

## B. GENERAL TESTS

### Microbial Limit Tests

The Microbial Limit Tests are designed to perform the qualitative and quantitative estimations of specific viable microorganisms present in samples. It includes tests for total viable count (bacteria and fungi) and *Escherichia coli*. The most care must be taken in performing the tests, so that microbial contamination from the outside can be avoided. When test samples have antimicrobial activity or when they include antimicrobial substances, these antimicrobial properties must be eliminated by dilution, filtration, neutralization, inactivation, or other appropriate means. The tests should be conducted for samples prepared by mixing multiple portions randomly chosen from individual ingredients or products. When samples are diluted with fluid medium, the tests must be conducted quickly. Due attention must be paid to the effective quality control and the prevention of biohazard.

#### 1. Total viable aerobic count

This test is to determine 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 they exist in a significant number. There are four methods for this test: membrane filtration method, pour plate method, spread plate method, and serial dilution method (most probable number method). An appropriate method should be taken from among these four, depending on purposes. If automated methods are comparable or superior in sensitivity and accuracy to the methods given here, they may be used. Different media and incubation temperature are required for the growth of bacteria and fungi (molds and yeasts). The serial dilution method is applicable only to bacteria.

#### Preparation of test fluids

To dissolve or dilute the sample, use phosphate buffer (pH 7.2), sodium chloride-peptone buffer solution, or fluid medium used for the test. Unless otherwise specified, use 10 g or 10 ml of the sample. However, a different weight or volume of the sample should be used, depending on the nature of the sample. Adjust the test fluid to pH 6 - 8. Use the test fluid within one hour after preparation.

Fluid samples or soluble solid samples Take 10 g or 10 ml of the sample and mix with the buffer or fluid medium given above to make 100 ml. Use this mixture as the test fluid. For a fluid sample including insoluble substances, shake well just before mixing make a homogeneous suspension.

Insoluble solid samples Take 10 g of the sample, grind to a fine powder, and suspend it in the buffer or fluid medium given above to make 100 ml. Use this suspension as the sample fluid. A larger volume of the buffer or fluid medium than specified here may be used to make a suspension, depending on the nature of the

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sample. If necessary, a blender may be used to disperse the insoluble particles well in the suspension. Also, an appropriate surfactant (e.g., 0.1% w/v polysorbate 80) may be added to help dissolve the sample.

Fatty samples For semisolid samples and liquids consisting mainly of lipid, take 10 g or 10 ml of the sample, emulsify the sample in the buffer or fluid medium given above using a surfactant such as polysorbate 20 or polysorbate 80, and make to 100 ml. Use this emulsified sample as the sample fluid. If necessary, warm at a temperature not exceeding 45 °C to emulsify the sample. Avoid warming for not longer than 30 minutes.

### Procedure

#### (1) Membrane Filtration Method

This method is applied to the sample which contains antimicrobial substances.

Use membrane filters of an appropriate material with a pore size of 0.45 µm or less. Filters about 50 mm across are recommended, but other sizes may be used. Sterilize the filters, filtration apparatus, media, and other apparatus used. Usually, measure two test fluids of 10 ml each, pass each sample through a separate filter. Dilute the pretreated test fluid if the bacteria concentration is high, so that 10 - 100 colonies can develop per filter. After filtration, wash each filter three times or more with an appropriate liquid such as phosphate buffer, sodium chloride-peptone buffer, or fluid medium. The volume of the washings should be about 100 ml each. If the filter used is not about 50 mm in diameter, use an appropriate volume of washing, depending on the size of the filter. If the sample includes lipid, polysorbate 80 or an appropriate emulsifier may be added to the washings. After filtration, for bacteria detection, place the two filters on a plate of soybean-casein digest agar medium, and for fungi detection, add an antibiotic to the medium and place them on a plate of one of Sabouraud glucose agar, potato-dextrose agar, or GP agar media. Incubate the plates at least for 5 days at 30-35 °C for bacteria detection and at 20-25 °C for fungi detection, and count the number of colonies. If counts obtained are considered to be reliable in shorter incubation time than 5 days, these counts may be adopted for calculation of the viable count.

#### (2) Pour Plate Method

Use petri dishes 9-10 cm in diameter. Use at least 2 agar media for each dilution. Take 1 ml of the test fluid or its dilution into each petri dish aseptically, add to each dish 15 - 20 ml of sterilized agar medium, previously melted and kept below 45 °C, and mix. For bacteria detection, use soybean-casein digest agar medium and for fungi detection, use one of Sabouraud glucose agar, potato-dextrose agar, and GP agar media, to which antibiotic has previously been added. After the agar solidifies, incubate at least for 5 days at 30 - 35 °C for bacteria detection and at 20 - 25 °C for fungi detection. If a large number of colonies develop, calculate viable counts based on counts obtained from plates with not more than 300 colonies per plate for bacteria

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detection and from plates with not more than 100 colonies per plate for fungi detection. If counts are considered to be reliable in a shorter incubation time than 5 days, these counts may be adopted.

### (3) Spread Plate Method

Place 0.05-0.2 ml of the test fluid on the solidified and dried surface of the agar medium and spread it uniformly using a spreader. Proceed under the same conditions as for the Pour Plate Method, especially about petri dishes, agar media, incubation temperature and time, and calculation method.

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

Use 12 test tubes: 9 containing 9 ml of soybean-casein digest medium each and 3 containing 10 ml of the same medium each for control. Prepare dilutions using the 9 tubes. First, add 1 ml of the test fluid to each of three test tubes and mix to make 10-times dilutions. Second, add 1 ml of each of the 10-times dilutions to each of another three test tubes and mix to make 100-times dilutions. Third, add 1 ml of each of the 100-times dilutions to each of the remaining three test tubes and mix to make 1,000-times dilutions. Incubate all 12 test tubes for at least 5 days at 30 - 35 °C. No microbial growth should be observed for the control test tubes. If the determination of the result is difficult or if the result is not reliable, take a 0.1ml fluid from each of the 9 test tubes and place it to an agar medium or fluid medium, incubate all media for 24 - 72 hours at 30 - 35 °C, and check them for the absence or presence of microbial growth. Calculate the most probable number of microorganisms per ml or gram of the sample, using the table given below.

The number of test tubes in which microbial growth is observed, when the amount of the sample given below (per test tube) is added			The most probable number of microorganisms per gram or ml
0.1 g or 0.1 ml	0.01 g or 0.01 ml	1 mg or 1 µl	
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

Note: When the number of test tubes showing microbial growth is not more than

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two for test tubes containing 0.1 g or 0.1 ml of a sample, the most probable number of microorganisms is possibly 100 or less per gram or ml of the sample.

### **Effectiveness of culture media and confirmation of anti-microbial substances**

Use the following strains or their equivalents for tests: *Escherichia coli* (ATCC 8739, NCIB 8545), *Bacillus subtilis* (ATCC 6633, NCIB 8054), *Staphylococcus aureus* (ATCC 6538, NCIB 8625), *Candida albicans* (ATCC 2091, ATCC 10231). Use soybean-casein digest medium and incubate at 30-35 °C for bacteria and at 20 - 25 °C for *Candida albicans*.

Dilute each of the prepared cultures using sodium chloride-peptone buffer or phosphate buffer to prepare a sample suspension containing 50-200 viable microorganisms per ml. When the prepared medium is inoculated with 1 ml of the sample suspension and incubated for 5 days at a specified temperature, clear evidence of growth should be observed and good recovery of the microorganisms should be obtained. If the ratio of the number of microorganisms for the test medium to control medium does not fall between 0.2 to 1 and 5 to 1, then the cause of this disparity must be eliminated using appropriate means such as dilution, filtration, neutralization, and inactivation. Use the sodium chloride-peptone buffer or phosphate buffer used for confirmation as the control in order to verify the sterility of the medium and diluent and the aseptic performance of the test.

### **2. Coliform test**

This test is to determine *Escherichia coli*. *Escherichia coli*, target for this test, becomes an important index to evaluate microbial contamination in ingredients and intermediate products as well as in the finished products. *Escherichia coli* should not be present in each of them.

#### **Preparation of test fluid**

Unless otherwise specified, proceed as directed in the preparation of test fluid for total viable aerobic count. When a fluid medium is used to dissolve or dilute the sample, use lactose broth medium or BGLB unless otherwise specified.

#### **Procedure**

Take 10 g or 10 ml of the sample, add lactose broth medium to make 100 ml, and incubate for 24 - 72 hours at 30 - 35 °C. When the growth of microorganisms in the tube is observed, shake the tube slightly, take a portion of the fluid using an inoculating loop, streak it on MacConkey agar medium, and incubate for 18 - 24 hours at 30 - 35 °C. Examine the plate for suspicious colonies. If red-brick colonies of Gram-negative rods surrounded by a reddish precipitation zone are not found, the sample is determined to be negative. If colonies meeting the above description are found, then transfer the suspicious colonies individually on the surface of EMB agar medium and incubate for 18 - 24 hours at 30 - 35 °C. Upon examination, if no colonies exhibit a metallic sheen or a blue black color under transmitted light, the sample is

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determined to be negative. For suspected colonies on the plate, conduct the IMViC tests (Indole production test, Methyl red reaction test, Voges-Proskauer test, and Citrate utilization test). When the result given in 1 or 2 in the table below for the four tests is obtained, determine the colonies to be *Escherichia coli*. Rapid detection kits for *Escherichia coli* may be used.

	Indole production test	Methyl red reaction test	Voges-Proskauer test	Citrate utilization test
1	Positive	Positive	Negative	Negative
2	Negative	Positive	Negative	Negative

### Effectiveness of culture media and confirmation of anti-microbial substances

For the confirmation test, incubate ATCC 8739, NCIB 8545, or their equivalents as strain in lactose broth medium for 18 - 24 hours at 30 - 35 °C. Dilute the incubated cultures with a sodium chloride - peptone buffer solution, phosphate buffer, or lactose broth agar medium, to make a suspension containing 1,000 viable microorganisms per ml. If necessary, add 0.1 ml of a separately prepared *Escherichia coli* suspension containing about 1,000 viable microorganisms per ml. Confirm the effectiveness of medium and the presence of anti-microbial substances both with and without the sample.

#### Confirmation

If uncertain result is obtained, conduct the test again with 25 mg or 25 ml of the sample, proceeding as directed in Procedure. If the amount of the sample used is increased, use additional amounts of medium and reagents in proportion to an increase of the sample.

### 3. Buffer solutions and media

Use the buffer solutions and media, given below, for the Microbial Limit Test. Other media may be used if they include similar nutritive ingredients and have similar selectivity and growth-promoting ability toward the microorganism to be tested.

#### (1) Buffer solutions

##### (i) *Phosphate buffer* (pH 7.2)

Stock solution: Dissolve 34 g of monopotassium phosphate in about 500 ml of water, add about 175 ml of sodium hydroxide (4.3 g/100) to adjust to pH 7.1 - 7.3, and add water to make 1,000 ml. Use this solution as the stock solution. Autoclave and cool this solution. Store it at a cool place. For use, dilute the stock solution with water to 800 times its original volume and autoclave for 15 - 20 minutes at 121 °C.

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### (ii) *Sodium chloride-peptone buffer (pH 7.0)*

Monopotassium phosphate	3.56 g
Disodium phosphate	18.23 g
Sodium chloride	4.30 g
Peptone	1.0 g
Water	1,000 ml

Mix all the ingredients and autoclave for 15 - 20 minutes at 121 °C. Its pH becomes 6.9 - 7.1 after autoclaving. 0.1 - 1.0%w/v polysorbate 20 or polysorbate 80 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	1,000 ml

Mix all the ingredients autoclave for 15-20 minutes at 121 °C. Its pH becomes 7.1 - 7.3. after autoclaving.

#### (ii) *Fluid soybean-casein digest medium*

Casein peptone	17.0 g
Soybean peptone	3.0 g
Sodium chloride	5.0 g
Dipotassium phosphate	2.5 g
Glucose	2.5 g
Water	1,000 ml

Mix all the ingredients and autoclave for 15 - 20 minutes at 121 °C. Its pH becomes 7.1 - 7.5.

#### (iii) *Antibiotics-added Sabouraud glucose agar medium*

Peptone (derived from meat and casein)	10.0 g
Glucose	40.0 g
Agar	15.0 g
Water	1,000 ml

Mix all the ingredients and autoclave for 15 - 20 minutes at 121 °C. Its pH becomes 5.4 - 5.8. Immediately before using, to above medium, add the sterilized solution containing 0.10 g of benzylpenicillin potassium and 0.10 g of tetracycline per liter of medium. Benzylpenicillin potassium and tetracycline may be replaced by 50 mg of chloramphenicol per liter of medium.

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### (iv) *Antibiotics-added potato-dextrose agar medium*

Potato extract	4.0 g
Glucose	20.0 g
Agar	15.0 g
Water	1,000 ml

Mix all the ingredients and autoclave for 15 - 20 minutes at 121 °C. Its pH becomes 5.4 - 5.8. Immediately before using, to above medium, add the sterilized solution containing 0.10 g of benzylpenicillin potassium and 0.10 g of tetracycline per liter of medium. Benzylpenicillin potassium and tetracycline may be replaced by 50 mg of chloramphenicol per liter of medium.

### (v) *Antibiotics-added GP (glucose-peptone) agar medium*

Glucose	20.0 g
Yeast extract	2.0 g
Magnesium sulfate	0.5 g
Peptone	5.0 g
Monopotassium phosphate	1.0 g
Agar	15.0 g
Water	1,000 ml

Mix all the ingredients and autoclave for 15 - 20 minutes at 121 °C. Its pH becomes 5.6 - 5.8. Immediately before using, to above medium, add the a sterilized solution containing 0.10 g of benzylpenicillin potassium and 0.10 g of tetracycline per liter of medium. Benzylpenicillin potassium and tetracycline may be replaced by 50 mg of chloramphenicol per liter of medium.

### (vi) *Fluid lactose broth medium*

Meat extract	3.0 g
Gelatin peptone	5.0 g
Lactose	5.0 g
Water	1,000 ml

Mix all the ingredients and autoclave for 15 - 20 minutes at 121 °C. Its pH becomes 6.7 - 7.1. Cool immediately after autoclaving.

### (vii) *BGLB (brilliant green lactose bile) medium*

Peptone	10.0 g
Lactose	10.0 g
Powdered Cattle Bile	20.0 g
Brilliant green	0.0133 g
Water	1,000 ml

Mix all the ingredients and autoclave for 15 - 20 minutes at 121 °C. Its pH becomes 7.0 - 7.4.

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### (viii) *MacConkey agar medium*

Gelatin peptone	17.0 g
Casein peptone	1.5 g
Meat peptone	1.5 g
Lactose	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	1,000 ml

Mix all the ingredients, boil for 1 minute, and autoclave for 15 - 20 minutes at

121 °C. Its pH becomes 6.9 - 7.3.

### (ix) *EBM (Eosin-methylene blue) agar medium*

Gelatin peptone	10.0 g
Dipotassium phosphate	2.0 g
Lactose	10.0 g
Agar	15.0 g
Eosine	0.40 g
Methylene blue	0.065 g
Water	1,000 ml

Mix all the ingredients and autoclave for 15 - 20 minutes at 121 °C. Its pH becomes 6.9 - 7.3.