

**National Standard  
of the People's Republic of China**

GB4789.40—2010

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**National food safety standard**  
**Food microbiological examination: *Enterobacter sakazakii***

Issued on 26-03-2010

Implemented on 01-06-2010

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**Issued by the Ministry of Health  
of the People's Republic of China**

## Preface

This standard replaced GB/T 4789.40-2008 “Microbiological examination of food hygiene—Examination of *Enterobacter sakazakii*”.

In comparison with GB/T 4789.40-2008, the major differences are as follows:

- Revised the Chinese and English name of this standard;
- Deleted the A.3 part in appendix A.

Both Appendix A and B in this standard are normative appendix.

This standard is proposed by and interpreted by the Ministry of Health of P.R. China.

This standard replaces all previous standard as follows:

- GB/T 4789.10-2008.

# **National food safety standard**

## **Food microbiological examination: *Enterobacter sakazakii***

### **1. Scope**

This standard stipulates the testing method of *Enterobacter sakazakii* in foods.

This standard applies to the examination of *Enterobacter sakazakii* in infant formula foods, dairy, dairy products and their raw material.

### **2. Apparatus and Materials**

In addition to the conventional apparatus for sterilization and incubation in microbiological laboratory, other apparatus and materials are as follows:

- 2.1 Constant temperature incubator: 25 ± 1℃, 36 ± 1℃, 44 ± 0.5℃.
- 2.2 Refrigerator: 2~5℃.
- 2.3 Constant temperature water bath: 44 ± 0.5℃.
- 2.4 Balance: weighing to 0.1g.
- 2.5 Homogenizer.
- 2.6 Oscillator.
- 2.7 Sterile pipette: 1ml with graduation of 0.01ml, 10 ml with graduation of 0.1ml, or micropipette and pipette tips.
- 2.8 Sterile conical flask: 100 ml, 200ml and 2000ml.
- 2.9 Sterile petri dish: diameter 90mm.
- 2.10 pH meter, pH colorimetric tubes or precise pH test paper.
- 2.11 VITEK automatic microbiological identification system.

### **3. Culture medium and reagents**

- 3.1 Buffer Peptone Water (BPW): see A.1.
- 3.2 Modified lauryl sulfate tryptose broth-vancomycin medium, mLST-Vm : see A.2.
- 3.3 Druggan-forsythe-iversen, DFI.
- 3.4 Trypticase Soy Agar, TSA: see A.3.
- 3.5 API 20E biochemical identification kit.
- 3.6 Oxidase reagents: see A.4.

- 3.7 L-Lysine Decarboxylation Medium: see A.5.
- 3.8 L-Ornithine Decarboxylation Medium: see A.6.
- 3.9 L-Arginine Dihydrolase Medium: see A.7.
- 3.10 Media for fermentation of carbohydrates: see A.8.
- 3.11 Simmons citrate medium: see A.9.

## Method I Detection of *Enterobacter sakazakii*

### 4. Detection Procedure

Refer to Figure 1.

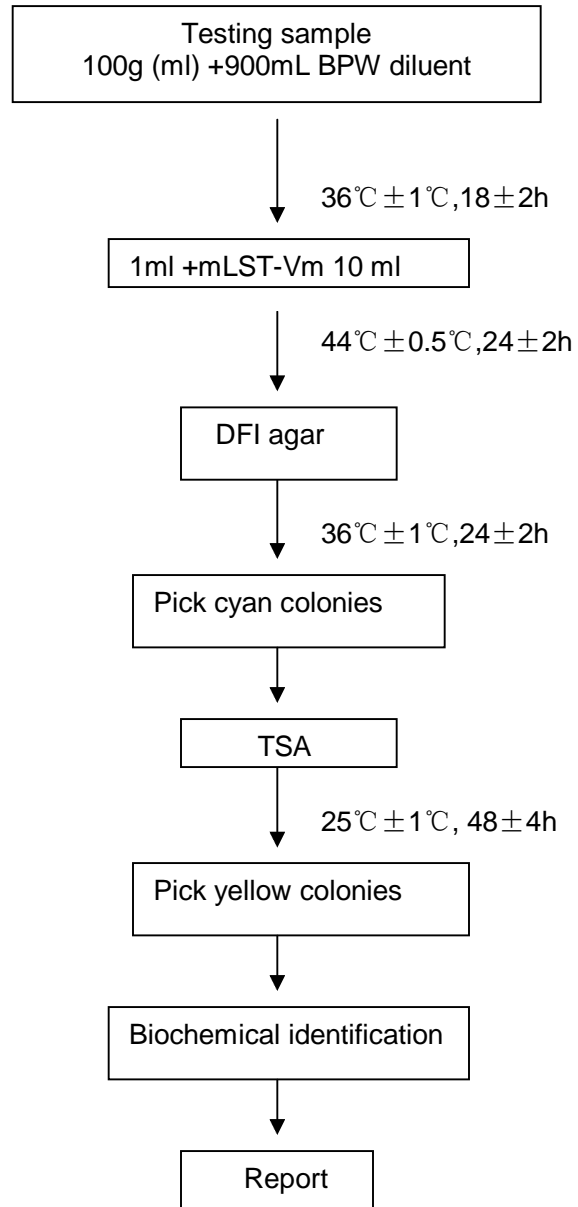


Figure 1 Detection procedure of *Enterobacter sakazakii*

### 5. Procedure

#### 5.1 Pre-enrichment & Enrichment

Add 100g (ml) of the test sample to conical flask with 900ml BPW medium pre-heated to 44°C. Gently and slowly shake until dissolved. Incubate at 36±1°C for 18±2h. Then transfer 1ml of the incubated broth into 10ml mLST-Vm medium. Incubate at 44±0.5°C for 24±2h.

## 5.2 Isolation

5.2.1 Gently mix the mLST-Vm broth culture that has been incubated. Streak each loop of duplicate enriched culture onto 2 DFI plates respectively, incubate at 36±1 °C for 24±2 h.

5.2.2 After incubation, pick 1-5 suspicious colonies, streak to TSA plate and incubate at 25±1 °C for 48±4h.

## 5.3 Identification

Pick yellow suspicious colonies from the TSA plate and carry out biochemical identification. The main biochemical properties of *Enterobacter sakazakii* are shown in Table1. Use biochemical identification system, such as API 20E Biochemical kit or VITEK automatic microbiological identification system.

**Table 1. Main biochemical properties of *Enterobacter sakazakii*.**

Biochemical Test		Characteristic Reaction
Yellow color production		+
Oxidase		-
L-Lysine decarboxylase		-
L-Ornithine decarboxylase		(+)
L-Arginine dihydrolase		+
Citric acid hydrolyse		(+)
Fermentation	D-sorbitol	(-)
	D-Rhamnose	+
	D-Sucrose	+
	D-Melibiose	+
	Amygdalin	+
Note: "+">99% is positive; "-">99% is negative; "(+)"90% ~ 99% is positive; "(-)"90% ~ 99% is negative.		

## 6. Report

Integrate the colonial morphological and biochemical characteristic, report *Enterobacter sakazakii* is detected or not detected in 100g (ml) sample.

## Method 2 Enumeration of *Enterobacter sakazakii*

### 7. Procedure

#### 7.1 Dilution of sample

7.1.1 Solid and semisolid sample: aseptically weigh out 100g, 10g and 1g of the sample and add to conical flasks with 900ml, 90ml and 9ml BPW which were preheated to 44°C respectively. Gently shake until uniformly dissolved. Incubate at 36±1°C for 18±2h. Take 1 ml from each of the flask respectively and transfer to 10ml mLST-Vm broth, incubate at 44±0.5°C for 24±2 h.

7.1.2 Liquid sample: aseptically weigh out 100ml, 10ml and 1ml of the sample and add to conical flasks with 900ml, 90ml and 9ml BPW which were preheated to 44°C respectively. Gently shake until uniformly dissolved, to get homogeneous sample solution of 1:10. Incubate at 36±1°C for 18±2h. Take 1 ml from each of the flask respectively and transfer to 10ml mLST-Vm broth, incubate at 44±0.5°C for 24±2 h.

#### 7.2 Isolation and Identification

Same with 5.2 and 5.3.

### 8. Report of Enumeration of *Enterobacter sakazakii*

Integrate the colonial morphological and biochemical characteristic, according to the number of confirmed *Enterobacter sakazakii* positive control, look up in MPN index and report the MPN number of *Enterobacter sakazakii* in 100g(ml) of sample (see Table B.1 in appendix B).

**Appendix A**  
**(Normative appendix)**  
**Culture mediums and reagents**

**A.1 Buffer Peptone Water (BPW)**

A.1.1 Composition

Peptone	10.0g
NaCl	5.0g
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O	9.0g
KH <sub>2</sub> PO <sub>4</sub>	1.5g
Distilled water	1000ml
pH 7.2	

A.1.2 Preparation method

Heat and stir to completely dissolve all the ingredients, adjust the pH and conduct autoclave sterilization at 121°C for 15min.

**A.2 Modified lauryl sulfate tryptose broth-vancomycin medium, mLST-Vm**

A.2.1 mLST broth

A.2.1.1 Composition

NaCl	34.0g
Trypticase	20.0g
Lactose	5.0g
KH <sub>2</sub> PO <sub>4</sub>	2.75g
K <sub>2</sub> HPO <sub>4</sub>	2.75g
Sodium lauryl sulfate	0.1g
Distilled water	1000ml
pH 6.8 ± 0.2	

A.2.1.2 Preparation method

Heat and stir to completely dissolve all the ingredients, adjust the pH, separately put 10ml into each test tube and conduct autoclave sterilization at 121°C for 15min.

A.2.2 Vancomycin solution

A.2.2.1 Composition

Vancomycin	10.0mg
Distilled water	10.0ml



#### A.2.2.2 Preparation method

Dissolve the 10.0mg vancomycin into 10.0ml distilled water, filtrate to remove bacteria. Vancomycin solution can be stored for 15d at 0~5°C.

#### A.2.3 Modified lauryl sulfate tryptose broth-vancomycin medium, mLST-Vm

Add 0.1ml of the above vancomycin solution into each 10ml of mLST broth, the final concentration of vancomycin in the mixture is 10µg/ml.

Note: mLST-Vm should be used within 24h.

### A.3 Trypticase Soy Agar, TSA

#### A.3.1 Composition

Trypticase	15.0g
Phytone	5.0g
NaCl	5.0g
Agar	15.0g
Distilled water	1000ml
pH 7.3 ± 0.2	

#### A.3.2 Preparation method

Heat and stir to completely dissolve all the ingredients, keep boiling for 1min, adjust the pH and conduct autoclave sterilization at 121°C for 15min.

### A.4 Oxidase reagents

#### A.4.1 Composition

N, N, N', N'-tetramethyl-p-phenylenediamine hydrochloride	1.0g
Distilled water	100.0ml

#### A.4.2 Preparation method

Freshly confected in small amount, stored in refrigerator in dark, it should be used within 7d.

#### A.4.3 Testing method

Pick individual characteristics of colonies with glass rod or one-time vaccination needle, coated in the oxidase reagent wetted filter paper plate. If the filter paper does not turn to amaranth, purple or deep blue within 10s, the oxidase test is negative; otherwise it is positive.

Note: Do not use nickel/chromium material during detection.

### A.5 L-Lysine Decarboxylation Medium

### A.5.1 Composition

L-lysine monohydrochloride	5.0g
Yeast extract	3.0g
Glucose	1.0g
Bromcresolpurple	0.015g
Distilled water	1000ml
pH $6.8 \pm 0.2$	

### A.5.2 Preparation method

Heat and stir to completely dissolve all the ingredients, adjust the pH if necessary. Separately put 5ml into each test tube and conduct autoclave sterilization at  $121^{\circ}\text{C}$  for 15min.

### A.5.3 Testing method

Pick the culture onto L-Lysine Decarboxylation Medium, just below the surface of liquid medium. Incubate at  $30 \pm 1^{\circ}\text{C}$  for  $24\text{h} \pm 2\text{h}$ , observe the results. L-Lysine Decarboxylation Medium shows purple if the result is positive, yellow if negative.

## A.6 L-Ornithine Decarboxylation Medium

### A.6.1 Composition

L-ornithine monohydrochloride	5.0g
Yeast extract	3.0g
Glucose	1.0g
Bromcresolpurple	0.015g
Distilled water	1000ml
pH $6.8 \pm 0.2$	

### A.6.2 Preparation method

Heat until completely dissolve all the ingredients, adjust the pH if necessary. Separately put 5mL into each test tube and conduct autoclave sterilization at  $121^{\circ}\text{C}$  for 15min.

### A.6.3 Testing method

Pick the culture onto L-Ornithine Decarboxylation Medium, just below the surface of liquid medium. Incubate at  $30 \pm 1^{\circ}\text{C}$  for  $24\text{h} \pm 2\text{h}$ , observe the results. L-Ornithine Decarboxylation Medium shows purple if the result is positive, yellow if negative.

## A.7 L-Arginine Dihydrolase Medium

### A.7.1 Composition

L-arginine monohydrochloride	5.0g
Yeast extract	3.0g
Glucose	1.0g
Bromcresolpurple	0.015g
Distilled water	1000ml
pH $6.8 \pm 0.2$	

### A.7.2 Preparation method

Heat until completely dissolve of all the ingredients, adjust the pH if necessary. Separately put 5ml into each test tube and conduct autoclave sterilization at 121 °C for 15min.

### A.7.3 Testing method

Pick the culture onto L-Arginine Dihydrolase Medium, just below the surface of liquid medium. Incubate at  $30 \pm 1$  °C for  $24 \pm 2$ h, observe the results. L-Arginine Dihydrolase Medium shows purple if the result is positive, yellow if negative.

## **A.8 Media for fermentation of carbohydrates**

### A.8.1 Basal medium

#### A.8.1.1 Composition

Casein (enzyme digestion)	10.0g
NaCl	5.0g
Phenolsulfonphthalein	0.02g
Distilled water	1000ml
pH $6.8 \pm 0.2$	

#### A.8.1.2 Preparation method

Heat until completely dissolve of all the ingredients, adjust the pH if necessary. Separately put 5ml into each test tube and conduct autoclave sterilization at 121 °C for 15min.

### A.8.2 Sugar solution (D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, amygdalin)

#### A.8.2.1 Composition

Sugar	8.0g
Distilled water	100ml

#### A.8.2.2 Preparation method

Weight 8g of D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, amygdalin respectively, dissolve in 100ml distilled water, filtrate to get rid of bacterial, then get the sugar solutions with concentration 80mg/ml.

### A.8.3 Complete medium

#### A.8.3.1 Composition

Basal medium	875ml
Sugar solution	125ml

#### A.8.3.2 Preparation method

Aseptically adding each sugar solution into basal medium, mixing; put each 10ml into aseptic tubes

#### A.8.4 Testing method

Pick the culture onto media for fermentation of carbohydrates, just below the surface of liquid medium. Incubate at  $30\pm 1^\circ\text{C}$  for  $24\text{h}\pm 2\text{h}$ , observe the results. Media for fermentation of carbohydrates shows yellow if the result is positive, red if negative.

### A.9 Simmons citrate medium

#### A.9.1 Composition

Sodium citrate	2.0g
NaCl	5.0g
$\text{K}_2\text{HPO}_4$	1.0g
$\text{NH}_4\text{H}_2\text{PO}_4$	1.0g
$\text{MgSO}_4$	0.2g
Bromo thymol blue	0.08g
Agar	8.0g~18.0g
Distilled water	1000ml
pH $6.8\pm 0.2$	

#### A.9.2 Preparation method

Heat and stir to completely dissolve all the ingredients, adjust the pH if necessary. Separately put 10ml into each test tube and conduct autoclave sterilization at  $121^\circ\text{C}$  for 15min. Make slope.

#### A.9.3 Testing method

Pick the culture onto medium slope, Incubate at  $30\pm 1^\circ\text{C}$  for  $24\text{h}\pm 2\text{h}$ , observe the results. The medium shows blue if the result is positive.

**Appendix B  
(Normative Appendix)**

***Enterobacter sakazakii* MPN Index Table**

MPN Index of *Enterobacter sakazakii* in 100g(ml) sample is shown in Table B.1.

Positive Tube			MPN	95% Confidence Limits		Positive Tube			MPN	95% Confidence Limits	
100	10	1		Lower Limit	Upper Limits	100	10	1		Lower Limit	Upper Limits
0	0	0	<0.3	—	0.95	2	2	0	2.1	0.45	4.2
0	0	1	0.3	0.015	0.96	2	2	1	2.8	0.87	9.4
0	1	0	0.3	0.015	1.1	2	2	2	3.5	0.87	9.4
0	1	1	0.61	0.12	1.8	2	3	0	2.9	0.87	9.4
0	2	0	0.62	0.12	1.8	2	3	1	3.6	0.87	9.4
0	3	0	0.94	0.36	3.8	3	0	0	2.3	0.46	9.4
1	0	0	0.36	0.017	1.8	3	0	1	3.8	0.87	11
1	0	1	0.72	0.13	1.8	3	0	2	6.4	1.7	18
1	0	2	1.1	0.36	3.8	3	1	0	4.3	0.9	18
1	1	0	0.74	0.13	2	3	1	1	7.5	1.7	20
1	1	1	1.1	0.36	3.8	3	1	2	12	3.7	42
1	2	0	1.1	0.36	4.2	3	1	3	16	4	42
1	2	1	1.5	0.45	4.2	3	2	0	9.3	1.8	42
1	3	0	1.6	0.45	4.2	3	2	1	15	3.7	42
2	0	0	0.92	0.14	3.8	3	2	2	21	4	43
2	0	1	1.4	0.36	4.2	3	2	3	29	9	100
2	0	2	2	0.45	4.2	3	3	0	24	4.2	100
2	1	0	1.5	0.37	4.2	3	3	1	46	9	200
2	1	1	2	0.45	4.2	3	3	2	110	18	410
2	1	2	2.7	0.87	9.4	3	3	3	>110	42	—

Note 1: Three dilution factors are adopted in this table [100g (or 100mL), 10g (or 10mL) and 1g (or 1mL)]. For each dilution factor, three tubes should be inoculated.

Note 2: If the sample volumes listed in the table are changed to 1000g (or 1000mL), 10g (or 10mL) and 1g (or 1mL), the numbers in table should be decreased by 10 times; if it is changed to 10g (or 0.01mL), 1g (or 1mL) and 0.1g (or 0.1mL), the numbers in table should be increased by 10 times and so forth.