# Manual
# of
# Methods of Analysis-
# Microbiological Examination
# of Food and Water

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*Note: The test methods given in the manuals are validated/ standardized test methods. However, it would be the responsibility of the respective testing laboratory to confirm that the above methods are verified in their laboratory and gives proper result in their laboratory.*

# ABBREVIATIONS

| Sr. No. | Abbreviation | Expanded Form |
|---|---|---|
| 1. | % | Percentage |
| 2. | µm | Micro meter |
| 3. | $^0$C | Degree Celsius |
| 4. | Hr | Hour |
| 5. | L | Liter |
| 6. | min | Minutes |
| 7. | mL | Milliliter |
| 8. | mm | millimeter |
| 9. | µL | Microliter |
| 10. | IS | Indian Standard |
| 11. | ppb | Parts per billion |
| 12. | ppm | Parts per million |
| 13. | ISO | International Organization for Standardization |
| 14. | gm | Gram |
| 15. | mg | Milligram |
| 16. | Cfu | Colony forming unit |
| 17. | sp. | species |
| 18. | µg | Microgram |
| 19. | UV | Ultra Violet |

# Chapter 1

# Equipment

## Scope

This chapter covers general requirements and guidance for handling, maintenance, performance check and calibration/validation of equipment used in the food microbiology laboratory for conducting the microbiological examinations, to help achieve homogeneous results in different laboratories.

## General

All the equipments in microbiology laboratory should be verified as fit for the intended purpose and its performance monitored during use, where appropriate. Where necessary, equipment and monitoring devices should be calibrated to traceable national standards. The laboratory has to define frequency of calibration/validation and performance checks/intermediate checks of equipments, unless specified frequency. Equipments should be regularly checked and maintained to ensure safety and fitness for use. Equipments should be monitored according to the working conditions and the accuracy demanded for the results.

## Calibration of equipment

The laboratory must establish a programme for the calibration and performance verification of equipment which has a direct influence on the test results. The frequency of such calibration and performance verification will be determined by documented experience and will be based on need, type and previous performance of the equipment. Intervals between calibration and verification shall be shorter than the time the equipment has been found to take to drift outside acceptable limits.

## Caution

When conducting microbiological examinations, it is especially important that and working environment shall be free from contamination and only those microorganisms which are present in the samples are isolated and enumerated. To achieve this, it is necessary to choose the correct equipment for food microbiology examination.

**Apparatus/Instruments**

**1. Protective cabinets (Biosafety Cabinet/Laminar Air Flow)**

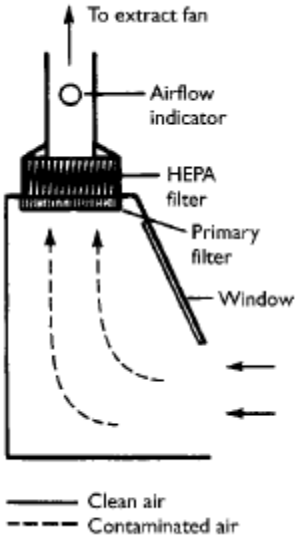
**1.1. Description**

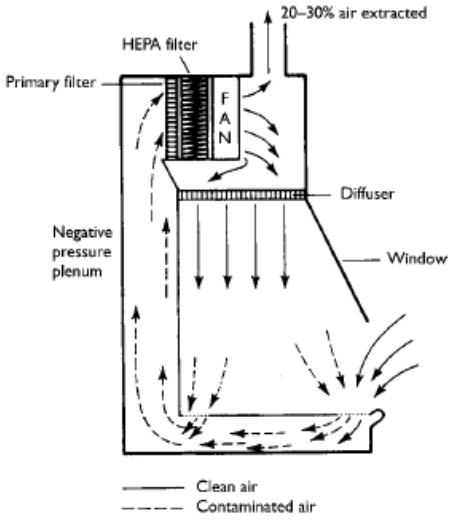
Protective cabinets are enclosed workspaces with a ventilated hood designed to contain pathogenic microorganisms, dust and other particles during food microbiological examination. These cabinets are equipped with HEPA (high-efficiency particular air filters) and a shortwave ultraviolet germicidal lamp that sterilizes the workstation. HEPA filters remove 99.97% of the particles having a size of more than 0.3 μm. For cabinets used in food microbiology, the number of particles shall not exceed 4 000 per cubic metre.

These are intended to capture and retain infected airborne particles released during the food analysis and to protect the food analyst from the infection that may arise from inhaling them. Four types of cabinet are use in food microbiology laboratory.

a) Class I is the most basic cabinet that protects the environment and the laboratory personnel. However, does not provide protection to the product. Biosafety cabinets of this class are either ducted (connected to the building exhaust system) or un-ducted (recirculating filtered exhaust back into the laboratory). The unsterilized room air is drawn in through opening, over the work surface. An airflow of between 0.7 and 1 m/s must be maintained through the front of the cabinet. Advanced cabinets have airflow indicators and alarming devices. The filters must be changed when the airflow falls below this level. They are not recommended for work with risk category 3 pathogens because of the difficulties in maintaining and ensuring appropriate operator protection.
b) Class II safety cabinets protect the product, the operator, and the environment. Class II BSCs are designed with an open front with inward airflow (personnel protection), downward HEPA-filtered laminar airflow (product protection) and HEPA-filtered exhaust air (environmental protection). These cabinets are further differentiated by types based on construction, airflow and exhaust systems. The types include A1, A2, B1, B2 and C1. They are suitable for work with risk category 2 and 3 pathogens.
c) Horizontal laminar outflow cabinets protect the work from contamination but blow any aerosols generated into the operator's face. Therefore, they are not suitable for handling inoculated cultures or preparation of tissue culture.
d) Vertical laminar air flow cabinets protect the product by the use of the vertical laminar flow of HEPA-filtered air. They also protect the operator by the use of

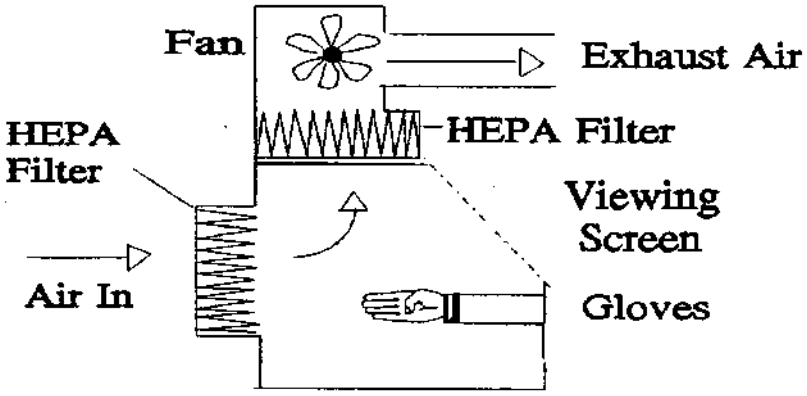
internally recirculated air. They are particularly suitable for providing an aseptic environment for handling sterile products and for protecting the operator when handling powders.




A B

Fig. A- Class I microbiological safety cabinet B- Class II microbiological safety cabinet




C - Class III microbiological safety cabinet

**1.2. Use**

These cabinets are intended to protect the food analyst from airborne infection. They do not protect from the consequences of poor techniques like spillage. The cabinet should not be overloaded with unnecessary equipment. Work shall be done in the middle to the rear of the cabinet, not near the front, and the worker should avoid bringing hands and arms out of the cabinet while working.

After each sample analysis before withdrawing the hands, the analyst should wait for 2–3 min to allow any aerosols to be spanned into the filters. The use of a gas burner or wire incinerator is not recommended in protective cabinets. If it is necessary, the gas burner should have a small flame so that the airflow is not disturbed. The use of disposable equipment (loops, pipettes, etc.) is recommended.

Cabinets should be kept as free of equipment as possible. Where practicable, place everything needed inside the cabinet before starting work to minimize the number of arm movements into and out of the working aperture. Position the equipment and materials so as to minimize disturbance to the airflow at the working aperture. Operators should be adequately trained in the correct use of cabinets to ensure their safety and the integrity of the product or culture.

### 1.3. Performance checks

The efficiency of a protective cabinet shall be checked by a qualified person on receipt and thereafter at regular intervals as recommended by the manufacturer, as well as after any repair or modification. Periodic verification of freedom from any microbial contamination should be carried out by a check of the working surface and walls of the cabinet. A periodic verification of the number of airborne microorganisms present should be carried out during operation of the filters using the usual equipment. For example, expose several open Petri dishes containing a non-selective agar culture medium (e.g. PCA) in each cabinet for 30 min. Plates are incubated at 37° C and examined at 24 and 48 hours. No colonies should be present on the plates. If one or more plates have colonies, the test must be repeated to determine if contamination is due to a malfunctioning hood or by analyst technique. If three individual trials consistently indicate contamination, it is assumed that the hood is malfunctioning, and a service technician should be contacted to check the airflow rate and determine if there are any holes in the filters or in the hood itself.

### 1.4. Maintenance

Clean the surface of cabinet with 70% ethanol/IPA or other disinfectant specified befoe and after completion of work. Regularly examine wire grids protecting prefilters and wipe clean with a disinfectant-soaked cloth. For laminar flow cabinets, the filter face should be vacuum cleaned regularly, taking care not to damage the filter medium. The filters should be checked monthly for plugging or obvious dirt accumulation and replaced as needed. Safety cabinets should be fumigated before filter changing or servicing. After cleaning of the cabinets, UV lamps may be used for disinfection.

The plug should be removed from the outlet and the fluorescent lamps cleaned every 2 weeks with a soft cloth moistened with ethanol.

UV lamps should be regularly cleaned and replaced in accordance with the manufacturer's instructions or every 3 months the ultraviolet lamps should be tested with a light meter. If the lamp emits less than 80% of its rated output, it should be replaced. Because ultraviolet rays do not penetrate, hoods must be empty for the ultraviolet lamp to be effective. The ultraviolet lamp should be turned on for 10 minutes before the hood is used and the same procedure repeats after analysis.

## 2. Autoclave

### 2.1. Description

An autoclave is a pressurized chamber used for sterilization by combining three factors: time, pressure, and steam. It uses steam under pressure as its sterilization agent at approx. 121 °C temperature and 15 lb./in2 pressure for about 15–30 min.

The autoclave must be able to maintain an internal temperature of 121° under a pressure of 1 bar (15 psi); it should be equipped with a calibrated temperature probe to measure the temperature within the sterilizing chamber; a calibrated pressure gauge (0-20 psi range), timer and safety valves. The autoclave should preferably be equipped with a temperature recorder to provide a permanent record of the sterilizing cycle.


**Fig: - Autoclave**

### 2.2. Use

Air removal is the important step before the pressure build-up. If the autoclave is not fitted with an automatic evacuation device, it is necessary to remove the air until a continuous jet of steam is emitted.

During the same sterilization cycle, do not use the autoclave to sterilize clean equipment (and/or culture media) and at the same time to decontaminate used equipment (and/or used culture media). It is preferable to use separate autoclaves to sterilize clean equipment and culture media and used equipment and culture media. After autoclaving, all materials and equipment should be allowed to cool within the autoclave before removal. For safety reasons, do not remove the contents until the temperature has dropped below approximately 80 °C.

### 2.3. Performance checks

The autoclave shall be kept in good operating condition and shall be regularly inspected by competent qualified personnel in accordance with the manufacturer's instructions. Keep the monitoring instruments in good working order and verify them regularly. Initial validation should include performance studies for each operating cycle and each load configuration used in practice. This process should be repeated after significant repair or modification. Sufficient temperature sensors should be positioned within the load to demonstrate adequate heat penetration at all locations. Validation and revalidation should consider the suitability of heat-up and cool-down times as well as the sterilization temperature.

Each cycle of autoclaving should have a heat-sensitive indicator such as autoclave indicator tape or strip attached to the outside of the bag. At least once a month, performance of autoclaves that are used to decontaminate waste or sterilization of media should be tested by using a biological indicator, such as endospores from the bacterium *Geobacillus stearothermophilus.*

### 2.4. Maintenance

Daily:

Clean door gasket with a soft cloth. The gasket should be clean and smooth.

Weekly:

Take out the tray holder and trays. Clean the tray holder and trays with a cleaning agent & water and with a cloth sponge.

Clean the outer parts of the autoclave with a soft cloth.

Clean the electrode with a soft cloth

Once a week, or after 20cycles (whichever comes first), drain the water from the reservoir and refill with fresh mineral-free water or distilled water.

Monthly:

Clean the strainer once

Once every month activate the safety valve

## 3. Sterilizing oven

### 3.1. Description

**Hot air ovens** are equipment that uses dry heat to sterilize. They are capable of maintaining a temperature of 160 °C to 180 °C for the destruction of microorganisms by dry heat. Also known as dry-heat sterilizers, they are thermostatically controlled and fitted with circulating fans to ensure even temperatures in all parts of the equipment.

### 3.2. Use

The hot air oven is used for sterilizing most laboratory glassware and not suitable for heat-sensitive materials such as many plastic and rubber items. Since air is not a good conductor of heat, overloading of the oven is not recommended. If volumetric glassware is sterilized in the sterilizing oven, verify regularly the accuracy of marked volumes. The temperature shall be uniform throughout the chamber. The oven shall be equipped with a thermostat and a thermometer or temperature-recording device of suitable accuracy.

It should be equipped with a duration indicator, programmer, or timer. Once the operating temperature is reached, the sterilizing procedure shall last for at least 1 h at 170 °C or an equivalent time/temperature combination. After sterilization, to prevent cracking, glassware should be allowed to cool in the oven before removal.

### 3.3. Performance checks

Check the stability and homogeneity of the temperature throughout the oven before initial use and after any repair or modification which might have an effect on the temperature control.

The oven shall be fitted with a calibrated thermometer, thermocouple or temperature-recording device of suitable accuracy which is independent of the automatic temperature-regulation system. The monitoring device shall have a resolution of 1 °C or better at the oven temperature used. The temperature of the oven should be monitored and recorded during each use.

Equipment should be calibrated with thermocouples at regular intervals. Commercially available indicators can also be used forverification with each load.

### 3.4. Maintenance

Interior surfaces should be cleaned with a mild detergent solution, rinsed with tap water, and dried.

## 4. Incubators

### 4.1. Description

An incubator consists of an insulated chamber which enables the temperature to be kept stable and uniformly distributed to within the maximum permissible temperature error specified in the test method. BOD incubator has both heating and cooling option and should be used in case of temperature requirement of less than $30^0$C. For the growth of anaerobic bacteria, CO2 incubators are available that have an internal atmosphere of 5–8% $CO_2$.

### 4.2. Use

Separate incubator shall be used for each incubation temperature requirement. Incubators should be kept separately in the laboratory where controlled environment temperature i.e. 25 ± 2 °C, relative humidity of 50 ± 10% is maintained. Incubators should never be overloaded.

### 4.3. Performance checks

Check the temperature stability and the homogeneity of the temperature distribution at the working temperature(s) throughout the working volume of the incubator through simultaneous use of several thermometers or thermocouples of known accuracy and appropriate temperature range. Check the incubator temperature at least every working day. For this purpose, each incubator shall incorporate at least one working measurement device, whose bulb can be immersed in glycerol (or other appropriate heat sink) contained in a sealed bottle.

### 4.4. Maintenance

Clean and sanitize regularly the inner and outer walls of the incubator and, if appropriate, remove dust from the ventilation system. Any spillage within the incubator should be cleaned and disinfected immediately to prevent subsequent cross-contamination. All interior surfaces should be cleaned with a mild detergent solution, rinsed, and dried thoroughly with a soft

cloth.

If stainless steel surfaces become discoloured by iron rust, a solution of 20% nitric acid and 1.5% hydrofluoric acid or a 2-5% solution of warm oxalic acid may be used to swab the affected area. After 1-2 minutes the area should be flushed with clean water to remove all of the acids and then dried thoroughly. When using these acid solutions, the analyst's hands must be adequately protected with rubber gloves, and the room must be well ventilated.

## 5. Anaerobic incubation

### 5.1. Description

Anaerobic incubation can be carried out in jars specially designed to maintain an anaerobic atmosphere for the growth of anaerobic bacteria. The anaerobic atmosphere is developed by the addition of hydrogen to the jar containing a platinum catalyst, in the presence of which the oxygen reacts with the hydrogen/ carbon dioxide/nitrogen gas mixture to produce water.

Alternatively, the hydrogen may be produced by a gas generation sachet to which water is added. The jar is placed in an incubator to achieve the correct incubation temperature.

### 5.2. Use

The anaerobic jar is an instrument used in the Microbiology laboratory, for the generation of anaerobic condition (anaerobiosis) to culture obligate anaerobes such as *Clostridium* spp.

### 5.3. Performance checks

The anaerobic atmosphere is verified by the growth of a strict anaerobe such as *Bacteroides melaninogenicus* and the failure of growth of a strict aerobe such as *Pseudomonas aeruginosa.*

Methylene blue indicator strips, which turn colorless in anaerobic conditions, may also be used.

### 5.4. Maintenance

Regularly clean and sanitize the equipment.

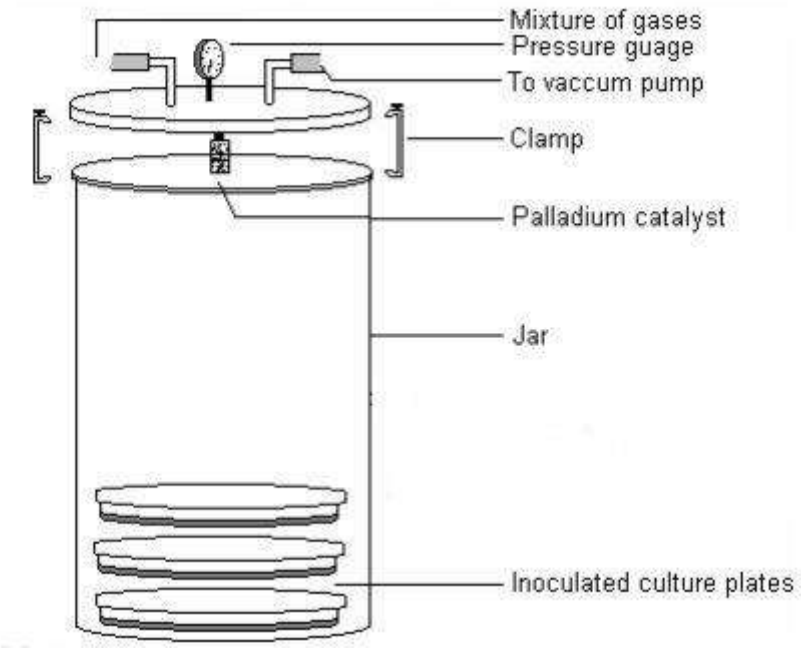



**Fig. Anaerobic jar**

## 6. Microwave oven

### 6.1. Description

Microwave oven, also called electronic oven, that allows heating of items using high-frequency electromagnetic waves called microwaves.

### 6.2. Use

A microwave oven is used to heat liquid and melting solidified agar media (but not for sterilization). Do not use metal equipment, including metal closures. Loosen bottle caps or stoppers before heating. Set microwave power and time to minimum settings. Take care to avoid media bubbling over.

A standing time of at least 5 min is recommended after the heating process before removal from the microwave oven.

### 6.3. Performance checks

The media melted in the microwave oven can be checked for quality performance with those obtained by conventional techniques.

### 6.4. Maintenance

Clean the oven immediately any spillage occurs, as well as at regular intervals dependent on usage. Ovens door seals should be inspected for integrity and the oven checked for radiation leakage at regular intervals.

## 7. Balances and gravimetric diluters

### 7.1. Description

The microbiology laboratory should be equipped with two top-loading balances, one with a capacity of 2,000 g and a sensitivity of 0.1 g, and a second with a capacity of 100-200 g and a sensitivity of 1 mg. Gravimetric diluters are electronic instruments consisting of a balance and programmable liquid dispenser and are used during the preparation of initial sample suspensions; they function by adding diluent to a subsample at a set ratio. The subsample is then weighed to the tolerance specified in the application, and the diluter set to dispense sufficient diluent for the ratio required (e.g. 9 to 1 for decimal dilutions).

### 7.2. Use

Balance shall be placed on a stable horizontal surface, adjusted as necessary to ensure that it is level and protected from vibration and draughts. Balances are mainly used for weighing the test portion of the sample to be examined and the components of the culture media and reagents. In addition, they may be used for carrying out measurements of dilution fluid volumes by mass.

Always wear Gloves, Face mask, and Apron while weighing the media taking care of the possible health hazards i.e. its carcinogenicity, disorders by inhalation of the media or allergic reactions, etc. A food microbiology laboratory shall be equipped with balances of the required range and measurement uncertainty for the different products to be weighed. Unless otherwise stated, the maximum permissible errors should be 1 % or better when weighing out test samples.

### 7.3. Performance checks

The performance of the balance system shall be regularly verified during use and after cleaning with check weights by a trained person. Calibration shall be checked across the entire range by a qualified person at a frequency dependent on use.

Check balance routinely (preferably daily before use) using at least two working weights that

bracket the normal usage range. The accuracy of all high-precision analytical balances should be checked at least every 3 to 6 months with a series of calibrated weights. The Tare mechanism must be checked before loading and after unloading.

### 7.4. Maintenance

Before each use, clean balance and tare weight before adding any material to weigh. If two or more balances are being cleaned simultaneously, the analyst should be certain not to interchange the pan supports and weighing pans of different balances.

Balances should be always clean. Weights should be cleaned regularly and must be protected from dust or dirt. The cleaning procedure should be done at a regular time.

## 8. pH meter

### 8.1. Description

ApH meter should preferably be fitted with a built-in temperature compensation system and with two regulating facilities for adjustment. It shall be capable of measuring to an accuracy of 0.05 pH units and its resolution shall be 0.01 pH units.

Use a digital meter, graduated in 0.1 pH units or less, that includes the theoretical slope of temperature compensation because the electrode pH response is temperature-dependent. Use electrodes suitable for a wide temperature range, and use a flat-head electrode to measure solid agar media.

The pH meter shall be equipped with either manual or automatic temperature compensation.

### 8.2. Use

A pH meter is used to measure the pH value of culture media and reagents to check if the adjustment is needed during preparation and as a quality check after sterilization. It may also be used to measure the pH value of samples and sample suspensions

Keep the probes clean and store the electrode immersed in the manufacturer-recommended solution.

Adjust the pH meter as indicated in the manufacturer’s manual to measure the pH value at a standardized temperature, e.g. 25 °C. Read the pH value after stabilization has been reached. Record the value to two decimal places.

**NOTE:** The reading may be considered stable when the pH value measured over a period of 5 s varies by not more than 0.02 pH units. Using electrodes in good condition, equilibrium is

normally achieved within 30 s.

### 8.3. Performance checks

The pH meter must be calibrated at least once every day before use. This can be done in accordance with the manufacturer's instructions, using at least two, and preferably three, standard buffer solutions. Buffer solutions at pH values of 4.00, 7.00, and 10.00 are most commonly used in the food microbiology laboratory. Define maximum permissible errors for this verification, depending on the use.

To verify that the pH meter is functioning properly, measure and record its slope after standardization daily. Most meters provide slope values automatically. If the pH meter does not calculate the slope automatically but can provide the pH in millivolts (mV), use the following formula to calculate the slope:

1) Read the mV potential generated by an electrode in the calibration buffer.

2) Determine the mV potential being generated per pH unit.

3) Divide this number by the theoretical maximum (59.16 mV/pH unit @ 25$^{o}$C) and multiply by 100.

Example:

pH electrode generated –15 mV in pH 7.01 buffer and +160 mV in pH 4.01 buffer.

160 mV – (-15 mV) = 175 mV

175mv/3 = 58.33 mV/pH unit

58.33/59.16 x 100 = 98.6% slope

If the slope is <95% or >105%, the electrode or meter may need maintenance. If all three buffers are used in sequence to standardize the meter (three-point standardization), analysts may provide both slopes and an average.

The electrodes are subject to ageing especially when solid media are used. This becomes apparent when there is a slow response during calibration. The final pH signal must be apparent within one minute with a stable response (±0.03 pH units) and sensitivity better than 95%.

The sensitivity of electrodes can be checked by measuringthe difference in mV potential between pH 4.00 and pH 7.00. The difference should be 172-171 mV. If the difference is less than 172mV but higher than 150 mV, the electrodes should be regenerated.

### 8.4. Maintenance

Rinse the electrodes with distilled or deionized water before and after each use. Replace pH buffer supply containers by the expiration date, preferably 6 months after opening because the solution may absorb carbon dioxide. Inspect the pH meter electrode for scratches, cracks, salt crystals build-up, or membrane/ junction deposits. Rinse off any salt build-up with distilled water.

Glass electrodes should be stored in a pH 7 buffer or a slightly acidic solution. Reference electrodes should be maintained in a 0.1 M KC1 solution to keep the junction moist and free-flowing. The levelof filling solution in the reference electrode should always be maintained above the level of both test and soaking solutions to provide a positive head pressure, thereby forcing the filling solution out through the junction.

To restore electrode efficiency, the tip and other contaminated surfaces should be cleaned. Pepsin or 0.1 M HC1 may be used to remove protein layers. To remove inorganic deposits, the electrode tip may be washed with ethylenediamine tetraacetic acid. To remove grease and oily films, the electrode tip may be cleaned with acetone, methanol, or diethylether. For electrode cleaning, a pepsin solution (commercially available) can be used.

## 9. Homogenizers, blenders and mixers

### 9.1. Description:

This equipment is used to prepare the initial suspension from the test sample of non-liquid products.

The following apparatus may be used:

i. a peristaltic blender (stomacher) with sterile bags, possibly with a device for adjusting speed and time; or
ii. a rotary homogenizer (blender), the notional speed of which is between 8 000 r/min and 45 000 r/min inclusive, with sterilizable glass or metals bowls equipped with covers; or
iii. a vibrational mixer (pulsifier) with sterile bags; or

### 9.2. Use

The usual operating time of a peristaltic homogenizer is 1 min to 3 min unless otherwise specified in standard method.

The rotary homogenizer shall operate for a duration such that the total number of revolutions

is between 15 000 r/min and 20 000 r/min inclusive. Even with the slowest homogenizer, this time shall not exceed 2,5 min.

Homogenizers, blenders, and mixers cannot be used for products that can puncture the bag due to the presence of sharp, hard or dry particles or products difficult to homogenize

### 9.3. Maintenance

Clean and disinfect peristaltic homogenizers and vibrational mixers regularly and after any bag spillage or leakage. For rotary homogenizers, clean and sterilize the glass or metal bowl after each use. Whenever spillage occurs, the exterior should be disinfected immediately to prevent contamination.

## 10. Thermostatically controlled Water baths

### 10.1. Description:

Water Bath is a conventional device that required a controlled environment at a constant temperature. A sensor in the device transfers water temperature to a reference value which is then amplified and a control system generates a signal for the heating system which heats the water to the desired temperature. A cover is recommended to prevent evaporation and to facilitate temperature control.

### 10.2. Use

The main uses are as follows:

- incubation at a constant temperature of inoculated culture media;
- maintenance of sterile molten agar media during media preparation;
- tempering of sterile molten agar media for use in specific methods;
- preparation of initial sample suspensions or solutions at a controlled temperature;
- heat treatment of initial sample suspensions at a controlled temperature (e.g. pasteurization).

Where precise temperature control is required, the bath shall be equipped with a circulating-water pump and an automatic temperature-regulation system. Any agitation of the liquid shall not cause droplet dispersal.

Care must also be taken to control possible cross-contamination during incubation. Water

baths with efficient control of temperature (±0.1°C) are used for the incubation of culture media or for heat treatment. Water baths (±1°C) are used for melting and/or maintaining melted agar bases**.** For incubation of inoculated media, maintain the liquid level so that the top of the test medium is at least 2 cm below the liquid level in the bath throughout the incubation.

### 10.3. Performance checks

Check the stability and homogeneity of the temperature throughout the bath before initial use and after any repair or modification having an effect on the temperature control.

Monitor each bath with a thermometer, thermocouple or temperature-recording device of suitable minimum measurement uncertainty, and independent of the automatic temperature-regulation system.

A digital display may also be used, provided that its accuracy and resolution are verified.

Monitor the temperature of the bath during each use and at least daily for periods of extended incubation.

The actual temperature and the possible fluctuations in water baths must be checked with calibrated thermometers having a suitable scale.

### 10.4. Maintenance

Water bath should be filled only with distilled/deionized water. Maintain water level on regular basisso it is above the upper level of the medium in either tubes or flasks. Use only stainless steel, plastic-coated, or other corrosion-proof racks. Empty and clean bath as needed to prevent buildup of salts and microbial growth, and disinfect before refilling. Removal of glass, remnants, and media remains must be made regularly. The water bath is cleaned by heating to 80°C for one hour, and after allowing for cooling, the water is removed by siphon. The bath is cleaned with citric acid 0.3% or sorbic acid 0.1%, debris is removed and the bath refilled with distilled water and reset.

## 11. Media preparatory

### 11.1. Description:

Media preparators ensure sterility is accomplished while minimizing the alteration to the nutrient components of media and improves the overall quality of the media. It consists of a heating vessel, water jacket, and continuous stirring device. The equipment shall also be

fitted with a temperature probe, pressure gauge, timer, and safety valve.

### 11.2. Use

The media preparator is principally designed for the sterilization of large volumes of media volume greater than 1 L. In a media preparator, the entire process takes place within the apparatus. The calculated quantity of dehydrated media is added to the water in the preparation vessel and then dissolved under continuous stirring and heating. Subsequent sterilization ensures a standard procedure. After preparation, the plate pouring should be checked to ensure an even surface of the agar.

### 11.3. Performance checks

Initial performance checks of the equipment are undertaken before being placed into service. Performance checks may also be necessary when deviations are noted within the course of the process that falls outside the tolerances.

Initial validation should include performance studies for each operating cycle and each load size used in practice. This process should be repeated after significant repair or modification. Two temperature probes, one adjacent to the control probe and another remote from it, may be used to demonstrate uniform heating. The temperature and duration of each cycle should be checked.

Calibration of temperature probe, pressure gauge, timer shall be done.

### 11.4. Maintenance

The preparation vessel must be carefully cleaned after each cycle of sterilization. Wash the preparator and rinse thoroughly with purified water between each media batch.

## 12. Membrane filtration/syringe driven filter

### 12.1. Description:

Many types of membrane filter and ready-for-use syringe driven filter are commercially available of 0.22/0.45 µm. Filter sterilization is used for:

1) liquids that cannot withstand high temperatures as they may be chemically changed or inactivated;

2) components in media that undergo undesirable reactions during heat sterilization with other substances.
3) Filtration of water samples

### 12.2. Use

For the membrane filter, filtration assembly unit must be sterile; this can be achieved by moist sterilization.

Disposable syringe filters sterilized by the manufacturer are used with disposable syringes for the sterilization of small volumes.

### 12.3. Performance checks

The performance of membrane filter can be checked by testing the filtrate for sterility by determining the pour- or spread-plate method.

### 12.4. Maintenance

Follow the manufacturer instruction for the storage and handling.

## 13. Membrane filtration apparatus

### 13.1. Description:

Membrane filtration assembly is used to a measured known volume of a liquid, usually water, drawn by vacuum through a membrane of sufficiently small pore size (0.45 micron) to trap all the microorganisms in the sample.

Apparatus consists of a manifold accommodating one or multiple filter funnels which are easily removable and a vacuum source connected by silicone tubing. The porous support for the membrane must provide sufficient support and prevent damage to the membrane.

### 13.2. Use

Before initial use, assemble filtration units and check for leaks. Cross-contamination during filtration is prevented by using loose-fitting lids on the funnels and is controlled between samples by disinfecting or sterilizing the removable filter funnels and lids between each analysis. The filter mounting and vacuum flask should be packed separately in laminate bags or sterilization paper.

### 13.3. Performance checks

The filter funnels will require calibration of the volume held, as the markings do not always correspond to the precise volume. A performance check is carried out by pouring a measured or weighed volume into the funnel and correcting the markings as necessary.

### 13.4. Maintenance

Filter assembly first cleans with tap water then 0.1% liquid soap again clean with tap water and finally rinse with distilled water, wrap in nontoxic paper or foil, and sterilize.

## 14. Thermometers

### 14.1. Description

Thermometers and thermocouples are used for temperature measurement for calibration and performance checks for various instruments in the microbiology laboratory. Liquid-in-glass thermometers may be of high accuracy, namely in the order of 0.02°C. Ordinary thermometers achieve a maximum error of usually ± 1°C.

### 14.2. Use

Use thermometers with temperature increments of 0.5°C or less, as appropriate. Thermometers used in refrigerators or sample-receipt areas may have temperature increments of 1 or 0.5°C. If using liquid-based thermometers to measure temperatures in incubators and refrigerators, keep the thermometer bulb in liquid paraffin or polypropylene glycol to buffer against heat loss when the door is opened and provide a stable reading.

Another option is to equip incubators, water baths, etc. with temperature-recording instruments that continuously monitor the operating temperature. These wired or wireless data-logging systems can be downloaded into a computerized or printed record. Data-logging units must meet the same requirements as temperature-sensing devices.

The measurement uncertainty of the temperature-monitoringdevice should be four times smaller than the range of the requested maximum permissible error. For example, for a target maximum permissible error of ± 1 °C, the measurement uncertainty should be ± 0.25 °C; for a target maximum permissible error of ± 0.5 °C, the measurement uncertainty should be ± 0.125 °C. The measurement uncertainty of the reference thermometer calibration should also be taken into account when determining the operating temperature

### 14.3. Performance checks

Reference thermometers and other temperature-monitoring devices shall be calibrated and maintain metrological traceability of its measurement results by means of a documented unbroken chain of calibrations. They shall be used for reference purposes only and shall not be used for routine monitoring. Reference thermometers shall be calibrated across the entire range against traceable national or international standards before initial use and at least every 5 years. Intermediate single-point (e.g. ice point) calibration shall be performed to verify performance.

Performance of the working thermometers and other temperature-recording devices shall be done in a way to maintain metrological traceability of its measurement results. Perform three-point verification at, below, and above the temperature at which the temperature-sensing device will be used. Intermediate checks shall be made against a reference thermometer to verify performance.

### 14.4. Maintenance

Maintain thermometers and thermocouples in a clean and sound condition.

Maintain other temperature-monitoring devices by the manufacturer's instructions.

**WARNING — Mercury is hazardous to health. Remove spillages in accordance with national regulations.**

## 15. Deionizing and distillation apparatus

### 15.1. Description

For the preparation of culture media, the water must be free from inhibitory substances which can affect the growth of microorganisms. This can be achieved by distillation, demineralization, reverse osmosis, or a combination of these purification procedures.

### 15.2. Use

It is used to obtain distilled water required for preparation of solutions and media and for final rinsing of glassware.

The distilled water shall be stored in tightly closed containers made from an inert material (neutral glass, polyethylene, etc.) which shall be free from all inhibitory substances. It is however recommended that the water is used as soon as produced.

### 15.3. Performance checks

The conductivity of freshly produced water from a deionizing or distillation apparatus must be monitored monthly. An efficient installation should deliver water with a conductivity of ≤ 2 mhos/cm (µm siemens/cm) at 25°C.

Microbial contamination should not exceed $10^3$ colony forming units (cfu)/ml and preferably be below $10^2$cfu /ml. Microbial contamination should be regularly monitored according to ISO 6222 with incubation at 22°C ± 1 °C for 68 h ± 4 h or using an equivalent method.

### 15.4. Maintenance

Multiplication of microorganisms in stored distilled/deionized water can be avoided by cleaning the storage tank regularly The distillation apparatus should be cleaned regularly.

## 16. Refrigerator/Cold Storage Room

### 16.1. Description

Refrigerators are used for:

i. To store test samples
ii. To store prepared media, agar plates or their perishable supplements and ingredients.
iii. Antisera
iv. Rapid Kits

The temperature to be maintained by refrigerators should be indicated on the exterior.

### 16.2. Use

For the storage of food samples for analysis, the temperature shall be 3 °C ± 2 °C. For other uses, the temperature, unless otherwise specified, shall be 5 °C ± 3 °C. Load refrigerators, chillers and cold-storage rooms in such a way that appropriate air circulation is maintained and the potential for cross-contamination is minimized.

To avoid cross-contamination, use different chambers, or at least different containers, to achieve physical separation, for the storage of

- uninoculated culture media and reagents,
- test samples, and
- microorganism cultures and incubated media.

### 16.3. Performance checks

Check the temperature of each chamber each working day using a thermometer or a permanently installed probe. The accuracy required of the temperature-monitoring device is dependent on the purpose for which the unit is used.

### 16.4. Maintenance

Defrost as required and discard outdated materials monthly. Cleanliness should be maintained by removing unused media orliquids and, depending on the type of refrigerator, by defrosting atregular intervals.

Exteriors should be cleaned with a damp cloth at least monthly cleaning and sanitization of the inside of the chambers.

## 17. Freezer and deep freezer

### 17.1. Description

A freezer is a chamber that allows frozen storage to be guaranteed. The temperature, unless otherwisespecified, shall be below -15 °C, preferably below -18 °C for food samples.

A deep freezer is a chamber which allows deep-frozen storage to be guaranteed. The temperature, unless otherwise specified, shall be below -70 °C.

### 17.2. Use

#### 17.2.1. **Freezer**

Different chambers, or at least different containers, shall be available to achieve physical separation for thestorage of uninoculated reagents, samples for analysis, andmicroorganism cultures. Load the freezer in such a way that a sufficiently low temperature is maintained, in particular when unfrozen products are introduced.

#### 17.2.2. **Deep freezer**

The principle use is the storage of microorganisms, reference and/or working cultures, and reagents. Load the freezer in such a way that a sufficiently low temperature is maintained and cross-contamination between microorganisms and reagents is prevented.

### 17.3. Performance checks

Check the temperature of each chamber regularly using a suitable temperature-monitoring device.

### 17.4. Maintenance

Defrost and clean at regular interval; discard outdated materials. Remove dust from the motor blades and from the external heat-exchange plates (if accessible)

## 18. Colony counters

### 18.1. Description

This may be simple pen-like devices or may consist of an illuminated stage with a calibrated grid for the plate and a magnifying screen to aid colony detection. Automated electronic colony counters, incorporating image analysers, operate by a combination of hardware and software systems incorporating the use of a camera and a monitor. Colony counter device helps with recording the colony count, which is usually done by marking the colonies (on the Petri dishes) to avoid double counting of the same colonies.

### 18.2. Use

Follow the manufacturer's instructions. Adjust the sensitivity of an automated counter to ensure that all target colonies are counted.

### 18.3. Performance checks

Checks should be made manually on a regular basis to ensure that accurate counts are obtained using a colony counter. In addition, automated colony counters should be checked every day of use with a calibration plate containing a known number of countable particles or colonies.

### 18.4. Maintenance

Clean the surface of the instrument by dry cloth before and after every use. Keep equipment clean and free of dust; avoid scratching of surfaces that are an essential element of the counting process. In case of automated electronic colony counters, programme regular maintenance of electronic counters incorporating image analysers as specified by the manufacturer, at a suitable frequency.

## 19. Microscopes

### 19.1. Description

The compound microscope should preferably be binocular with a 1.8 mm oil immersion objective, a sub-stage actuated by a rack and pinion carrying an Abbe condenser with a numerical aperture of at least 1.25, and iris diaphragm, a flat mirror if the light is not an essential part of the microscope or mounted on the base, a mechanical stage, and oculars providing magnifications of 100X, 400X, and 1,000X.

### 19.2. Use

Set up the optics of the microscope in accordance with the manufacturer’s instructions.

### 19.3. Maintenance

Follow the manufacturer's instructions concerning storage, cleaning and servicing. Servicing, preferably by the manufacturer is desirable.

Each day or after use, remove oil from the immersion lenses and related parts using lens tissue. When the microscope is not in use keep it covered with the dust cover. Prevent condensation occurring where humidity is high as this may lead to deterioration of lens quality. Microscopes should be positioned on a vibration-free surface and maintained at one location. The movement of microscopes from location to location is not recommended.

## 20. Centrifuges

### 20.1. Description

Centrifuge capable of holding 15-ml and 50-ml buckets and working at a maximum speed of 15000 rpm is adequate. Centrifuges are mechanical or electronically operated devices that use centrifugal force to separate suspended particles, including microorganisms, from fluids depending upon their size or density

### 20.2. Use

Centrifuge tubes should be of identical lengths and thickness and should be used always in pairs, opposite to one another and to have the approximately same quantity and weight to avoid ‘head wobble’ i.e. bursting during operation.

### 20.3. Performance checks

Where the speed of centrifuging is critical to or specified in the application, the speed indicator or settings against a calibrated and independent tachometer should be checked regularly and after significant repairs or modifications.

### 20.4. Maintenance

Check the balancing carefully. Improperly balanced tubes will cause ‘head wobble’ and spin-off accidents. Take necessary precautions toprevent aerosol generation and cross contamination.

Clean and disinfect centrifuges regularly and after any spillage involving microbial cultures or potentially contaminated samples. Centrifuges should be serviced regularly.

## 21. Gas burner or Electric Bunsen burners

### 21.1. Description

Gas burner is used for flame sterilization of inoculating loops, forceps, scissors, the mouth of the flask, test tubes, etc. during the analysis.

Wire incinerators use gas or electricity to achieve red heat without a flame for sterilizing loops and straight wires used for manipulating cultures.

### 21.2. Use

Gas burner is commonly used for micro-loop sterilization. While flaming the conical flasks or test tubes, cotton plugs should be kept at the proper distance to avoid fire. Alcohol or another disinfectant should also be used while taking proper vigilance.

Both the loop and straight wire must be flamed immediately after use to avoid contamination. The use of a gas burner, within the protective cabinets should be avoided.

### 21.3. Maintenance

Regularly clean and disinfect burners and covers on electric Bunsen burners.

## 22. Dispenser for culture media and reagents

### 22.1. Description

A media dispenser or a culture media dispenser is a device for repeatedly delivering small

fixed volumes (typically between 1 ml and 50 ml) of culture media into bottles and tubes.

### 22.2. Use

Clean equipment used for dispensing culture media and reagents shall be free of inhibitory substances. Use separate tubing for selective media to minimize leaching/carryover of such substances. If aseptic distribution of sterile culture media and reagents is required, all parts of the dispensing equipment in contact with the product shall be sterile.

### 22.3. Performance checks

Check accuracy of volumes dispensed with a graduated cylinder at the start of each volume change and periodically throughout extended runs. If the unit is used more than once per day, pump a large volume of hot reagent water through the unit to rinse between runs. The volume delivered by the dispenser for media shall have a tolerance not exceeding ±5% of the volume.

### 22.4. Maintenance

At the end of the workday, disassemble into parts, wash, rinse with reagent water, and dry. Lubricate parts according to manufacturer's instructions or at least once per month.

## 23. Vortex mixer

### 23.1. Description

A vortex mixture is one of the basic technologies used for the mixing of samples, dilutions in glass tubes or flasks. Motorized draft shafts present on the mixer oscillates and transfers the movement to the sample tubes causing the sample fluids to undergo turbulent flow.

### 23.2. Use

It is a useful device for mixing the contents of test tubes. These should be operated in Biosafety cabinets. Adequate mixing is evidenced by the appearance of a vortex throughout the depth of the liquid during the mixing operation.

### 23.3. Maintenance

Keep equipment clean. If spillage occurs, decontaminate the equipment using an appropriate laboratory disinfectant.

## 24. Hotplate and heating mantle

### 24.1. Description

Hotplates and heating mantles are thermostatically controlled heating devices. Some hotplates and heating mantles incorporate magnetic stirring systems.

### 24.2. Use

Hotplates and heating mantles equipped with magnetic stirring systems are used for heating relatively large volumes of liquid such as media. Do not use hotplates and heating mantles without stirring systems for the preparation of media.

### 24.3. Maintenance

Clean up any spillages as soon as the unit is cool.

## 25. Pipettes and Automatic pipettes and tips

### 25.1. Description

Pipettes are glass or disposable plastic devices used to deliver volumes of liquid or viscous materials. Pipettes have typically consisted of a narrow tube into which fluid is drawn by suction (as for dispensing or measurement) and retained by closing the upper end. Pipettors are high-precision laboratory instruments for dispensing extremely small volumes. Fixed or adjustable volumes can be delivered by automatic pipettes provided with plastic conic tips where the liquid is aspirated by moving an air piston.

### 25.2. Use

In case of auto pipette set the desired volume to be dispensed before use. Avoid over-dialing the recommended range of the micropipettor to avoid mechanical damage. The tips must be chosen so as to match the pipetting device without a leak, and must be discarded and decontaminated after use.

In case of glass pipette, if the tip is broken, the pipette should be discarded. Do not perform mouth pipetting in microbiological facilities, except for non-contaminated liquids. Bulbs used on Pasteur or graduated pipettes and the tips for pipettors shall be of the correct size to prevent leakage and ensure efficient operation

### 25.3. Performance checks

Perform intermediate gravimetric checks using distilled or deionized water to ensure that volumes dispensed remain within the maximum permissible errors. Test new pipettors before use, and at regular intervals depending on the frequency and nature of use, to confirm that they respect the maximum permissible errors.

### 25.4. Maintenance

Follow manufacturer's instructions to perform routine maintenance, such as cleaning, seal replacement, and re-lubrication.

All Pipettes and pipettors must be kept clean, free of residues of culture media. If the barrels or pistons of automatic pipettors become contaminated in use, disassemble them for decontamination and cleaning. After re-assembly, recalibrate them. Where it is not possible for this to be done in the laboratory, return the pipettors to the manufacturer for re-assembly and recalibration.

## 26. Densitometer

### 26.1. Description

Densitometer is designed for measurement of cell suspension's turbidity in the range of 0.0–6.0 McFarland units ($0 - 180 \times 10^7$ cells/ml). Densitometer provides the opportunity to measure solution turbidity in a wider range (up to 15.0 McFarland units). The operation principle is based on optical density measurement with digital result representation in McFarland units.

### 26.2. Use

A densitometer is used for measurement of cell concentration (bacterial, yeast cells).

### 26.3. Performance checks

Calibration is required at the unit by 2–6 points in 0.0–6.0 McFarland unit range.

### 26.4. Maintenance

All maintenance and repair operations must be performed only by qualified and specially trained personnel.

Standard ethanol (75%) or other cleaning agents recommended for cleaning of laboratory

equipment can be used for cleaning and decontamination of the densitometer.

**References:**


1. ISO 6887 (all parts), Microbiology of food and animal feeding stuffs -Preparation of test samples, initial suspension and decimal dilutions for microbiological examination.

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3. ISO 11133, Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media.

4. Neusely da Silva, Marta H. Taniwaki, et al Microbiological Examination Methods of Food and Water: A Laboratory Manual, 2nd Edition, CRC Press/Balkema, Taylor & Francis Group (2018).

5. AbhishekChauhan, Tanu Jindal - Microbiological Methods for Environment, Food and Pharmaceutical Analysis-Springer International Publishing_Springer (2020).

6. N.F. Lightfoot and E.A. Maier (Eds.) - Microbiological Analysis of Food and Water. Guidelines for Quality Assurance (1998, Elsevier) - libgen.lc.

7. Collins and Lyne's, Microbiological Methods, Eighth Edition, 2004, Arnold.

## Chapter 2

## Glassware/Plasticware

### Scope

This chapter gives general guidance for sterilization, handling, storage, decontamination and the requirements of performance test of glassware and plastic wares used in the food microbiology laboratory.

### Caution

Most disinfectants have some toxic effects. Wear gloves and eye protection when handling concentrated disinfectant. A cleaning agent may be hazardous and must be handled carefully as per instruction given by the manufacturer.

### Laboratory Glassware and Plastic ware

Laboratory 'glassware' mainly comprises glass or plastics. Glass containers can be made of:

(i) soft glass (soda-lime glass);

(ii) borosilicate glass;

(iii) heat-resistant borosilicate

Borosilicate is preferred for laboratory glassware over silica glass or soda-lime glass. Borosilicate glass has a low thermal expansion coefficient and is almost entirely inert. Soft-glass items should not be used because they release alkali and change the pH of the media. Some are lined with a polyphosphate film and can be used for some purposes, once only. They cannot be re-used because the protecting coat will be destroyed by autoclaving.

The glassware shall examine before use for chipped edges or etched inner surfaces. The glassware shall be discarded if found defective. Volumetric glassware must be of class A and must withstand repeated sterilization without significant volume change. All glassware must be kept clean and free from residue of media & detergents.

Plasticware like beaker, centrifuge tubes, dilution tubes, test tube stand should be used as per method requirement.

Disposable plastic ware may be used instead of re-usable equipment and materials like

glassware, Petri dishes, pipettes, bottles, tubes, loops, spreaders, etc. subjected to the specifications are similar. Disposable plastic wares are for single use only and must be discarded after use. All glassware must be kept clean, free of residues of culture media.

**Cleaning procedures**

Microbiology glassware should not be mixed with glassware for chemistry. Often, nevertheless, glassware is left with residues that are difficult to remove, especially if they have been allowed to dry onto the glass. Dichromate cleaning solution (chromic acid) is one of the most powerful cleaning agents for such purposes. Chromic acid is prepared by adding one liter of concentrated sulfuric acid slowly to 35 ml saturated sodium dichromate solution. Extreme care must be employed in the preparation or use of this mixture.

A warm solution of 2% ethanolic or aqueous sodium hydroxide is also useful with any greases or oils coating the glass. In either case, the item should be immersed for approximately 15 minutes, then rinsed with tap water, followed by successive rinses with distilled water. Any new glassware should be soaked in 1% hydrochloric acid overnight to neutralize any free alkali and then washed before using. The cleaning procedure must include a sequence with detergent wash at about 70°C, a rinse with clean (soft) water at about 80°C, a final rinse in distilled water, or equivalent and drying.

The washed glassware should be sparkling clean, free from acidity, alkalinity, and toxic residues. Contaminated vessels should be autoclaved prior to cleaning.

Any new glassware should be soaked in 1% hydrochloric acid overnight to neutralize any free alkali and then washed with distilled water before using.

Because acid or alkali residue may remain on glassware after cleaning, the pH of random batches of glassware must be checked by adding a few drops of 0.04% Bromthymol Blue (BTB) and observing the color reaction. This indicator dye is yellow (acid) to blue-green (neutral) to blue (alkaline) in the pH range of 6.5 to 7.3. The acceptance criteria for the Bromthymol Blue (BTB) test should be blue-green for cleaned glassware. The 0.04% bromthymol blue solution is prepared by adding 16 ml 0.01 N NaOH to 0.1 g bromthymol blue and diluting to 250 ml with distilled water.

**Test for inhibitory residues on glassware and plastic ware**

In addition to the pH reaction, cleaned glassware should be annually checked for bacteriostatic or bactericidal residue which may have adhered to the surface. The procedure is detailed below:

1. Wash six petri dishes according to the normal washing routine of the laboratory, and designate these dishes as Group A.
2. Wash six additional dishes as above, rinse 12 times with distilled water, and designate as Group B.
3. Wash another six dishes according to the laboratory's normal procedure, dry without further rinsing, and designate as Group C.
4. Sterilize petri dishes in Groups A, B, and C by the laboratory's normal procedure.
5. If testing of pre sterilized plastic petri dishes is desired, designate six sterile dishesas Group D.
6. To each petri dish add 1 ml of a pure culture dilution of *Enterobacter aerogenes*that will yield 50-150 colonies per plate.
7. To each plate, add 20 ml of plate count agar and mix thoroughly with inoculum.
8. After solidification, incubate plates at 37° for 48 ± 2 hours and then count the number of colonies.
9. Interpretation of counts**:**
    a. Less than 15% difference in the average plate counts for plates of Groups A, B, C, and D indicates no detergent residual with bacteriostatic or bactericidal properties or that the pre sterilized plates are acceptable.
    b. A difference in colony counts of more than 15% between Groups A and B or D and B indicates the presence of an inhibitory detergent residue.
    c. A difference in counts of less than 15% between Groups A and B and more than 15% between Groups A and C indicates that the detergent has inhibitory properties that are removed during routine washing.

**Sterilization by dry heat**

Heating in a dry oven for 1 hour at 170°C is convenient for empty glassware. Ground glass stoppers should be separated from the neck by a paper tape or a piece of string to avoid blocking during cooling.

**Sterilization by moist heat**

Autoclaving in moist heat is convenient but requires a loose closure, to allow the steam to replace all the air during the temperature arise The temperature of the autoclave chamber shall remain at 121 °C for at least 15 min. The screw caps must be tightened after sterilization.

**Sterility test for glassware**

The laboratory glassware should be tested for sterility on a routine basis. Sterilized Petri dishes may be tested by pouring plate count agar or nutrient agar into randomly selected plates, incubating the solidified plates at 37 ± 2° C for 48 ± 2hrs, and examining them for growth.

Glassware or plastic ware like sampling dilution bottles, and pipettes may be checked for sterility by rinsing with Butterfield's phosphate buffer and filtering the buffer rinsing through a membrane. The membrane filter is placed on a non selective medium and incubated under conditions prescribed by the method. Sterilized test tubes may be checked by adding fluid thioglycollate broth and observing growth after incubation.

**Decontamination with chemical compounds**

Use chemical compounds (e.g. chlorine-based products, alcohols, quaternary ammonium compounds) at appropriate concentrations and for an appropriate contact time. Ensure that chemical residues will not affect the recovery of microorganisms.

**Storage of clean glassware and materials**

To dry completely washed glassware and plasticware must either be placed with their mouth downwards or they should be made to dry in an oven at 105 C. Keep clean glassware in a cabinet to protect it from dust. Keep washed, cleaned, and sterilized glassware pieces in racks and at a distance to prevent any breakage.

**Decontamination of glassware and materials after use**

All contaminated glassware, plasticware, and laboratory waste (used media, disposable plasticware) shall be disposed of with autoclavable plastic bags. Autoclaving is the preferred method for all decontamination processes (at least 30 min at 121 °C). The autoclave should be loaded in a way that heats penetration into the load, (e.g. without over packing) and taking care to loosen caps/lids and open bags. After decontamination laboratory waste should therefore be made safe before handed over to approved external agency for final disposal. The same autoclave should never be used for sterilizing media and decontaminating microbiological waste in the same load. After decontamination, reusable glassware/plastic ware shall be used after cleaning procedure.

**References:**


1. ISO 7218, Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations.

2. Neusely da Silva, Marta H. Taniwaki, et al Microbiological Examination Methods of Food and Water: A Laboratory Manual, 2nd Edition, CRC Press/Balkema, Taylor & Francis Group (2018).

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5. Manual of food quality control, Quality assurance in the food control microbiological laboratory, FAO Food and Nutrition Paper 14/12, Food and Agriculture Organization of the United Nations Rome, 1991

6. Quality Assurance/Quality Control, 9020, American Public Health Association,1999

## Chapter 3

## Media/ Reagents and Reference culture

### A. Introduction

Microbiological examinations used a vast variety of culture media, the formulation of which varies as a function of the microorganism(s) that will be cultivated and the tests for which they are intended. The formulation is the complete set of ingredients that, in well-balanced and adequate proportions, will confer to the culture medium their required distinct characteristics.

The ingredients used to formulate culture media are generally commercially available in dehydrated form, and include sources of nutrients, selective agents, differential agents, reducing agents, buffering agents, chromogenic and fluorogenic substrates and agar (gelling agent). The ingredients of the formulation are dissolved in water, the quality of which is critical for the good performance of the media to be prepared.

Sufficient testing should be carried out to demonstrate

a) the acceptability of each batch of medium,

b) that the medium is “fit for purpose”, and

c) that the medium can produce consistent results.

### B. Scope

This chapter defines terms related to quality assurance of culture media and specifies the requirements for the preparation of culture media intended for the microbiological analysis of food.

### C. Terms and Definitions

i. **Batch of culture medium/ lot of culture medium:** Homogeneous and fully traceable unit of a medium referring to a defined amount of bulk, semi-finished product or end product, which is consistent in type and quality and which has been produced within one defined production period, having been assigned the same batch (or lot) number.

ii. **Chromogenic substrate and fluorogenic substrate:** substrate containing a chromophore/fluorophore group and a substrate utilizable by bacteria or fungi.

iii. **Performance of culture medium:** response of a culture medium to challenge by test organisms under defined conditions.

iv. **Target microorganism:** microorganism or group of microorganisms to be detected or enumerated.

v. **Non-target microorganism:** microorganism that is suppressed by the medium and/or conditions of incubation or does not show expected characteristics of the target microorganism.

vi. **Productivity of culture medium:** level of recovery of a target microorganism from the culture medium under defined conditions.

vii. **Selectivity of culture medium:** degree of inhibition of a non-target microorganism on or in a selective culture medium under defined conditions.

viii. **Specificity of culture medium:** demonstration, under defined conditions, that non-target microorganisms do not show the same visual characteristics as target microorganisms.

ix. **Culture medium:** formulation of substances, in liquid, semi-solid or solid form, which contain natural and/or synthetic constituents intended to support the multiplication, (with or without inhibition of certain microorganisms), identification or preservation of viability of microorganisms.

x. **Liquid medium:** culture medium consisting of an aqueous solution of one or more constituents, such as peptone water and nutrient broth.

xi. **Solid medium and semi-solid medium:** liquid medium containing solidifying substances (e.g. agar-agar, gelatin) in different concentrations.

xii. **Pre-enrichment medium or enrichment medium:** generally liquid medium which, due to its composition, provides particularly favourable conditions for multiplication of microorganisms. eg: Tryptone soya broth.

xiii. **Selective enrichment medium:** enrichment medium that allows the multiplication of specific microorganisms whilst partially or totally inhibiting the growth of other microorganisms. Eg: Rappaport-Vassiliadis soya peptone medium(RVS).

xiv. **Non-selective enrichment medium:** enrichment medium that allows the growth of a wide variety of microorganisms. Eg: Brain heart infusion broth.

xv. **Isolation medium:** solid or semi-solid medium that allows the growth of microorganisms.

xvi. **Selective isolation medium:** isolation medium that allows growth of specific target microorganisms, while inhibiting, totally or partially, other microorganisms. eg:

Modified charcoal cefoperazone deoxycholate agar (mCCD agar).

xvii. **Non-selective isolation medium:** isolation medium that is not intended to selectively inhibit microorganisms. eg: Nutrient agar.

xviii. **Chromogenic/fluorogenic selective culture medium:** chromogenic/fluorogenic culture medium that also contains selective compounds which inhibit, totally or partially, accompanying flora occurring in test materials and thus support the precise detection of target microorganisms. eg: TBX agar, MUG/EC medium.

xix. **Differential medium/characterization medium:** medium that permits the testing of one or more physiological/biochemical characteristics of the microorganisms for their identification. eg: TBX agar, Lactose agar with tergitol 7 and TTC.

Note 1 to entry: Differential media that can be used as isolation media are referred to as isolation/differential media, e.g. Xylose lysine deoxycholate (XLD) agar, lactose TTC agar.

xx. **Identification medium:** medium designed to produce a specific identification reaction which usually does not require any additional confirmatory test. eg: Bile aesculin azide agar.

xxi. **Enumeration medium:** selective or non-selective culture medium that enables a quantification of the microorganisms. eg: Baird-Parker agar, Yeast extract agar

xxii. **Confirmation medium:** medium that contributes to the identification or characterization of the microorganism following a preliminary resuscitation and/or enrichment and/or isolation step. eg: Kligler iron agar.

xxiii. **Ready-to-use medium:** liquid, solid or semi-solid medium that is supplied in plates, bottles, tubes or other containers, in ready to use form or ready-to-use after remelting or ready-to-use after remelting and supplementing.

xxiv. **Finished culture medium:** medium in a form that is ready for inoculation.

xxv. **Ready-to-use medium after remelting:** medium to be remelted, for instance for use in the pour-plate technique or to be poured into Petri dishes.

xxvi. **Ready-to-use medium after remelting and supplementing:** medium to be remelted, supplemented and dispensed before use. (incomplete ready-to-use medium). eg: Tryptose sulphite cycloserine (TSC) agar, Baird- Parker or Rabbit Plasma Fibrinogen (RPF) agar.

xxvii. **Medium prepared from commercially dehydrated formulations:** medium in dry form which requires rehydration and processing before use, resulting in one of two kinds of media:

— a complete medium;

— an incomplete medium to which supplements are added before use

eg: powders, compacted granules, lyophilized products

xxviii. **Medium prepared from individual components:** medium produced by a microbiology laboratory entirely from its individual ingredients.

xxix. **Test organism:** microorganism generally used for performance testing of culture media.

xxx. **Reference strain:** microorganism obtained directly from a reference culture collection.

xxxi. **Reference stock:** set of separate identical cultures obtained by a single subculture from the reference strain either in the laboratory or from a supplier.

xxxii. **Stock culture:** primary subculture from a reference stock.

xxxiii. **Working culture:** subculture from a reference stock or stock culture or a reference material, certified or not.

xxxiv. **Reference material (RM):** material containing a quantity of revivable microorganisms, sufficiently homogenous and stable with respect to quantity of revivable microorganisms, which has been established to be fit for its intended use in a measurement process.

xxxv. **Certified reference material (CRM):** reference material characterized by a metrologically valid procedure for the quantity of revivable microorganisms, accompanied by a certificate that provides the value of the specified quantity of revivable microorganisms, its associated uncertainty and a statement of metrological traceability.

## D. Quality assurance management- Culture Media

### i. Documentation from manufacturer or producer

The following information shall be available from the manufacturer or producer (commercial or noncommercial bodies supplying media to third parties):

- Name of the medium, individual components and any supplements and, if possible, their product codes;
- Technical data sheet, e.g. formulation, intended use, filling quantity if applicable, references;
- Safety and/or hazard data where needed;

- Batch number;
- Target pH of the complete medium;
- Storage information and expiry date;
- Assigned shelf-life;
- Quality control certificate showing test organisms used and results of performance testing with criteria of acceptance.

ii. **Delivery acceptance of products**

For each batch of product (ingredient or culture medium), check the following:

- Identification of the product;
- Integrity of packaging;
- Expiry date of the product;
- Documentation supplied;
- Number of units received.

Record the date of receipt.

iii. **Storage**

In all cases, follow the manufacturer's instructions.

iv. **Handling of dehydrated media and supplements**

Media are delivered as dehydrated powders or in compacted granular form in sealed containers. Supplements of different selective or diagnostic substances are supplied in either the lyophilized, powder or liquid state. Purchases should be planned to encourage a regular turnover of stock (i.e. first in, first out). When a new container is opened

- Check the seal,
- Record date of first opening, and
- Visually assess the contents of opened containers.

After opening a new container, the quality of the medium will depend on the storage environment. Loss of quality of dehydrated media is shown by change in flow characteristics of the product, homogeneity, caking, colour changes etc. Any dehydrated medium that has absorbed moisture or shows obvious changes in physical appearance shall be discarded.

When a bottle of dehydrated medium is opened, date the container and indicate a maximum storage time.

## E. Laboratory preparation of media

Follow good laboratory practice and the manufacturer's instructions regarding the handling of dehydrated media and other components, particularly those containing hazardous materials i.e. bile salts, sodium azide, antibiotics or other selective agents.

When media are prepared from dehydrated commercial formulations, follow the manufacturer's instructions precisely. Document all relevant data, e.g. code, lot number, mass/volume, pH, date of preparation, sterilization conditions, operator.

For media prepared from individual components, follow the formulation precisely. Record all details as before and, in addition, the full identity (i.e. code, lot number and expiry date if available) of all the components used.

### i. Quality of basic medium components

Formulation of basic media components is described in the specific International Standards (see the Bibliography). When available, the molecular mass and the CAS1) number of a chemical substance should be stated in the formulation.

It is sometimes the case that a particular ingredient (for example those listed below) specified in the formulation has to be modified to achieve constant and consistent performance of the medium.

— peptones and meat or yeast extracts variable in their nutritive properties;

— agar variable in its gelling properties;

— buffering substances;

— bile salts, bile extract and deoxycholate, antibacterial dyes, depending on their selective properties;

— indicator dyes;

— antibiotics, depending on their activity and interactions with other ingredients.

### ii. Water used for media preparation

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.

The purified water shall be stored in tightly closed containers made from an inert material (neutral glass, polyethylene, etc.) which shall be free from all inhibitory substances. It is however recommended that the water is used as soon as produced.

Microbial contamination should not exceed $10^3$ colony forming units (cfu) /ml and preferably be below $10^2$ cfu /ml. Microbial contamination should be regularly monitored with an incubation at 22 °C ± 1 °C for 68 h ± 4 h or using an equivalent method.

The conductivity of water used in the laboratory shall be no more than 25 μScm−1 (equivalent to a resistivity ≥ 0.04 MΩ cm) and preferably below 5 μScm−1 (grade 3 water, at 25 °C, unless otherwise required by design. The conductivity of the water should be checked before use.

iii. **Weighing and rehydration of culture media**

Following the appropriate safety precautions, carefully weigh the required amount of dehydrated medium or individual ingredients and progressively mix with the required amount of water to avoid formation of lumps. Use a balance of sufficient discrimination; the maximum permissible errors should be 1% or better. Unless otherwise stated, the ingredients are added to the volume of water specified, rather than making up to that volume.

iv. **Dissolution and dispersion**

Dehydrated media need rapid dispersion by instant and repeated or continuous stirring followed by heating, if necessary, to dissolve. Media containing agar should be allowed to soak for several minutes before heating with mixing to dissolve and then dispensing if necessary before autoclaving. Avoid overheating the medium.

v. **Measurement and adjustment of pH**

Measure the pH using a pH meter and adjust before sterilization if necessary, so that after sterilizing and cooling to 25 °C the medium is at the required pH ± 0.2 pH units, unless otherwise stated. The adjustment is normally carried out using a sodium hydroxide solution of approximately 40 g/l [*c* (NaOH) = 1 mol/l] or dilute hydrochloric acid of approximately 36,5 g/l [*c* (HCl) about 1 mol/l]. If adjustment is performed after sterilization, use a sterilized solution.

NOTE: Commercially manufactured media can show significant changes in pH before and after autoclaving. However, provided good quality distilled or deionized water is used, pH adjustments before autoclaving are usually not necessary.

vi. **Dispensing**

Dispense the medium into appropriate containers ensuring that sufficient headspace is left to avoid boiling over during the cooling process after heat treatment by autoclaving or remelting, or overflowing after addition of supplements.

vii. **Sterilization**

Sterilize the prepared culture media on the day of preparation. The sterilization of culture media and of reagents is generally carried out by moist heat or by filtration. Certain media do not need autoclaving but can be used following boiling. For example, media for *Enterobacteriaceae* containing brilliant green are particularly sensitive to heat and light and should be rapidly cooled after boiling and protected from strong light. Some reagents can be used without sterilization. In all cases, make reference to appropriate the manufacturer's instructions.

- **Sterilization by moist heat**

  Sterilization by moist heat is performed in an autoclave or media preparator. For containers with volumes of media greater than 1000 ml, adapt the sterilization cycle of theautoclave as necessary to ensure adequate heat treatment. In all cases, follow the instructions given in the appropriate International Standard or the manufacturer's instructions.

  NOTE: Overheating can occur when large volumes of media (>1000 ml) are processed in an autoclave.

  After heating, it is essential that media are cooled in a manner to prevent boiling over. This is particularly important for media in large volumes and for media containing heat sensitive ingredients, e.g. media containing brilliant green.

  Sterilization by heat should be evaluated using F0 values, taking into account the heat treatment during heating and cooling. F0 value is used to determine the exposure time for sterilization at particular temperature. The heat treatment should be defined for the particular load to be treated to ensure suitable treatment for containers irrespective of placement in the autoclave.

- **Sterilization by filtration**

  Sterilization by filtration can be performed under vacuum or pressurized conditions. Use sterile equipment and membranes with a pore diameter of 0.2 μm.

Sterilize the filtration apparatus or use pre-sterilized equipment. Some filter membranes might retain proteins or other substances (such as antibiotics). In order to obtain the correct concentration, the user should choose a suitable membrane type, e.g. low protein-binding membrane, and pre-wet the filter.

viii. **Preparation of supplements**

- Take appropriate safety precautions and follow the manufacturer's instructions when preparing solutions.
- Do not use beyond their stated shelf-life which, for antibiotic working solutions, is generally the same day. Under certain circumstances, antibiotic solutions may be stored frozen in suitable aliquots but should not be re-frozen after thawing. The potential loss of activity due to freezing shall be established with the manufacturer or tested by the user.

  **CAUTION:** Supplements containing toxic agents, particularly antibiotics, shall be handled with care avoiding dispersion of powder, which may give rise to allergic or other reactions in laboratory personnel.

ix. **Storage and shelf-life of prepared media**

- **Commercially supplied media**

  Follow the manufacturer's instructions regarding storage conditions, expiry date and use.

- **Laboratory prepared media**

  Identify all media in a way that ensures traceability.

  The shelf-life of different media varies. The frequency of verification shall be specified by the laboratory. Store the media under conditions which prevent any modification of their composition, namely protected from light and desiccation. If not used immediately or specified otherwise in the specific standard, store in a refrigerator at 5 °C ± 3 °C.

  If refrigerated, it is generally recommended not to exceed two to four weeks of storage for plates and three to six months for sealed bottles and tubes, unless otherwise specified in specific standards or results of the laboratory shelf-life evaluation indicate a longer shelf-life.

  It is recommended that media to which labile supplements have been added should be used on the day of preparation, unless otherwise specified in specific standards or results of laboratory shelf-life evaluation indicate a longer shelf-life

is suitable. Solid media containing chemically reactive and/or labile substances should not be stored in bulk for remelting. Prior to use or before further heating, it is recommended that the culture media be equilibrated to ambient temperature.

- **Storage of media in Petri dishes**

  Use the solidified medium immediately or store inverted under conditions which prevent deterioration and dehydration, i.e. in the dark and/or in the refrigerator at 5 °C ± 3 °C. Label the plates on the base or side with date of preparation and/or expiry date and identity. Alternative coding systems meeting these requirements may be used.

  The shelf-life of poured plates will be prolonged by storage in sealed plastic or cellophane bags. In order to minimize condensation, the plates shall be cool before being placed into bags. Do not dry the surface of agar plates before chill storage.

x. **Preparation for use**

- **Melting of agar culture media**

  Melt culture media by placing in a boiling water bath or by any other process which gives identical results (e.g. a steam flow-through autoclave). Media that have previously been autoclaved should be reheated for a minimum time to maintain media quality. Avoid over-heating and remove when they have melted. Stand on a heat-resistant surface at room temperature for a short time, e.g. 2 min, before putting in a water bath to cool to avoid glass breakage. Caps of containers should be loosened before heating and tightened after removal from heat.

  Cool the molten medium to 47 °C to 50 °C in a thermostatically controlled water bath. The time needed to reach 47 °C to 50 °C depends on the type of medium, the volume and the number of units in the water bath. Molten medium should be used as soon as possible, but it is recommended that it should not be retained for more than 4 h. In the case of particularly sensitive media, the holding time of molten media shall be shortened. Unused medium shall not be re-solidified and reused.

- **De-aeration of culture media**

  If necessary to provide the correct air/oxygen content, heat the culture medium just prior to use in boiling water or under a flow of steam for 15 min, with lids or caps loose; after heating, tighten the caps and cool down rapidly to the operating temperature.

- **Addition of supplements**

  Heat-labile supplements should be added to the medium after it has been cooled to below 50 °C. If the medium contains agar allow the sterile supplement to equilibrate to at least room temperature before adding it to the agar medium. Addition of cold liquid supplements may cause agar to gel or form transparent flakes and prevent proper dispersion. Follow the manufacturer's instructions. Mix all supplements into the medium gently and thoroughly, then distribute into the final containers as quickly as possible.

- **Preparation of solid media in Petri dishes**

  Pour the molten agar culture medium into Petri dishes so as to obtain a thickness of at least 3 mm (e.g. for 90 mm diameter dishes, 18 ml to 20 ml of agar are normally required) or as specified in the appropriate International Standard. If plates are stored or if incubation is extended beyond 72 h or incubation temperature is above 40 °C, more culture medium may be required. Allow the agar to cool and solidify by placing the plates with lids in place on a cool, horizontal surface.

  Commercially prepared ready-to-use agar plates should be stored and used according to the manufacturer's instructions.

- **Preparation of plated media for inoculation**

  For surface inoculation of solid culture media, dry the plates shortly before use until the droplets have disappeared from the surface of the medium. Do not over-dry the plates.

  For the drying of the plates, the following points are of importance.

  - The degree of humidity in culture media is important because optimum growth of bacteria will depend on the humidity conditions in and on the medium. Extensive moisture loss can lead, for example, to an increase in the concentrations of inhibitors in selective culture media and a reduction in the water activity at the surface of the medium.
  - When bacteria that do not spread rapidly are cultured, and the plates look dry after acclimatization, the circumstances are such that drying is not always necessary. In that case, drying may be omitted, as it only increases the likelihood of contamination and unnecessary moisture loss.
  - Select the temperature and drying time so that the likelihood of

contamination is kept as low as possible and heating will not negatively affect the quality of the culture medium. The drying time will depend on the degree to which condensation is present in the Petri dish, but shall be kept as short as possible.

- In order to avoid contamination, and if the plates are not dried in a laminar-flow cabinet, plates shall always be dried with the surface of the culture medium to be inoculated turned downwards.

In practice, the plates can be dried by placing them with the agar surfaces downwards and with half open lids in a cabinet set at a temperature of between 25 °C and 50 °C. Dry the plates until the droplets have disappeared from the surface of the lids. Do not dry any further. The agar plates can also be dried with the agar surface facing upwards in a laminar-flow safety cabinet (at room temperature) for 30 min to 60 min, or overnight at room temperature with the lids in place.

- **Incubation of solid media in Petri dishes**

  During incubation, agar media will lose moisture. This can affect the growth of microorganisms in some circumstances. Factors influencing water loss are medium composition, amount of medium in the plates, the type of incubator i.e. fan-assisted or otherwise, humidity of the atmosphere in the incubator, the position and number of the plates in the incubator and the incubation temperature. Water loss can be reduced by putting the plates, in piles of up to six, in open-topped plastic bags (to avoid excessive condensation). Alternatively, the humidity of air in incubators may be increased by placing an open container of water in the bottom. The water should be changed and containers disinfected frequently to avoid fungal contamination.

xi. **Disposal of media**

Both contaminated and unused media shall be disposed of in a manner that is safe and meeting the requirements of state or national regulations.

## F. Test organisms for performance testing

Performance testing of culture media, including the specification of control strains and acceptance criteria, should be as per the requirements of **Annex I**.

i. **Selection of test organisms**

A set of test organisms should contain microorganisms with stable characteristics

representative of their species and which have been shown to be reliable for the demonstration of optimal performance of a particular prepared medium. The test organisms should primarily comprise strains that are widely available in reference culture collections, but well-characterized strains isolated by the laboratory may also be included. It is preferable to use strains which have originated from foods or water, although not all culture collections provide such information on strain origin.

The relevant culture characteristics of the reference stock shall be examined and recorded by the laboratory. If strain variability is encountered, investigate the possible effects of the culture medium by obtaining the same medium from a different manufacturer, and obtain an additional reference culture from the culture collection in which it was originally deposited.

The test microorganisms for each medium may include:

— robust positive strains with typical characteristics of the target organism;

— weakly positive strains;

— negative strains not showing expected characteristics of the target organism (negative characteristics);

— partly or completely inhibited strains.

ii. **Preservation and maintenance of test organisms**

There are several methods available for the successful preservation and maintenance of all microorganisms relevant to food and water microbiology, e.g. Lyophilization, storage on beads at –70 °C, or using liquid nitrogen. One method might not be appropriate for all strains. Additional methods for preservation of microorganisms are given.

The number of transfers of test organisms should be documented to prevent excessive sub culturing that increases the risk of phenotypic alteration. One passage is defined as the transfer from a viable culture to a fresh medium with growth of the microorganisms. Any form of subculturing is considered to be a form of transfer/passage.

- **Test microorganisms from commercial sources**

  Test organisms or Reference cultures can be procured from approved culture collection centers with traceability to ATCC/MTCC/NCTC/WDCM. The manufacturer's directions for their cultivation and use shall be followed. The laboratory should ascertain whether the strain supplied is a reference strain or

reference stock and how many passages have taken place before receipt and document the information. Reference cultures shall be verified for their characteristics on receipt as per the details in the certificate provided by the culture collection and/or as per the requirements of the test method or activity.

Laboratories should maintain records of all their reference culture maintenance activities, including certificates from the reference culture Collection, verification records, and sub-culturing records including any purity/verification checks.

- **Laboratory prepared reference stocks**

  Reference stock cultures prepared from reference strains for performance testing purposes shall be maintained and handled in a manner that minimizes the opportunity for cross contamination, mutation or alteration of typical characteristics. Reference stocks should be stored in multiple portions, usually either deep-frozen, e.g. below –70 °C, or lyophilized. At a higher temperature, duration of viability might be reduced and genetic modification might occur.

  Their growth characteristics should be fully documented for each medium on/in which they will be utilized as test microorganisms.

  Reference stocks shall not be used to prepare reference strains.

- **Stock cultures**

  Stock cultures are usually prepared from lyophilized or deep-frozen reference stocks. Aliquots shall be handled in a manner that avoids possible cross-contamination of the reference stock and/or its deterioration. Stock cultures should be prepared by re-suspending a portion of the reference stock in or on a non-selective growth medium; incubate to yield a stationary phase culture for storage and documentation requirements. Reference stocks shall be used to prepare working stocks for routine work. Stock cultures, if sub cultured should be done only up to a defined number of generations which is recommended up to five passages from the original reference culture.

  For commercially available preservation systems, the manufacturer's instructions shall be rigorously followed.

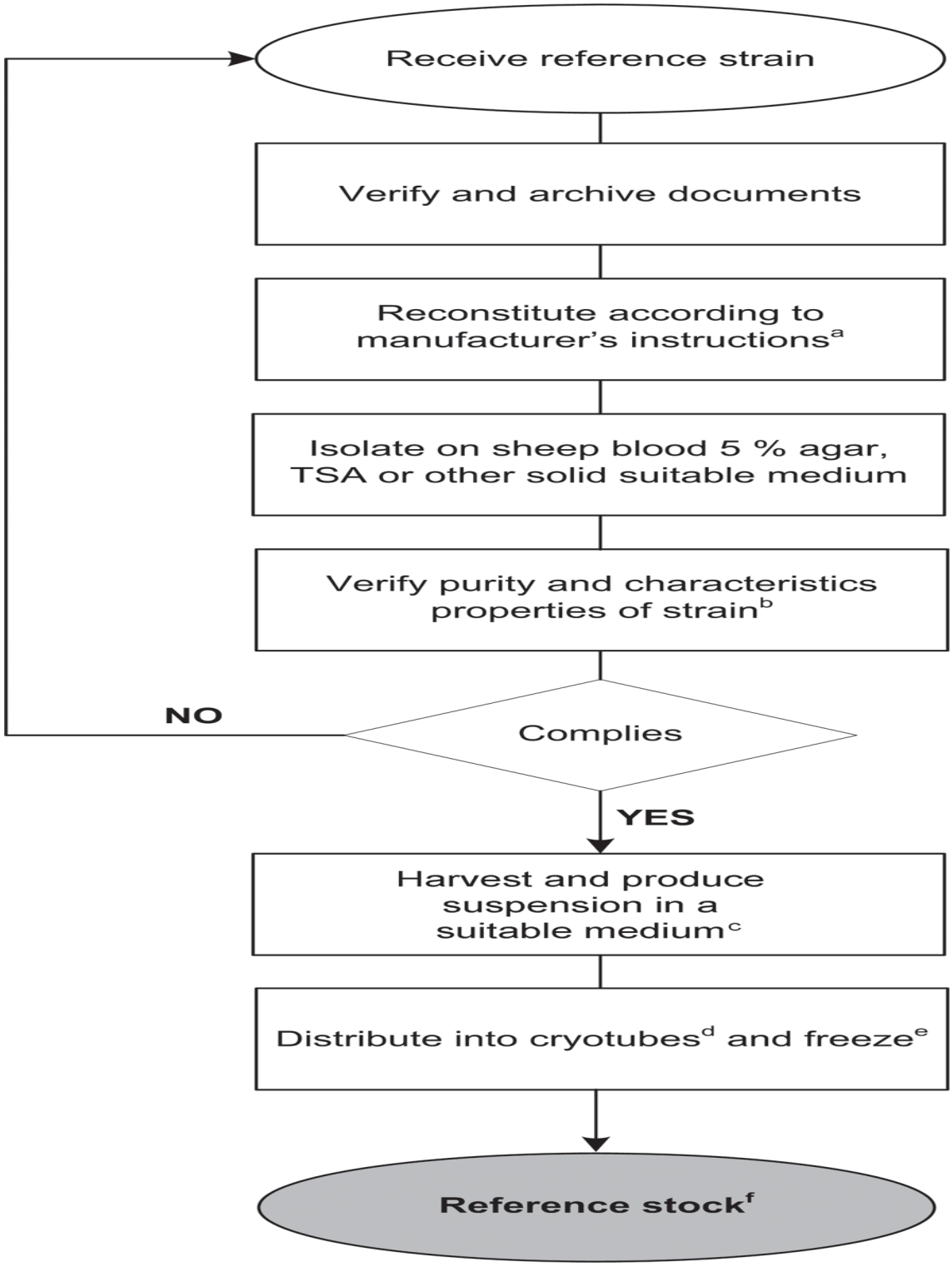
  Stock cultures shall not be used to prepare reference strains or reference stocks.

- **Working cultures**

  Working cultures are prepared from stock cultures or reference stocks and used

to prepare inocula for the tests. Working cultures shall not be used to prepare reference strains, reference stocks or stock cultures, or to make further working cultures.

**Flow chart of Preparation of reference stock from a reference strain**



In general, resuspension in a nutrient broth and holding time for resuscitation.

a Verify morphology of colonies and Gram staining or identify using biochemical tests.

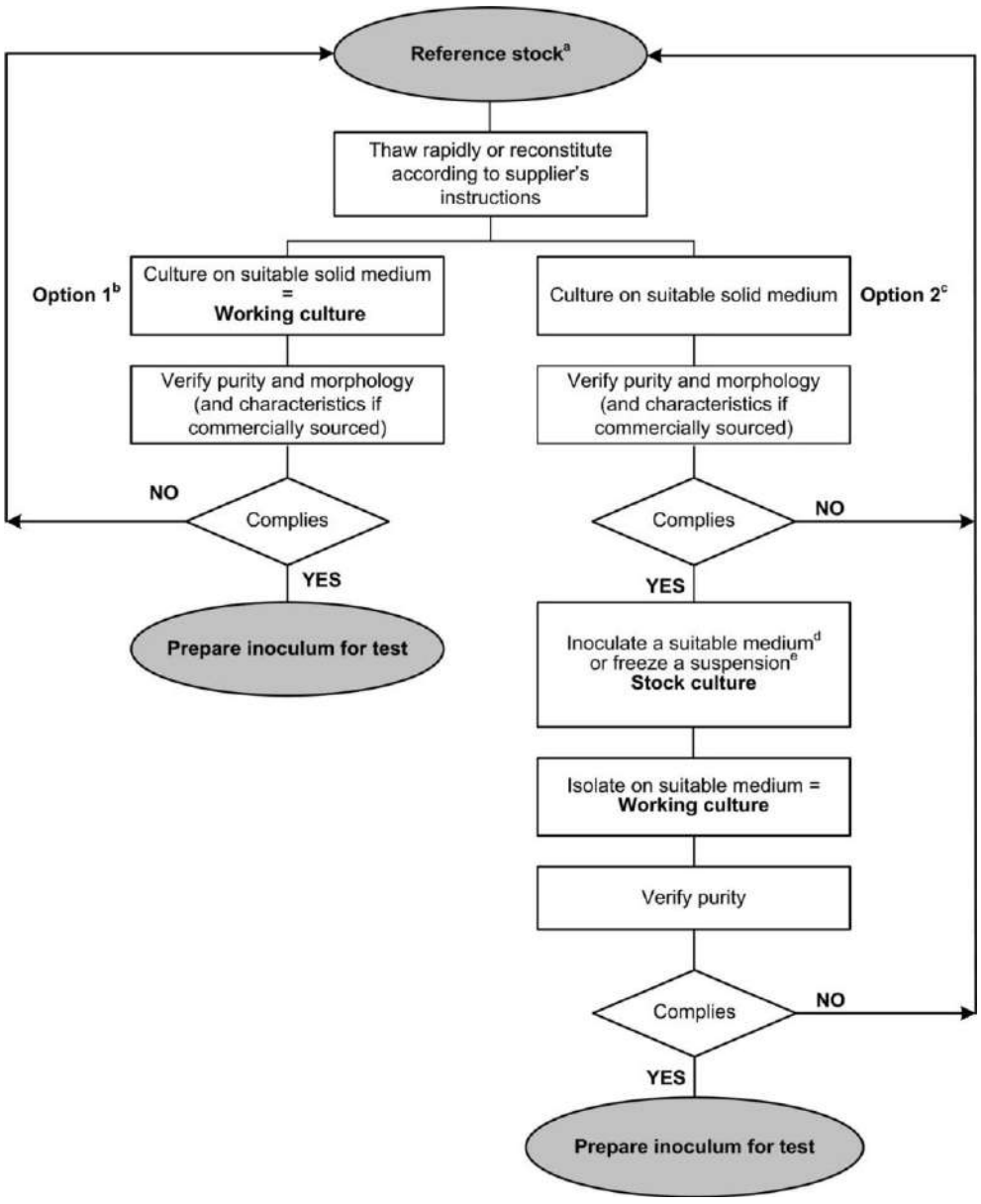
b For example, a cryoprotective medium, such as TSB supplemented with 0% to 15% glycerol volume fraction.

c Cryotubes may contain beads.

d Freezing at a temperature below −70°C enables extended storage. Storage life at a higher temperature is limited

f May also be used directly as a working culture.

**Flowchart of Preparation of working culture from reference stock**



a Verify and archive documents, including check on traceability to reference strain and relevant characteristics, if reference stock is obtained from outside source.

b This procedure is preferable.

c This procedure may be necessary for some strains, e.g. for quantitative tests. Document all stages.

d For example, inoculate a slant of TSA or sheep blood TSA or other suitable medium, incubate for 24 h and store at a suitable temperature (18°C to 25°C or 2°C to 8°C depending on the microorganisms) for upto four weeks.

e For example, a cryoprotective medium, such as TSB supplemented with 10% to 15% volume fraction glycerol. Freezing at a temperature below −70°C enables extended storage. Storage life at a higher temperature is limited.

## G. Microorganisms for performance testing

The volumes of inocula and numbers of organisms used are critical.

The following guidance is given as an example of procedures suitable for producing standardized inocula for quality control of media. These procedures apply in the general case but some organisms can require special conditions for preparation, e.g. anaerobes, moulds, halophilic, osmophilic or xerophilic organisms, and those with special growth or nutritional requirements.

### i. Preparation of stock cultures

When required inoculate a solid medium e.g. Tryptone Soya agar (TSA) or Blood TSA, from reference stock in a way to achieve single colonies. Incubate under appropriate conditions, e.g. for most aerobic bacteria 18 h to 24 h at 37 °C. Inspect this solid stock culture for purity and use it for a specified time (e.g. for 14 days at an appropriate temperature to prevent significant change according to the organism).

### ii. Preparation of working cultures

Working cultures shall be prepared from the reference stock (or when required the stock culture) as a pure stationary phase culture in a non-selective broth. For most aerobic bacteria this is normally achieved by incubation for 18 h to 24 h. The working culture can be prepared from a commercial reference material, RM or CRM, or be prepared by the laboratory. The concentration of the prepared

suspension shall be stable and homogeneous during its period of use. Different techniques may be used, but shall guarantee the purity of the inoculum, as well as its standardization, which allows it to be used at a later stage.

Depending on the size of the colonies, take one to two colonies from the stock culture medium with an inoculation loop. The use of a 1 µl loop is recommended in order to avoid too heavy an inoculum.

Transfer the inoculum to a non-selective liquid medium, e.g. Tryptone Soya broth (TSB), and mix carefully using a vortex mixer. Incubate under appropriate conditions and for an appropriate time (e.g. for most aerobic bacteria 18 h to 24 h at 37 °C).

Use this working culture for a specified time (e.g. for maximum three days at an appropriate temperature to prevent significant change according to the organism).

iii. **Preparation of suspensions (inocula) for the test**

Prepare serial dilutions in a suitable diluent (e.g. quarter-strength Ringer's solution, peptone salt solution) and use the most suitable dilution step for the desired number of organisms (cfu) in a specified volume. The suitable dilution to use as a test inoculum should be determined from previous tests conducted under strictly standardized conditions for all steps.

Use the suspensions (inocula) within a specified time (e.g. up to 2 h at room temperature or within 24 h if stored at 5 °C ± 3 °C; longer storage periods may be acceptable if validated. Frozen inocula may be used if it can be shown that the microorganism can survive for the chosen period.

iv. **Volumes of inocula**

Volumes of inocula used for quantitative performance testing shall reflect those used under test conditions for the relevant media.

For diluents and liquid media used for quantitative testing, the volume of the inoculum shall be in the same ratio as used in the relevant International Standard, usually 10 % of the medium under test.

- **Inoculum level for productivity testing**
  - **Quantitative testing**

    For the quantitative enumeration test, a level of around $10^2$ cfu is necessary to achieve sufficient precision (see Table 1). This may necessitate the use of more than one plate replicate.

A practicable range of 80 cfu to 120 cfu per plate with a minimum number of 50 cfu per plate should be used. For filters, the same number of cfu is needed using one or more filters. Table 1shows the 95 % confidence intervals associated with colony counts. For quantitative tests of diluents and liquid transport media, an inoculum level of $10^3$ to $10^4$ cfu is needed to achieve an inoculum of around 100 cfu in the volume spread on the plates.

**Table 1 — Approximate** 95 % **confidence intervals for numbers of colonies assuming agreement with Poisson distribution**

| **Number of colonies counted** | **Limiting precision (to nearest percentage)**% | **Approximate** 95 % **confidence limits** |
|---|---|---|
| 500 | ± | 455 to 545 |
| 400 | ±10 | 360 to 440 |
| 320 | ±11 | 284 to 356 |
| 200 | ±14 | 172 to 228 |
| 100 | ±20 | 80 to 120 |
| 80 | ±22 | 62 to 98 |
| 50 | ±28 | 36 to 64 |
| 30 | ±37 | 19 to 41 |
| 20 | ±47 | 11 to 29 |
| 16 | ±50 | 8 to 24 |
| 10 | ±60 | 4 to 16 |
| 6 | ±83 | 1 to 11 |

- **Qualitative testing**

The volume used for testing should contain

— $10^3$ to $10^4$ cfu for qualitative tests of plate media,

— ≤ 100 cfu for productivity tests of pre-enrichment and enrichment media,

— $10^4$ to $10^6$ cfu for qualitative tests of solid transport media.

- **Inoculum level for selectivity testing**

For selectivity testing of culture media, a suspension of the non-target microorganism containing $10^4$ to $10^6$ cfu is inoculated on to the plate or into the tube of medium.

- **Inoculum level for specificity testing**

  For qualitative tests of plate media, for specificity an inoculum level of 103 to 104 cfu is needed.

v. **Incubation**

Incubate the inoculated culture media in accordance with the conditions described in the relevant Test methods in manual.

## H. Quality control and performance testing of culture media

The following describe requirements for all culture media. They are applicable whatever the size of the batch.

In practice, samples may contain stressed microorganisms. The suitability of the medium with respect to the recovery of stressed cells should be taken into account.

The quality of culture media depends on the quality of the basic ingredients, correct formulation, and quality of preparation procedures, elimination of microbial contamination and appropriate packaging and storage conditions.

The quality control of the culture media shall be adapted to the use for which the media are intended (e.g. qualitative or quantitative). Before use, the performance of each batch of culture medium shall be tested according to the media categories. If testing before use is not possible due to the lability of the medium or supplement, parallel performance testing alongside the sample testing shall be performed.

i. **Physical and chemical quality control**

Finished culture media shall comply with the physico-chemical characteristics as specified in the corresponding test methods. Furthermore, quality assessment by visual inspection shall ensure that each culture medium conforms to stated recommendations, e.g.

- fill volume and/ or thickness,
- appearance, colour and homogeneity,
- gel consistency, and
- moisture content,

In addition, pH value shall be determined.

The individual components and any nutritive or selective supplements shall also undergo suitable quality assessment procedures.

ii. **Microbiological quality control**

The microbiological performance tests shall be carried out on a sample which is representative of a batch of end product.

- **Reference medium**

  In order to ensure there liability of results of performance testing, the reference medium used shall be of consistent high quality.

  Examples of aspects to be considered by the user are the following:

  — Use of a quantitative RM containing a well-defined number of organisms when evaluating a reference medium;
  — use of a defined production process including remelting, if applicable;
  — useofthesamemanufacturer/sourceforprovisionofthemedium/ingredients;
  — useofalargerrangeoftestorganismswhencommissioning(tocovertherang eoforganismssought);
  — the choice of "reference medium" for evaluation purposes;
  — appropriate procedures for assuring the quality when in use as a reference medium.

  It might not be necessary to include all of the above aspects when evaluating the suitability of the reference medium.

  Suitable test organisms, method of control and acceptance criteria for the reference medium Tryptone Soya agar (TSA) are described in Annexure I. Other non-selective reference media may be used if the above criteria are satisfied.

- **Microbial contamination for performance verification**

  An appropriate quantity, depending on the size of the batch of culture medium, shall be tested for absence of microbial contamination (sterility) by incubation under appropriate conditions.

  The samples to be tested shall be at least one plate or tube for small batches (<100 units). For larger batches, producers shall draw up specifications, e.g.

based on media components, process parameters and limits and type of packaging using appropriate acceptable quality limits.

Acceptance criteria shall be established and justified for each medium

iii. **General requirements for microbiological performance testing**

- To evaluate a batch of complete culture medium, nutrient components or supplements, growth shall be appropriately assessed by either quantitative or qualitative methods as described in this manual.
- Solid, semi-solid or liquid culture media shall be inoculated with an appropriate volume of the working culture of each of the defined test microorganisms using an appropriate device and following the inoculation technique described in relevant test methods in the manual; see Annexure I.
- Examples of quantitative and qualitative testing methods for solid culture media and liquid media are described in this manual.
- When culture media are intended for enumeration purposes, quantitative testing methods shall be performed.
- When a new medium or a new manufacturer is being evaluated, quantitative testing methods are recommended to provide additional information to support the change.
- In liquid media, the interactions leading to the successful growth of microorganisms are more complex, hence defining performance testing methods is less straightforward than for solid media.
- Suitable test microorganisms, methods of control and acceptance criteria are listed in Annexure I.
- The testing frequency shall be justified by the end user, taking into account the extent of preparation in the end user's laboratory and the level of quality assurance in place.
- **Ready-to-use media**

  Manufacturers of commercially available ready-to-use media, especially if according to ISO 9001, will have a quality programme in place and might issue a quality certificate with the media they supply. Under those conditions, the user might not need to carry out extensive testing on such media, but shall ensure that storage conditions are maintained as recommended by the manufacturers.

  For ready-to-use finished media to which supplements have been added, and

which have been controlled by the manufacturer in accordance with this International Standard, at least a qualitative test is recommended.

The user shall ensure that manufacturers of commercially available ready-to-use media have a quality programme in place for this range of products and issue quality control certificates meeting the requirement, specifying the expected and obtained results. The user laboratory shall also check documentary evidence to ensure that the manufacturers' acceptance criteria for performance testing satisfy their own internal requirements.

Periodic checks shall be carried out in order to demonstrate that the quality of media has been maintained during transport.

Checks shall also be performed following storage and further handling in the user's laboratory, e.g. melting of solid media. The frequency shall be justified.

For incomplete media to which supplements are added by the user laboratory, an additional check should be carried out either by checking the production records or by performing a qualitative test to ensure that the correct supplement has been added.

- **Media prepared from commercially available dehydrated formulations**

  For enumeration media, quantitative testing shall be performed. For other media, qualitative testing may be sufficient. Quantitative tests will give greater assurance of media quality.

- **Media not specified in Annexure I**

  For those media not described in Annexure I, quality control should be specified according to the following recommendations.

**Table 2 — Type of media and performance criteria**

| S.No | Type of media | Performance criteria to be checked |
|---|---|---|
| 1 | Selective enumeration broth | Productivity (method: quantitative)<br>Selectivity (qualitative) |
| 2 | Selective enumeration agar | Productivity (quantitative)<br>Selectivity (qualitative)<br>Specificity (qualitative) |

| | | |
|---|---|---|
| 3 | Selective enrichment broth | Productivity (qualitative)<br>Selectivity (qualitative) |
| 4 | Selective detection agar | Productivity (qualitative)<br>Selectivity (qualitative)<br>Specificity (qualitative) |
| 5 | Non-selective enumeration agar | Productivity (quantitative) |
| 6 | Non-selective enrichment broth | Productivity (qualitative) |
| 7 | Non-selective dilution broth | Productivity (quantitative) |
| 8 | Non-selective detection agar | Productivity (qualitative) |

- **Media prepared from basic individual components**

  In addition to the requirements stated in media prepared from commercially available dehydrated formulations, quantitative testing shall be performed in order to monitor trends in quality of basic materials, productivity of the medium and in-house laboratory production protocols.

iv. **Performance evaluation and interpretation of results**

A batch of culture medium performs satisfactorily if all the test microorganisms used perform according to the given specifications. It shall be accepted if both general and microbiological quality criteria are met.

If satisfactory performance is not achieved, carry out root cause analysis

v. **Confirmation media and reagents**

- **Confirmation media**

The performance of culture media used for confirmation tests shall be verified before use. Appropriate positive and negative test organisms shall be used for verification in a similar way to that described in the specific chapters in Manual of Methods of

Analysis – Microbiological Testing in Foods and Water.

- **Confirmation reagents**

Performance of Gram stain solutions, reagents, such as Kovacs, VP, nitrite, oxidase, catalase and other reagents used to demonstrate a biochemical characteristic, shall be verified before use. Appropriate positive and negative strains shall be used for verification and a shelf-life should be established. It is recommended that analytical grade reagents be used for confirmatory tests. If commercially prepared reagents are used, follow the manufacturer's instructions for storage and use.

## I. Methods for performance testing of solid culture media

This clause describes quantitative and qualitative performance testing for solid culture media specified in Manual of Methods of Analysis – Microbiological Testing in Foods and Water. These are general methods suitable for most culture media. They might not be suitable for testing some types of media for recovery of moulds.

### i. Test Criteria

- **Productivity**

For quantitative methods, the productivity ratio, $P_R$ is determined using Formula(1):

$$P_R = N_S/N_O$$

Where,

$N_S$ is the total count of colonies obtained on or in the culture medium under test, e.g. colony count on plates;

$N_O$ is the total count of colonies obtained on or in the defined reference culture medium, obtained from one or more plates, and shall be around 100 cfu

- **Selectivity**

Selective culture media and an on-selective reference medium are inoculated with different dilutions of non-target organism(s). Non target microorganisms shall be partially or totally inhibited.

The selectivity factor, $S_F$, is calculated as given by Formula(2):

$$S_F = D_O - D_S$$

Where,

$D_O$ isthehighestdilutionshowinggrowthonthenon-selectivereferencemedium;

$D_S$ isthehighestdilutionshowingcomparablegrowthontheselectivetestmedium;

$S_F$, $D_O$ and $D_S$ are expressed in log10 units.

For example, if $D_O\ 10^{-4} = \log_{10}4{,}0$ and $D_S\ 10^{-3} = \log_{10}3{,}0$ then the selectivity factor $S_F$= 1,0.

ii. **Quantitative method for solid culture media**

This protocol requires the use of a quantified bacterial suspension (which may be a quantitative reference material/test suspension) with an appropriate concentration of a target strain. The recovery from the new batch of culture medium will be compared to the recovery from a non-selective culture medium (reference medium) or, in special cases, a previously accepted batch of the same media composition.

- Use working cultures and inocula of known appropriate concentration of a target strain and where appropriate also of non-target strain or suitable RM.
- One or more plates per organism should be used. The number used will depend on the size of the batch, the confidence in the quality assurance production procedure and the reliability and level of the organism in the test suspension. The user laboratory shall justify the number used.
- Ensure that the surfaces of the plates are adequately dried.
- Inoculate by spreading the inoculum on the media or by the membrane filtration method to give counts that fall within the recommended limits for quantitative testing.
- The modified Miles-Misra surface drop method, other dropping systems or a spiral plater may also be used to give countable colonies on the plates.
  - The pour plate method shall be used for culture media normally used for enumeration in this way.
  - Inoculate reference medium or plates from a previously accepted batch in the same way.
  - Incubate the plates under the conditions defined in the individual methods in Manual of Methods of Analysis – Microbiological Testing in Foods and Water.
  - Count the colonies present on each plate. Assess the size and appearance of

the colonies on or in the medium under test by comparison with the recovery on a non-selective culture medium (reference medium) or a previously accepted batch of the same media composition.

- **Calculation and interpretation of results**
  - For the quantitative enumeration test, a level of around 100 cfu is necessary to achieve sufficient precision (see Table 1). This may necessitate the use of more than one plate per replicate. The results will be accepted as valid if the following conditions are satisfied:
    - Each replicate shall give a positive quantitative result (target bacterial growth.
    - Each single reported result is included in the standard range of analysis (up to 100 colonies for filtration methods and up to 150 colonies for surface methods).
  - To interpret the results, calculate the productivity ratio, PR and where appropriate, the selectivity factor, SF.
  - The PR shall be $\geq 0.50$ for comparison of a selective medium with the non-selective reference medium specified in Annexes I. The PR shall be $\geq 0.70$ for comparison of a non-selective medium with a non-selective reference medium or as specified in the standard or Annexes I. This also applies to special cases where comparison is made with the previous batch.
  - If the $P_R$ exceeds 1.4 identify the reason.
  - The $S_F$ of non-target microorganisms is at least 2.

- **Testing of culture media used for membrane filtration**
  - The quality of the membrane filters used shall be previously evaluated to demonstrate their suitability for use
  - To test the performance of a culture medium for use in membrane filtration, use working cultures and inocula as described above in Microorganisms for performance testing. Inoculate the suspension medium e.g. dilution fluid, sterile water, with a suitable inoculum level.
  - Filter the liquid according to the requirements of the specific test methods in

Manual of Methods of Analysis – Microbiological Testing in Foods and Water. Place the membrane on the surface of the agar under test. Inoculate sufficient membranes/plates to obtain a total of approximately 100 cfu for productivity testing. Repeat with a new membrane and place the second membrane on the surface of the reference medium, using dilutions if required for selectivity testing. Incubate the plates according tithe specific test method.

- Repeat the process each time the batch of membranes changes as well as each new batch of medium.
- If necessary, to evaluate the influence of the membrane on the result also spread the test inoculum on to the test medium and reference medium without the membranes.

iii. **Methods for qualitative tests**

- **Qualitative streaking Procedure**
  - Use working cultures and inocula as described above in Microorganisms for performance testing.
  - For productivity and specificity, use a plate of test medium and streak each test microorganism in a way to obtain discrete colonies.
  - For selectivity, use one plate of test medium and streak each test microorganism as a single straight line using a 1 µl loop on the surface of the test medium. Several test microorganisms can be streaked on the same plate as parallel lines without crossing; streaks should be distinguishable to allow observation of typical morphology. Other standardized streaking methods can be used.
  - Incubate the plates under the conditions defined in the specific test methods in Manual of Methods of Analysis – Microbiological Testing in Foods and Water.

  - **Interpretation of results**

    The amount of growth on the plates after incubation is assessed as follows:

    — 0 -corresponds to no growth;

    — 1 -corresponds to weak growth (either reduction in amount of

growth or colony size);

— 2 -corresponds to good growth.

Target microorganisms shall score 2 and have typical appearance, size and colony morphology. For selectivity tests, the degree of inhibition depends on the type of medium. The growth of non-target microorganisms shall be partly or completely inhibited.

- **Determination of specificity**

  The specificity of the culture medium is given by essential indicative physiological characteristics to differentiate related organisms by the presence, absence and/or grade of expression of biochemical responses and colony sizes and morphology.

**J. Methods for performance testing of liquid culture media**

This clause describes quantitative and qualitative methods for performance testing of liquid culture media.

i. **Quantitative tube method for performance testing of liquid enrichment media (dilution to extinction method)**

This method is a general method that may be used for non-selective or selective liquid media. It is also suitable for performance testing of liquid media used for enumeration, e.g. in most probable number methods.

- **Preparation of the dilution series**
  - Select a representative number of tubes.
  - Prepare a suitable dilution series from the working culture of the target or non-target organism in a suitable diluent as to achieve absence of organisms in the highest dilution (extinction), e.g. from 10−1 to 10−10. A decimal dilution series is most commonly used, but 1/5 or 1/2 dilution steps are also suitable.
  - Use the dilution series within a specified time.
  - At the time of use, transfer a known volume, e.g. 0.1 ml of each dilution to the surface of a non- selective agar plate and spread.
  - Incubate under appropriate conditions for the organism concerned.
  - Count the number of colonies on the agar plates at the lowest dilution

containing up to 150 colonies and the number of colonies on higher dilutions than this and record.

- **Procedure for testing the liquid medium**
  - Select the same number of tubes of medium under test to correspond to the number of tubes in the dilution series.
  - Using the dilutions prepared according to preparation of the dilution series and starting with the highest dilution, inoculate a known volume of the test organism suspension, e.g. 0.1 ml into the corresponding tube of medium.
  - Incubate the tubes under the conditions described in the relevant test methods in Manual of Methods of Analysis – Microbiological Testing in Foods and Water.
  - After incubation, use a separate 10 µl loop for each tube of incubated medium to subculture to a non-selective agar medium.
  - Incubate the inoculated plates under conditions appropriate for the organism.
  - After incubation, examine each plate for the presence or absence of growth.

    NOTE: For the target organism, it is usually sufficient to use the 10−5 to 10−8 dilutions. For non-target organisms, it is usually sufficient to use the 10−1 to 10−4 dilutions.

- **Calculation and interpretation of results**
  - Productivity of the liquid enrichment medium is satisfactory if good growth (at least 10 cfu from a 10 µl loopful) of the target microorganism is obtained from the dilution producing fewer than 100 cfu (in 0.1 ml) on the plate.
  - For selective liquid media, determine the selectivity factor, SF, from the highest dilution of the working culture showing good growth (at least 10 cfu) on the agar plate and the highest dilution of the inoculated selective liquid medium showing no growth (or less than 10 cfu) of the non-target microorganism on the non-selective agar plate. The SF should be at least 2.

ii. **Qualitativetubemethodforperformancetestingofselectiveliquidmedia**

This method uses target, non-target, or a mixture of target and non-target organisms in the same tube.

**Procedure**

- Select a number of tubes each containing 10 ml of medium or 10 ml portions from each batch to be tested.
- **Inoculation of target organisms:** Inoculate one tube of test broth with an inoculum containing ≤ 100 cfu of target microorganism and mix.
- **Inoculation of non-target microorganisms**: Inoculate one tube of test broth per microorganism with an inoculum containing a higher number (>1000 cfu) and mix.
- **Inoculation of target and non-target organisms in the same tube** when required in Annexes II and III or when a new medium or new manufacturer is being evaluated. Inoculate one tube of test broth with ≤ 100 cells of target microorganism and the same tube with a higher number of non-target microorganisms (≥1000 cells for every tube) and mix.
- Incubate the tubes under the conditions defined in the individual International Standard.
- Remove one loopful (10 µl) from the tube containing the target organism and streak on a plate of a non-selective medium (e.g. TSA).
- If a mixed culture of target and non-target organisms has been used, remove one loopful (10 µl) and streak on a plate of the specific medium for the target microorganism.
- Remove one loop (10 µl) from the culture of non-target microorganism and streak on a plate of a selective medium (e.g. XLD).
- Incubate the plates under the conditions defined in the individual International Standards.
- If a larger volume of medium is used the user may choose whether to adjust the inoculum size proportionately in order to achieve equivalent results.
- **Calculation and interpretation of results**
  - Productivity of the liquid test broth is satisfactory if good growth (at least 10 cfu or a line of confluent growth) of the target microorganism is obtained on the specific medium for that organism.
  - Selectivity of the liquid test broth is satisfactory if no growth (or less than 10 cfu) of non-target microorganisms occur on the non-selective agar plate.

iii. **Qualitative single tube method (turbidity) for performance testing of liquid**

**media**

- The method is suitable for performance testing of non-selective liquid culture media and selective media used for confirmation testing, e.g. Brilliant green bile lactose (BGBLB) broth. The method is only qualitative and scores are therefore only indicative. Inherently turbid media can only be tested by this method if subcultured to a solid medium to demonstrate growth. For clear media, the following notation is used:
  - 0 equals no turbidity;
  - 1 equals slight turbidity;
  - 2 equals good turbidity.
- **Pre-enrichment media**
  - Select a number of tubes each containing 10 ml of medium or 10 ml portions from each batch to be tested.
  - For performance testing of pre-enrichment media, e.g. buffered peptone water (BPW), inoculate the medium with an appropriate inoculum volume containing≤100 cfu directly into the medium under test.
  - Incubate the tube under the conditions defined in the specific International Standard;
  - Examine the medium for growth.
- **Confirmation media**
  - For performance testing of liquid confirmation media inoculate the medium under test with the working culture suspension (containing > 106 cfu/ml) using a 1 µl loop.
  - Incubate the tube under the conditions defined in the individual International Standards; see 5.4.2.6.
  - If the uninoculated medium is inherently turbid subculture to a solid medium, incubate the plates under the conditions defined in the individual standards and examine for growth.
- Interpretation of results
  - Qualitative evaluation shall be carried out visually by looking for good turbidity (i.e. 2) representing good growth. Qualitative evaluation of opaque media when produced is indicated by the presence of growth on the solid medium.

NOTE: Sometimes the growth of microorganisms can only be observed as a cell aggregation/deposit at the base of the tube or bottle. In this case, careful shaking can improve assessment and interpretation.

## K. Methods for performance testing of diluents

### i. Quantitative Testing:

The method determines the ability of the diluent to support the survival of microorganisms without undue multiplication or reduction during the period of contact before plating on to agar or inoculation into liquid media.

- **Procedure**
  - Inoculate a test portion (e.g. 9 ml) of the diluent with 1 ml of the test microorganism suspension containing around $10^4$ cfu/ml and mix; for preparation of the inoculum. Immediately remove 0.1 ml of inoculated diluent and spread over the surface of a non-selective agar (reference medium) such as TSA (t0).
  - Hold the inoculated diluent at ambient temperature for the time lapse between the end of preparation of the initial suspension and the moment when the inoculum comes into contact with the culture medium (usually 45 min). Mix and then remove the same volume (0.1 ml) and plate again on the reference medium (t1).
  - Incubate the reference medium at an appropriate temperature and time e.g. 30 °C/72 h.

- **Reading and interpretation of results**

  After incubation count the colonies on the plates $t_0$ and $t_1$.

  The number of microorganisms, $t_1$, after incubation of the diluent shall be within ±30% of the initial count ($t_0$).

## L. Documentation of test results

### i. Information provided by the manufacturer

The manufacturer or supplier of the culture media shall provide, on request, the specific

microbiologicalgrowthcharacteristicsandgeneralinformationrelatingtothespeci ficbatchofculturemedium.

ii. Traceability

All the data from routine performance testing should be documented inanappropriate way and kept for a sufficient period of time according to the quality system in use.

**Reference**

1. ISO 11133: Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media

# Chapter 4

**Quality Assurance: Assuring Quality of Test Results in Microbiology Laboratory**

## A. Introduction

Quality assurance (QA) and quality control (QC) measures are required in order to have confidence in both analytical test results and the equipment and processes used to derive those results. These measures include media negative control, positive control, method blank, sterility testing, matrices spiking etc. Additionally, there is a requirement to document and record all QA/QC criteria to ensure consistent test results and analyst performance. The details of these procedures, their performance frequency, and expected ranges of results should be documented in respective standard operating procedures (SOPs). Each method used in the laboratory should include acceptance criteria. If these criteria are not readily available, the laboratory should determine its own criteria by control-charting techniques.

Certified Reference cultures should be used to verify the test methods as well as for quality control activity. The laboratory must participate in proficiency testing (PT) performance evaluation (PE) studies in an annual, or preferably a semi annual and covering all scope of testing in a certain period of time. An acceptable result on a sample of this type is a strong indication that a test protocol is being followed correctly. If an unsuccessful result is obtained, the laboratory should perform corrective action that includes a root cause analysis to determine the cause of any failed PT/PE sample.

## B. Definitions

1) ***Batch***—Samples that are prepared and/or analyzed together using the same process and personnel along with the same lot(s) of reagents.
2) **Demonstration of capability (initial or ongoing*)*** - A documented process whereby an analyst uses single-blind sample(s) and performs the QC requirements of the method, laboratory SOP, client specifications, and/or any additional laboratory standards. Test results must be within the limits of the laboratory's QC requirements.
3) **Laboratory fortified blank**— Also referred to as a spiked blank, QC check, or laboratory positive or negative control sample. This sample is the matrix with no target microorganisms present, which is then spiked with a known concentration of a verified microorganism.

4) **Matrix—**The substrate of the test sample
5) **Matrix spike**—A sample of matrix that is spiked with a known amount of organisms and processed as a typical sample, either quantitatively or qualitatively. Matrix spikes are often performed to determine if the matrix will have an effect on the outcome of the test.
6) **Positive and negative culture controls—**Cultures of known microorganisms that will or will not produce a reaction in known media and under known test conditions. Certified reference cultures should be used, when available.
7) **Proficiency test sample—**A blinded sample with a known concentration and/or population of microorganisms that is provided to test whether the laboratory can produce analytical results within the specified acceptance criteria.
8) **Quality Assurance—**A management system that includes laboratory activities, such as planning, implementation, assessment, reporting, and quality improvement to ensure that a process or service is of the type and quality needed and expected by the client.
9) **Quality Control—**Technical activities that measure the attributes and performance of a process, item, or service against defined standards to verify that they meet the stated requirements established by the customer.
10) **Sterile or sterility**—Free from viable microorganisms.

## C. Scope: This procedure is applicable to Microbiology laboratory

## D. QC Practices

**1) Demonstration of Capability (DOC):**

Laboratory must demonstrate initial and ongoing capability for each analysis performed. These results must be documented. Any potential problems must be identified, corrected, and documented. The intent is to prove both the reliability and integrity of the laboratory's test results. There are two types of DOC: initial and ongoing.

a. **Initial DOC**—an initial DOC is performed before any test method is used, and any time there is a change in instrument type, personnel or test method. Prior to the use of a new method one of the two following options must be selected.

i. If validation data are available from the manufacturer and/ or regulatory agency, analyze four spiked samples in matrices similar to the normal laboratory samples.

ii. If validation data are not available from the manufacturer and/or regulatory agency, analyze 10–20 spiked samples in matrices similar to the routine laboratory matrix samples.

Analyze at least one PT sample, if available, from a PT provider certified or approved by a regulatory agency or accreditation authority. Observe analyst performance and analysis of known and unknown samples, and confirm that results meet laboratory criteria before allowing analyst(s) to conduct routine samples.

b. **Ongoing DOC—** Analyze single-blinded sample for colony count methods, determine analyst colony counting variability. Replicate counts for the same analyst should be within 5%, and replicate counts between analysts should be within 10%. Determine the precision of duplicate counts, and repeat counts on one or more positive samples at least monthly.

c. **Documentation—** Documentation includes SOPs, Raw data sheets**,** employee's training record, authorization to perform analysis, PT/PE records, summary discussion of results involving conversion to logarithmic values, and comparison to method published results or to established and documented values and documentation of review by management

**2) Method Blanks and Sterility Checks**

Sterility testing and the use of method blanks ensure that test samples have not been cross contaminated due to improper handling or preparation, inadequate sterilization, or environmental exposure.

a. **Method blanks—**Method blanks demonstrate that equipment, media, reagents, and sample containers were properly sterilized and were not contaminated while in storage or during the testing process. A method blank is typically a sterile sample consisting only of water, or other blank matrix, that is treated and processed exactly the same as an unknown sample to determine if any method-

specific reagents or equipment has interfered with the test sample results. At least one method blank should be run with each batch of samples. In the event that the blank sample shows contamination or unexpected results, discard the affected test sample(s) and request re-sampling.

b. **Sterility checks**—Sterility checks ensure that the processes used for sterilization are valid, and are done before running the method. Sterility checks for all media, reagents, buffers, and dilution/reagent water may be performed using no selective growth media and should be performed on each new lot of media or equipment before use. In the event that the sterility check sample shows contamination or unexpected results, discard the affected material. These tests may be done by a contract laboratory.

c. **Documentation—** Documentation includes SOPs, Raw data sheets/registers. The SOPs should include corrective action steps for nonconforming materials.

**3) QC Samples/Laboratory Fortified Blank (LFB)**

a. LFBs may also be referred to as QC samples, or negative and positive controls. They are used to ensure that growth media or other method reagents/materials are capable of supporting proper growth and/or analytical results. LFB samples may be used to establish intra laboratory or analyst-specific precision and bias. They may also be used for initial DOC and ongoing DOC.

b. A QC sample/LFB is typically a sterile aliquot of reagent water or blank matrix to which a known quantity of a single verified microorganism is added. Use a low concentration inoculum level to duplicate normal environmental conditions. The added organism may be either typically positive or negative for a specific method. Add only one type or strain of organism to one sample. These samples are processed and analyzed exactly as a test sample.

c. *Reference Cultures*

i. Reference cultures obtained from an accredited reference culture provider, a recognized national collection centre.

ii. Reference cultures should be maintained in the laboratory with documented procedures that demonstrate the continued purity and viability of the organism.

Add a known amount of organism to sterile reagent water or blank matrix. This sample may be used for initial and/or ongoing demonstration of capability or to assess multiple method attributes, such as selectivity, sensitivity, growth promotion, and growth inhibition.

d. **Documentation—**SOPs for reference culture maintenance, preparation of QC samples and controlled results sheets or registers etc.

**4) Matrix Spike for Difficult Matrices:**

The matrix being tested can have a profound and often unknown effect on resulting data. To mitigate unusable data, suspected difficult matrices should be spiked with known concentrations of organisms to determine recoverability. Some methods may routinely require a matrix spike and matrix spike duplicate.

a. **Matrix spike—**Add a known concentration of microorganism(s) at an anticipated ambient level to a field sample collected from the same site as the original. Process using the same conditions and criteria as a typical sample. Invalidate any sample if organisms are not recovered at the expected level from the matrix spike, then re-evaluate processes. Follow this process for any required matrix spike duplicate.

b. **Documentation—**Describe the process for analyzing a matrix spike for difficult matrices in a laboratory's SOP. Record all conditions and materials or strains used in the laboratory, including test results.

**5) Calibration of Microbiological Equipment (Initial and Continuing) Performance Qualification:**

The laboratories must have all relevant equipment and instrumentation of appropriate quality for each analytical method covered under scope. Test equipment and instrumentation before initial use and during continual usage in the laboratory to demonstrate that they perform consistently (continued qualification), thereby meeting user's needs and suitability for their intended purpose.

a. **Calibration—**Calibration is mandatory to determine performance capability of all major equipment and instrumentation before first use and after periodic interval. Laboratory has to make calibration frequency plan a per NABL guidelines. Use

reference standards; e.g., National Institute of Standards and Technology (NIST) traceable thermometers, NIST Class S/ American Society for Testing and Materials Class 1 weights, and certified or otherwise qualified personnel, to perform calibrations. Conduct equipment maintenance on a routine basis to ensure continued performance as directed by standards and/or manufacturers' recommendations, using internal staff experts or experienced experts obtained by contract. Review these activities to detect any deviations from accepted protocol. For details refer Part A chapter 1: Use and Handling of Common Microbiological Instruments/ Equipment

b. **Documentation**—Record written procedures on the use and operation, calibration, maintenance, and acceptance limits on all relevant equipment or instrumentation in the form of SOPs. Retain all critical manufacturers' manuals and document their location for easy retrieval. Record reference standards used and their calibration if applicable. Document initial and ongoing calibration and ongoing maintenance activities and results. Finally, document any problems found and its resolution.

**6) Control Charts and Trend Analyses of QC Results**

a. The laboratory should demonstrate equipment, instrumentation, or analytical changes over time. These trends in process control are best demonstrated in tabular form, graphs, or charts and show that the laboratory is operating under control and with the expected variations of the analyses. If trends exceed control limits, corrective action must be initiated.

b. *Steps to manage and trend QC results* —Follow SOPS for critical equipment and instrumentation, e.g., autoclave performance for timing, temperature, pressure and usage, and temperature recording device(s) calibration; glassware washing, including inhibitory detergent residue checks; balance calibrations, etc.

c. Follow SOPs for each analytical method being used, and follow all QC checks for steps of the analytical process, e.g., sample, dilution, sample bottle checks, volumetric checks, media preparation, and culture control testing. Calculate

precision of replicate analyses for each different type of sample examined, e.g., by performing duplicate results on the first 15 positive samples of each matrix type, with each set of duplicates analyzed by a single analyst. If there is more than one analyst, include all analysts regularly running the tests, with each analyst performing an approximately equal number of tests. Thereafter, analyze 10% of routine samples in duplicate or one per test run. Develop control charts with the initial 20 assays, then measure changes over time after developing mean and upper and lower control limits.

d. **Documentation**—Record results for calibration, verification, and QC of all critical equipment and instrumentation and analytical method activities. Record routine analyst(s) performance:

i. For routine performance evaluation, compare counts between analysts testing the same samples.

ii. Replicate counts for the same analyst should agree within 5% (within analyst repeatability of counting) and those between analysts should agree within 10% (between analysts reproducibility of counting). If they do not agree, initiate investigation and necessary corrective action.

iii. Record duplicate analyses of the first 15 positive samples of each matrix type and record as D1, or D2 if a second analyst is also conducting these tests. Calculate the logarithm of each result. If either of a set of duplicate results is <1, add 1 to both values before calculating the logarithms. Calculate the range (R) for each pair of transformed duplicates and the mean ($\bar{R}$) of these ranges.

iv. With the routine samples run in duplicate or one per batch or test run, transform the duplicates and calculate their range as above. If the range is >3.27($\bar{R}$) , there is greater than 99% probability that the laboratory variability is excessive; in such a case, discard all analytical results since the last precision check. Identify and resolve the analytical problem before making further analyses.

## 7) Corrective Action and Root Cause Analysis (RCA)

The objective of a QA manual is to ensure that the laboratory produces data of

known and documented quality, thus ensuring a high quality of laboratory performance. Both internal and external audits of the laboratory operations and procedures allow early identification of any weaknesses, including training needs, opportunities to improve documentation and recordkeeping, review of reporting systems, and ensuring compliance with regulations and client requirements. However, events that result in either incorrect or questionable data results can still occur. When this happens, it is important to have established and implemented a systematic process to uncover the root cause of the issue and a plan of action to prevent the situation from occurring again. These two processes are defined here, but will require modification depending upon the type and severity of the initial problem.

a. *RCA*—RCA is a structured problem-solving process that involves identification of a specific procedural step or process that led to a faulty or unexpected outcome. The purpose of performing an RCA is to address, correct, or eliminate root causes, as opposed to merely addressing the obvious symptoms.

*b.* *Corrective actions*—Corrective actions are directed corrective measures aimed at preventing specific issues uncovered during RCA. It is likely that recurrence can be prevented if specific, measurable, corrective actions are put in place after a root cause is identified.

**General Process of RCA and Corrective Actions**:

i. The following steps and questions can be used to help the laboratory develop and implement both a RCA and corrective action plans. Not all parts will pertain to every laboratory, and other processes not mentioned here may be worthy of adding. The RCA and corrective action development will be specific to a laboratory and the processes and steps that are followed there. Be prepared to document your investigations and elicit a team to help ensure objectivity.
ii. Define the problem factually —include the quantitative and qualitative properties of the outcome or issue, the nature of the issue, and the magnitude, locations, and timing.
iii. *Classify and document*—what are the steps that must be taken to get to an end

result similar to the current issue? List these steps and any associated training or other requirements for each step. Classify causes into causal factors that relate to an event in the sequence and root causes that if eliminated or changed, probably interrupted that step of the sequence chain. Examples of steps and processes that should to be captured, classified, and documented for RCA include, but are not limited to: sampling, including hold time and temperatures; sterility checks; equipment checks; training requirements and updates; performing methods correctly; and supplier documentation. If there are multiple root causes, which is often the case, document these clearly for later optimum selection. Identify all other harmful factors that have equal or better claim to be called root causes.

iv. Identify corrective action(s) that will with certainty prevent recurrence of each harmful effect, including outcomes and factors. Check that each corrective action would, if implemented before the event, have reduced or prevented specific harmful effects.

v. Identify effective solutions that prevent recurrence, and with reasonable certainty and consensus agreement of the group, are within your control, meet your goals and objectives, and do not cause or introduce other new, unforeseen problems. Implement the recommended root cause correction(s), and ensure effectiveness by observing the implemented recommendation solutions in action, typically by internal audit.

vi. *Documentation*—All steps in the determination of root cause, the corrective actions identified, corrective steps taken, and success of these changes should be documented. Modify any internal documents, such as SOPs or work instructions, to reflect changes made upon RCA and corrective action.

**8) *QC Acceptance Criteria:***

a. QC acceptance criteria are used to determine if test results are acceptable, and must be established to monitor the daily operation during laboratory testing processes.

b. *Establishing criteria* —QC acceptance/rejection criteria are established for the following:

| S.NO | QC Practice | QC Acceptance Criteria |
|---|---|---|
| 1 | Glassware Cleaning | Refer Part A Chapter 2: Glassware/Plastic ware |
| 2 | Quality check of reagent water | a) Microbial load: maximum 1000 cfu/ml after incubation at 22±1$^0$C for 68±4 hr<br>b) Conductivity: Not more than 25µScm$^{-1}$ |
| 3 | Membrane Filter Sterility | No Growth after Incubation at 35±0.5$^0$C for 24hr in non selective media (Nutrient broth, Tryptone soya Agar etc ) |
| 4 | Diluent sterility Test | |
| 6 | Media/Supplements Performance | Refer Part A Chapter 3: Media/ Reagents and Reference culture |
| 7 | Reference Culture | |

Established analytical methods include: variability of colony counting between analysts; precision of quantitative methods; and verification of results, including both positive and negative control. The purpose of verification is to determine if the analytical method is performing as expected. Follow manufacturers' or regulatory acceptance criteria when possible. When no method or regulatory criteria exists, the laboratory should have procedures for the development of acceptance/rejection criteria. Any new method must be validated to establish if the performance criteria provide reliable data.

i. *Qualitative test methods*—the selection of criteria should ensure the accuracy, precision, specificity/selectivity, detection limit (1 CFU/100 mL for presence/absence samples), robustness, and repeatability of the test.

ii. *Quantitative test methods*—The selection of criteria should ensure the accuracy, precision/repeatability, precision/reproducibility, recovery/sensitivity, detection limit, upper counting limit, and range of the test. Determine in advance what action is needed if QC acceptance criteria

fails. Possible actions include repeating test, recalibrating, rejection of test batch, RCA, and corrective action.

c. *Documentation*—Document QC acceptance/rejection criteria for established tests in SOPs. Record criteria results and pertinent information for all SOPs with acceptance/rejection criteria. QC results are reviewed on an ongoing basis by the laboratory manager or designee. QC acceptance/rejection criteria for new methods should be documented. Document actions to be taken if acceptance criteria are not met. When criteria are not met, record the root cause and corrective action(s).

**9) *QC Checks of Laboratory Equipment:***

To ensure precise and consistent results, laboratory equipment must be installed, maintained, and calibrated properly. For details refer Part A chapter 1: Equipment

**Reference**

1. Root et al.: Journal of AOAC International Vol. 97, No. 2, 2014 Microbiological Water Methods: Quality Control Measuresfor Federal Clean Water Act and Safe Drinking Water ActRegulatory Compliance

## Chapter 5

# Good Microbiology Laboratory Practices including Biosafety and Biohazard Management.

## A. Good Microbiology Laboratory Practices

### Scope

A microbiology laboratory is a unique environment that requires special practices referred as good practices and containment facilities to properly protect persons working with microorganisms. Human mistake, improper laboratory techniques and mismanagement of equipment cause the improper results and majority of laboratory injuries. The elements of containment for Good Laboratory Practices are 1. Safety design 2. Safety equipments 3. Personnel and 4. Aseptic techniques

### 1. Safety Design:

Safety design of laboratory is prepared as per the risk analysis and ability of a microorganism that is likely or unlikely to cause human or animal disease with various severity. Laboratory facilities are designated as basic – Biosafety Level 1 and Level 2 (preferably used in food safety laboratory), containment – Biosafety Level 3, and maximum containment – Biosafety Level 4. Biosafety level designations are created on a composite of the design structures, construction, containment facilities, equipment, practices and working procedures required for working with agents from the various risk groups. Each laboratory should adopt a safety or operations manual that identifies known and potential hazards, and specifies practices and procedures to eliminate or minimize such hazards.

#### 1.1. General requirements

**1.1.1.** There should be adequate suitable space with separate storage locations for e.g. biological indicators, reference organisms, samples and media etc.

**1.1.2.** The Lab should be away from restrooms etc to prevent cross contamination.

**1.1.3.** The air supply to the microbiology laboratory should be through separate air-handling units and other provisions.

**1.1.4.** The quality of the air supplied to the laboratory should be appropriate and not be a source of contamination.

**1.1.5.** Laboratory equipment used in the microbiology laboratory should not be used

outside the microbiology area and support equipment (e.g. autoclaves, Laminar floor, Biosafety cabinet etc) glassware) should be dedicated and physically separated from other areas.

**1.1.6.** Only authorized persons should be allowed to enter the laboratory working areas. The international biohazard warning symbol and sign must be displayed on the doors of the rooms where microorganisms of higher risk groups are handled

**1.1.7.** Appropriate entry and exit procedures including gowning

**1.1.8.** Special attention should be given to conditions that are known to pose safety problems like, aerosol formation, large volume of samples, over-crowding of equipments pest infestation and Workflow: use of specific samples and reagents etc.

**1.1.9.** An environmental monitoring programme should be in place which covers, use of air settlement plates and surface swabbing, temperature and pressure monitoring.

## 1.2. Laboratory Design topographies:

**1.2.1.** Sufficient space must be provided for the safe conduct of laboratory work.

**1.2.2.** Laboratory should have clearly designated areas, for the following activity:

- Receipt and storage of samples;
- Preparation and sterilization of culture media and equipment
- Sample Preparation
- Inoculation
- Incubation
- Storage of reference and other strains;
- Storage of culture media and reagents;
- Decontamination;
- Documentation and reporting
- cleaning of glassware and other equipment;
- storage of hazardous chemicals, preferably kept in specially designated cabinets, cupboards, rooms or buildings.

**1.2.3.** Laboratory should have uni-directional flow of activity

**1.2.4.** Walls, ceilings and floors should be smooth, easy to clean, impermeable to

liquids and resistant to the chemicals and disinfectants and Floors should be slip-resistant.

**1.2.5.** Bench tops should be impervious to water and resistant to disinfectants, acids, alkalis, organic solvents and moderate heat.

**1.2.6.** Adequate Illumination should be for all activities. Provide Open spaces between and under benches, cabinets and equipment should be accessible for cleaning.

**1.2.7.** Facilities for eating and drinking and for rest should be provided outside the laboratory working areas.

**1.2.8.** Hand-washing basins should be provided in each laboratory room. First-aid areas or rooms suitably equipped to tackle the emergencies.

**1.2.9.** Doors should have view panels and preferably be self closing.

**1.2.10.** An autoclave for decontamination should be available in proximity to the laboratory.

**1.2.11.** Consideration should be given to the provision of mechanical ventilation systems that provide an inward flow of air or if there is no mechanical ventilation, windows should be able to be opened and fitted with arthropod-proof screens.

**1.2.12.** A supply of good quality water is essential. There should be no cross connections between sources of laboratory, processing and drinking-water supplies.

**1.2.13.** There should be a reliable and adequate electricity supply and emergency lighting to permit safe exit and stand-by generator or power supply for essential equipments

**1.2.14.** Physical and fire security must be considered.

**1.2.15.** Poisonous and hazardous chemicals must be kept under safe custody

## 1.3. Maintaining Laboratory working areas

**1.3.1.** The laboratory should be kept neat, clean and free of materials that are not pertinent to the work

**1.3.2.** Work surfaces must be decontaminated before and after handling of samples and at the end of the working day.

**1.3.3.** All contaminated materials, food samples and cultures must be

decontaminated before disposal or cleaning for reuse.

**1.3.4.** Packing and transportation of sample must follow as per FSSAI regulations.

**1.3.5.** Reagent solution/standard solutions shall be prepared in established manner with use of relevant reference.

**1.3.6.** Prepared chemicals, they should be stored in appropriate storage condition i.e. protected from light, tightly stoppered, refrigerated etc with proper labelling. Wherever, it is recommended reagents are to be prepared freshly.

**1.3.7.** The precautions to be taken to prevent the contamination of media and broth in the laboratories.

**1.3.8.** All reference standards maintenance shall follow "Standard Operating Procedures" to maintain proper storage, transport, security, integrity, avoid mishandling etc.

**1.3.9.** Laboratory activities, such as sample preparation, media and equipment preparation and enumeration of microorganisms, should be segregated by space or at least time, so as to minimize risks of cross-contamination and false positives

**1.3.10.** The relevant records are also to be maintained.

**1.3.11.** Sterility testing should always be performed in a dedicated area.

### 1.4. Cleaning, disinfection and hygiene programme

**1.4.1.** Well documented cleaning and disinfection programme should be in place.

**1.4.2.** Appropriate procedure should be available for dealing with spillages.

**1.4.3.** Adequate hand-washing and hand sanitization facilities should be available.

## 2. Safety of equipments:

**2.1.** The laboratory should, ensure that adequate equipment is provided and that it is used properly.

**2.1.1.** Equipment should be selected to take account of certain general principles, i.e.

**2.1.2.** Equipments should be designed to prevent or limit contact between the operator and the hazardous material

**2.1.3.** Made up of materials that are, resistant to corrosion and meet structural requirements

**2.1.4.** Equipments should be designed, constructed and installed to facilitate simple operation

**2.1.5.** Provide for ease of maintenance, cleaning, decontamination and certification testing.

**2.1.6.** Maintain a logbook of operating hours for each rotor and a preventive maintenance programme to reduce risk of mechanical failure.

**2.1.7.** Electrical equipment should be handled with great care.

**2.2. Point to be considered while handling some basic equipments to reduce hazards**

**2.2.1.** Centrifuges: Use sealable buckets (safety cups) or sealed rotors to avoid aerosols

**2.2.2.** Anaerobic jars: Ensure integrity of wire capsule around catalyst to avoid dispersing of infectious materials.

**2.2.3.** Homogenizer or tissue grinders: Operate and open equipment in a biological safety cabinet and before opening the blender bowl, wait 30 min. to allow the aerosol cloud to settle.

**2.2.4.** Sonicators: Ensure insulation to protect against sub harmonics and Wear gloves to protect skin against chemical effects of detergents.

**2.2.5.** Water baths: Ensure regular cleaning and disinfection. Do not use sodium azide for preventing growth of organisms.

**2.2.6.** Pipetting aids – to avoid mouth pipetting.

**2.2.7.** Validation: Equipment such as autoclaves and biological safety cabinets must be validated with appropriate methods before being taken into use.

**2.2.8.** Decontamination: Autoclaves or other appropriate means to decontaminate infectious materials

## 3. Personnels:

Personnel should be advised of special hazards, and trained on safety or operations manual. All the working personnel's must follow standard practices and procedures.

**3.1. Basic GLP Practices to be followed by working personnel**:

**3.1.1.** The personnel should be technically competent to perform their duties as

operating on specific equipment's / performing tests / evaluating results / signing the reports.

**3.1.2.** Qualification for doing specific tasks shall be judged based on their education, training, specific experience and demonstrated skill.

**3.1.3.** Regular and refresher training should be organized

**3.1.4.** Each personnel should be defined with their role and responsibility

**3.1.5.** Personnel should wear proper uniform and protective clothing's, etc as required (Aprons, gloves, marks, headcap etc)

**3.1.6.** No phone calls/ cell calls should be attended in working areas.

**3.1.7.** The personnel at the time of working in the laboratory should be alert and concentrate on their work only.

**3.1.8.** Supervisory officer should randomly watch the analysis activity and guide from time to time to increase the competency of analyst.

**3.1.9.** Eating habits should be avoided in the laboratory.

**3.1.10.** Long hair should be secured behind head to minimize fire hazards or contamination of experiments

**3.1.11.** During odd times person should avoid working lonely.

**3.1.12.** Competency of the personnel should be judged regularly by giving unknown samples.

**3.1.13.** No external or internal pressure should be put on analyst.

**3.1.14.** Output should not be linked with quantum of work. More emphasis should be on quality output or results.

**3.1.15.** In case of contractual appointment, technical competency of the personnel should be evaluated, and they should be put on job only after they are trained and their competency in the respective field is established

**3.1.16.** Personnel should be medically fit depending upon the test method he is deployed to avoid any hazards.

**3.1.17.** Laboratory worker must be vaccinated and insured in order to cover health / financial risks.

**3.1.18.** When handling chemicals, note the hazard code on the bottle and take the appropriate precautions indicated.

**3.1.19.** All spills, accidents and potential exposures to infectious materials must be reported to a senior member of staff and entered in the ACCIDENT BOOK

### 3.2. Validating the Performance of analyst:

**3.2.1.** Normally blank determination along with the known-standards must be carried out in duplicate/ replicate to check the accuracy of the results obtained and include human error.

**3.2.2.** All the analysis records must be documented either through hardcopy or through soft copy to demonstrate that the tests are really been carried out.

**3.2.3.** Random checking of the result should be done inter-laboratory and intra-laboratory to check the proficiency of the personnel.

**3.2.4.** In case of hazardous analysis, special precautions as provided in the methods should be followed for self and surroundings.

**3.2.5.** Alternative arrangement of personnel should exist in case one is not available but not at the cost of their technical competency.

**3.2.6.** Special precaution should be taken by the personnel during break time to ensure that tests are carried out as per prescribed method and no relaxation is given in the test method.

**3.2.7.** Calculation / records should be rechecked on random basis by the supervisor.

### 3.3. Personal hygiene

**3.3.1.** Personal hygiene and safety in a microbiology lab are always to be practiced preventing self-infection or cross- contamination and quality of results are in direct relation to standards of personal hygiene.

**3.3.2.** Laboratory safety measures undoubtedly contribute to an accident free environment, but good hygiene practices supplement it with higher productivity and health of laboratory workers.

**3.3.3.** Hand washing is the most significant part of personal hygiene majorly while operating in a laboratory.


Fig.1 Hand washing procedure.

### 3.4. Personal Protective Equipments (PPE) for safety of Personnel's:

**3.4.1.** Gloves- protect hands, act as a barrier. Must be removed and disposed of when contaminated. Never re-use contaminated gloves

**3.4.2.** Lab coats/solid-front gowns- protect street clothing and skin

**3.4.3.** Eye Protection- protect against splashes

**3.4.4.** Sleeve covers,

**3.4.5.** Shoe Covers, booties

**3.4.6.** Full face protection (Shields)

## 4. Aseptic Techniques followed in laboratory:

**4.1. General Aseptic Techniques:** Aseptic technique or sterile techniques is a set of routine measures that are taken to prevent contamination of cultures, sterile media stocks, and other solutions from unwanted microorganisms (i.e., sepsis). following are some points to considered for GLP asepsis

**4.1.1.** Cleaning and disinfecting lab surfaces prior and after use limiting the duration that cultures or media are uncapped and exposed to the air keeping petri dishes closed whenever possible,

**4.1.2.** Effectively sterilizing inoculating loops and other equipment that comes into contact with cultures or media, and

**4.1.3.** Avoiding breathing on cultures or sterile instruments.

### 4.2. General rules to follow for microbial aseptic techniques:

**4.2.1.** To avoid disturbances of air close windows and doors to reduce draughts and

prevent sudden movements.

**4.2.2.** Make transfers over a disinfected surface. Ethanol disinfection may be used because of its rapid action.

**4.2.3.** Assemble all the apparatus and materials before start the operations

**4.2.4.** Complete all operations as quickly as possible with care and following appropriate SOP.

**4.2.5.** Vessels must be open for the minimum amount, must be done close to a Bunsen burner flame where air currents are drawn upwards. While opening a test tube or bottle, the neck must be immediately warmed by flaming.

**4.2.6.** Limit exposure of the sterile inner surfaces of petri plates to contamination from the air.

**4.2.7.** The parts of sterile pipettes must not be touched or allowed to come across with other non-sterile surfaces.

**4.2.8.** All items which encounter microorganisms must be sterilized before and after each such exposure.

## 4.3. Specific Aseptic Techniques

### 4.3.1. Sterile Handling

- Always wipe your hands and work area with 70% ethanol.
- It is recommended to wear personal protective equipments (gloves, marks, apron etc).
- Mouth pipetting is prohibited.
- Sterilize the outside of the containers, flasks, plates, and dishes with 70% ethanol before use.
- Avoid pouring media and reagents directly from bottles or flasks.
- Use sterile glass or disposable plastic pipettes and do not unwrap sterile pipettes until they are to be used.
- Always cap the bottles and flasks after use and seal multi-well plates with tape or place them in resalable bags. Never uncover a sterile flask, bottle, Petri dish, etc. until use
- Carry out the transfer of cultures as quickly as possible for the minimum length of time.
- Work very close to the Bunsen burner flame if lid of the Petri dish may be

removed for longer periods than normal, work very close to the Bunsen burner flame

- While using a wire loop, hold the handle of the wire loop close to the top, as you would hold a pen, at an angle that is almost vertical. This ensures that any liquid culture on the loop will run down into the flame.
- Sterilize a wire loop by heating to red hot in a roaring blue Bunsen burner flame before and after use. and allow to cool for a few seconds in the air, then use immediately.
- Sterile graduated or dropping (Pasteur) pipettes are used to transfer cultures, sterile media and sterile solutions.
- Immediately after use put the contaminated pipette into a nearby discard pot of disinfectant.
- Remove the teat only once the pipette is within the discard pot otherwise drops of culture will contaminate the working surface.
- Passing the mouth of the bottle through a flame produces a convection current away from the opening and helps to prevent contamination.

### 4.3.2. Aseptic Sampling Techniques

- An aseptic technique implies that you do not add any organisms to the sample when it is collected. It does not imply that the sample is aseptic or free of microorganisms.
- Extraneous microorganisms from the environment, hands, clothing, sample containers, and sampling devices may lead to erroneous analytical results.
- The use of aseptic sampling techniques and clean and sanitized equipment is of utmost importance.
- The purpose of aseptically collecting a sample is to prevent contamination of the sample or the surrounding product/product contact area.

### 4.3.3. Tools Used for Maintaining Aseptic Conditions

#### A) Burner

- Probably the easiest way to create a relatively sterile environment on the laboratory bench is by using a simple gas-powered burner to create a cone of hot air above and around the laboratory bench.

- The ability of the Bunsen burner flame to heat things very quickly also makes it an ideal choice for sterilizing inoculating loops, warming glass bottle necks, or igniting alcohol on culture spreaders.
- A Bunsen burner is not practical in some situations, e.g., within a laminar flow unit where the heat will disrupt airflow.
- A micro incinerator may be used as an alternative. This consists of a circular heating element. Placing an inoculating loop or needle within the ring will quickly heat and sterilize the loop/needle.

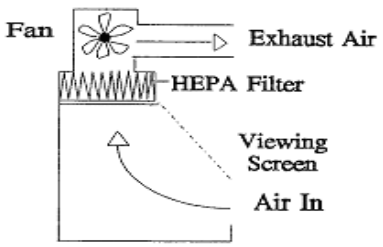
**B) The Laminar Flow Unit**

- A laminar flow unit (or hood or biosafety cabinets) is a sophisticated appliance that can further help prevent contamination of reagents and biological cultures it provides the work space with clean, ultra-filtered air.
- The most important part of a laminar flow hood is a high-efficiency bacterium-retentive filter, i.e., the HEPA (high-efficiency particulate air) filter. A certified HEPA filter must capture a minimum of 99.97% of dust, pollen, mold, bacteria, and any airborne particles with a size of >0.3 μm at 85 liters/min.

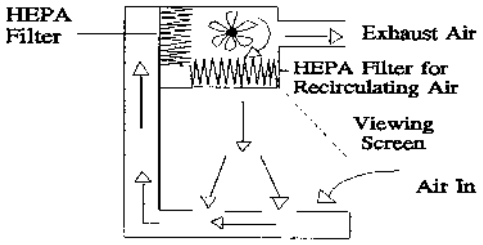
**C) Biological Safety Cabinet (BSC):** Primary containment device which utilizes HEPA filtered directional airflows to contain potentially infectious materials during experimental procedures. The BSC provides protection for the surrounding environment, research personnel and research materials being manipulated

- Three Classes of cabinets are Class I, Class II and Class III

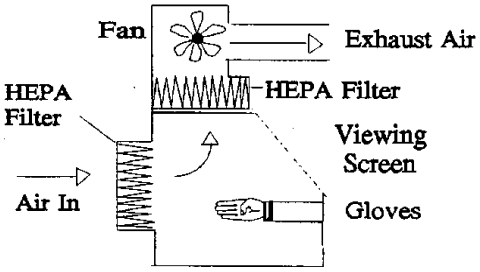
**Class I Cabinet**: **Inward airflow protects worker and exhaust to outside (w/wo high efficiency particulate air (HEPA) filter)**



**Class II Cabinet**: **Protect worker, product, environmental. "sterile" work area useful for work with aerosol-transmissible microorganisms**




**Class III Cabinet**: **It is totally enclosed, ventilated, air-tight and suitable for work with BSL3/4 agents**




## B. Biosafety & Biohazard Management

Safety is the first and foremost aspect in a Microbiology lab. Biosafety programs help to reduce or eliminate exposure of individuals and the environment to potentially hazardous biological agents.

**Biohazard:** An agent of biological origin that has the capacity to produce deleterious effects on humans, i.e. microorganisms, toxins and allergens derived from those organisms; and allergens and toxins derived from higher plants and animals.

**Biosafety:** The application of combinations of laboratory practice and procedure, laboratory facilities, and safety equipment when working with potentially infectious microorganisms

Biosafety is achieved by implementing various degrees of laboratory control and containment, through laboratory design and access restrictions, personnel expertise and training, use of containment equipment, and safe methods of managing infectious materials in a laboratory setting. Most importantly, we are concerned with those that pose threat to humans. However, biohazards may also potentially harm animals, aquatic life, and plants.

Special attention is required in the procedures for handling of biohazardous materials, intermediates, products and waste, and the attendant protective equipment in laboratories engaged in analysis of biological products. The pertinent safety information and hazard assessment procedures, standard laboratory practices must be identified, maintained and disseminated in the form of a manual. Good laboratory practices with critical requirements mentioned above will definitely help to achieve biosafety in laboratory.

**Biohazard Waste Management**

Waste management process consist of Waste collection, Segregation, Transportation and storage, Treatment & Disposal, Transport to final disposal site, Final disposal by application of Personal Protective Equipment (PPE). Biohazard waste treatment & disposal is done by different methods like Incineration Technology, Non-Incineration Technology, Autoclaving, Chemical Methods, Microwave Irradiation and Plasma Pyrolysis. The solid biohazardous waste treatment is biological inactivation in a manner that reduces hazardous exposure risk for lab personnel and the environment. This is generally achieved by autoclave treatment of waste or treatment and disposal through a waste disposal contractor who will autoclave or incinerate the waste. Wastes are to be "rendered non-infectious by sterilization techniques prior to disposal". This means that all items contaminated with a potentially infectious material must be autoclaved.

The latest biomedical waste (BMW) management guidelines which have been introduced in 2016 can be easily followed by various health/laboratory agencies. As per the rules microbiological and other clinical laboratory waste is to be pretreated by sterilisation to Log 6 or disinfection to Log 4, before packing and sending it to the common BMW treatment facility.

Biohazardous waste must be packaged so that personal protective equipment (PPE) is not needed during transport. Bagged Biohazardous waste transported must be closed, surface decontaminated, and placed inside secondary containment prior to transport. Autoclave tape should be used on biohazard bags to show that the waste has been treated. Bagged waste can be placed in the regular waste container for the laboratory and disposed as per the Table mentioned below (BMW, 2016).

| Sr. No | Category | Type of Bag and Container Used | Type of Waste | Treatment and Disposable options |
|---|---|---|---|---|
| 1 | Yellow | Yellow coloured Non chlorinated plastic bags | **A) Animal Anatomical Waste:**<br>Experimental animal carcasses, body parts, organs, tissues, including the waste generated from animals used in experiments or testing in veterinary hospitals or colleges or animal houses.<br>**B) Soiled Waste:**<br>Items contaminated with blood, body fluids like dressings, plaster casts, cotton swabs and bags containing residual or discarded blood and blood components<br>**C) Microbiology, Biotechnology and other clinical laboratory waste:**<br>Blood bags, Laboratory cultures, stocks or specimens of microorganisms, live or attenuated vaccines, human and animal cell cultures used in research, industrial laboratories, production of biological, residual toxins, dishes and devices used for cultures. | Pre-treat to sterilize with non chlorinated chemicals on-site using Autoclave safe plastic bags or containers<br>Incineration/plasma pyrolysis/deep burial |

| | | | | |
|---|---|---|---|---|
| | | | **D) Chemical Liquid Waste:** Liquid waste generated due to use of chemicals in production of biological and used or discarded disinfectants, discarded Formalin, infected secretions, liquid from laboratories and floor washings, cleaning, house-keeping and disinfecting activities etc. | Separate collection system leading to effluent treatment system After resource recovery, the chemical liquid waste shall be pre-treated before mixing with other wastewater. The combined discharge shall conform to the discharge norms given in Schedule-III BMW Rules 2016 |
| 2 | Red | Red coloured non-chlorinated plastic bags or containers | **Contaminated Waste (Recyclable)** Wastes generated from disposable items such as tubing, bottles, intravenous tubes and sets, catheters, urine bags, syringes (without needles and fixed needle syringes) and vaccutainers with their needles cut) and gloves PPE kits | Autoclaving or micro-waving/ hydroclaving followed by shredding or mutilation or combination of sterilization and shredding. Treated waste to be sent to registered or authorized recyclers or for energy recovery or plastics to diesel or fuel oil or for road making, whichever is possible. Plastic waste should not be sent to landfill sites. |
| 3 | White (Translucent) | Puncture proof, Leak proof, tamper proof containers | **Waste sharps including Metals:** Needles, syringes with fixed needles, needles from needle tip cutter or burner, scalpels, lades, or any other contaminated sharp object that | Autoclaving or Dry Heat Sterilization followed by shredding or mutilation or encapsulation in metal container or cement concrete; combination of shredding cum |

| | | | | |
|---|---|---|---|---|
| | | | may cause puncture and cuts. This includes both used, discarded and contaminated metal sharps | autoclaving; and sent for final disposal to iron foundries |
| 4 | Blue | Cardboard boxes with blue colored marking | **(a) Glassware:** Broken or discarded and contaminated glass including medicine vials and ampoules | Disinfection (by soaking the washed glass waste after cleaning with detergent and Sodium Hypochlorite treatment) or through autoclaving or microwaving or hydroclaving and then sent for recycling. |
| Disposal by deep burial is permitted only in rural or remote areas where there is no access to common bio-medical waste treatment facility. This will be carried out with prior approval from the prescribed authority and as per the Standards specified in Schedule-III. The deep burial facility shall be located as per the provisions and guidelines issued by Central Pollution Control Board from time to time. | | | | |

**C. References:**

i. Bio Medical Waste Management Rules - 2016

# Part – B

## Methods of Analysis for Foods

| | | | |
|---|---|---|---|
| | **Method for Enumeration of Aerobic Plate Count (APC)** | | |
| **Method No.** | FSSAI 15.001:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | The Total Aerobic Mesophilic Plate Count, usually called Aerobic Plate Count (APC) or Standard Plate Count, is the most commonly used general indicator of bacterial populations in foods. This method does not differentiate types of bacteria, and is only used to obtain general information on the sanitary quality of products, manufacturing practices, raw materials, processing conditions, handling practices and shelf life. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | Precautions should be taken while dealing with suspected food outbreak samples. | | |
| **Principle** | A specified quantity of homogenised food sample is either dispensed into an empty Petri dish, mixed with a specified molten agar medium or surface plated on a solid agar culture medium. Other plates are prepared under the same conditions using decimal dilutions of the test sample or of the initial suspension. The plates are incubated under aerobic conditions at 30 °C for 72 h. The number of microorganisms per gram or millilitre of sample is calculated from the number of colonies obtained on the plates containing fewer than 300 colonies for 90-100mm plates and 730 colonies for 140mm plates. | | |
| **Equipment** | 1. Laminar airflow<br>2. Hot air oven<br>3. Autoclave<br>4. pH meter with measuring accuracy ±0.1 | | |

<table>
<tr><td></td><td>5. Incubator (at 30 ± 1°C)<br>6. Water bath (at 44 °C to 47 °C)<br>7. Refrigerator (at 2 °C – 8 °C)<br>8. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>9. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>10. Micropipette with tips<br>11. Tubes and glass bottles<br>12. Vortex<br>13. Mechanical stirrer<br>14. pH meter with measuring accuracy ±0.1<br>15. Colony counter (optional)<br>16. Spreader (Sterile glass or plastic)<br>17. Spiral plater/rotator</td></tr>
<tr><td>Culture Media and Reagents</td><td>Diluent<br>The following diluents can be used for preparation of initial suspension and subsequent serial dilution:<br><br>
<table>
<tr><th>Purpose</th><th>Diluent</th></tr>
<tr><td>General Use</td><td>Peptone salt solution</td></tr>
<tr><td></td><td>Buffered peptone water</td></tr>
<tr><td>Special Purpose</td><td></td></tr>
<tr><td>Highly acidic products of pH ≥ 3.5 to pH < 4.5</td><td>Double-strength buffered peptone water</td></tr>
<tr><td>Gelatine</td><td>Phosphate buffered diluent</td></tr>
</table>
Plate Count Agar (PCA)<br>For dairy products, add skimmed milk powder (free from inhibitory substances) at a level of 1.0g/litre to Plate Count Agar.<br>For pour plate technique. In case the steriled medium to be used immediately, cool it to 44 °C to 47 °C in a water bath before use.<br>If the stored sterilized medium to be used, before starting the microbiological examination, completely melt the medium, then cool it to 44 °C to 47 °C in a water bath. The final pH of the media to be maintained at 7.0 For surface plating technique, pour 15-20 ml of the</td></tr>
</table>

<table>
<tr><td></td><td>medium into sterile petri dishes and solidify. Dry the plates either in a laminar flow or in a drying cabinet/incubator. While drying in laminar flow, dry the plates with agar surface facing upwards (at room temperature) for 30-60 min or overnight at room temperature with the lids in place. While drying in drying cabinet/incubator, keep the plates with agar surface facing downwards with half opened lids at 25-50 °C till the disappearance of water droplets from the surface of the lids.</td></tr>
<tr><td>Reference Cultures</td><td>Bacillus subtilis subsp. spizizenii (WDCM 00003a)<br>Escherichia coli (WDCM 00012a or WDCM 00013)<br>Staphylococcus aureus (WDCM 00032 or WDCM 00034)</td></tr>
<tr><td>Sample Preparation</td><td>Prepare the test sample in accordance with Chapter 2.</td></tr>
<tr><td>Procedure</td><td>Two different procedures can be followed for determination of APC in food matrix.
<table>
<tr><td>Procedure</td><td>Applicability</td></tr>
<tr><td>Pour Plate Technique</td><td>• Products that require lower LOD (< $10^2$/g or ml for liquid samples and <$10^3$/g for solid samples)<br>• Products expected to contain spreading colonies (e.g. Bacillus spp.; Proteus spp.)</td></tr>
<tr><td>Surface Plating Technique (Spread Plate/Spiral Plate technique)</td><td>• Chilled and frozen foods, dried foods, other foods that may contain heat-sensitive organisms;<br>• Products likely to contain significant proportion of obligate aerobic bacteria<br>• (e.g. Pseudomonas spp.)<br>• Products containing small particulate matter which from are difficult to distinguish from colonies in pour plate<br>• Products with strong colour that</td></tr>
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| | prevents recognition of colonies in pour plate |

**Test portion, initial suspension and dilutions**

Weigh or measure the test portion, to a tolerance of ±5 %, into a sterile container or plastic bag. A mass of m g or a volume of V ml (minimum 10 g or 10 ml, unless otherwise stated) representative of the laboratory sample shall be used. Add a quantity of diluent equal to 9 × m g or 9 × V ml to prepare a primary decimal dilution. Homogenize the sample with a peristaltic blender or rotary homogenizer or vibrational mixer as mentioned in Chapter 1. This corresponds to $10^{-1}$ dilution.

For further decimal dilution, transfer, using a pipette, 1 ml ± 0.02 ml of the initial suspension into a tube containing 9 ml ± 0.2 ml of sterile diluent. Mix thoroughly, preferably by using a mechanical stirrer for 5 s to 10 s, to obtain a $10^{-2}$ dilution. If necessary, repeat these steps using the $10^{-2}$ and subsequent dilutions and a new sterile pipette or tip for each operation, to obtain sufficient ($10^{-3}$, $10^{-4}$ etc.) dilutions to enumerate the appropriate number of microorganisms.

**Inoculation and incubation**

Pour Plate Technique

Transfer 1 ml of test sample if liquid, or 1 ml of the initial suspension ($10^{-1}$ dilution) in the case of other products to sterile petri dishes in duplicate. If more than one dilution is to be plated, the number of dishes can be reduced to one. Similarly transfer 1ml of suspension from subsequent dilutions ($10^{-2}$, $10^{-3}$ or higher) to separate sterile petri dishes using a new sterile pipette for each decimal dilution.

If appropriate and possible, select only the critical dilutions steps (at least two consecutive decimal dilutions) for the inoculation of the Petri dishes that will give colony counts of between 10 and 300 colonies per plate.

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| | Pour about 12 ml to 15 ml of the plate count agar (PCA) at 44 °C to 47 °C into each Petri dish. The time elapsed between sample preparation and plating shall not exceed 45 min. Carefully mix the inoculum with the medium by rotating the Petri dishes horizontally and allow the mixture to solidify by leaving the Petri dishes standing on a cool horizontal surface. In case there is possibility that the product contains microorganisms which will overgrow or swarm on the surface of the plate, pour an overlay of 4-5 ml of PCA and allow to solidify.<br><br>Incubate the plates (in inverted condition) in incubator at 30 ± 1 °C for 72 ± 3 h.<br><br><u>Surface Plating Technique</u><br><u>PCA plates</u><br>Pour about 15 ml to 20 ml of the plate count agar (PCA) at 44 °C to 47 °C into each Petri dish and allow it to solidify.<br>The plates may be stored at 5 °C ± 3 °C for upto 4 weeks.<br>Immediately before use, these agar plates should be dried.<br>Label all petri plates with the sample number, dilution, date and any other desired information.<br><br><u>Inoculation and Incubation</u><br>Using a sterile pipette, transfer 0.1 ml of test sample if liquid, or 0.1 ml of the initial suspension ($10^{-1}$ dilution) in the case of other products to the centre of each two agar plates. If more than one dilution is to be plated, the number of dishes can be reduced to one. Similarly transfer 0.1ml of suspension from subsequent dilutions ($10^{-2}$, $10^{-3}$ or higher) to separate agar plates using a new sterile pipette/ sterile micro pipette tip for each decimal dilution.<br><br>For counting low number of microorganisms, 1.0 ml of suspension can be transferred to surface of large agar plate (140 mm diameter) or three small agar plates (90 mm diameter). In both cases, use duplicate plates for surface plating (i.e. 2 large plates or 6 small plates). |

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| | Spread the inoculum uniformly and as quickly as possible over the surface of the agar plate, without touching the sides of the petri dish with a spreader (glass, metallic or disposable type). Same spreader can be used for all the dilutions of one sample, provided they are used progressively from highest to lowest dilution (containing greatest amount of test material). Allow the inoculum to be absorbed for 15 minutes.<br><br>The time elapsed between sample preparation and plating shall not exceed 45 min. Incubate the plates (in inverted condition) in incubator at (30 ± 1) °C for (72 ± 3) h.<br>**Counting of colonies**<br>After the specified incubation period of (72 ± 3) h, select the agar plates with, if possible, fewer than 300 colonies. If plates of 140mm diameter have been used, select plates up to 730 colonies. Count the colonies with visually or with a colony counter. Special care has to be taken to distinguish pinpoint colonies from food particles.<br>Plates with spreading colonies may be avoided if the swarming colony occupies more than 1/4th of plate area. |
| **Expression of Results** | Calculate the number N of microorganisms present in the test sample as a weighted mean from two successive dilutions using the following formula:<br>$N = \frac{\sum C}{Vx1.1xd}$<br>where<br>$\sum C$ is the sum of the colonies counted on the two dishes retained from two successive dilutions, at least one of which contains a minimum of 10 colonies;<br>V is the volume of inoculum placed in each dish, in millilitres;<br>d is the dilution corresponding to the first dilution retained [d = 1 when the undiluted liquid product (test sample) is tested]. |

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| | Round off the calculated result to two significant figures. When doing this, if the third figure is less than 5, do not modify the preceding figure; if the third figure is greater than or equal to 5, increase the preceding figure by one unit.<br><br>Express the result preferably as a number between 1.0 and 9.9 multiplied by the appropriate power of 10, or a whole number with two significant figures.<br>Report the result as the number N of microorganisms per millilitre (liquid products) or per gram (other products).<br>Special Cases<br>**i.** *When one dish (test sample or initial suspension or first dilution) contains less than 10 colonies*<br>If the plate contains less than 10 colonies, but at least four, calculate the result using the formula:<br>$N_E = \frac{C}{Vxd}$<br>Report it as the estimated number $N_E$ of microorganisms per millilitre (liquid products) or per gram (other products).<br>If the total is from 3 to 1, the precision of the result is too low and the result shall be reported as: "Microorganisms are present but less than 4/Vd per gram or per ml"<br>**ii.** *When the dish (test sample or initial suspension or first dilution) contains no colonies*<br>Report the result as follows:<br>"less than 1/Vd microorganisms per millilitre" (liquid products) or<br>"less than 1/Vd microorganisms per gram" (other products)<br>where<br>d is the dilution factor of the initial suspension or of the first dilution inoculated or retained<br>V is the volume of the inoculum used in each dish, in millilitres<br>**iii.** Where the counting of colonies for each one of the dishes for all inoculated dilutions produces a number greater than 300 (or 730 for |

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| | 140 mm diameter plates), report the result as follows:<br>"more than 300/Vd"<br>where<br>d is the dilution of the last inoculated dilution;<br>V is the volume of the inoculum used in each dish, in milliliters |
| **Reference** | 1) ISO 4833-1 (2013): Microbiology of the food chain —Horizontal method for the enumeration of microorganisms —Part 1: Colony count at 30 °C by the pour plate technique<br>2) ISO 4833-2 (2013) (corrigendum 2014): Microbiology of the food chain —Horizontal method for the enumeration of microorganisms —Part 2: Colony count at 30°C by the surface plating technique.<br>3) IS 5402 (2012): Microbiology of Food and Animal Feeding Stuffs — Horizontal Method for the Enumeration of Microorganisms — Colony-Count Technique at 30°C (Second Revision)<br>4) ISO 6887-1 (2017): Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Enumeration of *Bacillus cereus*** | | |
| **Method No.** | FSSAI 15.002:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | *Bacillus cereus* is a pathogenic bacterium, which causes foodborne diseases classified by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002) in Risk Group III: "diseases of moderate hazard usually not life threatening, normally of short duration without substantial sequelae, causing symptoms that are self-limiting but can cause severe discomfort". They are Gram-positive rods, spore forming, facultative anaerobic and each species is differentiated from *B. cereus* by, basically, for one single characteristic. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | Precautions should be taken while dealing with suspected food outbreak samples. | | |
| **Principle** | A specified quantity of homogenised food sample (liquid/initial suspension) is surface plated on a selective culture medium on to a petri dish. Other plates are prepared under the same conditions using decimal dilutions of the test sample or of the initial suspension. The plates are incubated under aerobic conditions at 30 °C for 18 to 48 h. The number of microorganisms per gram or millilitre of sample is calculated from the number of colonies confirmed on plates at dilution levels and further confirmed according to the test specified. | | |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven | | |

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| | 4. Autoclave<br>5. Incubator (at 30 ± 1°C, 37 ± 1°C and 55 ± 1°C)<br>6. Water bath (at 44 °C to 47 °C)<br>7. Refrigerator (at 2°C – 8 °C)<br>8. pH meter with measuring accuracy ±0.1<br>9. Microscope<br>10. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>11. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>12. Micropipette with tips<br>13. Tubes and glass bottles<br>14. Vortex<br>15. Mechanical stirrer<br>16. pH meter with measuring accuracy ±0.1<br>17. Colony-counter (optional)<br>18. Spreader (glass or plastic)<br>19. Inoculation loops and straight wire<br>20. Spiral plater/rotator<br>21. Drying cabinet or incubator at 37 ± 1°C and 55 ± 1°C |
| **Culture Media and Reagents** | i) Dilution fluid<br>ii) Complete medium (MYP agar) having Basal Medium<br>iii) Polymyxin B solution<br>iv) Egg yolk emulsion<br>v) Sheep blood agar<br>Details of preparation given in Chapter 3 |
| **Reference Cultures** | *Bacillus cereus* ATCC 14579, ATCC 10876, ATCC 11778<br>Further details of culturing given in Chapter 3 |
| **Sample Preparation** | Sample preparation based on the product categories are given in Chapter 2 |
| **Procedure** | **Test portion, initial suspension and dilutions**<br>Make a 1:10 dilution of the well mixed sample, by aseptically transferring sample to the desired volume of diluent. Aseptically |

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| | weigh 10 gm of solid or semi-solid sample into a sterile blender jar or into a stomacher bag. Add 90 mL of sterile diluent. Blend for 2 min at low speed (approximately 8000 rpm) or mix in the Stomacher for 30-60 sec. Powdered samples may be weighed and directly mixed with the diluent. Shake vigorously. In most of the samples particulate matter floats in the dilution water. In such cases allow the particles to settle for two to three minutes and then draw the diluent from that portion of dilution where food particles are minimum and proceed.<br><br>**Dilution (decimal dilution)**<br>Prepare decimal dilutions from initial suspension as follows. Shake each dilution 25 times in 30 cm arc. For each dilution use fresh sterile micropipette tips. Alternately use auto pipette. Pipette 1 ml of food homogenate (Initial suspension) into a tube containing 9 ml of the diluent and mix properly by using vortex/Cyclomixer. From the second dilution transfer 1mL tube containing 9 ml of the diluents and mix properly by using vortex/Cyclomixer.<br><br>Repeat the above to prepare the further dilutions using different diluent tubes, until the desired dilution is obtained.<br><br>**Inoculation and Incubation**<br><u>MYP Agar plates</u><br>Pour about 15 ml to 20 ml of the plate count agar (MYPA) at 44 °C to 47 °C into each Petri dish and allow it to solidify.<br>The plates may be stored at 5 °C ± 3 °C for upto 4 weeks.<br>Immediately before use, these agar plates should be dried<br>Label all Petri plates with the sample number, dilution, date and any other desired information.<br>1. Pipette 0.1 ml of the test sample (if product is liquid), or of the initial suspension (in case of other products), of such dilutions which have been selected, for plating (spread |

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| | plate technique) on MYP Agar, in duplicate.<br>2. In some samples where it is desirable to estimate low numbers of B. cereus, the limits of detection may be raised by a factor of 10 by examining 1.0 ml of the test sample if the initial product is liquid, or 1.0 ml of the initial suspension for the other products. Pipette 1 ml of inoculum either over the surface of three small dishes (90 mm) or on the surface of a large Petri dish (140 mm). In both the cases, prepare duplicates by using two large plates or six small plates.<br>3. Spread the inoculum over surface of agar plate, using a sterile spreader. Keep the plates in upright position until inoculum is absorbed by the agar.<br>4. Invert the inoculated plates and incubate them for 18 h to 24 ±2 h at 30± 1 °C. Reincubate the plates for an additional 24 h, if colonies are not clearly visible, prior counting.<br><br>**Enumeration**<br>After completion of incubation period, select only those plates (preferably at two successive dilutions) that contain less than 150 colonies (typical and/or atypical colonies) for enumeration.<br>If there are less than 15 characteristic colonies present on plates inoculated with the liquid product or the lowest dilution of other products, it is possible to make an estimated count as described in the expression of results below.<br><br>The presumptive colonies are large, pink (indicating absence of mannitol fermentation) and generally surrounded by a zone of precipitation (indicating the production of lecithinase).<br>• Numerous mannitol fermenting microbes lead to the production of acid, then the characteristic pink colour of *B. cereus* colonies may be reduced or disappear entirely.<br>• Some strains of *B. cereus* produce little or no lecithinase. |

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| | Colonies of these strains will not be surrounded by a precipitation zone. These colonies should also be subjected to confirmation tests.<br><br>**Confirmation**<br>Select 5 presumptive colonies from each plate for confirmation. If the plates are overcrowded, streak 5 presumptive colonies on MYP plates. After incubation at 30 °C for 18 h to 24 h select the well isolated colony for confirmation.<br><br>**Haemolysis test on sheep blood agar**<br>Streak, stab or spot the selected colonies onto the surface of sheep blood agar and incubate at 30 °C for 24 h ±2 h. Interpret the haemolysis reaction.<br><br>**Biochemical interpretation**<br>**Test Result confirming presumptive *Bacillus cereus***<br>• MYP agar - Formation of pink colonies surrounded by precipitate<br>• Haemolysis - Positive reaction |
| **Expression of Results** | When the method used requires identification, a given number A (generally 5) of presumptive colonies is identified from each of the dishes retained for the colony counting. After identification, calculate, for each of the dishes, the number of colonies complying with identification criteria, using Equation:<br>$a = \frac{b}{A} X C$<br>where<br>b is the number of colonies complying with identification criteria among the identified colonies A;<br>C is the total number of presumptive colonies counted on the dish.<br>Calculate the number N of identified microorganisms present in the test sample by replacing ΣC by Σa in the equation, |

$$N = \frac{\Sigma a}{VX(n1 + 0.1X\ n2)Xd}$$

Where

∑a is the sum of the colonies confirming presumptive *Bacillus cereus identified o all the dishes selected,*

V is the volume of inoculum placed in each dish, in millilitres;

n1 is the number of dishes selected at the first dilution;

n2 is the number of dishes selected at the second dilution; is the dilution rate corresponding to the first dilution selected (the initial suspension is a dilution).

d is the dilution corresponding to the first dilution retained [d = 1 when the undiluted liquid product (test sample) is retained.

EXAMPLE:

A count of a product after inoculation with 0.1 ml of product gave the following results:

- for the first dilution selected ($10^{-2}$): 65 typical colonies and 85 typical colonies and no atypical colonies

- for the second dilution selected ($10^{-3}$): 3 typical colonies and 7 typical colonies and no atypical colonies.

he following numbers were stabbed:

- from 65 colonies, 5 colonies were stabbed and all 5 proved to be biochemical confirmed, giving a = 65;

- from 85 colonies, 5 colonies were stabbed, 3 of which proved to be biochemical confirmed, giving a = 51;

- from 3 colonies, all 3 were stabbed and proved to be biochemical confirmed, giving a = 3;

- from 7 colonies, 5 colonies were stabbed and all 5 proved to be biochemical confirmed, giving a = 7.

$$N = \frac{65+51+3+7}{0.1X(2+0.1X\ 2)X1\ 0-2} = 57272$$

The result, after rounding off is 5.7 X $10^4$ cfu/gm or ml

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| | *No. of presumptive Bacillus cereus is expressed in cfu*/g or ml |
| **Reference** | 1) ISO 7932:2004-Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of presumptive Bacillus cereus — Colony-count technique at 30˚C.<br>2) IS 5887-6 - 2012 - Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Enumeration of Presumptive Bacillus cereus, Part 6 Colony-count Technique at 30°C.<br>3) ISO 6887-1 (2017): Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions. |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Determination of *Campylobacter* spp.** | | |
| **Method No.** | FSSAI 15.003:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | *Campylobacter* is one of the major causes of diarrhoea in humans and *C. jejuni* subsp. jejuni and *C. coli* are the species most frequently associated with acute foodborne gastroenteritis. Food-borne diseases caused by *C. jejuni* subsp. Jejuni include gastroenteritis, septicemia, meningitis, abortion and the Guillain-Barré Syndrome (GBS). GBS is classified by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002) into risk group IB: "diseases of severe hazard for restricted population; life threatening or resulting in substantial chronic sequelae or presenting effects of long duration". | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Camplylobacter* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. It is also the responsibility of the user to establish appropriate safety and health practices. | | |
| **Principle** | The detection of *Campylobacter* species involves enrichment in a selective liquid medium that is incubated at 37°C for 4-6 h followed by 41.5°C for 44 h. The enrichment culture is inoculated onto a blood free selective agar medium, which are incubated microaerobically at 41.5°C for24 h and examined for characteristic colonies.<br>Confirmation of suspect colonies of *Campylobacter* species is by | | |

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| | means of biochemical, morphological and physiological tests. |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven<br>4. Autoclave<br>5. Incubator (Operating at 25 ± 1 °C, 37 ± 1 °C and 41.5 ± 1°C)<br>6. $CO_2$ incubator<br>7. Water bath (at 44 °C to 47 °C)<br>8. pH meter with measuring accuracy ±0.1<br>9. Microscope<br>10. Refrigerator (at 2°C – 8°C)<br>11. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>12. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>13. Micropipette with tips<br>14. Tubes and glass bottles<br>15. Vortex<br>16. Mechanical stirrer<br>17. pH meter with measuring accuracy ±0.1<br>18. Spreader (glass or plastic)<br>19. Inoculation loops and straight wire<br>20. Spiral plater/rotator<br>21. Apparatus suitable for creating microaerophilic conditions |
| **Culture Media and Reagents** | **Obligatory**<br>Bolton Broth<br>Modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA)<br>Columbia Blood Agar (CBA)<br>Oxidase Kovacs Reagent<br>**Optional**<br>Mueller Hinton Blood Agar |

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| | 3% Hydrogen Peroxide<br>Nalidixic acid (30 μg) discs<br>Cephalothin (30 μg) discs<br>Sodium Hippurate Solution<br>Ninhydrin Solution<br>Indoxyl acetate discs (2.5 to 5.0 mg)<br>Bolton broth<br>Sterile lysed horse blood<br>Antibiotic solution 1<br>Antibiotic solution 2<br>Antibiotic solution 3<br>Preston broth<br>Modified charcoal cefoperazonedeoxychlolate agar (mCCD agar)<br>Colombia blood agar<br>Sterile sheep or horse blood<br>Reagent for oxidase activity<br>Reagent for catalase activity<br>Reagent for hydrolysis of hippurate<br>Indoxyl acetate discs<br><br>**Details of preparation given in Chapter 3** |
| **Reference Cultures** | Details given in Chapter 3<br>*Campylobacter jejuni* WDCM 00005 (positive control and *Escherichia coli* WDCM 00013 (negative control). |
| **Sample Preparation** | Details given in Chapter 2 |
| **Procedure** | Depending on the type of sample and the purpose of the test, one or more of three different detection procedures is/are used:<br>**1. Detection procedure A**<br>For samples with low numbers of campylobacters and low levels of background microflora and/or with stressed campylobacters |

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| | (e.g., cooked or frozen products), homogenize 10 g or 10 mL of sample with 90 mL of Bolton broth. Incubate at 37°C for 4 to 6 hours, then at 41.5 °C for 44 ± 4 hours, in a microaerobic atmosphere (oxygen content of 5 ± 2%, carbon dioxide 10 ± 3%, optional hydrogen ≤10%, with the balance Nitrogen).<br><br>**2. Detection procedure B**<br>For samples with low numbers of campylobacters and high level of background microflora (e.g. raw meats, including poultry): Homogenize 10 g or 10 mL of sample with 90 mL of Preston broth. Incubate at 41.5 ± 1°C for 24 ± 2 hours, in a microaerobic atmosphere.<br><br>**3.Detection procedure C**<br>For samples with high numbers of campylobacters, enrichment is not done.<br><br>**Isolation (Selective-differential plating)**<br>In general, an amount of test portion is mixed with enrichment media to yield a ten-fold dilution.<br><br>**1. Detection procedure A**<br>Using the enrichment culture obtained in Bolten Broth after Procedure A, two selective solid media are inoculated: 1. — modified Charcoal Cefoperozone Deoxycholate agar (mCCD agar); 2. — any other solid selective Campylobacter medium using different selective principles from those in mCCD agar.<br>**2. Detection procedure B**<br>Using the enrichment culture obtained after Procedure B, the selective mCCD agar is inoculated.<br>**3. Detection procedure C**<br>Using the enrichment culture obtained after Procedure C, the test portion is plated directly or after suspending in an appropriate |

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| | amount of liquid onto the selective mCCD agar.<br><br>Incubate the plates at 41.5 ± 1 °C/44 ± 4 h in a microaerobic atmosphere. Incubate the second selective isolation medium plates according to the manufacturers' instructions.<br><br>After the incubation period, examine the plates for typical colonies of *Campylobacter*.<br>The typical colonies on mCCDA are grayish, often with a metallic sheen, with a tendency to spread. Other forms of colonies may occur.<br>Follow the manufacturers' instructions to select typical colonies on the second isolation medium.<br><br>As *Campylobacter* rapidly loses culturability in air, follow the procedure of confirmation without delay.<br>For a clear distinction between positive and negative confirmation reactions, it is helpful to verify this with well characterized positive and negative control strains. *Campylobacter jejuni* WDCM 00005 (positive control) and *Escherichia coli* WDCM 00013 (negative control).<br>In addition, to the confirmation and identification tests described in here other tests (PCR tests, serological methods, matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF-MS) analysis, etc.) can be used, providing the suitability of the alternative procedure is verified (see ISO 7218).<br>**Confirmation:**<br>For confirmation, take a typical colony from each plate and a further four colonies if the first is negative. Store the isolated plates at 5°C preferably at micraerophilic conditions for confirmation<br>Streak each colony onto a Columbia Blood Agar (CBA) plate in |

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| | order to allow the development of well-isolated colonies and incubate the plates in a microaerobic atmosphere at 41.5 ± 1 °C for 24–48 h. Use the pure cultures obtained on CBA for examination of morphology, motility using microscope, oxidase activity, microaerobic growth at 25°C, aerobic growth at 41.5°C.<br>NOTE The suspect colony could be previewed for characteristic morphology and motility before streaking on CBA.<br><br>• **Morphology and motility:** From the Columbia Blood Agar (CBA) plate examine the fresh colony for morphology and motility using a microscope. Cultures showing curved bacilli with a spiralling "corkscrew" motility should be retained for the confirmatory tests below.<br>• **Growth at 25°C (microaerobic):** Inoculate the culture from the CBA plate onto the surface of a new CBA plate. Incubate the plate at 25 ± 1°C for 44 ± 4 h in a microaerobic atmosphere and examine for growth of Campylobacter colonies.<br>• **Growth at 41.5°C (aerobic):** Inoculate the culture from the CBA plate onto the surface of a new CBA plate. Incubate the plate at 41.5 ± 1°C for 44 ± 4 h in an aerobic atmosphere and examine for growth of Campylobacter colonies.<br>• **Oxidase test:** Using a platinum/iridium loop or glass rod, take a portion of a well-isolated colony from each individual CBA plate and streak it onto a filter paper moistened with the Oxidase Kovacs Reagent. The appearance of a mauve, violet or deep blue color within 10 s indicates a positive reaction. If a commercially available oxidase test kit (must be approved by FSSAI under RAFT scheme) is used, follow the manufacturer's instructions.<br>**Interpretation:** *Campylobacter spp.* the cultures exhibiting the following characteristics: small curved bacilli with a spiraling "corkscrew" motility, microaerobic growth at 25°C negative, |

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| | aerobic growth at 41.5°C negative, oxidase positive. |
| **Expression of results** | Based on the observations and interpretation of the results report presence or absence of *Campylobacter* spp. in test portion specifying the mass in grams or mililitres of the sample taken.<br>***Campylobacter* spp.**= present or absent/ gm or mL. |
| **Reference** | ISO 10272-1:217-Microbiology of food chain — Horizontal method for detection and enumeration of *Campylobacter* spp. - Part 1: Detection method. |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Enumeration of Coliforms** | | |
| **Method No.** | FSSAI 15.004:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | Coliforms are a broad group of aerobic or facultative anaerobic, rod-shaped, gram-negative non-spore forming bacteria which can ferment lactose with the production of acid and gas when incubated at 32–37°C. Coliform are members of *Enterobacteriacae* family. Most common genera are *E. coli*, *Citrobacter*, *Enterobacter*, *Klebsiella* and *Hafnia*. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good laboratory practices to avoid cross contamination. | | |
| **Principle** | Violet red bile lactose agar (VRBL) medium is used for enumeration of coliforms. VRBL contains selective inhibitors - inhibit the accompanying gram-positive and unrelated flora. Coliforms rapidly ferment lactose and produce red colonies surrounded by red purple halo. Lactose non-fermenters and late lactose fermenters produce pale colonies. Confirmation is carried out in brilliant green bile broth medium, which is also having selective components for the growth of coliforms. | | |
| **Equipment** | Refer Chapter 1 for general equipment.<br>1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven<br>4. Autoclave | | |

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| | 5. Incubator (Operating at 30 ± 1°C or 37± 1°C)<br>6. Water bath (at 44 °C to 47 °C or at 100 °C)<br>7. pH meter with measuring accuracy ±0.1<br>8. Microscope<br>9. Refrigerator (at 2°C – 8 °C)<br>10. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>11. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>12. Micropipette with tips<br>13. Tubes and glass bottles<br>14. Durham tubes<br>15. Vortex<br>16. Mechanical stirrer<br>17. pH meter with measuring accuracy ±0.1at 25°C<br>18. Spreader (glass or plastic)<br>19. Inoculation loops and straight wire<br>20. Spiral plater/rotator<br>21. Colony counter (optional) |
| **Culture Media and Reagents** | Primary Diluent<br>Violet Red Bile Lactose Agar (VRBL)<br>Brilliant Green Lactose Bile Broth (BGBB) ISO 4832:2006<br><br>For media preparation and other details refer Chapter 3 |
| **Reference Cultures** | Specific strains or equivalent member of coliform group (*E. coli* etc.) |
| **Sample Preparation** | Refer Chapter 2 |
| **Procedure** | **Test portion, initial suspension and dilution**<br>Weigh or measure the test portion, to a tolerance of ±5 %, into a sterile container or plastic bag. A mass of m g or a volume of V ml (minimum 10 g or 10 ml, unless otherwise stated) representative of the laboratory sample shall be used. Add a quantity of diluent equal to 9 × m g or 9 × V ml to prepare a primary decimal |

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| | dilution. Homogenize the sample with a peristaltic blender or rotary homogenizer or vibrational mixer as mentioned in Chapter 1. This corresponds to $10^{-1}$ dilution.<br><br>For further decimal dilution, transfer, using a pipette, 1 ml ± 0.02 ml of the initial suspension into a tube containing 9 ml ± 0.2 ml of sterile diluent. Mix thoroughly, preferably by using a mechanical stirrer for 5 s to 10 s, to obtain a $10^{-2}$ dilution. If necessary, repeat these steps using the $10^{-2}$ and subsequent dilutions and a new sterile pipette or tip for each operation, to obtain sufficient ($10^{-3}$, $10^{-4}$, etc.) dilutions to enumerate the appropriate number of microorganisms.<br><br>**Inoculation and Incubation**<br><br>Label all Petri dishes with the sample code, dilution, date and any other information.<br>1. Pipette 1ml of the test sample (if the product is liquid), or 1 ml of primary suspension (if prepared) to the centre of each petri dishes. Similarly prepare plates from subsequent dilution as required.<br>2. Pour approximately 15 ml of the molten VRBL agar, (cooled at 44 °C to 47 °C) into each petri dishes. Time elapse between inoculation and addition of agar into plates shall not exceed 15 mins.<br>3. Carefully mix the inoculum with the medium and allow the medium to solidify.<br>**Also prepare a control plate with 15 ml of the medium for checking its sterility.**<br>4. After complete solidification, pour about 4 ml of molten VRBL agar (cooled at 44 °C to 47 °C) onto the surface of inoculated medium and allow to solidify. Invert the inoculated plates and incubate them at 30 °C or 37 °C for 24 ± 2 h. |

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| | **Enumeration**<br>After completion of incubation period, count purplish red colonies with a diameter of at least 0.5 mm (sometimes surrounded by a reddish zone of precipitated bile). Consider all these as typical colonies of coliform and do not require further confirmation. Count other atypical colonies (smaller size) also and all colonies derived from milk products that contain sugar other than lactose, immediately after the incubation and confirm.<br>**Confirmation**<br>Select 5 colonies of each atypical types and inoculate into tubes of brilliant green lactose bile broth and incubate at 30 °C or 37 °C for 24 ± 2 h. Consider all colonies as coliforms that show gas formation in Durham tubes. Take the results into account in the calculation. |
| **Calculation** | 1. Select petri dishes having 10 to 150 colonies for enumeration.<br><br>2. Use the following formula for calculation<br>$$N = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2) \times (d)}$$<br>N is Number of colonies per ml or g of product<br>$\sum$ C is the sum of colonies counted on all the dishes retained<br>n1 is the no. of dishes retained in the first dilution<br>n2 is the no of dishes retained in the second dilution<br>d is the dilution factor corresponding to first dilution<br><br>In case petri dishes have total/typical colonies less than 10, calculate the results using the following formula:<br>N= C/(v × d)<br>N is Number of colonies per ml or g of product<br>C is the average of colonies on the petri dishes retained<br>v is the volume of inoculums used in each dish<br>d is the dilution corresponding to the dilution retained. |

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| **Expression of Results** | Results shall be expressed as a number between 1.0 and 9.9 multiplied by 10 x, where x is power of 10.<br>If plates from all dilutions have no colonies, the result is expressed as less than 1 cfu/ml or 10 cfu/g or mL (if primary suspension prepared) |
| **Reference** | 1) ISO 4832:2006: Microbiological of food and animal feeding stuffs- Horizontal method for the enumeration of coliforms – Colony count technique<br>2) IS 5401(Part 1): 2012: Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of coliforms: Part 1 colony count technique (Second Revision)<br>3) ISO 6887-1 (2017): Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA<br>Inspiring Trust, Assuring Safe & Nutritious Food<br>Ministry of Health and Family Welfare, Government of India | **Method for Commercial Sterility Test for Sterilized/UHT Milk /Flavored Milk / Evaporated Milk** | | |
| **Method No.** | FSSAI 15.005:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | Sterilization and ultra-high temperature treatment are used in the production of milk, flavoured milk, and evaporated milk to protect them from pathogens and spoilage bacteria while maintaining the product's organoleptic and nutritional quality. Because absolute sterility is impossible to achieve without severely compromising food integrity, thermal processed products must meet the commercial sterility criterion before they can be sold. Commercial sterility is defined as the absence of microorganisms capable of growing in food under normal non-refrigerated conditions under which the food is likely to be held during distribution and storage. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | -- | | |
| **Principle** | UHT processing and aseptic filling are intended to achieve commercial sterility, microbial defects can occur at any stage of production due to insufficient heat treatment or high contamination of raw materials, resulting in food spoilage by spore-forming microorganisms. In commercial testing, the products are incubated in their final packaging for seven to thirteen days at 30 °C to allow surviving spores or contaminating microorganisms to recover and grow to detectable levels. When the products are intended to be stored at higher temperatures (> 40 °C), additional incubation at 55 °C for five to seven days is also used. A second step focuses on detecting viable microorganisms that grew in the product using pH and acidity measurement. | | |

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| **Equipment** | Refer Chapter 1 for general equipment |
| **Chemicals and Reagents** | Dilute rosaniline acetate solution<br>Phenolphthalein solution<br>Sodium hydroxide solution<br>pH indicator strips |
| **Reference Cultures** | -- |
| **Sample Preparation** | |
| **Procedure** | **a) pH**<br>1. pH test is performed to assess the variation in pH levels during the incubation period of 0 and 7 days incubation.<br>2. pH variation can be an indicator of microbial activity which may be leading to acid production.<br>3. The determination of pH shall be done as per IS 1479 (Part 1) using indicator strips.<br>4. Sample which does not show any physical alteration during incubation at 55±1 °C for 7 days and where the pH does not show a difference of more than 0.3 unit from the initial pH, is considered sterile.<br>**b) Titratable acidity**<br>1. Titratable acidity test is performed to assess the variation in developed acidity levels during the incubation period of initial $0^{th}$ day and final after 7 days incubation.<br>2. Excessive variation can be an indicator of microbial activity which may be leading to acid production.<br>3. The determination of titratable acidity shall be done as per IS 1479 (Part 1)<br>4. Sample which does not show any physical alteration during incubation at 55 ± 1 °C for 7 days and where the acidity does not show a difference of more than 0.02 percent from the initial acidity is considered sterile. |
| **Calculation** | -- |
| **Expression of Results** | a) Based on observation made on pH strip where pH difference is not more than 0.3 from the initial pH is regarded as sterile |

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| | b) Based on observation made on titratable acidity wherein the difference in TA not more than 0.02% lactic acid is considered as sterile |
| **Reference** | 1) IS4238: 2020: Sterilized and Ultra High Temperature Sterilized Milk —Specification<br>2) IS 1479 (Part 1): 2016 (RA 2021): Methods of Test for Dairy Industry Part 1 Rapid Examination of Milk (First Revision) |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA<br>Inspiring Trust, Assuring Safe & Nutritious Food<br>Ministry of Health and Family Welfare, Government of India | **Method for Commercial Sterility Test for Sterilized/UHT Cream** | | |
| **Method No.** | FSSAI 15.006:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | Sterilization and ultra-high temperature treatment are used in the production of cream to protect them from pathogens and spoilage bacteria while maintaining the product's organoleptic and nutritional quality. Because absolute sterility is impossible to achieve without severely compromising food integrity, thermal processed products must meet the commercial sterility criterion before they can be sold. Commercial sterility is defined as the absence of microorganisms capable of growing in food under normal non-refrigerated conditions under which the food is likely to be held during distribution and storage | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | -- | | |
| **Principle** | UHT processing and aseptic filling are intended to achieve commercial sterility, microbial defects can occur at any stage of production due to insufficient heat treatment or high contamination of raw materials, resulting in food spoilage by spore-forming microorganisms. In commercial testing, the products are incubated in their final packaging for seven to thirteen days at 30 °C to allow surviving spores or contaminating microorganisms to recover and grow to detectable levels. When the products are intended to be stored at higher temperatures (> 40 °C), additional incubation at 55 °C for five to seven days is also used. A second step focuses on detecting viable microorganisms that grew in the product using acidity measurement and incubation | | |

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| | test by storing the pack at 38ºC for 14 days to check bulging of cans and other product characteristics like curdling, sliminess, etc. |
| **Equipment** | Incubator, 2 white porcelain basins,l0-ml burette fitted with a soda-lime guard tube, porcelain basins |
| **Culture Media and Reagents** | Dilute rosaniline acetate solution<br>Phenolphthalein solution<br>Sodium hydroxide solution |
| **Reference Cultures** | -- |
| **Sample Preparation** | -- |
| **Procedure** | **A) Titratable acidity**<br>a. Weigh 10.0 g cream into each of two white porcelain basins of approximately 60-ml capacity; add to both, 10 ml of water and stir to disperse the cream.<br>b. Prepare from one dilution a colour control by adding and stirring 2 ml dilute rosaniline acetate solution.<br>c. Stir 2 ml phenolphthalein solution into the other dilution and, while stirring, vigorously, add as rapidly as possible sodium hydroxide solution, from a 10-ml burette fitted with a soda-lime guard tube, until the colour matches the pink colour of the control.<br>d. The titration shall be preferably done in north daylight or under illumination from a daylight lamp.<br><br>**B) Incubation test**<br>Incubate the cans/retort pouches at a temperature of 38 ºC for 14 days.<br>The samples shall pass the test if:<br>a) the cans do not show any bulge due to positive pressure within, and<br>b) the product inside the can has not curdled or thinned and is free from any objectionable taste or odour, sliminess, etc. |
| **Calculation** | -- |

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| **Expression of Results** | a. Based on observation made on titratable acidity wherein the titratable acidity is not more than 0.15% lactic acid is considered as satisfactory for the assigned test.<br>b. After 14 days of incubation, the cans do not show bulging of cans, not curdling or thinned and free from objectionable odour, or odour and free from objectionable taste or odour, sliminess, etc is considered as it meets the sterility requirement of sterilized cream. |
| **Reference** | IS4884: 2021 Sterilized/UHT Sterilized Cream — Specification |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Determination of *Cronobacter* spp.** | | |
| **Method No.** | FSSAI 15.007:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | *Cronobacter* spp. *(Enterobacter sakazakii*) causes a foodborne disease classified by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002) in Risk Group IB: "diseases of severe hazard for restricted population; life threatening or resulting in substantial chronic sequelae or presenting effects of long duration". | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Cronobacter* spp. are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. It is also the responsibility of the user to establish appropriate safety and health practices. | | |
| **Principle** | A test portion is inoculated into BPW, then incubated between 34 °C and 38 °C for 18 h ± 2 h. The selective enrichment medium is inoculated with the culture and incubated at 41.5 °C ± 1 °C for 24 h ± 2 h. The chromogenic agar is streaked for isolation with the enrichment culture and incubated at 41.5 °C ± 1 °C for 24 h ± 2 h. Typical colonies are selected from the chromogenic agar, purified on a non-selective agar such as TSA and biochemically characterized. | | |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven | | |

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| | 4. Autoclave<br>5. Incubator (Operating at 34 °C to 38 °C, 37°C ± 1°C and 41.5 ± 1°C)<br>6. Water bath (at 47°C and 50°C and 37°C ± 1°C)<br>7. pH meter with measuring accuracy ±0.1<br>8. Microscope<br>9. Refrigerator (at 2 °C– 8 °C)<br>10. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>11. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (ClassA)<br>12. Micropipette with tips<br>13. Tubes and glass bottles<br>14. Vortex<br>15. Homogenizer<br>16. pH meter with measuring accuracy ± 0.1<br>17. Spreader (glass or plastic)<br>18. Inoculation loops and straight wire<br>19. Spiral plater/rotator<br>20. Inoculation loops and straight wire<br>21. Spectrophotometer |
| **Culture Media and Reagents** | • Buffered peptone water<br>• Cronobacter selective broth (CSB)<br>• Chromogenic Cronobacter isolation (CCI) agar<br>• Tryptone soya agar (TSA)<br>• Media and reagents for biochemical characterization<br>Details of preparation given in Chapter 3 |
| **Reference Cultures** | Details given in Chapter 3 |
| **Sample Preparation** | Details given in Chapter 2 |
| **Procedure** | **Pre enrichment**<br>Add 10 g or 10 ml of the test sample to 90 ml of pre warmed (room temperature) pre-enrichment medium (BPW), to yield a tenfold dilution. For specific products, follow the procedures |

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| | specified in chapter 3.<br>For preparing quantities larger than 10 g, BPW should be pre-warmed between 34 °C - 38 °C before inoculated with the test portion.<br>**Pre enrichment**<br>Incubate the inoculated pre-enrichment medium set temperature between 34 °C and 38 °C for 18 h ± 2 h.<br><br>**Enrichment**<br>After incubation, mix the inoculated pre-enrichment medium and transfer 0.1 ml of the obtained culture above into 10 ml of CSB and mix well. Incubate at 41.5 °C for 24 h ± 2 h.<br><br>**Isolation of presumptive *Cronobacter* spp.**<br>From enrichment culture, mix well and with the help of a streak onto the surface of the CCI agar (Brought to room temperature if they are stored at a lower temperature) to obtain well-separated colonies.<br>Incubate the plate at 41.5 °C for 24 h ± 2 h.<br>After incubation, examine the chromogenic plate for the presence of typical colonies of presumptive *Cronobacter*.<br>Typical presumptive *Cronobacter* colonies on CCI are small to medium-sized (1 mm to 3 mm) and blue to blue-green in colour. Non-*Cronobacter* colonies are often white or white with a green centre, grey or black, yellow or red.<br><br>**Confirmation**<br>Purification of colonies: Select 5 typical colonies from the CCI medium. In case colonies are not well separated, streak a typical colony onto the CCI agar again.<br>If on the dish there are fewer than five typical colonies, take all the marked colonies for confirmation.<br>Streak the selected colonies onto the non-selective agar (e.g.: |

TSA) to gain well-isolated colonies.

Invert and incubate the plates between 34 °C to 38 °C for 21 h ± 3 h.

If the cultures on the non-selective agar are mixed, sub-culture the suspect colony onto a plate of the non-selective agar further and incubate between 34 °C to 38 °C for 21 h ± 3 h to obtain a pure culture.

If positive, subject to biochemical confirmation tests. If negative, progress through the other selected colonies until either all are negative or a positive is found.

Strains can be kept on the non-selective agar at 5 °C, but cannot be stored for more than 7 days.

**Biochemical confirmation**

Fresh subcultures of the colonies should be obtained before performing confirmation tests.

**Table showing confirmation tests for *Cronobacter* spp.**

| Oxidase | Acid from: |
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| Hydrolysis of 4-Nitrophenyl α-D-glucopyranoside substrate | D-Arabitol |
| L-Lysine decarboxylase | D-Sorbitol |
| L-ornithine decarboxylase | D-Sucrose |
| Methyl Red (Optional) | A-Methyl-D-glucoside (optional) |
| Voges-Proskauer (Optional) | |

Select one yellow pigmented colony from each TSA plate for further biochemical characterization below. Miniaturized biochemical identification kits (must be approved by FSSAI under RAFT scheme) may be used.

**Oxidase test:** Using a inoculation loop or glass pasteur pipette, place a portion of the isolated colony on a filter paper moistened

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| | with the Oxidase Kovac's Reagent. The appearance of a mauve, violet or deep blue color within 10 s indicates a positive reaction.<br>If a commercially available oxidase test kit (must be approved by FSSAI under RAFT scheme) is used, follow the manufacturer's instructions.<br><br>**Citrate test:** Streak the selected colonies onto the slant surface of Simmons Citrate Agar. Incubate the tubes at 30 ± 1°C for 24 ± 2 h. Positive test is indicated by presence of growth and color change from green to blue. Negative test is indicated by no growth or very little growth and no color change.<br><br>**Arginine dihydrolase and lysine/ornithine decarboxylase tests:** Inoculate the culture into tubes of Decarboxylation Medium (with 0.5% L-lysine, L-ornithine or L-arginine). Incubate the tubes at 30 ± 1°C/24 ± 2 h. A violet color after incubation indicates a positive reaction. A yellow color indicates a negative reaction.<br><br>**Hydrolysis of 4 Nitrophenyl (PNP) α-D-glucopyranoside substrate**<br>Using a loop or wire, suspend an individual colony grown on the non-selective agar such as TSA in 2ml of physiological salt solution. 0.85% NaCl. Add 2ml of the α-Glucosidase enzymatic assay solution. Incubate in a water bath at 37˚C for 4h and measure the formation of yellow colouration in a spectrophotometer at 405 nm. A minimal absorption of 0.3 at 405 nm after 4h, equivalent to 16 mM PNP, can be considered positive.<br><br>**Carbohydrate fermentation tests**: Inoculate the culture into tubes of Carbohydrate Fermentation Medium (with 1% D-sorbitol, L-rhamnose, D-sucrose, D-melibiose or amygdaline). Incubate the |

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| | tubes at 30 ± 1°C/ 24 ± 2 h. A yellow color after incubation indicates a positive reaction. A red color indicates a negative reaction.<br><br>**Methyl Red (Optional)**<br>Inoculate the prepared glucose peptone medium and incubate at 37°C for 24-48h. Add 2 drops of methyl red solution prepared by dissolving 0.04g of methyl red in 40 ml of absolute ethanol and dilute with water to make up to 100 ml. A positive reaction is indicated by red colour and a negative reaction by yellow colour.<br><br>**Voges-Proskauer (VP) (Optional)**<br>Inoculate the prepared glucose peptone medium and incubate at 37°C for 24-48 h. To 1ml of the growth, add 0.6 ml of α-naphthol solution prepared as 5% solution in ethanol, shake and add 0.2 ml of 40% aqueous potassium hydroxide. Shake and slope the tube and observe for up to 4h for appearance of a pink colour which indicates a positive reaction. |
| **Interpretation**<br>**Expression of results:** | Based on the observations and interpretation of the results, report presence or absence of *Cronobacter spp*. in test portion specifying the mass in grams or mililitres of the sample *Cronobacter*spp.= present or absent/ 10 gm or ml. |
| **Reference** | ISO 22964:2017 -Microbiology of food chain — Horizontal method for the detection of *Cronobacter* spp. |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Enumeration of Sulfite Reducing Clostridia based on ISO 15213** | | |
| **Method No.** | FSSAI 15.008:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | Sulphite-reducing clostridia are Gram-positive anaerobic spore-forming rod-shaped bacteria which have ability of reducing sulphite to sulphide under anaerobic conditions within 24-48 hours. Anaerobic sulfite-reducing bacteria are generally considered as indicators of Clostridia contamination in food products. Clostridium is a genus of Gram-positive bacteria, which includes several significant human pathogens, including C*lostridium botulinum*, *Clostridium perfringens*, *Clostridium difficile,* etc. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good laboratory practices to avoid cross contamination. | | |
| **Principle** | Iron Sulfite Agar is used for sulfite reducing clostridia. The medium contain disodium sulfite and Iron ammonium citrate. Disodium sulfite is reduced by sulfite reductase enzyme released by bacteria. So when $H_2S$ is produced from sulfite reduction, the colony becomes black due to the formation of iron sulfide from citrate. As incubation is done under anaerobic conditions, only anaerobic bacteria having ability of sulfite reduction are isolated and enumerated. Clostridia colonies are confirmed by doing respiratory and spore forming test. | | |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven<br>4. Autoclave<br>5. Anerobic Jar for creating anerobic atmosphere and a system to check the anerobic condition | | |

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| | 6. Incubator (Operating at 37 °C ± 1°C and 50 °C ± 1°C)<br>7. Water bath (at 44 °C to 47 °C)<br>8. pH meter with measuring accuracy ±0.1<br>9. Microscope<br>10. Refrigerator (at 2 °C– 8 °C)Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>11. Micropipette with tips<br>12. Test tubes (16 x 160 mm) and flasks or bottles of capacity 500 ml.<br>13. Vortex<br>14. Mechanical stirrer<br>15. pH meter with measuring accuracy ±0.1<br>16. Spreader (glass or plastic)<br>17. Inoculation loops and straight wire<br>18. Spiral plater/rotator<br>19. Inoculation loops and straight wire |
| **Culture Media and Reagents** | • Saline Peptone Diluent/ or buffered peptone water (BPW)<br>• ron Sulfite Agar<br>• Ellner's Medium<br>For media preparation and other details refer Chapter 3 |
| **Reference Cultures** | *Clostridium* spp (*Clostridium sporogenes*) |
| **Sample Preparation** | Refer Chapter 2 |
| **Procedure** | **Preparation of the samples and serial dilutions**.<br>a). Following the procedures described in Chapter 2<br>Heat treatment of the initial suspension is necessary to eliminate vegetative forms of spore forming bacteria. Temperature and heating time may vary accrding to actual need from combinations producing definite pasteurization effect a moderate heat acivaion effect (e.g 75 C for 20 min o boiling to several minues)., Homogenize 10g of sample with 90 mL of saline peptone water (SPW) or buffered peptone water (BPW) ($10^{-1}$ dilution). From this first dilution prepare serial decimal dilutions, |

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| | **b) Inoculation**.<br><br>Take two sterile Petri dishes. Using a sterile pipette/ micropipette tip, transfer to the dish 1 ml of the test sample if the product is liquid, or 1 ml of the initial suspension in case of other products. Repeat the procedure described with the further dilutions, if necessary, using a fresh sterile pipette/ micropipette tip for each dilution.<br><br>Pour Approx. 18 to 20 ml molten iron sulfite agar, previously cooled to $44^0$C to $47^0$C in water bath.<br>Time elapse between inoculation and addition of agar into plates should not exceed 15 mins. The time between the end of the preparation of the initial suspension and pour plating shall not exceed 45 min.<br>Carefully mix the inoculums into medium by horizontal movements and allow the medium to solidify.<br>After solidification, add 5-10 ml of iron sulfite agar into dishes as overlay.<br>**c) Incubation**<br>1. Incubate all the petri dishes in anaerobic jar at $37^0$C±$1^0$C for 24 to 48h.<br>2. If thermophillic bacteria are suspected, prepare a second set of petri dishes and incubate this set at $50^0$C±$1^0$C.<br>• **Observation**<br>1. Read the results after 24 to 48 h, depending on the bacterial growth. Count all black colonies, possibly surrounded by a black zone as sulfite reducing bacteria.<br>• **Confirmation**<br>1. For confirmation of clostridia, pick 5 characteristics colonies colonies from each dish and proceed the confirmatory test i.e respiratory test and spore forming test.<br>2. In respiratory test allow to grow characteristic colonies aerobically to check their growth in presence of oxygen. |

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| | 3. Spore forming test can be done by inoculating spore inducing medium i.e. Ellner's medium and observe the spore production. |
| **Calculation** | 1. Select petri dishes having less than 300 total colonies and less than 150 typical colonies for calculation.<br>Use the following formula for calculation<br>$N = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2) \times (d)}$<br>N is Number of colonies per ml or g of product<br>$\sum$ C is the sum of colonies counted on all the dishes retained<br>n1 is the no. of dishes retained in the first dilution<br>n2 is the no of dishes retained in the second dilution<br>d is the dilution factor corresponding to first dilution<br>In case petri dishes have total/typical colonies less than 10, calculate the results using the following formula:<br>N= C/ (v × d)<br>N is Number of colonies per ml or g of product<br>C is the average of colonies on the petri dishes retained<br>v is the volume of inoculums used in each dish<br>d is the dilution corresponding to the dilution retained. |
| **Expression of Results** | • Results shall be expressed as a number between 1.0 and 9.9 multiplied by 10x, where x is power of 10.<br>• If plates from all dilutions have no colonies, the result is expressed as less than 1 cfu/ml or 10 cfu/g or mL (if primary suspension prepared) |
| **Reference** | ISO 15213 (2003): Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of sulfite-reducing bacteria growing under anaerobic conditions. |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Methods Isolation and Identification of *Clostridium perfringens* and *Clostridum botulinum* and enumeration of *Clostridium perfringens* based on IS 5887-Part 4** | | |
| **Method No.** | FSSAI 15.009:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | Several micro-organisms contaminating food give rise to clinical symptoms, such as abdominal pain, nausea, vomitting, diarrhoea and sometimes pyrexia. A well-known exception is that of botulism where the symptoms are those of difficulty in swallowing, diplopia, aphonia and difficulty in respiration. Poisoning through food is characterized by symptoms of explosive nature which occur in otherwise healthy individuals. Such explosive nature of food poisoning helps in differentiating conditions from those of out-breaks of food-borne infectious diseases which generally spread over a period of several days. This part of the standard covers the method for isolation and identification of some Clostridium species responsible for food poisoning. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | The test must be carried out under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good laboratory practices to avoid cross contamination. Utmost biosafety precautions to be taken while performing the test for *C. botulinum*. | | |
| **Principle** | *Clostridium perfringens*: The method of identification of food-poisoning strains of *Clostridium perfringens* is based on colonial characters, morphology and the Nagler reaction. The frequency of spore-bearing *Clostridium perfringens* is low and reduces the diagnostic values of this criterion.<br>*Clostridium perfringens:* The method is based on growth on blood agar medium associated with haemolysis and on egg-yolk medium, *C.* | | |

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| | *botulinum* colonies produce opalescence and a pearly layer and are lactose negative. Followed by demonstration of toxin by *in vivo* test in guinea pigs/mice. |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven<br>4. Autoclave<br>5. Incubator (Operating at 30 °C ± 1°C and 37°C ± 1°C)<br>6. Anerobic jar<br>7. Water bath (at 44 °C to 100 °C)<br>8. pH meter with measuring accuracy ±0.1<br>9. Microscope<br>10. Refrigerator (at 2 °C– 8 °C)<br>11. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>12. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>13. Micropipette with tips<br>14. Tubes and glass bottles<br>15. Vortex<br>16. Mechanical stirrer<br>17. pH meter with measuring accuracy ±0.1<br>18. Spreader (glass or plastic)<br>19. Inoculation loops and straight wire<br>20. Spiral plater/rotator<br>21. Inoculation loops and straight wire<br>22. Colony counter |
| **Medium** | i) Cooked meat medium<br>ii) Willis and Hobb's medium with neomycin (Egg yolk Medium)<br>iii) Medium for *C. botulinum* type E<br>iv) Nutrient broth<br>v) Nutrient agar<br>vi) Spore Inducing Medium (Ellner's Medium)<br>vii) Animals<br>viii) Mice |

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| | ix) Guinea Pig<br>x) Blood agar with neomycin<br>xi) *C. perferingens* antitoxin<br>xii) *C. botulinum* antitoxin |
| **Procedure** | **Isolation of *C. perfringens***<br>The sample is blended in a sterile blender/jar for 2 minutes using approximately 200 ml of diluting fluid per approximately 25 g of the sample. The diluting fluid should be 0.1 percent peptone in water sterilized at 120°C for 20 min, final pH6.8 ±0.1or 3.4 percent of potassium dihydrogen phosphate ($KH_2PO_4$) in water, pH adjusted to 7.2 and sterilized at 120°C for 20 min.<br>An aliquot of the specimen is inoculated into cooked meat medium and the inoculated tube heated in a steamer at 100°C for one hour and incubated over- night at 37°C.<br>An aliquot of the specimen is also inoculated directly on to blood agar edium and the egg-yolk medium and incubated in an anaerobic jar at 37°C overnight.<br>Subcultures are made from the growth in medium on to the two solid media (i.e Blood agar with Neomycin and Willis and Hobb's Medium with Neomycin) and incubated in an anaerobic jar at 37°C overnight.<br><br>**Isolation of *C. botulinum***<br>Preheat the sample at 80°C for 30 min and inoculate into cooked meat medium and the two solid media (Blood agar with neomycin and Wilfis and Hobb's medium with neomycin). The solid media are incubated anaerobically and all the three inoculated media are incubated at 37°C for 5 to 10 days.<br><br>**Isolation of *C. botulinum* type E strains:**<br>*C. botulinum*type E strains exhibit low thermal resistance and are missed in specimens which have been heated prior to inoculation. Inoculate the specimen in duplicate tubes of the medium (Medium for *C. botulinum type* E) and incubate at 30°C for 3 days |

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| | 1) In sterile test tubes take aliquots of 2 ml samples of growth and mix with equal volume of absolute ethanol. Let stand at 25°C for one hour with occasional mixing.<br>2) Streak onto Willis and Hobb's medium with neomycin and inoculate into medium for *C. botulinum* type E. Incubate overnight at 37°C the solid medium being incubated in an anaerobic jar.<br>3) Examine the solid medium for presence of colonies with opalescence zones indicating growth of *C .botulinum* Type E. If such colonies are present, carry out test for toxin using the growth in medium for *C. botulinum* type E inoculated with ethanol treated culture.<br><br>**Identification of *C. perfringens***<br>1) **Grams stain:** Test from liquid culture and solid media - Gram-positive rods, large and stout withstraight sides and rounded ends. Spores are oval, central or subterminal and distend the bacillary body.<br>2) **Colonial Characters:** On blood agar medium growth is associated with haemolysis which may not be larger than the colony. On Willis and Hobb's medium with neomycin, colonies produce opalescence and a pearly layer and are lactose negative.<br>3) **Spore prduction**<br>Inoculate growth from any of the media as in Blood agar with Neomycin and Willis and Hobb's medium with neomycin into spore inducing medium. The growth in cooked meat medium may also be inoculated into medium.<br>NOTE — Some workers have noted reduced heat resistance of spores when cultures are grown in spore inducing medium.<br><br>**4) Nagler reactin – *In vitro* Test for alpha Toxin**<br>One half of a plate of Willis and Hobb's Medium with Neomycin is spread over with two or three drops of standard *Clostridium perfringens* antitoxin and dried. The area is demarcated. The two halves of the plate are inoculated with the suspect strain and |

incubated at 37°C anaerobically. Lecithinase activity is shown by precipitates around colonies in the half without antitoxin and this reaction is inhibited in the other half with specific antitoxin. The production of the enzyme lecithinase C, as demonstrated in the Nagler reaction by all types of *C. perfringens* is used to distinguish *C. perfringens* from other species of Clostridia. However, *C. bifermentes* also produce lecithinase and maybe differentiated from *C. perfringens* by *C. bifermentes* showing proteolytic activity, ready sporulation and non-fermentation of lactose. Lactose fermentation is carried out in 1 percent peptone water sugar medium incubated anaerobically at 37°C.

**Identification of *C. botulinum***

**1) Gram's Stain**

**2) Colonial Characters**

By growth on blood agar medium and egg-yolk medium, as described and for Type E strains.

3) ***In vivo* Test for Toxin**

Grow suspect strain in cooked meat medium for to 10 days. Obtain filtrate and divide into two portions, one of which is heated at 100°C for 10 min. Use three guinea pigs for intraperitonal injection with filtrate as follows:

a) One animal is protected with polyvalent botulinum antitoxin and injected with 2 ml of unheated filtrate;

b) One animal as injected with 2 ml of unheated filtrate and is unprotected; and

c) One animal is injected with 2 ml of heated filtrate.

Death with paralytic symptoms of the unprotected animal receiving unheated filtrate and survival of the other two animals diagnose the presence of botulinum toxin.

**Demonstration of Toxin of *C. botulinum* Type E**

The procedure as in *In vivo Test for Toxin* may fail to demonstrate toxin of *C. botulinum*Type E. For such strains the procedure shall be as follows.

1) To filtrate from growth in medium as obtained after procedure described above trypsin is added to a final concentration of 0.1 percent. Incubate at 37°C for 60 min.
2) Dilute specific type E antitoxin 1 in 5 with 0.1 M phosphate buffer of pH 6.5 containing 0.2 percent gelatin.
3) To 1.5 ml of diluted antitoxin, add equal volume of trypsinized filtrate mix and keep at room temperature for 30 min.
4) Inject 1 ml of the mixture intra-peritoneally into a pair of white mice. Also inject a pair of mice with 0.5 ml of the filtrate heated at 100°C for 10 min and another pair of mice with 0.5 ml of unheated trypsinized filtrate.

   Observe the mice up to 96 h. Death of the unprotected mice and survival of the mice receiving neutralized toxin and the heated toxin diagnose toxin of *C. botulinum*Type E.

Since bacteriological diagnosis of food-poisoning due to *C. botulinum* is based on the demonstration of the toxin in the food or intestinal content, the presence of the toxin in such materials need to be demonstrated.

**Procedure**

The material is soaked overnight in equal volume of sterile normal saline. The suspension is centrifuged and the supernatant sterilized by filtration. This is then directly used to note the presence of botulinum toxin by animal inoculation as with culture filtrate described *in vivo* test and demonstration of *C. botulinum* Type E toxin.

**SEROTYPING**

Food poisoning strains of *Clostridium perfringens* may be serotyped by slide agglutination using colonies from blood agar and testing with specific agglutinating sera, if these are available.

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| | **ENUMERATION**<br>*Clostridium perfringens* 25 to 50 g of the sample is taken in a sterile blender/jar and to this is added diluting fluid to have dilution of $10^{-1}$. Blend at 8,000 to 10,000 rev/min for 2 min. Make serial ten-fold dilutions with the diluting fluid in duplicate series up to $10^{-7}$. Streak 0.1 ml from each tube evenly on to blood agar medium and also on to egg-yolk medium .Incubate in an anaerobic jar at 37°C for 18 to 24 h. It is useful to incubate aerobically duplicate plates similarly inoculated for comparison. The suspect colonies of *Clostridium perfringens* are counted and the number of viable colonies per gram of sample determined by multiplying by the dilution factor(s) and dividing by the mass of the sample. |
| **Reference** | IS 5887 (Part 4): Methods for Detection of Bacteria Responsible for Food Poisoning, Part 4: Isolation and Identification of *Clostridium Perfringens* (*Clostridium Welchii*) and *Clostridium Botulinum* and Enumeration of *Clostridium Perfringens* (Second Revision) |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Enumeration of *Enterobacteriacae*** | | |
| **Method No.** | FSSAI 15.010:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | *Enterobacteriaceae* are a large family of gram - negative non spore forming rod shape bacteria, with the ability to ferment glucose. Members of the *Enterobacteriaceae* are classified into coliform and non coliform group based on ability to ferment lactose. Common food borne genera of *Enterobacteriaceae* family are *Salmonella, Yersinia, Escherichia coli, Shigella, Citrobacter, Ervinia, Hafnia, Klebsiella, Proteus, Providencia, Morganella, Serratia* etc. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good laboratory practices to avoid cross contamination. | | |
| **Principle** | The quantification of *Enterobacteriaceae* can be achieved by the standard plate count method usingviolet red bile glucose (VRBG) agar as the culturemedium. Characteristic *Enterobacteriaceae* colonies on VRBG are glucose fermenting are show oxidae negative reaction. | | |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven<br>4. Autoclave<br>5. Incubator (Operating at 30 °C ± 1°C)<br>6. Water bath (at 44 °C to 47 °C) | | |

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| | 7. pH meter with measuring accuracy ±0.1<br>8. Microscope<br>9. Refrigerator (at 2 °C– 8 °C)<br>10. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>11. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>12. Micropipette with tips<br>13. Tubes and glass bottles<br>14. Vortex<br>15. Mechanical stirrer<br>16. pH meter with measuring accuracy ±0.1<br>17. Spreader (glass or plastic)<br>18. Inoculation loops and straight wire<br>19. Spiral plater/rotator<br>20. Colony counter (optional) |
| **Culture Media and Reagents** | For Colony count technoque<br>Diluent: Saline peptone water (SPW) or buffered peptone water (BPW)<br>(Refer Chapter 3 to check on special cases in which either the type or volume of diluent vary as a function of the sampleto be examined).<br>Violet Red Bile Glucose Agar (VRBG)<br>Nutrient Agar<br>Glucose OF medium<br>oxidase reagent (N,N,N',N'- Tetramethyl-p-phenylenediamine dihydrochloride) / Buffered brilliant green bile glucose broth (SS and double stenghth for MPN Method)<br>For media preparation and other details refer Chapter 3 |
| **Reference Cultures** | Member of *Enterobacteriacae* (*E.coli*, etc) |
| **Sample Preparation** | Refer Chapter 2 |
| **Procedure** | **Preparation of the samples and serial dilutions:** |

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| | Following the procedures described in Chapter 2, homogenize 10 g of sample with 90 mL of saline peptone water (SPW) or buffered peptone water (BPW) ($10^{-1}$ dilution). From this first dilution prepare serial decimal dilutions.<br>**Most probable number (MPN) technique**<br>I. This technique is recommended when the number sought is expected to be in the range 1 to 100 per millitre or per gram of the test sampleTake three tubes of double-strength medium. Transfer to each of these tubes, using a pipette, 10 ml of the test sample if the product is liquid, or 10 ml of the initialsuspension in the case of other products.<br>II. Take three tubes of single-strength medium. Transfer to each of these tubes, using another pipette 1 ml of the test sample if the product is liquid, or 1 ml of the initial suspension in the case of other products.<br>III. Take three more tubes of single-strength medium. Transfer to each of these tubes, using another pipette 1 ml of the first decimal dilution ($10^{-1}$) (of the test sample if the product is liquid, or 1 ml of the first decimal dilution of the initial suspension ($10^{-2}$) in case of other products.<br>Incubate these nine tubes at 35 °C or 37°C for 24 h.<br>**Isolation**<br>Streak a loopful from each of the nine incubated cultures on the violet red bile gucose agar and incubate the plates at 35 or 37 °C for 24 h.<br>**Selection of colonies for confirmation**<br>From each of the plates incubated on which typical pink to red colonies (with or without precipitation haloes or coloufless, mucoid colonies have developed, select at random five such colonies for biochemical confirmation and subculture on nutrient |

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| | agar paltes. Keep these plates at 35°C or 37°C for 24 h. Select a well isolated colon for biochemical confirmation from the number of confirmed positive tubes, calculation of the most probable number of *Enterobacteriaceae* per ml or per gm of Test sample using the MPN table (**Appendix A**).<br><br>**Colony Count Techniques;**<br>**Inoculation and Incubation:**<br>Label all petri dishes with the sample code, dilution, date and any other information.<br>1. Take two pert dishes. Pipette 1ml of the test sample (if the product is liquid), or 1 ml of primary suspension (if prepared) to the centre of each petri dish. Similarly prepare plates from subsequent dilution as required<br>2. Pour approximately 15 ml of the molten VRBG agar, (cooled at 44 °C) into each petri dish. Time elapse between inoculation and addition of agar into plates shall not exceed 15 min.<br>3. Carefully mix the inoculum with the medium and allow to solidify.<br>4. After complete solidification, pour about 5 ml to 10 ml of molten VRBG agar (over lay) onto the surface of inoculated medium and allow to solidify.<br>5. Incubate the plates in an inverted position at 35°C or 37 ±1 °C for 24 ±2 hours.<br><br>**Enumeration and selection of colonies**<br>After incubation, select the plates that contain less than 150 characteristic colonies for enumeration.<br>Characteristic colonies are pink to red or purple in color with or without precipitation haloes.<br><br>**Confirmation** |

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| | Select 5 colonies from each dish. If there are less than 5 colonies on the plate, take all presumptive colonies present for confirmation. When there is no characteristic colony present, take five whitish colonies for confirmation. Streak the selected colonies onto the non selective agar medium (e.g. nutrient agar) and incubate these plates at 35 or 37 °C for 24 ±2 hours.<br>Select isolated colonies for biochemical confirmation.<br><br>**Biochemical confirmation Tests**<br><br>1. **Oxidase Test**:<br>Using an inoculation loop or glass rod, take a portion of well isolated colony and streak onto a filter paper moistened with the oxidase reagent.<br>Consider the test negative, when the colour of the filter paper does not turn dark blue purple within 10s.<br>2. **Fermentation Test:**<br><br>Using an inoculation loop or glass rod, stab the same colonies selected in oxidase test, into a tube of Glucose OF medium and overlay the surface with minimum of 1 cm of sterile mineral oil. Incubate at 37 °C for 24 ± 2 h. If yellow colour developed in the tube throughout, consider as glucose fermentation positive.<br><br>**Interpretation of biochemical tests**<br><br>The colonies that are oxidase negative and glucose positive are confirmed as *Enterobacteriaceae.* |
| **Calculation** | **Calculation of the most probable number (MPN)**<br><br>I. Count the number of tubes giving a positive reaction for each dilution.<br>II. One of the selected typical colonies of a subculture is oxidase-negative and glucose-positive; the tube from which the subculture is derived shall be regarded as being |

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| | positive.<br>III. Using the MPN table (see Appendix A), determine from the number of positive tubes in the different dilutions, the most probable number (MPN) index.<br>IV. In the case of liquid products, the number of *Enterobacteriaceae* per millilitre is calculated by dividing the MPN index by 10. In the case of other products for which initial suspensions are prepared, the number per gram is equal to the MPN.<br>1518<br>**Colony count Method**<br>Select petri dishes having 10 to 150 characteristics colonies for enumeration.<br>Spreading colony shall be considered as single colony.<br>Use the following formula for calculation<br>$$N = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2) \times (d)}$$<br>N is Number of colonies per ml or g of product<br>$\sum$ C is the sum of colonies counted on all the dishes retained<br>$n_1$ is the no. of dishes retained in the first dilution<br>$n_2$ is the no of dishes retained in the second dilution<br>d is the dilution factor corresponding to first dilution<br>In case petri dishes have total/typical colonies less than 10, calculate the results using the following formula:<br>N= C/(v × d)<br>N is Number of colonies per ml or g of product<br>C is the average of colonies on the petri dishes retained<br>v is the volume of inoculums used in each dish<br>d is the dilution corresponding to the dilution retained. |

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| **Expression of Results** | Results shall be expressed as a number between 1.0 and 9.9 multiplied by 10 x, where x is power of 10.<br>If plates from all dilutions have no colonies, the result is expressed as less than 1 cfu/ml or 10 cfu/g or mL (if primary suspension prepared). |
| **Reference** | 1) ISO 21528-2:2017 Microbiologyof the food chain — Horizontal method for the detection and enumeration of *Enterobacteriaceae* — Part 2: Colony-count technique<br>2) IS 7402: Microbiology - General Guidance for the Enumeration of *Enterobacteriaceae* without Resuscitation MPN Technique and Colony-count Technique |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Enumeration of *Escherichia coli* based on ISO : 16649-2** | | |
| **Method No.** | FSSAI 15.011:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | *E. coli* is included both in the group of total coliforms as in that of thermo tolerant coliforms. Its natural habitat is the intestinal tract of hot-blooded animals, although it may also be introduced into foods via non faecal sources. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time.<br>This method specifies a horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* in products intended for human consumption. It uses a colony-count technique at 44 °C on a solid medium containing a chromogenic ingredient for detection of the enzyme β-glucuronidase. | | |
| **Caution** | Precautions should be taken while dealing with suspected food outbreak samples. | | |
| **Principle** | Duplicate plates of tryptone-bile-glucouronic medium (TBX) are inoculated with the specified quantity of the test sample/initial suspension. Under the same conditions, using decimal dilutions of the test sample/initial suspension, two plates per dilution are inoculated. The dishes are incubated then examined to detect the presence of colonies which, from their characteristics, are considered to be β-glucuronidase-positive *Escherichia coli*. The number of colony-forming units (CFU) of β-glucuronidase-positive *Escherichia coli* per gram or per milliliter of sample is calculated. | | |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven | | |

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| | 4. Autoclave<br>5. Incubator (Operating at 44 °C ± 1°C)<br>6. Water bath (at 44 °C to 47 °C)<br>7. pH meter with measuring accuracy ±0.1<br>8. Microscope<br>9. Refrigerator (at 2 °C– 8 °C)<br>10. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>11. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>12. Micropipette with tips<br>13. Tubes and glass bottles<br>14. Vortex<br>15. Mechanical stirrer<br>16. pH meter with measuring accuracy ±0.1<br>17. Inoculation loops and straight wire<br>18. Spiral plater/rotator<br>19. Inoculation loops and straight wire |
| **Culture Media and Reagents** | Diluent<br>Culture medium: Tryptone-bile-glucuronic medium (TBX)<br>Details of preparation given in Chapter 3 |
| **Reference Cultures** | Further details of culturing given in Chapter 3 |
| **Sample Preparation** | Sample preparation based on the product categories are given in Chapter 2 |
| **Procedure** | **Test portion, initial suspension and dilution**<br>Aseptically weigh 10 gm of solid or semi-solid sample into a sterile blender jar or into a homogenizer bag. Add 90 mL of sterile diluent. Blend for 2 minutes at low speed (approximately 8000 rpm) or mix in the homogenizer for 30-60 seconds. Powdered samples may be weighed and directly mixed with the diluent with vigorous shaking (50 times through 30 cm arc). In most of the samples particulate matter floats in the diluent. In such cases allow the particles to settle for two to three minutes and then draw the portion of dilution where food particles are minimum and proceed. |

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| | **Inoculation**<br>Using a sterilepipetteor a micropipette, transfer to a Petri dish, 1 ml of the test sample (if liquid), or 1 ml of the initial dilution ($10^{-1}$) in the case of other products. Inoculate two plates per dilution. Repeat the procedure with the further decimal dilutions, if necessary, using a new sterile pipette / micropipette tip for each dilution. Pour into each Petri dish approximately 15 - 20 ml of the TBX medium (kept at 44 °C to 47 °C). Carefully mix the inoculum with the medium and allow the mixture to solidify, with the Petri dishes standing on a cool horizontal surface. Time elapse between inoculation and addition of agar into plates shall not exceed 15 min.<br><br>**Incubation**<br>Incubate the plates in an inverted position at 44 °C for 18 h to 24 h**.** The total incubation time shall not be longer than 24 h.<br><br>**Enumeration**<br>After incubation count the typical blue-green colonies of β-glucuronidase-positive *Escherichia coli* in each dish containing less than 150 typical CFU and less than 300 total CFU (typical and non typical). |
| **Expression of Results** | The calculation given below takes into account those cases most frequently encountered when conducting tests in accordance with good laboratory practice. Some special, fairly improbable, cases can arise (e.g. very different CFU numbers between the two dishes from the same dilution, or very different proportions from that of the dilution factor between the dishes from two successive dilutions). It is then necessary that the counting results be examined, interpreted and possibly rejected by a competent microbiologist.<br><br>For a valid result, in general it is considered that it is necessary to count the CFU on at least one dish containing minimum 15 blue CFU. |

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| | Calculate *N*, the number of CFU of β-glucuronidase-positive *Escherichia coli* present in the test sample per milliliter or per gram, as the weighted mean from two successive dilutions using the following equation:<br>$$N = \frac{\Sigma a}{V(n1 + 0.1\ n2)d}$$<br>Where<br>∑a -the sum of the CFU counted on all the dishes retained from two successive dilutions, at least one of which contains a minimum 15 blue CFU.V -Volume of inoculum placed in each dish, in millilitres;<br>n1 - Number of dishes considred inthe first dilution;<br>n2 - Number of dishes considred in the second dilution;<br>d -Dilution factor corresponding to the first dilution retained [d = 1 when the undiluted liquid product (test sample) is retained].<br><br>Round off the results to two significant figures. Express the result as the number of β-glucuronidase-positive *Escherichia coli* per milliliter (liquid products) or per gram (other products) as a whole number to two significant figures (if below 100) or as a number between 1.0 and 9.9 multiplied by the appropriate power of 10.<br><br>***Estimation of low numbers***<br>If the two dishes [at the level of the test sample (liquid products) or of the initial suspension (other products) or of the first inoculated or retained dilution] contain less than 15 blue CFU, calculate *N*E, the number of CFU of β-glucuronidase-positive *Escherichia coli* present in the test sample, as the arithmetical mean from two parallel plates using the following equation:<br><br>$N_E = \frac{\Sigma c}{V \times n \times d}$where<br>Σc - Sum of the blue CFU counted on the two dishes;<br>V -Volume of the inoculum, in millilitres, applied to each dish; |

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| | n -Number of dishes retained (n = 2 in this case);<br>d -Dilution factor corresponding to the initial suspension [d = 1 in the case of liquid products where the directly inoculated test sample isconsidered].<br><br>Round off the results to two significant figures<br><br>Express the result as follows:<br>Estimated number of β-glucuronidase-positive *Escherichia coli* per millilitre (liquid products) or per gram (other products): *N*E = *Y*.<br><br>If the two dishes at the level of the test sample (liquid products) or the initial suspension (other products) of the first inoculated or retained dilution do not contain any blue CFU, express the result as follows:<br>- less than 1/*d* of β-glucuronidase-positive *Escherichia coli* per milliliter (liquid products) or per gram (other products), where *d* is the dilution factor of the initial suspension or the first inoculated or retained dilution [*d* = 1 in the case (liquid products) where the directly inoculated test sample is retained].<br><br>If for the two dishes from the first dilution *d*1 the total number of blue and non-typical CFU is higher than 300 with visible blue CFU, and if for the two dishes from the subsequent dilution *d*2 containing less than 300 colonies, no blue CFU can be counted, express the result as follows:<br>- less than 1/*d*2 and more than 1/*d*1 β-glucuronidase positive *Escherichia coli* per milliliter (liquid products) or per gram (other products), where *d*1 and *d*2 are the dilution factors corresponding to dilutions *d*1 and *d*2.<br><br>If for the two dishes from the first dilution *d*1 the total number of typical CFU and non-typical CFU is higher than 300 without visible blue CFU, and if for the two dishes from the subsequent dilution *d*2 |

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| | containing less than 300 colonies, no blue CFU can be counted, express the result as follows:<br>- less than 1/*d*2 CFU of β-glucuronidase-positive *Escherichia coli* per millilitre (liquid products) or per gram (other products),<br>where *d*2 is the dilution factor corresponding to dilution *d*2.<br><br>***Method of calculation: Special cases***<br>In the case where the number of blue CFU is higher than 150 for the two dishes from the first dilution d1, with a number of blue CFU below 15 for the two dishes from the subsequent dilution d2:<br>-if the number of blue CFU on each of the two dishes from dilution d1 is within the range 167 to 150 (upper part of the confidence interval of a weighted mean equal to 150), use the calculation method for the general case;<br>-if the number of blue CFU on each of the two dishes from dilution d1 is higher than 167 (upper limit of the confidence interval of a weighted mean equal to 150 CFU), only take into account the result of the counts of dilution d2 and carry out a low number count.<br>- In the case where counting the blue CFU on each of the dishes from all the inoculated dilutions gives a number higher than 150, express the result as follows:<br>more than 150/d -glucuronidase-positive Escherichia coli per milliliter (liquid products) or per gram (other products), where d is the dilution factor of the last inoculated dilution.<br>- In the case where only the two dishes from the lowest dilution (highest concentration) contain less than150 typical CFU, calculate the number N' of -glucuronidase-positive Escherichia coli present in the test sample as the arithmetical mean of the colonies counted on the two dishes, using the following equation:<br><br>$N' = \frac{\Sigma c}{VX\ n\ Xd}$ |

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| | where<br>$\Sigma c$ is the sum of the blue CFU counted on the two dishes;<br>V is the volume of the inoculum, in millilitres, applied to each dish;<br>n is the number of dishes retained (n = 2 in this case);<br>d is the dilution factor to the initial suspension or the first inoculated or retained dilution [d = 1 in the case (liquid products) where the directly inoculated test sample is retained].<br>Round off the results to two significant figures |
| **Reference** | ISO: 16649-2- Microbiology of food and animal feeding stuffs- Horizontal method for the enumeration of β –glucuronidase-positive Escherichia coli- Part 2 Colony-count technique at 44°C using 5-brom-4 chloro-3-indolyl β-D-glucuronide. |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Enumeration of *Escherichia coli* based on IS 5887-Part 1** | | |
| **Method No.** | FSSAI 15.012:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | *E. coli* is included both in the group of total *coliforms* as in that of thermo tolerant *coliforms*. Its natural habitat is the intestinal tract of hot-blooded animals, although it may also be introduced into foods via non faecal sources. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | Precautions should be taken while dealing with suspected food outbreak samples. | | |
| **Principle** | **A: Spread Plate Method:** A specified quantity of test sample (homogenised or serially diluted) is surface plated on a solid agar culture medium. After incubation period typical or suspected colonies are identified, enumerated and reported after confirmation.<br><br>**B: MPN Method:** The test sample to be tested is serially diluted and inoculated in MacConkey Broth Medium tubes in 3 replicates for each dilution. *E. coli* present in the sample, ferment lactose in the medium to produce acid and gas. The presence of acid is shown by a change in the medium's colour, whilst the presence of gas is indicated by the collection of gas bubbles in an inverted Durham tube present in the media. The pattern of positive tests (growth) in the replicates and statistical probability tables are used to determine the count (most probable number) of E.coli and reported after confirmation. | | |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven<br>4. Autoclave | | |

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| | 5. Incubator (Operating at 37 °C and 44 °C ± 1°C)<br>6. Water bath (at 44 °C to 47 °C)<br>7. pH meter with measuring accuracy ±0.1<br>8. Microscope<br>9. Refrigerator (at 2 °C– 8 °C)<br>10. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>11. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>12. Micropipette with tips<br>13. Tubes and glass bottles<br>14. Vortex<br>15. Mechanical stirrer<br>16. pH meter with measuring accuracy ±0.1<br>17. Inoculation loops and straight wire<br>18. Test Tube Racks |
| **Culture Media and Reagents** | Diluent (0.1 % Peptone and final pH adjusted 6.8 ± 0·1, or 3·4 percent of potassium diyhdrogen phosphate ($KH_2PO_4$) in water, pH adjusted to 7.2)<br>Culture medium: Tergitol -7 agar<br>Nutrient Broth<br>Nutrient Agar<br>MacConkey Broth Medium<br>• Single strength<br>• Double strength<br>Eosin Methylene Blue Lactose Agar Medium<br>TSI Medium<br>Medium for carbohydrate fermentation test<br>Simmon, Citarate Agar<br>Medium for Indole production<br>Medium for Urease Test<br>Medium for Motility Test<br>Slides<br>Gram Stain |

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| | Methyl Red and Voges-Proskauer Test. –<br>Details of preparation given in Chapter 3 |
| **Reference Cultures** | Further details of culturing given in Chapter 3 |
| **Sample Preparation** | Sample preparation based on the product categories are given in Chapter 2 |
| **Procedure** | **Test portion, initial suspension and dilution**<br>Preparation of Sample –<br>Take 25 g of the sample in a sterile blender jar and to this add 200 ml of diluting fluid (sterile 0.1 % Peptone and final pH adjusted 6.8 ± 0.1, or 3·4 percent of potassium diyhdrogen phosphate ($KH_2PO_4$) in water, pH adjusted to 7.2).<br>Homogenise the sample properly. Make serial ten-fold dilutions with the diluting fluid (0.1% peptone water), in duplicate series, up to $10^{-6}$.<br><br>**Method of Isolation**<br>1. Inoculate 1 ml of the homogenised sample into 10 ml of single strength MacConkey broth medium.<br>2. If the numbers of organisms are assumed to be very small, then inoculate 10 ml of double strength MacConkey broth medium.<br>3. Also streak loopfuls on to MacConkey agar medium and to eosin methylene blue lactose agar and if available Tergitol-7 agar.<br>4. Incubate all the inoculated media at 37°C overnight.<br>5. If there is growth with fermentation of lactose in' the MacConkey broth medium, then streak out a loopful on to each of the solid media and incubate at 37°C overnight.<br><br>**TESTS FOR IDENTIFICATION**<br>Take as many suspect colonies from the solid media as possible, but not less than 5, to investigate. The suspect colonies are smooth and are lactose fermenting on MacConkey agar and on eosin methylene blue lactose agar and are yellow colonies surrounded by yellow zones on |

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| | Tergitol-7 agar medium<br>***Escherichia coli*** - Suspect when conforming to the characters mentioned below and tested<br>1. Gram Stain: Gram negative rod shape bacteria<br>2. Motility test: Motile<br>3. Indole: Positive<br>4. $H_2S$ production in TSI medium: Negative<br>5. MR: Positive<br>6. VP: Negative<br>7. Citrate: Not Utilized<br>8. Urease: Negative<br>9. Salicin: Acid and gas production variable<br>10. Sucrose: Acid and gas production variable<br>11. Acid and gas formation in MacConkey broth: Positive with acid and gas at 44°Cfor 2 days<br><br>**Confirmation Test**<br>**1. Gram Stain**<br>Perform gram staining with 24 hr growth culture from Nutrient agar and observe the presence of gram negative rod shape bacteria<br><br>**2. Motility Test**<br>Inoculate by stabbing with a straight wire into the top of the motility test medium with the colonies to be tested, inside the glass tubing to a depth of about 5 mm. Take care that inoculation is not made on to the surface of the medium outside the glass tubing. Incubate at 37°C for 18 to 24 hours. Motile strains shall be found to show growth on the surface of the medium outside the 'inner glass tube' having travelled through the entire medium inside this inner tube. If negative on the first day, keep the inoculated tube at room temperature for a further 4 to 6 days to see if evidence of motility is present.<br><br>**3. Test for Indole Production** |

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| | Inoculate tubes of tryptone water with loopful of 24 hr growth culture of typical/suspected colonies or MPN positive tubes (from Nutrient Agar). Incubate the inoculated tubes at 37°C for 24 h. Add 0.5 m1 of the Kovac's reagent to the tubes. Mix well and shake tubes gently. The appearance of red color indicates the presence of indole. The appearance of yellow color is a negative reaction.<br><br>**4. Test for $H_2S$ Production in TSI Agar**<br>With help of inoculating wire or a needle inoculate the TSI agar (streaking slant and stabbing butt) with the culture obtained from the nutrient agar plates and incubate at 37°C for upto 7 days. The absence of blackening in the butt of TSI indicates a negative reaction.<br><br>**5.Test for Methyl Red (MR)**<br>Inoculate the MR-VP medium and incubate at 37°C for 2 days. Add 2 drops of methyl red solution prepared by dissolving 0.04 g of methyl red in 40 ml of absolute ethanol and diluting with water to make up to 100 ml. A positive reaction is indicated by red colour and a negative reaction by yellow colour.<br><br>**6. Test for Voges-Proskauer Reaction (VP)**<br>Inoculate the MR-VP medium and incubate at 37°C for 2 days. To 1 ml of the growth add 0.6 ml of alpha-naphthol solution prepared as 5 percent solution in ethanol. Shake and add 0.2 ml of 40 percent aqueous solution of potassium hydroxide. Shake and slope the tube and observe for up to 4 hours for the appearance of a pink colour which indicates a positive reaction.<br><br>**7. Test for Citrate Utilization**<br>Inoculate the strain on to SCA medium with a young nutrient agar slant culture using a straight wire. Incubate at 37°C for up to 4 days for growth of the organism. No medium color change indicates a negative reaction and medium color change to blue indicate a positive reaction. |

**8. Urease Test**

Inoculate the organisms from the 24hour incubated nutrient broth culture heavily over the entire slope of urea slant and incubate at 37°C for 18 to 24 hours. A positive urease is shown by the medium becoming, pink or red on incubation. If negative, continue incubation for at least 4 days. Proteus species gives a positive result and may be used as 'positive control'.

**9. Test for Carbohydrate fermentation**

Inoculate the Andrade Peptone water medium and the carbohydrates i.e sucrose or salicin @ 1 % and incubate at 37°C for 18 hours. Record the presence of acid from pink colour and that of gas in the Durham's Tube. Alternatively, readymade carbohydrate disc may be used.

**Enumeration of *Escherichia coli***

**Preparation of Sample** –

Take 25 to 50 g of the sample in a, sterile blender jar and to this add diluting fluid to have dilution of 10-1. Blend at 8000 to 10000 rev/min for 2 minutes. Make serial ten-fold dilutions with the diluting fluid (0.1% peptone water), in duplicate series, up to $10^{-6}$.

**A) Plate Count**

Spread out 0.1 ml from each dilutin tube, evenly n Tergitol-7 agar, and incubate at 37°C for 24 hours. Enumerae the colonies of *E. coli* which are of yellow clour surrounded by yellow zone and confirm these as being *E. coli* by the tests specified in this method. The number of viable colonies of *E. coli* per gram of sample shall be determined by multiplying by the dilution factor(s) and dividing by the mass of sample. Tergitol-7 agar plates/MacConkey agar plates or eosin methylene blue lactose agar plates may be used

B) **Determination'of the Most Probable Number of *E. coli***

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| | I. Obtain serial dilutions of the sample with a fresh sterile pipette, a measured volume of 1 ml of the homogenized mixture and of the five following serial-dilutions of both dilution series in triplicate to the tubes of 10 ml of single strength MacConkey broth containing Durham's tube for collection of gas.<br>II. Start with highest dilution and proceed to the lowest, filling and emptying the pipette three times before transferring the 1 ml portions to the tubes of medium.<br>III. When the number of *E. coli* is assumed to be very small, start by transferring 1.0 ml of the homogenized mixture in triplicate to $10^{-1}$ ml of double strength MacConkey broth medium containing Durham's tube for collection of gas, using a sterile 10 ml pipette.<br>IV. Incubate in a water-bath at 44°C for 48 hours.<br>V. Examine the tubes showing production of acid and gas, and using Table, obtain the most probable number (MPN) of *E. coli* per gram of the sample.<br>VI. Use for the calculation the results from three dilutions, selecting the highest dilution showing three positive tubes below which no sets with a smaller number of positive tubes occur, and the two following higher dilutions.<br>VII. The number obtained from Table 1 of Appendix A has to be multiplied by the lowest dilution factor, namely that of the first set of tubes, to obtain the most probable number of *E. coli* per gram of the sample.<br>For example, when dilution $10^0$ (= 10 ml of macerate), $10^{-1}$ and $10^{-2}$ are found to give the following numbers of positive tubes: 2, 2, 1, the MPN is 2.8 bacteria per gram, and when the dilutions $10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$ are found to give the following numbers of positive tubes: 3, 3, 3, 2, 0, 0, the MPN is 9.3 (3, 2, 0), multiplied by the dilution factor 102, that is, 9.3 X $10^2$ bacteria per gram. The MPN is reported as the average of the results obtained from each of the duplicate dilution series. |

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| | Refer **APPENDIX A** for MPN calculations |
| **Expression of Results** | **Plate count Method**<br>The number of viable colonies of *E. coli* per gram of sample shall be determined by multiplying by the dilution factor(s) and dividing by the mass of sample.<br>**MPN**<br>The MPN is reported as the average of the results obtained from each of the duplicate dilution series. |
| **Reference** | IS 5887 (Part 1): Methods for Detection of Bacteria Responsible for Food Poisoning, Part 1: Isolation, Identification and Enumeration of *Escherichia Coli* |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Detection, Isolation and Identification of Pathogenic *E. coli* in Food based on IS 14397** | | |
| **Method No.** | FSSAI 15.013:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | Apart from serving as indicator of faecal contamination, *E. coli* is also known to cause gastrointestinal disturbances especially in infants and children in addition to traveller's diarrhoea. Hence, presence of these organisms in food assumes greater importance. These pathogenic *E. coli* are classified into five groups, namely, Enteropathogenic *E. coli* serotypes, Enterotoxigenic *E. coli* serotypes, Enteroinvasive *E. coli* serotypes, Enterohaemorrhagic *E. coli* and Enteroaggregate (Enteroadhesive) *E. coli*. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | Precautions should be taken while dealing with suspected food outbreak samples. | | |
| **Principle** | The scheme for detection, isolation and identification of EPEC, ETEC, EIEC, EHEC and EAEC involves following steps: a) Presumptive coliform test; b) Test for identification of typical coliform bacilli (*E. coli* or faecal coli); c) Serological identification of EPEC, ETEC, EIEC; and d) Confirmation of various strains. | | |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven<br>4. Autoclave<br>5. Incubator (Operating at 44°C ± 1°C)<br>6. Water bath (at 44 °C to 47 °C)<br>7. pH meter with measuring accuracy ±0.1<br>8. Microscope | | |

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| | 9. Refrigerator (at 2 °C– 8 °C)<br>10. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>11. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>12. Micropipette with tips<br>13. Tubes and glass bottles<br>14. Vortex<br>15. Mechanical stirrer<br>16. pH meter with measuring accuracy ±0.1<br>17. Spreader (glass or plastic)<br>18. Inoculation loops and straight wire<br>19. Spiral plater/rotator<br>20. Inoculation loops and straight wire |
| **Culture Media and Reagents** | Diluent<br>Culture medium: Tryptone-bile-glucuronic medium (TBX)<br>Nutrient Broth<br>Nutrient Agar<br>MacConkey Broth Medium<br>• Single strength<br>• Double strength<br>Tryptone Water<br>Kovac's Reagent<br>Brilliant Green Lactose Bile Broth<br>Details of preparation given in Chapter 3 |
| **Reference Cultures** | Further details of culturing given in Chapter 3 |
| **Sample Preparation** | Sample preparation based on the product categories are given in Chapter 2 |
| **Procedure** | **ISOLATION**<br>**Presumptive Coliform Test**<br>Prepare serial dilutions of the sample; The choice of dilutions depends upon the type of samples to be tested. Transfer 1 ml of the sample and its dilutions (1:10 and 1:100) into Mac Conkey's broth tube in triplicate. |

***Production of Acid and Gas***

Incubate the tubes for 24 h at 37 °C and observe for the production of acid and gas. The production of acid is indicated by change of color of the medium. Production of gas is observed in the Durham's tubes which may be partially or completely filled with gas. If no change is observed incubate for another period of 24 h and record the observation.

**TEST FOR IDENTIFICATION OF TYPICAL COLIFORM BACTERIA (FAECAL OR E. COLI)**

**Elikman's Test**

Inoculate BGLB broth tubes with a loopful of the culture from a positive presumptive coliform tube in triplicate. Incubate the tubes for 24-48 h at 44°C and observe for the production of acid and gas. Only faecal *E. coli* strains are capable of producing acid and gas in BG LB broth.

**Test for Indole Production**

Incubate 3 tubes of tryptone water eachwith loopful of culture from positive presumptive coliform test tube.

Incubate the inoculated tubes at 44°C for 24 h. Add 0.5 m1 of the Kovac's reagent to the tubes. Mix well and examine after 1 min. The appearance of red color indicates the presence of indole. The appearance of yellow color is a negative reaction.

**SEROLOGICAL IDENTIFICATION OF *E. COLI* STRAINS**

Serological identification of *E. coli* strains depends upon the detection of O, H and K antigens, though most often only O antigen is detected.

**Determination of O antigen of Test Strains**

This needs inactivation of K antigen (which cause O inagglutinability).

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| | Suspend the growth from an agar slant culture in saline to give fairly light suspension (about 7.5 x $10^8$ organisms/ml). Heat the suspension at 121°C for 2 h 30 min to inactivate K antigens (where K antigens are of B type, heating at 100°C for 1 h will be sufficient). Transfer loopfuls of the antigen suspension on to a clean glass slide. Add loopful of different pool of O, K sera (O sera if available) to the drops of antigen on the slide. Mix and rock gently. Observe for agglutination which should be strong and clearly visible within one min. Weak and late agglutination reactions should not be taken into account. Always a saline control should be used in carrying out slide agglutination reactions.<br><br>If the agglutination is seen with any one of the pools of O-K antisera (or O antisera), then the same antigen should be checked with all the individual factor sera that constitute the pool to arrive at the 'O' antigen of the strain in question.<br>The results of slide agglutination should be confirmed by tube agglutination.<br><br>**Tube Agglutination**<br>Make serial dilutions of the antiserum (identified as above) in 0.5 ml volumes in saline from 1:10 to 1:640 (round bottomed glass tubes of approximately 9 mm x 85 mm size are suitable). To each, add 0.5 ml of the antigen suspension. This doubles the dilution of the serum. A control tube should be set up containing only the antigen and saline (0.5 ml each). Shake the tubes and incubate in water bath (50 °C) overnight. Examine for agglutination. Ag- agglutination titres 1 in 20 are insignificant. Titres at or near the stated titre of the serum are significant.<br><br>**CONFIRMATION OF ENTEROPATHOGENICITY OF EPEC STRAINS**<br>As the mode of pathogenicity of EPEC strain is not clearly understood, |

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| | no standard methods are available for determination of enteropathogenicity except serotyping.<br><br>**CONFIRMATION OF ENTEROTOXIGENCITY OF ETEC STRAINS**<br>ETEC are known to produce two types of toxins; (i) heat labile toxin (LT), and (ii) heat stable toxin (ST). Heat labile toxin (LT) is detected by both in-vitro methods (Biken test and tissue culture methods) and in-vivo method (Rabbit ileal loop assay method).<br>**Biken Test**<br>This test holds promise of being the simplest and most practical for laboratories with limited facilities while at the same time being reliable**.**<br><br>***Principle***<br>Thespecific antitoxin reacts with toxin liberated by an actively growing organism on a special medium and produces a line of precipitation at the sites where they meet in optimal proportion.<br>**Apparatus**<br>The Biken test kit:<br>Media and Reagents:<br>Biken agar No. 2<br>Composition (for 100 ml):<br>Casamino acid: 2.0g<br>Yeast extract: 1.0 g<br>NaCl: 0.25 g<br>$K_2HP0_4$: 1.5 g<br>Glucose: 0.5 g<br>Trace Salts Solution (5% $MgSO_4$, 2% $CoC_{16}.6H_20$ and 0.05 ml 0.5% $FeCl_3$)<br>Noble agar or agarose: pH to 7.5 with 1 M NaOH. Sterilize by autoclaving at 121°C for 15 ml and distribute 15 ml in test tubes for each plate. |

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| | *Lincomycin:* 2.7 mg/ml (it increases toxin production).<br>Polymixin B disc: 6 mm discs containing about SOC IU (it helps toxin release).<br>*Anti-LT serum:* The optimal dilution of the antitoxin has to be predetermined by testing two-fold dilutions (for example 1:2 to 1:32) of the antitoxin against the toxin antigen by the gel-diffusion technique. This is carried out in a Petri-dish using a media containing 1.5 g of Noble agar in 100ml of phosphate buffer solution (0.01 M) with 0.25 percent sodium azide. Punch one central well for the toxin antigen and other well around it at an equal distance for different dilutions of antitoxin. The optimal dilution lies between the two highest dilutions showing lines of precipitation after overnight incubation, for example, if the highest dilutions showing precipitation are 1:16 and 1:8, the optimal dilution for working purposes is 1:12.<br>Pure LT for determining the optimal working dilution of antitoxin.<br>Gel puncher: 4 mm diameter.<br>Template for inoculation of strains.<br><br>***Procedure***<br>Transfer 0.5 ml of lincomycin solution to a sterile Petri-dish 90 mm in diameter.<br>Dissolve Biken agar No. 2 on a boiling water bath. Cool the medium to about 50 to 60°C, and pour into the Petri-dish containing lincomycin solution. Mix well by rotating the plate at least 10 times. Prepared plates can be stored at 4°C for 3-5 weeks if kept in a plastic bag to prevent drying. Before using freshly prepared media, dry the agar surface.<br><br>***Inoculation of test cultures***<br>Spot inoculate the test strains to ensure a fairly large area of confluent growth around the site where the central well will be punched. Allow for a distance of about 4 mm between the inner edge of the growth and the margin of the site of the central well and take care that the strains |

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| | do not touch each other when they grow during the incubation period. Four test cultures, or three test and one positive control culture, may be inoculated around each central well**.**<br>After 48 h of incubation, put a polymyxin B disc on top of the growth of each strain. Incubate for 6 h.<br>Punch a well (4 mm in diameter) in the centre of the area so that the distance between the well and the edges of the growth is about 4 mm. Put 20 µl of the optimal dilution of the antiserum against LT into the central well. Incubate again for 20-24 h.<br><br>Examine for lines of precipitation in the zone between the growth and the central well. The lines may not always be very distinct at this time, but are seen better after further incubation for 15-20 h, or when the plates are placed on a light box with black background. The precipitation bands developed can be stained with commassie blue (0.1 percent) to make them more prominent.<br><br>**Rabbit ligated Ileal Loop Test**<br>***Reagents and Apparatus***<br>In addition to the usual microbiological apparatus, the following are required**.**<br>*Animal operating board*<br>*Clippers*<br>*Hypodermic syringe with 25-gauge needle*<br>*Stainless steel wound clips, 0.9 cm.*<br>*Surgical thread*<br>*Sodium pentabarbitone, 3 percent.*<br>*Ether*<br>*Normal saline*<br>*Han's F-JO Nutrient mixture*<br><br>**Procedure**<br>Select three albino rabbits weighing about 2 kg each. Supply them with |

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| | water but no food for 24-48 h before surgery. Restrain rabbits on their back using a small animal operating board. Remove abdominal fur with clippers. Using the clean technique and employing general anaesthesia (1 ml of 3 percent sodium pentabarbitone/kg body weight followed by ether inhalation), make a midline incision, identify and gently lift out the small bowel from the abdominal cavity, taking care to keep it moist with sterile saline. Tie a single cotton suture around the proximal end of the small bowel 5 cm below the stomach.<br>Inject 10 ml of sterile saline into the upper small bowel using a 25-gauge needle. Gently manipulate the saline between two fingers down the bowel and into the caecum. Place another tie above the ileo-caecal junction.<br><br>Tie off a series of 5 cm segments, starting proximally, with 2 cm segments separating each 5 cm segment. As many as 10-15 of the 5 cm segments can be isolated in a 2 kg rabbit. Do not injure blood vessels or interrupt the blood supply to the intestine. Inject positive control filtrates, saline controls, and test supernates or filtrates, 1 ml per 5 cm segments, using a sterile tuberculin syringe. and 25-gauge needle [or each injection. Whole live cultures can be used as a screening test for enterotoxin; however, positive results must be confirmed using filtrates. Inject the culture filtrates in triplicate into three rabbits. Randomize the injection pattern so that test filtrates and controls are present in proximal middle, and distal regions of the small bowel. Injection should be made into the anti-mesenteric border of the intestine near the separating ligature so that the site can be tied off with another ligature. Close the peritoneum with suture and close the skin with sutures or 0.9 cm stainless steel wound clips.<br><br>After 18 h, sacrifice each rabbit with intravenous phenobarbital or by 5 ml of air rapidly injected by the same route. Open the abdomen and excise the small bowel with ligated segments intact. Positive loops appear angry and distended with clear to brownish, or rarely, |

haemorrhagic fluid, and the negative loops remain collapsed. Measure the volume of fluid in each positive segment by aspirating into a syringe and dispensing into a graduated cylinder. Measure the length of the empty segment in cm.

***Interpretation of the results***

Determine the ratio of fluid accumulation (ml) to the length (cm) of the loop. An average value for the three rabbits is calculated. A value of 1.0 or greater is considered positive in the 18 h test. Exclude results in a rabbit if the saline-control segment contains; measurable fluid with a ml/cm ratio of LO at 18 h.

**Y-1 Adrenal Cell Tissue Culture Assay**

The Y-l adrenal cells are maintained in monolayer culture in Ham's F-I0 nutrient mixture (HAM). Prepare the medium from a dry powder or from a 10X concentrate. In either case, each litre of medium is supplemented with 150 ml horse serum, 25 ml foetal calf serum, 40 mg of gentamicin, and 100 000 units of penicillin. Adjust the pH with $NaHCO_2$ to 7.2. Sterilize by pressure filtration through a 0.22 µ membrane filter. Aliquot the medium using sterile technique and freeze at – 20 °C until use. Once thawad it may be stored at 4°C for up to 1 week, if kept sterile bacterial quality control should be done by adding 0.2 ml of medium to Mueller-Hinton broth and incubating for two weeks. Observe for turbidity indicating contamination.

Routinely, tissue culture flasks with a 75 $cm^2$ growth area are filled with 25 ml of medium and incubated at 37°C in a 5 percent $CO_2$ humidified atmosphere. All cell manipulations are done in a laminar flow hood, and cells are examined using a sub stage phase microscope.

***Weekly Procedures for Tissue Culture Assay and Maintenance***

*Day 1*

Aseptically suction the F-10 medium from the confluent monolayer (one week of growth in flask). Wash the cell monolayer with 5 ml

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| | sterile phosphate-buffered saline (PBS) and remove with suction.<br>Add 1.5 ml of 0.2 percent trypsin to the flask and leave the trypsin covered monolayer at room temperature until cells begin to loosen from the plastic surface (5-10 min).<br>Add 5 ml F-10 medium to the flask to neutralize the trypsin (The Ham's F-10 is stored at - 20°C and should be thawed and brought to 37°C in a water bath prior to all medium changes and cell sub culturing). If any monolayer remains, scrape it from the flask surface with a sterile rubber scrubber or suitably aspirate and flush with medium.<br>Transfer the suspended cells (approximately 6.5 ml) to a sterile control centrifuge tube and centrifuge at 500 to 1,000 g for 5 min.<br>Suction off the supernatant leaving a sediment of Y-1 adrenal cells in the centrifuge tube. Re-suspend the cells in 5 ml fresh F-10 medium with a Pasteur pipette. Using a Pasteur pipette dispensed six drops of the cell suspension into each flask to which has been added 25 ml fresh F-10 medium. As a general rule 2 flasks are carried.<br>For the toxin assay, which is run in flat-bottomed 96 well micro titre plates, make a 1:10 to 1:100 dilution of the cell suspension. The suspension is dispensed, approximately 0.15 ml per well. Therefore, one flask usually can fill 12 to 25 plates. Stack the' plates and cover the top plate to prevent contamination and evaporation. Place flask (with loose caps) and/or micro titre plates in the $CO_2$ incubator.<br><br>*Day 2*<br>Check flasks and/or wells under the microscope for evidence of cell growth**.**<br><br>*Day 3*<br>Change F-10 medium in the stock flasks by suctioning off the old medium and replacing it with 25 ml fresh F-10. Do not change the medium in the assay plates. |

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| | *In the Late Afternoon of Third Day* - Using a Pasteur pipette, inoculate each well of the micro titre plates containing a monolayer ofY-1 adrenal cells with 2 drops of the toxin supernatants or whole live broth cultures (including controls). Be sure the micro titre plates and appropriate record sheets are coded before the actual transfer. After 15 to 30 min, suction the medium from each well of the plate, being careful to touch only the corner of the well so as not to damage the monolayer. Wash once with medium and remove with suction. Finally, add 0.15 ml of medium to each well. During the washing procedure wash only half of the plate at one time to prevent drying of the monolayer. Restack micro titre plates and incubate in $CO_2$ at 37°C overnight.<br><br>*Day 4 (morning) read assay*<br>L T causes rounding of Y -1 adrenal cells.<br><br>**Chinese Hamster Ovary (CHO) Cell Assay**<br><br>***Preparation of Medium***<br>The CHO cells are grown in Eagle's minimum essential medium (MEM). To prepare the medium, follow manufacturer's instructions for dissolving powdered MEM. Add 100 µg/ml streptomycin and 100 units/ml penicillin G per litre. Mix well. Filter through a 0.22µ Millipore membrane filter. Aseptically, aliquot two 90 ml amounts into 280 ml screw cap flasks. Add 10 ml of sterile calf serum (CS) to 1 flask and 10 ml of CS and 1 ml of sterile foetal 1 calf serum (FCS) to the other. The two flasks containing 10 percent CS and 10 percent CS plus 1 percent FCS and the remaining MEM with antibiotic can be stored at 4°C for not more than 3 weeks.<br><br>***Maintenance of Tissue Culture***<br>Obtain Chinese hamster ovary cell culture from a laboratory that is routinely conducting this assay. Cells must be frozen in dry ice during |

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| | shipment. Using Pasteur pipette, add a drop of cell suspensions to 4.5 ml of the MEM medium with 10 percent calf serum (CS) and antibiotics in a 25 cm'" tissue culture flask. Two or more flasks are routinely carried. Maintenance of the tissue culture requires weekly passage to fresh medium. To determine the time for passage, observe cells for confluence in the monolayer using a sub stage phase microscope.<br>***Passage of cells***<br>Cells are passaged at least once a week and 2 flasks are routinely maintained. Fresh MEM with 10 percent CS and antibiotics should be added the day before passage. Simply decant the old medium and add 4.5 ml of the fresh medium.<br>Decant MEM with 10 percent CS and antibiotics from confluent cells. Add 2 ml of 0.25 percent trypsin, wash over cells for about 5 min and decant. Incubate at 37°C in 6% $CO_2$ for 20 min with the monolayer side of the flask up. Add 2 ml MEM with 10 percent S and antibiotics to stop trypsin digestion and agitate to suspend all cells and break up any clumping. Take the cell suspension into a sterile centrifuge tube and spin down at 800 rpm for 5 min at room temperature<br>Add desired quantity of cell suspension to a new tissue culture flask with fresh MEM with 10 percent CS and antibiotics. The amount added may be adjusted to achieve new monolayer cell confluency at a specified time point Usually one drop of the cell suspension from a Pasteur pipette into 4.5 ml of medium should reach confluency in 3 days. Incubate at 37°C in 6% $CO_2$.<br>***Assay for Enterotoxin***<br>Two to four old cultures are preferred for LT assay. Fresh MEM with 10 percent CS plus 1 percent FCS and antibiotics should be added the day before the assay. Simply decant the old medium and add 4.5 ml of the fresh medium. Decant MEM with 10 percent CS 1 percent FCS and antibiotics from confluent cells. Add 2 ml of 0.25 percent trypsin, wash over cells, and decant quickly. Incubate at 37°C in 6 percent $CO_2$ for 20 min with the monolayer side of the flask up. |

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| | Add 2 ml MEM with 10 percent CS plus 1 percent FCS and antibiotics to stop trypsin digestion and agitate to suspend all cells and break up any clumping. Dilute the cell suspension further in MEM with 10 percent CS plus 1 percent FCS and antibiotics to a concentration of approximately $2x10^4$ cells/ml Dispense 0.20 ml of cells into each Lab. Tek chamber (eight chamber slide), Add proximately 0.02 ml toxin or *E. Coli* culture supernatants to the Lab Tek Chamber. Each test should include filtrates from both positive and negative control cultures, incubate in 6 percent $CO_2$ at 37°C.<br>Read assay at 20-24 h and not later than 24 h, after pouring off all fluid and fixing the cells in 10 percent buffered formalin or 100 percent methanol for 5 min; some prefer to stain with giemsa (1:40) for 20 min. Lab Tek chamber slides are dissembled and treated as slides that is stained in Coplin jars.<br><br>**Result**<br>The number of elongated cells in each 100 cells may be counted and recorded, or a '+ 'or '-' interpretation may be made when the reaction is not questionable. Elongation is defined as bipolar and three times longer than wide. Greater than 10 percent elongated cells is considered positive.<br>Infant Mouse Test for Detection of Heat distinguished and identified biochemically from Stable (ST) Enterotoxin<br>Select 2-3 days old infant suckling mice. Inject 0.1 ml of the test inoculum mixed with 50 µl of 0.5 percent Evans blue dye intragastrally into each mouse separately, the control being injected by the same amount of peptone water. Sacrifice the animals after 4-5 h and examine the gastrointestinal tract. Record the distension of G.I. Tract if any. Determine fluid accumulation ratio by dividing weight of the G.I. Tract with the total body weight of the mouse. A ratio of 0.09 or more is considered as a positive reaction. The *E. coli* strain producing this value is considered enterotoxigenic. |

**CONFIRMATION OF EIEC STRAINS (SERENY TEST)**

A few strains of *E. coli* cause dysenteric symptoms like shigellosis frequently with blood and mucus in the stool. These *E. coli* strains are usually non-motile, lysine negative, slow in fermenting lactose and produce little or no gas. Invasive *E. coli* found so far belongs to a limited number of serotypes, but as the antisera are not generally - available, such strains are identified by the Sereny test, which is very reliable.

**Procedure**

Inoculate the test strain into a heart infusion agar (HIA) plate and streak without flaming the loop between quadrants on the plate so that maximum growth will be obtained. Incubate the plate at 37°C for 18-24 h. Remove the growth with a cotton swab and suspend it in 1 ml of physiological saline. Inoculate a drop (50µl) of this suspension into a guinea pig's eye, using a sterile Pasteur pipette. Be sure not to traumatize the eye. Inoculate only one eye. The other eye may serve as negative control. Known positive shigella strains may be used in a separate guinea pig as a positive control. Observe the guinea pig's eye daily for 72 h for development of keratoconjuctivitis. Guinea pigs with redness and swelling of the eye are considered positive. If the test is positive, report: invasive *E. coli*.

NOTE - Positive guinea pigs cannot be reused. They are infectious and their carcasses should be disinfected by autoclaving or by other means before they are discarded. Guinea pigs may be reused, however, if they were negative in previous tests, using the eye not used previously

**ISOLATION OF ENTEROHAEMORRHAGIC *E. COLI* (EHEC)**

Apart from the ordinary characteristic of *E. coli*, Enterohaemorrhagic *E. coli* O157:H7 can be distinguished and identified biochemically from *E. coli* as it is 100% sorbitol non-fermenter and 100% ducitol and raffinose fermenter. These characters are used as screening tests for

identifying the organism.

Non fermentation of sorbitol is observed on Sorbitol Mac Conkey Agar Medium (SMAC) and on Sorbitol Liquid Broth.

**Preparation of Sorbitol MacConkey Agar Medium (SMAC)**

The SMAC media is prepared with MacConkey agar base 40.0 g (without lactose) and D-sorbitol 10.0 g.

MacConkey Agar Base Contains

Peptone - 2.0 percent

Sodium taurocholate - 0.5 percent

Agar - 3.0 percent

Distilled Water - 100 ml.

**Procedure**

At first peptone and taurocholate is dissolved in the water by shaking and heating. Agar is added in it and is dissolved in the steamer or autoclaved. If necessary, clear by filtration, pH is adjusted to 75. Then 40.0 g of MacConkey agar base, 1 percent D-sorbitol and neutral red is added and mixed properly in 1,000 ml of distilled water. Heat in the autoclave with free steam (100 °C) for 1 h then at 115°C for 15 min. Pour in the plate and preserve the plate in refrigerator till use.

Sorbitol MacConkey Agar Medium is used, as it is a differential and selective medium, for Enterohaemorrhagic *E. coli* O157:H7. Here instead of lactose, sorbitol is used because EHEC O157 H7 do not ferment sorbitol that is EHEC O157:H7 is non-sorbitol fermenter (NSF). In contrast, other *E. coli* ferment sorbitol. Sorbitol Mac Conkey agar plate is incubated at 37 °C for overnight after the primary inoculation. Non-sorbitol fermenting *E. coli* colonies are picked up and subcultured to obtain the pure NSF growth.

**Characterisation of Enterohaemorrhagic *E. coli* (EHEC)**

***Colony Characteristics***

On primary isolation, colonies are translucent, large, thick, moist, smooth. The colonies are similar in appearance to non- lactose

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| | fermenting colony on Mac Conkey agar that is colourless and contrasted well with bright pink colonies of sorbitol positive organism of the faecal flora. EHEC colonies therefore are easily recognisable on SMAC medium culture where as they are indistinguishable from faecal flora in Mac Conkey agar culture medium.<br><br>***Preparation of Sorbitol Liquid Media***<br>At first base media is prepared by adding the following ingredients:<br>Peptone – 1%<br>Sodium chloride - 0.5%<br>Distilled water - 100 ml<br>Above ingredients are mixed and autoclaved at 121°C, for 15 min. Add 1 percent Andrade's indicator and adjust the pH to 7.6. Then 1% D-sorbitol is added in the base media which is again steamed in the autoclave under free steam for one hour after dispensing in small tubes with Durham's tube.<br>***Procedure of the Sorbitol Fermentation -Test***<br>Inoculate the media with the organism and incubate at 37°C overnight to observe the fermentation. EHEC is sorbitol non-fermenter.<br>***Preparation of Dulcitol and Raffinose Liquid Media***<br>Here base media is prepared as in the case of sorbitol liquid media. After adjusting the pH and addition of Andrade's indicator add 1 percent raffinose and 1% Dulcitol separately to the base media. The media is again sterilised by free steaming after dispensing in small tubes with Durham's tube.<br>***Procedure of the Dulcitol and Raffinose Fermentation Test***<br>Inoculate both the media with the organism and incubate at 37°C overnight and observe for fermentation. EHEC *E. coli* O157:H7 is 100 percent positive to Dulcitol and Raffinose fermentation test.<br>***Slide Agglutination Test for Confirmation of EHEC***<br>Now for confirmation of the organism as Enterohaemorrhagic *E. Coli* the slide agglutination test may be performed by O157 and H7 antiserum, respectively. |

***Procedure of the Slide Agglutination Test***

*E. coli* colonies are emulsified in saline on a grease free, dry microscopic slide. This is kept at the left side of the slide as control. Another emulsion is prepared in the same way and is kept on the right side of the slide. One loopful of the O157 antisera, is added to the emulsion to be tested, end is observed for agglutination.

Procedure of the slide agglutination test with H7 antiserum is same as previous one.

**IDENTIFICATION OF ENTEROADHERENT *E. COLI* (EAEC)**

Some EPEC tend to adhere to intestinal mucosa in vivo. Majority of EPEC strains isolated from out breaks showed mannose resistant adhesion to HEp2, whereas normal flora of *E. Coli* rarely adhered.

Two distinct pattern of EPEC adherence is noted

a) Localised adhesion (LA), and

b) Diffuse adhesion (DA).

LA pattern is characterised by organisms attaching to one or two small areas of the cell surface in micro colonies, whereas DA pattern attaches in hybridization with the probe correlates well with scattered pattern to the whole of cell surface.

Cell Lines used - HEp-2

Hela

It is thought that identical results are obtained in adhesion assays. However, some workers report that some isolates adhere to Hela and not to Hep-2 cells.

The adhesion property is thought to be plasmid mediated (size 50-70 M Da) and known as EPEC adhesive factor (EAF).

A DNA probe composed of 1 K Da portion of EAF plasmid has been developed by isolating plasmid fragment of adhesion. This probe has been used to detect mid fragment of adhesion. This probe has been used to detect EPEC in epidemiological studies and hybridization with the probe correlates well with production of LA.

The ability of EPEC to adhere to HEp-2 cells, in localised manner and to hybridize with EAF was most commonly found amongst EPEC

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| | serogroups 055, 0111, 0119, 0127, 0128 and 0142 (called Class-I EPEC). These strains are most commonly associated with outbreaks of infantile diarrhoea.<br>Strains showing DA and no adhesion to HEp-2 cells belong to serogroups less commonly incriminated in out breaks of diarrhoea and belong to EPEC SWEROGROUPS $O_{44}$, $O_{86,}$ $O_{144}$ (CLASS II EPEC). |
| **Expression of Results** | Pathogenic *E.coli* Present or absent |
| **Reference** | IS 14397: Detection, Isolation and Identification of Pathogenic *E. Coli* In Food |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Detection and confirmation of *Listeria monocytogenes* in Food based on IS: 14988-Part 1** | | |
| **Method No.** | FSSAI 15.014:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | *Listeria monocytogenes* is an intracellular, foodborne pathogen potentially lethal for humans and animals. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | While testing *L. monocytogene*s it is recommended that a properly equipped laboratory under supervision of skilled Microbiologist is done. The material used during testing is carefully disposed off after sterilization. Pregnant personnel may be asked to avoid handling of *L. monocytogenes* cultures and undertaking the tests. | | |
| **Principle** | *Listeria monocytogenes* may be present in small numbers and are often accompanied by considerably larger numbers of other microorganisms, therefore selective enrichment is necessary. It is also necessary to detect injured and stressed *Listeria monocytogenes* and the primary selective enrichment medium, with reduced inhibitor concentration, fulfils at least part of this function.<br><br>The detection of *Listeria monocytogenes* requires 4 successive stages viz. primary enrichment, secondary enrichment, identification and confirmation.<br><br>**Primary enrichment in a selective liquid enrichment medium with reduced concentration of selective agents (half-Fraser broth):**<br><br>Inoculation of a selective primary enrichment medium containing half the concentrations of acriflavine and nalidixic acid (half-Fraser broth and incubation of the initial suspension at 30 °C for 24 h to 26 h.<br><br>**Secondary enrichment with a selective liquid enrichment medium** | | |

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| | **with full concentration of selective agents (Fraser broth):**<br><br>Inoculation of full-strength secondary liquid enrichment medium (Fraser broth) with a culture obtained from 3.1. Incubation of the Fraser broth at 37 °C for 24 h.<br><br>**Plating out and identification:**<br><br>From the cultures obtained from Primery and secondary enrichment, plating out on the two selective solid media:<br>• Oxford Agar<br>• PALCAM Agar<br><br>Incubation of the paltes at 30°C, 35°C or at 37 °C for a total of 48 h for presence of characteristic colonies.<br><br>**Confirmation**<br><br>Subculturing of the colonies of presumptive *L. monocytogenes* plated out, and confirmation by means of appropriate morphological and biochemical tests. |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven<br>4. Autoclave<br>5. Incubator (Operating at 25°C ± 1°C, 30 °C ± 1°C and 35°C to 37°C ± 1°C)<br>6. Anerobic jar or anerobic workstation<br>7. Gas Mixure for microaerobic incubation (5% -12 % $CO_2$, 5% -15 % $O_2$ and 75 % $N_2$<br>8. Water bath (at 47 °C ± 2°C)<br>9. pH meter with measuring accuracy ±0.1<br>10. Microscope<br>11. Refrigerator (at 2 °C– 8 °C)<br>12. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>13. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A) |

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| | 14. Micropipette with tips<br>15. Tubes and glass bottles<br>16. Vortex<br>17. Mechanical stirrer<br>18. pH meter with measuring accuracy ±0.1<br>19. Spreader (glass or plastic)<br>20. Inoculation loops and straight wire<br>21. Spiral plater/rotator<br>22. Inoculation loops and straight wire<br>23. Colony counter<br>24. Equipment for Henry Illumination Test |
| Culture media and reagents | i) Phosphate buffered peptone water<br>ii) Listeria enrichment broth<br>iii) Half Frazer broth<br>iv) Frazer broth<br>v) Modified Oxford Agar<br>vi) PALCAM Agar<br>vii) Tryptone Soya Yeast Extract Agar<br>viii) Tryptone Soya Yeast Extract Broth<br>ix) Sheep Blood Agar<br>x) Carbohydrate utilization broth (Rhamnose and Xylose)<br>xi) Motility Agar<br>xii) CAMP Medium and test organisms<br>xiii) Hydrogen peroxide solution |

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| **Procedure** | **Preparation of sample and primary enrichment**<br><br>Aseptically open the sample container and weigh x gm sample into a sterile mixture bag/ empty wide mouth container with screw cap or suitable closure or take x ml of liquid sample. Add x ml of sterile half-Fraser broth to obtain a ration of test portion to selective primary medium of 1/10 (mass to volume or volume to volume). Make a uniform suspension by blending if necessary and incubate at 30 °C for 24 ±2 h.<br><br>**Secondary enrichment:**<br>Inoculate 0.1 ml of the culture from Half Fraser Broth onto 10 ml tubes of Fraser Broth. Incubate the tubes at 35 °C or 37 °C for 48 h<br><br>**Selective differential plating:**<br>From the culture obtained in the primary enrichment (Half Fraser Broth after 24 ± 2 h at 30 °C) inoculate (streaking) the surface of the selective isolation medium, Oxford agar and PALCAMplates.<br><br>From the culture obtained in the secondary enrichment (Fraser Broth after 48 h ± 2 h at 35°C or37 °C) inoculate (streaking) the surface of the selective PALCAM and Oxford agar plates.<br>Incubate the plates at 30 °C, 35 °C or 37 °C and observe for typical colonies after 24 h. PALCAM agar plates are incubated microaerobically.<br>Incubation of Oxford agar at 30 °C is suitable for food stuffs only lightly contaminated by a supplementary flora for products heavily contaminated by supplementary flora, incubate Oxford agar plates at 37 °C. If no suspect colonies are evident or if the growth is poor after 24 h, re-incubate the plates for an additional 24 h and read again. |
| **Appearance of colonies** | After the incubation period, examine the plates for typical *Listeria monocytogenes* colonies.<br>On PALCAM agar and Oxford agar - gray to black colonies surrounded by a black halo. |

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| **Confirmation of Listeria species** | Select five typical colonies from one plate of each medium. If presumed colonies are less than five on a plate, take all of them.<br><br>Purify each culture by streaking the selected colonies from each plate on to the surface of a well dried Tryptic Soya Yeast Extract Agar (TSYEA) for obtaining well separated colonies. Invert the plates and incubate at 35 °C or 37 °C for 18 to 24 hr or until the growth is satisfactory.<br><br>Typical colonies are 1 mm to 2 mm in diameter, convex, colorless and opaque with an entire edge. Carry out the following tests from colonies of a pure culture on the TSA YE.<br><br>**Catalase reaction:**<br>With the help of loop pick up an isolated colony and place it in $H_2O_2$ solution on a glass slide. Immediate production of gas bubbles indicates catalase positive reaction.<br><br>**Gram staining:**<br>Perform Gram staining on a colony, Listeria are Gram positive slim short rods.<br><br>**Motility Test:**<br>Take colony from TSYEA plate and suspend it TSYE broth. Incubate at 25°C for 8 to 24 hr until cloudy medium is observed. Take a drop o f culture and place it on a glass slide. Cover the top with a cover slip and observe under a microscope. *Listeria* is seen as slim rods with a tumbling motility (cultures grown above 25°C fail to show this motion. Compare them with a known culture – *cocci* or large rods with rapid motility are not *Listeria.*<br><br>As an alternative stab motility agar tube with an isolated colony from TSYEA and incubate at 25 °C for 48 h Typical umbrella like appearance around the stab indicate motility positive culture. If growth is not |

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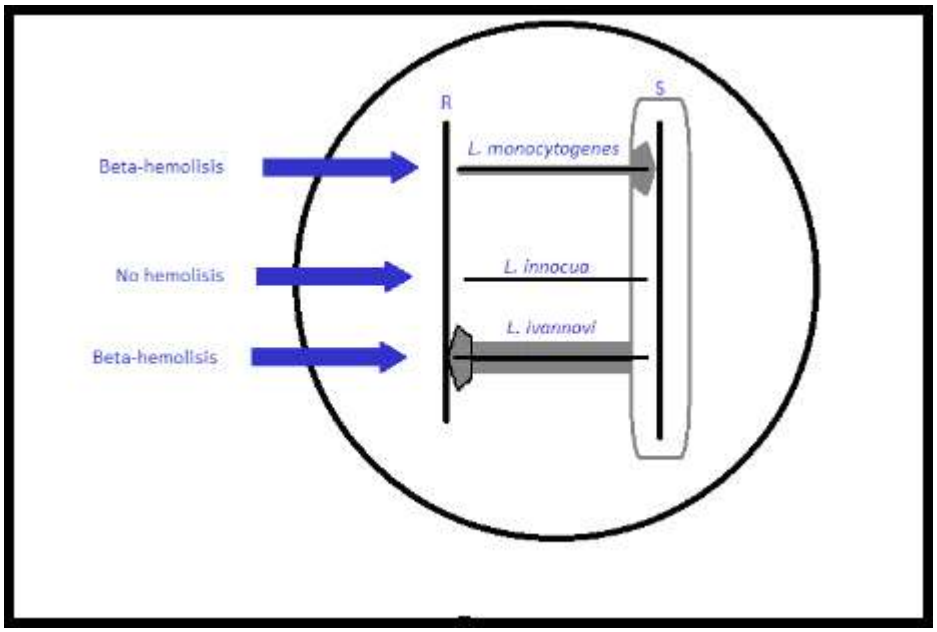
| | positive incubate up to five days and observe for the stab again. |
| **Confirmation of *Listeria monocytogenes*** | **Heamolysis test:**<br>Take a colony from TSA YE and stab it on a well dried surface of sheep blood agar plate. Simultaneously stab positive (*L. monocytogenes*) and negative (*L. innocua*) control cultures. Invert the plates and incubate at 35 °C or 37 °C for 24 ± 2 h. Examine the plates.<br>*L. monocytogenes* show clear light zones of beta haemolysis. *L. innocua* does not show any haemolysis. Examine the plates in a bright light to compare test cultures with the controls.<br><br>**Carbohydrate utilization:**<br>Inoculate each of the carbohydrate utilization broths (rhamnose and xylose) with a culture from TSYE broth and incubate at 35°C or 37°C for upto 5 days. Appearance of yellow color indicates a positive reaction within24 to 48 hr.<br><br>**CAMP test**<br>On a well dried surface of sheep blood agar or CAMP Medium streak each of the *Staphylococcus aureus* and *Rhodococcus equi* cultures in single lines and parallel to each other and diametrically opposite, a thin even innoculum is required.<br>Streak the test strain separated in a similar manner at right angles to these cultures as that the test strain and *S. aureus* and *R.equi* cultures do not touch but their closest are about 1 mm or 2 mm apart. Several test strains can be streaked on the same plate. Simultaneously streak control cultures of *L. monocytogene*s, *L. innocua* and *L. ivanovii*. Incubate blood agar plates at 35 to 37°C for 18 to 24 hr and CMP medium for 35 or 37°C for 16 to 18 hr.<br>Observe plates against bright light. In *L. monocytogenes* case there is enhanced zone of beta haemolysis at the intersectiono of *S. aureus*.<br>*L. innocua* does not show any enhanced zone of haemolysis with *S. aureus* or *R. equi*.<br>In case of *L. ivanovii* enhamced beta zone of haemolysis is seen on *R.* |

*equi* side.




Inoculate thin agar plates as in diaram. Vertical lines represent streaks of S. aureus (S) and R. equi (R). Horizontal lines represent sraks of test cultures. Hatched area indicates the location of enhanced haemolysis.
The dotted area indicates the zone of influence of the S. aureus culture.
**Figure 1 Inoculation and Interpretation of CMP test plates**

**Henry oblique transmitted illumination (Optional)**

Examine TSAYE plates using a source of beamed white light, striking the bottom of the palte at a 45° angle. When examined under this transmitted illumination from directly above the plates *Listeria* colonies are bluish in color with granular surface.

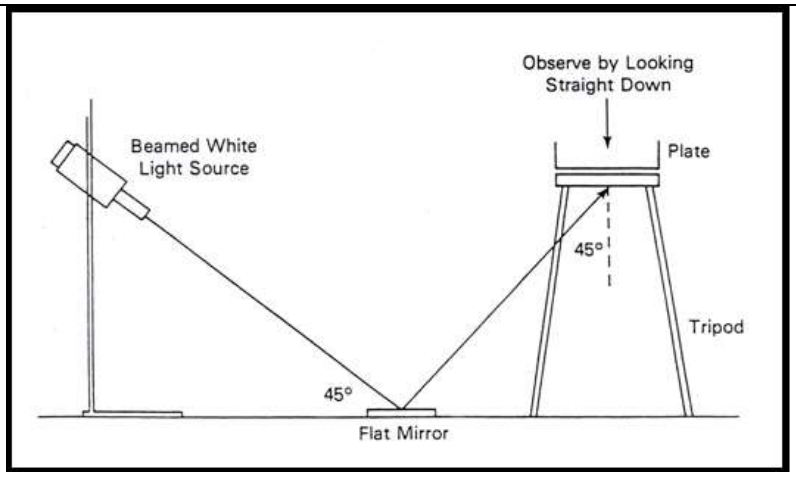



**Figure 2 Examination of plates for suspect colonies (Henry illumination test)**

**Interpretation of results**

All Listeria species are small, Gram positive rods that demonstrate motility and catalase positive reaction. *L. monocytogenes* are distinguished from other species by the characteristics listed in table given below.

| **Species** | **Haemolysis** | **Production of acid with Rhamnose** | **Production of acid with Xylose** | **CAMP Test** | |
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| | | | | ***S. aureus*** | ***R. equi*** |
| *L. monocytogenes* | + | + | - | + | - |
| *L. innocua* | - | V | - | - | - |
| *L. ivanovii* | + | _ | + | - | + |
| *L. seeligeri* | (+) | - | + | (+) | - |
| *L. welshmeri* | - | V | + | - | - |
| *L. grayi* subspecies *grayi* | - | - | - | - | - |
| *L. grayi* subspecies *murrayi* | - | V | - | - | - |

V is variable reaction

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| | (+) weak reaction<br>(-) no reaction<br>+ is >90% of positive |
| **Expression of results** | Based on the observations and interpretation of the results report presence or absence of *L. monocytogenes* in test portion specifying the mass in grams or mililitres of the sample taken.<br>*L. monocytogenes* =present or absent/ gm or mL or 25/gm or mL. |
| **References** | IS 14988-1: Microbiology of Food and Feeding Stuffs - Horizontal method for Detection and Enumeration of *Listeria Monocytogenes*, Part 1: Detection Method |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Detection and confirmation of *Listeria monocytogenes* in Food based on ISO 11290-1** | | |
| **Method No.** | FSSAI 15.015:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | *Listeria monocytogenes* is an intracellular, foodborne pathogen potentially lethal for humans and animals.<br>For pregnant women and immunocompromised persons it is classified in group IB:"diseases of severe hazard for restricted population; life threatening or resulting in substantial chronic sequelae or presenting eff ects of long duration." | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | While testing *L. monocytogene*s it is recommended that a properly equipped laboratory under supervision of skilled Microbiologist is done. The material used during testing is carefully disposed off after sterilization. Pregnant personnel may be asked to avoid handling of *L. monocytogenes* cultures and undertaking the tests. | | |
| **Principle** | *Listeria monocytogenes* may be present in small numbers and are often accompanied by considerably larger numbers of other microorganisms, therefore selective enrichment is necessary. It is also necessary to detect injured and stressed *Listeria monocytogenes* and the primary selective enrichment medium, with reduced inhibitor concentration, fulfils at least part of this function.<br><br>The detection of *Listeria monocytogenes* requires 4 successive stages viz. primary enrichment, secondary enrichment, identification and confirmation.<br><br>**Primary enrichment in a selective liquid enrichment medium with reduced concentration of selective agents (half-Fraser broth):**<br><br>Inoculation of a selective primary enrichment medium containing half | | |

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| | the concentrations of acriflavine and nalidixic acid (half-Fraser broth and incubation of the initial suspension at 30 °C for 24 h to 26 h.<br><br>**Secondary enrichment with a selective liquid enrichment medium with full concentration of selective agents (Fraser broth):**<br><br>Inoculation of full-strength secondary liquid enrichment medium (Fraser broth) with a culture obtained from 3.1. Incubation of the Fraser broth at 37 °C for 24 h.<br><br>**Plating out and identification:**<br><br>From the cultures obtained from Primery and secondary enrichment, plating out on the two selective solid media:<br><br>• Agar *Listeria* according to Ottaviani and Agosti (AOAL Agar)<br><br>• Any other solid selective Media at the choice of the laboratory complementary to Listeria agar according to Ottaviani and Agosti.<br><br>Incubation of the Agar *Listeria* according to Ottaviani and Agosti and secondary solid selective agar at 37 °C for a total of 48 h. If colonies of presumptive *L. monocytogenes* are evident at 24 h.The incubation may be stopped at this stage.<br><br>**Confirmation**<br><br>Subculturing of the colonies of presumptive *L. monocytogenes* plated out, and confirmation by means of appropriate morphological and biochemical tests. |
| **Equipment** | 1) Laminar airflow<br>2) Biosafety cabinet<br>3) Hot air oven<br>4) Autoclave<br>5) Incubator (Operating at 30 °C ± 1°C and 37°C ± 1°C)<br>6) Anerobic jar<br>7) Water bath (at 44 °C to 47 °C)<br>8) pH meter with measuring accuracy ±0.1 |

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| | 9) Microscope<br>10) Refrigerator (at 2 °C– 8 °C)<br>11) Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>12) Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>13) Micropipette with tips<br>14) Tubes and glass bottles<br>15) Vortex<br>16) Mechanical stirrer<br>17) pH meter with measuring accuracy ±0.1<br>18) Spreader (glass or plastic)<br>19) Inoculation loops and straight wire<br>20) Spiral plater/rotator<br>21) Inoculation loops and straight wire<br>22) Colony counter |
| **Culture media and reagents** | 1. Phosphate buffered peptone water<br>2. Listeria enrichment broth<br>3. Half Frazer broth<br>4. Frazer broth<br>5. Agar Listeria Ottaviani and Agosti (ALOA)<br>6. Modified Oxford Agar<br>7. PALCAM Agar<br>8. Tryptone Soya Yeast Extract Agar<br>9. Tryptone Soya Yeast Extract Broth<br>10. Sheep Blood Agar<br>11. Carbohydrate utilization broth (Rhamnose and Xylose)<br>12. Motility Agar<br>13. CAMP Medium and test organisms<br>14. Hydrogen peroxide solution |
| **Preparation of test sample** | **Preparation of sample and primary enrichment**<br>Aseptically open the sample container and weigh 25 gm sample into a sterile mixture bag/ empty wide mouth container with screw cap or |

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| | suitable closure or take 25 ml of liquid sample. Add 225 ml of sterile half-Fraser broth (pre warm at room temperature). Make a uniform suspension by blending if necessary and incubate at 30 °C ± for 25 ± 1 h.<br><br>**Secondary enrichment:** Inoculate 0.1 ml of the Half Fraser Broth into 10 ml tubes of Fraser Broth. Incubate the tubes at 37 °C for 24 h ± 2 h for *Listeria monocytogenes*.<br><br>**Selective differential plating:**<br>From the culture obtained in the primary enrichment (Half Fraser Broth after 24 ± 2 h at 30 °C) inoculate (streaking) the surface of the selective isolation medium, Agar *Listeria* Ottaviani and Agosti (ALOA) and second selective medium agar plates.<br>From the culture obtained in the secondary enrichment (Fraser Broth after 24 h ± 2 h at 37 °C) inoculate (streaking) the surface of the selective isolation medium, Agar *Listeria* Ottaviani and Agosti (ALOA) and second selective medium agar plates of choice.<br>Incubate the plates at 37 °C and observe for typical colonies after 24 h. Second selective medium agar plates are incubated as per manufacture instructions. If no suspect colonies are evident or if the growth is poor after 24 h, re-incubate the plates for an additional 24 h and read again. After incubation plates can be kept at 5°C for a maximum of 48 h before reading. |
| **Appearance of colonies** | After the incubation period, examine the plates for typical*Listeria monocytogenes* colonies.<br>On Agar *Listeria* Ottaviani and Agosti (ALOA) - blue-green colonies surrounded by an opaque halo (typical colonies).<br>Consider as presumptive Listeria spp all blue green colonies with and without holo. |
| **Confirmation of Listeria species** | Select at least one presumptive typical colony from one plate of each medium.<br>Purify each culture by streaking the selected colonies from each plate on to the surface of a well dried TSYEA for obtaining well separated |

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| | colonies. Invert the plates and incubate at 37 °C for 18 to 24 h or until the growth is satisfactory.<br>Typical colonies are 1 mm to 2 mm in diameter, convex, colorless and opaque with an entire edge. Carry out the following tests from colonies of a pure culture on the TSAYE.<br><br>**Catalase reaction:**<br>With the help of loop pick up an isolated colony and place it in $H_2O_2$ solution on a glass slide. Immediate production of gas bubbles indicates catalase positive reaction.<br><br>**Gram staining:**<br>Perform Gram staining on a colony, *Listeria* are Gram positive slim short rods.<br><br>**Motility Test:**<br>Take colony from TSYEA plate and suspend it TSYE broth. Incubate at 25°C for 8 to 24 hr until cloudy medium is observed. Take a drop of culture and place it on a glass slide. Cover the top with a cover slip and observe under a microscope. *Listeria* is seen as slim rods with a tumbling motility (cultures grown above 25°C fail to show this motion. Compare them with a known culture – *cocci* or large rods with rapid motility are not *Listeria.*<br>As an alternative stab motility agar tube with an isolated colony from TSA YE and incubate at 25 °C for 48 h Typical umbrella like appearance around the stab indicate motility positive culture. If growth is not positive incubate up to five days and observe for the stab again.<br><br>**Heamolysis test on blood agar:**<br>Take a colony from TSA YE and stab it on a well dried surface of sheep blood agar plate. Simultaneously stab positive (*L. monocytogenes*) and negative (*L. innocua*) control cultures. Invert the plates and incubate at 35 °C or 37 °C for 24 ± 2 h. Examine the plates. |

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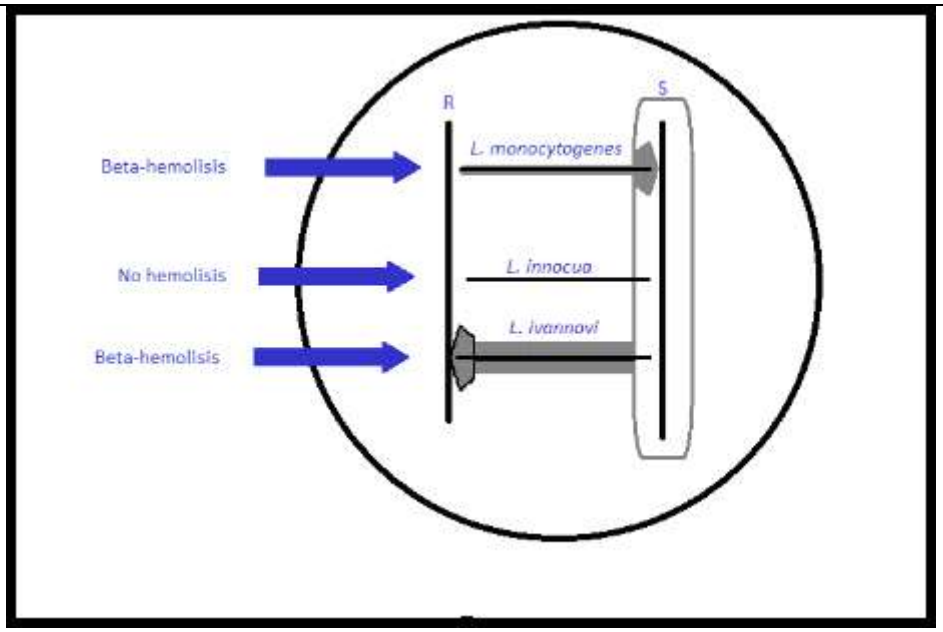
| | *L. monocytogenes* show clear light zones of beta haemolysis. *L. innocua* does not show any haemolysis. Examine the plates in a bright light to compare test cultures with the controls.<br>**Heamolysis reaction using red blood corpuscles**<br><br>**CAMP test (optional)**<br>On a well dried surface of sheep blood agar streak each of the *Staphylococcus aureus* and *Rhodococcus equi* cultures in single lines and parallel to each other and diametrically opposite, a thin even innoculum is required.<br>Streak the test strain separated in a similar manner at right angles to these cultures as that the test strain and *S. aureus* and *R. equi* cultures do not touch but their closest are about 1 mm or 2 mm apart. Several test strains can be streaked on the same plate. Simultaneously streak control cultures of L *monocytogene*s, L innocua and *L. ivanovii.* Incubate plates at 35 to 37°C for 18 to 24 hr.<br>Observe plates against bright light. In *L. monocytogenes* case there is enhanced zone of beta haemolysis at the intersectiono of *S. aureus*.<br>*L. innocua* does not show any enhanced zone of haemolysis with *S. aureus* or *R. equi.*<br>In case of *L. ivanovii* enhamced beta zone of haemolysis is seen on *R. equi* side. |




**Carbohydrate utilization:**

Inoculate each of the carbohydrate utilization broths (rhamnose and xylose) with a culture from TSYE broth and incubate at 35°C or 37°C for upto 5 days. Appearance of yellow color indicates a positive reaction within24 to 48 hr.

**Interpretation of results**

All *Listeria* species are small, Gram positive rods that demonstrate motility and catalase positive reaction. *L. monocytogenes* are distinguished from other species by the characteristics listed in table given below.

| **Species** | **Haemolysis** | **Production of acid with Rhamnose** | **Production of acid with Xylose** | **CAMP Test** | |
|---|---|---|---|---|---|
| | | | | ***S. aureus*** | ***R equi*** |
| *L.monocytogenes* | + | + | - | + | - |
| *L. innocua* | - | V | - | - | - |
| *L. ivanovii* | + | _ | + | - | + |
| *L. seeligeri* | (+) | - | + | (+) | - |
| *L welshmeri* | - | V | + | - | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| | *L. grayi* subspecies *grayi* | - | - | - | - | - |
| | *L. grayi* subspecies *murrayi* | - | V | - | - | - |
| **Expression of results** | Based on the observations and interpretation of the results report presence or absence of *L. monocytogenes* in test portion specifying the mass in grams or mililitres of the sample taken.<br>*L. monocytogenes* = present or absent/ gm or mL | | | | | |
| **Reference** | ISO 11290-1 (2017): Microbiology of the food chain — Horizontal method for the detection and enumeration of *Listeria monocytogenes* and of *Listeria spp*. — Part 1: Detection method. | | | | | |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis | | | | | |

| | | | |
|---|---|---|---|
| | **Method for Detection and Confirmation of *Salmonella*based on IS: 5887 Part3** | | |
| **Method No.** | FSSAI 15.016:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | *Salmonella* is a genus belonging to the family *Enterobacteriaceae*. The family is defined as rod-shaped, Gram-negative, non-spore-forming, facultative anaerobic and oxidase-negative bacteria. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Salmonella spp.* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist and that great care is taken in the disposal of all incubated materials. It is also the responsibility of the user to establish appropriate safety and health practices in the laboratory. | | |
| **Principle** | The detection of S*almonella* requires 4 successive stages viz. pre-enrichment, enrichment, differential selective and confirmation<br>**Pre-enrichment in non-selective broth**:<br>*Salmonella* may be present in small numbers and often accompanied by larger number of other *Enterobacteriaceae* members and other bacteria. Therefore, selective enrichment is necessary, furthermore, pre-enrichment is necessary to permit detection of *Salmonella*. The objectiveof this step is to recover injured cells, which can be obtained by incubating the sample in non-selective conditions, for at least 16-20 hours. The most commonly used media is buffered peptone water (BPW).<br><br>**Enrichment in selective broth**:<br>The objective of this step is to inhibit the multiplication of the accompanying microbiota and preferentially promote the increase of the | | |

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|---|---|
| | number of *Salmonella* cells, by incubating the pre-enriched sample in selective broth, for 24 h. In this step, it is recommended that two differentenrichment media be used, because the resistance of *Salmonella* to selective agents varies from strain to strain. The most recommended media for this purpose are modified Rappaport-Vassiliadis broth (RV) and Selenite/cystine medium.<br><br>**Differential selective plating**: The objective of this step is to preferentially promote the development of *Salmonella* colonies exhibiting typical characteristics that distinguish them from competitors, for subsequent serological and biochemical confirmation. Phenol red or brilliant green agar and any other second medium as per the choice of testing lkaboratory uness there is specific international standards relating to the product to be examined, which specifies the composition of this second medium.<br>**Confirmation**: The objective of this step is to confirm the *Salmonella* colonies by means of biochemical and serological assays. |
| **Equipment** | 1. Laminar airflow<br>2. Biosaftey cabinet<br>3. Hot air oven<br>4. Autoclave<br>5. Incubator (Operating at 35 °C ± 1°C, 37 °C ± 1°C and 42 °C ± 1 °C)<br>6. Water bath (at 42 °C, 45 °C, 55 °C and 70 °C)<br>7. pH meter with measuring accuracy ± 0.1 at 25 °C<br>8. Microscope<br>9. Refrigerator (at 2 °C– 8 °C)<br>10. Petri dishes (Glass or plastic of 90-100 mm diameter or 140 mm)<br>11. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>12. Tubes and glass bottles<br>13. Vortex<br>14. Mechanical stirrer<br>15. Spreader (glass or plastic) |

| | |
|---|---|
| | 16. Inoculation loops and straight wire<br>17. Spiral plater/rotator<br>18. Inoculation loops and straight wire made up of platinum/iridium or nickel/chromium of dia ≈ 3 mm |
| **Culture Media** | i) Nutrient Agar<br>ii) Lactose broth<br>iii) Reconstituted Non-Fat Dry Milk<br>iv) 1% aqueous Brilliant Green Dye Solution<br>v) Rappaport- Vassiliadis magnesium chloride/malachite reen medium (RV –medium)<br>vi) Triple Sugar Iron (TSI) Agar<br>vii) Lysine Iron Agar (LIA)<br>viii) Urea Agar<br>ix) Phenol Red Dulcitol Broth<br>x) Phenol Red Lactose Broth<br>xi) Tryptone Broth<br>xii) Buffered Glucose (MR-VP) Medium<br>xiii) Buffer Peptone Water<br>xiv) Selenite/cysteine Media<br>xv) Brillient reen /phenol red gar<br>xvi) Semi Solid Nutrient agar<br>xvii) L-Lysine decaroxylation Medium<br>xviii) Saline Solutin<br>xix) VP Medium<br>xx) Kovacs reagent<br>xxi) Saline solution<br>xxii) Toluene<br>xxiii) β galactosidase reaent<br>xxiv) TSI medium<br>xxv) Urea agar<br>xxvi) *Salmonella* antisra |

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|---|---|
| **Procedure** | **Preparation of sample and pre-enrichment**<br>Aseptically add 25 g/ml of sample into a sterile empty wide mouth container with suitable closure. Add 225 ml of sterile Buffer peptone water for pre-enrichment.<br>In case prescribed portion of test sample is other than 25, use necessary quantity of pre-enrichment media to yield approximately 1/10 dilution (mass to volume)<br>Make a uniform suspension by blending.<br>Incubate at 35 ℃ or 37 ℃ for 16 to 20 h<br><br>**Selective enrichment**<br>Mix the incubated sample by gentle shaking and transfer 0. 1 ml to 10 ml of RV broth<br>Incubation Temperature 42 °C for 24 h<br>Add additional of 10 ml to 100 ml of selenite or cysteine medium and incubate at 35 °C to 37 °C for 24 h and further 24 h.<br><br>**Selective media plating**<br>After incubation (24 h), gently mix and streak loopful of incubated RV medium and selenite/ cystine broth onto selective media plates of BGA (first selective media)/ or any other second selective media. Repeat the same process from selenite/ cystine broth of 48 h incubation.<br>Incubate the plates at 35 °C or 37 °C for 20 h to 24 h.<br>Re incubate the plates for a further 18 h to 24 h if no typical colonies of *Salmonella* are present.<br>Use a big petri plates (140 mm) or 2 small petri plates (90 mm) to get isolated colonies.<br><br>**<u>Observe plates for typical *Salmonella* colonies.</u>**<br>Typical colonies of *Salmonella* rown on phenol red/brilliant green agar cause the colour of the medium to change from pink to red.<br>Observe the secondary selective plates for typical *Salmonella* colony.<br><br>**Confirmation** |

**Treatment of typical or suspected colonies**

Take suspected five colonies (if present) from each of the selective plates and streak onto Nutrient agar plates. Incubate at 35 °C or 37 °C for 18 to 24 h.

Use this culture for biochemical and serological confirmation.

**Biochemical tests**

By means of inoculation wire inoculate following medium from cultures of the colonies selected.

**TSI Agar**

With help of inoculating wire or a needle inoculate the media TSI (streaking slant and stabbing butt) with the culture obtained from the nutrient agar plates

Incubate TSI slants at 37 °C for 24 ±2 hand 48 ±2 h respectively.

**Table: Typical *Salmonella* reactions are:**

| | **TSI** |
|---|---|
| ***Slant*** | Alkaline (red) |
| ***Butt*** | Acid (Yellow) |
| **$H_2S$ production** (blackening in butt) | + or - |

A culture is treated as presumptive postive if the reactions are typical on TSI slants.

Using a sterile inoculating needle or wire inoculate a portion of the positive culture into the following broths.

Incubate at 35 °C or 37°C for 24 h and read for *Salmonella* typical reactions.

**Biochemical tests**

| **Broth/ Media** | **Time of incubation** | **Results** |
|---|---|---|
| Urea broth | 35 °C or 37 °C 24 ±2 h | Negative (no change in yellow colour of medium) |

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| L-Lysine decarboxylation medium | 35 °C or 37 °C for 24 ±2 h | Positive (Purple color) |
| B-galactosidase | 35 °C or 37 °C for few min | Negative |
| Indole Test | 35 °C or 37 °C for 24 h | Negative (Yellow brown reaction) |
| MR-VP medium | 35 °C or 37 °C 24±2 h | Negative for VP test but positive for MR test. (Red for positive) |

**Serological Tests**

**Elimination of auto agglutinable strains**

Place 1 drop of saline on clean glass slide. Take a part of colony to be tested to make homogenate suspension. Tilt the slide gently for 1 min. Observe the result for any clumps which shows presence of auto agglutinable strain.

Auto-agglutinable strains shall not be submitted for following tests.

**Examination of H-antigens**

**Incubation**

Inoculate the colony on NA and incubate at 35 °C or 37 °C for 18 to 24h. Take a part of colony from NA on glass slide and add 1 drop of anti H serum on it. Mix well, if agglutination occur, the reaction is positive.

**Examination of Vi-antigens**

Add 1 drop of culture suspension to clean glass slide. Add a drop of anti-Vi- serum. If agglutination occurs, the reaction is positive.

**Monovalent/Polyvalent somatic (O) test**.

Add 1 drop of culture suspension to clean glass slide. Add a drop of anti O serum. If agglutination occurs, the reaction is positive.

<table>
<tr><td></td><td>
Interpretation of agglutination tests<br>
In place of serum, use normal saline as negative control.<br><br>
Interpretation of Biochemical and Serologiocal Reactions
<table>
<tr><th>Biochemical Reactions</th><th>Auto-agglutination</th><th>Serologiocal Reactions</th><th>Interpretation</th></tr>
<tr><td>Typical</td><td>No</td><td>O-, Vi-, H- antigen positive</td><td>Strains considered to be Salmonella</td></tr>
<tr><td>Typical</td><td>No</td><td>All reactions negative</td><td rowspan="3">May be Salmonella</td></tr>
<tr><td>Typical</td><td>Yes</td><td>Not tested</td></tr>
<tr><td>No Typical Reactions</td><td>No</td><td>O-, Vi-, H- antigen positive</td></tr>
<tr><td>No Typical Reactions</td><td>No</td><td>All reactions negative</td><td>Not considered to be Salmonella</td></tr>
</table>
In accordance with the results of the interpretation, indicate the presence and absence of Salmonella in a test portion of x of product.<br>
Definitive Confirmation<br><br>
Strains which are considered to be Salmonella shall be sent to reconised Salmonella reference centre for definitive typing.
</td></tr>
<tr><td>Calculation</td><td>NA</td></tr>
<tr><td>Expression of Result</td><td>Salmonella = Present/Absent per 25 gm or 25 ml</td></tr>
<tr><td>Reference</td><td>IS 5887-3: Methods for Detection of Bacteria Responsible for Food Poisoning, Part 3: General Guidance on Methods for the Detection of Salmonella</td></tr>
<tr><td>Approved by</td><td>Scientific Panel on Methods of Sampling and Analysis</td></tr>
</table>

| | | | |
|---|---|---|---|
| | **Method for Detection and Confirmation of *Salmonella species*based on ISO:6579-I** | | |
| **Method No.** | FSSAI 15.017:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | *Salmonella* is a genus belonging to the family *Enterobacteriaceae.* The family is defined as rod-shaped, Gram-negative, non-spore-forming, facultative anaerobic and oxidase-negative bacteria.. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Salmonella spp.* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist and that great care is taken in the disposal of all incubated materials. It is also the responsibility of the user to establish appropriate safety and health practices in the laboratory. | | |
| **Principle** | The detection of S*almonella* requires 4 successive stages viz. pre-enrichment, enrichment, differential selective and confirmation<br>**Pre-enrichment in non-selective broth**:<br>*Salmonella* may be present in small numbers and often accompanied by larger number of other Enterobacteriaceae members and other bacteria. Therefore, selective enrichment is necessary, furthermore, pre-enrichment is necessary to permit detection of Salmonella.<br>The objective of this step is to recover injured cells, which can be obtained by incubating the sample in non-selective conditions, for at least 16-20 hours. The most commonly used media are buffered peptone water (BPW).<br>**Enrichment in selective broth**:<br>The objective of this step is to inhibit the multiplication of the accompanying microbiota and preferentially promote the increase of the number of *Salmonella* cells, by incubating the pre-enriched sample in | | |

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| | selective broth, for 24 h. In this step, it is recommended that two different enrichment media be used, because the resistance of *Salmonella* to selective agents varies from strain to strain. The most recommended media for this purpose are modified semi-solid Rappaport-Vassiliadis broth (MSRV) or Rappaport Vassiliadis Soya broth (RVS) and Muller- Kauffmann tetrathionate- novobiocin broth (MKTTn broth).<br><br>**Differential selective plating**:<br>The objective of this step is to preferentially promote the development of *Salmonella* colonies exhibiting typical characteristics that distinguish them from competitors, for subsequent serological and biochemical confirmation. The most commonly used media is xylose lysine desoxicolate (XLD) agar that differentiate *Salmonella* through its incapacity to ferment lactose and the concomitant ability to produce $H_2S$,<br>**Confirmation**: The objective of this step is to confirm the *Salmonella* colonies by means of biochemical and serological assays. |
| **Equipment** | 1. Laminar airflow<br>2. Biosaftey cabinet<br>3. Hot air oven<br>4. Autoclave<br>5. Incubator (Operating at 34°C to 38 °C ,37 °C ± 1°C and 41.5 °C ± 1 °C)<br>6. Water bath (at 42 °C to 50 °C and 37 °C ± 1°C)<br>7. pH meter with measuring accuracy ± 0.1 at 20 °C to 25 °C<br>8. Microscope<br>9. Refrigerator (at 2 °C– 8 °C)<br>10. Petri dishes (Glass or plastic of 90-100 mm diameter or 140 mm)<br>11. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>12. Tubes and glass bottles<br>13. Vortex<br>14. Mechanical stirrer |

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|---|---|
| | 15. Spreader (glass or plastic)<br>16. Inoculation loops and straight wire<br>17. Spiral plater/rotator<br>18. Inoculation loops and straight wire made up of platinum/iridium or nickel/chromium of dia ≈ 3 mm |
| **Culture Media** | i) Nutrient Agar<br>ii) Rappaprt-Vassilliadis medium with Soya (RVS broth)<br>iii) Modified Semi Solid Rappaprt-Vassilliadis (MSRV agar)<br>iv) Muller Kauffmann tetrathionate broth (MKTT Broth)<br>v) Xylose Lysine Deoxycholate (XLD) Agar<br>vi) Triple Sugar Iron (TSI) Agar<br>vii) Lysine Iron Agar (LIA)<br>viii) Urea Agar<br>ix) Phenol Red Dulcitol Broth<br>x) Phenol Red Lactose Broth<br>xi) Tryptone Broth<br>xii) Buffered Glucose (MR-VP) Medium<br>xiii) Brain Heart Infusion (BHI) Broth<br>xiv) Buffer Peptone Water<br>xv) TSI agar<br>xvi) Kovacs reagent<br>xvii) Saline solution<br>xviii) Toluene<br>xix) β galactosidase reaent<br>xx) TSI medium<br>xxi) Urea agar<br>xxii) *Salmonella* antisra |

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|---|---|
| **Procedure** | **Preparation of sample and pre-enrichment**<br>Aseptically add 25 g/ml of sample into a sterile empty wide mouth container with suitable closure. Add 225 ml of sterile or Buffer peptone water for pre-enrichment. Make a uniform suspension by blending. Incubate at 34 to 38℃ for 18 ± 2 h.<br>After incubation this pre-enrichment can be stored for a maximum of 72 h at 5 °C.<br><br>**Selective enrichment**<br>**General**<br>Allow the pre-enrichment media to came at room temperature.<br>Gently shake the incubated sample avoiding particulate material from pre-enrichment medium.<br>After incubation this enrichment can be stored for a maximum of 72 h at 5 °C.<br><br>**Procedure for food samples from production area**<br>Transfer 0. 1 ml to 10ml of RVS broth or to the surface of MSRV agar plate with one to three equally spaced spots.<br>Add 1 ml of the pre enrichment culture to a tube of 10 ml of MKTTn broth.<br>Incubate RVS and MSRV at 41.5 °C for 24 h± 3h.<br>**Do not invert MSRV agar plates.**<br>Incubate MKTTn broth at 37 °C for 24 h ± 3h<br>Suspect MSRV plates will show grey white, turbid zone extending out from the inoculated drop.<br>An additional incubation of 24 h ± 3h may be required for dried milk products and cheese where *Salmonella* may be sublethally injured.<br>An additional incubationtime may also be beneficial for some products, while investigating outbreaks.<br><br>**Procedure for food samples from pre production area**<br>Inoculate the MSRV agar plate with 0.1 ml of pre enriched culture as one to three equally spaced spots. |

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|---|---|
| | Incubate MSRV at 41.5 °C for 24 h± 3h.<br>**Do not invert MSRV agar plates.**<br>Suspect MSRV plates will show grey white, turbid zone extending out from the inoculated drop.<br>In case plates are negative after 24 h, re-incubate for a further 24 h ± 3h.<br><br>**Selective media plating**<br>Gently mix and streak loopful of incubated selective enriched culture (RVS broth or MSRV agar and MKTTn broth) onto two selective isolation agar media.<br>The first isolation medium is Xylose Lysine Deoxycholate (XLD) agar.<br>The second isolation medium is chosen by the testing laboratory. Choose a second selective plating medium which is complementary to XLD agar (and is based on different diagnostic characteristics to those of XLD agar to facilitate detection of (for instance, lactose positive or $H_2S$negative) *Salmonella*.<br>From the incubated RVS broth, inoculate (by means of a 10 µl loop) the surface of an XLD plate so that well-isolated colonies will be obtained. Proceed in the same way with the second selective platingout medium.<br>From the MSRV agar, determine the furthest point of opaque growth from the inoculation points and dip a 1 µl loop just inside the border of the opaque growth. Withdraw the loop ensuring that no large lumps of MSRV agar are extracted.<br>Inoculate the surface of an XLD plate so that well-isolated colonies will be obtained. Proceed in the same way with the second selective plating-out medium.<br>From the MKTTn broth, inoculate (by means of a 10 µl loop) the surface of an XLD plate so that well-isolated colonies are obtained. Proceed in the same way with the second selective plating-out medium.<br>Invert and incubate the XLD plates at 37 °C for 24 ± 3 h<br>Incubate the second selective plating-out medium in accordance with the manufacturer's instructions.<br>If the selective enrichment media have been incubated for an additional |

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| | 24 h, follow the same plating-out procedure as described above.<br>Typical colonies of *Salmonella* on XLD agar have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator.<br>*Salmonella* $H_2S$ neative strains are pink with darker pink centre. Lactose positive *Salmonella* grown on XLD are yellow with or without blackening.<br>Check the second selective plating medium after appropriate incubation for the presence of colonies which from their characteristics are found to be presumptive *Salmonella*.<br>**Confirmation**<br>Combination of biochemical and serological test results indicate whether an isolate belons to enus *Salmonella*. Fr characterization of *Salmonella* strains, fully serotypingg is needed.<br><br>**Treatment of typical or suspected colonies**<br>Mark suspect colonies on each palte. Take at least one suspected colony for subculture and confirmation, if this is neative take four more suspect colonies (if present) from each of the different selective isolation medium and streak onto non selective medium *e.g.* Nutrient agar plates. Incubate between 34 °C and 38 °C for 24 h ± 3 h.**Use this culture for biochemical and serological confirmation.**<br><br>Alternatively, if well-isolated colonies (of a pure culture) are available on the selective plating media, the biochemical confirmation can be performed directly on a suspect, well-isolated colony from the selective plating medium.<br><br>**Biochemical tests**<br>**TSI Agar**<br>With help of inoculating wire or a needle inoculate the TSI agar (streaking slant and stabbing butt) with the culture obtained from the nutrient agar plates |

Incubate TSI slants at 37 °C for 24 ± 3 h

**Table: Typical *Salmonella* reactions are:**

| | **TSI** |
|---|---|
| ***Slant*** | Alkaline (red) |
| ***Butt*** | Acid (Yellow) |
| **$H_2S$ production** (blackening in butt) | + or - |

A culture is treated as presumptive postive if the reactions are typical on TSI slants.

**Biochemical tests**

Using a sterile inoculating needle or wire inoculate a portion of the positive culture into the following broths and read for *Salmonella* typical reactions.

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**Biochemical tests**

| **Broth/ Media** | **Time of incubation** | **Results** |
|---|---|---|
| Urea broth | 37 °C for 24 h | Negative (no change in yellow colour of medium) |
| L-Lysine decarboxylation medium | 37 °C for 24 ± 3 h | Positive (Purple color) |
| B-galactosidase | 37 °C for 24 h | Negative |
| MR-VP medium | 35 °C or 37 °C 24 ± 2 h | Negative for VP test but positive for MR test. |
| Indole test | 37 °C for 24 h± 3 h | Negative (Yellow brown reaction) |

**Serological Tests**

**Elimination of auto agglutinable strains**

Place 1 drop of saline on clean glass slide. Take a part of colony to be tested to make homogenate suspension. Tilt the slide gently for 1 min. Observe the result for any clumps which shows presence of auto agglutinable strain.

Auto-agglutinable strains shall not be submitted for following tests.

**Examination of H-antigens**

Take a part of colony on glass slide and add 1 drop of anti H serum on it. Mix well, if agglutination occurs, the reaction is positive.

**Examination of Vi-antigens**

Add 1 drop of culture suspension to clean glass slide. Add a drop of anti-Vi- serum. If agglutination occurs, the reaction is positive.

**Monovalent/Polyvalent somatic (O) test**.

Add 1 drop of culture suspension to clean glass slide. Add a drop of anti O serum. If agglutination occurs, the reaction is positive.

**Interpretation of agglutination tests**

In place of serum, use normal saline as negative control.

**Interpretation of Biochemical and Serologiocal Reactions**

| **Biochemical Reactions** | **Auto-agglutination** | **Serologiocal Reactions** | **Interpretation** |
|---|---|---|---|
| Typical | No | O- and H- antigen positive(and Vi positive if tested) | Strains considered to be *Salmonella* |
| Typical | No | O- and H- antigen negative | May be *Salmonella* |
| Typical | Yes | Not tested | |
| No Typical Reactions | _ | _ | Not considered to be *Salmonella* |

**Serotyping:**

Strans that are confirmed as *Salmonella* spp, can be further serotyped.

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| **Calculation** | NA |
| **Expression of Result** | *Salmonella* = Present/Absent per 25 gm or 25 ml |
| **Reference** | ISO 6579-1 (2017): Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of *Salmonella* — Part 1: Detection of *Salmonella* spp. |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Detection and Confirmation of *Shigella*based on ISO: 21567** | | |
| **Method No.** | FSSAI 15.018:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | Shigellosis is an infectious disease spread most commonly by person to person transmission. The genus consists of 4 species: *Sh. dysenteriae* (Subgroup A), *Sh. flexneri* (Subgroup B), *Sh. boyvdii* (Subgroup C) and *Sh. sonnei* (Subgroup D). These species are distinguishable by their biochemical and serological reactions | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Shigella spp.* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. It is also the responsibility of the user to establish appropriate safety and health practices. | | |
| **Principle** | The detection of *Shigella spp* requires 4 successive stages *viz.* pre-enrichment, enrichment, biochemical and serological confirmation<br>**Pre-enrichment selective broth**:<br>The objective of selective enrichment is to inhibit the competing microflora present in the samples, favoring at the same time multiplication of the target microorganism. This is achieved by using selective agents (0.5 µg/ml of Novobiocin) and/ or restrictive conditions (anerobically incubated at 41.5 ± 1 °C)for the growth of the competing microflora<br>**Differential selective plating**: The objective of this step is to preferentially promote the development of *Shigella* spp. colonies exhibiting typical characteristics that distinguish them from competitors, for subsequent serological and biochemical confirmation. | | |

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| | **Confirmation** by means of biochemical assays and serological assays:<br>The objective of this step is to verifywhether the colonies obtained on the plates are actually*Shigella spp* colonies, by means of biochemical and serologicaassays |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven<br>4. Autoclave<br>5. Incubator (Operating at 37 °C ± 1°C and 41°C ± 1°C)<br>6. Water bath (at 44 °C to 47 °C)<br>7. pH meter with measuring accuracy ±0.1<br>8. Microscope<br>9. Glass slides<br>10. Refrigerator (at 2 °C– 8 °C)<br>11. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>12. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>13. Micropipette with tips<br>14. Glass tubes, flasks and glass bottles<br>15. Vortex<br>16. Modified atmospheric jar /Anaerobic Jar /anerobic incubation cabinet<br>17. Mechanical stirrer<br>18. pH meter with measuring accuracy ±0.1<br>19. Spreader (glass or plastic)<br>20. Inoculation loops and straight wire<br>21. Spiral plater/rotator<br>22. Inoculation loops and straight wire<br>23. Colony counter |
| **Culture media** | (i) Nutrient Broth (NB)<br>(ii) MacConkey agar medium<br>(iii) Deoxycholate citrate Agar (DCA) medium<br>(iv) Triple Sugar Iron (TSI) Agar slants |

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| | (v) Urea Broth<br>(vi) Acetate Agar Slants<br>(vii) Carbohydrate Fermentation Media<br>(viii) Tryptone Broth (for Indole test)<br>(ix) Buffered Glucose (MR-VP) Medium<br>(x) Koser's Citrate Broth<br>(xi) Decarboxylase Test Media with Lysine or Ornithine<br>(xii) Motility Test Medium<br>(xiii) Thornley's Semi-Solid Arginine Medium |
| **Procedure** | **Preparation of sample and enrichment**<br>**Pre enrichment**<br>Aseptically open the sample container and weigh x gm sample into a sterile empty wide mouth container with screw cap or suitable closure or take x ml of liquid sample. Add x ml of sterile Shigella broth containing 0.5 µg/ml of novobiocin to make 1 in 10 dilution of sample. Make a uniform suspension by blending if necessary and incubate the *Shigella* broth under anaerobic conditions with caps and closures loose, or with equipment giving an equivalent effect, so that gas exchange can readily occur without contamination at 41.5 ± 1 °C for 16 h to 20 h.<br><br>**Plating out and colony selection**<br>**Isolation**<br>Using the cultures obtained in pre enrichment, gently mix the contents and allow the larger particles to settle. Inoculate (streaking), by means of a loop, the surface of the selective agars to obtain well-isolated colonies on MacConkey agar (Low selectivity), XLD agar (Moderate selectivity) and Hektoen enteric agar and incubate the plates at 37 ± °C for between 20 h and 24 h.<br><br>**Identification**<br>After the incubation period, examine the selective agar plates for typical colonies on<br>MacConkey agar: Colourless to pale pink, translucent, lactose negative. |

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| | XLD agar: Translucent with red/ cerise center, same colour as the agar<br>Hektoen enteric agar: Green and moist raised colonies.<br>If no typical colonies are seen, reincubate the plates for further 24 h for typical S*higella* colonies.<br><br>**Colony selection and purification:** Select five typical colonies of *Shigella* from each selective agar. If there are fewer than 5 typical or suspect colonies, then take all the marked colonies for confirmation. For purification, streak the selected colonies onto the surface of Nutrient Agar (NA) plates. Incubate the plates at 37 °C ± 1 °C for 24 ± 3 h.<br><br>**Biochemical confirmation:**<br>Use pure cultures from the NA plates for biochemical and serological tests. As an alternative to conventional biochemical tests, commercial biochemical kits (must be approved by FSSAI under RAFT scheme) can be used.<br>1. **Gram's Stain**:<br>Take one loop full culture and spread evenly over the drop of normal saline on a clean glass slide to form a smooth smear. Heat fix the smear by gently passing the slide over the flame. Add crystal violet for 60 seconds. The stain was poured off and slide was gently washed under slightly running tap water. Add Gram's Iodine for 60 seconds. Iodine solution was decanted and slide was washed under slightly running tap water. Then slide was de-colourized with alcohol for 1 second and subsequently washed under slightly running tap water. Add Safranin as counter stain for 30seconds, then wash with slightly running tap water and air dry. Examine stained slide under microscope at 100X.<br><br>2. **Test for Catalase**:<br>Take clean glass slide. Take an isolated colony and suspended it in a drop of 3 % (w/w) Hydrogen peroxide ($H_2O_2$) solution on the slide. Presence of effervescences, caused by the liberation of free oxygen as gas bubbles, indicated the presence of catalase and was considered positive. |

3. **Test for Oxidase**:

In freshly grown Nutrient Agar slant of culture, add few drops of freshly mixed test reagent, 1% solution of α-naphthol in 95% ethanol and equal amount of 1% solution of para-amino-dimethylaniline hydrochloride in water. A positive reaction is indicated by the appearance of blue colour within two minutes.

**4. Hugh-Leifson's test:**

The strain from fresh nutrient agar growth is stabbed into two tubes of Hugh-Leifson's media, one of which is then layered over with a small amount of sterile liquid paraffin. Incubate both tubes at 37°C and observe up to 4 days. Acid formation, yellow colour, in the tube without paraffin indicates oxidative utilization of glucose. Acid in both tubes indicates fermentative reaction. Lack of acid in either tube indicates the strain as not being able to utilize glucose oxidatively or fermentatively.

**5. Urease Test**

Streak the organisms over agar surface and incubate at 37 °C for 18 to 24 h and examine at intervals.

If urea is hydrolysed, a rose-pink to deep cerise colour develops from the release of ammonia by the decomposition of the urea with a change in the colour of the pH indicator. There is no change in colour of the agar with a negative reaction. *Shigella* species do not hydrolyse urea.

6. **Test for $H_2S$ Production TSI reactions**:

Inoculate each suspect culture into Triple Sugar Iron Agar (TSI) tubes by streaking the slant and stabbing the butt. Incubate at 37 ± 1°C/24 ± 3 h. Cap the tubes loosely to maintain aerobic conditions. After the incubation period, examine the tubes for typical *Shigella* reactions

**Butt:** Yellow Glucose fermented: positive. Red or unchanged Glucose not fermented: negative Black Formation of hydrogen sulfide: positive,

| | |
|---|---|
| | Bubbles or cracks Gas formation.<br><br>**Slant surface:** Yellow Lactose and/or sucrose utilized: positive. Red or unchanged Lactose and sucrose not utilized: negative<br><br>Typical *Shigella* cultures show a yellow butt (acid formation) and no gas bubbles, there is no change in thecolour of the slant (no utilization of lactose or sucrose) and no hydrogen sulphide production.<br><br>**7. L-Lysine decarboxylase medium**<br>Inoculate below the surface of the liquid broth. Incubate at 37 ±1 °C for 24 ± 3h. Turbidity and a purple colour after incubation indicate a positive reaction; yellow indicates a negative result. *Shigella* species do not decarboxylate lysine.<br><br>**NOTE** The use of a paraffin overlay in the tubes can help to ensure anaerobic conditions.<br>8. **L-Ornithine decarboxylase medium**<br>Inoculate below the surface of the liquid broth. Incubate at 37 ± 1°C for 24 ± 3h. If a purple colour develops, the test is positive; a yellow colour means a negative result. *Shigella sonnei* decarboxylates ornithine, but other *Shigella* species do not.<br><br>**9. Detection of indole formation**<br>Inoculate a tube containing 5 ml of tryptone/tryptophan medium (B.9.1) with the pure culture. Incubate at 37 °C ± 1 °C for 24 ± 3 h.<br>After incubation, add 1 ml of Kovac's reagent. The formation of a red ring within 10 min indicates indole formation, and a yellow/brown colour indicates a negative reaction. *Shigella sonnei* is negative whilst other strains give variable reactions.<br><br>**10. Detection of β-galactosidase**<br>Suspend a loopful of the purified culture from the nutrient agar into 0.25 ml of saline solution in a screw cap bottle or test tube. Add one drop of |

toluene and shake to mix well. Put the tube in an incubator set at 37 °C and leave for several minutes. Add 0.25 ml of the complete reagent and mix. Replace in the incubator set at 37 °C and leave for 24 h ± 3 h, examining at intervals.

A yellow colour indicates the formation of β-galactosidase, which can occur in as little as 20 min.

*Shigella sonnei* is positive. *S. dysenteriae* and *S. boydii* give variable reactions and *S. flexneri* is negative.

**11. Utilization of carbohydrates**

Inoculate each of the prepared carbohydrate broths with a small inoculum. Incubate at 37 °C for 18 to 48 h

A positive reaction when carbohydrate is utilized gives a change in the pH indicator from purple to yellow.

**Interpretation of biochemical results**

Strains within some *Shigella* species vary in their biochemical reactions (see Table 1), therefore interpretation based only on biochemical results is difficult and serotyping is essential to establish identity.

*Shigella* are Gram-negative bacilli, 2 μm to 4 μm by 0.5 μm in size, but often show a tendency to shorter cocco-bacillary forms and typically do not produce gas from glucose.

They are non-motile, do not produce hydrogen sulfide or decarboxylate lysine, and are lactose negative at 24 h. The other tests described above give variable reactions or differing reactions according to the species.

Within the genus *Shigella,* mannitol discriminates *Shigella dysenteriae* (negative*)* from other species andL-ornithine decarboxylase differentiates *Shigella sonnei* (positive) from other species.

**Table 1**

| **Test** | ***Shigella sonnei*** | ***Shigella flexneri*** | ***Shigella dysenteriae*** | ***Shigella boydii*** |
|---|---|---|---|---|
| $H_2S$ from TSI | - | - | | |

| | | | | |
|---|---|---|---|---|
| Gas from glucose (TSI) | | | - | - |
| Motility | | | | |
| Urease | | | | |
| L-Lysine decarboxylase | | | | - |
| L-Ornithine decarboxylase | | | | |
| Indole formation | | V[d](61 %) | V[d](44 %) | V[d](29 %) |
| β-Galactosidase | + (95 %) | - | V[f](50 %) | V[f](11 %) |
| Acid from: | | | | |
| Dulcitol | | V[g](9,4 %) | V[g](4.5 %) | V[g](6,7 %) |
| Glucose | + (100 %) | + (100 %) | + (100 %) | + (100 %) |
| Lactose | -c | -a | | - a |
| Mannitol | + (99 %) | + b(94 %) | | + (98 %) |
| Melibiose | | V | V | V |
| Raffinose | (2,5 %) | V (53 %) | | |
| Salicin | | | | |
| Sorbitol | | V (31 %) | V (29 %) | V (42 %) |
| Sucrose | -c (1,5 %) | | | |
| Xylose | | | V[h](4,0 %) | V (57 %) |

[a] some strains of *S. flexnerri* serovars 2a and *S. boydii* 9 produce acid

[b] some strains of *S. flexnerri* serovars 4 and 6b do not produce acid

[c] *S. sonnei* produces acid after several days of incubation

**Additional biochemical differentiation**

**General**

It is recommended to carry out additional biochemical differentiation tests for a better identification of the

strains: some strains of *Escherichia coli* and *Shigella* species are similar.

**A) Sodium acetate**

Streak the slope of the sodium acetate medium with the pure culture.

Use a straight wire to minimize the amount of culture medium transferred with the inoculum, or use an inoculation needle. Incubate under aerobic conditions for 2 days at 37 ±1°C.

Examine the green medium for growth: a positive result is found when the medium turns blue.

Look for the growth, a blue colour indicates a positive reaction. If no growth occurs, incubate the culture for 2 additional days at 37 ±1°C. Examine the medium again. *Shigella* species do not grow or grow very poorly. Strains of *E. coli* give blue colonies with the surrounding medium blue/green.

B) **Chrisensen's Citrate**

Inoculae the slant surface of Chrisensens Cirate Medium wih pure culure using an inoculating needle. Incubae aerobically for 2 days at 37 ±1°C. Examine to check cream pink growh. In case no growh observed incubate furher 24 h. Shigella species do not grow.

C) **Sodium mucate**

Inoculae the test broth and contol broh wih pure culure. Incubate aerobically for 2 days at 37 ±1°C. Examine to check growh. In case no growh is observed incubate furher for 24 h. Blue colur indicaes negative reaction and yellow colour indicaes posiive reaction.

All Shigella sp except *Shigella sonnei* give negative result.

**Table 2 - Additional biochemical tests[a] to differentiate some strains of *Shigella* spp.**

| Species | Biochemical reactions (growth) for a determined period after incubation | | | | | |
|---|---|---|---|---|---|---|
| | Sodium acetate | | Christensen's citrate | | Sodium mucate | |
| | + % at 2 days | + % at 2 days | + % at 2 days | + % at 2 days | + % at 2 days | + % at 2 days |
| S. dysenteriae | -(0) | -(0) | -(0) | -(0) | -(0) | -(0) |

| | | | | | | |
|---|---|---|---|---|---|---|
| *S. flexneri* | -(0) | -(0) | -(0) | -(0) | -(0) | -(0) |
| *S. boydii* | -(0) | -(0) | -(0) | -(0) | -(0) | -(0) |
| *S. sonnie* | -(0) | -(0) | -(0) | -(0) | V(6,4) | V(36,7) |
| *E. coli* | V (83,8) | + (93,5) | V (>15,3) | V (>34,2) | + (91,6) | + (93,0) |

\+ >90% strains positive.
\- >90% strains negative
V Variable results with between 10% to 89% of strains positive.
% Percentage of positive strains after determined incubation.
[a] From *Bacteriological Analytical Manual*, 8th Edition (Revised 1997), FDA, USA.

**Serological confirmation**

**Antigenic differentiation**

Shigella species are non-motile and therefore do not have flagella antigens. Differentiation within and between species depends upon the analysis of distinct somatic group "O" and specific "O" type antigens (see Table 3).

Growth from a fresh culture on nutrient agar is required. Carry out agglutination tests on clean glass slides or plates of glass of the appropriate size.

Table 3 — Antigenic differentiation within the *Shigella* species

| ***Shigella* species** | **Antigenic group** | **Serovars (specific antigen designation)** |
|---|---|---|
| *S. dysenteriae* | A | 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 |
| *S. flexneri* | B | 1a, 1b, 2a, 2b, 3a, 3b, 3c, 4a, 4b, 5a, 5b, 6, X, Y |
| *S. boydii* | C | 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 |
| *S. sonnei* | D | 1 |

| | |
|---|---|
| | NOTE 1 The group antigens (A, B, C, D) can contain minor antigens that may cross react with other group antigens; this is avoided by the use of absorbed antisera and/or its dilution to a stipulated level. Some species, particularly *Shigella dysenteriae,* have envelope antigens that will mask the group and serovar antigens which prevent agglutination with specific type antisera. The envelope antigen is removed by heating a suspension at 100 °C for 15 min to 60 min.<br>NOTE2 The *Shigella sonnei* group D antigen is present in both the smooth and rough colony types and has no crossreactivity with the other *Shigella* group antigens. Unlike some other Enterobacteriaceae, therough colony types of<br>*S. sonnei* do not necessarily auto-agglutinate. *Shigella sonnei* has no envelope antigen.<br><br>**Agglutination tests**<br>Follow precisely the instructions given by the manufacturer for preparing antisera and conducting agglutination tests.<br><br>Place one drop of the group antiserum and one drop of saline solution separately on a glass slide. Disperse part of the colony to be tested in the saline and part of the colony in the antiserum solution so as to obtain a homogeneous and turbid suspension in each. Rock the slide gently for 30 s to 60 s. Observe the result against a dark background, if necessary with the aid of a magnifying lens.<br><br>If the bacteria in the antiserum have clumped into more or less distinct particles and there is no agglutination in the saline, the isolate is positive for the group tested. If there is agglutination in the saline, the strain is considered to auto-agglutinate, and shall not be used further tests. The testing of other colonies from the same culture and the other isolates selected for examination from the original selective agar and giving biochemical reactions indicative of *Shigella* should then be tested.<br><br>**Expression of results:**<br>Based on the observations and interpretation of the results report |

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|---|---|
| | presence or absence of *Shigella spp.* in test portion specifying the mass in grams or mililitres of the sample taken. |
| **Expression of Results** | ***Shigella spp***= present or absent/ gm or ml. |
| **Reference** | ISO 21567 (2004): Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Shigella* spp. |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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|---|---|---|---|
| | **Method for Detection and Confirmation of *Shigella* based on IS 5887-Part 7** | | |
| **Method No.** | FSSAI 15.019:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | Shigellosis is an infectious disease spread most commonly by person to person transmission. The genus consists of 4 species: *Shigella dysenteriae* (Subgroup A), *Shigella flexneri* (Subgroup B), *Shigella boydii* (Subgroup C) and *Shigella sonnei* (Subgroup D). These species are distinguishable by their biochemical and serological reactions | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Shigella spp.* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. It is also the responsibility of the user to establish appropriate safety and health practices. | | |
| **Principle** | The detection of *Shigella spp* requires. pre-enrichment, isolation on differential selective medium and confirmation.<br><br>**Pre-enrichment in non-inhibitory medium**<br>**Differential selective plating**: The objective of this step is to preferentially promote the development of *Shigella spp.* colonies exhibiting typical characteristics that distinguish them from competitors, for subsequent serological and biochemical confirmation.<br>**Confirmation**:The objective of this step is to verifywhether the colonies obtained on the plates are actually*Shigella spp* colonies, by | | |

| | |
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| | means of biochemical and serologicl assays. |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven<br>4. Autoclave<br>5. Incubator (Operating at 37 °C ± 1°C and 41°C ± 1°C)<br>6. Anaerobic chamber<br>7. Water bath (at 44 °C to 47 °C)<br>8. pH meter with measuring accuracy ±0.1<br>9. Microscope<br>10. Glass slides<br>11. Refrigerator (at 2 °C– 8 °C)<br>12. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>13. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>14. Micropipette with tips<br>15. Glass tubes, flasks and glass bottles<br>16. Vortex<br>17. Modified atmospheric jar /Anaerobic Jar /anerobic incubation cabinet<br>18. Mechanical stirrer<br>19. Spreader (glass or plastic)<br>20. Inoculation loops and straight wire<br>21. Spiral plater/rotator<br>22. Inoculation loops and straight wire<br>23. Colony counter |
| **Culture media** | i) Nutrient Broth (NB)<br>ii) MacConkey agar medium<br>iii) Deoxycholate citrate Agar (DCA) medium<br>iv) Triple Sugar Iron (TSI) Agar slants<br>v) Urea Broth<br>vi) Acetate Agar Slants<br>vii) Carbohydrate Fermentation Media<br>viii) Tryptone Broth (for Indole test) |

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| | ix) Buffered Glucose (MR-VP) Medium<br>x) Koser's Citrate Broth<br>xi) Decarboxylase Test Media with Lysine or Ornithine<br>xii) Motility Test Medium<br>xiii) Thornley's Semi-Solid Arginine Medium |
| **Expression of Results** | ***Shigella spp***= present or absent/ gm or ml. |
| **Reference** | IS 5887 (Part 7): Methods for Detection of Bacteria Responsible for Food Poisoning, Part 7: General Guidance on Methods for Isolation and Identification of *Shigella*. |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Enumeration of Coagulase Positive *Staphylococci* based on ISO 6888-1/AMD 1 and IS 5887- Part 8 (Sec 1)** | | |
| **Method No.** | FSSAI 15.020:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | *Staphylococci* are aerobic Gram-positive bacteria, with diameters of 0.5 – 1.5 µm and characterized by individual cocci, in grape-like clusters. They are non-motile, non-spore forming facultative anaerobes. Some of species of Staphylococcus genera have ability to produce coagulase enzyme i.e *Staphylococcus aureus, Staphylococcus hyicus, Staphylococcus intermedius* etc. Coagulase positive *Staphylococci*produce Enterotoxins, which is responsible for food-poisoning if contaminated food is consumed. *S. aureus* is the species with the more pathogenic of the genus *Staphylococcus.* | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good laboratory practices to avoid cross contamination. | | |
| **Principle** | Enumeration medium BPA, contain Lithium chloride and potassium tellurite which inhibit most of the contaminating micro flora except *Staphylococci*. *Staphylococci* can reduce tellurite to telluride, which results in grey to black coloration of the colonies. With the addition of egg yolk, the medium becomes yellow, slightly opaque. A clear halo develops around colonies from coagulase positive *Staphylococci*. Grey-black colonies and a halo on this medium are presumed to be indicative of coagulase positive *Staphylococci* which are confirmed by doing coagulase test. | | |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven | | |

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| | 4. Autoclave<br>5. Incubator (Operating at 35°C ± 1°C or 37 °C ± 1°C, 25 °C ± 1°C ,44 °C ± 1°C and 50°C ± 1°C)<br>6. Water bath (at 44 °C to 47 °C ± 2 °C)<br>7. pH meter with measuring accuracy ±0.1<br>8. Microscope<br>9. Refrigerator (at 2 °C– 8 °C)<br>10. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>11. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>12. Micropipette with tips<br>13. Tubes and glass bottles<br>14. Vortex<br>15. Mechanical stirrer<br>16. Spreader (glass or plastic)<br>17. Inoculation loops and straight wire<br>18. Spiral plater/rotator<br>19. Inoculation loops and straight wire<br>20. Colony counter |
| **Culture Media and Reagents** | • Primary Diluent saline peptone water (SPW) or buffered peptone water (BPW)<br>• Baired Parker Agar (BPA) Medium<br>• Potassium tellurite solution<br>• Egg Yolk Medium<br>• Sulfamezathine Solution<br>• Brain Heart Infusion Broth (BHIB)<br>• Coagulase (Rabbit) Plasma<br><br>For media preparation and other details refer Chapter 3 |
| **Reference Cultures** | Staphylococcus aureus |
| **Sample Preparation** | Refer Chapter 2 |
| **Procedure** | **Preparation of the samples and serial dilutions**.<br>a). Following the procedures described in Chapter 2, make a 1:10 |

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| | dilution of the well mixed sample, by aseptically transferring sample to the desired volume of diluents saline peptone water (SPW) or buffered peptone water (BPW). Aseptically weigh 10 gm /10ml of solid or semi-solid sample into a sterile blender jar or into a stomacher bag. Add 90 mL of sterile diluent.<br>Prepare subsequent serial dilutions as required by pipetting 1 ml of previous dilution into a subsequent tube containing 9mL of the diluent. Shake each dilution. Vortex mixing can be performed to prepare homogenate.<br>Certain types of product result in viscous or thick initial suspensions when prepared with the usual 1 in 10 dilution and additional diluent may be necessary to facilitate further testing. In such cases, the diluent shall be added in other ratios (e.g. 1 in 20, 1 in 50, 1 in 100) until a satisfactory initial suspension for further operations is achieved. These non-standard ratios shall be taken into account in subsequent operations, particularly in the calculation and expression of results.<br>**Inoculation and Incubation**<br>**<u>BPA plates</u>**<br>Pour about 15 ml to 20 ml of the Baird- Parkar Agar Medium (BPA) at 44 °C to 47 °C into each Petri dish and allow it to solidify.<br>Thepaltes may be stored at 5 °C ± 3 °C for upto 24 h<br>Immedietletly before use, these agar plates should be dried<br>Label all petri plates with the sample number, dilution, date and any other desired information.<br>**Inoculation**<br>• Take two BPA plate prepared previously. Using sterile pipette/auto pipette, spread 0.1ml of test sample (liquid or primary suspension if prepared).<br>• Similarly prepare plates from subsequent dilution as required.<br>• Limit of detection can be raised by a factor of 10 by spreading 1 ml of liquid sample/primary suspension in case of food sample, on the surface of 3 petri dishes of 90 mm or using a single 140 mm petri dish. |

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| | • Carefully spread the inoculums as quickly as possible over the surface of agar medium, using the spreader, trying not to touch the sides of agar plate.<br>• Allow all the plates to dry with their lids on for 15 min at laboratory temperature.<br>**Incubation**<br>• Invert all petri dishes and incubate at35°C±1°C or 37°C±1°C for 24±2h. Then reincubate for a further 24h±2h.<br>**Selection of plates and Observation**<br>• Observe all plates for presence of typical or atypical colonies.<br>• Typical colonies appear as 1-2.5 mm diameter shiny black/grey color and surrounded by clear zone which may be partially opaque. Atypical colonies may appear as grey colonies free of clear zone or shiny black colonies with or without narrow white edge and free from clear zone.<br>**Confirmation**<br>• Select 5 typical and 5 atypical colonies from each plate selected for enumeration.<br>• In case of low count obtained (less than 15) on lowest dilution, select all colonies for confirmation.<br><br>**Coagulase test**<br>From the surface of each selected colony, transfer an inoculum with sterile inoculation loop/wire and transfer it to a tube of BHI broth. Incubate at 37°C±1°C for 24±2h. After that aseptically add 0.1 ml of this culture to a sterile test tube and add 0.3 ml of the rabbit plasma (or specified by manufacturer) and incubate at 37°C or 37°C ± 1°C. Put up conrol test tube wihout Rabbit plasma Examine for clotting of the plasma after 4 to 6h of incubation by tilting the tube, if the test is negative, re-examine at 24h of incubation. Consider the coagulase test to be positive if the volume of clot occupies more than half of original volume of the liquid. Control plasma shall show no sign of clotting. |
| **Calculation** | 1. Select petri dishes having 15 to 150 typical/atypical colonies for |

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| | enumeration.<br>2. Spreading colony shall be considered as single colony.<br>3. If a 1 ml inoculum was spread over three plates, treat these plates as one in all subsequent counting and confirmation procedures.<br>4. Use the following formula for calculation<br>$$N = \frac{\sum C}{[(1\times n_1)+(0.1\times n_2)\times(d)}$$<br>N is Number of colonies per ml or g of product<br>$\sum$ C is the sum of colonies counted on all the dishes retained<br>$n_1$ is the no. of dishes retained in the first dilution<br>$n_2$ is the no of dishes retained in the second dilution<br>d is the dilution factor corresponding to first dilution<br>5. In case petri dishes have typical/atypical colonies less than 15, calculate the results using the following formula:<br>N= C/ (v × d)<br>N is Number of colonies per ml or g of product<br>C is the average of colonies on the petri dishes retained<br>v is the volume of inoculums used in each dish<br>d is the dilution corresponding to the dilution retained. |
| **Expression of Results** | • Results shall be expressed as a number between 1.0 and 9.9 multiplied by 10x, where x is power of 10.<br>• In case of 0.1ml inoculum, if plates of initial dilution have no colonies, the result is expressed as less than 10 cfu/ml or 100 cfu/g or mL (if primary suspension prepared)<br>• In case of 1ml inoculum, if plates of initial dilution has no colonies, the result is expressed as less than 1 cfu/ml or 10 cfu/g or mL (if primary suspension prepared) |
| **Reference** | 1) ISO 6888-1:1999/AMD 1 (2003): Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) — Part 1: Technique using Baird-Parker agar medium — Amendment 1: Inclusion of precision data.<br>2) IS 5887-8-1 (2002): Methods for Detection of Bacteria Responsible |

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| | fo Food Poisoning, Part 8: Horizontal Method for Enumeration of Coagulase-Positive Staphylococci (Staphylococcus aureus and other Species), Section 1: Tecnique Using Baird-Parker Agar Medium. |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Detection of Coagulase Positive *Staphylococci*based on ISO 6888-2 and IS 5887- Part 8 (Sec 2)** | | |
| **Method No.** | FSSAI 15.021:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | *Staphylococci* are aerobic Gram-positive bacteria, with diameters of 0.5 – 1.5 µm and characterized by individual cocci, in grape-like clusters. They are non-motile, non-spore forming facultative anaerobesSome of species of Staphylococcus genera have ability to produce coagulase enzyme i.e *Staphylococcus aureus, Staphylococcus hyicus, Staphylococcus intermedius* etc. Coagulase positive *Staphylococci*produce Enterotoxins, which is responsible for food-poisoning if contaminated food is consumed.*S. aureus* is the species with the more pathogenic of the genus *Staphylococcus.* | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good laboratory practices to avoid cross contamination. | | |
| **Principle** | Pre enrichment of *Staphylococci* is carried out in salt medium. High salt content inhibit the growth of most of contaminating micro flora. After enrichment selective isolation is done on Baird Parker agar (BPA) and sheep blood agar. BPA contain Lithium chloride and potassium tellurite which inhibit most of the contaminating micro flora except *Staphylococci*. *Staphylococci* can reduce tellurite to telluride, which results in grey to black coloration of the colonies. With the addition of egg yolk, the medium becomes yellow, slightly opaque. A clear halo develops around colonies from coagulase positive *Staphylococci*. Grey-black colonies and a halo on this medium are presumed to be indicative of coagulase positive *Staphylococci* which | | |

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| | are confirmed by doing coagulase test. On blood agar, colonies appear as golden yellow colour with haemolytic activity. |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven<br>4. Autoclave<br>5. Incubator (Operating at 37 °C ± 1°C))<br>6. Water bath (at 44 °C to 47 °C)<br>7. pH meter with measuring accuracy ±0.1<br>8. Microscope and glass slides<br>9. Refrigerator (at 2 °C– 8 °C)<br>10. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>11. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>12. Micropipette with tips<br>13. Tubes and glass bottles<br>14. Vortex<br>15. Mechanical stirrer<br>16. Spreader (glass or plastic)<br>17. Inoculation loops and straight wire<br>18. Spiral plater/rotator<br>19. Inoculation loops and straight wire |
| **Culture Media and Reagents** | • Primary Diluent<br>• Baired Parker Agar (BPA)<br>• Sheep Blood Agar<br>• Nutrient Agar<br>• Cooked Meat Salt Medium<br>• Gram Stain kit<br>• Coagulase (Rabbit) Plasma<br>For media preparation and other details refer Chapter 3 |
| **Reference Cultures** | *Staphylococcus aureus* |
| **Sample Preparation** | Refer Chapter 2 |
| **Procedure** | **Enrichment**<br>1) Inoculate specified amount of food sample/primary suspension |

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| | prepared into blood agar and cooked meat salt medium (1:9) and on Baird Parker medium.<br>2) Incubate the blood agar and salt medium at 37 °C for 18-24 hr and Baird Parker medium at 37 °C for 30 h<br><br>**Selective Isolation**<br>Streak a loopful from primary suspension prepared from cooked salt medium onto BPA and sheep blood agar and incubate BPA plate at 37 °C for at least 30 hr and sheep blood agar plate at 37 °C for 18-24 hr.<br>**Observation**<br>On sheep blood agar, typical colonies appear as yellow color surrounded by clear zone of haemolysis.<br>On BPA, typical colonies appear as 1-2.5 mm diameter shiny black/grey color and surrounded by clear zone which may be partially opaque. Atypical colonies may appear as grey colonies free of clear zone or shiny black colonies with or without narrow white edge and free from clear zone.<br>**Confirmation**<br>Pick 5 suspected colonies from each plate and streak on nutrient agar, prior to confirmation. Perform Gram staining and coagulase test for confirmation.<br>**Gram Staining:** If there are typical/suspected colonies, pick up the colonies and confirm by gram staining. *Staphylococci* are Gram positive cocci which appear in clusters.<br>**Coagulase test:** The test may be performed using one of following method.<br>**(i) Slide method**: Emulsify a portion of suspected colony in normal saline or water on a clean slide. Mix it with a straight wire dipped in rabbit plasma. Coagulase positive *Staphylococci* produce visible clumping immediately.<br>Positive control with a known coagulase positive strain of *S. aureus* and a control of rabbit plasma without inoculum should be included in the test. |

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| | In case of nergative result, perform the test using tube method.<br>**(ii) Tube method**: Emulsify a portion of suspected colony from 24 hr growth on blood agar in 1 ml citrated rabbit plasma diluted 1 in 5 in 0.85% saline. Incubate at 37 °C preferably in a water bath or incubator. Observe every hour to observe clotting of plasma. Positive control with a known coagulase positive strain *S. aureus* and a control of rabbit plasma without inoculum should be included in the test. |
| **Expression of Results** | Results shall be expressed as Present/Absent per x ml/gm, where x is quantity of food sample enriched in salt medium. |
| **Reference** | 1) IS 5887-8-2 (2002): Methods for Detection of Bacteria Responsible for Food Poisoning, Part 8: Horizontal Method for Enumeration of Coagulase-Positive Staphylococci (Staphylococcus Aureus and other species), Section 2 Technique using rabbit plasma fibrinogen agar medium<br>2) ISO 6888-2 (1999): Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species) — Part 2: Technique using rabbit plasma fibrinogen agar medium |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Determination of *Vibrio cholera and Vibrio parahaemolyticus*** | | |
| **Method No.** | FSSAI 15.022:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | The members of the genus Vibrio are primarily aquatic bacteria, found both in freshwater and in seawater, in addition to being frequently associated with marine animals. Several species cause diarrhea or infections of the gastrointestinal tract but the most frequent enteric pathogens are *Vibrio cholerae* and *Vibrio parahaemolyticus*.<br>Cholera, a disease caused by strains of *V. cholerae* of serotypes O1 and O139, is classified by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002) into risk group IA: "diseases of severe hazard for general population; life threatening or resulting in substantial chronic sequelae or presenting effects of long duration".<br>The disease caused by *V. parahaemolyticus* is classified by the International Commission on Microbiological Specifications for Foods (ICMSF, 2002 ) into risk group III: "diseases of moderate hazard usually not life threatening, normally of short duration without substantial sequelae, causing symptoms that are self-limiting but can cause severe discomfort. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Special Caution** | In order to safeguard the health of laboratory personnel, it is essential that tests for detection of Vibrio spp., and particularly toxigenic *Vibrio cholerae*, *Vibrio parahaemolyticus* be conducted only in laboratories equipped for this purpose and under the supervision of an experienced microbiologist, and that great care is exercised in the disposal of contaminated material. | | |
| **Principle** | The detection of potentially enteropathogenic *Vibrio spp*. (*V. cholera, Vibrio parahaemolyticus*) requires four successive phases. Recovery of | | |

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| | certain *Vibrio spp*. from foodstuffs may be improved by the use of different incubation temperatures depending upon the target species or state of the food matrix. For example, recovery of *V. cholera and Vibrio parahaemolyticus* in fresh products is enhanced by enrichment at 41.5 °C whereas for deep frozen, dried or salted products, recovery is enhanced by enrichment at 37 °C.<br><br>If detection of *V. cholerae* and *Vibrio parahaemolyticus*is required, all specified incubation temperatures should be used. If detection of *V. cholera* and *Vibrio parahaemolyticus* together is not required, the specific procedure(s) maybe selected according to the species being sought. |
| **Equipment** | 1. Laminar airflow<br>2. Biosafety cabinet<br>3. Hot air oven<br>4. Autoclave<br>5. Incubator (Operating at 37°C ± 1°C)<br>6. Incubator (Operating at 41.5°C ± 1°C)<br>7. Water bath (at 44 °C to 47 °C)<br>8. pH meter with measuring accuracy ±0.1pH units at 25°C<br>9. Microscope<br>10. Refrigerator (at 2 °C– 8 °C)<br>11. Freezer, adjustable to <−15 °C<br>12. Micro-centrifuge tubes (capacity of 1.5 ml and 2.0 ml).<br>13. Micro-centrifuge, for reaction tubes (capacity of 1.5 ml and 2.0 ml) and capable of running at 10 000*g*.<br>14. Heating block (at 95 °C ± 2.0 °C).<br>15. Graduated pipettes and pipette filter tips, for volumes between 1 µl and 1000 µl.<br>16. Associated consumables for conventional or real-time PCR, e.g. optical plates and caps, optical plate holder, suitable for use with the selected PCR machine.<br>17. Conventional or real-time PCR machine, gel electrophoresis and UV |

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| | visualization equipment as appropriate.<br>18. Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>19. Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>20. Micropipette with tips<br>21. Tubes and glass bottles<br>22. Vortex<br>23. Mechanical stirrer<br>24. pH meter with measuring accuracy ±0.1<br>25. Spreader (glass or plastic)<br>26. Inoculation loops and straight wire<br>27. Spiral plater/rotator<br>28. Inoculation loops and straight wire |
| **Culture Media and Reagents** | • Enrichment medium: alkaline saline peptone water (ASPW)<br><br>***<u>Solid selective isolation media</u>***<br>• First medium: Thiosulphate Citrate Bile and Sucrose agar medium (TCBS)<br>• Second medium: The selection of the second medium is left to the choice of the test laboratory. Preparation of the medium should be strictly according to the manufacturer's instructions. (Saline nutrient agar) Reagent for detection of oxidase<br><br>***<u>Biochemical tests</u>***<br>• L-lysine decarboxylase saline medium (LDC)<br>• Arginine dihydrolase saline medium (ADH)<br>• Reagent for detection of β-galactosidase<br>• Saline medium for detection of indole<br>• Saline peptone waters<br>• Sodium chloride solution<br>• Oxidase Test<br>• Saline Nutient agar |

<table>
<tr><td></td><td>Details of preparation given in Chapter 3</td></tr>
<tr><td>Reference Cultures</td><td>Further details of culturing given in Chapter 3</td></tr>
<tr><td>Sample Preparation</td><td>Sample preparation based on the product categories are given in Chapter 2</td></tr>
<tr><td>Procedure</td><td>
Test portion and initial suspension<br>
Primary selective enrichment:<br>
Take 25 g or 25 ml of sample and homogenize in 225 ml of alkaline saline peptone water (ASPW). Incubate the flasks in the following conditions:<br>
Fresh products at 41.5 ±1°C for 6 ±1 hours;<br>
Processed (deep frozen, dried or salted) products at 37 ±1°C for 6 ±1 hours.<br><br>
<table>
<tr><td colspan="3">Target Vibrio spp. in fresh product</td></tr>
<tr><td>Incubation temperature</td><td>Vibrio parahaemolyticus</td><td>Vibrio cholerae</td></tr>
<tr><td>41.5 °C ± 1 °C</td><td>√</td><td>√</td></tr>
<tr><td>37 °C ± 1 °C</td><td>------</td><td>------</td></tr>
<tr><td colspan="3">Target Vibrio spp. in deep frozen, dried or salted product (such as bacalhau, stock fish, bonefish, katsuobushi, obambo)</td></tr>
<tr><td>Incubation temperature</td><td>Vibrio parahaemolyticus</td><td>Vibrio cholerae</td></tr>
<tr><td>41.5 °C ± 1 °C</td><td>-----</td><td>------</td></tr>
<tr><td>37 °C ± 1 °C</td><td>√</td><td>√</td></tr>
</table>
Primary incubation and target species/product state<br><br>
Secondary selective enrichment<br>
Transfer 1 ml of the Pre-enrichment Culture obtained from the surface into a tube containing 10 ml of ASPW. It is recommended that the sample is not agitated before taking the aliquot. Incubate the ASPW at 41.5 °C ± 1 °C for 18 h ± 1 h according to table below.<br><br>
<table>
<tr><td>Target Vibrio spp. in all product states</td></tr>
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| Incubation temperature | *Vibrio parahaemolyticus* | *Vibrio cholerae* |
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| 41.5 °C ± 1 °C | √ | √ |

**Isolation and identification**

From the cultures obtained in the **Secondary selective enrichment** ASPW, inoculate with a 1 µl sampling loop the surface of two selective agar paltes.

First selective agar plate is TCBS agar plate, so as to permit the development of well-isolated colonies.

Second selective agar plate must be chosen by laboratory which is complementary to TCBS using a fresh sampling loop.

Invert the agar plates and incubate as:

— for TCBS agar plates, incubate at 37 °C ± 1 °C for 24 h ± 3 h;

— for the second isolation medium, incubate according to the manufacturer's instructions.

After incubation, examine the TCBS and second selective medium for the presence of typical colonies of presumptive pathogenic *Vibrio spp.* Mark their position on the bottom of the plates.

On TCBS agar *V. parahaemolyticus*, and *V. cholerae* exhibit different typical colony morphologies:

— typical colonies of *V. parahaemolyticus* is smooth, green (negative sucrose) and of 2 mm to 3 mm in diameter;

— typical colonies of *V. cholerae* are smooth, yellow (positive sucrose) and of 1 mm to 2 mm in diameter.

For the second selective medium, examine for the presence of colonies, which, according to their characteristics, may be considered as possible isolates of *V. parahaemolyticus*, and/or *V. cholerae.*

**Confirmation**

Using this manual, *V. parahaemolyticus*, and *V. cholerae* can be confirmed by molecular PCR and/or biochemical approaches. Confirmation may be carried out at the end user laboratory or by a

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| | specialist reference laboratory. Not all *V. parahaemolyticus* possess pathogenicity traits. In order to confirm the pathogenic character of the strains, it is preferable to detect the presence of thermostable direct haemolysin (*tdh*) or TDH- related haemolysin (*trh*) genes. This should be carried out using PCR tests. PCR based confirmation also may reduce the subjective interpretation of biochemical identification tests and accelerate the identification process.<br><br>If shown to be reliable, commercially available biochemical test kits (must be approved by FSSAI under RAFT scheme) may be used to identify *Vibrio* to the species level provided they are inoculated with a suspension of the bacteria to be identified in a sufficiently saline medium or dilution fluid, and as long as the database or identification table for the product has been based on reactions obtained using similar media to those described in this document. These kits shall be used in accordance with the manufacturer's instructions.<br>Commercially available molecular detection kits (must be approved by FSSAI under RAFT scheme) may be used to identify *Vibrio* to the species level. These kits shall be used in accordance with the manufacturer's instructions.<br><br>**Selection of colonies for confirmation and preparation of pure cultures**<br>For confirmation, subculture from each selective medium at least one well isolated colony considered to be typical or similar to each of the potentially pathogenic *Vibrio spp.* sought. If the result of the first isolated colony tested is negative, a further four well isolated colonies should be tested. In samples where it is considered important to optimize the detection of potentially pathogenic *V. parahaemolyticus* based upon the presence of thermostable direct and thermostable direct related haemolysins, it is recommended that at least five and, where possible, all colonies exhibiting typical *V. parahaemolyticus* colony morphology are sub-cultured for downstream testing. Inoculate the colonies selected onto the surface of plates of saline nutrient agar (SNA) or suitable |

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| | medium of the laboratory's choice to obtain isolated colonies. Incubate at 37 °C ± 1 °C for 24 h ± 3 h.<br><br>**Tests for presumptive identification**<br>**Oxidase test**<br>Using an inoculating loop (platinum iridium straight wire) or glass rod, take a portion of the pure culture from the saline nutrient agar and streak onto the filter paper moistened with oxidase reagent or use a commercially available test, following the manufacturer's instructions. Neither a nickel-chromium sampling loop nor a metallic wire shall be used. Nickel or chrome wire sampling loops may give false-positive results, and should be avoided.<br><br>**Microscopic examination (optional)**<br>a) For each pure culture obtained, perform gram staining. Observe under a microscope and record the results.<br>b) Inoculate a tube of ASPW. Incubate at 37 °C ± 1 °C for 1 h to 6 h. Perform motility using concave glass slide, and examine for the motility under the microscope.<br>c) Note *Vibrio* cultures showing a positive result for motility.<br><br>**Selection of the cultures**<br>For confirmation, retain the oxidase-positive colonies.<br>For confirmation, also retain Gram-negative colonies which give a positive result in the motility test (if examined).<br><br>**Biochemical confirmation**<br>**Halotolerance test:** Prepare a suspension from each SNA cultures and inoculate a series of tubes of Halotolerance Saline Peptone Water (0, 2, 6, 8 and 10% NaCl). Incubate the tubes at 37 ± 1°C/24 ± 3 h. Growth (tolerance) at the corresponding NaCl concentration present in the tube is indicated by turbidity.<br>**L- Lysine decarboxylase and Arginine dehydrolasetests**: From each SNA cultures inoculate a tube of Saline Decaboxylase Broth with 0.5% |

of L-Lysine and a tube of Saline Decaboxylase Broth with 0.5% of Arginine. Cover both tubes with a layer (1 ml) of sterile mineral oil and incubate at 37 ± 1°C/24 ± 3 h. A violet color after incubation indicates a positive reaction. A yellow color indicates a negative reaction.

**β-Galactosidase test:** From each SNA cultures inoculate a tube containing 0.25 ml of sterile 1% aqueous NaCl solution. Add one drop of toluene, shake and incubate at 37 ± 1°C/5 min (water bath). Add 0.25 ml of the β-Galactosidase reagent (ONPG reagent), mix and incubate at 37 ± 1°C/24 ± 3 h (water bath). Examine the tubes periodically for the development of a yellow color (often after 20 min) indicative of positive reaction. If the yellow color is not observed after 24 h the test is considered negative.

**Indole test**: From each SNA cultures inoculate a 5 ml tube of Saline Tryptophan Broth and incubate at 37 ± 1°C/24 ± 3 h. Test for indole by adding 1 ml of Indole Kovacs Reagent to each 5 ml culture. Appearance of distinct red color in upper layer is a positive test. A yellow brown color is a negative test.

**Interpretation of biochemical tests**

| **Test** | ***Vibrio cholerae*** [a] | ***Vibrio parahaemolyticus*** [a] |
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| Oxidase | + | + |
| LDC(**Lysine decarboxylase**) | + | + |
| ADH (Arginine **dehydrolase**) | - | - |
| ONPG hydrolysis (**β-Galactosidase)** | + | - |
| Production of indole | + | + |
| Growth in peptone water with | | |
| 0 % NaCl | + | - |

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| | <table><tr><td>6 % NaCl</td><td>-</td><td>+</td></tr><tr><td>10 % NaCl</td><td>-</td><td>-</td></tr><tr><td colspan="3">[a] The sign + denotes 76 % to 89 % positive reactions. b Provided for reference purposes.</td></tr></table><br>• The reactions given in the table above are a guide to the identification of the listed species. Additional phenotypic tests can be required to fully distinguish these species from each other and from non pathogenic *Vibrio* spp.<br>• It is preferable to conduct serology for *V. cholerae* (at least to determine whether they are serogroups O1 or O139) or an appropriate PCR – based test to determine toxigenic strains such as those that carry the cholera toxin gene ctx. This can be carried out by the end user laboratory or a specialist reference laboratory. |
| **Expression of Results** | Depending on the interpretation of results, indicate that potentially enteropathogenic *Vibrio* spp. Is detected or not detected in a test portion of *x* grams or *x* ml of product, specifying the name of the relevant species and any pathogenicity characteristics if they have been tested. |
| **Reference** | ISO 21872-1- Microbiology of the food chain — Horizontal method for the determination of *Vibrio* spp. — Part 1: Detection of potentially enteropathogenic *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus* |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Enumeration of Yeast and Mould** | | |
| **Method No.** | FSSAI 15.023:2023 | **Revision No. & Date** | 0.0 |
| **Introduction** | Yeast and moulds are classified under the Kingdom Fungi. Yeast and mould are heterotrophic in nature and of great importance to the food industry. Yeast and mould can cause various degree of spoilage in food chain. Acid/alkaline requirements for yeast and mold growth in wide range food products are quite broad, ranging from pH 2 to above pH 9. | | |
| **Scope** | This method is applicable to those food product categories and their sub-categories as mentioned in the Appendix B tables of Food Safety and Standards (Food Products Standards and Food Additives) Regulations, 2011 and amendments (Gazette notifications) issued from time to time. | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good laboratory practices to avoid cross contamination. | | |
| **Principle** | Chloramphenicol Yeast Glucose Agar is a selective medium recommended for isolation and enumeration of Yeast & Moulds. The medium contains yeast extract, which provides nitrogenous nutrient and vitamin B complex. Dextrose is the energy source. Chloramphenicol a thermos table antibiotic, suppresses the bacterial flora. Aerobic incubation of plates is done at 25 °C ± 1 °C and count is taken on 3rd, 4th or 5th day. | | |
| **Equipment** | 1. Laminar airflow<br>2. Hot air oven<br>3. Autoclave<br>4. Incubator (Operating at 25 °C ± 1°C)<br>5. Water bath (at 44 °C to 47 °C)<br>6. pH meter with measuring accuracy ±0.1pH units at 25°C.<br>7. Microscope<br>8. Refrigerator (at 2 °C– 8 °C) | | |

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| | 9. Petri dishes (Glass or plastic of 90-100mm diameter Graduated pipettes (0.1 ml divisions) of capacity 1 ml (Class A)<br>10. Micropipette with tips<br>11. Tubes and glass bottles<br>12. Vortex<br>13. Mechanical stirrer<br>14. pH meter with measuring accuracy ±0.1<br>15. Spreader (glass or plastic)<br>16. Inoculation loops and straight wire<br>17. Spiral plater/rotator<br>18. Inoculation loops and straight wire<br>19. Colony counter |
| **Culture Media and Reagents** | • Primary Diluent<br>• Chloramphenicol Yeast Glucose Agar (CGYEA)<br>• Sodium Propionate<br>For media preparation and other details refer Chapter 3 |
| **Reference Cultures** | *Candida albicans, Saccharomyces, Aspergillus niger* |
| **Sample Preparation** | Refer Chapter 2 |
| **Procedure** | **Preparation of the samples and serial dilutions:** Follow the procedures as described in Chapter 2. Homogenize 10 g of sample with 90 ml of saline peptone water (SPW) or buffered peptone water (BPW) to make ($10^{-1}$ dilution). From this first dilution prepare serial decimal dilutions<br><br>Prepare subsequent serial dilutions as required by pipetting 1 ml of previous dilution into a subsequent tube containing 9ml of the diluent. Shake each dilution. Vortex mixing can be performed to prepare homogenate.<br><br>**Inoculation** |

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| | Label all petri dishes with the sample code, dilution, date and any other information.<br>**A) Spread plate**<br>Take plate of Chloramphenicol Yeast Glucose Agar prepared previously. Using sterile pipette/auto pipette, spread 0.1ml of test sample (liquid or primary suspension if prepared) over the surface of medium plate. Similarly prepare plates from subsequent dilution as required.<br>• Carefully spread the inoculums as quickly as possible over the surface of agar medium, using the spreader, trying not to touch the sides of agar plate.<br>• Allow all the plates to dry with their lids on for 15 min at laboratory temperature.<br><br>**B) Pour Plate**<br>• Transfer 1ml of test sample if the product is liquid, or 1 ml of primary suspension if prepared using a sterile pipette to a Petri plate in duplicate. Similarly prepare plates from subsequent dilution as required. If only yeast colonies are required, add 0.25% sodium propionate solution to the plate at the time of pouring to inhibit the growth of moulds. Pour approximately 15 to 20 ml molten Chloramphenicol Yeast Glucose Agar (CGYEA) agar (cooled at 44 $^0$C to 47 $^0$C in water bath).<br>Time elapse between inoculation and addition of agar into plates should not exceed 15 min. The total time between the end of the preparation of the initial suspension and pouring of plates (dishes) shall not exceed 45 min.<br>Carefully mix the inoculum with the medium by horizontal movements and allow to solidify.<br><br>**Incubation**<br>Incubate all the petri dishes at 25 °C ± 1 °C for 3 to 5 and 7 days in upright position. |

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| | **Observation**<br>Count the colonies on each plate after 3, 4 and 5 days of incubation. Yeast colonies grow as smooth, moist, elevated or surface colonies. Mould colonies grow as fuzzy, thread like profuse growth of hyphae and may appear in different color. |
| **Calculation** | 1. Select the Petri dishes having 10 to 150 colonies for enumeration.<br>2. Use the following formula for calculation<br>$$N = \frac{\sum C}{[(1 \times n_1) + (0.1 \times n_2) \times (d)}$$<br>N -Number of colonies per ml or g of product<br>$\sum$ C - Sum of colonies counted on all the dishes retained.<br>$n_1$ - Number of dishes considered for the first dilution<br>$n_2$ - Number of dishes considered forthe second dilution<br>d -Dilution factor corresponding to first dilution<br>3. In case Petri dishes have total/typical colonies less than 10, calculate the results using the following formula:<br>N= C/(v × d)<br>N -Number of colonies per ml or g of product<br>C -Average of colonies on the Petri dishes retained.<br>v -Volume of inoculum used in each dish.<br>d -Dilution from which first counts were obtained. |
| **Expression of Results** | • Results shall be expressed as number between 1.0 and 9.9 multiplied by 10x, where x is power of 10.<br>• If plates from all dilutions have no colonies, the result is expressed as less than 1 cfu/ml or 10 cfu/g or ml (if primary suspension prepared) |
| **Reference** | 1) ISO 6611 (2004) [IDF 94:2004] Milk and milk products — Enumeration of colony-forming units of yeasts and/or moulds — Colony-count technique at 25 degrees C<br>2) IS 5403: 1999 (RA 2013) Method for Yeast and Mould Count of Foodstuffs and animal feeds<br>3) ISO 21527-1 (2008): Microbiology of food and animal feeding |

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| | stuffs — Horizontal method for the enumeration of yeasts and moulds — Part 1: Colony count technique in products with water activity greater than 0.95<br>4) ISO 21527-2 (2008): Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and moulds — Part 2: Colony count technique in products with water activity less than or equal to 0.95 |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

# Part C

# ANALYSIS OF WATER

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| FSSAI FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA<br>Inspiring Trust, Assuring Safe & Nutritious Food<br>Ministry of Health and Family Welfare, Government of India | **Method for Enumeration of Aerobic Microbial count/Standard Plate Count** | | |
| **Method No.** | FSSAI 15.024:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good microbiology laboratory practices to avoid cross contamination. | | |
| **Principle** | Plate count agar (PCA) is a general purpose growth medium commonly used to assess "total" or "viable" bacterial growth of a water sample. The number of microorganisms per milliliter of sample is calculated from the number of colonies obtained on PCA plate from selected dilution. Poured plates are prepared using a specified culture medium and a specified quantity of the sample. The plates are aerobically incubated at two different temperatures i.e. 37 °C for 24 hr. and 20 – 22 °C for 72 hr. | | |
| **Apparatus/Instruments** | a) Laminar Air flow / Biosafety Cabinet<br>b) Universal Incubator (37 °C), and BOD Incubator 20± 2 °C)<br>c) Thermostatically controlled water bath (capable of being maintained at 44 °C to 47 °C)<br>d) Autoclave<br>e) Sterilizing oven<br>f) Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>g) Total delivery graduated pipettes, of nominal capacity 1 mL, graduated in 0.1 mL divisions (Class A), or Micropipette with sterile tips<br>h) Mechanical stirrer<br>i) Tubes, flasks or bottles, of appropriate capacity<br>j) Colony-counting device (optional)<br>k) Sterile Glass or plastic spreaders | | |
| **Culture Media and Reagents** | 1) Plate count Agar (PCA)<br>2) Buffered Peptone water (BPW)<br>3) Overlay Medium | | |

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| | (Overlay medium : 12 to 18 g Agar + 1000 ml water) |
| **Preparation of Reagents** | Not Applicable |
| **Sample Preparation** | Not Applicable |
| **Method of analysis** | 1) Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution.<br>2) Aseptically inoculate 1 mL of the water sample using sterile pipette into sterile petri plates in duplicate in two sets. The petri plates should be labeled with the sample number, date and any other desired information.<br>3) Pour into each plate 15–18 mL of the molten sterilized PCA media (cooled to 44 °C–47 °C). Avoid pouring of molten medium directly onto the inoculum. Mix the media and inoculum by swirling gently clockwise and anti-clockwise so as to obtain homogenous distribution of inoculum in the medium.<br>4) Allow to cool and solidify. In case, where in sample microorganism having spreading colonies is expected, add 4 mL of overlay medium onto the surface of solidified plates.<br>5) After complete solidification, invert the prepared plates and incubate one set at 37 °C for 24 hr. and second set at 20 – 22 °C for 72 hr.<br>6) After specified incubation period count all colonies including pinpoint colonies. Spreading colonies shall be considered as single colony. If less than one quarter of dish is overgrown by spreading, count the colonies on the unaffected part of the dish and calculate corresponding number in the entire dish. If more than one quarter is overgrown by spreading colonies discard the plate. |
| **Calculation with units of expression** | **Case 1:** Plates having microbial count between 10 and 300cfu<br>$N = \frac{ColoniesPlate1 + ColoniesPlate2}{2}$<br>**Case 2:** Plates having microbial count less than 10cfu but at least 4.<br>**Calculate the results as given in Case 1**.<br>**Case 3:** If microbial load is from 3 to 1 then reporting of results shall be:<br>**"Microorganisms are present, but, less than 4 per mL"** |

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| | **Case 4:** When the test sample/plates contains no colonies then reporting of results shall be:<br>**"Less than 1 cfu/ mL"**. |
| **Inference (Qualitative Analysis)** | NA |
| **Reference** | 1) IS 5402:2012 - Microbiology of Food and Animal Feeding Stuffs — Horizontal Method for the Enumeration of Microorganisms — Colony-Count Technique at 30°C (Second Revision)<br>2) ISO 7218:2007 Amd. 1: 2013 - Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations — Amendment 1<br>3) IS 14543:2016 - Packaged Drinking Water (Other Than Packaged Natural Mineral Water) - Specification (Second Revision) |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| FSSAI FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA<br>Inspiring Trust, Assuring Safe & Nutritious Food<br>Ministry of Health and Family Welfare, Government of India | **Method for Detection of *Escherichia coli* and Coliform** | | |
| **Method No.** | FSSAI 15.025:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good microbiology laboratory practices to avoid cross contamination. | | |
| **Principle** | A test portion of water sample is passed through a membrane filter, which is then placed for incubation on Chromogenic coliform agar plate. All presumptive coliform bacteria grow as pink to red colonies which are than confirmed by negative oxidase test. Due to β-D-galactosidase and β-D-glucuronidase activity *E. coli* bacteria appear as Dark blue to violet in color. This method is not applicable to β-D-glucuronidase negative *E. coli* strains like *E.coli* O157. | | |
| **Apparatus/Instruments** | a) Protective cabinet with vertical laminar airflow<br>b) Membrane Filtration Assembly<br>c) Incubators (36±2°C)<br>d) Thermostatically controlled water bath (capable of being maintained at 44 °C to 47 °C)<br>e) Autoclave<br>f) Sterilizing oven<br>g) Petri dishes (Glass or plastic of 90 – 100mm diameter or 140mm)<br>h) Tubes, flasks or bottles, of appropriate capacity<br>i) Colony-counting device (optional) | | |
| **Culture Media and Reagents** | a) Chromogenic coliform Agar (CCA)<br>b) Tryptone Soya agar (Soyabean Casein digest agar) | | |
| **Preparation of Reagents** | a) Oxidase Reagent (1% α-nepthol in 95% ethanol and equal amount of para-amino diamethylaniline hydrochloride in water) or Oxidase Disc commercially available | | |
| **Sample Preparation** | Not Applicable | | |
| **Method of analysis** | a) Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve | | |

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| | uniform distribution<br><br>b) Filter the sample (required volume) through a sterile membrane filter (0.45µm pore size) and place the filter in CCA plate and Incubate overnight at 36±2°C for 18 – 24hr.<br><br>a) Count all pink to red colonies as presumptive coliform.<br>b) Confirm all presumptive colonies by negative oxidase test.<br>c) Count all dark blue to violet colonies as *E. coli*.<br>d) **Oxidase test:** Add2-3 drops of Oxidase reagent on to a filter paper in a Petridish. Take a colony to be confirmed by inoculation loop on pretreated filter paper. A positive reaction is indicated by the appearance of blue color within 30 s. commercially available oxidase disc can be used as an alternative<br>e) Further subculture the presumptive colonies on non-selective agar (Tryptone Soy Agar) and incubate at 36±2°C for 21±2 hr. to carry out oxidase test.<br>f) Consider all colonies giving negative oxidase reaction as Coliform. |
| **Calculation with units of expression** | Presence/Absence of *E. coli* and Coliform given after confirmation of presumptive colonies in the sample examined.<br>Test for *E.coli* and Coliform= Present/absent per X mL of sample |
| **Reference** | 1) ISO 9308-1:2014 Amd. 1:2016 - Water quality — Enumeration of Escherichia coli and coliform bacteria — Part 1: Membrane filtration method for waters with low bacterial background flora — Amendment 1<br>2) IS 15185:2016 - Water Quality - Detection and Enumeration of *Echerichia Coli* and Coliform Bacteria - Membrane Filtration Method For Water With Low Bacterial Background Flora (First Revision)<br>3) IS 14543:2016 - Packaged Drinking Water (Other Than Packaged Natural Mineral Water) - Specification (Second Revision)<br>4) IS 15188:2022 - Water quality — General requirements and guidance for microbiological examinations by culture |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| FSSAI FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA<br>Inspiring Trust, Assuring Safe & Nutritious Food<br>Ministry of Health and Family Welfare, Government of India | **Method for Detection of Coliform (Alternative method)** | | |
| **Method No.** | FSSAI 15.026:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good microbiology laboratory practices to avoid cross contamination. | | |
| **Principle** | A test portion of water sample is passed through a membrane filter, which is then placed for incubation on Violet red Bile lactose Agar plate. The medium is selective due to the presence of the inhibitors - bile salts and crystal violet. Crystal violet inhibits gram-positive microorganisms especially Staphylococci. Organisms which rapidly ferment lactose produce red colonies surrounded by red purple halo. Lactose non-fermenters and late lactose fermenters produce pale colonies. | | |
| **Apparatus/Instruments** | a) Protective cabinet with vertical laminar airflow<br>b) Membrane Filtration Assembly<br>c) Incubators (36±1°C)<br>d) Thermostatically controlled water bath (capable of being maintained at 44 °C to 47 °C)<br>e) Autoclave<br>f) Sterilizing oven<br>g) Petri dishes (Glass or plastic of 90 – 100mm diameter or 140mm)<br>h) Tubes, flasks or bottles, of appropriate capacity<br>i) Colony-counting device (optional) | | |
| **Culture Media and Reagents** | a) Violet Red Bile Lactose Agar (VRBL) | | |
| **Preparation of Reagents** | Not Applicable | | |
| **Sample Preparation** | Not Applicable | | |
| **Method of analysis** | a) Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution | | |

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| | b) Filter the sample (required volume i.e X) through a sterile membrane filter (0.45µm pore size) and place the filter in VRBL agar plate and Incubate overnight at 36±1°C for 24±2hr.<br>c) Coliform bacteria grow as purplish red colonies surrounded by a reddish zone of precipitated bile.<br>d) Count all purplish red colonies as coliform. |
| **Calculation with units of expression** | Presence/Absence of Coliform given in per unit of the sample examined.<br>Test for Coliform= Present/absent per X mL of sample |
| **Reference** | 1) IS 5401 (Part 1): 2012 - Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of coliforms: Part 1 colony count technique (Second Revision)<br>2) IS 14543:2016 - Packaged Drinking Water (Other Than Packaged Natural Mineral Water) - Specification (Second Revision)<br>3) IS 15188: 2022 - Water quality — General requirements and guidance for microbiological examinations by culture |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Determination of *Enterococci* (Faecal *Streptococci*)** | | |
| **Method No.** | FSSAI 15.027:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | a) Sodium Azide is highly toxic and mutagenic; precautions shall be taken to avoid contact with it, especially through the inhalation of fine dust during the preparation of media.<br>b) Azide containing media should not be mixed with strong inorganic acids, as toxic hydrogen azide ($HN_3$) may be produced.<br>c) Solution containing azide can also form explosive compounds when in contact with metal pipework e.g. from sinks. | | |
| **Principle** | Slantez and Bartley medium contains sodium azide (to suppress the growth of gram negative bacteria) and 2,3,5-triphenyltetrazolium chloride that is reduced to red formazon by Enterococci. Confirmation is done by transfer of membrane with all the colonies on Bile Aesculin Azide agar where asculin is hydrolyzed within 2 hr. to form 6,7-dihydroxycoumarin, and combines with iron (III) to give tan colored to black compound which diffuses into the medium. | | |
| **Apparatus/Instruments** | a) Protective cabinet with vertical laminar airflow<br>b) Membrane Filtration Assembly<br>c) Incubators (36±1°C, 44 ± 0.5°C)<br>d) Thermostatically controlled water bath (capable of being maintained at 44 °C to 47 °C)<br>e) Autoclave<br>f) Sterilizing oven<br>g) Petri dishes (Glass or plastic of 90 – 100mm diameter or 140mm)<br>h) Tubes, flasks or bottles, of appropriate capacity<br>i) Colony-counting device (optional) | | |
| **Culture Media and Reagents** | a) Slantez and Bartley medium<br>b) Bile-aesculin-azide agar | | |
| **Preparation of Reagents** | Not Applicable | | |

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| **Sample Preparation** | Not Applicable |
| **Method of analysis** | a) Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution<br>b) Filter the sample (requisite volume) through a sterile membrane filter (0.45µm pore size) and place the filter on Slantez and Bartley medium plates.<br>c) Incubate the plates at 36±2°C for 44±4hr. After incubation observe the plates showing red, maroon or pink colonies as presumptive *Enterococci*.<br>d) If there are presumptive colonies, confirmation can be done by transferring the membrane with sterile forceps without inverting it on plate of bile-aseculin-azide agar which has been preheated to 44°C.<br>e) Incubate at 44 ± 0.5°C for 2hr and observe the plates immediately.<br>f) Consider all colonies showing tan to black color as *Enterococci.* |
| **Calculation with units of expression** | Presence/Absence of *Enterococci* is given after confirmation on Bile - aesculin-azide agar and given as.<br>*Enterococci*: Present/Absent per X mL of sample. |
| **Reference** | 1) IS 15186:2002 - Water Quality - Detection and Enumeration of Intestinal Enterococci - Membrane Filtration Method<br>2) IS 14543:2016 - Packaged Drinking Water (Other Than Packaged Natural Mineral Water) - Specification (Second Revision)<br>3) IS 15188: 2022 - Water quality — General requirements and guidance for microbiological examinations by culture - |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| FSSAI FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA<br>Inspiring Trust, Assuring Safe & Nutritious Food<br>Ministry of Health and Family Welfare, Government of India | **Method for Detection of *Enterococci* (Faecal *Streptococci*) (Alternative method)** | | |
| **Method No.** | FSSAI 15.028:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | a) Sodium Azide is highly toxic and mutagenic; precautions shall be taken to avoid contact with it, especially through the inhalation of fine dust during the preparation of media.<br>b) Azide containing media should not be mixed with strong inorganic acids, as toxic hydrogen azide ($HN_3$) may be produced.<br>c) Solution containing azide can also form explosive compounds when in contact with metal pipework e.g. from sinks. | | |
| **Principle** | Ethyl violet azide dextrose Agar contain sodium azide and ethyl violet that inhibit gram-positive bacilli and gram-positive cocci other than *Enterococci*. Confirmation is done by Gram stain reaction and colony chracterstics on MacConkey Agar at 44°C. | | |
| **Apparatus/Instruments** | a) Protective cabinet with vertical laminar airflow<br>b) Membrane Filtration Assembly<br>c) Incubators (36±1°C, 44 ± 0.5°C)<br>d) Thermostatically controlled water bath (capable of being maintained at 44 °C to 47 °C)<br>e) Autoclave<br>f) Sterilizing oven<br>g) Petri dishes (Glass or plastic of 90 – 100mm diameter or 140mm)<br>h) Tubes, flasks or bottles, of appropriate capacity<br>i) Colony-counting device (optional) | | |
| **Culture Media and Reagents** | i. Ethyl Violet Azide Dextrose Agar<br>ii. MacConkey Agar<br>iii. Nutrient Agar | | |
| **Preparation of Reagents** | Not Applicable | | |

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| **Sample Preparation** | Not Applicable |
| **Method of analysis** | a) Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution<br><br>b) Filter the sample (requiste volume) through a sterile membrane filter (0.45µm pore size) and place the filter on Ethyl Violet Azide Dextrose Agar plates.<br><br>c) Incubate the plates at 37±1°C for 48hr. After incubation observe the plates showing dark red colonies or colonies having red or pink centres as presumptive *Enteroccci*.<br><br>d) If there are presumptive colonies, confirmation can be done by Gram stain reaction and colony chracterstics on MacConkey Agar at 44°C.<br><br>e) On MacConkey agar Enterococci appear as small pink colonies. Streak suspected or typical colonies on nutrient agar prior to Gram staining.<br><br>f) Enterococci usually appear as Gram positive cocci in pairs or short chains. |
| **Calculation with units of expression** | Presence/Absence of *Enterococci* is given after confirmation as.<br>*Enterococci*: Present/Absent per X mL of sample. |
| **Reference** | 1) IS 5887 (Part 2): 1976 - Methods for detection of bacteria responsible for food poisoning: Part 2 Isolation, identification and enumeration of staphylococcus aureus and faecal streptococci (First Revision)<br>2) IS 14543:2016 - Packaged Drinking Water (Other Than Packaged Natural Mineral Water) - Specification (Second Revision)<br>3) IS 15188: 2022 - Water quality — General requirements and guidance for microbiological examinations by culture |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Detection of *Salmonella*** | | |
| **Method No.** | FSSAI 15.029:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable Packaged Drinking water | | |
| **Caution** | In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Salmonella spp.* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist and that great care is taken in the disposal of all incubated materials. It is also the responsibility of the user to establish appropriate safety and health practices in the laboratory | | |
| **Principle** | Detection of *Salmonella* is based on pre-enrichment, selective enrichment followed by isolation on selective media. Presumptive *Salmonella* colonies are confirmed by biochemical and serological tests. Pre-enrichment broth is necessary to enable injured cells to grow. Further selective enrichment is done to increase the proportion of *Salmonella* in relation to background flora. Selective media are used for further isolation and preliminary confirmation. | | |
| **Apparatus/Instruments** | a) Protective cabinet with vertical laminar airflow<br>b) Incubators (36±2°C**,** 41.5±1.0°C**)**<br>c) Thermostatically controlled water bath (capable of being maintained at 44 °C to 47 °C)<br>d) Membrane filtration unit (Filter size 0.45 µ)<br>e) Autoclave<br>f) Sterilizing oven<br>g) Petri dishes (Glass or plastic of 90 – 100mm diameter or 140mm)<br>h) Total delivery graduated pipettes, of nominal capacity 1 mL, graduated in 0.1 mL divisions (Class A), or Micropipette with sterile tips<br>i) Mechanical stirrer<br>j) Tubes, flasks or bottles, of appropriate capacity | | |
| **Culture Media and Reagents** | a) Buffered peptone water<br>b) Rappaport-Vassiliadis (RVS) broth | | |

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| | c) Brilliant green/phenol red lactose agar (BGA) or any additional selective Medium<br>d) Xylose lysine deoxycholate agar (XLD)<br>e) Nutrient agar<br>f) MkTTn Broth |
| **Preparation of Reagents** | As per manufacturer instructions |
| **Sample Preparation** | Not Applicable |
| **Method of analysis** | a) Pre-enrichment: Aseptically clean the surface of bottle/pouch/cups containing sample with 70% ethanol and filter 250 mL (or as specified) of water sample through a membrane filter of 0.45µm pore size using sterile membrane filtration assembly. Place the filter disk in 50 mL buffered peptone water and incubate at 36±2°C for 16-20 hrs.<br>b) Selective enrichment: Transfer 0.1mL of the pre-enrichment culture to 10mL (RVS broth) and incubate at 41.5±1.0°C for 24±3h. Transfer 1 mL of the pre-enrichment culture to 10mL MkTTn Broth and incubate at 36±2.0°C for 24±3h. Confirmation on Selective agar media: After incubation streak a loopful from RVS broth on selective medium i.e. BGA and XLD. Incubate the plates at 36±2°C for 24±3 hr.<br>c) In order to detect slow growing *Salmonella* reinoculate BGA, XLD and BSA (optional) after continued incubation of RVS broth for further 24 hr.<br>d) **Observation:** Colonies on BGA are red or slightly pink-white and opaque with red surrounding. Colonies on XLD agar are colorless but appear red usually with a black centre. *Salmonella* $H_2S$ negative strains appear on XLD agar as pink with a darker pink center. Lactose positive *Salmonella* strains grow on XLD agar as yellow with or without blackening<br>e) **Confirmation:** If there are typical/suspected colonies, plate at least 1 selected colony from each positive agar medium and further four colonies if the first colony tests negative on nutrient agar plates and |

incubate at 36±2°C for 18-24 hr. and thereafter proceed for further biochemical & serological confirmation.

f) **Biochemical confirmation:**

i. **Lactose/Glucose fermentation & Hydrogen Sulphide formation:** Streak a colony on Iron/two-sugar agar (Kligler Iron agar slant) and stab the butt. Incubate at 36±2°C for 24 hr. Typical *Salmonella* show red slant with gas formation and yellow butts with blackening of agar.

ii. **Urea degradation:** Incubate a colony on slant of urea agar and incubate at 36±2°C for 24hr. Typical *Salmonella* culture show a negative reaction i.e. no rose pink color followed by deep cerise.

iii. **Lysine decarboxylase medium:** Inoculate a colony just below the surface of the liquid Lysine decarboxylase medium. Overlay the medium with sterile liquid paraffin or oil. Incubate at 36±2°C for 24hr. Typical *Salmonella* show a purple color.

**Table1 Biochemical characterization of *Salmonella***

| Sr. No. | Biochemical test | Reaction | Observation |
|---|---|---|---|
| 1. | Lactose | - | Red slant with gas formation and yellow butts with blackening of the agar. |
| 2. | Glucose | + | |
| 3. | Hydrogen sulfide | + | |
| 4. | Urea | - | No rose-pink color followed by deep cerise. |
| 5. | Lysine decarboxylase | + | Development of purple color. |

Note: (+) means positive reaction, (-) means negative reaction

g) **Serological confirmation:** Carry out slide agglutination test to check the presence of Salmonella O-, Vi- and H-antigens with the appropriate antisera from pure colonies on Nutrient agar as per manufacturer's instructions after elimination of auto-agglutinable strain.

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| **Calculation with units of expression** | Presence/Absence of Coliform given in per unit of the sample examined.<br><br>Test for *Salmonella*= Present/absent per X mL of sample |
| **Reference** | 1) IS 15187:2016 - Water Quality - Detection of Salmonella Species (First Revision)<br>2) IS 14543:2016 - Packaged Drinking Water (Other Than Packaged Natural Mineral Water) - Specification (Second Revision)<br>3) IS 15188: 2022 - Water quality — General requirements and guidance for microbiological examinations by culture |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| FSSAI FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA<br>Inspiring Trust, Assuring Safe & Nutritious Food<br>Ministry of Health and Family Welfare, Government of India | **Method for Detection of *Shigella*** | | |
| **Method No.** | FSSAI 15.030:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Shigella spp.* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. It is also the responsibility of the user to establish appropriate safety and health practices. | | |
| **Principle** | Detection of *Shigella* is based on pre enrichment, selective enrichment followed by isolation on selective media. Presumptive *Shigella* colonies are identified by biochemical and serological test. Pre enrichment broth enables injured cells to grow. A selective enrichment is necessary to increase the proportion of *Shigella* sp. in relation to background flora. | | |
| **Apparatus/Instruments** | a) Protective cabinet with vertical laminar airflow<br>b) Incubators (36±2°C**)**<br>c) Thermostatically controlled water bath (capable of being maintained at 44 °C to 47 °C)<br>d) Membrane filtration unit (Filter size 0.45 µ)<br>e) Autoclave<br>f) Sterilizing oven<br>g) Petri dishes (Glass or plastic of 90 – 100mm diameter or 140mm)<br>h) Total delivery graduated pipettes, of nominal capacity 1 mL, graduated in 0.1 mL divisions (Class A), or Micropipette with sterile tips<br>i) Mechanical stirrer<br>j) Tubes, flasks or bottles, of appropriate capacity | | |
| **Culture Media and Reagents** | a) Nutrient broth<br>b) Kauffmann-Muller's Tetrathionate broth<br>c) Selenite F broth<br>d) Deoxycholate citrate agar | | |

| | |
|---|---|
| | e) MacConkey agar<br>f) Nutrient agar No. 2 |
| **Preparation of Reagents** | Commercially available reagents/Disc |
| **Sample Preparation** | Not Applicable |
| **Method of analysis** | a) Pre-enrichment: Aseptically clean the surface of bottle/pouch/cups containing sample with 70% ethanol and filter 250mL (or as specified) of water sample through a membrane filter of 0.45µm pore size using sterile membrane filtration assembly and place the filter in 50mL of Nutrient broth. Incubate at 37°C for 18-24 hr.<br>b) Selective enrichment: Transfer 1mL from pre-enriched nutrient broth to each of 100mL Selenite-F broth and tetrathionate broth. Incubate at 37°C for 24hr.<br>c) After incubation streak out a loopful from Selenite F broth and tetrathionate broth deoxycholate citrate agar (DCA). Incubate the plates at 37°C for 24 hr. If there is no typical/suspected growth on DCA plate, reincubate further for 24 hr.<br>d) **Observations:** *Shigella* colonies on deoxycholate citrate agar (DCA) appear opaque with a ground-glass appearance and with even margins.<br>e) **Confirmation:** If there are typical/suspected colonies, pick at least 5 colonies from each plate and streak on MacConkey agar Incubate the plate(s) overnight at 37°C to isolate pure colonies. Pure colonies are transferred on to Nutrient agar No. 2 prior to biochemical and serological confirmation.<br>f) **Biochemical Confirmation:**<br>i. **Gram staining:** Typical colonies of *Shigella* are gram negative rods.<br>ii. **Motility:** Nutrient agar tubes are inoculated by stabbing with a straight wire to a depth of 5 mm and incubated at 37°C for 18-24 hr to check the motility. Out growth in whole tube indicates motility. If no motility is observed within 24 hr. the tubes are incubated at room temperature for further 4-6 days to check the Motility. |

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| | *Shigella* is non-motile.<br>iii. **Catalase Test:** Streak a colony on nutrient agar slant and incubate at 37°C for 24 hr. Add 1 mL of hydrogen peroxide over the growth. *Shigella* is Catalase positive and show release of oxygen, as bubbles of hydrogen peroxide indicating the presence of catalase.<br>iv. **Oxidase Test:** Streak a colony on nutrient agar slant and incubate at 37 °C for 24 hr. To nutrient agar slant containing the culture, add a few drops of mixed test reagents (1% α-nepthol in 95% ethanol and equal amount of para-amino diamethylaniline hydrochloride in water). *Shigella* being an Oxidase negative does not develop blue color within two minutes of addition of reagent.<br>v. **Hugh-Leifson's test:** Stab a fresh culture from nutrient agar slant into two tubes of Hugh Leifson medium. One tube is over layered with a small amount of sterile paraffin liquid to create anaerobic conditions, whereas, other is incubated as such. Both are incubated at 37°C and examined daily up to 4 days. In case of *Shigella* Acid formation in both the tubes indicates fermentative reaction.<br>vi. **Test for $H_2S$ Production:** Inoculate Triple sugar iron agar (TSI) by stabbing the butt and streaking the slope. Incubate at 37°C and examine daily up to 7 days. In case of $H_2S$ production blackening of butt is observed.<br>vii. **Test for Urease:** Inoculate culture in Nutrient broth and incubate for 37°C for 24hr. Add inoculated culture from Nutrient broth to urea agar slant and incubate at 37°C for 18-24 hr. A positive urease is shown by the medium becoming pink or red on incubation. If no color formed, continue incubation for 4 days and record for color development.<br>viii. **Phenyl pyruvic acid production:** Suspend overnight grown culture in 0.5 mL of normal saline and transfer to a test tube (dia 1.5 cm). Add 0.5 mL of 0.2% di-phenylalanine and mix. Keep it in horizontal position for at least 3 hr. at room temperature. Add few drops of half saturated ferric chloride solution. A positive reaction is indicated by the formation of immediate deep color which fades on keeping. |

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| | ix. **Test for Citrate utilization:** Inoculate the culture on Simmons citrate agar slant using a straight wire. Incubate at 37°C and examine daily up to 4 days. No change in color of media indicates negative reaction.<br>x. **Test for Indole:** Inoculate Peptone water medium with a loopful of 24hr grown culture in Nutrient broth and incubate at 37°C for 48 hr. Add 0.5mL of KOVAC'S reagent and shake the tube gently. Observe for the appearance of red color which indicates the presence of Indole.<br>xi. **Test for Fermentation of carbohydrates:** Inoculate each of Andrade peptone water medium tubes for carbohydrates i.e. Glucose, Lactose, Sucrose, Salicin, Dulcitiol, Mannitol (@ 1% concentration) with freshly grown culture from Nutrient broth/agar and incubate at 37°C for 18-48 hr. Record the presence of acid from pink color and that of gas in Durham tube.<br>xii. **Test for Dihydrolase & decarboxylase activity:** Inoculate each of the tubes of Dihydrolase and Decarboxylase medium through liquid paraffin with freshly grown culture from Nutrient agar. Incubate at 37ºC and examine upto 4 days. Medium first become yellow due to acid production from the glucose. Later if dehydrolation or decarboxylation of respective amino acid occur, the medium become violet in color.<br>xiii. **Test for utilization of Malonate:** Inoculcate the medium with freshly grown culture from Nutrient broth/agar for Malonate test and incubate at 37°C for 24hr. Positive Malonate function is indicated by deep blue color and negative reaction by unchanged greenish or yellow color of medium.<br>xiv. **Gelatin liquefaction test:** Inoculate the culture into gelatin liquefaction test medium and as a stab culture. Incubate at 22°C for 4 days in an upright position. Before taking reading keep the tube at refrigerated temperature to check liquefaction of the media. |

**Table 1 Biochemical characteristics of *Shigella sp***

| S. No. | Tests | Positive Test observations | *Sh. dysenteriae* | *Sh. Flexneri, Sh. boydii* | *Sh. sonnei* |
|---|---|---|---|---|---|
| **1.** | Gram reaction | Gram negative, Rods | *Gram negative, rods* | *Gram negative, rods* | *Gram negative, rods* |
| **2.** | Motility | Growth present outside of inner glass tube | - | - | - |
| **3.** | Catalase | Oxygen released as bubble | + | + | + |
| **4.** | Oxidase | Blue color within 2 min | - | - | - |
| **5.** | Hugh-Leifson's test | Fermentative (Acid & yellow color formation in both tube) | *F* | *F* | *F* |
| **6.** | TSI for H2S | Blackening of Butt | - | - | - |
| **7.** | Urease | Medium color change to pink or red | - | - | - |
| **8.** | Phenyl pyruvic acid | Deep green color formed | - | - | - |
| **9.** | Citrate | Medium color change to Blue | - | - | - |
| **10.** | Glucose fermentation | Acid formation (Pink color), gas + | + | + | + |

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|---|---|---|---|---|---|---|
| | **11.** | Lactose(1%) fermentation | | - | - | *(++)* |
| | **12.** | Sucrose fermentation | | - | - | *(++)* |
| | **13.** | Salicin fermentation | | - | - | - |
| | **14.** | Mannitol fermentation | | - | + | + |
| | **15.** | Lysine | First yellow color(Acid formation) then purple color due to decarboxylation of amino acid | - | - | - |
| | **16.** | Ornithine | | - | -/+ | + |
| | **17.** | Malonate | Medium color change to deep Blue | - | - | - |
| | **18.** | Gelatin | Gelatin is liquefied | - | - | - |

*Note: (+) means positive reaction, (-) means negative reaction, (++) means late positive, (F) means fermentative reaction*

**xv.** **Serotyping:** Use slide agglutination with the appropriate antisera from discrete single colonies on Nutrient agar as per manufacturer's instructions after elimination of auto-agglutinable strain. *Shigella* strain is confirmed by Polyvalent *Shigella* sera, on the basis of serotyping.

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| **Calculation with units of expression** | Presence/Absence of *Shigella* species given after Biochemical & Serological confirmation of presumptive colonies in the sample examined. Test for *Shigella* = Present/absent per X mL of sample |
| **Reference** | 1) IS 5887 (Part-7):1999 (Reaffirmed 2018) - Methods for detection of |

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| | bacteria responsible for food poisoning: Part 7 general guidance on methods for isolation and identification of Shigella<br>2) IS 14543:2016 - Packaged Drinking Water (Other Than Packaged Natural Mineral Water) - Specification (Second Revision)<br>3) IS 15188: 2022 - Water quality — General requirements and guidance for microbiological examinations by culture |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| FSSAI FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA<br>Inspiring Trust, Assuring Safe & Nutritious Food<br>Ministry of Health and Family Welfare, Government of India | **Method for Detection of *Pseudomonas aeruginosa*** | | |
| **Method No.** | FSSAI 15.031:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good microbiology laboratory practices to avoid cross contamination. | | |
| **Principle** | Detection of *Pseudomonas aeruginosa* is based on selective enrichment followed by isolation on confirmation media. Asparagine proline broth is used as selective medium for cultivation of *P. aeruginosa.* Milk Agar is used for selective isolation of *Pseudomonas aeruginosa*. Strains of *Pseudomonas aeruginosa* are identified by their pigment i.e. pyocyanin production. *Pseudomonas aeruginosa* hydrolyzes casein and produces a yellow to green diffusible pigment on Milk agar. | | |
| **Apparatus/Instruments** | a) Protective cabinet with vertical laminar airflow<br>b) Incubators (36±2°C, 42±0.5°C**)**<br>c) Thermostatically controlled water bath (capable of being maintained at 44 °C to 47 °C)<br>d) Membrane filtration unit (Filter size 0.45 µ)<br>e) Autoclave<br>f) Sterilizing oven<br>g) Petri dishes (Glass or plastic of 90 – 100mm diameter or 140mm)<br>h) Total delivery graduated pipettes, of nominal capacity 1 mL, graduated in 0.1 mL divisions (Class A), or Micropipette with sterile tips<br>i) Mechanical stirrer<br>j) Tubes, flasks or bottles, of appropriate capacity | | |
| **Culture Media and Reagents** | a) Pseudomonas asparagine proline broth<br>b) Milk agar with cetrimide | | |
| **Preparation of Reagents** | Commercially available reagents/Disc | | |
| **Sample Preparation** | Not Applicable | | |

**Method of analysis**

a) Aseptically clean the surface of bottle/pouch/cups containing sample with 70% ethanol and filter 250 mL (or as specified) of water sample through a membrane filter of 0.45µm pore size using sterile membrane filtration assembly and place the filter in 50 mL of concentrated Asparagine proline broth. Incubate at 37±1°C for 48 hr.

b) After incubation examine the medium showing either growth or fluorescence under U.V. light (360±20 nm).

c) Subculture a loopful on Milk agar plate and incubate for 24h at 42±0.5°C.

d) **Observation:** Observe the plates for culture growth, pigment production and casein hydrolysis (clearing of medium around the colonies).

**Table 1 Characteristics *of Pseudomonas aeruginosa* on Milk agar**

| S. No. | Reaction | *Pseudomonas aeruginosa* characteristics | |
|---|---|---|---|
| | | Typical | Atypical |
| 1. | Casein hydrolysis | + | + |
| 2. | Growth at 42 °C | + | + |
| 3. | Fluorescence under UV light | + | - |
| 4. | Pyocyanine (Blue/green) pigment production | + | - |

*Note: (+) means positive reaction, (-) means negative reaction*

i. Culture showing either growth or fluorescence in Asparagine proline broth, which further produce colonies on Milk agar plates and show pigment production and casein hydrolysis are regarded positive for presence of *Pseudomonas aeruginosa.*

ii. The colonies (atypical) showing casein hydrolysis, but no florescence or pigment productions are further confirmed by biochemical tests.

**e) Confirmation of Atypical colonies:** If there are Atypical/suspected colonies, streak out at least 5 selected colonies further on Milk agar medium and incubate at 37±1°C for 24 hr. Proceed for further

biochemical confirmation.

i. **Catalase Test:** Streak a colony on Nutrient agar slant and allow to grow at 37°C for 24 hr. Add 1 mL of hydrogen peroxide over the growth in slanting position. Release of oxygen, as bubbles, from hydrogen peroxide indicates presence of catalase.

ii. **Oxidase Test:** Streak a colony on Nutrient agar slant and allow to grow at 37°C for 24 hr. Add a few drops of mixed test reagents (1% alpha nepthol in 95% ethanol and equal amount of para-amino diamethylaniline hydrochloride in water) to Nutrient agar slant of culture. A positive reaction is indicated by the appearance of blue color within two minutes of addition of reagent.

iii. **Hugh-Leifson's test:** Stab a fresh culture from Nutrient agar slant into two tubes of Hugh Leifson medium, one of which is then layered over with a small amount of sterile liquid paraffin. Incubate at 37°C and examine daily up to 4 days. Acid formation, in both the tubes indicates fermentative reaction.

iv. **Nitrate reduction:** AddReagent A (Sulfanilic acid) and Reagent B (N,N-dimethyl-1-naphthylamine) to 18hr old culture inoculated in Nitrate broth. Formation of red color indicates nitrate reduction. If no change in color is observed, add Zinc granules to tube, and observe color change. If the broth turns to red, test is negative but if no red color is developed after addition of zinc granules, then test is positive.

v. **Gelatin liquefaction test:** Inoculate the strain into Gelatin liquefaction test medium as a stab culture. Incubate at 22°C for 4 days in an upright position. Before taking reading keep the tube at refrigerated temperature to check liquefaction of the media.

**Table 2 Biochemical characteristics *Pseudomonas aeruginosa***

| S. No. | Tests | Observation |
|---|---|---|
| 1. | Catalase test | + |
| 2. | Oxidase test | + |
| 3. | Growth in Hugh and Liefson medium | Oxidative reaction |

| | | | |
|---|---|---|---|
| | 4. | Nitrate reduction to ammonia | + |
| | 5. | Gelatine Liquefication | + |
| **Calculation with units of expression** | Presence/Absence of *Pseudomonas* sp. is given after Biochemical & Serological confirmation of presumptive colonies in the sample examined.<br>Test for *Pseudomonas aeruginosa* = Present/ absent per X mL of sample | | |
| **Reference** | 1) IS 13428(Annex D):2005 - Packaged Natural Mineral Water - Specification (Second Revision)<br>2) IS 14543:2016 - Packaged Drinking Water (Other Than Packaged Natural Mineral Water) - Specification (Second Revision)<br>3) IS 15188: 2022 - Water quality — General requirements and guidance for microbiological examinations by culture | | |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis | | |

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| FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA<br>Inspiring Trust, Assuring Safe & Nutritious Food<br>Ministry of Health and Family Welfare, Government of India | **Method for detection of Yeast & Moulds** | | |
| **Method No.** | FSSAI 15.032:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good microbiology laboratory practices to avoid cross contamination. | | |
| **Principle** | Chloramphenicol Yeast Glucose Agar is a selective medium recommended for isolation and enumeration of Yeast & Moulds. The medium contains yeast extract, which provides nitrogenous nutrient and vitamin B complex. Dextrose is the energy source. Chloramphenicol a thermostable antibiotic, suppresses the bacterial flora. Aerobic incubation of plates is done at 25±1$^{o}$C and count is taken on 3$^{rd}$, 4$^{th}$ or 5$^{th}$ day. | | |
| **Apparatus/Instruments** | a) Protective cabinet with vertical laminar airflow<br>b) Incubators (25±1$^{o}$C)<br>c) Thermostatically controlled water bath (capable of being maintained at 44 °C to 47 °C)<br>d) Autoclave<br>e) Sterilizing oven<br>f) Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>g) Total delivery graduated pipettes, of nominal capacity 1 mL, graduated in 0.1 mL divisions (Class A), or Micropipette with sterile tips<br>h) Mechanical stirrer<br>i) Tubes, flasks or bottles, of appropriate capacity<br>j) Colony-counting device (optional)<br>k) Sterile Glass or plastic spreaders<br>l) Spiral plater | | |
| **Culture Media and Reagents** | Chloroamphenicol Glucose Yeast Extract Agar (CGYEA) | | |

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| **Preparation of Reagents** | Not Applicable |
| **Sample Preparation** | Not Applicable |
| **Method of analysis** | a) Aseptically clean the surface of bottle/pouch/cups containing sample with 70% ethanol and filter 250 mL (or as specified) of water sample through a membrane filter of 0.45µm pore size using sterile membrane filtration assembly.<br>b) Place the filter on CGYEA media and incubate at 25±1°C<br>c) **Observation:** Observe the plates for colonies of Yeast & Molds on $3^{rd}$, $4^{th}$ and $5^{th}$ days of incubation.<br>d) It is advisable to examine the plates at the end of three days for yeast colonies, which are smooth, moist, elevated or surface colonies. Mold colonies are easily recognized by the profuse growth of hyphae. |
| **Calculation with units of expression** | Presence/ Absence of yeast & molds given after examining the plates of sample.<br>Test for Yeast and molds = Present/absent per X mL of sample |
| **Reference** | 1) IS 5403:1999 (Reaffirmed 2018) - Method for yeast and mould count of foodstuffs and animal feeds (First Revision)<br>2) IS 14543:2016 - Packaged Drinking Water (Other Than Packaged Natural Mineral Water) - Specification (Second Revision)<br>3) IS 15188: 2022 - Water quality — General requirements and guidance for microbiological examinations by culture |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA<br>Inspiring Trust, Assuring Safe & Nutritious Food<br>Ministry of Health and Family Welfare, Government of India | **Method for Detection of Sulphite-Reducing anaerobes (*Clostridia*)** | | |
| **Method No.** | FSSAI 15.033:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good microbiology laboratory practices to avoid cross contamination. | | |
| **Principle** | Detection of sulphite reducing anaerobes by inoculating 50 mL of sample into equal volume of double strength of Differential Reinforced Clostridial Medium (DRCM), followed by anaerobic incubation at 37±1°C for 44±4 hr. As a result of reduction of sulphite and the precipitation of iron (II) sulphide in medium developing, Black color in medium, sample is considered as positive. | | |
| **Apparatus/Instruments** | a) Protective cabinet with vertical laminar airflow<br>b) Incubators (37±1°C, 75±5°C)<br>c) Thermostatically controlled water bath (capable of being maintained at 44 °C to 47 °C)<br>d) Autoclave<br>e) Sterilizing oven<br>f) Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>g) Total delivery graduated pipettes, of nominal capacity 1 mL, graduated in 0.1 mL divisions (Class A), or Micropipette with sterile tips<br>h) Mechanical stirrer<br>i) Tubes, flasks or bottles, of appropriate capacity | | |
| **Culture Media and Reagents** | Differential Reinforced Clostridial Medium (DRCM) | | |
| **Preparation of Reagents** | Not Applicable | | |
| **Sample Preparation** | Not Applicable | | |

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| **Method of analysis** | a) Aseptically clean the surface of the bottle (1lt. /5lt. /20lt.) or water pouch/cups containing sample with 70% ethanol. Aseptically withdraw 100 mL sample to sterile bottle and heat at 75±5°C for 15 min.<br>b) Add 50 mL of sample after heat shock to 100 mL bottle containing 50 mL of the double strength DRCM. Cap the bottles tightly and incubate under anaerobic conditions at 37±1°C for 44±4hr. Iron wire, heated to redness can be placed in the medium before inoculation to enhance anaerobic conditions. |
| **Calculation with units of expression** | Test for Sulphite-Reducing anaerobes (*Clostridia*): = Present/ absent per X mL of sample |
| **Reference** | 1) IS 13428(Annex C):2005 - Packaged Natural Mineral Water - Specification (Second Revision)<br>2) IS 14543:2016 - Packaged Drinking Water (Other Than Packaged Natural Mineral Water) - Specification (Second Revision) |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Detection of *Vibrio parahaemolyticus*** | | |
| **Method No.** | FSSAI 15.034:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | In order to safeguard the health of laboratory personnel, it is essential that tests for detection of *Vibrio* spp., and particularly toxigenic *Vibrio cholerae*, *Vibrio parahaemolyticus* be conducted only in laboratories equipped for this purpose and under the supervision of an experienced microbiologist, and that great care is exercised in the disposal of contaminated material. | | |
| **Principle** | Glucose salt teepol broth is used to enrich *Vibrio parahaemolyticus.* Glucose is utilized by the organism while teepol inhibits the migration of halophilic organisms and the growth of the gram-positive organisms. After overnight incubation at 35±2°C, inoculation on TCBS agar plates gives round green or bluish colonies. | | |
| **Apparatus/Instruments** | a) Protective cabinet with vertical laminar airflow<br>b) Incubators (36±2°C**)**<br>c) Thermostatically controlled water bath (capable of being maintained at 44 °C to 47 °C)<br>d) Sterile membrane filter (0.45µm pore size)<br>e) Autoclave<br>f) Sterilizing oven<br>g) Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>h) Total delivery graduated pipettes, of nominal capacity 1 mL, graduated in 0.1 mL divisions (Class A), or Micropipette with sterile tips<br>i) Mechanical stirrer<br>j) Tubes, flasks or bottles, of appropriate capacity | | |
| **Culture Media and Reagents** | Glucose-salt-teepol broth<br>Thiosulphate-Citrate-Bile salts-Sucrose (TCBS) | | |
| **Preparation of Reagents** | Commercially available reagents/Disc | | |
| **Sample Preparation** | Not Applicable | | |
| **Method of analysis** | a) Disinfect the surface of the bottle/pouch/cups containing sample with | | |

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| | 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution<br>b) Filter the sample (required volume) through a sterile membrane filter (0.45µm pore size) and place the filter in 50 mL of Glucose-salt-Teepol broth. Incubate overnight at 37°C.<br>c) After incubation streak a loopful from pre-enrichment culture on TCBS prepared plates and incubate for 18hr at 37°C.<br>d) Suspicious colonies of *V. parahaemolyticus* on TCBS medium are 2-3 mm and round with green or blue centers.<br>**e) Identification:** Suspicious growth of *V. parahaemolyticus* is confirmed through biochemical confirmation.<br>f) **Biochemical tests:** If there are typical/suspected colonies, plate out at least 5 selected colonies from each positive agar medium on nutrient agar plates and incubate at 36±2°C for 18-24 hr. Proceed for further biochemical & serological confirmation<br>i. **Gram Staining**: Gram stain the isolated colony. Typical *Vibrio parahaemolyticus* are Gram negative rods.<br>ii. **Test for Oxidase:** Streak a colony on Nutrient agar slant (with 2-3% added NaCl) and allow to grow for 24 hr. To Nutrient agar slant of culture, add a few drops of mixed test reagents (1% alpha nepthol in 95% ethanol and equal amount of para-amino diamethylaniline hydrochloride in water). A positive reaction is indicated by the appearance of blue color within two minutes of addition of reagent.<br>iii. **Hugh-Leifson's test:** Stab a fresh culture from Nutrient agar slant into two tubes of Hugh Leifson medium (with 2-3% added NaCl), one of which is then over layered with a small amount of sterile liquid paraffin. Incubate at 37°C and examine daily up to 4 days. Acid formation, shown by yellow color in the tube without paraffin indicates oxidative utilization of glucose. Acid in both tubes indicates fermentative reaction.<br>iv. **Test for Fermentation of carbohydrates:** Inoculate each of Andrade Peptone water medium tubes for carbohydrates i.e. Sucrose, Mannitol (@ 1% concentration) and incubate at 37°C for 18-48 hr. observe color change for carbohydrate fermentation |

v. **TSI for $H_2S$ production:** Inoculate Triple Sugar Iron Agar (TSI) medium (with 2-3% added NaCl) by stabbing into the butt and streaking the slope. Incubate at 37°C and examine daily up to 7 days. In case of $H_2S$ production blackening of butt is observed.

vi. **Test for Dihydrolase & Decarboxylase activity:** Inoculate each of the tubes of Dihydrolase & Decarboxylase medium (with 2-3% added NaCl) through liquid paraffin from freshly grown culture on Nutrient agar medium. Incubate at 37°C and examine up to 4 days. Medium first turns yellow due to acid production from glucose and later if dehydration or decarboxylation of respective amino acids occur, the medium changes to violet in color.

vii. **Test for Voges-Proskauer reaction:** Inoculate the medium with added NaCl (2-3%) and incubate for 48hr at 37°C. To 1 mL of growth, add 0.6mL of 5% alpha nephthol. Shake and add 0.2 mL of KOH solution 40%. Shake and slope the tube for upto 4 hr. for color change. Pink color indicate positive reaction.

viii. **Test for the growth in Tryptone broth:** Inoculate the culture in Tryptone broth with different concentration (0%, 1%, 8% & 10%) of Sodium chloride.

ix. Growth in Tryptone broth with added NaCl incubated at 42°C for 24 hr shows positive growth. Growth in 1% Tryptone broth, with added 8% NaCl and incubated at 37°C is positive. Growth in 1% Tryptone broth, with added 10% NaCl and incubated at 37°C is negative. Growth in 1% Tryptone broth, without NaCl and incubated at 37°C is negative

x. **Kanagawa test:** Grow the culture in Trypticase-Soy-Sodium Chloride broth by incubating for 18hr at 37°C. Further streak on blood agar and incubate at 37°C for not more than 24hr. A positive result in case of *V. parahaemolyticus* consists of a zone of transparent clearing of the red blood cells around the colony. It is important that the reading is not taken beyond 24hr of incubation as any haemolysis seen beyond this time is not to be recorded as Kanagawa positive.

**Table 1Biochemical characteristics of *Vibrio parahaemolyticus***

| S. | Tests | Reaction |
|---|---|---|

<table>
<tr><td></td><td>
<table>
<tr><td>No.</td><td></td><td></td></tr>
<tr><td>1.</td><td>Gram Reaction</td><td>Gram Negative , Rods</td></tr>
<tr><td>2.</td><td>Oxidase test</td><td>+</td></tr>
<tr><td>3.</td><td>Hugh-Leifson’s test</td><td>F</td></tr>
<tr><td>4.</td><td>H2S production</td><td>-</td></tr>
<tr><td>5.</td><td>Glucose fermentation</td><td>+ (without gas)</td></tr>
<tr><td>6.</td><td>Mannitol fermentation</td><td>+</td></tr>
<tr><td>7.</td><td>Sucrose fermentation</td><td>-</td></tr>
<tr><td>8.</td><td>Voges –Proskauer test</td><td>-</td></tr>
<tr><td>9.</td><td>Lysine decarboxylase</td><td>+</td></tr>
<tr><td>10</td><td>Arginine dihydrolase</td><td>-</td></tr>
<tr><td>11</td><td>Ornithine decarboxylase</td><td>+</td></tr>
<tr><td>12</td><td>Growth in 1% tryptone brothwith added NaCl at $42^0$C</td><td>+</td></tr>
<tr><td>13</td><td>Growth in 1% tryptone broth + 8% NaCl</td><td>+</td></tr>
<tr><td>14</td><td>Growth in 1% tryptone broth + 10% NaCl</td><td>-</td></tr>
<tr><td>15</td><td>Growth in 1% tryptone broth without naCl</td><td>-</td></tr>
</table>
Note: (+) means positive reaction, (-) means negative reaction, (F) means fermentative reaction
</td></tr>
<tr><td>Calculation with units of expression</td><td>Presence/Absence of V. parahaemolyticus given after confirmation of presumptive colonies in the sample examined.<br>Test for V. parahaemolyticus = Present/absent per X mL of sample.</td></tr>
<tr><td>Reference</td><td>1) IS 5887 (Part-5) -1976 (Reaffirmed 2018) - Methods for detection of bacteria responsible for food poisoning, Part 5: Isolation, identification and enumeration of Vibrio cholerae and Vibrio parahaemolyticus<br>2) IS 14543:2016 - Packaged Drinking Water (Other Than Packaged Natural Mineral Water) - Specification (Second Revision)<br>3) IS 15188: 2022 - Water quality — General requirements and guidance for microbiological examinations by culture</td></tr>
<tr><td>Approved by</td><td>Scientific Panel on Methods of Sampling and Analysis</td></tr>
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| | **Method for Detection of *Vibrio cholerae*** | | |
| **Method No.** | FSSAI 15.035:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | In order to safeguard the health of laboratory personnel, it is essential that tests for detection of Vibrio spp., and particularly toxigenic *Vibrio cholerae*, *Vibrio parahaemolyticus* be conducted only in laboratories equipped for this purpose and under the supervision of an experienced microbiologist, and that great care is exercised in the disposal of contaminated material. | | |
| **Principle** | Bile Salt agar and Thiosulphate Citrate Bile salt sucrose agar (TCBS) is used selectively for the isolation, identification and enumeration of *Vibrio cholerae*. *Vibrio* species grow in the presence of relatively high levels of bile salts which inhibit the growth of gram-positive microorganisms. TCBS has a very high pH (8.5-9.5) which suppresses growth of intestinal flora other than *Vibrio* sp. *V. cholerae* ferment sucrose, which results in a pH shift and production of yellow colonies. | | |
| **Apparatus/Instruments** | a) Protective cabinet with vertical laminar airflow<br>b) Incubators (36±2°C**)**<br>c) Thermostatically controlled water bath (capable of being maintained at 44 °C to 47 °C)<br>d) Sterile membrane filter (0.45µm pore size)<br>e) Autoclave<br>f) Sterilizing oven<br>g) Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>h) Total delivery graduated pipettes, of nominal capacity 1 mL, graduated in 0.1 mL divisions (Class A), or Micropipette with sterile tips<br>i) Mechanical stirrer<br>j) Tubes, flasks or bottles, of appropriate capacity | | |
| **Culture Media and Reagents** | a) Alkaline peptone water<br>b) Thiosulphate-Citrate-Bile salts-Sucrose agar (TCBS)<br>c) Bile salt agar (BSA) | | |

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| | d) Nutrient agar No. 2 |
| **Preparation of Reagents** | Commercially available reagents/Disc |
| **Sample Preparation** | Not Applicable |
| **Method of analysis** | a) Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution.<br>b) Filter the sample (requisite volume) through a sterile membrane filter (0.45µm pore size) and place the filter in 50 mL of alkaline peptone water. Incubate at 37°C overnight.<br>c) After incubation streak a loopful on TCBS and BSA medium plates and incubation at 37°C overnight.<br>d) **Observation:** Suspicious colonies of *V. cholerae* on TCBS appear as opaque yellow colored with entire round margins.<br>*V. cholerae* colonies show a distinctive appearance on BSA medium.<br>**e) Biochemical Confirmation:** If there are typical/suspected colonies, plate out at least 5 selected colonies from each positive agar medium on nutrient agar No. 2 plates and incubate at 36±2°C for 18-24 hr. Proceed for further biochemical & serological confirmation<br>i. **Gram Staining:** Gram stain the isolated colony. Typical *Vibrio cholerae* are Gram negative curved shaped rods when observed under microscope.<br>ii. **Test for Motility:** Nutrient agar for motility is used to check the motility of *V. cholera.* Inoculate by stabbing with a straight wire into glass tube to a depth of 5 mm and incubate at 37°C for 18-24 hr. Motile strain shall be found to show growth on the surface of the medium, outside the inner glass tube; travelled through entire medium. If no motility observed keep at room temperature for 4-6 days to see if any evidence of motility is present.<br>iii. **Test for Catalase:** Streak a colony on Nutrient agar slant and allow to grow for 24 hr. at 37°C. Add 1 mL of hydrogen peroxide over the growth in slanting position. Release of oxygen, shown as bubbles, from hydrogen peroxide indicates presence of catalase. |

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| | **iv. Test for Oxidase:** Streak a colony on Nutrient agar slant and allow to grow at 37°C for 24 hr. To the slant culture, add a few drops of mixed test reagents (1% α-napthol in 95% ethanol and equal amount of para-amino diamethylaniline hydrochloride in water). A positive reaction is indicated by the appearance of blue color within two (2) minutes of addition of reagent.<br>**v. Hugh-Leifson's test:** Stab a fresh culture from Nutrient agar slant into two tubes of Hugh Leifson medium, one of which is then layered over with a small amount of sterile paraffin liquid. Incubate at 37°C and examine daily up to 4 days. Acid formation, shown by yellow color in the tube without paraffin indicates oxidative utilization of glucose. Acid in both tubes indicates fermentative reaction.<br>**vi. Test for Fermentation of carbohydrates:** Inoculate each of Andrade Peptone water medium tubes for carbohydrates i.e. Glucose, inositol, Mannitol (@ 1% concentration) and incubate at 37°C for 18-48 hr. observe color change for carbohydrate fermentation. Ferment glucose without gas production and in case of mannitol ferment with acid production<br>**vii. TSI for $H_2S$ production:** Inoculate Triple Sugar Iron agar (TSI) medium by stabbing into the butt and streaking the slope. Incubate at 37°C and examine daily upto 7 days. In case of $H_2S$ production blackning of butt is observed.<br>**viii. Test for the growth in 1% Tryptone Broth:** Inoculate the culture in Tryptone broth (without sodium chloride) and incubate at 37°C for 18hr. *V. cholerae* grows in 1% tryptone broth.<br>**ix. Test for Dihydrolase & Decarboxylase activity:** Inoculate each of the tubes of Dihydrolase & Decarboxylase medium through liquid paraffin from freshly grown culture from Nutrient agar. Incubate at 37ºC and examine up to 4 days. Medium first become yellow due to acid production from the glucose. Later if dehydration or decarboxylation of respective amino acid occur, the medium become violet in color. |

**Table 1 Biochemical characteristics of *Vibrio cholera***

| S. No. | Tests | Reaction |
|---|---|---|
| 1. | Gram Reaction | Gram Negative , Rods |
| 2. | Motility test | + |
| 3. | Catalase test | + |
| 4. | Oxidase test | + |
| 5. | Hugh-Leifson's test | F |
| 6. | H2S production | - |
| 7. | Glucose fermentation | + (without gas) |
| 8. | Mannitol fermentation | + |
| 9. | Inositol fermentation | - |
| 10. | Growth in 1% tryptone broth | + |
| 11. | Lysine decarboxylase | + |
| 12 | Arginine dihydrolase | - |
| 13 | Ornithine decarboxylase | + |

*Note: (+) means positive reaction, (-) means negative reaction, (F) means fermentative reaction*

f) **Serological Confirmation:** Suspicious growth of *V. cholera* is tested by slide agglutination using polyvalent cholera typing serum (high titre serum of combined Ogawa & Inaba Serotypes). The growth is emulsified in a drop of normal saline and smooth suspensions mixed with a drop of Cholera serum. Positive reaction is shown by the appearance of clumps within 30 seconds.

**Calculation with units of expression**

Presence/Absence of *V. cholerae* species given after confirmation of presumptive colonies in the sample examined.

Test for *V. cholerae* = Present/absent per X mL of sample filtered

**Reference**


1) IS 5887 (Part-5): 1976 (Reaffirmed 2018) - Methods for detection of bacteria responsible for food poisoning, Part 5: Isolation, identification and enumeration of *Vibrio cholerae* and *Vibrio parahaemolyticus*
2) IS 14543: 2016 - Packaged Drinking Water (Other Than Packaged Natural Mineral Water) - Specification (Second Revision)
3) IS 15188: 2022 - Water quality — General requirements and guidance

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| | for microbiological examinations by culture |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Detection of *Staphylococcus aureus*** | | |
| **Method No.** | FSSAI 15.036:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good microbiology laboratory practices to avoid cross contamination. | | |
| **Principle** | Baird Parker agar is used for the isolation and differentiation of coagulase-positive *Staphylococci*. *Staphylococci* can reduce tellurite to telluride, which results in grey to black coloration of the colonies. With the addition of egg yolk, the medium becomes yellow, slightly opaque. A clear halo develops around colonies from coagulase positive *Staphylococcusaureus*. Grey-black colonies and a halo on this medium are presumed to be indicative of coagulase positive staphylococci. | | |
| **Apparatus/Instruments** | a) Protective cabinet with vertical laminar airflow<br>b) Incubators (36 ±2°C**)**<br>c) Thermostatically controlled water bath (capable of being maintained at 44 °C to 47 °C)<br>d) Sterile membrane filter (0.45µm pore size)<br>e) Autoclave<br>f) Sterilizing oven<br>g) Petri dishes (Glass or plastic of 90-100mm diameter or 140mm)<br>h) Total delivery graduated pipettes, of nominal capacity 1 mL, graduated in 0.1 mL divisions (Class A), or Micropipette with sterile tips<br>i) Mechanical stirrer<br>j) Tubes, flasks or bottles, of appropriate capacity | | |
| **Culture Media and Reagents** | a) Cooked Salt Meat medium<br>b) Baired-Parker agar<br>c) Blood agar | | |
| **Preparation of Reagents** | Commercially available reagents | | |

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| **Sample Preparation** | Not Applicable |
| **Method of analysis** | a) Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution<br>b) Filter the sample (requisite volume) through a sterile membrane filter (0.45µm pore size) and place the filter in 50 mL of Cooked Salt Meat medium. Incubate overnight at 37°C.<br>c) After incubation streak a loopful from Cooked Salt Meat medium on Baired-Parker agar for at least 30 hr. at 37°C and overnight on Blood agar at 37°C.<br>d) *S. aureus* show shiny black colonies with or without grey-white margins on Baired-Parker agar<br>e) Usually golden yellow colonies on Blood agar.<br>f) **Identification:**<br>i. **Gram Staining:** If there are typical/suspected colonies, pick up the colonies and confirm by gram staining. *S. aureus* are Gram positive cocci which appear in clusters.<br>ii. **Coagulase test:** The test may be performed using one of following method.<br>**1. Slide method**: Emulsify a portion of suspected colony in normal saline water on a clean slide. Mix it with a straight wire dipped in rabbit plasma. Coagulase positive staphylococci produce visible clumping immediately. Positive control with a known coagulase positive strain of *S. aureus* and a control of rabbit plasma without inoculum should be included in the test.<br>2. **Tube method**: Emulsify a portion of suspected colony from 24 hr growth on blood agar in 1 mL citrated rabbit plasma,diluted 1 in 5, 0.85% saline. Incubate at 37°C, preferably in a water bath. Observe every hour to observe clotting of plasma. Positive control with a known coagulase positive strain *S. aureus* and a control of rabbit plasma without inoculum should be included in the test. Tube method shall be preferred |

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| **Calculation with units of expression** | Presence/Absence of *S. aureus* given after confirmation of colonies in the sample examined.<br>Test for *S. aureus*= Present/absent per X mL sample |
| **Reference** | 1) IS 5887 (Part-II): 1976 (Reaffirmed 2018) - Methods for Detection of bacteria responsible for Food Poisoning- Part II Isolation, Identification and Enumeration of *Staphylococcusaureus* and faecal *Streptococci* (First Revision)<br>2) IS 14543: 2016 - Packaged Drinking Water (other than packaged natural mineral water)- Specification (first revision)<br>3) IS 15188: 2022 - Water quality — General requirements and guidance for microbiological examinations by culture |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Detection of Viruses** | | |
| **Method No.** | FSSAI 15.037:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good microbiology laboratory practices to avoid cross contamination. | | |
| **Principle** | MS2 Phages are indicator of viral contamination in drinking water. Detection procedure is based on RNA extraction followed by c-DNA synthesis, which is identified after PCR amplification. | | |
| **Apparatus/Instruments** | **1.1 CONCENTRATION OF DRINKING WATER**<br>**Apparatus**<br>Pressure Pump<br>Membrane Filter Assembly with 144mm Diameter with Tripod Stand<br>Pressure Vessel (50 litre capacity) with Pressure Gauge<br>Inter-connecting Pressure Tubes<br>**RNA EXTRACTION**<br>**Apparatus**<br>Cooling Centrifuge<br>Deep Freezer (-20°C)<br>Vortex Mixer<br>Pipette Man<br>**COMPLEMENTARY DNA (cDNA) SYNTHESIS**<br>**Apparatus**<br>PCR Machine<br>Deep Freezer (-20°C)<br>**PCR AMPLIFICATION**<br>PCR Machine<br>Deep Freezer (-20°C)<br>Micropippette<br>**AGROSE GEL ELECTROPHORESIS** | | |

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| | Micropippette<br>Electrophoresis Apparatus<br>Gel Documentation System |
| **Culture Media and Reagents** | **CONCENTRATION OF DRINKING WATER**<br>a) Autoclaved double distilled water<br>b) Aluminum Chloride<br>c) HCl/NaOH Urea (Extra Pure)<br>d) Disodium Hydrogen Phosphate ($Na_2HPO_4.2H_2O$) - 0.2M filter sterilized.<br>e) Sodium Dihydrogen Phosphate ($NaH_2PO_4.2H_2O$) - 0.2M filter sterilized.<br>f) Citric Acid - 0.1M filter sterilized.<br>g) L-Arginine - 0.5M filter Sterilized.<br>h) Urea-Arginine Phosphate Buffer (U-APB)<br>i) Magnesium Chloride ($MgCl_2$) - 1M.<br>j) McII Vaines Buffer (pH 5.0)<br>**RNA EXTRACTION**<br>a) Cetyl Trimethyl Ammonium Bromide (CTAB) Buffer CTAB:<br>b) Phenol, Chloroform and Isoamylalcohol in the ratio of 25:24:1 (PCI)<br>c) Ethanol<br>d) TE Buffer (pH 8.0)<br>**COMPLEMENTARY DNA (c DNA) SYNTHESIS**<br>cDNA synthesis kit<br>**PCR AMPLIFICATION**<br>a) Primers for EV and HAV - EV sense primer 5' – TCC TCC GGC CCC TGA ATG CG – 3'; antisense primer 5'- ATT GTC ACC ATA AGC AGC CA - 3'; HAV sense primer 5' –GTTTTGCTCCTCTTT ATCATGCTATG- 3'; antisense primer 5'- GGA AATGTC TCAGGT ACTTTCTTTG-3'<br>b) PCR Master Mix<br>c) Mineral Oil<br>**AGROSE GEL ELECTROPHORESIS**<br>a) Running Buffer |

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| | b) Tracking Dye – 6X bromophenol blue.<br>c) Ethidium Bromide – 0.5 µg/mL |
| **Preparation of Reagents** | **a)** Urea-Arginine Phosphate Buffer (U-APB)- Mix 4.5 gm of urea with 2mL of 0.2M $NaH_2PO_4$ and 2mL of 0.5 M L-Arginine and make up the volume to 50mL with sterile distilled water. The pH of the eluent shall be 9.0.<br>**b)** McII Vaines Buffer (pH 5.0)- Mix 9.7 mL of 0.1 M citric acid with 10.3mL of 0.2M $Na_2HPO_4.2H_2O$ under sterile conditions.<br>c) Cetyl Trimethyl Ammonium Bromide (CTAB) Buffer CTAB: 1 percent; Sodium Dodecyl Sulphate (SDS): 1 percent EDTA: 20 mM; Sodium Chloride: 1 M<br>d) TE Buffer (pH 8.0) - Tris base:1M; EDTA: 0.5 M; Sodium Acetate: 3M.<br>d) Running Buffer – 50X TAE buffer -Tris base/ Tris buffer: 121.00 gm; Glacial acetic acid: 28.55 mL; 0.5 M EDTA: 50.00 mL; Distilled water: 300.45 mL<br>Make the final volume up to 1000mL with deionized distilled water, sterilize and store at 4°C. The final concentration for the preparation of agarose gel and to run gel shall be 1X. |
| **Sample Preparation** | **CONCENTRATION OF DRINKING WATER:** Filter 100 litre of drinking water sample through membrane filter assembly using either positively charged membrane of 144mm diameter or 0.22micron diameter pore size nitrocellulose membrane. For positively charged membrane the test water pH need not be adjusted. But for the 0.22 µ nitrocellulose membrane adjust the pH to 3.5 after adding the aluminium chloride as a coagulant to a final concentration of 0.0005M. At lower pH pass the water through the membrane. The flow rate shall be 40lt/h approximately. After the completion of the filtration, elute the adsorbed particles using 100 mL of urea-arginine phosphate buffer using 1 mL of magnesium chloride (1M). Dissolve the resultant precipitate centrifuged out of the sample in 800 - 1.0 mL of McII vaines buffer. The processed sample can be stored at refrigerator until required. |
| **Method of analysis** | a) **RNA EXTRACTION:** Treat 300µL of concentrated water sample with equal volume of CTAB and 1/10$^{th}$volume of PCI. Vortex and centrifuge |

at 5000 x gm for 30 min at 4°C. Add 1/10$^{th}$volume of 3M sodium acetate and double the volume of cold ethanol to the aqueous layer. Keep the mixture at either -20°C for overnight or in liquid nitrogen for 2 – 5 min. Centrifuge at 10000 X gm for 30 min at 4°C. Discard the supernatant and air dry the pellet and dissolve it in 20 µL TE buffer.

b) **COMPLEMENTARY DNA (cDNA) SYNTHESIS:** Suspend the extracted RNA in 20 µL of cDNA reaction mixture, which consists of 4µL of 5X reverse transcriptase reaction buffer [250mM TRIS – HCl (pH 8.5), 40mM KCl, 150mM $MgCl_2$, 5mM dithiothreithol (DTT)], 0.5 L of 10mM deoxynucleotide phosphate (dNTP), 2µl of hexa nucleotide mixture, 1µL of 25U of Maloney Murine Leukaema Virus (M-MuLV) reverse transcriptase, 0.5µL of 20U of human placental RNase inhibitor. Heat the reaction mixture to 95°C for 5 min and rapidly chill on ice this is followed by the additional of 1 µL (25 U/µL) of M-MuLV reverse transcriptase. Incubate the reaction mixture as given by the manufacturer of the kit quickly chill the reaction tube on ice.

**c) PCR Amplification:**

**i. PCR Amplification for Hepatitis A Virus (HAV):** In 5µL of cDNA, add 95µL of a PCR Master Mix (10Mm tris-HCL (pH8.3), 50mMKCl, 2.5mM $MgCl_2$, 0.01 percent gelatin (1X PCR buffer), 200µM of each dNTP, 1.5U of Thermus Aquaticus polymerase). Add 25 pico moles of sense and antisense oligonucleotide primers of HAV and overlay with mineral oil. Appropriate positive and negative controls shall be included with each run. Set the following reaction at thermo cycler:

Denaturation at 94°C for 2 min

Denaturation for 1.0 min at 94°C
Annealing for 1.0 min at 57°C,
Extension for 1.3 min at 72°C
} 35 cycles

Final extension at 72°C for 7 min

ii. **PCR Amplification for Enterovirus:** In 5µL of cDNA, add 95µL of a PCR Master Mix (10 mM TRIS-HCL (pH 8.3), 50mM KCl,

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| | 2.5mM $MgCl_2$. 0.01 percent gelatin (1XPCR buffer), 200µM of each dNTP, 1.5 U of Thermus aquaticus polymerase). Add 25 picomoles of sense and antisense oligonucleotide primers of EV and overlay with mineral oil. Appropriate positive and negative controls shall be included with each run. Set the following reaction at thermo cycler:<br>Denaturation at 94°C for 2min<br>Denaturation for 1.0 min at 94°C<br>Annealing for 1.0 min at 42°C, } 35 cycles<br>Extension for 2.0 min at 72°C<br>Final extension at 72°C for 7 min.<br>**AGAROSE GEL ELECTROPHORESIS:** Run the PCR amplified product of EV and HAV on 1.5 percent agarose gel using 1X TAE buffer. Load 10µL of amplified product after mixing it with 1µL 10X loading Dye. Run the molecule weight marker along with the samples. Run the electrophoresis at 100V for 30 min. Stain the gel with ethidium bromide (0.5µL/mL) for 20 min. Wash it with distilled water and view under UV transilluminator and photograph the gel to analyse the band pattern. EV gives the band as 155 base pair and the HAV give band as 225 base pair. |
| **Calculation with units of expression** | Entero Virus = Present/Absent<br>HAV virus = Present/Absent |
| **Reference** | IS 10500:2012: Drinking Water — Specification (Second Revision) |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Method for Detection of MS2 Phage in Water by Enrichment Spot Assay Technique** | | |
| **Method No.** | FSSAI 15.038:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | Carry out the test under the control of skilled microbiologist and great care shall be taken in the disposal of all the incubated material. Follow safe and good laboratory practices to avoid cross contamination. | | |
| **Principle** | This is a qualitative, presence- absence method. It employs an initial enrichment step to amplify coliphage numbers in samples, prior to assay with a spot technique. When a suspension of an infective phage (e.g. MS2 phage) is spotted over the lawn of host bacterial cells (e.g. *Escherichia coli),* the phage attaches the bacterial cell, replicate inside it, which is indicated by the formation of a zone of clearing or plaque within the lawn of bacteria. In the absence of lytic phage, the bacteria form a confluent lawn of growth. | | |
| **Apparatus/Instruments** | a) Biosafety cabinet<br>b) Universal Incubator (36.5 ± 2°C**)**<br>c) Water bath (set temp. 45± 2 °C)<br>d) Centrifuge (0.5-1.0 mL sample capacity, 5-10,000x *g* performance capability)<br>e) Autoclave<br>f) Sterile membrane filter (0.22µm pore size)<br>g) Erlenmeyer flasks, 125 mL, 250 mL and 2 L<br>h) Glass bottles, capped, 100 ml, 1L capacity<br>i) Graduated cylinders, 100 mL and 500 mL<br>j) Inoculating loop<br>k) Pipettes (1mL, 5mL and 10 mL)<br>l) Petri dishes (100 x 15 mm, 150 x 15 mm) | | |
| **Culture Media and Reagents** | a) Tryptone enrichment broth<br>b) Tryptone agar slants | | |

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| | c) Tryptone broth<br>d) Tryptone spot agar<br>e) Ampicillin solution<br>f) Streptomycin solution<br>g) Beef extract<br>h) Calcium chloride solution |
| **Preparation of Reagents** | a) **Tryptone enrichment broth** — Add 10.0 g Tryptone, 1.0 g yeast extract, 1.0 g glucose, 8.0 g NaCl, and 0.022 g $CaCl_2$ per each 100 mL of $dH_2O$. Sterilize by autoclaving at 121°C for 20 min.<br><br>After autoclaving, cool and add 0.1 mL of filtered ampicillin solution and 0.1 mL of Streptomycin solution per 100 mL of broth. Store at 4°C.<br><br>b) **Tryptone agar slants** — with gentle mixing, add 1.0 g Tryptone, 0.1 g yeast extract, 0.1 g glucose, 0.8 g NaCl, 0.022 g $CaCl_2$, and 1.2 g of Bacto-agar to a total volume of 100 mL of $dH_2O$ in a 250 mL flask. Further dissolve and sterilize by autoclaving at 121°C for 20 min.<br><br>After autoclaving, allow the agar to equilibrate in water bath set at 44.5 °C and then add 0.1 mL of filtered ampicillin solution and 0.1 mL of filtered streptomycin solution. Dispense 8 mL aliquots into 16 × 150 mm test tubes with. Prepare slants by allowing the agar to solidify with the tubes at about a 20° angle. Slants may be stored at 4°C for up to three months.<br><br>c) **Tryptone broth** — Add 1.0 g Tryptone, 0.1 g yeast extract, 0.1 g glucose, 0.8 g NaCl, and 0.022 g $CaCl_2$ per each 100 mL of $dH_2O$. Sterilize by autoclaving at 121°C for 20 min.<br><br>After autoclaving, cool and add 0.1 mL of filtered ampicillin solution and 0.1 mL of Streptomycin cin solution per 100 mL of broth. Store at 4°C.<br><br>d) **Tryptone spot agar dishes** — Prepare one day prior to sample analysis using the ingredients and concentrations listed for Tryptone agar slants, except use 0.75 g of Bacto-agar for each 100 ml. After autoclaving, place in water bath set at 44.5 °C and allow |

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| | equilibrating.<br><br>Add 0.1 mL of filtered ampicillin solution and 0.1 mL of filtered streptomycin solution to the 100 mL volume of warm agar. With gentle mixing, add 2 mL of a 4 h culture of host *E. coli*. Pour the well mixed suspension into five sterile 100 × 15 mm petri dishes (approximately 20 mL per dish), swirl gently, and allow the agar to harden. Store the dishes inverted at 4°C overnight and warm to room temperature for 1 h before use.<br><br>e) **Ampicillin solution** — Dissolve 1.5 g of ampicillin in 100 mL of $dH_2O$ and filter with the 0.22 µm filter and store at 4°C.<br><br>f) **Streptomycin solution** — Dissolve 1.5 g of streptomycin sulfate in 100 mL of $dH_2O$ and filter with the 0.22µm filter and store at 4°C.<br><br>g) **Beef extract** — prepare buffered 1.5% beef extract by dissolving 1.5 g of beef extract powder and 0.375 g of glycine (final glycine concentration =0.05 M) in 90 mL of $dH_2O$. Adjust the pH to 7.0 - 7.5. Autoclave at 121°C for 15 min and use at room temperature. If beef extract solutions are prepared for use at a later, store at 4°C.<br><br>h) **Calcium chloride solution** — Add 0.22 g of $CaCl_2$ to 50 mL of $dH_2O$ and sterilize by autoclaving at 121°C for 15 min. Use at room temperature. |
| **Reference Cultures** | **a) Host Bacteria: *E. coli* Famp (ATCC #700891)**<br><br>Inoculate 5 mL of Tryptone broth with host *E. coli* from a slant using a sterile inoculating loop and incubate for 16 h at 36.5 ± 2°C. Transfer 1.5 mL of the 16 h culture to 30 mL of Tryptone broth in a 125 mL flask and incubate for 4 h at 36.5 ± 2°C with gentle shaking. The amount of inoculum and broth used can be proportionally altered according to need.<br><br>**b) Coliphage MS2 (ATCC # 15597- B1)**<br><br>Rehydrate the ATCC coli-phage MS2 and store at 4°C. Prepare a 30 mL culture of the appropriate host as described above. Incubate culture for 2 h at 36.5 ± 2°C with shaking. Add 1 mL of |

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| | rehydrated phage stock and incubate for an additional 4 hours at 36.5 ± 2 °C. Filter the culture through a beef extract-treated 0.22µm filter. Prepare $10^{-7}$, $10^{-8}$ and $10^{-9}$ dilutions of the filtrate using tryptone dilution tubes. (These three dilutions should be sufficient in most cases). Add 3 mL of melted tryptone top agarheld in the 44.5 ± 1°C water bath to fifteen 16 × 150 mm test tubes. These test tubes should be kept in the heated water bath to avoid premature solidifying of the agar. Add 0.1 mL of the host culture to each of the 15 test tubes. Add 1 mL of the $10^{-9}$ dilution into each offive test tubes. Add 1 mL of the $10^{-8}$ dilution into five additional tubes and 1 mL of the $10^{-7}$ dilution into the remaining five tubes. Be sure the tubes are labelled with the appropriate dilution. For each tube, mix and immediately pour the contents over the bottom agar of a petridish labelled with the dilution assayed. Rotate the dish to spread the suspension evenly over the surface of the bottom agar and place it onto a level surface to allow the agar to solidify. Invert and incubate the inoculated dishes at 36.5 ± 2°C overnight and examine for plaques the following day. Count the number of plaques on each of the 15 dishes. Five dishes from one of the assayed dilutions should yield plaque counts of 20 to 100 plaques. Average the plaque counts on these five dishes and multiply the result by the reciprocal of the dilution to obtain the titer of the undiluted stock. For use as a positive control in the coliphage assay, dilute the filtrate to 30 to 80 PFU/mL in tryptone broth. Store the original filtrate and the diluted positive control preparation at 4°C. Before using the positive control preparation for the first time, assay 10 mL by adding 1 mL volumes of the preparation to ten test tubes containing agar and host culture, and pouring their contents into ten petri dishes. Count the plaques on all dishes and divide by 10. If the result is not 30 to 80, adjust the dilution of the positive controls ample and assay again. |
| **Sample Preparation** | Not Applicable |

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| **Procedure** | **A. Enrichment:**<br><br>1. To 1000 ml of water sample add 5ml of 4h active E. coli culture, 12.5ml $CaCl_2$, 50ml Tryptone enrichment broth, 1ml streptomycin and 1ml ampicillin solution.<br><br>2. Forpositive control, first spike 100ml of sterile distilled water with 500 µl MS2 phage positive control. To the spiked sample add 500 µl of 4h E. coli culture, 1.25ml $CaCl_2$, 5ml Tryptone enrichment broth, 100µl streptomycin and 100µl ampicillin solution.<br><br>3. For the negative control, to 100ml of sterile distilled water add 500 µl of 4h *E. coli* culture, 1.25ml $CaCl_2$, 5ml Tryptone enrichment broth, 100µl streptomycin and 100µl ampicillin solution.<br><br>Incubate the water samples including positive and negative control overnight at 36.5 ± 2°C.<br><br>**B. Spot assay:**<br><br>1. Remove enrichment cultures from incubator and mix well.<br>2. Draw 2ml of enriched culture from each sample (including positive and negative control)<br>3. Take 10µl and carefully deposit the volume to a preselected area on a Tryptone spot agar dish.<br>4. Allow the sample to absorb into agar for up to 60 min.<br>5. Incubate the spot agar plates overnight at 36.5 ± 2°C.<br>6. Examine dish for lysis (clearing) at the spot where the enrichment inoculum was applied.<br>7. Sometime interfering bacterial growth may be observed at spot area and can prevent accurate assessment of lysis zone formation. Such interference may be addressed by including filtration via beef extract treated 0.45um sterile filtration or by centrifugation at 5000-10000x g for 10 mins. |
| **Expression of results** | Presence/Absence of *MS2 Phage* given after confirmation of zone of lysis in the sample examined. |

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| | Test for *MS2 Phage*= Present/absent per 1000 mL water sample |
| **Reference** | USEPA Manual of Methods for Virology, Chapter 16, June 2001 |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

<table>
<tr><td>  </td><td colspan="3">Isolation and Identification of Giardia and Cryptosporidium in Water-Method A</td></tr>
<tr><td>Method No.</td><td>FSSAI 15.039:2023</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">This method is applicable to Packaged Drinking water (volume 10L to 1000 L)</td></tr>
<tr><td>Caution</td><td colspan="3">a) This method is not appropriate in case of turbid water.<br>b) Cartridge filtration can be used for small volumes of water (typically 10 L) but, based on the cost of these filters, this may be an expensive approach in some circumstances.</td></tr>
<tr><td>Principle</td><td colspan="3">a) Concentration from water: The isolation of Cryptosporidium and Giardia from water requires the use of a procedure which allows the volume of the sample to be reduced whilst retaining any oocysts and cysts. The concentration procedure used however, is dependent upon the water type which is to be analysed, the volume of sample and the amount of particulate material in the sample.<br>Table 1 — Membrane filters/filtration systems used for the concentration of parasites from water samples Membrane filter/filtration system
<table>
<tr><td>Membrane filter/filtration system</td><td>Application</td></tr>
<tr><td>Pall EnvirochekTM STD * or equivalent</td><td>Concentration of 10-litre to 200-litre (or more) samples of water</td></tr>
<tr><td>Pall EnvirochekTM HV or equivalent</td><td>Concentration of 10-litre to 1000-litre samples of water</td></tr>
<tr><td>IDEXX Filta-Max® or equivalent</td><td>Concentration of 10-litre to 1000-litre samples of water</td></tr>
<tr><td colspan="2">* It has been shown by some laboratories that this technique may be used successfully for larger volumes of water although the manufacturers’ instructions may only include volumes up to 200 litres.</td></tr>
</table>
b) Purification and further concentration: After concentration of</td></tr>
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| | particulate material from filter eluates, oocysts and cysts are isolated using immunomagnetic separation (IMS). Oocysts and cysts are attached to para-magnetic beads coated with specific antibody, the beads are separated from the unwanted particulate material using a magnet and then the oocysts and cysts are dissociated from the beads using acid and neutralized using alkali before immunostaining.<br>**c) Detection of Cryptosporidium and Giardia:** After IMS, organisms are labelled with monoclonal antibody (mAb) conjugated to a fluorochrome, usually fluoroscein isothiocyanate (FITC). In addition, any nuclear material is labelled with a nucleic acid stain to aid identification. Each sample is then examined for the presence of labelled Cryptosporidium oocysts and Giardia cysts using epifluorescence and differential interference contrast (DIC) microscopy. |
| **Apparatus/Instruments** | **a) Scientific apparatus**, required for concentration using Pall EnvirochekTM STD or HV<br>i) Sampling capsule, EnvirochekTM STD or HV<br>ii) Peristaltic pump, capable of a flow rate of 2 l/min.<br>iii) Silicon tubing, for use with the peristaltic pump.<br>iv) Seeding container, 10 l, if seeding filters is required.<br>v) Wrist-action shaker, with arms for the agitation of the EnvirochekTM STD or HV sample capsules.<br>vi) Centrifuge, capable of a minimum of 1100 g.<br>vii) Centrifuge tubes, conical, plastic, screwtop, 250 ml capacity.<br>viii) Centrifuge tubes, conical, plastic, screwtop, 50 ml capacity.<br>**NOTE** A flow meter and flow restrictor are required for taking water samples with the filter.<br>**b) Specific apparatus**, required for concentration using IDEXX Filta-Max® or equivalent.<br>i) Sampling housing, Idexx Filta-Max® or equivalent.<br>ii) Sampling module, Idexx Filta-Max® or equivalent.<br>iii) Filter membranes, Idexx Filta-Max® or equivalent.<br>iv) Laboratory pump, capable of supplying 500 kPa (5 bar) pressure.<br>v) Peristaltic pump, capable of flow rate of 4 l/min. |

vi) Silicon tubing, for use with peristaltic pump.

vii) Seeding container, 10 L, if seeding filters is required.

viii) Wash station, automatic or manual, and wash station clamp set, Idexx Filta-Max® or equivalent.

ix) Vacuum set, includes plastic hand pump, waste bottle, tubing and magnetic stirring bar. Idexx Filta-Max® or equivalent.

x) Tubing set, includes elution tube, and middle section, concentrator tube and base, with line tap and steel rod Idexx Filta-Max® or equivalent.

xi) Membrane, for tubing set.

xii) Plastic bag, for washing membrane.

xiii) Centrifuge, capable of 1100 g.

xiv) Centrifuge tubes, conical, plastic, 50 ml capacity.

xv) Forceps.

**NOTE** A flow meter and flow restrictor are required for taking water samples with the filter

**c) General apparatus**

i) Incubator, at (36 ± 2) °C.

ii) Refrigerator, at (5 ± 3) °C

iii) Magnetic stirrer, and magnetic stirring bars.

iv) Vortex mixer.

v) Wash bottles, polypropylene, 250 ml.

vi) Calibrated micropipettes, adjustable: 1 µl to 10 µl with 1 µl to 10 µl tips; 20 µl to 200 µl with 10 µl to 200 µl tips; 200 µl to 1000 µl with 100 µl to 1000 µl tips.

vii) pH meter.

viii) Magnetic particle concentrators, with suitable tubes.

ix) Well microscope slides, with special hydrophobic coating and coverslips.

x) Epifluorescence microscope, with a UV filter (350 nm excitation, 450 nm emission), FITC filter (480 nm excitation, 520 nm emission) filters, TM differential interference contrast (DIC) optics and an eye piece graticule. Total magnification 1000x.

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| | xi) Microscope stage micrometer, 1 mm, ruled in 100 units.<br>xii) Eyepiece graticule, ruled in 100 units.<br>xiii) Humidity chamber, e.g. consisting of a tightly sealed plastic container containing damp paper towels on which the slides are placed.<br>xiv) 10 L containers, graduated in 1 L.<br>xv) Neubauer haemocytometer slide. |
| **Culture Media and Reagents** | **a)** Reagents required for eluting Pall EnvirochekTM STD capsule filters<br>i) Deionized water, 0.2 µm filtered at the point of use.<br>ii) Laureth 12 detergent.<br>iii) Tris buffer, pH 7.4<br>iv) EDTA solution, 0.5 mol/l, pH 8.0.<br>v) Antifoam A.<br>vi) Elution buffer<br>**b)** Reagents required for eluting Pall EnvirochekTM HV capsule filters<br>i) Deionized water, 0.2 µm filtered at point of use.<br>ii) Pre-treatment buffer<br>iii) Laureth 12 detergent<br>iv) Tris buffer, pH 7.4<br>v) EDTA solution, 0.5 mol/l, pH 8.0<br>vi) Antifoam A.<br>vii) Elution buffer<br>**c)** Reagents required for eluting IDEXX Filta-Max® filters or equivalent<br>i) Phosphate buffered saline (PBS)<br>ii) Polyoxyethylene(20)sorbitan monolaurate (Tween 20). Store at room temperature (20 ± 5) °C. Expiry date one year.<br>iii) Elution buffer<br>**d)** Concentration and detection reagents<br>i) Methanol, analytical grade.<br>ii) Magnetic beads, for the detection of Cryptosporidium and Giardia.<br>iii) Fluorescently labelled monoclonal antibodies (mAbs) against |

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| | Cryptosporidium and Giardia. Store at (5 ± 3) °C. Expiry date as stated by the manufacturer. When stains are prepared from concentrated material using a diluent supplied by the manufacturer, the prepared solution is stored at (5 ± 3) °C for no longer than 6 months.<br>iv) Immunofluorescence mounting medium<br>v) 4′,6′-Diamidino-2-phenylindole dihydrochloride dihydrate (DAPI) freeze dried reagent.<br>vi) DAPI stock solution<br>vii) DAPI working solution<br>viii) Phosphate buffered saline (PBS)<br>ix) Non-fluorescing immersion oil. Store at room temperature (20 ± 5) °C.<br>x) Stock suspensions of Cryptosporidium parvum oocysts and Giardia lamblia cysts: Store at (5 ± 3) °C, never allow the suspension to freeze and check quality regularly. Ideally, suspensions of oocysts and cysts should be no more than 3 months old. Stock suspensions should be checked microscopically to confirm that they are monodispersed and discarded if clumps or aggregates are detected. In addition, if mAb and DAPI staining become weak and oocysts become deformed, they should also be discarded<br>xi) Parasite storage medium |
| **Preparation of Reagents** | **a) Tris Buffer:** To prepare, dissolve 121.1g Tris in 700 ml of deionized water and adjust the pH to 7.4 with 1,0 mol/l HCl or NaOH. Make up to 1000 ml with deionized water. Filter sterilization is not necessary. Store at room temperature (20 ± 5) °C. Expiry date 3 months.<br>**b) EDTA solution:** To prepare, dissolve 186.1g EDTA in 800 ml of hot deionized water. Cool to room temperature (20 ± 5) °C and adjust pH to 8.0 with 6.0 mol/l NaOH for initial adjustment and 1,0 mol/l HCl or NaOH for final adjustment. Make up to 1000 ml with deionized water. Store at room temperature (20 ± 5) °C. Expiry date 3 months.<br>**c) Elution buffers for Gelman EnvirochekTM STD and HV capsule** |

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| | Laureth12 1 g<br>Tris buffer 10 ml<br>EDTA solution 2 ml<br>Antifoam A 150 µl<br>Deionized water 1000 ml<br>To prepare, weigh Laureth-12 in a glass beaker and add 100 ml of filtered deionized water. Heat the beaker to melt the Laureth-12 (approximately 1 min) and transfer the solution to a 1 000 ml graduated cylinder. Rinse the beaker several times to ensure the transfer of the detergent to the cylinder. Add 10 ml of Tris solution, pH 7.4, 2 ml of EDTA solution, pH 8.0 and 150 µl of Antifoam A. Dilute to 1 000 ml with filtered deionized water. Store the solution at room temperature (20 ± 5) °C, expiry 2 months.<br>**d) Pre-treatment buffer**<br>Sodium polyphosphate $(NaPO_3)_n$ 5 g<br>Deionized water 1000 ml<br>To prepare, dissolve the sodium polyphosphate in the water. Store at room temperature (20 ± 5) °C. Expiry date 1 week.<br>**e) Phosphate buffered saline (PBS) for IDEXX Filta-Max® filters or equivalent**<br>Sodium chloride 8,0 g<br>Di-sodium hydrogen phosphate ($Na_2HPO_4$) 1.15 g<br>Potassium di-hydrogen phosphate ($KH_2PO_4$) 0.2 g<br>Potassium chloride 0.2 g<br>Deionized water 1000 ml<br>Dissolve the ingredients in the water and adjust the pH to 7.3 ± 0.2 with 1,0 mol/l HCl or NaOH. Store at room temperature (20 ± 5) °C. Expiry date 3 months.<br>**f) Elution buffer for IDEXX Filta-Max® filters or equivalent**<br>Polyoxyethylene sorbitan monolaurate (Tween 20) 1 ml<br>PBS 10 L<br>To prepare, add approximately 8 L of PBS to a 10 L container (with tap). Stir this liquid using a magnetic stirrer and stir bar. Dispense 1 |

ml of Tween 20 to a 50 ml centrifuge tube and dissolve in approximately 10 ml of warm deionized water.

Carefully pour the contents of the centrifuge tube into the 10 L container.

Rinse the tube twice with 10 ml of deionized water, adding the contents of the tube to the 10 L container each time.

Finally, fill the 10 L container to the 10 L mark using PBS. Store at room temperature (20 ± 5) °C, expiry date 1 month.

**g) Immunofluorescence mounting medium**

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| 1,4-Diazabicyclo[2.2.2]octane (DABCO) | 2.0 g |
| Glycerol | 60 ml |
| PBS | 40 ml |

To prepare, dissolve the DABCO in the glycerol and the PBS. Adjust the pH to 7.1 ± 0.2 with 0.1 mol/l HCl or NaOH.

Store vials in use at room temperature (20 ± 5) °C. Store additional vials at (5 ± 3) °C.

**h) DAPI stock solution**

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| DAPI | 1 mg |
| Methanol | 0.5 ml |

To prepare, add 0.5 ml of methanol to a vial containing 1 mg of DAPI.

Store at (5 ± 3) °C. Expiry date 1 month.

**i) DAPI working solution**

Prepare by diluting 10 µl of DAPI stock solution in the 50ml of PBS.

Store at (5 ± 3) °C. Expiry date one day.

**j) Parasite storage medium — stock solution**

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| Sodium azide ($NaN_3$) | 100 mg |
| Deionized water | 5 ml |

To prepare, dissolve the sodium azide in the water. Store at (5 ± 3) °C. Expiry date 1 month.

**k) Parasite storage medium — working solution**

To prepare, add 1 ml of the parasite storage medium stock solution to 100 ml of deionized water. Store at (5 ± 3) °C. Expiry date 1 month.

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| **Sample Preparation** | **a) Sampling and Transport**<br><br>i) Take small volume grab samples (10 L) and transport them to the laboratory in the dark at ambient temperature. Once at the laboratory, samples should be stored at (5 ± 3) °C unless they are to be analysed immediately. Samples should be analysed preferably within 24 h of collection and no longer than 4 d.<br>ii) If the samples are filtered in the field (in case of large volume), connect the device in-line with the water supply, making sure that the flow through the filter is in the direction indicated on the housing by the manufacturer. A flow meter should be included with the filter and this should be read before and after sampling. Transport the filters in the dark at ambient temperature. Once at the laboratory, samples should be stored at (5 ± 3) °C unless they are to be analysed immediately. Samples should be analysed preferably within 24 h of collection and no longer than 4 d. If filters are stored at (5 ± 3) °C, they shall be allowed to warm to room temperature before elution starts.<br>iii) A pre-treatment step using sodium polyphosphate before the elution buffer was introduced to improve the removal of particulate material bound to the filter.<br><br>**NOTE 1** The EnvirochekTM STD or equivalent filter consists of a pleated polyether sulfone membrane sealed in a polycarbonate shell. The filter is supported on a loose polypropylene support. It is supplied packaged with two end caps which can be used to seal the filter. The filter can be connected to a water supply by connecting to a ribbed inlet and the direction of flow through the filter is clearly marked. The flow through the filter should not exceed 2.3 l/min and the differential pressure across the filter should not exceed 210 kPa (2.1 bar).<br><br>**NOTE 2** The EnvirochekTM HV capsule or equivalent is comprised of 1 µm pore size polyester track-etched membrane permanently enclosed in a |

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| | polycarbonate housing. The polyester membrane is directly laminated to a polypropylene support which offers a significant strength improvement over the standard EnvirochekTM STD. The capsule housing burst strength exceeds 1 000 kPa (10 bar) and the differential pressure across the filter membrane is rated to 410 kPa (4.1 bar). Each EnvirochekTM HV capsule is 100 % integrity tested after assembly to ensure product performance. The effective filtration area of the EnvirochekTM HV is 1 300 $cm^2$. The filter is supplied with two end caps which can be used to seal the filter for transport to the laboratory. The filter can be supplied with a tamper evident label containing a unique identification number. The flow through the EnvirochekTM HV or equivalent should not exceed 3.4 l/min.<br><br>**NOTE 3** The Filta-Max® or equivalent filter consists of a foam filter module comprising 60 open cell reticulated foam discs with an external diameter of 55 mm and an internal diameter of 15 mm. The discs are sandwiched between two retaining plates and compressed by tightening a retaining bolt to give a nominal porosity of 1 µm. The filter module fits into a filter housing which has a screw top and seal. The filter housing has stainless steel barbed inlet and outlet ports. The sample enters through the lid of the housing and exits through the base. Water flows into the housing, through the compressed foam rings into the centre of the module and through the outlet port. Removal of the retaining bolt during the elution stage allows the filter to expand during washing. Filter housings are supplied with two tools for the removal of the top and two rubber bungs to seal water in the sample. After sampling, Filta-Max® should be kept wet during storage and transportation. If stored or transported in the filter housing, the inlet and outlet should be securely plugged with the rubber stoppers provided. During transportation or storage, the filter module may be removed from the housing and aseptically placed in an airtight container along with several milliliters of additional deionized water.<br><br>**b) Sample Concentration** |

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| | i. **Pall EnvirochekTM STD or equivalent Filtration:** Support the filter vertically with the white bleed valve uppermost. Remove the two end caps and allow any water in the sample to drain out through the filter. Replace the bottom end cap, fill the cartridge with elution buffer through the inlet fitting until it covers the filter by approximately 1 cm. Replace the upper end cap and secure the cartridge horizontally into the wrist shaker with the white bleed valve in the 12 o'clock position. Shake at 600 cycles per minute (cpm) ± 25 cpm for 5 min ± 30 s. Remove the upper end cap and pour the washings into a 250 ml conical centrifuge tube. Add a further aliquot of elution buffer into the capsule, replace the upper end cap and shake for a further 5 min ± 30 s. Ensure that the white bleed valve is in the 3 o'clock or 9 o'clock position. After 5 min of shaking, remove the upper end cap and decant the washings into the 250 ml centrifuge tube and centrifuge at 1 100 × g for 15 min without braking during the deceleration phase. Record the pellet volume (volume of solids) immediately after centrifugation. A second centrifugation step may be required in a 50 ml centrifuge tube in order to measure the volume. Alternatively, 50 ml centrifuge tubes may be used to concentrate the particulate material eluted from the filter. Using a pipette and a vacuum source of less than 20 kPa (0.2 bar), carefully aspirate off the supernatant leaving 2 ml to 5 ml above the pellet. If no pellet is visible, extra care shall taken to avoid aspirating oocysts and cysts during this step. Add deionized water to the centrifuge tube to bring the total volume to 9 ml. Vortex the tube for 10 s to 15 s to resuspend the pellet and either store the sample at (5 ± 3) °C for future or proceed directly for IMS. If the pellet volume exceeds that recommended by the manufacturer of the IMS test kit, centrifuge the sample a second time in a tube that permits the pellet volume to be measured accurately. Subdivide the sample into aliquots for IMS such that each aliquot represents the maximum pellet volume recommended by the manufacturer and make up each aliquot to 9 ml with deionized water. |

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| | ii. **Pall EnvirochekTM HV or equivalent Filtration:** Support the filter vertically with the white bleed valve uppermost. Remove the two end caps and allow any water in the sample to drain out through the filter. Replace the bottom end cap, fill the cartridge with pre-treatment buffer through the inlet fitting until it covers the filter by approximately 1 cm. Replace the upper end cap and secure the cartridge horizontally into the wrist shaker with the white bleed valve in the vertical position. Shake at 600 cycles per minute (cpm) ± 25 cpm for 5 min ± 30 s. Secure the filter vertically with the white bleed valve uppermost, remove the end caps and allow the pre-treatment buffer to drain out through the filter. Replace the bottom end cap and fill the cartridge as above with deionized water. Replace the upper end cap and rinse the membrane by gently rotating the filter for 30 s. Secure the filter vertically, remove the end caps and allow the deionized water to drain out through the filter. Replace the bottom end cap, fill the cartridge with elution buffer through the inlet fitting until it covers the filter by approximately 1 cm. Replace the upper end cap and secure the cartridge into the wrist shaker with the white bleed valve in the 12 o'clock position. Shake at 600 cpm ± 25 cpm for 5 min ± 30 s. Remove the upper end cap and pour the washings into a 250 ml conical centrifuge tube. Add a further aliquot of elution buffer into the capsule, replace the upper end cap and shake for a further 5 min ± 30 s. Ensure that the white bleed valve is in the 4 o'clock position. After 5 min of shaking, turn the filter such that the white valve is in the 8 o'clock position and shake for a further 5 min. Remove the upper end cap and decant the washings into the 250 ml centrifuge tube and centrifuge at 1100 g for 15 min without braking during the deceleration phase. Record the pellet volume (volume of solids) immediately after centrifugation. A second centrifugation step may be required in a 50 ml centrifuge tube in order to measure the volume. Alternatively, 50 ml centrifuge tubes may be used to concentrate the particulate material eluted from the filter. Using a pipette and a vacuum source of less than 20 kPa (0.2 bar), carefully |

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| | aspirate off the supernatant leaving 2 ml to 5 ml above the pellet. If no pellet is visible, extra care shall be taken to avoid aspirating oocysts and cysts during this step. Add deionized water to the centrifuge tube to bring the total volume to 9 ml. Vortex the tube for 10 s to 15 s to re-suspend the pellet and either store the sample at (5 ± 3) °C for IMS or proceed directly to IMS. If the pellet volume exceeds that recommended by the manufacturer of the IMS test kit, centrifuge the sample a second time in a tube that permits the pellet volume to be measured accurately. Subdivide the sample into aliquots for IMS such that each aliquot represents the maximum pellet volume recommended by the manufacturer and make up each aliquot to 9 ml with deionized water.<br><br>**NOTE** Warming all the elution solutions to 37 °C improves the removal of particulate material. Elution is also helped by increasing the shaking speed to 900 cpm.<br><br>**iii. Idexx Filta-Max® or equivalent Filtration:** The elution apparatus consists of an upper and lower wash tube, a wash station and a vacuum set designed to reduce the volume of the eluate to 50 ml through a membrane. The elution procedure is as follows: Place a membrane filter (rough surface uppermost) in the base of the lower wash tube and put the tube into the base. Make sure that the membrane is held securely and that the tap on the base is closed. Unscrew the housing top using the tools provided, remove the filter module from its housing and screw it into the plunger head of the wash station. Pour any residual water in the filter housing into the lower wash tube. Place the upper part of the wash tube into the jaws of the wash station and lower the filter module down through the tube. Using the key provided, remove the retaining screw from the filter module. The filter should begin to expand. Screw the stainless steel tube into the base of the upper wash tube. Pour approximately 600 ml of wash buffer into the lower wash tube and run a small volume of buffer through the membrane by opening the tap on the |

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| | base of the lower wash tube. Attach the lower wash tube to the upper wash tube. Pump the plunger up and down four or five times to help the filter module expand. If the filter does not expand, leave it soaking in elution buffer for 5 min, occasionally pumping the plunger to help filter expansion. Pump the plunger up and down as far as it will travel 20 times only. Disconnect the lower wash tube, pressing the plunger 5 times to remove any residual elution buffer from the foam rings. Rinse the stainless steel tube with elution buffer and plug the end with the small rubber bung provided. Place the elution tube on a magnetic stirrer. Locate the magnetic stirring bar into the top of the elution tube and set the stirrer such that the liquid in the tube is mixed. Connect the vacuum pump and open the tap on the base of the wash tube. If the sample has little turbidity and the catch bottle is placed below the wash tube, liquid will flow by gravity through the membrane. For turbid liquids, apply a vacuum of no greater than 40 kPa (30 cm of mercury) to filter the washings through the membrane. Should the membrane become blocked, decant washings into a clean bottle, remove the membrane to a plastic bag and place a fresh membrane into the lower wash tube with the smooth surface uppermost. Pour the liquid back into the wash tube, rinsing the bottle, and continue the filtration process. Each membrane shall be washed in a separate bag. When the washings fall to approximately half way up the stirring bar (approximately 30 ml), close the tap and disconnect the vacuum pump and water trap. Pour the liquid in the wash tube into a 50 ml centrifuge tube. Add a further 600 ml of elution buffer to the lower wash tube and attach it to the wash station. Repeat the washing procedure using only 10 strokes of the plunger. Remove the lower wash tube, rinsing the stainless steel tube, place it on the stirrer and attach the stirring bar. Concentrate the filter washing down to approximately one inch above the stirring bar as described above. Add the contents of the first elution to the wash tube and continue reducing the volume of eluate until it is again half way up the magnetic bar. Remove the stirring bar and pour the filter |

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| | washings (approximately 30 ml) into the centrifuge tube. Unscrew the wash tube from the base and carefully remove the membrane filter with fine forceps. Add the filter to the bag provided together with 5 ml of elution buffer. Seal the bag and wash the filter by rubbing between fingers and thumb for (60 ± 10) s. Pipette off the washings and add to the 50 ml centrifuge tube. Repeat the wash procedure and add the second washings to the centrifuge tube. Make up the volume in the tube to 50 ml with elution buffer. Centrifuge the 50 ml tube at 1100x g for 15 min without braking during the deceleration phase. Record the pellet volume (volume of solids) immediately after centrifugation. Using a Pasteur pipette and a vacuum source of less than 20 kPa (0.2 bar), carefully aspirate off the supernatant leaving 2 ml to 5 ml above the pellet. If no pellet is visible, extra care shall be taken to avoid aspirating oocysts and cysts during this step. Add deionized water to the centrifuge tube to bring the total volume to 9 ml. Vortex the tube for 10 s to 15 s to re-suspend the pellet and either store the sample at (5 ± 3) °C for IMS or proceed directly to IMS. If the pellet volume exceeds that recommended by the manufacturer of the IMS test kit, centrifuge the sample a second time in a tube that permits the pellet volume to be measured accurately. Subdivide the sample into aliquots for IMS such that each aliquot represents the maximum pellet volume recommended by the manufacturer and make up each aliquot to 9 ml with deionized water.<br><br>**NOTE:** Clean the wash tubes with hot water and detergent followed by thorough rinsing in warm water and filtered deionized water.<br><br>**NOTE:** Where a number of samples from different sources are examined routinely, it is advantageous to have a separate wash tube set and plunger dedicated to each site to minimize cross contamination. |
| **Method of analysis** | **a) Immunomagnetic separation (IMS):**<br>i) This technique involves the attachment of oocysts and cysts to magnetic beads coated with antibodies to either Cryptosporidium |

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| | or Giardia.<br>ii) The oocyst or cyst-bead complex is separated from interfering particles in the water concentrate by using a magnet.<br>iii) After separation, the oocysts and cysts are dissociated from the beads by acid treatment.<br>iv) Oocysts and cysts are transferred in suspension to a microscope slide and the magnetisable beads are discarded.<br>**Note:** Commercial test kits are the only validated methods available for IMS. The test kits shall be used according to the manufacturer's instructions.<br>**b) Sample staining:**<br>i) Label an appropriate well slide with the sample number and the sample volume analysed (the whole of the sample should be analysed).<br>ii) After addition of NaOH to the wells of the slide, distribute aliquots of the suspension containing the separated oocysts and cysts onto the wells.<br>NOTE: The volume of the NaOH and the sample added to each well will depend on the size of the wells.<br>iii) Prepare two separate well slides with positive and negative controls. The positive control shall consist of a suspension of Cryptosporidium and Giardia containing a known number of parasites. The negative control shall consist of filtered deionized water or PBS. Further positive and negative controls shall be included with each batch of samples stained.<br>iv) Place the well slides containing the samples in an incubator at (36 ± 2) °C or no higher than 42 °C and evaporate to dryness.<br>v) Apply a drop of methanol to each well containing the dried sample and allow to air dry at (20 ± 5) °C. Overlay the sample well with FITC fluorescently labelled monoclonal antibodies (mAb).<br>vi) Place the slides in a humidity chamber, if required, and incubate at (36 ± 2) °C for the time specified by the manufacturer of the |

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| | conjugated antibodies.<br>NOTE: The exact volumes and times depend on the type of antibodies and well slides used.<br>vii) After incubation, remove the slides from the humidity chamber and gently aspirate excess labelled mAb from the side of each well. When performing this step, ensure that the pipette tip does not touch the well surface.<br>viii) Apply 1 drop of 4′,6′-diamidino-2-phenylindole (DAPI) solution to each well. Allow to stand at room temperature (20 ± 5) °C for 2 min.<br>NOTE: This timing applies only to slides that have been methanol fixed and subsequently dried.<br>ix) Remove excess DAPI solution by aspiration (as described above). Apply a drop of filtered deionized water to each well and then aspirate the excess deionized water (as described above).<br>NOTE: An additional washing step using 0.01 M PBS, pH 7.2 is sometimes used before washing with deionized water.<br>x) Allow slides to dry at room temperature (20 ± 5) °C or in an incubator at (36 ± 2) °C. Store the slides in the dark at (5 ± 3) °C until ready for examination.<br>xi) The slides should be examined as soon after processing as possible and shall be examined the next day.<br>NOTE: Slides have been kept for up to three months in the dark and retained their fluorescence. No detailed investigations have been carried out, however, concerning the loss of fluorescence or DAPI staining upon storage. Keep the slide dry and mount it before examination.<br>xii) Before the examination, apply approximately 20 µl of slide mountant to the edge of the well on the sample slide, taking care not to touch the slide with the pipette tip. Place a coverslip onto the slide, taking care not to create bubbles in the slide mountant. Seal the edges of the coverslip with clear nail polish.<br>xiii) Alternatively, the mounting medium may be pipetted onto the |

centre of the coverslip and the slide carefully inverted and placed on the coverslip. The slide can then be carefully turned over with the coverslip uppermost. Take care to avoid trapping air bubbles between the slide and the coverslip.

**c) Microscopy**

**i) General:**

- Use an epifluorescence microscope fitted with DIC for analysis of all sample preparations. Use objectives and eyepieces to a total magnification of 200x or 400x and 1000x. Refer to the manufacturer's instruction manual for details of microscope configuration.
- Calibrate the eyepiece graticule at regular intervals.
- Use a magnification of 800x to 1000x for the confirmation of oocysts and cysts.
- Within this procedure, oocyst and cyst detection relies upon the manual examination of sample preparations using epifluorescence/DIC microscopy. Although this technique is widely employed, it is time consuming, can cause operator fatigue and, as a result, is open to human error. Consequently, a reliable automated procedure is of considerable benefit. Presently, several instruments that can automatically scan sample preparations are available (e.g. laser scanning cytometry) or are in development. When properly validated, such equipment may be employed.

**ii) Examination of fluorescent sample preparations using epifluorescence microscopy**

- Using the epifluorescence microscope and a 200x or 400x magnification, examine the stained control slides to ensure that, on the positive control slide, oocysts and cysts have been correctly labelled by the mAb and that the negative control slide is free from oocysts and cysts. Repeat this examination at 1000x magnification to confirm the staining, the size and appearance of the oocysts and cysts. Examine the contents using the UV

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| | excitation filter to ensure that the nuclear material has been correctly labelled by DAPI.<br>• If the positive control slide is negative, repeat the stain before any samples are processed. If the negative control slide is positive, undertake an investigation to determine the source of the contamination. Prepare fresh reagents and stain the control slides again before any samples are processed.<br>• Providing that these checks are satisfactory, examine the samples by scanning each well systematically using epifluorescent microscopy (FITC). Use a side-to-side or top-to-bottom scanning pattern.<br>• When a horizontal row has been completed, identify a feature situated at the bottom centre of the field of view (i.e. sample debris or the edge of the well slide coating). Move the microscope stage so that this feature appears near the top of the field of view. If the scanning has been carried out using a top-to-bottom pattern (vertical rows), then identify a feature situated at the right hand side, centre of the field of view. Move the microscope stage so that this feature appears near the left hand side of the field of view.<br>– With side-to-side scanning, move the stage horizontally so that the boundary of the well is completely in view, then scan horizontally back across the well.<br>– With top-to-bottom scanning, move the stage vertically so that the boundary of the well is completely in view, then scan up or down the well as necessary.<br>• Repeat until the whole well has been scanned. Scan using a magnification of 200 × or 400 × and note the number of objects which are presumptive Cryptosporidium or Giardia. Where there are only one or two objects, examine each object at 800 × to 1 000 × using water or oil immersion objectives to confirm that they are oocysts or cysts. Where there are more presumptive oocysts or cysts, examine the whole slide at 800 × to 1 000 × and |

confirm each object. This process is easier than switching from a dry low power objective to a high power objective to examine each suspect body.

- All objects with typical characteristics of Cryptosporidium or Giardia should be further examined and measured using DAPI and DIC
- When labelled with FITC-mAb and examined using epifluorescence microscopy (FITC, filter block), organisms should exhibit the following characteristics.

| **Cryptosporidium oocysts Giardia cysts** | **Cryptosporidium oocysts Giardia cysts** |
|---|---|
| Apple green fluorescence (often with bright edges) | Apple green fluorescence (often with bright edges) |
| Spherical or slightly ovoid in shape<br>Some oocysts will exhibit creases, splits and suture lines | Spherical or slightly ovoid in shape<br>Some cysts will exhibit creases and folds |
| Diameter of 4 µm to 6 µm | Dimensions of (8 µm to 12 µm) × (7 µm to 10 µm) |

- Count badly distorted and damaged objects with care, particularly when no typical oocysts or cysts are observed on a slide.

NOTE: The majority of Cryptosporidium oocysts appear spherical or slightly ovoid with brighter even staining around the entire circumference. Some oocysts can deviate from this description. Those which have been in the environment for some time can be weakly stained or appear fuzzy. They may still have contents and sporozoite nuclei can be identified. Often oocysts are split as if a segment has been removed. Under these circumstances, the oocyst may have ruptured during drying on the slide and sporozoites and sporozoite nuclei may be evident adjacent to the oocyst. In addition, oocysts, especially those without contents, may appear to be distorted or partially folded.

NOTE: The majority of Giardia cysts appear ovoid (8 µm to 12 µm × 7 µm to 10 µm), however, on occasion, cysts may appear spherical with dimensions of approximately 10 µm × 10 µm. Cysts which have been in the environment for some time may stain weakly and be badly distorted,

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| | especially those without contents.<br>NOTE: A number of species of both Cryptosporidium and Giardia have been classified (Annex G). The given size ranges target primarily C. parvum and G. intestinalis. However other species may be in that size range which may or may not be pathogenic to humans. Alternatively, other species or single bodies of the target species may not be identified as Cryptosporidium or Giardia due to their size being larger or smaller than the given size range and pathogenicity to humans cannot be ruled out.<br>• When an apple green fluorescent event is observed which is characteristic of a Cryptosporidium oocyst or Giardia cyst, examine the object with the UV filter block for DAPI staining and subsequently with DIC. Other objects (e.g. algae) may mimic the size, structure and staining of Cryptosporidium and Giardia. It is therefore important to further confirm presumptive cells (bodies) by DAPI and DIC.<br>**iii) Identification of Cryptosporidium oocysts and Giardia cysts: DAPI**<br>• Each presumptive oocyst or cyst should be examined to confirm the presence of DAPI staining nuclei using a 100x oil or water immersion objective. Switch over to the UV filter block on the microscope for DAPI visualization.<br>**NOTE** The nuclei of DAPI stained oocysts and cysts appear sky blue upon examination with the epifluorescence microscopy (DAPI UV filter block).<br>• If the object exhibits one of the following characteristics, consider it to be a Cryptosporidium oocyst or Giardia cyst:<br>– two to four distinct, sky blue nuclei within a single body;<br>– nuclear material that may be slightly diffuse giving it a fuzzy or ragged appearance;<br>– diffuse blue internal staining where distinct nuclear material cannot be identified.<br>• Include the two sub-groups in the total count unless they show atypical morphological characteristics such as greater than four nuclei or where one or two large intensely stained nuclei are |

visible within the object.

**iv) Identification of Cryptosporidium oocysts and Giardia cysts: DIC**

- Having examined the object using the FITC and DAPI filter blocks, close the light stop for the UV light and switch on the transmitted light source ensuring that the substage condenser is in place. Ensure that the substage condenser turret plate has the correct ICT condenser prism in place.

**IMPORTANT — It is important that the light from the mercury vapour lamp is blocked as UV light can damage the DIC filter.**

- Slide the DIC filter and prism into position and optimize the image by adjusting the light intensity and/or turning the adjustment screw on the prism.
- Using DIC, measure the size and look for external or internal morphological characteristics typical of a Cryptosporidium oocyst or Giardia cyst. Confirmation of size and internal contents should only be done at a magnification of 1000x (using a 100x oil or water immersion objective).
- Cryptosporidium oocysts should exhibit one of the following characteristics.

  − Spherical or slightly ovoid with a convex central area, the surface of which is irregular in appearance. In addition, a thickened oocyst wall may be observed. This is indicative of an oocyst which contains sporozoites. It may be possible to see sporozoites inside the oocyst as well as a distinct refractile point which is the residual body.

  − Spherical or slightly ovoid objects with a thickened oocyst wall. In addition, a refractile residual body may be observed. This can be indicative of an oocyst after excystation.

  − Spherical or slightly ovoid object that is flat and indistinct. In addition, a thickened oocyst wall is observed. This can also be indicative of an oocyst after excystation.

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| | • Giardia cysts should exhibit one of the following characteristics.<br>– Ovoid with a thickened cyst wall and a convex central area. This is indicative of a cyst with contents. In addition, the nuclei demonstrated by DAPI staining may be observed together with remnants of flagellar axonemes and the median body.<br>– Ovoid with a thickened cyst wall, the central area appearing flat and indistinct. This is indicative of an empty cyst.<br><br>**NOTE:** The identification of organisms using DIC requires much experience. The characteristics given can only be used as guidelines for identification purposes. Misidentification of objects that mimic oocysts and cysts can occur even at this stage of identification.<br>**NOTE:** Cryptosporidium- and Giardia-like bodies can show external or internal morphological characteristics atypical of oocysts or cysts (e.g. spikes, stalks, appendages, pores, one or two large nuclei filling the cell, red fluorescing chloroplasts, crystals, spores, etc.). The presence of such features indicates that the object is not an oocyst or cyst. Be aware that oocyst contents stained by Evans Blue (present in many mAb preparations) can also fluoresce red.<br>• Under some circumstances, the examination of samples using DIC may not be possible due to the presence of interfering debris. In such circumstances, this should be reported and a decision on the identity of the event should be based on the characteristics of FITC-mAb and DAPI labelling |
| **Calculation with units of expression** | a) The whole of the sample pellet should be examined.<br>b) Give the number of Cryptosporidium oocysts and/or Giardia cysts per volume of sample examined. Then calculate the concentration of Cryptosporidium oocysts and/or Giardia cysts for the standard volume usually given (e.g. 10 L or 1000 L). Absence of the organism, i.e. none detected, shall be expressed as "not detected" in the sample volume examined.<br>**NOTE:** The numbers of oocysts or cysts in different aliquots of the pellet can vary considerably. Calculation of the numbers of oocysts and/or cysts in |

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| | the total volume from a number found in an aliquot of the pellet can therefore result in an over- or underestimation of oocyst or cyst concentration. |
| **Inference (Qualitative Analysis)** | - |
| **Reference** | a) IS 10500:2012: Drinking Water — Specification (Second Revision)<br>b) ISO 15553:2006: Water quality — Isolation and identification of Cryptosporidium oocysts and Giardia cysts from water |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

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| | **Isolation and Identification of Giardia and Cryptosporidium in Water- Method B** | | |
| **Method No.** | FSSAI 15.040:2023 | **Revision No. & Date** | 0.0 |
| **Scope** | This method is applicable to Packaged Drinking water | | |
| **Caution** | Flocculation technique is not suitable for particle-free water. Water containing a substantial amount of particulate material can be difficult to process by membrane filtration. | | |
| **Principle** | This method is based on chemical flocculation. Three different concentration techniques are described here. These are calcium carbonate flocculation, ferrous (II) sulfate flocculation and membrane filtration. These techniques allow the analysis of small volumes of water which can be collected and delivered to the laboratory in a relatively short period of time. The principles of IMS and staining and detection described in part A method can be followed once oocysts and cysts have been concentrated from water samples. | | |
| **Apparatus/Instruments** | **a) General apparatus:** As given in Part A method<br>**b) Specific apparatus required for concentration using calcium carbonate flocculation / iron (II) sulfate flocculation**<br>i) Measuring cylinders, 10 ml, 100 ml, 1 000 ml.<br>ii) Aspiration tubes.<br>iii) Vacuum source, with vacuum gauges and vacuum catch bottles/reservoirs.<br>iv) Centrifuge, capable of 7 200 g and 1 100 g.<br>v) Centrifuge bottles, plastic, screwtop, 1 000 ml capacity.<br>vi) Centrifuge tubes, conical, plastic, 50 ml capacity.<br>vii) Flocculation container, 10 l carboy.<br>**c) Specific apparatus required for concentration using 142 mm membrane filtration**<br>i) 142 mm stainless steel filter housing.<br>ii) 142 mm filter membranes, cellulose acetate, no greater than 2.0 µm pore size. | | |

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| | iii) Peristaltic pump, capable of a flow rate of 1 l/min.<br>iv) Silicon tubing, for use with the peristaltic pump.<br>v) Seeding container, 10 l, if seeding filters is required.<br>vi) Suitable polythene bag, for washing the filter, e.g. Stomacher bag.<br>vii) Centrifuge, capable of 1100 g.<br>viii) Centrifuge tubes, conical, plastic, capacity 50 ml. |
| **Culture Media and Reagents** | **a) Reagents required for use with calcium carbonate flocculation**<br>i) Deionized water, 0.2 µm filtered at the point of use.<br>ii) Calcium chloride dihydrate, 1 mol/l.<br>iii) Sodium hydrogen carbonate, 1 mol/l.<br>iv) Sodium hydroxide, 1 mol/l,<br>v) Sulfamic acid, 10 %.<br>vi) Polyoxyethylene(20)sorbitan monolaurate (Tween 20), volume fraction 0.01 %.<br>**b) Reagents for use with iron (II) sulfate flocculation**<br>i) Deionized water, 0.2 µm filtered at point of use.<br>ii) Sodium hydroxide, 1 mol/l.<br>iii) Iron(II) sulfate, 1 mol/l.<br>iv) Oxalic acid, 10 % (mass/volume).<br>v) Polyoxyethylene(20)sorbitan mono-oleate (Tween 80), 0.1 % volume fraction.<br>vi) PBS-Tween, 10 mmol/l PBS (pH 7.4) and 0.1 % Tween 80.<br>vii) Polyoxyethylene(20)sorbitan monolaurate (Tween 20), 0.01 % volume fraction.<br>**c) Reagents required for eluting 142 mm membrane filters**<br>i) Deionized water, 0.2 µm filtered at point of use.<br>ii) Tween 80 in deionized water, 0.1 % volume fraction. |
| **Preparation of Reagents** | **a) Calcium chloride dihydrate, 1 mol/l:** Add 1470 g of $CaCl_2 \cdot 2H_2O$ to 10 L of water. Store at room temperature (20 ± 5) °C. Expiry date 3 months.<br>**b) Sodium hydrogen carbonate, 1 mol/l:** Add 840 g of $NaHCO_3$ to 10 L of water. Store at room temperature (20 ± 5) °C. Expiry date |

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| | 3 months.<br>**c) Sodium hydroxide, 1 mol/l:** 400 g of NaOH per 10 Lof water. Store at room temperature (20 ± 5) °C. Expiry date 3 months.<br>**d) Sulfamic acid, 10 %:** Add 1000 g of $NH_2SO_3H$ to10 L of water. Store at room temperature (20 ± 5) °C. Expiry date 3 months.<br>**e) Iron(II) sulfate**, 1 mol/L: Add 2780 g $FeSO_4 \cdot 7\ H_2O$ to 10 L of water. Store at room temperature (20 ± 5) °C. Expiry date 3 months. |
| **Sample Preparation** | **a) Sampling and Transport:** As given in Part A method<br><br>**b) Sample Concentration**<br><br>**i) Procedure for concentration using calcium carbonate flocculation**<br>• To a 10 L water sample, add 100 ml of 1 mol/L $CaCl_2$ and 100 ml of 1 mol/L $NaHCO_3$ and then shake the container to mix. Add 100 ml of 1 mol/L NaOH and then shake the container to mix.<br>• Allow the contents to stand at room temperature for a minimum of 4 h. Samples shall be left for no longer than 24 h. After the floc has settled, aspirate the supernatant to just above the floc precipitate using a suction vacuum pressure of no greater than 20 kPa (0.2 bar). Take care not to disturb the precipitate.<br>• Add sufficient (100 ml to 200 ml) 10 % sulfamic acid to dissolve the floc completely. Swirl the contents of the container to mix and pour into a 1 L centrifuge bottle labelled with the sample number.<br><br>NOTE: Larger deposits may require the use of two centrifuge bottles.<br>• Add (200 ± 20) ml of 0.01 % volume fraction Tween 20 to the container, shake vigorously to rinse and add to the 1 L centrifuge bottle.<br>• Add a further (200 ± 20) ml of 0.01 % volume fraction Tween 20 |

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| | to the container, rotate the container slowly to pick up froth from around the edges and add to the 1 L centrifuge bottle.<br><br>• Carefully adjust the contents of the 1 L centrifuge bottle to pH 6 to 6.5 by the addition of 1 mol/L NaOH. Ensure that the pH does not exceed this level as this will result in re-formation of the floc.<br><br>• Balance 1 L centrifuge bottles in pairs to within 1 g using filtered deionized water. Centrifuge at 7200 g maximum for 12 min without braking during the deceleration phase.<br><br>• Immediately after centrifugation, remove the bottles from the centrifuge and carefully aspirate the supernatant liquid to just above the pellet using a vacuum pressure of no greater then 20 kPa (0.2 bar).<br><br>• Shake the remaining liquid vigorously to re-suspend the deposited material and transfer to a 50 ml centrifuge tube.<br><br>• Using a wash bottle add (20 ± 2) ml of 0.01 % volume fraction Tween 20 to the 1 L centrifuge bottle to suspend any remaining sample debris and transfer to the 50 ml centrifuge tube. Make up the volume in each tube to approximately 50 ml with filtered deionized water.<br><br>• Centrifuge the 50 ml tubes at 1100x g for 15 min without braking during the deceleration phase. Record the pellet volume (volume of solids) immediately after centrifugation.<br><br>NOTE: IMS test kits usually have a defined pellet volume to be used for the test, e.g. between 0.5 ml and 2 ml.<br><br>• Using a Pasteur pipette and a vacuum source of less than 20 kPa (0.2 bar), carefully aspirate off the supernatant leaving 2 ml to 5 ml above the pellet. If no pellet is visible, take extra care to avoid aspirating oocysts and cysts during this step. |

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| | • Add deionized water to the centrifuge tube to bring the total volume to 10 ml. Vortex the tube for 10 s to 15 s to re-suspend the pellet and either store the sample at (5 ± 3) °C for IMS.<br><br>• If the pellet volume exceeds that recommended by the manufacturers of the IMS test kit, centrifuge the sample a second time in a tube which permits the pellet volume to be measured accurately. Sub-divide the sample into aliquots for IMS such that each aliquot represents no more than the maximum pellet volume recommended by the manufacturer and make each aliquot up to 10 ml with filtered deionized water<br><br>**ii) Procedure for concentration using iron(II) sulfate flocculation**<br>• Fill a flocculation container with 10 L of surface water. Adjust the pH to 9.0 ± 0.2 with 1 mol/L NaOH. Add 20 ml of the ferrous sulfate solution while stirring and mix for 5 min at 280 rev/min.<br><br>• After overnight sedimentation (18 h) at room temperature (20 ± 5) °C, remove the supernatant carefully until 4 cm to 5 cm above the **p**ellet (sediment).<br><br>• Place a beaker under the flocculation tank and collect the sediment (600 ml to 1200 ml according to the surface water used).<br><br>• Rinse the flocculation tank with a sufficient volume (100 ml to 150 ml) of 10 % oxalic acid in order to allow a complete dissolution of the ferric sulfate precipitate.<br><br>• Rinse the tank with 150 ml of the 0.1 % volume fraction Tween 80 solution and add this solution to the sediment.<br><br>• Centrifuge washings at 1500 g for 10 min in 250 ml or 500 ml conical centrifuge tubes (without braking). Carefully aspirate the supernatant until 1 cm above the pellet. Do not disturb the |

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| | pellet as the supernatant is discarded.<br><br>• Measure the pellet volume and re-suspend the pellet with 5 volumes of 0.1 % Tween 80 (5 volumes of Tween 80 for 1 volume of pellet). Repeat the last washing-centrifugation step.<br><br>• Centrifuge at 1500 g for 10 min and resuspend the pellet with 1 volume of PBS-Tween.<br><br>NOTE: The volume of PBS-Tween added is equivalent to the volume of the pellet, total volume 60 ml to 80 ml usually.<br><br>• Control the pH of the water concentrate and adjust it to pH 7.2 to 7.4 with PBS (10 mmol/l pH 7,4) if necessary.<br><br>• Transfer the suspension to 50 ml centrifugation tubes. Add (20 ± 2) ml of 0.01 % volume fraction Tween 20 to the 1 L centrifuge bottle to suspend any remaining sample debris and transfer to the 50 ml centrifuge tubes. Make up the volume in each tube to approximately 50 ml with filtered deionized water.<br><br>• Centrifuge the 50 ml tubes at 1100 g for 15 min without braking during the deceleration phase. Record the pellet volume (volume of solids) immediately after centrifugation.<br><br>• Using a Pasteur pipette and a vacuum source of less than 20 kPa (0.2 bar), carefully aspirate off the supernatant leaving 2 ml to 5 ml above the pellet. If no pellet is visible, extra care shall be taken to avoid aspirating oocysts and cysts during this step.<br><br>• Add deionized water to the centrifuge tube to bring the total volume to 10 ml. Vortex the tube for 10 s to 15 s to re-suspend the pellet and either store the sample at (5 ± 3) °C for IMS.<br><br>• If the pellet volume exceeds that recommended by the manufacturer of the IMS test kit, centrifuge the sample a second time in a tube that permits the pellet volume to be measured accurately. Subdivide the sample into aliquots for IMS such that |

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| | each aliquot represents the maximum pellet volume recommended by the manufacturer and make up each aliquot to 10 ml with deionized water.<br><br>**iii) Procedure for concentration using membrane filtration**<br>• Place the membrane filter into the housing and clamp on the upper part. Pump the water sample through the filter at a rate of no greater than 1.5 L/min. Rinse the container with 2 L of filtered deionized water and pump the washings through the filter.<br>• Remove the filter from the filter housing and place into a suitable clean polythene bag (e.g. a stomacher bag). Add 25 ml of 0.1 % volume fraction Tween 80 and gently rub the surface of the filter for 1 min through the bag to remove particulate material.<br>• Decant the washings into a 50 ml centrifuge tube. Repeat the wash procedure with a further 25 ml of 0.1 % volume fraction Tween 80 and add this to the centrifuge tube.<br>• Centrifuge the tube at 1100x g for 15 min.<br>• Using a Pasteur pipette and a vacuum source of less than 20 kPa (0.2 bar), carefully aspirate off the supernatant leaving 2 ml to 5 ml above the pellet. If no pellet is visible, extra care shall be taken to avoid aspirating oocysts and cysts during this step.<br>• Add deionized water to the centrifuge tube to bring the total volume to 10 ml. Vortex the tube for 10 s to 15 s to re-suspend the pellet and either store the sample at (5 ± 3) °C for IMS.<br>• If the pellet volume exceeds that recommended by the manufacturer of the IMS test kit, centrifuge the sample a second time in a tube that permits the pellet volume to be measured accurately. Subdivide the sample into aliquots for IMS such |

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| | each aliquot represents the maximum pellet volume recommended by the manufacturer and make up each aliquot to 9 ml with deionized water. |
| **Method of analysis** | As given in Method A |
| **Calculation with units of expression** | As given in Method A |
| **Inference (Qualitative Analysis)** | As given in Method A |
| **Reference** | 1) IS 10500:2012 - Drinking Water — Specification (Second Revision)<br>2) ISO 15553:2006 - Water quality — Isolation and identification of Cryptosporidium oocysts and Giardia cysts from water |
| **Approved by** | Scientific Panel on Methods of Sampling and Analysis |

# APPENDIX A

*Table 1—MPN values per gram of sample and 95% confidence limits*

(when three test portions of 1g, three of 0.1 g and three of 0.01g are used)

| Number positive results for inoculums volume, ml or g | | | MPN /ml or /g | 95% Confidence limits | |
|---|---|---|---|---|---|
| 1.00 | 0.10 | 0.01 | | Lower | Upper |
| 0 | 0 | 0 | 0 | 0 | 1.1 |
| 0 | 1 | 0 | 0.30 | 0.04 | 2.3 |
| 1 | 0 | 0 | 0.36 | 0.05 | 2.7 |
| 1 | 0 | 1 | 0.72 | 0.17 | 3.0 |
| 1 | 1 | 0 | 0.74 | 0.18 | 3.1 |
| 1 | 2 | 0 | 1.1 | 0.35 | 3.7 |
| 2 | 0 | 0 | 0.92 | 0.21 | 4.0 |
| 2 | 0 | 1 | 1.4 | 0.42 | 4.8 |
| 2 | 1 | 0 | 1.5 | 0.43 | 5.0 |
| 2 | 1 | 1 | 2.0 | 0.69 | 6.0 |
| 2 | 2 | 0 | 2.1 | 0.71 | 6.2 |
| 3 | 0 | 0 | 2.3 | 0.55 | 9.7 |
| 3 | 0 | 1 | 3.8 | 0.93 | 16 |
| 3 | 1 | 0 | 4.3 | 0.95 | 19 |
| 3 | 1 | 1 | 7.5 | 1.9 | 30 |
| 3 | 1 | 2 | 12 | 3.6 | 37 |
| 3 | 2 | 0 | 9.3 | 2.2 | 40 |
| 3 | 2 | 1 | 15 | 4.4 | 51 |
| 3 | 2 | 2 | 21 | 7.2 | 64 |
| 3 | 3 | 0 | 24 | 5.6 | 100 |
| 3 | 3 | 1 | 46 | 9.6 | 220 |
| 3 | 3 | 2 | 110 | 25 | 480 |
| 3 | 3 | 3 | ∞ | 36 | ∞ |

**Table 2 – Most Probable Number (MPN) of organisms present per 100 ml of Sample and Confidence Limits using 5 tubes of 10 ml, 5 tubes of 1 ml and 5 tubes of 0.1 ml**

| Number of Positive Tubes | | | Most Probable Number (MPN) per 100 ml | Limits within which MPN per 100 ml can lie | |
|---|---|---|---|---|---|
| 10 ml Tubes | 1 ml Tubes | 0.1 ml Tubes | | Lower Limit | Upper Limit |
| (1) | (2) | (3) | (4) | (5) | (6) |
| 0 | 0 | 1 | 2 | <0.5 | 7 |
| 0 | 0 | 2 | 4 | <0.5 | 11 |
| 0 | 1 | 0 | 2 | <0.5 | 7 |
| 0 | 1 | 1 | 4 | <0.5 | 11 |
| 0 | 1 | 2 | 6 | <0.5 | 15 |
| 0 | 2 | 0 | 4 | <0.5 | 11 |
| 0 | 2 | 1 | 6 | <0.5 | 15 |
| 0 | 3 | 0 | 6 | <0.5 | 15 |
| 1 | 0 | 0 | 2 | <0.5 | 7 |
| 1 | 0 | 1 | 4 | <0.5 | 11 |
| 1 | 0 | 2 | 6 | <0.5 | 15 |
| 1 | 0 | 3 | 8 | 1 | 19 |
| 1 | 1 | 0 | 4 | <0.5 | 11 |
| 1 | 1 | 1 | 6 | <0.5 | 15 |
| 1 | 1 | 2 | 8 | 1 | 19 |
| 1 | 2 | 0 | 6 | <0.5 | 15 |
| 1 | 2 | 1 | 8 | 1 | 19 |
| 1 | 2 | 2 | 10 | 2 | 23 |
| 1 | 3 | 0 | 8 | 1 | 19 |
| 1 | 3 | 1 | 10 | 2 | 23 |
| 1 | 4 | 0 | 11 | 2 | 25 |
| 2 | 0 | 0 | 5 | <0.5 | 13 |
| 2 | 0 | 1 | 7 | 1 | 17 |
| 2 | 0 | 2 | 9 | 2 | 21 |
| 2 | 0 | 3 | 12 | 3 | 28 |
| 2 | 1 | 0 | 7 | 1 | 17 |
| 2 | 1 | 1 | 9 | 2 | 21 |
| 2 | 1 | 2 | 12 | 3 | 28 |
| 2 | 2 | 0 | 9 | 2 | 21 |
| 2 | 2 | 1 | 12 | 3 | 28 |
| 2 | 2 | 2 | 14 | 4 | 34 |
| 2 | 3 | 0 | 12 | 3 | 28 |
| 2 | 3 | 1 | 14 | 4 | 34 |

| Number of Positive Tubes | | | Most Probable Number (MPN) per 100 ml | Limits within which MPN per 100 ml can lie | |
|---|---|---|---|---|---|
| 10 ml Tubes | 1 ml Tubes | 0.1 ml Tubes | | Lower Limit | Upper Limit |
| (1) | (2) | (3) | (4) | (5) | (6) |
| 2 | 4 | 0 | 15 | 4 | 37 |
| 3 | 0 | 0 | 8 | 1 | 19 |
| 3 | 0 | 1 | 11 | 2 | 25 |
| 3 | 0 | 2 | 13 | 3 | 31 |
| 3 | 1 | 0 | 11 | 2 | 25 |
| 3 | 1 | 1 | 14 | 4 | 34 |
| 3 | 1 | 2 | 17 | 5 | 46 |
| 3 | 1 | 3 | 20 | 6 | 60 |
| 3 | 2 | 0 | 14 | 4 | 34 |
| 3 | 2 | 1 | 17 | 5 | 46 |
| 3 | 2 | 2 | 20 | 6 | 60 |
| 3 | 3 | 0 | 17 | 5 | 46 |
| 3 | 3 | 1 | 21 | 7 | 63 |
| 3 | 4 | 0 | 21 | 7 | 63 |
| 3 | 4 | 1 | 24 | 8 | 72 |
| 3 | 5 | 0 | 25 | 8 | 75 |
| 4 | 0 | 0 | 13 | 3 | 31 |
| 4 | 0 | 1 | 17 | 5 | 46 |
| 4 | 0 | 2 | 21 | 7 | 63 |
| 4 | 0 | 3 | 25 | 8 | 75 |
| 4 | 1 | 0 | 17 | 5 | 46 |
| 4 | 1 | 1 | 21 | 7 | 63 |
| 4 | 1 | 2 | 26 | 9 | 78 |
| 4 | 2 | 0 | 22 | 7 | 67 |
| 4 | 2 | 1 | 26 | 9 | 78 |
| 4 | 2 | 2 | 32 | 11 | 91 |
| 4 | 3 | 0 | 27 | 9 | 80 |
| 4 | 3 | 1 | 33 | 11 | 93 |
| 4 | 3 | 2 | 39 | 13 | 106 |
| 4 | 4 | 0 | 34 | 12 | 96 |
| 4 | 4 | 1 | 40 | 14 | 108 |
| 4 | 5 | 0 | 41 | 14 | 110 |
| 4 | 5 | 1 | 48 | 16 | 124 |
| 5 | 0 | 0 | 23 | 7 | 70 |
| 5 | 0 | 1 | 31 | 11 | 89 |
| 5 | 0 | 2 | 43 | 15 | 114 |

| Number of Positive Tubes | | | Most Probable Number (MPN) per 100 ml | Limits within which MPN per 100 ml can lie | |
|---|---|---|---|---|---|
| 10 ml Tubes | 1 ml Tubes | 0.1 ml Tubes | | Lower Limit | Upper Limit |
| (1) | (2) | (3) | (4) | (5) | (6) |
| 5 | 0 | 3 | 58 | 19 | 144 |
| 5 | 0 | 4 | 76 | 24 | 180 |
| 5 | 1 | 0 | 33 | 11 | 93 |
| 5 | 1 | 1 | 46 | 16 | 120 |
| 5 | 1 | 2 | 63 | 21 | 154 |
| 5 | 1 | 3 | 84 | 26 | 197 |
| 5 | 2 | 0 | 49 | 17 | 126 |
| 5 | 2 | 1 | 70 | 23 | 168 |
| 5 | 2 | 2 | 94 | 28 | 219 |
| 5 | 2 | 3 | 120 | 33 | 281 |
| 5 | 2 | 4 | 148 | 38 | 366 |
| 5 | 2 | 5 | 177 | 44 | 515 |
| 5 | 3 | 0 | 79 | 25 | 187 |
| 5 | 3 | 1 | 109 | 31 | 253 |
| 5 | 3 | 2 | 141 | 37 | 343 |
| 5 | 3 | 3 | 175 | 44 | 503 |
| 5 | 3 | 4 | 212 | 53 | 669 |
| 5 | 3 | 5 | 253 | 77 | 788 |
| 5 | 4 | 0 | 130 | 35 | 302 |
| 5 | 4 | 1 | 172 | 43 | 486 |
| 5 | 4 | 2 | 221 | 57 | 698 |
| 5 | 4 | 3 | 278 | 90 | 479 |
| 5 | 4 | 4 | 345 | 117 | 999 |
| 5 | 4 | 5 | 426 | 145 | 1161 |
| 5 | 5 | 0 | 240 | 68 | 754 |
| 5 | 5 | 1 | 348 | 118 | 1005 |
| 5 | 5 | 2 | 542 | 180 | 1405 |

**Table 3: Most Probable Number (MPN) of Organisms present per 100 ml of Sample and Confidence Limits using 5 tubes of 10 ml, 5 tubes of 1 ml and 5 tubes of 0.1 ml**

| Combination of Positives | MPN Index/ 100 ml | 95 percent Confidence Limits | | Combination of Positives | MPN Index/ 100 ml | 95 percent Confidence Limits | |
|---|---|---|---|---|---|---|---|
| | | Lower | Upper | | | Lower | Upper |
| 0-0-0 | <2 | - | - | 420 | 22 | 9.0 | 56 |
| 0-0-1 | 2 | 1.0 | 10 | 421 | 26 | 12 | 65 |
| 0-1-0 | 2 | 1.0 | 10 | 430 | 27 | 12 | 67 |
| 0-2-0 | 2 | 1.0 | 13 | 431 | 33 | 15 | 77 |
| | | | | 440 | 34 | 16 | 80 |
| | | | | 500 | 23 | 9.0 | 86 |
| 1-0-0 | 2 | 1.0 | 11 | 501 | 30 | 10 | 110 |
| 1-0-1 | 4 | 1.0 | 15 | 502 | 40 | 20 | 140 |
| 1-1-0 | 4 | 1.0 | 15 | 510 | 30 | 10 | 120 |
| 1-1-1 | 6 | 2.0 | 18 | 511 | 50 | 20 | 150 |
| 1-2-0 | 6 | 2.0 | 18 | 512 | 60 | 30 | 180 |
| 2-0-0 | 4 | 1.0 | 17 | 520 | 50 | 20 | 170 |
| 2-0-1 | 7 | 2.0 | 20 | 521 | 740 | 30 | 210 |
| 2-1-0 | 7 | 2.0 | 21 | 522 | 90 | 40 | 250 |
| 2-1-1 | 9 | 3.0 | 24 | 530 | 80 | 30 | 250 |
| 2-2-0 | 9 | 3.0 | 25 | 531 | 110 | 40 | 300 |
| 2-3-0 | 12 | 5.0 | 29 | 532 | 140 | 60 | 360 |
| 3-0-0 | 8 | 3.0 | 24 | 533 | 170 | 80 | 410 |
| 3-0-1 | 11 | 4.0 | 29 | 540 | 130 | 50 | 390 |
| 3-1-0 | 11 | 4.0 | 29 | 541 | 170 | 70 | 480 |
| 3-1-1 | 14 | 6.0 | 35 | 542 | 220 | 100 | 580 |
| 3-2-0 | 14 | 6.0 | 35 | 543 | 280 | 120 | 690 |
| 3-2-1 | 17 | 7.0 | 40 | 544 | 350 | 160 | 820 |
| | | | | 550 | 240 | 100 | 940 |
| 4-0-0 | 13 | 5.0 | 38 | 551 | 300 | 100 | 1300 |
| 4-0-1 | 17 | 7.0 | 45 | 552 | 500 | 200 | 2000 |
| 4-1-0 | 17 | 7.0 | 46 | 553 | 900 | 300 | 2900 |
| 4-1-1 | 21 | 9.0 | 55 | 554 | 1600 | 600 | 5300 |
| 4-1-2 | 26 | 12 | 63 | 555 | 1600 | - | - |

Annexure 1 — Test microorganisms and performance criteria for culture media commonly used in food microbiology

| Media[a] | Type[e] | Microorganism | Function | Incubation | Control strain | WDCM number[c] | Reference media | Method of control | Criteria | Characteristic reaction |
|---|---|---|---|---|---|---|---|---|---|---|
| Agar Listeria according to Ottaviani and Agosti | S | *Listeria monocytogenes* | Productivity | (44 ± 4) h/ (37 ± 1) °C | *Listeria monocytogenes* 4b<br>*Listeria monocytogenes* 1/2a | 00021[b]<br>00109 | TSA | Quantitative | $P_R \geq 0,5$ | Blue green colonies with opaque halo |
| | | | Selectivity | | *Escherichia coli*[d]<br>*Enterococcus faecalis*[d] | 00012 or 00013<br>00009 or 00087 | — | Qualitative | Total inhibition (0) | — |
| | | | Specificity | | *Listeria innocua* | 00017 | — | Qualitative | — | Blue green colonies without opaque halo |
| Baird-Parker | S | Coagulase-positive staphylococci | Productivity | (24 ± 2) h to (48 ± 2) h/ (37 ± °1) °C | *Staphylococcus aureus* | 00034[b]<br>00032 | TSA | Quantitative | $P_R \geq 0,5$ | Black or grey colonies with clear halo (egg yolk clearing reaction) |
| | | | Selectivity | (48 ± 2) h/ (37 ± 1) °C | *Escherichia coli*[d] | 00012<br>00013 | — | Qualitative | Total inhibition (0) | — |
| | | | Specificity | (24 ± 2) h to (48 ± 2) h/ (37 ± 1) °C | *Staphylococcus saprophyticus*<br>*Staphylococcus epidermidis* | 00159[b]<br>00036 | — | Qualitative | — | Black or grey colonies without egg yolk clearing reaction |
| BGBLB | L | Coliforms | Productivity | (24 ± 2) h to (48 ± 2) h/ (30 ± 1) °C | *Escherichia coli*<br>*Citrobacter freundii* | 00012[b]<br>00013<br>00006 | — | Qualitative | Turbidity (2)[f] and gas in Durham tube | Gas production and turbidity |
| | | | Selectivity | | *Enterococcus faecalis*[d] | 00009<br>00087 | — | Qualitative | Partial inhibition without gas production | — |
| CFC | S | *Pseudomonas spp.* | Productivity | (44 ± 4) h/ (25 ± 1) °C | *Pseudomonas fluorescens*<br>*Pseudomonas fragi* | 00115[b]<br>00116 | TSA | Quantitative | $P_R \geq 0,5$ | — |
| | | | Selectivity | | *Escherichia coli*[d] | 00012<br>00013 | — | Qualitative | Total inhibition (0) | — |

Annexure **1** — (*continued*)

| **Media**[a] | **Type**[e] | **Microorganism** | **Function** | **Incubation** | **Control strain** | **WDCM number**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reaction** |
|---|---|---|---|---|---|---|---|---|---|---|
| DG18 | S | Yeasts and moulds | Productivity | 5 days/ (25 ± 1) °C | *Saccharomyces cerevisiae*<br>*Wallemia sebi*<br>*Aspergillus restrictus*<br>*Eurotium rubrum* | 00058[b]<br>00182[b]<br>00183<br>00184 | SDA | Quantitative | $P_R \geq 0,5$ | Characteristic colony/propagules according to each species |
| | | | Selectivity | | *Escherichia coli*<br>*Bacillus subtilis subsp. spizizenii* | 00012 or 00013[g]<br>00003 | — | Qualitative | No growth | — |
| DRBC | S | Yeasts and moulds | Productivity | 5 days/ (25 ± 1) °C | *Saccharomyces cerevisiae*<br>*Aspergillus brasiliensis*<br>*Candida albicans*<br>*Mucor racemosus* | 00058[b]<br>00053[b]<br>00054<br>00181 | SDA | Quantitative | $P_R \geq 0,5$ | Characteristic colony/propagules according to each species |
| | | | Selectivity | | *Escherichia coli*<br>*Bacillus subtilis subsp. spizizenii* | 00012 or 00013[g]<br>00003 | — | Qualitative | No growth | — |
| EC | L | *Escherichia coli* | Productivity | (24 ± 2) h to (48 ± 2) h/ (44 ± 1) °C | *Escherichia coli* | 00012[b] 00013 | — | Qualitative | Turbidity (2)[f] and gas in Durham tube | Gas production and turbidity |
| | | | Selectivity | | *Pseudomonas aeruginosa* | 00025 | — | Qualitative | No growth | — |
| IS (“TS”) | S | Sulfite-reducing bacteria | Productivity | (24 ± 3) h to (48 ± 2) h/ (37 ± 1) °C anaerobic atmosphere | *Clostridium perfringens* | 00007[b] 00080 | TSA or other non-selective medium for anaerobes | Quantitative | $P_R \geq 0,5$ | Black colonies |
| | | | Specificity | | *Escherichia coli*[d] | 00012 00013 | — | Qualitative | — | No blackening |

Annexure **1** — (*continued*)

| **Media**[a] | **Type**[e] | **Microorganism** | **Function** | **Incubation** | **Control strain** | **WDCM number**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reaction** |
|---|---|---|---|---|---|---|---|---|---|---|
| LST | L | Coliforms | Productivity | (24 ± 2) h to (48 ± 2) h/ (30 ± 1) °C | *Escherichia coli*<br>*Citrobacter freundii* | 00012[b]<br>00013<br>00006 | — | Qualitative | Turbidity (2)[f] and gas in Durham tube | Gas production and turbidity |
| | | | Selectivity | | *Enterococcus faecalis*[d] | 00009<br>00087 | — | Qualitative | No growth | — |
| | | *Escherichia coli* | Productivity | (24 ± 2) h to (48 ± 2) h/ (37 ± 1) °C | *Escherichia coli* | 00012[b]<br>00013 | — | Qualitative | Turbidity (2)[fi] and gas in Durham tube | Gas production and turbidity |
| | | | Selectivity | | *Enterococcus faecalis*[d] | 00009<br>00087 | — | Qualitative | No growth | — |
| mCCDA | S | *Campylobacter* | Productivity | (44 ± 4) h/ (41,5 ± 1) °C microaerobic atmosphere | *Campylobacter jejuni*<br>*Campylobacter coli* | 00156[b]<br>00005<br>00004 | Blood agar | Quantitative | $P_R \geq 0,5$ | Greyish, flat and moist, sometimes with metallic sheen |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total or partial inhibition (0-1) | — |
| | | | | | *Staphylococcus aureus* | 00034 | — | Qualitative | Total inhibition (0) | — |
| MRS | S | Lactic acid bacteria | Productivity | (72 ± 3) h/ (30 ± 1) °C | *Lactobacillus sakei*<br>*Lactococcus lactis*<br>*Pediococcus pentosaceus* | 00015[b]<br>00016[b]<br>00158 | Media batch MRS already validated | Quantitative | $P_R \geq 0,7$ | Characteristic colonies accordingto each species |
| | | | Selectivity | (72 ± 3) h/ (30 ± 1) °C | *Escherichia coli*[d]<br>*Bacillus cereus* | 00012 or 00013<br>00001 | — | Qualitative | Total inhibition (0) | — |
| MYP | S | *Bacillus cereus* | Productivity | (24 ± 3) h to (44 ± 4) h/ (30 ± 1) °C | *Bacillus cereus* | 00001 | TSA | Quantitative | $P_R \geq 0,5$ | Pink colonies with precipitation halo |
| | | | Selectivity | (44 ± 4) h/ (30 ± 1) °C | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibition (0) | — |
| | | | Specificity | | *Bacillus subtilis subsp. spizizenii* | 00003 | — | Qualitative | — | Yellow colonies without precipitation halo |

Annexure **1** — (*continued*)

| **Media**[a] | **Type**[e] | **Microorganism** | **Function** | **Incubation** | **Control strain** | **WDCM number**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reaction** |
|---|---|---|---|---|---|---|---|---|---|---|
| RPFA | S | Coagulase-positive staphylococci | Productivity | (24 ± 2) h to (48 ± 2) h/ (37 ± 1) °C | *Staphylococcus aureus* | 00034[b] 00032 | TSA | Quantitative | $P_R \geq 0,5$ | Black or grey colonies with opacity halo |
| | | | Selectivity | (48 ± 2) h/ (37 ± 1) °C | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibition (0) | — |
| | | | Specificity | (24 ± 2) h to (48 ± 2) h/ (37 ± 1) °C | *Staphylococcus saprophyticus* | 00159[b] | — | Qualitative | — | Black or grey colonies without opacity halo |
| | | | | | *Staphylococcus epidermidis* | 00036 | | | | |
| PPA | S | *Pseudomonas spp.* | Productivity | (48 ± 2) h/ (25 ± 1) °C | *Pseudomonas fluorescens* | 00115[b] | TSA | Quantitative | $P_R \geq 0,5$ | — |
| | | | | | *Pseudomonas aeruginosa* | 00025 | | | | |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibition (0) | — |
| TBX | S | β-d-Glucuronidase-positive *Escherichia coli* | Productivity | (21 ± 3) h/ (44 ± 1) °C | *Escherichia coli*[h] | 00012[d] 00013[d] 00202[b] | TSA | Quantitative | $P_R \geq 0,5$ | Blue colonies |
| | | | Selectivity | | *Enterococcus faecalis*[d] | 00009 00087 | | Qualitative | Total inhibition (0) | — |
| | | | Specificity | | *Citrobacter freundii* | 00006[b] | | Qualitative | — | White to green-beige colonies |
| | | | | | *Pseudomonas aeruginosa* | 00025 | | | | |
| TSC (SC) | S | *Clostridium perfringens* | Productivity | (20 ± 2) h/ (37 ± 1) °C anaerobic atmosphere | *Clostridium perfringens* | 00007[b] 00080 | TSA or other non-selective medium for anaerobes | Quantitative | $P_R \geq 0,5$ | Black colonies |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibition (0) | — |

Annexure **1** — (*continued*)

| **Media**[a] | **Type**[e] | **Microorganism** | **Function** | **Incubation** | **Control strain** | **WDCM number**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reaction** |
|---|---|---|---|---|---|---|---|---|---|---|
| VRBG | S | *Enterobacteriaceae* | Productivity | (24 ± 2) h/ (37 ± 1) °C | *Escherichia coli*<br>*Salmonella* Typhimurium[d,i]<br>*Salmonella* Enteritidis[d,i] | 00012[b] 00013<br>00031<br>00030 | TSA | Quantitative | $P_R \geq 0,5$ | Pink to red colonies with or without precipitation halo |
| | | | Selectivity | | *Enterococcus faecalis*[d] | 00009 00087 | — | Qualitative | Total inhibition (0) | — |
| VRBL | S | Coliforms | Productivity | (24 ± 2) h/ (30 ± 1) °C | *Escherichia coli* | 00012[b] 00013 | TSA | Quantitative | $P_R \geq 0,5$ | Purplish-red colonies with or without precipitation halo |
| | | | Selectivity | | *Enterococcus faecalis*[d] | 00009 00087 | — | Qualitative | Total inhibition (0) | — |
| | | | Specificity | | *Pseudomonas aeruginosa* | 00025 | | Qualitative | — | Colourless to beige colonies |
| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM numbers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions** |
| PCA<br>MPC<br>A | S | Colony count | Productivity | (72 ± 3) h/ (30 ± 1) °C | *Bacillus subtilis subsp. spizizenii*<br>*Escherichia coli*<br>*Staphylococcus aureus* | 00003[b]<br>00012[b] 00013<br>00034 | TSA | Quantitative | $P_R \geq 0,7$ | — |

Annexure **1** — (*continued*)

| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM num-bers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions of target microorganism** |
|---|---|---|---|---|---|---|---|---|---|---|
| Bolton | L | *Campylobacter* | Productivity | (5 ± 1) h/ (37 ± 1) °C then (44 ± 4) h/ (41,5 ± 1) °C microaerobic atmosphere | *Campylobacter jejuni*[d] | 00156 or 00005 | — | Qualitative | > 10 colonies on mCCDA | Greyish, flat and moist, sometimes with metallic sheen |
| | | | | | + *Escherichia coli*[d] | 00012 or 00013 | | | | |
| | | | | | + *Proteus mirabilis* | 00023 | | | | |
| | | | | | *Campylobacter coli* | 00004 | | | | |
| | | | | | + *Escherichia coli*[d] | 00012 or 00013 | | | | |
| | | | | | + *Proteus mirabilis* | 00023 | | | | |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibition (0) on TSA | — |
| | | | | | *Proteus mirabilis* | 00023 | | | | |
| EE | L | *Enterobacteriaceae* | Productivity | (24 ± 2) h/ (37 ± 1) °C | *Escherichia coli* | 00012[b] 00013 | — | Qualitative | > 10 colonies on VRBG | Pink to red colonies with or without precipitation halo |
| | | | | | + *Enterococcus faecalis*[d] | 00009 or 00087 | | | | |
| | | | | | *Salmonella* Typhimurium[d,i] *Salmonella* Enteritidis[d,i] | 00031 or 00030 | | | | |
| | | | | | + *Enterococcus faecalis*[d] | 00009 or 00087 | | | | |
| | | | Selectivity | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | Total inhibition (0) on TSA | — |

Annexure **1** — (*continued*)

| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM num-bers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions of target microorganism** |
|---|---|---|---|---|---|---|---|---|---|---|
| Fraser | L | *Listeria monocytogenes* | Productivity | (48 ± 2) h/ (37 ± 1) °C | *Listeria monocytogenes* 4b | 00021[b] | — | Qualitative | > 10 colonies on Agar Listeria according to Ottaviani and Agosti | Blue green colonies with opaque halo |
| | | | | | + *Escherichia coli*[d] | 00012 or 00013 | | | | |
| | | | | | + *Enterococcus faecalis*[d] | 00009 or 00087 | | | | |
| | | | | | *Listeria monocytogenes* 1/2a | 00109 | | | | |
| | | | | | + *Escherichia coli*[d] | 00012 or 00013 | | | | |
| | | | | | + *Enterococcus faecalis*[d] | 00009 or 00087 | | | | |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibition (0) on TSA | — |
| | | | | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | < 100 colonies on TSA | — |
| Giolitti Cantoni | L | Coagulase-positive staphylococci | Productivity | (24 ± 2) h to (48 ± 2) h/ (37 ± 1) °C | *Staphylococcus aureus* | 00034[b] | — | Qualitative | > 10 colonies on Baird Parker or RPFA | Characteristic colonies accordingto each medium for Baird Parker and RPFA) |
| | | | | | + *Escherichia coli*[d] | 00012 or 00013 | | | | |
| | | | | | *Staphylococcus aureus* | 00032 | | | | |
| | | | | | + *Escherichia coli*[d] | 00012 or 00013 | | | | |
| | | | Selectivity | (48 ± 2) h/ (37 ± 1) °C | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibition (0) on TSA | — |

Annexure **1** — (*continued*)

| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM numbers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions of target microorganism** |
|---|---|---|---|---|---|---|---|---|---|---|
| Half-Fraser | L | *Listeria monocytogenes* | Productivity | (24 ± 2) h/ (30 ± 1) °C | *Listeria monocytogenes* 4b | 00021[b] | — | Qualitative | > 10 colonies on Agar Listeria according to Ottaviani and Agosti | Blue green colonies with opaque halo |
| | | | | | + *Escherichia coli*[d] | 00012 or 00013 | | | | |
| | | | | | + *Enterococcus faecalis*[d] | 00009 or 00087 | | | | |
| | | | | | *Listeria monocytogenes* 1/2a | 00109 | | | | |
| | | | | | + *Escherichia coli*[d] | 00012 or 00013 | | | | |
| | | | | | + *Enterococcus faecalis*[d] | 00009 or 00087 | | | | |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibition (0) on TSA | — |
| | | | | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | < 100 colonies on TSA | — |
| ITC | L | *Yersinia enterocolitica* | Productivity | (44 ± 4) h/ (25 ± 1) °C | *Yersinia enterocolitica* | 00038[b] | — | Qualitative | > 10 colonies on CIN or SSDC | Characteristic colonies accordingto each medium |
| | | | | | + *Escherichia coli*[d] | 00012 or 00013 | | | | |
| | | | | | + *Pseudomonas aeruginosa* | 00025 | | | | |
| | | | | | *Yersinia enterocolitica* | 00160 | | | | |
| | | | | | + *Escherichia coli*[d] | 00012 or 00013 | | | | |
| | | | | | + *Pseudomonas aeruginosa* | 00025 | | | | |
| | | | Selectivity | | *Pseudomonas aeruginosa* | 00025 | — | Qualitative | Total inhibition (0) on TSA | — |
| | | | | | *Proteus mirabilis* | 00023 | | | | |

Annexure **1** — (*continued*)

| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM num-bers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions of target microorganism** |
|---|---|---|---|---|---|---|---|---|---|---|
| MKTTn | L | *Salmonella* | Productiv-ity | (24 ± 3) h/ (37 ± 1) °C | *Salmonella* Enteritidis[d,i]<br>*Salmonella* Typhimurium[d,i]<br>+ *Escherichia coli*[d]<br>+ *Pseudomonas aerugi-nosa* | 00030<br>00031<br>00012 or 00013<br>00025 | — | Qualitative | > 10 colonies on XLD or other medium of choice | Characteristic colonies accordingto each medium |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Partial inhi-bition ≤ 100 colonies on TSA | — |
| | | | | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | < 10 colonies on TSA | — |
| MSRV[k] | SS | *Salmonella* | Productiv-ity | 2 × (24 ± 3) h/ (41,5 ± 1) °C | *Salmonella* Enteritidis[d,i]<br>*Salmonella* Typhimurium[d,i] | 00030<br>00031 | — | Qualitative | Grey-white, turbid zone extending out from inoculated drop(s). After 24–48 h, the turbid zone(s) will be (almost) fully migrated over the plate. | Possible extra: characteristic colo-nies after subculturing on XLD[k] |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Possible growth at the place of the inocu- lated drop(s) without a turbid zone. | — |
| | | | | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | No growth | — |

Annexure **1** — (*continued*)

| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM num-bers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions of target microorganism** |
|---|---|---|---|---|---|---|---|---|---|---|
| MMG | L | β-d-Glucuronidase-positive *E. coli* | Productivity | (24 ± 2) h/ (37 ± 1) °C | *Escherichia coli* | 00012[b] 00013 | — | Qualitative | Acid production | Colour change to yellow |
| | | | Selectivity | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | No growth | — |
| PSB | L | *Yersinia enterocolitica* | Productivity | 3 to 5 days/ (25 ± 1) °C | *Yersinia enterocolitica* | 00038[b] | — | Qualitative | > 10 colonies on CIN or SSDC | Characteristic colonies accordingto each medium |
| | | | | | + *Escherichia coli*[d] | 00012 or 00013 | | | | |
| | | | | | + *Pseudomonas aeruginosa* | 00025 | | | | |
| | | | | | *Yersinia enterocolitica* | 00160 | | | | |
| | | | | | + *Escherichia coli*[d] | 00012 or 00013 | | | | |
| | | | | | + *Pseudomonas aeruginosa* | 00025 | | | | |
| | | | Selectivity | | *Pseudomonas aeruginosa* | 00025[b] | — | Qualitative | Total inhibition (0) on TSA | — |
| | | | | | *Proteus mirabilis* | 00023 | | | | |
| RVS | L | *Salmonella* | Productivity | (24 ± 3) h/ (41,5 ± 1) °C | *Salmonella* Enteritidis[d,i] *Salmonella* Typhimurium[d,i] | 00030 00031 | — | Qualitative | > 10 colonies on XLD or other medium of choice | Characteristic colonies accordingto each medium |
| | | | | | + *Escherichia coli* [d] | 00012 or 00013 | | | | |
| | | | | | + *Pseudomonas aeruginosa* | 00025 | | | | |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Partial inhibition ≤ 100 colonies on TSA | — |
| | | | | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | < 10 colonies on TSA | — |

Annexure **1** — (*continued*)

| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM num-bers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions of target microorganism** |
|---|---|---|---|---|---|---|---|---|---|---|
| TSPB | L | *Bacillus cereus* | Productivity | (48 ± 4) h/ (30 ± 1) °C | *Bacillus cereus* | 00001 | — | Qualitative | > 10 colonies on PEMBA orMYP | Characteristic colonies accordingto each medium |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibition (0) on TSA | — |
| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM num-bers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions** |
| BHI | L | Coagulase-positive staphylococci | Productivity | (24 ± 2) h/ (37 ± 1) °C | *Staphylococcus aureus* | 00034 | — | Qualitative | Turbidity (1–2)[f] | — |
| Brucella | L | *Campylobacter* | Productivity | 2 to 5 days/ (41,5 ± 1) °C microaerobic atmosphere | *Campylobacter jejuni*[d]<br>*Campylobacter coli*[d] | 00156 00005<br>00004 | — | Qualitative | Turbidity (1–2)[f] | — |
| Diluents for special purposes e.g.<br>BPW with bromo-cresol-purple | L | Dilution liquids | Diluent | 45 min – 1 h/ 20 °C to 25 °C | *Escherichia coli*[d]<br>*Staphylococcus aureus* | 00012 or 00013<br>00034[b] | TSA | Quantitative | ±30 % colonies/ $T_0$ (±30 % of original count) | — |
| Quarter-strength Ringer's<br>Peptone solution<br>Peptone-salt<br>Phosphate buffer solution | L | Dilution liquids | Diluent | 45 min – 1 h/ 20 °C to 25 °C | *Escherichia coli*[d]<br>*Staphylococcus aureus* | 00012 or 00013<br>00034[b] | TSA | Quantitative | ±30 % colonies/ $T_0$ (±30 % of original count) | — |
| Thioglycollate | L | *Clostridium perfringens* | Productivity | (21 ± 3) h/ (37 ± 1) °C | *Clostridium perfringens* | 00007 | — | Qualitative | Turbidity (1–2)[f] | — |
| TSYEB | L | *Listeria monocytogenes* | Productivity | (21 ± 3) h/ (25 ± 1) °C | *Listeria monocytogenes* 4b<br>*Listeria monocytogenes* 1/2a | 00021[b]<br>00109 | — | Qualitative | Turbidity (1–2)[f] | — |

Annexure **1** — (*continued*)

| Media[a] | Type[e] | Micro-organisms | Function | Incubation | Control strains | WDCM num-bers[c] | Reference media | Method of control | Criteria | Characteristic reactions |
|---|---|---|---|---|---|---|---|---|---|---|
| Agar Listeria according to Ottaviani and Agosti[j] | S | *Listeria monocytogenes* | Productiv-ity | (44 ± 4) h/ (37 ± 1) °C | *Listeria monocytogenes* 4b<br>*Listeria monocytogenes* 1/2a | 00021[b]<br>00109 | — | Qualitative | Good growth (2) | Blue green colonies with opaque halo |
| | | | Selectivity | | *Escherichia coli*[d]<br>*Enterococcus faecalis*[d] | 00012 or 00013<br>00009 or 00087 | — | Qualitative | Total inhibi-tion (0) | — |
| | | | Specificity | | *Listeria innocua* | 00017 | — | Qualitative | — | Blue green colonies without opaque halo |
| mCCDA[j] | S | *Campylobacter* | Productiv-ity | (44 ± 4) h/ (41,5 ± 1) °C microaerobic atmosphere | *Campylobacter jejuni*<br>*Campylobacter coli* | 00156[b] 00005<br>00004 | — | Qualitative | Good growth (2) | Greyish, flat and moist colonies, sometimes with metallic sheen |
| | | | Selectivity | | *Escherichia coli*[d] | 000012 or 00013 | — | Qualitative | Total or partial inhi-bition (0 − 1) | No characteristic colonies |
| | | | | | *Staphylococcus aureus* | 00034 | — | Qualitative | Total inhibi-tion (0) | — |
| CT-SMAC | S | *Escherichia coli* O157 | Productiv-ity | (21 ± 3) h/ (37 ± 1) °C | *Escherichia coli* O157:H7 | 00014 (non-toxigenic strain) | — | Qualitative | Good growth (2) | Transparent colonies with a pale yellowish-brown appearance anda diameter ~1 mm |
| | | | Selectivity | | *Staphylococcus aureus*[d] | 00032 or 00034 | — | Qualitative | Total inhibi-tion (0) | — |
| | | | | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Partial inhi-bition (1) | Growth of some pink colonies |

Annexure **1** — (*continued*)

| Media[a] | Type[e] | Micro-organisms | Function | Incubation | Control strains | WDCM num-bers[c] | Reference media | Method of control | Criteria | Characteristic reactions |
|---|---|---|---|---|---|---|---|---|---|---|
| CPC<br>mCP<br>C | S | *Vibrio spp.* other than *Vibrio para-haemolyticus/ V. cholerae* | Productiv-ity | (24 ± 3) h/ (37 ± 1) °C | *Vibrio vulnificus* | 00187[b] | — | Qualitative | Good growth (2) | Yellow colonies surrounded by a yellow coloration in the medium |
| | | | | | *Vibrio cholerae* non-O1/non-O139 | 00203[b] | — | Qualitative | Good growth (2) | Purple colonies surrounded by a purple coloration in the medium |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 or 00090 | — | Qualitative | Total inhibi-tion (0) | — |
| MYP[j] | S | *Bacillus cereus* | Productiv-ity | (21 ± 3) h to 48 h/ (30 ± 1) °C | *Bacillus cereus* | 00001 | — | Qualitative | Good growth (2) | Pink colonies with precipitation halo |
| | | | Selectivity | (44 ± 4) h/ (30 ± 1) °C | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibi-tion (0) | — |
| | | | Specificity | | *Bacillus subtilis subsp. spizizenii* | 00003 | — | Qualitative | — | Yellow colonies without precipita-tion halo |
| PEMBA | S | *Bacillus cereus* | Productiv-ity | (21 ± 3) h to (44 ± 4) h/ (37 ± 1) °C | *Bacillus cereus* | 00001 | — | Qualitative | Good growth (2) | Turquoise-blue colonies with precipitation halo |
| | | | Selectivity | (44 ± 4) h/ (37 ± 1) °C | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibi-tion (0) | — |
| | | | Specificity | | *Bacillus subtilis subsp. spizizenii* | 00003 | — | Qualitative | — | White colonies without precipita-tion halo |
| SDS | S | *Vibrio spp.* other than *Vibrio para-haemolyticus/ V. cholerae* | Productiv-ity | (24 ± 3) h/ (37 ± 1) °C | *Vibrio vulnificus* | 00187[b] | — | Qualitative | Good growth (2) | Purple/green colonies with an opaque halo |
| | | | | | *Vibrio cholerae* non-O1/non-O139 | 00203[b] | — | Qualitative | Good growth (2) | Yellow colonies with an opaque halo |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 or 00090 | — | Qualitative | Total inhibi-tion (0) | — |

Annexure **1** — (*continued*)

| Media[a] | Type[e] | Micro-organisms | Function | Incubation | Control strains | WDCM numbers[c] | Reference media | Method of control | Criteria | Characteristic reactions |
|---|---|---|---|---|---|---|---|---|---|---|
| TBX[j] | S | β-d-Glucuronidase-positive *Escherichia coli* | Productivity | (21 ± 3) h/ (44 ± 1) °C | *Escherichia coli*[h] | 00012[d] 00013[d] 00202[b] | — | Qualitative | Good growth (2) | Blue colonies |
| | | | Selectivity | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | Total inhibition (0) | — |
| | | | Specificity | | *Citrobacter freundii* | 00006[b] | — | Qualitative | — | White to green-beige colonies |
| | | | | | *Pseudomonas aeruginosa* | 00025 | | | | |
| TCBS | S | *Vibrio parahaemolyticus / V. cholerae* | Productivity | (24 ± 3) h/ (37 ± 1) °C | *Vibrio parahaemolyticus* | 00185[b] | — | Qualitative | Good growth (2) | Green colonies (sucrose negative) |
| | | | | | *Vibrio furnissii* | 00186[b] | — | Qualitative | Good growth (2) | Yellow colonies (sucrose positive) |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 or 00090 | — | Qualitative | Total inhibition (0) | — |
| VRBG[j] | S | *Enterobacteriaceae* | Productivity | (24 ± 2) h/ (37 ± 1) °C | *Escherichia coli* | 00012[b] 00013 | — | Qualitative | Good growth (2) | Pink to red colonies with or without precipitation halo |
| | | | | | *Salmonella* Typhimurium[d,i] | 00031 | | | | |
| | | | | | *Salmonella* Enteritidis[d,i] | 00030 | | | | |
| | | | Selectivity | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | Total inhibition (0) | — |

**Table E.1 —** (*continued*)

| Media[a] | Type[e] | Micro-organisms | Function | Incubation | Control strains | WDCM numbers[c] | Reference media | Method of control | Criteria | Characteristic reactions |
|---|---|---|---|---|---|---|---|---|---|---|
| XLD | S | *Salmonella* | Productivity | (24 ± 3) h/ (37 ± 1) °C | *Salmonella* Typhimurium[d,i]<br>*Salmonella* Enteritidis[d,i] | 00031<br>00030 | — | Qualitative | Good growth (2) | Colonies with black centre anda lightly transparent zone of reddish colour due to the colourchange of the medium |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Growth or partial inhibition (0 − 1) | Yellow colonies |
| | | | | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | Total inhibition (0) | — |
| **Media[a]** | **Type[e]** | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM numbers[c]** | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions** |
| Nutrient agar[l] | S | *Enterobacteriaceae* | Productivity | (24 ± 2) h/ (37 ± 1) °C | *Escherichia coli* | 00012[b] 00013 | — | Qualitative | Good growth (2) | — |
| | | *Salmonella* | | (24 ± 2) h/ (37 ± 1) °C | *Salmonella* Typhimurium[d,i]<br>*Salmonella* Enteritidis[d,i] | 00031<br>00030 | | | | |
| | | *Yersinia enterocolitica* | | (24 ± 2) h/ (30 ± 1) °C | *Yersinia enterocolitica* | 00038[b] 00160 | | | | |
| TSYEA | S | *Listeria monocytogenes* | Productivity | (21 ± 3) h/ (37 ± 1) °C | *Listeria monocytogenes* 4b<br>*Listeria monocytogenes* 1/2a | 00021[b]<br>00109 | — | Qualitative | Good growth (2) | — |

Annexure **1** — (*continued*)

| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM num-bers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions** |
|---|---|---|---|---|---|---|---|---|---|---|
| BPW[m] | L | Diluent for all enumerations of microorganisms | Dilution | 45 min – 1 h/ 20 °C to 25 °C | *Escherichia coli*<br>*Staphylococcus aureus* | 00012[b]<br>00013<br>00034[b] | TSA | Quantitative | ±30 % colonies/ $T_0$ (±30 % of original count) | — |
| | | Diluent for *Listeria monocytogenes* enumeration | Dilution | (1 h ± 5°min) / (20 ± 2) °C | *Listeria monocytogenes* 4b<br>*Listeria monocytogenes* 1/2a | 00021[b]<br>00109 | TSA | Quantitative | ±30 % colonies/ $T_0$ (±30 % of original count) | — |
| | | Pre-enrichment for *Salmonella* detection | Productivity | (18 ± 2) h/ (37 ± 1) °C | *Salmonella* Typhimurium[d,i]<br>*Salmonella* Enteritidis[d,i] | 00031<br>00030 | — | Qualitative | Turbidity (1–2)[f] | — |
| | | Pre-enrichment for *Enterobacteriaceae* detection | Productivity | (18 ± 2) h/ (37 ± 1) °C | *Escherichia coli*<br>*Salmonella* Typhimurium[d,i]<br>*Salmonella* Enteritidis[d,i] | 00012[b]<br>00013<br>00031<br>00030 | — | Qualitative | Turbidity (1–2)[f] | — |

Annexure **1** — (*continued*)

| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM numbers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions** |
|---|---|---|---|---|---|---|---|---|---|---|
| Blood agar | S | *Campylobacter* | Productivity | (44 ± 4) h/ (41,5 ± 1) °C | *Campylobacter jejuni*[d]<br>*Campylobacter coli*[d] | 00156<br>00005<br>00004 | Media batch blood agar already validated | Quantitative | $P_R \geq 0,7$ | — |
| TSA[n] | S | Colony count | Productivity | As specified in the method in which TSA is used as reference medium | *Bacillus cereus*<br>*Bacillus subtilis subsp. spizizenii*<br>*Escherichia coli*<br>*Listeria monocytogenes* 4b<br>*Staphylococcus aureus* | 00001<br>00003<br>00012<br>00021<br>00034 | Media batch TSA already validated | Quantitative | $P_R \geq 0,7$ | Characteristic colony according to each species |
| SDA | S | Colony count | Productivity | As specified in the method in which SDA is used as reference medium | *Saccharomyces cerevisiae*<br>*Aspergillus brasiliensis* | 00058[b]<br>00053[b] | Media batch SDA already validated | Quantitative | $P_R \geq 0,7$ | Characteristic colony/propagules/ germs according to each species |

**Annexure 1 —** (*continued*)

[a] Full names of media abbreviated terms.

[b] Strains to be used as a minimum.

[c] Make reference to the reference strain catalogue available on http://www.wfcc.info for information on culture collection strain numbers and contact details.

[d] Strain free of choice; one of the strains has to be used as a minimum.

[e] L: liquid medium, S: solid medium, SS: semi-solid medium.

[f] Growth/turbidity is categorized as: 0 — no growth/no turbidity; 1 — weak growth/slight turbidity; 2 — growth/good turbidity

[g] *Escherichia coli* WDCM 00013 is given by the specific standard.

[h] *Escherichia coli* WDCM 00013 is a strong β-d-glucuronidase producer and WDCM 00202 is a weak β-d-glucuronidase producer.

[i] Some national restrictions and directions may require the use of a different serovar. Make reference to national requirements relating to the choice of *Salmonella* serovars.

[j] In case of both quantitative and qualitative use for the medium, only results of the quantitative tests are required .

[k] More details for quality control of MSRV medium including final concentration of the inoculum and criteria are given in manual.

[l] If nutrient agar is used for two or three of these different applications: perform the *Salmonella* growth test as a minimum (if laboratory tests for this organism).

[m] If BPW is used for two or three of these different applications: perform the *Salmonella* enrichment test as a minimum (if laboratory tests for this organism).

[n] Choose the strain(s) according to the method for which TSA is used as a reference medium.

Table 2 — Abbreviated terms for media used in Annexure 1

| Abbreviated media term | Full name of the media |
|---|---|
| Baird –Parker | Baird-Parker agar |
| BGBLB | Brilliant green lactose bile broth |
| BHI | Brain heart infusion broth |
| Bolton | Bolton broth |
| BPW | Buffered peptone water |
| Brucella | Brucella broth |
| CFC | Cephalothin fucidin cetrimide agar |
| CIN | Cefsulodin, Irgasan novobiocin agar |
| CPC | Cellobiose polymyxin B colistin agar |
| CT-SMAC | Cefixime tellurite sorbitol MacConkey agar |
| DG18 | Dichloran glycerol agar |
| DRBC | Dichloran-rose bengal chloramphenicol agar |
| EC | EC broth |
| EE | Buffered brilliant green bile glucose broth |
| Fraser | Fraser broth |
| Half-Fraser | Half Fraser broth |
| IS (“TS”) | Iron sulfite agar (“Tryptose sulfite agar”) |
| ITC | Irgasan, ticarcillin chlorate broth |
| LST | Lauryl sulfate broth, lauryl tryptose broth |
| mCCDA | Modified charcoal cefoperazone deoxycholate agar |
| mCPC | Modified cellobiose polymyxin B colistin agar |
| MKTTn | Muller-Kauffmann tetrathionate novobiocin broth |
| MMG | Minerals-modified glutamate medium |
| MPCA | Plate count agar with skimmed milk/ milk platecount agar |
| MRS | MRS medium (de Man, Rogosa and Sharpe) |
| MSRV | Modified semi-solid Rappaport-Vassiliadis medium |
| MYP | Mannitol egg yolk polymyxin agar |
| PCA | Plate count agar |
| PEMBA | Polymyxin pyruvate egg yolk mannitol bromothy-mol blue agar |
| PPA | Penicillin and pimaricin agar |
| PSB | Peptone, sorbitol and bile salts broth |
| RPFA | Rabbit plasma fibrinogen agar |

**Table .2** *(continued)*

| Abbreviated media term | Full name of the media |
|---|---|
| RVS | Rappaport-Vassiliadis soya peptone broth |
| SDA | Sabouraud dextrose agar |
| SDS | Sodium dodecyl sulfate polymyxin sucrose agar |
| SSDC | Salmonella Shigella deoxycholate calcium agar |
| TBX | Tryptone bile X-glucuronide agar |
| TCBS | Thiosulfate citrate bile salts sucrose agar |
| Thioglycollate | Fluid thioglycollate medium |
| TSA | Tryptone soya agar |
| TSC/SC | Sulfite cycloserine agar/ tryptose sulphite cycloserine agar without egg yolk |
| TSPB | Tryptone soya polymyxin broth |
| TSYEA | Tryptone soya yeast extract agar |
| TSYEB | Tryptone soya yeast extract broth |
| VRBG | Violet red bile glucose agar |
| VRBL | Violet red bile lactose agar |
| XLD | Xylose lysine deoxycholate agar |

Annexure II — Test microorganisms and performance criteria for culture media commonly used in water microbiology

| Media[a] | Type[e] | Micro-organisms | Function | Incubation | Control strains | WDCM numbers[c] | Reference media | Method of control | Criteria | Characteristic reactions |
|---|---|---|---|---|---|---|---|---|---|---|
| Colilert-18 | L | *Escherichia coli* /coliform bacteria | Productivity | (20 ± 2)°h/ (36 ± 2) °C | *Escherichia coli* | 00013[b] 00090 | TSA | Quantitative | $P_R \geq 0,5$ | Yellow colour and fluorescence for *E. coli* |
| | | | | | *Klebsiella pneumoniae* | 00206 | TSA | Quantitative | $P_R \geq 0,5$ | Yellow colour equal or greater than the comparator for coliform bacteria |
| | | | Selectivity | | *Pseudomonas aeruginosa*[d] | 00024 or 00025 | — | Qualitative | Total inhibition (0) | Less yellow than the comparator |
| GVPC[f] | S | *Legionella* | Productivity | 2-5 days/ (36 ± 2) °C | *Legionella pneumophila* | 00107[b] 00180 | BCYE | Quantitative | $P_R \geq 0,5$ | White-grey-blue-purple colonies with an entire edge and exhibiting a characteristic ground-glass appearance |
| | | | | 5-10 days/ (36 ± 2) °C | *Legionella anisa* | 00106 | | | | |
| | | | Selectivity | 3 days/ (36 ± 2) °C | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | Total inhibition (0) | — |
| | | | | | *Pseudomonas aeruginosa*[d] | 00026 or 00025 | — | Qualitative | Total or partial inhibition (0-1) | — |
| | | | | | *Escherichia coli*[d] | 00012 or 00013 | | | | |
| Lactose TTC | S | *Escherichia coli* / coliform bacteria | Productivity | (21 ± 3) h/ (36 ± 2) °C | *Escherichia coli* | 00179[b] 00012 00013 | TSA | Quantitative | $P_R \geq 0,5$ | Yellow colour in the medium under the membrane |
| | | | | | *Enterobacter aerogenes* | 00175 | | | | |
| | | | | | *Citrobacter freundii* | 00006 | | | | |
| | | | Selectivity | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | Total inhibition (0) | — |
| | | | Specificity | | *Pseudomonas aeruginosa*[d] | 00025 or 00026 | — | Qualitative | — | Red colonies, blue colour in the medium |
| mCP | S | *Clostridium perfringens* | Productivity | (21 ± 3) h/ (44 ± 1) °C anaerobic atmosphere | *Clostridium perfringens* | 00007[b] 00080 00174 | TSA or other non-selective medium for anaerobes | Quantitative | $P_R \geq 0,5$ | Yellow colonies; Phophatase test positive |
| | | | Specificity | | *Clostridium bifermentans* | 00079 | — | Qualitative | — | Blue colonies; Phosphatase test negative |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibition (0) | — |

Annexure II — (*continued*)

| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM numbers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions** |
|---|---|---|---|---|---|---|---|---|---|---|
| Pseudo-monas CN | S | *Pseudomonas aeruginosa* | Productiv-ity | (44 ± 4) h/ (36 ± 2) °C | *Pseudomonas aeruginosa* | 00024[b] 00025 00026 | TSA | Quantita-tive | $P_R \geq 0,5$ | Blue-green colonies with fluores-cence under UV light (360 ± 20 nm) |
| | | | Selectivity | | *Escherichia coli*[d] *Enterococcus faecalis*[d] | 00012 or 00013 00009 or 00087 | — | Qualitative | Total inhibi-tion (0) | — |
| Slanetz and Bartley | S | Intestinal enterococci | Productiv-ity | (44 ± 4) h/ (36 ± 2) °C | *Enterococcus faecalis* *Enterococcus faecium*[d] | 00009[b] 00087 00176 00177 00178 | TSA | Quantita-tive | $P_R \geq 0,5$ | Red-maroon-pink colonies |
| | | | Selectivity | | *Escherichia coli*[d] *Staphylococcus aureus*[d] | 00012 or 00013 00032 or 00034 | — | Qualitative | Total inhibi-tion (0) | — |
| Sulfite Iron Tryptose Sulfite (TS) | S | Sulfite-reducing anaer-obes (clostridia) | Productiv-ity | (44 ± 4) h/ (37 ± 1) °C anaerobic atmosphere | *Clostridium perfringens* | 00007[b] 00080 | TSA or Blood agar or other non-selective medium for anaerobes | Quantita-tive | $P_R \geq 0,5$ | Black colonies |
| | | | Specificity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | — | No blackening |
| TSC | S | *Clostridium perfringens* | Productiv-ity | (21 ± 3) h/ (44 ± 1) °C anaerobic atmosphere | *Clostridium perfringens* | 00007[b] 00080 00174 | TSA or Blood agar or other non-selective medium for anaerobes | Quantita-tive | $P_R \geq 0,5$ | Black colonies |
| | | | Selectivity | | *Bacillus subtilis subsp. spizizenii* | 00003 | — | Qualitative | Total inhibi-tion (0) | — |

Annexure II — (*continued*)

| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM numbers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions** |
|---|---|---|---|---|---|---|---|---|---|---|
| Colilert-18 | L | *Escherichia coli* /coliform bacteria | Productivity | (20 ± 2)h/ (36±2) °C | *Escherichia coli* | 00013[b] 00090 | previously validated batch Colilert | Quantitative | $P_R \geq 0,7$ | Yellow colour and fluorescence for *E. coli* |
| | | | | | *Klebsiella pneumoniae* | 00206 | previously validated batch Colilert | Quantitative | $P_R \geq 0,7$ | Yellow colour equal or greater than the comparator for coliform bacteria |
| | | | Selectivity | | *Pseudomonas aeruginosa*[d] | 00024 or 00025 | — | Qualitative | Total inhibition (0) | Less yellow than the comparator |
| GVPC[f] | S | *Legionella* | Productivity | 2-5 days/ (36 ± 2) °C | *Legionella pneumophila* | 00107[b] 00180 | Media batch GVPC already validated | Quantitative | $P_R \geq 0,7$ | White-grey-blue-purple colonies with an entire edge and exhibit- ing a characteristic ground-glass appearance |
| | | | | 5-10 days/ (36 ± 2) °C | *Legionella anisa* | 00106 | | | | |
| | | | Selectivity | 3 days/ (36 ± 2) °C | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | Total inhibition (0) | — |
| | | | | | *Pseudomonas aeruginosa*[d] | 00026 or 00025 | — | Qualitative | Total or partial inhibition (0 - 1) | — |
| | | | | | *Escherichia coli*[d] | 00012 or 00013 | | | | |
| Lactose TTC | S | *Escherichia coli* / coliform bacteria | Productivity | (21 ± 3) h/ (36 ± 2) °C | *Escherichia coli* | 00179[b] 00012 00013 | Media batch Lactose TTC already validated | Quantitative | $P_R \geq 0,7$ | Yellow colour in the medium under the membrane |
| | | | | | *Enterobacter aerogenes* | 00175 | | | | |
| | | | | | *Citrobacter freundii* | 00006 | | | | |
| | | | Selectivity | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | Total inhibition (0) | — |
| | | | Specificity | | *Pseudomonas aeruginosa*[d] | 00025 or 00026 | — | Qualitative | — | Red colonies, blue colour in the medium |

Annexure II — (*continued*)

| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM numbers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions** |
|---|---|---|---|---|---|---|---|---|---|---|
| mCP | S | *Clostridium perfringens* | Productivity | (21 ± 3) h/ (44 ± 1) °C anaerobic atmosphere | *Clostridium perfringens* | 00007[b] 00080 00174 | Media batch mCP already validated | Quantitative | $P_R \geq 0,7$ | Yellow colonies; Phophatase test positive |
| | | | Specificity | | *Clostridium bifermentans* | 00079 | — | Qualitative | — | Blue colonies; Phosphatase test negative |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibition (0) | — |
| Pseudomonas CN | S | *Pseudomonas aeruginosa* | Productivity | (44 ± 4) h/ (36 ± 2) °C | *Pseudomonas aeruginosa* | 00024[b] 00025 00026 | Media batch Pseudomonas CN already validated | Quantitative | $P_R \geq 0,7$ | Blue-green colonies with fluorescence under UV light (360 ± 20 nm) |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibition (0) | — |
| | | | | | *Enterococcus faecalis*[d] | 00009 or 00087 | | | | |
| Slanetz and Bartley | S | Intestinal enterococci | Productivity | (44 ± 4) h/ (36 ± 2) °C | *Enterococcus faecalis* | 00009[b] 00087 00176 | Media batch Slanetz and Bartley already validated | Quantitative | $P_R \geq 0,7$ | Red-maroon-pink colonies |
| | | | | | *Enterococcus faecium*[d] | 00177 00178 | | | | |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Total inhibition (0) | — |
| | | | | | *Staphylococcus aureus*[d] | 00032 or 00034 | | | | |

Annexure II — (*continued*)

| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM numbers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions** |
|---|---|---|---|---|---|---|---|---|---|---|
| Sulfite Iron<br>Tryptose Sulfite (TS) | S | Sulfite-reducing anaerobes (clostridia) | Productivity | (44 ± 4) h/ (37 ± 1) °C anaerobic atmosphere | *Clostridium perfringens* | 00007[b]<br>00080 | Media batch Sulfite iron or TS already validated | Quantitative | $P_R \geq 0,7$ | Black colonies |
| | | | Specificity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | — | No blackening |
| TSC | S | *Clostridium perfringens* | Productivity | (21 ± 3) h/ (44 ± 1) °C anaerobic atmosphere | *Clostridium perfringens* | 00007[b]<br>00080<br>00174 | Media batch TSC already validated | Quantitative | $P_R \geq 0,7$ | Black colonies |
| | | | Selectivity | | *Bacillus subtilis subsp. spizizenii* | 00003 | — | Qualitative | Total inhibition (0) | — |
| **Media**[a] | **Type**[e] | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM numbers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions** |
| YEA | S | Total flora | Productivity | (44 ± 4) h/ (36 ± 2) °C | *Escherichia coli*[d]<br>*Bacillus subtilis subsp. spizizenii* | 00012 or 00013<br>00003 | Media batch YEA already validated | Quantitative | $P_R \geq 0,7$ | — |

Annexure II —
(*continued*)

| Media[a] | Type[e] | Micro-organisms | Function | Incubation | Control strains | WDCM numbers[c] | Reference media | Method of control | Criteria | Characteristic reactions |
|---|---|---|---|---|---|---|---|---|---|---|
| Bolton<br>Preston | L | *Campylobacter* | Productivity | (44 ± 4) h/<br>(37 ± 1) °C<br>microaerobic atmosphere | *Campylobacter jejuni* | 00156[b]<br>00005 | — | Qualitative | > 10 colonies on mCCDA | Small, flat or convex colonies with a glossy surface |
| | | | | | + *Escherichia coli*[d] | 00012<br>or 00013 | | | | |
| | | | | | + *Proteus mirabilis* | 00023 | | | | |
| | | | | | *Campylobacter coli* | 00004[b] | | | | |
| | | | | | + *Escherichia coli* [d] | 00012<br>or 00013 | | | | |
| | | | | | + *Proteus mirabilis* | 00023[b] | | | | |
| | | | Selectivity | | *Escherichia coli*[d] | 00012<br>or 00013 | — | Qualitative | Total inhibition on TSA (0) | — |
| | | | | | *Proteus mirabilis* | 00023 | | | | |
| MUG/EC[g] | L | *Escherichia coli* / coliform bacteria | Productivity | 48 h/<br>(44 ± 0,5) °C | *Escherichia coli* | 00179 | Details for method of control and quality criteria of MUG/EC medium | | | |
| MUD/SF[h] | L | Intestinal enterococci | Productivity | (44 ± 4) h/<br>(44 ± 0,5) °C | *Enterococcus faecalis* | 00176 | Details for method of control and quality criteria of MUD/SF medium | | | |
| | | | | | *Enterococcus hirae* | 00089 | | | | |
| | | | | | *Enterococcus faecium* | 00178 | | | | |
| | | | Selectivity | | *Aerococcus viridans* | 00061 | | | | |
| | | | | | *Lactococcus lactis* | 00016 | | | | |
| | | | | | *Staphylococcus epidermidis* | 00132 | | | | |

Annexure II —
(*continued*)

| Media[a] | Type[e] | Micro-organisms | Function | Incubation | Control strains | WDCM numbers[c] | Reference media | Method of control | Criteria | Characteristic reactions |
|---|---|---|---|---|---|---|---|---|---|---|
| RVS | L | *Salmonella* | Productivity | (24 ± 3) h/ (41,5 ± 1) °C | *Salmonella* Enteritidis[d,i]<br>*Salmonella* Typhimurium[d,i]<br>+ *Escherichia coli*[d]<br>+ *Pseudomonas aeruginosa* | 00030<br>00031<br>00012 or 00013<br>00025 | — | Qualitative | > 10 colonies on XLD or other medium of choice | Characteristic colonies accordingto each medium (see standard) |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Partial inhibition ≤ 100 colonies on TSA | — |
| | | | | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | < 10 colonies on TSA | — |
| **Media[a]** | **Type[e]** | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM numbers[c]** | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions** |
| DRCM | L | Sulfite-reducing anaerobes (clostridia) | Productivity | (44 ± 4) h/ (36 ± 1) °C anaerobic atmosphere | *Clostridium perfringens* | 00007[b]<br>00080 | — | Qualitative | Turbidity<br>(1-2)[j] | Blackening |
| | | | Specificity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Turbidity (0-1)[j] | No blackening |
| Saline solution<br>Peptone diluent<br>Peptone salt solution<br>Ringer's solution (1/4 strength)<br>Phosphate buffer solution | L | Dilution liquids | Diluent | 45 min – 1 h/ 20 °C – 25 °C | *Escherichia coli*[d]<br>*Staphylococcus aureus* | 00012 or 00013<br>00034[b] | TSA | Quantitative | +/- 30 % colonies/ $T_0$ (+/- 30 % of original count) | — |

Annexure II —
(*continued*)

| Media[a] | Type[e] | Micro-organisms | Function | Incubation | Control strains | WDCM numbers[c] | Reference media | Method of control | Criteria | Characteristic reactions |
|---|---|---|---|---|---|---|---|---|---|---|
| mCCDA | S | *Campylobacter* | Productivity | (44 ± 4) h/ (41,5 ± 1) °C microaerobic atmosphere | *Campylobacter jejuni*<br>*Campylobacter coli* | 00156[b] 00005<br>00004 | — | Qualitative | Good growth (2) | Small, flat or convex colonies with a glossy surface |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 or 00179 or 00090 | — | Qualitative | Total or partial inhibition (0–1) | No characteristic colonies |
| | | | | | *Staphylococcus aureus*[d] | 00032 or 00034 | — | Qualitative | Total inhibition (0) | — |
| XLD | S | *Salmonella* | Productivity | (24 ± 3) h/ (36 ± 2) °C | *Salmonella* Typhimurium[d,i]<br>*Salmonella* Enteritidis[d,i] | 00031<br>00030 | — | Qualitative | Good growth (2) | Colonies with black centre and a lightly transparent zone of reddish colour due to the colour change of the medium |
| | | | Selectivity | | *Escherichia coli*[d] | 00012 or 00013 | — | Qualitative | Growth or partial inhibition (0–1) | Yellow colonies |
| | | | | | *Enterococcus faecalis*[d] | 00009 or 00087 | — | Qualitative | Total inhibition (0) | — |
| **Media[a]** | **Type[e]** | **Micro-organisms** | **Function** | **Incubation** | **Control strains** | **WDCM numbers[c]** | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions** |
| BPW[k] | L | Diluent for enumerations of all microorganisms | Dilution | 45 min – 1 h/ 20 °C to 25 °C | *Escherichia coli*[d]<br>*Staphylococcus aureus* | 00012 or 00013<br>00034 | TSA | Quantitative | +/- 30 % colonies/$T_0$ (+/- 30 % of original count) | — |
| | | Pre-enrichment for *Salmonella* detection | Productivity | (18 ± 2) h/ (36 ± 2) °C | *Salmonella* Typhimurium[d,i]<br>*Salmonella* Enteritidis[d,i] | 00031<br>00030 | — | Qualitative | Turbidity (1-2)[j] | — |

Annexure II —
(*continued*)

| Reference media for enumeration of microorganisms | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Media**[a] | **Type**[e] | **Micro-organisms** | | **Function** | **Incubation** | **Control strains** | **WDCM numbers**[c] | **Reference media** | **Method of control** | **Criteria** | **Characteristic reactions** |
| BCYE | S | Colony count | | Productiv-ity | 2-5 days / (36 ± 2) °C | *Legionella pneumophila* | 00107[b] | Media batch BCYE already validated | Quantita-tive | $P_R \geq 0,7$ | White-grey-blue-purple colonies with an entire edge and exhibit- ing a characteristic ground-glass appearance |
| TSA[l] | S | Colony count | | Productiv-ity | As specified in the method in which TSA is used as refer-ence medium | *Escherichia coli*[d]<br>*Clostridium perfringens*<br>*Pseudomonas aeruginosa*<br>*Enterococcus faecalis* | 00012<br>00013<br>00090<br>00179<br>00007<br>00024<br>00087 | Media batch TSA already validated | Quantita-tive | $P_R \geq 0,7$ | Characteristic colony according to each species |

[a] Full names of media abbreviated terms are given.

[b] Strains to be used as a minimum.

[c] Make reference to the reference strain catalogue available on http://www.wfcc.info for information on culture collection strain numbers and contact details.

[d] Strain free of choice; one of the strains has to be used as a minimum.

[e] L: liquid medium, S: solid medium, SS: semi-solid medium.

[f] More details for quality control of *Legionella* media including storage of the control strains are given

[g] More details for quality control and quality criteria of MUG/EC medium

[h] More details for quality control and quality criteria of MUD/SF medium are given.

[i] Some national restrictions and directions may require the use of a different serovar. Make reference to national requirements relating to the choice of *Salmonella* serovars.

[j] Growth/turbidity is categorized as: 0 — no growth/turbidity; 1 — weak growth/turbidity; 2 — good growth/turbidity

[k] If BPW is used for two of these different applications: perform the *Salmonella* enrichment test as a minimum (if laboratory tests for this organism).

[l] Choose the strain(s) according to the method for which TSA is used as a reference medium.

Annexure II — Abbreviated terms for media used

| Media abbreviated term | Full name of the media |
|---|---|
| BCYE | Buffered charcoal yeast extract agar medium |
| Bolton | Bolton broth |
| BPW | Buffered peptone water |
| DRCM | Differential reinforced clostridial medium |
| GVPC | Buffered charcoal yeast extract agar with glycine, van-comycin, polymyxin B, cycloheximide |
| Lactose TTC | Lactose triphenyltetrazolium chloride agar with sodiumheptadecylsulfate |
| mCCDA | Modified charcoal cefoperazone deoxycholate agar |
| mCP | Membrane clostridium perfringens agar |
| MUD/SF | 4-methylumbelliferyl-α-D glucoside /SF medium |
| MUG/EC | 4-methylumbelliferyl-β-D glucuronide /EC medium |
| Preston | Preston broth |
| Pseudomonas CN | Pseudomonas cetrimide nalidixic acid agar |
| RVS | Rappaport-Vassiliadis soya peptone broth |
| Slanetz and Bartley | Slanetz and Bartley medium |
| Sulfite Iron | Iron Sulfite agar |
| Tryptose Sulfite (TS) | Tryptose sulphite agar |
| TSA | Tryptone soya agar |
| TSC | Tryptose sulphite cycloserine agar (without egg yolk) |
| XLD | Xylose lysine deoxycholate agar |
| YEA | Yeast extract agar |