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Report Name: National Food Safety Standard Microbiological Examination in Foods - Listeria monocytogenes Testing

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Report Highlights:

On March 27, 2025, China's NHC and the SAMR jointly released the National Food Safety Standard Microbiological Examination in Foods - Listeria monocytogenes Testing (GB 4789.30-2025). This updated standard applies to the testing of Listeria monocytogenes in foods. The final standard will enter into force on September 16, 2025, and will replace the current standard of GB 4789.30-2016. At the time of this report, China hadn't notified the WTO of this revised standard. This report provides unofficial translation of the final standard. Stakeholders should conduct their own review of the regulations to assess any market or regulatory effect on their business.

FAS China provides this analysis and reporting as a service to the United States agricultural community, and to our farmers, ranchers, rural communities, and agribusiness operations in support of a worldwide agricultural information system and a level playing field for U.S. agriculture.

Report Summary:

On March 27, 2025, The People's Republic of China (China's) National Health Commission (NHC) and the State Administration for Market Regulation (SAMR) jointly released the National Food Safety Standard Microbiological Examination in Foods *Listeria monocytogenes* Testing (GB 4789.30-2025) (link in Chinese).

The updated standard applies to the testing of Listeria Monocytogenes bacteria in foods. The final standard will enter into force on September 16, 2025, and replace the current implementing standard GB 4789.30-2016, which has been implemented since June 2017. At the time of the report, China hadn't notified the WTO of this revised standard.

This report provides an unofficial translation of the final standard. Stakeholders should conduct their own review of the regulations to assess any market or regulatory effect on their business.

BEGIN TRANSLATION

Foreword

This standard replaces GB 4789.30-2016 "National Food Safety Standard Microbiological Examination in Foods *Listeria monocytogenes* Testing."

Compared with GB 4789.30-2016, the main changes in this standard are as follows:

- Modified the scope of application;
- Modified the culture medium and reagents and added OA Listeria chromogenic medium formula;
- Modified method I: qualitative test enrichment solution, selective medium, test procedures, identification methods, etc. of *Listeria monocytogenes*;
- Modified method II: sample inoculation, colony count, and confirmation, result count and result report of plate counting method of *Listeria monocytogenes*;
- Modified method III: sample inoculation for MPN counting of *Listeria monocytogenes*.

National Food Safety Standard

Microbiological Examination in Foods

Listeria Monocytogenes Testing

1. Scope

This standard specifies the test methods for *Listeria monocytogenes* in foods.

Method I of this standard is applicable to the qualitative testing of *Listeria monocytogenes* in foods; Method II is applicable to the counting of *Listeria monocytogenes* in foods with high content of *Listeria monocytogenes*; Method III is applicable to the counting of *Listeria monocytogenes* in foods with low content of *Listeria monocytogenes*.

2. Equipment and Materials

In addition to the conventional sterilization and culture equipment in the microbiology laboratory, other equipment and materials are as follows.

2.1 Refrigerator: 2C~8°C.

2.2 Constant temperature incubator: 30°C±1°C, 36°C±1°C, 25°C~30°C.

2.3 Homogenizer.

2.4 Microscope:100×~1 000 ×.

2.5 Electronic balance: the sensitivity is 0.1 g, 0.1 mg.

2.6 Conical flask: 100mL, 500 mL.

2.7 Sterile pipette: 1 mL (with 0.01mL scale), 10mL (with 0.1mL scale) or pipette (with scales of 0.1mL, 1 mL, 10 mL) and sterile pipette tip.

2.8 Sterile Petri dish: 90 mm in diameter.

2.9 Sterile test tube: 16 mm×160 mm.

2.10 Centrifuge: 4 000 r/min.

2.11 Sterile centrifuge tube: 30 mm×100 mm.

2.12 Sterile syringe: 1 mL.

2.13 Oil lens or phase contrast microscope.

2.14 Sterile coating rod.

2.15 *Listeria monocytogenes* ATCC 19111 or CMCC 54004 or other equivalent strains.

2.16 *Listeria innocua* ATCC 33090 or other equivalent strains.

2.17 *Listeria ivanovii* ATCC 19119 or other equivalent strains.

2.18 *Listeria seeligeri* ATCC 35967 or other equivalent strains.

2.19 *Staphylococcus aureus* ATCC 25923 or other equivalent strains, required to produce *β*-hemolytic rings.

2.20 *Rhodococcus equi* ATCC 6939 or NCTC 1621 or other equivalent strains.

2.21 Mice: ICR strain, weight 18g~22g.

2.22 Microbial biochemical identification system.

2.23 Sterile filter device.

3. Medium and Reagents

3.1 Trypticase soy broth with 0.6% yeast extract (TSB-YE): see A.1 in Appendix A.

3.2 Trypticase soy agar with 0.6% yeast extract (TSA-YE): See A.2.

3.3 Fraser enrichment broth (FB_1, FB_2): See A.3.

3.4 OA Listeria chromogenic medium: See A.4.

3.5 PALCAM medium: See A.5.

3.6 Gram stain solution: See A.6.

3.7 SIM dynamic medium: See A.7.

3.8 Buffered Glucose Peptone Water [for methyl red (MR) and acetyl methyl alcohol (VP) test]: See A.8.

3.9 Sheep blood agar: See A.9.

3.10 Sterile phosphate buffer saline: See A.10.

3.11 Sterile saline: See A.11.

3.12 Sugar fermentation tube: See A.12.

3.13 Hydrogen peroxide reagent: See A.13.

3.14 Microbial biochemical identification kit.

Method I Qualitative Test of *Listeria monocytogenes*

4. Testing Procedure

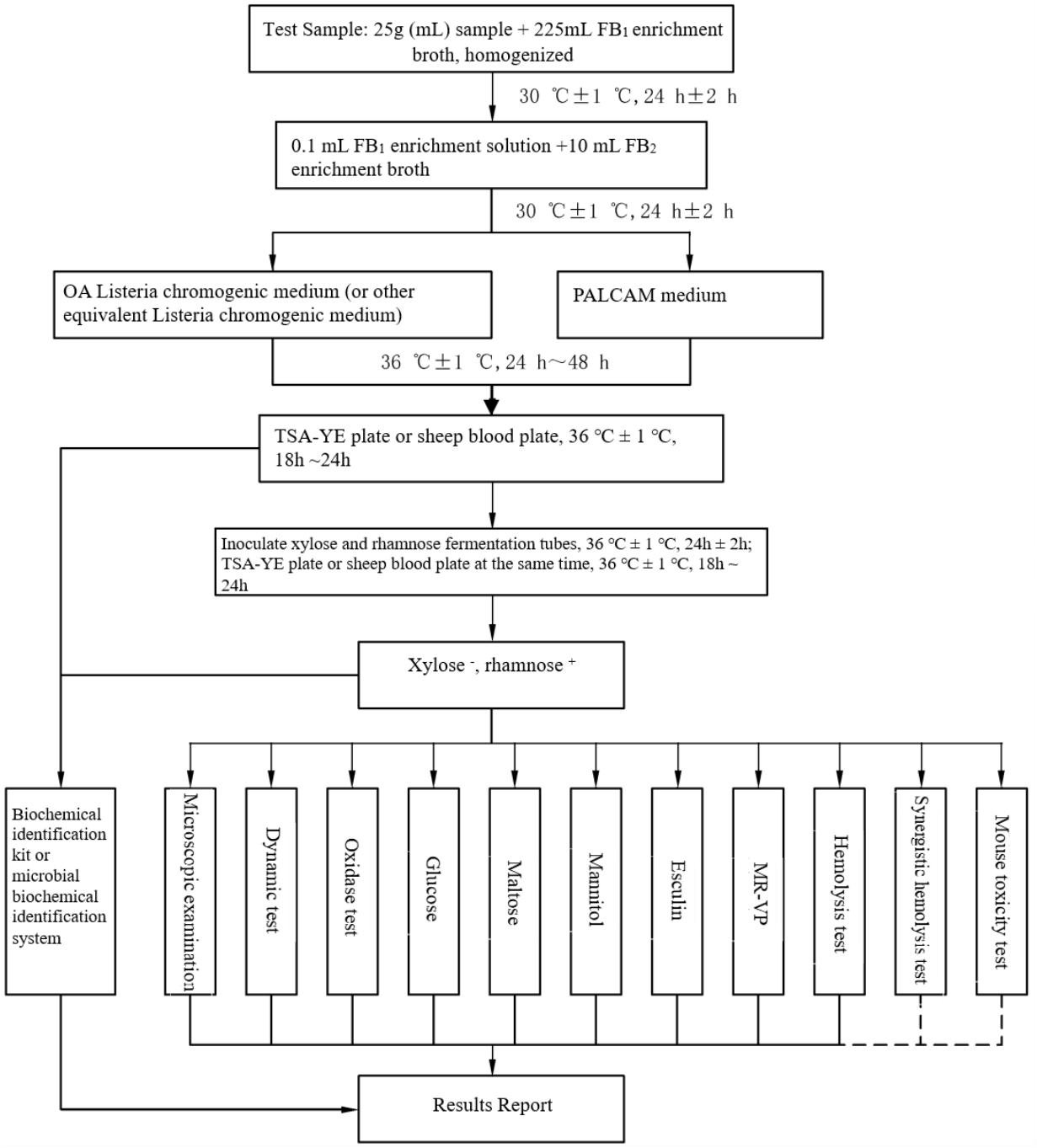

Figure 1: Qualitative testing procedure of Listeria monocytogenes

5. Operational Steps

5.1 Enrichment

Take 25 g (mL) of sample in sterile operation, put it into a sterile homogenization cup containing 225 mL FB_1 enrichment broth, homogenize it at 8 000 r/min~ 10 000 r/min for 1 to 2 minutes, or put it into a sterile homogenization bag containing 225 mL FB_1 enrichment broth, use a slapping homogenizer to beat for 1 to 2 minutes, and make a 1:10 sample homogenization solution. If the sample is liquid, it can also be mixed by shaking or stirring. Incubate at 30 ℃± 1 ℃ for 24 h ± 2 h. Mix well, take 0.1 mL FB_1 enrichment broth, and transfer it into 10 mL FB_2 enrichment broth. Incubate at 30 ℃± 1 ℃ for 24 h ± 2 h.

5.2 Separation

Take the mixed FB_2 enrichment broth and inoculate it into OA Listeria chromogenic medium (or other equivalent Listeria chromogenic medium) plate and PALCAM medium plate respectively. Culture it at 36 ℃± 1 ℃ for 24 to 48 hours and observe the colonies growing on each plate. The typical colonies form a round blue-green colony with a diameter of 1 mm~3 mm on the Listeria OA chromogenic medium plate, surrounded by an opaque halo. Typical colonies formed round gray green colonies with a diameter of 1 mm~3 mm on the PALCAM medium plate, surrounded by brown-black hydrolysis circles. After 48 h of culture, some colonies formed black spots and pits in the center. The colony characteristics of other equivalent Listeria chromogenic medium plates shall be determined according to the product description.

Note 1: the halo around some Listeria monocytogenes on the OA Listeria chromogenic medium is not obvious or even absent. The halos around some monocytogenes colonies on OA Listeria chromogenic medium appeared slowly, sometimes taking more than 4 days to appear.

Note 2: the colony morphology of Listeria ivanovii on OA Listeria chromogenic medium is similar to that of *Listeria monocytogenes*.

5.3 Pure culture

Select 3-5 typical or suspicious colonies (if it is less than 3, all shall be selected) from each plate (plates meeting the requirements of 5.2), draw lines on TSA-YE plate or sheep blood plate, and incubate at 36 ℃± 1 ℃ for 18 to 24 hours. *Listeria monocytogenes* on TSA-YE plate or sheep blood plate is grayish white, translucent, with neat edges, dew drop like colonies, and the diameter is 1 mm~2 mm.

5.4 Preliminary identification

Pick a single colony on TSA-YE plate or sheep blood plate, inoculate it into xylose and rhamnose fermentation tubes, and culture at 36℃ ± 1℃ for 24h ± 2h; at the same time, draw a line on TSA-YE plate or sheep blood plate, and culture at 36℃ ± 1℃ for 24h ± 2h to obtain the

pure culture for the next identification. Then, pure cultures negative for xylose and positive for rhamnose were selected for further identification.

5.5 Identification

Note: one strain of bacteria can be selected from each plate (the plate meeting the requirements of Section 5.2) for identification test. If it is identified as Listeria monocytogenes, the detection results can be reported directly according to the provisions of Section 6 listed in this method; the result of "not detected" can only be reported after 3 to 5 typical or suspicious colonies (all selected if fewer than 3) picked according to the requirements of Section 5.3, are all identified as non-Listeria monocytogenes.

5.5.1 Microscopic examination: select a single colony of pure culture incubated for 18 to 24 hours for Gram staining and microscopic examination. *Listeria* species appear as Gram-positive short rods, with a size of (0.4 μm~0.5 μm) × (0.5 μm~2.0 μm). Prepare a bacterial suspension using physiological saline and observe it under an oil immersion lens or phase-contrast microscope, and the bacteria exhibit slight rotational or tumbling motility.

5.5.2 Dynamic test: select a single colony of pure culture incubated for 18 to 24 hours by stabbing into a semi-solid or SIM motility medium and incubate at 25 °C to 30 °C for 48 ± 2 hours. *Listeria* species show irregular, cloud-like diffusion growth extending outward along the stab line. A characteristic umbrella-shaped (or spindle-shaped) growth appears 3 mm to 5 mm below the surface of the medium. If the umbrella-shaped (or spindle-shaped) growth is not clearly visible, continue incubation and observe the results once daily for up to 5 days.

5.5.3 Biochemical identification: select a single colony of pure culture for 18 to 24 hours and carry out catalase test. The colony with catalase positive reaction continues to conduct sugar fermentation test and MR-VP test. The main biochemical characteristics of *Listeria monocytogenes* are as shown in Table 1.

5.5.4 Hemolysis test: divide the underside of the fresh sheep blood agar plate into 20 to 25 small cells, select a single colony of pure culture for 18 to 24 hours to puncture the blood plate, and puncture one colony in each cell, with the positive control strains (*Listeria monocytogenes*, *Listeria ivanovii*, and *Listeria seeligeri*) and negative control strain (*Listeria innocua*). During stabbing, insert the inoculating needle as close to the bottom as possible without touching it, and avoid cracking the agar. Incubate at 36 ℃± 1 ℃ for 24 to 48 hours. Observe in well-lit area, *Listeria monocytogenes* produces narrow, clear, and bright zone of hemolysis, *Listeria seeligeri* shows a narrow and weak hemolytic ring around the stab site, *Listeria innocua* shows no hemolytic zone, and *Listeria ivanovii* produces a wide, well-defined β-hemolytic zone.

5.5.5 Collaborative hemolysis test CAMP (optional): inoculate *Staphylococcus aureus* and *Rhodococcus equi* on sheep blood agar plate with parallel lines, select a single colony of pure culture for 18 to 24 hours and inoculate it with vertical lines between the parallel lines. The two ends of the vertical line should not touch the parallel line, with distance of 1 mm ~2 mm. At the same time, inoculate *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, and *Listeria seeligeri*, and culture it at 36℃± 1℃ for 24 to 48 hours. The enhanced hemolysis area of *Listeria monocytogenes* is about 2 mm near *Staphylococcus aureus*, and *Listeria seeligeri* also has a weak enhanced hemolysis area; *Listeria ivanovii* has an "arrow shaped" enhanced hemolysis area of 5 mm~10 mm near *Rhodococcus equi*, and *Listeria innocua* does not produce hemolysis. If the result is not obvious, it can be put into refrigerator at 2℃ ~8℃ for 24 to 48 hours to observe again.

Note: 5 percent to 8 percent of *L. monocytogenes* can form slight hemolysis enhancement (about 1 mm) at one end of *Rhodococcus equi*, which is not the case of hemolysis enhancement.

Table 1: Main Identification Characteristics of Listeria Monocytogenes and its Differences from Other Listeria

Strain	Xylose	Rhamnose	Glucose	Maltose	Mannitol	Esculin	MR-VP	Hemolysis Test
L.*monocytogenes*	-	+	+	+	-	+	+ / +	+
L.*grayi*	-	-	+	+	+	+	+ / +	-
L.*seeligeri*	+	-	+	+	-	+	+ / +	+
L.*welshimeri*	+	V	+	+	-	+	+ / +	-
L.*ivanovii*	+	-	+	+	-	+	+ / +	+
L.*innocua*	-	V	+	+	-	+	+ / +	-
Some *Listeria monocytogenes* is not hemolytic. Some *Listeria monocytogenes* serotypes are unable to ferment rhamnose.								
Note: - indicates that 90%~100% of strains are negative; + indicates that 90%~100% of strains are positive; V indicates uncertain reaction.								

If a biochemical identification kit or microbial biochemical identification system is selected, suspicious colonies can be directly picked from TSA-YE plate or sheep blood plate without

preliminary determination specified in 5.4, and the bacteria suspension with appropriate turbidity can be prepared with regular saline, and the microbial biochemical identification kit or microbial biochemical identification system can be used for determination.

5.6 Mouse toxicity test (optional)

The pure culture that meets the above characteristics was inoculated into TSB-YE, cultured at 36℃ ± 1℃ for 24 hours, centrifuged at 4,000 r/min for 5 minutes, the supernatant was discarded, and the bacterial suspension with a concentration of 1010 cfu/ml was prepared with sterile saline. This bacterial suspension was intraperitoneally injected into three to five mice, each 0.5 ml, and the death of mice was observed at the same time. It shall be the pathogenic strain when the mouse died within 2 to 5 days. The test sets up pathogenic strains of *Listeria monocytogenes* and a sterile saline control group. *Listeria monocytogenes* and *Listeria ivanovii* are pathogenic to mice.

6. Results and Reports

According to the identification results described in 5.4 and 5.5, whether *Listeria monocytogenes* is detected or not detected in 25 g (mL) sample shall be reported. If *Listeria monocytogenes* was not detected in 25 g (mL) sample, it can be reported as 0/25 g (mL).

Method II: *Listeria monocytogenes* Plate Count Method

7. Testing Procedures

The plate counting procedure of *Listeria monocytogenes* is shown in Figure 2.

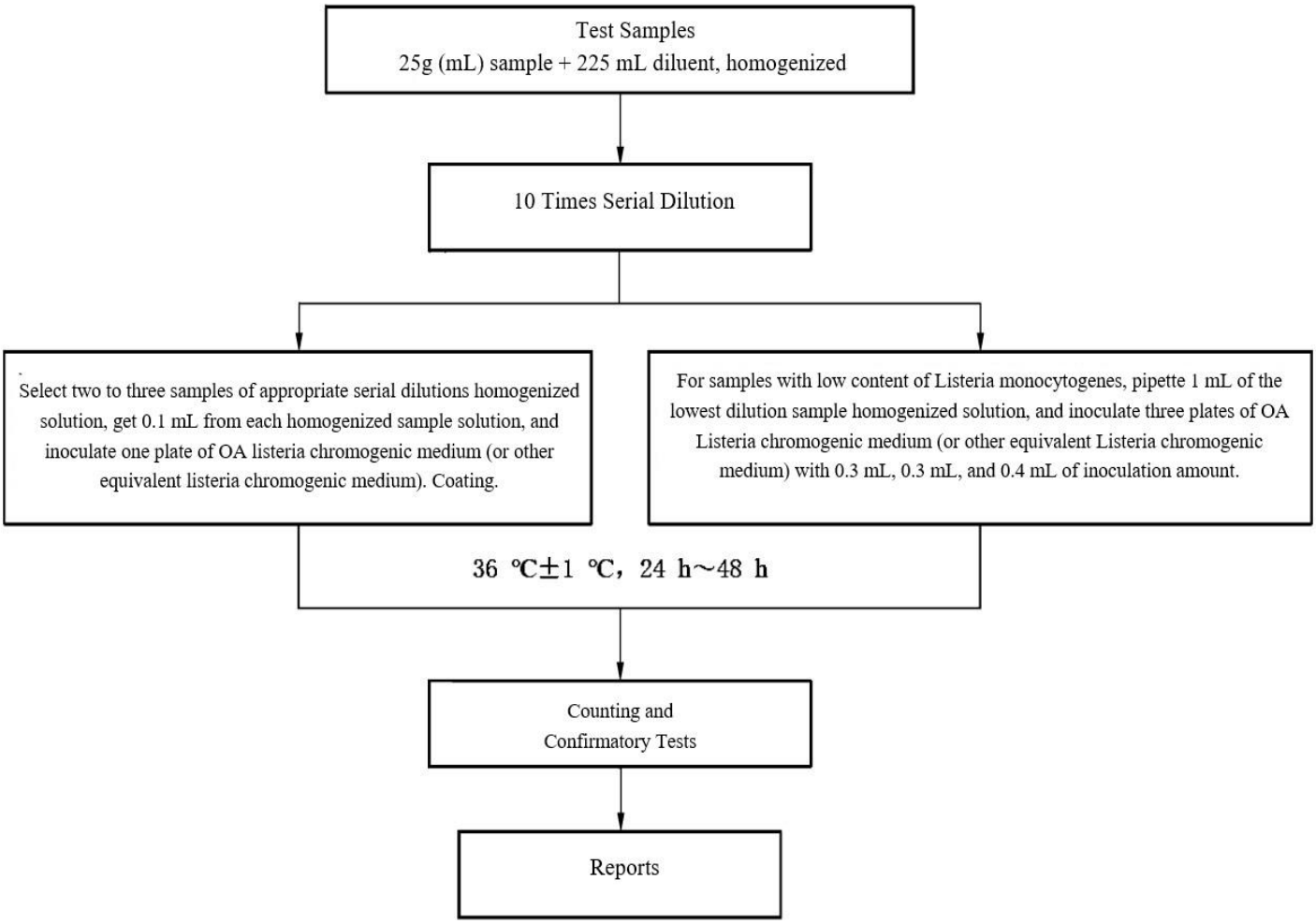

Figure 2: Plate Counting Procedures of *Listeria monocytogenes*

8. Operational Steps

8.1 Dilution of samples

8.1.1 Take 25 g (mL) of sample with aseptic operation, put it into a sterile homogenization cup containing 225 mL of phosphate buffer or Fraser enrichment broth without additives, homogenize it at 8,000 r/min to 10,000 r/min for 1 to 2 minutes, or put it into a sterile homogenization bag containing 225 mL of the above dilutions, hit it with a tapping homogenizer for 1 to 2 minutes, and make a 1:10 sample homogenization solution. If the sample is liquid, it can also be mixed by shaking or stirring to make a 1:10 sample homogenization solution.

8.1.2 Pipette 1 mL of 1:10 sample homogenization solution with a sterile pipette or pipette of 1 mL, and slowly inject it along the tube wall into a sterile test tube containing 9 mL of phosphate buffer or Fraser enrichment broth without additives (note that pipette or tip of the pipette should not touch the diluted liquid level), mix well, and make a 1:100 sample homogenization solution.

8.1.3 Follow the procedure in 8.1.2 to prepare 10-fold serial dilutions of the sample. Use a new 1 mL sterile pipette or pipette tip for each incremental dilution.

8.2 Inoculation of samples

8.2.1 According to the estimation of sample contamination, select two to three sample homogenization solution with appropriate serial dilutions (liquid samples can include stock solution), pipette 0.1 mL of sample homogenization solution for each dilution, and inoculate one plate of Listeria OA chromogenic medium (or other equivalent Listeria chromogenic medium). And apply the entire plate with a sterile coating rod, taking care not to touch the edge of the plate. Before use, if there are water drops on the surface of the agar plate, it can be dried in an incubator at a temperature of 25℃~50℃ until the water drops on the surface of the plate disappear.

Note: If necessary, repeat the test for each dilution.

8.2.2 For food samples with low content of *Listeria monocytogenes*, take 1 mL sample homogenization solution of the lowest dilution and apply it to three plates of OA Listeria chromogenic medium (or other equivalent Listeria chromogenic medium) with inoculation amount of 0.3 mL, 0.3 mL, and 0.4 mL. The coating method is the same as 8.2.1

8.2.3 The time from sample dilution to sample inoculation shall not exceed 45 minutes.

8.3 Culture

8.3.1 Generally, after coating, place the plate on the horizontal table for 10 to 20 minutes, turn the plate over, put it into the incubator for culture, and incubate at 36 ℃± 1 ℃ for 24 to 48 hours.

8.3.2 If the sample solution is not easy to be absorbed, the plate can be placed upright in the incubator for 1 hour at 36 ℃± 1 ℃, and then the plate can be turned over after the sample homogenization solution is absorbed, place invertedly in the incubator, and continue to be cultured at 36 ℃± 1 ℃ for 24 to 48 hours.

8.4 Typical colony count and confirmation

8.4.1 Select a plate where typical or suspicious *L. monocytogenes* colony is growing, if:

a) If the total number of typical or suspicious colonies on the plate with only one dilution is between 15 CFU and 150 CFU, the number of typical or suspicious colonies on the plate with this dilution should be counted;

b) If the total number of typical or suspicious colonies on of all dilutions are less than 15 CFU, and the number of typical or suspicious colonies on the plate with the lowest dilution should be counted;

c) If the total number of typical or suspicious colonies of all dilutions are greater than 150 CFU, the number of typical or suspicious colonies on the plate with the highest dilution shall be counted;

d) If the total number of typical or suspicious colonies on the plates of all dilutions is not between 15 CFU and 150 CFU, but some are less than 15 CFU or greater than 150 CFU, the number of typical or suspicious colonies on the plates of dilutions closest to 15 CFU or 150 CFU should be counted;

If it conforms to conditions of a) to d), it shall be calculated according to formula (1) specified in 9.1.1.

e) The total number of typical or suspicious colonies of the plate with two serial dilutions are between 15 CFU and 150 CFU, which is calculated according to formula (2) specified in 9.1.2.

8.4.2 Select five typical or suspicious colonies (if it is less than 5, all shall be selected) from each plate (plates meeting the requirements of 5.2) and identify them according to 5.3, 5.4, and 5.5.

9. Results and Reports

9.1 Counting method

9.1.1 Equation (1):

$$T = \frac{AB}{CVd} \qquad \text{..... (1)}$$

In which:

T - The number of colonies of Listeria monocytogenes in the sample [CFU/g (mL)];

A - The total number of typical or suspicious colonies of the count dilution;

B - The number of colonies of Listeria monocytogenes confirmed for the count dilution;

C - The number of colonies used in the confirmation test of Listeria monocytogenes for the count dilution;

V - The inoculum of the count dilution, in milliliter (mL);

d - Dilution factor.

9.1.2 Equation (2):

$$T = \frac{A_1 B_1 / C_1 + A_2 B_2 / C_2}{Vd} \quad \text{..... (2)}$$

In which:

T - The number of colonies of Listeria monocytogenes in the sample [CFU/g (mL)];

A_1 - the total number of typical or suspicious colonies of Listeria monocytogenes at the first count dilution (low dilution);

A_2 - the total number of typical or suspicious colonies of Listeria monocytogenes at the second count dilution (high dilution);

B_1 - The number of colonies of Listeria monocytogenes in the identification result at the first count dilution (low dilution);

B_2 - The number of colonies of Listeria monocytogenes in the identification result at the second count dilution (high dilution);

C_1 - The number of colonies used for the identification of Listeria monocytogenes at the first count dilution (low dilution);

C_2 - The number of colonies used for the identification of Listeria monocytogenes at the second count dilution (high dilution);

V - The inoculum of the first count dilution plus 1/10 of the inoculum of the second count dilution, in milliliter (mL), for example: the *V* value of the inoculum of 0.1 mL method is 0.11; The *V* value of using methods of 0.3 mL, 0.3 mL, and 0.4 mL was 1.1;

D - Dilution factor (the first dilution).

9.2 Results report:

9.2.1 When the colony number is less than 100 CFU, it shall be rounded off according to the principle of "rounding off" and reported as an integer.

9.2.2 When the colony number is greater than or equal to 100 CFU, the third digit is rounded off according to the principle of "rounding off," and the first two digits are taken, followed by 0 instead of digits. It can also be expressed in the form of an index of 10. After rounding off, two significant digits are retained.

9.2.3 Report the colony number of *Listeria monocytogenes* in each g (mL) sample, in CFU/g (mL); If the *T* value is 0, the inoculum volume shall be reported as less than 10 multiplied by the minimum dilution factor using 0.1 mL method. The inoculum of 0.3 mL, 0.3 mL, 0.4 mL is reported as less than 1 multiplied by the minimum dilution factor.

Method III: *Listeria monocytogenes* MPN Counting Method

10. Testing Procedures

See Figure 3 for the testing procedure of *Listeria monocytogenes* MPN counting method.

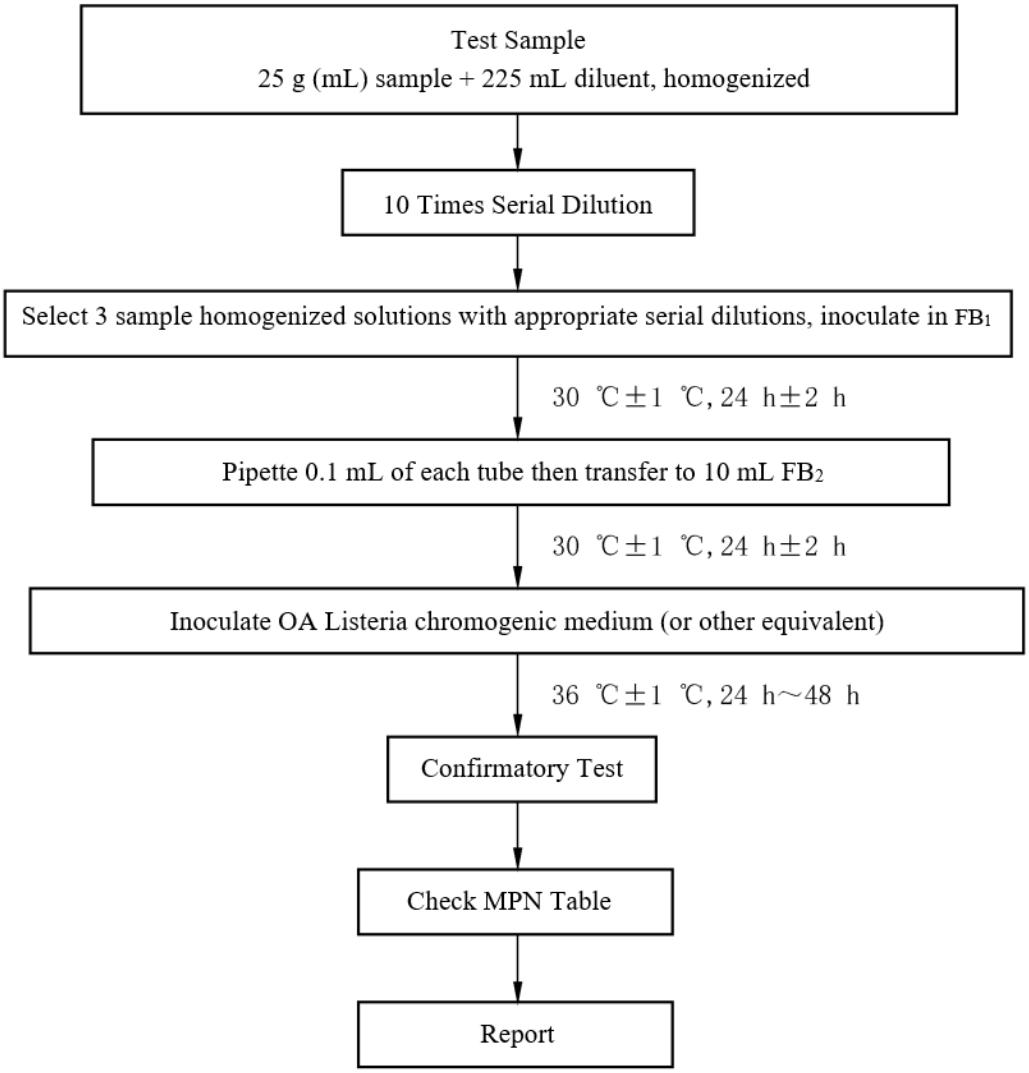

Fig. 3: Inspection procedure of Listeria monocytogenes MPN counting method

11. Operational Steps

11.1 Dilution of samples

Phosphate buffer was used for sample dilutions. The sample dilution method is the same as described in 8.1.

11.2 Inoculation and culture

11.2.1 According to the evaluation on the contamination of samples, select homogenized sample solution (liquid samples can include stock solution) with three appropriate serial dilutions and inoculate them in 10 mL FB_1 enrichment broth. Inoculate three tubes with each dilution and 1 mL in each tube. If the inoculation volume is 10 mL, inoculate to 10 mL double FB_1 enrichment broth. Incubate at 30 ℃ ± 1 ℃ for 24 ± 2 hours. Draw 0.1 mL from each tube, transfer and inoculate into 10 mL FB_2 enrichment broth, and incubate at 30℃ ± 1℃ for 24 ± 2 hours.

11.2.2 Take 1 ring from each tube of FB_2 enrichment broth with inoculation ring, inoculate OA Listeria chromogenic medium (or other equivalent Listeria chromogenic medium) plate, and incubate at 36 ℃± 1 ℃ for 24 to 48 hours.

11.3 Confirmatory test

Select 3 to 5 typical or suspicious colonies (when it is less than 3, all shall be selected) from each plate (plates meeting the requirements of 5.2) and make determination according to conditions in 5.3, 5.4, and 5.5.

12. Results and reports

According to the number of tubes confirmed to be positive for L. monocytogenes, check the MPN key table (see Appendix B) and report the most probable number of L. monocytogenes per gram (mL) of sample, expressed in MPN/g (mL).

Appendix A

Culture Medium and Reagents

A.1 Tryptone soy broth (TSB-YE) with 0.6% yeast extract powder

A.1.1 Composition

Tryptone	17.0 g
Multivalent peptone	3.0 g
Yeast extract powder	6.0 g
Sodium chloride	5.0 g
Dipotassium phosphate	2.5 g
Glucose	2.5 g
Distilled water	1 000 mL

A.1.2 Preparation

Heat, stir, and dissolve the above ingredients, adjust the pH value, if necessary, dispense, autoclave at 121 ℃ for 15 minutes, and set aside. The pH value of the medium at 25 ℃ after sterilization was 7.2 ± 0.2.

A. 2 Tryptone soy agar (TSA-YE) containing 0.6% yeast extract powder

A.2.1 Composition

Tryptone	17.0 g
Multivalent peptone	3.0 g
Yeast extract powder	6.0 g
Sodium chloride	5.0 g
Dipotassium phosphate	2.5 g
Glucose	2.5 g
Agar	15.0 g
Distilled water	1 000 mL

A.2.2 Preparation

Heat, stir, and dissolve the above ingredients, adjust the pH value to 7.2 ± 0.2, if necessary, subpackage, autoclave at 121 C for 15 minutes, then cool to 47 ℃ ~50 ℃, pour into a sterile plate, and set aside. The pH value of the medium at 25℃ after sterilization was 7.2 ± 0.2.

A. 3 Fraser enrichment broth (FB_1, FB_2)

A. 3.1 Basic medium

A.3.1.1 Composition

Tryptone	5.0 g
Beef extract powder	5.0 g
Yeast extract powder	5.0 g
Peptone	5.0 g
Sodium chloride	20.0 g
Potassium dihydrogen phosphate	1.35 g
Disodium hydrogen phosphate	12.0 g
Lithium chloride	3.0 g
Esculin	1.0 g
Distilled water	1000 mL

A.3.1.2 Preparation

Heat, stir, and dissolve the above ingredients, adjust the pH value, if necessary, dispense, autoclave at 121 ℃ for 15 minutes, and cool to 47 ℃ ~50 ℃. The pH value of the medium at 25℃ after sterilization was 7.2 ± 0.2.

A. 3.2 Additives

A. 3.2.1 1% Nalidixic acid solution

Dissolve 0.5g of nalidixic acid sodium in 50 mL of 0.05 mol/L sodium hydroxide, filter through 0.45 μm sterilized filter membrane, and sterilize.

A. 3.2.2 0.25% Acriflavine hydrochloride solution

Dissolve 0.25 g of acridine yellow hydrochloride in 100 mLof distilled water, filter with 0.45 μm sterile filter membrane, and sterilize.

A. 3.2.3 5% Ammonium ferric citrate solution

Dissolve 5 g of ammonium ferric citrate in 100 mL of distilled water, filter with 0.45 μm sterilized filter membrane, and sterilize.

A. 3.3 Complete medium

A. 3.3.1 FB_1 enrichment broth

Basic medium (A.3.1)	984 mL
1% Nalidixic acid solution (A.3.2.1)	1 mL
0.25% Acriflavine hydrochloride solution (A.3.2.2)	5 mL
5% Ammonium ferric citrate solution (A.3.2.3)	10 mL

A. 3.3.2 FB_2 enrichment broth

Basic medium (A.3.1)	978 mL
1% Nalidixic acid solution (A.3.2.1)	2 mL
0.25% Acriflavine hydrochloride solution (A.3.2.2)	10 mL
5% Ammonium ferric citrate solution (A.3.2.3)	10 mL

A. 4. Agar Listeria according to Ottaviani and Agosti

A.4.1 Basic medium

A.4.1.1 Composition

Peptone	18.0 g
Tryptone	6.0 g
Yeast extract powder	10.0 g
Sodium pyruvate	2.0 g
Glucose	2.0 g
Magnesium glycerophosphate	1.0 g
Magnesium sulfate (anhydrous)	0.5 g
Sodium chloride	5.0 g
Lithium chloride	10.0 g
Disodium hydrogen phosphate (anhydrous)	2.5 g
(5-Bromo-4-chloro-3-indolyl-β-D-glucopyranoside)	0.05 g
Agar	15.0 g
Distilled water	930 mL*

Note: * indicates that if amphotericin B is used to replace cycloheximide, the preparation volume of basic medium is changed to 925 mL.

A.4.1.2 Preparation

Heat, stir, and dissolve the above ingredients, adjust the pH value, if necessary, dispense, autoclave at 121 ℃ for 15 minutes, and cool to 47 ℃ to 50 ℃. The pH value of the medium at 25 ℃ after sterilization was 7.2 ± 0.2.

A. 4.2 Additives

A. 4.2.1 0.4% Nalidixic acid solution

Dissolve 0.02 g of nalidixic acid sodium in 5ml of sodium hydroxide, filter through 0.45 μm sterile filter membrane, and sterilize.

A. 4.2.2 0.4% Ceftazidime solution

Dissolve 0.02 g of ceftazidime in 5 mL of distilled water, filter with 0.45 μm sterile filter membrane, and sterilize.

A.4.2.3 Polymyxin B solution

Dissolve 76 700 IU polymyxin sulfate B in 5 mL distilled water, filter it with a 0.45 μm sterile filter membrane, and sterilize.

A. 4.2.4 2% Cycloheximide solution

Dissolve 0.05 g cycloheximide in 2.5 mL absolute ethanol, add 2.5 mL distilled water, filter with 0.45 μm sterile filter membrane, and sterilize.

A. 4.2.5 0.1% amphotericin B solution (alternative to cycloheximide solution)

Mix 2.5 mL hydrochloric acid (1mol/L) and 7.5 mL dimethylformamide (DMF) into HCl/DMF solution, add 0.01 g amphotericin and dissolve, filter with 0.45 μm sterile filter membrane, and sterilize.

A. 4.2.6 L- α - phosphatidylinositol solution

Dissolve 2 g of L-α- phosphatidylinositol in 50 mL of distilled water (2 g of soybean lecithin containing 9% - 15% of unfractionated phosphatidylinositol can be used instead of L- α - phosphatidylinositol) and stir for about 30 minutes until a uniform suspension is obtained. Autoclave at 121 ℃ for 15 minutes and then cool to 47 ℃ to 50 ℃.

A. 4.3 Complete medium

A.4.3.1 Composition

Basic medium (A.4.1)	930 mL
0.4% Nalidixic acid solution (A.4.2.1)	5 mL
0.4% Ceftazidime solution (A.4.2.2)	5 mL
Polymyxin B solution (A.4.2.3)	5 mL
2% Cycloheximide solution (A.4.2.4)	5 mL
Or 0.1% Amphotericin B solution (A.4.2.5)	10 mL
L - a - phosphatidylinositol solution (A.4.2.6)	50 mL

When the basic medium (A.4.1) is cooled to 47 ℃ to 50 ℃, add nalidixic acid, ceftazidime, polymyxin B, cycloheximide or amphotericin B, and L -a - phosphatidylinositol solution successively. Each addition requires immediate and thorough mixing. The pH value of complete medium should be 7.2 ± 0.2 at the temperature of 25 ℃, and the medium should be uniform and slightly milky white. After mixing, pour it into a sterile plate. Pour 18 mL~20 mL into each dish and set aside for use after solidification.

A. 5 PALCAM medium

A. 5.1 Composition

Yeast extract powder	8.0 g
Glucose	0.5 g
Esculin	0.8 g
Ferric ammonium citrate	0.5 g
Mannitol	10.0 g
Phenol red	0.1 g
Lithium chloride	15.0 g
Casein trypsin digest (casein peptone)	10.0 g
Cardiopancreatic enzyme digest	3.0 g
Corn starch	1.0 g
Stomach enzymatic digests of meat	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1 000 mL

A. 5.2 Preparation

Heat, stir, and dissolve the above ingredients, adjust the pH value, if necessary, dispense, autoclave at 121 ℃ for 15 minutes. The pH value of the medium at 25 ℃ after sterilization was 7.2 ± 0.2.

A. 5.2.1 PALCAM selective additive

Polymyxin B	10.0 mg
Acriflavine hydrochloride	5.0 mg
Ceftazidime	20.0 mg
Sterile distilled water	2 mL

A.5.2.2 Preparation

Cool the PALCAM basic medium to 47 ℃ to 50 ℃, add 2 mL of PALCAM selective additive, mix well and pour it into sterilized dishes. Pour 18 mL to 20 mL into each dish and set it aside for use after solidification.

A. 6 Gram stain solution

A. 6.1 Crystal violet stain solution

A.6.1.1 Composition

Crystal violet	1.0 g
95% ethanol	20.0 mL
1% ammonium oxalate aqueous solution	80.0 mL

A.6.1.2 Preparation

Completely dissolve the crystal violet in ethanol and then mix with ammonium oxalate aqueous solution.

A. 6.2 Gram iodine solution

A.6.2.1 Composition

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300 mL

A.6.2.2 Preparation

First mix iodine and potassium iodide, add a little distilled water, shake well, and then add distilled water to 300 mL after it is completely dissolved.

A. 6.3 Sand yellow counterstain

A.6.3.1 Composition

Sand yellow	0.25 g
95% ethanol	10.0 mL
Distilled water	90.0 mL

A.6.3.2 Preparation

Dissolve the sand yellow in ethanol and then dilute with distilled water.

A. 6.4 Staining method

A. 6.4.1 After the smear is fixed with flame, add crystal violet stain solution, react for 1 minute, and wash it with water.

A. 6.4.2 Add gram iodine solution dropwise, react for 1 minute, and wash it with water.

A. 6.4.3 Add 95% ethanol to decolorize it for 15 to 30 seconds until the stain solution is washed away. Do not bleach excessively, wash it with water.

A. 6.4.4 Add the counterstain solution, counterstain for 1 minute, wash it with water, set aside for drying, and conduct microscopic examination.

A.7 SIM dynamic medium

A.7.1 Composition

Tryptone	20.0 g
Multivalent peptone	6.0 g
Ammonium ferric sulfate	0.2 g
Sodium thiosulfate	0.2 g
Agar	3.5 g
Distilled water	1 000 mL

A.7.2 Preparation

Heat, stir, and dissolve the above ingredients, adjust the pH value, if necessary, dispense, autoclave at 121 ℃ for 15 minutes, and set aside for use. The pH value of the medium at 25 ℃ after sterilization was 7.2 ± 0.2.

A.8 Buffered Glucose Peptone Water [for methyl red (MR) and acetyl methyl alcohol (VP) test]

A.8.1 Composition

Polyvalent peptone	7.0 g
Glucose	5.0 g
Dipotassium phosphate	5.0 g
Distilled water	1 000 mL

A.8.2 Preparation

Heat, stir, and dissolve the above ingredients, adjust the pH value, if necessary, dispense and add 1 mL in each tube, autoclave at 121 ℃ for 15 minutes, and set aside for use. After sterilization with buffered Glucose Peptone Water, the pH value at 25℃ is 7.2 ± 0.2.

A. 8.3 Methyl red (MR) test

A. 8.3.1 Methyl Red Reagent

A.8.3.1.1 Composition

Methyl red	10 mg
95% ethanol	30 mL
Distilled water	20 mL

A.8.3.1.2 Preparation

Dissolve 10 mg of methyl red in 30 ml of 95% ethanol and then add 20 mL distilled water.

A. 8.3.1.3 Test method

Take an appropriate amount of agar culture and inoculate it in buffered glucose peptone water, and culture it at 36 ℃± 1 ℃ for 2 to 5 days. Add 1 drop of methyl red reagent and observe the results immediately. Bright red is positive and yellow color is negative.

A. 8.4 Acetylmethyl methanol (VP) test

A.8.4.1 6% α - naphthol - ethanol solution

Composition and preparation: take 6.0 g of α - naphthol, add anhydrous ethanol to dissolve, and make the volume to 100 mL.

A. 8.4.2 40% potassium hydroxide solution

Composition and preparation: take 40 g of potassium hydroxide, add distilled water to dissolve, and make the volume to 100 mL.

A. 8.4.3 Test method

Take an appropriate amount of agar culture and inoculate it in buffered glucose peptone water, incubate it at 36 ℃± 1 ℃ for 2 to 4 days, add 0.5 mL of 6% α – naphthol - ethanol solution and 0.2 mL of 40% potassium hydroxide solution, shake the test tube, and observe the results. If it is positive, the solution turns red immediately or within a few minutes. If it is negative, it should be cultured at 36 ℃± 1 ℃ for 1 hour before observation.

A. 9 Sheep blood agar

A. 9.1 Composition

Peptone	1.0 g
Beef paste	0.3 g
Sodium chloride	0.5 g
Agar	1.5 g
Distilled water	100 mL
Defibrillated sheep blood	5 mL~8 mL

A. 9.2 Preparation

Except for fresh defibrillated sheep blood, heat to dissolve the other ingredients mentioned above, autoclave at 121 ℃ for 15 minutes, cool to 50 ℃, then add fresh defibrillated sheep blood in sterile operation, shake well, and pour into the plate.

A. 10 Sterile phosphate buffer

A. 10.1 Composition of storage solution

Potassium dihydrogen phosphate (KH_2PO_4)	34.0g
1 mol/L sodium hydroxide	About 175 mL
Distilled water	1 000 mL

A.10.2 Preparation

A. 10.2.1 1 mol/L sodium hydroxide: weigh 20.0 g of sodium hydroxide and dissolve it in 500 mL of distilled water.

A. 10.2.2 Storage solution: weigh 34.0 g of potassium dihydrogen phosphate and dissolve it in 500 mL of distilled water, adjust the pH value to 7.2 with about 175 mL of 1 mol/L sodium hydroxide solution, make the volume to 1,000 mL with distilled water, then store it in the refrigerator.

A. 10.2.3 Working solution: take 1.25 mL of storage solution, dilute it to 1,000 mL with distilled water, dispense it in a suitable container, and autoclave at 121 ℃ for 15 minutes.

A. 11 Sterile saline

A.11.1 Composition

Sodium chloride	8.5 g
Distilled water	1 000mL

A.11.2 Preparation

Weigh 8.5g of sodium chloride and dissolve it in 1, 000 mL of distilled water, dispense it in a suitable container, and autoclave it at 121 ℃ for 15 minutes.

A. 12 Sugar fermentation tube

A. 12.1 Sugar fermentation base broth

Beef paste	5.0 g
Peptone	10.0 g
Sodium chloride	3.0 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)	2.0 g

0.2% bromothymol blue solution	12.0 mL
Distilled water	1 000 mL

A. 12.2 Preparation

A. 12.2.1 Heat, stir, and dissolve the above ingredients, adjust the pH value, if necessary, dispense, autoclave at 121 ℃ for 15 minutes, and set aside. The pH value of the sugar fermentation broth at 25 ℃ after sterilization was 7.4 ± 0.2.

A. 12.2.2 Glucose fermentation tube: prepare glucose into 10% solution with distilled water and autoclave at 121 ℃ for 15 minutes. Aseptically take 5 mL of glucose solution into 100 mL sugar fermentation broth prepared according to A.12.2.1, mix well, and then dispense it into a small test tube with aseptic operation, and put aside for use. The pH value of glucose fermentation tube at 25 ℃ was 7.4 ± 0.2.

A. 12.2.3 Other sugar fermentation tubes: other sugar fermentation tubes can be prepared according to the preparation method of glucose fermentation tubes in A.12.2.2.

A. 12.3 Test method

Take an appropriate amount of pure culture and inoculate it in the sugar fermentation tube. Culture it at 36 ℃± 1 ℃ for 24 to 48 hours, then observe the results: blue is negative and yellow is positive.

A. 13 Hydrogen peroxide reagent

A.13.1 Reagents

Prepare 3% hydrogen peroxide solution for use.

A.13.2 Catalase test method

Pick up a single colony with a fine glass rod or a disposable inoculation needle, place it in a clean glass plate, add 2 drops of 3% hydrogen peroxide solution, and observe the results.

A.13.3 Results

Those with bubbles within 0.5 minutes are positive, and those without bubbles are negative.

Appendix B
Listeria Monocytogenes Most Probable Number (MPN) Table

The most probable number (MPN) of Listeria monocytogenes per gram (mL) of sample is retrieved in Table B.1.

Table B.1: Most Probable Number (MPN) of Listeria Monocytogenes

Number of positive tubes			MPN	95% confidence		Number of positive tubes			MPN	95% confidence	
0.10	0.01	0.001		Lower limits	Upper limits	0.10	0.01	0.001		Lower limits	Upper limits
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	1 100	180	4 100
2	1	2	27	8.7	94	3	3	3	>1 100	420	-

Note 1: This table uses three dilutions [0.1g (mL), 0.01g (mL), and 0.001g (mL)] and inoculates three tubes at each dilution.
Note 2: If the sample volume listed in the table is changed to 1g (mL), 0.1g (mL), and 0.01g (mL), the number in the table should be reduced by 10 times accordingly; If using 0.01g (mL) ,0.001g(mL),0.0001g(mL), then the number in the table should be increased by 10 times, and so on.

END TRANSLATION

Attachments:

GB 4789.30-2025 Listeria Monocytogenes Testing.pdf