Guidance Document

Microbiological Process Validation & Surveillance Program

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The AATB recognizes the efforts of the following individuals who generously donated their time and expertise to creating this document.

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TABLE OF CONTENTS

I. INTRODUCTION

A. History and Purpose

AATB's *Standards for Tissue Banking (Standards)* and various global regulatory authorities with oversight of cell and tissue establishments require validation of procedures related to tissue processing. Lack of stepwise instructions for validating such procedures is a recognized gap that exists worldwide. This Guidance Document provides detailed information to assist tissue banks in development of a comprehensive microbiological surveillance program and describes steps to consider when validating processing of 'conventional' allograft tissue (e.g., skin, bone, cartilage, ligaments, tendons, dura mater, amnion, vessels, heart valves, and cellular tissue), similar autograft tissue, and allografts regulated as biologics, medical devices or combination products. Scenarios (see examples and annexes) are included to further illustrate expectations.

Due to evolving information regarding the critical role of microbiological test methods used for human tissue recovered for transplantation, as well as a need to establish microbiological sampling plans that can sufficiently establish a validated process's capabilities, updates were issued to *Standards* during early 2011 accompanied by an AATB Interim Guidance Document (i.e., No. 5, Standard K2.210 Pre-Sterilization/Pre-Disinfection Cultures). The guidance was referenced within *Standards* and became required (see AATB Bulletin #11-01, January 4, 2011). It described a more detailed document would be issued in the future; this guidance document replaces that interim guidance.

AATB-accredited tissue banks are expected to use the following guidelines when validating microbial surveillance methods and when validating processing methods and steps. Documentation, including relevant data, is expected to support decisions and must be made available to AATB when requested. **The advisory nature of this guidance will eventually become mandatory.**

B. Definitions

Terms used throughout this Guidance Document are defined here and are generally accepted definitions. Where applicable, the source of the definition is referenced.

Allograft: Tissue intended for transplantation into another individual of the same species. (1)

Aseptic Processing: The processing of tissue using aseptic techniques when tissue, containers and/or devices are handled in a controlled environment in which the air supply, materials, equipment and personnel are regulated to prevent microbial contamination of tissue.

Note: This term is used in different ways in various industries, including the tissue banking profession.

The primary differences between uses of the term aseptic process for tissue versus applicability to other products are:

- Generally, for aseptically manufactured healthcare products that do not contain human tissue, all components and products are sterilized in some manner prior to assembly and packaging in an aseptic process; sterility of the components and products are maintained during assembly and packaging. (2)
- For aseptically manufactured products that contain human tissue, the non-tissue components are sterile prior to entry into the aseptic process, but the tissue may not be sterile entering the aseptic process. Thus, the aseptic process may include bioburden reduction steps for the tissue, and the aseptic process environment prevents any subsequent contamination of the tissue and maintains the sterility of the other components.

Bioburden: Population of viable microorganisms on or in tissue and/or the sterile barrier system (packaging). (3)

Note: Testing for bioburden can be performed before, during or after tissue processing.

Bioburden reduction: The act of reducing the number of viable microorganism on surfaces and/or tissue.

Cleaning (tissue): The initial step of the tissue disinfection process that can include the removal of extraneous tissue, blood, lipids, certain proteins and many microorganisms. (4)

Companion tissue: Tissue used for microbiological destructive testing that is co-processed from the same donor and is of the same type (e.g., tendon for tendon; bone for bone, and skin for skin) as finished tissue from the same lot. It is not intended for transplant.

Decontamination: Cleaning the environment, facilities, and/or surfaces (sanitation), or instruments, supplies, and equipment (sanitization), with intent to remove or reduce pathogenic microbes. (4, 8, 39)

Destructive testing: When tissue used for microbiological testing is destroyed and can no longer be used as a result of performing the testing.

Disinfectant: An agent (e.g., heat, a chemical) capable of reducing the number of viable microorganisms. A disinfectant might not kill spores. Use of antimicrobials in tissue processing is included here.

Disinfection: A process that reduces the number of viable microorganisms on tissue, but may not destroy all microbial forms, such as spores and viruses. Use of antimicrobials in tissue processing is included here.

Note: In this document the term disinfection has been specified for use on tissue and decontamination has been specified for use on surfaces, however, disinfectants are used with either application. For example one can use a disinfectant to decontaminate surfaces or a disinfectant to disinfect tissue.

e.g.: abbreviation for *exempli gratia*; for example, such as; the list is not finite.

Finished tissue: Tissue that has been fully processed, enclosed in its final container, labeled,

and released to distribution inventory. (1)

Fluid extraction: A solution that has been exposed to tissue and subsequently used for microbiological testing. It is highly preferred that the solution be the last fluid the tissue contacts during packaging for finished allograft end point culture testing.

HCT/P: FDA term for "human cells, tissues, or cellular or tissue-based products"; see 21 CFR Part 1271.3(d) for more information. (5)

i.e.: abbreviation for *id est*; that is; indicates a finite list.

Log reduction: The reduction in number of viable microorganisms, expressed in logarithmic units. (6)

Note: The log reduction value is not equal to a sterility assurance level (SAL) of the same value. For example, if a tissue product has a bioburden of 10^4 CFU, a 3 log reduction will result in a bioburden of 10^1, **not** an SAL of 10^{-3}.

Method suitability test [also called the bacteriostasis/fungistasis (B/F) test]: A test performed with selected microorganisms to demonstrate the presence or absence of substances that inhibit the multiplication of microorganisms. Note: This is often performed using the approach described in USP <71> (7) under Method Suitability Test.

Microorganism: A microscopic organism, including bacteria and fungi; viruses, while sometimes included in this classification, are not included here. (10)

Process validation: Establishing by objective evidence that a process consistently produces a result or product meeting predetermined specifications. (8, 9)

Processing (FDA 21 CFR Part 1271.3 (ff)): any activity performed on an HCT/P, other than recovery, donor screening, donor testing, storage, labeling, packaging, or distribution, such as testing for microorganisms, preparation, sterilization, steps to inactivate or remove adventitious agents, preservation for storage, and removal from storage. (9)

Quality system: The organizational structure, responsibilities, procedures, processes, and resources for implementing quality management. (1)

Sterile: For tissue, the absence of detectable, viable, microorganisms (10). For reagents, supplies, materials and equipment, free from viable microorganisms.

Sterility assurance level (SAL): The probability of a single viable microorganism occurring on an item after sterilization.

Note: The term SAL is a quantitative value, generally 10^{-6} or 10^{-3}. When applying this quantitative value to assurance of sterility, an SAL of 10^{-6} has a lower value but provides greater assurance of sterility than an SAL of 10^{-3}. (3, 8)

Sterilization: A validated process used to render tissue free from viable microorganisms including spores. (10, 11)

Terminal sterilization: A validated process whereby tissue within its final sterile barrier system (e.g., package, container) is sterilized. (10, 11)

Note: Use of this term is restricted to sterilization that occurs when the tissue is in its sealed sterile barrier system (i.e., primary package).

Validation: Confirmation through the provision of documented objective evidence that predefined specifications have been fulfilled and can be consistently reproduced. (9)

Verification: The confirmation by examination and provision of documented objective evidence that specified requirements have been fulfilled. (8)

II. MICROBIOLOGICAL ASPECTS OF PROCESS CHARACTERIZATION

A. Introduction

This section provides detail regarding validation of processes aimed at reducing or eliminating microorganisms from allografts. Additional aspects of process validation (e.g., physical effects on tissue, chemical residues) are not addressed in this document although some of the concepts may apply.

Successful process validation is heavily dependent on knowledge of the tissue type and the process. A process must be capable of delivering a set of defined conditions to which the tissue is exposed. Additionally, attributes of the tissue must be well understood such that the response of the tissue to the conditions is both consistent and acceptable. Thus, the investigator must verify the critical attributes of both the tissue and the process.

Historical process and knowledge of the tissue type, literature reviews, feasibility studies, and process and allograft characterization are all means by which one can verify critical attributes and process parameters. Verification of critical attributes and process parameters will allow one to define appropriate tissue and process specifications for process validation.

B. Process Characterization; Preparing for Process Validation

Prior to process validation it is important to understand each individual step of the process. This effort is called process characterization. Process characterization is often iterative in nature, intent on defining and optimizing the steps in the process. Steps may include defining the process range by gathering available, relevant data in literature, gathering historical information from subject matter experts, and/or performing feasibility studies.

Each step of the process must be evaluated so that critical steps can be differentiated from non-critical steps (e.g. a step with specific log reduction activities versus a rinse step with no specific outcome). When evaluating a step in the process, it is necessary to identify variables that affect the process step and determine if they can produce an unacceptable outcome for the finished tissue. Critical steps for a process must be well defined, controlled, and documented. Non-critical steps may not require a high level of control, and may not be as well defined.

Annex A provides an example of steps taken in process characterization. Annex B describes how information from process characterization can be used in process validation.

Oftentimes, verification of the tissue quality and performance of equipment are outputs of process characterization. This information may become part of equipment installation qualification (IQ), which is discussed later in this document.

If a step is determined to be non-critical (e.g., tissue transfer, storage step), a rationale should be written describing how that decision was made. Failure to control a seemingly inconsequential step in the process may result in an undesirable or unacceptable outcome.

> *Example: The physical act of transporting tissue from one location to another may have little impact on the tissue. The transport conditions however, (time, temperature range, exposure to environment) may have a significant impact on the tissue from microbiological and functional perspectives. A seemingly inconsequential step is critical to preserving the attributes of the tissue.*

Characterization of a process may include evaluation of parameters such as sensitivity, specificity, linearity, precision, accuracy, and ruggedness. Some parameters are more applicable to an analytical test than to a microbiological test. When designing protocols, it is important to assess which parameters are relevant to the process.

> *Example: If a process step may be performed by a number of personnel and actions of the personnel involved can have significant impact on the finished tissue, then determination of ruggedness by including multiple personnel may be prudent.*

When performing process characterization, it is important to understand the effect of using multiple variables for critical aspects of a step (e.g., temperature, time, concentration). If the number of variables to be evaluated can be minimized, it is easier to determine if optimization of individual steps is necessary. These data may be used to establish proper ranges for individual processing steps.

> *Example: Process step #2 has four variables (temperature, time, concentration, and life expectancy of a chemical). The tissue processer has determined, through literature and experience, that the optimal working temperature is between 18-25°C, and the life expectancy of the chemical is 8 hours at that temperature. They decide to not perform extensive testing of those two variables, but to focus on optimization of the time and concentration variables. Therefore, during process characterization, the temperature range is set at 18-25°C and time is tested at the maximum life expectancy of 8 hours. However, concentrations of 0.08, 0.1, 0.12 and 0.14 are used, as well as times of 20, 40, 60 and 90 minutes.*

When evaluating process steps, it is important to characterize or evaluate microbial reduction as well as the impact of the process steps on the physical and functional aspects of the tissue. A process step may be implemented which is primarily intended to have a physical effect on the tissue, but which also has a microbial reduction aspect as a secondary benefit, or vice versa.

Once a process is well understood/characterized, writing validation requirements becomes a straightforward process because the critical process parameters and the acceptable variability around those parameters have been defined during process characterization.

III. MICROBIOLOGICAL PROCESS VALIDATION

This section addresses process validation for assessment of microbiological aspects of processing. Process validation is performed to demonstrate with a high level of confidence that a consistent finished tissue outcome will occur when the process is properly applied. Process validation is required in instances where a critical process cannot be 100% verified. Documentation of Process Validation (e.g., the protocol and final report) is expected. See AATB Standard K1.100 Basic Elements (at K1.000 Quality Assurance Program) and the relevant definition in A2.000 (1). A validation protocol must be written that includes procedures, rationale, acceptance criteria, etc.

A. Distinguishing Validation of Methods from Validation of Processes

Discussion of validation work in this section is in context of validating a process step rather than validating a test method.

To validate a process step, the proposed process step is applied to representative samples and the samples are tested to demonstrate the ability of the step to consistently provide the desired effect. The process is typically performed multiple times to show reproducibility of the process. Inconsistent data may indicate that changes should be made to the process to provide better or more consistent outcomes.

To validate a test method, representative samples are selected and tested to demonstrate performance of the proposed test method. Data may indicate that changes to the test method should be made so that test parameters (e.g., accuracy, precision, sensitivity, specificity) are improved. See Section XII. Validation and Qualification of Test Methods.

B. Three Stages of Process Validation

An FDA guidance document (9) describes process validation activities are expected to occur in three stages:

- Process Design
- Process Qualification
- Continued Process Verification

This approach entails evaluation of the process during the entire life cycle of the tissue, not just at a single time point.

The process should be designed based on an understanding of the desired outcomes of the process. If specific outcomes of the proposed process are established and documented it will be easier to tailor the process with those outcomes in mind. Previous experience in tissue processing can also be used to assist in designing the process.

In the process qualification stage, testing is performed to evaluate the quality and reproducibility of the established process. This could include testing of individual steps in the process (also known as process characterization) as well as testing of the entire process. During the course of process qualification it might be determined that aspects of the process should be adjusted to result in a more optimized or improved process. In fact, going into process qualification it

should be understood that the proposed process is still somewhat fluid and might change depending on results obtained.

The stage of continued process verification is a reminder that qualification or validation of a process does not guarantee proper implementation or function of that process for its entire life. Process design and qualification stages can assist in determining particular aspects of the process; these are good indicators of continued quality and reproducibility. Those indicators are often called process monitors and should be performed regularly enough to demonstrate maintained control.

C. Step-wise Approach

An individual performing process validation may choose to validate the entire process or may first choose to validate individual steps within the process.

Some aspects of a process step may be validated as a group (if a particular process step has multiple aspects which must be validated), and others may have to be validated individually. If aspects of a step or steps of a process are to be validated together, an important consideration is the number of variables involved. If issues arise from the validation data, the number and complexity of variables involved may hinder determination of the root cause of a validation problem.

Annex B provides an example of the stepwise approach to process validation.

Validating every aspect of each process step can be unmanageable due to time, cost and personnel constraints. It is acceptable to specify some of the aspect details rather than perform validations to optimize all of them. If this approach is used, a rationale detailing how the decision was made and supporting information must be documented.

The scope of a process validation should be well defined prior to initiating the validation. It is often helpful to identify validation limitations and validation related issues that are addressed in other protocols or portions of the Quality System. In validation there are some aspects that are not further tested by the tissue bank.

> *Example: If your Quality System dictates that sterile water is purchased from an approved vendor whose quality system has been audited and has been deemed acceptable, a requirement to test the specifications of the sterile water may not be necessary if verification can be performed effectively (e.g., a compliance certificate, certificate of analysis).*

In evaluating various aspects of a process step, the term 'address' may be used rather than 'validate.' It may not be necessary or possible to 'validate' every potential impact (e.g., microbiological and physical) that every process step may have on tissue. Process engineers should determine, based on the intended purposes and criticality of each step, which aspects should be tested.

D. Validation of the Overall Process

After assessing individual process steps, process validation should be completed by microbiologically challenging the entire process (e.g., with inoculated tissue) and evaluating the

tissue post processing. The results of step-based validations will assist in determining how evaluation of the entire process should be done.

A decision to be made is whether to perform a qualitative or a quantitative analysis after the inoculated tissue goes through the process (see Section IV. General Considerations For Microbiological Testing at B. Qualitative vs. Quantitative Testing).

In a very robust process (e.g., where six or more log reductions are expected during the process) a qualitative analysis of the inoculated tissue post-processing is likely best due to its greater sensitivity. In a less robust process (e.g., fewer than six log reductions are expected during the process) a quantitative analysis of the inoculated tissue post-processing is likely the best approach due to its ability to provide microorganism counts.

> *Example: If it is known from process characterization that Step A provides a 3 log reduction and Step B provides a 4 log reduction, it is possible that the two processes together would provide enough log reduction to reduce a 10^6 inoculum to almost zero. This means that if tissue is inoculated with the selected microorganism(s) at a level of 10^6 CFU and then processed, the remaining microorganism count would likely be very low. In this situation, a sterility test of the inoculated tissue post-processing might be best.*
>
> *Example: If it is known from process characterization that one process applied to the tissue, Step A, only provides a 2.5 log reduction there will likely be a high number of microorganisms remaining post-processing if the initial inoculum is 10^6 CFU. In this situation a bioburden test of the inoculated tissue post-processing might be best due to its ability to provide the remaining number of CFUs.*

One reason to microbiologically evaluate the overall process is to verify assumptions regarding the potentially additive microbial reduction nature of the individual steps. Generally, log reductions cannot be automatically added together to determine the overall log reduction capability of a process because the modes of action of the various steps in the process might overlap (12). For example:

- if the mode of action of Step A and Step B **are the same** (e.g., both are alcohol-based steps) then log reductions from Step B might not be additive;
- if the mode of action of Step A and Step B **are different** (e.g., alcohol-based and peroxide) then log reductions from Step B could be additive.

Also, evaluation of the overall process can be used to verify the absence of unforeseen interactions between steps of the process that may be detrimental to the finished tissue, either microbiologically or physically.

In validation of the overall process it is best to limit the quantity of variables to be addressed in order to limit the quantity of experiments that must be performed. Process characterization and previous validation of the individual steps play a large role in establishing the conditions of these variables. In characterization of the individual steps, one can determine which variables to use as 'worst case' or 'typical case' scenario (e.g., high or low temperature, minimum and maximum soak times, high or low concentration). The conditions used in the validation and the rationale for their use should be documented in the validation protocol.

Annex C provides an example of the potential challenges when performing validation on the entire process rather than first on individual steps.

E. Acceptance Criteria

It is important to establish acceptance criteria for process validation *prior to* initiation of the validation. Acceptance criteria must be written in support of the validation protocol. Acceptance criteria are determined by the individual tissue bank and will vary, minimally based on the following:

1. Label claims (e.g., the label claim of 'sterile' carries a higher regulatory requirement than other claims);
2. What process characterization data suggests the overall process is capable of; and
3. Statements the individual tissue bank intends to make regarding final tissue attributes (e.g., marketing literature statements must be supported).

Oftentimes components of a tissue process are licensed or purchased pre-validated. If a tissue bank chooses to purchase and use this validated process for its own tissue, the process must be verified for use in their establishment.

Consideration should be given when adopting a previously validated process to a new material or load configuration. Changes to a validated process must be reviewed and evaluated and revalidation performed where appropriate.

Annex D provides an example of a limited validation.

IV. GENERAL CONSIDERATIONS FOR MICROBIOLOGICAL TESTING

A. Introduction

The outcome of a disinfection or sterilization process is related to the capability of that process to reduce or eliminate an expected level and mix of microorganisms on the particular tissue type being exposed to the process. If pre-sterilization/pre-disinfection microbiological load exceeds what the process has been validated to remove or inactivate, there is a lack of assurance the process will result in an expected reduction of microorganisms. Thus, it is imperative that test methods used to identify microorganism contamination at critical, predetermined steps produce accurate results. Pre-sterilization/pre-disinfection microbiological cultures play a critical role in indicating the capability of the validated process will not be exceeded. It is equally important that in-process and final culture methods are not inhibited or influenced by residual processing agents, test material, or other factors.

B. Qualitative vs. Quantitative Testing

Often microbiological evaluation of a process or process step is performed by inoculating tissue with known numbers and types of microorganisms, performing process step(s) on the tissue, and determining if viable microorganisms remain on the tissue after treatment.

A qualitative test might be performed by enumerating the starting microbial population, processing the tissue, immersing the processed tissue in culture media, then looking for turbidity as an indication of growth. The results obtained are limited to either positive or negative for growth. In this approach, statements that can be made are limited regarding the presence or

absence of microorganisms. Turbidity visualized in positive tests may be due to the growth of 1 surviving microorganism or 1,000,000 surviving microorganisms. Exact enumeration of residual microbes is not possible.

A quantitative test might be performed by extracting microorganisms from the tissue and performing plate counts to enumerate starting and ending bioburden. The results obtained provide the quantity of CFU on the tissue. Thus, plate counts allow a description to be made regarding reduction of the microbial population due to the treatment. However, a notable limitation of the quantitative approach is that it requires extraction of organisms from tissue. Extraction methods are not 100% efficient (e.g., counts obtained may be the result of incomplete extraction rather than reduction of the organisms in the process).

Quantitative analysis usually provides better microbiological reduction data for an individual step and is better suited to evaluating different variables of that step and understanding lethality kinetics of treatment.

> *Example: An evaluation of two or three time points of a process step, using quantitative analysis provides data which can be used to optimize the microbial reduction potential of that step. Microbial counts obtained in the testing make it easier to determine which time point is providing the best microbial reduction. Negative or positive results obtained using a qualitative test would not provide useful data for optimizing the process step.*
>
> *Additionally, quantitative analysis can assist in determining if the microbial reduction mechanism is linear with respect to time (e.g., the longer the process step is applied the more microbiological reduction occurs) and whether the microbial reduction is consistent with respect to time.*

C. Selection of Microorganisms for Process Validation

When performing process validation, relevant microorganisms and microorganism loads must be selected. This determination is based upon knowledge of:

- What the protocol is attempting to achieve (i.e., disinfection or sterilization);
- The type and quantity of bioburden present on the tissues being processed; and
- The capability or limitations of the microbial reduction process being validated.

For low-level disinfection, it is generally acceptable to achieve up to 99% reduction of naturally occurring bioburden. The intent of this approach is to reduce vegetative bioburden and prevent any "blooms" of bioburden during the process. Low-level disinfection may not control or eradicate resistant forms of microbial flora such as spores.

For high level disinfection, it is generally acceptable to achieve $\geq$ 99.99% reduction of naturally occurring bioburden. The intent of this approach is to reduce vegetative bioburden, spores and fungi so as to provide a very clean material.

For sterilization, the focus changes from "reducing bioburden" on tissue to "predicting the probability of a non-sterile unit" resulting from the process. The probability of a non-sterile unit is more commonly referred to as a Sterility Assurance Level (SAL).

Generally, for grafts labeled "Sterile," an SAL of $1x10^{-6}$ has been achieved by the process on the final tissue configuration. This is often referred to as a "Terminal Sterilization Process" because the tissue is in its final configuration and will not be further manipulated or packaged after sterilization is complete.

The level of disinfection or sterilization takes into account the amount of bioburden reduction and the type of bioburden being reduced. If the tissue bioburden is known to include spore-formers like *Bacillus sp. or Clostridium sp.,* the use of a general disinfectant may not provide significant reduction of the bioburden.

Capabilities or limitations of the microbial reduction potential of a process are often supported by literature searches, consultation with experts in the field, and/or process characterization.

1. Using Representative Challenge Microorganisms

Challenge microorganisms are often chosen to understand the effectiveness of individual steps of the overall process across multiple microorganism types. For example, a species from each primary category of microorganism may be selected:

- Gram negative bacilli (e.g., *E. coli, P. aeruginosa, Serratia species*)
- Gram positive bacilli – usually a spore-former (e.g., *Bacillus species*)
- Gram positive cocci (e.g., *Staphylococcus aureus*)
- Mold (e.g., *Aspergillus species*)
- Yeast (e.g., *Candida species*)
- Anaerobe (e.g., *Clostridium species* for a spore-former or *P. acnes* for a non-spore-former)

Additionally, it is common to add microorganisms that occur on tissue or in the environment, such as air-, surface- and water-borne microorganisms. In some cases, microorganisms can be selected to meet multiple criteria (e.g., *E. coli* may represent gram negative bacilli and may also be found on tissue).

When selecting microorganisms, it may be tempting to omit select microorganisms because they have not been previously demonstrated to be of concern. While this is an option, if a potentially pathogenic microorganism category emerges in the future, it may be necessary to perform additional evaluations with that microorganism type. It is usually best to initially evaluate a broad range of microorganism categories and species.

There are no specified criteria for selection of microorganisms in FDA documents related to tissue. Other documents, however, provide guidance regarding this topic. ISO Standards 14160 (12) and ISO 14937 (13) provide guidance on selecting appropriate microorganisms for evaluating a sterilization process. These concepts are similar for evaluating a disinfection process such as those commonly applied to tissue.

2. Using Microbial Screening Studies

Another option for selecting microorganisms is to implement a screening method for process-resistant microorganisms. This is often performed by applying the process or the process step to tissue, using a shorter time and/or lesser concentration than typically specified, then testing the

tissue for surviving microorganisms. Surviving microorganism types must then be included in the process validation.

3. Use of Microorganisms in Validation

When performing validations of a step or an overall process it may be possible to use initial test data to reduce the need for testing all microorganisms under consideration. Using only the more resistant microorganisms (for a worst-case challenge) allows for a greater number of process variables to be challenged with similar resources due to the reduction of replications and tissue samples involved compared to if all microorganisms needed to be evaluated.

> *Example: Initial research and development (R&D) testing or process characterization may indicate that Step B easily kills gram negative rods, strict anaerobes, gram positive cocci and yeasts. If all critical aspects of Step B (as determined by risk analysis and the initial R&D tests) remain the same, it may be possible to perform the validation of the step using only the more resistant microorganisms (i.e., a gram positive rod and a mold). Testing fewer microorganism types might then allow for testing of additional time points to optimize Step B for microbial reduction.*

Note that the data used to reduce the number of microorganism types for Step B may not apply to other steps in the process because the mechanism of kill for other steps may be different. It is likely that this type of evaluation will need to be performed individually for each step in a process.

D. Determination of Microbiological Surveillance Components

Analysis of process characterization or validation data of an individual step or of the entire process can indicate that certain steps or certain aspects of a step in a process should be monitored (tested) at some established frequency. Every step of a process will not require monitoring on a routine basis unless there is little or no process validation in place. The criticality of a process step should be considered in selecting steps to be monitored routinely. Correctly established process monitors may give a more sensitive and accurate representation of finished tissue integrity because only a limited number can be tested.

Thorough process validation and data review can also demonstrate that there are aspects of the process which are comparable or better indicators of the microbiological status of the finished tissue than testing the finished tissue itself. Although this is not a common practice, it should not be ruled out as an option when substantial data are available to support it.

In performing this determination one should consider whether there is a terminal sterilization step in the process. Inclusion of a terminal sterilization step in a process may result in different surveillance components compared to a process that does not include terminal sterilization (e.g., aseptic processing only). Inclusion of a terminal sterilization step should never result in a loosely controlled process, but it may result in routine testing of fewer components due to the additional safety provided by the sterilization step.

Annexes E & F provide scenarios where microbiological surveillance might be applied.

E. Establishing Tissue Bioburden Criteria

Evaluation of process validation data can be used to establish bioburden specifications as applicable, and to understand the potential impact an excursion can have on the tissue. Risk analysis and risk management can be used to assist in setting bioburden criteria and addressing excursions. These may be established initially based on process validation data, and periodically evaluated to assure they are still properly established.

Annex E provides an example of establishing bioburden alert and action levels.

A thorough knowledge of the capability of the process can also assist in determining the microbiological acceptability of tissue coming into the process. A process that is not very robust may require more stringent rules regarding the microbiological status of tissue subjected to that process. A very robust process may allow for more flexibility regarding microbiological status. In current guidance (8), FDA recommends the following: "Discard all musculoskeletal HCT/Ps from a donor that has any musculoskeletal pre-processing cultures positive for *Clostridium, Streptococcus pyogenes* (group A strep), or any other microorganisms that you have determined to be difficult to eliminate, unless you have a terminal sterilization process validated to a sterility assurance level (SAL) of 10^{-6}." This is reflected in AATB Standard K2.310 Pre-Sterilization/Pre-Disinfection Cultures.

F. Change control

Upon completion of a process step validation or a complete process validation it is critical that an effective change control system be established. Changes to any aspect of the process may alter the effectiveness of the process and must be evaluated carefully. Evaluations must be thorough and documented, and may require some degree of testing to verify assumptions made regarding the impact the change may have on the finished tissue.

V. METHODS FOR SAMPLING AND CULTURING

Many methods for sampling and culturing are available and it is not the intent of this guidance to specify that a particular method be used. The intent is to clearly explain some of the common methods being used and discuss their advantages and disadvantages. Whether 100% of the tissue is tested (e.g., recovery cultures or pre-processing cultures) or a percentage (post-processing or other types of cultures), these concepts will usually apply.

A. Sampling Methods

The term sampling methods refers to techniques and tests applied to the tissue in order to determine the numbers and/or types of microorganisms on the tissue.

1. Swabbing: Swabbing has been the most common sampling method for obtaining pre-processing cultures and has also been used to obtain post-processing cultures. Its advantages are a long history of use, the lack of equipment needed for the test, and the ease of training and use.

Swab culturing has historically been shown to have low accuracy, sensitivity, and reliability (14-25). Establishing quantifiable bioburden (in colony forming units per tissue) can be accomplished via filter-culturing and fluid-extraction techniques (14). However, obtaining

accurate quantifiable bioburden via swabbing can prove difficult due to the limitations of swabbing techniques and protocols often used (26).

A primary disadvantage to swabbing is lack of microbiological sensitivity. Data have been published regarding the low sensitivity of the swab method. Sensitivity of the swab method is both user and method dependent, and can be understood by evaluating use of swabs in the actual process. To accomplish this, a recovery efficiency test can be performed to determine the effectiveness of recovering microorganisms from the tissue when using a swab. See Section XII. Validation and Qualification of Test Methods at B. Test Method Validation, listing 1. Bioburden Recovery Efficiency.

2. Elution: The term elution in this context means that a fluid is used to remove (elute) microorganisms from the tissue after which the fluid is tested to determine the number and/or type of microorganisms present in the fluid.

Many methods can be employed in the elution technique and some are described below:

Extraction with surfactants: Extraction fluids (eluents) that contain surfactants (e.g., Fluid D as described in USP) might be used as they can result in a more complete removal of the microorganisms from the tissue. However, surfactants can often leave unacceptable residues on the tissue after the test, so this method will usually result in discarding the tissue that was used for testing unless a validated cleaning technique can be performed on the tissue.

Extraction with fluid: It's advised to use extraction fluids that do not leave residues on the tissue (e.g., saline) because the tissue used for testing can continue though processing and be released for clinical use. Removal of microorganisms might not be as complete due to the lack of surfactants, but since the tissue can be used after the test is performed it may result in allowing for more tissues to be tested.

Testing of tissues: Regardless of which type of extraction fluid is used, the tissues may be tested either individually or multiple tissues from the same donor can be combined. If tissue is tested individually, data are obtained which can demonstrate the consistency of the tissue bioburden on a tissue-by-tissue basis. Depending on use of results, combining multiple tissues from the same donor might be appropriate. Such results could be used to establish or monitor incoming bioburden but should not be used for tissue release to distribution. See Section IV. General Considerations For Microbiological Testing at E. Establishing Tissue Bioburden Criteria, and Section IX. Acceptance Criteria at B. Alert And Action Levels.

Much of this information is explained in ANSI/AAMI/ISO 11737-1 (27) and AAMI TIR 37-2007 (28). It is also important to determine the recovery efficiency of these methods, as previously described (29, 30).

3. Destructive Testing: Destructive testing refers to tissues immersed in a growth medium and incubated to determine if viable microorganisms are present on the tissue. It is often used in sterility tests. It is the most sensitive of tests in that it does not rely on removal of microorganisms from the tissue. However it does not allow for testing of a high percentage of tissue because the tissue must be discarded after testing. Destructive testing does not provide a count of microorganisms (quantitative test), it only provides a positive or negative result (qualitative test). It is common to characterize any growth that occurs (e.g., Gram stains, identification to genus and/or species, etc.).

4. Automated systems and rapid microbiological methods (RMM): These types of microbiological test systems are becoming more common and might be applicable to testing of tissue. In the end, they provide similar types of results as the methods described above, so extensive discussion is not needed here. It is important to ensure that the automated or rapid method used has the proper level of sensitivity and specificity so that the results gathered provide the information needed. In growth-based RMMs, an evaluation for inhibitory substances (e.g., growth promotion or a bacteriostasis/fungistasis test) is also required. See FDA draft guidance (31).

VI. NEUTRALIZATION

In any test system it must be demonstrated that proper neutralization of inhibitory substances has occurred. Inhibitory substances introduced by disinfected tissue may result in lower numbers of microorganisms being seen (for elution methods) or in false negative results (for destructive test or sterility test methods). Inhibition can also be naturally caused by viable or functional cells in the tissue being cultured (e.g., skin, cardiac and vascular tissue), so the fact that the tissue does not come into contact with antibiotics or inhibitory chemicals does not mean that the testing to demonstrate neutralization is unnecessary. Neutralization must be demonstrated with any type of sample (e.g., a piece of tissue, an extract of the tissue or a swab).

Validation of neutralization in a sterility test (i.e., destructive test) is usually called a Method Suitability test [aka bacteriostasis/fungistasis test (B/F test)]. The Method Suitability test procedures described in USP <71> (7) have traditionally been used with tissue allografts. This test does not determine the effectiveness of microorganism recovery, since microorganisms are not removed from the tissue. This test determines whether proper neutralization has occurred in the test system such that false negative test results do not occur.

Determination of proper neutralization and potential remedies for inhibitory substances are discussed in ANSI/AAMI/ISO 11737-1 (27), 11737-2 (32) and USP <71> (7) and USP <1227> (33).

There can be circumstances where inclusion of other microorganisms, in addition to those called out in the previously mentioned documents, may be appropriate. Evaluating this need might be accomplished by comparing the microorganisms that the tissue bank has experienced or those that are expected to occur (e.g., anaerobic microorganisms) with those to be used in the neutralization study to ensure that relevant, general microorganism types are included. Another example might be in testing skin to demonstrate that the microorganisms from the criteria list for skin are all capable of growing in the neutralized test system. The rationale for inclusion or exclusion of any additional microorganisms should be documented.

VII. TISSUES AND FLUIDS USED FOR TESTING

Various types of tissues and fluid should be tested, and must be representative of the different tissue types processed. Variation in bioburden count and inhibition are commonly encountered when testing different types of tissues (i.e., bone, soft tissue, cardiac, skin, etc.). Some common approaches to sampling are described below.

Sample Type	Description	Advantages	Disadvantages
Companion Tissue	Similar in bioburden to tissues in the batch	Use increases the quantity of available finished tissue	Obtaining sufficient sample size may be difficult; Not actual finished tissue
Finished Tissue Sample	Sample of finished tissue (fully processed, enclosed in its final container)	Direct analysis of finished tissue	Obtaining sufficient sample size may be difficult; reduces quantity of available finished tissue
Fluid Extraction	Fluid has had contact with entire batch of tissue	Assessment of entire batch of tissue	Debris from the tissue can clog the filter

For all sample types described above there are two test methods to select from. A quantitative test (i.e., bioburden) will provide a count of viable microorganisms from the tissue. The validation for a quantitative test is a recovery efficiency (discussed in Section XII. Validation and Qualification of Test Methods at B. Test Method Validation, listing 1. Bioburden Recovery Efficiency). A qualitative test (i.e., sterility) will provide a presence or absence result for viable microorganisms. The validation for a qualitative test is a method suitability test (also called a B/F test, discussed in Section VI. Neutralization).

A. Companion Tissue

Companion tissue refers to tissue which is recovered from the donor but which will not be used for transplantation. It can be comprised of portions of recovered tissue that are not acceptable for transplantation (i.e., not meeting tissue quality specifications such as discoloration, high porosity, tendon fiber separation, conduits with calcific atheroma, etc.), or portions trimmed from transplantable tissue during recovery and/or processing stages.

Companion tissue goes through every step of processing with tissue intended to become finished tissue (e.g., same processing events, containers, solutions, incubators, etc.). Because the tissue is destined for the same cleaning and disinfection process and is of the same type, it is reasonable to expect the bioburden type and numbers to be representative of the finished tissue. This is confirmed through validation or verification that the size/volume yields bioburden that, per unit area, is representative of the finished tissue. A rationale, supported by data, should be in place to address any differences in size between the companion tissue used for testing and the transplantable tissue. Companion tissue is discarded after testing.

See Annex G for an example when validating use of companion tissue.

B. Fluids

A fluid extraction refers to a fluid-based removal of microorganisms from tissues (i.e., elution) and performing microbiological testing on the fluid. These fluids may be tested to represent the microbiological quality of the tissues provided that proper validation has occurred (e.g., recovery efficiency or Method Suitability testing depending on the test to be performed).

Fluid extraction can be performed with any type of tissue (e.g., transplantable, non-transplantable, etc.) and at any point during the tissue process (e.g., early steps, intermediate, final packaging steps, final packaged tissues, etc.).

Fluid extractions taken from static rinses (e.g., transport tissue solutions) are typically relying on the long-term contact of the rinse fluid with the tissue whereby microorganisms are liberated from the tissue via non-mechanical means. Fluid extractions taken from static rinses are usually a method with higher variability associated with recovery efficiency and can be difficult to show repeatability.

Fluid extractions taken from mechanical rinses (e.g., in-process tissue steps, final extraction step prior to tissue packaging) are usually short rinse steps coupled with mechanical energy (e.g., shaking, stomaching, ultrasonics and vortexing) to liberate microorganisms into the fluid extraction. Mechanical rinsing will have less extraction efficiency variability than static rinses and can be validated for repeatability.

Considerations when validating a fluid extraction methodology are:

1. Worst-case attributes of extraction process
    a. Worst-case attributes can be the maximum amount of tissue in the extraction step, shortest time of step (if a range is specified), lowest setting on mechanical component of the step or other variables determined during characterization studies.
2. Tissue types (e.g., bone, soft tissue, soft tissue with bone attached)
3. Tissue inoculation sites (if validated via the inoculation method)
4. Inoculum contact time (the amount of time to allow the inoculum to absorb prior to testing)
    a. The higher the inoculum volume, the longer the contact time will need to be
5. Fluid extraction volume
6. Mechanical energy utilized for fluid extraction
7. Amount of time allowed for fluid extraction
8. Required neutralization for test method
9. Characterization surrounding the extraction methodology to understand variability associated with the method, tissue type and donor to donor variability
10. Appropriate number of replicates
11. Method of determining repeatability/acceptance criteria (e.g., recovery efficiency, calculations)

Validation of fluid extraction processes is usually based on the concept of recovery efficiency. Recovery efficiency details are outlined in Section XII. Validation and Qualification of Test Methods at B. Test Method Validation, listing 1. Bioburden Recovery Efficiency.

See Annex H for an example when validating a fluid extraction method, and Annex I for an example when calculating fluid extraction results.

C. Sample Size

It is not possible to establish a sample size representative of all situations. Sample size should be based on a documented rationale. Historically, 10% of a finished lot (considering variations in samples) has been a common quantity tested and appears to have functioned well. USP <71> (7), Table 3 provides some options on sample sizes for aseptically processed pharmaceutical

products. Under certain circumstances, a sample size of 10% of the finished batch is recommended for testing in each medium with a minimum sample size of four and a maximum of 10 in each medium. Table 2 provides options for whether each test sample can be split into two portions for testing (resulting in 10% of the finished batch being tested) or whether each test sample must be tested in its entirety in each medium (resulting in 20% of the finished batch being tested). Although the intent behind USP <71> (aseptically manufactured pharmaceuticals) meets the intent behind tissues which are not terminally sterilized, there must be some adaptation since in most cases a finished batch of tissue does not result in all pieces being equivalent in properties (e.g., size and shape).

Determination of sample size can be made in many ways.

Note that many statistical sampling methods only consider numbers and do not consider validation work, or controls and monitoring schemes. Process validation data should be able to assist in determining proper sample sizes, perhaps even better than purely statistical analyses. This approach will result in tissue banks using different sample sizes, and that is appropriate based on the level of validation performed and the number of controls and/or monitors in place.

> *For example: During a process validation it is noticed that the tissue bioburden after Step #3 is representative of the fully processed tissue bioburden. It is determined to use the rinse solution after Step #3 as an indication of the fully processed tissue bioburden. The recovery efficiency of the rinse solution is determined.*
>
> *Concurrent testing of rinse solution from Step #3 and fully processed tissue from the same batch demonstrates consistent results, both in numbers and types of microorganisms. This concurrent testing is performed using tissue from donors having authorization for research use, where 100% of tissue from six batches of the same tissue type were tested after completion of processing. This evaluation was performed using different processing personnel and over six weeks of time.*
>
> *In this situation the consistency of the results between testing the Step #3 rinse solution and the fully processed tissue allows for some flexibility in how much tissue is tested at the end of the process. If the Step #3 rinse solution is tested for every batch, that may allow for testing of only a few fully processed tissues at first (e.g., 3) and testing of no fully processed tissue after data is gathered over time (e.g., one year) if the consistency of the data continues.*

This example does not provide a hard number of samples to be tested on a routine basis. Any number or percentage established in this guidance may be too restrictive for some and provide too much allowance for others, depending on the robustness of the quality system, process validation and other controls/monitors in place.

For terminally sterilized tissue grafts, the sample size situation may be different. In some cases (e.g., radiation sterilization) the sample sizes for some types of testing are established by the procedure being followed such as Method 1, Method 2 or VDmax (34, 35). For a process that is validated to a sterility assurance level of 10^{-6}, terminal sterilization should never replace or diminish the need for good overall process validation and good controls/monitoring of the process. However, if validated terminal sterilization is being used, it may allow for testing of fewer tissue samples on a routine basis, or may allow for more quickly reducing the quantity tested due to the additional safety provided in the sterilization process.

VIII. SAMPLING FREQUENCY

It is required to obtain data regarding the microbiological status of tissue from each donor prior to processing. Historically, the extent of the required data has ranged from testing some of the tissues from a donor (skin) to testing a sample of every tissue (cardiac and vascular) from a donor (pre-processing cultures).

Current requirements, see AATB Standard K2.310 (1), are that the sampling plan must provide an accurate microbiological representation of the tissues. This allows for flexibility to test all of the tissues from a donor (the default position) or to test a specific set (sampling) of tissues from the donor based on sufficient validation data and a documented rationale.

Regardless of the sampling plan details, it is required to obtain microbiological data on each donor prior to processing, see AATB Standard K2.310 (1). Whether this testing is qualitative (e.g., swab testing) or quantitative (e.g., extraction method), any method used must be followed by identification of the microorganisms. There is a classically accepted set of identification methods available (either automated or conventional methods), so the critical issue is that the sampling plan selected must adequately represent the clinically usable tissue.

Historically, it has been required to test a percentage of tissue post-processing. In the case of a (validated) *terminal sterilization* process, it is not necessary to perform pre-sterilization or post-sterilization tissue testing on a regular basis.

This concept is similar to that of sample size and is previously covered in the example under Sample Size.

IX. ACCEPTANCE CRITERIA

A. Specified microorganisms

The AATB Standards provide acceptance criteria for particular tissues and microorganisms. Those acceptance criteria (i.e., the list of specified microorganisms) are a minimum requirement and not meant to be all-inclusive. The numbers and types of microorganisms should be considered for each batch of tissue and the data should be used to determine their acceptability for release.

One option for addressing this concept is to establish acceptance criteria based on the results of the tissue bank's process validation. It may not be possible to establish a specific acceptance criterion for every microorganism of concern, but it may be possible to establish these criteria for some relevant microorganisms and include different microorganism types (e.g., Gram negative rod, Gram positive cocci, etc.).

Usually each microorganism type is included in a tissue process validation. Based on data obtained from the validation it is possible to determine how much of each microorganism type the process can effectively eliminate. These same numbers can be used to establish acceptance criteria for the microorganism types.

If a particular microorganism is demonstrated to consistently be on the tissue in numbers (amounts or with frequency) that cause concern, and if it is not clear whether the process validation covers this microorganism, then challenging the process using this specific microorganism may be necessary.

This does not require a full revalidation but can be performed using one microorganism included in the initial validation as a control along with the microorganism in question. It may also not require an evaluation of the entire process. Based on knowledge of the process, which the validation provides, it can be determined if the evaluation must be performed on the entire process or only a certain part(s) of the process.

B. Alert And Action Levels

Another aspect of acceptance criteria is the allowable quantity of microorganisms permitted on tissue pre-processing or post-processing. These values are often called alert and action levels. Alert and action levels will be different for pre-processing and post-processing evaluations. Pre-processing levels are based on process efficiency and post-processing levels are based on assessment of risk. It is not required that these levels be established when evaluating bioburden data (e.g., establishment of two levels can be appropriate if desired). Information is provided here regarding three levels to explain how they might be used as part of a bioburden monitoring system. Just as bioburden levels are commonly used for environmental monitoring, they should also be used for evaluation of tissue bioburden.

Validation of a process should generate data that verify the capability of the entire process to eliminate microorganism types. These data can be used to establish a bioburden level at which there should be concern that may not require corrective action (alert level) and a level where corrective action must occur (action level) as well as a level where it is known that the process is incapable of reducing the bioburden to the desired level (bioburden specification). Bioburden counts at these levels might vary widely depending on the process in place. A robust tissue process that provides many log reductions (e.g., demineralization process with acid and alcohol) could allow for higher pre-processing bioburden counts compared to a minimal tissue process that only has one or two log reductions (e.g., soft tissue process with detergents and antibiotics). Additionally, a validated terminal sterilization process (e.g., irradiation) might allow even higher alert and action level values.

There are no standard terms that must be used, nor are there standardized approaches to establishing bioburden levels. The concept of these levels however, is critical. Once the capability of a tissue process is understood, it will also be understood that there are bioburden levels that might overwhelm the process and render it ineffective. The purpose of establishing bioburden levels and trending bioburden data is to ensure that all tissue treated using the process is acceptable for transplantation. Bioburden levels should be established keeping this safety concept in mind.

Generally, the alert level can be established to demonstrate when a bioburden count is substantially above the typical counts obtained. This level might not require corrective action but might result in additional testing or a heightened awareness during review of subsequent test data.

The action level can be established to demonstrate when a bioburden count is set at a level low enough not to challenge the capability of the process. This level requires corrective action,

which often includes additional testing, and requires quarantine of tissue until the issue can be investigated and resolved.

Bioburden levels should be established using substantial data. It is commonly suggested that the data should also represent a long period of time (e.g., 6-12 months) to include seasonal or other variations that may occur. While the proper amount of data is being gathered it is appropriate to establish temporary levels, which also should be based on actual data.

At no time should bioburden alert and action levels be established randomly or arbitrarily, as this might result in failure of expectations of the process resulting in unsafe tissue, or an overreaction resulting in discard of tissue which could be rendered safe by the process.

As is the case with specified microorganisms previously discussed, if terminal sterilization is in place then supportive data from its validation studies must be taken into account when establishing bioburden levels.

X. CULTURE NEGATIVE RESULTS

Historically, obtaining culture negative results of processed tissue was often the primary, and in some cases the only, indication that the tissue was microbiologically acceptable for use. "Culture negative results" refers to tests performed where the results of the test are negative for growth (usually a destructive or "sterility" test).

Under current expectations, this approach is no longer acceptable. Culture negative results of processed tissue are meant to be a component of the overall process control system. Culture negative results are only useful in the context of proper process validation and validation of the test method performed, including evaluation of adequate neutralization studies for the test.

XI. ENVIRONMENTAL MONITORING

This section discusses general concepts and recommended practices regarding the establishment of environmental monitoring (EM) plans and interpretation of EM data. In the context of this chapter, use of the word "environment" includes all surfaces, water, personnel and the air in the area(s) being monitored.

Standardized values or requirements, cleanroom classification, and non-viable particulates are not described here. Guidance is already provided in national and international standards [e.g., USP <1116> (36), ISO 14644 series (37), and PDA TR13 (38).

A. Introduction

Contamination in the environment (air, surfaces and water) has the potential to contaminate tissue during processing operations. Because of this, EM must be employed in any environment that can possibly have a microbiological impact on tissue. Note that the primary intent of EM is to evaluate the quality of the manufacturing environment and to demonstrate acceptable trends over time. EM data should always be reviewed and considered prior to making an HCTP

available for distribution, but an EM excursion does not necessarily mean that tissue has been impacted/affected.

There are few specific regulatory requirements relating to EM (39):

> § 1271.190(b)(1):
> *Facility cleaning and sanitation*.
> You must maintain any facility used in the manufacture of HCT/Ps in a clean, sanitary, and orderly manner, to prevent the introduction, transmission, or spread of communicable disease.
>
> § 1271.195(a) and (c):
> *Environmental control*.
> Where environmental conditions could reasonably be expected to cause contamination or cross-contamination of HCT/Ps or equipment, or accidental exposure of HCT/Ps to communicable disease agents, you must adequately control environmental conditions and provide proper conditions for operations.
>
> *Environmental monitoring*.
> You must monitor environmental conditions where environmental conditions could reasonably be expected to cause contamination or cross-contamination of HCT/Ps or equipment, or accidental exposure of HCT/Ps to communicable disease agents. Where appropriate, you must provide environmental monitoring for microorganisms.

Note that these requirements only state what must be accomplished, not how to accomplish it. Because of this, there is flexibility regarding how the requirements should be met. Some guidance is provided in different standards or documents and the more notable of these are provided in the following text.

Environmental control does not ensure acceptability of processed tissue because environmental control is not the sole aspect of an overall quality system. Demonstration of consistent, acceptable EM is usually the outcome of a well-established and well-implemented quality system. An exact or specific level of required "control" has not been defined. The requirement is that the level of control provided by the procedure consistently provides an environment that does not negatively impact the tissue. If EM data or tissue bioburden data demonstrate that the environment is impacting the tissue, the procedure and/or process must be updated to improve safety conditions. This also requires the evaluation of impact to tissue that may have been processed under questionable conditions.

B. Environment/Tissue Connection

The relationship between the microbiological status of the environment and the microbiological status of tissue is not always clear. This relationship, if any, will vary depending on a number of aspects, including but not limited to the:

- environment (level of classification);
- personnel in the environment;
- training program and staff adherence to it;
- amount of contact between the tissue and critical areas in the room;
- amount of time the tissue is exposed to the environment;
- processes being performed in the room and their impact on the environment; and

- effectiveness of cleaning protocols applied in the environment.

It cannot automatically be assumed that the microbiological status of the environment will have an impact on tissue. The level of impact that different aspects of the environment might have on tissue must be understood and sufficiently addressed. Since there is not an automatic association between the two, it should not be automatic that an excursion in the environment causes discard of the tissue. An EM excursion should always cause a documented investigation and determination of the potential impact on tissue, but it does not always follow that the tissue has been affected.

Due to the level of cleanliness in most processing rooms, it may be the case that the tissue may contaminate the environment rather than *vice versa*. In the early stages of tissue processing it might be more likely that the tissue would contaminate the environment, and in the later stages, that the environment might contaminate the tissue.

In general, sequential movement of tissue from the uncontrolled environment to cleaner environments, will necessitate stricter levels of environmental control and monitoring. During all such movements of tissue, strict segregation of donor lots shall be observed as well as thorough cleaning of the area between handling of tissue from different donors.

A clear definition of the activities that are conducted during each phase of the process and the associated environmental requirements for each phase of the process will often make the processor's approach more readily understood.

C. Temperature And Humidity Monitoring In The Tissue Processing Environment

In the United States, there are no standard requirements for temperature and humidity in the tissue processing environment. General guidance regarding acceptable ranges for temperature and humidity can be found [36, 37, 39 see 1271.195 (a)], however, there are no standard levels or values provided for tissue establishments. Tissue establishments must develop their own levels and values.

The primary purposes for temperature and humidity controls are for the control of contamination and cross-contamination, control of manufacturing conditions (tissue sensitivity), for the comfort of personnel, and/or for reduction of electrostatic charges (if this is critical to the tissue or if it poses a fire hazard).

For purposes of tissue processing, generally it would not be necessary to have tight specifications for temperature or humidity. However, it might be necessary to have a target specification and to routinely monitor one or the other. The rationale for temperature and humidity specifications or monitoring (or reasons for not needing them) must be documented.

When addressing temperature and humidity levels, consideration is given to operator comfort. If an operator is perspiring due to environmental conditions, they will be more likely to contaminate the environment with perspiration through attempts at wiping off the perspiration.

Also, certain types of microbes are more likely to thrive at high humidity levels (e.g., greater than 70%). Specifically, fungi could become a significant contributor to the environmental flora due to an increase in humidity in the working environment. These organisms in a high humidity

environment (especially where visible moisture is present) can rapidly grow and sporulate and contaminate the facility.

D. Tissue Sterilization Versus Aseptic Processing

Environmental controls and monitoring should be maintained to assure that processing conditions are controlled to the extent necessary to prevent risk of infectious disease transmission. For terminally sterilized tissue subjected to a validated sterilization technology, the controls must assure that *Bioburden* limits used to determine validation parameters and sterilization cycles are met. In the case of tissues not subject to a sterilization process, environmental controls and monitoring need to be established to control risk of adventitious *Bioburden* introduced during processing. In either case, controls should be adequate to prevent the risk of cross contamination.

E. Decontamination Effectiveness Studies for Cleanroom Surfaces

Disinfectant (cleaning agent) effectiveness studies are generally straightforward since there is usually one step involved (e.g., a spray or a wipe of a surface with one disinfectant). Standards are available which provide a good framework for evaluation of disinfectants [e.g., ASTM E2614-08 (40), USP <1072> (41), and AAMI TIR 12 (42)].

There are two phases to evaluating disinfectants. The first phase is usually performed by the disinfectant manufacturer, to determine the efficacy of the disinfectant against a panel of microorganisms, some of which may be considered "compliant" and some "non-compliant" when applied to tissue processing functions.

The second phase is usually performed by the user of the surface disinfectant (e.g., a tissue bank) against a selected panel of additional microorganisms, including types that have been recovered in the processing facility or from the tissue. In these tests, it is important to evaluate various surfaces on which the disinfectant will be used. Testing is usually performed on coupons of the various types of material to be decontaminated so that test microorganisms are not being introduced to the processing environment. This type of testing must include a neutralization study to ensure that media used for testing will allow growth of surviving microorganisms.

Title 21 CFR Part 1271 Subpart D, Current Good Tissue Practice (39) does not require that companies perform cleaning validation, only that they be able to demonstrate that their disinfection process is effective in preventing the potential contamination or cross-contamination of tissue. A tissue bank should have a documented rationale for the choice made regarding the disinfectant and any associated testing.

Other healthcare industries have different expectations. It is common for pharmaceutical companies to validate the cleaning process by properly performing cleanroom surface disinfectant effectiveness studies even though the manufacturer can provide information regarding effectiveness. Historically, medical device companies have referenced manufacturer information to justify use of a particular disinfectant, but this practice is evolving to be more consistent with the approach used by pharmaceutical companies.

In a situation where there is very little or no disinfection of tissue during tissue processing it might be more important for a tissue bank to perform the cleanroom surface disinfectant cleaning

validation themselves, using coupons of their surfaces and the microorganisms they carefully select.

It is not the intent of this document to establish a required log reduction for cleanroom surface disinfectants. Certainly, acceptance criteria must be established if a cleaning validation is to be performed, and the acceptance criteria must be determined by each tissue bank. Note that USP <1072> (41) targets at least a 3 log reduction of vegetative microorganisms and at least a 2 log reduction for bacterial spores.

F. Initial Qualification of Processing Areas

Whether the processing area is a classified cleanroom or controlled environment the same concepts apply, even though the requirements are stricter for a classified cleanroom. Although the requirements for particulates in a cleanroom are well established, that is not the case for viable microorganisms in the air or on surfaces. USP <1116> (36) provides guidance that, although it is only guidance, has proven to be attainable and generally acceptable to regulatory agencies. There is no need to require that tissue banks establish more rigorous requirements than those provided in USP. Requirements that are less rigorous might be allowable with written justification (e.g., terminal sterilization).

Additionally, there is no defined requirement regarding how much data must be obtained prior to initiation of use of the cleanroom. The quantity of data obtained for air and surface microorganisms should be sufficient to demonstrate consistent microbiological control of the area. This usually consists of demonstration over a period of time that the environmental monitoring test results meet the acceptability criteria established by the company, or that they are consistent and low if criteria have not yet been established. One approach would be monitoring over the course of an entire day and over the course of a week or two, including periods of inactivity.

Initially a tissue bank may not establish EM alert/action levels until monitoring data from the cleanrooms are obtained. In this instance, the acceptability criteria might be that the EM results be consistently low. Then when cleanroom use begins, if tissue testing is providing acceptable results, this indicates that the environment is not negatively impacting tissue.

USP <1116> (36) provides suggestions regarding contamination recovery rates in cleanrooms (see Tables 2 and 3). For example, an ISO 5 room classification a <1% contamination recovery rate for active air sample, settle plate, contact plate or swab, and glove or garment is advised. PDA TR13 (38) Table 1, provides a summary of microbial level suggestions which includes EU recommendations compared to USP recommendations. These suggestions (provided to the pharmaceutical industry) may be implemented in a tissue bank, but they should not be blindly implemented without data that suggests they are applicable.

It is wise to initially gather air and surface data over a period of time (to be determined by the tissue bank) with the cleanroom fully equipped but at rest (i.e., static or not in operation). This provides baseline data on the ability of the cleanroom to remain in control over time.

A second set of data can be obtained with the cleanroom in use (active or dynamic). The sampling plan for this data could be the same as that used with the cleanroom at rest. This data can be compared to the “at rest” data to determine the impact that people and processes have on the controlled environment.

The last step demonstrates the effect that people and processes have on the room over time. Generally it is wise to perform monitoring more frequently initially (e.g., with every processing session, once a day or once a week) to demonstrate control, followed by a reduction in the frequency for the long term (e.g., daily or weekly depending on the criticality of the process in the room and the quantity of data available). See the section entitled Frequency of Sampling for more information on this topic.

The decision to gather initial dynamic data while processing actual tissue or while processing surrogate tissue should be documented by the tissue bank.

Note that viable microorganism counts will usually increase when the cleanroom is active. There is not an expectation that the dynamic data be identical to the static data, but the dynamic data should still demonstrate that the room is in control.

G. Types of Tests Commonly Performed

Following are types of EM tests commonly performed and a basic description of each. For all EM tests performed it is important that the media types and incubation conditions are appropriate for the microorganisms that could be present in the environment.

1. Nonviable air (particulates): This test determines the number and size of particles in the cleanroom air. It is performed as part of cleanroom certification and should be repeated at a specified interval. Limits are established by national and international standards [e.g., ISO 14644 series (37) and USP <1116> (36)].

2. Viable air (microorganisms): This test determines the number of viable microorganisms in the air. Active air samplers are used to test a particular quantity of air (e.g., 10-100 m3) and impinge the microorganisms onto an agar surface that is incubated for growth. Passive air sampling consists of the use of an agar plate left open on a surface for a specified length of time (e.g., 2-4 hours) and incubated for growth.

No limits are established but some recommended levels are provided in USP <1116> (36). Company-specific levels are usually established after an initial data-gathering period (e.g., 6 months to one year).

3. Viable surface (microorganisms): This test determines the number of viable microorganisms on the surfaces in a cleanroom (e.g. tabletops and equipment). It is performed using agar plates (e.g., Rodac® or Hygicult®) where the agar is lightly pressed against the surface for a short time (e.g., 5-10 seconds) and incubated for growth. Cleaning of residual agar on the surface tested must be performed after such sampling. Other surfaces that will not have tissue contact (e.g., floors and walls) may also be tested; if used to check cleaning effectiveness, these areas may have different acceptance criteria due to the lack of tissue contact.

Swabbing is sometimes performed on irregular-shaped surfaces followed by removal of the microorganisms from the swabs (usually with a water-based solution), filtration of the solution, and plating and incubating for growth.

No limits are established but some recommended levels are provided in USP <1116> (36).

Establishment-specific levels are usually established after an initial data-gathering period (e.g., 6 months to one year).

4. Water (microorganisms): This test determines the number of viable microorganisms in water used for processing. It is usually performed using membrane filtration of a pre-determined quantity (e.g., 100 mL) followed by plating and incubation for growth. Testing should ensure the water meets required specifications for tissue processing operations and, where appropriate, must be sterile [39 see 1271.210 (b)].

5. Water (endotoxin): This test determines the number of endotoxin units (EUs) in a water system and is usually performed using the Limulus Amebocyte Lysate (LAL) test. Endotoxin comes from the cell walls of Gram negative microorganisms. The EU result of a water sample does not equate to a viable microorganism count because endotoxins can be present whether microorganisms are dead or alive. Endotoxin limits are published for different USP grades of water (e.g., USP sterile water for irrigation has a limit of 0.25 EU per mL) but limits should be established by the tissue bank based on historical values and intended use of the water.

6. Personnel (microorganisms): This test determines the number of microorganisms on personnel in the cleanroom (e.g., gowns and gloves). The time when sampling is performed (e.g., beginning of process/shift, in-process, and/or at end of process/shift) should be established and justified. It is usually performed using the same types of agar plates used in surface sampling (e.g., Rodac® or Hygicult®), or by using conventional agar plates. After touching the plates to sampling areas, the plates are incubated for growth. Microorganism levels are published for personnel [e.g., PDA TR13 (38), and USP <1116> (36)] but they should be established by the tissue bank based on historical values, and on the purpose for testing. There should be consideration of the potential for residual agar remaining on the sampled area, and covering or discarding the garb may be indicated.

H. Selection of Sampling Sites

Initially all surfaces and areas which may have tissue contact or exposure should be monitored to assist in establishing an environmental monitoring baseline. After initial data are gathered, the number of sites can often be reduced to those having greatest potential for critical impact on tissue.

Generally it is not necessary to perform routine EM on surfaces or areas not intended to have direct or indirect contact with tissue. For example it may be necessary to monitor the classified room where tissue packaging occurs, but may not be necessary to monitor the room where shipping boxes are packed for distribution.

I. Alert and Action Levels

Note that many of these concepts are similar to those described for determination of alert and action levels for tissue bioburden.

An EM excursion is said to occur when an EM value is above a specified level (e.g., alert and/or action level). It is important that alert and action levels are properly established so that appropriate action is triggered when necessary. It is not within the scope of this document to provide a recommended mathematical method to establish alert and action levels, however, some concepts provided in standards [e.g., PDA TR13 (38) and USP <1116> (36)] are shared.

Alert and action levels should not be established with a limited amount of data. Initial values can be established during the cleanroom qualification stage and the values should be revisited after more time has passed (e.g., several months to a year) in order to affix permanent values.

Typically, there is not a direct correlation between EM bioburden and tissue bioburden. Although EM results are a part of the overall control process, they are not a direct indicator of whether tissue is suitable for use. An excursion in EM results might initiate an investigation into the appropriateness of particular batches of tissue, and EM results should be considered as tissue is released. However, a higher than normal count from an air sample or from the floor of the cleanroom should not automatically result in discard of tissue. The EM program is used less for determination of tissue release and more for demonstration of continued control over the environment and recognition of shifts from the norm. Evaluation of an excursion of the level of control can affect the determination of tissue disposition on a case-by-case basis (i.e., voluntary recall or withdrawal). Because of this typical separation between EM bioburden and tissue bioburden, the idea of establishing an EM bioburden specification is not addressed, although a tissue impact assessment must be performed.

Alert Level:
It is generally agreed that an alert level indicates that microbial counts have increased outside of the norm, but not to a point where there is immediate concern for tissue processed in the affected environment. An investigation into the excursion can be initiated, and appropriate corrective actions taken. Such actions may include, but are not limited to, review of the cleaning protocol, re-cleaning and resampling affected areas, review of personnel protocol and activities, or review of the processing operation for unusual events.

Action Level:
A value at or above the action level indicates that the microbial counts are outside of the norm and that they might be at a point where tissue could be impacted. An investigation is required, including a root cause analysis, followed by appropriate corrective and preventive action, to include an evaluation of impact on processed tissue.

Determination of alert and action levels is always accomplished by some mathematical means. Whether those means are as simple as use of standard deviations and averages or as sophisticated as complex statistical calculations is not the point of this document. The important concept to grasp is that the levels should assist a tissue bank in understanding when the environment might be getting out of control or might be impacting tissue. This means that trending EM data is critical to maintenance of a cleanroom and to demonstration of continued control.

Often the exact cause of an EM excursion cannot be determined. This does not mean that nothing should be done. In these cases, those involved must make educated decisions regarding what the potential cause or causes might have been, and determine and implement reasonable action for those causes.

J. Trending

There is no specified requirement for when EM alert and action levels must be revised and recalculated. However a formal review to determine if the current levels are still applicable is necessary at some determined interval. While many companies reassess levels annually, each tissue bank must determine an appropriate frequency for itself.

Since alert levels in EM are used largely to determine if the environment is in a state of control, alert levels should be evaluated to assure that a departure from historical trends can be readily detected and acted upon. If alert levels are set too high, changes in trending from historical norms could be occurring for some time prior to the alert being exceeded. Often, excursions of this nature are hard to investigate and resolve because of extended time that has already passed. Trending of the EM values in these instances will often show a continuous drift upward toward the alert level.

K. When to Sample

EM samples can be taken prior to processing, during processing or after processing. Each option provides a different set of data for a different purpose.

EM samples taken prior to processing provide the tissue bank with information relating to the cleanliness of the area before introduction of equipment and tissue, and also provide information on what may have happened to the area since the last time it was cleaned (e.g., during periods of inactivity). These samples can be used to establish baseline EM data for the room in question.

EM samples taken during processing or after processing (but before cleaning) assist in understanding what the microbiological effects of personnel, equipment and tissue have been to the environment, as well as what affect those might have had on the tissue. When performed during processing, care must be taken to ensure that performing EM does not potentially cause contamination of the tissue. For example, it may not be wise to perform active air sampling directly over tissue during processing.

L. Frequency of Sampling

USP <1116> (36), Table 2, recommends testing in each operating shift for a Class 100 (ISO Class 5) room. It has been common for some tissue banks to perform EM while processing tissue from each donor or batch. EM testing performed during processing can be used to understand the effect that processing tissue has on the environment in addition to understanding how the environment might affect the tissue. The frequency of EM should be dictated by the type of tissue that's processed and the amount of data obtained to support the desired frequency.

A terminally sterilized tissue process may not require that EM be performed while processing tissue from each donor or batch because of the additional safety the sterilization process provides. Tissue being released under an aseptic process may require more frequent EM because of the greater potential impact the environment may have on the finished tissue.

Determination of the frequency of testing should begin with a risk analysis for the tissue and the process and an evaluation of the potential impact the environment may have on the finished tissue. Generally, all EM plans should begin with frequent testing until enough data are gathered to justify reducing the frequency. The minimum frequency permissible must be determined by the tissue bank based on potential risk, which is identified in a documented risk assessment analysis.

M. Typical culturing conditions

Culture conditions are provided in many standards and documents relating to EM [USP<1116> (36), (11)]. However, use of standard media (e.g., soybean casein digest medium) at standard incubation parameters (e.g., 20-25^{0}C for not less than four days and up to seven days for molds and yeasts or 30-35^{0}C for not less than two or three days and up to seven days for bacteria) is appropriate. Other incubation conditions can certainly be used. It is important that there be a rationale supporting the incubation conditions used (e.g. based on a particular standard or on validation data).

N. Interpreting EM Data

Since the primary purpose of EM is to evaluate the quality of the processing environment, it is critical that EM data be reviewed by appropriate personnel and trended to determine continued control.

Full identification of microorganisms can be beneficial when establishing an initial baseline, when evaluating the potential impact of a process change, or investigating an EM excursion. In an ISO Class 5 environment or other critical environment, full identification is expected (i.e., genus and species). However, it would not be necessary to identify all microorganisms obtained on a routine basis, especially if the results are within specification. There are many cases where colony morphology and a Gram stain provide sufficient information (e.g. when gathering data over the course of a year to establish a baseline) and other cases where merely having the microorganism count is acceptable (e.g. after years of data have already been gathered and no changes are occurring to the process). The level of identification performed should be determined by the purpose for gathering the data.

Interpretation of EM data will be more useful if performed with EM trends in mind. Trending of EM data will assist the company in understanding their EM baseline as well as identifying potential issues on the horizon.

O. Tissue Impact for EM Excursions

In the event of EM excursions, it is critical to determine the potential impact, if any, on tissue. It should not be automatically assumed that an EM excursion had an impact on tissue; rather, an investigation should determine whether an impact was likely.

Ideally, EM should not be the only monitor or control involved in day-to-day processing. The investigation of potential impact of an EM excursion on tissue should include more than the EM results; it should also include the results of the batch-to-batch or day-to-day in-process controls. It might be possible, based on the entire amount of data available, to determine that the EM excursion had no impact on tissue, even without any additional testing. Herein lies the benefit of proper process validation and determination of appropriate microbiological surveillance. Note that testing additional tissue for sterility in the event of an EM excursion should not be considered a default approach.

> *Example: An EM excursion is obtained from the touch plate of a processing technician's gloves. The alert level is established at 3 CFU, action level is set at 9, and the result is 10 CFU. Identification is performed and the microorganisms are determined to be two*

different species of Staphylococcus. In the same processing session, the following additional controls and monitors are performed and found to be within specification:

Transport solution microbial count
EM of one surface and cleanroom air (settling plate during processing)
Negative controls of the processing solutions
Sterilization cycles for equipment

Process validation data indicates that the process is very effective at killing Staphylococcus microorganisms (it provides a 5.6 log reduction). The aseptic packaging process validation indicates that the technician in question can properly package the tissue without adding contamination. The touch plate of the gloves was performed post-processing and prior to packaging, and the technician changed gloves prior to the packaging step. Therefore, the packaging process is not suspect. Lastly, the technician does not have a history of EM excursions.

All available data suggests that the contaminated gloves would have potentially impacted tissue only at the processing stage, and not at the packaging stage. Also, the process has been demonstrated to reduce contamination of this microorganism at the levels in question.

If the tissue is released under aseptic processing (i.e., no terminal sterilization), finished tissue from the batch is tested (cultured). If all other pre-established release specifications are met, a risk assessment analysis must be performed and documented, and used to justify release of finished tissue.

If the bank is releasing tissue using terminal sterilization, there would likely not be a need to perform any additional testing based on the strength of the data that no additional contamination likely occurred, and based on the microbial reduction that terminal sterilization provides.

XII. VALIDATION AND QUALIFICATION OF TEST METHODS

A. Introduction

There are two important stages in evaluating test methods.

- Validation to ensure that the test is functioning properly and providing the desired data. This means demonstration that the test method is capable of providing consistent and correct results (e.g. if testing the tissue for anaerobic microorganisms, the data represent only anaerobes and not strict aerobes).
- Qualification to ensure that the test method is functioning properly for a specific tissue type. In this instance it is already known that the test method provides consistent and correct data (because #1 above has already been completed), but different tissue types may affect the test method (e.g., if residual antibiotics in soft tissue are present in the test system, contaminating microorganisms may not be able to replicate causing a false negative test). The term "verification" is often used interchangeably with "qualification".

Although it is common to use the term "test method validation" for both aspects, it is best to distinguish the two aspects by using different terminology. In other industries, use of the terms

"test method validation" and "test method qualification" assist in distinguishing between them; these terms are also appropriate in the tissue industry.

B. Test Method Validation

In validating a test method the tissue industry is not very different from other industries. There are different parameters evaluated during the validation for a qualitative test versus a quantitative test. For example, qualitative methods generally require evaluation of fewer parameters (e.g., specificity, limit of detection, ruggedness/repeatability, and equivalence if applicable). By comparison a quantitative method may require evaluation of additional parameters such as accuracy, precision, limit of quantitation, linearity, and range.

Guidance is provided in three chapters of USP, depending on the type of validation needed. Below is a discussion of all three.

Note that in most cases it will not be necessary for a tissue bank to perform full validation of a microbiological test method. Examples of the need for a full test method validation are included in the discussions below.

1. Bioburden Recovery Efficiency

When tissue bioburden is low, the recovery efficiency test must be performed by inoculating the tissue with a known number of microorganisms followed by performing the test method, and enumerating the microorganisms. The selection of the microorganism(s) to use for inoculation can be based on those types expected or demonstrated to be on the tissue. Typically, a single microorganism is chosen for the test.

Note that the recovery efficiency test of a swab culture method involves two phases:

First: The removal of the microorganisms from the tissue onto the swab (the swabbing step)

Second: The removal of the microorganisms from the swab into the test system. This is accomplished by either:

a. Direct plating of the swab onto agar plates (i.e., swabbing the agar plate with the swab to transfer the microorganisms from the swab to the plate), or
b. Applying an extraction method to the swab to remove the microorganisms into a liquid which can be filtered, or plated and cultured.

Both phases should be evaluated to understand the sensitivity of the swab method. The removal of microorganisms from the tissue has already been explained above. The removal of microorganisms from the swab is similar except that the swab is inoculated rather than the tissue. Both phases can be evaluated in a single experiment, but if improvement is needed, the data will not be available to determine which phase of the test must be changed.

When determining a recovery efficiency using the inoculation method, the percent recovery is determined by dividing the number recovered in the test by the number placed onto the tissue and the result being multiplied by 100. A correction factor can be determined by calculating the reciprocal of the percent recovery with the percent as a decimal. Either a recovery efficiency or a correction factor can be used as they both provide the same answer when used to adjust bioburden.

Example:
Recovery Efficiency = (# Recovered in Test / # Placed onto Tissue) X 100
If:
Recovered in Test = 18 CFU and
Placed onto Tissue = 87 CFU

Then:
Recovery Efficiency = (18 / 87) X 100 = 20.7%

Correction Factor = 1 / Percent Recovery as a Decimal
If:
Percent Recovery = 20.7%

Then:
Correction Factor = 1 / 0.207 = 4.8

If it is expected or understood that the naturally occurring bioburden is not low, a repetitive rinse recovery efficiency may be performed rather than inoculating the tissue. This test is similar but the tissue is not inoculated with microorganisms; rather, the same tissue is swabbed multiple times. The goal is that the final count recovered from the tissue be significantly lower than the initial count, ideally zero. The percent recovery is determined by dividing the count obtained from the first swab by all counts obtained from all swabs.

Example:
Recovery Efficiency =
(# Recovered From First Swab / # Recovered From All Swabs) X 100

If:
Swab 1 = 23 CFU
Swab 2 = 5 CFU
Swab 3 = 0 CFU
Swab 4 = 0 CFU

Then:
Recovery Efficiency = (23 / 28) X 100 = 82.1%

The correction factor in this example would be: 1 / .821 = 1.2

2. USP <1223> Validation of Alternative Microbiological Methods

This chapter (43) describes validation of a test method that is an alternative to the standard test methods in USP. For example, a tissue bank may wish to employ a modified (7) sterility test, and to incubate for 7 rather than 14 days.

This chapter would not be applicable for adding a neutralizer in the sterility test media to overcome inhibition in the test system.

This chapter may be of limited use to tissue banking since many of the test methods used are already standardized by USP or AAMI/ISO. If an alternative test method would be

advantageous to the tissue bank however, this would be the proper USP chapter to follow. Additional guidance can also be found in PDA TR33 (44).

3. USP <1225> Validation of Compendial Procedures

This chapter (45) specifies requirements for information that must be gathered in order to submit a new test method for inclusion in USP. For example, a tissue bank would reference this chapter if it develops a new rapid microbiological test method and would like this method to be included in the next version of USP. This chapter provides general information about test method validation, although performance of all aspects of this chapter may be overkill for the more traditional methods used in the tissue industry.

4. USP <1226> Verification of Compendial Procedures

This chapter (46) provides information on the verification procedure to be applied when setting up testing specified in USP [e.g., USP <71> Sterility Tests (7)]. The chapter clarifies that it is not required to perform full validation for test procedures outlined in USP, rather it states to select relevant aspects from USP <1225> (45) for evaluation. Note that this chapter uses the term "verification" rather than validation. Use of "verification" is appropriate considering the scope of what is being done.

This same approach can be used for test procedures outlined in AAMI/ISO methods [e.g., 11737-1 (27) for bioburden testing and 11737-2 (32) for sterility testing].

C. Test Method Qualification

When test method qualification is being performed it is already known that the test method functions properly under typical circumstances (e.g. when testing non-inhibitory tissue types with simple configurations, or if the validation was performed using a tissue surrogate such as a plastic or stainless steel coupon). It is necessary, however, to demonstrate that the tissue being tested falls under those "typical" circumstances. If it is found that the tissue does not fall under those circumstances then the test method must be altered so that the test is valid for that tissue. Examples of common alterations are the addition of neutralizing substances to media to eliminate the effect of residuals (e.g., for bioburden or sterility testing) or the use of a specialized filter material (for membrane filtrations).

Most compendial methods include a qualification process. For example, USP <71> (7) on sterility testing contains details for performing a growth promotion test and a Method Suitability test (also called the bacteriostasis/fungistasis test or B/F test), which is the qualification study for that test. Other tests in USP also provide the necessary qualification steps for that method (sometimes called verification). The AAMI/ISO tests also provide guidance on appropriate qualification tests (e.g., recovery efficiency and test for inhibitory substances for bioburden and Method Suitability).

When tissue banks are using test methods provided in compendia, the associated qualification studies provided are usually sufficient. If the test method is not compendial, as with an alternative test method, then an appropriate qualification study must be determined and performed.

Qualification studies that confirm proper neutralization of the test article usually follow the same general concepts:

1. Perform the intended neutralization step
2. Perform the intended culturing step
3. Inoculate the filter or test media with a low number (e.g., <100 CFU) of microorganisms, followed by incubation
4. Compare the inoculated test sample results to controls which had no contact with the tissue

An example of a neutralization qualification study is found in USP <71> (7), in the "Method Suitability Test" section.

There is no established percent recovery considered acceptable by tissue banking professionals. In USP, values of 50% and 70% are considered acceptable, depending on the type of test being performed.

A common misconception is that if the graft has properties that interfere with the growth of microorganisms during testing, the graft will behave in the same manner when transplanted into a graft recipient. The fallacy of this logic is that the inhibition displayed by the graft during testing may be transient. When implanted into a recipient the inhibiting compound may be diluted out or neutralized, allowing for microorganisms to reproduce. Failure to appropriately verify the accuracy of culturing methods can potentially put at risk the safety of the graft recipient by allowing inadequately sterilized or disinfected tissue into the marketplace.

Generally, it is not required to characterize what is causing inhibition in a test. It is usually acceptable to simply demonstrate that neutralization is occurring. Characterization of the inhibitory substance might be recommended as part of an investigation if typical neutralization techniques are not providing acceptable results. Guidance on neutralization can be found in USP <61> (47), <71> (7) and <1227> (33).

See Annex J for an example of test method qualification.

XIII. TISSUE LABELING

A. Labeling

Labeling should be clear, simple and consistent so the end user can understand the treatment status of the finished tissue. In an effort to achieve this, the following options are provided by AATB for labeling regarding the microbial processing status of the tissue.

- Aseptically processed
- Sterile

A description of *aseptic processing* is provided in the definitions in this document.

For sterile labeling, it is expected that a validated SAL, with an appropriately justified SAL value, is justified and used (see the section entitled SAL and the definition for *terminal sterilization*). If the term *sterile* is used on labeling, immediately following it there should be a description of the sterilization process.

It is not a requirement to provide the SAL value on the labeling. Providing the SAL on the labeling has not been a practice in the medical device industry, the SAL is not necessary for the user to know.

Additionally, information regarding bacterial or viral reduction or clearance is not necessary, but can be described in supportive documentation, including validation data.

Variations to these labeling requirements may exist where required and/or approved by other jurisdictions/regulatory agencies outside the United States.

B. Sterility Assurance Level (SAL)

The term SAL generally must only be used in a situation where a sterilization process is being applied to the tissue in its final packaging. If a sterilization process has been applied to tissue, but the tissue is then packaged or manipulated in some other way, the SAL associated with the sterilization process has potentially been compromised and is no longer assured without additional validation work. In the situation where the tissue is somehow manipulated post-sterilization, the appropriate terms to use would be *aseptic processing*.

In *aseptic processing*, typical approaches are used to assure disinfection is appropriate (e.g., disinfection using washing and/or chemicals during processing).

The log reduction value of a process does not equal an SAL of the same value. For example, if a tissue process can provide a 6 log reduction of microorganisms, this does not equal a 10^{-6} SAL. As mentioned previously, the term SAL should not be used to describe sterility for validated tissue process that does not include *terminal sterilization*.

Typically, a minimum SAL of 10^{-6} is used for health care products labeled sterile, and this is the usual expectation for tissue as well. The sterilization validation will clearly identify that an SAL has been demonstrated, and this is the SAL that should be referenced in documentation supported by data.

In the United States, an ANSI/AAMI standard allows for some flexibility in the SAL requirement (10). If tissue is sensitive to the sterilization process there are criteria provided which allow use of a higher SAL (e.g., 10^{-3}, 10^{-4} or 10^{-5}), and a sterile claim can be made on labeling. Refer to the standard for details on how to use this option.

Realistically, regardless of the SAL value of the tissue, the cleanliness of the tissue upon opening the package is reduced to the cleanliness of the environment in which it is opened. At that point, the environment dictates the tissue cleanliness more than the SAL value while the allograft was in the packaging.

XIV. ANNEXES

A. Characterizing a Process

Scenario:	A feasibility study has been conducted in which a 30 minute soak of 100 grams

	of tissue in 0.1g/L Gentamicin solution results in <1 CFU gram negative organisms / 10 grams of tissue. The study has been repeated twice and has a high level of confidence that it will pass validation. The validation manager indicated that the process would have to be performed three times in order to demonstrate reproducibility. The intention is to perform the process a third and final time, and then transfer the process to production.
Issues:	The range has not been determined for antibiotic concentration, soak time, or quantity of tissue that will allow the process to achieve <1 CFU / 10 grams of tissue. Since there is no range for concentration, exposure time, and quantity of tissue, a specification range cannot be set by which the processing team can run the process. This would steer the processing team toward failure, because the processing step is set to be exactly a "30 minute soak in 0.1g/L Gentamicin solution for 100g of tissue." The process will produce acceptable outcomes when performed; however it is so narrowly defined that production teams will be plagued with process deviations.
Evaluation:	After speaking to the production manager regarding processing capacity, a matrix study was performed that evaluated: Gentamicin concentration: 0.06 – .14 g/L Tissue weight: 100g – 300g Soak time: 30 – 360 minutes Minimum and maximum parameters for concentration, exposure time, and quantity of tissue produced the desired outcome of <1 CFU of Gram negative organisms / 10 g of tissue. The specifications that were written and validated as a result of characterizing the process were: Gentamicin concentration: 0.08 – .12 g/L Tissue weight: 150g – 250g Soak time: 60 – 300 minutes In expanding the processing range, a process specification was provided that production could manage and, when occasional process excursions occurred, data was available to help evaluate acceptability of affected tissue. In addition, QC sent the metrology group a memo asking them to determine the accuracy of production equipment used to measure the weights and times.

B. Stepwise Approach to Process Validation

This annex describes the processing steps in a tissue process then provides items that were considered during process characterization. It illustrates how initial process characterization can simplify process validation.

1. Sonicate tissue in sterile water for 10 minutes

a. Process of cleaning/decontaminating/sterilizing the equipment
    i. Validated as a separate step rather than addressing it in process validation
b. Determination that 10 minutes provides the required results
    i. Addressed in process characterization rather than in process validation
c. Determination of temperature requirements
    i. Process characterization showed no considerable affect based on temperature. A predetermined temperature range will be used during process validation.

2. Soak tissue in antibiotic cocktail A for 25 minutes
    a. Process of cleaning/decontaminating the equipment
        i. Validated as a separate step rather than addressing it in process validation
    b. Required antibiotic cocktail concentrations
        i. Initial work was performed in process characterization and a concentration has been established for the process validation
    c. Shelf life of antibiotic cocktail potency after preparation
        i. Addressed in process characterization rather than in process validation
    d. Determination that 25 minutes provides the required results to the tissue
        i. Initial work was performed in process characterization but two time points (25 minutes and 40 minutes) will be used in the process validation to determine the optimal time
    e. Determination of temperature requirements, if any
        i. Addressed in process characterization rather than in process validation
3. Soak tissue in antibiotic cocktail B for 15 minutes
    a. Same as above for cocktail A
4. Rinse tissue in sterile water for 15 minutes
    a. Same as above for initial sterile water sonication
5. Package tissue
    a. Process of cleaning/decontaminating the equipment
        i. Validated as a separate step rather than addressing it in process validation
    b. Qualification of package sealer
        i. Performed as separate qualification previous to the process validation
    c. Package validation
        i. Includes: Integrity/strength, tissue/package interactions during shipping and package shelf life:
            a) Performed as separate validation previous to or concurrent with the process validation

C. Validation of an Entire Process

This annex demonstrates the importance of performing process characterization and/or validation on individual process steps prior to validating an entire process. Assuming that process characterization has not occurred, consider the following:

In validation of a process intended to reduce bioburden on tissue, the following steps are used:

1. Sonicate tissue in sterile water for 10 minutes
2. Soak tissue in antibiotic cocktail A for 25 minutes
3. Soak tissue in antibiotic cocktail B for 15 minutes
4. Rinse tissue in sterile water for 15 minutes
5. Package tissue

Inoculating tissue with 10^6 CFU of specified microorganisms and testing the tissue for those microorganisms at the end of the process may result in recovery of 0 CFU. In this instance, it is impossible to determine the full capability of the process.

Alternatively, this approach may result in a final recovery of 10^4 CFU of the microorganisms. In this instance, there is no data to suggest which steps of the process might be optimized. The following questions have not been answered:

1. Is 25 minutes of cocktail A too much time or too little?
2. Is 15 minutes of cocktail B too much time or too little?
3. What concentration of cocktails A and B must be used?
4. What is the acceptable range (variation) for concentration, time or temperature of the cocktails?

D. Limited Validation of Existing Process

Process Q, which is marketed by Company ABC, has been validated to provide a specified reduction of microorganisms and a specified physical effect on tissue.

If a tissue bank chooses to purchase and use this validated process on its own tissue, the process must be qualified for use:

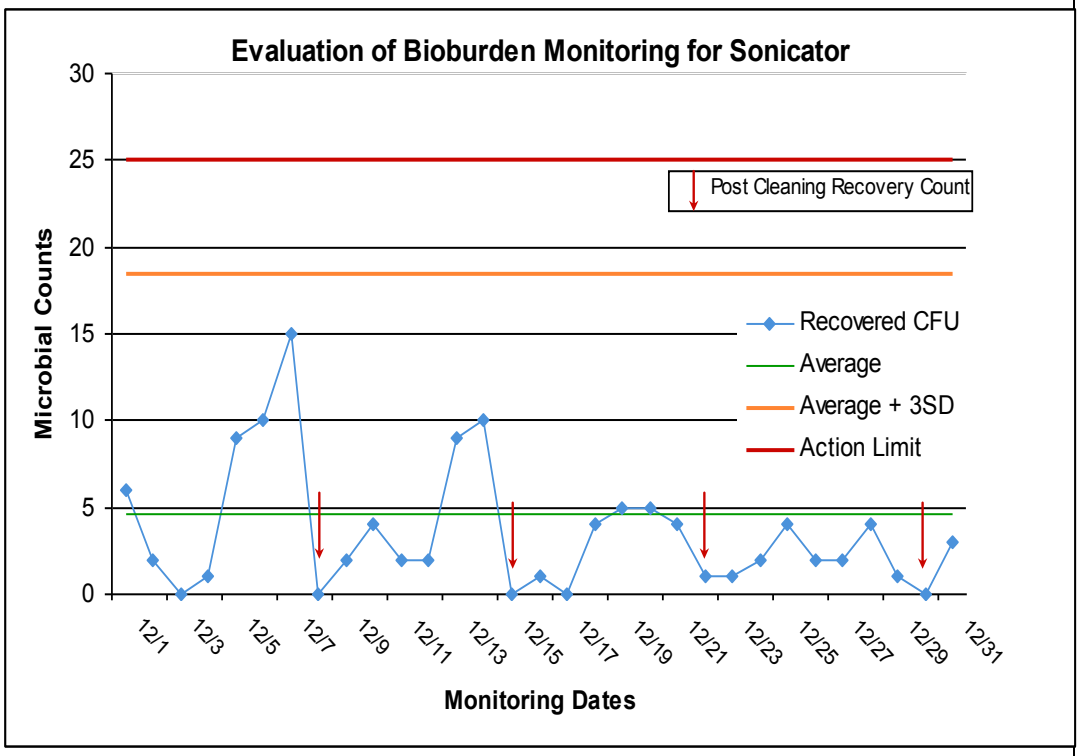
1. in their establishment;
2. on their tissue; and
3. by their personnel.

This does not mean that an entire validation must be performed, but it may mean that verification should be performed to assure the validation performed by Company ABC is applicable to their tissue. The verification may include the following:

1. Perform and document a risk analysis to determine the critical steps in the process.
2. Determine worst-case microorganisms and worst-case loads to be tested. This may have already been determined by Company ABC.
3. Determine the acceptance criteria for the test. This may be established with assistance from Company ABC.
4. Verify that appropriate results are obtained when using this validated process (i.e., similar results as Company ABC are obtained).
    a. Demonstrate the suitability of the test method to be used (e.g., B/F test, see Section VI. Neutralization).
    b. Inoculate tissue with the microorganisms at the appropriate loads and apply the process to the tissue.
    c. Perform the proper neutralization step and test to verify the effectiveness of the process in eliminating the microorganisms.
    d. Compare these results to the published results of Company ABC.

E. Applying Microbiological Surveillance – Example 1

Scenario:	It was noted in the validation of the initial sterile water sonication step that the water is prone to contamination and growth after about four hours of use. It was also noted that the speed of contamination was somewhat variable; sometimes by four hours the water microbial count was 0 CFU per mL and other times it ranged between 10 and 25 CFU per mL. It was determined that up to 25 CFU per mL

	was acceptable, but it was suspected that the variability could be even more pronounced over time than it was during the validation. The water is changed and a simple cleaning is performed with every processing run, but the frequency of a thorough cleaning and of testing the water is in question. It was also noted that none of the other process components were prone to microorganism growth and/or concentration change over the time period validated. The initial test data would confirm whether there was a specific day of the week that should be monitored when the frequency was reduced to weekly or if the day of the week was irrelevant. After three months of weekly testing it would be determined if the frequency could be reduced to monthly.
Issues:	It was determined, due to criticality of impact to tissue and based on the validation data, that the initial water sonication step should be monitored routinely. It was determined to monitor the water once per day for the first four weeks. This data would be used to set alert and action levels for the process, verify cleaning frequency for the equipment, and determine the frequency with which action levels are exceeded.
Evaluation:	 After plotting the data for one month of monitoring, the following observations were made: • The reduction of the monitoring frequency to weekly is supported by the fact that 3 standard deviations (9%) of the values for the process fall below 18.5 CFU per mL and the action level is 25 CFU per mL. • The cleaning frequency is appropriate given that the bioburden does not exceed

	the action level during the interval between cleanings and the bioburden after cleaning is near zero in all instances. • The process appears to be in a state of control in that after plotting the average plus 3 standard deviations (18.5) this value falls below the action limit (25 CFU) where a potential impact on the tissue could be expected. • The average plus 3 standard deviations (18.5) would be an appropriate alert level for indicating that the process might no longer be in a state of control while still allowing corrective action to be taken to restore a state of control before an impact on the tissue (action level) would be expected. • Data indicate that the process can be monitored weekly for the next three months (as planned) and that it should be performed sometime during the week, and not immediately after changing the water. Some of the data indicate that testing a day or two prior to the thorough clean provides some of the higher results, and this also seems logical. Thus the weekly samples will be pulled on day five or six after the thorough cleaning for the next three months, followed by a similar evaluation as described above. • In this situation a bioburden specification was not determined because there is not always a direct connection between environmental bioburden and tissue bioburden. Any value at or above the action level will be evaluated to determine whether there was likely impact to the tissue and what the resulting actions should be.

F. Applying Microbiological Surveillance – Example 2

Scenario:	In the initial validation of the last step of a process (Step F: final rinse of the batch of tissue in sterile water and sonicated for 10 minutes) it was discovered that if there were microorganisms remaining on some of the tissues after the process step (via sterility testing, aka destructive testing), the rinsate (the solution which remained after the sonication process) consistently contained the same microorganisms. This was determined because during the initial validation, plate count testing was performed on the water from the sonicator (post sonication) as well as sterility testing of the finished tissue (i.e., destructive testing). Based on this initial connection between the finished tissue and the Step F rinsate, it was determined to pay special attention to this situation during subsequent validation activities.
Issues:	In the validation activities, it was substantiated that the rinsate consistently contained the microorganisms which were present on the finished tissue (again based on rinsate plate count results compared to sterility testing (i.e., destructive testing) of finished tissue. It was noted that although some of the finished tissues would carry a particular microorganism, it would not be present on all of them. This means that testing a percentage of the finished tissue may or may not represent the entire batch of tissue. This information led the tissue bank to conclude that testing of the rinsate may actually be a better indicator of the microbiological nature of the batch of tissue than testing finished portions of tissue

	from the batch.
Evaluation:	It was determined that for some period of time after the validation, side-by-side testing of both the finished tissue (sterility testing of 10% of the batch, aka destructive testing) and the Step F rinsate would be performed and trended. Based on the results of the testing over time it would be determined if the rinsate provides as sensitive or a more sensitive detection method for identifying the presence of microorganisms on tissue compared to sterility testing (i.e., destructive testing). The tissue bank may then have options regarding the necessity of testing finished tissue from each batch as an indication of the microbiological status of the batch.

G. Companion Tissue Validation Example

To qualify a companion tissue for end point culture testing, the following approach can be used:

1. Identify the largest and most difficult graft to disinfect and document the rationale for why it is worst case.
2. Identify the desired companion tissue size(s) for end point culture testing.
3. Validate a bioburden extraction method, by seeding the tissue with approximately 100-200 CFU of *Bacillus atropheous* or other appropriate organism from experience. Reference ISO 11737-1, Annex C (27) and AAMI TIR 37 (28).
    a. Develop a recovery efficiency number to use to correct the bioburden count [(CFU recovered/CFU control plates)*100)]
    b. Perform this validation for the largest graft and the potential companion tissue
4. Perform bioburden testing on UNPROCESSED tissues, largest and potential companion samples.
    a. The tissue may require debriding and non-chemical gross lipid removal to facilitate filtration of the extract for subsequent bioburden testing; pour plate is an alternative option to a filtration sample.
    b. Unprocessed tissue is used to evaluate the bioburden equivalency between the largest and companion sample as unprocessed tissue is either culture negative or has very low bioburden which will make reproducible detection for a validation almost impossible.
    c. It is recommended to evaluate bioburden from at least 10 different donors to account for donor derived variability.
    d. Assess the bioburden from a companion sample(s) made from the same lot and type of tissue that will have the bioburden from the largest graft tested.
        i. It's desirable to test at least three replicates of the companion and largest grafts per donor.
    e. Normalize the corrected bioburden by surface area, mass or volume – provide rationale for its appropriateness.
5. Acceptance criteria
    a. Recovery efficiency – it is desirable to have at least 50%, but this is not always achievable.
    b. The normalized bioburden amount from the companion sample should be comparable to largest graft in the lot.
        i. Comparable can be defined as but not limited to: within 1 log, 2 standard deviation or within 30%.

H. Validating a Fluid Extraction Method

This example uses a final rinse of transplantable tissue (i.e., cortical bone tissue) following a cleaning/disinfection process where all tissues were exposed to a mechanical-based extraction.

1. Pre-Validation

   Establishment of the extraction method for a test should be based on appropriate characterization of the test variables. Examples of the variables to consider are:

   a. Time
   b. Temperature
   c. Extraction fluid (e.g., Fluid A, Fluid D, saline)
   d. Extraction type (e.g., orbital, stomaching, ultrasonic)
   e. Filter type (e.g., cellulose, polyethersulphone, nylon, hydrophobic edge)

   The following example is based on evaluating and validating the recovery efficiency of the mechanical-based extraction selected. The tissue inoculation method was selected because historical data shows tissue bioburden at this stage is too low to use the repetitive recovery method of validating recovery efficiency.

   a. Utilizing a tissue inoculation method [Reference ISO 11737-1 Annex C (27)], determine recovery efficiency of fluid extraction methodology considering variables above.
   b. Add *Bacillus atrophaeus* spores using a target of 100 CFU per tissue tested to selected bone tissues (e.g., cortical rings) at the selected inoculation sites. Allow time to absorb, 10 minutes is generally sufficient, within a biological safety cabinet.
   c. At the time of inoculation, also inoculate agar plates with the same volume to determine the suspension titer.
   d. Test tissue that is not inoculated to understand naturally occurring bioburden on the tissue.
   e. Add inoculated ring(s) to rinse vessel with other cortical tissue (to represent a worst-case amount).
   f. Add solution and perform mechanical rinse step (worst-case parameters for volume, speed and time).
   g. Decant solution from tissue and filter solution using standard microbiological techniques, apply neutralization step (if applicable) and plate on appropriate media. Incubate plates in appropriate environment for appropriate amount of time [reference ISO 11737-1 (27) and USP<61> (47)].
      i. Count colonies and determine recovery efficiency as a function of amount of microorganisms.
   h. Count colonies and determine recovery efficiency as a function of amount of microorganisms added to tissue vs. amount of microorganisms recovered on filter.
      i. Ensure that the colonies counted are the challenge microorganism.
   i. Perform above test on the selected number of replicates to factor in system variability.
   j. Analyze data for variability using the selected approach.
   k. Define acceptance criteria for validation (overall recovery efficiency). Refer to pre-validation example immediately below.

2. Validation
    a. Perform recovery efficiency runs (e.g., n ≥3 donors) using nominally defined variables (typical rinse procedure, tissue amounts, etc.) and the microbiological techniques outlined in pre-validation work.
    b. Compare determined recovery efficiency data from validation runs to acceptance criteria to demonstrate repeatability of fluid extraction methodology.
    c. Calculate correction factor to normalize the data for tracking purposes. Refer to validation example immediately below.

I. Fluid Extraction Calculation Example

1. Pre-Validation
    a. Worst-Case Extraction Parameters
    b. Establish Acceptance Criteria for Validation

Inoculum Check	CFU Count
Rep 1	90
Rep 2	98
Rep 3	92
Rep 4	91
Rep 5	101
Average ± Std Dev	94 ± 4

Test Article Recovery Runs	CFU Count	Recovery Efficiency	Bioburden CFU Correction Factor*
Rep 1	45	45 / 94 (x100) = 48%	1 / 0.42 = 2.4 *for use in quantitative testing
Rep 2	42	42 / 94 (x100) = 45%	
Rep 3	38	38 / 94 (x100) = 40%	
Rep 4	52	52 / 94 (x100) = 55%	
Rep 5	22	22 / 94 (x100) = 23%	
Average ± Std Dev	40 ± 11	42% ± 12	

2. Validation
    a. Nominal Extraction Parameters
    b. Three Operator Runs
    c. Demonstrate Reproducibility of Extraction Methodology
    d. Target = All Validation Runs should average ≥ 42%; in this validation example it was determined that results should be equal to or better than the average of 42%

Pre-Validation Average Recovery Efficiency	Validation Run 1 Average Recovery Efficiency (5-10 samples)	Validation Run 2 Average Recovery Efficiency (5-10 samples)	Validation Run 3 Average Recovery Efficiency (5-10 samples)
42%	44%	52%	50%
Validation Result	Pass	Pass	Pass

Note: If a Validation Run fails, the pre-validation should be re-evaluated.

J. Test Method Qualification

Scenario:	A process validation is being performed to determine the ability of a solution to reduce naturally occurring bioburden on a tissue by 99.9% (three logs). The solution being tested contains an antibiotic that is selective for Gram negative microorganisms. Data from a previous study is available where the bioburden of the tissue was identified. The predominant organisms are Gram negative facultative anaerobes, with less than 2% of the bioburden being fungi, Gram positive cocci and Gram positive rods. The test laboratory has already performed validation of the general bioburden test method for its use. There are additional tests that must be performed specific to this tissue and this bioburden reduction study to ensure that valid results are obtained. The process validation will consist of the following steps: 1. Inoculating tissue with selected microorganisms 2. Treating some of the tissue with the intended process 3. Extracting the microorganisms remaining from both treated tissue and untreated tissue 4. Filtering the extracts through a 0.45 um filter 5. Placing the filter on trypticase soy agar (TSA) media plates 6. Incubating at 30-35°C for 48-96 hours 7. Counting the CFUs from the treated and untreated tissue media plates The difference in the microorganism counts from the treated and untreated tissue will be used to determine the effectiveness of bioburden reduction. The physical removal of bioburden from the tissue (the bioburden extraction method) had been previously determined to be 62% using a repetitive recovery approach. This bioburden recovery method was reviewed and found appropriate to use for this bioburden reduction study.
Issues:	1. (Optimal media type) The media type and incubation conditions stated above have not been previously qualified for culturing the microorganisms to be used in the bioburden reduction study. It is desired to use one media type (TSA) and one incubation parameter (30-35^{0}C for 48-96 hours) rather than using the media types and incubation parameters usually considered optimal for these microorganisms. 2. (Antibiotic neutralization) When the bioburden extraction is performed, residual antibiotics may also be extracted into the fluid. When the extraction fluid is filtered through a membrane filter, in addition to the microorganisms trapped on the filter, the residual antibiotics may also remain on the filter and inhibit microorganism growth. It must be demonstrated that the test method

	being used provides proper neutralization for growth of the microorganisms in question.
Evaluation:	1. (Optimal media type) In order to assess the validity of the culturing conditions, three different Gram negative facultative anaerobes, a Gram positive rod, and a yeast (the same microorganisms which will be used in the process validation) are placed separately in quantities of 10-100 CFU onto both the optimal test media and the desired test media. The different media types are incubated as outlined in the table below and the colonies are counted. If, after incubating at the various conditions, the quantity of CFUs from the desired media and incubation conditions is comparable to the CFUs from the optimal media and incubation conditions, the desired media and incubation conditions may be considered valid. The tissue bank has established as acceptance criteria that the desired culturing condition CFUs must not be less than 70% of the optimal condition CFUs [per USP <1227> (33)]. 2. (Antibiotic neutralization) Neutralization is demonstrated by performing the bioburden extraction method on portions of tissue that have undergone the antibiotic soaking process. The extraction solution and the established number of rinses are filtered. As the final rinse is performed, the rinse solution is inoculated with 10-100 CFU of the same microorganisms as listed above. The filter is placed onto TSA and incubated at 30-35°C for 48-96 hours. As a positive control, a solution containing the same number of microorganisms is filtered and incubated. If after incubation, the number of recovered CFUs from the test samples and the controls are comparable (again to 70%), the neutralization method may be considered valid. Note that it is stated to inoculate *the final rinse* of the tissue extraction solution prior to filtering. If the tissue is directly inoculated, there are other variables involved that make the test results difficult to interpret.

Organism	Optimal Culturing Conditions	Desired Culturing Conditions
Gram negative facultative anaerobes	BHI w/ 5% Sheep's Blood incubated at 30-35°C for 48-72 hours under aerobic and anaerobic conditions	TSA incubated at 30-35°C for 48-96 hours under aerobic conditions
Yeast	Potato dextrose agar incubated at 20-25°C for 48-72 hours	
Gram positive rod	TSA w/ 5% Sheep's Blood incubated at 30-35°C for 48-72 hours	

XV. REFERENCES

(1) AATB *Standards for Tissue Banking,* current edition, McLean, Va.

(2) ANSI/AAMI/ISO 13408-1:2008, Aseptic Processing of Health Care Products – Part 1: General Requirements.

(3) ANSI/AAMI/ISO TIR 11139:2006, Sterilization of Health Care Products – Vocabulary.

(4) Jorgensen JH, Pfaller MA, et al., Decontamination, Disinfection, and Sterilization. Manual of Clinical Microbiology, 11th edition, Volume 1, ASM Press 2015, Chapter 13.

(5) CFR - Code of Federal Regulations Title 21, Part 1271 -- Human Cells, Tissues, and Cellular and Tissue-Based Products, Subpart A-- General Provisions, Sec. 1271.3 How does FDA define important terms in this part?

(6) ANSI/AAMI/ISO 14161:2009, Sterilization of Health Care Products – Biological Indicators – Guidance for the Selection, Use, and Interpretation of Results. (AAMI/Draft Standard-2).

(7) USP <71> Sterility Tests. United States Pharmacopeia (current), Rockville, MD.

(8) U.S. Department of Health and Human Services, Food and Drug Administration, Guidance for Industry, Current Good Tissue Practice (CGTP) and Additional Requirements for Manufacturers of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps), December 2011.

(9) U.S. Department of Health and Human Services, Food and Drug Administration, Guidance for Industry, Process Validation: General Principles and Practices; Current Good Manufacturing Practices (CGMP), Revision 1; January 2011.

(10) ANSI/AAMI ST67:2011, Sterilization of Health Care Products – Requirements for Products Labeled "Sterile".

(11) U.S. Department of Health and Human Services, Food and Drug Administration, Sterile Drug Products Manufactured by Aseptic Processing – Current Good Manufacturing Practice, Final Guidance for Industry, September 2004.

(12) ANSI/AAMI/ISO 14160:1998 / R 2008, Sterilization of Single-use Medical Devices Incorporating Materials of Animal Origin – Validation and Routine Control of Sterilization by Liquid Chemical Sterilants.

(13) ANSI/AAMI/ISO 14937:2009 (R2013) Sterilization of healthcare products - General requirements for characterization of a sterilizing agent and the development, validation and routine control of a sterilization process for medical devices.

(14) Ronholdt CJ, Bogdansky S, A Fluid Extraction Technique for Detection of Microbial Contamination on Human Allografts. AATB Posters and Abstracts, 28th Annual Meeting (2004).

(15) von Garrel T, Knaepler H, Mutters B, Quality of Microbiological Testing Methods for Evaluation of Bacterial Contamination of Allogeneic Cancellous Bone Grafts. AATB Abstracts, 16th Annual Meeting (1993), and, in *Transfusion*, Vol. 33, No. 7, 1993.

(16) Veen MR, Petit P, Bloem RM, Sensitivity of Swab Cultures in Musculoskeletal Allograft Procurement. AATB Abstracts, 16th Annual Meeting (1993), and, in *Transfusion*, Vol. 33, No. 7, 1993.

(17) Taylor AK, Zanzi KP, Burchardt H, Forsell JH, The Reliability of Using a Dry Swab Culturing Method to Validate Tissue Sterility After Lyophilization. AATB Abstracts, 16th Annual Meeting (1993), and, in *Transfusion*, Vol. 33, No. 7, 1993.

(18) Veen MR, Bloem RM. Petit PL. Sensitivity and Negative Predictive Value of Swab Cultures in Musculoskeletal Allograft Procurement. *Clinical Orthop*, 259-63, 1994.

(19) Perry JL, Assessment of Swab Transport Systems for Aerobic and Anaerobic Organism Recovery. *Journal of Clinical Microbiology*, May 1997, p. 1269-1271.

(20) Perry JL, Inhibitory Properties of a Swab Transport Device. *Journal of Clinical Microbiology*. Vol. 35, p. 3367-3368, 1997.

(21) Wilson DA, Tuohy MS, Procop GW, Hall GS, Effects of Storage Temperature on the Recovery of Bacteria from Three Swab Transport Systems… Abstracts, American Society for Microbiology, 101st General Meeting, May 2001.

(22) Mills AR, Roberts MR, Evaluation of Culturing Methods at Predicting Allograft Sterility for Aseptically Processed Tissue. AATB Posters and Abstracts, 25th Annual Meeting (2001).

(23) Vehmeyer SB, Bloem RM, Petit PL. Microbiological screening of post-mortem bone donors—two case reports. J Hosp Infect 47:193–197, 2001.

(24) Ronholdt CJ, Bogdansky S. Quantitative Microbial Comparison of Swab Culturing Systems on Human Allografts. AATB Posters and Abstracts, 28th Annual Meeting (2004).

(25) Dennis JA, Martinez OV, Landy DC, Malinin TI, Morris PR, Fox WP, Buck BE, Temple HT. A comparison of two microbial detection methods used in aseptic processing of musculoskeletal allograft tissue. Cell Tissue Bank. 12:45–50, 2009.

(26) AATB Guidance Document No. 2, v2, Prevention of Contamination and Cross-Contamination at Recovery: Practices & Culture Results, May 29, 2007.

(27) ANSI/AAMI/ISO 11737-1:2006 Sterilization of Health Care Products – Microbiological Methods – Part 1: Determination of the population of microorganisms on product.

(28) AAMI TIR 37-2007, Sterilization of health care products, Radiation, Guidance on sterilization of human tissue-based products. AAMI International (current).

(29) Kowalski JB, Mosley GA, Merritt K, Osborne J. Assessment of bioburden on human and animal tissues: Part 1—Results of method development and validation studies. Cell Tissue Bank (2012) 13:129–138.

(30) Kowalski JB, Mosley GA, Merritt K, Osborne J. Assessment of bioburden on human and animal tissues: Part 2—Results of testing of human tissue and qualification of a composite sample for routine bioburden determination. Cell Tissue Bank (2012) 13:431–439.

(31) U.S. Department of Health and Human Services, Food and Drug Administration, Validation of Growth-Based Rapid Microbiological Methods for Sterility Testing of Cell and Gene Therapy Products. Draft Guidance, February 2008.

(32) ANSI/AAMI/ISO 11737-2:2009, Sterilization of Medical Devices – Microbiological Methods – Part 2: Tests of Sterility Performed in the Definition, Validation and Maintenance of a Sterilization Process.

(33) USP <1227> Validation of Microbial Recovery from Pharmaceutical Articles. United States Pharmacopeia (current), Rockville, MD.

(34) ANSI/AAMI/ISO 11137-2:2013, Sterilization of health care products—Radiation-Part 2: Establishing the sterilization dose.

(35) AAMI TIR 33:2005, Sterilization of health care products – Radiation Substantiation of a selected sterilization dose – Method VDmax.

(36) USP<1116> Microbiological Control and Monitoring of Aseptic Processing Environments, United States Pharmacopeia (current), Rockville, MD.

(37) ANSI/IEST/ISO 14644-4:2001, Cleanrooms and associated controlled environments - Part 4: Design, construction and start-up.

(38) PDA TR13:2014, Fundamentals of an Environmental Monitoring Program. Parenteral Drug Association.

(39) US FDA Title 21 CFR Part 1271, Subpart D. Current Good Tissue Practice, Final Rule, Federal Register Vol. 69, No. 226, November 24, 2004.

(40) ASTM E2614-08, Standard Guide for Evaluation of Cleanroom Disinfectants. AAMI International (current).

(41) USP<1072> Disinfectants and Antiseptics. United States Pharmacopeia (current), Rockville, MD.

(42) AAMI TIR 12:2010 Designing, testing and labeling reusable medical devices for reprocessing in health care facilities: A guide for medical device manufacturers. AAMI International (current).

(43) USP <1223> Validation of Alternative Microbiological Methods, United States Pharmacopeia (current), Rockville, MD.

(44) PDA TR33:2013 Evaluation, Validation and Implementation of Alternative and Rapid Microbiological Methods. Parenteral Drug Association.

(45) USP <1225> Validation of Compendial Procedures, United States Pharmacopeia (current), Rockville, MD.

(46) USP <1226> Verification of Compendial Procedures, United States Pharmacopeia (current), Rockville, MD.

(47) USP<61> Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests, United States Pharmacopeia (current), Rockville, MD.