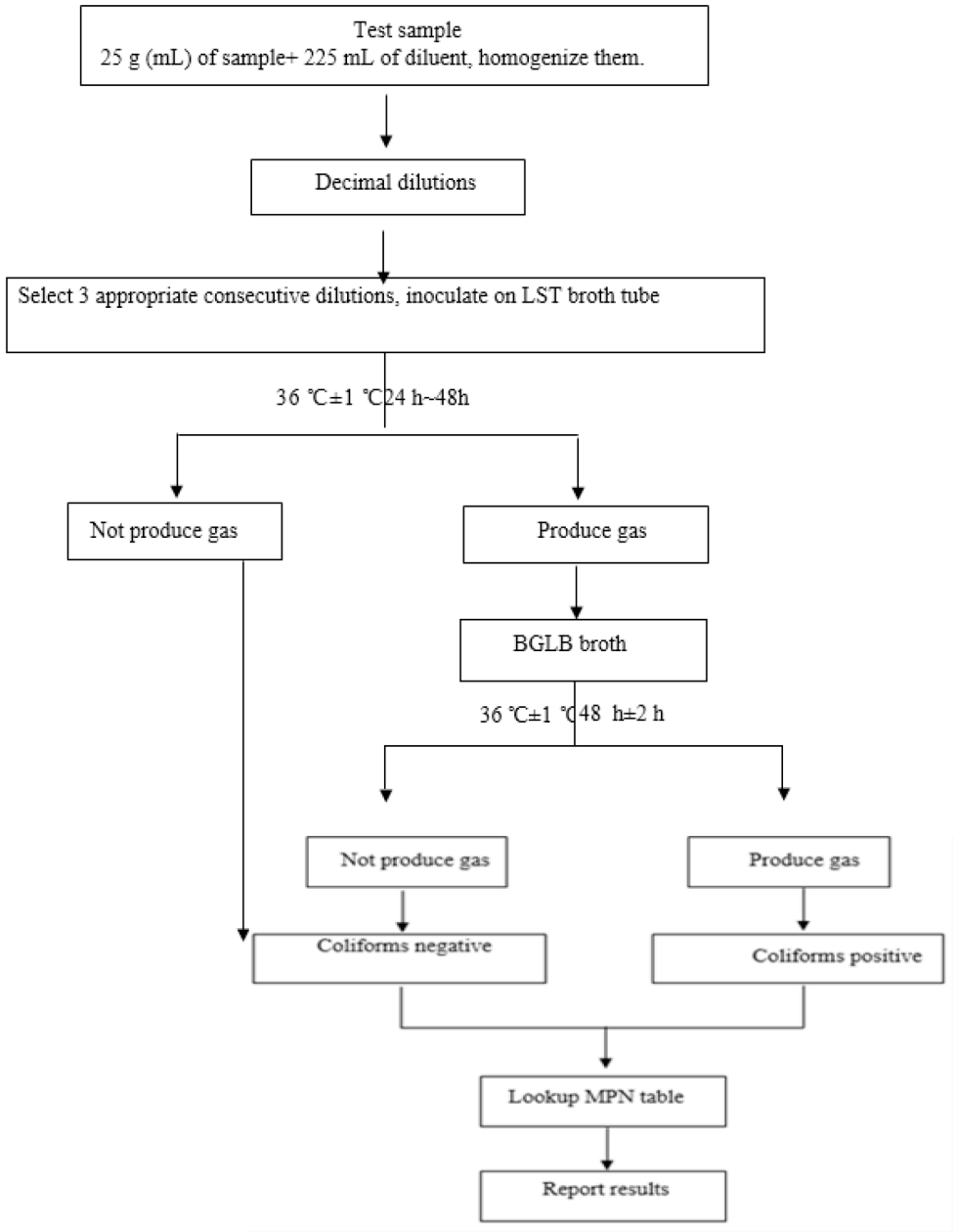

Figure 1 Examination procedures of Coliforms MPN count

7. Operation procedures

7.1 Dilution of sample

7.1.1 Solid and semi-solid sample: Weigh 25g of sample and put it into a sterile homogenizing cup which contains 225mL of phosphate buffer solution or physiological saline solution, and homogenize at 8000r/min~10000r/min for 1min~2min or put it into a sterile homogenizing bag which contains 225mL of phosphate buffer solution or physiological saline solution and homogenize it for 1min~2min in a slapping homogenizer to get a 1:10 homogeneous sample solution.

7.1.2 Liquid sample: Pipette 25mL of sample with a sterile pipette, put it into a sterile conical flask (an appropriate amount of sterile glass beads should be put into the flask in advance) which contains 225mL of phosphate buffer solution or physiological saline solution, or in other sterile container for adequate shaking or put it into mechanical oscillator for shaking, and mix them well to get a 1:10 homogeneous sample solution.

7.1.3 The pH of the homogenous sample solution should be within the range of 6.5 and 7.5. Adjust the pH with 1mol/L sodium hydroxide (NaOH) or 1mol/L hydrochloric acid (HCL), when necessary.

7.1.4 Pipette 1mL of the above 1:10 homogenous sample solution with a 1mL sterile pipette or micropipette, slowly pour it into a sterile tube which contains 9mL of phosphate buffer solution or physiological saline solution along the tube wall (Make sure that the pipette or pipette tip do not touch the diluent), shake or blow and beat with another 1mL sterile pipette again and again to mix them well and a 1:100 homogeneous sample solution is produced.

7.1.5 Based on the estimate of sample contamination status and according to the operation procedures above, prepare decimal ascending dilutions. Use a new 1mL sterile pipette or pipette tip for each dilution. During the process from preparing the homogenous sample solution to finishing the inoculation, only 15min or less is allowed.

7.2 Primary fermentation test

For each sample, select three appropriate consecutive dilutions (test sample may be selected in case of liquid sample). Inoculate on 3 tubes of lauryl sulfate tryptone (LST) broth for each dilution, 1mL for each tube (if the inoculation amount exceeds 1 mL, double-strength LST broth should be put into use). Incubate at 36℃±1℃ for 24h±2h and observe whether bubbles are generated in the inverse tubes. If there are bubbles, perform secondary fermentation test. If there are no any bubbles, continue incubating to 48h±2h. Tubes without bubbles are coliforms negative and tubes with bubbles take secondary fermentation test.

7.3 Secondary fermentation test

Take 1 loop of culture from each of the gas-producing LST broth tubes with inoculation loops, transfer inoculate them to brilliant green lactose bile (BGLB) broth tubes. Incubate at 36℃±1℃ for 48h±2h and observe bubble-generation. Gas-producing tubes are recorded as coliforms positive.

7.4 Report on most probable number (MPN) of coliforms

Based on the number of tubes which are coliforms B G L B positive verified in accordance with the procedures in Section 7.3, search the MPN Table (see Annex B) and report MPN of coliforms in each gram (or mL) of sample.

Method II Coliforms Plate Count

8. Examination procedures

See Figure 2 for the examination procedures of coliforms plate count.

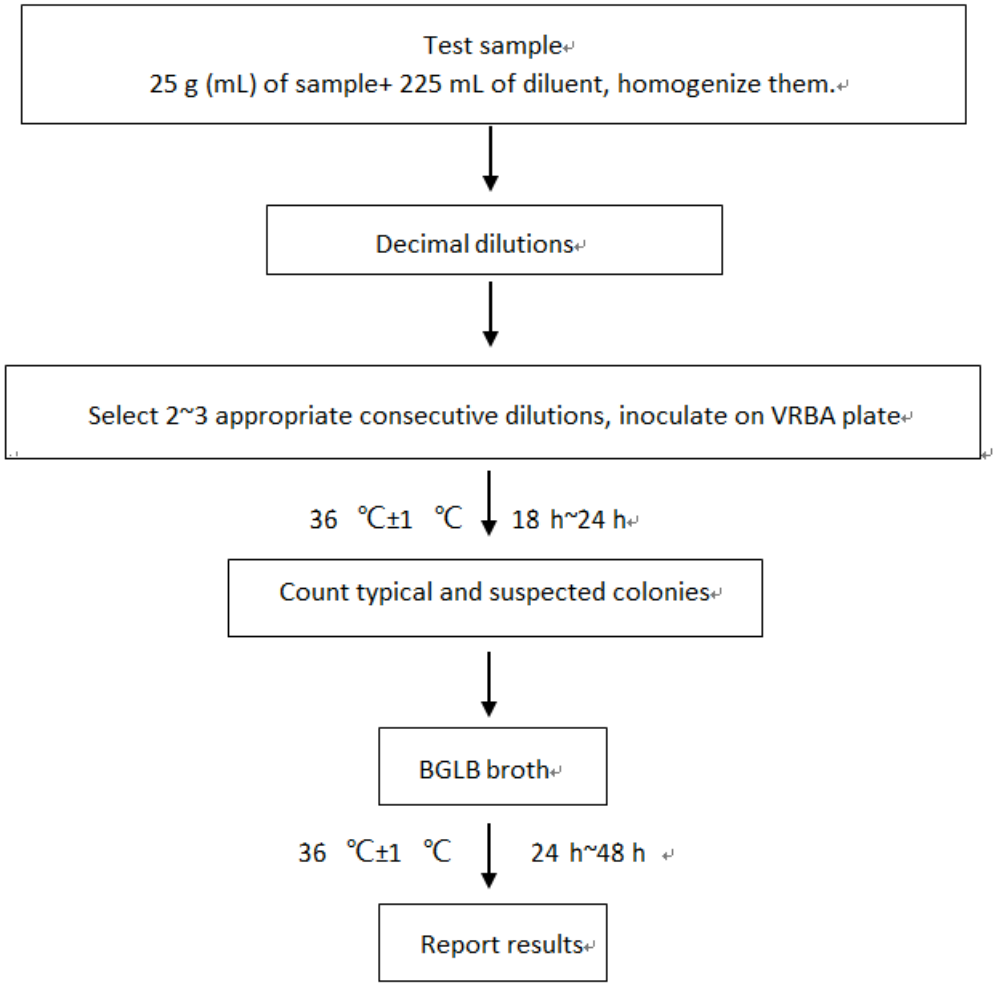

Figure 2 Examination procedures of coliforms plate count

9. Operation procedures

9.1 Dilution of sample

Perform on the basis of 7.1.

9.2 Plate count

9.2.1 Select 2~3 proper consecutive dilutions, inoculate on 2 sterile plates for each dilution, 1mL for each plate. Meanwhile, add 1mL of physiological saline solution into a sterile plate acting as blank control.

9.2.2 Pour 15mL~20mL of violet red bile agar (VRBA) which has melted and kept temperature of 46℃ into each plate in time. Rotate the plates with caution to make the culture medium and the sample solution mix well. After the agar is coagulated, add 3mL~4mL of VRBA more to cover the plate surface. Inverse the plate and incubate at 36℃±1℃ for 18h~24h.

9.3 Selection of colony plate count

The selected the plates shall be those whose colony counts are within the range of 15 CFU-150 CFU, and the typical and suspected coliforms colonies appearing on plates shall be respectively counted (for example, its colony diameter is smaller than typical one's). Typical colonies are purple red with red bile salt precipitation ring around and their diameter is 0.5 mm or bigger. For the plates whose minimum dilution degree is lower than 15 CFU, specific number of coliforms colonies should be recorded.

9.4 Verification test

Pick 10 typical and suspected colonies of different types from the VRBA plates, and if there are less than 10

coliforms colonies pick all typical and suspected colonies for those plates. Transfer and inoculate them into the BGLB broth tubes respectively. Incubate at 36 ℃ ±1 ℃ for 24h~48h and observe bubble generation. All gas-producing BGLB broth tubes can be reported as coliforms positive.

9.5 Report on coliforms plate count

The percentage of the test tubes finally verified to be coliforms positive multiplies by the number of the plate colonies counted in Section 9.3 and the dilution ratio, that's the number of coliforms in each gram (ml) of the sample. For example, 1mL of 10^{-4} diluted sample solution can produce 100 typical and suspected colonies on VRBA plates, and 10 of them are selected to inoculate into the BGLB broth tubes, and 6 tubes are verified to be positive. Thus the number of coliforms in this sample is: $100\times6/10\times10^{4}$/g (mL) = 6.0×10^{5} CFU/g (mL). If there is no coliforms colony cultured on the plate of all the dilution degree (including the sample liquid), then it multiplies the minimum dilution degree by a coefficient less than 1.

Annex A

Culture mediums and reagents

A.1 Lauryl sulfate tryptose (LST) broth

A.1.1 Composition

Typtone or Trypticase	20.0g
Sodium chloride	5.0g
Lactose	5.0g
Potassium hydrogen phosphate (K_2HPO_4)	2.75g
Potassium dihydrogen phosphate (KH_2PO_4)	2.75g
Lauryl sodium sulfate	0.1g
Distilled water	1000mL

A.1.2 Preparation method

Dissolve all the above components in distilled water and adjust the pH to 6.8±0.2. Dispense 10mL of the solution into each test tube which possesses small glass backward tubes, and sterilize for 15min in the autoclave set at 121 ℃.

A.2 Brilliant green lactose bile (BGLB) broth

A.2.1 Composition

Peptone	10.0g
Lactose	10.0g
Oxgall or oxbile solution	200.0mL
0.1% brilliant green water solution	13.3mL
Distilled water	800mL

A.2.2 Preparation method

Dissolve peptone and lactose in about 500mL of distilled water, add 200mL of oxgall solution (dissolve 20.0g of dehydrated oxgall powder in 200mL of distilled water, and adjust the pH to 7.0~7.5), dilute to 975mL with distilled water, adjust the pH to 7.2±0.1, and then add 13.3mL of 0.1% brilliant green water solution, dilute to 1000mL with distilled water, filter the solution through cotton, and then dispense 10mL of the solution into each test tube which possesses small glass backward tubes. Sterilize for 15min in the autoclave set at 121℃.

A.3 Violet red bile agar (VRBA)

A.3.1 Composition

Peptone	7.0g
Yeast extract	3.0g
Lactose	10.0g
Sodium chloride	5.0g
Bile salt or No.3 bile salt	1.5g
Neutral red	0.03g
Crystal violet	0.002g
Agar	15g~18g
Distilled water	1000mL

A.3.2 Preparation method

Dissolve all the above components in distilled water, stand for a few minutes, stir fully, and adjust the pH to 7.4±0.1. Boil for 2 min, melt and keep the temperature of the culture medium within the range of 45℃~50℃ and

pour plate. Prepare it immediately before use, within 3 hours.

A.4 Phosphate buffer solution

A.4.1 Composition

Potassium dihydrogen phosphate (KH_2PO_4)	34.0g
Distilled water	500mL

A.4.2 Preparation method

Stock solution: Weigh 34.0g of potassium dihydrogen phosphate and dissolve in 500mLof distilled water. Adjust the pH to 7.2±0.2 with about 175mL of 1mol/L sodium hydrochloride solution, dilute to 1000mL with distilled water, and store it in a refrigerator. Dilute solution: Take 1.25mL of the stock solution, dilute to 1000mL with distilled water, dispense the solution into suitable containers, and sterilize for 15min in the autoclave set at 121℃.

A.5 Sterile physiological saline

A.5.1 Composition

Sodium chloride	8.5g
Distilled water	1000mL

A.5.2 Preparation method

Weigh 8.5g of sodium chloride, dissolve in 1000mL of distilled water, and sterilize for 15 min in the autoclave set at 121℃.

A.6 1mol/L NaOH

A.6.1 Composition

NaOH	40.0g
Distilled water	1000mL

A.6.2 Preparation method

Weigh 40g of sodium hydroxide, dissolve in 1000mL of sterile distilled water.

A.7 1mol/L HCl

A.7.1 Composition

HCl	90mL
Distilled water	1000mL

A.7.2 Preparation method

Take 90mL of concentrated hydrochloric acid, dilute to 1000mL with sterile distilled water·

Annex B

Coliforms most probable number (MPN) index table

B.1 Coliforms most probable number (MPN) index table

See Table B.1 for the most probable number (MPN) of coliforms per gram (or millimeter) of the test sample.

Table B.1 Coliforms most probable number (MPN) index table

Number of positive Tubes			MPN	% confidence limit		Number of positive Tubes			MPN	% confidence limit	
0.10	0.01	0.001		Lower limit	Upper limit	0.10	0.01	0.001		Lower limit	Upper limit
0	0	0	<3.0	--	9.5	2	2	0	21	4.5	42
0	0	1	3.0	0.15	9.6	2	2	1	28	8.7	94
0	1	0	3.0	0.15	11	2	2	2	35	8.7	94
0	1	1	6.1	1.2	18	2	3	0	29	8.7	94
0	2	0	6.2	1.2	18	2	3	1	36	8.7	94
0	3	0	9.4	3.6	38	3	0	0	23	4.6	94
1	0	0	3.6	0.17	18	3	0	1	38	8.7	110
1	0	1	7.2	1.3	18	3	0	2	64	17	180
1	0	2	11	3.6	38	3	1	0	43	9	180
1	1	0	7.4	1.3	20	3	1	1	75	17	200
1	1	1	11	3.6	38	3	1	2	120	37	420
1	2	0	11	3.6	42	3	1	3	160	40	420
1	2	1	15	4.5	42	3	2	0	93	18	420
1	3	0	16	4.5	42	3	2	1	150	37	420
2	0	0	9.2	1.4	38	3	2	2	210	40	430
2	0	1	14	3.6	42	3	2	3	290	90	1,000
2	0	2	20	4.5	42	3	3	0	240	42	1,000
2	1	0	15	3.7	42	3	3	1	460	90	2,000
2	1	1	20	4.5	42	3	3	2	1100	180	4,100
2	1	2	27	8.7	94	3	3	3	>1100	420	--

Note 1: Three dilutions [0.1g (or 0.1mL), 0.01g (or 0.01mL) and 0.001g (or 0.001mL) are adopted in this table, for which, three tubes are inoculated.

Note 2: If the tested amounts as shown above in this table are changed to 1g (or 1mL), 0.1g (or 0.1mL) and 0.01g (or 0.01mL), figures in this table should be decreased by 10 times correspondingly; if the tested amounts are changed to 0.01g (or 0.01mL), 0.001g (or 0.001mL) and 0.0001g (or 0.0001mL), figures in this table should be increased by 10 times accordingly, so on and so forth.