Sargramostim for Injection: “Sterility <71>—It meets the requirements when tested as directed for Membrane Filtration under Test for Sterility of the Product to be Examined.”

What is another common means of referring to sterility testing requirements from within monographs?
Morphine Sulfate Injection: “Other Requirements—It meets the requirements under Injections.”

From <1> Injections:

“Sterility”
Sterility Tests—Preparations for injection meet the requirements under Sterility Tests <71>”

What is “Sterility”?  
American Heritage's View of Sterility:

“Free from bacteria or other microorganisms”
What is “Sterility”? 

The view from *USP <1211> Sterilization and Sterility Assurance of Compendial Articles*:

- “Within the strictest definition of sterility, a specimen would be deemed sterile only when there is complete absence of viable microorganisms from it.”

Is it possible to demonstrate complete absence of microorganisms from a product?

The Principal Difficulty for Sterility Testing

- Not unless you’re not interested in having product left to sell, because you can’t demonstrate absolute sterility without the complete destruction of every article from the lot.

- What is the probability of passing the sterility test despite having contaminated units within the batch?
If you can’t evaluate every article in a lot, then what is the basis for a sterility determination?

Sterility is determined using probabilistic terms. A determination is made of the probability of finding a contaminated article among all the containers from a given lot.

The goal is to make that likelihood acceptably remote.

OK, so you can’t prove an entire batch of article is sterile by passing the sterility test.

What can you prove by an article passing the sterility test?
Flow of the Sterility Test

1. Media and Bacteriostasis/Fungistasis Testing
2. Eliminate any bacteriostatic/fungistatic properties
3. Determine number of articles, quantity from each, to test
4. Incubate the samples
5. Examine test articles for signs of growth
6. Examine suspect tubes microscopically for signs of growth
7. Subculture if necessary
8. Write the report!

USP Education

USP <71> Sterility Tests: Media for Testing

Three types of bacteriological media described, one is:

**Fluid Thioglycollate Medium**

- L-Cystine: 0.5 g
- Sodium Chloride: 2.5 g
- Dextrose ($C_6H_{12}O_6 \cdot H_2O$): 5.5/5.0 g
- Agar, granulated (moisture content not exceeding 15%): 0.75 g
- Yeast Extract (water-soluble): 5.0 g
- Pancreatic Digest of Casein: 15.0 g
- Sodium Thioglycollate: 0.5 g
  - or Thioglycolic Acid: 0.3 mL
- Resazurin Sodium Solution (1 in 1000), freshly prepared: 1.0 mL
- Purified Water: 1000 mL

USP Education
Fluid Thioglycollate Medium is primarily intended for the culture of anaerobic bacteria. However, it will also detect aerobic bacteria. Fluid Thioglycollate Medium is to be incubated at 30 –35°.

For products containing a mercurial preservative that cannot be tested by the membrane filtration method, Fluid Thioglycollate Medium incubated at 20 –25° may be used instead of Soybean–Casein Digest Medium provided that it has been validated as described in Growth Promotion Test of Aerobes, Anaerobes, and Fungi.

If the medium is stored, store at a temperature between 2 and 25° in a sterile, airtight container. If more than the upper one-third of the medium has acquired a pink color, the medium may be restored once by heating the containers in a water-bath or in free-flowing steam until the pink color disappears and by cooling quickly, taking care to prevent the introduction of nonsterile air into the container. (Note: with repeated reheating, toxic oxygen radicals are formed)
Alternate Thioglycollate Medium

- Where prescribed or justified and authorized, the alternative thioglycollate medium might be used. Prepare a mixture having the same composition as that of the Fluid Thioglycollate Medium, but omitting the agar and the resazurin sodium solution.
- Used for sterility testing of viscous products and for devices having tubes with small lumen.

**Alternative Thioglycollate Medium**

- L-Cystine: 0.5 g
- Sodium Chloride: 2.5 g
- Dextrose (C$_6$H$_{12}$O$_6$. H$_2$O): 5.5/5.0 g
- Yeast Extract (water-soluble): 5.0 g
- Pancreatic Digest of Casein: 15.0 g
- Sodium Thioglycollate or Thioglycolic Acid: 0.5 g or 0.3 mL
- Purified Water: 1000 mL
### Soybean Casein Digest Medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic Digest of Casein</td>
<td>7.0 g</td>
</tr>
<tr>
<td>Papaic Digest of Soybean Meal</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Dibasic Potassium Phosphate</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Dextrose Monohydrate/Anhydrous</td>
<td>2.5/2.3 g</td>
</tr>
<tr>
<td>Purified Water</td>
<td>1000 mL</td>
</tr>
</tbody>
</table>

### Soybean–Casein Digest Medium

Soybean–Casein Digest Medium is suitable for the culture of both fungi and aerobic bacteria.
Incubate a portion of each required medium at specified temperature for 14 days, and look for turbidity.

From Table 1:
- *Staphylococcus aureus*
- *Pseudomonas aeruginosa*
- *Clostridium sporogenes*
- *Bacillus subtilis*
- *Candida albicans*
- *Aspergillus brasiliensis*

What types of microorganisms are these, and what is the purpose of including so many?
Growth promotion test:

- Add ≤ 100 cfu of Staphylococcus aureus, Pseudomonas aeruginosa, Clostridium sporogenes to Fluid Thioglycollate Medium
- Add ≤ 100 cfu of Bacillus subtilis, Candida albicans, Aspergillus brasiliensis to Soybean-Casein Digest Medium
- Incubate for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi.
- Look for signs of growth

Why so few cfu?
What would “growth” look like?

Bacteriostasis/fungistasis (BF) testing:

- It is important to remove, as far as possible, any bacteriostatic and fungistatic activity inherent in the article to be tested.

Why?
How to test for B/F:

- Prepare dilute cultures of bacteria and fungi from the required microorganisms to obtain a final concentration of microorganisms in the product of less than 100 cfu.

- Use the membrane filtration or the direct transfer methods as appropriate for your product (or monograph requirement).

Membrane filtration method:

- Filter the article under test through a microorganism-retentive filter (not > 0.45 µM).
- Inoculate the final rinse with less than 100 cfu.
- Repeat the rinse procedure with filter not exposed to test article (positive control).
USP <71> Sterility Tests: BF Testing

Membrane filtration method:

- Place the filter into 100-mL volumes of the test medium
- Remember you will do this for each of the 6 test microorganisms as appropriate
- Incubate the containers at the appropriate temperature for not more than 5 days

Interpretation:

- If growth, as evidenced by turbidity, is comparable to the controls, things are good

- If not, you need to modify the conditions of the test such that antimicrobial activity is eliminated

How can you do that?
**USP <71> Sterility Tests: BF Testing**

**Rinsing:**

“Do not exceed a washing cycle of 5 times 100 mL, even if during validation it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity.”

Chemical additives:

**USP <1227> Validation of Microbial Recovery from Pharmacopeial Articles**

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**USP <71> Sterility Tests: BF Testing**

**Direct transfer method:**

- Use for articles that cannot be tested by membrane filtration (or if monograph requires it)

- Inoculate two containers of each sterility test medium with less than 100 colony-forming units (one set of containers for each required microorganism)
**USP <71> Sterility Tests: BF Testing**

**Direct transfer method:**

- Add the article under test to one of the inoculated containers of each medium. The other inoculated container is the positive control.

- Incubate the containers at the appropriate temperature for not more than 5 days.

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**USP Education**

**USP <71> Sterility Tests: BF Testing**

**Interpretation:**

- If growth, as evidenced by turbidity, is comparable to the controls, things are good.

- If not, you need to modify the conditions of the test such that antimicrobial activity is eliminated.

   **How can you do that?**
With a test method dependent upon microbial growth, it’s important to eliminate bacteriostatic or fungistatic properties.

- Can employ specific neutralizers, dilution, a combination of washing and dilution, or by any combination of these methods.
- Estimating the number of colony forming units.

1. Media and Bacteriostasis/Fungistasis Testing
2. Eliminate any bacteriostasis/fungistatic properties
3. Determine number of articles, quantity from each, to test
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5. Examine test articles for signs of growth
6. Examine suspect tubes microscopically for signs of growth
7. Subculture if necessary
8. Write the report!
USP <71> Sterility Tests: General Procedure

How much of the article needs to be tested?

- Table 2 specifies the quantity of article to be removed from each container, e.g., Liquids (other than antibiotics): Less than 1mL—The whole contents of each container

- Table 3 specifies the number of articles to be tested, e.g., Number of items in the batch: More than 500 containers—2% or 20 containers, whichever is less

USP Education

USP <71> Sterility Tests: General Precaution

Opening articles:

- Personnel must be properly trained and qualified
- The test for sterility is carried out under aseptic conditions
- “The working conditions in which the tests are performed are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.”
1. Media and Bacteriostasis/Fungistasis Testing
2. Eliminate any bacteriostatic/fungistatic properties
3. Determine number of articles, quantity from each, to test
4. Incubate the samples
5. Examine test articles for signs of growth
6. Examine suspect tubes microscopically for signs of growth
7. Subculture if necessary
8. Write the report!

Incubation conditions:

- Incubate for not less than 14 days at 32.5 (±2.5) °C for the Fluid Thioglycollate Medium or at 22.5 (±2.5) °C for the Soybean-Casein Digest Medium
1. Media and Bacteriostasis/Fungistasis Testing
2. Eliminate any bacteriostatic/fungistatic properties
3. Determine number of articles, quantity from each, to test
4. Incubate the samples
5. Examine test articles for signs of growth
6. Examine suspect tubes microscopically for signs of growth
7. Subculture if necessary
8. Write the report!

**Observations:**

- Observe the tubes of media on a periodic basis over the 14 days of incubation
- Does the observation period have to be 14 days, or could it be less?
**USP <71> Sterility Tests: General Procedure**

**Observations:**

- Not if the test specimen is positive before 14 days of incubation

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**USP <71> Sterility Tests: So Where Are We Now?**

1. Media and Bacteriostasis/Fungistasis Testing
2. Eliminate any bacteriostatis/fungistatic properties
3. Determine number of articles, quantity from each, to test
4. Incubate the samples
5. Examine test articles for signs of growth
6. Examine suspect tubes microscopically for signs of growth
7. Subculture if necessary
8. Write the report!
Why is this required? Isn’t the presence of turbidity sufficient to indicate that the article under examination is contaminated (barring legitimate invalidation of the results)?

If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 mL) of the medium to fresh vessels of the same medium, and then incubate the original and transfer vessels for not less than 4 days.
USP <71> Sterility Tests: So Where Are We Now?

1. Media and bacteriostasis/fungistasis Testing
2. Eliminate any bacteriostatis/fungistatic properties
3. Determine number of articles, quantity from each, to test
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8. Write the report!

Invalidating a Sterility Test

The test may be considered invalid only if one or more of the following conditions are fulfilled:

- The data of the microbiological monitoring of the sterility testing facility show a fault. A review of the testing procedure used during the test in question reveals a fault. Microbial growth is found in the negative controls. After determination of the identity of the microorganisms isolated from the test, the growth of this species (or these species) may be ascribed unequivocally to faults with respect to the material and or the technique used in conducting the sterility test procedure.

- If the test is declared to be invalid, it is repeated with the same number of units as in the original test. If no evidence of microbial growth is found in the repeat test, the product examined complies with the test for sterility. If microbial growth is found in the repeat test, the product examined does not comply with the test for sterility.

USP Education
When microbial growth is detected in a pharmaceutical or medical device product, the product is considered non-sterile, pending an investigation. Because of the public health importance of a non-sterility finding, preliminary results should be reported by your laboratory management, without delay.

Concurrently, a laboratory review should be conducted to answer the following question: Was the result true product contamination or was there a clear laboratory error that caused contamination of the sample during the analysis? The Out-of-Specification (OOS) investigation will review and document that the test results are based on sound laboratory operation.

Investigating a Sterility Test Failure

Whenever a sterility positive occurs, lab supervisors are responsible for starting the investigation immediately. Four factors should be evaluated in the basic investigation:

1. Equipment: Determine whether equipment malfunctioned or was not operated properly. If a malfunction occurred, determine whether it was likely to cause the contamination. Determine if any checklists or logs indicate that the ISO 5 device was in good state of repair at the time of the sterility test. Be aware of the most likely failure modes in the equipment (e.g., laminar flow hood, glovebox, or isolator) used.

2. Adherence to Analytical Method: Determine whether there were any anomalies or deviations from the analytical method. Adherence to method should be verified at the time of analysis, and any major breach of sterility test procedure should also be documented at that time. If any method breaches occurred, determine whether it was likely to cause the contamination. Be aware of any possible weaknesses in the test method (e.g., kit, manifold, etc.) used.
1. Analyst: Evaluate the analyst’s qualifications, including proficiency, training record, and experience. Also note whether the sterility testing practice of the analyst was observed during this or a recent analysis.

2. Cleanroom and ISO 5 (Class 100) Environmental Conditions:

3. Determine if disinfection/decontamination of the ISO 5 device was properly done. Determine whether there was adverse environmental data. Note that a negative control failure, on its own, is not necessarily cause for invalidating a result.

4. If a negative control was contaminated, consider whether the microbe identified is similar to, or the same as, the sterility test isolate and also consider whether there are other adverse environmental trends.

5. If an investigation finds that the conduct of the analysis included errors or events that caused the test specimens to be contaminated by the lab environment, the Sterility Test result would be invalid and the substandard laboratory practice should be corrected to prevent this problem from recurring.