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*Draft Indian Standard*

# Methods of Sampling and Microbiological Examination of Water and Wastewater

(*Second Revision of IS 1622*)

*भारतीय मानक मसौदा*

# जल के नमूने लेने एवम सूक्ष्म जैविक परीक्षा की पध्दतियाँ

(*दूसरा पुनरीक्षण*)

ICS 13.060.70, 07.100.20

| **Water Quality Sectional Committee, CHD 36** | **Last date for Comments: 29.09.2025** |
|---|---|

FOREWORD

(*Formal clause to be added later*)

Microbiological examination of water and wastewater includes both bacteriological and biological examinations. Bacteriological examination of water is necessary for determining its fitness for use for human consumption, and for use in industries such as food processing and dairy, photofilm, etc. Water used for drinking, food processing and dairying should be free from faecal or sewage contamination because microorganisms causing water-borne diseases such as typhoid and paratyphoid fevers, food poisoning, gastroenteritis, cholera; dysentery and diarrhoea are excreted in the faeces of individuals suffering from the disease. The detection of these pathogenic organisms in a sample of water is difficult and may not always be accomplished with certainty. Bacterial organisms of the coliform and faecal *Streptococci* groups, however, inhabit the intestinal tract of man and animals in great abundance and are readily detectable. Hence their presence in a sample of water is looked upon as an indication of the probable presence of intestinal pathogenic organisms, while their absence from water usually precludes the presence of such pathogens. Tests for the presence of *Clostridium welchii* are also carried out on samples of water for obtaining supplementary evidence of faecal pollution.

The presence of organisms belonging to the groups such as iron bacteria, sulphur bacteria, sulphate reducing bacteria, slime-forming bacteria and gelatin liquefying bacteria is undesirable in water used for drinking purposes, air-

conditioning, paper manufacture and many other industrial uses. Some of these organisms are known to cause corrosion.

Algae and other microscopic plants and animal-life in water may cause odour and taste problems and also affect suitability of the water for use in various industries. However, this standard includes only microscopic examination and enumeration of these organisms.

This standard prescribes laboratory preparation of culture media. However, dehydrated media commercially available may also be employed. Since preparation of culture media and solutions is a critical aspect of water quality testing, the date of receipt of media (dehydrated powder medium) and the date of opening should be recorded. Where practical, appropriate capacity bottles should be used to ensure minimum exposure.

When a new lot of media is used, the contents should be tested for expected performance. It should be stored in a cool, dry place away from sunlight.

This standard was first published in 1964. The first revision of this standard was published in 1981 incorporating two amendments issued to IS 1622, the membrane filter technique for coliforms and faecal *Streptococci (Enterococci)*, test for faecal coliforms, delayed incubation method for total coliforms and spore staining technique for *Clostridium welchii*.

In this second revision following changes have been incorporated:

a) Amendment 1 to 4 published have been incorporated;
b) Deleted the existing method of test for slime forming bacteria as this method has become outdated and not being used in labs anymore;
c) Annex C has been updated;
d) References, and ICS No. have been updated; and
e) Other editorial changes have been done to bring the standard in the latest style and format of Indian Standards.

For the preparation of culture media, use only purified water, i.e. distilled, demineralized, deionized or produced by reverse osmosis, or of equivalent quality free from substances likely to inhibit or influence the growth of the microorganisms under the test conditions e.g. traces of chlorine, traces of ammonia and traces of metal ions.

In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS 16122: 2013 ‘Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations’.

## 1 SCOPE

This standard prescribes methods of sampling and microbiological examination of water and wastewater.

## 2 REFERENCES

The standards listed in Annex A contain provisions which, through reference in this text, constitute provisions of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of these standards.

## 3 SAMPLING

### 3.1 Sampling for Microbiological Examination

**3.1.1** *Sampling Bottles*

The sampling for microbiological examination shall be done as per IS 17614 (Part 25).

**3.1.2** *Size of the Sample*

The volume of the sample shall be sufficient for carrying out all the tests required. The sampling bottle should not be filled up to the brim and 2 cm to 3 cm space should be left for effective shaking of the bottle.

**3.1.3** *Preservation and Storage*

The preservation and storage of water and wastewater samples for microbiological analysis shall be done as specified in clause **5** and Annex B of IS 17614 (Part 25).

**3.1.4** *Identifying Data*

All samples shall be legibly marked with the source of the sample, date and time of collection, and the name and designation of the person collecting the sample. As results of laboratory examination of the sample shall always be considered in conjunction with the sanitary survey of the water supply system, it is important that when submitting a sample for analysis, complete and accurate data of the nature and source of the supply, topography of the water shed, possibility of pollution gaining access to the source, methods of treatment adopted, the condition of the distribution system, and such other information as would be relevant from sanitary viewpoint is furnished. It shall be ascertained whether the tap from where the sample is collected is supplying water from a service pipe directly connected with the main or with a cistern or a storage tank. Specimen form for such information is given in Annex B.

**3.2** *Procedure for Sampling*

**3.2.1** The sample of natural plankton producing water shall be collected in clean, neutral glass bottles of 2 litre capacity, fitted with ground glass stopper. The bottle shall not be filled completely and a small air space shall be left below the stopper.

**3.2.2** A concentrated sample or catch of the plankton organisms and other particulate suspended matter in the water shall be collected with the aid of a plankton net. The net shall be conical in shape, of suitable size, with a circular mouth and made of bolting silk cloth with more than 6 000 meshes/cm$^2$. The net shall be hauled through the water in an oblique or horizontal direction for a certain distance, lifted from the water, allowed to drain and the organisms in the net washed down into a container by splashing water on the outer surface of the net. The catch shall then be made up to a known volume with the water. Nets provided with closing devices shall be used for collecting samples of plankton from different depths.

**3.2.3** For quantification of the plankton it is recommended to strain known volume of water through plankton net. Number of organisms caught in the catch may be used to calculate back the original number per unit volume of the sample.

**3.2.4** Sampling phytoplankton with nets provides data of limited value since the total count, volume, bio-mass and species composition are not measurable. Because of selectivity of mesh size the smaller plankton (nano-planktons) which may contribute as much as 60 percent of the total bio-mass are not collected. In such situation sedimentation membrane filtration or centrifugation are recommended.

**3.2.5** The samples shall be examined within 2 h to 3 h after collection, when the organisms are alive. If this is not possible, the samples shall be preserved in ice/ice packs or in the refrigerator (3 °C to 4 °C) for a few days taking care not to allow it to freeze. If the examination is to be made later, the samples shall be preserved as follows:

**3.2.5.1** To each 100 ml of the sample, add about 3 ml of 2 percent formaldehyde solution (made by diluting 5 ml of 37 percent to 40 percent aqueous formaldehyde solution to 100 ml with distilled water), 0.5 ml of 20 percent detergent solution made by diluting 20 ml of liquid detergent to 100 ml with distilled water and 5 drops to 6 drops of copper sulphate solution 21 percent (*v/v*). This preservative maintains cell colouration and is effective indefinitely. Store the preserved sample in the dark.

## 4 MICROBIOLOGICAL EXAMINATION

### 4.1 General Equipment

**4.1.1** *Equipment*

**4.1.1.1** *General*

It is essential for accurate and satisfactory laboratory work that good equipment in proper working order be provided. Thus, the minimum laboratory equipment listed, must be available in an approved laboratory and all items should meet the minimum requirements given. Additional items of equipment not listed, will be required in an approved laboratory and they should meet similar standards of quality and operation.

**4.1.1.2** *Vertical Laminar air flow unit or bio-safety cabinets*

For use of pouring and plating of sterile media and testing of samples to avoid contamination.

**4.1.1.3** *Incubators*

**4.1.1.3.1** Incubators shall maintain a uniform and constant temperature 35 °C to 37 °C or 44 °C to 45 °C at all times in all parts.

**4.1.1.3.2** Suitable temperature monitoring devices with least count 0.1 °C, should be installed within the incubator, to record the daily readings of the temperatures.

**4.1.1.3.3** It is desirable, where ordinary room temperatures very excessively, that laboratory incubators be kept in special rooms which may be maintained at a few degrees below the recommended incubator temperature.

**4.1.1.4** *Water-baths*

Water-baths are useful for carrying out the 44 °C fermentation test. They should be capable of maintaining a temperature of 44 °C to 45 °C. Suitable temperature controlling cum monitoring devices with least count of 0.1 °C should be used. Water baths should be adequately insulated against heat loss.

**4.1.1.5** *Sterilizers*

The ovens and autoclaves shall be operated as per IS 16122.

**4.1.1.5.1** *Ovens*

Hot-air sterilizing ovens should be of sufficient size to prevent crowding of the interior and constructed to give uniform and adequate sterilizing temperatures and equipped with suitable thermometers capable of registering accurately in the range 170 °C ± 10 °C for at least 1 h. The use of temperature-recording instrument is optional.

**4.1.1.5.2** *Autoclaves*

Autoclaves should be of size sufficient to prevent crowding of the interior, and constructed to provide uniform temperatures within chambers up to and including the sterilizing conditions of 121 °C ± 3 °C for at least 15 min and equivalent pressure 15 psig ± 0.5 psig for at least 15 min. They should be equipped with pressure gauges, properly adjusted safety valves, and accurate temperature controlling cum indicator devices.

NOTE — Timer as well as pressure and temperature recording devices are optional.

**4.1.1.6** *Glassware and plasticwares*

**4.1.1.6.1** Clean glassware is critical to ensure valid results. Previously used or new glassware should be thoroughly cleaned with only purified water, i.e. distilled, demineralized, deionized or produced by reverse osmosis, or of equivalent quality free from substances likely to inhibit or influence the growth of the microorganisms under the test conditions e.g. traces of chlorine, traces of ammonia and traces of metal ions. General glassware such as pipettes, dilution bottles, test tubes and Petri dishes may be used. Disposable plasticware such as micropipettes tips and sterilized Petri dishes may be used.

**4.1.1.6.2** If test tubes are used for culture work, these shall be plugged with non-absorbent cotton or caps and shall be sterilized by autoclaving at 121 °C ± 3 °C for at least 15 min and equivalent pressure 15 psig ± 0.5 psig for at least 15 min.

**4.1.1.6.3** *Pipettes*

**4.1.1.6.3.1** Pipettes may be of appropriate volume (generally 0.1 ml to 10 ml) provided it is found by actual test that they deliver accurately the required amount in the way they are used. Calibrated pipettes should be used.

**4.1.1.6.3.2** If glass pipettes are used, it shall be sterilized by dry heat sterilization process using hot air oven at 170 °C ± 10 °C for at least 1 h or by autoclaving at 121 °C ± 3 °C for at least 15 min and equivalent pressure 15 psig ± 0.5 psig for at least 15 min.

**4.1.1.6.3.3** If plastic pipettes or tips of micropipettes are used, they shall be sterilized by autoclaving at 121 °C ± 3 °C for at least 15 min and equivalent pressure 15 psig ± 0.5 psig for at least 15 min.

**4.1.1.6.4** *Dilution bottles or tubes*

Bottles or tubes of resistant glass, preferably borosilicate, closed with caps equipped with or without liners that do not produce toxic or bacteriostatic compounds on sterilization, should be used. Cotton plugs may be used as closures. Graduation levels may be indelibly marked on the side. Sterile disposable or reusable plastic tubes may be used.

**4.1.1.6.5** *Petri dishes*

**4.1.1.6.5.1** Petri dishes with appropriate dimensions, vented or without vent made of glass or plastic should be used. The bottoms of the dishes should be free from bubbles and scratches and should be flat, so that the medium will be of uniform thickness throughout the plate.

**4.1.1.6.5.2** If glass Petri dishes are used, it shall be sterilized by dry heat sterilization process using hot air oven at 170 °C ± 10 °C for at least 1 h or by autoclaving at 121 °C ± 3 °C for at least 15 min and equivalent pressure 15 psig ± 0.5 psig for at least 15 min. When available, sterilized disposable plastic petri dishes may be used as an alternative.

**4.1.1.7** *Inoculating needle and loop*

Inoculating needle and loop of appropriate dimensions made of nichrome, platinum or platinum-iridium alloy should be used. It shall be sterilized before use. Alternatively, sterile disposable needles and loops may be used.

**4.1.1.8** *Refrigerators*

Laboratory should have a refrigerator of sufficient capacity for the required work load and capable of maintaining a continuous temperature in range 5 °C ± 3 °C.

**4.1.1.9** *Colony counter*

An effective device for examining colonies, providing a magnification of 10×, should be available.

**4.2 Standard Plate Count**

**4.2.1** *General*

**4.2.1.1** Standard plate count (which is an empirical method) serves to indicate the efficiency of certain processes in water treatment, particularly coagulation, filtration and disinfection and the cleanliness of the mains, reservoirs, etc. It provides an estimate of the general hygienic quality of water, which is important where large scale preparation of food and drink is concerned. Low counts are of importance for avoiding food spoilage, while higher plate counts give the earliest sign of contamination.

**4.2.1.2** The standard plate count method is a direct measurement of the viable aerobic and facultative anerobic bacteria in a water environment capable of growth on the selected plating medium. The procedure does not allow the more fastidious aerobes or obligate anaerobes to develop. Also, the bacteria of possible importance in water such as *Crenothrix*, *Sphaerotilus* and Actinomycetes will not develop within the incubation period specified for potable water. Clumps of organisms in the water sample which are not broken up by shaking, results in under estimation of bacterial density, since an aggregate of cells will appear as one colony on the growth medium. The number of types of bacteria that develop are influenced by the time and comparative temperature of incubation, the pH of the medium, the level of oxygen, the presence of specific nutrients on the growth medium, competition among cells for nutrients, antibiosis, mediation, etc.

**4.2.2** In solid medium counting of organisms depends on the fact that living cells will proceed to multiply and in time will produce sufficient progeny to form a colony visible to naked eye. Since bacteria occur in water as single cells, pairs, groups, chains or even dense clumps, not every individual living cell will develop into a separate colony on incubation. Therefore, the number of colonies appearing on a plate does not necessarily represent the total number of organisms present in test volume. The results are expressed as colony forming unit (CFU) per ml.

**4.2.3** *Medium and Reagent*

**4.2.3.1** *Plate count agar*

**4.2.3.1.1** *Composition*

The composition of Plate count agar shall be as specified in Table 1.

**Table 1 Composition of Plate Count Agar**

(*Clause* 4.2.3.1.1**)**

| S. No. | Parameter | Requirements |
|---|---|---|
| (1) | (2) | (3) |
| i) | Enzymatic digestion of casein | 5.0 g |
| ii) | Yeast extract | 2.5 g |
| iii) | Glucose, anhydrous ($C_6H_{12}O_6$) | 1.0 g |
| iv) | Agar (*see* Note) | 9 g to 18 g |
| v) | Water | 1 000 ml |

NOTE — Depending on the gel strength of the agar.

**4.2.3.1.2** Dissolve the components or the dehydrated medium in the 1 000 ml water, by heating if necessary. Mix thoroughly and leave to stand for several minutes. Adjust the *p*H, if necessary, so that after sterilization it is 7.0 ± 0.2 at 25 °C. Dispense the medium into tubes, flasks or bottles of suitable capacity. Sterilize in an autoclave at 121 °C for 15 min. lf the medium is to be used immediately, cool it to 44 °C to 47 °C in a water bath before use. If not, store it in the dark at a temperature of (5 ± 3) °C for no longer than 3 months, under conditions which do not allow any change in its composition and properties. Before beginning the microbiological examination, completely melt the medium, then cool it to 44 °C to 47 °C in a water bath before use. Use the molten agar as soon as possible; it should not be retained for more than 4 h.

**4.2.3.2** *Diluents*

The diluents as specified in clause **6.3** of IS 15188 should be used.

**4.2.4** *Procedure*

**4.2.4.1** *Preparation and dilution*

**4.2.4.1.1** Shake the samples appropriately to ensure homogeneity. Withdraw required portion with a sterile pipette and introduce into the sterile petri dish or dilution tube.

**4.2.4.1.2** Clean and dirty water should be separated and processed using separate equipment in separate areas to reduce cross-contamination risk where possible. Alternatively, process batches of clean waters before dirty water. Before examination, mix the sample thoroughly by agitation to achieve uniform distribution of microorganisms and other particles. This can be achieved by inversion of the sample or by a to-and fro motion. Depending on the nature of the water and the microbial content anticipated, make any necessary dilutions at this stage. For plate counts, ten-fold dilutions are usually used. For ten-fold dilutions, aseptically measure nine volumes of the diluent and one volume of the water sample into sterile dilution bottles or tubes.

**4.2.4.1.3** Alternatively, volumes of diluent pre-sterilized in screw-capped bottles are used and volumes verified after autoclaving. One or more ten-fold dilutions are made by transferring one volume of water sample to nine volumes of diluent. Mix the solution thoroughly with a fresh pipette or by mechanical means and transfer one volume of this dilution to another nine volumes of diluent. Repeat these steps as many times as required. Prepare sufficient volumes of each dilution for all the tests to be carried out on each water sample. For dilutions of other magnitudes, the volume of diluent to volume of sample is adjusted accordingly. For example, four-fold dilutions can be made as described above for ten-fold dilutions, only in this case one volume of water sample is mixed with three volumes of diluent. Another approach is to use a tenfold dilution series, but filter both 10 ml and 30 ml volumes. If the concentration of the target organism is expected to be high, hundred-fold dilution steps may be used by mixing one volume of water sample with 99 volumes of diluent, but such large intervals between measurements can adversely affect the reliability of the test results.

**4.2.4.2** *Plating*

Place 1.0 ml, or 1.0 ml of other suitable dilution to be used for plating in the petri dish first. Then add to the petri dish 10 ml to 15 ml of melted plate count agar medium at a temperature of 44 °C to 47 °C (tolerable to the skin). The plate count agar and the sample shall be thoroughly mixed over the bottom of the petri dish by tilting and rotating the dish several times. Allow the plate to solidify and place immediately in the incubator in an inverted position.

**4.2.4.3** *Incubation*

Incubate the plates at 37 °C for 24 h to 48 h.

NOTE — The incubation may be carried out for 72 h where necessary.

**4.2.4.4** *Counting*

In preparing plates, take original sample and appropriate diluted samples to obtain measurable bacterial counts from 30 CFU per plate to 300 CFU per plate. Always have two or more plates for each dilution. Report the result as the average of all plates falling within limits. It is not desirable to plant more than 1.0 ml in a plate. If the colonies are more than 300 or less than 30 from 1 ml sample, disregard it. In practice, counts less than 30 occur when chlorinated water samples are plated. When the number of colonies is more than 300 in a plate, report the count at 'TNTC ' (too numerous to count). Counting shall be done with an approved counting aid, such as colony counter, Record the number of incubation and days and temperature of incubations.

**4.2.4.5** The expression of results shall be as per clause **9.1.8** of IS 15188.

**4.3 Test for Coliforms, Fecal (Thermotolerant) Coliform, and *E. coli***

**4.3.1** *Test for Coliforms*

The Coliform group includes all of the aerobic and facultative anaerobic gram negative, non-spore forming rod shaped bacteria which ferment lactose with gas formation within 48 h at 37 °C ± 1°C. The standard test for the estimation of number of the coliform groups, Feacal coliform, and *E. coli* may be carried out either by the multiple tube dilution test (presumptive test, confirmed test, or completed test) or by the membrane filtration technique as specified in IS 15185 or Enzyme substrate method as specified in IS 17819.

**4.3.1.1** *Multiple tube dilution test* (MTD)

The presumptive, confirmed and completed tests are presented as total independent procedures. In using these procedures, the worker must know what is to be the stage at which the test is to be ended, and details of the procedure throughout. Thus, if the worker knows that the test will end at the presumptive or confirmed test, the test can be stopped at that test stage itself. All the necessary information regarding the sample should be recorded. It is convenient to express the results of the examination of replicate tubes and dilutions in terms of the most probable number. This term is actually an estimate based on certain probability formulae. The most satisfactory information is obtained when the largest portion examined shows no gas in all or a majority of the tubes. The Most Probable Number (MPN) value for a given sample is obtained by the use of MPN tables. Standard practice in water analysis is to plant five tubes for each dilution and a minimum three different dilutions are employed. The results are to be recorded in the proper form.

The Table of MPN are given in Annex C. Alternatively, MPN calculator as specified in clause **9.2.7.4** of IS 15188 may also be referred.

**4.3.1.1.1** *Media and reagents*

**4.3.1.1.1.1** *Dilution water — see* **4.2.3.2.**

**4.3.1.1.1.2** *MacConkey broth*

**4.3.1.1.1.2.1** This is used as a presumptive medium for the enumeration of Coliform bacteria in water samples. The composition of MacConkey broth is as specified in Table 2.

**Table 2 Composition of MacConkey Broth**

(*Clause* 4.3.1.1.2.1)

| S. No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | Peptone | 20 g |
| ii) | Lactose | 10 g |
| iii) | Sodium chloride | 5 g |
| iv) | Bile salt | 5 g |
| v) | Distilled water | 1 000 ml |

**4.3.1.1.1.2.2** In place of bile salt, which is a commercial product, sodium taurocholate or sodium tauroglycocholate may be used.

**4.3.1.1.1.2.3** Dissolve all the ingredients and adjust the *p*H to 7.4 ± 0.2. After adjusting the *p*H, add 1 ml of 1 percent alcoholic solution of bromocresol purple or 5 ml of 1 percent aqueous solution of neutral red. This will be the single strength medium. Distribute 10 ml of the medium into 150 mm × 15 mm test tubes and add a Durham's tube (25 mm × 5 mm) in an inverted position. Plug the tubes with non-absorbent cotton or sterilizable plastic caps and sterilize by autoclaving at 121 °C ± 3 °C for atleast 15 min and equivalent pressure 15 psig ± 0.5 psig for atleast 15 min. This medium is used for 1 ml and the decimal dilutions of the water and wastewater sample. For 10 ml and larger aliquots a double strength medium is used. For the double strength medium add the above ingredients in double the quantities in 1 000 ml of distilled water. This medium is dispensed into 10 ml quantities in 150 mm × 18 mm test tubes added with Durham's tube and sterilized.

**4.3.1.1.1.2.4** Alternatively, Lauryl sulphate tryptose broth may be used.

**4.3.1.1.1.3** *Lauryl sulfate tryptose broth*

**4.3.1.1.1.3.1** *Composition*

The composition of Lauryl sulfate tryptose broth is given at Table 3.

**Table 3 Composition of Lauryl Sulfate Tryptose Broth**

(*Clause* 4.3.1.1.3.1)

| S. No. | Parameter | Requirements | |
|---|---|---|---|
| | | Double-strength medium | Single-strength medium |
| (1) | (2) | (3) | (4) |

| | | | |
|---|---|---|---|
| i) | Enzymatic digest of milk and animal proteins | 40 g | 20 g |
| ii) | Lactose ($C_{12}H_{22}O_{11}.H_2O$) | 10 g | 5 g |
| iii) | Dipotassium hydrogen phosphate ($K_2HPO_4$) | 5.5 g | 2.75 g |
| iv) | Potassium dihydrogen phosphate ($KH_2PO_4$) | 5.5 g | 2.75 g |
| v) | Sodium chloride | 10 g | 5 g |
| vi) | Sodium lauryl sulfate | 0.2 g | 0.1 g |
| vii) | Water | 1 000 ml | 1 000 ml |

**4.3.1.1.1.3.2** *Preparation*

**4.3.1.1.1.3.2.1** Dissolve the different components or the dehydrated complete medium in the water, by heating if necessary.

**4.3.1.1.1.3.2.2** Adjust the *p*H, if necessary, so that after sterilization it is 6.8 ± 0.2 at 25 °C.

**4.3.1.1.1.3.2.3** Dispense the media in quantities of 10 ml into tubes of dimensions of approximately 16 mm × 160 mm containing Durham tubes in the case of single-strength medium, and into test tubes of dimensions of approximately 20 mm × 200 mm (not containing Durham tubes) in the case of the double-strength medium. Sterilize in an autoclave set at 121 °C ± 3 °C for 15 min. The Durham tubes shall not contain air bubbles after sterilization.

**4.3.1.1.1.4** *Brilliant Green lactose bile broth (BGB)*

**4.3.1.1.1.4.1** This medium is used as confirmatory test for Coliforms as well as for faecal Coliforms. The composition of Brilliant Green lactose bile broth (BGB) is as specified in Table 4.

**Table 4 Composition of Brilliant Green Lactose bile Broth (BGB)**

(*Clause* 4.3.1.1.4.1)

| S. No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | Enzymatic digest of casein | 10 g |
| ii) | Lactose ($C_{12}H_{22}O_{11}\cdot H_2O$) | 10 g |
| iii) | Dehydrated ox bile | 20 g |
| iv) | Brilliant green | 0.0 133 g |
| v) | Water | 1 000 ml |

**4.3.1.1.1.4.2** Dissolve the components or the dehydrated complete medium in the water, by heating if necessary. Adjust the *p*H, if necessary, so that after sterilization it is 7.2 ± 0.2 at 25 °C. Dispense the medium in quantities of 10 ml in test tubes of approximately 16 mm × 160 mm containing Durham tubes. Sterilize in an autoclave set at 121 °C ± 3 °C for 15 min. The Durham tubes shall not contain air bubbles after sterilization.

**4.3.1.1.1.5** *Peptone water*

**4.3.1.1.1.5.1** This is used for indole tests or for preparing a liquid culture of an organism. The composition of Peptone water is as specified in Table 5.

**Table 5 Composition of Peptone Water**

(*Clause* 4.3.1.1.5.1)

| S No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | Peptone | 10 g |
| ii) | Sodium chloride | 5 g |
| iii) | Distilled water | 1 000 g |

**4.3.1.1.1.5.2** Dissolve all the ingredients. Adjust the *p*H to 7.4. Dispense 4 ml medium into 100 mm × 12 mm tubes and plug with non-absorbent cotton. Sterilize in the autoclave at 121 °C ± 3 °C for at least 15 min and 1.02 kg/cm$^2$ ± 0.03 kg/cm$^2$ gauge pressure (15 psig ± 0.5 psig) for 15 min.

**4.3.1.1.1.6** *Tryptone water*

**4.3.1.1.1.6.1** *Composition*

The composition of tryptone water is as specified in Table 6.

**Table 6 Composition of Tryptone Water**

(*Clause* 4.3.1.1.6.1)

| S. No. | Reagent | Requirements |
|---|---|---|
| (1) | (2) | (3) |
| i) | Tryptone | 20 g |
| ii) | Sodium chloride (NaCl) | 5 g |
| iii) | Reagent grade water | 1 000 ml |

**4.3.1.1.1.6.2** Add ingredients to water and mix thoroughly until dissolved. Adjust *p*H to 7.5. Dispense 5 ml portion into tubes, cap, and sterilize for 10 min at 121 °C ± 3 °C.

**4.3.1.1.1.7** *Tryptone/tryptophan medium*

**4.3.1.1.1.7.1** *Composition*

The composition of *Tryptone/tryptophan medium* is as specified in Table 7.

**Table 7 Composition of Tryptone/tryptophan medium**

(*Clause* 4.3.1.1.7.1)

| S. No. | Reagent | Requirements |
|---|---|---|
| (1) | (2) | (3) |
| i) | Tryptone | 10 g |
| ii) | Sodium chloride (NaCl) | 5 g |
| iii) | DL-Tryptophan | 1 g |

| | | |
|---|---|---|
| iv) | Reagent grade water | 1 000 ml |

**4.3.1.1.1.7.2**

Dissolve the components in the boiling water. Adjust the *p*H, if necessary, so that after sterilization, it is 7.5 ± 0.2 at 25 °C. Dispense the medium into tubes in quantities of 5 ml. Sterilize for 15 min in the autoclave set at 121 °C. Store the poured tubes at 5 °C for up to three months.

**4.3.1.1.1.8** *MacConkey agar*

**4.3.1.1.1.8.1** The medium is used for the completed test. The composition of *MacConkey agar* is as specified in Table 8.

**Table 8 Composition of MacConkey agar**

(*Clause* 4.3.1.1.8.1)

| S. No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | Peptone | 20 g |
| ii) | Lactose | 10 g |
| iii) | Sodium chloride | 5 g |
| iv) | Bile salt | 5 g |
| v) | Distilled water | 1 000 ml |

**4.3.1.1.1.8.2** Dissolve all the ingredients and adjust the *p*H to 7.4 ± 0.2. Add 10 ml of 1 percent aqueous solution of neutral red indicator and 15 g of agar. Steam the medium for 15 min to 30 min so that agar is dissolved properly and sterilize in autoclave at 121 °C ± 3 °C for atleast 15 min for 15 min. After sterilization, cool to 45 °C and prepare the plates by pouring 15 ml of melted agar per plate.

**4.3.1.1.1.8.3** Allow to solidify, invert and incubate at 37 °C ± 1 °C for drying as well as for sterility test.

**4.3.1.1.1.9** *Crystal violet neutral red bile lactose (VRBL) agar*

**4.3.1.1.1.9.1** *Composition*

The composition of crystal violet neutral red bile lactose (VRBL) agar is as specified in Table 9.

**Table 9 Composition of Crystal Violet Neutral Red Bile Lactose (VRBL) Agar**

(*Clause* 4.3.1.1.9.1)

| S No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | Enzymatic digest of animal tissues | 7 g |
| ii) | Yeast extract | 3 g |
| iii) | Lactose ($C_{12}H_{22}O_{11}·H_2O$) | 10 g |
| iv) | Sodium chloride (NaCl) | 5 g |
| v) | Bile salts | 1.5 g |

| | | |
|---|---|---|
| vi) | Neutral red | 0.03 g |
| vii) | Crystal violet | 0.002 g |
| viii) | Agar (*see* Note) | 12 g to 18 g |
| ix) | Water | 1 000 ml |

NOTE — Depending on the gel strength of the agar.

**4.3.1.1.1.9.2** *Preparation*

Proceed as follows to conserve the selectivity power and specificity of the medium. Thoroughly mix the components or the dehydrated complete medium in the water and leave to stand for several minutes. Adjust the pH so that, after boiling, it is 7.4 ± 0.2 at 25 °C. Heat until boiling, stirring from time to time. Allow to boil for 2 min. Immediately cool the medium in the water bath at to 44 °C to 47 °C. To avoid overheating, do not heat the medium for too long nor reheat it. Consequently, do not sterilize it in the autoclave, and check the sterility of the medium at the time of use. Use the medium within 4 h of its preparation.

**4.3.1.1.1.10** *Nutrient agar slants*

Prepare the plate count agar as prescribed in clause **4.2.3.1**. Dispense while in the melted condition about 10 ml quantity into each tube (150 mm × 15 mm). Sterilize in the autoclave at 121 °C ± 3 °C for at least 15 min. After sterilization the slants are prepared by keeping the tubes in a slanting position and allow them to solidify. Unless they are to be used, they should be stored in a refrigerator.

**4.3.1.1.1.11** *Kovac's reagent*

**4.3.1.1.1.11.1** It is used for indole test. The composition of Kovac's reagent is as specified in Table 10.

**Table 10 Composition of Kovac's Reagent**

(*Clause* 4.3.1.1.11.1)

| S. No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | para-Dimethyl- amino-benzaldehyde | 5 g |
| ii) | Amyl alcohol or n-butanol | 75 ml |
| iii) | Concentrated hydrochloric acid | 25 ml |

**4.3.1.1.1.11.2** Dissolve para-Dimethyl- amino-benzaldehyde in amyl alcohol and then add 25 ml of hydrochloric acid. The reagent should be yellowish in colour. Store in amber coloured glass stoppered bottle.

**4.3.1.1.1.12** *Gram staining reagents*

**4.3.1.1.1.12.1** Crystal violet is used as a primary stain.

| | | |
|---|---|---|
| Solution A | Crystal violet (85 percent dye content) | 2 g |
| | Ethyl alcohol (95 percent) | 20 ml |
| Solution B | Ammonium oxalate | 0.8 g |
| | Water | 80 ml |

**4.3.1.1.1.12.2** Mix the two solutions A and B and age for 24 h before use. Filter through paper in staining bottle. If the solution A and solution B are mixed in equal parts, and it does not decolorize Gram negative bacteria properly, then dilute solution A as much as ten times. Use 20 ml of this diluted solution and mix with solution B.

**4.3.1.1.1.12.3** *Lugol's iodine*

Dissolve 1 g of iodine crystals and 2 g of potassium iodide in 300 ml of distilled water.

**4.3.1.1.1.12.4** Safranin is used as a counter stain. Dissolve 25 g of safranin dye in 100 ml of 95 percent ethyl alcohol. Add 10 ml of the solution to 100 ml of distilled water.

**4.3.1.1.1.12.5** *Ethyl alcohol*, 95 percent.

NOTE — Lugol's iodine is used as mordant and ethyl alcohol is used as a decolourizer.

**4.3.1.2** *Procedure*

Shake the water and wastewater samples thoroughly before making dilutions or before inoculation. When analyzing non-potable waters, inoculate a series of tubes with appropriate decimal dilution of the water (multiples of 10 ml) based on the probable Coliform density.

**4.3.1.2.1** *Presumptive test*

**4.3.1.2.1.1** Use MacConkey broth or Lauryl tryptose broth LST. Inoculate a series of fermentation tubes with appropriate measured quantities of the water and wastewater to be tested. The concentration of nutritive ingredients in the mixture should be sufficient and according to requirements. The 10 ml and above aliquots should be inoculated in double strength and 1 ml and its dilution should be inoculated into single strength medium.

**4.3.1.2.1.2** Incubate all tubes at 37 °C ± 1 °C for 24 h to 48 h. Examine each tube at the end of (24 ± 2) h for gas production and if no gas has been formed, re-incubate for another 24 h and at the end of 48 h, examine again. Record the presence of or absence of gas at each examination of the tubes regardless of the amount.

**4.3.1.2.1.3** Formation of the gas within (48 ± 3) h in any amount, in the inner fermentation tubes, constitutes a positive presumptive test. The absence of gas formation at the end of (48 ± 3) h of incubation constitutes a negative test.

**4.3.1.2.2** *Confirmed test*

**4.3.1.2.2.1** The medium used for confirmed test is brilliant green lactose bile broth (BGB).

**4.3.1.2.2.1.1** Submit all primary fermentation tubes showing any amount of gas at the end of 24 h incubation to the confirmed test. If additional primary fermentation tubes show gas at the end of 48 h incubation, these too shall be submitted to the confirmed test. Use a sterile metal or disposable plastic loop of 3 mm to 4 mm in diameter to transfer one or two loopful of medium from the presumptive positive tubes to a tube of BGB broth. When making such transfers, gently shake the tube first or mix by rotating. Incubate the inoculated tubes at 37 °C ± 1 °C for (48 ± 3) h.

**4.3.1.2.2.1.2** The formation of gas in any amount in the Durham's tubes of BGB tube at any time within (48 ± 3) h constitutes a positive confirmed test.

**4.3.1.2.3** *Completed test*

**4.3.1.2.3.1** It may be applied to positive BGB tubes. Shake the tube, and streak with the help of a loop on the MacConkey agar plates or violet red bile lactose agar plates as soon as possible in such a way so as to get discrete colonies. Incubate the plates at 37 °C ± 1 °C for (24 ± 2) h.

**4.3.1.2.3.2** From each plate pick up typical or atypical colonies and inoculate lactose broth and nutrient agar slants. Incubate at 37 °C ± 1 °C for 24 h to 48 h.

**4.3.1.2.3.3** Nutrient agar slants can be used for Gram-staining. If organisms are Gram negative, non-spore forming bacilli and if gas is produced in lactose broth, the test is considered completed and the presence of coliform organisms is demonstrated.

**4.3.1.2.3.4** *Gram-staining technique*

**4.3.1.2.3.4.1** Prepare a thin smear of the growth from the agar slant on a clean glass slide. Air dry, fix by passing the slide through a flame, and stain for 1 min with ammonium oxalate-crystal violet solution. Wash the slide in water, immerse in Lugol's iodine solution for 1 min. Wash the slide in water, blot dry, then decolorize with ethyl alcohol for 30 s, using gentle agitation. Blot and cover with counter stain for 10 s with safranin, then wash, dry and examine under oil immersion.

**4.3.1.2.3.4.2** Cells which decolorize and accept the safranin stain are pink in colour and defined as Gram-negative in reaction. Cells which do not decolorize but retain the crystal violet stain, are deep blue in colour, and are defined as Gram-positive.

NOTE — The results are acceptable only when the controls of Gram positive and Gram negative organisms have given typical or characteristic reactions.

**4.3.2** *Test for Faecal Coliform*

**4.3.2.1** *General*

This procedure is used to differentiate coliforms of faecal origin from those of non-faecal origin. Faecal coliforms are those coliforms which can ferment lactose at 44.5 °C ± 0.2 °C within (24 ± 2) h with the production of gas. Use brilliant green lactose bile broth or EC broth for this test.

**4.3.2.2** Subculture all presumptive positive tubes of the coliform test, at the end of 24 h and 48 h into BGB medium or EC broth (s*ee clause* **4.3.1.1.2**) and incubate at 44.5 °C ± 0.2 °C for 24 h in a water bath. Gas formation within 24 h is considered a positive reaction for faecal coliforms.

**4.3.2.3** *EC broth (selective medium)*

**4.3.2.3.1** The composition EC broth is as specified in Table 11.

**Table 11 Composition of Kovac's Reagent**

(*Clause* 4.3.2.3.1)

| S No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | Enzymatic digest of casein | 20.0 g |
| ii) | Lactose | 5.0 g |
| iii) | Bile salt no. 3 | 1.5 g |
| iv) | Potassium monohydrogen phosphate ($K_2HPO_4$) | 4.0 g |
| v) | Potassium dihydrogen phosphate ($KH_2PO_4$) | 1.5 g |
| vi) | Sodium chloride | 5.0 g |
| vii) | Water | 1 000 ml |

**4.3.2.3.2** *Preparation*

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary. Adjust the *p*H, if necessary, so that after sterilization it is 6.8 ± 0.2 at 25 °C. Dispense the medium in quantities of 10 ml into tubes of dimensions 16 mm × 160 mm containing Durham tubes. Sterilize for 15 min in an autoclave set at 121 °C. The Durham tubes shall not contain air bubbles after sterilization.

**4.3.3** *Test for E.coli*

**4.3.3.1** *E.coli* is one of the members of faecal coliforms which ferments lactose with the production of gas at 44.5 °C ± 0.2 °C within 24 h as well as produce indole from Tryptophan medium at 44.5 °C ± 0.2 °C within 24 h.

**4.3.3.2** Subculture from all the positive tubes of BGB broth or EC Broth at 44.5 °C ± 0.2 °C (faecal coliforms) into tubes of peptone or Tryptone/Tryptophan medium. Incubate at 44.5 °C ± 0.2 °C for (24 ± 2) h. At the end of the incubation period test for indole production by adding a few drops of Kovac's reagent. Positive test will give pink colour while negative test will give yellow colour.

**4.3.3.3** *Computing and recording of MPN*

The number of positive findings of Coliform group organisms (either presumptive, confirmed, or completed), *Faecal* Coliform, and *E. coli* resulting from the multiple portion decimal dilution planting should be computed as combination of the positives and recorded in terms of the Most Probable Number. The Most Probable Number for a variety of planting series and results is given in Annex C.

**4.3.4** *Membrane Filter (MF) Technique*

**4.3.4.1** For especially suitable for waters with low bacterial numbers that will cause less than 100 total colonies on chromogenic coliform agar (CCA). These may be drinking water, disinfected pool water, or finished water from drinking water treatment plants refer to IS 15185.

**4.3.4.2** *Standard total Coliform membrane filter procedure using m-Endo medium*

**4.3.4.2.1** *Outline of method*

**4.3.4.2.1.1** The membrane filter technique in water analysis is becoming more and more popular due to its advantages over the multi tube dilution technique. Results are obtained within 24 h or 48 h as compared to 48 h to 96 h by multi tube dilution technique. A much larger volume and hence more representative sample can be tested. Results are obtained with much greater precision and require less laboratory space; equipment is not bulky and involves less labor. The limitations of this technique are few. Samples with high turbidity and less indicator bacterial count will be difficult to examine. Samples having a high number of non-indicator organisms will give less count.

**4.3.4.2.1.2** On m-Endo media, typical coliforms ferment lactose to produce aldehydes, which react with Sodium sulfite and basic fuchsin in the medium to five rise to red colonies with a green or gold metallic sheen. Non-coliform bacteria unable to ferment lactose appear as colorless, pink, blue or white colonies.

**4.3.4.3** *Description of MF assembly*

**4.3.4.3.1** There are many varieties of MF assemblies. The filter-holding assembly (constructed of glass, autoclavable plastic, porcelain, stainless steel, or disposable plastic) consists of a seamless funnel fastened to a base via a locking device or magnetic force.

**4.3.4.3.2** The design must permit the membrane filter to be held securely on the receptacle’s porous plate without mechanical damage and allow all fluid to pass through the membrane during filtration. Discard plastic funnels with deep scratches on inner surface or glass funnels with chipped surfaces.

**4.3.4.3.3** For filtration, mount receptacle of filter-holding assembly on a 1 litre filtering flask with a side arm or other suitable device (e.g. manifold to hold 3 to 6 filter assemblies). Connect the flask to a vacuum line.

**4.3.4.3.4** Only those filter membranes may be used which have been found, through complete laboratory tests certified by manufacturer, to provide full bacterial retention, stability in use, freedom from chemicals inimical to the growth and development of bacteria, and satisfactory speed of filtration, no increase in number of confluent colonies or spreaders compared to control membrane filters. They should preferably be grid marked in such a way that bacterial growth is neither inhibited nor stimulated along the grid lines. The membrane filters of 47 mm in diameter and 0·45-micron pore size is used, for complete retention of bacteria.

NOTE — Membrane filters to be verified before use for recovery of targeted organism. The sterility to be verified, in case of pre-sterilized membrane filters.

**4.3.4.3.5** Use grid-marked membranes to facilitate colony counting. Ensure that bacterial growth is neither inhibited nor stimulated along the grid lines when membrane with entrapped bacteria are incubated on a suitable medium. It is recommended to use stocks of membrane filters before expiry date. They should be stored in an environment without extremes of temperature and humidity. If the membranes are sterilized in the laboratory, autoclave for 15 min at 121 °C ± 3 °C. At the end of the sterilization period, let the steam escape rapidly to minimize accumulation of water of condensation on filters. Alternatively, the recommendations given by manufacturers may be followed.

**4.3.4.3.6** Absorbent pads for nutrients should consist of discs or filter paper or other materials known to be of high quality and free of sulphites or other substances that could inhibit bacterial growth. These should be approximately 48 mm in diameter and of thickness sufficient to absorb 1.8 ml to 2.2 ml of nutrient. Sterilize pads with resealable kraft envelopes, or separately in other suitable containers for 15 min in an autoclave (same temperature and tolerance). Pads may be pre-sterilized individually wrapped filters.

**4.3.4.3.7** Use sterile filtration units at the beginning of each filtration series as a minimum precaution to avoid accidental contamination. A filtration series is interrupted when an interval of 30 min or longer elapses between sample filtrations. After such interruption, treat any further sample filtration as a new filtration series and sterilize all membrane filter holders in use. Wrap the assembly (as a whole or separate parts) in heavy wrapping paper or aluminum foil, or place in commercially available autoclave bags, sterilize via autoclaving, and store until use.

**4.3.4.4** *Selection of sample size*

**4.3.4.4.1** The size of the sample is governed by the expected bacterial density. An ideal quantity of number of colonies in the ideal range for quantitative determinations are as specified Table 12. If water is heavily contaminated use less quantity of water. When less than 20 ml is to be filtered, dilute the portion to a minimum of 30 ml before filtration. The suggested sample volumes for MFT Coliform Test are as specified in Table 13.

**Table 12 Suggested number of colonies in the ideal range for Quantitative determinations**

(*Clause* 4.3.4.4.1)

| S. No. | Test | Colony counting range | |
|---|---|---|---|
| | | Minimum | Maximum |
| (1) | (2) | (3) | (4) |
| i) | Total coliform | 20 | 80 |
| ii) | Fecal coliform | 20 | 60 |
| iii) | *E.coli* | 20 | 80 |

**Table 13 Suggested sample volumes for MFT Coliform Test**

(*Clause* 4.3.4.4.1)

| S. No. | Water Source | Volume to be filtered (ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 100 | 50 | 10 | 1 | 0.1 | 0.01 | 0.001 | 0.0001 |

| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) |
|---|---|---|---|---|---|---|---|---|---|
| i) | Drinking water | X | | | | | | | |
| ii) | Swimming pools | X | | | | | | | |
| iii) | Wells, springs | X | X | X | | | | | |
| iv) | Lakes, reservoirs | X | X | X | | | | | |
| v) | Water supply intake | | | X | X | X | | | |
| vi) | Bathing beaches | | | X | X | X | | | |
| vii) | River water | | | | X | X | X | X | |
| viii) | Chlorinated sewage | | | | X | X | X | | |
| ix) | Raw sewage | | | | | X | X | X | X |

**4.3.4.4.2** Analyze drinking waters by filtering 100 ml to 1 000 ml, or by filtering replicate smaller sample volumes (e.g., duplicate 50 ml portions or four replicates of 25 ml portions). Analyze other waters by filtering 3 different volumes (diluted or undiluted), depending on the expected bacterial numbers. When < 10 ml of sample (diluted or undiluted) is to be filtered, add approximately 10 ml sterile buffered dilution water to the funnel and then add sample followed by another 25 ml to 50 ml dilution water before filtration or pipet the sample volume into sterile dilution water and then filter the entire contents of the dilution bottle. This increase in water volume aids in uniform dispersion of the bacterial suspension over the entire effective filtering surface.

**4.3.4.5** *Filtration of sample*

**4.3.4.5.1** Using sterile forceps, place a sterile filter over the porous membrane holding plate of any suitable material. Place the funnel unit carefully over the receptacle and lock it in place. Thoroughly mix the sample by vigorously shaking to break up clumps of bacteria, which is crucial for a microbial quantitative method. If the sample bottle lacks enough headspace for adequate mixing, pour the sample into a larger sterile vessel to mix appropriately. Then pass the sample through the filter under the vacuum. Rinse the filter by filtration two to three times with 20 to 30 ml of sterile buffer water. Unlock the funnel. Remove the filter with sterile forceps and place it on the sterile pad or agar with a rolling motion to avoid the entrapment of air.

**4.3.4.5.2** Incorrect filter placement is at once obvious, because patches of unstained membrane indicate entrapment of air. Where such patches occur, carefully reseat filter on the agar surface. Place only one membrane filter per plate. Place the dish in upright position and incubate at 36 ± 2 °C for 22 to 24 h for Chromogenic Coliform agar (for water with low bacterial background flora, refer IS 15185) or m-Endo medium (for all types water) for potable water violent red bile agar media. If loose-lidded plates are used, incubate them in a humid container (i.e. sealed container or bag with moist paper towel or cheesecloth). Differentiation of some colonies may be lost if cultures are incubated > 24 h. Place plates in the incubator within 30 min of filtration.

**4.3.4.5.3** To prevent carryover between non-potable water samples, rinse and sanitize funnel with UV radiation for 2 min between sample filtrations.

**4.3.4.6** *m-Endo broth medium*

**4.3.4.6.1** Medium used for enumerating coliforms by membrane filter technique is known as *m*-Endo broth. The composition of the medium is specified in Table 14.

**Table 14 Composition of m-Endo Broth**

(*Clause* 4.3.4.6.1)

| S No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | Yeast extract | 1.2 g |
| ii) | Casitone or trypticase | 3.7 g |
| iii) | Thiopeptone or thiotone | 3.7 g |
| iv) | Tryptose | 7.5 g |
| v) | Lactose | 9.4 g |
| vi) | Dipotassium hydrogen phosphate (K2HPO4) | 3.3 g |
| vii) | Potassium dihydrogen phosphate (KH2PO4) | 1.0 g |
| viii) | Sodium chloride (NaCl) | 3.7 g |
| ix) | Sodium desoxycholate | 0.1 g |
| x) | Sodium lauryl sulfate | 0.05 g |
| xi) | Sodium sulfite (Na2SO3) | 1.6 g |
| xii) | Basic fuchsin | 0.8 g |
| xiii) | Agar | 15.0 g |
| xiv) | Reagent grade water | 1 000 ml |

**4.3.4.5.2** Dissolve the above ingredients in 1 000 ml of distilled water containing 20 ml of ethyl alcohol (95 percent). Heat the medium to boiling point. Do not heat for a long time or do not submit to steam under pressure. Cool to between 45 ℃ and 50 °C. The final *p*H should be between 7.1 to 7.3 or instruction provided with media may be referred. Refrigerate inverted plates of finished medium in the dark and discard unused agar after 2 weeks or sooner if there is evidence of moisture loss, medium contamination, medium deterioration or surface sheen formation.

CAUTION – Do not use denatured ethanol. Do not sterilize by autoclaving.

**4.3.4.6** *Procedure*

Saturate the pad in a small Petri dish with M. Endo broth and place the filter on it. Remove excessive medium by tilting. Invert the plate and incubate at 36 °C ± 2 °C for 24 h under the humid chamber. All colonies which produce a dark red colony with a metallic shine within 24 h incubation are considered members of coliform group and are counted. The count is made by a low-power (10 to 15 magnification) optical device, with a cool white, fluorescent light source directed to provide optimal viewing of sheen.

**4.3.4.7** *Counting*

**4.3.4.7.1** The angle of light on the colony affects the detection of sheen for coliform colonies growing on m-Endo plates. Rocking and turning the petri plate reflects light at different angles and aids in detecting sheen on the colony. The typical coliform colony on m-Endo media has a pink to dark-red color with a metallic surface sheen. Count both

typical and atypical coliform colonies. The sheen area may vary in size from a small pinhead to complete coverage of the colony surface. Atypical coliform colonies can be dark red, mucoid, or nucleated without sheen. Generally pink, blue, white, or colorless colonies lacking sheen are considered non-coliforms. The total count of colonies (coliform and non-coliform) on Endo-type medium has no consistent relationship to the total number of bacteria present in the original sample. A high count of non-coliform colonies may interfere with the maximum development of coliforms. After 22 h incubation, refrigerate cultures with high densities of non-coliform colonies for 0.5 h to 1 h before counting may deter spread of non-coliform growth while aiding sheen discernment.

**4.3.4.7.2** On CCA plate count all pink to red colonies as presumptive colonies. Confirm all presumptive colonies by negative oxidase test.

**4.3.4.7.3** Coliform bacteria grow as purplish red colonies surrounded by a reddish zone of precipitated bile. Count all purplish red colonies as coliforms. Countable plates are those with a range of 20 colonies to 80 colonies. Plates with more than 200 colonies are considered too crowded to be counted. Select the membranes with acceptable number of coliform colonies (20 CFU to 80 CFU) and ≤ 200 CFU of all types per membrane, by the following equation:

$$Coliform\ density\ (coliforms/100\ ml)\ =\ \frac{Coliform\ colonies\ \times 100}{Volume\ in\ ml\ of\ the\ sample\ filtered}$$

**4.3.4.7.3.1** For drinking water sample. If no total coliform colonies are observed, report the coliform colonies counted as <1 CFU/100 ml or report total coliform bacteria absent per 100 ml sample.

**4.3.4.8** *Thermotolerant (Fecal) coliform membrane filter procedure*

**4.3.4.8.1** *Test portion*

**4.3.4.8.1.1** The maximum volume of the test portion depends on the filterability of the water sample and on the membrane filters used. This technique is suitable for waters that contain little particulate or colloidal (e.g. iron) matter in suspension, e.g. water intended for drinking. It may be possible to filter several litres of such water, achieving a high level of test sensitivity.

**4.3.4.8.1.2** The test volume of the sample or dilution should be chosen so that the expected number of colonies formed on a membrane filter of 47 mm to 50 mm in diameter is less than 80, and the number of target colonies is greater than 10. Recommended sample volumes for thermotolerant coliforms and *E.coli* are as specified in Table 15.

**Table 15 Suggested sample volumes for membrane filter Thermotolerant (Faecal) Coliform or *E.coli* Test**

(*Clause* 4.3.4.8.1.2)

| **S. No.** | | **Volume (X) to be filtered (ml)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Water Source** | **100** | **50** | **10** | **1** | **0.1** | **0.01** | **0.001** | **0.0001** |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) |
| i) | Drinking water | X | | | | | | | |
| ii) | Lakes, reservoirs | X | X | | | | | | |
| iii) | Wells, Springs | X | X | | | | | | |
| iv) | Water supply intake | | X | X | X | | | | |
| v) | Natural bathing waters | | X | X | X | | | | |
| vi) | Sewage treatment plant | | | X | X | X | | | |
| vii) | Farm ponds, rivers | | | | X | X | X | | |
| viii) | Storm water runoff | | | | X | X | X | | |
| ix) | Raw municipal sewage | | | | | X | X | X | |

| | | | | | |
|---|---|---|---|---|---|
| x) | Feedlot runoff | X | X | X | |
| xi) | Sewage sludge | | X | X | X |

**4.3.4.8.2** *Filtration apparatus — see* **4.3.4.3**

**4.3.4.8.3** *Filtration — see* **4.3.4.5**

**4.3.4.8.4** *Transfer of membrane filter*

**4.3.4.8.4.1** Remove the funnel after closing the stopcock and transfer the membrane filter with sterile flat-ended forceps in one of the following ways, ensuring that no air bubbles are trapped between the membrane filter and the medium:

a) grid upwards onto an agar medium in a Petri dish;
b) grid downwards or upwards into a Petri dish, or onto 5 ml to 10 ml agar medium in a 50 mm or 90 mm Petri dish (for anaerobes); then overlay the membrane filter with molten agar medium (44 °C to 47 °C) as quickly as possible to avoid the membrane filter drying out and excessive contact with air.

**4.3.4.8.4.2** For different volumes of the same sample, the funnel may be re-used without disinfection provided that the smallest volumes and/or the most diluted sample are filtered first. To filter another sample, either a separate sterile apparatus shall be used, or where appropriate the funnel can be disinfected, for example, by direct flaming or immersion in a boiling water bath. Alternatively, the manufacturer's instructions for disinfection should be followed.

**4.3.4.8.5** *Membrane transfer techniques using liquid media or diluents*

**4.3.4.8.5.1** As an alternative to an agar plate, if stated by the relevant standard, the membrane filter may be placed grid upwards onto a sterile absorbent pad, previously saturated with a liquid medium or a dehydrated medium pad reconstituted with sterile water in a Petri dish. To avoid confluent growth, any excess liquid should be poured off, preferably before placing the membrane filter on the pad.

**4.3.4.8.5.2** Certain methods, such as MPN culture and qualitative methods, may require the membrane filter to be transferred directly into a pre-enrichment or enrichment broth.

The membrane filter may also be used as part of a concentration system and may be washed (eluted) with the help of a diluent to obtain a new test portion to spread over the dry surface of an agar medium.

**4.3.4.8.6** *Incubation*

**4.3.4.8.6.1** Invert the inoculated agar plates and place them either in an incubator or in a water-tight container in a water bath. If necessary, pack the plates in plastic bags or other containers to prevent desiccation of the medium (e.g. when a fan-assisted incubator is used). Do not stack Petri dishes more than six high to ensure that all plates reach incubation temperature rapidly and leave space between the stacks for air circulation. However, plates may be stacked higher in specially designed racks or jars when fan-assisted incubators are used and the correct incubation temperatures for the required times have been verified.

**4.3.4.8.6.2** Plates containing membrane filters on absorbent pads place lids uppermost in an air or water-tight container to prevent desiccation of the medium. Plates for spore-forming microorganisms above the agar surface (such as molds or actinomycetes) shall also not be inverted.

NOTE — Molds spores can spread from Petri dishes to the surrounding dishes or the incubator when moving the Petri dishes. Sealing the lid of the Petri dish to the base can help to prevent this.

**4.3.4.8.6.3** If anaerobic incubation is required, the inoculated agar plates should be placed in an anaerobic incubator, or an air-tight anaerobic jar containing an anaerobic generator of a suitable size for the volume of the jar. In addition, a suitable means of establishing that anaerobic conditions have been achieved should also be included in the jar or incubator.

**4.3.4.8.6.4** Choose the duration and temperature of incubation after reference to the specific standard method as these will depend on the microorganisms, or groups of microorganisms, sought.

**4.3.4.8.6.5** Membrane filters are sometimes incubated for a limited period (e.g. 2 h or 4 h) on a resuscitation medium and then transferred to another medium, which is usually selective, for further incubation.

**4.3.4.8.7** *Media for Thermotolerant/Faecal coliforms*

**4.3.4.8.7.1** The composition of m-FC medium is as specified in Table 16.

**Table 16 Composition of m-FC medium**

(*Clause* 4.3.4.8.7.1)

| S. No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| | Part A | |
| i) | Tryptose or biosate | 10.0 g |
| ii) | Proteose peptone or polypeptone | 5.0 g |
| iii) | Yeast extract | 3.0 g |
| iv) | Sodium chloride, NaCl | 5.0 g |
| v) | Lactose | 12.5 g |
| vi) | Bile salts or bile salts mixture | 1.5 g |
| vii) | Aniline blue | 0.1 g |
| viii) | Agar (optional) | 15.0 g |
| | Part B | |
| i) | Rosolic acid | 10 g |
| ii) | 0.2 M NaOH | 100 ml |

Note for Rosolic acid preparation: Do not autoclave. Refrigerate the stock solution in the dark and discard after 2 weeks, or sooner if its color changes from dark red to muddy brown.

Note for plate preparation: Prepare medium in 1 litre reagent grade water containing 10 ml of 1 percent rosolic acid (dilute 10 percent stock in 0.2 M NaOH). Heat medium to near boiling, promptly remove from heat, and cool to <50 °C. Do not autoclave. The final *p*H must be 7.4 ± 0.2. Refrigerate plates inverted in the dark in sealed plastic bags or other containers to reduce moisture loss. Discard after 2 weeks.

**4.3.4.8.7.2** *Procedure for Thermotolerant/Faecal coliforms (MF)*

Filter selected test portion of sample and place the membrane filter in a Petri dish with m-FC agar, avoiding air bubbles. Invert the plate and incubate at 44.5 °C ± 0.2 °C for 24 h in incubator or water bath. Colonies produced by thermotolerant/Faecal coliforms bacteria on mFC medium are various shades of blue. Nonfecal coliform colonies are

typically gray to cream-coloured. Confirm typical blue colonies by inoculating in EC broth and incubate at 44.5 °C ± 0.2 °C in incubator or water bath for 24 h. Growth in EC broth with gas formation in durham tubes constitutes positive test for Faecal coliforms.

**4.4 Test for Faecal Streptococci**

The test terms faecal streptococci and enterococci have been used somewhat synonymously by many in recent years. The faecal streptococci group are indicators of faecal pollution of water because the general habitat of these organisms is the intestine of man and animals. They are gram positive cocci and ferment glucose with the production of acid only and are capable of growing in the presence of 40 percent bile and at 45 °C. On the basis of newer concepts of speciation of faecal streptococci, it is suggested that the terms faecal streptococci and Lancefields group D streptococcus be considered synonymous. The standard test for the estimation of number of the faecal streptococci may be carried out either by the multiple tube dilution technique or by the membrane filter technique.

**4.4.1** *Multiple Tube Dilution Technique*

Multiple tube dilution technique employs presumptive test procedures and confirmed test procedures.

**4.4.1.1** *Media*

**4.4.1.1.1** *Dilution water — see* **4.2.3.2**

**4.4.1.1.2** *Azide dextrose broth (ADB)*

**4.4.1.1.2.1** This is a presumptive test medium used for enumerating faecal streptococci in the water samples. Dissolve the following ingredients as specified in Table 17 and adjust the *p*H to 7.2 ± 0.2:

**Table 17 List of Ingredients**

**(***Clause* 4.4.1.1.2)

| S No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | Tryptone or Polypeptone | 15 g |
| ii) | Beef extract | 4.5 g |
| iii) | Glucose | 7.5 g |
| iv) | Sodium chloride | 7.5 g |
| v) | Sodium azide | 0.2 g |
| vi) | Distilled water | 1 000 ml |

**4.4.1.1.2.2** Dispense 6 ml to 7 ml of medium into 150 mm × 15 mm test tubes and plug with non-absorbent cotton (other alternative as mentioned above). Sterilize in the autoclave at 121 °C ± 3 °C temperature approximately for 15 min. This a single strength medium and used for 1 ml aliquotes and decimal dilution when 10 ml sample or more has to be inoculated use double strength medium. This is prepared by using double the quantities given above in 1 000 ml of water 10 ml of this double strength medium is put into each 150 mm × 18 mm test tube.

**4.4.1.1.3** *Ethyl violet azide broth (EVA)*

**4.4.1.1.3.1** The Confirmatory medium used for enumerating faecal streptococci is as specified in Table 18.

**Table 18 Confirmatory Medium used for Enumerating Faecal Streptococci**

(*Clause* 4.4.1.1.3.1)

| S. No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | Tryptone or biosate | 20 g |
| ii) | Sodium chloride | 5 g |
| iii) | Glucose | 5 g |
| iv) | Di-potassium hydrogen phosphate | 2.7 g |
| v) | Sodium azide | 0.4 g |
| vi) | Distilled water | 1 000 ml |
| vii) | Potassium Di-hydrogen phosphate | 2.7 g |

**4.4.1.1.3.2** Dissolve all the ingredients and adjust the *p*H to 7.1. Add 1 ml of 0.083 percent alcoholic solution of ethyl violet. Dispense 10 ml medium into 150 mm × 18 mm test tubes and plug with non-absorbent cotton. Sterilize in the autoclave at (1.02 ± 0.03) kg/cm$^2$ gauge pressure [(15 ± 0.5) psi gauge pressure, 120 °C temperature approximately] for 15 min.

**4.4.1.2** *Procedure*

Shake the water sample thoroughly before making dilution or before inoculation.

**4.4.1.2.1** *Presumptive test*

Inoculate a series of tubes of azide dextrose broth with appropriate graduated quantities of the water to be tested (follow the same procedure as given for coliforms). Incubate inoculated tubes at 37 °C. Examine each tube at the end of 24 h for the presence of turbidity. If no definite turbidity is present re-inoculate and read at the end of 48 h.

**4.4.1.2.2** *Confirmed test*

All azide dextrose broth tubes showing turbidity after 24 h or 48 h incubation must be subjected to the confirmed test. Transfer three loopfuls of growth from each azide dextrose broth to ethy1 violet azide broth tubes. Incubate the inoculated tubes for 48 h at 37 °C. The presence of streptococci is indicated by the formation of a purple button at the bottom of the tube, or occasionally by a dense turbidity. Find out the MPN value from Annex C. Record the result as MPN per 100 ml of the sample.

**4.4.2** *Membrane Filler Technique*

For detection and enumeration of Faecal Streptococci in water and wastewater refer to IS 15186.

**4.5 Test for Sulphite Reducing Anaerobe**

The sulphite Reducing Anaerobe shall be tested in accordance with the method given in Annex C of IS 13428.

**4.6 Test for Clostridium perfringens**

**4.6.1** *General*

**4.6.1.1** *Clostridium perfringens* are large rod-shaped, non-motile anaerobic bacteria which form spores which are relatively resistant to heat, drying and ordinary bacterial agents.

**4.6.1.2** Presumptive *Clostridium perfringens* (ISO/TS 15213-3-2024) are spore-forming bacteria forming typical colonies on a specific selective medium under obligatory anaerobic conditions.

**4.6.1.3** Confirmed *Clostridium perfringens* (ISO/TS 15213-3-2024) are bacteria that produce characteristic colonies on a specific selective medium under obligatory anaerobic conditions and either possess the enzyme acid phosphatase, or able to produce sulfite, are not able to produce indole and are not motile (SIM agar).

**4.6.1.4** Detection of *Clostridium perfringens* is determination of confirmed *Clostridium perfringens* in a particular mass, volume of the products, on a surface area or object, when specified test is conducted.

**4.6.2** *Equipment and Apparatus*

**4.6.2.1** Apparatus for dry sterilization (oven) and wet sterilization (autoclave).

**4.6.2.2** Incubator(s) capable of operating at 37 °C ± 1 °C, 46 °C ± 1 °C

**4.6.2.3** *p*H meter, having an accuracy of calibration of ± 0.1 *p*H unit at 25 °C

**4.6.2.4** Refrigerator capable of operating at 5 °C ± 3 °C

**4.6.2.5** Sterile bottles, flasks or tubes: of appropriate capacity with non-toxic metallic or plastic screwcaps may be used.

**4.6.2.6** Sterile graduated pipettes or automatic pipettes of nominal capacities of 10 ml and 1ml.

**4.6.2.7** Sterile loops of appropriate diameter of 3 mm (10 µl volume) and of 1 µl volume, or inoculation needle or wire.

**4.6.2.8** Sterile Petri dishes, with a diameter of approximately 90 mm or 100 mm.

**4.5.2.9** Thermostatically controlled water bath, capable of operating at 44 ℃ to 47 °C.

**4.6.2** *Medium and Reagent for Procedure A*

**4.6.2.1** *Litmus milk medium*

Keep fresh raw milk of low bacterial content in the refrigerator for (18 ± 1) h so that the cream may separate. Remove the cream and add 10 percent litmus solution to the milk to give a purplish blue colour. Distribute in tubes in 10 ml quantities. Add a mixture (melting point approximately 45 °C) of equal parts of paraffin wax and petroleum jelly to form a layer about 2 mm to 5 mm thick of the surface of the medium. Steam for 30 min on 3 successive days. Test for sterility by incubation at (37.0 ± 0.5) °C for (48 ± 3) h.

**4.6.3** *Procedure*

Inoculate varying quantities of the sample into bottles or tubes containing freshly boiled (in a water-bath) and rapidly cool litmus milk medium. Heat the tubes in a controlled water-bath at (80 ± 1) °C for 15 min, remove from water bath, cool to approximately 37 °C and place the tubes in an incubator at (37 ± 0.5) °C for 5 days. Examine the tubes every day for signs of stormy fermentation which indicates a positive test. Further confirmation may be done by the spore staining technique. The presence and absence of ***Clostridium perfringens*** may be reported based on the volume of the sample taken.

**4.6.4** *Confirmation by Spore Staining Technique*

**4.6.4.1** *General*

Stormy fermentation is a presumptive indication for the presence of *clostridium welchii*; spore staining technique can be used for confirmation. The positive test eliminates doubt about non-spore forming rods and cocci and the position of the spore gives added information, for example, terminal, sub-terminal, central etc.

**4.6.4.2** *Reagents*

**4.6.4.2.1** *Zichl Neelsen's Carbol Fuchsin (ZNCF)*

The composition of Zichl Neelsen's Carbol Fuchsin (ZNCF) is as specified in Table 19.

**Table 19 Composition of Zichl Neelsen's Carbol Fuchsin (ZNCF)**

(*Clause* 4.6.4.2.1)

| S. No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | Basic Fuchsin | 5 g |
| ii) | Phenol | 25 g |
| iii) | Ethanol (95 percent) | 50 ml |
| iv) | Distilled water | 500 ml |

**4.6.4.2.2** *Sulphuric acid*, 0.5 percent.

**4.6.4.2.3** *Methylene blue*, 1 percent.

**4.6.4.3** *Procedure*

Prepare a smear and fix it. Stain with ZNCF and beat the preparation until steam rises. Wash with water and treat with 0.5 percent sulphuric acid for 1 min to 2 min. Wash with water and counter stain with 1 percent aqueous methylene blue for 3 min. Wash, dry and examine under oil immersion, using a compound microscope. The cell will have bulging in the middle due to central spore. The stains are bright red and the protoplasm of bacilli blue.

**4.6.5** *Procedure B*

**4.6.5.1** *Test portion and initial suspension*

Add 1 ml of the liquid sample or 1 ml of the initial suspension to 9 ml of rapid perfringens medium (RPM). Alternatively, 10 ml of the liquid sample or of the initial suspension is added to 90 ml of the RPM.

**4.6.5.2** *Selective enrichment*

Incubate the selective enrichment broth RPM in closed tubes or bottles at 46 °C ± 1 °C for 18 h ± 2 h.

**4.6.5.3** *Isolation*

**4.6.5.3.1** From the selective enrichment obtained above, inoculate by mean of 10 µl loop the surface of a Petri dish containing the selective medium tryptose sulfite cycloserine agar (TSC agar) and a Petri dish containing the selective medium Lactose egg-yolk neomycin agar (LENA).

**4.6.5.3.2** Incubate the TSC agar plates anaerobically in an incubator at 37 °C ± 1°C for 24 h ± 2 h. Incubate the LENA plates anaerobically in an incubator at 46 °C ± 1 °C for 24 h ± 2 h.

NOTE — After inoculation of the TSC agar plates, an overlay of TSC agar can be used to prevent the development of spreading colonies on the surface of the medium. Pour about 5 ml of the TSC medium as overlay and allow them to solidify by leaving the Petri dishes standing on a cool horizontal surface.

**4.6.5.4** *Confirmation of Clostridium perfringens*

**4.6.5.4.1** Selection of colonies for confirmation: Typical colonies on TSC agar are black or grey to yellow-brown staining, even if the colour is faint. Typical colonies on LENA show yellow colour (acid fermentation from lactose) and precipitation (lecithinase reaction). Upon removal of the TSC agar plates from the anaerobic atmosphere, the red plates within 30 min as the colour of the colonies rapidly fade and disappear upon exposure to oxygen. If anaerobic jars are used, check the plates jar by jar or in small portions if the incubation is performed in an anaerobic incubator. For confirmation, take five presumptive *Clostridium perfringens* colonies from each dish containing typical colonies. If more than one morphology is present among the colonies, select one of each morphology for structure and confirmation.

**4.6.5.4.2** Streak each of the selected colonies with a sterile loop onto one non-selective blood agar plate, e.g. columbia blood agar or Tryptone soya agar or Brain heart infusion agar or nutrient agar, taking care in streaking to obtain well-isolated colonies. Incubate the plates in anaerobic atmosphere in an incubator at 37 °C ± 1°C for 20 h ± 2 h. Perform confirmation by the acid phosphatase test or by the sulfite indole motility (SIM) agar test.

**4.6.5.4.3** *Acid phosphatase test*

Transfer colonies grow anaerobically on blood or nutrient agar plates on filter paper, spread and add 2 to 3 drops of the acid phosphatase reagent onto the colonies. A purplish colour developed within 3 min to 4 min is considered a positive reaction.

**4.6.5.4.4** Sulfite indole motility (SIM) agar test

Inoculate colonies grown anaerobically on blood or nutrient agar plates into SIM agar tubes by stabbing with sterile needle wire. Incubate the tubes at 37 °C ± 1 °C for 24 h ± 2 h, in an anaerobic atmosphere with the caps of the SIM agar tubes loosened. After the incubation, read the tubes for:

a) Sulfite production: tubes showing blackening are positive;
b) Motility: tubes showing growth outside the inoculation stab are positive; and
c) Indole production: tubes giving a red coloured ring directly after adding Kovacs reagent are positive. Clostridium perfringens is positive for sulfite production and negative for indole production and motility.

**4.6.5.4.5** *Expression of results*

In accordance with the interpretation of results, indicate *Clostridium perfringens* detected or not detected in a test portion of x g or x ml of product/sample.

**4.6.6** The enumeration of Clostridium perfringens using membrane filtration technique shall be done as specified in IS 18771.

**4.7 Test for Iron Bacteria**

**4.7.1** *General*

Iron bacteria are considered to be capable of withdrawing iron present in their aqueous habitat and of depositing it in the form of hydrated ferric hydroxide on or in their mucilaginous secretion. Their presence may cause pitting and tuberculations in pipes and render the water unsuitable for domestic and industrial purposes. Bacteria of this type, to obtain energy, oxidize ferrous to ferric iron which is precipitated as ferric hydrate. Iron may be obtained from the pipe itself or from the water being carried. The amount of ferric hydrate deposited is very large in comparison with the enclosed cells. The main types are *Gallionella ferruginea*, *Leptothrix, Crenothrix polyspora*, *Sphaerotilus natans* and *Thiobacillus ferrooxidants*. *Leptothrix* and *Crenothrix* are filamentous forms which deposit iron in their sheaths and the family *Gallionella* consists of stalked bacteria.

**4.7.2** *Procedure*

It is generally sufficient to ascertain the presence or absence of these organisms in a water sample. For this purpose, the centrifuged deposit from the sample spread over a glass slide shall be examined under a microscope. The ferric deposits which may obscure the structure of the organisms may be removed by adding dilute hydrochloric acid to the smear on the slide and staining the organisms with Lugol's iodine solution (prepared by dissolving 1 g of iodine and 2 g of potassium iodide in 300 ml of water).

**4.7.3** *Cultivation of Iron Bacteria*

When necessary the iron bacteria in the sample shall be cultivated and enriched by the method given in **4.7.3.1** and **4.7.3.2**.

**4.7.3.1** *Gallionella*

**4.7.3.1.1** *Medium*

Mix equal volumes of 10 percent solution of ferrous sulphate in water and 3 percent solution of agar at 45 °C. Distribute in screw cap tubes and sterilize in the autoclave at (1.02 ± 0.03) kg/cm$^2$ gauge pressure [(15 ± 0.5) psi gauge pressure, 121 °C ± 3 °C] for 20 min. Then prepare the agar slants. Dissolve 1.0 g of ammonium chloride, 0.5 g of dipotassium phosphate, 0.2 g of magnesium sulphate and 0.1 g of calcium chloride in 1 litre of water. Sterilize in the autoclave for 25 min at (1.02 ± 0.03) kg/cm$^2$ gauge pressure [(15 ± 0.5) psi gauge pressure, 121 °C ± 3 °C]. Bubble carbon dioxide through this medium for 10 s to 15 s. Place in each agar tube a quantity of this liquid medium sufficient to cover the agar completely.

**4.7.3.1.2** *Procedure*

Inoculate the tubes containing the medium with a drop of the suspension of the organisms in the centrifuged deposit of the sample and incubate at room temperature. Examine after 18 h to 36 h. White deposits on the sides of the culture tube indicate the presence of *Gallionella* colonies. Pick the deposit with a sterile needle, spread over a slide, and examine under the microscope as in **4.7.2**.

**4.7.3.2** *Leptothrix*

**4.7.3.2.1** *Medium*

Dissolve 1.0 g of ammonium sulphate, 0.5 g of magnesium sulphate, 0.1 g dibasic potassium phosphate, and 0.02 g of calcium nitrate in 1 litre of water. Place 100 ml quantities in conical flasks and sterilize at (1.02 ± 0.03) kg/cm$^2$ gauge pressure [(15 ± 0.5) psi gauge pressure, 121 °C ± 3 °C] in an autoclave for 20 min. Place in each flask 0.05 g of sterilized iron filings.

**4.7.3.2.2** *Procedure*

Inoculate the medium prepared above with a suspension of the centrifuged deposit of the sample and incubate at room temperature. Examine after three days under the microscope as specified in **4.7.2**.

**4.8 Test for Sulphate Reducing Bacteria**

**4.8.1** *General*

The sulphate reducing bacteria effect a direct reduction of sulphates. They are widely distributed in nature and are common in soils, canals and lake waters, sewage, marine sediment, etc. The water used for sealing petroleum or gas tanks generally harbor these bacteria. They may also be present in water cooling systems of industrial plants. These bacteria cause blackening of pulp in a paper mill and corrode concrete sewage pipes and pipe surfaces. The most common of these organisms is *Desulphovibrio desulphuricans*. Some strains are mesophilic and grow best at 25 °C to 40 °C while others are thermophilic and grow at 45 °C to 60 °C. They are curved rods and are strictly anaerobic.

**4.8.2** *Apparatus*

**4.8.2.1** Incubator at 20 °C to 30 °C. For thermophilic organisms, use 45 °C incubator. An anaerobic jar can be used along with incubator.

**4.8.2.2** Pipets, sterile 1 ml and 10 ml, calibrated to deliver.

**4.8.2.3** Test tubes with close fitting or airtight caps, 16 mm by 150 mm and 20 mm by 150 mm.

**4.8.2.4** Test tube racks of sufficient size to contain 16 mm and 20 mm tubes.

**4.8.3** *Culture Media and Reagents*

**4.8.3.1** *Starkey's Medium A*

The composition of Starkey's Medium A is as specified in Table 20.

**Table 20 Composition of Starkey's Medium A**

(*Clause* 4.8.3.1)

| S. No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | Sodium lactate ($C_3H_5NaO_3$) | 3.5 g |
| ii) | Ammonium chloride ($NH_4Cl$) | 1.0 g |
| iii) | Dipotassium hydrogen phosphate ($K_2HPO_4$) | 0.5 g |
| iv) | Magnesium sulfate ($MgSO_4.7H_2O$) | 2.0 g |
| v) | Sodium sulfate ($Na_2SO_4$) | 0.5 g |
| vi) | Calcium chloride ($CaCl_2.2H_2O$) | 0.1 g |
| vii) | Thioglycollic acid | 0.1 g |
| viii) | Ammonium ferrous sulfate or ferrous ammonium sulfate $(NH_4)_2SO_4.FeSO_4.6H_2O$) | 0.001 g |
| x) | Distilled water | 1 000 ml |

Heat to dissolve and dispense 9 ml of medium per single strength tube, and 10 ml per double strength tube. Prepare double strength medium (2×) as above except 500 ml of water are used instead of 1 litre. Tubes should be of sufficient capacity to contain 1 ml of inoculum plus 9 ml of single strength medium or 10 ml of inoculum plus 10 ml of 2× medium. pH of medium should be 7.2 after autoclave sterilization, at 121 °C ± 3 °C for 15 min.

**4.8.3.2** *Starkey's Medium B*

The medium is similar to that described above with the following modification. Water collected from the sample collection site is filtered (1.2 micron membrane filter) to remove particulates and the *p*H is recorded. After preparing Medium B and prior to dispensing, check and adjust *p*H, if necessary to that of the original water used, then filter sterilize the medium by passage through 0.2 micron filter and aseptically dispense into pre-sterilized tubes.

**4.8.3.3** *Sulfate reducing agar medium for plate count method.*

The composition of *Sulfate reducing agar medium* is as specified in Table 21.

**Table 21 Composition of Sulfate reducing agar medium**

(*Clause* 4.8.3.3)

| S. No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | Trypticase soy agar | 40.0 g |
| ii) | Agar | 5.0 g |

| | | |
|---|---|---|
| iii) | 60 percent sodium lactate (0.4 percent *v/v*) | 4 ml |
| iv) | Magnesium sulfate ($MgSO_4.7H_2O$) | 2.0 g |

**4.8.3.4** *Sulfate reducing medium for MPN Method*

The composition of *Sulfate reducing agar medium* is as specified in Table 22.

**Table 22 Composition of Sulfate reducing medium**

(*Clause* 4.8.3.4)

| S. No. | Parameter | Requirement |
|---|---|---|
| (1) | (2) | (3) |
| i) | Sodium lactate | 3.5 g |
| ii) | Beef extract | 1.0 g |
| iii) | Peptone | 2.0 g |
| iv) | Magnesium sulfate ($MgSO_4.7H_2O$) | 2.0 g |
| v) | Sodium sulfate ($Na_2SO_4$) | 1.5 g |
| vi) | Dipotassium hydrogen phosphate ($K_2HPO_4$) | 0.5 g |
| vii) | Ferrous ammonium sulfate [$(NH_4)_2SO_4.FeSO_4.6H_2O$] | 0.392 g |
| viii) | Calcium chloride ($CaCl_2$) | 0.10 g |
| ix) | Sodium ascorbate | 0.10 g |
| x) | Distilled water | 1 000 ml |
| Prepare medium (excluding ferrous ammonium sulfate and sodium ascorbate), dispense in screw capped test tubes, and sterilize by autoclaving at 121 °C ± 3 °C for 15 min. The final *p*H is 7.5 ± 0.3. Use completely filled tubes. Separately sterilize extra medium to be added to tubes for filling. On the day of use, prepare separate solutions of ferrous ammonium sulfate (3.92 g/100 ml) and sodium ascorbate (1.00 g/100 ml), filter sterilize through 0.45 µm membrane filter, and aseptically add 0.1 ml of each solution to 10 ml basal medium. | | |

**4.8.3.5** *Hydrogen sulfide test reagent*

**4.8.3.5.1** *Ferric chloride stock solution ($FeCl_3.6H_2O$)*

Dissolve 13.5 g of ferric chloride in a mixture of 250 ml of water and 250 ml of HCl (sp gr 1.19). Store in an airtight amber container. Prepare fresh monthly.

**4.8.3.5.2** *p-Aminodimethylaniline dihydrochloride stock solution*

*p*-Aminodimethylaniline dihydrochloride ($C_8H_{12}N_2.2HCl$) 1.0 g HCl (6 N) 500 ml. Dissolve 1 g of p-aminodimethylaniline dihydrochloride in 500 ml of 6 N HCl. Store for up to 1 month in an amber airtight container.

**4.8.3.6** Liquid Paraffin, Heavy, sterile, or sterile mineral oil.

**4.8.3.7** *Buffered dilution water, stock solution*

Dissolve 34.0 g of $KH_2PO_4$ in 500 ml of water, adjust *p*H to 7.2 with 1 N NaOH and dilute to 1 litre with distilled water. This is called the stock phosphate solution. Dissolve 38 g of $MgCl_2$ in 1 litre of distilled water.

**4.8.3.8** *Buffered dilution water, working solution*

Add 1.25 ml of stock buffered dilution water and 5 ml of $MgCl_2$ solution to 500 ml of water. Bring to 1 litre with water. Mix well and dispense as 90 ml dilution blanks in screw-capped bottles. Sterilize by autoclaving at 121 °C for 15 min.

**4.8.4** *Detection of Sulphate Reducing Bacteria*

**4.8.4.1** Add 10 ml of the sample to 10 ml double strength sterile test tube(s). Cover the surface of each test tube by sterile liquid paraffin to a depth of 1 mm to 2 mm. Incubate the tubes at 28 °C to 30 °C for 5 days to 7 days.

**4.8.4.2** The production of black colour in medium will indicate the presence of Sulphate Reducing Bacteria (SRB).

**4.8.4.3** Expression of results: Report presence or absence of sulfate reducing bacteria in 1ml or 10ml of sample.

**4.8.5** *Enumeration of Sulphate Reducing Bacteria (MPN)*

**4.8.5.1** *General*

Water and water deposit samples and dilutions of these samples are dispensed into tubes of Starkey's medium (A or B) following five tube MPN procedures. The tubes are sealed with liquid paraffin and incubated at 20 °C for 21 days. Positive reactions are indicated by the deposit of a black precipitate. For the isolation and enumeration of thermophilic sulfate-reducing bacteria encountered in waters associated with oil and gas production, all broths, dilution blanks, and incubations must be maintained at temperatures of at least 45 °C and preferably within 5 °C at the sample temperature. The sensitivity of these test methods can be increased by purging the dilution blanks and tubes of media with nitrogen immediately prior to use.

**4.8.5.2** *Procedure*

**4.8.5.2.1** Clean and disinfect the area with a cleaning solution that leaves no residue. Set out and label five replicate tubes of 10 ml double strength Starkey's medium, A or B, in the test tube rack. Set out and label five replicate tubes of 10 ml single strength Starkey's medium, A or B, for each ml of sample or ml of sample dilution to be tested. Use two sets of five replicate 10 ml tubes, each to contain 1 ml of sample or 1 ml of 1/10 dilution of sample. Prior to sample inoculation, heat tubes of media and dilution blanks in a water bath to 60 °C then cool rapidly to 20 °C to ensure minimal oxygen levels.

**4.8.5.2.2** Alternative sulfate reducing medium (**4.8.3.4**) may also be used for MPN.

**4.8.5.2.3** Shake sample thoroughly, at least 25 times; make dilutions starting with 10 ml of sample into one 90 ml dilution blank.

**4.8.5.2.4** Incubate at room temperature (28 °C to 30 °C) After incubation, observe and note down the tubes showing black colour. Compare the tubes showing black colour from double strength and single strength media. With the help of MPN Table, quantify the number of SRB in the water sample.

**4.8.5.2.5** When more than three series of tubes are employed in a decimal series of dilutions, use the results from only three of these used in computing the MPN, for example:

| 10 ml | 1 ml | 0.1 ml | 0.01 ml | | Number of positive tubes | | MPN/100 ml |
|---|---|---|---|---|---|---|---|
| 5/5 | 5/5 | 2/5 | 0/5 | = | 5-2-0 × 10 | = | 490 |
| 5/5 | 4/5 | 2/5 | 0/5 | = | 5-4-2 | = | 220 |
| 5/5 | 3/5 | 2/5 | 1/5 | = | 5-3-2 | = | 140 |
| 5/5 | 0/5 | 0/5 | 0/5 | = | 5-0-0 | = | 23 |

**4.8.5.3** *Expression of results*

Report the results as number of sulfate reducing bacteria per 100 ml of sample (MPN/100 ml).

NOTE — If soil sample is to be analyzed, then take 1 000 mg, 100 mg, 10 mg in five test tubes each. Add 10 ml of the double strength medium in the 1 000 mg tubes and 5 ml of the single strength medium in each of the rest 10 test tubes. Follow the rest of the procedure as described above and report results as MPN/gram.

**4.9 Test for Sulphur Bacteria**

**4.9.1** *General*

The organisms belonging to the group sulphur bacteria are autotrophic bacteria which oxidize elemental sulphur or reduce sulphur compounds, obtaining their carbon requirements from carbon dioxide. They are undesirable in water used in many industrial processes, and the acid produced during their metabolism may be destructive to concrete and other structures, *Thiobacittus thioparus* and *Thiobacillus thiooxidans* arc the more common forms belonging to this group.

**4.9.2** *Test for T. thioparus*

**4.9.2.1** *Medium*

Dissolve 10.0 g of sodium thiosulphate pentahydrate, 2.0 g of dibasic potassium phosphate, 0.1 g of magnesium sulphate heptahydrate, 0.1 g of calcium chloride, 0.1 g of ammonium chloride, 0.02 g of ferric chloride hexahydrate and 0.02 g of manganese sulphate in 1 litre of water, Place 50 ml quantities in sterilized 250 ml conical flasks and sterilize by autoclaving at (1.02 ± 0.03) kg/cm$^2$ gauge pressure [(15 ± 0.5) psi gauge pressure, 121 °C ± 3 °C for 15 min] for about 15 min but not exceeding 30 min.

**4.9.2.2** *Procedure*

Inoculate a shallow layer of the medium with a known volume of the sample (50 ml or 10 ml or less) and incubate at about 30 °C for 2 days to 3 days. In the presence of *T. thioparus* bacteria the surface of the inoculated medium becomes covered with sulphur from the autotrophic oxidation of the thiosulphate,

**4.9.3** *Test for T. Thiooxidans*

**4.9.3.1** *Medium*

Dissolve 0.2 g of ammonium sulphate, 0.5 g of magnesium sulphate heptahydrate, 3.0 g of monobasic potassium phosphate, 0.25 g of calcium chloride and 0.01 g of ferrous sulphate in 1 litre of water. Weigh 1.0 g of elemental sulphur in a 250 ml flask and add to it 100 ml of this solution.

NOTE — Ferrous sulphate solution should be sterilized by filtration through a millipore membrane filter of 0.45 µm and then mixed aseptically to the rest of the basal medium. Heating, steaming or autoclaving of ferrous sulphate solution may result in oxidation and hydrolysis of the salt. Solution of potassium phosphate should be sterilized separately and then added aseptically to the basal medium. Sulphur should be sterilized by steaming for 30 min on three successive days before adding to the medium. Rest of the medium should be sterilized in a steam sterilizer for 30 min on 3 successive days.

**4.9.3.2** *Procedure*

Inoculate flasks containing the medium with 10 ml or less of the sample and incubate at 25 °C to 30 °C for 4 days to 5 days. The sulphur sinks to the bottom, the reaction of the medium decreases to *p*H 2.0 and in the presence of T, thiooxidans the medium will become turbid.

**4.10 Test for Gelatin Liquefying Bacteria**

**4.10.1** *General*

Certain micro-organisms are capable of producing proteolytic ferments which digest and liquefy gelatin. The presence of these organisms in process water is of importance in industries such as the manufacture of photographic films, edible gelatin, glue and in food processing.

**4.10.2** *Nutrient gelatin Medium*

Dissolve 3 g of beef extract, 5 g of peptone and 120 g of gelatin in 1 litre of water over a water-bath. Cool to about 50 °C and distribute into tubes in quantities of 10 ml to 15 ml. Sterilize in the autoclave for 20 min at (1.02 ± 0.03) kg/cm$^2$ gauge pressure [(15 ± 0.5) psi gauge pressure, 121 °C ± 3 °C for 15 min]. The final reaction of the medium shall be *p*H 6.8 ± 0.2.

**4.10.3** *Procedure*

**4.10.3.1** Place 1 ml of the well shaken sample in a sterile Petri dish. Add the molten medium at (30.0 ± 0.2) °C and mix thoroughly by careful rotation and to and fro movement of the dish placed on a flat table. Not more than 20 minutes shall elapse between placing the sample in the Petri dish and adding the medium. Incubate the dish at (20.0 ± 0.2) °C and examine every day for seven days for evidence of liquefaction of the medium around the colonies of micro-organisms. If the atmospheric temperature is above 20 °C the examination of the dish should be made as soon as it is taken from the incubator before the media starts thawing. The negative dishes shall be incubated further up to a period of at least 21 days. It is preferable to inoculate at least 5 dishes for each sample.

**4.10.3.2** *Confirmation*

**4.10.3.2.1** If further confirmation of the gelatin liquefying property of the organisms is desired, the following procedure shall be adopted:

**4.10.3.2.2** *Preparation of gelatin liquefying medium*

Dissolve in 1 000 ml water, 3 g meat extract (*see* IS 6851), 5 g peptone (*see* IS 6853), 120 g gelatin (*see* IS 7590 ) and 30 g sodium chloride. Distribute into sterilized test tubes and sterilize at 120°C for 12 minutes. The final pH should be 7.0.

**4.10.3.2.2.1** Place the gelatin medium tubes at (20.0 ± 0.2) °C for the medium to harden and take out only just before use. Pick out a suspected colony from the petri dish culture obtained in **4.10.3.1** using a straight needle and prepare a stab inoculation in the tube. Incubate the tubes at (20.0 ± 0.2) °C and examine for evidence of liquefaction as in **4.10.3.1**.

**4.10.3.3** If a 20 °C incubator is net available, the test for presence of gelatin liquefying organisms may be carried out by inoculating 1 ml of the sample into a tube of nutrient gelatin medium and incubating at (37.0 ± 0.2) °C. Examine every day for seven days for evidence of liquefaction by placing the tube in cooled water (below 20 °C) and observing whether the medium hardens or not. An un-inoculated tube of nutrient gelatin medium shall also be incubated and tested as a control.

**4.10.3.4** *Expression of result*

Report as present or absent in 1 ml or 10 ml.

**Annex A**

(*Clause* 2)

**LIST OF REFERED STANDARDS**

| *IS No.* | *Title* |
|---|---|
| IS 6851 : 1973 | Specification for meat extract, microbiological grade |
| IS 6853 : 1973 | Specification for peptone, microbiological grade |
| IS 7590 : 2023 | Gelatin, microbiological grade — Specification (*first revision*) |
| IS 13428 : 2024 | Packaged natural mineral water — Specification (*third revision*) |
| IS 15185 : 2016/ ISO 9308-1 : 2014 | Water quality — Detection and enumeration of *echerichia coli* and coliform bacteria — Membrane filtration method for water with low bacterial background flora (*first revision*) |
| IS 15186 : 2002/ ISO 7899-2 : 2000 | Water quality — Detection and enumeration of intestinal enterococci — Membrane filtration method |
| IS 15188 : 2022/ ISO 8199 : 2018 | Water quality — General requirements and guidance for microbiological examinations by culture (*second revision*) |
| IS 16122 : 2013/ ISO 7218 : 2007 | Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations |
| IS 17614 (Part 25) : 2022/ ISO 19458 : 2006 | Water quality — Sampling Part 25 Microbiological analysis |
| IS 17819 : 2022/ ISO 9308-2 : 2012 | Water quality — Enumeration of *escherichia coli* and coliform bacteria — Most probable number method |
| IS 18771 : 2024/ ISO 14189 : 2013 | Water Quality — Enumeration of *clostridium perfringens* — Method using membrane filtration |

## ANNEX B

(*Clause* 3.1.5)

## PARTICULARS TO BE SUPPLIED ALONG WITH SAMPLES

**B-1** While submitting samples the following particulars shall be supplied along with sample:

a) Name and address of person requesting the examination;
b) Name, designation or other identification particulars of the person drawing samples;
c) Date and time of collection and dispatch;
d) Reasons for examination and whether it is a routine sample or otherwise;
e) Source of water (well, spring, stream, public supply, etc.);
f) Exact place from which sample was taken. If from a tap whether the sample was drawn through a cistern: or directly from the mains;
g) The method of purification and sterilization used, if any; details of dose of chemicals, point of application, quantity treated, etc.;
h) Temperature of the sample;
j) Weather at the time of collection and particulars of recent rainfall;
k) Whether the water becomes affected in appearance, odour or taste after heavy rains;
m) If the sample has been taken from a well, then:
   1) Depth of well, and of water surface from ground level;
   2) Whether covered or uncovered, and nature, material and construction of the cover;
   3) Whether newly constructed or with any recent alterations which might affect the condition of the water;
   4) Type of construction — i) bricks set dry or in cement; ii) cement or cylinder lined) and whether puddled outside the lining; iii) depth of lining; iv) whether bricked above ground surface, if so, height of coping; v) presence and extent of apron; and vi) method of pumping or other means of raising water;
   5) Proximity of drains. cesspools or other possible sources of pollution, and distance from source;
   6) Any discoloration of the sides of the well or other visible indication of pollution;
   7) Nature of subsoil and water-bearing stratum.

NOTE — When available, a section or drawing of the well and general surroundings should be furnished.

n) If the sample has been taken from a spring, then
   1) Stratum from which it issues;
   2) Whether the sample has been taken direct from the spring or from a collecting chamber. If from the latter, the mode of construction of chamber;
p) If the sample has been taken from a river or stream, then;
   1) Depth below surface at which the sample was taken;
   2) Whether the sample was taken from the middle or side;
   3) Whether the level of water is above or below the average;
   4) Condition of weather at the time of sampling and particulars of any recent rainfall or flood eonditloas;
   5) Observations with reference to any possible sources of pollution in the vicinity and approximate distance from sampling point; and
n) Results of field tests made on the sample.

## ANNEX C

(*Clause* 4.3.1.1, 4.3.3.3, *and* 4.4.1.2.2)

## TABLES OF MOST PROBABLE NUMBERS

**Table 23 Most Probable Number (MPN) Index and 95 percent Confidence Limits for All Combinations of Positive and Negative Results When Five 20 ml Portions Are Used**

| No. of Tubes Giving Positive Reaction Out of 5 (20 ml each) | MPN Index/100 ml | 95 percent Confidence Limits (Exact) | |
|---|---|---|---|
| | | Lower | Upper |
| (1) | (2) | (3) | (4) |
| 0 | <1.1 | - | 3.5 |
| 1 | 1.1 | 0.051 | 5.4 |
| 2 | 2.6 | 0.40 | 8.4 |
| 3 | 4.6 | 1.0 | 13 |
| 4 | 8.0 | 2.1 | 23 |
| 5 | >8.0 | 3.4 | - |

**Table 24 Most Probable Number (MPN) Index and 95 percent Confidence Limits for All Combinations of Positive and Negative Results When Ten 10 ml Portions Are Used**

| No. of Tubes Giving Positive Reaction Out of 10 (10 ml each) | MPN Index/100 ml | 95 percent Confidence Limits (Exact) | |
|---|---|---|---|
| | | Lower | Upper |
| (1) | (2) | (3) | (4) |
| 0 | <1.1 | - | 3.4 |
| 1 | 1.1 | 0.051 | 5.9 |
| 2 | 2.2 | 0.37 | 8.2 |
| 3 | 3.6 | 0.91 | 9.7 |
| 4 | 5.1 | 1.6 | 13 |
| 5 | 6.9 | 2.5 | 15 |
| 6 | 9.2 | 3.3 | 19 |
| 7 | 12 | 4.8 | 24 |
| 8 | 16 | 5.8 | 34 |
| 9 | 23 | 8.1 | 53 |
| 10 | >23 | 13 | - |

**Table 25 Most Probable Number (MPN) Index and 95 percent Confidence Limits for All Combinations of Positive Results When Five Tubes Are Used per Dilution (10 ml, 1.0 ml, and 0.1 ml)**

| Combination of Positives | MPN Index/100 ml | Confidence Limits | |
|---|---|---|---|
| | | Low | High |
| (1) | (2) | (3) | (4) |
| 0-0-0 | <1.8 | - | 6.8 |
| 0-0-1 | 1.8 | 0.090 | 6.8 |
| 0-1-0 | 1.8 | 0.090 | 6.9 |
| 0-1-1 | 3.6 | 0.70 | 10 |
| 0-2-0 | 3.7 | 0.70 | 10 |
| 0-2-1 | 5.5 | 1.8 | 15 |
| 0-3-0 | 5.6 | 1.8 | 15 |
| 1-0-0 | 2.0 | 0.10 | 10 |
| 1-0-1 | 4.0 | 0.70 | 10 |
| 1-0-2 | 6.0 | 1.8 | 15 |
| 1-1-0 | 4.0 | 0.71 | 12 |
| 1-1-1 | 6.1 | 1.8 | 15 |
| 1-1-2 | 8.1 | 3.4 | 22 |
| 1-2-0 | 6.1 | 1.8 | 15 |
| 1-2-1 | 8.2 | 3.4 | 22 |
| 1-3-0 | 8.3 | 3.4 | 22 |
| 1-3-1 | 10 | 3.5 | 22 |
| 1-4-0 | 11 | 3.5 | 22 |
| 2-0-0 | 4.5 | 0.79 | 15 |
| 2-0-1 | 6.8 | 1.8 | 15 |
| 2-0-2 | 9.1 | 3.4 | 22 |
| 2-1-0 | 6.8 | 1.8 | 14 |
| 2-1-1 | 9.2 | 3.4 | 22 |
| 2-1-2 | 12 | 4.1 | 26 |
| 2-2-0 | 9.3 | 3.4 | 22 |
| 2-2-1 | 12 | 4.1 | 26 |
| 2-2-2 | 14 | 5.9 | 36 |
| 2-3-0 | 12 | 4.1 | 26 |

| Combination of Positives | MPN Index/100 ml | Confidence Limits | |
|---|---|---|---|
| | | Low | High |
| (1) | (2) | (3) | (4) |
| 2-3-1 | 14 | 5.9 | 36 |
| 2-4-0 | 15 | 5.9 | 36 |
| 3-0-0 | 7.8 | 2.1 | 22 |
| 3-0-1 | 11 | 3.5 | 23 |
| 3-0-2 | 13 | 5.6 | 35 |
| 3-1-0 | 11 | 3.5 | 26 |
| 3-1-1 | 14 | 5.6 | 36 |
| 3-1-2 | 17 | 6.0 | 36 |
| 3-2-0 | 14 | 5.7 | 36 |
| 3-2-1 | 17 | 6.8 | 40 |
| 3-2-2 | 20 | 6.8 | 40 |
| 3-3-0 | 14 | 6.8 | 40 |
| 3-3-1 | 21 | 6.8 | 40 |
| 3-3-2 | 24 | 9.8 | 70 |
| 3-4-0 | 21 | 6.8 | 40 |
| 3-4-1 | 24 | 9.8 | 70 |
| 3-5-0 | 25 | 9.8 | 70 |
| 4-0-0 | 13 | 4.1 | 35 |
| 4-0-1 | 17 | 5.9 | 36 |
| 4-0-2 | 21 | 6.8 | 40 |
| 4-0-3 | 25 | 9.8 | 70 |
| 4-1-0 | 17 | 6.0 | 40 |
| 4-1-1 | 21 | 6.8 | 42 |
| 4-1-2 | 26 | 9.8 | 70 |
| 4-1-3 | 31 | 10 | 70 |
| 4-2-0 | 22 | 6.8 | 50 |
| 4-2-1 | 26 | 9.8 | 70 |
| 4-2-2 | 32 | 10 | 70 |
| 4-2-3 | 38 | 14 | 100 |
| 4-3-0 | 27 | 9.9 | 70 |

| Combination of Positives | MPN Index/100 ml | Confidence Limits | |
|---|---|---|---|
| | | Low | High |
| (1) | (2) | (3) | (4) |
| 4-3-1 | 33 | 10 | 70 |
| 4-3-2 | 39 | 14 | 100 |
| 4-4-0 | 34 | 14 | 100 |
| 4-4-1 | 40 | 14 | 100 |
| 4-4-2 | 47 | 15 | 120 |
| 4-5-0 | 41 | 14 | 100 |
| 4-5-1 | 48 | 15 | 120 |
| 5-0-0 | 23 | 6.8 | 70 |
| 5-0-1 | 31 | 10 | 70 |
| 5-0-2 | 43 | 14 | 100 |
| 5-0-3 | 58 | 22 | 150 |
| 5-1-0 | 33 | 10 | 100 |
| 5-1-1 | 46 | 14 | 120 |
| 5-1-2 | 63 | 22 | 150 |
| 5-1-3 | 84 | 34 | 220 |
| 5-2-0 | 49 | 15 | 150 |
| 5-2-1 | 70 | 22 | 170 |
| 5-2-2 | 94 | 34 | 230 |
| 5-2-3 | 120 | 36 | 250 |
| 5-2-4 | 150 | 58 | 400 |
| 5-3-0 | 79 | 22 | 220 |
| 5-3-1 | 110 | 34 | 250 |
| 5-3-2 | 140 | 52 | 400 |
| 5-3-3 | 170 | 70 | 400 |
| 5-3-4 | 210 | 70 | 400 |
| 5-4-0 | 130 | 36 | 400 |
| 5-4-1 | 170 | 58 | 400 |
| 5-4-2 | 220 | 70 | 440 |
| 5-4-3 | 280 | 100 | 710 |
| 5-4-4 | 350 | 100 | 710 |

| Combination of Positives | MPN Index/100 ml | Confidence Limits | |
|---|---|---|---|
| | | Low | High |
| (1) | (2) | (3) | (4) |
| 5-4-5 | 430 | 150 | 1100 |
| 5-5-0 | 240 | 70 | 710 |
| 5-5-1 | 350 | 100 | 1100 |
| 5-5-2 | 540 | 150 | 1700 |
| 5-5-3 | 920 | 220 | 2600 |
| 5-5-4 | 1600 | 400 | 4600 |
| 5-5-5 | >1600 | 700 | - |