National Standard of the People's Republic of China

GB 4789.10-2016

National Food Safety Standard

Food Microbiological Examination: Staphylococcus Aureus

食品安全国家标准

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Foreword

This standard replaces GB 4789.10-2010 “National food safety standard Food Microbiological Examination: *Staphylococcus Aureus*”, SN/T 0172-2010 “Determination of *Staphylococcus aureus* in import and export foods” and SN/T 2154-2008 “Determination of coagulase-positive *staphylococci* in import and export food: Technique using rabbit plasma fibrinogen agar medium”.

Compared with GB 4789.10-2010, major changes of this standard are as follows:

- The enrichment solution for test shall be 7.5 % sodium chloride broth.

National Food Safety Standard

Food Microbiological Examination: Staphylococcus Aureus

1.Scope

This standard specifies the examination method for *Staphylococcus aureus* in foods.
Method I of this standard is applicable to the qualitative test of *Staphylococcus aureus* in foods; Method II is applicable to the enumeration of *Staphylococcus aureus* in foods with high content of *Staphylococcus aureus*; Method III is applicable to the enumeration of *Staphylococcus aureus* in foods with low content of *Staphylococcus aureus.*

2.Apparatus and Materials

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

2.1 Constant temperature incubator: 36 ℃±1 ℃.

2.2 Refrigerator: 2 ℃~5 ℃.

2.3 Constant temperature water bath: 36 ℃~56 ℃.

2.4 Balance: with sensitivity of 0.1 g.
2.5 Homogenizer.
2.6 Oscillator.
2.7 Sterile pipette: with normal capacities of 1 mL (graduated in 0.01 mL division) and 10 mL (graduated in 0.1 mL division) or micropipette and pipette tips.
2.8 Sterile conical flask: with nominal capacities of 100 mL and 500 mL.
2.9 Sterile culture dish: with diameter of 90 mm.
2.10 Spreading rod
2.11 pH meter or pH colorimetric tube or precise pH test paper.

3.Culture Mediums and Reagents

3.1 7.5 % sodium chloride broth: see A.1.
3.2 Blood agar plate: see A.2.
3.3 Baird-Parker agar plate: see A.3.
3.4 Brain heart infusion (BHI) broth: see A.4.
3.5 Rabbit plasma: see A.5.
3.6 Diluent: phosphate buffer solution: see A.6.
3.7 Nutrient agar small slant: see A.7.
3.8 Gram stain solution: see A.8.
3.9 Sterile saline solution: see A.9.

Method I Qualitative examination of *Staphylococcus aureus*

4.Examination Procedures

See Figure 1 for the qualitative examination procedures of *Staphylococcus aureus.*

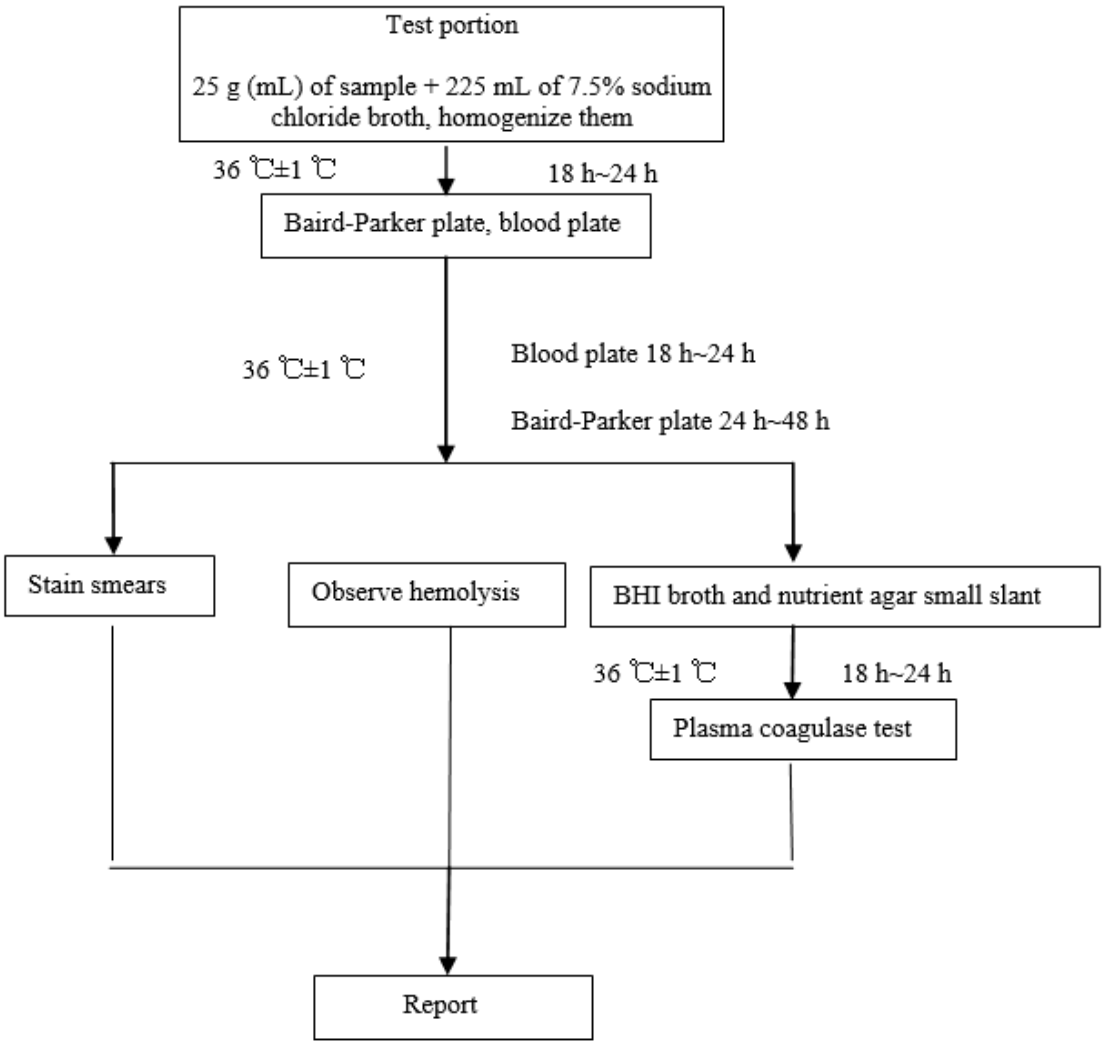

Figure 1 Examination procedures of *Staphylococcus aureus*

5.Operation Steps

5.1 Sample treatment

Weigh 25 g of sample, put into a sterile homogenization cup that contains 225 mL of 7.5% sodium chloride broth, homogenize it at 8000 r/min~10000 r/min for 1 min~2 min, or put the sample into a sterile homogenization bag that contains 225 mL of 7.5% sodium chloride broth, and slap it for 1 min~2 min with a slapping homogenizer. If the sample is liquid, pipette 25 mL of the sample, and put it into a sterile conical flask that contains 225 mL of 7.5% sodium chloride broth (proper amount of sterile glass beads may be pre-placed in the flask), shake and mix well.

5.2 Enrichment

Incubate the above homogeneous sample solution at 36 ℃±1 ℃ for 18 h~24 h.

Staphylococcus aureus shows turbid growth in 7.5% sodium chloride broth.

5.3 Isolation

Respectively streak-inoculate the above cultures after enrichment on Baird-Parker plate and blood plate. Incubate at 36 ℃±1 ℃ for 18 h~24 h on blood plate and incubate at 36 ℃±1 ℃ for 24 h~48 h on Baird-Parker plate.

5.4 Preliminary identification

Staphylococcus aureus appears to be round on the Baird-Parker plate with smooth, convex and moist surface. The

colonies are gray black to black with gloss and light (not white) margin with a diameter of 2 mm~3 mm. The margin is surrounded by a non-transparent circle (precipitate) with a distinct edge. When touching the colonies with an inoculating needle, it feels sticky like butter. Non-lipolytic strains may occur on occasion, which basically look like the same except they have no non-transparent circle and distinct edge. Compared to the typical colonies, the colonies which are isolated from frozen or dehydrated foods kept for a long time is lighter black, and they may be rougher on appearance and drier in texture. Colonies forming on the blood plate are larger, round, smooth, convex and moist in golden yellow (occasionally in white), surrounded by complete transparent hemolytic circles. Pick the suspected colonies mentioned above for Gram staining microscopic examination and plasma coagulase test.

5.5 Confirmatory identification

5.5.1 Staining microscopic examination: *Staphylococcus aureus* is a Gram-positive coccus with a diameter of 0.5 μm~1 μm, which is arranged in grape shape without spores and capsules

5.5.2 Plasma coagulase test: pick up at least 5 suspected colonies (select all if there are less than 5) from Baird-Parker plate or blood plate, inoculate on 5 mL of BHI and nutrient agar small slant respectively, and incubate at 36 ℃±1 ℃ for 18 h~24 h.

Take 0.5 mL of fresh-prepared rabbit plasma and place into a small test tube, then add 0.2 mL~0.3 mL of BHI culture, shake and mix well, place it into the incubator or water bath at 36 ℃±1 ℃, and observe it every half an hour for 6 h. If coagulation occurs (i.e., coagulation can be watched when the tube is tilted or inverted) or coagulative volume is more than half of the initial volume, it is judged to be positive. At the same time, use the broth culture of the plasma coagulase positive and *staphylococcus* strains negative as control. Commercial reagents can also be used for plasma coagulase test in accordance with the operating instruction.

If the result is suspicious, pick up the colonies on the nutrient agar small slant into 5 mL of BHI, incubate at 36 ℃ ±1 ℃ for 18 h~48 h, and repeat the test.

5.6 Examination of staphylococcal enterotoxin (optional)

For the identification of food samples which are suspected of poisoning or *Staphylococcus aureus* strains producing staphylococcal enterotoxin, staphylococcal enterotoxin testing is needed in accordance with Annex B.

6.Results and Report

6.1 Result determination: the strains in accordance with Section 5.4 and 5.5 can be determinedas*Staphylococcus aureus*.

6.2 Result reporting: *Staphylococcus aureus* is detected or not in 25 g (mL) of sample.

Method II Plate count of *Staphylococcus aureus*

7.Examination Procedures

See Figure 2 for the examination procedures for plate count of *Staphylococcus aureus*.

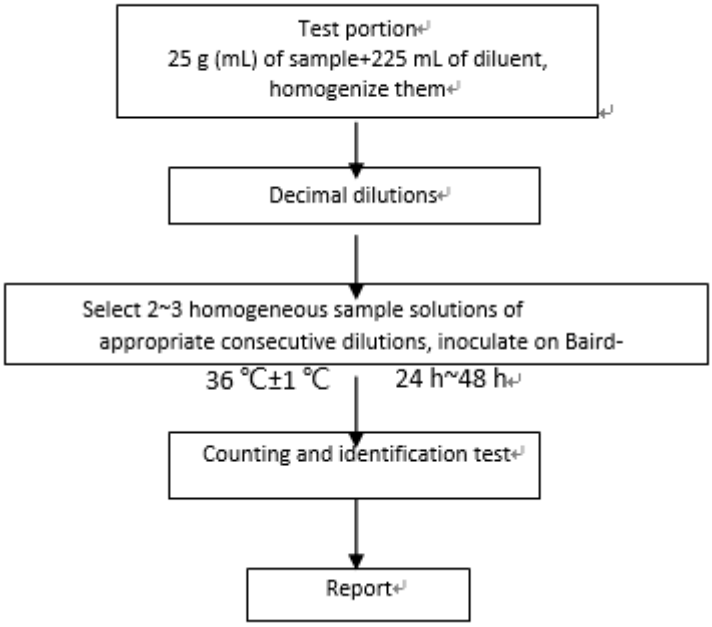

Figure 2 Examination procedures for plate count of *Staphylococcus aureus*

8.Operation Steps

8.1 Dilution of sample

8.1.1 Solid and semi-solid sample: weigh 25g of sample, place it in a sterile homogenization cup that contains 225 mL of phosphate buffer solution or normal saline, homogenize it at 8000 r/min~10000 r/min for 1 min~2 min; or place into a sterile homogenization bag that contains 225 mL of phosphate buffer solution, and slap it for 1 min~2 min with a slapping homogenizer to produce a 1:10 homogeneous sample solution.

8.1.2 Liquid sample: pipette 25 mL of the sample with a sterile pipette, place in a sterile conical flask (proper amount of sterile glass beads may be pre-placed in the flask) containing 225 mL of phosphate buffer solution or normal saline, and thoroughly shake and mix well to produce 1:10 homogeneous sample solution.

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

8.1.4 According to the operation procedures above in Section 8.1.3, prepare decimal dilutions of homogeneous sample solution. Use a new 1-mL sterile pipette or pipette tip for each dilution.

8.2 Sample inoculation

Based on the estimation of sample contamination status, 2~3 homogeneous sample solutions of appropriate dilutions (for liquid sample, the original sample can be included) can be selected. While preparing decimal dilutions, respectively pipette 1mL of sample for each dilution, inoculate on three Baird-Parker agar plates with inoculation amount of 0.3 mL, 0.3 mL and 0.4 mL respectively, and then spread all over the plate with a sterile spreading rod, and avoid touching the plate edge. If there are drops of water on surface of Baird- Parker agar plates before use, dry it in incubator at 25 ℃~50 ℃ till they disappear.

8.3 Incubation

Generally, let the plates stand for 10 min after spreading. If the sample solution is difficult to be absorbed, incubate the plates in an incubator at 36 ℃±1 ℃ for 1h. Invert the plates after the solution is fully absorbed, and incubate at 36 ℃±1 ℃ for 24 h~48 h.

8.4 Enumeration and verification of typical colonies

8.4.1 *Staphylococcus aureus* appears to be round on the Baird-Parker plate with smooth, convex and moist surface. The colonies are gray black to black with gloss and light (not white) margin with a diameter of 2 mm~3 mm. It is usually surrounded by a non-transparent circle (precipitate) with a distinct edge. When touching the colonies with an inoculating needle, it feels sticky like butter. Non-lipolytic strains may occur on occasion, which basically look like the same except they have no non-transparent circle and distinct edge. Compared to the typical colonies, the colonies isolated from frozen or dehydrated foods kept for a long time is lighter black, and they may be rougher on appearance and drier in texture.

8.4.2 Select the plates with typical *Staphylococcus aureus* colonies and that whose total colonies of 3 plates for same dilution within the range of 20 CFU and 200 CFU, count the number of typical colonies.

8.4.3 Select at least 5 suspected colonies from typical ones (select all if there are less than 5), and perform identification test. Respectively perform staining microscopic examination and plasma coagulase test (see 5.5); streak-inoculate on blood plate, incubate at 36 ℃±1 ℃ for 18 h~24 h and observe colonial morphology. *Staphylococcus aureus* colonies are larger, round, smooth and convex, moist in golden yellow (occasionally white) surrounded by visible completely transparent hemolysis ring.

9.Calculation

9.1 If only one dilution's plate count is within the range of 20 CFU-200 CFU, then count typical colonies on plates of this dilution in accordance with Formula (1).

9.2 If the lowest dilution's plate count is less than 20 CFU, then count typical colonies on plates of this dilution in accordance with Formula (1).

9.3 If a certain dilution's plate count is larger than 200 CFU, but there are no typical colonies on the next dilution's plates, then count typical colonies on plates o this dilution in accordance with Formula (1).

9.4 If a certain dilution's plate count is larger than 200 CFU, and there are typical colonies on the next dilution's plates but the count is not within the range of 20 CFU-200 CFU, then count typical colonies on plates of this dilution in accordance with Formula (1).

9.5 If 2 consecutive dilutions' plates count is within the range of 20 CFU-200 CFU, then calculate in accordance with Formula (2).

9.6 Calculation formula

Formula (1):

$$T = \frac{AB}{Cd} \quad \cdots\cdots (1)$$

Where:

T——the count of *Staphylococcus aureus* colonies in the sample;

A——the total count of typical colonies for a certain dilution;

B——the count of positive colonies for a certain dilution;

C——the count of colonies used for test for a certain dilution;

d——the dilution factor.

Formula (2):

$$T = \frac{A_1B_1/C_1 + A_2B_2/C_2}{1.1d} \quad \cdots\cdots (2)$$

Where:

T——the count of *Staphylococcus aureus* colonies in the sample;

A_1——the total count of typical colonies for the first dilution (low dilution ratio);

B_1——the count of positive colonies for the first dilution (low dilution ratio);

C_1——the count of colonies used for identification test for the first dilution (low dilution ratio);

A_2——the total count of typical colonies for the second dilution (high dilution ratio);

B_2——the count of positive colonies for the second dilution (high dilution ratio);

C_2——the count of colonies used for identification test for the second dilution (high dilution ratio);

1.1——the calculation coefficient;

d——the dilution factor (the first dilution).

10.Report

The result is calculated based on the Formula in Section 9, and report the number of *Staphylococcus aureus* per g (mL) sample, expressed as CFU/g (mL); if T=0, report the result as less than "1 multiplying by the lowest dilution ratio".

Method III MPN Enumeration of *Staphylococcus aureus*

11.Examination Procedures

See Figure 3 for the examination procedures for MPN enumeration of *Staphylococcus aureus*.

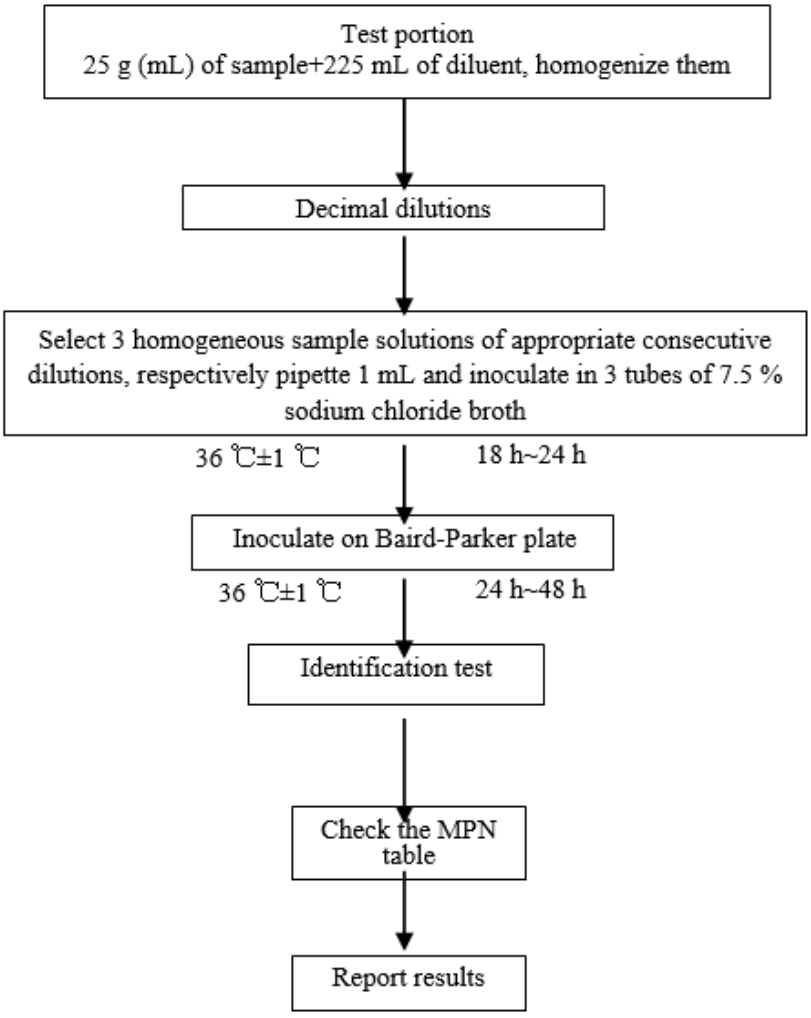

Figure 3 Examination procedures for MPN enumeration of *Staphylococcus aureus*

12.Operation Procedures

12.1 Dilution of sample

It shall be implemented in accordance with 8.1.

12.2 Inoculation and incubation

12.2.1 Select 3 homogenous sample solution of proper consecutive dilutions for liquid sample, the original sample

can be included) based on the estimation of sample contamination status. While preparing decimal dilutions, respectively inoculate 1 mL of homogeneous sample solutions into 7.5% sodium chloride broth for each dilution (use double-strength 7.5% sodium chloride broth if the inoculation amount is required to be more than 1 mL). Inoculate 3 tubes of each dilution. And incubate the above culture for 18 h~24 h at 36 ℃±1 ℃.

12.2.2 Respectively take 1 loop of culture from 7.5% sodium chloride broth after incubation with inoculating loop, inoculate on the Baird-Parker plate and incubate for 24 h~48h at 36 ℃±1 ℃.

12.3 Verification of typical colonies

It shall be implemented in accordance with 8.4.1 and 8.4.3.

13.Results and Report

Based on the number of test tubes determined as *Staphylococcus aureus*-positive, search the MPN index table (see Annex C), and report the most probable number of *Staphylococcus aureus* per g (mL) sample expressed as MPN/g (mL).

Annex A

Culture mediums and reagents

A.1 7.5% sodium chloride broth

A.1.1 Composition

Peptone	10.0 g
Beef extract	5.0 g
Sodium chloride	75 g
Distilled water	1 000 mL

A.1.2 Preparation method

Heat to melt the above components, and adjust pH to 7.4±0.2. Dispense them into bottles with the amount of 225 mL, and sterilize for 15 min in the autoclave set at 121 ℃.

A.2 Blood Agar Plate

A.2.1 Composition

Soya flour agar (pH 7.5±0.2)	100 mL
Defibrinated Sheep Blood (or rabbit blood)	5 mL~10 mL

A.2.2 Preparation method

Heat to melt the agar and cool down to 50 ℃. Aseptically add defibrinated sheep blood, shake well and pour into plate.

A.3 Baird-Parker Agar Plate

A.3.1 Composition

Tryptone	10.0 g
Beef extract	5.0 g
Yeast extract	1.0 g
Sodium pyruvate	10.0 g
Glycine	12.0 g
Lithium chloride ($LiCl \cdot 6H_2O$)	5.0 g
Agar	20.0 g
Distilled water	950 mL

A 3.2 Preparation method of enrichment reagents

Mix 50 mL of 30% yolk saline with 10 mL of 1 % potassium tellurite solution which was filtered and sterilized by filter membrane with a bore diameter of 0.22 μm, and store the above solution in the refrigerator.

A.3.3 Preparation method

Add each component into distilled water and heat to boil to dissolve thoroughly. Adjust pH to 7.0 ± 0.2. Dispense

them into bottles with the amount of 95 mL, and sterilize for 15 min in the autoclave set at 121 ℃. Heat and melt the agar just before use, cool down to 50 ℃, and add 5 mL of yolk potassium tellurite emulsion which is preheated to 50 ℃ for each 95 mL of agar, shake well and pour into the plate. The medium shall be dense and non- transparent. And it shall be stored in the refrigerator for less than 48 h before use.

A.4 Brain Heart Infusion (BHI) Broth

A.4.1 Composition

Tryptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate ($12H_2O$)	2.5 g
Glucose	2.0 g
Beef heart infusion	500 mL

A 4.2 Preparation method

Heat and dissolve, adjust pH to 7.4±0.2, and dispense them into 16 mm×160 mm test tubes with the amount of 5 mL. Sterilize for 15 min in the autoclave set at 121 ℃.

A.5 Rabbit Plasma

Take 3.8 g of sodium citrate, add 100 mL of distilled water, filter after dissolution, fill in a bottle and sterilize for 15 min in the autoclave set at 121 ℃. Preparation of rabbit plasma: take one portion of 3.8 % sodium citrate solution, add 4 portions of rabbit whole blood, mix well and let it stand (or centrifuge for 30 min at 3 000 r/min) to make the blood cell to decrease, and then the plasma can be produced.

A.6 Phosphate Buffer Solution

A.6.1 Composition

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

A.6.2 Preparation method

Stock solution: weigh 34.0 g of potassium dihydrogen phosphate, dissolve it in 500 mL of distilled water, adjust pH to 7.2 with some 175 mL of 1 mol / L sodium hydroxide solution, dilute to 1 000 mL with distilled water and store it in the refrigerator.

Diluent: take 1.25 mL of the stock solution, dilute to 1 000 mL with distilled water, dispense it into proper containers, and sterilize for 15 min in the autoclave set at 121 ℃.

A.7 Nutrient Agar Small Slant

A.7.1 Composition

Peptone	10.0 g
Beef extract	3.0 g
Sodium chloride	5.0 g
Agar	15.0 g~20.0 g

Distilled water	1 000 mL

A.7.2 Preparation method

Dissolve all the components except agar into distilled water, and add 2 mL of 15% sodium hydroxide solution to adjust the pH to 7.3±0.2. Add agar, heat and boil to melt the agar, dispense into 13 mm×130 mm test tubes, and sterilize for 15 min in the autoclave set at 121 ℃.

A. 8 Gram Stain Solution

A.8.1 Crystal Violet Staining Solution

A.8.1.1 Composition

Crystal violet	1.0 g
95% ethyl alcohol	20.0mL
1% ammonium oxalate water solution	80.0mL

A.8.1.2 Preparation method

Thoroughly dissolve the crystal violet into ethanol and then mix with ammonium oxalate solution.

A.8.2 Gram iodine solution

A.8.2.1 Composition

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300 mL

A.8.2.2 Preparation method

Firstly mix the iodine and potassium iodide, add a little amount of distilled water, thoroughly shake, then add distilled water to 300 mL after complete dissolution.

A.8.3 Safranine counterstaining solution

A.8.3.1 Composition

Safranine	0.25 g
95% ethyl alcohol	10.0 mL
Distilled water	90.0 mL

A.8.3.2 Preparation method

Dissolve the safranine into ethyl alcohol and then dilute with distilled water.

A.8.4 Staining method

a) Fix the smear on the flame, dropwise add crystal violet staining solution, stain for 1 min and wash with water.
b) Dropwise add gram iodine solution, and react for 1 min and wash with water.
c) Dropwise add 95% ethyl alcohol and decolor for 15 s~30 s until the staining solution is washed off. Excessive decoloration and water washing is banned.
d) Dropwise add counterstaining solution, counter stain for 1 min and wash with water, stay for drying and take

microscopic examination.

A.9 Sterile Normal Saline

A.9.1 Composition

Sodium chloride	8.5 g
Distilled water	1 000 mL

A.9.2 Preparation method

Weigh 8.5 g of sodium chloride and dissolve into 1 000 mL of distilled water, sterilize for 15 min in the autoclave set at 121℃.

Annex B

Examination of *Staphylococcal* Enterotoxin

B.1 Reagents and Materials

Unless otherwise provided, the purity of reagents used in this standard shall be analytically pure. The water used in the test shall meet the requirements of Grade 1 water in GB/T 6682.

B.1.1 ELISA detection kit for typing of A, B, C, D and E - type *Staphylococcus aureus* enterotoxin.

B.1.2 pH test paper within the range of 3.5~8.0 (accurate to 0.1).

B.1.3 Tris buffer (0.25 mol/L, pH 8.0): dissolve 121.1 g of Tris into 800 mL of deionized water. After cooling down to ambient temperature, add 42 mL of concentrated HCl to adjust pH to 8.0.

B.1.4 Phosphate buffer (pH 7.4): weigh 0.55 g of $NaH_2PO_4 \cdot H_2O$ (or 0.62g of $NaH_2PO_4 \cdot 2H_2O$), 2.85 g of $Na_2HPO_4 \cdot 2H_2O$ (or 5.73 g of $Na_2HPO_4 \cdot 12H_2O$) and 8.7 g of NaCl, dissolve them into 1000 mL of distilled water, and mix well.

B.1.5 Heptane

B.1.6 10% sodium hypochlorite solution.

B.1.7 Enterotoxin toxin-producing medium.

B.1.7.1 Composition

Peptone	20.0 g
Pancreatic digest of casein	200 mg (amino acid)
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	1.0 g
Potassium dihydrogen phosphate	1.0 g
Calcium chloride	0.1 g
Magnesium sulfate	0.2 g
Niacin	0.01 g
Distilled water	1 000 mL
pH 7.3±0.2	

B.1.7.2 Preparation method

Mix all components in water, adjust pH after dissolution, and sterilize for 30 min in the autoclave set at 121 ℃.

B.1.8 Nutrient agar

B.1.8.1 Composition

Peptone	10.0 g
Beef extract	3.0 g
Sodium chloride	5.0 g
Agar	15.0 g~20.0 g
Distilled water	1 000 mL

B.1.8.2 Preparation method

Dissolve all the components except agar into distilled water, add 2 mL of a 15% sodium hydroxide solution to adjust pH to 7.3±0.2. Add agar, heat and boil to melt the agar. Dispense them into flasks, and sterilize for 15 min in the autoclave set at 121 ℃.

B.2 Apparatus and Equipment

B.2.1 Electronic balance: with sensitivity of 0.01 g.

B.2.2 Homogenizer.

B.2.3 Centrifugal machine: with rotation rate of 3000 *g*~5000*g*.

B.2.4 Centrifugal tube: with norminal capacity of 50 mL.

B.2.5 Filter: with a bore diameter of filter membrane of 0.2 μm.

B.2.6 Microscale sample injector: with nominal capacities of 20 μL~200 μL and 200 μL~1000 μL.

B.2.7 Microscale multichannel sample injector: with nominal capacities of 50 μL~300 μL.

B.2.8 Automatic plate washer (optional).

B.2.9 ELIASA: with a wave length of 450 nm.

B.3 Principle

The method can be implemented by using enzyme-linked immunosorbent assay kit for typing of A, B, C, D and E-type *Staphylococcus aureus* enterotoxin. The determination of this method is based on enzyme-linked immunosorbent assay (ELISA). The A-E wells of each micro well strip of the 96-well ELISA plate were coated with A, B, C, D and E type *Staphylococcus aureus* enterotoxin antibodies respectively. The Well H is positive control coated with mixed type of S*taphylococcus aureus* enterotoxin antibody, while Well F and Well G are negative control coated with the antibody of non-immune animal. If the *Staphylococcus aureus* enterotoxin is present in the sample, the free *Staphylococcus aureus* enterotoxin will bind to the specific antibody coated in each micro well to form an antigen-antibody complex, and the remaining unbound compositions are washed off during plate-washing process; the antigen-antibody complex then binds to a peroxidase marker (secondary antibody), and the unbound peroxidase marker is washed off during the plate-washing process; add enzyme substrate and color developing agent and incubate, the peroxidase-catalyzed substrate on the enzyme marker is decomposed to make the colorless reagent into blue; add reaction terminating solution to change the color from blue to yellow and terminate the enzyme reaction; measure the absorbance value of the micro well solution by a ELIASA with a wave length of 450 nm, and the *Staphylococcus aureus* enterotoxin in the sample is in proportion to the absorbance value.

B.4 Detection Procedures

B.4.1 Method for detection of *Staphylococcus aureus* enterotoxin from culture of isolated strains

Inoculate the strains to be tested on nutrient agar slant (test tube 18 mm×180 mm), incubate for 24 h at 36 ℃. Wash the colonies with 5 mL of normal saline, pour into 60 mL of toxigenic medium, shake it at 36 ℃ with a vibration velocity of 100 times/min to incubate for 48h, pipette out the bacteria solution, centrifuge for 20 min at 8 000r/min, heat to 100 ℃ for 10 min, take the supernatant and take 100 μL of sample solution after dilution to perform test.

B.4.2 Methods for extraction and detection of *Staphylococcus aureus* enterotoxin from foods

B 4.2.1 Milk and milk powder

Dissolve 25 g of milk powder into 125 mL of Tris buffer (0.25 M, pH 8.0), mix well, and then prepare in accordance with the following steps as in the same with liquid milk. Centrifuge at 3500 *g* for 10 min at 15 ℃. Remove the fat layer formed on the surface to change it into skim milk. Dilute it with distilled water (1:20) and take 100 μL of diluted sample solution to perform test.

B.4.2.2 Food with fat content less than 40%

Weigh 10 g of sample, triturate and add into 15 mL of PBS solution with pH 7.4 to homogenize. Shake for 15min. Centrifuge at 3 500 *g* for 10 min at 15 ℃. Remove the fat layer formed on the surface if necessary. Take the supernatant to perform filtration and sterilization. Take 100 μL of the percolate to perform test.

B.4.2.3 Food with fat content more than 40%

Weigh 10 g of sample, triturate and add into 15 mL of PBS solution with pH 7.4 to homogenize. Shake for 15min. Centrifuge at 3 500 *g* for 10 min at 15 ℃. Pipette 5 mL of the upper layer suspension liquid, transfer to another centrifuge tube, then add 5 mL of heptane, and mix well for 5 min. Centrifuge at 3 500 *g* for 5 min at 15 ℃. All the upper organic phase (heptane layer) shall be discarded, and note that there shall be no heptane left in the process. Perform filtration and sterilization for the lower water phase layer. Take 100 μL of the percolate to

perform test.

B.4.2.4 It may refer to the food processing methods mentioned above as appropriate for other foods.

B.4.3 Detection

B.4.3.1 All operations shall be implemented at ambient temperature (20 ℃~25 ℃), and the temperature of all reagents in ELISA detection kit of somatotyped A, B, C, D, E - type *Staphylococcus aureus* enterotoxin shall be put into use only after returning to ambient temperature. The pipette tip shall be replaced when pipetting different reagents and sample solution in test, and the used tips and waste solution shall be soaked in 10% sodium hypochlorite solution overnight before treatment.

B.4.3.2 Insert the micro well strips of required number into the frame (one micro well strip for each sample). Add the sample solution into Well A to G of micro well strip, 100 μL per well. Add 100 μL of positive control in Well H, pat the micro well plates to thoroughly mix with hand, seal the micro well with adhesive paper to prevent the solution from volatilizing, and incubate for 1h at room temperature.

B.4.3.3 Pour the liquid in the well into a container that contains 10% sodium hypochlorite solution and pat for several times on absorbent paper to guarantee that there is no liquid left in the wells. Inject 250 μL of washing liquor to each well with multichannel sample injector, and then pour out and pat dry on the absorbent paper.

Repeat the above operations of washing plates for 4 times. This step can also be finished by an automatic microplate washer.

B.4.3.4 Add 100 μL of enzyme-labeled antibody to each well,pat the microwell plate with hand to mix well, and incubate at ambient temperature for 1 h.

B.4.3.5 Repeat the plate washing procedures as described in B.4.3.3.

B.4.3.6 Add 50 μL of TMB substrate and 50 μL of colour former to each microwell, mix well by patting, and incubate for 30 min in a dark place at room temperature.

B.4.3.7 Add 100 μL of 2 mol/L sulfuric acid stop solution, mix well by patting, measure the OD value of each micro well with ELIASA under a wavelength of 450 nm less than 30 min.

B.4.4 Calculation and expression of the results

B.4.4.1 Quality control

The OD value of positive control of test results shall be greater than 0.5, and the OD value of negative control of test results shall be less than 0.3.If it cannot meet the above requirements meanwhile, the test results shall not be recognized. Positive results shall be excluded from the interference of endogenous peroxidase.

B.4.4.2 Calculation of critical value

The Well F and Well G of each micro well strip are negative control, and the average value of the two negative control OD values plus 0.15 is the critical value.

E.g.: negative control 1=0.08

negative control 2=0.10

average value=0.09

critical value=0.09+0.15=0.24

B.4.4.3 Result expression

The sample well with OD value less than the critical value is judged to be negative, expressed as certain type of *Staphylococcus aureus* enterotoxin not detected in a sample; the sample well with OD value greater than or equal to the critical value is judged to be positive, expressed as certain type of *Staphylococcus aureus* enterotoxin detected in a sample.

B.5 Biosafety

Due to the fact that the existence of other potential infectious substances cannot be ruled out in the sample, the wastes shall be disposed of based on GB 19489 “Laboratories-General Requirements for Biosafety”.

Annex C

Most Probable Number (MPN) index table for *Staphylococcus Aureus*

See Table C.1 for index of *Staphylococcus Aureus* MPN in per g (mL) of test portion.

Table C.1 Most probable number (MPN) index table for *Staphylococcus Aureus*

Number of positive tubes			MPN	95% confidence		Number of positive tubes			MPN	95% confidence	
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	1000
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	-

Note1: 3 dilutions [0.1 g (mL), 0.01 g (mL) and 0.001 g (mL)] are adopted in this table. For each dilution, 3 tubes shall be inoculated.

Note 2: if the quantities of test portion listed in the table are changed to 1 g (mL), 0.1 g (mL) and 0.01 g (mL), the numbers listed in the Table shall be declined by 10 times accordingly; if they are changed to 0.01 g (mL), 0.001g (mL) and 0.0001 g (mL), the numbers listed in the Table shall be increased by 10 times, and so forth.