transferring the specified test organism. Incubate the inoculated slant at 32 to 37°C for 16 to 24 hours. The subculture should be performed at least three times.

Inoculate the subcultured test organism onto another slant of the agar medium (described above), and incubate the slant at 32 to 37°C for 16 to 24 hours. After incubation, prepare the stock suspensions of the test organism as follows:

(1) Preparation of a stock suspension of test organism [1]

Suspend the test organism in approximately 10 mL of the liquid medium for suspending the test organism. Add approximately 150 mL of the same medium to give approximately 85% transmittance at a wavelength of 650 nm. Before use, prepare the suspension of the test organism by adding 4.0 mL of this suspension to 100 mL of the liquid medium for suspending the test organism. The liquid medium should be maintained at not more than 15°C.

(2) Preparation of a stock suspension of the test organism [2]

Suspend the test organism in approximately 10 mL of the liquid medium for suspending the test organism. Add approximately 150 mL of the same medium to give approximately 70% transmittance at a wavelength of 650 nm. Before use, prepare a suspension of the test organism by adding 5.0 mL of this suspension to 100 mL of the liquid medium for suspending the test organism. The liquid medium should be maintained at not more than 15°C.

(3) Preparation of a stock suspension of the test organism [3]

Suspend the test organism in a little amount of the liquid medium for suspending the test organism. Add approximately 150 mL of the same medium to give approximately 85% transmittance at a wavelength of 650 nm. Before use, prepare the suspension of the test organism by adding 6.0 mL of this suspension to 100 mL of the liquid medium for suspending the test organism. The liquid medium should be maintained at not more than 15°C.

(4) Preparation of a stock suspension of the test organism [4]

Suspend the test organism in 5 mL of water, and add this suspension dropwise onto the slant of agar medium, which was prepared for transferring the specified test organism, contained in a suitable vessel such as a Roux bottle. Spread the suspension of organisms evenly over the agar surface with the aid of sterile glass beads, and then incubate the Roux bottle at 32 to 37°C for 16 to 24 hours.

Scrape away the resulting growth from the agar surface, and suspend in water, adjusting to give approximately 65% transmittance at a wavelength of 650 nm. Store the stock suspension at a temperature not exceeding 5°C, and use it within 14 days. Before use, prepare the suspension of the test organism by adding 6.0 mL of this stock suspension to 100 mL of the liquid medium for suspending the test organism. The liquid medium should be maintained at not more than 15°C.

5. Standard solutions

Use the standard solutions specified in the individual monograph. Unless otherwise specified, prepare the standard solutions before use.

6. Sample solutions

Use the sample solutions specified in the individual monograph. Unless otherwise specified, prepare the sample solutions before use.

7. Procedure

Unless otherwise specified, proceed as follows:

Distribute 1.0 mL of each concentration of the standard solution, the sample solution, and water used as a control, into each set composed of 3 test tubes (about 14 mm in inside diameter and about 13 cm in length). Add 9.0 mL of the suspension of the test organism to each tube, cover with a suitable lid or a cotton plug, and then incubate in a water bath maintained at 35 to 37°C for 3 to 4 hours. After incubation, add 0.5 mL of dilute formaldehyde (1 in 3) to each tube, and read each transmittance or absorbance at a wavelength of 530 nm.

8. Estimation of potency

Average the transmittance or absorbance values of each concentration of the standard solution, the sample solution and water used as a control, respectively. Generate the standard curve based on the average values of transmittance or absorbance of each concentration of the standard solution, and estimate the potency of the sample solution from its average value of transmittance or absorbance using the obtained standard curve.

If the standard dilutions of five concentrations in geometric progression are used, calculate the L and H values from the following equations. Plot point L and point H on graph paper and construct a straight line for the standard curve.

$$L = \frac{3a + 2b + c - e}{5} \qquad H = \frac{3e + 2d + c - a}{5}$$

where:

L = calculated value of transmittance or absorbance for the lowest concentration of the standard curve.

H = calculated value of transmittance or absorbance for the highest concentration of the standard curve.

a, b, c, d, e = average transmittance or absorbance values for each standard dilution, where a is the value from the lowest concentration standard solution, b, c and d are the values from each geometrically increased concentration standard solution, respectively, and e is the value from the highest concentration standard solution.

35. Microbial Limit Test

This chapter provides tests for the qualitative and quantitative estimation of viable microorganisms present in pharmaceutical articles. It includes tests for total viable count (bacteria and fungi) and specified microbial species (*Escherichia coli, Salmonella, Pseudomonas aeruginosa* and *Staphylococcus aureus*). Microbial limit test must be carried out under conditions designed to avoid accidental microbial contamination of the preparation during the test. When test specimens have antimicrobial activity, or contain antimicrobial substances, any such antimicrobial properties must be eliminated by means of procedures such as dilution, filtration, neutralization or inactivation. For the test, use a mixture of several portions selected at random from the bulk or from the contents of a sufficient number of containers. If test specimens are diluted with fluid medium, the test should be performed quickly. In performing the test, precau-

tions must be taken to prevent biohazard.

1. Total viable aerobic count

This test determines the mesophilic bacteria and fungi which grow under aerobic conditions. Psychrophilic, thermophilic, basophilic and anaerobic bacteria, and microorganisms which require specific ingredients for growth, may give a negative result, even if a significant number exists in the test specimens. The test may be carried out using one of the following 4 methods, i.e., membrane filtration method, pour plate method, spread plate method or serial dilution method (most probable number method). Use an appropriate method depending on the purpose. An automated method may be used for the test presented here, provided it has been properly validated as giving equivalent or better results. Different culture media and temperature are required for the growth of bacteria and fungi (molds and yeasts). Serial dilution method is applicable only to the enumeration of bacteria.

Preparation of the test solution

Phosphate Buffer (pH 7.2), Buffered Sodium Chloride-Peptone Solution or fluid medium used for the test is used to dissolve or dilute the test specimen. Unless otherwise specified, 10 g or 10 mL of the test specimen is used for the test, but another quantity or volume may be used according to the nature of the test specimen. The pH of the solution is adjusted to between 6 and 8. The test solution must be used within an hour after preparation.

Fluid specimens or soluble solids: Take 10 g or 10 mL of the test specimen, mix with the buffer or fluid medium specified to make 100 mL, and use this as the test fluid. A fluid specimen containing insoluble materials must be shaken well, just prior to mixing, to effect fine suspension.

Insoluble solids: Take 10 g or 10 mL of the test specimen, reduce the substance to a fine powder, suspend it in the buffer or fluid medium specified to make 100 mL, and use this as the test fluid. A larger volume of the buffer or fluid medium than indicated may be used for the suspension, depending on the nature of the test specimen. The suspension may be dispersed well using, if necessary, a mechanical blender. A suitable surface-active agent (such as 0.1 w/v% polysorbate 80) may be added to aid dissolution.

Fatty products: For water-immiscible fluids, ointments, creams, waxes, and lotions which consist mainly of lipid, take 10 g or 10 mL of the test specimen, emulsify it in the buffer or fluid medium specified with the aid of a suitable surface-active agent such as polysorbate 20 or 80 to make 100 mL, and use this as the test fluid. An emulsion may be made by warming to a temperature not exceeding 45°C, but do not maintain this temperature for more than 30 minutes.

Test procedures

(1) Membrane Filtration Method

This method is applicable especially to specimens which contain antimicrobial substances. Use membrane filters of appropriate materials, having a normal pore size not greater than 0.45 μm. Filter discs about 50 mm in diameter are recommended, but filters of a different diameter may also be used. Filters, the filtration apparatus, media, etc., should be sterilized well. Usually, take 20 mL of the test fluid (containing 2 g of test specimen), transfer 10 mL of the solution to each of two membrane filters, and filter. If necessary, dilute the pretreated preparation so that a colony count of 10 to 100 may be expected. After the filtration of the test fluid, wash each membrane by filtering through it three or more times with a suitable liquid such as Buffered Sodium Chloride-Peptone Solution, Phosphate Buffer, or the fluid medium to be used. The volume of the washings to be used is approximately 100 mL each, but in case filter disc is significantly different from 50 mm in diameter, the volume may be adjusted according to the size of the filter. For fatty substances, the washings may contain a suitable surface-active agent such as polysorbate 80. Put one of the membrane filters, intended primarily for the enumeration of bacteria, on the surface of a plate of Soybean-Casein Digest Agar and the other, intended primarily for the enumeration of fungi, on the surface of a plate of one of Sabouraud Glucose Agar, Potato Dextrose Agar, or GP Agar Medium (each contains antibiotics). After incubation of the plates at least for 5 days, between 30°C and 35°C in the test for the detection of bacteria and between 20°C and 25°C in the test for fungi, count the number of colonies that are formed. If a reliable count is obtained in a shorter incubation time than 5 days, this may be adopted.

(2) Pour Plate Method

Use petri dishes 9 to 10 cm in diameter. Use at least two petri dishes for each dilution. Pipet 1 mL of the test fluid or its diluted solution onto each petri dish aseptically. Promptly add to each dish 15 to 20 mL of sterilized agar medium that has previously been melted and kept below 45°C, and mix. Primarily for the detection of bacteria, use Soybean-Casein Digest Agar Medium, and, primarily for the detection of fungi, use one of Sabouraud Glucose Agar, Potato Dextrose Agar, and GP Agar Medium (each contains antibiotics). After the agar solidifies, incubate the plates for at least 5 days, between 30°C and 35°C for bacteria and between 20°C and 25°C for fungi. If too many colonies are observed, dilute the fluid as described above so that a colony count of not more than 300 per plate may be expected in the case of bacteria, and not more than 100 per plate in the case of fungi. If a reliable count is obtained in a shorter incubation time than 5 days, this may be adopted.

(3) Spread Plate Method

On the solidified and dried surface of the agar medium, pipet 0.05 to 0.2 mL of the test fluid and spread it on the surface with a spreader. The diameter of petri dishes, the kind and volume of the medium to be used, the temperature and time of incubation, and the method for calculation of total viable count are the same as described in the Pour Plate Method section.

(4) Serial Dilution Method (Most Probable Number Method)

Prepare a series of 12 tubes each containing 9 to 10 mL of Fluid Soybean-Casein Digest Medium. Use three tubes for each dilution. To each of the first three tubes add 1 mL of the test fluid (containing 0.1 g or 0.1 mL of specimen), resulting in 1 in 10 dilution. To the next three tubes add 1 mL of a 1 in 10 dilution of the fluid, resulting in 1 in 100 dilution. To the next three tubes add 1 mL of a 1 in 100 dilution of the fluid, resulting in 1 in 1000 dilution. To the last three tubes add 1 mL of the diluent as a control. Incubate the tubes between 30°C and 35°C for not less than 5 days. The control tubes should show no microbial growth. If the reading of the results is difficult or uncertain, transfer about 0.1 mL to a liquid or solid medium and read the results after a further period of incubation between 30°C and 35°C for 24 to 72 hours. Determine the most probable number of microorganisms per mL of the specimen from Table 1.

Table 1. Most probable number of microorganisms

Number of tubes in which microbial growth is observed for each quantity of the specimen			Most probable number of microorganisms per g or per mL
0.1 g or 0.1 mL per tube	0.01 g or 0.01 mL per tube	1 mg or 1 μL per tube	
3	3	3	>1100
3	3	2	1100
3	3	1	500
3	3	0	200
3	2	3	290
3	2	2	210
3	2	1	150
3	2	0	90
3	1	3	160
3	1	2	120
3	1	1	70
3	1	0	40
3	0	3	95
3	0	2	60
3	0	1	40
3	0	0	23

If, for the first column (containing 0.1 g or 0.1 mL of specimen), the number of tubes showing microbial growth is two or less, the most probable number of microorganisms per g or per mL is likely to be less than 100.

Effectiveness of culture media and confirmation of antimicrobial substances

Use microorganisms of the following strains or their equivalent. Grow them in Fluid Soybean-Casein Digest Medium between 30°C and 35°C for bacteria and between 20°C and 25°C for *Candida albicans*.

Escherichia coli, such as ATCC8739, NCIB8545
Bacillus subtilis, such as ATCC6633, NCIB8054
Staphylococcus aureus, such as ATCC6538, NCIB8625
Candida albicans, such as ATCC2091, ATCC10231

Dilute portions of each of the cultures using Buffered Sodium Chloride-Peptone Solution, or Phosphate Buffer to prepare test suspensions containing about 50 to 200 viable microorganisms per mL. Growth-promoting qualities are tested by inoculating 1 mL of each microorganism into each medium. The test media are satisfactory if clear evidence of growth appears in all inoculated media after incubation at indicated temperature for 5 days. When a count of the test organisms with a test specimen differs by more than a factor of 5 from that without the test specimen, any such effect must be eliminated by dilution, filtration, neutralization or inactivation. To confirm the sterility of the medium and of the diluent and the aseptic performance of the test, carry out the total viable count method using sterile Buffered Sodium Chloride-Peptone Solution or Phosphate Buffer as the control.

2. Test for the detection of specified microorganisms

Escherichia coli, Salmonella, Pseudomonas aeruginosa and *Staphylococcus aureus* are included as the target strains of the test. These four species of microorganisms are important for the evaluation of microbial contamination not only in the finished products, but also in the bulk or intermediate of the production process, and are representative of the microorganisms which should not exist in these materials.

Preparation of the test fluid

If necessary, refer to the paragraph on Preparation of the Test Solution in Total viable aerobic count. When test specimens are dissolved in or diluted with a fluid medium, use the medium designated in each test, unless otherwise specified.

Test procedure

(1) *Escherichia coli*

To 10 g or 10 mL of the test specimen add a volume of Fluid Lactose Medium to make 100 mL, and incubate between 30°C and 35°C for 24 to 72 hours. Examine the medium for growth, and if growth is present, mix by gentle shaking, take a portion by means of an inoculating loop, and streak it on the surface of MacConkey Agar Medium. Incubate between 30°C and 35°C for 18 to 24 hours. If brick-red colonies of Gram-negative rods surrounded by a reddish precipitation zone are not found, the specimen meets the requirements of the test for absence of *Escherichia coli.* If colonies matching the above description are found, transfer the suspect colonies individually to the surface of EMB Agar Medium, and incubate between 30°C and 35°C for 18 to 24 hours. Upon examination, if none of the colonies exhibits both a characteristic metallic sheen and a blue-black appearance under transmitted light, the specimen meets the requirements of the test for the absence of *Escherichia coli.* Confirm any suspect colonies on the plate by means of the IMViC test (Indole production test, Methyl red reaction test, and Voges-Proskauer test, and Citrate utilization test), and the colonies which exhibit the pattern of either [+ + − −] or [− + − −] are judged as *Escherichia coli.* Rapid detection kits for *Escherichia coli* may also be used.

(2) *Salmonella*

As in the case of the detection of *Escherichia coli*, to 10 g or 10 mL of the test specimen add a volume of Fluid Lactose Medium to make 100 mL, and incubate between 30°C and 35°C for 24 to 72 hours. Examine the medium for growth, and if growth is present, mix by gentle shaking, pipet 1 mL portions, respectively, into 10 mL of Fluid Selenite-Cystine Medium and Fluid Tetrathionate Medium, and incubate for 12 to 24 hours. 10 mL of Fluid Selenite-Cystine Medium may be replaced by the same volume of Fluid Rappaport Medium. After the incubation, streak portions from both the fluid media on the surface of at least two of Brilliant Green Agar Medium, XLD Agar Medium, and Bismuth Sulfite Agar Medium, and incubate between 30°C and 35°C for 24 to 48 hours. Upon examination, if none of the colonies conforms to the description given in Table 2, the specimen meets the requirements of the test for the absence of the genus *Salmonella.* If colonies of Gram-negative rods matching the description in Table 2 are found, transfer suspect colonies individually, by means of an inoculating wire, to a slant of TSI Agar Medium using both surface and deep inoculation. Incubate between 35°C and 37°C for 18 to 24 hours. The presence of genus *Salmonella* is confirmed if, in the deep culture but not in the surface culture, there is a change of color from red to yellow and usually a formation of gas with or without production of hydrogen sulfide. Precise identification and typing of genus *Salmonella* may be carried out by using appropriate biochemical and serological tests additionally including an identification kit.

Table 2. Morphologic characteristics of *Salmonella* species on selective agar media

Medium	Description of colony
Brilliant Green Agar Medium	Small, transparent and colorless, or opaque, pink or white (often surrounded by a pink to red zone)
XLD Agar Medium	Red, with or without black centers
Bismuth Sulfite Agar Medium	Black or green

(3) *Pseudomonas aeruginosa*

To 10 g or 10 mL of the specimen add Fluid Soybean-Casein Digest Medium, or another suitable fluid medium without antimicrobial activity, to make 100 mL. Fluid Lactose Medium is not suitable. Incubate between 30°C and 35°C for 24 to 48 hours. If, upon examination, growth is present, use an inoculating loop to streak a portion of the medium on the surface of Cetrimide Agar Medium or NAC Agar Medium and incubate between 30°C and 35°C for 24 to 48 hours. If no growth of microorganisms is detected, the specimen passes the test. If growth of colonies of Gram-negative rods with a greenish fluorescence occurs, streak suspect colonies from the agar surface of Cetrimide Agar Medium on the agar surfaces of Pseudomonas Agar Medium for Detection of Fluorescin and Pseudomonas Agar Medium for Detection of Pyocyanin, and incubate between 30°C and 35°C for 24 to 72 hours. The production of yellowish fluorescence on the surface of the former medium shows the production of fluorescin, and the production of blue fluorescence on the latter medium indicates the production of pyocyanin. Confirm any suspect colonies as *Pseudomonas aeruginosa* by means of the Oxidase test. Transfer each of the suspect colonies to filter paper that has previously been impregnated with *N*,*N*-dimethyl-*p*-phenylenediammonium dichloride. If the colony changes to purple within 5 to 10 seconds, the oxidase test is judged as positive. If the oxidase test is judged to be negative, the specimen meets the requirements of the test for the absence of *Pseudomonas aeruginosa*. The presence of *Pseudomonas aeruginosa* may also be confirmed by using appropriate biochemical tests including an identification kit.

(4) *Staphylococcus aureus*

To 10 g or 10 mL of the specimen add Fluid Soybean-Casein Digest Medium, or another suitable fluid medium without antimicrobial activity to make 100 mL. Incubate the fluid containing the specimen between 30°C and 35°C for 24 to 48 hours. If, upon examination, growth is present, use an inoculating loop to streak a portion of the medium on the surface of one of the Vogel-Johnson Agar Medium, Baird-Parker Agar Medium, or Mannitol-Salt Agar Medium, and incubate between 30°C and 35°C for 24 to 48 hours. Upon examination, if no colonies of Gram-positive cocci having the characteristics listed in Table 3 are found, the specimen meets the requirements of the test for the absence of *Staphylococcus aureus*. Confirm any suspect colonies as *Staphylococcus aureus* by means of the coagulase test. With the aid of an inoculating loop, transfer suspect colonies to individual tubes, each containing 0.5 mL of mammalian, preferably rabbit or horse, plasma with or without suitable additives. Incubate in a thermostat at 37±1°C. Examine the coagulation after 3 hours and subsequently at suitable intervals up to 24 hours. Test positive and negative controls simultaneously. If no coagulation is observed, the specimen meets the requirements of the test for the absence of *Staphylococcus aureus*.

Table 3. Morphologic characteristics of *Staphylococcus aureus* on selective agar media

Medium	Colonial characteristics
Vogel-Johnson Agar Medium	Black surrounded by yellow zone
Baird-Parker Agar Medium	Black, shiny, surrounded by clear zones
Mannitol-Salt Agar Medium	Yellow colonies with yellow zones

Effectiveness of culture media and confirmation of antimicrobial substances

Grow the test strains listed in Table 4 in the media indicated between 30°C and 35°C for 18 to 24 hours. Dilute portions of each of the cultures using Buffered Sodium Chloride-Peptone Solution, Phosphate Buffer, or medium indicated for each bacterial strain to make test suspensions containing about 1000 viable microorganisms per mL. As occasion demands, using a mixture of 0.1 mL of each suspension of *Escherichia coli, Salmonella, Pseudomonas aeruginosa* and *Staphylococcus aureus* containing about 1000 viable microorganisms, the validity of the medium and the presence of antimicrobial substances are tested in the presence or absence of the specimen.

Table 4. Bacterial strains and media used for confirmation of the effectiveness of culture medium and validity of the test for specified microorganisms

Microorganisms	Strain number	Media
Escherichia coli	ATCC8739, NCIB8545 or equivalent strains	Fluid Lactose Medium
Salmonella	No strain number is recommended*	Fluid Lactose Medium
Pseudomonas aeruginosa	ATCC9027, NCIB8626 or equivalent strains	Fluid Soybean-Casein Digest Medium
Staphylococcus aureus	ATCC6538, NCIB8625 or equivalent strains	Fluid Soybean-Casein Digest Medium

**Salmonella* strains of weak or no pathogenicity may be used. *Salmonella* Typhi may not be used.

Retest

For the purpose of confirming a doubtful result, a retest is conducted using 25 g or 25 mL of test specimen. Proceed as directed under Test procedure, but make allowance for the larger specimen size, for example by adjusting the volume of the medium.

3. Buffer solution and media

Buffer solution and media used for the microbial limit test are described below. Other media may be used if they have similar nutritive ingredients, and selective and growth-promoting properties for the microorganisms to be tested.

(1) Buffer solution

(i) Phosphate Buffer (pH 7.2)

Stock Solution: Dissolve 34 g of potassium dihydrogen-

phosphate in about 500 mL of water. Adjust to pH 7.1 to 7.3 by the addition of 175 mL of sodium hydroxide TS, add water to make 1000 mL, and use this solution as the stock solution. After sterilization by heating in an autoclave, store under refrigeration. For use, dilute the Stock Solution with water in the ratio of 1 to 800, and sterilize at 121°C for 15 to 20 minutes.

(ii) Buffered Sodium Chloride-Peptone Solution (pH 7.0)

Potassium dihydrogenphosphate	3.56 g
Disodium hydrogenphosphate 12-water	18.23 g
Sodium chloride	4.30 g
Peptone	1.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 6.9 - 7.1. Polysorbate 20 or 80 (0.1 to 1.0 w/v%) may be added.

(2) Media

(i) Soybean-Casein Digest Agar Medium

Casein peptone	15.0 g
Soybean peptone	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.1 - 7.3.

(ii) Fluid Soybean-Casein Digest Medium

Casein peptone	17.0 g
Soybean peptone	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogenphosphate	2.5 g
Glucose	2.5 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.1 - 7.5.

(iii) Sabouraud Glucose Agar Medium with Antibiotics

Peptones (animal tissue and casein)	10.0 g
Glucose	40.0 g
Agar	15.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 5.4 - 5.8. Just prior to use, add 0.10 g of benzylpenicillin potassium and 0.10 g of tetracycline per liter of medium as sterile solutions or, alternatively, add 50 mg of chloramphenicol per liter of medium.

(iv) Potato Dextrose Agar Medium with Antibiotics

Potato extract	4.0 g
Glucose	20.0 g
Agar	15.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 5.4 - 5.8. Just prior to use, add 0.10 g of benzylpenicillin potassium and 0.10 g of tetracycline per liter of medium as sterile solutions or, alternatively, add 50 mg of chloramphenicol per liter of medium.

(v) GP (Glucose-peptone) Agar Medium with Antibiotics

Glucose	20.0 g
Yeast extract	2.0 g
Magnesium sulfate heptahydrate	0.5 g
Peptone	5.0 g
Potassium dihydrogenphosphate	1.0 g
Agar	15.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 5.6 - 5.8. Just prior to use, add 0.10 g of benzylpenicillin potassium and 0.10 g of tetracycline per liter of medium as sterile solutions or, alternatively, add 50 mg of chloramphenicol per liter of medium.

(vi) Fluid Lactose Medium

Meat extract	3.0 g
Gelatin peptone	5.0 g
Lactose monohydrate	5.0 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 6.7 - 7.1. After sterilization, cool immediately.

(vii) MacConkey Agar Medium

Gelatin peptone	17.0 g
Casein peptone	1.5 g
Animal tissue peptone	1.5 g
Lactose monohydrate	10.0 g
Sodium desoxycholate	1.5 g
Sodium chloride	5.0 g
Agar	13.5 g
Neutral red	0.03 g
Crystal violet	1.0 mg
Water	1000 mL

Mix all the components, boil for 1 minute to effect solution and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 6.9 - 7.3.

(viii) EMB (Eosin-Methylene Blue) Agar Medium

Gelatin peptone	10.0 g
Dipotassium hydrogenphosphate	2.0 g
Lactose monohydrate	10.0 g
Agar	15.0 g
Eosin Y	0.40 g
Methylene blue	0.065 g
Water	1000 mL

Mix all the components, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 6.9 - 7.3.

(ix) Fluid Selenite-Cystine Medium

Gelatin peptone	5.0 g
Lactose monohydrate	4.0 g
Trisodium phosphate 12-water	10.0 g
Sodium acid selenite	4.0 g
L-Cystine	0.010 g
Water	1000 mL

Mix all the components, and heat to effect solution. Final pH: 6.8 - 7.2. Do not sterilize.

(x) Fluid Tetrathionate Medium

Casein peptone	2.5 g
Animal tissue peptone	2.5 g
Sodium desoxycholate	1.0 g
Calcium carbonate	10.0 g
Sodium thiosulfate pentahydrate	30.0 g
Water	1000 mL

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

(xi) Fluid Rappaport Medium

Soybean peptone	5.0 g
Sodium chloride	8.0 g
Potassium dihydrogenphosphate	1.6 g
Malachite green oxalate	0.12 g
Magnesium chloride hexahydrate	40.0 g
Water	1000 mL

Dissolve malachite green oxalate and magnesium chloride hexahydrate, and the remaining solids separately in the water, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. For the use, mix the both solutions after sterilization. Final pH: 5.4 - 5.8.

(xii) Brilliant Green Agar Medium

Peptones (animal tissue and casein)	10.0 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Lactose monohydrate	10.0 g
Sucrose	10.0 g
Phenol red	0.080 g
Brilliant green	0.0125 g
Agar	20.0 g
Water	1000 mL

Mix all the components, and boil for 1 minute. Sterilize just prior to use by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 6.7 - 7.1. Cool to about 50°C and pour to petri dishes.

(xiii) XLD (Xylose-Lysine-Desoxycholate) Agar Medium

D-Xylose	3.5 g
L-Lysine monohydrochloride	5.0 g
Lactose monohydrate	7.5 g
Sucrose	7.5 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red	0.080 g
Sodium desoxycholate	2.5 g
Sodium thiosulfate pentahydrate	6.8 g
Ammonium iron (III) citrate	0.80 g
Agar	13.5 g
Water	1000 mL

Mix all the components, and boil to effect solution. pH after boiling: 7.2 - 7.6. Do not sterilize in an autoclave or overheat. Cool to about 50°C and pour to petri dishes.

(xiv) Bismuth Sulfite Agar Medium

Meat extract	5.0 g
Casein peptone	5.0 g
Animal tissue peptone	5.0 g
Glucose	5.0 g
Trisodium phosphate 12-water	4.0 g
Iron (II) sulfate heptahydrate	0.30 g
Bismuth sulfite indicator	8.0 g
Brilliant green	0.025 g
Agar	20.0 g
Water	1000 mL

Mix all the components, and boil to effect solution. pH after boiling: 7.4 - 7.8. Do not sterilize in an autoclave or overheat. Cool to about 50°C and pour to petri dishes.

(xv) TSI (Triple Sugar Iron) Agar Medium

Casein peptone	10.0 g
Animal tissue peptone	10.0 g
Lactose monohydrate	10.0 g
Sucrose	10.0 g
Glucose	1.0 g
Ammonium iron (II) sulfate hexahydrate	0.20 g
Sodium chloride	5.0 g
Sodium thiosulfate pentahydrate	0.20 g
Phenol red	0.025 g
Agar	13.0 g
Water	1000 mL

Mix all the components, and boil to effect solution. Distribute in small tubes and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.1 - 7.5. Use as a slant agar medium. The medium containing 3 g of meat extract or yeast extract additionally, or the medium containing ammonium iron (III) citrate instead of ammonium iron (II) sulfate hexahydrate may be used.

(xvi) Cetrimide Agar Medium

Gelatin peptone	20.0 g
Magnesium chloride hexahydrate	3.0 g
Potassium sulfate	10 g
Cetrimide	0.30 g
Glycerin	10 mL
Agar	13.6 g
Water	1000 mL

Dissolve all solid components in the water, and add the glycerin. Heat, with frequent agitation, boil for 1 minute, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.0 - 7.4.

(xvii) NAC Agar Medium

Peptone	20.0 g
Dipotassium hydrogenphosphate	0.3 g
Magnesium sulfate heptahydrate	0.2 g
Cetrimide	0.2 g
Nalidixic acid	0.015 g
Agar	15.0 g
Water	1000 mL

Final pH: 7.2 - 7.6. Do not sterilize. Dissolve by warming.

(xviii) Pseudomonas Agar Medium for Detection of Fluorescin

Casein peptone	10.0 g
Animal tissue peptone	10.0 g
Dipotassium hydrogenphosphate	1.5 g
Magnesium sulfate heptahydrate	1.5 g
Glycerin	10 mL
Agar	15.0 g
Water	1000 mL

Dissolve all the solid components in the water, and add the glycerin. Heat, with frequent agitation, boil for 1 minute, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.0 - 7.4.

(xix) Pseudomonas Agar Medium for Detection of Pyocyanin

Gelatin peptone	20.0 g
Magnesium chloride hexahydrate	3.0 g
Potassium sulfate	10.0 g
Glycerin	10 mL
Agar	15.0 g
Water	1000 mL

Dissolve all the solid components in the water, and add the glycerin. Heat, with frequent agitation, boil for 1 minute, and sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.0 – 7.4.

(xx) Vogel-Johnson Agar Medium

Casein peptone	10.0 g
Yeast extract	5.0 g
D-Mannitol	10.0 g
Dipotassium hydrogenphosphate	5.0 g
Lithium chloride	5.0 g
Glycine	10.0 g
Phenol red	0.025 g
Agar	16.0 g
Water	1000 mL

Mix all the components, and boil for 1 minute to effect solution. Sterilize by heating in an autoclave at 121°C for 15 to 20 minutes, and cool to between 45°C and 50°C. pH after sterilization: 7.0 - 7.4. To this solution add 20 mL of sterile potassium tellurite solution (1 in 100), and mix.

(xxi) Baird-Parker Agar Medium

Casein peptone	10.0 g
Meat extract	5.0 g
Yeast extract	1.0 g
Lithium chloride	5.0 g
Glycine	12.0 g
Sodium pyruvate	10.0 g
Agar	20.0 g
Water	950 mL

Mix all the components. Heat the mixture with frequent agitation, and boil for 1 minute. Sterilize by heating in an autoclave at 121°C for 15 to 20 minutes, and cool to between 45°C and 50°C. pH after sterilization: 6.6 - 7.0. To this solution add 10 mL of sterile potassium tellurite solution (1 in 100) and 50 mL of egg-yolk emulsion. Mix gently, and pour into petri dishes. Prepare the egg-yolk emulsion by mixing egg-yolk and sterile saline with the ratio of about 30% to 70%.

(xxii) Mannitol-Salt Agar Medium

Casein peptone	5.0 g
Animal tissue peptone	5.0 g
Meat extract	1.0 g
D-Mannitol	10.0 g
Sodium chloride	75.0 g
Phenol red	0.025 g
Agar	15.0 g
Water	1000 mL

Mix all the components. Heat with frequent agitation, and boil for 1 minute. Sterilize by heating in an autoclave at 121°C for 15 to 20 minutes. pH after sterilization: 7.2 – 7.6.

36. Microbial Limit Test for Crude Drugs

This chapter provides tests for the qualitative and quantitative estimation of viable microorganisms present in crude drugs. It includes tests for total viable count (aerobic bacteria and fungi) and specified microbial species (Enterobacteria and other gram-negative bacteria, *Escherichia coli, Salmonella*, and *Staphylococcus aureus*). Microbial limit test must be carried out under conditions designed to avoid accidental microbial contamination of the preparation during the test. When test specimens have antimicrobial activity, or contain antimicrobial substances, any such antimicrobial properties must be eliminated by means of procedures such as dilution, filtration, neutralization or inactivation. For the test, use a mixture of several portions selected at random from the bulk or from the contents of a sufficient number of containers. If test specimens are diluted with fluid medium, the test should be performed quickly. In performing the test, precautions must be taken to prevent biohazard.

1. Total viable aerobic count

This test determines mesophilic aerobic bacteria and fungi (molds and yeasts) which grow under aerobic conditions. Psychrophilic, thermophilic, basophilic, and anaerobic bacteria, and microorganisms which require specific ingredients for growth, may give a negative result, even if a significant number exists in the test speciments. The test may be carried out using one of the following 4 methods, i.e., pour plate method, spread plate method, serial dilution method (most probable number method) or membrane filtration method. Use an appropriate method depending on the purpose. An automated method may be used for the test presented here, provided it has been properly validated as giving equivalent or better results. Different culture media and temperature are required for the growth of bacteria and fungi (molds and yeasts). The serial dilution method is applicable only to the enumeration of bacteria.

Sampling and Preparation of the test specimens

Unless otherwise specified, samples should be taken by the following methods.

(1) When crude drugs to be sampled are small-sized, cut or powdered, 50 to 250 g of sample should be taken after mixing thoroughly.

(2) When crude drugs to be sampled are large-sized, 250 to 500 g of sample should be taken after mixing thoroughly and cutting.

(3) When the mass of each single piece of the crude drug is not less than 100 g, not less than 5 pieces should be taken for a sample, or not less than 500 g of the sample should be taken after cutting to a suitable size and mixing thoroughly. If necessary, cut more for use.

(4) When crude drugs to be sampled are in the form of a solution or a preparation, the sample should be taken after mixing thoroughly.

(5) An insoluble solid should be taken after reducing the substance to a moderately fine powder.

Preparation of the test fluid

Phosphate Buffer, pH 7.2, Buffered Sodium Chloride-Peptone Solution, pH 7.0 or fluid medium used for the test is used to suspend or dilute the test specimen. Unless otherwise specified, usually take 10 g or 10 mL of the test specimen, and suspend or dissolve it in 90 mL of the buffer or fluid medium specified. A test specimen as a suspension must be shaken for 10 minutes. If necessary, for crude drugs to which microorganisms might adhere, repeat the same method and use this as the test fluid. A different quantity or volume may be used if the nature of the test specimen requires it. The pH of the test fluid is adjusted to between 6 and 8. The test fluid must be used within an hour after preparation.