Media

Brain-heart infusion agar medium or Fluid brain-heart infusion medium

Bovine brain extract powder*1	An amount equivalent to 200 g of calf brain
Bovine heart extract powder*2	An amount equivalent to 250 g of the material
Peptone	10.0 g
Glucose	2.0 g
Sodium chloride	5.0 g
Disodium hydrogenphosphate 12-water	2.5 g
Agar	15.0 g
Water	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.2 - 7.6.

Fluid cooked meat medium

Bovine heart extract powder*2	An amount equivalent to 450 g of the material
Peptone	20.0 g
Glucose	2.0 g
Sodium chloride	5.0 g
Water	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.2 - 7.6.

Glucose peptone agar medium or Fluid glucose peptone medium

See Microbial Limit Test. Antibiotic is added if necessary.

Nutrient agar medium or Fluid nutrient medium

Meat extract	3.0 g
Peptone	5.0 g
Agar	15.0 g
Water	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 6.6 - 7.0.

Potato-dextrose agar medium or Fluid potato-dextrose medium

See Microbial Limit Test. Antibiotic is added if necessary.

Reinforced clostridial agar medium or Fluid reinforced clostridial medium

Meat extract	10.0 g
Peptone	10.0 g
Yeast extract	3.0 g
Soluble starch	1.0 g
Glucose	5.0 g
L-Cystein hydrochloride monohydrate	0.5 g
Sodium chloride	5.0 g
Sodium acetate trihydrate	3.0 g
Agar	15.0 g
Water	1,000 mL

For fluid medium, add 0.5 g of agar. Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 6.7 - 6.9.

Sabouraud dextrose agar medium or Fluid sabouraud dextrose medium

See Microbial Limit Test. Antibiotic is added if necessary.

Soybean-casein digest agar medium or Fluid soybean-casein digest medium

See Microbial Limit Test.

Thioglycolate agar medium or thioglycolate medium I for sterility test

See Sterility Test. The agar concentration of Thioglycolate agar medium is about 1.5%.

*1 **Bovine brain extract powder** Dried extract of bovine fresh brain. A yellow-brown powder having a characteristic odor.

Loss on drying: not more than 5%.

*2 **Bovine heart extract powder** Dried extract of bovine fresh heart. A yellow-brown powder having a characteristic odor.

Loss on drying: not more than 5%.

Rinsing Liquids

Buffered sodium chloride-peptone solution (pH 7.0)

See Microbial Limit Test.

LP liquid

Casein peptone	1.0 g
Soybean lecithin	0.7 g
Polysorbate 80	1.0 - 20.0 g
Water	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.2.

Phosphate buffered solution (pH 7.2)

See Microbial Limit Test.

Ringer's solution, 1/4 concentration

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride dihydrate	0.16 g
Water	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 7.0.

Thiosulfate-Ringer's solution

Sodium thiosulfate pentahydrate	0.8 g
Ringer's solution, 1/4 concentration	1,000 mL

Sterilize by heating in an autoclave at 121°C for 15 to 20 min. pH after sterilization: 6.6.

9. Mycoplasma Testing for Cell Substrates used for the Production of Biotechnological/ Biological Products

This document describes the currently available methods of mycoplasma testing that should be performed for cell substrates that are used in the manufacture of biotechnological/biological products.

Methods suggested for detection of mycoplasma are, A. culture method, B. indicator cell culture method, and C.

polymerase chain reaction (PCR) method.

Mycoplasma testing should be performed on the master cell bank (MCB) and the working cell bank (WCB), as well as on the cell cultures used during the manufacturing process of the product. For the assessment of these cells, mycoplasma testing should be performed using both methods A and B. Method B, however, does not detect only DNA derived from mycoplasma. Therefore, if a positive result is obtained only from method B, method C can be used to determine whether mycoplasma is actually present. When method C is used, it is necessary to demonstrate the rationale for determining a negative result. In such a case, the sensitivity and specificity of the method, the appropriateness of the sample preparation, and the suitability of the selection of the test method, including selection of reagents, reaction conditions and primers should be taken into account.

Prior to mycoplasma testing, the sample should be tested to detect the presence of any factors inhibiting the growth of mycoplasma. If such growth-inhibiting factors are detected they should be neutralized or eliminated by an appropriate method, such as centrifugation or cell passage.

If the test will be performed within 24 hours of obtaining the sample, the sample should be stored at a temperature between 2°C and 8°C. If more than 24 hours will elapse before the test is performed, the sample should be stored at −60°C or lower.

If mycoplasma is detected, additional testing to identify the species may be helpful in determining the source of contamination.

A. Culture Method

1. Culture Medium

Both agar plates and broth are used. Each lot of agar and broth medium should be free of antibiotics except for penicillin. Refer to the Minimum Requirements for Biological Products regarding selection of the culture media. Other culture media may be used if they fulfill the requirements described in the following section 2.

2. Suitability of Culture Medium

Each lot of medium should be examined for mycoplasma growth-promoting properties. To demonstrate the capacity of the media to detect known mycoplasma, each test should include control cultures of at least two known species or strains of mycoplasma, one of which should be a dextrose fermenter (i.e., *M. pneumoniae* strain FH or equivalent species or strains) and one of which should be an arginine hydrolyser (i.e., *M. orale* CH 19299 or equivalent species or strains). The mycoplasma strains used for the positive control tests should be obtained from an official or suitably accredited agency, and handled appropriately. Inoculate the culture medium with no more than 100 colony-forming units (CFU).

3. Culture and Observation

1) Inoculate no less than 0.2 mL of test sample (cell suspension) in evenly distributed amounts over the surface of each of four or more agar plates. After the surfaces of the inoculated plates are dried, one half of the plates should be incubated under aerobic conditions in an atmosphere of air containing 5 to 10 percent carbon dioxide and adequate humidity, and the other half under anaerobic conditions in an atmosphere of nitrogen containing 5 to 10 percent carbon dioxide and adequate humidity at 36 ± 1°C for no less than 14 days.

2) Inoculate no less than 10 mL of the test sample (cell suspension) into each of two vessels containing 100 mL of broth medium. One vessel should be incubated under aerobic conditions, and the other under anaerobic conditions.

If the culture medium for the sample cells contains any growth-inhibiting factors, such as antibiotics, these factors must be removed. A method such as centrifugation is recommended for this purpose.

3) Subculture 0.2 mL of broth culture from each vessel on the 3rd, 7th, and 14th days of incubation onto two or more agar plates. The plates inoculated with aerobic or anaerobic broth cultures should be incubated aerobically or anaerobically, respectively at 36 ± 1°C for no less than 14 days.

4) Examination of all plates for mycoplasma colonies should be done microscopically on the 7th and 14th day at 100 times magnification or greater.

B. Indicator Cell Culture Method

Using Vero cell culture substrate, pretest the suitability of the method using an inoculum of no more than 100 CFU of *M. hyorhinis* DBS 1050 or *M. orale* CH 19299.

An equivalent indicator cell substrate and suitable mycoplasma strains may be acceptable if data demonstrate at least equal sensitivity for the detection of known mycoplasma contaminants. The mycoplasma strains should be obtained from an official or suitably accredited agency, and handled appropriately. The cell substrate used should be obtained from a qualified cell bank and certified to be mycoplasma free. The acquired cells should be carefully cultured and propagated, and sufficient volumes of seed stock should be prepared with the proper precautions to avoid mycoplasma contamination. The stock should be tested for mycoplasma contamination using at least one of the methods described in this document, then frozen for storage. For each test a new container from the stock should be thawed and used within 6 passages.

Indicator cell cultures should be grown on cover slips submerged in culture dishes or equivalent containers for one day. Inoculate no less than 1 mL of the test sample (cell culture supernatant) into two or more of the culture dishes.

The test should include a negative (non-infected) control and two positive mycoplasma controls, such as *M. hyorhinis* DBS 1050 or *M. orale* CH 19299. Use an inoculum of no more than 100 CFU for the positive controls.

Incubate the cell cultures for 3 to 6 days at 36 ± 1°C in an atmosphere of air containing 5 percent carbon dioxide.

Examine the cell cultures after fixation for the presence of mycoplasma by epifluorescence microscopy (400 to 600 times magnification or greater) using a DNA-binding fluorochrome, such as bisbenzimidazole or an equivalent stain. Compare the microscopical appearance of the test cultures with that of the negative and positive controls.

Procedure

1) Aseptically place a sterilized glass cover slip into each cell culture dish (35 mm diameter).

2) Prepare Vero cell suspension in Eagle's minimum essential medium containing 10 percent bovine calf serum at a concentration of 1×10^4 cells per 1 mL. The bovine calf serum should be tested and confirmed to be free from mycoplasma prior to use.

3) Inoculate aliquots of 2 mL of the Vero cell suspension into each culture dish. Ensure that the cover slips are completely submerged, and not floating on the surface of the culture medium. Incubate the cultures at 36 ± 1°C in an

atmosphere of air containing 5 percent carbon dioxide for one day, so that the cells are attached to the glass cover slip.

4) Replace 2 mL of the culture medium with fresh medium, then add 0.5 mL of the test sample (cell culture supernatant) to each of two or more culture dishes. Perform the same procedure for the positive (2 types of mycoplasma, such as *M. hyorhinis* and *M. orale*) and negative controls.

5) Incubate the cultures for 3 to 6 days at 36 ± 1°C in an atmosphere of air containing 5 percent carbon dioxide.

6) Remove the culture medium from the culture dishes, and add 2 mL of a mixture of acetic acid (100) and methanol (1:3) (fixative) to each dish; then, allow them to stand for 5 minutes.

7) Remove the fixative from each dish, then add the same amount of fixative again, and leave the dishes to stand for 10 minutes.

8) Remove the fixative and then completely air-dry all the dishes.

9) Add 2 mL of bisbenzamide fluorochrome staining solution to each culture dish. Cover the dishes and let them stand at room temperature for 30 minutes.

10) Aspirate the staining solution and rinse each dish with 2 mL of distilled water 3 times. Take out the glass cover slips and dry them.

11) Mount each cover slip with a drop of a mounting fluid. Blot off surplus mounting fluid from the edges of the cover slips.

12) Examine by epifluorescence microscopy at 400 to 600 times magnification or greater.

13) Compare the microscopic appearance of the test sample with that of the negative and positive controls.

14) The test result is judged to be positive if there are more than 5 cells per 1000 (0.5%) that have minute fluorescent spots that appear to surround, but are outside, the cell nucleus.

C. Polymerase Chain Reaction (PCR) Detection Method

The PCR method is a highly specific method that enables the detection of trace amounts of mycoplasma DNA, and has come to be widely used in recent years as a means of detecting mycoplasma contamination. However, the sensitivity and specificity depend on the procedure employed, and a positive result from PCR does not always indicate the presence of viable mycoplasma.

The PCR method is based on amplifying DNA extracted from the cell culture with specific primers so that the presence of the target DNA is detected. A two-step PCR (nested PCR) is recommended in order to increase sensitivity and specificity. The tests should include both a positive control (such as *M. hyorhinis* of 100 CFU or less) and a negative control.

Mycoplasma DNA from the sample of cells or cell cultures is amplified using primers which should be able to amplify some common conserved mycoplasma DNA sequence. The amplification should be performed using an appropriate heat-resistant DNA polymerase, and suitable conditions. The amplified DNA can be identified after agarose gel electrophoresis, followed by ethidium bromide staining and UV irradiation of the gel.

For this method, it is important to use primers that are specific to mycoplasma by choosing base sequences that are well-conserved for a wide range of mycoplasma species, for example, the spacer region between the 16S-23S ribosome genes.

It is recommended that a two-step PCR using nested primers should be performed to increase the sensitivity and specificity, if the one-step PCR is negative.

The primers to be selected for the second stage of a two-step PCR are nested primers from the inner portion of the sequence. The outer and inner primers should have proven effectiveness and specificity as described in publications or be validated experimentally.

It is possible to increase the accuracy of the detection of mycoplasma DNA by performing PCR tests after cultivation of mycoplasma that may be present in samples using Vero cells.

The following is an example of a two-step PCR procedure. The reagents and reaction conditions in this example are not exclusive. If the suitability of other reagents and conditions is verified, they may be used. If another procedure is used, the procedure should be justified and documented in detail, and the information provided should include the sensitivity and specificity of the method.

Example Procedure

1. Preparation of template

1) Place 600 μL of the test cell suspension (if necessary, subcultured with Vero cells) in a tube and dissolve the cells with 0.1% SDS or an equivalent. Add an equal volume (600 μL) of TE (10 mmol/L tris-hydrochloric acid (pH 8.0), 1 mmol/L EDTA) buffer-saturated phenol, and mix.

2) Centrifuge at 15,000 min^{-1} for 5 minutes at room temperature.

3) Transfer 400 μL of the supernatant to another tube, and add 10 μL of 3 mol/L sodium acetate.

4) Add 1 mL (2.5 volumes) of ethanol (95) and stir thoroughly. Ice the mixture for 15 minutes, then centrifuge at 15,000 min^{-1} for 10 minutes at 4°C.

5) Discard the supernatant and rinse the precipitate once or twice with 200 to 300 μL of 80% ethanol. Remove the rinse solution using a pipette. Centrifuge at 15,000 min^{-1} for 10 minutes at 4°C, then remove the supernatant thoroughly and dry up the precipitate.

6) Dissolve the precipitate in 40 μL of distilled water.

2. Perform the same procedure for the positive and negative controls

3. First stage of a two-step PCR

1) Make a mixture of the heat-resistant DNA polymerase, dNTP solution, outer primer, and reaction buffer solution (including Mg ions), and place 90 μL in each tube.

2) Add 10 μL of the template prepared as above to each tube containing the first stage PCR solution (90 μL).

3) Perform the DNA amplification by repeating 30 cycles of denaturation at 94°C for 30 seconds, annealing at an appropriate temperature for the primer (55°C for the primer in this example), and elongation at 72°C for 2 minutes. Add drops of mineral oil or suitable equivalent as needed during the reaction to prevent evaporation.

4. Second stage of a two-step PCR

1) Make a mixture of the heat-resistant DNA polymerase, dNTP solution, inner primer, and reaction buffer solution (including Mg ions), and place 99 μL in each tube.

2) Add 1 μL of the first stage PCR product from each tube to a tube containing the second stage PCR solution (99 μL).

3) Perform the DNA amplification by repeating 30 cycles of denaturation at 94°C for 30 seconds, annealing at an appropriate temperature for the primer (55°C for the primer

in this example), and elongation at 72°C for 2 minutes. Add drops of mineral oil or suitable equivalent as needed during the reaction to prevent evaporation.

5. Agarose gel electrophoresis

1) Mix 10 μL of each of the first stage and second stage PCR products with 2 μL of an appropriate dye as a migration marker, and perform 1% agarose gel electrophoresis.

2) Stain the gel with ethidium bromide and take a photograph under UV irradiation.

3) The test is judged to be positive if a DNA band is detected.

[An Example of Primer]

For mycoplasma detection

Outer primer

F1:5′-ACACCATGGGAG(C/T)TGGTAAT-3′

R1:5′-CTTC(A/T)TCGACTT(C/T)CAGACCCAAGG-CAT-3′

Inner primer

F2:5′-GTG(G/C)GG(A/C)TGGATCACCTCCT-3′

R2:5′-GCATCCACCA(A/T)A(A/T)AC(C/T)CTT-3′

( ) indicates a mixture.

[PCR reaction solution]

	[First stage]	[Second stage]
dNTP solution (each 1.25 mol)	16 μL	16 μL
Primer (10 pmol/μL)	F1 2 μL	F2 2 μL
Primer (10 pmol/μL)	R1 2 μL	R2 2 μL
Heat-resistant DNA polymerase (1 U/μL)	2 μL	2 μL
Reaction buffer solution	68 μL	77 μL
25 mmol/L magnesium chloride hexahydrate	8 μL	8 μL
10-fold buffer solution*	10 μL	10 μL
Sterile distilled water	50 μL	59 μL

*Composition of 10-fold buffer solution

2-amino-2-hydroxymethyl-1,3-propanediol-hydrochloric acid (pH 8.4)	100 mmol/L
Potassium chloride	500 mmol/L
Magnesium chloride hexahydrate	20 mmol/L
Gelatin	0.1 g/L

[Method of cultivating mycoplasma within Vero cells]

1) Use at least two cell culture dishes for each of the test sample, positive control and negative control.

2) Into each cell culture dish (diameter 35 mm), inoculate 2 mL of the Vero cell suspension (1×10^4 cells per 1 mL) in Eagle's minimum essential medium containing 10 percent bovine calf serum (tested in advance using the PCR method to verify that it does not contain any detectable mycoplasma DNA). Incubate the cultures at 36 ± 1°C in an atmosphere of air containing 5 percent carbon dioxide for one day.

3) Replace the culture media with fresh media, and add 0.5 mL of the test sample (cell culture supernatant) to each of two or more Vero cell culture dishes. Perform the same procedure for the positive (such as 100 CFU or less *M. hyorhinis*) and negative controls.

4) Incubate the Vero cell culture dishes for the test sample, positive and negative controls for 3 to 6 days at 36 ± 1°C in an atmosphere of air containing 5 percent carbon dioxide.

10. pH Test for Gastrointestinal Medicine

In this test, medicine for the stomach and bowels, which is said to control stomach acid, is stirred in a fixed amount of the 0.1 mol/L hydrochloric acid for a fixed duration, and the pH value of this solution is obtained. The pH value of a stomach medicine will be based on the dose and the dosage of the medicine (when the dosage varies, a minimum dosage is used) and expressed in the pH value obtained from the test performed by the following procedure.

Preparation of Sample

Solid medicine which conforms to the general regulations for medicine (the powdered medicine section) can be used as a sample. When the medicine is in separate packages, the content of 20 or more packages is accurately weighed to calculate the average mass for one dose and mixed evenly to make a sample. For granules and similar types in separate packages, among the solid medicine which does not conform to the general regulations for medicine (the powdered medicine section), the content of 20 or more packages is accurately weighed to calculate the average mass for one dose and is then powdered to make sample. For granules and similar types not in separate packages, among solid medicine which does not conform to the general regulations for medicine (the powdered medicine section), 20 doses or more are powdered to make a sample. For capsules and tablets, 20 doses or more are weighed accurately to calculate the average mass for one dose or average mass and then powdered to make a sample.

Liquid medicine is generously mixed to make a sample.

Procedure

Put 50 mL of the 0.1 mol/L hydrochloric acid with the molarity coefficient adjusted to 1.000, or equivalent 0.1 mol/L hydrochloric acid with its volume accurately measured in a 100-mL beaker. Stir this solution with a magnetic stirrer and a magnetic stirrer rotator (35 mm length, 8 mm diameter) at the speed of about 300 revolutions per minute. While stirring, add the accurately weighed one-dose sample. After 10 minutes, measure the pH value of the solution using the pH Determination. The solution temperature should be maintained at 37 ± 2°C throughout this operation.

11. Plastic Containers for Pharmaceutical Products

Various kinds of plastics are used in the manufacture of containers for pharmaceutical products. Such plastics should not alter the efficacy, safety or stability of the pharmaceutical products. In selecting a suitable plastic container, it is desirable to have full information on the manufacturing processes of the plastic container including the substances added. Since each plastic has specific properties and a wide variety of pharmaceutical products may be stored in containers made from it, the compatibility of plastic containers with pharmaceutical products should be judged for each combination of container and the specific pharmaceutical product to be contained therein. This judgement should be