Sections in This Document

1. Purpose

The purpose of this Pharmaceutical Microbiology Manual (PMM) is to collectively clarify, standardize, and communicate useful analytical procedures that are not specifically addressed in the microbiology methods chapters in the United States Pharmacopeia. In addition, some sections of this manual can serve as a technical reference when conducting microbiological inspections of drug, biotechnology and medical device manufacturers. The contents of this PMM were collaboration between ORS and CDER in order to maximize the efficiency of our analytical results to support CDER's goal to assure the safety and reliability of commercially distributed medical products.

2. Introduction

The Pharmaceutical Microbiology Manual (PMM) evolved from the Sterility Analytical Manual and is a supplement to the United States Pharmacopeia (USP) for pharmaceutical microbiology testing, including antimicrobial effectiveness testing, microbial examination of non-sterile products, sterility testing, bacterial endotoxin testing, particulate matter, device bioburden and environmental monitoring testing. The goal of this manual is to provide an ORS/CDER harmonized framework on the knowledge, methods and tools needed, and to apply the appropriate scientific standards required to assess the safety and efficacy of medical products within ORS testing laboratories. The PMM has expanded to include some rapid screening techniques along with a new section that covers inspectional guidance for microbiologists that conduct team inspections.

This manual was developed by members of the Pharmaceutical Microbiology Editorial Board and includes individuals with specialized experience and training.

The instructions in this document are guidelines for ORS analysts. When available, analysts should use procedures and worksheets that are standardized and harmonized across all ORS labs, along with the PMM, when performing analyses related to product testing of pharmaceuticals and medical devices. When changes or deviations are necessary, documentation should be completed per the laboratory's Quality Management System. Generally, these changes should originate from situations such as new products, unusual products, or unique situations.

This manual was written to reduce compendia method ambiguity and increase standardization between ORS laboratories. By providing clearer instructions to ORS labs, greater transparency can be provided to both industry and the public.

However, it should be emphasized that this manual is a supplement and does not replace any information in USP or applicable FDA official guidance references. The PMM does not relieve any person or laboratory from the responsibility of ensuring that the methods being employed from the manual are fit for use, and that all testing is validated and/or verified by the user.

The PMM will continually be revised as newer products, platforms and technologies emerge or any significant scientific gaps are identified with product testing.

Reference to any commercial materials, equipment, or process in the PMM does not in any way constitute approval, endorsement, or recommendation by the U.S. Food and Drug Administration.

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5. Chapter 1: Antimicrobial Effectiveness Testing

Antimicrobial Effectiveness testing is described in USP <51>. Previously this chapter was known as “Preservative Effectiveness Testing”. Detailed procedure for the performance of the test can be found in USP <51>.

A. Media

For the cultivation of the test organisms, select agar medium that is favorable to the rigorous growth of the respective stock culture. The recommended media are Soybean Casein Digest Agar/Broth and Sabouraud’s Dextrose Agar/Broth. Add a suitable inactivator (neutralizer) for the specific antimicrobial properties in the product to the broth and/or agar media used for the test procedure whenever needed.

B. Growth Promotion of the Media

Media used for testing needs to be tested for growth promotion by inoculating the medium with appropriate microorganisms. It is preferable that test microorganisms be chosen for growth promotion testing (Section D).

Solid media tested for growth promotion is to be set up using the method that will be used to analyze the product (pour plate or spread plate) to determine a microbial plate count (CFU) which must be ≥ 50% of the microorganism inoculum’s calculated value.

C. Suitability of the Counting Method in the Presence of Product

For all product types, follow current USP methodology in chapter <51>, with the following additional instructions.

Prior to the Antimicrobial Effectiveness testing, determine if any antimicrobial properties exist by performing a Suitability testing utilizing microorganisms used for product testing (section D). Should the Suitability Test fail the results of Suitability test are invalid and will need to be repeated with proper method modification to neutralize the inhibiting property.

If multiple samples of the same product from the same manufacturer (same amount and form) are collected, one sample may be used for method suitability for all the samples collected.

D. Test Organisms

All cultures must be no more than 5 passages removed from the original stock culture.

1. *Candida albicans* (ATCC No. 10231)
2. *Aspergillus brasiliensis* (ATCC No. 16404)
3. *Escherichia coli* (ATCC No. 8739)
4. *Pseudomonas aeruginosa* (ATCC No. 9027)
5. *Staphylococcus aureus* (ATCC No. 6538)

E. Preparation of Inoculum

Preparatory to the test, inoculate the surface of the appropriate agar medium from a recently grown stock culture of each of the above test microorganisms. Use Soybean-Casein Digest agar for *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* ATCC 6538 and incubate at 32.5 ± 2.5° C for 3 – 5 days. Use Sabouraud Dextrose agar for *Candida albicans* ATCC 10231 and *Aspergillus brasiliensis* ATCC 16404 and incubate at 22.5 ± 2.5° C for 3 – 5 days for *Candida albicans* and 3 - 7 days for *Aspergillus brasiliensis*.

Harvest the cultures by washing the growth with sterile saline to obtain a microbial count of about $1x10^8$ CFU/mL (see *Microbial Enumeration Tests* <61> and *Tests for Specified Microorganisms* <62>). For the *A. brasiliensis* ATCC 16404 culture, use sterile saline containing 0.05% polysorbate 80.

Alternatively, cultures may be grown in a liquid medium, i.e. Soybean Casein Digest Broth or Sabouraud's Dextrose Broth, (except for the A. brasiliensis ATCC 16404 culture) and harvested by centrifugation, washing and suspending in sterile saline to obtain a count of about 1 X 108 colony forming units (CFU) per mL.

The estimate of inoculum concentration may be obtained by turbidimetric procedures for the challenge microorganisms and later confirmed by plate count.

Refrigerate the suspension if not used within 2 hours at 2-8° C.

Determine the number of CFU/mL in each suspension using the appropriate media and recovery incubation times to confirm the CFU/mL estimate.

Use bacterial and yeast suspensions within 24 hr. of harvest. The mold preparation may be stored under refrigeration (2-8° C) for up to 7 days.

Note: Alternative commercially available standardized cultures may be used in lieu of in-house prepared cultures.

F. Procedure

The procedure requires that the test be conducted with a suitable volume of product. It is advisable to begin with at least 20 mL of product. Use the original product containers whenever possible or five sterile, capped bacteriological containers of suitable size into which a suitable volume of product has been transferred. If the diluted product exhibits antimicrobial properties, specific neutralizers may need to be incorporated into the diluents or the recovery media. For purposes of testing, products have been divided into four categories:

Category 1 – Injections, other parenteral including emulsions, otic products, sterile nasal products, and ophthalmic products made with aqueous bases or vehicles.

Category 2 – Topically used products made with aqueous bases or vehicles, non-sterile nasal products, and emulsions, including those applied to mucous membranes.

Category 3 – Oral products other than antacids, made with aqueous bases or vehicles.

Category 4 – Antacids made with aqueous bases or vehicles.

Inoculate each container with one of the prepared and standardized inoculums and mix. The volume of the suspension inoculums used is 0.5% to 1.0% of the volume of the product. The concentration of the test organisms added to the product for Categories 1, 2 and 3 is such that concentration of the test preparation immediately after inoculation is between $1x10^5$ and $1x10^6$ colony forming organisms (CFU) per mL of product. *If no suitable neutralizing agent or method is found and method suitability requires significant dilution, a higher level of inoculum (e.g., 10^7-10^8) may be used so that a 3-log unit reduction can*

be measured. For category 4 products (antacids) the final concentration of the test organisms is between $1x10^3$ and $1x10^4$ CFU/mL of product.

Immediately determine the concentration of viable organisms in each inoculum suspension and calculate the initial concentration of CFU/mL by the plate count method (see *Microbial Enumeration Tests* <61>).

Incubate the inoculated containers between 22.5 ±2.5°C in a controlled environment (incubator) and sample the container at specified intervals. The container sampling intervals include: Category 1 products are sampled at 7, 14, and 28 days and Category 2 – 4 products are sampled at 14 and 28 days. Refer to table 3 within USP <51>. Record any changes in appearance of the product at these intervals. Determine the number of viable microorganisms per mL present at each of these sampling intervals by the plate count method utilizing media with suitable inactivator (neutralizer). Calculate the change in log10 values of the concentration per mL based on the calculated concentration in CFU/mL present at the start of the test for each microorganism at the applicable test intervals and express the changes in terms of log reductions.

NOTE: The USP does not require a specific volume of product to be added to each of the five sterile tubes. It is recommended that 20 mL/tube be used to standardize testing for all ORS laboratories.

NOTE: All plate counts should be performed in duplicate (2 plates per dilution), and in a dilution series to detect growth inhibited by the preservative system at the lower dilutions. Carrying the test to the 10-3 dilution would be sufficient in most cases to overcome preservative inhibition.

G. Interpretation

The criteria for microbial effectiveness are met if the specified criteria are met, see table below. No increase is defined as not more than 0.5 log10 unit higher than the previous value measured.

Category 1 Products	
Bacteria:	Not less than 1.0 log reduction from the initial calculated count at 7 days, not less than 3.0 log reduction from the initial count at 14 days, and no increase from the 14-day count at 28 days.
Yeast and Molds:	No increase from the initial calculated count at 7, 14, and 28 days.
Category 2 Products	
Bacteria:	Not less than a 2.0 log reduction from the initial count at 14 days, and no increase from the 14-day count at 28 days.
Yeast and Molds:	No increase from the initial calculated count at 14 and 28 days.
Category 3 Products	
Bacteria:	Not less than a 1.0 log reduction from the initial count at 14 days, and no increase from the 14-day count at 28 days.
Yeast and Molds:	No increase from the initial calculated count at 14 and 28 days.
Category 4 Products	
Bacteria, Yeast and Molds:	No increase from the initial calculated count at 14 and 28 days.

6. Chapter 2: Microbial Examination of Non-Sterile Products

This section contains supplemental information for the quantitative enumeration of viable microorganisms and the determination of the absence of specified microorganisms in finished pharmaceutical products and raw materials, previously referred to as Microbial Limits Testing (MLT). The detailed procedures for these tests are not addressed in this PMM chapter because they are found in USP <60> MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: TESTS FOR *BURKHOLDERIA CEPACIA* COMPLEX, USP <61> MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: MICROBIAL ENUMERATION TESTS and <62> MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: TESTS FOR SPECIFIED MICROORGANISMS.

USP Chapter <60> is a test to detect the presence of *Burkholderia cepacia* species in a substance or preparation. Products for inhalation use or aqueous preparations for oral, oromucosal, cutaneous, or nasal USP <61> describes the methods for enumeration of microorganisms from pharmaceuticals and includes membrane filtration, conventional plate count (including pour-plate method, surface- spread method), and the Most-Probable-Number (MPN). USP Chapter <62> describes specific enrichment procedures depending on

the target specified microorganism that must be absent, as required by a product monograph. Products which are insoluble or immiscible in water must be appropriately treated to obtain a suspension suitable for the test procedures. USP <1111> is used to identify organism recommendations for various drug dosage forms. use—are of concern.

It is important to note that even though the USP delineates methods for the recovery and identification of specified microorganisms based on monograph requirements, it is normally necessary to determine if any other microorganisms may also be present in the product(s) that may be objectionable and report these microorganisms on worksheets. In many cases, these may be opportunistic or emerging pathogens not targeted for recovery by USP <62>. Identification methods, such as VITEK, should be used to identify any microbes recovered during USP <62> testing. Alternative methods, advanced molecular methods (i.e. PCR, sequencing, etc.) or the use of additional general enrichment agar plates or broth without selective properties, may better suit the screening of test samples. The application of these additional agars or methods may need to be considered based on the target population of the drug or product under analysis and may require a dialogue with the laboratory supervisor for additional instructions.

A. Product Storage and Handling

Samples are to be held under the same storage conditions required by the package label or insert.

1. Prior to product testing, the exterior of the unit container should be disinfected before transfer to the work station or HEPA filtered laminar flow hood. If the product container is not hermetically sealed do not soak the product container in a disinfection solution which may allow the ingress of bactericidal solution into the product.
2. The work area for opening the unit container should be either a HEPA filtered laminar flow hood or an alternate controlled environment to safeguard the exposure of open media and product to either environmental or personnel contamination.
3. If the sample is an aqueous based product, the unit(s) should be shaken prior to transfer to work area to maximize microbial dispersement.
4. All subsequent manipulation of test tubes with product or sub-culturing can be conducted on the laboratory work bench or within a Biological Safety Cabinet (BSC) if filamentous fungi are suspected.

B. Gowning Requirements

When conducting the testing, the analyst should wear a clean lab coat, sterile sleeves and sterile gloves. Gloves should be frequently disinfected especially between opening and handling sample (product) units.

Depending on the type of Laminar Flow Hood or equipment barriers in a particular laboratory, it might be beneficial to also wear a surgical mask and hair net.

C. Growth Promotion, Indicative and Inhibitory Properties of the Media

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients for growth promotion and where appropriate, for indicative and inhibitory properties, following USP <60>, <61> and <62>. Each chapter provides guidance on test strains to be used for each type of media, refer to Table 1 of USP <60>, <61> and <62>. Ensure that seed-lot cultures used are not more than five passages removed from the original master seed-lot. Test strains suspensions should be used within 2 hours, or within 24 hours if refrigerated between 2ºC and 8ºC. Spore suspensions (A. *brasiliensis*, B. *subtilis, and C. sporogenes)* refrigerated between 2ºC and 8ºC may be kept for a validated period. If commercially available ready-to-use bacteria or fungal suspension are used, then the manufacturer's instructions should be followed with respect to preparation and storage requirements. Additionally, all bacterial and spore suspensions should be prepared to yield ≤100 CFU. Growth promotion (and suitability test) plates and tubes should not be incubated in the same incubators used for product testing. If this cannot be avoided because of limited space, it is preferable to store the "spiked samples" in the lower half of the incubator below the sample inoculated plates and tubes.

D. Suitability of the Test Method

Suitability demonstrates that the products tested do not exhibit inhibitory effects on the growth of microorganisms under the conditions of the tests. Although the intent is to perform the suitability test before performing the analysis of the product, it is acceptable to run the product test and the suitability test concurrently. However, it should be noted that if the suitability test is run concurrently with the product test and the suitability test should fail, the results of the product test are invalid and the suitability test as well as the product test will need to be repeated with proper method modification to neutralize the inhibiting property.

Neutralizing agents may be used to neutralize the activity of antimicrobial agents in products, see USP <61> Table 2 for a list of potential neutralizing agents/methods. The appropriate neutralizing agent should be added preferably before sterilization of the media. Include a blank control with neutralizer and without product to demonstrate efficacy and absence of toxicity for microorganisms.

USP <60>, <61> and <62> describe the suitability tests necessary for each analysis. The correct inoculum of not more than 100 CFU is required in addition to specific incubation temperatures and durations. Ensure that seed-lot cultures used are not more than five passages removed from the original master seed-lot. For in-house prepared test strain suspensions of vegetative bacteria and yeast should be used within 2 hours, or within 24 hours if refrigerated between 2ºC and 8ºC. Spore suspensions (*A. brasiliensis*, *B. subtilis,* and *C. sporogenes*) can be prepared and maintained between 2ºC and 8ºC for up to seven days. Additionally, all bacterial and spore suspensions should be prepared to yield ≤100 CFU. If commercially available ready-to-use bacteria or fungal suspension are used, then the manufacturer's instructions should be followed with respect to preparation and storage requirements. USP <60>, <61> and <62> require a control which is without test material to be included in the suitability test. The following viable ATCC derived cultures may be used. Please be aware that under the Microbiological Examination of Nonsterile Products chapters <60>, <61> and <62> users are allowed alternative sources of the below listed strains. The organisms below are recommended for ORS use in order to have a consistent and standard worksheet format:

USP <60>:

Burkholderia cepacian (ATCC 25416)

Burkholderia cenocepacia (ATCC BAA-245)

Burkholderia multivorans (ATCC BAA-247)

USP <61>:

Pseudomonas aeruginosa (ATCC 9027)

Staphylococcus aureus (ATCC 6538)

Bacillus subtilis (ATCC 6633)

Candida albicans (ATCC 10231)

Aspergillus brasiliensis (ATCC 16404)

USP <62>:

Pseudomonas aeruginosa (ATCC 9027)

Staphylococcus aureus (ATCC 6538)

Escherichia coli (ATCC 8739)

Salmonella enterica (ATCC 14028)

Candida albicans (ATCC 10231)

Clostridium sporogenes (ATCC 11437)

USP <60>, <61> and <62> each contain the acceptance criteria for their respective suitability test. USP <60> requires that the BCC microorganisms must be detected with the indicated reactions. Indicated reactions are indicated by the growth of greenish-brown colonies with yellow halos, or white colonies surrounded by a pink-red zone on BSCA. For USP <61> the Results and Interpretation section requires the inoculated product to have a mean count of any of the test organisms not differing by a factor greater than 2 from the control which was without test material. For example, if the control count is 80 CFU the acceptable counts need to be greater or equal to 40 CFU. USP <62> requires the specified microorganisms to be detected with the indicated reactions.

E. Test Procedure

Prepare the sample in a manner to achieve a uniform solution or suspension. This is critical because microbial contamination is not evenly dispersed throughout a lot or sample of product. Use conventional mechanical and shaking methods to the extent that original numbers and types of microorganisms are not altered in the product.

Use the following general procedures to prepare and handle samples.

1. Analyze samples as soon as possible after receipt. Inspect each unit visually for integrity of primary container and note any irregularities. Do not use the product container if it has been compromised or damaged without supervisor approval. Testing of a compromised or damaged container should be evaluated on a case by case basis. Discuss with supervisor if compromised unit containers need to be tested for forensic purposes (i.e. product tampering).
2. Identify units to be tested with Analyst's initials, date, subsample number, and sample number.
3. Cleanse outer surfaces of sample containers with sterile wipes using a validated effective antimicrobial agent. Place on a disinfected tray or surface in a properly disinfected laminar flow hood or biosafety cabinet. Allow containers to dry.
4. Aseptically open containers and perform weighing procedures in a laminar flow hood or biological safety cabinet if possible.

5. Appropriate environmental controls such as air exposure plates should be used in accordance with local quality procedures.
6. Appropriate negative controls should be run concurrently with the sample.

F. Interpretation of the Results

USP <60>

The possible presence of Bcc is indicated by the growth of greenish-brown colonies with yellow halos, or white colonies surrounded by pink-red zone on BCSA. Any growth on BCSA is confirmed by identification tests. Isolates should be identified to genus and, if possible, species using rapid identification kits or DNA sequencing. The product complies with test if no growth is observed or if the confirmatory identification tests are negative.

USP <61>

Regarding USP <61> the acceptance criterion for microbiological quality as it pertains to quantitative analyses has an allowable variability of the final colony forming units (CFUs). There is a two-fold tolerance in the final results. For example, if the monograph requires a 100 cfu/ml limit, the acceptable upper limit for these results would be 200 cfu/ml. Additional information is included in the "Interpretation of the Results" section of USP <61> that should be read and understood when reviewing quantitative test results.

USP <62>

Bile-Tolerant Gram-Negative

The product complies with the test if there is no growth. Any isolates should be identified to genus and, if possible, species using rapid identification kits or DNA sequencing.

Escherichia coli

Growth of colonies indicates the possible presence of *E. coli.* This is confirmed by identification test. Isolates should be identified to genus and, if possible, species using rapid identification kits or DNA sequencing. The product complies with the test if no colonies are present or if the identification tests are negative.

Salmonella

The possible presence of *Salmonella* is indicated by the growth of well-developed, red colonies, with or without black centers. This is confirmed by identification tests. Isolates should be identified to genus and, if possible, species using rapid identification kits or DNA

sequencing. The product complies with the test if no growth is observed or if the confirmatory identification tests are negative.

Pseudomonas aeruginosa

Growth of colonies indicates the possible presence of *P. aeruginosa.* This is confirmed by identification test. Isolates should be identified to genus and, if possible, species using rapid identification kits or DNA sequencing. The product complies with the test if no colonies are present or if the identification tests are negative.

Staphylococcus aureus

The possible presence of *S. aureus is* indicated by the growth of yellow or white colonies surrounded by a yellow zone. This is confirmed by identification tests. Isolates should be identified to genus and, if possible, species using rapid identification kits or DNA sequencing. The product complies with the test if no growth is observed or if the confirmatory identification tests are negative.

Clostridia

The occurrence of anaerobic growth of rods with or without endospores, giving a negative catalase reaction indicates the presence of *Clostridia.* This is confirmed by identification tests. Isolates should be identified to genus and, if possible, species using rapid identification kits or DNA sequencing. The product complies with the test if no growth is observed or if the confirmatory identification tests are negative.

Candida albicans

Growth of white colonies may indicate the presence of *C. albicans*. This is confirmed by identification test. Isolates should be identified to genus and, if possible, species using rapid identification kits or DNA sequencing. The product complies with the test if no colonies are present or if the identification tests are negative.

*Note: Regarding USP <60> and <62>, each isolate should be identified to genus and, if possible, species using rapid identification kits or DNA sequencing.

7. Chapter 3: Sterility Testing

A. Method Suitability Test

For all product types, follow current USP methodology in <71>, with the following additional instructions.

In all cases, even if the product does not include a preservative, the product itself may have growth inhibiting properties. All products should undergo a prescribed Method Suitability test.

Units selected for suitability testing should be subjected to the same disinfection procedure utilized in the sample analysis.

When developing the testing protocol for method suitability the volume of product as well as the concentration of the product should be evaluated such that the highest volume of product and the highest concentration of product should be used for the method suitability testing.

If multiple samples of the same product from the same manufacturer (same dosage and form) are collected, one sample may be used for method suitability for all the samples collected.

1. When to run Method Suitability:
    a. Run the method suitability test prior to conducting the sterility test in accordance with USP requirements under the following conditions:
        i. If insufficient information about the product exists to judge its probable growth inhibiting activity.
        ii. In all cases, when there is sufficient analytical time available, i.e., survey type samples.
    b. Run the method suitability test concurrently with product sterility tests when time is critical, and problems associated with 1. above have been resolved. However, it should be noted that if the Method Suitability Test is run concurrently with the product sterility test and the Method Suitability Test should fail, the results of the product test are invalid and the Method Suitability Test as well as the product test will need to be repeated with proper method modification to neutralize the inhibiting property.
    c. If an insufficient amount of product is collected and the analysis is critical, the suitability test can be conducted at the end of the 14-day incubation period. Be sure to use best judgment and maximum neutralization approach when initially conducting the product sterility test. If the suitability results indicate inhibition then the results, if negative, are invalid. However, if the product test results indicate microbial presence and the suitability test shows inhibition, the results are still valid.
2. Method Suitability Test Procedures

Method Suitability and positive culture control tests which require the use of viable microorganisms, should be performed outside the clean room or isolator, in a biosafety cabinet or equivalent.

a. Membrane filtration

   i. Pass product fluid through filter membrane. Rinse the membrane with three 100 ml portions (or more if applicable) of specified rinse fluid. Do not exceed a washing cycle of five times 100mL per filter. This step hopefully will neutralize and remove any antimicrobial residue on the filter membrane.

   ii. Add indicated test organisms in specified numbers (less than 100 CFU) into the last 100 ml rinse fluid used.

   iii. Filter the rinse fluid and aseptically cut the filter membrane into two equal parts, transfer one half into each of two suitable media. If conducting the sterility test using a closed canister system, rinse each canister with the inoculated rinse fluid.

   iv. If the available number of test vessels is insufficient for a complete challenge test for each individual microorganism, then the test organisms may be composited as necessary. However, confirmation of growth for the composited microorganisms will need to be performed.

   v. Confirm composited microorganisms by Gram stain, microscopic examination, and identification after the completion of incubation.

   vi. See step c. below for additional considerations.

b. Direct inoculation:

For direct inoculation, add the test microorganisms to separate test vessels of product and culture media if sufficient product is available. See step c. below for additional considerations.

c. The following test procedures apply to Direct Inoculation and Membrane Filtration:

   i. Inoculate the same microorganism using the same medium without the product as a positive control.

   ii. For bacteria and fungi, incubate test vessels according to USP requirements. Ensure that seed-lot cultures used are not more than five passages removed from the original master seed-lot. For in-house prepared test strain suspensions of vegetative bacteria and yeast should be used within 2 hours,

or within 24 hours if refrigerated between 2ºC and 8ºC. Spore suspensions (*A. brasiliensis*, *B. subtilis,* and *C. sporogenes*) can be prepared and maintained between 2ºC and 8ºC for up to seven days. Additionally, all bacterial and spore suspensions should be prepared to yield ≤100 CFU.

iii. If growth comparable to that of the positive control vessel without product is obtained, then you may proceed with the sterility test. If comparable visible growth is not obtained, the antimicrobial activity of the product has not been eliminated under the conditions of the test. Modify the test conditions and repeat the Method Suitability test.

d. If product is found to exhibit growth inhibiting activity when determined concurrently with product testing, the sterility test must be repeated using a neutralizing agent (or increase media volume) to modify the conditions in order to eliminate the antimicrobial activity.

e. Cultures used for the method suitability test can be purchased commercially, ready to use, or can be prepared and maintained locally. Either procedure requires quantitative verification of actual CFU's inoculated at time of use.

B. Sample Analysis

1. Sample Containers

a. Open the outer sample packaging on a laboratory bench disinfected with a sporicidal antimicrobial agent. Refer to appropriate literature for choosing suitable antimicrobial agents for use in your facility.

b. Count the number of units received. Compare this number with the number of units collected.

c. Inside the clean room preparation area located outside the ISO 5 area (if available) remove all outer packaging from subsample units that will be tested without compromising the sterile integrity of the product. Remove sample units and place them on a tray or cart disinfected with an effective antimicrobial agent.

Note: One or more units can be sacrificed to aid in the determination for how to aseptically remove test material if the number of the units received is sufficient.

d. Examine all units visually for container closure integrity, for the presence of any foreign matter in the product and other container closure defects. Note findings on analyst's worksheet.

e. If foreign matter is observed within the primary container, discuss with supervisor the employment of ORS procedure Document ORA-LAB.015 entitled “Screening Protocol for Direct Staining on Products with Appearance of Visible Contamination” (see QMiS for Procedure).

2. Sample Identification

If sample units are not identified by the collector, the analyst should identify unit with sample #, initials, date, and sub sample # as appropriate for sample traceability. Otherwise, date and initial each unit.

3. Unit Container Disinfection

a. Cleanse the exterior of all product primary containers using antimicrobial/sporicidal agents.

Depending on the clean room design, immediately move the sample to the clean room on a disinfected designated stainless-steel cart or place it inside the clean room pass thru for final preparation. If conducting the sterility test in an isolator, place the sample on a designated stainless-steel cart. Allow exposure of the sample to the disinfectant for appropriate time before further handling. All units should be disinfected appropriately. The suggested disinfection procedures can be performed on commonly encountered units as follows:

i. Ampoules can be wiped with lint free sterile towel/wipes saturated with disinfectant. Ampoules may be soaked in disinfectant/sporicidal following manufacturer’s guidance or laboratory SOP.

ii. Vials should not be soaked due to the possibility of migration of disinfectant under the closure and into the product.

iii. Laminated Tyvek package composed of polyethylene/plastic laminate can be disinfected with sterile towel/wipes soaked in disinfectant. Tyvek portion lightly scrubbed with sterile particle free dry wipe and air dry in a HEPA filtered laminar flow hood before testing.

iv. Paper Packages can be disinfected with UV light if possible. Wipe where applicable with sterile particle free dry wipes and air dry as above.

b. Number of units and/or amount of product tested:
Follow the current edition of the USP to determine the correct number of units to be tested and the amount of product to be analyzed from each unit. It is preferable to test the entire contents of each unit if possible. Follow laboratory policy if it requires testing more units than the USP requires.

c. If the number of units collected is less than the USP requirements, discuss with the laboratory supervisor before proceeding. Samples collected in a for-cause situation may be analyzed with a number of units less than the USP requirements.

C. Preparation for the Analysis

1. Media and Rinsing Fluid Preparation:
Follow current USP when preparing media used for sample analysis. Commercially purchased media may also be used for the analysis. Both prepared and purchased media must meet the requirements of the USP growth promotion test of aerobes, anaerobes and fungi. Media used are:

a. Fluid Thioglycollate medium (FTM) This medium should be prepared in a suitable container to provide a surface to depth ratio so that not more than the upper half of the medium has undergone a color change indicative of oxygen uptake at the end of the incubation period. If more than the upper third of the medium has acquired a pink color, the medium may be restored once by heating until the pink color disappears. Care should be taken to prevent the ingress of non-sterile air during cooling.

b. Soybean Casein Digest medium (SCD medium) This media must be incubated under aerobic conditions

c. Alternative Thioglycollate medium This type of media must be incubated under anaerobic conditions.

d. Media for Penicillin and Cephalosporin containing drugs. Add sufficient quantity of sterile Beta-lactamase to the media to inactivate the effect of these antibiotics.

e. Diluting and rinsing fluids. These fluid rinses may be filtered before sterilization to avoid clogging of the filter membrane during testing.

2. Media storage
For laboratory prepared media, do not use medium for longer storage period than has been validated.

For commercially purchased media, follow the manufacturer's recommended storage requirements and expiration date.

3. Media qualification:
   Perform the following tests on the prepared media before use:

   a. Sterility: The media batch may be used if the sterilization cycle is validated and monitored with the use of a biological indicator, and the batch passes other quality control testing. Also, if possible, prior to otherwise concurrently, incubate a portion of the media at the specified temperature for 14 days.

   b. Growth promotion test; follow the current USP using recommended strains of organisms (Table 1, USP <71>). Do not use cultures that are more than five passages removed from the original master seed lot. Commercially prepared and standardized stable suspension cultures of the recommended organisms can also be used. Test strains suspensions of vegetative bacteria or yeast should be used within 2 hours, or within 24 hours if refrigerated between 2ºC and 8ºC. Spore suspensions (*A. brasiliensis, B. subtilis*, and *C. sporogenes*) refrigerated between 2ºC and 8ºC may be kept for a validated period of time. If using commercially prepared organisms, follow the manufacturer's instructions. Additionally, all bacterial and spore suspensions should be prepared to yield ≤100CFU. All bacterial counts must be verified at time of use.

4. Equipment Preparation
   Analytical equipment and tools used in sterility analysis and suitability should be cleaned and sterilized using a validated sterilization procedure. Commercially purchased equipment and tools should be labeled sterile and accompanied by a certificate of analysis for sterility.

D. Clean Room Activities

1. Gowning
   Personnel are critical to the maintenance of asepsis in the controlled environment. Thorough training in aseptic techniques is required. Personnel must maintain high standards each time they deal with sterile product.

   a. Personnel gowning qualification should be performed by any analyst that enters the aseptic clean room. Personnel gowning qualification must consist of:

      i. Training of gowning techniques by a qualified trainer.

ii. Observation of trainee by trainer while gowning.

iii. General growth media touch plates utilized to analyze if the trainee gowned correctly without contaminating the sterile outer gown, sterile gloves and sterile head cover.

b. Some consideration should be taken before entering the clean room (see below). Follow applicable specific procedures for the facility.

i. Proper gowning immediately prior to entry the clean room is required of all personnel without exception.

ii. Non-linting clean room scrubs that cover as much skin as possible is the ideal inner-suit to wear before gowning up for an aseptic clean room. Street clothes are not permitted.

iii. Remove jewelry and makeup.

iv. Scrub hands (and arms when possible) before gowning.

v. Non-shedding sterile uniform components should be used all the time.

vi. Use aseptic gowning procedure to don sterile uniform components.

vii. Care should be taken to choose gowning that does not expose any skin to the aseptic clean room environment.

viii. An appropriate sporicidal/disinfectant is used to sanitize the gloves.

ix. If possible, post the gowning procedures in the gowning room or area to help individuals follow the correct order of gowning.

x. Should an analyst find it necessary to leave the room, he/she should discard all gowning components and put on new ones upon re-entry.

xi. If an individual scheduled to enter the clean room for analysis feels sick or has compromised skin, he/she should talk to his/her supervisor to postpone entry into the clean room until fully healed.

2. Sample Preparation
Repeat disinfection procedure using appropriate disinfectant/sporicidal immediately prior to placing product primary containers in a working certified laminar flow hood. Allow all disinfected containers to completely air dry in the laminar flow hood prior to opening for analysis. Alternatively, if conducting the testing in an isolator, place the disinfected items into the isolator and proceed

with the local procedures for the proper decontamination of the interior of the isolator.

3. Room Cleaning After Analysis
   a. Remove inoculated tubes of media and all controls from the analytical area by putting them in the pass-thru or on a stainless-steel cart used for transporting materials in and out of the clean room.
   b. After analysis, all sample containers, equipment wrap, used equipment and tools are to be removed from the clean room before the analyst exits.
   c. Sample containers used in the analysis should be returned to the original outer containers for storage as part of the reserve sample.
   d. Disinfect working area before exiting the clean room.
4. Clean room disinfection and surface monitoring must be conducted for both aerobic and anaerobic microorganisms on a routine basis. The frequency is to be determined by the local laboratory policy.

E. Method of Analysis

1. Membrane Filtration
   Follow the current edition of the USP for the amount of sample to be tested.
2. Direct Inoculation
   Follow the current edition of USP for the amount of sample and media to be used. For example: Use 200 ml of each medium when analyzing solid form products. If the membrane filter method is unsuitable, certain liquids may be tested by direct inoculation method.
3. Devices
   All devices with only the pathways labeled as sterile are to be tested by the pathway with sterile Fluid D and testing the Fluid D via membrane filtration.
4. Incubation of Sterility Test Media
   a. Incubate Fluid Thioglycollate (THIO) at 32.5 ± 2.5^{o}C. Do not shake or swirl test media during incubation or during examination to minimize aeration of this broth.

b. Incubate Soybean-Casein Digest Broth (SCD) at 22.5 ± 2.5°C. Gentle swirling, on occasion is acceptable to increase aeration of media.

c. Incubation period for THIO and SCD:

i. Not less than 14 days except for products sterilized using ionizing radiation. If tubes are not read on day 14 due to holiday or weekend then record the results, even if positive, on the first available day to observe the tubes.

ii. Additional incubation time may be warranted if the analyst is made aware of sterilization processes other than heat or filtration (e.g. 30 days (at minimum) for products sterilized using ionizing radiation). This is to allow repair of DNA of microorganisms injured by ionizing radiation, if any, that may be present).

F. Analysis of Medical Devices (ex. Purified Cotton, Gauze, Sutures and Surgical Dressings)

The USP method for analysis of surgical dressing/cotton/gauze (in packages) calls for a minimum quantity of 100 mg, to be tested in each medium. It is recommended that an entire unit shall be tested in each medium for individually packaged single-use articles.

1. Gauze, Purified Cotton, Sutures and Surgical Dressings

a. Using media containers as large as quart jars analyze entire unit of product.

b. If unit is too large for the container, analyze as much of unit as can be placed in container and covered by the medium.

2. Compositing of Medical Devices

a. Devices may be tested in composites (2 – 4 units/composite) as long as they meet the specifications of Chapter 71 of the current USP with regards to minimum quantity of a test unit and minimum number of units to be tested. All composited units must be the same lot number.

b. Devices may be composited only if they successfully pass the Method Suitability test. If composited units do not pass Method Suitability test, then the product cannot be composited.

G. Control Systems

The objective of a control system is to ensure the sterility, within designated limits, of all items, media, rinsing fluids, and equipment used in a sterility test.

The control systems which will accompany all sterility analyses are outlined below.

1. System Control

A "system control" is used to demonstrate maintenance of sample integrity during all analytical manipulations. Any piece of equipment that comes in contact with the product under analysis, along with any manipulations by the analysts, must be controlled. Thus, all equipment, fluids, and culture media for the "system control" must be handled in a manner which duplicates, as closely as possible, the manipulations of the actual sample being analyzed. All materials used as system controls must be sterilized by the analyzing laboratory. However, the method of sterilization need not be the same as for the product, but they must render the material sterile.

The first choice for the system control is the actual product, if enough test units are available. When complex medical devices must be sacrificed in order to design a suitable sterility test, consider using them for a system control after cleaning, repacking and sterilizing.

When there are viable alternatives, a product unit should not be sacrificed for use as a system control if this will reduce the number of units available for sterility testing below USP requirements or ORS policy requirements, except as provided in the preceding paragraph. If using a product unit would reduce the subsamples examined below the number required by USP or ORS policy, the analyzing laboratory should prepare a control from other material than a unit of the sample product whenever possible.

a. Membrane Filtration: A filter funnel from the vacuum source connection on each manifold used in the test is used for the system control. Alternatively, if a closed canister system is used to conduct the sterility test a canister set from the same lot used during the analysis should be used for the system control.

i. Filterable Materials (liquids, soluble solids, etc.)

Use a material similar to the product under test. The control material must be of the same volume, and similarly packaged as the test product. Filter-sterilized and autoclaved Peptone water (USP Fluid A) may be useful for this purpose in many cases.

ii. Devices with sterile Fluid Pathway

Use tubing or other containers similarly fitted with needles, valves, connectors, etc., as the product under test. Use USP Fluid D to flush lumens.

b. Materials tested by direct inoculation (devices, insoluble solids, and other non-filterable materials)

Use materials similar in size, shape, and texture, and similarly packaged as product under test. Replicate as nearly as possible pertinent, unusual features that may reflect on the credibility of the sterility test.

In designing "system controls" for sterility testing, care must be taken to duplicate the sample product for most aspects, as nearly as possible. Be novel and innovative to meet this requirement and make the system control meaningful.

2. Equipment Controls

All equipment items used in the analysis listed below will be controlled individually. One item from each autoclave lot of equipment is tested in each medium used in the test. Therefore, for a sample tested in THIO and SCD, one item from each sterilizer load (oven or autoclave) is tested in each medium giving a total of two controls for each forceps, syringe, etc., used in the test.

Forceps

Syringes

Scissors

Scalpels

Swabs

Pipettes

Membranes (dry, directly from the package). If membranes are sterilized in place, this control may be omitted.

Hemostats

Other special items that may be required by a specific test.

3. Media and Rinse Fluid Controls

   a. An uninoculated media and rinse fluid control are analyzed to ensure sterility at time of use.

Alternatively, controls for these materials are accomplished as part of the "system control" for each manifold. This will also include membrane cutters, and other items that contact the product but cannot be individually controlled.

4. Environmental Controls

   a. Open Media Controls

Tubes of each medium (THIO and SCD) used in the sterility analysis are exposed to the immediate environment of the test (e.g., laminar flow hood) for

the duration of the test. Alternatively, a laboratory may use agar settling plates as detailed in section b.

b. Agar Settling Plates

Plastic Petri dishes containing an effective non-selective medium (based on test requirements) are exposed in the hood for a period not to exceed four hours during the analysis. After four hours, plates should be replaced to continue monitoring (as appropriate).

Plates should be incubated for 48 hours at 35° C, and an additional 5 days at 25°C in order to detect mold contamination.

c. Controls within an Isolator

When conducting the sterility test within an isolator, if it has been designed to allow for a connection to an air sampler and particle counter this sampling may be performed for the duration of the sample analysis in lieu of the environmental samples described above. If the isolator is unable to accommodate an air sampler and/or particle counter or the instruments are unavailable the environmental controls described in section a. and b. should be used. Isolator gloves should be examined before and after a testing session to ensure integrity of the gloves were maintained. This examination should be documented. Additionally, prior to each decontamination cycle a leak test of the isolator system must be performed with passing results.

5. Personnel Monitoring

Personnel monitoring must be performed after analysts conclude sterility testing and prior to exiting the aseptic clean room. The analyst shall use general media touch plates to monitor the sterile condition of their clean room attire and to ensure aseptic techniques were followed.

For example, a minimum of five touch plates should be used for the following personnel gowning sites:

RH glove finger tips.

LH glove finger tips.

Chest

Left Forearm

Right Forearm

General media touch plates will be incubated for 5 days at 30-35ºC.

NOTE: The numerical values for personnel monitoring limits and specifications are established on the basis of a review of actual findings within the facility. All isolates are to be identified by local laboratory procedure to ensure that the

analyst did not contaminate the sample. Analysts should be sanitizing their gloves throughout the sterility analysis and changing gloves when needed. However, changing gloves prior to performing personnel monitoring is unacceptable. Each laboratory is required to monitor and trend data to ensure compliance and detect any abnormalities.

H. Sub-culturing Primary Media

Daily observations of primary test media (THIO and SCD) containing product should be performed without unnecessary disturbance. All handling of positive tubes, streaked plates, or subsequent inoculations of additional media will be done outside the clean room. These culture transfers are to be performed within a HEPA filtered biosafety cabinet or equivalent outside the ISO5 area which has been cleansed with an effective sporicidal/disinfectant anti-microbial agent. The analyst should be gowned with at least sterile gloves, sterile sleeves and a mask to minimize any possible cross contamination.

1. Record on Analyst's worksheets the day that the primary isolation media, Fluid Thioglycollate Broth (THIO), or Soybean-Casein Digest Broth (SCD) is turbid and inform supervisor. Streak tubes on the day they first appear positive and again at 14 days to determine the presence of other possible slow-growing (i.e., fungi) microorganisms.
2. Within a HEPA filtered biosafety cabinet or equivalent outside the clean room, streak turbid tubes onto Modified Soybean-Casein Digest Medium [SCD broth + 1.5% agar] (Modified SCDA) or other non-selective agar plate.
3. All streaked plates are incubated for a period at least as long as required for growth in original isolation media (THIO or SCD) not to exceed seven days.
4. Subculturing from Fluid Thioglycollate Broth (THIO)
    a. Subculture Thioglycollate broth to general medium agar plates in duplicate. Streak two plates; incubate one aerobically, and one anaerobically, each at 32.5 ± 2.5 ºC. NOTE: It is suggested to transfer an aliquot of media from close to the bottom of the tube to maximize the recovery of strict anaerobes.
        i. Note if any growth is observed on the anaerobic plate which differs from growth on the aerobic plate. Pick a single representative colony and perform an aero-tolerance test in order to determine if a strict anaerobe has been recovered. Proceed with identification of any strict anaerobes recovered when isolation is complete.

ii. Note if any growth is observed on aerobic plate and compare to growth on anaerobic plates. Proceed with identification when isolation is complete.

5. Subculturing from Soybean Casein Digest broth (SCD)

a. Sub culture SCD broth to general growth medium and incubate aerobically. Streak one plate; incubate aerobically at 22.5 ± 2.5 ^{o}C.

i. Note if any growth is observed on general growth medium plate. Proceed with identification when isolation is complete.

ii. Each organism should be identified to genus and species, if possible, using rapid identification kits or DNA sequencing.

I. Product-Induced Turbidity in Primary Test Media

When product-induced turbidity prevents the confirmation of visual observation of growth, the following instructions apply

1. Record "T" for any subsample which is turbid due to product-medium mixture.
2. On the daily observation page, indicate the meaning of "T" as: "T = product induced turbidity".
3. At the end of the initial 14 days of incubation, transfer portions of the medium (not less than 1 ml) to a fresh container of the same medium and then incubate the original and transferred containers for not less than 4 days. Note: Follow the current edition of the USP for any changes concerning subculturing and incubation of turbid samples.
4. Examine original product inoculated media and the subcultured media for growth daily when possible for not less than 4 days of incubation and record the results on a new daily observation continuation sheet.

8. Chapter 4: Investigating USP Sterility Testing Failure

A. Introduction

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, to ORS and the appropriate Center (e.g., Office of Compliance/OCTEC). 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 whether the test results are based on sound laboratory operation.

B. Investigations

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 monitoring records and any checklists or logs indicate that the ISO 5 environment was in good state of control (serious environmental quality or equipment repair issues) 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.

3. Analyst:

Evaluate the analyst's qualifications, including proficiency, personnel monitoring results, training record, and experience. Also note whether the sterility testing practice of the analyst was observed during this or a recent analysis.

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

Determine if disinfection/decontamination of the ISO 5 environment 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. 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.

It is advisable to summarize this review process in a standard report and maintain a sufficient record to reflect that these areas were investigated. In

addition to the four considerations listed above, overall cleanroom design is also an important consideration. There are differences in the construction, configuration, and material flow of laboratory cleanrooms. There may be differences in size, number of rooms, shape, air handling system, pass through autoclaves, gowning room accommodations (sink, HEPA filtration, adequate space, bench, etc.). Proper practices and conditions should be assured by mitigating contamination hazards potentially presented by layout and other design provisions. These include appropriate procedures for room and material disinfection, proper cleanroom uniforms (disposable or reusable), sample preparation area, etc. Cleanroom risks can be mitigated by the design of ISO 5 testing environment. Equipment that provides barrier protection can mitigate risks of the surrounding cleanroom environment.

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. For more information on how to judge investigational findings to make this evaluation, see Section XI.C1.and 2 of FDA's guidance on Sterile Drug Products Produced by Aseptic Processing for principles and expectations for investigating a sterility positive.

http://www.fda.gov/downloads/Drugs/.../Guidances/ucm070342.pdf

Also see the current version of USP <71>, which provides some guidance limited on investigations under Interpretation of Data.

C. Conclusion

This suggested list of areas and conditions to review should not be considered as comprehensive. Additional areas of review may need to be added based on some of the unique features or procedures employed by individual ORS laboratories.

9. Chapter 5: Bacterial Endotoxin Testing

Bacterial endotoxin is a lipopolysaccharide found in the cell membrane of Gram-negative bacteria which may cause adverse events in patients such as, but not limited to fevers, headaches, inflammation, nausea, chills, vomiting, hypotension, lung toxicity, Toxic Anterior Segment Syndrome, abortion or death. Therefore, all sterile drugs, medical devices and combination products must meet bacterial endotoxin specifications. Historically, *in vitro* Limulus Amoebocyte Lysate (LAL) assays have been used to detect and quantify bacterial endotoxin in pharmaceutical products.

NOTE: The Gel-Clot Method is considered the "referee method" per the USP. If there is a disagreement between the photometric turbidimetric or chromogenic methods, the gel-clot method results are reported.

This chapter of the Sterility Analytical Manual is intended to supplement the methodology procedures found in the USP <85> BACTERIAL ENDOTOXINS TEST and USP <161> MEDICAL DEVICES-BACTERIAL ENDOTOXIN AND PYROGEN TESTS. The USP <85> test is based on a reaction between bacterial endotoxin and the LAL reagent. Requirements for the LAL test includes optimal pH, ionic strength, temperature and incubation time. The USP <85> methods include the gel-clot LAL method and photometric technique methods such as the turbidimetric and chromogenic kinetic LAL methods. The USP <161> chapter describes the bacterial endotoxin extraction procedure for medical devices, if required. After the medical device extraction is performed, the USP <85> chapter is followed to detect and quantify any bacterial endotoxin present in the sample.

The Gel-Clot Method is a qualitative assay that detects Gram-negative bacterial endotoxin based upon a reaction between lysate and endotoxin which results in a firm clot formation. For samples with endotoxin, the endotoxin amount present in a test sample is calculated by diluting the sample to determine the assay endpoint where a clot does not form. If no clot forms in the verified dilution from the inhibition and enhancement testing, the sample does not contain detectable endotoxin.

Photometric techniques include the turbidimetric and chromogenic endpoint and kinetic assays. The assays involve a change in turbidity or color (depending on the assay reagents used) over time for kinetic assays or at the end of the incubation period for endpoint assays. For kinetic assays, the onset time for turbidity or color change is inversely proportional to the concentration of endotoxin in the solution, i.e. more rapid change at higher endotoxin concentrations. An instrument is used to read the changes. The analytical test results are calculated based on the linear relationship between the endotoxin concentration and the turbidity or color development. The standard curve plots the log onset time against the log endotoxin concentration. These assays are rapid and sensitive, allowing large numbers of samples to be assayed quickly. However, the gel-clot method is the reference method per USP and should be used if there are any doubts or disputes, unless otherwise indicated in the monograph for the product tested.

New microbiologists should review the references at the end of this chapter.

A. Gel-Clot Method

1. Reference Standard Endotoxin and Control Standard Endotoxins

The potency of the control standard endotoxin (CSE) with respect to the reference standard endotoxin (RSE) is determined by the CSE manufacturer. This information is found in the associated package insert and need not be repeated.

NOTE: Follow manufacturers' recommendations for the storage, reconstitution, and preparation of CSEs, lysates, and other Limulus Amoebocyte Lysate (LAL) reagents. In case of a dispute, final decision is based on results obtained with the USP Endotoxin RSE.

The field laboratories are encouraged to mix the reconstituted CSE for at least 5 minutes and for at least 1 minute between dilutions.

2. Negative Controls

Run appropriate negative controls with each sample tested. This assures that the equipment and solutions used in the test contain no extraneous detectable endotoxin. This assay requires two negative controls: negative water control and negative system accessory control. The negative water control (blank) consists of the pyrogen-free water (Water for BET or LAL Reagent Water) used in the assay and is tested undiluted. Run a system negative (accessory) control to test the detectable endotoxin level, if any, of accessories used in the assay (i.e. beaker full of pyrogen- free test tubes stored for an extended period, pipette tips, pipettes, sample tubes, syringes, spatulas, etc.). Rinse all accessories used in the assay with pyrogen-free water and test undiluted prior to completing all testing for a particular sample. If an alternative diluent is used, run an alternative diluent control prior to completing all testing for a particular sample. Record these results on the worksheets.

When using commercially purchased pyrogen-free water for product dilutions, it is recommended to transfer a working volume from the original stock container to an individual pyrogen-free test tube or flask in order to minimize back contamination. Run a negative control for the working volume for each sample run.

NOTE: Pyrogen free pipettes, micropipettor tips, test tubes, and other accessories are commercially available.

3. Test for Confirmation of Labeled LAL Reagent Sensitivity

Prior to use in the test, the labeled LAL reagent sensitivity must be confirmed. Prepare a control standard endotoxin dilution series having at least four concentrations equivalent to 2 λ, λ, 0.5 λ, and 0.25 λ. Inoculate four replicates from each control standard endotoxin tube with equal amounts of reconstituted lysate per manufacturer's recommendation. Multiple dilution series are not required. The geometric mean of the endpoints must be within the limits of

labeled claim. The acceptable variation is one half (0.5 λ) to two times (2 λ) the labeled sensitivity (λ).

4. Inhibition or Enhancement Test/Test for Interfering Factors

The suitability of the test results for bacterial endotoxin require an adequate demonstration that specimens of the article or of solutions to which the test is to be applied do NOT of themselves inhibit or enhance the reaction or otherwise interfere with the test.

USP states to perform this test "on aliquots of the specimen… in which there is no detectable endotoxin". However, this characteristic of the product cannot be ascertained prior to the analysis because the specimens are unknown samples. Because of this limitation, any positive result below the 0.5 lambda level may not be an enhancement trait of the product, but instead a positive reaction due to contamination in the sample. The evidence for this conclusion should be obvious with the results of the assay tubes containing product only.

A large percentage of small volume parenterals appear to be inhibitory to the LAL gel- clot method because of low pH, or some excipient / active component of the product. In order to expedite the neutralization of this interfering trait, determine the lowest product dilution overcoming the interference but still within the Maximum Valid Dilution (MVD). The detailed description of this protocol is delineated in LIB No. 2433 (July 25, 1980), "A condensed procedure for diluting product in determining compatibility with the Limulus Amebocyte Lysate test for endotoxin". In addition, the use of neutralizers such as sodium laurel sulfate or Pyrosperse™ has also been described (see references).

LAL manufacturers recommend the test sample to have a pH range of 6.0 to 8.0 for optimal assay performance. Since the lysate is buffered, sample dilutions in pyrogen-free water may be enough to test the sample with the LAL assay. Determine the pH of the sample with the added lysate and document the results. If pH adjustment is needed, use pyrogen-free acid, base or buffers.

NOTE: Contact LAL manufacturers for recommendation of commercially available neutralizing buffer to be used with their LAL kits.

5. Test Procedure

The storage and mixing of samples prior to analysis may affect recovery of endotoxin contamination. Sample (product) bottles should be vigorously shaken prior to analysis, preferably on a vortex (see reference for supporting evidence for this step). A minimum of 30 seconds to 1 min on the vortex is recommended for each product unit.

6. Endotoxin Calculation

Calculate endotoxin concentration per the USP Bacterial Endotoxins test chapters <85> and <161>. For additional information refer to USP <1085>.

NOTE: Adjust the final endotoxin value taking into account the volume of the rinse solution used in the extraction procedure.

7. Compositing Samples

The Bacterial Endotoxin test <85> does not directly address the issue of combining product units (compositing/pooling). The risk of unit composites is that one unit (vial, ampoule, etc.) may have bacterial endotoxin contamination at a higher level but the dilution of this one unit with endotoxin-free units of product may reduce the detectable level of endotoxin below the sensitivity of the lysate or dilute the level of endotoxin below the acceptable monograph level. Therefore, **when using a composite format for screening drug products for endotoxin it is important to adjust the MVD calculation to account for this reduced lysate sensitivity**. Secondly, **when compositing is performed for product screening, if a positive result is detected a repeat test is acceptable under the conditions stated by the Interpretation section of the USP chapter**.

It would be advisable when performing the repeat test from a composite mixture that, if remaining product is available and had been opened aseptically under controlled conditions, the repeat test be performed on the original individual units. It is strongly advised that the individual units be adequately shaken to assure that the endotoxin is re-suspended back into solution before taking the sample test aliquot. **If any of the original individual units fail the USP test at this point, the compendium does not allow any additional repeat testing unless the test can be proven not to be suitable as defined by the USP chapter**.

8. Relevant excerpts from Guidance for Industry Pyrogen and Endotoxin testing: Questions and Answers)

Question 1: Can FINISHED product units (vials, ampoules, pre-filled syringes, etc.) be "Pooled" into a composite and screened for bacterial endotoxin?

Response 1:

Yes. With some exceptions (see below), finished drug product units may be pooled into a composite sample and assayed for bacterial endotoxins. The composite sample may be represented by the entire unit or partial aliquots (equal volumes) of finished product containers from one manufactured lot of aqueous-based pharmaceuticals. Pooling would generally be accepted for small-volume parenterals (those with volumes of 100 mL or less) as long as the MVD is adjusted to a proportional, lower value because of the potential for diluting a unit containing harmful levels of endotoxins with other units

containing lower, less harmful, levels of endotoxins. This “adjusted MVD” is obtained by dividing the MVD computed for an individual sample by the total number of samples to be pooled. FDA suggests pooling no more than three units per composite in keeping with the concept of testing representative beginning, middle, and end finished product containers. If this reduction in MVD results in an inability to overcome product-related assay interference because of an insufficient dilution, then the samples should be tested individually.

Finished medical devices may also be pooled into a composite sample and assayed for bacterial endotoxins. Testing for medical devices should be conducted using rinsing/eluting and sampling techniques as described in ISO 10993-1 and ISO 10993-12, as also used for inhibition/enhancement. Sampling can be adjusted for special situations. After a suitable eluate/extract pool is obtained from a finished production lot, this pooled extract should be kept under conditions appropriate for stability until it is tested in duplicate.

FDA recommends that pooled samples be a composite of aseptically removed aliquots (after at least 30 seconds of vigorous mixing) from each of the product containers. In this way, the original, individual containers will be available for possible retesting in the event the pooled sample displays an OOS result.

Some product types should not be pooled. Two examples are drug products that have an initial low MVD (see discussion above of “adjusted MVD”) and products that are manufactured as a suspension, because sample aliquot homogeneity may present significant interference issues.

Question 2: Can INTERMEDIATE (IN-PROCESS) sample aliquots be "pooled" into a composite and screened for bacterial endotoxin?

Response 2:

FDA does not recommend pooling in-process samples from different in-process stages of the manufacturing process because it may be difficult to ensure the homogeneity of these materials.

Question 3: Retesting when test failure occurs:

Response 3:

When conflicting results occur within a test run, the analyst should consult USP Chapter <85>, Gel-Clot Limits Test, Interpretation, for guidance on repeat testing. As specified in Chapter <85>, if the test failure occurred at less than the maximum valid dilution (MVD), the test should be repeated using a greater dilution not exceeding the MVD. A record of this failure should be included in the laboratory results. If a test is performed at the MVD and an out-of-specification (OOS) test result occurs that cannot be attributed to testing error,

continue product dilution until the actual endotoxin concentration can be calculated. These results should be recorded on your worksheets.

B. Photometric Quantitative Techniques

Endpoint and kinetic assays are photometric quantitative assays used for the detection of bacterial endotoxins. Endpoint and kinetic assays may utilize turbidimetric or chromogenic formats. Endpoint assays measure endotoxin the increase in turbidity (turbidimetric) or color (chromogenic) at the end of the incubation time.

This section provides procedural information that can be applied to kinetic assays. The Kinetic Chromogenic and Turbidimetric reagents are commercially available. Assay may be purchased as a kit. A certificate of analysis should be maintained for the control standard endotoxin, Limulus Amebocyte Lysate (LAL), and pyrogen free water used in the assay. Other materials such as pyrogen free pipettes, micropipettor tips, test tubes, and 96-well microplates may be purchased from various vendors. Certificates of analysis indicating these materials are pyrogen-free should also be maintained by the laboratory.

1. Kinetic Assays: The kinetic bacterial endotoxin detection software is designed to run the following assays.
    a. Initial qualification of a lysate/Initial qualification of the testing analyst
    b. RSE/CSE assesses potency of control standard endotoxin (CSE) in terms of reference standard endotoxin (RSE)
    c. Test for Interfering Factors
    d. Sample Test
    e. Instrument Calibration Tests

The initial qualification assay verifies the proficiency of the analyst operating the Kinetic software and equipment. The initial qualification assay may also be used to qualify each new lot of kinetic lysate and control standard endotoxin. Requirements of USP <85> must be followed with respect to the number of endotoxin standards and the number of replicates required for a valid assay.

The RSE/CSE assay may be used to compare the potency of the CSE with the concentration of the RSE. Normally, the RSE/CSE assay does not need to be performed, unless there is reason to believe the values in the manufacturer's certificate of analysis (COA) are not correct.

The test for interfering factors must be run for each sample.

The routine assay is designed to test unknown samples for bacterial endotoxins. Samples collected for LAL analysis should be run using the routine assay, after taking the other three assays into consideration.

NOTE: Contact LAL manufacturer to qualify the kinetic readers (IQ/OQ/PQ) prior to use. Furthermore, laboratories should follow scheduled preventative maintenance (PM) and/or calibration to ensure the readers perform properly for regulatory sample testing.

2. Procedure

Perform the assay according to the instructions that are included with the LAL test kit or LAL lysate, in accordance with USP <85>. Additional instructions may be found in reference 8 listed below.

C. Medical Devices

This section applies to medical devices or assemblies or fluid pathways of medical devices or assemblies (i.e. solid medical devices such as disposable syringes, cartridges, transfusion and infusion assemblies, implants, intravenous catheters, dialysis tubing; liquid medical devices such as saline, heparin and dialysate; and gel medical devices such as demineralized bone matrices and hyaluronic acid devices) that are labeled sterile and nonpyrogenic that are in contact directly or indirectly with the cardiovascular system, lymphatic system or cerebrospinal fluid. Follow USP <161> for testing requirements

If only the device pathways are labeled as sterile and nonpyrogenic, the device pathways must come in contact with the extraction fluid for the entire course of the extraction per USP<161>.

Liquid medical devices do not require extractions.

The standard extracting, rinsing and soaking fluid for medical devices is pyrogen-free water. The analytical approach for testing medical devices is covered in USP. If the extraction fluid shows endotoxin interfering results, modification of testing may be necessary depending on the product configuration. Alternate diluents must be verified to not interfere with the endotoxin assay prior to use.

NOTE: The rinse or extract volume may be adjusted for the size and configuration of the device. The device or components may be cut into smaller pieces using sterile, nonpyrogenic equipment such as forceps, scissors, wire cutters, etc. prior to the extraction.

A collaborated method prepared by an ORS laboratory is available for extraction of endotoxin from devices and may be used if necessary. Analytical verification of the final version should be conducted by the responsible

laboratory. The ORS laboratory protocol is summarized below for convenience.

Perform medical device extractions and final endotoxin concentration calculations per USP <161> and USP <1085> Guidelines on the Endotoxins Test.

Alternatively, if deemed necessary, perform the ORS laboratory modified extraction and analysis of Endotoxin from Medical Devices:

a. Preparation of 1% SLS solution

Prepare a 1% stock solution by placing one (1) gram of sodium lauryl sulfate (SLS) into a depyrogenated glass flask and add 99 ml of pyrogen-free water. Allow the SLS to fully dissolve. This should be followed by filtration through a 10,000 MW depyrogenation membrane filter into a pyrogen-free glass or plastic container.

b. Equipment needed: Ultrasonic bath with a range of 150 to 480 watts.

c. Extraction procedure

i. Dilute 2mL of 1% SLS stock solution to 20mL (0.1%) using LAL reagent water in a 20 x 150 mm screw-cap tube.

ii. Dilute 1.5mL of the 0.1% SLS solution to 15 mL (0.01%) using LAL reagent water in a 20 x 150 mm screw-cap tube.

iii. Prepare the appropriate number of tubes (one tube for each device) and one as a negative / system control. Preheat in a water bath to 37°C ±1°C.

iv. Aseptically remove the device from its packaging and cut it diagonally into pieces less than 5mm in length. Small metal and plastic pieces such as needles and luer-locks should be tested whole. Pyrogen-free fluid pathways should be flushed with extract solution.

Note: Extract volume may need to be adjusted depending on medical device size.

v. Place all pieces into the 20 x 150 mm tube containing 15 mL of preheated (37°C ±1°C) 0.01% SLS rinse solution.

vi. Vortex the tubes for 30 – 60 seconds or until all pieces of the device are immersed in the rinse solution.

vii. Sonicate the test containers for 60 minutes (wattage range 150 – 480 watts) at 37°C ±1°C. Do not sonicate more tubes than can be vortexed within 15 minutes of completion of the sonication. Make sure the water in the sonicator covers the

rinse solution in the 20 x 150 mm tubes. Do not allow the water in the sonicator to exceed 38°C.

viii. Vortex the tubes for 2 minutes. Remove a portion of the eluate (5 – 10 mL) for LAL testing. If the eluates are not tested immediately for endotoxin, they should be refrigerated. All eluates must be tested within 24 hours of extraction. Prior to analysis vortex at least one minute.

2. Alternative Diluent Examples (Verify prior to use):

Diluent/Buffer	Interfering Factor
Pyrosperse™	Endotoxin Binding or Masking
B-G-Blocker®	Enhancement due to β-glucans
Tris Buffer 50mM solution	Strongly Acidic (pH < 6) or Basic (pH > 8)
Magnesium chloride ($MgCL_2$) 10mM solution	Chelating
Note: Always use LRW as the diluent for the CSE standard serial dilutions.	

3. Test the sample eluate from the medical device extraction, using the bacterial endotoxins test (BET) assay parameters, procedures, standards, and controls for the gel-clot, kinetic chromogenic or turbidimetric, or endpoint assays, as directed in USP<85> and USP<161>.

D. Endotoxin References

1. United States Pharmacopeia (USP) Chapter <85> Bacteria Endotoxins Test. Official, Current Version.

2. USP <161> Medical Devices – Bacterial Endotoxin and Pyrogen Tests, Current Version.

3. USP <1085> Guidelines on the Endotoxins Test, Current Version.

4. The U.S. Food and Drug Administration, Guidance for Industry, Pyrogen and Endotoxins Testing: Questions and Answers, June 2012

5. Guilfoyle, D.E., Yager, J.F. and S.L. Carito. 1989. The effect of refrigeration and mixing on detection of endotoxin in parenteral drugs using the Limulus Amebocyte Lysate (LAL) test. J. Parenteral Science & Technology. Vol 43, No 4, p. 183-187

6. Novitsky, T.J., Schmidt-Gengebach, J. and J.F. Remillard. 1986. Factors affecting recovery of endotoxin adsorbed to container surfaces. J. Parenteral Science & Technology. Vol 40, No 6, P. 284-286

7. Twohy, C.W., Duran, A.P. and J.T. Peeler. 1986. Extraction of bacterial endotoxin from medical devices. J. Parenteral Science & Technology. Vol 40, No 6, p. 287-291

8. Guilfoyle, D.E. and T.E. Munson. 1982. Procedures for improving detection of endotoxin in products found incompatible for direct analysis with Limulus amebocyte lysate, in Endotoxins and Their Detection with the Limulus Amebocyte Lysate Test., Watson, S.W., Levin, J., and Novitsky, T.J. (eds), Alan R. Liss Inc., NY, p. 79-90

9. Guilfoyle, D.E., Munson, T. and J.P. Schrade. 1981. Use of Pyrosphere™ for Reducing Product Interference in the Limulus Amebocyte Lysate Test, Laboratory Information Bulletin, U.S. Food and Drug Administration, Washington D.C., No. 2503 (115), p. 1-8

10. Tepedino, A., Guilfoyle, D.E. and Munson, T. 1980. A Condensed Procedure for Diluting Product in Determining Compatibility (Inhibition / Enhancement test) with the Limulus Amebocyte Lysate Test for Endotoxin, Laboratory Information Bulletin, U.S. Food and Drug Administration, Washington D.C., No. 2433, p. 1-17

10. Chapter 6: Particulate Matter

This chapter is intended to supplement the methodology procedures found in the USP <788> PARTICULATE MATTER IN INJECTIONS and USP <789> PARTICULATE MATTER IN OPHTHALMIC SOLUTIONS. In addition, USP <1788> DETERMINATION OF PARTICULATE MATTER provides useful guidance for these assays. Where appropriate the laboratory wide procedure, ORA-LAB.019 “HIAC 9703+ Liquid Particle Analyzer” is referenced to address specific requirements for the use of the HIAC instrument.

Particulate matter consists of mobile, randomly-sourced extraneous substances, other than gas bubbles, that cannot be quantitated by chemical analysis due to the small amount of material that it represents and heterogeneous composition. Injectable solutions, including solutions constituted from sterile solids intended for parenteral use, are essentially free from particulate matter observable on visual inspection. The tests described herein are physical tests performed for the purpose of enumerating sub-visible extraneous particles within specific size ranges.

All large-volume injections for single-dose infusion and those small-volume injections for which the monographs or product specifications specify such requirements are subject to the particulate matter limits set forth for the test being applied, unless otherwise specified in the individual monograph or product specification.

Not all injection formulations can be examined for particles using the light obscuration method. Any product that is not a pure solution having clarity and viscosity approximating those of water may provide erroneous data when analyzed by the light obscuration counting method. Refer to specific monographs when a question of test applicability occurs. The microscope counting method may be used to analyze such materials. In some instances, the viscosity of a material to be tested may be sufficiently high so as to preclude its analysis by either method. In this event, a quantitative dilution with an appropriate diluent may be made to decrease viscosity, as necessary, to allow the analysis to be performed.

A. Light Obscuration Particle Count Test

The test applies to large-volume injections labeled as containing more than 100 mL and single-dose or multiple-dose small-volume injections labeled as containing 100 mL or less that are either in solution or in solution constituted from sterile solids, where a test for particulate matter is specified in the individual monograph or drug product specification. It counts suspended particles that are solid or liquid.

1. Test Apparatus

The apparatus is an electronic, liquid-borne particle counting system that uses a light- obscuration sensor with a suitable sample feeding device. Critical operational criteria consist of the following:

a. Sensor Concentration Limits

Use an instrument that has a concentration limit (the maximum number of particles per ml) identified by the manufacturer that is greater than the concentration of particles in the test specimen to be counted.

b. Sensor Dynamic Range

The dynamic range of the instrument used (range of sizes of particles that can be accurately sized and counted) must include the smallest particle size to be enumerated in the test articles.

2. Instrument Calibration

The instrument must be calibrated periodically according to the manufacturer's recommendation. The following are parameters that should be evaluated as

part of the periodic instrument calibration in addition to other tests recommended or routinely performed by the instrument manufacturer.

a. Sample Volume Accuracy

The accuracy of the sample volume must be assessed and found to be within the manufacturer's recommended range.

b. Sample Flow Rate

Verify that the flow rate is within the manufacturer's specifications for the sensor used.

c. Calibration and Resolution of the sensor

Verify that the sensor resolution is within the manufacturer's specifications for spherical particles of known size between 10µm and 25µm.

3. Test Environment
   a. Glassware, closures and other equipment must be suitably cleaned so that the level of particles added has a negligible effect on the outcome of the test.

      Note: Glassware can be purchased in particle free packages which are a suitable particulate free vehicle for compositing liquid samples.

   b. Perform the test in an environment that does not contribute any significant amount of particulate matter. Preparation of the test specimen, glassware, closers and other equipment should be performed in an environment protected by HEPA filters.

   c. The analyst should don non-shedding garments and powder-free gloves throughout the preparation of samples.

4. Instrument Start-up and Environment Blank
   a. Following instrument start-up perform an instrument flush using Milli-Q water or equivalent in accordance with ORA-LAB-.019.

   b. Prior to beginning a test procedure an environment blank must be prepared and tested according to ORA-LAB.019. After passing results have been obtained the instrument can now be used to perform the test procedure.

5. Test Procedure
   a. For containers having volumes of less than 25 mL, test a solution pool of 10 or more units to obtain a volume of no less than 25

mL. Single units of small-volume injections may be tested individually if the individual unit volume is 25 mL or greater.

b. Prepare the test specimens as described in USP <788> and <789>. Following the preparation of the test specimen the sample can be assayed in accordance with USP <788> and <789> in addition to the procedures found in ORA-LAB.019.

c. Containers with removable stoppers may be sampled directly by removing the closure. For test specimens that require the contents of the container to be removed for testing withdraw the contents of the container in the normal or customary manner of use, or as instructed in the package labeling. When test specimens are to be pooled remove the closure and empty the contents into a suitably cleaned container (preferably from particle free glassware vendor).

d. Following the completion of the test the instrument will generate a report. The report will include the raw data counts, calculated values and state if the test specimen met the USP limits for the particular test performed.

Note: If the average number of particles exceeds the USP limits the prepared specimen must be tested by the Microscopic Particle Count Test.

B. Microscopic Particle Count Test

The microscope particulate matter test may be applied to large-volume and small volume parenteral injections and to ophthalmic solution products.

The test Apparatus is described in USP <788> with additional information found in USP <1788>.

1. Test Environment and Environmental Blank:

Refer to Section A.3 for the requirements of the test environment and preparation of the specimen, glassware and equipment used in the assay.

Prior to initiating the test sequence with a specimen, a blank determination is required and must be carried out according to USP <788>. The environmental blank must meet the requirements set forth in USP <788> in order to initiate testing of the specimen.

2. Test Procedure and Interpretation of Data

a. For large volume parenterals, single units are tested. For small volume parenterals less than 25 ml in volume, the contents of 10 or more units are combined in a cleaned container; the test

solution may be prepared by mixing the contents of a suitable number of vials and diluting 25 ml with particle-free water or with an appropriate particle-free solvent when particle-free water is not suitable. Small volume parenterals having a volume of 25 ml or more may be tested individually.

b. Proceed with the test as delineated in USP <788> recording the number of particles that are equal to or greater than 10µm and the number of particles that are equal to or greater than 25µm. As an alternative a partial membrane filter count and determination of the total filter count by calculation is allowed. Once a particle count is determined the mean number of particles for the examined specimen is calculated. Note: For test specimens which are covered by USP <789> the number of particles that are equal to or greater than 50µm must also be counted and a calculated average reported.

c. The calculated data is evaluated according to USP <788> or USP <789> considering the solution type and the container volume.

11. Chapter 7: Antibiotic Potency Testing

A. General Information

USP <81> Antibiotics- Microbial Assays, is the primary reference for bioassay of human antibiotic potency testing. Prior to testing, the current USP <81> and respective drug monograph should be reviewed for testing requirements, acceptance criteria and applicability. USP <81> lists specific human antibiotics to be tested for microbial potency; this list can change at any time. All other human antibiotics not listed in USP <81> are typically tested using CFR 21 Parts 300-499 or USP HPLC methods. Lastly, animal antibiotics are typically tested using chemistry methods as published in JOAC, AOAC, Laboratory Information Bulletins (LIBs) and/or manufacturer's methodology.

Improved manufacturing technology (e.g. purification methods) has evolved potency testing from a simple biological assay to different chemical assays. Most chemical assays are based in the segregation and quantification of antibiotic components through the use of high-performance liquid chromatography (HPLC). However, chemical assays do not demonstrate biological activity and antimicrobial efficacy of a test antibiotic, particularly an antibiotic which may contain numerous active components, each exhibiting different antimicrobial activities. Chemical potency assay does not require the use of a live test microorganism. For the remainder of this chapter, antibiotic potency refers to USP <81> testing only.

Antibiotic potency testing is a biological assay whereby varying concentrations of antibiotic are tested against a live microorganism. The resulting biological response is measured and evaluated against a median reference standard [S3] and standard curve [S1], [S2], [S4] and [S5]. The biological response is referred to as antibiotic activity or potency. Antibiotic potency is dependent upon antibiotic-microorganism specificity and is physically expressed by the inability of a microorganism to grow under optimal conditions in the presence of a specific test antibiotic.

Antibiotic potency testing is a multi-variable test dependent on a variety of factors. Factors may include: 1) Test microorganism growth requirements and inoculum levels 2) Test antibiotic dose and 3) Technical competency in preparation and/or use of equipment, growth media, reagent, test organism and antibiotic standards. Potency testing requires a basic knowledge of laboratory safety, analytical chemistry, microbiology and aseptic techniques. Potency testing is a manual, multi-step and multi-day process performed with common laboratory equipment. At minimum, two employees are required for preparation and sample setup. Due to the multiple stages of preparation and testing, only qualified (i.e. initial and routine trainings and evaluations) laboratory personnel should be authorized to perform USP <81> testing.

Antibiotic potency testing is performed either by the plate (cylinder-plate or diffusion) or tube (turbidimetric) method. Both plate and tube methods demonstrate measurable levels of growth inhibition. For example, zones of inhibition (ZOIs) are observed and measured during cylinder-plate testing. And, turbidity is observed and measured during tube testing. Growth inhibition measurements are tabulated and integrated into a linear regression curve, resulting in extrapolated antibiotic potency values.

Potency is denoted in units (U) or µg of activity and may or may not be exact in equivalence to the µg (weight) of the active compound. The following three reasons may explain this weight-activity discrepancy: 1) activity may be caused by the antibiotic's free base or salt form and activity is denoted in either form 2) the antibiotic may contain similar chemical components but differ in activity or 3) the antibiotic activity is represented by a heterogeneous family of antibiotics and not a single analog.

An internal quality control is built into each plate and tube tested. Since antibiotics have different listed dosages per label, the test antibiotic/unknown sample is diluted to a known sample concentration ([U3]). The unknown sample [U3] has an equivalent concentration of the median reference standard ([S3]). The median reference standard ([S3]) is the median concentration (i.e. mid-point) of the five-point standard curve ([S1], [S2], [S4] and [S5]). The five-

point standard functions as the testable detection range and is prepared from a traceable and within expiry USP Reference Standard (RS).

The USP <81> states the following criteria for both the Plate and Tube Methods:

1. The calculated potency of the test antibiotic/unknown sample ([U_3]) must be 80% to 125% of the median reference standard ([S_3]);
2. Relative standard deviation for all measured (i.e. millimeters or absorbance) and calculated data (e.g. averages) is NMT 10%; and,
3. Testing is performed in triplicate over a period of three independent test runs

The USP <81> Plate Method further states:

1. The percentage coefficient of determinations (%R^2) for each standard curve will be NLT 95% (i.e. correlation coefficient of NLT 0.9750); and,
2. ZOIs for all media reference standard ([S_3] will measure between 14-16 mm).

The USP <81> Tube Method further states:

1. The percentage coefficient of determinations (%R^2) for each standard curve will be NLT 90% (i.e. correlation coefficient of NLT 0.950); and,
2. Absorbance values of the media reference standard ([S_3]) are predetermined per antibiotic. Refer USP <81> for testing parameters and acceptable data requirements.

B. Equipment

Antibiotic potency testing is a quantitative test dependent upon the preparation and use of growth media, reagents, test organism and antibiotic standards; therefore, Class A volumetric glassware is used in the preparation of the test antibiotic/unknown sample, reference standard and dilutions of the reference standard. Class A glassware is physically labeled either “to deliver” (TD) or “to contain” (TC). An understanding of Class A glassware prior to laboratory use is required. For example, a high viscous liquid (e.g. antibiotic ointment) cannot be delivered from a “to deliver” Class A pipette. “To deliver” Class A pipettes solely rely on gravity to assist in evacuation. In the case of the antibiotic ointment, the ointment would remain in the Class A pipette at time of evacuation. However, USP <81> states sterile and disposable glassware can also be used for any measurement of test antibiotic/unknown sample, reference standard and dilutions of the reference standard.

Standard laboratory glassware such as beakers, funnels, flasks, roux and 1-2 liter bottles are also required. Sterile and disposable equipment such as test

tubes, petri-plates and serological pipettes may be used so long as the use of this equipment does not affect the quantitative aspect of potency testing. Additional equipment includes a stainless steel penicylinders, penicylinder dropper, cuvettes, pipettor and micropipette, pH meter, hot plate, adjustable-temperature water bath, incubator and a manual/automatic plate reader or UV-VIS spectrophotometer.

Equipment in direct contact with the test microorganism should be clean (i.e. residue free) and sterile. Residues (e.g. antibiotic or detergent) may interfere with antibiotic potency testing. Methods of equipment cleaning and dry and heat sterilization should have appropriate validation and verification checks to ensure glassware is clean and sterile. See USP <81> for penicylinder cleaning instructions and USP <1051> Cleaning Glass Apparatus, for glassware cleaning instructions.

Equipment used to provide a unit of measurement should have the appropriate validation and frequent verification checks to ensure reliable and reproducible results; examples of such equipment include a weight scale, pH meter, autoclave, micrometer, manual/automatic plate reader and UV-VIS spectrophotometer. For more information on manual and automatic plate readers, see section titled Antibiotic Potency Testing: Plate Method.

Testing can be performed on a laboratory benchtop and does not require setup in a clean room or a laminar flow hood. However, it is important to exercise aseptic technique when working with general growth media and test organisms to prevent cross contamination.

When performing the plate method, it is important to use a laboratory bench top that has been checked with a level. The bench top will be used to prepare single and bi-layer agar plates. If the bench top is not level, liquid agar could potentially pool unevenly within a petri plate. Unevenly distributed agar causes uneven antibiotic diffusion. Zones of inhibition formed after diffusion should be uniform in shape (i.e. circular). Irregular shaped ZOIs do not have a diameter that can be measured accurately. Irregular shapes include ovoid, elliptical and/or any shape without a uniform and defined perimeter.

C. Test Organism, Inoculum Preparation and Standardization

Antibiotic potency is dependent upon antibiotic-microorganism specificity. USP <81> identifies specific microorganisms and correlating antibiotics for testing. Prior to use in test, the test microorganism must be characterized as pure and robust. Primary and working cultures must be aseptically prepared and dedicated to preventing contamination of the primary test microorganism. If the primary and/or working culture becomes contaminated, perform a visual inspection and basic microscopy for typical growth characteristics and morphology. Additionally, AOAC approved rapid identity testing methods such

as API or VITEK should be performed. Contaminated primary and/or working cultures cannot be used for antibiotic testing; likewise, all antibiotic potency data generated using contaminated cultures will be considered as invalid.

A test inoculum is prepared from a working culture. Inoculum preparation is a multi-step and multi-day process. Preparation of the inoculum requires a basic knowledge of microbiology, aseptic technique and laboratory safety. Refer to USP <81> Antibiotics- Microbial Assays, for test organism and inoculum preparation. The test inoculum is a solution containing the live test microorganism diluted with a method specific diluent. To be viable, the microorganisms used in the test inoculum must be within 5 passages of the primary test microorganism.

Verification of the test inoculum is performed prior to sample testing. Verification is a preliminary test which evaluates the potency, purity and robustness of the test inoculum when challenged against a known median reference standard ([S_3]) and standard curve ([S_1], [S_2], [S_4] and [S_5]). Refer to USP <81> for inoculum verification, testing parameters and acceptable data requirements and Section D, Antibiotic Standard and Sample Solution Preparation. As previously stated, acceptance testing requirements and acceptance criteria are as follows:

The USP <81> states the following criteria for both the Plate and Tube Methods:

1. The calculated potency of the test antibiotic/unknown samples ([U_3]) must be 80% to 125% of the median reference standard ([S_3]);
2. Relative standard deviation for all measured (i.e. millimeters or absorbance) and calculated data (e.g. averages) is NMT 10%; and,
3. Testing is performed in triplicate over a period of three independent test runs

The USP <81> Plate Method further states:

1. The percentage coefficient of determinations (%R^2) for each standard curve will be NLT 95% (i.e. correlation coefficient of NLT 0.9750); and,
2. ZOIs for all media reference standard ([S_3] will measure between 14-16 mm).

The USP <81> Tube Method further states:

1. The percentage coefficient of determinations (%R^2) for each standard curve will be NLT 90% (i.e. correlation coefficient of NLT 0.950); and,

2. Absorbance values of the media reference standard ([S_3]) are predetermined per antibiotic. Refer USP <81> for testing parameters and acceptable data requirements.

Primary and working cultures should be identified with the microorganism specie name, ATCC (American Type Culture Collection) number and preparation and expiration date. Recommended storage requirements for primary and working inoculum stock are specified in USP <81>. To reduce assay variability, a primary stock culture of ≤ 14 days should be used to prepare a working stock culture; also, a working stock culture of ≤ 7 days should be used to prepare a test inoculum. The test microorganism bioactivity is known to decrease over time and adjustments to the test inoculum volume may be required to meet testing acceptance criteria (e.g. ZOI diameters between 14-16 mm as exhibited by the median reference standard ([S_3]). Therefore, to reduce assay variability, it is recommended to use fresh (e.g. ≤ 2 day old) primary and working cultures when possible.

D. Antibiotic Reference Standards ([S_1] – [S_5]) and Unknown Sample ([U_3]) Preparation

Antibiotic potency testing requires the use of a standard curve to test an unknown sample. The reference standard (RS) must be from a verified source such as U.S. Pharmacopeia (USP). Procedures for reference standard preparation can be found in USP <81>. A single RS will be used to prepare a five-point ([S1] – [S5]) standard curve. Typically, the five standard solutions increase in concentration by a ratio of 1:1.25. For example, [S1] – [S5] standards can be represented in test as the following standard concentrations: 6.40 µg/mL, 8.00 µg/mL, 10.0 µg/mL, 12.5 µg/mL and 15.6 µg/mL. See Diagram 1 for further details. Labeling accompanying the RS will contain preparation, storage and expiration information; this information contains specific handling instructions and should be followed in order to achieve reproducible and reliable potency test data. A new standard curve must be prepared each day of inoculum verification (i.e. test plates) and/or each of the three independent test runs.

Antibiotic potency samples will vary in physical form, dose and administration; examples of differing sample types include tablets, powders, solutions or semi-solids. Regardless of these physical and chemical attributes, the test sample and reference standard must be diluted prior to testing. Each dilution for the test sample and reference standard must be considered. The dilution factor (for each dilution) and the total dilution (for multiple dilutions) are data required to calculate potency.

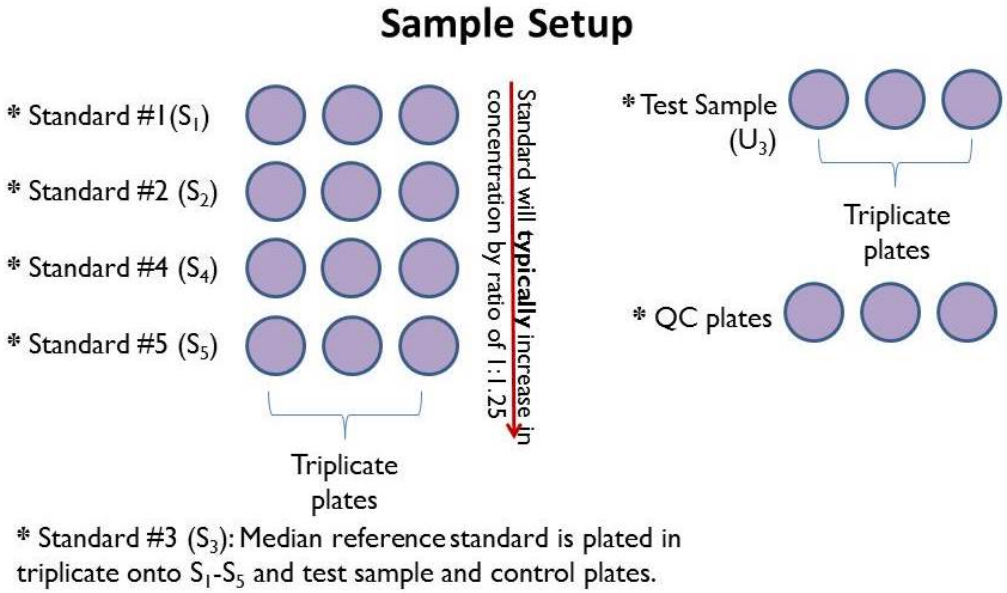

Diagram 1. Example of the Plate Method and placement of the standard curve and unknown sample onto triplicate agar plates. 6/19/17 MBB.

Prior to testing, the unknown sample [U3] must be diluted to a known concentration. See USP <81> for the list of recommended concentrations of median reference standards used in test. The target concentration is typically equivalent to the median reference standard [S3] of the standard curve. Diluting the unknown sample [U3] in this manner ensures a detection limit within the linear portion of the standard curve. For example, if the median reference standard [S3] has a concentration of 10.0 ug/mL, the unknown sample [U3] will also be diluted to a concentration of 10.0 ug/mL. See Diagram 1 for an example of one independent test run for the Plate Method and placement of the standard curve and unknown sample onto triplicate agar plates.

Quality control (QC) plates may include a test of respective diluents used for the reference, standard curve ([S1]-[S5]) and/or test/unknown sample [U3]. One of three QC plates should be dedicated for the evaluation of the inoculum used in test; specifically, this plate will only contain the test inoculum, will be free of penicylinders and should exhibit uniform growth (i.e. lawn) on top and within the agar. Microbial growth other than the lawn indicates the inoculum used in test may be contaminated and/or the technique used in preparing the single and agar plates may have been compromised the agar plates. The remaining two of three QC plates should be dedicated to each specific diluent used in test; for example, Water for Injection (WFI) and Buffer No. 4 are used in Vancomycin testing and therefore two QC plates, each containing WFI or

Buffer No. 4 are prepared. Diluents are specific to each antibiotic and are listed in USP <81>.

E. Growth Media and Additional Test Solutions

In order to culture a pure and robust test organism, proper use and preparation of growth media (agar or broth), buffers and diluents is required. Components or final preparations of growth media, reagents or diluents may be prepared on-site and/or purchased from outside sources. Regardless of origin, any final product made from growth media components and/or preparations must be verified for identification, expiry, sterility and growth promotion, prior to use in test. Refer to USP <81> for preparation, use and storage of all growth media, buffers and diluents.

F. Antibiotic Potency Testing: Plate Method

The Plate Method is the most commonly used USP <81> test. The Plate Method uses a solid medium (agar) to demonstrate antibiotic activity (i.e. zones of inhibitions (ZOIs)). This method requires the use of a five-point standard curve [S1] – [S5], median reference standard [S3], test antibiotic/ unknown sample [U3], verified inoculum, stainless steel penicylinders and petri-plates containing growth agar. See Diagram 1 for an example of one independent test run for the Plate Method and placement of the standard curve and unknown sample onto triplicate agar plates.

Depending upon the antibiotic, either a single layer or bi-layer agar plates are used in test. See USP <81> for preparation for single and bi-layer agar plates.

1. Single Layer Plates: Prior to solidification, the growth agar is inoculated with a known volume of the verified test microorganism. The inoculated agar is thoroughly mixed, poured into a petri-plate and allowed to solidify.
2. Bi-layer Plates: Prior to solidification, a portion of the growth agar is poured into the base of a petri-plate and allowed to solidify; this is the base layer of the test plate. The remaining portion of growth agar is further cooled and inoculated with a known volume of verified test organism. This inoculated agar is thoroughly mixed, poured onto the cooled base layer and allowed to solidify.

Once the agar solidifies (either single layer or bi-layer agar plate), stainless steel penicylinders are applied to the agar surface using a penicylinder dispenser. Penicylinders are applied in an equidistant and upright fashion. Each penicylinder should be immediately dosed with two concentrations of antibiotic, the diluted unknown sample [U3] or the median reference standard (S3). Further, both antibiotics are dosed in equal volume into alternating penicylinders. See Diagram 2 for further details.

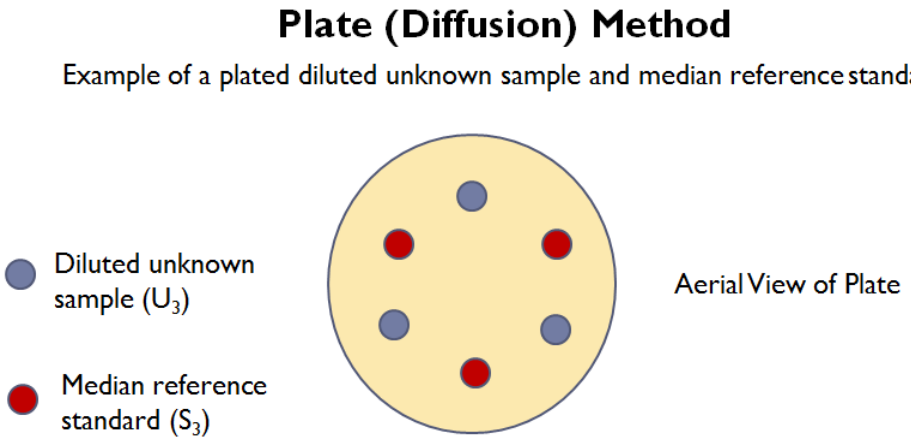

Diagram 2. Example of plated and dosed penicylinders containing the diluted unknown sample and median reference standard. 6/19/17 MBB.

After dosing, all plates prepared in one independent test run are incubated at the same time. As the plates incubate, the antibiotics diffuse through the agar, creating a zone of clearing below and around penicylinder perimeter; this clearing is referred to as the zone of inhibition (ZOI). The ZOI demonstrates the antibiotic activity of the diluted unknown sample [U3] and the median reference standard [S3]. See Diagram 3 for further details.

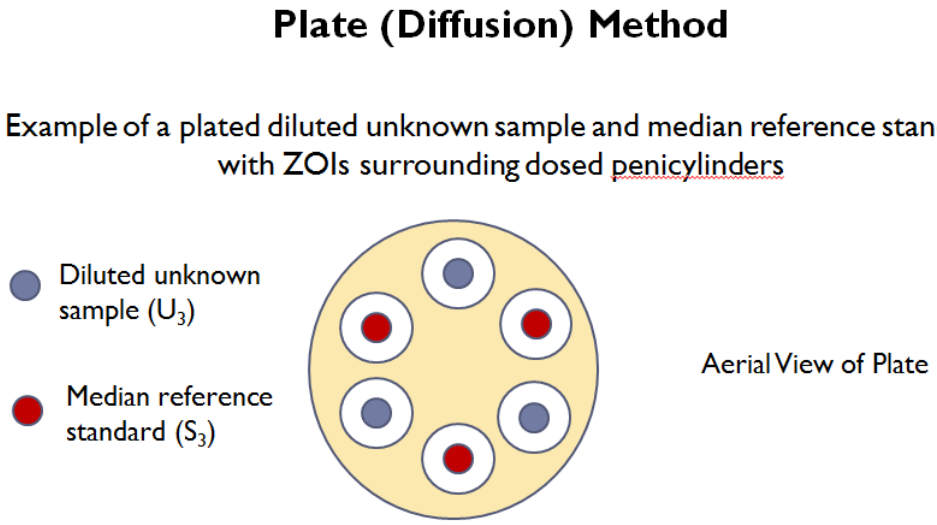

Diagram 3. Example of plated and dosed penicylinders on incubated plates showing zones of inhibition. 6/19/17 MBB.

After the specified incubation time, the penicylinders are removed, decontaminated (i.e. autoclaved) and washed with soap and water. The penicylinders are then heat sterilized prior to re-use in test. The diameter of each ZOI is measured with a manual/automatic plate reader. ZOIs should only be measured by qualified laboratory personnel. ZOI measurements are taken with the use of a manual or automatic plate reader. Examples of a manual reader include a Fisher-Lily zone reader or manual/electronic calipers; manual reads require data entry (e.g. handwritten and/or electronic) onto hardcopy and/or into electronic logbooks and/or spreadsheets. Examples of automated plate readers include OMNICON or Trinity V3. Unlike manual reads, automated plate readers allow a computerized system to measure the plates and do not require data entry of raw data.

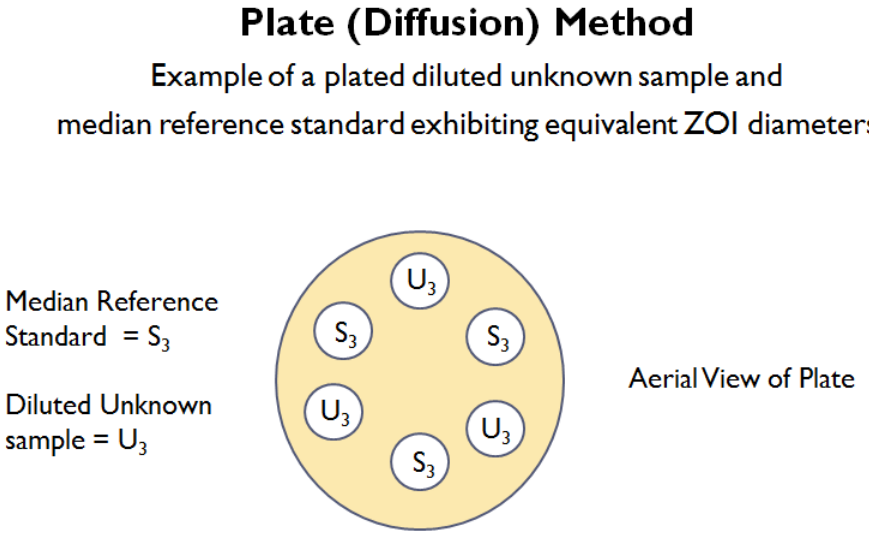

Diagram 4. Example of a plated diluted unknown sample and median reference standard exhibiting equivalent ZOI diameters. 6/19/17 MBB.

The zones of inhibition exhibited by the unknown sample [U3] and the median reference standard [S3] should be approximately equivalent. See Diagram 4 for further details. The ZOI comparison between the diluted unknown [U3] and the median reference standard [S3] can be described mathematically as the percentage of reference concentration. The calculated potency of the unknown sample [U3] must have a percentage of reference concentration of 80% - 125%. If the concentration of the diluted unknown sample [U3] falls outside this range of 80%-125%, the same unknown sample must be retested using an estimated dilution to obtain an equivalent concentration of the median reference standard [S3]. Diluting the unknown sample [U3] in this manner

ensures a final detection limit within the linear portion of the standard curve. Test sample preparation, storage and expiration require the dose and administration information (as per label claim) and procedures prescribed in USP <81> and the appropriate USP antibiotic monograph.

USP <81> states the following criteria for both the Plate and Tube Methods: 1) The calculated potency of the test antibiotic [U3] must be 80% to 125% of the median reference standard [S3] 2) Relative standard deviation for all measured and calculated data is NMT 10% and 3) Testing is performed in triplicate over a period of three independent test runs. The Plate Method further states: 1) The percentage coefficient of determinations (%R2) for each standard curve will be NLT 95% (i.e. correlation coefficient of NLT 0.9750) and 2) ZOIs for all media reference standard (S3 will measure between 14-16 mm). Refer to USP <81> for testing parameters and acceptable data requirements.

Diagram 5 below summarizes the operations prior, during and after testing using the Plate (Diffusion) Method (Hewitt and Vincent 1989).

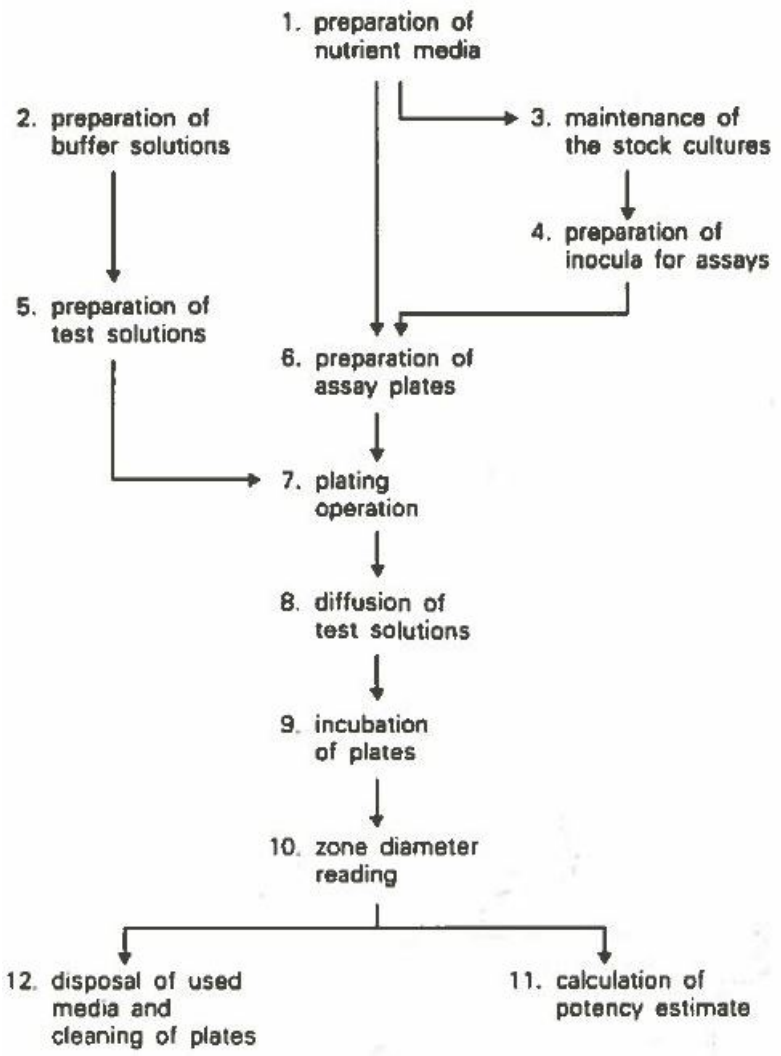

Diagram 5. Example of operations conducted prior, during and after testing using the Pate (Diffusion) Method (Hewitt and Vencent 1989).

G. Antibiotic Potency Testing: Tube Method

Of the two USP <81> methods, the Tube Method is less commonly used. The tube method uses a liquid medium (growth broth) to demonstrate antibiotic activity. This method requires the use of a test antibiotic/unknown [U3], five-point (minimum) standard curve ([S1]-[S5]) and verified inoculum. Growth inhibition is measured by qualified laboratory personnel with the use of a UV-VIS spectrophotometer. Test antibiotic will be analyzed in triplicate over a period of three independent test runs. Refer to USP <81> for Tube Method procedures.

Test antibiotic/unknown [U3] tubes will contain the growth broth, inoculum and the test antibiotic/unknown [U3] in triplicate. The standard curve ([S1]-[S5]) tubes will contain the growth broth, inoculum and each of the respective standard curve concentrations [S1], [S2], [S3], [S4], and [S5] in triplicate. The QC tubes will contain additional median reference standard [S3], will be used to perform preliminary absorbance checks and serve as a quality check for diluents and inoculum used in test. See Diagram 6 for an example of one independent test run for the Tube Method and placement of the standard curve and unknown sample into triplicate tubes.

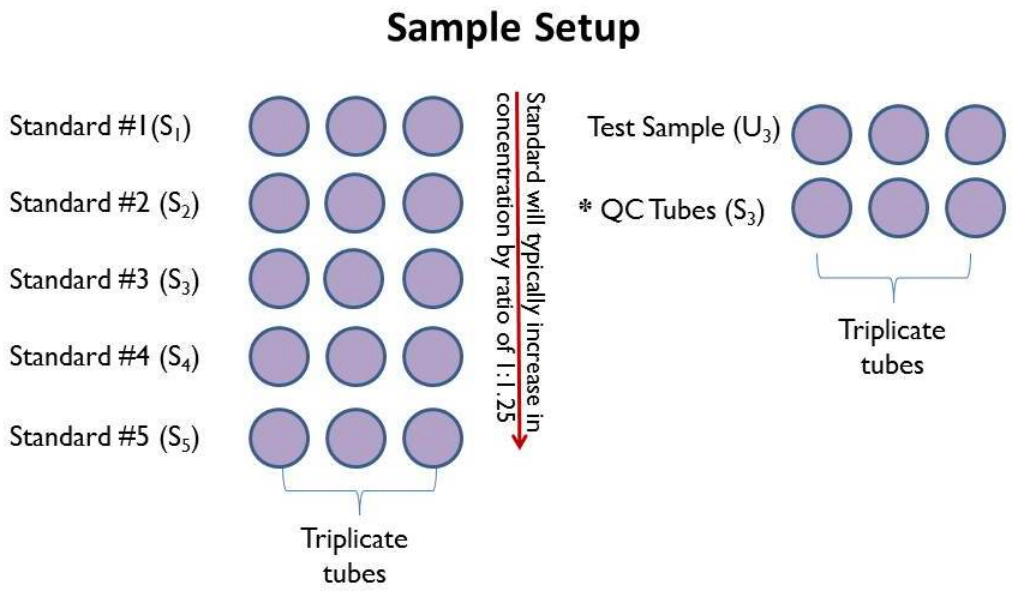

Diagram 6. An example of one independent test run for the Tube Method and placement of the standard curve and unknown sample into triplicate tubes. 6/22/17 MBB.

When a microorganism is placed into a broth containing the appropriate nutrients to support growth, the microorganism flourishes and the broth becomes turbid. Turbidity is typically a simple visual indicator of microbial growth; this growth can be quantified by a UV-VIS spectrophotometer by measuring values absorbance or transmittance exhibited by the broth.

Test tubes containing growth broth, inoculum, test/unknown sample and standard curve ([S1] – [S5]), will be prepared and analyzed the same day. The test tubes are then placed into a circulating water bath for NMT 5 hours to reach a specified turbidity (i.e. absorbance). It is recommended, one or more QC tubes be used to perform an absorbance check at timed interval to assure the required absorbance is not exceeded; this activity may require the preparation of more than three QC tubes. After the specified absorbance is

achieved, formaldehyde or a heat treatment is immediately added/applied to each test tube to inhibit additional microbial growth prior to absorbance reading and use of the UV-VIS. The absorbance or transmittance is read ad 580 nm or 530 nm.

USP <81> states the following criteria for both the Plate and Tube Methods: 1) The calculated potency of the test antibiotic [U3] must be 80% to 125% of the median reference standard [S3] 2) Relative standard deviation for all measured and calculated data is NMT 10% and 3) Testing is performed in triplicate over a period of three independent test runs. The Tube Method further states: The percentage coefficient of determinations (%R2) for each standard curve will be NLT 90% (i.e. correlation coefficient of NLT 0.950) and 2) Absorbance values of the media reference standard [S3] are predetermined per antibiotic. Refer USP <81> for testing parameters and acceptable data requirements.

Diagram 7 below summarizes the operations prior, during and after testing using the Tube Method (Hewitt and Vincent 1989).

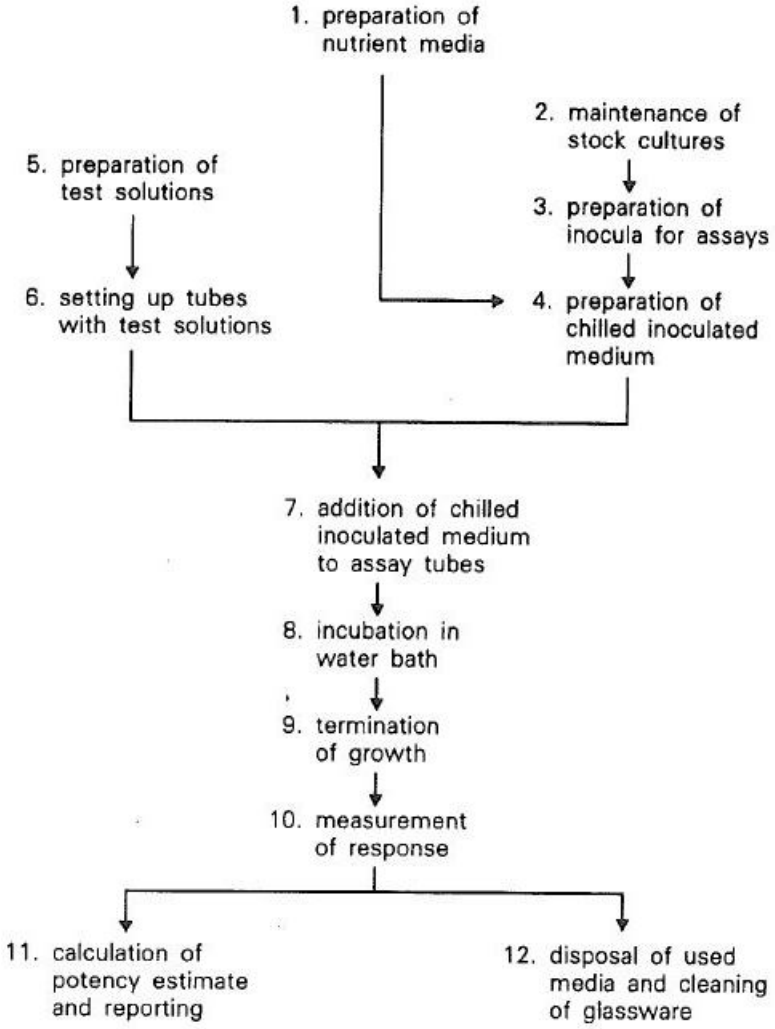

Diagram 7. Example of operations conducted prior, during and after testing using the Tube (Turbidimetric) Method (Hewitt and Vencent 1989).

H. Calculations

Potency calculations are listed in USP <81> and are specific to either the Plate or Tube Method. Calculations may be performed by traditional hand math or by electronic spreadsheet such as Microsoft Excel. In order to achieve reliable and reproducible results, electronic spreadsheets should be validated. Likewise, automated readers performing calculations should also be validated prior to use in testing.

I. Inspectional Objectives

When conducting an inspection of a human antibiotic manufacturer , contract manufacturer or test laboratory, inspectional objectives should include

coverage of high risk product/s (unless stated otherwise through special assignment or investigation) and a comparison of both USP <81> and USP monograph requirements and acceptance criteria to three types of in-house practice and procedures related to: 1) General laboratory and analytical operations 2) Initial training and requalification and 3) Data generation, review and archival. Employee practices should be documented in established written procedures. And, the original test data should match the final and/or released test results. All laboratory practices, procedures and test data should be based in sound science and meet the requirements and acceptance criteria of both USP <81> and related USP monograph.

Specifically, a CSO and/or Analyst should conduct observations and make a procedural comparison of media, reference standard, standard curve, culture control, unknown sample and equipment preparation and use; also, a review or practice and procedure of equipment calibration, maintenance (preventative and major) and use should be conducted. Practice and procedure comparisons should be made for laboratory personnel involved with data entry, transcription and/or review should be contemporaneously observed, followed by an immediate review of related documents for thoroughness and accuracy; this includes data transcription from handwritten and/or electronic data into logbooks, batch records and/or certificates of analyses. Additionally, it is necessary to understand which document serves as the original record; request for the most responsible person employed at the firm to identify the original record/s containing the raw data.

If the firm utilizes contract manufacturing services, is a contract manufacturer and/or a contract test laboratory, an active Quality Agreement, as established by the firm and customer, may provide insight to the roles and responsibilities of each party for testing and data generation, review and archival.

J. References:

1. Hewitt, W. & Vincent, S. (1989) Theory and Application of Microbiological Assay, Academic Press, Inc.

12. Chapter 8: Bioburden Estimation for Medical Devices

According to FDA Compliance program 7382.845, *Inspections of Medical Device Manufacturers, Part IV*, - "Bioburden testing is to be performed in accordance with the guidance provided in *ISO 11737-1, Sterilization of medical devices – Microbiological methods – Part I: Estimation of population of microorganisms on products.* The methodology used for estimating the bioburden is to be validated. Twenty units are to be tested." [1]

The term "bioburden" is commonly used to describe the population of microorganisms present on unsterilized material or products. The bioburden

quantity and types of bioburden organisms present can impact the sterilization process of the material or product. It is important to develop procedures which provide accurate, precise, and reproducible measurement of the bioburden population associated with the material or product. There are several approaches to remove microorganisms from a medical device. Some examples of these recovery methods include: filtration followed by plating; ultrasonic/shaking followed by filtration then placing on an agar medium; stomaching/rinsing/flushing followed by filtration and plating on an agar medium; if all else fails perform a direct swabbing or contact plate.

The bioburden estimation of a medical device generally consists of four distinct stages:

1. Collection of microorganisms from the medical device. (See Annex A and B)
2. Enumeration of the collection sample containing recovered microorganisms.
3. Bioburden characterization.
4. Application of the correction factor(s) determined during bioburden recovery studies in order to calculate the bioburden estimate from the raw presterilization count. The correction factor is derived from the determination of the recovery efficiency of a method. The calculation of a correction factor is illustrated in Appendix C, Section C.2.

It is not possible to define a single microbial collection technique because of the wide variety of materials used in health care products. Furthermore, the selection of conditions for enumeration will be influenced by the types of microbial contamination which may be anticipated.

The current method *"ANSI/AAMI/ISO 11737-1:2018/(R) 2011 sterilization of health care products – Microbiological methods – Part 1: Determination of the population of microorganisms on product"* has the latest revisions and provides a great deal of information that will guide an analyst to the method needed for a particular type of product.

Annex A contains a decision tree "that addresses designing a bioburden method based on the nature of the product being tested and includes guidance for choosing such things as agitation techniques or filtration versus direct plating."[2] Annex A also addresses the procedures (repetitive recovery method, product inoculation method) available for the validation of the method for determining bioburden.

Annex B has a comprehensive list of the different removal techniques that can be employed and alternatives for samples where removal of microorganisms by elution is not used.

Annex C has a more in-depth explanation of the validation of the repetitive recovery and product inoculation methods.

A. References:

1. FDA Compliance Program 7382.845 Inspections of Medical Device Manufacturers, February 2, 2011.
2. ANSI/AAMI/ISO 11737-1:2018/(R) 2011, Sterilization of health care products – Microbiological methods – Part 1: Determination of the population of microorganisms on product.
3. PDA Technical Report No. 21, Bioburden Recovery Validation. 1990

13. Chapter 9: Environmental Monitoring

ORS microbiologists may be required to assist CSOs during on-site inspections of pharmaceutical facilities to perform environmental monitoring (EM) sampling of those facilities to assess the microbiological bioburden of critical surfaces. This chapter describes a suggested procedure for conducting this activity. This should only serve as a guide with some modifications depending on the specific facility or special instructions from ORS Headquarters. Be sure to confirm with ORS Headquarters which ORS laboratory was designated to receive the EM samples. These procedures allow for a qualitative and quantitative assessment of environmental monitoring samples for the microbial presence in critical processing or laboratory area(s) being monitored.

A. Materials/Equipment

1. Sampling Materials (Disinfect all materials being brought into the ante-room, cleanroom, or area designated for compounding/processing by the firm.)
   a. Sterile polyester or cotton swab with a sterile transport media solution.
   b. Sterile sponge with detachable handle
   c. *Hycheck or equivalent surface samplers
   d. *RODAC/contact plates (Replicate Organism Detection And Counting)

   **Use media containing lecithin and tween neutralizers*

e. Sterile Whirl-pak® bags.
f. Sterile water for irrigation or sterile saline held in screw cap containers.
g. Dey/Engley (D/E) neutralizing broth
h. Letheen Broth
i. Sterile Disinfectant in a spray bottle
j. Sterile 70% alcohol spray bottle or wipes
k. Black marker (permanent, fine point).
l. Digital Camera

2. Testing Equipment and Materials
   a. Biological Safety Cabinet (BSC) with HEPA filtration
   b. Laminar Flow Hood (LFH) with HEPA filtration
   c. 10% Bleach or appropriate disinfectant/sporicidal
   d. Sterile 70% ethanol (ETOH) or Isopropyl Alcohol (IPA)
   e. Sterile Sleeves
   f. Sterile Gloves
   g. Hair Net
   h. Lab Coat/Sterile Gown
   i. Beard Cover and/or Mask
   j. Incubator set at 32.5ºC ± 2.5ºC. Incubator set at 22.5ºC± 2.5ºC.
   k. Modified Letheen Broth (MLB)
   l. Modified Letheen Agar (MLA)
   m. Sabouraud Dextrose Broth
   n. Sabouraud Dextrose Agar
   o. Malt Extract Agar w/chlorotetracycline
   p. TSA w/5% Sheep Blood Agar
   q. MacConkey Agar
   r. RODAC plates
   s. Hycheck slides
   t. Soybean Casein Digest Agar (TSA)

u. Soybean Casein Digest Broth (with neutralizers)

v. Neutralizers (i.e. lecithin, tween, etc...)

B. Sampling Preparation

1. Don appropriate personal protective equipment (PPE) as follows:

Note: *All ORS full gowning and procedures should be performed prior to conducting the collection. The firm's procedures/guidelines should be adhered to when entering classified area; however, it is the expectation that the microbiologists entering the classified area, in order to perform sampling, will don sterile garments. If there is a dispute regarding the PPE to be donned for aseptic sampling, ORS Headquarters should be contacted prior to commencing gowning.*

a. Nonsterile Hair net and beard cover (if needed)

b. Sterile mask

c. Shoe covers (non-sterile)

d. Sterile Gloves

e. Sterile Disposable Coverall

f. Sterile Hood

g. Sterile Goggles

h. Sterile Boot Covers

2. Sampling Equipment Controls

a. Aseptic Technique Control: Place one (1) sterile Dacron or cotton swab into sterile water and place back into its sterile transport media solution. Place this negative control into a sterile Whirl-pak® bag.

Note: *This control may not be needed if the sponges and/or swabs are received pre-moistened. There is no surface contact for this control.*

b. Swab/Sponge Sterility Control: Place an intact unused swab (or sponge) unit into a sterile Whirl-pak® bag.

c. RODAC/Hycheck Sterility Control: Place an unused RODAC plate and/or Hycheck plate within a sterile Whirl-pak® bag.

d. Whirl-pak® bag Sterility Control: Include one unopened Whirl-pak® bag as a closed control.

e. Glove Sterility Control: If the sampler uses ORS sterile gloves then have an intact unit containing gloves placed into a sterile plastic bag and sent as a control.

f. Sterile Equipment Control: Include any other sterile equipment used during EM sampling (i.e. sterile specimen cup, sterile media, etc.). (Coveralls, masks, and boot covers do not need to be included).

C. EM Sampling Procedure

It is recommended that the investigative team bring equipment for both qualitative and quantitative EM methods. Qualitative methods utilizing sponges/ swabs are used for hard to reach areas. RODACs or Hychecks are employed for the quantitative method to enumerate microbes on open flat work surfaces. Each analyst is to perform the same role throughout the collection. (Ex. One analyst collects swabs and second analyst serves as an assistant.) See suggested sampling locations listed in section E of this procedure.

1. Disinfect gloved hands with a suitable sanitizing agent (i.e. sporicidal agent or sterile 70% alcohol).
   a. Repeat this step between each EM sample.
   b. Allow gloves to air dry so no disinfectant is dripping from gloves.
   c. Some swab/sponge sampling packages include a secondary set of sterile gloves. In these instances, the secondary glove can be aseptically used on top of the primary gloves to expedite the sampling process. The secondary gloves should not be removed, but additional gloves should be added and disinfected as needed.
   d. When sampling an ISO 5 location.
      i. Verify that the LFH/BSC certification is current.
      ii. Allow LFH/BSC to run approximately 10 minutes before initiating sampling
      iii. Wipe down all outer sampling containers with a suitable sanitizing agent before placement in the LFH/BSC
      iv. Do not open sampling materials outside the LFH/BSC
2. Qualitative Swabbing
   a. Open a sterile swab (or sponge). Dampen with wetting agent (sterile water, saline, or D/E neutralizing broth) and squeeze off excess by pressing against the inside of the container holding the wetting agent.

b. Apply swab (or sponge with handle) to surface (or equipment) being monitored with **firm** application pressure.

c. When sampling (monitoring) flat surfaces allow the swab (or sponge with handle) to firmly rub an area of approximately 24 to 30 cm^2.

d. Apply the swab (or sponge) within this contact area in both a horizontal and vertical direction for approximately 10 seconds.

e. Place the swab (or sponge) back into the carrier container (if it came with one) and place into the additional sterile Whirl-pak® bag. Be sure to break off the handle portion of the sponge applicator stick.

3. Quantitative RODAC/Hycheck Sampling

a. Carefully remove the lid of RODAC plate or loosen the cap on the Hycheck slide tube. Take care not to touch the agar surface.

Note: *Examine agar for contamination and or dehydration*

b. Gently but firmly touch the RODAC agar surface against the area being sampled, exert moderate, even, vertical pressure and then carefully replace lid. Avoid using rubbing motions of the plate at the sample site as this may break the agar.

c. When using Hycheck press down on the spike to bend the paddle at the hinge line gently lowering the slide and press the agar to the surface with firm and even pressure. Repeat this step using the 2nd agar surface on an area adjacent to the initial test site. Replace slide in container and close tightly.

4. Sampling of Irregular Surfaces

A classified area may have an exposed irregular surface (i.e. particle board, wood, broken laminate, etc.) that requires sampling. In these situations, a colorless transport media such as letheen broth, saline or sterile water should be used to wet the sponge or swab prior to sampling. The microbiologist should avoid the use of D/E neutralizing broth which is a dark purple color and may discolor the irregular surface.

At the microbiologist's discretion, it may be determined that a RODAC plate should be used to sample the irregular surface. In this situation, a RODAC plate comprised of colorless agar such as tryptic soy agar would be appropriate. A colored agar such as D/E agar should be avoided.

5. Place the EM sample into a sterile Whirl-pak® bag and identify the bag immediately after.

6. Assign a consecutive number to the sample (i.e.1, 2, 3, etc.), in addition include the date, location of sample site (be specific) and your initials. Record in your inspectional record book the swab number and the location of the swab site.
7. After the sampling, the sampled area should be disinfected with sterile 70% alcohol. A sterile lint free wipe may be used to help remove sampling residue and expedite drying of the area.
8. As soon as possible, place the double-bagged subs inside an insulated cooler, with pre-frozen gel packs to keep the samples cold, but not frozen, and transport/ship the sample to the servicing lab for analysis, within 24 hours of collection.
9. Place the sample into a suitable mailing container to prevent crushing or physical damage to the swabs. The container should have some insulation capacity to prevent extreme temperature (freezing or excessive heat).
10. Contact with the receiving laboratory in advance regarding pending samples. This will ensure they have appropriate personnel and materials for sample set up within 48 hours of collection.

D. Recommended Environmental Monitoring Sites

When on an inspection, do not allow the firm to disinfect the work area prior to sampling. The facility and the equipment should be sampled during an in-process state as determined by the firm. The presence of disinfectant on the swab may reduce the microbial bioburden or increase inhibition during broth incubation. When collecting EM samples start in locations that are under the greatest control (ISO 5- HEPA filtered LFH/BSC or Isolator) and move to lesser controlled areas (areas outside the work station but still within the room).

1. Swab the frequently utilized surfaces within the controlled work station such as:
   a. Hooks for intravenous bags in the LFH or isolator
   b. Center of work surface
   c. Fingertips & sleeves of Isolator gloves
   d. Peristaltic pumps
   e. Storage bins inside work station
   f. Shelving inside work station or any other stationary items
   g. Equipment control panels including on/off switches of LFH/BSC
   h. Flexible plastic curtains used to separate multiple workstations

2. Swab corner crevices inside the HEPA Filtered work station.
3. Swab the handle, squeeze-trigger and nozzle of any bottle kept in the clean room or work station used for spraying (i.e., 70% alcohol, disinfectant solutions, etc.).
4. Swab the underside of the chair in front of the work station. Specifically, on the front bottom rim where personnel would hold to pull up the chair.
5. Swab tables or benches within the controlled room where product container(s) or post sterilized product may be held outside of the HEPA filtered workstation.
6. Swab the air in-take grid on each of the HEPA filtered work stations. Usually located on top of the unit holding the course filters.
7. Swab the exhaust (return) grid for the room air handling system that is connected to the facility air supply where the product manufacturing or compounding occurs.
8. Swab the light switch and door knob or handles leading into and out of the clean room and the phone or intercom base.
9. Swab any cardboard boxes, handles of plastic containers, tools (crimpers) or scissors, key pads on weighing scales, calculators or computers kept in the cleanrooms, keyboard, mouse and touch screen monitors.
10. Swab the exterior cuffs of the used lab coats worn by personnel during manufacturing or compounding. They may be hanging in the entry (ante) gowning room.
11. Swab the bottom horizontal window sill within the clean room.
12. Swab any area under open or dislodged ceiling panels.
13. Sample areas of discoloration, stains or water and oil droplets.
14. Use your discretion to sample any other high-risk surface locations.
15. Photograph surfaces or equipment that display gross signs of contamination (i.e., particulate matter, fungi, discoloration, etc.). Try to include a distant picture of the targeted area along with a focused close-up. Be sure to sample this location, as well.

E. Analysis Preparation conducted by ORS laboratory

1. All processing of swabs must be aseptically performed within a HEPA Filtered II Biological Safety Cabinet (BSC) or HEPA Filtered

Laminar Flow Hood (LFH) with an air classification at the same rating or better than that from which the swab was collected.

2. All surfaces within the BSC or LFH must be thoroughly disinfected with a sporicidal disinfectant followed by filter sterile 70% ethanol or IPA prior to placing swabs under the hood and beginning analysis.
3. In order to assure that the BSC/LFH and media are free of microbial contamination, standard open and closed controls used for sterility testing should be performed concurrently with analysis.
4. Don as appropriate the following PPE:
    a. Hair net
    b. Disposable lab coat
    c. Sterile disposable sleeves
    d. Sterile gloves
    e. Mask/beard covers
    f. Lab Safety Glasses
5. Sterile gloves must be decontaminated between the processing of each individual swab. Sterile gloves and sleeves should be discarded and replaced as needed.
6. Sample Preparation
    a. Examine swab containers for closure integrity to ensure tampering, leakage, or potential cross contamination has not occurred.
    b. Carefully disinfect exterior of each swab container and place into the sanitized BSC/LFH and allow to air dry.
7. Media Selection
    a. Neutralizing additives (i.e. Tween/Polysorbate, Lecithin, etc.) are utilized to neutralize inhibitory disinfectant residues transferred to the swab during sampling that might inhibit microbial growth.
    b. For a broad-spectrum recovery of microorganisms, utilize a nutrient rich general-purpose media containing neutralizers (i.e. MLB, MLA, etc.).
    c. When targeting fungal populations only, it is necessary to use an appropriate fungal media such as Sabouraud Dextrose or Malt Extract media. It is beneficial to use an antibiotic (i.e. Chlortetracycline) which will help to selectively inhibit bacterial

growth and restrict the size and height of colonies of more rapidly growing molds.

d. TSA w/5% Sheep Blood Agar is beneficial for cultivating fastidious microorganisms.

e. MacConkey Agar is used for the isolation and differentiation of Gram negative and enteric organisms.

f. RODAC plates and Hycheck slides are used for the detection and quantification of microbiological contamination.

F. Analytical Procedure

1. Approximately 100 ml of sterile media, MLB or other suitable media, should be aseptically added to each plastic bag containing a square sponge swab. Mix or swirl thoroughly.
2. Approximately 10 ml of sterile media, MLB or other suitable media, should be aseptically added to a sterile container to which the swab and its transport media are added. Mix or swirl thoroughly.
3. All swabs are incubated at 25ºC- 30ºC for at least 14 days to allow for the resuscitation of potentially stressed microbes.
4. Hycheck slides and RODAC plates should promptly be incubated at 30-35ºC for 2 days and then 20 ºC -25 ºC for 5 days. Longer incubation times may be required when contaminants are suspected to be slow growing. Check plates daily for colony formation to minimize obscuring visualization of smaller colonies by over growth.
   a. Count and record the number of colony forming units for RODAC plates. A representative of each colony type should be picked and re-streaked for purity and subsequently identified.
   b. Count the number of colonies on both sides of the paddle for the Hycheck slide. Report the colony counts for each side of the paddle and a representative of each all colony type should be picked and re-streaked for purity and subsequently identified.
5. Check all swabs/sponges daily for turbidity and subculture for isolation as turbidity is observed. All subculturing must be performed under LFH or BSC. If container does not allow for turbidity observation to be made, then subculture all environmental samples between Day 5 and 7. All environmental samples will be subcultured following day 14 incubation regardless of previous subculturing.

6. Subculture all EM samples onto a combination of non-selective media (i.e. MLA, etc.) and selective/differential media (i.e. MacConkey agar, MEA, etc.). It is recommended to include TSA w/5% Sheep Blood Agar as one of the differential medias for subculturing. A minimum of two agars should be utilized, 1 must be a nonselective agar.
    a. Fungal media should be incubated at 20º to 25º for 5 to 7 days. In some cases, extended incubation times may be appropriate, but generally not beyond 14 days unless there is a specific scientific justification.
    b. All other culture media should be incubated at 30º to 35º for 2 to 3 days.
7. Re-incubate all cultured swabs until the full incubation (14 days) timeframe are met.
8. Incubate all negative controls, such as system controls, media controls, under the same conditions as the sample.
9. Process and incubate submitted collector's controls under the same conditions as the sample.
10. Perform microbial characterization and identification following USP <1113> Microbial characterization, Identification and Strain Typing, as guidance. Typically, rapid identification systems (i.e. VITEK) are employed after primary screening and characterization are performed. Other identification platforms such as DNA sequencing may be beneficial if acceptable identification is not obtained through biochemical testing.

14. Chapter 10: Inspectional Guidance

A. Microbiological Issues for Inspection of Pharmaceutical Laboratories

The following topics should be reviewed and evaluated during an inspection of a pharmaceutical microbiology laboratory.

1. Finished product testing using USP or Non-compendia method
2. Review the original results for the following: sterility, bacterial endotoxin, microbiological examination of nonsterile products: specified microorganisms and enumeration, antimicrobial-effectiveness test, bioburden determination, water quality control testing.
3. Method Suitability (sterility), preparatory test (bacterial endotoxin), validation of method used for bioburden and water analysis

4. Reagents and media- proper storage, expiration date, and growth promotion
5. Equipment and Instrumentation- (Steritest, manifold, automated/molecular identification system, Vitek, isolator and bio-decontamination system) review calibration, maintenance, validation (IQ, OQ, PQ)
6. Sterility testing area design, operational procedures, monitoring, aseptic technique, gowning procedures, proper sample container disinfection, surface/air monitoring, HEPA filter certification, etc.
7. Method description, modifications, and verification along with recording of sample results and appropriate review and evaluation by management
8. Qualifications, training and identification of the personnel conducting each step of the analysis
9. Qualification and training of management to critically review data and interpret its significance
10. Microbial specifications set for raw material, finished product, water bioburden, and EM for analytical areas. Note: During pre-approval inspections questions about the appropriateness of finished product, raw material and in-process specifications should be discussed with the Center reviewer of the application.
11. Integrity and accuracy of the laboratory information management system (LIMS) for microbiology data entry, review and approval
12. Selection, handling, and storage of Biological Indicators (BIs)
13. Private (contract) testing laboratory quality agreements, data review, and associated problems; Have there been any changes in contract labs and why?
14. Proper use and control of In-vitro diagnostic test kits, positive and negative controls, interpretation and reliability of results
15. Risk assessment of microbiological results for non-sterile products
16. A list of the entire laboratory's microbiological data deviations (Out of Specification (OOS)/ (Out of Limits (OOL) results) and Corrective Action Preventative Actions (CAPA) since last FDA on-site inspection
17. Stability Testing – sample storage conditions, missed sampling dates, etc.

B. Microbiological Issues for Inspection of Pharmaceutical Manufacturing Facilities

The following topics should be reviewed and evaluated by a microbiologist although some aspects will also be covered by the Consumer Safety Officer during the inspection.

1. Product sterilization or bioburden reduction stage and validation- aseptic/filtration, steam, ETO, radiation, and other chemical processes
2. Depyrogenation- dry heat ovens for glass containers, wash/rinse for stoppers, adequacy of validation using spiked endotoxin, recovery studies before depryrogenation, filtration and column applications
3. Environmental monitoring- Types of equipment, calibration, operation and maintenance; surface, air, personnel and water; critical work areas for aseptically filled products (ISO 5, isolators, etc.); surface contact, surface sanitizer neutralizing media (e.g., TSA w/ Lecithin & Polysorbate 80), observe sampling technique, sample must represent dynamic/operational conditions, and trending/CAPA.
4. Process simulation (media fills) studies - growth promotion testing to include when is it performed and which microorganisms are included, who is responsible for reading turbidity, volume adequacy in the product containers and accountability of product containers during and after incubation periods.
5. Disinfection and sanitization- agents used (sporicidal?), preparation problems (over dilution); applicator (i.e., mop, spray. aerosol), time of exposure, areas of contact, supervision; residues, UV lights, water systems, filling equipment, work surfaces, process columns, verification and validation
6. Room design and Equipment- accessibility for disinfection and cleaning; aseptic filling critical area; HEPA filter certification and maintenance, air flow patterns/smoke studies, change evaluation/re-certification (rearranging cleanroom, adding equipment, HVAC, etc.), test during dynamic/operational conditions with maximum number of personnel in place, personnel and equipment flow, room differential pressure and temperature; adequacy of primary and secondary barriers
7. Water purification and delivery system: vulnerability of distillation process, RO, deionizers, cartridge filters, etc.; UV lights, dead legs, biofilm; corrosion (heat exchangers); waterborne microorganisms

(nanobacteria) and endotoxin production; cold system problems, disinfection problems

8. Personnel- training procedures for aseptic technique, gowning procedures, cleaning and maintenance personnel training for ISO 5 room entry; glove and garment monitoring procedures
9. Product sampling: a representative sample is selected based on lot size as per USP <71> guidelines; quantity per container & units per batch, sample storage (time and temperature), sampling port sanitization or sterilization problems; be aware of skip lot testing on raw material.
10. Maintenance records- determine dates, and location of equipment failures or out-of- service equipment that may have an impact on microbial contamination of product; looks for signs of roof leaks and water stains on ceiling panels, the degree of dirt and dust accumulation on supply and exhaust vents. Ask about new construction, plumbing or air handling system and the reason for change.
11. Compressed air systems—sterile process air, microbial particulate filtration (0.2 µm, hydrophobic), condensate causing blockage and microbial growth, routine point-of-use sampling, maintenance, filter integrity test

C. Sample Data Review – When all results are negative

The following should be reviewed when all the firm's sterility, nonsterile product and/or environmental monitoring test results indicate no microbial growth for validity and/or accuracy:

1. Media- growth promotion; adequacy of the documented pH; low agar or broth volume in container, incubator temperature not set correctly; improper medium storage after QC (crystals from freezing, inadequate mixing prior to dispensing, agar plates dried out during incubation, etc.)
2. Method suitability testing- validation for sterility; preparatory testing (BET). Appropriateness of neutralizers, product dilution, filtration; presence of toxic chemical contaminants in the water used to prepare the buffers, Not following method (excess of product added to broth during test but not during suitability testing, etc.)
3. Improper tube or agar plate examination- check filter surface on submerged filter membrane (mold budding); surface film, light hazy growth in Thio broth, microbes settle to bottom of tube, pinpoint

colonies (microaerophilic); medium not inoculated. Review disinfection process to assess the potential of antimicrobial residue being introduced during sample preparation; Review gas used for Isolator sterilization with medium inside chamber to assess the potential penetration into liquid broth and/or test product packaging.

4. Bioburden - Review the products bioburden for the presence of fastidious microorganisms which may require inclusion of special additives in the medium, such as halophilic contaminants in bicarbonate or high salt products need medium supplements with essential salts for survival. Where appropriate, the laboratory extend incubation time to improve the detection and recovery of fastidious microorganisms as part of their environmental monitoring program or part of investigation.
5. Water test method- Evaluate the length of storage and storage temperature for collected samples prior to testing. Evaluate the type of collection bottles and their compatibility with the testing, for example endotoxin testing.
6. Adherence to methods - Review data for adequacy of incubation time (for example 14 days for USP <71>), temperature (USP required temperatures) or appropriate media usage.
7. Worksheets - Review possible falsification or incorrect entry onto worksheets or Laboratory LIM system.

   Compare LIMs database entries to the analyst's laboratory notebook; Phrase the question "When you get a positive test result…." not "If you get a positive test result..." Inspect the laboratory refrigerator or freezer for evidence of stored sample isolates. If they lyophilize sample isolates, ask to review the spread sheet data storage directly from the computer screen; hard copies could be obtained later. Review the Vitek or Micro Id isolate log book for all microorganisms identified and work backwards to the product lot number, filling rooms, equipment used, components or raw material used for that lot. This may allow you to find other lots associated with the contaminated lot.
8. Personnel- review training records, personnel qualifications and experience. Observe analysts during sample collection, preparation, etc., and look for errors which may inhibit microbial recovery.
9. Laboratory - Visit the microbiology laboratory and look in the refrigerators, incubators, look at discarded plates from that day's work or request speciation log book and determine if microbial

recovery has occurred, but not recorded on official worksheets or entered into LIMs. Determine where plates or other growth media are stored pending microbial identification. Review available plates or growth media to determine if the findings match those documented in the laboratory records.

D. Sample Data Review – When Microbial Growth Is Indicated

When you encounter inspectional evidence that the firm has manufactured a microbiologically contaminated product, the few suggestions listed below should help you evaluate and proceed with this information. It is recommended that this evidence be communicated with the lead Consumer Safety Officer on the inspection so that appropriate communications with the responsible compliance office and the Center can occur. In addition, the Consumer Safety Officer can assist with the inspection of the manufacturing operations.

1. Documentation- Review and obtain copies of all records for lots indicating contamination; determine if there are other lots manufactured either before or after the “bad” lot(s). Review all associated activity and equipment related to the contaminated lot. There may be common water sources, mixing tanks, piping, raw material, sterilizers, filters, etc. that may have been cross contaminated and transferred microbes to subsequent lots of products.
2. Validation - Review current established validation studies for product/component sterilization (disinfection for non-sterile products); has there been any equipment changed or modified; has there been a change of personnel or training; any new source material for equipment (vent filters, gaskets, filter manufacturer, etc.); any processing changes or room modifications, construction elsewhere in the facilities, etc.
3. Environmental Monitoring (EM) - Review of environmental monitoring (EM) procedures and results for manufacturing and laboratory area- would the product contaminant grow on the EM medium; was the product contamination found in the manufacturing area; growth promotion potential of contaminant in other medium (i.e., TSB and Thio)
4. Speciation- Record and copy the method of identification (i.e. API, Vitek, etc.). Determine if there were possible secondary contaminants that were not identified or recorded (check original plates, or isolates); verify accuracy of entry into the LIM system.

5. Source of potential contamination – Determine the potential source of contamination, which may include *Staphylococcus* (skin, insect, etc.); *Pseudomonas* (water, plants, etc.); yeast and mold (spores) (environmental)
6. Investigation Report – Review firm's investigation report of microbiological out of specification (OOS) test results; source of contamination; where there corrective action(s) taken; if any repeat testing performed; batch disposition; does it include related lots and ancillary systems? Was the product rejected or released? If released ask why? Evaluate justification.
7. Product Test Failure - When a firm has a final product or in-process test result that indicates a failure (USP test failure, OOS, etc.) ask– Were the results due to laboratory error or a true process contamination?
8. Focus Areas - During the investigation, there are two areas for the review to focus: the manufacturing site and the laboratory that determined the OOS result. The investigations may run concurrently between manufacturing and the laboratory. For ease of review the questions listed in #9 (below) deal with laboratory data and those in Section E concern the manufacturing review. The laboratory section covers those questions that should be asked for a critical review of the microbiological data accumulated for sterility failures, non-sterile medical product failures, etc. Section E covers the manufacturing area and is divided into aseptic manufacturing (high risk) and terminally sterilized products (low risk).
9. Laboratory Facility and Analytical Review
    a. Review QC records for proper/validated sterilization of all equipment and media used during the sterility test method: manifold/ Steritest; rinse fluid, culture media, canister kits, etc.
    b. Review the EM data acquired during sterility testing (i.e., settling plates, RODAC), simulation system controls, etc. What are the microbial species and their determined normal habitat (i.e., water, plants, people, etc.?)
    c. Review training records and qualification of analysts performing the test; interview and/or observe analysts
    d. Review the qualification of the bio-clean room facilities or isolator chamber used during testing. Are there any leaks in the gloves, improper sanitization of product container before placement into

work station or isolator? Has the isolator been evaluated for leaks?

e. Review cleaning and sterilization requirements for reusable glassware and equipment. Poorly cleaned glassware will make sterilization of equipment more difficult and possibly shelter trapped microbes from the killing effect of the sterilant.

f. Review laboratory areas used for sub-culturing the sterility test medium onto enrichment plates. Cluttered work space or un-sanitized surfaces may cause plate contamination.

g. Check the original plates used for isolation for possible pre-existing contamination (i.e. growth in non-streaked locations on the agar surface, subsurface growth)

h. Check to see if the medium had been recalled or has had past problems with contamination during manufacturing.

i. It may be necessary to perform a genotype identification on the two isolates (product source and manufacturing area isolate) if they are the same species.

E. Manufacturing Facility Review

1. Aseptically filled pharmaceuticals

a. Check environmental monitoring (EM) data taken from production areas and the testing environment (i.e., S-T-A, settling plates, RODAC, etc.) for microbial contamination that matches the microbe isolated from the finished product sterility test

If no microorganisms are detected, check the adequacy of the EM method used during manufacturing for proper sensitivity and applicability, for example

i. Are they using proper medium (i.e. non-selective medium)?
ii. Have they performed growth promotion?
iii. Did they use appropriate incubation time and temperatures?
iv. Are they sampling in the appropriate room locations, during dynamic conditions, longest time between cleanings/sanitation and at frequency to assure reliability of the results?
v. If they recovered an anaerobic bacterium from the sterility test, (Thioglycollate broth) do they perform EM for anaerobic bacteria?

b. Have they performed a filter integrity test on the membrane used for the product sterilization? Review the products pre-filtration bioburden levels to assure that the concentration of bacteria in the bulk has not exceeded the membrane filtration capacity that was determined in their validation studies. Have they changed the source or model for the membrane filter cartridge used in the process?

c. Has the firm manipulated or excluded some of the data used in the final QC report? Perhaps raw data was averaged to bring the bioburden count below the alert or action levels. It can be helpful to request electronic Excel sheet version of data, to allow sorting (by frequency of organism, location, etc.) and trend analysis; hard copies can be requested later, if necessary.

d. Review the media simulation studies. Did the microbial species recovered in past simulation studies match the microbe(s) recovered from the current product test failure?

e. Has there been a change or breach in the personnel barrier system to protect the product? Were there any interventions by maintenance or other staff personal during the manufacturing of the contaminated lots? Review glove/uniform monitoring results.

f. Review the antimicrobial effectiveness challenge studies for the product if it multiple dose. Where there any changes to the container/closure component source or requirements?

g. Were there any changes to the disinfection procedure, reagents use, new personnel, application, equipment (mops, aerosols, etc.) etc.?

2. Terminally sterilized drug product

a. Check autoclave validation studies for sterilization process- cold spot, heat penetration (challenged inside dry tubing, connectors/caps/stoppers, largest liquid volume, etc.), changes in chamber load configuration, etc.

b. Check maintenance records for house steam, records for autoclave repair, new plumbing

c. Check Biological Indicator (BI) information- improper storage of BIs; changes in the culture (inoculum level and/or BI organism species) and incubation parameters

d. Evaluate the heat resistance characteristics of the microbial isolate found in the product during sterility testing and determine

if it can survive during the process conditions, review product container/closure integrity data and possible recent supply source changes to vials or rubber stoppers; check possible post sterilization package integrity problems- mostly medical device issue.

F. Inspectional Elements listed in the six (6) Inspectional Systems covered by the CP 7356.002 that cover ONLY Microbiological Issues

Some of the key coverage elements in five of the six Inspectional Systems listed in FDA Compliance Program Guidance Manual Program 7356.002 that relate to **microbiological** issues. Some examples are included for clarification. (Labeling system not included).

1. Quality System
    a. Discrepancy and failure investigations related to manufacturing and testing: are they documented, evaluated and investigated in a timely manner; including corrective actions where appropriate.
    b. Validation: status of required validation/revalidation (e.g., computer, manufacturing process, laboratory methods).
    c. Training/qualification of employees in quality control unit functions.
2. Facilities and Equipment System
    a. Facilities
        i. Cleaning and maintenance
        ii. Facility layout and air handling systems for prevention of cross-contamination (e.g. penicillin, beta-lactams, steroids, hormones, cytotoxics, etc.)
        iii. Specifically, designed areas for the manufacturing operations performed by the firm to prevent contamination or mix-ups
        iv. General air handling systems
        v. Lighting, potable water, washing and toilet facilities, sewage and refuse disposal
        vi. Sanitation of the building, use of rodenticides, fungicides, insecticides, cleaning and sanitizing agents
    b. Equipment
        i. Adequacy of equipment design, size, and location
        ii. Equipment surfaces should not be reactive, additive, or absorptive

iii. Appropriate use of equipment operations substances, (lubricants, coolants, refrigerants, etc.) contacting products/containers/etc.
iv. Cleaning procedures and cleaning validation
v. Controls to prevent contamination, particularly with any pesticides or any other toxic materials, or other drug or non-drug chemicals
vi. Qualification, calibration and maintenance of storage equipment, such as refrigerators and freezers for ensuring that standards, raw materials, reagents, etc. are stored at the proper temperatures

3. Materials System
   a. Representative samples collected, tested or examined using appropriate means
   b. Testing or validation of supplier's test results for components, containers and closures
   c. Rejection of any component, container, closure not meeting acceptance requirements. Investigate the firm's procedures for verification of the source of components.
   d. Appropriate retesting/reexamination of components, containers, closures
   e. Water and process gas supply, design, maintenance, validation and operation
   f. Containers and closures should not be additive, reactive, or absorptive to the drug product
   g. Documented investigation into any unexpected discrepancy
4. Production System
   a. Training/qualification of personnel
   b. Validation and verification of cleaning/sterilization/ depryrogenation of containers and closures
   c. Established time limits for completion of phases of production (i.e. microbial growth potential of product)
   d. Implementation and documentation of in-process controls, tests, and examinations (e.g., bioburden determination pH, adequacy of mixing)

e. Justification and consistency of in-process specifications and drug product final specifications

f. Prevention of objectionable microorganisms in non-sterile drug products

g. Equipment cleaning and use logs

h. Process validation, including validation and security of computerized or automated processes (i.e. simulation studies)

i. documented investigation into any unexpected discrepancy

5. Laboratory Control System

a. Training/qualification of personnel

b. Adequacy of staffing for laboratory operations

c. Adequacy of equipment and facility for intended use

d. Calibration and maintenance programs for analytical instruments and equipment

e. Validation and security of computerized or automated processes

f. Reference standards; source, purity and assay, and tests to establish equivalency to current official reference standards as appropriate

g. System suitability checks on chromatographic systems (e.g., GC or HPLC)

h. Specifications, standards, and representative sampling plans

i. Adherence to the written methods of analysis

j. Validation/verification of analytical methods

k. Control system for implementing changes in laboratory operations

l. Required testing is performed on the correct samples

m. Documented investigation into any unexpected discrepancy

n. Complete analytical records from all tests and summaries of results

o. Quality and retention of raw data (e.g., chromatograms and spectra)

p. Correlation of result summaries to raw data; presence of unused data

q. Adherence to an adequate Out of Specification (OOS) procedure which includes timely completion of the investigation

r. Adequate reserve samples; documentation of reserve sample examination

s. Stability testing program, including demonstration of stability indicating capability of the test methods (i.e. container/closure, AET)

G. Sample Collection During an Establishment Inspection

Samples of defective product constitute persuasive evidence that significant CGMP problems exist. Physical samples may be an integral part of a CGMP inspection where control deficiencies are observed. Physical samples should be correlated with observed control deficiencies. The investigator should consult Center and/or ORS Headquarters for guidance on quantity and type of samples (in-process or finished) to be collected. Documentary samples may be submitted when the documentation illustrates the deficiencies better than a physical sample. Divisions may elect to collect, but not analyze, physical samples, or to collect documentary samples to document CGMP deficiencies. Physical sample analysis is not necessary to document CGMP deficiencies.

When a large number of products have been produced under deficient controls, collect physical and/or documentary samples of products which have the greatest therapeutic significance, narrow range of toxicity, or low dosage strength. Include samples of products of minimal therapeutic significance only when they illustrate highly significant deficiencies.

H. Appendix A: Literature and Resources

A Comprehensive List of Only Microbiological Regulatory and Scientific Literature Resources

The scope of this reading material will ONLY include microbiological scientific and regulatory publications or websites for conventional drugs, biologics and combinatorial products. Some references to medical device regulations will be included if relevant during an FDA investigation that covers microbiology.

1. Legal requirements and regulations

a. CFR 210 & 211 “cGMPs for finished Pharmaceuticals”

b. CFR 210 & 211 amended effective Dec 2008 (several changes that include microbiological requirements of aseptically filled products)

c. CFR 610 General Biological Product standards” (Not covered during this review)

d. CFR 820 “Quality Systems Regulation (Devices, not covered)

e. CFR 314.81(b)(3)(ii)- Applications for FDA approval to market a new drug (revised April 1, 2008) For submission of an alternate microbiological method with a comparability study

f. CFR 1271 Human cells, tissues, and cellular and tissue-based products

2. FDA Compliance Program Guidance Manuals

a. FDA Compliance program Guidance Manual for FDA Staff: Drug Manufacturing Inspections program 7356.002

During an inspection this program designated six (6) critical systems for review. They include: Quality System (always covered during an FDA inspection); Facilities &Equipment; Material; Production; Packaging and labeling; and Laboratory control systems.

b. FDA Compliance program Guidance Manual for FDA Staff: Sterile Drug Process Inspections 7356.002A

The sections entitled “Inspectional” and “Analytical” and “Attachment A” are pertinent to an FDA microbiologist.

3. Compliance Policy Guides

a. Sec. 100.550- Status and Responsibilities of Contract Sterilizers Engaged in the Sterilization of Drugs and Devices (CPG 7150.16) (Oct 2006)

b. Sec. 490.100 Process Validation Requirements for Drug Products and Active Pharmaceutical Ingredients Subject to Pre-Market Approval (CPG 7132c.08) (3/2004)

c. Manual of Policies and Procedures, CDER, MAPP 5040.1

Product Quality Microbiology Information in the Common Technical Document - Quality (CTD- Q)

d. Compliance Policy Guidance for FDA Staff- Sec. 280.110 Microbiological Control Requirements in Licensed Anti-Human Globulin and Blood Grouping Reagents

4. US Pharmacopeia (USP) Compendium

Review all relevant product monographs (not all have microbiological requirements); the following are regulatory chapters that contain enforceable microbiology requirements

General Notices and Requirements (page 1-13), Chart 10- Microbiology

a. <1> Injections
b. <51> Antimicrobial Effectiveness test
c. <55> Biological Indicators-Resistance Performance tests
d. <60> Microbiological Examination of Nonsterile Products: Tests for Burkholderia cepacia complex
e. <61> Microbiological Examination of Nonsterile products: Microbial enumeration tests
f. <62> Microbiological Examination of Nonsterile products: Tests for Specified Microorganisms
g. <63> Mycoplasma
h. <71> Sterility Tests
i. <81> Antibiotics-Microbial Assays
j. <85> Bacterial Endotoxins Test
k. <151> Pyrogen Test
l. <161> Transfusion and Infusion Assemblies and Similar Medical Devices
m. <171> Vitamin B12 Activities Assay
n. <797> Pharmaceutical Compounding-Sterile Preparations

Dietary Supplements General Chapters Information

a. <2021> Microbial Enumeration Test-Nutritional and Dietary Supplements
b. <2022> Microbiological Procedures for Absence of Specified Microorganisms- Nutritional and Dietary Supplements
c. <2023> Microbiological Attributes of Non-sterile Nutritional and Dietary Supplements

USP Informational chapters<1000>through <1999> are informational chapters and provide guidance to the industry. These Informational chapters will help explain or expand on scientific principles established in the regulatory chapters.

a. <1035> Biological Indicators for Sterilization
b. <1072> Disinfectants and antiseptics

c. <1111> Microbiological Examination of Nonsterile Products: Acceptable Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use

d. <1112> Application of water activity Determination to Non-sterile pharmaceutical products

e. <1113> Microbial Characterization, Identification, and Strain Typing

f. <1116> Microbiological evaluation of clean rooms and other controlled environments

g. <1117> Microbiological Best Laboratory Practices

h. <1207> Sterile Product Packaging—Integrity Evaluation

i. <1208> Sterility Testing –Validation of Isolator Systems

j. <1209> Sterilization—Chemical and Physicochemical Indicators and Integrators

k. <1211> Sterilization and Sterility Assurance of Compendial Articles

l. <1223> Validation of alternative microbiological methods

m. <1227>Validation of Microbial Recovery from Pharmacopeial Articles

n. <1237> Virology Test Methods

5. AOAC international Includes chapters on disinfectants evaluation (i.e., Phenol coefficient Methods; Hard surface carrier test methods; Use-Dilution Method)

6. Association for the Advancement of Medical Instrumentation (AAMI)/ International Organization for Standardization (ISO).

There are over fifty (50+) documents available through AAMI/ISO on the topic of “Sterilization Processes and Validation”. These are internationally recognized standards and procedures recognized by FDA and Industry.

AAMI/ISO Guidance documents- are available at the FDA intranet.

7. FDA Inspection Guidance documents-

Listed below are all the FDA guidance documents that contain only microbiological information relevant to inspection. In most cases these will be listed in the general website for CBER or CBER guidelines.

a. Submission of Documentation in Applications for Parametric Release of Human and Veterinary Drug Products Terminally Sterilized by Moist Heat Processes (draft 8/2008)

b. Validation of Growth-Based Rapid Microbiological Methods for Sterility Testing of Cellular and Gene Therapy Products (draft guidance, 2/2008)

c. Guidance for Industry- Container and Closure system Integrity Testing in Lieu of Sterility Testing as a Component of the Stability Protocol for Sterile Products (2/2008)

d. Guidance for Industry- Quality Systems Approach to Pharmaceutical cGMP Regulations (9/2006)

e. Draft Guidance for Industry and FDA Staff- Nucleic Acid Based In Vitro Diagnostic Devices for Detection of Microbial Pathogens (12/2005) Docket number 2005D-0434

f. Guidance for Industry- Manufacturing Biological Drug Substances, Intermediates, or Products using Spore-forming Microorganisms (2/2005)

g. Sterile Drug Products Produced by Aseptic Processing — Current Good Manufacturing Practice, 9/2004

h. Comparability Protocols - Chemistry, Manufacturing, and Controls Information, Required for industry interested in substituting an automated/Rapid Microbiological method in place of the USP compendial method cited in their original application (2/2003)

i. Guidance for Industry- Sterility Requirement for Aqueous- Based Drug Products for Oral Inhalation—Small Entity Compliance Guide (11/2001)

j. Guide to Inspections of Quality Systems-Medical Device (8/1999)

k. Guidance for Industry-Content and Format of Chemistry, Manufacturing and Controls Information and Establishment Description Information for a Vaccine or Related Product (1/1999)

l. Guide to Inspections of Lyophilization of Parenterals (10/18/97)

m. Guide to Inspections of Cosmetic Product manufacturers (2/1995)

n. Guide to Inspections of Sterile Drug Substance Manufacturers, (7/1994)

o. Guide to Inspections of Topical Drug Products (7/1994)

p. Guidance for Industry- Submission Documentation for Sterilization Process Validation in Applications for Human and Veterinary Drug Products (11/1994)

q. Guideline for the manufacture of In Vitro Diagnostic Products (1/1994)

r. Guide to Inspections of Microbiological Pharmaceutical Quality Control Laboratories (7/1993)

s. Guide to Inspections of High Purity Water Systems, (7/1993)

t. Guide to Inspections of Validation of Cleaning Processes (7/1993)

u. FDA Biotechnology Inspection Guide, Reference materials and training aids (11/1991)

v. Guidance for Industry, Pyrogen and Endotoxins Testing: Questions and Answers (June 2012)

8. Inspectors technical guidance (ITG)-

The following are the ITGs that were written regarding microbiological issues.

a. PYROGENS, STILL A DANGER (1/12/79 Number: 32)

b. HEAT EXCHANGERS TO AVOID CONTAMINATION (7/31/79 Number: 34)

c. REVERSE OSMOSIS (10-21-80 Number: 36)

d. BACTERIAL ENDOTOXINS/PYROGENS (3/20/85 Number: 40)

e. LYOPHILIZATION OF PARENTERALS (4/18/86 Number: 43)

f. WATER FOR PHARMACEUTICAL USE (12/31/86 Number: 46)

g. MICROBIOLOGICAL CONTAMINATION OF EQUIPMENT GASKETS WITH PRODUCT CONTACT (12/31/86 Number: 48)

9. Miscellaneous FDA Documents and References

This list of references may not be entirely microbiology but very important none the less.

a. FDA Inspectional Operational Manual

b. FDA Warning Letters and Responses

c. FDA Bacteriological Analytical Manual (BAM) (1/2001)

10. Important Government and International organizations:

a. National Institute of Health (www.nih.gov)

b. Center for Disease Control and Prevention (www.cdc.gov)

c. CDC report on environmental monitoring (http://www.cdc.gov/ncidod/dhqp/gl_environinfection.html)

d. World Health Organization (www.who.org) International pharmaceutical regulations along with monitoring of disease outbreaks around the globe may be important if assigned to work in a high-risk area.

11. Industry Technical references-

Parenteral Drug Association (PDA) Technical Reports- Although the scientific recommendations in these technical reports are not enforceable by FDA, they do contain industry current manufacturing practices and scientifically sound principles that support regulatory concerns. The following is a selection of PDA technical reports that may be useful:

Report No.	Title	Date
1	Validation of Moist Heat Sterilization Processes: Cycle Design, Development, Qualification and Ongoing Control	July 2007
3	Validation of Dry Heat Processes Used for Sterilization and Depyrogenation	1981
4	Design Concepts for the Validation of Water-for-Injection Systems	1983
5	Sterile Pharmaceutical Packaging: Compatibility and Stability	1984
7	Depyrogenation	1985
11	Sterilization of Parenterals by Gamma Radiation	1988

13	Fundamentals of an Environmental Monitoring Program	1990 (Revised 2001)
15	Industrial Perspective on Validation of Tangential Flow Filtration in Bio-pharmaceutical Application	1992
20	Report on Survey of Current Industry Gowning Practices	1990
21	Bioburden Recovery Validation	1990
22	Process Simulation Testing for Aseptically Filled Products	2011
23	Industry Survey on Current Sterile Filtration Practices	1996
26	Sterilizing Filtration of Liquids	2008
28	Process Simulation Testing for Sterile Bulk Pharmaceutical Chemicals	2006 (revised)
29	Points to Consider for Cleaning Validation	2012
30	Parametric Release of Pharmaceuticals Terminally Sterilized by Moist Heat	1999
33	Evaluation, Validation and Implementation of New Microbiological Testing Methods	2000
34	Design and Validation of Isolate Systems for the Manufacturing and Testing of Health Care Products	2001
35	A Proposed Training Model for the Microbiological Function in the Pharmaceutical Industry	2001
36	Current Practices in the Validation of Aseptic Processing -- 2001	2002
40	Sterilization Filtration of Gases	2005
41	Virus Filtration	2005
45	Filtration of Liquids using Cellulose-based depth filter	2008
57	Analytical Method Validation and Transfer for Biotechnology products	2012

61	Steam in place	2013

12. Books and Commercial Trade reports:
    a. ASM, Manual of Clinical Microbiology;
    b. Disinfection, Sterilization, and Preservation, by S Block; Bergey's manual systematic Bacteriology
    c. Remington's Pharmaceutical Sciences
    d. F-D-C Monthly Reports

Excellent summary of conferences, FDA regulation changes, Key Industry personnel and often a list of the most recent Product Recalls and regulatory actions by FDA. Need to sign up for email membership. Instructions for membership enrollment are available at FDA website below.

(http://inside.fda.gov/Library/ElectronicResourcesWebLERN/Alphabeticallist/index.htm)

The "Gold Sheet"- Pharmaceutical & Biotechnology Quality Control The "Pink Sheet"- Prescription Pharmaceuticals and Biotechnology The "Gray Sheet"- Medical Devices Diagnostics & Instrumentation The "Silver Sheet"- Medical Device Quality Control reports

13. Free Trade publications available on line-

Pharmaceutical Technology (www.pharmtech.com)

Controlled Environments (www.cemag.us)

American Pharmaceutical Review (www.americanpharmaceuticalreview.com)

International BioPharm (www.biopharminternational.com/)

XIV. Professional memberships

These organizations have available searchable references.

International Society of Pharmaceutical Engineers (www.ispe.org)

American Society for Microbiology (www.asm.org)

Parenteral Drug Association (www.pda.org)

15. Glossary/Definitions

None

16. Records

None

17. Supporting Documents

None

18. Document History

Revision #	Status* (D, I, R)	Date	Author Name and Title	Approving Official Name and Title
1.1	I	4/25/2014	Angele C. Smith, Microbiologist	
1.2	R	3/30/2015	Angele C. Smith, Microbiologist	
1.3	R	6/7/2016	Angele C. Smith, Microbiologist	Selen Stromgren, Deputy Director MPTSS
1.4	R	1/31/2018	Angele C. Smith, Microbiologist	George Salem, Staff Director OMPSLO
02	R	7/27/2020	Angele C. Smith, Microbiologist	Bryan Gamble, Acting Deputy Associate Director OMPSLO

* - D: Draft, I: Initial, R: Revision

19. Change History

Revision #	Change
1.1	ii.- updated Chapter 3. A. 2. a. iv. – revised Chapter 8. D. 1st paragraph – revised Chapter 10. H. 5. – author information updated
1.2	Chapter 3, D. 1. a. - revised Chapter 7 – Antibiotic Potency Assay (inserted)
1.3	Chapter 9, A. 1. i – revised Chapter 9, B, B.1.a, C.4, C.7, C.9, C.10, E.1, E.2 – revised Chapter 10, A.6 – removed Appendix A, removed links
1.4	Updated highlighted (grey) in document
1.5	Updated to new template Updated author information Updated to 508 Compliance Updated Chapter 2 – revised to match new USP method Updated Chapter 3 – revised for clarification Updated Chapter 4 – revised for clarification Updated Chapter 5 – revised for clarification; added two USP references Updated Chapter 9 – revised for clarification Updated Chapter 10 – revised for clarification

20. Attachments

None