Observation and interpretation of results. Conventional microbiological/biochemical techniques are generally satisfactory for identification of micro-organisms recovered from a sterility test. However, if a manufacturer wishes to use condition (d) as the sole criterion for invalidating a sterility test, it may be necessary to employ sensitive typing techniques to demonstrate that a micro-organism isolated from the product test is identical to a micro-organism isolated from the test materials and/or the testing environment. While routine microbiological/biochemical identification techniques can demonstrate that 2 isolates are not identical, these methods may not be sufficiently sensitive or reliable enough to provide unequivocal evidence that two isolates are from the same source. More sensitive tests, for example, molecular typing with RNA/DNA homology, may be necessary to determine that micro-organisms are clonally related and have a common origin.

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2.6.2. MYCOBACTERIA

If the sample to be examined may be contaminated by micro-organisms other than mycobacteria, treat it with a suitable decontamination solution, such as acetylcysteine-sodium hydroxide solution or sodium laurilsulfate solution.

Inoculate 0.2 ml of the sample in triplicate onto each of 2 suitable solid media (Löwenstein-Jensen medium and Middlebrook 7H10 medium are considered suitable). Inoculate 0.5 ml in triplicate into a suitable liquid medium. Incubate all media at 37 °C for 56 days.

Establish the fertility of the media in the presence of the preparation to be examined by inoculation of a suitable strain of a *Mycobacterium* sp. such as BCG and if necessary use a suitable neutralising substance.

If contaminating micro-organisms develop during the first 8 days of incubation, repeat the test and carry out at the same time a bacteriological sterility test.

If at the end of the incubation time no growth of mycobacteria occurs in any of the test media, the preparation complies with the test.

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2.6.7. MYCOPLASMAS

Where the test for mycoplasmas is prescribed for a master cell bank, for a working cell bank, for a virus seed lot or for control cells, both the culture method and the indicator cell culture method are used. Where the test for mycoplasmas is prescribed for a virus harvest, for a bulk vaccine or for the final lot (batch), the culture method is used. The indicator cell culture method may also be used, where necessary, for screening of media.

CULTURE METHOD

CHOICE OF CULTURE MEDIA

The test is carried out using a sufficient number of both solid and liquid media to ensure growth in the chosen incubation conditions of small numbers of mycoplasmas that may be present in the product to be examined. Liquid media must contain phenol red. The range of media chosen is shown to have satisfactory nutritive properties for at least the organisms shown below. The nutritive properties of each new batch of medium are verified for the appropriate organisms in the list.

Acholeplasma laidlawii (vaccines for human and veterinary use where an antibiotic has been used during production)

Mycoplasma gallisepticum (where avian material has been used during production or where the vaccine is intended for use in poultry)

Mycoplasma hyorhinis (non-avian veterinary vaccines)

Mycoplasma orale (vaccines for human and veterinary use)

Mycoplasma pneumoniae (vaccines for human use) or other suitable species of D-glucose fermenter

Mycoplasma synoviae (where avian material has been used during production or where the vaccine is intended for use in poultry).

The test strains are field isolates having undergone not more than fifteen subcultures and are stored frozen or freeze-dried. After cloning the strains are identified as being of the required species by a suitable method, by comparison with type cultures, for example:

A. laidlawii	NCTC 10116	CIP 75.27	ATCC 23206
M. gallisepticum	NCTC 10115	CIP 104967	ATCC 19610
M. hyorhinis	NCTC 10130	CIP 104968	ATCC 17981
M. orale	NCTC 10112	CIP 104969	ATCC 23714
M. pneumoniae	NCTC 10119	CIP 103766	ATCC 15531
M. synoviae	NCTC 10124	CIP 104970	ATCC 25204

INCUBATION CONDITIONS

Divide inoculated media into two equal parts and incubate one in aerobic conditions and the other in microaerophilic conditions; for solid media maintain an atmosphere of adequate humidity to prevent desiccation of the surface. For aerobic conditions, incubate in an atmosphere of air containing, for solid media, 5 to 10 per cent of carbon dioxide. For microaerophilic conditions, incubate in an atmosphere of nitrogen containing, for solid media, 5 to 10 per cent of carbon dioxide.

NUTRITIVE PROPERTIES

Carry out the test for nutritive properties for each new batch of medium. Inoculate the chosen media with the appropriate test organisms; use not more than 100 CFU (colony-forming units) per 60 mm plate containing 9 ml of solid medium and not more than 40 CFU per 100 ml container of the corresponding liquid medium; use a separate plate and container for each species of organism. Incubate the media in the conditions that will be used for the test of the product to be examined (aerobically, microaerophilically or both, depending on the requirements of the test organism). The media comply with the test for nutritive properties if there is adequate growth of the test organisms accompanied by an appropriate colour change in liquid media.

INHIBITORY SUBSTANCES

Carry out the test for nutritive properties in the presence of the product to be examined. If growth of the test organisms is notably less than that found in the absence of the product to be examined, the latter contains inhibitory substances that must be neutralised (or their effect otherwise countered, for example, by dilution) before the test for mycoplasmas is carried out. The effectiveness of the neutralisation or other process is checked by repeating the test for inhibitory substances after neutralisation.

TEST FOR MYCOPLASMAS IN THE PRODUCT TO BE EXAMINED

For solid media, use plates 60 mm in diameter and containing 9 ml of medium. Inoculate each of not fewer than two plates of each solid medium with 0.2 ml of the product

to be examined and inoculate 10 ml per 100 ml of each liquid medium. Incubate at 35 °C to 38 °C, aerobically and microaerophilically, for 21 days and at the same time incubate an uninoculated 100 ml portion of each liquid medium for use as a control. If any significant pH change occurs on addition of the product to be examined, restore the liquid medium to its original pH value by the addition of a solution of either sodium hydroxide or hydrochloric acid. On the first, second or third day after inoculation subculture each liquid culture by inoculating each of two plates of each solid medium with 0.2 ml and incubating at 35 °C to 38 °C aerobically and microaerophilically for not less than 21 days. Repeat the procedure on the sixth, seventh or eighth day and again on the thirteenth or fourteenth day of the test. Observe the liquid media every 2 or 3 days and if any colour change occurs subculture immediately. Observe solid media once per week.

If the liquid media show bacterial or fungal contamination, repeat the test. If, not earlier than 7 days after inoculation, not more than one plate at each stage of the test is accidentally contaminated with bacteria or fungi, or broken, that plate may be ignored provided that on immediate examination it shows no evidence of mycoplasmal growth. If, at any stage of the test, more than one plate is accidentally contaminated with bacteria or fungi, or broken, the test is invalid and must be repeated.

Include in the test positive controls prepared by inoculating not more than 100 CFU of suitable species such as *M. orale* and *M. pneumoniae*.

At the end of the incubation periods, examine all the inoculated solid media microscopically for the presence of mycoplasmas. The product passes the test if growth of mycoplasmas has not occurred in any of the inoculated media. If growth of mycoplasmas has occurred, the test may be repeated once using twice the amount of inoculum, media and plates; if growth of mycoplasmas does not occur, the product complies with the test. The test is invalid if the positive controls do not show growth of the relevant test organism.

INDICATOR CELL CULTURE METHOD

Cell cultures are stained with a fluorescent dye that binds to DNA. Mycoplasmas are detected by their characteristic particulate or filamentous pattern of fluorescence on the cell surface and, if contamination is heavy, in surrounding areas.

VERIFICATION OF THE SUBSTRATE

Using a Vero cell culture substrate, pretest the procedure using an inoculum of not more than 100 CFU (colony-forming units) of a strain growing readily in liquid or solid medium and demonstrate its ability to detect potential mycoplasma contaminants such as suitable strains of *Mycoplasma hyorhinis* and *Mycoplasma orale*. A different cell substrate may be used, for example the production cell line, if it has been demonstrated that it will provide at least equal sensitivity for the detection of potential mycoplasma contaminants.

Test method

Take not less than 1 ml of the product to be examined and use it to inoculate in duplicate, as described under Procedure, indicator cell cultures representing not less than 25 cm^2 of cell culture area at confluence.

Include in the test a negative (non-infected) control and two positive mycoplasma controls, such as *M. hyorhinis* and *M. orale*. Use an inoculum of not more than 100 CFU for the positive controls.

If for viral suspensions the interpretation of results is affected by marked cytopathic effects, the virus may be neutralised using a specific antiserum that has no inhibitory effects on mycoplasmas or a cell culture substrate that does not allow growth of the virus may be used. To demonstrate the absence of inhibitory effects of serum, carry out the positive control tests in the presence and absence of the antiserum.

Procedure

1. Seed culture at a regular density (2×10^4 to 2×10^5 cells/ml, 4×10^3 to 2.5×10^4 cells/cm^2) and incubate at 36 ± 1 °C for at least 2 days. Inoculate the product to be examined and incubate for at least 2 days; make not fewer than one subculture. Grow the last subculture on coverslips in suitable containers or on some other surface suitable for the test procedure. Do not allow the last subculture to reach confluence since this would inhibit staining and impair visualisation of mycoplasmas.

2. Remove and discard the medium.

3. Rinse the monolayer with *phosphate buffered saline pH 7.4 R*, then with a mixture of equal volumes of *phosphate buffered saline pH 7.4 R* and a suitable fixing solution and finally with the fixing solution; when *bisbenzimide R* is used for staining, a freshly prepared mixture of 1 volume of *glacial acetic acid R* and 3 volumes of *methanol R* is a suitable fixing solution.

4. Add the fixing solution and allow to stand for 10 min.

5. Remove the fixing solution and discard.

6. If the monolayer is to be stained later, dry it completely. (Particular care is needed for staining of the slides after drying because of artefacts that may be produced.)

7. If the monolayer is to be stained directly, wash off the fixing solution twice with sterile water and discard the wash.

8. Add *bisbenzimide working solution R* or some other suitable DNA staining agent and allow to stand for 10 min.

9. Remove the stain and rinse the monolayer with water.

10. Mount each coverslip, where applicable, with a drop of a mixture of equal volumes of *glycerol R* and *phosphate-citrate buffer solution pH 5.5 R*; blot off surplus mountant from the edge of the coverslip.

11. Examine by epifluorescence (330 nm/380 nm excitation filter, LP 440 nm barrier filter) at 100-400 × magnification or greater.

12. Compare the microscopic appearance of the test cultures with that of the negative and positive controls, examining for extranuclear fluorescence. Mycoplasmas give pinpoints or filaments over the cytoplasm and sometimes in intercellular spaces.

The product to be examined complies with the test if there is no evidence of the presence of mycoplasmas in the test cultures inoculated with it. The test is invalid if the positive controls do not show the presence of the appropriate test organisms.

The following section is published for information.

RECOMMENDED MEDIA FOR THE CULTURE METHOD

The following media are recommended. Other media may be used providing their ability to sustain the growth of mycoplasmas has been demonstrated on each batch in the presence and absence of the product to be examined.

RECOMMENDED MEDIA FOR THE DETECTION OF MYCOPLASMA GALLISEPTICUM

Liquid medium

Beef heart infusion broth (1)	90.0 ml
Horse serum (unheated)	20.0 ml

Yeast extract (250 g/l)	10.0 ml
Thallium acetate (10 g/l solution)	1.0 ml
Phenol red (0.6 g/l solution)	5.0 ml
Penicillin (20 000 IU/ml)	0.25 ml
Deoxyribonucleic acid (2 g/l solution)	1.2 ml

Adjust to pH 7.8.

Solid medium

Prepare as described above replacing beef heart infusion broth by beef heart infusion agar containing 15 g/l of agar.

RECOMMENDED MEDIA FOR THE DETECTION OF MYCOPLASMA SYNOVIAE

Liquid medium

Beef heart infusion broth (1)	90.0 ml
Essential vitamins (2)	0.025 ml
Glucose monohydrate (500 g/l solution)	2.0 ml
Swine serum (inactivated at 56 °C for 30 min)	12.0 ml
β-Nicotinamide adenine dinucleotide (10 g/l solution)	1.0 ml
Cysteine hydrochloride (10 g/l solution)	1.0 ml
Phenol red (0.6 g/l solution)	5.0 ml
Penicillin (20 000 IU/ml)	0.25 ml

Mix the solutions of β-nicotinamide adenine dinucleotide and cysteine hydrochloride and after 10 min add to the other ingredients. Adjust to pH 7.8.

Solid medium

Beef heart infusion broth (1)	90.0 ml
Ionagar (3)	1.4 g

Adjust to pH 7.8, sterilise by autoclaving then add:

Essential vitamins (2)	0.025 ml
Glucose monohydrate (500 g/l solution)	2.0 ml
Swine serum (unheated)	12.0 ml
β-Nicotinamide adenine dinucleotide (10 g/l solution)	1.0 ml
Cysteine hydrochloride (10 g/l solution)	1.0 ml
Phenol red (0.6 g/l solution)	5.0 ml
Penicillin (20 000 IU/ml)	0.25 ml

RECOMMENDED MEDIA FOR THE DETECTION OF NON-AVIAN MYCOPLASMAS

Liquid medium

Hanks' balanced salt solution (modified) (4)	800 ml
Distilled water	67 ml
Brain heart infusion (5)	135 ml
PPLO Broth (6)	248 ml
Yeast extract (170 g/l)	60 ml
Bacitracin	250 mg
Meticillin	250 mg
Phenol red (5 g/l)	4.5 ml
Thallium acetate (56 g/l)	3 ml
Horse serum	165 ml
Swine serum	165 ml

Adjust to pH 7.4 - 7.45.

Solid medium

Hanks' balanced salt solution (modified) (4)	200 ml
DEAE-dextran	200 mg
Ionagar (3)	15.65 mg

Mix well and sterilise by autoclaving. Cool to 100 °C. Add to 1740 ml of liquid medium as described above.

(1) *Beef heart infusion broth*

Beef heart (for preparation of the infusion)	500 g
Peptone	10 g
Sodium chloride	5 g
Distilled water to	1000 ml

Sterilise by autoclaving.

(2) *Essential vitamins*

Biotin	100 mg
Calcium pantothenate	100 mg
Choline chloride	100 mg
Folic acid	100 mg
i-Inositol	200 mg
Nicotinamide	100 mg
Pyridoxal hydrochloride	100 mg
Riboflavine	10 mg
Thiamine hydrochloride	100 mg
Distilled water to	1000 ml

(3) *Ionagar*

A highly refined agar for use in microbiology and immunology prepared by an ion-exchange procedure which results in a product having superior purity, clarity and gel strength.

It contains about:

Water	12.2 per cent
Ash	1.5 per cent
Acid-insoluble ash	0.2 per cent
Chlorine	0
Phosphate (calculated as P_2O_5)	0.3 per cent
Total nitrogen	0.3 per cent
Copper	8 ppm
Iron	170 ppm
Calcium	0.28 per cent
Magnesium	0.32 per cent

(4) *Hanks' balanced salt solution (modified)*

Sodium chloride	6.4 g
Potassium chloride	0.32 g
Magnesium sulphate heptahydrate	0.08 g
Magnesium chloride hexahydrate	0.08 g
Calcium chloride, anhydrous	0.112 g
Disodium hydrogen phosphate dihydrate	0.0596 g
Potassium dihydrogen phosphate, anhydrous	0.048 g
Distilled water to	800 ml

(5) *Brain heart infusion*

Calf-brain infusion	200 g
Beef-heart infusion	250 g
Proteose peptone	10 g
Glucose monohydrate	2 g
Sodium chloride	5 g
Disodium hydrogen phosphate, anhydrous	2.5 g
Distilled water to	1000 ml

(6) *PPLO broth*

Beef-heart infusion	50 g
Peptone	10 g
Sodium chloride	5 g
Distilled water to	1000 ml

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2.6.8. PYROGENS

The test consists of measuring the rise in body temperature evoked in rabbits by the intravenous injection of a sterile solution of the substance to be examined.

Selection of animals. Use healthy, adult rabbits of either sex weighing not less than 1.5 kg, fed a complete and balanced diet not containing antibiotics, and not showing loss of body mass during the week preceding the test. A rabbit is not be used in a pyrogen test:

a) if it has been used in a negative pyrogen test in the preceding 3 days, or

b) if it has been used in the preceding 3 weeks in a pyrogen test in which the substance under examination failed to pass the test.

Animals' quarters. Keep the rabbits individually in a quiet area with a uniform appropriate temperature. Withhold food from the rabbits overnight and until the test is completed; withhold water during the test. Carry out the test in a quiet room where there is no risk of disturbance exciting the animals and in which the room temperature is within 3 °C of that of the rabbits' living quarters, or in which the rabbits have been kept for at least 18 h before the test.

Materials. Glassware, syringes and needles. Thoroughly wash all glassware, syringes and needles with water for injections and heat in a hot-air oven at 250 °C for 30 min or at 200 °C for 1 h.

Retaining boxes. The retaining boxes for rabbits whose temperature is being measured by an electrical device are made in such a way that the animals are retained only by loosely fitting neck-stocks; the rest of the body remains relatively free so that the rabbits may sit in a normal position. They are not restrained by straps or other similar methods which may harm the animal. The animals are put into the boxes not less than 1 h before the first record of the temperature and remain in them throughout the test.

Thermometers. Use a thermometer or electrical device which indicates the temperature with a precision of 0.1 °C and insert into the rectum of the rabbit to a depth of about 5 cm. The depth of insertion is constant for any one rabbit in any one test. When an electrical device is used it may be left in position throughout the test.

Preliminary test. After selection of the animals, one to three days before testing the product to be examined, treat those animals that have not been used during the previous 2 weeks by intravenous injection of 10 ml per kilogram of body mass of a pyrogen-free 9 g/l solution of *sodium chloride R* warmed to about 38.5 °C. Record the temperatures of the animals, beginning at least 90 min before injection and continuing for 3 h after the injection of the solution. Any animal showing a temperature variation greater than 0.6 °C is not used in the main test.

Main test. Carry out the test using a group of three rabbits.

Preparation and injection of the product. Warm the liquid to be examined to approximately 38.5 °C before the injection. The product to be examined may be dissolved in, or diluted with, a pyrogen-free 9 g/l solution of *sodium chloride R* or another prescribed liquid. Inject the solution slowly into the marginal vein of the ear of each rabbit over a period not exceeding 4 min, unless otherwise prescribed in the monograph. The amount of the product to be injected varies according to the product to be examined and is prescribed in the monograph. The volume injected is not less than 0.5 ml per kilogram and not more than 10 ml per kilogram of body mass.

Determination of the initial and maximum temperatures. The "initial temperature" of each rabbit is the mean of two temperature readings recorded for that rabbit at an interval of 30 min in the 40 min immediately preceding the injection of the product to be examined. The "maximum temperature" of each rabbit is the highest temperature recorded for that rabbit in the 3 h after the injection. Record the temperature of each rabbit at intervals of not more than 30 min, beginning at least 90 min before the injection of the product to be examined and continuing 3 h after the injection. The difference between the maximum temperature and the initial temperature of each rabbit is taken to be its response. When this difference is negative, the result is counted as a zero response.

Rabbits showing a temperature variation greater than 0.2 °C between two successive readings in the determination of the initial temperature are withdrawn from the test. In any one test, only rabbits having initial temperatures which do not differ from one another by more than 1 °C are used. All rabbits having an initial temperature higher than 39.8 °C or less than 38.0 °C are withdrawn from the test.

Interpretation of results. Having carried out the test first on a group of three rabbits, repeat if necessary on further groups of three rabbits to a total of four groups, depending on the results obtained. If the summed response of the first group does not exceed the figure given in the second column of the Table 2.6.8.-1, the substance passes the test. If the summed response exceeds the figure given in the second column of the table but does not exceed the figure given in the third column of the table, repeat the test as indicated above. If the summed response exceeds the figure given in the third column of the table, the product fails the test.

Table 2.6.8.-1

Number of rabbits	Product passes if summed response does not exceed	Product fails if summed response exceeds
3	1.15 °C	2.65 °C
6	2.80 °C	4.30 °C
9	4.45 °C	5.95 °C
12	6.60 °C	6.60 °C

Rabbits used in a test for pyrogens where the mean rise in the rabbits' temperature has exceeded 1.2 °C are permanently excluded.