Pharmaceutical microbiology

confidence is critical

Whether performing testing for microbial limits, sterility, environmental monitoring or mycoplasma: companies need a partner that can deliver science, service, and confidence to get the job done.

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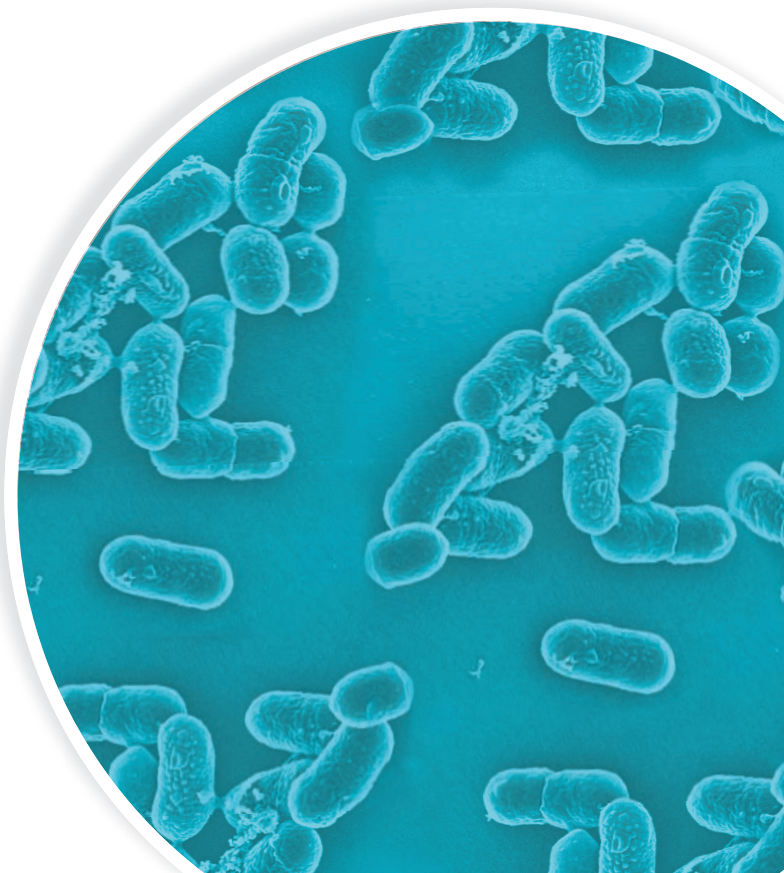
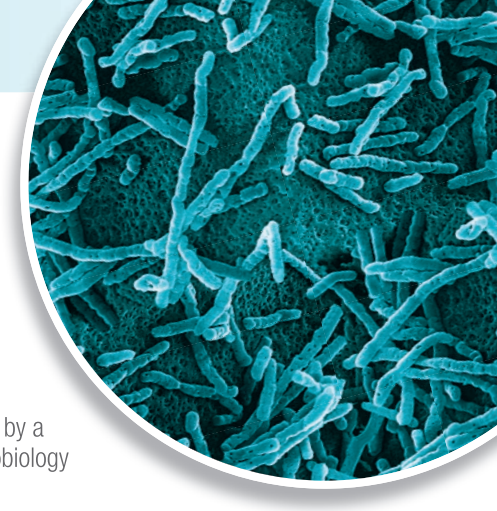


This guide contains consolidated algorithms provided by Dr. Scott Sutton, as an outline for detection of specific pathogens isolated in the pharmaceutical microbiology laboratory.

For more detailed information, please consult the appropriate U.S. Pharmacopeia methods.



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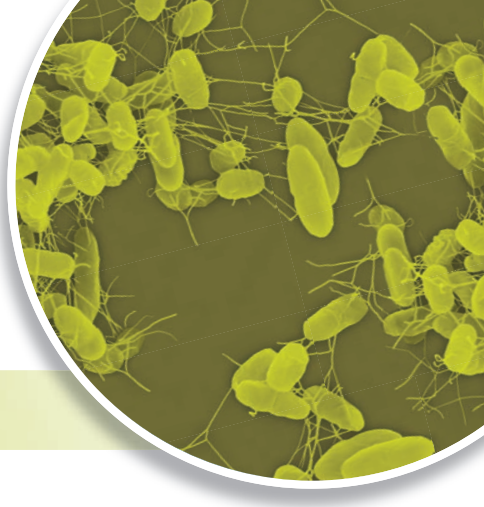


Sterility Test: Membrane Filtration

Filter appropriate number of product containers through 0.45µm filter

Validated volume of appropriate diluent through 0.45µm filter

Filter, then remove filter to appropriate recovery medium

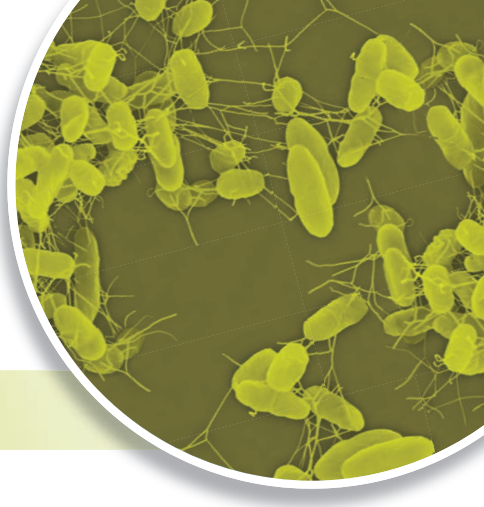
Incubate for growth for 14 days
Fluid Thioglycollate Media: 30–35°C
Soybean-Casein Digest Agar[Δ]: 20–25°C

Do the media show evidence of growth?

NO → Pass

YES → Fail

[Δ] *Soybean-Casein Digest Broth = Tryptic Soy Broth (TSB)*



Method Suitability Study Design

for Sterility Test: Membrane Filtration

Grow Cultures of

- *B. subtilis*
- *P. aeruginosa*
- *S. aureus*
- *C. albicans*
- *A. brasiliensis*
- *C. sporogenes*

From national stock cultures at 30–35°C in Fluid Thioglycollate Media for 3 days (anaerobic) or 20–25°C in Soybean-Casein Digest Broth[Δ] media for 3 days (bacteria) or 5 days (yeast & mold)

Filter appropriate number of product containers through 0.45µm filter

Filter two 100mL volumes of diluent through 0.45µm filter

Add a 100mL volume of diluent to filtration apparatus. Inoculate to <100 CFU.

Filter, then remove filter to appropriate recovery medium

Incubate for growth

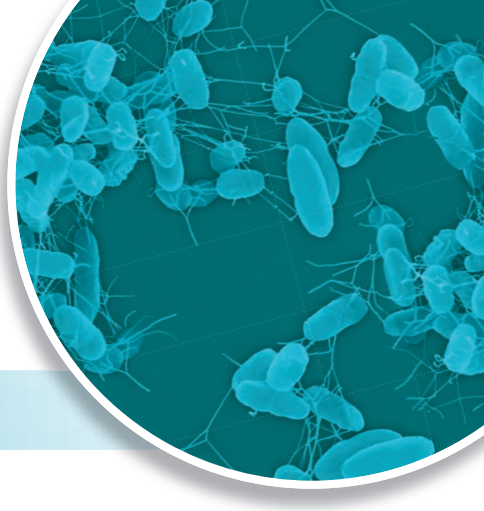
- Fluid Thioglycollate Media: *C. sporogenes, P. aeruginosa, S. aureus* at 30–35°C for 3 days
- Soybean-Casein Digest Agar[Δ]: *A. brasiliensis, B. subtilis, C. albicans* at 20–25°C for 3 days (bacteria) or 5 days (yeast & mold)

Do the media show evidence of growth?

NO → Pass

YES → Fail → Repeat, altering recovery conditions

[Δ] *Soybean-Casein Digest Broth = Tryptic Soy Broth (TSB)*



Sterility Test: Direct Transfer

Add validated amount of product to achieve an appropriate product:recover medium ratio. Test all required units of product in both Soybean-Casein Digest Broth[Δ] and Fluid Thioglycollate Media.

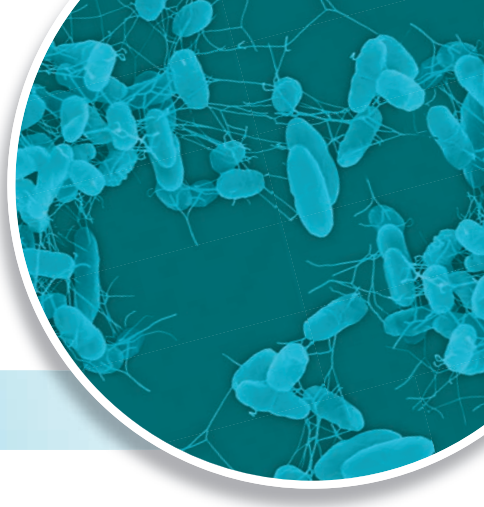
Incubate for growth 14 days
Fluid Thioglycollate Media: 30–35°C
Soybean-Casein Digest Broth[Δ]: 20–25°C

Do the media show evidence of growth?

NO → Pass

YES → Fail

[Δ] *Soybean-Casein Digest Broth = Tryptic Soy Broth (TSB)*



Method Suitability Study Design

for Sterility Test: Direct Transfer

Grow Cultures of

- *B. subtilis*
- *P. aeruginosa*
- *S. aureus*
- *C. albicans*
- *A. brasiliensis*
- *C. sporogenes*

From national stock cultures at 30–35°C in Fluid Thioglycollate Media for 3 days (anaerobic) or 20–25°C in Soybean-Casein Digest Broth[Δ] media for 3 days (bacteria) or 5 days (yeast & mold)

Add correct amount of product to achieve an appropriate product:recovery medium ratio

Inoculate to <100 CFU

Incubate for growth

- Fluid Thioglycollate Media: *C. sporogenes*, *P. aeruginosa*, *S. aureus* at 30–35°C for 3 days
- Soybean-Casein Digest Agar[Δ]: *A. brasiliensis*, *B. subtilis*, *C. albicans* at 20–25°C for 3 days (bacteria) or 5 days (yeast & mold)

Do the media show evidence of growth?

NO → Pass

YES → Fail → Repeat, altering recovery conditions

[Δ]*Soybean-Casein Digest Broth = Tryptic Soy Broth (TSB)*

Sterility Testing

Product	Type/Fill	Ref #
Fluid Thioglycollate Medium	10mL tube, 20/pk	R07174
	100mL bottle w/septum	R112646
	500mL bottle w/septum	R112642
	100mL serum bottle	R112641
	100mL wide mouth bottle	R112640
Fluid Thioglycollate Medium, Dehydrated	100g	R453451
	500g	R453452
	2.5kg	R453454
Tryptic Soy Broth (TSB)[Δ]	10mL tube, 20/pk	R117834
	100mL bottle w/septum	R112745
	500mL bottle w/septum	R112732
	1000mL bottle	R112740
	100mL serum bottle	R112731
	100mL wide mouth bottle	R112730
Vegetable Peptone Broth (VPB)	500g	VG0101B
	5kg	VG0101T
Cold Filterable TSB	500g	CM1065B
	5kg	CM1065T
Cold Filterable Vegetable Peptone Broth	500g	VG0104B
	5kg	VG0104T
Fluid A	300mL bottle w/septum	R112312
	1000mL polypropylene bottle	R112314
	100mL serum bottle	R112490
	300mL serum bottle	R112311
Fluid D	100mL bottle	R112323
	300mL bottle w/septum	R112322
	300mL serum bottle	R112321
	100mL wide mouth bottle	R112325
Fluid K	100mL serum bottle	R112332

Product	Type/Fill	Ref #
MicroTest™ M4®	3mL/tube, 12/pk	R12502
	3mL tube, 72/pk	R12500
MicroTest™ M5®	3mL tube, 12/pk	R12516
	3mL tube, 72/pk	R12515
MicroTest™ M6®	1.5mL tube, 12/pk	R12535
	1.5mL tube, 72/pk	R12530
10B Arginine Broth	1.8mL tube (15x45mm)	R20305
A-8 Agar	10/pk monoplate	R20205
A-8 Agar, selective	10/pk monoplate	R20204
SP4 Glucose Agar/Broth	10/pk monoplate	R20276
	100mL clear square bottle	R112585
PPLO Agar/Broth	10/pk monoplate	R20261
	15x60mm plate	R20260
	5mL tube	R20360
Mycoplasma Broth Base	500g	R454172
Mycoplasma Agar Base	500g	CM0401B
Mycoplasma Broth, Frey	500g	R454162
Staphylococcus aureus ATCC® 6538™*	10 tests/kit	R4737016
	100 tests/kit	R4717016
Bacillus subtilis ATCC® 6633™*	10 tests/kit	R4731221
	100 tests/kit	R4711221
Pseudomonas aeruginosa ATCC® 9027™*	10 tests/kit	R4735210
	100 tests/kit	R4715210
Clostridium sporogenes ATCC® 19404™*	100 tests/kit	R4711700
Candida albicans ATCC® 10231™*	10 tests/kit	R4731503
	100 tests/kit	R4711503
Aspergillus brasiliensis ATCC® 16404™*	10 tests/kit	R4731100
	100 tests/kit	R4711100

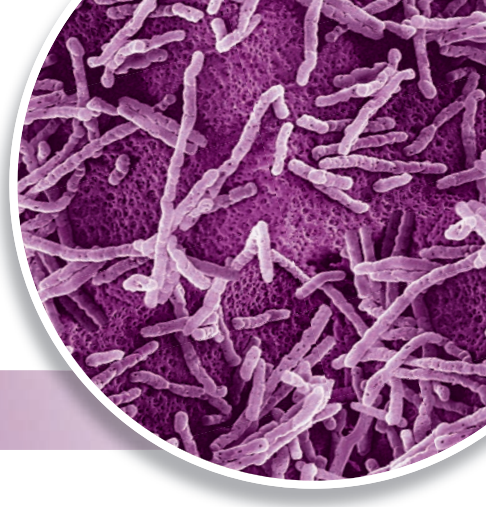
Category	Product	Type/Fill	Ref #
Contact Plates[†]	Sterile D/E Neutralizing Agar	10/pk, Double + bag	R111803
		100/pk, Double + bag	R111804
	Sterile Sabouraud Dextrose Agar w/Lecithin	10/pk, Double + bag	R111805
		100/pk, Double + bag	R111806
	Sterile Tryptic Soy Agar w/Lecithin, Polysorbate 80	10/pk, Double + bag	R111800
		100/pk, Double + bag	R111802
Settling Plates[†]	Sterile Tryptic Soy Agar	10/pk, Double + bag	R111870
		100/pk, Double + bag	R111872
Swabs	Sani-Cult™	5mL, 100/pk	R723141

	Product	Type/Fill	Ref #
Isolator Wrap™ Sterile Contact Plates	Sterile D/E Neutralizing Agar	10/pk, Foil barrier wrap + bag	R111823
		100/pk, Foil barrier wrap + bag	R111824
	Sterile Sabouraud Dextrose Agar w/Lecithin, Polysorbate 80	10/pk, Foil barrier wrap + bag	R111825
		100/pk, Foil barrier wrap + bag	R111826
	Sterile Tryptic Soy Agar w/Lecithin, Polysorbate 80	10/pk, Foil barrier wrap + bag	R111820
		100/pk, Foil barrier wrap + bag	R111822

[Δ] *Soybean-Casein Digest Broth = Tryptic Soy Broth (TSB)*

[†] *Not intended for IVD use*

Absence of Clostridium



1:10 dilution (≥1g PRODUCT) as in "Enumeration". Take 2 equal aliquots (≥1g PRODUCT).

Heat 80°C for 10 minutes; cool rapidly

Hold

Transfer 10mL of each to separate containers of 100mL Reinforced Clostridial Media

Incubate anaerobically at 30–35°C for 48 hours

Streak each culture onto Columbia Agar

Incubate anaerobically at 30–35°C for 48 hours

Anaerobic growth of rods?

NO

Pass

YES

Catalase positive?

YES

NO

Fail

Absence of Clostridium

Test	Medium	Property	Type/Fill	Ref #

Reinforced Clostridial Medium (RCM)

Hirsch and Grinstead developed Reinforced Clostridial Medium (RCM) for the cultivation and enumeration of clostridia. Reinforced Clostridial Medium MLT is a nonselective enrichment medium that supports the growth of various anaerobic and facultative bacteria when incubated anaerobically. It is formulated in conformance with harmonized United States Pharmacopeia (USP)/European Pharmacopeia (EP) guidelines for use in testing for the presence of *Clostridium* spp.

Mode of Action

Peptone and beef extract are sources of carbon, nitrogen, vitamins, and minerals essential for bacterial growth. Yeast extract supplies B-complex vitamins which stimulate bacterial growth. Dextrose is an energy source. Sodium chloride maintains osmotic equilibrium. Sodium acetate is a buffering agent. Starch acts as a protective colloid against toxic materials present in the medium. Cysteine hydrochloride is a reducing agent. A small amount of agar is added to impede the diffusion of oxygen.

Classical Formula

Beef extract	10.0g
Yeast extract	3.0g
Peptone	10.0g
Soluble starch	1.0g
Dextrose	5.0g
Cysteine hydrochloride	0.5g
Sodium chloride	5.0g
Agar	0.5g
Sodium acetate	3.0g
Demineralized water	1000.0mL

Columbia Agar (Base)

Columbia agar was developed by Ellner et al. at Columbia University. Prior to that time, traditional bases were made from either casein hydrolysate or meat infusion media. Ellner combined peptones from both animal and vegetable proteins, resulting in a base that supports the growth of both fastidious and nonfastidious organisms. Columbia Agar MLT is formulated in conformance with harmonized United States Pharmacopeia (USP)/ European Pharmacopeia (EP) guidelines for use in testing for the presence of *Clostridium* spp.

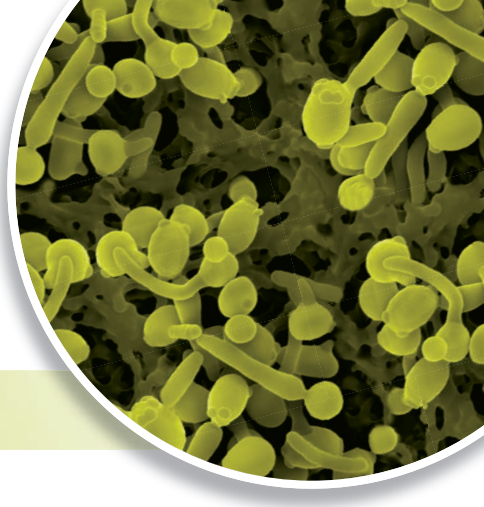
Mode of Action

Peptones supply growth factors such as nitrogen, carbon, vitamins, and trace elements essential for bacterial growth. Com starch serves as an energy source and yeast extract supplies B-complex vitamins.

Classical Formula

Pancreatic digest of casein	10.0g
Heart pancreatic digest	3.0g
Meat peptic digest	5.0g
Com starch	1.0g
Sodium chloride	5.0g
Agar	12.5g
Yeast extract	5.0g
Demineralized water	1000.0mL

Absence of Candida albicans



1:10 dilution (≥1g PRODUCT) as in "Enumeration". Inoculate ≥1g PRODUCT into 100mL Sabouraud Dextrose Broth.

Incubate at 30–35°C for 3–5 days

Streak culture on Sabauraud Dextrose Agar

Incubate at 30–35°C for 24–48 hours

Growth of white colonies?

NO

YES

Perform identification tests

Identity confirmed as *C. albicans*?

NO

YES

Pass

Fail

Absence of Candida albicans

Test	Medium	Property	Type/Fill	Ref #
Test for *Candida albicans*	Sabouraud Dextrose Broth	Nutritive for *C. albicans*	15x103mm tube, 5mL	R064410
	Sabouraud Dextrose Agar 5.6	Nutritive & indicative for *C. albicans*	500mL 500g	R112551 R454462

Sabouraud - 2% Dextrose Broth

Sabouraud Dextrose Broth was described by Sabouraud in 1892. Emmons modified Sabouraud's formulation by reducing the dextrose from 40g/L to 20g/L. Sabouraud Dextrose Broth (2%) is formulated in conformance with harmonized United States Pharmacopeia (USP)/European Pharmacopeia (EP) guidelines.

Mode of Action

Casein and meat peptones supply nitrogenous compounds and amino acids necessary for the growth of yeasts and fungi. Dextrose is a ready source of energy. The low pH of the medium is favorable to the growth of fungi, especially dermatophytes, while also inhibiting bacteria.

Classical Formula

Dextrose	20.0g
Casein peptone	5.0g
Meat peptone	5.0g
Demineralized water	1000.0mL

Sabouraud Dextrose Agar pH 5.6 w/ and w/o Chloramphenicol

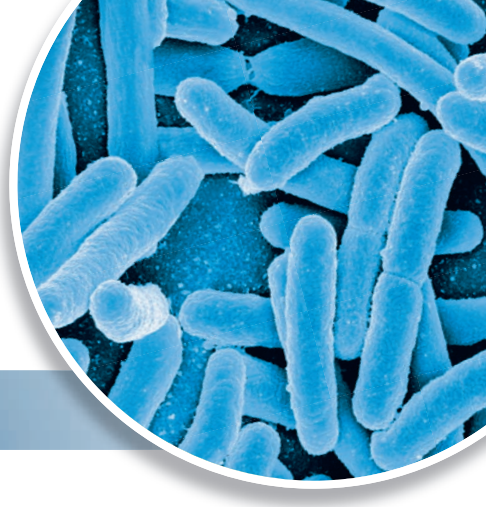
Sabouraud Dextrose Agar was developed by Sabouraud in 1892 for cultivation of dermatophytes. The low pH of 5.6 enhances the growth of fungi, especially dermatophytes, and is slightly inhibitory to bacteria in clinical specimens. This medium is recommended by the U.S. Pharmacopeia for mold and yeast counts. It is also recommended by the Association of Official Analytical Chemists (AOAC) and the American Public Health Association (APHA). The addition of chloramphenicol to the base agar makes the medium more selective.

Mode of Action

Casein and meat peptones provide nitrogen, amino acids, and peptides necessary for the growth of fungi. Dextrose is an energy source. Chloramphenicol is a selective agent which is inhibitory to most bacteria.

Classical Formula

Casein peptone	5.0g
Dextrose	40.0g
Meat peptone	5.0g
Agar	15.0g
Demineralized water	1000.0mL



Absence of Escherichia coli

1:10 dilution (≥1g PRODUCT) as in "Enumeration", add equivalent to 1g to Soybean-Casein Digest Broth[Δ]

↓

Incubate at 30–35°C for 18–24 hours

↓

Subculture 1mL to 100mL MacConkey Broth

↓

Incubate at 42–44°C for 24–48 hours

↓

Subculture on MacConkey Agar

↓

Incubate at 30–35°C for 18–72 hours

↓

Any growth?

NO ← Pass

↓ **YES**

Identity confirmed as *E. coli*?

NO ← Pass

↓ **YES**

Fail

[Δ]*Soybean-Casein Digest Broth = Tryptic Soy Broth (TSB)*

Absence of Escherichia coli

This is a Gram-negative bacterium and is an indicator for fecal contamination. Such contamination could arise from poor hygiene of operators, contamination from feral animals, cats, birds or a low quality water supply amongst others. *Escherichia coli* is capable of causing diarrhea and sickness and some strains are capable of producing a potent verotoxin.

Test	Medium	Property	Type/Fill	Ref #
Test for *Escherichia coli*	MacConkey Broth	Nutritive for *E. coli* & selective for *S. aureus*	15x103mm tube, 5mL 500g	R061336 R453822
	MacConkey Agar	Nutritive & indicative for *E. coli*	Monoplate 500g	R01550 R453802

MacConkey Broth

MacConkey Broth is a modification of the original bile salt broth first described by MacConkey in 1900, which contained litmus as an indicator and sodium taurocholate to inhibit Gram-positive organisms. Oxgall in the medium serves to inhibit growth of Gram-positive organisms and replace sodium taurocholate used in the original formulation. MacConkey Broth is formulated in conformance with harmonized United States Pharmacopeia (USP)/European Pharmacopeia (EP) guidelines.

Mode of Action

Peptone provides nitrogenous compounds and amino acids necessary for bacterial growth. Lactose is a carbon source for energy. The selective agent, oxgall, inhibits most Gram-positive organisms. Lactose and bromcresol purple indicator enable the differentiation of lactose-fermenting Gram-negative bacilli. Lactose-fermenters (i.e. coliforms) cause the medium to change from purple to yellow and produce gas bubbles.

Classical Formula

Gelatin peptone	20.0g
Oxgall	5.0g
Lactose	10.0g
Bromcresol purple	0.01g
Demineralized water	1000.0mL

MacConkey Agar

In 1900, MacConkey first described a neutral red bile salt medium for cultivation and identification of enteric organisms. A detailed description of the selective and differential properties of the medium was published in 1905. Over the years, MacConkey's original formula has been modified; the agar content has been reduced, the concentration of bile salts and neutral red has been adjusted, and sodium chloride has been added. The modification of MacConkey Agar which resulted has demonstrated improved inhibition of swarming by *Proteus* spp.

Mode of Action

Peptones provide nitrogenous nutrients and amino acids necessary for bacterial growth. Lactose is a carbon source for energy. Sodium chloride supplies essential electrolytes and maintains osmotic equilibrium. Crystal violet and bile salts are selective agents which inhibit most Gram-positive organisms. Differentiation of Gram-negative bacilli is accomplished by addition of lactose and neutral red which is an indicator.

Classical Formula

Gelatin peptone	17.0g
Meat peptone	1.5g
Lactose	10.0g
Neutral red	30.0mg
Sodium chloride	5.0g
Crystal violet	1.0mg
Bile salts	1.5g
Agar	13.5g
Casein peptone	1.5g
Demineralized water	1000.0mL

Absence of Salmonella

1:10 dilution (≥1g PRODUCT) as in "Enumeration", add equivalent to 1g to Soybean-Casein Digest Broth[Δ]

Incubate at 30–35°C for 18–24 hours

Subculture 0.1mL to 10mL Rappaport Vassiladis Salmonella Enrichment Broth

Incubate at 30–35°C for 18–48 hours

Subculture on Xylose-Lysine-Desoxycholate Agar

Incubate at 30–35°C for 18–48 hours

Any well-developed, red colonies?

NO → Pass

YES

Confirmed as *Salmonella* with biochemical tests?

NO → Pass

YES

Fail

[Δ] *Soybean-Casein Digest Broth = Tryptic Soy Broth (TSB)*

Absence of Salmonella

This microorganism is of fecal origin and can cause severe diarrhea and sickness with the resultant dehydration potentially fatal in children and the elderly who are at greatest risk.

Test	Medium	Property	Type/Fill	Ref #
Test for *Salmonella enterica* spp. Typhimurium	Rappaport Vassiliadis Salmonella Enrichment	Broth Nutritive for *S. enterica* spp. Typhimurium	500g	R455432
	XLD Agar	Nutritive & indicative for *S. enterica* spp. Typhimurium	Monoplate 500g	R01980 R459902

RVS (Rappaport-Vassiliadis Salmonella) Enrichment Broth MLT

Rappaport et al. formulated an enrichment medium for selective recovery of *Salmonella* spp. Vassiliadis et al. modified the formula by reducing the concentration of malachite green and magnesium chloride, creating Rappaport-Vassiliadis (RV) Broth. RVS Enrichment Broth MLT is formulated in conformance with harmonized United States Pharmacopeia (USP)/ European Pharmacopeia (EP) guidelines for use in testing for the presence of *Salmonella* spp.

Mode of Action

Soya Peptone is the source of carbon and nitrogen, magnesium chloride raises the osmotic pressure, and potassium dihydrogen phosphate acts as a buffer. Malachite green is a selective agent which is inhibitory to organisms other than *Salmonella* spp. The low pH of RVS Enrichment Broth MLT combined with the presence of malachite green and magnesium chloride creates an environment which facilitates selective recovery of *Salmonella* spp. from contaminated sources.

Lowering the pH to 5.2 increases selectivity.

Classical Formula

Magnesium chloride	13.6g
Potassium dihydrogen phosphate	0.6g
Sodium chloride	5.0g
Dipotassium phosphate	0.4g
Soya peptone	4.5g
Malachite green	36.0mg
Demineralized water	1000.0mL

XLD Agar (Xylose Lysine Desoxycholate)

This medium was developed by Taylor for selective isolation and differentiation of enteric pathogens, especially *Shigella.*

XLD Agar has since been found to be a satisfactory medium for the recovery of *Salmonella* spp. from clinical specimens.

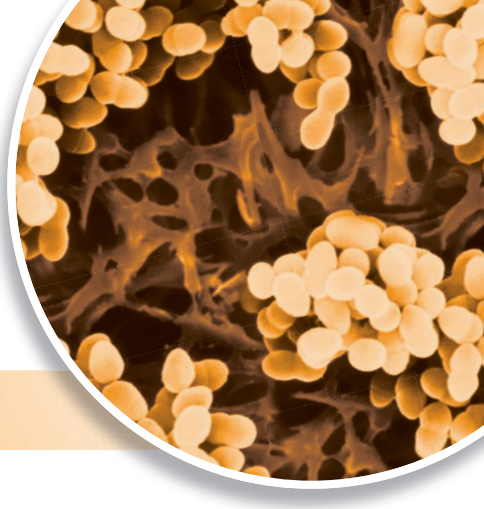
Mode of Action

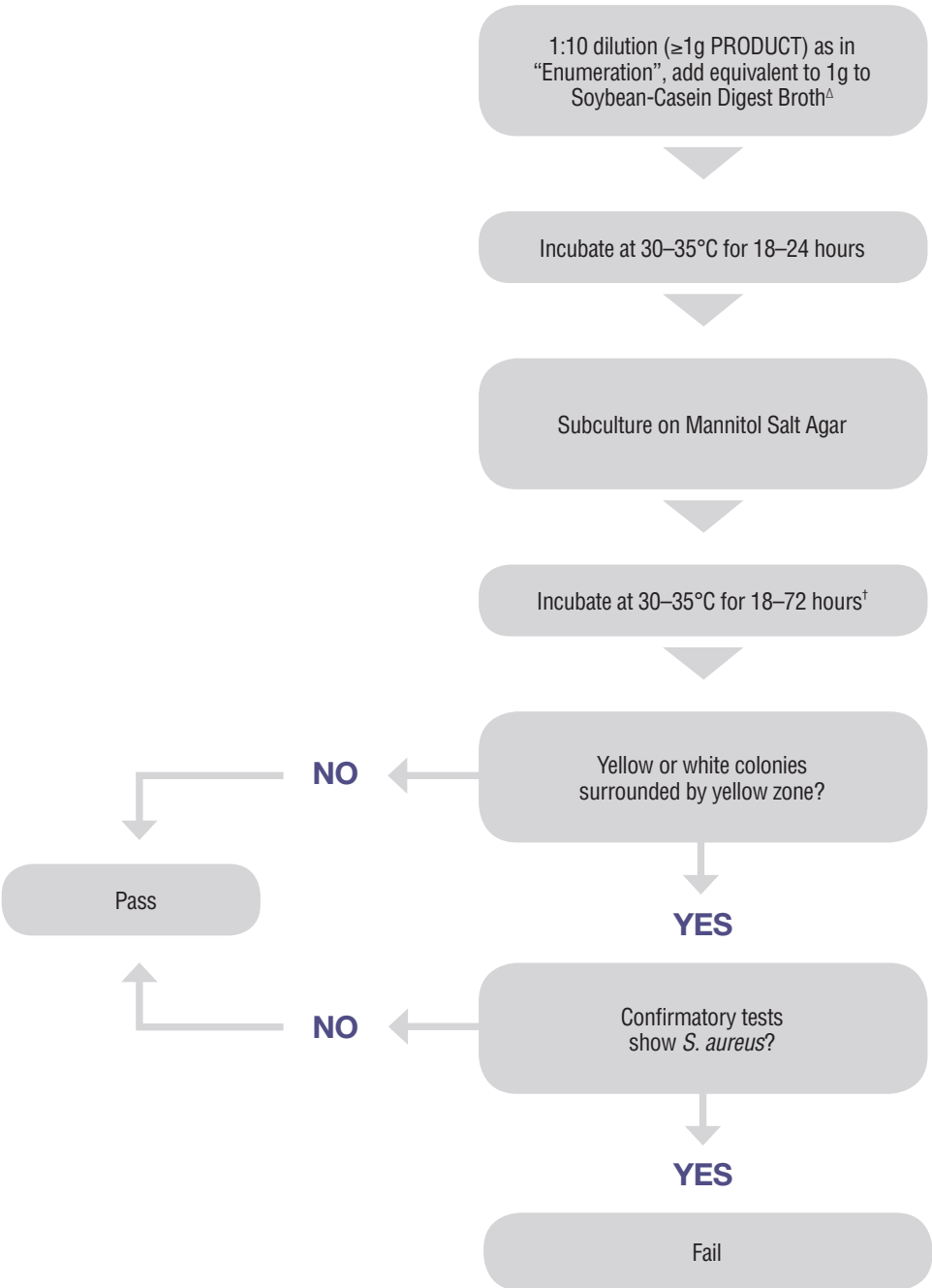
Xylose is rapidly fermented by most enteric Gram-negative bacilli other than *Shigella* spp., producing red colonies. Lysine provides for differentiation of *Salmonella* spp. from nonpathogenic enteric Gram-negative bacilli. *Salmonella* produces lysine decarboxylase which causes the pH to revert to alkaline after xylose is fermented, producing red colonies. Sodium thiosulfate, a sulfur source, and ferric ammonium citrate, an indicator, are added to enable organisms which form hydrogen sulfide (H,S) to produce black-centered colonies under alkaline conditions. Such organisms include *Salmonella* spp. Organisms which ferment xylose, lactose, or sucrose and are lysine negative cause an acid pH and produce yellow colonies. Desoxycholate is a selective agent which inhibits Gram-positive organisms.

Classical Formula

Lactose	7.5g
Yeast extract	3.0g
Sucrose	7.5g
Sodium desoxycholate	2.5g
Sodium thiosulfate	6.8g
Ferric ammonium citrate	0.8g
L-lysine	5.0g
Phenol red	0.08g
Sodium chloride	5.0g
Agar	13.5g
Xylose	3.5g
Demineralized water	1000.0mL

Absence of Staphylococcus aureus





[Δ] *Soybean-Casein Digest Broth = Tryptic Soy Broth (TSB)*

[†] *Incubation conditions not defined in USP. JP instructs incubation at 30–35°C for 24–48 hours for all plate incubations.*

Absence of Staphylococcus aureus

Staphylococcus aureus is a human skin commensal and, if present, is highly likely to be associated with operator-associated contamination. It is undesirable as at high levels (10^5 cfu/g) it is capable of producing an endotoxin. The toxin is heat stable and can cause severe effects, such as stomach cramps and severe vomiting. Dehydration may also be a problem. *Staphylococcus aureus* is an opportunistic pathogen and can cause severe systemic infections, such as meningitis. It can also be the infective agent for skin lesions and can cause spots and boils. Although not as severe as *Salmonella* spp., the effects are undesirable consequences from the ingestion of a medicinal product.

Test	Medium	Property	Type/Fill	Ref #
Test for *Staphylococcus aureus*	Mannitol Salt Agar	Nutritive for *S. aureus* & selective for *E. coli*	Monoplate 500g	R01580 R453902

Mannitol Salt Phenol-red Agar

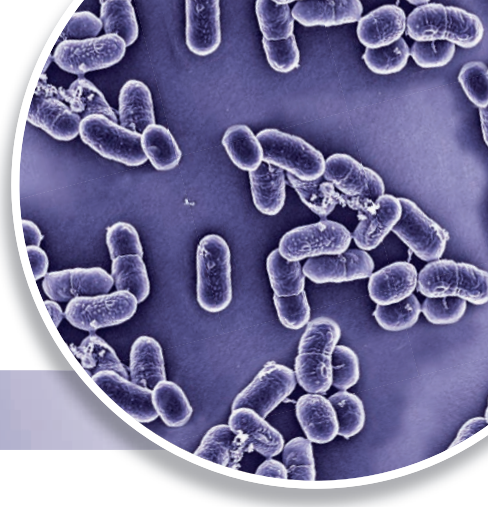
In 1942, Koch reported the use of 7.5% sodium chloride as a selective agent for the isolation of staphylococci. Chapman confirmed the results of Koch and suggested the addition of 7.5% sodium chloride to phenol-red mannitol agar. Most strains of coagulase-positive staphylococci grow on Mannitol Salt Agar, producing yellow zones as a result of mannitol fermentation. Coagulase-negative strains of staphylococci produce small colonies with red-colored zones in the surrounding medium.

Mode of Action

Casein and meat peptones supply nitrogen, amino acids, and peptides necessary for bacterial growth. Sodium chloride in a concentration of 7.5% is a selective agent which inhibits many bacteria other than staphylococci. Phenol red is a pH indicator which causes a color change in the medium from red-orange to yellow when acid is produced. Staphylococci colonies that ferment mannitol will be surrounded by a yellow zone, while those that do not ferment mannitol will have a red zone.

Classical Formula

Sodium chloride	75.0g
Beef extract	1.0g
D-mannitol	10.0g
Phenol red	25.0mg
Casein peptone	5.0g
Agar	15.0g
Meat peptone	5.0g
Demineralized water	1000.0mL



Absence of Bile-tolerant Gram-negative Bacteria

1:10 dilution (≥1g PRODUCT) as in "Enumeration", add equivalent to 1g to Soybean-Casein Digest Broth[Δ]

↓

Incubate at 20–25°C for 2–5 hours

↓

≥1g PRODUCT into Enterobacteria Enrichment Broth - Mossel

↓

Incubate at 30–35°C for 24–48 hours

↓

Subculture on Violet Red Bile Glucose Agar

↓

Any growth?

NO → Pass

YES

↓

Fail

[Δ] *Soybean-Casein Digest Broth = Tryptic Soy Broth (TSB)*

Absence of Bile-tolerant Gram-negative Bacteria

These microorganisms are usually associated with aqueous environments and are indicators of poor hygiene or poor water quality. These organisms are of concern as many species are opportunistic pathogens or may cause spoilage of the product.

Test	Medium	Property	Type/Fill	Ref #
Test for Bile-tolerant Gram-negative bacteria	Mossel Enterobacter Enrichment Broth	Nutritive for *E. coli* & *P. aeruginosa;* selective for *S. aureus*	100mL Bottle 500g	R112281 R453332
	Violet Red Bile Glucose Agar	Nutritive & indicative for *E. coli* & *P. aeruginosa*	15x100mm Plate 500g	R110097 R455302

EE Broth Mossel

EE Broth Mossel is a modification of brilliant green bile broth, which contains purified ox bile (oxgall), in place of bile salts, and disodium phosphate to improve the buffering capacity of the medium and encourage early growth of indicator organisms. EE Broth Mossel is recommended by the United States Pharmacopeia (USP) for use in testing for the presence of bile-tolerant, Gram-negative bacteria by the American Public Health Association for use in the most probable number assay, and as an enrichment broth in straight enrichment procedures. EE Broth Mossel is formulated in conformance with harmonized United States Pharmacopeia (USP)/European Pharmacopeia (EP) guidelines.

Mode of Action

The undesired, accompanying bacterial flora is almost completely inhibited by brilliant green and ox bile. Dextrose favors the growth of all Enterobacteriaceae. The strong buffering capacity of the culture medium prevents the formed acid from killing the culture.

Classical Formula

Oxgall	20.0g
Peptone	10.0g
Disodium phosphate	8.0g
Dextrose	5.0g
Monopotassium phosphate	2.0g
Brilliant green	15.0mg
Demineralized water	1000.0mL

VRBG Agar (Violet Red Bile Glucose Agar)

Mossel et at. modified Violet Red Bile Agar by adding glucose to enable detection of nonlactose-fermenting Enterobacteriaceae. Further research demonstrated that lactose could be omitted, resulting in the formulation known as Violet Red Bile Glucose Agar (VRBGA). VRBGA is formulated in conformance with harmonized United States Pharmacopeia (USP)/ European Pharmacopeia (EP) guidelines for use in testing for the presence of bile-tolerant, Gram-negative bacilli.

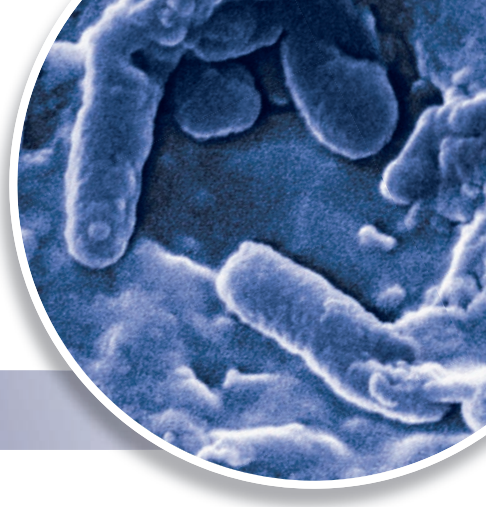
Mode of Action

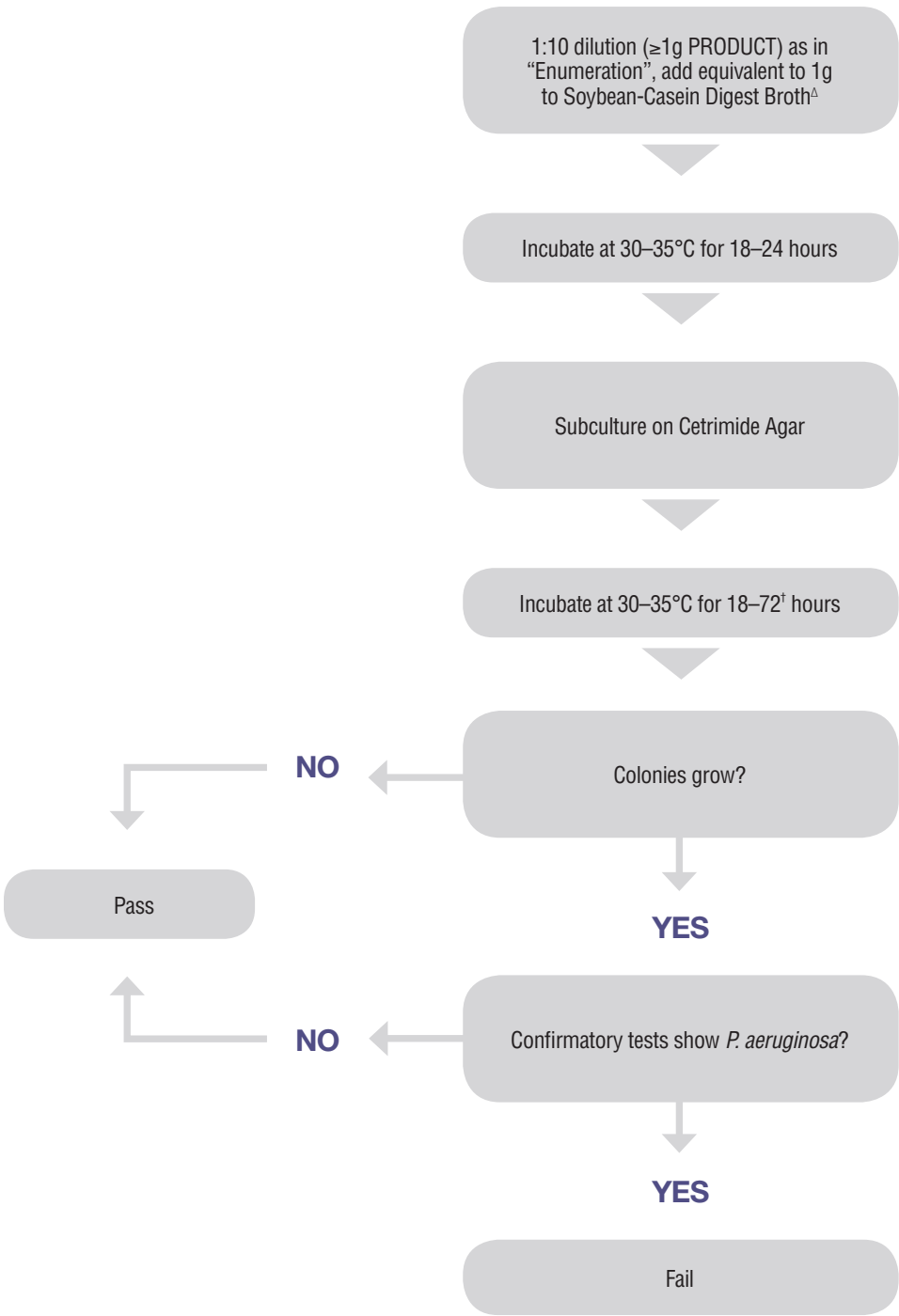
Gelatin peptone supplies amino acids, peptides, and nitrogenous compounds essential for bacterial growth. Yeast extract provides essential B-complex vitamins and glucose is a carbon energy source. Sodium chloride maintains osmotic equilibrium. Bile salts and crystal violet are selective agents which inhibit the growth of Gram-positive organisms. Neutral red is an indicator of acid production. Gram-negative organisms which ferment glucose form colonies that are pink to red in color.

Classical Formula

Glucose	10.0g
Bile salts	1.5g
Gelatin peptone	7.0g
Neutral red	30.0mg
Sodium chloride	5.0g
Crystal violet	2.0mg
Yeast extract	3.0g
Agar	15.0g
Demineralized water	1000.0mL

Absence of Pseudomonas aeruginosa





[Δ] *Soybean-Casein Digest Broth = Tryptic Soy Broth (TSB)*

[†] *Incubation conditions not defined in USP. JP instructs incubation at 30–35°C for 24–48 hours for all plate incubations.*

Absence of Pseudomonas aeruginosa

As a Gram-negative microorganism, *Pseudomonas aeruginosa* is usually associated with water contamination. It is an opportunistic pathogen and has been linked with severe infections in the eye and wounds caused by burns. It is also very adaptable to its environment and is known to be able to develop resistance to some disinfectants.

Test	Medium	Property	Type/Fill	Ref #
Test for *Pseudomonas aeruginosa*	Cetrimide Agar	Nutritive for *P. aeruginosa* & selective for *E. coli*	Monoplate 500g	R01292 R452802

Cetrimide Agar

(Pseudomonas Selective Agar, Base)

This medium complies with the recommendations of the harmonized method in the European Pharmacopeia 6.0 and the United States Pharmacopeia 29 (2006).

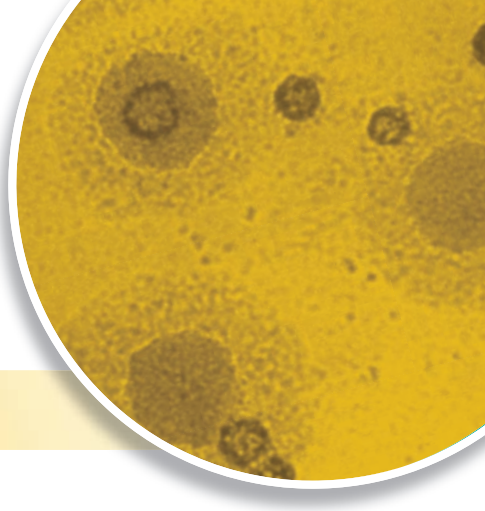
Mode of Action

The use of cetrimide (cetyltrimethylammonium bromide) was recommended by Lowbury (1951) and other authors; this compound largely inhibits the growth of the accompanying microbial flora. According to Lowbury and Collins (1955), a concentration of 0.3g/L inhibits the accompanying organisms satisfactorily and minimizes interference with the growth of *P. aeruginosa*. The pigment production of *P. aeruginosa* is not inhibited when grown on this medium. Goto and Enomoto (1970) recommended the addition of 15µg/mL nalidixic acid to improve the inhibition of the accompanying microbial flora.

Classical Formula (g / litre)

Peptone from gelatin	20.0
Magnesium chloride	1.4
Potassium sulfate	10.0
N-cetyl-N,N, N-trimethylammoniumbromide (cetrimide)	0.3
Agar-agar	13.6
Also to be added:	
Glycerol	10mL

Routine Mycoplasma Testing



Two testing methods:

Direct Cultivation Method

- 10mL of product inoculated into mycoplasma media
- Incubate media in tightly stoppered containers at 36 ± 1°C
- Transfer media to agar plates in duplicate on day 3. Examine plates microscopically at day 17.
- Transfer media to agar plates in duplicate on day 7. Examine plates microscopically at day 21.
- Transfer media to agar plates in duplicate on day 14. Examine plates microscopically at day 28.
- Transfer media to agar plates in duplicate on day 21. Examine plates microscopically at day 28.

Cell Culture Method

- 1mL of product inoculated into VERO cell plates in duplicate
- Incubate plates 3–5 days
- Harvest and put onto coverslips
- Incubate plates 3–5 days
- Coverslips are stained with DNA Fluorochrome stain

Control plates utilizing Mycoplasma orale and Mycoplasma pneumoniae are prepared alongside the test products.

The test passes if Mycoplasma is not detected using the direct and cell culture methods. The controls must exhibit growth when inoculated with <100 CFU per organism. All negative controls must be free of Mycoplasma contamination.

Routine Mycoplasma Testing

Mycoplasmas are a significant problem for the biopharmaceutical industry, because they are a very common cause of contamination in cell cultures used in research laboratories and in industrial processes. The bacteria thrive in cell culture media and can reach very high numbers without causing visible changes in the culture, or affecting its viability, and so remain undetected.

The purpose of the test is to determine the presence or absence of mycoplasmal contaminants in the cell cultures samples.

The FDA requires that both assays are performed in order to ensure a high degree of certainty in confirming the presence or absence of *Mycoplasma* contamination.

The testing challenges consist of the two methods on the previous page.

Test	Medium	Type/Fill	Ref #
PPLO Broth	w/horse serum, yeast extract. For cultivation of *Mycoplasma* spp.	15x103mm tube, 5mL 20/pk	R20360
PPLO Agar	w/horse serum, amphotericin B, penicillin, thallium acetate. For selective isolation of *Mycoplasma* spp.	15x60mm plate 10/pk	R20260
SP4 Glucose Broth	w/thallium acetate, penicillin. For isolation of *Mycoplasma* spp. Penicillin inhibits growth of Gram-positive bacteria. REF R112585 not intended for IVD use.	15x103mm tube, 5mL 20/pk	R20376
SP4 Glucose Agar	w/thallium acetate, penicillin. For isolation of *Mycoplasma* spp. Penicillin inhibits growth of Gram-positive bacteria.	15x60mm plate 10/pk	R20276

Table 1. Methodological differences between the new USP chapter <63>, EP 2.6.7, and the 1993 PTC

Requirements	USP<63>	EP 2.6.7	1993 PTC
Nutritive properties	The solid medium complies with the test if a count within 0.5-log unit range of the inoculate amount is found for each test microorganism	The solid medium complies with the test if growth obtained does not for differ by a factor >5 from value calculated with respect to the inoculum	Not addressed; reference made to the 21 CFR 610.30
Inhibitory substances	If plates inoculated with the test article/material are not within a 0.5-log unit range of the number of colonies of those without, inhibitory substances are present	If plates inoculated with the product to be examined have fewer than 1/5 of the number or colonies of those inoculated without the product, inhibitory substances are present	Not addressed
Quality Control strain organisms	At least two *Mycoplasma* species should be included as positive controls (one dextrose fermenter and one arginine hydrolyzer)	At lease one of the species listed will be included as a positive control	At least two *Mycoplasma* species should be included as positive controls (one dextrose fermenter and one arginine hydrolyzer)
Quality Control strain organisms storage	Stored frozen (at -20°C or lower) or freeze-dried	Stored frozen or freeze-dried	Not addressed
Number of subcultures	Positive control organisms to be used not more than 15 passages from isolation	Positive control organisms to be used not more than 15 subcultures from isolation	Positive control organisms to be used not more than 15 passages from isolation
Incubation conditions	Tightly stoppered containers at 36 ± 1°C	Tightly stoppered containers at 35–38°C	36 ± 1°C

Notes

Notes

Notes

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