8

STERILITY TESTING: PHARMACEUTICAL PRODUCTS

- Introduction
- Test for Sterility: Pharmaceutical Products
- Sampling: Probability Profile
- Overall conclusions

8.1 INTRODUCTION

A sterility test may be defined as — ‘a test that critically assesses whether a sterilized pharmaceutical product is free from contaminating microorganisms’.

According to Indian Pharmacopoeia (1996) the sterility testings are intended for detecting the presence of viable forms of microorganisms in or on the pharmacopoeal preparations.

In actual practice, one invariably comes across certain absolutely important guidelines and vital precautionary measures that must be adhered to strictly so as to accomplish the utmost accuracy and precision of the entire concept of sterility testing for life-saving secondary pharmaceutical products (drugs). A few such cardinal factors, guidelines, and necessary details are as enumerated under:

(a) Sterility testing, due to its inherent nature, is intimately associated with a statistical process wherein the portion of a batch is sampled almost randomly*; and, therefore, the chance of the particular batch (lot) duly passed for actual usage (consumption) solely depends upon the ‘sample’ having passed the stringent sterility test.

(b) Sterility tests should be performed under conditions designed to avoid accidental contamination of the product (under investigation) during the test. Nevertheless, such particular precautions precisely taken for this purpose must not, in any case, adversely affect any microbes that should be revealed in the test ultimately.

(c) Working environment wherein the sterility tests are meticulously carried out must be adequately monitored at regular intervals by sampling the air and the surface of the working area by performing necessary control tests.

* From a thorough investigative study, it has been duly proposed that the random sampling must be judiciously applied to: (a) such products that have been processed and filled aseptically; and (b) with products heat-sterilized in their final containers must be drawn carefully from the potentially coolest zone of the load.
Sterility tests are exclusively based upon the principle that in case the bacteria are strategically placed in a specific medium that caters for the requisite nutritive material and water, and maintained duly at a favourable temperature (37 ± 2°C), the microbes have a tendency to grow, and their legitimate presence may be clearly indicated by the appearance of a turbidity in the originally clear medium. Extent of probability in the detection of viable microorganisms for the tests for sterility usually increases with the actual number supposedly present in a given quantity of the preparation under examination, and is found to vary according to the species of microorganisms present. However, extremely low levels of contamination cannot be detected conveniently on the basis of random sampling of a batch.*

In case, observed contamination is not quite uniform throughout the batch, random sampling cannot detect contamination with absolute certainty. Therefore, compliance with the tests for sterility individually cannot certify absolute assurance of freedom from microbial contamination. Nevertheless, greater assurance of sterility should invariably originate from reliable stringent manufacturing procedures vis-a-vis strict compliance with Good Manufacturing Practices (GMPs).

Tests for sterility are adequately designed to reveal the presence of microorganisms in the ‘samples’ used in the tests. However, the interpretation of results is solely based upon the assumption that the contents of each and every container in the batch, had they been tested actually, would have complied with the tests. As it is not practically possible to test every container, a sufficient number of containers must be examined to give a suitable degree of confidence in the ultimate results obtained of the tests.

It has been duly observed that there exists no definite sampling plan for applying the tests to a specified proportion of discrete units selected carefully from a batch is capable of demonstrating that almost all of the untested units are in fact sterile absolutely. Therefore, it is indeed quite pertinent that while determining the number of units to be tested, the manufacturer must have adequate regard to the environment parameters of manufacture, the volume of preparation per container together with other special considerations specific to the preparation under investigation. For this Table 8.1 records the guidance on the exact number of items recommended to be tested with regard to the number of items in the batch on the assumption that the preparation has been duly manufactured under specified stringent parameters designed meticulously to exclude any untoward contamination.

**Table : 8.1. Profile of Guidance** : Number of Items in a Batch Vs Minimum Number of Items Recommended to be Tested

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Product Variants</th>
<th>Number of Items in a Batch</th>
<th>Minimum Number of Items Recommended to be Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Injectable Preparations</td>
<td>(a) Not more than 100 containers</td>
<td>Either 10% or 4 containers whichever is greater. 10 containers.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) More than 100, but not more than 500 containers.</td>
<td>Either 2% or 20 containers whichever is less.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) More than 500 containers.</td>
<td></td>
</tr>
</tbody>
</table>

* Batch : A batch may be defined for the purposes of these tests a — ‘a homogeneous collection of sealed containers prepared in such a manner, that the risk of contamination is the same for each of the units present in it’.

** Adapted from : Indian Pharmacopoeia, Vol. II, Published by the Controller of Publications, New Delhi, 1996.
8.2. TEST FOR STERILITY: PHARMACEUTICAL PRODUCTS

In a broader perspective the wide-spectrum of the pharmaceutical products, both pure and dosage forms, may be accomplished by adopting any one of the following two well-recognized, time-tested, and universally accepted methods, namely:

(a) Membrane Filtration, and
(b) Direct Inoculation.

These two methods stated above shall now be treated individually in the sections that follows:

8.2.1. Membrane Filtration

The membrane filtration method has gained and maintained its glorious traditional recognition to not only circumvent but also to overcome the activity of antibiotics for which there exist practically little inactivating agents. However, it may be duly extended to embrace legitimately a host of other relevant products as and when deemed fit.

Importantly, the method emphatically requires the following characteristic features, namely:

■ an exceptional skill,
■ an in-depth specific knowledge, and
■ rigorous routine usage of positive and negative controls.

As a typical example of a suitable positive control with respect to the appropriate usage of a known 'contaminated solution' essentially comprising of a few microorganisms of altogether different nature and types*.

Salient Features: The salient features of the 'membrane filtration' method are as enumerated under:

* Approximately ten bacterial cells in the total volumes are employed.
(1) The solution of the product under investigation is carefully filtered via a hydrophobic-edged membrane filter that would precisely retain any possible contaminating microorganisms.

(2) The resulting membrane is duly washed in situ to get rid of any possible ‘traces of antibiotic’ that would have been sticking to the surface of the membrane intimately.

(3) Finally, the segregated microorganisms are meticulously transferred to the suitable culture media under perfect aseptic environment.

**Microorganisms for Positive Control Tests:** There are, in fact, four typical microorganisms that are being used exclusively for the positive control tests along with their respective type of specific enzymatic activity mentioned in parentheses:

- (a) *Bacillus cereus*: [Broad spectrum];
- (b) *Staphylococcus aureus*: [Penicillinase];
- (c) *Klebsiella aerogenes*: [Penicillinase + Cephalosporinase]; and
- (d) *Enterobacter species*: [Cephalosporinase].

Interestingly, the microorganisms invariably employed for the positive control tests together with a particular product containing essentially an ‘antimicrobial agent’ must be, as far as possible, explicitly sensitive to that agent, in order that the ultimate growth of the microbe solely indicates three vital and important informations, namely:

- satisfactory inactivation,
- satisfactory dilution, and
- satisfactory removal of the agent.

**Specific Instances of Pharmaceutical Products:** Virtually all the ‘Official Compendia’ viz., Indian Pharmacopoea (IP); British Pharmacopoea (BP), United States Pharmacopoea (USP); European Pharmacopoea (Eur. P), and International Pharmacopoea (Int. P.) have duly provided comprehensive and specific details with regard to the ‘tests for sterility’ of parenteral products (e.g., IV and IM injectables), opthalmic preparations (e.g., eye-drops, eye-ointments, eye-lotions etc.); besides a plethora of non-injectable preparations, such as: catgut, dusting powder, and surgical dressings.

**Test Procedures:** In a broader perspective, the membrane filtration is to be preferred exclusively in such instances where the substance under investigation could any one of the following four classes of pharmaceutical preparations:

- (i) an oil or oil-based product,
- (ii) an ointment that may be put into solution,
- (iii) a non-bacteriostatic solid that does not become soluble in the culture medium rapidly, and
- (iv) a soluble powder or a liquid that essentially possesses either inherent bacteriostatic or inherent fungistatic characteristic features.

The membrane filtration must be used for such products where the volume in a container is either 100 mL or more. One may, however, select the exact number of samples to be tested from Table 8.1; and subsequently use them for the respective culture medium suitably selected for microorganisms and the culture medium appropriately selected for fungi.
Precautionary Measures: In actual practice, however, the tests for sterility must always be carried out under highly specific experimental parameters so as to avoid any least possible accidental contamination of the product being examined, such as:

(a) a sophisticated laminar sterile airflow cabinet (provided with effective hepa-filters),

(b) necessary precautionary measures taken to be such so as to avoid contamination that they do not affect any microbes which must be revealed duly in the test.

(c) ensuing environment (i.e., working conditions) of the laboratory where the ‘tests for sterility’ is performed must always be monitored at a definite periodical interval by:

- sampling the air of the working area,
- sampling the surface of the working area, and
- perforing the stipulated control tests.

Methodology: In usual practice, it is absolutely urgent and necessary to first clean meticulously the exterior surface of ampoules, and closures of vials and bottles with an appropriate antimicrobial agent; and thereafter, the actual access to the contents should be gained carefully in a perfect aseptic manner. However, in a situation where the contents are duly packed in a particular container under vacuum, introduction of ‘sterile air’ must be done by the help of a suitable sterile device, for instance: a needle duly attached to a syringe barrel with a non-absorbent cotton.

Apparatus: The most suitable unit comprises of a closed reservoir and a receptacle between which a properly supported membrane of appropriate porosity is placed strategically.

- A membrane usually found to be quite suitable for sterility testing essentially bears a nominal pore size not more than 0.45 μm, and diameter of nearly 47 mm, the effectiveness of which in the retention of microbes has been established adequately.
- The entire unit is most preferably assembled and sterilised with the membrane in place prior to use.
- In case, the sample happens to be an oil, sterilize the membrane separately and, after thorough drying, assemble the unit, adopting appropriate aseptic precautionary measures.

Diluting of Fluids: In the ‘test for sterility’ one invariably comes across with two different types of fluids which will be treated individually in the sections that follows:

(a) Fluid A—Digest 1 g of peptic digest of animal tissue* or its equivalent in water to make up the volume upto 1L, filter or centrifuge to clarify, adjust to pH 7.1 ± 0.2, dispense into flasks in 100 mL quantities, and finally sterilize at 121° C for 20 minutes (in an ‘Autoclave’).

Note: In a specific instance, where Fluid A is to be used in carrying out the tests for sterility on a specimen of the penicillin or cephalosporin class of antibiotics, aseptically incorporate an amount of sterile penicillinase to the Fluid A to be employed to rinse the membrane(s) sufficient to inactivate any residual antibiotic activity on the membrane(s) after the solution of the specimen has been duly filtered.

* Such as: Bacteriological Peptone.
(b) **Fluid B**: In a specific instance, when the test sample usually contains either oil or lecithin*, use Fluid A to each litre of which has been added 1 mL of Polysorbate 80**, adjust to pH 7.1 ± 0.2, dispense into flasks and sterilize at 121° C for 20 minutes (in an ‘Autoclave’).

**Note**: A sterile fluid shall not have either antimicrobial or antifungal properties if it is to be considered suitable for dissolving, diluting or rinsing a preparation being examined for sterility.

**Quantum of Sample Used for ‘Tests for Sterility’**: In fact, the exact and precise quantities of sample to be used for determining the ‘Tests for Sterility’ are quite different for the injectables and ophthalmics plus other non-injectables; and, therefore, they would be discussed separately as under:

(a) **For Injectable Preparations**: As a common routine practice and wherever possible always use the whole contents of the container; however, in any case not less than the quantities duly stated in Table : 8.2, diluting wherever necessary to 100 mL with an appropriate sterile diluent e.g., Fluid A.

(b) **For Ophthalmic and other Non-injectable Preparations**: In this particular instance exactly take an amount lying very much within the range prescribed in Column (A) of Table : 8.3, if necessary, making use of the contents of more than one container, and mix thoroughly. For each specific medium use the amount duly specified in column (B) of Table : 8.3, taken carefully from the mixed sample.

**Table : 8.2. Quantities of Liquids/Solids per Container of Injectables Vs Minimum Quantity Recommended for Each Culture Medium.**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Type of Preparation</th>
<th>Quantity in Each Container of Injectables</th>
<th>Minimum Quantity Recommended for Each Culture Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>For Liquids</td>
<td>(a) Less than 1 mL</td>
<td>Total contents of a container</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) 1 mL or more but &lt; 4 mL</td>
<td>Half the contents of a container 2 mL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) 4 mL or more but &lt; 20 mL</td>
<td>10% of the contents of a container unless otherwise specified duly in the ‘monograph’.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(d) 20 mL or more but &lt; 100 mL</td>
<td>Not less than half the contents of a container unless otherwise specified in the ‘monograph’.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(e) 100 mL or more</td>
<td>Total contents of a container.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Half the contents of a container.</td>
</tr>
<tr>
<td>2</td>
<td>For Solids</td>
<td>(a) Less than 50 mg</td>
<td>Total contents of a container.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) 50 mg or more but &lt; 200 mg</td>
<td>Half the contents of a container.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(c) 200 mg or more</td>
<td>100 mg.</td>
</tr>
</tbody>
</table>

* Lecithin: A phospholipid (phosphoglyceride) that is found in blood and egg-yolk, and constitute part of cell membranes.
** Polysorbate-80: Non-ionic surface-active agents composed of polyoxyethylene esters of sorbitol. They usually contain associated fatty acids. It is used in preparing pharmaceuticals.
Table : 8.3. Type of Preparation Vs Quantity to be Mixed and Quantity to be Used for Each Culture Medium

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Type of Preparation</th>
<th>Quantity to be Mixed (A)</th>
<th>Quantity to be Used for Each Culture Medium (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ophthalmic Solutions: Other non-injectable liquid preparations.</td>
<td>10—100 mL</td>
<td>5—10 mL</td>
</tr>
<tr>
<td>2</td>
<td>Other Preparations: Preparations soluble in water or appropriate solvents; insoluble preparations to be suspended or emulsified duly (e.g., creams and ointments).</td>
<td>1—10 g</td>
<td>0.5—1 g</td>
</tr>
<tr>
<td>3</td>
<td>Absorbent cotton</td>
<td></td>
<td>Not less than 1 g*</td>
</tr>
</tbody>
</table>

Method of Actual Test: In reality, the method of actual test may be sub-divided into the following four categories, namely:

(i) Aqueous Solutions,
(ii) Liquids Immiscible with Aqueous Vehicles and Suspensions
(iii) Oils and Oily Solutions, and
(iv) Ointments and Creams.

These three aforesaid types of pharmaceutical preparations shall be treated separately as under:

[I] Aqueous Solutions: The following steps may be followed sequentially:

1. Prepare each membrane by transferring aseptically a small amount (i.e., just sufficient to get the membrane moistened duly) of fluid A on to the membrane and filtering it carefully.

2. For each medium to be employed, transfer aseptically into two separate membrane filter funnels or two separate sterile pooling vessels prior to transfer not less than the quantity of the preparation being examined which is duly prescribed either in Table : 8.2 or Table : 8.3.

3. Alternatively, transfer aseptically the combined quantities of the preparation being examined prescribed explicitly in the two media onto one membrane exclusively.

4. Suck in the ‘liquid’ quickly via the membrane filter with the help of a negative pressure (i.e., under vacuum).

5. In case, the solution being examined has significant antibacterial characteristic features, wash the membrane(s) by filtering through it (them) not less than three successive quantities, each of approximately 100 mL of the sterile fluid A.

6. Precisely, the quantities of fluid actually employed must be sufficient to permit the adequate growth of a ‘small inoculum of microorganisms’ (nearly 50) sensitive to the antimicrobial substance in the presence of the residual inhibitory material retained duly on the membrane.

* In one lot only.
(7) Once the filtration is completed, aseptically remove the membrane(s) from the holder, cut the membrane in half, if only one is used, immerse the membrane or 1/2 of the membrane, in 100 mL of the ‘Fluid Soyabean-Casein Digest Medium’*, and incubate at 20–25°C for a duration of seven days.

(8) Likewise, carefully immerse the other membrane, or other half of the membrane, in 100 mL of ‘Fluid Thioglycollate Medium’**, and incubate duly at 30–35°C for not less than seven days.

[II] Liquids Immiscible with Aqueous Vehicles and Suspensions: For this one may carry out the ‘test’ as stipulated under [I] Aqueous Solutions, but add a sufficient amount of fluid A to the pooled sample to accomplish fast and rapid rate of filtration.

Special Features: These are as stated under:

(1) Sterile enzyme preparations, for instance:

- Penicillinase
- Cellulase

...can be incorporated to fluid A to help in the dissolution of insoluble substances.

(2) In a situation when the substance under test usually contains lecithin, always make use of fluid B for dilution.

[III] Oils and Oily Solutions: The various steps that are essentially involved in treating oils and oily solutions for carrying out the ‘test for sterility’ are as enumerated under:

(1) Filter oils or oily solutions of sufficiently low viscosity as such i.e., without any dilution via a dry membrane.

(2) It is absolutely necessary to dilute viscous oils as necessary with an appropriate sterile diluent e.g., isopropyl myristate which has been proved beyond any reasonable doubt not to exhibit any antimicrobial activities under the prevailing parameters of the test.

### Fluid Soyabean-Casein Digest Medium*

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Ingredients</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pancreatic digest of casein</td>
<td>17.0</td>
</tr>
<tr>
<td>2</td>
<td>Papaic digest of soyabean meal</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>4</td>
<td>Diabasic potassium phosphate [K₂HPO₄]</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>Dextrose monohydrate [C₆H₁₂O₆ . H₂O]</td>
<td>2.5</td>
</tr>
<tr>
<td>6</td>
<td>Distilled water to</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

Dissolve the solids in distilled water, warming slightly to effect solution. Cool to room temperature and add, if necessary sufficient 0.1 M NaOH to give a final pH of 7.1 ± 0.2 after sterilization. Distribute into suitable containers and sterilize in an autoclave at 121°C for 20 minutes.

### Fluid Thioglycollate Medium**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Ingredients</th>
<th>Quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L-Cystine</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>Sodium chloride</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>Dextrose [C₆H₁₂O₆ . H₂O]</td>
<td>5.5</td>
</tr>
<tr>
<td>4</td>
<td>Granular agar [moisture &lt; 15% w/w]</td>
<td>0.75</td>
</tr>
<tr>
<td>5</td>
<td>Yeast-extract (water-soluble)</td>
<td>5.0</td>
</tr>
<tr>
<td>6</td>
<td>Pancreatic digest of casein</td>
<td>15.0</td>
</tr>
<tr>
<td>7</td>
<td>Sodium thioglycollate or</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>Thioglycolic acid</td>
<td>0.3 (mL)</td>
</tr>
<tr>
<td>9</td>
<td>Resazurin [0.1% fresh solution]</td>
<td>1.0 (mL)</td>
</tr>
<tr>
<td>10</td>
<td>Distilled water to</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

For procedure: Please refer to Appendix 9.5.

Indian Pharmacopea Vol. II, 1996 (p-A : 118)
(3) Permit the ‘oil’ to penetrate the membrane, and carry out the filtration by the application of gradual suction (with a vacuum pump).

(4) Wash the membrane by filtering through it at least 3/4 successive quantities, each of nearly 100 mL of sterile fluid B or any other appropriate sterile diluent.

(5) Complete the test as described under [I] Aqueous Solutions from step (7) onwards.

[IV] Ointments and Creams : The various steps involved are as stated under :

(1) Dilute ointments carefully either in a ‘fatty base’ or ‘emulsions’ of the water-in-oil (i.e., w/o) type to yield a fluid concentration of approx. 1% w/v, by applying gentle heat, if necessary, to not more than 40°C with the aid of an appropriate sterile diluent e.g., isopropyl myristate previously adequately sterilized by filtration via a 0.22 μm membrane filter which has been shown not to possess antimicrobial activities under the prevailing conditions of the test.

(2) Carry out the filtration as rapidly as possible as per details given under ‘Oils and Oily Solutions’ [Section III] from step (4) onwards.

(3) However, in certain exceptional instances, it would be absolutely necessary to heat the substance to not more than 45°C, and to make use of ‘warm solutions’ for washing the membrane effectively.

Note : For ointments and oils that are almost insoluble in isopropyl myristate one may employ the second method viz., ‘Direct Inoculation’ [Section 2.2].

[V] Soluble Solids : For each individual culture medium, dissolve not less the quantity of the substance being examined, as recommended in Tables : 8.2 and 8.3, in an appropriate sterile solvent e.g., fluid A, and perform the test described under Section (I) i.e., Aqueous Solutions, by employing a membrane suitable for the selected solvents.

[VI] Sterile Devices : Pass carefully and aseptically a sufficient volume of fluid B via each of not less than 20 devices so that not less than 100 mL is recovered ultimately from each device. Collect the fluids in sterile containers, and filter the entire volume collected via membrane filter funnel(s) as described under Section (I), Aqueous Solutions.

8.2.2. Direct Inoculation [or Direct Inoculation of Culture Media]

The three usual methods being used for performing the ‘tests for sterility’ are as enumerated under :

(a) Nutrient Broth,

(b) Cooked Meat Medium and Thioglycollate Medium, and

(c) Sabouraud Medium.

These methods shall now be treated individually in the sections that follows :

8.2.2.1. Nutrient Broth

Importantly, it is exclusively suitable for the ‘aerobic microorganisms’.

- Oxidation-reduction potential (Eh) value of this medium happens to be quite high to enable the growth of the anaerobes specifically.
Importantly, such culture media that particularly allow the growth of festidious microorganisms, such as: soyabean casein digest broth, Hartley’s digest broth.*

8.2.2.2. Cooked Meat Medium and Thioglycollate Medium

These two different types of media are discussed briefly as under:

(a) **Cooked Meat Medium:** It is specifically suited for the cultivation (growth) of *Clostridia**.**

(b) **Thioglycollate Medium:** It is particularly suited for the growth of anaerobic microbes. It essentially comprises of the following ingredients, namely:

- **Glucose and Sodium thioglycollate**— that invariably serve as:
  - an inactivator of mercury compounds,
  - to augment and promote reducing parameters, and
  - an oxidation-reduction indicator.

- **Agar**—to cause reduction of the ensuing ‘convection currents’.

8.2.2.3. Sabouraud Medium

It is a medium specifically meant for fungal species. It essentially bears two vital and important characteristic features, such as:

- an acidic medium, and
- contains a rapidly fermentable carbohydrate *e.g., glucose or maltose.*

**Note:** (1) All the three aforesaid media must be previously assessed adequately for their nutritive characteristic features *i.e.*, in fertility tests to ascertain the growth of specified microorganisms.

(2) Duly incubated at the stipulated temperature(s).

The direct inoculation method shall now be dealt with in a sequential manner under the following three categories, such as:

- Quantities of sample to be employed,
- Method of test, and
- Observation and Interpretation of Results.

**Quantities of Sample to be used:** In actual practice, the precise quantum of the substance or pharmaceutical preparation under investigation, that is required to be used for inoculation in the respective culture media usually varies justifiably as per the amount present in each particular container, and is stated clearly in Table : 8.2 together with the exact volume of the culture medium to be employed.

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* Hartley’s Digest Broth: It is prepared by the tryptic digestion of defatted ox heart.

** Clostridium: A genus of bacteria belonging to the family *Bacillaceae.* They are found commonly in the soil and in the intestinal tract of humans and animals, and are frequently found in wound infections. However, in humans several species are pathogenic in nature, being the primary causative agents of gas gangrene.
Method of Test: The ‘method of test’ varies according to the substance to be examined, for instance:

(a) Aqueous Solutions and Suspensions: The actual tests for microbial contamination are invariably performed on the same sample of the preparation under investigation by making use of the above-stated media (Section 2.2.1 through 2.2.3). In certain specific instance when the amount present in a single container is quite insufficient to carry out the stipulated ‘tests’, the combined contents of either two or more containers may be employed to inoculate the above-stated media.

Methodology: The various sequential steps involved are as given under:

1. Liquid from the ‘test containers’ must be removed carefully with a sterile pipette or with a sterile syringe or a needle.
2. Transfer aseptically the requisite prescribed volume of the substance from each container to a vessel of the culture medium.
3. Mix the liquid with the medium carefully taking care not to aerate excessively.
4. Incubate the ‘inoculated media’ for not less than 14 days (unless otherwise specifically mentioned in the monograph*) at: 30–35°C for ‘Fluid Thioglycollate Medium’, and 20–25°C for ‘Soyabean-Casein Digest Medium’.

Special Points: The following special points may be noted meticulously:

(i) In case, the substance under investigation renders the culture medium turbid whereby the presence or absence of the actual microbial growth may not be determined conveniently and readily by sheer ‘visual examination’, it is always advisable and recommended that a suitable transfer of a certain portion of the medium to other fresh vessels of the same medium between the 3rd and 7th days after the said test actually commenced.

(ii) Subsequently, continue the incubation of the said ‘transfer vessels’ for not less than 7 additional days after the transfer, and for a total of not less than 14 days.

(b) Oils and Oily Solutions: For carrying out the required tests for the bacterial contamination of oils and oily solutions it is recommended to make use of culture media to which have been incorporated duly:

Octylphenoxy polyethoxyethanol (I) : 0.1% (w/v) [or Octoxynol]

\[
\text{Octylphenoxy polyethoxyethanol} = \text{CH}_{15}\text{OH} + \text{C}_3\text{H}_7\text{OH} + \text{OS} + \text{CH}_{5n} + \text{CH}_{5n}
\]

Polysorbate 80**: 1% (w/v)

However, these emulsifying agents should not exhibit any inherent antimicrobial characteristic features under the prevailing parameters of the ‘test’.

* Official Compendia i.e., BP ; USP ; Int. P., Ind. P., ; Eur. P.,

** An emulsifying agent or other appropriate emulsifying agent in a suitable concentration.
The required test must be carried out as already described under Section (a) above i.e., Aqueous Solutions and Suspensions.

Precautionary Measures: The following two precautionary measures should be taken adequately:

(i) Cultures essentially comprising of ‘oily preparations’ should be shaken gently every day.

(ii) Importantly, when one employs the fluid thioglycollate medium for the ultimate detection of the anaerobic microorganisms, shaking or mixing must be restricted to a bear minimum level so as to maintain perfect anaerobic experimental parameters.

(c) Ointments: The following steps may be adopted in a sequential manner:

(1) Carefully prepare the ‘test sample’ by diluting ten times in a sterile diluent, for instance: Fluid B or any other suitable aqueous vehicle which is capable of dispersing the test material homogeneously throughout the ‘fluid mixture’.*

(2) Mix 10 mL of the fluid mixture thus obtained with 80 mL of the medium, and subsequently proceed as per the method given under Section (a) i.e., Aqueous Solutions and Suspensions.

(d) Solids: The various steps involved are as stated under:

(1) Transfer carefully the requisite amount of the preparation under examination to the quantity of culture medium as specified in Table : 8.3, and mix thoroughly.

(2) Incubate the inoculated media for not less than 14 days, unless otherwise mentioned in the monograph at 30–35°C in the particular instance of fluid thioglycollate medium, and at 20–25°C in the specific case of soyabean-casein digest medium.

(e) Sterile Devices: For articles of such size and shape as allow the complete immersion in not more than 1 L of the culture medium test the intact article, using the suitable media; and incubating as stated under Section (a) i.e., Aqueous Solutions and Suspensions.

(f) Transfusion or Infusion Assemblies: For transfusion or infusion assemblies or where the size of an item almost renders immersion impracticable, and exclusively the ‘liquid pathway’ should be sterile by all means, flush carefully the lumen of each of twenty units with a sufficient quantum of fluid thioglycollate medium and the lumen of each of 20 units with a sufficient quantum of soyabeancasein digest medium to give an ultimate recovery of not less than 15 mL of each medium. Finally, incubate with not less than 100 mL of each of the two media as prescribed under Section (a) i.e., Aqueous Solutions and Suspensions.

Exception: Such ‘medical devices’ wherein the lumen is so small such that fluid thioglycollate medium will not pass through easily, appropriately substitute alternative thioglycollate medium instead of the usual fluid thioglycollate medium and incubate that duly inoculated medium anaerobically.

Note: In such situations where the presence of the specimen under examination, in the culture medium critically interferes with the test by virtue of the ensuing bacteriostatic or fungistatic action, rinse the article thoroughly with the bare minimum quantum of fluid A. Finally recover the rinsed fluid and carry out the ‘test’ as stated under ‘Membrane Filtration’ for Sterile Devices.

* Before use, test the dispersing agent to ascertain that in the concentration employed it clearly exerts absolutely no significant antimicrobial activities during the time interval for all transfers.
Observation and Interpretation of Results: In the case of ‘direct inoculation’ the various observation and interpretation of results may be accomplished by taking into consideration the following cardinal factors, such as:

1. Both at intervals during the incubation period, and at its completion, the media may be examined thoroughly for the critical macroscopic evidence of the bacterial growth.

2. In the event of a negative evidence, the ‘sample’ under examination passes the ‘tests for sterility’.

3. If positive evidence of microbial growth is found, reserve the containers exhibiting this, and unless it is amply proved and adequately demonstrated by any other means that their (microorganisms) presence is on account such causes unrelated to the ‘sample’ being examined; and, therefore, the tests for sterility are pronounced invalid. In such cases, it may be recommended to carry out a ‘retest’ employing an identical number of samples and volumes to be tested, and the media as in the original test.

4. Even then, if no evidence of microbial growth is duly observed, the ‘sample’ under investigation precisely passes the ‘tests for sterility’.

5. In case, reasonable evidence of bacterial growth is observed, one may go ahead with the isolation and subsequent identification of the organisms.

6. If they are found to be not readily distinguishable from those (microbes) growing in the containers reserved in the very First Test, the ‘sample’ under investigation fails the ‘tests for sterility’.

7. If the microorganisms are readily distinguishable from the ones actually growing in the containers reserved in the ‘First Test’, it is very much advisable to carry out a ‘Second Retest’ by employing virtually twice the number of samples.

8. Importantly, if no evidence of bacterial growth is observed in the ‘Second Retest’, the sample under examination legitimately passes the ‘tests for sterility’.

9. Contrarily, if evidence of growth of any microorganisms is duly observed in the ‘second retest’, the sample under investigation obviously fails the ‘tests for sterility’.

8.3. SAMPLING: PROBABILITY PROFILE

Sampling refers to—‘the process of selecting a portion or part to represent the whole’.

In usual practice, a sterility test attempts to infer and ascertain the state (sterile or non-sterile) of a particular batch; and, therefore, it designates predominantly a statistical operation.

Let us consider that ‘p’ duly refers to the proportion of infected containers in a batch, and ‘q’ the proportion of corresponding non-infected containers. Then, we may have:

\[ p + q = 1 \]

or

\[ q = 1 - p \]

Further, we may assume that a specific sample comprising of two items is duly withdrawn from a relatively large batch containing 10% infected containers. Thus, the probability of a single item taken at random contracting infection is usually given by the following expression:
whereas, the probability of such an item being non-infected is invariably represented by the following expression:

\[ q = 1 - p = 1 - 0.1 = 0.9 \]

**Probability Status**—The probability status of the said two items may be obtained virtually in three different forms, such as:

(a) When both items get infected : \( p^2 = 0.01 \)

(b) When both items being non-infected : \( q^2 = (1 - p)^2 = (0.9)^2 = 0.81 \), and

(c) When one item gets infected and the other non-infected : \( 1 - (p^2 + q^2) \)

or \[ = 1 - (0.01 + 0.81) = 1 - 0.82 \]

or \[ = 0.18 \]

i.e., \[ = 2pq \]

**Assumption**: In a particular ‘sterility test’ having a ‘sample’ size of ‘n’ containers, the ensuing probability \( p \) of duly accomplishing ‘n’ consecutive ‘steriles’ is represented by the following expression:

\[ q^n = (1 - p)^n \]

Consequently, the ensuing values for various levels of \( p^* \) having essentially a constant sample size are as provided in the following. Table 8.4A, that evidently illustrates that the ‘sterility test’ fails to detect rather low levels of contamination contracted/present in the ‘sample’.

Likewise, in a situation whereby different sample sizes were actually used**, it may be emphatically demonstrated that as the sample size enhances, the probability component of the batch being passed as sterile also gets decreased accordingly.

**Table 8.4 : Sampling in Sterility Testing**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Percentage of Infected Items in Batch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>1</td>
<td>( p )</td>
</tr>
<tr>
<td>2</td>
<td>( q )</td>
</tr>
<tr>
<td>3</td>
<td>Probability ( p ) of drawing 20 consecutive sterile items</td>
</tr>
<tr>
<td></td>
<td>( B^2 \times 0.99 )</td>
</tr>
</tbody>
</table>

1. **A : First Sterility Test** : Calculated from \( P = (1 - p)^{20} = q^{20} \)

2. **B : First Re-Test** : Calculated from \( P = (1 - p)^{20} [2 - (1 - p)^{20}] \)


* i.e., the proportion of infected containers present duly in a Batch.

** It is also based upon \( (1 - p)^{20} \) factor.
In actual practice, however, the additional tests, recommended by BP (1980), enhances substantially the very **chances of passing a specific** batch essentially comprising of a proportion or part of the **infected items** (see Table : 8.4B). Nevertheless, it may be safely deduced by making use of the following mathematical formula :

\[(1 - p)^n [2 - (1 - p)^n]\]

that provides adequate chance in the **First Re-Test** of passing a batch comprising of a proportion or part ‘\(p\)’ of the **infected containers**.

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**8.4. OVERALL CONCLUSIONS**

The various techniques described in this chapter essentially make a sincere and benevolent attempt to accomplish to a reasonably large extent, the stringent control and continuous monitoring of a specific sterilization process. However, it is pertinent to state here that the **sterility test** on its own fails to provide any **guarantee** with respect to the specific **sterility of a batch**. Nevertheless, it categorically accounts for an **additional check**, besides a continued compliance and offer sufficient cognizable confidence pertaining to the degree of an aseptic process or a sterilization technique being adopted.

Interestingly, an absolute non-execution of a prescribed (as per the ‘**Official Compendia**’) **sterility test** of a particular batch, despite the equivocal major criticism and objection of its gross inability and limitations to detect other than the gross contamination, could tantamount to both **moral consequences** and **important legal requirements**.

US-FDA promulgates and strongly advocates the adherence of USP-prescribed requirements for the **sterility test** for parenterals as the most authentic, reliable, and trustworthy **guide for testing the official sterile products**.

On a broader perspective, it may be observed that the **sterility test** is not exclusively intended as a **thoroughly evaluative test** for a product duly subjected to a known sterilization method of unknown effectiveness. Nevertheless, it is solely meant primarily as an intensive **check test** on the ensuing probability that :

- a previously validated sterilization process has been repeated duly, and
- to provide adequate assurance **vis-a-vis** its continued effectiveness legitimately.

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**FURTHER READING REFERENCES**

4. Indian Pharmacopoea : Published by the Controller of Publications, Delhi, Vol. II, 1996.