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# 〈2022〉 MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS

## INTRODUCTION

Good manufacturing practices require that objectionable organisms be absent from nonsterile nutritional and dietary products. A microorganism can be considered objectionable if it represents a potential health hazard to the user who is using the product as directed, or if it is capable of growing in the product. Objectionable microorganisms are defined as contaminants that, depending on the microbial species, number of organisms, dosage form, intended use, and patient population, would adversely affect product safety. Additionally, microorganisms may be deemed objectionable if they adversely affect product stability or if they may damage the integrity of the container closure system.

This chapter describes the testing of nutritional and dietary articles for specified microorganisms, which are specified in the individual monographs or whose absence is recommended by the guidance under [Microbiological Attributes of Nonsterile Nutritional and Dietary Supplements \(2023\)](#). When objectionable microorganisms are not specified in the individual monograph, it is the manufacturers' responsibility to determine which microorganisms in their products are objectionable. It is not intended that all nonsterile nutritional and dietary articles be tested for the absence of all of the microorganisms mentioned in this chapter, nor is the testing of relevant microorganisms restricted to those presented in this chapter.

Alternative microbiological, physicochemical, and biotechnological methods, including automated methods, may be substituted for these tests, provided they have been validated as being equivalent in their suitability for determining compliance.

**Change to read:**

## BUFFER AND MEDIA

### General Considerations

See *Buffer Solution and Media* under [Microbial Enumeration Tests—Nutritional and Dietary Supplements \(2021\)](#). The appropriateness of each medium for the intended purpose is to be assessed. Control sets of *Fluid Soybean—Casein Digest Medium for Preparatory Testing* are also used to assess the appropriateness of these media in the growth promotion of the specified microorganisms. For other media, streak agar plates to obtain isolated colonies of appropriate microorganisms, and inoculate the fluid media with the appropriate microorganisms at a final concentration of less than 100 cfu per mL. Observe the growth to establish the appropriateness of the media.

### Buffer

#### BUFFER STOCK SOLUTION and PH 7.2 PHOSPHATE BUFFER

Proceed as directed under [Microbial Enumeration Tests—Nutritional and Dietary Supplements \(2021\)](#).

### Media

#### FLUID SOYBEAN—CASEIN DIGEST MEDIUM

Prepare as directed under [Microbial Enumeration Tests—Nutritional and Dietary Supplements \(2021\)](#).

#### MANNITOL—SALT—AGAR MEDIUM

Pancreatic Digest of Casein	5.0 g
Peptic Digest of Animal Tissue	5.0 g
Beef Extract	1.0 g
D-Mannitol	10.0 g
Sodium Chloride	75.0 g

Agar	15.0 g
Phenol Red	0.025 g
Water	1000 mL

Mix, then heat with frequent agitation, and boil for 1 minute to effect solution.  
pH after sterilization:  $7.4 \pm 0.2$ .

## FLUID TETRATHIONATE MEDIUM

Pancreatic Digest of Casein	2.5 g
Peptic Digest of Animal Fat	2.5 g
Bile Salts	1.0 g
Calcium Carbonate	10.0 g
Sodium Thiosulfate	30.0 g
Water	1000 mL

Heat to boiling. Do not autoclave; use the same day. Immediately before use, add a solution prepared by dissolving 5 g of potassium iodide and 6 g of iodine in 20 mL of water. Then add 10 mL of a solution of brilliant green (1 in 1000), and mix. Do not heat after adding the brilliant green solution.

## BRILLIANT GREEN—AGAR MEDIUM

Yeast Extract	3.0 g
Peptic Digest of Animal Tissue	5.0 g
Pancreatic Digest of Casein	5.0 g
Lactose	10.0 g
Sodium Chloride	5.0 g
Sucrose	10.0 g
Phenol Red	80.0 g
Agar	20.0 g
Brilliant Green	12.5 mg
Water	1000 mL

Boil for 1 minute. Sterilize just prior to use, melt, pour into Petri dishes, and allow to cool.  
pH after sterilization:  $6.9 \pm 0.2$

## XYLOSE—LYSINE—DESOXYCHOLATE—AGAR MEDIUM

Xylose	3.5 g
L-Lysine	5.0 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium Chloride	5.0 g

Yeast Extract	3.0 g
Phenol Red	80 mg
Agar	13.5 g
Sodium Desoxycholate (as Bile Salts)	2.5 g
Sodium Thiosulfate	6.8 g
Ferric Ammonium Citrate	800 mg
Water	1000 mL

Heat, with swirling, just to the boiling point. Do not overheat or sterilize. Transfer at once to a water bath maintained at about 50°, and pour into Petri plates as soon as the *Medium* has cooled.

Final pH: 7.4 ± 0.2.

#### HEKTOEN ENTERIC AGAR MEDIUM

Protease Peptone	12.0 g
Yeast Extract	3.0 g
Lactose	12.0 g
Sucrose	2.0 g
Salicin	9.0 g
Bile Salts No. 3	9.0 g
Sodium Chloride	5.0 g
Sodium Thiosulfate	5.0 g
Ferric Ammonium Citrate	1.5 g
Acid Fuchsin	0.1 g
Bromothymol Blue	65 mg
Agar	14.0 g
Water	1000 mL

Mix, and allow to stand for 10 minutes. Heat gently, and allow to boil for a few seconds to dissolve the agar. Do not sterilize. Cool to 60°, and pour into Petri dishes.

Final pH: 7.5 ± 0.2.

#### TRIPLE SUGAR—IRON—AGAR MEDIUM

Pancreatic Digest of Casein	10.0 g
Pancreatic Digest of Animal Tissue	10.0 g
Lactose	10.0 g
Sucrose	10.0 g
Dextrose	1.0 g
Ferrous Ammonium Sulfate	200 mg

Sodium Chloride	5.0 g
Sodium Thiosulfate	200 mg
Agar	13.0 g
Phenol Red	25 mg
Water	1000 mL

pH after sterilization:  $7.3 \pm 0.2$ .

#### MACCONKEY AGAR MEDIUM

Pancreatic Digest of Gelatin	17.0 g
Pancreatic Digest of Casein	1.5 g
Peptic Digest of Animal Tissue	1.5 g
Lactose	10.0 g
Bile Salts Mixture	1.5 g
Sodium Salts Mixture	5.0 g
Agar	13.5 g
Neutral Red	30 mg
Crystal Violet	1.0 mg
Water	1000 mL

Boil for 1 minute to effect solution.

pH after sterilization:  $7.1 \pm 0.2$ .

#### LEVINE EOSIN—METHYLENE BLUE—AGAR MEDIUM

Pancreatic Digest of Gelatin	10.0 g
Dibasic Potassium Phosphate	2.0 g
Agar	15.0 g
Lactose	10.0 g
Eosin Y	400 mg
Methylene Blue	65 mg
Water	1000 mL

Dissolve pancreatic digest of gelatin, dibasic potassium phosphate, and agar in water, with warming, and allow to cool. Just prior to use, liquefy the gelled agar solution, and add the remaining ingredients, as solutions, in the following amounts: for each 100 mL of the liquefied agar solution, add 5 mL of lactose solution (1 in 5), 2 mL of the eosin Y solution (1 in 50), and 2 mL of methylene blue solution (1 in 300). Mix. The finished *Medium* may not be clear.

pH after sterilization:  $7.1 \pm 0.2$ .

#### BAIRD—PARKER AGAR MEDIUM

Pancreatic Digest of Casein	10.0 g
Beef Extract	5.0 g

Yeast Extract	1.0 g
Lithium Chloride	5.0 g
Agar	20.0 g
Glycine	12.0 g
Sodium Pyruvate	10.0 g
Water	950 mL

Heat with frequent agitation, and boil for 1 minute. Sterilize, cool to between 45° and 50°, and add 10 mL of sterile potassium tellurite solution (1 in 100) and 50 mL of egg yolk emulsion prepared as follows. Disinfect the surface of whole-shell eggs, aseptically crack the eggs, transfer intact yolks to a sterile graduated cylinder, add sterile saline TS to obtain a 3 to 7 ratio of egg yolk to saline, add to a sterile blender cup, and mix at high speed for 5 seconds. Mix all ingredients well but gently, and pour into plates.

pH after sterilization: 6.8 ± 0.2.

#### VOGEL–JOHNSON AGAR MEDIUM

Pancreatic Digest of Casein	10.0 g
Yeast Extract	5.0 g
Mannitol	10.0 g
Dibasic Potassium Phosphate	5.0 g
Lithium Chloride	5.0 g
Glycine	10.0 g
Agar	16.0 g
Phenol Red	25.0 mg
Water	1000 mL

Boil for 1 minute. Sterilize, cool to between 45° and 50°, and add 20 mL of sterile potassium tellurite solution (1 in 100).

pH after sterilization: 7.2 ± 0.2.

#### FLUID SELENITE–CYSTINE MEDIUM

Pancreatic Digest of Casein	5.0 g
Lactose	4.0 g
Sodium Phosphate	10.0 g
Sodium Acid Selenite	4.0 g
L-Cystine	10.0 ▲mg▲ (ERR 1-Nov-2019)
Water	1000 mL

Mix, and heat to effect solution. Then heat in flowing stream for 15 minutes. Do not sterilize.

Final pH: 7.0 ± 0.2.

#### REINFORCED MEDIUM FOR CLOSTRIDIA

Beef Extract	10.0 g
Peptone	10.0 g

Yeast Extract	3.0 g
Soluble Starch	1.0 g
Glucose Monohydrate	5.0 g
Cysteine Hydrochloride	0.5 g
Sodium Chloride	5.0 g
Sodium Acetate	3.0 g
Agar	0.5 g
Water	1000 mL

Dissolve agar in water by heating to boiling, while stirring continuously. Adjust the pH if necessary, and sterilize. pH after sterilization:  $6.8 \pm 0.2$ .

## COLUMBIA AGAR

Pancreatic Digest of Casein	10.0 g
Meat Peptic Digest	5.0 g
Heart Pancreatic Digest	3.0 g
Yeast Extract	5.0 g
Cornstarch	1.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Water	1000 mL

Dissolve agar in water by heating to boiling and with continuous stirring. If necessary, adjust the pH. Sterilize, and allow to cool to 45° to 50°. Add, when necessary, gentamicin sulfate, equivalent to about 20 mg of gentamicin base, and pour into Petri dishes.

Pre-reduction of the medium is recommended.

pH after sterilization:  $7.3 \pm 0.2$ .

## RAPPAPORT VASSILIADIS SALMONELLA ENRICHMENT BROTH

Soya Peptone	4.5 g
Magnesium Chloride Hexahydrate	29.0 g
Sodium Chloride	8.0 g
Dipotassium Phosphate	0.4 g
Potassium Dihydrogen Phosphate	0.6 g
Malachite Green	0.036 g
Purified Water	1000 mL

Dissolve, warming slightly. Sterilize in an autoclave using a validated cycle, at a temperature not exceeding 115°.

The pH is  $5.2 \pm 0.2$  at 25° after heating and autoclaving.

**PREPARATORY TESTING**

Proceed as directed for *Preparatory Testing* under [Microbial Enumeration Tests—Nutritional and Dietary Supplements \(2021\)](#).

For enrichment broth, selective media, and differential media use an inoculating loop to transfer the inoculum of each test organism to the plated or liquid media being tested. If a plated medium is being tested, streak the surface of plate with the loop in four directions to obtain a pattern of isolated colonies. Incubate the media, and examine the plated or liquid media for the characteristic growth of the inocula (See [Tables 1, 2, 3, and 4](#)).

### SAMPLING

Proceed as directed for *Sampling* under [Microbial Enumeration Tests—Nutritional and Dietary Supplements \(2021\)](#).

### TEST PROCEDURES

**Test Preparation**—Prepare as directed for *Sampling*. Transfer to a suitable container with 100 mL of *Fluid Soybean–Casein Digest Medium (FSCD)*. Mix by shaking gently. [NOTE—On the basis of results for *Preparatory Testing*, modify the *Test Preparation* as appropriate.]

#### Test for Absence of *Staphylococcus aureus*

Incubate at 30° to 35° for 18 to 24 hours. Streak a loopful from *FSCD* onto the surface of one or more of the following media: *Vogel–Johnson Agar Medium (VJ Agar)*, *Mannitol–Salt–Agar Medium (MS-Agar)*, and *Baird–Parker Agar Medium (BP Agar)*. Cover the Petri plates, invert them, and incubate at 30° to 35° for 24 to 48 hours.

Examine the plates of *VJ Agar*, *MS-Agar*, and/or *BP Agar*, and interpret the results with reference to [Table 1](#): if no plate contains colonies having the characteristics described, the test specimen meets the requirement for the absence of *Staphylococcus aureus*. If characteristic colonies are present, perform coagulase test as follows. Transfer representative colonies to separate tubes containing 0.5 mL of rabbit plasma, horse plasma, or any other mammalian plasma. Incubate in a water bath at 37°. Examine for coagulation after 3 hours of incubation and at suitable intervals up to 24 hours. Comparing with positive and negative controls, the absence of a coagulase reaction indicates the absence of *Staphylococcus aureus* in the tested article.

**Table 1. Characteristics of *Staphylococcus aureus* on Specified Agar Media**

Agar Medium	Colonial Morphology	Gram Stain
<i>Vogel–Johnson</i>	Black surrounded by yellow zone	(+), cocci
<i>Mannitol–Salt</i>	Yellow colonies with yellow zone	(+), cocci
<i>Baird–Parker</i>	Black, shiny surrounded by 2–5-mm clear zones	(+), cocci

#### Test for Absence of *Salmonella* Species

Incubate at 30° to 35° for 18 to 24 hours. From *FSCD*, pipet a 1-mL aliquot into 10 mL of *Rappaport Vassiliadis Salmonella Enrichment Broth*, mix, and incubate at 30° to 35° for 18 to 24 hours. Streak a loopful from both incubated media onto individual surfaces of one or more of following media: *Brilliant Green Agar Medium (BG-Agar)*, *Xylose–Lysine–Desoxycholate–Agar Medium (XLDC-Agar)*, and *Hektoen Enteric Agar Medium (HE Agar)*. Cover, invert the plates, and incubate at 30° to 35° for 24 to 48 hours. Examine the inoculated plates of *BG-Agar*, *XLDC-Agar*, and/or *HE Agar*, and interpret the results with reference to [Table 2](#): if no colonies having the characteristics described are observed, the test specimen meets the requirement for the absence of *Salmonella* species. If colonies with characteristics described in [Table 2](#) are present, the suspect colonies are transferred to a slant of *Triple Sugar–Iron–Agar Medium (TSI)* using an inoculating wire, by first streaking the surface of the slant, and then stabbing the wire well beneath the surface. Incubate at 30° to 35° for 24 to 48 hours. If the tubes do not have red alkaline slants and yellow acid butts, with or without concomitant blackening of the butts from hydrogen sulfide production, the test specimen meets the requirement for the absence of *Salmonella* species.

**Table 2. Characteristics of *Salmonella* Species on Specified Agar Media**

Agar Medium	Colonial Morphology	Gram Stain
<i>Brilliant Green</i>	Small, transparent and colorless; or opaque, pink or white (often surrounded by pink to red zone)	(–), rods
<i>Xylose–Lysine–Desoxycholate</i>	Red, with or without black centers	(–), rods
<i>Hektoen Enteric</i>	Blue-green, with or without black centers	(–), rods

#### Test for Absence of *Escherichia coli*

Incubate at 30° to 35° for 24 to 48 hours. From *FSCD*, pipet a 1-mL aliquot into a container containing 10 mL of *MacConkey Broth*, mix, and incubate at 42° to 44° for 24 to 48 hours. Streak a loopful from both incubated media onto individual surfaces of *MacConkey Agar Medium (MC Agar)*, and incubate at 30° to 35° for 18 to 24 hours. Examine the inoculated *MC Agar* plate, and interpret the results with reference to

**Table 3:** if no colonies having the characteristics described are observed, the test specimen meets the requirement for the absence of *Escherichia coli*. Suspect colonies showing the characteristics described in **Table 3** are transferred individually, using an inoculating loop, to the surface of a plate with *Levine Eosin–Methylene Blue–Agar Medium (LEMB-Agar)*. If a large number of suspect colonies are to be transferred, divide the surface of each plate into quadrants, each quadrant being inoculated with a different colony. Cover the plates, invert, and incubate at 30° to 35° for 24 to 48 hours. If none of the colonies exhibit a characteristic metallic sheen under reflected light, and if none exhibit a blue-black appearance under transmitted light, the test specimen meets the requirement for the absence of *Escherichia coli*.

**Table 3. Characteristics of *Escherichia coli* on MacConkey Agar Medium**

Colonial Morphology	Gram Stain
Brick red, may have surrounding zone of precipitated bile	(-), rods

### Test for Absence of *Clostridium* Species

#### TEST PREPARATION

Prepare as directed for *Sampling*. [NOTE—On the basis of results for *Preparatory Testing*, modify the *Test Preparation* as appropriate.]

**PROCEDURE**—Take two equal portions of the *Test Preparation*, heat one to 80° for 10 minutes, and cool rapidly. Transfer 10 mL of each portion to separate containers, each containing 100 mL of *Reinforced Medium for Clostridia*, and incubate under anaerobic conditions at 35° to 37° for 48 hours. After incubation, subculture each specimen on *Columbia Agar Medium* to which gentamicin has been added, and incubate under anaerobic conditions at 35° to 37° for 48 hours. Examine the plates, and interpret with reference to **Table 4**: if no growth of microorganisms is detected, the test specimen meets the requirement for the absence of *Clostridium* species.

**Table 4. Characteristics of *Clostridium* Species on Specified Media**

Medium	Gram Stain	Catalase
<i>Reinforced Medium for Clostridia</i>	(+), rods	
<i>Columbia Agar</i>	(+), rods	Negative

If growth occurs, subculture each distinct colony on *Columbia Agar Medium*, and separately incubate in aerobic and in anaerobic conditions at 35° to 37° for 48 hours. The occurrence of only anaerobic growth of gram-positive bacilli, giving a negative catalase reaction, indicates the presence of *Clostridium sporogenes*. To perform the catalase test, transfer discrete colonies to glass slides, and apply a drop of dilute hydrogen peroxide solution: the reaction is negative if no gas bubbles evolve. If the test specimen exhibits none of these characteristics, it meets the requirement for the absence of *Clostridium* species.

#### RETEST

For the purpose of confirming a doubtful result by any of the procedures outlined in the foregoing tests following their application to a 10 g specimen, a retest on a 25 g specimen of the nutritional or dietary supplement may be conducted. Proceed as directed under *Procedure*, but make allowances for the larger specimen size.

**Auxiliary Information** - Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
<2022> MICROBIOLOGICAL PROCEDURES FOR ABSENCE OF SPECIFIED MICROORGANISMS—NUTRITIONAL AND DIETARY SUPPLEMENTS	<a href="#">Leslie Furr</a> Associate Scientific Liaison	GCM2022 General Chapters - Microbiology 2022

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