

# ⟨62⟩ MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: TESTS FOR SPECIFIED MICROORGANISMS

## INTRODUCTION

The tests described hereafter will allow determination of the absence of, or limited occurrence of, specified microorganisms that may be detected under the conditions described.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopeial method has been demonstrated.

## GENERAL PROCEDURES

The preparation of samples is carried out as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61).

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralized as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61).

If surface-active substances are used for sample preparation, their absence of toxicity for microorganisms and their compatibility with any inactivators used must be demonstrated as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61).

## GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA, SUITABILITY OF THE TEST AND NEGATIVE CONTROLS

The ability of the test to detect microorganisms in the presence of the product to be tested must be established. Suitability must be confirmed if a change in testing performance or a change in the product that may affect the outcome of the test is introduced.

### Preparation of Test Strains

Use standardized stable suspensions of test strains as stated below. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot.

### AEROBIC MICROORGANISMS

Grow each of the bacterial test strains separately in containers containing *Soybean–Casein Digest Broth* or on *Soybean–Casein Digest Agar* at 30° to 35° for 18 to 24 hours. Grow the test strain for *Candida albicans* separately on *Sabouraud Dextrose Agar* or in *Sabouraud Dextrose Broth* at 20° to 25° for 2 to 3 days.

<i>Staphylococcus aureus</i>	such as ATCC 6538, NCIMB 9518, CIP 4.83, or NBRC 13276
<i>Pseudomonas aeruginosa</i>	such as ATCC 9027, NCIMB 8626, CIP 82.118, or NBRC 13275
<i>Escherichia coli</i>	such as ATCC 8739, NCIMB 8545, CIP 53.126, or NBRC 3972
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium or, as an alternative,	such as ATCC 14028
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Abony	such as NBRC 100797, NCTC 6017, or CIP 80.39
<i>Candida albicans</i>	such as ATCC 10231, NCPF 3179, IP 48.72, or NBRC 1594

Use *Buffered Sodium Chloride–Peptone Solution pH 7.0* or *Phosphate Buffer Solution pH 7.2* to make test suspensions. Use the suspensions within 2 hours or within 24 hours if stored at 2° to 8°.

### CLOSTRIDIA

Use *Clostridium sporogenes* such as ATCC 11437 (NBRC 14293, NCIMB 12343, CIP 100651) or ATCC 19404 (NCTC 532 or CIP 79.3). Grow the clostridial test strain under anaerobic conditions in *Reinforced Medium for Clostridia* at 30° to 35° for 24 to 48 hours. As an alternative to preparing and then diluting down a fresh suspension of vegetative cells of *Cl. sporogenes*, a stable spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2° to 8° for a validated period.

## Negative Control

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of microorganisms. A negative control is also performed when testing the products as described under *Testing of Products*. A failed negative control requires an investigation.

## Growth Promotion and Inhibitory Properties of the Media

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Verify suitable properties of relevant media as described in *Table 1*.

**Table 1. Growth Promoting, Inhibitory, and Indicative Properties of Media**

Test/Medium	Property	Test Strains
<i>Test for bile-tolerant Gram-negative bacteria</i>		
Enterobacteria Enrichment Broth Mossel	Growth promoting	<i>E. coli</i>
		<i>P. aeruginosa</i>
	Inhibitory	<i>S. aureus</i>
Violet Red Bile Glucose Agar	Growth promoting + Indicative	<i>E. coli</i>
		<i>P. aeruginosa</i>
<i>Test for Escherichia coli</i>		
MacConkey Broth	Growth promoting	<i>E. coli</i>
	Inhibitory	<i>S. aureus</i>
MacConkey Agar	Growth promoting + Indicative	<i>E. coli</i>
<i>Test for Salmonella</i>		
Rappaport Vassiliadis Salmonella Enrichment Broth	Growth promoting	<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar <i>Typhimurium</i> or
		<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar <i>Abony</i>
	Inhibitory	<i>S. aureus</i>
Xylose Lysine Deoxycholate Agar	Growth promoting + Indicative	<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar <i>Typhimurium</i> or
		<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar <i>Abony</i>
<i>Test for Pseudomonas aeruginosa</i>		
Cetrimide Agar	Growth promoting	<i>P. aeruginosa</i>
	Inhibitory	<i>E. coli</i>
<i>Test for Staphylococcus aureus</i>		
Mannitol Salt Agar	Growth promoting + Indicative	<i>S. aureus</i>
	Inhibitory	<i>E. coli</i>
<i>Test for Clostridia</i>		
Reinforced Medium for Clostridia	Growth promoting	<i>Cl. sporogenes</i>
Columbia Agar	Growth promoting	<i>Cl. sporogenes</i>
<i>Test for Candida albicans</i>		
Sabouraud Dextrose Broth	Growth promoting	<i>C. albicans</i>
Sabouraud Dextrose Agar	Growth promoting + Indicative	<i>C. albicans</i>

## TEST FOR GROWTH-PROMOTING PROPERTIES, LIQUID MEDIA

Inoculate a portion of the appropriate medium with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Clearly visible growth of the microorganism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

## TEST FOR GROWTH-PROMOTING PROPERTIES, SOLID MEDIA

Perform *Surface-Spread Method* (see *Plate-Count Methods* under *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61)), inoculating each plate with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the microorganism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

## TEST FOR INHIBITORY PROPERTIES, LIQUID OR SOLID MEDIA

Inoculate the appropriate medium with at least 100 cfu of the appropriate microorganism. Incubate at the specified temperature for not less than the longest period of time specified in the test. No growth of the test microorganism occurs.

## TEST FOR INDICATIVE PROPERTIES

Perform *Surface-Spread Method* (see *Plate-Count Methods* under *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61)), inoculating each plate with a small number (not more than 100 cfu) of the appropriate microorganism. Incubate at the specified temperature for a period of time within the range specified in the test. Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

## Suitability of the Test Method

For each new product to be tested perform sample preparation as described in the relevant paragraph under *Testing of Products*. At the time of mixing, add each test strain in the prescribed growth medium. Inoculate the test strains individually. Use a number of microorganisms equivalent to not more than 100 cfu in the inoculated test preparation.

Perform the test as described in the relevant paragraph under *Testing of Products* using the shortest incubation period prescribed.

The specified microorganisms must be detected with the indication reactions as described under *Testing of Products*.

Any antimicrobial activity of the product necessitates a modification of the test procedure (see *Neutralization/Removal of Antimicrobial Activity* under *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61)).

For a given product, if the antimicrobial activity with respect to a microorganism for which testing is prescribed cannot be neutralized, then it is to be assumed that the inhibited microorganism will not be present in the product.

## TESTING OF PRODUCTS

### Bile-Tolerant Gram-Negative Bacteria

#### SAMPLE PREPARATION AND PRE-INCUBATION

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), but using *Soybean-Casein Digest Broth* as the chosen diluent, mix, and incubate at 20° to 25° for a time sufficient to resuscitate the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 hours but not more than 5 hours).

#### TEST FOR ABSENCE

Unless otherwise prescribed, use the volume corresponding to 1 g of the product, as prepared in *Sample Preparation and Pre-Incubation*, to inoculate *Enterobacteria Enrichment Broth Mossel*. Incubate at 30° to 35° for 24 to 48 hours. Subculture on plates of *Violet Red Bile Glucose Agar*. Incubate at 30° to 35° for 18 to 24 hours.

The product complies with the test if there is no growth of colonies.

#### QUANTITATIVE TEST

**Selection and Subculture**—Inoculate suitable quantities of *Enterobacteria Enrichment Broth Mossel* with the preparation as directed under *Sample Preparation and Pre-Incubation* and/or dilutions of it containing respectively 0.1 g, 0.01 g, and 0.001 g (or 0.1 mL, 0.01 mL, and 0.001 mL) of the product to be examined. Incubate at 30° to 35° for 24 to 48 hours. Subculture each of the cultures on a plate of *Violet Red Bile Glucose Agar*. Incubate at 30° to 35° for 18 to 24 hours.

**Interpretation**—Growth of colonies constitutes a positive result. Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result. Determine from *Table 2* the probable number of bacteria.

**Table 2. Interpretation of Results**

Results for Each Quantity of Product			Probable Number of Bacteria per g or mL of Product
0.1 g or 0.1 mL	0.01 g or 0.01 mL	0.001 g or 0.001 mL	
+	+	+	more than 10 <sup>3</sup>
+	+	–	less than 10 <sup>3</sup> and more than 10 <sup>2</sup>

**Table 2. Interpretation of Results** (continued)

Results for Each Quantity of Product			Probable Number of Bacteria per g or mL of Product
0.1 g or 0.1 mL	0.01 g or 0.01 mL	0.001 g or 0.001 mL	
+	–	–	less than 10 <sup>2</sup> and more than 10
–	–	–	less than 10

### ***Escherichia coli***

#### SAMPLE PREPARATION AND PRE-INCUBATION

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), and use 10 mL or the quantity corresponding to 1 g or 1 mL, to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean–Casein Digest Broth*, mix, and incubate at 30° to 35° for 18 to 24 hours.

#### SELECTION AND SUBCULTURE

Shake the container, transfer 1 mL of *Soybean–Casein Digest Broth* to 100 mL of *MacConkey Broth*, and incubate at 42° to 44° for 24 to 48 hours. Subculture on a plate of *MacConkey Agar* at 30° to 35° for 18 to 72 hours.

#### INTERPRETATION

Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests. The product complies with the test if no colonies are present or if the identification tests are negative.

### ***Salmonella***

#### SAMPLE PREPARATION AND PRE-INCUBATION

Prepare the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), and use the quantity corresponding to not less than 10 g or 10 mL to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean–Casein Digest Broth*, mix, and incubate at 30° to 35° for 18 to 24 hours.

#### SELECTION AND SUBCULTURE

Transfer 0.1 mL of *Soybean–Casein Digest Broth* to 10 mL of *Rappaport Vassiliadis Salmonella Enrichment Broth*, and incubate at 30° to 35° for 18 to 24 hours. Subculture on plates of *Xylose Lysine Deoxycholate Agar*. Incubate at 30° to 35° for 18 to 48 hours.

#### INTERPRETATION

The possible presence of *Salmonella* is indicated by the growth of well-developed, red colonies, with or without black centers. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

### ***Pseudomonas aeruginosa***

#### SAMPLE PREPARATION AND PRE-INCUBATION

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean–Casein Digest Broth*, and mix. When testing transdermal patches, filter the volume of sample corresponding to one patch of the preparation (see *Transdermal Patches* under *Preparation of the Sample* in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61)) through a sterile filter membrane, and place in 100 mL of *Soybean–Casein Digest Broth*. Incubate at 30° to 35° for 18 to 24 hours.

#### SELECTION AND SUBCULTURE

Subculture on a plate of *Cetrimide Agar*, and incubate at 30° to 35° for 18 to 72 hours.

## INTERPRETATION

Growth of colonies indicates the possible presence of *P. aeruginosa*. This is confirmed by identification tests. The product complies with the test if colonies are not present or if the confirmatory identification tests are negative.

### ***Staphylococcus aureus***

#### SAMPLE PREPARATION AND PRE-INCUBATION

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under *Suitability of the Test Method*) of *Soybean–Casein Digest Broth*, and homogenize. When testing transdermal patches, filter the volume of sample corresponding to one patch of the preparation (see *Transdermal Patches* under *Preparation of the Sample* in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61)) through a sterile filter membrane, and place in 100 mL of *Soybean–Casein Digest Broth*. Incubate at 30° to 35° for 18 to 24 hours.

#### SELECTION AND SUBCULTURE

Subculture on a plate of *Mannitol Salt Agar*, and incubate at 30° to 35° for 18 to 72 hours.

#### INTERPRETATION

The possible presence of *S. aureus* is indicated by the growth of yellow or white colonies surrounded by a yellow zone. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

### ***Clostridia***

#### SAMPLE PREPARATION AND HEAT TREATMENT

Prepare a sample using a 1 in 10 dilution (with a minimum total volume of 20 mL) of not less than 2 g or 2 mL of the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61). Divide the sample into two portions of at least 10 mL. Heat one portion at 80° for 10 minutes, and cool rapidly. Do not heat the other portion.

#### SELECTION AND SUBCULTURE

Use 10 mL or the quantity corresponding to 1 g or 1 mL of the product to be examined of both portions to inoculate suitable amounts (determined as described under *Suitability of the Test Method*) of *Reinforced Medium for Clostridia*. Incubate under anaerobic conditions at 30° to 35° for 48 hours. After incubation, make subcultures from each container on *Columbia Agar*, and incubate under anaerobic conditions at 30° to 35° for 48 to 72 hours.

#### INTERPRETATION

The occurrence of anaerobic growth of rods (with or without endospores) giving a negative catalase reaction indicates the presence of *Clostridia*.

This is confirmed by identification tests. The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

### ***Candida albicans***

#### SAMPLE PREPARATION AND PRE-INCUBATION

Prepare the product to be examined as described in *Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests* (61), and use 10 mL or the quantity corresponding to not less than 1 g or 1 mL, to inoculate 100 mL of *Sabouraud Dextrose Broth*, and mix. Incubate at 30° to 35° for 3 to 5 days.

#### SELECTION AND SUBCULTURE

Subculture on a plate of *Sabouraud Dextrose Agar*, and incubate at 30° to 35° for 24 to 48 hours.

#### INTERPRETATION

Growth of white colonies may indicate the presence of *C. albicans*. This is confirmed by identification tests. The product complies with the test if such colonies are not present or if the confirmatory identification tests are negative.

## RECOMMENDED SOLUTIONS AND CULTURE MEDIA

[NOTE—This section is given for information.]

The following solutions and culture media have been found satisfactory for the purposes for which they are prescribed in the test for microbial contamination in the Pharmacopeia. Other media may be used provided that their suitability can be demonstrated.

**Stock Buffer Solution**—Transfer 34 g of potassium dihydrogen phosphate to a 1000-mL volumetric flask, dissolve in 500 mL of Purified Water, adjust with sodium hydroxide to a pH of  $7.2 \pm 0.2$ , add Purified Water to volume, and mix. Dispense in containers, and sterilize. Store at a temperature of  $2^\circ$  to  $8^\circ$ .

**Phosphate Buffer Solution pH 7.2**—Prepare a mixture of Purified Water and *Stock Buffer Solution* (800:1 v/v), and sterilize.

Buffered Sodium Chloride–Peptone Solution pH 7.0	
Potassium Dihydrogen Phosphate	3.6 g
Disodium Hydrogen Phosphate Dihydrate	7.2 g (equivalent to 0.067 M phosphate)
Sodium Chloride	4.3 g
Peptone (meat or casein)	1.0 g
Purified Water	1000 mL

Sterilize in an autoclave using a validated cycle.

Soybean–Casein Digest Broth	
Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean	3.0 g
Sodium Chloride	5.0 g
Dibasic Hydrogen Phosphate	2.5 g
Glucose Monohydrate	2.5 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $7.3 \pm 0.2$  at  $25^\circ$ . Sterilize in an autoclave using a validated cycle.

Soybean–Casein Digest Agar	
Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $7.3 \pm 0.2$  at  $25^\circ$ . Sterilize in an autoclave using a validated cycle.

Sabouraud Dextrose Agar	
Dextrose	40.0 g
Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein (1:1)	10.0 g
Agar	15.0 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $5.6 \pm 0.2$  at  $25^\circ$ . Sterilize in an autoclave using a validated cycle.

Potato Dextrose Agar	
Infusion from potatoes	200 g
Dextrose	20.0 g
Agar	15.0 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $5.6 \pm 0.2$  at  $25^\circ$ . Sterilize in an autoclave using a validated cycle.

Sabouraud Dextrose Broth	
Dextrose	20.0 g
Mixture of Peptic Digest of Animal Tissue and Pancreatic Digest of Casein (1:1)	10.0 g
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $5.6 \pm 0.2$  at  $25^\circ$ . Sterilize in an autoclave using a validated cycle.

Enterobacteria Enrichment Broth Mossel	
Pancreatic Digest of Gelatin	10.0 g
Glucose Monohydrate	5.0 g
Dehydrated Ox Bile	20.0 g
Potassium Dihydrogen Phosphate	2.0 g
Disodium Hydrogen Phosphate Dihydrate	8.0 g
Brilliant Green	15 mg
Purified Water	1000 mL

Adjust the pH so that after heating it is  $7.2 \pm 0.2$  at  $25^\circ$ . Heat at  $100^\circ$  for 30 minutes, and cool immediately.

Violet Red Bile Glucose Agar	
Yeast Extract	3.0 g
Pancreatic Digest of Gelatin	7.0 g
Bile Salts	1.5 g
Sodium Chloride	5.0 g
Glucose Monohydrate	10.0 g
Agar	15.0 g
Neutral Red	30 mg
Crystal Violet	2 mg
Purified Water	1000 mL

Adjust the pH so that after heating it is  $7.4 \pm 0.2$  at  $25^\circ$ . Heat to boiling; do not heat in an autoclave.

MacConkey Broth	
Pancreatic Digest of Gelatin	20.0 g
Lactose Monohydrate	10.0 g
Dehydrated Ox Bile	5.0 g
Bromocresol Purple	10 mg
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $7.3 \pm 0.2$  at  $25^\circ$ . Sterilize in an autoclave using a validated cycle.

MacConkey Agar	
Pancreatic Digest of Gelatin	17.0 g
Peptones (meat and casein)	3.0 g
Lactose Monohydrate	10.0 g
Sodium Chloride	5.0 g
Bile Salts	1.5 g
Agar	13.5 g
Neutral Red	30.0 mg
Crystal Violet	1 mg

<b>MacConkey Agar</b>	
Purified Water	1000 mL

Adjust the pH so that after sterilization it is  $7.1 \pm 0.2$  at  $25^\circ$ . Boil for 1 minute with constant shaking, then sterilize in an autoclave using a validated cycle.

<b>Rappaport Vassiliadis Salmonella Enrichment Broth</b>	
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  $115^\circ$ . The pH is to be  $5.2 \pm 0.2$  at  $25^\circ$  after heating and autoclaving.

<b>Xylose Lysine Deoxycholate Agar</b>	
Xylose	3.5 g
L-Lysine	5.0 g
Lactose Monohydrate	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 Deoxycholate	2.5 g
Sodium Thiosulfate	6.8 g
Ferric Ammonium Citrate	0.8 g
Purified Water	1000 mL

Adjust the pH so that after heating it is  $7.4 \pm 0.2$  at  $25^\circ$ . Heat to boiling, cool to  $50^\circ$ , and pour into Petri dishes. Do not heat in an autoclave.

<b>Cetrimide Agar</b>	
Pancreatic Digest of Gelatin	20.0 g
Magnesium Chloride	1.4 g
Dipotassium Sulfate	10.0 g
Cetrimide	0.3 g
Agar	13.6 g
Purified Water	1000 mL
Glycerol	10.0 mL

Heat to boiling for 1 minute with shaking. Adjust the pH so that after sterilization it is  $7.2 \pm 0.2$  at  $25^\circ$ . Sterilize in an autoclave using a validated cycle.

<b>Mannitol Salt Agar</b>	
Pancreatic Digest of Casein	5.0 g
Peptic Digest of Animal Tissue	5.0 g
Beef Extract	1.0 g



Mannitol Salt Agar	
D-Mannitol	10.0 g
Sodium Chloride	75.0 g
Agar	15.0 g
Phenol Red	0.025 g
Purified Water	1000 mL

Heat to boiling for 1 minute with shaking. Adjust the pH so that after sterilization it is  $7.4 \pm 0.2$  at  $25^\circ$ . Sterilize in an autoclave using a validated cycle.

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
Purified Water	1000 mL

Hydrate the agar, and dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is about  $6.8 \pm 0.2$  at  $25^\circ$ . Sterilize in an autoclave using a validated cycle.

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
Maize Starch	1.0 g
Sodium Chloride	5.0 g
Agar, according to gelling power	10.0–15.0 g
Purified Water	1000 mL

Hydrate the agar, and dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilization it is  $7.3 \pm 0.2$  at  $25^\circ$ . Sterilize in an autoclave using a validated cycle. Allow to cool to  $45^\circ$  to  $50^\circ$ ; add, where necessary, gentamicin sulfate corresponding to 20 mg of gentamicin base, and pour into Petri dishes.